GLOBAL CHANGE, CLONAL GROWTH, AND BIOLOGICAL INVASIONS BY PLANTS

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GLOBAL CHANGE, CLONAL GROWTH, AND BIOLOGICAL INVASIONS BY PLANTS

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The introduced clonal plant *Alternanthera philoxeroides* invades an abandoned crop field in China. Figure by F-H Yu.

There are few more active frontiers in plant science than helping understand and predict the ecological consequences of on-going, global changes in climate, land use and cover, nutrient cycling, and acidity. This collection of research papers and reviews focuses on how these changes are likely to interact with two important factors, clonal growth in plants and the introduction of species into new regions by humans, to reshape the ecology of our world. Clonal growth is vegetative reproduction in which offspring remain attached to the parent at least until establishment. Clonal growth is associated with the invasiveness of introduced species, their tendency to spread after introduction and negatively affect other species. Will changes in climate, land cover, or nutrients further increase biological invasions by introduced, clonal plants? The articles in this book seek to address this question with new research and theory on clonal growth and its interactions with invasiveness and other components of global change.

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EDITORIAL
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Editorial: Global Change, Clonal Growth, and Biological Invasions by Plants

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Keywords: climate change, clonality, environmental heterogeneity, genotypic and phenotypic diversity, invasiveness, rapid evolution

The Editorial on the Research Topic

Global Change, Clonal Growth, and Biological Invasions by Plants

Global changes in climate, land use, nutrient availability, acidity, populations of harvested or undesired species, and concentrations of toxins are now widely evident. Their ecological and evolutionary consequences are likely to be great but are often hard to identify or anticipate because of the multiple interactions that shape most ecological systems. One potentially important set of interactions involves the properties of clonal growth in plants. Clonal growth is common in plants and in ecosystems around the world and appears to be associated with the invasiveness of introduced plant species. Emergent clonal traits such as resource sharing and signaling between connected plants within clones, selective positioning of plants during clonal growth, stores of energy or nutrients that can be reallocated between connected plants, meristem banks that can be initiated in response to clonal integration, and tradeoffs between clonal and sexual reproduction might contribute to plant invasiveness and community invasibility, and global change may affect the expression, fitness effects, and evolution of these traits. This research topic assembles articles that deal explicitly with interactions between clonal growth and plant invasion or global change, and additional papers that advance understanding of aspects of clonal growth likely to affect invasion or response to global change.

Four papers consider the evolution of clonality in plants. Epigenetic variation may provide an alternative means of adaptation to changing environments and play a key role during the invasion of some introduced clonal plants that cannot successfully reproduce sexually in the invaded range. In a forum paper, Dodd and Douhovnikoff propose how epigenetic variation may potentially enable clonal plants to adjust to changes in means and extremes of climate. Castro et al. report that plants of *Oxalis pes-caprae* from the native range show higher sexual fitness, while those from the invasive range have higher asexual fitness, suggesting that evolution has favored asexual over sexual reproduction during invasion. Roiloa et al. compare a different suite of clonal growth properties in *Carpobrotus edulis* from the native range in South Africa and the invaded range in Spain and Portugal. Plants from different ranges did not differ in degree of division of labor (i.e., specialization to acquire and share locally abundant resources), but the beneficial effect of clonal integration on the dry mass of apical ramets was higher in the introduced populations, suggesting that this clonal trait may have been selected for in the invaded range. Holmes et al. suggest that local adaptation in response to salinity has enabled clones of *Phragmites australis* from highly saline areas to function better under saline conditions in Australia. Their examination of gene expression using transcriptomics found clear differences in responses to salinity between clones sampled from areas with low and high salinity.

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Reliance on asexual reproduction has generally been seen as a likely constraint on evolution in clonal organisms. Two papers shed new light on the question of whether asexual and sexual reproduction conflict. Huber et al. examine the implications of intraspecific variation in the size of ramets in *Trifolium repens* and find evidence for a tradeoff between net production of biomass and rate of vegetative reproduction. Evolutionary shifts in the genetic composition of clonal populations may thus depend on whether ramet size or ramet number is under stronger selection. The picture may be quite different at higher taxonomic levels. Xie et al. compile data on 115 species from 139 publications and find no consistent tradeoff between allocation to clonal organs and to sexual reproduction.

Three papers focus on the intensively studied, highly invasive, amphibious, clonal herb *Alternanthera philoxeroides,* which has been introduced from South America to Asia, Australia, and North America. *A. philoxeroides* shows lower genetic diversity and phenotypic plasticity in China than in Argentina or the USA (Geng et al.). Since relatively high plasticity has often been found to be associated with invasiveness, this may mean than capacity for clonal growth can to some extent substitute for plasticity in invasions by plants. You et al. simulate invasion by *A. philoxeroides* into both open areas and stands of a native wetland clonal plant, *Jussiaea repens*; propagule pressure and connection between plants both facilitate invasion, supporting a role for physiological integration in the invasiveness of clonal plants. Zhang et al. caution that this may depend upon nutrient availability: connection between ramets within pairs in *A. philoxeroides* decreased their combined growth in mass when the level of nutrients was high and decreased the production of new ramets when the level of nutrients was low.

Three papers introduce novel concepts or properties of clonal plants. Martínková and Klimešová present the concept of enforced clonality, vegetative reproduction due to damage in normally non-clonal plants. Root fragmentation increased fitness in *Barbarea vulgaris* but not *B. stricta*, suggesting that enforced clonality may shift the relative fitness of species of some non-clonal plants in frequently disturbed habitats. Waters and Watson provide evidence for clonal foraging in response to volatiles released from soil. Ramets of *Fragaria vesca* produced more root mass when exposed to air above non-sterilized soils than when exposed to air above sterilized soils, and stolons tended to grow toward non-sterilized soils rather than sterilized soils. This could provide a mechanism for detection of soil resource levels prior to rooting. In what may be the first study of the potential role of endophytes in invasion by clonal plants, Dai et al. show that infection by the endophyte *Bacillus* sp. promotes the growth of the invasive clonal plant *Wedelia trilobata* but not that of the congeneric, native, clonal plant *W. chinensis*.

One of the best studied aspects of clonality is physiological integration between connected ramets. Liu et al. review impacts of clonal integration on tolerance of stress, invasiveness, competitive interactions, community structure, and productivity. One gap in knowledge of clonal integration has been a lack of studies on epiphytic clonal plants. Lu et al. report that clonal integration increased the performance of two rhizomatous, epiphytic ferns in a subtropical, montane, moist forest in China. One next step in the study of clonal integration is to understand how effects scale up to the level of the population. Wang, Y.-J. et al. compare effects of positively and negatively correlated patchiness of resources on intraspecific interactions in the rhizomatous herb *Iris japonica* to show that both the arrangement and the scale of patches can significantly affect competitive intensity.

Mechanisms and consequences of synchronous flowering in clonal bamboos are of great basic and practical interest. Wang, W. et al. present data on stand structure, seed bank dynamics, seedling regeneration, culm characteristics, and energy allocation in patches of an arrow bamboo, *Fargesia qinlingensis,* in patches that did and did not flower during five years after a mass flowering event. Results suggest that habitat modification was most likely responsible for the delayed flowering.

The interaction between clonality and invasion has received considerable attention, and interaction between clonal growth and climate change has begun to attract interest. Researchers are just beginning to tackle three-way interaction between clonality, invasion, and climate change. Clonal plants are widely represented in nature and dominate a number of plant communities and ecosystems around the world, evidence of the ecological importance of clonal growth. Understanding interactions that involve clonal growth will help us predict the impacts of ongoing, rapid change at a global scale in natural environments. We hope that publication of this research topic will stimulate more studies on this important nexus in the coming years.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial and direct intellectual contributions to the work and approved it for publication.

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ORIGINAL RESEARCH
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Enforced Clonality Confers a Fitness Advantage

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In largely clonal plants, splitting of a maternal plant into potentially independent plants (ramets) is usually spontaneous; however, such fragmentation also occurs in otherwise non-clonal species due to application of external force. This process might play an important yet largely overlooked role for otherwise non-clonal plants by providing a mechanism to regenerate after disturbance. Here, in a 5-year garden experiment on two short-lived, otherwise non-clonal species, *Barbarea vulgaris* and *Barbarea stricta*, we compared the fitness of plants fragmented by simulated disturbance ("enforced ramets") both with plants that contemporaneously originate in seed and with individuals unscathed by the disturbance event. Because the ability to regrow from fragments is related to plant age and stored reserves, we compared the effects of disturbance applied during three different ontogenetic stages of the plants. In *B. vulgaris*, enforced ramet fitness was higher than the measured fitness values of both uninjured plants and plants established from seed after the disturbance. This advantage decreased with increasing plant age at the time of fragmentation. In *B. stricta,* enforced ramet fitness was lower than or similar to fitness of uninjured plants and plants grown from seed. Our results likely reflect the habitat preferences of the study species, as *B. vulgaris* occurs in anthropogenic, disturbed habitats where body fragmentation is more probable and enforced clonality thus more advantageous than in the more natural habitats preferred by *B. stricta*. Generalizing from our results, we see that increased fitness yielded by enforced clonality would confer an evolutionary advantage in the face of disturbance, especially in habitats where a seed bank has not been formed, e.g., during invasion or colonization. Our results thus imply that enforced clonality should be taken into account when studying population dynamics and life strategies of otherwise non-clonal species in disturbed habitats.

Keywords: bud bank, disturbance, fitness, life strategy, ontogeny, ramet, root fragment, seed number

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INTRODUCTION

Clonality is defined as the production of new, genetically identical ramets with potential to become independent of their mother (Klimeš et al., 1997). It has been repeatedly documented that clonal growth brings benefits including resource acquisition, maternal support for new offspring, higher competitive abilities, independence from mates, and high ability of vegetative regeneration (Meloni et al., 2013; Wang et al., 2013; Fukui and Araki, 2014; Elgersma et al., 2015; Glover et al., 2015). Splitting of a clone into ramets is usually spontaneous and takes months to decades to be completed (Klimeš et al., 1997). However, it may be also suddenly realized by external force that

fragments a plant (Wehsarg, 1954; Klimešová and Martínková, 2004). When such resulting plant fragments are capable of surviving and regenerating we term this process of fragmentation and subsequent regeneration "enforced clonality." Although this process is not so essential for plants that otherwise split into ramets spontaneously, it might be crucial for otherwise non-clonal plants from disturbed habitats (Sosnová et al., 2014).

Enforced clonality relies upon fragmentation of the plant body. However, successful survival and growth subsequent to such severe intrusion on the plant's integrity is dependent on the ability of fragments to form missing tissues (Groff and Kaplan, 1988). Therefore, a root fragment may provide the foundation for a new ramet only when the root is able to form an adventive bud from which a shoot can emerge. Similarly, a shoot fragment must bear an axillary bud to continue growth and must also be able to produce adventive roots. A leaf fragment would need to form adventive buds that give rise to both shoot and roots to become a new individual. Although all types of successfully regenerating fragments can be observed in nature, those based on leaves are extremely rare (see examples for woody plants in Sagers, 1993 and for herbs in Klimešová and Klimeš, 2007). In aquatic conditions, plants use water as a substrate; thus, formation of roots is not necessary and body fragmentation is a common way of clonal growth in such habitat (Barrat-Segretain and Bornette, 2000; Boedeltje et al., 2003; Campbell, 2003). In terrestrial ecosystems, enforced clonality has been reported from arable land, with species displaying it receiving attention as weeds (Kefford and Caso, 1972; McIntyre, 1972; Klimešová et al., 2008). It has also been documented in ruderal habitats, where short-lived species survive severe disturbance and some new individuals establish from root fragments (Klimešová et al., 2008; Martínková et al., 2016). Enforced clonality, however, has importance in other contexts as well, including habitats with natural soil disturbances such as landslides, scree, and water erosion (Hess, 1909), colonization of new areas, spread of invasive plants (Bailey et al., 2009; Lin et al., 2012; Monty et al., 2015), and vegetatively propagated crops and ornamentals (Shepherd et al., 2013; de Souza et al., 2014; Birlanga et al., 2015).

We can summarize that according to empirical observations enforced clonality exists and has ecological importance. However, the evolutionary importance of enforced clonality would depend on whether, and to what extent, it confers a fitness advantage over alternative regeneration modes. Even though there are cases when enforced clonality is the only one way to regenerate and thus its advantage is not questionable (e.g., cultivated varieties of seedless crops, Roberts-Nkrumah, 2006, or naturally in *Armoracia rusticana* in Central Europe, Sampliner and Miller, 2009), after disturbance plants more generally regenerate from seed banks. Thus, to identify the evolutionary significance of enforced clonality it is necessary to determine whether there are situations in which ramets generated by enforced clonality have higher fitness than plants that emerge from the seed bank.

The regeneration of plant fragments is affected by external and internal factors as in any other plant vegetatively regenerating after injury. In addition to the ability to form missing tissues, to regenerate, a fragment must also have sufficient storage carbohydrates to provide energy and carbon for body renewal. Because carbohydrate storage fluctuates with phenology and ontogeny (Sosnová and Klimešová, 2009; Kaur et al., 2012; Bazot et al., 2013). Especially in short-lived monocarpic species, carbohydrate storage can negatively affect the success of vegetative regeneration since stored reserves are exhausted by generative reproduction and vegetative regeneration is thus limited (Klimešová et al., 2007; Martínková et al., 2008; Tolsma et al., 2010). Similarly, the ontogenetic phase of the mother plant at the time of fragmentation may affect the fitness of resulting ramets since regrowth of newly established ramets is probably stored reserves dependent. Nutrient availability and depth in the soil are other factors influencing fragment regeneration outcomes (Dietz et al., 2002; Li et al., 2013; Thomsen et al., 2013).

Regeneration from the seed bank after disturbance have also some limitations. Seed bank is not necessarily formed as in the case of species not forming seed bank (Fenner, 1995) bud also species have no seed bank at the new locality during colonization or invasion process (Gioria et al., 2012) or due to recurrent disturbance (Noble and Slatyer, 1980), low level of resources or lack of signals for flower initiation due to climatic conditions hindering successful seed production (Simpson et al., 1999). In the case when seed bank is formed, seeds can germinate only when conditions are suitable and signals for germination triggering are present (Bewley, 1997; Baskin and Baskin, 1998). Therefore, ecological and evolutionary significance of enforced clonality is related to seed bank and habitat attributes, but fundamentally to presence or absence of the seed bank.

To investigate whether enforced clonality confers an evolutionary advantage on injured individuals via increased fitness and to explore how fitness of enforced ramets is affected by ontogeny of the mother plant at the time of fragmentation, we established a 5-year garden experiment with *Barbarea vulgaris* and *Barbarea stricta*, two short-lived species with potential for enforced clonality. Whole-life seed production, whole-life viable seed production, ratio of viable seeds to all seeds and annual immediate reproduction served as a proxy of fitness. The experiment allowed us to address the following specific questions: (i) whether the fitness of enforced ramets is higher than the fitness of plants that regenerated from the seed bank at the time of disturbance; (ii) whether the fitness of enforced ramets is higher than the fitness of an unfragmented plant; and (iii) whether fitness of enforced ramets decreases with the ontogenetic phase of the mother plant at the time of fragmentation, i.e., decreases during generative reproduction of the mother plant at the time of fragmentation.

MATERIALS AND METHODS

Study Species

B. vulgaris R. Br. and *B. stricta* ANDRZ. (Brassicaceae) are common European species. *B. vulgaris* occupy man-made, ruderal habitats (e.g., arable land, urban habitats, roadside ditches) that are subjected to frequent, severe anthropogenic disturbance, whereas *B. stricta* occurs in more natural habitats (i.e., pond banks, river alluvia, (Dvořák, 1992), that experience naturally occurring disturbance). Both are short-lived herbs

typically reproducing once in their lifetime and behaving as biennials, but in certain conditions reproducing repeatedly and behaving as short-lived perennials (MacDonald and Cavers, 1991; Dvořák, 1992; Martínková et al., 2016). During the first year of life, these plants remain vegetative, with rosettes overwintering to the next growing season, when they form leafy flowering stalks. Both species are usually non-clonal, but enforced clonality has been reported from them (Martínková et al., 2008, 2016). In particular, after fragmentation of the root system, they are able to form adventitious buds on roots, successfully regrow and finish the reproductive cycle. *B. vulgaris* regenerates from roots more vigorously than *B. stricta* (Martínková et al., 2016). Both species form persistent seed banks and are able to germinate throughout the year (Hintikka, 1988; Baskin and Baskin, 1989; Martínková, pers. obs.).

Experiment

Seeds for the experiment were collected during the year 2003 from South-Bohemian natural populations (15 populations for *B. vulgaris* and three for *B. stricta*). To minimize any effects of seed origin, for each of these species, the seed from all populations was mixed. During the winter, seeds were kept in dark, dry storage at room temperature.

Mother Plants

In the spring of 2004, for each species, hundred 2.5 l containers were filled with a garden substrate-sand 2:3 mixture and five seeds were sown per container. The containers were placed outdoors in a random design in the experimental garden of the Institute of Botany in Třeboň, Czech Republic. One week after seedlings emergence, the number of seedlings was reduced to only one per container. Containers were then randomly assigned to four groups. Of these, three groups were set up to simulate establishment of enforced ramets originating from different ontogenetic phases of mother plants, number of replicates was 20 plants per group. In the first group, mothers were subjected to fragmentation during the first-year rosette phase (R1). The second group represented mothers subjected to fragmentation during the second-year rosette phase (R2). The third group represented mothers subjected to fragmentation during the reproductive phase (REP), number of replicates was 30. The fourth group comprised unfragmented plants (NO INJURY) and served to represent the scenario without disturbance. During the whole cultivation of all four groups, plants were regularly fertilized with NPK commercial solution without hormone addition and watered when necessary.

Enforced Ramets—Root Fragmentation

Fragments were cut once in 2004 (fragmentation of R1 group) and twice in 2005 (fragmentation of R2 and REP groups), thus yielding three groups of enforced ramets: FRG R1, FRG R2, and FRG REP (**Figure 1**, **Table 1**). Each maternal plant served as the source of two root fragments, since we simulated a scenario in which severe disturbance establishes only two vegetative offspring from one mother to set the lowest level of possible multiplication. These two root fragments were each 6 cm long but differed in diameter and position in the root system. Six centimeters fragments were found to be able to successfully regenerate in both species (Martínková et al., 2016). Thus, the first fragment would be cut from the main root, specifically from the topmost part of the root directly under the hypocotyl. The second fragment would be cut from the first lateral root, directly behind its branching from the main root. Immediately after cutting, each fragment was placed horizontally into a 2.5 l container filled with a substrate:sand 2:3 mixture. Containers were placed in the experimental garden in a random design, regularly fertilized with NPK commercial solution without hormone addition and watered when necessary.

Simulation of Establishment from the Seed Bank

At the same time that enforced ramets were obtained, once in 2004 and twice in 2005, we did "parallel sowing," i.e., sowing seeds at the same time that the fragmentation was done to the mothers of enforced ramets. This was done to simulate the situation in which regeneration after disturbance is possible only from the seed bank (**Figure 1**, **Table 1**), and would allow for comparison of plants that originated from mother plant fragmentation at various ontogenetic stages with plants that originated from seed approximately contemporaneously. Five seeds were sowed per 2.5 l container for each species separately in 20 replicates. One week after seedling emergence, the number of seedlings was reduced to only one per container. This yielded three groups of plants that originated from seeds: S R1, S R2, and S REP (**Figure 1**, **Table 1**). Containers were placed outdoors in the experimental garden, set in a random design, fertilized, and watered.

Seed Collection and Assessment of Fitness Variables

Seed collection and germination testing were done on all groups of plants every year of the experiment except the first, because both *Barbarea* species start reproducing in the second year of their lives. All seeds from each plant were trapped by wrapping reproducing plants in light white cloth when all their flowers had terminated flowering. The weight of all trapped seeds for each reproducing plant was evaluated after seed maturation. The average weight of one seed and the total number of seeds per plant were calculated from the weight of all trapped seeds and from the average weight of 30 seeds assessed from three replicates.

Germination tests were done every autumn following the reproductive season in standardized conditions of a chamber that followed a regime of 15 vs. 8 h and 23°C vs. 15°C for "day" vs. "night," respectively. For each reproducing plant, 30 seeds in three replicates were placed on wet sand in Petri dishes. Over the course of the next 21 days, the number of germinated seeds would be recorded.

During in May, 2009, the experiment was terminated because the majority of plants had died out and the rest were so weak that high probability of death without reproduction was obvious.

For all plants, the following characteristics as a proxy of fitness were recorded: whole-life seed production (the sum of all seeds), whole-life viable seed production, ratio of number of viable to all seeds (VIABLE/ALL SEEDS) and annul immediate reproduction (ANNUAL REP). The numbers of all seeds and viable seeds were calculated as respective sums of the numbers of seeds produced by both enforced ramets of each mother plant. Annual

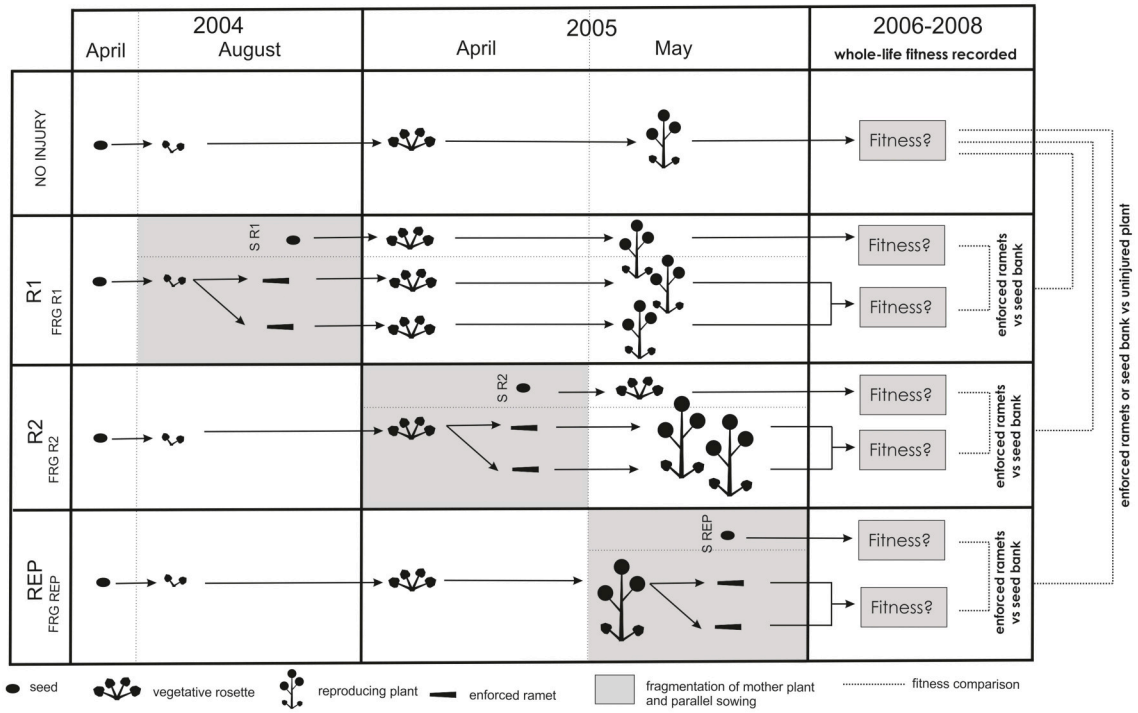

FIGURE 1 | Time and fitness comparison diagram of the experiment on *Barbarea vulgaris* and *Barbarea stricta*. Enforced ramets were established from mother plants in three ontogenetic stages. Parallel sowings were done at the same time as fragmentations of mother plants, to simulate regeneration from the seed bank after disturbance. FRG, enforced ramets; S, seed bank—parallel sowing; R1, seeds sowed or roots of mother plant fragmented in first-year rosette phase; R2, seeds sowed or roots of mother plant fragmented in second-year rosette phase; REP, seeds sowed or roots of mother plant fragmented in reproductive phase. NO INJURY, control unfragmented plants. Full descriptions of regeneration types and ontogenetic stages (and their abbreviations) are in **Table 1**.

immediate reproduction was calculated as an annual average, by first multiplying the mean number of viable seeds for each year and by that year's average seed weight, and next dividing this total by the number of reproductive years. VIABLE/ALL SEEDS and ANNUAL REP were both calculated as means of the both enforced ramets from each mother plant.

Statistical Analysis

As our data distributions do not fulfill the assumptions of traditional ANOVA (e.g., many of the plants do not produce any seed at all) we used analogous permutation tests in the program PERMANOVA+ for PRIMER (Anderson et al., 2008). Using these permutation tests, the pseudo-F ratio was calculated in a manner similar to the F ratio in traditional methods, but does not correspond to Fisher's F distribution, and the appropriate distribution which would be generated by a true null hypothesis is obtained by the permutation procedure (Anderson et al., 2008). The number of all seeds, the number of viable seeds, the ratio of viable/all seeds and annual immediate reproduction were treated as dependent variables in the analyses. Species affiliation, regeneration type, and ontogenetic phase of the mother plant were fixed factors. All tests were done for both species together in order to find general patterns between dependent variables and factors and were also done for each species separately to identify effects of regeneration type and ontogenic stage within individual species. We also performed permutation pairwise comparisons, which correspond to parametric *t*-tests (Anderson et al., 2008), to compare fitness characteristics among enforced ramets, parallel sowings, and uninjured plants.

RESULTS

Number of All Seeds

Whole-life seed production was significantly influenced by all tested factors and their interactions (**Table 2**). When looking at the species separately, in *B. vulgaris*, whole-life seed production of enforced ramets was higher than seed production of plants from parallel sowings if fragmentation occurred during the vegetative phase of mother plants, regardless of whether it was in the first or the second year of the plant's life (**Table 3**, **Figure 2A**). When comparing enforced ramets and uninjured plants, whole-life seed production of enforced ramets was higher when fragmentation had occurred during the first year of the mother's life. Whole-life seed production of plants from the seed bank was lower than seed production of uninjured plants (**Table 3**, **Figure 2A**).

In *B. stricta*, whole-life seed production of enforced ramets was higher than seed production of plants from the seed bank if fragmentation had occurred during the second-year vegetative phase of the mother plants (**Table 3**, **Figure 2A**). Seed production of enforced ramets and plants from the simulated seed bank

TABLE 1 | Overview of procedures used in experiment on *Barbarea vulgaris* and *Barbarea stricta* to yield different regeneration types as well as offspring from different mother plant ontogenetic stages.

Plant group	Fragmentation or sowing	Description
Mother plants	**Sowing**	**Plants serving as source of enforced ramets**
R1	2nd of April 2004	Mothers, subjected to fragmentation in first-year rosette phase, of enforced ramets
R2	2nd of April 2004	Mothers, subjected to fragmentation in second-year rosette phase, of enforced ramets
REP	2nd of April 2004	Mothers, subjected to fragmentation in reproductive phase, of enforced ramets
Enforced ramets	**Fragmentation**	**Plants simulating enforced clonality**
FRG R1	18th of August 2004	Enforced ramets established from R1 mothers
FRG R2	6th of April 2005	Enforced ramets established from R1 mothers
FRG REP	20th of May 2005	Enforced ramets established from REP mothers
Seed bank	**Sowing**	**Plants simulating regeneration after disturbance from the seed bank**
S R1	18th of August 2004	Plants originating from seeds sowed concurrently with FRG R1
S R2	6th of April 2005	Plants originating from seeds sowed concurrently with FRG R2
S REP	20th of May 2005	Plants originated ing from seeds sowed concurrently with FRG REP
Control	**Sowing**	**Plants simulating conditions of no disturbance**
NO INJURY	2nd of April 2004	Plants of mother generation not subjected to fragmentation

were in most cases lower than seed production of control unfragmented plants (**Table 3**, **Figure 2A**).

Number of Viable Seeds

If only viable seeds are taken into account, whole-life seed production was significantly influenced by the tested factors and their interactions (**Table 2**), similarly to production of all seeds. In *B. vulgaris*, seed production of enforced ramets was higher than seed production of plants from the seed bank if fragmentation had occurred during the first year of the mother's life. The same relationship is found when comparing seed production of enforced ramets and uninjured plants (**Table 3**, **Figure 2B**).

In *B. stricta,* seed production of enforced ramets was equal to control plants and also to plants from the simulated seed bank in most cases (**Table 3**, **Figure 2B**). Seed production of plants from the seed bank was significantly higher than seed production of uninjured plants if fragmentation had occurred during the first year of the mother's life (**Table 3**, **Figure 2B**).

Ratio Viable/All Seeds

In *B. vulgaris*, the ratio of viable seeds to all seeds did not differed among regeneration types or ontogenetic stages (**Table 3**, **Figure 2C**). On the other hand, in *B. stricta*, the ratio was significantly higher in plants from the seed bank than in enforced ramets and also, in the case of fragmentation during the first year of the mother's life, than in uninjured plants (**Table 3**, **Figure 2C**). In enforced ramets, the ratio decreased with the ontogeny of the mother plant (**Figure 2C**).

Annual Immediate Reproduction

Annual immediate reproduction was significantly influenced by the tested factors and their interactions in both species (**Table 2**). In *B. vulgaris*, reproduction of enforced ramets and plants from the seed bank did not differ (**Table 3**, **Figure 2D**), but decreased if fragmentation occurred during the second year of the mother's life.

In *B. stricta*, annual immediate reproduction was lower in enforced ramets than reproduction of plants from the seed bank and did not differ from reproduction of uninjured plants. In enforced ramets, annual immediate reproduction decreased if fragmentation had occurred during the reproductive phase of the mother plant (**Table 3**, **Figure 2D**). Plants from parallel sowing showed the highest annual immediate reproduction in the treatment that simulated their germination from the seed bank during the first year of the mother's life (**Figure 2D**).

DISCUSSION

In *B. vulgaris,* enforced ramets showed higher fitness than uninjured plants if their mothers were fragmented early in their ontogeny, and also higher fitness than plants originating in the seed bank. In *B. stricta,* enforced ramet fitness was lower than or similar to fitness of unfragmented plants and plants grown from seed. Fragmentation of the plant body therefore showed adaptive value, however the importance of enforced clonality reflected the individual species' habitat preferences. In particular, although the habitat of *B. stricta* does experience natural disturbances, it is not as frequently or severely disturbed as the man-made habitat of *B. vulgaris*, with the latter species therefore experiencing root system fragmentation more often. Our finding of increase in fitness by body fragmentation supports the idea of enforced clonality as a significant strategy in disturbed habitats.

Enforced Ramets

In our study, we used two enforced ramets from each mother to compare their fitness with that of one unfragmented individual or of one plant established from the seed bank. This setup affected our results because disturbance may lead to an even higher degree of fragmentation or, on the other hand, results only in injury to the plant body without causing fragmentation. Simple removal of aboveground biomass does not increase fitness of *B. vulgaris* (Martínková et al., 2016) in comparison with unfragmented plants; however, as we have shown here, formation of two enforced ramets of root origin significantly increases fitness. It is obvious that the advantage of enforced

TABLE 2 | Summary of fitted models for fitness characteristics obtained from experiment on *Barbarea vulgaris* and *Barbarea stricta*.

B. vulgaris + *B stricta*	*d.f.*	F_{ps}	*p*	*B. vulgaris*	*d.f.*	F_{ps}	*p*	*B. stricta*	*d.f.*	F_{ps}	*p*
A. NUMBER OF ALL SEEDS											
Species (SP)	1	69.51	***	Regeneration type (REG)	1	31.86	***	Regeneration type (REG)	1	0.42	n.s.
Regeneration type (REG)	1	25.64	***	Ontogenic stage (O)	3	8.08	***	Ontogenic stage (O)	3	13.12	***
Ontogenic stage (O)	3	17.84	***	REGxO	2	3.39	*	REGxO	2	5.52	**
SPxREG	1	19.00	***								
SPxO	3	1.03	n.s.								
REGxO	2	3.99	*								
SPxREGxO	2	3.99	*								
B. NUMBER OF VIABLE SEEDS											
Species (SP)	1	131.19	***	Regeneration type (REG)	1	22.44	***	Regeneration type (REG)	1	1.87	n.s.
Regeneration type (REG)	1	12.48	***	Ontogenic stage (O)	3	11.27	***	Ontogenic stage (O)	3	5.17	***
Ontogenic stage (O)	3	10.76	***	REGxO	2	6.48	**	REGxO	2	2.70	n.s.
SPxREG	1	23.04	***								
SPxO	3	8.67	***								
REGxO	2	2.58	n.s.								
SPxREGxO	2	8.62	***								
C. RATIO VIABLE/ALL SEEDS											
Species (SP)	1	434.64	***	Regeneration type (REG)	1	2.78	n.s.	Regeneration type (REG)	1	40.845	***
Regeneration type (REG)	1	9.79	**	Ontogenic stage (O)	3	0.87	n.s.	Ontogenic stage (O)	3	12.115	***
Ontogenic stage (O)	3	7.34	***	REGxO	2	0.36	n.s.	REGxO	2	0.8219	n.s.
SPxREG	1	31.01	***								
SPxO	3	5.02	**								
REGxO	2	0.78	n.s.								
SPxREGxO	2	0.36	n.s.								
D. ANNUAL IMMEDIATE REPRODUCTION											
Species (SP)	1	166.40	***	Regeneration type (REG)	1	0.27	n.s.	Regeneration type (REG)	1	54.63	***
Regeneration type (REG)	1	10.77	***	Ontogenic stage (O)	3	11.61	***	Ontogenic stage (O)	3	29.95	***
Ontogenic stage (O)	3	21.81	***	REGxO	2	1.84	n.s.	REGxO	2	26.89	***
SPxREG	1	5.41	*								
SPxO	3	6.38	***								
REGxO	2	9.56	***								
SPxREGxO	2	1.28	n.s.								

In the first column, both species are tested together; in the second and third columns, B. vulgaris and B. stricta, respectively, are tested separately. Effects of regeneration type—enforced ramets, seed bank, and control, as well as ontogenetic stage and species were tested on the following characteristics: ***(A)*** *Number of all seeds* ***(B)*** *Number of viable seeds* ***(C)*** *Ratio viable/all seeds* ***(D)*** *Annual immediate reproduction. Degrees of freedom (d.f.), pseudo-F values (F_{ps}) and significance range are shown. *p < 0.05; **p < 0.01, ***p < 0.00, n.s.—non-significant. Error d.f.: full model with both species = 296; partial model B. vulgaris = 151, partial model B. stricta = 145.*

clonality is thus related to the type and also the severity of the particular disturbance. Thus, to maximize the gain from enforced clonality it is necessary to encounter appropriate disturbance severity. Indeed, if disturbance results in smaller fragments, their successful establishment and fitness may be reduced as it is dependent on stored reserves (Leakey et al., 1977; Klimešová and Klimeš, 2007). Species possessing enforced clonality are therefore probably preferring certain disturbance regime in order to encounter disturbance severe enough to increase their fitness but not too severe to kill them.

Higher fitness of enforced ramets is caused by removal of apical dominance during fragmentation, leading to production of numerous flowering shoots on fragments (see also Martínková et al., 2016). However, these shoots are usually shorter and less branched than uninjured shoots (Bartušková and Klimešová, 2010). Even though disturbance usually also severely affects competitors, if disturbance injures only some individuals within a community, their lower height and smaller root system might disadvantage them in competition. Unfortunately, competition was not simulated in our experiment. Nevertheless, the effect of competition on enforced clonality is not probably so strong since enforced clonality is disturbance-dependent and disturbance results in reduced competitive pressure (Wilson and Tilman, 1993). Another possible problem of enforced ramets may be delayed ontogeny and thus the inability to successfully set seeds during the year of fragmentation and postponement of reproduction to subsequent ones (Huhta et al., 2009; Piippo et al., 2009; Martínková et al., 2016). However, postponement of reproduction seems disadvantageous only when we consider situation in which not all individuals are fragmented, since

TABLE 3 | Results of pair-wise tests of fitness characteristics for individual regeneration types and ontogenic stages: (A) Number of all seeds; (B) Number of viable seeds; (C) Ratio viable/all seeds; and (D) Annual immediate reproduction *B. vulgaris* and *B. stricta*, were tested separately.

	B. vulgaris		*B. stricta*	
	t_{ps}	*p*	t_{ps}	*p*
A. NUMBER OF ALL SEEDS				
FRG R1 vs. S R1	5.20	***	1.57	n.s.
FRG R2 vs. S R2	3.21	***	2.17	*
FRG REP vs. S REP	1.87	n.s.	1.84	n.s.
FRG R1 vs. NO INJURY	3.37	**	2.08	*
FRG R2 vs. NO INJURY	0.54	n.s.	0.48	n.s.
FRG REP vs. NO INJURY	0.85	n.s.	6.14	***
S R1 vs. NO INJURY	1.82	n.s.	0.51	n.s.
S R2 vs. NO INJURY	3.45	***	4.60	***
S REP vs. NO INJURY	2.40	*	5.01	***
B. NUMBER OF VIABLE SEEDS				
FRG R1 vs. S R1	4.79	***	1.96	n.s.
FRG R2 vs. S R2	1.16	n.s.	0.74	n.s.
FRG REP vs. S REP	1.81	n.s.	1.94	n.s.
FRG R1 vs. NO INJURY	4.07	***	1.30	n.s.
FRG R2 vs. NO INJURY	2.13	*	1.28	n.s.
FRG REP vs. NO INJURY	0.19	n.s.	2.37	*
S R1 vs. NO INJURY	1.63	n.s.	3.51	***
S R2 vs. NO INJURY	3.07	**	0.50	n.s.
S REP vs. NO INJURY	1.90	n.s.	0.05	n.s.
C. RATIO VIABLE/ALL SEEDS				
FRG R1 vs. S R1	0.36	n.s.	4.76	***
FRG R2 vs. S R2	1.38	n.s.	3.31	**
FRG REP vs. S REP	0.95	n.s.	2.92	*
FRG R1 vs. NO INJURY	1.17	n.s.	0.88	n.s.
FRG R2 vs. NO INJURY	0.74	n.s.	3.08	**
FRG REP vs. NO INJURY	0.12	n.s.	3.78	***
S R1 vs. NO INJURY	0.57	n.s.	5.37	***
S R2 vs. NO INJURY	1.12	n.s.	0.41	n.s.
S REP vs. NO INJURY	1.23	n.s.	0.87	n.s.
D. ANNUAL IMMEDIATE REPRODUCTION				
FRG R1 vs. S R1	1.66	n.s.	6.79	***
FRG R2 vs. S R2	1.14	n.s.	0.84	n.s.
FRG REP vs. S REP	0.67	n.s.	2.80	*
FRG R1 vs. NO INJURY	0.24	n.s.	0.16	n.s.
FRG R2 vs. NO INJURY	3.03	**	0.84	n.s.
FRG REP vs. NO INJURY	3.45	***	3.20	***
S R1 vs. NO INJURY	1.01	n.s.	6.16	***
S R2 vs. NO INJURY	2.29	*	0.10	n.s.
S REP vs. NO INJURY	2.16	*	0.44	n.s.

*Degrees of freedom (d.f.), pseudo t-test values (t_{ps}) and significance range are shown. *$p < 0.05$; **$p < 0.01$, ***$p < 0.00$, n.s.—non-significant. FRG, enforced ramets; S, seed bank; R1, seeds sowed or roots of mother plant fragmented in first-year rosette phase; R2, seeds sowed or roots of mother plant fragmented in second-year rosette phase. REP, seeds sowed or roots of mother plant fragmented in reproductive phase. NO INJURY, control unfragmented plants. Full descriptions of regeneration types and ontogenetic phases (and their abbreviations) are in* ***Table 1****.*

the relevant comparison is between enforced ramets and unfragmented plants. Unfragmented plants set seeds in current year while enforced ramets a year later. Enforced ramets would face a disadvantage if recurrent disturbance comes before they finish reproductive cycle as unfragmented plants have already finished theirs. If all individuals are fragmented and enforced ramets are compared with plants established from seeds, they have an advantage due to the higher amount of stored reserves for faster regrowth, and postponement of their reproduction does not play a role as ramets reproduce at the same time as the plants established from seeds. Based on consideration of these scenarios, we can see that significance of enforced clonality is probably higher in situations in which disturbance fragmentizes all rather than only some individuals within a community.

Besides fitness increase, another advantage of enforced clonality is the deceleration of senescence. Enforced ramets of *B. vulgaris* survived 1 year longer than uninjured plants (Martínková et al., 2016). Indeed, enforced clonality is able to rejuvenate plants by resetting the aging clock in *B. vulgaris* (Martínková et al., 2016). Moreover, even though the number of reproductive events was the same due to postponement of reproduction in enforced ramets, whole-life seed production was higher in enforced ramets than in unfragmented plants. Since both *Barbarea* species start to reproduce during the second year of life and they behaved as polycarpic species (Martínková et al., 2016), advantage of enforced clonality may be more pronounced in annuals. In annual species with enforced clonality such as *Rorippa palustris* (Klimešová et al., 2008), injury-induced prolongation of the vegetative phase from 1 to 2 years could lead to a higher amount of stored reserves for generative reproduction in comparison to uninjured plants which germinate, reproduce and die within 1 year. Thus, the significance of enforced clonality probably varies among life-history strategies.

Seed Bank

In our experiment, disturbance timing was designed in relation to the ontogeny of maternal plants, and this could result in less-than- ideal timing for germination from the seed bank. Although seeds of the two study species are able to germinate during the whole year, the usual time for seedling establishment is the spring (MacDonald and Cavers, 1991), so that plants attain maximal size in the first growing season and are thus well prepared for flowering the next year. The signal for flowering in these species is low temperature during winter, not size, which plays this role in other short-lived plants; additionally, the length of the first growing season is an important influence on the seed production (Collins, 1981; Galen and Stanton, 1991). Therefore, it is not surprising that in our experiment plants from the seedbank frequently had lower fitness than both enforced ramets and uninjured plants, since fragmentation and simulation of establishment from the seed bank were not done exactly at the beginning of growing season. The timing of disturbance and hence the timing of germination affects fitness output. Nevertheless, disturbance is unpredictable, especially those caused by humans, which could occur at any time during the year. Thus, the design of our experiment actually reflects

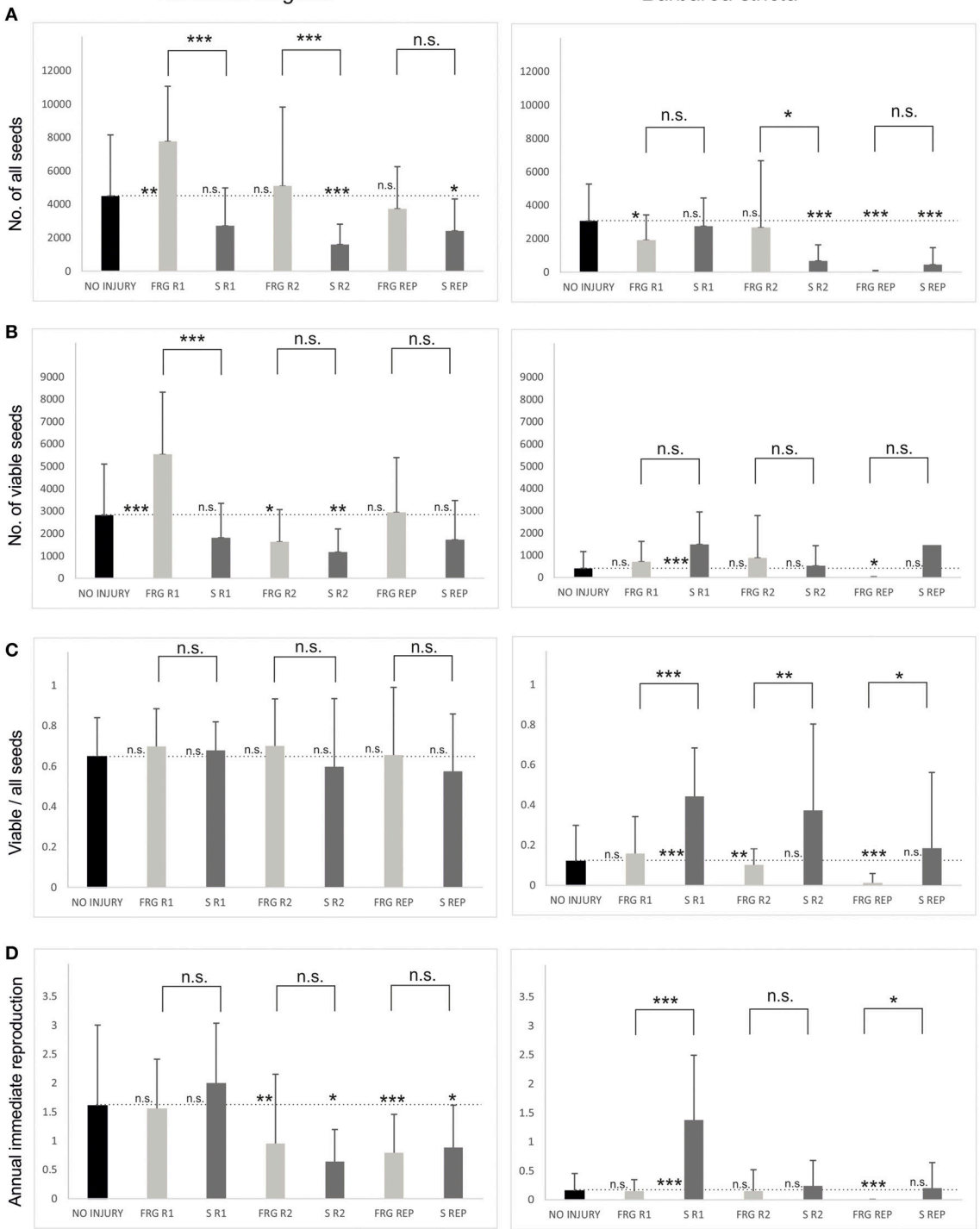

FIGURE 2 | Box diagrams of fitness characteristics obtained from experiment on *Barbarea vulgaris* and *Barbarea stricta* for individual regeneration types and ontogenetic stages in which the disturbance was inflicted. (A) Number of all seeds; **(B)** Number of viable seeds; **(C)** Ratio viable/all seeds; **(D)** Annual immediate reproduction. FRG, enforced ramets; S, seed bank; R1, seeds sowed or roots of mother plant fragmented in first-year rosette phase; R2, seeds sowed or roots of mother plant fragmented in second-year rosette phase. REP, seeds sowed or roots of mother plant fragmented in reproductive phase. NO INJURY, control plants. Full descriptions of regeneration types and ontogenetic stages (and their abbreviations) are in **Table 1**. Means and 95% confidence intervals are plotted and significance range of pair-wise tests are shown. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.00$, n.s.—non-significant.

reality, and suggests that indeed enforced clonality is a useful strategy in highly unpredictably disturbed habitats.

Advantage of Enforced Clonality in Establishing New Populations

Enforced clonality as a regenerative mode has a clear advantage in the situation in which disturbance hits a population that has not yet formed a seed bank. Furthermore, enforced clonality is especially important for short-lived otherwise non-clonal plants establishing as pioneer species on a new substrate, on places where vegetation was destroyed or during invasive or other colonization processes. Pioneer species can have quite small populations, and enforced clonality can greatly reduce their vulnerability to disturbances that would otherwise wipe them out. Overcoming such bottlenecks may in fact be the reason for retaining enforced clonality even though it does not seem to be so advantageous compared to regeneration from the seed bank in some situations. This may hold true for *B. stricta*, which occurs on natural habitats where experiencing body fragmentation is much less probable than on the man-made disturbed habitats inhabited by *B. vulgaris* (Dvořák, 1992). Since buds for regrowth after fragmentation are either formed adventitiously on roots only after injury or are a standard part of plant ontogeny, enforced clonality does not incur any costs for species. Therefore, once they attain the ability of resprouting it can be further kept without expense.

Another important aspect of enforced clonality is the ability of short-lived, non-clonal plants to survive disturbance when germination from the seed bank is not possible or is less successful due to unfavorable germination conditions (Bewley, 1997). More generally, enforced clonality could serve as insurance for species with problematic germination. Furthermore, enforced clonality may also have ecological effects even when enforced ramets are not capable of setting seeds (e.g., due to lack of mates or pollinating vectors or to insufficient growing season length) as these ramets can still play important roles such as competing for resources and serving as a source of litter (e.g., *Reynoutria* taxa, Bímová et al., 2003).

In conclusion, enforced clonality can increase the fitness of some short-lived species and thus bring a life history advantage. It is advantageous in habitats where a seed bank has not yet been formed. Our results imply that enforced clonality should be taken into account when studying population dynamics and life strategies of short-lived species from disturbed habitats.

AUTHOR CONTRIBUTIONS

JM as a first author substantially contributed to the conception and design of the work. She was responsible for the experiment, analyzed data, interpreted results and wrote the manuscript. JK substantially contributed to the conception and design of the work and also to interpretation of data. She critically revised work and did final approval of the manuscript.

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Live substrate positively affects root growth and stolon direction in the woodland strawberry, *Fragaria vesca*

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Studies of clonal plant foraging generally focus on growth responses to patch quality once rooted. Here we explore the possibility of true plant foraging; the ability to detect and respond to patch resource status **prior to rooting**. Two greenhouse experiments were conducted to investigate the morphological changes that occur when individual daughter ramets of *Fragaria vesca* (woodland strawberry) were exposed to air above live (non-sterilized) or dead (sterilized) substrates. Contact between daughter ramets and substrate was prohibited. Daughter ramet root biomass was significantly larger over live versus dead substrate. Root:shoot ratio also increased over live substrate, a morphological response we interpret as indicative of active nutrient foraging. Daughter ramet root biomass was positively correlated with mother ramet size over live but not dead substrate. Given the choice between a live versus a dead substrate, primary stolons extended preferentially toward live substrates. We conclude that exposure to live substrate drives positive nutrient foraging responses in *F. vesca*. We propose that volatiles emitted from the substrates might be effecting the morphological changes that occur during true nutrient foraging.

Keywords: plant nutrient foraging, clonal plants, woodland strawberry, *Fragaria vesca*, root biomass, stolon trajectory

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Introduction

Optimal foraging theory (OFT) proposes that organisms forage for nutrients in a way that maximizes energy intake per unit time (MacArthur and Pianka, 1966; Charnov, 1976; Norberg, 1977; Oaten, 1977). Resources often occur in patches within an environment and the theory predicts that there is an optimum pattern of visitation that provides an organism with maximum benefits for minimum output of energy. Application of the theory requires two conditions: (1) that individuals can move through and explore an environment and (2) that individuals can distinguish between and respond to patches of varying quality. The theory includes factors regarding both within-patch ("exploitation", i.e., how long to remain, how to efficiently capture resources) and between-patch ("true foraging", i.e., patterns of locating resources, time spent searching) behavior (Charnov, 1976; Oaten, 1977). Optimal adjustment of these factors results in an increased uptake of energy, and thus improves fitness.

Optimal foraging theory was originally used as a means of understanding and predicting animal behavior. It posited that animals adjust both foraging time and patch visitation order to maximize energy acquisition (MacArthur and Pianka, 1966; Charnov, 1976; Pyke et al., 1977; Pyke, 1984; McNamara et al., 2006). However, complex thought in animals involving predation risk

(Brown, 1988; Higginson et al., 2012), food choice (Houston et al., 2011; Cressman et al., 2014), and memory (Freidin and Kacelnik, 2011) confounds the theory (Perry and Pianka, 1997).

The theory has also been applied to clonal plants (Slade and Hutchings, 1987; Birch and Hutchings, 1994; Cain, 1994; de Kroon and Hutchings, 1995; Grime and Mackey, 2002). Clonal plants are unique in the plant world for the ability of genets (aggregates of plants that are the products of a single seed) to change their location over time, and therefore explore and effectively exploit a heterogeneous environment for light and nutrients. They do this through the production of ramets, or potentially physiologically independent genetically identical units (**Figure 1**). Compared to most animals, plant movement is slow; it occurs via growth processes and benefits accrue due to maintenance of connections between sister ramets. As new ramets are produced and extend into the environment, older ramets die, essentially moving the genet through space. Ramets remain connected via stolons or rhizomes for variable lengths of time (Jónsdóttir and Watson, 1997) and these connections allow for transport of nutrients and hormones between the mother and daughter ramets (Alpert and Mooney, 1986; Jónsdóttir and Watson, 1997; Hutchings, 1999). Thus, clonal plants fulfill the first requirement for application of OFT through their ability to move. But, can they do this in a selective way? Can they distinguish between and respond to patches of differing quality in a heterogeneous environment?

For plants to forage for light or nutrients, they must be able to sense, interpret, and respond to environmental signals that specify habitat quality in a way that results in the non-random placement of individual ramets in appropriate patches. Thus, clonal plant foraging can be said to occur if placement of daughter ramets occurs more frequently in high quality than in low quality patches (Cain, 1994). In stoloniferous plants (those with above-ground connections between ramets), the means of sensing and responding to light patches in a heterogeneous environment has been well studied (Slade and Hutchings, 1987; Methy et al., 1990; Kemball et al., 1992; Dong, 1993, 1995; Dong and Pierdominici, 1995; Stoll and Schmid, 1998; Grime and Mackey, 2002; Lepik et al., 2004; Dauzat et al., 2008). Detection of differences in red/far-red ratios via phytochromes and other photoreceptors induces plant morphological responses such as enhanced elongation rates or increased leaf area in response to low photosynthetically active radiation (PAR; Ballare et al., 1997; Smith, 2000; Franklin and Quail, 2010). These responses assist daughter ramets in locating and then occupying high light patches. Similarly, plants utilize red/far-red radiation ratios to assess areas of high vs. low density (i.e., neighbor sensing) (Schmitt and Wulff, 1993; Ballare, 1999; Marcuvitz and Turkington, 2000; Smith, 2000; Franklin and Quail, 2010). The majority of studies focused on morphological changes in leaf area and shoot biomass to differing light conditions, while others suggest that plasticity of spacers in length and branching intensity play a more critical role in light foraging, particularly in keeping ramets in light-rich patches (Kemball et al., 1992; Oborny, 1994; de Kroon and Hutchings, 1995; Dong, 1995; Dauzat et al., 2008).

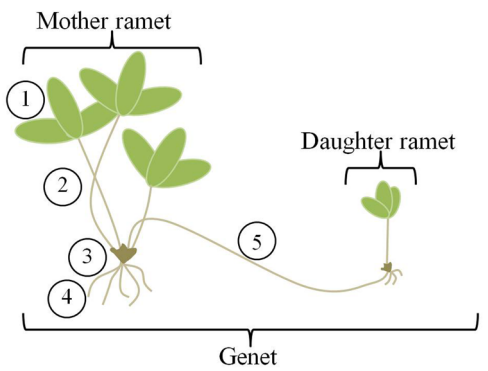

FIGURE 1 | Clone morphology. Strawberry genets are made up of mother and daughter ramets connected by aboveground stolons. Individual mother and daughter ramets consist of five main organs (1) leaves, (2) petioles, (3) crown or stem, (4) roots, and (5) stolons. For analysis, we have combined the petioles and crown, labeling them "shoots".

Far less is known about the capacity of plants to detect nutrient-rich environments. Early studies focused on the proliferation of roots **after** plant establishment ("exploitation") (Birch and Hutchings, 1994; Cain, 1994; de Kroon and Hutchings, 1995; van Kleunen and Fischer, 2001). Studies found that lateral root elongation is highly responsive to the presence of nitrates (Zhang and Forde, 2000), and results in an abundance of root mass in richer patches (Leyser and Fitter, 1998; Jansen et al., 2006). Connected ramets in complementary environments increase the size of organs that obtain the most abundant resource (Stuefer et al., 1996). In a light-rich environment clonal ramets increase the mass of shoots and leaves, whereas in a nutrient-rich environment, root growth is increased. While an overall increase in biomass indicates that ramets are situated in an abundance of resources, evidence of clonal ramet foraging arises when the ratio of root:shoot biomass increases or decreases in response to an increase in nutrients or light, respectively. These morphological changes indicate a preferential allocation of resources to specific organs specialized for the capture of the abundant resource (Tuomi et al., 1983). These studies mirror those related to light in that they indicate that once plants enter a rich environment they alter their morphology in ways that enhance exploitation. Evidence of between-patch foraging – the ability of a developing stolon to distinguish between nutrient-rich or nutrient-poor patches – also exists.

Precision of foraging depends on "the ability of a species to perceive the heterogeneity and respond to it" (Wijesinghe et al., 2001) and there is evidence of this ability in many clonal plants. Salzman (1985) demonstrated that when given a choice between saline or non-saline soils, *Ambrosia psilocstachya* placed 67% of its rhizomes in non-saline soils. While it may be argued that the salinity suppressed plant growth, similar patterns of nutrient patch detection also have been found in stoloniferous plants. *Cuscuta subinclusa* exhibited coiling responses prior to physiological connection and exploitation of its host, indicating an ability to survey and interpret its surroundings and adjust development appropriately (Kelly, 1990). To date, the most striking example of patch recognition and differentiation was reported by Roiloa and Retuerto (2006). They found that

offspring ramets of *Fragaria vesca*, when given a choice of six soils of varying quality, preferentially grew into higher quality soils first. Only after these higher quality soils were colonized did the newest formed ramets colonize lower quality patches. These findings were in stark contrast to the homogeneous control, where daughter ramet placement was random. This experiment was particularly interesting because unlike *A. psilocstachya*, *F. vesca* is stoloniferous, demonstrating that clonal plants are capable of precision foraging aboveground. *While the study demonstrated that F. vesca are able to detect and respond to nutrient-rich patches, it did not investigate the morphological changes that occur when the ramet encounters a nutrient-rich patch, and raises the question: are there changes and, if so, are they consistent with optimal nutrient foraging?*

We conducted a series of experiments designed to investigate the morphological changes that occur in developing ramets ***prior to rooting*** in response to unsterilized (live) versus sterilized (dead) field substrate. Our goal was to determine whether air-borne signals are able to alter development such that newly developing ramets can be placed into favorable nutrient patches.

First, we examined local root growth and development of *F. vesca* daughter ramets exposed to live versus dead, nutrient-rich field substrates. Once a ramet roots, it can no longer move, so if true foraging is to occur, there must be air-borne clues signaling soil quality. Therefore, during the experiments, rooting into the substrate was prevented. We hypothesized that **unrooted** ramets exposed to air above live substrates would exhibit an increase in root biomass and root:shoot ratio compared to dead controls.

Because prior studies demonstrated that ramets of *F. vesca* are placed into higher quality soils first (Roiloa and Retuerto, 2006), we also developed two experiments to look at the trajectory of stolon extension when given the choice of nutrient-rich versus nutrient-poor patches. We hypothesized that there will be a positive response in the direction of growth of the extending stolon, such that it grows toward the rich substrate. Both parameters, a positive alteration of stolon direction and an increase in the root biomass and root:shoot ratio of **unrooted** ramets, would be taken as a positive indicators of nutrient foraging.

Materials and Methods

Experimental Species

Fragaria vesca, the woodland strawberry, is an herbaceous perennial native to the northern hemisphere. Growth occurs clonally via production of stolons; distribution of ramets within a colony indicates a guerrilla growth form (Angevine, 1983).

Substrate Collection

In order to determine responsiveness to airborne signals from live substrate, we collected soil and litter from a site colonized by *F. vesca* in Aurora, IN (N 39 05.225 W 084 55.663) in March and September 2012. Prior to collection, all strawberries were removed from the substrate. Leaf and stick litter was harvested and placed in plastic bags. Field soil no more than three inches deep was collected and stored in plastic bags. Any residual root mass in the soil was removed prior to storage. Soil and litter were stored in cool, dry, dark conditions until the experiment was initiated. At the beginning of each experiment, half of the collected field soil and litter was autoclaved, the other half was not.

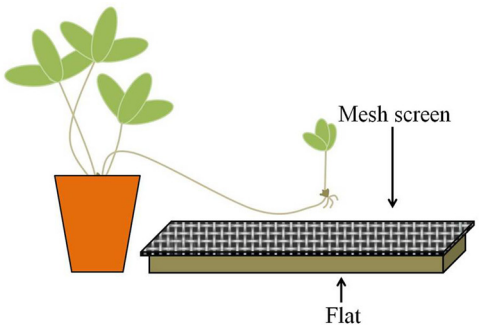

FIGURE 2 | Experimental scheme. Strawberry daughter ramets were allowed to grow over flats filled with non-sterilized (live) or sterilized (dead) substrates. Mesh screens were placed on top of the flats, approximately two centimeters above the substrate, to prevent contact between the developing ramet and the substrate.

Fragaria Vesca Propagation

Woodland strawberries were propagated from a single clone in the greenhouse at Indiana University, Bloomington, IN, USA in the spring of 2012, the fall of 2012, the fall of 2013 and again in the spring of 2014. These genetically identical ramets were individually potted in 12 cm diam. pots filled with SunGro Metro-Mix and watered daily.

Root Growth Experimental Design and Plant Data Collection

In the spring of 2012, 15 flats (27.3 cm × 54.0 cm × 6.1 cm) each were filled with 1L of field soil and covered with 0.5 L of leaf litter; an additional 15 contained dead (autoclave-sterilized) field substrate (soil plus litter). In order to prevent direct contact between developing ramets and the substrate, a sheet of aluminum screen mesh was placed over each flat; the mesh was situated approximately two centimeters above the substrate (**Figure 2**). Treatments were randomly placed along both sides of a bench in the greenhouse so that half faced east and half faced west.

Thirty potted strawberries with new daughter stolons at least 35 cm in length were randomly assigned to a treatment (live or dead) and were placed at the short edge of each flat, one per flat. The daughter stolon was directed toward and allowed to extend over the mesh-covered substrate. Developing stolons and ramets were not allowed to root into or come into direct contact with the substrate. Both the mother plants and substrate flats were watered daily with tap water and subjected to a 16-h light cycle. The identical experiment was repeated in the fall of 2012 with 80 individual ramets of the same genotype.

Primary stolon length was measured daily. All other newly emerging stolons were clipped from the mother over the

duration of the experiment. Dates of initiation of daughter ramet development and root formation were recorded. Initiation of ramet development was identified by the upward curling of the stolon tip, accompanied by leaf production. Root formation was defined by the presence of primordial root hairs extending from the base of the developing daughter ramet. Three days after root formation on the daughter ramet, the entire assemblage (mother and daughter ramet) was removed from the experiment, harvested and separated into organs; for each ramet, the stem and petioles were combined and labeled "shoot" (**Figure 1**). Leaves were scanned into tif files and Image J (U.S. National Institutes of Health, Bethesda, MD, USA) was used to measure leaf area. All plant organs, including leaves were dried at 60°C for 3 days and weighed to mean ± 1 mg.

Statistical Analysis

The statistical analyses were designed to determine if there was an effect of substrate type (live versus dead) on organs of the daughter ramet. Because previous studies indicated that the size of the mother ramet can affect growth responses (e.g., Cain, 1990), we also examined the effect of mother ramet size. Data sets were analyzed for normality based on QQ plots and Kolmogorov–Smirnov test values: for the spring 2012 data, all factors were log transformed to establish normality; for the fall 2012 data only daughter ramet aboveground dry mass, daughter ramet leaf dry mass, daughter ramet root dry mass and stolon length were log transformed, while daughter shoot dry mass, stolon dry mass and total mother dry mass were not. We performed a series of ANCOVAS on the daughter ramet leaf dry mass, daughter ramet shoot dry mass (later combined as aboveground dry mass), daughter ramet root dry mass, stolon dry mass and stolon length. Mother ramet total dry mass (maternal effect) was analyzed as a covariate for all factors. We used partial Eta squared (η^2) to estimate the effect size. All analyses were performed using SPSS (IBM Corp., Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.,).

Stolon Trajectory Experimental Design and Plant Data Collection

In the fall of 2013, forty-three flats (27.3 cm × 54.0 cm × 6.1 cm) each were filled with 1 L of live field soil and covered with 0.5 L of leaf litter (live substrate). An additional 43 flats (27.3 cm × 54.0 cm × 6.1 cm) each were filled with autoclaved play sand (Hardscapes by Quikrete). Treatments consisted of a runway made of three glass blocks (6 in. × 8 in. × 4 in., Pittsburgh Corning Premiere). Each runway was flanked by one flat filled with live substrate and one flat filled with sand; distribution of live substrate on the left versus right side was randomized (**Figure 3**). Treatments were randomly placed along both sides of a bench in the greenhouse so that approximately half faced east and half faced west. Forty-three potted strawberries with new daughter stolons at least 35 cm in length were randomly assigned to treatments and placed at the short edge of the runway, one per runway. The daughter stolon was directed toward and allowed to extend along the glass runway. Both mother plants and substrate flats were watered daily with tap water and subjected to a 16-h light cycle. The identical experiment was repeated in the spring of 2014 with the following changes: (1) there were 46 individual strawberries (23 per treatment) and (2) the sand treatment was replaced with sterilized field substrate and litter (dead substrate).

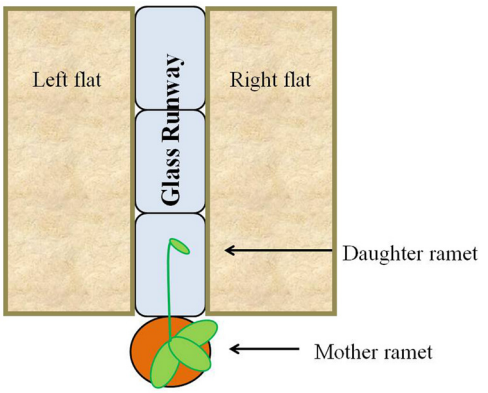

FIGURE 3 | Experimental scheme. Strawberry daughter ramets were allowed to grow over a glass runway flanked with non-sterilized (live) or sand/sterilized (dead) substrates. Distribution of live and dead substrate on the left or right side of the plant was randomized.

Primary stolon growth was monitored daily. Once a developing stolon extended beyond the edge of the glass blocks, either in the direction of the live or the dead substrate, or off the end of the runway, the date was recorded and the individual was removed from the experiment. All other newly developing stolons were clipped from the mother over the duration of the experiment.

Statistical Analysis

These statistical analyses were designed to determine if there was an effect of substrate type (live versus dead/sand) on the direction of stolon extension. Because there was an equal probability of the strawberry stolon extending into the live substrate, the dead substrate, or growing past the end of the glass runway (no choice), we performed a series of chi-square analyses with the final choice as the categorical variable. All analyses were performed using SPSS (IBM Corp., Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.,).

Results

Root Experiment: Spring (Table 1)

Exposure to live substrate affected individual plant organs to different degrees. Most notably, average root dry mass was nearly three times greater on daughter ramets exposed to live versus dead substrates ($p < 0.001$). There was no significant difference in size of leaves, shoots or stolons in plants growing over live versus dead substrate. Because we considered changes in the root:shoot ratio as indicative of nutrient foraging, we analyzed the difference in root:shoot ratio between ramets exposed to the two substrate treatments. We found a significantly higher root:shoot ratio over live (0.02) versus dead substrate (0.01) [$F(1,28) = 42.56$, $p \ll 0.001$).

TABLE 1 | Analysis from the spring experiment.

	Mean		Treatment effect		Maternal effect		
Factor	**Live substrate treatment**	**Dead substrate treatment**	***F*(1,27)**	***P***	***F*(1,27)**	***P***	**Effect size (η^2)**
Leaf dry mass (mg)	139.56 ± 15.32	150.40 ± 12.59	0.55	0.46	12.82	**^{2}0.001**	0.32
Shoot dry mass (mg)	49.88 ± 5.27	54.64 ± 4.07	1.03	0.32	22.39	**<0.001**	0.45
Root dry mass (mg)	2.94 ± 0.24	1.06 ± 0.08	68.05	**<0.001**	2.93	0.10	0.10
Stolon dry mass (mg)	218.73 ± 28.44	242.69 ± 32.16	0.17	0.69	28.95	**<0.001**	0.52
Stolon length (mm)	296.47 ± 16.23	302.53 ± 20.47	0.00	0.96	8.05	**0.01**	0.23

Effects of live versus dead substrate (treatment effect) and total dry mass of the mother ramet (maternal effect) on the organs of the daughter ramet. Bold face highlights effects significant at $p < 0.05$.

Maternal effect differed between substrate treatments. Daughter ramet root biomass over dead substrate was independent of maternal size, whereas over live substrate, there was a strong correlation between the two ($r^2 = 0.561$; $n = 15$; $p = 0.015$; **Figure 4A**) In contrast, aboveground dry mass (leaf + shoot) was significantly correlated with mother ramet size over both live ($r^2 = 0.656$, $n = 15$; $p = 0.004$) and dead ($r^2 = 0.599$; $n = 15$; $p = 0.009$) substrate*s* (**Figure 4B**).

Root Experiment: Fall (Table 2)

Similar to the results from the spring experiment, root dry mass was significantly greater when daughter ramets were exposed to live substrate ($p < 0.001$). Substrate treatment had a significant effect on shoot dry mass ($p = 0.03$). Leaf dry mass was only marginally affected ($p = 0.06$) although daughter ramets produced more over live than dead substrate. Neither stolon dry mass nor stolon length was affected by substrate treatment. These results differ from those obtained in the spring, when only root dry mass was significantly affected by substrate type. As before, root:shoot ratio was significantly higher in daughter ramets exposed to live (0.02) versus dead substrate (0.01) [$F(1,76) = 29.74$, $p \ll 0.001$]. Interestingly, the root:shoot ratios were similar between spring and fall.

Consistent with results from the spring experiment, daughter ramet root biomass over dead substrate was independent of maternal size, while over live soils the two factors were marginally correlated ($r^2 = 0.242$; $n = 38$; $p = 0.072$; **Figure 4C**). Mother ramet size was significantly correlated with aboveground dry mass over dead ($r^2 = 0.179$; $n = 40$; $p = 0.003$) but not live substrate (**Figure 4D**).

Stolon Trajectory Experiment

In order to rule out any developmentally predetermined directional growth of the stolon, we analyzed the frequency of the stolon extending to the left versus the right of the glass blocks and found no statistically significant preference for growth direction ($\chi^2 = 2.78$; df = 1; $p = 0.096$).

When *F. vesca* stolons were given the choice of live substrate or sand, 67.4% grew into the live substrate, 16.3% grew into the sand, and 16.3% extended beyond the glass runway ($\chi^2 = 22.52$; df = 2; $p < 0.001$; **Figure 5**). When the experiment was repeated using live versus dead substrate, 56.5% grew into live substrate, 21.7% grew into dead substrate, and 21.7% extended beyond the glass runway ($\chi^2 = 11.13$; df = 2; $p < 0.05$; **Figure 6**).

Discussion

We hypothesized that in order to grow into nutrient rich patches, the developing daughter ramet must be able to: (1) detect the patch and (2) respond to the presence of the patch. We suggest two types of responses indicative of nutrient foraging: (1) an increase in root biomass and root:shoot ratio prior to rooting and (2) an alteration of stolon growth trajectory in the direction of a nutrient rich patch. As a first step in testing this hypothesis, we asked whether root growth differs when a ramet extends over live versus dead substrate.

We found a consistent effect of substrate (live versus dead) on the daughter ramet root dry mass; daughter ramets produced more root biomass over live substrate (**Tables 1** and **2**). We also saw an increase in root:shoot ratio, an indicator of the relative allocation of biomass. Not only were our results consistent with our hypothesis, but also ratios in the spring and fall for both treatments were nearly identical. Because ramets were not allowed to come into contact with the substrate, this suggests that the consistency in root:shoot ratio is a programmed response of the daughter ramet to the presence of volatiles emitted from the substrate. The increase in root biomass correlated with maternal size over live (but not dead) substrate (**Figure 3**). One possible explanation for this pattern is that exposure to live substrates initiates a cascade of events increasing the distribution of resources from mother to daughter ramet.

In terms of stolon trajectory, we expected to see a higher frequency of growth toward nutrient rich versus nutrient poor patches. In both experiments, our results were consistent with our hypothesis, in that the majority of *F. vesca* individuals extended stolons into the live substrate (**Figures 4** and **5**). Furthermore, in both experiments, the frequency of stolon extension into nutrient-poor flats (sand or dead substrate) occurred equally, suggesting no differential influence from the less ideal patch.

The experiments in this study strongly demonstrate that ramets of *F. vesca* can identify and respond to nutrient-rich substrate patches, however, the mechanisms behind this capacity are less clear. Because the ability of a new ramet to explore an environment ends once rooting occurs, it is fundamentally important for the plant to be able to predict (based on environmental cues) the quality of the surrounding substrate. Thus, we designed our experiments in such a way to highlight morphological responses to nutrient-rich

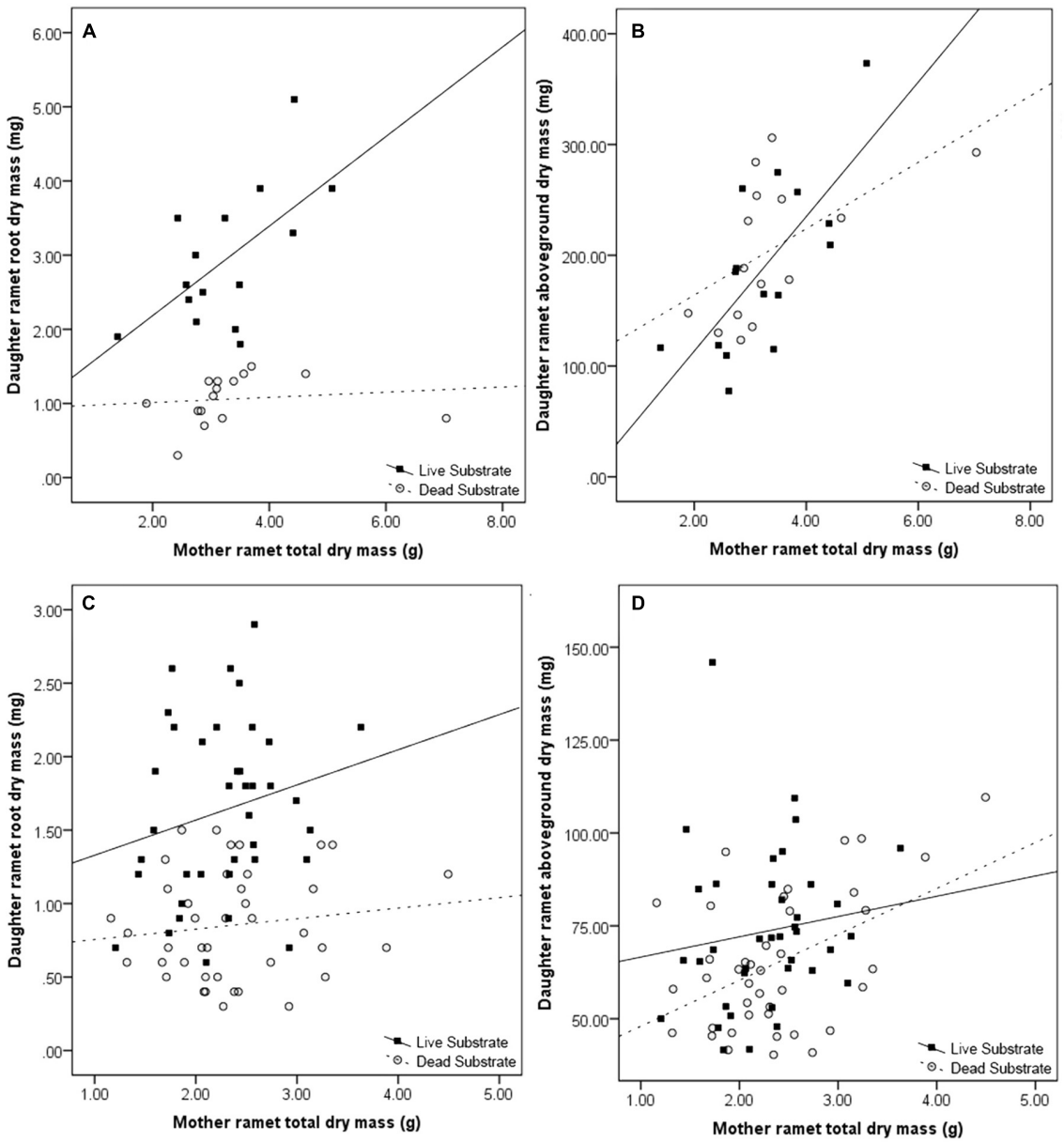

FIGURE 4 | Effects of maternal ramet size on daughter ramet root dry mass (A,C) and daughter ramet aboveground dry mass (B,D) in the spring (A,B) and fall (C,D). Filled boxes (■) represent ramets exposed to live substrate and open circles (○) represent ramets exposed to dead substrates.

TABLE 2 | Analysis from the fall experiment.

	Mean		Treatment effect		Maternal effect		
Factor	**Live substrate treatment**	**Dead substrate treatment**	***F*(1,75)**	***P***	***F*(1,75)**	***P***	**Effect size (η^2)**
Leaf dry mass (mg)	49.79 ± 2.72	43.75 ± 2.12	3.56	0.06	6.22	**0.01**	0.08
Shoot dry mass (mg)	23.77 ± 0.94	21.15 ± 0.91	4.70	**0.03**	5.04	**0.03**	0.06
Root dry mass (mg)	1.63 ± 0.09	0.85 ± 0.06	48.74	**<0.001**	1.96	0.17	0.03
Stolon dry mass (mg)	285.56 ± 10.67	267.75 ± 11.22	2.37	0.13	21.97	**<0.001**	0.28
Stolon length (mm)	469.18 ± 11.87	464.43 ± 12.59	0.19	0.66	1.99	0.16	0.03

Effects of live versus dead substrate (treatment effect) and total dry mass of the mother ramet (maternal effect) on the organs of the daughter ramet. Bold face highlights effects significant at $p < 0.05$.

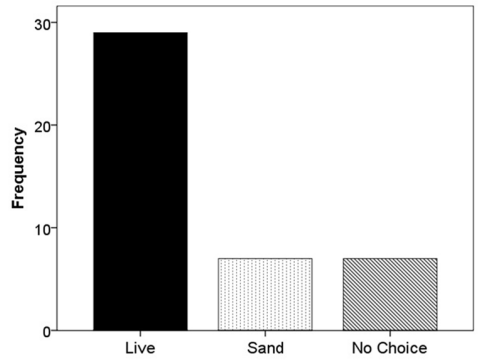

FIGURE 5 | Frequency of selection of extending stolons between live substrate (■), sand (⬚), or no choice (▨).

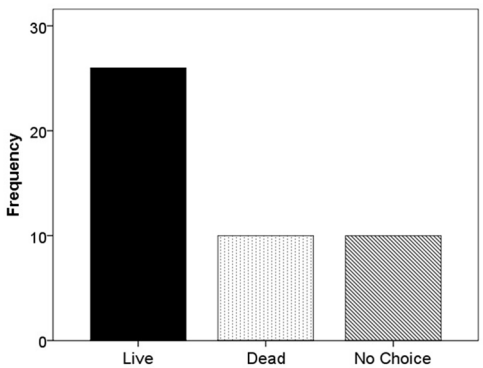

FIGURE 6 | Frequency of selection of extending stolons between live substrate (■), dead substrate (⬚), or no choice (▨).

and nutrient-poor substrates independent of soil contact. Our positive results, specifically the increase in root:shoot ratio of developing ramets and the alteration of stolon trajectory, have led us to propose the following mechanism of patch detection.

The soil environment is highly heterogeneous, and the nature of the soil environment is primarily determined by its inhabitants; a nutrient-rich environment also is a substrate environment rich in microflora, microinvertebrates or larger fauna (Chaparro et al., 2012). Belowground volatile emission can influence the community (Wenke et al., 2010; Tumlinson, 2014) by controlling the bacterial and fungal population (Fiddaman and Rossall, 1993; Mackie and Wheatley, 1999; Kai et al., 2007; Vespermann et al., 2007), attracting herbivores (Neveu et al., 2002; Rasmann et al., 2005; Johnson and Gregory, 2006; Ali et al., 2010), and moderating plant growth (Ryu et al., 2003; Splivallo et al., 2007). It is highly likely that the soil inhabitants produce volatile organic compounds (VOCs) that could be detected by a foraging clonal plant. In this scenario, a developing ramet at the terminal end of an extending stolon would have an opportunity to effectively sample the nutrient environment without the morphological commitment to rooting. This would increase the likelihood of the plant establishing roots in a nutrient-rich/high-quality patch and would explain how a stoloniferous clonal plant might identify and grow into these patches.

Volatile organic compounds are naturally produced chemicals that are critical in influencing ecological interactions both above and belowground (Hughes and Sperandio, 2008; Faure et al., 2009; Kai et al., 2009; Insam and Seewald, 2010; Wenke et al., 2010; Tumlinson, 2014). They are produced by a large variety of organisms, including microbes (Zhang et al., 2007; Kai et al., 2009; Ortiz-Castro et al., 2009), fungi (Splivallo et al., 2007; Tarkka and Piechulla, 2007; Morath et al., 2012; Hung et al., 2015), and plants (Niinemets et al., 2004; Kant et al., 2009; Zhao et al., 2011). Along with mediating communication between different species, they also are byproducts released in response to temperature changes (Asensio et al., 2007; Zhao et al., 2011; Hartikainen et al., 2012), herbivory (Farmer, 2001; Rasmann et al., 2005; Poelman et al., 2013), pathogens (Huang et al., 2012; Panka et al., 2013), and drought (Asensio et al., 2012; Bourtsoukidis et al., 2014; Copolovici et al., 2014).

Volatile organic compounds are often implicated in the promotion of secondary responses, including plant growth. One highly cited example demonstrated that compounds emitted from *Bacilus subtilis* GB03 and *B. amyloliquefaciens* IN937a significantly increased the growth of *Arabidopsis* seedlings as compared to a non-growth promoting strain of *E. coli* and water controls (Ryu et al., 2003). In a similar study, Kai and Piechulla (2009) looked at the effects of *Serratia odorifera* volatiles on growth in *Arabidopsis* in an open system; they concluded that presence of volatiles significantly increased plant growth and a possible role of bacterially emitted CO_2 was suggested. More recently, Minerdi et al. (2011) found that volatiles emitted from *Fusarium oxysporum*, specifically β-Caryophyllene, increased root and shoot length as well as fresh biomass of *Lactuca sativa*. They concluded that this increased growth was the result of the upregulation of seven expansin proteins.

Increase in root growth as a result of exposure to VOCs has been widely discussed in the literature (Zhang et al., 2007; Minerdi et al., 2011; Zamioudis et al., 2013) and is a likely explanation of the results in the current study. VOCs may also explain how *F. vesca* were able to locate nutrient-rich patches in past experiments (Roiloa and Retuerto, 2006). While not directly addressed in the current study, this mechanism of patch-detection might also contribute to the increased root-foraging plasticity in aggressive invaders (Keser et al., 2014), which are perhaps more sensitive or more responsive to volatile clues of nutrient availability. Our on-going studies seek to elucidate mechanisms governing plant foraging by examining the ability of individuals to respond to specific volatile cues emitted from substrates and how these volatiles might elicit specific responses in plant foraging and invasion. We want to determine whether specific growth promoting volatiles are emitted by live *versus* dead substrates and whether they affect stolon trajectory given that establishing a growth trajectory toward a nutrient-rich patch is a necessary precursor to colonization and successful nutrient foraging.

