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Mosaic distribution of cytotypes in a mixed-ploidy plant species, *Jasione montana*: nested environmental niches but low geographical overlap

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Polyploids often have divergent geographical ranges compared to their diploid progenitors, but the causes of such differentiation are poorly understood. The geographical ranges of cytotypes within polyploid complexes may be caused by multiple factors, including historical events, interactions among cytotypes and divergent environmental tolerances, but the fine-scale geographical arrangement of cytotypes is rarely known for most mixed-ploidy species. In this study, we assessed cytotype diversity and distribution patterns in the *Jasione montana* polyploid complex and examined whether environmental factors can explain the occurrence of tetraploids. Specifically, we reviewed all chromosome counts available in the literature, examined cytotype distributions in a large-scale population survey using flow cytometry (N = 278 populations, N = 3396 plants), and used niche modelling to compare cytotype environmental associations. Two cytotypes were detected: diploids, which are widespread across Europe, and tetraploids, restricted to the north-west quadrant of the Iberian Peninsula. The two cytotypes were distributed in a mosaic with areas dominated by diploids intermixed with those dominated by tetraploids, rarely forming mixed-ploidy populations (1.4%). Although having low geographical overlap, the tetraploid niche is fully nested within the diploid niche breadth and occupies only a subset of the environmental envelope of the diploid progenitor, suggesting that polyploidization has not caused niche expansion due to novel environmental preferences. The mosaic diploid-tetraploid contact zones and the lack of mixed-ploidy populations suggest that frequency-dependent selection may play a role in excluding minority cytotypes. Under this scenario, tetraploids would have to disperse to places unoccupied by diploids to successfully establish. The aggregation of tetraploid populations in areas suitable for diploids suggests that tetraploids may also outcompete diploids in certain areas. Collectively, our results indicate that environmental sorting has played a role, at least on a broader scale, in the successful establishment of polyploids in *J. montana*.

ADDITIONAL KEYWORDS: diploids – exclusion – flow cytometry – minority cytotype – niche modelling – polyploidy – tetraploids.

INTRODUCTION

The duplication of whole chromosome sets is a common event in nature (Wood *et al.*, 2009; Husband, Baldwin & Suda, 2013; Marques *et al.*, 2018), giving rise to new polyploids that might establish and spread within diploid/lower-ploidy progenitor populations. The recurrent formation of polyploids has been documented multiple times throughout the evolutionary history of particular plant groups (Wood *et al.*, 2009; Otto & Whitton, 2000; Soltis *et al.*, 2010), but also in extant plant populations (e.g. Maceira *et al.*, 1992; Burton & Husband, 2001; Ramsey, 2007; Castro *et al.*, 2018). This is probably due to the frequent production of unreduced gametes in nature (Bretagnolle & Thompson, 1995; Ramsey, 2007; Brownfield & Köhler, 2011), although successful polyploid establishment

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depends on numerous factors (Levin, 1975; Rodriguez, 1996). Because polyploids arise frequently and can have significant ecological and evolutionary effects, polyploidy is currently recognized as a major mechanism of sympatric speciation (Otto & Whitton, 2000; Soltis *et al.*, 2010), being considered an important mechanism of diversification of flowering plants (Soltis & Soltis, 1999). Given its significant contribution, the factors involved in the successful establishment of polyploid lineages have received increased attention in the last decades.

Immediately after polyploid formation, the new cytotype is at a numerical disadvantage within the population of its diploid/lower-ploidy progenitor. Theoretical models suggest that the new polyploid can establish within the population of the progenitor only if it has the necessary conditions to increase its numbers; otherwise, it will be excluded from the population due to frequency-dependent selection (Levin, 1975; Rodriguez, 1996; Husband & Schemske, 2000). Polyploid establishment will be favoured by features that increase the probability of successful mating, such as recurring formation of polyploids, spatial clustering, perenniality, increased selfing and/or increased competitive ability (Fowler & Levin, 1984; Felber, 1991; Rodriguez, 1996; Husband & Schemske, 2000; Barringer, 2007). Assortative mating, enforced by various reproductive barriers, may also promote the coexistence of polyploids and their progenitors [e.g. *Chamerion angustifolium* (L.) Holub, Husband & Sabara 2004; *Aster amellus* L., Jersáková *et al.*, 2010; Castro *et al.*, 2011; *Gladiolus communis* L., Castro *et al.*, 2018]. Alternatively, polyploids might disperse elsewhere, thereby escaping minority cytotype exclusion, and establish new populations beyond the environmental limits of the parental individuals (niche shift hypothesis; Levin, 1975, 2004; Husband & Schemske, 2000).

Different geographical patterns have been documented in diploid–polyploid complexes (namely sympatric, parapatric or allopatric cytotype distributions; e.g. Petit, Bretagnolle & Felber, 1999; Baack, 2004; Kolář *et al.*, 2009; Castro *et al.*, 2012). An association between ploidy and differences in environmental and spatial boundaries is frequently observed (Levin, 2002), with polyploids often having different geographical ranges from their diploid progenitors (e.g. Balao *et al.*, 2009; Kolář *et al.*, 2009; Sonnleitner *et al.*, 2010; Laport *et al.*, 2013; Casazza *et al.*, 2017). The geographical range occupied by each cytotype is the result of several potentially interacting forces, including historical patterns of origin or migration, interactions among cytotypes and divergence in environmental tolerances (Husband *et al.*, 2013). Historical processes, including the timing of polyploid emergence, dispersal and colonization patterns, are among the factors determining geographical patterns (Lowry & Lester, 2006). Recurrent polyploid formation generates primary contact zones and mosaic distributions, whereas secondary contact zones originate after allopatric divergence and subsequent migration, bringing cytotypes into contact and creating allopatric and parapatric distributions (Petit, Bretagnolle & Felber, 1999). Additionally, changes in cell size and gene expression associated with whole genome duplication may have broad-scale impacts on developmental processes and environmental tolerances that might change the fitness of polyploids (Levin, 1983; Adams & Wendel, 2005). These differences have been linked with increased ecological tolerances, niche partitioning and/or wider geographical ranges (e.g. Levin, 1975; Husband & Schemske, 2000; Buggs & Pannell, 2007; Ramsey, 2011; Hao *et al.*, 2013) and may determine the likelihood of polyploid establishment and subsequent spread. Consequently, knowing the geographical arrangement of diploid–polyploid complexes in nature provides essential information for inferring the processes involved in polyploid establishment, coexistence and divergence (e.g. Levin, 2002; Petit *et al.*, 1999; Lexer & van Loo, 2006; Castro *et al.*, 2018).

