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EVOLUTIONARY ECOLOGY OF POLYPLOIDS:

UNDERSTANDING SPECIES COEXISTENCE AT THE CONTACT ZONES

Tese no âmbito do Doutoramento em Biociências, especialização em Ecologia,
orientada pelo Professor Doutor João Carlos Mano Loureiro Castro,
Doutora Sílvia Raquel Cardoso Castro Loureiro e Professor Doutor Brian C. Husband,
apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia
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Evolutionary ecology of polyploids: understanding species coexistence at the contact zones

Ecologia evolutiva dos poliploides: compreender a coexistência de espécies em zonas de contacto

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List of Abbreviations

1C*x* – monoploid genome size

2C – holoploid genome size

2n – sporophytic

2*x* – diploid

3*x* – triploid

4*x* – tetraploid

6*x* – hexaploid

8*x* – octoploid

9*x* – nonaploid

10*x* – decaploid

12*x* – duodecaploid

AA – Ana Afonso (collector)

alt – altitude

AM – Ana Martins (collector)

am – (L. *ante meridiem*) in the morning

An. – aneuploid

aspect – exposition

AUC – area under the curve

Bio_14 – precipitation of driest month

Bio_15 – precipitation seasonality

Bio_4 – temperature seasonality

Bio_5 – maximum temperature of warmest month

BR – bibliographic reports

bs_top – base saturation of the tops

C – sugar content

cf. – (L. *confer*) compare with

Cont. – to be continued

CS – Carlos Silva (collector)

CV – coefficient variation

CZ – contact zone

D – Schoener's *D* value

d – density sugar

ddH_2O – double-distilled water

DI – DNA index

dist – distance from the sea

DNA – deoxyribonucleic acid

DT – Daniela Tavares (collector)

e.g. – (L. *exempli gratia*) for example

EA – Elisabete Almeida (collector)

EDTA $Na_2.2H_2O$ – Disodium ethylenediaminetetraacetate dihydrate

EM – Elisabete Marchante (collector)

ENM – environmental niche modelling

et al. – (L. *et alia*) and other

F1 – first filial generation

FL – fluorescence pulse integral

FM – Filomena Morgado (collector)

FS – forward light scatter

G.s. – genome size

GBIF – Global Biodiversity Information Facility

GI – geographic isolation index

GLM – generalized linear model

GLMM – generalized Linear Mixed Models

GMT – Greenwich Mean Time

GPS – Global Positioning System

HCl – hydrogen chloride

HSD – honest significant difference

i.e. – (L. *id est*) that is

IC – continentality index

ID code – identification code

Ios3 – sumer ombrothermic index

ITC – compensated thermicity index

IUCN – International Union for Conservation of Nature

JC – Joana Costa (collector)

JCO – José Cerca (collector)

JMC – José Miguel (collector)

L0 – electrical conductivity after autoclaving

Lat – latitude

lito – lithology

LM – Lucie Mota (collector)

log – logarithm

Long – longitude

Lt – electrical conductivity after 24 hours

M – mean maximum temperature of the coldest month of the year

m – mean minimum temperature of the of the coldest month of the year

m a.s.l. – meters above sea level

Max – maximum

MaxEnt – Maximum Entropy Modelling

MC – Mariana Castro (collector)

MCD – Maria Cristina Duarte (collector)

MG – Marta Golçalves (collector)

$MgCl_2.6H_2O$ – magnesium chloride hexahydrate

Min – minimum

MS – Miguel Serrano (collector)

n – gametophytic

N – sample size

n.s. – not-significant

NaCl – sodium chloride

Neo4*x* – neotetraploid

ni – number of individuals visited

n_{ind} – number of individuals

no. – number of

n_{pot} – number of pots

P – probability

P.s. – *Pisum sativum* 'Ctirad'

PCA – principal component analyses

pg – pictograms

PI – propidium iodide

pm – (L. *post meridiem*) in the afternoon

POP – population

pp – mean annual precipitation

PVP – polyvinylpyrrolidone

RI – reproductive isolation index

$RI_{behavioral}$ – behavioral reproductive isolation index

$RI_{gametic}$ – gametic reproductive isolation index

$RI_{morphological}$ – morphological reproductive isolation index

$RI_{phenological}$ – phenological reproductive isolation index

RNase – ribonuclease

ROC – receiver operating characteristic

ROS – reactive oxygen species

s – nectar sugar content

S.l. – *Solanum lycopersicum* 'Stupické'

s.l. – (L. *sensu lato*) in the broad sense

S:O – seed/ovule ratio

SANT – Herbarium of the Universidade de Santiago de Compostela

SC – Sílvia Castro (collector)

SD – standard deviation

SE – standard error

Sect. – Section

slope – slope

slprng – slope range

sp. – species

spp. – subspecies

SS – side light scatter

T50 – 50% of total germination rate

Tmax – mean temperature of the hottest month of the year

Tmed – mean temperature in hottest months

Tmin – mean temperature of the coldest month of the year

tpi – topographic position index

Tris.HCl – tris(hydroxymethyl)aminomethane

txsrfdo – dominant surface textural class of the STU

V – nectar production

vs – *versus*

WGD – whole genome duplication

WPB – woody plant buffer

x – haploid

δ – degrees of freedom

NOTE: All units used follow the SI (Système International d'Unités)

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Abstract

Cytogenetic diversity is widely observed in numerous organisms. In flowering plants, diversification and evolution are intimately related with polyploidization events throughout their entire evolutionary history. Polyploid formation is frequent in nature, however, the establishment of new entities is not always successful. The changes generated by polyploidization have profound consequences in the genetics, morphology, physiology and mating system of a plant, affecting plant performance and leading to divergence. After formation, polyploids may be eliminated from parental populations due to selective pressures against rare cytotypes, or polyploidization may have conferred an advantage allowing polyploids to escape frequency-dependent selection. This advantage might enable polyploids to outcompete their progenitors and/or suit the new polyploid with broader environmental tolerances enabling the dispersal to new habitats. Cytotype coexistence will be possible if a set of barriers promote assortative mating. The main goal of this PhD Thesis was to understand the role of polyploidization in plant diversification by focusing on the ecological processes involved with the successful establishment and spread of polyploid lineages. I have focused in three different levels: *in situ* cytogeographical patterns and environmental association in different polyploid complexes; interaction between cytotypes at contact zones; direct consequences of whole genome duplication *versus* post-polyploidization adaptation.

Large-scale screenings to determine DNA-ploidy levels were performed along the distribution range of *Jasione maritima* and *J. montana*, in the latter mostly focusing in the contact zone at the northwest of the Iberian Peninsula, and environmental requirements of each cytotype were assessed. Diploids were reported for the first time in *J. maritima*, with the cytotypes being allopatrically distributed: diploids occur in northern dunes, while tetraploids are present in drier and hotter locations of central and south areas of the species distribution. The broader environmental niche shown by tetraploids suggests that polyploidization may have changed the ecological requirements, allowing the colonization and establishment in southern areas, partially explaining the current distribution patterns. The restricted distribution of diploids highlights the need for cytotype targeted conservation measures. In *J. montana*, the cytotypes were parapatrically distributed forming several contact zones, with diploids having broader environmental and geographic niches than tetraploids. In this polyploid complex, polyploidization does not seem to have driven shifts in ecological preferences of tetraploids, and thus other factor are expected to be involved with the current distribution patterns.

The tetraploid-octoploid *Gladiolus communis* was used to explore cytotype interactions at contact zones using a high-ploidy level complex. First, cytogenetic diversity, distribution patterns and environmental requirements were explored. Tetraploids and octoploids were dominant and, despite a high degree of geographic isolation, it was also shown that they grow in sympatry in several populations. Environmental requirements between cytotypes were similar, suggesting that polyploidization does not seem to have generated a shift in environmental preferences. The detection of hexaploids and mixed-ploidy populations suggested that hybridization and unreduced gamete production are frequent events, which points for recurrent polyploid formation and gene flow. Consequently, reproductive barriers between cytotypes enabling coexistence were explored, in particular, temporal, behavioral, mechanical and gametic barriers. Pre-pollination barriers were weak, while post-pollination interactions were strong and variable depending on pollen loads, and consequently a reflection of population structure. Controlled pollinations suggest that, after formation, a lower fitness will exclude the polyploid from the population, unless unreduced gametes formation ameliorate this disadvantage, while in later stages, strong post-zygotic barriers may enable cytotype coexistence.

Considering that *J. maritima* cytotype distribution was only partially explained by environmental variables, the role of polyploidization driving different competitive abilities between cytotypes was explored. The direct consequences of whole genome duplications were evaluated using diploids, neotetraploids and established tetraploids, being the use of neotetraploids a key innovation. As it was not possible to detect neotetraploids in natures, a protocol was established to successfully synthetize neotetraploids in the laboratory, using colchicine treatments applied to natural diploids. Afterwards, diploids, neotetraploids and tetraploids were grown with and without competition in controlled conditions. Results showed that polyploidization did not affect competitiveness, and thus, it may have not played a significant role for polyploid establishment. However, similar competitive abilities at the contact zone may be responsible for the maintenance of a stable contact zone. Also, differential competitive abilities of cytotypes across the distribution area of *J. maritima*, possibly linked with adaptations to environmental gradients, could have contributed for the current allopatric distribution of this species.

In conclusion, the consequences of polyploidization were broad and variable, being highly species-specific. With this PhD Thesis, I observed that polyploidization may partially change ecological requirements of polyploid entities and broaden the niches of cytotypes, allowing the colonization of new environments. However, this was not observed in all studied

complexes. Indeed, in some cases, other factors were involved with polyploid establishment and spread. Reproductive barriers between cytotypes were determinant in inter-cytotype interactions and crucial for the establishment of the new cytotype at the contact zones. Also, genome duplications drove some developmental changes, but shifts in competitiveness were not so clear, despite differences in competitive ability between diploids and established tetraploids enabled to explain current distribution patterns. The results obtained in this PhD Thesis open several avenues for research regarding polyploidy. Clearly, more studies focusing on the ecological processes, both in natural populations and under controlled conditions, are needed to understand the conditions responsible for the successful establishment and spread of polyploids and, consequently to understand the pervasive occurrence of polyploidization in flowering plants and its role in plant evolution and diversification.

Keywords: competitive ability, cytogeographic patterns, environmental requirements, neopolyploids, polyploidization, reproductive barriers, sympatric speciation.

Resumo

A diversidade citogenética é amplamente observada em inúmeros organismos. Nas plantas com flor, a diversificação e a evolução estão intimamente relacionadas com eventos de poliploidização ao longo de toda a sua história evolutiva. A formação de poliploides é frequente na natureza, contudo, o estabelecimento de novas entidades nem sempre é bem-sucedido. As alterações geradas pela poliploidização têm profundas consequências na genética, morfologia, fisiologia e sistema reprodutivo da planta, afetando o êxito da planta e levando a fenómenos de divergência. Depois da sua formação, os indivíduos poliploides podem ser eliminados da população parental através de pressões seletivas que afetam o citotipo minoritário, ou, por sua vez, a poliploidização pode conferir uma vantagem que permite ao poliploide escapar da seleção dependente da frequência. Esta vantagem pode permitir ao poliploide excluir os seus progenitores e/ou capacitá-lo com tolerâncias ambientais mais amplas que permitirão a sua dispersão para novos habitats. A coexistência dos citotipos será possível se um conjunto de barreiras reprodutivas promoverem cruzamentos seletivos. O principal objetivo desta Tese de Doutoramento foi perceber o papel da poliploidização na diversificação das plantas, focando-se nos processos ecológicos envolvidos no estabelecimento e na dispersão com sucesso de linhagens poliploides. Para o efeito, foquei-me a três níveis diferentes: nos padrões citogeográficos *in situ* e associações ambientais em diferentes complexos poliploides; na interação entre citotipos em zonas de contacto; nas consequências diretas da duplicação do genoma em oposição a adaptações após a poliploidização.

Estudos em larga escala para determinar os níveis de ploidia foram realizados ao longo da área de distribuição de *Jasione maritima* e *J. montana*, na última com foco maioritário na zona de contacto no noroeste da Península Ibérica, e os requisitos ambientais de cada citotipo foram avaliados. Indivíduos diploides foram reportados pela primeira vez em *J. maritima*, sendo que os citotipos se encontravam distribuídos alopatricamente: os diploides ocorrem nas dunas do Norte, enquanto os tetraploides estão presentes em populações mais secas e quentes das regiões Centro e Sul da distribuição da espécie. O nicho ambiental mais amplo observado nos tetraploides sugere que a poliploidização pode ter alterado os requisitos ambientais deste citotipo, permitindo a colonização e seu estabelecimento em áreas do Sul e explicando, em parte, o seu atual padrão de distribuição. A distribuição restrita dos diploides revela a necessidade de se desenvolverem medidas de conservação focadas nos citotipos. Em *J. montana*, os citotipos encontravam-se distribuídos parapatricamente, formando várias zonas de contacto, com os diploides a apresentarem nichos ambientais e geográficos mais amplos que os

tetraploides. Neste complexo poliploide, a poliploidização não parece ter causado alterações nas preferências ecológicas dos tetraploides, e por isso é expectável que outros fatores estejam envolvidos nos padrões de distribuição atual.

O complexo tetraploide-octoploide *Gladiolus communis* foi usado para explorar as interações dos citotipos em zonas de contacto, usando um complexo de espécies que possui níveis de ploidia elevados. Primeiro, a diversidade citogenética, os padrões de distribuição e os requisitos ambientais foram explorados. Os citotipos tetraploides e octoploides foram dominantes e, apesar do elevado grau de isolamento, foi igualmente observado que ambos os citotipos crescem em simpatria em várias populações. As exigências ambientais entre citotipos foram similares, sugerindo que a poliploidização não parece ter gerado alterações nas preferências ambientais. A deteção de hexaploides e populações de ploidia mista sugeriram que a hibridização e a formação recorrente de gâmetas não reduzidos são eventos frequentes, o que aponta para recorrente formação de poliploides e fluxo de genes. Consequentemente, a existência de barreiras reprodutivas entre os citotipos que permitam a sua coexistência foram exploradas, em particular, barreiras temporais, comportamentais, mecânicas e gaméticas. Observou-se que as barreiras pré-polinização foram fracas, enquanto que as interações pós-polinização foram fortes e variáveis dependendo das cargas de pólen, refletindo, consequentemente, a estrutura da população. Polinizações controladas sugerem que, após formação, uma menor capacidade excluirá o poliploide da população, a menos que a formação de gâmetas não reduzidos minore esta desvantagem, enquanto que em fases posteriores, as barreiras pós-zigóticas fortes poderão permitir a coexistência dos citotipos.

Considerando que a distribuição dos citotipos de *J. maritima* foi apenas parcialmente explicada pelas variáveis ambientais, foi explorado o papel da poliploidização como motor de diferentes capacidades competitivas entre citotipos. As consequências diretas de duplicações do genoma foram avaliadas usando diploides, neotetraploides e tetraploides estabelecidos, sendo a utilização de neotetraploides uma inovação importante desta Tese. Como não foi possível detetar neotetraploides na natureza, foi estabelecido um protocolo para sintetizar com sucesso neotetraploides em laboratório, usando diferentes tratamentos com colquicina aplicados a diploides naturais. De seguida, diploides, neotetraploides e tetraploides de *J. maritima* foram cultivados com e sem competição em condições controladas. Os resultados mostraram que a poliploidização não afetou a capacidade competitiva, e por isso, este fator pode não ter desempenhado um papel significativo no estabelecimento dos polipoides. Contudo, capacidades competitivas similares na zona de contacto podem ser responsáveis pela manutenção de uma zona de contacto estável. Além disso, diferentes capacidades competitivas

dos citotipos ao longo da área de distribuição de *J. maritima*, possivelmente ligadas com adaptações a gradientes ambientais, podem ter contribuído para a atual distribuição alopátrica desta espécie.

Em conclusão, as consequências da poliploidização foram amplas e variáveis, sendo altamente especificas de cada espécie. Com esta Tese de Doutoramento, observei que a poliploidização pode alterar, parcialmente, os requisitos ambientais das entidades poliploides e ampliar o nicho de cada citotipo, permitindo a colonização de novos habitats. Contudo, isto não foi observado em todos os complexos estudados. De facto, em alguns casos, outros fatores estiveram envolvidos com o estabelecimento e dispersão dos poliploides. As barreiras reprodutivas entre os citotipos foram determinantes nas interações entre citotipos e cruciais para o estabelecimento do novo citotipo nas zonas de contacto. Além disso, as duplicações de genoma levaram a algumas alterações em características de desenvolvimento das plantas, contudo mudanças na capacidade competitiva não foram tão claras, apesar das diferenças existentes entre diploides e tetraploides estabelecidos terem permitido explicar os padrões de distribuição atuais. Os resultados desta Tese de Doutoramento apresentam várias perspetivas futuras no estudo da poliploidia. Claramente, são necessários mais estudos focados nos processos ecológicos, tanto em populações naturais como em condições controladas, de forma a perceber quais as condições responsáveis pelo estabelecimento e dispersão, com sucesso, dos poliploides e, consequentemente, compreender a ocorrência universal da poliploidização nas plantas com flor e o seu papel na evolução e diversificação das plantas.

Palavras chave: barreiras reprodutivas, capacidade competitiva, especiação simpátrica, neopoliploides, padrões citogeográficos, poliploidização, requisitos ambientais.

Chapter 1 – General introduction

Ecosystems result from complex associations between its biotic and abiotic elements, linked by energy flows (Tansley 1935; Golley 1993; Carpenter and Turner 1998) in dynamic and continuous interactions towards an equilibrium stage (Jackson 2011). The ecosystems are frequently subjected to natural (and currently also anthropogenic) pressures that create negative imbalances. These changes may decrease biodiversity both at species and population levels but at the same time might also create new opportunities for diversification. One of the mechanisms pointed out to contribute to the genesis and diversification of organisms is polyploidization.

Polyploidization or whole genome duplication (WGD) is the hereditary capacity to have more than two sets of chromosomes per nucleus and it is widely considered as a key mechanism for plant diversification (Ramsey and Schemske 1998; Soltis and Soltis 1999). In fact, studies based in the cytology, fossil and genetic analyses of angiosperms, suggested that 47% up to 100% of the flowering plants had suffered a WGD event during its evolutionary history (Grant 1981; Masterson 1994; Cui *et al.* 2006; Soltis *et al.* 2009; Albert *et al.* 2013). However, despite polyploidization is broadly recognized as a dynamic and recurrent process in the natural history of many groups of organisms, little is known about the array of effects resulting from polyploidization events, their role in polyploid lineages establishment and spread and the evolutionary processes after genome duplication.

Current incidence of polyploidy

Since the mid of the 20th century that polyploidy started to receive attention from the scientific community, with the first studies devoted to estimate the incidence of this speciation mechanism in plants being published. Indeed, estimates of current polyploidy incidence ranged from 20-40% (*e.g.*, Stebbins 1938, 1950; Wood *et al.* 2009). In 2017, Marques and co-authors (Marques *et al.* 2017), compiled the information about polyploidy in plants, available for the Mediterranean region and the Iberian Peninsula in particular due to an extensive data availability and recent taxonomic treatment of the flora for the region, and obtained estimates of 48% of polyploid species in the Iberian Peninsula. Also, similarly to what was observed in the previous studies (*e.g.*, Grant 1981; Barker 2013), in this study Pteridophytes was the plant group with higher incidence of polyploidy (75%), followed by Angiosperms (47%) and Gymnosperms (6%; Figure 1.1) (Marques *et al.* 2017).

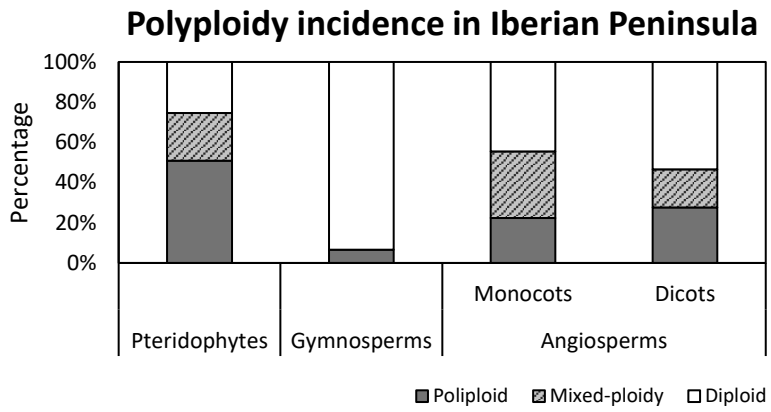


Figure 1.1. Polyploidy incidence in the Iberian Peninsula according to three of the major plant groups: Pteridophytes, Gymnosperms and Angiosperms. White bars represent the percentage of diploid taxa, dark grey bars represent polyploid taxa and light grey bars with diagonal bands mixed-ploidy taxa. Adapted from Marques *et al.* (2017).

In Pteridophytes, studies that have followed a more conservative approach suggested that 44% of the ferns and allies are polyploid (Vida 1976), while Grant (1981) suggested that 95% of the Monilophytes (a group of Pteridophytes) resulted from polyploidization events, and Barker (2013) considered the Pteridophytes as the plant group with the highest possibility of chromosomal diploidization events. The presence of mixed-ploidy taxa was estimated to range from 17 to 34% (Wood *et al.* 2009 and Marques *et al.* 2017, respectively). By contrast, Gymnosperms are long considered the plant group with the lowest polyploidy incidence (Murray 2013) with no mixed-ploidy taxa being reported in the Mediterranean region (Marques *et al.* 2017), altogether suggesting that polyploidization is not a stable process in this plant group (Ahuja 2005; Husband *et al.* 2013). In Angiosperms, the percentage of polyploidy incidence ranged from 30 to 80%, depending of the methodology used (Otto and Whitton 2000; Wood *et al.* 2009; Husband *et al.* 2013; Marques *et al.* 2017) or the region studied (*e.g.*, Levin 2002; Brochmann *et al.* 2004; Thompson 2005; Vamosi and McEwen 2013). Nevertheless, some patterns have emerged. For example, both Otto and Whitton (2000) and Marques *et al.* (2017) estimated that the percentage of polyploidization events was bigger in monocots than in dicots (32 and 56% for monocots, and 18 and 47% for dicots, respectively). Also, a large occurrence of mixed-ploidy taxa was observed in the flowering plants, with 40% of the taxa that grow in the Iberian Peninsula presenting two or more ploidy levels (Marques *et al.* 2017). Nevertheless, Marques *et al.* (2017) observed a slightly larger incidence of polyploids in comparison with Wood *et al.* (2009).

Most of the information used in these estimations was collected from karyologic studies. However, with the development of new methodologies, such as flow cytometry, that allowed faster and more efficient analyses of plant tissues (Galbraith *et al.* 1983), the number of studies focused on the cytogenetic diversity of plant species is steadily increasing in the past years (Husband *et al.* 2013; *e.g.*, *Chamerion angustifolium*, Husband and Schemske 1998; *Ranunculus adoneus*, Baack 2004; *Dianthus broteri*, Balao *et al.* 2009; *Aster amellus*, Castro *et al.* 2012; *Limonium* spp., Caperta *et al.* 2017; *Erysimum mediohispanicum*, Muñoz-Pajares *et al.* 2017; Chapters 2-4). Despite the increasing number of reports on the occurrence of polyploidy, little is known about the origin, establishment and spread of polyploids in natural populations (Thompson and Lumaret 1992; Bretagnolle and Thompson 1995; reviewed in Soltis *et al.* 2010). Therefore, more studies are needed to quantify the true contribution of polyploidy to evolution and diversification of plants.

Unreduced gametes and polyploid formation

As referred above, the occurrence of polyploid complexes in nature is widely reported and cytogenetic diversity studies have stressed their importance to investigate the origin, establishment and persistence of polyploids (Thompson and Lumaret 1992), although the ecological processes involved in these stages have received less attention (Soltis *et al.* 2010). The first step is the emergence of new entities. The duplication of somatic cells was pointed as one way of polyploids formation, still, although largely unexplored in natural populations, the production of unreduced gametes is seen the key element in the formation of new polyploids (but see Thompson and Lumaret 1992; Kreiner *et al.* 2017a, and references therein).

As a result of the meiotic process, haploid gametes (or reduced gametes – n) with half the number of chromosomes than that of the somatic cells are formed. However, due to meiotic errors that interfere with chromosomal segregation or cell division, unreduced gametes (2n) with the somatic chromosome number can be formed (Bretagnolle and Thompson 1995; Brownfield and Köhler 2010). Previous studies have reported the occurrence of unreduced gametes both in the production of female and male gametes (ovules and pollen grains, respectively; *e.g.*, Kron and Husband 2009; Herben *et al.* 2016; Kreiner *et al.* 2017b), although more emphasis has been given to the male component (reviewed by Kreiner *et al.* 2017a). In fact, the direct detection of unreduced gametes is mostly performed through volumetric analyses (De Storme *et al.* 2013) and flow cytometry (Bino *et al.* 1990; Kron and Husband 2012, 2015), based in male gametes quantification. The formation of unreduced female gametes has been indirectly quantified through analyses of the ploidy level of seeds resulting from controlled

pollinations (*e.g.*, De Haan *et al.* 1992; Maceira *et al.* 1992; Kron and Husband 2009; Chapter 5). As highlighted by Herben *et al.* (2016), the direct quantification of female unreduced gametes formation is difficult and when assessing indirectly through controlled pollinations can be problematic if the frequency of production and contribution of ovules and pollen grains is different.

But, how can unreduced gametes give rise to a neopolyploid? As mentioned above, individuals can produce reduced (n) and unreduced gametes (2n), being the later important for the polyploidization process. In that sense, neopolyploids can arise from two distinct pathways: through a one-step pathway (bilateral polyploidization), or through a two-step pathway (unilateral polyploidization) (Figure 1.2). In the one-step pathway, the neopolyploid results from the fusion of two unreduced gametes (one unreduced ovule fused with an unreduced pollen grain) and the neopolyploid is originated in only one generation. Contrarily, the two-step pathway is a longer process that involves an intermediary cytotype (triploid bridge) and at least two generations. In the first generation, one reduced gamete and one unreduced gamete fuse together, originating a triploid (in the case of diploid entities) that in most cases is partially or totally sterile (Ramsey and Schemske 1998). However, in the meiotic process, the triploid can sometimes produce viable gametes with variable ploidy, namely with one, two or three sets of chromosomes (*e.g.*, Vignoli 1937; Signorini *et al.* 2013; Costa *et al.* 2014) that, through crossing with the gametes pool of the population can originate a neotetraploid in the second generation (Ramsey and Schemske 1998; Kreiner *et al.* 2017a).

Independently of the pathway of formation, the genetic provenance of the gametes may bear a high impact. Neopolyploids can result from the fusion of genomes of the same species (*i.e.*, autopolyploids) or from the fusion of genomes from different species through an interspecific hybridization (*e.g.*, allopolyploids) (Kihara and Ono 1926; see also Figure 1 in Marques *et al.* 2017). Depending on the entities involved in the process, the neopolyploid may acquire different characteristics. The autopolyploids form multivalents during meiosis and have a polysomic inheritance, while allopolyploids have a behavior like diploids in the meiosis, with the formation of bivalents and a disomic inheritance (Stebbins 1947; Jackson and Casey 1982; Ramsey and Schemske 1998). Therefore, as allopolyploidization supposes a hybridization event, several traits observed in neoallopolyploids are not the consequence of genome duplication *per se*, but the cumulative effect of hybridization and polyploidization. In autopolyploids, the differences in comparison with the progenitor, may be attributed to WGD, and thus natural or synthetic neoautopolyploids are considered the most appropriate entities to study the effects and consequences of polyploidy (Ramsey and Schemske 2002; Ramsey 2007; Chapter 6).

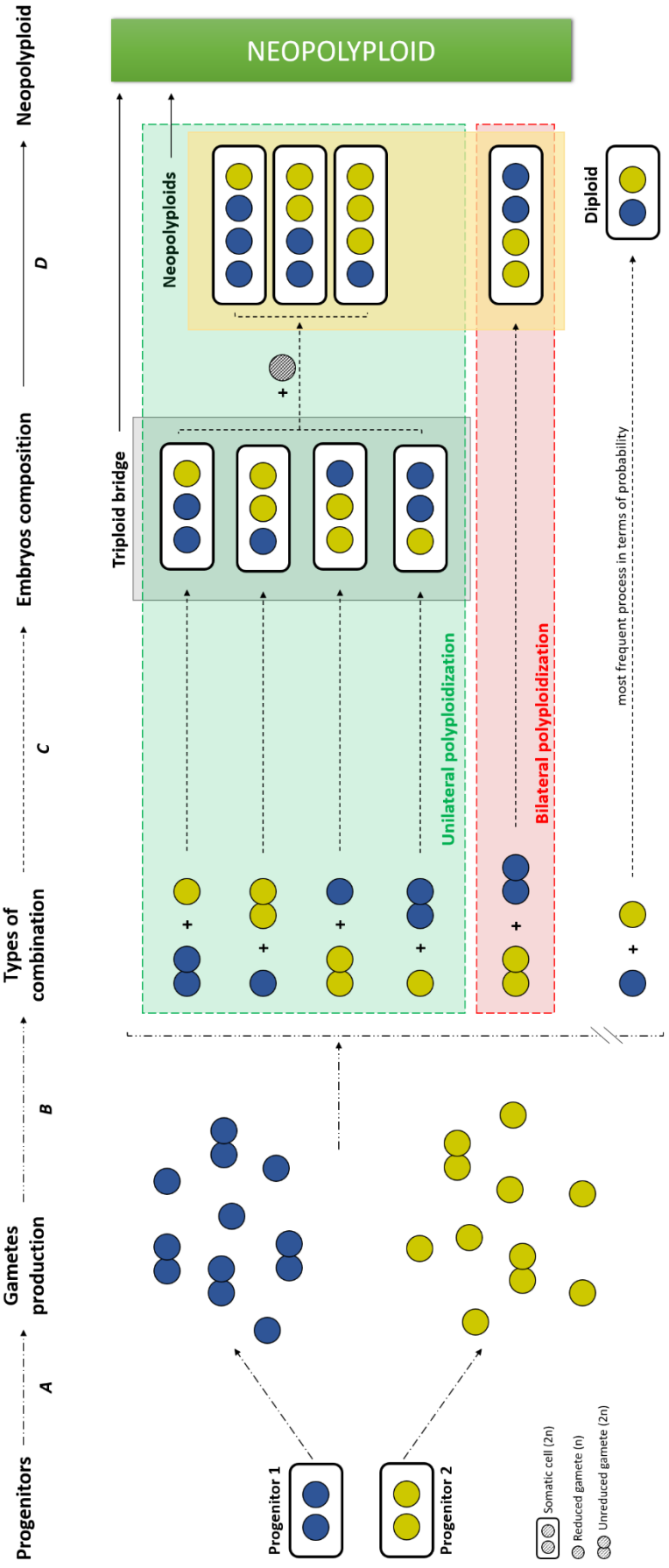


Figure 1.2. Pathways for neopolyploid formation in plants. Blue and yellow circles represent a set of chromosomes from progenitor 1 and 2, respectively. Somatic cells (2n) are represented by circles within a box and reduced (n) and unreduced gametes (2n) are represented by one or two closed circles, respectively. The four steps that influence the emergence of neopolyploids are also presented: A – Meiosis (that determine the frequency of unreduced gamete production); B – Pollination; C – post-pollination and pre-fertilization processes; D - Seed viability.

Therefore, the emergence of neopolyploids in nature greatly depends on the frequency of unreduced gametes formation during the meiosis (A in Figure 1.2). However, the fate of unreduced gametes and subsequent neopolyploid formation, is dependent also on other factors such as: gametes mobility (*i.e.*, pollen dispersal efficiency) and selfing ability; prezygotic processes after pollination; and seed viability (Figure 1.2; reviewed in Kreiner *et al.* 2017a).

The production of unreduced gametes through a failed meiosis is the first challenge in neopolyploid formation. Yet, with exception of hybrid species, the ratio between unreduced and reduced gametes varies greatly among species, populations and even among individuals in natural populations (Kreiner *et al.* 2017a, and references therein). Current estimates of unreduced gametes formation point for frequencies between 0.1 and 2.0%, with most of individuals producing unreduced gametes in very low numbers (near 0.1%) and only a small number of individuals producing more than 10% (*e.g.*, *Anthoxanthum alpinum*, 9.0 up to 12.3% individuals producing 0.5 up to 39.5% of unreduced pollen grains, Bretagnolle 2001; *Achillea borealis*, mean values ranging between 0.03% and 0.54%, with some individuals with up to 15.8% of unreduced gametes; Ramsey 2007). Indeed, in a review of the published studies, Kreiner *et al.* (2017a) shows that only three species expressed means of unreduced gametes production highly superior to 2% at the population level [*Malus coronaria* (11.6%), Kron and Husband 2009; *Turnera sidoides* (2.44%), Kovalsky and Neffa 2016; and *Pilosella echioides* (83.8% for triploid plants and 5.77% for tetraploid plants), Herben *et al.* 2016]. Curiously, in these three species, unreduced gametes were formed through the female component. The authors also suggested that unreduced gametes production could be associated with the reproductive strategies, as species with average production higher than 2% are apomict (Kron and Husband 2009) or rarely sexual reproductive (Kreiner *et al.* 2017b). However, more studies are needed to support these estimations and relations, and to evaluate the real frequency of unreduced gametes in nature (Ramsey and Schemske 1998; Kreiner *et al.* 2017a). Other studies showed that the production of unreduced gametes is a hereditary trait (Bretagnolle and Thompson 1995; Brownfield and Köhler 2010), being possible to increase their frequency in a few generations (*Trifolium pretense*, Parrott and Smith 1986; *Medicago sativa*, Tavoletti *et al.* 1991). Besides the genetic base, unreduced gametes frequency can be also governed by environmental conditions (*e.g.*, Mason *et al.* 2011; Vanneste *et al.* 2014; Sora *et al.* 2016). Several studies have shown a relation between the production of unreduced gametes and environmental stress (reviewed in Ramsey and Schemske 1998), in particular in response to changes in temperature. In that sense, unreduced gamete production was shown to significantly increase after temperature fluctuations (including both cold and heat conditions; *e.g.*, Manson *et al.* 2011; Pecrix *et al.* 2011;

De Storme *et al.* 2012). This suggests that natural environmental changes (*e.g.*, altitudinal gradients), as well as large-scale climate changes (as shown in Kürschner *et al.* 2013 and Vanneste *et al.* 2014), could substantially alter the dynamics of polyploid evolution, or at least fuel the opportunities for establishment through the recurrent formation of new entities. This has been one of major explanations for the rates of polyploidy observed for example in the Artic Flora (Brochmann *et al.* 2004) and in the Mediterranean (Marques *et al.* 2017). Unreduced gamete formation is one important factor to be considered not only in neopolyploid formation but also in their establishment (see below; Felber 1991; Rausch and Morgan 2005; Suda and Herlen 2013). Therefore, regardless of the pathway of formation (Figure 1.2), the occurrence of unreduced gametes in sufficient amounts is the first step for neopolyploid genesis (Felber 1991).

After an unreduced gamete is formed, it needs to merge with other (un)reduced gamete (pollination – B in Figure 1.2). Pollen dispersal will determine the fate of unreduced gametes and consequently the formation of polyploids. In predominantly self-pollinated plants, selfing can promote neopolyploid emergence (Grant 1956). Yet, in predominantly outcrossing plants, polyploids tend to be formed mostly through triploid intermediate (Ramsey and Schemske 1998; Burton and Husband 2000) because the variable frequency of unreduced gametes production between individuals and the random pollen flow results in high gametes loss.

Additionally, a successful pollination does not necessary lead to the formation of a viable embryo. After pollination, the pollen grain needs to be recognized by the pistil, develop a pollen tube and fuse with the ovule (C in Figure 1.2). Many processes occur after pollination and before fertilization that can facilitate or prevent fertilization (Gantait *et al.* 2018). Most of the information available comes from crosses between established entities with ploidy levels, in which different pollen tube growth rates and siring abilities were reported in pollen competition experiments (Williams *et al.* 1999; Ishizaki *et al.* 2013). In these studies, regardless of the direction of the cross, conspecific crosses had always higher siring success. In *Chamerion angustifolium* (diploid-tetraploid complex), diploid pollen grains produced by tetraploid individuals presented similar or higher siring success than haploid gametes from diploids plants when mixed-ploidy loads were applied to diploids and tetraploids flowers, respectively. The asymmetric success of pollen according with their ploidy conferred a unilateral advantage to diploid pollen grains when compared with haploid pollen (Husband *et al.* 2002; Baldwin and Husband 2011) that might suggest that unreduced gametes might also have an advantage. Most probably, this pattern results from the often-larger diameter of polyploid gametes in comparison with haploid gametes (Masterson 1994), which can ensure more resources, thus allowing farther and/or faster pollen tube growing in the style (Cruzan 1990). Therefore,

although the scarcity of studies directly with unreduced gametes, the patterns observed in crosses with mixed-ploidy loads are controversial, suggesting that post-pollinations and pre-fertilization mechanisms were species-specific (Williams *et al.* 1999; Husband *et al.* 2002; Baldwin and Husband 2011). Therefore, more studies are needed to evaluate the real impact of heteroploidy pollen grain in pollen-pistil interactions and in prezygotic mechanisms.

Finally, neopolyploids often need to overcome fertility problems (D in Figure 1.2). At this stage, a set of mechanisms are determinant for the emergence of polyploids, from the successful development of the seeds until fertility of the offspring (post-zygotic reproductive barriers). One of the main problems to seed development after interploidy cross is the unbalanced ratio of maternal-paternal genomes in the endosperm which has severe consequences to embryo development (Endosperm Balance Number hypothesis, Johnston *et al.* 1980; Haig and Westoby 1991; Scott *et al.* 1998; von Wangenheim and Peterson 2004). Embryo and endosperm growth were observed when the parental genome was the double of maternal, while an excess in maternal genome led to the inhibition of endosperm development, resulting in small embryos (Haig and Westoby 1991; Scott *et al.* 1998; Sutherland and Galloway 2017). This suggests higher fruit and seed sets when the higher-ploidy comes from the paternal progenitor. Consequently, offspring fitness might also be affected by the interploidy cross direction. Despite, seeds from interploidy cross with higher ploidy of the maternal genome presented a higher percentage of germination (Scott *et al.* 1998; Dilkes and Comai 2004; Stoute *et al.* 2012; Sutherland and Galloway 2017), Sonnleitner *et al.* (2013) observed that seedlings that resulted from the fusion of one reduced maternal gamete with one unreduced parental gamete grew faster than interploidy crosses with an excess of maternal genome, resulting in larger seedling sizes. In the fusion of two unreduced gametes, no imbalance between maternal and paternal genomes is verified, and thus, the one-way pathway formation is favorited.

In conclusion, despite unreduced gametes are the result of meiotic errors, they are extremely important in evolutionary and diversification process through polyploidization. The rate of unreduced gametes formation is the first and crucial step for the successful emergence and establishment of neopolyploids (reviewed by Kreiner *et al.* 2017a). However, as highlighted above, the subsequent steps are also crucial for a successful neopolyploid formation, although the available information is still scarce. In the only study that assessed the contribution of each of these steps, it was verified that from the 9.60% of unreduced gametes produced by diploid individuals of *Anthoxanthum alpinum* (estimated according with pollen size), only 0.21% of the seeds were triploid or tetraploid (ranging from 0.12% and 0.49%) (Bretagnolle 2001).

Polyploids establishment

Neopolyploids are frequent in nature, however, the successful establishment of the newly formed polyploid depends on the formation and extinction rates (Soltis *et al.* 2007, 2010). In the diploid progenitor population (diploid being here used in general to represent the lower-ploidy progenitor), the newly generated polyploids are in numeric disadvantage. Under this scenario, the neopolyploids are in direct competition with the progenitors for resources and reproductive partners, being subjected to strong constraints to their establishment (Levin 1975). The new formed cytotype will cross almost exclusively with diploid individuals and thus form mostly triploids offspring, theoretically sterile (*i.e.*, triploid block), leading to frequency-dependent selection against the rare cytotype (minority cytotype exclusion principle, Levin 1975).

However, some studies focused on neopolyploid establishment revealed the existence of a series of mechanisms that may allow the neopolyploid to overcome of frequency-dependence selection against rare cytotype, making neopolyploid establishment less restrictive than initially expected. Such studies focused on the role of the mating system, inbreeding depression, niche differentiation, assortative mating and increased competition as advantages for polyploid establishment (*e.g.*, Fowler and Levin 1984; Felber 1991; Rodriguez 1996a, 1996b; Li *et al.* 2004; Husband and Sabara 2004; Rausch and Morgan 2005; Marchant *et al.* 2016; Karunarathne *et al.* 2018). As revealed above, before establishment, the frequency of unreduced gametes mediates polyploid formation (Felber and Bever 1997), frequently through the production of triploid intermediates. In some cases, these intermediate triploid individuals are not completely sterile as previously assumed (Levin 1975; Fowler and Levin 1984; Felber 1991; Rodríguez 1996a; Rausch and Morgan 2005) and may contribute to increase the probability of production of neotetraploid progeny, *i.e.*, working as triploid bridge (Ramsey and Schemske 1998). Computational simulations showed that triploid success is one of the elements that can balance the establishment process (Husband 2004).

Besides the direct contribution of unreduced gametes by diploids and the contribution of triploid intermediates, other reproductive traits such as the mating system may ameliorate the initial stages after neopolyploid emergence. For example, if the neopolyploid is apomictic, the number of tetraploids increases at a faster rate in the population, without the need of fertilization (Caperta *et al.* 2016; Keiner *et al.* 2017a). In predominant asexual species the selection against unreduced gametes is usually low, resulting in the establishment and maintenance of polyploids (Keiner *et al.* 2017b). Selfing can also be advantageous in this stage

by increasing the probability of unreduced gametes fusion, by allowing to bypass the absence of compatible mates and by decreasing the loss of gametes in interploidy crosses (*e.g.*, Levin 1975; Rausch and Morgan 2005; Barringer 2007; Oswald and Nuismer 2011a). However, self-fertilization also bears some disadvantages, such as inbreeding depression. Still, the consequences of inbreeding depression are more severe in diploid progenitors than in neopolyploids, as the extra genome set in neopolyploids can more easily mask the effect of deleterious alleles (Soltis and Soltis 2000; Rausch and Morgan 2005; Oswald and Nuismer 2011a; Husband 2016). Increased fecundity of polyploids or a perennial habit that allows the neopolyploid to wait for opportunities for mating can also be advantageous (Rodriguez 1996b).

Assortative mating may also be an important factor for the establishment of the newly formed polyploid. Within the parental population, the neopolyploid needs to avoid interploidy crosses to ensure that its 2n gametes are not lost in the production of inviable offspring. Differences in phenology, flower morphology and physiology, and pollinator's composition and fidelity can mediate assortative mating in mixed-ploidy populations before pollination takes place (*e.g.*, Segraves and Thompson 1999; Husband and Sabara 2004; Castro *et al.* 2011; Jersáková *et al.* 2010). These pre-pollination barriers, usually in combination, may contribute to reproductive isolation between cytotypes. According with the contribution of each barrier, assortative mating can have a similar effect on the establishment as selfing, however, without the disadvantages of self-pollination (Rausch and Morgan 2005).

Another factor that can contribute for the establishment of the neopolyploid is the environmental preferences of the cytotypes, *i.e.*, the existence of niche differentiation after genome duplications (Li *et al.* 2004; Baack and Stanton 2005). Within the population, environmental conditions may not be homogeneous, which can lead to spatial segregation of the cytotypes within the population (Li *et al.* 2004). Genome duplication may result in different environmental adaptions and tolerances, allowing polyploids to occupy partially different niches, dispersing to habitats that are less suitable for diploids (niche shift hypothesis; Levin 1975, 2004; Husband and Schemske 2000). Many studies showed spatial segregation between established cytotypes (Husband and Schemske 1998; Sonnleitner *et al.* 2010; Balao *et al.* 2009; Kolář *et al.* 2009; Castro *et al.* 2012; Casazza *et al.* 2016). Besides that, at the populational level, pollen and seed dispersal may also interfere with the population structure (Li *et al.* 2004; Baack 2005). As discussed above, in the absence of pre-pollination reproductive barriers, random pollen dispersion may lead to large rates of interploidy crosses. However, under limited seed dispersion, individuals from each cytotypes will be distributed in clumps and, thus, limited pollen dispersal will favor intraploidy crosses (assortative mating), allowing the maintenance of

polyploids in the population (Li *et al.* 2004; Baack 2005). The available models suggest that habitat heterogeneity and limited dispersal, even with a low number of unreduced gametes production, may enable the maintenance of polyploids in the diploid population (Li *et al.* 2004; Baack 2005). This mosaic pattern of distribution is often observed in contact zones between cytotypes within a population (developed in next section). However, limited seed dispersal may be disadvantageous in subsequent colonization processes, besides promoting some level of inbreeding depression (Baack 2005).

Also, superior competitive ability by neopolyploids is recurrent indicated as one key mechanism in the establishment process, in addition to its importance in the successful spread and colonization of new habitats by the neopolyploid (Husband 2000; Levin 2002; Treier *et al.* 2009; Schlaepfer *et al.* 2010; Hahn *et al.* 2012; te Beest *et al.* 2011; Rey *et al.* 2017). However, the few available studies that had experimentally demonstrated the effect of competition showed contrasting results hindering the interpretation of general patterns. In some studies, the tetraploids are more competitive and excluded diploids from mixed-ploidy population (*e.g.*, *Dactylis glomerata,* Maceira *et al.* 1993), in others competition ability varies over the distribution area, with no differences being observed in contact zones where diploids and tetraploids coexist, while in areas dominated by tetraploids they revealed to be more competitive than diploids (*Centaurea stoebe*, Collins *et al.* 2011). In other systems, diploids were competitively superior than higher ploidies (*Mercurialis annua*, Buggs and Pannell 2006, 2007). No differences in competitive performances between cytotypes have also been reported (Münzbergová 2007; Thompson *et al.* 2015). Still, polyploids can also present different performances depending on the surrounding individuals (inter-species competition; Rodriguez 1996b; Thébault *et al.* 2011). To date, only one study evaluated the performance of diploids and tetraploids competing with dense multi-species neighborhoods, with the results suggesting that tetraploids were more competitive than diploids (allopolyploid *Brachypodium* complex, Rey *et al.* 2017). Therefore, competition ability, as other characteristics, seems to be species-specific and more studies are necessary to evaluate the role of competition in neopolyploid establishment and on current cytogeographical patterns.

In conclusion, after formation, the neopolyploid is subjected to strong frequency-dependent selection within the lower ploidy parental population (Levin 1975). To overcome this disadvantageous scenario, computational models suggest the existence of a series of variables which will drive the fate of the newly formed entity. Each variable may have a different contribution to polyploid success according with the species and evolutionary history of the polyploid complex. More experimental studies involving cytotype composition, their frequency

and spatial configuration and interactions in the population are thus necessary to disentangle the contribution of each of these variables to neopolyploid establishment.

Breeding barriers in stable cytotypes co-occurrence

The existence of cytotype diversity is more common than initially envisaged, suggesting that polyploids successful establish and spread in nature. Many studies reported the presence of different cytotypes within the same polyploid complex (reviewed by Kolář *et al.* 2017), with some of them reporting the co-occurrence of multiple cytotypes within the same population (*e.g.*, Baack 2004; Kolář *et al.* 2009; Ståhlberg 2009; Trávníček *et al.* 2010; Castro *et al.* 2012; Zozomová-Lihová *et al.* 2015; among others). The rates of neopolyploid formation and the process involved with their establishment and spread, together with the life history of the complex will all determine the spatial structure and cytotype diversity in nature.

The cytotype composition of polyploid complexes can be highly variable. However, most of the complexes are dominated by diploids and tetraploids (around 60% of the known polyploids, Kolář *et al.* 2017) or by the combination of diploids, tetraploids and hexaploids (around 9%, Kolář *et al.* 2017). *In situ*, cytotypes usually interact with each other, and only 4% of the polyploids complexes described to date present an allopatric distribution (Kolář *et al.* 2017). In the remaining polyploid complexes, cytotypes grow in proximity forming contact zones (Figure 1.3; Petit *et al.* 1999; Kolář *et al.* 2017). Cytotypes can contact in limited geographic ranges (*i.e.*, having a large-scale parapatric distribution), in numerous single populations across its distribution (*i.e.*, mosaic parapatry) or even in at a higher proximity within mixed-ploidy populations (*i.e.*, sympatry). Although the classification in nature is more difficult, examples of different distribution patterns can be found in Chapters 2-4: *Jasione maritima* cytotype being mostly allopatric but still having a contact zone thus showing a large-scale parapatry (Chapter 3), *Jasione montana* cytotypes having a mosaic parapatry (Chapter 2), and *Gladiolus communis* presenting a complex contact zone characterized by frequent sympatry of different cytotypes (Chapter 4). Life history also influences the dynamics of the contact zone, depending of the time of neopolyploid formation and on the levels of recurrent formation of new polyploids: mixed-ploidy areas where neopolyploids are recurrently formed are considered primary contact zones, while secondary contact zones result from migration after allopatric divergence (Petit *et al.* 1999). One type of contact zone does not necessarily exclude the other. For example, in *Melampodium* spp. (Stuessy *et al.* 2004) and *Knautia arvensis* agg. (Kolář *et al.* 2009) the two types of contact zones were observed in the same complex. Therefore, cytotype distribution

patterns are the combination of whole genome duplication dynamics, ecological preferences and cytotype interactions (Petit and Thompson 1999; Husband *et al.* 2012). Consequently, and because polyploids arise within parental populations, contact zones are recognized as natural laboratories to study the processes involved with the emergence, successful establishment and subsequent spread of the new polyploid (Hewitt 1988; Harrison 1993; Petit *et al.* 1999; Lexer and van Loo 2006).

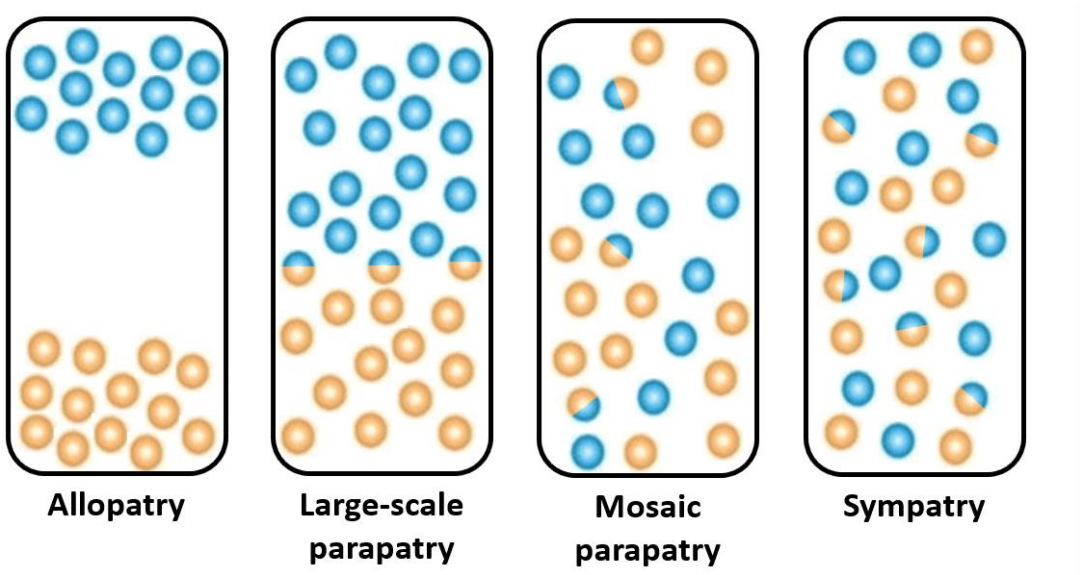


Figure 1.3. Spatial distribution patterns within and among population at regional scale. Different cytotypes can grow in allopatry, parapatry or sympatry, in case cytotypes grow disjunct, adjacent or intermixed, respectively. Pure color balls (orange or blue) represent pure-ploidy populations from different cytotypes, while balls with more than one color represent mixed-ploidy populations composed by the two cytotypes. Adapted from Kolář *et al.* (2017).

Populations where different cytotypes grow together can be considered as 1) a transitory step where polyploids are recurrently formed within the population or dispersed from neighboring populations (Felber 1991; Kolář *et al.* 2009) or 2) regarded as a stable step where several mechanisms ameliorate fitness disadvantages and enable cytotype co-existence (*e.g.*, Rieseberg and Willis 2007; Paun *et al.* 2009; Thompson and Merg 2008; Jersáková *et al.* 2011; Sonnleitner *et al.* 2016). Among the conditions necessary to enable cytotype coexistence are the recurrent unreduced gamete formation, migration from other populations, micro-habitat niche segregation and plant clumping, and the existence of barriers to interploidy reproduction (Figure 1.4) (Li *et al.* 2004; Baack 2005; Kreiner et al 2017; Kolář *et al.* 2017; Segraves 2017).

Spatial segregation (habitat isolation – Figure 1.4A) was already observed in several polyploid complexes, ranging from altitudinal gradients and large-scale geographic gradients

(*e.g.*, Felber-Girard *et al.* 1996; Husband and Schemske 1998; Buggs and Pannell 2007; Hülber *et al.* 2009; Ramsey 2011; Martin and Husband 2013; Muñoz-Pajares *et al.* 2017) to the micro-habitat segregation within the population (*e.g.*, Baack 2004; Kolář *et al.* 2009; Manzaneda *et al.* 2012; Hao *et al.* 2013). Recently, with the development of niche modeling tools, several studies associated cytotypes segregation with different environmental preferences (*e.g.*, Glennon *et al.* 2014; Thompson *et al.* 2014; Visger *et al.* 2016; Muñoz-Pajares *et al.* 2017), suggesting the occurrence of niche differentiation between different cytotypes. Levin (2002) proposed that polyploids are less impacted by stressful conditions, having a higher tolerance to low nutrient levels, drought and cold temperatures. Therefore, in a population with heterogeneous environmental conditions, spatial segregation due to both different micro-habitat preferences and limited seed/pollen dispersal (as mentioned above) will mediate the clumping of individuals of the same cytotype. Under a clumping distribution of the cytotypes, generalist pollinators will visit more neighboring individuals, promoting assortative mating (Segraves and Thompson 1999; Husband and Schemske 2000; Baak 2005; Nuismer and Cunningham 2005; Thompson and Merg 2008). Nevertheless, more field studies, including reciprocal transplants (*e.g.*, Baack and Stanton 2005; Martin and Husband 2013), are still needed to experimentally evaluate habitat segregation at several scales.

Besides the small-scale habitat segregation, there are other pre-pollination barriers to interploidy pollen mating that can act within mixed-ploidy populations to promote assortative mating (Figure 1.4). Phenological segregation in time is one of the mechanisms to avoid the loss of gametes through failed interploidy crosses (temporal isolation – Figure 1.4B). Many studies reported differences in flowering time between cytotypes (*e.g.*, van Dijk and Bijlsma 1994; Petit *et al.* 1997; Segraves and Thompson 1999; Husband and Sabara 2004, Nuismer and Cunningham 2005; Martin and Husband 2012; Laport *et al.* 2016), ranging from limited overlapping (*Arrhenatherum elatius*; Petit *et al.* 1997; *Gymnadenia conopsea* complexes, Jersáková *et al.* 2010) to total overlapping (*Aster amellus*, Castro *et al.* 2011; *Gladiolus communis*, Castro *et al.* 2018 – Chapter 4). Besides being suggested as direct consequence of polyploidization, differences between cytotypes in phenology may be also consequence of selection processes after polyploidization to allow cytotype coexistence (*e.g.*, *Plantago media*, van Dijk and Bijlsma 1994; *H. grossulariifolia*, Nuismer and Cunningham 2005). Also, shifts in morphology are another trait commonly associated with polyploidization events (*e.g.*, Melaragno *et al.* 1993; Li *et al.* 1996; Maherali *et al.* 2009; Ramsey 2011; Hao *et al.* 2013; Madlung 2013). Morphological and physiological differences in floral traits such as changes in the size and color of flowers and inflorescences (Husband and Schemske 2000; Kennedy *et al.* 2006; Borges *et al.* 2012; Hao *et al.*

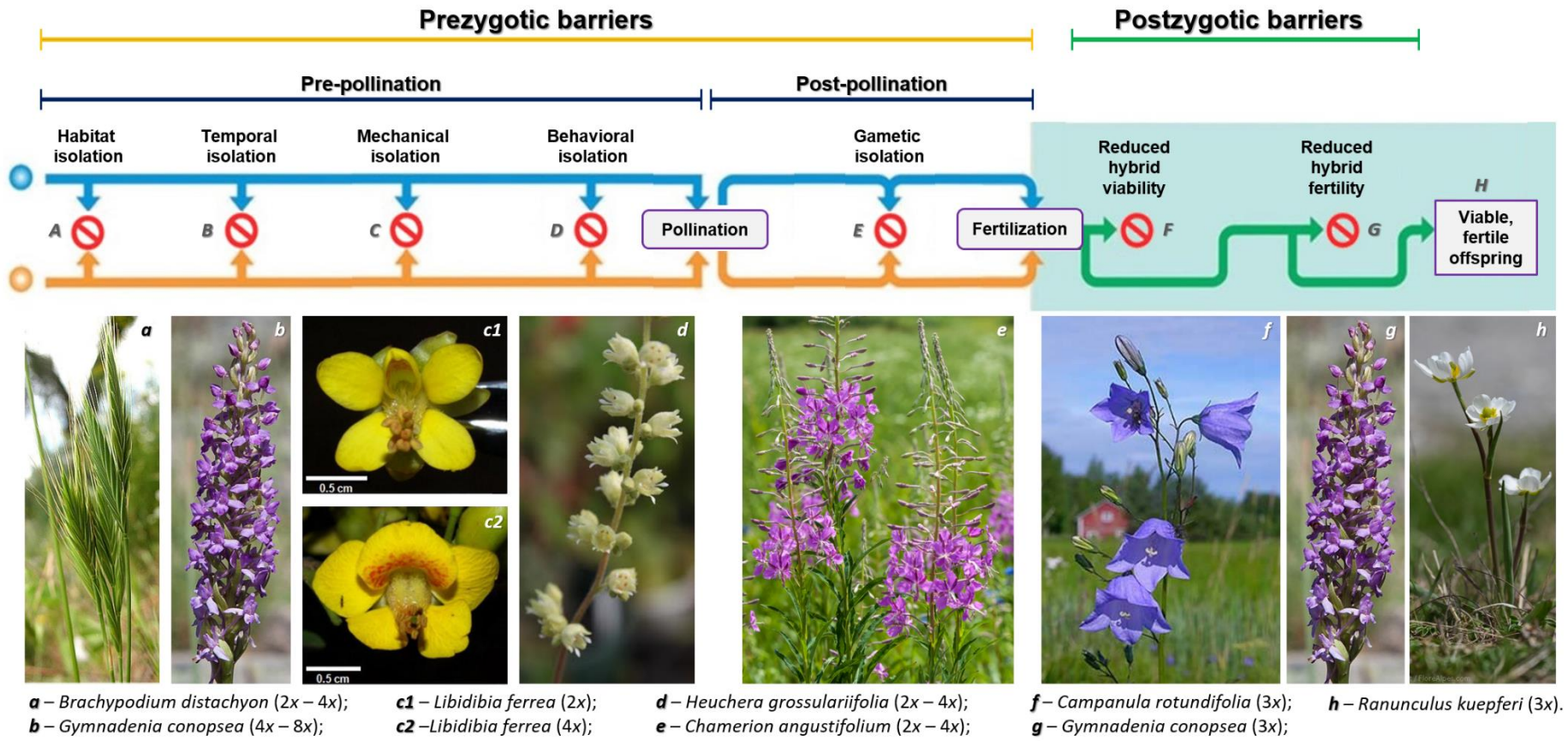


Figure 1.4. Breeding barriers that may interfere with gene flow within polyploid complexes, with some examples of polyploid complexes where such barriers were observed being given. Images sources: a, b, g, h – http://www.flora-on.pt; c – Borges *et al.* 2012; d – https://sites.google.com/site/ryanheuchera/heuchera-grossulariifolia; e – http://www.lhseeds.com/; f – http://www.luontoportti.com/suomi/en/kukkakasvit/harebell and h – http://www.florealpes.com/fiche_renonculekupfer.php.

2013; McCarthy *et al.* 2015; Gross and Schiestl 2015) or in nectar composition and scent (Jersáková *et al.* 2010) were also reported in some polyploids. These differences may contribute to mechanical isolation among cytotypes by for example resulting in deposition of pollen in different parts of the insect body (Grant 1994; Figure 1.4C) or may drive different behavior by the main pollinators. Indeed, floral traits are extremely important in pollinator's attraction and for their preferences and behavior (behavioral isolation – Figure 1.4D), greatly determining the patterns of pollen flow within and among cytotypes. Different flower characteristics, combined or not with different flowering times, can lead to the attraction of different pollinator sets resulting in increased levels of pre-pollination reproductive isolation (*e.g.*, Segraves and Thompson 1999; Roccaforte *et al.* 2015). Therefore, pre-pollination barriers can reduce the reproductive costs related with gametes losses (Harder and Wilson 1998; Barrett 2002), being fundamental for the maintenance of the neopolyploids and cytotype coexistence.

Despite of the numerous examples given above regarding the importance of pre-pollination barriers, in other polyploid complexes there is no evidences of assortative mating before pollination takes place (*e.g.*, *Gymnadenia* complex, Jersáková et al 2010; *Aster amellus* Castro *et al.* 2011; *Gladiolus communis*, Chapter 5). However, in such cases, post-pollination barriers may prevent fertilization (gametic isolation – Figure 1.4E). For example, competition between pollen grains in mixed-ploidy pollen loads and subsequent male-female gametophyte interactions will determine cytogenetic composition of the offspring (Cavanah and Alexander 1963; Husband *et al.* 2002; Peckert and Chrtek 2006). This has been suggested also to drive the results observed in mixed-ploidy population of *Gladiolus communis* (Chapter 5). Theoretical models suggested that hybrid zygotes will be inviable or have low viability when compared with progenitors (triploid block; Marks 1966; Levin 1975; Fowler and Levin 1984; Felber 1991; Rodríguez 1996a; Rausch and Morgan 2005), however, as explained above, this is not always the case and viable and fertile intermediate cytotypes can contribute to the maintenance of cytogenetic diversity within mixed-ploidy populations, governing the dynamics of the contact zone (Ramsey and Schemske 1998; Schinkel *et al.* 2017; Figure 1.4H).

The different breeding barriers can work together governing gene flow between cytotypes and determining the offspring cytogenetic composition, and consequently cytotype distribution patterns. Advantageous traits resulting from WGD process may be selected allowing the persistence of minority cytotypes and subsequently their divergence and speciation. However, the patterns of distribution and the interactions between cytotypes at contact zones are very species-specific and thus, detailed ecological studies in natural populations are needed (reviewed by Segraves and Anneberg 2016; Segraves 2017).

Polyploid-progenitor differences: direct consequence of genome duplications and/or post-polyploidization selection

Whole genome duplications can result in immediate shifts in morphology, ecological tolerances and/or reproduction (Levin 1983), that can ameliorate the numeric advantage and overcome the minority cytotype exclusion (Levin 1975). Physically, increases on cell size are pointed to be one of the direct effects of genome size increase associated with polyploidization, resulting in many cases in significant increases in the size of many plant organs, such as the leaves, flowers, fruits and seeds ("gigas" effect; *e.g.*, Stebbins 1971; Buggs and Pannell 2007; Hoya *et al.* 2007; Ramsey and Ramsey 2014). Genetically, besides interfering with gene expression, the extra genome set(s) can minimize the effect of deleterious recessive mutation through heterozygosity (Adam and Wendel 2005; Comai 2005). These advantages have long been acknowledged and used. For example, induction of synthetic polyploids has been frequently used in crop improvement programs (Levin 2002; Dar *et al.* 2017), as many selected polyploids present higher biomass and are more robust when compared with their diploid/lower ploidy progenitors (*e.g.*, Müntzing 1936; Smith 1946; Masterson 1994; Levin 2002; Ramsey and Schemske 2002; Ramsey and Ramsey 2014). In many studies, higher vigor of polyploids is associated with higher competitive capacity. For example, the larger seeds of polyploids may increase germination rate (Bretagnolle *et al.* 1995; Hoya *et al.* 2007) and produce larger seedlings (Moles and Westoby 2004; Ortega-Olivencia and Devesa 1997; Westoby *et al.* 1996) favoring polyploids under competition conditions (Liancourt *et al.* 2009). As referred above, a higher competitive capacity may allow the establishment of neopolyploid within progenitors' population (Fowler and Levin 1984; Levin 2002), governing the interactions between cytotypes in the contact zone (Petit *et al.* 1999; Laport *et al.* 2013) and consequently determining their distribution patterns (Maceira *et al.* 1993; Buggs and Parnnell 2007).

Beside the changes in competitive ability, genome duplications also modify polyploids physiology driving different requirements and tolerances and allowing them to colonize novel niches (Levin 1975; Hao *et al.* 2013; Ramsey 2011). For example, drought tolerance is suggested to be higher in polyploids than in the diploid progenitors (Maherali *et al.* 2009; Hao *et al.* 2013). Increases in stomata size and vessel diameter result in a more efficient water use which might be particularly advantageous in soils with low humidity (Maherali *et al.* 2009). However, the increase in hydraulic conductivity can also increase the risk of cavitation due enlargement of xylem (Maherali *et al.* 2009). Therefore, water transport efficiency and safety need to be balanced, so that polyploids have a higher drought tolerance than diploids (Hao *et al.* 2013). Increases in leaf thickness (Laere *et al.* 2011) or in epidermis thickness and pubescence (Li *et al.*

1996, 2009) by polyploids, are also efficient strategies to reduce water loss. Besides changes in leaves morphology, Li *et al.* (1996) observed that polyploids presented a trade-off between the number of stomata and their size, *i.e.*, polyploids presented bigger stomata in lower frequency, promoting the maintenance of photosynthetic rates at lower water potential and turgor, which confers an advantage under water stressful environments. This trade-off between size and number of stomate was further observed in other polyploid complexes (Maherali *et al.* 2009; Oswald and Nuismer 2011b; Green *et al.* 2013). Stressful conditions also increase the production of reactive oxygen species (ROS) increasing the oxidative status of the plant (Mittler 2002). After damage, the plant reacts and triggers an antioxidant response and differences in antioxidant response between cytotypes were also observed in polyploid complexes. For example, tetraploids of *Arabidopsis thaliana* presented a higher capacity of defense to different stresses (*e.g.*, NaCl and drought) probably due to polyploidization (del Pozo and Ramirez-Parra 2014). In *Dioscorea zingiberensis*, a higher antioxidant enzyme activity was observed in tetraploids than in diploids when exposed to stressful conditions (Zhang *et al.* 2010). Therefore, polyploidization was pointed to cause changes in antioxidant system due to up-regulation of genes, allowing a better response of polyploids under stressful conditions (*e.g.*, del Pozo *et al.* 2015; Tan *et al.* 2015; Kong *et al.* 2017). Also, the capacity to adapt to colder environments, as well as, the competitive superiority of polyploids in comparison with their diploid counterparts, suggests that polyploids could be more efficient during invasion processes (te Beest *et al.* 2011), as observed in *Solidago gigantea* (Schlaepfer *et al.* 2010), *Centaurea solstitiallis* (Hahn *et al.* 2012) and *Oxalis pes-caprae* (Castro *et al.* 2016a; Tavares 2014).

