UNIVERSIDADE D
COIMBRA

Ana Sofia dos Santos Afonso

THE ROLE OF POLYPLOIDY IN SHAPING THE DIVERSITY WITHIN *LINUM SUFFRUTICOSUM S.L.* (LINACEAE)

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 Juan Arroyo, apresentada ao Departamento de Ciências da Vida Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Abril de 2022

The role of polyploidy in shaping the diversity within *Linum suffruticosum s.l.* (Linaceae)

O papel da poliploidia na génese de diversidade em *Linum suffruticosum s.l.* (Linaceae)

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Thesis in the scope of the Doctorate in Biosciences, specialization in Ecology, supervised by Professor João Carlos Mano Castro Loureiro (Department of Life Sciences, University of Coimbra), Doctor Sílvia Raquel Cardoso Castro Loureiro (Department of Life Sciences, University of Coimbra) and Professor Juan Arroyo (Department of Plant Biology and Ecology, University of Seville), presented to the Department of Life Sciences of the Faculty of Sciences and Technology of the University of Coimbra.

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I declare that this thesis was written and organized by me, and I confirm that it has not been previously submitted, in whole or in part, to obtain another academic degree. I confirm that the work described was done by me and by the co-authors, in the case of joint publications, as indicated in chapter II. In these cases, my contribution is explicitly indicated below:

The work presented in chapter II was conceived by me and João Loureiro, Sílvia Castro and Juan Arroyo. I performed all laboratory work with the support of João Loureiro and Sílvia Castro, and the field work with the support of Juan Arroyo, João Loureiro and Erika Olmedo-Vicente. I analysed all the data, wrote the first draft of the paper, and incorporated the suggestions made by all co-authors.

The work presented in chapters III, IV and V was designed by me with the support of all supervisors. In Chapter III, I performed all field work with the support of Juan Arroyo, João Loureiro, Erika-Olmedo Vicente, Sara Lopes, Daniela Tavares and Guillaume Papuga. I also perfomed all niche modelling and data analysis with the support of Mariana Castro and Albano Figueiredo. In Chapter IV, I performed all field work with the support of Mariana Castro and Catarina Siopa. I also performed all laboratory work and data analysis. In Chapter V, I performed all laboratory work and data analysis, with the support of Alejandra de Castro in laboratory work, of Elena Varela-Álvarez in sequence alignment, of Marcial Escudero in phylogenetic analysis and of José Ruiz-Martin in genetic data sequences.

Declaro que esta tese foi elaborada por mim e confirmo não ter sido previamente submetida, total ou parcialmente, para obtenção de outro grau académico. Confirmo que o trabalho descrito foi realizado por mim e pelos co-autores, no caso de publicações conjuntas, como indicado no capítulo II. Nestes casos, a minha contribuição está explicitamente indicada abaixo:

O trabalho apresentado no capítulo II foi concebido por mim e por João Loureiro, Sílvia Castro e Juan Arroyo. Realizei todo o trabalho laboratorial com a ajuda de João Loureiro e Sílvia Castro, e o trabalho de campo com o apoio de Juan Arroyo, João Loureiro e Erika Olmedo-Vicente. Escrevi a primeira versão do artigo e incorporei as sugestões dos outros autores.

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“Le véritable voyage de découverte ne consiste pas à chercher de nouveaux paysages,
mais à avoir de nouveaux yeux.”
Marcel Proust

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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

10*x* – decaploid

14*x* – tetradecaploid

a - anthers of long-styled morph

A – anthers of short-styled morph

AICc – Akaike Information Criterion corrected

an. – aneuploidy

auct. – (L. Auctorum) authors

aw – soil water capacity at 15 cm

BIC – Bayesian Information Criterion

bio11 – mean temperature of the coldest quarter

bio14 – precipitation of the driest month

bio16 – precipitation of the wettest quarter

bio2 – mean diurnal range;

bio3 – isothermality

BioNJ algorithms – biological neighbor-joining algorithms

BP – before present

cat – cation exchange capacity at 15 cm

clay – clay content at 15 cm

Cod – Code

COI – Herbarium of the University of Coimbra

comb. nov. – (L. *combinatio nova*) new combination

CV – coefficient variation

Cont. – to be continued

D – Schoener's *D* value

dcoast – distance to the coast

DI – DNA index

DNA – deoxyribonucleic acid

dNTPs – Deoxynucleotide triphosphates

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

EDTA $Na_2.2H_2O$ – Disodium ethylenediaminetetraacetate dihydrate

ele – elevation

ENM – environmental niche modelling

et al. – (L. *et alia*) and other

FL – fluorescence pulse integral

frag – fragment content at 15 cm

FS – forward light scatter

G – gamma distribution

G test - goodness-of-fit test

G value - goodness-of-fit value

G.s. – genome size

GBIF – Global Biodiversity Information Facility

GLM – generalized linear model

GTR - general time reversible (model)

H – herkogamy

HCl – hydrogen chloride

HKY – Hasegawa-Kishino-Yano (model)

I – proportion of invariable sites

i.e. – (L. *id est*) that is

Ina-High – inaccuracy high organs

Ina–Low – inaccuracy low organs

InL – log-likelihood

IR – reciprocity index

IRI – index of reproductive isolation

ITS – internal transcribed spacer

JC – Jukes-Cantor (model)

K2 – Kimura 2-parameter (model)

L – long-styled morph

log – logarithm

Ma – million years

Max – maximum

MaxEnt – Maximum Entropy Modelling

$MgCl_2$ – magnesium chloride

Min – minimum

MLC – Maximum Composite Likelihood

n – gametophytic

N – sample size

n.s. – not-significant

na – not available

NaCl – sodium chloride

ndhA - *ndhA* intron

ndhF - *ndhF-rpL32* spacer

NE – northeast

no. – number of

No. Chro – number of chromosome counts

non – (L. *non*) other than

NW – northwest

P – probability

PCA – Principal Component Analyses

PCR – Polymerase Chain Reaction

pg – pictograms

ph - soil pH at 30 cm

PI – propidium iodide

PL – ploidy level

POP – population

PVP – polyvinylpyrrolidone

r – correlation coefficient

RNase – ribonuclease

ROC – receiver operating characteristic

S – short-styled morph

St – stigmas of long-styled morph

st – stigmas of short-styled morph

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

S.l. – Solanum lycopersicum 'Stupické'

s.s. – sensu stricto

sand –sand content at 15 cm

SD – standard deviation

SE – standard error

Sect. – Section

SEV – Herbarium of the University of Seville

sp. – species

SS – side light scatter

Stat. nov. – (L. *status novus*) new status

subsp. – subspecies

SW – south-west

SYBR Green – Synergy Brands, Inc. Green

T – average organ measure

T92 – Tamura 3-parameter (model)

TAE – Tris-acetate buffer

text – soil texture at 15 cm

TN93 – Tamura-Nei 93-parameter (model)

Tota-Ina – total inaccuracy

Tris.HCl – tris(hydroxymethyl)aminomethane

USDA system – soil system from United States Department of Agriculture

UV – ultraviolet radiation

v% – volumetric fraction

Var – variances

var. – variety

vs – versus

w% – mass fraction

WGD – Whole Genome Duplication

WPB – Woody Plant Buffer

x – haploid

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

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Chapter V - Multiple events of genome duplications in the differentiation of the polyploid complex *L. suffruticosum s.l.*

Abstract

Whole genome duplications (WGDs) bear broad-scale consequences on gene expression and developmental processes, potentially leading to immediate morphological, reproductive and physiological shifts. Changes in reproductive traits will be particularly relevant in species with complex breeding systems, such as heterostyly, a polymorphism that promotes outcrossing and reduces sexual self-interference. Cytotypes frequently have distinct characteristics that allow to grow in habitats different from their parentals and/or expand to new areas, leading to spatial segregation. In the Mediterranean region, the high frequency of polyploidy is likely a consequence of its dynamic paleogeographic and climatic history. Despite the advances in the genetic and epigenetic consequences of WGDs, the ecological factors driving polyploidy and its evolutionary consequences are poorly understood.

The main goal of this study was to evaluate the role of WGDs as one of the factors shaping the evolution of flowering plants, using *Linum suffruticosum s.l.* as study system. This species complex comprises heterostylous populations with long- and short-styled morphs individuals and a heteromorphic self-incompatibility system. It was also described as a polyploid complex, but nothing was known about its current cytogeographical patterns. First, I have focused on the cytogenetic and cytogeographical patterns, then, on the impacts of WGDs on environmental attributes and the reproductive morphology and reproductive system, and, finally, on the phylogenetic and phylogeographic relationships among cytotypes.

Using flow cytometric analyses and chromosome counts, I investigated cytotypes diversity and distribution patterns in most of the distribution range. High cytogenetic diversity was found with five major cytotypes being detected (diploids, tetraploids, hexaploids, octoploids and decaploids) and new ploidy levels were reported for the first time. The different ploidy levels were distributed parapatrically with several contact zones, and a few mixed-ploidy populations were observed. These results showed that WGDs are one of the key mechanisms governing the diversification of *L. suffruticosum s.l.*

The environmental niches of the five main cytotypes were studied across the distribution range, using niche modelling tools. Differences in the ecological requirements were observed, with polyploids being associated with dry and harhs habitat. Diploids presented the widest environmental niche, with polyploids occupying part of the diploid niche. Although some polyploids have equivalent potential ecological niches, the cooccurrence of cytotypes was not frequent in nature. The different ecological requirements could not entirely explain the distribution of cytotypes, but they have played a role in the mosaic distribution of ploidy levels of *L. suffruticosum s.l.*

To evaluate the impact of WGDs on reproductive traits and reproductive relationships, morph frequencies were recorded, and the sexual organs were measured in flowers from populations of each cytotype. Crosses within cytotypes (selfing, and intra- and inter-morph crosses) and crosses between diploids and tetraploids were performed. Most of the populations were isoplethic, and the reciprocity indexes were maintained in all cytotypes. The size of sexual organs increased with ploidy level, and there was an overlap in reciprocal positions among cytotypes. *Linum. suffruticosum s.l.* was strongly self- morph incompatible across all ploidy levels. However, crosses between diploid and tetraploids produced similar pollen tube development to intra-cytotype crosses. These results suggest that pollen and gene flow is possible among cytotypes and that such flow is prevented by geographical distance between populations.

Finally, DNA extractions from populations of all cytotypes were made and plastid and nuclear markers were used to estimate a haplotype-ribotype network and infer the phylogenetic and phylogeographic relationships among the cytotypes. Much higher variability of ribotypes and haplotypes were found in diploid populations from the east Pyrenees to northern Italy than in diploid and polyploid populations from Spain and Morocco. These results showed that several WGDs and differentiation events occurred along the distribution area.

In conclusion, the geographical overlap and high cytogenetic diversity suggest multiple origins of the polyploids. The ecological requirements played a role in the mosaic distribution of cytotypes, and the results provide solid data for future reciprocal experiments. There is no evidence of the breakdown of the incompatibility system, but pollen flow between cytotypes appears to be possible. Despite the impossibility to disentangle the origin of polyploids, the diversity observed provides valuable data for future phylogeny and phylogeography studies and should be accounted for when studying this complex's biosystematics.

Key words: *Linum suffruticosum s.l.*; ecology; heterostyly; niche differentiation; polyploidy.

Resumo

As duplicações do genoma podem ter consequências em larga escala na expressão genética e nos processos de desenvolvimento, podendo levar a potenciais alterações morfológicas, reprodutivas e fisiológicas imediatas. Alterações nas características reprodutivas são particularmente relevantes em espécies com sistemas de reprodução complexos, como a heterostilia, um polimorfismo que promove a polinização cruzada e reduz a interferência dos órgãos sexuais. Os citotipos novos apresentam, frequentemente, características distintas que lhes permitem crescer em habitats diferentes dos seus progenitores e/ou expandirem-se para novas áreas, levando à segregação espacial. Na região do Mediterrâneo, a alta frequência de poliploidia é provavelmente consequência da uma história dinâmica paleogeográfica e climática. Apesar dos avanços nas consequências genéticas e epigenéticas das duplicações de genoma, os fatores ecológicos que conduzem à poliploidia e as suas consequências evolutivas são ainda pouco compreendidos.

O principal objetivo deste estudo foi avaliar o papel das duplicações do genoma como um dos fatores que influenciam a evolução das angiospérmicas, usando *Linum suffruticosum s.l*. como sistema de estudo. Este complexo de espécies é composto por populações heterostilas com indivíduos de morfotipos longos e curtos e um sistema de auto-incompatibilidade heteromórfica. Também foi descrito como um complexo poliploide, mas pouco se sabia sobre os seus padrões citogeográficos atuais. Primeiro, foquei-me nos padrões citogenéticos e citogeográficos, depois nos impactos das duplicações do genoma nos atributos ambientais e na morfologia reprodutiva e sistema reprodutivo e, por fim, nas relações filogenéticas e filogeográficas entre citotipos.

Usando a citometria de fluxo e contagens cromossómicas, investiguei a diversidade de citotipos e os padrões de distribuição na maior parte da área de distribuição. Foi encontrada uma elevada diversidade citogenética, com cinco citotipos principais detectados (diploides, tetraploides, hexaploides, octoploides e decaploides) e a descoberta de novos níveis de ploidia. Os diferentes níveis de ploidia distribuíram-se parapatricamente com várias zonas de contato, e foram detetadas algumas populações de vários citotipos. Esses resultados mostraram que as duplicações do genoma são um dos principais mecanismos que influenciam a diversificação de *L. suffruticosum s.l.*

Os nichos ambientais dos cinco principais citotipos foram estudados em toda a área de distribuição, usando ferramentas de modelação de nicho. Foram observadas diferenças nos atributos ecológicos, com os poliploides a serem associados a habitats secos e agrestes. Os diploides apresentaram o nicho ambiental mais amplo, com os poliploides a ocupar parte do

nicho diploide. Embora alguns poliploides tenham nichos ecológicos potenciais equivalentes, a coocorrência de citotipos não é frequente na natureza. As diferentes exigências ecológicas não podem explicar inteiramente a distribuição dos citotipos, mas desempenharam um papel na distribuição em mosaico dos níveis de ploidia de *L. suffruticosum s.l.*

Para avaliar o impacto das duplicações do genoma nas características reprodutivas e relações reprodutivas, frequências de morfotipos foram registadas e os órgãos sexuais foram medidos em flores de populações de cada citotipo. Cruzamentos dentro de citotipos (autopolinização, e cruzamentos intra- e inter-morfos) e cruzamentos entre diploides e tetraploides foram realizados. A maioria das populações demonstrou ser isoplética e os índices de reciprocidade foram mantidos em todos os citotipos. O tamanho dos órgãos sexuais aumentou com o nível de ploidia e houve sobreposição nas posições recíprocas entre os citotipos. *Linum suffruticosum s.l.* é fortemente auto- e morfo-incompatível em todos os níveis de ploidia. No entanto, nos cruzamentos entre diploides e tetraploides foi obtido um desenvolvimento de tubos polínicos semelhante aos cruzamentos intra-citotipo. Estes resultados sugerem que o fluxo genético e de pólen é possível entre citótipos e que este fluxo é evitado pela distância geográfica entre as populações.

Finalmente, extrações de ADN de populações de todos os citotipos foram realizadas e marcadores plastidiais e nucleares foram usados para estimar redes de haplotipos-ribotipos e inferir as relações filogenéticas e filogeográficas entre os citotipos. Foi encontrada uma variabilidade muito maior de ribotipos e haplotipos em populações diploides desde o Leste dos Pirenéus ao norte da Itália do que em populações diploides e poliploides de Espanha e Marrocos. Esses resultados mostraram que vários eventos de duplicação de genoma e de diferenciação ocorreram ao longo da área de distribuição.

Em conclusão, a sobreposição geográfica e a elevada diversidade citogenética sugerem múltiplas origens dos poliploides. As exigências ecológicas desempenharam um papel na distribuição em mosaico de citotipos e os resultados fornecem dados sólidos para futuras experiências recíprocas. Não há evidência de quebra do sistema de incompatibilidade, mas o fluxo de pólen entre citotipos parece ser possível. Apesar da impossibilidade de desvendar a origem dos poliploides, a diversidade observada fornece dados valiosos para futuros estudos de filogenia e filogeografia e deve ser considerada no estudo da biossistemática desse complexo.

Palavras chave: *Linum suffruticosum s.l.*; diferenciação de nicho; ecologia; heterostilia; poliploidia.

Chapter I – General Introduction

Evolutionary forces acting on plant variation and shaping diversity in the Mediterranean region

The Mediterranean basin region has been considered among the most important biodiversity hotspot, with about 25,000 plant species, of which about 50% are endemic (Myers *et al.* 2000). The geological and climatic history has greatly impacted the distribution of species in the Mediterranean basin, often leading to isolated populations and/or contact with populations of closely related taxa. The climate characteristics of the Mediterranean basin, with mild, rainy winters and hot, dry summers, and its evolution and oscillation had a considerable impact on the survival or emergence of species, as well as, on species ranges (Figure 1.1; Jansson and Dynesius 2002; Thompson 2020). Therefore, the climate, the rocky topography, and the isolation, both in mountains and islands, and the geological heterogeneity and historical dynamics, constitute the main factors for the rapid diversification and speciation in this region (Figure 1.1; Rundel *et al.* 2016). Furthermore, the closure of the marine gateways that existed between the Atlantic Ocean and the Mediterranean Sea, which led to the drying of the Mediterranean Sea (Messinian Salinity Crisis- 5.96–5.33 Mya), and the formation of the current insular system, played an important role in species expansion reported during the Oligocene–Miocene (Figure 1.1; Steininger and Rögl 1984; Krijgsman 2002; Meulenkamp and Sissingh 2003). More recently, human activities have led to a change in the selective pressures. This impact is particularly significant in the Mediterranean basin, given the long history of human presence and activity (Figure 1.1; Thompson 2020). Altogether, these factors create the opportunity for lineage divergence and speciation, increasing genetic and species diversity (Hewitt 2011; Nieto-Feliner 2014; Thompson 2020).

The mosaic of the Mediterranean basin influences plant ecology and evolution, including plant reproductive traits. In fact, the diversity in floral morphology can contribute to reproductive isolation and, consequently, to speciation (Thompson 2020). Considering that most plants are visited by different pollinators, for floral adaptations to occur, there must be a variation in the strength of the plant-pollinator interaction (Schemske and Horvitz 1984). Therefore, spatial and temporal variations in the abundance and composition of pollinators coupled with pollination efficiency in response to variation in floral characteristics can lead to selective pressures on the adaptive responses of flowers to pollinators (Thompson 2020). This is particularly important in species with complex sex polymorphisms, such as distyly, tristyly, or stigma-anther dimorphism (heterostyly and related conditions, Figure 1.2). The heterostylous syndrome is estimated to occur in 1,608 species worldwide (reviewed in Costa 2017). The Iberian

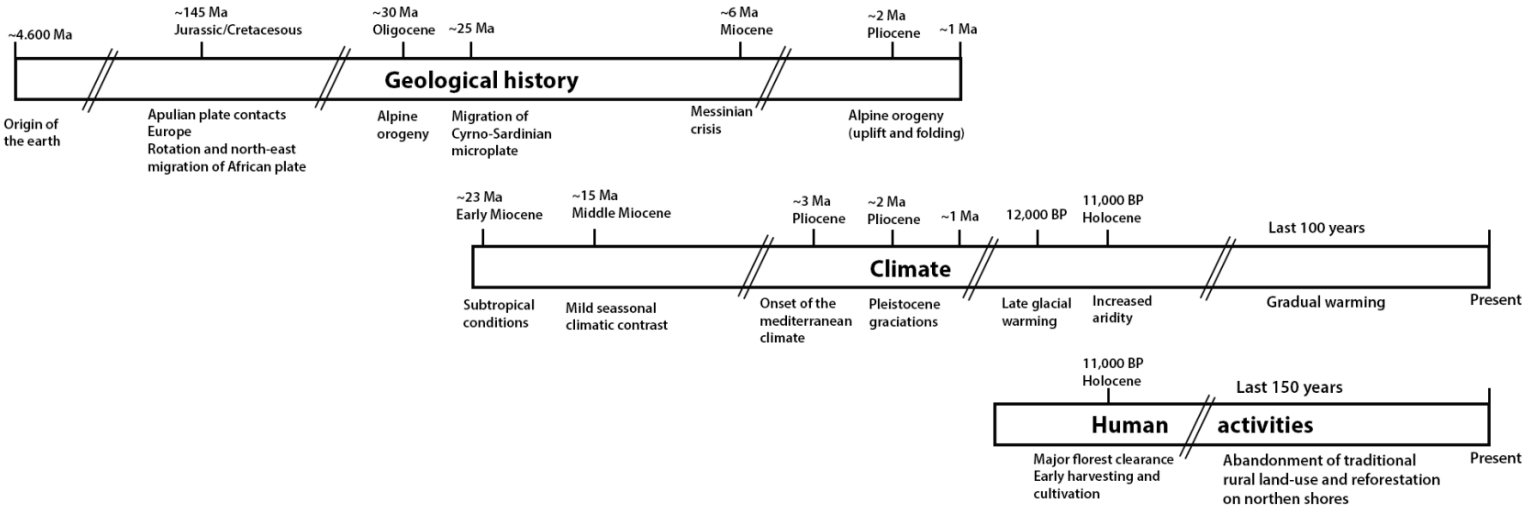

Figure 1.1. Key factors that impacted plant evolution in the Mediterranean basin (adapted from Thompson 2020).

Peninsula alone comprises 2.5% of the total number of heterostylous species described so far (Costa 2017).

Another consequence of the Mediterranean basin's dynamic paleogeographic and climatic history (Thompson 2020), ecogeographical heterogeneity, and human influence (Blondel *et al.* 2010) is the high frequency of polyploidy. The mountain ranges that allowed multiple refugia and the recurrent connections and disconnections with Northern Africa contributed to the emergence of allopatric and parapatric clades (Hewitt 2011). Therefore, multiple events of whole-genome duplications (WGDs) occurred on both sides of the Mediterranean Sea (*e.g.*, Bougoutaia *et al.* 2021). About 12.0-13.0% of the angiosperm species were estimated to be ploidy variable, supporting that polyploidy is a dynamic and ongoing process in nature (Ramsey and Schemske 1998; Soltis 2005; Wood *et al.* 2009). In the Mediterranean region, a polyploid incidence of 36.5% was detected, with higher values detected for the Iberian Peninsula (48.8%; Marques *et al.* 2018).

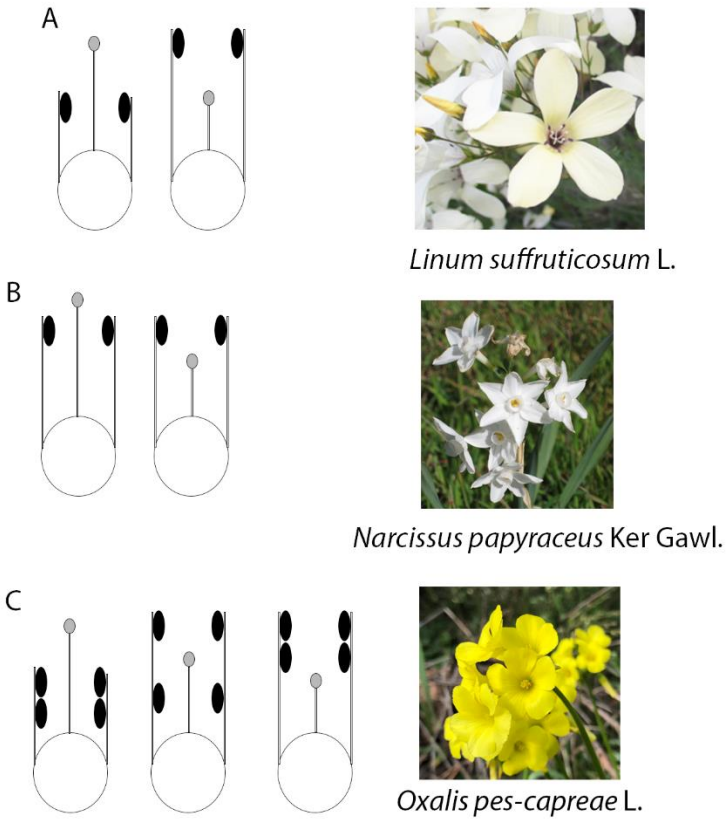

Figure 1.2. Representation of distyly (A), stigma-height dimorphism (B), and tristyly (C) with representative species for each type of floral polymorphism [distyly: *Linum suffruticosum* L. (Linaceae); stigma-height dimorphism: *Narcissus papyraceus* Ker Gawl. (Amaryllidaceae); tristyly: *Oxalis pes-capreae* L. (Oxalidaceae)].

General considerations about polyploidy

Polyploidy or whole-genome duplications (WGDs), *i.e.*, the existence of more than two sets of chromosomes, plays a significant role in the evolution and diversification of flowering plants and is recognized as an important speciation mechanism (Ramsey and Schemske 1998; Segraves *et al.* 1999). This widespread phenomenon is observed in up to 100% of flowering plants, appearing in several lineages (Soltis 2005), and polyploidy has been correlated with bursts of species diversity (Soltis *et al.* 2009). According to Wood *et al.* (2009), 15.0% of the speciation events in Angiosperms have been associated with ploidy increase. Stebbins (1971) estimated that about 35.0% of angiosperms had polyploids in their ancestry, and Grant (1981) estimated that 47.0% of angiosperms were polyploids. In the Arctic flora, 51.1% of taxa are exclusively polyploids, and 9.6% are diploid-polyploid complexes (Brochmann *et al.* 2004).

Among Angiosperms, it has been estimated that the percentage of polyploidization events is bigger in monocots than in dicots (Otto and Whitton 2000; Marques *et al.* 2018). On the other hand, Gymnosperms are considered the plant group with the lowest polyploidy incidence (Murray 2013), with no mixed-ploidy taxa reported in the Mediterranean region (Marques et al., 2017). This suggests that polyploidization is not a stable process in this plant group (Ahuja 2005; Husband *et al.* 2013). Pteridophytes are the plant group with the highest incidence of polyploidy (75.0%), followed by Angiosperms (47.0%) and Gymnosperms (6.0%) (Marques *et al.* 2018).

Until a few decades ago, research and reports about the incidence of polyploidy were mostly based on karyological studies. However, the application of flow cytometry to plants coupled with the development of fast and straightforward methodologies (Kron *et al.* 2007; Loureiro 2007; Suda *et al.* 2007) enabled to map cytotype distribution for the entire range of distribution of many polyploid complexes (*e.g.*, Husband and Schemske 1998; Kron *et al.* 2007; Jersáková *et al.* 2010; Castro *et al.* 2011, 2019; Husband *et al.* 2013; Mandák *et al.* 2016; Kolář *et al.* 2017). This enabled the development of detailed studies and provided excellent opportunities to understand the evolution and dynamics of polyploidy complexes.

The results of large scale studies using flow cytometric analyses revealed that polyploidy is a common phenomenon, not only among different species but also within species and populations, with minority cytotypes and contact zones being detected in numerous polyploid complexes (*e.g.*, Jersáková *et al.* 2010; Castro *et al.* 2011, 2018; Husband *et al.* 2013). These contact zones and mixed-ploidy populations are described as “natural laboratories” for studying evolutionary transitions as they provide excellent opportunities to understand the evolution and dynamics of polyploidy complexes (Lexer and van Loo 2006). Nevertheless, in most cases, ploidy inference using flow cytometry should be complemented with other cytological techniques,

such as chromosome counts. This is particularly important when genome size variation is considerable, and it is difficult to assign a specific genome size range to a given ploidy level (Baack 2004; Balao *et al.* 2009; Castro *et al.* 2012a; Kim *et al.* 2012a; Prančl *et al.* 2018; Castro *et al.* 2019). Furthermore, by combining the two techniques, it is possible to detect differences in the DNA content in organisms with the same number of chromosomes (Dimitrova *et al.* 1999; Mahelka *et al.* 2005).

Despite the significant amount of new studies and advances made in several polyploidy complexes, the consequences of WGDs for the overall success of a given species and/or cytogenetic entity have been seldom explored.

Polyploid formation

The origin of polyploids can be quite complex to disentangle, as it involves the duplication of the genome from the same taxon (autopolyploidy) or the combination of genomes from two parental taxa (allopolyploidy) (Ramsey and Schemske 1998). Both an auto- and an allopolyploid can face different challenges. After neopolyploid formation, the architecture of the cell is modified, the mitosis and meiosis 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.* 2016).

Multiple origins of a polyploid from genetically distinct parent individuals and the rapid genomic changes that occur immediately after polyploid formation may contribute to the genetic diversity of polyploid plants (Wagner 1983). The duplication of chromosomes could be due to somatic events or the fusion of unreduced gametes. In the first case, zygomatic doubling or meristematic chromosome doubling occurs. However, it has been considered a rare phenomenon in nature (DeWet 1980). Thus, the primary mechanism of polyploid emergence is through the formation of unreduced gametes (2n gametes) (Ramsey and Schemske 1998; Levin 2002). One or both male and female gametes can be unreduced (*i.e.*, unilateral or bilateral polyploidization; Ramsey and Schemske 1998). There is a tendency for a higher frequency of unreduced pollen than unreduced eggs; however, this could be because it is much simpler to study and detect unreduced pollen than unreduced ovules (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998).

Although the estimated percentage of unreduced gametes in angiosperms is 0.56%, this frequency can vary significantly among species or even within the same species (Ramsey and Schemske 1998). For example, in *Trifolium pratense*, the frequency is 3.0%, but it can vary between 1.0 and 84.0% (Parrott and Smith 1986), while in *Dactylis glomerata*, the overall

frequency of unfreduced gametes is 60.0% (Maceira *et al.* 1993). Yet, in other studies for the latter species the frequency varied between 0.1% and 26.0% for unreduced eggs (De Haan *et al.* 1992), and between 0.1% and 14.0% for unreduced pollen (Maceira *et al.* 1993). However, in most cases, hybridization was not considered, and it is known that hybrids produce higher amounts of unreduced gametes (on average, hybrids produced 27.5% of unreduced gametes; Levin 2002). Finally, it is important to state that environmental conditions, such as temperature-cycling, temperature stress, and low nutrient stress, might influence the production of unreduced gametes (Levin 2002; Mason *et al.* 2011; Zhou *et al.* 2015a; Loginova and Silkova 2017).

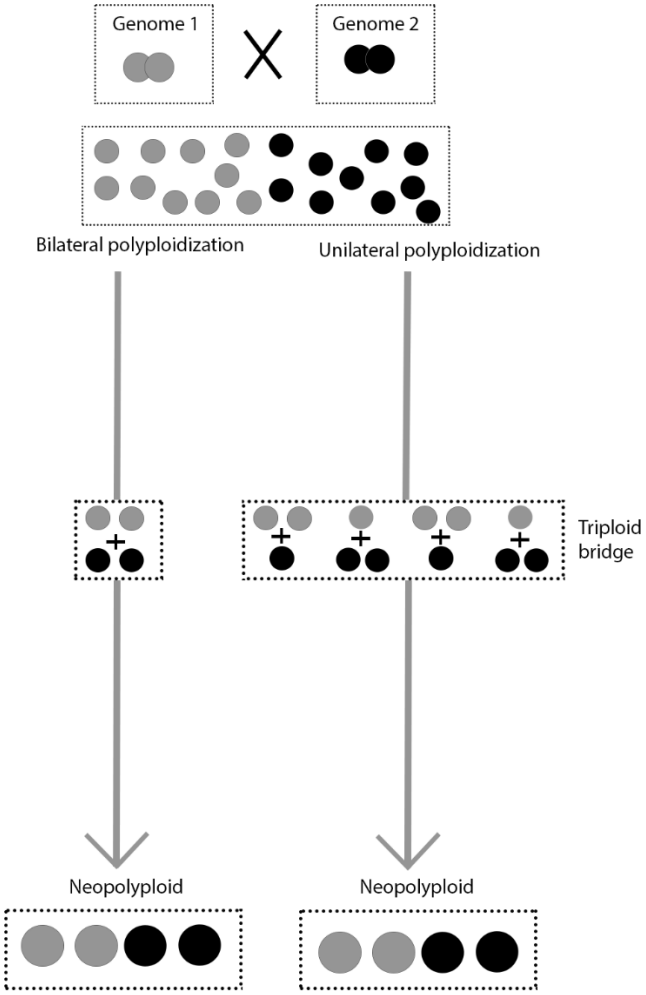

Figure 1.3. Pathways for neopolyploid formation in plants. Grey and black circles represent a set of chromosomes from progenitors 1 and 2, respectively. Reduced (n) and unreduced gametes (2n) are represented by one or two closed circles, respectively.

The production of a neopolyploid could occur in two different ways, by bilateral or unilateral polyploidization. Bilateral polyploidization occurs through the fusion of two unreduced gametes (2n), originating a polyploid individual (Figure 1.3; Bretagnolle and

Thompson 1995; Ramsey and Schemske 1998). Additionally, the ongoing formation of 2n gametes by diploids may allow the formation of tetraploid offspring through crosses between diploids with reduced gametes and tetraploids (Figure 1.3; Bretagnolle and Lumaret 1995). In unilateral polyploidization, the fusion occurs between an unreduced gamete (2n) and a reduced one (n) with the formation of a triploid bridge (3n). The triploids can give origin to tetraploids when they cross with unreduced gametes of diploids or with another triploid (Figure 1.3) (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). Additionally, the production of unreduced gametes usually is heritable. This may lead to the formation of gametes with different ploidy levels, leading to the production of neopolyploid offspring (Husband 2000; Levin 2002; Ramsey and Schemske 2002; Husband *et al.* 2013). However, after their emergence, neopolyploids have to overcome a series of pre- and post-zygotic barriers to establish themselves and overcome minority cytotype exclusion (Segraves and Thompson 1999; Husband and Schemske 2000; Husband *et al.* 2002; Baack and Stanton 2005; Glennon *et al.* 2012; Hao *et al.* 2013; Laport *et al.* 2016; Castro *et al.* 2018; Muñoz-Pajares *et al.* 2018).

Consequences of WGDs and polyploid establishment

When a neopolyploid arises in a population it will be in very low number, and thus, the majority of pollen that it will receive is from the progenitor, leading to the production of unviable odd ploidy offspring. This is expected to significantly impact the reproductive fitness of the neopolyploid and lead to its exclusion from the population (minority cytotype exclusion theory; Levin 1975). However, in some cases, along time, the neopolyploid can replace their parentals or disperse to new areas. For this to happen, the new polyploid must have advantages over its progenitor. Genome duplications are known to lead to a profound genetic change that can manifest in biochemical, cytological, reproductive, morphological and physiological traits of the plant (Husband *et al.* 2002; Marques *et al.* 2007; Laport *et al.* 2016). Furthermore, ecological factors might also play a role in the establishment of polyploids (Levin 2002; Glennon *et al.* 2012; Thompson *et al.* 2014; Castro *et al.* 2018; Muñoz-Pajares *et al.* 2018).

Among the direct effects of polyploidy is an increase in cell size and potentially in the overall size of the organs (Segraves and Thompson 1999; Levin 2002). Such “giga effect” may have a significant impact on the structure of the sexual organs such as the flower parts, potentially affecting the interactions with pollinators (Segraves and Thompson 1999; Marques *et al.* 2007) and, consequently, the reproductive success of such individuals (Husband and Schemske 2000). Differences in phenology, flower morphology, and physiology can also impact the establishment of the neopolyploid since they can promote their assortative mating (*e.g.*,

Segraves and Thompson 1999; Husband and Sabara 2004; Jersáková *et al.* 2010; Castro *et al.* 2011). Many studies reported differences in flowering time between cytotypes (Van Dijk and Bijlsma 1994; Petit *et al.* 1997; Segraves and Thompson 1999; Husband and Sabara 2004; Martin and Husband 2012; Laport *et al.* 2016), and morphological and physiological differences in floral traits such as changes in the size and color of flowers and inflorescences (Husband and Schemske 1998; Kennedy *et al.* 2006; Borges *et al.* 2012; Hao *et al.* 2013; Gross and Schiestl 2015) and in nectar composition and scent (Jersáková *et al.* 2010). High ovule number per flower (Husband 2000), or a low number of seeds of larger dimensions are also common in polyploids and may confer an advantage regarding the number of propagules produced or germination and seedling survival (Bretagnolle and Thompson 1995). Changes in breeding systems are also common after WGDs. For example, self-fertilization can be favored to increase the reproduction of the new polyploid (Levin 1975; Rausch and Morgan 2005; Barringer 2007). Although this can also lead to negative consequences due to inbreeding depression, several studies reported that these adverse effects could be minor in polyploids (Soltis and Soltis 1999; Rausch and Morgan 2005; Husband *et al.* 2016; Siopa *et al.* 2020).

Finally, polyploidy has been long regarded as a mechanism that confers increased ecological tolerances and competitive ability, niche partitioning and/or broader geographic ranges (Fowler and Levin 1984; Otto and Whitton 2000; Levin 2002). For example, the ability to tolerate low nutrient levels, drought, and cold temperatures have been proposed in several studies (Levin 2002; Maherali *et al.* 2009; Hao *et al.* 2013; Thompson *et al.* 2014). Many studies have characterized the abiotic factors of polyploid populations and evaluated cytotype environmental preferences, predicting the possible existence of niche shift (Thompson *et al.* 2014; Visger *et al.* 2016; Muñoz-Pajares *et al.* 2018; López-Jurado *et al.* 2019; Castro *et al.* 2020) or niche conservatism (McIntyre 2012; Laport *et al.* 2013; Glennon *et al.* 2014; Castro *et al.* 2019) among polyploids and their progenitors. Therefore, the successful establishment of polyploid lineages has long been associated with niche divergence or niche partitioning (Levin 1975; Glennon *et al.* 2014; Thompson *et al.* 2014; Muñoz-Pajares *et al.* 2018). However, the spatial distribution of cytotypes results from several interacting, often complex, processes occurring in natural populations, including not only ecological preferences but also formation and migration patterns, reproductive interactions, and competitive and dispersal abilities (Levin 2002; Godsoe *et al.* 2013; Laport *et al.* 2013; Kolář *et al.* 2017; Castro *et al.* 2018; Morgan *et al.* 2020).

Evolution of heterostyly: occurrence and prevalence within polyploids

The impact of WGDs in the breakdown of self-incompatibility mechanisms is well documented (Miller and Venable 2000; Baack and Stanton 2005; Rausch and Morgan 2005). It

has been suggested that polyploids self-fertilize more than their diploid relatives and that polyploidization may attenuate the levels of inbreeding depression, favoring selfing and its reproductive assurance advantage (Ramsey and Schemske 1998; Mable 2004; Barringer 2007). This can allow the polyploid to spread in the initial stages of polyploid establishment and deal with the minority cytotype exclusion (Levin 1975; Ramsey and Schemske 1998; Miller and Venable 2000; Baack 2005). Later, after polyploid establishment and spread, the advantages of selfing can dissipate (Mable 2004; Husband *et al.* 2008).

As already referred, WGDs have been related to changes in incompatibility relationships and floral traits. These changes will be particularly relevant in species with complex breeding systems, such as plants with heterostyly and other related stylar polymorphisms (*e.g.*, distyly, tristyly, stigma-height dimorphism, Figure 1.2). On one hand, the variability in floral morphology may contribute to reproductive isolation between polyploids and their diploid progenitor(s) (*e.g.*, Petit *et al.* 1997; Husband and Schemske 1998; Segraves and Thompson 1999; Laport *et al.* 2016; Casazza *et al.* 2017; Muñoz-Pajares *et al.* 2018). On the other hand, changes in the incompatibility system such has the breakdown of the self- and/or morph-incompatibility in stylar polymorphic species may ameliorate the lack of compatible mates at the initial stages of polyploid emergence (*e.g.*, Kelso 1992; Tamari *et al.* 2001; Arroyo *et al.* 2002; Ferrero *et al.* 2012; Naiki 2012; Castro *et al.* 2012b; Simón-Porcar *et al.* 2015; Zhou *et al.* 2015b).

Heterostyly is reported from approximately 28 Angiosperm families, and studies of heterostyly have concentrated mainly on a few well-characterized taxa (*e.g.*, *Primula*, *Linum*, *Lythrum*) originally studied by Darwin (1877) (Barrett 1992, 2019; Barrett and Shore 2008). *Primula* is often represented in the literature as the model system for heterostyly (Mast *et al.* 2006) and in fact it was one of the first studied (Hildebrand 1863; Van Dijk 1943; Gilmartin 2015). This floral polymorphism had been considered genetically controlled by a single genetic region, the S locus, that controls the existence of two morphs, *i.e.*, distyly (long- and short-styled morphs), or by two diallelic loci that control the existence of three morphs, *i.e.*, tristyly (long-, mid- and short-styled morphs), being epistatic to the M-locus (Lewis and Jones 1992). However, recent molecular studies in several systems had found that the S locus supergene involves several genes that will determine the floral phenotypes *(e.g.*, *Linum*, Ushijima *et al.* 2012; *Primula*, Li *et al.* 2016; Kappel *et al.* 2017; Huu *et al.* 2020). In taxonomy, style polymorphisms are normally classified as heterostylous (distyly, tristyly) or homostylous which refers to to any style monomorphic condition (*i.e.*, single anther level at the same height as the stigma – non-herkougamus, or one of the two anther levels in a flower at the same height as the stigma – style monomorphic). Nevertheless, what phenotypically defines a heterostylous plant is the presence of a reciprocal arrangement of sex-organ heights in the floral morphs, also known as

reciprocal herkogamy (Webb and Lloyd 1986). Thus, the outcrossing and self-interference reduction is achieved by reciprocal positioning of stamens and stigmas within a single flower (Darwin 1877; Barrett 2002a; b). The style-stamen polymorphism is usually linked to a sporophytically controlled, diallelic self-incompatibility system that prevents self- and intra-morph fertilizations (Lewis and Jones 1992), sometimes accompanied by other morphological polymorphisms (*e.g.*, stigmas and pollen morphologies; Barrett 1992; Dulberger 1992).