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ORIGINAL RESEARCH
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Different Growth Promoting Effects of Endophytic Bacteria on Invasive and Native Clonal Plants

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The role of the interactions between endophytes and alien plants has been unclear yet in plant invasion. We used a completely germ-free culture system to quantify the plant growth-promoting (PGP) effects of endophytic bacteria *Bacillus* sp. on aseptic seedlings of *Wedelia trilobata* and of its native clonal congener *W. chinensis*. The endophytic bacteria did not affect the growth of *W. chinensis*, but they significantly promoted the growth of *W. trilobata*. With the PGP effects of endophytic bacteria, relative change ratios of the clonal traits and the ramets' growth traits of *W. trilobata* were significantly greater than those of *W. chinensis*. Our results indicate that the growth-promoting effects of endophytes may differ between invasive and native clonal plants, and the endophytes of invasive plant may be host-specific to facilitate plant invasion.

Keywords: repeatable aseptic culture system, bio-invasion, clonal plant, plant–microbe interaction, endophytic bacteria

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INTRODUCTION

In the past century, with the rapid development of global economic trade and cultural exchanges, a large number of plant species have broken their natural geographical barriers and have been introduced to new habitats (van Kleunen et al., 2015). Some of them have become successful invasive plant, which naturalize successfully and cause damage to ecosystem, economy and society (Bai et al., 2013). Therefore, understanding the mechanisms of plant invasion is dramatically important and contributes to their control management.

Plants may harbor abundant microorganisms in rhizosphere, rhizoplane, and endosphere (Hardoim et al., 2008; Compant et al., 2010). Soil biota is critical to enhance the plant's capability of achieving resources from soil (Peiffer et al., 2013). Therefore, interactions between invasive plant and soil biota are hot topics in ecological researches. Rhizosphere microbiota is usually recommended as drivers in successful plant invasions owing to their plant growth promoting effects (Reinhart and Callaway, 2006; Coats and Rumpho, 2014). Invasive plants may change soil biota community to facilitate the plants' invasion (Si et al., 2013), known as the "plant-soil feedback hypothesis" (Klironomos, 2002). This hypothesis suggests that facilitating effect could be achieved by encountering strong mutualism (Sun and He, 2010), by being released from soil-borne enemies (Callaway et al., 2004), or by inhibiting beneficial soil biota of native plants (Bozzolo and Lipson, 2013). Another important hypothesis involving the interactions of rhizosphere microbes and invasive plants, enhanced mutualisms hypothesis suggests that invasive plants may acquire

better soil mutualists in their introduced ranges to enhance their competition ability or disrupting beneficial soil mutualists of native plants (Reinhart and Callaway, 2006). However, previous studies involving enhanced mutualisms hypothesis focused on the roles of rhizosphere microbiota on invasive plants (Sun and He, 2010).

Another important type of soil mutualist biota, endorhizosphere microbiota (abbreviated as endophytes), which live inside plants for at least part of their life cycle without causing any obvious symptoms (Hardoim et al., 2008), may also promote plant invasion like rhizosphere (Rout et al., 2013). It has been reported that endophytic bacteria may enhance the invasion ability of *Sorghum halepense* by changing soil biogeochemistry (Rout and Chrzanowski, 2009; Rout et al., 2013).

Many studies have investigated the interaction between microbes (e.g., rhizosphere microbes or endophytes) and invasive plants (Rout and Chrzanowski, 2009; Sun and He, 2010; Rout et al., 2013; Li et al., 2014). However, they did not use complete aseptic seedlings as studied material, which might cause a bias due to the interference of intrinsic endophytes in plants. Moreover, the behavior and ecological roles of rhizosphere microbes and endophytes for exotic plants' invasion may vary across different environmental conditions (Long et al., 2008; Rout and Callaway, 2012) due to the potential inferences of different soil chemistry and soil biota (Reinhart and Rinella, 2016). Therefore, it is of great importance to use a uniform aseptic culture system to explore the interactions of plant–symbiont or plant-rhizosphere microbiota, in order to understand the mechanisms of plant invasion.

Here, we explore the interactions between endophytic bacteria and invasive plant by using a completely sterile pure culture system of repeatable conditions for invasive clonal plant *Wedelia trilobata* and its endophytic bacteria. We isolated the endophytic bacteria of *W. trilobata* and compared the promoting effects of the endophytic bacteria on aseptic seedlings of *W. trilobata* and its native congener *W. chinensis*. We aim to test whether the promoting effects of the endophytic bacteria are different on the aseptic seedlings of invasive *W. trilobata* and native *W. chinensis*.

MATERIALS AND METHODS

Plant Materials

Wedelia trilobata (L.) Hitchc. (Asteraceae), native to tropical America, is one of the 100 worst invasive species in the world (IUCN, 2001). *W. trilobata* spreads rapidly by strong stolon growth (**Figure 1**) and often overgrows with thick litter layer (Qi et al., 2014a). It is notorious to natural ecosystems in South China (Qi et al., 2014b). *W. trilobata* plants were randomly collected from its invading habitat Haikou, China. *W. chinensis* (Osbeck.) Merr. (Asteraceae) is the native congener of *W. trilobata* in China (Song et al., 2010). Both *W. trilobata* and *W. chinensis* are typical clonal plant. These two *Wedelia* plants were propagated in a greenhouse at Jiangsu University, Zhenjiang, China (Dai et al., 2016). The work has been conducted in conformity with the ethical standards of the field, and did not involved human subjects or animals.

Endophytic Bacteria Strain Isolation from *W. trilobata*

The stems near root (∼3 cm) of healthy *W. trilobata* were collected and were cleaned with running tap water. Under sterile conditions, the stems samples were surface sterilized by stepwise washing in 70% ethanol for 1 min, rinsing with sterile water two times, and then with sodium hypochlorite solution (2% available Cl) for 10 min, followed by five rinses in sterile distilled water. A 100 μl sample of distilled water from the final rinse was planted on Luria–Bertani (LB) agar (Sambrook and Russell, 2001) to confirm that the disinfection process was successful.

After surface disinfection, the stem tissues were cut into approximately 0.5 cm pieces, then slit into two pieces. The wound was stuck to solid LB medium plate. The plates were incubated at 30°C and monitored daily for bacterial colony development over 5 days. Bacterial colonies were isolated and purified by streaking and selection based on phenotypic characteristics, e.g., colony color and morphology (Gagne-Bourgue et al., 2013).

Cell Morphology Observation of Endophytic Bacteria of *W. trilobata* by SEM

The endophytic bacteria (50 ml LB liquid medium culture in 250 ml triangular flasks) were incubated separately at 30°C with shaking (200 rpm) for 16 h. After centrifugation at 10,000 rpm for 15 min, the substrate cells were harvested and washed three times with phosphate buffer solution (PBS, pH 7.2). The collected cells were fixed with 2.5% glutaraldehyde at 4°C for 24 h, then washed three times with PBS. After eliminating solution, the dehydration process was conducted with 30, 50, 70, 80, and 95% of alcohol for 15 min each step, and 100% of alcohol for two times (Ahmad Barudin et al., 2014). After freeze drying for 12 h in the vacuum freeze dryer (Lyoquest-55, Azbil Telstar Technologies S.L.U. Spain), the bacterial cells were harvested and coated with gold under vacuum for examination by a scanning electron microscope (SEM) (S-3400N, HITACHI, Japan) with an acceleration voltage of 10 kV.

Phylogenetic Analysis of Endophytic Bacteria of *W. trilobata*

Bacterial genomic DNA was extracted from pure cultures using the Microbial DNA Isolation Kit (MoBio). The universal bacterial primers, Bac8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Fierer and Jackson, 2006), were used to amplify the 16Sr-DNA. The DNA PCR amplification was performed with initial denaturing at 94°C (5 min), followed by 30 PCR cycles of 1 min denaturing at 95°C, 1 min annealing at 54°C, 1 min extension at 72°C and ended with 10 min extension at 72°C. The PCR products were subjected to electrophoresis on 1.5% agarose gels in 1× TBE buffer and stained with ethidium bromide to verify the target size. The PCR products were purified to remove non-target products using the PCR purification kit (Axygen Bioscience Inc., USA), and then sequenced by Sangon Biotech (Shanghai) Co. Ltd., (China). The

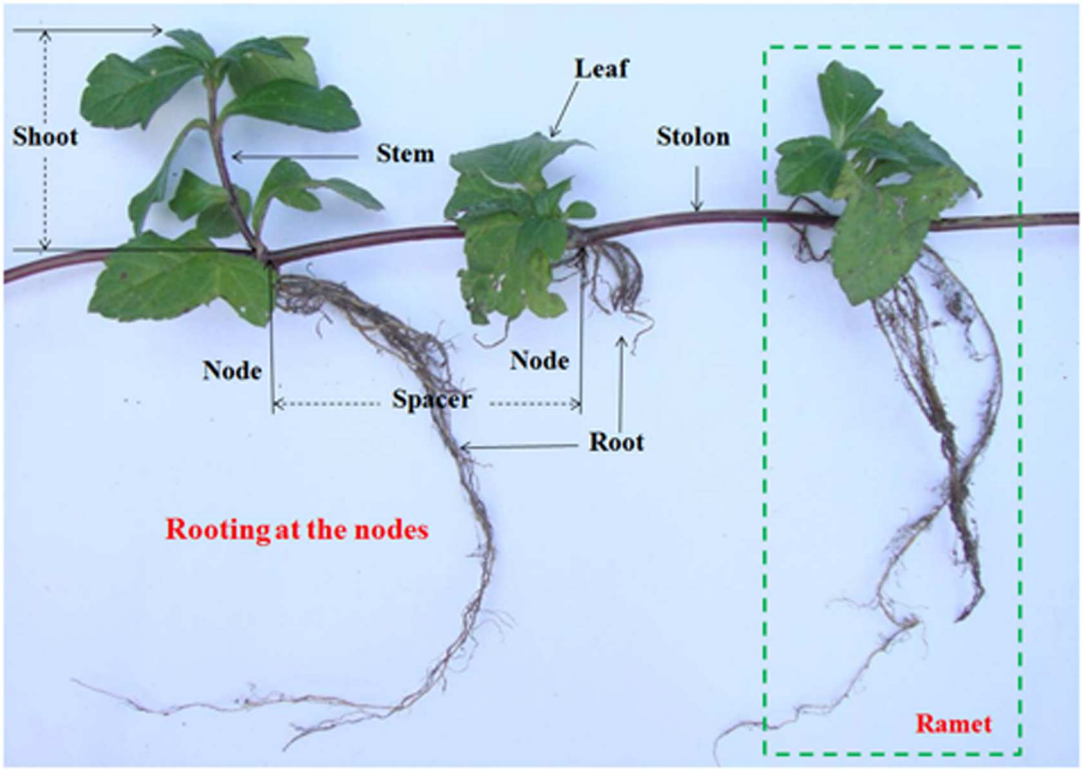

FIGURE 1 | Clonal fragment of *W. trilobata* plant.

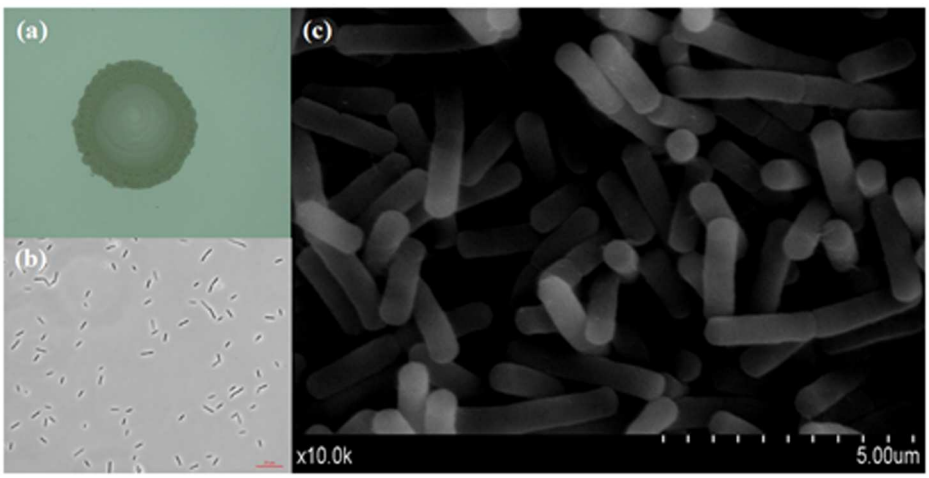

FIGURE 2 | Morphology of endophytic bacteria WtEB-JS040 of *W. trilobata*. (a) Bacterial colony, **(b)** normal light microscope, **(c)** scanning electron microscope (SEM).

sequences were subjected to a BLAST search[1], aligned, and built phylogenetic tree using MEGA 6 with neighbor–joining (NJ) method (Dai et al., 2015).

[1]http://blast.ncbi.nlm.nih.gov/Blast.cgi

Aseptic Culture System

In this study, an aseptic culture system, which contained uniform aseptic seedlings and sterile culture environment of nutrient control, was used to investigate the effects of endophytic bacteria on invasive plant. Aseptic seedlings were produced by fresh

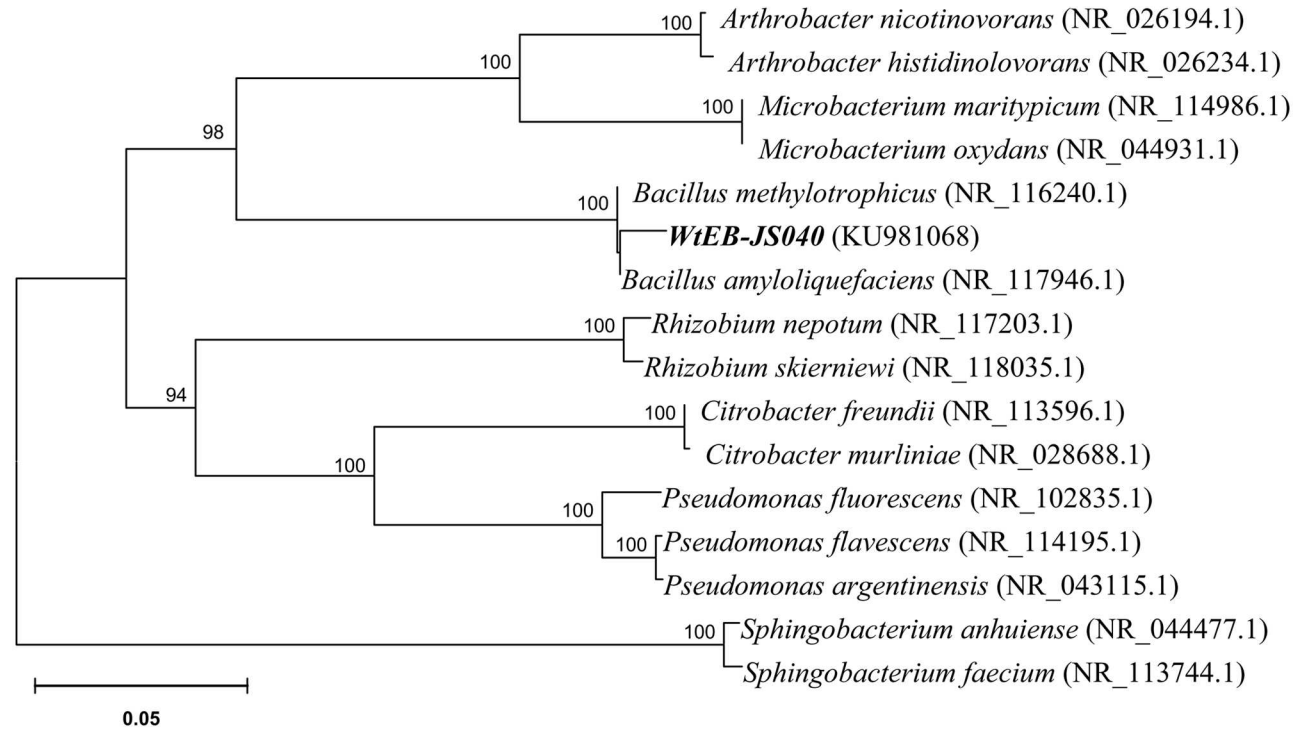

FIGURE 3 | Phylogenetic tree of endophytic bacteria WtEB-JS040 of *W. trilobata* based on 16Sr-DNA gene sequence.

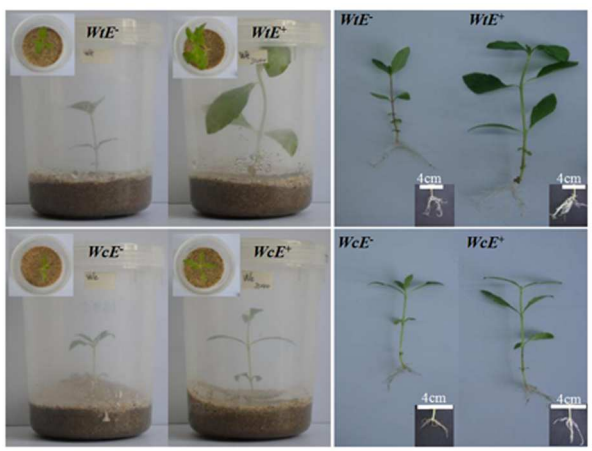

FIGURE 4 | The growth of *W. trilobata* (*Wt*) and *W. chinensis* (*Wc*) aseptic seedlings inoculated with (*E*$^+$) / without (*E*$^-$) WtEB-JS040 strain.

sprouts in ramets of *W. trilobata* and *W. chinensis*. Fresh apical buds of *W. trilobata* and *W. chinensis* were surface-sterilized with 5% sodium hypochloride solution for 10 min and washed thoroughly five times with sterilized distilled water. Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.8 mg·l^{-1} 6-benzylaminopurine, 0.1 mg·l^{-1} 1-naphthaleneacetic acid, and 0.8 mg·l^{-1} silver nitrate. Media were adjusted to pH 6.5 before sterilization by autoclaving for 20 min at 115°C. All the cultures were kept in a culture room at 24°C under a 16 h day and 8 h night photoperiod with 450 μmol·m^{-2}·s^{-1}. The aseptic seedlings were confirmed as complete aseptic seedlings using the coating plate method and 16S-rDNA PCR method (Data sheet 1.doc). These aseptic seedlings were subsequently used as explants, and cultured *in vitro* as follows.

Multiple axillary buds proliferated after approximately 50 days (**Supplementary Figure S1A**). Apical shoots (∼3 cm length) cut from axillary buds (**Supplementary Figure S1B**) were cultured in glass culture bottles (250 ml) containing 35 ml of MS medium for approximately 3∼5 days to obtain aseptic seedling with roots (**Supplementary Figure S1C**). Aseptic seedlings in similar sizes were transferred from MS medium into aseptic culture environment (**Supplementary Figure S1D**), which contained 150 g sterilized water-clean sand and 40 ml sterilized half-strength Hoagland liquid nutrient solution (Hoagland and Arnon, 1950) in an incubator (temperature: 28 ± 2°C; photoperiod: 16 h light and 8 h dark; light intensity: 450 μmol · m^{-2}·s^{-1}).

Endophyte Experiments

The endophytic bacteria strain were grown in LB medium for 16 h (OD_{660} = 1) at 30°C with shaking (200 rpm). Then the endophytic bacteria cells were collected by centrifugation (13,000 rpm for 15 min at 4°C). Cells were re-suspended with sterile 0.05 mM PBS (pH 7.0) at a final concentration of

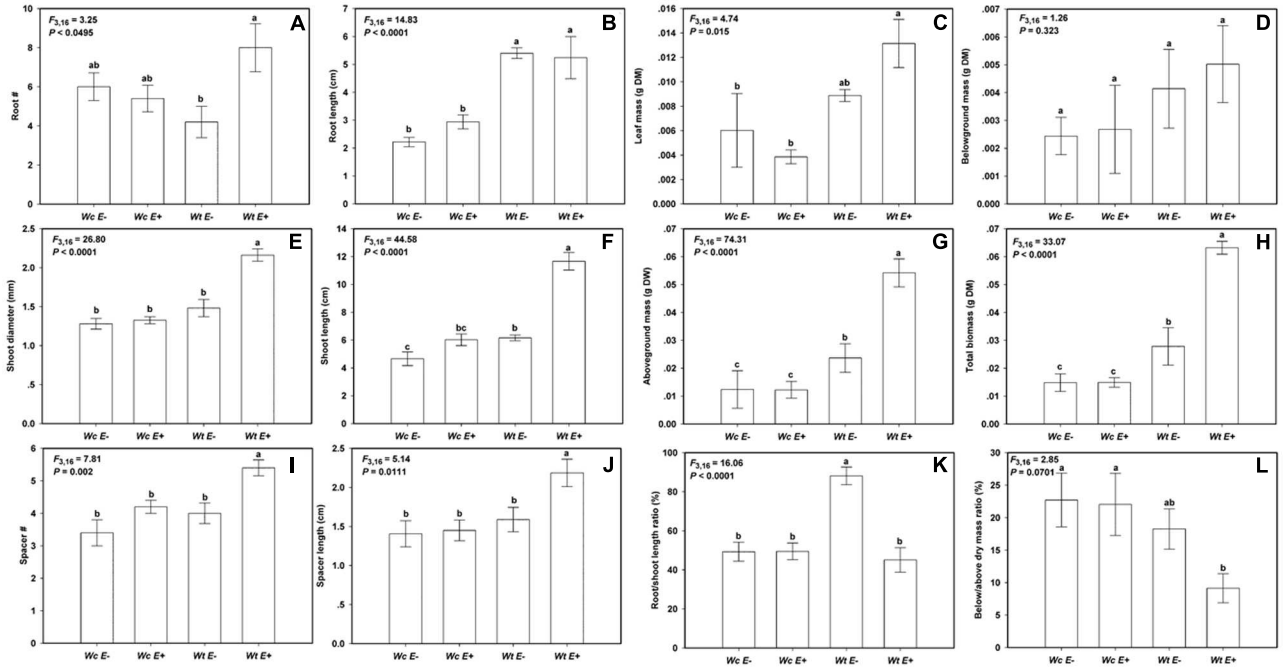

FIGURE 5 | The growth of *W. trilobata* (*Wt*) and *W. chinensis* (*Wc*) aseptic seedlings inoculated with (E^+) / without (E^-) WtEB-JS040 strain. Growth phenotype: **(A)**-root number, **(B)**-root length, **(C)**-shoot diameter, **(D)**-shoot length; Clonal traits: **(E)**-spacer number, **(F)**-spacer length; Biomass: **(G)**-leaf mass, **(H)**-belowground mass, **(I)**-aboveground mass, **(J)**- total mass; Allocation strategy: **(K)**-root/shoot length ratio, **(L)**- root/shoot mass ratio. Different letters indicate significant growth difference of seedlings between the endophytes treatments. Bars represent standard errors ($n = 5$).

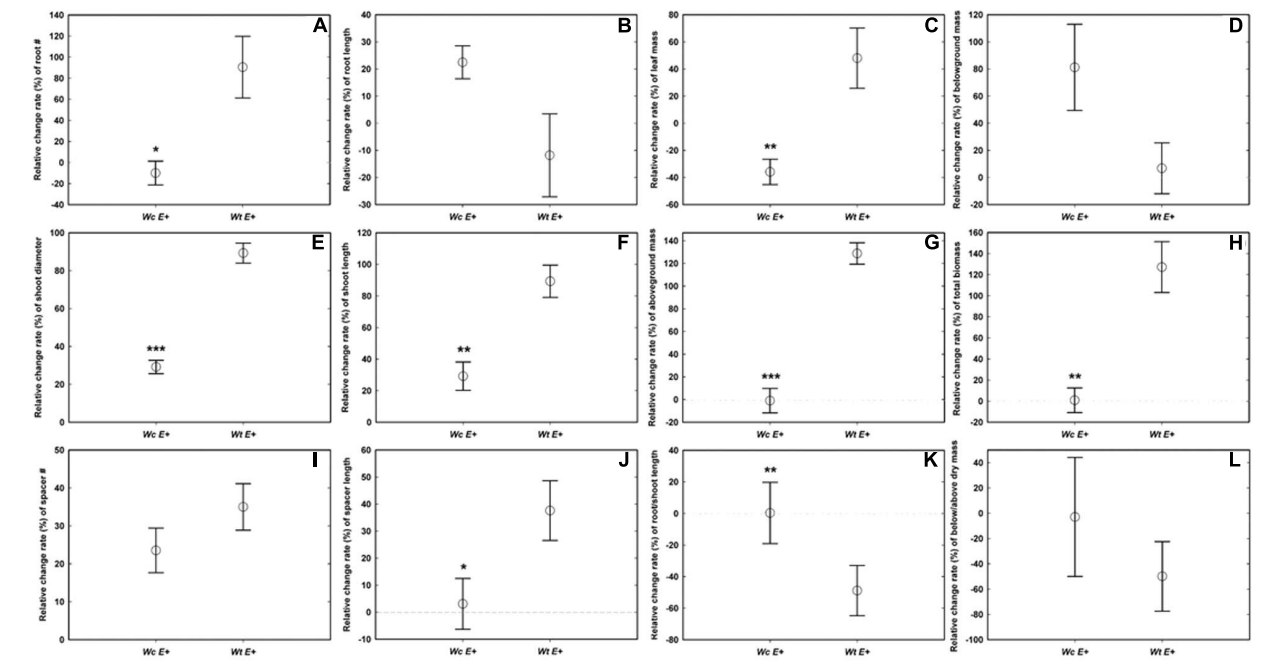

FIGURE 6 | The relative change ratio (RCR) of *W. trilobata* (*Wt*) and *W. chinensis* (*Wc*) aseptic seedlings inoculated with (E^+) WtEB-JS040 strain. Growth phenotype: **(A)**-root number, **(B)**-root length, **(C)**-shoot diameter, **(D)**-shoot length; Clonal traits: **(E)**-spacer number, **(F)**-spacer length; Biomass: **(G)**-leaf mass, **(H)**-belowground mass, **(I)**-aboveground mass, **(J)**- total mass; Allocation strategy: **(K)**-root/shoot length ratio, **(L)**- root/shoot mass ratio. * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) indicate the difference of the RCR of seedlings between the endophytes treatments. Bars represent standard errors ($n = 5$).

TABLE 1 | One-Way ANOVAs for the effects of endophytic bacteria WtEB-JS040 on the relative change rate of the growth of invasive *W. trilobata* and native *W. chinensis*.

Traits	Source	df_1	df_2	*F*	*P*
Growth phenotype	Root #	1	8	10.32	0.012*
	Root length	1	8	4.33	0.071
	Shoot diameter	1	8	44.47	0.0002***
	Shoot length	1	8	19.52	0.002**
Clonal traits	Spacer #	1	8	1.82	0.214
	Spacer length	1	8	5.64	0.045*
Biomass	Leaf mass	1	8	12.07	0.008**
	Belowground mass	1	8	0.07	0.802
	Aboveground mass	1	8	82.08	< 0.0001***
	Total mass	1	8	22.22	0.002**
Allocation strategy	Root/shoot length ratio	1	8	19.22	0.002**
	Root/shoot mass ratio	1	8	3.71	0.090

*Significance levels: ***P < 0.001, **P < 0.01, *P < 0.05.*

10^7 CFU·ml^{-1} to uniform population of bacteria for seedlings inoculation (Taghavi et al., 2009).

After all aseptic seedlings grown stably for 2 days in the aseptic culture system, 2 ml phosphate buffer of the endophytic bacteria cells (E^+) were added around the roots of aseptic seedlings of *W. trilobata* (*Wt*) and *W. chinensis* (*Wc*). Two milliliters of phosphate buffer of heat-killed endophytic bacteria cells were added as negative control treatment (E^-). Thus, there were four treatments for two plant species and two endophytic bacteria addition treatments: (1) *Wc*E^-, (2) *Wc*E^+, (3) *Wt*E^-, and (4) *Wt*E^+. The colonization of endophytic bacteria in aseptic seedlings were identified using coating plate method and 16S-rDNA sequences method (Data sheet 2.doc). Each treatment was repeated five times. Six weeks after bacterial inoculation, the phenotypic growth (root #, root length, shoot diameter, shoot length), clonal growth (spacer #, spacer length), and dry biomass (the second pair of leaves mass, below and aboveground mass, total mass) were measured.

Data Analysis

Root vs. shoot length/mass ratio were calculated to evaluate resource allocation strategy of *W. trilobata* and *W. chinensis*. Two-way ANOVAs were used to compare means of growth traits between treatments using Duncan's multiple-range test ($\alpha = 0.05$) in the endophyte experiments. To eliminate the potential interference of background value from the plant species, the relative change ratio (RCR) of the indices was also calculated as follow: $RCR\,(\%) = [(E^+ - E^-)/E^-] \times 100\%$. One-way ANOVAs was performed to quantify the effects of endophytic bacteria on plants between *W. trilobata* and *W. chinensis*, using Duncan's multiple–range test ($\alpha = 0.05$).

RESULTS

Identification of Endophytic Bacteria of *W. trilobata*

The endophytic strain isolated from *W. trilobata* and used in this study was identified as *Bacillus* sp. WtEB-JS040 (naming scheme: Wt – *Wedelia trilobata*, EB – endophytic bacteria, JS – Jiangsu; hereinafter abbreviated as JS040) based on its phenotypic characteristics (**Figure 2**) and 16Sr-DNA gene sequence (**Figure 3**; Supplementary Table S1; GenBank accession no. KU981068). A fragment (1,152 Bp) of 16Sr-DNA was amplified by PCR from strain JS040 (**Supplementary Figure S2**). Sequence analysis and homology comparison of 16Sr-DNA gene sequence showed that strain JS040 had a similarity of 99% with *Bacillus amyloliguefaciens* (GenBank no. NR_117946.1) and *Bacillus methylotrophicus* (GenBank no. NR_116240.1).

Effects of JS040 on the Aseptic Seedling of *W. trilobata* and *W. chinensis*

The endophytic bacteria JS040 inoculation showed significant promoting effects on *W. trilobata* (**Figure 4**). The phenotypic growth (root #, shoot diameter, and shoot length) (**Figures 5A,C,D**), clonal growth (spacer # and spacer length) (**Figures 5E,F**), and biomass (aboveground mass and total mass) (**Figures 5I,J**) of *W. trilobata* were greatly increased with the JS040 inoculation. However, JS040 reduced the resource cost in *W. trilobata* roots (**Figures 5K,L**). JS040 did not significantly change the growth phenotype, clonal growth, biomass and resource allocation in *W. chinensis* (**Figures 5A–L**).

Compared with *W. chinensis*, JS040 significantly increased the RCR of root # (**Figure 6A**), shoot diameter (**Figure 6C**), shoot length (**Figure 6D**), spacer length (**Figure 6F**), leaf mass (**Figure 6G**), aboveground mass (**Figure 6I**), total mass (**Figure 6J**) of *W. trilobata*, whereas JS040 decreased the RCR of root vs. shoot of *W. trilobata* (**Figure 6K**) (**Table 1**).

DISCUSSION

Different Effects of Endophytic Bacteria WtEB-JS040 between *W. trilobata* and *W. chinensis*

The effects of soil biota on plants may be estimated inappropriately owing to the variation of culture environment

gradients, plant genetics, and even soil biota across very small spatial scales (Reinhart and Rinella, 2016). In this study, we suggest using a completely aseptic culture system containing uniform aseptic seedlings and repeatable environment (**Supplementary Figure S1**), to explore the effects of microorganism on invasive plants. As this aseptic culture system eliminates other microorganism in growth media and intrinsic endophytes in target plants, our results contribute to our understanding of the real functions of microbes on plant invasion.

Previous studies have shown that auxin regulates initiation and emergence of root (Ljung et al., 2005; Overvoorde et al., 2010), and indoleacetic acid (IAA) synthesized by plant-associated bacteria plays a major role in the development of the host plant root system (Patten and Glick, 2002; Tchinda et al., 2016). In the present study, the endophytic bacteria JS040 showed significant promoting effects on its host invasive clonal plant *W. trilobata*. Although the root length and mass of *W. trilobata* did not increase after endophytes inoculation, endophytic JS040 increased the growth performance of *W. trilobata*, which might be due to the increase of the root number and vitality stimulated by bacteria-excreted auxin to enhance the nutrient uptake capability. Considering the fast dispersal of *W. trilobata* through clonal reproduction (**Figure 1**; Qi et al., 2014a; Si et al., 2014; Dai et al., 2016), the increase of shoot length, spacer number, spacer length (**Figures 5D–F**) will potentially enhance the expansion ability of ramet population of *W. trilobata*. In addition, we also found endophytic JS040 may help invasive *W. trilobata* to allocate less resource to below-ground system (**Figure 5K**) in the meantime of keeping its shoot growth dominance, which may be because that JS040 was isolated from stems.

As for the native congener *W. chinensis*, endophytic bacteria JS040 did not have significant promoting effects (**Figure 5**). After inoculation of JS040, the RCR of clonal traits (spacer length) and potential ramets' growth traits (root #, shoot diameter and length, leaf mass, aboveground mass, total mass) of *W. trilobata* were higher than that of *W. chinensis* (**Figure 6**; **Table 1**), suggesting that the growth-promoting effects of endophytic bacteria JS040 may differ between invasive and native clonal plants. Therefore, our results indicate that endophytes of *W. trilobata* may be host-specific to increase the growth of *W. trilobata*, which provides the preliminary supports for enhanced mutualisms hypothesis (Reinhart and Callaway, 2006) from endosymbiosis using complete aseptic culture system (**Supplementary Figure S1**).

Implications for Future Researches

The plant–microbe interactions are important in plant fitness and adaptability, which have evolved in direct association with microbes functioning as both agonists and antagonists in terms of plant development and defense activities (Coats and Rumpho, 2014). The interactions between plant and rhizosphere microbe have been well studied in plant invasion ecology (Klironomos, 2002; Callaway et al., 2004; Sun and He, 2010; Rout and Callaway, 2012; Si et al., 2013). However, our understanding of the complex interactions between endophytes and their host plants is not clear yet, and our finding of the promoting growth effect mediated by endophytes contributes to this area. This promoting growth effects could be important to the invasion process of alien invasive plants. Future works can be launched to investigate the interactions between invasive plant and endophytes as follows:

(1) The roles of more endophytes on host plants, endophytes from different tissues of host invasive plants and host native plants. These aspects make the cross inoculation experiments of endophytes from different host plants essential to understand the contributions of endophytes to plant invasion; (2) Microbial colonization of rhizoplane and endosphere in invasive plants. Efficient colonization in the roots of hosts is endophytes' first step to function in the plant–microbe interactions (Long et al., 2008; Compant et al., 2010); (3) Mechanisms of endophytes promoting the growth of hosts and the hosts' regulation of endophytes. In this way, we need to explore the feedback system between invasive plants and endophytes. Since researchers do not fully understand the mechanisms by which bacterial endophytes promotes the growth of host plant, we still need to conduct in-depth researches in the field of plant invasion. What is more, we shall pay more attention in exploring the regulation mechanisms of invasive plants on endophytes in future.

AUTHOR CONTRIBUTIONS

Z-CD, S-SQ, and D-LD designed the study. Z-CD, WF, and L-YW carried out the experiment work for strain isolation and identification. S-SQ and H-HC carried out the experiment work for aseptic seedlings culture. WF and NW carried out the experiment work for endophyte experiments and data selection. Z-CD and D-LD did the data analysis. Z-CD and S-SQ wrote the manuscript. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00706

FIGURE S1 | Aseptic culture system for endophytes research. (A) multiple axillary buds, **(B)** apical shoots cut from axillary buds, **(C)** aseptic seedling with roots, **(D)** aseptic seedling system.

FIGURE S2 | Amplification of 16S-rDNA from WtEB-JS040 strain. Lane M, DL2000 LADDER; Lane 1, 16S-rDNA fragment amplified by PCR.

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Adjusting to Global Change through Clonal Growth and Epigenetic Variation

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The earth is experiencing major changes in global and regional climates and changes are predicted to accelerate in the future. Many species will be under considerable pressure to evolve, to migrate, or be faced with extinction. Clonal plants would appear to be at a particular disadvantage due to their limited mobility and limited capacity for adaptation. However, they have outlived previous environmental shifts and clonal species have persisted for millenia. Clonal spread offers unique ecological advantages, such as resource sharing, risk sharing, and economies of scale among ramets within genotypes. We suggest that ecological attributes of clonal plants, in tandem with variation in gene regulation through epigenetic mechanisms that facilitate and optimize phenotype variation in response to environmental change may permit them to be well suited to projected conditions.

Keywords: clonal plants, epigenetic variation, transgenerational, climate acclimation, plasticity

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INTRODUCTION

Rapid and extreme climate changes are predicted, raising questions as to the capacity of plants to adjust to and survive the new environments. In clonal plants, limited dispersal and lack of recombination as a source of new gene combinations might compromise their capacity to migrate or evolve fast enough. Recent work in epigenetics has revealed an alternative path to adaptation involving variation in gene regulation, whereby genotypes can respond to environmental change without genetic recombination (Richards, 2006; Jablonka and Raz, 2009; Massicotte and Angers, 2012), that has consequences for clonal plants (Verhoeven and Preite, 2014; Douhovnikoff and Dodd, 2015). We suggest that ecological advantages to the clonal growth strategy, in tandem with epigenetically regulated accommodation through plasticity (acclimation) could position many clonal plant species well for future ecological success.

The most recent IPCC report (IPCC, 2014) predicts increasing temperatures among a range of carbon emission scenarios, with greatest changes occurring at higher latitudes and elevations. A second prediction from the IPCC report is increased frequency of extreme climatic events, such as heat, droughts, floods and storm damage. Indirect effects of these extreme events will include reduced plant defenses, increased attacks from pests and diseases and subsequent episodes of fire and soil erosion. Rates of climate change predicted by the climate models for the next century are unprecedented and may exceed by an order of magnitude the rates of climate warming during the Holocene deglaciation (Diffenbaugh and Field, 2013). The last major period of climatic change, during the Pleistocene, saw major species' distributional shifts (Hewitt, 2004), and changes in community structure as a result of unequal species' responses (Huntley, 1999). Ecologists have

already monitored plant responses to recent climate change that include (1) colonization, as altitudinal and latitudinal displacements occur (Parmesan and Yohe, 2003) and (2) adaptation, such as spring-time advances in phenological processes (Hughes, 2000). Currently, mortality through increased attacks by pests and diseases following drought stress is putting some ecosystems at risk (Woods et al., 2005; Hicke et al., 2006). Because of the anticipated rates of climate change, population re-locations through dispersal and colonization are expected to be more successful responses than survival through adaptation *in situ* (Aitken et al., 2008). Community structure will likely change as species respond unequally; modeling suggests that community diversity decreases and non-analog communities are most likely to form when dispersal differences among species are high (Urban et al., 2012). Those species with limited ranges, or low dispersal potential are the most likely to face extinction.

The sedentary nature of plants imposes constraints on the velocity of response to rapidly changing environmental conditions. The potential for plants to track climate change depends on long distance dispersal events that allow colonization of new habitat and added genetic variation on the colonizing front (Kremer et al., 2012). For many clonal plants that spread vegetatively from attached organs (rhizomes, roots, stem bases), the opportunity for populations to track environmental change through long distance dispersal will be limited (Winkler and Fischer, 2002; Winkler and Stöcklin, 2002). If clonal plants are limited in their ability to disperse, will species that depend on this mode of reproduction be able to take advantage of *in situ* adaptation as a response to climate change? In the absence of meiotic recombination, adaptation requires fitness-beneficial mutations (mitotic recombination is unlikely to provide a sufficient source of genotypic variation that could be selectively advantageous.). Accumulation of beneficial mutations is slow and, for phenotypic traits, antagonistic interactions among traits are likely to impede adaptation (Etterson and Shaw, 2001). Nevertheless, clonal plants have persisted for thousands, or even millions of years during past environmental changes (Neiman et al., 2009), and clonal plants have been successful in colonizing new habitats (Ahmad et al., 2008; Zhang et al., 2013), occupying broad geographic ranges (Geng et al., 2007; Ganie et al., 2016) and have come to dominate some ecosystems (Hollingsworth and Bailey, 2000).

So, how do the classic paradigms for clonal plants fail to capture their ecological potential in a dynamic world? First, obligate clonality is rare in nature (Savidan, 2010). Even in extreme environments where clonal reproduction is expected to predominate (Eckert, 2002), high genetic diversity can be maintained by episodes of sexual recruitment (de Witte and Stöcklin, 2010). Second, clonal growth permits a range of advantageous ecological strategies including resource sharing (Alpert, 1990), niche specialization (Gómez and Stuefer, 2006; Louâpre et al., 2012) and rapid vegetative growth, particularly in pioneer habitats. Thirdly, plastic phenotypic responses may include an adaptive component that to some extent substitutes for, and can be far more rapid than adaptation through genetic selection (Verhoeven and Preite, 2014; Douhovnikoff and Dodd, 2015). Accommodation through plasticity has commonly been invoked as important in permitting clonal plants to respond to heterogeneous environments (Parker et al., 2003; Geng et al., 2007). Furthermore, phenotypic accommodation to changing environments ultimately can lead to evolutionary change through selection on elevations and slopes of norms of reaction (West-Eberhard, 2005; Lande, 2009). Recently, an increasing number of studies have shown that plastic responses can be mediated through epigenetic modifications (Jaenisch and Bird, 2003; Richards et al., 2006), the most commonly studied being DNA methylation that results in changes in gene expression. These epigenetic marks may be stable across somatic generations (Bossdorf et al., 2008; Castonguay and Angers, 2012) and across germlines (Jablonka and Raz, 2009; Richards et al., 2012; Verhoeven and Preite, 2014; Douhovnikoff and Dodd, 2015); the latter known as transgenerational (Boyko et al., 2010). Stable epigenetic variations that result in phenotypic variation are thought to offer both short and long-term possibilities for plants to be buffered against their environment (Richards et al., 2006; Nicotra et al., 2010; Verhoeven et al., 2010; Zhang et al., 2013) and provide an alternative, or are complementary to genomic adaptations. Because epigenetic variations can be heritable and reversible (Bossdorf et al., 2008; Richards et al., 2008, 2012; Jablonka and Raz, 2009; Nicotra et al., 2010; Verhoeven and Preite, 2014; Douhovnikoff and Dodd, 2015), they offer a potentially flexible mechanism for plant adaptation. Therefore, epigenetic diversity could provide a crucial source of adaptive potential in asexual plants (Castonguay and Angers, 2012; Verhoeven and Preite, 2014; Douhovnikoff and Dodd, 2015).

Here, we provide some perspectives on the possible success of clonal plants under the predictions of future climate change. We examine how epigenetic changes and their inheritance may contribute to resilience of clonal plants under progressive climate change and climatic extremes.

Colonization on the Advancing Front

Studies of plant responses to past climate changes suggest that rare long-distance seed dispersal events have been important in facilitating population migrations (Clark et al., 2003; Petit et al., 2004). The success of these founder populations depends on the chance that suitable genotypes are among the migrant pool and that sufficient genetic diversity can rapidly be incorporated in the population (Kremer et al., 2012). The latter requires a continuous supply of migrants, or the capability of the founder individuals to support environmental conditions through phenotypic plasticity for long enough that genetic variation can accumulate (Fischer et al., 2000; Petit et al., 2003; Mimura and Aitken, 2007). Contrary to the common belief that clonal plants have low dispersal capability, elevated levels of genotypic diversity in many clonal species suggest recruitment from seedlings (Douhovnikoff et al., 2004), even at altitudinal and elevational extremes (de Witte and Stöcklin, 2010), allowing opportunities for long distance dispersal. The maintenance and growth of a population founded by a single propagule and subsequent variations in plastic response, as it accommodates to the new environment, constitutes an important ecological advantage. Two sources of epigenetic variation help founder populations to adjust. The

first is through environmentally-induced epigenetic change and the second is through stochastic epimutations (Verhoeven and Preite, 2014).

Environmentally-induced epigenetic changes are direct responses to environmental stresses that are heritable across generations and distinct from common environmental effects such as on gametes or seeds subject to the same environment as the parent. Such effects have been shown for salt-stressed invasive knotweeds (Richards et al., 2008), environmentally-stressed dandelions (Verhoeven et al., 2010) and pathogen-infected *Arabidopsis* (Dowen et al., 2012). Because these epigenetic changes are likely to be directional, as long as epigenetic changes are rapid enough and are stable across generations, serial epimutations could maintain populations at the same fitness level as the environment progressively changes. High rates of methylation of cytosine at the CG position, five times greater than genetic mutations in *Arabidopsis,* were estimated by Ossowski et al. (2010). Although lack of stability resulting from high epimutation rates has been argued as a reason that epigenetic changes are unlikely to have long-term evolutionary consequences (Furrow, 2014), van der Graaf et al. (2015), have shown that epimutation-selection equilibria are comparable to those expected for genetic mutations, suggesting their potential role in long-term evolution. In addition, a form of midterm acclimation, the dosage regulation of genes in addition to simple activation and silencing, and reversals at different rates allows for a broad range of fine-tuned modifications (Bräutigam et al., 2013). This increased epigenetic variation permits plants to adjust to change as they encounter new conditions through space, or changing conditions over time; two highly likely circumstances for clonal plants that are often broadly distributed and long lived (Douhovnikoff and Dodd, 2015).

Stochastic epimutations can be beneficial, or deleterious, and provided that they are stable across enough generations, can be acted upon in similar ways as genetic mutations (Slatkin, 2009). Indeed, there is evidence that stochastic epimutations accumulate in a clock-like manner, resembling the accumulation of genetic mutations (Hagmann et al., 2015). Elevated levels of epimutations have been associated with environmental stress (Rapp and Wendel, 2005; Verhoeven et al., 2010) and therefore provide an important source of variation under climate change (Platt et al., 2015). For clonal plants, the higher rate of epimutations over genetic mutations offers an increased possibility for different epialleles to be selected for among ramets of the same founding genotype, permitting both spatial and temporal scales of heterogeneity to develop that may be exploited rapidly by clonal spread (Douhovnikoff and Dodd, 2015). For example, at a spatial scale, as clones forage across the landscape, ramets could become differentially specialized in the uptake of resources (Hutchings and de Kroon, 1994; Wang et al., 2011). At a temporal scale, serial epigenetic mutations can lead to a greater range of phenotypes available for response to climatic variations; high forward and backward epimutation rates leads to much greater variation in epialles than genetic alleles (van der Graaf et al., 2015).

The rapidity at which epigenetic changes can occur and their stability will confer considerable advantages to both sexual and asexual plants under rapid environmental changes, relative to the slower accumulation of genetic diversity through mutations and recombination. Is there any evidence that epigenetic variation has indeed played a role in response to climatic variations across species ranges or in response to climate changes? Preite et al. (2015) tested for a signal of DNA methylation with northward colonization following the last glaciation in apomictic dandelions. Although they failed to detect any signal that would confirm clinal epigenetic adaptation, some epigenetic variants were uncorrelated with genetic variation and may have been a source for local phenotypic adaptations. They concluded that clinal epigenetic changes may be transitory and, over long periods of time genetic adaptation would erase any epigenetic signal. In the California oak *Quercus lobata*, seed dispersal distances are limited and post-glacial advances have probably been over relatively short distances. Platt et al. (2015) showed significant CpG methylation associated with climatically distinct populations of valley oak, suggesting epigenetic adaptation, or that the epialleles were tightly linked to adaptive genes. An interesting role for epigenetics on transcriptomic variations according to clone history was reported for *Populus* by Raj et al. (2011). Cuttings from clonally propagated *Populus* trees that had been planted in different geographic locations were grown in a common environment and exposed to drought stress. Transcriptome-level patterns were paralleled by differences in genome-wide DNA methylation and were most pronounced in clones with the longest time since establishment and last common propagation. This suggests an epigenomic basis for the transcriptome variation in drought stress response associated with clone history. The synchronization of plant phenology with environmental conditions is under strong selection pressure because of the need to maximize the growing season, while minimizing the risks of cold injury (Savolainen et al., 2004) and is important in the adaptation of plants during range shifts. Epigenetic-based "memory" of environmental conditions during seed development has been shown to determine bud phenology in progeny of *Picea abies* (Yakovlev et al., 2011). Below normal temperatures during seed development led to earlier bud phenology and above normal temperatures led to later bud phenology. Only recently, have we begun to examine variations in epigenetic responses to environmental variations in non-model plants, but these and other examples are beginning to emerge that point to an important role that epigenetic modifications may play in plant acclimation to climatic variation.

Extreme Climatic Events

Extreme climatic events can be the most devastating for plant survival by pushing systems beyond thresholds of tolerance (Feder et al., 2000). The predicted increase in extreme events will place many organisms under stress (i.e., drought) and impose episodes of extreme environmental events (i.e., fire) and potentially devastating biotic interactions (i.e., pests and diseases). It has been suggested that extreme events may have impacts on ecosystems even before the progressive changes in temperature or rainfall (Gaines and Denny, 1993). Evolutionary

responses to select for stress-tolerant genotypes are unlikely to be rapid enough to protect populations against extinction in the face of extreme stresses. However, the potential for epigenetic responses to stress may provide the phenotypic variation necessary to sustain populations during events that could push plants past threshold tolerance levels. We now know that environmental stresses can elicit changes in DNA methylation. Examples mostly from *in vitro* tests on well-studied systems such as crop plants or *Arabidopsis* have been reviewed by Chinnusamy and Zhu (2009). In many cases, these involve histone mediated epigenetic changes that are reversed when the environmental cue is removed. The reversibility of epigenetic changes can provide an important additional source of variation. It would be interesting to test whether reversals can provide a pre-adaptation to future change. In other words, once an epigenetic change has occurred, even if reversed, could it be easier for the same change to occur again in the future (a form of hormesis, Douhovnikoff and Dodd, 2015). Further studies of stress-induced DNA methylation in genetically identical apomictic dandelions revealed more than 75% of epigenetic modifications to be transmitted to offspring not exposed to the environmental stress (Verhoeven et al., 2010). These epigenetic changes may be stress-targeted or random (subject to natural selection), but in either case they contribute to the overall response to environmental stimuli and indicate an added potential for phenotypic diversity (Verhoeven and Preite, 2014).

Although the potential for stress-related epigenetic changes may occur in both sexual and asexual plants, the combination of these responses and the ecology of clonal species can explain in part the success of clonality in environments subject to severe stresses. Many species are particularly vulnerable during the seed development, germination and seedling establishment phase, particularly in drought-prone habitats. Indeed, the switch from sexual to asexual reproduction is commonly associated with the risks associated with sexual reproduction (Eckert, 2002). Clonal reproduction provides an escape from the seedling phase, coupled with rapid vegetative growth because of the existing root system. The moderating effect epigenetic variation has on reduced genetic recombination, to some extent tempers the genetic disadvantage of this sex-avoidance strategy. Increased fire frequencies and severity are expected to be the new norm in many parts of the world as a result of higher temperature and increased drought (Westerling et al., 2011; Brando et al., 2014). Although some sexual reproductive systems are fire-adapted, in a great many resprouting systems, clonal reproduction provides the most rapid recovery after fire (Bond and Midgley, 2001). Another effect of drought-stress is the increased incidence of disease and pest outbreaks (Woods et al., 2005; Hicke et al., 2006). The search for heritable variation in disease resistance traits usually assumes variations in DNA sequence. However, recently Latzel et al. (2012) reported the epigenetic inheritance of response to the defense hormones, jasmonic acid and salicylic acid, in *Arabidopsis thaliana* (Latzel et al., 2012). The role of epigenetics in plant defense to pests and diseases is an area that deserves much more attention as it seems likely to hold considerable promise in understanding disease dynamics in natural populations. The assumption that resistance is genetically based would suggest that clonal genotypes may be at a disadvantage as disease spreads through a population. However, if stress-related epimutations arise in populations, it is possible that disease outbreaks could induce defense responses regardless of the host genotype.

CONCLUSIONS

The velocity of future climate change is commonly viewed as necessitating rapid plant movements as natural selection cannot operate fast enough to generate novel beneficial gene combinations. However, the range of origins of epigenetic variation could provide phenotypic variation that would buffer against all but the most extreme climatic events. Clonal plants will continue to be an important component of ecosystems because of the attributes that they offer under heterogeneous environments, including rapid vegetative growth and multiplication, resource sharing and niche specialization among connected individuals. With our improved understanding of epigenetic systems and their mode of transmission among clonal copies and across sexual generations, we are uncovering only the superficial skin of a layer of complexity that drives phenotypic responses to the environment. The epigenome is likely to be particularly important in biological systems that lack genetic recombination, and under environmental changes, when the velocity of change exceeds the adaptation possible through natural selection. The added phenotypic diversity offered through epigenomic change should provide the buffer against environmental change that will permit more stable genetic systems to evolve.

AUTHOR CONTRIBUTIONS

RD and VD contributed equally to the ideas expressed here and to the writing of the manuscript.

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Invasion Fosters Change: Independent Evolutionary Shifts in Reproductive Traits after *Oxalis pes-caprae* L. Introduction

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Biological invasions offer optimal scenarios to study evolutionary changes under contemporary timescales. After long-distance dispersal, exotic species have to cope with strong mate limitation, and shifts toward uniparental reproduction have been hypothesized to be selectively advantageous. *Oxalis pes-caprae* is a clonal tristylous species native to South Africa, and invasive in Mediterranean regions worldwide. It reproduces sexually and asexually but the importance of each strategy differs between ranges. Native populations reproduce mostly sexually while in invasive ones asexual reproduction is the prevailing strategy due to the dominance of pentaploid monomorphic populations. Nevertheless, two contrasting scenarios have been observed after introduction: transition toward clonality, and re-acquisition of sexuality fueled by multiple introductions of compatible mates. Here, we aimed to assess evolutionary changes of reproductive traits in *O. pes-caprae* invasive populations and evaluate whether these traits could be related with invasion success and prevalence of certain forms in the western Mediterranean basin. Sexual and asexual reproduction traits were quantified under optimal conditions in a common garden experiment including native and invasive sexual, predominately asexual, and obligated asexual individuals. Different reproductive, ecological, and genetic constraints created by long-distance dispersal seem to have generated different selective pressures in sexual and asexual traits, with our results supporting evolutionary changes in invasive populations of *O. pes-caprae*. Native plants had higher sexual fitness, while a transition toward clonality was clear for invasive forms, supporting clonal reproduction as a major trait driving invasion. Differences were also observed among invasive plants, with sexual forms having increased dispersal potential; thus, they are expected to be in advantage in comparison with predominantly asexual and obligated asexual plants, and may become widespread in the future. Historical processes, like the initial introduction of predominantly asexual forms followed by sexual

forms more recently, could be in the origin of current distribution patterns of *O. pes-caprae* in the western Mediterranean. This study shows that invasion processes are very dynamic and that ecological and genetic constraints determined by the invasion process may originate different reproductive strategies that are likely to determine invasion success.

Keywords: clonality, evolution of reproduction, Mediterranean regions, pentaploid, polyploidy, reproductive strategy, sexual and asexual reproduction, tristyly

INTRODUCTION

Biological invasions are a serious threat to biodiversity and have long been recognized to comprise significant ecological and evolutionary consequences, not only for the communities being invaded, but also for the invasive species themselves (Brown and Eckert, 2005; Barrett et al., 2008; Pyšek et al., 2012; Oduor, 2013). For these reasons, since the seminal works of Elton (1958) and Baker and Stebbins (1965), biological invasions have attracted much attention of researchers in an attempt to identify traits that might confer an advantage during colonization of new habitats (Pyšek and Richardson, 2007; Hayes and Barry, 2008; van Kleunen et al., 2010) and to understand the biotic and abiotic factors that determine invasion success (e.g., Souza et al., 2011; Wisz et al., 2013). It also became clear that the introduction of sub-populations in new ecological scenarios generates valuable oportunities to study evolutionary transitions over contemporary time scales (e.g., Sakai et al., 2001; Brown and Eckert, 2005; Barrett et al., 2008; Prentis et al., 2008). These studies contribute to a better understanding of the factors triggering a successful invasion, and provide new insights on the evolutionary history of specific traits, such as those related to the reproductive system (Barrett et al., 2008; Barrett, 2011).

Reproduction is one of the key factors involved in the successful establishment and spread of a given organism after long-distance dispersal (e.g., Sakai et al., 2001; Barrett et al., 2008; Hayes and Barry, 2008; Castro-Díez et al., 2014; Moravcová et al., 2015). Reproductive modes determine the production, dispersal and genetic composition of propagules, thus influencing the genetic and demographic structure of populations, as well as the dispersal ability and evolutionary potential of introduced individuals or sub-populations, that, by its turn, will also determine the reproductive strategy (Sakai et al., 2001; Novak and Mack, 2005; Barrett et al., 2008; Ness et al., 2010). Flowering plants exhibit an outstanding diversity of reproductive strategies, from sexual to asexual modes and from self-compatible to obligated outcrossers (reviewed in Barrett, 2002), that frequently occur in combination and reveal liability under certain ecological and genetic stressful conditions (Dorken and Eckert, 2001; Eckert, 2002; Goodwillie et al., 2005; Herben et al., 2015). Thus, the relative contribution of each strategy to the fitness of a population/individual is expected to vary under the novel conditions and will play a major role in the establishment and spread of the introduced individual(s) (e.g., Brown and Eckert, 2005; Lui et al., 2005; Barrett et al., 2008; Silvertown, 2008). Sexual reproduction provides the possibility for increasing genetic diversity through recombination, thus contributing not only to ameliorate loss of genetic diversity due to founder events, but also to fuel the opportunities for local adaptation and the ability of colonizers to respond to unpredictable environmental fluctuations in the new range(s) (Eckert, 2002; Novak and Mack, 2005; reviewed in Barrett, 2011). Despite the clear advantages of sexuality, asexual reproduction might be favored under unreliable circumstances, as it provides reproductive assurance and enables the persistence of individuals in unfavorable habitats for sexual reproduction or avoids the costs associated with sexual reproduction, allowing small populations and adaptive genotypes to rapidly establish and spread (Eckert, 2002; Barrett, 2015; reviewed in Vallejo-Marín et al., 2010).

Long-distance dispersal is frequently associated with strong founder effects and loss of genetic diversity, thus exposing founder individual(s) to strong mate limitation both at the establishment of the first viable population(s) and during range expansion (Baker, 1955, 1965; Stebbins, 1957). This is particularly relevant in obligated outcrossing species, such as self-incompatible or heterostylous plants, in which compatible mates might be lost during long-distance dispersal (e.g., Ornduff, 1987; Hollingsworth and Bailey, 2000; Barrett et al., 2008; Zhang et al., 2010). Under this scenario, a switch to uniparental reproduction, either through self-fertilization or increased asexual reproduction, might be selectively advantageous and foster invasion (reviewed in Pannell et al., 2015). Transitions to asexual reproduction or selfing have been documented for several introduced species, such as the clonals *Fallopia japonica* in the UK (Hollingsworth and Bailey, 2000), *Eichhornia crassipes* in China (Zhang et al., 2010), *Oxalis pes-caprae* in Mediterranean regions (Baker, 1965; Ornduff, 1987), and *Arundo donax* in Australia (Haddadchi et al., 2013), and the self-compatibles *Echinochloa microstachya* in Australia (Barrett and Husband, 1990), *Echium plantagineum* in Australia and Canary Islands (Petanidou et al., 2012), and *Gomphocarpus physocarpus* in Australia (Ward et al., 2012). Additionally, higher rates of uniparental reproduction in introduced and in invasive species compared with natives or with species that failed to establish, have been reported by several studies (Mulligan and Findlay, 1970; Rambuda and Johnson, 2004; Silvertown, 2008; van Kleunen et al., 2008; Marco et al., 2010). Nevertheless, comparative studies of plant reproductive strategies in native and invaded ranges are scarce (but see Brown and Eckert, 2005; Lavergne and Molofsky, 2007; Petanidou et al., 2012).

Sexual and asexual reproductive strategies frequently co-occur in flowering plants and, although this dual strategy was proven to be advantageous (Silander, 1985; Bengtsson and Ceplitis, 2000;

Van Drunen et al., 2015), it can also lead to allocation trade-offs and antagonist interactions between reproductive modes, such as the interference generated by clonal growth in the opportunities for mating (Handel, 1985; Vallejo-Marín et al., 2010; Barrett, 2015; Van Drunen et al., 2015). By reducing the number of mating partners and by increasing the opportunities for geitonogamous pollen dispersal, clonal growth interferes with sexual reproduction in reducing not only the offspring sired, but also its quality and fitness (e.g., Handel, 1985; Charpentier, 2002; Somme et al., 2014; but see Van Drunen et al., 2015). Allocation trade-offs occur when the production of sexual and asexual structures compete for the resources available from the total resource pool (van Kleunen et al., 2002; Thompson and Eckert, 2004; Liu et al., 2009), or through the replacement of sexual structures by asexual ones or vice-versa (e.g., production of inflorescences instead of vegetative shoots, Geber et al., 1992; production of bulbils in the inflorescences instead of flowers, Ronsheim and Bever, 2000; or production of flowers from meristems that in previous years resulted in vegetative tissue, Savinykh, 2003). Therefore, it is expected that differential fitness of the two strategies will affect the balance between sexual and asexual reproduction in the population over time (Silvertown, 2008; Vallejo-Marín et al., 2010; Van Drunen et al., 2015). If strong trade-offs between investment in sexual and asexual reproduction occur, rapid clonal expansion may limit allocation to flowering and seed production (Vallejo-Marín et al., 2010). However, evidence for fitness trade-offs between sexual and asexual reproduction is ambiguous (Van Drunen et al., 2015). Although several studies support a trade-off between the two strategies (e.g., van Kleunen et al., 2002; Thompson and Eckert, 2004; Liu et al., 2009; Van Drunen and Dorken, 2012), studies at the genet level are scarce and many of them failed to detect such trade-offs between reproductive strategies (Vallejo-Marín et al., 2010; Van Drunen and Dorken, 2012).

Oxalis pes-caprae is a clonal tristylous species native to South Africa and invasive throughout all Mediterranean regions of the world. This species reproduces by two contrasting strategies: asexually through the profuse production of bulbs (Pütz, 1994; Vilà et al., 2006) and sexually trough a highly specialized mechanism, tristyly and heteromorphic self-incompatibility system (Ornduff, 1987) that promote cross-fertilization and increased genetic diversity (Barrett, 2002). Sexual and asexual reproduction occurs in both native and invaded areas, but the contribution of each reproductive mode differs between ranges (Castro et al., 2007, 2013; Ferrero et al., 2015). In the native range, isoplethic populations occur (Ornduff, 1987; Turketti, 2010; Ferrero et al., 2015), i.e., populations with similar proportions of the three reciprocal style morphs (long-, mid-, and short-styled morphs, hereafter L-, M-, and S-morph, respectively), indicating that populations are in equilibrium and that sexual reproduction is expected to be the main reproductive mode. In the invaded range, two different scenarios appear to be occurring. Until very recently, the main scenario was a transition toward clonality in which the pentaploid (5*x*) S-morph was the dominant form, and thus asexual reproduction through bulbs has been pointed as the prevailing mechanism of reproduction and spread (Baker, 1965; Ornduff, 1987; Castro et al., 2007). Additionally, a complete sterile double-flowered form was also reported to be successfully spreading in south western Iberian Peninsula (Castro et al., 2007). However, we have recently detected the re-acquisition of sexual reproduction likely fueled by multiple introductions of compatible mating partners [tetraploid (4*x*) L-, M-, and S-morph individuals; Castro et al., 2013; Ferrero et al., 2015]. This is the first study exploring the role of reproductive traits in the invasion success of *O. pes-caprae*.

The objective of this study was to quantify changes in reproductive traits in invasive populations of *O. pes-caprae* and evaluate whether these differences could explain the prevalence of some floral forms in the invaded range of the western Mediterranean basin and be involved in the invasion success of this species. We compared the investment in sexual and asexual reproduction between native and invasive individuals, and among sexual (4*x* L-, M-, and S-morph), predominately asexual (5*x* S-morph) and obligated asexual individuals (4*x* sterile double-flowered form) found in the invaded range. Based on the invasion history of *O. pes-caprae* and on a trade-off hypothesis between allocation to sexual and asexual reproduction, we expected that, in the invaded range, selection has promoted individuals with an increased capacity for investment in asexual reproduction in detriment of sexual reproduction, especially among the mostly clonal forms; in sexual forms the trade-off between the two strategies might be more dependent of the environmental context. Still, the low sexual success of sexual forms in the invaded area (due to low mate availability and/or genetic depauperated populations; Castro et al., 2013; Ferrero et al., 2015) may generate a context promoting asexuality in comparison with sexual forms from native populations. Thus, we hypothesized that asexual forms would have significantly higher asexual potential than sexual forms in order to become dominant in the invaded range, and that both would have significantly higher asexual potential than sexual forms from the native area where sexual reproduction prevails. Our findings are discussed in the light of biological invasions and of the role of reproductive traits in successful invasion.

MATERIALS AND METHODS

Plant Species

Oxalis pes-caprae L. (Oxalidaceae), Bermuda buttercup, is a geophyte with a deeply buried annual bulb that produces subterranean stems bearing a rosette of leaves and several inflorescences of yellow flowers arranged in umbellate cymes (Vilà et al., 2006; Sánchez-Pedraja, 2015). It is a tristylous species with a heteromorphic self-incompatibility system (Ornduff, 1987). Thus, the production of viable offspring only occurs after legitimate pollination between individuals with reciprocal style morphs. Double-flowered sterile individuals have also been frequently observed in the western Mediterranean basin (Castro et al., 2007) and sporadically in South Africa (Salter, 1944; Suda and Oberlander, personal communication). The Bermuda buttercup has a high capacity for asexual reproduction through a profuse production of bulbs. The main bulb produces a

fasciculate root with contractile properties that grows deeper in the soil some centimeters each year (Pütz, 1994), and later in the season or under stressful conditions (e.g., soil perturbation), the subterranean stems produce a high number of small bulbs (Young, 1968; Verdaguer et al., 2010; authors personal observations). Furthermore, *O. pes-caprae* is a polyploid species, with diploid ($2n = 2x = 14$ chromosomes), tetraploid ($2n = 4x = 28$ chromosomes), and pentaploid ($2n = 5x = 35$ chromosomes) individuals. In South Africa, all cytotypes have been reported, although the $5x$ cytotype appears to be extremely rare (Ornduff, 1987; te Beest et al., 2012; Ferrero et al., 2015). Contrarily, the $5x$ is the dominant cytotype in the invaded ranges worldwide, although in Australia and recently in the western Mediterranean region, the $4x$ has also been reported (Symon, 1961; Michael, 1964; Castro et al., 2007, 2013).

The Bermuda buttercup was introduced into the Mediterranean basin in the end of the 18th century, most probably multiple times (Vignoli, 1937; Galil, 1968; Signorini et al., 2011), and spread widely afterward. The species was soon recognized as a weed in several Mediterranean areas (e.g., Sicily, Hildebrand, 1887; Canary Islands, Morris, 1895; Algeria, Ducellier, 1914; Balearic Islands, Knoche, 1922; Malta and neighbor islands, Borg, 1927; Tunisia, Chabrolin, 1934), including Portugal where it was described as abundant in orchards (Henriques, 1920; Vasconcelos and Moreira, 1976). Early introductions of the plant occurred due to its ornamental value, and later through soil movement in agriculture, horticulture, and gardening (Michael, 1964; Signorini et al., 2011). Still, the routes of (repeated) introduction to the Mediterranean basin and other invaded regions are not clear.

Field Sampling

Extensive field sampling for bulb harvesting was conducted during February and March 2010 in the invaded range of the western Mediterranean basin (MB), and during August 2011 in the native area, South Africa (SA). All the necessary permits for plant collection were obtained. In the invaded range, an additional effort was made to sample throughout the regions where trimorphic populations and the sterile double-flowered form are more common (Castro et al., 2007, 2013). All floral forms and cytotypes found in this invaded range were included in our study, i.e., the $4x$ L-morph, $4x$ M-morph, $4x$ S-morph, $5x$ S-morph, and the $4x$ sterile double-flowered individuals (Supplementary Table 1). In the native range, the field sampling was conducted across most of the latitudinal and longitudinal distribution of the species (Salter, 1944; Supplementary Table 1). Our extensive sampling in the native range confirmed previous results showing that the $5x$ S-morph and the double-flowered individuals are extremely rare in South Africa (Ferrero et al., 2015). For this reason, only $4x$ L-morph, $4x$ M-morph, and $4x$ S-morph native plants were included in this comparative study. In each population, we sampled bulbs from 10 individuals per floral form, separated at least 5-m apart to avoid re-sampling clones of the same individual. Sampled populations were characterized for style morph frequency and cytotype composition as described in Castro et al. (2013).

Common Garden Experiment

To investigate if there were differences in sexual and asexual reproductive traits between native and invasive plants, we conducted a common garden experiment at the Botanical Garden of the University of Coimbra, where individual plants from both areas were grown outdoors under similar optimal conditions. To remove potential maternal effects, bulbs from SA and the MB were grown for one and two generations, respectively, before sexual and asexual investment traits were measured. In June 2012, all bulbs were harvested and stored in paper bags. During September 2012, they were weighed and weight values were recorded as initial bulb weight. The analysis of the dispersion of the initial bulb weight allowed us to select one bulb per individual matching similar overall mean weights (mean $\pm$ SD, 0.463 ± 0.086 g). In total, 338 bulbs were selected, representing 29 populations and 137 individuals from the native area, and 13 populations and 201 individuals from the invaded range (Supplementary Table 1). This selection reflected the different reproductive strategies found in SA and MB: sexual ($4x$ L-, M-, and S-morphs), predominately asexual ($5x$ S-morph), and obligated asexual ($4x$ sterile double-flowered form).

Bulbs were individually planted $\sim$2.0 cm below the soil surface in 2 L plastic pots (9.6 cm $\times$ 9.6 cm $\times$ 21.5 cm) filled with commercial substrate, and pots were randomized at the beginning of the experiment. Before flowering, plants were covered with a mosquito net to avoid undesirable pollination. To characterize sexual and asexual reproduction performance, we measured the following traits: (a) bulb viability, (b) occurrence of flowering; (c) floral display; (d) biomass invested in sexual and asexual structures; and (e) production of diaspores through sexual and asexual means (fruit, seed, and bulb production). During the flowering peak, we classified each individual as either vegetative or reproductive, and we collected one flower per inflorescence, when produced, into individual paper bags for later estimation of: (a) mean flower weight and (b) total weight investment in flowers per plant. Inflorescences were periodically monitored, and were collected when senescent, allowing us to simultaneously assess: (a) the total number of flowers produced per plant, i.e., floral display, and (b) the total investment in inflorescences measured as dry weight. Total and average investments in the production of sexual structures were estimated for each plant. Fruit and seed production were obtained by cross pollinating three flowers per plant using reciprocal style morphs of the same area of origin. Fruit set was calculated as the proportion of flowers that developed into fruits and seed production as the mean number of seeds per fruit. We calculated a measure of sexual potential for each plant by multiplying the total number of flowers produced by the mean fruit and seed production. Bulbs were harvested by the end of the season. The investment in asexual structures was quantified by assessing the number of bulbs produced per plant, and total and mean bulb weight per plant.

Statistical Analysis

Data was grouped according to the following criteria: (a) area of origin (South Africa native range, SA, and invaded range of

the Mediterranean Basin, MB) and (b) reproductive strategy. We defined reproductive strategy as: *sexual*, including 4*x* plants with morphologically functional flowers (Sex); *predominantly asexual*, 5*x* S-morph plants that reproduce mostly asexually despite some sporadic ability to produce viable offspring (Asex; Castro et al., 2013; Costa et al., 2014, in press); and, *obligated asexual*, 4*x* double-flowered sterile mutants (St). Accordingly, all individuals were assigned to one of the following groups: South African 4*x* sexual forms (SA4*x*Sex), Mediterranean basin 4*x* sexual forms (MB4*x*Sex), Mediterranean basin 5*x* predominantly asexual form (MB5*x*Asex) and Mediterranean basin 4*x* obligated asexual form (MB4*x*St, double-flowered sterile mutant).

The above groups were defined as fixed factor in generalized linear mixed models (GLMM) to assess differences in sexual and asexual traits. GLMMs enabled us to model variables that did not completely fulfill the assumptions of a standard linear model and had the advantage to allow the incorporation of random factors in the models (Bolker et al., 2009). Although the initial bulb weight was fairly homogenous, this variable was included as covariate to account for possible differences caused by bulb weight. Population and individual were defined as random factors and these were removed from the models whenever their variance was lower than the variance of the residuals (Bolker et al., 2009). When both random factors were removed, a generalized linear model (GLM) was used instead (Supplementary Table 2). A binomial distribution with a logit link function was used to model bulb viability and probability of flowering; a Poisson distribution with a log link function was used to model the number of flowers, inflorescences and bulbs per plant; and a Gaussian distribution with an identity link function was used to model the mean number of flowers per inflorescence, mean flower and inflorescence weight, total flower and inflorescence weight per plant, total weight of sexual structures per plant, fruit set (arcsine transformed), mean seed production, sexual potential, mean and total bulb weight. In all cases, differences between least-square means were tested pairwise through multiple comparisons. To evaluate the existence of trade-offs between sexual and asexual investments, correlations between the amount of biomass invested in sexual and asexual structures were calculated for the entire dataset and for each group separately. All statistical analyses were performed in R version 3.1.1 (R Core Team, 2014) using the packages "car" for GLMs and Type-III analysis of variance (Fox and Weisberg, 2015), "nlme" for linear and non-linear mixed models (Pinheiro et al., 2015), and "multcomp" for multiple comparisons after Type-III analysis of variance (Hothorn et al., 2008), and "stats" for GLMs (R Core Team, 2014).

RESULTS

Bulb Viability and Probability of Flowering

Results from all statistical analyses are summarized in Supplementary Table 2. Bulb viability was high, varying between 88% in invasive sexual individuals (MB4*x*Sex) and 94% in native sexual individuals (SA4*x*Sex), with no statistically significant differences being observed among groups ($\chi^2_{3,338} = 3.49$, $P = 0.322$; Supplementary Figure 1A).

The probability of producing floral structures differed among groups ($\chi^2_{3,305} = 11.23$, $P = 0.011$), with the obligated asexual individuals (MB4*x*St) having a significantly higher probability to remain vegetative than the other invasive groups (i.e., MB4*x*Sex and MB5*x*Asex; $P < 0.05$), while native sexual plants had intermediate values between the two extremes (Supplementary Figure 1B).

Sexual Traits: Floral Display

Floral display differed among groups in terms of the number of flowers per inflorescence ($\chi^2_{3,276} = 19.07$, $P < 0.001$), number of inflorescences per plant ($\chi^2_{3,276} = 47.89$, $P < 0.001$) and total number of flowers per plant ($\chi^2_{3,276} = 31.68$, $P < 0.001$; **Figures 1A–C**). The native sexual plants and the invasive predominantly asexual plants (MB5*x*Asex) produced inflorescences with more flowers than the invasive sexual individuals (MB4*x*Sex; $P < 0.05$), while the obligated asexual form (MB4*x*St) had a lower and highly variable mean value not differing from the other three groups (**Figure 1A**). The groups with ability for sexual reproduction (SA4*x*Sex and MB4*x*Sex), even if only sporadically (MB5*x*Asex), produced significantly more inflorescences than the obligated asexual individuals, resulting in larger total floral display per plant ($P < 0.05$; **Figures 1B,C**).

Significant differences among groups were also observed in the biomass invested for the production of sexual structures (**Figures 1D–F**, Supplementary Figure 1), namely in the mean flower and inflorescence weight ($\chi^2_{3,276} = 820.80$, $P < 0.001$ and $\chi^2_{3,276} = 51.78$, $P < 0.001$, respectively; **Figures 1D–E**), total flower and inflorescence weight per plant ($\chi^2_{3,276} = 28.08$, $P < 0.001$ and $\chi^2_{3,276} = 44.39$, $P < 0.001$, respectively; Supplementary Figures 1C,D), and total weight of sexual structures per plant ($\chi^2_{3,276} = 32.30$, $P < 0.001$; **Figure 1F**). Obligated asexual individuals (MB4*x*St) had significantly heavier flowers, followed by the MB5*x*Asex, and the native and invasive sexual individuals had lower flower weights ($P < 0.05$; **Figure 1D**). The same trend was observed for total flower weight per plant, except for the obligated asexual individuals (MB4*x*St) which produced less inflorescences (**Figure 1B**) and consequently less flowers (**Figure 1C**), lower total flower weight (**Figure 1F**) and reduced investment in total flower biomass (Supplementary Figure 1C). A different scenario was found for mean inflorescence weight, which was significantly lower for the obligated asexual individuals and sexual native plants. Sexual invasive individuals presented intermediate inflorescence weight, and invasive predominantly asexual plants had significantly heavier inflorescences ($P < 0.05$; **Figure 1E**; a similar pattern is observed for the total inflorescence weight per plant; Supplementary Figure 1D). Despite the differences in the number and biomass of reproductive structures among all groups, the total investments in the production of sexual structures per plant did not differ among groups except for the MB5*x*Asex, which presented significantly higher weights ($P < 0.05$; **Figure 1F**).

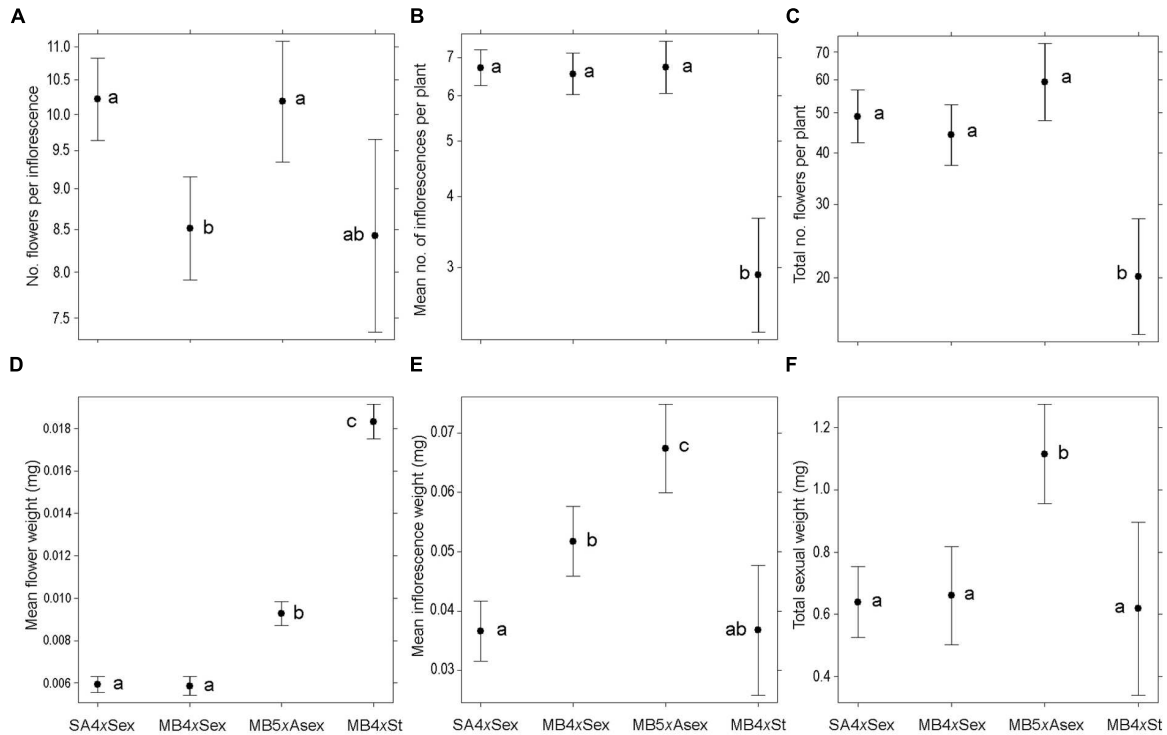

FIGURE 1 | Floral display and biomass invested in sexual reproductive structures among different forms of *Oxalis pes-caprae* from its native and invaded areas and with distinct reproductive strategies: South African 4*x* sexual forms (SA4*x*Sex), Mediterranean basin 4*x* sexual forms (MB4*x*Sex), Mediterranean basin 5*x* predominantly asexual form (MB5*x*Asex), and Mediterranean basin 4*x* obligated asexual form (MB4*x*St, sterile double-flowered form). (A) Mean number of flowers per inflorescence; **(B)** Mean number of inflorescences per plant; **(C)** Total number of flowers per plant; **(D)** Mean flower weight (mg); **(E)** Mean inflorescence weight (mg); **(F)** Total weight of sexual structures per plant (mg). Values are given as model-adjusted back-transformed least-square means and 95% confident intervals. Significant differences among factors are indicated with different letters ($P < 0.05$).

Production of Dispersal Units: Sexual and Asexual Strategies

Sexual fitness differed significantly among groups (fruit set: $\chi^2_{2,251} = 47.38$, $P < 0.001$; seed production: $\chi^2_{2,246} = 89.44$, $P < 0.001$; and sexual potential: $\chi^2_{2,251} = 15.77$, $P < 0.001$). Fruit and seed production were significantly lower in invasive plants than in native ones and, among invasive groups, it was significantly lower in the predominantly asexual individuals ($P < 0.05$; **Figures 2A,B**). The calculation of a measure of sexual potential revealed that native sexual individuals had a significantly higher success than invasive plants ($P < 0.05$); however, no significant differences were detected in sexual potential between sexual invasive and predominantly asexual invasive individuals (**Figure 2C**).

Asexual traits also differed significantly among groups, either measured as number of bulbs ($\chi^2_{3,305} = 350.13$, $P < 0.001$), mean bulb weight ($\chi^2_{3,305} = 27.36$, $P < 0.001$) or total bulb weight per plant ($\chi^2_{3,305} = 211.13$, $P < 0.001$). Invasive sexual plants produced more bulbs per plant than predominantly asexual individuals, which also produced more bulbs than native sexual and invasive obligated asexual individuals ($P < 0.05$; **Figure 2D**). However, predominantly asexual individuals had significantly heavier bulbs than native and invasive sexual plants ($P < 0.05$), while obligated asexuals had fairly heavy, but highly heterogeneous bulbs that did not differ significantly from the other invasive groups (**Figure 2E**). There was a clear and significantly higher investment in total bulb weight by the invasive sexual and predominantly asexual plants than the other groups, as well as in the obligated asexual individuals in comparison with native sexual plants ($P < 0.05$; **Figure 2F**).

Trade-off between Sexual and Asexual Investment

No trade-off was observed in the biomass invested in sexual and asexual structures. On the contrary, the production of sexual structures was positively correlated with the biomass invested in the production of bulbs, except for invasive sexuals and obligated asexuals (total: $r = 0.214$, $P < 0.001$; analyses by group: SA4*x*Sex: $r = 0.286$, $P = 0.0182$; MB4*x*Sex: $r = -0.0223$, $P = 0.800$; MB5*x*Asex: $r = 0.449$, $P < 0.001$; MB4*x*St: $r = 0.183$, $P = 0.381$; **Figure 3**).