Genome doubling may also have consequences at mating system level. Several studies associated polyploidy with increases in self-fertilization rates (Rodriguez 1996a; Barringer 2007; Husband *et al.* 2008) and clonal reproduction (Gustafsson 1948; Kao 2007). Chromosome doubling may break down incompatible-systems, allowing self-fertilization (Grant 1956; Stebbins 1957; *e.g.*, Petit *et al.* 1997; Castro *et al.* 2011; Borges *et al.* 2012). Recently, in a large comparative study, Barringer (2007) observed that selfing rates are superior in polyploids than in diploids. A similar pattern was observed by Husband *et al.* (2008) in 10 diploid-polyploid species pairs. Besides that, Husband *et al.* (2008) observed that the mode of origin of the polyploid (*i.e.*, allopolyploid *vs* autopolyploid) was linked with different responses in mating system: allopolyploids being predominantly self-compatible (observed also by Grant 1956; Stebbins 1957), while autopolyploids having higher rates of outcrossing. However, such association between ploidy and incompatibility was not observed by Mable (2004). Some studies pointed that the breakdown in incompatible systems after polyploidization is a transitory

process (Husband *et al.* 2008). Therefore, it can be suggested that at initial stages, when the probability of mating of the neopolyploids is low, self-fertilization is crucial to avoid their reproductive disadvantage, while mixed or outcrossing mating systems are favored afterwards by natural selection (Mable 2004; Husband *et al.* 2008). It has also been suggested that incompatible system breakdown and consequent inbreeding depression due to polyploidization may be involved with sexual dimorphism development (Miller and Venable 2000). Besides the changes in sexual mating systems, ploidy is also associated with increased asexual reproduction (Gustafsson 1948). Clonality can allow the persistence of neopolyploid in the population (Stebbins 1938; Otto and Whitton 2000), minimizing the effect of minority cytotype exclusion. In polyploid complexes with sexual and asexual strategies of reproduction, asexuality can maintain neopolyploid in the population until a reproductive compatible individual emerges in the population, having a similar effect of the changes towards a perennial life-cycle strategy (Rodriguez 1996b). Apomixis is also important in several polyploid complexes. Stebbins (1941) pointed that numerous apomictic plants were also polyploid and in the literature it is possible to find numerous transitions towards apomixis in polyploid complexes (*e.g.*, Quarin *et al.* 2001; Krahulcová and Rotreklová, 2014). However, apomixis is a very complex process, and little is still known about dynamics and relation between apomixis and polyploidy (Kao 2007), and thus further efforts should be done in future studies.

Despite of their importance, the majority of the examples given above (*e.g.*, Husband and Sabara 2004; Ramsey 2011; Hao *et al.* 2013; Laport *et al.* 2016; Segraves and Annaberg 2016) compared polyploid progenitors with established polyploids, aggregating the direct effects of genome duplication and the effects of post-polyploidization ecological adaptations (Levin 1983; Ramsey and Ramsey 2014; Soltis *et al.* 2014). The interaction of these two processes needs to be considered when studying polyploidy within an evolutionary context, as the consequences of genome duplication *per se* can only be assessed using neopolyploids (Ramsey 2011; Husband *et al.* 2012; Chapters 6 and 7). Also, only when comparing neopolyploids and established tetraploids it is possible to understand the selective pressures that may have acted during the evolutionary process. One good example is the case study of *Heuchera grossulariifolia*. In the field, where cytotypes grow in sympatry, tetraploids flowered earlier than diploids (Segraves and Thompson 1999). However, in the greenhouse, synthetic neotetraploids were shown to flower later than diploids, suggesting that selection after polyploidization can mask or even change the effects of genome duplications (Oswald and Nuismer 2011b). Besides this work, only a few other studies used this approach to evaluate the role of genome duplications *per se* (*e.g.*, *Chamerion angustifolium*, Husband *et al.* 2008, Maherali *et al.* 2009;

Tragopogon species, Tate *et al.* 2009; *Achillea borealis*; Ramsey 2011; *Vicia craca*, Pavlíková *et al.* 2017). In ecological studies, synthetic tetraploids are produced using c-mitotic agents when not found in nature, as for example was the case of *Jasione maritima* where synthetic neotetraploids were directly obtained from seedlings (Chapter 6) among others (*e.g.*, *Chamerion angustifolium*, Husband *et al.* 2008, Maherali *et al.* 2009; Baldwin and Husband 2011, Husband *et al.* 2016; *Tragopogon* species, Tate *et al.* 2009; *Heuchera grossulariifolia*, Oswald and Nuismer 2011; *Vicia craca*, Pavlíková *et al.* 2017). These synthetic neotetraploids can be then used to explore the effects of genome duplications by comparing their performance with diploid progenitors and established tetraploids (*e.g.*, Chapter 7, Maherali *et al.* 2009; Husband *et al.* 2016; Pavlíková *et al.* 2017). Given the informative relevance of including neopolyploids, more studies including these key players are needed to quantify the genome duplication consequences and respective evolution afterwards.

Objectives and structure of the PhD Thesis

Polyploidization is a very complex process and a major sympatric speciation mechanism in flowering plants. Still, the ecological determinants involved with the successful establishment and spread of polyploid lineages is still poorly studied (Thompson and Lumaret 1992; Soltis *et al.* 2010). Therefore, the main goal of this thesis was to understand the role of polyploidization in plant diversification by evaluating the ecology of different polyploid complexes, from geographical patterns in nature to the interactions between cytotypes and the environment, interaction between cytotypes at contact zones and responses in controlled experiments using neopolyploids. For this, this PhD thesis was organized in three main parts: Part I, focused on large-scale geographic distribution of cytotypes and environmental determinants that could explain the observed patterns (Chapters 2 and 3); Part II, focused on cytotype interactions and coexistence at contact zones (Chapters 4 and 5); and, Part III, focused on direct consequences of whole genome duplications in cytotype competitive ability using diploids, neotetraploids and established tetraploids (Chapters 6 and 7). In Chapter 8, the general conclusions are presented, as well as the future perspectives of the results obtained in this Thesis.

In Part I, two polyploid complexes formed by diploid and tetraploid plants, *Jasione maritima* (Chapter 2) and *J. montana* (Chapter 3), were used with the objective of evaluating the relationships between the observed geographic distribution and the ecological requirements of each cytotype. For that, large-scale sampling and flow cytometric screenings were performed in the Iberian Peninsula to assess cytotype distribution patterns, detect rare cytotypes and diploid-

tetraploid contact zones, and evaluate environmental and soil requirements of each cytotype using niche modeling tools. In both systems, based on the observed geographic distribution pattern, it was possible to build hypotheses that could explain the establishment and maintenance of tetraploids within or beyond the diploid range, thus providing insights on some of the factors involved in successful polyploid establishment.

Part II is focused on the areas where cytotypes coexist and interact, *i.e.*, contact zones, with the objective to evaluate the dynamics in these areas. For this, the tetraploid-octoploid *Gladiolus communis* polyploid complex was used as study system to describe contact zones and assess reproductive barriers between cytotypes. First, in Chapter 4, the cytotype diversity and distribution within a complex contact zone was studied in detail, with the objective to evaluate if different cytotypes can grow in close proximity and how strong was the geographic barrier to cytotype coexistence and gene flow. Furthermore, using niche modelling tools, this study also aimed to evaluate if cytotypes coexistence is facilitated by different environmental relations between cytotypes, and to underpin the production of unreduced gametes and/or hybridization processes in natural populations by the detection of intermediate cytotypes. In Chapter 5, the role of phenological, morphological, behavioral, and gametic barriers between the dominant cytotypes (*i.e.*, tetraploid and octoploid) were evaluated in natural populations and in controlled conditions.

Finally, in Part III, the direct consequences of whole genome duplications were evaluated using the diploid-tetraploid *J. maritima* as study system. Because neopolyploids were not found in natural populations of *J. maritima*, the first goal was to synthetize neopolyploids in the laboratory using a c-mitotic agent, colchicine, taking in consideration the variability coming from population of origin and mother plant (Chapter 6). Afterwards, using seeds of synthetic neopolyploids, natural diploids and established tetraploids, the second goal was to evaluate the performance of the three cytotypes growing with and without competition (Chapter 7). This enabled to assess the contribution of genome duplications *per se* to cytotype differentiation and in particular for increased competitive ability, and to evaluate if natural tetraploids presented adaptions that have emerged after polyploidization. The analyses of the competitive ability of cytotypes along the distribution range of the species also enabled to evaluate the role of competition in the maintenance of current distribution of *J. maritima* cytotypes.

PART I – Large-scale cytogeographic distribution and environmental determinants

Chapter 2 – Is allopatric distribution of a diploid-tetraploid complex an indicator of different environmental preferences?

Chapter section submitted as an original article to Frontiers in Plant Sciences:
Castro, M., Loureiro, J., Figueiredo, A., Tavares, D., Serrano, M., Husband B. and Castro, S. Is allopatric distribution of a diploid-tetraploid complex an indicator of different environmental preferences?

ABSTRACT

Polyploidization is tightly correlated with evolution in flowering plants. Knowing the cryptic diversity within polyploid complexes and its geographic distribution in nature is the first step to unravel the ecological consequences of polyploidization and the processes involved in the successful establishment and spread of polyploids. This study aimed to investigate the occurrence of different ploidy levels, access cytotype distribution patterns and explore the ecological preferences of each cytotype in the endangered species *Jasione maritima*. Chromosome counts and large-scale cytotype screenings in the entire distribution range in the Iberian Peninsula were performed to characterize the species cytogenetically. Environmental requirements of each cytotype were performed using niche modelling tools to assess if the observed cytotype distribution patterns could be explained by environmental variables. *Jasione maritima* is described for the first time as a polyploid complex harboring both diploid and tetraploid individuals. Diploids and tetraploids grow in geographically segregated pure-ploidy populations, resulting in an allopatric distribution, with diploids occupying northern areas and tetraploids being present in central and southern areas of the species distribution range. Environmental requirements of diploids and tetraploids were distinguishable and could, at least partially, explain the observed geographic distribution of each cytotype. Tetraploids grow in areas that tend to be more exposed, being drier and hotter than those occupied by diploids. Although diploid and tetraploid presented similar niches, tetraploids clearly have a broader environmental niche than diploids. This might suggest that polyploidization could have provided an advantage to tetraploids that enabled them to colonize southern areas. Still, the similitude in the environmental niches between both cytotypes and the absence of diploids in suitable areas suggest that other factors could also be involved in the establishment and spread of tetraploids. The importance of assessing cytogenetic diversity and understanding cytotype distribution patterns for the conservation of endangered species, such as *J. maritima*, is discussed.

Keywords: Allopatric distribution, cryptic diversity, cytotypes, *Jasione maritima*, niche modeling.

INTRODUCTION

Plant evolution and diversification is driven by several governing forces (Stebbins 1950; Levin 2002). Polyploidization events, *i.e.*, duplications of chromosome sets, are one of the mechanisms widely accepted as an important motor of the genesis of new entities, and subsequent evolutionary divergence (Soltis and Soltis 1999; Jiao *et al.* 2011; Soltis *et al.* 2010). Polyploidy incidence has been reported in many living organisms, with Angiosperms incidence rates ranging from 35% (Wood *et al.* 2009), 37-47% in Mediterranean Basin (Marques *et al.* 2017), to 69-87% in the Arctic Flora (Brochmann 2004). The available bibliographic reviews focused in the incidence of polyploidy are based mostly in chromosomal data obtained while evaluating the occurrence of polyploid taxa. However, due to the difficulty of obtaining chromosomal plates from a large number of individuals, karyological studies are usually based in only a few counts per species, hindering the detection of multiple cytotypes in numerous species (Bennett 1998; Soltis *et al.* 2007; Marques *et al.* 2017). With the development of other techniques, such as flow cytometry, the number of studies focused in polyploid incidence and in cytogenetic distribution patterns has extensively increased in the last decades (Kron *et al.* 2007; Husband *et al.* 2013; examples provided in Chapter 1), suggesting that the incidence of polyploidy might still be underestimated (Marques *et al.* 2017).

The high performance of flow cytometry, enabling the measurement of light scatter and fluorescence parameters in thousands of particles (*e.g.*, cellular organelles) at high speed, is one of the main advantages of this technique when compared with classical approaches, such as chromosome counts and Feulgen microdensitometry (Greilhuber 1988). This technique has been initially developed for biomedical studies, with the protocols to isolate plant nuclei being later adapted for its successful application to study plant genomes (Galbraith *et al.* 1983). The developed protocol enables to isolate plant nuclei with ease and rapidly, using small amounts of tissue and without the need of tissues with dividing cells (Loureiro 2007). In flowering plants, in most cases, this protocol can be used with success to analyze a multitude of tissues, including leaves (more commonly used in the majority of the studies), seeds or even pollen grains (Doležel *et al.* 2007; Pan *et al.* 2004; Castro *et al.* 2018 – Chapter 4). This versatility is very important when studying polyploid complexes as it enables to investigate intermediate life-cycle stages fundamental for understanding polyploid emergence and establishment (*e.g.*, Burton and Husband 1999, 2001; Kron and Husband 2012; Chapter 4-5). However, it should be bear in mind that, for many studies, flow cytometry should be complemented with other cytological techniques, as chromosome counts and fluorescence and genomic in situ hybridization, that are fundamental for ascertaining the results obtained (Bennett and Leitch 2005; *e.g.*, Chapter 4).

The advent of flow cytometry enabled many researchers to study polyploid complexes in detail, providing novel insights on cytotype diversity and its geographic patterns, from the individual and population level to the entire geographical range of the species (*e.g.*, *Aster amellus*, Castro *et al.* 2012; *Chamerion angustifolium*, Husband and Schemske 1998; *Erysimum mediohispanicum*, Muñoz-Pajares *et al.* 2017; *Knautia arvensis* agg., Kolář *et al.* 2009; *Mercurialis annua*, Buggs and Pannell 2007; *Ranunculus adoneus*, Baack 2004). These large-scale studies performed between and within species revealed a wide variety of cytotype compositions and variable geographic distributions, confirming that polyploidization is a common process in nature. According with the classification of Petit *et al.* (1999), the majority of cytotypes in polyploid complexes grow in proximity, at least in part of their distribution range, forming parapatric or sympatric contact zones, depending on how close they grow from one another (adjacent or intermixed, respectively; *e.g.*, Chapter 3-4). Indeed, a recent review has shown that only 4% of the polyploid complexes known in detail have a disjunct distribution, *i.e.*, cytotypes grow in allopatry (Kolář *et al.* 2017). The *in situ* distribution patterns of the different cytotypes is the result of multiple processes, from polyploid formation rates and evolutionary history of the complex, to cytotype ecological preferences, competitive and dispersal abilities and inter-cytotype breeding barriers, among other factors (Levin 2002; Petit *et al.* 1999; Lexer and Loo 2006; Kolář *et al.* 2017). Therefore, assessing cytotype distribution patterns is the base for subsequent evolutionary studies on polyploid lineages (Petit *et al.* 1999).

Polyploidization can provide novel traits potentially suiting polyploids with different environmental adaptation and tolerances (Levin 1975; Husband and Schemske 2000; Ramsey 2011). Changes in physiological and ecological traits may promote the colonization of new habitats by the polyploids, surpassing the distribution limits of their progenitor(s). For example, polyploids have been proposed to be less impacted by stressful conditions, tolerating better low nutrient levels, drought and cold temperatures than their diploid progenitor(s) (Levin, 2002; Maherali *et al.* 2009; Hao *et al.* 2013). These differences may have significant ecological implications (Ramsey and Schemske 2002), allowing ecological niche expansion of polyploid lineages (Niche shift hypothesis; Levin 1975; Husband and Schemske 2000). Recently, with the development of niche modeling tools such ecological niche modelling (ENM; Warren *et al.* 2008) and multivariate analyses of niche variables (Broennimann *et al.* 2012), several studies have examined large-scale cytotype distribution patterns and associated cytotype segregation with different environmental preferences (*e.g.*, Glennon *et al.* 2012; Godsoe *et al.* 2013; Thompson *et al.* 2014; Visger *et al.* 2016; Muñoz-Pajares *et al.* 2017). These studies relate the occurrences of polyploid and diploid populations with abiotic factors to evaluate cytotype environmental

preferences, predicting the possible existence of niche shifts or niche conservation between cytotypes (Warren *et al.* 2008, 2010). These predictions are highly informative and enable to build hypotheses to be tested in manipulative experiments such as, reciprocal transplants in natural populations (*e.g.*, C*hamerion angustifolium*, Martin and Husband 2013).

The genus *Jasione* L. belongs to Campanulaceae, a family rich in polyploid entities (*e.g.*, over 52% of polyploid taxa in the Iberian Peninsula, from which 41% are ploidy variable; Marques *et al.* 2017), and it is composed by 16 species distributed in Europe and in the Mediterranean region (Pérez-Espona *et al.* 2005). The Iberian Peninsula is the center of maximum morphological variability of the genus, which comprises 10 accepted species (Sales and Hedge 2001b) that vary in several morphological traits, but also in plant habit (from annuals to perennials), ploidy composition (from diploid taxa to polyploid entities and diploid-polyploid complexes), and habitat preferences (from dune systems to alpines mountains) (Sales and Hedge 2001b; Rubido-Bará *et al.* 2010; Chapter 3). Among the taxa of interest is *J. maritima*, an endemic plant from the west coast of the Iberian Peninsula, classified as endangered by the IUCN Red List of Threatened Species (under the name *J. lusitanica*; Bilz 2001). The species occurs in dune systems across a latitudinal gradient from the northern coast of Galicia exposed to the high humidity levels of the Atlantic Ocean, to south until Aveiro in the Central Portuguese Atlantic coast, a region clearly marked by a drier environment. Despite, *J. maritima* has been previously classified as a tetraploid species (2n = 2*x* = 24 chromosomes, Lago Canzobre and Castroviejo 1992), being further assumed as homogenously tetraploid in the latest taxonomic review of the genus (Sales and Hedge 2001b), a recent study observed the presence two genome size categories in some Galician populations, namely 2C = 3.44 ± 0.04 pg in the northern locations and 2C = 6.62 ± 0.23 pg in the southern ones (Rubido-Bará *et al.* 2010). Despite the authors presented a different interpretation, to our best comprehension this suggests the presence of different ploidy levels in this species, which could be potentially linked with a latitudinal gradient.

Considering all this, the main objective of this study was to investigate the occurrence of different ploidy levels in *J. maritima*, access the distribution pattern of the different cytotypes detected in natural populations, and explore cytotype ecological preferences. For this, chromosome counts and large-scale cytotype screening over the entire distribution area (central to northwest coast of the Iberian Peninsula) were performed to characterize the species cytogenetically. Furthermore, statistical analyses of environmental requirements using niche modelling tools were performed to assess if the observed cytotype distribution patterns could be explained by environmental variables. Assessing the cytogenetic diversity and understanding the patterns of distribution in nature of this endangered species may provide useful information

not only from a conservation point of view, enabling to account for cryptic diversity in conservation plans, but also constitutes an ideal study system to understand the role of genome duplications in driving niche differentiation and in shaping cytotype distribution in nature.

MATERIALS AND METHODS

Study system

Jasione maritima is a perennial herb that grows in dune systems, between the primary and the secondary dune, occurring from Ferrol (Galicia, Spain) to Aveiro (Portugal) (Sales and Hedge, 2001a). The plant forms rosettes of leaves in the winter and produces blue to lilac glomerular inflorescences in the summer, each producing hundreds of very small seeds that germinate from autumn to late winter (Sales and Hedge, 2001a; M. Castro, field observations). To date, *J. maritima* has been described as tetraploid with 2n = 4*x* = 24 chromosomes (Lago Canzobre and Castroviejo 1992; Sales and Hedge, 2001a; Rubido-Bará *et al.* 2010). However, two different genome sizes were reported in populations from the northwest of Galicia (2C = 3.44 pg and 6.62 pg; Rubido-Bará et al, 2010) with two different ploidy levels in face of a 2:1 ratio in genome size.

Field sampling

Field collections were carried out during *J. maritima* flowering season (June and July), from 2013 to 2015. Field surveys were carried out within and beyond the distribution limits of *J. maritima* to guarantee that the entire range of the species in the Iberian Peninsula was covered. In each population, fresh leaves were sampled to access genome size and DNA ploidy levels, and seeds from selected locations were collected to perform chromosome counts. Stems with 5-6 leaves belonging to 4-30 individuals were collected randomly in each population, stored in hermetic bags, and maintained at 4 to 8 °C in a refrigerator until flow cytometric analysis. Seeds from up to 15 plants of locations selected based on preliminary genome size estimates, including one population of each genome size category [Appendix 2.1], were collected into paper bags and left to air-dry. Geographic coordinates of all the populations sampled were obtained and detailed information for all collection sites is provided in Appendix 2.1.

Chromosome counts

For chromosome counts, the protocol of Goldblatt *et al.* (1993) was followed with some modifications. Briefly, seeds from the selected populations [one of each genome size category, Appendix 2.1] were germinated and grown in 1L pots with commercial soil in an experimental garden. Actively growing root tips were harvested and pre-treated in 0.002M aqueous 8-hydroquinoline at room temperature for 4h30; afterwards, root tips were fixed in a solution of 3:1 of 95% ethanol and glacial acetic acid, for at least 24 h at 4 °C. Roots tips were then hydrolyzed in 1N HCl at 60 °C in a sand bath for 5 min, submerged in Schiff reagent (based in Greilhuber and Ebert 1994) for 1h30, washed in Sulphur water three times for 10 min periods, and finally squashed under a glass cover in aseptic orcein 2%. Chromosome spreads were observed using a Nikon Eclipse 80i light microscope and photographed using a Nikon Plan Apo VC 100×/1.40 oil-immersion lens, with a Q Imaging Retiga 2000R Fast 1394 digital camera and Q-Capture Pro v.7 software. Chromosome counts were assigned to a genome size category, enabling to estimate the DNA ploidy level of the remaining populations analyzed through flow cytometry.

Genome size and DNA ploidy level estimates using flow cytometry

Genome size and DNA ploidy level were assessed using flow cytometry. Galbraith *et al.* (1983) methodology was used to obtain nuclear suspensions. In brief, 50 mg of plant material of the study species was chopped with 50 mg of leaves of an internal reference standard (*Solanum lycopersicum* 'Stupické', hereafter S.l., with 2C = 1.96 pg; Doležel *et al.* 1992) using a sharp razor blade in a glass Petri dish with 1 ml of WPB buffer (0.2 M Tris-HCl, 4 mM $MgCl_2.6H_2O$, 1 % Triton X-100, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM metabisulfite, 1 % PVP-10, pH adjusted to 7.5 and stored at 4-8 °C; Loureiro *et al.* 2007). The nuclear suspension was filtered through a 50 µm nylon filter and 50 µg ml^{-1} propidium iodide (PI; Fluka, Buchs, Switzerland) and 50 µg ml^{-1} RNAse (Fluka) were added to stain the DNA and avoid the staining of dsRNA, respectively. After 5 min of incubation, the samples were analyzed in a Partec CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW; Partec GmbH., Görlitz, Germany). The results were acquired using Partec FloMax software v2.4d (Partec GmbH, Münster, Germany) in the form of four graphics: histogram of fluorescence pulse integral in linear scale (FL); forward light scatter (FS) vs. side light scatter (SS), both in logarithmic (log) scale; FL vs. time; and FL vs. SS in log scale. To remove debris, a polygonal region was defined in FL vs. SS histogram and subsequently applied to all graphics. At least 1,300 nuclei in both sample and standard G1 peaks were analyzed

per sample (Suda *et al.* 2007). Only coefficient of variation (CV) values of 2C peaks below 5% were accepted, otherwise a new sample was prepared and analyzed until such quality standards were achieved (Greilhuber *et al.* 2007). In 12 populations (six diploid and six tetraploid populations), 2 to 10 individuals were analyzed individually, enabling to estimate genome size. For the remaining individuals and populations, the pooled sample strategy was followed (5–6 individuals plus the reference standard) enabling only to access DNA ploidy level. The holoploid genome size (2C in pg; *sensu* Greilhuber *et al.* 2005) was calculated using the formula:

$$\textit{J. maritima}\text{ 2C nuclear DNA content (pg)} = \frac{\textit{J. maritima}\text{ G1 peak mean}}{\text{S.l. G1 peak mean}} \times \text{S.l. genome size.}$$

The DNA ploidy level was inferred for each sample based on the chromosome counts and genome size estimates obtained for a few selected populations.

The monoploid genome size (1C*x*; *sensu* Greilhuber *et al.* 2005) was calculated in mass values (pg) by dividing the holoploid genome size (2C) by the assigned DNA ploidy level. Populations were characterized according to the ploidy levels of its individuals and mapped.

Descriptive statistics of holoploid genome size were calculated for each cytotype (mean, standard deviation of the mean, coefficient of variation of the mean and range of variation) based only on individual flow cytometry estimates. Mean and standard deviation of the mean were also calculated for the monoploid genome size. To access differences between diploids and tetraploids in holoploid and monoploid genome sizes, Generalized Linear Models were used (Bolker *et al.* 2009) with a Gaussian distribution and a log link function to model the responses. Cytotype was used as factor and genome size as response variable. Statistical analyses were performed in R software version 3.0.1 (R Core Development Team 2016), using the packages "car" for Type-III analysis of variance (Fox *et al.* 2015), "lme4" for generalized linear models (GLMs; Bates *et al.* 2014) and "multcomp" for multiple comparisons after Type-III analysis of variance (Hothorn *et al.* 2017).

Environmental niche modelling

Environmental preferences of *J. maritima* cytotypes were accessed using GLM analyses and niche modelling tools. Taking into account the habitat of the species (dune species with limited extension), a high resolution was used for the variables included in the model (100 m). The following set of variables were explored: altitude (http://srtm.csi.cgiar.org); topographic variables, such as aspect, slope, slope range and topographic position index

(http://www.jennessent.com/arcview/tpi.htm); climatic variables, such as mean temperature in hottest months (June to August) and mean annual precipitation (http://www.opengis.uab.es/wms/iberia/index.htm); lithology (http://datos.gob.es/es); and distance to coast (based on http://www.jennessent.com/arcview/tpi.htm). Additionally, latitude and longitude were also included (Table 2.1). The values of all the variables were extracted for all sampled population using R package "dismo" (Hijmans *et al.* 2017). The obtained dataset was explored using GLM's to assess for differences between diploid and tetraploid populations, defining ploidy level as factor and each variable as response variable. A Poisson distribution with a log link function was used for discrete variables (lithology and solar radiation incidence) and a Gaussian distribution with a log link function was used for the remaining variables (continuous variables). Correlations between the variables were obtained using Pearson coefficient, and variables with correlation values higher than 0.7 were excluded. In the end, a set of four non-correlated variables relevant for the species were selected for niche modelling analyses: slope, mean temperature in hottest months, mean annual precipitation and distance to coast (Table 2.1).

Table 2. 1. Selected environmental variables used to characterize the environment of diploid and tetraploid populations of *Jasione maritima*. For each cytotype, mean and standard error of the mean (mean ± SE), *F* value and significance levels are given. Different letters correspond to statistically significant differences between cytotypes for a given variable (ns, nonsignificant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.01$). Variables highlighted in bold correspond to the variables used in niche modelling.

Variables	**CODE**	**Diploids** mean ± SE, N =12	**Tetraploids** mean ± SE, N =23	**ANOVA** *F* value
Altitude	alt	9.75 ± 2.24 [a]	5.35 ± 0.58 [b]	0.07 *
Exposition	aspect	174.23 ± 30.38	210.43 ± 20.33	1.03 ns
Distance from the sea	dist	197.49 ± 40.07	433.57 ± 188.64	0.80 ns
Lithology	lito	41.08 ± 2.48	37.00 ± 1.56	2.10 ns
Mean annual precipitation	pp	1744.28 ± 33.16	1637.58 ± 60.76	1.48 ns
Slope	slope	4.71 ± 1.29 [a]	1.61 ± 0.27 [b]	9.75 ***
Slope range	slprng	78.67 ± 6.10	69.52 ± 3.89	1.75 ns
Mean temperature in hottest months	Tmed	13.90 ± 0.07 [a]	14.43 ± 0.10 [b]	13.92 ***
Topographic position index	tpi	-1.11 ± 0.78	-0.74 ± 0.28	0.29 ns
Latitude	Lat	43.07 ± 0.05 [a]	42.04 ± 0.15 [b]	23.08 ***
Longitude	Long	-9.11 ± 0.04 [a]	-8.85 ± 0.03 [b]	31.06 ***

Niche modeling analyses were performing in R package "biomod2" (Thuiller *et al.* 2016). Spatial predictive models were calibrated based on the four selected variables and on presence/absence data. A buffer of 300 m around each sampled population was applied and 5,000 points were randomly selected within the remaining study area (defined as background points). A total of 5,035 points were used in presence/absence dataset (12 diploids, 23

tetraploids and 5,000 background points): background points were used as absences in the datasets of both diploids and tetraploids; additionally, in the diploid dataset, diploid populations were considered as presences and tetraploid populations as absences, and vice-versa in the tetraploid dataset.

The final model of each cytotype resulted from the combination of different modelling techniques. To reduce uncertainty and to produce robust models, each technique was replicated 30 times using random subsets obtained from each ploidy level dataset. The dataset of each ploidy level was divided randomly into training (70%) and test (30%) subsets (Phillips *et al.* 2006; Araújo and New 2007). All subsets were statistically independent, since in each replication each occurrence was used only once, as training or as test occurrence (Phillips 2008). Models were evaluated based on the independent accuracy measure AUC of ROC (area under the curve of the receiver operating characteristic), using AUC > 0.7 as a threshold to produce the final model of each cytotype. The final model of each cytotype was conserved in a binary format and used to calculate the suitable habitat of each cytotype and consequently the niche overlap between diploids and tetraploids.

Cytotype niche overlap was quantified using the proportional similarity of the distribution (Schoener's *D*; Schoener 1970). This metric ranges from zero to one, with zero corresponding to "no overlap" and one to "complete overlap". Niche identity and similarity tests were performed (Warren *et al.* 2008; Broennimann *et al.* 2012) using "ecospat" (Broennimann *et al.* 2012) and "raster" (Hijmans *et al.* 2017) R packages. In niche identity tests it was evaluated if the observed *D* value fall within the 95th percentile of the simulated *D* values, while in the niche similarity test it was evaluated if the environmental niches of diploids and tetraploids are distinguishable one from another (Broennimann *et al.* 2012). In both cases, the procedure was replicated 100 times to obtain confidence intervals that enabled to evaluate the null hypothesis. All models and analyses were performed in R environment (R Development Core Team 2016).

RESULTS

Cytogenetic diversity in *Jasione maritima*

Each genome size category revealed to correspond to different chromosome numbers (Figure 2.1; Table 2.2): individuals with 12 chromosomes presented average genome sizes of 2.98 pg/2C (Figure 2.1A, B), while individuals with 24 chromosomes had average genome sizes of 6.06 pg/2C (Figure 2.1A, C), corresponding to diploid and tetraploid cytotypes, respectively.

Table 2.2. Genome size estimates in *Jasione maritima*. DNA ploidy level, chromosome number, mean, standard deviation of the mean (SD), coefficient of variation (CV, in %), and 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*), and total number of populations and individuals analyzed are also presented for each cytotype. Two ploidy levels were observed: diploids (2*x*) and tetraploids (4*x*). Different letters correspond to statistically significant differences at $P < 0.05$.

Ploidy level	Chromosome number	Holoploid genome size (2C, pg)					Monoploid genome size (1C*x*, pg)		Populations (individuals)
		Mean	SD	CV (%)	Min	Max	Mean	SD	
2*x*	12	2.98[a]	0.07	2.4%	2.84	3.10	0.25[a]	0.01	6 (24)
4*x*	24	6.06[b]	0.11	1.9%	5.80	6.36	0.25[a]	0.01	6 (38)

Flow cytometric histograms were of very good quality, with all samples used to assess genome size having CV values below 5% (Figure 2.1A; Table 2.2) [Appendix 2.2]. Genome sizes were obtained for a total of 62 individuals from 12 populations [Appendix 2.2], with diploids having a small genome of 2C = 2.98 ± 0.07 pg (ranging from 2.84 pg to 3.10 pg), while tetraploids presented an intermediate genome size of 2C = 6.06 ± 0.11 pg (ranging from 5.80 pg to 6.36 pg) (Table 2.2). Holoploid genome size was statistically different between cytotypes ($F_{1,60} = 14061$, $P < 0.001$), while no statistically significant differences were observed between diploids and tetraploids regarding monoploid genome size ($F_{1,60} = 0.46$, $P = 0.502$; Table 2.2).

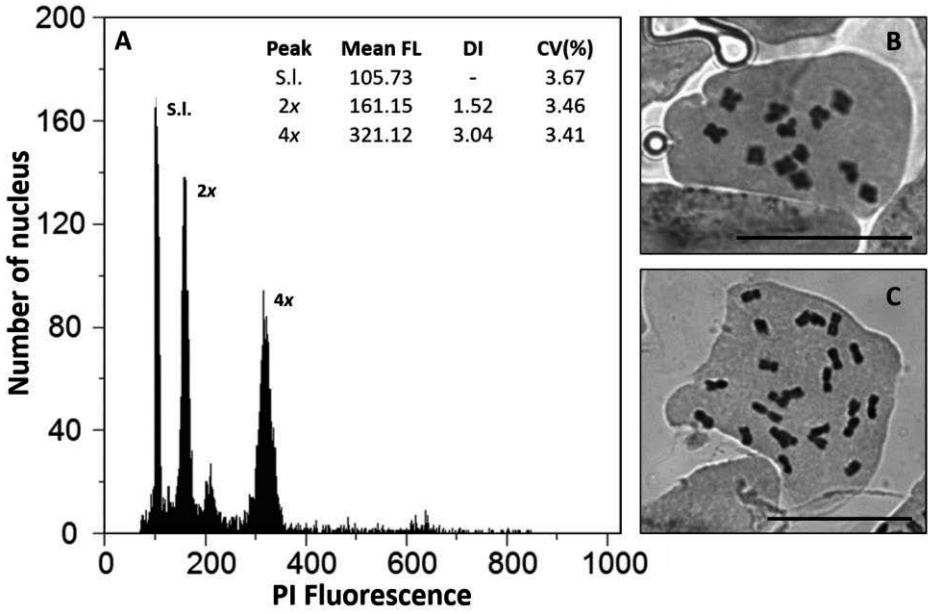


Figure 2.1. Cytogenetic diversity in *Jasione maritima*: A) Flow cytometric histogram of relative propidium iodide fluorescence intensity (PI Fluorescence) of nuclei isolated from fresh leaves of *Solanum lycopersicum* 'Stupické' (S.l.; reference standard with 2C = 1.96 pg) and of *Jasione maritima* diploid (2*x*) and tetraploid (4*x*) cytotypes; B) Chromosome plate of a diploid individual from population SC73 (2n = 2*x* = 12 chromosomes; bar = 20 µm); C) Chromosome plate of a tetraploid individual from population MC293 (2n = 4*x* = 24 chromosomes; bar = 20 µm). In A, for each peak, the mean relative fluorescence (Mean FL), DNA index (DI, Mean FL of *J. maritima* peak/Mean FL of the reference standard) and coefficient of variation of the peak (CV, in %) are provided.

Cytotype distribution patterns

Flow cytometry analyses enabled to assess the DNA ploidy level of 964 individuals from 35 natural populations, covering the entire distribution range of *J. maritima* [Appendix 2.1]. The large-scale screening revealed the occurrence of diploids and tetraploids, only. Additionally, only pure-ploidy populations were found in nature. These pure-ploidy populations were distributed in a clear allopatric pattern across a latitudinal gradient, with diploids occurring in the north, from Casas da Hermida to Lariño (Spain), while tetraploids were revealed to grow in the center and south of the distribution area, from Ventim (Spain) to Torreira (Portugal) (Figure 2.2) [Appendix 2.1]. It was also evident that tetraploids occupy a wider area than diploids (Figure 2.2).

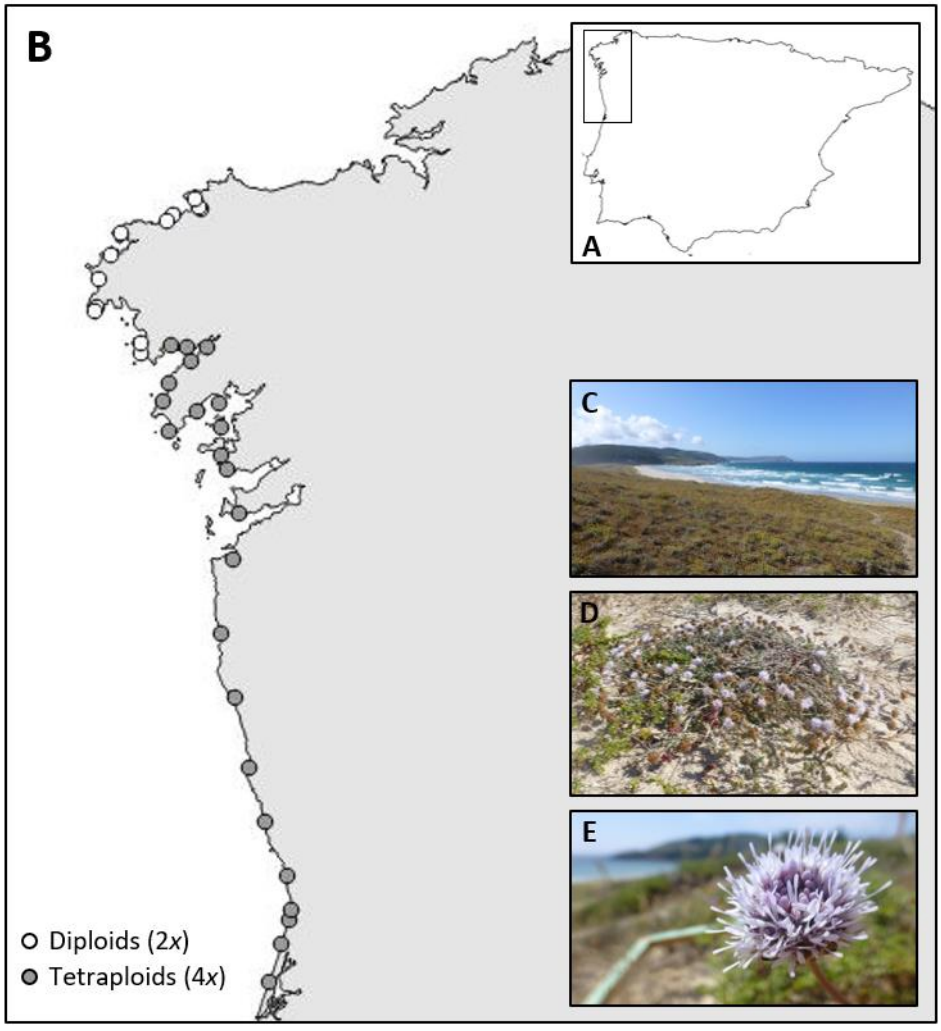


Figure 2.2. *Jasione maritima* large-scale cytotype distribution. A) illustrative images of the screening area, B) cytotype distribution in screening area, C) dune habitat, D) plant size and F) flower morphology are given. White circles represent pure-ploidy populations of diploids (2*x*), and grey circles correspond to pure-ploidy populations of tetraploids (4*x*).

Cytotype niche overlap

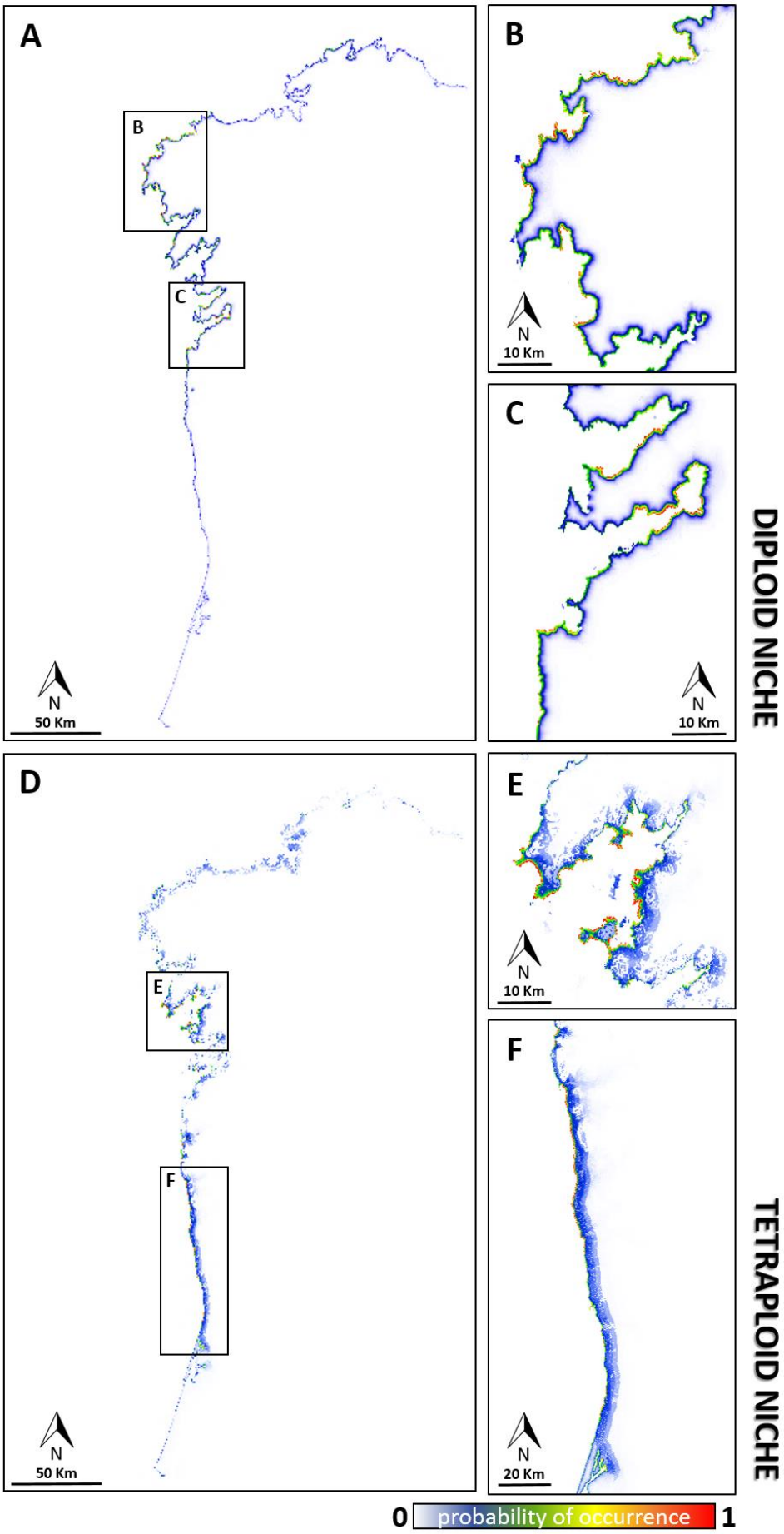
The allopatric distribution observed in the field could be partially explained by different environmental requirements (Table 2.1). Diploids colonized northern areas, which present slightly higher altitude, steeper slopes and lower mean temperatures in the hottest months than the areas where tetraploids are present ($P < 0.05$; Table 2.1). By other way, the southern areas colonized by tetraploids are marked by higher exposures (lower slopes) and hotter and drier environments than those occupied by diploids ($P < 0.05$; Table 2.1).

The predicted distribution models revealed high ROC values (2*x* - 0.93 ± 0.09; 4*x* - 0.91 ± 0.09; mean ± SE) and relatively low omission rates (2*x* - 0.13 ± 0.19; 4*x* - 0.16 ± 0.18; mean ± SE); the binary projections of the final model of each cytotype also predicted the occurrences with high accuracy, with all presences being correctly predicted as presence and with omission rates presenting a value of zero for both cytotypes.

Based on the selected variables, the geographical niches of the two cytotypes were distinguishable (Figure 2.3). The final models predicted that diploids were restricted to dune areas (Figure 2.3A-C), while tetraploids presented a broader distribution area that can go beyond the dune system (Figure 2.3D-F). Excluding the range between Pontevedra and Baiona where diploids had a higher probability to occur (Figure 3C), tetraploids presented a higher probability to occur from Corrubedo (Spain) to Torreira (Portugal, Figure 2.3D-F). Diploids also presented a high probability to occupy northern dune systems, from Lira to Casas da Hermida (Spain, Figures 2.3A and 2.3B).

PCA analyses revealed that the selected variables explained 66.0% (36.7% in Axis 1 and 29.3% and Axis 2) of the variance in cytotype distribution, with mean temperature in the hottest months and slope being particularly relevant (Figure 2.4A). Geographically, diploid and tetraploid niches presented low overlap ($D = 0.01$), while environmental niches revealed that tetraploids presented a broader niche than that of the diploids. Consequently, the tetraploids environmental conditions overlapped in 28% with those of the diploid, while diploids overlapped with 95% of the environmental conditions of the tetraploids (Figure 2.4B). Despite these differences, environmental niches were equivalent ($P = 0.96$) and similar ($P = 0.18$ for both comparisons, *i.e.*, diploids growing in tetraploid niches and vice-versa).

Figure 2.3. Predictive geographic niches for each cytotype of *Jasione maritima* (diploids – A-C, and tetraploids – D-F). Cold temperature colors represent habitats with low probability of occurrence of the cytotype, and hot temperature colors habitats with high probability of occurrence. ⇨


A
B
C
N
50 Km
B
N
10 Km
C
N
10 Km
DIPLOID NICHE
D
E
F
N
50 Km
E
N
10 Km
F
N
20 Km
TETRAPLOID NICHE
0
probability of occurrence
1

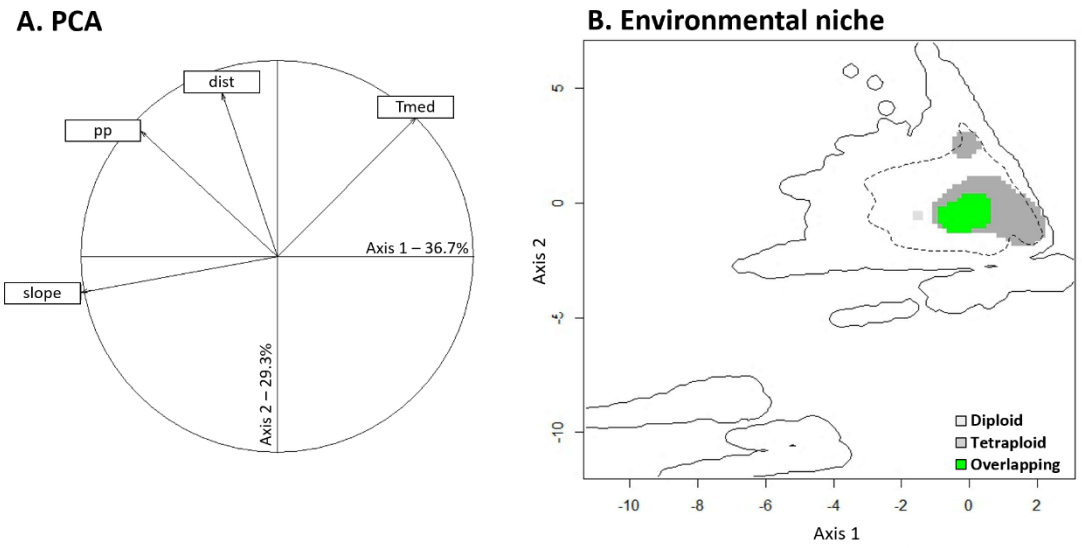


Figure 2.4. Ecological niche models for *Jasione maritima* cytotypes. A) contribution of each selected variable in the first two axes of principal component analyses (PCA) and percentage of variance explained by each axis. B) Environmental niche of diploids (light grey) and tetraploids (dark grey), with overlapping areas between cytotypes being highlighted in green); the continuous line corresponds to the whole climatic space, while the dashed line indicates the 75th percentile.

DISCUSSION

This study provided detailed cytogeographical information for *Jasione maritima* across its entire distribution range, and allowed to explore the relationships between environmental requirements of each cytotype and the distribution patterns observed in nature. The results showed that: 1) *J. maritima* is a polyploid complex composed by diploid and tetraploid individuals, with diploids being here reported for the first time; 2) only pure-diploid or pure-tetraploid populations have been found and the two cytotypes distribute allopatrically, with diploids growing in northern dune systems and tetraploids growing in a wider area in central and southern regions of the species distribution; 3) diploids grow in areas with slightly higher altitude, steeper slopes and lower mean temperatures than tetraploids, while tetraploids colonized areas with higher exposures, hotter and slightly drier environments than diploids; 4) predictive models suggested low geographic niche overlap (0.01), although the models suggest equivalent and similar environmental niches; 5) the environmental niche of the tetraploid was broader, representing only 28% of the environmental niche of the diploids, while diploids shared 95% of their environmental niche with tetraploids; 6) tetraploids currently seem to occupy the entire predicted environment, while diploids are restricted to their northern suitable predicted environment, not being able to reach suitable regions in southern more locations.

Polyploidization is a common phenomenon in numerous groups of the flowering plants (Otto and Whitton 2000), with a huge percentage of the current species harboring polyploid individuals (Marques *et al.* 2017). The genus *Jasione* L. is not an exception as it presents several polyploid taxa with a basic chromosome number of *x* = 6 (*e.g.*, *J. sessiliflora*; Favarger 1980; *J. montana*, *J. laevis*, *J. maritima*, *J. crispa*; Leitão and Paiva 1988; Sales and Hedge 2001a; Rubido-Bará *et al.* 2010). *Jasione maritima* is one of such polyploid species. Being initially described as exclusively tetraploid with 2n = 4*x* = 24 chromosomes (Lago Canzobre and Castroviejo 1992; Rubido-Bará *et al.* 2010), the chromosome counts of this study revealed, for the first time, the occurrence of diploid individuals with 2n = 2*x* = 12 chromosomes, corresponding to the lower category of genome size estimates obtained (2.98 ± 0.07 pg/2C *vs.* 6.06 ± 0.11 pg/2C, obtained for tetraploid individuals). Consequently, this species constitutes a diploid-tetraploid complex, here investigated in detail for the first time.

The large-scale cytogenetic analyses of natural populations sampled over the species entire range revealed a very clear geographical distribution pattern of its cytotypes. The different ploidy levels distributed allopatrically, with a contact zone between diploids and tetraploids in Lariño, in which the populations of each cytotype are separated by 8 kms approximately (in straight line). Diploids were observed to the north of this region, while tetraploid colonized southern locations, over an area that is clearly larger than the one occupied by the diploids. Spatial segregation between cytotypes has been observed in several other polyploid complexes (*e.g.*, Husband and Schemske 1998; Sonnleitner *et al.* 2010; Balao *et al.* 2009; Kolář *et al.* 2009; Castro *et al.* 2012; Casazza 2017; Wefferling *et al.* 2017), although very few complexes have been described to have a clear allopatric distribution of its cytotypes (reviewed by Kolář *et al.* 2017). Spatial segregation reduces inter-cytotype interactions and constitutes a physical barrier that prevents gene flow between cytotypes (Segraves and Thompson 1999; Husband and Schemske 2000; Baak 2005; Nuismer and Cunningham 2005), being pointed as one of the most effective barriers for successful polyploid establishment (Levin 2002; Li *et al.* 2004; Baack and Stanton 2005). The spatial and, consequently, reproductive isolation observed between diploid and tetraploid populations of *J. maritima* might promote the accumulation of differences between the two cytotypes and drive evolutionary divergence, especially if the two cytotypes are subjected to different selective pressures across the latitudinal range occupied by the species. In the long term, this could result in the formation of two different species (Otto and Whitton 2000; Soltis *et al.* 2010).

Polyploidization has been shown to have significant consequences in genetic, phenotypic and physiologic traits that can drive, for example, different habit requirements and broader

environmental tolerances (Levin 1975; Husband 2000; Comai 2005; Buggs and Pannell 2007; Ramsey 2011; Hao *et al.* 2013). The capacity of polyploids to grow in habitats that differ from their progenitor(s) has been suggested as one of the factors that allow polyploid lineages to overpass the minority cytotype exclusion at initial stages (Levin 1975; Fowler and Levin 1984; Felber 1991; Hao *et al.* 2013) and to expand to new areas where their lower ploidy parental(s) are absent. For example, increased stomata size and vessel diameter in tetraploid *Chamerion angustifolium* (Maherali *et al.* 2009), more efficient water transport by tetraploid *Atriplex canescens* (Hao *et al.* 2013) and increased leaf thickness in tetraploid *Spathiphyllum wallisii* (Laere *et al.* 2011) were shown to provide an advantage to the polyploids of these species in the colonization of drier soils in comparison with their diploid counterparts. Niche modeling tools have also shown a strong association between the spatial distribution of cytotypes and their environmental requirements in several polyploid complexes (*e.g.*, Glennon *et al.* 2014; Thompson *et al.* 2014; Visger *et al.* 2016; Muñoz-Pajares *et al.* 2017). The distribution patterns of *C. angustifolium* is, among other factors, partially justified by differences in cytotype requirements, with tetraploids occur in drier areas than diploids (Thompson *et al.* 2014). In *J. maritima*, although the niches of the two cytotypes revealed to be equivalent and similar, individual analyses of environmental variables and niche modelling indicate that habitat requirements of tetraploids and diploids can be distinguishable. Interestingly, we observed that the southern areas where tetraploids grow tend to be more exposed, drier and hotter than the northern areas occupied by diploids. Tetraploids also seem to grow in more heterogeneous environmental habitats, suggesting that polyploidization may have conferred them the capacity to occupy wider or at least different niches from those occupied by diploids. Indeed, niche modelling revealed that tetraploids occupy the majority of the environmental area predicted as suitable for them, while diploids seem to be restricted to the northern areas not suitable for tetraploids. In all, this might suggest that tetraploids could have broader environmental tolerances and/or be more tolerant to drought than diploids, which enabled them to colonize areas beyond those suitable for the diploids. Further studies involving reciprocal transplant experiments are needed to experimentally test this hypothesis.

However, despite the differences observed in some environmental variables, diploids and tetraploids presented similar environmental niches. This suggests that other factors, besides some differentiation in environmental requirements, might be involved in the current distribution patterns. As mentioned above, polyploidization frequently has consequences in plant traits, among which is competitive ability (Levin 2002). Increased competitive ability of polyploids has been frequently referred as an important advantage that allows polyploids to

overpass frequency-dependent selection (Levin 1975; Fowler and Levin 1984; Rodríguez 1996a), although the few experimental studies available to date showed contrasting results (*e.g.*, Maceira *et al.* 1993; Collins *et al.* 2011; Thompson *et al.* 2015). The results of these studies varied from superior competitive ability of tetraploid *Dactylis glomerata* in comparison with diploids (Maceira *et al.* 1993), to similar competition abilities between diploid and tetraploid *Chamerion angustifolium* (Thompson *et al.* 2015), or even variable competitive capacity across the distribution range in the diploid-tetraploid *Centaurea stoebe* (Collins *et al.* 2011). In *J. maritima*, competitive ability might also be involved with current distribution patterns. In case the two cytotypes present similar competitive abilities, then the contact zone might be maintained stable. By opposition, different competitive abilities of diploids and tetraploids are expected to generate a moving contact zone (*e.g.*, Maceira *et al.* 1993), towards the south if the diploids of *J. maritima* are more competitive, or to the north if the tetraploids are the more competitive cytotype, in any case expanding until each cytotype reach their environmental limit. Therefore, competition experiments are needed to evaluate the role of competitive ability in shaping current cytotype distribution patterns in *J. maritima*.

Detailed knowledge regarding cytogenetic diversity within a species, usually classified as cryptic diversity, is crucial to delineate guidelines for conservation plans (Carroll and Fox 2008), as it allows to establish conservation priorities (Iriondo *et al.* 2008). As suggested by other authors (*e.g.*, Bennett 1998; Soltis *et al.* 2007; Marques *et al.* 2017), in many species, the incidence of polyploidy and the *in situ* distribution patters are still poorly known. Combining the information of large-scale cytogenetic analyses with environmental preferences, grants conservation biologists with the necessary information to delineate specific measures that take in consideration the current and future scenarios of distribution of a given species. In the case *of J. maritima*, tetraploids showed broader environmental niche requirements, while diploids seemed to be more restrictive. Additionally, if the tetraploids are indeed more tolerant to drought, in face of the current scenarios of climate change, diploid populations may be more severely affected in the future. Therefore, considering that *J. maritima* is only protected in Portugal (Directiva Habitats 1992), it is of pivotal importance that a conservation status is granted for the diploid populations in Galicia (Spain).

CONCLUSIONS

Jasione maritima was shown to be a diploid-tetraploid complex, in which cytotypes are geographically segregated, resulting in an allopatric distribution. Environmental requirements of diploids and tetraploids were distinguishable and could, at least partially, explain the observed geographic distribution of each cytotype. Although diploid and tetraploid environmental niches revealed to be equivalent and similar, tetraploids clearly presented a broader niche that allowed them to colonize southern areas of the distribution range of the species. This might suggest that polyploidization could have provided an advantage to tetraploids in comparison with diploids. Still, the similitude in the environmental niches and the absence of diploids in suitable areas suggest that other factors could be involved in the establishment and spread of tetraploids. More studies, such as reciprocal transplants and competition experiments, are needed to test these hypotheses.

APPENDICES

Appendix 2.1. Geographic information of the *Jasione maritima* populations sampled in this study. For each population, an ID code, estimated DNA ploidy level, sample size (N), and information on the location and geographical coordinates (angular) are given. Two cytotypes were observed: diploids (2*x*) and tetraploids (4*x*). The two locations marked with an asterisk (*) constitute the origin population of seed used in chromosome counts.

ID code	DNA Ploidy level (N)	Location	Geographic coordinates	
			Latitude	Longitude
SC244	2*x* (7)	Casas da Hermida, La Coruña, Spain	43.26401	-8.95120
SC243	2*x* (12)	Balarés, Pontecesso, La Coruña, Spain	43.24197	-8.94148
MS046	2*x* (37)	Monte Branco, Pontecesso, La Coruña, Spain	43.23429	-8.93088
MS001	2*x* (30)	Lage, Soesto, La Coruña, Spain	43.21240	-9.02343
SC242	2*x* (12)	Boaño, La Coruña, Spain	43.19168	-9.04252
MS002	2*x* (30)	Pedrosa beach, Mourín, La Coruña, Spain	43.15818	-9.19126
MS003*	2*x* (30)	Lourido, La Coruña, Spain	43.08677	-9.22109
SC150	2*x* (4)	Neriña, Talón, La Coruña, Spain	43.00983	-9.26141
SC077	2*x* (33)	Fisterra, Afora beach, La Coruña, Spain	42.90851	-9.27328
SC076	2*x* (30)	Fisterra, Rostro beach, La Coruña, Spain	42.91861	-9.26416
SC074	2*x* (32)	Lira, La Coruña, Spain	42.80479	-9.12781
SC073	2*x* (30)	Lariño, La Coruña, Spain	42.77103	-9.12227
SC072	4*x* (35)	Ventim, Abelheira, La Coruña, Spain	42.79917	-9.02685
SC071	4*x* (49)	Esteiro, La Coruña, Spain	42.79029	-8.97947
SC070	4*x* (30)	Testal, Taramancos, La Coruña, Spain	42.79078	-8.91341
SC078	4*x* (29)	Cans, La Coruña, Spain	42.74260	-8.96409
SC079	4*x* (30)	Tarela, La Coruña, Spain	42.67273	-9.03290
SC080	4*x* (35)	Basoña, La Coruña, Spain	42.61898	-9.05401
MC369	4*x* (30)	Couso, La Coruña, Spain	42.52006	-9.03848
SC083	4*x* (30)	Caiños, La Coruña, Spain	42.58534	-8.94885
SC084	4*x* (30)	Fonte de Mouro, La Coruña, Spain	42.61228	-8.87213
SC085	4*x* (30)	Con Cerrado, Illa Arousa, Pontevedra, Spain	42.53166	-8.86943
SC113	4*x* (27)	A Lanzada, O Grove, Spain	42.44249	-8.87156
SC114	4*x* (30)	Barbeito, Pontevedra, Spain	42.39955	-8.85051
SC116*	4*x* (35)	Liméns, Pontevedra, Spain	42.26023	-8.81370
SC117	4*x* (35)	Baiona, Pontevedra, Spain	42.11335	-8.82828
SC118	4*x* (29)	A Praia, Pontevedra, Spain	41.87318	-8.86698
MC220	4*x* (30)	Anha, Viana do Castelo, Portugal	41.66749	-8.82249
MC219	4*x* (30)	Aguçadeira, Porto, Portugal	41.44315	-8.77734
SC028	4*x* (30)	Angeiras, Porto, Portugal	41.26942	-8.72622
MC218	4*x* (30)	Marinha, Vila Nova de Gaia, Portugal	41.09783	-8.65881
MC217	4*x* (30)	Sisto, Esmoriz, Portugal	40.98698	-8.64463
MC216	4*x* (3)	Esmoriz, Portugal	40.95983	-8.65245
MC293	4*x* (10)	Furadouro, Aveiro, Portugal	40.87816	-8.67341
MC215	4*x* (30)	Torreira, Aveiro, Portugal	40.75708	-8.71291

Appendix 2.2. Genome size estimated in *Jasione maritima*. In each population, DNA ploidy level estimation and mean, standard deviation of the mean (SD), coefficient of variation (CV, in %) and minimum (Min) and maximum (Max) values of holoploid genome size (2C, in pg) are given. Information about the number of individuals analyzed in each population (N) and mean monoploid genome size (1C*x*, in pg) are also presented. Only pure-ploidy populations were observed, either composed by diploids (2*x*) or by tetraploids (4*x*) individuals.

ID code	Ploidy level	Holoploid genome size (2C)						Monoploid Genome size (1C*x*)
		Mean	SD	CV (%)	Min	Max	N	
SC244	2*x*	3.07	0.04	1.20	3.03	3.10	3	0.25
SC243	2*x*	3.02	0.04	1.50	2.91	3.06	10	0.25
SC242	2*x*	3.01	0.05	1.60	2.98	3.05	2	0.25
MS003	2*x*	2.93	0.01	0.40	2.92	2.94	3	0.25
SC077	2*x*	2.89	0.04	1.30	2.84	2.91	3	0.25
SC073	2*x*	2.92	0.05	1.90	2.88	2.98	3	0.24
SC072	4*x*	6.11	0.09	1.50	5.97	6.22	10	0.25
SC071	4*x*	6.03	0.14	2.30	5.80	6.36	10	0.25
SC080	4*x*	6.11	0.05	0.80	6.06	6.17	5	0.25
MC369	4*x*	6.11	0.16	2.60	6.00	6.29	3	0.25
SC116	4*x*	6.03	0.08	1.40	5.93	6.16	5	0.25
SC117	4*x*	5.98	0.11	1.80	5.86	6.09	5	0.25

Chapter 3 – Parapatric distribution of *Jasione montana* cytotypes: similar environmental niches but low geographic overlap

Chapter section submitted as an original article to *Ecology and Evolution*:
Castro, M., Loureiro, J., Serrano, M., Husband B. and Castro, S. Parapatric distribution of *Jasione montana* cytotypes: similar environmental niches but low geographic overlap.

ABSTRACT

Polyploidization is a phenomenon that gives rise to new genomic variants that might establish and spread within and/or beyond progenitor populations. Current geographical ranges of cytotypes within polyploid complexes result from intricate interacting forces, including historical processes, interactions among cytotypes and relations between cytotypes and the environment. Although essential to build hypotheses on the process of emergence, establishment and spread of polyploid lineages, the geographical arrangement of cytotypes is still largely unknown for most polyploid complexes. This study aimed to access cytotype diversity and distribution patterns in the *Jasione montana* polyploid complex and explore if environmental factors could explain the successful establishment and spread of tetraploids. We reviewed all chromosome counts available in the bibliography, examined cytotype distributions throughout the Iberian Peninsula, in particular at the diploid-tetraploid contact zone, including 279 populations and 3396 plants, and used niche modelling to compare ecological requirements of each cytotype at two spatial scales. Diploids are widespread across Europe, while tetraploids are restricted to the northwest of the Iberian Peninsula arranged in two nuclei, with no additional cytotypes being detected. The two cytotypes presented a parapatric distribution with areas dominated by diploids being alternated with some areas dominated by tetraploids, thus forming several contact zones. Still, mixed-ploidy populations were rarely found (1.4%). Despite having low geographical overlap (D = 0.05 and 0.11, for the Iberian Peninsula and the contact zone, respectively), the cytotypes had similar niches at both spatial scales, although the amplitude of the environmental niche of the diploids was larger than that of tetraploids. Contrary to other polyploid complexes, in *J. montana*, diploids and tetraploids had similar environmental niches, suggesting that polyploidization did not change the environmental preferences of the tetraploids. The aggregation of tetraploid populations in some areas may indicate that tetraploids might outcompete diploids, gradually excluding them from the population. Additionally, the extensive contact zones between cytotypes together with the absence of mixed-ploidy populations suggest that frequency-dependent selection might be an important force driving the exclusion of the minority or less fit cytotype from populations.

Keywords: Cytotypes, diploids, *Jasione montana*, minority cytotype exclusion, niche modeling, polyploidy, tetraploids.

INTRODUCTION

The duplication of whole chromosome sets (WGD), is a common event in nature (Wood *et al.* 2009; Husband *et al.* 2012; Marques *et al.* 2017), giving rise to new polyploids that might establish themselves and spread within the diploid/lower-ploidy progenitor population. The recurrent formation of polyploids has been documented multiples times throughout the evolutionary history of particular plant groups (Wood *et al.* 2009; Otto and Whitton 2000; Soltis *et al.* 2010), but also in extant plant populations (*e.g.*, Maceira *et al.* 1992; Burton and Husband 2001; Ramsey 2007; Castro *et al.* 2016b, 2018). This is likely due to frequent production of unreduced gametes in nature (Bretagnolle and Thompson 1995; Ramsey 2007; Brownfield and Kohler 2010), although successful polyploid establishment is assumed to be much less frequent (Soltis *et al.* 2014 reply to Mayrose *et al.* 2011). Therefore, polyploidy is currently recognized as a major mechanism of sympatric speciation (Otto and Whitton 2000; Soltis *et al.* 2010) and considered an important mechanism of evolutionary diversification of flowering plants (Soltis and Soltis 1999). For these reasons, 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 progenitor's populations only if it has the necessary conditions to increase its number, otherwise it will be excluded from the population due to frequency-dependent selection (Levin 1975; Rodriguez 1996; Husband and Schemske 2000). Polyploid establishment will be favored by ecological 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 and Levin, 1984; Felber 1991, Rodriguez 1996; Husband and Schemske 2000, Barringer 2007). Assortative mating, enforced by various reproductive barriers, may also promote coexistence of polyploids and their progenitors (*e.g.*, *Chamerion angustifolium*, Husband and Sabara 2004; *Aster amellus*, Jersáková *et al.* 2010; Castro *et al.* 2011; *Gladiolus communis*, Chapter 5). Alternatively, polyploids might disperse outside parental populations, escaping minority cytotype exclusion and establishing new populations outside of the environmental tolerances of the parental individuals (niche shift hypothesis; Levin 1975, 2004; Husband and Schemske 2000).

The geographical range occupied by each cytotype is the result of complex interacting forces, including the historical processes of the polyploid complex, the interactions among cytotypes and the relations between the cytotypes and the environmental conditions (Husband

et al. 2013). Different geographical patterns have been documented, namely sympatric, parapatric or allopatric cytotype distribution depending on whether cytotypes grow intermixed, adjacent or disjunct, respectively (Petit *et al.* 1999; Chapter 1). Life history is one of the factors determining these patterns, depending on the time of polyploid formation and on the levels of recurrent polyploid formation, generating primary contact zones when polyploids are recurrently formed or secondary contact zones after allopatric divergence and subsequent migration (Petit *et al.* 1999). Contact zones are frequent in most polyploid complexes enabling cytotype interactions, however, mixed-ploidy populations are expected to be rare because frequency-dependent selection will exclude the minority cytotype (Levin 1975; Rodriguez 1996a). Consequently, mixed-ploidy populations will reflect a transitory stage, unless cytotypes are ecologically and reproductively isolated on a small spatial scale enabling cytotype coexistence (*e.g.*, Kolář *et al.* 2009; Jersáková *et al.* 2010). Since its discovery, polyploidy has also been postulated to have broad-scale impacts on gene regulation and developmental processes that might change the fitness of polyploids (Levin 1983; Adams and Wendel 2005). These differences have been linked with increased ecological tolerances, niche partitioning and/or wider ranges (*e.g.*, Levin 1975; Husband and Schemske 2000; Ramsey 2011; Buggs *et al.* 2007; Ramsey 2011; Hao *et al.* 2013), thus being determinant for cytotype distribution. Knowing the geographic arrangement of diploid-polyploid complexes *in situ* provides essential information for inferring the processes involved in polyploid establishment, coexistence and divergence (*e.g.*, Levin 2002; Petit *et al.* 1999; Lexer and van Loo 2006; Castro *et al.* 2018).