Heterostyly has been described as a mechanism to avoid self-pollination and promote efficient cross-pollination (i.e, low gamete wastage; Darwin 1877; Barrett 1992; Barrett and Shore 2008). Despite several models have been proposed through time, two of them are quantitative models that predict particular intermediate stages and conditions driving the transition between them, namely the Charlesworth and Charlesworth (1974) and the Lloyd and Webb (1992a; b) models. In the first, the self-incompatibility mechanism is proposed to occur first and the polymorphism would evolve later to favor pollen transfer, thus the major and initial selective force would be selfing avoidance through self-incompatibility (Figure 1.4A) (Charlesworth and Charlesworth 1974). However, Darwin (1877) suggested that the difference in the height of pistils and anthers was an adaptation for the efficient transport of pollen by pollinators. Thus, more recently, Lloyd and Webb (1992a; b) proposed a quatitative selection model where the evolution of heterostyly aimed at promoting efficient cross-pollination rather than preventing self-pollination (Figure 1.4B). In this model, the ancestral condition would be an approach herkogamy, followed by recirprocal herkogamy, and the incompatibility and the ancillary characters would evolve independently of the floral polymorphism, in order to avoid pollen and ovule discounting (Lloyd and Webb 1992a; b).

At equilibrium, heterostylous populations should have an 1:1 ratio of style morphs for distylous species (Lewis and Jones 1992). Changes from this equilibrium are often associated with the breakdown of the distylous incompatibility system and with random stochastic events, such as fluctuations in population size or founder events (*e.g.*, Brys *et al.* 2008; Costa *et al.* 2016; Eckert and Barrett 1992; Zhou *et al.* 2012, 2017; Castro *et al.* 2013; Ferrero *et al.* 2020). Thus, in a heterostylous species, disassortative mating must exceed assortative mating to maintain the polymorphism by negative frequency-dependent selection (Lloyd and Webb 1992a; Barrett *et al.* 1996; Baker *et al.* 2000). Deviations from reciprocity (*i.e.*, the similar position of reciprocal organs) can lower the probability of disassortative mating (*i.e.*, outcrossing) and seed production or facilitate a breakdown of the floral polymorphism (Keller *et al.* 2014; Zhou *et al.* 2015b; Wu *et al.* 2018; Brys and Jacquemyn 2020). Therefore, usually, the breakdown of heterostyly is associated with the breakdown of the incompatibility system, or at least the breakdown of self-incompatibility. This can lead to close proximity of the sexual organs, and populations can

become homostylous (Barrett and Shore 2008). In some cases, only the breakdown of morph-incompatibility occurs, and the polymorphism is maintained since the inter-morph crossing is higher than intra-morph crossing (Simón-Porcar *et al.* 2015; Zhou *et al.* 2015b).

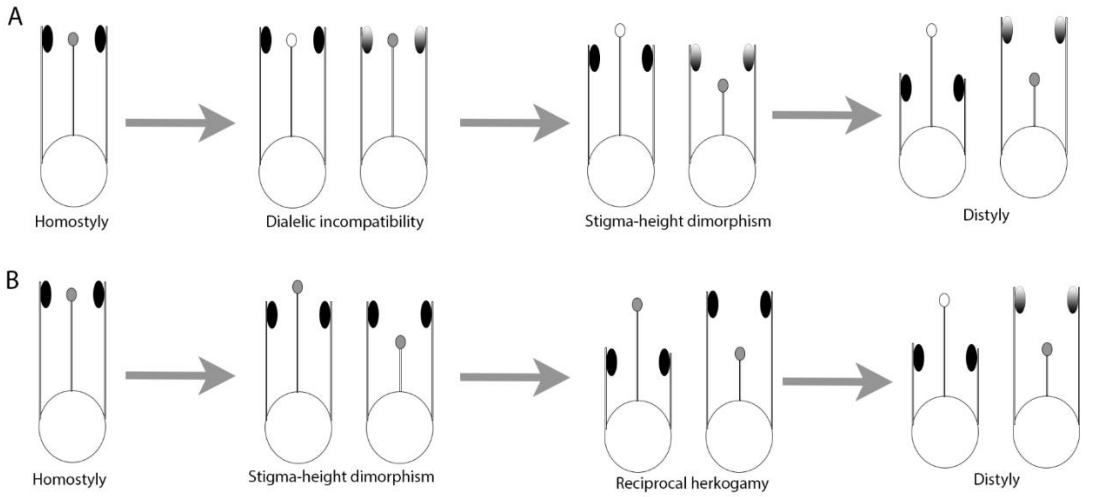

Figure 1.4. Models for the evolution of distyly**:** (A) Model proposed by Charlesworth and Charlesworth (1974) of selfing avoidance; (B) Model of pollen transfer proposed by Lloyd and Webb (1992a; b). Flowers with black anthers and grey stigmas are self-compatible; flowers with shaded anther and white stigmas are self-incompatible (adapted from Thompson 2020 and Costa 2017).

Studies in heterostylous polyploid species had demonstrated a high self-compatibility. Also, WGDs have been associated with the loss of this polymorphism (Kelso 1992; Tamari *et al.* 2001; Naiki 2012). A negative correlation was found between the occurrence of distyly and polyploidy in *Damnacanthus* (Rubiaceae) (Naiki and Nagamasu 2004) and *Ophiorrhiza japonica* (Rubiaceae) (Nakamura *et al.* 2007). In higher polyploids of *Primula* (6*x*, 8*x*, 14*x*), there was a tendency towards the evolution of homostylous flowers. Tetraploids could bear homostylous or distylous flowers, and no homostylous flowers were found in diploids (Kelso 1992; Guggisberg *et al.* 2006). Homostyly can be important, from an ecological point of view, as neopolyploids are low in frequency, and their successful establishment depends on overcoming the minority cytotype exclusion (Levin 1975). Therefore, homostyly is strongly associated with self-compatibility (Charlesworth and Charlesworth 1974; Barrett and Shore 1987) and could be advantageous for neopolyploids. Because WGDs attenuate the adverse effects of inbreeding depression, homostylous and self-compatible flowers may thus be favored (Mable 2004; Husband *et al.* 2008). However, no clear correlation between the breakdown of distyly and polyploidization was found by Naiki (2012). Furthermore, it was suggested that distylous polyploids are restricted to those of autotetraploid origin, and allopolyploids show self-compatible homostyly, only with some exceptions (Shore *et al.* 2006). Despite this, a breakdown

in morph-incompatibility can allow the neopolyploid to strive and persist and, at the same time, avoid inbreeding depression and maintain the reciprocal herkogamy (Arroyo *et al.* 2002; Ferrero *et al.* 2012; Costa *et al.* 2014; Simón-Porcar *et al.* 2015; Zhou *et al.* 2015b).

Linum as a study system since Charles Darwin

Linaceae DC. ex Perleb is a large family that comprises 22 genera and approximately 300 species (Ockendon and Walters 1968; Hickey 1988; Heywood 1993; McDill *et al.* 2009; McDill and Simpson 2011). *Linum* L. is one of the most diverse genera of this family. It is the largest genus within the family Linaceae (Rogers 1982; McDill and Simpson 2011; Dressler *et al.* 2014), comprising more than 200 species, being particularly diverse in the Mediterranean basin (Diederichsen and Richards 2003; McDill *et al.* 2009; Ruiz-Martín *et al.* 2018; Maguilla *et al.* 2021a). In addition, Section *Linopsis* (Rchb.) Engelm. is the largest and probably the most widespread, with about 85 species (Rogers 1982; Heywood 1993).

Distyly is widespread and very common in *Linum* (about 40 % of the species are heterostylous; Rogers 1979). Each of the five generally accepted sections of the genus, with the exception of the monospecific, homostylous Section *Cathartolinum*, have both distylous and style monomorphism species, indicating that floral polymorphisms have evolved several times in the genus (Rogers 1979; McDill *et al.* 2009; Ruiz-Martín *et al.* 2018; Maguilla *et al.* 2021a). Heterostyly in *Linum* was first reported in the works of Darwin (1863, 1877) and Hildebrand (1864). Species of *Linum* were later used to study the inheritance of heterostyly. Several studies have shown that style polymorphism and heteromorphic incompatibility appear to be linked (Lewis 1943; Dulberger 1992; Lewis and Jones 1992; Ushijima *et al.* 2012). Long- and short-styled plants present some differences in morphological and micromorphological characters, including the color, number, and size of pollen grains, stamens shape, shape and color of stigma, and surface of stigmatic papillae (Dulberger 1992; Richards and Barrett 1992; Talebi *et al.* 2012). In some heterostylous species of *Linum* such as, *L. perenne*, *L. grandiflorum* and *L. alpinum*, long- and short- styled plants differ in the sculpturing of the exine (Dulberger 1981). Furthermore, the presence of heterostyly has been considered a crucial taxonomic trait, having been used as a key character to identify some species (Ockendon and Walters 1968; Ornduff 1969; Ockendon 1971; Martínez-Labarga and Garmendia 2015; Ruiz-Martín *et al.* 2015). This has been valuable to characterize species and conduct evolutionary reconstructions of the trait (McDill *et al.* 2009). In Section *Linopsis* this feature has been important as a key identification trait, particularly in the Mediterranean area (Rogers 1982). In Appendix 1.1 is summarized the floral polymorphism for the most representative species of Section *Linopsis* in the Mediterranean basin.

Furthermore, the ancestor species of the Section is believed to be a heterostylous species of the Mediterranean area (Rogers 1982).

Over the years, multiple ploidy has been reported in some species of *Linum* (*e.g.*, Nilsson and Lassen 1971; Rogers *et al.* 1972; Chennaveeraiah and Joshi 1983). However, most studies about the genetics and cytogenetics of *Linum* have been done in economically relevant groups (*e.g.*, *L. usitatissimum* L. from Section *Dasylinum* (Plach.) Juz, and the group of *L. perenne* L.; Ockendon 1968; Chennaveeraiah and Joshi 1983; Bolsheva *et al.* 2015), whereas other sections, such as sect. *Linopsis* have received less attention, and their diversity remains largely unknown. Overall, in the genus, the chromosome numbers range from 12 to 72, with a chromosome base number varying between n = 9 to n = 10 (Darlington *et al.* 1955; Rogers *et al.* 1972; Xavier *et al.* 1980; Rogers 1982; Muravenko *et al.* 2010). The chromosomes are small (1–4 µm) and morphologically similar (Muravenko *et al.* 2003). Section *Linopsis* is highly diverse, and its species are characterized by having very small chromosomes, which makes the karyological characterization challenging. In Appendix 1.1 is summarized the chromosome reports available for the most representative species of Section *Linopsis* in Mediterranean basin.

Linum genus presents a wide geographic distribution, but most of the diversity appears in the Mediterranean (Darwin 1877; Armbruster *et al.* 2006; Ruiz-Martín *et al.* 2018). Despite this, the diversity of Mediterranean species of *Linum* species remains poorly explored. Within the *Linopsis* Section, a group of white-flowered plants generated a lot of controversy over the years due to its morphological and karyological diversity. Over time, this group has been called *L. suffruticosum* complex or *L. tenuifolium* complex and has always had a complex taxonomy. The complex has been described as having monomorphic and heterostylous races, with diploids and tetraploids in the heterostylous race. In some cases, two species were recognized inside the complex: *L. tenuifolium* (monomorphic) and *L. suffruticosum* (heterostylous), with some infraspecific taxa being described (Rogers *et al.* 1972; Rogers 1979; Nicholls 1985a; b, 1986). Currently most of the treatments recognize *L. tenuifolium* (Ockendon and Walters 1968; López González 1979; Martínez-Labarga and Garmendia 2015), and most of the confusion and diversity being included within *L. suffruticosum*.

Objectives and structure of the Thesis

The Mediterranean basin is classified as a hotspot of biodiversity for conservation purposes (Myers *et al.* 2000), with a high incidence of heterostylous and polyploid species (Costa 2017; Marques *et al.* 2018). The main objective of this Thesis was to explore the relationship between polyploidy and reproductive and ecological traits driving plant divergence.

For that, I used *L. suffruticosum s.l.* group, which is comprised of perennial herbs distributed through the Mediterranean Basin and presents a complex and variable reproductive strategy. Sexual reproduction is obligate (vegetative propagation is almost negligible), and populations are heterostylous, comprising both long- and short-styled morphs (Figure 1.5). However, the reciprocity of sex organs in *L. suffruticosum s.l.* has been described to be in three dimensions (contrarily to the most common distribution of anthers and stigmas in one dimension, *i.e.*, height). This is the result of differences in the angle of divergence of the styles and stamens from the central axis of the flower and of the degree of rotation of the styles and filaments. The stigmas of short-styled morph contact the ventral side of the pollinator and the stigmas of long-styled morph contact the dorsal side. By opposition, the pollen from the short-styled morph is placed in the dorsal side of the pollinator, while the pollen from long-styled morph is placed in the ventral side (Armbruster *et al.* 2006). In the Iberian Peninsula, the group has been described as a polyploid complex that includes diploids (2n = 2*x* = 18 chromosomes), tetraploids (2n = 4*x* = 32) and octoploids (2n = 8*x* = 72) (Appendix 1.1). The lack of strong/unambiguous diagnostic characters and the high diversity have been the main reasons for the different taxonomic treatments of *L. suffruticosum s.l* over the years. A recent treatment recognized more than 20 taxa for the Iberian Peninsula alone (Martínez-Labarga and Garmendia 2015), while in most of the previous treatments, only three taxa have been consensually accepted as distinct species (*L. salsoloides* Lam., *L. appressum* Caball. and *L. suffruticosum,* Appendix 1.2), with some varieties being described in *L. suffruticosum* (Appendix 1.2, Jahandiez and Maire 1932; Ockendon and Walters 1968; López González 1979).

Figure 1.5. *Linum suffruticosum s.l.* in the natural habitat and long-styled morph and short-styled morph.

Linum suffruticosum complex is considered to be very recent, having originated probably at the beginning of Pleistocene, while the genus originated and began to diversify in the early Oligocene to late Miocene in Western Palearctic (Ruiz-Martín *et al.* 2018; Maguilla *et al.* 2021a). Despite some recent phylogenetic studies, internal phylogenetic relationships are still unclear for this complex (McDill *et al.* 2009; McDill and Simpson 2011; Schneider *et al.* 2016; Ruiz-Martín *et al.* 2018). This group's complexity and geographical setting offer a unique opportunity to explore the relationship between polyploidy and reproductive strategies, namely, to understand how polyploidy relates with floral polymorphisms (heterostyly), breeding system and ability to hybridize.

To achieve the main objective, this PhD Thesis was organized into five chapters in addition to this introductory chapter (Capter I): Chapter II is focused on cytogenetic diversity and the large-scale geographic distribution of *L. suffruticosum s.l.* cytotypes, to understand how WGDs shaped the distribution of the complex in nature; Chapter III is focused on the environmental factors that might explain the observed cytotype distribution patterns; Chapter IV is focused on the consequences of WGDs in the polymorphism and reproductive strategies of five different cytotypes; and Chapter V is focused in disentangling the phylogeographic relationships in this group. Finally, in Chapter VI, the general conclusions are presented, as well as the future perspectives after this Thesis.

Chapter II: the main objective was to explore the cytogenetic diversity of *L. suffruticosum s.l.* For that, I used flow cytometric analyses complemented with chromosome counts to investigate cytotype diversity and distribution patterns in 151 populations covering most of the distribution range in the Iberian Peninsula, south-east France, north-west Italy and north Morocco. I found a high cytogenetic diversity with new ploidy levels being reported for the first time. Five major cytotypes were detected, namely diploids, tetraploids, hexaploids, octoploids and decaploids, distributed parapatrically and having a geographical structure and several contact zones. The observed geographical overlap, high cytogenetic diversity and different genome sizes suggest that polyploidization is one of the key mechanisms, alone or involving hybridization, governing the diversification of *L. suffruticosum s.l.*

Chapter III: the main objective was to evaluate the role of polyploidy driving environmental niche divergence and explore relationships between the observed geographic distribution and the ecological requirements of each cytotype. For that, I used niche modelling tools to assess the environmental and soil requirements of each cytotype. Differences in the ecological requirements of *L. suffruticosum s.l.* cytotypes were observed, with diploids

presenting the widest environmental niche, and polyploids occupy part of the diploid niche. Some polyploids have equivalent potential ecological niches but cytotypes do not co-occur in nature. Although the different ecological requirements played a role in the distribution of cytotypes, the mosaic distribution could not be entirely explained by the environmental variables. Based on the observed geographic distribution pattern and environmental conditions of each cytotype, it was possible to build hypotheses that could explain the establishment and maintenance of cytotypes of *L. suffruticosum s.l.* Furthermore, this study provides important data on the niche requirements of each cytotype for further competition and reciprocal transplant experiments.

Chapter IV: the main objective was to assess the impact of WGDs on the reproductive traits of *Linum suffruticosum s.l.* and assess the reproductive relationships among some of the cytotypes. For that, I assessed morph frequencies in 92 populations, characterized the floral polymorphism in 86 populations and performed experimental crosses within the five cytotypes (selfing and intra- and inter-morph crosses) and crosses between diploids and tetraploids from a parapatric contact zone. I found no evidence for the breakdown of the recriprocal herkogamy nor the incompatibility system (both morph- and self- incompatibility) after WGDs. Additionally, there was pollen tube development in intercytotype crosses possibly enabling inter-cytotype hybridization. This results provide important insigths for investigation on reproductive traits and on evolutionary paths to understand the polyploid and heterostylous lineages of this complex.

Chapter V: the main objective was to reconstruct the historical setting of the polyploid complex throughout its range in Western Mediterranean Basin and its biogeographic and climatic transitions (*i.e.*, from the arid Mediterranean to temperate Europe), taking into consideration shifts in ploidy level. For that, I extracted DNA from 61 populations of all cytotypes and used two plastid markers and one nuclear marker to estimate a haplotype-ribotype network. The results showed a higher variability of haplotypes and ribotypes in diploid populations of the homogeneously diploid zone than in remaining diploid and polyploid populations from Spain and Morocco. Although the results failed to ascertain, unambiguously, the ancestral condition of the polyploids, they showed that polyploids of *L. suffruticosum s.l.* could have multiple origins. From the results of the study it was possible to integrate all these results in a geographical and ecological context of the western Mediterranean Basin and suggest the conditions under which polyploidy most likely evolved.

Appendices

Appendix 1.1. Breeding systems and chromosome reports for the most representative species of Section *Linopsis* in the Mediterranean basin.

Taxon	Breeding system	References	2n	References
Linum tenuifolium L.	Homostylous (style-monomophic with horizontal herkogamy)	Ockendon DJ, Walters SM. 1968. Linum L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, eds. Flora Europaea, Vol. 2. Cambridge: Cambridge University Press, 206–211	16	BAKSAY L. 1956. Cytotaxonomical studies on the flora of Hungary. *Annales Historico-Naturales Musei Nationalis Hungarici* 7: 321-334.
			18	Rogers CM. 1980. In IOPB chromosome number reports LXVII. *Taxon* 29:347.
			18	Magulaev A. 1984. Cytotaxonomic study of some flowering plants from the Northern Caucasus. *Botanicheskii zhurnal.*
			18	Nilsson O and Lassen P.1971. Chromosome numbers of vascular plants from Austria, Mallorca and Yugoslavia. *Botaniska notiser*.
			18	Van Loon, JC and Snelders HCM. 1979. In IOPB chromosome number reports LXV. *Taxon* 6: 627-637.
			18	Rogers CM, Mildner R; Harris BD. 1972: Some additional chromosome numbers in the Linaceae. *Brittonia* 24: 313-316.
			18	Petrova AV. 1972. IOPB chromosome number report XXXV. *Taxon* 21: 164
			18	Májovský J. 1970: Index of chromosome numbers of Slovakian flora (Part 1). *Acta Facultatis Rerum Naturalium Universitatis Comenianae*, Bot 16: 1-26.
Linum suffruticosum L:	3D Distyly	Armbruster WS, Pérez-Barrales R, Arroyo J, Edwards ME, Vargas P. 2006. Three-dimensional reciprocity of floral morphs in wild flax (Linum suffruticosum): a new twist on heterostyly. New Phytologist 171:581-590.	18	Nicholls MS. 1986. Variation and evolution in *Linum tenuifolium* (Linaceae). *Plant Systematics and Evolution* 153: 243–258.
			36	Rogers CM. 1981. A note on Linum in Spain. *Anales del Jardin Botanico de Madrid* 38:302.
			36	Nicholls MS. 1986. Variation and evolution in L*inum tenuifolium* (Linaceae). *Plant Systematics and Evolution* 153: 243–258.
			36	Rogers CM. 1980. In IOPB chromosome number reports LXVII. *Taxon* 29:347.
			72	Lorenzo-Andreu A, García Sanz P. 1950. Cromosomas somáticos de plantas espontâneas en la estepa de Aragón. II. *Anales de la Estación Experimental de Aula Dei* 2:12-63.
			72	Elena Rosselló JA, González Zapatero MA, Navarro Andrés F. 1985. Sobre los niveles de ploidía y otras particularidades cromosómicas de algunos vegetales castellano-leoneses de preferencias calcícolas. *Studia Botanica Universidad de Salamanca* 4: 109-115.

⇩Cont.

Linum maritimum L.	Distyly	Ockendon DJ, Walters SM. 1968. Linum L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, eds. Flora Europaea, Vol. 2. Cambridge: Cambridge University Press, 206–211.	18	Nagao,S. 1941). Cytogenetics in the genus *Linum. Japanese Journal of Genetics* 17: 109-116.
			20	Joshi,KK and Chennaveeraiah MS. 1980. Cytological studies in some species of *Linum* L. *Cytology and Genetics* 15: 128–133.
			20	Rogers CM. 1980. In IOPB chromosome number reports LXVII. *Taxon* 29:347.
			20	Chennaveeraiah MS and Joshi KK. 1983. Karyotypes in cultivated and wild species of Linum. *Cytologia* 48: 833–841.
			20	Rogers CM, Mildner R; Harris BD. 1972: Some additional chromosome numbers in the Linaceae. *Brittonia* 24: 313-316.
			20	Vilmorin R and Simonet M. 1927. Nombre des chromosomes dans les genres Lobelia, *Linum,* et chez quelques autres espèces végétales. *Comptes rendus des séances et mémoires de la Société de biologi*e 96: 166-168
Linum corymbulosum Rchb.	Homostyly	Ockendon DJ, Walters SM. 1968. Linum L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, eds. Flora Europaea, Vol. 2. Cambridge: Cambridge University Press, 206–211.	18	Joshi,KK and Chennaveeraiah MS. 1980. Cytological studies in some species of *Linum* L. *Cytology and Genetics* 15: 128–133.
			18	Petrova AV. 1972. IOPB chromosome number report XXXV. *Taxon* 21: 161-166.
Linum trigynum L.	Homostyly	Ockendon DJ, Walters SM. 1968. Linum L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, eds. Flora Europaea, Vol. 2. Cambridge: Cambridge University Press, 206–211.	20	González Zapatero, MA, Elena-Roselló JÁ, Andrés FN. 1989. Números cromosomáticos de plantas occidentales, 527--532. *Anales del Jardín Botánico de Madrid* 45: 505–508.
			20	Petrova AV. 1972. IOPB chromosome number report XXXV. *Taxon* 21: 161-166.
			20	Rogers CM, Mildner R; Harris BD. 1972: Some additional chromosome numbers in the Linaceae. *Brittonia* 24: 313-316.
Linum setaceum Brot.	Homostyly	Ockendon DJ, Walters SM. 1968. Linum L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, eds. Flora Europaea, Vol. 2. Cambridge: Cambridge University Press, 206–211.	18	Rogers, C. M. 1980. In IOPB chromosome number reports LXVII. *Taxon* 29:347.

⇩Cont.

Linum corymbiferum Desf.	Distyly	Quezel, P and Santa, S. 1963. Nouvelle flore de l'Algerie: et des regions desertiques meridionales. CNRS, Paris, pp 1170.	18	Rogers CM, Mildner R; Harris BD. 1972: Some additional chromosome numbers in the Linaceae. *Brittonia* 24: 313-316.
			30	Kikichi M. 1926. Studies on the difference of chromosome numbers in Linum species. *Journal of the Sapporo Agricultural College* 81: 26-37.
Linum strictum L.	Homostyly	Ockendon DJ, Walters SM. 1968. Linum L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, eds. Flora Europaea, Vol. 2. Cambridge: Cambridge University Press, 206–211.	18	Nilsson O and Lassen P.1971. Chromosome numbers of vascular plants from Austria, Mallorca and Yugoslavia. Botaniska notiser 124: 270-276.
			18	González Zapatero, MA, Elena-Roselló JÁ, Andrés FN. 1989. Números cromosomáticos de plantas occidentales, 527--532. *Anales del Jardín Botánico de Madrid* 45: 505–508.
			30	SEETHARAM A. 1972. Interspecific hybridization in *Linum. Euphytica* 21: 489-495.
			32	Chennaveeraiah MS and Joshi KK. 1983. Karyotypes in cultivated and wild species of Linum. *Cytologia* 48: 833–841.
Linum tenue Desf.	Distyly	Ockendon DJ, Walters SM. 1968. Linum L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, eds. Flora Europaea, Vol. 2. Cambridge: Cambridge University Press, 206–211.	20	Pastor J, Diosdado JC, Bárbara CS, Vique J, Pérez E. 1990. Números cromosómicos para la flora Española. 556--591. *Lagascalia* 15: 269–282.
			20	Rogers CM, Mildner R; Harris BD. 1972: Some additional chromosome numbers in the Linaceae. *Brittonia* 24: 313-316.
			40	Rogers CM, Mildner R; Harris BD. 1972: Some additional chromosome numbers in the Linaceae. *Brittonia* 24: 313-316.
			30	SEETHARAM A. 1972. Interspecific hybridization in *Linum. Euphytica* 21: 489-495.

Appendix 1.2. Synonyms reported for *L. suffruticosum s.l.* (in bold: taxa from López González 1979).

***L. salsoloides* Lam.**, synonims considered: *L. suffruticosum* subsp. *salsoloides* (Lam.) Rouy; *L. tenuifolium* subsp. *salsoloides* (Lam.) Fiori; *L. ortegae* Planchon; *L. salsoloides* subsp. *ortegae* (Planchon) Nyman; *L. tenuifolium* subsp. *ortegae* (Planchon) O. Bolós & Vigo; *L. suffruticosum* subsp. *ortegae* (Planchon) Rivas Goday & Borja ex Rivas-Martínez; *L. tenuifolium* var. *ericoides* Willk; *L. ortegae* var. *tenuissimum* Caballero; *L. suffruticosum* Ortega MS, non L.

***L. appressum* Caballero**, synonims considered: *L. tenuifolium* subsp. *appressum* (Caballero) Rivas-Martínez; *L. salsoloides* subsp. *appressum* (Caballero) Rivas Goday & Rivas-Martínez; *L. suffruticosum* subsp. *appressum* (Caballero) Rivas-Martínez; *L. tenuifolium* subsp. *salsoloides* var. *appressum* (Caballero) O. Bolós & Vigo; *L. salsoloides* auct. non Lam.; *L. tenuifolium* auct. non L.

***L. suffruticosum* L.**, synonims considered: *L. ramosissimum* Willk.¸ *L. tenuifolium* subsp. *suffruticosum* (L.) Litard

L. suffruticosum* var. *suffruticosum, synonims considered: *L. tenuifolium* subsp. *suffruticosum* var. *suffruticosum* (L.) O. Bolós & Vigo; *L. diferens* Pau; *L. suffruticosum* subsp. *diferens* (Pau) Rivas Goday & Rivas-Martínez; *L. tenuifolium* subsp. *suffruticosum* var. *diferens* (Pau) O. Bolós & Vigo;

***L. suffruticosum* var. *milletii* (Sennen & Gonzalo) G. López stat. & comb. nov**. , synonims considered: *L. suffruticosum* subsp. *milletii* (Sennen & Gonzalo) Romo; *L. tenuifolium* subsp. *milletii* (Sennen) Bólos, Vigo, Masalles & Ninot; *L. milletii* Sennen & Gonzalo; *L. ortegae* Sennen, non Planchon;

***L. suffruticosum* var. *tejedense* Pau;**

***L. suffruticosum* var. *carratracensis* (Rivas Goday & Rivas-Martinez) G. Lopéz stat. nov.**, synonims considered: *L. suffruticosum* subsp. *carratracensis* Rivas Goday & Rivas-Martinez;

***L. suffruticosum* var. *angustifolium* Lange**, synonims considered: *L. jimenezii* Pau; *L. squarrosum* var. *jimenezii* (Pau) Pau; L. suffruticosum subsp. *jimenezii* (Pau) Rivas Goday & Rivas-Martinez; *L. tenuifolium* subsp. *suffruticosum* var. *jimenezii* (Pau) O. Bolós & Vigo; *L. tenuifolium* subsp. *marianorum* Bellot & Rivas Goday; *L. suffruticosum* subsp. *marianorum* (Bellot & Rivas Goday) Rivas Goday & Rivas-Martinez; *L. squarrosum* Munby.

Chapter II - Cytogenetic diversity in the polyploid complex *Linum suffruticosum s.l.* (Linaceae)

Chapter section published as an original article to *Botanical Journal of the Linnean Society*

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Abstract

Polyploidy plays a significant role in the evolution and diversification of flowering plants. In several polyploid complexes, high morphological variability and plasticity coupled with cytogenetic diversity, makes it difficult to disentangle their evolutionary history. The main goal of this study was to gain insights into the role of whole genome duplications as one of the factors shaping the evolution of flowering plants. *Linum suffruticosum s.l.* has been described as a polyploid complex, with high morphological variability, but nothing is known about current cytogeographical patterns. We investigated cytotype diversity and distribution patterns in 151 populations covering most of the distribution range, in the Iberian Peninsula, south-east France, north-west Italy and Morocco, using flow cytometric analyses complemented with chromosome counts. A remarkably high cytogenetic diversity was found with five major cytotypes being detected, namely diploids, tetraploids, hexaploids, octoploids and decaploids, and with new ploidy levels being reported for the first time. The different ploidy levels were distributed parapatrically, having a geographical structure and several contact zones. Most of the populations comprised one cytotype, but a few mixed-ploidy populations were observed. Our results suggest that whole genome duplications are one of the key mechanisms, alone or together with hybridization, governing the diversification of *L. suffruticosum s.l.* Genome size and/or chromosome counts might be useful tools for identifying specimens of *L. suffruticosum s.l.* Also, geographical overlap and high cytogenetic diversity suggest multiple origins of the polyploids. The diversity observed here has been mostly neglected to date and should be accounted when studying the biosystematics of this complex.

Key words: chromosome counts, contact zones, evolutionary history, genome size, Mediterranean plants, ploidy level.

Introduction

Polyploidy plays a significant role in the evolution and diversification of flowering plants (Ramsey and Schemske 1998; Soltis and Soltis 1999; Otto and Whitton 2000; Blanc and Wolfe 2004; Madlung 2013). This widespread phenomenon is observed in the evolutionary history of virtually all flowering plants, being frequent in several plant lineages (Soltis 2005) and correlated with explosions in species diversity (Soltis *et al*. 2009). Estimates suggest that a high percentage of speciation events in Angiosperms has been associated with ploidy increases (Wood *et al.* 2009) and there is evidence that some polyploid taxa have multiple origins (Soltis *et al.* 1992; Kolář *et al*. 2009; Chelaifa *et al.* 2010; Castro *et al.* 2018; Wan *et al.* 2019). Estimates of the incidence of polyploidy in current floras also reveal high levels of polyploid taxa in certain regions (*e.g.*, 37% in the Mediterranean region and 49% in the Iberian Peninsula; Marques *et al*. 2018). The Mediterranean Basin is considered a cradle where polyploidy has frequently occurred through the evolutionary history of plants groups thriving in these territories, linked to its dynamic palaeogeographic and climatic history (*e.g.*, Late Miocene Salinity Crisis, spread of Mediterranean-type climate at the Pliocene, Pleistocene Ice Ages; Thompson 2005). Additionally, the detection of taxa with multiple ploidy levels (*e.g.*, Buggs and Pannell 2007; Balao *et al*. 2009; Kolář *et al*. 2009; Castro *et al.* 2012a; Castro *et al*. 2018, 2019; Kim *et al*. 2012a; Muñoz-Pajares *et al*. 2018; Prančl *et al*. 2018) supports that polyploidy is a dynamic and ongoing process in nature (Ramsey and Schemske 1998, 2002; Soltis 2005; Wood *et al*. 2009; Marques *et al*. 2018).

Whole genome duplications generate a new entity reproductively isolated from the progenitor(s) and, thus, have been described as an important mechanism of sympatric speciation (Otto and Whitton 2000; Soltis *et al*. 2010). Polyploids arise through the duplication of genomes from the same species (autopolyploidy) or by the combination of genomes from two species (allopolyploidy; Ramsey and Schemske 1998). The recognition of the origin of polyploids is however difficult in many occasions. While allopolyploids typically have phenotypes differentiated from their progenitors and may be more easily detected as hybrids, autopolyploids may be nearly indistinguishable from their progenitors (Brochmann *et al*. 2004; Doyle *et al*. 2004; Soltis *et al*. 2010; Spoelhof *et al.* 2017). Multiple origins and recurrent hybridization and introgression may also increase the complexity of certain taxa in natural populations and generate intricate series of polyploids (*e.g.*, Segraves *et al*. 1999; Soltis and Soltis 1999; Sampson and Byrne 2012). Additionally, in several plant groups, the taxonomic identification of polyploids is problematic due to the lack of reliable diagnostic characters, high morphological variability and phenotypic plasticity (Brochmann *et al*. 2004; Doyle *et al*. 2004; Prančl *et al*. 2018). These traits allied with polyploidization events significantly increase the

difficulty to understand the evolutionary history of certain plant taxa. In this context, genome size can be an additional diagnostic character, helpful to recognize polyploid series, with potential to be a relevant tool for identifying different evolutionary histories and/or independent polyploidization events (Balao *et al*. 2009; Kolář *et al*. 2009). Still, we know little about many polyploid complexes that bear major taxonomic problems and that have not been studied systematically throughout their entire distribution range, even though polyploidy has already been identified to have played an important role in their evolution.

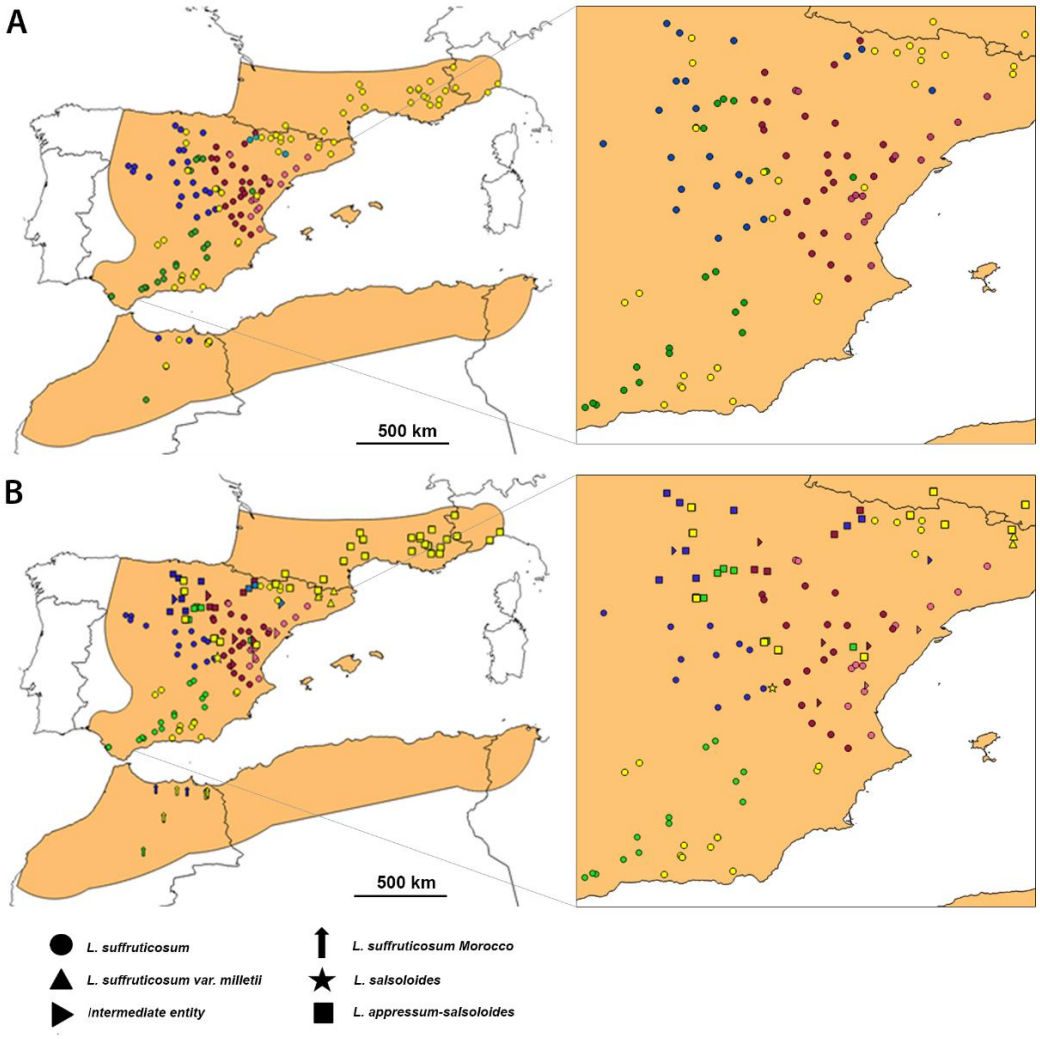

Figure 2.1. A, Geographical distribution of *L. suffruticosum s.l.* (orange) and of all populations sampled in the study area depicted with the respective ploidy level (diploid: yellow; tetraploid: green; hexaploid with low genome size: dark blue; hexaploid with high genome size: light blue; octaploid: dark red; decaploid: pink). B, Geographical distribution of *L. suffruticosum s.l.* taxonomic entities (*L. salsoloides*: star; *L. suffruticosum*: circles; *L. suffruticosum* var. *milletii*: triangles; *L. appressum-salsoloides*: squares; intermediate entity: lying triangles, *L. suffruticosum* Morocco: arrows) with ploidy levels presented with different colors as in A. The base map was downloaded from https://www.diva-gis.org/gdata.