DISCUSSION

Our results indicate the occurrence of evolutionary changes in the reproductive traits of invasive populations of *O. pes-caprae*. Indeed, most of the traits evaluated, differed between native

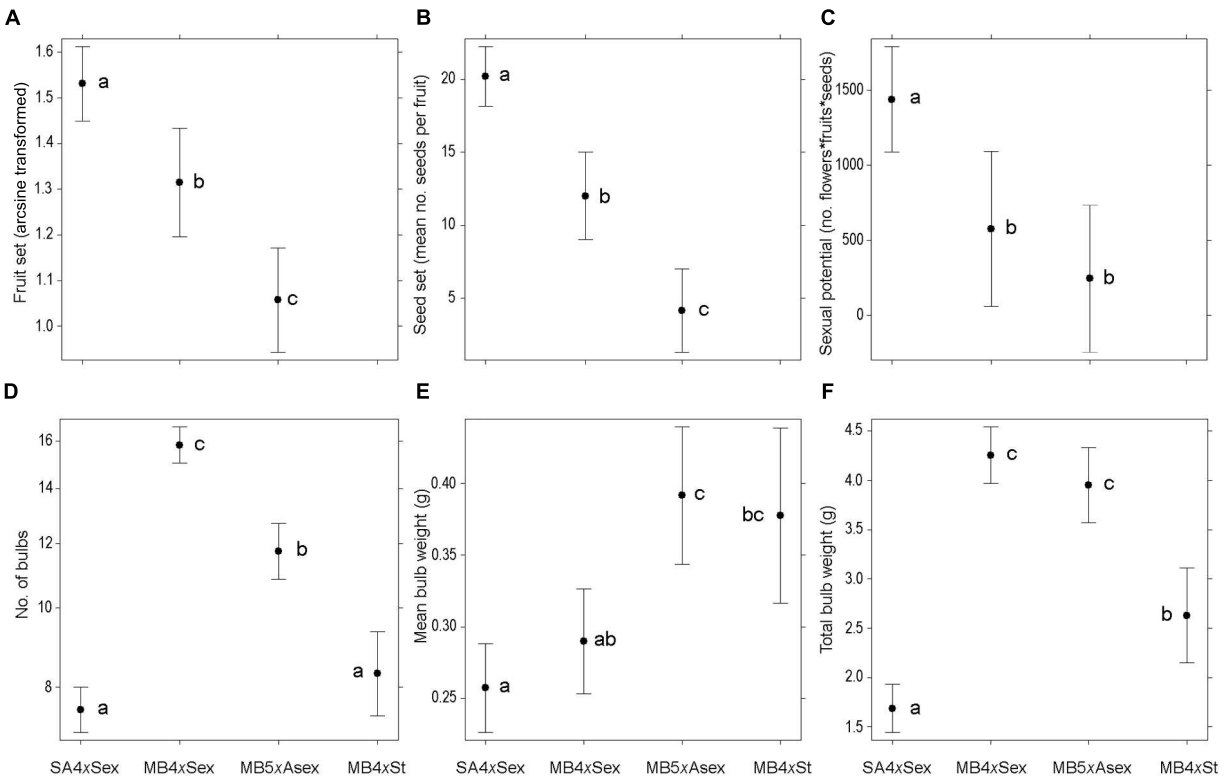

FIGURE 2 | Production of sexual and asexual diaspores among different forms of *Oxalis pes-caprae* from its native and invaded areas and with distinct reproductive strategies: South African 4*x* sexual forms (SA4*x*Sex), Mediterranean basin 4*x* sexual forms (MB4*x*Sex), Mediterranean basin 5*x* predominantly asexual form (MB5*x*Asex) and Mediterranean basin 4*x* obligated asexual form (MB4*x*St, sterile double-flowered form). (A) Fruit set, given as arcsine of the proportion of flowers developing into fruit; **(B)** Seed production, given as the mean number of seeds per fruit; **(C)** Sexual potential, given as a measure of sexual ability of the plant obtained multiplying the number of flower produced in a plant by the fruit and seed production; **(D)** Mean number of bulbs per plant; **(E)** Mean bulb weight (mg); **(F)** Total weight of the bulbs produced per plant (mg). Values are given as model-adjusted back-transformed least-square means and 95% confident intervals. Significant differences among factors are indicated with different letters ($P < 0.05$).

and invasive populations and among individuals with different reproductive strategies when grown in the same environment. In particular, we found that: (1) overall, plants with sexual ability (including the 5*x* S-morph) had higher probability of flowering and larger floral displays than the sterile forms; (2) the total investment in the production of floral structures was significantly higher in the 5*x* form than in the remaining forms; this suggests an effect of the ploidy level in the overall size of the structures and, in the case of the sterile double-flowered form, a trade-off between the number of flowering structures and the resources needed to produce them (i.e., heavier sterile flowers resulting in lower number of inflorescences); (3) differences in the production of bulbs and seeds revealed that native plants had higher sexual fitness, while a transition toward clonality was clear for the invasive forms; (4) differences were also observed among invasive individuals, with the sexual forms producing more dispersal units (seeds and small bulbs), the predominantly asexual form producing an inter-medium number of large bulbs, and the sterile form being apparently less aggressive and producing less, yet large, bulbs; (5) finally, no trade-off between sexual and asexual investments was observed. Below, we discuss our results in light of the complex invasion history of *O. pes-caprae* and draw hypotheses on how reproductive traits could have been involved in the invasion success and in the prevalence of some forms in the invaded range of the western Mediterranean region.

Reproduction: Traits and Strategies

Reproduction determines the number and genetic composition of dispersal units, being vital for the establishment and spread of plant populations after long-distance dispersal (e.g., Sakai et al., 2001; Ness et al., 2010). The relative contribution of different reproductive modes varies depending on the ecological and genetic factors under which colonizers are subjected (e.g., Dorken and Eckert, 2001; Eckert, 2002; Herben et al., 2015). Our results showed remarkable differences in several reproductive traits between ranges and among forms with different reproductive strategies. Overall, a transition to clonality was observed among invasive plants: native individuals had a higher sexual fitness than invasive ones, which in contrast had higher asexual fitness than natives. These observations matched our expectations mainly by two reasons, described below.

Firstly, genetic diversity of native populations is higher than that of invasive ones (Ferrero et al., 2015), and this is

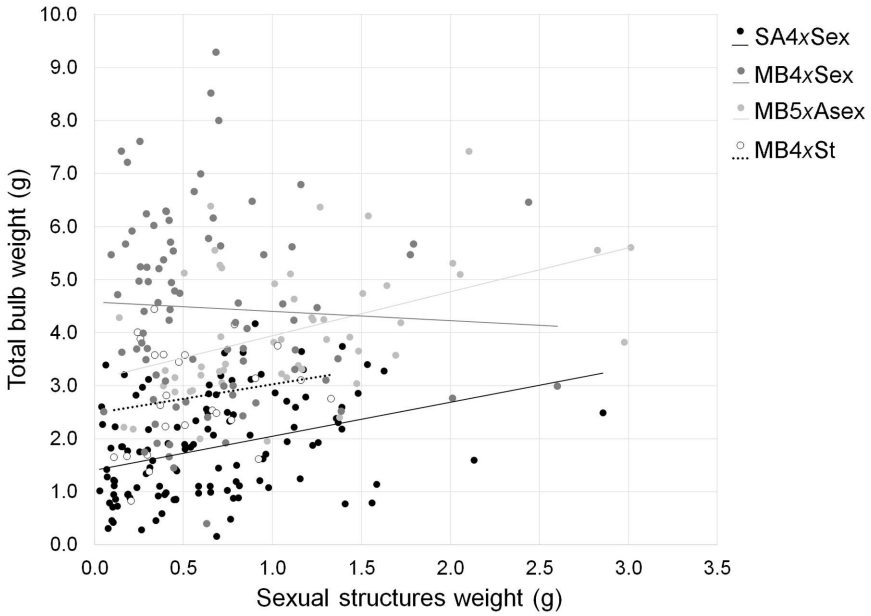

FIGURE 3 | Correlation between biomass invested in the production of sexual and asexual structures among different forms of *Oxalis pes-caprae* from its native and invaded areas and with distinct reproductive strategies: South African 4*x* sexual forms (SA4*x*Sex), Mediterranean basin 4*x* sexual forms (MB4*x*Sex), Mediterranean basin 5*x* predominantly asexual form (MB5*x*Asex) and Mediterranean basin 4*x* obligated asexual form (MB4*x*St, sterile double-flowered form).

expected to affect the sexual fitness. Sexual reproduction is the main mechanism of reproduction in the native range, where floral polymorphism promotes outcrossing and thus genetic diversity and frequency dependent selection governs isoplethic populations (Ornduff, 1987; Turketti, 2010; Ferrero et al., 2015). Contrarily, invasive populations are highly constrained by the scarcity of compatible mates (Castro et al., 2007, 2013), being dominated by 5*x* S-morphs with residual sexual reproduction (Costa et al., 2014, in press), and thus with low recombination probabilities. Additionally, strong founder effects led to a decrease in genetic diversity of invasive populations (Ferrero et al., 2015). Altogether, these factors significantly impacted genetic composition of invasive populations and, consequently, plant fitness under outcross pollinations. Genetic depauperation after long distance dispersal has been described in several other invasive species (e.g., Dlugosch and Parker, 2008; Zhang et al., 2010), and although multiple introductions can ameliorate their consequences (Novak and Mack, 2005; Dlugosch and Parker, 2008; Simberloff, 2009), negative impacts of low genetic diversity for plant reproduction have been shown (Barrett, 2002; Crawford and Whitney, 2010). However, genetic bottlenecks do not necessarily hinder the adaptive potential of invasive species (Barrett et al., 2008; Dlugosch and Parker, 2008; Rollins et al., 2013).

Secondly, under unfavorable conditions for sexual reproduction in invasive populations, i.e., strong mate limitation (either due to monomorphic populations or due to the predominance of asexual 5*x* individuals; Baker, 1965; Ornduff, 1987; Castro et al., 2007, 2013; Ferrero et al., 2015), we expected that selection would benefit individuals with an increased capacity for investment in asexual reproduction. Indeed, uniparental reproduction has been proposed to be selectively advantageous under scenarios of strong mate limitation, such as invasions, rapid range expansion, island colonization and meta-population dynamics (Baker's Law; Baker, 1965; Pannell et al., 2015). Our results corroborate this prediction: regardless of the reproductive strategy, invasive *O. pes-caprae* forms invested significantly more in asexual reproduction than natives. An enhancement in clonal reproduction in invasive populations in comparison with natives has also been observed in several other species. For example, invasive *Butomus umbellatus* individuals were more likely to produce bulbils than native individuals (Brown and Eckert, 2005); rapid selection of genotypes with stronger vegetative growth was observed in *Phalaris arundinacea* (Lavergne and Molofsky, 2007), and greater vegetative reproduction in the invasive ranges of *Achillea millefolium* and *Hypericum perforatum* than on their native ranges (Beckmann et al., 2009). Interestingly, differences in bulb production have also been observed among invasive populations of *O. pes-caprae* in the Mediterranean basin, with insular populations having higher dispersal potential than continental ones (Vilà and Gimeno, 2006). Clonal reproduction was one of the traits identified by Baker (1965) for the "ideal weed" and there are several studies addressing invasive species traits that support this reproductive strategy as one of the features involved with successful invasions (e.g., Pyšek and Richardson, 2007; Silvertown, 2008; Marco et al., 2010).

As expected, individuals with the ability to reproduce sexually, including the predominantly asexual plants, invested more in the production of floral structures than the sterile form. This is in accordance with a strategy to promote sexual reproduction either by investing in attractive floral display (i.e., larger inflorescences, inflorescences with larger flower displays) or by producing more sexual potential units (Barrett, 2002). A trade-off between the number of inflorescences produced and the considerable amount of energy necessary to produce double-flowers might explain the reduced floral display of the sterile double-flowered form (see below). Interestingly, the 5*x* form produced larger flowers and larger inflorescences that resulted in a higher biomass investment without a detrimental impact in the floral display. The production of larger reproductive structures is likely related with the ploidy level, since polyploidy is hypothesized to drive significant changes in cell size and, consequently, in overall organ size (Levin, 2002). Apparently, this higher investment did not lead to allocation trade-offs since the 5*x* cytotype produced similar floral display to other sexual forms.

Besides the differences in the production of sexual and asexual diaspores detected between ranges, different strategies were also observed among invasive forms. As expected, sexual fitness was higher in the 4*x* sexual forms than in the 5*x* form, and null in the sterile double-flowered form where the sexual organs were replaced by petals due to a mutation in the genes responsible for the floral development (Weigel and Meyerowitz, 1994). The lower sexual fitness of the 5*x* individuals after outcrossing is mainly due to its odd ploidy level; although these 5*x* individuals are able to produce some viable gametes, they also produce unviable gametes with variable ploidies (Vignoli, 1937; Signorini et al., 2013; Costa et al., 2014), diminishing significantly the production of offspring through seed. However, and as described above, the 5*x* individuals produced slightly larger floral displays and bigger floral structures; these features increase the number of gametes and the attractiveness of the plants for pollinators, which might contribute to ameliorate the low sexual potential of the 5*x* individuals. Additionally, differences in the asexual traits were also detected among invasive forms. Under optimal resource conditions, the obligated asexuals invested significantly less in bulb production than the other invasive forms, producing larger bulbs but in smaller amounts (like native plants) than the other invasive forms. Interestingly, the sexual and predominately asexual forms allocated a similar amount of energy to the production of bulbs; however, while the former invested resources in producing many small bulbs, the later invested in less but larger bulbs. These patterns agree with trade-off models for propagule number and size which predict that in optimal environments it is preferable to maximize offspring quantity, whereas in stressful conditions (such as the limitation of sexual partners) it is preferable to invest in offspring quality (Smith and Fretwell, 1974; Sadras, 2007). Based on this model, we could hypothesize that selective pressures during the invasion of the predominantly asexual form might have benefited larger bulbs, while selective pressures over asexual propagule production are not expected to be so strong for the sexual forms that have an additional reproductive mode (Costa et al., unpublished results). *Oxalis pes-caprae* bulb weight has been pointed as an important feature of the invasion process, especially under stressful conditions (Lane, 1984; Sala et al., 2007), with parental bulb weight significantly impacting plant biomass in shaded environments, as evident by the production of significantly more leaves in plants originated from bigger bulbs than from smaller ones (Verdaguer et al., 2010). However, although fitting nicely the results, there are several lines of evidence that do not completely support this hypothesis and make our findings difficult to interpret. First, it is difficult to disentangle the effects of ploidy level from those related with evolutionary changes. Although, bulb size of the offspring of 4*x* sterile double-flowered form and the 5*x* S-morph were similar, the larger bulbs in the 5*x* individuals might be driven by ploidy, similarly to the pattern observed in the flowering structures (results herein) and to the patterns observed in other polyploid complexes (Levin, 2002). Second, 5*x* S-morph individuals showed that parent bulb weight has a small overall effect on *O. pes-caprae* plant biomass (Sala et al., 2007; Verdaguer et al., 2010). In general, bulbs emerged successfully and vigorously regardless of their sizes (Vilà et al., 2006; Verdaguer et al., 2010), still, parent bulb size might be particularly important for plant emergence and initial development, depending on the conditions where the plant is growing (Vilà et al., 2006; Sala et al., 2007; Verdaguer et al., 2010). Regardless of the effects in early stages, bulb size was not determinant for the development of adult plants and subsequent offspring production, possibly because further plant growth might become independent of this storage organ once the plant starts to photosynthesize. Finally, the production of bulbs in *O. pes-caprae* was shown to be plastic and highly dependent of nutrient availability (Sala et al., 2007). If bulb weight has no fitness advantage, then producing many small bulbs would be advantageous, especially when mate limitation is strong and no allocation trade-off between sexual and asexual investment is observed (see below). In this context, sexual invasive forms have a higher dispersal potential, through both sexual and asexual means than the other invasive forms and might become widespread in the future.

Bermuda Buttercup Invasion History: What Have We Learned So Far?

The Bermuda buttercup is a classic example in biological invasions, known as a strictly asexual form that successfully spread in Mediterranean climate regions of the world (5*x* S-morph; Baker, 1965). However, the origin of this invasive form is still unclear and the colonization history revealed to be more complex and dynamic than previously envisaged. Native populations are composed of the 4*x* cytotype, with the 5*x* S-morph being extremely rare (Michael, 1964; te Beest et al., 2012; Signorini et al., 2013; Ferrero et al., 2015). Contrarily, the 5*x* S-morph dominates all Mediterranean climate regions, except in Australia where both asexual (monomorphic 5*x* S-morph populations) and sexual populations (4*x* trimorphic populations) have been reported (Symon, 1961; Michael, 1964; Ferrero, personal observations). The most accepted hypothesis is that the 5*x* S-morph has been originated from 4*x* individuals in the introduced range and subsequently introduced in several areas of

the world (Krejčíková et al., 2013; Signorini et al., 2013; Ferrero et al., 2015; most probably multiple times in the Mediterranean basin, Signorini et al., 2011), including South Africa, where it was recently reported in a new semi-natural location for the first time (Ferrero et al., 2015). The combination of several factors, including strong heteromorphic incompatibility system, lack of compatible mates and odd ploidy, constrained the production of dispersal units mostly to asexual means, and consequently the successful spread of this form in introduced ranges became dependent on bulb production (Baker, 1965; Ornduff, 1987). Our results strongly support this hypothesis showing a clear selection toward clonality through a significantly increase in the number of bulbs as well as in their size (the latter driven or not exclusively by the ploidy level) in comparison with natives. Producing more bulbs would be selectively advantageous since it increases the number of propagules, while larger bulbs may confer significant advantages under stressful environments, allowing faster plant emergence and providing more reserves, which will translate into larger plants (Vilà and Gimeno, 2006; Sala et al., 2007; Verdaguer et al., 2010; Tavares, 2014).

However, the story does not end here. In the western Mediterranean region, invasive populations seem to be changing very rapidly (Castro et al., 2007, 2013; Costa et al., 2014, in press). Molecular studies have shown an invasion punctuated by multiple introductions of other floral morphs comprised of the tetraploid ploidy level (Ferrero et al., 2015), and field surveys detected a reacquisition of sexual reproduction in this region (Castro et al., 2013; Costa et al., 2014, in press). Although the introduction or incipient occurrence of M- and L-morphs would constitute a source of compatible mates, these individuals are still under a scenario of strong mate limitation due to the dominance of the predominantly asexual 5*x* S-morph. Thus, they will be subjected to similar strong selective pressures toward uniparental reproduction. Our results clearly support that these new individuals have also diverged from native populations and present an inversion toward uniparental reproduction via asexual reproduction (results herein), but also via changes in the strength of the incompatibility system (Costa et al., unpublished results). Additionally, our results show that these sexual forms have superior reproductive fitness in comparison with the 5*x* S-morph and the 4*x* sterile double-flowered form. So, how can we explain the distribution patterns in the western Mediterranean basin? Based on the reproductive traits, the current distribution patterns can only be explained under a scenario of different introduction timings, first with the introduction and spread of the 5*x* individuals and more recently with the introduction of 4*x* sexual plants that are starting to become more dominant than previously documented. Given the superior reproductive fitness of the 4*x* sexual individuals, they are expected to become more dominant in the future. Still, other life-history transition stages and ecological responses, including bulb viability and emergence, competitive ability, resistance to herbivory and response to soil disturbance, need to be addressed in future studies in order to fully characterize the fitness of each form.

The successful spread of the sterile double-flowered form in south–west Iberian Peninsula is particularly intriguing. This form had the lowest dispersal potential among invasive forms and therefore it is likely under a competitive disadvantage with other floral forms. Recent molecular studies have shown a close relationship between these individuals and native plants, supporting the occurrence of several multiple introductions. These multiple introductions might have provided a sufficiently high number of propagules to mediate a successful invasion process. Although species traits are extremely important, several studies have shown that propagule pressure is also a determinant factor for successful invasion (Novak and Mack, 2005; Colautti et al., 2006; Dlugosch and Parker, 2008; Simberloff, 2009). Interestingly, propagule pressure was also shown to be important in colonization by *O. pes-caprae* along altitudinal gradients within invaded areas (Ross et al., 2008). Additionally, besides ecological and life history traits, human mediated dispersal (e.g., in earlier stages as ornamental plant, and currently through soil movements in agriculture, horticulture and gardening, or through land translocations during road constructions; Michael, 1964; Signorini et al., 2011; Castro et al., 2013) might have also promoted the dispersal of this invasive form, as well as the others (Pyšek and Richardson, 2007).

Trade-offs between Sexual and Asexual Strategies

No trade-off between the production of sexual and asexual structures has been detected in *O. pes-caprae*. These observations agree with studies in other species (Vallejo-Marín et al., 2010; Van Drunen and Dorken, 2012) and with previous experiments with *O. pes-caprae* (Vilà and Gimeno, 2006; Verdaguer et al., 2010). This lack of a trade-off might be explained by the particular developmental processes of the plant as the production of flowering structures and bulbs are asynchronous in *O. pes-caprae* likely reducing the competition for resources between both reproductive processes. In the first half of the plants' life cycle, most of the energy is redirected to growth and flowering, and only afterward, when the aboveground part of the plant starts to senesce, energy is directed to the production of underground structures, namely to the production of bulbs (Pütz, 1994; Verdaguer et al., 2010). Indeed, we observed the opposed pattern, with a positive correlation between bulb and flower biomass. This could simply be a reflection of plant size rather than resource management strategies.

CONCLUSION

Different sexual and asexual reproductive traits were quantified between native and invasive populations, as well as among different forms within invasive populations. Different reproductive strategies and ecological and genetic contexts created by long-distance dispersal seem to generate divergent selective pressures in several sexual and asexual reproductive traits. The introduction process seems to have promoted clonal reproduction and this is most probably the major trait driving the invasion success of *O. pes-caprae*; however, invasive sexual forms have increased dispersal potential and additional means to produce dispersal units and promote heterozygosity. Consequently, invasive sexual forms are expected to be in

competitive advantage in relation to the predominately asexual and obligated asexual plants, and thus could become widespread in the invaded range in the future. Historical processes, with the introduction of the predominantly asexual 5*x* S-morph first and more recently of the 4*x* sexual morphs, were probably important in establishing the current distributional patterns of the different forms in the western Mediterranean basin. This study shows that invasion processes can be incredibly complex and dynamic, while the interaction between ecological and genetic constraints determined by the invasion process might result in different reproductive strategies which in turn determine the success of invasive populations.

AUTHOR CONTRIBUTIONS

SC, VF, LN, and JL designed the experiment; SC, VF, JC, and JL conducted field collections; MC, with the collaboration of all the authors, conducted the common garden experiment; SC analyzed the data with the other authors participating in the discussion of the results; SC and MC, with contribution of all the authors, wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00874

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Division of Labor Brings Greater Benefits to Clones of *Carpobrotus edulis* in the Non-native Range: Evidence for Rapid Adaptive Evolution

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Why some species become invasive while others do not is a central research request in biological invasions. Clonality has been suggested as an attribute that could contribute to plant invasiveness. Division of labor is an important advantage of clonal growth, and it seems reasonable to anticipate that clonal plants may intensify this clonal attribute in an invaded range because of positive selection on beneficial traits. To test this hypothesis, we collected clones of *Carpobrotus edulis* from native and invasive populations, grew pairs of connected and severed ramets in a common garden and under negative spatial covariance of nutrients and light to induce division of labor, and measured biomass allocation ratios, final biomass, and photochemical efficiency. Our results showed that both clones from the native and invaded range develop a division of labor at morphological and physiological level. However, the benefit from the division of labor was significantly higher in apical ramets from the invaded range than in ramets from the native area. This is a novel and outstanding result because it provides the first evidence that the benefit of a key clonal trait such as division of labor may have been subjected to evolutionary adaptation in the invaded range. The division of labor can therefore be considered an important trait in the invasiveness of *C. edulis*. An appropriate assessment of the influence of clonal traits in plant invasions seems key for understanding the underlying mechanisms behind biological invasions of new environments.

Keywords: biological invasions, biomass allocation, *Carpobrotus edulis*, chlorophyll fluorescence, clonal integration, division of labor, local adaptation, spectral reflectance

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INTRODUCTION

The establishment of invasive alien species modifies the stability and functioning of populations, communities, and ecosystems, displaces native species and, as consequence, promotes a loss of biodiversity (Vitousek et al., 1996; Mack et al., 2000; Strayer, 2012). In a globalized world, biological invasions and their negative impacts increased dramatically during the last decades and represent

one of the most important threats to the conservation of biodiversity worldwide (Vitousek et al., 1996; Mack et al., 2000). The study of biological invasions is a rapidly developing field in modern ecology, and a crucial request in this research area is to determine the traits underlying the invasion process. However, this issue remains unsolved (Roy, 1990; Groves and di Castri, 1991; Lodge, 1993; Rejmánek and Richardson, 1996; Alpert et al., 2000; Levine et al., 2003) and more effort needs to be devoted to identify the mechanisms that explain the success of invasive species (Alpert et al., 2000; Levine et al., 2003; Blackburn et al., 2011).

It seems reasonable to assume that some plant characteristics might better explain the success of invasive species than others. In particular, clonal propagation has been suggested as an attribute that could contribute to plant invasiveness (Pyšek, 1997; Liu et al., 2006; Wang et al., 2008; Song et al., 2013). In fact, many of the most aggressive invasive plant species show clonal growth, and a recent study has highlighted the importance of traits related to clonal propagation in successful invaders (Song et al., 2013). Key aspects such as physiological integration or the capacity for a division of labor make a significant contribution to the success of clonal plants in a wide range of habitats (Hartnett and Bazzaz, 1983; Slade and Hutchings, 1987; Alpert and Stuefer, 1997; Kliměs et al., 1997; Saitoh et al., 2002; Roiloa et al., 2007). Here, we aim to add new evidence that may help to elucidate the role of clonal traits in plant invasions.

A pivotal task in plant invasions is to clarify how exotic plants adapt to the new environments that they are invading. Three mechanisms could explain the adaptation to the invaded range: (i) 'pre-adaptation', when species show similar trait standards in native and invaded ranges, indicating that the trait is a successful strategy in either area (Fridley and Sax, 2014); (ii) 'phenotypic plasticity', when responses allow plants to adjust their morphological and physiological responses very precisely to the challenges presented by particular environmental conditions, promoting resource acquisition and success in a new habitat (Grime and Mackey, 2002; Valladares et al., 2007; Mommer et al., 2011); and (iii) 'local adaptation', whenever there is a rapid adaptive evolution due to new selection pressures, resulting in improved fitness in the introduced environment (Maron et al., 2004; Cano et al., 2008; Xu et al., 2010; Buswell et al., 2011). Common garden experiments comparing plants from native and invaded ranges are required to test whether trait shifts are due to phenotypic plasticity or local adaptation (Wolfe et al., 2004; Erfmeier and Bruelheide, 2005; Güsewell et al., 2006; Zou et al., 2007). Trait differences between populations from native and invaded ranges grown alongside in a common garden would be indicative of an episode of local adaptation to the introduced range. This result would support the evolution of invasiveness hypothesis where rapid genetic changes are driven by natural selection pressures in the invaded environment (Lee, 2002; Stockwell et al., 2003).

One of the most striking attributes of clonal growth is the capacity for a 'division of labor' (i.e., specialization to acquire locally abundant resources, thereby increasing clone's overall performance). Stolon and rhizome internodes allow resource sharing between the connected ramets of a clonal system, which therefore are physiologically integrated. Resources are generally transferred from ramets growing under conditions of high resource supply to ramets located in areas where resource supply is low, following a source-sink system (Hartnett and Bazzaz, 1983; Roiloa and Retuerto, 2005). As result of this integration, supported ramets benefit in terms of growth and survival (e.g., Hartnett and Bazzaz, 1983; Slade and Hutchings, 1987; Saitoh et al., 2002; Roiloa and Retuerto, 2006). Heterogeneous distribution of essential resources, at spatial and temporal scale, is a characteristic of many natural environments (Chapin et al., 1987; Lechowicz and Bell, 1991; Caldwell and Pearcy, 1994). In this sense, natural habitats posses both favorable and unfavorable patches which are often negatively correlated in the space (Stuefer and Hutchings, 1994). This patchy distribution of resources commonly conducts to situations in which the resource-acquiring structures of clonal plant occupy sites that differ in quality (Hutchings and Wijesinghe, 1997). When the availabilities of two essential resources are negatively correlated in space, physiological integration can induce a plastic response in which one ramet specializes to acquire the resource that is locally abundant to it but scarce to other ramets (Friedman and Alpert, 1991; Birch and Hutchings, 1994; Stuefer et al., 1996; Alpert and Stuefer, 1997). Because the resource acquisition is expected to be more economical at high concentrations, the subsequent reciprocal transfer of resources between ramets should increase the overall performance of the clone (Stuefer et al., 1996; Alpert and Stuefer, 1997; Hutchings and Wijesinghe, 1997; Stuefer, 1998). This specialization to acquire locally abundant resources is termed 'division of labor' (Alpert and Stuefer, 1997; Hutchings and Wijesinghe, 1997; Stuefer, 1998), and appears to be a singular trait of clonal species. In comparison, non-clonal plants or disconnected ramets in a clonal system, typically respond to resources availability by following the optimal partitioning theory that predicts an increase in the relative allocation of biomass to structures specialized in acquiring the most limiting resource (Thornley, 1972; Bloom et al., 1985).

Plant invaders usually show faster growth rates in the introduced range than in the native area (Elton, 1958; Leger and Rice, 2003; Jakobs et al., 2004; Bossdorf et al., 2005). Division of labor could be accepted as an important advantage of clonal propagation, both at the native and invaded range. However, as the invasion process can be considered as a continuum with a number of filters that the successful invader must overcome (Richardson et al., 2000), it seems reasonable to anticipate that clonal plants may intensify this clonal attribute in the invaded range given a positive selection on beneficial traits, resulting in a rapid adaptive evolution for this trait (Roiloa et al., 2015). To test this hypothesis, we (i) collected clones of *Carpobrotus edulis* from native and invasive populations, (ii) grew pairs of connected and severed ramets in a common garden experiment, under negative spatial covariance of nutrients and light, in order to induce division of labor, and (iii) measured biomass allocation ratios, final biomass, and photochemical efficiency to quantify ramet specialization and performance at morphological and physiological level. Our specific hypothesis is that the capacity for division of labor, and consequent benefit, would be greater in populations from the invaded range (i.e., Portugal and

Spain) than in those from the native range (i.e., South Africa). Thus, we predict that (i) connection would induce an increase in proportional root mass and a decrease in photochemical efficiency in ramets under low light and high nutrients, and that this specialization to acquire below-ground resources will be greater in ramets from the invaded range than in those from the native range; (ii) connection would induce a decrease in proportional root mass and an increase in photochemical efficiency in ramet under high light and low nutrients, and that this specialization to acquire above-ground resources will be greater in ramets from the invaded range than in ramets from the native range; and (iii) the subsequent reciprocal transfer of resources between connected ramets should increase overall performance of the clone, and this benefit would be greater in clones from the invaded range than in clones from the native range.

Many experiments have compared relative performance in invasive and native or exotic non-invasive species (Pyšek and Richardson, 2007; Garcia-Serrano et al., 2009). Nonetheless, relatively few studies have determined whether invaders change their functional strategies from the native to the introduced range (Zou et al., 2007; Leishman et al., 2014; Heberling et al., 2015). Our study appears to be the first in testing these differences for a key clonal trait such as division of labor in an aggressive invader. Assessing the influence of clonal traits in plant invasions is key for understanding the underlying mechanisms behind biological invasions and, therefore, for predicting future invasion scenarios as well as for designing efficient control strategies in invaded areas. Additionally, this information is likewise important for a better understanding of how plants respond and evolve in new environments.

MATERIALS AND METHODS

Study Species

Carpobrotus edulis (L.) N.E. Br., is a mat-forming succulent clonal plant, native to the Cape Region (South Africa), and an aggressive invader in coastal ecosystems of all the Mediterranean climate regions around the world, including Australia, Europe and America (D'Antonio and Mahall, 1991; Traveset et al., 2008; Vilà et al., 2008). *C. edulis* forms dense mats and spreads horizontally by the production of numerous apical ramets that remain physiologically integrated by stolon connections (Wisura and Glen, 1993). Ramets produce roots after direct contact with the substrate and can survive if disconnection from the parent ramet occurs. This type of vegetative growth allows *C. edulis* a very effective colonization of the surrounding area, competing aggressively with local species and affecting negatively the diversity of the native flora (D'Antonio and Mahall, 1991; Traveset et al., 2008). Previous experiments have studied several aspects of the ecology of *C. edulis* as plant–pollinator networks, plant–soil feedbacks, or hybridization studies (Vilà and D'Antonio, 1998; Bartomeus et al., 2008; de la Peña et al., 2010). However, the importance of clonal traits in the expansion of this aggressive invader has been generally overlooked (but see Roiloa et al., 2010, 2013, 2014a,b).

Sampling Protocol

Four spatially separated populations of *C. edulis* were sampled in the native (Cape Region, South Africa) and four in the invaded range (Iberian Peninsula, South Europe) (see **Figure 1**). Plant material was collected in the native and in the invaded range from coastal sand dune systems where *C. edulis* typically inhabits. To have a more comprehensive illustration of the genetic variability, we selected 36 separated clumps in each of the eight populations sampled. Within a population, selected clumps were separated at least 25 m from the others. *C. edulis* forms compact clumps (Wisura and Glen, 1993) and it is reasonable to assume that each separated clump represents a different genotype. However, no genetic analyses were performed and the clumps might or might not differ in genotype. Four-member un-rooted clonal fragments were excised at the edge of each clump. Clonal fragments contained the first four units or modules (ramets *sensu* Harper, 1977) from the apices, and thus we ensured that all the experimental plant material had the same developmental stage. Plant material from the native range was collected at mid-January 2015 and maintained in the greenhouse during 3 months before the experiment began to minimize maternal environmental effects.

Experimental Design

In April 2015, 48 ramet pairs comprised by the third and fourth ramets from the apices were selected for size uniformity from the plant stock. Initial size of ramet pairs was estimated by fresh mass. Preliminary analysis showed that the initial sizes of ramet pairs from the native and the invaded range did not differ significantly (ANOVA $F_{1,46} = 1.787$, $P = 0.188$). The experimental design consisted of two crossed factors with region (native, invaded) and connection (connected, severed) as main factors. The region factor included ramet pairs from coastal sand dunes of the native (South Africa) and invaded range (Europe), as explained above. In the connection factor, ramets within each pair were either left connected (division of labor allowed) or severed (division of labor prevented). Ramets were severed by cutting the connecting stolon halfway between them. We did not observe any immediate negative side effect of cutting the stolon (e.g., sudden death or disease). From the 36 clumps sampled in each population, we randomly selected 6 clumps to obtain the 48 ramet pairs used in the experiment (8 populations $\times$ 6 clumps). Ramet pairs from each of the eight populations sampled in the field were equally represented and randomly assigned to each combination of region by connection treatments.

Each pair was subjected to a regime of resource availability known to induce division of labor via changes in allocation of mass between roots and shoots (Roiloa et al., 2014b). Older ramets (the "fourth" modules) were subjected to low light and high nutrient conditions, and younger ramets (the "third" modules) were exposed to high light and low nutrients conditions (see **Figure 2**). This regime of resource availability mimics the natural conditions of *C. edulis* growing at coastal sand dune habitats, where older ramets usually grow shaded by shrubs, which enrich the nutrient content of the soil, whereas developing

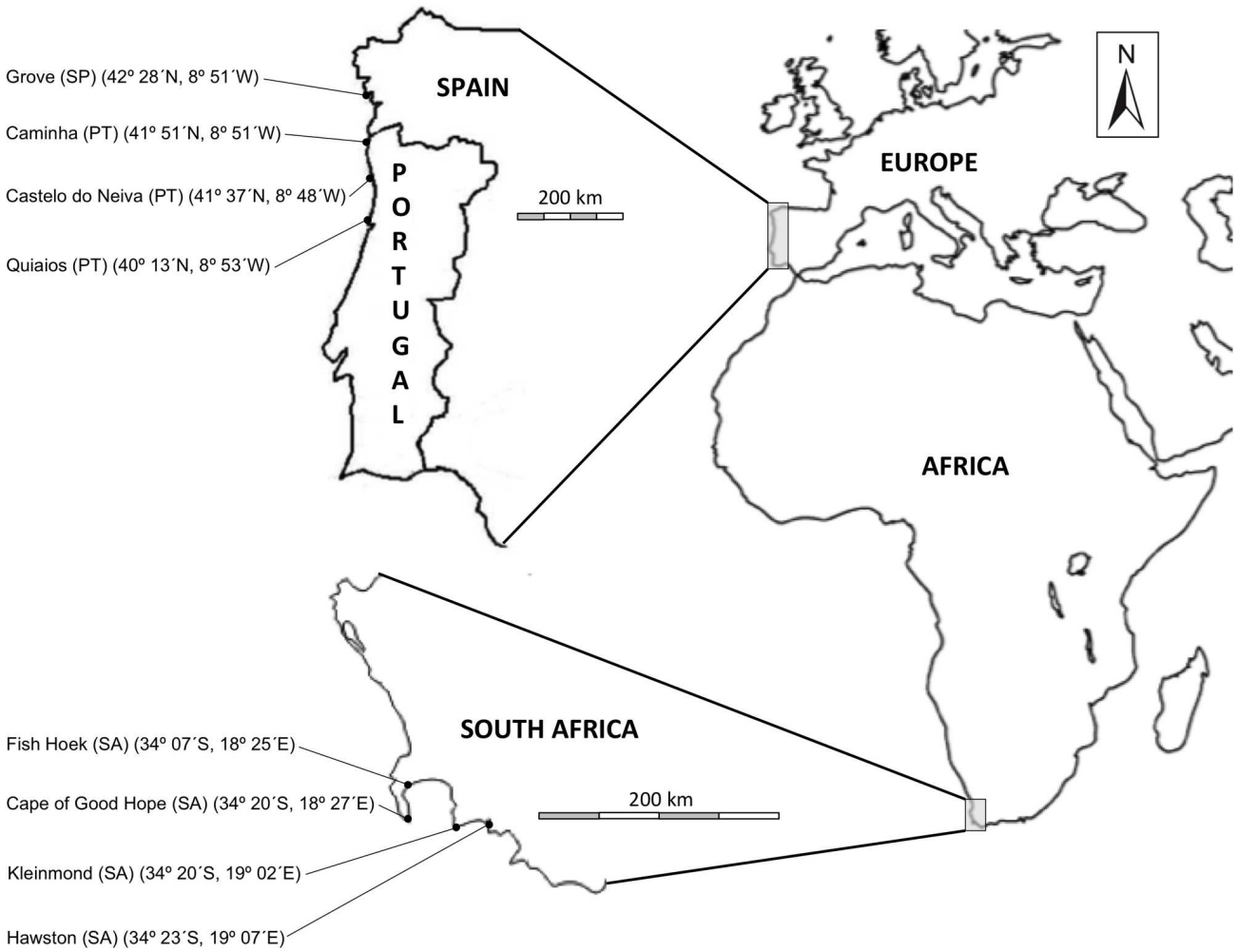

FIGURE 1 | Site location (latitude and longitude) of *Carpobrotus edulis* populations sampled from the native (Cape Region in South Africa) and the invaded (Portugal and Spain in Europe) regions. (SA, South Africa; PT, Portugal; SP, Spain).

younger ramets spread into the non-shaded sand with low nutrient content (S. R. Roiloa, personal observation). High nutrient conditions consisted of a 3:1 mixture of potting compost and sand. In the low nutrient conditions, ramets grew in sand. Low light conditions were created using a polypropylene shade cloth that reduces ambient light to 10%. Ramets in the high light treatment were left unshaded. Each pair of ramet was planted in a single 5L plastic pot hermetically divided into two equal compartments by plastic barriers to avoid root interactions (see **Figure 2**). None of the ramets had roots at the start of the experiment. Each treatment was replicated 12 times ($n = 12$). The common garden experiment was carried out in a open-end greenhouse at the University of Santiago de Compostela (Spain) (42° 52′ 26.65″ N, 8° 33′ 31.64″ W). In order to avoid confounding effect of position within the greenhouse, the four types of treatments were interspersed randomly. Plants were watered regularly with as much water as necessary to maintain soil moisture. Treatments began on 10 April 2015 and continued for 90 days.

Measurements

Spectral Reflectance

Leaf spectral reflectance parameters were measured 30, 60, and 90 days after treatment application using a portable spectrometer (UniSpec Spectral Analysis System, PP Systems, Haverhill, MA, USA). Specifically, we determined the photochemical reflectance index (PRI), that was calculated as $(R_{539}-R_{570})/(R_{539} + R_{570})$, where R_{539} and R_{570} are reflectances at 539 and 570 nm, respectively (Filella et al., 1996). This index correlates with both net CO_2 uptake and photosynthetic radiation-use efficiency (mol CO_2/mol photons) (Peñuelas et al., 1995; Filella et al., 1996; Gamon et al., 1997).

Chlorophyll Fluorescence

Immediately after reflectance measurements, chlorophyll fluorescence parameters were determined by the saturation pulse method (Schreiber et al., 1998) using a portable pulse-amplitude-modulate fluorometer (MINI-PAM photosynthesis yield analyser; Walz, Effeltrich, Germany). In particular, we

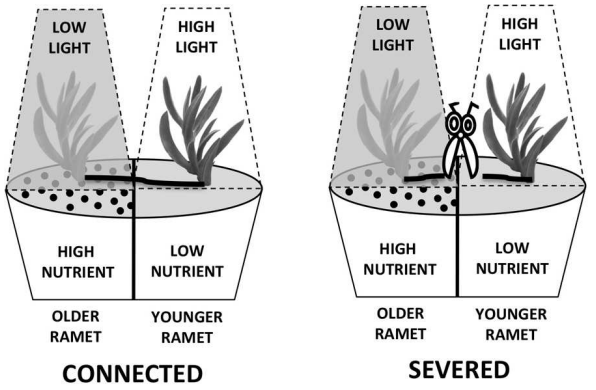

FIGURE 2 | Scheme of the experiment, showing levels of light and nutrients given to pairs of connected or severed ramets. Older ramets were exposed to low light and high nutrient conditions. Younger ramets were exposed to high light and low nutrient conditions. See text for experimental design details.

measured the maximum quantum yield of photosystem II (PSII), $F_v/F_m = (F_m - F_0)/F_m$, where F_m and F_0 are the maximum and minimum fluorescence yield, respectively, of dark-adapted samples, after a saturation pulse (>5000 μmol photons m^{-2} s^{-1} of actinic white light) (see Bolhàr-Nordenkampf et al., 1989). The maximum PSII quantum yield (Fv/Fm) characterizes the photosynthetic process associated with electron transport (light reactions), and provides information on the efficiency of excitation energy capture by open PSII reaction centres (Butler and Kitajima, 1975). F_v/F_m correlates with the amount of carbon gained per unit of light absorbed (Bolhàr-Nordenkampf and Öquist, 1993). This variable was measured after a 30-min dark adaptation period, which allowed the PSII reaction centers of the leaf to be fully open.

Growth

At the end of the experiment, each ramet was separated into shoots (including leaves and stolons) and roots, dried at 80°C for 72 h, and weighed. The total dry mass (shoot dry mass + root dry mass) and the proportional biomass allocated to roots (root-shoot ratio, RSR = root dry mass/shoot dry mass) were calculated for older and younger ramets separately. Total dry mass at whole clone level (older + younger ramets) was also calculated.

Statistical Analysis

Prior to analyses, variables were transformed as necessary to meet the assumptions of parametric tests. Thus, the root/shoot (RSR) of older ramets were square root transformed. We analyzed differences in the total dry mass and the proportional biomass allocated to roots (RSR) by two-way analysis of variance (ANOVA) with region (native, invaded) and connection (connected or severed) as fixed effects. Separates analyses were conducted for older (with low light and high nutrients) and younger (with high light and low nutrients) ramets. Similarly, total dry mass at whole clone level (older + younger ramets) was compared by two-way ANOVA with region and connection as main factors. Changes in leaf spectral reflectance (PRI) and chlorophyll fluorescence (F_v/F_m) over time were analyzed with two-way analyses of variance with repeated measures (ANOVAR), using region and connection as between-subject effects. For these variables, we conduced separates analyses for older and younger ramets. Three ramets (one older and two younger) died during the experiment, and the corresponding pairs (older + younger) were excluded from the

TABLE 1 | Results of two-way analyses of variance (ANOVA) to examine the effects of region and connection on root to shoot ratio (RSR) of the older and younger ramets.

	Root/shoot (RSR)			Total dry mass		
Effect	**df**	***F***	***P***	**df**	***F***	***P***
Older ramet						
Region	1	0.127	0.723	1	0.318	0.576
Connection	1	163.595	**<0.001**	1	2.089	0.156
Region × connection	1	0.229	0.635	1	12.498	**0.001**
Error	41			41		
Younger ramet						
Region	1	0.898	0.349	1	3.817	0.058
Connection	1	28.124	**<0.001**	1	107.025	**<0.001**
Region × connection	1	0.122	0.729	1	4.894	**0.033**
Error	41			41		
Whole clone						
Region	nd	nd	nd	1	1.135	0.293
Connection	nd	nd	nd	1	63.965	**<0.001**
Region × connection	nd	nd	nd	1	0.111	0.740
Error	nd			41		

*ANOVAs to detect effects of treatments on total dry mass of older and younger ramets, and at whole clone level (older + younger ramets). nd, non-determined. Values of P < 0.05 are in boldface. See **Figures 3** and **4** for data.*

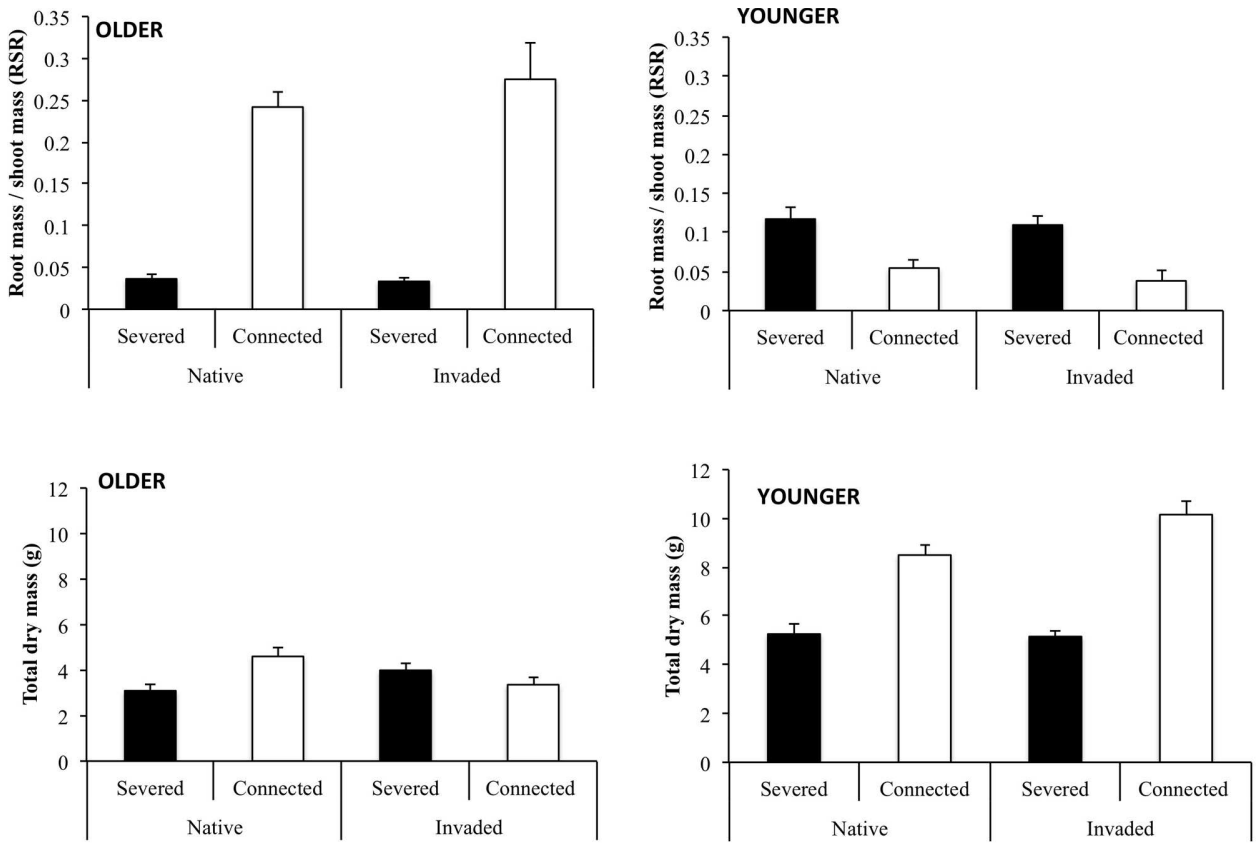

FIGURE 3 | Final measurements (mean +SE) of the proportional dry mass allocated to roots (determined as the root to shoot ratio, RSR) and the total dry mass (g) of connected and severed older (left) and younger (right) ramets from native and invaded regions. See **Table 1** for ANOVA results.

analyses. This reduced the number of replicates used in the different analyses, as indicated by the error degree of freedom. Significance level was set at $P < 0.05$. Statistical tests were performed with SPSS Statistics 19.0 (IBM, Armonk, New York, USA).

RESULTS

Growth

The proportion of biomass allocated to roots by older and younger ramets, as determined by the root to shoot ratio (RSR), was significantly affected by the connection treatment (**Table 1**). Connection significantly increased the proportion of dry mass allocated to roots (RSR) in older ramets but it was decreased in younger ramets (**Figure 3**). The effect of region and the interaction between region and connection on root to shoot (RSR) was not significant neither in older nor in younger ramets (**Table 1**).

The final total dry mass of older ramets was significantly affected by the interaction between region and habitat (**Table 1**). Connection significantly increased the total dry mass in the genotypes from the native region. However, this effect was not maintained in the genotypes from the invaded region, where we detected a decrease of the total dry mass in the connected older ramets (**Figure 3**). On the other hand, connection significantly increased the final dry mass of younger ramets (**Table 1**; **Figure 3**). This positive effect of connection on the final dry mass of younger ramets was stronger in genotypes from the invaded

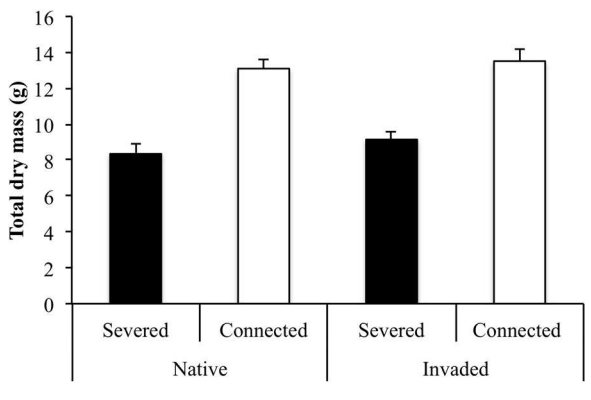

FIGURE 4 | Final measurements (mean +SE) of the total dry mass (g) of the whole clones (older + younger ramets) from the native and invaded regions in the connected and severed treatments. See **Table 1** for ANOVA results.

region, as denoted by the significant effect of the interaction between factors region and connection (**Table 1**; **Figure 3**). This result indicates that the benefit of physiological integration was significantly higher in invasive populations than in native populations. At the whole clone level (older + younger ramets), our results showed a significant effect of connection, with an increase of the final total dry mass due to connection both in the genotypes from the native and the invaded region (**Table 1**; **Figure 4**). Neither the region nor the interaction between region and connection had a significant effect on total dry mass at the whole clone level (**Table 1**).

Leaf Spectral Reflectance and Chlorophyll Fluorescence

Older ramets from the invaded region showed a significantly higher PRI than older ramets from the native region (**Table 2**; **Figure 5**). Our results also showed a significant effect of the connection treatment on the PRI of older ramets (**Table 2**). Thus, there was a significant decrease in PRI of connected older ramets, regardless of whether they came from the native or the invaded region (**Figure 5**). We detected a significant effect of the interaction between region and connection in the maximum quantum yield of photosystem II (F_v/F_m) of older ramets (**Table 2**). Connection significantly increased F_v/F_m in older ramets of the genotypes from the native region, but decreased it in the older ramets of genotypes from the invaded region (**Figure 5**).

Connection significantly affected the PRI of younger ramets (**Table 2**). PRI values were significantly higher in connected than in severed younger ramets both in genotypes from the native and the invaded region (**Figure 5**). Although we did not detect a significant between-subject effect of connection in younger ramets in terms of maximum quantum yield of photosystem II (F_v/F_m) (**Table 2**; **Figure 5**), this variable was significantly affected by the interaction of connection by time (within-subject effect) (**Table 2**). Thus, the F_v/F_m values of younger ramets changed with time, and this change was dependent on the connection treatment. Connected and severed younger ramets significantly inverted their F_v/F_m values during the experiment, regardless of their region of origin. At day 30 F_v/F_m values were higher in connected than in severed younger ramets, whereas severed younger ramets showed greater F_v/F_m values than connected ramets at days 60 and 90 (**Figure 6**).

DISCUSSION

Our results support the existence of division of labor, both at morphological and at physiological level, in native and invaded

TABLE 2 | Results of two-way repeated-measure analysis of variance (ANOVAR) with region and connection as between-subject effects, for differences in the photochemical reflectance index (PRI) and the maximum quantum yield of photosystem PSII (F_v/F_m) of older and younger ramets.

	PRI			F_v/F_m		
Effect	**df**	***F***	***P***	**df**	***F***	***P***
Older ramet						
Between-subject effects						
Region	1	4.933	**0.032**	1	0.135	0.715
Connection	1	7.933	**0.007**	1	0.230	0.634
Region × connection	1	0.615	0.437	1	5.994	**0.019**
Error	41			41		
Within-subject effects						
Time	2	41.226	**0.001**	2	17.732	**0.001**
Region × time	2	2.154	0.123	2	1.081	0.344
Connection × time	2	0.879	0.419	2	0.012	0.988
Region × connection × time	2	0.408	0.666	2	1.049	0.355
Error	82			82		
Younger ramet						
Between-subject effects						
Region	1	0.008	0.928	1	1.315	0.258
Connection	1	4.635	**0.037**	1	0.579	0.451
Region × connection	1	0.397	0.532	1	0.305	0.584
Error	41			41		
Within-subject effects						
Time	2	18.015	**0.001**	2	22.316	**0.001**
Region × time	2	0.012	0.988	2	0.154	0.857
Connection × time	2	0.853	0.430	2	5.749	**0.005**
Region × connection × time	2	1.62	0.204	2	0.220	0.803
Error	82			82		

*Values of $P < 0.05$ are in boldface. See **Figures 5** and **6** for data.*

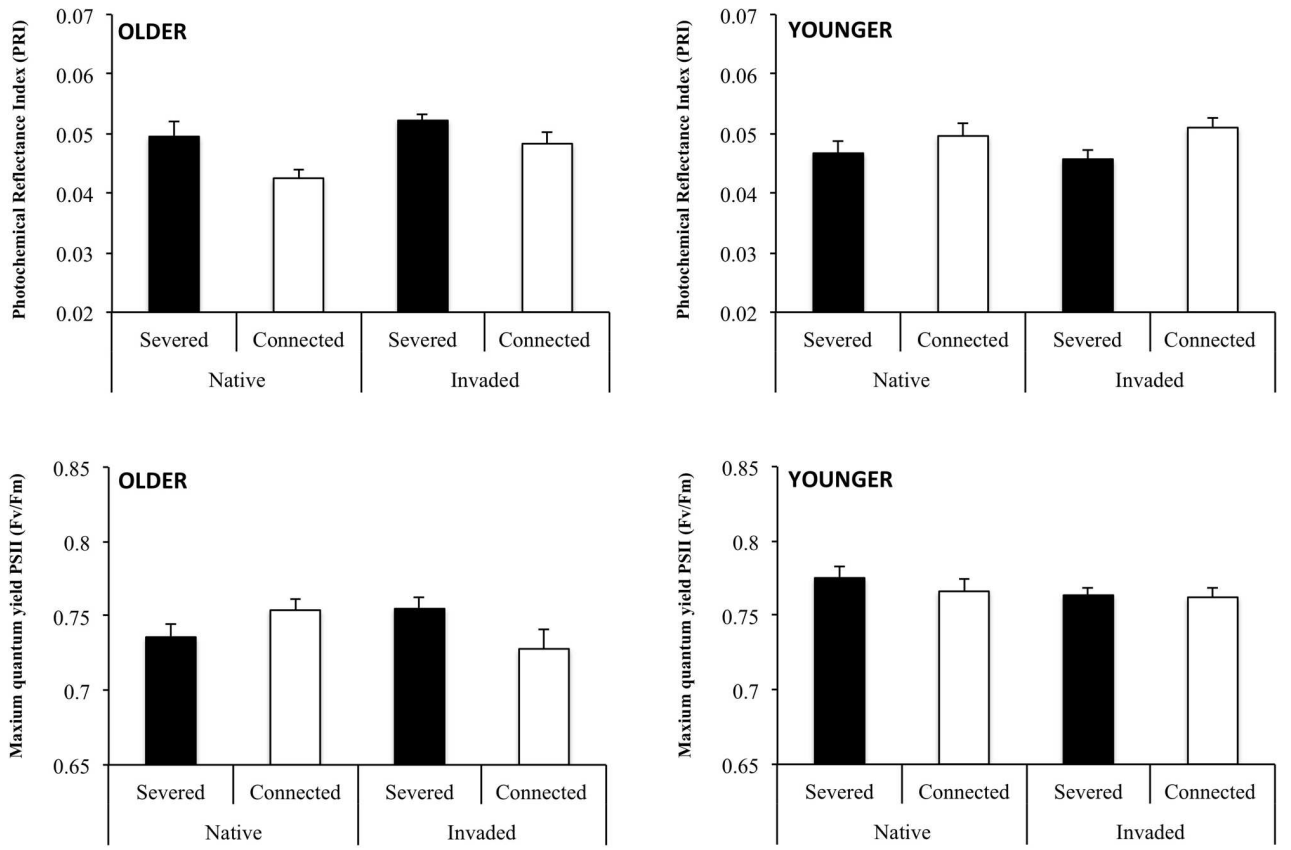

FIGURE 5 | Mean values (+SE) of photochemical reflectance index (PRI) and the maximum quantum yield of photosystem II (Fv/Fm) for connected and severed older (left) and younger (right) ramets from the native and invaded regions. See **Table 2** for ANOVA results.

populations of *C. edulis*. As proposed in our hypotheses, we found that connection significantly increase the mass allocated to roots (RSR) in older ramets subjected to high nutrients and low light conditions, denoting a specialization to acquire the relatively abundant below-ground resource. On the other hand, connection significantly increased the photosynthetic radiation-use efficiency (as estimated by the PRI and reduced the proportional root mass (RSR) in younger ramets, which were subjected to high light and low nutrients conditions, indicating a specialization to acquire above-ground resources. In populations from both the native and the invaded range, resource sharing mediated by stolon connection significantly increased the final biomass at the whole clone level. These results provide strong evidence that ramets of clones of *C. edulis* have a capacity for division of labor, and that this specialization for acquiring the most abundant resource reports a benefit for the whole clone. However, our results do not support the prediction that clones from the invaded range may have a greater capacity for division of labor than those from the native range. Since resource acquisition is expected to be more economical where the resource is more abundant, the specialization to acquire the relatively rich resource and the subsequent reciprocal resource sharing between connected ramets would increase the overall performance of the clone (Stuefer et al., 1996; Alpert and Stuefer, 1997; Hutchings and Wijesinghe, 1997; Roiloa et al., 2014b). Similar results showing a capacity for division of labor at morphological and physiological level were recently reported by Roiloa et al. (2014b) in clones of *C. edulis* in the invaded range. Likewise, Roiloa et al. (2007) reported environmental-induced division of labor at morphological and physiological levels in clones of the stoloniferous *Fragaria chiloensis*. Both studies showed greater capacity for division of labor in clones from patchier habitats, where essential resources were negatively correlated. In these conditions, the division of labor between ramets would be specially beneficial, suggesting an adaptive division of labor induced by the environment (Roiloa et al., 2007, 2014b). Previous studies with other clonal plants also reported differences between genotypes for several clonal traits, including division of labor, resource sharing, or sexual/asexual shift, which suggest a potential for local adaptation (Lotscher and Hay, 1997; Alpert, 1999; Prati and Schmid, 2000; Alpert et al., 2003; Roiloa et al., 2007; Nilsson and D'Hertefeldt, 2008; D'Hertefeldt et al., 2014), and are in agreement with the evolutionary theory that predicts that populations evolve to generate traits to gain an advantage under their local conditions (Willians, 1966).

Although our results showed no differences in the capacity for division of labor between invasive and native populations, we found, however, significant differences in the benefits obtained

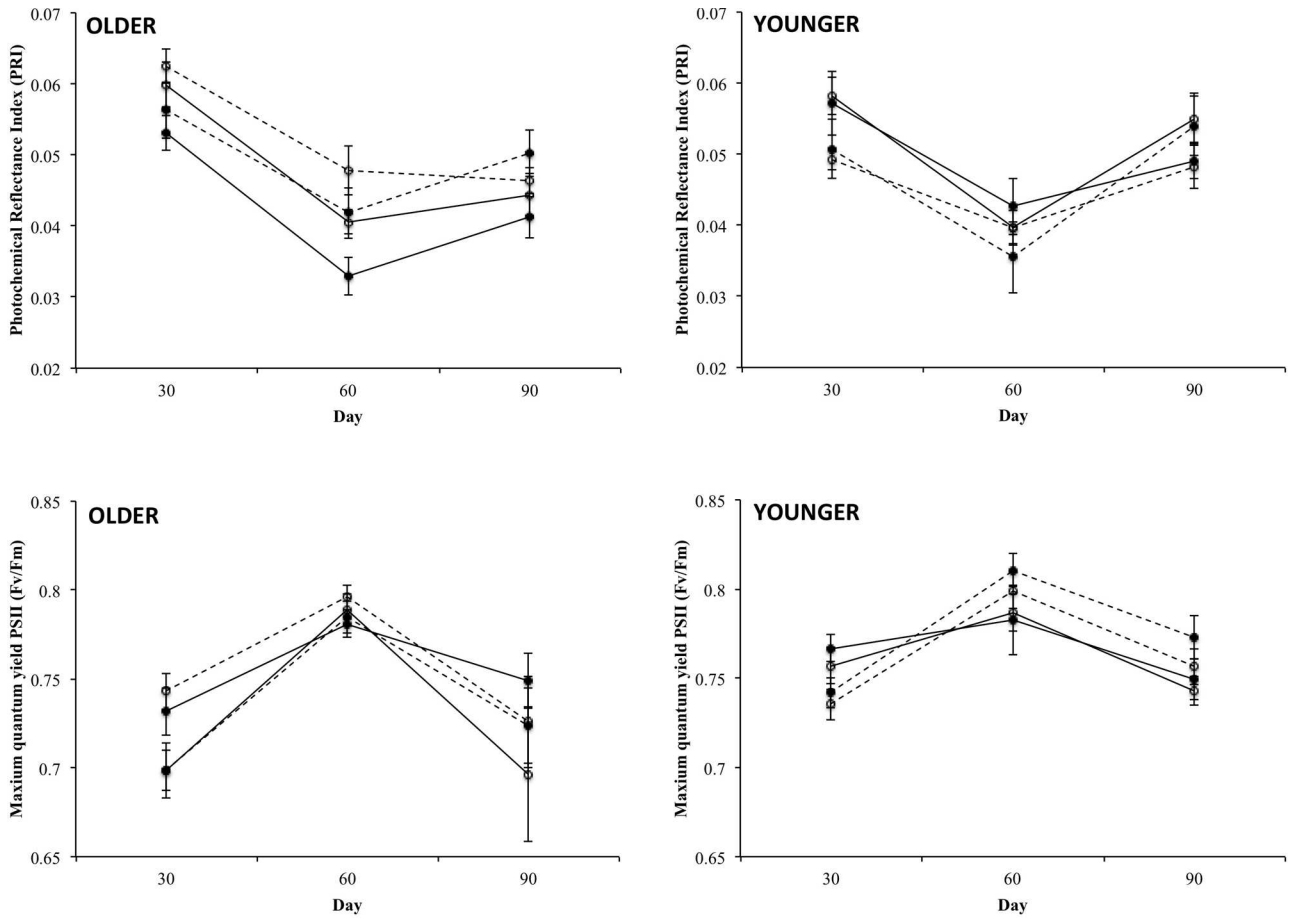

FIGURE 6 | Time course of mean values (±SE) of photochemical reflectance index (PRI) and the maximum quantum yield of photosystem II (Fv/Fm) for connected (solid lines) and severed (dashed lines) treatments of older (left) and younger (right) ramets from the native (closed symbols) and invaded (open symbols) regions. See **Table 2** for ANOVA results.

by the division of labor among ramets. As reported, benefits were significantly higher in younger ramets from invasive populations than in those from native populations. This is a novel and outstanding result because it provides the first evidence that the benefit of a key clonal trait, as division of labor, may be subjected to evolutionary adaptation in the invaded range. Therefore, our results indicate that rapid genetic changes linked to selection pressures might contribute to the invasion of *C. edulis* in the introduced range. Rapid adaptive evolution of introduced populations could explain invasion success in a new environment (Maron et al., 2004; Sax et al., 2007). In this line, it seems reasonable to predict that positive selection of beneficial traits such as division of labor could be favoring the expansion of *C. edulis* in the introduced range and, therefore, promoting its invasiveness.

Interestingly, the benefit of division of labor detected in younger ramets at the invaded range seems to be obtained at the cost of the older ramets. This is, in native populations the reciprocal transport of resources between the connected older and younger ramets seems to be balanced and, consequently provided mutual benefits to both ramets. However, our results for the invasive populations showed a significant benefit of the connection for younger ramets, but a significant cost for older ramets, indicating an unbalanced share of resources. This result seemingly indicates that there was a unidirectional transport of resources from the older to the younger ramet in the population from the invaded range. As a result, growth increase was more pronounced in invasive younger ramets than in native ones, where resources transport appeared to be bidirectional, as described in previous works (e.g., Stuefer et al., 1996; Alpert and Stuefer, 1997; Hutchings and Wijesinghe, 1997). This trait shift in the introduced range could be promoting the expansion of younger ramets, contributing to the expansion of invasive plants. *C. edulis* spreads horizontally by the production of abundant younger ramets that remain integrated by stolon connections. Roiloa et al. (2010) found an association between the increase in total biomass and the horizontal expansion of apical ramets of *C. edulis* when colonizing a natural dune system in the invaded range. Thus, our finding of an intensification of the benefits derived from division of labor for apical ramets in populations from introduced range supports the idea that adaptation after introduction may favor the expansion of this aggressive invader.

Our pioneer results showing an unbalanced benefit for older and younger ramets from division of labor in populations from the invaded range could be explained within the evolution of increased competitive ability (EICA) hypothesis. The latter proposes that exotic plants, once established in the introduced range and liberated from their natural enemies, invest more in fast-growing and less in defence (Blossey and Nötzold, 1995; Mooney and Cleland, 2001; Hierro et al., 2005; Alba et al., 2012). Under this supposition, older ramets in the invasive range can reallocate resources from defensive traits to support connected younger ramets favoring the expansion of this aggressive invader. In any case, we recognize that our results are not robust enough to confirm this statement, and more experiments are necessary to elucidate if the unbalanced transport of resources described in our study could be explained by EICA hypothesis.

In addition, our results showed that photosynthetic efficiency (estimated by the PRI, which is correlated with net CO_2 uptake and photosynthetic radiation-use efficiency, Peñuelas et al., 1995; Filella et al., 1996; Gamon et al., 1997) was significantly higher in older ramets from the invaded range than in older ramets from the native area. Previous studies have detected that invasive plants show greater growth rates in their introduced range than in the native range (Elton, 1958; Leger and Rice, 2003; Jakobs et al., 2004; Bossdorf et al., 2005). In another common garden experiment, Zou et al. (2007) also found that invasive populations of *Sapium sebiferum* showed significantly higher photosynthetic activity, in terms of net CO_2 assimilation (determined directly by gas exchange measurements), than populations from their native range. Eco-physiological results also showed a significant increase in the PRI in connected younger ramets, both in the native and invaded range. This increases in photochemical efficiency was translated into a significant increase in the total mass, denoting a positive correlation between PRI and growth. However, and differing to the pattern obtained for total mass, we did not detect a more accentuate increase in photochemical activity in younger ramets from invaded range in comparison with values obtained in native populations. Probably, the significantly higher benefit in younger ramets from invasive populations was due to the support received from their connected older ramets. In this sense, the unidirectional transport of resources from the older to the younger ramet in the population from the invaded range increased more pronounced the growth in invasive younger ramets than in native ones, in spite of not showing differences in photochemical activity.

CONCLUSION

This is the first study reporting differences between native and invaded populations in the benefits derived from a key clonal trait such as division of labor. The results from this study seem consistent with a rapid adaptive evolution of the clonar invader *C. edulis* with a positive selection of the benefits from division of labor in the invaded range. The benefits from division of labor could therefore be considered an important trait in the invasiveness of *C. edulis*. However, whether this is the case for all clonal plant invaders or not remains to be clarified, and future research should include more clonal species. The contribution of clonal traits to the capacity to establish in new environments represents an exciting research field for understanding the mechanisms underlying plant invasions, and for a better knowledge of how plants respond and evolve in new habitat. In addition, experiments testing for differences between invasive and exotic non-invasive species are mandatory to understand the role of clonal traits in plant invasions. Understanding the influence of clonal life-history traits in plant invasions seems key for predicting future invasion scenarios and for devising efficient strategies of control and restoration of invaded areas.

AUTHOR CONTRIBUTIONS

SR was involved in designing the experiment, field samples collection, set up the experiment, data collection, and analysis, manuscript preparation and submission. RR field samples collection, set up the experiment, data collection, and co-prepared manuscript. JC set up the experiment, data collection and co-prepared manuscript. AN and RB field samples collection and co-prepared manuscript.

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Using Transcriptomics to Identify Differential Gene Expression in Response to Salinity among Australian *Phragmites australis* Clones

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Common Reed (*Phragmites australis*) is a frequent component of inland and coastal wetlands in temperate zones worldwide. Ongoing environmental changes have resulted in the decline of this species in many areas and invasive expansion in others. In the Gippsland Lakes coastal waterway system in south-eastern Australia, increasing salinity is thought to have contributed to the loss of fringing *P. australis* reed beds leading to increased shoreline erosion. A major goal of restoration in this waterway is to address the effect of salinity by revegetating with a genetically-diverse range of salt-tolerant *P. australis* plants. This has prompted an interest in examining the variation in salinity tolerance among clones and the underlying basis of this variation. Transcriptomics is an approach for identifying variation in genes and their expression levels associated with the exposure of plants to environmental stressors. In this paper we present initial results of the first comparative culm transcriptome analysis of *P. australis* clones. After sampling plants from sites of varied surface water salinity across the Gippsland Lakes, replicates from three clones from highly saline sites (>18 g L^{-1} TDS) and three from low salinity sites (<6 g L^{-1}) were grown in containers irrigated with either fresh (<0.1 g L^{-1}) or saline water (16 g L^{-1}). An RNA-Seq protocol was used to generate sequence data from culm tissues from the 12 samples allowing an analysis of differential gene expression. Among the key findings, we identified several genes uniquely up- or down-regulated in clones from highly saline sites when irrigated with saline water relative to clones from low salinity sites. These included the higher relative expression levels of genes associated with photosynthesis and lignan biosynthesis indicative of a greater ability of these clones to maintain growth under saline conditions. Combined with growth data from a parallel study, our data suggest local adaptation of certain clones to salinity and provide a basis for more detailed studies.

Keywords: clonality, common reed, differential gene expression, *Phragmites australis*, salinity, salt tolerance, transcriptomics

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INTRODUCTION

Phragmites australis (Cav.) Trin. ex Steud or "Common Reed" is a rhizomatous perennial grass found in fresh and saline wetland systems throughout temperate regions of the world. The species is genetically complex with a range of ploidy levels including 2n = 3x, 4x, 8x, 10x, and 12x (Clevering and Lissner, 1999; Lambertini et al., 2006). Several studies have reported high levels of genetic diversity within, and among, populations of this species (see Lambertini et al., 2008; Achenbach et al., 2012; and references therein) which may confer a high level of phenotypic plasticity in response to environmental variability (Hansen et al., 2007; Achenbach et al., 2012; Eller and Brix, 2012). In addition, *P. australis* can reproduce both sexually and asexually further contributing to its success in establishing and persisting under a range of environmental conditions. However, its local abundance is affected by several environmental factors including variable levels of nutrients, water and salinity, niche availability and its genetic make-up (Eller et al., 2014). For example, Achenbach et al. (2013) found substantial differences in salt tolerance between clones of *P. australis* supporting the idea that varied clonal responses have a genetic basis.

The rapid expansion of *P. australis* across North America in recent decades has been due almost exclusively to a Eurasian lineage introduced in the late 19th century (Haplotype 'M' *sensu* Saltonstall, 2002). Salinity tolerance (coupled with clonal reproduction) is thought to underpin the invasiveness of this genetic lineage (Vasquez et al., 2005, 2006). DNA sequence variants and varied expression levels of genes associated with salinity tolerance have been observed among *Phragmites* lineages (Zhao et al., 2004; Takahashi et al., 2007a,b, 2009) and while salinity tolerance has been reported more generally for *P. australis*, study results vary in the effect of salinity levels on growth response (e.g., Pagter et al., 2009; Gorai et al., 2011; Yang et al., 2014).

The Gippsland Lakes in south-eastern Australia is an extensive Ramsar-listed wetland system of >60,000 ha, that experienced chronic salinization following the construction of a permanent channel to the sea in the late 19th century to improve boat access (Boon et al., 2015a). The effects were exacerbated in the mid-late 20th century when fresh water from inflowing rivers was increasingly regulated and extracted. This process has led to substantial ecological impacts on the Lakes' ecosystem including a marked decline in the area of fringing *P. australis* beds (Bird, 1961, 1966; Boon et al., 2008, 2015a; Sinclair and Boon, 2012). However, an extensive field survey undertaken in 2014 suggested that *P. australis* has re-appeared in parts of the system from which it seems to have been formerly precluded (Bird, 1961; Boon et al., 2015b). This raises the question as to why reed beds can now grow in these areas and whether there has been an adaptive response to increased salinity.

Shoreline erosion and retreat is a serious problem for the Gippsland Lakes (Hart, 1921; Bird, 1966, 1983; Bird and Rosengren, 1974; Sjerp et al., 2002). Shoreline degradation is expected to become even more pronounced with projected rises in eustatic sea levels coupled with an increased frequency and severity of storm surges, both linked to climate change. *Phragmites australis* is arguably the plant species most capable of protecting shorelines in the Gippsland Lakes from these processes (Boon et al., 2015b,c). Accordingly there is currently great interest in rehabilitating shoreline vegetation, often focussing on the restoration of *Phragmites* beds. However, the species has displayed mixed responses to increasing salinity making it difficult to pinpoint the causes and to identify the best germplasm for restoration. This has prompted an interest in the rapid identification of salt tolerant lineages of *P. australis* that could be used in the rehabilitation of wetlands and lake foreshores in the Gippsland Lakes, but also more generally across coastal wetland systems. The Gippsland Lakes provide an ideal opportunity to compare the responses of clones to salinity depending on the salinity of source populations.

One approach to help understand the mechanisms involved in the response of organisms to stressors is to compare genes that are up- or down-regulated under controlled conditions. Identifying the gene transcripts in *P. australis* that correlate with salinity tolerance will provide a basis for understanding this ecologically important trait.

In this study, we utilize next-generation sequencing technology to identify culm-expressed genes in *P. australis* associated with exposure to saline water. Six clones were obtained from areas of low or high salinity across the Gippsland Lakes and grown in pot trials. Paired-samples of the clones from each site were irrigated with either fresh-, or highly saline water. Using an RNA-Seq approach, we sequenced transcriptomes from the culms of each of these twelve samples and identified genes differentially expressed among treatments.

We predicted that plants from low salinity sites when irrigated with highly saline water would display higher levels of up-regulation of stress response genes than would plants from high salinity sites. Plants from high salinity sites irrigated with fresh water should display the opposite response. We also hypothesized that because of local adaptation, plants from high salinity sites grown in saline water would display minimal changes to gene expression states compared with plants from low salinity sites grown in fresh water. In summary, in this paper we address two main questions: (1) which genes are expressed in the culms of *P. australis* in response to growth in highly saline water? (2) which genes are differentially expressed in the culms of plants sourced from low salinity sites compared to plants from high salinity sites when grown in saline water?

MATERIALS AND METHODS

Growth Conditions

Sections of rhizome from six *P. australis* clones were harvested in September 2014 from several sites across the Gippsland Lakes area of south-eastern Australia (**Figure 1**; **Table 1**). These sites are described in Boon et al. (2015c). Rhizome sections were stored at 4°C in damp hessian bags until potted out. The surface-water salinities of the sites (**Table 1**) were determined using the total dissolved solids (TDS) function of a TPS WP-81 water-quality meter with a $k = 10$ temperature-compensating conductivity sensor (TPS Instruments, Brisbane,

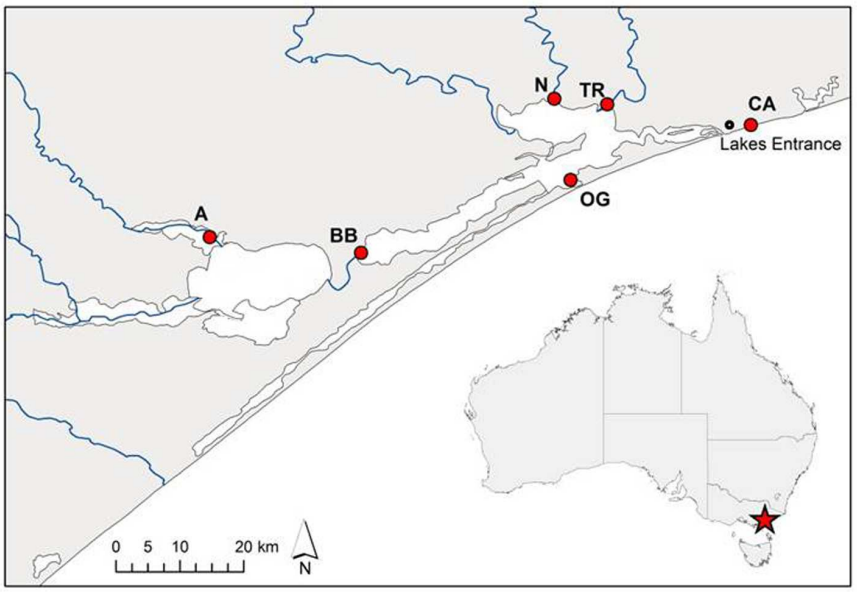

FIGURE 1 | Source sites of *Phragmites australis* clones from the Gippsland Lakes, south-eastern Australia used in this study. Population codes are as listed in **Table 1**. The artificial opening of the lake system to Bass Strait is immediately south-west of the Lakes Entrance township while blue lines indicate inflowing rivers.

QLD, Australia). Although once-off 'spot' measurements, the surface-water salinities measured at each site agreed closely with long-term spatial patterns of water salinity across the Gippsland Lakes reported by Webster et al. (2001). To check whether *P. australis* plants could persist in apparently saline environments by accessing shallow lenses of fresh ground water, we also measured interstitial water salinity at various depths in the sediment at each site. These data will be reported in a separate paper. In summary, there was a good correlation ($r^2 = 0.41$, $n = 37$) between salinity in surface water and interstitial water across the sites, strongly suggesting that the plants from saline sites were not subsidized by ground water lower in salinity than the overlaying water column (Boon et al., 2015c). Rhizomes of each clone were divided into short sections (typically 5-10 cm) and planted into 150 mm diameter plastic pots containing commercial potting medium with no added nutrients (Richgro All-purpose Potting mix, Richgro Garden Products, Jandakot, WA, Australia). Each pot was placed in a 9 L plastic bucket and positioned randomly in an outdoor trial plot at Victoria University, Werribee (Melbourne) in mid-October 2014. Six replicates of each of the six clones were prepared for each salinity treatment. During a preliminary four-week plant establishment phase, pots were kept wet by maintaining 1–2 cm of mains-sourced fresh water (<0.1 g L^{-1} TDS) in each bucket. This initial low water level was required to facilitate the development of the young plants (Doug Frood, pers. comm.). After the establishment period, water levels were increased to the height of the potting medium (i.e., the plants were inundated but not fully flooded). Nine weeks after planting, the water in each pot was replaced with mains water containing a dissolved salt mix of Oceanpure synthetic sea salt OP-50 (Commodity Axis, Camarillo, CA, USA) in a progression of 0, 2, 4, 8, or 16 g L^{-1}. The highest salt concentration applied was approximately half of that found in typical sea water. At the same time, 5 g of slow-release fertilizer (Osmocote all-purpose, Scotts Australia, Bella Vista, NSW, Australia; 13.4% N, 1.0% P, 5.2% K, 7.5% S, 2.2% Ca, 0.3% Mg, 1.7% Fe, plus trace elements) was added to each pot. Water levels within each bucket were maintained by topping up with fresh water every two to three days and the water completely replaced with fresh solutions of the appropriate salinity every two weeks.

TABLE 1 | Details of *Phragmites australis* source population within the Gippsland Lakes including surface water salinities measured as g L^{-1} total dissolved solids (TDS).

Population	Code	Water salinity (g L^{-1} TDS)
Low salinity ('L')		
Avon River	A	1.2
Tambo River	TR	3.1
Nicholson River	N	5.7
High salinity ('H')		
Bandon Bay	BB	18.6
Ocean Grange	OG	23.7
Cunninghame Arm	CA	31.2

Populations are grouped by salinity level classes (low = < 6 g L^{-1}; high = > 18 g L^{-1}).

Sampling for Transcriptomics

After 8 weeks of exposure to salinity regimes, tissue samples from six clone pairs (one sample from the fresh; <0.1 g L^{-1} and one from the highly saline; 16 g L^{-1}, irrigation treatments) were

harvested on the same day between 11:15 and 13:00. No technical replicates were included. For each plant, a 2 cm-long culm section was sampled from within the leaf sheath directly below the topmost ligule from each of four actively growing stems. The tissue type and sampling position were chosen to obtain material at a comparable stage of development and to minimize external contaminants. The material was immediately immersed in 2 ml of chilled Qiagen RNAlater (Ambion Inc, Austin, TX, USA) and stored in darkness at 4°C until processed. Rhizome-tip samples were also harvested from one clone pair (TR) to aid in the assembly of a more complete reference transcriptome (see below) but are not reported.