Recently, the development of niche modelling tools has enabled researchers to characterize ecological niches and to compare niches between different taxa. Ecological niche modelling (Warren *et al.* 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 calculate and to compare environmental niches. This approach has been used in related diploid-polyploid species (*e.g.*, *Houstonia* species, Glennon *et al.* 2012; *Leucanthemum* Iberian taxa, Oberprieler *et al.* 2012; *Claytonia perfoliata* complex, McIntyre, 2012; *Primula* sect. *Aleuritia* complex, Theodoridis *et al.* 2013; allopolyploid complexes, Marchant 2016; *Tolmeia* species, Visger *et al.* 2016), or in the analysis of different cytotypes within a species (autopolyploid complexes, such as: *Houstonia pururea* and *H. longifolia*, Glennon *et al.* 2012; *Heuchera cylindrica*, Godsoe *et al.* 2013; C*hamerion angustifolium*, Thompson *et al.* 2014; *Erysimum mediohispanicum*, Muñoz-Pajares *et al.* 2018), to evaluate the niche shift hypothesis. These comparisons enabled researchers to identify the potential environmental constraints on the distribution of different taxa and have

been important for disentangling the role of ecological preferences caused by polyploidization *versus* biotic interactions and colonization history in the establishment and spread of polyploid lineages. Modelling tools have also highlighted new hypotheses involved in polyploid establishment that could be tested experimentally in the field or in controlled conditions through reciprocal transplants or competition experiments (*e.g.*, *Ranunculus adoneus*, Baack and Stanton 2005; C*hamerion angustifolium,* Martin and Husband 2013; *Jasione maritima*, Chapter 7).

Jasione L. (Campanulaceae) is a small genus distributed in Europe, North Africa and Southwest Asia, with most of its species having restricted distributions and with the center of morphological diversity being localized in the Iberian Peninsula (Tutin 1973; Sales and Hedge 2001b; Pérez-Espona *et al.* 2005). Phylogenetic analyses using ITS suggest a recent origin of the species within the genus (Sales *et al.* 2004; Pérez-Espona *et al.* 2005). *Jasione* comprises several diploid taxa (*e.g.*, *J. foliosa*, *J. corymbosa*; Silveira 1986, Parnell 1987), but it is also rich in polyploid complexes including tetraploid species (*e.g.*, *J. sessiliflora*; Favarger 1980) and species with several ploidy levels (*e.g.*, *J. montana*, *J. laevis*, *J. maritima*, *J. crispa*; Sales and Hedge 2001a; Rubido-Bará *et al.* 2010; Chapter 2). Among the latter is the widespread *J. montana* which has been formerly described as diploid throughout its distribution range across Europe (*e.g.*, Kovanda 1968; Bjorkqvist *et al.* 1969; Kliphuis and Wieffering 1972; Ubera 1980), until Leitão and 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 much morphological variability, with diversity of habit, growth form and organ size (Parnell 1985, 1987; Bokhari and Sales 2001; Sales *et al.* 2004). Consequently, the taxonomic treatment within *J. montana* varies greatly depending on the author, with the most recent taxonomic review of the genus recognizing a continuum in morphological traits, although no consideration has been given to the cytogenetic diversity within the species (Sales and Hedge 2001a). More recently, Rubido-Bará *et al.* (2010) detected some morphological differences in plant size, root thickness and leaf size as well as in characters related to reproductive fitness (*e.g.*, number of seeds per capsule) between diploids and tetraploids from Galicia. These differences have led to the recognition of two subspecies, each corresponding to one cytotype (Rubido-Bará *et al.* 2010). While these authors did characterize the cytogenetic diversity within some populations in Galicia (Spain), 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 in detail the diversity and distribution of cytotypes within the *Jasione 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 geographic distribution of the 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 requirements that could explain the observed geographical distribution. 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 requirements of each cytotype at two spatial scales, throughout the Iberian Peninsula and within the contact zone. We hypothesize that polyploidization drives shifts in environmental preferences and, thus, diploids and tetraploids colonize different environmental niches according with their requirements resulting in low geographic overlap. The information about cytotype diversity, geographical patterns and environmental associations enabled us to explore the factors involved with the establishment and spread of *J. montana* tetraploid individuals in nature.

MATERIALS AND METHODS

Study system

Jasione montana L. (Campanulaceae) is a widespread species distributed through most of Europe, from the Mediterranean to approximately 62°N in upland regions, western Asia and North Africa (Flora Europae; Tutin 1973). It grows on rocks or in rocky grounds, heaths and grasslands with thin soil layers, preferentially in acid soils, being absent from limestone regions (Horwood 1919). Individuals of this species exhibit high morphological variability and may be annual, biennial or perennial. *Jasione montana* plants frequently form a rosette of leaves during the winter, emitting erect to ascending stems in the spring, each ending in a capituliform inflorescence of bluish flowers (Parnell 1980; Sales and Hedge 2001a). The species comprises diploids with 2n = 2*x* = 12 chromosomes through most of its distribution area (*e.g.*, Kovanda 1968; Ubera 1980; Luque and Mejas 1986; Pastor Diaz *et al.* 1990; Rubido-Bará *et al.* 2010), while tetraploids with 2n = 4*x* = 24 chromosomes have been reported in the northwestern region of the Iberian Peninsula (Leitão and Paiva, 1988; Rubido-Bará *et al.* 2010). The genome size of the two cytotypes has also been estimated with diploids (2C = 3.24 pg) being roughly half that of tetraploids (2C = 6.58 pg; Rubido-Bará *et al.* 2010).

An extensive literature review on the karyology of *J. montana* was made to compile all the geographically determined chromosome counts and subsequently map the distribution of the cytotypes. A total of 40 references including 89 identified localities with karyological information were compiled [Appendix 3.1] and mapped (white diamonds in Figure 3.1A).

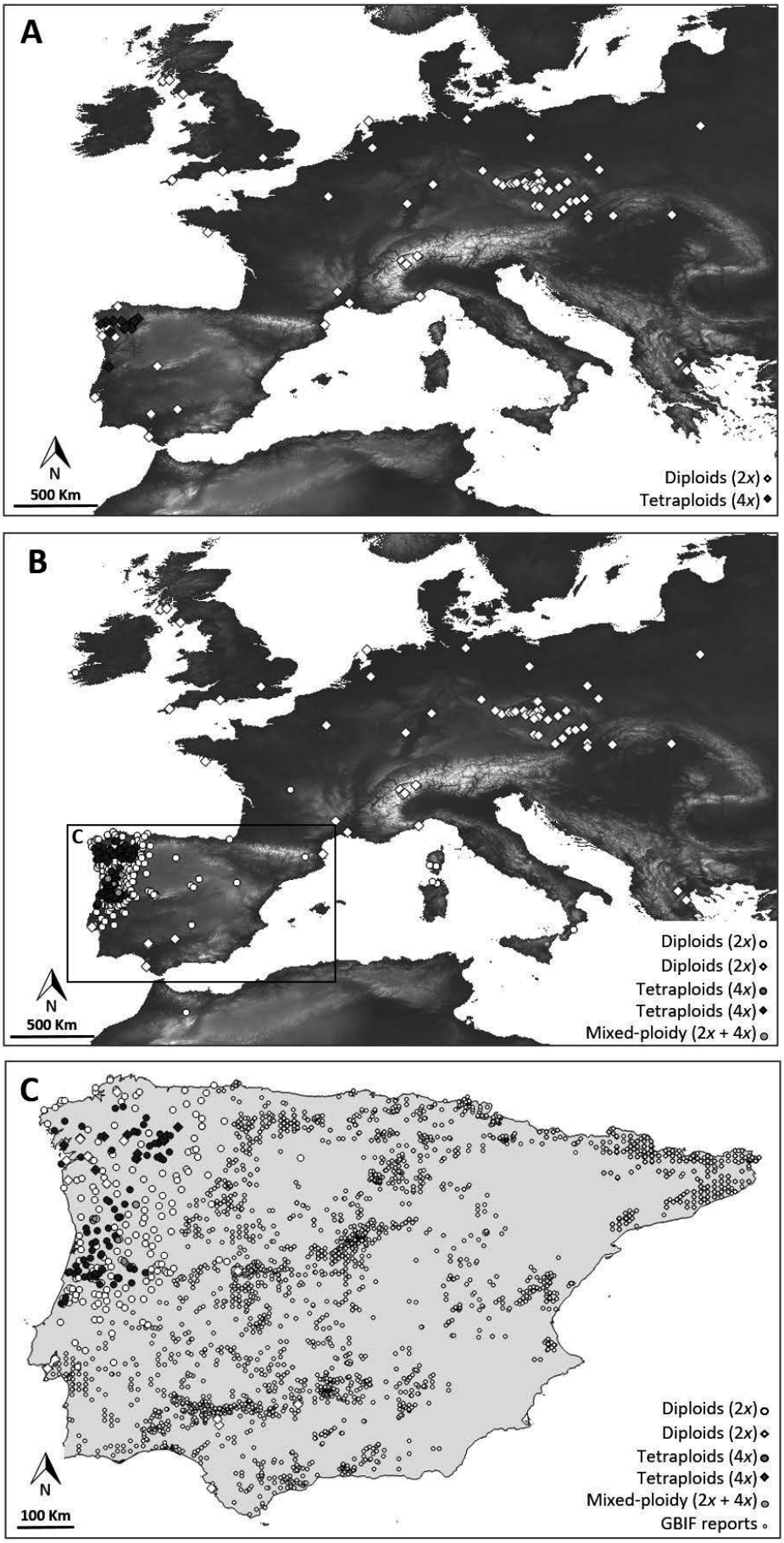
Field sampling

The sampling was mostly focused in the Iberian Peninsula, in particular in the northwestern region where the tetraploids have been previously reported (Leitão and Paiva, 1988; Rubido-Bará *et al.* 2010). Sampling through the Iberian Peninsula and other Mediterranean areas was also performed to confirm the diploid dominance reported in the bibliography. In total, 288 populations were sampled, including 279 in the Iberian Peninsula and 8 elsewhere (France, Ireland, Italy and Morocco). In detail, during spring and summer of 2013-2016, fresh leaves were collected in the field into 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, covering the entire population [Appendix 3.2]. When the harvesting of fresh material was impractical, mature seeds were collected (13 populations). Geographic coordinates of each population were recorded, and all populations were mapped in Quantum-GIS version 2.18.3 by importing points as *x/y* coordinates [in decimal format, Appendix 3.2]. Herbarium vouchers were also collected for species confirmation, being deposited in SANT herbarium.

Flow cytometric analyses

Fresh leaves were analyzed using flow cytometry to estimate genome size and DNA ploidy of each individual sampled. In brief, in a Petri dish, 50 mg of both sample material and the reference standard (*Solanum lycopersicum* 'Stupické', 2C = 1.96 pg; Doležel *et al.* 1992), were co-chopped in 1 ml of Woody Plant Buffer (WPB: 0.2 M Tris-HCl, 4 mM $MgCl_2.6H_2O$, 1 % Triton X-100, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM metabisulfite, 1 % PVP-10, pH adjusted to 7.5 and stored at 4-8 °C; Loureiro *et al.* 2007) to obtain a nuclear suspension (adapted from

Figure 3.1. *Jasione montana* reports. A) bibliographic information from chromosome counts in Europe (namely, diploids and tetraploids reports); B) all records obtained from bibliographic information and from this study; C) detail of the Iberian Peninsula with all reports, including also GBIF occurrences. Diamonds – records from the bibliography; circles – populations screened in this study; small circles – GBIF occurrences; Ploidy levels: white – diploids (2*x*); grey – tetraploids (4*x*); green – mixed-ploidy populations. ⇨


A
N
500 Km
Diploids (2x)
Tetraploids (4x)
B
C
N
500 Km
Diploids (2x)
Diploids (2x)
Tetraploids (4x)
Tetraploids (4x)
Mixed-ploidy (2x + 4x)
C
N
100 Km
Diploids (2x)
Diploids (2x)
Tetraploids (4x)
Tetraploids (4x)
Mixed-ploidy (2x + 4x)
GBIF reports

Galbraith *et al.* 1983). Nuclear suspensions were filtered through a 50 µm nylon filter, and 50 µg ml^{-1} of propidium iodide (PI; Fluka, Buchs, Switzerland) and 50 µg ml^{-1} of RNAse (Fluka) were added to stain the DNA and to digest the double-stranded RNA, respectively. After a 5 min incubation period, samples were analyzed in 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 analyzed (Suda *et al.* 2007). Partec FloMax software v2.4d (Partec GmbH, Münster, Germany) was used to acquire the results in the form of four graphics: histogram of fluorescence pulse integral in linear scale (FL); forward light scatter (FS) vs. side light scatter (SS), both in logarithmic (log) scale; FL vs. time; and FL vs. SS in log scale. A polygonal region was defined in FL *vs* SS histogram to electronically remove the debris. Following Greilhuber *et al.* (2007), only when the coefficient of variation (CV) value of the 2C peak of *J. montana* was below 5% it was considered acceptable, otherwise a new sample was prepared and analyzed until such quality standard was achieved.

Given the large number of individuals collected in each population, 1-9 randomly selected individuals (mean = 2.4 individuals/population) were analyzed individually to estimate their genome size, while the remaining were analyzed for DNA ploidy only using the pooled sample strategy (2-6 individuals in each pool plus the reference standard). For 14 populations, we analyzed the ploidy level directly from the seeds following the protocol above and the pooled sample method (adapted from Castro *et al.* 2018 – Chapter 4).

The holoploid genome size (2C; *sensu* Greilhuber *et al.* 2005) of each individual sample was calculated using the following formula: Holoploid genome size (pg) = $\frac{\textit{J. montana}\text{ G1 peak mean}}{\textit{S. lycopersicum}\text{ G1 peak mean}} \times$ *S. lycopersicum* genome size. The monoploid genome size (1C*x*; *sensu* Greilhuber *et al.* 2005) of each individual sample was also calculated by dividing the holoploid genome size (2C) by the ploidy level of each cytotype. Samples were classified as diploid or tetraploid according to the estimates of genome size and their range of variation: diploid for values ranging between 2.80 and 3.08 pg/2C, and tetraploid for values ranging between 5.63 and 6.06 pg/2C. The non-overlapping ranges of genome size enabled always a clear assignment of the ploidy levels. Subsequently, populations were classified according with the DNA ploidy level composition of its individuals, as pure-ploidy or mixed-ploidy and mapped.

Descriptive statistics of holoploid and monoploid genome sizes were calculated for each cytotype based on the individual flow cytometry estimates. Differences between diploids and tetraploids in holoploid and monoploid genome sizes were tested using cytotype as factor and genome size as response variable. In both analyses, GLMs with a Gaussian distribution and a log

link function were used. The analyses were performed in R software version 3.0.1 (R Core Development Team 2016), using the packages "car" for Type-III analysis of variance (Fox and 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).

Ecological niche modelling

Two spatial scales were used to evaluate the environmental requirements of each cytotype: the first encompassed the distribution of *Jasione montana* in the Iberian Peninsula, while the second encompassed the diploid-tetraploid contact zone in the northwestern region of the Iberian Peninsula (39.6° to 43.7.6° in latitude, and from -6.1° to -9.2° in longitude).

For the abiotic parameters, 19 bioclimatic variables (Bio1-Bio19) plus altitude, latitude and longitude at a 1 km resolution were extracted from Worldclim database (http://www.worldclim.org/). To improve the quality of the niche environmental predictions, the following six variables related with soil properties were 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 STU – txsrfdo, and first soil adjective code of the STU – wrbadj1 (Panagos et al, 2012; European Soil Data Centre: esdac.jrc.ec.europa.eu). Environmental values of each variable were extracted for all records using the "dismo" package in R (Hijmans *et al.* 2017). Correlations between all variables were assessed and highly correlated variables were excluded. Additionally, based on the variance explained in the PCA and the expected biological importance in *J. montana* life cycle, four environmental variables (Bio4, Bio5, Bio14 and Bio15) and two soil parameters (bs_topand txsrfdo) were selected (Table 3.1). These variables were subsequently used in both approaches.

Table 3.1. Selected environmental variables using sampled populations from the diploid-tetraploid contact zone of *Jasione montana*. For each cytotype, mean and standard error of the mean (Mean ± SE) are given. Different letters represent statistically significant differences between cytotypes at $P < 0.05$.

Variables	**CODE**	**Diploid**	**Tetraploid**
		Mean ± SE, N =170	Mean ± SE, N =73
Temperature seasonality	**Bio_4**	4820.89 ± 56.43[a]	4583.34 ± 70.79[b]
Maximum temperature of warmest month	**Bio_5**	267.62 ± 1.76[a]	258.66 ± 2.04[b]
Precipitation of driest month	**Bio_14**	16.65 ± 0.75[a]	17.95 ± 0.98[a]
Precipitation seasonality	**Bio_15**	49.57 ± 0.57[a]	50.02 ± 0.69[a]
Base saturation of the tops	**bs_top**	1.78 ± 0.04[a]	1.92 ± 0.03[b]
Dominant surface textural class of the STU	**txsrfdo**	1.74 ± 0.04[a]	1.88 ± 0.04[b]

In the first approach, *i.e.*, for the Iberian Peninsula, we combined: 1) the occurrences from our intensive field sampling (39.6° to 43.7.6° in latitude, -6.1° to -9.2° in longitude) and the occurrences from Rubido-Bará et al (2010; based on chromosome counts and flow cytometric analyses) for this area, with 2) the occurrences for *J. montana* downloaded from GBIF database (http://gbif.org) beyond this area. In the first group of occurrences, points were classified as diploids and tetraploids based on our estimates and in Rubido-Bará et al (2010). In the second group of occurrences, points were classified as diploids based on the extensive literature review on the karyology of *J. montana* and on estimates obtained here. The dataset was filtered to include only one presence per square kilometer. Additionally, a filter of 10 km was used to remove GBIF reports that were separated by less than this distance to avoid oversampling in the area beyond the contact zone. The final dataset comprised 871 diploid and 88 tetraploid points. Niche modelling was performed with maximum entropy modelling (MaxEnt) using the R software package "dismo" (Hijmans *et al.* 2017) with default parameters, except for the number of replicates (30), percentage of random tests (30), and maximum number of background points (5,000). MaxEnt, a model based only on presence records, was used because we did not have true absence records for the area outside the contact zone. The Area Under the Curve (AUC) was used to evaluate model accuracy. Finally, MaxEnt results were converted to binary projections for further statistical analyses (see below).

In the second approach, *i.e.*, spatially restricted to the contact zone, only our sampled populations from the contact zone were incorporated in the models. Once again, only one occurrence per square kilometer was used, and the final dataset comprised 180 diploid and 76 tetraploid points. Calibration of the spatial predictive models was based on presence/absence records collected in the field. For the diploid dataset, diploid populations were recorded as presence and tetraploid populations as absence, and vice-versa for the tetraploid dataset. Presence/absence models were used in this approach because our sampling enabled us to assign a true absence of a given cytotype in pure populations of the other cytotype. Niche modelling of diploids and tetraploids was performed using R package "biomod2" (Thuiller *et al.* 2016), with the final model of each cytotype resulting from the combination of different modeling techniques, each one replicated 30 times after splitting data in training (70%) and testing (30%) subsets, randomly selected to reduce the uncertainty of the model (Phillips *et al.* 2006; Araújo and New 2007). To guarantee statistical independence of all replicates, each specific occurrence was used only once in each run, either as training or as test data without replacement (Phillips 2008). Models were evaluated based on the independent accuracy measure, AUC. In the

ensemble forecasting process, only models with an AUC > 0.7 were used to produce the final model of each cytotype.

Tests of niche equivalence and similarity

The Schoener's *D* metric, a measure of niche similarity (Schoener, 1970), was used to quantify niche overlap in the geographic distribution of diploids and tetraploids. This metric ranges from 0, representing no overlap, to 1, representing a complete overlap. The analysis was run with "ecospat" (Broennimann *et al.* 2012) and "raster" (Hijmans *et al.* 2017) R packages using the binary projections. Both niche identity and similarity tests were computed to test whether predicted distributions were significantly different between cytotypes (classification by Smith and Donoghue 2010; Warren *et al.* 2008; Broennimann *et al.* 2012).

The niche identity test determines if the distribution models produced for the two cytotypes differ in their environmental attributes by polling diploid and tetraploid records and by randomly sampling from the polled occurrences to create a pseudo-replicate dataset of equal size that was then used for *D* calculation (simulated values). This process was repeated 100 times to obtain confidence intervals for the evaluation of the null hypothesis. For this, the simulated *D* values were compared with the observed *D* value and cytotypes niches were considered equivalent if the observed *D* value fell within the 95th percentile of the simulated *D* values (Broennimann *et al.* 2012).

The niche similarity test determines whether the environmental niche of diploids and tetraploids are distinguishable from each other by comparing the records of one cytotype with random points from the geographic range of the other cytotype. As in the identity test, the process was repeated 100 times to obtain confidence intervals.

All analyses were performed in R software version 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 ploidy levels, DNA diploid or DNA tetraploid, to all analyzed plants (Figure 3.2). Diploids had an average genome size of 2C = 2.92 ± 0.07 pg (mean ± SD), ranging from 2.80 to 3.08 pg, while tetraploids had an average genome

size of 2C = 5.86 ± 0.14 pg (mean ± SD), varying between 5.63 and 6.06 pg (Table 3.2, Figure 3.2) [Appendix 3.3]. Holoploid genome size differed significantly between cytotypes ($F_{1,249}$ = 42233.00, P < 0.001), while no statistically significant differences between cytotypes were observed in monoploid genome size ($F_{1,249}$ = 0.40, P = 0.525).

Table 3.2. Genome size estimates in *Jasione montana* according with each cytotype. DNA ploidy level 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 analyzed are also presented for each cytotype. Two ploidy levels were observed: diploids (2*x*) and tetraploids (4*x*). Different letters correspond to statistically significant differences at P < 0.05.

DNA-Ploidy level	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[a]	0.04	84 (205)
4*x*	5.86[b]	0.14	3.3%	5.63	6.06	1.46[a]	0.04	24 (46)

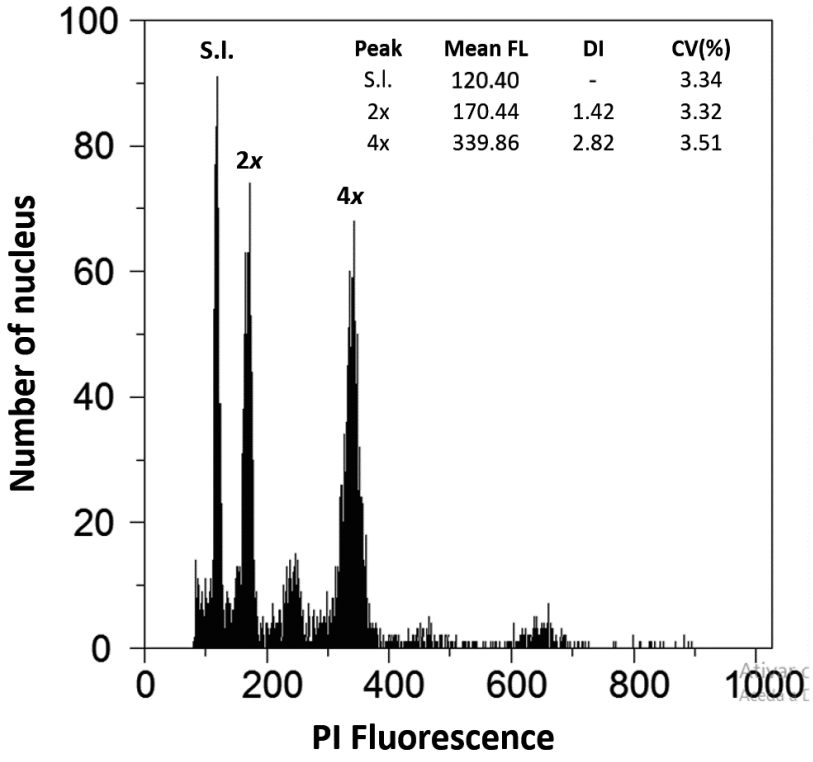


Figure 3.2. Flow cytometric histogram of relative propidium iodide fluorescence intensity (PI fluorescence) of nuclei isolated from fresh leaves of *Solanum lycopersicum* 'Stupické' (S.l.; reference standard with 2C = 1.96 pg) and of *Jasione montana* diploid (2*x*) and tetraploid (4*x*) cytotypes. For 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 the peak (CV, in %) are provided.

Cytotype distribution

The literature review on the karyology of *J. montana* revealed a widespread distribution of the diploids across Europe (white diamonds in Figure 3.1A) [Appendix 3.1]. This result was extended by the additional estimates provided in our study (white circles in Figure 3.1B). Conversely, our sampling confirmed the distribution of tetraploids as restricted to the northwestern Iberian Peninsula, in particular to Central and Northern regions of Portugal, and to Galicia in Spain (grey circles in Figs. 3.1B-C) [Appendix 3.2], as preliminarily suggested by the bibliographic records (grey diamonds in Figure 3.1A).

A total of 279 populations were sampled in the Iberian Peninsula. The great majority of the populations were pure-ploidy populations (98.6%, from which 71.3% were pure diploid and 27.3% were pure tetraploid), with only four localities (1.4%) harboring both diploid and tetraploid individuals (green circles in Figure 3.1C) [Appendix 3.2]. In the northwestern Iberian Peninsula, the diploid and tetraploid populations appeared intermingled in space, although most areas are dominated by diploids, with only some areas being dominated by tetraploids (Figure 3.1C). The tetraploid populations seem to be clustered in two regions, one in Central Portugal and another in Galicia (Spain), creating several areas of contact between diploids and tetraploids, including sympatric areas where cytotypes coexist and form a few mixed-ploidy populations (Figure 3.1C). The structure 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 [Appendix 3.2]. Despite a large sample size (N =3396), especially in the contact zone, no other cytotype was detected.

Cytotype niche overlap

The selected variables explained a high percentage of variance in the cytotype distribution in the first two axes, in both approaches (Figure 3.3): 69.9% (48.0% in Axis 1 and 21.9% in Axis 2; Table 3.3; Figure 3.3A) in the Iberian Peninsula, and 72.0% in the contact zone (52.3% in Axis 1 and 19.7% in Axis 2; Table 3.3; Figure 3.3C). Model evaluation revealed high AUC values both in the Iberian Peninsula (2*x*: 0.62 ± 0.21; 4*x*: 0.96 ± 0.00) and in the contact zone (2*x*: 0.69 ± 0.12; 4*x*: 0.69 ± 0.08), and relatively low omission rates in the final models (Iberian Peninsula – 2*x*: 0.23 and 4*x*: 0.08; Contact zone – 2*x*: 0.18 and 4*x*: 0.08), indicating that the models could predict cytotype occurrences with high accuracy.

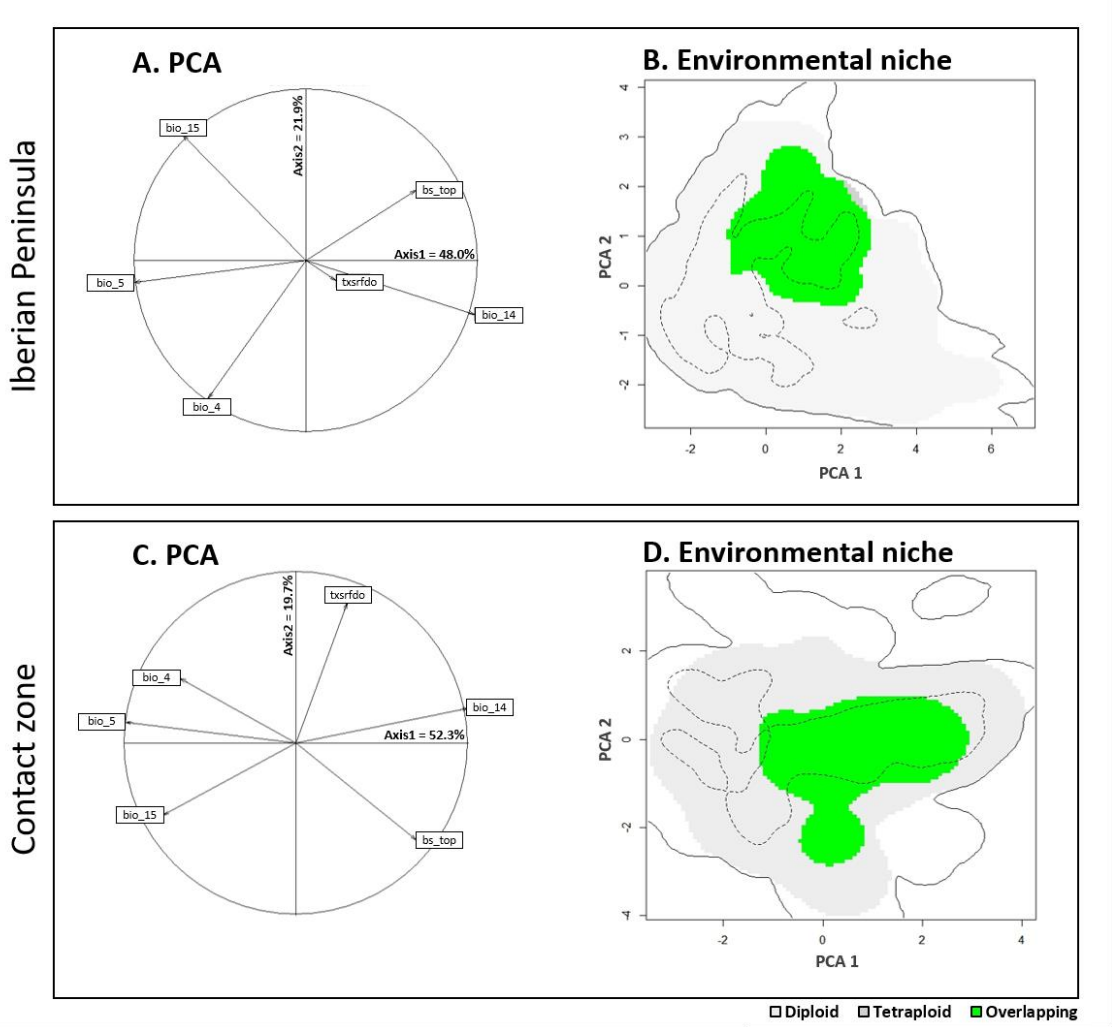


Figure 3.3. Ecological niche models for *Jasione montana* cytotypes at the Iberian Peninsula, and at the contact zone. A) and C) represent the contribution of climatic and soil variables in the first two axes of the principal component analyses (PCA) and the percentage of variance explained by each axis. B) and C) represent the environmental niche of diploids and tetraploids, respectively, based on the PCA of selected variables; colored areas represent suitable habitats as follows: light grey – diploids, dark grey – tetraploids, and green – overlapping areas between diploids and tetraploids environmental niches; the continuous line corresponds to the whole climatic space, while the dashed line indicates the 75th percentile.

In the Iberian Peninsula approach, the visual inspection of the distribution models revealed a high predicted suitability of diploids over most of the region, with exception of eastern calcareous areas, where its probability to occur is very low (Figure 3.4A). In contrast, the predicted tetraploid distribution was essentially restricted to the northwestern regions of the Iberian Peninsula, where this cytotype is currently found, and near the eastern coast of Valencia (Figure 3.4B). The amplitude of the environmental niche of diploids was larger than that of tetraploids, with tetraploids presenting very similar environmental requirements in comparison with diploids (98.5% of overlapping), while only 23.2% of diploids presented the same environmental niche conditions of tetraploids (Figure 3.3B). The observed niche overlap

between the two cytotypes was low (Schoener's *D* metric, *D* = 0.05). However, the niche identity test indicated that the observed value of overlap for diploid and tetraploid distribution models fell within the distribution of expected values of similarity (*P* = 1.00; Table 3.3), indicating that the climatic niches of diploids and tetraploids are equivalent (Glor and Warren 2011). Additionally, the comparisons of the cytotype ranges with the niche similarity test also indicated that diploids and tetraploids were climatically similar, since the observed value of overlap was not significantly different from the range of pseudo-replicate comparisons between sites of a given cytotype and the random occurrences extracted from the range of the other cytotype (*P* = 0.39 and *P* = 0.34, for diploids within tetraploid range and tetraploids within diploid range, respectively; Table 3.3).

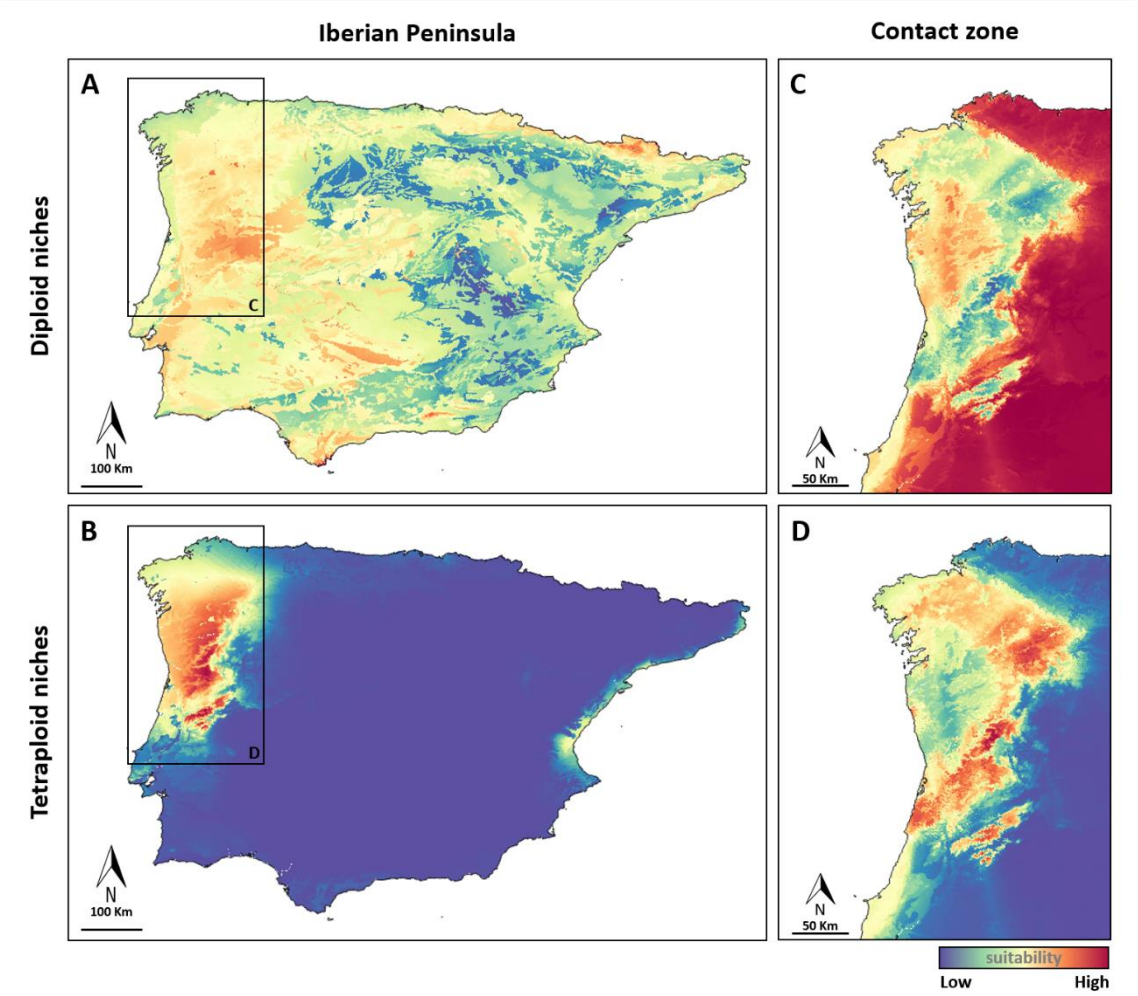


Figure 3.4. Predictive suitable niche for each cytotype (diploids – A and C, and tetraploids – B and D) of *Jasione montana* in the Iberian Peninsula (A and B) and in the contact zone (C and D). Cold temperature colors represent habitats with low suitability and hot temperature colors habitats with high suitability.

In the contact zone approach, the patterns were similar to what was observed for the Iberian Peninsula (Figure 3.4C-D). Tetraploids present a restricted distribution (Figure 3.4D), while diploids can occur in the entire range modelled, although with a lower probability within

the areas inhabited by tetraploids (Figure 3.4C). However, the environmental niche of the tetraploid overlapped completely with that of the diploid despite it occupied only 30.6% of the diploid environmental space (Figure 3.3D). As observed for the Iberian Peninsula, cytotypes presented low geographic niche overlap (D = 0.11, Table 3.3), although the environmental niches were equivalent (*P* = 1.00, Table 3.3) and similar (*P* = 0.32 and *P* = 0.39, for diploids within tetraploid range and tetraploids within diploid range, respectively; Table 3.3).

Table 3.3. Environmental niche analyses in *Jasione montana*. For each approach (Iberian Peninsula and the contact zone), the variance explained by the first two axes of the principal component analyses, and the equivalency (*D* and respective *P* values) and similarity (*P* value) tests (2*x* → 4*x*: diploids within tetraploid range; 4*x* → 2*x*: tetraploids within diploid range), are presented.

Studied area	Axis 1	Axis 2	Equivalence test		Similarity test (*P* value)	
			D value	*P* value	2*x* → 4*x*	4*x* → 2*x*
Iberian Peninsula	48.0%	21.9%	0.05	1.00	0.39	0.34
Contact zone	52.3%	19.7%	0.11	1.00	0.32	0.39

DISCUSSION

In this cytogeographical study of the polyploid *Jasione montana* we provide novel insights on the diversity and distribution patterns of diploids and tetraploids, which enabled us to explore the factors involved with the establishment and spread of tetraploids in nature. In particular we observed that: 1) diploids are the widespread cytotype across Europe, while tetraploids are restricted to the northwest of the Iberian Peninsula, apparently arranged in two nuclei, one in Central Portugal and another in Galicia (Spain), with no additional cytotypes being detected; 2) in the northwest Iberian Peninsula, the two cytotypes present a mosaic parapatric distribution with areas dominated by diploids being alternated with some areas dominated by tetraploids, thus harboring several contact zones; 3) mixed-ploidy populations were seldom found, with diploids and tetraploids growing in sympatry in just 4 out of 279 sampled populations (1.4%); 4) despite the low geographical overlap directly observed in the field and confirmed by environmental niches models, cytotypes revealed similar niches at the two spatial scales studied.

The genus *Jasione*, although it still bears unresolved phylogenetic relationships within Campanulaceae (Haberle *et al.* 2009), has been suggested as an old genus in which speciation within the crown group occurred recently, possibly linked with the last glaciation period in Europe (Sales *et al.* 2004; Pérez-Espona *et al.* 2005). Among the factors possibly involved in the genesis of new species is polyploidy, a phenomenon regarded as frequent during glaciation

periods (Thompson 2005; Marques *et al.* 2017) due to the effect of temperature fluctuations in the generation of unreduced gametes (Ramsey and Schemske 1998; Mason *et al.* 2011). Indeed, in *Jasione*, polyploidization is regarded as a frequent phenomenon, with half of the species within the center of diversity of the genus, *i.e.*, the Iberian Peninsula, being polyploid or harboring multiple cytotypes (Sales and Hedge 2001a; Rubido-Bará *et al.* 2010; Marques *et al.* 2017). One of such polyploid complexes is the widespread *J. montana*, the study species of this work. Until very recently, the extensive chromosome counts available for *J. montana* reported diploid occurrences throughout all Europe (compiled in Table 3.2 and Figure 3.2A), with tetraploids having been reported in one locality in the center of Portugal (Leitão and Paiva 1988) and, more recently, also in Galicia, Spain (Rubido-Bará *et al.* 2010). Our field sampling and flow cytometric screenings corroborated the presence of tetraploids in previous reported areas and enabled to determine their distribution range. Currently, tetraploids are restricted to the northwest of the Iberian Peninsula, and interestingly, they appear to be concentrated in two areas, one in central-north Portugal and another in Galicia. This spatial arrangement might suggest that tetraploids might have more than once within the species. Multiple polyploid emergence is frequent and has been found in numerous polyploid complexes (*e.g.*, Soltis and Soltis 1993, 1999; Segraves and Thompson 1999; Sampson and Byrne 2011), although further molecular studies are needed to confirm this hypothesis in *J. montana*.

Polyploidy has been suggested to mediate changes in environmental requirements and tolerances that might allow the polyploid to occupy different niche amplitudes in comparison to their progenitors (Husband and Schemske 2000; Baack and Stanton 2005; Buggs and Pannell 2007; Ramsey 2011). This is particularly advantageous as it enables the neopolyploid to escape frequency-dependent selection and minority cytotype exclusion (Levin 1975; Fowler and Levin 1984; Felber 1991; Hao *et al.* 2013). Spatial segregation between cytotypes has been observed in numerous polyploid complexes (*e.g.*, Husband and Schemske 1998; Sonnleitner *et al.* 2000; Balao *et al.* 2009; Kolář *et al.* 2009; Castro *et al.* 2012; Casazza 2017) and in some studies the distribution of cytotypes has been strongly associated with environmental variables (*e.g.*, Glennon *et al.* 2014; Thompson *et al.* 2014; Visger *et al.* 2016; Muñoz-Pajares *et al.* 2017), suggesting the occurrence of niche differentiation, either mediated by genome duplications or by post-polyploidization selection. Surprisingly, regardless of the spatial scale studied, ecological niche modelling of *J. montana* cytotypes indicate very similar environmental niches between the two cytotypes. This suggests that, in this species, polyploidization does not seem to result in differentiation in environmental requirements, despite the two cytotypes not occurring together. The absence of differentiation can result from a recent tetraploid formation, with no

changes in traits related with environmental tolerances being driven by polyploidization. Alternatively, tetraploids might not have had time to diverge from their progenitor and/or recurrent gene flow between cytotypes is occurring in nature (Godsoe *et al.* 2013; Laport *et al.* 2016; Chapter 5). The lack of niche differentiation has been observed in some polyploid complexes, with other factors being pointed out as being involved in the observed distribution patterns (Godsoe *et al.* 2013; Laport *et al.* 2013, 2017; Castro *et al.* 2018 – Chapter 4). For example, in *Heuchera cylindrica*, climatic niche differentiation did not explain current parapatric distribution of diploids and tetraploids, with the latter occurring in environments that are predicted to be suitable to diploids (Godsoe *et al.* 2013). In this species, the authors suggested that tetraploids might have expanded to sites unoccupied by diploids after glacial retreat. Similarly, tetraploid and octoploid individuals of *Gladiolus commnunis* did not differ in environmental requirements, growing in similar habitats, with current cytotype distribution patterns being shaped by historical patterns of migration, colonization and selection against the minority cytotype (Castro *et al.* 2018). In *J. montana*, future studies with reciprocal transplants are needed to experimentally test the lack of niche differentiation.

Regardless of the similar environmental niches observed between diploids and tetraploids of *J. montana*, niche modelling also indicated a very low geographical niche overlap between cytotypes, which was corroborated in the field by the presence of very few mixed-ploidy populations. This means that other factors rather than environmental requirements would have to be involved with the current cytotype distribution in nature. At present, the tetraploids and diploids growing in the northwest of the Iberian Peninsula form a complex parapatric mosaic, with some areas dominated by tetraploids being intermingled with areas dominated by diploids. This mosaic creates several contact zones between diploids and tetraploids. In this scenario, and in the absence of niche differentiation, cytotype interactions are expected to be frequent (Hewitt 1988; Harrison 1993; Petit *et al.* 1999; Lexer and van Loo 2006; Castro *et al.* 2018). Thus, either the tetraploids have a higher fitness in comparison with diploids and are able to increase their numbers within the diploid populations (Felber 1991; Burton and Husband 2000; te Beest 2011; Ramsey and Ramsey 2014) and/or they are able to disperse to vacant places in the landscape to avoid the minority cytotype exclusion (Godsoe *et al.* 2013; Thompson *et al.* 2014; Visger *et al.* 2016; Muñoz-Pajares *et al.* 2017). An aggregation of the tetraploid populations in some regions suggest that tetraploids might be able to outcompete diploids, excluding them from populations. If this is true, tetraploids may be able to expand their distribution. Fitness advantages such has increased competitive ability (Maceira *et al.* 1993; Laport *et al.* 2013), asymmetric assortative mating (Husband and Sabara 2004; Buggs

and Pannell 2006, 2007; Laport *et al.* 2016), and/or high rates of unreduced gametes formation (Ramsey and Schemske 1998; Ramsey 2007; Husband 2016) among other factors, have been reported to play crucial roles in the success of polyploid lineages. For example, early flowering and a higher flower production in the tetraploid *Larrea tridentata*, combined with higher plant densities, conferred a reproductive advantage for the tetraploids over sympatric and parapatric diploid and hexaploid individuals, and might explain the distribution of tetraploids in areas suitable for diploid populations (Laport *et al.* 2013, 2016). Also, in *Mercurialis annua*, although the cytotypes are ecologically differentiated, the diploids present a reproductive advantage displacing the hexaploids and driving a moving contact zone as a result of asymmetrical reproductive interference and pollen swamping generated by different reproductive systems (Buggs and Pannell 2006, 2007). Differences in competitive ability have also been observed between diploid and tetraploid *Dactylis glomerata*, with tetraploids having a competitive superiority, which gradually led to the exclusion of diploid plants from mixed-ploidy populations (Maceira *et al.* 1993). In *J. montana*, some differences between cytotypes have been observed for several morphological traits that might be linked with plant fitness and competitive ability. Although being very variable, tetraploids seem to be bigger plants, presenting larger leaves and inflorescences and producing more seeds than diploids, although diploids produce a higher number of inflorescences (Rubido-Bará *et al.* 2010). Further studies are thus needed to unravel possible fitness differences that could explain cytotype interactions at contact zones and the current distribution patterns.

The coexistence of different cytotypes in sympatry is possible when a set of reproductive barriers mediate assortative mating (Levin 1975; Husband and Sabara 2004; Kolár *et al.* 2017; Husband *et al.* 2016). Thus, the profuse contact zones between diploid and tetraploid *J. montana* with the lack of mixed-ploidy populations also suggest that reproductive barriers between the two cytotypes might be weak and that frequency-dependent selection might be an important force excluding the minority cytotype or the cytotype in disadvantage. Although having been tested experimentally only by Husband (2000) in *Chamerion angustifolium*, minority cytotype exclusion has been referred as an important mechanism that drives cytotype distribution patterns in numerous contact zones (*e.g.*, Levin 2002; Baack 2004; Španiel *et al.* 2008; Castro *et al.* 2011).

CONCLUSIONS

The geographical patterns observed in nature suggest that tetraploids might have arisen multiple times in *Jasione montana*. Contrary to what has been observed in other polyploid complexes, environmental niche associations indicate similar environmental niches between the two cytotypes, suggesting that polyploidization in *J. montana* has not generated shifts in the environmental preferences of the tetraploids. Under this scenario, either the tetraploids have higher fitness in comparison with diploids and increase their number within the diploid populations and/or are able to disperse themselves to places of the landscape unoccupied by their progenitors, thus avoiding minority cytotype exclusion. Indeed, the aggregation of the tetraploid populations in some areas suggest that tetraploids might outcompete diploids, excluding them from the population. The profuse contact zones between diploids and tetraploids with the lack of mixed-ploidy populations also suggest that, in the absence of any fitness advantage, frequency-dependent selection might be an important force excluding the minority cytotype. Future molecular and experimental studies such as reciprocal transplants and competition experiments will allow us to test the hypotheses arising from this study.

APPENDICES

Appendix 3.1. Chromosome counts of *Jasione montana* available in the bibliography. Information about the original name reported in each reference, number of chromosomes (n, gametophytic; 2n, sporophytic), ploidy level (2*x*, diploid) and reference are given. References highlighted in bold provide geographic information about the material used for the chromosome counts.

Original name	n	2n	Ploidy	References
Jasione montana L.		12	2*x*	Anchev 1976
Jasione blepharodon Boiss. & Reut.		12	2*x*	**Bjorkqvist *et al.* 1969**
Jasione montana L.		12	2*x*	Brullo *et al.* 1977
Jasione montana L.	6	12	2*x*	**Contandriopoulos 1966**
Jasione montana L.	6		2*x*	Delay 1969
Jasione montana L.		12	2*x*	**Dobea *et al.* 1996**
Jasione montana L.		12	2*x*	**Gadella 1966**
Jasione montana L.	6	12	2*x*	Gadella and Kliphuis 1966
Jasione montana L.		12	2*x*	**Gadella and Kliphuis 1968**
Jasione montana L.		12	2*x*	**Gadella and Kliphuis 1970**
Jasione montana L.		12	2*x*	**Kliphuis and Wieffering 1972**
Jasione montana L.		12	2*x*	**Kovanda 1968**
Jasione montana L.		12	2*x*	Kovanda 1983
Jasione montana L.		12	2*x*	**Králik and Hrozienčik 2000**
Jasione montana L.		12	2*x*	Lago Canzobre and Castroviejo 1992
Jasione montana L.		12	2*x*	Lövkvist and Hultgård 1999
Jasione montana L.	6		2*x*	Luque and Mejas 1986
Jasione montana L.		12	2*x*	**Majovsky 1970**
Jasione echinata Boiss. & Reut.		12	2*x*	Ottonello *et al.* 1986
Jasione montana L.		12	2*x*	Parfenov and Dmitrieva 1985
Jasione montana L.		12	2*x*	Parfenov and Dmitrieva 1988

⇩Cont.

Jasione montana L.	6		2*x*	Parnell 1982
Jasione montana		12	2*x*	Parnell 1986
Jasione echinata Boiss. & Reut. Nyman	6	12	2*x*	Pastor 1990
Jasione montana L.		12	2*x*	Poddubnaja-Arnoldi 1933
Jasione montana L.		12	2*x*	**Podlech 1963**
Jasione montana L.	6	12	2*x*	Pogan *et al.* 1980
Jasione montana L.		12	2*x*	Rohweder 1937
Jasione montana L.		12	2*x*	Rosén 1932
Jasione montana L.		12	2*x*	Sugiura 1940
Jasione montana L.		12	2*x*	Sugiura 1942
Jasione montana L.	6		2*x*	Ubera 1980
Jasione montana L.		12	2*x*	Uhríková and Králik 2000
Jasione montana L.		12	2*x*	Van Den Brand 1979
Jasione montana L.	6	12	2*x*	Van Loon and Snelders 1979
Jasione montana L.		12	2*x*	Wcislo 1983
Jasione montana L.		12	2*x*	Wisskirchen and Haeupler 1998
Jasione montana L.		12	2*x*	**Wulff 1937**
Jasione montana		24	4*x*	**Leitão and Paiva 1988**
Jasione montana L.	6, 12		2*x* and 4*x*	**Rubido-Bará *et al.* 2010**

Appendix 3.2. Geographic information of the *Jasione montana* populations sampled in this study. For each population, an ID code, estimated DNA ploidy level, sample size (N) and information on the location and GPS coordinates (angular) are presented. Two ploidy levels were observed: diploids (2*x*) and tetraploids (4*x*). Populations were divided in two groups according with their ploidy composition: pure-ploidy populations and mixed-ploidy populations. The five locations marked with an asterisk (*) constitute unpublished chromosome counts by Miguel Serrano. [S] denote the populations where DNA-ploidy level was assessed directly from seeds.

ID code	DNA Ploidy level (N)	Location	Geographic coordinates	
			Latitude	Longitude
Pure-ploidy populations				
MS16-006 [S]	2*x* (15)	Jbel Tazekka, Taza, Marrocos	34.08691	-4.18341
MS16-015	2*x**	Pazzano, Calabria, Italy	38.46803	16.41768
AA002	2*x* (6)	Pegões, Setúbal, Portugal	38.65242	-8.61802
MC357	2*x* (5)	Arraiolos, Évora, Portugal	38.72755	-7.98722
MS16-007 [S]	2*x* (15)	Aldea del Rey, Cuidad Real, Spain	38.73722	-3.87583
MC356	2*x* (7)	Coruche, Santarém, Portugal	38.94409	-8.49414
MC353	2*x* (17)	Muge, Salvaterra de Magos, Portugal	39.06865	-8.66193
MC358	2*x* (3)	Domingão, Ponte de Sor, Portugal	39.24543	-8.02592
SC122	2*x* (4)	Jardim, Marvão, Portugal	39.39334	-7.38971
SC121	2*x* (5)	Quinta Mão do Novilheiro, Castelo de Vide, Portugal	39.42844	-7.48734
DT017	2*x* (3)	Chãos, Alcobertas, Portugal	39.42958	-8.92472
MC341	2*x* (19)	Rio de Moinhos, Abrantes, Portugal	39.47490	-8.23640
MC342	2*x* (6)	Constância, Santarém, Portugal	39.48597	-8.33026
MC340	2*x* (25)	Penedo, Oleiros, Portugal	39.66357	-8.12899
MC221	2*x* (30)	Peral, Proença-a-Nova, Portugal	39.69370	-7.79880
MC346	2*x* (26)	Pias, Ferreira do Zêzere, Portugal	39.71898	-8.32942
SC191	2*x* (19)	Vale de Urso, Proença-a-Nova, Portugal	39.74483	-7.89155

⇩Cont.

MC309	2*x* (23)	Albergaria, Marinha Grande, Portugal	39.74603	-8.88242
LM014	2*x* (6)	Dornes, Ferreira do Zêzere, Portugal	39.76843	-8.26169
MC362	2*x* (13)	Janardo, Leiria, Portugal	39.78695	-8.77387
MC350	2*x* (29)	Vale da Meda, Ourém, Portugal	39.79381	-8.53367
SC192	2*x* (16)	Sertã, Portugal	39.80863	-8.11590
SC190	2*x* (3)	Silveira dos Figos, castelo Branco, Portugal	39.83898	-7.67476
MC360	2*x* (1)	Vermoil, Pombal, Portugal	39.84320	-8.66282
SC189	2*x* (3)	Castelo Branco, Portugal	39.84571	-7.47026
LM009	2*x* (25)	Souto de Carpalhosa, Leiria, Portugal	39.84595	-8.83335
MC368	2*x* (16)	Granja, Bajouca, Portugal	39.85191	-8.87618
MC361	2*x* (9)	Valeirão, Pombal, Portugal	39.87102	-8.75234
EM001	2*x* (24)	Pedrógão Pequeno, Sertã, Portugal	39.89622	-8.13783
SC193	2*x* (14)	Poço Negro, Figueiró-dos-Vinhos, Portugal	39.91965	-8.25304
LM005	2*x* (6)	Casal Fernão João, Pombal, Portugal	39.91980	-8.64870
LM003	2*x* (5)	Casal Fernão João, Pombal, Portugal	39.92007	-8.65293
MC330	2*x* (20)	Pedrogão Grande, Portugal	39.92688	-8.15120
MC336	2*x* (22)	Cancinos, Oleiros, Portugal	39.92747	-7.92447
MC329	2*x* (18)	Fato, Figueiró dos Vinho, Portugal	39.92843	-8.32886
MC371	2*x* (18)	Matos do Carriço, Pombal, Portugal	39.98608	-8.79657
SC188	2*x* (9)	São Miguel da Acha, Idanha-a-Nova, Portugal	40.00324	-7.33108
MC354	2*x* (1)	Redinha, Pombal, Portugal	40.00532	-8.58961
MC334	2*x* (12)	Alvares, Góis, Portugal	40.00980	-8.10677
MC298	2*x* (11)	Casal da Rola, Pombal, Portugal	40.02076	-8.71501
SC184	2*x* (11)	Bogas de Baixo, Oleiros, Portugal	40.03460	-7.77846

⇩Cont.

MC369	2*x* (5)	Torneira, Pombal, Portugal	40.03966	-8.75807
SC182	2*x* (18)	Vilarinho, Lousão, Portugal	40.12720	-8.20791
AM002	2*x* (4)	Vale, Lousã, Portugal	40.13018	-8.23468
AM001	2*x* (6)	Vale, Lousã, Portugal	40.13082	-8.23518
SC187	2*x* (16)	Penamacor, Portugal	40.13903	-7.20258
SC186	2*x* (15)	Fatela, Fundão, Portugal	40.16101	-7.42409
MC271	2*x* (10)	Caneiro, Miranda do Corvo, Portugal	40.17904	-8.31525
MC292	2*x* (12)	Torre do Mondego, Coimbra, Portugal	40.19314	-8.38946
SC179	2*x* (2)	Madorno, Montemor-o-Velho, Portugal	40.19499	-8.65544
MS16-009 [S]	2*x* (15)	San Martín de Trevejo, Cáceres, Spain	40.20992	-6.78700
MC307	2*x* (9)	Santo António dos Olivais, Coimbra, Portugal	40.21064	-8.40051
SC180	2*x* (10)	Meães, Montemor-o-Velho, Portugal	40.21384	-8.60850
AA001	2*x* (4)	Salgueiro, Fundão, Portugal	40.22853	-7.33197
MC272	2*x* (6)	Quinta Grande, Coimbra, Portugal	40.23437	-8.42012
MC306	2*x* (10)	Secarias, Arganil, Portugal	40.24692	-8.03352
SC225	2*x* (10)	Caria, Belmonte, Portugal	40.29917	-7.35661
SC230	2*x* (10)	Malcata, Sabugal, Portugal	40.29997	-7.05845
SC231	2*x* (7)	Quadrazais, Sabugal, Portugal	40.31300	-7.00049
SC229	2*x* (7)	Sabugal, Portugal	40.33050	-7.09721
SC227	2*x* (4)	Azenha, Sabugal, Portugal	40.33134	-7.22465
MS16-011 [S]	2*x* (15)	Candelario, Salamanca, Spain	40.33659	-5.76651
MC281	2*x* (9)	Cácemes, Mortágua, Portugal	40.33928	-8.34688
MS15-062	2*x* (6)	Montemayor del Río, Salamanca, Spain	40.34574	-5.88048
MC287	2*x* (12)	Almacinha, Mortágua, Portugal	40.35560	-8.19451

⇩Cont.

SC241	2*x* (3)	Catraia do Buraco, Belmonte, Portugal	40.37214	-7.32858
SC232	2*x* (3)	Alfaiates, sabugal, Portugal	40.37808	-6.94432
MC283	2*x* (17)	Freixo, Mortágua, Portugal	40.38542	-8.19956
MC282	2*x* (14)	Barracão, Mortágua, Portugal	40.38915	-8.28630
MC269	2*x* (9)	Várzea, Mealhada, Portugal	40.39001	-8.38288
MC285	2*x* (12)	Santa Comba Dão, Portugal	40.39751	-8.12613
SC127	2*x* (3)	Senhora do Espinheiro, Seia, Portugal	40.41127	-7.67038
SC169	2*x* (27)	Póvoa da Forcada, Carregal do Sal, Portugal	40.41677	-8.03776
JCO001	2*x* (20)	Quintãzinha do Mouratão, Guarda, Portugal	40.41750	-7.70591
SC239	2*x* (3)	Quinta do Souto, Belmonte, Portugal	40.42413	-7.25116
SC238	2*x* (4)	A-de-Moura, Guarda, Portugal	40.45907	-7.2212
SC236	2*x* (7)	Adão, Guarda, Portugal	40.46016	-7.14943
SC208	2*x* (11)	Vila Verde, Seia, Portugal	40.46337	-7.77766
MC273	2*x* (23)	Santo Amaro, Tondela, Portugal	40.50142	-8.07658
MS15-065	2*x* (6)	Villanueva del Conde, Salamanca, Spain	40.50603	-6.01425
SC176	2*x* (19)	Palhaça, Oliveira do Bairro, Portugal	40.51136	-8.59473
SC170	2*x* (21)	Nelas, Portugal	40.53123	-7.86441
MS15-069	2*x* (6)	Peña de Francia, Salamanca, Spain	40.53424	-6.14993
MG001	2*x* (11)	Caramulo, Tondela, Portugal	40.57402	-8.17512
MC248	2*x* (17)	Granja, Guarda, Portugal	40.60071	-7.10506
SC210	2*x* (4)	Lajeosa do Mondego, Guarda, Portugal	40.63036	-7.35552
MC249	2*x* (28)	Germil, Penalva do Castelo, Portugal	40.65076	-7.73303
SC171	2*x* (28)	Abraveses, Viseu, Portugal	40.68324	-7.92715
MC296	2*x* (2)	Vale, Estarreja Portugal	40.70659	-8.55917

⇩Cont.

MS16-020	2*x**	Soto del Real, Madrid, Spain	40.75042	-3.79266
MC291	2*x* (14)	Parada, Sever do Vouga, Portugal	40.77002	-8.29246
FM001	2*x* (10)	Falachos, Trancoso, Portugal	40.77475	-7.31173
MC247	2*x* (26)	Palhais, Trancoso, Portugal	40.81355	-7.43169
MC295	2*x* (6)	Torre, Ovar, Portugal	40.82751	-8.57485
MS16-008 [S]	2*x* (15)	Rascafria, Madrid, Spain	40.84123	-3.89195
MC253	2*x* (25)	Vila Nova de Paiva, Portugal	40.86050	-7.74368
EA001	2*x* (11)	Folgosa, Castro Daire, Portugal	40.89789	-7.89528
MC246	2*x* (9)	Devesas, Mêda, Portugal	40.90534	-7.22070
MS16-014 [S]	2*x* (15)	Luras, Sardenha, Italy	40.93919	9.19006
MC255	2*x* (23)	Beselga, Penedono, Portugal	40.94033	-7.41715
MC294	2*x* (16)	Gamoal, Santa Maria da Feira, Portugal	40.95436	-8.59781
MC275	2*x* (1)	Carvalhosa, Castro Daire, Portugal	40.95601	-7.97051
MC254	2*x* (22)	Moimenta da Beira, Portugal	40.96929	-7.60611
MC265	2*x* (20)	Bigorne, Lamego, Portugal	41.00491	-7.88631
MS16-018	2*x**	Galllocanta, Zaragoza, Spain	41.01189	-1.48764
SC211	2*x* (16)	Almendra, Vila Nova de foz Côa, Portugal	41.02128	-6.99439
MS16-016	2*x**	Li Cossi, Trinitá d'Agultu, Italy	41.04651	8.93621
MC256	2*x* (20)	Sebadelhe, Vila Nova de Foz Côa, Portugal	41.05878	-7.28996
MC276	2*x* (18)	Fornelos, Resende, Portugal	41.10547	-7.97133
MC264	2*x* (15)	Varais Lamego, Portugal	41.14550	-7.77917
MC257	2*x* (14)	Ervedosa do Douro, São João da Pesqueira, Portugal	41.17167	-7.47849
MS16-010 [S]	2*x* (5)	Cerezo de Arriba, Segóvia, Spain	41.20521	-3.47356
SC220	2*x* (16)	Cancelas, Paredes, Portugal	41.22045	-8.35026

⇩Cont.

SC221	2*x* (16)	Gondalães, Paredes, Portugal	41.22737	-8.33442
SC222	2*x* (14)	Vales, Vizela, Portugal	41.24451	-8.29639
SC219	2*x* (21)	Grifão, Paredes, Portugal	41.24545	-8.34648
SC218	2*x* (15)	Sobroso, Paredes, Portugal	41.25119	-8.32535
SC194	2*x* (9)	Via Pouca, Vizela, Portugal	41.25188	-8.29536
MC258	2*x* (9)	Alijo, Portugal	41.26786	-7.45585
MC394	2*x* (8)	Fontiela, Trofa, Portugal	41.28733	-8.56441
MC259	2*x* (9)	Vila Flor, Portugal	41.30274	-7.15472
MC263	2*x* (19)	Bairro da Carvalha, Vil Real, Portugal	41.31980	-7.73189
MC245	2*x* (31)	Alfândega da Fé, Vinhais, Portugal	41.36845	-6.95750
MC262	2*x* (15)	Seixo, Murça, Portugal	41.40044	-7.44749
MC388	2*x* (17)	Infantas, Guimarães, Portugal	41.42072	-8.24202
MC260	2*x* (15)	Golfeiras, Mirandela, Portugal	41.48038	-7.20335
MC244	2*x* (30)	Bairro de Santa Luzia, Miranda do Douro, Portugal	41.49838	-6.29006
MC243	2*x* (29)	Macedo de Cavaleiros, Portugal	41.52176	-6.97683
MC385	2*x* (9)	Vargens, Valpaços, Portugal	41.57491	-7.44362
MC239	2*x* (29)	Rita, Povoa de Lenhoso, Portugal	41.58439	-8.32088
MC393	2*x* (10)	Rugem, Barcelos, Portugal	41.61484	-8.56782
MC384	2*x* (10)	Torre de Dona Chama, Mirandela, Portugal	41.65511	-7.14501
SC099	2*x* (16)	Outeiro, Montalegre, Portugal	41.68602	-7.94060
MC391	2*x* (12)	Bouças, Terras do Bouro, Portugal	41.72944	-8.30498
MC382	2*x* (19)	Mosca, Baçal, Portugal	41.76454	-6.80383
MC381	2*x* (17)	Bolideira, Chaves, Portugal	41.77525	-7.32473
SC120	2*x* (28)	Praia de Afife, Viana do Castelo, Portugal	41.78630	-8.87042

⇩Cont.

MC392	2*x* (12)	Salvador, Ponte da Barca, Portugal	41.80221	-8.35737
SC104	2*x* (18)	Torneiros, Ourense, Spain	41.85503	-8.11290
SC100	2*x* (21)	Pitões das Júnias, Montalegre, Portugal	41.86905	-7.95285
MS16-012 [S]	2*x* (15)	Zicavo, Córsega, França	41.87789	9.15295
MC237	2*x* (30)	Coussourado, Paredes de Coura, Portugal	41.92381	-8.63805
MS16-013 [S]	2*x* (1)	Campo dell'Oro, Córsega, France	41.92438	8.782612
SC119	2*x* (13)	As Eiras, Pontevedra, Spain	41.92591	-8.78854
SC097	2*x* (9)	Parâmio, Braçal, Portugal	41.92869	-6.88478
MC380	2*x* (4)	Pazos, Ourense, Spain	41.93630	-7.46737
SC151	2*x* (5)	Porreiras, Paredes de Coura, Portugal	41.94870	-8.55439
SC105	2*x* (27)	Guxinde, Ourense, Spain	41.97209	-8.15188
SC098	2*x* (7)	Castrelos, Zamora, Spain	42.00266	-6.89702
MS15-049	2*x* (7)	Mombuey, Zamora, Spain	42.01321	-6.34481
MS15-051	2*x* (8)	A, Gudiña, Ourense, Spain	42.04722	-7.13633
MS15-046	2*x* (6)	Riego de Loma, Zamora, Spain	42.07390	-6.68260
SC107	2*x* (11)	Fiães, Melgaço, Portugal	42.09481	-8.19062
MC379	2*x* (13)	Trandeiras, Ourense, Spain	42.10841	-7.66274
SC108	2*x* (22)	San Bieito, Pontevedra, Spain	42.11550	-8.31584
MS14-061	2*x* (2)	Lagoa de Samabria, Zamora, Spain	42.11619	-6.72132
MC378	2*x* (17)	Mandrás, Ourense, Spain	42.14466	-7.94348
SC109	2*x* (12)	Vixiáns, Pontevedra, Spain	42.26487	-8.33807
SC110	2*x* (13)	O Piñeiro Da Igrexa, Pontevedra, Spain	42.27738	-8.37261
SC111	2*x* (30)	O Piñeiro Da Igrexa, Pontevedra, Spain	42.28235	-8.38272
MS16-003 [S]	2*x* (15)	Carrión de los Condes, Paléncia, Spain	42.31130	-4.72234

⇩Cont.