Linum suffruticosum s.l. (Linaceae) is composed by perennial, variable woody plants with a complex floral dimorphism and breeding system (Nicholls 1985b; Armbruster *et al*. 2006), distributed from the Iberian Peninsula, south-east France and north-west Italy, to north-west Africa (Figure 2.1). The taxonomy of the group is very complex and, consequently, it has been subjected to different taxonomic treatments over the years. This difficulty is mainly due to the high morphological variability observed in the group and to the lack of strong/unambiguous diagnostic characters. The most recent treatment of the group of *Linum suffruticosum s.l.* describes a high variability and recognizes morpho-geographical divisions with numerous transitional areas, leading to a taxonomic treatment comprising over 20 taxa, only for the Iberian Peninsula (Martínez-Labarga and Garmendia 2015). However, while this exhaustive division could be functional on a regional scale, it does not always work at a wider scale given the continuum of morphological variability. Among the previously available taxonomic treatments, three taxa have been consensually accepted as distinct species, namely *L. salsoloides* Lam., *L. appressum* Caball. and *L. suffruticosum* L., with some varieties being described in the last taxon (*e.g.*, Jahandiez and Maire 1932; Ockendon and Walters 1968; López González 1979). Additionally, the group is monophyletic with uncertain phylogenetic relationships between all its entities (Ruiz-Martín et al. 2018). *Linum suffruticosum s.l.* has been described as a polyploid complex with a basic chromosome number of 9, bearing diploids (2n = 2*x* = 18 chromosomes), tetraploids (2n = 4*x* = 36) and octoploids (2n = 8*x* = 72), all in the Iberian Peninsula (Appendix 2.1). The available chromosome counts describe *L. salsoloides* and *L. appressum* as diploids, and *L. suffruticosum s.s.* as a polyploid complex harbouring tetraploid and octoploid individuals (Appendix 2.1). The available records already pointed for some segregation among cytotypes at a regional scale, namely tetraploids occurring in the south of Spain and octoploids in the northern regions (Nicholls 1985b; c, 1986). Still, information about the prevalence of each cytotype, as well as its diversity and distribution patterns across the distribution range of the group is scarce due to poor sampling and limited cytogenetic information. The main goal of this study was to explore whole genome duplications as one of the factors shaping the evolution of the apparently self-incompatible, style dimorphic *L. suffruticosum s.l.* complex (Nicholls 1985c, 1986). For that, we explored in detail the cytotype diversity and distribution patterns in *L. suffruticosum s.l.* throughout most of its distribution range (NW Italy, S France, Iberian Peninsula, North Morocco). Flow cytometric analyses complemented with chromosome counts were used to address the following specific objectives: (1) describe the diversity of chromosome numbers, ploidy levels and genome sizes within the group; (2) explore the geographic distribution and variation of ploidy levels, including dominant and rare cytotypes, across the entire distribution range of the group; (3) explore the potential existence of contact zones

among ploidy levels and mixed-ploidy populations; and (4) evaluate the potential of genome size and ploidy level as additional diagnostic characters for future taxonomical treatments. This study offers novel insights in the cytogenetic diversity of this complex with new key diagnostic characters, namely ploidy level and genome size, and opens new avenues for understanding the complex evolutionary pathways within *L. suffruticosum s.l.*

Materials and methods

Field sampling

In SW Europe, field sampling was done in two periods, *i.e*., in flowering and fruiting seasons (May-July in the southerly most locations, and August in most of the northern locations) of 2016 and 2017. Sampling in each population included the collection of herbarium vouchers for taxonomic confirmation, and flower buds or recently open flowers for flow cytometric analyses of the petals. We used this tissue because, contrarily to other plant organs, petals did not have mucilaginous compounds, which hampered flow cytometric analyses as samples clogged the flow cytometer. Fresh flower buds or petals were collected in individual plastic bags and stored in a portable or a conventional refrigerator (up to 7 days) until flow cytometric analyses. Up to 30 individuals (mean ± SD: 16 ± 11) were sampled per population. In the fruiting season, targeted populations encompassing all morphological and cytogenetic entities were revisited for the collection of seeds to be used for chromosome counting. Seeds from 30 individuals were collected in selected populations and stored in individual paper bags. In Morocco, field sampling was made in the flowering season of 2018, and included the collection of herbarium vouchers and petals. Geographic coordinates of all sampled localities were obtained and detailed information about all sites is provided in Appendix 2.2. In total, 151 populations were sampled throughout most of the distribution range of the group (Figure 2.1). Voucher specimens are deposited in COI and SEV herbaria.

Field sampling was designed to record most of regions were the group is present, and the morphological variability described by taxonomic treatments. All specimens collected in the field were identified according to López González (1979) and Fennane *et al*. (2007) and assigned to four taxa: *L. suffruticosum* var. *milletii* (Sennen and Gonzalo) G. López, *L. suffruticosum*, *L. salsoloides* and *L. appressum-salsoloides*, the latter including those plants not clearly assigned to either of these two species. With exception of *L. suffruticosum* var. *milletii* (a very distinguishable variety from Catalonia), it was not possible to determine unambiguously the lower rank categories (varieties) of *L. suffruticosum* due to the occurrence of intermediate characters. Additionally, we were unable to use the taxonomic treatment of López González

(1979) to identify the plants from Morocco, thus these plants were classified as *L. suffruticosum* following the available literature for this region (Jahandiez and Maire 1932; Emberger and Maire 1941; Quézel and Santa 1962; Fennane *et al*. 2007; Valdés *et al*. 2007). In one locality, only, the individuals were unambiguously identified as *L. salsoloides*, following López González (1979). For the remaining populations, individuals had intermediate characters between *L. appressum* [considered by López González (1979) as an Iberian endemism] and *L. salsoloides* (distributed in France and Italy; Ockendon and Walters 1968), and thus we treated those specimens as *L. appressum-salsoloides*. Also, in a few populations in Spain, the identification of some specimens was dubious due to the occurrence of intermediate morphological characters between *L. suffruticosum*, *L. salsoloides* and *L. appressum* and, thus, these individuals were classified as intermediate entities.

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, approximately 50 mg of petal tissue of *Linum* L. was chopped together with 50 mg of leaves of an internal reference standard (*Solanum lycopersicum* L. 'Stupické', hereafter S.l., with 2C = 1.96 pg; Doležel, Sgorbati and Lucretti 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°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 (Doležel *et al.* 2007). 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 analysed per sample (Suda *et al.* 2007). Only samples with coefficient of variation values of 2C peaks below 5% were accepted (data not shown), otherwise a new sample was prepared and analysed until such quality standards were achieved (Greilhuber, Temsch and Loureiro 2007).

In all populations, 3 to 6 individuals were analysed individually, enabling to estimate the genome size of the population. For the remaining individuals, a pooled sample strategy was followed (5–6 *Linum* individuals plus the reference standard) enabling to access the DNA ploidy level. The holoploid genome size (2C in pg; *sensu* Greilhuber, Temsch and Loureiro 2007) was calculated using the formula:

$$\textit{Linum}\ 2C\ \text{nuclear DNA content (pg)} = \frac{\textit{Linum}\ G_1\ \text{peak mean} \times \textit{S.l.}\ \text{genome size}}{\textit{S.l.}\ G_1\ \text{peak mean}}$$

The DNA ploidy level of each individual was inferred from chromosome counts (see section Chromosome counts) and genome size estimates obtained for the individuals in the population. The monoploid genome size (1Cx; *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.

Chromosome counts

For counting the number of chromosomes, seeds from the selected populations (at least one for each genome size category; Appendix 2.2) were germinated in Petri dishes. Actively growing root tips were harvested and pre-treated with ice at 4 ˚C in the dark for 24 h; afterwards, root tips were fixed in a solution of 3:1 of 95% ethanol and glacial acetic acid, for 48 h at room temperature. Root tips were then washed two times for 5 min with distilled water and incubated in acetic carmine for at least 48 h at room temperature. Finally, chromosomes were squashed under a glass cover in 45% acetic acid. 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 analysed using flow cytometry.

Statistical analyses

Descriptive statistics of holoploid genome size were calculated for each cytotype (mean, standard deviation of the mean, and maximum and minimum values) based only on individual flow cytometric estimates. Coefficient of variation (CV, in %) was calculated for each ploidy level and taxon/entity as the ratio between standard deviation and the mean. To assess differences among cytotypes in holoploid and monoploid genome sizes, Generalized Linear Models were used (Bolker *et al.* 2009), with a Gaussian distribution and an identity 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 3.6.1 (R Core Development Team 2019), using the packages *car* for Type-III analysis of variance (Fox *et al.* 2005), *glm* for generalized linear models (Hastie and Pregibon 1992) and *multcomp* for multiple comparisons after Type-III analysis of variance (Hothorn *et al.* 2017). Spatial correlation analysis for all cytotypes was evaluated with a Mantel test using the package *ade4* (Dray and Dufour 2007). The same analysis was performed for each cytotype individually. The Mantel test provides a correlation coefficient between the two data matrices, namely the geographic distance matrix and the genome size distance matrix, with $P < 0.05$ indicating significant correlation between them and positive r values indicating positive association, *i.e.*, more similar genome sizes are found geographically together.

Results

Cytogenetic diversity in *Linum suffruticosum s.l.*

A remarkably large variation in genome size was observed, with 2C values ranging from 1.33 to 7.76 pg (Table 2.1, Figure 2.2, n = 729 individuals, n = 151 populations). However, with a few exceptions, the variation was not continuous (Figure 2.2; Table 2.1) and, together with data on chromosome counts (n = 134 individuals, 53 populations), we were able to clearly assign the DNA ploidy level to most genome size ranges (Figures 2.3-2.5, Appendices 2.3-2.5). In total, five main cytotypes were detected, namely diploids (2*x*), tetraploids (4*x*), hexaploids (6*x*), octoploids (8*x*) and decaploids (10*x*), with occasional triploids (3*x*) and aneuploids (an.) being also found (Table 2.2). The holoploid (2C) and monoploid (1C*x*) genome sizes of the dominant cytotypes differed significantly (2C – $F_{4, 751} = 5123.2$, $P < 0.001$; 1Cx – $F_{4,751} = 211.12$, $P < 0.001$; Figure 2.2).

Variation in chromosome number and genome size was also observed within most of the ploidy levels (Table 2.1). Diploids comprised individuals with either 16 or 18 chromosomes and genome sizes with 9% variation (ranging from 1.33 to 1.78 pg; Figures 2.2, 2.3, 2.4A and 2.5A, Appendix 2.5A). Tetraploids had 2n = 36 chromosomes, rarely 32 or 38, and genome size showed a 25% variation (ranging from 2.63 to 3.63 pg; Figures 2.2, 2.4C and 2.5B, Appendix 2.5B). The genome size variation in the hexaploids was even higher, 51% (ranging from 3.64 to 5.51 pg), with 2n = 54 chromosomes, and occasionally 48 chromosomes (Figures 2.2, 2.4D and 2.5C, Appendices 2.4A and 2.5C). Notably, some of the genome size values from hexaploids fall within the range observed for octoploids, but their ploidy level was confirmed through chromosome counting (Figures 2.2 and 2.5D, Appendix 2.4B). Octoploids had a lower genome size variation when compared with some of the other ploidy levels (22%; ranging from 4.61 to 5.67 pg) and all analysed individuals had 2n = 72 chromosomes (Figures 2.2, 2.4E and 2.5E,

Appendix 2.4C). Finally, decaploids exhibited the highest genome size (52%) and intra-population variation (ranging from 5.75 to 7.76 pg), with individuals having 2n = 90 chromosomes (Figures 2.2 and 2.5D, Appendix 2.4D). Clearly, two basic chromosome numbers were observed, n = 8 and 9, with the former being rare.

Table 2.1. Ploidy levels detected and observed chromosome numbers in *L. suffruticosum s.l.*. Abbreviations: 2*x*, diploid; 3*x*, triploid; 4*x*, tetraploid; 6*x*, hexaploid; 8*x*, octoploid; 10*x*, decaploid; N, number of individuals analyzed; 1C*x* G.s, mean and standard deviation of the mean of the monoploid genome size in picograms (pg); 2C G.s. mean and standard deviation of the mean of the holoploid genome size in picograms (pg); Range, maximum and minimum values in pictograms (pg); CV, coefficient of variation calculated as the ratio of the standard deviation to the mean (in %); no. chro., number of chromosomes (individual chromosome numbers are separated by comma; rare chromosome counts are presented in parenthesis).

Ploidy level	N	1Cx G.s. (pg)	2C G.s. (pg)	Range (pg)	CV (%)	No. chro.
2*x*	1144	0.80 ± 0.04	1.59 ± 0.09	1.33 - 1.78	5.57%	16, 18
3*x*	3	0.86 ± 0.04	2.59 ± 0.00	2.58 - 2.59	0.16%	27
4*x*	496	0.78 ± 0.06	3.11 ± 0.25	2.63 - 3.63	8.06%	(32) , 36, (38)
6*x*	642	0.70 ± 0.09	4.21 ± 0.51	3.64 - 5.51	12.11%	(48) , 54
8*x*	417	0.64 ± 0.03	5.13 ± 0.22	4.61 - 5.67	4.28%	72
10*x*	219	0.66 ± 0.05	6.64 ± 0.52	5.75 - 7.76	7.83%	90

Table 2.2. Number of populations and individuals observed with different ploidy levels of *L. suffruticosum s.l.*. Abbreviations: 2*x*, diploid; 3*x*, triploid; 4*x*, tetraploid; 6*x*, hexaploid; 8*x*, octoploid; 10*x*, decaploid; an., aneuploidy; N pop, number of populations analyzed; N total, number of individuals analyzed.

Ploidy level	N pop (N total)
2*x*	53 (962)
2*x* + 3*x*	3 (107+3)
2*x* + 4*x*	3 (75+16)
4*x*	27 (464)
6*x*	19 (428)
6*x* + 4*x*	6 (175+16)
6*x* + an.	1 (29+1)
8*x*	19 (238)
8*x* + 6*x*	5 (151+10)
8*x* + an.	1 (26+3)
10*x*	10 (160)
10*x* + 8*x*	2 (16+2)
10*x* + an.	2 (43+2)
Total	151 (2927)

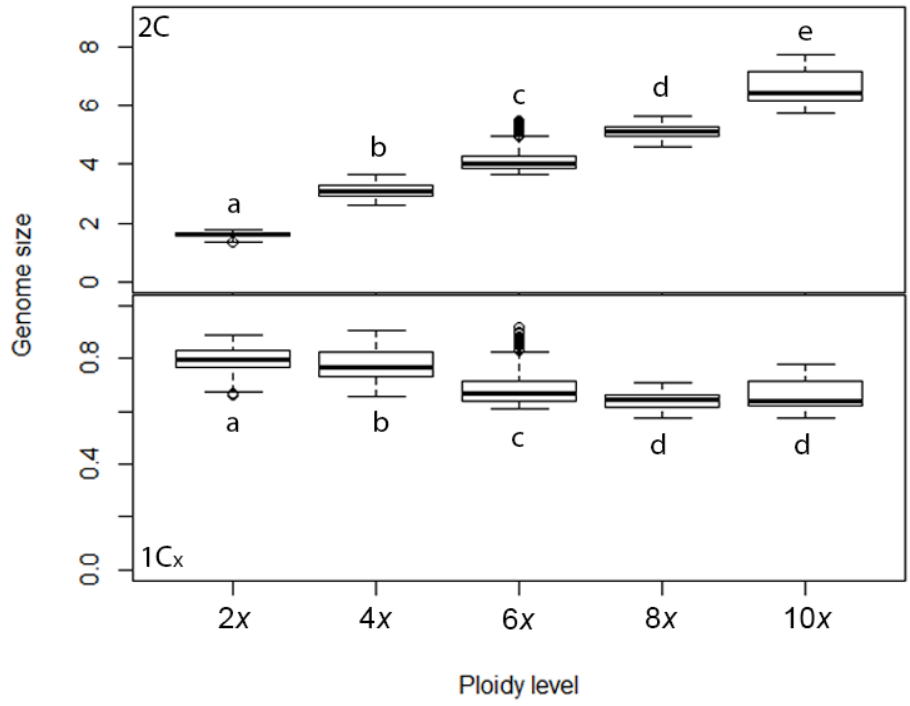

Figure 2.2. Holoploid and monoploid genome size range and mean (black line) of diploid, tetraploid, hexaploid, octoploid and decaploid population**s.** Abbreviations: 2C, holoploid genome size; 1C*x*, monoploid genome size; 2*x*, diploid; 4*x*, tetraploid; 6*x*, hexaploid; 8*x*, octoploid; 10*x*, decaploid. Outliers are given as white circles. Different letters correspond to statistically significant differences at $P < 0.05$.

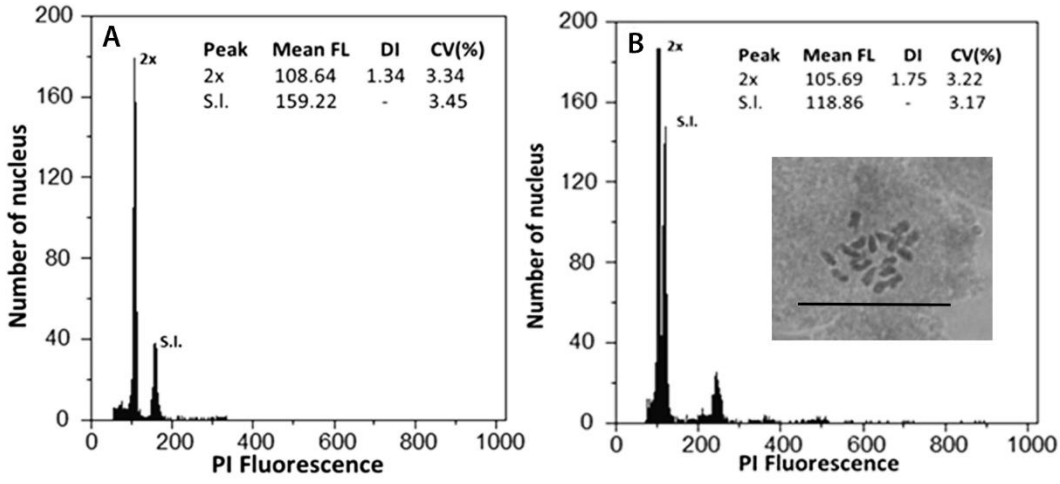

Figure 2.3. Genome size estimation and chromosome number of A, *L. salsoloides* and of B, *L. suffruticosum* var. *milletii*. Abbreviations: 2*x*, diploid; S.l., *Solanum lycopersicum*; Mean FL, mean relative fluorescence in picograms; DI, DNA index; CV(%), coefficient of variation of the peak in %. Scale bar: 20 µm (black line).

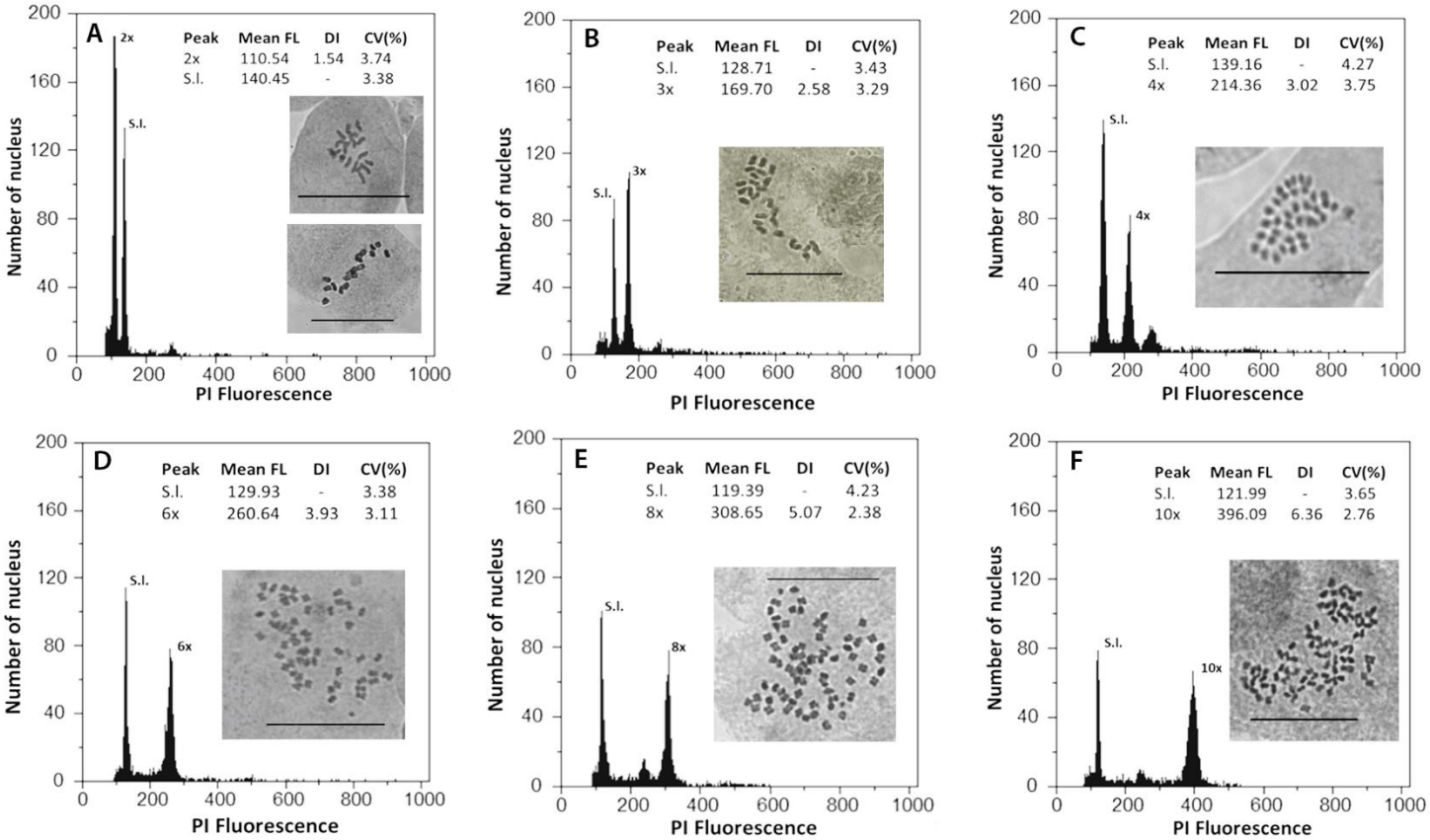

Figure 2.4. Genome size estimation and chromosome number of *L. suffruticosum* **A,** diploid; **B,** triploid; **C,** tetraploid; **D,** hexaploid; **E,** octoploid; **F,** decaploid. Abbreviations: 2*x*, diploid; 3*x*, triploid; 4*x*, tetraploid; 6*x*, hexaploid; 8*x*, octoploid; 10*x*, decaploid; S.l., *Solanum lycopersicum*; Mean FL, mean relative fluorescence in picograms; DI, DNA index; CV(%), coefficient of variation of the peak in %. Scale bar: 20 µm (black line).

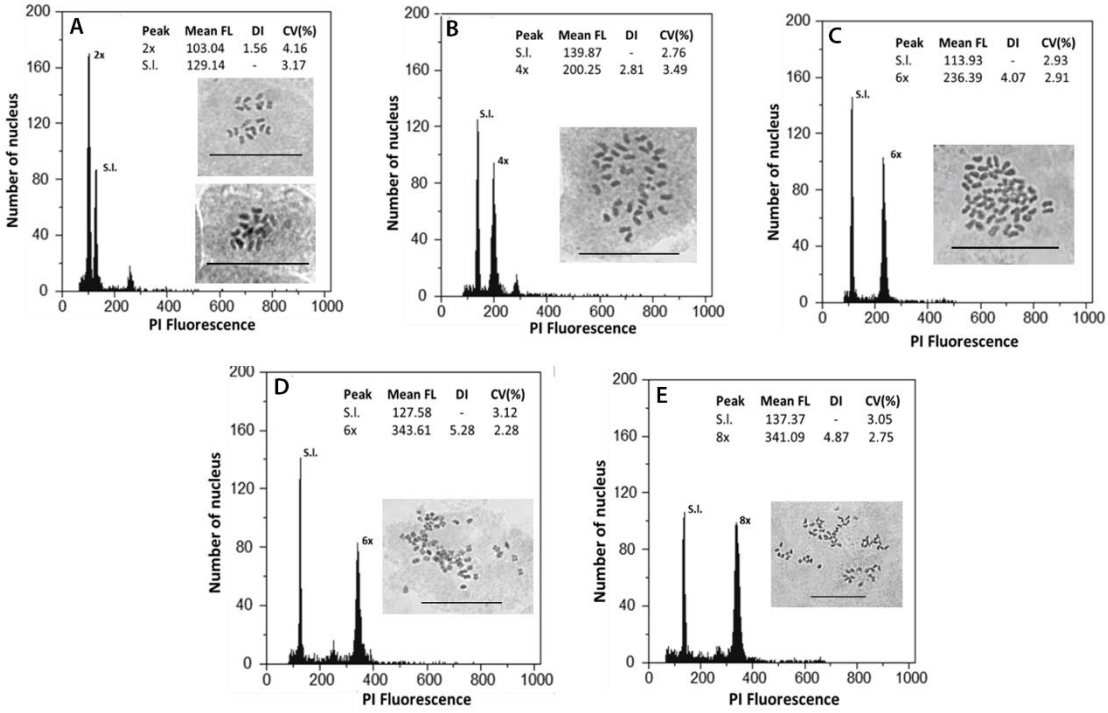

Figure 2.5. Genome size estimation and chromosome number of *L. appressum-salsoloides.* **A,** diploid; **B,** triploid; **C,** tetraploid; **D,** hexaploid; **E,** octoploid. Abbreviations: 2*x*, diploid;4*x*, tetraploid; 6*x*, he*x*aploid; 8*x*, octoploid; S.l., *Solanum lycopersicum*; Mean FL, mean relative fluorescence in picograms; DI, DNA index; CV(%), coefficient of variation of the peak in %. Scale bar: 20 µm (black line).

Cytotype distribution patterns in *Linum suffruticosum s.l.*

Cytotypes had different distribution patterns across the range. Diploids were scattered through the entire distribution area of *Linum suffruticosum s.l.* (Figure 2.1A). In contrast, polyploids were found mostly in the Iberian Peninsula (namely, 4*x*, 6*x*, 8*x* and 10*x*) and Morocco (namely, 4*x* and 6*x*), although complex contact zones are observed in central and northern regions of Spain (Figure 2.1A). In the Pyrenees, France and Italy, all populations were found to be diploid; in Spain, diploids were detected mostly in mountainous regions (one cluster in the Pyrenees and another in southern regions of Spain; Figure 2.1A), with only a few populations being found in low altitudes. Tetraploids were found mostly in the South of Spain (Figure 2.1A). Hexaploids have a more northern distribution in Spain, and some populations reaching western Pyrenees (Figure 2.1A). Octoploids occur at lower altitude and hotter regions in eastern ranges (Figure 2.1A). Finally, decaploids occur in areas nearer the NE coast of Spain, but not necessarily at low altitudes, although some populations were also found in more inland areas in NE Spain (Figure 2.1A). Although this differential pattern of distribution of cytotypes, there is a non-significant spatial correlation of genome size for the whole sample of populations across the

cytotypes (Figure 2.1A, Mantel test: $r = 0.0252$, $P > 0.05$). This is probably due to the weight of non-significant correlation of diploid populations scattered through the entire range (Figure 2.1A, Mantel test: $r = -0.032$, $P > 0.05$). On the contrary, most of polyploid populations showed significant positive correlations within their more restricted ranges (Figure 2.1A, Mantel tests: tetraploids, $r = 0.845$, $P < 0.05$; hexaploids, $r = 0.180$, $P < 0.05$; octoploids: $r = 0.165$, $P < 0.05$). The non-significant correlation of decaploid populations (Figure 2.1A, Mantel test: $r = -0.054$, $P > 0.05$) is probably due to the low number of populations found.

Most populations comprised only one cytotype (85% single-ploidy populations), however, some mixed-ploidy populations were also detected (Table 2.2, Appendix 2.6). Most mixed-ploidy populations are characterized by a dominant cytotype growing with another cytotype occurring in low frequency, such as, a few triploids or tetraploids growing in diploid populations (3 localities each), a few tetraploids growing in hexaploid populations (6 localities), a few hexaploids growing in octoploid populations (5 localities) and a few octoploids in decaploid populations (2 localities). The exceptions were one diploid-tetraploid population (AA87, with 19 individuals 2*x* and 11 4*x* individuals) and one tetraploid-hexaploid population (DP1980, with five 6*x* individuals and five 4*x* individuals) (Appendix 2.2) where the two cytotypes occur in more even proportions.

Cytogenetic diversity in taxonomic and geographical entities

Diversity was also observed within and among taxonomic groups of *Linum suffruticosum s.l.* (Figures 2.3-2.6, Appendices 2.4-2.6) with significant differences being found in genome size among most taxonomic entities and cytotypes (2C – $F_{14, 1695.25} = 4521.5$, $P < 0.001$; 1Cx – $F_{14, 1695.25} = 336.54$, $P < 0.001$; Figure 2.6).

Diploids. Two diploid taxa were detected, *L. salsoloides* and *L. suffruticosum* var. *milletii* (Figure 2.3). *Linum salsoloides* was observed in central Spain, and revealed to be homogenously diploid, having the lowest values and the smallest variation in genome size (Figure 2.3A, Appendix 2.3). Because we were unable to grow seedlings from this species, information on the number of chromosomes is available only from the literature, with 2n = 18 chromosomes (Appendix 2.1). *Linum suffruticosum* var. *milletii* grows in the eastern Pyrenees and was revealed to be homogenously diploid (Figures 2.1B and 2.3B). In contrast to *L. salsoloides*, this taxon had the largest genome size values within the diploid level, bearing also 18 chromosomes (Figure 2.3 and 2.6, Appendix 2.3).

Polyploids. Two polyploid series were observed, *L. suffruticosum* and *L. appressum-salsoloides*, with high variability in genome size and chromosome numbers. *Linum suffruticosum* was the taxon with the highest levels of variability (including 2*x*, 4*x*, 6*x*, 8*x*, 10*x* and a few 3*x* and

aneuploids), and genome size estimates enabled us to unambiguously assign ploidy levels to all individuals analysed (Figure 2.4, Appendix 2.3). Diploids with 2n = 16 and 18 chromosomes and 2C and 1Cx genome size values intermediate to the two diploid taxa, were found growing in mountain regions from Southern Spain and in central Spanish Pyrenees (Figure 2.1B). Tetraploid *L. suffruticosum* with 2n = 32 or 36 chromosomes and the double value of the diploid genome size (Figures 2.4C and 2.6, Appendix 2.3) was found mostly in Southern Spain in a parapatric distribution with diploids (Figure 2.1B). The highest ploidy cytotypes were found mostly in the North of Spain, with an increase of ploidy level from west to east (Figure 2.1B). Hexaploids were mostly found in central Spain with variable chromosome numbers (2n = 48, 54, Figures 2.4D and 2.6, Appendix 2.3), octoploids were found from Valencia to Zaragoza all with 2n = 72 (Figures 2.4E and 2.6, Appendix 2.3) and, finally, decaploids with 2n = 90 were found close to the coast in the NE Iberian Peninsula, although a few populations were also found in La Rioja and Teruel mountain regions (Figures 2.4F and 2.6, Appendix 2.3). Interestingly, the increase in genome size among cytotypes is not proportional. As a result, the genome size of octoploids and decaploids did not differ statistically, and in addition, 1Cx values of *L. suffruticosum* in Spain decreased with increased ploidy levels (Figure 2.6). In Morocco, *L. suffruticosum* was rarer, still, diploids, tetraploids and hexaploids were detected (Figure 2.1B); diploids showed genome sizes similar to those found in diploid *L. suffruticosum* in Europe, while tetraploids and hexaploids showed genome sizes two and three times bigger than diploids, respectively, showing less evidence of genome downsizing (Figure 2.6, Appendices 2.3, 2.5).

Linum appressum-salsoloides, whose populations are distributed in the North of Spain, France and Italy, harbour all cytotypes except decaploids (Figure 2.1B). Diploid populations with 2n = 16 and 18 (Figure 2.5A, Appendix 2.3) were mainly found in the Pyrenees and through France and Italy, with only a few populations being found in north and central Spain (Figure 2.1B). In contrast, polyploids were concentrated in Northern Spain, overall at higher latitudes than *L. suffruticosum* polyploids (Figure 2.1B). The 2C values of diploids are similar to those found in diploid *L. suffruticosum* (Figure 2.6), however, such specimens are morphologically distinct. Tetraploids were found from central to northern Spain and had 2n = 36 (occasionally with 2n = 38 chromosomes) (Figures 2.1B and 2.5B, Appendix 2.3). Within hexaploids, two distinct groups based on genome size were found (Figures 2.5C-D and 2.6, Appendix 2.3). One of the groups was found in northwestern Spain with a genome size falling within the hexaploid range of values (2C = 4.24 ± 0.35 pg) and 2n = 54 (Figure 2.5C, Appendix 2.3). The other group was found in the western Pyrenees and had significantly higher genome sizes falling within the range of octoploid estimates (2C = 5.23 ± 0.15 pg; Figure 2.5D), with homogenous chromosome counts of 2n = 54 (Figure 2.5D, Appendix 2.3). Consequently, the latter group of hexaploids had

very distinctive 1Cx values (Figure 2.6), being an exception to the pattern of genome downsizing with increasing ploidy within this taxon. Finally, octoploids with 2n = 72 were found from the western Pyrenees to Soria (Figure 2.1B and 2.5E, Appendix 2.3).

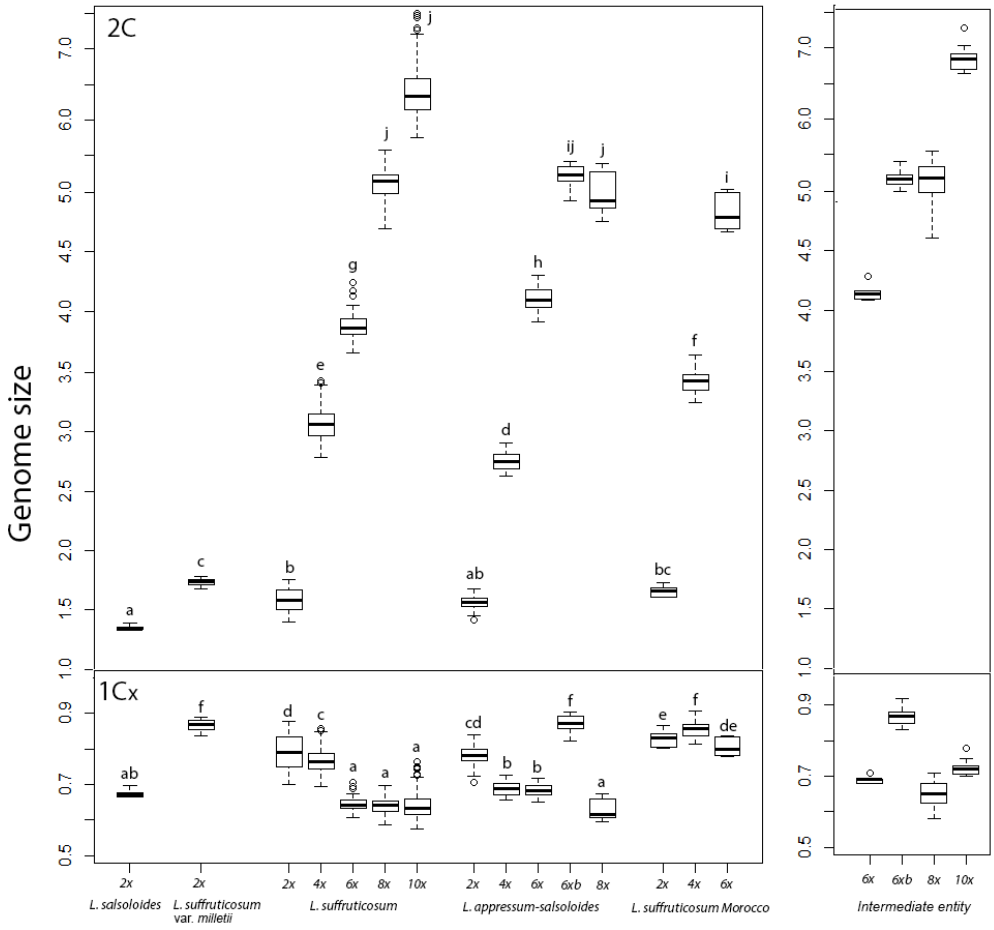

Figure 2.6. Holoploid and monoploid genome size range and mean (black line) of diploid, tetraploid, hexaploid, octoploid and decaploid populations of each taxonomic entity. Abbreviations: 2C, holoploid genome size; 1C*x*, monoploid genome size; 2*x*, diploid; 4*x*, tetraploid; 6*x,* 6*x*b, hexaploid; 8*x*, octoploid; 10*x*, decaploid. Outliers are also given as white circles. Different letters correspond to statistically significant differences at $P < 0.05$.

Intermediate entities. Specimens classified as intermediate (nine populations) also showed variability in both chromosome numbers and genome size estimates, although such populations comprise mostly higher ploidy levels, namely hexaploids, octoploids and decaploids (Figure 2.6, Appendix 2.3 and 2.4). As in *L. appressum-salsoloides*, we observed hexaploids with distinct genome sizes (2C = 4.15 ± 0.07 pg and 2C = 5.22 ± 0.15 pg, Appendix 2.4), both with 2n = 54 chromosomes (Appendix 2.3 and 2.4). Interestingly, the intermediate populations were detected in regions of contact among cytotypes and taxa (Figure 2.1B).

Discussion

Cytogenetic diversity in *Linum suffruticosum s.l.*

The genus *Linum* comprises over 200 species and is particularly diverse in the Mediterranean region (McDill *et al.* 2009; Ruiz-Martín *et al.* 2018). Economically relevant groups have been studied in detail, including its genetic and cytogenetic diversity (*e.g.*, *L. usitatissimum* L. from sect. *Dasylinum* (Plach.) Juz, and the group of *L. perenne* L.; Ockendon 1968; Chennaveeraiah and Joshi 1983; Bolsheva *et al.* 2017), while other sections, highly diverse and with complex reproductive features, have received less attention, and their diversity remains largely unknown (*e.g. Linum* sect. *Linopsis* (Rchb.) Engelm., Nicholls 1985b; McDill *et al.* 2009; Muravenko *et al.* 2010). *Linum suffruticosum s.l.* has been described as a polyploid group, but our study reveals the occurrence of wider cytogenetic variability within the complex than previously thought. Here, we observed that variation occurs at three levels, namely in chromosome number, ploidy level and genome size. First, we observed two basic chromosome numbers (n = 8 and 9; with the former being reported here for the first time in *L. suffruticosum s.l.*) and consequently, we observed different chromosome numbers within the same cytotype (namely for diploids, tetraploids and hexaploids). This suggests the occurrence of chromosomal rearrangements, such as chromosome loss or gain in chromosome pairing, which may have played a role in promoting the cytogenetic diversity of the group. Processes of chromosome fusion, translocations and/or inversions have been described in other species of the genus (Muravenko *et al.* 2010; Bolsheva *et al.* 2015). Second, we observed new ploidy levels within the group, with triploids, hexaploids and decaploids being described here for the first time. Although multiple ploidy levels have been reported in some species of *Linum* (*e.g.* Nilsson and Lassen 1971; Rogers, Mildner and Harris 1972; Chennaveeraiah and Joshi 1983), none encompasses the amount of variability observed here (including five dominant cytotypes). This large variation in ploidy level is probably the highest known for this genus, where ploidy has been reported in about ¼ of the taxa with available data, usually with only diploid and tetraploid levels (Ruiz-Martín *et al.* 2018). A recent review on mixed-ploidy species revealed that most of the well-studied polyploid complexes harbour two (77%) or three ploidy levels (14%), and more rarely additional ploidies (9%) (Kolář *et al.* 2017). This supports the idea that whole genome duplications (alone or together with hybridization events) are one of the key mechanisms in the diversification of *L. suffruticosum s.l.* Finally, we observed variation in genome sizes within ploidy levels and, consequently, variation in 1Cx values (*e.g.*, for hexaploids and diploids or between *L. suffruticosum* individuals from Spain and Morocco), supporting different evolutionary histories (discussed below).

Cytogeographical patterns

Our large-scale sampling revealed complex geographical patterns of *L. suffruticosum s.l.* cytotypes. The different ploidy levels were distributed parapatrically, with several contact zones among cytotypes and with mixed-ploidy populations being rarely found. Polyploids were found in the Iberian Peninsula and Northern Africa with the remaining areas of the species distribution in Europe being characterized by homogenously diploid populations, only. In Northern Africa, we found diploid, tetraploid and hexaploid populations, with the species being less abundant there than in Europe. In the Iberian Peninsula, although cytotype distribution was complex, cytotypes had a spatially structured distribution. Diploid populations were more abundant in Southern Spain and in the Pyrenees; this wide distribution of diploids may reflect a complex history of movements and lineage sorting across the range since the early Pleistocene, when the group originated (Ruiz-Martín *et al.* 2018) and explain the lack of Mantel correlation between geographic distance and genome size. In contrast, tetraploid populations were scarce, being concentrated in the South where a clear contact zone of diploid-tetraploid populations was found. The majority of the higher polyploids were found in Central and Northern Spain, with spatial segregation and some contact zones. Hexaploid populations are distributed in Central and Northeastern Spain, but some populations can also be found in the Pyrenees. The majority of octoploid and decaploid populations are found in lower and arid zones of the eastern part of Spain, with the latter being found near the coast. In general, the more recent polyploid populations had a narrower range. Thus the Mantel correlation of the genome size is significant and positive, except for the scarcer decaploid populations. Spatial segregation has been shown in several polyploid complexes (Husband and Schemske 1998; Balao *et al.* 2009; Kolář *et al.* 2009; Sonnleitner *et al.* 2010; Castro *et al.* 2012b), and has been proposed as one of the most effective barriers for successful polyploid establishment (Levin, 2002; Li, Xu and Ridout 2004; Baack and Stanton 2005). The capacity to disperse and colonize new niches escaping competition with the progenitor individuals increases the probability of establishment by reducing the minority cytotype disadvantage (Levin 1975; Ramsey 2011; Hao *et al.* 2013). Polyploidization has been shown to have consequences in the ability of polyploids to grow in habitats that differ from their progenitors, enabling polyploids to expand to new areas (Levin 1975; Buggs and Pannell 2007; Ramsey 2011; Hao *et al.* 2013). In *L. suffruticosum s.l.* the current distribution patterns may be associated with niche differentiation among cytotypes that promotes spatial segregation and consequently reproductive isolation, enabling the establishment and maintenance of polyploid lineages. A strong association between the spatial distribution of cytotypes and their environmental requirements has been explored using niche

modelling tools in several polyploid complexes (Glennon, Ritchie and Segraves 2014; Thompson, Husband and Maherali 2014; Visger *et al.* 2016; Muñoz-Pajares *et al.* 2018). Further studies to model niche preferences in *L. suffruticosum s.l.* are already being developed in order to disentangle the ecological requirements that might explain polyploid success and the current distribution patterns.