RNA Isolation

For each plant, an equal amount of tissue from each of the four sampled culms was pooled to give a total of 60–70 mg. This tissue was ground to a powder under liquid nitrogen using a mortar and pestle. While the sample was frozen, RLY buffer from an Isolate II RNA Plant Kit (Bioline, London, UK) was added and the material was allowed to thaw to a slurry before additional grinding was performed. Total RNA was subsequently isolated following the manufacturer's protocol which included an on-column DNA digestion using DNase I. The RNA was eluted with 60 μl RNase-free water and the flow-through reapplied to the spin column for a second round of elution. Quality and quantity was assessed by electrophoresis on a 1.5% agarose gel and spectrophotometry using a NanoDrop 1000 v 3.7 (ThermoFisher Scientific, Wilmington, DE, USA). RNA was also isolated as above for small sections of the rhizome samples.

cDNA Library Preparation and Sequencing

For each of the 14 samples (12 culms, 2 rhizomes), a polyA cDNA library was prepared from 4 μg of total RNA using a TruSeq Stranded mRNA LT Kit (Illumina, San Diego, CA, USA) following the manufacturer's low sample (LS) protocol. A chemical fragmentation step of 30 s at 94°C as described in the Illumina protocol was used to prepare insert lengths between 130 and 340 bp with an aim of producing a final library size of c.450 bp. Fourteen complimentary adapters (Illumina) were chosen with the aid of a barcode diversity calculator[1] and ligated to the sample inserts. For each of the 14 cDNA libraries, the fragment size average and range was assessed using a Bioanalyzer and associated DNA1000 reagent kit (Agilent Technologies, Santa Clara, USA) and the concentration determined using a Qubit 1.0 fluorometer (ThermoFisher Scientific, Wilmington, USA).

The concentration of each cDNA library was normalized to 10 nM before being pooled for processing. Paired-end sequencing of the libraries was undertaken at La Trobe University (Melbourne, VIC, Australia) on a HiSeq^{TM}1500 platform after preparation with a TruSeq PE Cluster Kit v3-cBot-HS and a TruSeq SBS v3 kit (Illumina, San Diego, USA). The libraries were run across a proportion (c.74%) of two lanes on a flow cell.

Transcriptome Assembly and Data Analysis

FastQ files from the sequencing run were de-multiplexed using CASAVA 1.8.2 software (Illumina). Read quality as determined by 'phred' scores was assessed using FastQC v0.11.4 (Andrews, 2010) on the LIMS High Performance Computing cluster (La Trobe University, Bundoora, Australia). High quality reads for all treatments, including the rhizome reads, were *de novo* assembled using Trinity version r20140717 (Grabherr et al., 2011) with stranded data and a minimum k-mer coverage of one to produce a reference transcriptome. Reads from individual samples were then mapped back to the reference using Bowtie (Langmead et al., 2009). The number of reads per gene model (hereafter referred to as a gene) was determined using RSEM (Li and Dewey, 2011) before annotation performed in Trinotate[2]. BLAST searches included Blastp and Blastx against the SwissProt database and Blastx nr and Blastn nt against the GenBank database.

Analysis of differential gene expression among treatments for culm samples was undertaken using *edgeR* v3.10.5 (Robinson et al., 2010) as implemented in Degust v0.2 with the False Discovery Rate (FDR) cut-off initially set to 1.0 and then to 0.05 for subsequent analyses. Using the latter FDR value, differences in expression levels of genes common to different treatments were assessed for samples grouped by source site salinity class (**Table 1**) and by irrigation treatment. Throughout the Results section, 'L' refers to plants sourced from low salinity sites and 'H' refers to plants sourced from high salinity sites as per **Table 1**. Salinity treatments were coded as '0' for freshwater and '16' for 16 g L^{-1} TDS. Gene expression levels from plants sourced from low salinity sites and irrigated with fresh water (L-0) were used as a baseline for comparison. Output from Degust was used to construct four-way Venn diagrams using Venny v.2.0.2 (Oliveros, 2015) to highlight differentially expressed genes common (or unique) to comparisons between specific pair-wise interactions of different treatments. The comparisons presented below highlight informative differences between plants sourced from 'L' and 'H' salinity sites and their response to irrigation with '0' and '16' treatments.

RESULTS

RNA Isolation, Sequencing, and *De Novo* Assembly

Total RNA quality and yield was generally high with little degradation observed after electrophoresis and 260/280 nm absorbance ratios ranging between 2.11 and 2.19. Yields ranged between 10 and 41 μg from 60 to 70 mg of tissue. Sequencing of 12 pooled culm cDNA libraries resulted in approximately 28.6 Gb of data from 286 million 100-bp indexed reads (ave. 23.8 million reads/sample). Over 90% of the reads had phred scores ≥Q30 and 20% were ≥Q40. Reads from the two rhizome samples were used to improve the quality of subsequent assembly but are not reported further. *De novo* assembly of the reads

[1]https://nematodegenetics.wordpress.com/protocols/

[2]http://trinotate.github.io

from culm libraries resulted in the construction of 130,521 contigs across all samples ranging between 201 and 13,436 bp (ave. 732.1 bp). Included among the contigs with the highest raw read counts (c46264_g1 and c43507_g1) were sequences identified as the 18S ribosomal subunit and the 26S ribosomal subunit, respectively, indicative of incomplete non-polyA RNA exclusion during library preparation. Other sequences highly expressed across all samples include those identified as putatively coding for horcolin, phenylalanine ammonia-lyase, YLS9, dehydrin COR410, catalase isozyme 3, probable polyamine oxidase 2, mitochondrial glycine dehydrogenase (decarboxylating), chloroplastic cystathionine gamma-synthase 1, ethylene-responsive transcription factor RAP2-13-like, heavy metal-associated isoprenylated plant protein 26, NAC domain-containing protein 2, chloroplastic photosynthetic NDH subunit of lumenal location 5, cytochrome c, uncharacterized protein ycf68, chloroplastic glutamine synthetase, protein translation factor SUI1 homolog, metallothionein-like protein 4A, and rhodopsin.

Differential Expression Analysis

In total, 62,526 expressed 'genes' were shared between two or more treatment regimes when the FDR cut-off was set to 1.0 (i.e., all genes regardless of their differential expression level significance). The similarity between this suite of genes is represented in multi-dimensional space in **Figure 2A**. The MDS, very similar to a PCA plot, shows groupings of clones and condition when looking at all genes. When data from all clones treated with fresh water were combined and compared to those treated with saline water (L-0 + H-0 cf. L-16 + H-16) at an FDR cut-off of 0.05, 705 (1.13%) genes displayed significant differential expression levels. Of these, 349 were up-regulated in the 'L-16 + H-16' group and 356 were down-regulated (see **Supplementary Data Sheet 1**).

When data from each individual treatment regime were compared (L-0 cf. H-0 cf. L-16 cf. H-16) at an FDR of 0.05, 1832 (2.93%) genes displayed significant differential expression levels in one or more of the regimes (**Figures 2B** and **3**). The clustering of clones sourced from Nicholson River (N) with Cunninghame Arm (CA) and Bandin Bay (BB) in **Figures 2A,B** was unexpected given the large differences in salinity at their source sites (**Table 1**). While the number of genes shared in the 'L-0 cf. L-16' comparison that showed significant up- and down-regulation were similar (53 and 60, respectively), the number of up-regulated genes shared in 'H-0 cf. H-16' was much greater than those down-regulated (54 and 9, respectively; **Figure 4**). In contrast, *P. australis* clones in the 'L-0 cf. H-0' comparison displayed a much lower number of shared up-regulated genes than those down-regulated (15 and 118, respectively).

There were no individual genes present in all four main pair-wise treatment regime comparisons (L-0 cf. L-16, H-0 cf. H-16, L-0 cf. H-0, and L-16 cf. H-16) that showed significant up- or down-regulation as indicated in the central overlapping segments in **Figures 4A,B**. The only treatment comparisons that showed up-regulated genes in common (**Figure 4A**) were 'L-0 cf. L-16' and 'H-0 cf. H-16' (22 genes) and 'L-0 cf. H-0' and 'L-16 cf. H-16' (one gene). Treatment comparisons that showed down-regulated genes in common (**Figure 4B**) were: 'L-0 cf. L-16' and 'H-0 cf. H-16' (3 genes); 'L-0 cf. H-0' and 'L-16 cf. H-16' (eight genes); 'L-0 cf. L-16' and 'L-0 cf. H-0' (six genes).

Differentially expressed genes in culm tissues representative of a general response to saline conditions independent of genotype are likely to include those common to the 'L-0 cf. L-16' and 'H-0 cf. H-16' comparisons. Of the 22 significantly up-regulated genes, notable Blast hits included six genes for which the products are chloroplastic (outer envelope pore protein 16-2; ATP-dependent zinc metalloprotease; glutamate synthase 2 [NADH]; ATP-dependent zinc metalloprotease; glucose-1-phosphate adenylyltransferase large subunit 1; 1,4-alpha-glucan-branching enzyme), Heat stress transcription factor C-2a, NADP-dependent malic enzyme, arginine decarboxylase 2, EID1-like F-box protein 3 and metal transporter Nramp5 (see **Supplementary Data Sheet 2** for further details). The three down-regulated genes found in both 'L-0 cf. L-16' and 'H-0 cf. H-16' comparisons were identified as similar to anthocyanidin 3-O-glucosyltransferase 2-like mRNA (*Setaria italica*), proline-rich protein (PRP) gene (*Saccharum* hybrid) and an unannotated sequence from *Oryza sativa* subsp. *japonica* chromosome 7. We also compared genes showing significant expression level differences in the combined ('L-0 + H-0') cf. ('L-16 + H-16') pair-wise data comparison which is likely to highlight the general response of *P. australis* to salinity (**Supplementary Data Sheet 1**).

Reponses to high salinity that are most likely to be associated with genotypic differences between *P. australis* clones are those unique to the 'L-16 cf. H-16' comparison. A total of 23 genes showed significantly different expression levels for this comparison and, of these, 14 were found only in this pair-wise comparison (**Table 2**). Of these, the two up-regulated genes returned Blast hits to the genes DIR1 (coding for dirigent protein 1) and CB48 (chlorophyll a/b binding protein 48). The 12 down-regulated genes included PER45 (Peroxidase 45), PCO1 (Plant cysteine oxidase 1), LAC15 (Laccase 15), PECT1 (ethanolamine-phosphate cytidylyltransferase), NO93 (early nodulin-93-like), and ankyrin repeat domain-containing protein 65-like.

Despite initial expectations that *P. australis* from various sites in the Gippsland Lakes would not grow at salinities >10 g L^{-1} (based on field data presented in Bird, 1961), specimens from all six sites grew well (albeit often with reduced vigor) at the highest salinity used in the trial, 16 g L^{-1} (Boon et al., 2015c). Specimens collected from the most saline site - Cunninghame Arm - were unaffected by the highest salinity used in the growth trials. At 8 g L^{-1} and 16 g L^{-1} their final above-ground biomass was indistinguishable from plants grown at 0 g L^{-1}. Plants collected from Ocean Grange, another highly saline site, were unaffected by salinity over the range 0-8 g L^{-1}, but showed a 59% reduction in biomass at 16 g L^{-1}. Similar responses were observed when plant performance was gauged in terms of plant height. When *P. australis* was grown in freshwater conditions, plants ranged in height from ~0.6–1.2 m at the end of the growth trials. Mean plant height was little affected by salinity up to 4 g L^{-1}, and at higher salinities, effects were dependent upon the plants' provenance. The height of specimens from Ocean Grange, for example, was little affected at 8 g L^{-1} whereas heights of plants from the three low salinity river sites – Avon, Tambo

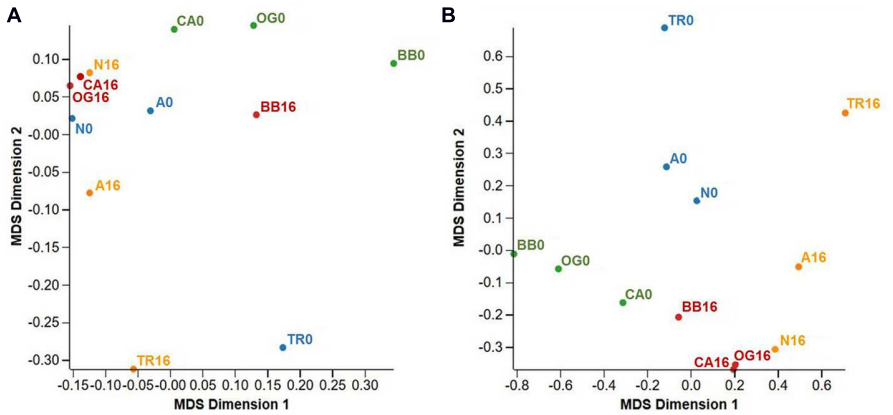

FIGURE 2 | Multi-dimensional scaling (MDS) plots summarizing gene expression profiles from *Phragmites australis* clones shared across two conditions (fresh and 16 g L^{-1} TDS salt water). Blue labels: low salinity source site/fresh water irrigation (L-0); orange labels: low salinity source site/salt water irrigation (L-16); green labels: high salinity source site/fresh water irrigation (H-0); red labels: high salinity source site/salt water irrigation (H-16). **(A)** Represents all genes $n = 62526$, FDR cut-off = 1.0. **(B)** Includes only differentially expressed genes across treatments $n = 1832$, FDR cut-off = 0.5. Population codes are as per **Table 1**.

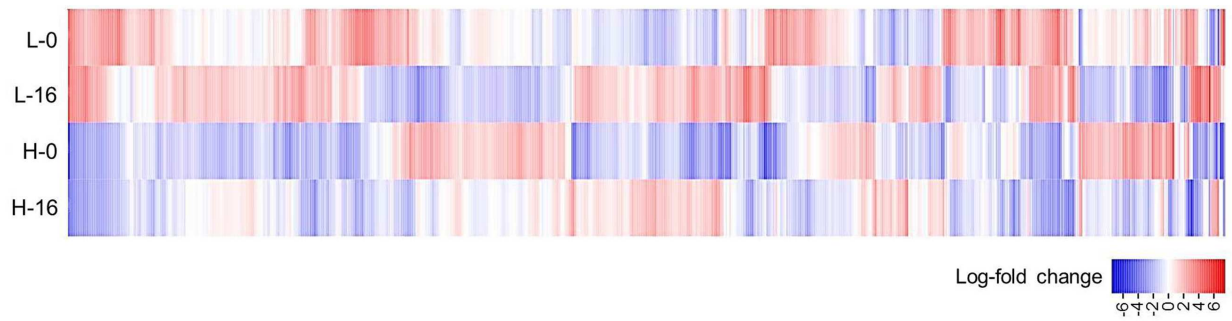

FIGURE 3 | Heat-map of *Phragmites australis* transcript data for plants sourced from low salinity sites (L) versus high salinity sites (H) and irrigated with fresh (0), versus 16 g L^{-1} TDS salt water (16). The figure displays $\log_2$-fold change in average expression of 1832 gene models (horizontal axis) showing significant differential expression across the four data sets (FDR cut-off = 0.05). Genes up-regulated from the average are shown in red and genes down-regulated are shown in blue.

and Nicholson – were reduced by ∼50–60% relative to the 0 g L^{-1} controls. At the highest salinity, mean plant heights were reduced by ∼60–70%, except for specimens collected from Ocean Grange (35%).

DISCUSSION

General Response of *Phragmites* to Salinity

In this study, we present the first comparative transcriptome data for culms of *Phragmites australis* and the first transcript data from southern hemisphere clones of this species. We found that a broad suite of genes was significantly up- or down-regulated in *P. australis* culms in response to irrigation with freshwater, relative to saline water. Changed levels of expression were found in *P. australis* in genes including those identified in other plant species as being responsive to osmotic stress such as DHN1 and DHN3 (coding for dehydrin proteins) (Singh et al., 2015).

However, in our study the expression levels of many of the genes commonly associated with a response to salt stress in plants were not significantly different in *P. australis* clones irrigated with saline compared to fresh water (**Table 3**). These genes included those previously identified as varying between salt tolerant and salt sensitive plants of *P. australis* (Zhao et al., 2004; Takahashi et al., 2007a,b,c, 2009; Eller et al., 2014) and in other species (e.g., Munns and Tester, 2008) including the HAK/HKT gene family. Several genes that have previously been linked to salt stress in other plant species were significantly down-regulated, or the expression did not differ between *P. australis* plants exposed to saline water compared to fresh water while we had predicted significant up-regulation. Some of the discrepancy between our results and those of other researchers might be explained by our comparison of genotypes that are more closely related than those compared in other studies.

This was the case for clones from both low- and high-salinity populations within the Gippsland Lakes. A possible explanation for this discrepancy is that these genes are more

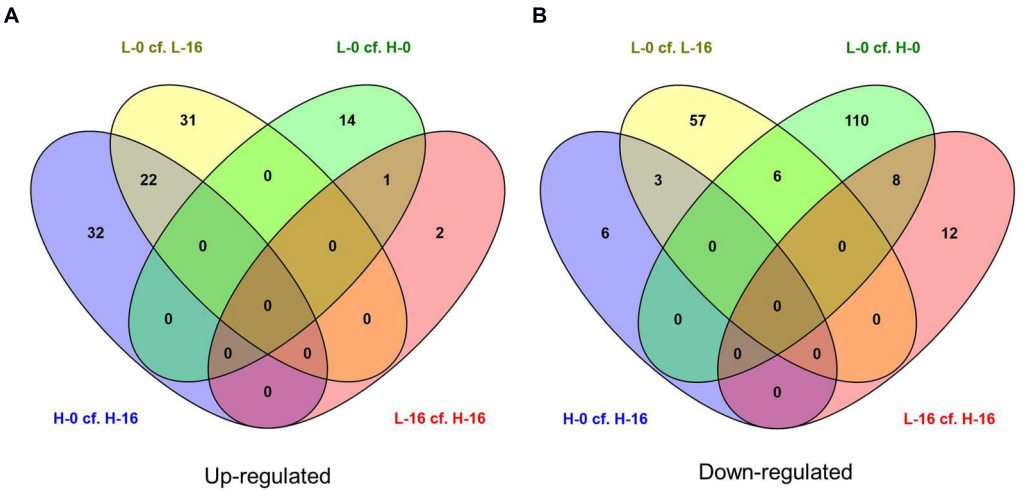

FIGURE 4 | Venn diagrams showing the number of (A) up-regulated and (B) down-regulated genes (FDR cut-off = 0.05) in salinity treatment comparisons for *Phragmites australis* clones from sites with differing water salinity levels (L = low salinity sites, H = high salinity sites; 0 = freshwater irrigation, 16 = 16g L^{-1} salt water irrigation). Figures in the overlapping sections indicate the number of differentially expressed genes common to multiple pair-wise treatment regime comparisons. Lists of corresponding genes are presented in **Supplementary Data Sheet 2**.

strongly expressed in tissues other than culms (e.g., roots and rhizomes) when the *P. australis* clones are exposed to salt stress. In addition, there are likely to be changes in the suite of genes expressed, or their expression levels, related to the period of exposure to salt stress and the stage of physiological response (see Munns and Tester, 2008). Another possible source of variation is that surface-water measurements did not always represent the salinity to which plants were exposed. This can be largely discounted because, as noted earlier, there was a good correlation between surface- and interstitial-water salinities at the various field-collection sites. This indicates that plants growing in saline sites were unlikely to be subsidized by a shallow lens of fresh ground water. It remains possible that some interconnections persist via rhizomes between widely spaced stems in large clones and that sections of a genet growing in lower salinity could alleviate stressors in those sections exposed to higher salinity. However, this scenario seems unlikely due to the regular and consistent salinity regimes that existed within a given site. While this varied intra-genet salinity exposure may occur where a clone expands across a strong salinity gradient, it seemed not to be the case in any of the sites from which plants were collected for this study.

Differential Response of *Phragmites* Clones to Salinity

We found clear differences in gene expression responses to salinity treatment between *P. australis* clones sourced from low salinity areas compared to those sourced from highly saline sites within the Gippsland Lakes. This suggests *in situ* local adaptation of clones within this species to varied salinity levels.

When irrigated with highly saline water, *P. australis* clones with a highly saline provenance (BB, CA, and OG) displayed considerably higher expression levels for genes coding for Dirigent 1 protein (DIR1) and Chlorophyll a/b-binding protein 48 (CB48), than clones from low salinity sites (A, N, TR). DIR proteins are hypothesized to play a role in lignan biosynthesis in the presence of laccase (Davin et al., 1997) and Chlorophyll a/b binding protein 48 is part of the antenna system of the photosynthetic apparatus (Knight et al., 1992; Jansson, 1994). This result suggests that plants from highly saline sites in the Gippsland Lakes are able to maintain higher levels of photosynthesis and biomass production than those from low salinity sites under such conditions, and is supported by growth data for these plants collected in a parallel study (Boon et al., 2015c). Of note, a field-based study of *P. australis* in the Gippsland Lakes indicated reduced photosynthetic efficiency of plants from high salinity sites relative to those in low salinity sites as measured by leaf fluorescence (*Fv*/*Fm*) (Hurry et al., 2013). Their data indicated that plants in highly saline conditions were exhibiting at least some degree of stress response regardless of provenance; a finding paralleled in a greenhouse-based study by Achenbach et al. (2013).

Achenbach et al. (2013) also found differential growth/photosynthesis response to salinity in *P. australis* clones sourced from a range of ploidy levels and geographic areas. However, these authors were looking at global scale differences with plant material sourced thousands of kilometers apart. In the current study, such differences were observed for plants of a (putative) single ploidy level at a scale of tens of kilometers in a single lake system.

While we observed different responses to salt exposure, Hurry et al. (2013) found only moderate genetic structure based on neutral genetic markers (microsatellites) in *P. australis* across the Gippsland Lakes and no correlation between genetic structure and water salinity. In contrast, Gao et al. (2012) found genetic structure based on microsatellite markers in *P. australis* in the Yellow River Delta associated with soil salinity and over a similar

TABLE 2 | Differently expressed genes (FDR cut-off = 0.05) identified in *Phragmites australis* clones sourced from high salinity sites compared to low salinity sites when irrigated with highly saline water (16 g L^{-1} TDS).

Contig sequence ID	UniProt annotation	FDR	Log_2-fold expression change	Gene product and organism	GO terms
Up-regulated					
c29327_g1*	DIR1[1]	0.017	4.59	Dirigent protein 1 (*Arabidopsis thaliana*)	Cellular component; apoplast
c12017_g1*	CB48[1]	0.021	3.94	Chlorophyll a–b binding protein 48, chloroplastic (*Zea mays*)	Biological process: photosynthesis, light harvesting; protein–chromophore linkage Molecular function: chlorophyll binding; metal ion binding Cellular component: chloroplast thylakoid membrane; integral component of membrane; photosystem I; photosystem II
c54138_g2	–	0.019	2.80	Hypothetical protein, mRNA (*Sorghum bicolor*)[4]	(Similar to Putative stress resistance-related protein)
Down-regulated					
c43433_g1	NUD14[1]	0.047	–2.67	Nudix hydrolase 14, chloroplastic (*Arabidopsis thaliana*)	Molecular function: ADP-glucose pyrophosphohydrolase activity; ADP-ribose pyrophosphohydrolase activity; ADP-sugar diphosphatase activity; metal ion binding Cellular component: chloroplast; chloroplast stroma
c45711_g1*	–	0.030	–2.68	Uncharacterized protein, predicted (*Setaria italica*)[4]	–
c41377_g1*	PER45[2]	0.019	–2.83	Peroxidase 45 (*Arabidopsis thaliana*)	Biological process; hydrogen peroxide catabolic process; response to oxidative stress Molecular function: metal ion binding; peroxidase activity Cellular component: extracellular region
c26253_g1*	–	0.012	–3.16	ATP-dependent 6-phosphofructokinase 6-like mRNA – predicted (*Setaria italic*)[4]	–
c51225_g3	DIV[1]	0.045	–3.68	Transcription factor DIVARICATA (*Antirrhinum majus*)	Biological process: DNA binding Molecular function: determination of dorsal/ventral asymmetry; flower development; regulation of transcription, DNA-templated; transcription, DNA-templated Cellular component: nucleus
c56784_g6*	–	0.002	–4.45	Phosphoethanolamine cytidylyltransferase (*Hordeum vulgare*)[3]	–
c55866_g4	RNHX1[1]	0.004	–4.53	Putative ribonuclease H protein (*Arabidopsis thaliana*)	Molecular function; metal ion binding; nucleic acid binding; RNA–DNA hybrid ribonuclease activity
c46015_g2	GSTX3[1]	0.019	–4.91	Probable glutathione *S*-transferase	Biological process: auxin-activated signaling pathway Molecular function: glutathione transferase activity
c61575_g1	–	0.019	–5.18	Predicted protein mRNA (*Hordeum vulgare*)[4]	–
c57868_g1	PLP3[2]	0.009	–5.81	Patatin-like protein 3 (*Oryza sativa* subsp. *japonica*)	Biological process: defense response; lipid catabolic process Molecular process: hydrolase activity
c26482_g3*	–	0.049	–5.91	No significant hits	–
c71221_g1*	–	0.004	–6.15	No significant hits	–
c29596_g1*	PCO1[1]	0.047	–6.20	Plant cysteine oxidase 1 (*Arabidopsis thaliana*)	Biological process: anaerobic respiration; detection of hypoxia; peptidyl-cysteine oxidation; response to hypoxia Molecular function: cysteine dioxygenase activity; metal ion binding Cellular process: cytosol; nucleus
c48421_g1*	NO93[1]	0.029	–6.38	Early nodulin-93 (*Glycine max*)	Biological process: Nodulation
c14700_g1*	–	0.004	–6.50	Ankyrin repeat domain-containing protein 65-like mRNA – predicted (*Setaria italica*)[4]	–
c35884_g1*	–	0.047	–7.10	Hypothetical protein, mRNA (*Sorghum bicolor*)[4]	–

(Continued)

TABLE 2 | Continued

Contig sequence ID	UniProt annotation	FDR	Log_2-fold expression change	Gene product and organism	GO terms
c56558_g7	IMK2[1]	7.83e–[5]	–7.39	Probably inactive leucine-rich repeat receptor-like protein kinase (*Arabidopsis thaliana*)	Biological process: hormone mediated signaling pathway; protein autophosphorylation; Molecular function: ATP binding; peptide receptor activity; transmembrane receptor protein serine/threonine kinase signaling pathway; ubiquitin protein ligase binding Cellular component: cell wall; integral component of membrane; plant-type cell wall; plasma membrane; plasmodesma
c52051_g2*	LAC15[2]	0.019	–7.47	Laccase-15 (*Oryza sativa* subsp. *japonica*)	Biological process: lignin catabolism Molecular function: copper ion binding, hydroquinone:oxygen oxidoreductase activity Cellular component: apoplast
c56238_g3	–	0.047	–9.50	B2 protein-like (*Setaria italica*)	–
c5429_g1	LECH[1]	0.004	–12.55	Horcolin (*Hordeum vulgare*)	Molecular function: mannose binding Cellular component: apoplast

*Annotation and/or product descriptions are based on Blast searches (E-value ≤ 0.05). Gene Ontology (GO) terms as given in UniProtKB where available. Sequences marked * are those unique to the 'L-16 cf. H-16' treatment comparison shown in* ***Figure 3****.* [1]*Blastp Swiss-Prot;* [2]*Blastx Swiss-Prot;* [3]*Blastx nr GenBank;* [4]*Blastn nt GenBank.*

geographic scale. The cause of this discrepancy is unclear, but may be related to the suite of markers used, the degree of gene-flow or clonal spread amongst sites, or factors unaccounted for such as temporal changes in salinity. This highlights the need to identify genetic responses affecting biological functions that are likely to be responsive to directional selection.

As the Gippsland Lakes complex is over 120 km long and is fed by seven major inflowing freshwater rivers (**Figure 1**), there exists a wide range of soil/water salinity conditions to which *P. australis* can be exposed and which may vary temporally. When rhizomes were collected for our study in spring 2014, surface water salinity across the system ranged from 1.2 to 36 g L^{-1} TDS and was positively correlated with the salinity of subsurface soils (Boon et al., 2015c). The degree and frequency of changes to salinity levels at these sites is uncertain but will have an impact on the growth of clones and favor those whose response can minimize the detrimental effects on growth.

When plants are grown in highly saline water, a transcript showing a close affinity with the gene coding for Horcolin (*Hordeum vulgare* coleoptile lectin) was strongly up-regulated in clones from two of the three low salinity sites (A and N) relative to clones from high salinity sites but down-regulated slightly in the clones from the third low-salinity site (TR). This protein is hypothesized to be a mannose binding lectin (Grunwald et al.,

TABLE 3 | Examples of genes associated with salt stress response in plants and their relative expression levels in culms of *Phragmites australis* for the 'L-0 cf. L-16' comparison in this study.

Gene	Product	Organism	Contig sequence ID	Log_2-fold change	FDR
CIPK24	CBL-interacting serine/threonine-protein kinase 24 (SOS2)	*Oryza sativa* subsp. *indica*	c58974_g1	–1.81	0.66
CNBL4	Calcineurin B-like protein 4 (SOS3)	*O. sativa* subsp. *indica*	c43983_g1	–0.51	0.94
FLA4	Fasciclin-like arabinogalactan protein 4 (SOS5)	*Arabidopsis thaliana*	c67150_g1	1.18	0.9
GPX4	Glutathione peroxidase	*A. thaliana*	c51621_g4	–0.79	0.73
HAK26	Potassium transporter 26	*O. sativa* subsp. *japonica*	c36769_g1	–3.35	0.33
HKT7	Probable cation transporter	*O. sativa* subsp.*japonica*	c73767_g1	–3.63	0.62
HSP26	26.7 kDa heat-shock protein	*O. sativa* subsp. *japonica*	c49650_g1	4.53	0.02
MSD2	Superoxide dismutase [Mn] 2, mitochondrial	*A. thaliana*	Not found	–	–
MYB4	Myb-related protein	*O. sativa* subsp. *japonica*	c51737_g4	–4.78	0.25
NHX7	Sodium/hydrogen exchanger 7 (SOS1)	*A. thaliana*	c34293_g2	0.44	0.9
PERK2	Proline-rich receptor-like protein kinase	*A. thaliana*	c46152_g3	–1.45	0.64
PGKH	Phosphoglycerate kinase, chloroplastic	*Triticum aestivum*	c36562_g1	–0.51	0.93
PhaNHA1	Na^+/H^+ antiporter	*Phragmites australis*	Not found	–	–
PK	Pyridoxal kinase (SOS4)	*A. thaliana*	c32545_g1	–1.09	0.72
SALT	Salt-stress-induced protein	*O. sativa* subsp. *indica*	c72546_g1	2.89	0.72

Where a gene is part of a family (e.g., HAK), the copy with the lowest False Discovery Rate (FDR) and most extreme log_2-fold change is listed. FDR ≤ 0.05 is considered significant.

2007) and may play a role in the context of stress signaling in plants (Yang et al., 2013). The significance of this putative stress response in *P. australis* is unclear, particularly given the varied response among clones from low salinity sites. Variation in response at the clonal level as well a treatment level highlights the difficulty in identifying the underlying genetic mechanisms of salinity response under field conditions.

The transcriptome data presented here, coupled with the field-based experimental design, provides a deeper understanding of the complex responses to salt stress in *P. australis*. Our results have shown that a transcriptomics approach provides useful new data, although there are limitations to what can be inferred. We have only examined variation in gene expression levels in *P. australis* culms whereas much of the primary physiological response to salt stress may be specific to tissues within the roots and rhizomes. Response may also vary temporally (see Munns and Tester, 2008) so a comparison of responses in different tissues and at different times after exposure to salinity is needed to provide a clearer understanding of differences in salt tolerance among *P. australis* clones.

While we found strong evidence of differential gene expression in culms among *P. australis* clones and salinity treatments, the underlying reasons for this variation are likely to be complex and involve many gene-by-environment interactions. In addition, the variable response of clones to salinity may be influenced by other factors including endophytic or mycorrhizal relationships. The beneficial role of symbionts in imparting increased salinity tolerance for grasses has been demonstrated by several researchers (e.g., Feng et al., 2002; Saleh Al-Garni, 2006; Baltruschat et al., 2008). Supporting these findings, Ma et al. (2013) showed differences in bacterial and archaeal endophyte assemblages in the tissues of *P. australis* growing along a salinity gradient and Kowalski et al. (2015) have recently investigated the potential for indirectly controlling invasive *P. australis* growing around the Great Lakes in North America by manipulating the occurrence of beneficial microfungal endophytes. Epigenetic variation may also influence variation in salt tolerance among, and within *P. australis* clones in the absence of underlying sequence variation (e.g., Richards et al., 2012; Foust et al., 2016). Epigenomic analysis of different clones could be used in conjunction with transcriptomic data to determine whether non-sequence-based differences influence salinity tolerance and are heritable.

Our study is one step toward developing a series of genetic markers that can be used to select genetically diverse, salt tolerant germplasm for restoration purposes where salinity has been identified as a risk factor. Revegetation projects involving *P. australis* could be more effective if they utilize multiple salt-tolerant clones so that high levels of genetic diversity are maintained to enable adaptive responses. Plant response to environmental change is complex and further study is required to understand how genetic and environmental factors interact to influence salinity tolerance and adaptive capacity in *P. australis*.

While we have focussed on restoration of *P. australis* in salinizing wetlands, the ability to identify genes that confer salinity tolerance also has implications for understanding why particular lineages may be more competitive under saline conditions. We have seen differences in the relative levels of gene expression and this approach provides a means to investigate lineages that are declining or becoming invasive. For example, different gene expression levels may provide insight into the invasiveness in North America of the introduced Eurasian lineage (haplotype M, *sensu* Saltonstall, 2002, 2003) which has a faster growth rate under saline conditions compared to the native North American subspecies (Vasquez et al., 2005, 2006; Howard et al., 2007). At the same time, the approach of using salt tolerant germplasm for restoration must be applied with caution, as there is still a risk that specific native clones and/or lineages could become invasive and dominate sites, particularly if few clones are used widely for restoration.

In our study, clonal differences in gene expression suggest that those from highly saline areas in the Gippsland Lakes are better able to maintain effective physiological functions under saline conditions relative than those from freshwater areas. This has implications for biological conservation and restoration of *P. australis* in temperate coastal wetlands worldwide where increasing salinization is a consequence of environmental changes.

AVAILABILITY OF SUPPORTING DATA

The transcriptome sequence dataset supporting the results in this article is available from the NCBI Sequence Read Archive, accession SRR3233385-SRR3233398. The GenBank BioProject Accession number is PRJNA314710. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GEKX00000000. The version described in this paper is the first version, GEKX01000000.

AUTHOR CONTRIBUTIONS

PB and EJ conceived the broad experiment and were the recipients of GLEF project funds; EJ conceived the molecular analysis. GH, EJ, and PB collected samples. PB prepared and supervised the growth trial. AG provided laboratory space, equipment, and reagents. GH undertook cDNA library preparation and data analysis. NH facilitated sequencing of the cDNA libraries and undertook bioinformatics. GH, EJ, and PB prepared the manuscript.

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provided advice during cDNA library preparation. This work was funded by the Gippsland Lakes Environmental Fund (GLEF).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00432

DATA SHEET 1 | List of putative genes (contigs) with significantly different relative expression levels (FDR cut-off ≤ 0.05) identified in *Phragmites australis* clones sourced from sites in the Gippsland Lakes with high (H) or low (L) water salinity and irrigated *ex situ* with either fresh (0) or 16 g L^{-1} TDS saline water (16). Each worksheet lists different pair-wise comparisons of assembled transcript data. Relative expression levels are given as $\log_2$-fold change and read numbers for the individual samples listed as counts per million reads. Population codes are as listed in **Table 1**.

DATA SHEET 2 | Lists of putative genes (contigs) with significantly different relative expression levels (FDR cut-off ≤ 0.05) that were common to multiple pair-wise treatment regimes in *Phragmites australis* clone culms. These details correspond to the results displayed in Venn diagrams presented in **Figure 4** of the main text. Data in the Blastp and Blastx columns represent significant Blast hits against the Swiss-Prot/UniProtKB databases and display UniProt annotations while Blastx nr and Blastn nt represent significant (*E*-value ≤ 0.05) hits against GenBank.

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Genotypic and Phenotypic Diversity Does Not Affect Productivity and Drought Response in Competitive Stands of *Trifolium repens*

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Clonal plants can form dense canopies in which plants of different genetic origin are competing for the uptake of essential resources. The competitive relationships among these clones are likely to be affected by extreme environmental conditions, such as prolonged drought spells, which are predicted to occur more frequently due to global climate change. This, in turn, may alter characteristics of the ecological system and its associated functioning. We hypothesized that the relative success of individual clones will depend on the size of the ramets as ramets with larger leaves and longer petioles (large ramets) were predicted to have a competitive advantage in terms of increased light interception over smaller-sized ramets. Under drier conditions the relative performances of genotypes were expected to change leading to a change in genotype ranking. We also hypothesized that increased genotypic and phenotypic diversity will increase stand performance and resistance to drought. These hypotheses and the mechanisms responsible for shifts in competitive relationships were investigated by subjecting genotypes of the important pasture legume *Trifolium repens* to competition with either genetically identical clones, genetically different but similarly sized clones, or genetically as well as morphologically different clones under well-watered and dry conditions. Competitive relationships were affected by ramet size with large genotypes outperforming small genotypes in diverse stands in terms of biomass production. However, large genotypes also produced relatively fewer ramets than small genotypes and could not benefit in terms of clonal reproduction from competing with smaller genotypes, indicating that evolutionary shifts in genotype composition will depend on whether ramet size or ramet number is under selection. In contrast to our hypotheses, diversity did not increase stand performance under different selection regimes and genotype ranking was hardly affected by soil moisture, indicating that increasing fluctuations in water availability result in few short-term effects on genotypic diversity in this stoloniferous grassland species. Communities dominated by stoloniferous herbs such as *T. repens* may be relatively resilient to environmental change and to low levels of genetic diversity.

Keywords: clonal growth, competition, drought, genotypic selection, genetic and phenotypic diversity, size hierarchies, *Trifolium repens*

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INTRODUCTION

Ecosystem productivity and resilience to environmental fluctuations are generally believed to increase with increasing diversity (Cardinale et al., 2013; de Mazancourt et al., 2013; Gross et al., 2014). As different species can occupy different ecological niches, complex interactions of different trophic levels exist and negative feedback loops driven by, e.g., soil pathogens are less likely to affect species performance in diverse ecosystems as large species- or genotype-specific pathogen populations rarely build up (Hendriks et al., 2013; van der Putten et al., 2013; Bardgett and van der Putten, 2014; Brotherton and Joyce, 2015). While these concepts have long been applied to species diversity, increasing evidence exists that within-species genetic diversity can be equally important (Reusch et al., 2005; Hughes et al., 2008; Ellers et al., 2011; Grettenberger and Tooker, 2015). The effects of intraspecific genetic diversity may be the consequence of different processes that enable genotypes to occupy slightly different niches, e.g., by association with different microbial communities or herbivores (Ofek-Lalzar et al., 2014; Simonsen et al., 2014; Grettenberger and Tooker, 2015) or due to variation in other functional traits (Ellers et al., 2011; Hughes, 2014). Increased persistence under and resilience to environmental stress in genotypically diverse populations would require among-genotype variation in performance under different selection regimes, as different genetically determined traits interact in their specific way with the environment depending on the specific trait values, ultimately leading to a change in the relative abundance of genotypes (Hughes et al., 2008; Engelhardt et al., 2014). However, the interactions between genotypic and functional diversity and environmental fluctuations are largely unresolved to date.

Species capable of vegetative propagation are an important component in most herbaceous plant communities (Schmid and Harper, 1985; Hamilton et al., 1987; van Groenendael et al., 1996). In contrast to species relying solely on sexual reproduction, vegetatively reproducing species can maintain, or even multiply specific genotypes over prolonged periods of time. Seed set is usually associated with genetic recombination, leading to a new set of genotypes characterized by new trait combinations. While sexual reproduction is one of the preconditions creating the necessary variation for selection to act upon, it may also slow down or weaken the response to selection and adaptation to changed environmental conditions as the genes are reshuffled each generation (Becks and Agrawal, 2012). Clonal species, however, do not require flowering and seed production for population maintenance. Clonal propagation can therefore be expected to lead to fast evolutionary shifts if, in a genetically diverse population, variation in relative fitness among different genotypes exists (Gladieux et al., 2015).

Changes in environmental conditions are bound to alter the consequences of trait variation on competitive outcomes, thereby leading to shifts in genotype performance (Stuefer et al., 2009). In clonal plant populations short-term responses of genetic diversity may thus be enhanced as clonal propagation allows for relatively fast and specific selection for the best adapted genotypes. However, this may also lead to reduced genetic and phenotypic diversity within populations if selection pressures are strong, environmental changes slow and input from new genetic material by sexual reproduction low. This in turn could potentially reduce the ability to adapt to new environmental challenges. Alternatively, environmental effects on genotypic diversity may be mitigated through high levels of phenotypic plasticity, enabling multiple genotypes to buffer environmental fluctuations and maintain genetically diverse populations over time (Nicotra et al., 2010); or because genotypes have constitutively different relative performance in different environmental conditions. As changes in genetic diversity within populations can translate into variation at higher trophic levels (Reusch et al., 2005; Barton et al., 2015), whether environmental fluctuations maintain diversity or not can have a large influence on community processes.

Over the last decades environmental conditions are increasingly changing, in many parts of the world leading to a higher frequency of extreme weather conditions such as early or prolonged drought events (IPCC, 2014). Drought conditions may, directly or indirectly, select for a different suite of traits as compared to well-watered conditions. Profuse growth under favorable soil conditions will lead to asymmetric competition for light (Anten and Hirose, 2001). In the common stoloniferous species *Potentilla reptans* taller, heavier leaves placed at the top of the canopy captured a disproportionate amount of light per unit invested biomass compared to leaves with a lower investment that were placed lower down (Vermeulen et al., 2008b). Hence in competition, genotypes with large ramets can maintain their position in high light conditions through their high investment in leaves, while genotypes that produce smaller ramets can have a strongly reduced productivity due to shading if the height growth of the competitive surrounding is fast enough (Vermeulen et al., 2008a). Because there is a trade-off between investment in large ramets and the number of ramets (Huber and Wiggerman, 1997; Vermeulen and During, 2010), competition for light should lead to selection for genotypes producing large ramets with long petioles, but with a lower number of ramets.

The strong selective pressure on aboveground competitive ability will become lower when water shortage leads to reduced biomass development. Under such conditions, selection can be expected to be more strongly driven by belowground competition, which is usually assumed to be less dependent on size, i.e., symmetric (Schwinning and Weiner, 1998; Casper et al., 2003). In addition, selection gradients on leaf size were found to differ between low and high water availability in *Cakile edentula* (Dudley, 1996). This leads to the prediction that depending on the soil water availability different genotypes will be selected for, depending on their specific suite of traits and the potential to show adaptive responses to the respective environmental conditions.

In this study, we artificially manipulated genetic and functional diversity, by creating genetically homogeneous stands, genetically diverse, but phenotypically similar stands and phenotypically as well as genetically diverse stands. By using genotypes that differed up to 2.5-fold in the ecologically relevant key-trait ramet size we manipulated the potential range of phenotypic diversity within the experimental plots. The study was performed to test (I) the effects of water availability and

phenotypic characteristics on relative genotype performance, (II) the consequence of inherent genetic variation in trait expression for phenotypic variation in competitive stands, and (III) the consequence of genetic and phenotypic variation for population performance.

(I) We expected shifts in genotype abundance, and the direction of the shift to be dependent on water availability. The relative shift in genotype abundance was hypothesized to be driven by the phenotypic characteristics of the genotypes. Generally we expected genotypes characterized by large ramets to be competitively superior over genotypes characterized by smaller ramets, due to different positioning of leaves within the canopy. This relative advantage was expected to be reduced under drought conditions, as overall leaf area of the stands will be smaller and light gradients less steep. (II) We expected that competition would lead to shifts in variation in trait expression. Variation could decrease due to more uniform trait expression, if e.g., all petioles assume a similar length in competitive stands, or, alternatively, could increase due to asymmetric competition having stronger negative effects on genotypes which are positioned lower within a size hierarchy. (III) We also hypothesized that genetically and phenotypically diverse stands would have a higher overall performance and be more resistant (Grimm and Wissel, 1997) to drought as different genotypes may differ in their drought tolerance.

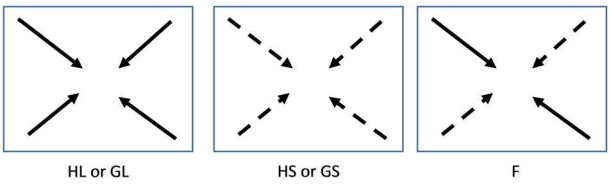

FIGURE 1 | Schematic drawing of experimental set up. Four lateral cuttings consisting of approximately five ramets were planted 5 cm from the corners of the trays and the stolons of the plants were directed toward the center of the trays. H: genetically and phenotypically homogeneous monostands; G: genetically diverse but phenotypically similar stands; F: phenotypically diverse stands; S and dashed arrows: Small size class; L and solid arrows: Large size class. A total of 768 plants were used for the experiment. Number of replicates per genotype per treatment: HL and HS: 24; GL, GS, and F: 12

MATERIALS AND METHODS

Plant Material

The experiment was performed with the clonal species *Trifolium repens*. This species grows by means of aboveground monopodial stolons along which single-leaved rooted ramets are positioned at regular distances. The plants can produce extended integrated clonal systems which can face high intensity of competition at different organizational levels. Each ramet consists of an internode, a leaf, one axillary meristem which can give rise to either a flower or a new lateral stolon, and roots (Hutchings et al., 1997; Huber and During, 2000). The size of the modular units (ramets) can vary greatly among genotypes, which is of pivotal importance for the relative performance of the genotypes in their respective environments (Weijschedé et al., 2006, 2008). Ramet size reflects an internally determined covariation of leaf size, internode length, and petiole length, with a positive correlation among these traits (Weijschedé et al., 2006). Previous research has shown that *T. repens* is characterized by a high genetically determined variability of trait expression, strongly responds to competition and that this response may be different depending on whether the competitors are from the same genotype or genetically and morphologically different (Turkington and Burdon, 1983; Hutchings et al., 1997; Lotscher and Hay, 1997; Bittebiere et al., 2012). In addition, in this species leaf size has been shown to differently affect drought tolerance and recovery to drought (Annicchiarico and Piano, 2004; Li et al., 2013). The formation of dense communities under natural conditions with high incidence of inter- and intra-clonal competition, high levels of plasticity, fast ramet production rates and large inter-genotypic variation makes *T. repens* an ideal species to experimentally study the effect of genetic and functional diversity on population performance.

For the present experiments eight genotypes differing in ramet size were selected from 36 genotypes used by Weijschedé et al. (2006, 2008). The genotypes used in this experiment were originally collected in a riverine grassland along the river Waal (Ewijk, the Netherlands). Distance between plants was at least 5 m and plants were identified as being genetically different by means of AFLP analyses (J.L. Peters, unpublished results; Supplementary Figure 1 in Supplementary Data Sheet 1). We chose four genotypes characterized by small ramets and four genotypes characterized by large ramets (**Table 1**). The choice of the genotypes was primarily based on petiole and leaf length, as these traits are important for determining the competitive hierarchies in dense stands (Weiner, 1985; Vermeulen et al., 2008a; Weijschedé et al., 2008).

Experiment

Genotypes were collected more than 5 years prior to the experiment to minimize potential environmentally induced carry

TABLE 1 | Phenotypic ramet characteristics of genotypes used in this experiment.

Genotype identifier	Size class	Petiole length (mm)	Leaf length (mm)	Internode length (mm)
A25 ○	Large	53.8	11.8	14.5
A4 ●	Large	48.5	13.2	21.9
B15 ▼	Large	52.7	16.5	22.9
B4 △	Large	49.5	14.3	19.3
B7 ■	Small	18.8	8.7	13.7
D18 □	Small	22.1	8.0	15.8
D21 ♦	Small	20.8	8.5	13.5
D30 ◊	Small	22.2	9.4	12.3
Average initial variation				
Phenotypically similar		6.2	10.5	14.8
Phenotypically diverse		48.5	29.3	24.9

-over effects. During summer genotypes were maintained under outdoor conditions in the experimental garden of Nijmegen University in containers (l × w × h = 0.4 m × 0.3 m × 0.3 m) filled with a 1:1 mixture of commercial potting compost and sand with an addition of 4gr slow release fertilizer (Osmocote+, Scott Sierra International B.V. Heerlen, the Netherlands, 9–10 months) per liter soil. Plants were watered regularly and repotted each year into new containers. During winter plants were moved to a heated greenhouse where the experiment was also performed. Incident light was supplemented by high pressure sodium lamps (Hortilux Schreder, 600 Watt) whenever light availability fell below 400 μmol m^{-2} s^{-1} between 6:00 h and 22:00 h. Before the experiment, 16 lateral cuttings (hereafter referred to as clonal fragments) containing one rooted ramet with a lateral stolon consisting of three to five newly produced ramets were made for each genotype. The cuttings were distributed over 32 trays (l × w × h = 0.22 m × 0.18 m × 0.05 m) filled with 1 l substrate consisting of five parts loamy sand and one part sieved potting compost. Three grams slow release fertilizer (Osmocote+ Scott Sierra International, 3–4 months) were added to each tray. Trays were filled and kept wet for 2 weeks before the onset of the experiment for nutrient release to commence prior to the experiment. Each tray contained four clonal fragments. Each clonal fragment was planted in one of the corners of the trays, with the rooted ramets approximately 5 cm from the corner and the stolon apices directed toward the center of the tray to facilitate competition among the four plants (**Figure 1**). Throughout the experiment the stolons were prevented from growing out of the trays by bending them back to the tray in order to increase competitive interactions within the trays and avoid support of clones in the trays by ramets subjected to favorable light conditions outside the tray borders.

The clonal fragments were subjected to three types of competition treatments, in which they were allowed to compete above- as well as belowground (**Figure 1**). In the monostands four clonal fragments of the same genotype were competing with each other (one tray per genotype per block). In the phenotypically similar stands clonal fragments of either the four small or the four large genotypes were competing with each other. This treatment was repeated twice per size class per block. In the phenotypically diverse stands two small and two large genotypes were grown in each tray. This treatment was repeated four times per block with a different combination of two large and two small genotypes in each tray. Each genotype was thus used twice with three different competitors. In this treatment, the two large or the two small genotypes were grown in opposite corners, resulting in each phenotype having two neighbors of the opposite phenotype. All combinations were subjected to either well-watered (moist) or drought (dry) conditions. For the well-watered conditions soil moisture was kept at 35% (v:v) and for the drought treatments soil moisture was kept at 10–15% (v:v). The volumetric water content in the soil was monitored every other day by measuring at four different locations in each tray using a theta probe (HH2, Moisture Meter version 2; Delta-T Devices, Cambridge, UK) after which the trays were supplemented with water to regain 35% and 15% soil moisture, respectively. Drought treatments started 2 weeks after planting and lasted for 4 weeks. The experiment took place in the greenhouse of Nijmegen University in the period April–June. Treatments were repeated in six temporal blocks starting at 1-week intervals. The experiment contained a total number of 192 trays and 768 plants. For the monocultures a total of 24 clonal fragments per genotype were used (spread over six trays) and for the phenotypically similar and phenotypically diverse stands a total of 12 clonal fragments were used per genotype (spread over 12 trays).

At harvest all plants were washed carefully free of substrate to allow for separation of the roots. As the stolon connections of *T. repens* are long-lived and were still intact at the end of the experiment, the four original plants could be separated. For each clonal fragment the length of the main stolon, number of ramets and lateral branches, total dry weight as well as allocation to roots, stolons and leaves were measured. In addition the fourth youngest ramet of the main stolon was harvested separately to determine ramet architecture (internode length, petiole length, and leaf size). To get information about leaf turnover, the number of leaves on the main stolon was counted as well. Plant parts were dried for at least 48 h at 72°C before determining dry weights.

Statistical Analyses

Overall production of the trays in terms of total ramet number and biomass was analyzed by means of two-way ANOVA, with water availability and competition type as the main factors. Growth, ramet architecture and biomass allocation of individual clonal fragments were analyzed by means of a three-way nested ANOVA with competition treatment, genotype size class and soil moisture treatment as main effects. Genotypes were nested within size class and the temporal block was added as a random factor. As performing ANOVA's on absolute trait values can impede the interpretation of biologically relevant relative trait responses to treatments in traits which are characterized by inherently different trait values (Huber, 1996) we also log-transformed ramet size to test whether small and large sized genotypes responded differently to moisture treatments.

In order to test whether plants assume similar phenotypes if grown in mixtures or, alternatively, phenotypic variation among plants gets reinforced due to, e.g., effects of size hierarchies, phenotypic diversity was determined by calculating the realized coefficient of variation (CV) of phenotypic traits among the four competing clonal fragments. A two-way ANOVA was performed on these CVs with competition treatment and moisture treatment as main effects to test whether phenotypic diversity was affected by the type of competition treatment and soil moisture conditions.

To test whether genetically and phenotypically diverse stands had a higher overall performance than can be expected on the basis of their monostands, we used the additive partitioning method (Loreau and Hector, 2001). This method calculates a net effect of treatments on biomass (ΔY) as the differences between observed yield and the expected yield of the mixture. The expected yield is defined as the average monostand yield of the genotypes in the mixture. Because of significant block effects, we used monostand within blocks to calculate this expected yield (see Van Ruijven and Berendse, 2009). ΔY was then decomposed into a complementarity effect (whether the genotype yields in

mixtures are higher or lower than expected on the basis of the weighted average monostand yields) and a selection effect (the covariance between the monostand yield of the genotype and the difference between expected yield in monostand and the observed yield in the mixtures of the genotypes -ΔRY-). In addition, a multiple regression was performed for each moisture treatment separately. Total ramet number or biomass were the dependent variables and regressed against the CV of the different phenotypic characteristics.

We performed a correspondence analysis (CA) as implemented in PC-Ord 6.03 (option RA; McCune and Mefford, 2011) to get an overview of the results and insight into the mutual relationships between the measured traits in the face of the two water availability treatments and the three competition treatments. We analyzed the datasets of the measured traits of the plants in both water treatments separately and in combination, with the major directions of variation correlated to treatments, block effect, type of competition and individual genotypes calculated afterward and added as supplementary variables in the ordination graphs.

RESULTS

Relative Genotype Abundance

Neither competition type nor moisture treatments had strong effects on the genotype ranking, but the relative variation among genotypes with respect to total biomass or ramet number varied conspicuously among competition treatments (**Figure 2**, **Table 2**). Variation in plant weight was up to fourfold larger in the phenotypically diverse stands than in the other two competition treatments. The biomass of large and small plants responded differently to competition treatments (**Table 2**). This was mainly due to the positive response of the large sized genotypes B15 and B4 to increased diversity, and a negative response for most of the small genotypes in the phenotypically diverse treatment (**Figure 2**). Small-sized genotypes produced on average twice as many ramets as large-sized genotypes did. Contrary to the results on biomass, variation in ramet number was greater in monostands than in phenotypically diverse stands (**Figure 2**). The number of ramets produced by large and small genotypes responded differently to competition treatments (**Figure 2**, **Table 2**). The small-sized genotypes showed on average a stronger reduction in ramet number in response to diversity, while the large-sized genotypes showed a slight increase in ramet number in response to increasing diversity. In addition, within size classes there was significant variation in total ramet number in response to competition treatments, with the small-sized genotype B7 showing the strongest decrease and the large sized genotype B15 the strongest increase in ramet number in response to increasing diversity.

Consequences of Treatments for Phenotypic Characteristics

Competition type and size class hardly affected mean ramet dry weight or ramet architecture (**Figures 2** and **3**, **Table 2**). Large-sized genotypes produced on average heavier ramets characterized by longer petioles and larger leaves. There was a very slight, but significant increase of the ramet weight of large-sized genotypes with increasing diversity, which coincided with a similarly slight decrease of ramet weight of small-sized genotypes. The relative allocation to different plant structures was fairly constant over competition treatments and size classes, with exception of allocation to roots (**Figure 4**, **Table 2**), which was on average 50% larger in large genotypes than in genotypes characterized by small ramets. This coincided with a marginally significantly reduced allocation to stolons. Across the competition treatments, the allocation pattern remained rather constant.

Soil moisture consistently affected all traits (**Figures 2–4**, **Table 2**). Plants subjected to dry conditions produced considerably less biomass, fewer and smaller ramets, and allocated more mass to roots and less to aboveground structures. Generally, the different genotypes responded similarly to soil water availability across size classes and competition treatments (**Figures 2–4**, **Table 2**). The apparently different response of ramet weight across and within size class to soil moisture was mainly due to absolute size differences and disappeared after log transformation. Within moisture conditions, ramets of large-sized genotypes remained on average twice as heavy as ramets from small-sized genotypes (moisture * size: $F = 4.7$, $p = 0.07$; moisture * genotype(size): $F = 0.7$, $p = 0.6$). The same held for the response of leaf size across size classes to water availability. This result was also confirmed when testing for potentially different effects of ramet size or petiole length on relative total biomass and ramet number under different soil moisture conditions within competition treatment, which revealed that there was no significant interaction between ramet size or petiole length and moisture availability (F-values ranging between 0.3 and 3.4).

Correspondence analysis of the whole dataset revealed that variation along the first axis corresponded very well with the separation of the genotypes according to ramet size ('Large' vs. 'Small'); genotypes B7 ('Small') and B15 ('Large') in particular contributed to this correlation. Variation along axis 2 mainly corresponded with the contrast between the two water treatments (factor 'Dry'). The third axis represented other aspects of the water treatment, with a strong correlation between allocation to roots and drought (see Supplementary Data Sheets 1 and 2). Interestingly, the treatments with different types of competition were not correlated to any of the first three axes (Pearson $r < 0.01$ in all cases), suggesting, that any effects of these treatments were unrelated to the main directions of variation in the experiment. The patterns of variation of plant traits in the two water availability treatments were remarkably similar (see Supplementary Data Sheets 1 and 2), in spite of the considerable effect of drought on the plants.

Plant traits tended to cluster in groups (**Figure 5**, Supplementary Data Sheet 2), which largely remained the same in the three analyses (moist treatment only, dry treatment only, and complete dataset – see Supplementary Data Sheet 2). One group consisted of traits measuring various aspects of individual ramets (leaf size, petiole length, and dry weight of ramet, lamina, and petiole). A second, more dispersed group

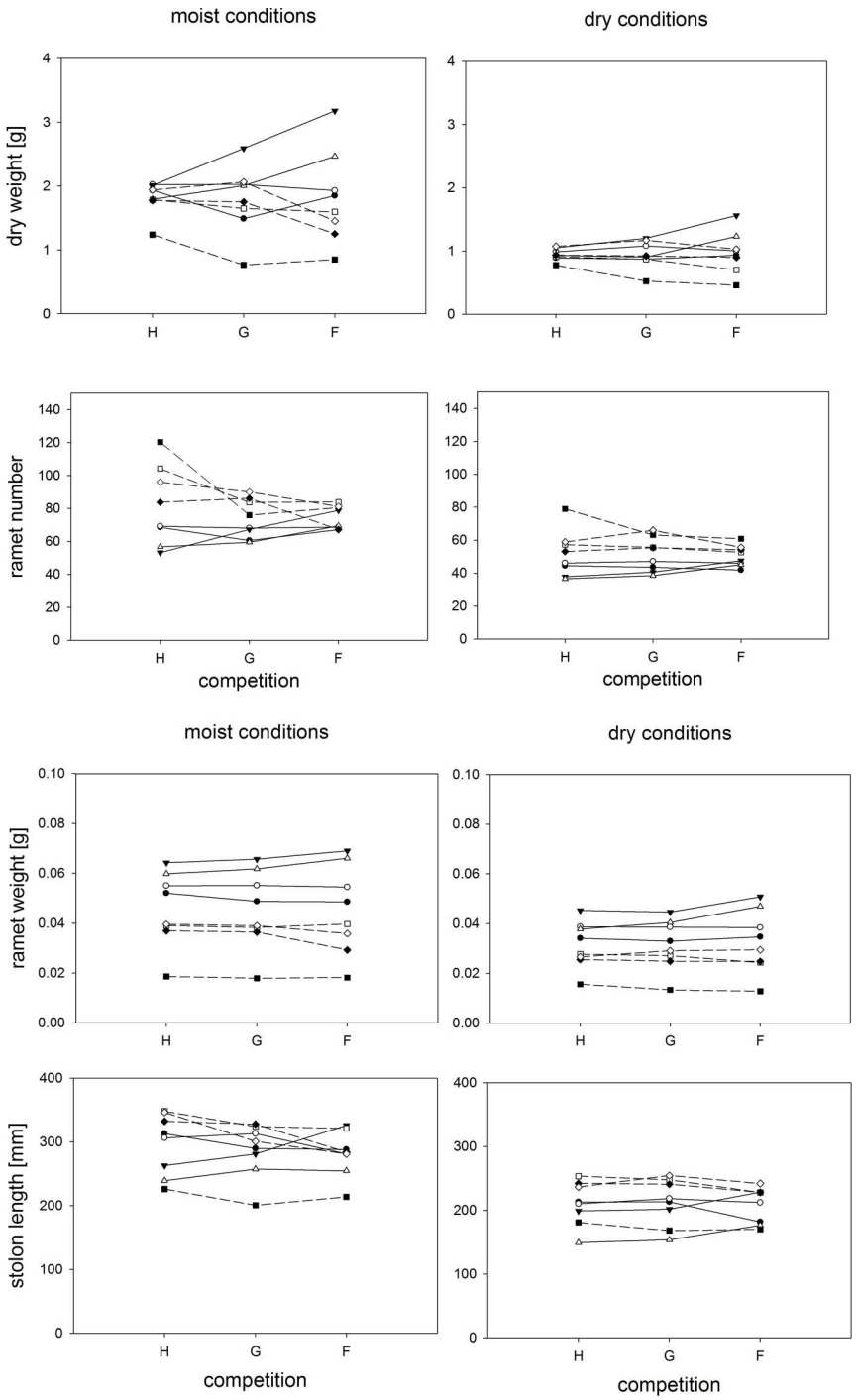

FIGURE 2 | Clonal fragments: Response of plant biomass, ramet number, average ramet weight, and stolon length to competition and soil moisture treatments. Each line represents the mean value of a specific genotype in the respective treatments. Solid lines represent large genotypes, dashed lines small genotypes. Competition type abbreviations: H: homogeneous monostands; G: phenotypically similar stands; F: phenotypically diverse stands. For significances, see **Table 2**.

consisted of traits of the whole clonal fragment such as allocation to the different organs, number of primary ramets, leaf turnover and side branches. A small third group consisted of total ramet number, lateral ramet number, and overall stolon weight. Allocation to roots was higher for genotypes with big ramets and in the drought treatment, allocation to leaves correlated strongly with the big-ramet group of plants, and allocation to stolons correlated with the drought treatment.

TABLE 2 | Results (*F*-values and their significance) of a three-way nested ANOVA testing for the effects of competition treatment, genotype size class and soil moisture treatment on growth, ramet architecture and biomass allocation.

	df	Dry weight [g]	Ramet number [n]	Ramet weight [g]	Stolon length [mm]	Internode length [mm]	Petiole length [mm]	Leaf size [cm^2]	Root allocation [%]	Stolon allocation [%]	Leaf allocation [%]
Competition (C)	2	0.3^{ns}	0.2^{ns}	0.2^{ns}	0.9^{ns}	0.7^{ns}	0.4^{ns}	1.1^{ns}	0.2^{ns}	0.1^{ns}	0.1^{ns}
Size class (S)	1	1.7^{ns}	**41.7***	**17.4****	0.5^{ns}	0.3^{ns}	**23.8***	**37.4*****	**28.6****	*4.4*$^{\$}$	0.1^{ns}
C * S	2	**5.1*****	**4.7***	**4.1***	*3.1*$^{\$}$	*2.9*$^{\$}$	0.1^{ns}	2.3^{ns}	1.6^{ns}	*3.4*$^{\$}$	1.4^{ns}
Genotype(size)	6	**13.0*****	1.5^{ns}	**19.2*****	**14.0*****	**12.2****	**5.1***	**4.4***	**33.8****	**64.7*****	**50.1*****
C * Gen(S)	12	*2.5*$^{\$}$	**4.7****	2.1^{ns}	2.1^{ns}	1.0^{ns}	1.6^{ns}	**3.5***	1.0^{ns}	1.5^{ns}	**2.7***
Moisture (M)	1	**412.6*****	**354.2*****	**175.1*****	**210.1*****	**180.8*****	**308.8*****	**114.6*****	**352.0*****	**34.1*****	**566.3*****
M * C	2	0.1^{ns}	1.1^{ns}	1.4^{ns}	1.4^{ns}	1.3^{ns}	0.9^{ns}	*3.3*$^{\$}$	**4.3***	1.3^{ns}	0.4^{ns}
M * S	1	*4.1*$^{\$}$	1.0^{ns}	**20.2****	3.5^{ns}	1.6^{ns}	2.8^{ns}	**16.4****	0.2^{ns}	0.2^{ns}	0.7^{ns}
M * Gen(S)	6	0.8^{ns}	1.3^{ns}	**4.2***	2.1^{ns}	**2.9***	**3.4***	**15.7*****	1.2^{ns}	**3.8***	1.5^{ns}
M * C * S	2	0.2^{ns}	**4.0***	0.0^{ns}	1.6^{ns}	2.2^{ns}	2.0^{ns}	1.6^{ns}	1.2^{ns}	0.2^{ns}	1.3^{ns}
M * C * Gen(S)	12	0.9^{ns}	0.4^{ns}	0.6^{ns}	1.0^{ns}	1.3^{ns}	1.1^{ns}	0.5^{ns}	0.8^{ns}	0.7^{ns}	0.6^{ns}
Block	5	**6.7***	**8.0*****	**15.3*****	**8.5*****	**4.4*****	**32.2*****	**23.2*****	**6.9*****	**30.3*****	**29.5*****

Genotypes were nested within size class. Significances are as follows: $^{ns}p > 0.10$; $^{\$}0.10 < p < 0.05$; $^{*}0.01 < p < 0.05$; $^{**}0.05 < p < 0.01$; $^{***}p < 0.001$. *Significant values are highlighted in bold, marginally significant values in italics.*

Phenotypic Diversity

Generally the realized phenotypic diversity among competing plants in the treatments with several genotypes was similar to or larger than the phenotypic diversity expressed by plants grown in monocultures. The overall variation among plants was highest for the performance parameters and lowest for biomass allocation patterns and stolon length, where the realized phenotypic variation among plants was similar to the predicted range based on the traits expressed in monocultures (**Figure 6**, Supplementary Table 2 in Supplementary Data Sheet 1). There was an almost linear increase with increasing diversity for CV of whole plant and individual ramet weight. Phenotypic, but not genetic diversity led to increased among-plant variation for leaf size and allocation to roots, while genetic and phenotypic diversity increased among-plant variation for all stolon parameters and allocation to leaves to a similar extent. Phenotypic diversity hardly affected biomass and ramet production (**Table 3**) with a few exceptions: Under moist conditions high variation in stolon length was negatively and variation in root allocation was positively associated with biomass production. Ramet number only showed negative associations with stolon allocation under dry and moist conditions.

Stand Performance

The type of competition significantly affected overall ramet and biomass production by the whole stand (**Figure 7**, **Table 4**). If grown in monocultures stands consisting of large genotypes produced significantly fewer ramets and a higher total biomass than monocultures consisting of small genotypes. This result was similar for phenotypically similar stands consisting of the four genotypes characterized by large ramets. Overall, low soil moisture availability significantly reduced overall biomass by up to 50% and ramet number by up to 25% (**Figure 7**, **Table 4**). The negative effect of low moisture availability on ramet number depended on the type of competition. Monocultures consisting of small sized genotypes responded most strongly to the drought treatments, while phenotypically diverse stands showed the weakest response to drought treatments.

No evidence of overyielding of the mixtures was found. The statistical analysis (see **Table 5**) indicated that the net diversity effect did not significantly differ from 0. This was true for both, populations subjected to different competition treatments and to different soil moisture availability. In addition, the variation among traits hardly explained variation in biomass and ramet production (**Table 3**). Across competition treatments variation in the different traits explained only total biomass in stands subjected to moist conditions (**Table 3**). Under these conditions high variation in stolon length was negatively associated with overall biomass production, while high variation in allocation to roots was positively associated with overall biomass production.

DISCUSSION

Our results clearly show that the relative performance of phenotypically different genotypes was neither affected by the competitive environment nor by the prevalent environmental

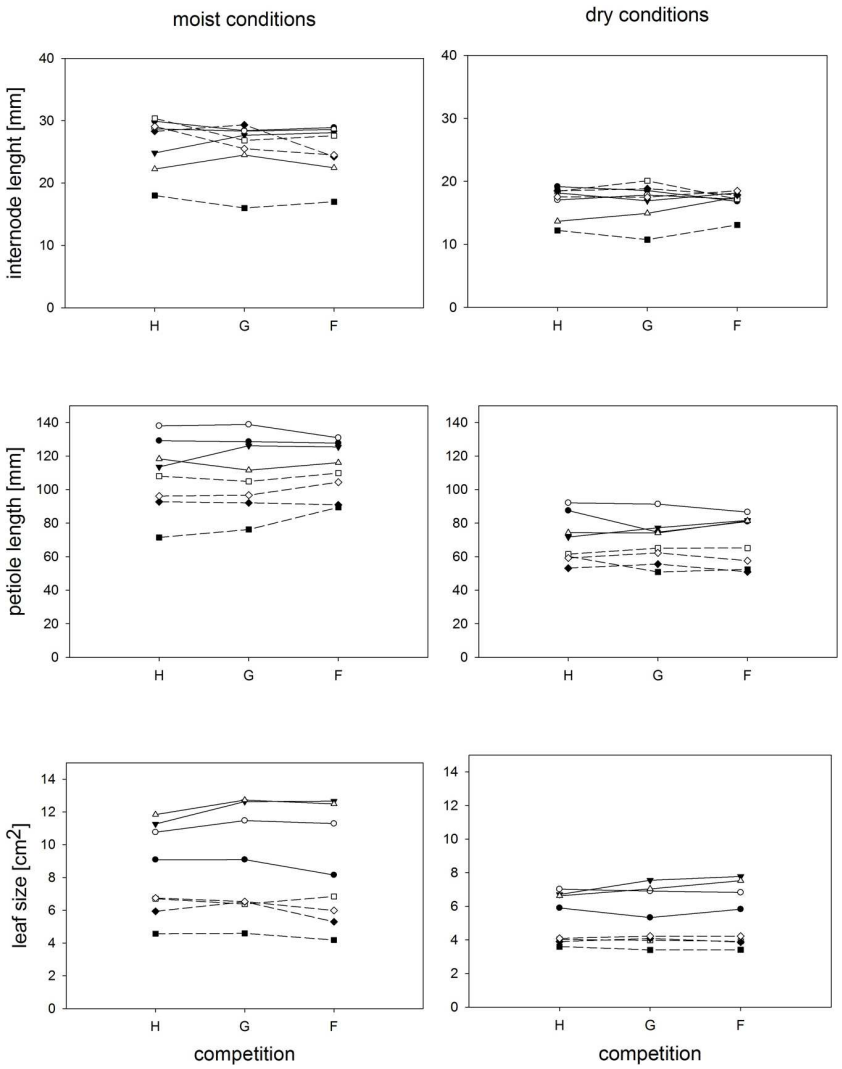

FIGURE 3 | Individual ramets: Response of parameters representing ramet architecture to competition and soil moisture treatments. Each line represents the mean value of a specific genotype in the respective treatments. Solid lines represent large genotypes, dashed lines small genotypes. Competition type abbreviations: H: homogeneous monostands; G: phenotypically similar stands; F: phenotypically diverse stands. For significances, see **Table 2**.

conditions, resulting in an almost constant ranking among genotypes. They further show that highly diverse populations did not outperform monocultures in terms of increased ramet or biomass production or increased resistance to environmental fluctuations. However, the increased variation in biomass production and decreased variation in ramet production with increasing diversity showed that these competition treatments affected genotypes differently. This latter pattern was consistent across different levels of water availability. These results may be explained by a highly conservative, evolutionarily fixed, set of character combinations. Generally evolutionary changes and genotypic diversity are not independent. Increased variation among genotypes creates a higher potential for selection to act upon, and will also eventually result in faster evolutionary changes. Based on the results from the present experiment, diversity can be hypothesized to be lost at different speeds depending on the main trait under selection, i.e., biomass or ramet number; but this loss of genetic diversity appears to be largely unaffected by water availability. These results are in contrast to the common expectation that diversity increases resistance to environmental change and that environmental variation can lead to a maintenance of genetic diversity in populations. Below, we discuss our results in the light of community productivity and resilience.

Relative Genotype Performance Does Not Change across Water Treatments

Contrary to our expectation, the relative performance of genotypes was not affected by water availability. Our expectation was based on the idea that in highly competitive herbaceous communities the performance of plants strongly depends on

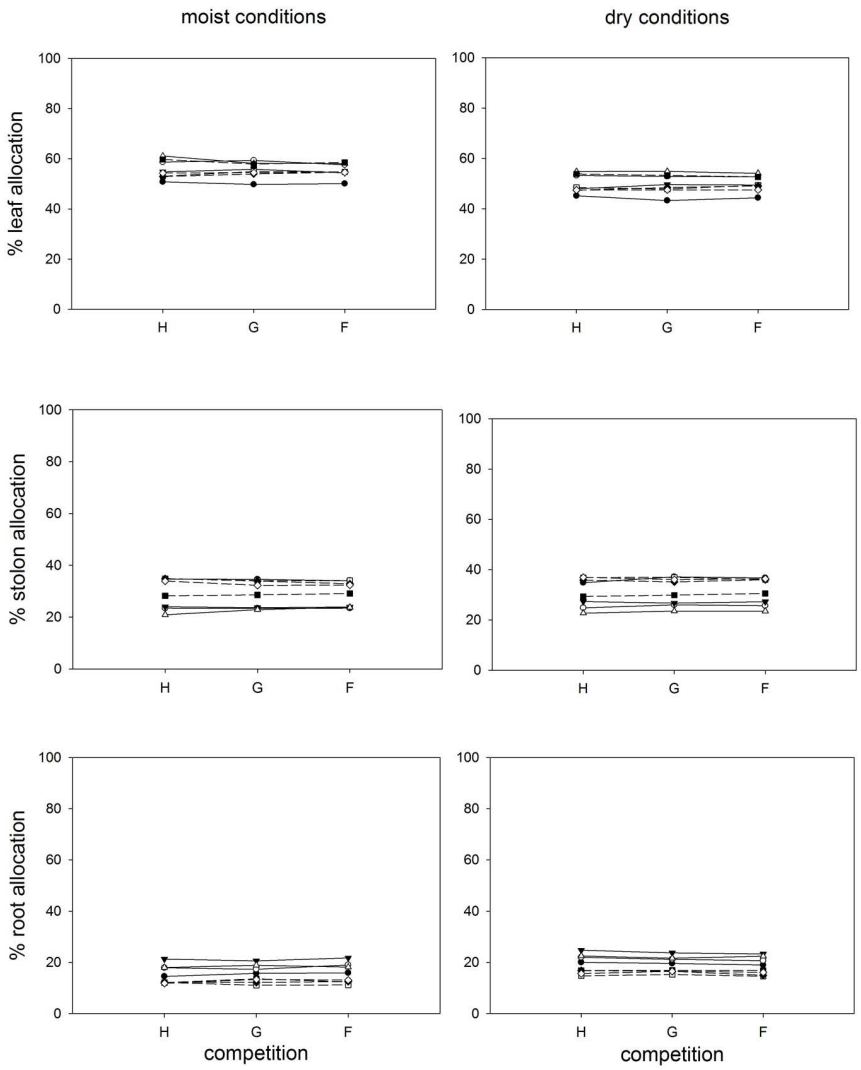

FIGURE 4 | Clonal fragments: Response of biomass allocation pattern [calculated as (g g^{-1})*100] to competition and soil moisture treatments. Each line represents the mean value of a specific genotype in the respective treatments. Solid lines represent large genotypes, dashed lines small genotypes. Competition type abbreviations: H: homogeneous monostands; G: phenotypically similar stands; F: phenotypically diverse stands. For significances, see **Table 2**.

the relative positioning of leaves within the canopy (Anten and Hirose, 1998; Anten et al., 2005), leading to a competitive advantage of taller individuals over shorter ones (Weiner, 1985, 1990). As *T. repens* had formed dense canopies throughout the experiment, resulting in an up to fivefold elongation of the vertically oriented petioles in moist conditions, we had expected genotypes with inherently larger ramets to outperform genotypes with inherently smaller ramets if subjected to moist conditions, but not under dry conditions where canopy density was lower and the light gradient less steep. Interestingly, even though plants responded to moisture treatments with altering petiole elongation, the relative positioning of the leaves within the canopy remained constant across moisture treatments, as petiole elongation of small and large ramets was similarly reduced by the drought treatment, and thus the relative height distribution across genotypes did not change much. This constant distribution of leaves of different genotypes may explain why, even though the leaf area index was lower under drought treatments, no shift in genotype ranking took place despite the large differences in standing crop biomass and why there was no shift in genotypic and phenotypic selection across different drought levels.

The riverine populations of *T. repens* from which the genotypes used in this experiment originated were characterized by a 97% within-population molecular variance as compared to a 3% among-population variance (J.L. Peters, unpublished results). This is in line with results on other clonal plant species characterized by high within-population variation exceeding among-population genetic variation (Huber et al., 2014). Spatiotemporal environmental heterogeneity has been argued to be an important driver explaining the high genetic variation in many clonal plant populations, as environmental perturbations, with slight advantages or disadvantages for given genotypes,

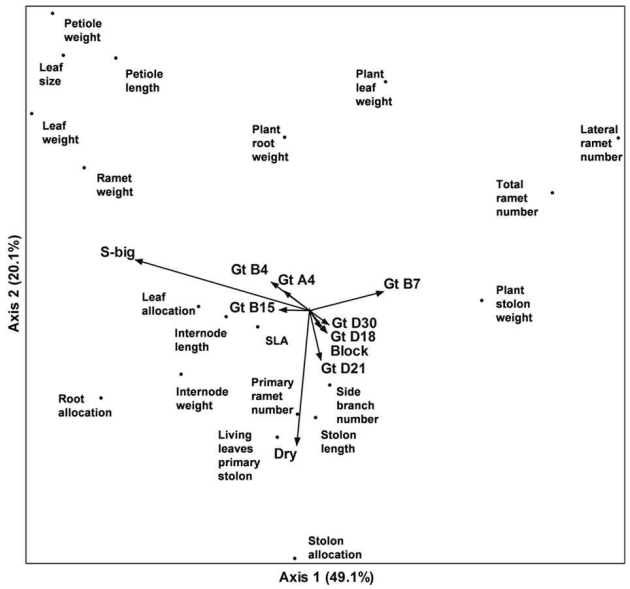

FIGURE 5 | Ordination diagram of the first two axes of a Correspondence Analysis of plant traits of eight genotypes of *Trifolium repens* with big (4) or small (4) ramets grown in three different competitive settings (within-genotype, within functional group, i.e., phenotypically similar stands, and between functional groups, i.e., phenotypically diverse stands) and with or without drought treatment. Genotype identities, ramet size class, drought treatment, competitive setting (not visible in graph), and block were added as passive variables.

can be expected to reduce the likelihood of potential genotypic variation loss. The proliferation of well-adapted genotypes and associated loss of less well-adapted genotypes can result in a decreased genetic variation over time (Stuefer et al., 2009; Gladieux et al., 2015). This, however, is not supported by the results of our experiment: genotype ranking remained relatively constant across environmental conditions, despite the strongly altered growth and phenotype in dry as compared to moist conditions.

Maintenance of genetic variation is thought to be important for community stability and resilience to environmental change. However, proliferation of well-adapted genotypes and associated loss of less well-adapted genotypes can result in a decreased genetic variation over time (Stuefer et al., 2009; Gladieux et al., 2015). In our experiment, the different genotypes were characterized by different performance across competition treatments, which is likely to result in loss of genotypic variation, especially as the same genotypes were favored across environmental conditions. This may indicate a potentially high short term resistance of population structure in response to environmental variation in *T. repens*. In a similar, long-term experiment with the clonal species *P. reptans* it has been found that over time the community became dominated by a few genotypes while other genotypes completely disappeared (Stuefer et al., 2009). In concert these results imply that while genetic diversity can be lost in stable conditions on the long run, environmental variation does not have to lead to maintenance of genetic diversity, as different environmental conditions do not necessarily favor different genotypes.

Diversity Does Not Affect Overall Population Performance

In our experiment, overall yield was not affected by genotypic or phenotypic diversity. This is in contrast to the hypothesis that genetically and phenotypically diverse stands would have a higher overall performance and be more persistent (Grimm and Wissel, 1997) to drought as different genotypes may occupy different ecological niches. Several studies have shown that in multispecies communities functional diversity of stands positively affects community growth and stability (van Ruijven and Berendse, 2005; Gross et al., 2014; Brotherton and Joyce, 2015; Venail et al., 2015), which has been attributed to various mechanisms such as niche differentiation, positive effects of high diversity of associated soil and herbivore communities, and the lower accumulation of species specific soil pathogen loads (de Kroon et al., 2012; Hendriks et al., 2013; Bardgett and van der Putten, 2014). Within-species genetic diversity has been shown to have similar positive effects on population growth and stability to environmental variation both, for plant and insect populations (Reusch et al., 2005; Ellers et al., 2011; Drummond and Vellend, 2012; Hughes, 2014). The positive effects of genetic diversity on productivity have been attributed to niche complementarity among phenotypically diverse genotypes. While genetic diversity has led to increased community performance in some cases, our experiment supports other studies performed under controlled and (semi)natural conditions where no such positive effect was found (Vellend et al., 2010; Tomimatsu et al., 2014; Williams et al., 2014). In line with the predictions of Drummond and Vellend (2012) large-sized genotypes in our experiment did produce a higher total biomass if grown in monocultures or in competition with other large-sized genotypes, while small-sized genotypes performed better in terms of ramet number if grown in monoculture or in communities consisting of small sized genotypes. However, this difference did not lead to a relative overyielding in terms of, respectively, biomass or ramet number if plants were grown in phenotypically diverse communities. While genotypes did maintain their relative position across treatments, overall productivity was not higher in phenotypically diverse stands than predicted from the average performance in homogeneous stands. This indicates that complementarity and increased productivity may not be the main mechanism favoring genetically diverse stands in stoloniferous grassland species like *T. repens*.