Recently, ecological niche modelling (Warren, Glor & Turelli, 2008) and multivariate analyses of niche variables (Broennimann *et al.*, 2012) using cytotype occurrence data and various abiotic factors (e.g. precipitation, temperature, soil characteristics and elevation) have been used to characterize ecological niches and to compare environmental niches between different taxa. This approach has been used to evaluate the niche shift hypothesis in related diploid–polyploid species [e.g. species of *Houstonia* Gronov. (Glennon, Rissler & Church, 2012); Iberian taxa of *Leucanthemum* Mill. (Oberprieler *et al.*, 2012); the *Claytonia perfoliata* Donn ex Willd. complex (McIntyre, 2012); the *Primula* L. section *Aleuritia* Duby complex (Theodoridis *et al.*, 2013); species of *Tolmiea* Torr. & A.Gray (Visger *et al.*, 2016)], or in the analysis of different cytotypes within a species [*Houstonia purpurea* L. and *H. longifolia* Gaertn. (Glennon *et al.*, 2012); *Heuchera cylindrica* Douglas (Godsoe *et al.*, 2013); *Chamerion angustifolium* (Thompson, Husband & Maherali, 2014); *Erysimum mediohispanicum* Polatschek (Muñoz-Pajares *et al.*, 2018)]. The characterization of the ecological niches enables researchers, not only to identify the potential environmental constraints on the distribution of different cytotypes, but also to unmask other factors potentially affecting cytogeographic patterns, such as inter-cytotype biotic interactions and historical processes (e.g. Godsoe *et al.*, 2013; Laport *et al.*, 2013; Glennon, Ritchie & Segraves, 2014; Laport, Minckley &

Ramsey, 2016; Castro, 2018; Mairal *et al.*, 2018). Thus, modelling tools have enabled researchers to formulate hypotheses about the successful establishment of certain polyploid complexes and, subsequently, to test these hypotheses experimentally in the field or in controlled conditions, through reciprocal transplants or competition experiments [e.g. *Ranunculus adoneus* A.Gray (Baack & Stanton, 2005); C*hamerion angustifolium* (Martin & Husband, 2013); *Jasione maritima* (Duby) Merino, Castro, 2018].

Jasione L. (Campanulaceae) is a small genus distributed in Europe, North Africa and South-West Asia, with its centre of diversity in the Iberian Peninsula and most of its species showing restricted ranges (Tutin, 1973; Sales & Hedge, 2001a, 2001b; Pérez-Espona *et al.*, 2005). Phylogenetic analyses using ITS suggest a recent origin of the species in the genus, possibly linked with Pleistocene glaciations (Sales *et al.*, 2004; Pérez-Espona *et al.*, 2005), although the evolutionary history of the genus is still unknown. Polyploidy is often expected to be frequent during glaciation periods (Thompson, 2005; Marques *et al.*, 2018), because of the association between the production of unreduced gametes and temperature fluctuations (Ramsey & Schemske, 1998; Mason *et al.*, 2011). *Jasione* comprises several diploid taxa (e.g. *J. foliosa* Cav., *J. corymbosa* Poir.: Silvestre, 1986; Parnell, 1987), but it is also rich in polyploid complexes, including tetraploid species (e.g. *J. sessiliflora* Boiss. & Reut.; Favarger, Galland & Kupfer, 1980) and species with several ploidy levels [e.g. *J. montana* L., *J. laevis* Lam., *J. maritima*, *J. crispa* (Pourr.) Samp.; Sales & Hedge, 2001a; Rubido-Bará, Horjales Luaces & Villaverde, 2010]. Among the latter is the widespread *J. montana*, which was described as diploid throughout its distribution range across Europe (e.g. Kovanda, 1968; Bjorkqvist, 1969; Kliphuis & Wieffering, 1972; Ubera, 1980), until Leitão & Paiva (1988) reported, for the first-time, tetraploid plants in central Portugal, and, more recently, Rubido-Bará *et al.* (2010) described the occurrence of tetraploids in Galicia (Spain). The species exhibits high morphological variability, with diversity of habits, growth form and organ size (Parnell, 1985, 1987; Bokhari & Sales, 2001; Sales *et al.*, 2004). Although some minor morphological differences in plant size, root thickness and leaf size have been reported between diploids and tetraploids from Galicia (Rubido-Bará *et al.*, 2010), the morphological traits reveal a high variability and overlap between the two entities, hindering the identification of the cytotypes in the field. This morphological similarity suggests an autopolyploid origin of the tetraploid *J. montana*, although allopolyploidization cannot be excluded. Although the cytogenetic diversity has been characterized in some populations in Galicia, the geographical distribution of the tetraploids and the environmental niche preferences of each cytotype are still unknown.

The main objective of this study was to explore the diversity and distribution of cytotypes within the *J. montana* polyploid complex and identify possible factors involved in the successful establishment and spread of tetraploids. In particular, the goals of the present study were to: (1) delineate the geographical distribution of tetraploids in the Iberian Peninsula; (2) identify minority cytotypes, mixed-ploidy populations and contact zones between cytotypes and (3) determine whether the cytotypes have different ecological associations that could explain their observed geographical distributions. To accomplish this, we sampled populations throughout the Iberian Peninsula, in particular at detected contact zones, to determine DNA ploidy levels using flow cytometry and assess the distribution patterns of each cytotype. Niche modelling tools were then used to explore the ecological associations of each cytotype in the Iberian Peninsula. We hypothesize that polyploidization results in shifts in environmental preference, which allows tetraploids to establish by colonizing different environmental niches than diploids, thus exhibiting low geographical overlap. The information about cytotype diversity, geographical patterns and environmental associations enabled us to explore the factors involved in the establishment and spread of tetraploid individuals of *J. montana* in nature.

MATERIAL AND METHODS

Study system

Jasione montana is distributed throughout Europe, from the Mediterranean to *c.* 62°N, and from western Asia to North Africa (Tutin, 1973). It grows on rocky ground, heaths and grasslands with thin soil and prefers acid soils rather than limestone regions (Horwood, 1919). Individuals vary morphologically and may be annual, biennial or perennial; however, no clear association between ploidy and plant habit has been found. Plants of *J. montana* form a rosette of leaves during the winter and produce erect or ascending stems in the spring, each ending in a capituliform inflorescence of bluish flowers (Parnell, 1980; Sales & Hedge, 2001a). The species comprises diploids ($2n = 2x = 12$ chromosomes) through most of its distribution (e.g. Kovanda, 1968; Ubera, 1980; Luque & Mejas, 1986; Pastor, 1990; Rubido-Bará *et al.*, 2010), whereas tetraploids ($2n = 4x = 24$ chromosomes) have been reported in Coimbra (central Portugal; Leitão & Paiva, 1988) and in Galicia (Spain; Rubido-Bará *et al.*, 2010). The genome size of the diploid cytotypes has been estimated to be 2C = 3.24 pg, which is roughly half that of tetraploids, 2C = 6.58 pg (Rubido-Bará *et al.*, 2010).

Chromosome counts from the literature

We reviewed the literature on the karyology of *J. montana* (available in Chromosome Counts database, http://ccdb.tau.ac.il/, Rice *et al.*, 2015; Floras and published manuscripts), compiled all chromosome counts with reference to the locality of sampling and mapped the distribution of cytotypes. Forty references including 89 identified localities with karyological information were compiled (Table S1) and mapped.