Although rare, mixed-ploidy populations were also detected (15%), usually with one of the cytotypes in low frequency in the populations. These populations were mostly found at zones of contact between different ploidy levels but were also observed in areas dominated by a cytotype. Contact zones are frequent in most polyploid complexes and enable cytotype interactions; still, mixed-ploidy populations are considered a transitory stage and are expected to be rare because positive frequency-dependent selection will exclude the cytotype in minority (Levin 1975). Interestingly, in most *L. suffruticosum s.l.* mixed-ploidy populations the lower ploidy was rarer. This may reflect dynamic contact zones where: 1) new polyploids are formed or disperse to existing localities, 2) between-ploidy hybridization occurs or 3) a decline in the frequency of a once dominant cytotype is ongoing. For example, in diploid regions, diploid populations with a few triploids suggest the production of unreduced gametes and emergence of new cytotypes, but in contact zones with tetraploids or hexaploids it may reflect inter-cytotype hybridization; in contrast, mixed-ploidy populations such as tetraploid-hexaploid, hexaploid-octoploid and octoploid-decaploid where the lower ploidy occurs in low frequency suggest a successful expansion of higher ploidies over lower ones. To sum up, the detection of mixed-ploidy populations in *L. suffruticosum s.l.* clearly supports that cytotype interactions at contact zones exist and that such populations are very dynamic. The levels of interaction will influence the genetic structure and diversity at contact zones. Future studies at mixed-ploidy populations involving controlled pollinations and plant fitness assessment will provide insights into the dynamics of this polyploid complex at contact zones.

Genome size and chromosome number as informative characters

By analysing extensively the ploidy levels and genome sizes in the group, our study reveals cryptic diversity that has not been taken into consideration in previous studies, but that constitutes a strong reproductive barrier. Some of the specimens are easily distinguishable morphologically, such *as L. suffruticosum* var. *millettii* and *L. salsoloides*, and are here distinguished also by different 2C and 1Cx genome size values, the former taxon having the highest genome sizes of diploids and the latter one the lowest values. Noteworthy, most of the ploidy variability was found in the Iberian Peninsula, and polyploids are not restricted to *L. suffruticosum* as previously described in the literature (Appendix 2.1), but also occur in *L.*

appressum-salsoloides. The latter can be found as a diploid throughout France and Italy, but in central and northern Spain it comprises diploid and polyploid populations, while *L. suffruticosum* appears to be more constrained to central and southern Spain, and its cytotypes have a clear geographical structure. Within *L. suffruticosum* we also found different 1Cx values for Spanish and Moroccan populations, with genome downsizing being observed in the former region, but not in the latter. This may reflect different evolutionary trajectories, as observed in several other plant groups (*e.g.* Hohmann *et al.* 2014; Mandák *et al.* 2016; Krahulcová *et al.* 2017), especially across the Strait of Gibraltar (Rodríguez-Sánchez *et al.* 2008) and supports the need for an extensive review of the group in its entire distribution range, as well as dated phylogenetic and phylogeographic studies.

Additionally, another source of complexity lies in contact zones (*e.g.*, between *L. suffruticosum* and *L. appressum-salsoloides*) where populations of higher ploidies and intermediate characteristics are found (here treated as intermediate entities). Hybridization and introgression processes have been suggested to be involved in creating the variability found in *Linum* in the Iberian Peninsula and, in some cases, generate populations of individuals with contiguous characters among closely related taxa (Martínez-Labarga and Garmendia 2015; Ruiz-Martín 2017), making the taxonomy of this group even more difficult. Previous works revealed the importance of cytogenetic traits for taxonomic and relationship evaluations in complex plant groups (*e.g.*, Murray 2005; Hohmann *et al.* 2014; Habibi *et al.* 2018; Prančl *et al.* 2018) and in this genus (Nicholls ,1985c; McDill *et al.* 2009; Muravenko *et al.* 2010; Bolsheva *et al.* 2015; Talebi *et al.* 2015). Still, while the latest review for the Flora Iberica segregates groups that are hardly distinguishable morphologically in the field (Martínez-Labarga and Garmendia 2015), our results also reveal that previous treatments (*e.g.*, Ockendon and Walters 1968; López González 1979) do not accommodate all the diversity found in natural populations. All this cytogenetic information is useful as a tool to define geographic units that are hardly distinguishable morphologically, although it cannot be used alone as taxonomic character. Thus, in combination with morphological characterizations and dated phylogenetic relationships based on molecular data, our results can be a helpful tool for clarifying the taxonomy of *L. suffruticosum s.l.* in future studies.

Can cytogenetic data provide insight on the origin of *Linum suffruticosum s.l.* cytotypes?

The *L. suffruticosum s.l.* group is monophyletic, but internal phylogenetic relationships are still unclear (Ruiz-Martín *et al.* 2018). Multiple origins of polyploids from the same and/or different progenitors and rapid genomic changes immediately after polyploid formation, may have contributed to the diversity in *L. suffruticosum s.l.* Our results support, at least for some of

the cytotypes, different origins and evolutionary pathways. First, geographical overlap and high cytogenetic diversity detected in natural populations suggest that unreduced gamete formation and hybridization events seem frequent in this complex and might be involved in recurrent auto- and/or allopolyploid formation and in gene flow among cytotypes. This agrees with molecular analyses within the group (Ruiz-Martín 2017) and with the occurrence of morphologically intermediate individuals (*e.g.*, Martínez-Labarga and Garmendia 2015). Second, differences in monoploid genome size for different geographical areas have been described in several plant groups (Balao *et al.* 2009; Kolář *et al.* 2009) and reflect dissimilar evolutionary relationships among polyploids (*e.g.* Hohmann *et al.* 2014; Mandák *et al.* 2016; Krahulcová *et al.* 2017). Indeed, the differences in 2C and 1Cx genome sizes observed for populations from different geographical areas support distinct origins, as the one detected in hexaploid individuals of *L. suffruticosum s.l.* Additionally, differences in 1Cx genome sizes between Spanish and Moroccan populations of *L. suffruticosum* support different evolutionary histories, in Morocco likely involving autopolyploidy, while northern Spain would harbour a melting pot where different taxa occur and allopolyploidy and/or multiple origins were likely involved. Finally, *L. suffruticosum s.l.* bears a rare three-dimensional reciprocal heterostyly with associated heteromorphic self-incompatibility (Armbruster *et al.* 2006). Our observations throughout the whole geographic range suggest that there is a constant presence of this 3-D heterostyly in all populations of the group. Moreover, most of the examined populations showed a 1:1 ratio of style morphs (A. Afonso and J. Arroyo, field observations), suggesting that this complex breeding system with heteromorphic self-incompatibility is maintained (Barrett 2002a) across the range, irrespective of ploidy. Breeding systems are hypothesized to change along polyploid complexes, particularly when hybridization is involved (Naiki and Nagamasu 2004; Guggisberg *et al.* 2006), while in outcrossing plants, polyploids tend to be formed mostly through autopolyploid events (Ramsey and Schemske 1998). Still, it is difficult to distinguish between the different processes without appropriate molecular markers, and further phylogenetic and phylogeographic studies are needed to confirm these hypotheses.

Conclusions

This study revealed complex geographical distribution patterns of *L. suffruticosum s.l.* cytotypes. The large-scale screening showed an outstanding cytogenetic diversity, with triploids, hexaploids and decaploids being described here for the first time. The different ploidy levels were distributed parapatrically, thus having a geographical structure and several contact zones. Genome size and/or chromosome counts might be useful tools for identifying individuals of *L. suffruticosum s.l.* However, the complexity and morphological variability of the group requires additional taxonomic studies accounting with the diversity found here. In addition, the origin of the polyploids is not easy to disentangle. The geographical overlap and high cytogenetic diversity detected here suggest multiple origins of the polyploids from the same and/or different progenitors. Future phylogenetic and phylogeographic studies coupled with niche modelling analyses are needed to understand the relationships among *L. suffruticosum* entities, and to disentangle the ecological requirements that might explain the success of polyploids and their current distribution patterns.

Appendices

Appendix 2.1. Chromosome number reported for *L. suffruticossum s.l.*

Chromosome number reported	**Taxon** (original publication)	**Taxon** (following López González 1979)	**Locality**	**Reference**
2n=18	*Linum tenuifolium* subsp. *appressum* (Caball) Rivas Goday & Rivas-Mart.	*L. appressum* Caball.	France	Nicholls MS. 1986. Variation and evolution in *Linum tenuifolium* (Linaceae). *Plant Systematics and Evolution* 153: 243–258.
2n=18	*Linum tenuifolium* subsp. *appressum* (Caball) Rivas Goday & Rivas-Mart.	*L. appressum* Caball	Spain (Cuenca, Guadalajara)	Nicholls MS. 1986. Variation and evolution in *Linum tenuifolium* (Linaceae). *Plant Systematics and Evolution* 153: 243–258.
2n=18	*Linum suffruticosum* subsp. *salsoloides* (Lam.) Rouy	*L. salsoloides* Lam.	Spain	Mugnier C. 1983. In IOPB chromosome number reports LXXIX. *Taxon* 32: 323.
2n=18	*Linum tenuifolium* L.	Difficult to assign	Spain (Lérida)	Rogers CM. 1980. In IOPB chromosome number reports LXVII. *Taxon* 29:347.
2n=18	*Linum tenuifolium* subsp. *salsoloides* (Lam.) R. Lit.	*L. salsoloides* Lam.	Spain (Cuenca)	Nicholls MS. 1986. Variation and evolution in *Linum tenuifolium* (Linaceae). *Plant Systematics and Evolution* 153: 243–258.
2n=36	*Linum tenuifolium* subsp. *suffruticosum* (L.) R. Lit.	*Linum suffruticosum* var. *suffruticosum*	Spain (Albacete, Jaén, Malaga, Valencia)	Nicholls MS. 1986. Variation and evolution in *Linum tenuifolium* (Linaceae). *Plant Systematics and Evolution* 153: 243–258.
2n=36	L. *suffruticosum* var. *angustifolium* Lange	L. *suffruticosum* var. *angustifolium* Lange	Spain (Albacete, Granada).	Rogers CM. 1981: A note on *Linum* in Spain. *Anales del Jardín Botánico de Madrid* 38, 302.
2n=36	*Linum suffruticosum* L.	*Linum suffruticosum* L.	Spain (Albacete)	Rogers CM, Mildner R; Harris BD. 1972: Some additional chromosome numbers in the Linaceae. *Brittonia* 24: 313-316.
2n=36	*Linum suffruticosum* L.	*Linum suffruticosum* L.	Spain (Granada)	Rogers CM. 1980. In IOPB chromosome number reports LXVII. *Taxon* 29:347.
2n=72	*Linum suffruticosum* L.	*Linum suffruticosum* L.	Spain (Zaragoza)	Lorenzo-Andreu A, García Sanz P. 1950. Cromosomas somáticos de plantas espontâneas en la estepa de Aragón. II. *Anales de la Estación Experimental de Aula Dei* 2:12-63.
2n=72	*Linum suffruticosum* L.	*Linum suffruticosum* L.	Spain (Zamora)	Elena Rosselló JA, González Zapatero MA, Navarro Andrés F. 1985. Sobre los niveles de ploidía y otras particularidades cromosómicas de algunos vegetales castellano-leoneses de preferencias calcícolas. *Studia Botanica Universidad de Salamanca* 4: 109-115.

Appendix 2.2. Specimens and populations of *L. suffruticosum s.l.* analyzed in this study**.** Information about the ploidy level of each population (Ploidy level), mean, standard deviation of the mean (SD), and minimum (min) and maximum (max) values of the holoploid (2C) and monoploid (1C*x*) genome size in picograms (pg) are given. The coefficient variation of the mean holoploid genome size (CV; calculated as the ratio of the standard deviation to the mean) in percentage (%), number of individuals with ploidy level estimations (N ploidy level), number of individuals with genome size estimates (N G.s.,), chromosome numbers (2n; individual chromosome counts are separated by a comma; "~" denotes approximate counts due to low availability of material), and number of individuals with chromosome counts (N no. chro) are provided. Finally, information about country, locality, coordinates, date of collection, collector, collector number and herbarium where specimen has been deposited are also given.

Taxonomic entity	Ploidy level	2C (pg)				1Cx (pg)				CV (%)	N ploidy level	N G.s.	2n	N no. chro.	Country	Locality	Coordinates	Date	Collector	Herbarium
		mean	SD	min	max	mean	SD	min	max											
L. salsoloides	2	1.35	0.02	1.33	1.39	0.674	0.012	0.665	0.696	1.74%	30	6	-	-	Spain	Cuenca, Monteagudo de las Salinas	39.81750, -1.98131	15-06-2016	J. Arroyo; 85JAM	COI
L. suffruticosum var. *milletii*	2	1.75	0.03	1.71	1.78	0.876	0.013	0.856	0.889	1.51%	30	6	-	-	Spain	Barcelona, Bagà	42.25406, 1.87108	08-06-2016	A. Afonso; E. Olmedo-Vicente, AA41	COI
L. suffruticosum var. *milletii*	2	1.73	0.04	1.68	1.78	0.866	0.020	0.838	0.890	2.26%	30	6	18	3	Spain	Barcelona, Cercs	42.12848, 1.86359	08-06-2016	A. Afonso; E. Olmedo-Vicente, AA42	COI
L. suffruticosum var. *milletii*	2	1.74	0.02	1.71	1.76	0.868	0.010	0.856	0.880	1.16%	13	6	-	-	Spain	Barcelona, Villalleons	41.88472, 2.32464	09-06-2016	A. Afonso; E. Olmedo-Vicente, AA43	COI
L. suffruticosum var. *milletii*	2	1.73	0.03	1.70	1.76	0.863	0.013	0.851	0.879	1.56%	8	5	-	-	Spain	Girona, Rocabruna	42.32937, 2.46187	09-06-2016	A. Afonso; E. Olmedo-Vicente, AA44	COI
L. suffruticosum	4	3.31	0.06	3.26	3.41	0.828	0.016	0.815	0.853	1.89%	5	5	-	-	Spain	Cádiz, Puerto Real	36.51330, -6.13980	12-06-2018	J. Arroyo, AA114	SEV
L. suffruticosum	4	3.18	0.11	3.05	3.40	0.795	0.027	0.763	0.850	3.41%	21	13	36	3	Spain	Cádiz, Jarana	36.51683, -6.13983	04-05-2016	J. Arroyo, AA1	SEV
L. suffruticosum	4	3.13	0.05	3.07	3.18	0.784	0.012	0.767	0.794	1.53%	30	4	36	6	Spain	Málaga, Carratraca	36.84642, -4.80506	09-05-2016	A. Afonso; E. Olmedo-Vicente, AA2	COI
L. suffruticosum	4	3.16	0.20	2.97	3.43	0.790	0.050	0.743	0.857	6.26%	9	4	36	4	Spain	Málaga, Ardales,	36.86330, -4.86258	09-05-2016	A. Afonso; E. Olmedo-Vicente, AA3	COI

⇩Cont.

L. suffruticosum	4	2.98	0.02	2.95	3.02	0.746	0.006	0.738	0.755	0.82%	16	6	36	9	Spain	Málaga, Archidona	37.07613, -4.36787	13-05-2016	A. Afonso; E. Olmedo-Vicente, AA4	COI
L. suffruticosum	2	1.54	0.03	1.51	1.56	0.771	0.013	0.757	0.780	1.63%	7	3	18	2	Spain	Granada, Lentegí	36.84307, -3.72026	27-05-2017	A. Afonso; AA62	COI
L. suffruticosum	4	3.09	0.05	3.07	3.15	0.774	0.012	0.767	0.787	1.53%	8	3	36	2	Spain	Granada, Loja	37.19358, -4.12204	28-05-2017	A. Afonso; AA63	COI
L. suffruticosum	4	3.15	0.07	3.04	3.22	0.788	0.018	0.761	0.806	2.27%	18	6	-	-	Spain	Málaga, El Burgo	36.79416, -4.99011	31-05-2017	J. Arroyo, AA28	COI
L. suffruticosum	2	1.56	0.02	1.54	1.59	0.781	0.011	0.768	0.796	1.36%	36	6	18	3	Spain	Almería, Enix	36.89150, -02.61966	14-05-2016	A. Afonso; E. Olmedo-Vicente, AA5	COI
L. suffruticosum	2	1.53	0.02	1.51	1.54	0.765	0.008	0.757	0.772	1.02%	5	3	-	-	Spain	Granada, Monachil	37.11266, -3.43547	26-06-2016	A. Afonso; S. Lopes, AA98	COI
L. suffruticosum	2	1.56	0.06	1.50	1.67	0.782	0.029	0.749	0.833	3.66%	24	6	-	-	Spain	Granada, Monachil	37.11958, -3.43561	09-06-2016	J. Arroyo, 81JAM	COI
L. suffruticosum	2	1.50	0.02	1.47	1.52	0.748	0.010	0.734	0.760	1.31%	31	6	-	-	Spain	Granada, El Pocico	37.27697, -2.98046	01-06-2016	A. Afonso; E. Olmedo-Vicente, AA29	COI
L. suffruticosum	2	1.48	0.01	1.45	1.49	0.738	0.007	0.726	0.745	0.91%	9	6	-	-	Spain	Granada, Baza	37.43552, -2.87157	01-06-2016	A. Afonso; E. Olmedo-Vicente, AA30	COI
L. suffruticosum	2	1.45	0.03	1.40	1.49	0.726	0.016	0.700	0.744	2.18%	26	6	18	1	Spain	Granada, Baza	37.43008, -2.87095	01-06-2016	A. Afonso; E. Olmedo-Vicente, AA31	COI
L. suffruticosum	2	1.53	0.06	1.49	1.61	0.766	0.028	0.745	0.806	3.72%	15	5	-	-	Spain	Granada, Diezma	37.31229, -3.40837	02-06-2016	A. Afonso; E. Olmedo-Vicente, AA32	COI
L. suffruticosum	2	1.53	0.05	1.47	1.60	0.767	0.025	0.735	0.802	3.30%	33	6	-	-	Spain	Granada, Güéjar Sierra	37.13917, -3.45667	09-06-2016	J. Arroyo, 80JAM	COI
L. suffruticosum	4	3.03	0.03	2.99	3.06	0.758	0.007	0.748	0.766	0.88%	30	6	-	-	Spain	Granada, Puebla de Don Fadrique	37.99434, -2.46491	14-06-2016	J. Arroyo, 83JAM	COI
L. suffruticosum	4	3.09	0.03	3.05	3.14	0.772	0.008	0.761	0.785	1.04%	30	6	-	-	Spain	Córdoba, La Concepción	37.44153, -4.1538	09-06-2016	J. Arroyo, 82JAM	COI
L. suffruticosum	4	3.01	0.06	2.92	3.11	0.753	0.015	0.731	0.777	2.03%	19	11	-	-	Spain	Jaén, Pegalajar	37.74808, -3.63973	23-05-2016	A. Afonso; E. Olmedo-Vicente, AA26	COI
L. suffruticosum	4	2.97	0.06	2.92	3.06	0.743	0.014	0.730	0.765	1.89%	38	7	-	-	Spain	Jaén, Cárchel	37.66194, -3.64153	23-05-2016	A. Afonso; E. Olmedo-Vicente, AA27	COI

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L. suffruticosum	2	1.69	0.01	1.68	1.71	0.846	0.006	0.838	0.853	0.73%	4	8	16, 18	2, 2	Spain	Murcia, Yecla	38.57439, -1.24025	25-05-2017	A. Afonso; AA61	COI
L. suffruticosum	2+4	1.66	0.03	1.63	1.69	0.831	0.014	0.815	0.843	1.72%	30	3	16, 18	3, 7	Spain	Murcia, Jumilla	38.5065, -1.27158	25-05-2017	A. Afonso; AC1	COI
		-	-	-	-	-	-	-	-	-	-	-	32	2						
L. suffruticosum	4	2.93	0.03	2.90	2.96	0.732	0.006	0.725	0.741	0.87%	33	6	-	-	Spain	Albacete, Riópar	38.47679, -2.44495	14-06-2016	J. Arroyo, 84JAM	COI
L. suffruticosum	4	3.05	0.09	2.95	3.13	0.762	0.023	0.737	0.783	3.07%	4	3	-	-	Spain	Jaén, La Hueta	38.32924, -2.57487	24-06-2017	A. Afonso; S. Lopes, AA97	COI
L. suffruticosum	8	4.87	0.10	4.69	4.98	0.608	0.012	0.586	0.622	2.03%	33	6	~72	1	Spain	Albacete, Casas de Ves	39.26055, -1.34936	15-05-2016	A. Afonso; E. Olmedo-Vicente, AA8	COI
L. suffruticosum	2	1.69	0.02	1.66	1.71	0.844	0.012	0.832	0.856	1.41%	3	3	-	-	Spain	Ciudad Real, Puertollano	38.62872, -4.11755	28-05-2017	A. Afonso; AA64	COI
L. suffruticosum	4	2.98	0.02	2.96	3.00	0.744	0.005	0.739	0.750	0.71%	8	3	-	-	Spain	Ciudad Real, Ruidera	38.97051, -2.89594	29-05-2017	A. Afonso; AA65	COI
L. suffruticosum	4	2.84	0.06	2.78	2.94	0.710	0.014	0.695	0.735	2.00%	34	6	32, 36	3, 6	Spain	Ciudad Real, Alhambra	38.88722, -3.05270	21-05-2016	A. Afonso; E. Olmedo-Vicente, AA25	COI
L. suffruticosum	2+3	1.69	0.03	1.66	1.75	0.847	0.016	0.829	0.877	1.93%	35	8	16, 18	2, 4	Spain	Ciudad Real, Fuencaliente	38.46912, -4.35216	02-06-2016	A. Afonso; E. Olmedo-Vicente, AA33	COI
		2.58	-	-	-	0.860	-	-	-	-	1	1	-	-						
L. suffruticosum	8	5.24	0.05	5.19	5.30	0.655	0.007	0.649	0.662	1.03%	3	3	-	-	Spain	Cuenca, Pajaroncillo	39.92318, -1.75872	29-05-2017	A. Afonso; AA66	COI
L. suffruticosum	8	5.16	0.11	5.08	5.29	0.645	0.014	0.635	0.661	2.20%	5	3	-	-	Spain	Cuenca, Salvacañete	40.09732, -1.43841	29-05-2017	A. Afonso; AA67	COI
L. suffruticosum	6	3.73	0.04	3.66	3.76	0.622	0.007	0.610	0.627	1.15%	30	6	48, 54	3, 7	Spain	Cuenca, Mota del Cuervo	39.50835, -2.85839	21-05-2016	A. Afonso; E. Olmedo-Vicente, AA24	COI
L. suffruticosum	6+4	3.86	0.08	3.75	4.02	0.643	0.013	0.625	0.670	2.09%	34	8	~54	6	Spain	Cuenca, La Almarcha	39.68468, -2.39875	03-06-2016	A. Afonso; E. Olmedo-Vicente, AA34	COI
		-	-	-	-	-	-	-	-	-	-	-	36	2						
L. suffruticosum	6+an.	3.91	0.08	3.83	4.05	0.652	0.013	0.639	0.675	2.05%	26	8	54	3	Spain	Cuenca, Olmeda del Rey	39.80865, -2.13021	03-06-2016	A. Afonso; E. Olmedo-Vicente, AA35	COI
		5.76	-	-	-	-	-	-	-	-	1	1	-	-						
L. suffruticosum	8	5.21	0.07	5.13	5.32	0.651	0.009	0.642	0.665	1.37%	10	5	72	3	Spain	Cuenca, Minglanilla	39.54205, -1.51656	24-05-2017	A. Afonso; AA57	COI
L. suffruticosum	6	3.86	0.07	3.78	3.94	0.643	0.012	0.630	0.657	1.93%	30	7	-	-	Spain	Guadalajara, Valdearenas	40.81540, -2.99018	19-05-2016	A. Afonso; E. Olmedo-Vicente, AA19	COI

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L. suffruticosum	6	3.95	0.04	3.91	4.01	0.659	0.007	0.651	0.668	1.00%	30	6	54	6	Spain	Guadalajara, Salmeroncillos	40.52493, -2.52060	20-05-2016	A. Afonso; E. Olmedo-Vicente, AA20	COI
L. suffruticosum	6	3.80	0.07	3.75	3.88	0.633	0.012	0.625	0.647	1.95%	8	3	-	-	Spain	Madrid, Cotos de Monterrey	40.80028, -3.61283	11-06-2017	A. Afonso; S. Lopes, AA83	COI
L. suffruticosum	6	3.84	0.09	3.77	3.95	0.640	0.016	0.628	0.658	2.45%	8	3	-	-	Spain	Cuenca, Priego	40.43013, -2.35204	12-06-2017	A. Afonso; S. Lopes, AA84	COI
L. suffruticosum	6	3.90	0.09	3.81	4.04	0.650	0.015	0.635	0.674	2.30%	31	6	54	4	Spain	Guadalajara, Zorita de los Canes	40.34458, -2.88111	20-05-2016	A. Afonso; E. Olmedo-Vicente, AA21	COI
L. suffruticosum	6+4	3.86 -	0.04 -	3.82 -	3.92 -	0.644 -	0.007 -	0.637 -	0.653 -	1.01% -	35 -	7 -	54 36	5 2	Spain	Madrid, Arganda del Rey	40.28493, -3.45061	20-05-2016	A. Afonso; E. Olmedo-Vicente, AA22	COI
L. suffruticosum	6	3.76	0.08	3.64	3.89	0.627	0.013	0.607	0.648	2.11%	31	6	48, 54	1, 6	Spain	Toledo, Ocaña	39.95684, -3.52719	21-05-2016	A. Afonso; E. Olmedo-Vicente, AA23	COI
L. suffruticosum	6+4	3.82 -	0.06 -	3.71 -	3.87 -	0.636 -	0.010 -	0.619 -	0.644 -	1.54% -	10 -	6 -	48, 54 36	1, 6 3	Spain	Valladolid, Castronuño	41.39400, -5.26770	15-05-2016	Maria Montserrat Ortega, MO6136	COI
L. suffruticosum	6	4.07	0.16	3.79	4.24	0.678	0.026	0.631	0.707	3.86%	10	6	-	-	Spain	Ávila, Arévalo	41.02213, -4.69442	23-05-2016	Ana Afonso, DC705	COI
L. suffruticosum	6	3.82	0.15	3.59	3.91	0.637	0.025	0.599	0.652	4.00%	11	4	-	-	Spain	Zamora, Valdefinjas	41.42613, -5.49754	15-05-2016	Maria Montserrat Ortega, MO6135	COI
L. suffruticosum	6+4	3.92 -	0.08 -	3.86 -	4.02 -	0.653 -	0.013 -	0.643 -	0.670 -	1.94% -	11 -	4 -	54 36	4 2	Spain	Zamora, Castrillo de la Guareña	41.23768, -5.30493	23-05-2016	Maria Montserrat Ortega, MO6137	COI
L. suffruticosum	10+8	6.76 -	0.13 -	6.58 -	6.87 -	0.676 -	0.013 -	0.658 -	0.687 -	1.96% -	5 -	5 -	90 72	2 1	Spain	Zaragoza, El Buste	41.87410,-1.61061	07-07-2016	A. Afonso; AA54	COI
L. suffruticosum	10	7.41	0.15	7.22	7.64	0.741	0.015	0.722	0.764	2.05%	31	7	-	-	Spain	Valencia, Gátova	39.76209, -0.52134	16-05-2016	A. Afonso; E. Olmedo-Vicente, AA10	COI
L. suffruticosum	8+6	4.89 -	0.05 -	4.83 -	4.98 -	0.612 -	0.007 -	0.604 -	0.623 -	1.11% -	35 -	6 -	72 54	2 2	Spain	Valencia, Ayora	39.07773, -1.05370	15-05-2016	A. Afonso; E. Olmedo-Vicente, AA7	COI

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L. suffruticosum	10	6.33	0.08	6.24	6.42	0.633	0.008	0.624	0.642	1.25%	9	5	-	-	Spain	Valencia, Chiva	39.53413, -0.77518	24-05-2017	A. Afonso; AA58	COI
L. suffruticosum	10	6.42	0.24	6.15	6.58	0.642	0.024	0.615	0.658	3.74%	4	3	~90	3	Spain	Valencia, Carcaixent	39.06750, -0.40326	25-05-2017	A. Afonso; AA59	COI
L. suffruticosum	8	5.17	0.12	5.00	5.29	0.646	0.015	0.625	0.661	2.36%	7	4	-	-	Spain	Valencia, Mogente	38.85921, -0.77324	25-05-2017	A. Afonso; AA60	COI
L. suffruticosum	8	5.25	0.16	5.07	5.55	0.656	0.020	0.633	0.693	3.09%	17	6	-	-	Spain	Teruel, Calanda	40.99976, -0.21913	05-06-2016	A. Afonso; E. Olmedo-Vicente, AA37	COI
L. suffruticosum	10+an.	6.31	0.10	6.18	6.42	0.631	0.010	0.618	0.642	1.60%	7	4	-	-	Spain	Teruel, Venta del Aire	40.13747, -0.72671	13-06-2017	A. Afonso; S. Lopes, AA88	COI
		9.25	-	-	-	-	-	-	-	-	1	1	-	-						
L. suffruticosum	10+8	6.28	0.10	6.16	6.35	0.628	0.010	0.616	0.635	1.64%	3	3	90	6	Spain	Teruel, Rubielos de Mora	40.18681, -0.65115	13-06-2017	A. Afonso; S. Lopes, AA89	COI
		-	-	-	-	-	-	-	-	-	-	-	72	2						
L. suffruticosum	10	6.03	0.13	5.88	6.13	0.603	0.013	0.588	0.613	2.21%	7	3	90	3	Spain	Teruel, Cortes de Arenoso	40.18121, -0.54764	13-06-2016	A. Afonso; S. Lopes, AA90	COI
L. suffruticosum	8	5.21	0.20	5.08	5.43	0.651	0.025	0.635	0.679	3.79%	8	3	-	-	Spain	Teruel, Montalbán	40.82729, -0.79369	15-06-2017	A. Afonso; S. Lopes, AA93	COI
L. suffruticosum	8	5.24	0.08	5.15	5.29	0.655	0.010	0.644	0.661	1.45%	8	3	-	-	Spain	Teruel, Torre los Negros	40.83581, -1.10473	15-06-2017	A. Afonso; S. Lopes, AA94	COI
L. suffruticosum	8	5.13	0.06	5.08	5.19	0.641	0.007	0.635	0.649	1.14%	6	3	-	-	Spain	Guadalajara, Castellar de la Muela	40.82104, -1.76417	15-06-2017	A. Afonso; S. Lopes, AA95	COI
L. suffruticosum	8	5.17	0.02	5.15	5.19	0.646	0.003	0.643	0.649	0.44%	8	3	-	-	Spain	Teruel, Torre de Arcas	40.76718, -0.05773	31-05-2017	A. Afonso; AA73	COI
L. suffruticosum	10	6.11	0.07	6.06	6.19	0.611	0.007	0.606	0.619	1.10%	1	3	-	-	Spain	Teruel, La Cerollera	40.83116, -0.02805	31-05-2017	A. Afonso; AA74	COI
L. suffruticosum	8	5.26	0.11	5.18	5.38	0.657	0.014	0.648	0.673	2.07%	8	3	-	-	Spain	Teruel, Vilastar	40.27082, -1.15967	30-05-2017	A. Afonso; AA68	COI
L. suffruticosum	8	5.41	0.14	5.33	5.58	0.677	0.018	0.666	0.697	2.65%	8	3	72	4	Spain	Teruel, Corbalán	40.39623, -1.00594	30-05-2017	A. Afonso; AA68	COI
L. suffruticosum	10	6.07	0.17	5.75	6.21	0.607	0.017	0.575	0.621	2.78%	30	6	-	-	Spain	Tarragona, Ascó	41.12289, 0.53928	17-05-2016	A. Afonso; E. Olmedo-Vicente, AA13	COI
L. suffruticosum	10	6.16	0.08	6.09	6.25	0.616	0.008	0.609	0.625	1.31%	5	3	~90	4	Spain	Tarragona, Vallclara	41.36270, 0.97867	14-06-2017	A. Afonso; S. Lopes, AA91	COI
L. suffruticosum	10	6.64	0.09	6.59	6.75	0.664	0.009	0.659	0.675	1.42%	8	3	-	-	Spain	La Rioja, Santuario de Misericordia	41.86026, -1.58001	02-06-2017	A. Afonso; AA79	COI

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L. suffruticosum	8+6	5.05 -	0.13 -	4.93 -	5.27 -	0.631 -	0.017 -	0.617 -	0.659 -	2.67% -	12 -	6 -	72 54	3 5	Spain	Zaragoza, Torrehermosa	41.24220, -2.13033	19-05-2017	A. Afonso; E. Olmedo-Vicente, AA18	COI
L. suffruticosum	8	5.04	0.05	5.01	5.10	0.630	0.007	0.626	0.638	1.03%	5	3	-	-	Spain	Zaragoza, Fuendetodos	41.34113, -0.98877	15-06-2017	A. Afonso; S. Lopes, AA92	COI
L. suffruticosum	8+6	5.03 -	0.14 -	4.79 -	5.21 -	0.629 -	0.018 -	0.599 -	0.651 -	2.85% -	35 -	10 -	72 54	5 1	Spain	Zaragoza, Morata de Jalón	41.45468, -1.46656	19-05-2016	A. Afonso; E. Olmedo-Vicente, AA16	COI
L. suffruticosum	8	5.37	0.04	5.34	5.40	0.672	0.006	0.668	0.676	0.82%	2	2	-	-	Spain	Zaragoza, Pozuel de Ariza	41.32335, -2.15162	19-05-2016	A. Afonso; E. Olmedo-Vicente, AA17	COI
L. suffruticosum	2+3	1.64	0.04	1.58	1.70	0.822	0.020	0.791	0.848	2.48%	31	6	16, 18 27	4, 8 1	Spain	Huesca, Azanuy-Alins	41.97099, 0.30046	18-05-2016	A. Afonso; E. Olmedo-Vicente, AA15	COI
L. suffruticosum	2	1.66	0.03	1.63	1.70	0.831	0.017	0.817	0.850	2.09%	8	3	18	2	Spain	Huesca, Graus	42.34691, 0.38497	01-06-2017	A. Afonso, AA75	COI
L. suffruticosum	2	1.66	0.01	1.65	1.66	0.829	0.003	0.826	0.832	0.35%	4	3	-	-	Spain	Huesca, Seira	42.49577, 0.40497	01-06-2017	A. Afonso, AA76	COI
L. suffruticosum	2	1.66	0.03	1.64	1.69	0.830	0.013	0.820	0.845	1.62%	6	3	16	1	Spain	Huesca, Jánovas	42.46925, 0.00080	01-06-2017	A. Afonso, AA77	COI
L. suffruticosum	2	1.66	0.03	1.63	1.68	0.829	0.013	0.815	0.841	1.61%	8	3	-	-	Spain	Huesca, Sardas	42.50355, -0.3489	01-06-2017	A. Afonso, AA78	COI
L. suffruticosum	10	6.30	0.12	6.06	6.41	0.630	0.012	0.606	0.641	1.97%	30	6	~90	2	Spain	Barcelona, Tora	41.77531, 1.44332	05-06-2016	A. Afonso; E. Olmedo-Vicente, AA36	COI
L.suffruticosum Morocco	4	3.54	0.11	3.40	3.63	0.884	0.028	0.851	0.908	3.18%	8	5	-	-	Morocco	Khenifra, Ait Ayach	32.61611, -4.79861	08-06-2018	A. Afonso, J. Loureiro, A. Figueiredo; AA105	COI
L.suffruticosum Morocco	2	1.64	0.04	1.61	1.68	0.818	0.021	0.806	0.841	2.52%	13	3	-	-	Morocco	Taza, Tmourghout	33.87222, -4.03000	08-06-2018	A. Afonso, J. Loureiro, A. Figueiredo; AA106	COI
L.suffruticosum Morocco	2	1.67	0.02	1.66	1.69	0.835	0.008	0.830	0.845	0.99%	19	3	-	-	Morocco	Taza, Tmourghout	33.90750, -4.03139	08-06-2018	A. Afonso, J. Loureiro, A. Figueiredo; AA107	COI
L.suffruticosum Morocco	2+4	1.65 3.25	0.05 -	1.61 -	1.68 -	0.823 0.813	0.024 -	0.806 -	0.840 -	2.92% -	16 3	2 1	- -	- -	Morocco	Berkane, Tghasroutte	34.82006, -2.37532	06-05-2018	A. Afonso, J. Arroyo; AA102	COI
L.suffruticosum Morocco	4	3.28	0.02	3.26	3.31	0.820	0.006	0.815	0.826	0.71%	20	3	-	-	Morocco	Berkane, Ihoufay	34.78492, -2.38274	06-05-2018	A. Afonso, J. Arroyo; AA100	COI

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L.suffruticosum Morocco	2	1.62	0.01	1.60	1.63	0.808	0.007	0.802	0.815	0.81%	20	3	-	-	Morocco	Berkane, Souk Alhad	34.75336, -2.43578	06-05-2018	A. Afonso, J. Arroyo; AA101	COI
L.suffruticosum Morocco	4	3.45	0.02	3.43	3.47	0.864	0.005	0.859	0.868	0.55%	16	3	-	-	Morocco	Berkane, Jbel Dals	34.79528, -2.42917	09-06-2016	A. Afonso, J. Loureiro, A. Figueiredo; AA108	COI
L.suffruticosum Morocco	4	3.47	0.02	3.45	3.49	0.868	0.005	0.862	0.871	0.57%	9	3	-	-	Morocco	Berkane, Ihoufay	34.79694, -2.39972	09-06-2016	A. Afonso, J. Loureiro, A. Figueiredo; AA109	COI
L.suffruticosum Morocco	4	3.38	0.03	3.35	3.42	0.846	0.007	0.838	0.855	0.85%	4	4	-	-	Morocco	Berkane, Tagma	34.83306, -2.41111	09-06-2016	A. Afonso, J. Loureiro, A. Figueiredo; AA110	COI
L.suffruticosum Morocco	6	4.95	0.13	4.80	5.03	0.824	0.021	0.800	0.838	2.53%	20	3	-	-	Morocco	Nador, Afsou	34.87556, -3.14889	09-06-2016	A. Afonso, J. Loureiro, A. Figueiredo; AA111	COI
L.suffruticosum Morocco	2	1.69	0.03	1.67	1.73	0.846	0.013	0.837	0.865	1.51%	18	4	-	-	Morocco	Midar, Beni Touzine	34.90083, -3.54694	10-06-2016	A. Afonso, J. Loureiro, A. Figueiredo; AA112	COI
L.suffruticosum Morocco	6	4.72	0.05	4.67	4.78	0.786	0.009	0.779	0.796	1.14%	20	3	-	-	Morocco	Al Hoceima, Targuist	34.95000, -4.33444	10-06-2016	A. Afonso, J. Loureiro, A. Figueiredo; AA113	COI
L. appressum-salsoloides	2	1.56	0.03	1.53	1.60	0.779	0.014	0.766	0.798	1.85%	14	4	-	-	Italy	Savona, Voze	44.20705, 8.39293	14-06-2016	E. Olmedo-Vicente, A. Afonso, EO6	SEV
L. appressum-salsoloides	2	1.53	0.02	1.51	1.55	0.765	0.009	0.757	0.774	1.15%	24	4	-	-	Italy	Genoa, Bosio	44.52783, 8.78101	14-06-2016	E. Olmedo-Vicente, A. Afonso, EO7	SEV
L. appressum-salsoloides	2	1.59	0.05	1.53	1.66	0.795	0.027	0.766	0.829	3.38%	30	4	-	-	Italy	Torino, Mompantero	45.15230, 7.05635	15-06-2016	E. Olmedo-Vicente, A. Afonso, EO8	SEV
L. appressum-salsoloides	2	1.54	0.03	1.51	1.57	0.771	0.015	0.754	0.783	1.98%	12	3	-	-	Italy	Cuneo, Chiappi	44.37383, 7.14801	16-07-2015	J. arroyo, 68JAM	SEV
L. appressum-salsoloides	2	1.63	0.03	1.60	1.67	0.817	0.017	0.802	0.835	2.02%	31	3	-	-	France	Occitanie, Pardailhan	43.43327,2.81627	11-06-2016	E. Olmedo-Vicente, A. Afonso, EO5	SEV
L. appressum-salsoloides	2	1.51	0.02	1.50	1.53	0.756	0.008	0.748	0.764	1.10%	30	3	-	-	France	Provence-Alpes-Côte d'Azur, Saint André-les-Alpes	43.94977, 6.51158	21-06-2016	E. Olmedo-Vicente, A. Afonso, EO17	SEV
L. appressum-salsoloides	2	1.55	0.02	1.52	1.57	0.774	0.009	0.762	0.783	1.11%	11	4	-	-	France	Provence-Alpes-Côte d'Azur, Bauduen	43.72676, 6.15751	22-06-2016	E. Olmedo-Vicente, A. Afonso, EO19	SEV