Relative Performance Depends on Whether Biomass Increment or Ramet Number is under Selection

Interestingly, there was a much higher variation in performance among genotypes in phenotypically diverse stands if performance was expressed in terms of dry weight, than if expressed in terms of ramet number. This was conspicuously different from the genotype performance predicted by growth in monocultures, which were characterized by a relatively constant biomass, but

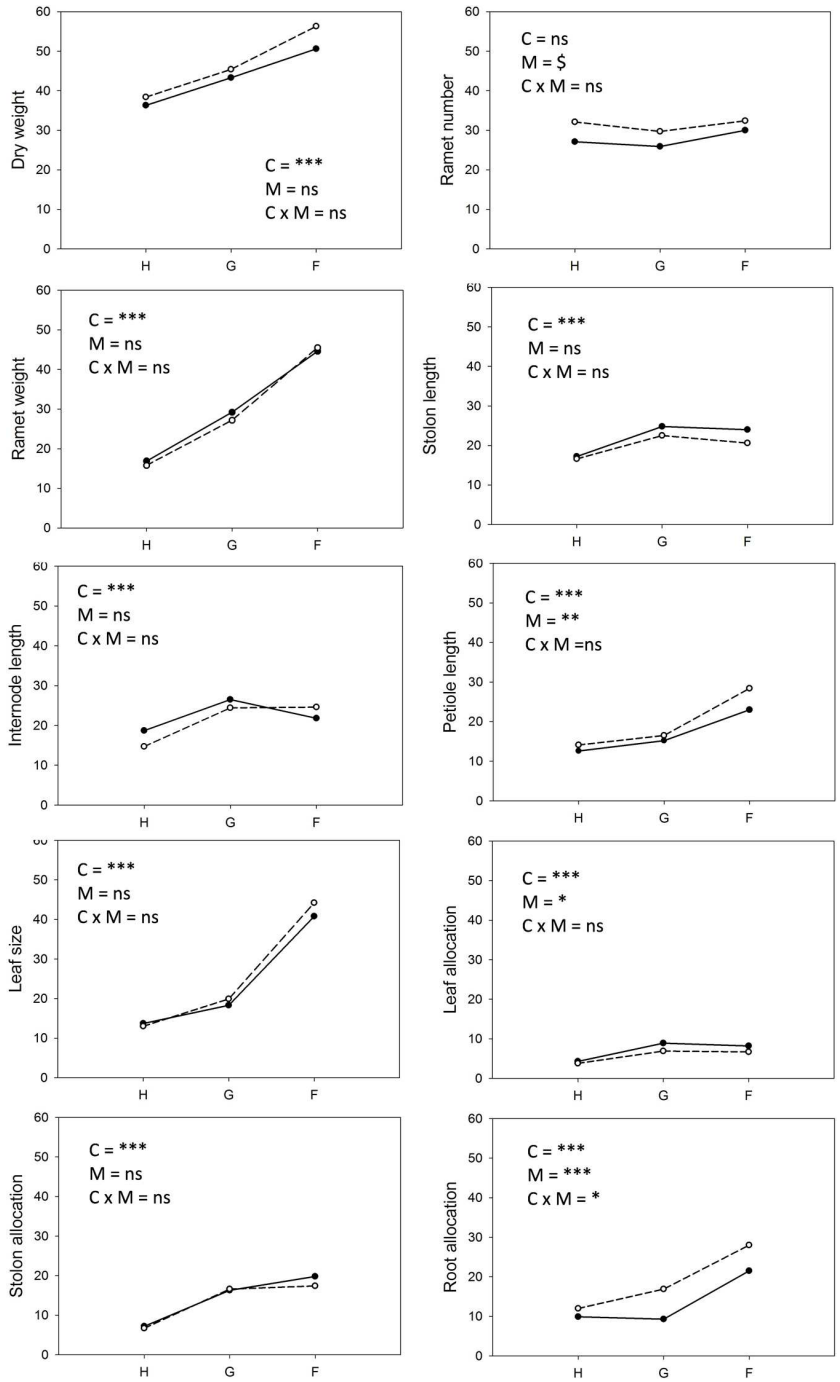

FIGURE 6 | Whole stand: Realized coefficient of variation of performance parameters, ramet and plant architectural traits and allocation pattern within stands. Solid lines and closed symbols indicate dry conditions, dashed lines with open symbols indicate moist conditions. Competition type abbreviations: H, homogeneous monostands; G, phenotypically similar; F, phenotypically diverse. Treatment abbreviations: C, competition; M, soil moisture. Significance levels are as in **Table 2**.

highly variable ramet number. Different genotypes had different mechanisms to reach the same biomass production, either by producing many small or few large ramets, a typical pattern for organisms characterized by size-number trade-offs (Huber and Wiggerman, 1997; Vermeulen and During, 2010). However, in phenotypically diverse populations the relative advantage of genotypes with large ramets over genotypes with small ramets led to a shift in the rate of ramet production, resulting in a convergence of ramet production rate across phenotypes. Depending on the prevalent conditions, either high reproduction

TABLE 3 | Effects of phenotypic diversity on total biomass and ramet number per tray subjected to either moist or dry conditions.

	Biomass		Ramet number	
	Moist	**Dry**	**Moist**	**Dry**
Model	**2.86****	1.16^{ns}	0.83^{ns}	1.29^{ns}
Ramet weight	0.15^{ns}	–0.13^{ns}	–0.04^{ns}	*0.34*$^{\$}$
Stolon length	**–0.32****	–0.06^{ns}	0.06^{ns}	0.13^{ns}
Internode length	–0.07^{ns}	–0.12^{ns}	–0.06^{ns}	–0.19^{ns}
Petiole length	–0.15^{ns}	–0.11^{ns}	0.05^{ns}	0.09^{ns}
Leaf size	–0.27^{ns}	0.14^{ns}	0.10^{ns}	*–0.31*$^{\$}$
Root allocation	**0.30***	0.16^{ns}	–0.06^{ns}	0.16^{ns}
Stolon allocation	0.08^{ns}	–0.17^{ns}	*–0.35*$^{\$}$	**–0.44***
Leaf allocation	0.09^{ns}	0.27^{ns}	0.12^{ns}	0.18^{ns}

*A positive regression coefficient indicates that high variability of a specific traits is associated to higher biomass or ramet number, a negative coefficient indicates that high variability of a trait is associated to relatively lower biomass or ramet number. Data were analyzed by means of multiple regression. For the overall model the F-value, for the individual traits the standardized regression coefficients and their significance are given. Significances are as follows: $^{ns}p > 0.10$; $^{\$}0.10 < p < 0.05$; *$0.01 < p < 0.05$; **$0.05 < p < 0.01$; ***$p < 0.001$. Significant values are highlighted in bold, marginally significant values in italics.*

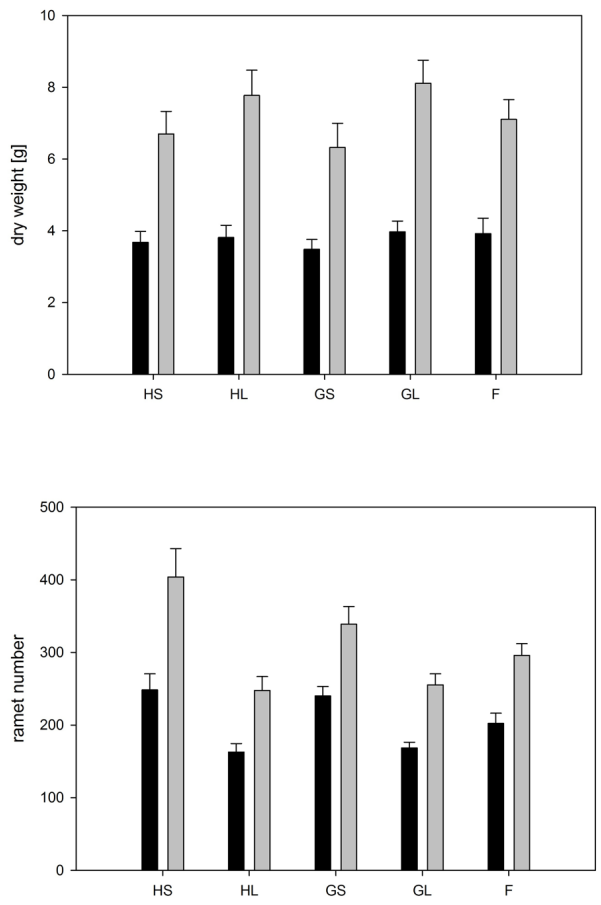

FIGURE 7 | Whole stand: Overall dry weight and ramet number per tray for each competition treatment and water availability. Dark bars represent trays subjected to dry conditions, light bars trays subjected to moist conditions. Treatment abbreviations are as follows: H: homogeneous monostands; G: phenotypically similar stands consisting of four different genotypes with similar phenotype; F: phenotypically diverse stands consisting of two large and two small genotypes; L: genotypes with large ramets; S: genotypes with small ramets. For significances, see **Table 4**.

TABLE 4 | Results of a two-way ANOVA testing for the effects of competition treatment and soil moisture on overall production parameters per tray.

	df	Overall biomass per tray	Overall ramet number per tray
Competition	4	**4.5****	**49.2*****
Soil moisture	1	**383.3*****	**206.6*****
Competition * moisture	4	1.9^{ns}	**4.4****
Block	5	**9.9*****	**8.8*****

*F-values and their significance are given. Significances are as follows: $^{ns}p > 0.10$; $^{\$}0.10 < p < 0.05$; *$0.01 < p < 0.05$; **$0.05 < p < 0.01$; ***$p < 0.001$. Significant values are highlighted in bold, marginally significant values in italics.*

TABLE 5 | Results of a two-way ANOVA testing for the effects of competition treatment and soil moisture on the increase in total mass in the mixtures compared to the expected yield from the monocultures (net diversity effect, Δy) and its components, the complementarity effect (CE) and the selection effect (SE).

	df	Δy	CE	SE
Intercept	1	0.05^{ns}	0.01^{ns}	0.01^{ns}
Competition	2	2.39^{ns}	2.14^{ns}	0.11^{ns}
Soil moisture	1	1.47^{ns}	0.02^{ns}	0.00^{ns}
Competition * moisture	2	0.55^{ns}	0.50^{ns}	0.66^{ns}
block	5	**2.72***	**4.64****	**2.92***

*F-values and their significance are given. Significances are as follows: $^{ns}p > 0.10$; $^{\$}0.10 < p < 0.05$; *$0.01 < p < 0.05$; **$0.05 < p < 0.01$; ***$p < 0.001$. Significant values are highlighted in bold, marginally significant values in italics.*

or high biomass may be favored by selection. Producing many ramets may be favorable in highly disturbed environments, where ramets may serve as a bud bank, enabling plants to regrow after part of the vegetation has been removed. In relatively stable, highly competitive environments genotypes with large ramets maintaining high biomass production may be characterized by higher performance which might lead to a loss of smaller sized genotypes over time, even if they have a potentially higher ramet production rate (Stuefer et al., 2009; Vermeulen et al., 2013).

Testing this hypothesis would require to subject genotypes from different positions along the ramet number-ramet size trade-offs gradient to selection regimes differing in disturbance and plant density.

Trifolium repens is Characterized by Constant Character Combinations

While the phenotypes of a given genotype largely remained similar across competition treatments, drought treatments induced conspicuously different phenotypes. However, the relationship among the different traits remained surprisingly constant over drought treatments. Selection driven by heterogeneous conditions would allow for genotypes to co-exist if the rank order of performance varies under different conditions, or if genotypes show different plastic alterations of traits associated with increased performance. In our experiment, neither the relative performance of genotypes was different among environments, nor were the within-genotype trait relations changed. In addition, even across the different competition treatments the trait values remained relatively constant, indicating a highly integrated phenotype, where most traits appear to respond in concert. This is also in line with the rigid structure of this species, which results in limited opportunities for changed growth and allocation pattern. This rigidity in character combination may explain the lack of shift in genotypes across environmental conditions as well as the extraordinarily high within population genetic variation in natural populations, as there may be little potential for selection on genotypes with a specific, highly favorable, sets of traits.

CONCLUSION

A clear pattern emerges from our experiment. In contrast to our hypotheses, genotype size and architecture neither affected the overall ranking of the different genotypes in response to the different competitive environments, nor in response to water availability. These results were consistently supported across the extensive set of different analyses: as can be seen by the lack of a significant association between type of competition and the first two axes in the CA, as well as the absence of over-yielding, which would indicate a difference in relative genotype performance under a given set of environmental conditions. These results may indicate that increased frequency of drought spells, as predicted by global change scenarios, will not necessarily lead to immediate shifts in genotype abundance of this important pasture legume. However, the genotypes did differ in their relative performance, which implies that some genotypes may disappear in the long run, a process which does not seem to be affected by drought. The speed of this process is likely to depend on competitive environments, as the relative difference in fitness expressed by the eight genotypes did vary across competition environments. Depending on the main trait under selection, overall biomass production or ramet number, the long term changes may take at a different speed. The convergence of ramet number in diverse mixtures implies that diversity can be maintained if ramet number is under selection. Alternatively if total biomass is the main trait under selection, genotypes may get lost on the long run. However, the eventual loss in genetic diversity may not be associated to immediate negative consequences. This is supported by our results (i.e., the lack of correlation between diversity and overall stand performance) which show that phenotypic and genetic diversity are unlikely to contribute to increased productivity or increased resistance to drought in stands dominated by white clover. These results suggest that communities dominated by *T. repens* may be relatively resilient in changing environments characterized by higher frequency of drought events.

AUTHOR CONTRIBUTIONS

This paper is the result of a close cooperation of the authors with significant contribution of all authors in different parts of the process. HH was involved in all stages of the experiment, including planning, analyses and writing of the manuscript. FB performed the experiment and the initial analyses of the data and wrote a master thesis on which this paper is based. HD, NA, and PV were involved in conceptual interpretation of the data, data analyses, and writing of the manuscript.

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frontiers in Plant Science

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Biomass Allocation of Stoloniferous and Rhizomatous Plant in Response to Resource Availability: A Phylogenetic Meta-Analysis

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Resource allocation to different functions is central in life-history theory. Plasticity of functional traits allows clonal plants to regulate their resource allocation to meet changing environments. In this study, biomass allocation traits of clonal plants were categorized into absolute biomass for vegetative growth vs. for reproduction, and their relative ratios based on a data set including 115 species and derived from 139 published literatures. We examined general pattern of biomass allocation of clonal plants in response to availabilities of resource (e.g., light, nutrients, and water) using phylogenetic meta-analysis. We also tested whether the pattern differed among clonal organ types (stolon vs. rhizome). Overall, we found that stoloniferous plants were more sensitive to light intensity than rhizomatous plants, preferentially allocating biomass to vegetative growth, aboveground part and clonal reproduction under shaded conditions. Under nutrient- and water-poor condition, rhizomatous plants were constrained more by ontogeny than by resource availability, preferentially allocating biomass to belowground part. Biomass allocation between belowground and aboveground part of clonal plants generally supported the optimal allocation theory. No general pattern of trade-off was found between growth and reproduction, and neither between sexual and clonal reproduction. Using phylogenetic meta-analysis can avoid possible confounding effects of phylogeny on the results. Our results shown the optimal allocation theory explained a general trend, which the clonal plants are able to plastically regulate their biomass allocation, to cope with changing resource availability, at least in stoloniferous and rhizomatous plants.

Keywords: biomass allocation, clonal reproduction, ontogenetic drift, optimal allocation theory, phylogenetic meta-analysis, trade-off, sexual reproduction, vegetative growth

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INTRODUCTION

How plants allocate limiting resources among different life functions, e.g., growth vs. reproduction, survival vs. future growth, in response to the variation in their environments has been a central question in plant ecology for half a century. Bloom et al. (1985) vividly drew a parallel between a plant and a business firm, and articulated that plants like businesses must engage in long-term

(i.e., reproduction) as well as short-term (i.e., growth) planning on resource allocation. The theory of allocation, borrowed from microeconomics, was firstly introduced to biology by Levins and MacArthur (cited in Cody, 1966) to describe the resource partitioning mode of iteroparous organisms and extended to the study of plants (Harper and Ogden, 1970). Subsequently, optimal allocation theory was proposed and suggested that plants regulate allocation of resources to their organs in response to variation in the environment in order to optimize the capture of resources (e.g., nutrients, light and water) essential for survival, growth and reproduction in a manner that maximizes fitness in changing environments (Bloom et al., 1985; McConnaughay and Coleman, 1999). According to the optimal allocation theory, plants should allocate resources to increase their uptake of the resource that is most limiting growth. It has been widely tested in different species (Bloom et al., 1985; Robinson, 1986; Johnson and Thornley, 1987; Levin et al., 1989; Hilbert, 1990; McConnaughay and Coleman, 1999). For example, plants allocated more biomass to leaf under low light intensity (Shipley and Meziane, 2002), and more biomass to root under low soil nutrients or water (Ericsson, 1995; Mony et al., 2007; Gonzáles et al., 2008). However, the optimal allocation theory has also been questioned for ignoring "ontogenetic drift," which described the phenomenon of a trait changing in a predictable way as a function of plant growth or development (Evans, 1972; McConnaughay and Coleman, 1999). For instance, Coleman et al. (1994) found that plant biomass allocation was size-dependent and supported by the subsequent studies (Pino et al., 2002; Ogawa, 2003; Weiner, 2004; Huang et al., 2009).

The optimal allocation theory means that plastic resource allocation patterns result from environmental changes and are size-independent (Bloom et al., 1985). In this view, allocation is considered as a proportional process: "partitioning," as in a pie chart, and usually analyzed as ratios (e.g., root mass: shoot mass, reproductive mass: vegetative mass) or fractions of total biomass (e.g., root mass: total mass, reproductive mass: total mass; Poorter and Nagel, 2000; Weiner, 2004). While the "ontogenetic drift" means that variable resource allocation patterns are genetically determined and size-dependent (Coleman et al., 1994; McConnaughay and Coleman, 1999). In this perspective, plant growth is allometric (allocation changing with size), and is generally showed by allometric analyses (Müller et al., 2000). However, we still do not know which theory can explain the general patterns of multiple species with their phylogenetic relations.

Around the above theories, there have been numerous studies focusing on resource allocation strategy of clonal plants, such as biomass allocation between growth and reproduction (Delph et al., 1993; Salonen, 1994; Li et al., 2001a; Van Zandt et al., 2003), between sexual and clonal reproduction (Hartnett, 1990; Cheplick, 1995; van Kleunen et al., 2002; Thompson and Eckert, 2004; Van Drunen and Dorken, 2012; Wang et al., 2013), between aboveground and belowground (Aerts et al., 1991; Cao and Ohkubo, 1998; King et al., 1999; Yang et al., 2009). Since clonal plants are mostly perennial and possess two modes of regeneration (Barrett, 2015), namely sexual reproduction by seed and clonal reproduction through a form of clonal growth (Richards et al., 2004), it is more complicated to understand resources allocation strategy of clonal plants. Resource allocation among vegetative growth, clonal reproduction and sexual reproduction may be age-related and not necessarily mutually exclusive in life history (Cheplick, 1995). Furthermore, higher plasticity of clonal plants allows them to modify the growth and development in response to changes in environmental conditions (Strand and Weisner, 2004). So, are there any general patterns of resource allocation of clonal plants in response to variation in availability of resources? If so, do these patterns depend on ontogeny, or the environment experienced by the clonal plants? Although, numbers of previous reviews have focused on various aspects of resource allocation (Bloom et al., 1985; Lovett Doust, 1989; de Kroon and Schieving, 1990; Reekie, 1999; Weiner, 2004), there has been no consistent conclusion drawn from quantitative analysis yet. The accumulation of studies in this topic, along with the development of meta-analysis, offers us an opportunity to examine the general trends of biomass allocation of clonal plant in response to changing resource availability.

Most studies have looked at allocation of biomass, as it generally reflects other resource available to an individual (Reekie and Bazzaz, 1987). In this study, we considered ramet biomass as a measure of resource allocated to vegetative growth, and further divided into two parts: aboveground part (shoot, leaf, and stem) and belowground part (root). Because some studies found that stolons and rhizomes have partly different functions (Dong and de Kroon, 1994) and because rhizomatous plants are less plastic than stoloniferous plants in response to changes in resource availability (Dong and de Kroon, 1994; de Kroon and Hutchings, 1995; Xie et al., 2014), we considered that biomass allocated to reproduction consisted of two parts: biomass allocated to clonal reproduction (rhizomes or stolons) and biomass allocated to sexual reproduction (flowers, seeds, and fruits; **Table 1**). Additionally, we analyzed the allocation patterns from two perspectives: absolute and relative biomass (Reekie and Bazzaz, 1987). The absolute biomass allocation to a component or activity (e.g., reproductive biomass) was a measure of the total quantity of the component or activity and was in relation to plant size, while the relative biomass allocation to that (e.g., reproductive biomass: total biomass) was a measure of the proportion of biomass devoted to it and was size-independent (Bazzaz et al., 2000). To take the evolutionary relationships of the multiple species involved into account, we adopted phylogenetic meta-analysis (PMA), an emerging method incorporating phylogenetic information into traditional meta-analysis (Lajeunesse, 2009), to address the following questions: Is there any general pattern of biomass allocation of clonal plants (i) between vegetative growth and reproduction, (ii) between aboveground and belowground, (iii) between clonal reproduction and sexual reproduction, in response to change in resources (e.g., light intensity, nutrient level and water availability)? Do they vary among different types of clonal organ? Whether, biomass allocation of clonal plants is genetically determined or responsive to environment?

TABLE 1 | Trait categories.

Trait category	Trait sub-category	Traits
Vegetative growth (VG)	Aboveground (AG)	Aboveground biomass (or shoot biomass): leaf DW and stem DW
	Belowground (BG)	Belowground biomass (or root biomass): root DW
Reproduction (RE)	Clonal reproduction (CR)	Clonal reproductive biomass (or spacer biomass): rhizome DW or stolon DW
	Sexual reproduction (SR)	Sexual reproductive biomass: flower DW, fruit DW, and seed DW
Vegetative proportion (VGP)	Aboveground proportion (AGP)	Aboveground biomass proportion (or shoot biomass proportion): leaf DWP and stem DWP
	Belowground proportion (BGP)	Belowground biomass proportion (or root biomass proportion): root DWP
Reproductive proportion (REP)	Clonal reproductive proportion (CRP)	Clonal reproductive biomass proportion (or spacer biomass proportion): rhizome DWP or stolon DWP
	Sexual reproductive proportion (SRP)	Sexual reproductive biomass proportion: flowers DWP, fruits DWP, and seeds DWP

DW, dry weight; DWP, dry weight proportion.

MATERIALS AND METHODS

Literature Survey and Data Selection Criteria

To perform a comprehensive literature survey, we conducted an exhaustive search primarily relying on the internet search engine, Google Scholar (Beckmann and von Wehrden, 2012), and supplemented by additional searches based on main databases (i.e., ISI Web of Knowledge, Science Direct, Wiley-Blackwell, Springer Link, CNKI [China National Knowledge Infrastructure], etc.) with keywords of "trade-off," "biomass allocat*" in combination with "clonal plant*," obtained 477 literatures. To identify studies specific to our questions, we did a separate search on papers which referred to "clonal plant*," finally 449 literatures fit the topic of our meta-analysis.

For each literature, we recorded the title, author(s), year, location, and some other information (see the Supplementary Table S1) and examined their potential for meeting the selection criteria for inclusion in review. Foremost, only experimental studies in greenhouse, common garden or field were taken into account, while reviews, models and other studies were excluded. Secondly, we only included studies that reported traits (**Table 1**) related to biomass allocation strategy in response to resource availability (i.e., light intensity, nutrient level and water availability). Furthermore, we excluded the studies in which the means, standard deviations and sample sizes for the treatment and control group were neither reported nor able to be inferred (or calculated) from other information (Gurevitch et al., 1992). The final data set contained 139 literatures published in 50 journals between 1973 and 2013, from which we extracted data for the meta-analyses (Supplementary S2).

Data Assembly

For each study, we extracted the means, the statistical variation (usually SE or SD) and the sample size values for treatment and control groups for each responsive variable (trait). We regarded multiple results within a single paper as different results from independent studies when they contained different species and/or treatments (Wolf, 1986; Gurevitch et al., 1992; Bolnick and Preisser, 2005; Marczak et al., 2007), while only extracted data once from the same experimental results in different papers (Gurevitch et al., 2001). When the study set up experiments on several treatment levels, each "treatment level" was paired with "control" to calculated effect size firstly and would be pooled later. Resource treatments (light intensity, nutrient level and water availability) used in the studies followed the explanation in Xie et al. (2014). All data were extracted from tables or digitized from graphs with the software GetData v2.22 (http://www.getdata-graph-digitizer.com). A total of 2308 comparisons containing 115 clonal plant species from 87 genera in 33 families were involved in analysis at last. For each comparison, we calculated Hedges' *d* as effect size of experimental treatment (Lajeunesse and Forbes, 2003; van Kleunen et al., 2010). The absolute value of Hedges' *d* showed the magnitude of the treatment impact. Positive or negative *d*-values signified an increase or decrease effect of the treatment, respectively. Zero meant no difference between treatment and control group (Rosenberg et al., 2000).

With regard to the comparisons from experiments on multiple treatment levels, we pooled effect sizes and variances of each trait per species and study by doing a separate meta-analysis to avoid pseudo-replication (see also Leimu et al., 2006; van Kleunen et al., 2010; Song et al., 2013). The pooled mean effect sizes and mean variances were used in the final datasets containing 229 cases in light treatment, 380 cases in nutrient treatment and 93 cases in water treatment. For all analyses, we chosen the random-model setting, as we assumed that differences among comparisons and among studies are not only due to sampling errors but also due to true random variations, as is the rule for ecological data (Gurevitch and Hedges, 2001). All effect size calculations were carried out with the software MetaWin, version 2.1 (Rosenberg et al., 2000).

In order to apply PMA, we created phylogenetic trees (Supplementary Figure S3) with branch lengths through the Phylomatic (http://phylodiversity.net/phylomatic/), with option (Phylomatic tree R20120829 for plants) and Phylocom software (Webb and Donoghue, 2005; Webb et al., 2008), and generated a subset tree for each trait category per species. As the restriction of input files executed on phyloMeta v1.3 software (Lajeunesse, 2011), we pooled again those multiple effect sizes on the

same species from different studies. This resulted in only one accumulated weighted effect size and variance for each species within a given trait category on one hand, and inevitably resulted in smaller sample sizes for each trait category on the other hand ($N_{effect-size} = N_{species}$) (Carmona et al., 2011; Nakagawa and Santos, 2012). The pooling was also carried out with a random-effect model on MetaWin, version 2.1 (Rosenberg et al., 2000).

Data Analysis

Before all planned analysis, we explored the possibility of publication bias graphically (funnel plot and normal quantile plot; Wang and Bushman, 1998; Gates, 2002), statistically (Spearman rank correlation test; Begg, 1994), and also by calculating a fail-safe number (Rosenthal, 1991; Rosenberg, 2005). As a result, the funnel plot of effect size vs. sample size showed no skewness; the plot of standardized effect sizes against normal quantiles revealed a straight line; the result of Spearman rank-order correlation test on effect size vs. sample size was not significant ($R = 0.050$, $P = 0.187$); the fail-safe number 100,896 was much greater than 3520 (i.e., $5n + 10$; n was the number of cases in our dataset, Supplementary Table S1). All results of those tests indicated that there was no evidence of publication bias (Supplementary Figures S4, S5).

We categorized each trait into trait category and trait subcategory (**Table 1**), for example, sexual reproductive biomass, if there was a species with multiple traits, e.g., flower biomass, fruit biomass, we calculated the sum of them. For each trait category, we calculated the overall effect sizes ($d++$) of light, nutrient and water separately on the relevant responsive variables. The overall effect sizes were cumulative effect sizes per species (Hedges and Olkin, 1985; Lajeunesse, 2009). For each part of biomass allocation, we analyzed the response of clonal plants in two aspects: absolute biomass value and its proportion to total value (**Table 1**). The data might be not independent because the absolute value and relative value could share some data. To detect the differences between stoloniferous plants and rhizomatous plants, we considered the type of clonal organs as moderator

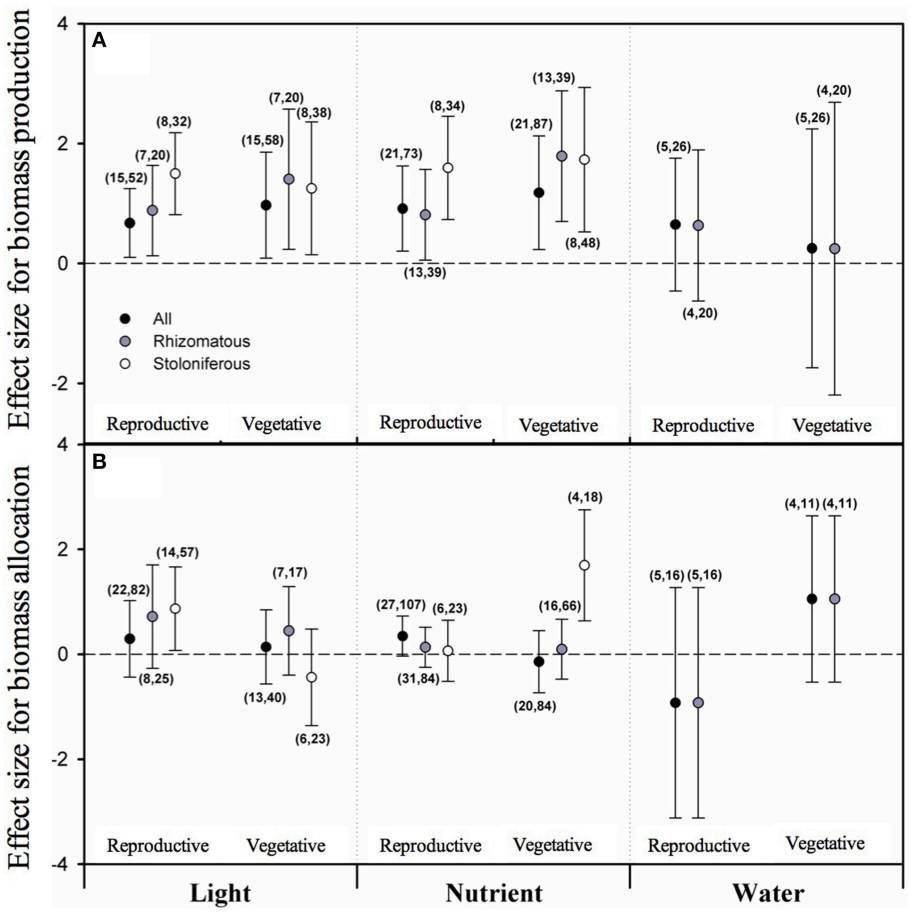

FIGURE 1 | The mean effect sizes of three types of resources (light, nutrient and water) on biomass production (A) and biomass allocation (B) between vegetative growth and reproduction for all plants (black circles), rhizomatous plants (gray circles), and stoloniferous plants (white circles) from random-model in PMA by software phyloMeta v1.3. The bars around the means denote bias-corrected 95% bootstrap confidence intervals, and a mean effect size is significantly different from zero when its 95% confidence interval does not include zero. The first and second numbers in brackets are number of species and number of studies, respectively.

variable. In this paper we just concerned the two types of clonal plants. The analyses were performed on the software phyloMeta v1.3 (Lajeunesse, 2011).

RESULTS

Biomass Allocation between Vegetative Growth and Reproduction of Clonal Plants

According to the PMA results, the overall effect sizes of light intensity on biomass allocated to both vegetative growth (VG) and reproduction (RE) were positive and significantly different from zero, and so did those of nutrient level on VG and RE (**Figure 1A**). But the grand mean effect size of water availability neither on VG nor on RE was different from zero. Considering clonal organ types, the mean effect sizes of light intensity and nutrient level on VG and RE were still positive and significantly different from zero for both rhizomatous and stoloniferous plants (**Figure 1A**). The mean effect sizes of water availability on VG and RE of rhizomatous plants were not different from zero. The data of stoloniferous plants were too few to analyze.

From the perspective of biomass proportion, none of the overall effect sizes of light intensity, nutrient level and water availability on biomass proportion allocated to vegetative growth (VGP) or reproduction (REP) were significant (**Figure 1B**). None of mean effect sizes of rhizomatous plants in response to light intensity, nutrient level and water availability on VGP or REP were significant either. However, the mean effect size of stoloniferous plants in response to light intensity on REP and that of nutrient level on VGP were positive (**Figure 1B**).

Biomass Allocation between Aboveground and Belowground Part of Clonal Plants

None of the overall effect sizes of light intensity, nutrient level and water availability on biomass allocated to aboveground (AG) or belowground (BG) was significant irrespective of clonal organ types. Taking clonal organ type into account, however, the mean effect sizes of light intensity and nutrient level on both AG and BG of stoloniferous plants were positive and significant; also positive and significant were the mean effect sizes of light intensity on BG and of nutrient level on AG and BG in rhizomatous plants (**Figure 2A**). However, the mean effect sizes of water availability on AG and BG in either rhizomatous or stoloniferous plant were not statistically significant (**Figure 2A**).

In biomass proportion perspective, only the overall effect size of water availability on biomass proportion allocated to belowground (BGP) was significantly negative irrespective of clonal organ types (**Figure 2B**). For rhizomatous plants, the mean effect sizes of nutrient level and water availability on biomass proportion allocated to aboveground (AGP) were significant and positive and those on BGP were significant and negative, but the effect size of light intensity neither on AGP nor on BGP was significant (**Figure 2B**). Whereas, only the mean effect size of light intensity on AGP was significant and negative for stoloniferous plants, the mean effect sizes of nutrient level and water availability on AGP or BGP were not significant, and the data were not sufficient to analyze effect of water availability on BGP (**Figure 2B**).

In addition, we conducted supplementary analyses for the effects of light intensity, nutrient level and water availability on the ratio of root to shoot (R/S), and the results indicated that the effect sizes of light intensity on R/S were significant and positive, for rhizomatous plants: $d+ = 0.939$, $N = 12$, 95% $CI =$ [0.257, 1.622] (N: number of species; CI: confidence interval), and for stoloniferous plants: $d+ = 1.379$, $N = 12$, 95% $CI =$ [0.615, 2.143]. And the effect sizes of nutrient level on R/S were significant and negative, for rhizomatous plants: $d+ = -0.891$, $N = 18$, 95% $CI = [-1.608, -0.174]$, and for stoloniferous plants: $d+ = -1.272$, $N = 14$, 95% $CI = [-1.885, -0.659]$. The effect sizes of water availability on R/S were only significant and negative for stoloniferous plants ($d+ = -1.082$, $N = 5$, 95% $CI = [-2.057, -0.106]$), but not significant for rhizomatous plants.

Biomass Allocation between Clonal and Sexual Reproduction of Clonal Plants

Based on the results of PMA, none of the overall effect sizes of light intensity, nutrient level and water availability on biomass allocated to clonal reproduction (CR) or sexual reproduction (SR) were significant irrespective of clonal organ type (**Figure 3A**). As taking clonal organ type into account, although the mean effect sizes for rhizomatous plants were not significant yet, the effect sizes of light intensity and nutrient level on CR for stoloniferous plants were significant and positive (**Figure 3A**). There were not sufficient data for analyze the effect of water availability on CR or SR of stoloniferous plants.

For biomass proportion, the overall effect sizes of nutrient level on biomass proportion allocated to sexual reproduction (SRP) was significant and positive (**Figure 3B**). Only the mean effect size of light intensity on SRP of stoloniferous plants was significant and positive, and that of water availability on biomass proportion allocated to sexual reproduction (SRP) of rhizomatous plants was significant and negative (**Figure 3B**). The data were not sufficient to analyze the effects of nutrient level and water availability on SRP of stoloniferous plants and that of water availability on CRP of stoloniferous plants.

DISCUSSION

Conclusions from individual species or single experiment were highly diverse, depending on species and environmental factors. This paper reported the overall results of PMA to draw the general pattern of biomass allocation in clonal plants in response to resource availability. A coherent picture of some aspects (i.e., growth vs. reproduction, aboveground vs. belowground part, and clonal vs. sexual reproduction) of biomass allocation strategy emerges.

Biomass Allocation to Growth vs. Reproduction in Clonal Plants

Growth and reproduction are among the most fundamental activities in plants. Once plant initiates reproductive machinery via growth, its biomass allocation requires investment trade-offs,

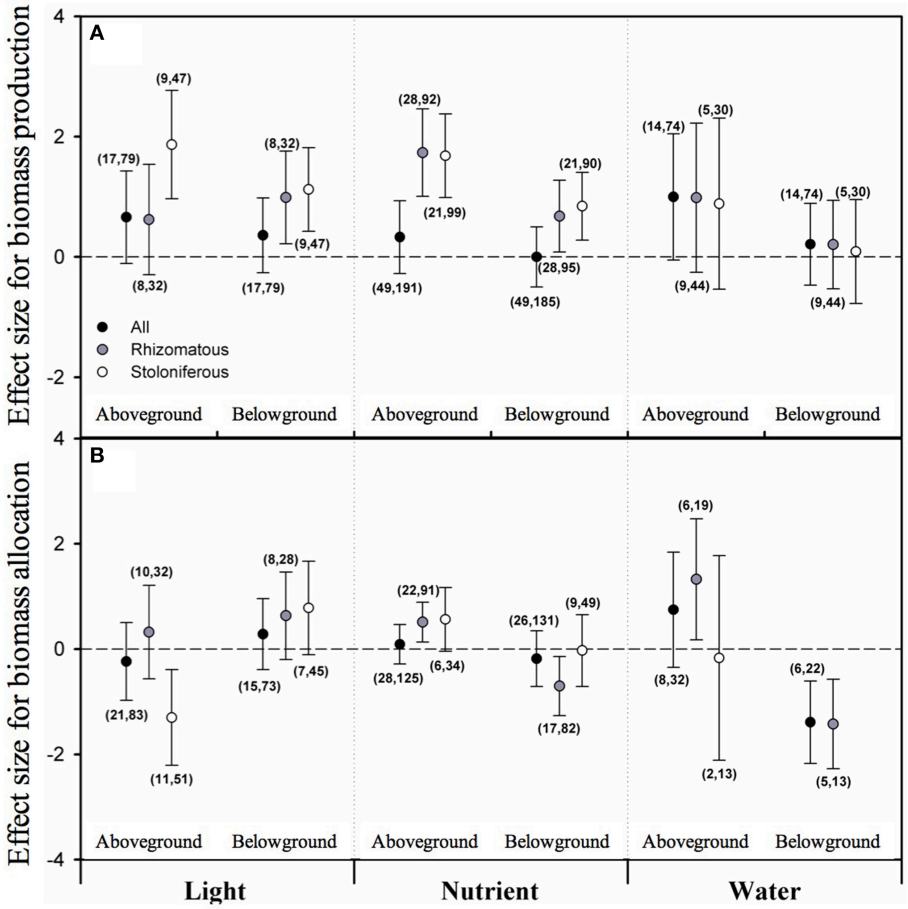

FIGURE 2 | The the mean effect sizes of three types of resources (light, nutrient, and water) on biomass production (A) and biomass allocation (B) between aboveground and belowground for all plants (black circles), rhizomatous plants (gray circles), and stoloniferous plants (white circles) from random-model in PMA by software phyloMeta v1.3. The bars around the means denote bias-corrected 95% bootstrap confidence intervals, and a mean effect size is significantly different from zero when its 95% confidence interval does not include zero. The first and second numbers in brackets are number of species and number of studies, respectively.

as resources allocated to one function or organ are unavailable for other functions or organs (Weiner, 2004; Weiner et al., 2009). But the expectation that trade-offs would occur between the competing functions of vegetative growth and reproduction is not upheld in this study. Our results show that clonal plants simultaneously increase biomass allocated to vegetative growth and reproduction with light intensity and nutrient level increased, no matter of the clonal organ types. This is unsurprising and complies with common sense, and also can be regarded as a result of individual growth (Coleman et al., 1994). The results further shown that the biomass proportions allocated to vegetative growth and reproduction of rhizomatous plants neither increased with light intensity, nutrient level or water availability increased, which suggests that changes of resource level had not impact on pattern of biomass allocation between growth and reproduction in rhizomatous plants (Bazzaz et al., 2000). While for stoloniferous plants, the biomass proportion allocated to reproduction increased with the increasing light intensity, and the biomass proportion allocated to vegetative growth increased with the increasing nutrient level. These results imply that stoloniferous plants would decrease biomass proportion allocated to reproduction in shaded environment and decrease biomass proportion allocated to vegetative growth under nutrient-poor conditions. This might be explained as that stoloniferous plants would rather sacrifice reproduction to maintain the vegetative growth to seek light resource, and rather ensure reproduction at the cost of vegetative growth to get away from the infertile habitat. So by comparison, the biomass allocation in rhizomatous plants is much more determined by ontogeny while that of stoloniferous plants is more susceptible to environmental changes.

Regarding to effect of water availability on biomass allocation, our present data on stoloniferous plants are not sufficient to be analyzed. The results of rhizomatous plants indicate that water availability has no significant effects on the biomass allocation. Although, this may be due to the limiting data, similar results

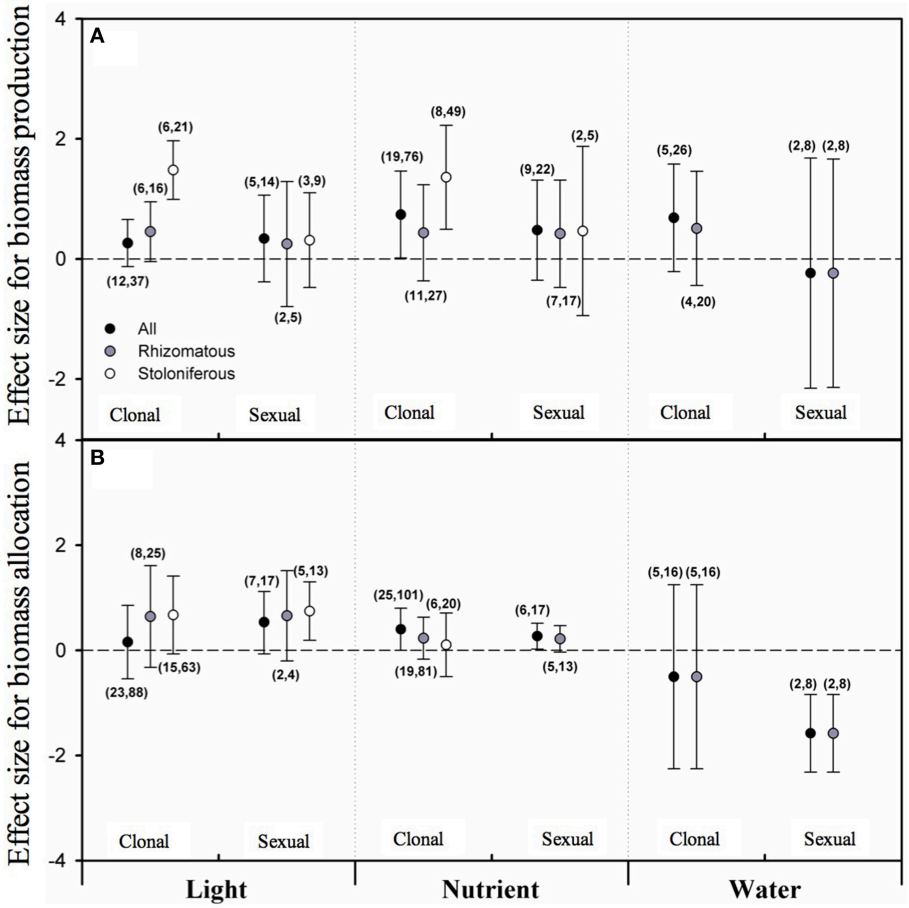

FIGURE 3 | The mean effect sizes of three types of resources (light, nutrient and water) on biomass production (A) and biomass allocation (B) between clonal reproduction and sexual reproduction for all plants (black circles), rhizomatous plants (gray circles), and stoloniferous plants (white circles) from random-model in PMA by software phyloMeta v1.3. The bars around the means denote bias-corrected 95% bootstrap confidence intervals, and a mean effect size is significantly different from zero when its 95% confidence interval does not include zero. The first and second numbers in brackets are number of species and number of studies, respectively.

have been reported in previous studies (McConnaughay and Coleman, 1999). Explicit and credible conclusions need more experimental studies to test the effect of water availability on biomass allocation of stoloniferous plants.

Biomass Allocation to Aboveground vs. Belowground in Clonal Plants

Numerous studies on biomass allocation of plants have focused on aspects of between above- and below-ground biomass (i.e., root vs. shoot). And the mechanisms underlying the observed partitioning responses of plants have always been the debate center (Müller et al., 2000; Poorter and Nagel, 2000; Shipley and Meziane, 2002). Given the ontogenetic drift and optimal allocation theories, biomass allocation was analyzed and interpreted in terms of size (e.g., aboveground biomass) and proportion (e.g., aboveground or belowground biomass to total biomass) in this study. The ontogenetic drift theory stressed preferential allocation to shoot over root as plant grew larger regardless of environmental conditions (Coleman et al., 1994; Müller et al., 2000), which contradicted our results. Our results suggest that with light intensity and nutrient level increased, stoloniferous plants increased aboveground and belowground biomass almost simultaneously, but decreased the biomass allocated to aboveground with light intensity increased, not belowground (McConnaughay and Coleman, 1999). In comparison with stoloniferous plants, the rhizomatous was not sensitive to light intensity and only increased belowground biomass with light intensity increased, but more susceptible to nutrient availability. With nutrient level increased, rhizomatous plants increased aboveground and belowground biomass simultaneously, but increased aboveground biomass proportion and decreased belowground biomass proportion. So we can infer that the opposite is true: with nutrient level decreased, rhizomatous plants would reduce biomass proportion allocated to aboveground and increase biomass proportion allocated to belowground to search the limiting nutrients, which

profoundly supports the optimal allocation theory (Poorter and Nagel, 2000; Shipley and Meziane, 2002). With water availability increased, both rhizomatous and stoloniferous plants had no significant changes in terms of size, but the rhizomatous responded in the same way as they did with nutrient level increased (Gonzáles et al., 2008; Huang et al., 2013).

The results of R/S ratio are also compliance with the optimal allocation theory: R/S in both rhizomatous and stoloniferous plant were increased with light intensity, which may be confounded by ontogenetic drift; and R/S were decreased with nutrient level, which is obviously inconsistent with ontogenetic drift in this point, as according to ontogenetic drift theory, the ratios should be increased with increasing individual size irrespective of the nutrient level (Müller et al., 2000). In addition, it should not be ignored that our results from PMA have excluded the confounding effects of phylogeny.

In general, the optimal allocation theory found in many ecological models involving plant biomass allocation, in which biomass is preferentially allocated to the plant part obtaining the resource that is essential but limiting for growth, appears to be a reasonable explanation of the biomass allocation strategies of clonal plants suggested by our results (Poorter and Nagel, 2000; Shipley and Meziane, 2002). In addition, the results of this study also prove that in terms of biomass allocation, stoloniferous plants are more sensitive to light condition, while rhizomatous plants are more sensitive to nutrient condition.

Biomass Allocation to Clonal vs. Sexual Reproduction in Clonal Plants

Clonal plants possess two modes of reproduction (clonal and sexual), and each mode has its own pros and cons (Jackson et al., 1985; Wu et al., 2010; Barrett, 2015). How resources are divided between two modes of reproduction has been considerable interest to researchers (Willson, 1983; Bazzaz et al., 1987; Lovett Doust, 1989; Reekie, 1991). A trade-off between clonal and sexual reproduction has been commonplace in clonal plants (Watson, 1984; Silvertown et al., 1993; Svenning, 2000; Wu et al., 2010). This prediction derives from assumption that allocation among competing functions is mutually exclusive, as a plant has a given amount of resources at any point in time, so different allocation patterns reflect different adaptive strategies of clonal plant in response to variable environment (Sutherland and Vickery, 1988; Weiner, 2004; Liu et al., 2009). Some previous studies confounded clonal reproduction and vegetative growth, which might confuse the real trade-off between clonal and sexual reproduction. However, when separated clonal reproduction from vegetative growth, the trade-off patterns of biomass allocation between clonal and sexual reproduction were not detected in this study (Reekie, 1991). Our results exhibit that increasing light intensity, nutrient level and water availability had no significant effect on biomass allocated to clonal and sexual reproduction of rhizomatous plants, as well as biomass proportion. Only one exception to these trends was that biomass proportion allocated to sexual reproduction of the rhizomatous decreased with water availability increased, which means that the rhizomatous preferentially allocate biomass to the sexual reproduction under low-water condition (Li et al., 2001b). This is in line with the notion that sexual reproduction may allow escape from poor conditions and produce genetically diverse offspring that may be better able to cope with harsh conditions (Eriksson, 1997; Gardner and Mangel, 1999; van Kleunen et al., 2002). As to stoloniferous plants, high light intensity and nutrient level resulted in increased biomass of clonal reproduction without concomitant decreases of sexual reproduction, and inversely high light intensity resulted in increased biomass proportion of sexual reproduction without concomitant decreases of clonal reproduction. The former can be explained as that clonal reproduction may be beneficial as a means to remain in benign environments (Abrahamson, 1975; van Kleunen et al., 2002), and the later one is consistent with the earlier result of this study that stoloniferous plants would rather sacrifice reproduction to maintain the vegetative growth to capture light resource in shaded environment (Svenning, 2000). Therefore, these results reveal that biomass allocation of clonal plants to reproduction is much more constrained by ontogeny or heredity than by environments, and that stoloniferous plants are relatively more susceptible to environments than rhizomatous plants in biomass allocation between clonal and sexual reproduction.

According to current results, there might be no trade-offs between vegetative growth and reproduction, clonal and sexual reproduction in biomass allocation of clonal plants (Pitelka et al., 1985; Reekie, 1991; Mendoza and Franco, 1998; Svenning, 2000). The two critical preconditions of trade-off are that the resource is in fixed supply and that allocation among competing functions is mutually exclusive (Watson, 1984; Bazzaz et al., 2000). But in its application to the study of reproductive strategies, these two preconditions may be not always true for two principal reasons. Firstly, some processes, such as the consecutive photosynthesis of plants, can lead to an increase in total resource supply associated with reproduction, because light, nutrients and other resources are supplied continuously. Secondly, plant structures can contribute to more than one function and may well not be mutually exclusive (Bazzaz et al., 2000). As a result, measures of allocation to different structures or functions do not always exhibit the trade-off patterns.

In conclusion, our study used PMA to analyze the response of functional traits related to biomass allocation of clonal plant to changing environments. Here, we summarize several general patterns based on the PMA results: (i) clonal plants exhibit higher plasticity of vegetative growth traits than reproduction traits in response to resource levels; (ii) in response to resources constrains biomass allocation patterns between belowground and aboveground parts of clonal plants conform to optimal allocation theory; (iii) no evidence was found of trade-off patterns between clonal and sexual reproduction. All biomass allocation strategies of clonal plants obey the tenet that tends to maximize genet fitness, whether conforming to optimal allocation theory or constrained by ontogeny. The optimal allocation theory explained the "true plasticity" of clonal plants to cope with changing environments, while the ontogeny drift theory emphasized the genetic influence on plant growth. In this paper, we just tried to clarify the trade-off strategies of clonal plants in changing environments using PMA method with which

phylogenetic effect was avoided. Our results profoundly support the optimal allocation theory rather than disprove the ontogeny drift theory. Besides, this study analyzed trade-off strategies in terms of biomass allocation in clonal plants, but allocations of some other resources, such as meristems (Bazzaz et al., 2000) were not considered due to data limitation. Meanwhile, the results related to water availability must be interpreted with caution because the outcomes are perhaps caused by data shortage. So we highlight that experimental studies are essential and indispensable whatsoever in the past, present or future.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MD, YS. Performed the experiments: XX, YH. Contributed to the writing of the manuscript: XX, YH, XP, FL, YS, and MD.

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The funder had no role in study design, data collection and analysis, decision to publish, or preparation the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00603

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REVIEW
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Ecological Consequences of Clonal Integration in Plants

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Clonal plants are widespread throughout the plant kingdom and dominate in diverse habitats. Spatiotemporal heterogeneity of environment is pervasive at multiple scales, even at scales relevant to individual plants. Clonal integration refers to resource translocation and information communication among the ramets of clonal plants. Due to clonal integration, clonal plant species possess a series of peculiar attributes: plasticity in response to local and non-local conditions, labor division with organ specialization for acquiring locally abundant resources, foraging behavior by selective placement of ramets in resource-rich microhabitats, and avoidance of intraclonal competition. Clonal integration has very profound ecological consequences for clonal plants. It allows them to efficiently cope with environmental heterogeneity, by alleviating local resource shortages, buffering environmental stresses and disturbances, influencing competitive ability, increasing invasiveness, and altering species composition and invasibility at the community level. In this paper, we present a comprehensive review of research on the ecological consequences of plant clonal integration based on a large body of literature. We also attempt to propose perspectives for future research.

Keywords: clonal plants, environmental heterogeneity, physiological integration, ramet/genet, resource translocation

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INTRODUCTION

Modularity occurs in almost all vascular plants (Watkinson and White, 1986; Dong, 1996a,b; Clarke, 2012). Plants can be regarded as an assembly of many modules (de Kroon et al., 2005). When their modules are capable of iterating themselves in a spontaneous way, and thus produce potentially independent offspring through vegetative propagation, the plants are referred to as clonal plants (Mogie and Hutchings, 1990; Dong, 1996a,b, 2011; de Kroon and van Groenendael, 1997; Hutchings and Wijesinghe, 1997). In clonal plants, the clonally formed offspring are specifically referred to 'ramets,' a term coined by Harper (1977). The whole plant, which is often comprised of a number of ramets of the same clone, is referred to as a 'genet' (Harper, 1977). Different ramets belonging to the same genet will have actually developed from a single zygote, and thus share the same genotype (Harper, 1977, 1985; Clarke, 2012). Within a genet, each ramet has the potential to perform all biological functions as an independent, non-clonal plant, even if it is separated from the rest of the genet (Hutchings and Wijesinghe, 1997). In this respect, a grown-up ramet can be regarded as an individual.

It is well-understood that various materials including external resources absorbed by plants, hormones, photosynthates, and secondary metabolites can be translocated among different parts

of an intact plant individual via its interconnected vascular structures (Bray, 1954; van Groenendael and de Kroon, 1990). Ramets of clonal plants normally stay connected through horizontal connecters (stolons and rhizomes) for an extended period, enabling physiological integration between ramets (Dong, 2011). Experiments using isotope tracers (^{13}C or ^{14}C, and D) and acid fuchsine to investigate the patterns of resource translocation within clonal plant species have provided direct evidence to confirm this possibility (Guttridge, 1959; Qureshi and Spanner, 1971; Ong and Marshall, 1979; Ashmun et al., 1982; Schellner et al., 1982; Salzman, 1985; D'Hertefeldt and Jónsdóttir, 1994; Zhang et al., 2002; Liu et al., 2007). Clonal integration is referred to as the physiological integration that takes place among different ramets in clonal plants, and includes resource translocation and information communication (Dong, 1996a,b, 2011). In terms of its evolutionary implications and adaptive significance, it is one of the most specific and important characteristics possessed by clonal plant species (Dong, 2011). Owing to clonal integration, the patterns of materials translocation within clonal plants are more complicated than those of non-clonal plants (Dong, 1996a,b, 2011).

Spatiotemporal heterogeneity of environments is pervasive in all natural habitats (Wiens, 1976; Smith and Vrieze, 1979; Turkington and Klein, 1991; Turkington et al., 1991; Magyar et al., 2007). External resources like light, water, and mineral nutrients, which are essential for plants, and environmental conditions such as temperature and moisture, are distributed heterogeneously at various scales, including at scales relevant to individual plants (Dong, 2011). How to cope with environmental heterogeneity is one of the foremost problems that plants have to solve (Magyar et al., 2007). Connected ramets of clonal plants often experience different environmental conditions: for example, some ramets may be located in microsites with an abundant resource supply and/or without a particular stress or disturbance, while other ramets of the same genet are located in unfavorable microsites with scarce resources and/or severe stresses or disturbances. If there is resource translocation or information sharing within a clonal plant, donor ramets will help resource-poor or otherwise adversely situated ramets to alleviate their shortages and/or to resist stress and disturbances, resulting in an increase in the performance of the recipient ramets, and sometimes for that of the whole plant (Song et al., 2013). Most studies on clonal plants that spread through horizontal connecters (for example, stolons or rhizomes) support this notion. It is widely accepted that clonal integration – as one of the most important adaptive functional traits of clonal plants in dealing with environmental heterogeneity – came into being during the long-term evolution of plant clonality (Dong, 2011).

In this paper, we present a comprehensive review of research on clonal integration. We place a particular focus on plant environmental-adaptation strategies that derive specifically from clonal integration and also consider the ecological consequences of these strategies. We also propose perspectives for future research on this topic.

CLONAL INTEGRATION AS A CORE CONCEPT FOR CLONAL PLANT RESEARCH

In an effort to obtain an overall picture of the importance of the topic of clonal integration in research relating to "clonal plants," we downloaded the full metadata (including tiles, authorship, abstracts, keywords, keywords plus, and citations, as well as cited references) of clonal plant-related papers from the ISI Web of Science Core Collection for the publication period of 1900–2016. We used the search key terms "clonal plant*," and excluded any document defined as note, correction/addition, editorial material or meeting abstract. In total, 1369 articles (henceforth, the "clonal plants dataset") were obtained.

Terms parsed from articles can be seen to be representative of key focal points of scientific research (Cui et al., 2012). We used CiteSpaceIII software[1] to generate knowledge maps of key words from the clonal plants dataset. CiteSpace, developed by Chaomei Chen at Drexel University, USA (see Chen, 2004, 2006; Chen et al., 2010), is a Java application that supports visual exploration with knowledge discovery based on bibliographic information, and has been widely used in various research domains over the last decade. We set the relevant CiteSpace parameters as follows: time slicing (years 1991 and 2016), years per slice (3 years), term source (title, abstract, and author keywords, and keywords plus), and node type (keywords/term). The reason we chose 1991 as the beginning year for time slicing is that prior to that time only a few (less than three) relevant papers were published each year.

Figure 1 provides an overview of these terms, from which we can see that the terms of "clonal integration" and "physiological integration" occupy the core position. Environmental heterogeneity is another highly frequent term (**Figure 1**), which is related very closely with clonal integration.

ECOLOGICAL STRATEGIES TO DEAL WITH ENVIRONMENTAL HETEROGENEITY

Integrating Local and Non-local Responses

The traits of plants in nature are determined by both genetics and environmental conditions. The term plasticity is used to describe the fact that a given genotype may express a range of phenotypes under different environmental conditions (Schlichting, 1986; Bradshaw and Stettler, 1995; de Kroon et al., 2005). That is to say, plastic responses of plants are induced by variations in their surrounding environments, such as the quality and or quantity of essential resources (de Kroon et al., 2005). Such variations in environmental factors are normally present at multiple scales in many natural ecosystems (Hutchings and Wijesinghe, 1997). For example, the quality and/or quantity of soil resources in Southern Quebec forests vary remarkably over a distance of

[1] http://cluster.cis.drexel.edu/~cchen/citespace/

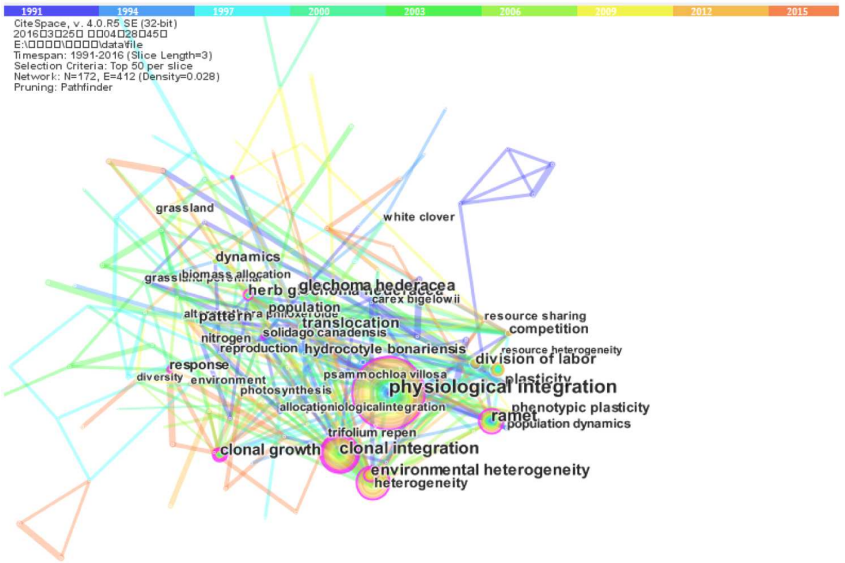

FIGURE 1 | The visualization of highly frequently occurring terms extracted from articles with the topic of "clonal plants" indexed by Thomson Routers. This knowledge map was done using CiteSpace.

only 10s of centimeters (Lechowicz and Bell, 1991). In chalk grassland, light availability varies at a scale of 1 cm (Silvertown, 1981). This means that the physically interconnected ramets of a clonal plant may experience a variety of microhabitats with different environmental conditions (Stuefer and Hutchings, 1994; Hutchings and Wijesinghe, 1997; Hutchings et al., 1997; de Kroon et al., 2005). Ramets are the behavioral unit of plastic responses in clonal plant species (de Kroon et al., 2005). However, the local plastic responses of a ramet can be modified via clonal integration. In other words, responses can be induced both by the local environmental conditions that the ramets experience and by exchanges with interconnected ramets (Dong, 1996a,b; de Kroon et al., 2005). According to de Kroon et al. (2005), clonal integration changes the plant's plastic responses at the ramet-level in three different ways, enhancing responses to local environment for a ramet, diminishing local responses to local environment for a ramet, and triggering new responses that are absent without integration. According to the model described by Magyar et al. (2007), such kind of plasticity is beneficial the most to plants that grow in spatially heterogeneous, yet temporally stable environments. When environmental conditions change at a short temporal scale, a non-plastic strategy becomes favorable (Magyar et al., 2007). However, most of previous studies on relationship between integration and plastic responses of clonal plants did not distinguish the particular effects exerted by spatial variation and temporal variation. Separating the effects of spatial and temporal environmental variation could provide more insights.

Division of Labor

If the distribution patterns of two or more essential resources are not consistent within the area that a clonal plant covers, it is hard to determine whether or not the microhabitat of a ramet is good. Occasionally, the microhabitat is abundant in one resource but scarce in another, causing the availabilities of different resources to be negatively correlated with each other within patches (Alpert and Stuefer, 1997; Magyar et al., 2007). For instance, clonal herbs in the forest edge often experience negatively correlated light and soil resource conditions. For example, some ramets grow in a microsite with high light intensity and low soil nutrient availability, while interconnected ramets have complementary resource conditions, with low light intensity and high soil nutrient status (Cook, 1983; Alpert and Mooney, 1986; Friedman and Alpert, 1991; Stuefer and Hutchings, 1994; Stuefer et al., 1994, 1996; Alpert and Stuefer, 1997). This resource distribution pattern is called "reciprocal patchiness of resources (RPR)" (Alpert and Stuefer, 1997; Yu et al., 2002). Because it is least costly for a plant to acquire resources when they are abundant (Bloom et al., 1985; Stuefer et al., 1996), ramets of clonal plants subjected to an RPR-environment tend to specialize in acquiring whichever resource is locally abundant (Hutchings et al., 1997). Complementary resources are then reciprocally exchanged among the ramet systems in a bi-directional manner via clonal integration (Evans and Whitney, 1992; Alpert and Stuefer, 1997; van Kleunen and Stuefer, 1999). In doing so, each locally abundant resource can be acquired to the maximum extent by the whole genet This behavior, called "division of labor induced by environments," is unique to clonal plants, and only exists among interconnected ramets (Stuefer, 1998). Non-clonal plants or single ramets usually display specialization for the uptake of the most limited resource (specialization for scarcity; Dong, 1996a,b; Hutchings and Wijesinghe, 1997).

Another phenomenon known as "developmentally programmed division of labor" (Stuefer, 1998) takes place in various rhizomatous plant species that can form large clonal fragments consisting of a number of interconnected ramets in

environments with poor soils (Callaghan, 1976, 1984; Jónsdóttir and Callaghan, 1988, 1990; Schmid, 1990; Jonsson et al., 1996; Jónsdóttir and Watson, 1997). When clonal fragments grow, the above-ground parts (shoot) of some older mature ramets may die, but the below-ground parts (root system) can remain alive, while connected young ramets have active leaves yet have poorly developed roots. The leafless mature ramets often specialize in uptake of water and nutrients from the soil, while the young ramets are specialized for capturing light. At the same time, integration allows extensive translocation of light and light, water and nutrients through the entire fragment. Thus, either type of ramet undertakes specific responsibilities for acquiring different resources, and enjoys the benefits from resource sharing (Stuefer, 1998). This kind of "labor division among different-aged ramets" seems not to be associated with the spatial distribution pattern of resources, but is rather inherently driven by the plant's own programmed development (Stuefer, 1998).

Foraging for Resources

Glechoma hederacea was shown to have short internodes, copious branching, and a great number of ramets with large leaves when growing under either nutrient-rich soil (Slade and Hutchings, 1987c) or high light intensity conditions (Slade and Hutchings, 1987b); which could indicate that clonal plants forage for key resources (Salzman, 1985; Slade and Hutchings, 1987a,b,c; Alpert, 1991; Evans, 1991, 1992; Kelly, 1992; Hutchings and de Kroon, 1994; Bergamini et al., 2001). Some other herbs were observed to behave similarly, such as *Ranunculus repens* (Ginzo and Lovell, 1973), *Ipomoea phillomega* (Penalosa, 1983), *Trifolium repens* (Harper, 1983), and *Solidago canadensis* (Hartnett and Bazzaz, 1983). When subjected to heterogeneous environmental conditions, clonal plants prefer to inhabit favorable patches and avoid unfavorable patches via selective placement of ramets (Slade and Hutchings, 1987a,b,c; Kelly, 1992; Adam et al., 2003). The adaptive implication of this is quite clear: in order to ensure performance and fitness of the whole clone, more resource-acquiring structures are produced in resource-rich sites to exploit the abundant essential resources in an intensive way. However, foraging responses are more obvious when clones expanded from resource-poor microsites toward resource-rich microsites. For instance, when individual clones of *G. hederacea* in low-light and low-nutrient conditions grew into high-light and high-nutrient conditions, the morphology of the newly developed ramets changed significantly. In comparison, the change in ramet morphology was less conspicuous when clones grew from the rich into poor conditions (Slade and Hutchings, 1987a). Likely, foraging responses (e.g., development of more ramets with larger leaves and longer petioles) were reinforced when *G. hederacea* developed from low light patches toward high light ones; these responses were likely suppressed if expanded from high light patches toward low light ones (Wijesinghe and Hutchings, 1996). A recent meta-analysis conducted by Xie et al. (2014) showed that some typical foraging traits of clonal plants such as spacer length, specific-spacer length, branch intensity, and branch angle responded to light intensity but did not respond to nutrient or water availability. They also found that stoloniferous plants foraged resources more significantly than rhizomatous plants (Xie et al., 2014).

Coordinating Inter-ramet Relations

Clonal integration contributes greatly to the establishment of new ramets, leading to a genet or a clonal fragment composed of a great number of interconnected ramets (Zahner and Debyle, 1965; Hartnett and Bazzaz, 1983; Bullock et al., 1994). Since each ramet is a potentially fully functional individual, a genet or a clonal fragment can be seen as a population of ramets (Herben et al., 1994). Ramet coexistence may have negative consequences in the form of inter-ramet for resources. Based on the fact that many clonal invertebrates avoid kin competition within taxonic families (Sebens, 1984; Ayre and Grosberg, 1995; Ishii and Saito, 1995) and the fact that plants tend to place their roots away from their neighbors (Brisson and Reynolds, 1994; Schenk, 1999), Holzapfel and Alpert (2003) evaluated the hypothesis that physiological integration could enable clonal plants to minimize interference among connected ramets of the same genet via root segregation. Their experiment with *Fragaria chiloensis* showed that in connected ramet pairs, less root biomass was placed between the pair, and more was placed on the sides away from each other, in comparison to separated ramet pairs. Due to root separation, the performance of the genets increased considerably (Holzapfel and Alpert, 2003). It is likely that physiological integration between connected ramets resulted in the avoidance of self-competition in *F. chiloensis* (Holzapfel and Alpert, 2003). This experiment provided clear evidence to support the hypothesis that integration in at least some clonal plant species can facilitate cooperation and reduce inter-competition among ramets thereby enhancing the overall performance of the genet or of the clonal fragment. *Carpobrotus edulis* was also observed to avoid competition for resources via physiological integration by adjusting the biomass allocation to roots among connected ramets (Roiloa et al., 2014). However, whether this phenomenon is a broadly general among most species or is specific to a given species will require further studies.

ECOLOGICAL CONSEQUENCES OF CLONAL INTEGRATION

Alleviating Resource Shortage

Interconnected ramets of a genet in clonal plant species are often located in different microhabitats, resulting in a source-sink gradient along ramets in terms of resource quality (Dong et al., 2007). The resources acquired by ramets growing in favorable microhabitats can be transported to ramets growing where resources are scarce, thanks to clonal integration. Clonal integration can therefore alleviate local shortages of resources for ramets in poor microsites (Hutchings and Wijesinghe, 1997). In this case, recipient ramets benefit directly from the import of resources that they lack, while those same resources are relatively ample for donor ramets. On the other hand, the donor ramets may have to bear loss to some extent due to the export of their resources. Using cost-benefit analysis, many empirical studies have found that the benefits frequently outweigh the costs.

The biomass of ramets, the number of newly born ramets and the number of seeds of *Hydrocotyle bonariensis* in low N availability, are all significantly increased when connected to ramets with a high N supply. Even when clonal growth and reproduction of N-rich ramets were impaired, the overall benefit was still higher than the cost (Evans, 1988). Disconnected clones of *Potentilla simplex* weighed significantly less than connected clones under under heterogeneous nutrient conditions, but differed little under homogeneous conditions (Wijesinghe and Handel, 1994). In addition, a field-based experiment on a rhizomatous grass, *Psammochloa villosa,* supported the hypothesis that water translocation could alleviate water shortage often experienced by *P. villosa* (Dong and Alaten, 1999). For *Potentilla reptans* and *Potentilla anserina* in heterogeneous light quality conditions, specifically when there was a connection between ramets in full-light and ramets in shaded patches, shade did not negatively impact the performance of either of the ramets (Stuefer et al., 1994). Clonal integration has been shown to alleviate different types of resource shortage, such as shading, nutrient depletion and drought in many clonal plant species in numerous experiments (Guttridge, 1959; Ginzo and Lovell, 1973; Hartnett and Bazzaz, 1983; Salzman, 1985; Alpert, 1991, 1999a,b; de Kroon et al., 1996; Dong and Alaten, 1999; Alpert et al., 2003). By combining a large body of empirical evidence, Magyar et al. (2007) found that a modular cooperation strategy was most advantageous when environmental conditions varied spatially. Since the resources necessary for plant growth (such as light, water, and mineral nutrients) are distributed unevenly in all habitats, clonal integration is viewed as being beneficial for clonal plant species.

Buffering Stress and Disturbance

In addition to the uneven distribution of essential resources, clonal plant species may also suffer various local biotic and abiotic stresses and disturbances. Since some case studies can fall under both the topics of "resource shortage" and "stressful environment," e.g., water shortage or drought stress, we limited "stress" in the present review to those adverse conditions that were not directly caused by the lack of essential resources. 'Disturbance' here refers to a change in environmental conditions that disrupt the ecosystem, community, or population structure and bring about a change in resources, substrate availability, or the physical environment (Pickett and White, 1985). Salzman and Parker (1985) found that ramets under salt stress connected with unstressed ramets accumulated more biomass than ramets connected with other stressed ramets in *Ambrosia psilostachya*. In the sandy grasslands of Inner Mongolia, China, a series of experiments to test the hypothesis that clonal integration may help local native plant species to withstand frequently occurring stresses and disturbances, such as sand burial, wind erosion, and grazing (Yu et al., 2001, 2004, 2009; Liu F.H. et al., 2006; Liu et al., 2009; Xu et al., 2012). Their results showed that the connection through rhizomes conferred a considerable increase in performance to *P. villosa* during both sand burial and wind erosion. This positive effect was more obvious when heavy sand burial and sand erosion were imposed on the plant (Yu et al., 2004, 2008). Similarly, another dominant rhizomatous semi-shrub, *Hedysarum laeve,* and a stoloneferous herb, *P. anserina,* both benefited from clonal integration when subjected to sand burial (Yu et al., 2001; Liu F.H. et al., 2006). In addition, when *Bromus ircutensis* and *P. villosa* were subjected to heavy clipping (simulating grazing), clonal integration was found to function as an additional compensatory mechanism, greatly improving the performance of both species (Liu et al., 2009). When considering the simultaneous occurrence of both trampling and defoliation caused by grazing, clonal integration did not impact the response to defoliation, but did alleviate the trampling-induced damage to the root-suckering clonal tree *Populus simonii* (Xu et al., 2012). From the above results, it is reasonable to conclude that clonal integration indeed plays a key role in the long-term persistence of clonal plant species in the inland dune regions of northern China. In a few cases, though, this has not been consistently found. For example, in *Fargesia qinlingensis*, a bamboo species known for being a primary food of panda bears, clonal integration was shown to not be a compensatory response to herbivore feeding (Wang et al., 2007). Similarly, rhizome severing (cutting off clonal integration) did not significantly affect rhizome growth, ramet growth, or vegetative bud outgrowth of the ramet population in *Leymus chinensis* (Wang et al., 2004). One explanation could be that the experimental duration was not long enough to observe the real functions of clonal integration. Regardless, most of the existing case studies empirically suggest that clonal integration is an adaptive strategy, conferring advantages to clonal plant species to improve stress and disturbance tolerance.

Increasing Competitive Ability

Given that clonal integration can increase the performance of clonal plants in a range of habitats, it seems to be advantageous for successful competition over non-clonal plants (van Groenendael et al., 1996; de Kroon and van Groenendael, 1997; Yu et al., 2010). However, many previous studies did not find that clonal integration strongly contributes to the interspecific competition ability of clonal plant species. Schmid and Bazzaz (1987) reported that cutting off the connection of *Aster* had very little influence on their intraspecific competition capacity, neither did for *Solidago*. Similar results were observed in *Brachypodium pinnatum* and *Carex flacca* (de Kroon et al., 1992). Based on the finding that the clonal tree *Populus tremuloides* did not profit from a rhizome connection when invading the native prairie, Peltzer (2002) suggested that clonal integration might be functional for exploiting patchy resources or tolerating stressful environments rather than for improving the competitive ability of clonal plant species (Salzman, 1985; Cain, 1994; Evans and Cain, 1995; Shumway, 1995; Brewer and Bertness, 1996; Dong, 1996a,b, 1999; Stuefer et al., 1996; Stoll and Schmid, 1998; Dong and Alaten, 1999; Yu et al., 2002, 2004, 2009; Zuidema et al., 2007). Wang et al. (2011) found that clonal integration enhanced the disturbance and drought resistance ability rather than the competitive ability of the rhizomatic *Eremosparton songoricum*. Pennings and Callaway (2000) compared the role of clonal integration in six salt

marsh plant species and found that clonal integration was most important for plants invading stressful habitats, moderately important for plants invading sites with neighbor plants clipped, and least important for plants invading habitats with intact vegetation. These results were strongly in support of Peltzer's (2002) conclusions. An invasive plant *Alternanthera philoxeroides* was also shown to benefit little from a stolon connection in invading a population of *Schedonorus phoenix* (Wang et al., 2008). In contrast, a few studies found that clonal integration promoted the competitive abilities in some clonal plants. The response of *S. canadensis* ramets to interspecific competition was affected by the ramet connection: connected ramets of *S. canadensis* responded more or less equally in their shoot growth, reproduction, and clonal growth while separated ramets exhibited large differences when grown together with different neighboring species (Hartnett and Bazzaz, 1985). The notion of benefits from clonal integration also holds true for an aquatic plant *Vallisneria spiralis*, for which stolon connection improved its ability to invade vegetated habitats (Xiao et al., 2011).

According to previous empirical evidence, we can say that the positive effects of clonal integration on the competitive ability of clonal plants are not as common as were once expected, but are very likely species-specific and strongly rely on environmental conditions. We propose that, when a clonal plant encounters competitors, it rapidly grows and forages to occupy new adjacent microhabitats, rather than competing directly. A meta-analysis compiling all existing case studies may provide more insights.

Maintaining Community Biodiversity and Productivity

Whether and how the effects of physiological integration on clonal plant species are scaled to the community level is another interesting question (Oborny et al., 2000, 2012; Wilsey, 2002; Yu et al., 2009, 2010; Eilts et al., 2011). However, even though a tremendous amount of research has focused on the individual level, much less has attempted to measure the consequences of clonal integration on ecological processes at the community level (Eilts et al., 2011). Among the limited community-level studies, two coherent aspects were examined. One is whether the effects of clonal integration on genet growth can be translated to influence the productivity of the community (Wilsey, 2002; Yu et al., 2010). This is particularly relevant in the case of communities where clonal plant species are dominant and produce most biomass (Wilsey, 2002; Yu et al., 2009, 2010; Eilts et al., 2011). In the Serengeti grassland that has a high frequency of perennial rhizomatous and stoloniferous plants, all connecters between ramets severing significantly reduced the net primary productivity at a community level, suggesting that plants grew better when ramets remained connected (Wilsey, 2002). The other question, arguably more important, is whether the effects at the individual clonal plant species level further impact the species composition and diversity of a community. Spatial heterogeneity in soil resources can facilitate a species' coexistence by enhancing the diversity of a community (MacArthur, 1972). Clonal plants, especially those that spread extensively, are thought to have the capacity to override fine-grain spatial heterogeneity (Hutchings and de Kroon, 1994) and thereby even the heterogeneity out via extensive resource translocation within the ramet network (Gough et al., 2002). Therefore, it has been supposed that clonal integration can impair the positive effect of resource heterogeneity on plant species richness. In a field experiment carried out over 6 years, rhizomatous clonal plants were found to have a strong negative effect on species richness (Eilts et al., 2011). In particular, the effect was strengthened when the spatial scale of nutrient heterogeneity within the scale at which rhizomatous clones could potentially integrate across resource patches (Eilts et al., 2011). This may result both from physiological integration and from the high competitive ability of clonal plants. Further study will be needed to disentangle the particular effects of clonal integration and those competitive ability traits *per se*. Experiments that keep the rhizome connected or not can be used to explore the particular effects resulting from clonal integration. Of note, in the Mu Us Sandland of northern China, rhizome connection neither increased growth of the dominant half-shrub *Hedysarum laeve* nor exerted any conspicuous influence on species composition (Yu et al., 2010).

The provisionary conclusions drawn from a limited number of cases cannot be generalized. More studies dealing with effects of clonal integration on community processes, particularly productivity, species coexistence, and diversity, will be needed.

Enhancing Invasiveness and Invasibility

Plant invasion has become a significant threat to biodiversity for environments and economies, both globally and locally (Mack et al., 2000; Liu et al., 2005; Pysek and Richardson, 2010). There are a considerable number of invasive plant species that are capable of vigorous clonal propagation, and their invasiveness may be related to clonal integration (Liu J. et al., 2006; Yu et al., 2009). Many studies have shown that clonal integration can enhance plant invasion success in alien plants (Reichard and Hamilton, 1997; Liu et al., 2008; Aguilera et al., 2010; Roiloa et al., 2010, 2014). Song et al. (2013) compiled 84 case studies covering 57 taxa, to provide a synthetic analysis of the effects of clonal integration on the performance of clonal plants. The results showed that clonal taxa for which recipient ramets in unfavorable patches benefited more from integration, were also more invasive on a global scale. A few more recent experiments provided additional support. You et al. (2014a) found that the invasive clonal plant *A. philoxeroides* benefits from clonal integration more than the co-occurring native species *Jussiaea repens*, suggesting that the invasiveness of *A. philoxeroides* may be closely related to clonal integration in heterogeneous environments. They also found that clonal integration can help *A. philoxeroides* to respond to defoliation (You et al., 2014b). The non-native invasive taxa *Typha angustifolia* and *T. x glauca* benefited more from increased maternal resource availability than the native congeneric counterpart *T. latifolia*, which strongly suggested that clonal integration confers advantages for the invasion of *T. angustifolia* (Elgersma et al., 2015). Physiological integration of resources might improve the establishment

of juvenile ramets of *Ludwigia hexapetala* across variable light environments during early colonization and thus contribute to its invasiveness (Gover et al., 2015). Similar results were also obtained by Wolfer and Straile (2012), Tuya et al. (2013), Roiloa et al. (2014), and Liu et al. (2016), but see Peltzer (2002).

Despite the existing evidence that clonal integration contributes to the invasive ability of clonal plants, what if any role it plays when clonal plants invade new habitats remains unclear. It is possible that the relationship(s) between clonal integration and invasion success is species-specific and/or stage-dependent. Considering that clonal integration does not necessarily improve the competition ability of clonal plants (see Increasing Competitive Ability), clonal integration might support the survival and growth of new ramets of invasive clonal plants at their introduction stage (but see Liu et al., 2010).

CONCLUSION AND FUTURE RESEARCH

Since pioneer studies that began in the 1970s (Qureshi and Spanner, 1973), clonal integration has attracted considerable attention in the field of plant ecology. There is a large body of literature exploring the mechanisms of physiological integration between ramets in clonal plant species, and its ecological and evolutionary consequences have been widely considered. The previous studies arrived at the common viewpoint that clonal integration allows translocation of materials within a whole or partial clone. It can help clonal plants adjust the plastic responses, helps clonal plants avoid possible inter-ramet competition, and favor stressed or damaged ramets. Overall, clonal integration can enhance the performance of clonal plant species at the ramet, or sometimes at the genet level(s), especially in the context of environmental variation (Dong, 2011). Recent studies have provided new insights on this topic, suggesting that research on clonal integration is far from over. Here, we propose four possible research directions for open discussions:

(1) Du et al. (2009) found that arbuscular mycorrhizal fungi (AMF) reduced the effects of physiological integration in *Trifolium repens*. It provides the first evidence for interactions between colonization by AMF and effects of physiological integration in a clonal plant (Du et al., 2009). Since symbiotic associations between AMF and plant roots are common in natural environments (Gosling et al., 2006), it is necessary to take such interactions into consideration in future research. Results are expected to provide more insights on ecological importance of clonal growth in the spatial and temporal composition of plant communities.

(2) Dong et al. (2015) found that clonal integration increased performance in a homogeneous resource-rich environment when connected ramets of *A. philoxeroides* differed in external resource uptake ability. The sibling ramets always developed in a successive order along the runner (stolon or rhizome), and resource uptake ability therefore usually differs from one ramet to another. Thus, the result produced from this study is likely not just an isolated case. We need more evidence to test this idea.

(3) Theoreticaly, clonal plants have to incur large costs, including potentially evolving slower owing to reduced sexual reproduction, risking the accumulation of mutations because absence in recombination and the possibility to create genetic variation in offspring, and the lack of the benefits of DNA repair mechanisms in comparison of plants with sexual reproduction (Douhovnikoff and Dodd, 2015). However, clonal plants are widespread throughout the plant kingdom and are found in diverse habitats (Price and Marshall, 1999), and many clonal plants both bear clonal growth and sexual reproduciton. To explain the contradiction between the theory and the facts, Douhovnikoff and Dodd (2015) proposed that epigenetic mechanisms might help clonal plants outweigh the evolutionary costs, and clonal plants may use epigenetic acclimation over long stretches of evolutionary time to adapt to environmental variation. What role, if any, that clonal integration may playin epigenetics processes is also one of the far-reaching and promising topics for future research.