Field sampling

Our sampling was mostly focused on the Iberian Peninsula, particularly the north-west quadrant, where tetraploids have been previously reported (Leitão & Paiva, 1988; Rubido-Bará *et al.*, 2010). We also sampled other Mediterranean areas to confirm the diploid dominance reported in the literature. In total, 287 populations were sampled, including 279 in the Iberian Peninsula and eight elsewhere (France, Ireland, Italy and Morocco; Table S2). During spring and summer of 2013–2016, fresh leaves were collected from each population, placed in hermetic plastic bags and stored at 4 °C for flow cytometric analyses. In each population, up to 38 individuals (mean = 12 individuals/population) were randomly sampled, throughout the entire population (Table S2). When the harvesting of fresh material was impractical, mature seeds were collected (procedure followed in 14 populations). Geographical coordinates of each population were recorded, and all populations were mapped in Quantum-GIS v.2.18.3 (Table S2). Herbarium specimens were collected for species confirmation in populations with high morphological variability and vouchers were deposited in the SANT herbarium (Table S2).

Flow cytometric analyses

Fresh leaves were analysed using flow cytometry to estimate genome size and DNA ploidy (hereafter referred as ploidy) of each individual sampled. In brief, 50 mg of sample material and reference standard (*Solanum lycopersicum* L. 'Stupické', 2C = 1.96 pg; Doležel, Sgorbati & Lucretti, 1992), were co-chopped in 1 mL Woody Plant Buffer (Loureiro *et al.*, 2007) to obtain a nuclear suspension (Galbraith *et al.*, 1983). Nuclear suspensions were filtered through a 50 µm nylon filter, and 50 µg mL^{-1} of propidium iodide and 50 µg mL^{-1} of RNAse (Fluka, Buchs, Switzerland) were added, incubated for 5 min and analysed using a Partec CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW; Partec GmbH, Görlitz, Germany). For each sample, at least 1300 nuclei in both the G_1 peaks of the sample and standard were analysed (Suda *et al.*, 2007). Partec FloMax software v.2.4d (Partec GmbH, Münster, Germany) was used to acquire the results as described in Castro *et al.* (2018). Samples were considered acceptable when the coefficient of variation (CV) of the 2C peak of *J. montana* was < 5%; otherwise, a new sample was prepared and analysed until sufficient quality was achieved (Greilhuber, Temsch & Loureiro, 2007).

Given the large number of individuals collected in each population, one to nine randomly selected individuals (mean = 2.4 individuals/population) were analysed individually to estimate their genome size, and the remaining samples were analysed for ploidy only by pooling two to six samples (plus the reference standard). For 14 populations, we analysed ploidy directly from seeds following the protocol above and the pooled sample method (adapted from Castro *et al.*, 2018).

The holoploid genome size (2C; *sensu* Greilhuber *et al.*, 2005) of each individual sample was calculated using the following formula:

$$\textit{holoploid genome size (pg)} = \frac{\text{J. montana } G1 \textit{ peak mean}}{\text{S. lycopersicum } G1 \textit{ peak mean}} \times \text{S. lycopersicum } \textit{genome size}.$$

The monoploid genome size (1Cx; *sensu* Greilhuber *et al.*, 2005) of each individual sample was also calculated by dividing the holoploid genome size (2C) by the ploidy of each cytotype. Samples were classified as diploid or tetraploid in line with Rubido-Bará *et al.* (2010) and with our estimates of genome size and their range of variation: diploids range between 2.80 and 3.08 pg/2C, and tetraploids range between 5.63 and 6.06 pg/2C. The fact that genome size ranges of the two cytotypes do not overlap enabled a confident identification of the ploidy. Subsequently, populations were classified according to the ploidy composition of its individuals, as single-ploidy or mixed-ploidy.

Descriptive statistics of holoploid and monoploid genome sizes were calculated for each cytotype based on the individual flow cytometric estimates. Diploid and tetraploid genome sizes (holoploid and monoploid) were compared using GLMs, with Gaussian distribution and identity link function and with cytotype as a fixed factor and genome size as the response variable. The analyses were performed in R software v.3.0.1 (R Core Development Team, 2016), using the packages 'car' for Type-III analysis of variance (Fox & Weisberg, 2015), 'lme4' for generalized linear models (Bates *et al.*, 2014) and 'multcomp' for multiple comparisons after Type-III analysis of variance (Hothorn *et al.*, 2017).

Environmental niche modelling

Abiotic environmental associations with each cytotype were evaluated for the Iberian Peninsula populations.

Nineteen bioclimatic variables (Bio1-Bio19) plus elevation, latitude and longitude at a 1 km resolution were extracted from the WorldClim database (http://www.worldclim.org/). To improve the quality of the niche environmental predictions, six soil-related variables were also obtained at the same resolution: base saturation of the topsoil (bs_top), topsoil cation exchange capacity (cec_top), topsoil organic carbon content (oc_top), slope, dominant surface textural class of the soil topological unit (txsrfdo) and first soil adjective code of the soil topological unit (wrbadj1) (Panagos *et al.*, 2012; European Soil Data Centre). Values for each variable were extracted for all *J. montana* records using the 'dismo' package in R (Hijmans *et al.*, 2017). Correlations between all variables were assessed. The selection of the variables was based on the variance explained in an exploratory principal component analysis (PCA; data not shown), excluding highly correlated variables (Spearman's rho correlation > 0.7). In the end, four bioclimatic variables (Bio4 – temperature seasonality, Bio5 – maximum temperature of the warmest month, Bio14 – precipitation of the driest month and Bio15 – precipitation seasonality) and two soil parameters (bs_top – base saturation of the topsoil and txsrfdo – dominant surface textural class of the soil topological unit) were selected (Table S3). Differences between diploid and tetraploid populations with respect to these variables were tested using GLMs, with cytotype as fixed factor and each variable as a response variable.

The *J. montana* population records (presence/absence of each ploidy) used in this analysis were from multiple sources: (1) our intensive field sampling in the north-west quadrant of the Iberian Peninsula (39.6° to 43.7° latitude, –6.1° to –9.2° longitude) and ploidy occurrences (from chromosome counts and flow cytometric analyses) from Rubido-Bará *et al.* (2010) in Galicia; (2) occurrences downloaded from GBIF database (http://gbif.org) beyond the area of intensive sampling in the north-west quadrant of the Iberian Peninsula and (3) randomly selected points from the Iberian Peninsula (background records). Population reports from our sampling and from Rubido-Bará *et al.* (2010) were classified as diploid and tetraploid according with the ploidy or chromosome numbers available, whereas GBIF points were classified as diploid according to the literature and our own observations (Tables S1 and S2). Records lying within 10 km of a given record were excluded from the dataset to avoid oversampling, resulting in a dataset of 462 diploid and 53 tetraploid records. To obtain the background points, we applied a buffer of 20 km to all the points from the presence dataset and randomly obtained 5000 points beyond this buffer; additionally, a filter of 20 km was used to remove background points that were separated by less than this distance, again to avoid oversampling. This resulted in a final dataset with a total of 739 records (462 diploid, 53 tetraploid and 224 background points). For the diploid dataset, diploid populations were recorded as presences and tetraploid populations as absences and vice versa for the tetraploid dataset. Background points were used as absences in both analyses.