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L. appressum-salsoloides	2	1.54	0.00	1.54	1.54	0.771	0.001	0.770	0.771	0.08%	29	3	-	-	France	Provence-Alpes-Côte d'Azur, Malijai	44.00856, 6.04698	22-06-2016	E. Olmedo-Vicente, A. Afonso, EO20	SEV
L. appressum-salsoloides	2	1.53	0.03	1.50	1.56	0.767	0.015	0.751	0.781	1.95%	24	3	-	-	France	Provence-Alpes-Côte d'Azur, Comps-sur-Artuby	43.71774, 6.48123	21-06-2016	E. Olmedo-Vicente, A. Afonso, EO18	SEV
L. appressum-salsoloides	2	1.56	0.02	1.54	1.59	0.781	0.012	0.770	0.794	1.55%	21	3	-	-	France	Provence-Alpes-Côte d'Azur, Vercoiran	44.30601, 5.39480	26-06-2016	E. Olmedo-Vicente, A. Afonso, EO33	SEV
L. appressum-salsoloides	2	1.55	0.01	1.54	1.57	0.777	0.007	0.772	0.784	0.87%	14	3	-	-	France	Provence-Alpes-Côte d'Azur, Montfort	44.06909, 5.92536	22-06-2016	E. Olmedo-Vicente, A. Afonso, EO21	SEV
L. appressum-salsoloides	2	1.55	0.02	1.53	1.58	0.776	0.010	0.766	0.790	1.32%	19	4	-	-	France	Provence-Alpes-Côte d'Azur, Barret-sur-Méouge	44.28092, 5.73385	23-06-2016	E. Olmedo-Vicente, A. Afonso, EO24	SEV
L. appressum-salsoloides	2	1.59	0.02	1.58	1.61	0.795	0.008	0.788	0.805	1.05%	18	3	-	-	France	Provence-Alpes-Côte d'Azur, Ancelle	44.61944, 6.17424	25-06-2016	E. Olmedo-Vicente, A. Afonso, EO29	SEV
L. appressum-salsoloides	2	1.52	0.01	1.51	1.53	0.759	0.004	0.756	0.763	0.46%	26	3	-	-	France	Provence-Alpes-Côte d'Azur, Trescleoux	44.36697, 5.73211	24-06-2016	E. Olmedo-Vicente, A. Afonso, EO25	SEV
L. appressum-salsoloides	2	1.52	0.01	1.51	1.53	0.759	0.004	0.756	0.763	0.50%	7	3	-	-	France	Occitanie, Campagna-de-Sault	42.75700, 2.05698	26-06-2015	J. Arroyo, 104JAM	SEV
L. appressum-salsoloides	2	1.54	0.05	1.50	1.60	0.769	0.027	0.750	0.801	3.57%	7	3	-	-	France	Occitanie, Troubat	42.96936, 0.58719	27-06-2015	J. Arroyo, 106JAM	SEV
L. appressum-salsoloides	2	1.59	0.01	1.58	1.60	0.795	0.006	0.789	0.801	0.79%	30	3	-	-	France	Occitanie, Saint-Paul-et-Valmalle	43.63136, 3.63502	11-06-2016	E. Olmedo-Vicente, G. Papuga, G13	SEV
L. appressum-salsoloides	2	1.54	0.01	1.52	1.55	0.769	0.007	0.760	0.773	0.93%	18	3	-	-	France	Occitanie, Lapanouse-de-Cernon	43.98292, 3.07511	11-06-2016	E. Olmedo-Vicente, G. Papuga, G28	SEV
L. appressum-salsoloides	2	1.55	0.01	1.54	1.57	0.776	0.006	0.770	0.783	0.83%	17	3	-	-	France	Occitanie, Barjac	44.49721, 3.44122	11-06-2016	E. Olmedo-Vicente, G. Papuga, G20	SEV
L. appressum-salsoloides	2	1.64	0.04	1.60	1.66	0.819	0.019	0.798	0.832	2.27%	6	3	-	-	France	Provence-Alpes-Côte d'Azur, Vaugines	43.81130, 5.41673	11-07-2015	J. Arroyo, 49JAM	SEV

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L. appressum-salsoloides	2	1.60	0.03	1.57	1.63	0.802	0.014	0.787	0.814	1.77%	5	3	-	-	France	Occitanie, Vebron	44.23643, 3.55383	06-07-2015	J. Arroyo, 34JAM	SEV
L. appressum-salsoloides	2	1.53	0.05	1.48	1.59	0.767	0.024	0.742	0.795	3.12%	7	4	-	-	France	Provence-Alpes-Côte d'Azur Entraunes	44.25253, 6.75378	15-07-2015	J. Arroyo, 63JAM	SEV
L. appressum-salsoloides	2	1.59	0.03	1.57	1.63	0.796	0.014	0.786	0.814	1.74%	31	6	-	-	Spain	Huesca, Salinas	42.58480, 0.21635	18-06-2016	J. Arroyo, 88JAM	SEV
L. appressum-salsoloides	6	5.23	0.18	4.93	5.42	0.872	0.031	0.821	0.904	3.50%	30	6	-	-	Spain	Huesca, Salinas de Jaca	42.41919, -0.79235	06-06-2016	A. Afonso; E. Olmedo-Vicente, AA38	SEV
L. appressum-salsoloides	6	5.23	0.11	5.10	5.40	0.872	0.019	0.851	0.899	2.16%	31	6	~54	1	Spain	Huesca, Jaca	42.52736, -0.55612	06-06-2016	A. Afonso; E. Olmedo-Vicente, AA39	SEV
L. appressum-salsoloides	8	4.85	0.08	4.75	4.95	0.606	0.009	0.594	0.619	1.55%	29	8	-	-	Spain	Huesca, Borau	42.67286, -0.57986	18-06-2016	J. Arroyo, 89JAM	SEV
L. appressum-salsoloides	8	5.07	0.12	4.94	5.17	0.634	0.015	0.618	0.646	2.30%	10	3	-	-	Spain	Zaragoza, Orés	42.27650, -0.99220	18-06-2016	N. López González, NLG154	SEV
L. appressum-salsoloides	2	1.65	0.04	1.61	1.68	0.823	0.018	0.803	0.839	2.24%	6	4	-	-	Spain	Lleida, Irgo	42.43985, 0.77179	24-06-2015	J. Arroyo, 5JAM	SEV
L. appressum-salsoloides	2+3	1.61	0.02	1.59	1.66	0.806	0.012	0.795	0.828	1.50%	23	6	-	-	Spain	Girona, Urus	42.35238, 1.84263	24-06-2016	J. Arroyo; 95JAM	SEV
		2.59	-	-	-	0.863	-	-	-	-	1	1	-	-						
L. appressum-salsoloides	6	4.01	0.07	3.91	4.07	0.668	0.011	0.651	0.679	1.63%	11	6	54	2	Spain	Burgos, Lano	42.67366, -2.61219	06-06-2016	A. Afonso; E. Olmedo-Vicente, AA40	COI
L. appressum-salsoloides	6	4.24	0.06	4.15	4.30	0.707	0.010	0.692	0.717	1.43%	21	8	-	-	Spain	Burgos, Contreras	42.02212, -3.36847	05-07-2016	A. Afonso; AA48	COI
L. appressum-salsoloides	2	1.59	0.05	1.55	1.68	0.796	0.024	0.777	0.840	2.97%	31	6	-	-	Spain	Burgos, Valmala	42.304065, -3.26406	05-07-2016	A. Afonso; AA49	COI
L. appressum-salsoloides	6	4.07	0.08	3.99	4.18	0.679	0.013	0.666	0.697	1.86%	10	7	54	4	Spain	Burgos, Panizares	42.79345, -3.47700	06-07-2016	A. Afonso; AA50	COI
L. appressum-salsoloides	6	4.05	0.05	3.95	4.11	0.676	0.009	0.659	0.684	1.36%	22	7	54	2	Spain	Burgos, Villasopliz	42.94284 ,-3.67850	06-07-2016	A. Afonso; AA51	COI
L. appressum-salsoloides	2	1.60	0.03	1.56	1.64	0.800	0.017	0.781	0.822	2.07%	30	6	18	1	Spain	Burgos, Frías	42.71386, -3.27968	06-07-2016	A. Afonso; AA52	COI
L. appressum-salsoloides	6	4.06	0.04	4.01	4.09	0.677	0.007	0.669	0.682	1.08%	10	3	-	-	Spain	Burgos, Moradillo de Roa	41.55525, -3.80580	21-05-2016	D. Pinto,; DP2020	COI
L. appressum-salsoloides	4	2.83	0.07	2.78	2.91	0.708	0.017	0.694	0.727	2.41%	7	3	36	5	Spain	Teruel, Allepuz	40.48512, -0.69683	30-05-2017	A. Afonso; S. Lopes, AA70	COI
L. appressum-salsoloides	2	1.54	0.04	1.50	1.57	0.770	0.018	0.750	0.786	2.32%	8	3	16	1	Spain	Teruel, Linares de Mora	40.32056, -0.51911	30-05-2017	A. Afonso; S. Lopes, AA71	COI

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L. appressum-salsoloides	8+6	5.34 -	0.05 -	5.28 -	5.39 -	0.667 -	0.006 -	0.660 -	0.673 -	0.94% -	9 -	4 -	72 54	7 1	Spain	Soria, Noviercas	41.69526, -2.06456	02-06-2017	A. Afonso; S. Lopes, AA80	COI
L. appressum-salsoloides	4	2.76	0.04	2.71	2.79	0.689	0.009	0.679	0.697	1.35%	8	3	-	-	Spain	Soria, Golmayo	41.70171, -2.60745	02-06-2017	A. Afonso; S. Lopes, AA81	COI
L. appressum-salsoloides	6+4	4.11 -	0.07 -	4.03 -	4.16 -	0.685 -	0.011 -	0.672 -	0.693 -	1.63% -	17 -	3 -	~54 ~36	5 5	Spain	Soria, San Esteban de Gormaz	41.58345, -3.20358	21-05-2016	D. Pinto; DP1980	COI
L. appressum-salsoloides	4	2.74	0.07	2.68	2.82	0.685	0.017	0.670	0.704	2.55%	9	3	36	3	Spain	Soria, Golmayo	41.73766, -2.77594	21-05-2016	D. Pinto; DP1995	COI
L. appressum-salsoloides	8	4.98	0.14	4.87	5.13	0.622	0.017	0.608	0.642	2.80%	10	3	-	-	Spain	Soria, Ojuel	41.72451, -2.27836	21-05-2016	D. Pinto; DP2008	COI
L. appressum-salsoloides	4	2.69	0.02	2.66	2.72	0.672	0.006	0.664	0.681	0.88%	9	5	-	-	Spain	Soria, Torreblacos	41.66796, -2.86945	02-06-2017	A. Afonso; S. Lopes, AA82	COI
L. appressum-salsoloides	2	1.49	0.02	1.47	1.50	0.745	0.008	0.737	0.751	1.01%	5	3	-	-	Spain	Cuenca, Beteta	40.56234, -2.12199	12-06-2017	A. Afonso; S. Lopes, AA85	COI
L. appressum-salsoloides	4	2.68	0.04	2.65	2.73	0.670	0.011	0.662	0.683	1.67%	8	3	36	2	Spain	Cuenca, Beteta	40.57291, -2.08928	12-06-2017	A. Afonso; S. Lopes, AA86	COI
L. appressum-salsoloides	2+4	1.50 2.88	0.08 -	1.41 2.78	1.62 2.98	0.752 0.720	0.040 -	0.706 0.695	0.810 0.745	5.29% -	19 11	5 2	- -	- -	Spain	Cuenca, El Perchel	40.42815, -1.89412	12-06-2017	A. Afonso; S. Lopes, AA87	COI
L. appressum-salsoloides	2	1.56	0.04	1.48	1.59	0.781	0.019	0.742	0.797	2.44%	30	7	18	3	Spain	Guadalajara, Villacadima	41.26267, -3.20973	04-07-2016	A. Afonso; AA45	COI
L. appressum-salsoloides	4	2.80	0.05	2.70	2.86	0.701	0.014	0.674	0.716	1.96%	13	7	36, 38	1, 3	Spain	Guadalajara, Somolinos	41.26203, -3.09311	04-07-2016	A. Afonso; AA46	COI
L. appressum-salsoloides	4	2.69	0.07	2.63	2.77	0.673	0.018	0.658	0.693	2.69%	5	3	-	-	Spain	Guadalajara, Villacadima	41.28480, -3.20559	16-06-2017	A. Afonso; S. Lopes, AA96	COI
Intermediate entity	6	4.15	0.07	4.08	4.28	0.691	0.012	0.680	0.714	1.76%	28	6	-	-	Spain	Burgos, Retuerta	42.02040, -3.52026	05-07-2016	A. Afonso; AA47	COI
Intermediate entity	8	5.49	0.10	5.40	5.67	0.686	0.012	0.675	0.709	1.75%	26	6	-	-	Spain	La Rioja, Turruncún	42.15736, -2.10916	07-07-2016	A. Afonso; AA53	COI
Intermediate entity	8	5.24	0.21	5.02	5.67	0.655	0.026	0.627	0.709	4.03%	27	10	-	-	Spain	Teruel, Alfambra	40.55088, -1.09680	15-06-2016	A. Afonso; 86JAM	COI
Intermediate entity	8+an.	5.21 6.30	0.16 -	4.91 -	5.33 -	0.651 -	0.020 -	0.613 -	0.666 -	3.09% -	26 3	6 3	- -	- -	Spain	Teruel, La Iglesuela del Cid	40.48900, -0.35974	31-05-2017	A. Afonso; S. Lopes, AA72	COI
Intermediate entity	8+6	4.80 -	0.16 -	4.60 -	5.03 -	0.600 -	0.020 -	0.575 -	0.629 -	3.27% -	36 -	8 -	72 54	7 1	Spain	Valencia, Utiel	39.58482, -1.15701	16-05-2016	A. Afonso; AA9	COI

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Intermediate entity	10+an.	7.25	0.23	6.98	7.76	0.725	0.023	0.698	0.776	3.24%	33	9	~90	3	Spain	Castellón, Castellnovo	39.86288, -0.44650	16-05-2016	A. Afonso; AA11	COI
		10.27	-	-	-	-	-	-	-	-	1	1	-	-						
Intermediate entity	10	7.21	0.21	6.98	7.46	0.721	0.021	0.698	0.746	2.88%	23	6	-	-	Spain	Tarragona, Mas de Barberans	40.75941, 0.39276	17-05-2016	A. Afonso; AA12	COI
Intermediate entity	6+4	5.22	0.15	4.99	5.51	0.870	0.026	0.832	0.919	2.94%	33	10	54	8	Spain	Lleida, Santa Ana	41.86960, 0.56566	18-05-2016	A. Afonso; AA14	COI
		-	-	-	-	-	-	-	-	-	-	-	36	2						

Appendix 2.3. Genome size and chromosome numbers among *L. suffruticosum s.l.* entities/taxa. Information about the mean, standard deviation of the mean (SD), and minimum and maximum (2C range) values of the holoploid (2C) and monoploid (1C*x*) genome size in picograms (pg) are given. The coefficient variation of the mean holoploid genome size (CV; calculated as the ratio of the standard deviation to the mean) in percentage (%), number of populations analyzed (N pop.), number of individuals with genome size estimates (N G.s.), chromosome numbers (no. chro, individual chromosome counts are separated by a comma; "~" denotes approximate counts due to low availability of material; rare chromosome counts are presented in parenthesis; * Denotes chromosome numbers reported in the literature; na indicates data not available), number of individuals with chromosome counts (N no. chro.), and information about geographic distribution are also given. Different letters correspond to statistically significant differences at $P < 0.05$.

Taxonomic entity and ploidy level	2C Mean	2C SD	2C range	CV (%)	1Cx mean	1Cx SD	1Cx range	N pop.	N G.s.	no. chro.	N no. chro.	N total	Geographic distribution
Linum salsoloides													
2*x*	1.35	0.02	1.33 - 1.39	1.74%	0.674	0.012	0.665 - 0.696	1	6	18*	-	30	Spain - Cuenca
Linum suffruticosum* var. *milletii													
2*x*	1.74	0.03	1.68 - 1.78	1.65%	0.868	0.014	0.838 - 0.890	4	23	18	3	84	Spain – Barcelona, Girona
Linum suffruticosum													
2*x*	1.59	0.09	1.40 - 1.75	5.58%	0.794	0.044	0.700 - 0.877	18	87	16, 18	11, 30	356	Spain - Almería, Granada, Murcia, Ciudad Real, Huesca
3*x*	2.58	-	-	-	0.860	-	-	2	1	27	1	2	Spain - Ciudad Real
4*x*	3.07	0.14	2.78 - 3.43	4.63%	0.769	0.036	0.695 - 0.857	20	89	(32) , 36	6, 36	345	Spain - Cádiz, Málaga, Ciudad Real, Jaén, Córdoba, Granada, Albacete
6*x*	3.87[A]	0.11	3.64 - 4.24	2.84%	0.645	0.018	0.607 - 0.707	17	80	(48) , 54	5, 55	365	Spain - Guadalajara, Madrid, Toledo, Cuenca, Ávila, Valladolid, Zamora
an. 1	5.76	-	-	-	-	-	-	1	1	-	-	1	Spain - Cuenca

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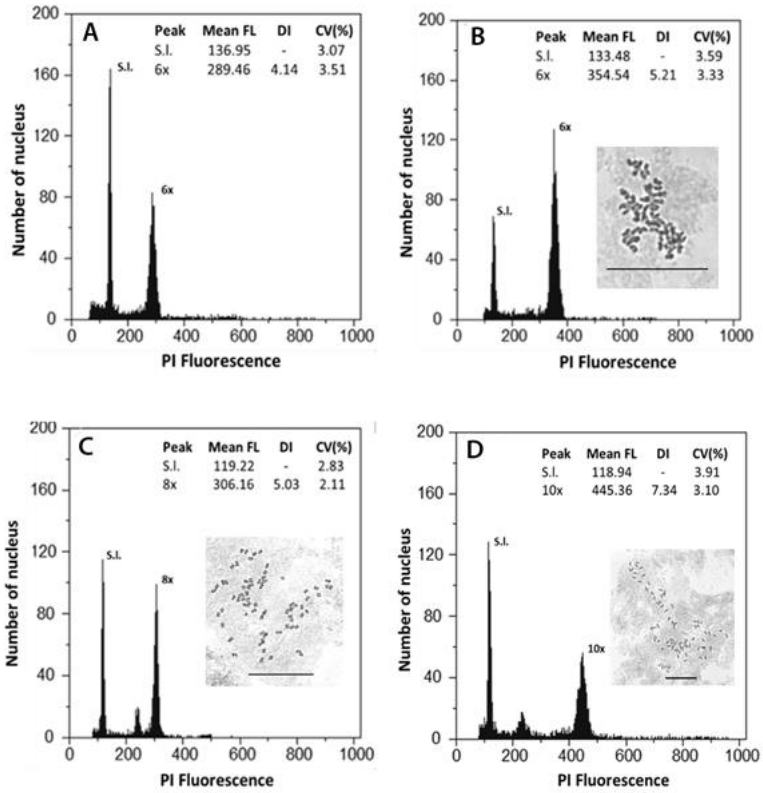
8*x*	5.12[a]	0.18	4.69 - 5.58	3.49%	0.640	0.022	0.586 - 0.697	19	72	72	20	230	Spain - Valencia, Albacete, Teruel, Zaragoza, Cuenca, Guadalajara
10*x*	6.46[Bb]	0.45	5.75 - 7.64	6.94%	0.646	0.045	0.575 - 0.764	12	51	90	20	160	Spain - Barcelona, Tarragona, Valencia, La Rioja, Teruel
an. 2	9.25	-	-	-	-	-	-	1	1	-	-	1	Spain - Teruel
***Linum suffruticosum* (Morocco)**													
2*x*	1.66	0.04	1.60 - 1.73	2.29%	0.828	0.019	0.802 - 0.865	5	15	na	-	86	Morocco
4*x*	3.43	0.11	3.26 - 3.63	3.24%	0.857	0.028	0.815 - 0.908	6	19	na	-	60	Morocco
6*x*	4.83 [C]	0.15	4.67 - 5.03	3.02%	0.805	0.024	0.779 - 0.838	2	6	na	-	40	Morocco
Linum appressum-salsoloides													
2*x*	1.56	0.05	1.41 - 1.68	3.15%	0.782	0.025	0.706 - 0.840	31	118	16, 18	1, 4	588	France; Italy; Spain - Huesca, Lleida , Girona, Burgos, Guadalajara, Cuenca, Teruel
3x	2.59	-	-	-	0.863	-	-	1	1	-	-	1	Spain - Girona
4*x*	2.75	0.07	2.63 - 2.98	2.44%	0.682	0.017	0.658 - 0.745	9	29	36, (38)	16, (3)	89	Spain - Soria, Guadalajara, Cuenca, Teruel
6*x* a	4.09[iB]	0.10	3.91 - 4.30	8.27%	0.706	0.058	0.651 - 0.717	7	34	54	14	105	Spain - Burgos, Soria
6*x* b	5.23[D]	0.15	4.93 - 5.42	2.78%	0.872	0.024	0.821 - 0.904	2	12	54	1	62	Spain - Huesca
8x	5.04[a]	0.22	4.75 - 5.39	4.36%	0.631	0.028	0.593 - 0.673	4	18	72	7	65	Spain - Huesca, Soria, Zaragoza
Intermediate entity													
6*x* a	4.15[B]	0.07	4.08 - 4.28	1.76%	0.691	0.012	0.680 - 0.714	2	6	54	1	29	Spain - Burgos
4*x* b	-	-	-	-	-	-	-	1	-	36	2	2	Spain - Lleida

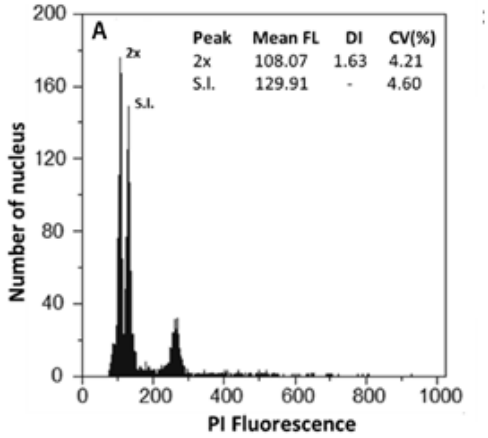
⇩Cont.

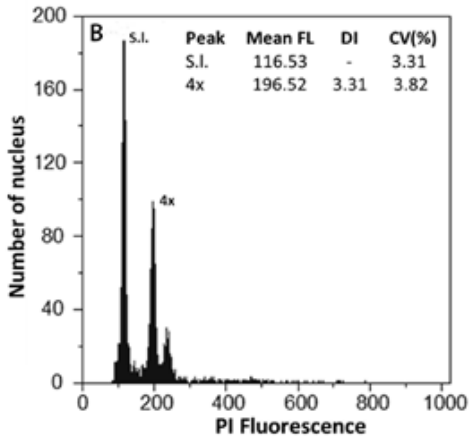
6*x* b	5.22[D]	0.15	4.99 - 5.51	2.94%	0.870	0.026	0.832 - 0.919	1	10	54	8	41	Spain - Lleida
8*x*	5.19[a]	0.28	4.60 - 5.67	5.47%	0.649	0.036	0.575 - 0.709	4	31	72	7	122	Spain - Teruel, Valencia, La Rioja
an. 3	6.31	0.66	5.92 - 7.07	10.43%	-	-	-	1	3	-	-	3	Spain - Teruel
10*x*	7.23[Aa]	0.22	6.98 - 7.76	3.01%	0.723	0.022	0.698 - 0.776	2	15	~90	3	59	Spain – Castellón, Tarragona
an. 4	10.27	-	-	-	-	-	-	1	1	-	-	1	Spain - Castellón, Tarragona

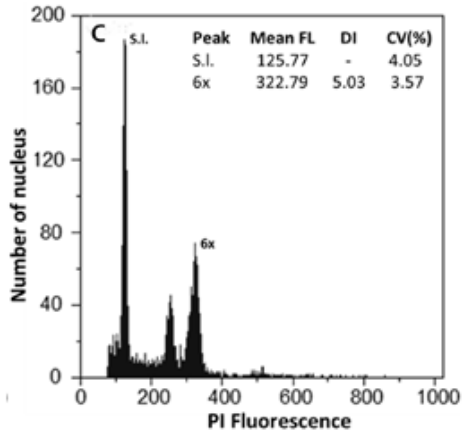
Appendix 2.4. Genome size estimation and chromosome number of the intermediate entity. **A** and **B**, hexaploid; **C**, octoploid; **D**, decaploid populations. Abbreviations:6*x*, hexaploid; 8*x*, octoploid; 10*x*, decaploid; S.l., *Solanum lycopersicum*; Mean FL, mean relative fluorescence in picograms; DI, DNA index; CV(%), coefficient of variation of the peak in %. Scale bar: 20 µm (black line).



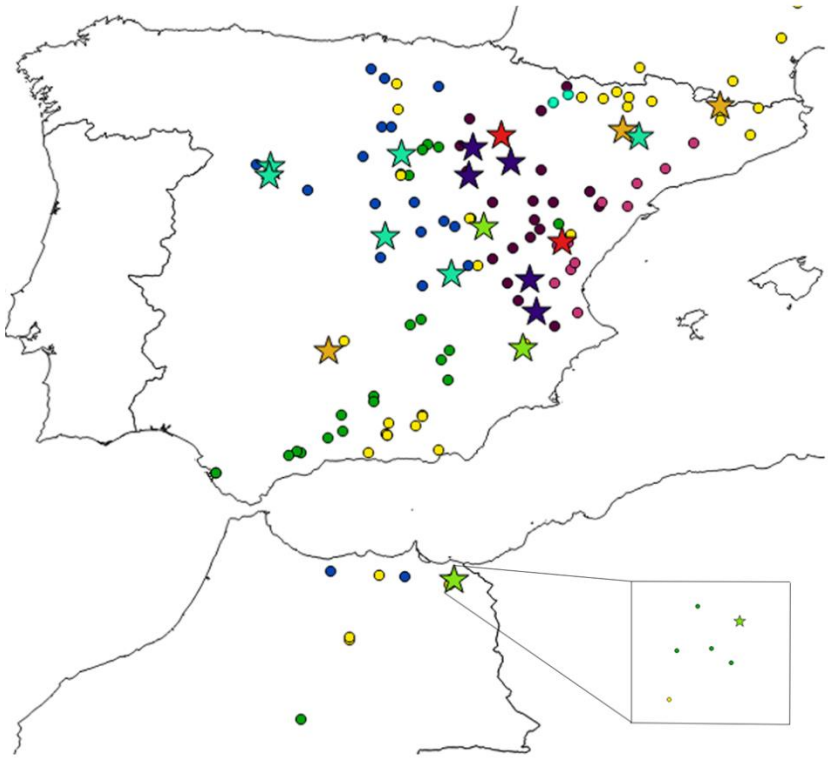
Appendix 2.5. Genome size estimation of *L. suffruticosum* from Morocco. **A**, diploid; **B**, tetraploid; **C**, hexaploid. abbreviations: 2*x*, diploid; 4*x*, tetraploid; 6*x*, hexaploid; S.l., *Solanum lycopersicum*; Mean FL, mean relative fluorescence in picograms; DI, DNA index; CV(%), coefficient of variation of the peak in %.







Appendix 2.6. Pure-ploidy populations sampled in Iberian Peninsula and Morocco with the respective ploidy levels (diploid: yellow; tetraploid: green; hexaploid with low genome size: dark blue; hexaploid with high genome size: light blue; octaploid: red; decaploid: pink) and with mixed-ploidy populations with the respective ploidy levels composition (diploid-triploid: yellow star; diploid-tetraploid: green, hexaploid-tetraploid: blue star, octoploid-hexaploid: purple star, decaploid-octoploid: red star). The base map was downloaded from https://www.diva-gis.org/gdata.



Chapter III - Ecological niche of the polyploid complex *L. suffruticosum s.l.*

Abstract

The high frequency of polyploidy in the evolutionary history of many plant groups occurring in the Mediterranean region is likely a consequence of its dynamic paleogeographic and climatic history. The spatial distribution of cytotypes results from several interacting, often complex, processes occurring in natural populations. Cytotypes frequently have distinct reproductive and competitive characteristics that allow them to overcome the minority cytotype exclusion. Such traits may enable polyploid individuals to grow in habitats different from their parentals and/or expand to new areas, leading to spatial segregation. Therefore, the successful establishment of polyploid lineages has long been associated with niche divergence or niche partitioning and the ability of polyploids to cope with different, often more stressful, conditions. In this study, we aimed to explore the role of environmental variables associated with the current distribution patterns of cytotypes within the polyploid complex *L. suffruticosum s.l.* The distribution and environmental niches of the five main cytotypes of *L. suffruticosum s.l.* (diploids, tetraploids, hexaploids, octoploids and decaploids) were studied across the distribution range. Potential environmental requirements of each cytotype were determined using niche modelling tools, such as maximum entropy modelling and niche equivalency and similarity tests. Differences in the ecological requirements of *L. suffruticosum s.l.* cytotypes were observed, with polyploids being associated with habitats of increased drought, temperature ranges and soil pH, and decreased soil water capacity and cation exchange capacity. However, diploids present the widest environmental niche, and polyploids occupy part of the diploid niche. Although some polyploids have equivalent potential ecological niches, cytotypes do not co-occur in nature. Additionally, the ecological niche of this polyploid complex is different between continents, with North African habitats being characterised by differences in soil texture, higher pH, low cation exchange capacity, precipitation and soil water capacity and higher temperatures than habitats in southwest Europe. The different ecological requirements played a role in the distribution of cytotypes, but the mosaic distribution could not be entirely explained by the environmental variables included in this study. Other factors, such as, reproductive isolation and competitive interactions among cytotypes, could further explain the current diversity and distribution patterns in white flax. This study provides important data on the niche requirements of each cytotype for further competition and reciprocal transplant experiments.

Keywords: ecological niche, *Linum*, Mediterranean region, niche modelling, polyploids.

Introduction

Polyploidization is a widespread mechanism of plant evolution and diversification (Soltis and Soltis 1999; Soltis *et al.* 2010; Castro and Loureiro 2014). Whole genome duplications (WGD) have occurred multiple times during the evolutionary history of angiosperms (Grant 1981; Soltis 2005), with studies suggesting that 47% to 100% underwent a WGD event during its evolutionary history (Grant 1981; Masterson 1994; Cui *et al.* 2006; Soltis *et al.* 2009). Due to its broad-scale consequences on gene expression and developmental processes, WGDs are known to lead to remarkable shifts in genetic, phenotypic and physiological traits that can confer advantages to the newly formed polyploids (Ramsey and Schemske 1998; Levin 2002; Husband *et al.* 2013; Barker *et al.* 2016). The spatial distribution of cytotypes results from several, often complex, interacting processes occurring in natural populations, such as cytotype origin, formation rates, inter-cytotype reproductive interactions, ecological preferences, and competitive and dispersal abilities (Levin 2002). In nature, for a polyploid to establish, it must have distinct reproductive and competitive characteristics that allow the polyploid to overcome the numerical disadvantage within the progenitor's population (minority cytotype exclusion; Levin 1975; Fowler and Levin 1984; Husband 2000; Levin 2002). In many polyploid complexes, such traits have enabled polyploid individuals to grow in habitats different from their parentals and/or expand to new areas, leading to spatial segregation (Balao *et al.* 2009; Kolář *et al.* 2009; Glennon *et al.* 2012). Among the traits that might have played a significant role in spatial segregation is the ability of polyploids to cope with more stressful conditions. For example, the capacity to tolerate low nutrient levels, drought, and cold temperatures has been proposed in several studies (Levin 2002; Maherali *et al.* 2009; Hao *et al.* 2013; Thompson *et al.* 2014). Therefore, the successful establishment of polyploid lineages has long been associated with niche divergence or niche partitioning (Levin 1975; Glennon *et al.* 2014; Thompson *et al.* 2014; Muñoz-Pajares *et al.* 2018).

The Mediterranean Basin is known for its complex geological and palaeoclimatic history. It is an extensive territory around the Mediterranean Sea characterized by a Mediterranean climate, *i.e.*, mild, rainy winters and hot, dry summers (Thompson 2020). The Mediterranean region is considered a biodiversity hotspot, with estimates of polyploidy incidence of 36.5%, with higher values being detected for the Iberian Peninsula (48.8%; Marques *et al.* 2018). The high frequency of polyploids in the evolutionary history of many plants groups from this region is likely a consequence of its dynamic paleogeographic and climatic history (*e.g.*, Late Miocene Salinity Crisis, the spread of Mediterranean-type climate at the Pliocene, Pleistocene Ice Ages) (Thompson 2020), of a high percentage of species with narrow distribution ranges (Thompson

2020), of ecogeographical heterogeneity and human influence (Blondel *et al.* 2010). In the Iberian Peninsula, the determinant factors of evolution of plant lineages and polyploid complexes include the existence of mountain ranges that promoted multiple refugia and produced allopatric and parapatric clades and the recurrent connections and disconnections with Northern Africa (Hewitt 2011; Nieto-Feliner 2014; Thompson 2020).

The development of niche modelling tools such as ecological niche modelling (ENM; Warren *et al.* 2008) and multivariate analyses of niche variables (Broennimann *et al.* 2012), enables to explore environmental preferences of different cytotypes and to study patterns of spatial segregation. These tools are based on a quantitative assessment of ecological divergence related to geographic distribution and statistical comparison of the overlap of the niche occupied by different cytogenetic entities. Therefore it allows building hypotheses for the mechanisms involved in cytotype establishment and subsequent spread (Warren *et al.* 2008; Broennimann *et al.* 2012). Many studies have characterized the abiotic factors of polyploidy populations and evaluated cytotype environmental preferences, predicting the possible existence of niche shifts (Thompson *et al.* 2014; Visger *et al.* 2016; Muñoz-Pajares *et al.* 2018; López-Jurado *et al.* 2019; Castro *et al.* 2020) or niche conservatism (McIntyre 2012; Laport *et al.* 2013; Glennon *et al.* 2014; Castro *et al.* 2019), among them and their progenitors. For example, in *Chamerion angustifolium,* it was shown that tetraploids occupied warmer and drier habitats than diploid progenitors (Thompson *et al.* 2014), while in *Erysimum mediohispanicum* tetraploids grow in habitats with higher levels of precipitation than diploids (Muñoz-Pajares *et al.* 2018). However, other studies showed no niche differentiation, suggesting that other factors such as competitive and dispersal abilities and intercytotype breeding barriers were involved in the success of polyploids (Levin 2002; Godsoe *et al.* 2013; Laport *et al.* 2013; Kolář *et al.* 2017; Castro *et al.* 2018; Morgan *et al.* 2020).

Linum suffruticosum s.l. is a polyploid complex distributed through the western Mediterranean basin (Rogers 1979; Nicholls 1985b; c, 1986; McDill *et al.* 2009). Recent detailed studies have shown that *L. suffruticosum s.l.* harbors a high cytogenetic diversity, with five major cytotypes (namely diploids (2*x*), tetraploids (4*x*), hexaploids (6*x*), octoploids (8*x*) and decaploids (10*x*)) being detected in nature (Afonso *et al* 2021 in Chapter II). The different ploidy levels are distributed parapatrically, geographically structured, and comprise several contact zones with very few mixed-ploidy populations (15.0%, 23 out of 151 populations), usually with one of the cytotypes in lower frequency in the populations than the other(s). Most of the cytogenetic diversity was found in the Iberian Peninsula, with the remaining areas of the species distribution in Europe being characterized by homogeneously diploid populations, only (Afonso *et al.* 2021 in Chapter II). Thus, this study system has a high cytogenetic diversity with a complex mosaic

distribution distributed along the Mediterranean basin. Considering all this, *L. suffruticosum s.l.* constitutes an ideal system to explore the role of niche divergence to explain current distribution patterns of different cytotypes.

Therefore, the main objective of this study was to explore the role of environmental variables in the current distribution patterns of cytotypes within the polyploid complex *L. suffruticosum s.l.*. Given the group's cytogenetic variability and wide distribution range and the potential impact of WGDs in physiological traits and environmental tolerances, we hypothesized that polyploidization may have led to shifts in environmental preferences. Thus, diploids and polyploids are expected to colonize different environmental niches, resulting in low geographic overlap. By coupling information about cytotype diversity, geographical patterns, and environmental preferences, this study will formulate ecologically-driven hypotheses that might help explain the establishment and spread of *L. suffruticosum s.l.* cytotypes and polyploid lineages in general.

Materials and methods

Study system and occurrence data

Linum suffruticosum s.l. occurs in limestone soils or limestone derivatives, from the Mediterranean to Atlantic climate areas in the mountains to lowlands and dry regions. The geographic distribution comprises the western Mediterranean basin, from the Iberian Peninsula to the North of Italy, and the North of Africa, where it is less abundant (A. Afonso, field observations). The group is composed of obligate outcrossers due to a heteromorphic self-incompatibility system associated with a floral dimorphism (Rogers 1979; Chapter IV). *Linum suffruticosum s.l.* is a diploid-polyploid complex with high cytogenetic and morphological variability (*e.g.*, Rogers *et al.* 1972; Elena Roselló *et al.* 1985; Nicholls 1986a; Afonso *et al.* 2021 in Chapter II). Furthermore, this group revealed a complex taxonomy due to its high morphological variability and lack of reliable diagnostic characters. Here, all the entities for the cytotypes of *L. suffruticosum s.l.* considered by Afonso et al., 2021 (in Chapter II - *L. salsoloides* Lam.*, L. suffruticosum* var. *milletii* (Sennen & Gonzalo) G.López*, L. suffruticosum, L. appressum-salsoloides* and the *intermediate taxon*) were studied. The chromosome base number can be n = *x* = 6 or n = *x* = 8, with the latter being the most common. Diploid populations cover a larger area, being detected throughout all sampled distribution areas, and it is the only cytotype found north and northeast of the Pyrenees. Most of the cytogenetic diversity is found in the Iberian Peninsula and North Africa, with tetraploids, hexaploids, octoploids, and decaploids having been detected (Afonso *et al.* 2021 in Chapter II). For this study, occurrence records of *L. suffruticosum s.l.* populations and its ploidy level were mostly based in Afonso *et al. (*2021). Several new

populations were sampled, with field sampling and ploidy level of the sampled individuals being estimated (when possible) using flow cytometry following Afonso *et al.* (2021), in Chapter II. Further occurrences for North Africa were obtained from the GBIF database (http://gbif.org). In total, 13 occurrences from GBIF and 323 natural, field collected, populations – 137 diploid, 26 tetraploid, 24 hexaploid, 25 octoploid and 14 decaploid populations, 2 diploid-tetraploid mixed-ploidy populations and 1 tetraploid-hexaploid population – were selected, covering most of the distribution area of *L. suffruticosum s.l.* (Appendix 3.1, Figure 3.1A and Afonso *et al.* 2021 in Chapter II).

Environmental data

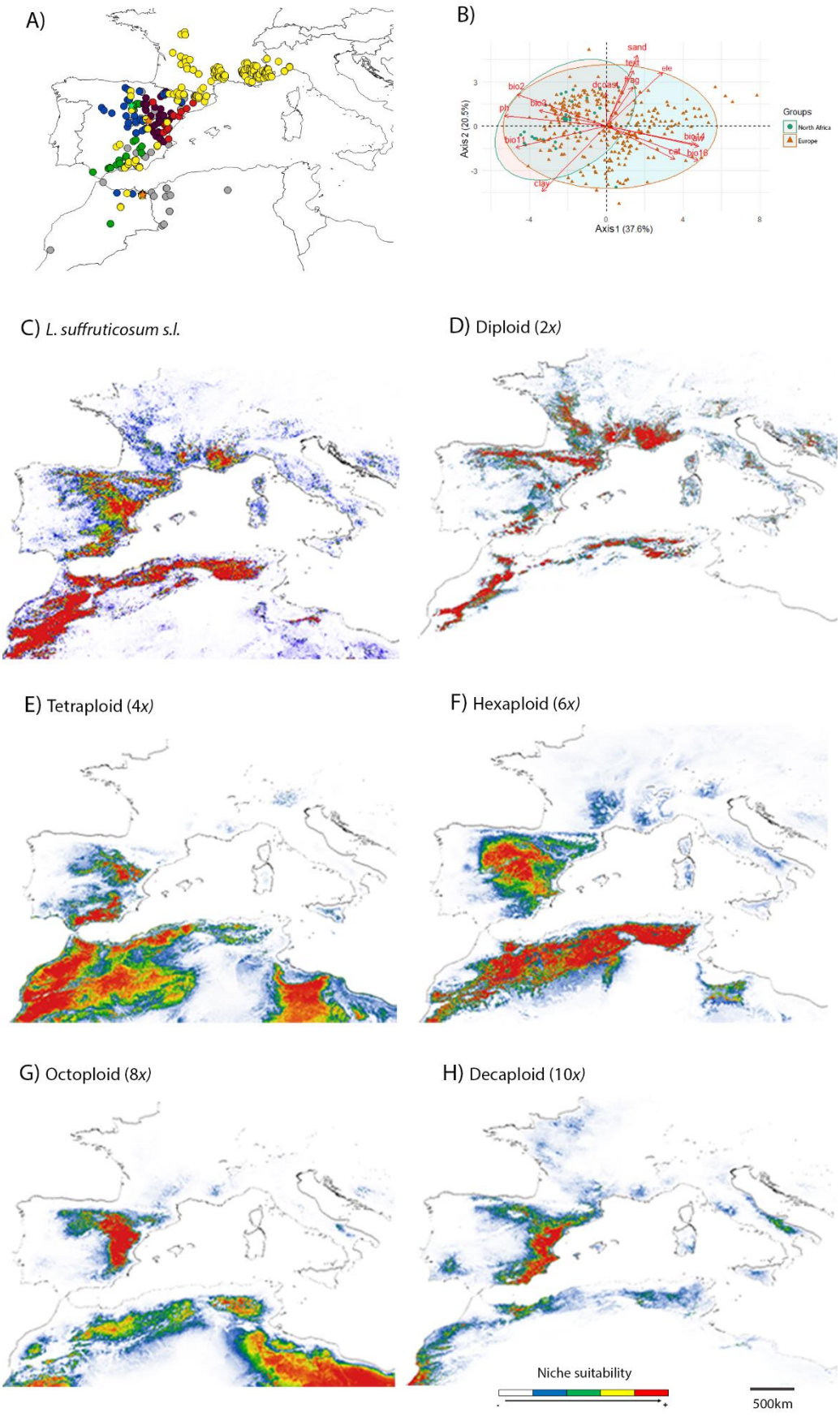
In this study a Grinnellian niche concept was used, with only environmental, abiotic variables considered to define each cytotype niche. Given that it was not analyzed the whole spectrum of environmental and community conditions where the cytotypes might spread, it was assumed a realized niche concept for the cytotypes, which resulted from interactions with other species and cytotypes (Soberón 2007).