MS14-126	2*x* (1)	Saceda, León, Spain	42.32858	-6.52247
MS16-004 [S]	2*x* (15)	Queralbs, Girona, Spain	42.35393	2.16396
MS14-052	2*x* (4)	O Rego, Ourense, Spain	42.38071	-8.00801
MS14-050	2*x* (2)	Casanova, Ourense, Spain	42.41356	-8.04025
MS14-125	2*x* (1)	Pombriego, León, Spain	42.41944	-6.69567
SC162	2*x* (17)	Montes de Valdueza, León, Spain	42.44958	-6.59802
SC164	2*x* (6)	San Clemente de Valdueza, León, Spain	42.46647	-6.54864
SC283	2*x* (2)	Pradorrey, León, Spain	42.48694	-6.11456
SC284	2*x* (3)	Catro Camiños, Lugo, Spain	42.55973	-7.51190
MS14-110	2*x* (7)	Alto do Cadám, A Noveliza, Pontevedra, Spain	42.60561	-8.26591
MS15-120	2*x* (5)	Moreda do Courel, Lugo, Spain	42.62507	-7.11880
SC271	2*x* (11)	Fresnedo, León, Spain	42.65532	-6.57830
MS15-122	2*x* (5)	Pedrachantada, Lugo, Spain	42.75522	-7.38493
SC267	2*x* (4)	Pedrachantada, Lugo, Spain	42.75539	-7.38570
MS14-129	2*x* (1)	Pico Sacro, Pontevedra, Spain	42.80715	-8.44611
SC272	2*x* (10)	Palacios del Sil, León, Spain	42.87388	-6.45031
SC273	2*x* (2)	Caboalles de Abajo, León, Spain	42.87388	-6.45031
MS14-121	2*x* (6)	O leboreiro, A Coruña, Spain	42.88406	-7.98270
SC075	2*x* (12)	Gures, A Coruña, Spain	42.91249	-9.14875
SC276	2*x* (16)	Villasecino, León, Spain	42.96125	-6.05127
SC274	2*x* (5)	Brañas de Arriba, Asturias, Spain	43.01310	-6.44518
SC275	2*x* (10)	Pontarás, Asturias, Spain	43.08285	-6.54761
MS13-004	2*x* (1)	Cabo Vilon, LA Coruña, Spain	43.15536	-9.20540
MS14-118	2*x* (1)	Vegadécima, Asturias, Spain	43.15547	-6.95791

⇩Cont.

SC265	2*x* (3)	Toiral, Lugo, Spain	43.17893	-7.61298
MS14-048	2*x* (6)	Soesto, La Coruña, Spain	43.20609	-9.01620
MS14-117	2*x* (4)	Lago, Asturias, Spain	43.25826	-6.71970
MS16-005 [S]	2*x* (15)	Oiartzun, Gipuzkoa, Spain	43.27755	-1.85519
MS15-074	2*x* (3)	Praia da Barda, A Coruña, Spain	43.28271	-8.92655
SC261	2*x* (27)	As Toxeiras de Riba, Lugo, Spain	43.30417	-7.86336
SC245	2*x* (2)	Beo, La Coruña, Spain	43.32059	-8.84373
MS14-045	2*x* (28)	Faro de Punta Nariga, A Coruña, Spain	43.32071	-8.91042
MS14-116	2*x* (4)	La Estrella, Asturias, Spain	43.32573	-6.46229
SC259	2*x* (19)	O Reguengo, Lugo, Spain	43.39240	-7.80027
MS14-112	2*x* (5)	Vilariño, Lugo, Spain	43.41257	-7.60451
MS14-115	2*x* (3)	Castañedo, Asturias, Spain	43.44945	-6.37391
MS14-113	2*x* (2)	Serra de Xistral, Lugo, Spain	43.46848	-7.54026
SC250	2*x* (3)	Cariño, A Coruña, Spain	43.47066	-8.31559
MS14-114	2*x* (5)	Caoña, Asturias, Spain	43.50813	-6.74835
SC257	2*x* (7)	A Agraxoiba, A Coruña, Spain	43.52127	-7.94350
SC253	2*x* (6)	Cabo Prior, A Coruña, Spain	43.55296	-8.31155
MS16-002 [S]	2*x* (15)	Vivigo, Asturias, Spain	43.59356	-6.24420
SC256	2*x* (8)	A Areosa, A Coruña, Spain	43.68170	-8.04729
SC254	2*x* (14)	Punta Candieiro, A Coruña, Spain	43.70746	-8.05096
MS16-017	2*x**	Ambazac, Limoges, France	45.95951	1.39128
MS16-001[S]	2*x* (15)	Anascaul Lake, Kerry, Ireland	52.17875	-10.06399
MC367	4*x* (6)	Amieira, Marinha Grande, Portugal	39.76900	-8.91144
SC038	4*x* (37)	Amor, Leiria, Portugal	39.79586	-8.85416

⇩Cont.

SC042	4*x* (34)	Corucho, Bajouca, Portugal	39.86877	-8.83948
SC183	4*x* (13)	Moradias, Pampilhosa da Serra, Portugal	40.09120	-7.99071
MC323	4*x* (8)	Nossa Senhora da Piedade, Lousã, Portugal	40.09878	-8.23438
SC185	4*x* (13)	São Martinho, Fundão, Portugal	40.12071	-7.68483
JMC002	4*x* (26)	Parrozelos, Arganil, Portugal	40.20324	-7.90134
JC002	4*x* (20)	Mata da Margaraça, Arganil, Portugal	40.21075	-7.92777
JC001	4*x* (21)	Relva Velha, Arganil, Portugal	40.21613	-7.90580
JMC001	4*x* (11)	Mata da Margaraça, Arganil, Portugal	40.21738	-7.92158
SC181	4*x* (5)	São João do Campo, Coimbra, Portugal	40.23508	-8.50015
MC290	4*x* (7)	Caneiro, Penacova, Portugal	40.23770	-8.31345
SC168	4*x* (28)	Ponte, Penacova, Portugal	40.27446	-8.27590
MC268	4*x* (16)	Mata, Montemor-o-Velho, Portugal	40.28449	-8.67892
SC299	4*x* (5)	Gavinhos, Coimbra, Portugal	40.28935	-8.31675
MC289	4*x* (6)	Miro, Penacova Portugal	40.29552	-8.24626
MC280	4*x* (26)	Casalito, Penacova, Portugal	40.29764	-8.31378
AA004	4*x* (7)	Terlamonte, Covilhã, Portugal	40.29845	-7.44582
SC285	4*x* (5)	Casal do Céu, Figueira da Foz, Portugal	40.29999	-8.78180
MC267	4*x* (5)	Casal do João, Cantanhede, Portugal	40.32617	-8.75311
SC178	4*x* (20)	Lemede, Cantanhede, Portugal	40.33496	-8.61014
MC286	4*x* (1)	Almacinha, Mortágua, Portugal	40.36062	-8.19430
SC131	4*x* (3)	Lagoa Seca, Serra da Estrela, Portugal	40.37139	-7.63578
SC145	4*x* (5)	Ponte Jugais, Seia, Portugal	40.38427	-7.70510
JC011	4*x* (32)	Seia, Portugal	40.41750	-7.70591
MC266	4*x* (6)	Cabeços, Vagos, Portugal	40.42994	-8.65257

⇩Cont.

MC270	4*x* (14)	S. Lourenço do Bairro, Anadia, Portugal	40.44003	-8.47327
MC297	4*x* (2)	Bunheira, Oliveira do Bairro, Portugal	40.52468	-8.51850
SC175	4*x* (5)	Bolfiar, Águeda, Portugal	40.56382	-8.39382
SC174	4*x* (30)	Arca, Oliveira de Frades, Portugal	40.60769	-8.21392
SC173	4*x* (21)	Agros, Vouzela, Portugal	40.63570	-8.19801
MC141	4*x* (29)	Ponte de S. Tiago, Sever do Vouga, Portugal	40.70100	-8.37497
SC172	4*x* (17)	Vilar, São Pedro do Sul, Portugal	40.77453	-8.07215
MC301	4*x* (14)	Paredes, Vale de Cambra, Portugal	40.81652	-8.37489
DT001	4*x* (6)	Paço de Mato, Vale de Cambra, Portugal	40.85100	-8.31014
LM004	4*x* (7)	Drave, Arouca, Portugal	40.85963	-8.11861
MC252	4*x* (13)	Ribolhos, Castro Daire, Portugal	40.88682	-7.92844
MC275	4*x* (31)	Carvalhosa, Castro Daire, Portugal	40.95807	-7.96297
MC279	4*x* (32)	Canedo, Santa Maria da Feira, Portugal	41.01160	-8.45774
MC278	4*x* (32)	Santa Cecília, Castelo de Paiva, Portugal	41.04545	-8.27445
MC277	4*x* (11)	Cidadelhe, Cinfães, Portugal	41.07827	-8.08915
SC223	4*x* (19)	Boialvo, Gondomar, Portugal	41.08075	-8.47271
SC091	4*x* (18)	Fragas, Amarante, Portugal	41.26528	-7.90186
SC152	4*x* (5)	Cotorinho, Vila Real, Portugal	41.27313	-7.87249
SC089	4*x* (17)	Candemil, Amarante, Portugal	41.27966	-7.91785
MC387	4*x* (13)	Veade, Celorico de Basto, Portugal	41.41861	-7.97825
MC241	4*x* (29)	Portela de Santa Eulália, Ribeira de Pena, Portugal	41.50069	-7.79296
MC389	4*x* (11)	Figueiró do Monte, Fafe, Portugal	41.55806	-8.07242
MC390	4*x* (11)	Pombal, Vieira do Minho, Portugal	41.57658	-8.11106
MC377	4*x* (16)	Barracão, Montalegre, Portugal	41.76378	-7.70449

⇩Cont.

DM001	4*x* (1)	Illa do Faro, Galiza, Spain	42.21562	-8.90836
SC115	4*x* (10)	Limens, Pontevedra, Spain	42.25865	-8.81608
MS14-060	4*x* (1)	As Ermidas, Ourense, Spain	42.28400	-7.12801
MS14-053	4*x* (2)	Vale de Marcelle, Ourense, Spain	42.30219	-7.61265
MS15-113	4*x* (7)	Soutomaior, Pontevedra, Spain	42.33273	-8.56529
MS14-057	4*x* (1)	Mendoia, Ourense, Spain	42.33795	-7.22352
MS14-055	4*x* (3)	Piñeira, Ourense, Spain	42.36158	-7.40242
SC112	4*x* (19)	Forzáns, Pontevedra, Spain	42.36261	-8.45016
MS14-059	4*x* (2)	O Barco, Ourense, Spain	42.42409	-7.01482
MS15-043	4*x* (6)	Fontaíñas, Ourense, Spain	42.45200	-8.02619
MS15-118	4*x* (5)	A Frieira, Lugo, Spain	42.52176	-7.36829
RS001	4*x* (5)	Vilamor, Lugo, Spain	42.56537	-7.23140
MS15-119	4*x* (4)	Parada dos Montes, Lugo, Spain	42.57203	-7.27245
MS14-049	4*x* (9)	Taboada, Pontevedra, Spain	42.68757	-8.22030
SC269	4*x* (7)	Pedrafita do Cebreiro, Lugo, Spain	42.71282	-7.00560
SC268	4*x* (6)	Fillobal, Lugo, Spain	42.73892	-7.18958
MS15-123	4*x* (4)	Paradela, Lugo, Spain	42.76807	-7.56978
MS15-124B	4*x* (3)	Paradela, Lugo, Spain	42.77391	-7.62728
MS15-124A	4*x* (4)	Paradela, Lugo, Spain	42.77436	-7.62736
SC072	4*x* (7)	Ventim, A Coruña, Spain	42.80037	-9.02645
MS14-123	4*x* (2)	Santiago de Compostela, A Coruña, Spain	42.87518	-8.55576
SC266	4*x* (10)	Sabarei de Abaixo, Lugo, Spain	42.89582	-7.47154
SC195	4*x* (6)	Parada, A Coruña, Spain	42.96313	-8.84410
MS14-119	4*x* (2)	Gondar, Lugo, Spain	43.02258	-7.41816

⇩Cont.

MS14-111	4*x* (5)	O Porto Velho, Lugo, Spain	43.18596	-7.95939
SC263	4*x* (7)	San Salvador, Lugo, Spain	43.20826	-7.83605
Mixed-ploidy populations				
SC203	2*x* (1), 4*x* (15)	Vila Rosa, Seia, Portugal	40.50062	-7.81098
MC274	2*x* (1), 4*x* (25)	Fareja, Castro Daire, Portugal	40.89768	-7.91486
MC235	2*x* (19), 4*x* (14)	Sobroso, Paredes, Portugal	41.25087	-8.33041
MC386	2*x* (8), 4*x* (1)	Nozedo, Vila Pouca de Aguiar, Portugal	41.50717	-7.62201

Appendix 3.3. Genome size estimates in *Jasione montana*. In each population, DNA ploidy level estimation and mean, standard deviation of the mean (SD), coefficient of variation (CV, in %) and minimum (Min) and maximum (Max) values of holoploid genome size (2C, in pg) are given. Information about the number of individuals analyzed in each population (N) and mean monoploid genome size (1C*x*, in pg) are also presented. In bold are highlighted the mixed-ploidy populations.

ID code	Holoploid genome size (2C)						Monoploid genome size (1C*x*)
	Mean	SD	CV (%)	Min	Max	N	
Diploids	**2.92**	**0.07**	**2.2**	**2.80**	**3.08**	**213**	**1.46**
DT017	2.91	0.02	0.7	2.89	2.93	3	1.45
JCO001	2.96	0.01	0.3	2.95	2.97	3	1.48
LM009	2.86	0.03	1.1	2.83	2.88	2	1.43
LM014	2.89	0.02	0.5	2.88	2.90	2	1.45
MC246	2.89	0.03	1.1	2.84	2.92	7	1.44
MC247	2.81	-	-	-	-	1	1.40
MC248	2.81	0.00	0.2	2.81	2.82	2	1.41
MC249	2.88	0.08	2.7	2.80	3.00	7	1.44
MC253	2.91	0.04	1.2	2.88	2.96	4	1.45
MC254	2.85	0.05	1.6	2.81	2.96	9	1.42
MC263	2.83	-	-	-	-	1	1.42
MC274	2.97	-	-	-	-	1	1.49
MC275	2.90	-	-	-	-	1	1.45
MC276	2.91	0.03	1.1	2.88	2.93	3	1.46
MC283	2.94	-	-	-	-	1	1.47
MC298	2.85	-	-	-	-	1	1.42
MC306	2.95	-	-	-	-	1	1.47
MC307	2.87	-	-	-	-	1	1.43
MC309	2.88	0.04	1.6	2.85	2.93	3	1.44
MC329	2.88	0.06	2.0	2.84	2.95	3	1.44
MC330	2.97	0.01	0.3	2.96	2.98	3	1.48
MC334	3.00	0.02	0.7	2.98	3.02	3	1.50
MC235	2.91	0.07	2.5	2.81	3.00	8	1.45
MC336	2.90	0.02	0.7	2.88	2.92	3	1.45
MC340	2.87	0.00	0.0	2.87	2.87	2	1.44
MC341	2.91	0.09	3.2	2.84	3.01	3	1.45
MC346	2.89	0.06	2.1	2.84	2.96	3	1.45
MC350	2.95	0.01	0.3	2.94	2.95	3	1.47
MC354	2.85	-	-	-	-	1	1.43
MC360	2.87	-	-	-	-	1	1.43
MC378	3.00	-	-	-	-	1	1.50
MC378	2.89	-	-	-	-	1	1.44
MC386	2.92	0.04	1.5	2.89	2.95	2	1.46
MS13-004	3.05	-	-	-	-	1	1.53
MS14-118	2.87	-	-	-	-	1	1.44
MS14-121	2.92	0.04	1.3	2.87	2.95	3	1.46
MS14-125	2.85	-	-	-	-	1	1.42

⇩Cont.

MS14-126	2.88	-	-	-	-	1	1.44
MS14-129	2.99	-	-	-	-	1	1.49
MS15-046	2.82	0.01	0.4	2.81	2.83	3	1.41
MS15-049	2.82	0.03	1.0	2.80	2.84	2	1.41
MS15-051	2.86	0.08	2.9	2.81	2.96	3	1.43
MS15-062	2.90	0.07	2.5	2.84	2.98	3	1.45
MS15-065	2.88	0.06	2.0	2.84	2.94	3	1.44
MS15-069	2.85	0.05	1.7	2.81	2.90	3	1.42
MS15-074	3.06	0.01	0.5	3.05	3.07	2	1.53
SC120	2.87	0.03	1.2	2.83	2.89	3	1.43
SC164	2.84	0.03	1.0	2.82	2.87	3	1.42
SC169	2.91	-	-	-	-	1	1.45
SC203	2.83	-	-	-	-	1	1.41
SC208	2.90	-	-	-	-	1	1.45
SC210	2.91	-	-	-	-	1	1.45
SC211	2.99	-	-	-	-	1	1.49
SC221	2.97	0.04	1.2	2.93	3.01	3	1.49
SC225	2.87	0.04	1.5	2.84	2.90	2	1.44
SC227	2.98	-	-	-	-	1	1.49
SC229	3.03	-	-	-	-	1	1.52
SC230	2.95	-	-	-	-	1	1.48
SC231	2.92	-	-	-	-	1	1.46
SC232	3.00	-	-	-	-	1	1.50
SC236	2.91	-	-	-	-	1	1.46
SC238	3.02	-	-	-	-	1	1.51
SC239	2.91	0.01	0.4	2.90	2.92	2	1.46
SC241	2.96	-	-	-	-	1	1.48
SC245	3.02	0.02	0.7	3.01	3.04	2	1.51
SC250	2.99	0.03	1.1	2.96	3.02	3	1.50
SC253	2.99	0.02	0.7	2.96	3.03	6	1.49
SC254	3.04	0.03	0.9	3.01	3.08	7	1.52
SC256	3.04	0.03	1.1	3.00	3.08	4	1.52
SC257	3.05	0.03	1.1	3.01	3.07	3	1.52
SC259	3.02	0.03	1.1	3.00	3.04	2	1.51
SC261	3.00	0.02	0.5	2.99	3.02	3	1.50
SC265	2.94	0.02	0.7	2.93	2.96	2	1.47
SC267	2.93	0.03	1.1	2.90	2.97	4	1.46
SC271	2.90	0.04	1.4	2.86	2.95	5	1.45
SC272	2.92	0.01	0.5	2.90	2.93	5	1.46
SC273	2.98	0.02	0.5	2.97	2.99	2	1.49
SC274	2.94	0.03	0.9	2.92	2.97	3	1.47
SC275	2.96	0.01	0.3	2.95	2.97	3	1.48
SC276	2.88	0.04	1.4	2.82	2.92	5	1.44
SC283	2.90	0.02	0.7	2.89	2.92	2	1.45
SC284	2.93	0.03	0.9	2.91	2.96	3	1.46
SC75	3.03	0.03	0.8	3.02	3.06	3	1.52

⇩Cont.

SC97	2.83	0.01	0.4	2.82	2.83	2	1.41
SC98	2.82	-	-	-	-	1	1.41
Tetraploids	**5.86**	**0.12**	**2.1**	**5.63**	**6.06**	**47**	**1.47**
AA004	5.97	0.06	1.0	5.93	6.01	2	1.49
JC011	6.29	-	-	-	-	1	1.51
MC274	6.10	0.07	1.1	6.03	6.16	1	1.48
MC286	6.23	0.10	1.6	6.12	6.31	1	1.49
MC323	5.91	-	-	-	-	3	1.46
MC235	5.95	-	-	-	-	1	1.49
MC386	5.97	-	-	-	-	1	1.51
MS14-057	6.24	-	-	-	-	1	1.49
MS14-060	5.85	0.07	1.2	5.78	5.93	1	1.43
MS15-043	6.22	-	-	-	-	3	1.48
MS15-113	6.02	-	-	-	-	1	1.51
MS15-118	5.97	-	-	-	-	3	1.41
MS15-119	5.72	-	-	-	-	3	1.43
MS15-123	6.08	0.20	3.4	5.80	6.29	2	1.44
MS15-124A	6.02	-	-	-	-	1	1.46
MS15-124B	5.65	0.03	0.6	5.63	5.69	2	1.42
RS001	5.70	0.07	1.2	5.63	5.74	3	1.46
SC131	5.78	0.09	1.5	5.71	5.84	3	1.49
SC203	5.82	-	-	-	-	5	1.43
SC230	5.68	0.02	0.4	5.67	5.70	1	1.49
SC263	5.86	0.06	1.1	5.78	5.90	3	1.48
SC266	5.97	0.07	1.2	5.89	6.03	2	1.49
SC269	5.72	0.09	1.5	5.65	5.87	1	1.52
SC285	6.19	-	-	-	-	1	1.51
SC299	6.29	-	-	-	-	1	1.47

PART II - Cytotype interactions and coexistence at contact zones

Chapter 4 – Complex cytogeographical patterns reveal a dynamic tetraploid-octoploid contact zone

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ABSTRACT

The distribution of cytotypes in mixed-ploidy species is crucial for evaluating ecological processes involved in the establishment and evolution of polyploid taxa. Here, we use flow cytometry and chromosome counts to explore cytotype diversity and distributions within a tetraploid-octoploid contact zone. We then use niche modeling and ploidy seed screening to assess the roles of niche differentiation among cytotypes and reproductive interactions, respectively, in promoting cytotype coexistence. Two cytotypes, tetraploids and octoploids, were dominant within the contact zone. They were most often distributed parapatrically or allopatrically, resulting in high geographic isolation. Still, 16.7% of localities comprised two or more cytotypes, including the intermediate hexaploid cytotype. Tetraploids and octoploids had high environmental niche overlap and associated with similar climatic environments, suggesting they have similar ecological requirements. Given the geographical separation and habitat similarity among cytotypes, mixed-ploidy populations may be transitional and subject to the forces of minority cytotype exclusion which lead to pure-ploidy populations. However, seed ploidy analysis suggests that strong reproductive barriers may enforce assortative mating which favors stable cytotype coexistence. High cytogenetic diversity detected in the field suggests that unreduced gamete formation and hybridization events seem frequent in the studied polyploid complex and might be involved with the recurrent polyploid formation, governing, as well, the gene flow between cytogenetic entities.

Keywords: Tetraploid, hexaploid, octoploid, contact zone, distribution patterns, hybridization, niche modeling, niche overlapping, *Gladiolus communis*.

INTRODUCTION

Polyploidization, duplication of complete chromosome sets, is widely considered an important mechanism of plant evolution (Soltis and Soltis 1999; Jiao *et al.* 2011) and sympatric speciation (Otto and Whitton 2000; Soltis *et al.* 2010). Based on recent molecular and fossil studies, polyploidy has been linked with radiations in species diversity throughout evolutionary history (Soltis *et al.* 2009) and associated with 15% of speciation events in extant angiosperms (Wood *et al.* 2009). Consequently, polyploidy is pervasive in flowering plants. The standing incidence of polyploid species is estimated at 35% (Wood *et al.* 2009), with higher values being observed in specific geographic regions such as the Mediterranean basin (ranging between 37 up to 47%; Marques *et al.* 2017) and the Arctic region (69 up to 87%; Brochmann *et al.* 2004).

The geographic distribution of polyploids is useful for inferring mechanisms of polyploid evolution, coexistence and divergence. The spatial arrangement of cytotypes *in situ* is the result of several interacting processes operating in natural populations including formation and migration; ecological preferences, and competitive and dispersal abilities; and reproductive interactions, among others (Levin 2002; Petit *et al.* 1999; Lexer and van Loo 2006). Cytotype distributions can be characterized as sympatric, parapatric or allopatric depending on whether the different cytotypes grow intermixed, adjacent or disjunct, respectively (Petit *et al.* 1999; and illustrated in Figure 2 of Mallet *et al.* 2009, which can be applied to polyploid complexes). Theoretical models predict that within zones of sympatry, mixed-ploidy populations are expected to be rare and evolutionarily unstable because frequency-dependent selection will drive the exclusion of the minority cytotype (Levin 1975; Rodriguez 1996a; Husband and Schemske 2000). Still, numerous studies have documented mixed-ploidy populations (reviewed in Husband *et al.* 2013; and examples below). The presence of multiple cytotypes in the same population can reflect either a transitory stage, in which neopolyploids are recurrently formed, or a persistent stage such as when cytotypes are ecologically and reproductively isolated on a small spatial scale (*e.g.*, Kolář *et al.* 2009; Jersáková *et al.* 2010). In this context, assessing the distribution of cytotypes within and among natural populations is crucial to build and test hypotheses that account for the successful establishment of polyploids.

Contact zones, areas with two or more cytotypes growing in close proximity, are thus considered natural laboratories within which to study evolutionary transitions through polyploidy. In recent years, an increasing number of studies have provided insights into ploidy-mediated processes occurring in contact zones (*e.g.*, Husband *et al.* 2013; Ramsey and Ramsey 2014). Significant advances in this field have been largely fueled by the ability to rapidly and

easily screen thousands of individuals using flow cytometry (Kron *et al.* 2007). This approach has resulted in a proliferation of cytogeographical studies (*e.g.*, Baack 2004; Kolář *et al.* 2009; Ståhlberg 2009; Trávníček *et al.* 2010; Castro *et al.* 2012; Zozomová-Lihová *et al.* 2015; Wefferling *et al.* 2017; reviewed in Ramsey and Ramsey 2014), which detect extensive cytogenetic diversity and, in several cases, occurrence of mixed-ploidy populations (*e.g.*, Baack 2004; Kolář *et al.* 2009; Trávníček *et al.* 2010; Castro *et al.* 2012; Zozomová-Lihová *et al.* 2015; Wefferling *et al.* 2017), rare cytotypes (*e.g.*, Kolář *et al.* 2009; Trávníček *et al.* 2010), production of unreduced gametes (*e.g.*, Maceira *et al.* 1992; Burton and Husband 2001; Ramsey 2007; Castro *et al.* 2016*a*) or recurrent occurrence of gene flow (*e.g.*, Husband 2004; Kolář *et al.* 2009; Castro *et al.* 2011). Particularly interesting are polyploid complexes with higher ploidies, such as diploid-hexaploid (*e.g.*, *Aster amellus*, Castro *et al.* 2012) or tetraploid-octoploid complexes (*e.g.*, *Gymnadenia conopsea*, Jersáková *et al.* 2010), that can produce even-ploidy hybrids, which are potentially more stable and lead to highly dynamic contact zones. Regardless of the increasing number of studies at contact zones, the available information is still scarce and insufficient for many plant groups and regions (Soltis *et al.* 2010, 2016; Marques *et al.* 2017).

Gladiolus communis L. (Iridaceae) is a Mediterranean polyploid complex with high morphological variation (Alonso and Crespo 2010). Multiple ploidy levels have been described for the complex, namely tetraploids (2n = 4*x* = 60 chromosomes; Fernandes *et al.* 1948; Fernandes 1950; Nilsson and Lassen 1971; Queirós 1980; Fernández and Pastor Días, 1985) and octoploids (2n = 8*x* = 120; Fernandes and Queirós 1971; Löve and Kjellqvist 1973; Queirós 1980), although hexaploids (2n = 6*x* = 90) and duodecaploids (2n = 12*x* = 180) have also been occasionally reported in the Mediterranean basin (Darlington and Whylie 1955). The Iberian Peninsula seems to harbor this diversity (Fernandes *et al.* 1948; Fernandes and Queirós 1971; Queirós 1979) and areas of close contact between tetraploids and octoploids have been detected, for example, in calcareous regions from Central Portugal (Castro *et al.* 2016*b*). Occasionally, *G. communis* grows with another congeneric species, namely *G. italicus*, which, in the Iberian Peninsula, is represented by duodecaploid individuals (Queirós 1979; Pérez and Pastor 1994; although octoploids have also been described in the Mediterranean basin, *e.g.*, Susnik and Lovka 1973; Strid and Franzen 1981; van Raamsdonk and de Vries 1989; Kamari *et al.* 2001). The high morphological variation of the group has led taxonomists to accept multiple taxonomic entities within the *G. communis* complex (*e.g.*, Gussone 1832; van Raamsdonk and de Vries 1989), although morphologically intermediate forms are found in natural populations, and many characters used to distinguish each taxon are extremely variable and largely overlap, even within populations (Hamilton 1980; revised in Alonso and Crespo 2010). Consequently,

recent morphological reviews and preliminary molecular analyses failed to support the previous taxonomic delimitations and the species is currently accepted as a complex formed by three ploidy levels (Buchanan 2008; Alonso and Crespo 2010). Regardless of the variability detected in the species, nothing is known about the role of genome duplications generating diversity within this polyploid complex. Exploring cytotype diversity and distribution patterns, especially at contact zones, is thus crucial to understand ecological processes, such as ecological preferences and reproductive interactions, driving current diversity patterns at natural contact zones.

In this study, we explore in detail the cytotype diversity and distribution patterns in a tetraploid-octoploid *G. communis* contact zone. In particular we pose the following specific questions: 1) what are the dominant cytotypes and how are they distributed in the contact zone? 2) Do cytotypes coexist and at which spatial scale? 3) Is coexistence facilitated by differences in environmental associations between cytotypes? And finally, 4) is there evidence for the production of unreduced gametes and/or cytotype hybridization? To address our questions, cytotype diversity was studied at several spatial scales, namely, 1) across the contact zone, to characterize the most dominant cytotypes and their environmental preferences within areas of contact; 2) within mixed-ploidy populations, to measure micro-habitat segregation; and 3) among offspring from plants in pure and mixed-ploidy populations, to detect cytotype diversity at early stages. Flow cytometric analyses complemented with chromosome counts were used to assess ploidy levels of all the sampled individuals. The reproductive success of pure and mixed-ploidy populations was also quantified in natural conditions to depict fitness differences between cytotypes. The spatial arrangement of cytotypes in the contact zone was analyzed with niche modeling tools to determine if differences in environmental requirements could explain cytotype distribution. If cytotypes differ in environmental requirements, we expect a mosaic contact zone with tetraploids and octoploids fairly isolated within a given spatial scale and with plants growing in different habitats or micro-habitats. If no environmental differences are observed, we expect a tension zone where sympatric cytotype co-occurrence is possible, where intermediate cytotypes are detected, and where other processes such as reproductive barriers, competition or dispersal abilities are expected to play major roles in driving distribution patterns.

METHODS

Study system and studied region

Gladiolus communis is a perennial species that is widespread on the Iberian Peninsula and throughout the Mediterranean basin. The species produces an ovoid bulb, relatively thick roots, a cylindrical glabrous stem, and linear leaves with typical parallel ribs. The pink bisexual flowers are zygomorphic and usually grouped in one spiked inflorescence per individual. A second *Gladiolus* species, *G. italicus*, is found on the Iberian Peninsula and occurs in sympatry with *G. communis* in some places. Although very similar morphologically, these two species are easily distinguished based on inflorescence architecture, anther and filament lengths, and seed morphology. *G. communis* has a unilateral inflorescence, anthers equaling or shorter than the filaments, and broadly winged seeds, while *G. italicus* usually has a weakly distichous inflorescence, anthers longer than the filaments, and polyhedric apterous seeds (Hamilton 1980; Alonso and Crespo 2010).

In the Iberian Peninsula, *G. communis* is recognized as a polyploid complex comprising tetraploids (2n = 4*x* = 60 chromosomes), hexaploids (2n = 6*x* = 90) and octoploids (2n = 8*x* = 120) (*e.g.*, Fernandes *et al.* 1948; Fernandes and Queirós 1971; Alonso and Crespo 2010) with duodecaploids being described elsewhere in the Mediterranean region (Darlington and Whylie 1955). The high morphological resemblance among *G. communis* cytotypes (Alonso and Crespo 2010; Cantor and Tolety 2011) suggest a putative autopolyploid origin. The species is common in the calcareous regions from Central Portugal, where preliminary field sampling revealed the presence of tetraploid and octoploid populations growing in close proximity. This study focused on this contact zone, an area extending from 39.3° to 40.6° in latitude, and from 7.8° to 9.4° in longitude. This territory is dominated by calcareous rocks and presents a Mediterranean climate that exhibits a strong influence from the Atlantic Ocean, an attribute identified on the significant values of annual precipitation (1000-1300 mm). However, the dominance of poor soils determines a low water storage capacity, which, combined with a long and hot summer, determines the dominance of evergreen vegetation. Allied to such climatic conditions, human pressure contributed to current dominance of shrubby communities in the landscape, and constrained forests (evergreen and semi-deciduous) to very small patches, favoring the wide presence of open habitats. These open habitats are also characterized by the presence of limestone outcrops exposed to stressful ecological conditions that limit the installation of higher vegetation covers.

Although not exhaustive, additional sampling was extended beyond this area to determine the dominant cytotypes within the species. Also, because *G. communis* coexist with *G. italicus*, hybridization might occur and generate additional cytogenetic diversity, the duodecaploid *G. italicus* (Queirós 1979; Pérez and Pastor 1994) was also sampled whenever growing with *G. communis*.

Field sampling

Field collections were carried out during the flowering and fruiting seasons (mid-April to July) of *G. communis* from 2012 to 2015. Individual plants or clusters of plants were easily detected when blooming because of the tall inflorescences growing above the remaining vegetation. We sampled 81 populations across the contact zone where both tetraploid and octoploid cytotypes have been previously detected in close proximity. An additional group of 27 populations covering the western distribution of the species around the contact zone were also sampled to depict the dominant cytotypes [Appendix 4.1]. In each of the 108 populations, we collected about 3 cm^2 of fresh leaf of up to 53 individuals of *G. communis* (with an average of 20 individuals per locality, excluding two particularly large localities where more intensive sampling was done, with 106 and 454 plants being screened), and of *G. italicus* whenever detected growing with *G. communis* (up to 32 individuals, averaging 13 plants per locality). The sampled individuals were randomly selected, covering the extension of the population. Leaves were stored in labeled hermetic bags and maintained at 4 °C for later flow cytometric analysis (see section *Genome size and ploidy level estimates*). Geographic coordinates of the population were recorded. Bulbs of nine localities identified in preliminary surveys as DNA tetraploid, DNA hexaploid and DNA octoploid populations (Castro *et al.* 2016*b*) were also collected, potted and maintained at the common garden for chromosome counts (see section *Chromosome counts*).

In addition, we sampled in mixed-ploidy populations more intensively to test for micro-habitat segregation. Three mixed-ploidy populations containing tetraploids, hexaploids and/or octoploids were revisited and all adult, individuals (both vegetative and reproductive individuals) were mapped with *x/y* coordinates, tagged and sampled for ploidy level analyses using flow cytometry (see section *Genome size and ploidy level estimates*). To delimit the clusters of plants growing in sympatry, screenings for *Gladiolus* plants were made around a radius of over 150 m around the cluster of plants initially detected or until an anthropogenic or natural barrier was observed. Additional mixed-ploidy populations were not sampled because they were disturbed by grazing or human activities.

Finally, we screened offspring from plants in pure and mixed-ploidy populations to examine the production of unreduced gametes and/or hybridization events by the detection of rare cytotypes that might not reach the adult stage. For this, four tetraploid, two hexaploid, four octoploid and one mixed tetraploid-octoploid populations were revisited and individual plants with known ploidy were sampled to determine reproductive success and screen ploidy of the seeds (see section *Reproductive success in natural populations*).

Chromosome counts

Chromosome counts were used to calibrate genome size estimates, obtained using flow cytometry, to a given ploidy level. For this, the plants grown from bulbs collected in the selected natural populations and maintained in the common garden were used simultaneously for genome size estimates and chromosome counting. For chromosome counts, we followed the protocol of Goldblatt and Takei (1993), with some adjustments. Briefly, actively growing root tips were harvested and pre-treated in 0.002M aqueous 8-hydroquinoline at room temperature for 4h30min, and fixed in 95% ethanol and glacial acetic acid (in a ratio of 3:1) for at least 48h at 4 °C. Roots tips were hydrolysed in 1M hydrogen chloride at 60 °C in a sand bath for 40 min, submerged in Schiff reagent (Greilhuber and Ebert 1994) for 1h30min, washed in sulphur water for three periods of 10 min and finally squashed under a glass cover in a drop of acetic orcein 2%. Chromosome spreads were observed using a Nikon Eclipse 80i light microscope and photographed using a Nikon Plan Apo VC 100×/1.40 oil-immersion lens with a Q Imaging Retiga 2000R Fast 1394 digital camera and Q-Capture Pro v.7 software. A total of 40 individuals from nine populations were used to access chromosome number and genome size: 4*x* – populations MC147 (N =10 individuals), MC193 (N =1), MC195 (N =4), MC201 (N =1), and MC212 (N =2); 6*x* – population MC211 (N =4); 8*x* – populations MC032 (N =8), MC143 (N =3), MC190 (N =4), MC193 (N =2), and MC201 (N =1) [see Appendix 4.1].

Genome size and DNA ploidy level estimates

To estimate genome size and DNA ploidy levels, fresh leaves collected in natural populations were analyzed using flow cytometry. Nuclear suspensions were prepared following Galbraith *et al.* (1983) by chopping the plant material of the sampled species together with leaf tissue of an internal reference standard. In the case of *Gladiolus* nuclear suspensions, 100 mg of leaf tissue or 2-5 seeds were co-chopped with 50 mg of leaf of *Solanum lycopersicum* 'Stupické'

(2C = 1.96 pg; Doležel *et al.* 1992) or *Pisum sativum* 'Ctirad' (2C = 9.09 pg; Doležel *et al.* 1998). *Solanum lycopersicum* was used as the internal standard in most cases, except when unavailable, with *P. sativum* being used in those situations. Sample and standard were co-chopped in 1 ml of WPB buffer (WPB: 0.2 M Tris-HCl, 4 mM $MgCl_2.6H_2O$, 1% Triton X-100, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM metabisulfite, 1% PVP-10, pH adjusted to 7.5 and stored at 4 °C, Loureiro *et al.* 2007) using a razor blade. The resulting nuclear suspension was filtered through a 50 µm nylon filter and 50 µg ml^{-1} propidium iodide (Fluka, Buchs, Switzerland) and 50 µg ml^{-1} RNAse (Fluka) were added to the sample, to stain the DNA and avoid staining of double-stranded RNA, respectively. After 5 min of incubation, DNA fluorescence of the sample was analyzed using a Partec CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW; Partec GmbH., Görlitz, Germany). Using Partec FloMax software v2.4d (Partec GmbH, Münster, Germany) the following four histograms were obtained: fluorescence pulse integral in linear scale (FL); forward light scatter (FS) vs. side light scatter (SS), both in logarithmic (log) scale; FL vs. time; and FL vs. SS in log scale [see Appendix 4.2]. To digitally remove some of the debris, the FL histogram was gated using a polygonal region defined in the FL vs. SS histogram [see R1 in Appendix 4.2] and was further applied to all the other graphics. At least 1,300 nuclei in both sample and standard G_1 peaks were analyzed per sample (Suda *et al.* 2007). Only CV values of G_1 peak of *G. communis* below 5% were considered acceptable [see examples in Appendix 4.2], otherwise a new sample was prepared and analyzed until this quality standard was achieved (Greilhuber *et al.* 2007).

Genome size was estimated in 41 populations by analyzing 3 plants per population and cytotype individually (rarely less, unless there were no more plants in the locality, while in a few populations up to 30 individuals were analyzed for genome size) [see Appendix 4.3]. For the remaining individuals and populations, only DNA ploidy level information was gathered following the pooled sample strategy (5–6 individuals plus the reference standard). A total of 108 natural populations of *G. communis* and 2,665 individuals were sampled and analyzed [see Appendix 4.1].

We used flow cytometry to measure DNA ploidy of offspring produced by plants of known ploidy. A total of 1,252 seeds from 178 individuals from four tetraploid, two hexaploid and four octoploid pure-ploidy populations and one tetraploid-octoploid mixed population were analyzed. We sampled 10 to 15 seeds per maternal individual, and 7 to 15 individuals per population and cytotype. For pure-ploidy populations of tetraploids and octoploids and mixed-ploidy population, 5 seeds were chopped simultaneously with the internal reference standard (pooled sample strategy) following the protocol described above, producing easy to interpret

histograms. When analyzing the seeds, at least two peaks (plus the peak of the internal standard) were always obtained, corresponding to the peak of the embryo and that of the endosperm. Consequently, the interpretation of each histogram was made with particular caution, determining the ploidy levels of all the peaks obtained in the histogram. Preliminary analyses revealed that hexaploid populations presented higher variability and thus only two seeds were pooled, in order to unambiguously assign the DNA ploidy levels of each seed.

The holoploid genome size (2C in pg; *sensu* Greilhuber *et al.* 2005) was obtained using the following formula:

$$\text{Holoploid genome size (pg)} = \frac{\textit{G. communis}\ \text{G1 peak mean}}{\text{reference standard G1 peak mean}} \times \text{reference standard genome size.}$$

Based on the chromosome counts obtained in this study and respective genome sizes, as well as the four chromosome numbers described in the literature for *G. communis* and *G. italicus*, DNA ploidy levels were inferred for each sample and individual. Populations were then characterized according to their DNA ploidy composition.

Descriptive statistics of holoploid genome size were calculated for each cytotype and species (mean, standard deviation of the mean, coefficient of variation of the mean, maximum and minimum values) based on the individual flow cytometric estimates. Mean and standard deviation of the mean were also calculated for the monoploid genome size (1Cx; holoploid genome size divided by inferred DNA ploidy level, *sensu* Greilhuber *et al.* 2005). Differences in holoploid and monoploid genome sizes among species and cytotypes were investigated using linear models (hereafter GLM) performed in R software version 3.0.1 (R Core Development Team, 2016), using the packages “car” for Type-III analysis of variance (Fox *et al.* 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).

The geographical isolation index (GI) between the two dominant cytotypes (*i.e.*, tetraploids and octoploids) at the contact zone was calculated according to the following formula (Husband *et al.* 2016), where only pure-ploidy and mixed-ploidy populations of tetraploids and octoploids from the contact zone were considered:

$$\text{GI} = 1 - \frac{\text{no. mixed-ploidy populations}}{\text{total no. populations}}$$

Environmental preferences

The environmental associations of the two dominant cytotypes were evaluated through GLM, and spatial predictive models were produced based on niche modeling tools, aiming to assess niche overlap. To explore niche overlapping, two approaches were used considering two different spatial scales: 1) one with an extension encompassing the contact zone in Central Portugal; and 2) the other extension encompassing the entire territory of mainland Portugal.

Variables were extracted from the following sources with a resolution of approximately 111 meters: 1) bioclimatological data from http://home.isa.utl.pt/~tmh/aboutme/Informacao_bioclimatologica.html (methodology to obtain variables in Monteiro-Henriques *et al.* 2016); and 2) data for soil conditions from: http://epic-webgis-portugal.isa.ulisboa.pt/. Values for climatic and soil variables were extracted for all the surveyed populations using the R package "dismo" (Hijmans *et al.* 2017). Then, GLMs were used to explore climatic and soil variables and assess differences between tetraploid and octoploid populations (Table 4.1), namely for climatic variables [mean annual total precipitation (*PP*), mean temperature of the hottest month of the year (*Tmax*), mean temperature of the coldest month of the year (*Tmin*), mean maximum temperature of the coldest month of the year (*M*), mean minimum temperature of the of the coldest month of the year (*m*)], bioclimatic indexes [continentality index (*IC*), compensated thermicity index (*ITC*), summer ombrothermic index (*Ios3*)], soil conditions [texture (*txt*) and pH] and altitude. Correlation between variables was explored using Pearson coefficient for continuous variables and Spearman's rho for categorical variables, to assist variable selection by removing variables with correlation values higher than 0.7. The final set of variables selected included the following four which were also important descriptors of the type of habitat where the species grows: mean annual total precipitation, mean temperature of the hottest month, soil texture and pH (highlighted in bold in Table 4.1).

Spatial predictive models were calibrated based on presence/absence records collected in the field and the selected environmental and soil variables (Table 4.1). For the tetraploid dataset, tetraploid populations were recorded as presences and octoploid populations as absences, and vice-versa for the octoploid dataset. Mixed-tetraploid-octoploid populations were considered as presences for both cytotypes. For the contact zone (Central Portugal) we used data from 76 sampling points (including 33 tetraploid, 40 octoploid and 3 tetraploid-octoploid populations), corresponding to all the known occurrences of *G. communis* with a minimum distance between populations of 600 meters. For the territory of Portugal, and aiming

Table 4.1. Characterization of the climatic and soil variables for tetraploid and octoploid populations of *Gladiolus communis* in the contact zone of Central Portugal. The mean, standard error of the mean (se) and statistical tests (comparison between cytotypes) are provided for each variable and cytotype. Significance levels: *** $P < 0.01$; * $0.05 < P < 0.01$; n.s., non-significant. In bold the variables used in niche modelling are highlighted.

Variables	CODE	Tetrapoid mean ± se, N = 43	Octoploid mean ± se, N = 36	ANOVA $F_{1,78}$ value
Precipitation	**PP**	1096.11 ± 21.97 [a]	1106.89 ± 21.74 [a]	0.12 n.s.
Mean temperature of the hottest month of the year	**Tmax**	20.51 ± 0.13 [a]	20.95 ± 0.19 [b]	4.04 *
Mean temperature of the coldest month of the year	Tmin	9.06 ± 0.10 [a]	8.91 ± 0.12 [a]	1.10 n.s.
Mean max. temp. of the coldest month of the year	M	13.52 ± 0.11 [a]	13.42 ± 0.12 [a]	0.38 n.s.
Mean min. temp. of the of the coldest month of the year	m	4.61 ± 0.08 [a]	4.50 ± 0.09 [a]	0.84 n.s.
Continentality index	IC	11.44 ± 0.13 [a]	12.04 ± 0.19 [b]	6.95 *
Compensated thermicity index	ITC	327.12 ± 2.78 [a]	325.81 ± 3.22 [a]	0.10 n.s.
Summer ombrothermic index	Ios3	1.10 ± 0.03 [a]	1.09 ± 0.03 [a]	0.05 n.s.
Soil texture	**Texture**	2.14 ± 0.29 [a]	2.25 ± 0.13 [a]	0.11 n.s.
Soil pH	**pH**	308.60 ± 46.51 [a]	99.44 ± 19.69 [b]	15.02 ***
Altitude	alt	198.61 ± 18.63 [a]	169.23 ± 17.38 [a]	0.94 n.s.
Latitude	Lat	-8.47 ± 0.05 [a]	-8.58 ± 0.03 [a]	3.14 ns
Longitude	Long	39.98 ± 0.05 [a]	40.01 ± 0.04 [a]	0.18 ns

Variables were extracted from the following sources with a resolution of approximately 111 meters: 1) bioclimatological data from http://home.isa.utl.pt/~tmh/aboutme/Informacao_bioclimatologica.html (methodology to obtain variables in Monteiro-Henriques *et al.* 2016); and 2) data for soil conditions from: http://epic-webgis-portugal.isa.ulisboa.pt/.

to reduce the bias effect of spatial clustering associated with our intense screening in the contact zone, only occurrences that had a minimum distance of 10 km between them were selected, based on radon selection, resulting in a subset of 66 sampling points (including 35 tetraploid, 19 octoploid and 6 tetraploid-octoploid populations).

Environmental Niche Modeling (ENM) of tetraploids and octoploids was created using R package "biomod2" (Thuiller *et al.* 2016). Final model for each cytotype is based on the combination of results from different modeling techniques, each one replicated thirty times after data splitting into training (70%) and testing (30%) subsets based on random selection, aiming to reduce uncertainty and to produce robust models (Phillips *et al.* 2006; Araújo and New 2007). In the resampling replication, each specific occurrence was used only once in each run, as training or as test without replacement, making all replicates statistically independent (Phillips 2008). Models were evaluated based on the independent accuracy measure AUC of ROC (Area Under the Curve of the Receiver Operating Characteristic), and only those with AUC > 0.7 where used in the ensemble forecasting procedure, the approach used to produce the final model for each cytotype.

Model evaluation revealed high ROC values (contact zone: 4*x* - 0.79 ± 0.01 and 8*x* - 0.79 ± 0.01; Portugal: 4*x* - 0.77 ± 0.01 and 8*x* - 0.76 ± 0.01) and relatively low omission rates (contact zone: 4*x* - 0.19 ± 0.02 and 8*x* - 0.28 ± 0.02; Portugal: 4*x* - 0.23 ± 0.01 and 8*x* - 0.28 ± 0.01). However, when considering the binary projections, the omission rates decrease to 0.10 and 0.09 for the tetraploid and octoploid models in the contact zone, respectively, and 0.17 and 0.04 in Portugal (tetraploids and octoploids, respectively), demonstrating that the models were able to predict the occurrences with high accuracy, namely for octoploids. The binary projection produced by the final model of each cytotype was used to calculate niche overlap.

Cytotype niche overlap was quantified through the metric of proportional similarity of the distribution of both cytotypes, using Schoener's *D* (a measure of niche similarity; Schoener 1970). This metric ranges from zero (no overlap) to one (complete overlap). The "ecospat" (Broennimann *et al.* 2012) and "raster" (Hijmans *et al.* 2017) packages were used to perform niche identity and similarity tests (Warren *et al.* 2008; Broennimann *et al.* 2012). In niche equivalency (identity test), the points of both cytotypes were pooled and randomly split in two groups according to size of the original dataset. This new dataset was used in *D* calculation, and the process was repeated 100 times (to obtain confidence intervals that enable evaluation of the null hypothesis). The resulting *D* values (simulated values) were compared with the observed *D* value, and cytotype niches were considered equivalent if the observed *D* value fell within the

95th percentile of the simulated *D* values (Broennimann *et al.* 2012). In niche similarity (similarity test), we evaluate if the environmental niches of the two cytotypes were distinguishable from each other. In this case, the comparison was between the points of one cytotype and random points from the geographic range of the other cytotype. As in the identity test, the process was repeated 100 times and *D* values were calculated. The results revealed if niche overlap between the cytotypes is greater (niche conservation) or lower (niche divergence) than expected, according to the geographic region of the other cytotype. All the models and analyses were performed in R software version 3.0.1 (R Development Core Team 2016).

Reproductive success in natural populations

The reproductive success of each cytotype was evaluated in 11 natural populations, namely 10 pure-ploidy populations (including four tetraploid, two hexaploid and four octoploid populations) and one mixed-ploidy population composed by tetraploid and octoploid individuals (MC201). In each population, 11 to 20 individuals of known ploidy level were labeled and infructescences collected in individually labeled bags. The number of fruits was counted for each inflorescence and fruit set calculated as the proportion of flowers that developed into fruit. The number of morphologically viable seeds (based in their size and shape) was assessed in all fruits, and the seed-ovule ratio (S:O ratio) was calculated by dividing the number of morphologically viable seeds by the number of ovules. The total reproductive success of populations and cytotypes was also calculated by multiplying the S:O ratio by the fruit set. Descriptive statistics were calculated for each population type.

Differences in fruit set, S:O ratio and total reproductive success between the three cytotypes (tetraploids, hexaploids and octoploids) within pure-ploidy populations, differences between tetraploids and octoploids in the mixed-ploidy population, and differences between pure- and mixed-ploidy populations (excluding hexaploid ones) were assessed using GLM. Mixed models with individual and population as random factors were initially used, but the random factors were further removed due to low variance in comparison with residuals (Bolker *et al.* 2009). A binomial distribution with a logit link function was used for fruit set, and a Gaussian distribution with an identity link function was used for S:O ratio and total reproductive success after transformation with the arcsine of the square root. When significant differences were obtained, post-hoc tests for multiple comparisons were performed.

All analyses were performed in R software version 3.0.1 (R Core Development Team 2016), using the packages “car” for Type-III analysis of variance (Fox and 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).

RESULTS

Genome size and cytogenetic diversity

Based on chromosome counts and flow cytometric analyses, we detected three ploidy levels in *G. communis*: tetraploids with 2n = 4*x* = 60 chromosomes (Figure 4.1A) and an average genome size of 2.69 ± 0.06 pg/2C (mean ± SD); hexaploids with 2n = 6*x* = 90 chromosomes (Figure 4.1B) and an average genome size of 4.07 ± 0.07 pg/2C; and octoploids with 2n = 8*x* = 120 chromosomes (Figure 4.1C) and an average genome size of 5.42 ± 0.14 pg/2C (Table 4.2; Figure 4.2A-B) [see Appendix 4.3].

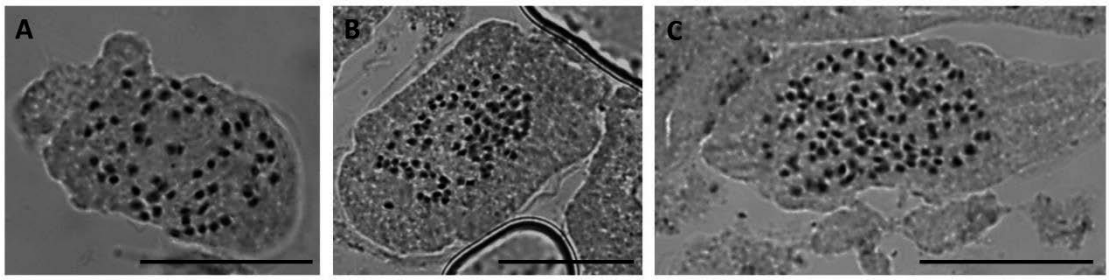


Figure 4.1. *Gladiolus communis* chromosome counts. A) tetraploid (2n = 4*x* = 60 chromosomes), B) hexaploid (2n = 6*x* = 90) and C) octoploid (2n = 8*x* = 120) individuals. Bar = 20 µm.

Genome size estimates also suggest the occurrence of nonaploid *G. communis* individuals, characterized by genome sizes with nine times the monoploid genome size (1C*x*) values obtained for the other ploidy levels, and had mean genome size of 6.10 ± 0.18 pg/2C (Table 4.2; Figure 4.2C). These individuals were rare, and we were unable to confirm their ploidy using chromosome counts. *Gladiolus italicus* had a higher genome size (2C = 7.27 ± 0.17 pg) than *G. communis*, consistent with duodecaploids, as described for the species (Table 4.2; Figure 4.2D). The holoploid genome sizes (2C) of the five cytotypes differed significantly ($F_{4,175}$ = 7691.3, P < 0.001; Table 4.2). Monoploid genome size values were conserved within *G. communis*, with no significant differences being observed between cytotypes ($F_{3,155}$ = 7691.3, P = 0.5272; Table 4.2). However, monoploid genome size of *G. communis* (0.67 ± 0.03 pg) was significantly higher than for *G. italicus* (0.61 ± 0.01 pg; $F_{1,178}$ = 7691.3, P < 0.001).

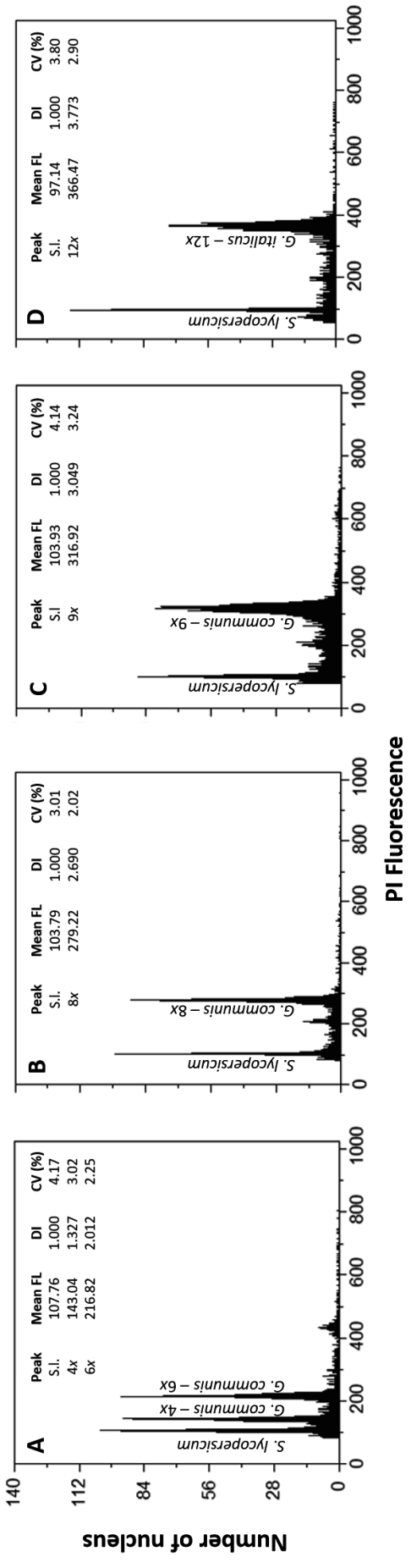


Figure 4.2. Flow cytometric histograms of relative propidium iodide fluorescence from nuclei isolated from fresh leaves of *Solanum lycopersicum* 'Stupické' (S.I.) and different cytotypes and/or species of *Gladiolus*: A) tetraploid (4*x*) and hexaploid (6*x*), B) octoploid (8*x*), and C) nonaploid (9*x*) individuals of *G. communis*, and D) duodecaploid (12*x*) individual of *G. italicus*.

Table 4.2. Genome size and DNA ploidy levels estimations for *Gladiolus communis* and *G. italicus*. Holoploid genome size (G.s.; 2C) is provided for each species and cytotype as mean and standard deviation (SD) in picograms (pg), followed by coefficient of variation (CV, %), DNA range (minimum, Min, and maximum, Max, genome size values); mean and standard deviation of the mean is also provided for the monoploid genome size (1C*x*); total number of individuals (N total) and populations (N pop) analyzed for genome size are also provided for each ploidy level. Chromosome numbers (Chr. number) for each species based on chromosome counts of this study and bibliographic records are also provided. DNA ploidy levels: tetraploid (4*x*), hexaploid (6*x*), octoploid (8x), nonaploid (9*x*) and duodecaploid (12*x*). Different letters denote statistically significant differences at $P < 0.05$.

Species	DNA ploidy level	Chr. number	Holoploid G.s. (2C, pg)					Monoploid G.s. (1C*x*, pg)		N total	N pop
			Mean	SD	CV (%)	Min	Max	Mean	SD		
G. communis	4*x*	60[1,2]	2.69 [a]	0.06	2.28	2.58	2.86	0.67 [a]	0.02	57	16
G. communis	6*x*	90[1,2]	4.07 [b]	0.07	1.76	3.93	4.19	0.68 [a]	0.01	9	3
G. communis	8*x*	120[1,2]	5.42 [c]	0.14	2.53	5.13	5.73	0.68 [a]	0.02	91	21
G. communis	9*x*	135[3]	6.10 [d]	0.18	2.89	5.98	6.23	0.68 [a]	0.02	2	1
G. italicus	12*x*	180[2]	7.27 [e]	0.17	2.31	6.97	7.55	0.61 [b]	0.01	21	10

[1] Chromosome numbers detected in this study; [2] Chromosome counts documented in the bibliography; [3] DNA ploidy level extrapolated based on the genome size values obtained here and on the chromosome counts available from other ploidy levels.

Geographic distribution of cytotypes

Tetraploids and octoploids were prevalent across the geographic area sampled, both occurring in pure- and in mixed-ploidy populations (Figure 4.3). No marked segregation pattern of cytotype arrangement in space was observed: tetraploids seem to occur across the entire area surveyed, and octoploids in the center and south of the surveyed area, forming broad contact zones.

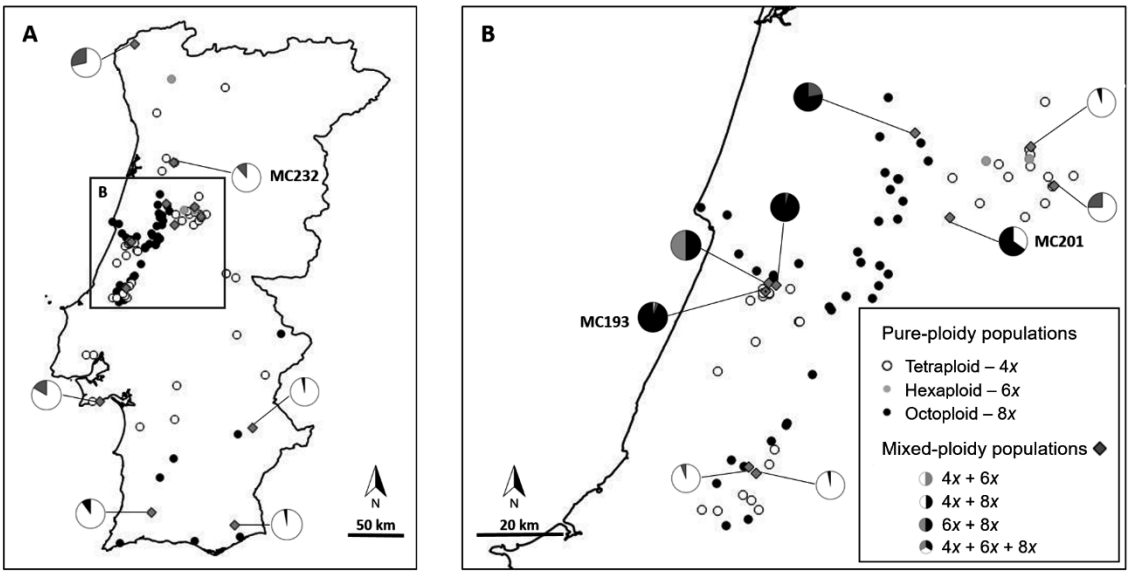


Figure 4.3. *Gladiolus communis* cytotype screening: A) all studied area (Portugal); and B) detail of the contact zone studied (Central Portugal). White, grey and black circles represent pure tetraploid, hexaploid and octoploid populations, respectively. Mixed-ploidy populations are represented by a grey diamond and each population is accompanied by a pie diagram reflecting cytotype composition. One sole population harbouring also two nonaploid individuals (not included in the pie diagram) is denoted by a dotted grey diamond, namely population MC193. Populations identified with ID code correspond to the populations where all the individual plants were sampled in detail (see Figure 4.4). DNA ploidy levels: tetraploid (4*x*), hexaploid (6*x*), octoploid (8*x*).

Minority cytotypes were also detected, namely hexaploids, which were observed growing with other cytotypes and occasionally found forming pure-populations (Figure 4.3). A few nonaploids in a mixed-ploidy population harboring all cytotypes of *G. communis* were also detected (Figure 4.3) [see Appendix 4.3]. Most populations were cytogenetically uniform (*i.e.*, pure-ploidy populations, 86.1%) and, in the majority of cases, were composed of either tetraploid or octoploid individuals (43.5% and 39.8%, respectively). Hexaploids were detected growing alone in three locations (2.8%) (Figure 4.3). Populations harboring two or more cytotypes (*i.e.*, mixed-ploidy populations) represented 13.9% of all sampled populations. The mixed-ploidy populations presented different cytotype compositions: tetraploids and hexaploids (4.6%), in which the former is more frequent than the latter; tetraploids and

octoploids (5.6%) again, in which tetraploids are generally more abundant than octoploids, except in one population; tetraploids, hexaploids, octoploids and nonaploids (0.9%; one population), where octoploids are the dominant cytotype; and hexaploids and octoploids (2.8%), in which octoploids are dominant, except in one location where only two plants, one of each cytotype, were found [see Appendix 4.4].

Within the contact area (Figure 4.3B), most localities contained a single ploidy of either tetraploids (42.0%), octoploids (44.4%), or rarely hexaploids (2.5%). These populations were distributed mostly in parapatry; still, cytotypes were found growing in sympatry in some locations (11.1%) (Figure 4.3B). Octoploid populations occur from north to south, resulting in cytogenetically diverse contact zones with tetraploids to the east, south and southwest. At these contact zones, areas with different types of mixed-ploidy populations were detected. Hexaploids were frequent in the contact zones between tetraploids and octoploids, although they were also detected in other places of the screened area, growing with tetraploid individuals. Tetraploids and octoploids, the two main cytotypes, were observed growing together in 4 locations out of the 81 populations at the contact zone (4.9%), resulting in a total GI of 0.95, with tetra- and octoploids presenting a similar individual geographical isolation index (GI_{4x} = 0.90, GI_{8x} = 0.91).

The detailed screening of three selected mixed-ploidy populations revealed variable patterns of cytotype distribution within each population (Figure 4.4).

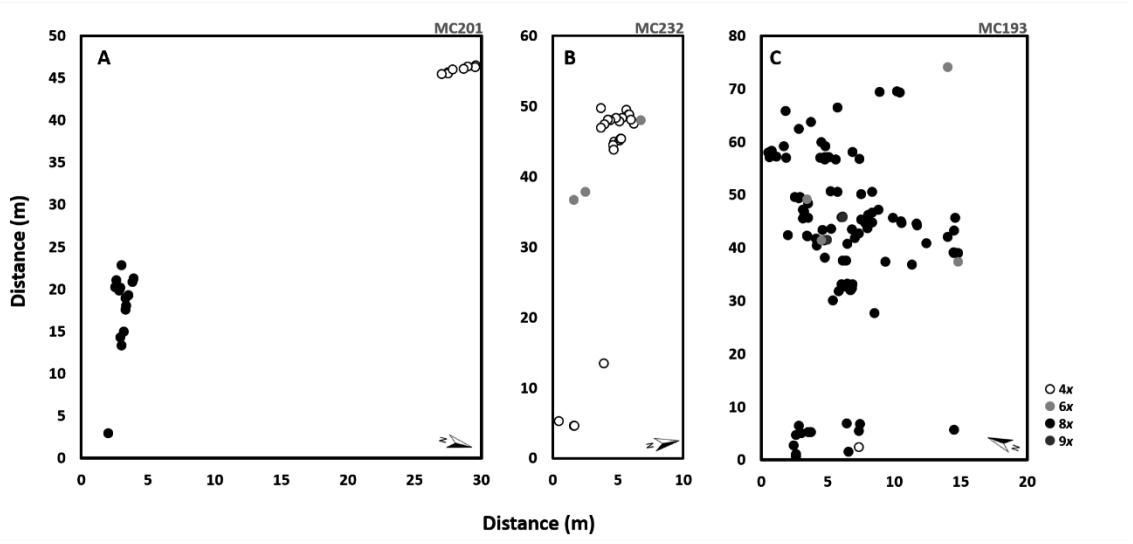


Figure 4.4. Fine-scale distribution of *Gladiolus communis* individuals within three mixed-ploidy populations: A) tetraploid and octoploid mixed-ploidy population (MC201), B) tetraploid and hexaploid mixed-ploidy population (MC232); and C) tetraploid, hexaploid, octoploid and nonaploid mixed-ploidy population (MC193). Each point represents one individual plant mapped in a *x/y* system where distance is given in meters (m): tetraploids (4*x*), hexaploids (6*x*), octoploids (8*x*) and nonaploids (9*x*) individuals are represent by white, grey, black and dark grey points, respectively.

In the tetraploid-octoploid population (MC201), cytotypes were distributed in two well-defined clusters separated by more than 20 m, with tetraploids being restricted to the northeast side and octoploids to the southwest of the population (Figure 4.4A). The mixed ploidy population with tetraploids and hexaploids (MC232) was dominated by tetraploid individuals, with a few hexaploids growing intermingled (Figure 4.4B). The population with the highest cytogenetic diversity (MC193) revealed to be dominated by octoploids individuals with a few tetraploid, hexaploid and nonaploid plants growing intermingled (Figure 4.4C). While MC201 and MC193 were located in the contact zones, MC232 is located in an otherwise tetraploid zone (Figure 4.3).

Environmental preferences

Niche geographic overlap between tetraploids and octoploids at both the contact zone (Schoener's *D* metric, *D* = 0.03) and Portugal (*D* = 0.01) was low (Table 4.3). However, and despite little geographical overlap, there was no statistical evidence that the environmental niches differed, *i.e.*, neither niche equivalency nor niche similarity were rejected (Table 4.3). This indicates that environmental niche of the dominant cytotypes was equivalent within the suitable ranges of both tetraploids and octoploids, and that environmental niche of each cytotype was similar to the suitable range of the other cytotype.

Table 4.3. Niche analyses in *Gladiolus communis*. For each region studied, equivalency (*D* and *P* values) and similarity (*P* value) tests for suitable habitat are given.