Niche modelling was performed using maximum entropy (MaxEnt) and the R software package 'biomod2' (Thuiller *et al.*, 2016). Spatially predictive models were calibrated using the six selected variables and the presence/absence datasets. To reduce uncertainty and produce robust models, the models were replicated 30 times after splitting the data into randomly selected training (70%) and testing (30%) subsets (Phillips, Anderson & Schapire, 2006; Araújo & New, 2007). To guarantee statistical independence of all replicates, each occurrence was used only once in each run, either as training or as test occurrence (Phillips, 2008). Models were evaluated based on the independent accuracy measure (AUC) of the curve of the receiver operating characteristic. The models for diploid and tetraploid cytotypes were obtained based on the ensemble forecasting procedure. Only models with AUC > 0.7 were considered for the final model. Model evaluation revealed high AUC values (2*x*: 0.75 ± 0.03; 4*x*: 0.92 ± 0.02) and relatively low omission rates in the final models (2*x*: 0.29 ± 0.07 and 4*x*: 0.00 ± 0.02), indicating that the models could predict cytotype occurrences with high accuracy. The final models were converted into a binary format (threshold of 0.5 as defined by default, package 'biomod2'; Thuiller *et al.*, 2016) to calculate the suitable habitat of each cytotype and assess niche overlap between diploids and tetraploids.

Niche equivalence and similarity tests

The statistical framework used to compare niches between cytotype measures the niche overlap using the metric *D* of proportional similarity of the distribution (Schoener, 1970). This metric ranges from 0, representing 'no overlap', to 1, representing a 'complete overlap'. We performed niche equivalency and similarity tests (Warren *et al.*, 2008; Broennimann *et al.*, 2012) and the analyses were run using 'ecospat' (Di Cola *et al.*, 2017) and 'raster' (Hijmans *et al.*, 2017) R packages using binary projections. Both niche equivalency and similarity tests were computed to evaluate whether predicted distributions were significantly different between cytotypes (classification by Warren *et al.*, 2008; Smith & Donoghue, 2010; Broennimann *et al.*, 2012).

The 'ecospat' R package was used to compare cytotype niches with an ordination approach using a PCA calibrated with environmental values (Di Cola *et al.*, 2017). The PCA calculates the occurrence density and environmental factor density along environmental (principal component) axes for each pixel, maximizing the ecological variance of the areas of the cytotypes. Then the PCA scores of the two cytotype distributions were projected onto a grid of cells bounded by the maximum and minimum PCA scores, which allows the visual assessment of the overlap and dynamics of the environmental niches of diploids and tetraploids.

The niche equivalency test determines whether the environmental niche occupancies of the cytotypes in their ranges are equivalent. For that, diploid and tetraploid records were pooled into a total database; this total database was divided randomly into two groups, each having a sample size equal to the diploid and tetraploid original databases, to obtain a simulated *D* value. This process was repeated 100 times to obtain confidence intervals for the evaluation of the null hypothesis, i.e. the niche overlap is constant when randomly reallocating the occurrences of both cytotypes among the two ranges. Thus, if the observed value of D falls within the 95th percentile of the simulated *D* values the null hypothesis of niche equivalency cannot be rejected and the niches of the two cytotypes are equivalent. The niche similarity test assesses whether the observed overlap of the two cytotypes is greater than the overlap between the range of one cytotype and niches selected at random from the range of the other cytotype, i.e. whether the two cytotypes share a greater portion of their environmental volume that would be expected by chance. As in the equivalency test, the observed *D* value is compared with the simulated *D* values after repeating the process 100 times to obtain confidence intervals for null hypothesis evaluation (Broennimann *et al.*, 2012).

All analyses were performed in R software v.3.0.1 (R Development Core Team, 2016). Quantum-GIS was used to observe and build the distribution maps.

RESULTS

Flow cytometric analyses

Using flow cytometry, we were able to assign ploidies to all analysed plants (Fig. 1). Diploids had an average genome size of 2C = 2.92 ± 0.07 pg (mean ± SD, range: 2.80–3.08 pg), whereas tetraploids had an average genome size of 2C = 5.86 ± 0.14 pg (range: 5.63–6.06 pg; Table 1, Fig. 1; Table S4). Holoploid genome size differed significantly between cytotypes ($F_{1,249}$ = 42233.00, P < 0.001), but there was no statistically significant difference in monoploid genome size ($F_{1,249}$ = 0.40, P = 0.525).

Cytotype distribution

The literature review on the karyology of *J. montana* revealed that diploids are widespread across Europe (white diamonds in Fig. 2A, Table S1). This result was reinforced by the additional samples in our study (white circles in Fig. 2A).

In the Iberian Peninsula, we sampled 279 populations and the great majority of the populations sampled were single-ploidy populations (98.6%, from which 71.3% were pure diploid and 27.3% were pure tetraploid); only four localities (1.4%) harboured both diploid and tetraploid individuals (black circles in Fig. 2B; Table S2). Our sampling confirmed that tetraploids are restricted to the north-west quadrant of the Iberian Peninsula, in particular to central and northern regions of Portugal, and to Galicia in Spain (grey circles in Fig. 2B; Table S2), as initially suggested by records in the literature (grey diamonds in Fig. 2A).

In the north-west quadrant of the Iberian Peninsula, the diploid and tetraploid populations appeared intermingled, although most areas are dominated

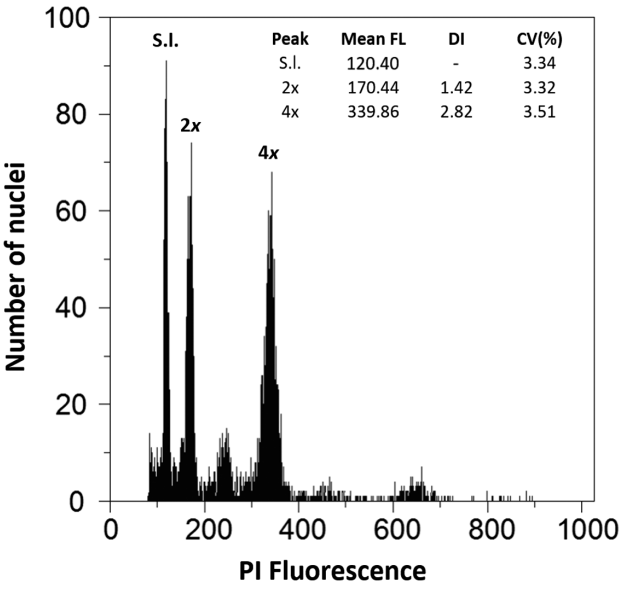

Figure 1. Flow cytometric histogram of relative propidium iodide fluorescence intensity (PI fluorescence) of nuclei isolated from fresh leaves of *Solanum lycopersicum* 'Stupické' (S.l.; reference standard with 2C = 1.96 pg) and of diploid (2*x*) and tetraploid (4*x*) plants of *Jasione montana*. For each peak, the mean relative fluorescence (mean FL), DNA index (DI, mean FL of *J. montana* peak/mean FL of the reference standard) and coefficient of variation of each peak (CV, in %) are provided.