To explore the environmental niches of *L. suffruticosum s.l.* cytotypes, 20 bioclimatic variables from the WorldClim database (www.worldclim.org), and 19 topographic and soil conditions variables at two different depths (15 and 30 cm) from the World Soil Information (www.isric.or) were extracted at a resolution of 30 arc-seconds (approx. 1 km) for most of the distribution area of *L. suffruticosum s.l.* (27.0 ° to 51.0 N latitude, -13.0 ° to 18.0 ° longitude). To evaluate the contribution of each variable for the total reported variance, exploratory principal component analyses (PCA) were done, and correlations between the variables were obtained using Pearson or Spearman coefficients (for variables with continuous measurements or with ordinal scale, respectively). Only one variable was selected for pairs of variables with correlation values higher than 0.7. Therefore, the following noncorrelated variables were used in niche modelling analyses: mean diurnal range (bio2); isothermality (bio3), mean temperature of the coldest quarter (bio11), precipitation of the driest month (bio14), precipitation of the wettest quarter (bio16), elevation (ele), distance to the coast (dcoast); furthermore, seven soil variables at two standard depths predicted using the global compilation of soil ground observations (accuracy assessment of the maps is available in Hengl *et al.* 2017) were used: soil water capacity at 15 cm in volumetric fraction (aw), clay content at 15 cm in mass fraction (clay), cation exchange capacity at 15 cm (cat), fragment content at 15 cm in volumetric fraction (frag), sand content at 15 cm in mass fraction (sand), soil pH at 30 cm (ph), and soil texture at 15 cm (text-texture class in USDA system - www.nrcs.usda.gov). Values for climatic and soil variables were

extracted for all the *L. suffruticosum s.l.* populations using the R package 'dismo' (Hijmans *et al.* 2017). To explore bioclimatic and soil variables and assess differences between continents (Europe vs North Africa), generalized linear models (GLMs) were used with continent as a fixed factor and each variable as a response variable. Furthermore, to assess differences among cytotype's environmental variables, generalized linear models (GLMs) were used with cytotype as fixed factor, and each variable as a response variable. A Gaussian distribution with an identity link function was used for continuous variables and a Poisson distribution with a log link function was used for discrete variables. Soil water capacity, clay content, fragment content and sand content are proportions and, thus, were transformed with the arcsine of the square root. Statistical analyses were performed in R software v.3.6.1 (R Core Development Team 2019), using the packages *car* for Type-III analysis of variance (Fox *et al.* 2005), *glm* for generalized linear models (Hastie and Pregibon 1992) and *multcomp* for multiple comparisons after Type- III analysis of variance (Hothorn *et al.* 2017).

Ecological niche modelling

Niche modelling tools were used to explore the ecological requirements of 1) *L. suffruticosum s.l.*, and 2) each of the five cytotypes of *L. suffruticosum s.l.*. Niche modelling analyses were performed with maximum entropy modelling (MaxEnt; package "biomod2"; (Thuiller *et al.* 2016). In both approaches, spatial predictive models were calibrated based on the selected variables and presence/absence data using European reports, as reports from North Africa were scarce for such a vast and heterogeneous area. Field and GBIF records were used to build the presence dataset. Duplicate occurrences were removed, and locally dense sampling was reduced by thinning the records to one per grid cell size. To obtain pseudo-absences (background points), we applied a buffer of 10 km around each reported population from the presence dataset, and 5000 points were randomly selected beyond this buffer; additionally, a filter of 1 km was used to remove pseudo-absences that were separated by less than this distance to avoid oversampling. In the first approach, all European populations were used as presences and background points as absences. In contrast, in the second approach, populations of a given cytotype were recorded as presences and the populations of the other cytotypes and the background points were recorded as absences. Finally, mixed-ploidy populations were considered as presences for both cytotypes. Models were replicated 30 times after splitting data in training (70%) and testing (30%) subsets (Phillips *et al.* 2006; Araújo and New 2007). To guarantee statistical independence of all the replicates, each occurrence was used only once in each run, either as training or as a test occurrence (Phillips 2008). Models were


A)
B)
sand
text
ele
bio2
ph
bio3
bio11
bio14
cal
bio18
clay
Axis2 (20.5%)
Axis1 (37.6%)
Groups
North Africa
Europe
C) L. suffruticosum s.l.
D) Diploid (2x)
E) Tetraploid (4x)
F) Hexaploid (6x)
G) Octoploid (8x)
H) Decaploid (10x)
Niche suitability
500km

⇩Cont.

Figure 3.1. Distribution of *L. suffruticosum s.l.* cytotypes: diploids, yellow circles; tetraploids, green circles; hexaploids, blue circles; octoploids, purple circles; decaploids, red circles; diploid-tetraploid mixed-ploidy population, orange star; hexaploid-tetraploid mixed-ploidy population, green star; without cytotype information, grey circles (A); PCA of all variables (Precipitation of Driest Month, bio14; Precipitation of Wettest Quarter, bio16; Mean Temperature of Coldest Quarter, bio11; Mean Diurnal Range, bio2; Isothermality, bio3; elevation, ele; Distance to the coast, dcoast; soil water capacity at 15 cm, aw; Fragment content at 15 cm, frag; Clay content at 15 cm, clay; Soil pH at 30 cm, ph; Sand content at 15 cm, sand; Cation exchange capacity at 15 cm, cat; Soil texture at 15 cm, text) for Europe, and North Africa (B); and habitat suitability for *L. suffruticosum s.l.* (C), and for diploids (D); tetraploids (E); hexaploids (F); octoploids (G) and decaploids (H) separately.

evaluated based on the independent accuracy measure of receiver operating characteristic (ROC). Only models with ROC > 0.7 were considered for the final model. The evaluation of each model revealed high ROC values (mean ± SE; *L. suffruticosum s.l.*: 0.94 ± 0.01; 2*x*: 0.93 ± 0.01; 4*x*: 0.97 ± 0.04; 6*x*: 0.97 ± 0.02; 8*x*: 0.98 ± 0.02; 10*x*: 0.98 ± 0.02) and relatively low omission rates (mean ± SE; *L. suffruticosum s.l.*: 0.10 ± 0.04; 2*x*: 0.13 ± 0.05 and 4*x*: 0.04 ± 0.06; 6*x*: 0.01 ± 0.01; 8*x*: 0.01 ± 0.03; 10*x*: 0.00 ± 0.01), indicating that the models could predict occurrences with high accuracy. In both approaches, and assuming that the environmental requirements of the species are similar over the Mediterranean basin, we used the final model and the 14 selected variables to project suitable areas of *L. suffruticosum s.l.* for North of Africa and predict the total suitable habitat of the species and of each cytotype in that region. In the second approach, the final model of each cytotype was converted into a binary format (using the default threshold of 0.5), to calculate the suitable habitat of each cytotype and assess niche overlap (package "biomod2"; Thuiller *et al.* 2016).

Niche comparisons: equivalence and similarity tests

Niche equivalency and similarity tests (Warren *et al.* 2008; Broennimann *et al.* 2012), using the Schoener's *D* metric (Schoener 1970) were applied to quantify niche overlap in the geographic distribution of cytotypes of *L. suffruticosum s.l.* in Europe. This metric ranges from 0 (no overlap) to 1 (complete overlap). The analyses were run with "ecospat" (Broennimann *et al.* 2012) and "raster" (Hijmans *et al.* 2017) packages using the binary projections. The "ecospat" R package was used to compare cytotype niches with an ordination approach using a PCA calibrated with environmental values (Di Cola *et al.* 2017). The PCA calculates the occurrence density and environmental factor density along environmental (principal component) axes for each pixel, maximizing the ecological variance of the areas of the cytotypes. Then, PCA scores of the two cytotype distributions being compared were projected onto a grid of cells bounded by

the maximum and minimum PCA scores, which allowed the visual assessment of the overlap and dynamics of the environmental niches of cytotypes. Both niche equivalency and similarity tests were computed for each pair of cytotypes to test whether predicted distributions were significantly different between cytotypes (classification by Warren *et al.* 2008; Smith and Donoghue 2010; Broennimann *et al.* 2012). The niche identity test determines if the distribution models produced for the two cytotypes being compared differ in their environmental attributes by pooling records of two different cytotypes and by randomly sampling from the pooled 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 evaluating the null hypothesis. For this, the simulated *D* values were compared with the observed *D* value, and cytotype's niches were considered equivalent if the observed *D* value fell within the 95th percentile of the simulated *D* value (Broennimann *et al.* 2012). The niche similarity test determines whether the environmental niche of two different cytotypes are distinguishable 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

Ecological requirements of *L. suffruticosum s.l.*

L. suffruticosum s.l. is found in habitats with highly variable ecological attributes (Table 3.1). It is located in a high range of elevation (46-2599 m, Table 3.1), precipitation ranges (bio 14 [1-69 mm] and bio16 [73-492 mm], Table 3.1) as well as close (2.09 m, Table 3.1) and distant to the coast (379.36 km, Table 3.1). For temperature variables, the range of values for isothermality (26-44) are more variable than the mean diurnal range (0.6-1.4 ºC) and the mean temperature of the coldest quarter (-0.5-1.2 ºC, Table 3.1). Regarding soil attributes such as fragment, sand and clay content, soil pH, soil water capacity, soil texture and cation exchange capacity, this complex can be found in habitats with variable ranges of these attributes (Table 3.1). Concerning the comparison between European and North African habitats of *L. suffruticosum s.l.*, some differences in ecological attributes related with soil properties and climatic differences were found. In North Africa, *L. suffruticosum s.l.* populations are found, on average, at a significantly higher elevation than in Europe, while in Europe, the plant occurs in a broader range of elevation (Table 3.1). Statistically significant differences were found in precipitation and minimum temperatures, with precipitation being scarcer in North Africa and

with temperatures not reaching values as low as in Europe (Table 3.1). Significant differences in soil properties were also found; in North Africa, the soil pH is significantly more basic, and the soil water capacity is significantly lower than in Europe, while sand content values are significantly higher (Table 3.1). Significantly higher values of soil texture were also found in Europe compared to North Africa (Table 3.1). No statistically significant differences were observed for the remaining variables (Table 3.1).

The two first PCA axes explained 58.1% (axis 1: 37.6%, axis 2: 20.5%) of the environmental variance in European and North Africa distribution. They revealed that the environmental values of North Africa were overlapped within European environmental values, being the ecological attributes of European populations broader than those of North Africa. The latter overlapped only partially with the cluster of European populations, being skewed along axis 1 due to higher values of pH, mean temperature of the coldest quarter (bio11) and mean diurnal range (bio2), clay content (clay), and lower levels of precipitation of the driest month (bio14), precipitation of wettest quarter (bio16), sand content (sand), soil texture (text), cation exchange capacity (cat) and soil water capacity (aw) (Figure 3.1B).

Ecological attributes of *L. suffruticosum s.l.* cytotypes

When comparing environmental variables among cytotypes, significant differences were observed for all variables, except elevation, soil texture and clay and sand content (Table 3.2 and Appendix 3.2). A gradient was observed for several variables with increased ploidy associated with increasing mean diurnal temperature range, isothermality, mean temperature of coldest quarter, and soil pH, and decreasing precipitation of wettest quarter soil water capacity, and cation exchange capacity (Table 3.2 and Appendix 3.2). Diploid individuals in Europe grow in habitats with significantly higher precipitation levels than the other cytotypes and soils with the highest water retention and cation exchange values and the lowest pH levels (Table 3.2 and Appendix 3.2). Diploids also grow in areas with significantly lower mean of temperature in the coldest quarter, low-temperature diurnal range and low isothermality than polyploids Table 3.2 and Appendix 3.2). Environmental variables for polyploids largely overlap, although some trends are observed. Tetraploids tend to occur in habitats with higher values of precipitation in the wettest quarter, higher levels of cation exchange capacity and lower values of precipitation in the driest month than higher ploidy levels (Table 3.2 and Appendix 3.2). The

Table 3.1. Mean and standard error of the mean (mean ± SE) and minimum and maximum (min-max) values of selected variables used to characterize the niche of *L. suffruticosum s.l.* in Europe and North Africa. Number of populations (N), and F value and significance levels (n.s., $P > 0.05$, * $P < 0.05$, ** $P < 0.01$,*** $P < 0.001$), for the comparison between continents, are also provided.

Variables	CODE	*L. suffruticosum s.l.*		Europe		North Africa		$F_{1,226}$ value
		mean ± SE, N = 336	min-max	mean ± SE, N = 306	min-max	mean ± SE, N = 30	min-max	
Elevation (metres)	ele	896.54 ± 23.17	46.00-2599.00	880.45 ± 23.66	46.00-2599.00	1060.67 ± 91.63	269.00-2149.00	4.67*
Distance to the coast (km)	dcoast	131.33 ± 4.83	2.09-379.36	131.63 ± 4.93	2.09-379.36	128.30 ± 20.47	3.60-366.73	0.15 n.s
Mean Diurnal Range (°C)	bio2	1.07 ± 0.01	0.60-1.40	1.06 ± 0.01	0.60-1.40	1.11 ± 0.03	0.60-1.30	1.49 n.s
Isothermality (* 100)	bio3	38.28 ± 0.15	26.00-44.00	38.28 ± 0.14	28.00-44.00	38.30 ± 0.09	26.00-44.00	0.07 n.s
Mean Temperature of Coldest Quarter (°C)	bio11	0.45 ± 0.01	-0.50-1.20	0.42 ± 0.01	-0.50-1.20	0.74 ± 0.05	0.20-1.20	54.07***
Precipitation of Driest Month (mm)	bio14	25.65 ± 0.96	1.00-69.00	27.78 ± 0.97	1.00-69.00	3.97 ± 0.42	1.00-9.00	60.83***
Precipitation of Wettest Quarter (mm)	bio16	219.33 ± 4.12	73.00-492.00	227.92 ± 4.07	73.00-492.00	131.73 ± 11.24	73.00-392.00	52.95***
Soil water capacity (v%)	aw	13.80 ± 0.10	10.00-18.00	13.98 ± 0.10	10.00-18.00	11.93 ± 0.21	10.00-14.00	38.13***
Cation exchange capacity (cmolc/kg)	cat	19.52 ± 0.16	13.00-30.00	19.62 ± 0.17	13.00-30.00	18.47 ± 0.55	14.00-24.00	3.67 n.s
Soil pH (pH)	ph	7.02 ± 0.03	5.50-8.10	6.96 ± 0.03	5.50-8.10	7.61 ± 0.08	6.10-8.10	33.73***
Clay content (w%)	clay	24.51 ± 0.19	13.00-34.00	24.40 ± 0.20	13.00-34.00	25.57 ± 0.43	21.00-29.00	3.27 n.s
Fragment content (v%)	frag	18.25 ± 0.22	8.00-28.00	18.18 ± 0.23	8.00-28.00	18.90 ± 0.73	8.00-24.00	0.79 n.s
Sand content (w%)	sand	39.04 ± 0.3	23.00-59.00	38.72 ± 306.01	23.00-59.00	42.33 ± 0.66	36.00-51.00	12.30***
Soil texture (USDA system)	text	6.46 ± 0.06	4.00-9.00	6.50 ± 0.07	4.00-9.00	6.03 ± 0.25	4.00-7.00	3.95*

Table 3.2. Mean and standard error of the mean (SE) of selected variables used to characterize the niche of *L. suffruticosum s.l.* cytotypes. Number of populations (N), and F value and significance levels (n.s., $P > 0.05$, * $P < 0.05$, ** $P < 0.01$,*** $P < 0.001$), for the comparison among cytotypes, are also provided.

Variables	Code	Diploids	Tetraploids	Hexaploids	Octoploids	Decaploids	$F_{4, 214}$
		mean ± SE, N = 139	mean ± SE, N = 29	mean ± SE, N = 25	mean ± SE, N = 25	mean ± SE, N = 14	value
Elevation (metres)	**ele**	866.89 ± 41.22	962.07 ± 66.88	796.64 ± 34.9	880.68 ± 54.44	601.00 ± 72.97	$1.94^{n.s.}$
Distance to the coast (km)	**dcoast**	111.16 ± 5.35^{ad}	146.74 ± 18.71^{ac}	229.69 ± 18.86^{b}	157.83 ± 13.21^{cd}	73.96 ± 17.74^{d}	16.45***
Mean Diurnal Range (°C)	**bio2**	0.99 ± 0.01^{a}	1.17 ± 0.02^{bc}	1.14 ± 0.02^{b}	1.24 ± 0.02^{c}	1.20 ± 0.03^{bc}	45.11***
Isothermality (* 100)	**bio3**	37.31 ± 0.19^{a}	40.21 ± 0.34^{bc}	39.2 ± 0.29^{b}	40.96 ± 0.32^{c}	41.14 ± 0.52^{c}	32.60***
Mean Temperature of Coldest Quarter (°C)	**bio11**	0.36 ± 0.02^{a}	0.6 ± 0.05^{b}	0.53 ± 0.02^{b}	0.48 ± 0.03^{ab}	0.64 ± 0.06^{b}	11.02***
Precipitation of Driest Month (mm)	**bio14**	35.88 ± 1.39^{a}	9.48 ± 1.67^{b}	17 ± 2.21^{bc}	21.48 ± 1.36^{c}	19.43 ± 1.66^{bc}	30.65***
Precipitation of Wettest Quarter (mm)	**bio16**	264.83 ± 5.22^{a}	183.34 ± 11.68^{b}	163.52 ± 7.98^{b}	155.52 ± 5.76^{b}	157.07 ± 6.08^{b}	44.80***
Soil water capacity (v%)	**aw**	14.85 ± 0.14^{a}	12.07 ± 0.19^{b}	12.96 ± 0.27^{b}	12.84 ± 0.19^{b}	12.57 ± 0.25^{b}	34.98***
Cation exchange capacity (cmolc/kg)	**cat**	20.8 ± 0.25^{a}	19.48 ± 0.48^{a}	16.84 ± 0.47^{b}	17.44 ± 0.50^{b}	17.93 ± 0.59^{b}	18.11***
Soil pH (pH)	**ph**	6.72 ± 0.04^{a}	7.259 ± 0.09^{b}	7.388 ± 0.13^{b}	7.56 ± 0.10^{b}	7.44 ± 0.07^{b}	25.75***
Clay content (w%)	**clay**	24.53 ± 0.33	25.59 ± 0.61	24.2 ± 0.69	24.20 ± 0.45	25.93 ± 0.47	$1.21^{n.s.}$
Fragment content (v%)	**frag**	17.99 ± 0.36^{ab}	18.59 ± 0.67^{b}	15.64 ± 0.55^{a}	17.36 ± 0.66^{b}	18.57 ± 0.89^{ab}	2.47*
Sand content (w%)	**sand**	37.94 ± 0.46	40.41 ± 0.81	40.76 ± 1.48	39.24 ± 0.85	36.64 ± 1.14	$2.89^{n.s.}$
Soil texture (USDA system)	**text**	6.36 ± 0.11	6.17 ± 0.25	6.4 ± 0.26	7.00 ± 0.00	6.14 ± 0.38	$1.91^{n.s.}$

ecological requirements of the niche of hexaploids have similarities to all cytotypes, presenting high geographical segregation with the remaining cytotypes. Moreover, these populations had the highest distance to the coast (Figue 3.1A; Table 3.2 and Appendix 3.2). Octoploids and decaploids occurred in a wide range of elevations. Its habitats were characterized by the highest values of isothermality and the lowest levels of precipitation of the wettest quarter. The distance to the coast of decaploids populations was the lowest among cytotypes. In addition, the soil texture (text) of octoploid populations was the highest of all cytotypes (Table 3.2 and Appendix 3.2).

Ecological niche modelling

The predicted ecological niche of *L. suffruticosum s.l.* confirmed the distribution patterns in Europe, and the variables with the highest contribution for the model were isothermality, elevation and soil pH (Appendix 3.3; Figure 3.1A and C). However, despite the predicted area overlap with the observed distribution, the predicted suitable area is larger than the area where the plant was found, particularly in North Africa (Figure 3.1A and C).

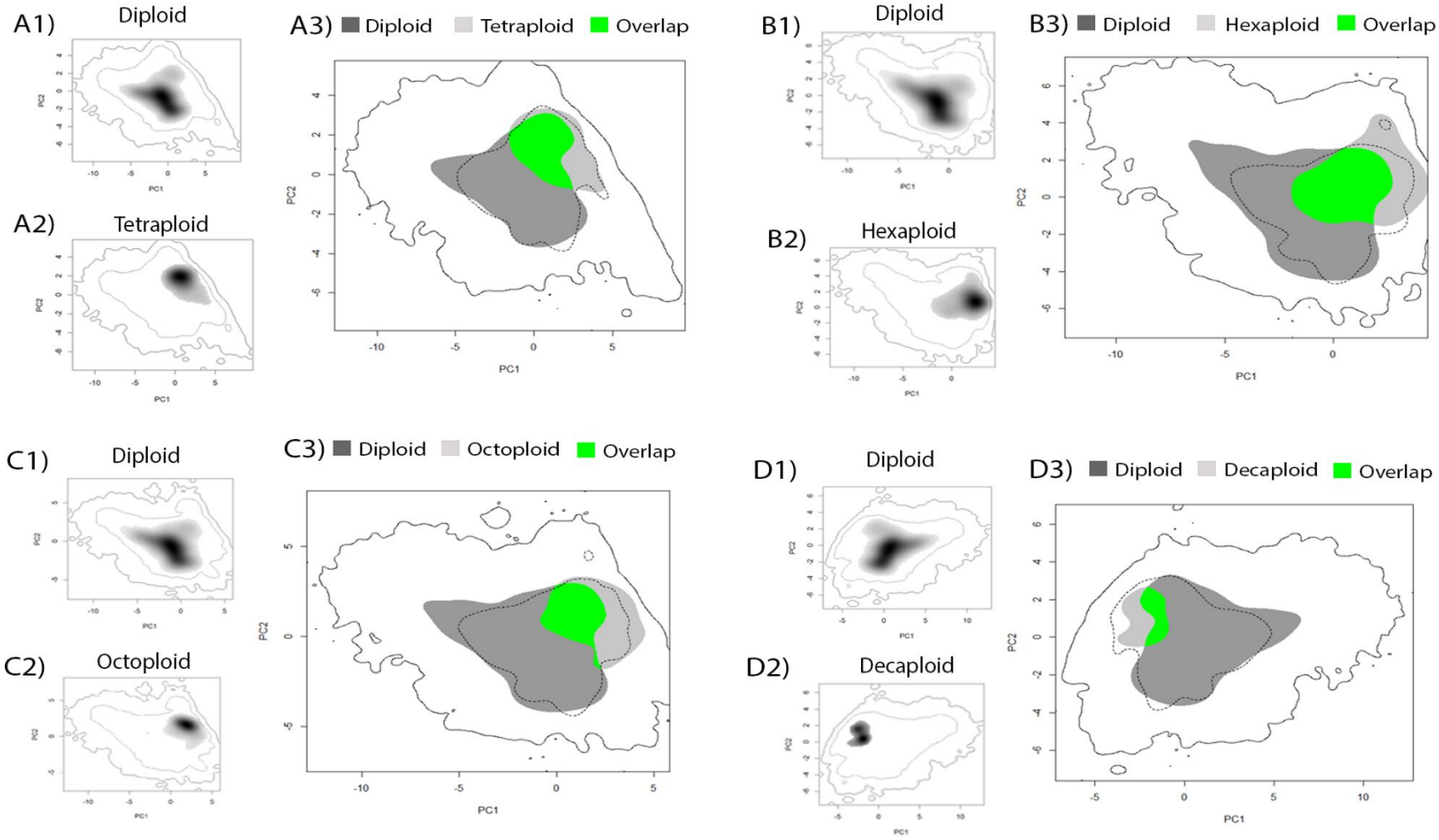
Overall, each cytotype's predicted distribution of niches confirmed the parapatric distribution of *L. suffruticosum s.l.* cytotypes (Figure 3.1A, D-H). According to the models, diploids could be found in the Iberian Peninsula, but mainly in the south of France, where diploid populations occupy the widest continuous area (Figure 3.1A and D). In North Africa and the Iberian Peninsula, diploids have a high probability of occurrence in mountainous regions (Figure 3.1D). The areas with the highest suitability of occurrence, were also those where most diploid populations were found (Figure 3.1A and D). The variables that mainly explained the predicted distribution of diploids were isothermality, precipitation in the wettest quarter, and soil pH (Appendix 3.3). The high contribution of soil pH is in line with the significantly lower soil pH observed in natural populations (Figure 3.1D; Table 3.2 and Appendix 3.2).

For polyploids, the predicted niche confirmed the distribution found in the Iberian Peninsula and North Africa but suggested a high potential of expansion beyond their current range, as the areas with a high probability of occurrence were more extensive than the observed distributions (Figure 3.1A, E-H). Furthermore, the predicted niches suggested the occurrence of suitable niches for octoploids and, to a less extent, decaploids in North Africa (Figure 3.1A, G-H). In tetraploids, the model was primarily explained by elevation and precipitation in the driest month, and in hexaploids by elevation, precipitation in the wettest quarter and isothermality (Appendix 3.3). Octoploids were distributed primarily in dry inland regions. Still, the predicted niche showed a larger area with a high probability of occurrence that expands to the coast and

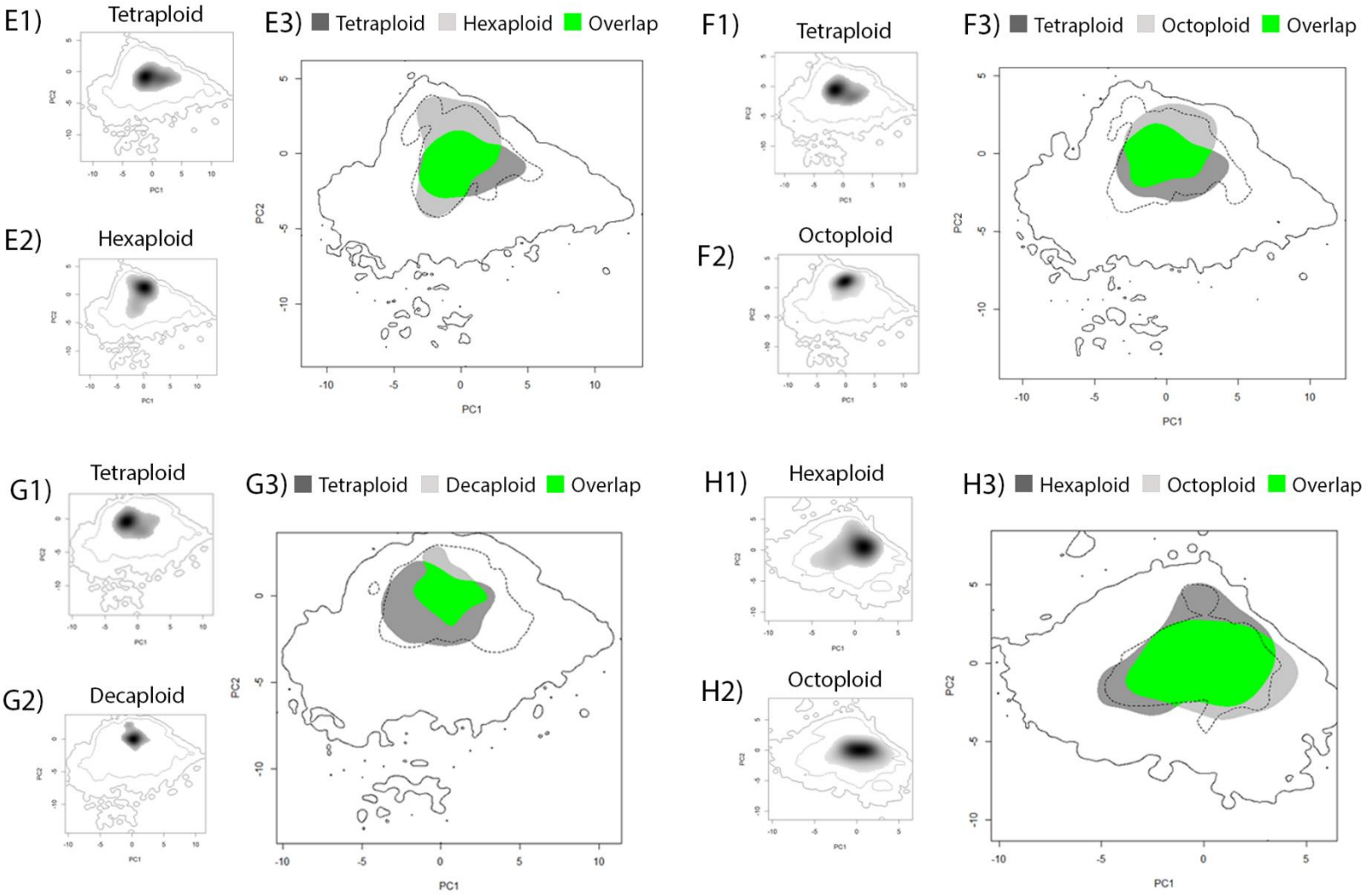
the north of the Iberian Peninsula. Also, in North Africa, where no octoploids populations were found, high suitability was detected in inner lands, especially in the eastern areas (Figure 3.1A and G). For decaploids, the model predicted suitable niches along the coast, which agrees with the distribution found in nature in the Iberian Peninsula. In North Africa, despite no decaploid populations being found, a few areas with high habitat suitability were also found along the coast (Figure 3.1A and H). In octoploid and decaploid cytotypes, habitats were strongly influenced by temperature and water availability since variables related to precipitation and isothermality presented a high contribution to the model's prediction. Fragment content was also a relevant factor in predicting the niche of decaploids (Appendix 3.3).

Niche comparisons: equivalence and similarity tests

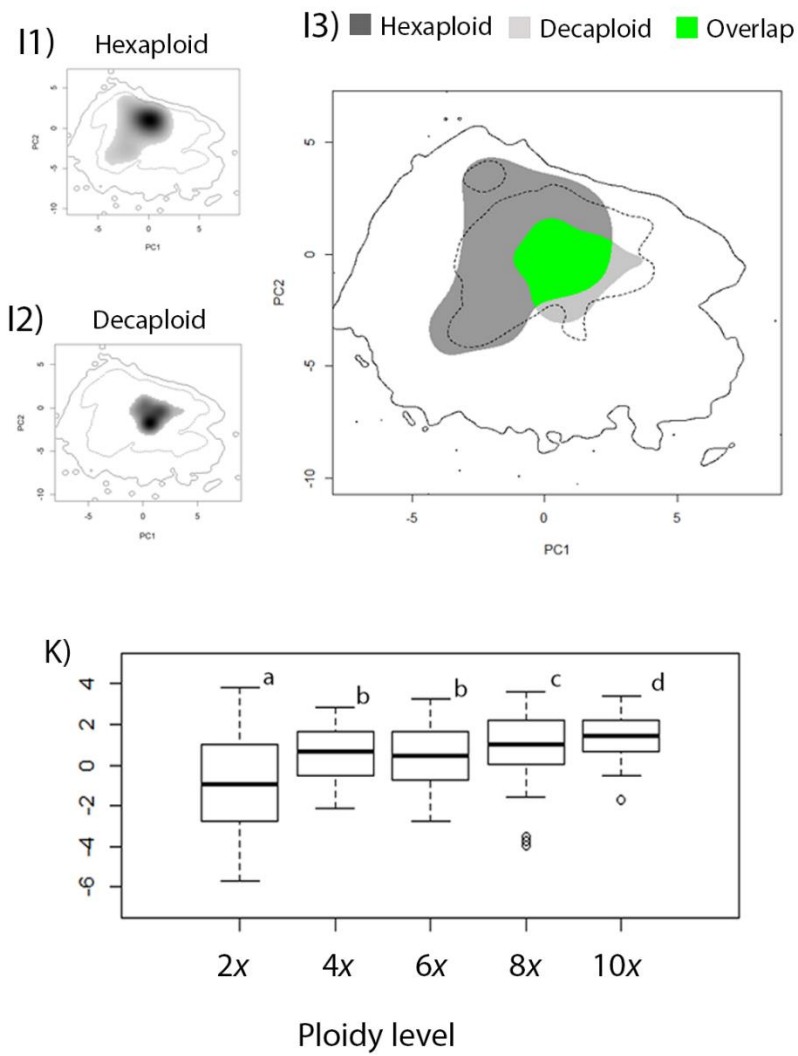
The amplitude of the niche of diploids was larger than that of the higher-ploidy cytotypes and presented low environmental niche overlap (diploid niche overlaps with other cytotype niches; 4*x*: 28.1%; 6*x*: 32.3%, 8*x*: 21.3%, 10*x*: 9.0%; Table 3.3; Figure 3.2). Thus, the ecological range of the polyploid's niches was smaller than that of diploids (Figure 3.2K and L), and a high percentage of the polyploid's environmental niches occurred within the diploid one (cytotype niche overlap with diploid niche; 4*x*: 58.9%; 6*x*: 60.1%; 8*x*: 51.4%; 10*x*: 51.7%; Table 3.3; Figure 3.2A1-D3). Comparing the diploid environmental niche with that of the polyploids demonstrated that the occurrence density in the ecological space was different, as showed by a low, statistically significant, *D* metric in the equivalence tests (Table 3.3; Figure 3.2A1-A2; B1-B2; C1-C2; D1-D2). Thus, polyploid's niches occupy areas of the diploid niche with reduced density, which corresponds to less optimal conditions for diploids (Figure 3.2). In addition, the first two axes of the PCA of the comparisons with the diploid niche explained a high percentage of the ecological variance (4*x*: 58.3%; 6*x*: 60.4%; 8*x*: 59.8%; 10*x*: 59.3%; Table 3.3; Figure 3.2A1-D3). The niche magnitude of tetraploids, hexaploids and octoploids differed in the environmental space: in the PCA1 axis, the magnitude of octoploids niche was different from that of the other cytotypes, and in the PCA2 axis, the magnitude of the hexaploid niche was different from that of the other cytotypes. The magnitude of the environmental niche of decaploids was lower than the other cytotypes for PCA2 axis (Figure 3.2K and L). The environmental niche of tetraploids and hexaploids largely overlapped (66.7% and 58.7%, Table 3.3; Figure 3.2E1-E3), but the occurrences density in the ecological space of each cytotype was different, as demonstrated by the equivalency test (D = 0.15, $P < 0.05$, Table 3.3; Figure 3.2E1-E2). Comparing the environmental niches of tetraploids and octoploids revealed that the climatic niches are equivalent ($P > 0.05$, Table 3.3; Figure 3.2E1-E3). Indeed, the environmental niche of tetraploids


A1)
Diploid
A2)
Tetraploid
A3)
Diploid
Tetraploid
Overlap
B1)
Diploid
B2)
Hexaploid
B3)
Diploid
Hexaploid
Overlap
C1)
Diploid
C2)
Octoploid
C3)
Diploid
Octoploid
Overlap
D1)
Diploid
D2)
Decaploid
D3)
Diploid
Decaploid
Overlap
PC1
PC2

⇩Cont.


E1)
Tetraploid
E2)
Hexaploid
E3)
Tetraploid
Hexaploid
Overlap
F1)
Tetraploid
F2)
Octoploid
F3)
Tetraploid
Octoploid
Overlap
G1)
Tetraploid
G2)
Decaploid
G3)
Tetraploid
Decaploid
Overlap
H1)
Hexaploid
H2)
Octoploid
H3)
Hexaploid
Octoploid
Overlap
PC1
PC2

⇩Cont.


I1) Hexaploid
I2) Decaploid
I3) Hexaploid Decaploid Overlap
PC1
PC2
J1) Octoploid
J2) Decaploid
J3) Octoploid Decaploid Overlap
K)
a b b c d
2x 4x 6x 8x 10x
Ploidy level
L)
a b c b d
2x 4x 6x 8x 10x
Ploidy level

⇩Cont.

Figure 3.2. Ecological niche models for *L. suffruticosum s.l.*, based on the PCA of selected variables; colored areas represent suitable habitats for cytotype 1 and cytotype 2 (light grey and dark grey, respectively) and overlapping areas (green). The continuous line corresponds to the whole climatic space, while the dashed line indicates the 75th percentile. Boxplot of the magnitude of ecological niches of each cytotype (K, occupation of niches in PCA1 axis and L, occupation of niches in PCA2 axis). Abbreviations: 2*x*, diploids; 4*x*, tetraploids; 6*x*, hexaploids; 8*x*, octoploids; 10*x*, decaploids.

Table 3.3. Equivalency (*D* and *P* values) and similarity (*P* value) tests for suitable habitat for each pair of cytotypes of *L. suffruticosum s.l.* Percentage of niche overlap and variance explained by the first two axes of the principal component analyses (PCA) are also presented. 2*x*: diploids; 4*x*: tetraploids; 6*x*: hexaploids; 8*x*: octoploids; 10*x*: decaploids.

A vs B	Equivalence test		Similarity test (*P* values)		% Niche overlap		% PCA	
	***D* value**	***P* value**	**A--» B**	**B --» A**	**A--» B**	**B --» A**	**% PCA1**	**% PCA2**
2*x* vs 4*x*	**0.14**	**0.010**	0.822	0.812	28.1%	58.9%	35.8%	22.5%
2*x* vs 6*x*	**0.11**	**0.010**	0.782	0.851	32.3%	60.1%	36.4%	24.0%
2*x* vs 8*x*	**0.07**	**0.010**	0.822	0.752	21.3%	51.4%	37.0%	22.8%
2*x* vs 10*x*	**0.02**	**0.010**	0.733	0.653	9.0%	51.7%	39.1%	20.2%
4*x* vs 6*x*	**0.15**	**0.010**	0.822	0.792	66.7%	58.7%	30.5%	18.4%
4*x* vs 8*x*	0.21	0.059	0.723	0.673	58.8%	68.7%	31.8%	17.5%
4*x* vs 10*x*	**0.19**	**0.050**	0.881	0.851	35.8%	80.8%	33.8%	16.4%
6*x* vs 8*x*	0.39	0.594	0.842	0.931	72.2%	79.3%	28.2%	21.8%
6*x* vs 10*x*	**0.11**	**0.010**	0.802	0.772	26.6%	70.9%	27.7%	22.3%
8*x* vs 10*x*	0.25	0.129	0.891	0.851	45.9%	89.3%	29.3%	21.8%

and octoploids overlap (58.8% and 68.7%, Table 3.3; Figure 3.2E3). The environmental niches of tetraploids and decaploids are not equivalent ($D = 0.19$, $P < 0.05$, Table 3.3; Figure 3.2F1-F3). Even though the environmental niche of tetraploids had low overlap with that of decaploids (35.8%, Table 3.3; Figure 3.2K and L), the niche of decaploids was within that of tetraploids (80.8%, Table 3.3; Figure 3.2F3). Hexaploids and octoploids have an environmental overlap (72.2% and 79.4%, Table 3.3), and their geographic niche is equivalent ($D = 0.39$, $P > 0.05$, Table 3.3), being also evident by similar occurrence densities (Figure 3.2H1-H2). By opposition, the geographic niche of hexaploids and decaploids was not equivalent, and their occurrences density was significantly different ($D = 0.11$, $P < 0.05$, Table 3.3; Figure 3.2I1-I2). Hexaploid niche has a low geographic niche overlap within the decaploid niche (26.6 %, Table 3.3; Figure 3.2I3); still, the ecological requirements of decaploids largely fall within the niche of the hexaploids (70.9%, Table 3.3; Figure 3.2I3). Octoploids and decaploids presented geographic niche overlap, being equivalent ($D = 0.25$, $P > 0.05$). A high percentage of the environmental niche of decaploids is like the niche of octoploids (89.3%), but only 45% of octoploid's niche fall within the niche of the decaploid (Table 3.3; Figure 3.2J1-J3).