Suitable habitat	**Equivalence test**		**Similarity test (*P* values)**	
	D value	*P* value	Tetra → Octo	Octo → Tetra
Contact zone	0.034	0.960	0.406	0.337
Portugal	0.009	0.515	0.535	0.515

At the contact zone, the selected climatic and soil variables explained 62.98% of the variance in the distribution (Figure 4.5A), and a high environmental overlap of a given cytotype within the niche of the opposite cytotype was observed (74.87% and 61.95% for tetraploids and octoploids, respectively; Figure 4.5B). A similar pattern was observed for Portugal, although the climatic and soil variables explained higher variance than at the contact zone (74.78%; Figure 4.5C). A high environmental overlap between cytotypes was also observed (91.51% and 47.96% for tetraploids and octoploids, respectively; Figure 4.5D).

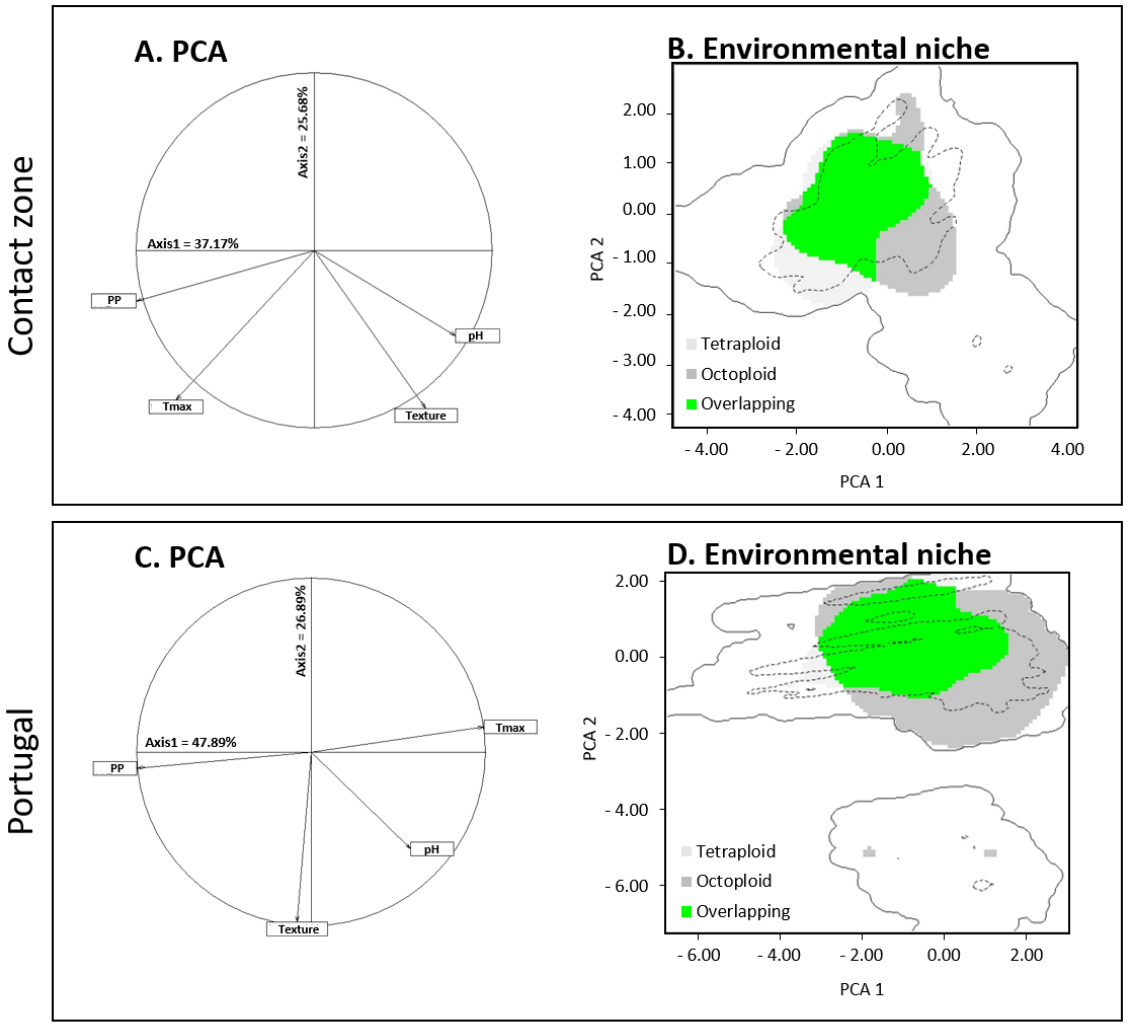


Figure 4.5. Results of ecological niche models for *Gladiolus communis* polyploid complex at A-B) the contact zone in Central Portugal, and C-D) Portugal. A) and C) represent the contribution of climatic and soil variables in the first two axes of the principal component analyses (PCA) and the percentage of variance explained by each axis. B) and C) represent the environmental niche of each cytotypes based on the PCA of selected variables; coloured areas represent suitable habitats as follows: light grey – tetraploids, dark grey – octoploids, and green – overlapping areas between tetraploids and octoploids; the continuous line corresponds to the whole climatic space, while the dashed line indicates the 75th percentile.

Reproductive success in natural populations and offspring cytogenetic composition

Plants in all the natural populations successfully formed fruits and seeds. However, the success differed according to the cytotype and population type. Pure-ploidy populations (excluding the hexaploid populations) had higher reproductive success compared to the mixed-ploidy population for all parameters (Fruit set: $F_{1,1033} = 15.51$, $P < 0.001$; S:O ratio: $F_{1,706} = 4.62$, $P = 0.032$; reproductive success: $F_{1,1033} = 21.04$, $P < 0.001$; Figure 4.6). Within pure-ploidy populations, significant differences between cytotypes were observed for all the variables (Fruit set: $F_{2,1087} = 4.96$, $P = 0.007$; S:O ratio:$F_{2,770} = 100.18$, $P < 0.001$; reproductive success: $F_{2,1087}$ =

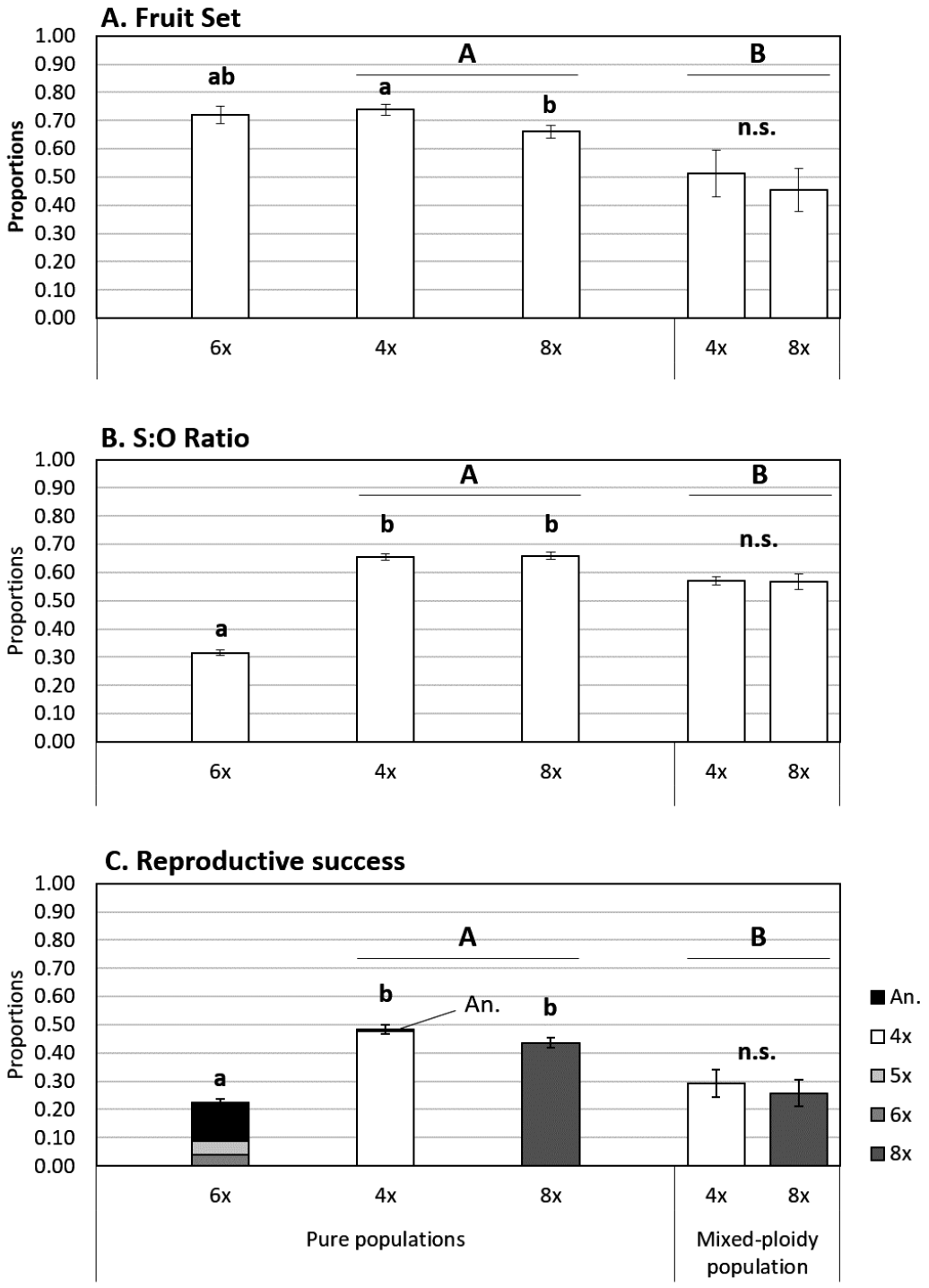


Figure 4.6. Reproductive fitness of natural pure- and mixed-ploidy populations of *Gladiolus communis*: A) fruit set; B) S:O ratio (number of viable seeds divided by the number of ovules); and C) reproductive success (fruit set multiplied by S:O ratio). In C) the proportion of DNA ploidy levels detected in the offspring is also given. DNA ploidy levels: tetraploid (4*x*), pentaploid (5*x*), hexaploid (6*x*), octoploid (8*x*); seeds with genome size values out of the range of variation of each ploidy levels were assumed as aneuploids (An.). Different letters correspond to statistically significant differences as follows: 1) differences between population type (pure- versus mixed-ploidy populations, excluding 6*x*) are denoted by upper case letters; and 2) differences between ploidy levels within population type (among 4*x*, 6*x* and 8*x* from pure-populations, and between 4*x* and 8*x* from the mixed-ploidy population) are denoted by lower case letters (Tukey HSD; $P < 0.05$); n.s correspond to non-significant differences ($P > 0.05$).

28.34, $P < 0.001$), with octoploids having lower fruit set than tetraploids. S:O ratio and reproductive success were similar in tetraploids and octoploids ($P > 0.05$), but significantly higher than for hexaploids ($P < 0.05$; Figure 4.6B-C). Within the mixed-ploidy population, no significant differences were observed between the cytotypes for any of the reproductive variables (Fruit set: $F_{1,79} = 0.27$, $P = 0.603$; S:O ratio: $F_{1,37} = 0.01$, $P = 0.934$; reproductive success: $F_{1,79} = 0.15$, $P = 0.698$).

The analyses of offspring ploidy revealed that tetraploid and octoploid individuals, in both pure-ploidy and mixed-ploidy populations, produced seeds with the same ploidy as the mother plants (Figure 4.6C). Tetraploid plants in pure populations produced a few aneuploids (<1% of the offspring; Figure 4.6C) [see Appendix 4.5]. In contrast, the flow cytometric analyses of the seeds from hexaploid individuals pointed out highly variable genome sizes, the analyses of the genome size estimates suggest the following DNA ploidy levels: 62% of seeds were aneuploid, 20% were pentaploids and 18% were hexaploid, although further confirmation is needed.

DISCUSSION

This study corroborates the existence of high cytogenetic diversity within the *G. communis* polyploid complex. Two dominant cytotypes, tetraploids and octoploids, were observed along with two minority cytotypes, mostly hexaploids, and rarely nonaploids. Tetraploids and octoploids have been well documented on the Iberian Peninsula through chromosome counts (Fernandes *et al.* 1948; Fernandes 1950; Fernandes and Queirós 1971; Nilsson and Lassen 1971; Löve and Kjellqvist 1973; Queirós 1980; Fernández and Pastor Días 1985). Also, hexaploids have been previously reported in the Mediterranean basin (Darlington and Whylie 1955). We observed them in 11% of the sampled localities (12 of 105 localities), commonly growing with one of the dominant cytotypes and occasionally in pure-ploidy populations. Nonaploids are reported here for the first time and were detected in the most diverse mixed-ploidy population.

Despite the cytogenetic diversity reported in *G. communis*, almost nothing was known about the geographic distribution of the cytotypes, or the presence and structure of its contact zones. Based on our survey, tetraploids occurred throughout the sampling area, although they were more common in the north and central regions of Portugal. Octoploids occurred in south and central regions of Portugal, but not in the north, notwithstanding the fact that more extensive surveys are needed to confirm this pattern. Although several mixed-ploidy

populations were found, the geographical isolation index between tetraploids and octoploids is high, reflecting the fact that most of the populations contain a single cytotype. These populations distribute in space allopatrically or parapatrically, forming several contact zones between tetraploids and octoploids. However, despite that tetraploids and octoploids have non-overlapping distributions, they can inhabit similar environmental niches. Niche identity and similarity tests showed that tetraploids and octoploids occupy similar niches and are not differentiated in their environmental niches, showing niche conservation. These results contrast with other polyploid complexes for which niche differentiation, driven either by the direct effects of polyploidy or by subsequent selection, underlies the spatial separation of cytotypes and allows them to escape the minority cytotype disadvantage (*e.g.*, Glennon *et al.* 2012; Thompson *et al.* 2014; Visger *et al.* 2016; Muñoz-Pajares *et al.* 2017). Still, the absence of environmental niche differences might not be completely unexpected as polyploids might not differ from their lower ploidy ancestors, either because they have been formed recently and the new polyploids did not have time to diverge from their progenitors, because genome duplications did not generate significant direct physiological changes, and/or because they might have been subjected to recurrent gene flow (Godsoe *et al.* 2013; Laport *et al.* 2016). Also, the effect of other environmental parameters on the distribution patterns observed in *G. communis* cannot be completely ruled out, nor the fact that niche differentiation might occur at a special resolution higher than that used in our study, although we did not find any clear evidence of differentiation in the field, namely considering the type of vegetation or the type of substrate in the mixed-ploidy populations detected (M. Castro, field observations).

Considering that *G. communis* cytotypes do not differ in suitable habitat, there should be historical processes and other ecological determinants shaping their distributional patterns, similarly to what has been observed in several polyploid complexes (*e.g.*, Baack 2004, 2005; Pannell *et al.* 2004; Baack and Stanton 2005; Godsoe *et al.* 2013; Münzbergová *et al.* 2013; Wefferling *et al.* 2017). Contact zones are generated by direct emergence of neopolyploids in lower ploidy parental populations or through secondary contact of previously allopatric distributions in which cytotypes colonized the area separately in dissimilar ways and at different timings (Petit *et al.* 1999; Lexer and van Loo 2006). Although we still do not know the origin of *G. communis* contact zones, the different cytotype compositions found in natural populations provide significant insights into the processes that might be occurring at these areas (*e.g.*, Husband and Schemske 1998; reviewed in Husband *et al.* 2013; Suda *et al.* 2013). One of the main observations is the fairly few mixed-ploidy populations (10 *versus* 90% of mixed- and pure-ploidy populations), all composed of unbalanced number of tetraploid and octoploid plants

(either dominated by tetraploid or by octoploids). In the absence of environmental differences, and regardless of the origin of the contact zone, *G. communis* mixed tetraploid-octoploid populations are expected to be more common at contact areas than detected here (4.9% in the contact zone and 6.5% from the total), since cytotypes might disperse to areas of the other cytotype and/or new cytotypes might be formed. Consequently, the high geographical isolation observed between *G. communis* cytotypes suggests that the mixed-ploidy populations might be transitory because strong frequency-dependent selection is expected to eliminate the minority cytotype as a result of fitness disadvantage generated by its lower number. This selection will ultimately drive the occurrence of pure-ploidy populations at contact zones (Levin 1975; Husband 2000).

However, tetraploid-octoploid populations may persist in nature. The regular production of unreduced gametes and the presence of reproductive barriers promoting assortative mating might lessen the magnitude of frequency-dependent selection and enable cytotype coexistence (*e.g.*, Felber 1991; Segraves and Thompson 1999; Husband 2004; Husband and Sabara 2004; Kennedy *et al.* 2006). Octoploids might emerge directly in tetraploid populations through the union of two unreduced gametes (n = 4*x*) or might result from seed dispersal from neighboring octoploid populations. Unreduced gamete production has been detected in controlled pollinations in tetraploid *G. communis* (Castro *et al.* in preparation) and in screenings in natural populations through the detection of hexaploid individuals (see below). The rates at which unreduced gametes are produced might feed the population of octoploids enabling their maintenance within tetraploid populations (Felber 1991; Husband 2004). Additionally, seed ploidy analyses in a tetraploid-octoploid population suggest that strong reproductive barriers may enforce assortative mating, further favoring cytotype coexistence. Reproductive barriers driven, for example, by phenological and/or morphological mismatch, different pollinator assemblages or preferences, and/or gametic isolation will, thus, play a major role for overcoming minority cytotype exclusion in mixed-ploidy populations. Therefore, the fate of octoploids might depend not only on the rates of unreduced gamete formation but also on the reproductive isolation levels. Additionally, differences in other traits, such as perenniality or asexual reproduction, could compensate for the minority cytotype disadvantage (*e.g.*, Rodriguez 1996; Kao 2007; Castro *et al.* 2016*a*). In other polyploid complexes, traits such as the production of bulbs represented an advantage, enabling new cytotypes to persist at initial stages and spread within lower ploidy populations (*e.g.*, *Allium oleraceum*, Duchoslav *et al.* 2010; *G.* × *sulistrovicus*, Szczepaniak *et al.* 2016). If, through some of these traits, the number of octoploids can surpass the number of tetraploids, at some time octoploids might even outcompete tetraploids and

exclude them from the population, as observed in other polyploid complexes (*e.g.*, Buggs and Pannell 2007). Indeed, octoploids were observed as the dominant cytotype in some mixed-ploidy populations of the contact zone. Future studies on the contribution of all the above-mentioned processes, and on the relative contribution of sexual *versus* asexual reproduction for the maintenance of the populations, are needed to fully understand the dynamics of mixed-ploidy populations.

The cytotype composition of *G. communis* natural populations also revealed that hexaploid plants might be more common than previously thought. These hexaploids might have originated through two different pathways. Hexaploids may originate from tetraploids through the union of reduced (n = 2*x*) and unreduced (n = 4*x*) gametes (Ramsey and Schemske 1998). Indeed, unreduced gamete formation is an important pathway for new polyploid emergence and has been shown to be common in nature (Felber 1991; Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Husband 2004; Ramsey 2007). This might explain the detection of hexaploid plants frequently found in otherwise tetraploid populations. Alternatively, hexaploids may form as a result of hybridization events between tetraploid and octoploid *G. communis* individuals. *Gladiolus communis* is pollinated by generalist pollinators that seem to have no cytotype preferences and might move pollen within mixed-ploidy populations or between populations in close proximity (Chapter 5). Additionally, controlled pollinations between tetraploid and octoploid plants were also successful in producing hexaploid offspring (Chapter 5). Either one of these pathways, *i.e.*, unreduced gamete formation or hybridization, may operate in natural populations, being difficult to distinguish them without genetic markers. However, the relative abundance of tetraploid-hexaploid populations and paucity of tetraploid-hexaploid-octoploid populations suggests that the majority of the hexaploids are formed through unreduced gametes in tetraploid populations. Additionally, unreduced gamete production has been frequently detected in controlled pollinations involving tetraploid *G. communis* (Chapter 5), supporting it as a probable pathway for new cytotype emergence in natural populations. Quantifying unreduced gamete production in natural populations will provide significant insights on how frequent this process could be involved with hexaploid emergence.

Interestingly, hexaploid individuals were also found forming pure-ploidy populations, showing that this cytotype can successfully establish and spread beyond parental populations, although their sexual reproductive fitness was revealed to be lower in comparison with tetraploids and octoploids. Regardless of the lower fitness, recurrent unreduced gamete formation and asexual reproduction might enable to compensate for this disadvantage (*e.g.*,

Husband 2004; Kao 2007; Castro *et al.* 2016*a*). The successful establishment of hexaploid plants further contributes to the diversification of the complex. Ultimately, contact zones result from the combination of several factors, including historical factors, unreduced gamete formation, pollen flow and hybridization events, and seed dispersal, among others (Petit *et al.* 1999; Levin 2002; Lexer and van Loo 2006). Future studies reconstructing the history of the complex and quantifying unreduced gamete production, and its ability to hybridize, would provide significant insights on the dynamics of the distribution of *G. communis*.

The genome size of *G. italicus* suggests that this species is duodecaploid in the studied area, which is in accordance with chromosome counts for the Iberian Peninsula (Queirós 1979; Pérez and Pastor 1994), and contrasts with the dominance of the octoploids elsewhere in the Mediterranean basin (Susnik and Lovka 1973; Strid and Franzen 1981; van Raamsdonk and de Vries 1989; Kamari *et al.* 2001). Interestingly, the variation in monoploid genome size within *G. communis* cytotypes was very low and differed significantly from that of *G. italicus* (about 9%). Given the magnitude of the differences between *G. italicus* and *G. communis*, both in ploidy levels and in monoploid genome sizes, holoploid genome size might be an important tool to detect hybridization (*e.g.*, Kolář *et al.* 2009; Agudo 2017). In our study, *G. italicus* and *G. communis* were found growing in sympatry in 13% of localities; however, all the *G. italicus* individuals were duodecaploid. In most of the cases, the duodecaploid *G. italicus* was found growing with the octoploid *G. communis* (12 out of 14 localities); still, no decaploids were observed in these localities. When growing with the tetraploid *G. communis,* no octoploid individuals with lower genome size resulting from the hybridization between the two species (~5.00 pg based on the monoploid genome sizes of each species) were observed. Although hybridization has been suggested to occur in these and in other *Gladiolus* species (*e.g.*, van Raamsdonk and de Vries 1989; Mifsud and Hamilton 2013; Szczepaniak *et al.* 2016), we were not able to detect hybrids between *G. italicus* and *G. communis*. This suggests that, in the studied range, hybridization between them might be less common, either because of assortative mating or hybrid offspring inviability. Monoploid genome size also suggests a close relationship between the cytotypes of *G. communis*, pointing to an autopolyploid origin of the complex in the studied area. This is also supported by the high morphological resemblance between *G. communis* cytotypes (Alonso and Crespo 2010; Cantor and Tolety 2011) and by the lack of evidence supporting hybridization between *G. communis* and *G. italicus* in this region. Still, the origin of *G. communis* polyploid complex needs to be properly evaluated in future studies.

CONCLUSIONS

In this study we find a complex cytogeographical pattern in *G. communis*, which opens several hypotheses that might explain the formation and maintenance of its tetraploid-octoploid contact zone. According to our results, tetraploids and octoploids do not differ in their environmental requirements, potentially growing in similar habitats. Without differences in habitat requirements, mixed-ploidy populations were expected to be frequent; however, a high geographical isolation index was obtained. The high geographical isolation observed in nature, along with habitat similarity, suggests that the cytotype distribution in *G. communis* reflects historical patterns of migration and colonization, and further selection against minority cytotype, and does not result from different environmental requirements, creating a tension zone of contact. Still, in areas of contact, reproductive barriers might mediate assortative mating and enable cytotype coexistence. Nevertheless, the high cytogenetic diversity detected in the field suggests that unreduced gamete formation and hybridization events seem frequent in this complex and might be involved with recurrent polyploid formation and with gene flow between cytogenetic entities. Future studies involving reciprocal transplants will provide significant insights into the dynamics of this polyploid complex.

APPENDICES

Appendix 4.1. Geographic information of sampled *Gladiolus* populations. ID code, DNA ploidy level, sample size and information about the location and geographical coordinates (angular) are given for each population. ID codes in bold represent populations with *Gladiolus communis* (Gc) and *Gladiolus italicus* (Gi) growing together. *Gladiolus communis* presented four cytotypes (4*x* – tetraploids, 6*x* – hexaploids, 8*x* – octoploids and 9*x* – nonaploids), while *G. italicus* presented only duodecaploid (12*x*) individuals. Populations marked with * represent populations used in chromosome counts.

ID code	DNA Ploidy level (n)		Location	Geographical coordinates	
	Gc	Gi		Latitude	Longitude
MC153	4*x* (44)		Valverde, Santarém, Portugal	39.45794	-8.85369
MC034	4*x* (26)		Amiais de Cima, Santarém, Portugal	39.46061	-8.75144
MC037	4*x* (30)		Casais Monizes, Alcobaça, Portugal	39.46080	-8.89088
MC150	4*x* (31)		Fonteinhas, Porto de Mós, Portugal	39.48230	-8.77042
MC149	4*x* (35)		Cabeça das Pombas, Porto de Mós, Portugal	39.49715	-8.79206
MC147 *	4*x* (42)		Casal Duro, Fátima, Portugal	39.57156	-8.72667
MC146	4*x* (3)	12*x* (2)	Casal do Suão, Fátima, Portugal	39.60689	-8.71474
DT11	4*x* (2)		Vila Velha de Rodão, Castelo Branco	39.65356	-7.69009
DT10	4*x* (2)		Idanha-a-Nova, Castelo Branco, Portugal	39.69477	-7.78098
MC222	4*x* (34)		Vale da Mua, Castelo Branco, Portugal	39.69477	-7.78098
SC037	4*x* (13)		Barreiros, Leiria, Portugal	39.79640	-8.85395
LM009	4*x* (24)		Valeirão, Pombal, Portugal	39.86840	-8.76110
LM001	4*x* (24)		Casal Fernão João, Pombal, Portugal	39.91580	-8.65211
LM006	4*x* (17)		Casal Fernão João, Pombal, Portugal	39.91789	-8.65620
SC043	4*x* (35)		Antões, Pombal, Portugal	39.96736	-8.77196
MC234	4*x* (30)		Moita do Boi, Pombal, Portugal	39.97942	-8.74266

⇩Cont.

MC194	4*x* (32)		Castelhanas, Soure, Portugal	39.98274	-8.72756
MC195 *	4*x* (39)		Castelhanas, Soure, Portugal	39.98665	-8.72668
SC044	4*x* (5)		Castelhanas, Soure, Portugal	39.98744	-8.74219
MC197	4*x* (41)		Vascos, Soure, Portugal	39.99569	-8.67563
MC192	4*x* (11)		Casal de Santo António, Soure, Portugal	39.99724	-8.74108
MC203	4*x* (21)		Regateira, Góis, Portugal	40.17067	-8.10327
MC202	4*x* (11)		Vale do Gueiro, Vila Nova de Poiares, Portugal	40.20516	-8.21416
MC204	4*x* (21)		Casal de S. José, Arganil, Portugal	40.20526	-8.05387
SC055	4*x* (35)		Secarias, Arganil, Portugal	40.24508	-8.03521
JC001	4*x* (6)		Penacova, Penacova, Portugal	40.26659	-8.28116
MC206	4*x* (22)		Vale do Matouco, Arganil, Portugal	40.26881	-8.14510
MC205	4*x* (31)		Pousadouros, Tábua, Portugal	40.26896	-8.04568
SC054	4*x* (13)		Coja, Arganil, Portugal	40.27031	-7.98444
MC212 *	4*x* (45)		Carapinha, Tábua, Portugal	40.29412	-8.09082
JC009	4*x* (6)		São Pedro De Alva, Coimbra, Portugal	40.30049	-8.16638
MC210	4*x* (8)		Covelo de Baixo, Tábua, Portugal	40.32391	-8.09137
MC209	4*x* (22)		Ázere, Tábua, Portugal	40.33529	-8.09207
MC208	4*x* (5)		Nagozela, Santa Comba Dão, Portugal	40.45014	-8.05349
MC187	6*x* (29)		Carvalhal, Penacova, Portugal	40.30821	-8.19913
MC211 *	6*x* (26)		Covelo de Baixo, Tábua, Portugal	40.3136	-8.09344
MC152	8*x* (4)	12*x* (32)	Feiteira, Santarém, Portugal	39.42256	-8.83183
MC151	8*x* (4)	12*x* (23)	Coutada de Cima, Santarém, Portugal	39.43799	-8.77790
MC154	8*x* (25)	12*x* (13)	Casais do Chão de Mendiga, Porto de Mós, Portugal	39.52468	-8.85429
MC032 *	8*x* (30)		Alcaria, Fátima, Portugal	39.56421	-8.78816

⇩Cont.

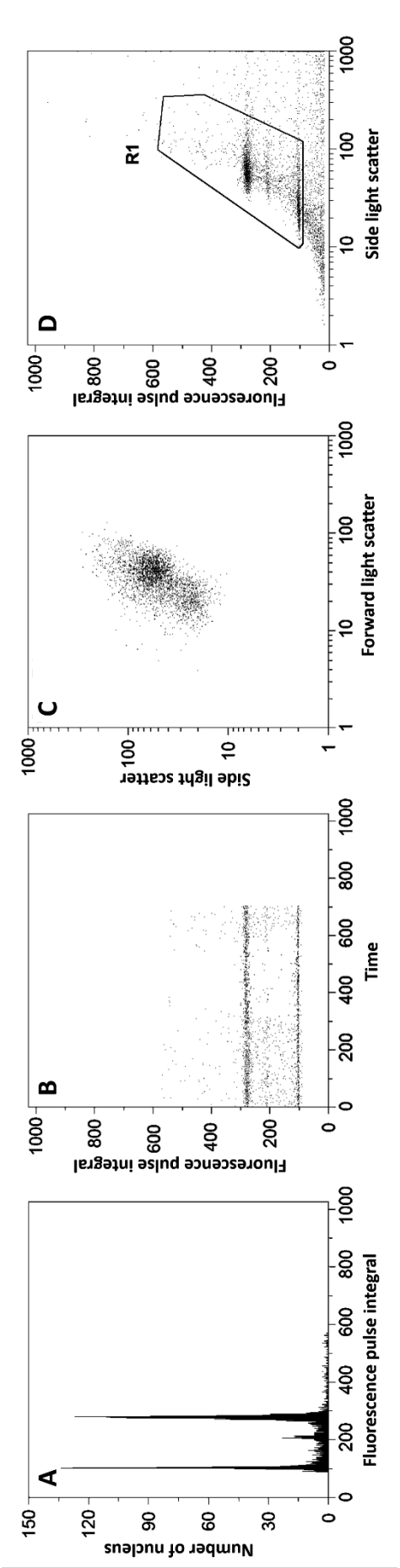
MC155	8*x* (9)	12*x* (22)	Pragais, Porto de Mós, Portugal	39.57958	-8.81942
MC145	8*x* (2)	12*x* (6)	Milheirice, Fátima, Portugal	39.62782	-8.72085
MC395	8*x* (24)		Santa Catarina da Serra, Fátima, Portugal	39.66640	-8.68534
MC027	8*x* (21)		Santa Catarina da Serra, Fátima, Portugal	39.67179	-8.68210
MC025	8*x* (3)		Ruge Água, Leiria, Portugal	39.78900	-8.62085
MC199	8*x* (36)		Vérigo, Soure, Portugal	39.94442	-8.57491
MC198	8*x* (3)		Salgueiro, Soure, Portugal	39.95214	-8.58133
MC200	8*x* (5)		Outeiro, Soure, Portugal	39.97424	-8.47408
SC158	8*x* (5)		Vale de Poios, Pombal, Portugal	39.97909	-8.53983
MC200A	8*x* (8)		Ribeira de Alcalamouque, Soure, Portugal	40.00444	-8.45882
MC190 *	8*x* (53)		Casal da Rola, Soure, Portugal	40.02146	-8.71541
SC045	8*x* (19)		Casal da Rola de Cima, Soure, Portugal	40.02876	-8.71737
MC015	8*x* (28)	12*x* (2)	Rabaçal, Penela, Portugal	40.03225	-8.43642
MC370	8*x* (4)		Cavadas, Pombal, Portugal	40.04072	-8.75673
MC013	8*x* (30)		Zambujal, Condeixa-a-Nova, Portugal	40.05251	-8.45642
SC050	8*x* (27)		Camparca, Soure, Portugal	40.05894	-8.65606
MC011	8*x* (24)		Casmilo, Condeixa-a-Nova, Portugal	40.06095	-8.50322
MC374	8*x* (5)		Vales, Figueira da Foz, Portugal	40.08155	-8.80047
MC006	8*x* (13)		Arrifana, Condeixa-a-Nova, Portugal	40.08765	-8.51106
MC157	8*x* (3)	12*x* (27)	Antanhol, Coimbra, Portugal	40.1619	-8.46086
JC008	8*x* (9)		Marcos dos Pereiros, Coimbra, Portugal	40.16665	-8.41494
SC002	8*x* (30)		Ferrugenta, Figueira da Foz, Portugal	40.17705	-8.82848
SC033	8*x* (23)		Serra da Boa Viagem, Figueira da Foz, Portugal	40.19532	-8.89348
MC144	8*x* (43)		Santo António dos Olivais, Coimbra, Portugal	40.20853	-8.40075

⇩Cont.

MC143 *	8*x* (38)		Relvinha, Coimbra, Portugal	40.23889	-8.43151
MC142	8*x* (41)		Lôgo de Deus, Coimbra, Portugal	40.26295	-8.41314
SC001	8*x* (29)		Brasfemes, Coimbra, Portugal	40.26413	-8.40991
MC182	8*x* (30)		Trouxemil, Coimbra, Portugal	40.27786	-8.44533
MC186	8*x* (23)		Telhado, Penacova, Portugal	40.30728	-8.33973
MC188	8*x* (30)		Louredo, Penacova, Portugal	40.35184	-8.35776
MC181	8*x* (30)		Mealhada, Coimbra, Portugal	40.36726	-8.45775
MC189	8*x* (6)		Anadia, Aveiro, Portugal	40.46232	-8.43689
MC213	4*x* (16), 6*x* (1)		Alcaria, Fátima, Portugal	39.5637	-8.77687
JMC001	4*x* (6), 6*x* (2)		Secarias, Arganil, Portugal	40.24689	-8.03339
MC148	4*x* (453), 8*x* (1)	12*x* (3)	Carrascos, Fátima, Portugal	39.54983	-8.75783
MC201 *	4*x* (8), 8*x* (15)		Covelos, Coimbra, Portugal	40.17012	-8.28642
MC207	4*x* (18), 8*x* (1)		Ázere, Tábua, Portugal	40.34179	-8.08996
MC196	6*x* (1), 8*x* (20)		Borda do Rio, Soure, Portugal	40.00512	-8.70998
MC191	6*x* (1), 8*x* (1)		Louriçal, Soure, Portugal	40.00975	-8.72784
JC010	6*x* (4), 8*x* (14)		Luso, Aveiro, Portugal	40.37578	-8.37109
MC193 *	4*x* (1), 6*x* (4), 8*x* (99), 9*x* (2)		Casal de Santo António, Soure, Portugal	39.98978	-8.73567
MC159	4*x* (28)		Grândola, Grândola, Portugal	38.20238	-8.62507
MC050	4*x* (2)		Algalé, Alcácer do Sal, Portugal	38.27983	-8.29287
MC046	4*x* (13)		Santana, Sesimbra, Portugal	38.45344	-9.09083
AM001	4*x* (16)		Paião, Montemor-o-Novo, Portugal	38.60961	-8.26568
MC178	4*x* (33)		Alandroal, Alandroal, Portugal	38.7058	-7.40776
MC041	4*x* (30)		Freixial, Loures, Portugal	38.90263	-9.15484

⇩Cont.

MC039	4*x* (24)		Romão Charneca, Loures, Portugal	38.90346	-9.08455
MC180	4*x* (35)		Vale da Seda, Fronteira, Portugal	39.09706	-7.68025
MC158	4*x* (37)		Foz, Sever do Vouga, Portugal	40.69068	-8.41344
MC250	4*x* (7)		Parada, Sever do Vouga, Portugal	40.77010	-8.2939
MC302	4*x* (4)		Paredes, Vale de Cambra, Portugal	40.81631	-8.37471
MC236	4*x* (11)		Agrela, Paços de Ferreira, Portugal	41.2562	-8.46647
MC242	4*x* (3)		Santa Eulália, Vila Pouca de Aguiar, Portugal	41.50069	-7.79299
MC240	6*x* (3)		Póvoa de Lenhoso, Braga, Portugal	41.58447	-8.32197
MC168	8*x* (9)	12*x* (24)	Budens, Vila do Bispo, Portugal	37.08206	-8.82402
MC169	8*x* (30)		Vila Moura, Faro, Portugal	37.09428	-8.09808
MC172	8*x* (2)	12*x* (15)	Tavira, Faro, Portugal	37.13949	-7.65385
MC162	8*x* (30)	12*x* (1)	Colos, Ourique, Portugal	37.71288	-8.43586
CS001	8*x* (3)	12*x* (3)	Pinheiro, Aljustrel, Portugal	37.89733	-8.29525
MC175	8*x* (1)	12*x* (7)	Peso, Vidigueira, Portugal	38.13894	-7.67163
MC223	8*x* (3)		Arronches, Portalegre, Portugal	39.11088	-7.25028
MCD001	4*x* (5), 6*x* (1)		Fojo dos Morcegos, Arrábida, Portugal	38.45607	-9.01801
MC232	4*x* (23), 6*x* (3)		Parada, Sever do Vouga, Portugal	40.77817	-8.29725
MC173	4*x* (33), 8*x* (1)		Borracheira, Tavira, Portugal	37.24766	-7.69962
MC164	4*x* (27), 8*x* (3)		Carvalho, Monchique, Portugal	37.37825	-8.5119
MC176	4*x* (31), 8*x* (1)		Alqueva, Portel, Portugal	38.19684	-7.53172
MC238	4*x* (5), 6*x* (2)		Chão, Vila Nova de Cerveira, Portugal	41.92757	-8.68125



Appendix 4.2. Flow cytometry graphics analyzed for each sample: A) fluorescence pulse integral in linear scale (FL); B) FL vs. time; C) forward light scatter (FS) vs. side light scatter (SS), both in logarithmic (log) scale; and D) FL vs. SS in log scale. R1 in D) presents a polygonal region that was applied to all the other graphics to digitally remove some of the debris.

Appendix 4.3. Genome size variation in *Gladiolus communis*. For each population and DNA ploidy level, the following values are given for the holoploid genome size (2C/pg): mean, standard deviation of the mean (SD), coefficient of variation (CV, %), minimum (Min) and maximum (Max). Sample size, Monoploid genome size (1C*x*/pg) and reference standard used in the analyses [*Solanum lycopersicum* (*S.l.*) *and Pisum sativum* (*P.s.*)] are also given. Genome values are presente in picograms (pg). DNA ploidy levels: tetraploid (4*x*), hexaploid (6*x*), octoploid (8*x*) and nonaploid (9*x*).

ID code	DNA Ploidy level	Holoploid genome size (2C)						Monoploid genome size (1C*x*)	Reference standard
		Mean	SD	CV (%)	Min	Max	N		
AM001	4*x*	2.73	0.05	1.81	2.78	2.65	6	0.68	*S.l.*
CS001	8*x*	5.41	-	-	-	-	1	0.68	*S.l.*
DT010	4*x*	2.78	0.09	3.13	2.79	2.63	3	0.68	*S.l.*
DT011	4*x*	2.66	0.02	0.70	2.69	2.65	3	0.67	*S.l.*
MC006	8*x*	5.40	0.11	1.96	5.44	5.24	3	0.66	*S.l.*
MC011	8*x*	5.34	0.15	2.75	5.51	5.24	3	0.67	*S.l.*
MC013	8*x*	5.30	0.03	0.62	5.33	5.27	3	0.66	*S.l.*
MC015	8*x*	5.34	0.04	0.70	5.36	5.31	2	0.64	*S.l.*
MC025	8*x*	5.31	0.03	0.01	5.34	5.27	3	0.66	*S.l.*
MC027	8*x*	5.45	0.10	0.02	5.54	5.35	3	0.68	*S.l.*
MC032	8*x*	5.57	0.09	0.02	5.62	5.47	3	0.70	*S.l.*
MC034	4*x*	2.68	0.03	0.01	2.70	2.64	3	0.67	*S.l.*
MC037	4*x*	2.68	0.01	0.00	2.69	2.67	3	0.67	*S.l.*
MC039	4*x*	2.69	0.03	0.01	2.72	2.67	3	0.67	*S.l.*
MC041	4*x*	2.66	0.02	0.01	2.69	2.64	3	0.67	*S.l.*
MC046	4*x*	2.74	0.04	0.02	2.78	2.70	3	0.68	*S.l.*
MC050	4*x*	2.74	0.03	0.01	2.76	2.71	2	0.68	*S.l.*

⇩Cont.

MC144	8*x*	5.21	-	-	-	-	1	0.65	*S.l.*
MC147	4*x*	2.67	-	-	-	-	1	0.67	*S.l.*
MC148	4*x*	2.66	0.06	2.14	2.76	2.62	5	0.66	*S.l.*
	8*x*	5.30	-	-	-	-	1	0.66	*S.l.*
MC151	8*x*	5.30	-	-	-	-	1	0.66	*S.l.*
MC152	8*x*	5.30	-	-	-	-	1	0.66	*S.l.*
MC154	8*x*	5.52	0.09	1.61	5.66	5.26	18	0.69	*S.l.*
MC193	4*x*	2.86	-	-	-	-	1	0.71	*S.l.*
	6*x*	4.07	0.03	0.01	4.09	4.02	4	0.68	*S.l.*
	8*x*	5.39	0.15	0.03	5.69	5.13	30	0.67	*S.l.*
	9*x*	6.10	0.18	0.03	6.23	5.98	2	0.68	*S.l.*
MC201	4*x*	2.62	0.01	0.00	2.62	2.61	2	0.65	*S.l.*
	8*x*	5.38	0.12	0.02	5.51	5.30	3	0.67	*S.l.*
MC232	4*x*	2.67	0.07	0.02	2.78	2.58	18	0.67	*S.l.*
	6x	4.01	0.07	0.02	4.06	3.93	3	0.67	*S.l.*
MC238	4*x*	2.71	0.02	0.01	2.73	2.67	5	0.68	*S.l.*
	6*x*	4.16	0.04	0.01	4.19	4.13	2	0.69	*S.l.*
MC302	4*x*	2.78	-	-	-	-	1	0.69	*S.l.*
MC370	8*x*	5.54	0.15	0.03	5.73	5.38	4	0.69	*P.s.*
MC395	8*x*	5.59	0.04	0.01	5.61	5.54	3	0.70	*S.l.*
SC001	8*x*	5.41	0.08	0.01	5.51	5.35	3	0.68	*S.l.*
SC002	8*x*	5.34	0.04	0.01	5.39	5.30	3	0.67	*S.l.*
SC298	4*x*	2.86	0.03	0.01	2.89	2.83	3	0.71	*P.s.*

Appendix 4.4. Mixed-ploidy populations of *Gladiolus communis*. For each population, the total number of analyzed individuals (N total) and percentage of each cytotype within the population are presented. Populations are identified by ID codes following Appendix 1 and are organized in groups according with their cytotype composition (4*x* + 6*x*, 4*x* + 6*x* + 8*x* + 9*x*, 4*x* + 8*x*, and 6*x* + 8*x*). DNA ploidy levels: tetraploids (4*x*), hexaploid (6*x*), octoploid (8*x*) and nonaploid (9*x*). Populations where all the individuals were sampled are underlined (results in Figure 4.3).

Mixed-ploidy populations	N total	Cytotypes (%)			
		4*x*	6*x*	8*x*	9*x*
4*x* + 6*x*					
JMC001	8	75.0	25.0		
MC213	17	94.1	5.9		
MC232	26	88.5	11.5		
MC238	7	71.4	28.6		
MCD001	6	83.3	16.7		
4*x* + 6*x* + 8*x* + 9*x*					
MC193	106	0.9	3.8	93.4	1.9
4*x* + 8*x*					
MC148	449	99.8		0.2	
MC164	30	90.0		10.0	
MC173	34	97.1		2.9	
MC176	32	96.9		3.1	
MC201	23	34.8		65.2	
MC207	19	94.7		5.3	
6*x* + 8*x*					
JC010	18		22.2	77.8	
MC191	2		50.0	50.0	
MC196	21		4.8	95.2	

Appendix 4.5. DNA ploidy levels of the offspring of pure- and mixed-ploidy populations of *Gladiolus communis*. For each population, the total number of seeds analyzed (N total) and percentage of each DNA ploidy level within the offspring are presented. DNA ploidy levels: tetraploids (4*x*), pentaploid (5*x*), hexaploid (6*x*), octoploid (8*x*) and aneuploid (An.).

Populations	N total	Offspring DNA ploidy level (%)				
		4*x*	5*x*	6*x*	8*x*	An.
Pure-ploidy						0.96
4*x*	515	99.04				0.96
6*x*	264		20.47	17.80		61.73
8*x*	540				100.00	
Mixed-ploidy						
4*x*	70	100.00				
8*x*	60				100.00	

Chapter 5 – Do reproductive barriers facilitate cytotype coexistence in *Gladiolus communis* contact zones

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ABSTRACT

Polyploids are considered an important mechanism of sympatric speciation; however, to successfully establish, the new polyploids must overcome strong positive frequency-dependent selection. Assortative mating is one mechanism that can enable polyploids to surpass this problem. Therefore, strategies promoting assortative mating will increase their fitness within parental populations. Here, we quantify the reproductive barriers contributing to assortative mating between tetraploid and octoploid *Gladiolus communis* in a contact zone in Western Iberian Peninsula. Geographical, temporal, behavioral, mechanical and gametic barriers were accessed in natural populations and common garden experiments. Tetraploid and octoploid *G. communis* have high overlap in flowering time, similar morphology and are both visited by generalist insects, enabling pollen flow between cytotypes in mixed ploidy arrays. Controlled pollinations revealed high inter-cytotype crossability and the production of hexaploid hybrids under pure-ploidy inter-cytotype crosses. Gametic selection was the most important reproductive barrier in this complex as pollen from the maternal ploidy most often fertilized ovules, thus restricting the production of hybrids in mixed ploidy pollinations (conspecific precedence). Our results show that low reproductive isolation in initial stages might inhibit the establishment of novel entities, although recurrent production of unreduced gametes might ameliorate this stage with recurrent polyploid formation; still, strong post-zygotic barriers in later stages might enable cytotype co-existence in sympatry. All these processes promote high cytotype diversity and dynamic contact zones with possibility for recurrent gene flow in this polyploid complex.

Keywords: contact zone, cytotypes, *Gladiolus communis*, hexaploid, octoploid, polyploidy, post-pollination reproductive barriers, pre-pollination reproductive barriers, tetraploid.

INTRODUCTION

Speciation is a slow process, however, in plants (and in some animal groups) there is a pervasive mechanism that can generate new entities within parental populations, *i.e.*, whole genome duplications or polyploidization (Ramsey and Schemske 1998). New polyploids are frequently formed in nature although their extinction rates are also expected to be high (Soltis *et al.* 2007, 2010) due to strong constraints on their establishment (Levin 1975). Still, polyploidization is a widely spread mechanism in the evolutionary history of flowering plants (Wood *et al.* 2009, Marques *et al.* 2017). Because polyploids arise within parental populations, contact zones are key to understand the processes involved in the emergence, successful establishment and subsequent spread of the new polyploid entity (Petit *et al.* 1999).

Generically, contact zones are defined as areas where two or more taxa meet and interact with each other (Haffer 1969; Hewitt 1988; Pratt 1991; Lexer and van Loo 2006), sometimes in asymmetrical relationships, such as the ones generated by different population sizes and different biological attributes (*e.g.*, see Buggs 2007, and references therein). Such contact enables mating and ecological interactions between the taxa that frequently generates hybrid zones (Harrison 1993). The dynamics of these zones will depend on the levels of interaction between the taxa and might influence their genetic structure and diversity, enable the transfer of genetic adaptations, lead to the breakdown or reinforcement of barriers to reproduction or even lead to the emergence of new entities (Barton and Hewitt 1989; Abbott 1992; Rieseberg 1997), and polyploids complexes were not one exception (Petit *et al.* 1999; Levin 2002). Thus, we may encounter primary contact zones where polyploids are (recurrently) formed within parental populations and expand their range afterwards (*e.g.*, Felber 1991; Kim *et al.* 2012), or secondary contact zones that result from allopatric emergence of polyploids [with displacement of the parental(s)] and subsequent contact with lower ploidy populations after range expansion (*e.g.*, Ståhlberg 2009; Mráz *et al.* 2012). Both structures can even occur within the same polyploid complex (*e.g.*, Stuessy *et al.* 2004; Kolář *et al.* 2009; Castro *et al.* 2018). Thus, contact zones have long been recognized as natural laboratories to study the patterns and processes involved in species divergence (Hewitt 1988; Harrison 1993; Lexer and van Loo 2006).

Recent studies of some polyploid plants have revealed surprisingly high cytogenetic diversity (*e.g.*, Baack 2004; Kolář *et al.* 2009; Ståhlberg 2009; Trávníček *et al.* 2010; Castro *et al.* 2012; Zozomová-Lihová *et al.* 2015, among any others) and made the distinction between dominant and minority cytotypes (*e.g.*, Kolář *et al.* 2009; Trávníček *et al.* 2010). In the majority of these studies, cytotypes formed contact areas where they can grow in close proximity

(reviewed in Husband *et al.* 2013). Cytotype co-existence in mixed-ploidy populations can be a transitional stage where cytotypes are recurrently formed *in situ* or co-occur through dispersal (Felber 1991; Kolář *et al.* 2009). Theoretical models predict that mixed-ploidy populations are unstable and frequency-dependent selection will eliminate the minority cytotype due to the formation of sterile odd ploidy offspring (Levin 1975; Rodriguez 1996a; Husband and Schemske 2000). However, cytotype coexistence is more common than previously hypothesized. Numerous studies have provided evidence that contact zones with occurrence of stable mixed-ploidy populations where cytotypes coexist are also possible if biological attributes, such as assortative matting within cytotype, large viability/fertility of polyploids and/or recurrent polyploid formation through unreduced gametes, can ameliorate fitness disadvantages (Rieseberg and Willis 2007; Paun *et al.* 2009; Thompson and Merg 2008; Jersáková *et al.* 2011).

Barriers to between-cytotype mating might have various ecological and reproductive causes which may act in isolation or in concert to reduce fertilizations between cytotypes (Husband 2000) and fitness disadvantages generated by the production of (theoretically) sterile progeny (Levin 1975). Among these barriers are, for example, micro-habitat segregation and phenological, mechanical and behavioral barriers. Differences in micro-habitat requirements or limited dispersal abilities might promote an aggregated distribution of plants of the same cytotype and thereby promote assortative pollen dispersal and mating (*e.g.*, Felber-Girard *et al.* 1996; Baack 2004; Kolář *et al.* 2009; Ståhlberg 2009; Richardson and Hanks 2011). In some polyploid complexes, flowering time overlap may be limited or non-existent, reducing the probability of pollen flow between cytotypes (phenological barrier, *e.g.*, Van Dijk and Bijlsma 1994; Petit *et al.* 1997; Segraves and Thompson 1999; Husband and Sabara 2004, Nuismer and Cunningham 2005, Jersáková *et al.* 2010; Martin and Husband 2012). In addition, morphological and/or physiological differences between cytotypes in flower characters may influence pollinator composition and foraging behavior (behavioral barrier, *e.g.*, Segraves and Thompson 1999; Husband and Schemske 2000; Husband and Sabara 2004; Kennedy *et al.* 2006). Differences in floral morphology might also affect pollen removal and deposition on the pollinator's body (mechanical barrier, Grant 1994), although this reproductive barrier has been poorly studied in polyploid complexes (Segraves and Thompson 1999; Jersáková *et al.* 2010; Borges *et al.* 2012). If pollen exchange is not precluded, gametic barriers might also prevent hybrid fertilizations and ameliorate the fitness disadvantage of inter-cytotype pollinations (*e.g.*, pollen competition in mixed-ploidy loads, Baldwin and Husband 2011; mentor effect, Mráz 2003; or reproductive strategies changes, Barringer 2007; Kao 2007). Finally, the sterility of inter-cytotype hybrids and their role in new cytotype establishment has been questioned. Several

studies show that inter-cytotype hybrids may be not completely sterile and produce viable gametes with a multitude of ploidies and might produce viable offspring (Ramsey and Schemske 1998; Husband 2004; Costa *et al.* 2014). Thus, these entities might actually function as bridges promoting the establishment of new cytotypes (triploid bridge; Husband 2004). Despite the increased detection of mixed-ploidy populations and species, the magnitude and influence of reproductive barriers on cytotype diversity and coexistence is poorly known and isolated to only a few case studies. Only a hand full of studies has actually quantified the strength and contribution of multiple barriers to reproductive isolation between cytotypes (*Arrhenatherum elatius*, Petit *et al.* 1997; *Aster amellus*, Castro *et al.* 2011; *Chamerion angustifolium*, Husband *et al.* 2016; *Gymnadenia conospsea*, Jersáková *et al.* 2010; *Heuchera grossulariifolia*, Segraves and Thompson 1999; *Plantago media*, Van Dijk *et al.* 1992; Van Dijk and Bijlsma 1994).

Gladiolus communis L. (Iridaceae) is a bulbous Mediterranean polyploid plant harboring high morphological and cytogenetic diversity (Hamilton 1980; revised in Alonso and Crespo 2010). In the past, this complex has been considered different species; however, recent morphological reviews and molecular analyses do not support previous taxonomic delimitations. Therefore, *G. communis* is currently considered as a complex formed by several cytotypes (Buchanan 2008; Alonso and Crespo 2010). In the Iberian Peninsula, it occurs most frequently as tetraploid ($2n = 4x = 60$ chromosomes) and octoploid cytotypes ($2n = 8x = 120$), but hexaploids ($2n = 6x = 90$) have also been observed (*e.g.*, Fernandes 1948; Fernandes and Queirós 1971; Castro *et al.* 2018 – Chapter 4). Detailed surveys of natural populations reveal complex cytogenetic patterns in nature (Chapter 4). Although tetraploids and octoploids are the dominant cytotypes, mixed ploidy populations were observed within and outside the contact zone and differ in the specific composition of cytotypes (Chapter 4). The distribution patterns of the dominant cytotypes, *i.e.*, tetraploids and octoploids, can be partially explained by some abiotic variables, but nothing is known about the reproductive barriers governing the dynamics of contact zones.

The intricate contact zones of *G. communis* cytotypes raises the question: can the two entities coexist? Following the theoretical models, either reproductive barriers mediate assortative mating enabling the stable coexistence of different cytogenetic entities in sympatry or the mixed-ploidy populations are transitional stages where minority cytotype exclusion ultimately drives the transition to pure-ploidy populations. In this study, we quantified the contribution of phenological, morphological, behavioral, and gametic barriers between tetraploid and octoploid *G. communis* at the contact zone in natural populations and common garden experiments. In particular we evaluated the reproductive isolation mediated by: 1)

differences in flowering phenology between the cytotypes; 2) differences in flower size that might mediate different pollinator preferences and/or segregate the pollen along the pollinator body; 3) differences in the behavior and/or cytotype preferences of pollinators; and 4) gametic selection against alternate cytotype pollen. We used a series of controlled pollinations to assess self-incompatibility differences and quantify the production of hybrids under pure- and mixed-ploidy pollen loads delivered by pollinators, including self-pollen deposition. Finally, the cumulative effects of all these reproductive barriers were quantified in an experimental mixed-ploidy population of tetraploid and octoploid individuals grown in common garden, controlling for resource limitation and cytotype frequency, to ultimately understand the patterns observed in nature.

MATERIALS AND METHODS

Study system

Gladiolus communis is a perennial bulbous polyploid species of the Mediterranean basin and Iberian Peninsula (Hamilton 1980; Alonso and Crespo 2010). The plant produces spike inflorescences, usually one per individual, of pink bisexual flowers. Flowers are zygomorphic and sessile, short lived, nectar rewarding and odorless. Perianth parts are fused in the base forming a short tube where nectar accumulates. The three lower tepals have white bands delimited by a strong pink band that point towards the flower entrance. The three stamens are unilateral, opening downwards, and are curved towards the upper tepal, such that pollen is deposited on the upper part of the insect's thorax during a visit. The pistil has a filiform three-lobed stigma that is exposed between the anthers and the upper petal, and a 3-lobule ovary with axial placentation (Hamilton 1980; Alonso and Crespo 2010). Flowering period is from mid-April to mid-July.

Study populations and general experimental design

Our study was conducted within the 4*x*-8*x* contact zone of central Portugal, where cytotypes occur in close proximity and occasionally in mixed-ploidy populations. (Castro *et al.* 2018 – Chapter 4). Here, we examined the barriers to between-cytotype mating in three pure-tetraploid and three pure-octoploid populations from this contact zone and in plants from the same populations growing in pots in a common garden at the Botanic Garden of the University of Coimbra (Table 5.1). Bulbs were collected in the field in 2013 and potted in 2 L pots with

commercial soil. DNA ploidy of these populations was assessed with flow cytometry by Castro *et al.* (2018 – Chapter 4), while DNA ploidy of all individuals growing in the experimental garden were also confirmed following the same protocol (data not shown). Bulbs in the experimental garden were grown for one generation to reduce maternal effects. These plants were used to: 1) assess flowering phenology under common conditions; 2) perform controlled pollinations and measure gametic isolation; and, 3) build experimental mixed-ploidy population where the cumulative effect of all the reproductive barriers was quantified. In the field, pollinator assemblage, preferences and behavior were assessed in all the selected populations. Flowering phenology and flower morphology were also assessed in two natural populations, one tetraploid and one octoploid (Table 5.1).

Table 5.1. Locality, DNA ploidy level (4*x*, tetraploid; 8*x*, octoploid) and geographic information of the studied *Gladiolus communis* populations in a tetraploid-octoploid contact zone. Populations marked with * were used to study flowering phenology.

Populations	DNA ploidy level	Longitude	Latitude	Altitude (m a.s.l.)
Secarias, Arganil	4*x*	40.24689	-8.03339	187
Antões, Pombal*	4*x*	39.96736	-8.77196	122
Casal Duro, Fátima	4*x*	39.57156	-8.72667	431
Trouxemil, Coimbra	8*x*	40.27874	-8.44585	56
Casal da Rola, Soure*	8*x*	40.02041	-8.71506	48
Alcaria, Fátima	8*x*	39.6664	-8.68534	345

Flowering phenology

Flowering phenology was evaluated in natural populations and in the common garden. In the field, 45 individuals in Antões (Pombal; 4*x*) and 45 in Casal da Rola (Soure, 8*x*; Table 5.1) were randomly selected and tagged before the beginning of the flowering season. After the opening of the first flower, these individuals were monitored daily during 20 consecutive days, covering the flowering period of each plant. This enabled to quantify flowering phenological patterns in *G. communis*. In each plant and flower, the flowering period to quantify flowering phenological patterns. The following variables were recorded for each plant and flower of the inflorescence: corolla opening timing, pollen dehiscence, stigmatic lobes opening, corolla wilting. With this information, we estimated the frequency of individuals flowering and number of open flowers on any given day. Additionally, it was also possible to calculate the following parameters for each cytotype: flower lifespan (mean number of days that a flower is open and accessible to pollinators), inflorescence size (total number of flowers per inflorescence), and

floral display (mean number of simultaneously open flower per individual). Flowering phenology was also assessed in plants from the selected populations growing in the common garden by monitoring daily for 50 days the number of open flowers of tetraploid and octoploid plants (N = 39 and N = 21, respectively).

Phenological reproductive isolation ($RI_{phenological}$) between tetraploids and octoploids was calculated for each cytotype individually when growing in natural populations and in the common garden, using,

$$RI_{phenological} = 1 - \frac{\text{no. of co-flowering days}}{\text{total no. of days flowering}}.$$

For the $RI_{phenological}$ index of tetraploids, the number of days that tetraploids co-flowered with octoploids was divided by the total number of flowering days for tetraploids, while for $RI_{phenological}$ index of octoploids, the number of days that octoploids co-flowered with tetraploids was divided by the total number of flowering days of the octoploids. The total $RI_{phenological}$ index was calculated, in this case, by dividing the number of co-flowering days of both cytotypes by the total number of flowering days (Husband and Sabara 2004).

Differences between tetraploids and octoploids in the variables measured in natural populations were tested using Generalized Linear Models (GLM) with cytotype as factor and flower duration (given in number of days), number of flowers per inflorescence and number of simultaneously open flowers, as response variables; population and individual were initially introduced as random factors (using Generalized Linear Mixed Models, GLMM). However, as these random factors presented a lower variance than residuals, they were removed from the analyses (Bolker *et al.* 2009). A Poisson distribution with a log link function was used in all models.

Flower morphology and nectar quantification

We studied floral morphology in all selected natural populations. Ten individuals were randomly selected in each population and one flower per individual was characterized with respect to 1) corolla traits, namely flower size, corolla opening, and corolla tube opening and length, and 2) sexual organ position and size, namely anther-lower tepal distance representing the space for pollinator entrance, stamen length, anther length, style and stigma length and stigmatic filament length (Figure 5.1).

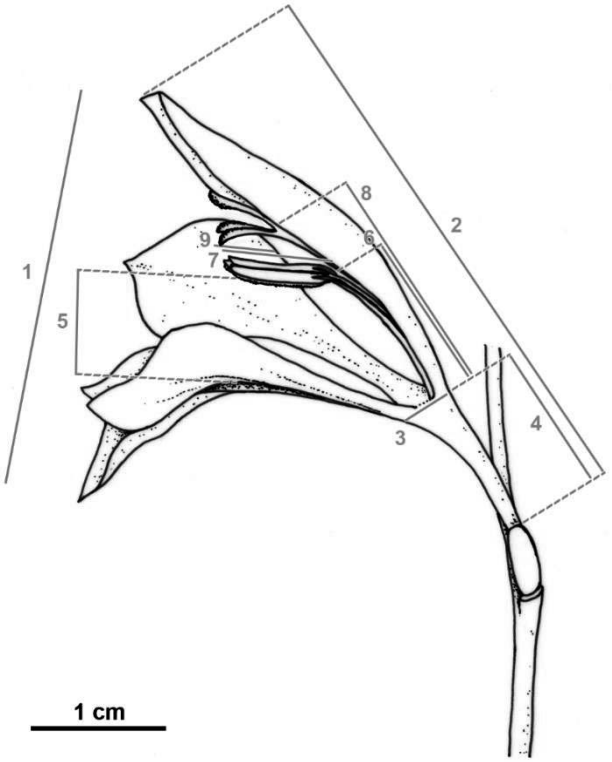


Figure 5.1. Morphological parameters measured in *Gladiolus communis* flowers: 1, flower opening; 2, flower length; 3, tube opening; 4, tube length; 5, anther-lower petal distance; 6, anther's position; 7, anther length; 8, stigma's position; 9, stigma length.

Differences between tetraploids and octoploids in flower traits were evaluated using generalized linear models with cytotype as factor and each measured variable as a response variable. Population, individual and flower position in the inflorescence were initially included in the model as random factors with a Gaussian distribution and an identity link function to model responses.

To quantify mechanical reproductive isolation, morphological reproductive isolation ($RI_{morphological}$) index was calculated following the same approach as described above, considering the overlap of both male and female functions:

$$RI_{morphological} = 1 - \frac{\text{no. flowers with overlap}}{\text{total no. measurements}}.$$

For the $RI_{morphological}$ index of tetraploids, we considered the number of flowers with overlap as the number of flowers where the stigma (female function) of the tetraploids overlapped in physical position with the anthers (male function) of the octoploids plus the number of flowers where the anthers of the tetraploids overlapped in physical position with the stigmas of the octoploids. The $RI_{morphological}$ index for the octoploids was calculated following the

same approach as that used in the tetraploids. Finally, the total $RI_{morphological}$ index was also calculated using the sum of the flowers where at least one of the functions overlapped with the complementary function of the other cytotype.

Additionally, nectar production and concentration were measure in plants growing in the common garden to evaluate floral rewards of each cytotype. At peak flowering, plants were bagged 24 hours before the measures. Nectar production (*V*) was measured with a 5 µl capillary micropipette, and sugar content (*C*, percentage by weight) with a portable refractometer. The density of sugar (*d*) was calculated following the Prys-Jones and Corbet (1987) formula. Finally, nectar sugar content (*s*, in mg) was calculated using $s = VC/d*100$.

Differences between cytotypes in nectar variables were assessed with cytotype as factor and each parameter (*V*, *C*, s and *d*) as response variables; flower position, flower age and collection date were initially used in the model as random factors. Random factors were removed whenever the variance was lower than the variance of the residuals (Bolker *et al.* 2009). A Poisson distribution with a log link function was used in all models.

Pollinator foraging behavior

Pollinator foraging were assessed by direct observations in the six selected natural populations. In each population, three randomly selected patches of approximately 2 m^2 were delimited, and insect visits to the individual plants were monitored during the whole day (09:00am to 16:00pm, GMT+0) for a total of 126 hours of surveillance evenly distributed among the populations. For each insect flight, the following variables were recorded: visitor species and number of flowering plants visited. All insects contacted the sexual organs except individuals from the family Lepidotera, which behaved as nectar thieves (following Castro *et al.* 2013; Inouye 1980). Insect specimens were collected for subsequent identification. This enabled us to assess the overall insect assemblage in the populations studied and to determine the main visitor species of *G. communis*. To describe the visitors of tetraploid and octoploid populations of *G. communis*, the following parameters were calculated for each visitor species and population: visitation rate (number of individual plants visited per hour), insect abundance (number of individuals of a given insect species per hour) and frequency of interaction (visitation rate multiplied by insect abundance).

Pollinator foraging behavior was also studied in artificial arrays composed of tetraploid and octoploid individuals. Each array comprised 10 inflorescences with similar number of open

flowers and height, five inflorescences of each cytotype, alternately arranged in a circle and separated by 20 cm. The arrays were displayed in each of six selected populations (three arrays per population) and monitored during the whole day (mean 21 hours of observation per population). During these observations, the insect species and the visitation sequence to each cytotype were recorded. This information was used to assess pollinator preferences (floral preference index) and behavior (floral constancy index) for the five most abundant pollinator species. Floral preference index of a given pollinator species was calculated as the ratio between the number of visits to a given cytotype and the total number of visits recorded for that pollinator species, *i.e.*, the proportion of visits to that cytotype. This index ranges from 0 to 1, where 0.5 indicates no preference by the pollinator and 0 or 1 shows selected preference for one of the cytotypes, namely for octoploids or tetraploids, respectively. Floral constancy index of a given pollinator species was calculated as the ratio between the number of movements within a cytotype and the total number of flights of the pollinator during the visit, considering only the movements made between individuals. A floral constancy index of 0 indicates an alternating foraging behavior (all flights are between cytotypes), a value of 0.5 indicates a random foraging behavior, while a value of 1 indicates complete foraging constancy within a cytotype. To calculate these parameters only visits that comprised the interaction with three or more inflorescences were considered. For both indexes and for each pollinator species, we tested for deviations from no preference (floral preference of 0.5) and floral constancy of 0.5 using Chi-square tests.

A behavioral reproductive isolation index ($RI_{behavioral}$) due to pollinator fidelity was calculated using:

$$RI_{behavioral} = 1 - \frac{\text{no. movements between cytotypes}}{\text{total no. movements}}.$$

A similar approach as for other RI indices was used, with three reproductive indexes being calculated, namely for tetraploids ($RI_{behavioral}$ using the number of movements between 4*x* plants and the total number of movements involving 4*x* plants), octoploids ($RI_{behavioral}$ using the number of movements between 8*x* plants and the total number of movements involving 8*x* plants), as well as the total $RI_{behavioral}$ (formula above).