Table 1. Genome size estimates in *Jasione montana* according with each cytotype. Ploidy and mean, standard deviation of the mean (SD), coefficient of variation (CV, in %), minimum and maximum values of holoploid genome size (2C, in pg) are given. Mean and standard deviation of the mean (SD) of estimated monoploid genome size (1C*x*, in pg) and the total number of populations and individuals analysed are also presented for each cytotype. Two ploidies were observed: diploids (2*x*) and tetraploids (4*x*). Different letters correspond to statistically significant differences at $P < 0.05$, NS denotes non-significant differences at $P > 0.05$.

Ploidy	Holoploid genome size (2C, pg)					Monoploid genome size (1C*x*, pg)		Populations (individuals)
	Mean	SD	CV (%)	Min	Max	Mean	SD	
2*x*	2.92[a]	0.07	2.9	2.80	3.08	1.46[NS]	0.04	84 (205)
4*x*	5.86[b]	0.14	3.3	5.63	6.06	1.46[NS]	0.04	24 (46)

by diploids (Fig. 2B). The tetraploid populations are mostly clustered in two regions, one in central Portugal and another in Galicia, creating several areas of contact between diploids and tetraploids, including a few sympatric areas where cytotypes coexist and form mixed-ploidy populations in central Portugal (Fig. 2B). The composition of the mixed-ploidy populations was variable: two populations were dominated by tetraploids, with only one diploid individual being detected in each population, one population had fairly similar cytotype proportions and one small population was dominated by diploids bearing only one tetraploid individual (Table S2). Despite the large sample size ($N = 3396$), especially in the contact zone, no other cytotypes were detected.

Cytotype environmental characteristics and niche overlap

Visual inspection of the distribution models reveals a high predicted suitability for diploids over most of the Iberian Peninsula, except for the eastern calcareous areas, where the probability of occurrence is very low (Fig. 3A). In contrast, the predicted tetraploid distribution was restricted to the north-west quadrant of the Iberian Peninsula, where this cytotype is currently found and to the eastern coast of Valencia (Fig. 3B).

The first two axes of the PCA explained a high percentage (74.8%) of ecological (climatic and edaphic) variance (51.3% and 23.5% for axes 1 and 2, respectively). Maximum temperature of the warmest month (Bio_5) and precipitation in the driest month (Bio_14) had the highest contributions to the first axis, followed by temperature and precipitation seasonality (Bio_4 and Bio_15, respectively). Two soil variables (base saturation of the topsoil, bs_top, and dominant surface textural class, txsrfdo) had lower contributions and mostly for the second axis (Fig. 4A). Based on the first and second principal components, the environmental niche of the tetraploid falls completely within the environmental range of the diploid (Fig. 4B, C). However, the reverse is not true; rather, the amplitude of the environmental niche of diploids was much larger than that of the tetraploids (Fig. 4B, C) and only 20.7% of the diploid environmental niche overlaps with the tetraploid niche (green area in Fig. 4B).

Even though the tetraploid niche is embedded in the diploid niche envelope (Fig. 4B), given the differences in occurrences density in the ecological space between cytotypes, the *D* metric of niche overlap was low ($D = 0.08$). The niche equivalency test rejected the null hypothesis ($P < 0.05$; Fig. 4D) and indicates that the climatic niches of diploids and tetraploids are not equivalent or identical (Glor & Warren, 2011). However, the niche similarity test revealed that the observed *D* value falls within the 95th percentile of the simulated *D* values, thus failing to reject the null hypothesis of niche similarity. Consequently, cytotype environmental niches were not more similar (or different) from one another than the expected after random sampling ($P > 0.05$, for both diploids growing in the tetraploid potential area, and tetraploids growing in the diploid potential area; Fig. 4E, F).

DISCUSSION

We found differences in both geographical distribution and environmental niche breadth between diploid and tetraploid individuals of *J. montana*. Tetraploids are restricted to a relatively small geographical area in the Iberian Peninsula, whereas diploids are widespread across Europe, a pattern consistent with previous reports (compiled in Table S1 and Fig. 2A; Leitão & Paiva, 1988; Rubido-Bará *et al.*, 2010). In the region of overlap, tetraploids and diploids mostly occur as a mosaic of single-ploidy populations. Consistent with their smaller geographical range, the environmental niche of tetraploids is more restricted