Although significant differences were observed in niche equivalency in some cytotype pairs, in niche similarity, the observed *D* values fall within the 95th percentile of the simulated values, which indicates that cytotypes were not more similar (or different) from one another than expected after random sampling (Table 3.3).

Discussion

This study revealed differences in the ecological attributes of *L. suffruticosum s.l.* cytotypes, with polyploids being associated with habitats with increased drought (low precipitation and high temperatures), increased temperature ranges (both isothermality and mean diurnal temperature), higher soil pH, and decreased soil water and cation exchange capacities. These results could be explained as an adaptation of polyploids to dry and harsh environments. Despite the absence of environmental niche differences among most of polyploids, the niche of the diploids differed significantly from that of the polyploids, being the widest among all cytotypes. Polyploids may have spread to environments less suitable for the diploids to escape competition. Additionally, in the two sides of the Mediterranean basin separated by the Strait of Gibraltar (SW Europe and NW Africa), the ecological niche of *L. suffruticosum s.l.* is different, as well as the niche of diploids and polyploids in each area. This study is important to understand the niche requirements of each cytotype and gives us relevant information for future competition and reciprocal transplant experiments. Below, we discuss the mechanisms underlying these results and their implications for understanding polyploid establishment and persistence.

Ecological differences between diploids and polyploids

Recent detailed field surveys enabled to map *L. suffruticosum s.l.* cytotypes through its entire distribution range. The results obtained here suggest that the parapatric distribution of cytotypes observed in the field (Afonso *et al*. 2021 in Chapter II) can be partly explained by differences in the ecological niche. The habitats where diploids occur presented ecological dissimilarities compared to those where polyploids were found. Diploids were found in habitats with high precipitation, low temperatures and isothermality, higher soil water retention, and lower soil pH and cation exchange capacity than polyploids. By opposition, polyploids grow in drier and harsher habitats (low precipitation and high temperatures), with high isothermality, mean diurnal temperature, and soil pH. In fact, pH is a key predictor for the occurrence of many plant species since it affects nutrient availability (Wagner *et al.* 2017). In the Mediterranean

region, the distribution patterns of other polyploid complexes have also been shown to be constrained by environmental variables, related to precipitation and temperature, imposed by the Mediterranean climate (Muñoz-Pajares *et al.* 2018; López-Jurado *et al.* 2019), given its high spatio-temporal dynamic nature (Nieto-Feliner 2014; Cook *et al.* 2016). Also, it has already been shown that polyploids tend to grow in more specialized niches in narrower and stressful habitats (Brochmann *et al.* 2004; Blaine Marchant *et al.* 2016; Hijmans *et al.* 2017).

Although both diploids and tetraploids grow in places with a mountain-influenced climate, diploids of *L. suffruticosum s.l.* were always found in populations at high elevation. Also, diploid plants are smaller than tetraploid ones (A Afonso, personal observations). Many studies have demonstrated niche differentiation across altitudinal gradients, with diploids growing at high elevation and polyploids at lower elevation (Stebbins 1971; Te Beest *et al.* 2012). Furthermore, we also observed that tetraploids are not as highly restricted in soil characteristics as diploids that only grow in habitats with the highest water retention and cation exchange values and low soil pH levels. This ability of tetraploids to colonize different soils could have allowed them to expand to areas beyond the suitable areas of their diploid parentals and overcome the minority cytotype exclusion. Tetraploids, hexaploids, and decaploids occupy different geographic niches, suggesting a possible niche specialization (Vamosi *et al.* 2014; Parisod and Broennimann 2016). However, hexaploid and octoploid populations presented equivalent and similar environmental niches. The same was true for tetraploid and octoploid populations and octoploid and decaploid populations. The absence of environmental niche differentiation among polyploids was not completely unexpected as the requirements of the higher-ploidy individuals might not differ from their lower-ploidy ancestors (Godsoe *et al.* 2013; Laport *et al.* 2016). As ecological preferences do not constitute a strong barrier in *L. suffruticosum s.l.* polyploids the gene flow between individuals of neighboring populations is still possible. Furthermore, it is shown in Chapter IV that cytotypes are not reproductively isolated.

Despite the differences in the ecological requirements, diploids have a broader environmental niche breadth than polyploids, and polyploids occupy a part of the diploid's niche. In practice, diploids and polyploids share the same environmental niche (niches were not more similar nor different than expected in a random sampling), and polyploids occur at marginal areas of the diploid niche, despite growing in different geographic areas (ecological niches were not equivalent). In young polyploid complexes (as *L. suffruticosum s.l.* seems to be, Ruiz-Martín *et al.* 2018; Maguilla *et al.* 2021a), polyploids may partially occupy the niche of their progenitors, thus growing in climatic conditions of diploids as they did not had time yet to disperse further, specialize and/or completely diverge in their niche (Felber 1991; Kim *et al.*

2012b; Glennon *et al.* 2014). Alternatively, polyploids could have diverged in their niche and later recolonized part of the diploid niche (Ståhlberg and Hedrén 2009; Glennon *et al.* 2014).

Nevertheless, previous works have suggested that spatial segregation reflects ecological niche divergence and is one of the requirements for the successful establishment of polyploid lineages (Lumaret *et al.* 1987; Levin 2002). Interestingly, as observed in *L. suffruticosum s.l.*, in other polyploid complexes, it was shown that the frequency of polyploid individual's increases at the periphery of parental ranges (Levin 1975; Fowler and Levin 1984; Felber 1991), suggesting environmental specialization (Knouft *et al.* 2012; Vamosi *et al.* 2014; Parisod and Broennimann 2016). Several studies also indicated that spatial segregation could have resulted from the ability of polyploids to tolerate low nutrient levels, drought, and cold temperatures and colonize areas unfavorable or less favorable to their lower-ploidy progenitors (Levin 2002; Maherali *et al.* 2009; Hao *et al.* 2013). Several examples of environmental niche divergence between cytotypes have been reported in several polyploid complexes (Glennon *et al.* 2014; Thompson *et al.* 2014; Visger *et al.* 2016; Muñoz-Pajares *et al.* 2018), although it is difficult to separate the direct effects of WGD from subsequent evolutionary divergence (Maherali *et al.* 2009). The absence of niche specialization of *L. suffruticosum s.l.* polyploids could be either because genome duplications did not generate significant direct physiological changes due to their recent origin (Ruiz-Martín *et al.* 2018; Maguilla *et al.* 2021a) or because they might have been subjected to recurrent gene flow (Godsoe *et al.* 2013; Laport *et al.* 2016). The latter hypothesis is discussed below.

Maintenance of the mosaic distribution of the polyploid complex

Previous field screenings of *L. suffruticosum s.l.* found that most populations were pure-ploidy populations. However, a few mixed-ploidy populations with minority cytotypes or aneuploids were also observed (Afonso *et al*., 2021 in Chapter II). Despite being rare, the occurrence of mixed-ploidy populations (two diploid-tetraploid and one tetraploid-hexaploid) and minority cytotypes (namely, triploid, tetraploid, hexaploid, octoploid, and aneuploid individuals) can give us clues about how dynamic this polyploid complex can be. Despite the observed mosaic distribution, there is a clear contact zone between diploids and tetraploids in southern Spain (where these two cytotypes are abundant) and some contact areas in northern Spain (where diploids and tetraploids are scarce) and in the north of Africa. The low number of diploid-tetraploid mixed populations (Afonso *et al*. 2021 in chapter II) suggests that the two cytotypes cannot occur in sympatry, likely because of the minority cytotype exclusion (Levin 1975, 2002; Fowler and Levin 1984; Husband 2000). Although long-distance pollen flow and hybridization between the two cytotypes cannot be excluded entirely, the presence of a few

triploids in diploid populations suggests that tetraploids likely arose from the fusion of unreduced gametes, leading to a primary contact zone. Current diploid-tetraploid distribution could thus result from the combined effect of differences in environmental preferences and minority cytotype exclusion. However, other processes may further contribute to the distribution pattern of diploids and tetraploids, such as competitive exclusion and/or divergent evolution.

Hexaploids occupy a large area, presenting the westernmost distribution in the Iberian Peninsula and being more geographically segregated from the others cytotypes in central Spain. There is a clear area of suitable habitats for hexaploids in central Spain, where most natural populations were found. Despite of the presence of a tetraploid-hexaploid mixed population, their ecological niches were not equivalent. Similar results were found for hexaploid and decaploid niches. No hexaploid-decaploid mixed-ploidy population was found, as they occur far apart from each other (Afonso *et al.* 2021 in Chapter II). These observations suggest that hexaploids seem to have suitable areas in regions not overlapping with the other cytotypes. Thus, they support an important role of environmental variables defining their distribution. Octoploids and decaploids also have a clear area of suitable habitats, with high overlap between them (with 89.3%of the environmental niche of decaploids in the niche of octoploids and 45% of octoploid's niche within the niche of the decaploid). Since there is no evidence of mixed-populations (Afonso *et al*., 2021 in Chapter II), their distribution could be explain by the minority cytotype exclusion (Levin 1975, 2002; Fowler and Levin 1984; Husband 2000). Different competitive abilities are expected to generate moving contact zones and lead to the expansion of cytotype area until the environmental limit of the strongest competitor is reached (Maceira *et al.* 1993). However, considering the reproductive system of this species (*i.e.*, distyly), with this polymorphism being present in all populations, with a strongly self- and morph-incompatible system (Chapter IV), the absence of compatible mates might be critical during colonization of new areas, contributing to more stable areas. To sum up, for some cytotypes there was a divergence of niche and a colonization of areas that were not favorable to the other cytotypes. Additionally, the existence of mostly pure populations in contact zones between cytotypes that showed a large overlap of suitable niche supports the existence of minority cytotype exclusion. Nevertheless, the forces that maintain the dynamics of each contact zone will also depend on other factors such as competition ability, or reproductive strategies. Further investigation about the polyploids' competitive abilities and reproductive strategies is needed in the future.

Different environmental requirements across geographic areas – evolutionary implications

The distribution patterns of *L. suffruticosum s.l.* in the Iberian Peninsula and North Africa could be associated with different ecological preferences related to soil properties and climatic differences. The morphological variability, geographical overlap, and high cytogenetic diversity detected in the field might indicate multiple origins of the polyploids from the same and/or from different progenitors (Nicholls 1986a; Ruiz-Martín 2017; Afonso *et al.* 2021 in Chapter II). Over the evolutionary history of *L. suffruticosum s.l.*, it seems that polyploids originated several times from diploid populations. These multiple events of WGDs may have occurred in both sides of the Mediterranean Sea, as reported in other polyploid complexes (Bougoutaia *et al.* 2021). The Mediterranean region has complex geological and paleoclimatic characteristics. The western Mediterranean was particularly active tectonically during the Oligocene (Rosenbaum *et al.* 2002), while the eastern Mediterranean area is more recent. Its present configuration results from the collision of the Arabian plate with stable Eurasia in the middle Miocene (Krijgsman 2002). In addition, the paleoclimatic history of the Mediterranean Basin included important long-term changes, such as the gradual global cooling and an aridification (Zachos *et al.* 2008), as well as cyclical climatic changes (Jansson and Dynesius 2002). The dynamic mosaic distribution of cytotypes could represent the result of different waves of colonization and retractions following ice ages. The Mediterranean Basin has served as a refugium for many species during the Tertiary and the Quaternary and it has been a reservoir for later colonization during interglacial periods (Thompson, 2020). However, this complex is very recent, having originated most probably at the beginning of the Pleistocene, while the genus originated and began to diversify in the early Oligocene to late Miocene (Ruiz-Martín *et al.* 2018; Maguilla *et al.* 2021a). The dispersion and diversification could be related to the dispersal through the strait of Gibraltar (open during the last ca. 5Myr) and a fast adaptation to new environments. Indeed, species expansion has been reported to occur during the Oligocene–Miocene, when the closure of the marine gateways that existed between the Atlantic Ocean and the Mediterranean Sea took place, leading to the dryness of the Mediterranean Sea (Messinian Salinity Crisis- 5.96–5.33 My; Steininger and Rögl 1984; Krijgsman 2002; Meulenkamp and Sissingh 2003). The recurrent and possible different origins of polyploids could explain the existence of both diploids and polyploids in both continents. As described above, changes in environmental requirements promoting eco-spatial segregation would increase the probability of establishment and persistence of neopolyploids (Felber 1991). Thus, ecological differentiation could have occurred not only among cytotypes between continents but also within continents since they might have different evolutionary histories and have been exposed to other selective pressures.

In Europe, polyploids only occur in the Iberian Peninsula, while in the rest of the European distribution, only diploids were detected. The potential niche of polyploids seems to restrict their distribution in Europe to the Southern side of the Pyrenees (with Mediterranean climate on the southern side in contrast with temperate climate on the northern one), suggesting that areas north and northeast of the Pyrenees are not suitable for polyploids (as also supported by the models presented here). In other polyploid complexes where diploids grow in a higher elevation than polyploids, this distribution pattern suggests that diploids are old and probably well adapted to different areas over the entire distribution area (Theodoridis *et al.* 2013; Dai *et al.* 2020). However, as mentioned before, other factors could have been involved in the interactions among cytotypes at contact areas. Furthermore, the Pyrenees could have acted as a geographic barrier for polyploids spread after their recent emergence in the Iberian Peninsula, leading to the sole existence of diploids beyond the mountain complex. Geographical barriers seem to have played a significant role in driving the emergence and establishment of polyploid complexes in the Mediterranean flora (Marques *et al.* 2018). Reciprocal transplant experiments are needed to test this hypothesis.

The potential niche projected for polyploids in North Africa is much higher than what is observed in nature and in the records from the literature. However, due to scarce information about the species occurrence in North Africa, which made it difficult to perform field sampling and resulted in a low number of populations with ploidy data, we used the European populations to project the potential ecological niche in North Africa. Due to possible differences in the origin and evolutionary history of the populations in North Africa, the niche projection may not be as accurate as envisaged, and the results should be considered with caution. The habitat suitability for *L. suffruticosum s.l.* in North Africa is much larger than what was sampled in the field for all cytotypes, with a high probability of habitat suitability even for cytotypes not reported for this area (*e.g.*, octoploid and decaploid). Fennane *et al.* (2007) reported the possible occurrence of the species further South in Morocco, but we did not find it during field sampling. Also, the sampled populations were very small, having a lower number of individuals than those usually found in the Iberian Peninsula. The climatic versus topographical heterogeneity in North Africa is much higher than in the Iberian Peninsula, which may be one of the reasons for the difficulty in correctly identifying the niche in this area. Furthermore, North African habitats are characterized by low precipitation, high minimum temperatures, and different soil attributes (higher soil pH, low cation exchange capability and water retention capacity, variable, and slightly higher soil texture). Soil texture mainly influences the soil water capacity, and therefore it is an essential factor in the adaptation to Mediterranean dry biomes (Saxton and Rawls 2006;

Padilla and Pugnaire 2007). Overall, these environmental variables might help explain the high suitability of *L. suffruticosum s.l.* polyploids in this region.

In addition, overall, the species range appears to be limited by the presence of limestone and related substrates combined with the Mediterranean climate. Consequently, the species is scarce in other soil types, with populations almost absent in the western half of the Iberian Peninsula, where limestone areas are restricted. Also, the species do not occur in the western Mediterranean islands (Balearic Islands, Corsica, Sardinia, Sicily), despite the presence of limestone soil and Mediterranean climate. This might be correlated with the phylogenetic evidence showing the recent origin of the complex's (Maguilla *et al.* 2021a). The geological and climatic context during the evolutionary history of *L. suffruticosum s.l.* and subsequent divergent evolution could have played a role in shaping its diversity. Biogeographical processes, including historical patterns of origin or migration, interactions among cytotypes, and divergence in levels of environmental tolerance have been reported as the main factors determining the success of populations with different ploidies (Husband *et al.* 2013). Despite we suggest that various polyploidization events have occurred in other geographical areas and biogeographical contexts, leading to differences in the predicted and observed niche of cytotypes in both sides of the Mediterranean Sea, molecular dating and biogeographical analyses along the distribution range of this complex are necessary to fully understand the evolutionary processes that have governed the current distribution patterns.

Conclusions

This study revealed variation between diploid and polyploid ecological niches with differences in precipitation and temperatures ranges. However, some higher-ploidy cytotypes had equivalent ecological niches but never co-occurred. In addition, differences among cytotypes of different geographical areas were found. Overall, these results support that particular ecological requirements played a role in the distribution of cytotypes, but the mosaic distribution could not be entirely explained based on environmental conditions. Reproductive and competitive interactions among cytotypes could have played a role in shaping the current diversity and distribution patterns.

Appendices

Appendix 3.1. Populations of *L. suffruticosum s.l.* used in this study. Information about the country, ploidy level, coordinates, and data source are provided. Abbreviations: 2*x*, diploid.

Country	Ploidy level	Coordinates (longitude, latitude)	Source
Algeria	-	35.00191,-1.69000	GBIF
Algeria	-	34.71121,-1.52919	GBIF
Algeria	-	32.47723,-0.88002	GBIF
Algeria	-	32.56142,-0.75479	GBIF
Algeria	-	34.67475,-0.62188	GBIF
Algeria	-	34.67438,-0.62158	GBIF
Algeria	-	35.29926,-0.55875	GBIF
Algeria	-	35.29924,-0.54844	GBIF
Algeria	-	33.55924,-0.31422	GBIF
Algeria	-	33.55934,-0.31375	GBIF
Algeria	-	33.55913,-0.31311	GBIF
Algeria	-	34.78790,-0.25771	GBIF
Algeria	-	35.15528,4.08760	GBIF
France	-	42.38316,0.79038	field data
France	-	42.14775,0.80809	field data
France	-	42.26159,1.55418	field data
France	-	42.2616,1.55418	field data
France	-	42.26391,1.5786	field data
France	-	42.34083,1.6773	field data
France	-	42.34104,1.71902	field data
France	-	42.12808,1.86343	field data
France	-	42.25406,1.87156	field data
France	-	41.88294,2.32505	field data
France	-	42.32940,2.46178	field data
France	-	44.26368,3.22603	field data
France	-	43.85580,3.40369	field data
France	-	43.85295,3.40748	field data
France	-	44.24528,5.13186	field data
France	-	43.81130,5.41738	field data
France	-	44.69188,5.67261	field data
France	-	43.33808,5.77677	field data
France	-	43.71561,6.48246	field data
France	-	43.95023,6.51151	field data
France	-	43.96913,6.77871	field data
France	-	43.81819,7.15852	field data
France	-	43.78156,7.25228	field data
France	2*x*	45.34233,0.53009	field data
France	2*x*	47.01359,0.54389	field data
France	2*x*	44.72748,0.55015	field data
France	2*x*	44.91382,0.91041	field data

⇩Cont.

France	2*x*	44.57694,0.95173	field data
France	2*x*	44.37685,0.99413	field data
France	2*x*	45.08731,1.21225	field data
France	2*x*	44.32375,1.45193	field data
France	2*x*	42.26168,1.55408	field data
France	2*x*	42.26406,1.57844	field data
France	2*x*	42.25878,1.60694	field data
France	2*x*	42.24306,1.65840	field data
France	2*x*	42.35431,1.68983	field data
France	2*x*	42.34280,1.71804	field data
France	2*x*	44.00648,1.88058	field data
France	2*x*	42.92768,2.22010	field data
France	2*x*	44.35223,3.04405	field data
France	2*x*	44.29072,3.11781	field data
France	2*x*	44.31679,3.18623	field data
France	2*x*	43.76217,3.19308	field data
France	2*x*	43.89537,3.27788	field data
France	2*x*	44.52045,3.30480	field data
France	2*x*	44.53441,3.31566	field data
France	2*x*	44.35740,3.39321	field data
France	2*x*	43.85575,3.40342	field data
France	2*x*	44.18306,3.42710	field data
France	2*x*	43.79252,3.43457	field data
France	2*x*	43.94450,3.75128	field data
France	2*x*	43.81401,3.76189	field data
France	2*x*	43.87777,3.79476	field data
France	2*x*	43.73861,3.86829	field data
France	2*x*	43.88470,3.87942	field data
France	2*x*	44.24543,5.13175	field data
France	2*x*	44.28870,5.15818	field data
France	2*x*	44.02568,5.20353	field data
France	2*x*	43.99158,5.24231	field data
France	2*x*	43.99573,5.26753	field data
France	2*x*	44.20839,5.30797	field data
France	2*x*	43.91538,5.36244	field data
France	2*x*	44.86936,5.55651	field data
France	2*x*	44.75780,5.60397	field data
France	2*x*	44.40102,5.60980	field data
France	2*x*	43.49727,5.61766	field data
France	2*x*	44.21340,5.82559	field data
France	2*x*	44.42599,5.92640	field data
France	2*x*	43.40018,5.95794	field data
France	2*x*	44.56877,5.99975	field data
France	2*x*	44.62321,6.05137	field data
France	2*x*	43.77365,6.25733	field data

⇩Cont.

France	2*x*	43.90471,6.26188	field data
France	2*x*	44.57872,6.28205	field data
France	2*x*	43.85326,6.29738	field data
France	2*x*	43.83488,6.32799	field data
France	2*x*	43.82121,6.33933	field data
France	2*x*	43.59993,6.47773	field data
France	2*x*	43.67915,6.80786	field data
France	2*x*	43.94288,6.91903	field data
France	2*x*	43.93502,6.94795	field data
France	2*x*	43.75534,7.08371	field data
France	2*x*	43.82441,7.14850	field data
France	2*x*	43.78105,7.25274	field data
France	2*x*	43.87132,7.39935	field data
France	2*x*	43.77050,7.41429	field data
Italy	-	45.15230,7.05635	field data
Italy	-	44.20828,8.39238	field data
Italy	-	44.55103,8.77351	field data
Italy	-	44.51626,8.79681	field data
Italy	2*x*	47.17707,0.83299	field data
Italy	2*x*	46.98093,0.87023	field data
Italy	2*x*	45.17736,0.89165	field data
Italy	2*x*	44.14883,6.74478	field data
Italy	2*x*	44.12930,6.91598	field data
Italy	2*x*	44.08788,6.95363	field data
Italy	2*x*	44.08885,7.03835	field data
Italy	2*x*	44.35820,7.16899	field data
Italy	2*x*	44.06465,7.25121	field data
Italy	2*x*	44.93493,7.94928	field data
Morocco	-	30.62055,-9.36000	field data
Morocco	-	35.75000,-5.37000	field data
Morocco	-	32.60058,-4.81533	field data
Morocco	-	33.87000,-4.02949	field data
Morocco	-	34.81744,-2.41999	field data
Spain	-	36.51626,-6.13829	field data
Spain	-	36.84303,-4.81689	field data
Spain	-	36.84772,-4.80372	field data
Spain	-	38.61821,-4.11421	field data
Spain	-	36.83497,-3.96445	field data
Spain	-	36.84573,-3.71784	field data
Spain	-	37.67548,-3.63504	field data
Spain	-	37.08130,-3.53616	field data
Spain	-	38.35197,-3.52645	field data
Spain	-	37.04302,-3.52627	field data
Spain	-	37.07966,-3.50738	field data
Spain	-	37.13986,-3.48191	field data

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ain	-	37.07730,-3.48177	field data
Spain	-	37.11288,-3.45447	field data
Spain	-	40.28628,-3.45067	field data
Spain	-	37.31237,-3.40771	field data
Spain	-	40.40841,-3.28319	field data
Spain	-	42.71315,-3.28087	field data
Spain	-	42.30792,-3.26693	field data
Spain	-	40.28126,-3.22350	field data
Spain	-	41.25421,-3.21485	field data
Spain	-	41.29013,-3.12433	field data
Spain	-	38.88706,-3.05306	field data
Spain	-	41.23544,-3.04183	field data
Spain	-	37.39097,-2.99911	field data
Spain	-	37.27691,-2.97741	field data
Spain	-	39.51558,-2.88366	field data
Spain	-	37.44014,-2.88298	field data
Spain	-	38.01059,-2.86330	field data
Spain	-	40.28111,-2.84901	field data
Spain	-	37.05358,-2.82561	field data
Spain	-	37.03333,-2.80352	field data
Spain	-	37.02997,-2.77830	field data
Spain	-	42.68585,-2.62159	field data
Spain	-	36.93636,-2.60633	field data
Spain	-	40.52624,-2.52711	field data
Spain	-	40.63177,-2.50335	field data
Spain	-	42.64925,-2.47542	field data
Spain	-	42.61402,-2.42155	field data
Spain	-	37.79578,-2.28389	field data
Spain	-	40.60276,-2.16080	field data
Spain	-	37.70116,-2.14920	field data
Spain	-	39.86344,-2.13530	field data
Spain	-	38.03207,-1.73343	field data
Spain	-	41.86741,-1.60224	field data
Spain	-	40.84743,-1.46141	field data
Spain	-	41.18116,-1.45741	field data
Spain	-	39.58531,-1.15620	field data
Spain	-	39.10466,-1.03229	field data
Spain	-	40.84296,-1.02938	field data
Spain	-	42.49427,-0.80719	field data
Spain	-	40.82986,-0.79818	field data
Spain	-	42.38981,-0.71527	field data
Spain	-	42.50463,-0.63880	field data
Spain	-	42.53272,-0.54955	field data
Spain	-	39.71479,-0.53845	field data
Spain	-	38.64907,-0.41454	field data

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Spain	-	39.88275,-0.37088	field data
Spain	-	41.98788,0.28381	field data
Spain	-	41.28413,0.52138	field data
Spain	-	41.83267,0.59080	field data
Spain	-	41.83264,0.59143	field data
Spain	-	42.46673,0.77404	field data

Appendix 3.2. Minimum (min) and maximum (max) values of selected variables used to characterize the niche of *L. suffruticosum s.l.* cytotypes.

Variables	CODE	Diploids (min-max)	Tetraploids (min-max)	Hexaploids (min-max)	Octoploids (min-max)	Decaploids (min-max)
Elevation (metres)	ele	52.00-2599.00	46.00-1738.00	438.00-1151.00	367.00-1315.00	32.00-966.00
Distance to the coast (km)	dcoast	2.09-307.12	3.13-306.31	27.43-379.36	61.70-293.48	2.09-221.50
Mean Diurnal Range (°C)	bio2	0.60-1.30	0.80-1.30	0.90-1.30	0.90-1.40	0.60-1.40
Isothermality (* 100)	bio3	28.00-43.00	0.00-44.00	36.00-43.00	38.00-44.00	28.00-43.00
Mean Temperature of Coldest Quarter (°C)	bio11	-0.50-1.00	0.20-1.20	0.30-1.10	0.30-0.90	0.40-1.00
Precipitation of Driest Month (mm)	bio14	2.00-69.00	1.00-33.00	2.00-44.00	10.00-41.00	1.00-32.00
Precipitation of Wettest Quarter (mm)	bio16	118.00-485.00	73.00-344.00	115.00-271.00	122.00-249.00	121.00-198.00
Soil water capacity (v%)	aw	11.00-18.00	10.00-14.00	11.00-16.00	12.00-16.00	10.00-14.00
Cation exchange capacity (cmolc/kg)	cat	13.00-30.00	14.00-25.00	13.00-21.00	14.00-23.00	14.00-22.00
Soil pH (pH)	ph	5.50-8.00	6.30-8.00	5.70-8.10	5.70-8.10	5.70-7.90
Clay content (w%)	clay	13.00-34.00	20.00-34.00	17.00-32.00	20.00-29.00	14.00-28.00
Fragment content (v%)	frag	8.00-28.00	14.00-26.00	11.00-21.00	13.00-25.00	10.00-24.00
Sand content (w%)	sand	25.00-52.00	27.00-47.00	30.00-59.00	28.00-46.00	23.00-43.00
Soil texture (USDA system)	text	4.00-8.00	4.00-7.00	4.00-9.00	7.00-7.00	4.00-7.00

Appendix 3.3. Contribution of variables in the models of *Linum suffruticosum s.l.* and of each cytotype**.** In bold are highlighted the variables with the highest contribution in each model. Abbreviations: 2*x*, diploids; 4*x*, tetraploids; 6*x*, hexaploids; 8*x*, octoploids; 10*x*, decaploids.

Variables	**CODE**	***L. suffruticosum s.l.***	**2*x***	**4*x***	**6*x***	**8*x***	**10*x***
Mean Diurnal Range (°C)	bio2	0.006	0.088	**0.256**	0.047	0.087	0.000
Isothermality (*100)	bio3	**0.312**	**0.469**	0.109	**0.212**	**0.435**	**0.530**
Mean Temperature of Coldest Quarter (°C)	bio11	0.159	0.189	0.000	0.092	0.000	0.158
Precipitation of Driest Month (mm)	bio14	0.032	0.055	**0.717**	0.082	**0.444**	0.114
Precipitation of Wettest Quarter (mm)	bio16	0.047	**0.206**	0.012	**0.250**	0.173	**0.584**
Elevation (m)	ele	**0.240**	0.172	**0.706**	**0.396**	0.141	0.074
Distance to the coast (km)	dcoast	0.062	0.125	0.169	0.031	**0.223**	**0.464**
Soil water capacity (v%)	aw	0.033	0.182	0.101	0.001	**0.225**	0.192
Cation exchange capacity (cmolc/kg)	cat	0.182	0.155	**0.310**	0.076	0.001	0.045
Clay content (w%)	clay	0.014	0.063	0.000	0.000	0.000	0.000
Fragment content (v%)	fra	0.100	0.038	0.000	0.054	0.043	**0.528**
Soil pH (pH)	ph	**0.203**	**0.345**	0.005	0.042	0.095	0.000
Sand content (w%)	sand	0.068	0.116	0.002	0.000	0.012	0.122
Soil texture (USDA system)	text	0.001	0.007	0.083	0.036	0.002	0.001

Chapter IV - Stylar polymorphism and incompatibility system are maintained across ploidy levels of *Linum suffruticosum s.l.* complex

Abstract

Polyploidization may have a significant effect on the structure of sexual organs in flowers and in associated traits. Whole genome duplication (WGS) is also known to break reproductive self-incompatibility mechanisms, allowing the polyploid to self-reproduce and counter act minority cytotype exclusion. Changes in reproductive traits will be particularly relevant in species with complex breeding systems, such as heterostyly, a polymorphism which promotes outcrossing and reduces sexual self-interference in hermaphroditic flowers. Here, *L. suffruticosum*, a polyploid complex from a genus with about 40% of heterostylous species, was used to evaluate the reproductive traits and how such traits vary with WGD. Additionally, the reproductive relationships among diploid and tetraploid cytotypes were also studied. Morph frequencies were recorded, and the sexual organs were measured in flowers from populations of each of the five cytotypes (diploids, tetraploids, hexaploids, octoploids and decaploids). Experimental crosses within cytotypes (selfing and intra- and inter-morph crosses) and crosses between diploids and tetraploids from a parapatric zone were performed. Results showed that most of the populations were isoplethic (90%), even being very variable in population size, and that the anisoplethic populations had intermediate population sizes. The size of sexual organs increased with ploidy level and there was a size overlap among cytotypes. However, the reciprocity indexes were maintained in all cytotypes. Pollen tube development was lower in self- and intra-morph crosses than in inter-morph crosses within cytotypes. Crosses between diploids and tetraploids produced similar pollen tube development to within-cytotype crosses. There was no evidence of breakdown of the incompatibility system and the results suggested higher successful inter-morph crosses than intra-morph crosses. The reciprocal herkogamy is maintained in all ploidy levels, as well morph- and self- incompatibility. Pollen tube development and overlap among reciprocal sexual organs suggest that pollen and gene flow is possible among cytotypes, and that this may be avoided only by geographical distance between populations. Further investigation on pollen transfer, reproductive fitness and phylogenetic and phylogeographic studies are still needed.

Key words: heterostyly, incompatibility system, *Linum*, style polymorphism, polyploidy, white flax.

Introduction

Whole genome duplications (WGDs) are known to lead to profound genetic changes that can be manifested in biochemical, cytological, reproductive, morphological, physiological and developmental traits of organisms (Bretagnolle and Thompson 1995; Husband and Schemske 2000; Buggs and Pannell 2007; Maherali *et al.* 2009; Hao *et al.* 2013; Clo and Kolář 2021). Events of WGD are particularly frequent in plant lineages, and may have putative important ecological implications that can, ultimately, act as an advantage, enabling polyploids spread, establish and diversify (Levin 2002). Different flowering phenologies and/or flower morphologies, or different growth rate and competitive ability may increase the probability of success of polyploids (Rodríguez 1996; Rausch and Morgan 2005; Rieseberg and Willis 2007; Castro and Loureiro 2014). Common and important changes found in polyploids are related with reproductive traits, because of their direct impacts in plant fitness (Grant 1956; Husband *et al.* 2008).

Changes in reproductive traits can involve changes in the morphology of floral traits and in physiological responses, such as incompatibility reactions. Among the direct effects of polyploidy are an increase in cell size and potentially in the overall size of the organs (Segraves *et al.* 1999; Levin 2002). This may have a significant effect on the structure of sexual organs in the flowers and, in animal-pollinated plants, the interactions with pollinators, the patterns of pollen deposition in pollinator's bodies, pollination efficiency and, consequently, the reproductive success of such individuals (Segraves and Thompson 1999). Changes in flower traits have been reported to occur along polyploid complexes. For example, in *Arrhenatherum elatius* flowering time was longer for tetraploids than diploids (Petit *et al.* 1997) and in *Larrea tridentata* differences in flowering time and number of flowers were also observed among diploids and tetraploids (Laport *et al.* 2016). Diploids of *Chamerion angustifolium* presented shorter petals and styles than diploids (Husband and Schemske 2000) and *Erysimum mediohispanicum* presented larger flowers in diploids (Muñoz-Pajares *et al.* 2018). Flowers from dodecaploids in *Primula* are reported to be larger than flowers from hexaploids (Casazza *et al.* 2017). Also, in *Heuchera grossulariifolia*, flowers from tetraploids and diploids had different sizes and shapes, as well different flowering times. Consequently, they can attracted a different set of pollinators (Segraves and Thompson 1999) and reduce opportunities for inter-cytotype mating (Husband and Schemske 2000; Laport *et al.* 2016).

Changes in floral traits will be particularly relevant in species with complex breeding systems, such as plants with heterostyly and other related stylar polymorphisms. This flower polymorphism is genetically based and determines the existence of two (distyly: long- and short-styled) or three (tristyly: long-, mid- and short-styled) morphs within populations. In

heterostylous plants, outcrossing and reduction of self-interference is achieved by reciprocal positioning of stamens and stigmas within a single flower (Darwin 1877; Barrett 2002b). Deviations from reciprocity (*i.e.*, similar position of reciprocal organs) can lower the probability of disassortative mating (*i.e.*, promote outcrossing) and seed production, or facilitate a breakdown of the floral polymorphism (Keller *et al.* 2014; Zhou *et al.* 2015b; Wu *et al.* 2018; Brys and Jacquemyn 2020). Despite some studies in polyploidy complexes demonstrated the impacts of WGD in organ size and the maintenance of the heterostylous system, to date, few studies explore how floral traits and levels of reciprocity are affected along ploidy levels. However, it has been demonstrated in hexaploids and dodecaploids of *Primula* that reciprocity is maintained among the ploidy levels (Casazza *et al.* 2017; but see Naiki 2012).

Additionally, WGDs are known to lead to a breakdown of self-incompatibility mechanisms, allowing the polyploid to self-reproduce in the initial stages of polyploid establishment, and, thus, better counter act minority cytotype exclusion (Levin 1975; Ramsey and Schemske 1998; Miller and Venable 2000; Baack 2005). Several studies associated polyploidy with increases in self-fertilization rates (Ramsey and Schemske 1998; Barringer 2007). Consequently, it has been suggested that polyploids self-fertilize more than their diploid relatives and that polyploidization may attenuate the levels of inbreeding depression, favoring selfing and its reproductive assurance advantage (Mable 2004; Barringer 2007). However, in some cases the advantages of selfing might only occur at the initial stages after polyploid emergence dissipate with repeated generations of self-fertilizations (Mable 2004; Husband *et al.* 2008), particularly if deleterious alleles are not purged. In *Chamerion angustifolium*, self-pollinated neotetraploids had lower inbreeding depression than established tetraploids. This can permit to neopolyploids siring offspring enough to reduce the problems of reproductive assurance in newly arisen polyploid individuals (Husband *et al.* 2008; Ozimec and Husband 2011; Siopa *et al.* 2020)

In heterostylous species, reciprocal herkogamy is usually associated with a heteromorphic self-incompatibility (SI) system that limits or prevents selfing and intra-morph mating. Consequently, compatible crosses occur only when stigmas are pollinated with the pollen grains of the other morph(s) (legitimate pollination; Darwin 1877; Dulberger 1992). Moreover, this SI is usually controlled by the sporophyte and has a diallelic system (Barrett 1992). Incompatibility responses in heterostylous plants can include lack of adhesion, hydration and germination of pollen, inability of pollen tubes to penetrate the stigmatic zone, and cessation of pollen tube growth in the style and ovary, although the initial stages are most frequent (Dulberger 1992). In distyly, the entire syndrome is controlled by a "supergene", with two alleles, and in tristyly the control is by genes at two loci, each with two alleles and epistatic

interaction (Lewis and Jones 1992). In most distylous species, the long-styled morph represents the recessive (ss) and the short-styled morph the heterozygous (Ss) genotype (Lewis and Jones 1992). Therefore, an advantageous short-styled variant should spread easily in populations, because all individuals with the dominant allele express the novel phenotype (Haldane 1926; Lewis and Jones 1992). The dominant homozygous conditions appears to be deleterious, which favor the expansion of the short-styled plants, but the genetic mechanism has not been fully resolved yet (Lewis and Jones 1992; Cocker *et al.* 2018; Yuan *et al.* 2019; Huu *et al.* 2020).

Heterostyly is maintained by negative frequency-dependent selection resulting from disassortative mating between the style morphs (Barrett and Shore 2008; Barrett 2013). Thus, at equilibrium, populations of distylous species are expected to show a 1:1 ratio of style morphs after full disassortative mating (Pannell *et al.* 2005). Biases in long- or short-styled morph ratios or populations fixed for a morph are associated with the breakdown of the distylous heteromorphic SI (*e.g.*, Yuan *et al.*; Arroyo *et al.* 2002; Ferrero *et al.* 2012; Simón-Porcar *et al.* 2015; Zhou *et al.* 2015b) and/or with random stochastic events, such as fluctuations in population size (*e.g.*, Brys *et al.* 2008), or founder events (*e.g.*, Eckert and Barrett 1992; Zhou *et al.* 2012, 2017; Ferrero *et al.* 2020). Some studies in heterostylous polyploid groups have demonstrated a high self-compatibility with genome duplications, although this is normally associated with the loss of the polymorphism (Kelso 1992; Tamari *et al.* 2001; Naiki 2012). Yet, it is also important to note that in some polyploid species of *Primula*, genome duplications did not lead to the breakdown of distyly (Casazza *et al.* 2017). A breakdown in morph-incompatibility or a different type of SI is also common (Arroyo *et al.* 2002; Ferrero *et al.* 2012; Costa *et al.* 2014; Simón-Porcar *et al.* 2015; Zhou *et al.* 2015b) and may allow the neopolyploid to strive and persist and at the same time to avoid inbreeding depression.

Distyly is widespread and very common in the genus *Linum*, being present in about 40% of the species (Rogers 1979). Also, since Darwin (1877), *Linum* is considered one the best examples to illustrate the functioning of floral polymorphisms. Distyly can be found in four out of five sections of the genus, namely *Linum*, *Syllinum*, *Dasylinum* and *Linastrum* (Rogers 1979; Dulberger 1981; Talebi *et al.* 2012; Maguilla *et al.* 2021a). Nevertheless, distyly in *Linum* is mostly restricted to Old-World Mediterranean regions (Maguilla *et al.* 2021a). In eastern Spain, *Linum suffruticosum s.l.* has been described as distylous and intramorph-incompatible (Rogers 1979). Besides being style polymorphic, a high cytogenetic diversity was found, with five major cytotypes, namely diploids, tetraploids, hexaploids, octoploids and decaploids being detected in nature (Afonso *et al.* 2020 in Chapter II). Most of the cytogenetic diversity was found in the Iberian Peninsula and less cytotypes in North Africa, with the remaining areas of the species distribution in Europe being characterized by homogeneously diploid populations, only. The

different ploidy levels are distributed parapatrically, being geographically structured and comprising several contact zones (Afonso *et al.* 2020 in Chapter II). However, due to the high morphological variability observed in natural populations the white flax group (*Linum tenuifolium s.l.*, to which *L. suffruticosum s.l.* belongs) has been subjected to several taxonomic treatments over the years (Jahandiez and Maire 1932; Emberger and Maire 1941; Quézel and Santa 1962; Ockendon and Walters 1968; López González 1979; Fennane *et al.* 2007; Valdés *et al.* 2007; Martínez-Labarga and Garmendia 2015). The most recent treatment of the group of *L. suffruticosum s.l.* records high levels of variability and recognizes morpho-geographical divisions in the Iberian Peninsula (Martínez-Labarga and Garmendia 2015). However, such treatment does not always work at a wider scale given the continuum of morphological variability.