(4) New findings by Ye et al. (2016) suggest that the resources from a donor microsite (here referring to the microsite where donor ramets are) could be translocated within a clonal network and then released into recipient microsites (here referring to the microsite where recipient ramets are) and these resources could subsequently be used by neighbor plants, resulting in resource redistribution at a community level. The findings of this study raise the very novel question of whether clonal integration could facilitate water and nutrient cycling, and therefore have implications for the whole ecosystem. More empirical evidence is needed to address this fascinating question.

AUTHOR CONTRIBUTIONS

FL, JL, and MD: proposed the outline of the manuscript. FL: wrote and revised the manuscript; MD: made a final check of the manuscript.

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Survival and Growth of Epiphytic Ferns Depend on Resource Sharing

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Locally available resources can be shared within clonal plant systems through physiological integration, thus enhancing their survival and growth. Most epiphytes exhibit clonal growth habit, but few studies have tested effects of physiological integration (resource sharing) on survival and growth of epiphytes and whether such effects vary with species. We conducted two experiments, one on individuals (single ramets) and another on groups (several ramets within a plot), with severed and intact rhizome treatments (without and with physiological integration) on two dominant epiphytic ferns (*Polypodiodes subamoena* and *Lepisorus scolopendrium*) in a subtropical montane moist forest in Southwest China. Rhizome severing (preventing integration) significantly reduced ramet survival in the individual experiment and number of surviving ramets in the group experiment, and it also decreased biomass of both species in both experiments. However, the magnitude of such integration effects did not vary significantly between the two species. We conclude that resource sharing may be a general strategy for clonal epiphytes to adapt to forest canopies where resources are limited and heterogeneously distributed in space and time.

Keywords: canopy-dwelling plants, clonal growth, clonal integration, forest canopy, habitat adaptation, montane moist forest, physiological integration

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INTRODUCTION

Environments are characterized by patchy distributions of abiotic and biotic factors (Alpert and Mooney, 1996; Chen et al., 2002; Jahnke et al., 2015). Clonal plants can integrate information about such environmental heterogeneity and respond accordingly (Louâpre et al., 2012; Wang et al., 2013; Oborny and Hubai, 2014; Chen et al., 2015; Saunders and Pezeshki, 2015). One strategy by which clonal plants cope with environmental heterogeneity is physiological integration, i.e., the capacity to share resources among interconnected ramets (Hutchings and Wijesinghe, 1997; Herben and Suzuki, 2001; Song et al., 2013; Roiloa et al., 2014; Dong et al., 2015). Physiological integration enables parent ramets to support offspring ramets (Matlaga and Sternberg, 2009; Oborny and Hubai, 2014; Roiloa et al., 2014; Glover et al., 2015) and ramets growing in favorable conditions to support those in unfavorable conditions (Roiloa et al., 2007; Xu L. et al., 2012; Kui et al., 2013; Tuya et al., 2013; Cornelissen et al., 2014; Luo et al., 2014).

Forest canopies house ca. 50% of terrestrial biodiversity (Ozanne et al., 2003; May, 2010; Lowman and Schowalter, 2012). As a key component of tropical and subtropical floras (Benzing, 2012; Zotz, 2013), canopy-dwelling epiphytes serve important ecological functions in forest hydrology and nutrient fluxes (Umana and Wanek, 2010; Zhang et al., 2015). However, epiphytic habitats are usually described as "harsh" because tree crowns are characterized by a limited storage capacity for available nutrients and water, sporadic and dilute nutrient inputs, low physical stability, extreme fluctuations in moisture and temperature, high wind speed, and severe and variable vapor pressure deficits (Théry, 2001; Zotz and Hietz, 2001; Benzing, 2012; Lowman and Schowalter, 2012). Significant variation in resource availability can occur at small spatial and temporal scales, and short-term drought can occur even in wet seasons of tropical rain forests (Zotz and Hietz, 2001; Watkins et al., 2007a). How epiphytes adapt to the harsh and heterogeneous environments of forest canopies remains one of the most fascinating questions in plant ecology (Benzing, 2012; Lowman and Schowalter, 2012; Reyes-García et al., 2012).

Almost all epiphytic bryophytes and lichens and many vascular epiphytes are capable of clonal growth (Jackson et al., 1985; During, 1990; de Kroon and van Groenendael, 1997; Benzing, 2012; Robinson and Miller, 2013). Different ramets within a clone are often interconnected via rhizomes, stolons or roots so that resource sharing (physiological integration) within the clone is possible (Eilts et al., 2011; Cornelissen et al., 2014; Weiser and Smycka, 2015). In the past decades, roles of physiological integration have been extensively documented in different species and in different habitats (Jackson et al., 1985; de Kroon and van Groenendael, 1997; Song et al., 2013). However, little is known about how physiological integration facilitates adaptation of epiphytes to forest canopies.

Recently, we selected one clonal, facultative, epiphytic fern to test effects of physiological integration in both epiphytic and terrestrial habitats in the dry season in a subtropical montane moist forest (Lu et al., 2015). We found that clonal integration contributed greatly to survival and growth of this species, and that the effect was more important in forest canopies than in forest understories (Lu et al., 2015). However, the target species possesses the unique aspects of facultative epiphytes and overwintering leaves (Lu et al., 2015), and is a common yet not dominant species in the forest. Furthermore, the experiment was carried out during the dry season when seasonal drought occurred, whereas most dominant epiphytes stop growing (shed leaves) in the dry season. Thus, it is still unknown whether clonal integration also plays an important role in dominant epiphytes and during the wet season. We hypothesize that (1) physiological integration can also increase survival and growth of dominant clonal epiphytes in the wet season.

In subtropical montane moist forests in Southwest China, eight of the nine dominant vascular epiphytes are ferns (Supplementary Table 1). Seven of these ferns produce long, creeping rhizomes that may potentially be investigated in the wet season (Xu and Liu, 2005; Ma, 2009). Because epiphytic ferns vary in morphology, physiology and phenology (Schneider et al., 2004; Watkins et al., 2007b), it is likely that these epiphytic species have adapted to habitats using various strategies. We thus hypothesize that (2) clonal epiphytes with divergent traits differ in their degree of dependence on clonal integration.

To test the hypotheses, we conducted two field experiments on two dominant epiphytes with divergent traits in a wet season in a subtropical montane moist forest in Southwest China. Specifically, we addressed two questions. (1) Does clonal integration increase survival and growth of dominant epiphytes during the wet season when water stress was seemingly weak? (2) If it does, does the effect of clonal integration on survival and growth differ between the two epiphytes with divergent traits? By addressing such questions in two dominant epiphytes and in growing (wet) seasons, we aim to test whether clonal integration is a general strategy for clonal epiphytes to adapt to forest canopies. The results obtained will deepen our understanding of the strategies of epiphytes dwelling on forest canopies.

MATERIALS AND METHODS

Study Site

We conducted the two field experiments in a primary subtropical montane moist forest in the Xujiaba region (24° 32′ N, 101° 01′ E) of Yunnan province, China, a core area covering 5100 ha of the northern crest of the Ailao Mountain National Nature Reserve. In this region, water loss occurs during the dry season, while water accumulates during the wet season (You et al., 2013a; Lu et al., 2015). During 2000–2010, the mean annual precipitation was 1874 mm, with 87% occurring in the wet season (May to October) and 13% in the dry season (November to April), the mean annual relative humidity was 84%, and the mean air temperature was 11.1°C (5.6°C in January and 15.3°C in July; Song et al., 2012). The forest is dominated by *Lithocarpus xylocarpus*, *Castanopsis wattii*, *L. chintungensis*, *Schima noronhae*, *Machilus viridis*, and *Hartia sinensis*, and also inhabited by a diverse community of epiphytes (Li et al., 2014).

Target Species

Polypodiodes subamoena (C. B. Clarke) Ching and *Lepisorus scolopendrium* (Ham. ex. D. Don) Menhra are two dominant vascular epiphytes in the montane moist forest (Xu and Liu, 2005; Ma, 2009). They mainly inhabit tree bark, junctions or rocks, and are capable of clonal growth via long, creeping rhizomes with adventitious roots (Zhang, 2012). The fronds of both ferns wither in the dry season, but their rhizomes can persist for several years. These two ferns exhibit different functional traits (i.e., morphology, physiology, and growth; **Table 1**). *P. subamoena* bears remote compound fronds and pinnatipartite (15–20 paired), herbaceous laminas and mainly occurs at 2400–3300 m a.s.l., whereas *L. scolopendrium* bears a close single frond and a herbaceous or papery lamina and occurs at 500–2800 m a.s.l. (Zhang, 2012).

Experiment Design

Individual Experiment

For each of the two species, we selected 60 mature ramets from the boles or crowns of 20 host trees (i.e., phorophytes) with diameter at breast height exceeding 30 cm. Ramet height of *P.*

TABLE 1 | Contrasting functional traits of the ramets of two species, *Polypodiodes subamoena* and *Lepisorus scolopendrium*.

Trait	*P. subamoena*	*L. scolopendrium*	*t*	*P*
Frond length (cm)	19.73 ± 0.74	16.14 ± 0.85	3.2	**0.002**
Frond width (cm)	4.61 ± 0.22	2.54 ± 0.10	8.7	**<0.001**
Frond thickness (mm)	0.33 ± 0.02	0.99 ± 0.04	−14.1	**<0.001**
F_v/F_m	0.74 ± 0.01	0.79 ± 0.01	−3.4	**0.001**
Aboveground mass per ramet (g)	0.24 ± 0.02	0.12 ± 0.01	5.6	**<0.001**
Belowground mass per ramet (g)	0.28 ± 0.02	0.25 ± 0.02	1.1	0.275
Total mass per ramet (g)	0.52 ± 0.04	0.37 ± 0.02	3.5	**0.001**
Ramet density (no. dm^{-2})	3.40 ± 0.11	6.50 ± 0.26	−11.1	**<0.001**

The given are mean ± SE of each species and results of t-tests.
Bold letters in column of "P" mean significant.

subamoena was 30.0 ± 0.4 cm (mean ± *SE*, ranging from 24.0 to 33.9 cm), and that of *L. scolopendrium* was 18.2 ± 0.3 cm (mean ± *SE*, ranging from 15.0 to 22.2 cm). Half of the ramets were randomly assigned to the severed-rhizome treatment and the other half to the intact-rhizome treatment. For the severed treatment, the rhizome internodes at both ends of the ramet were carefully exposed and cut off halfway from the ramet to prevent clonal integration. For the intact treatment, the rhizome internodes of the ramet were also carefully exposed, but no cutting was conducted so that physiological integration was allowed. The experiment started on July 26, 2013 and ended on October 26, 2013. At the end of the experiment, the survival status of all ramets was noted and the surviving ramets were harvested. A ramet was considered dead if all its fronds were shed, dried or withered. We measured frond length and width of the ramets with a ruler and frond thickness with calipers. Biomass was measured after drying the ramets at 70°C for 48 h. One day before harvest, we also measured maximum quantum yield of PS II (F_v/F_m) using a portable fluorometer (FSM-2; Hansatech, Norfolk, UK).

Group Experiment

For each species, we selected 20 plots, each with at least three ramets of the target species. Plots were located on 20 phorophytes (with diameter at breast height >30 cm). Half of the plots were randomly selected and subjected to the severed-rhizome treatment, and the remaining half to the intact-rhizome treatment. For the severed treatment, the rhizomes along the edges of each plot were carefully exposed by removing surrounding soil, humus, mosses and/or lichens, if any, and cut off with a sharp blade so that ramets inside the plot were disconnected from those outside the plot to prevent integration. For the intact treatment, the rhizomes along the edges of each plot were also carefully exposed, but were kept intact (i.e., not cut off) so that ramets inside the plot were connected with those outside to allow integration. The experiment started on July 30, 2013 and ended on October 30, 2013. At harvest, we counted number of surviving ramets and measured length, width, and thickness of the fronds of each surviving ramet in each plot. One day before harvest, we measured F_v/F_m using the FMS-2 on the fronds of two ramets in each plot. Biomass in each plot was measured after drying the plant materials at 70°C for 48 h.

Statistical Analyses

We analyzed the data from the two experiments separately. For the individual experiment, we used logistic regression to test the effect of rhizome severing (intact vs. severed) on survival of the ramets because the data of survival were binary (alive or dead) (McCullagh and Nelder, 1989). We used two-way ANOVA to test the effects of rhizome severing, species, and their interaction on growth (total biomass, aboveground, and belowground biomass), morphology (frond length, width and thickness), and physiology (F_v/F_m) of the individual ramets.

For the group experiment, we expressed the final biomass data on a per initial ramet basis because initial number of ramets differed greatly between the two species [*P. subamoena* vs. *L. scolopendrium*: 3.4 ± 0.11 vs. 6.5 ± 0.26 g (mean ± SE); $t = -11.07$, $P < 0.001$, $n = 40$]. We also calculated mean frond length, width and thickness and F_v/F_m of the ramets in each plot. We then used two-way ANOVA to test the effects of rhizome severing, species and their interactions on number of surviving ramets, growth, morphology and physiology in the group experiment. When needed, data were transformed to square root or natural logarithm to meet the ANOVA assumptions. Statistical analyses were carried out with SPSS 19.0 (IBM, Armonk, NY, USA) and R software (R Development Core Team, 2012).

RESULTS

Individual Experiment

In the individual experiment, rhizome severing significantly affected survival of the single ramets ($\chi^2 = 8.61$, $P = 0.003$), and such effects were not species-dependent (i.e., no interaction effect; $\chi^2 = 0.02$, $P = 0.893$). Survival rates of the single ramets were 86.7% for *P. subamoena* and 83.3% for *L. scolopendrium* when the rhizomes were intact, but were reduced to 63.3 and 60.0% when the rhizomes were severed (**Figure 1A**).

Rhizome severing significantly decreased total and belowground biomass (**Table 2**; **Figure 1B**) and maximum quantum yield of PS II (F_v/F_m; **Table 2**; **Figure C**) of the single ramets of both epiphytes. Such effects did not depend on species (no Se × Sp interaction; **Table 2**). Severing had no effect on frond length, width or thickness of the single ramets of either species (**Figures 1D–F**). Species significantly affected biomass, F_v/F_m, frond length, width and thickness (**Table 2**; **Figure 1**), affirming the contrasting growth, physiological and morphological traits of these two species (**Table 1**).

Group Experiment

In the group experiment, rhizome severing significantly reduced number of ramets, total biomass and belowground biomass of both epiphytes, and such effects did not depend on species (no Se × Sp interaction; **Table 3**; **Figures 2A,B**). Rhizome

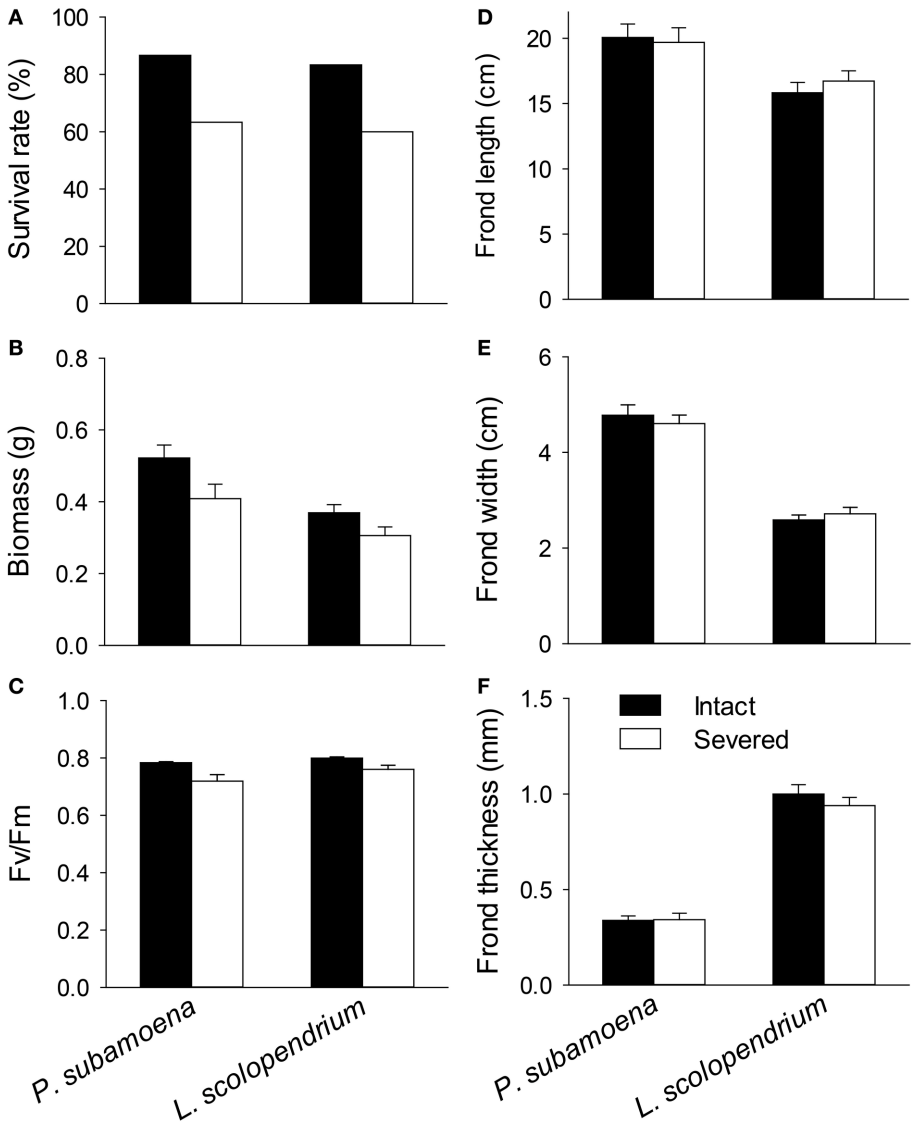

FIGURE 1 | Effects of rhizome severing on (A) survival, (B) biomass, (C) F_v/F_m, and (D–F) frond morphology of the two epiphytic ferns in the individual experiment. Error bars represent SEs.

severing did not significantly affect F_v/F_m, frond length, width or thickness of either species (**Table 3**; **Figures 2C–F**). Species significantly affected aboveground and belowground biomass, F_v/F_m, frond length, width and thickness (**Table 3**; **Figure 2**).

DISCUSSION

Both individual and group experiments showed that severing rhizomes decreased survival and growth of the two dominant epiphytic ferns in the wet season, supporting the first hypothesis that clonal integration (resource sharing) contributes to performance of epiphytes. These results agree with the findings on the facultative epiphytic fern *Selliguea griffithiana* (i.e., growing in both epiphytic and terrestrial habitats) conducted in a dry season in the same forest using similar approaches (Lu et al., 2015) and also those on the terrestrial fern *Diplopterygium glaucum* in a subtropical evergreen forest in China (Du et al., 2010). While numerous studies have tested effects of clonal integration (Song et al., 2013; Glover et al., 2015; Weiser and Smycka, 2015), very few have examined those on performance of epiphytes (Lu et al., 2015). This study of multiple species verified the key role of resource sharing for epiphytes in surviving and growing in the wet season.

Extraordinary heterogeneity is present because light intensity and temperature diminish downward through the forest canopy, whereas humidity and nutrients increase toward the ground (Benzing, 2012). Epiphytes also suffer from water shortage between rainfall events even in wet seasons in tropical forests

(Watkins et al., 2007a; Bartels and Chen, 2012). Our study site is characterized by a seasonal climate with variation in precipitation (You et al., 2013a). Although the forest is exposed to frequent rain and mist during the wet season, alternating wet and dry events occur daily and weekly (You et al., 2013a,b). Large trees have great microhabitat heterogeneity within their crowns and exhibit substantial changes from the inner to the outer crown in branch diameter, canopy humus cover, openness, and mean daily maximum vapor pressure deficits (Woods et al., 2015). Epiphytes dwelling in these large treetops must cope with microhabitat heterogeneity (Théry, 2001; Zotz and Hietz, 2001; Benzing, 2012). The findings of this study and the previous one (Lu et al., 2015) suggest that clonal epiphytes may evolve a high degree of clonal integration to alleviate resource stress in both wet and dry seasons. This may especially be the case for epiphytic ferns that exhibit poor water conservation owing to their limited hydraulic conductance and passive stomatal control (McAdam and Brodribb, 2012a,b).

Effects of clonal integration may differ among species (Song et al., 2013; Isogimi et al., 2014) and even among genotypes of the same species (Alpert et al., 2003; D'Hertefeldt et al.,

TABLE 2 | Individual experiment results of a two-way ANOVA for effects of species and rhizome severing on biomass, F_v/F_m, and frond morphology.

Trait	Species (Sp)	Severing (Se)	Se × Sp
Total mass[a]	13.18***	8.68**	0.17
Aboveground mass[a]	64.01***	0.74	0.58
Belowground mass	0.01	12.65**	2.06
F_v/F_m^a	5.47*	17.84***	1.05
Frond length	15.288***	0.08	0.48
Frond width[a]	141.98***	0.03	0.61
Frond thickness	251.74***	0.53	0.67

*F statistics are shown with significance levels (***P < 0.001; **P < 0.01; *P < 0.05).*
[a]Analysis performed on square-root transformed data.

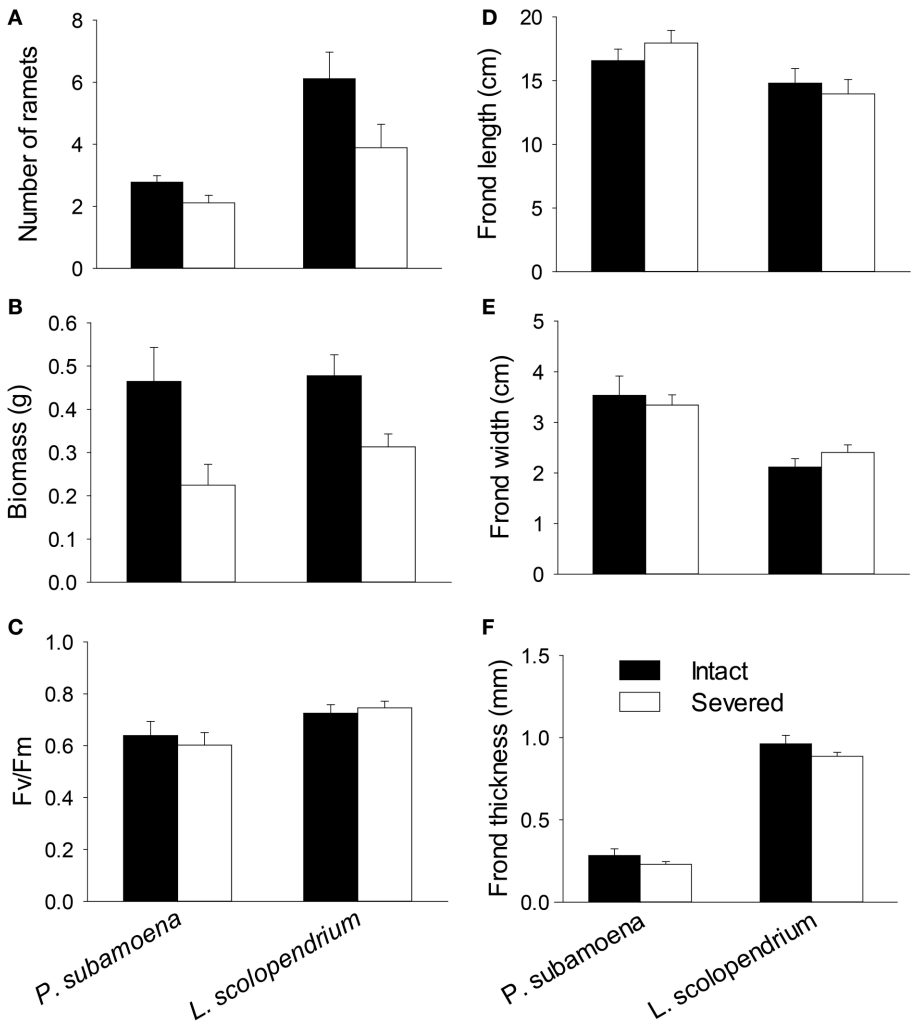

FIGURE 2 | Effects of rhizome severing on (A) ramet number, (B) biomass, (C) F_v/F_m, (D–F) and frond morphology of the two epiphytic ferns in the group experiment. Error bars represent SEs.

TABLE 3 | Group experiment results of a two-way ANOVA for effects of species and rhizome severing on the number of ramets, biomass, F_v/F_m, and frond morphology.

Trait	Species (Sp)	Severing (Se)	Se × Sp
Number of ramets[a]	0.72	6.36*	1.73
Total mass	0.88	13.99**	0.49
Aboveground mass	11.45**	2.39	1.55
Belowground mass[b]	4.77*	12.75**	0.12
F_v/F_m[b]	6.78*	0.04	0.43
Frond length	4.51*	0.04	0.67
Frond width	20.86***	0.03	0.87
Frond thickness[b]	308.86***	2.89	0.08

*F statistics are shown with significance levels (***P < 0.001; **P < 0.01; *P < 0.05).*
[a]Analysis performed on log transformed data.
[b]Analysis performed on square-root transformed data.

2014; Zhou et al., 2014). For instance, rhizomatous species may be more reliant on clonal integration than stoloniferous species (Song et al., 2013), and genotypes from sand dunes have shown a greater impact of clonal integration than those from grasslands (Alpert, 1999). Although, the two epiphytic ferns differ greatly in morphological, physiological, and growth traits (**Tables 1**–**3**, **Figures 1**, **2**), we found that the effects of clonal integration on ramet survival or growth did not differ significantly between the two epiphytes. These results thus do not support the second hypothesis, and suggest that clonal integration may be a general strategy for clonal epiphytes to survive and grow in forest canopies where resources are rather limited and also heterogeneously distributed in space and in time.

Clonal integration had a significant effect on F_v/F_m of epiphytes in the individual experiment, but not in the group experiment (**Tables 2, 3**, **Figures 1**, **2**). Previous studies also showed contrasting effects of clonal integration on photochemical activity of ramets (Luo et al., 2014; Roiloa et al., 2014). For instance, integration significantly affected photochemical activity of *Alternanthera philoxeroides* (Luo et al., 2014) and *Fragaria vesca* (Roiloa et al., 2014), but had little effect on that of the terrestrial fern *D. glaucum* (Du et al., 2010). Thus, effects of clonal integration on photochemical activity of the fronds may not be translated into the effects on survival and growth of the ramets. Data on survival and growth are more robust to evaluate the benefits of clonal integration.

We observed little impact of clonal integration on frond morphology of either of the epiphytes in either of the experiments (**Tables 2, 3**, **Figures 1**, **2**), agreeing with the findings of our previous study (Lu et al., 2015). However, many studies have shown a significant effect of clonal integration on morphological traits such as length and thickness of petioles and internodes of stolons and rhizomes (Alpert, 1999; Saitoh et al., 2002; Xu C. et al., 2012; Dong et al., 2015; Glover et al., 2015). Our results suggest that clonal epiphytes may not rely on integration-mediated changes in frond morphology to adapt to forest canopies.

CONCLUSIONS

Our results indicate that clonal integration (resource sharing) may have been selected for as a general trait for clonal epiphytes to adapt to the harsh and heterogeneous epiphytic habitats. While epiphytes have been shown to take different strategies to adapt to forest canopies (Benzing, 2012; Lowman and Schowalter, 2012; Reyes-García et al., 2012), our study suggests that resource sharing is an additional one for clonal epiphytes. Epiphytes are a key component of forest canopies and play important roles in maintaining biodiversity (e.g., fauna diversity; Ozanne et al., 2003; Ellwood and Foster, 2004; May, 2010) and ecosystem functioning (e.g., carbon and nutrient cycling; Umana and Wanek, 2010; Benzing, 2012; Lowman and Schowalter, 2012). Considering that many epiphytes are clonal and also most of the dominant epiphytes are clonal (Jackson et al., 1985; During, 1990; de Kroon and van Groenendael, 1997; Benzing, 2012; Robinson and Miller, 2013), we hypothesize further that resource sharing may also play important roles during the underlying processes by promoting survival and growth of clonal epiphytes. Therefore, further studies could be designed to examine whether effects of resource sharing within clones of epiphytes can be cascaded to affect biodiversity and ecosystem functioning.

AUTHOR CONTRIBUTIONS

WL and FY designed the project. HL, LS, and FY performed the experiments, analyzed the data and wrote the manuscript text. XX, YH, SL, ZF, and SGL analyzed some data and prepared some figures and tables. XS, WM, YC, and YW did some field work and collected data. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00416

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Effects of Spatial Patch Arrangement and Scale of Covarying Resources on Growth and Intraspecific Competition of a Clonal Plant

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Spatial heterogeneity in two co-variable resources such as light and water availability is common and can affect the growth of clonal plants. Several studies have tested effects of spatial heterogeneity in the supply of a single resource on competitive interactions of plants, but none has examined those of heterogeneous distribution of two co-variable resources. In a greenhouse experiment, we grew one (without intraspecific competition) or nine isolated ramets (with competition) of a rhizomatous herb *Iris japonica* under a homogeneous environment and four heterogeneous environments differing in patch arrangement (reciprocal and parallel patchiness of light and soil water) and patch scale (large and small patches of light and water). Intraspecific competition significantly decreased the growth of *I. japonica*, but at the whole container level there were no significant interaction effects of competition by spatial heterogeneity or significant effect of heterogeneity on competitive intensity. Irrespective of competition, the growth of *I. japonica* in the high and the low water patches did not differ significantly in the homogeneous treatments, but it was significantly larger in the high than in the low water patches in the heterogeneous treatments with large patches. For the heterogeneous treatments with small patches, the growth of *I. japonica* was significantly larger in the high than in the low water patches in the presence of competition, but such an effect was not significant in the absence of competition. Furthermore, patch arrangement and patch scale significantly affected competitive intensity at the patch level. Therefore, spatial heterogeneity in light and water supply can alter intraspecific competition at the patch level and such effects depend on patch arrangement and patch scale.

Keywords: clonal growth, *Iris japonica*, intraspecific interactions, reciprocal patchiness, pararell patchiness, patch scale

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INTRODUCTION

Spatial heterogeneity in supplies of essential resources (light, water, and soil nutrients) commonly occurs in nature, and different ramets of clonal plants interconnected by, e.g., rhizomes, stolons, and horizontal growing roots are often located in contrasting levels of resource availability (Hutchings and Wijesinghe, 1997; Hutchings and John, 2004; Liu et al., 2006, 2008; Bartels and Chen, 2010). Clonal plants can exhibit foraging responses, i.e., placing more resource-absorbing

organs (e.g., leaves, roots, or ramets) in high-quality patches than in low-quality ones, to efficiently utilize heterogeneously distributed resources of light and water (Hutchings and de Kroon, 1994; Hodge, 2004; Hutchings and John, 2004; de Kroon et al., 2005; Guo et al., 2011; Peng et al., 2013). Furthermore, ramets growing in high-quality patches can transport carbohydrates, water and minimal nutrients to those in low-quality ones by physiological integration via rhizomes, stolons, or roots (Alpert and Stuefer, 1997; Price and Marshall, 1999; He et al., 2010, 2011). Such a cooperative system can buffer effects of spatial heterogeneity (Roiloa et al., 2007) and enhance performance of the whole plant (Roiloa and Retuerto, 2007; Hutchings and Wijesinghe, 2008; Song et al., 2013; Zhang and Zhang, 2013; Dong et al., 2015). Spatial heterogeneity in resource supply may also affect plant–plant interactions (Fransen et al., 2001; Day et al., 2003; Moore and Franklin, 2012; Wang et al., 2012; Li H.L. et al., 2014; Dong et al., 2015). For instance, light heterogeneity increased intraspecific competition in *Duchesnea indica* (Wang et al., 2012), and soil nutrient heterogeneity increased intraspecific competition in *Briza media* and interspecific competition between *Festuca ovina* and *B. media* (Day et al., 2003). So far, however, studies testing effects of resource heterogeneity on plant–plant interactions considered spatial heterogeneity in the supply of only one single resource (light or soil nutrients), and little study has examined effects of spatial heterogeneity in two co-variable resources such as light and soil water on intraspecific competition of plants.

In nature, light and soil water commonly co-vary (Alpert and Mooney, 1996). In some habitats such as forest edges, grasslands and shrublands, high light intensity in open patches without vegetation is commonly accompanied with low soil water availability due to high evaporation, and low light intensity underneath dense vegetation is associated with high soil water availability due to low evaporation (Alpert and Mooney, 1996; Griffith, 2010; Li Q.Y. et al., 2014). In such environments with reciprocal patchiness of light and soil water, neither patches alone are ideal for plants growing in them (He et al., 2011; Zhang and Zhang, 2013; Li Q.Y. et al., 2014). In some other habitats such as wetlands or forest gaps opened by disturbance or mortality and dunes with dense shrubs, high light intensity may be associated with high soil water availability and low light intensity with low soil water availability (Prati and Schmid, 2000; Dyer et al., 2010). In such environments with parallel patchiness of light and soil water, patches with high light and high soil water are ideal for plants, whereas patches with low light and low water may not (He et al., 2011; Zhang and Zhang, 2013). Previous studies have shown that reciprocal and parallel patchiness may differently affect the growth of clonal plants (Alpert and Mooney, 1996; Prati and Schmid, 2000; Griffith, 2010; He et al., 2011; Zhang and Zhang, 2013; Li Q.Y. et al., 2014). However, no study has tested whether such patch arrangement (i.e., reciprocal vs. parallel patchiness) affects intraspecific competition of clonal plants. Furthermore, responses of intraspecific competition to resource heterogeneity may also vary with the scale of the patchiness, because foraging ability and thus the growth of plants depends on patch scale of heterogeneity (van der Waal et al., 2011; Wang et al., 2012; Peng et al., 2013; Dong et al., 2015).

To test effects of patch arrangement (reciprocal vs. parallel patchiness) and patch scale on intraspecific competition, we conducted a greenhouse experiment with a rhizomatous, clonal plant *Iris japonica.* We grew one (without intraspecific competition) or nine isolated ramets (with competition) of *I. japonica* under a homogeneous environment and four heterogeneous environments differing in patch arrangement (reciprocal vs. parallel patchiness of light and soil water) and patch scale (large vs. small patches of light and water). Specifically, we addressed the following questions: (1) Does spatial heterogeneity in light and soil water affect intraspecific competition of *I. japonica*? (2) Do reciprocal and parallel patch arrangements have different effects on intraspecific competition of *I. japonica*? (3) Does spatial scale of heterogeneity matter?

MATERIALS AND METHODS

Plant Material

Iris japonica Thunb. (Iridaceae) is a perennial clonal herb and widely distributed in forest understories, forest gaps, forest edges, and moist grasslands in Asia (Wang et al., 2013; Li Q.Y. et al., 2014). This species produce long slender rhizomes along which rooted ramets are formed. In the field, most rhizomes are distributed in the top soil of less than 5 cm deep. Inter-ramet distance (spacer length) is 5–15 cm (Wang et al., 2013). Rhizomes that connected ramets of the same genet can break due to disturbance or senescence so that genets become fragmented in the field. The blossoming time is from March to April, and viable seeds are produced from May to June. Clonal growth is the main means for the maintenance and spread of the populations (Wang et al., 2013).

In early January 2014, more than 1000 ramets of *I. japonica* were collected from five locations in an evergreen broad-leaved forest on Shizi Mountain in Hubei Province, China (N 30°28′-30°30′; E 114°20′-114°23′). Adjacent locations were at least 100 m apart so that ramets from different locations were likely to belong to different genotypes. Plants from different locations were mixed and propagated vegetatively in a greenhouse of Huazhong Agricultural University in Hubei Province, China. After 2 weeks of cultivation, we selected 424 similar-sized ramets of *I. japonica*, each with a node, three leaves and some roots. Of them, 24 ramets were randomly selected for measuring initial dry mass (0.389 ± 0.039 g, mean ± SE), and the other 400 were used for the experiment described below.

Experimental Design

The experiment was a factorial design with two levels of intraspecific competition (without and with intraspecific competition) and five levels of heterogeneity (homogeneous, reciprocal large patch, reciprocal small patch, parallel large patch, and parallel small patch), making a total of 10 treatments (**Figure 1**). In the treatments without competition, one ramet of *I. japonica* was planted in the center of a plastic container (50 cm long × 50 cm wide × 30 cm high) with sealed bottom,

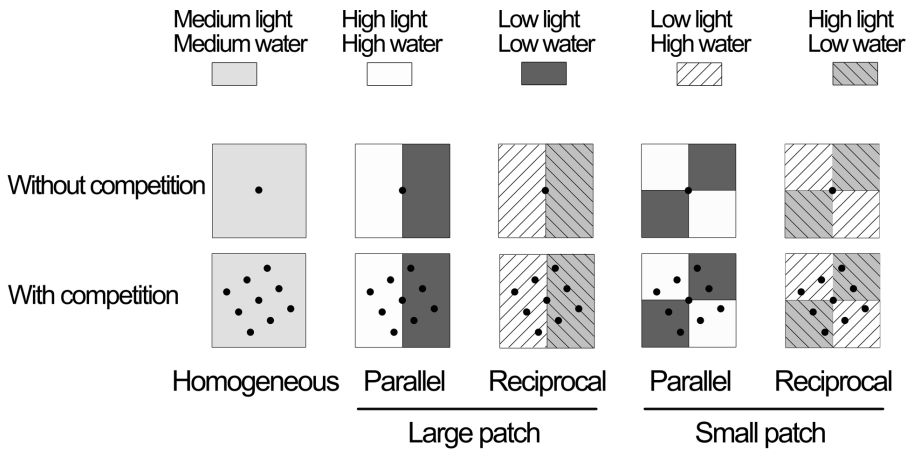

FIGURE 1 | Experimental design. The experiment had two competition treatments (without vs. with competition by growing one or nine ramets of *Iris japonica* in a container) crossed with five heterogeneity treatments, i.e., (i) homogeneous (all patches received moderate light and moderate water), (ii) parallel large patch (the container was divided into two large patches; one received high light and high water, and the other low light and low water), (iii) reciprocal large patch (the whole container was divided into two large patches; one patch received high light and low water, and the other low light and high water), (iv) parallel small patch (the container was divided into four small patches; two received high light and high water, and the other low light and low water) and (V) reciprocal small patch (the container was divided into four small patches; two received high light and low water, and the other low light and high water). The light and water content received by the plants in the heterogeneous treatments was the same as that in the homogeneous treatment.

and in the treatments with competition, nine ramets were planted (**Figure 1**). In the reciprocal large-patch treatments, each container was divided into two large patches (each measuring 50 cm × 25 cm), one of which was subjected to high light and low water and the other to low light and high water. In the reciprocal small-patch treatments, each container was divided into four small patches (each measuring 25 cm × 25 cm), two of which were subjected to high light and low water and the other two to low light and high water. In the parallel large-patch treatments, each container was divided into two large patches, one of which was subjected to high light and high water and the other to low light and low water. In the parallel small-patch treatments, each container was divided into four small patches, two of which were subjected to high light and high water and the other two to low light and low water. In the homogeneous treatments, each container was subjected to medium light and medium water. There were eight replicates in each treatment.

Each container was filled with a mix of sand and yellow–brown soil (1:1 v/v) homogeneously mixed with 20 g slow release fertilizer (Osmocote, N–P–K: 15–9–12, lasting for 5–6 months). Ramets were transplated to the containers on 14 February 2014 and allowed to recover and establish by supplying with sufficient water for 1 week. Then the soils were allowed to dry for 5 days without adding any water. High light was 100% of natural light in the greenhouse, without covering the patches with a shading net; medium and low light were 55 and 10% of natural light in the greenhouse, respectively, realized by covering the patches with black, neutral shading nets of 55 and 10% transmittances. During the experiment, we added 440 mL water to each container every one to four days depending on the weather conditions and thus how fast the soil dried. In the homogeneous treatments, we spayed 440 mL water evenly to the soil in each container to creat medium water availability. In the heterogeneous treatments with large patches, we supplied 400 mL water to the large patch of high water availability and 40 mL water to the large patch of low water availability in each container. In the heterogeneous treatments with small patches, we added 200 mL water to each of the two small patches of high water availability and 20 mL to each of the two small patches of low water availability in each container.

The bottom of the container was sealed so that there was no water leakage. We built physical barriers between patches inside each container. The barriers were 25 cm high and sealed to the containers (30 cm high) to prevent horizontal flow of water in the soil more than 5 cm deep between patches. Because in the top 5-cm-deep soil, there were no barriers the ramet in the central position in the container could be planted in the soil on the barrier (to be revised further). During watering, we also sprayed water slowly and carefully into each patch to avoid massive horizontal flow of water in the top soil of 5 cm deep. Because rhizomes of *I. japonica* are distributed within the top, 5-cm-deep soil (Wang et al., 2013), the physical barriers could not prevent rhizomes to grow across patches. Soil water content was monitored everyday in four replications during the experiment by a Soil Moisture Meter (TZS-II, HEB Biotechnology Co., Xi'an, China). Soil water content was about 32–37% in the high water patches, 20–25% in the medium water patches and 8–13% in the low water patches.

The experiment was conducted in the greenhouse at Huazhong Agricultural University. During the experiment, the mean temperature and mean relative humidity in the greenhouse were 25.1°C and 72.2%, respectively (measured by Amprobe TR300, Amprobe, Everett, WA, USA). Light intensity in the greenhouse was 85% of that outside. The experiment was started

on 26 February 2014 and ended on 1 July 2014, lasting for 125 days.

Measurements

At the end of the experiment, parent (original) ramets and offspring ramets were harvested separately. For the reciprocal patch treatments, we harvested offspring ramet located in the patches of high water and low light and patches of low water and high light separately. Similarly, for the parallel patch treatments, we harvested offspring ramets in the patches of high water and high light and patches of low water and low light separately. In each container, we pooled offspring ramets located in the same type of patches into one sample. For the homogeneous treatment, offspring ramets were harvested in a similar fashion, i.e., offspring ramets located in the imagined high and low water patches were harvested separately and those in the same type of imaged patches were pooled into one sample. The plants were then separated into leaves, stem, rhizomes, and roots, dried at 80°C for 48 h and weighed. Biomass in a container (at the container level) was the sum of biomass of the parent ramets, offspring ramets located in the high water patches and offspring ramets in the low water patches in that container. Similarly, we obtained number of ramets and rhizome length at the container level.

Data Analysis

The growth measures could not be compared directly because number of initial ramets of *I. japonica* differed between the two competition treatments (one vs. nine for the treatments without vs. with competition). Thus, we calculated biomass, number of ramets and rhizome length on the basis of per initial ramet in each container and also in each type of the patches, and these data were used for further analyses.

We used two-way ANOVAs to test effects of intraspecific competition (with and without competition) and spatial heterogeneity (homogeneous, reciprocal large and small patch, and parallel large and small patch) on the growth of *I. japonica* at the container level. If a significant effect of spatial heterogeneity was detected, then Tukey HSD tests to conducted to compare the means among the five heterogeneity treatments. The aim of these analyses was to examine whether there was an overall impact of spatial heterogeneity (homogeneous treatment vs. heterogeneous treatments of different types), as well as its interaction with intraspecific competition, so that the homogeneous treatments could be included. We further used three-way ANOVAs to examine effects of intraspecific competition, patch arrangement (reciprocal vs. parallel) and patch scale (small vs. large) on the growth at the container level, and in these analyses the homogeneous treatments were excluded. The aim of these analyses was to test the effect of patch scale and patch arrangement (and their interactions with competition), and the homogeneous treatments could not be included because they did not belong to either of the two patch sizes or patch arrangements. At the patch level, we employed three-way ANOVAs with repeated measures to test effects of intraspecific competition, spatial heterogeneity and patch type (high vs. low water patches) within a container on the growth of offspring ramets of *I. japonica* (Wang et al., 2012). If a significant effect of spatial heterogeneity was detected, then Tukey HSD tests to conducted to compare the means among the five treatments. We further used four-way ANOVAs with repeated measures to test effects of intraspecific competition, patch arrangement, patch scale and patch type within a container on the growth of offspring ramets at the patch level (Wang et al., 2012; Dong et al., 2015), and in these analyses the homogeneous treatments were excluded. Patch type within a container was used as a repeated variable because the two types of patches in a container were not independent (Potvin et al., 1990; Zar, 1999, p. 255).

To measure the intraspecific competitive intensity, we calculated the log response ratio (LnRR) of biomass as $\mathrm{LnRR} = \ln(B_o/B_w)$, where B_o is mean biomass of a treatment without competition across the eight replicates, and B_w is biomass of the treatment with competition in each replicate. Values of LnRR are symmetrical around zero (Hedges et al., 1999; Armas et al., 2004). Positive values indicate competition, negative values indicate facilitation and zero indicates neutral. At the container level, we used one-way ANOVA to examine the effect of spatial heterogeneity on LnRR. If a significant effect was detected, we further used two-way ANOVA to test the effects of patch arrangement and patch scale on LnRR. At the patch level, we used two-way ANOVA to examine the effect of spatial heterogeneity and patch type on LnRR. If a significant effect of spatial heterogeneity was detected, we further used three-way ANOVA to test the effects of patch arrangement, patch scale and patch type on LnRR. Patch type was treated as a repeated variable. All analyses were conducted using SPSS 13.0 (SPSS, Chicago, IL, USA).

RESULTS

Effects of Spatial Heterogeneity and Intraspecific Competition at the Container Level

Spatial heterogeneity in light and water significantly affected biomass and rhizome length of *I. japonica* at the container level (**Table 1**). Irrespective of competition, biomass and rhizome length were the highest in the heterogeneous treatments with large patches, smallest in the homogeneous treatments, and intermediate in the heterogeneous treatments with small patches (**Figures 2A,C**;

TABLE 1 | ANOVAs for effects of spatial heterogeneity (homogeneous vs. parallel large patch vs. reciprocal large patch vs. parallel small patch vs. reciprocal small patch) and intraspecific competition (without vs. with competition) on the growth of *Iris japonica* at the whole container level.

Effect	df	Biomass	Number of ramets	Rhizome length
Heterogeneity (H)	4, 80	**5.365****	1.277	**2.812***
Competition (C)	1, 80	**55.727*****	**10.992****	**31.977*****
H × C	4, 80	1.107	0.234	1.176

*Significance levels: ***P < 0.001, **P < 0.01, *P < 0.05.*

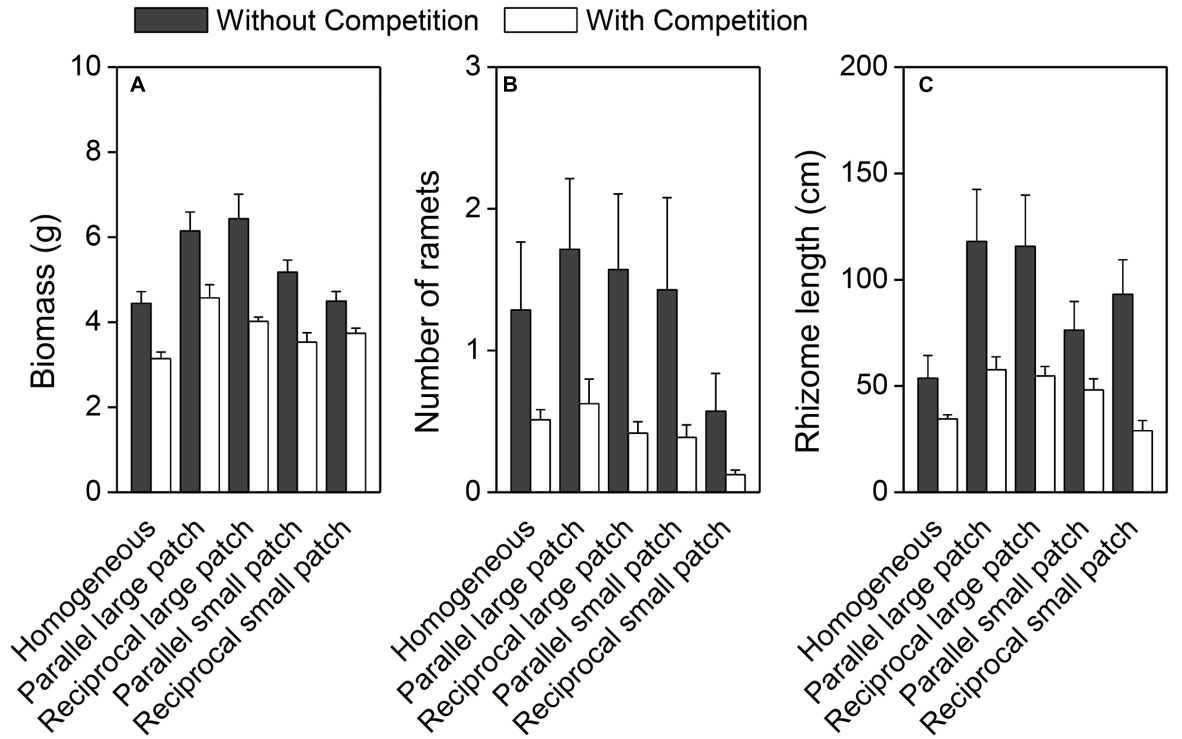

FIGURE 2 | Biomass (A), number of ramets (B), and rhizome length (C) of *I. Japonica* at the whole container level under the ten treatments. Error bars show +SE.

Appendices 1A,C; **Tables 1** and **2**). However, none of the three growth measures differ significantly between parallel and reciprocal patch arrangements (**Figure 2**; **Appendices 1A–C**; **Table 2**).

Intraspecific competition significantly decreased biomass, number of ramets and rhizome length of *I. japonica* at the container level (**Figure 2**; **Tables 1** and **2**). However, there were no significant interaction effects of competition by spatial heterogeneity (**Tables 1** and **2**; **Figure 2**), and no significant effect of spatial heterogeneity on the log response ratio of biomass (LnRR; **Figure 3A**, $F_{4,35} = 0.698$, $P = 0.599$), suggesting that spatial heterogeneity in light and water, irrespective of its patch arrangement or scale, did not alter intraspecific competitive intensity of *I. japonica* at the container scale.

TABLE 2 | ANOVAs for effects of patch arrangement (parallel vs. reciprocal), patch scale (large vs. small) and intraspecific competition (without vs. with competition) on the growth of *I. japonica* at the whole container level.

Effect	df	Biomass	Number of ramets	Rhizome length
Patch arrangement (P_a)	1, 64	1.373	0.894	0.032
Patch scale (P_s)	1, 64	**8.927****	**6.502***	**4.253***
Competition (C)	1, 64	**45.823*****	**8.716****	**28.749*****
$P_a \times P_s$	1, 64	0.171	0.028	0.001
$P_a \times C$	1, 64	0.822	0.024	0.857
$P_s \times C$	1, 64	0.940	1.743	0.053
$P_a \times P_s \times C$	1, 64	0.260	0.002	0.688

*Significance levels: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.*

Effects of Spatial Heterogeneity and Intraspecific Competition at the Patch Level

There were significant interaction effects of patch type × spatial heterogeneity (**Table 3**), patch type × spatial heterogeneity × competition (**Table 3**), patch type × patch arrangement (**Table 4**), patch type × patch arrangement × patch scale (**Table 4**), and patch type × patch arrangement × competition (**Table 4**) on the growth measures. Irrespective of competition, biomass, number of ramets and rhizome length were significantly larger in the high than in the low water patches in the heterogeneous treatments with large patches, but was statistically the same in the high and low water patches in the homogeneous treatments (**Figure 4**; **Tables 3** and **4**). For the heterogeneous treatments with small patches, the growth of *I. japonica* was not significantly affected by patch type in the absence of competition, but significantly larger in the high than in the low water patches in the presence of competition (**Figure 4**; **Appendices 1D–F**; **Tables 3** and **4**).

There were significant interaction effects of patch type × spatial heterogeneity ($F_{4,35} = 7.815$, $P < 0.001$), patch type × patch arrangement ($F_{1,28} = 17.634$, $P < 0.001$) and patch type × patch scale ($F_{1,28} = 6.705$, $P = 0.036$) on LnRR at the patch level (**Figure 3B**). LnRR was significantly larger in the low

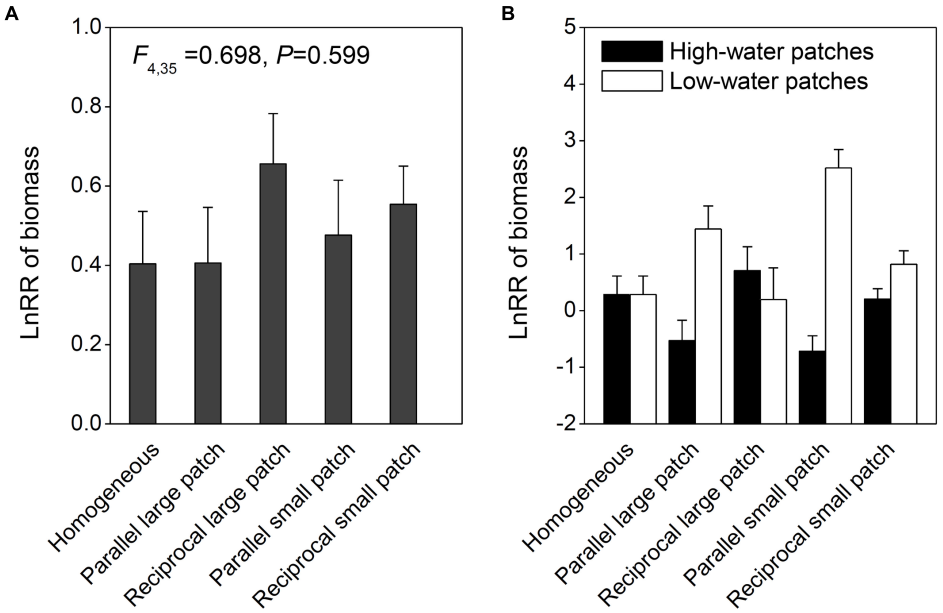

FIGURE 3 | Completive intensity as measured by log response ratio (LnRR) of biomass of *I. japonica* at the whole container level (A) and in the high and low water patches at the patch level (B) in the five treatments. Error bars show +SE.

TABLE 3 | ANOVAs for effects of heterogeneity (homogeneous vs. parallel large patch vs. reciprocal large patch vs. parallel small patch vs. reciprocal small patch), intraspecific competition (without vs. with competition) and patch type (high vs. low water patches) on the growth of *I. japonica* at the patch level.

Effect	df	Biomass	Number of ramets	Rhizome length
Heterogeneity (H)	4, 80	**5.401*** **	1.812	**6.716*** **
Competition (C)	1, 80	0.001	1.900	0.011
Patch type (P_t)	1, 80	**175.642*** **	1.658	0.445
H × C	4, 80	**6.936*** **	0.182	1.683
H × P_t	4, 80	**45.715*** **	**2.557***	**13.536*** **
C × P_t	1, 80	**63.164*** **	2.452	1.467
H × C × P_t	4, 80	**10.875*** **	1.559	**4.990****

*Significance levels: ***P < 0.001, **P < 0.01, *P < 0.05. Patch type was treated as a repeated variable.*

TABLE 4 | ANOVAs for effects of patch arrangement (parallel vs. reciprocal), patch scale (large vs. small), intraspecific competition (without vs. with competition) and patch type (high vs. low water patches) on the growth of *I. japonica* at the patch level.

Effect	df	Biomass	Number of ramets	Rhizome length
Patch arrangement (P_a)	1, 64	**4.096***	2.220	0.516
Patch scale (P_s)	1, 64	**11.594*** **	**4.303***	**3.879***
Competition (C)	1, 64	**4.517***	1.728	0.085
Patch type (P_t)	1, 64	**233.102*** **	1.104	**3.745***
P_a × P_s	1, 64	0.007	0.144	0.029
P_a × C	1, 64	3.230	0.021	3.359
P_a × P_t	1, 64	**58.490****	**6.249***	**27.417*** **
P_s × C	1, 64	0.001	0.001	1.411
P_s × P_t	1, 64	0.519	0.820	0.658
C × P_t	1, 64	**40.372*** **	**3.987***	2.368
P_a × P_s × C	1, 64	0.506	0.622	0.197
P_a × P_s × P_t	1, 64	**11.486*** **	1.797	**7.337****
P_a × C × P_t	1, 64	**36.669*** **	2.658	**14.505*** **
P_s × C × P_t	1, 64	0.558	0.379	0.009
P_a × P_s × C × P_t	1, 64	0.241	0.474	0.592

*Significance levels: ***P < 0.001, **P < 0.01, *P < 0.05. Patch type was treated as a repeated variable.*

than in the high water patches in the heterogeneous treatments with the parallel arrangement, but was statistically the same in the high and low water patches in the homogeneous treatments and the heterogeneous treatments with the reciprocal arrangement (**Figure 3B**). LnRR was significantly larger in the low than in the high water patches in the heterogeneous treatments with small patches, but was not significantly affected by patch type in the reciprocal arrangement with large patches (**Figure 3B**). Compared to the homogeneous treatments and the reciprocal arrangement treatments, the parallel arrangement treatments greatly decreased LnRR in the high water patches, but increased that in the low water patches (**Figure 3B**). These results suggest that patch type, patch arrangement and patch scale can alter intraspecific competitive intensity of *I. japonica* at the patch scale.

DISCUSSION

While many studies have tested effects of environmental heterogeneity in the supply of a single resource (e.g., light or soil nutrients) on intraspecific and/or interspecific interactions of plants (Fransen et al., 2001; Day et al., 2003; Moore and

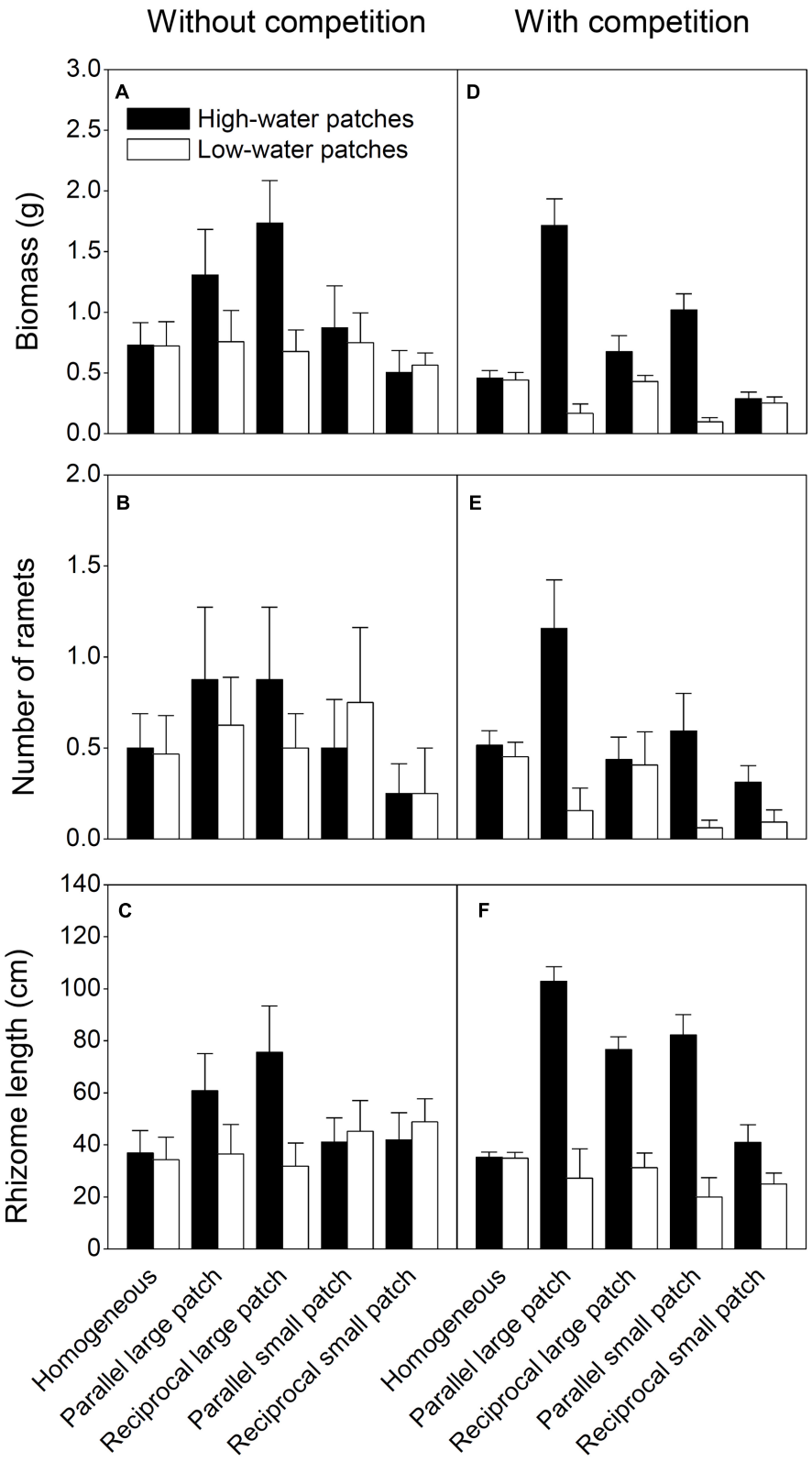

FIGURE 4 | Biomass (A,D), number of ramets (B,E) and rhizome length of (C,F) *I. japonica* in the high and low water patches under the ten treatments. Error bars show +SE.

Franklin, 2012; Wang et al., 2012; Li H.L. et al., 2014; Dong et al., 2015), none has examined those of heterogeneous distribution of two co-variable resources. Our results clearly showed that spatial heterogeneity in light and water availability could alter intraspecific competition at the patch level and that such effects depended on spatial patch arrangement and patch scale.

Spatial heterogeneity in light and water availability, irrespective of its patch arrangement or patch scale, did not significantly alter intraspecific competition intensity of *I. japonica* at the container scale. Previous studies have also showed that soil nutrient heterogeneity did not affect intraspecific competition in *Hydrocotyle vulgaris* (Dong et al., 2015), *Alternanthera philoxeroides* (Zhou et al., 2012) or *F. ovina* (Day et al., 2003) at the container level. It has been suggested that a significant effect of light and soil heterogeneity on competition may be caused by the differences between plants in their ability to concentrate ramets and/or roots where resource levels are high (Fransen et al., 2001; Zhou et al., 2012). In this study, *I. japonica* showed the relatively high and low ability to concentrate ramets and rhizome mass in high and low water patches, respectively. Consequently, a significant integrative effect of resource heterogeneity of light and water on the intraspecific interactions was not observed at the container level.

In the heterogeneous treatment with parallel patchiness, resource heterogeneity of light and water significantly decreased intraspecific competition intensity of *I. japonica* in the high water patches and increased that in the low water patches, but such effects were absent in the heterogeneous treatments with reciprocal patchiness. Thus, at the patch level, patchy distribution of light and water could alter intraspecific competition and patch arrangement mattered. Previous studies indicated that preferential ramet and root placements in resource-rich patches might greatly improve the efficiency and amount of resource capture and further increase their local growth (Roiloa and Retuerto, 2007; Wang et al., 2012). The efficiency of resource capture by these ramets in resource-rich patches can also benefit the growth of the whole plant by resource translocation from connected ramets in competition-free conditions (He et al., 2011; Zhou et al., 2012; Zhang and Zhang, 2013). In the presence of competition, plants prefer to concentrate more new ramets or new rhizomes in the high water and high light patches to promote the success of growth rather than in the low water and low light patches. These may result from the fact that resources were sufficient in high water and high light conditions and insufficient in low water and low light conditions for the competitive growth of *I. japonica*. Clonal integration might reduce the intensity of competition between ramets in the resource-rich patches by allowing internal transportation of resources from the ramets in the resource-rich patches to the connected ramets in the resource-poor patches, which can also be beneficial to plant growth in resource-rich patches (Novoplansky, 2009; Dong et al., 2015). In reciprocal patchiness, *I. japonica* might develop division of labor of plants between high water with low light and low water with high light conditions, which shared the risk of intense competition in both high and low water patches (Stuefer et al., 1996; Wang et al., 2011). That might be also a strategy for selecting advantageous patches and balancing the benefit between resource-poor and resource-rich patches in competition conditions.

Impacts of patchy distribution of two co-variable resources on intraspecific competition intensity of *I. japonica* also depended on patch scale. Patch scale had substantial effects on performance of clonal plants (Wijesinghe and Hutchings, 1997, 1999), and resource heterogeneity that affects plant performance at one scale may not do so at other scales. For instance, *Glechoma hederacea* clones growing in heterogeneous conditions with large patches produced greater biomass than those growing in heterogeneous conditions with small patches (Wijesinghe and Hutchings, 1997, 1999). However, spatial heterogeneity in light intensity increased intraspecific competition intensity of *D. indica* at both large and small patch scales (Wang et al., 2012). Impacts of patch scale on plant performance and interaction may be related to inter-ramet distance and also the size of root and shoot systems of ramets. If the patch size is too small or too large, then there will be no impact of heterogeneity (Zhou et al., 2012; Dong et al., 2015). In our study, enough space with sufficient resources in large patches with high water availability was benefit to the competitive growth of *I. japonica*. However, in small patches with high water availability, the rhizome growth and placement of new ramets of *I. japonica* was restricted, and nearly all space was overloaded. Thus the resource-rich patches might not always maintain equal suitability and gradually decline to the same level of suitability as the resource-poor patches. Therefore, in our study, plants in heterogeneous treatments with large patches produced more new ramets or new rhizome in the high water than low water patches in competitive conditions.

Our results also indicate that *I. japonica* exhibited foraging responses in the heterogeneous environment of both reciprocal and parallel patchiness, especially in competitive conditions. The possible reason can be the existence of a negative correlation between the space of plant growth and foraging precision (Wijesinghe et al., 2001; Cahill et al., 2010). If *I. japonica* grows alone, sufficient space may favor the fast growth of plants and decrease its foraging precision, which might lead to ignoring the heterogeneous resource distribution in the parallel patchiness (Rajaniemi and Reynolds, 2004; Mommer et al., 2012; Dong et al., 2015). In reciprocal patchiness, *I. japonica* might develop division of labor of plants between high water with low light and low water with high light conditions, which promoted the high potential benefits to enhance resource capture of clonal plants and thereby to increase their performance in heterogeneous habitats (Stuefer et al., 1996; Roiloa et al., 2007; Wang et al., 2011; Zhang and Zhang, 2013). Meanwhile, in the presence of intraspecific competition, limited space for the growth may enable *I. japonica* to show a higher foraging precision in response to resources (Cahill et al., 2010; Dong et al., 2015), especially in parallel patchiness with extremely rich and poor patches.

CONCLUSIONS

We conclude that environmental heterogeneity in the supply of two co-variable resources can affect intraspecific interactions of plants at some circumstances. Our results also suggest that competitive responses to spatial heterogeneity in resource availability may necessarily be adaptive and depend on resource combination and patch scale. Therefore, spatial heterogeneity in light and water availability may be of great importance in regulating population structure and dynamics of clonal plants (Hutchings et al., 2003; He et al., 2011; Wang et al., 2012; Zhang and Zhang, 2013).

AUTHOR CONTRIBUTIONS

Y-JW, X-PS, and F-HY designed the experiment. X-JW and X-FM performed the experiment. Y-JW wrote the first draft of the manuscript. Y-JW, F-LL, and F-HY did the statistical analysis. Y-JW and F-HY contributed substantially to the revisions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00753

DATA SHEET 1 | Biomass (A,D), number of ramets (B,E) and rhizome length (C,F) of *Iris japonica* at the whole container level (i) and at the patch level (ii) under the five heterogeneity treatments. Error bars show +SE. Letters show the differences between the treatments (Tukey HSD tests, $P = 0.05$).

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Delayed Flowering in Bamboo: Evidence from *Fargesia qinlingensis* in the Qinling Mountains of China

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Gregarious flowering of bamboo species impacts ecosystem properties and conservation, but documentation of these periodic events is difficult. Here, we compare the characteristics of flowering sites and un-flowered patches of an arrow bamboo (*Fargesia qinlingensis*) in the Qinling Mountains, China, over a 5-year period (2003–2007) after a mast flowering event (2003). We examined flowering culm and seedling characteristics in relation to questions regarding the evolution of delayed flowering. Density of live culms decreased over the 5 years in both flowering sites and un-flowered patches. New shoots regenerated only in un-flowered patches. Chemical constituent allocation varied among culm parts (stems, branches, and leaves). Crude protein and extract ether in branches and leaves were less in flowering culms than in un-flowered culms. Seedling density was lower than expected based on floret counts, suggesting predation of seeds. Seedling density was significantly greater in flowering sites than in un-flowered patches and decreased over time. Seedlings performed better in flowering sites than in un-flowered patches based on their height, leaf number per seedling, and average leaf length, while fertilization on flowering sites had no significant effect on seedling growth, suggesting a saturation of resources. This study suggested that the characteristics of bamboos and bamboo stands were dramatically altered during this flowering event, enhancing seedling establishment and growth, and supporting mostly the habitat modification hypothesis of delayed reproduction.

Keywords: biomass, clonal, delayed flowering, energy allocation, gregarious, habitat modification, predator satiation

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INTRODUCTION

Many bamboo species are semelparous, having an unusual life history including a long clonal growth phase (sometimes over 100 years), followed by mass synchronous flowering and subsequent death (Janzen, 1976; Tian, 1987; Qin et al., 1989; Taylor et al., 1991). The long inter-mast period has hindered documentation of stand changes (Widmer, 1998; Marchesini et al., 2009; Abe and Shibata, 2012; Austin and Marchesini, 2012) and a thorough understanding of bamboo regeneration mechanisms and patterns, because flowering events are relatively rare (but see Kakishima et al., 2011 and de Carvalho et al., 2013). Thus, the evolutionary selection for such long vegetative phases, and semelparity, in bamboos is still debated (Keeley and Bond, 1999; Saha and Howe, 2001; Franklin, 2004; Iler and Inouye, 2013). Bamboo forests also provide habitat throughout the world

(Franklin, 2004; Nath and Das, 2010), but sporadic die-off events result in the loss of habitat and diversity (Sertse et al., 2011) for several years threatening endangered species like the giant panda (Tian, 1987). In addition, bamboo flowering events are linked to heterospecific tree species and thus forest regeneration patterns (Kitzberger et al., 2007; Caccia et al., 2009; Montti et al., 2014; Caccia et al., 2015). For these reasons, it is imperative to advance our understanding of the patterns and mechanisms of bamboo flowering events. In this study, we examined changes of stand structure (*habitat modification hypothesis*), seed bank dynamics and seedling regeneration (*seed predator hypothesis*), and culm characteristics/energy allocation (*resource hypothesis*) during a flowering event of *Fargesia qinlingensis* T.P. Yi and J. X. Shao in order to better understand the biology of gregarious-flowering, semelparous long-lived bamboos, and subsequently manage for habitats of endangered species following die-offs (Nath and Das, 2010).

One theory for delayed reproduction is based on habitat modification. For example, increased soil nutrients may play a role in seedling performance following bamboo flowering, as in temperate forests dominated by *Sasa kurilensis* in Japan (Abe et al., 2001, 2002) and by *Bashania fangiana* in China (Taylor and Qin, 1988). Such resource change is at the heart of the intraspecific competition hypothesis (Janzen, 1976; Gadgil and Prasad, 1984; Taylor et al., 1991; Franklin and Bowman, 2003), which explains the long clonal phase of bamboos as habitat modification. Stearns (1980) habitat modification theory suggests delayed flowering is selected for when adult longevity changes environmental conditions such that successful seedling recruitment is increased. Bamboo expands vegetatively over a long period of time sequestering a large spatial patch, then flowers and dies, leaving ample resources and little competition in its wake for seedlings to germinate and grow. In addition, the sheer size of the genet may also play a role. The longer the clone waits, the larger the genet, and larger genets tend to produce more seed (Matsuo et al., 2014).

Another mechanism of delayed reproduction may be seed predation (e.g., rodent outbreaks following flowering; Jaksic and Lima, 2003), the so-called predator satiation hypothesis (Janzen, 1976; Gadgil and Prasad, 1984). Based on this hypothesis, a higher seedling establishment probability would be expected during gregarious flowering events because not all seeds are consumed. Kakishima et al. (2011) found evidence for selection of a 6-year semelparous cycle of *Strobilanthes flexicaulis* via pollinator activity and predator satiation; i.e., pollination was more likely and seed predation less likely when gregarious flowering occurred. Kitzberger et al. (2007) also found lower *Chusquea culeou* seed predation rates in flowered areas versus those that had not flowered, as did Abe et al. (2001, 2002) for *S. kurilensis* and Caccia et al. (2015) for *C. culeou*. One argument against the predator satiation hypothesis is that there is no clear selection force that would result in such long periods (i.e., multiple decades) of clonal growth (Keeley and Bond, 1999). In addition, there is mounting evidence of small-scale flowering events of bamboo species (Miyazaki et al., 2009; Kitamura and Kawahara, 2011) that may fail to satiate predators.

A third mechanism put forth to explain long vegetative periods prior to flowering is the resource hypothesis (Gadgil and Bossert, 1970), which suggests that a period of time is needed to acquire the necessary resources for flowering, and that the resources are exhausted during the flowering event. The latter explains the death of culms and how plants sacrifice growth for the sake of reproduction (Abrahamson and Caswell, 1982). The energy allocated to reproduction gradually increases as buds become flowers, flowers are fertilized and seeds mature (Harper and Ogden, 1970). Lupine [*Lupinus nanus* ssp. *latifolius* (Benth.) D. Dunn.] distributes up to 61% of its energy to reproductive tissues and 29% to seeds (Pitelka, 1977), but such studies are lacking for bamboo. One study shed a bit of light. Miyazaki et al. (2009) was able to show that non-flowering culms transferred carbon to flowering culms, suggesting an energy need was being met through physiological integration of the clonal bamboo. The increase of energy to reproductive organs may thus decrease energy allocated to vegetative organs, and it is reasonable to predict that bamboo may allocate a large proportion of energy to reproduction during flowering that leads to culm death.

The success of seedling establishment and growth relies on both biotic (birds, mammals, insects, etc.) and abiotic (light, water, nutrients, etc.) factors (Grubb, 1977; Harper, 1977; Grime, 1979). Several studies have highlighted the effects of flowering on both bamboo seedlings and heterospecific tree species. For some bamboo species, restoration of bamboo forest relies exclusively on regeneration from seeds after bamboo flowers and dies, because rhizomes die after culms flower (Tian, 1987; Keeley and Bond, 1999); although, this may not include the entire genet (Miyazaki et al., 2009). Whether large-scale gregarious flowering or small-scale events, bamboo regeneration is clearly greatest in the gaps left behind by flowering and subsequent death of culms (Marchesini et al., 2009).

The positive response of bamboo seedlings to flowering seems mainly driven by an increase in light to the forest floor following die-off of culms. For example, *S. kurilensis* and *C. ramosissima* seedling growth was positively related to increased light levels but showed little response to other environmental variables (Makita, 1992; Montti et al., 2011). Makita (1992) described the post-flowering regeneration response as three phases: establishment, density-stable, and thinning, where ramets eventually die due to competition with neighboring ramets.

The flowering of arrow bamboo *F. qinlingensis*, one of giant panda's main food resources in the Qinling Mountains, offers a valuable opportunity to examine delayed flowering mechanisms in bamboo. We monitored the flowering event for 5 years, with an analysis of energy availability in flowered and vegetative culms to elucidate exhaustion of resources, and a nutrient addition study to examine habitat modification.

Our objectives were to: (1) document bamboo internal energy resources during flowering, (2) document bamboo regeneration following die-offs, and (3) add evidence toward our understanding of selection for delayed flowering. In reference to hypotheses of delayed flowering, we expected seedlings to grow better in sites that had flowered and died with little advantage from additional fertilization, supporting mostly a habitat modification hypothesis.

MATERIALS AND METHODS

Study Area

This study was carried out at the Taibaishan National Natural Reserve (TNNR, 33° 49′ 30″ – 34° 05′ 35″ N, 107° 22′ 25″ – 107° 51′ 30″ E) in the Qinling Mountains, Shaanxi Province, China (**Figure 1**). TNNR is in the southern end of the warm temperate zone with four distinguished seasons. It is the northern range of giant panda's distribution. Mean annual temperature is 8.4°C; – 4.2°C in January and 20.4°C in July. Precipitation averages 945.5 mm/year with 50% falling between July and September. The soil in the flowering area is rhogosol brown soil (pH 6.2) that forms from granite. The lower part of the soil is semi-weathered parent material with pH 6.5.

Vegetation is diverse due to the coexistence of both northern and southern Chinese taxa. Forest communities cover 81% of TNNR, made up of four general vegetation types along an elevation gradient: oak forests (1500–2000 m), birch forests (2000–2500 m), coniferous forests (2500–3540 m), and alpine shrublands and meadows (3450–3700 m). Two main bamboo species are distributed in Qinling Mountains; *F. qinlingensis* predominates at higher elevations (1700–3000 m) and *B. fargesii* (Camus) Kenget Yi at lower elevations (900–1900 m).

Study Species

Fargesia qinlingensis has a pachymorph rhizome (Li et al., 2003), and its culm can grow up to 3.6 m in height and 13 mm in basal diameter. New shoots regenerate from rhizomes in May and June; during flowering years, flowers appear in April and seeds are set in June. Like most other bamboos in tropical and temperate regions (Janzen, 1976; Gadgil and Prasad, 1984), F. qinlingensis is a perennial monocarpic species which is known for its long period of vegetative growth followed by a mast seeding; it has a seeding cycle of ca. 50 years (Tian, 1987). In TNNR, sporadic flowering of F. qinlingensis began in 1999 in a watershed and mast flowered throughout this watershed in an area of ca. 300 ha from 2001 to 2003 (Yue and Li, personal communication). The temporal gregarious flowering with a few early, a few late, and most flowering during 1 year has been documented in other bamboo species (Abe and Shibata, 2012; de Carvalho et al., 2013). A small amount of un-flowered patches of mature culms, approximately elliptical in shape, grew within a matrix of dead and flowering culms. Similar mosaic patterns of live and dead culms have been reported for several other bamboos (Schaller et al., 1985; Taylor and Qin, 1987, 1988; Johnson et al., 1988; Reid et al., 1989; Taylor et al., 1991; Widmer, 1998; Marchesini et al., 2009; Mizuki et al., 2014).

Field Methods

Three sites with un-flowered patches were randomly selected for study. Unfortunately, un-flowered patch of site 2 flowered in 2003 and 2004, but only a few sporadic stems (i.e., it does not match the flowering sites we chose). Due to low sample size, we chose to maintain this site as a non-flowering patch and consider our analyses as conservative. The length and width of each patch was measured, and area calculated by using an elliptical formula. The areas of the three un-flowered patches were 240.33, 66.76, and 159.06 m^2, respectively. The three sites were within a flowering area of about 10 km^2 with elevation from 1850 to 1930 m, had similar topographic characteristics, canopy cover, tree composition and bamboo cover (**Table 1**). Sites ranged from 1 to 3 km apart. Herb cover was greatest in site 1. All three sites were in a broad-leaved forest mixed with a very small proportion of conifers. There were clearly defined layers of canopy, sub-canopy, and understory. Dominant canopy species consisted of *Quercus baronii* Skan, *Q. aliena* Bl. var. *acuteserrata* Maxim, and *Carpinus turczaninowii* Hance. Species in the shrub layer mainly included *Morus alba* Linn, *Lespedeza bicolor* Turcz, and *Symplocos paniculata* (Thunb) Miq. The understory herbaceous layer was mainly formed by *Oxalis acetosella* Linn, *Hippochaete hiemale* (Linn) Borner, and *Smilax riparia* A. DC, with *F. qinlingensis* dominating.

Habitat Modification Experiment

Six 1 m × 1 m plots were randomly set up in each of the three un-flowered patches. Another adjacent six 1 m × 1 m plots were randomly set up in each flowering area. Plots were at least 5 m apart. In each 1 m × 1 m plot, basal diameter of each culm (i.e., ramet) was measured to its nearest 0.01 mm in October 2003, and re-measured over the next 4 years (2004–2007). Culms were divided into four categories: new shoots, flowering culms, live culms, and dead culms. New shoots (<1 year old) and culms (>1 year old) specifically refer to vegetative reproduction, not seedlings. Seedlings have weak structure and more branches while new shoots are sturdier; seldom have braches, and adjacent to older bamboo shoots.

Total seedlings, all new shoots from seed, of *F. qinlingensis* were counted in each 1 m × 1 m plot from 2004 to 2007. No seedlings were established in 2003 or after 2004 (i.e., seedlings only emerged in 2004). In 2004–2007, ten randomly selected seedlings, or all seedlings if there were less than 10 seedlings in the 1 m × 1 m plot, were measured for height and leaf length to the nearest 0.1 cm.

In the fall of 2006, we performed a fertilization experiment with 1 m × 1 m plots (six per treatment) in a randomized block design with four treatments (+N, +P, N + P, and no fertilizer addition) in each flowering site (sites were blocks). This research examines one aspect of the habitat modification hypothesis: we would expect fertilization to have no effect if the die-off already provides ample resources for seedling recruitment, survival and growth. Measurements of bamboo seedlings (taken at the start and 1 year later in 2007) included density, height, number of branches, number of leaves, and the length of the three longest leaves (subsequently averaged). We calculated plot averages of each variable prior to running analyses, and analyzed the change over the 1 year period.

Seed Predation Experiment

Twenty flowering culms were randomly selected from each of the three flowering sites in October 2003; $N = 60$. Florets and actual seeds on each culm were counted. In addition, five 0.5 m × 0.5 m plots were randomly set up in each flowering site. Soil of 10 cm

FIGURE 1 | Study site location.

TABLE 1 | The characteristics of three un-flowered patches of *Fargesia qinlingensis* at the Taibaishan National Natural Reserve, Shaanxi Province, China.

Patch	Area (m^2)	Slope steepness (°)	Slope aspect (°)	Elevation (m)	Canopy cover (%)	Canopy height (m)	Herb cover (%)	Bamboo cover (%)	
								Non-flowering site	Flowering site
1	240.33	26	234	1850	85	18	70	75	45
2	66.76	18	162	1910	80	18	30	70	50
3	159.06	30	180	1880	70	17	25	60	45

deep in each plot was extracted and all viable seeds (with white embryo) were counted. We also randomly selected and marked 10 culms that started to flower in 2003 in each flowering site. They were revisited in August 2004 and June 2005 to determine survival.

Resource Experiment

In October 2003, ten flowering culms from each flowering site and un-flowered patch ($n = 10$; $N = 60$) were randomly collected, sorted into culm parts (stems, branches, and leaves; one sample of each treatment of rhizomes and seeds), and then dried for analysis. Dried culm samples were weighed and randomly split in two subsamples to garner enough material for chemical analyses (two sets of analyses). Chemical constituents in each culm part were analyzed at Mississippi State University, providing dry matter percentage of organic matter (OM), crude protein (CP), neutral detergent fiber (NDF; lignin, cellulose, and hemi-cellulose), acid detergent fiber (ADF; lignin and cellulose), hemi-cellulose (HC = NDF – ADF), and extract ether (EE; crude fat). CP and EE represent energy reserves in the plant while other variables represent structural components.