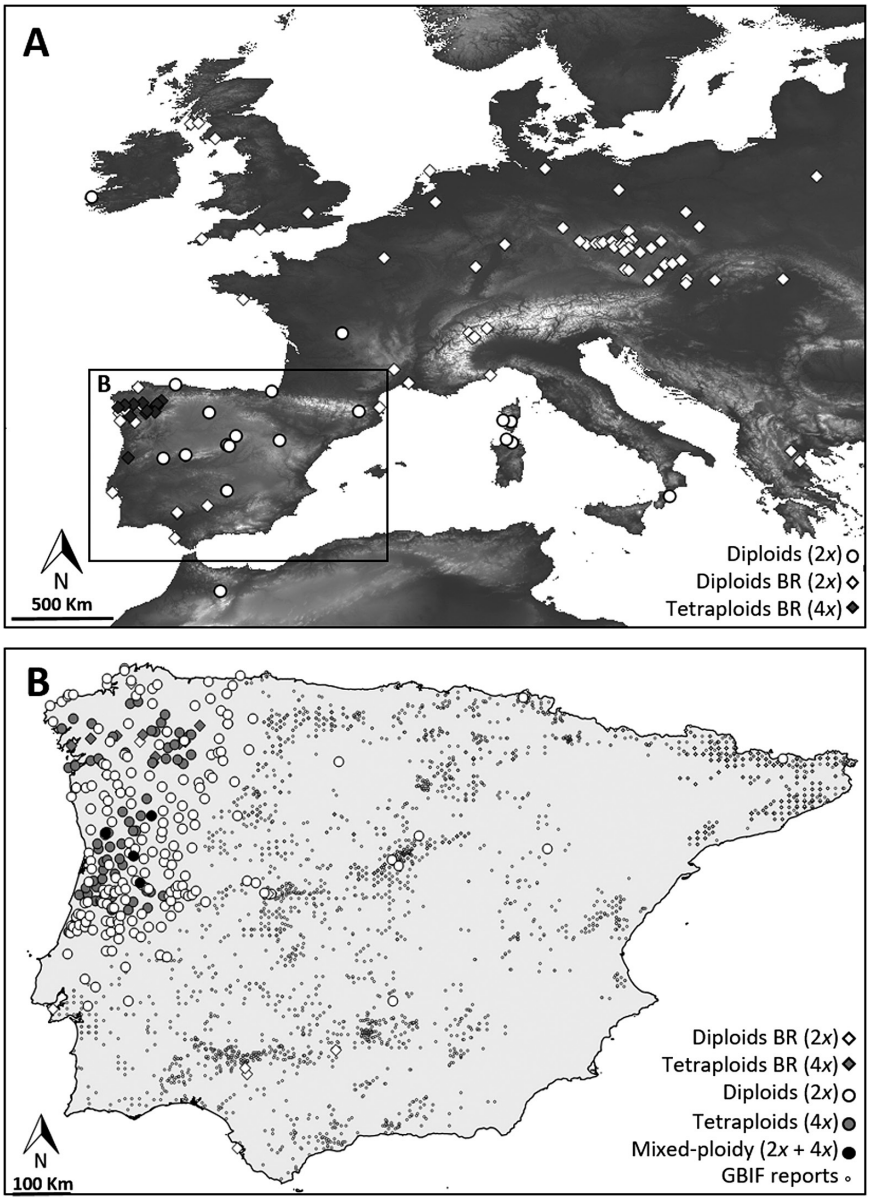

Figure 2. *Jasione montana* records. A, bibliographic information for chromosome counts and ploidies in Europe (diamonds) and additional estimates provided in this study (circles) and B, detail of the Iberian Peninsula, including estimates from this study (big circles) and GBIF occurrences (small circles). Diamonds: bibliographic records (BR); large circles: populations screened in this study; small circles: GBIF occurrences; ploidy: white = diploids (2*x*), grey = tetraploids (4*x*), black = diploid-tetraploid populations.

than, and completely nested within, that of diploids. Contrary to our hypothesis, these observations suggest that whole genome duplication in this species is not associated with the origin of novel environmental requirements; if anything, the tetraploid niche is a narrow subset of the extant diploid niche, a pattern seen in other taxa (Glennon *et al.*, 2014). Below, we discuss the mechanisms underlying these results and their implications for our understanding of polyploid establishment and persistence in plants.

Ploidy levels in *Jasione montana*

Our study confirms the presence of two cytotypes in *J. montana*, diploids and tetraploids. Polyploidization is common in *Jasione*, and approximately half of the *Jasione* spp. occurring in the Iberian Peninsula, the centre of diversity for the genus, are polyploid or harbour multiple cytotypes (Sales & Hedge, 2001b; Rubido-Bará *et al.*, 2010; Castro, 2018); *J. montana* is one such species. The co-occurrence of diploids

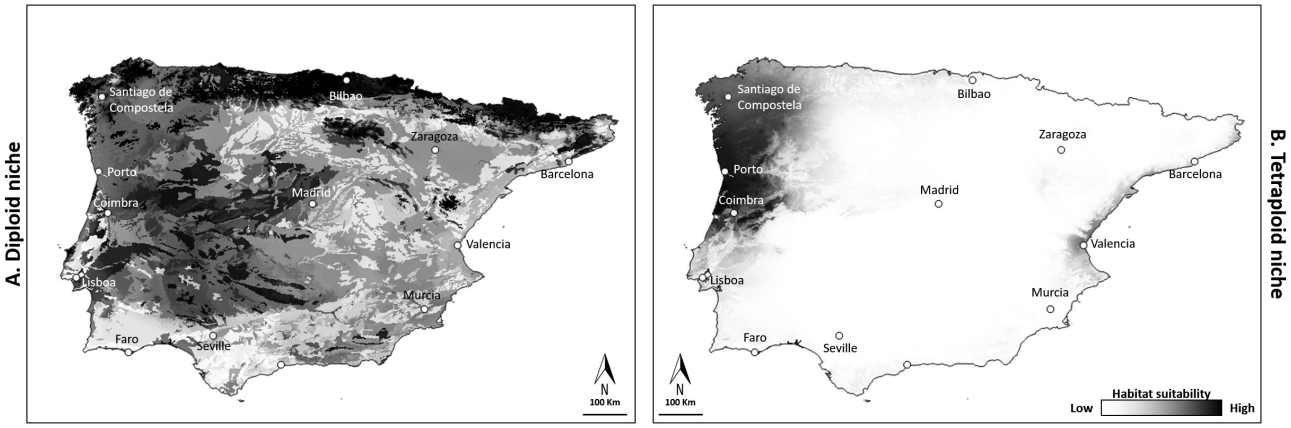

Figure 3. Predictive suitable niche for each cytotype: A, diploids and B, tetraploids of *Jasione montana* in the Iberian Peninsula. Light and dark shading represent habitats with low and high suitability, respectively.

and tetraploids is commonly observed in mixed-ploidy species (Kolář *et al.*, 2017) and is common among polyploid species in *Jasione*. For example, *J. laevis* and *J. maritima* (Sales & Hedge, 2001a; Rubido-Bará *et al.*, 2010; Castro, 2018) both consist of diploids and tetraploids, whereas *J. crispa* has diploids, tetraploids and hexaploids (Sales & Hedge, 2001a). More surprising is the absence of triploid hybrids, especially in the contact zone in the Iberian Peninsula. This pattern may reflect the spatial separation of cytotypes into single-ploidy populations or the presence of strong triploid block (discussed below). The absence of triploids in the four mixed-ploidy populations suggests that both factors may be operating in the diploid-tetraploid contact zones of *J. montana*.

Nested and asymmetrical geographical ranges: historical causes

The nested and asymmetrical geographical ranges of diploids and tetraploids, and the restricted geographical range of tetraploids in particular, may reflect the history of formation of tetraploids from diploid ancestors in this species. Tetraploids may have arisen and successfully colonized the north-west quadrant of the Iberian Peninsula relatively recently and, thus, have had limited time to expand beyond the site of origin, as has been suggested for other mixed-ploidy species (Godsoe *et al.*, 2013; Laport *et al.*, 2016). The limited geographical range also suggests that tetraploids have arisen a limited number of times across the entire geographical range. Although our sampling was extensive, more intensive screening for rare tetraploids throughout the diploid portion of the range is needed to determine whether tetraploids do in fact arise frequently but are unlikely to establish. Multiple origins of polyploids have been documented in numerous taxa and on a range of spatial scales (e.g. Soltis & Soltis, 1999; Segraves & Thompson, 1999; Sampson & Byrne, 2011). Therefore, we cannot exclude the possibility that the two clusters of tetraploid populations, one in Portugal and the other in Galicia (Spain), represent independent origins. To date, we have little evidence with which to evaluate these historical scenarios in *J. montana*. Phylogenetic analyses of *Jasione* positioned *J. montana* in a clade with other species currently occurring in the north-west quadrant of the Iberian Peninsula (Sales *et al.*, 2004; Pérez-Espona *et al.*, 2005). However, the internal relationships of the clade remain unresolved and the time of divergence of tetraploid *J. montana* relative to diploids is not known. Additional research on genetic divergence and phylogeography of tetraploids and diploids may help to shed light on the timing and frequency of whole genome duplication in this species.