Crossing ability under controlled conditions

Controlled hand-pollinations were performed to assess the levels of reproductive isolation and the ability of the two cytotypes to produce hybrids. Two pollination treatments, differing in the

composition of the pollen applied to the stigma, were performed: pure-ploidy pollen loads and mixed-ploidy pollen loads (Figure 5.2). The following pure-ploidy pollen load treatments were performed: 1) self-pollination (anthers of the same inflorescence were used as pollen donor), 2) outcross within cytotypes (anthers of different individuals of the same cytotype were used as pollen donor) and 3) outcross between cytotypes (anthers of the other cytotype were used as pollen donor) (Figure 5.2A). Also, the following mixed-ploidy pollen load treatments were performed: 4) mixed-ploidy outcross (mix of tetraploid and octoploid anthers were used as pollen donors) and 5) outcross between cytotypes and self-pollen (anthers of the recipient individual and anthers of individuals of the other cytotype were used as donors; Figure 5.2B). These treatments enabled us to assess self-incompatibility differences, quantify the production of hybrids and evaluate the effect of mixed-ploidy pollen loads delivered by pollinators in hybrid production, as well as the role of self-pollen deposition in hybrid production avoidance under mixed-ploidy pollen loads.

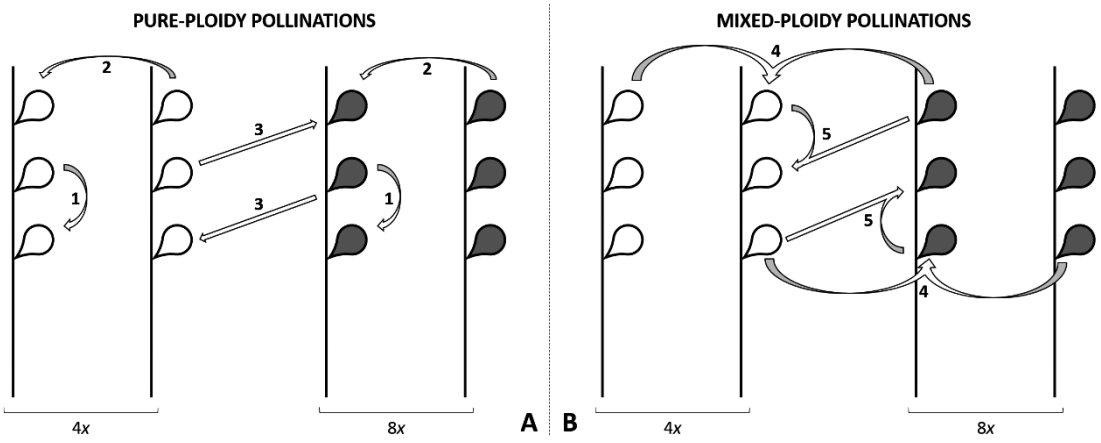


Figure 5.2. Controlled pollination experiments performed in tetraploid (white, 4*x*) and octoploid (grey, 8*x*) *Gladiolus communis* inflorescences. Two types of treatments were performed: A) pure-ploidy pollinations (1 – self pollinations, 2 – outcross within cytotypes, and 3 – outcross between cytotypes); and B) mixed-ploidy pollinations (4 – mixed outcross, and 5 – outcross between cytotypes and self-pollen). Arrows denote each pollination treatment, going from the donor plant(s) to the recipient one.

Pollination experiments were conducted during peak flowering in 2014 and 2015 (May) using a total of 102 plants growing in a common garden (treatment 1 – 35 and 18 for tetraploid and octoploid recipients, respectively; treatment 2 – 37 and 29; treatment 3 – 33 and 24; treatment 4 – 27 and 13; and treatment 5 – 33 and 14). Before flowers started to open, and until fruit collection, plants were protected with a nylon mesh to exclude pollinators. With the exception of treatment 1 (self-pollen), all flowers used as pollen recipients were emasculated before their stigmas were receptive. In pure-ploidy pollination treatments, anthers from three

individuals (one per individual) were gently rubbed directly on the stigmatic papillae until stigma saturation (except in self-pollination, where anthers belonging to the same plant were used). In mixed-ploidy pollination treatments, three anthers of each cytotype were collected in an Eppendorf tube and shaken, which was then applied to the stigmatic papillae with a needle. In these crosses, only the first four flowers of each inflorescence were used, to avoid different resource allocation within the inflorescence, since previous pollen-supplement experiments showed no differences in fruit and seed production for these positions (χ^2 = 0.02, P = 0.892; χ^2 = 0.08, P = 0.784, respectively; M. Castro, unpublished data). Fruits were collected when mature, immediately before fruit dehiscence, and the number of seeds was quantified under a binocular microscope. Fruit set (calculated as proportion of pollinated flowers that developed into fruit), S:O ratio (proportion of ovules that resulted in morphologically viable seeds) and reproductive success (combination of fruit set with S:O ratio) were calculated. The S:O ratio was calculated using the mean number of ovules per flower of each cytotype, assessed in 89 flowers from a total of 20 distinct individuals, following Dafni *et al.* (2005). The mean number of ovules per tetraploid flower was 44.2 ± 0.5 (mean ± SE) and for octoploids was 42.4 ± 0.5 ovules, with no statistically significant differences being found between them ($Z_{1,87}$ = -1.29, P = 0.198). Differences in fruit set, S:O ratio and reproductive success were assessed using GLM, with cytotype and pollination treatment defined as factors. Year, individual and flower position were initially used a random factor, but, again, these factors were removed due to low variance in comparison with residuals (Bolker *et al.* 2009). A binomial distribution with a logit link function was used to model fruit set, and a Gaussian distribution with an identity link function was applied for S:O ratio and reproductive success after transformation with the arcsine of the square root. Since the interaction between cytotype and pollination treatment was significant for S:O ratio and reproductive success, differences for each fixed factor were evaluated separately in these two variables, *i.e.*, differences between cytotypes for each pollination treatment and differences between pollination treatment for each cytotype were evaluated following a similar approach to the one described above. If a significant difference were observed between pollination treatments, a post-hoc test for multiple comparisons was performed.

The production of hybrids was evaluated by analyzing the DNA ploidy levels of the offspring obtained after controlled hand-pollinations using flow cytometry. For that, ten seeds per fruit were analyzed using the protocol of Galbrailth *et al.* (1983) with some adjustments (Castro *et al.* 2018 – Chapter 4). Briefly, two seeds per sample were simultaneously chopped with 0.5 cm^2 of leaf tissue of *Pisum sativum* (2C = 9.09 pg; Doležel *et al.* 1998) in Woody Plant Buffer (Loureiro *et al.* 2007). After that, the nuclear suspension was filtered and stained with

propidium iodide for 2-3 minutes, and the samples analyzed with a CyFlow Space flow cytometer (Partec GmbH., Görlitz, Germany). The DNA ploidy level was inferred for each seed following Castro *et al.* (2018). Differences in the proportion of hybrids (*i.e.*, 6*x* individuals) between treatments were assessed using GLM with cytotype and pollination treatment as factors (analyzed separately), and hybrid proportion as response variable, with a binomial distribution and a logit link function to model responses. Year, individual and flower position were initially considered as random factors but later removed due to low variance of the residuals (Bolker *et al.* 2009). When significant differences were detected, a post-hoc test for multiple comparisons was performed.

Gametic reproductive isolation ($RI_{gametic}$) index, resulting from gamete siring ability and zygote viability, was calculated following the same approach as the previous indices, as follows:

$RI_{gametic}$ = 1 – Reproductive success resulting from pollen flow between cytotypes.

The pollination treatments enabled us to calculate the $RI_{gametic}$ under several distinct scenarios. We used results of pure-ploidy cross-pollinations between cytotypes (treatment 3) or mixed-ploidy with selfing (treatment 5) to mimic the pollen pool immediately after polyploid formation or cytotype dispersal to a population of the other cytotype, and results of mixed-ploidy pollinations (treatment 4) to simulate when cytotype grow in sympatry. In pollinations using mixed pollen loads (treatments 4 and 5), only seeds that differed in DNA ploidy level from the mother plant were used for $RI_{gametic}$ calculation, since offspring with the same ploidy level of the mother was assumed to result from successful within-cytotype fertilization. Regardless of the scenario, the same approach as in the previous reproductive indexes was used, with individual $RI_{gametic}$ indices being calculated for tetraploids, octoploids, as well as, the total $RI_{gametic}$.

Cumulative effects of all reproductive barriers

The cumulative effect of the reproductive barriers studied was calculated, first by combining all the reproductive indexes calculated above, and second by studying the offspring production of tetraploids and octoploids growing in sympatry under controlled conditions. In the common garden, we created an experimental mixed-ploidy population with 1:1 proportions of tetraploid and octoploid plants, comprising 250 pots. Therefore, 125 tetraploid and 125 octoploid individuals were randomly displayed before flowering season. Unfortunately, not all the plants flowered, and, in the end, the artificial population was composed of 122 tetraploid (56%) and 94 octoploid (44%) individuals flowering simultaneously. These plants were left to be

open pollinated, subjected to the same pollinator's assemblage. After flowering season, 30 individuals per cytotype were randomly selected and fruits were collected (424 fruits from 60 individuals). Fruit and seed production were quantified, and the DNA ploidy levels of the offspring was assessed as described above. The results were analyzed statistically as described in the hand-pollination experiments.

All analyses were performed in R software version 3.0.1 (R Core Development Team 2016), using the packages "car" for Type-III analysis of variance (Fox and Weisberg 2015), "lme4" for generalized linear models and generalized linear mixed models (Bates *et al.* 2014), and "multcomp" for multiple comparisons after Type-III analysis of variance (Hothorn *et al.* 2017).

RESULTS

Flowering phenology

Flowering phenology of tetraploids and octoploids was almost completely synchronized, with both cytotypes flowering at the same time (Figure 5.3). In natural populations, the flowering period of tetraploids and octoploids did not differ significantly ($F_{1,38}$ = 0.11, P = 0.747) (Figure 5.3A). In the common garden, although the tetraploids tended to peak slightly earlier than the octoploids, no significant differences were observed between cytotypes in the proportion of open flowers per day ($F_{1,98}$ = 0.12, P = 0.735) (Figure 5.3B). These phenological patterns resulted in low RI indexes (Table 5.2). In natural populations, phenological reproductive isolation was 0.05, with octoploids always flowering in the presence of flowering tetraploid individuals ($RI_{phenological\ 8x}$ = 0.00), and tetraploids flowering alone on one day only ($RI_{phenological\ 4x}$ = 0.05) (Table 5.2, Figure 5.3). A similar pattern was observed in common garden plants; again, octoploids always flowered with tetraploids ($RI_{phenological\ 8x}$ = 0.00), while tetraploids flowered alone for a slightly longer time period, both at the beginning and at the end of the flowering season ($RI_{phenological\ 4x}$ = 0.14), resulting in a low total phenological reproductive isolation in *G. communis* of 0.14 (Table 5.2).

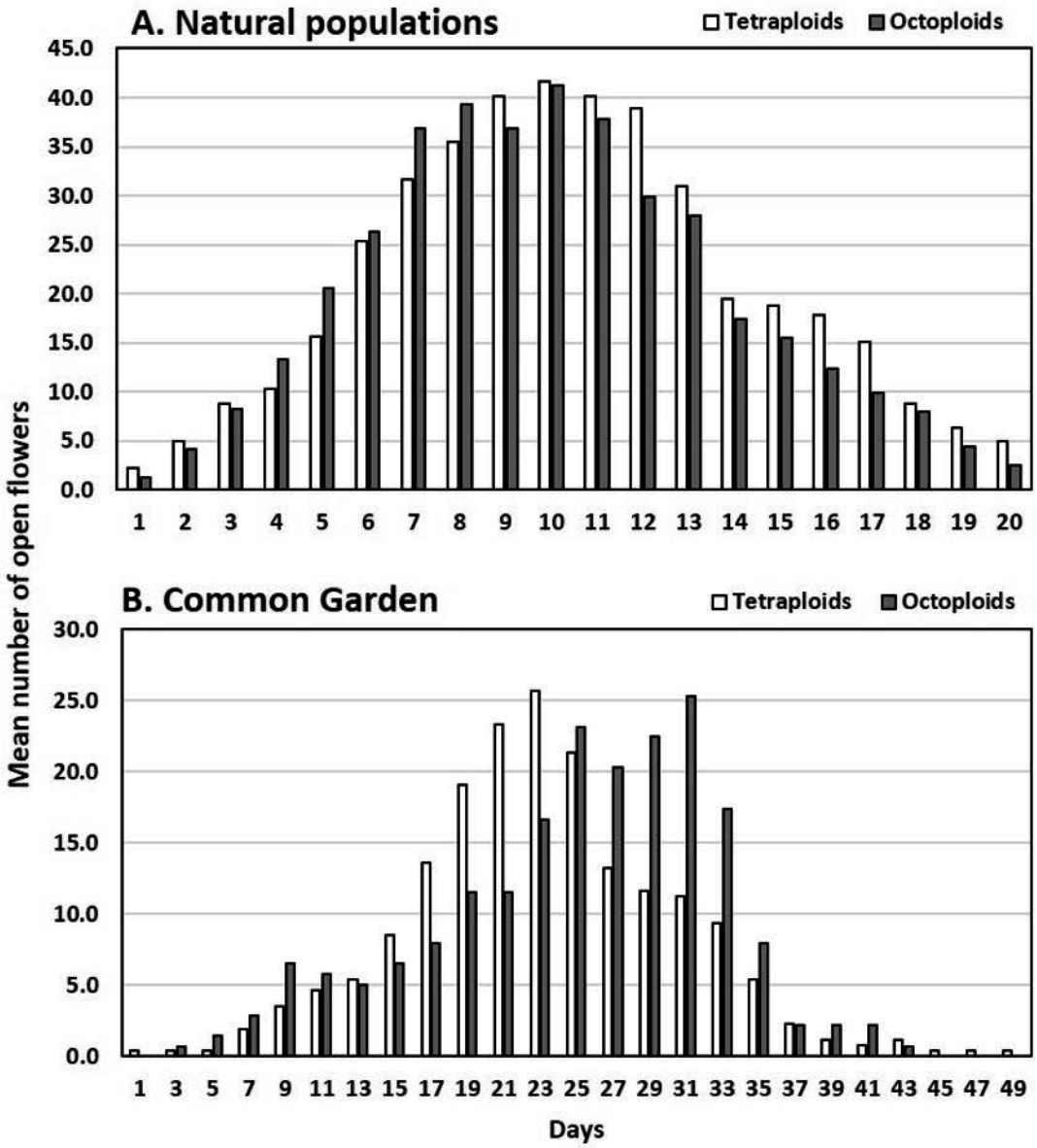


Figure 5.3. Flowering phenology of tetraploid (white) and octoploid (grey) *Gladiolus communis* cytotypes in: A) natural populations, and B) common garden. Values are given as mean number of open flowers per inflorescence per day, starting in the day of the first flower opening.

Field observations enabled also to characterize flower development. Flower lifespan did not differ significantly between cytotypes ($z_{1,597}$ = -0.73, P = 0.468) with flowers of both cytotypes being open for 3-4 days (mean ± SE: 3.6 ± 0.0 days). Male and female functions matured at different times along the flower lifespan: the flower opens, but the anthers become dehiscent only in the second day of the flower life; stigmatic branches harboring the stigmatic papillae started to open at the third day. The floral display, *i.e.*, the number of simultaneously open flowers in the inflorescence, as well as the total number of flowers per inflorescence did not

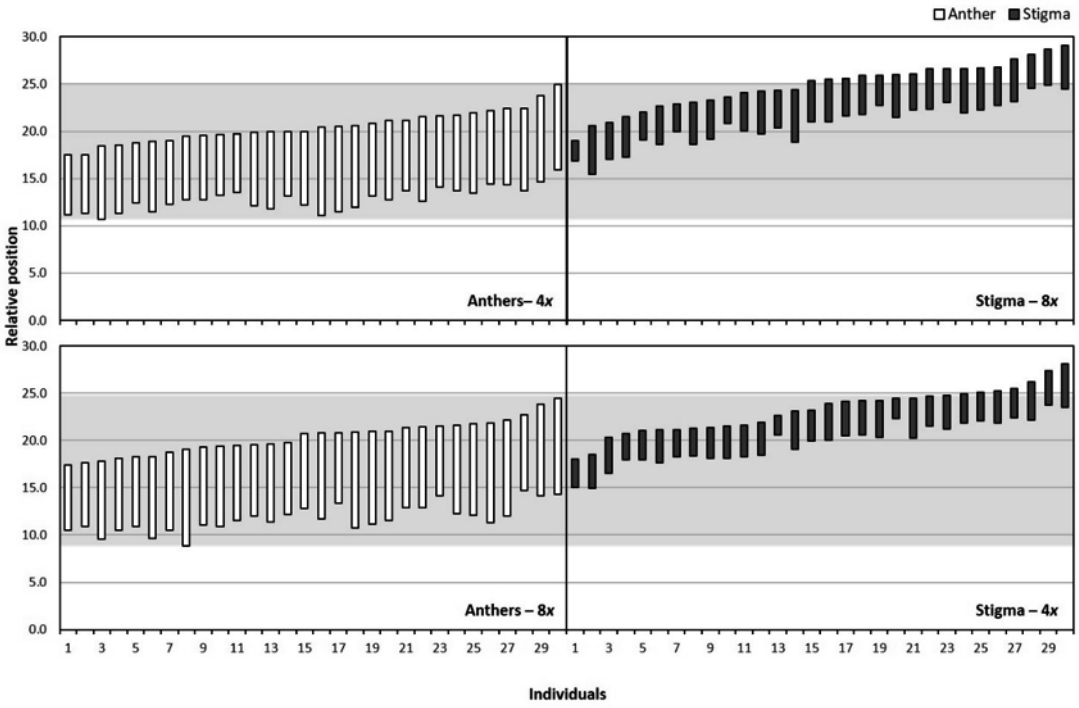
differ significantly between cytotypes (floral display: $z_{1,757}$ = 0.07, P = 0.438, mean ± SE: 2.6 ± 0.0 flowers; total number of flowers: $z_{1,88}$ = -0.16, P = 0.874, mean ± SE: 7.0 ± 0.2 flowers).

Table 5.2. Reproductive isolation indices in the *Gladiolus communis* polyploid complex. The isolation index is provided as total for the complex (RI total) and separately for each cytotype (tetraploids, RI 4*x*; octoploids, RI 8*x*). Cumulative effect of the studied reproductive barriers (phenological in natural populations, morphological, behavioral and gametic) was also calculated for each scenario studied using controlled pollinations (Cumulative RI).

Reproductive barriers	RI 4*x*	RI 8*x*	RI total	Cumulative RI
Phenological				
Natural populations	0.05	0.00	0.05	
Common Garden	0.14	0.00	0.14	
Morphological	0.00	0.00	0.00	
Pollinator behavior	0.13	0.21	0.29	
Gametic				
Pure-ploidy inter-cytotype pollination	0.64	0.56	0.60	0.73
Mixed-ploidy pollination	0.99	0.94	0.97	0.98
Mixed-ploidy pollination with selfing	0.96	0.64	0.80	0.87

Flower morphology and nectar quantification

The flowers of tetraploid and octoploids individuals were morphologically similar with no differences observed for the characters measured (Table 5.3).



Additionally, the relative position of the sexual organs (anthers in relation to stigmas) revealed an overlap between 4*x* anthers and 8x stigmas and between 8*x* anthers and 4*x* stigmas (Figure 5.4), leading to the absence of morphological RI between cytotypes ($RI_{morphological}$ = $RI_{morphological\ 4x}$ = $RI_{morphological\ 8x}$ = 0.00; Table 5.2). No statistically differences were observed between cytotypes in any of the nectar parameters studied (Table 5.3).

Table 5.3. Morphological characterization and nectar production of tetraploid and octoploid *Gladiolus communis* flowers. Flower traits are characterized by the mean and standard error of the mean, followed by the statistical test used to explore differences between cytotypes for each trait (degrees of freedom, *F* statistics, and *P* value).

Flower traits	**Tetraploids**	**Octoploids**	**δ**	***F***	***P***
Flower opening (mm)	33.20 ± 0.51	33.97 ± 0.69	1,58	0.37	0.577
Flower length (mm)	42.43 ± 0.47	46.21 ± 0.62	1,58	3.42	0.136
Tube opening (mm)	4.60 ± 0.09	5.11 ± 0.12	1,58	1.00	0.375
Tube length (mm)	10.34 ± 0.17	11.37 ± 0.25	1,58	1.82	0.247
Anther-lower petal distance (mm)	8.21 ± 0.11	8.66 ± 0.14	1,58	2.44	0.194
Anther' position (mm)	20.48 ± 0.22	20.34 ± 0.23	1,58	0.05	0.838
Anther length (mm)	7.70 ± 0.12	8.59 ± 0.14	1,58	2.54	0.186
Stigma' position (mm)	23.15 ± 0.31	24.80 ± 0.32	1,58	0.57	0.492
Stigma length (mm)	3.37 ± 0.07	4.02 ± 0.09	1,58	1.22	0.331
Nectar production (*V,* µl)	4.19 ± 0.51	5.14 ± 0.52	1,73	1.67	0.200
Sugar content (*C*, %)	38.75 ± 2.23	38.35 ± 1.57	1,40	0.02	0.882
Density of sugar (*d*)	1.17 ± 0.01	1.17 ± 0.01	1,40	0.01	0.917
Nectar sugar content (*s,* mg)	1.53 ± 0.19	1.75 ± 0.22	1,40	1.00	0.327

Pollinator foraging behavior

Gladiolus communis inflorescences were visited by insects belonging to 12 genera of Hymenoptera, all behaving as pollinators while foraging for nectar and pollen [Appendix 5.1]. Inflorescences were also visited by several Lepidoptera, all of which were behaving as nectar thieves, *i.e.*, these insects were able to collect nectar without touching the sexual organs; still, this group accounted for 1.4% of the interactions, only. Pollinator assemblage was variable among populations, with the tetraploid populations having a lower mean pollinator species

⇦ **Figure 5.4.** Relative position of sexual organ in of tetraploid (4*x*) and octoploid (8*x*) *Gladiolus communis* flowers. White boxes represent anthers length (4*x* on top, 8*x* on the bottom) and grey boxes represent stigma length (8*x* on top, 4*x* on the bottom). Light grey boxes represent the range of male organs meaning that stigma inside that box could be pollinated by the donor anthers.

richness than octoploid populations (60% and 85% of the total insects' species, respectively). Although the dominant pollinator species varied among populations, the following species were important in both tetraploid and octoploid populations: *Bombus* spp. [including *B. hortorum* (4.1%), *B. pascuorum* (48.3%) and *B. terrestris* (14.2%)], *Anthophora* sp. (14.0%) and *Colletes* sp. (7.0%). With the exception of *Anthophora* sp., all dominant pollinators presented higher visitation rates in octoploids than in tetraploids populations [Appendix 5.2]. *Anthidium florentinum* was also an important pollinator in octoploids populations.

Of the total species richness in natural populations, 65% of the species were observed visiting the artificial arrays, including the most frequent pollinators [Appendix 5.3]. The five most abundant pollinators presented similar preference and behavioral patterns. Overall, no significant differences among pollinator species were found for preference and constancy indices (Table 5.4). The number of plants visited ranged from 4.6 for *B. terrrestris* to 8.5 in *A. florentinum*; still, the mean number of visited plants did not differ significantly among pollinator species ($F_{4,193}$ = 1.50, P = 0.203). Preference indices did not differ statistically from 0.5 indicating a lack of preference for a specific cytotype by each pollinator species (Table 5.4). The constancy indices revealed that *Anthidium florentinum* presented an alternating foraging behavior (P = 0.044), while the remaining pollinator species presented a random foraging behavior (P > 0.05; Table 5.4). These values are in accordance with field observations in which the insects were observed visiting the nearest plant (personal observation, M. Castro). The lack of preferences and the random/alternated behavior by the pollinators resulted in RI values between cytotypes ($RI_{behavioral}$ = 0.29, $RI_{behavioral\ 4x}$ = 0.13, $RI_{behavioral\ 8x}$ = 0.21, Table 5.2).

Table 5.4. Pollinator preferences and behavior: preferences and constancy indices for the most abundant pollinator species of *Gladiolus communis*. Values are provided as mean and standard error of the mean (SE). The mean number of plants visited per foraging flight (Plants per visit), total number of visits (N) and total number of individuals visited (ni) are also given. The *P* value for deviations of preference and constancy indices from 0.5 are provided for each pollinator. Statistical significant P values are highlighted in bold. Comparisons between pollinators for the number of plants visited for foraging flight, preference and constancy index are also presented.

Taxa	Plants per visit	Preference index		Constancy index		N (ni)
		Mean ± SE	*P*	Mean ± SE	*P*	
Anthidium florentinum	8.4 ± 1.3	0.5 ± 0.0	0.811	0.2 ± 0.0	**0.044**	17 (142)
Anthophora sp.	5.2 ± 0.3	0.5 ± 0.0	0.838	0.3 ± 0.0	0.536	39 (203)
Bombus pascuorum	5.1 ± 0.2	0.5 ± 0.0	0.761	0.3 ± 0.0	0.498	106 (543)
Bombus terrestris	4.6 ± 0.3	0.4 ± 0.0	0.641	0.3 ± 0.0	0.352	20 (91)
Colletes sp.	6.5 ± 0.9	0.5 ± 0.1	0.997	0.3 ± 0.1	0.612	16 (104)
$F_{4,193}$, *P* values	1.50, 0.203	0.47, 0.757		0.87, 0.477		

Crossing ability under controlled conditions

All pollination treatments produced fruits and seeds; however, we observed significant differences between cytotypes, pollination treatments and/or their interactions for the studied reproductive variables (Table 5.5; Figure 5.5). Because the interaction between factors was significant for S:O and reproductive success variables, the effects of each factor were interpreted separately (Table 5.5).

No significant differences in fruit set were observed between cytotypes for each pollination treatment, but significant differences were observed among pollination treatments. Self-pollinations producing significantly lower fruit set than the remaining treatments (Figure 5.5A; Table 5.5).

No significant differences in S:O ratio were observed between cytotypes for each pollination treatment, except for treatment 5 (outcross between cytotypes and selfing; Table 5.5), with octoploids having significantly higher S:O ratios than tetraploids ($P < 0.05$; Figure 5.5B). Within each cytotype, significant differences were observed among pollination treatments (Table 5.5), with self-pollinations presenting significantly lower values and the outcross within cytotypes presenting significantly higher values of all treatments ($P < 0.05$); the remaining treatments, although having intermediate values, did not differ from self-pollinations for the octoploids. Described another way, for tetraploids, treatment 5 did not differ from selfing, while the remaining treatments presented significantly different S:O ratios, with intermediate values between selfing and outcrossing within cytotype (Figure 5.5B).

The results of the reproductive success were similar to the S:O ratio (Figure 5.5C), presenting, overall the same statistical patterns, except for the selfing, where the slightly higher fruit set and S:O ratio recorded in the octoploids resulted in a significantly higher reproductive success in comparison with tetraploids (Table 5.5; Figure 5.5).

The analyses of the DNA ploidy levels of the offspring (Figure 5.5C) revealed that the pollinations within the same cytotype (*i.e.*, selfing and outcross within cytotypes) produced mostly offspring with the same ploidy level of the parentals, *i.e.*, tetraploids in crosses between tetraploids, and octoploids in crosses between octoploids. Interestingly, the production of unreduced gametes was also detected, with the production of a few hexaploid seeds after selfing of tetraploid individuals.

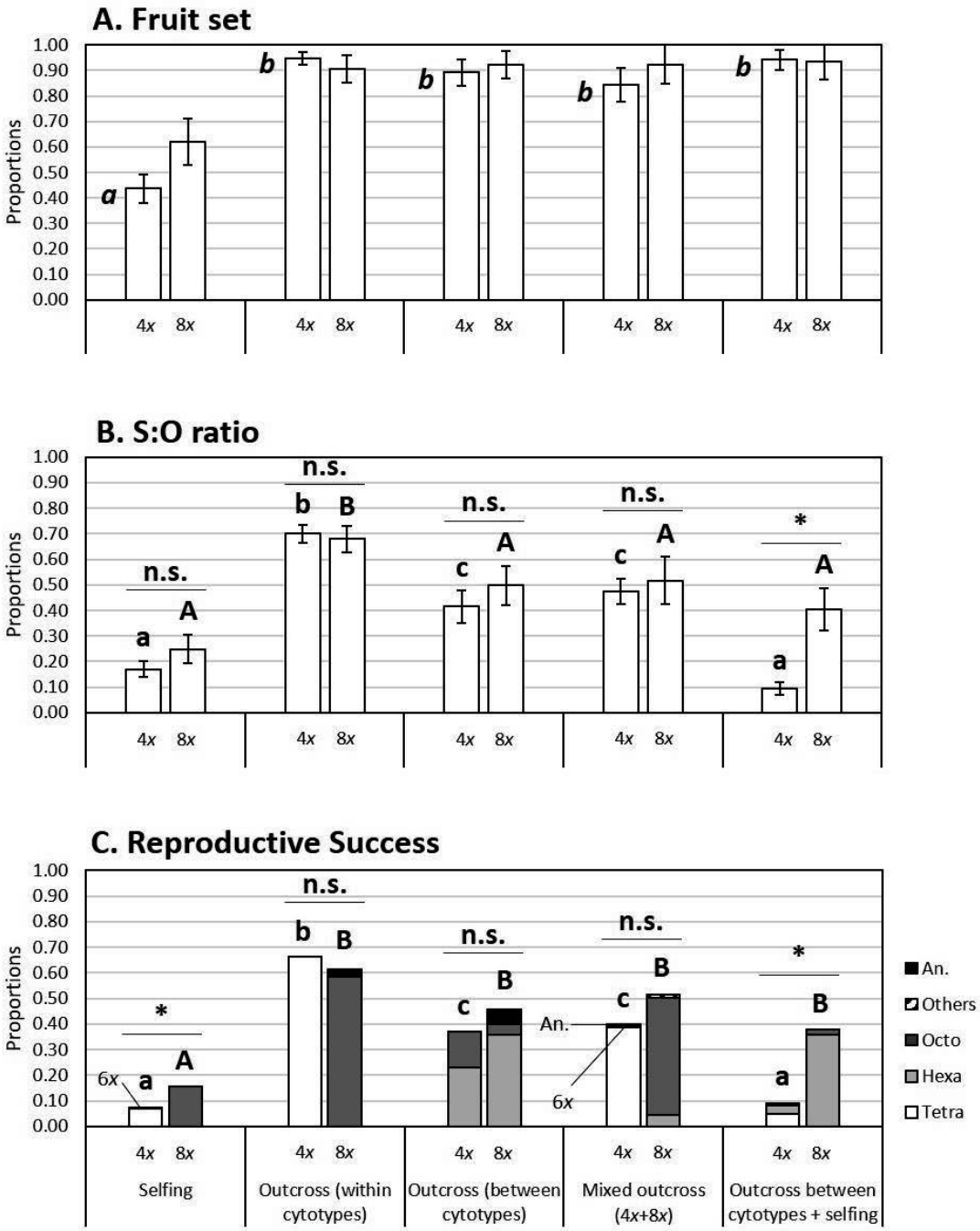


Figure 5.5. Reproductive variables after different pollination treatments in *Gladiolus communis*: A) Fruit set (proportion of flowers that developed into fruit; mean and standard error of the mean); B) S:O ratio (proportion of ovules that developed morphologically viable seeds; mean and standard error of the mean); and C) Reproductive Success (fruit set multiplied by the S:O ratio). In A) no differences were observed between ploidies (not shown), and statiscally differences between pollination treatments at $P < 0.05$ are denoted by different italic lower-case letters (for details see Table 5.5). In B) and C) statistical comparisons between ploidies within treatments are marked with * for significant at $P < 0.05$, and by n.s. for non-significant, while differences among treatments within cytotype are denoted by lower-case letters for tetraploids and upper-case letters for octoploids, with different letters representing significant differences at $P < 0.05$. In C), the bars provide the relative proportion of the ploidy-levels observed in the offspring of each treatment given by different colors: white – tetraploids (Tetra, 4x), light grey – hexaploids (Hexa, 6x), dark grey – octoploids (Octo, 8x), black diagonal stripes – decaploids and dodecaploids (Others, 10x and 12x, respectively), and black – aneuploids (An.). dark grey – octoploids (Octo), black diagonal stripes – decaploids and dodecaploids (Others), and black – aneuploids (An.).

Table 5.5. Generalized mixed-effect model or linear model analysis of the effect of ploidy or pollinator treatment in fruit set, S:O ratio and reproductive success after controlled hand-pollinations in *Gladiolus communis*. Ploidy levels: 4*x*, tetraploid; 8*x*, octoploid. Statistically significant differences are highlighted in bold.

Factors	Fruit set	S:O ratio	Reproductive success
Effect of ploidy and pollination treatment			
Ploidy	$\chi^2_1 = 1.144$, $P = 0.285$	$F_{1,289} = 3.980$, **$P = 0.047$**	$F_{1,289} = 5.210$, **$P = 0.023$**
Pollination treatment	$\chi^2_4 = 64.305$, **$P < 0.001$**	$F_{4,289} = 35.060$, **$P < 0.001$**	$F_{4,289} = 53.287$, **$P < 0.001$**
Pollination treatment*Ploidy	$\chi^2_4 = 2.488$, $P = 0.647$	$F_{4,289} = 2.403$, **$P = 0.050$**	$F_{4,289} = 2.522$, **$P = 0.041$**
Differences between cytotypes within pollination treatment			
Selfing	-	$F_{1,51} = 1.862$, $P = 0.178$	$F_{1,107} = 5.096$, **$P = 0.026$**
Outcross (between cytotypes)	-	$F_{1,100} = 0.188$, $P = 0.666$	$F_{1,107} = 0.632$, $P = 0.428$
Outcross (within cytotypes)	-	$F_{1,55} = 0.522$, $P = 0.473$	$F_{1,61} = 0.689$, $P = 0.410$
Mixed outcross (4*x*+8*x*)	-	$F_{1,38} = 0.045$, $P = 0.832$	$F_{1,43} = 1.025$, $P = 0.317$
Outcross between cytotypes + selfing	-	$F_{1,45} = 21.689$, **$P < 0.001$**	$F_{1,48} = 18.697$, **$P < 0.001$**
Differences among pollination treatments within cytotype			
Tetraploids	-	$F_{4,196} = 34.749$, **$P < 0.001$**	$F_{4,256} = 51.597$, **$P < 0.001$**
Octoploids	-	$F_{4,93} = 16.485$, **$P = 0.002$**	$F_{4,110} = 7.492$, **$P < 0.001$**

For pollinations between cytotypes (treatments 3 to 5), the production of hexaploid seeds differed significantly between treatments for each cytotype (tetraploids: $\chi^2_{2,186} = 33.872$, $P < 0.001$; octoploids: $\chi^2_{2,355} = 91.802$, $P < 0.001$). The outcross between cytotypes resulted in a high production of hybrids (*i.e.*, 6*x* seeds) in both cytotypes, with no significant difference between them, although octoploids produced more hybrids than the tetraploids ($\chi^2_{1,187} = 2.564$, $P = 0.11$; Figure 5.5C). This pollination treatment revealed, once again, the production of unreduced gametes by tetraploid individuals, via both female and male gametes, detected by the production of octoploid seeds in both tetraploid and octoploid individuals. The mixed-ploidy pollen load treatments produced offspring with dissimilar ploidy compositions, according with the origin of the pollen (Figure 5.5C). Specifically, octoploids produced significantly more hexaploid seeds than tetraploids in both treatments (treatment 4: $\chi^2_{1,186} = 4.198$, $P = 0.04$; treatment 5: $\chi^2_{1,168} = 69.927$, $P < 0.001$). When the mixed-ploidy pollen treatment involved an outcross 4*x* and 8*x* pollen mixture (treatment 4), the offspring produced had the ploidy level of the mother, revealing a higher success of its own ploidy pollen to fertilize the ovules; when the mixed-ploidy pollen treatment involved an outcross with the other cytotype plus its own pollen (selfing) (treatment 5), octoploids produced mostly hybrids, while reproductive success of tetraploids significantly decreased and resulted in the production of a few tetraploids through selfing and few hexaploid seeds (either resulting from the fusion of self-unreduced gametes and/or hybrids between cytotypes). Several aneuploid seeds were also observed in most crosses, in particular when octoploids were involved (Figure 5.5C).

The differences obtained in the inter-cytotype crosses (treatments 3-5) lead to different gametic isolation levels depending on the composition of the pollen loads (Table 5.2). When the mother-plant received a pure-ploidy load from the other cytotype, reproductive isolation was similar for both cytotypes with total $RI_{gametic}$ of 0.60 (Table 5.2). When the mother plant received a mixed pollen load the reproductive isolation increased in both cytotypes to a total of $RI_{gametic}$ of 0.97, which mediated an almost total reproductive isolation between cytotypes (Table 5.2). Finally, when the recipient plant received a mixed-ploidy load composed by pollen from the other cytotype and self-pollen, the pattern differed between cytotypes: while tetraploids revealed a gametic isolation similar to the other mixed-ploidy treatment ($RI_{gametic,4x} = 0.96$), the octoploid revealed lower gametic isolation values ($RI_{gametic,8x} = 0.64$), similar to the pure-ploidy pollination (Table 5.2).

Cumulative effect of all studied reproductive barriers

The cumulative effect of the studied barriers resulted in total reproductive isolation values ranging from 0.73 to 0.98 (Figure 5.6, Table 5.2).

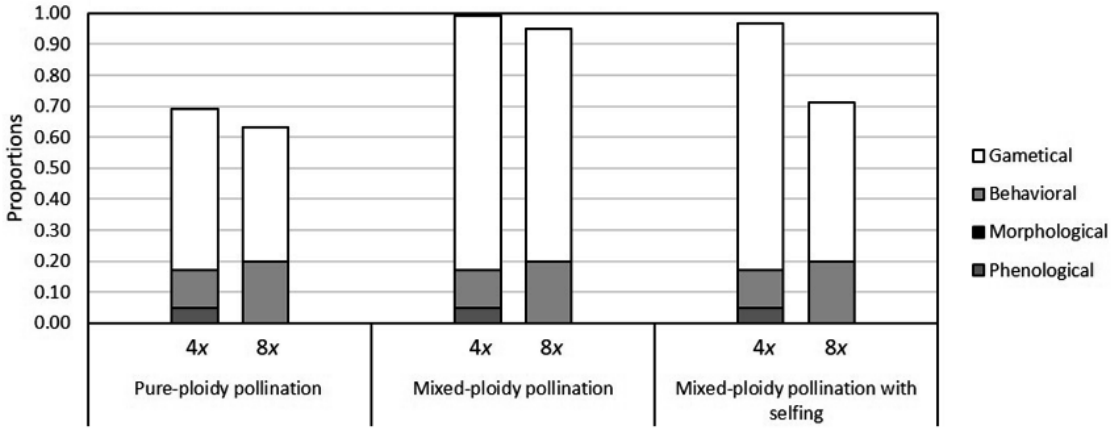


Figure 5.6. Relative contribution of the studied reproductive barriers for each cytotype of *Gladiolus communis* in three different scenarios (pure-ploidy pollination, mixed-ploidy pollinations and mixed-ploidy pollinations with selfing). Different colors represent different reproductive barriers: dark grey – Phenological; black – Morphological; light grey – Behavioral; white – Gametic.

The experimental mixed-ploidy population produced similar results (Figure 5.7) in comparison with the mixed-ploidy pollinations (treatment 4) described above. No statistical differences were observed in fruit set between cytotypes ($z_{1,409}$ = -0.08, P = 0.938, Figure 5.6A), and, although octoploids produced significantly more viable seeds than tetraploids ($z_{1,409}$ = -2.26, P = 0.02, Figure 5.7B), there were no significant differences in final reproductive success ($z_{1,409}$ = -1.34, P = 0.173, Figure 5.7C). Most of the offspring produced presented the ploidy level of the mother plant (Figure 5.7C). A few hexaploids were produced, but only by octoploids plants ($z_{1,624}$ = 0.01, P = 0.991). Finally, unreduced gametes were also observed (production of octoploids by tetraploids mothers and decaploids by octoploids), as well as the production of some aneuploids by both cytotypes.

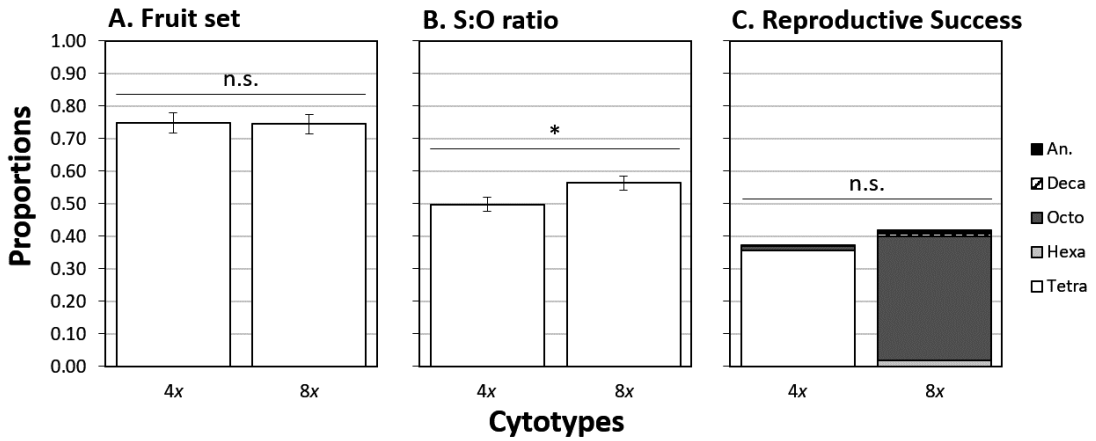


Figure 5.7. Reproductive variables in the experimental mixed tetraploid-octoploid population of *Gladiolus communis*: A) Fruit set (proportion of flowers that developed into fruit; mean and standard error of the mean); B) S:O ratio (proportion of ovules that developed morphologically viable seeds; mean and standard error of the mean); and C) Reproductive Success (fruit set multiplied by S:O ratio). Statistical comparisons between cytotypes are marked with * for significant results at $P < 0.05$ and with n.s. for non-significant values (4*x*, tetraploid; 8*x*, octoploid). In C), the bars provide the relative proportion of the ploidy-levels observed in the offspring of each cytotype by different colors: white – tetraploids (Tetra, 4*x*), light grey – hexaploids (Hexa, 6*x*), dark grey – octoploids (Octo, 8*x*), black diagonal stripes – decaploids (Deca, 10*x*), and black – aneuploids (An.).

DISCUSSION

Strong assortative mating between cytotypes reduced the loss of gametes in inter-cytotype cross, favoring pollen exchange between plants of the same cytotype, which can allow the coexistence of different cytotypes and the formation of mixed-ploidy populations in nature. We quantified the contribution of several reproductive barriers between tetraploid and octoploid plants of *G. communis* that co-occur in several areas forming complex contact zones (Castro *et al.* 2018 – Chapter 4). Our results revealed weak pre-pollination barriers and strong post-pollination interactions. In particular: 1) no differences in flowering phenology, flower morphology and display size were observed between cytotypes; and 2) both tetraploids and octoploids were visited by generalist pollinators without specific foraging preferences; by the contrary, 3) post-pollination isolation resulting from gametic isolation and post-zygotic viability was the strongest barrier to reproduction between cytotypes but the degree of isolation varied with pollen load composition.

The experimental manipulations of pollen load composition enabled us to evaluate different population scenarios. The results suggest lower isolation when the cytotype is rarer in the population, and higher isolation when cytotypes have similar opportunities to receive pollen from both ploidies. Selfing leads to different fitness outputs in each cytotype, with tetraploids

achieving higher degree of isolation but a significantly reduced fitness, while octoploids produced hybrid offspring. Additionally, our results showed that both tetraploids and octoploids are partially self-compatible, although the reproductive success of octoploid plants after selfing was higher than tetraploids.

The production of unreduced gametes was also detected in several treatments suggesting that this phenomenon might be common in natural populations and, together with hybrid production, contributes to the cytogenetic diversity of *G. communis* complex and to gene exchange between cytotypes, possibly contributing to the establishment and persistence of octoploid individuals in initial stages. All these results are discussed below based on the available bibliography and framed within the hypotheses proposed for polyploid evolution.

Pre-pollination reproductive barriers

To establish, neopolyploids need to overcome the minority cytotype disadvantage, usually by promoting assortative mating (Levin 1975; Husband and Saraba 2004). A diverse array of barriers can mediate assortative mating in mixed-ploidy populations before pollination takes place. We have addressed if assortative mating was mediated by differences on floral traits, like asynchronous flowering times, flower morphology and different pollinator foraging, in the tetraploid-octoploid *G. communis* contact zone. The results show complete overlap in flowering phenology of tetraploids and octoploids, and thus phenology by itself cannot prevent inter-cytotype crossing, exposing both cytotypes to the same window of pollination resources. The studies exploring phenological patterns in polyploid complexes have documented significant differentiation in flowering times among cytotypes, from total flowering divergence (Petit *et al.* 1997) to variable degrees of segregation (*e.g.*, Felber 1988; Bretagnolle and Thompson 1996; Husband and Schemske 2000; Ramsey 2011; Laport *et al.* 2016). In most cases, phenological barriers work in combination with other barriers to generate reproductive isolation. For example, differences in flowering time combined with unique pollinator assemblages influence reproductive isolation between the diploid *Erythronium mesochoreum* and the autotetraploid *E. albidum* at contact zones (Roccaforte *et al.* 2015); and differences in floral traits combined with different flowering time result in different pollinator assemblages and behavior in natural diploid and tetraploid *Heuchera grossulariifolia* populations (Segraves and Thompson 1999). Subsequent selection processes have also been shown to reinforce flowering time variation enabling cytotype co-existence, for example in *Plantago media* (van Dijk and Bijlsma 1994) and *H. grossulariifolia* (Nuismer and Cunningham 2005). Still, complete overlap in flowering

phenology, as the one observed here, has also been observed in other polyploid complexes such as *Aster amellus* (Castro *et al.* 2011) and *Gymnadenia conopsea* complexes (Jersáková *et al.* 2010). In these polyploid complexes, as well as in *G. communis*, flowering phenology can be ruled out as one of the mechanisms driving assortative mating in mixed-ploidy populations.

Floral traits might affect pollen flow in two different ways, either by differing in morphology and floral display which might lead to different pollinator preferences (*e.g.*, Segraves and Thompson 1999; Roccaforte *et al.* 2015), and/or driving differential pollen deposition on the insect body (Grant 1994). As described above polyploids may differ from their lower-ploidy progenitors in floral traits such as flower and inflorescence size and/or nectar and scent production (*e.g.*, Thompson and Merg 2008; Jersáková *et al.* 2010; Gross and Schiestl 2015; Sun *et al.* 2015). In *G. communis,* tetraploids and octoploids did not differ with respect to flower and inflorescence size and nectar production, nor for flower lifespan or floral display. Because these traits are linked with pollinator attraction and opportunities for pollen dispersal and reception (Grant 1994; Ramsey *et al.* 2003; Fulton and Hodges 1999; Sun *et al.* 2015), the lack of difference in floral traits was in accordance with lack of preferences by *G. communis* pollinators (see below), although some unstudied traits could also be involved with pollinator behavior and preferences (*e.g.*, nectar composition, Jersáková *et al.* 2010; flower color, Gross and Schiestl 2015). Additionally, the lack of differences in flower morphology is not surprising since recent reviews failed to detect subgroups of morphologically distinct individuals within the *G. communis* complex and rather a gradient in morphology was observed (Alonso and Crespo 2010). This was also clear from our field surveys in natural populations since no morphological trait could be used to differentiate between tetraploids and octoploids (M. Castro and S. Castro, field observations).

G. communis is visited by several generalist pollinator species that vary in abundance and distribution. Hymenoptera, in particular long- and short-tonged bees (*e.g.*, *Anthophora* and *Bombus* species), were the main pollinators of *G. communis*. These bees land on the lower tepals and collect the nectar accumulated at the base of the corolla tube, touching the anthers and stigmas with the upper part of their thorax. Flowers were also visited by smaller bees (*e.g.*, *Colletes* sp.) that behave differently as they moved around the anthers, touching the stigmas while collecting the pollen. Although accounting for a small fraction of the interactions, the flowers were also visited by several Lepidoptera; however, all these floral visitors behaved as nectar thieves, *i.e.*, collected nectar without touching the sexual organs while landing in the lower tepal (Inouye 1980; Castro *et al.* 2013). Few studies have addressed whether shifts in

plant-pollinator interactions might cause assortative mating in mixed-ploidy populations (reviewed in Segraves and Anneberg 2016).

Overall, the main pollinators showed no preference for a specific cytotype and randomly visited inflorescences in the mixed-ploidy arrays, revealing that pollinators do not discriminate between the two cytotypes. This behavior promotes pollen exchange between cytotypes and thus, similarly to phenology, pollinator behavior does not prevent hybridization between the two cytotypes of *G. communis* leading to low levels of reproductive isolation. The lack of preference may be due to the lack of differences in the floral traits related with pollinator attraction. Similar results were observed in other polyploid complexes visited by generalist pollinators or even by specific pollinator guilds (*e.g.*, *A. amellus*, Castro *et al.* 2011; *G. conopsea* complex, Jersáková *et al.* 2010; *Libidibia ferrea*, Borges *et al.* 2012). Still, the available studies showed a huge range of variation in pollinator's behavior: from similar pollinator assemblages to divergent communities and complete specialization of a pollinator to one cytotype (reviewed in Segraves and Anneberg 2016), or even asymmetric frequency of visitation to a given cytotype under similar pollinator communities (*e.g.*, Segraves and Thompson 1999; Kennedy *et al.* 2006). However, pollinators efficiency was not evaluated in these studies. Interestingly, we could observe a particular behavior of the pollinators, that is, pollinators tend to move to the nearest inflorescence (personal observation, M. Castro). Thus, if the cytotypes are clustered in natural populations, it is expected that pollinators might mediate higher levels of assortative mating than what we observed in our experiment (Segraves and Thompson 1999; Husband and Schemske 2000; Nuismer and Cunningham 2005; Thompson and Merg 2008). Similarly, under similar pollinator communities, tetraploid *C. angustifolium* was visited more frequently by specific pollinators likely due to the spatial arrangement of diploid and tetraploid plants within the population rather than driven by different cytotype preferences (Kennedy *et al.* 2006). Individual aggregation has also been shown to promote the maintenance of mixed-ploidy levels in *Ranunculus adoneus* (Baack 2004; Husband and Schemske 2000). Further studies in natural mixed-ploidy populations of *G. communis* are thus still needed to fully understand the role of pollinator behavior.

Although pre-pollinator barriers can facilitate the establishment of neopolyploids in populations from the progenitor ploidy and the maintenance of mixed-ploidy populations, *G. communis* shows very weak pre-pollination isolation leading to a random pollen flow within mixed-ploidy arrays. Similar flowering phenologies and floral traits between tetraploid and octoploid plants may result from recurrent polyploid formation and frequent gene flow between cytotypes at the contact zone, similarly to what has been proposed in *Larrea tridentata* (Laport

et al. 2016). This is supported by the fairly common production of unreduced gametes (results wherein), morphological resemblance (Alonso and Crespo 2010) and the occurrence of hexaploid plants in contact zones (Castro *et al.* 2018 – Chapter 4), with further studies being necessary to address these hypotheses.

Post-pollination reproductive barriers

Although pre-pollination barriers may significantly contribute to isolation (*e.g.*, Petit *et al.* 1997; Thompson and Merg 2008; Jersáková *et al.* 2010), post-pollination interactions are also important reproductive barriers between polyploids and lower ploidy parentals (Levin 1975; Castro *et al.* 2011; Borges *et al.* 2012; Pegoraro *et al.* 2016). Post-pollination processes can occur before or after ovule fertilization, ameliorating the fitness disadvantage created by inter-cytotype crosses (*e.g.*, Husband *et al.* 2002; Mráz 2003; Barringer 2007; Kao 2007). Still, early-acting post-pollination interactions are particularly important since they enable to reduce the resources allocated to the production of non-viable or sterile offspring (Burton and Husband 2001; Castro *et al.* 2011; Baack and Rieseberg 2007). Considering that *G. communis* present weak pre-pollination barriers, inter-cytotype pollen flow was expected in mixed-ploidy populations, and consequently one could hypothesize that post-pollination barriers would have to be strong otherwise the two cytotypes could not co-exist in sympatry. The controlled pollinations performed in a common garden enabled us to explore the interactions between the two cytotypes under diverse pollination scenarios. Interestingly, different pollination scenarios produced dissimilar results dependent on the ploidy of the mother plant and on the pollen load composition deposited in the stigmas, all discussed in continuation.

First, pure-ploidy pollinations enabled us to explore inter-cytotype cross-ability and quantify the production of hybrids excluding factors such as mixed-ploidy pollen loads and self-pollen deposition. Although the fitness of inter-cytotype crosses was lower when compared with intra-cytotype crosses, the crosses between cytotypes produced over 37% of morphologically viable seeds, with more than 60% of the seeds produced (62.2 and 78.3%, by tetraploids and octoploids, respectively) being detected as hexaploid hybrids. The differences in siring success between intra- and inter-cytotype crosses result most probably from pollen–pistil interactions that might affect pollen germination and pollen tube development along the style (as detected by Baldwin and Husband 2013) as well as from post-zygotic processes determining zygote development (*e.g.*, maternal-parental ratio and endosperm development, Müntzing 1933; Van Dijk *et al.* 1992; Burton and Husband 2000; Castro *et al.* 2011; reviewed in Lafon-Placette and

Köhler 2016). Still, the production of hexaploid offspring by both tetraploid and octoploid mothers suggests that pollen can germinate and successfully fertilize at least some of the ovules of the other cytotype after inter-cytotype crosses. These results suggest a weak barrier to reproduction between tetraploid and octoploid *G. communis* and will have major impacts, particularly at initial stages after the emergence of a new polyploid entity (discussed below). Post-pollination barriers were shown to be weak in other polyploid complexes, where triploids were observed in mixed-ploidy populations in diploid-tetraploid contact zones (*e.g.*, *Ranunculus adoneus*, Baack 2004; *Dactylorhiza maculata* s.l., Ståhlberg 2009). It is interesting to note that this controlled pollination also enabled to detect unreduced gamete formation by tetraploid plants, a process that might ameliorate minority cytotype disadvantages and feed the population with new polyploids (octoploids) emerging in parental populations (further discussed below).

Second, considering the lack of phenological shifts and pollinator preferences (results herein), controlled pollinations allowed us to explore the production of hybrids under mixed-ploidy pollen loads delivered by pollinators. Mixed-ploidy pollinations created a scenario of even proportions of pollen being delivered by the pollinators under random mating. Under this scenario, both tetraploid and octoploids mothers produced mostly offspring of its own ploidy. This result suggests that the pollen with the ploidy of the mother plant was more successful in fertilizing the ovules than the pollen from the opposite ploidy. A similar pattern was observed in tetraploid plants of *C. angustifolium* and it was attributed to a differential success of pollen tube development along the style, although this differential behavior allowed only a unilateral reproductive barrier with diploids less often failing to block triploid hybrid production (Husband *et al.* 2002). In interspecific interactions, pollen competition is considered a key reproductive barrier for hybridization (Carney *et al.* 1996; Diaz and Macnair 1999), being also observed in polyploid complexes. For example, Susiacue and Álvarez (1997) observed several differences in pollen germination and pollen tube growth in diploid and tetraploid plants of *Cucumis melo*. Haploid and diploid pollen grains had different germination requirements (Tanaka and Mukai 1955) which was then reflected in different pollen tube growths, with diploid pollen grains germinating slower (Susiacue and Álvarez 1997). Besides that, the behavior of pollen grains was also dependent of the ploidy level of the mother-plant, with inter-cytotypes crosses resulting in fruit production by tetraploids while no fruits were produced by diploids (Susiacue and Álvarez 1997). Like in *C. angustifolium*, pollen competition might have driven the different siring ability under mixed-ploidy pollinations in *G. communis*, although further studies of pollen tube development are needed. Still, the fitness of mixed-ploidy pollinations was also lower than intra-

cytotype crosses suggesting that some post-pollination interactions might also act to reduce the production of potentially unviable offspring. It is also interesting to note that octoploids were still able to produce some hexaploid individuals (in very low proportions). These results contrast with the high production of hybrid hexaploids detected in the pure-ploidy inter-cytotype crosses and suggest that when growing in sympatry and receiving mixed-pollen loads, post-pollination interactions are strong and lead to high reproductive isolation between tetraploid and octoploid plants, although some intermedium offspring that might serve as bridge for recurrent octoploid formation is still produced.

Third, pollination experiments enabled to assess self-incompatibility differences between the cytotypes and the role of self-pollen in mixed-ploidy pollen loads. Differences in the self-incompatibility could be involved in a fitness advantage at initial stages after polyploid emergence (Levin 1975; Barringer 2007) and were shown to be strategic mechanisms promoting neopolyploid establishment, despite being considered a short time solution (Husband 2016). *Gladiolus communis* revealed to be only partially self-compatible, and contrarily to hypothesis suggesting higher selfing ability in polyploid individuals compared with their lower-ploidy parentals (Barringer 2007; Borges *et al.* 2012), no differences were observed between cytotypes in the levels of self-compatibility when considering fruit and seed ovule ratio, although in general octoploids had a slightly higher self-compatibility than tetraploids (significant when analyzing the reproductive success). Additionally, we evaluated the role of self-pollination in escaping hybrid production through mixed-ploidy pollinations with self-pollen and pollen of the other ploidy. This treatment enabled to simulate initial stages after polyploid emergence where self-pollen deposition resulting from pollinator behavior when visiting the inflorescence (*i.e.*, all open flowers of the inflorescence in sequence) is possible and could represent an advantage. Contrasting results were obtained between cytotypes: while siring success was significantly lower in tetraploids and offspring was composed of few tetraploid and few hexaploid seeds, octoploids produced a significant amount of hexaploid offspring (similarly to inter-cytotype crosses). This suggest that although the octoploids had slightly higher reproductive success than tetraploid after selfing, the presence of self-pollen might not provide any benefit at the initial stages since it does not prevent the production of a high proportion of hexaploid hybrids. Contrarily, in tetraploids self-pollen deposition seems to significantly reduce the development of hybrid offspring, and consequently reduce the cost associated with its production. The significant decrease in hybrid offspring might have resulted from ovule blocking by self-pollen, ultimately leading to a significantly higher post-pollination barrier to reproduction than the one observed in octoploids. Still, although tetraploids achieved a higher degree of isolation than

octoploids they also presented significantly lower fitness. Octoploids by other way produced significantly more offspring of intermedium ploidy, which might serve as bridge for recurrent octoploid formation.

Regardless of the pollination scenario, it was clear that gametic barriers were the most important reproductive barrier in the polyploid *G. communis* complex and that the composition of the pollen load delivered by the pollinators greatly determined the production of hexaploid hybrids. Because the composition of the pollen load determines both cytotype fitness and offspring ploidy, the interactions between cytotypes are expected to be complex in natural contact zones.

Unreduced gamete formation

The production of unreduced gametes, *i.e.*, gametes with a somatic chromosome number, is a central feature for the emergence of new polyploid entities and a frequent phenomenon in nature (Harlan and Wet 1975; Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Bretagnolle 2001; Ramsey 2007). The controlled pollination experiments also enabled to detect the production of unreduced gametes after inter-cytotype crosses. Unreduced gametes were mainly produced by tetraploid plants, interestingly by both female and male gametes, while octoploids were more frequently involved with the production of aneuploids. The production of unreduced gametes by tetraploids might promote the recurrent polyploid formation and contribute to the diverse cytogenetic patterns observed in the complex.

In nature, unreduced gametes can occur in different scenarios and produce different cytogenetic entities (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). In *G. communis* tetraploid populations, the fusion of one reduced and one unreduced gamete will lead to the emergence of a “triploid bridge”, represented by the intermediate hexaploid cytotype. This is supported by the detection of hexaploid individuals within tetraploid populations outside tetraploid-octoploid contact areas (Castro *et al.* 2018 – Chapter 4). Also, the direct fusion of two unreduced gametes may lead to the emergence of octoploids plants that might establish in the population or spread to new areas. In sympatric areas, either generated by octoploid emergence within tetraploid populations or through secondary contact after range expansion, unreduced gametes produced by tetraploids might contribute to the establishment of octoploid cytotypes within the population. The production of unreduced gametes has been unequivocally attributed to the emergence of new entities (Bretagnolle and Thompson 1995), but unreduced gamete formation was also shown to play an important role in the subsequent

stages of establishment and spread of new polyploid entities (Felber 1991; Rausch and Morgan 2005; Suda and Herben 2013; reviewed by Kreiner *et al.* 2017a). In *C. angustifolium*, triploids play an important role in the establishment of tetraploids acting as "triploid bridge" (Husband 2004). A similar importance is given to the few triploids that can overcome the triploid block and become sexually successful (Burgess *et al.* 2014). In *G. communis*, the recurrent fusion of unreduced gametes may be sufficient to overcome the minority cytotype disadvantage and allow octoploid frequency to increase. Still, the information available on the rates of unreduced gamete formation is still insufficient and further studies are needed to understand the contribution of this process to the dynamics of *G. communis* contact zones.

Cytotype co-existence at contact zones

The quantification of the reproductive barriers potentially involved with isolation between tetraploid and octoploid *G. communis* individuals provided new insights into the processes operating at contact zones, not only at initial stages after polyploid emergence but also in later stages when the new polyploids increase their population size or come into contact with their lower-ploidy parentals.

What might be happening at initial stages? When a new cytotype emerges within the parental population (or arrives after dispersal), low reproductive isolation was observed between the cytotypes and thus, it is expected that the minority cytotype would be selectively excluded from the population. However, a fairly high proportion of unreduced male and female gametes produced by tetraploids suggest that polyploid formation might be frequent and may contribute to the establishment of octoploid plants within tetraploid populations (Felber 1991; Husband 2004; Suda and Herben 2013). Additionally, inter-cytotype crosses produced a significant amount of hexaploid seeds. The presence of flowering hexaploids in natural populations demonstrates that some hexaploids seeds are viable and that hexaploid plants can reach reproductive maturity (Castro *et al.* 2018 – Chapter 4), similarly to what was observed by Roccaforte *et al.* (2015). These hexaploid individuals may serve as a bridge ("triploid bridge" as defined by Ramsey and Schemske 1998), contributing to a recurrent polyploid formation and to their establishment in lower-ploidy populations. These hexaploid individuals were observed growing in pure-tetraploid populations, supporting the production of unreduced gametes in natural conditions, and in tetraploid-octoploid contact zones as well as after inter-cytotype crosses, suggesting that hybridization might also be occurring (Castro *et al.* 2018; results herein).

At contact zones, tetraploid plants might also disperse to an octoploid population, and under this scenario the minority cytotype is expected to be in disadvantage since individuals of this ploidy level mostly produce offspring of other ploidy levels, unless self-pollen is deposited by pollinators. Still, it remains unclear if the contribution of self-pollination is sufficient to overcome the minority cytotype disadvantage of the tetraploids.

What might be happening when cytotypes co-occur in similar proportions? When growing in sympatry and receiving mixed-pollen loads, post-pollination interactions were strong and lead to high reproductive isolation between tetraploid and octoploid plants. These interactions became clear when quantifying the cumulative effects of all the reproductive barriers in an experimental mixed-ploidy population, controlling for resource limitation and cytotype frequency. When tetraploids and octoploids where growing in similar proportions in the common garden they presented similar sexual reproductive success, produced offspring mainly of its own ploidy, and octoploids produced a few hexaploids (mostly probably after inter-cytotype mating), while tetraploids produced a few octoploids (mostly probably after inter-cytotype mating and unreduced gamete fusion). This might enable cytotype coexistence, although both cytotypes will be subjected to pollen and ovule discounting and some gene flow is still expected through the production of hexaploid hybrids or through the production of unreduced gametes by tetraploids. The stable coexistence of cytotypes were observed in *Tripleurospermum inodorum* diploid-tetraploid complex (Čertner *et al.* 2017), with mixed-ploidy populations being found in a secondary contact zone with diploids and tetraploids being reproductively isolated, and rarely produce triploids. This pattern was also founded in *Cardamine amara* (Zozomová-Lihová *et al.* 2015). Still, the dynamics of the populations depend on several other factors, and other life traits might influence plant fitness and drive cytotype frequencies within the population. The dynamic of contact zones of the polyploid *G. communis* complex is far from being completely understood and additional information on pollen tube growth rates and later acting barriers and life-history traits such as seed viability, dispersal capacity and asexual reproduction need to be evaluated to understand the entire picture.

CONCLUSIONS

While it has been largely accepted that pre-zygotic barriers are stronger than post-zygotic barriers in mediating species isolation (Ramsey *et al.* 2003; Coyne and Orr 2004), a different scenario might occur in polyploid complexes. The study of cytogenetic distribution patterns of *G. communis* has recently suggested that although the cytotypes are isolated geographically to some degree, they do not differ in niche requirements, being able to occur sympatrically. Indeed, cytotypes come into contact in several areas (Chapter 4) and because pre-pollination barriers appear weak, post-pollination interactions may constitute the most important barriers to hybrid formation. Still, the scenario in *G. communis* is far from simple. At the initial stage of polyploid formation, reproductive isolation between cytotypes is not complete, and unreduced gamete formation leading to a "triploid bridge" (here represented by hexaploid individuals) might contribute to octoploid establishment, while selfing might enable tetraploid persistence in the population, although significantly reducing its reproductive success. At later stages, when growing in similar proportions, post-pollination isolation was strong and might contribute to cytotype co-existence in sympatry, although equilibrium would depend on overall fitness of the cytotypes. Additionally, other traits such as micro-habitat segregation or asexual reproduction might also contribute to the maintenance of cytotypes in sympatry. The production of hexaploid hybrid offspring and unreduced gametes can thus suggest a dynamic contact area where polyploid formation and pollen flow is frequent. Further studies should focus on tetraploid-octoploid populations in variable proportions to test for minority cytotype exclusion and the factors involved in the establishment of polyploids, contributing to the understanding of polyploid success.

APPENDICES

Appendix 5.1. Pollinator assemblage and number of interactions for three tetraploid (4*x*) and three octoploid (8*x*) populations of *Gladiolus communis*. Total number of interactions as well as their relative percentage per population (given in parenthesis) are presented. Empty cells represent the absence of visits by a given visitor in the population. Taxa marked with * were observed visiting the artificial arrays. Populations: MC148 – Casal Duro, Fátima; MC212 – Secarias, Arganil; SC043 – Antões, Pombal; MC032 – Alcaria, Fátima; MC182 – Trouxemil, Coimbra; and MC190 – Casal da Rola, Soure.

Taxa	4*x*			8*x*			TOTAL
	MC148	MC212	SC043	MC032	MC182	MC190	
Order Hymenoptera	**789 (98.7)**	**432 (97.7)**	**345 (100.0)**	**1137 (99.0)**	**338 (94.9)**	**816 (99.3)**	**3857 (98.6)**
Amegilla quadrifasciata				3 (0.2)		30 (3.6)	33 (0.8)
Andrena haemorrhoa *						21 (2.6)	21 (0.6)
Anthidium florentinum *	16 (2.0)				211 (59.3)		227 (5.8)
Anthophora sp. *	52 (6.5)	383 (86.7)	50 (14.5)	64 (5.6)			549 (14.0)
Apis mellifera *				4 (0.3)		27 (3.3)	31 (0.8)
Bombus hortorum *	2 (0.3)			154 (13.4)		4 (0.5)	160 (4.1)
Bombus pascuorum *	706 (88.3)		15 (4.3)	514 (44.8)		655 (79.7)	1890 (48.3)
Bombus terrestris *	12 (1.5)		212 (61.5)	297 (25.9)		36 (4.4)	557 (14.2)
Colletes sp. *		38 (8.6)	68 (19.7)	35 (3.0)	94 (26.4)	40 (4.8)	275 (7.0)
Colletes sp.2 *					33 (9.3)		33 (0.8)
Eucera longicornis		5 (1.1)					5 (0.1)
Megachilidae *				63 (5.5)			63 (1.6)
Megascolia maculata spp. *flavifrons*						3 (0.4)	3 (0.1)
Panurgus sp.	1 (0.1)	6 (1.4)					7 (0.2)
Psithyrus cf. *campestris*				3 (0.3)			3 (0.1)

⇩Cont.