Data Analysis

Habitat Modification Experiment

We used a repeated measures ANOVA in a randomized complete block design to test the flowering effect (two levels: flowering site and un-flowered patch) and time effect (five levels: 2003, 2004, 2005, 2006, and 2007) on the density of live culms. Plots within patches were averaged and patches considered replicates. Flowering and time were considered fixed effects. If there was a significant interaction between the flowering effect and time effect, a multiple comparison with Bonferroni adjustments was performed. Density of seedlings, and height, number of leaves and average length of longest leaf of individual seedlings were analyzed with a similar repeated

measures analysis (using blocks as replicates), except that the time treatment had only four levels (2004–2007). These analyses were performed using the GLM procedure in SAS (SAS Institute, 2013).

For the fertilization experiment, a blocked (by site) MANOVA followed by ANOVAs examined the effects of fertilization on plant growth (change from 2006 to 2007) for stem height, number of branches, number of leaves, leaf length, and seedling density using the GLM procedure in SAS (SAS Institute, 2013).

Resource Experiment

A randomized complete block design with two replications in each experimental unit (bamboo culm) was analyzed by using the MIXED procedure in SAS/STAT (SAS Institute, 2013) to test for flowering effect (two levels: flowering site and un-flowered patch) and culm part effect (three levels: leaf, branch, and culm) on the six chemical constituent variables (OM%, CP%, NDF%, ADF%, HC%, and EE%). Two main effects (flowering and culm part) were considered fixed, and block effect was considered random. If there was a significant interaction between the two main effects, a multiple comparison with Bonferroni adjustments was performed to seek significant differences among six treatment combinations (flowering/stem; flowering/leaf; flowering/branch; un-flowered/stem; un-flowered/leaf; un-flowered/branch) for each dependent variable. These analyses were performed using the MIXED procedure in SAS (SAS Institute, 2013). Percentage data were arcsin square root transformed prior to analysis to meet the assumptions of normality and homogeneity of variances. For all analyses, differences were considered significant at $P < 0.05$ level.

RESULTS

Habitat Modification Experiment

Dynamics of Density and Characteristics of Culms

No new shoots regenerated from rhizomes in the flowering sites during the five studied years. However, new shoot regeneration occurred every year in the three un-flowered patches except site 2 that only flowered in 2003 and 2004 (**Figure 2**).

There was a significant effect of flowering on the density of live culms ($F = 14.09$, $P = 0.0199$), but no time ($F = 26.71$; $p > F = 0.1440$) or time*treatment effect ($F = 36.91$; $p > F = 0.1228$). Density decreased dramatically in the flowering site over the 5 years, but surprisingly, density of live culms in un-flowered patches also significantly decreased (**Figure 3A**). The density of live culms in un-flowered patches was significantly greater than that in flowering sites every year (**Figure 3A**). Four of 30 culms that started to flower in 2003 died in 2004; half of them (15 culms) were still alive in 2005, but none remained in 2006.

Number of flowering culms decreased from about 14/m^2 to zero within 5 years in the flowering sites, while un-flowered patches had limited flowering (<5 culms/m^2) in both 2004 and 2005 (**Figure 3B**). Live culm biomass decreased by about 86% in the flowering patches but only by about 52% in the un-flowered patches (**Figure 3C**).

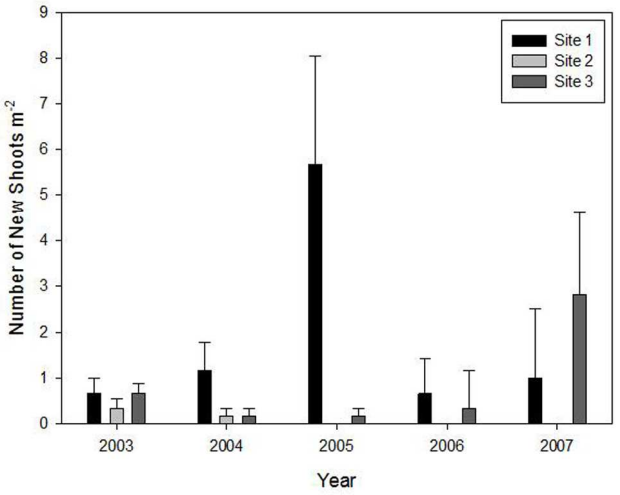

FIGURE 2 | Means (±1 SE) of density of new shoots of *Fargesia qinlingensis* in the three un-flowered patches (there were no new shoots found in flowering sites) during the 5-year period at the Taibaishan National Natural Reserve, Shaanxi Province, China.

Density and Characteristics of Seedlings

No seedlings were established until 2004. We found no significant effect of time and flowering interaction on either seedling density or average leaf length (**Table 2**). Both variables had a significant effect (greater in flowering areas; **Figures 4A,D**) and seedling density also decreased significantly over time (**Figure 4A**). Both seedling height (**Figure 4B**) and leaf number (**Figure 4C**) had a significant time*flowering effect, in that flowering and non-flowering areas showed a similar increase until the last time period when flowering site characteristics clearly diverged to being significantly greater (a trend also shown with average longest leaf). Survivorship from 2004 to 2007 was 32 and 40% in flowering and un-flowered patches, respectively.

Fertilization

No significant effects were found among fertilization treatments for change (growth between 2006 and 2007) in stem height ($F = 0.34$, $p > F = 0.7964$), number of branches ($F = 0.52$, $p > F = 0.6678$), number of leaves ($F = 0.45$, $p > F = 0.7177$), leaf length ($F = 0.69$, $p > F = 0.5603$), or seedling density ($F = 0.41$; $p > F = 0.7485$). Height growth increased by approximately 13 cm, number of branches and number of leaves increased by 4 and 12, respectively, and leaf length increased by 0.7 cm (**Figure 5**). Interestingly, ramet density also increased, averaging as little as 2.9 individuals m^{-2}in control plots, to 3.6, 5.4, and 6.6 in N, NP, and P plots, respectively.

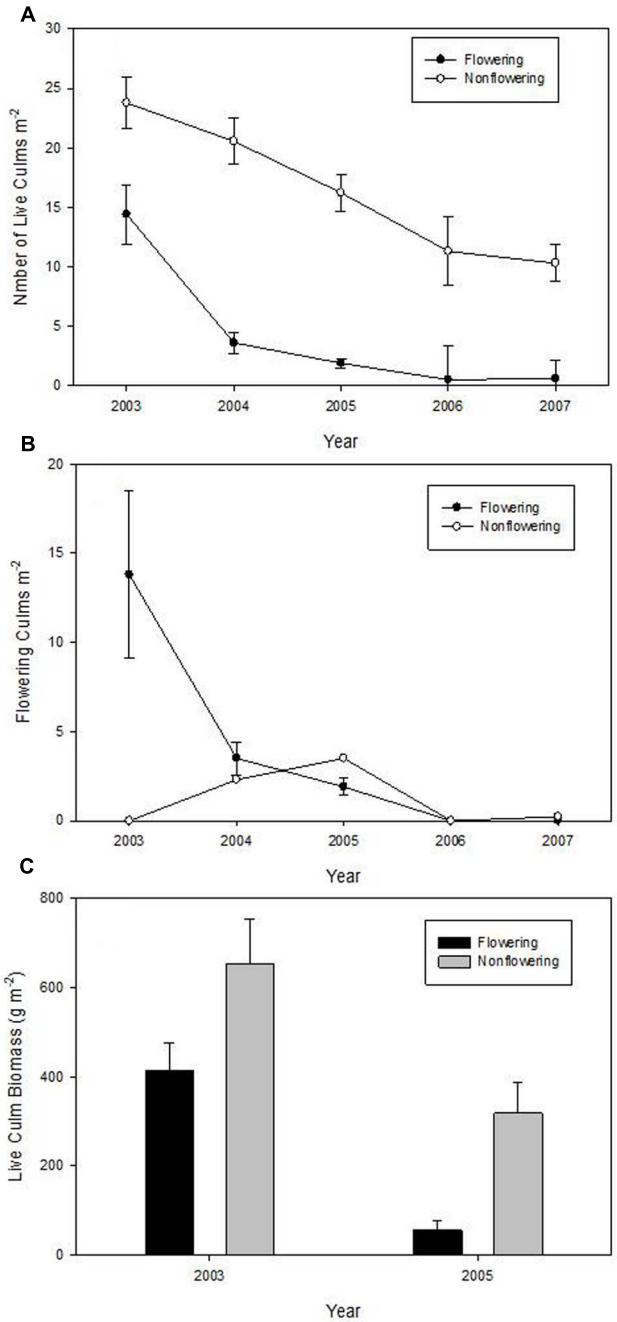

FIGURE 3 | Means (±1 SE) of the density of live culms of *Fargesia qinlingensis* in the flowering sites and un-flowered patches over 5-year period at the Taibaishan National Natural Reserve, Shaanxi Province, China. (A) Number of live culms, **(B)** flowering culms, **(C)** live culm biomass.

Seed Predation Experiment

Seed Density and Seedling Establishment

In the three flowering sites, average floret density (2687 ± 847 florets m^{-2}) was much greater than average density of actual seeds on culms (53 ± 20 seeds m^{-2}) and seeds in the soil seed bank (5 ± 2 seeds m^{-2}). Density of 1-year-old seedlings averaged 27 ± 2 seedlings m^{-2} (**Table 3**).

Resource Experiment

The interaction of flowering and culm part type was significant for all six chemical compounds (**Table 4**). OM% was significantly greater in flowering branches than in un-flowered branches (**Figure 6A**). Leaves contained much more CP% than branches and stems (16.1 and 13.0% compared to 5.4 and 4.2% in branch and 1.5 and 1.6% in stem for un-flowered culms and flowering culms, respectively), and CP% in leaves and branches was significantly greater for un-flowered culms than for flowering culms (**Figure 6B**). Two less-digestible fiber constituents (NDF% and ADF%) had the same trend: stems > branches > leaves. Fibers' percentages in stems were significantly greater for un-flowered culms than flowering culms, but the differences were not significant for branches and leaves (**Figures 6C,D**). Hemicellulose (HC%) was significantly different among culm parts: leaves > branches > stems, but there was no significant difference between flowering culms and un-flowered culms (**Figure 6E**). Leaves contained more crude fat (EE%) than branches and stems for un-flowered culms (13.9% compared to 12.5 and 10.4% in branches and stems), and crude fat in leaves and branches was significantly greater for un-flowered culms than flowering culms (**Figure 6F**).

Two rhizomes from a flowering site and an adjacent un-flowered patch were analyzed, and seeds from several flowering culms were also analyzed, rendering only enough material for one set of chemical constituent analyses each ($n = 1$). Greater amount of easily digestible crude fat and partially digestible hemicellulose were found in seeds compared to all other culm parts (**Figures 6E,F**). Chemical amounts in rhizomes were similar to branches and stems. Little difference was found between the flowered and un-flowered rhizome samples, but flowered rhizomes generally had lower chemical concentrations.

DISCUSSION

Understanding the dynamics of gregarious flowering events and their impacts on ecosystem structure and community dynamics is imperative for conservation management. In addition, the evolutionary basis of delayed and synchronized flowering is still debated and data are needed to compare contrasting views (Saha and Howe, 2001). We first answer general questions about the timing and structural changes during a *F. qinlingensis* flowering event and then interpret data in relation to hypotheses of delayed reproduction.

Flowering, Semelparity, and Reproduction

We first wanted to determine if flowering was gregarious. Flowering started in 1999 in the area, as documented by locals, but the peak flowering year was 2003 – word spread about the event which is what prompted us to begin our study. Our data documents much lower flowering occurrence since 2003. Thus, the gregarious flowering event can be

TABLE 2 | Repeated analysis of density, height, leaf number, and average leaf length of seedlings of *Fargesia qinlingensis* at the Taibaishan National Natural Reserve, Shaanxi Province, China.

Fixed Effects	Seedling Density		Seedling Height		Leaf Number		Leaf Length	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Flowering (F)	34.31	0.0042	27.52	0.0345	14.22	0.0637	35.26	0.0272
Time (T)	9.44	0.0018	5.28	0.0404	14.08	0.0040	3.16	0.1070
T × F	2.93	0.0769	4.93	0.0465	6.62	0.0248	2.94	0.1209

Variable flowering had two levels: flowering sites and non-flowering patches. Variable time had four levels: 1–4 years old.

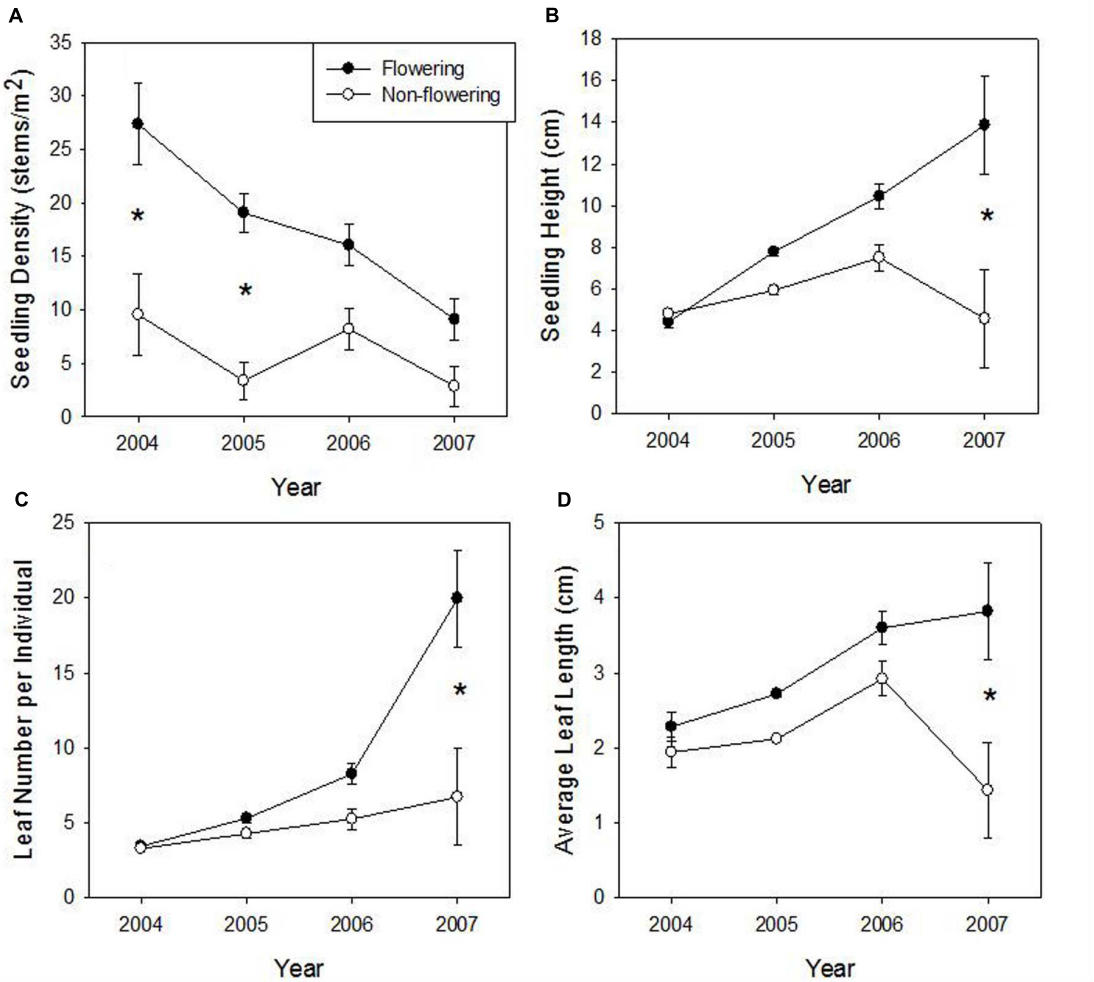

FIGURE 4 | Means (±1 SE) of density of seedlings (A) seedling height (B), seedling leaf number (C), and average seedling leaf length (D) at the Taibaishan National Natural Reserve, Shaanxi Province, China. Asterisks indicate significant difference in flowering and un-flowered sites for a particular year at $P < 0.05$ level with Bonferonni correction.

described more as an asymptote in time rather than a specific year, as has also been suggested by several other studies (Veblen, 1982; Gadgil and Prasad, 1984; Banik, 1995; Widmer, 1998; Kitamura and Kawahara, 2011) and is perhaps more typical than large-scale gregarious events for temperate bamboo species (Makita, 1998; Keeley and Bond, 1999).

We also wanted to document the semelparity of *F. qinlingensis*. We found that half of the flowering culms of *F. qinlingensis* survived for at least 2 years. A similar phenomenon has been reported for individuals of *S. veitchii* var. *hirsute* (Abe and Shibata, 2014) and *F. robusta* that lived for 2–3 years after flowering in the Min Mountains (Qin et al., 1993). In addition, new vegetative shoots stopped generating in flowering

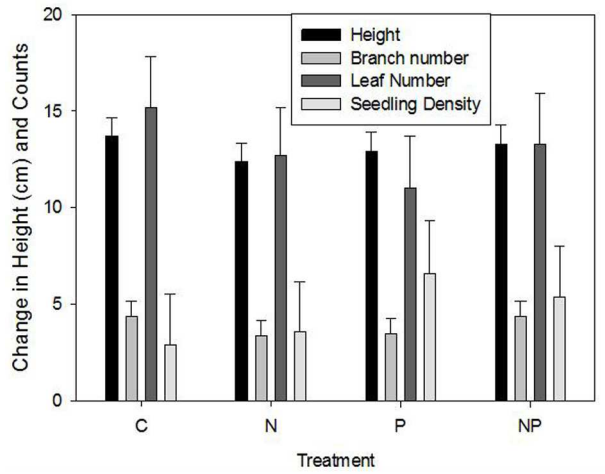

FIGURE 5 | Mean (±1 SE) height, branch number, leaf number, and seedling density for control (C), nitrate addition (N), phosphorus addition (P), and combined N + P addition. There were no significant differences.

sites, while in the nearby un-flowered patches, new shoots continued to regenerate every year, so rhizomes and roots had apparently died as well after flowering; thus, *F. qinlingensis* appears semelparous at least in relation to one flowering event. However, since we did not collect genetic data, we are unsure if we have genets flowering and dying or portions of genets, as shown with *Sasa* (Miyazaki et al., 2009), flowering and dying. For *Sasa*, non-flowering culms did not die and thus the genet was not semelparous. This remains to be tested for *F. qinlingensis*.

Our next goal was to document the timing of regeneration. Although flowering started from 1999, no seedlings were established until 2004, suggesting either delayed germination or much higher pollination success (greater number of seeds that escape predation) following the gregarious flowering peak in 2003 when we started data collection. No seedling establishment may be also due to seed limitation (no seed from rather poor seed production before 2003 arrived at the plots); predation or damage of the seeds (for instance by fungi) should also be contributory. Seeds of *B. fangiana* are reported to remain dormant for at least 2 years in the Qionglai Mountains (Taylor and Qin, 1988). Qin (1985) also reports that seeds of *F. scabrida* remain dormant in the soil up to 5 years in the Min Mountains. Recent work on *Chusquea* (González et al., 2002) and *Sasa* (Kitamura and Kawahara, 2011) also identified multi-year dormancy. However, seeds of *F. qinlingensis* showed no dormancy characteristics in the lab, germinating any time of year under both light and dark conditions (Wang et al., 2007). The second alternative, that pollination success is much greater during the peak of gregarious flowering events, is also supported in the literature (Widmer, 1998; Kakishima et al., 2011). Measurements of cross-fertilization success recorded during non-flowering years (at least no gregarious flowering, only local individuals) were much lower (<7%) in *Sasa* species (Mizuki et al., 2014). Regardless of the driver, our data suggest masting promoted seed production and seedling success, as also shown by Abe and Shibata (2014).

Seedlings grew slow in this study. Three-year-old seedlings had heights of 10.5 cm and 7.5 cm in flowering sites and un-flowered patches, respectively. Allelopathy and micronutrients might be considered as a mechanism slowing down regeneration in non-flowering sites, but support for these mechanisms is lacking. Slow growth also was noted for *C. tomentosa* (Stern et al., 1999). Qin et al. (1989) report that 3-year-old seedlings of *B. fangiana* averaged only 6.7 cm high. Reid et al. (1989) report

TABLE 3 | Flower and seed characteristics of *Fargesia qinlingensis* from three flowering sites in the Taibaishan National Natural Reserve, Shaanxi Province, China, in October 2003.

Site	Total florets / culm (±1 SE)	Actual seeds/ culm (±1 SE)	Florets/m^2 (±1 SE)	Actual seeds on culms/m^2 (±1 SE)	Seeds in soil/m^2 (±1 SE)	Seedlings/m^2 (±1 SE)
1	183.5 (30.4)	3.8 (1.5)	3303 (843.6)	68.4 (17.5)	8.8 (2.9)	29.8 (5.2)
2	299.6 (39.9)	6.2 (1.6)	3745 (253.6)	77.5 (5.25)	5.2 (2.3)	28.7 (13.2)
3	242.9 (44.9)	3.0 (0.8)	1012.1 (211.9)	12.5 (2.6)	1.8 (0.8)	23.6 (9.6)
Average	242.0 (33.5)	4.3 (1.0)	2686.7 (847.0)	52.8 (20.3)	5.3 (2.0)	27.4 (1.9)

TABLE 4 | Summary of six mixed linear models for chemical compound variable of *Fargesia qinlingensis* collected from the Taibaishan National Natural Reserve, Shaanxi Province, China.

Fixed effects	*F* value					
	OM%	CP%	NDF%	ADF%	HC%	EE%
Flowering (F)	$6.44_{(1,2)}$	$15.04_{(1,2)}$	$2.96_{(1,2)}$	$0.47_{(1,2)}$	$0.00_{(1,2)}$	$23.28_{(1,2)}^{*}$
Part (P)	$37.32_{(2,26)}^{***}$	$1178.81_{(2,26)}^{***}$	$670.52_{(2,26)}^{***}$	$1019.37_{(2,26)}^{***}$	$346.81_{(2,26)}^{***}$	$48.53_{(2,26)}^{***}$
F × P	$3.64_{(2,26)}^{*}$	$9.11_{(2,26)}^{**}$	$28.04_{(2,26)}^{***}$	$27.25_{(2,26)}^{***}$	$7.33_{(2,26)}^{**}$	$24.33_{(2,26)}^{***}$

*OM, organic matter; CP, crude protein; NDF, neutral detergent fiber (ligning, cellulose, and hemi-cellulose); ADF, acid detergent fiber (ADF; lignin and cellulose); HC, hemi-cellulose (HC = NDF – ADF); and EE, extract ether (crude fat). Variable Flowering had two levels: flowered site and un-flowered site. Variable Part had three levels: leaf, branch, and culm. *P < 0.05, **P < 0.01, ***P < 0.001. Numbers in parenthesis represent numerator and denominator df.*

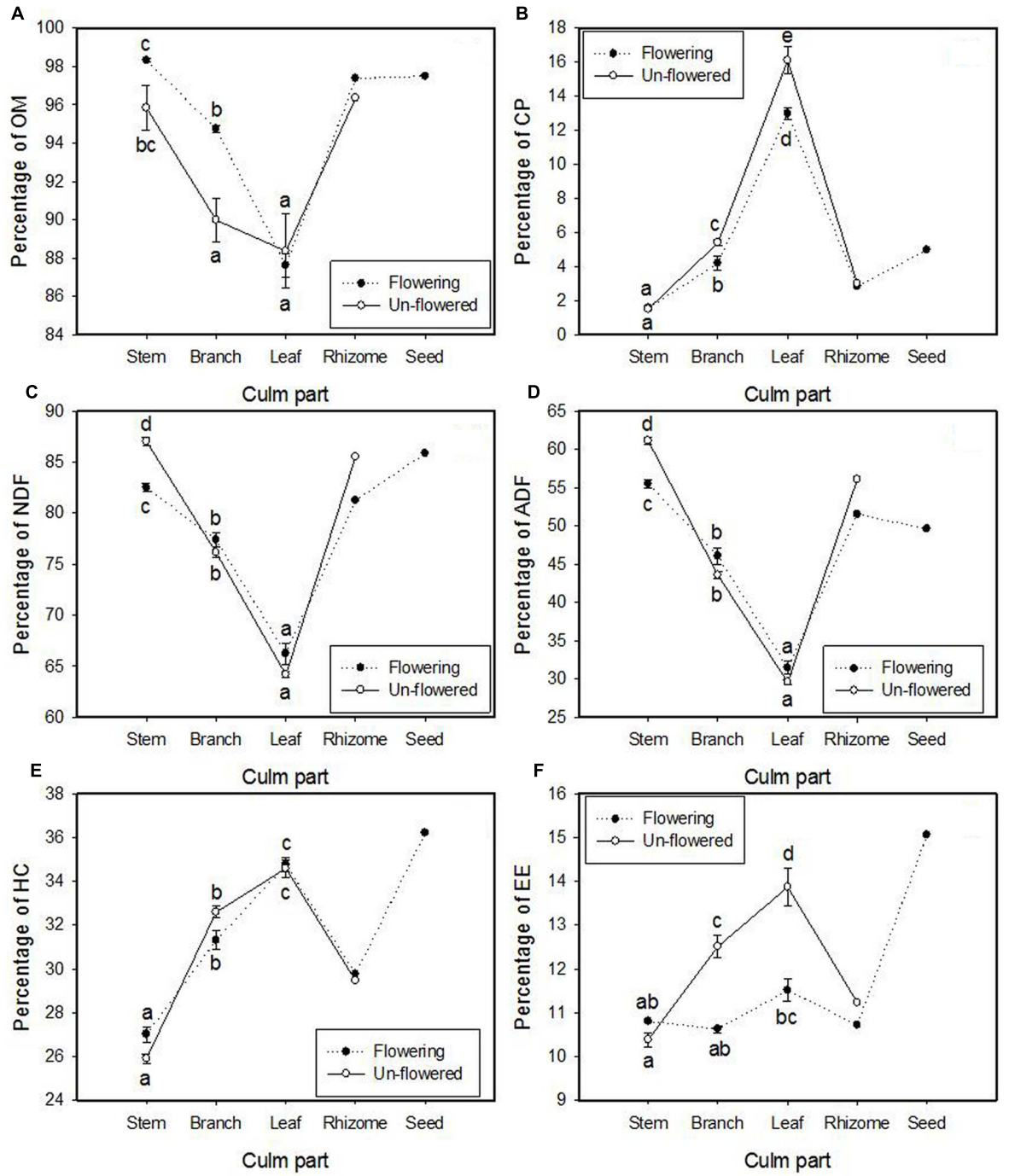

FIGURE 6 | The means (±1 SE) of treatment combinations for seven chemical compound variables: (A) organic matter (OM), (B) crude protein (CP), (C) neutral detergent fiber (NDF; lignin, cellulose, and hemi-cellulose), (D) acid detergent fiber (ADF) (lignin and cellulose), (E) hemi-cellulose (HC), and (F) ether extract (EE; crude fat) in culms of *Fargesia qinlingensis* collected from the Taibaishan National Natural Reserve, Shaanxi Province, China. Different letters indicate significant differences at $P < 0.05$ level. Two samples of rhizome (flowering and un-flowered) and one sample of seeds were also graphed for comparison but not included in analyses.

that *B. fangiana* 4-year-old seedlings were no more than 10-15 cm tall, matching 4-year-old seedlings in flowering sites of this study (14 cm). Researchers have estimated the time for bamboo seedlings to grow back to their original full size after a flowering event: 19 years for *F. qinlingensis* in the Qinling Mountains, Shaanxi, China (Tian, 1991); 15–20 years for *B. fangiana* and *F. scabrida* in the Qionglai Mountains, Sichuan, China (Qin et al., 1989; Taylor and Qin, 1993); 20 years for *S. kurilensis*

TABLE 5 | Predicted changes based on data collected from a *Fargesia qinlingensis* flowering event at the Taibaishan National Natural Reserve, Shaanxi Province, China, that would support hypotheses regarding delayed flowering.

Hypothesis	Flowering Culms	Seedling Survival	Seedling Growth	Culm Energetics	Vegetative Reproduction	+N, +P	Reproduction
Habitat Modification	Die	F > NF	F > NF	F≈NF	F = NF	**No effect**	Seed potential≈seed persistence
Resource	Die	F > NF	F > NF	**F < NF**	**F < NF**	Increased growth	Seed potential≈seed persistence
Predator Satiation	**Survive**	**F = NF**	**F = NF**	F≈NF	F = NF	Increased growth	**Seed potential > seed persistence**

*Predicted changes in bold represent unique responses for one hypothesis. *F indicates Flowering, and NF indicates Non-Flowering. If the main driver of delayed flowering follows the Habitat Modification hypothesis, the habitat after flowering should be more favorable (ample resources and little competition) for seedling survival and growth (F > NF), but we should not find significant effects on culm energetics, vegetative reproduction, and fertilizer addition. If the main driver of delayed flowering follows the Resource hypothesis, culm energetics and vegetative reproduction of Flowering should be less than that of Non-Flowering because all resources of the former will be used by flowering ramets. If the Predator Satiation hypothesis is the main driver of delayed flowering, the seed potential would be greater than persistence in Flowering areas but approximately equal in Non-Flowering areas while the performance of seedlings, culm energetics and vegetative reproduction should not be different.*

in the Hakkoda Mountains of northern Japan (Makita, 1992); and 16–19 years for *F. denudata* in the Min Mountains, Gansu, China (Huang, 1994). Data comparing *F. qinlingensis* areas that flowered in the early 1980s versus adjacent un-flowered areas (Wang et al. unpublished data) show little difference in density or culm diameter, suggesting the recovery occurs within 20 years following flowering in the Qinling Mountains. These remarkably similar estimates of recovery for temperate bamboo species may be used for management of bamboo forests.

Support for Hypotheses of Delayed Flowering

We expected seedlings to grow better in sites that had flowered and died with little advantage from additional fertilization. Our results show that flowering of *F. qinlingensis* significantly altered stand characteristics. Live culms dramatically declined in flowering areas from 2003 to 2007, and only a small amount of flowering culms remained in 2005. Surprisingly, live culms also decreased over the 5-year period in the un-flowered patches. One possible explanation is that some culms generate from rhizomes running from flowering areas, and indeed, some of the culms in un-flowered patches did flower in 2004 and 2005. Such small-scale spatial structure in flowering patterns have been shown in other bamboo species with genet-level flowering periodicity; i.e., different genets flower at different times (Mizuki et al., 2014) and even ramets within genets flower at different times (Miyazaki et al., 2009). A second explanation is a changed environment after culms die that affects neighboring un-flowered patches, such as increased light (Taylor and Qin, 1988), temperature fluctuations (Abe et al., 2001, 2002), and predator outbreaks (Jaksic and Lima, 2003).

Seedlings performed better in flowering sites than in un-flowered patches, but as expected the addition of nitrogen and phosphorus had no significant effects. Thus, the better growth of seedlings in flowering sites than in un-flowered patches likely resulted from the resource change, albeit we do not have resource change data, caused by death of culms and a saturation of resources in flowering sites (Taylor and Qin, 1988; Abe et al., 2001, 2002). Greater light levels especially (Makita, 1992; Marchesini et al., 2009) and reduction of mineral nutrient uptake from soils following rhizome death resulted in increased resources that could be used by bamboo seedlings in flowering sites (Taylor et al., 1991). Marchesini et al. (2009) also found higher survivorship of bamboo seedlings in senescent (after flowering) versus live sites. The better performance of seedlings in flowering sites suggests intraspecific competition (Janzen, 1976; Gadgil and Prasad, 1984; Taylor et al., 1991; Franklin and Bowman, 2003); few plants establish and survive beneath dense culms, because most resources for plant growth are used by established culms. Contrarily, Li et al. (2013) found the opposite trend for two *Fargesia* species, where dead culm litter apparently blocked seedling regeneration success. Thus, our data support the habitat modification hypothesis more so than other hypotheses.

We expected to find an exhaustion of resources in flowering culms that would support the resource allocation hypothesis. It has been shown that many plants sacrifice growth for the sake of reproduction (Abrahamson and Caswell, 1982; Iler and Inouye, 2013). The energy allocated to reproduction gradually increases as buds become flowers, flowers are fertilized and seeds mature (Harper and Ogden, 1970); and hence, allocation to vegetative organs decreases. We examine this hypothesis with the knowledge of vegetative propagation and allocation of energy within ramets. No new shoots were found in flowering areas even though culms were still alive. The reason why vegetative reproduction stopped when the sexual reproduction started is probably because of the reallocation of energy from culms to reproduction organs and subsequent death of culms; thus, allocation of all energy was to reproductive stems of *F. qinlingensis*.

Chemical constituents' allocation in different culm parts of *F. qinlingensis* were similar to other bamboos. Leaves of both flowering culms and un-flowered culms contained more CP than branches and stems. Similar results were reported in *Phyllostachys aureosulcata* McClure by Dierenfeld et al. (1982) and in *B. fangiana* by Qin et al. (1993). But CP% in leaves of these three bamboos is slightly different: *B. fangiana* (19.44%) > *F. qinlingensis* (16.1%, this study) > *P. aureosulcata* (13%). Two fibers' (NDF and ADF) allocation in *F. qinlingensis* had the same trend: stems > branches > leaves, and matching that of *F. scabrida*

(Schaller et al., 1990). Organic matter and hemi-cellulose also had the same trend (leaves > branches > stems) as *F. scabrida* (Schaller et al., 1990). Leaves of un-flowered culms had more crude fat than stems and branches, which is similar to *B. fangiana* (Qin et al., 1993), but crude fat content was much greater in *F. qinlingensis* (10.4, 12.5, and 13.9% in stems, branches, and leaves, respectively) than in *B. fangiana* (0.59, 2.4, and 3.4% in stems, branches, and leaves, respectively).

Organic matter in branches was greater in flowering culms than in un-flowered culms, while less-digestible fibers (NDF and ADF) in stems were greater in un-flowered culms than in flowered culms. CP and crude fat in leaves and branches were greater in un-flowered culms than in flowering culms. One explanation is that the allocation of energy to reproduction decreased energy allocated to vegetative modules as noted above (Leopold and Kriedemann, 1975). Crude fat had the greatest concentration in seeds, and both crude fat and CP were significantly less in branches and leaves of flowering plants, suggesting its reallocation. Albeit not extreme, significant differences (specifically crude fat and CP) always showed flowering culm amounts less than non-flowering culms and support a reallocation for reproduction. However, such energy allocation is true for iteroparous species as well, where there is not a complete exhaustion of resources that the hypothesis suggests. Our results corroborate such allocation based on lower percentages of especially CP and fat, but not complete exhaustion; thus, we do not consider our results as strong evidence for the resource allocation hypothesis.

Finally, we expected to find significant loss of seeds that would support the predator satiation hypothesis (Janzen, 1976). The number of seeds in the soil seed bank and in the culms was much less than the florets, suggesting that a large portion of seeds had been removed or eaten. Although most bamboos set large quantities of seeds (Janzen, 1976), seeds are preyed upon heavily by invertebrates (e.g., insects, Taylor and Qin, 1988; Zheng, 1994) and vertebrates (e.g., rodents and birds, Janzen, 1976). Therefore, our data on seed numbers may underestimate fecundity of *F. qinlingensis*, because rodent outbreaks often follow bamboo flowering (Gallardo and Mercado, 1999; Jaksic and Lima, 2003). We unfortunately do not know how many viable seeds were developed, but we cannot exclude the predator satiation hypothesis as a mechanism for delayed reproduction in *F. qinlingensis*.

Of course, these above hypotheses of delayed reproduction are not mutually exclusive as can be seen based on our predictions (**Table 5**). For example, by default of an exhaustion of resources, adult culms die and create the same patch for seedling reproduction as the habitat modification hypothesis. We found evidence for both hypotheses with further study: no effect of N and P fertilization supported the habitat modification theory and allocation of energy to reproduction supported the resource allocation theory. Of course, resource allocation can also occur in iteroparous species and the modest differences in energy between flowering and un-flowered culms do not strongly suggest an exhaustion of resources. Further, the apparently high loss of seeds supports the predator satiation hypothesis, although none of the other results suggest predator satiation as the main driver of delayed flowering. Taken together, the data most strongly support, and in fact never truly negate, the habitat modification hypothesis.

CONCLUSION

The characteristics of bamboos and bamboo stands were dramatically altered during this flowering event in terms of culm dynamics, seedling establishment, and chemical constituent allocation. We have shown evidence that allocation of energy toward growth is sacrificed for allocation toward flowering and fruiting, but the amount does not appear dramatic enough to fully support an exhaustion of resources hypothesis. Our data suggest slow seedling growth, enhanced growth in areas without a bamboo canopy, and a long regeneration time for bamboo stands, supporting the habitat modification hypothesis for semelparity. We have conflicting results regarding the predator satiation hypothesis. Seed dormancy would seem to argue against such a hypothesis, while the low number of regenerating individuals compared to the number of potential seeds from counted empty florets, and their being distributed into un-flowered areas, suggests predation was a factor. While we have no data on seed herbivory, we agree with Keeley and Bond (1999) that suggest delayed flowering for extended periods (c. 50 years for *F. qinlingensis*) seems too long for the sole purpose of off-setting herbivore populations. We also suggest that multiple forcing factors may have led to the development of delayed flowering, and that the more recent evidence that larger genets are more productive (Matsuo et al., 2014) should be added as a hypothesis.

AUTHOR CONTRIBUTIONS

WW: helped conceive research ideas, helped collect field data, helped analyze data, and write manuscript. ZL: helped conceive research ideas, helped collect field data, helped analyze data, and write manuscript. SF: helped conceived research ideas, helped with analysis, and writing manuscript. BR: helped conceive research ideas, performed all analysis of tissue energy, and edited manuscript.

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The Relative Importance of Genetic Diversity and Phenotypic Plasticity in Determining Invasion Success of a Clonal Weed in the USA and China

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Phenotypic plasticity has been proposed as an important adaptive strategy for clonal plants in heterogeneous habitats. Increased phenotypic plasticity can be especially beneficial for invasive clonal plants, allowing them to colonize new environments even when genetic diversity is low. However, the relative importance of genetic diversity and phenotypic plasticity for invasion success remains largely unknown. Here, we performed molecular marker analyses and a common garden experiment to investigate the genetic diversity and phenotypic plasticity of the globally important weed *Alternanthera philoxeroides* in response to different water availability (terrestrial vs. aquatic habitats). This species relies predominantly on clonal propagation in introduced ranges. We therefore expected genetic diversity to be restricted in the two sampled introduced ranges (the USA and China) when compared to the native range (Argentina), but that phenotypic plasticity may allow the species' full niche range to nonetheless be exploited. We found clones from China had very low genetic diversity in terms of both marker diversity and quantitative variation when compared with those from the USA and Argentina, probably reflecting different introduction histories. In contrast, similar patterns of phenotypic plasticity were found for clones from all three regions. Furthermore, despite the different levels of genetic diversity, bioclimatic modeling suggested that the full potential bioclimatic distribution had been invaded in both China and USA. Phenotypic plasticity, not genetic diversity, was therefore critical in allowing *A. philoxeroides* to invade diverse habitats across broad geographic areas.

Keywords: ***Alternanthera philoxeroides*, common garden experiment, genetic diversity, invasive species, molecular marker, phenotypic plasticity**

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INTRODUCTION

Since Charles Elton published his classic book on biological invasions in 1958, ecologists have been seeking to determine the factors that make a species an aggressive invader (Williamson, 1996; Nentwig, 2007; Van Kleunen et al., 2015). The ability of alien species to cope with new and heterogeneous environments is essential for their successful establishment in areas outside

their native ranges. Phenotypic plasticity, where one genotype can express different phenotypes, is frequently proposed as a characteristic that allows invaders to maintain components of fitness (e.g., growth, survival or fertility; Parker et al., 2003; Richards et al., 2006; Geng et al., 2007a) and ultimately overall fitness (Pichancourt and Van Klinken, 2012) in heterogeneous environments. Another hypothesis is local adaptation by post-invasion evolution (Lee, 2002; Maron et al., 2004; Colautti and Lau, 2015). In this scenario, rapid selection of adaptive genotypes, often facilitated by high levels of genetic diversity, can result in local adaption within the invaded range (Sakai et al., 2001; Lavergne and Molofsky, 2007; Xu et al., 2010a; Barrett, 2015). Both phenotypic plasticity and genetic diversity are effective in generating phenotypic variation in natural populations. Notably, these two mechanisms are not exclusive (Moroney et al., 2013; Si et al., 2014) and it is the total adaptive phenotypic variation, either due to phenotypic plasticity or due to genetic diversity, that will affect the realized performance of alien species in heterogeneous environments (Sultan, 1995; Falconer and Mackay, 1996). Indeed, phenotypic plasticity itself can be the target of natural selection and go through rapid evolution during the different phases of biological invasion (Lande, 2015). Many studies have highlighted the effects of local adaptation or phenotypic plasticity on invasiveness of alien species (Bossdorf et al., 2005; Davidson et al., 2011; Dlugosch et al., 2015), but few have examined the two factors simultaneously. As a result, the relative importance of the two adaptive strategies for invasive species remains largely unknown (Barrett, 2015; Bock et al., 2015).

Clonality is also proposed as an important characteristic in invasive alien plants (Pyšek, 1997). Alien plant populations have higher frequencies of clonality than native species and some of the world's most damaging invasive plants are clonal species (Silvertown, 2008). Furthermore, some clonal plants can occupy disturbed and dynamic habitats across broad geographic distributions (Geng et al., 2007a; Ganie et al., 2015). However, for clonal species, many of the physiologically separated individuals are asexual offspring of the same genet and thus share a common genotype (Ellstrand and Roose, 1987; Silvertown, 2008). Theory predicts that clonal plants will only evolve slowly, making local adaptation more difficult to occur (Barton and Charlesworth, 1998; Silvertown, 2008). Phenotypic plasticity is therefore likely to be an important mechanism allowing clonal species to rapidly invade new and diverse environments (Riis et al., 2010; Keser et al., 2014; Roiloa et al., 2014).

Alternanthera philoxeroides is native to South America and has become a problematic species in more than 30 countries (Holm et al., 1997). Interwoven stems can form large, dense monocultures, displacing native vegetation, blocking waterways, and causing significant economic impacts to agriculture (Wang and Wang, 1988; Sainty et al., 1998). In the introduced ranges (e.g., Australia, China, and the USA), *A. philoxeroides* rarely produces viable seeds, reproducing mainly through vegetative structures such as roots and broken stems (Julien, 1995; Holm et al., 1997; Dong et al., 2012). Clonal integration among different ramets is proposed as a mechanism that allow *A. philoxeroides* to colonize habitats that are spatially heterogeneous at fine scale (Liu et al., 2008; Wang et al., 2009; Yu et al., 2009; Xu et al., 2010b; Guo and Hu, 2012; You et al., 2014).

In China *A. philoxeroides* is widely distributed but genetically uniform DNA markers suggest genetic diversity is extremely low (Xu et al., 2003; Ye et al., 2003). This is consistent with its dominantly clonal reproduction. Despite this, *A. philoxeroides* occurs in diverse habitats in China, from fully aquatic (e.g., rivers, reservoirs) to terrestrial (e.g., roadside dry lands), and shows prominent phenotypic variation (Pan et al., 2006). Also, phenotypic plasticity, rather than local adaptation, is responsible for the phenotypic variation with relation to different water availabilities (Geng et al., 2007a). An interesting question is whether the species niche of *A. philoxeroides* in China is mainly determined by phenotypic plasticity and is not limited by low levels of genetic diversity. So far it is not known how the levels of genetic and phenotypic diversity observed in China relates to that present in it's native range and other introduced ranges. Direct comparison of the native and introduced clones is needed to determine the relative importance of genetic diversity and phenotypic plasticity during biological invasions.

In this study, we conducted a series of intercontinental comparisons using *A. philoxeroides* clones collected from both native (Argentina) and two introduced ranges (the USA and China). Our major aim was to examine the relative importance of phenotypic plasticity and genetic diversity in determining invasion extent of *A. philoxeroides* in the USA and China. Molecular marker analyses and a common garden experiment were performed to compare the genetic diversity and phenotypic plasticity of *A. philoxeroides* among the three regions. We expected that the genetic diversity in the introduced ranges was lower, and phenotypic plasticity was higher, than in native range. In addition, we used a bioclimatic model fitted against native range distribution data to examine whether the full potential distribution of the species in the introduced ranges were invaded. If genetic diversity had played an important role in determining the niche range of *A. philoxeroides,* we expected that the lower levels of genetic diversity would limit its potential distribution in the introduced ranges.

MATERIALS AND METHODS

Study Species and Sampling

Alternanthera philoxeroides (Mart.) Griseb., alligator weed, is a perennial stoloniferous herb. It can thrive in both terrestrial and aquatic habitats (**Figure 1**). High biomass allocation to root is an important factor determining the performance of *A. philoxeroides* in terrestrial habitats (Wilson et al., 2007; Geng et al., 2007a), including regeneration where cold winters damage most above-ground parts (**Figure 1**). In contrast, regeneration in aquatic habitats relies mainly on stems (**Figure 1**).

A. philoxeroides is native to South America and has invaded many tropical and subtropical areas across the world (Holm et al., 1997). In Argentina, *A. philoxeroides* is mainly distributed along the Rarana and Uruguay rivers in the north and along the San Borombon and Salado rivers in the center of Buenos Aires province (Sosa et al., 2003; **Figure 2**). In the USA, *A. philoxeroides* is distributed in several states in the southern coastal plains from

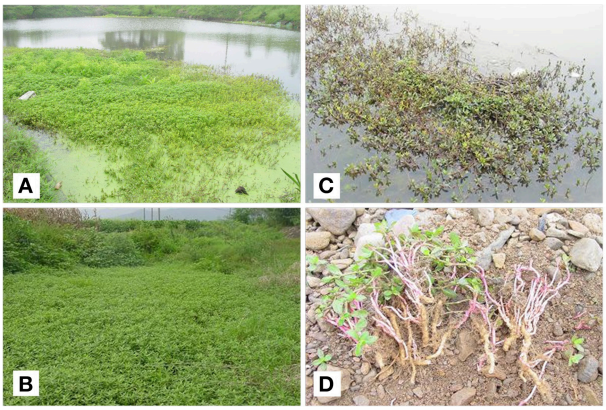

FIGURE 1 | *Alternanthera philoxeroides* invades diverse habitats in China and shows different asexual life cycles. (A) Monoculture in aquatic habitat in late summer; **(B)** Monoculture in terrestrial habitat in late summer; **(C)** New shoots grow from underwater stems in aquatic habitat in spring; **(D)** New shoots grow from underground storage roots in terrestrial habitat in spring.

Virginia to southern Florida, and westward along coastal areas to Texas and California (**Figure 2**). In China, *A. philoxeroides* is widely distributed, including in most provinces south of the Yellow River (**Figure 2**).

A total of 179 *A. philoxeroides* specimens were sampled from its distribution in Argentina (7 sites), the USA (9 sites), and China (9 sites) (Table S1, **Figure 2**). Specimens at a site were sampled from at least 10 m apart. For each individual, a stem fragment or thickened root was sampled in field. These were grown in a greenhouse in China (Shanghai) for about 6 months before the common garden experiment was performed.

Molecular Marker Analysis

To compare the genetic diversity measured by neutral molecular makers, all field-collected samples were analyzed using Inter Simple Sequence Repeat (ISSR) markers, which have proven effective in discriminating different clones of *A. philoxeroides* (Ye et al., 2003). In brief, we extracted total DNA using the cetyltrimethyl ammonium bromide (CTAB) protocol from fresh leaves of *A. philoxeroides* grown in the greenhouse and performed PCR using ISSR primers from the University of British Columbia primer set nine. Eight primers (UBC no. 811, 813, 823, 835, 840, 841, 880, and 887) were selected to genotype *A. philoxeroides*. For each sample, at least two PCR amplifications were performed to evaluate the reproducibility of the bands obtained. Each reaction was carried out in a total volume of 20 μl mixture consisting of 20 ng of template total DNA, 10 mM Tris-HCl (PH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.7 mM primer, 1.5 unit of Taq polymerase and double distilled water. PCR was performed with an Eppendorf Mastercycler programmed for 5 min at 94°C followed by 40 cycles of 45 s at 94°C, 45 s at the appropriate annealing temperature (48–52°C), and 2 min at 72°C. The last cycle was 7 min at 72°C, followed by a 4°C soak. Amplification products were resolved by electrophoresis on 1.5% agarose gels buffered with 1 × TAE.

Common Garden Experiment

A common garden experiment (Fudan University, Shanghai; E121°29′-N31°14′) was conducted to compare phenotypic plasticity of *A. philoxeroides* from all sampled ranges in response to different water treatments (aquatic and terrestrial habitat). Each habitat consisted of four rectangular plots (15 × 2 m). The aquatic habitat was simulated using 1 m deep ponds while the terrestrial habitat was simulated with raised garden beds. The aquatic and terrestrial plots were spatially alternated with each other, with adjacent pairs considered as replicates (blocks).

Regional-level phenotypic plasticity was compared by randomly selecting one clone from each sampling site (i.e., 9 from the USA, 9 from China, and 7 from Argentina). As no plants produced seeds in the greenhouse, thick root fragments were used to produce asexual plants as experimental replications. For each of the 25 clones, eight asexual plants with two pairs of leaves grown in pots (30 cm in diameter and 35 cm in depth, containing 1:1 mixture of loam and sand) were allocated randomly to eight plots (i.e., 2 water treatments × 4 replicates) to give a total of 200 pots. Aquatic plants were monitored daily to ensure that the water level remained nearly 2 cm above the pots in ponds. In terrestrial plots, plants received natural precipitation (1200 mm/year) plus supplementary irrigation in continuous sunny days (1L/pot when surface soil in >50% pots are dry).

Plants were harvested after 2 months of growth, which was before any flowers appeared. First, six morphological and physiological traits were measured following the protocol reported in Geng et al. (2007a): (1) leaf length, (2) stem diameter, (3) stem pith cavity diameter, (4) internode length (5) specific leaf area (SLA), and (6) relative chlorophyll content (measured using a chlorophyll meter, Minolta SPAD-502) which gives a value that is well correlated with chlorophyll content. In addition, each individual was separated into four parts: leaves, stems, thick storage roots, and fine roots (i.e. roots with diameter less than 1 mm). All plant materials were oven-dried at 80°C for 48 h and weighed. Then, two allocation traits were obtained, root/shoot ratio and storage root/fine root ratio. The whole experiment was performed in a closed garden equipped with weed mat to prevent plants from escaping into the field.

Data Analyses

Analysis of Genetic Variation in Molecular Markers and Quantitative Traits

Genetic diversity was assessed both by neutral molecular markers (then referred to as marker diversity) and quantitative traits under common garden conditions (then referred to as quantitative variation).

In the molecular marker analysis we recorded ISSR bands as present (1) or absent (0) for each sample. Bands of the same molecular weight were considered to represent the same allele at a given locus. This dataset was analyzed in two ways. First, we used Popgene 1.32 (Yeh et al., 1999) to examine the genetic diversity measured by molecular markers at a regional level, using the following genetic variables: the percentage of polymorphic loci (*P*), the Nei's genic diversity index (*He*), and the Shannon diversity index (*I*). We performed a re-sampling procedure to control the confounding effect of uneven sample

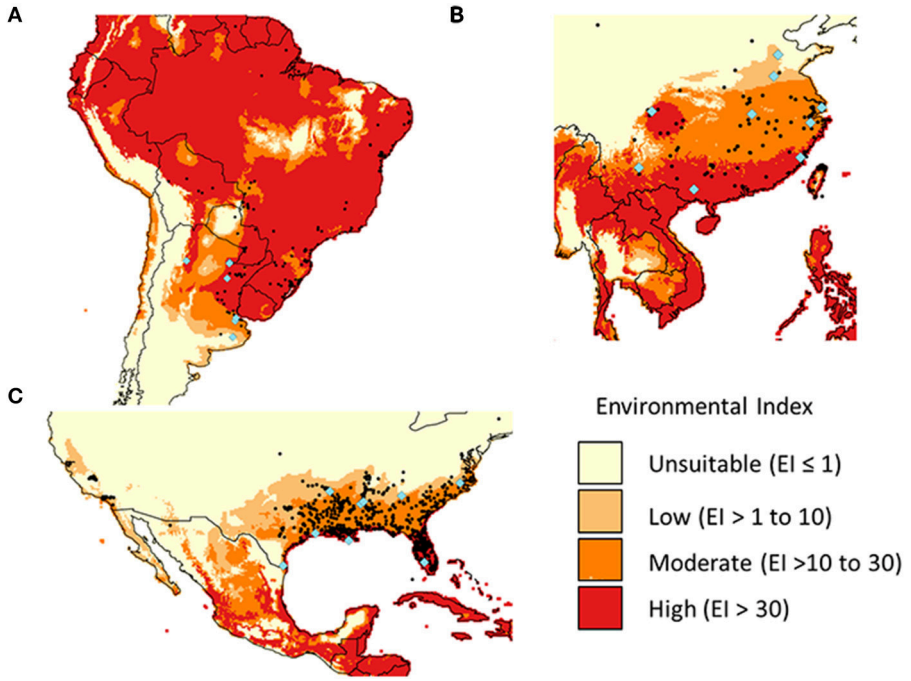

FIGURE 2 | The potential (Ecolimatic Index) and actual (black points) geographical distribution of *Alternanthera philoxeroides* in Argentina (A), China (B), and the USA (C). The bioclimatic model was fitted against the native-range distribution data. Sample sites are indicated by blue diamonds.

size (i.e., 21, 32, and 126 for Argentina, the USA and China, respectively). Specifically, we randomly sampled 21 individuals from the USA and China datasets respectively, from which we calculated the regional genetic parameters. This re-sampling procedure was then repeated 30 times, and the average value for each genetic variable based on the sub-dataset was reported along with average based on the whole dataset. Second, PAUP 4.0 (Swofford, 1998) was then used to determine the relationships among *A. philoxeroides* individuals from different geographical origins using neighbor-joining method. Estimates of similarity were calculated using the index of Nei and Li (1979). Bootstrap values for the neighbor-joining tree were calculated using 1000 replicated neighbor-joining searches.

For the quantitative traits in the common garden experiment, we calculated the coefficients of genetic variation (CVg, Houle, 1992) as our estimation of quantitative variation. For each region in each habitat, the coefficient of genetic variation is calculated as CVg = Sqrt (Vg)/M, where Vg was the genetic variance components among clones within a region, and M was the mean value of different clones within a region.

Analysis of Phenotypic Plasticity in Quantitative Traits

We used the dataset from the common garden experiment that simulated aquatic and terrestrial habitats to compare phenotypic plasticity between the three study regions (Argentina, China and the USA).

First, we examined the reaction norms at regional level by plotting the mean values of all clones from the same continent against two habitat treatments. We performed two-way nested ANOVAs to examine the effects of treatment, region, clone, and treatment-by-region interaction on each univariate trait, in which clone was nested in region as a random factor. The statistical model included the following terms: treatment, region, clone, treatment-by-region, treatment-by-clone, and error term. A significant effect of treatment suggests significant phenotypic plasticity of *A. philoxeroides* in terrestrial vs. aquatic growth conditions while the regional or clonal effect suggests differentiation of *A. philoxeroides* in phenotypic traits among different regions or clones. A significant treatment by region or treatment by clone interaction indicates that the level of phenotypic plasticity is different among regions or clones. We performed *F* tests by testing region effect over clone term; by testing both treatment and treatment-by-region effects over the treatment-by-clone interaction term; and testing treatment-by-clone effects over the error term. We first used Log (initial stem length) as a covariate to examine whether the covariate explained a significant amount of variation. When the covariate was not significant, we performed an ANOVA instead of ANCOVA, and examined the assumptions of homoscedasticity and performed data transformation where necessary.

Second, quantitative analyses of phenotypic plasticity were also performed based on the plasticity index (Schlichting, 1986). Specifically, for each trait of each clone, the plasticity index was calculated as: Ip = (Max (P1, P2) – Min (P1, P2))/Mean (P1, P2), where P1 and P2 were the average values of four replicates of the same clone under aquatic and terrestrial habitats, respectively. We performed one-way ANOVA on these indices to examine whether the effect of region was significant, with

clone as error term. When a significant region effect was detected, we conducted *post-hoc* comparisons based on Bonferroni test to examine whether or not the phenotypic plasticity of *A. philoxeroides* in China and USA was significantly different from those from Argentina. As all Chinese clones in common garden experiments proved to be the same multi-locus genotype (i.e., C-Dominant), the plasticity index for Chinese clones might not be independent to each other. To examine the potential effects of pseudoreplication, we also performed a nested ANOVA on plasticity indices, in which region was the main factor and clone was nested in region as a random factor. The overall mean plasticity for Chinese clones was used in this nested ANOVA. In addition we conducted multiple comparisons via *t*-tests. Specifically, the overall mean plasticity index for Chinese clones was used as a fixed value in two single-sample *t*-tests (i.e., USA vs. China mean and Argentina vs. China mean), separately. One two-sample *t*-test was performed when comparing the USA and Argentina. The results of one-way ANOVA, nested-ANOVA, and multiple *t*-tests were similar (see Supplementary Files). For simplification, we reported the result of one-way ANOVA in main text.

Third, to explore the effect of treatment on phenotypic correlation, we examined the Pearson's product correlations between paired traits in aquatic and terrestrial habitats, respectively. For each genotype, trait means were calculated per habitat. Based on the plasticity index, we examined the correlation of plastic responses across habitats among traits (i.e., plasticity integration). The critical probability levels for the correlation coefficients were Bonferroni corrected for multiple comparisons to $\alpha/36 = 0.0013$ (i.e., there were 36 paired comparisons).

Finally, to provide a multivariate perspective, we examined the overall phenotypic pattern of *A. philoxeroides* from different regions, using principal component analysis (PCA) conducted on the clone mean value of each trait ($n = 50$, 2 treatments $\times$ 25 clones). Trait data were standardized prior to PCA. We also performed constrained ordination analysis (e.g., redundancy analysis, RDA), in which two factors variables (i.e., treatment and region) were used as explanatory variables. The result of RDA was highly similar to that of PCA (see Supplementary Files). For simplification, we reported the result of PCA (biplot) in main text. All analyses were carried out with R (R Core Team, 2015).

Correlation between Genetic and Phenotypic Dissimilarity

To assess the correlation between molecular markers and quantitative traits, we further examined whether the differences among clones in their quantitative traits were related to their genetic marker dissimilarity. Specifically, we calculated the Euclidean distance matrix based on the quantitative traits of each genotype in terrestrial and aquatic habitats, respectively. The trait data were standardized prior to distance calculations. Mantel tests were then used to assess correlations between the trait matrix and genetic distance matrix based on molecular markers. In addition, the Euclidean distance matrix based on the phenotypic response to treatment (i.e., plasticity) for each genotype across terrestrial and aquatic habitats were also examined using the same method. The distance calculations and Mantel tests were done using the ecodist package (Goslee and Urban, 2007) in R (R Core Team, 2015).

Bioclimatic Modeling

A bioclimatic model was fitted against the native-range distribution of *A. philoxeroides*. It was then used to test whether the potential distribution in its introduced range (China and the USA) was fully invaded. The model was developed using CLIMEX Version 4.0 (Kriticos et al., 2015) and the world 10 min climate data set downloaded from CliMond (Kriticos et al., 2012). CLIMEX uses temperature and soil moisture data (calculated using rainfall and evaporation). Distribution data was obtained from Global Biodiversity Information Facility (GBIF, global species distribution dataset, http://www.gbif.org/species/3084923), supplemented by China distribution (NSII, China National Specimen Information Infrastructure, http://www.nsii.org.cn/), and the USA distribution (Early Detection and Distribution Mapping System, EDDMapS, http://www.eddmaps.org/distribution/viewmap.cfm?sub=2779). A previously published CLIMEX model for alligator weed (Julien et al., 1995) was modified (Table S2). Temperature parameters were adjusted so that stress only began once conditions were no longer suitable for growth (a CLIMEX requirement not adhered to in the original model), Moisture Index and Temperature Index parameters were tightened as much as possible without affecting the native-range fit, and the cold stress accumulation parameter reduced to better fit the southern-most distribution in Argentina. Outputs (Environmental Index scaled from 0 to 100) from the new model were plotted against distributional data for each region using QGIS 2.12 (Qgis Development Team, 2015).

RESULTS

Genetic Variation of Molecular Markers and Individual Traits

A total of 179 *A. philoxeroides* individuals from Argentina (21), the USA (32), and China (126) were analyzed using ISSR markers. The eight ISSR primers generated a total of 60 bands and 61 unique ISSR multi-locus genotypes. For samples from Argentina and the USA, each plant was characterized by a unique multi-locus genotype. In contrast, 94% (119) of the Chinese samples were identical (referred as to "C-Dominant" in **Figure 3**). The eight Chinese genotypes clustered together as a single well-supported clade in the neighbor-joining tree. This clade was closest to individuals from two sites in the USA (N4 and N8), although bootstrap value was low (**Figure 3**). In contrast, USA genotypes were represented in many clades, including ones that included Argentine genotypes. Individuals from the same site were usually grouped together, but there was no clear genetic structuring within each region.

All three genetic variables (*P, He,* and *I*) also showed much higher genetic diversity for clones from Argentina and the USA than those from China, even after the confounding effects of different sample size were controlled (**Table 1A**).

Genetic diversity as estimated using quantitative traits measure during the common garden experiment showed patterns

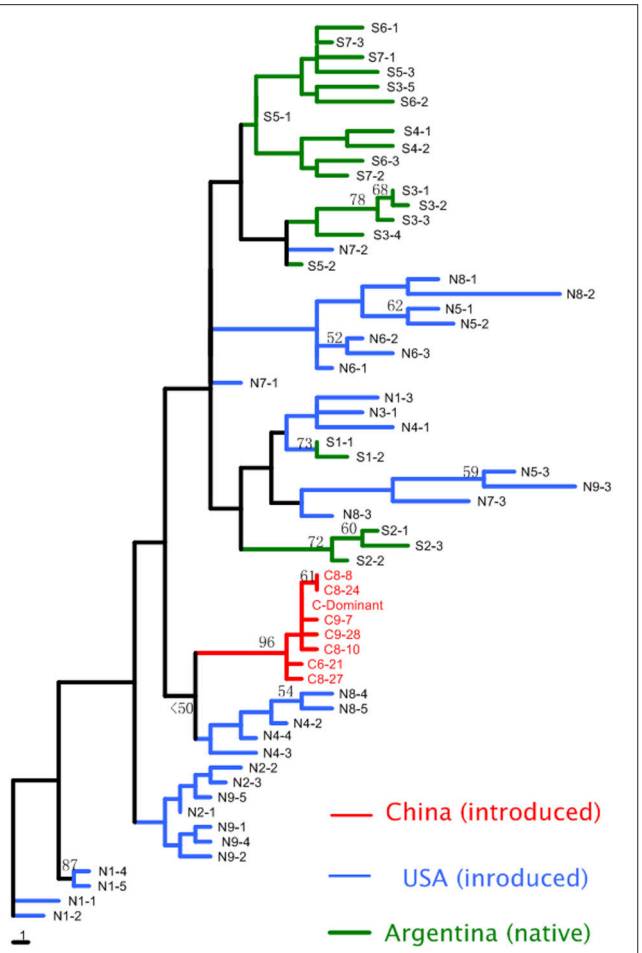

FIGURE 3 | Neighbor-joining tree of *Alternanthera philoxeroides* multi-locus genotypes from Argentina, China, and the USA. Numbers at nodes represent bootstrap support values (%, only values >50 are shown). All the individuals from China cluster together, with most having the same multi-locus genotype (C-dominance).

consistent with those revealed by neutral molecular markers. For both habitats, quantitative variation was much lower among Chinese clones than among clones from Argentina and the USA for most quantitative traits examined (**Table 1B**).

Phenotypic Plasticity of Individual Traits

Generally, plants in aquatic plots had longer leaves, longer internodes, thicker stems and larger stem pith cavity, larger specific leaf area, lower root/shoot ratio, lower relative chlorophyll content, and lower storage root/fine root ratio, than those in the terrestrial plots (**Figure 4**). The two-way ANOVA revealed significant effects of treatment on all the traits, indicating significant phenotypic plasticity across all regions (**Table 2**, treatment effect $P < 0.01$), despite significant differences among clones (**Table 2**, clonal effect $P < 0.01$).

Contrary to our expectation that clones in introduced ranges had higher phenotypic plasticity than those in the native range, the levels of phenotypic plasticity for examined traits in response to water availability (terrestrial vs. aquatic) were consistent across all three regions (**Figure 4**). The one-way ANOVA, nested ANOVA, and *t*-tests also found no significant differences among regions for most examined traits when comparing plasticity indices (**Figure 5**, Table S3). Consistent levels of phenotypic plasticity among regions were further supported by the non-significant effects of treatment by region interaction (**Table 2**). Although, plants had similar responses at region level, we did detect significant difference in plasticity among clones (**Table 2**). The clone-level reaction norm suggested that the slope of most traits varied greatly, especially for the clones from Argentina (Figure S1). Indeed, some Argentine clones were more plastic than those from USA and China (**Figure 5**, Figure S1).

Phenotypic correlation analyses showed that some traits were significantly correlated with each other in both habitats (Figure S2). For example, stem diameter was positively correlated with leaf length and stem-pith-cavity; specific leaf area (SLA) was negatively correlated with leaf length and stem diameter. The treatment changed the phenotypic correlation quantitatively (i.e., increased or decreased the correlation coefficients), but the overall correlation pattern remained unchanged (Figure S2). For the plasticity integration, only one pair of trait plasticity showed a negative correlation (i.e., the stem pith cavity and relative chlorophyll content, Figure S2).

Correlation between Genetic and Phenotypic Dissimilarity

The Mantel test found the molecular marker distance to be positively correlated with the dissimilarity of quantitative traits in terrestrial habitat ($r = 0.27$, $p = 0.04$) and aquatic habitat ($r = 0.23$, $p = 0.06$, Figure S3). However, we detected no significant correlation between marker distance and dissimilarity of phenotypic plasticity across terrestrial and aquatic habitats ($r = 0.15$, $p = 0.29$, Figure S3).

Multivariate (PCA) Pattern of Quantitative Genetic Variation and Phenotypic Plasticity

The PCA provided a multivariate perspective and indicated the similar pattern. The phenotypic variation among regions was mainly accounted for by the second principal component, which explained 19.53% of the total variation (**Figure 6**). Specifically, Chinese clones (red) formed a single cluster; in contrast, USA clones (blue) formed two discrete clusters and Argentina clones (green) were interspersed in the PCA space (**Figure 6**). The first principal component of PCA clearly separated aquatic and terrestrial treatments in PCA space, indicating that most (67.61%) of the phenotypic variation within the common garden experiment was a plastic response to habitat treatment.

Bioclimatic Modeling

Bioclimatic modeling suggested that the full potential distribution of the species in the introduced ranges were invaded in China and the USA. *A. philoxeroides* occurs in relatively diverse climates within its native range (Argentina), restricted largely by cold stress to the south and west, and heat stress in areas to the north of Argentina (**Figure 2**). Soil moisture had limited effect in the model as *A. philoxeroides* was present

TABLE 1 | Comparison of genetic diversity, measured both by molecular markers (A) and by quantitative traits (B), of *Alternanthera philoxeroides* from Argentina, China and the USA.

(A) MARKER DIVERSITY			
Population (sample size)	***P (%)***	***He***	***I***
Argentina (21)	60.00	0.1821	0.2759
USA (32)	69.33[a] (71.67[b])	0.2293 (0.2323)	0.3445 (0.3495)
China (126)	2.22 (11.67)	0.0043 (0.0144)	0.0071 (0.0260)

(B) QUANTITATIVE VARIATION									
	Biomass	**RSR**	**SFR**	**Leaf-L**	**LIN**	**Stem**	**SPC**	**RCC**	**SLA**
CV-Terrestrial									
Argentina	0.1585	0.5030	0.4391	0.2033	0.1036	0.2034	0.4487	0.1268	0.1290
USA	0.1147	0.1981	0.2392	0.1700	0.1575	0.2440	0.4073	0.1268	0.1541
China	0.0763	0.1577	0.2107	0.0807	0.1131	0.0312	0.1968	0.0870	0.0713
CV-Aquatic									
Argentina	0.3772	0.2194	0.8221	0.2300	0.1430	0.2335	0.2319	0.1215	0.1613
USA	0.3680	0.2771	0.5291	0.1889	0.1482	0.2116	0.2552	0.1178	0.1787
China	0.2377	0.1254	0.5354	0.0868	0.0930	0.0604	0.0897	0.0668	0.0749

SFR, storage roots/fine roots; RSR, root/shoot; LIN, length of internode; Leaf-L, leaf length; stem, stem diameter; SPC, stem pith cavity; RCC, relative chlorophyll content; SLA, specific leaf area.

P, percentage of polymorphic loci; He, Nei's genic diversity; I, Shanon's index.

[a] *Values based on re-sampling dataset with confounding effect of disproportional sample sizes controlled;*

[b] *Values based on original dataset. See details in Methods.*

CV-terrestrial (-aquatic), coefficients of genetic variation in terrestrial (aquatic) plot.

from wet to quite arid climates. The Environmental Index (EI) was high even in areas where *A. philoxeroides* has not been recorded in Argentina. These could not be excluded from the model by further restricting parameters without losing known locations, therefore suggesting non-climatic factors are also important.

The potential distribution in China and the USA was largely restricted by cold stress to the north and heat stress within the range and to the west. In both cases most distribution records occurred within the potential range, although records did extend into areas where cold stress was expected to be too high. There were no extensive areas where EI was moderate to high (above 10) in which *A. philoxeroides* has not yet been reported.

DISCUSSION

Phenotypic plasticity and genetic diversity have long been proposed to contribute to the invasion success of alien plants, especially clonal invaders, but few studies have tested their relative importance (Barrett, 2015; Bock et al., 2015). In this study, we found high levels of genetic diversity in *A. philoxeroides* in the native range (Argentina) and one introduced range (USA), but not in another introduced range (China). Specifically, genetic diversity in the USA was similar to that in Argentina. In contrast, the levels of genetic diversity in China were extremely low and many individuals collected from geographically distant sites shared the same multi-locus genotype. Contrary to our expectations that clones in introduced ranges had higher phenotypic plasticity than those in native range, the phenotypic plasticity in response to different water availability (terrestrial or aquatic) was similar across all three regions. Despite the different levels of genetic diversity, bioclimatic modeling suggests that the full potential bioclimatic distribution had been invaded in both China and USA. Taken together, our results suggest that the ability of *A. philoxeroides* to successfully invade heterogeneous habitats and broad geographic distributions is the consequence of phenotypic plasticity rather than genetic diversity.

Comparison of Genetic Diversity

In this study, we used both molecular markers and quantitative traits to assess the genetic diversity of *A. philoxeroides*. Mantel test suggested that the correlation of the two measures is significant but weak ($r = 0.27$ and 0.23 in terrestrial and aquatic habitat, respectively), which is similar with previous results ($r = 0.217$) based on meta-analysis (Reed and Frankham, 2001). Both molecular markers and quantitative traits revealed a clear pattern that Chinese clones had much lower levels of genetic diversity, in terms of both marker diversity and quantitative variation, than those from Argentina and the USA. The extremely low levels of marker diversity among analyzed Chinese clones are consistent with previously reported results (Xu et al., 2003; Ye et al., 2003; Geng et al., 2007a). The regional-level genetic diversity in USA and Argentina may be underestimated due to smaller sample size, which means the overall pattern of genetic diversity between China and the other two regions may be even more prominent. The levels of genetic diversity of alien species are often shaped by population history (e.g., foundering effect, and whether multiple-introduction had occurred). In this study, all the Chinese samples clustered as a single well-supported clade in the neighbor-joining tree. This suggests Chinese populations may be the result of

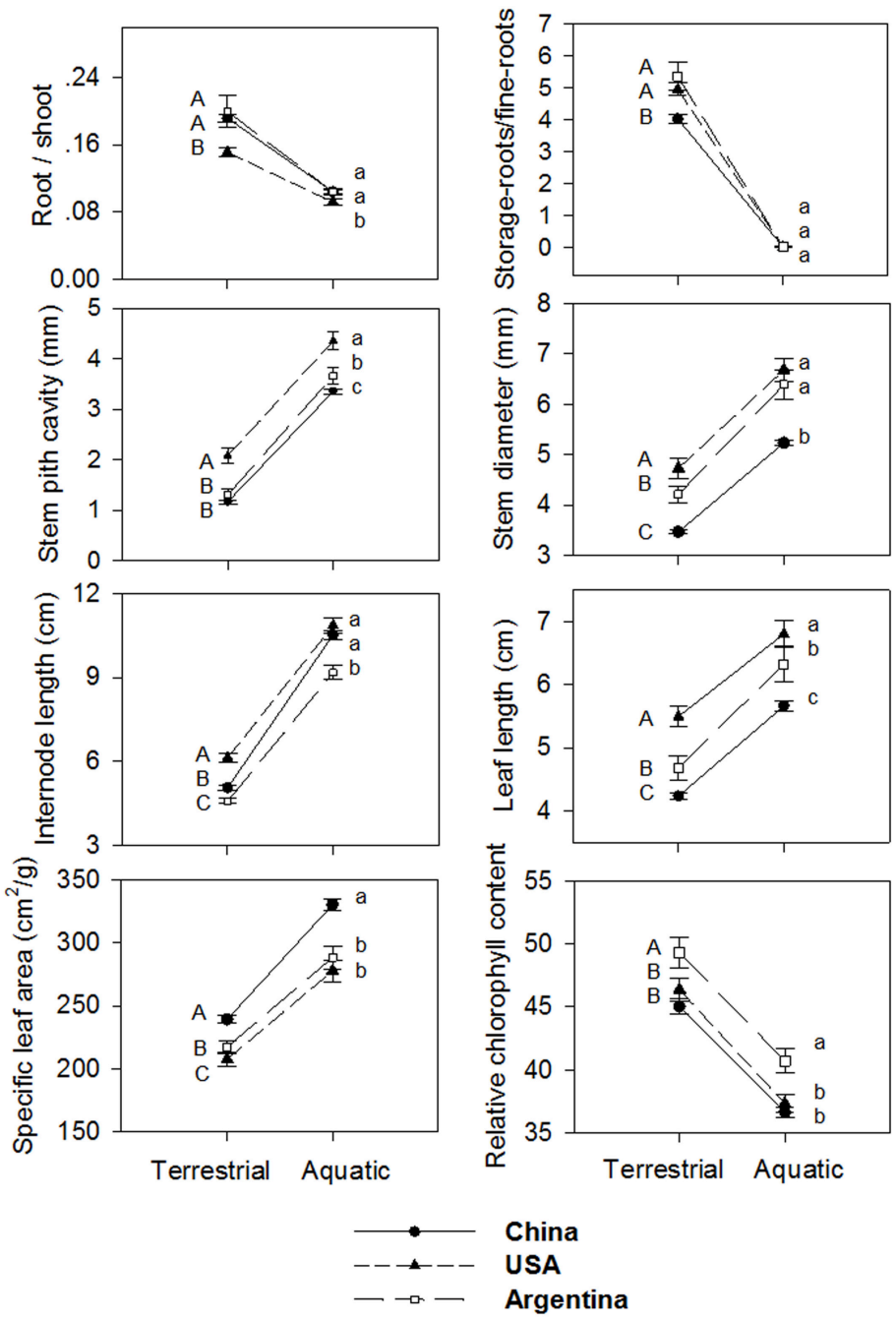

FIGURE 4 | Reaction norms of *Alternanthera philoxeroides* from Argentina, China and the USA against the two habitat treatments tested in the common garden experiment. Lines are the mean ± 1 SE. Results of *Post-hoc* comparison based on Bonferroni test are shown using capital letters (terrestrial plots) and small letters (aquatic plots). Values sharing the same letter do not differ significantly ($\alpha = 0.05$). Abbreviations are the same as **Table 1**.

a single introduction, with the low levels of genetic diversity among Chinese clones being the result of founding effects during invasion. In contrast, the USA clones were scattered on the neighbor-joining tree and were intermingled with Argentina clones, suggesting that the *A. philoxeroides* populations in the USA might have stemmed from multiple introductions. Indeed, the levels of genetic diversity of the clones in the USA were similar to that in Argentina, suggesting no obvious founding effect in the USA.

The level of genetic diversity had little effects on the invasion potential of *A. philoxeroides* to invade its potential distribution within either China (low diversity) or the USA (high diversity) as assessed by a bioclimatic model fitted against the native-range distribution. Especially, the genetic uniformity

TABLE 2 | Effects of treatment (aquatic and terrestrial), region (Argentina, China and the USA), clone and two-factor interaction on the allocation and morphological traits in *A. philoxeroides* in the common garden experiment.

Traits	Treatment	Region	Treatment × Region	Clone (Region)	Treatment × Clone
	df = 1,22	*df* = 2,22	*df* = 2,22	*df* = 22,112	*df* = 22,112
***F*-VALUES**					
Ln(biomass)	493.96**	0.78	2.84	9.24**	2.37**
Sqrt(SFR)	5435.36**	1.26	2.32	4.13**	1.87*
Sqrt(RSR)	58.38**	5.65*	0.54	6.43**	9.75**
LIN	876.78**	13.16**	2.60	5.75**	2.52**
Leaf-L	160.32**	4.21*	0.32	24.08**	2.53**
Stem	470.37**	4.68*	0.96	46.60**	2.30**
SPC	915.24**	4.97*	0.79	21.50**	1.79*
RCC	133.56**	2.29	0.09	8.78**	2.27**
SLA	237.72**	6.45*	2.25	9.91**	2.31**

*Clone was nested in region as a random factor. Significance levels are given by *P < 0.05, **P < 0.01. Abbreviations are the same as **Table 1**.*

of Chinese clones did not appear to restrict the geographic and ecological distribution of *A. philoxeroides*. Similar results have also been reported in a few other invasive alien plants in their introduced ranges, e.g., *Pennisetum setaceum* (Poulin et al., 2005), *Rubus alceifolius* (Amsellem et al., 2000), and *Fallopia japonica* (Hollingsworth and Bailey, 2000). Notably, most of these invaders are selfing, or apomixis clonal species, which can usually avoid genetic erosion through Allee effects (e.g., inbreeding depression). Therefore, it seems that the levels of genetic diversity may not be a critical factor limiting the distribution and abundance of clonal invasive plants. So far, several well-documented case studies on post-introduction evolution mainly involved out-crossing or mixed-crossing species, e.g., *Hypericum perforatum* (Hierro et al., 2005), *Phalaris arundinacea* (Lavergne and Molofsky, 2007), and *Sapium sebiferum* (Rogers and Siemann, 2004). Therefore, the role of genetic diversity in invasion success might be variable for plant species with different reproductive modes (e.g., mating system).

Comparison of Phenotypic Plasticity

Phenotypic plasticity is frequently envisaged as one of the characteristics that contribute to the adaptability and invasiveness of alien species (Parker et al., 2003; Richards et al., 2006) by allowing them to maintain or increase population growth rates across diverse environments (Pichancourt and Van Klinken, 2012). In this study, we found that all clones, regardless of their geographic origins, showed significant phenotypic plasticity in biomass allocation and morphological traits in response to varying water ability. This may play an important role in shaping its niche breadth in relation to water. In particular, there was no significant correlation between the dissimilarities of genetic markers and plasticity indexes among clones, suggesting the plastic response norm of *A. philoxeroides* in terrestrial vs. aquatic habitats is an inherent (species-level) acclimation to these habitats.

Although, it is not easy to rigorously confirm the adaptive significance of phenotypic plasticity in non-model species (Sultan, 1995), we did find evidence that the phenotypic plasticity of *A. philoxeroides* is not the passive result of growth allometry or resource shortage. First, in a previous study, Geng et al. (2007b) found the plastic root/shoot ratio in response to different water treatments were the result of developmentally active adjustment (i.e., true plasticity) rather than ontogenetic drift along the same developmental trajectory (i.e., apparent plasticity). Second, the phenotypic changes are largely functionally adaptive. For example, terrestrial plants allocated more biomass to roots, and produced smaller and thicker leaves, had shorter internodes, which help plants to better balance the absorption and transpiration of water. Aquatic plants had a much larger stem-pith-cavity, which can act as highly efficient aerenchyma (Voesenek et al., 2006) and also enables the stem mats to float on water (Julien, 1995). Notably, the pattern of trait correlation was qualitatively similar across terrestrial and aquatic habitats (Figure S2). We also detected a significant correlation in phenotypic plasticity between stem pith cavity and relative chlorophyll content. Such phenotypic integration may reflect adaptation within a certain environment (e.g., terrestrial or aquatic habitat) or could be by-products of the genetic/developmental constraints (Pigliucci, 2003), which may constrain the expression and evolution of phenotypic plasticity in dynamic environments (Gianoli and Palacio-Lopez, 2009).

Terrestrial plants allocated much more resources to storage roots than those in aquatic habitats, which may be critical for *A. philoxeroides* to invade into both terrestrial and aquatic habitats. Specifically, *A. philoxeroides* is susceptible to seasonal disturbances (e.g., winter frost) in terrestrial habitats, which often kill all the above-ground parts (**Figure 1**). Thus, the below-ground storage roots become the indispensable organs that allow plants to resprout and re-produce in terrestrial habitats (Wilson et al., 2007; Geng et al., 2007a). In contrast, regeneration of *A. philoxeroides* in aquatic habitats relies mainly on stems that can often survive winter (**Figure 1**). Indeed, manipulative experiments suggest that the storage roots had much lower resprout ability in aquatic habitats (Geng et al. unpublished data), suggesting decreased functional importance for storage roots in aquatic habitats. The observed phenotypic plasticity was consistent across the native and two introduced ranges, suggesting it is an inherent (species-level) acclimation pattern for growing in diverse habitats.