Asymmetrical ranges: environmental niche shifts?

Our results provide insight into the role of environmental differences between cytotypes in determining establishment and geographical distribution of polyploids. Tetraploid plants of *J. montana* occupy only a subset of the diploid environmental niche, as depicted in the PCA (Fig 4B, C), and the equivalency test indicates that climatic niches of diploids and tetraploids are not equivalent; however, cytotype environmental niches were not more similar (or different) from one another than expected by chance. Therefore, we conclude that there is no evidence that whole genome duplication, leading to tetraploidy, resulted in a shift in environmental niche beyond that of the diploid niche. Changes in environmental tolerance are often postulated as necessary for polyploid establishment (Husband & Schemske, 2000; Baack & Stanton, 2005;

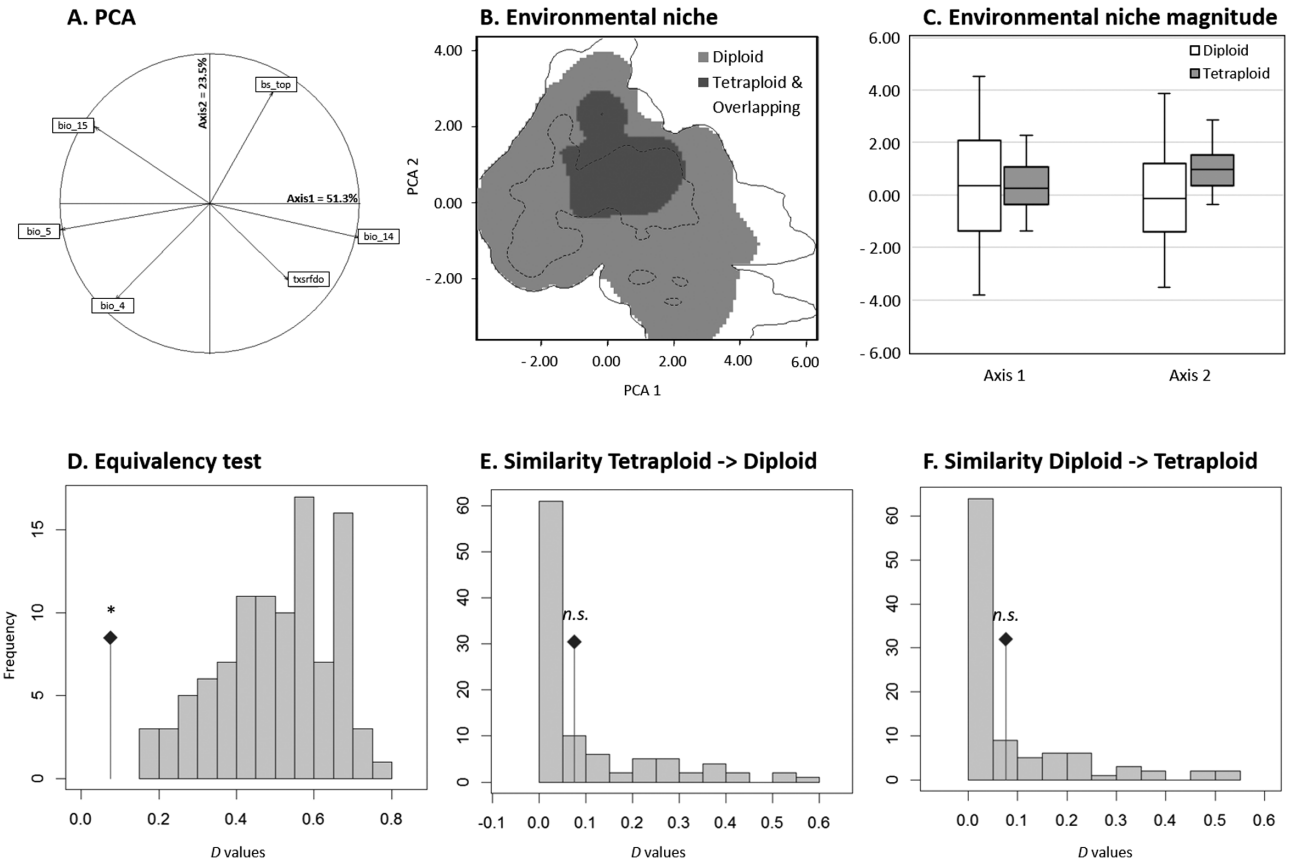

Figure 4. Ecological niche models for *Jasione montana* cytotypes in the Iberian Peninsula. A, Contribution of climatic and soil variables to the first two axes of the principal component analyses (PCA) and the percentage of variance explained by each axis. B, Environmental niche of diploids and tetraploids, respectively, based on the PCA of selected variables; coloured areas represent the following: grey: suitable habitats for diploids, dark grey: denotes both the suitable habitats for tetraploids and the overlapping areas between diploid and tetraploid environmental niches; the continuous line corresponds to the whole climatic space and the dashed line indicates the 75th percentile. C, Magnitude of environmental niches of each cytotype (white and dark grey represent the occupation of niches in PCA1 and PCA2, respectively). D, Equivalency test results. E, Similarity test results of diploids growing in the tetraploid environmental niche. F, Similarity tests of tetraploids growing in the diploid environmental niche. In D, E and F, dark grey diamonds represent the observed *D* values, grey bars correspond to the frequency of calculated *D* value resulting from a similarity test process.

Buggs & Pannell, 2007; Ramsey, 2011) as they promote spatial segregation and within-cytotype mating, allowing rare cytotypes to avoid frequency-dependent selection and exclusion (Levin, 1975; Fowler & Levin, 1984; Felber, 1991; Hao *et al.*, 2013). However, evidence in the literature for niche shifts that occur in association with whole genome duplication is inconsistent. Examples of environmental niche divergence between cytotypes have been reported in species such as *Chamerion angustifolium* (Thompson *et al.*, 2014), *Centaurea stoebe* L. (Glennon *et al.*, 2014), *Tolmiea diplomenziesii* Judd, Soltis & P.S.Soltis/T. *menziesii* Torr. & A.Gray (Visger *et al.*, 2016) and *Erysimum medio-hispanicum* Polatschek (Muñoz-Pajares *et al.*, 2018), although it is usually unclear whether this results directly from the duplication or subsequent evolutionary divergence (see Maherali *et al.*, 2009, for *C. angustifolium*). In contrast, a test of the niche shift hypothesis using 20 different diploid-polyploid species found that most pairs showed significant similarity or no difference in niche between pairs (Glennon *et al.*, 2014). Collectively, these results suggest that polyploids frequently occupy similar environmental conditions to their diploid progenitors, and that niche differentiation, measured as climatic variables, may not be a necessary requirement for polyploid establishment and persistence in nature (Glennon *et al.*, 2014).