Order Lepidoptera	**10 (1.3)**	**10 (2.3)**	**0 (0.0)**	**11 (1.0)**	**18 (5.1)**	**6 (0.7)**	**55 (1.4)**
Euphydryas aurinia	2 (0.3)						2 (0.1)
Gonepteryx cleopatra	8 (1.0)			2 (0.2)			10 (0.3)
Gonepteryx rhamni *					12 (3.3)		12 (0.3)
Macroglossum stellatarum *		8 (1.8)		9 (0.8)	2 (0.6)	5 (0.6)	24 (0.6)
Thymelicus sylvestris *		2 (0.4)			4 (1.1)	1 (0.1)	7 (0.2)
Total no. of interactions	**799**	**442**	**345**	**1148**	**356**	**822**	**3912**

Appendix 5.2. Visitation rates (number of individual plants visited per hour) of each pollinator in three tetraploid (4*x*) and three octoploid (8*x*) populations of *Gladiolus communis*. Empty cells present the absence of visits by a given taxa in the population. The highest visitation rates are highlighted in bold. Populations: MC148 – Casal Duro, Fátima; MC212 – Secarias, Arganil; SC043 – Antões, Pombal; MC032 – Alcaria, Fátima; MC182 – Trouxemil, Coimbra; and MC190 – Casal da Rola, Soure.

Taxa	4*x*			8*x*			TOTAL
	MC148	MC212	SC043	MC032	MC182	MC190	
Order Hymenoptera	38.488	22.154	17.250	51.101	15.721	37.953	30.856
Amegilla quadrifasciata				0.135		1.395	0.264
Andrena haemorrhoa						0.977	0.168
Anthidium florentinum	0.780				**9.814**		**1.816**
Anthophora sp.	2.537	**19.641**	2.500	2.876			**4.392**
Apis mellifera				0.180		1.256	0.248
Bombus hortorum	0.098			6.921		0.186	1.280
Bombus pascuorum	**34.439**		0.750	**23.101**		**30.465**	**15.120**
Bombus terrestris	0.585		**10.600**	13.348		1.674	**4.456**
Colletes sp.		1.949	3.400	1.573	4.372	1.860	**2.200**
Colletes sp.2					1.535		0.264
Eucera longicornis		0.256					0.040
Megachilidae				2.831			0.504
Megascolia maculata spp. flavifrons						0.140	0.024
Panurgus sp.	0.049	0.308					0.056
Psithyrus cf. *campestris*				0.135			0.024

⇩Cont.

Order Lepidoptera	0.488	0.513	0.000	0.494	0.837	0.279	**0.440**
Euphydryas aurinia	0.098						0.016
Gonepteryx cleopatra	0.390			0.090			0.080
Gonepteryx rhamni					0.558		0.096
Macroglossum stellatarum		0.410		0.404	0.093	0.233	0.192
Thymelicus sylvestris		0.103			0.186	0.047	0.056
Total visitation rates	**38.976**	**22.667**	**17.250**	**51.596**	**16.558**	**38.233**	**31.296**

Appendix 5.3. Frequency of interaction (visitation rates multiplied by the mean of insects per hour) between *Gladiolus communis* and each visitor in three tetraploid (4*x*) and three octoploid (8*x*) populations. Empty cells represent the absence of visits by the insect in each population. The highest frequencies of interaction are highlighted in bold. Populations: MC148 – Casal Duro, Fátima; MC212 – Secarias, Arganil; SC043 – Antões, Pombal; MC032 – Alcaria, Fátima; MC182 – Trouxemil, Coimbra; and MC190 – Casal da Rola, Soure.

Taxa	4*x*			8*x*			TOTAL
	MC148	MC212	SC043	MC032	MC182	MC190	
Order Hymenoptera	347.329	59.077	48.300	438.666	58.496	227.721	171.066
Amegilla quadrifasciata				0.006		0.519	0.019
Andrena haemorrhoa						0.182	0.005
Anthidium florentinum	0.228				**23.280**		**0.828**
Anthophora sp.	1.609	**37.268**	1.375	1.681			**2.600**
Apis mellifera				0.024		0.584	0.026
Bombus hortorum	0.005			3.422		0.026	0.154
Bombus pascuorum	**265.432**		0.038	**111.093**		**127.528**	**44.271**
Bombus terrestris	0.171		**11.130**	25.197		0.312	**2.602**
Colletes sp.		0.899	3.910	0.353	3.660	0.692	**1.109**
Colletes sp.2					0.785		0.023
Eucera longicornis		0.039					0.001
Megachilidae				1.018			0.032
Megascolia maculata spp. flavifrons						0.013	0.000
Panurgus sp.	0.002	0.047					0.002
Psithyrus cf. *campestris*				0.006			0.000

⇩Cont.

Order Lepidoptera	0.048	0.079		0.111	0.312	0.026	0.070
Euphydryas aurinia	0.005						0.000
Gonepteryx cleopatra	0.019			0.004			0.001
Gonepteryx rhamni					0.104		0.003
Macroglossum stellatarum		0.042		0.073	0.009	0.011	0.014
Thymelicus sylvestris		0.005			0.017	0.002	0.002
Total frequency of interaction	**355.534**	**63.932**	**48.300**	**454.504**	**67.773**	**232.952**	**178.512**

PART III – Direct consequences of whole genome duplication in competitive ability

Chapter 6 – Production of synthetic tetraploids in the dune species *Jasione maritima*

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ABSTRACT

Polyploidization has been traditionally considered a phenomenon that mediates ecological differentiation, however, the adaptive value of genome duplications has been seldom explored. Natural or synthetic polyploids offer unique opportunities to experimentally quantify the immediate consequences of genome duplications at distinct levels. *Jasione maritima* is a diploid-tetraploid complex ideal to explore the genome duplications role in the success of polyploid lineages, but neotetraploids have never been found in nature. In this study we aimed to develop a methodology to obtain synthetic tetraploids from wild diploid plants of *J. maritima*. We tested the effect of different colchicine concentrations and seedling's age in survival and polyploidization success of *J. maritima* seedlings, considering also the origin population. A methodology to synchronize seed germination was also explored. Synchronization of seed germination was best achieved using a cold treatment of two weeks and further transfer to a growth chamber. An overall survival rate of 11.5 ± 0.7% and further tetraploid conversion of 35.6 ± 2.9% was obtained using 3-days-old seedlings of *J. maritima*. Survival rates were variable depending on colchicine treatment (the highest the most lethal) and seedling origin (*i.e.*, population), while conversion rate was similar across these factors and high rates of tetraploid conversion were obtained. Considering that in *J. maritima* the main constrain was survival to the colchicine treatment, we suggest the use of the lowest colchicine concentration tested, *i.e.*, 0.1% colchicine. The use of older seedlings increased survival rates but, in many cases, compromised a complete tetraploid conversion, generating many diploid-tetraploid mixoploid plants.

Keywords: colchicine, conversion rate, neotetraploids, seed germination, seedling age, seedling survival.

INTRODUCTION

Polyploidy as long been recognized to play a significant role in the evolution and diversification of flowering plants (Soltis 2005), being correlated with explosions in species diversity (Soltis *et al.* 2009). Accordingly, 15% of the speciation events in Angiosperms have been associated with ploidy increase (Wood *et al.* 2009). After polyploidization the architecture of the cell is modified, cell division must adapt to the new nuclear DNA content and deal with changes in the homology of the chromosomes, gene expression and epigenetics (Comai 2005; Husband *et al.* 2013; Barker *et al.* 2015). Changes in gene expression and developmental processes due to genome duplications can potentially generate shifts in the morphology, reproduction and physiology of the polyploid individual (Levin 2002). Consequently, it may provide unique or transgressive tolerances and developmental patterns, which could confer an advantage to these newly formed polyploids to conditions that are beyond the limits of their diploid (or lower ploidy) progenitors (Petit and Thompson 1999; Manzaneda *et al.* 2012).

Polyploidization has been traditionally considered a phenomenon that mediates ecological differentiation; however, the adaptive value of genome duplications has been seldom explored. Despite its importance, the majority of the studies published so far compared pairs of congeneric species or established cytotypes of a given species, without considering the temporal scale, *i.e.*, the time that has passed since the formation of the polyploid (*e.g.*, Segraves and Thompson 1999; Jersáková *et al.* 2010; Ramsey and Schemske 2002; Husband and Sabara 2004; Baack *et al.* 2015). In that sense, those studies did not enable to determine if the observed differences (or their absence) were due to subsequent evolution of the polyploid lineage, or if they were exclusively due to the duplication of the genome. The detection of newly formed polyploids in natural populations (*i.e.*, neopolyploids) using flow cytometry (Kron *et al.* 2007), or, alternatively, the synthesis of polyploids in the laboratory using mutagenic anti-mitotic agents (Husband *et al.* 2008), enables the unique opportunity to experimentally quantify the immediate consequences of genome duplications at distinct levels. While the comparison between diploid (or lower ploidy) progenitors and neopolyploids (either naturally occurring or synthetized) enables to explore the consequences of polyploidy *per se*, the comparison between neopolyploids and established polyploids enables to explore the changes accumulated after their origin (*e.g.*, Bretagnolle and Lumaret 1995; De Kovel and De Jong 2002; Ramsey and Schemske 2002; Oswald and Nuismer 2011b; Ramsey 2011; Münzbergová 2017; Pavlíková *et al.* 2017). Therefore, neopolyploids allow for evaluating the immediate effects of genome duplication and its role in the establishment and persistence of the new cytotype, being a key component when studying the ecological processes involved in polyploid evolution.

Natural occurring neopolyploids have been detected in a few polyploid complexes and were used to study the role of genome duplications in the successful establishment of polyploid lineages (*e.g.*, *Achillea borealis*, Ramsey 2011; *Chamerion angustifolium*, Maherali *et al.* 2009). Still, its occurrence in nature represents a screenshot in the evolutionary history of a given polyploid group and might thus be confined to recently formed complexes. As an alternative, researchers have developed methodologies to synthetize polyploids in the laboratory. Indeed, synthetic polyploids have long been used for plant breeding because of the advantages and new features conferred by genome duplications (Semeniuk and Arisumi 1968; Lumaret 1988; Levin 2002; Tamayo-Ordonez *et al.* 2016). However, ecological studies using this approach are much more recent and restricted to a few polyploid complexes of autopolyploid origin (*Chamerion angustifolium*, Husband *et al.* 2008, 2016; Maherali *et al.* 2009; Martin and Husband 2012, 2013; *Heuchera grossulariifolia*, Oswald and Nuismer 2011b; *Spartina pectinata*, Kim *et al.* 2012; *Vicia cracca*, Münzbergová 2017, Pavlíková *et al.* 2017).

Synthetic polyploids can be obtained by applying anti-mitotic agents that block the cell cycle, such as colchicine, oryzalin or trifuralin (Semeniuk and Arisumi 1968; Lignowski and Scott 1972; Jaskani *et al.* 2005; Zlesak *et al.* 2005; Chen *et al.* 2006; Allum *et al.* 2007). From this, colchicine is the most commonly used agent in both biotechnological and ecological studies (*e.g.*, Chen *et al.* 2006; Stanys *et al.* 2006; Ascough *et al.* 2008; Husband *et al.* 2008; Münzbergová 2017). Colchicine causes the depolymerization of the microtubular cytoskeleton in the early phases of metaphase, blocking the separation of chromosomes in mitoses, consequently, leading to polyploidization of the cells. In higher concentrations, in a later stage, it induces polymerization of new tubulin-containing structures in c-metaphase cells, allowing the reconstitution of 4C nuclei and their progression into the cell cycle (Caperta *et al.* 2006). In crop improvement, the induction protocols are usually applied *in vitro* to selected elite clones subjected to different colchicine concentrations, in solid or liquid cultures (*Saccharum officinarum*, Heinz and Mee 1970; *Citrus*, Gmitter *et al.* 1991; *Miscanthus* x *giganteus*, Yu *et al.* 2009). Contrarily, in ecological studies, synthetic polyploids are usually induced from seeds or seedlings obtained in natural populations (*e.g.*, Husband *et al.* 2008; Thompson*et al.* 2010; Münzbergová 2017).

Jasione maritima (Duby) Merino (Campanulaceae) is an endemic plant from northwest dune systems of the Iberian Peninsula, closely related with *J. montana*, a widely distributed species in Europe (Sales and Hedge, 2001a). *Jasione maritima* is a diploid-tetraploid complex with cytotypes showing an allopatric distribution, harboring diploid populations (2n = 2*x* = 12 chromosomes) in the northern parts of the distribution range, while tetraploid populations (2n

= 4*x* = 24) occur in the southern area (Chapter 2). This system is ideal to address questions related with polyploid establishment, because the cytotypes are distributed across an environmental gradient (Chapter 2) and seem to bear some differences in morphological and fitness related traits (Lago and Castroviejo 1992; Rubido-Bará *et al.* 2010), thus raising the question on the role of genome duplications in driving different distributional patterns and successful establishment and spread of neopolyploids. However, since no neotetraploids have been found in natural diploid populations of *J. maritima* (Chapter 2), to study the effects of genome duplications *per se*, it is fundamental to synthetize tetraploids in the laboratory. For this, an optimal procedure to obtain synthetic tetraploids in this species needs to be developed.

Considering all the above, the main objective of this study was to develop a methodology to obtain synthetic tetraploids from wild diploid plants of *J. maritima*. Specifically, we wanted to address the following questions: 1) what is the effect of different colchicine concentrations in survival and polyploidization success of *J. maritima* seedlings? 2) What is the effect of different seedling ages, in seedling survival and successful induction of neotetraploids? 3) Do the differences in polyploidization success vary between populations? As a result of this study, besides the proposal of an innovative approach to induce synthetic polyploids, with prospects of being applied to other study systems, a methodology to synchronize seed germination is also presented.

MATERIAL AND METHODS

Study species and field sampling

Fruiting heads of *Jasione maritima* were collected in July 2013 in four natural populations previously confirmed to be homogenously diploid, namely, Population 1 – Lariño (POP1), Population 2 – Fisterra (POP2), Population 3 – Neriña (POP3), and Population 4 – Soesto (POP4), all in La Coruña, Spain (for more details see Chapter 2). Within each population, fruiting heads from 40 mother plants, separated at least 4 m apart, were collected to individual paper bags. Seeds were air dried, cleaned from fruiting heads and harvested in labeled microtubes. Several seeds per mother plant (hereafter denoted as seed family) and several mother plants per population were used to study germination rates and explore differences in polyploidization success between populations and seed families. POP1 and POP2 were used in both germination and induction studies, POP3 was only used in the germination studies, and POP4 was only used for induction assays, due to a low seed availability.

Seed germination

Basic information about germination patterns is fundamental to determine the correct seedling stage for induction. Because no information was available on the germination patterns of *Jasione maritima*, a preliminary germination trial focused in obtaining high germination rates and, more importantly, synchronized germination was made. For this, 30 seeds from 20 mother plants from three populations were placed to germinate in individual Petri dishes with moist filter paper (POP1, POP2 and POP3), and were subjected to four treatments varying in the exposure to cold: 1) conditioned directly in a growth chamber (without cold treatment); 2) conditioned for 3 days at 4 °C in the dark and then transferred to the growth chamber; 3) conditioned for one week at 4 °C in the dark and then transferred to the growth chamber; 4) conditioned for two weeks at 4 °C in the dark and then transferred to the growth chamber. The conditions of the growth chamber were: 16:8h (light/dark) photoperiod with 24 °C of temperature. Petri dishes were watered when necessary (usually needed after transference to the growth chamber, being watered every two days). Seed germination was monitored for one month, every day during the first 2 weeks after transference to the growth chamber, and every two days afterwards. Total germination rates were calculated for each population and treatment as the percentage of seeds that germinated from the total number of seeds placed in the Petri dish. The time needed to reach 50% of total germination rate (T50) was calculated for each mother plant, enabling to characterize each treatment and population regarding the rate and pace of seed germination: lower T50 values would indicate higher germination synchrony, while higher T50 values would imply a germination extended over longer periods of time. The protocol that resulted in a higher number of seedlings in similar development stages at the moment of polyploid induction was selected for synthetic polyploids induction.

Synthetic polyploid's induction

Synthetic polyploids were induced directly in young seedlings from three natural populations (POP1, POP2 and POP4), by submerging them in aqueous solutions of colchicine (Husband *et al.* 2008). Two induction trials were made, one testing different colchicine concentrations and another testing different seedling ages. In the first trial, 3-days-old seedlings (T1) presenting fully expanded cotyledons and exposing the apical meristem, as well as bearing sufficiently smaller roots that could be manipulated without damage, were used. Up to 30 seedlings per mother plant and 40 mother plants per population were submerged in 0.1%, 0.2%, 0.5% or 1.0% aqueous colchicine solutions and left in the fume hood overnight for 14h. An

additional set was submerged in sterile ddH_2O for control. The seedlings were then rinsed five times with sterile ddH_2O. In the second trial, we tested the success of polyploid induction when using seedlings with different ages. For this, 2-weeks-old seedlings (T2) were subjected to a second induction trial using 0.5% colchicine concentration, following the procedure described above, and subsequently compared with the 3-days-old seedlings subjected to the same colchicine concentration. In both cases, after induction, seedlings were carefully transplanted directly to a multi-pot tray containing commercial standard soil. The seedlings were maintained in the greenhouse, watered daily and seedling mortality was monitored weekly.

Flow cytometry analyses

All the plants that survived were analyzed with flow cytometry to estimate genome size and DNA ploidy levels. Fresh leaves were used to prepare the nuclear suspension following Galbraith *et al.* (1983) protocol, by simultaneously chopping the plant material of the sampled plant with leaf tissue of *Solanum lycopersicum* 'Stupické' (internal reference standard, 2C = 1.96 pg, S.l.; Doležel *et al.* 1992). Nuclei were isolated in 1 ml of Woody Plant Buffer (WPB; Loureiro *et al.* 2007) and filtered through a 50 µm nylon filter. Then, 50 µg ml^{-1} propidium iodide and 50 µg ml^{-1} RNAse were added to the sample, to stain the DNA and degrade double-stranded RNA, respectively. The sample was analyzed in Partec CyFlow Space flow cytometer (532 nm green solid-state laser, 30 mW; Partec GmbH., Görlitz, Germany). Partec FloMax software v2.4d (Partec GmbH, Münster, Germany) was used to obtain the following graphics: fluorescence pulse integral in linear scale (FL); forward light scatter (FS) vs. side light scatter (SS), both in logarithmic (log) scale; FL vs. time; and FL vs. SS in log scale. DNA ploidy levels were inferred for each individual plant based on the chromosome counts and respective genome sizes (cytotype: mean ± SD; diploids: 2n = 2*x* = 2.98 ± 0.07 picograms; tetraploids: 2n = 4*x* = 6.06 ± 0.11 pg; Figure 6.1A-B). According to this, each plant was classified as DNA diploid, DNA tetraploid, DNA octoploid, DNA aneuploid, diploid-tetraploid mixoploids and tetraploid-octoploid mixoploids.

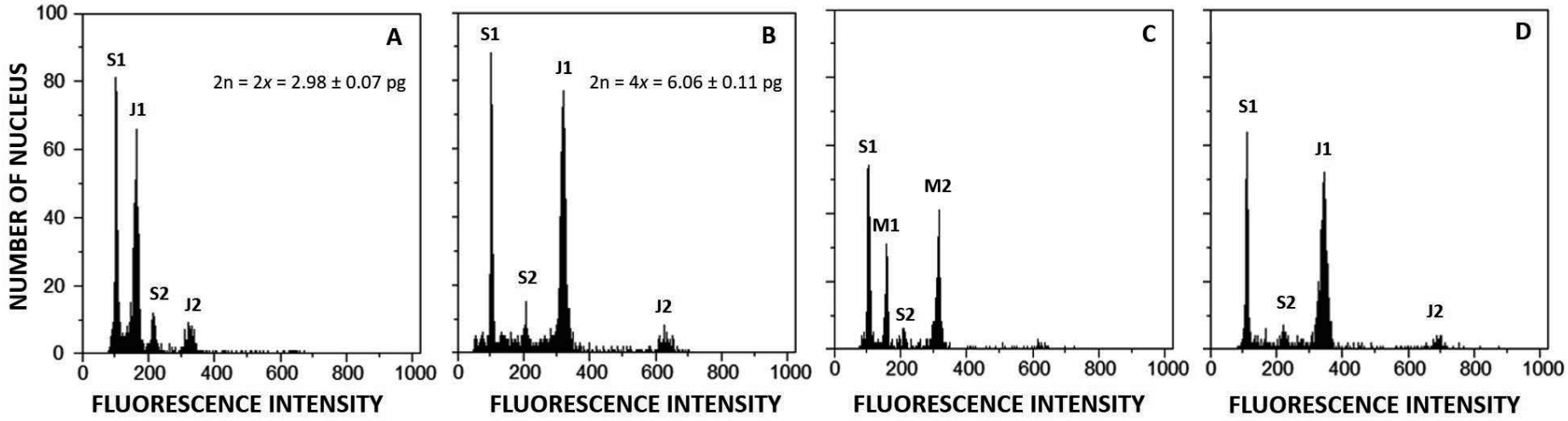


Figure 6.1. Flow cytometric histograms of *Jasione maritima* genome size analyses. Histograms of relative propidium iodide fluorescence intensity of nuclei obtained from fresh leaves of *Solanum lycopersicum* 'Stupické' (as reference standard, 2C = 1.96 pg) and *Jasione maritima*: **A.** natural diploid *J. maritima*; **B.** natural tetraploid *J. maritima*; **C.** diploid-tetraploid mixoploid individual obtained after colchicine treatment; and **D.** DNA tetraploid individual obtained after colchicine treatment. S1 and S2 correspond to G_1 and G_2 peaks of *S. lycopersicum*, respectively; J1 and J2 correspond to peak G_1 and G_2 of *J. maritima*, respectively; M1 and M2 correspond to nuclei with 2C and 4C genome size values, respectively. Mean genome size values (± SD) of natural diploid and tetraploid populations of *J. maritima* (Chapter 2) are provided in the respective histograms.

Statistical analyses

General Linear Models (GLMs) and Generalized Linear Mixed Models (GLMMs) were used to analyze differences in germination rates among treatments and populations. First, we explored overall differences in germination rates and in T50 among treatments, by defining germination treatment as fixed factor, population as random factor, and germination rate and T50 as response variables. Germination rates were arccosine transformed. A Gaussian distribution with an identity link function and a Poisson distribution with a log link function were used to model germination rate and T50, respectively. Differences among populations and colchicine treatments nested within population were also tested as fixed factors, with germination rate and T50 as response variables, as described above.

GLMMs were also used to analyze differences in survival and induction rates among colchicine treatments, populations and seedling ages. First, we explored overall differences in survival and induction success among colchicine treatments, defining colchicine concentration as fixed factor, population and mother plant as random factors, and survival and induction success as response variables. A binomial distribution with a logit link function was used to model response variables. Second, because population could impact the response of the plants, we also explored differences among populations and colchicine treatments nested within population as fixed factors, including, as above, mother plant as random factor and survival and induction success as response variables. Finally, a similar approach was used to explore differences in survival and induction success among seedlings with different ages and populations. When significant differences were observed, post hoc tests for multiple comparisons were performed.

The analyses were performed in R software version 3.0.1 (R Core Development Team, 2016), using the packages “car” for Type-III analysis of variance (Fox *et al.* 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).

RESULTS

Seed germination

Jasione maritima presented germination rates of 93.1 ± 0.5% (mean ± SE), on average. Overall, germination rates differed significantly among treatments ($F_{3,236}$ = 4.47, P < 0.001), with cold treatments increasing total germination rates (P < 0.05) [Appendix 6.1]. However, when analyzing in a nested design the differences became less evident: while significant differences were observed among populations ($F_{2,228}$ = 7.93, P < 0.001; population: mean ± SE, POP1: 89.6 ± 1.1%, POP2: 96.3 ± 0.6%, POP3: 93.3 ± 0.9%), among treatments within population the differences were near the significance level ($F_{9,228}$ = 1.93, P = 0.05), with the subsequent multiple comparison tests showing no significant differences (P > 0.05) [Appendix 6.1].

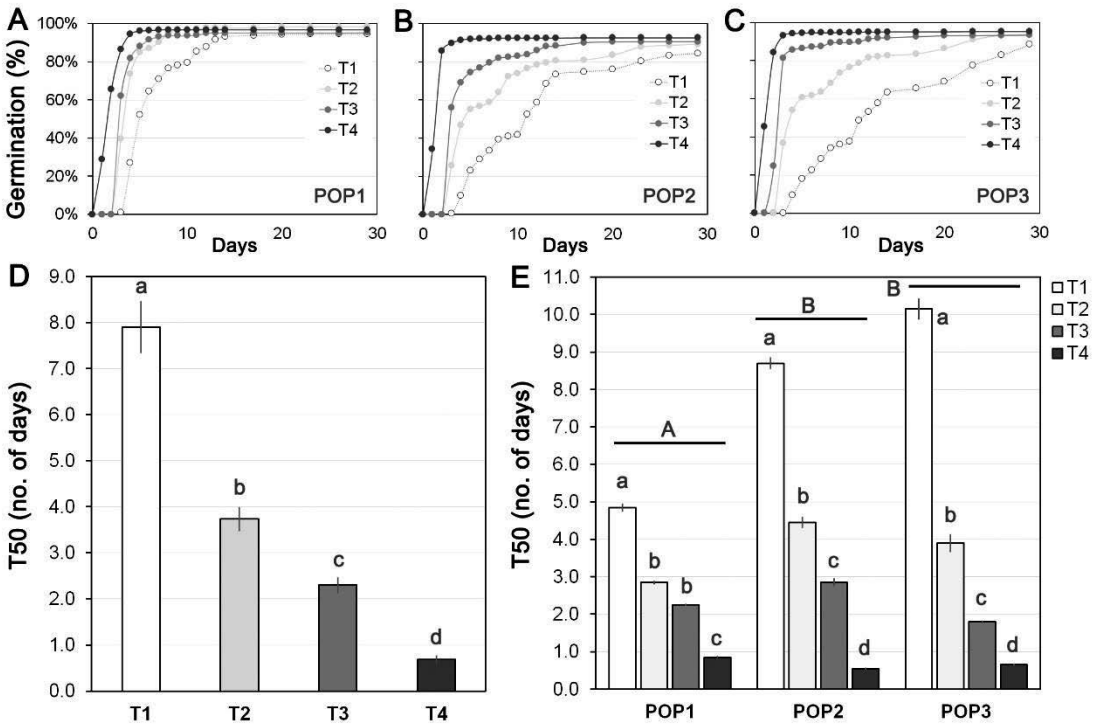


Figure 6.2. Seed germination of *Jasione maritima* diploid populations under different germination conditions. Seed germination (in %) along one month after different cold treatments is provided for: A. population 1 (POP1); B. population 2 (POP2); and C. population 3 (POP3). The time needed to reach 50% of total germination rate (T50; mean ± SE; in number of days) is given for: D. each treatment; and E. each treatment within each studied population. Treatment before transfer to the growth chamber: T1, without cold treatment; T2, 3 days at 4 °C; T3, one week at 4 °C; and T4, two weeks at 4 °C. Different lower-case letters denote significant differences among treatments, including the total comparison (in D.) and among treatments within population (in E.) at P < 0.05; different upper-case letters denote significant differences among populations at P < 0.05.

Despite the total germination rates were overall high among treatments, which enabled to easily obtain seedlings for the induction experiments, the largest differences were observed in the pace of germination, with the increased exposure to cold increasing the germination speed (Figure 6.2A-C). This pattern was reflected in the T50 values we obtained (Figure 6.2D-E). The T50 differed significantly among treatments (χ^2_3 = 362.83, P < 0.001), being as slow as 7.9 ± 0.6 days without cold treatment to as fast as 0.7 ± 0.1 days with the longest cold treatment (Figure 6.2D). Differences were also observed among populations (χ^2_2 = 36.59, P < 0.001), with POP2 having significantly lower T50 than the other two populations (P < 0.05; population: mean ± SE, POP1: 3.7 ± 0.02%, POP2: 2.7 ± 0.02, POP3: 4.1 ± 0.06), and among treatments within population (χ^2_9 = 363.40, P < 0.001), with T50 showing the same patterns, *i.e.*, significantly decreasing with the increased exposure to cold (P < 0.05; Figure 6.2E).

Synthetic polyploid's induction – Colchicine concentrations

An overall survival rate of 11.5 ± 0.7% and a tetraploid conversion rate of 35.6 ± 2.9% was obtained in *J. maritima*. Survival of control seedlings submerged in ddH_2O was 100%, thus indicating that mortality was mainly due to the colchicine treatment. Survival rates were variable depending on the colchicine treatment and seed origin (*i.e.*, population), while conversion rates were similar across these factors (*i.e.*, colchicine treatment and population), being surprisingly high.

Seedling survival varied between 4.5 ± 0.9% and 19.5 ± 1.5% (mean ± SE; for treatments with 1.0% and 0.1% of colchicine, respectively) and differed significantly among colchicine treatments (χ^2_3 = 35.69, P < 0.001), with survival significantly decreasing with increased colchicine concentration (P < 0.05) (Figure 6.3A). Differences were also observed among populations (χ^2_2 = 12.40, P = 0.002), with one of the populations (POP2: 18.6 ± 1.4%) having significantly higher survival rates than the remainder populations (POP1: 7.3 ± 0.9% and POP4: 8.3 ± 1.0%; P < 0.05), and among concentrations within population (χ^2_9 = 39.89, P < 0.001). Again, survival decreased with increased colchicine concentration, although this effect was only significant in POP1 and POP2 (P < 0.05; Figure 6.3B).

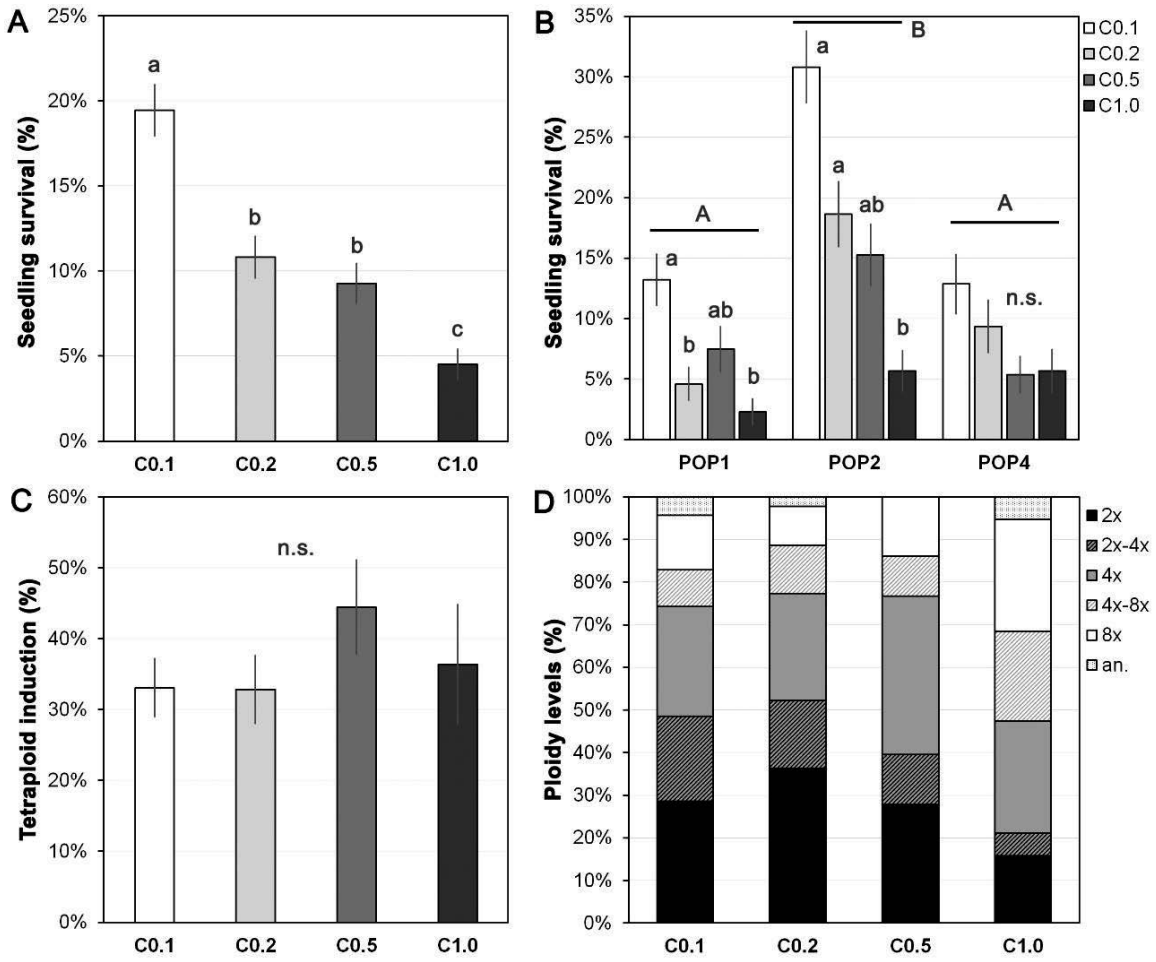


Figure 6.3. Colchicine treatment effect on seedling survival, synthetic tetraploid induction and ploidy levels of treated seedlings. A. Overall seedling survival for each treatment with different colchicine concentrations; B. Seedling survival for each treatment within each studied population; C. Synthetic tetraploid induction rate per treatment; and D. Ploidy levels of the plants after treating seedlings with different colchicine concentrations. Treatment with different colchicine concentrations: C0.1, colchicine at 0.1%; C0.2, colchicine at 0.2%; C0.5, colchicine at 0.5%; C1.0, colchicine at 1.0%. Ploidy levels: 2*x*, DNA diploid; 2*x*-4*x*, diploid-tetraploid mixoploids; 4*x*, DNA tetraploid; 4*x*-8*x*, tetraploid-octoploid mixoploids; 8*x*, DNA octoploid; an., DNA aneuploids. Values are given in percentage as mean and standard error of the mean (in A-C) or percentage from the total (in D). Different lower-case letters denote significant differences among treatments, including the total comparison (in A.) and among treatments within population (in B.) at $P < 0.05$; different upper-case letters denote significant differences among populations at $P < 0.05$; n.s. denote non-significant differences between treatments at $P > 0.05$.

The induction success, measured through the production of tetraploids, did not differ significantly between colchicine concentrations ($\chi^2_3 = 2.57$, $P = 0.463$), although there was a trend of increasing tetraploid induction (Figure 6.1C) with an increased colchicine concentration (Figure 6.3C-D). The lack of differences was also observed among populations ($\chi^2_2 = 0.59$, $P = 0.743$) and colchicine concentrations within population ($\chi^2_9 = 8.35$, $P = 0.499$). Still, although the proportion of synthetic tetraploids did not differ significantly between colchicine treatments, the ploidy levels detected in the seedlings were variable among colchicine treatments. The

higher concentration tested originated a lower percentage of DNA diploid and diploid-tetraploid mixoploid individuals (Figure 6.1D) and a higher percentage of individuals with higher ploidies, including DNA octoploids and tetraploid-octoploid mixoploid plants (Figure 6.3D).

Synthetic polyploid's induction – Seedling age

The age at which the seedling was manipulated affected significantly the survival rates (χ^2_1 = 23.60, P < 0.001), with younger seedlings having significantly lower survival rates than the older ones (mean ± SE, 9.3 ± 1.2% and 19.8 ± 1.1%, respectively; P < 0.05; Figure 6.4A). However, a high variability was also observed due to population, with significant differences being observed among populations (χ^2_2 = 17.43, P = 0.001), with one of the populations (POP4: 10.5 ± 3.6%) having lower survival rates than the other two (POP1: 16.3 ± 6.2% and POP2: 16.9 ± 1.1%; P < 0.05). Also, significant differences were observed between seedling ages within population (χ^2_3 = 35.24, P < 0.001) (Figure 6.4B), with the overall pattern of increasing survival with increased age being evident in each population. When populations were analyzed separately, the differences were only significant for POP1 and POP4 (P < 0.05; Figure 6.4B).

Once again, no differences were observed in the percentage of synthetic tetraploids obtained between the two groups varying in seedling age (χ^2_1 = 2.65, P = 0.104), although there was a pattern of lower percentage of tetraploid induction with increased age (Figure 6.4C). The lack of differences was also consistent among populations (χ^2_2 = 0.29, P = 0.865) and among colchicine concentrations within population (χ^2_3 = 4.83, P = 0.184). Despite no statistical differences were observed, older seedlings subjected to colchicine treatment seemed to produce a higher percentage of diploid-tetraploid mixoploids plants (marginally significant: χ^2_1 = 3.61, P = 0.057) instead of tetraploid individuals (Figure 6.4D).

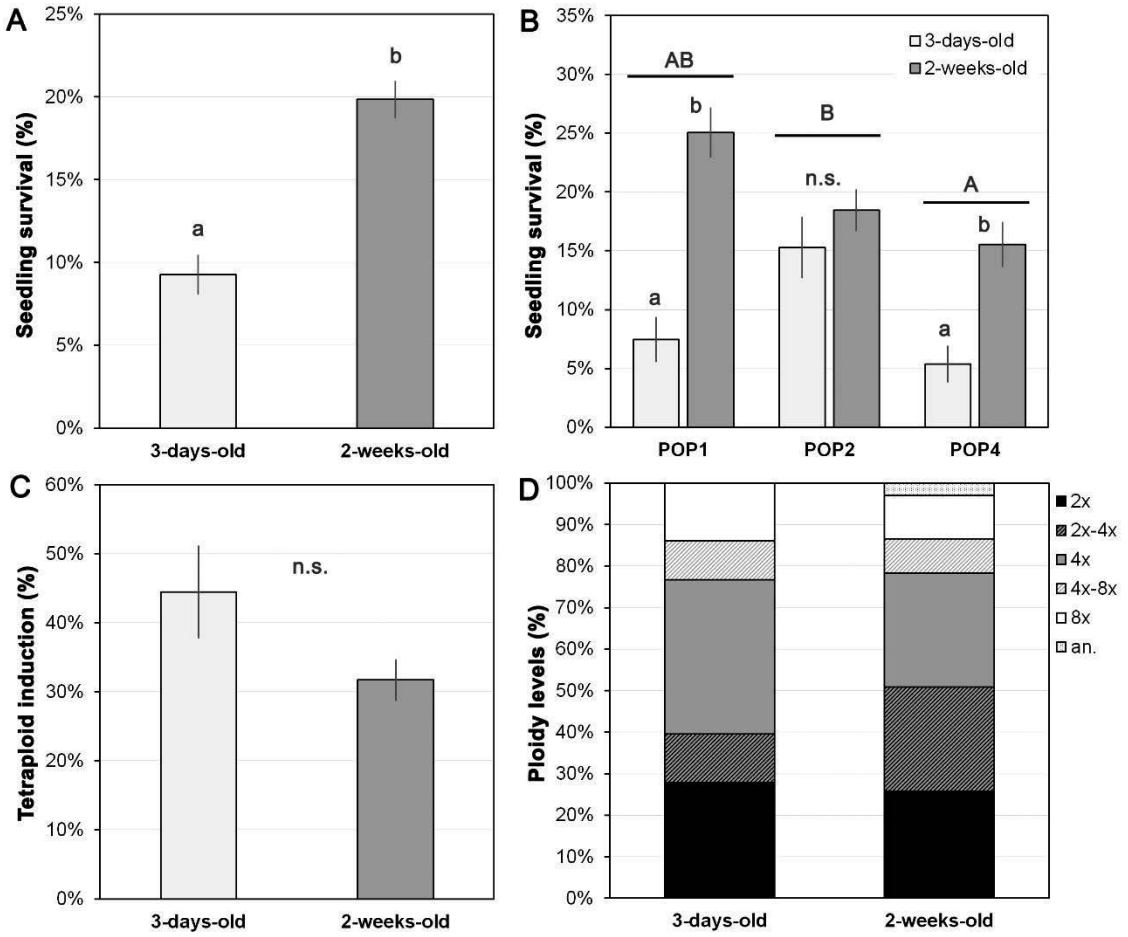


Figure 6.4. Seedling age effect on seedling survival, synthetic tetraploid induction and ploidy levels of treated seedings. A. Overall seedling survival for each age category; B. seedling survival for each age category within each studied population; C. Percentage of synthetic tetraploid induction per age category; and D. Ploidy level of the plants after treating seedlings with different ages with colchicine. Age categories denote the age at which the seedling was treated with colchicine: 3-days-old and 2-weeks-old seedlings. Ploidy levels: 2*x*, DNA diploid; 2*x*-4*x*, diploid-tetraploid mixoploids; 4*x*, DNA tetraploid; 4*x*-8*x*, tetraploid-octoploid mixoploids; 8*x*, DNA octoploid; an., DNA aneuploids. Values are given in percentage as mean and standard error of the mean (in A-C) or percentage from the total (in D). Different lower-case letters denote significant differences among treatments, including the total comparison (in A.) and among treatments within population (in B.) at $P < 0.05$; different upper-case letters denote significant differences among populations at $P < 0.05$; n.s. denote non-significant differences between treatments at $P > 0.05$.

DISCUSSION

Jasione maritima is a diploid-tetraploid complex ideal to explore the role of genome duplications in the success of polyploid lineages, but neotetraploids have never been found in nature. In this study we tested the effect of different colchicine concentrations and seedling ages in polyploid induction and the successful production of synthetic tetraploids from several populations and seed families of the diploid *J. maritima*.

The success of the polyploidization process depends of numerous factors such as, the type of explant, the anti-mitotic agent used and its ability to penetrate the cells, the concentration applied and the duration of the exposure (Ascough *et al.* 2008, and references therein). The protocols to induce polyploidy can depart from a wide variety of plant materials, in which, the presence of active meristems is fundamental to achieve successful polyploidization. However, in the case of ecological studies the most frequently used explants are seeds (Pringle and Murray 1992; Walters and Wehne, 2002; Thompson *et al.* 2010) or seedlings (Husband *et al.* 2008; Tate *et al.* 2009; Pavlíková *et al.* 2017), as such plant materials enable to introduce the variability of the diploid (or lower ploidy level) parental populations. The use of seeds and seedlings as explants are also particularly suitable for species with small seeds such as *Jasione* species. However, the use of seedlings requires the development of a protocol that enables the obtainment of a high number of explants ready for polyploidization induction, especially if polyploidization rates are low, and ideally with synchronized development. In this study, a protocol of synchronization of seed germination using a cold treatment is proposed, in which most of the seeds germinated within a few days after being transferred from cold conditions to optimal growing conditions. Despite we observed that the seeds of *J. maritima* germinated at room temperature, the cold treatment seemed to synchronize seed germination by holding the development of the seedling until the temperature conditions are favorable (Garcia *et al.* 2006; Ribeiro and Costa 2015). This enabled us to obtain a high number of seedlings in a similar developmental stage for induction of polyploidization. It is worth noticing that high levels of germination were observed in this species, averaging 93%, although values varied significantly between 84.9% to 98.5%, according with the population and treatment.

Colchicine has been successfully used with a wide range of concentrations depending on the studied species (*e.g.*, from very low concentrations, 0.00001% in *Lychnis senno*, Chen *et al.* 2006; to very high concentration, 1.5% in *Chaenomeles japonica*, Stanys *et al.* 2006). The literature suggests that while the treatments based on higher concentrations of colchicine and/or exposure for longer periods are necessary for successful polyploidization, it was also verified that these treatments are very aggressive, being in many cases lethal to the explants (*e.g.*, *Acacia crassicarpa*, Lam *et al.* 2014; *Chaenomeles japonica*, Stanys *et al.* 2006; *Cucumis* spp., Walters and Wehner 2002; *Lychnis senno*, Chen *et al.* 2006; *Watsonia lepida*, Ascough *et al.* 2008). Consequently, the ideal protocol must consider a balance between survival and successful polyploidization. Here, after testing a series of increasing concentrations, we observed that the most detrimental step to produce synthetic tetraploids in *J. maritima* was the lethality of the seedlings after the colchicine treatment. Survival of *J. maritima* seedlings was

low, being always lower than 19.5%, decreasing significantly with increased colchicine concentrations to 4.5%. This result was not surprising given the fragile nature of the seedlings. Similar patterns have been reported in the available studies (Chen *et al.* 2006; Stanys *et al.* 2006; Ascough *et al.* 2008; Ntuli and Zobolo 2008; Pavlíková *et al.* 2017). Also, expectedly, survival rates were also affected by seedling age with older seedlings presenting higher survival rates (increasing survival from 9.3% to 19.8% when subjected to the same colchicine concentration).

Despite of the low survival rates after exposure to the anti-mitotic agent, we obtained high polyploidization rates in comparison with other studies (*e.g.*, 11% in Ascough *et al.* 2008; 13% in Lam *et al.* 2014; 13% in Sakhanokho *et al.* 2009). Surprisingly, the conversion rates did not differ among colchicine treatments (35.6% of neotetraploids from the total surviving seedlings) or between seedling ages (34.0%). This result was surprising because most studies up to date showed an increased polyploidization success with increased concentrations or exposure to colchicine (*e.g.*, Chen *et al.* 2006; Stanys *et al.* 2006; Ascough *et al.* 2008; Ntuli and Zobolo 2008; Sakhanokho *et al.* 2009; Pavlíková *et al.* 2017). Additionally, some patterns have emerged when analyzing the ploidy level of the surviving plants. First, the seedlings subjected to higher colchicine concentrations tended to have lower percentages of diploid and of diploid-tetraploid mixoploids; instead, they presented higher percentages of tetraploid-octoploid mixoploids and even higher amounts of octoploid individuals than the set of seedlings subjected to the lower colchicine concentrations, with 0.5% colchicine being apparently the turnover point. Second, when comparing seedlings of different ages subjected to 0.5% of colchicine, older seedlings tended to result in lower percentages of tetraploid plants and higher percentages of diploid-tetraploid mixoploids, suggesting that older seedlings did not convert as efficiently as younger ones. Thus, selecting the ideal developmental stage and age to subject the seedling to the anti-mitotic agent seems important, despite such factor has seldom been tested. Instead, most studies using seedlings exposed the explants when the cotyledons are fully expanded (Omran and Mohammad 2008; Ye *et al.* 2010; Zhang *et al.* 2010) so that the meristems in active division are fully exposed to the colchicine solution. Because of the very few information on explant's age available in the low number of protocols that have used seedlings, it is difficult to compare polyploidization success rate across studies.

As referred above, in some of the concentrations, besides the formation of synthetic polyploids, many of the treated seedlings resulted in plants presenting tissues with mixed ploidy patterns, especially diploid and tetraploid in the lower colchicine concentrations, or tetraploid and octoploid in the highest concentration. The creation of chimeric individuals is quite common in polyploidy induction studies (Pringle and Murray 1999; Vainola and Repo 2001; Ascough *et al.*

2008), despite that at variable rates, and it has been attributed to the use of a multicellular tissue and to an uneven penetration of the colchicine to the seedling's tissue. Considering the relatively high rates of tetraploid induction in the seedlings that survived, the progress of those mixoploid plants was not followed.

So far, most of the studies that compared natural diploids and polyploids with synthetic polyploids were based in seeds or seedlings originated from a single population or did not account for this factor (Husband *et al.* 2008; Maherali *et al.* 2009; Griffin *et al.* 2012; Husband *et al.* 2016). Despite of that, differences between synthetic polyploids of a different origin can be expected, as it was revealed by Oswald and Nuismer (2011b) in *Heuchera grossulariifolia*. In our study, most of the population related differences were observed in seed germination and seedling survival. A recent study by Münzbergová (2017), also pointed for interactions between population and some of the traits that were compared in natural and synthetic tetraploids of *Vicia craca* (*e.g.*, plant size at 2 weeks, the measures of seed production and stomata size). This suggests that the colchicine effects are context dependent and probably result from the varying genetic composition of each population. Thus, the use of multiple populations should be regarded as an important aspect when developing a new protocol to induce polyploidy, at least, in ecological related studies.

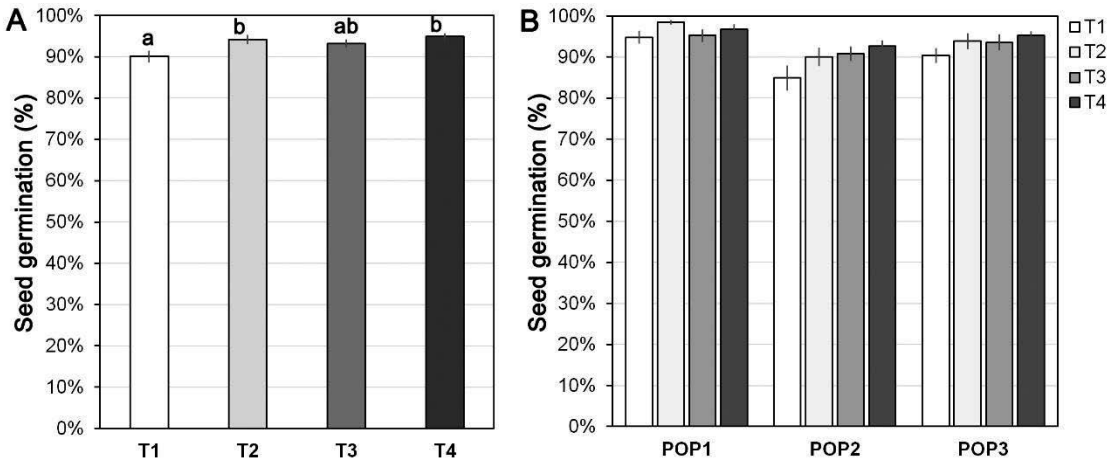
Although being an invaluable tool, in particular when no neopolyploids are not found in nature, the production of synthetic polyploids presents some drawbacks that should be highlighted. In some studies, diploids progenitors and synthetic polyploids expressed different phenotypes, including production of a higher amount of biomass, larger organs, higher amounts of secondary metabolites, among other effects (Hassan and Wazuddin 2000; Jaskani *et al.* 2005; Contreras *et al.* 2007; Cohen *et al.* 2013). However, these changes were pointed to result not only from polyploidization, but from the combination of polyploidization and of anti-mitotic compound effects (Ramsey and Schemske 2002). To circumvent this problem, Husband *et al.* (2008) suggested that the phenotypic effects of the anti-mitotic agents could be eliminated by using the second-generation of synthetic polyploids (either by selfing or by intercrossing two synthetic polyploids of the same population) and by analyzing the performance of the offspring of these parents. Still, it is not clear if the genetic effects of anti-mitotic substances persist. A recent study by Münzbergová (2017) suggested that plant performance can also be affected by colchicine treatment, even in the second generation.

CONCLUSIONS

The survival and polyploidization rates obtained here suggest that the lowest colchicine concentration (0.1%) applied to younger seedlings (3-days-old) was the most successful treatment, as it enabled a higher survival of the seedlings than the more aggressive concentrations, while maintaining a relative high induction rate, leading to an overall higher number of synthetic tetraploids. The use of older seedlings might increase survival rates, but it seems to compromise a complete tetraploid conversion by generating many diploid-tetraploid mixoploid plants.

APPENDICES

Appendix 6.1. Seed germination of *Jasione maritima* diploid populations under different germination conditions. Seed germination (mean ± SE; in %) for: A. different cold treatments; and B. each treatment within each studied population. Treatment before transfer to the growth chamber: T1, without cold treatment; T2, 3 days at 4 °C; T3, one week at 4 °C; and T4, two weeks at 4 °C. Different lower-case letters denote significant differences among treatments at $P < 0.05$.



Chapter 7 – Competitive ability does not favor neopolyploid establishment but explains the current distribution of a diploid-tetraploid plant complex

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ABSTRACT

Polyploidy is a pervasive phenomenon in nature and has significantly contributed to the adaptive evolution of plants. The conditions necessary for invasion of neopolyploids in populations of the diploid progenitor are limited; however, a superior competitive ability of neopolyploids may promote its establishment. Here, we assess the contribution of genome duplication *per se* to divergence of plant traits affecting competitive ability, which could explain the successful establishment and current geographic distribution of polyploids. We conducted a competition experiment using diploids, neotetraploids and established tetraploids of *Jasione maritima* to determine whether the cytotypes differ in phenological and physiological traits, and competitive ability, and to evaluate whether competitive abilities differ among populations in and outside the contact zone. Diploids and neotetraploids were similar over all traits measured. Competition impacted all cytotypes but diploids and neotetraploids were less affected than tetraploids. Diploids and neotetraploids performed better when grown with neighbors of their own cytotype, while tetraploids performed worse under competition regardless of the competitor. The tetraploid population from the contact zone are competitively similar to diploid populations, while those from outside the contact zone where less competitive. Finally, tetraploids outside of the contact zone had a significantly higher investment in belowground biomass, suggesting that root development might play an important role in colonizing southern locations. Our results do not support the hypothesis that neotetraploid plants of *Jasione maritima* are stronger competitors than diploids and, thus, competitive interactions might not account for the initial stages of polyploid establishment in *J. maritima*. Still, differential competitive abilities of cytotypes across their distribution range, possibly linked with adaptations to environmental gradients, could be responsible for the current geographical patterns. The similar competitive abilities of diploid and tetraploid plants in the contact zone may be responsible for the maintenance of the allopatric distribution of this species.

Keywords: competitive ability, contact zones, cytotypes, genome duplications, *Jasione maritima*.

INTRODUCTION

Polyploidization, *i.e.*, whole genome duplication, is an important mechanism of evolutionary divergence in plants and the factors determining the success of polyploid lineages has long attracted the attention of the scientific community (Soltis *et al.* 2010; Ramsey and Ramsey, 2014). Polyploidization often produces significant changes in cell functioning that can result in morphological and physiological changes with strong ecological and evolutionary consequences (*e.g.*, Melaragno *et al.* 1993; Li *et al.* 1996; Maherali *et al.* 2009; Ramsey 2011; Hao *et al.* 2013; Madlung 2013). Indeed, the dramatic changes in morphology and physiology resulting from polyploidization often contribute to the differentiation between cytotypes (Parisod *et al.* 2010). In the past years, several studies have reported differences between polyploids and their diploid (or lower ploidy) progenitors (Husband and Sabara 2004; Jersáková *et al.* 2010; Ramsey 2011; Hao *et al.* 2013; Laport *et al.* 2016), including changes in growth rates, secondary metabolism, cold tolerance, water relations or stress tolerance (Garbutt and Bazzaz 1983; McArthur and Sanderson 1999; Maherali *et al.* 2009; Schlaepfer *et al.* 2010; Liu *et al.* 2011; Coate *et al.* 2013). All of these changes can confer an advantage at the initial stages when the new polyploid is in low numbers within the parental population and subjected to frequency-dependent selection (minority cytotype exclusion; Levin 1975; Husband 2000).

New polyploids may overcome minority cytotype exclusion by having either different environmental requirements and tolerances than their progenitors, which lead to niche partitioning, or superior competitive ability for limiting resources (Levin 1975, 2002; Fowler and Levin 1984; Maceira *et al.* 1993; Soltis and Soltis 2000; Hao *et al.* 2013). Niche partitioning can occur at different spatial scales, from micro-habitat segregation within populations (*e.g.*, Baack 2004; Kolář *et al.* 2009; Manzaneda *et al.* 2012; Hao *et al.* 2013) to wider niche differentiation along altitudinal gradients or geographical ranges (*e.g.*, Felber-Girard *et al.* 1996; Husband and Schemske 1998; Buggs and Pannell 2007; Hülber *et al.* 2009; Ramsey 2011; Martin and Husband 2013; Muñoz-Pajares *et al.* 2017; Chapter 2). In contrast, competitive ability of polyploids has been included in theoretical models (Fowler and Levin 1984; Rodríguez 1996a) but has rarely been explored experimentally (but see Maceira *et al.* 1993; Collins *et al.* 2011; Thompson *et al.* 2015).

Polyploidization has been associated with increased competitive ability because of the direct effects on cell. The increase in cell size generated by genome duplications frequently leads to an overall increase in the plant's organs, such as the size of flowers, fruits, leaves, as well as an increase in the size of stomata (Sehepper *et al.* 2004; Leitch and Leitch 2008; Sun *et al.* 2009;

Allario *et al.* 2011; Van Laere *et al.* 2011; Tan *et al.* 2015), the so called "gigas effect" (Stebbins 1971; Masterson 1994; Buggs and Pannell 2007; Ramsey and Ramsey 2014). Additionally, heterosis generated by enforced homologous chromosomes pairing and maintenance of heterozygosity or by gene redundancy shielding polyploids from deleterious recessive mutations and enabling the diversification of gene function might also confer an advantage to polyploids by increasing plant vigor in comparison with the parental(s) (Comai 2005; Adam and Wendel 2005). Consequently, polyploids are frequently described to be taller and more robust plants, with higher biomass and overall stronger vigor when compared with their lower ploidy parental(s) (*e.g.*, Müntzing 1936; Smith 1946; Masterson 1994; Levin 2002; Ramsey and Schemske 2002; Ramsey and Ramsey 2014), traits that have been extensively used, for example, in crop improvement programs (Levin 2002; Dar *et al.* 2017). In nature, the differences described above can provide an increased competitive ability allowing polyploids to outcompete their diploid progenitors (*e.g.*, Maceira *et al.* 1993) or can enable polyploids to grow in stressful conditions such as in denser vegetation (Maceira *et al.* 1993; Hülber *et al.* 2009; Stahlberg 2009; but see Thompson *et al.* 2015). Greater allocation to vegetative growth and biomass has also been associated with successful invasion of polyploids (te Beest *et al.* 2011; *e.g.*, *Solidago gigantea*, Schlaepfer *et al.* 2010; *Centaurea solstitiallis*, Hahn *et al.* 2012; *Oxalis pes-caprae*, Castro *et al.* 2016a).

Different competitive abilities might be one of the factors involved in the initial establishment of newly emerged cytotypes (*e.g.*, Fowler and Levin 1984; Levin 2002). However, differences in competitive ability can also be important in subsequent stages, shaping the distribution patterns of the cytotypes at contact zones. The interactions between different cytotypes will drive the spatial dynamics at diploid-polyploid contact areas, generating stable or more dynamic zones of contact (Petit *et al.* 1999). Superiority of a given cytotype will provide an advantage and potentially lead to the displacement of the other cytotype generating transient mixed-ploidy populations, moving contact zones and spread of the fittest cytotype (Buggs and Parnnell 2007; Collins *et al.* 2011; Laport *et al.* 2013). In contrast, similar competitive performances between the cytotypes may increase the ability for the two cytotypes to coexist (Collins *et al.* 2011), although other factors need to be involved to assure cytotype coexistence. Competition between cytotypes has been proposed as an important driver of cytotype distribution in *Larrea tridentata* (Laport *et al.* 2013). Different competitive abilities across the distribution range have also been observed between diploid and tetraploid *Centaurea stoebe* with the authors linking the different performances to different distributional patterns (Collins *et al.* 2011). The contact zone between diploid and tetraploid *Dactylis glomerata* in the

northwest of the Iberian Peninsula (Galicia, Spain) also seems to be very dynamic, with tetraploids presenting greater competitive ability than diploids, competitively excluding diploids from mixed-ploidy populations over the course of only two years (Maceira *et al.* 1993). Taken all the above, it becomes clear that experiments quantifying competitive ability of different cytotypes will provide insights into not only the factors governing successful establishment of polyploid lineages but also the factors maintaining current geographical patterns.

Despite the recognized potential for polyploidization to cause instant phenotypic effects, only a few studies have able to test its ecological significance. Although the number of researchers studying polyploidy have increased over the last decades, the majority of studies focus on field observations and comparisons of established polyploids with their lower ploidy progenitor(s) (reviewed by Segraves 2017). Therefore, the immediate consequences of polyploidization *versus* post-polyploidization adaptation can hardly be unraveled (Ramsey 2002; Husband *et al.* 2008; Ramsey 2011). Therefore, the detection of neopolyploids in natural populations by large-scale screening methods or the production of synthetic neopolyploids in the laboratory using C-mitotic agents provide unique opportunities to quantify the immediate consequences of genome duplications (Ramsey 2011; Martin and Husband 2012). Only the comparisons between diploids, neotetraploids and established tetraploids can distinguish between polyploidization effects (*i.e.*, differences between diploids and neotetraploids) from the effect of evolutionary pressures after genome duplication (*i.e.*, differences between neotetraploids and stablished tetraploids). Regardless of its key importance, the inclusion of neopolyploids in ecological studies has only been considered recently and in a few polyploid complexes (*e.g.*, *Chamerion angustifolium*, Husband *et al.* 2008, Maherali *et al.* 2009; Baldwin and Husband 2011; Husband *et al.* 2016; *Tragopogon* species, Tate *et al.* 2009; *Achillea borealis*, Ramsey 2011; *Heuchera grossulariifolia*, Oswald and Nuismer 2011b; *Vicia craca*, Pavlíková *et al.* 2017). The introduction of neopolyploids in such comparisons are of major importance when studying the adaptive ecological potential of polyploid complexes.

Jasione maritima (Duby) Merino (Campanulaceae) is a mixed ploidy species occurring in the dune systems of the northwestern Iberian Peninsula. Ploidy cytotypes are allopatrically distributed, with diploids (2n = 2*x* = 12 chromosomes) located in the north of Galicia, from Ferrol to Lariño (Spain), and tetraploids (2n = 4*x* = 24) found from Lariño (Spain) to Aveiro, Portugal (Chapter 2). Morphological studies have shown the occurrence of some differences in a few plant traits between northern and southern populations, namely in plant ramification and inflorescence size (Rubio-Bará *et al.* 2010). Recent studies of cytogeographical patterns using niche modelling tools have suggested that, at present, tetraploids occupy their potential

environmental niche, while diploids are restricted to a smaller area when compared with their potential area (Chapter 2). This pattern suggests that tetraploids may have competitively excluded diploids from its populations, restricting diploids to northern areas where tetraploids are not able to succeed. This hypothesis could be formally tested using competition experiments and reciprocal transplants. Furthermore, given that neotetraploids have been successfully synthetized from diploid *J. maritima* (Chapter 6), including them in the comparisons will enable us to disentangle the role of genome duplications *per se* from the selection processes that operated along the evolutionary history of this polyploid complex.

The aim of this work was to assess the contribution of genome duplication *per se* to ecological divergence between diploid and tetraploid *Jasione maritima* and their current geographical distributions. For that, we compared diploids and established tetraploids to synthetic tetraploids (neotetraploids) in a common environment. In particular, we pose the following specific questions. First, do diploids, neotetraploids and established tetraploids differ in phenological, growth and physiological traits? We hypothesize that genome duplication produces changes in the newly arisen tetraploids; however, differences may have arisen through selection after the emergence of the polyploid. Comparisons of neotetraploids to diploids and established tetraploids under controlled conditions allow us to disentangle these possibilities. Second, does genome duplication increase the competitive ability of newly formed tetraploids compared to diploids or is the result of selective pressures that justify that present distribution? We hypothesize that increased competitive ability due to WGD has contributed to the successful establishment of newly formed tetraploids and explains the distribution of tetraploids in natural populations. To assess competitive ability, we grow each cytotype as a focal plant with neighbors of all possible cytotypes and quantify the performance. Third, does competitive ability of diploid and tetraploid plants depend on its geographical origin? We hypothesize that populations from the contact zone will maintain competitiveness, generating a stable contact zone, while populations outside of the contact zone will have decreased competitive ability. Thus, we can evaluate how genome duplications and evolutionary pressures affected the competitive ability of cytotypes and if competition is one of the factors involved in the successful establishment of neopolyploids and in the dynamics of the contact zone of *J. maritima*.

MATERIALS AND METHODS

Plant material

Three cytotypes were included in the experiment: diploids and established tetraploids (hereafter called tetraploids) collected from natural populations, and synthetic tetraploids (hereafter called neotetraploids) produced from diploids after treatments with colchicine (Chapter 6). In 2013 and 2014, seeds were collected from three diploid and three tetraploid populations (Table 7.1), distributed within and outside the contact zone (Chapter 2). In each population, infructescences were collected from 40 maternal parents, each separated by at least 4 m. In the laboratory, the inflorescences were air-dried, and seeds were removed, cleaned and stored in labeled microtubes.

Table 7.1. Locality, DNA ploidy level (2*x*, diploid; 4*x*, tetraploid) and geographic information of the natural *Jasione maritima* populations. Populations marked with * were consider as populations from contact zone.

Populations	DNA Ploidy level	Longitude	Latitude
Lourido, La Coruña	2*x*	43.08677	-9.22109
Fisterra, Afora beach, La Coruña	2*x*	42.90851	-9.27328
Lariño, La Coruña *	2*x*	42.77103	-9.12227
Ventim, Abelheira, La Coruña *	4*x*	42.79917	-9.02685
Barbeito, Pontevedra	4*x*	42.39955	-8.85051
Liméns, Pontevedra	4*x*	42.26023	-8.8137

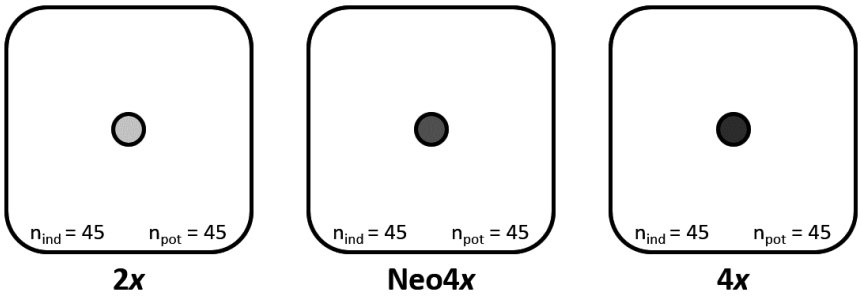
Synthetic neotetraploid plants were generated from plants of the same diploid populations described above (for methodological details see Chapter 6). Before flowering time, ploidy levels of synthetic plants were assessed through flow cytometry (Chapter 6). Each successfully transformed plant was cross-pollinated to yield a F1 seed generation. For this, neotetraploids were grown to flowering within an insect-free cage at the Botanic Garden of the University of Coimbra. Plants were reciprocally crossed with multiple randomly chosen neotetraploids from the same population. Each inflorescence served as pollen donor and pollen receiver and pollinations were performed by gently rubbing the inflorescences. Because inflorescences open gradually, each inflorescence was pollinated on at least three different days. Matured infructescences were harvested and air-dried, and seeds were removed, cleaned and stored in labeled microtubes.

Competition experiment

To assess competitive ability, we grew each *J. maritima* cytotype (*i.e.*, 2*x*, diploids; Neo4*x*, neotetraploids; 4*x*, tetraploids) with (competition) and without (no competition) a neighbor. For the competition treatment, the neighbor plant included all possible cytotypes (diploids, neotetraploids or tetraploids). Thus, each of the three cytotypes were subjected to four treatments (totaling 12 groups, Figure 7.1): no competition (*i.e.*, growing alone: 2*x*, Neo4*x* and 4*x*); competition with a diploid plant (focal plant + competitor: 2*x* + 2*x*, Neo4*x* + 2*x* and 4*x* + 2*x*); competition with a neotetraploid plant (2*x* + Neo4*x*, Neo4*x* + Neo4*x* and 4*x* + Neo4*x*); and competition with a tetraploid plant (2*x* + 4*x*, Neo4*x* + 4*x* and 4*x* + 4*x*). In the competition pots, both plants were used as focal plants and as competitors. Each treatment was replicated 15 times per population (with exception of competition with the same cytotype, which was replicated 16 times), including an even number of mother plants from the selected populations in every treatment, totaling 342 pots and 549 transplanted seedlings.

The experiment was conducted in the greenhouse of the Botanic Garden of the University of Coimbra from November 2nd 2015 to June 30th 2016. Ten days prior to the start of the experiment, 10 seeds from each of the 15-16 mother plants per population were placed in individual Petri dishes on filter paper moistened with distilled water and stored at 4 °C for 5 days to synchronize seed germination (Chapter 6). Petri dishes were then transferred to a growth chamber and incubated at 24 °C with a 16h:8h (light:dark) photoperiod. After five days, most seeds had germinated and produced fully expanded cotyledons. Seedlings were then transplanted into 1-L plastic pots (8.6 × 8.6 wide and 21.5 cm deep) filled with a 1:1 mixture of commercial soil and sand. One or two seedlings were transplanted to each pot according with the treatment, no competition or competition, respectively. All the pots were randomly assigned to a position in the greenhouse bench at the beginning of the experiment and re-randomized four more times across the experiment. All the seedlings that died within the first 2 weeks after the transplant were replaced and interpreted as losses due to the transplant process. No more seedlings were replaced afterwards. Plants were watered regularly, three times per week in the winter and daily in the spring and summer. The ploidy level of all the plants used in the experiment was confirmed through flow cytometry using the protocol described in Chapter 6.