Invasive species are expected to be more plastic than their native conspecifics (Parker et al., 2003; Richards et al., 2006), which particularly applies to *A. philoxeroides*, given the extremely low genetic variation and broad niche in Chinese populations. However in this study, we found no evidence of this. Although, plants from different regions had similar plastic responses, we did detect significant difference among clones. Clone-level reaction norms suggested that the slope (i.e., amount of plasticity) varied greatly, especially in the clones from Argentina (native region). Indeed, phenotypic plasticity levels of Chinese and USA clones fell within the variation ranges

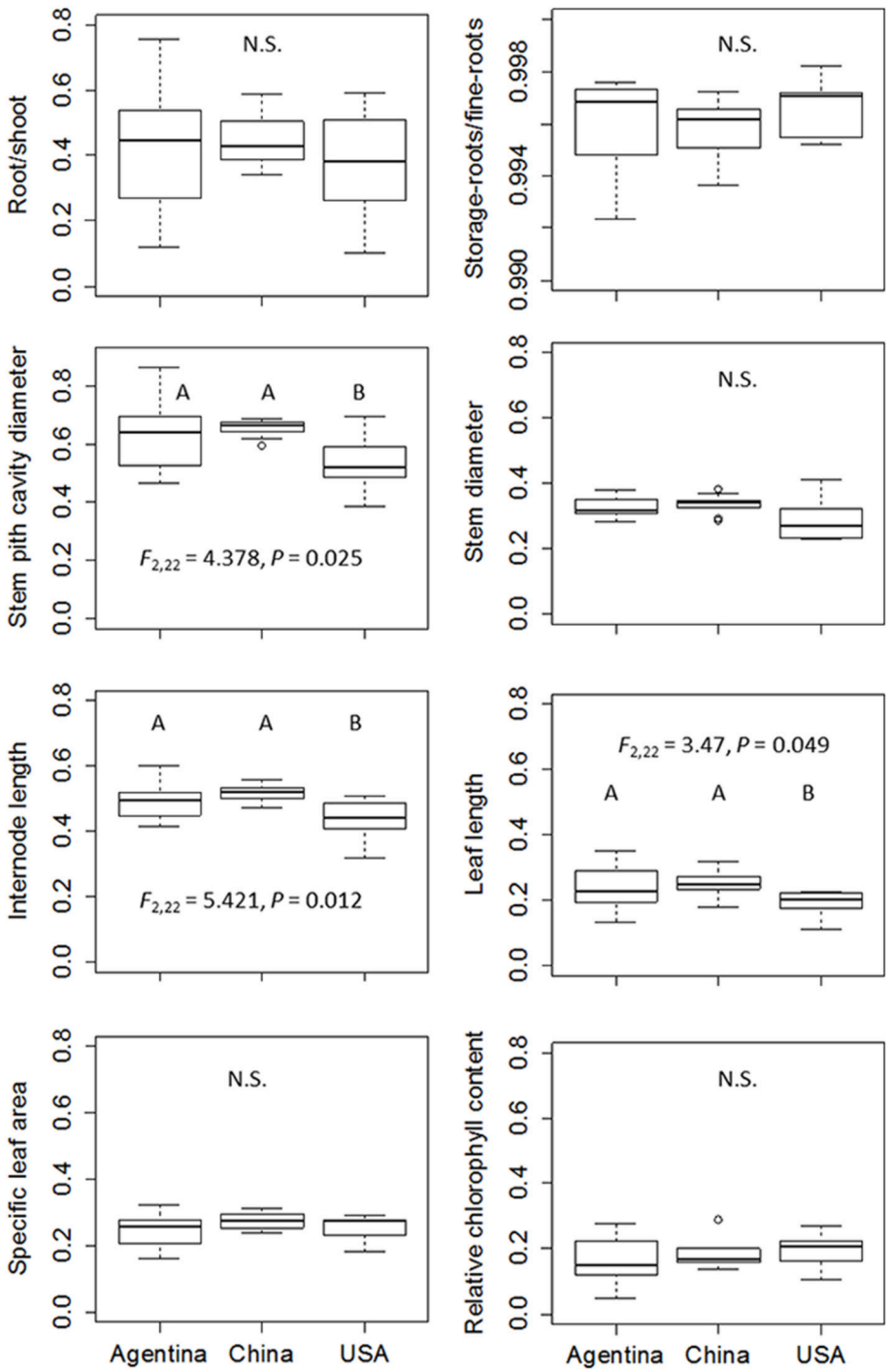

FIGURE 5 | Comparison of plasticity index of *Alternanthera philoxeroides* from Argentina, China and the USA. Results of *Post-hoc* comparison based on Bonferroni test are shown where significant at $\alpha = 0.05$. Abbreviations are the same as **Table 1**.

of Argentine clones. In other words, some native clones were even more plastic than the introduced clones. Previous studies on the comparison of phenotypic plasticity between native and introduced populations/species have produced mixed results. For example, Bossdorf et al. (2005) synthesized 10 case studies, of which half suggested that introduced populations were more plastic than native ones, while the other did not. In more recent meta-analysis studies, (Davidson et al., 2011) found that invasive species were more plastic than non-invasives, while (Palacio-López and Gianoli, 2011) found no significant difference between

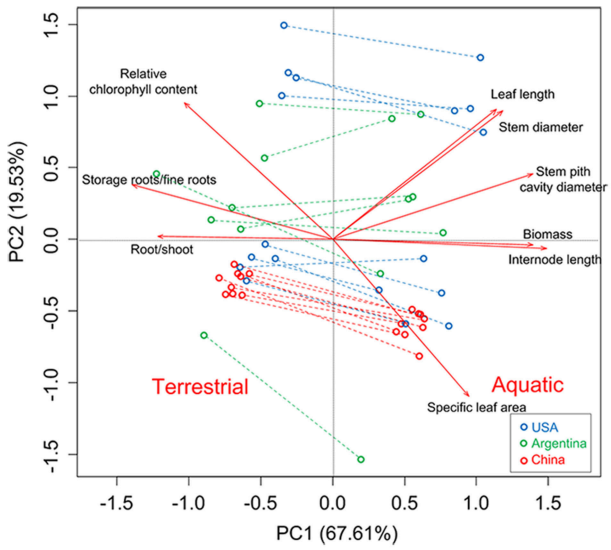

FIGURE 6 | Biplot for principal components analysis based on quantitative traits. Each point represents a single clone in a single treatment.

invasive and native species. Theoretically, it has been proposed that phenotypic plasticity may be favored by natural selection only in the initial phase of invasion, resulting in a transient increase in plasticity; in later invasion phases, plasticity will reduce due to plasticity costs because the novel habitat poses continuous directional selection on the optimum phenotype (Palacio-López and Gianoli, 2011; Lande, 2015). However, such a process of genetic assimilation is less likely to occur in asexual clonal species like *A. philoxeroides*. Indeed, the absence of different plasticity levels between native and introduced populations in *A. philoxeroides* does not seem to be the result of transient and reversible post-invasion evolution, but an inherent characteristic of *A. philoxeroides*.

Phenotypic plasticity may be much more important than genetic diversity in determining the success of clonal invasive species like *A. philoxeroides*. In non-clonal species with high levels of genetic diversity, local adaptation and post-invasion evolution are frequently observed (Lee, 2002; Maron et al., 2004; Novy et al., 2013; Turner et al., 2014). However, in clonal species, the efficiency of natural selection is often constrained and rapid evolution is more difficult to occur (Barton and Charlesworth, 1998; Silvertown, 2008). In the case of *A. philoxeroides*, phenotypic plasticity, rather than genetic diversity, may be critical for the potential to cope with heterogeneous habitats with variable water availabilities and climate conditions, i.e., the basic niche, which can translate into a broader realized niche in introduced ranges when the co-evolved competitors (Gurevitch, 1986) and natural enemies (Louda and Rodman, 1996) are absent. This is partially supported by the bioclimatic model result, which greatly overestimated the native-range distribution of *A. philoxeriodes*, suggesting that other factors such as topography and competition are important in limiting the distribution of *A. philoxeriodes* in the native range. Including other factors in the distribution model, as has been done for similar species (Murray et al., 2012), would therefore help to demonstrate the key factors that lead to the niche expansion of *A. philoxeroides* in introduced ranges.

A biogeographical approach is often proposed to compare the introduced populations with their native counterparts (Bossdorf et al., 2005; Hierro et al., 2005). If we regard biological invasion as a "natural experiment," the repeated invasion success by some global invaders (e.g., *A. philoxeroides*) provides valuable information akin to experimental replications. In cases where repeated invasions indeed share common features, comparisons among these replications may help identify the relative importance of different factors in determining invasion success. In this study, we compared the genetic diversity and phenotypic plasticity of *A. philoxeroides* across three regions. Our results revealed that the pattern of "lower genetic diversity" in one introduced range (i.e., China) was not found in another introduced range (i.e., the USA), reflecting the heterogeneous nature of biological invasions even for the same invader. In contrast, high levels of phenotypic plasticity were found across all three regions, highlighting the importance of phenotypic plasticity as a common feature underlying successful invasions of *A. philoxeroides*. Accordingly, this multi-region comparative approach, including two or more biogeographical replicates, may be especially indicative for understanding the relative importance of different factors underlying successful invasion.

AUTHOR CONTRIBUTIONS

YG, BL, JC, and CX designed the research. YG performed the wet lab work. RV performed the climate niche modeling. YG and CX performed the data analysis. YG, AS, CX participated in the sampling. YG, RV, BL, JC, and CX drafted and revised the manuscript. All authors carefully read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00213

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Propagule Pressure, Habitat Conditions and Clonal Integration Influence the Establishment and Growth of an Invasive Clonal Plant, *Alternanthera philoxeroides*

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Many notorious invasive plants are clonal, spreading mainly by vegetative propagules. Propagule pressure (the number of propagules) may affect the establishment, growth, and thus invasion success of these clonal plants, and such effects may also depend on habitat conditions. To understand how propagule pressure, habitat conditions and clonal integration affect the establishment and growth of the invasive clonal plants, an 8-week greenhouse with an invasive clonal plant, *Alternanthera philoxeroides* was conducted. High (five fragments) or low (one fragment) propagule pressure was established either in bare soil (open habitat) or dense native vegetation of *Jussiaea repens* (vegetative habitat), with the stolon connections either severed from or connected to the relatively older ramets. High propagule pressure greatly increased the establishment and growth of *A. philoxeroides*, especially when it grew in vegetative habitats. Surprisingly, high propagule pressure significantly reduced the growth of individual plants of *A. philoxeroides* in open habitats, whereas it did not affect the individual growth in vegetative habitats. A shift in the intraspecific interaction on *A. philoxeroides* from competition in open habitats to facilitation in vegetative habitats may be the main reason. Moreover, clonal integration significantly improved the growth of *A. philoxeroides* only in open habitats, especially with low propagule pressure, whereas it had no effects on the growth and competitive ability of *A. philoxeroides* in vegetative habitats, suggesting that clonal integration may be of most important for *A. philoxeroides* to explore new open space and spread. These findings suggest that propagule pressure may be crucial for the invasion success of *A. philoxeroides*, and such an effect also depends on habitat conditions.

Keywords: alligator weed, intraspecific interaction, interspecific interaction, plant invasion, propagule supply

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INTRODUCTION

Plant invasion has posed a great threat to biodiversity, environment, and economy both globally and locally (Mack et al., 2000; Vila et al., 2011). Previous studies have demonstrated that plant invasion is the outcome of complicated interactions that involve many biotic and abiotic factors (Davis et al., 2000; Lockwood et al., 2005; Melbourne et al., 2007; Chun et al., 2010), which can

be divided into three broad categories: propagule pressure (the number of propagules entering the new habitat), invasibility of the environment (habitat conditions) and the characteristics of the plant species (such as clonal traits; Davis et al., 2000; Simberloff, 2009; Ehrenfeld, 2010; Xie et al., 2013).

Propagule pressure (propagule supply) has been considered one of the most important factors for explaining the invasion success of plants (Levine and D'Antonio, 1999; Britton-Simmons and Abbott, 2008; Simberloff, 2009; Duncan, 2011). It has been proposed that the greater number of propagules arriving in a new environment gives a plant a higher chance to establish itself, persist, naturalize, spread, and invade (Rouget and Richardson, 2003; Lockwood et al., 2009; Simberloff, 2009). Indeed, many previous studies have showed a positive relationship between propagule pressure and invasion success of introduced plants (Rouget and Richardson, 2003; Colautti et al., 2006; Lockwood et al., 2009; Liu et al., 2014). Despite its acknowledged importance, propagule pressure has rarely been experimentally studied (but see Roiloa and Retuerto, 2005), and the interaction of propagule pressure with other factors (such as disturbance and habitat conditions) that influence invasion success is still not well understood (Lockwood et al., 2005; Britton-Simmons and Abbott, 2008; Liu et al., 2014).

According to the "ecological resistance hypothesis," resident native communities may indirectly control invasion success by reducing the input of propagules and resource availability, thereby inhibiting the establishment and spreading process of the introduced species (Levine et al., 2004; Xie et al., 2013). Therefore, the role of propagule pressure in shaping the invasion process of introduced plants may be closely related to habitat conditions (Rouget and Richardson, 2003; Warren et al., 2012; Liu et al., 2014). In habitats where resident native vegetation is scare and much space is available (open habitats), introduced plants may need a few propagules to ensure establishment and invasion success. On the contrary, introduced plants may need a larger number of propagules to overcome high interspecific competition and establish successfully in habitats where resident native vegetation is dense (D'Antonio et al., 2001). However, to our knowledge, relatively few experimental researches have investigated how habitat conditions affect the role of propagule pressure in the invasion process of alien invasive species, although several studies have addressed that the effects of propagule pressure on the invasion success of plants also depended on habitat suitability (or habitat conditions; Holle and Simberloff, 2005; Warren et al., 2012; Liu et al., 2014).

Another important factor for invasion success is the characteristics of the plant species such as clonal traits (Kolar and Lodge, 2001). As we know, many of the most notorious alien invasive plants have the capacity for vigorous clonal propagation (Kolar and Lodge, 2001; Xu et al., 2010; You et al., 2013). For instance, *Eichhornia crassipes* (water hyacinth), *Alternanthera philoxeroides* (alligator weed), and *Myriophyllum aquaticum* (parrotfeather) can grow and spread mainly by vegetative growth and clonal propagation (no seeds or seed sterility) in their introduced regions (Villamagna and Murphy, 2010; Schooler, 2012; Xie et al., 2013). Recently, some studies have pointed out that the invasiveness of alien clonal plants may be closely related to clonal traits such as clonal integration (i.e., the reciprocal translocation of resources between interconnected ramets; Wang et al., 2008; Song et al., 2013; You et al., 2014a). Clonal integration, driven by the source-sink relationship, can improve plants' exploitation of ubiquitous heterogeneous resources, help plants invade new environments and facilitate plants' spatial occupation of new habitats for both native and invasive clonal plants (Klimeš et al., 1997; Yu et al., 2009). For example, clonal integration may increase competitive ability of invasive plants when grown with resident native vegetation, thereby influence species co-existence, community structure, and ecosystem functioning (Yu et al., 2009; You et al., 2014a). Although several studies have addressed that clonal integration had positive effects on establishment and growth of the invasive clonal plants (such as *A. philoxeroides* and *Carpobrotus edulis*) in different habitat conditions (bare soil or vegetation; Wang et al., 2008; Yu et al., 2009; Roiloa et al., 2010; You et al., 2014a), unfortunately, all these studies ignored the role of propagule pressure in shaping this process with regard to vegetative propagules.

Alternanthera philoxeroides, originating from the Parana River region of South America, is a clonal weed that causes serious economic and environmental problems worldwide (Julien et al., 1995; Schooler, 2012). It is stoloniferous and amphibious, growing in both riparian and terrestrial habitats (Schooler, 2012). This species is one of the world's worst invasive weeds and is listed as one of the 16 worst alien invasive weeds in China (Julien et al., 1995; Ma and Wang, 2005). *A. philoxeroides* often suffers natural disturbances, such as herbivory, mowing and trampling, which may fragment its clones into pieces (Schooler et al., 2007; Dong et al., 2010, 2012; You et al., 2014b). In China, *A. philoxeroides* has extremely low genetic diversity (Xu et al., 2003; Wang et al., 2005), and clonal integration plays an important role in determining its growth and spread (Wang et al., 2008, 2009; Xu et al., 2010; You et al., 2014b). *Jussiaea repens* is a rooted emergent stoloniferous clonal plants and a fast-proliferating species in wetlands, naturally distributed in central and south China. In natural environments, these two species often co-exist in aquatic habitats or aquatic-terrestrial ecotones in south China (You et al., 2014a).

To investigate the effects of propagule pressure, habitat conditions and clonal integration on the establishment, growth and thus invasion success of introduced invasive clonal plants, we selected these two co-occurring stoloniferous clonal plants, *A. philoxeroides* (invasive) and *J. repens* (native). In an 8-week greenhouse experiment, we grew one fragment (low propagule pressure) or five fragments (high propagule pressure) of *A. philoxeroides* either in bare soil (open habitat) or dense native vegetation of *J. repens* (vegetative habitat), with the stolon connections either severed from or connected to the older ramets to test the effect of clonal integration. Specifically, we test the following hypotheses. (1) Increase in propagule supply will increase the establishment and growth of *A. philoxeroides*, especially when it grew in vegetative habitats. (2) Clonal integration will promote the growth and competitive ability

of *A. philoxeroides* under high propagule pressure. (3) High propagule pressure of *A. philoxeroides* with clonal integration will reduce the growth of *J. repens*.

MATERIALS AND METHODS

Plant Material

Given that genetic diversity of wetland clonal plants is relatively low (Sosnová et al., 2011), especially for *A. philoxeroides* in China (Xu et al., 2003; Wang et al., 2005), source material of *A. philoxeroides* and *J. repens* were collected in middle June 2014, from at least five locations at least 20 m apart in each of two wetlands in Gonghu Bay of the Taihu Lake in the Jiangsu province of China (N 31°25′–31°28′, E 120°15′–120°21′). Then plants from different locations were mixed and propagated in the greenhouse. After 2 weeks of adaptive culture, about 200 tip cuttings of *A. philoxeroides* and about 1000 tip cuttings of *J. repens* were selected and planted vertically into 20 plots (30 cm diameter × 15 cm height) with lake soil (Total nitrogen concentration 3.05 mg g^{-1}, total phosphorus concentration 0.16 mg g^{-1}) for continued culture.

Experimental Design

The growth experiment was conducted in a greenhouse under natural sunlight (about 14/10 day/night cycle) and ambient temperature at the Field Station of Jiangsu University. The experiment was conducted with a factorial design involving propagule pressure (low or high; i.e., one fragment or five fragments), habitat conditions (open or vegetative) and clonal integration (stolon connections were severed or intact; **Figure 1**). The tested plants used in this experiment were 120 similar-sized clonal fragments of *A. philoxeroides* (tip cuttings, 15.27 ± 0.20 cm in length, 0.51 ± 0.07 g in dry mass; means ± SE, measured by another 20 clonal fragments), each consisting of a stolon with five ramets. No differences between treatments were detected in initial size of this plants ($P > 0.05$, One-way ANOVA). Each clonal fragment was divided into two parts, one termed as 'basal part' consisting of three relatively old ramets (close to the mother ramets) and the other as 'apical part' consisting two relatively young ramets (distal to the mother ramets) and a stolon apex (You et al., 2014a).

There were 45 plastic containers (50 cm × 50 cm × 25 cm; length × width × height), each having two separated sections in this experiment (see **Figure 1**). The basal section was 20 cm long and the apical section was 30 cm long. Resources (nutrients and water) and roots in the two sections did not interfere with each other. All the containers in both sections were filled with a mixture of sand and lake mud at a volume ratio of 3:1 and with 2.0 g slow-release fertilizer (Osmocote, N–P–K: 16–8–12, 6 months). On July 5th 2014, the apical sections of 25 containers were planted vertically with cultured plant fragments (tip cuttings, 15.05 ± 0.22 cm in length, 0.55 ± 0.05 g in dry mass; means ± SE, measured by another 20 clonal fragments) of *J. repens* (monoculture) in the greenhouse to mimic natural plant populations (vegetative habitats), with a density of 200 plants m^{-2} (30 plants in each apical section; You et al., 2014a). The remaining 20 containers were kept with apical sections bare (open habitats).

After 4 weeks adaptive growth of *J. repens* populations in vegetative habitats, on August 3rd 2014, one (low propagule pressure) or five (high propagule pressure) fragments of *A. philoxeroides* were horizontally positioned in both habitat conditions of 40 containers (20 open habitats and 20 vegetative habitats in apical sections), the remaining five containers with vegetative habitats were used as a control for plant population growth of *J. repens* without competition. For each clonal fragment, three ramets of basal part were placed within basal section of a container and the other two ramets and apex of the apical part were within the apical section of the same container (**Figure 1**). The stolons of basal and apical ramets were both anchored to the soil surface to facilitating rooting. Five days later, when the clonal fragments were successfully rooted, the stolon connections between the apical and basal parts were severed in 20 containers, while the other 20 ones were kept intact. Therefore, each treatment was replicated five times (see **Figure 1**). The experiment was conducted for 8 weeks and ended on October 6th 2014. The experimental containers were randomly repositioned every 2 weeks to avoid the effects of possible environmental heterogeneity (such as light), and watered every other day to keep the soil in the containers wet. The mean light intensity at the top of the plant canopy was 1200–1400 μmol m^{-2} s^{-1} on the cloudless days, and the mean air temperature was 25–30°C during the experimental period.

Measurements

At the final harvest, the number of ramets and leaves were recorded, and total stolon length of *A. philoxeroides* was measured for the apical sections of all treatments. Then the plants of *A. philoxeroides* in the apical part of the container were harvested and separated into leaves, stolons and roots, and their biomass was determined after drying at 70°C for 72 h. Neighboring vegetation of *J. repens* (entire plants including roots) in the apical sections of each container were also harvested and their dry mass was also determined in the same way.

Data Analysis

Data were expressed as means ± SE. Growth measures (total biomass, ramet number, leaf number, and stolon length) of *A. philoxeroides* in the apical part were calculated at both individual level and population (container) level. Prior to analysis, data were log-transformed if necessary to meet the assumptions of normality and homoscedasticity.

The intraspecific relative competition intensity (RCI) of *A. philoxeroides* was calculated as intraspecific RCI = $(G_{low}-G_{high})/G_{low}$, where G_{low} is the mean growth measure of *A. philoxeroides* in low propagule pressure and G_{high} is that measure in high propagule pressure (Weigelt and Jolliffe, 2003). The index was calculated for each container and averaged for comparison between the two habitat conditions (open

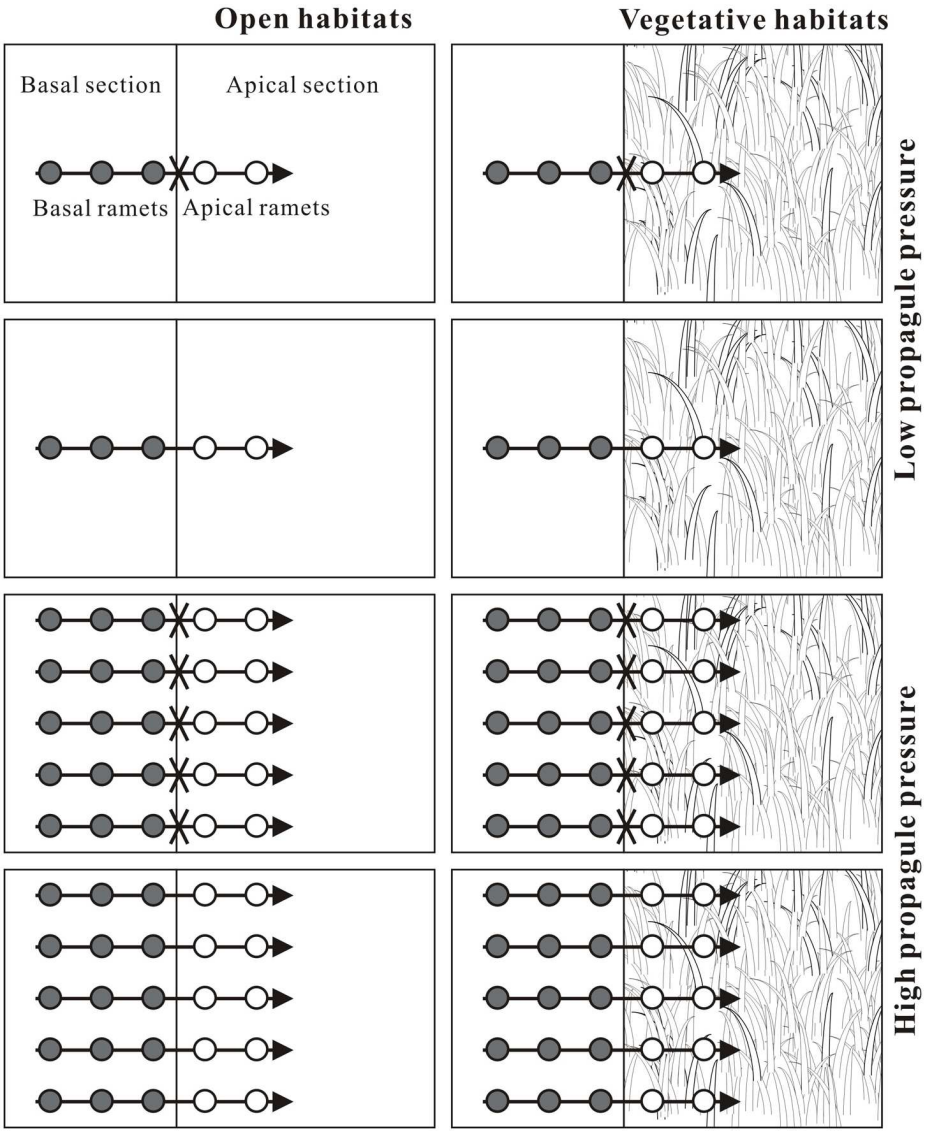

FIGURE 1 | Schematic representation of the experimental design. One clonal fragment (low propagule pressure) or five clonal fragments (high propagule pressure) of the invasive plant *Alternanthera philoxeroides*, each consisting of three basal ramets (dark gray circles) and two apical ramets (white circles) with a stolon apex (horizontal arrow), were grown either in open habitats or in vegetative habitats (*Jussiaea repens*), and with stolon connections between basal and apical ramets were either intact or severed (fork).

and vegetative) with the stolon connections either severed or intact. The interspecific RCI was calculated as interspecific RCI = $(G_{open}-G_{vegetative})/G_{open}$, where G_{open} is the mean growth measure of *A. philoxeroides* in open habitats and $G_{vegetative}$ is that measure in vegetative habitats (Weigelt and Jolliffe, 2003). The index was also calculated for each container and averaged for comparison between the low and high propagule pressure with the stolon connections either severed or intact. A positive value of RCI suggests competition and a negative one indicates facilitation (Armas et al., 2004; Liu et al., 2014). The RCI of *J. repens* was not considered because a significant competition effect was not observed (see Results).

Three-way ANOVA was used to assess the effects of propagule pressure, habitat conditions and clonal integration on the growth measures of *A. philoxeroides* in the apical section at individual level and container level. Two-way ANOVA was employed to investigate the effects of habitat conditions and clonal integration on intraspecific RCI of *A. philoxeroides*, and the effects of propagule pressure and clonal integration on interspecific RCI of *A. philoxeroides*. One-way ANOVA was used to test whether total biomass of vegetation (*J. repens*) in the apical section differed between the four competition treatments and the control. *Post hoc* pair-wise comparisons of the means were performed to examine differences between the treatments using Studentized Tukey's HSD for multiple

comparisons. Statistical significance was assigned at $P < 0.05$. All data analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Growth of *A. philoxeroides*

At the population (container) level, propagule pressure, habitat condition and clonal integration significantly affected all the growth measures (total biomass, ramet number, leaf number, and stolon length), and propagule pressure × habitat conditions, habitat conditions × clonal integration had also significant effects on growth of *A. philoxeroides* in the apical sections (**Table 1**). High propagule pressure greatly increased the growth of *A. philoxeroides* in both open and vegetative habitats (**Figure 2**), and the effect of propagule pressure on the growth of *A. philoxeroides* was more significant in vegetative habitats than in open habitats (**Figure 2**, 92.7–205.2% of growth increase in open habitats vs. 442–593% of growth increase in vegetative habitats). Moreover, vegetation of *J. repens* significantly suppressed the growth of *A. philoxeroides* in both low and high propagule supply (**Table 1**; **Figure 2**). In open habitats, clonal integration greatly promoted the growth of *A. philoxeroides* either in low propagule supply or in high propagule supply, however, such effect of clonal integration on plant growth disappeared when grown with *J. repens* (**Figure 2**).

At initial individual level, propagule pressure, habitat condition, clonal integration and their interactions had significant effects on the growth of *A. philoxeroides* in the apical sections (**Table 1**). The growth measures of *A. philoxeroides* were greatly higher in open habitats than in vegetative habitats (**Table 1**; **Figure 3**). There were no differences in growth of *A. philoxeroides* among the treatments in vegetative habitats (**Figure 3**). However, in open habitats, the growth measures were significantly lower in high propagule pressure than in low propagule pressure, and clonal integration significantly increased the growth of *A. philoxeroides* only in low propagule pressure (**Figure 3**).

Relative Competition Intensity (RCI) of *A. philoxeroides*

The intraspecific RCI of all the growth measures of *A. philoxeroides* were significantly affected by habitat conditions (vegetation), however, those values were not affected by clonal integration except for the intraspecific RCI on stolon length (**Table 2**). The values of the intraspecific RCI of biomass, ramet number, leaf number, and stolon length were significantly lower in vegetative habitats than in open habitats (**Figure 4**). Interestingly, the values of the intraspecific RCI of biomass and stolon length were negative in vegetative habitats (**Figures 4A,D**), indicating that the intraspecific interactions between *A. philoxeroides* individuals changed from competition in open habitats to facilitation in vegetative habitats.

The interspecific RCI of all the growth measures of *A. philoxeroides* were significantly affected by propagule pressure, whereas those values were not affected by clonal integration (**Table 2**). The values of the interspecific RCI of the four growth measures were significantly lower when propagule supply was high than when it is low (**Figure 5**), suggesting that interspecific competition that vegetation of *J. repens* posed on *A. philoxeroides* became weaker when propagule supply was higher.

Growth of *J. repens*

Total biomass of *J. repens* vegetation in the apical sections had no significant differences among all the treatments ($F_{4,20} = 1.15$, $P = 0.21$). Biomass in the apical sections of *J. repens* in five treatments (control, low propagule pressure, low propagule pressure with clonal integration, high propagule pressure and high propagule pressure with clonal integration) were 82.68 ± 4.30, 87.18 ± 5.78, 84.02 ± 3.74, 78.59 ± 6.12, and 80.82 ± 4.89 g (means ± SE), respectively.

TABLE 1 | Three-way ANOVA analyses for the effects of propagule pressure, habitat conditions (vegetation) and clonal integration (connection) on the growth measures of the invasive plant *Alternanthera philoxeroides* in the apical sections at container level and individual level.

Dependent variable	Propagule pressure (P)	Vegetation (V)	Connection (C)	P × V	P × C	V × C	P × V × C
Container level							
Biomass (g)	400.78***	428.46***	21.27***	33.01***	0.20	9.55**	0.68
Ramet number[1]	439.27***	474.48***	23.30***	55.34***	1.97	8.12**	0.07
Leaf number[1]	364.12***	332.46***	11.09**	23.40***	0.4	6.06*	0.05
Stolon length (cm)	282.95***	285.41***	6.03*	16.62***	0.32	4.39*	0.23
Individual level							
Biomass (g)	81.66***	411.16***	32.01***	93.73***	14.95**	19.15***	12.04**
Ramet number[1]	211.88***	626.36***	51.49***	170.08***	11.16**	24.20***	9.11**
Leaf number[1]	171.05***	572.86***	27.54***	136.28***	11.82**	15.08***	6.60*
Stolon length (cm)	65.69***	372.13***	14.88**	95.61***	9.01**	9.01**	4.52*
df	1	1	1	1	1	1	1
Error	32	32	32	32	32	32	32

[1]*Lg, transformed. *P < 0.05, **P < 0.01, ***P < 0.001.*

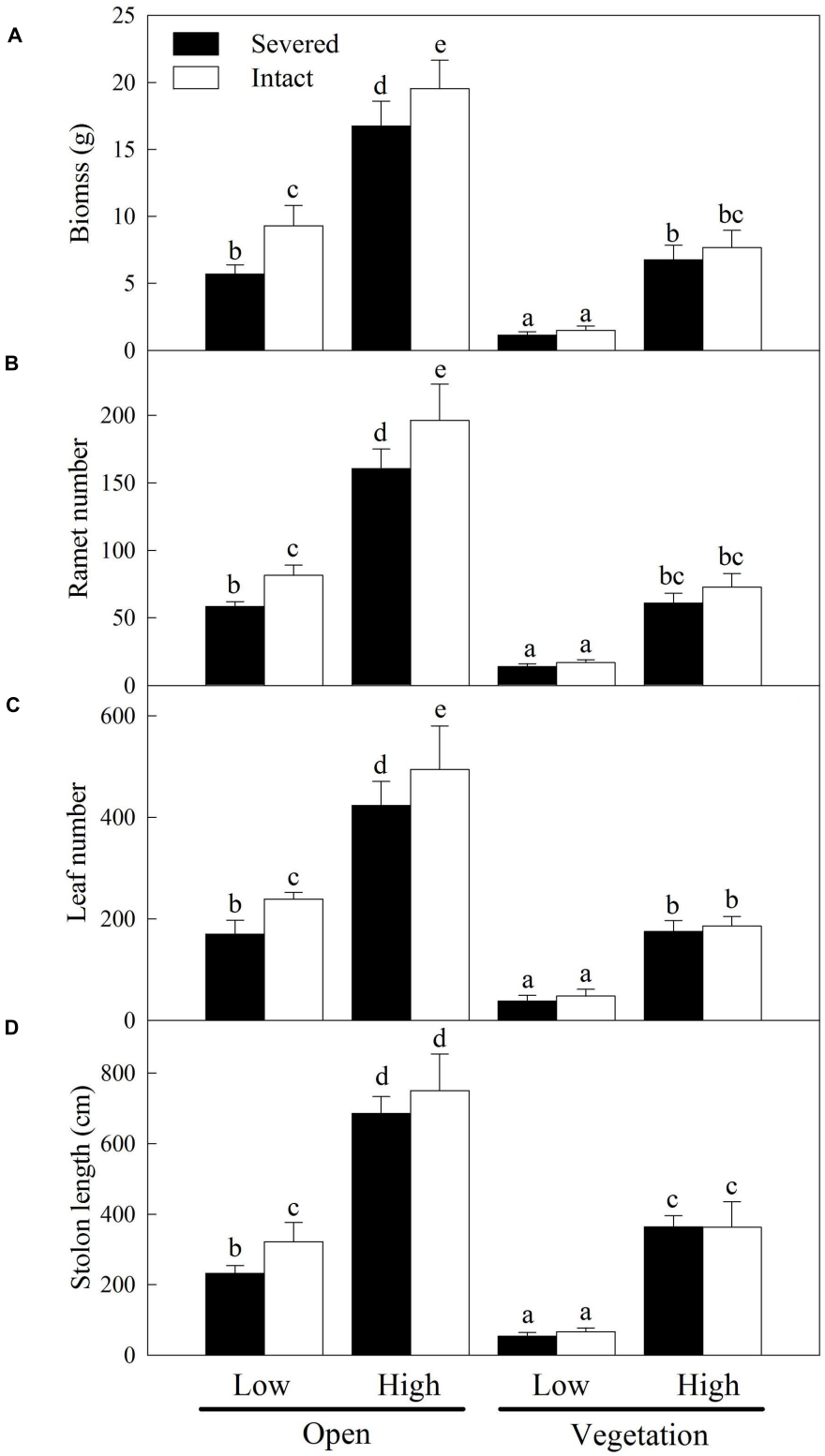

FIGURE 2 | Effects of experimental treatments on the growth measures of *A. philoxeroides* at container level. Total biomass **(A)**, ramet number **(B)**, leaf number **(C)**, and stolon length **(D)** of the invasive plant *A. philoxeroides* (low or high propagule pressure) in the apical sections, grown either in open habitats or in vegetative habitats (*J. repens*), and with stolon connections between basal and apical ramets were either intact or severed. Data indicate the means ± SE. Bars sharing the same letter are not significantly different at $P = 0.05$.

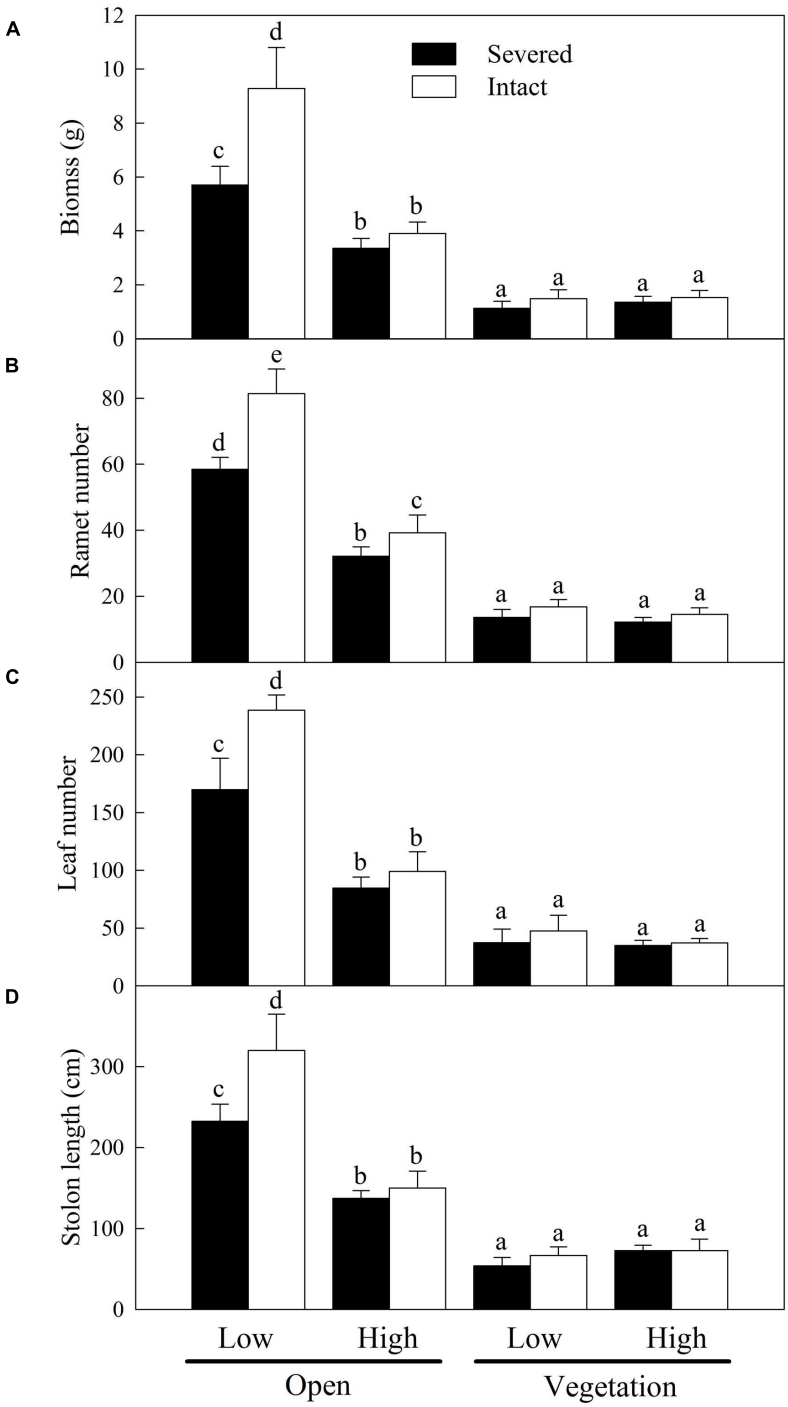

FIGURE 3 | Effects of experimental treatments on the growth measures of *A. philoxeroides* at initial individual level. Total biomass **(A)**, ramet number **(B)**, leaf number **(C)**, and stolon length **(D)** of the invasive plant *A. philoxeroides* (low or high propagule pressure) in the apical sections, grown either in open habitats or in vegetative habitats (*J. repens*), and with stolon connections between basal and apical ramets were either intact or severed. Data indicate the means ± SE. Bars sharing the same letter are not significantly different at $P = 0.05$.

DISCUSSION

As more propagules arrive in a new habitat, the probability of successful invasion increases due to either increased propagule numbers or increased frequency of arrival events (Lockwood et al., 2005; Simberloff, 2009). Therefore, propagule pressure may be the primary control parameter for preventing invasions (Lockwood et al., 2005; Liu et al., 2014). In our study,

TABLE 2 | Two-way ANOVA analyses for the effects of habitat conditions (vegetation) and clonal integration (connection) on the intraspecific relative competition intensity (RCI), and the effects of propagule pressure and clonal integration on the interspecific RCI of the growth measures of the invasive plant *A. philoxeroides* in the apical sections.

Source of variation	df	Error	Biomass (g)	Ramet number[1]	Leaf number[1]	Stolon length (cm)
Intraspecific RCI						
Vegetation (V)	1	16	48.46***	25.89***	25.92***	40.95***
Connection (C)	1	16	3.01	0.74	2.10	4.68*
V × C	1	16	0.01	0.03	0.18	1.14
Interspecific RCI						
Propagule pressure (P)	1	16	51.69***	44.17***	30.05***	51.20***
Connection (C)	1	16	0.70	0.35	0.41	0.46
P × C	1	16	1.00	0.24	0.05	0.02

[1]*Lg, transformed. *P < 0.05, ***P < 0.001.*

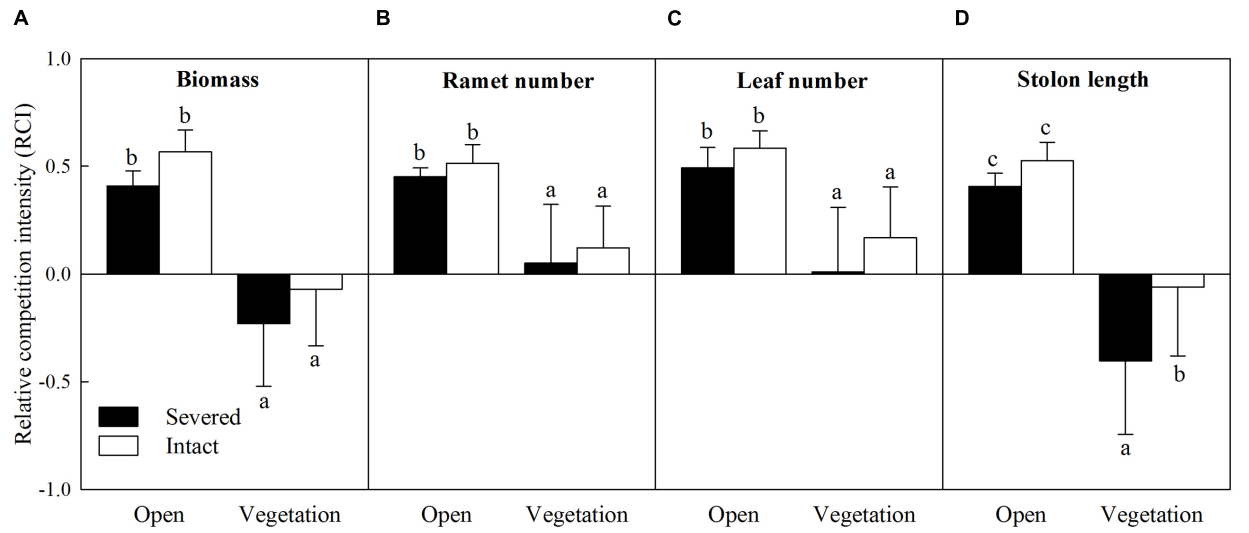

FIGURE 4 | Effects of experimental treatments on the intraspecific relative competition intensity (RCI) of *A. philoxeroides*. The intraspecific RCI of biomass **(A)**, ramet number **(B)**, leaf number **(C)** and stolon length **(D)** of the invasive plant *A. philoxeroides* in the apical sections, grown either in open habitats or in vegetative habitats (*J. repens*), and with stolon connections between basal and apical ramets were either intact or severed. Data indicate the means ± SE. Bars sharing the same letter are not significantly different at $P = 0.05$.

A. philoxeroides with high propagule supply grew better and thus probably had higher chance to establish and invade into new habitats than with low propagule supply in both open and vegetative habitats (Lockwood et al., 2009; Liu et al., 2014). In open habitats, high propagule supply increased the growth and clonal propagation (ramet number and stolon length) of *A. philoxeroides* at the expense of reducing the growth of individual plants (**Figure 3**) due to increase in intraspecific competition (intraspecific RCI was relatively high, **Figure 4**). Surprisingly, however, when grown with native vegetation, the enhanced performance of *A. philoxeroides* by high propagule supply did not sacrifice the growth of individual plants. These results support our first hypothesis, suggesting that the role of propagule pressure in the growth and invasion of *A. philoxeroides* population may be more important in vegetative habitats than in open habitats (Liu et al., 2014).

In open habitats, clonal integration significantly improved the growth of *A. philoxeroides* with both low and high propagule supply. This result occurred most likely because the relatively older ramets in the basal sections supported the growth of the interconnected young apical ramets and facilitated the production of new tissue due to acropetal (from basal ramets to apical ramets) translocation of carbohydrates, suggesting that clonal integration plays an important role in exploring new open space and rapid expansion for this invasive plant (Wang et al., 2008; You et al., 2013). Moreover, with low propagule supply, clonal integration also resulted in increased biomass and ramet production of the individual plants, suggesting that clonal integration may be crucial for growth and spread of *A. philoxeroides* in new habitats when propagule pressure is relatively low. However, clonal integration contributed little to the growth and competitive ability of *A. philoxeroides* in vegetative habitats, even with high propagule supply (**Figures 2–5**). This result does not support our second hypothesis, probably because the role of clonal integration in the invasion process of

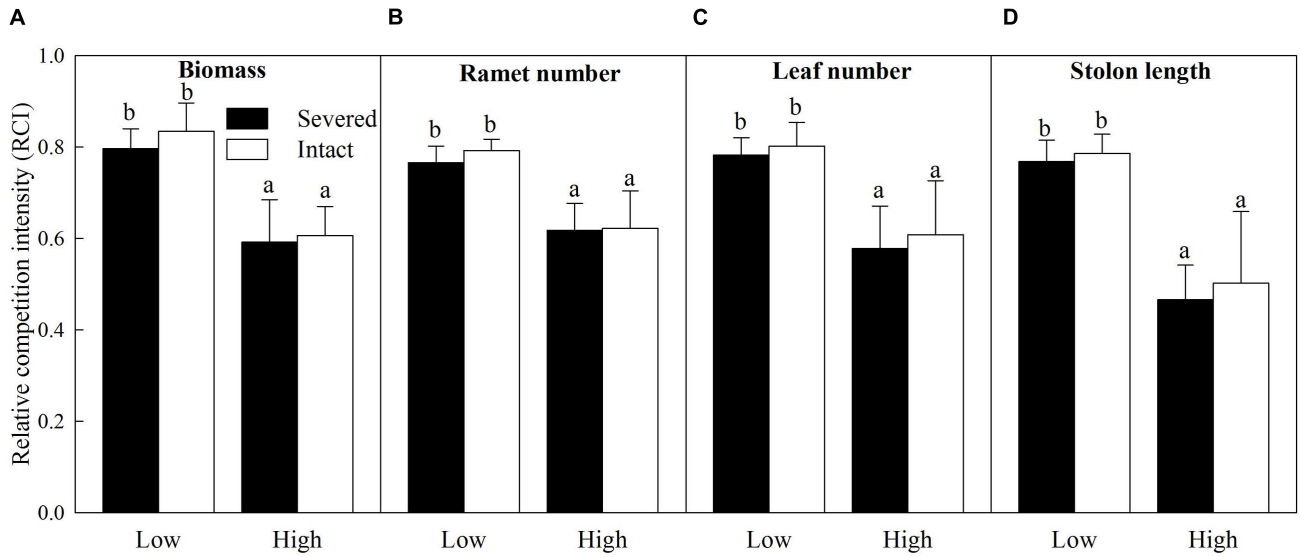

FIGURE 5 | Effects of experimental treatments on the interspecific RCI of *A. philoxeroides*. The interspecific RCI of biomass **(A)**, ramet number **(B)**, leaf number **(C)** and stolon length **(D)** of the invasive plant *A. philoxeroides* in the apical sections under either low or high propagule pressure, and with stolon connections between basal and apical ramets were either intact or severed. Data indicate the means ± SE. Bars sharing the same letter are not significantly different at $P = 0.05$.

A. philoxeroides is occupying open new space and spread, but not increasing competitive ability (Wang et al., 2008; You et al., 2014a).

Without native vegetation, the intraspecific interaction of *A. philoxeroides* was competition, as verified by the positive values of RCI (**Figure 4**). However, in vegetative habitats, there are both intraspecific and interspecific interactions (Mangla et al., 2011; Liu et al., 2014). Under such a habitat condition, the interspecific interaction on *A. philoxeroides* was competition (**Figure 5**, positive values of RCI), whereas the intraspecific interaction on *A. philoxeroides* was facilitation, as demonstrated by the negative values of RCI (**Figure 4**). The shift in the intraspecific interaction on *A. philoxeroides* from competition in open habitats to facilitation in vegetative habitats was also found by Liu et al. (2014), which showed a similar trend of intraspecific interaction on another introduced clonal plant, *Hydrocotyle vulgaris*. The shift in the intraspecific interaction occurred most likely because the native vegetation *J. repens* imposed a severe interspecific competition on *A. philoxeroides*, as shown by the relatively high positive values of RCI (Fajardo and McIntire, 2011). Therefore, the relatively importance of intraspecific and interspecific interactions of *A. philoxeroides* may directly affect the role of propagule pressure in its invasion to native plant community. For example, when propagules of *A. philoxeroides* are introduced into a habitat with dense native vegetation, if the relative effect of intraspecific interaction is lower than interspecific interaction or even shifts to facilitation, as the results showed in this investigation, then high propagule pressure will undoubtedly facilitate the invasion of *A. philoxeroides*. This finding may partially explain why invasions of *A. philoxeroides* are so wide in diverse habitat conditions. Hence, reducing propagule pressure in introduced regions may effectively control the invasion success of this clonal weed into native vegetation, thereby preventing biodiversity loss of native plant communities due to plant invasion (Lockwood et al., 2005; Warren et al., 2012). In the other case, if the relative effect of intraspecific interaction is higher than that of interspecific interaction, then the positive effects of high propagule pressure on invasion success of exotic species may be counteracted by the severe intraspecific competition of individual plants (Liu et al., 2014). In this situation, high propagule pressure will contribute little to the invasion of introduced plants to native plant communities, and controlling the propagule number may not be an effective way to prevent invasion for these species.

Interestingly, total biomass of native vegetation (*J. repens*) was not affected by the presence of *A. philoxeroides*. This result did not accord with our third hypothesis, suggesting that invasion of *A. philoxeroides* in present study did not suppress growth of native plant populations. This is most likely because that competition between apical ramets and native vegetation was asymmetrical because of low density of *A. philoxeroides* in this experiment and their biomass was too small to influence *J. repens* (You et al., 2014a). This observation was supported by the fact that biomass of apical parts of *A. philoxeroides* in vegetative habitats was sharply decreased to less than 35% as compared with that in open habitats. These findings suggest that the propagule pressure (propagule number) examined in this study did not reach or exceed a certain level to influence native vegetation, indicating that *A. philoxeroides* needed more propagules or more time to accumulate enough propagules to establish itself and then invade native plant communities (Lockwood et al., 2009; Simberloff, 2009).

CONCLUSION

In conclusion, increased propagule pressure greatly facilitated the growth and potential invasion of *A. philoxeroides*, especially when it grew in vegetative habitats. This is probably due to the shift in the intraspecific interaction on *A. philoxeroides* from competition in open habitats to facilitation in vegetative habitats. Moreover, clonal integration did not affect the growth and competitive ability of *A. philoxeroides* in vegetative habitats, even with high propagule supply, suggesting that clonal integration may be of most important for *A. philoxeroides* to explore new open space and spread, especially when propagule supply was low, but may contribute little to its competitive ability and invasion to native vegetation. Using a control experiment, we show that even a relatively small difference in the number of propagule supply can greatly affect the invasion success of invasive clonal plants, and such an effect also depends on habitat conditions. Furthermore, our study may add support to the argument that high propagule pressure may facilitate invasion (Lockwood et al., 2005; Simberloff, 2009), suggesting that the effects of propagule pressure on the establishment and growth of clonal plants may be an important component of plant risk assessment that is used to identify their potential invasiveness before introduced.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: W-HY and D-LD. Performed the experiments: W-HY, C-MH, and L-XF. Analyzed the data: W-HY and C-MH. Contributed reagents/materials/analysis tools: W-HY and D-LD. Wrote the paper: W-HY and DLD.

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Roles of Clonal Integration in both Heterogeneous and Homogeneous Habitats

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Many studies have shown that clonal integration can promote the performance of clonal plants in heterogeneous habitats, but the roles of clonal integration in both heterogeneous and homogeneous habitats were rarely studied simultaneously. Ramet pairs of *Alternanthera philoxeroides* (Mart.) Griseb were placed in two habitats either heterogeneous or homogeneous in soil nutrient availability, with stolon connections left intact or severed. Total biomass, total length of stolons, and number of new ramets of distal (relatively young) ramets located in low-nutrient environments were significantly greater when the distal ramets were connected to than when they were disconnected from proximal (relatively old) ramets located in high-nutrient environments. Total length of stolons of proximal ramets growing in low-nutrient environments was significantly higher when the proximal ramets were connected to than when they were disconnected from the distal ramets growing in high-nutrient environments, but stolon connection did not affect total biomass or number of new ramets of the proximal ramets. Stolon severing also did not affect the growth of the whole ramet pairs in heterogeneous environments. In homogeneous high-nutrient environments stolon severing promoted the growth of the proximal ramets and the ramet pairs, but in homogeneous low-nutrient environments it did not affect the growth of the proximal or distal ramets. Hence, for *A. philoxeroides*, clonal fragmentation appears to be more advantageous than clonal integration in resource-rich homogeneous habitats, and clonal integration becomes beneficial in heterogeneous habitats. Our study contributes to revealing roles of clonal integration in both heterogeneous and homogeneous habitats and expansion patterns of invasive clonal plants such as *A. philoxeroides* in multifarious habitats.

Keywords: *Alternanthera philoxeroides*, clonal integration, heterogeneous habitat, homogeneous habitat, nutrients, severing

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INTRODUCTION

Plant invasion has become a significant threat to biodiversity, environment, and economy both globally and locally (Mack et al., 2000; Liu et al., 2005; Vila et al., 2011). Many notorious invasive plants are clonal, with the capability of vigorous clonal propagation (Dong, 1996; Pysek et al., 2003; Liu et al., 2006; Wang et al., 2008; Keser et al., 2014). For example, *Alternanthera philoxeroides* (Mart.) Griseb is an invasive clonal plant, which has heavily invaded many areas of the world (Julien et al., 1995; Ye et al., 2003; Wang et al., 2008; Zhang et al., 2015). Clonal growth has been considered

an important trait for invasive clonal plants (Liu et al., 2008; Wang et al., 2009; Xu et al., 2010; Roiloa et al., 2013; Song et al., 2013; You et al., 2013). For instance, *A. philoxeroides* can expand from terrestrial to aquatic environments with the support of clonal integration (Wang et al., 2009). Clonal integration can also aid the spreading of *A. philoxeroides* and *Vallisneria spiralis* L. into competitive environments (Xiao et al., 2011; You et al., 2014).

Heterogeneity is common in nature (Hutchings and Wijesinghe, 1997; Alpert, 1999; Dong et al., 2015; Keser et al., 2015). Numerous studies have investigated the strategies of clonal plants to cope with habitats with heterogeneous distributions of, e.g., nutrients, light, space, and others (Liu et al., 2008; Wang et al., 2009; Xu et al., 2010; Song et al., 2013; Roiloa et al., 2014b). These studies have shown that in heterogeneous environments, ramets exposed to stressful environments commonly perform better when they are integrated with ramets located in non-stressful conditions (Wang et al., 2008; Xu et al., 2010; Song et al., 2013; You et al., 2013; Roiloa et al., 2014a).

Natural environments can also be homogeneous at the scale of plant growth (Stuefer, 1996; Dong et al., 2015). Theoretical models (Caraco and Kelly, 1991; Alpert, 1999) have predicted that clonal integration may be disadvantageous in environments with a homogeneous supply of resources. In a recent study, however, Dong et al. (2015) developed a conceptual model showing that clonal integration may also have a positive effect on the growth of clonal plants when connected ramets in resource-rich habitats have different uptake abilities. Some studies have tested the roles of clonal integration in homogeneous environments (e.g., Salzman and Parker, 1985; Evans and Whitney, 1992; Yu et al., 2002; He et al., 2011), but very few have detected a significant effect.

We conducted a greenhouse experiment on *A. philoxeroides* to test effects of clonal integration in both heterogeneous and homogeneous environments. We grew ramet pairs of *A. philoxeroides* in both homogeneous high and low soil nutrient conditions and heterogeneous conditions with a high and a low soil nutrient patch, with the stolon connecting the two ramets of a pair severed or left connected. We detected that clonal integration could play significant roles in both homogeneous and heterogeneous environments.

MATERIALS AND METHODS

Plant Materials

Alternanthera philoxeroides is a perennial weed from the family Amaranthaceae. It is native to South America but widespread in a variety of habitats around the world (Julien et al., 1995; Geng et al., 2007; Zhang et al., 2015). In most of the introduced regions, it reproduces asexually, primarily from stem nodes and shoot fragments (Julien et al., 1995; Geng et al., 2007). Despite the extremely low genetic diversity of *A. philoxeroides* in China (Xu et al., 2003; Wang et al., 2005), the species experienced massive vegetative propagation and rapid expansion in China (Xu et al., 2003; Ye et al., 2003; Wang et al., 2005; Zhu et al., 2015).

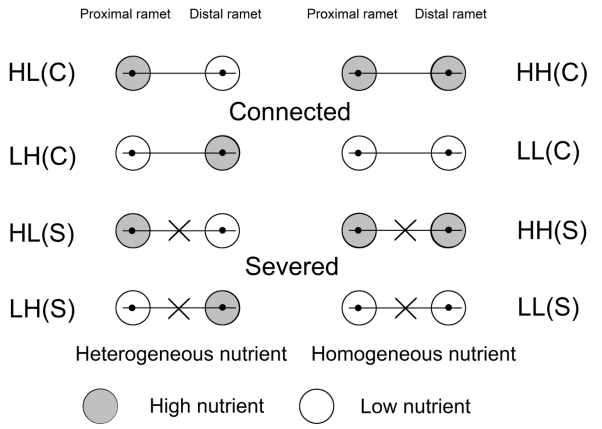

FIGURE 1 | Schematic representation of the experimental design. Ramet pairs of *Alternanthera philoxeroides*, consisting of proximal and distal ramets, were planted in either heterogeneous or homogeneous nutrient habitats, with stolon connections left intact (C) or severed (S). Proximal and distal ramets were separately placed in two pots. The gray and white circles represented high (H) and low (L) nutrients, respectively.

We collected eight *A. philoxeroides* plants from a cropland in Kunming, China, in April, and transplanted them into a greenhouse under natural sunlight and ambient temperature for propagation. After 4 months of cultivation, we selected newly produced ramet pairs that were similar in size. To eliminate possible effects of genotype, ramet pairs in every treatment were from the eight original plants. Also, genetic diversity of *A. philoxeroides* is extremely low in China (Xu et al., 2003; Wang et al., 2005). In each ramet pair, the two ramets were connected and were rooted in two plastic pots filled with river sand. The ramets were standardized to the same size (two leaves and 2-cm-long root). Standardization was conducted once a week and repeated three times.

Experimental Design

The treatments with different soil nutrient availabilities and stolon severing were performed on August 14. The ramets in each pair (64 pairs) were exposed to: (1) heterogeneous distribution of soil nutrients (high in proximal ramet and low in distal ramet, HL); (2) heterogeneous distribution of soil nutrients (low in proximal ramet and high in distal ramet, LH); (3) homogeneous distribution of high levels of soil nutrients (high in both proximal and distal ramets, HH); and (4) homogeneous distribution of low levels of soil nutrients (low in both proximal and distal ramets, LL). There were 16 ramet pairs for every nutrient treatment, and eight with connected stolons (C) and eight with severed stolons (S) (**Figure 1**). There were eight replicates in each of the eight treatments. The ramet pairs for the experiment were taken from eight plants and all treatments included ramet pairs originating from the same eight initial plants.

To create low level of nutrients, 0.1 g Peters Professional (20% N, 20% P, 20% K; The Scotts Company, LLC., Marysville, OH, USA) was added to the sand at the beginning of the experiment; this amount supports the survival of *A. philoxeroides* but restricts

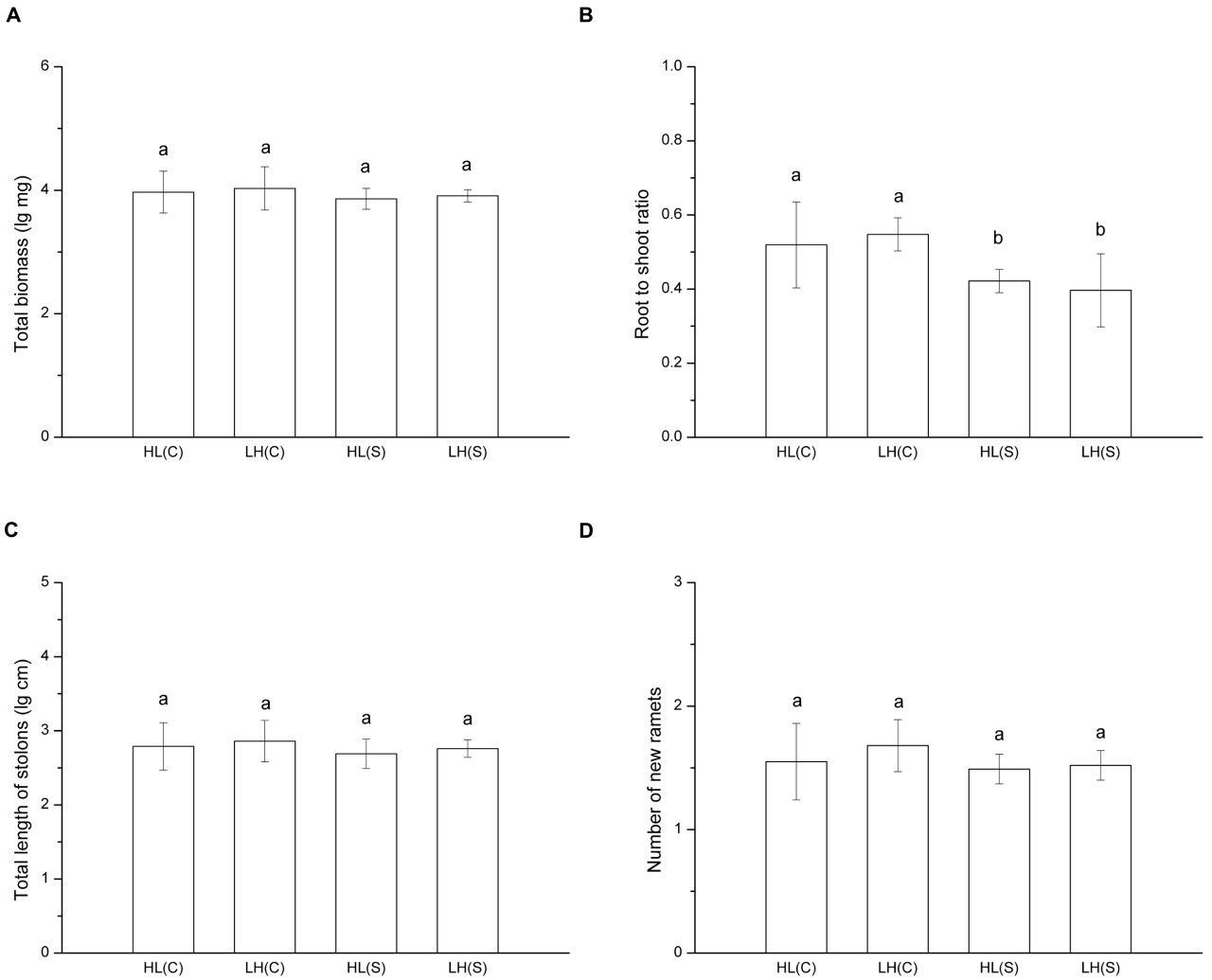

FIGURE 2 | Growth and biomass allocation of ramet pairs in heterogeneous habitats. (A) Total biomass, **(B)** root to shoot ratio, **(C)** total length of stolons, **(D)** number of new ramets. Values sharing the same letter are not significantly different at $\alpha = 0.05$. Total biomass, total length of stolons, and number of new ramets were log transformed to satisfy the normality and homogeneity of variance.

its growth. To create high level of nutrients, 0.1 g Peters Professional was added daily to each pot during the experimental period to promote plant growth. All pots were placed randomly in the greenhouse and watered daily with 100 mL of water every afternoon. The temperature in the greenhouse was about 20–35°C and the light intensity was 70% of full daylight during the experiment. The treatments were conducted for 2 months and the plants were harvested on October 15.

Measurement and Data Analysis

Before the harvest, number of new ramets for each proximal and distal ramet was counted. The roots were then washed by hand to remove sand and plants were harvested and stored in a refrigerator (5°C) for further measurements. The total length of each stolon was measured and the plants were separated into roots, leaves, and stolons and oven-dried at 70°C for 72 h to determine the dry weight.

Before analysis, the data that did not meet normality were log transformed. We analyzed the growth and biomass allocation of *A. philoxeroides* in heterogeneous and homogeneous habitats with respect to different effects of clonal integration and various nutrient availabilities in the two habitats. The data were analyzed by using one-way analysis of variance and Duncan test. All analyses were conducted in IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA).

RESULTS

Growth and Biomass Allocation in Heterogeneous Habitats

In treatments with heterogeneous distribution of nutrients, total biomass ($F = 0.630$, $p > 0.05$), total length of stolons ($F = 0.699$,

$p > 0.05$), and number of new ramets ($F = 1.311$, $p > 0.05$) of each ramet pair (proximal ramet plus distal ramet) did not show a significant difference among treatments (**Figures 2A,C,D**). However, root to shoot ratio of ramets exposed to HL(C) and LH(C) was significantly higher than that in HL(S) and LH(S) ($F = 6.262$, $p < 0.01$; **Figure 2B**).

Total biomass of proximal ramets did not differ significantly between HL(C) and HL(S), but total biomass of distal ramets was significantly higher in HL(C) than in HL(S) (**Figure 3A**). In contrast, total biomass of proximal or distal ramets did not show significant differences between LH(C) and LH(S) (**Figure 3A**). Root to shoot ratio of proximal ramets in LH(S) was the highest among the four treatments (**Figure 3B**). Root to shoot ratio of proximal ramets in HL(C) was similar to that in LH(C), but significantly higher than that in HL(S) (**Figure 3B**). Similarly, root to shoot ratio of distal ramets in HL(S) was the highest among the four treatments (**Figure 3B**). Root to shoot ratio of distal ramets in LH(C), similar to distal ramets in HL(C), exceeded that of distal ramets in LH(S) (**Figure 3B**). Total stolon length of proximal ramets in HL(C) and HL(S) was similar, whereas that of distal ramets was significantly greater in HL(C) than in HL(S) (**Figure 3C**). Likewise, total stolon length of distal ramets did not show significant difference between LH(C) and LH(S), whereas total stolon length of proximal ramets was greater in LH(C) than in LH(S) (**Figure 3C**). In addition, number of new ramets showed a similar trend to that of total biomass (**Figure 3D**).

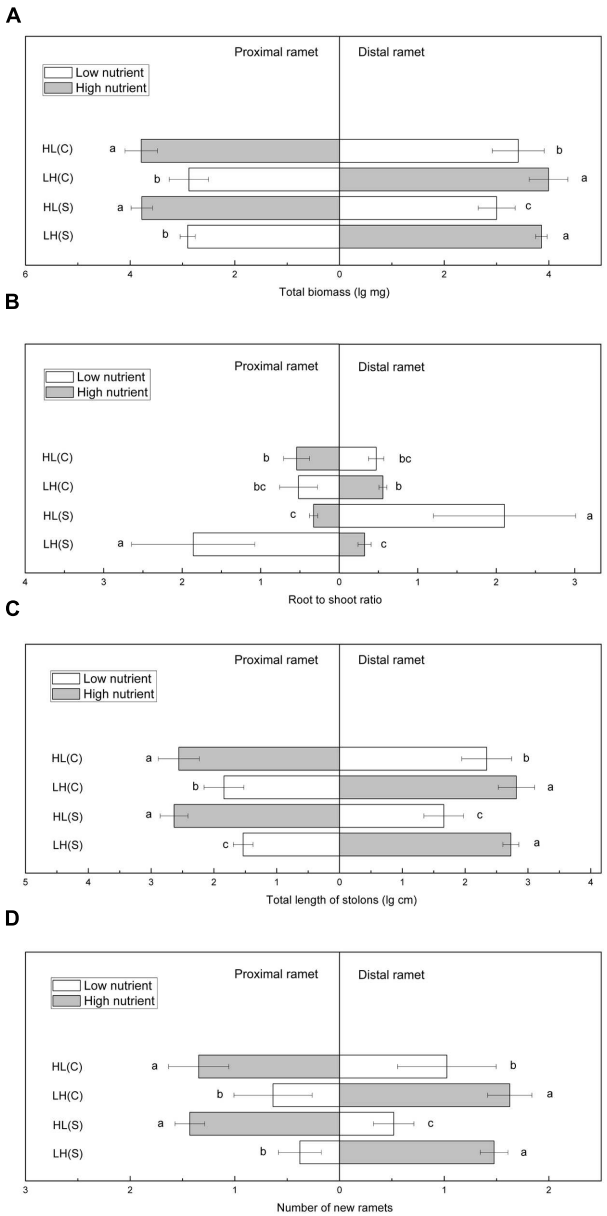

FIGURE 3 | Growth and biomass allocation of proximal and distal ramets in heterogeneous habitats. (A) Total biomass, **(B)** root to shoot ratio, **(C)** total length of stolons, **(D)** number of new ramets. Values sharing the same letter are not significantly different at $\alpha = 0.05$. Total biomass, total length of stolons, and number of new ramets were log transformed to satisfy the normality and homogeneity of variance.

Growth and Biomass Allocation in Homogeneous Habitats

In treatments with homogeneous nutrient availabilities, total biomass of ramet pairs (proximal ramet plus distal ramet) was significant smaller in HH(C) than in HH(S) and it did not differ between LL(C) and LL(S) ($F = 47.927$, $p < 0.001$; **Figure 4A**). Root to shoot ratio showed no difference between stolon-connected and stolon-severed treatments in high (HH[C] and HH[S]) or low (LL[C] and LL[S]) nutrient availabilities, while the ratio was much lower in high nutrient availabilities than in low nutrient availabilities ($F = 24.673$, $p < 0.001$; **Figure 4B**). Total stolon length of ramet pairs exhibited the same trends as total biomass among the four treatments ($F = 65.073$, $p < 0.001$; **Figure 4C**). Ramet pairs had similar number of new ramets between HH(C) and HH(S), but ramet pairs had less new ramets in LL(C) than in LL(S) ($F = 26.488$, $p < 0.001$; **Figure 4D**).

In homogeneous high nutrient conditions, total biomass of proximal ramets was significantly smaller in HH(C) than in HH(S), while total biomass of distal ramets in HH(C) and HH(S) did not show any difference (**Figure 5A**). In homogeneous low nutrient availabilities, total biomass of proximal or distal ramets showed no difference between LL(C) and in LL(S) (**Figure 5A**). Root to shoot ratio of proximal ramets was similar between HH(C) and HH(S) or between LL(C) and LL(S), and so it was for root to shoot ratio of distal ramets (**Figure 5B**). Total stolon length of proximal and distal ramets exhibited the similar trends to that of total biomass (**Figure 5C**). Number of new ramets of proximal ramets was significantly less in HH(C) than in HH(S), but that of distal ramets was not significantly different between HH(C) and HH(S) (**Figure 5D**). New ramets of proximal and distal ramets showed similar trends in low nutrient (LL[C] and LL[S]) availabilities to that in high nutrient availabilities (**Figure 5D**).

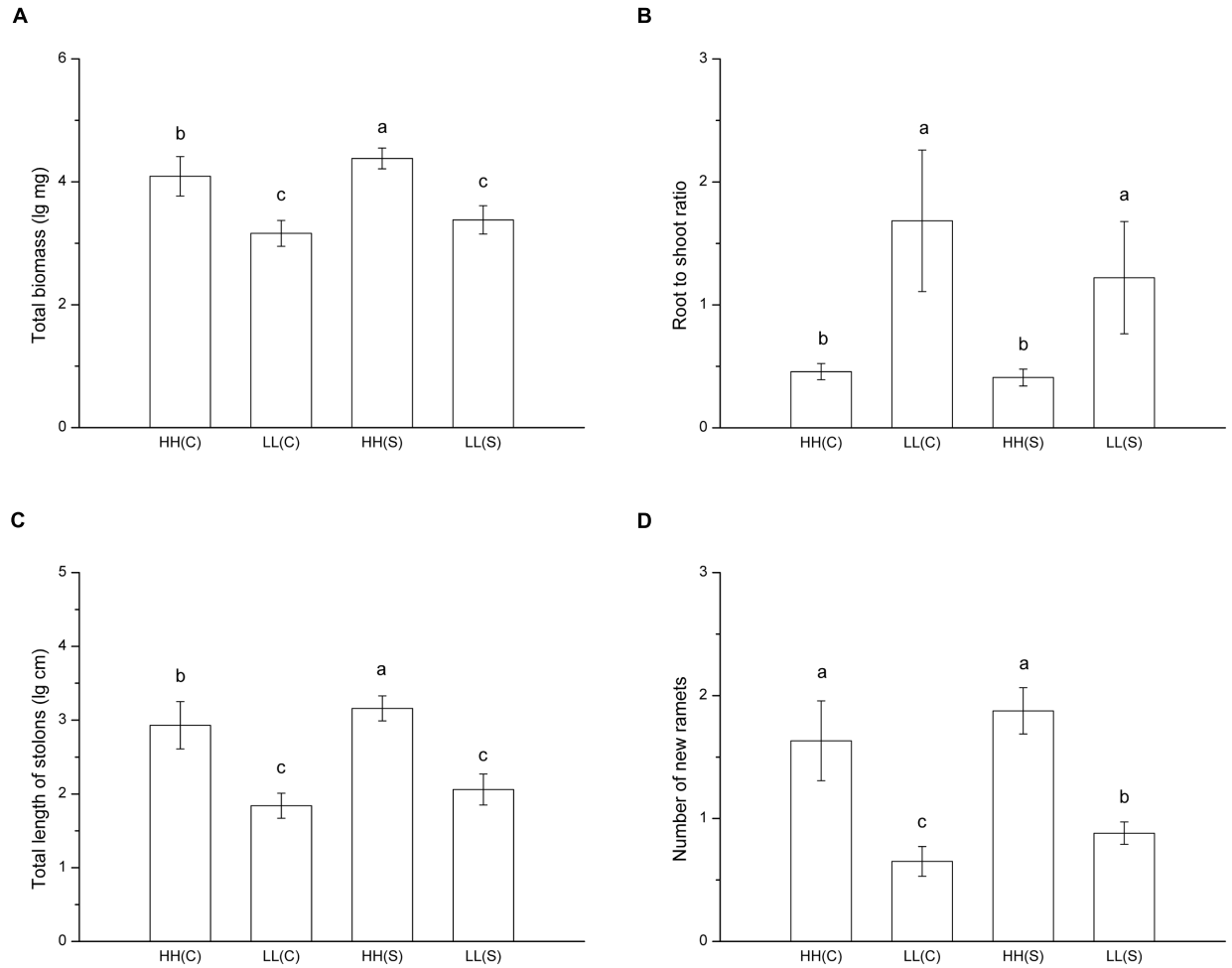

FIGURE 4 | Growth and biomass allocation of ramet pairs in homogeneous habitats. (A) Total biomass, **(B)** root to shoot ratio, **(C)** total length of stolons, **(D)** number of new ramets. Values sharing the same letter are not significantly different at $\alpha = 0.05$. Total biomass, total length of stolons, and number of new ramets were log transformed to satisfy the normality and homogeneity of variance.

DISCUSSION

Clonal Integration in Heterogeneous Habitats

In heterogeneous habitats, clonal plants translocate resources among ramets through clonal integration, promoting the growth of ramets in stressful habitats (Wang et al., 2008; Xu et al., 2010; Song et al., 2013; Roiloa et al., 2014a). In the present study, we confirmed that in heterogeneous soil nutrient conditions total biomass, total length of stolons, and number of new ramets of distal ramets were significantly greater when stolon connection between ramets were left intact than when it was severed. Clonal integration did not reduce the growth of proximal ramets, which was in agreement with previous reports (Pauliukonis and Gough, 2004; Song et al., 2013). However, clonal integration did not affect the growth of ramet pairs, disagreeing with previous findings of a meta-analysis (Song et al., 2013). This may be because performance of proximal ramets was much better than that of distal ramets in all ramet pairs under this nutrient condition.

When proximal ramets were grown in low nutrient conditions and distal ramets in high nutrient conditions, clonal integration increased total length of stolons but did not affect total biomass or number of new ramets in proximal ramets. Therefore, proximal ramets in low nutrient conditions benefited a little from distal ramets in high nutrient conditions, which agreed with the study on *Hydrocotyle peduncularis* (Peterson and Chesson, 2002). The performance of ramets in two heterogeneous habitats indicate that clonal integration of *A. philoxeroides* is bidirectional and differentiated. It is highly acropetal and lowly basipetal, and distal ramets can obtain more resources than proximal ramets when they are grown in low nutrient habitats and connected to ramets in high nutrient habitats. This effect may promote the escape of this species from barren habitats in heterogeneous nutrient environments (Zhou et al., 2012).

Clonal integration increased root to shoot ratio of ramet pairs in heterogeneous habitats. Specifically, clonal integration

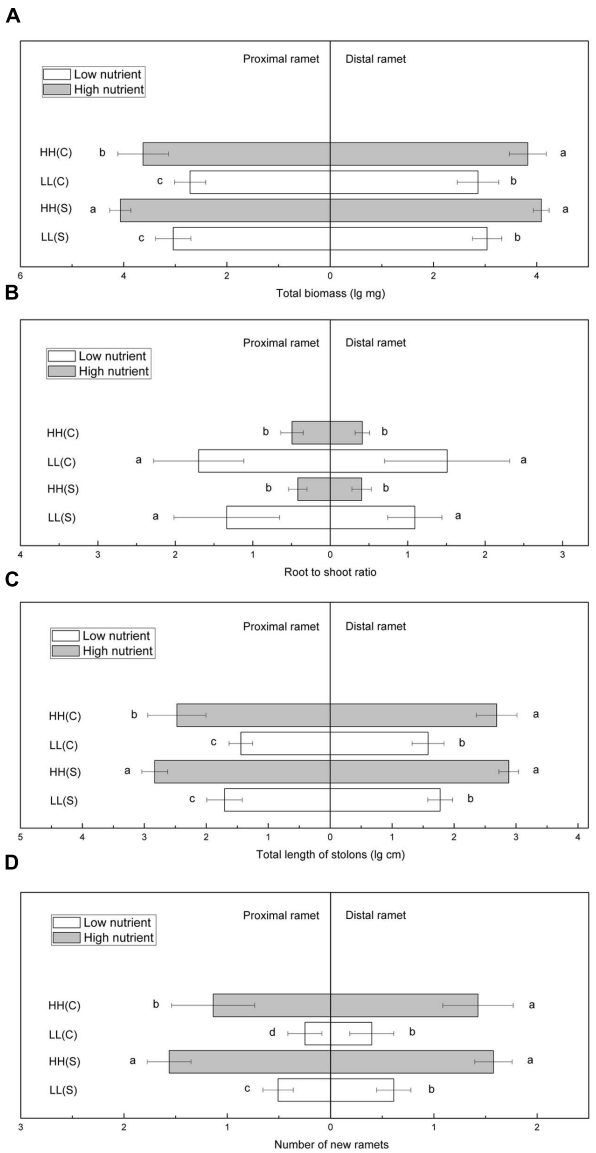

FIGURE 5 | Growth and biomass allocation of proximal and distal ramets in homogeneous habitats. (A) Total biomass, **(B)** root to shoot ratio, **(C)** total length of stolons, **(D)** number of new ramets. Values sharing the same letter are not significantly different at $\alpha = 0.05$. Total biomass, total length of stolons, and number of new ramets were log transformed to satisfy the normality and homogeneity of variance.

increased root to shoot ratio of ramets in high-nutrient conditions but decreased root to shoot ratio of ramets in low-nutrient conditions. As biomass of ramets was much larger in high-nutrient conditions than in low-nutrient conditions, root to shoot ratio of ramet pairs was dominated by ramets in the zone with high nutrient availabilities. These results indicated that labor division occurred in proximal and distal ramets in heterogeneous nutrient conditions (You et al., 2014). However, when stolon connections were severed, ramets located in low-nutrient habitats directed more biomass into roots to enhance absorption, and ramets in high-nutrient habitats allocated less biomass to roots. Therefore, *A. philoxeroides* has high plasticity in biomass allocation no matter whether stolon connection was severed or not, which may contribute to its invasiveness (Geng et al., 2007; Keser et al., 2014).

Effects of Stolon Severing in Homogeneous Habitats

Many studies showed that stolon severing decreases the growth of distal ramets and increases the performance of proximal ramets in heterogeneous habitats (Wang et al., 2009; Song et al., 2013). It is believed that clonal integration does not affect performance of clonal plants in homogeneous habitats (Evans and Whitney, 1992; Yu et al., 2002; He et al., 2011). Nevertheless, in our study, stolon severing played significant roles in homogeneous habitats. In habitats with homogeneous distribution of high nutrient availabilities, stolon severing increased biomass of proximal ramets and the whole ramet pairs but did not affect that of distal ramets. Similarly, stolon severing increased biomass of proximal ramets and the entire clone in *Pistia stratiotes* (Wang et al., 2014). It is considered that severing eliminates the effects of distal ramets on proximal ramets (such as resource transportation, metabolic costs, and apical dominance), resulting in increasing growth of proximal ramets (Pauliukonis and Gough, 2004; Wang et al., 2009, 2014). By comparison, in habitats with homogeneous distribution of low nutrient availabilities, severing did not affect biomass of proximal or distal ramets as well as whole ramet pairs. These results demonstrated that clonal fragmentation was more advantageous than clonal integration in high nutrient habitats (Oborny and Kun, 2002).

In homogeneous habitats, root to shoot ratio was significantly lower in high-nutrient conditions than in low-nutrient conditions no matter whether stolons were severed or not. Stolon severing increased total length of stolons and number of new ramets, especially in proximal ramets, under homogeneous high-nutrient conditions, which was different from the performance of *P. stratiotes* (Wang et al., 2014). This difference is mostly the result of species-specific plasticity, just as the effects of clonal integration on morphological traits of *A. philoxeroides* and *Phyla canescens* are species-specific (Xu et al., 2012). These results also confirmed high plasticity of *A. philoxeroides*, which partly answers why *A. philoxeroides* can live in diverse habitats (Geng et al., 2007).

CONCLUSION

Clonal integration, which is bidirectional and differentiated in *A. philoxeroides*, could not significantly promote biomass accumulation of ramet pairs of *A. philoxeroides* but increased total length of stolons and number of new ramets of proximal or distal ramets in stressful habitats, contributing to the spreading of *A. philoxeroides* in heterogeneous habitats. In habitats with homogeneous distribution of high nutrient availabilities, stolon severing—which often occurs due to natural and/or artificial disturbance—promoted growth of proximal ramets and ramet pairs, owing to the absence of the effects from distal ramets.

Hence, *A. philoxeroides*, with high plasticity, can employ different strategies to cope with various habitats. Our study contributes to revealing roles of clonal integration in both heterogeneous and homogeneous habitats and expansion patterns of invasive clonal plants such as *A. philoxeroides* in multifarious habitats.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JL, HZ, and FL. Performed the experiments: JL and HZ. Analyzed the data: JL, HZ, and RW. Wrote the paper: HZ, JL, FL, and RW.

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