Asymmetrical geographical ranges: environmental sorting

Although evidence for a significant environmental niche shift in tetraploids is lacking, environmental

sorting may still explain, in part, the differences in geographical range between cytotypes of *J. montana*. The smaller geographical range of tetraploids is congruent with their narrower environmental niche compared to diploids. This correlation suggests a possible causal relationship, specifically that the narrower niche of tetraploids is limiting the expansion and establishment on a larger geographical scale. Additionally, polyploids with smaller niche ranges than their diploid relatives may be well-adapted, or even specialized, to a given set of environmental conditions (Parisod & Broennimann, 2016). Thus, the restricted and spatially clustered distribution of the tetraploids could reveal areas harbouring a combination of environmental conditions where the tetraploid niche is at its optimum, allowing them to outcompete the diploids. In the case of *J. montana*, the tetraploid distribution coincides with the boundaries between four main biogeographic sectors (the Galicia and North Portugal sector, the North Lusitania Sierra sector, the Montemuro and Estrela Sierras sector and the north part of the Divisorio Portuguese sector; Rivas-Martínez *et al.*, 2017) and is characterized by a subatlantic-submediterranean climate and with similar floristic compositions (Rivas-Martinez *et al.*, 2017). This biogeographic region of contact between different sectors constitutes the interface between the temperate and Mediterranean biomes and harbours several species adapted to this transition (Amigo *et al.*, 2017). Niche relationships between closely related species or cytotypes in which the niche of one taxon/cytotype is much narrower than, and nested within, the niche of the other, have been interpreted as indicative of environmental specialization (Knouft *et al.*, 2006; Vamosi *et al.*, 2014; Parisod & Broennimann, 2016). To confirm this hypothesis, reciprocal transplant experiments are necessary to establish that tetraploids are, indeed, unable to thrive in diploid environments outside of their current geographical range.

What allows tetraploids to persist with diploids on the Iberian Peninsula?

If tetraploid *J. montana* is restricted in distribution to a small portion of the diploid range, what then allowed tetraploids to persist and coexist with diploids? Theoretical models predict that, in randomly mating populations, rare cytotypes will experience a frequency-dependent mating disadvantage, due to increased inter-cytotype mating, which is generally ineffectual. As a result, dominant cytotypes will preclude establishment of newly arising cytotypes (Levin, 1975; Rodriguez, 1996; Husband & Schemske, 2000) unless new cytotypes can disperse to unoccupied locations (e.g. Godsoe *et al.*, 2013; Thompson *et al.*, 2014; Visger *et al.*, 2016; Muñoz-Pajares *et al.*, 2018), resulting in a mosaic of single-ploidy populations on the landscape, each excluding the other from their respective populations through minority cytotype exclusion (Petit *et al.*, 1999; e.g. Baack, 2004; Kolář *et al.*, 2009; Castro *et al.*, 2012). In *J. montana*, the diploids and tetraploids indeed form a complex mosaic of single-ploidy populations in the north-west quadrant of the Iberian Peninsula, with areas dominated by diploids intermingled with areas dominated by tetraploids. Furthermore, the rarity of mixed populations suggests that reproductive barriers between the two cytotypes might be insufficient to prevent inter-cytotype mating, resulting in minority cytotype disadvantage (triploid block; Levin, 1975; Husband, 2000). Indeed, preliminary pollination experiments revealed that both cytotypes are unable to produce viable offspring after inter-cytotype crosses (M. Castro, unpublished data). Minority cytotype exclusion has been tested experimentally only by Husband (2000) in *Chamerion angustifolium* and by Baack (2005) in *Ranunculus adoneus*, but it has been referred as an important mechanism driving cytotype distribution patterns in numerous diploid–polyploid contact zones (e.g. Levin, 2002; Španiel *et al.*, 2008; Castro *et al.*, 2011).

Our results suggest that, to establish successfully, tetraploids of *J. montana* must disperse and colonize unoccupied areas in the landscape and, thus, avoid the minority cytotype exclusion (e.g. Godsoe *et al.*, 2013; Thompson *et al.*, 2014; Visger *et al.*, 2016; Muñoz-Pajares *et al.*, 2018). There is a tendency for tetraploid *J. montana* to occupy ruderal and more disturbed habitats than diploids, which can occupy both less disturbed areas as well as some ruderal habitats (M. Castro & M. Serrano, Field observations). This pattern has been observed in other polyploid complexes, in which tetraploids spread into ruderal habitats forming mosaic contact zones even without differences in climatic niche (e.g. Španiel *et al.*, 2008; Kolář *et al.*, 2016). Our observations remain to be confirmed, but they highlight the hypothesis that cytotype coexistence in the contact zone can be enabled by microhabitat differences between cytotypes within the existing climatic niche. Also, the aggregation of tetraploid populations in regions suitable for diploids may indicate an ability to outcompete diploids in specific environments, thereby excluding them from population. Fitness advantages such as increased competitive ability (Maceira, Jacquard & Lumaret, 1993; Laport *et al.*, 2013) and asymmetric assortative mating (Husband & Sabara, 2004; Buggs & Pannell, 2006, 2007; Laport *et al.*, 2016), among other factors, play crucial roles in the success of polyploid lineages. In *J. montana*, some morphological differences between cytotypes have been observed (Rubido-Bará *et al.*, 2010) that might be associated with plant fitness and competitive ability, such as increased plant size

and tendency for perenniality in the tetraploids (M. Serrano, field observations). Nevertheless, further studies are needed to unravel possible fitness differences that could explain cytotype interactions at contact zones

CONCLUSIONS

Our results indicate that polyploidization in *J. montana* has not caused expansion to novel environmental conditions. Under this scenario, tetraploids will be subjected to strong frequency-dependent selection and will be excluded by diploids unless they can disperse to sites unoccupied by diploids, where they in turn may exclude diploid colonists. The mosaic of diploid and tetraploid populations and a lack of mixed-ploidy populations is consistent with a frequency-dependent selection influencing distributions within the mixed-ploidy zones, although the influence of inter-cytotype competition cannot be ruled out. The interplay between both mechanisms is unclear and future molecular and experimental studies will be important for fully understanding the role of environmental sorting and evolutionary history in governing the distribution of cytotypes within the contact zones of *J. montana*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Chromosome counts of *Jasione montana* available in the bibliography.
Table S2. Geographical information of *Jasione montana* populations sampled in this study.
Table S3. Selected environmental variables for *Jasione montana* cytotype niche modelling.
Table S4. Genome size estimates in *Jasione montana*.