- **No competition**



- **Competition**

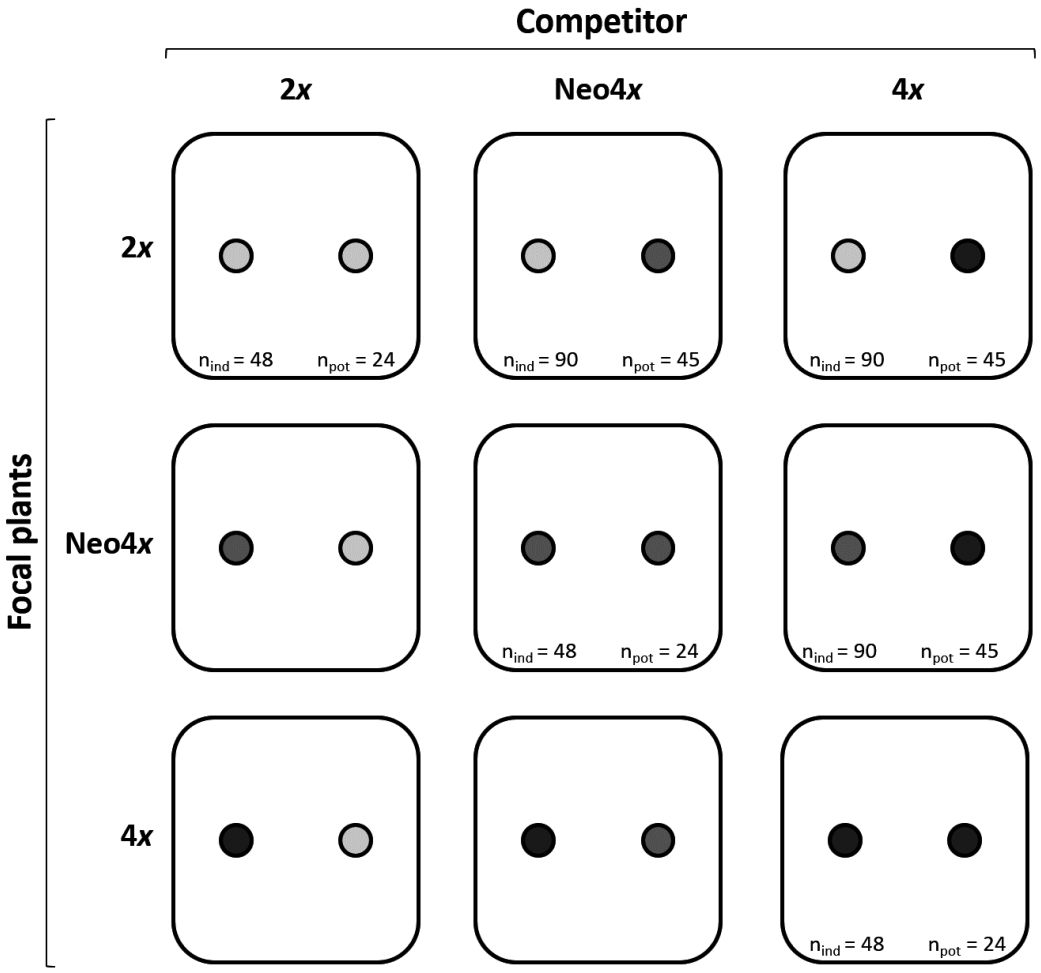


Figure 7.1. Experimental design of the competition experiment. Twelve treatments were included: cytotypes (2*x*, diploids; Neo4*x*, neotetraploids; 4*x*, tetraploids) growing alone (no competition) or with another plant (competition). In the competition treatment each focal plant was grown with a different competitor: competition with a diploid plant (2*x* + 2*x*, Neo4*x* + 2*x* and 4*x* + 2*x*), competition with a neotetraploid plant (2*x* + Neo4*x*, 4*x* + Neo4*x* and Neo4*x* + Neo4*x*), or competition with a tetraploid plant (2*x* + 4*x*, 4*x* + 4*x* and Neo4*x* + 4*x*). The number of individuals and pots (n_{ind} and n_{pot}, respectively) is given for each treatment.

Plant measurements

Survival was measured monthly after the first month. During spring, each individual was monitored every two days to record the beginning of flowering, expressed as the number of days from the beginning of the experiment to the day when the first inflorescence flowered. Eight months after the beginning of the experiment, at the peak of flowering, all the plants were harvested. Of the 547 plants that survived until the end of the experiment, 450 were used for measuring growth, and 97 were used for measuring physiological traits, in both cases plants were evenly distributed sample sizes across treatments.

Plants harvested for biomass were separated into aboveground and belowground components. Whenever possible, aboveground biomass was further divided into vegetative and reproductive parts (peduncles and inflorescences). Also, the number of inflorescences was counted before harvesting and placing them into paper bags. Roots were washed carefully to eliminate soil residuals. However, in the competition treatments, it was difficult to calculate belowground biomass, as the fine roots were often intertwined with the other plant roots in the pot. Therefore, only the taproot and main roots of each plant were considered. For a more correct comparison among treatments and cytotypes, the same procedure was followed with the plants growing alone. Above- and belowground plant material was dried at 60 °C for 48 h and weighed.

The following physiological parameters were measured: starch content, cell membrane leakage and total soluble sugars. To estimate starch and sugar content, fresh leaves were collected in individual aluminum foil envelopes, immediately frozen in liquid nitrogen, and stored at -80 °C until carbohydrate quantification. Total soluble sugars and starch content were extracted from leaf samples and quantified using the anthrone methods described in Irigoyen *et al.* (1992) and in Osaki *et al.* (1991), respectively. To measure cell membrane leakage, two to four leaves, depending on leaf dimensions, were collected, washed with deionized water, placed in closed microtubes with deionized water, and incubated overnight on a rotary shaker. Electrical conductivity on the solution was determined two times, one after 24 hours (Lt) and another after samples were autoclaved (L0) (samples were autoclaved at 120 °C for 20 min and the measures were obtained after cooling to 25 °C). Cell membrane leakage was then assessed following Lutts *et al.* (1996), and the electrolyte leakage was calculated as Lt/L0 and expressed as percentage.

For each variable the magnitude of the effect of competition was calculated to express each studied variable as a response to competition. For this, we calculated the difference

between the mean with neighbors and the mean without neighbors and expressed it as a proportion. Mean values were calculated for each cytotype and variable.

Statistical analyses

Preliminary statistical analyses detected a high complexity and frequently significant factor interactions [Appendix 7.1]. Thus, using generalized linear mixed models (GLMMs) we have followed a question based statistical approach. Below are presented the specific questions posed and the statistical tests performed to address each question:

1) Do cytotypes differ in the studied plant traits? We explored differences among cytotypes for each of the following treatments separately: plants growing alone and plants growing under competition. Cytotype was defined as fixed effect, plant traits as response variables, and population as random effect.

2) Does competition affect cytotype performance? We explored differences between cytotypes growing with and without competition. Cytotype and treatment (competition *versus* no competition) were defined as fixed effects, plant traits as response variables, and population as random effects. Because interactions between fixed effects were significant (Supplementary material), differences between plants growing with and without competition (competition *versus* no competition) were also assessed for each cytotype separately. Additionally, differences in the magnitude of the effect of competition among cytotypes were also explored following the approach described in question 1.

3) Does genome duplication produce differences that increase the competitive ability of neotetraploids? We explored differences between diploids and neotetraploids competing with diploids and neotetraploids (comparison C1). The cytotype of the focal plant and of the competitor (same ploidy of focal plant *versus* different ploidy) combined were used as fixed effect, plant traits as response variables, and population as random effect.

4) Are there differences that suggest adaptation only of tetraploid individuals? We explored differences between tetraploids and other cytotypes competing with each other as follows: neotetraploids and tetraploids (comparison C2) and diploids and tetraploids (comparison C3). A similar approach to question 3 was used: the cytotype of the focal plant and of the competitor combined were used as fixed effect, plant traits as response variables, and population as random effect.

5) Do diploid and tetraploid populations from the contact zone differ in their competitive ability in comparison with populations from outside the contact zone? We explored differences between the following groups: diploids far from contact zone (2*x*_out), diploids from the contact zone (2*x*_CZ), neotetraploids (Neo4*x*), tetraploids from the contact zone (4*x*_CZ) and tetraploids far from the contact zone (4*x*_out). These groups were defined as fixed effect, and plant traits as response variables.

The response variables were the following: reproductive, above- and belowground biomasses, total biomass, number of inflorescences, phenology, starch content, cell membrane leakage and total soluble sugars. Population was used as random factor. A Poisson distribution with a log link function was used when analyzing the number of inflorescences and phenology, and a Gaussian distribution with the identity link function was used when analyzing reproductive biomass, aboveground biomass, belowground biomass, total biomass, starch content, cell membrane leakage and total soluble sugars. Outlier values were inspected and excluded from the analyses. All analyses were performed in R software version 3.0.1 (R Core Development Team, 2016), using the packages "car" for Type-III analysis of variance (Fox and Weisberg, 2015), "lme4" for generalized linear models and generalized linear mixed models (Bates *et al.* 2014), "lsmeans" for least-squares means (Lenth 2016) and "multcomp" for multiple comparisons after Type-III analysis of variance (Hothorn *et al.* 2017).

RESULTS

Do cytotypes differ in phenological, growth and physiological traits? Cytotype performance when growing alone

When grown in the absence of neighbors, cytotypes differed significantly for only one biomass measure, *i.e.*, belowground biomass (white bars in Figure 7.2) [Appendices 7.2 and 7.3]. Tetraploids presented significantly higher belowground biomass than diploids and neotetraploids, which for this trait were similar to one another (Figure 7.2C) [Appendix 7.2]. Phenology did not differ significantly between cytotypes as flowering started approximately at the same time, largely overlapping between all the cytotypes [Appendices 7.2 and 7.3C].

Regarding the physiological parameters, significant differences were observed among the studied cytotypes for starch content and cell membrane leakage. When grown alone, diploids expressed lower cell membrane leakage and starch content than neotetraploids and tetraploids, despite this difference was only significant for diploids regarding starch content and

for tetraploids regarding cell membrane leakage (Figure 7.2D) [Appendices 7.2 and 7.3D]. No significant differences were observed for total soluble sugars [Appendices 7.2 and 7.3E].

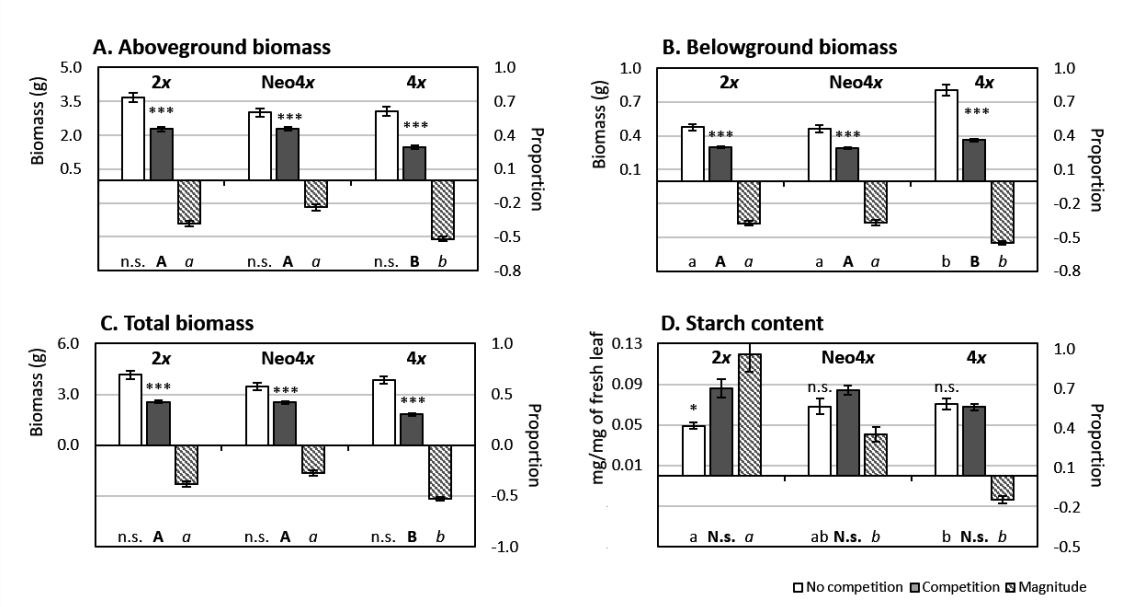


Figure 7.2. Mean (± SE) values of growth and physiological traits for diploid (2*x*), neotetraploid (Neo4*x*) and tetraploid (4*x*) focal plants of *Jasione maritima* grown in different competitive environments: A) Aboveground biomass; B) Belowground biomass; C) Total biomass; and D) Starch content. Treatments: no competition, white bars; growth with a neighbor, grey bars; and the magnitude of the impact of competition, striped bars. Lower case letters indicate differences ($P < 0.05$) between cytotypes when grown alone; upper case letters indicate differences between cytotypes grown with a neighbor; and, italic letters represent differences between the magnitude of the impact of competition. *, $0.01 < P < 0.05$; **$0.01 < P < 0.001$ and *** $P < 0.001$; n.s., nonsignificant.

<u>Does competition affect cytotype performance? Performance with and without competition</u>

Competition significantly affected all cytotypes for all growth variables (white vs. grey bars in Figure 7.2A-C) [Appendices 7.2 and 7.3A-C]. Diploid, neotetraploids and tetraploid plants growing under competition suffered significant decreases in every parameter compared with plants growing alone (Figures 7.2A-C) [Appendices 7.2 and 7.3A-B]. Although not significant for neotetraploids, diploid and tetraploid plants under competition started flowering slightly later than plants growing alone [Appendices 7.2 and 7.3C].

The magnitude of the impact of competition in growth traits varied among cytotypes (striped bars in Figure 7.2) [Appendices 7.2 and 7.3]. The neotetraploids and diploids exhibited a significantly lower effect of competition than tetraploids for all traits (Figure 7.2) [Appendices 7.2 and 7.3B].

The physiological traits were more variable (Figure 7.2D) [Appendix 7.3D-E). Diploids growing under competition increased their starch content significantly (Figure 7.2D) [Appendix 7.2]. Although not significant, a similar trend was observed for neotetraploids, while for tetraploids, values with and without competition were similar. Consequently, the impact of competition on starch content was high and positive for diploids, followed by neotetraploids, being negative for tetraploids. Neotetraploids and tetraploids growing alone showed a tendency to have a higher cell membrane leakage than under competition (only significant in tetraploids), while diploids showed an opposite trend, despite not significant [Appendices 7.2 and 7.3D]. A clear pattern was observed for total soluble sugars, with plants under competition having significant higher total soluble sugar amounts than plants growing alone [Appendix 7.3E; not significant for neotetraploids; Appendix 7.2]. Still, the magnitude of the impact of competition in cell membrane leakage and total soluble sugars did not differ significantly among cytotypes [Appendix 7.2], although the impact in cell membrane leakage presented opposite directions, being positive for diploids and negative for neotetraploids and tetraploids [Appendix 7.3D].

Do genome duplications produce differences that increase the competitive ability of neotetraploids? Performance of diploids and neotetraploids with different competitors

Diploids and neotetraploids under competition exhibited significant differences among competition levels for all growth traits, except belowground biomass (C1 in Figures 7.3A-C) [Appendices 7.2 and 7.4A-B]. No differences were observed in phenology [Appendix 7.4C]. Focal plants grown with a plant of its own cytotype (*i.e.*, 2*x* + 2*x* and Neo4*x* + Neo4*x*) performed better than when grown with another cytotype (*i.e.*, 2*x* + Neo4*x* and Neo4*x* + 2*x*), being significant for aboveground biomass and total biomass (Figures 7.3A and 7.3C) [Appendices 7.2], although the trend is also visible for inflorescence number and reproductive biomass [Appendices 7.2 and 7.4A-B].

For starch content and total soluble sugars, the focal plant exhibited lower values when competing against the same cytotype than when competing with a different cytotype (although not significant for diploids in starch content and for neotetraploids in total soluble sugars; Figure 7.3D) [Appendices 7.2 and 7.4E]. No significant differences were observed between competition levels for cell membrane leakage [Appendices 7.2 and 7.4D].

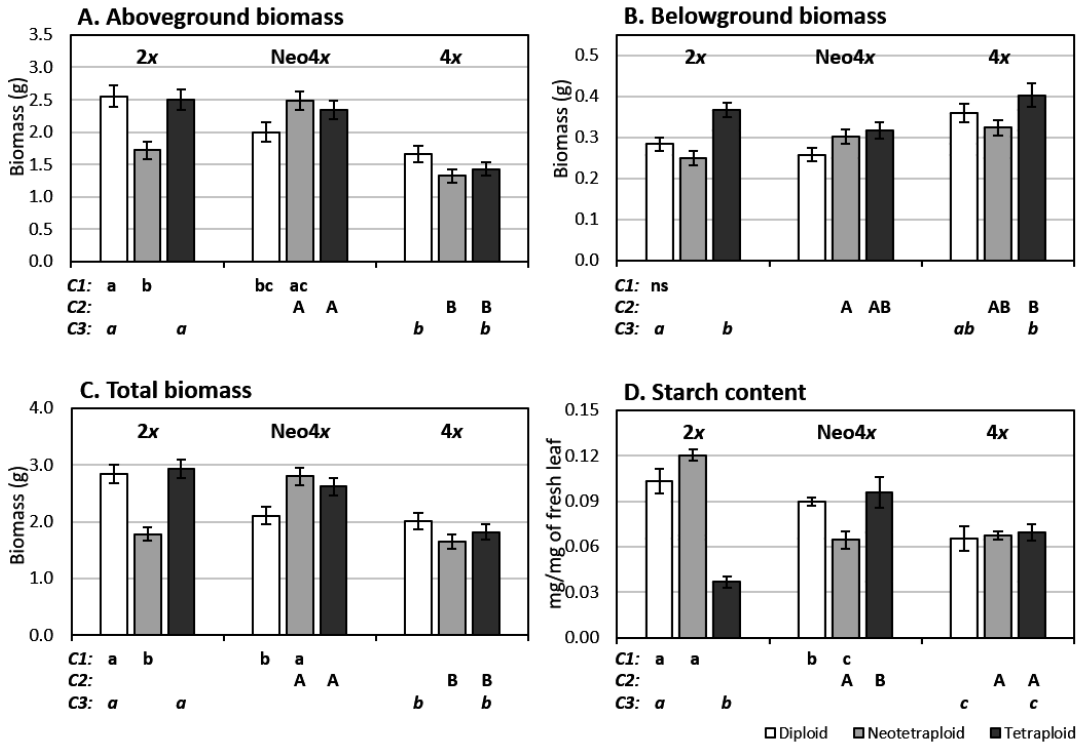


Figure 7.3. Mean (± SE) values of measured variables in plants of *Jasione maritima* under competition: A) Aboveground biomass; B) Belowground biomass; C) Total biomass; and D) Starch content. Focal plants competing with diploid plants (2*x*, white bars), competing with neotetraploids (Neo4*x*, grey bars) and competing with tetraploid plants (4*x*, dark grey bars). Comparisons: C1) comparison between diploids and tetraploids; C2) comparison between neotetraploids and tetraploids; and C3) comparison between diploids and tetraploids. Different letters correspond to statistically significant differences ($P < 0.05$): C1 – lower case letters, C2 – upper case letters and C3 – italic letters.

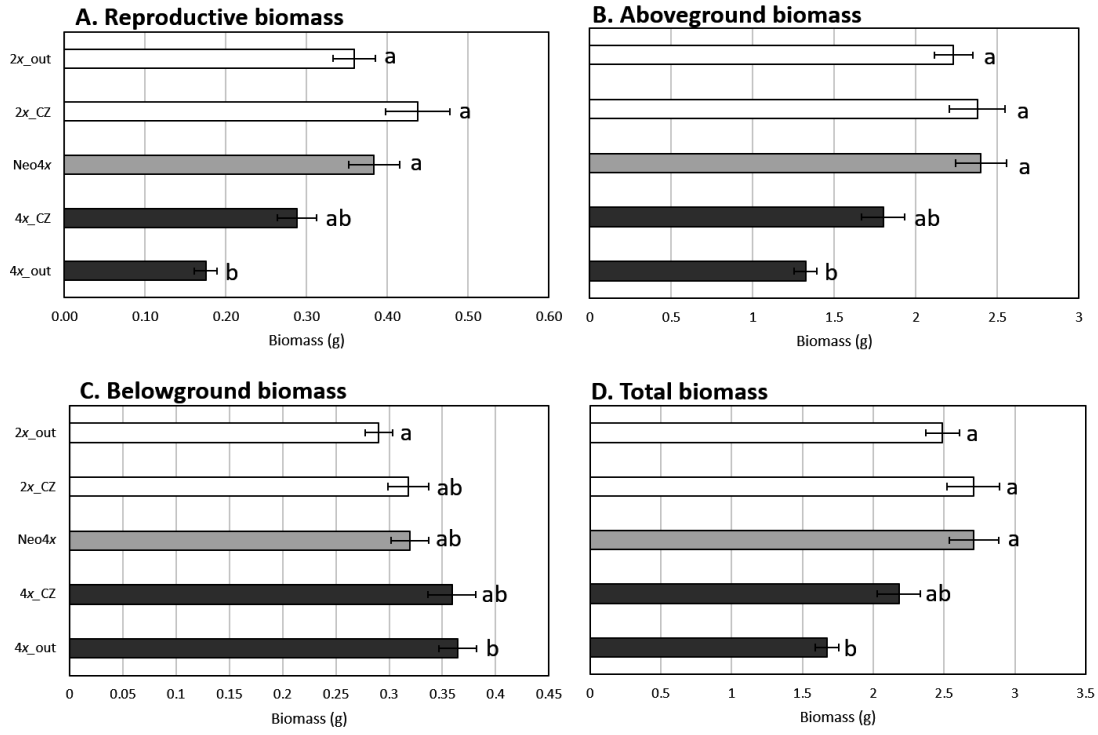
Are there differences that suggest adaption only of tetraploid individuals? Performance of tetraploids *versus* other cytotypes under competition

Tetraploids under competition performed worse than diploids and neotetraploids, regardless of the competitor identity (Figure 7.3) [Appendix 7.4]. This pattern was clear and significant for aboveground biomass and total biomass for which tetraploids presented lower values than the other cytotypes (comparisons C2 and C3 in Figures 7.3A, 7.3C) [Appendix 7.2]. Reproductive biomass revealed a similar pattern [Appendices 7.2 and 7.4B]. The exception was belowground biomass, which tended to be overall higher for tetraploids when compared with the other cytotypes (Figure 7.3B) [Appendix 7.2]. Tetraploids under competition tended to perform similarly to neotetraploids in some of the measured parameters (namely for inflorescence number) [Appendix 7.4A].

When grown with neighbors, tetraploids performed similarly to diploids and neotetraploids with respect to cell membrane leakage [Appendix 7.4D]. However, neotetraploids exhibited a significantly lower cell membrane leakage when competing with tetraploids than when competing with plants from its own cytotype [Appendix 7.2; C2 in Appendix 7.4D]. A more complex pattern was obtained for total starch content (Figure 7.3D) and total soluble sugars [Appendix 7.4E], with significant differences being observed for both variables [Appendix 7.2].

Do diploid and tetraploid populations from the contact zone differ in their competitive ability? Performance of populations from contact zone *versus* outside the contact zone

When the diploid and tetraploid populations from the contact zone are compared with populations outside this zone (Table 7.1), different patterns emerge among tetraploid populations but not among diploids (Figure 7.4) [Appendix 7.5]. Under competition, tetraploids from the contact zone performed similarly to diploid populations (in or outside of the contact zone). In contrast, tetraploid populations from southern locations (*i.e.*, more separated from the contact zone) exhibited lower reproductive, aboveground and total biomass than the other cytotypes (Figures 7.4A-B) [Appendix 7.5]. All tetraploid populations produced significantly higher belowground biomass, although they did not differ from diploid populations in the contact zone (Figure 7.4C) [Appendix 7.5].



Neotetraploids were similar to diploid populations for all traits and similar to the tetraploid population from the contact zone for reproductive, aboveground and total biomass (Figure 7.4) [Appendix 7.5].

DISCUSSION

Superior competitive ability of neopolyploids can promote their establishment within the progenitor populations. This idea has been invoked often to explain the spread of polyploids and colonization of new habitats (Husband 2000; Levin 2002; Treier *et al.* 2009; Schlaepfer *et al.* 2010; Hahn *et al.* 2012; te Beest *et al.* 2011; Rey *et al.* 2017). However, only a few studies have tested the effect of competition among cytotypes, including neopolyploids, and their importance for the establishment of new cytotypes and for the maintenance of contact zones (Maceira *et al.* 1993; Collins *et al.* 2011; Thompson *et al.* 2015; Pavlíková *et al.* 2017).

In this study, we assessed the performance of diploids, neotetraploids and established tetraploids growing with and without competition to evaluate if genome duplication *per se* confers a higher competitive ability or if differences result from post-polyploidization selective pressures. Our results show that: 1) when grown alone, cytotypes differed significantly only for belowground biomass, starch content and cell membrane leakage, with tetraploids having significantly higher belowground biomass than the other two cytotypes, and diploids presenting lower starch content and cell membrane leakage than the other two cytotypes; 2) larger differences emerged under competition; overall, competition reduced plant growth for all cytotypes, although diploids and neotetraploids presented similar biomass investments; tetraploid plants were smaller but maintained a high investment in belowground biomass; the effect of competition was also evident in the physiological traits, with plants under competition having significantly higher total soluble sugars than plants growing alone; 3) when accounting for the competitor's identity, diploids and neotetraploids competed better with their own cytotype than with each other, while, in general, tetraploids performed worse regardless of the competitor; and 4) tetraploid populations presented different competitive abilities depending with their geographic site of origin : the tetraploid population from the contact zone behaved

⇦ **Figure 7.4.** Mean (± SE) values of biomass across competition treatments for diploid and tetraploid plants of *Jasione maritima* located in (CZ) and outside (out) the contact zone: A) Reproductive biomass; B) Aboveground biomass; C) Belowground biomass; and D) Total biomass. Diploid populations (2*x*) are shown in white, neotetraploids (Neo4*x*) in grey and tetraploids (4*x*) in dark grey. Means with different letters correspond to statistically significant differences ($P < 0.05$).

similarly to diploid populations, while tetraploid populations far away from the contact zone where less competitive.

Below we discuss our results in light of the questions posed at the beginning of the study to unravel the role of competition in the successful establishment of neotetraploids and in the current interactions at contact zones, aspects that are crucial to understand the adaptive value of polyploidy and its widespread occurrence in nature.

Differences among cytotypes: effects of genome duplication?

Genome duplication has been suggested to drive significant genomic and phenotypic changes (Levin 1983; reviewed by Segraves 2017). Several comparative studies between polyploids and their diploid progenitors have shown differences ranging from cell size level (*e.g.*, stomatal cells, Bretagnolle and Lumaret 1995) to the interactions between the cytotypes and other organisms (*e.g.*, herbivores, Nuismer and Thompson 2001; pollinators, Segraves and Thompson 1999). However, only those studies that consider neotetraploids (either natural or synthetic) can effectively test the effect of genome duplications *per se* (Martin and Husband 2012; Ramsey 2011). Comparisons among the three cytotypes enable us to distinguish between differences due to genome duplications from differences due to natural selection after polyploidization: if divergence is due entirely to genome duplication, then neopolyploids should differ from diploids but resemble natural tetraploids. To disentangle these effects, in this study we explored the differences between diploids, neotetraploids and established tetraploids when growing alone under optimal conditions and when growing under competition.

Overall, diploids and neotetraploids performed very similarly for most traits, suggesting no direct effect of WGD on competitive ability. The overall similarity between diploids and neotetraploids suggests that genome duplication in *J. maritima* does not seem to cause broad shifts in plant traits, contrary to the observations made in other polyploid complexes (reviewed by Segraves 2017). Still, there are examples in the literature of polyploid complexes that report the lack of differences between cytotypes for several morphological traits (Münzbergová 2006, 2007b; Thompson *et al.* 2015; Pavlíková *et al.* 2017). The absence of differences between cytotypes under competition was also reported before (*e.g.*, *Allium oleraceum*, Fialová and Duchoslav 2014; *Aster amellus*, Münzbergova 2007; *Chamerion angustifolium*, Thompson *et al.* 2015; *Ranunculus adoneus*, Baack and Stanton 2005; *Senecio carniolicus,* Hulber *et al.* 2011). Similarly, our results showed that most divergence between diploids and tetraploids of *J.*

maritima arose after the duplication event, at least in the time-frame of the plant's life-cycle studied here.

Still, a few differences in physiological traits were observed and could be linked with different plant growth strategies of the cytotypes in subsequent stages of the life-cycle, such as, starch accumulation. Here we observed a negative correlation between starch content and plant growth in diploid plants, while this correlation disappears in neotetraploids (and tetraploids). Under favorable conditions, diploids seem to invest in plant growth, accumulating less amounts of starch than neotetraploids (and tetraploids), while under stressful conditions such as competition, growth decreases, and starch is accumulated in higher amounts. In contrast, in neotetraploids there is always a similar investment in starch accumulation regardless of the presence of competition. Interestingly, tetraploids maintain this trend of starch accumulation regardless of the presence of competition suggesting that this strategy could have been advantageous for the success of neotetraploids. *Jasione maritima* is a perennial dune plant that undergoes the winter period in the form of small rosettes produced in autumn after the energy demanding period of reproduction. Thus, for this plant having a higher amount of energetic reserves might be particularly advantageous in subsequent stages of the life-cycle (discussed below).

An interesting pattern was also observed for cell membrane permeability, used here as a biomarker of oxidative damages (cell membrane electrolyte; Demidchik *et al.* 2014) to assess plant stress under competition. Although the basal cell membrane permeability level was higher in neotetraploids (and tetraploids) than in diploids, under competition, neotetraploids (and tetraploids) reduced oxidative damages, while in diploids the cell membrane leakage increased (similar magnitude of response but in different directions). Taking into account that a low oxidative status is correlated with a high antioxidant response (Dias *et al.* 2018), our data suggest that competition may lead to increased capacity of defense in neotetraploids (and tetraploids). Similar patterns were observed when comparing diploids and tetraploids of *Arabidopsis thaliana* under stressful conditions (del Pozo *et al.* 2014), suggesting that polyploidization affected the expression of genes involved in stress response, which in turn provide a flexible and rapid response of tetraploids to external/internal stimuli. Polyploidization has been described to promote the antioxidant capacity, in part due to an up-regulation of genes related to the antioxidant system, reactive oxygen species (ROS) scavenging function and ROS signaling processes, making tetraploids more tolerant, especially, under stress conditions (*e.g.*, del Pozo *et al.* 2015, Tan *et al.* 2015, Kong *et al.* 2017). For example, tetraploids of *Dioscorea zingiberensis* presented lower levels of ROS (superoxide anions and hydrogen peroxide) and membrane

injuries (cell membrane permeability and lipid peroxidation) associated to a higher antioxidant enzyme activity (Zhang *et al.* 2010). Our results suggest that in *J. maritima*, genome duplications might be responsible for an increased antioxidant response, being this trait selected over the evolution. Still, it was not possible to establish the link between this and other measured traits.

Implications for the establishment of neotetraploids

Superior competitive ability in neopolyploids has been proposed as one of the mechanisms that can promote polyploid establishment within the progenitor population. However, the direct comparison of plant performance shows no evident advantage in competitive ability by neotetraploids. Therefore, at initial stages after polyploid formation, the fate of neotetraploids will be driven by other factors, and in the absence of any advantage, they are expected to be excluded from the diploid populations (minority cytotype exclusion; Levin 1975; Husband 2000). Still, it is interesting to note that the identity of the competitor affected the performance of both diploids and neotetraploids, with plants competing with their own cytotype performing better than when competing with a different cytotype. Consequently, when growing with their own cytotype, plants presented higher biomass. This might suggest that, although not differing in competitive ability, spatial segregation within the population may allow the overcome the minority cytotype exclusion (Levin 1975), promoting assortative mating as observed in other polyploid complexes (Baack 2005).

Therefore, in the absence of clear differences in competitive ability, neotetraploids would have to present other advantages that could enable them to overcome the minority cytotype exclusion. One of such advantages could be plant growth strategy. For example, perennial plants may persist in time within the parental population until opportunities for reproduction appear (Gustafsson 1948; Stebbins 1971; Levin 1983; Rodriguez 1996b), such as unreduced gamete formation by the diploid parental and emergence of new compatible polyploid mating partners (*e.g.*, Baack 2005; Rausch and Morgan 2005; Ramsey 2007; Kreiner *et al.* 2017a), or by having multiple opportunities for reproduction (*e.g.*, Muller 1989; Rosche *et al.* 2017). *Jasione maritima* is a perennial plant and thus, plant habit might constitute an advantage for neotetraploid establishment at initial stages. Additionally, as described above, *J. maritima* undergoes the winter period in the form of small rosettes. In this context, because starch accumulation could be indirectly related with plant biomass, having higher amount of starch as an energetic reserve could enable neotetraploids to re-direct to a higher production of rosettes and constitute an advantage in the following year. Indeed, some studies over longer time scales

or measuring long-term persistence traits reveal differences between cytotypes in growth strategies that affect plant performance. For example, tetraploids of *Centaurea stoebe* produce a greater number of rosettes than their diploid counterparts regardless of the competition regime, suggesting significant shifts in life cycle between cytotypes (Collins *et al.* 2011). Diploids and tetraploids of *Vicia cracca* presented different strategies along two years. While diploids presented a higher fitness than tetraploids in the first year, tetraploids grew faster in the second year, minimizing the differences between cytotypes (Eliášová *et al.* 2017). Thus, a better performance in traits associated with long-term persistence may allow neotetraploids to establish and outcompete diploids (Collins *et al.* 2011; Thébault *et al.* 2011), despite their initial numerical disadvantage. Consequently, studies over longer periods are needed to assess the fitness advantage of long-term persistence traits in *J. maritima*.

Trade-offs between the size and number of structures were observed in several polyploid complexes and might have significant consequences for plant performance (*e.g.*, Levin 2002; Oswald and Nuismer 2011b; Green *et al.* 2013; Castro *et al.* 2016a). Although genome duplication in *J. maritima* does not seem to influence the total biomass invested, it is interesting to note that neotetraploids produced fewer structures but with bigger sizes, such as the inflorescences (especially when growing under competition; Appendix 1A-B). The presence of bigger inflorescences can be advantageous for neotetraploids due to their effects on plant-pollinator's interactions. *Jasione maritima* is a self-incompatible plant, so the presence of bigger inflorescences may be more attractive and lead to changes in the behavior and preference of certain pollinator species, promoting assortative crossing between neotetraploids and increasing their fitness. Differences in floral traits and pollinator behavior between cytotypes have been linked with different levels of reproductive isolation between cytotypes growing in sympatry (Segraves and Thompson 1999; Husband and Schemske 2000; Husband and Sabara 2004) and are fundamental to understand interactions in initial stages of polyploid establishment.

Differences between cytotypes: changes after genome duplications?

The overall comparison between tetraploids and the other two cytotypes and the detailed studies of the populations at the contact zones revealed that tetraploids differed in two main aspects, competitive ability and belowground biomass. The patterns of variation in both traits were similar but acted in opposite directions. Also, the populations at the contact zone behaved similarly among all cytotypes, suggesting a differential response across the latitudinal

and environmentally dissimilar distributional range of the complex. First, in general, tetraploids presented lower competitive ability than diploids and neotetraploids; however, detailed studies at the population level showed that the tetraploid population from the contact zone behaved similarly to all the populations from the other cytotypes, while tetraploid populations more distant from the contact zone where less competitive than diploid (and neotetraploid) populations. This might indicate that competitive ability might not have been a key advantage during the colonization of southern most locations and suggest that tetraploids might have lost their competitive ability towards the south, while maintaining it at the zone of direct contact with diploids. Different performances between populations of polyploids have already been documented and were related with different geographical patterns, environmental gradients and contact zones of different natures (Collins *et al.* 2011; Thompson *et al.* 2015; Rey *et al.* 2017).

Second, overall, tetraploids showed a consistently higher belowground biomass in comparison with diploids and neotetraploids, investing always more in the production of roots regardless of the competition treatment. However, once again, populations at the diploid-tetraploid contact zone presented similarly high belowground biomass. The higher root development could thus be a trait already present in the southern diploid populations, being possibly linked with adaptations to environmental gradients. A higher allocation to the production of belowground structures has been reported for example for tetraploid *Solidago gigantea* (Schaepfer *et al.* 2010) and *C. stoebe* (Collins *et al.* 2011) in comparison with diploid individuals, and in some cases, it has been related with increased competitive ability (*e.g.*, Gaudet and Keddy 1988; Aerts *et al.* 1991), although this was not observed in *J. maritima*. A well-developed root might also enable tetraploid plants to explore water reserves in deeper layers of the soil and thus could have been particularly relevant in colonizing southern and drier locations where tetraploids currently occur. Indeed, tetraploids had a broader niche, preferring drier areas than diploids (Chapter 2). Environmental gradients and in particular, adaptation to drier environments, has been shown in other polyploid complexes, in which several polyploids colonized drier habitats (Leven 2002; te Beest *et al.* 2011; Manzaneda *et al.* 2015; Rey *et al.* 2017). For example, tetraploid *Brachypodium hybridum* was shown to present a drought-escape strategy having higher performances and colonizing drier places than the diploid parental *Brachypodium distachyon* found in more humid environments (Manzaneda *et al.* 2012, 2015).

Geographical patterns and dynamics at contact zones

Jasione maritima present an allopatric distribution of diploids in the north and tetraploids in the south (Chapter 2). This cytogeographical pattern is expected to be driven by historical and ecological factors (*e.g.*, Baack 2004, 2005; Pannell *et al.* 2004; Baack and Stanton 2005; Glennon *et al.* 2014; Godsoe *et al.* 2013; Münzbergová *et al.* 2013; Wefferling *et al.* 2017). Among the latter, and based on the differences observed here, traits such as competitive ability and belowground investment are expected to play an important role in the distribution ranges of diploid and tetraploid populations. From northern to southern locations it is possible to observe environmental gradients such as different water availability and different vegetation covers, with northern dune localities having higher moisture and lower temperatures and consequently a denser vegetation cover where competition is expected to be high. By other way, southern dune locations are drier and harbor sparser vegetation cover where competition and water availability are expected to be lower. In this scenario, the traits observed for diploids and tetraploids fit this environmental gradient, with more competitive diploids in the north, and with tetraploids presenting bigger root systems in the south. Experiments such as reciprocal transplants and drought tolerance experiments are being developed to test these hypotheses.

At the contact zone, the plants from diploid and tetraploid populations that are expected to be in direct competition, surprisingly, presented similar performances under competition. In the field, while the superiority of a given cytotype will generate dynamic contact zones and the displacement of the unfit cytotype, similar competitive abilities (in the absence of other advantages) may lead to stable contact zones (Maceira *et al.* 1993; Petit *et al.* 1999; Collins *et al.* 2011). Thus, the similitude between diploids and tetraploids at the contact zone is expected to maintain the contact zone. Different competitive abilities between diploids and tetraploids of *C. stoebe* across their geographical range have been linked with the current observed distribution patterns (Collins *et al.* 2011). In one hand, tetraploids of *C. stoebe* revealed a competitive superiority in Western Europe where they are dominant suggesting that they have led to competitive exclusion of diploids in this area, as probably happened also in North America where diploids and tetraploids were introduced (Treier *et al.* 2009). On the other hand, the lack of differences in competitive ability in Eastern Europe (Španiel *et al.* 2008; Treier *et al.* 2009) suggested that competition was small enough to enable the coexistence of the two cytotypes in this region, being detected several mixed-ploidy populations (Collins *et al.* 2011). In *J. maritima*, although the two cytotypes are similar, competitive ability at contact zone might be important to limit the expansion of diploids to the south or tetraploids to the north in the absence of other ecological determinants defining cytotype distribution patterns.

CONCLUSION

In the *J. maritima* polyploid complex, genome duplication *per se* does not seem to increase competitiveness (*i.e.*, in neopolyploids). Therefore, this important phenomenon in flowering plants does not seems to represent an advantage at initial stages of polyploid establishment, although changes in traits such as starch accumulation might be advantageous in the subsequent life-cycle stages of the plant. Still, differential competitive abilities of cytotypes across their distribution range, possibly linked with adaptations to environmental gradients, could be responsible for the observed geographical patterns. This highlights the importance of studying polyploids at the population level, including the study of populations from different geographical contexts. In the north, the high competitive ability of diploids might be an advantage in dunes with a dense vegetation cover, while towards south, tetraploids seem to lose competitive ability, investing more in belowground biomass. This could reflect the colonization of more open dune habitats, more severely affected by drought in comparison with the northern more locations where diploids occur. In the diploid-tetraploid contact zone, cytotypes present similar competitive abilities and the minority cytotype exclusion may be maintaining the allopatric distribution of the species. Similarly to recent studies, our results suggest that competition may not be a general mechanism involved in the initial stages of polyploid establishment, but that it may play an important role for maintaining the composition of contact zones.

APPENDICES

Appendix 7.1. Results of preliminary statistical analyses of fixed factors for each response variable. Three ploidy levels were considered (2*x*, Neo4*x* and 4*x*) and two types (with and without competition). Degree of freedom (δ), χ^2 and *P* values and sample size (N) are presented for each statistical test. Significant *P* values are highlighted in bold.

Response variable	δ	χ^2	*P* values	N
Inflorescence number				431
Ploidy	2	3.56	0.169	
Growing type	1	155.93	**< 0.001**	
Ploidy:Growing type	2	10.00	**0.01**	
Reproductive biomass				427
Ploidy	2	2.90	0.235	
Growing type	1	44.24	**< 0.001**	
Ploidy:Growing type	2	9.36	**0.01**	
Aboveground biomass				426
Ploidy	2	7.61	**0.022**	
Growing type	1	57.18	**<0.001**	
Ploidy:Growing type	2	12.71	**0.002**	
Belowground biomass				434
Ploidy	2	77.02	**<0.001**	
Growing type	1	35.73	**<0.001**	
Ploidy:Growing type	2	53.91	**<0.001**	
Total biomass				443
Ploidy	2	5.23	0.073	
Growing type	1	65.78	**<0.001**	
Ploidy:Growing type	2	14.93	**0.001**	
Phenology				510
Ploidy	2	3.63	0.163	
Growing type	1	8.15	**0.004**	
Ploidy:Growing type	2	3.49	0.175	
Membrane leakage				92
Ploidy	2	0.00	0.999	
Growing type	1	6.61	0.086	
Ploidy:Growing type	2	26.03	**<0.001**	

⇩Cont.

Total soluble sugars				87
Ploidy	2	0.433	0.805	
Growing type	1	7.063	**0.008**	
Ploidy:Growing type	2	0.932	0.628	
Starch content				84
Ploidy	2	3.245	0.197	
Growing type	1	11.203	**0.001**	
Ploidy:Growing type	2	7.113	**0.028**	

Appendix 7.2. Results of the generalized linear mixed models testing for difference in the measured variables. Degree of freedom (δ), and χ^2 and *P* values are presented for each statistical test. For each response variable, three types of comparisons were made: 1) differences among cytotypes ($C_{no\ competition}$), under competition ($C_{competition}$) and magnitude of the competition effect ($C_{magnitude}$) among cytotypes; 2) differences between growing alone and under competition for each cytotype (diploids, Neotetraploid and tetraploid, respectively; C_{2x}, C_{Neo4x} and C_{4x}); and 3) differences between pairs of cytotypes competing with each other: diploids and Neotetraploids competing with diploids and neotetraploids (C1), Neotetraploids and tetraploids (C2) and diploids and tetraploids (C3), tested with response variable values (C1, C2 and C3) and the magnitude of the effect ($C1_{magnitude}$, $C2_{magnitude}$ and $C3_{magnitude}$). Significant *P* values are highlighted in bold.

Response variable	Comparison	δ	χ^2	*P* values
Inflorescence number	1) Differences among cytotypes			
	$C_{no\ competition}$	2	1.50	0.474
	$C_{competition}$	2	6.43	**0.040**
	$C_{magnitude}$	2	2.57	0.277
	2) Alone *vs* competition			
	C_{2x}	1	155.91	**< 0.001**
	C_{Neo4x}	1	89.63	**< 0.001**
	C_{4x}	1	211.11	**< 0.001**
	3) Pairs of cytotypes			
	C1	3	14.30	**0.002**
	C2	3	11.36	**0.010**
	C3	3	19.48	**< 0.001**
	$C1_{magnitude}$	3	5.00	0.172
	$C2_{magnitude}$	3	15.07	**0.002**
	$C3_{magnitude}$	3	2.35	0.503
Reproductive biomass	1) Differences among cytotypes			
	$C_{no\ competition}$	2	0.78	0.676
	$C_{competition}$	2	15.69	**< 0.001**
	$C_{magnitude}$	2	12.66	**0.002**
	2) Alone *vs* competition			
	C_{2x}	1	30.57	**< 0.001**
	C_{Neo4x}	1	21.00	**< 0.001**
	C_{4x}	1	110.37	**< 0.001**

⇩Cont.

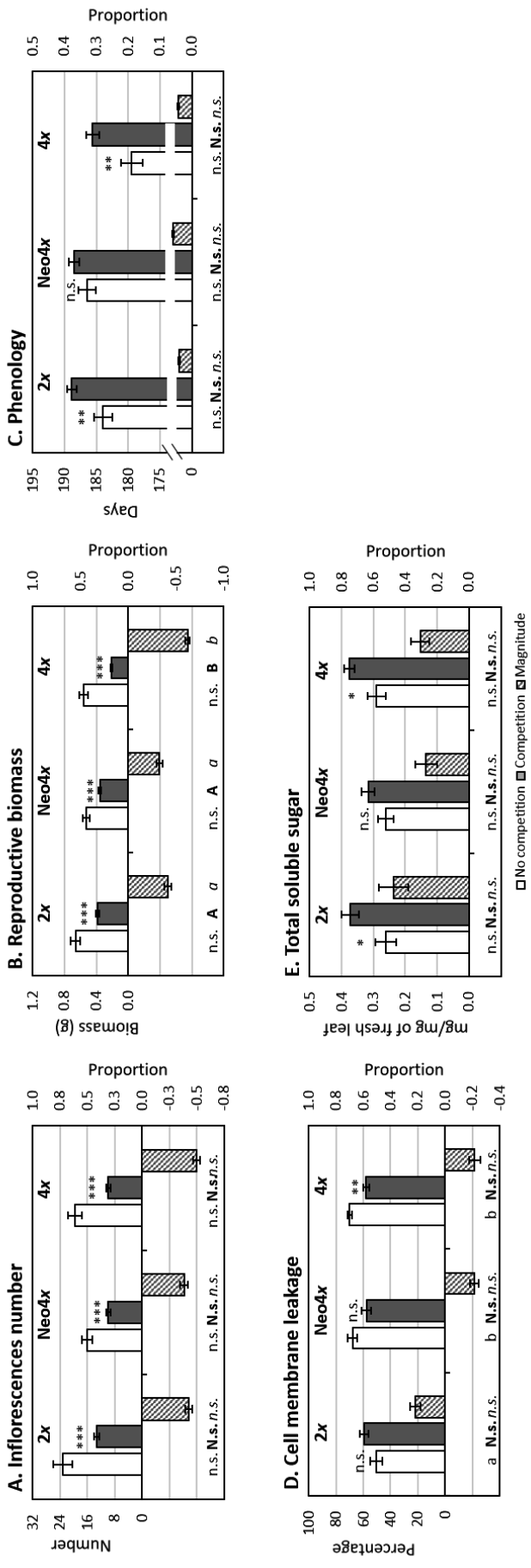
	3) Pairs of cytotypes			
	C1	3	17.54	**0.001**
	C2	3	23.34	**< 0.001**
	C3	3	13.20	**0.004**
	$C1_{magnitude}$	3	22.40	**< 0.001**
	$C2_{magnitude}$	3	31.30	**< 0.001**
	$C3_{magnitude}$	3	4.71	0.194
Aboveground biomass	1) Differences among cytotypes			
	$C_{no\ competition}$	2	2.79	**0.248**
	$C_{competition}$	2	33.19	**< 0.001**
	$C_{magnitude}$	2	17.88	**< 0.001**
	2) Alone *vs* competition			
	C_{2x}	1	45.03	**<0.001**
	C_{Neo4x}	1	16.37	**< 0.001**
	C_{4x}	1	106.53	**< 0.001**
	3) Pairs of cytotypes			
	C1	3	20.27	**< 0.001**
	C2	3	39.11	**< 0.001**
	C3	3	22.76	**< 0.001**
	$C1_{magnitude}$	3	45.53	**< 0.001**
	$C2_{magnitude}$	3	42.39	**< 0.001**
	$C3_{magnitude}$	3	4.30	**0.231**
Belowground biomass	1) Differences among cytotypes			
	$C_{no\ competition}$	2	25.37	**< 0.001**
	$C_{competition}$	2	21.16	**< 0.001**
	$C_{magnitude}$	2	32.38	**< 0.001**
	2) Alone vs competition			
	C_{2x}	1	52.43	**< 0.001**
	C_{Neo4x}	1	44.37	**< 0.001**
	C_{4x}	1	138.94	**< 0.001**
	3) Pairs of cytotypes			
	C1	3	6.34	0.096
	C2	3	9.341	**0.025**
	C3	3	15.62	**0.001**
	$C1_{magnitude}$	3	8.47	**0.037**
	$C2_{magnitude}$	3	88.15	**< 0.001**
	$C3_{magnitude}$	3	157.41	**< 0.001**
Total biomass	1) Differences among cytotypes			
	$C_{no\ competition}$	2	1.60	0.449
	$C_{competition}$	2	20.69	**< 0.001**
	$C_{magnitude}$	2	22.27	**< 0.001**
	2) Alone *vs* competition			
	C_{2x}	1	53.28	**< 0.001**
	C_{Neo4x}	1	24.17	**< 0.001**
	C_{4x}	1	129.66	**< 0.001**

⇩Cont.

	3) Pairs of cytotypes			
	C1	3	35.12	**< 0.001**
	C2	3	24.36	**< 0.001**
	C3	3	19.01	**< 0.001**
	$C1_{magnitude}$	3	62.12	**< 0.001**
	$C2_{magnitude}$	3	46.08	**< 0.001**
	$C3_{magnitude}$	3	9.44	**0.024**
Phenology	1) Differences among cytotypes			
	$C_{no\ competition}$	2	2.86	0.240
	$C_{competition}$	2	1.29	0.524
	$C_{magnitude}$	2	5.63	0.06
	2) Alone *vs* competition			
	C_{2x}	1	9.49	**0.002**
	C_{Neo4x}	1	1.43	0.232
	C_{4x}	1	10.75	**0.001**
	3) Pairs of cytotypes			
	C1	3	0.35	0.951
	C2	3	0.24	0.972
	C3	3	2.99	0.393
	$C1_{magnitude}$	3	1.273	0.736
	$C2_{magnitude}$	3	1.87	0.600
	$C3_{magnitude}$	3	1.91	0.590
Cell membrane leakage	1) Differences among cytotypes			
	$C_{no\ competition}$	2	9.71	**0.008**
	$C_{competition}$	2	0.28	0.871
	$C_{magnitude}$	2	0.17	0.921
	2) Alone *vs* competition			
	C_{2x}	1	2.74	0.098
	C_{Neo4x}	1	3.09	**0.079**
	C_{4x}	1	10.72	**0.001**
	3) Pairs of cytotypes			
	C1	3	1.28	0.735
	C2	3	7.46	**0.058**
	C3	3	2.39	0.496
	$C1_{magnitude}$	3	2.97	0.396
	$C2_{magnitude}$	3	7.13	0.068
	$C3_{magnitude}$	3	10.04	**0.018**
Total soluble sugars	1) Differences among cytotypes			
	$C_{no\ competition}$	2	0.60	0.741
	$C_{competition}$	2	4.69	0.096
	$C_{magnitude}$	2	4.15	0.125
	2) Alone *vs* competition			
	C_{2x}	1	4.93	**0.026**
	C_{Neo4x}	1	2.23	0.135
	C_{4x}	1	6.33	**0.012**

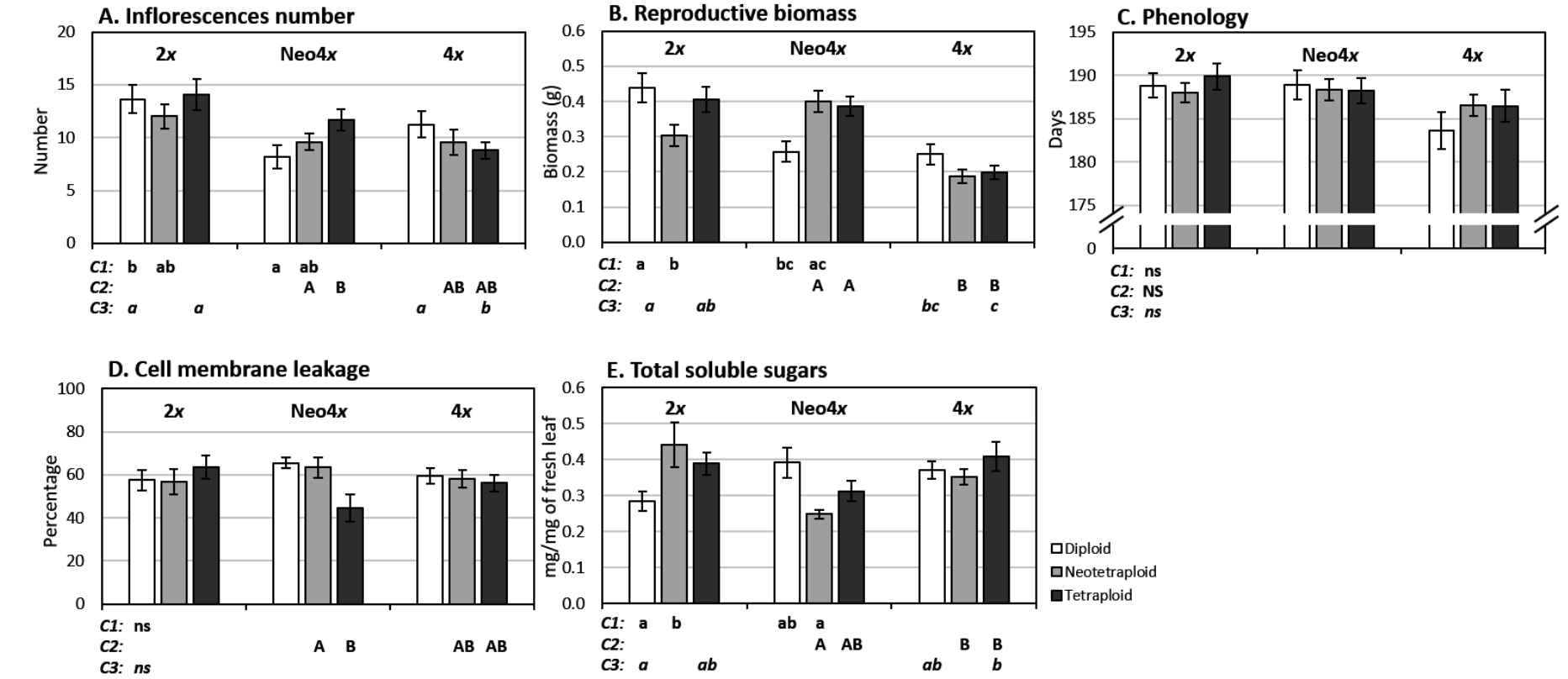
⇩Cont.

	3) Pairs of cytotypes			
	C1	3	15.76	**0.001**
	C2	3	17.88	**< 0.001**
	C3	3	8.40	**0.038**
	$C1_{magnitude}$	3	15.73	**0.001**
	$C2_{magnitude}$	3	11.44	**0.010**
	$C3_{magnitude}$	3	6.43	0.092
Starch content	1) Differences among cytotypes			
	$C_{no\ competition}$	2	6.89	**0.032**
	$C_{competition}$	2	6.29	**0.043**
	$C_{magnitude}$	2	49.56	**< 0.001**
	2) Alone *vs* competition			
	C_{2x}	1	5.85	**0.016**
	C_{Neo4x}	1	2.75	0.097
	C_{4x}	1	0.38	0.540
	3) Pairs of cytotypes			
	C1	3	51.03	**< 0.001**
	C2	3	13.79	**0.003**
	C3	3	43.26	**< 0.001**
	$C1_{magnitude}$	3	103.09	**< 0.001**
	$C2_{magnitude}$	3	15.27	**0.002**
	$C3_{magnitude}$	1	57.40	**< 0.001**



Appendix 7.3. Mean (± SE) values of measured phenological, growth and physiological variables in *Jasione maritima*: A) Inflorescence number; B) Reproductive biomass; C) Phenology; D) Cell membrane leakage; and E) Total soluble sugars. Treatments: no competition, white bars; growth with a neighbor, grey bars; and the magnitude of the impact of competition, striped bars. Lower case letters indicate differences ($P < 0.05$) between cytotypes when grown alone; upper case letters indicate differences between cytotypes grown with a neighbor; and, italic letters represent differences between the magnitude of the impact of competition. *, $0.01 < P < 0.05$; **$0.01 < P < 0.001$ and *** $P < 0.001$; n.s., nonsignificant.

Appendix 7.4. Mean (± SE) values of measured variables in plants of *Jasione maritima* in competition: A) Inflorescence number; B) Reproductive biomass; C) Phenology; D) Cell membrane leakage; and E) Total soluble sugars. Focal plants competing with diploid plants (2*x*, white bars), competing with neotetraploids (Neo4*x*, grey bars) and competing with tetraploid plants (4*x*, dark grey bars). Treatments: C1) comparison between diploids and tetraploids; C2) comparison between neotetraploids and tetraploids; and C3) comparison between diploids and tetraploids. Different letter corresponds to statistically differences ($P < 0.05$): C1 – lower case letters, C2 – upper case letters and C3 – italic letters.



Appendix 7.5. Results of the Generalized Linear Mixed Models testing for difference in the biomass between populations in the contact zone and outside this area. Degree of freedom (δ) and χ^2 and *P* values are presented for each statistical test. Significant *P* values are highlighted in bold.

Biomasses	**δ**	**χ^2**	***P* values**
Reproductive	4	12.25	**0.016**
Aboveground	4	52.24	**< 0.001**
Belowground	4	20.52	**< 0.001**
Total	4	32.47	**< 0.001**

Chapter 8 – General conclusions and future perspectives

General conclusions

Polyploidization has long been acknowledged as one of the major mechanisms responsible for flowering plants speciation (Ramsey and Schemske 1998; Soltis and Soltis 1999). Still, to date, the great majority of the studies focused on genetic and epigenetic effects of genome duplications, while little is known about the ecological processes involved with the emergence and successful establishment and spread of polyploids (Thompson and Lumaret 1992; Soltis *et al.* 2010). The work developed in this PhD thesis allows to increase the current knowledge about the role of polyploidization in plant evolution and diversification by studying several polyploid complexes and applying different approaches, from cytogeographical patterns across the entire distribution range and correlation with environmental requirements (Part I – Chapters 2 and 3), direct cytotype interactions at contact zones, potentially enabling cytotype coexistence (Part II – Chapters 4 and 5), to direct consequences of whole genome duplications in the performance of the cytotypes (Part III – Chapters 6 and 7).

The general conclusions resulting from the previous chapters are here summarized, discussed and listed. The main future perspectives opened by the results of this PhD thesis are also presented.

Part I – Large-scale cytogeographic distribution and environmental determinants

Understanding current distribution patterns of different cytotypes provides useful information to unravel the processes involved in cytotype emergence and establishment. The niche shift hypothesis suggested that if polyploidization changes the environmental tolerances of polyploid individuals, they might be able to disperse beyond parental populations and establish in novel habitats (Levin 1975, 2004; Husband and Schemske 2000). In Chapters 2 and 3, I investigated the geographical patterns of diploid and tetraploid populations in the entire distribution range of two congeneric polyploid species and observed that environmental variables explained in dissimilar ways the distribution patterns within polyploid complexes, leading to the construction of different hypotheses.

Two different *in situ* distribution patterns were observed: in *J. maritima*, cytotypes were distributed allopatrically, while in *J. montana*, cytotypes presented a mosaic parapatric distribution. Therefore, in *Jasione maritima* (Chapter 2), polyploidization seems to have broaden the environmental requirements of the tetraploid plants, being probably involved in the ability of the tetraploids to colonize a wider range towards southern and dryer areas than those that

are occupied by the diploids, which are restricted to northern areas of the distribution range of the species. However, environmental variables could only partially explain the currently observed patterns, and thus, it is suggested that other factors, such as competitive ability, might also be involved. Cytotype performance under competition was later tested in this PhD thesis (Chapter 7; see below), and reciprocal transplant experiments are currently being developed to test the hypotheses that resulted from this Chapter.

Contrarily, *J. montana* distribution patterns and niche analyses (Chapter 3) show that environmental preferences of diploids and tetraploids are similar, suggesting that environmental variables do not seem to be involved with the current distribution patterns, similarly as observed in Chapter 4. The paucity of mixed-ploidy populations also suggests that frequency dependent selection might be an important force driving current distribution patterns in both the *Jasione* polyploid complexes studied. The results obtained for *J. montana* open new scientific avenues, particularly the evaluation of direct ecological and reproductive interactions between the two cytotypes at contact zones. Future studies focused in testing reproductive isolation and minority cytotype exclusion, in quantifying unreduced gamete formation and in evaluating the competitive ability of each cytotype will be very informative.

Besides providing relevant information on the processes occurring in natural populations, the results of these Chapters also corroborated the need for more detailed studies in groups where polyploidization is frequent, such as the genus *Jasione*. Also, detailed knowledge about the cryptic diversity found within each species is fundamental for decision-making processes related with the establishment and/or maintenance of conservation plans.

Part II – Cytotype interactions and coexistence at contact zones

Contact zones where different cytotypes grow in proximity or within the same population are natural laboratories to study the establishment of cytotypes and its interactions. These zones are far more interesting and dynamic if they are composed by polyploid complexes bearing high ploidy-levels (*e.g.*, tetraploids and octoploids), as crosses between such cytotypes can result in potential viable offspring with an even ploidy level. In Chapters 4 and 5 of this PhD Thesis, I investigated the geographical patterns and interactions of the tetraploid-octoploid *Gladiolus communis* polyploid complex at contact zones and observed complex interaction patterns, with polyploidization and hybridization being frequent, providing new insights on the reproductive relationships between the dominant cytotypes.

The contact zone of *G. communis* revealed to be complex, with cytotypes being distributed parapatrically (Chapter 4). Still, environmental analyses suggested a high environmental niche overlap, and so the dynamics of the contact zones had to be driven by other factors. Geographical separation and habitat similarity among cytotypes suggest that the detected mixed-ploidy populations may be transitional due to minority cytotype exclusion process. However, the high diversity of cytotypes observed in the field suggests that recurrent polyploid formation and hybridization events are frequent processes in *G. communis* contact zones. These results motivated a detailed evaluation of the reproductive barriers between the dominant cytotypes of this polyploid complex, to understand the fate of new polyploids in natural populations.

Therefore, in Chapter 5, I tested the occurrence of reproductive barriers between tetraploid and octoploid individuals of *G. communis.* The results obtained revealed weak pre-pollination barriers, while post-pollination interactions were strong and may limit gene flow. However, such interactions were highly dependent on the pollen composition delivered by pollinators, and consequently, conditioned by the cytotype composition of the population. Therefore, the application of different pollination treatments enabled to recreate different scenarios that might contribute to explain the coexistence of both cytotypes in nature. If at initial stages, higher ploidy cytotypes may suffer strong frequency dependent selection, at later stages, strong post-zygotic barriers may enable cytotype coexistence. Factors such as, recurrent unreduced gametes formation, shown to be frequent in the complex, might be responsible for the establishment of the newly originated polyploid at initial stages, which accords with theoretical models produced.

Both studies suggest the occurrence of gene flow within the *G. communis* polyploid complex, resulting in dynamic contact zones bearing high cytogenetic diversity. Experimental studies testing the minority cytotype exclusion theory in tetraploid-octoploid populations varying in cytotype proportions are important to improve our knowledge about polyploid dynamics and interactions at contact zones.

Part III – Direct consequences of whole genome duplication in competitive ability

Different performance between diploids and polyploids driven, for example, by contrasting competitive abilities might have dramatic consequences in the successful establishment of polyploid lineages (Fowler and Levin 1984; Levin 2002). However, competitive ability has been studied in only a few polyploid complexes and showed highly species-specific

responses (Maceira *et al.* 1993; Collins *et al.* 2013; Thompson *et al.* 2015). Also, in such studies, only rarely the effects of whole genome duplications *per se* were decoupled from adaptation processes that operated after polyploidization (but see Ramsey 2002; Husband *et al.* 2008; Ramsey 2011). In Chapter 6 and 7, I synthetized neotetraploid individuals from diploid population to quantify the direct effects of genome duplications in the competitive ability of the diploid-tetraploid *J. maritima*, an approach rarely used so far.

Since no neotetraploids were detected in natural populations, as evident by the allopatric distribution of diploid and tetraploid *J. maritima* across the entire distribution range (Chapter 2), I have successful developed a methodology to synthetize tetraploids from wild diploid seedlings of *J. maritima* (Chapter 6). Several methodological approaches were tested to get the optimum synchronization of germination and to get the highest tetraploid induction rates. The best protocol for *J. maritima* has the potential for being applied to other wild species and enabled to obtain adult plants to experimentally quantify the immediate consequences of genome duplications in Chapter 7.

In Chapter 7, I tested the contribution of genome duplications *per se* to the divergence of plant traits affecting competitive ability using a novel approach involving diploids, neotetraploids and established tetraploids. I observed that, at the contact zone, cytotypes presented similar competitive abilities, suggesting that this trait might maintain a stable contact zone and that genome duplications did not seem to drive major changes in traits linked with competitive ability. Interestingly, tetraploids presented different competitive abilities across their distribution range, possibly linked with adaptations to an environmental gradient. Such differences may contribute to explain the current allopatric distribution of *J. maritima*.

Besides the key conclusions highlighted above, such studies reinforce the importance of incorporating neopolyploids in comparative experiments, as well as the need to consider population variation in ecological studies, as the obtained results were dependent on the context of each population. Future studies of drought tolerance, with and without competition, will help to clarify if polyploidization could be involved with changes in different water efficiencies, and if this factor could further explain the current distribution pattern of *J. maritima*.

Broader future perspectives

This PhD thesis clearly reveals the need for further ecological studies at different levels and using polyploid complexes with different characteristics. Overall, detailed large-scale cytogeographical information is fundamental when studying polyploid complexes. Niche modelling analyses revealed to be an excellent tool to understand the role of environmental variables in cytotype distribution and to build hypotheses on the factors generating the current geographical patterns observed in nature, and should be the basis when designing targeted manipulative experiments. Clearly, there is still very few information in higher-ploidy complexes where more complex interactions can occur and might generate higher levels of cytogenetic diversity. Also, interactions at contact zones are poorly understood and, although empirically inferred in numerous studies, the minority cytotype exclusion theory initially proposed by Levin in 1975 has been experimentally tested only once, thus requiring further studies in the field. Manipulative experiments involving reciprocal transplants and common garden experiments under competition and/or stressful condition are of pivotal importance. Ideally, such experiments should include all the necessary players, *i.e.*, diploids, neotetraploids and established tetraploids. Only this way, we might decouple the effects of genome duplications *per se* from evolutionary changes that occurred after polyploid emergence. By doing this, it will be possible to better understand the role of polyploidization in the genesis of plant diversity.

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