The main objectives of this study were to understand how the reproductive traits of *Linum suffruticosum s.l.* vary with WGDs and assess the reproductive relationships among some of the cytotypes. In particular, we addressed the following questions: 1) Is the floral polymorphism (in morphology and reproductive physiology) maintained with WGDs? I was interested in testing if genome duplications led to a predicted breakdown of polymorphisms, 2) Are populations in equilibrium, *i.e.*, isoplethic? I hypothesized that the breakdown of the distylous syndrome may drive biased morph frequencies, and that stochastic forces would likely contribute towards greater variance in morph ratios in smaller than larger populations, 3) Does WGDs drive changes in floral traits? Since genome duplication could led to a gigas effect, I hypothesized that genome duplications will lead to bigger flowers and sexual organs, 4) Do these differences mediate reproductive isolation among cytotypes? I hypothesized that if there are great differences between cytotypes, these differences could lead to morphological reproductive isolation between cytotypes, 5) are diploids and tetraploids from a parapatric contact zone able to cross? I hypothesized that inter-cytotype crosses would produce similar fitness results when compared with legitimate intra-cytotype crosses. To achieve these objectives, I recorded morph frequencies and performed floral morphometric measures in flowers from populations of each ploidy level to evaluate morphological differences. I also performed inter- and intra-morph crosses and self-pollination within each cytotype to evaluate possible impacts of WGDs in reproductive isolation, and inter-morph crosses between diploids and tetraploids to evaluate relationships between cytotypes.

Materials and methods

Study system

Linum suffruticosum s.l., a diploid-polyploid complex distributed throughout the Mediterranean Basin, has been included for a long time within the *L. tenuifolium* complex along

with the species *L. tenuifolium* (a monomorphic and apparently self-compatible specie; Ockendon and Walters 1968; López González 1979; Nicholls 1986a), having been later separated and undergoing several taxonomic changes over the years (Jahandiez and Maire 1932; Emberger and Maire 1941; Quézel and Santa 1962; Ockendon and Walters 1968; López González 1979; Fennane *et al.* 2007; Valdés *et al.* 2007; Martínez-Labarga and Garmendia 2015). This complex bears five main cytotypes [diploids (2n = 2*x* = 16 or 18 chromosomes), tetraploids (2n = 4*x* = 36 chromosomes, rarely 32 or 38), hexaploids (2n = 6*x* = 54 chromosomes, and occasionally 48 chromosomes), octoploids (2n = 8*x* = 72 chromosomes) and decaploids (2n = 10*x* = 90 chromosomes); Afonso *et al*., 2020 in Chapter II]. In contrast with *L. tenuifolium, L. suffruticosum s.l.* is described as obligate outcrosser (vegetative propagation is almost negligible) with heterostylous populations comprising both long- and short-styled morphs (Figure 4.1), which are strongly self-incompatible, although data were limited in the number of plants and populations analyzed (Rogers 1979; Nicholls 1986a; Ruiz-Martín 2017). As a result of a heteromorphic self-incompatibility system associated with distyly, successful crosses are only possible after pollination between different floral morphs (Figure 4.1) (Nicholls 1985c, 1986a). Additionally, it has been shown that the anthers and stigmas show reciprocity in three dimensions: styles in the center versus in outer parts of the sexual verticils of the flower, introrse versus extrorse anthers, in addition to the most common spatial arrangement of anthers and stigmas, *i.e.*, differing reciprocally in height (Armbruster *et al.* 2006). These authors suggested that the stigmas of the short-styled morph contact the ventral side of specific pollinators, while those from the long-styled morph contact the dorsal side of these pollinators. By opposition, the pollen from the short-styled morph is placed in the dorsal side of the pollinators, while that from the long-styled morph is placed in the ventral side. This is the result of differences, not only in the angle of divergence of the styles and stamens from the central axis of the flower, but also in the degree of rotation of the styles and filaments (Armbruster *et al.* 2006). The taxonomic treatment followed by Afonso *et al.* (2020), in Chapter II, that considers the high morphological diversity in association with cytogenetic variability of this complex, was used (Chapter II). Also, populations with more differentiating characters from the rest of the complex were excluded from this study, namely, *L. suffruticosum* var. *milletii* (Sennen & Gonzalo - G. López), an easily distinguishable variety from Catalonia, and *L. salsoloides*, described for France and Italy (Ockendon and Walters 1968). Therefore, the populations used in this study were those treated as *L. suffuticosum*, *L. appressum-salsoloides* or as intermediate individuals between these entities (Afonso *et al*., 2020 in Chapter II).

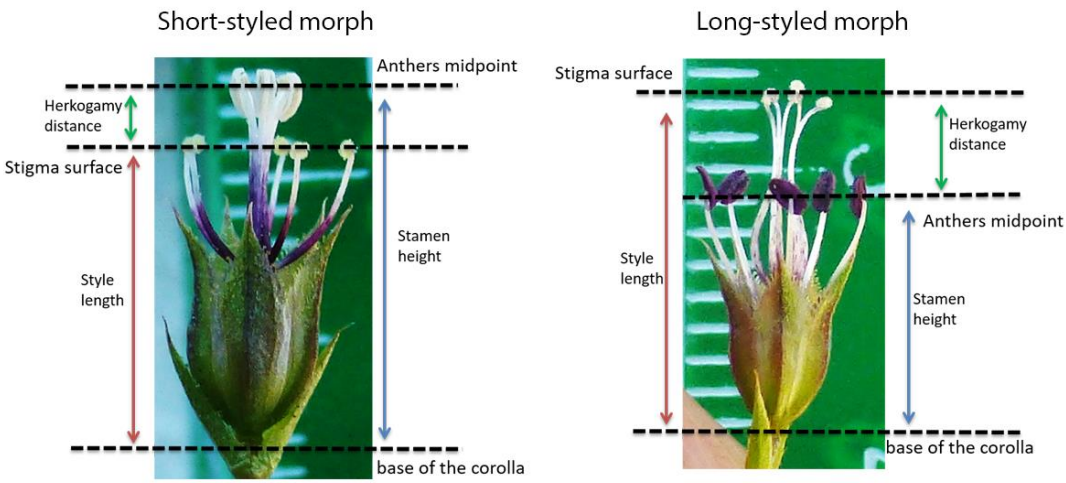

Figure 4.1. Picture of the two floral morphs with measures of style length, anther height and herkogamy distance for short- and long-styled morph**.**

Morph frequencies and morphometric analyses

A total of 92 populations of *L. suffruticosum s.l.* were sampled, including 21 diploid, 19 tetraploid, 23 hexaploid, 18 octoploid and 11 decaploid populations from Europe and Northwestern Africa (Figure 4.2). The cytotype of each population was obtained from Afonso *et* al. (2020), in Chapter II. Population sizes were characterized in the following categories: ≤50, >50 and ≤100, >100 and ≤500, >500 and ≤1000, >1000 and ≤5000, and >5000 and ≤10000 individual plants. Morph frequencies were recorded in each population by visually identifying the floral morph of up to 50 individuals (when possible). Deviations from isoplethy (*i.e.*, 1:1 proportion of short- and long-styled morphs) were tested using G-tests for goodness-of-fit with Yates correction (Zar 2010). We also investigated the relation between deviations from isoplethy and population size categories using a Spearman correlation. Descriptive statistics (mean and standard deviation) were calculated for proportion of short- and long styled morphs for each population and ploidy level.

Floral morphometric analyses were performed in 19 diploid, 19 tetraploid, 21 hexaploid, 17 octoploid and 10 decaploid populations. In the field, at least 30 flowers (one per individual) per morph (when available) were collected and stored in ethanol 70%. In the laboratory, up to 15 flowers per morph were photographed, and style length (from the base of the corolla up to the stigma), and stamen height (from the base of the corolla up to the midpoint of the anthers insertion, Figure 4.1) were measured using ImageJ software (Rasband 2008). Flowers stored in ethanol were used because of logistic constraints in sampling a high number of populations. Consequently, I did not examined variations in 3D distyly since ethanol storing may provoke variations in the position of styled and bending of anthers. Yet, the main goal was to compare

reciprocal levels between morphs and, thus, my approach still enabled to efficiently collect the necessary information. Herkogamy distances were calculated for both morphs by subtracting style length and anthers height. The reciprocity index was calculated for each population using the quantitative indexes from Sánchez *et al.* (2013) and from Armbruster *et al*. (2017). The reciprocity index from Sánchez *et al.* (2013) compares stigma–stamen height gaps for all potential crosses in the population. This index considers stigma–stamen distance as well as dispersion and it is not skewed by favoring the more prevalent morph. The index from Armbruster *et al*. (2017) calculates inaccuracy, which in reciprocity is estimated as the contribution of differences in mean length/height of reciprocal organs and imprecision (variance) between reciprocal organs (Armbruster *et al*., 2017). The adaptive optimum of an anther level is represented by the population mean of the reciprocal stigma and vice versa; if all anthers and stigmas of a population are at the same height, inaccuracy will be zero. Therefore, low values of inaccuracy correspond to high levels of reciprocity (low dispersion around the optimal values) (Armbruster *et al.* 2017). Population mean and variance of each organ type were used to calculate inaccuracy values for high organs [stigmas of long-styled morph (St) and anthers of short-styled morph (A)] and low organs [anthers of long-styled morph (a) and stigmas of short-styled morph (st)]. The results of high and low organ inaccuracies were summed to provide a total inaccuracy value per population. To compare levels of inaccuracy between populations, values were standardized by the squared mean of all anther and stigma heights recorded for each population and adjusted to a proportional scale (Armbruster *et al.* 2017).

To assess differences among cytotypes in morph frequency, style length, anther height, herkogamy and reciprocity index, separated generalized linear models were used for each response variable (Bolker *et al.* 2009), with a Gaussian distribution and an identity link function to model the responses. To test differences in morph frequency, cytotype was used as factor and the frequency of short- and of long-styled morphs as response variables. To test differences in the measures of sexual organs, cytotype, morph and sexual organ (style or anther) were used as factors and the measures of style length and anther height as response variables. To test differences in herkogamy, morph nested within cytotype were used as factors. Finally, to test differences in the reciprocity index and inaccuracy, cytotype was used as factor. Morph frequency and reciprocity indexes were transformed with the arcsine of the square root to achieve normality and homoscedasticity. Statistical analyses were performed in R software v.3.6.1 (R Core Development Team 2019), using the packages “stats” for Spearman correlation (Best and Roberts 1975), *car* for Type-III analysis of variance (Fox *et al.* 2005), *glm* for generalized linear models (Hastie and Pregibon 1992) and *multcomp* for multiple comparisons after Type- III analysis of variance (Hothorn *et al.* 2017). Descriptive statistics (mean and standard deviation of

the mean) were also calculated for style length, anther height, and inaccuracy of low organs, inaccuracy of high organs, total inaccuracy and standardized inaccuracies.

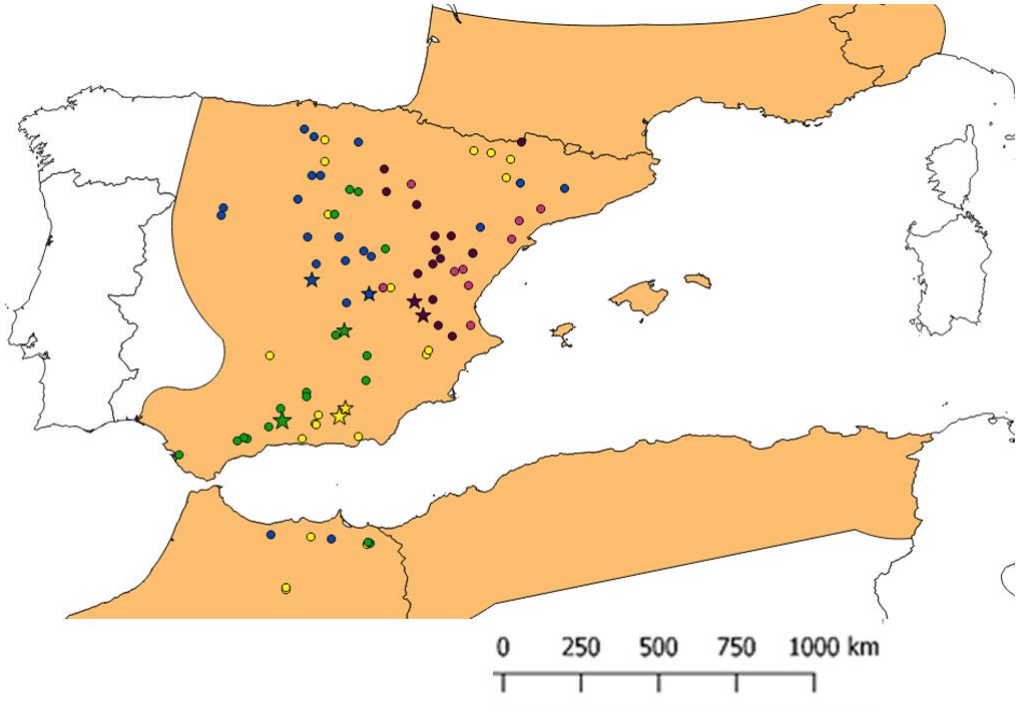

Figure 4.2. Geographical distribution of *Linum suffruticosum s.l.* (orange) and all populations sampled for flower measurements (circles) and crosses (stars) with the respective ploidy (diploid: yellow; tetraploid: green; hexaploid: blue; octoploid: purple; decaploid: pink). The base map was downloaded from https://www.diva-gis.org/gdata.

Self- and morph-incompatibility

Controlled hand-pollinations were carried in 2019, during the species flowering period (May-July), in 10 populations (2 populations per cytotype; Figure 4.3a), to quantify self- and morph-incompatibility levels of the different cytotypes. The following treatments were applied to plants collected in the field in each population: self-pollination – flower pollinated with its own pollen; intra-morph pollination – pollination with pollen from 1-3 different individuals of the same morph and population; and inter-morph pollination – pollination with pollen from 1-3 individuals of the other floral morph of the same population (Figure 4.3a). The pollination experiment had to be designed to accommodate the limitations related with plant biology and location of the populations from different cytotypes. In particular, we were unable to grow adult plants collected in the field and those obtained from seed germination died at young age, most probably due to inappropriate edaphoclimatic conditions. Also, populations of the different cytotypes are located relatively far away from each other, making it difficult to perform manipulations directly in the field in a representative number of populations. Thus, we designed

an experiment using flowering shoots maintained in a makeshift greenhouse and performed pollinations and collected samples within 24h to 48h after shoot collection to avoid methodological effects of using cuttings. During this period, the plant cutting maintained the vigor and flowers developed naturally. In this experimental design, our response variable was pollen tube development, which for heteromorphic sporophyte SI is adequate, as the incompatibility reaction commonly occurs at the stigma rather than along the style (Allen and Hiscock 2008).

In each population, plant shoots with flower buds about to open were cut and harvested, always in the early morning. The flowering shoots were identified and kept in nutrient solution and excluded from pollinators until the next day. In each population, at least 30 individuals (when possible) with various flowers were collected to serve as pollen donors, and at least 15 different individuals of each morph were collected to serve as pollen recipients. The latter were marked and carefully separated by individual plant. Crossings were always done the next day in a makeshift greenhouse and on freshly opened flowers. Crosses were done on virgin flowers when stigmas become receptive (*i.e.*, when stigmas start to swell), about 2-3 hours after flower opening. The petals of recipient flowers were removed, and the five stigmas were pollinated by detaching the anthers of the male donor and brushing them over the projecting stigmas of the recipient flower. For cross-pollinations, the recipient flowers were first emasculated. Preliminary observations revealed that flower development of the different cytotypes was different, with increased ploidy leading to increased robustness and longer flower lifespan (A. Afonso, personal observations). Thus, the timing for flower collection after pollination had to be adjusted to each cytotype and was defined based on the mean time needed for flower wilting for each cytotype. After 8h for diploid flowers and 20 to 24h for polyploid flowers, the ovary started to swell and the styles to wilt, and pistils were cut and harvested in ethanol 70% to assess pollen tube development in the style. In the laboratory, stigmas and styles were softened with 8 N sodium hydroxide for 10 to 60 min depending on the cytotype (diploids: 10 min; tetraploids: 20 min; hexaploids: 30 min; octoploids: 45 min and decaploids: 60 min), due to differences in flower size and robustness, which increased with increased ploidy level. Then, stigmas and styles were washed in distilled water and placed overnight in 0.05% (w/v) aniline blue prepared in 0.1 N potassium phosphate (Dafni *et al.* 2005). The styles were placed on a microscope slide with a drop of glycerin 50%, squashed beneath a coverslip and observed using a Nikon Eclipse 80i epifluorescence microscope (Nikon Instruments, Kanagawa, Japan) with the UV-2A filter cube. Pollen tube development along the style was assessed by counting the number of pollen tubes in three places of the style, namely in the top, middle and bottom level of the style (Figure 4.4). The mean number of ovules of each floral morph was also assessed in

at least 30 flowers from different individuals in each population. The incompatibility index (self-incompatibility index and morph-incompatibility index) was calculated for each treated flower and style as: 1- [expected pollen tubes/observed pollen tubes] that reached the bottom of the style (expected pollen tubes calculated as the mean number of pollen tubes at the bottom of the style for legitimate crosses in each cytotype). Following this index, values close to 1 indicate high incompatibility levels and values close to 0 indicate low incompatibility levels, *i.e.*, self or morph compatibility. This incompatibility index assumes that incompatible pollen fails to adhere to incompatible stigmas preventing pollen germination and pollen-tube growth; thus, it assumes that pollen tubes reaching the bottom of the style will sire viable seeds in healthy plants in the wild (Dulberger 1975a, 1992).

To assess differences among cytotypes and treatments in the number of pollen tubes for all treatments, separated generalized linear models were used (Bolker *et al.* 2009), with a Poisson distribution and a Log link function to model the responses. The number of pollen tubes was used as response variable in three different analyses. First, I tested the overall differences in the number of pollen tubes between treatments at each level of the style (*i.e.*, top, middle, bottom). Second, we tested differences in the number of pollen tubes between treatments nested within cytotype at the top, middle and bottom level of the style. And third, we tested differences in the number of pollen tubes among cytotypes, morphs and their interaction at the top, middle and bottom level of the style. Finally, to assess differences among cytotypes and self- and morph incompatibility indexes, separated generalized linear models were used (Bolker *et al.* 2009), with a Gaussian distribution and an identity link function to model the responses. Cytotype was used as factor and incompatibility index as response variable, previously transformed with the arcsine of the square root to achieve normality and homoscedasticity. Statistical analyses were performed in R software v.3.6.1 (R Core Development Team 2019), using the packages *car* for Type-III analysis of variance (Fox *et al.* 2005), *glm* for generalized linear models (Hastie and Pregibon 1992) and *multcomp* for multiple comparisons after Type- III analysis of variance (Hothorn *et al.* 2017). Descriptive statistics (mean and standard deviation of the mean) were also calculated for self- and morph incompatibility indexes.

Inter-cytotype cross ability

Crosses between one diploid and one tetraploid population were performed in the contact zone of these two cytotypes in the south of Spain (Figure 4.2). We performed four inter-cytotype crosses (recipient x donor plant): 1) 2*x* short-styled × 4*x* long-styled; 2) 4*x* short-styled × 2*x* long-styled; 3) 2*x* Long-styled morph × 4*x* short-styled; 4) 4*x* Long-styled morph × 2*x* short-styled (Figure 4.3b). Additionally, inter-cytotype crosses within the same morph were performed

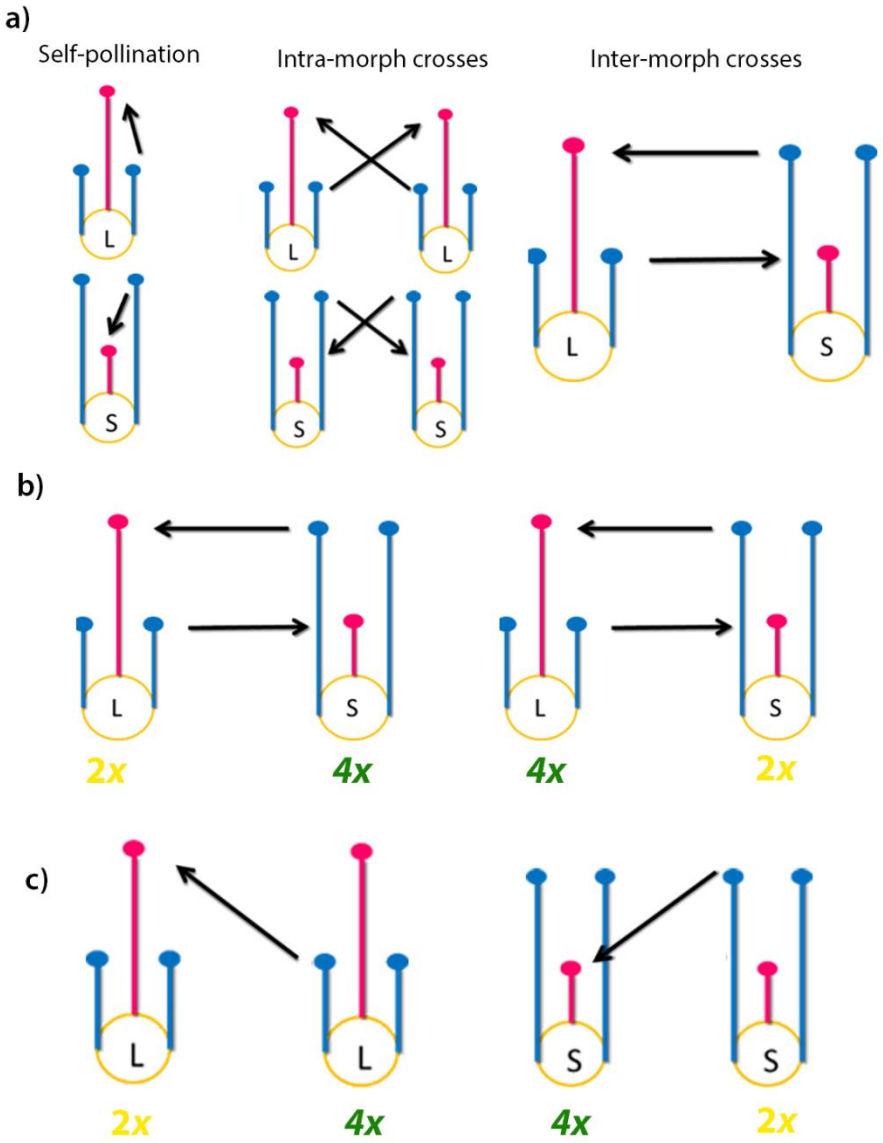

Figure 4.3. Design of crosses in *Linum suffruticosum s.l.* within the same cytotype (a): self-pollination – flower pollinated with its own pollen, intra-morph pollination – pollination with pollen from the same morph, and inter-morph pollination – pollination with pollen from the other floral morph; and crosses between diploids and tetraploids (b): inter-morph crosses between the long-styled morph of diploids and the short-styled morph of tetraploids, and between the long-styled morph of tetraploids and the short styled morph of diploids, (c) intra-morph crosses between the long-styled morph of diploids (recipient) and tetraploids (donor), and between the short-styled morph of diploids (donor) and tetraploids (recipient). Abbreviations: S, long-styled morph; L, short-styled morph; 2*x*, diploid; 4*x*, tetraploid. Ovary – yellow circles, pistils – pink lines, and anthers – blue lines.

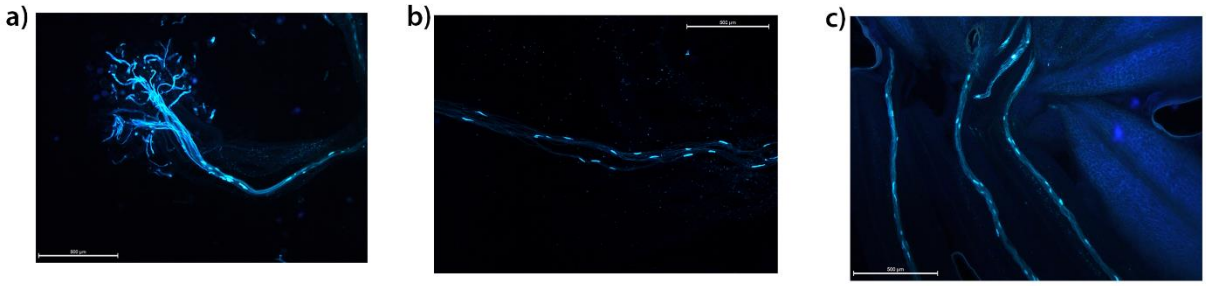

Figure 4.4. Pollen tube grow observations in the top (a), midlle (b) and bottom (c) of the style of *L. suffruticosum s.l.* (scale 500 µm).

(recipient x donor plant): 1) 2*x* long-styled × 4*x* long-styled and 2) 4*x* short-styled × 2*x* short-styled morph (Figure 4.3c). Pollinations and pistil processing were made as described above. Finally, an index of reproductive isolation (IRI) for the intercytotype crosses (*i.e.*, 2*x* × 4*x* and 4*x* × 2*x*) was calculated as: 1 - (number of pollen tubes of intercytotype crosses / number of pollen tubes of intracytotype crosses). This index ranges from 0 (no reproductive isolation) to 1 (high reproductive isolation).

To assess differences in the number of pollen tubes between treatments at each level of the style (*i.e.*, top, middle, bottom), generalized linear models were used (Bolker *et al.* 2009), with a Poisson distribution and a Log link function to model the response variables. Pollination treatments was used as factor and number of pollen tubes at each level of the style as response variable. Statistical analyses were performed in R software v.3.6.1 (R Core Development Team 2019), using the packages *car* for Type-III analysis of variance (Fox *et al.* 2005), *glm* for generalized linear models (Hastie and Pregibon 1992) and *multcomp* for multiple comparisons after Type- III analysis of variance (Hothorn *et al.* 2017). Descriptive statistics (mean and standard deviation) were calculated for pollen tubes in each style level.

Results

Morph frequency and sex organ morphometry

All the populations sampled maintained the style polymorphism regardless of the cytotype. Of the 92 populations sampled, 90% were isoplethic ($P > 0.05$, Appendix 4.1) and the frequencies of short- and long-styled morphs did not differ among cytotypes (long-styled morph: $F_{4, 87} = 0.74$, $P > 0.05$, short-styled morph: $F_{4, 87} = 0.74$, $P > 0.05$, Appendix 4.2, Table 4.1). Of the nine anisoplethic populations, six had a higher frequency of long-styled plants (four 2*x*, one 4x, and one 6*x*), and three had a higher frequency of short-styled ones (one 2*x*, and two 6*x*). All these populations had small-intermediate (>50 and ≤100 and >100 and ≤500) numbers of individuals; indeed, small (≤50 individual plants) and large (>500 and ≤1000, >1000 and ≤5000,

and >5000 and ≤10000 individual plants) populations had a tendency to be isoplethic, while intermediate populations presented more deviant values. No correlation between population size and frequency of morphs was found (r = -0.019, *P* > 0.05; Figure 4.5, Appendix 4.1).

Table 4.1. Population morph frequencies for each cytotype of *Linum suffruticosum s.l.*. The number of populations sampled (N population), mean and standard deviation of the frequency of short- (S frequency) and long-styled (L frequency) morph, and total number of individuals of each morph (N S, total number of short-styled morph individuals; N L total number of long-style morph individuals) are also provided. Abbreviations: n.s. non-significant differences at *P* > 0.05 for Type-III analysis of variance; 2*x*, diploid; 4*x*, tetraploid; 6*x*, hexaploid; 8*x*, octoploid; 10*x*, decaploid.

Ploidy level	N population	S frequency – mean ± SD	N S	L frequency – mean ± SD	N L
2*x*	21	0.49 ± 0.08 $^{n.s}$.	782	0.51 ± 0.08 $^{n.s}$	817
4*x*	19	0.50 ± 0.07 $^{n.s}$	730	0.50 ± 0.07 $^{n.s}$	714
6*x*	23	0.52 ± 0.08 $^{n.s}$	969	0.48 ± 0.08 $^{n.s}$	836
8*x*	18	0.52 ± 0.05 $^{n.s}$	637	0.48 ± 0.05 $^{n.s}$	593
10*x*	11	0.52 ± 0.06 $^{n.s}$	380	0.48 ± 0.06 $^{n.s}$	345

Significant differences were observed in stigma and anther lengths of short- and long-styled morphs and among cytotypes ($F_{1, 4837}$ = 113.43, *P* < 0.001; $F_{1, 4837}$ = 32.20, *P* < 0.001; $F_{4, 4837}$ = 314.04, *P* < 0.001, respectively, Appendix 4.2; Figure 4.6). The size of male and female organs increased with increased ploidy level, and this increase was more evident for the long-styled than for the short-styled morph (Figure 4.6, Table 4.2). Hexaploids showed the highest variability in the length of male and female organs. When comparing reciprocal levels (*i.e.*, stigma versus anther heights of long- and short-styled morphs, and anthers versus stigma heights of long- and short-styled morphs) within cytotype, most of them differed significantly (*P* < 0.05), except for the long-styled anther and short-styled stigma heights for tetraploids, hexaploids and octoploids *(P* > 0.05). When comparing reciprocal levels among cytotypes, similar heights were observed between different cytotypes; namely, stigma height of long-styled morph was similar to anthers height of short-styled morph between diploids and tetraploids, tetraploids and hexaploids, and hexaploids and octoploids, respectively; and stigma height of short-styled morph was similar to anthers height of long-styled morph between tetraploids and diploids, hexaploids and octoploids, and octoploids and hexaploids, respectively (Figure 4.5).

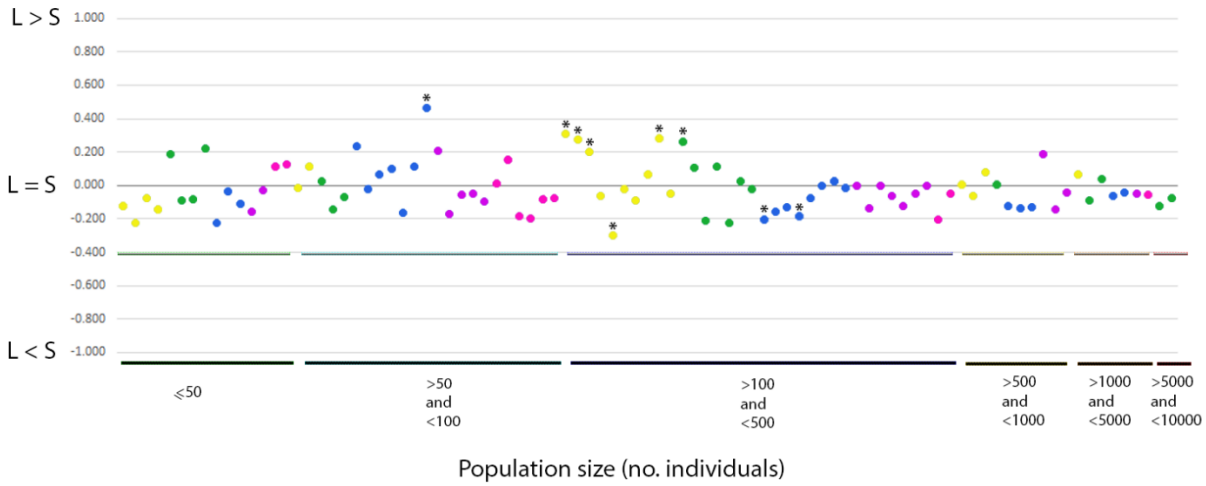

Figure 4.5. Proportion of morphs in *Linum suffruticosum s.l.* populations (calculated as the subtraction of frequency of long-styled morph (L) by the frequency of short-styled morph (S)), according with categories of population size and ploidy level (indicated by circle color: diploid – yellow, tetraploid – green, hexaploid – blue, octoploid – purple and decaploid – pink). *indicates anisoplethic populations ($P < 0.05$) after G-tests for goodness-of-fit with Yates correction.

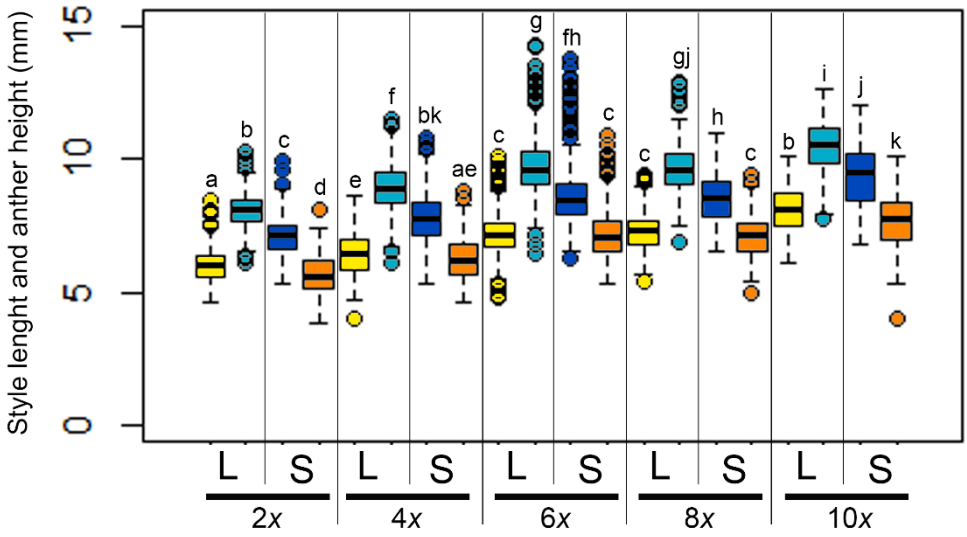

Figure 4.6. Style length (long-styled morph – light blue; short-styled morph – dark blue) and anthers height (long-styled morph – yellow; short-styled morph – orange) range with upper and lower quartiles and mean (black line) in short- (S) and long-styled (L) morphs of diploid (2*x*), tetraploid (4*x*), hexaploid (6*x*), octoploid (8*x*) and decaploid (10*x*) populations of *Linum suffruticosum s.l.*. Outliers are also given as circles. Different letters correspond to statistically significant differences at $P < 0.05$ for Type- III analysis of variance.

Table 4.2. Measurements of style length, anther height, herkogamy, reciprocity index by Sanchez *et al*. (2013), inaccuracy (Armbruster *et al*. 2017) of high and low organs and total inaccuracy (in units of mm^2 and percentage standardized), number of populations sampled of each cytotype, number of flowers sampled, mean and standard deviation for short- and long-styled morph of *Linum suffruticosum s.l.*. Abbreviations: 2*x*, diploid; 4*x*, tetraploid; 6*x*, hexaploid; 8*x*, octoploid; 10*x*, decaploid; N population, total number of populations; N flowers, total number of flowers (corresponding also to number of individual plants sampled).

Ploidy level	Morph	N population	N flowers	Style length mean ± SD	Anther lenght mean ± SD	Herkogamy distance mean ± SD	IR	Inacurrancy high organs mean ± SD (mm^2)	Standardized inacurrancy high organs mean ± SD (%)	Inacurrancy low organs mean ± SD (mm^2)	Standardized inacurrancy low organs mean ± SD (%)	Total inacurrancy mean ± SD (mm^2)	Standardized total inacurrancy mean ± SD (%)
2*x*	L	19	259	8.05 ± 0.68	6.00 ± 0.62	2.05 ± 0.30	0.67 ± 0.07	1.64 ± 0.92	3.72 ± 2.26	0.63 ± 0.28	1.43 ± 0.71	2.27 ± 1.09	5.15 ± 2.71
	S		248	5.62 ± 0.72	7.11 ± 0.76	1.49 ± 0.52							
4*x*	L	19	266	8.96 ± 0.88	6.45 ± 0.79	2.52 ± 0.67	0.61 ± 0.08	2.68 ± 1.47	5.25 ± 3.23	0.8 ± 0.42	1.53 ± 0.89	3.48 ± 1.74	6.78 ± 3.88
	S		258	6.25 ± 0.84	7.79 ± 1.02	1.54 ± 0.61							
6*x*	L	21	282	9.80 ± 1.21	7.30 ± 0.89	2.50 ± 0.64	0.63 ± 0.09	2.27 ± 0.62	3.44 ± 1.08	0.73 ± 0.20	1.11 ± 0.37	3.00 ± 0.69	4.55 ± 1.29
	S		263	7.21 ± 0.98	8.73 ± 1.31	1.52 ± 0.70							
8*x*	L	17	252	9.64 ± 0.91	7.28 ± 0.76	2.36 ± 0.58	0.62 ± 0.08	2.29 ± 1.02	3.54 ± 1.64	0.88 ± 0.40	1.33 ± 0.52	3.18 ± 1.29	4.87 ± 2.00
	S		254	7.08 ± 0.79	8.55 ± 0.92	1.47 ± 0.61							
10*x*	L	10	136	10.42 ± 1.00	8.03 ± 0.93	2.39 ± 0.57	0.58 ± 0.07	2.08 ± 0.73	2.8 ± 1.15	1.12 ± 1.02	1.46 ± 1.13	3.20 ± 1.69	4.27 ± 2.31
	S		141	7.63 ± 0.95	9.34 ± 1.12	1.70 ± 0.62							

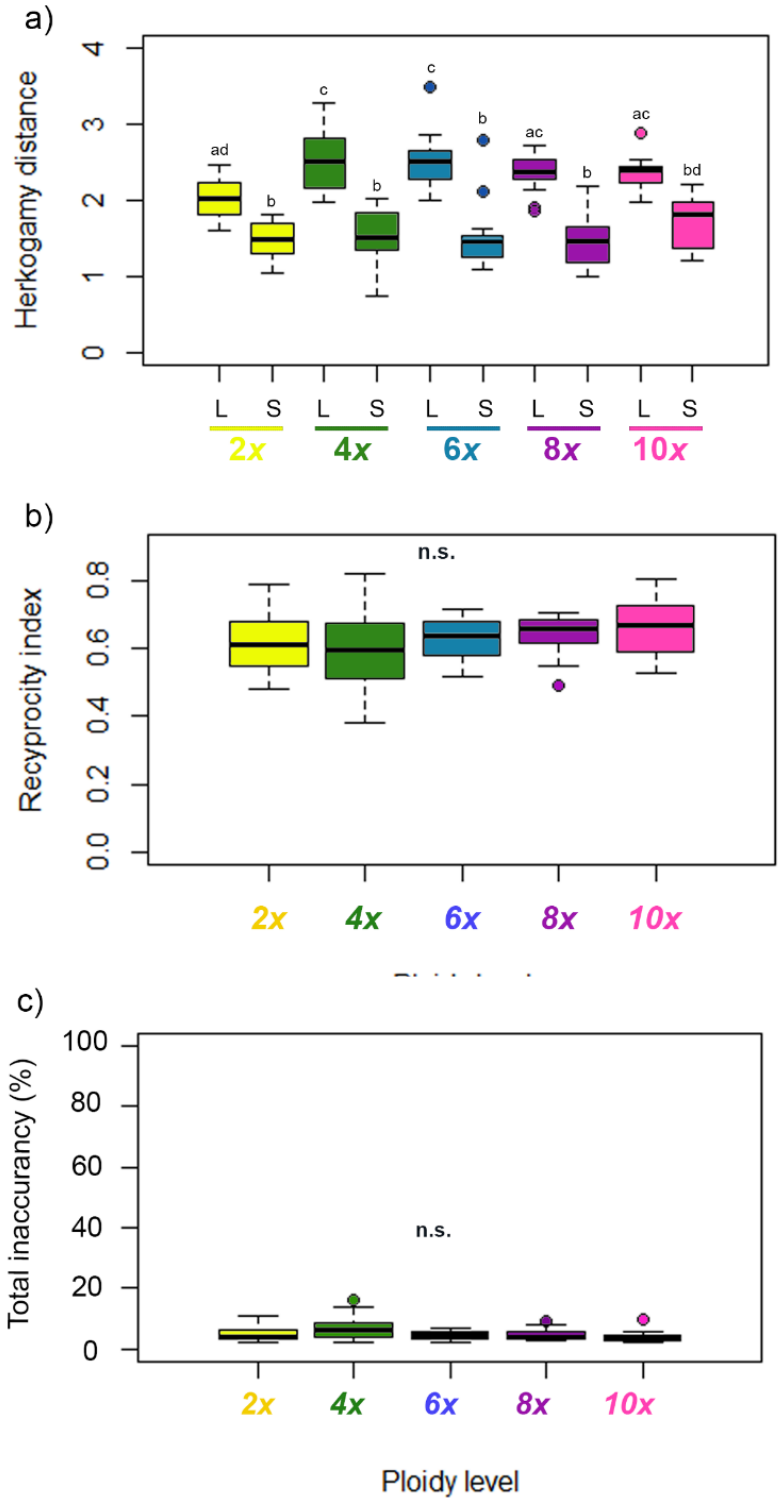

Figure 4.7. Herkogamy distance (anther-stigma separation) (a) reciprocity index of Sánchez et al (2013) (b), and total inaccuracy values following Armbruster *et al*. (2017) (c) of diploid (2*x*), tetraploid (4*x*), hexaploid (6*x*), octoploid (8*x*) and decaploid (10*x*) populations of *Linum suffruticosum s.l.*. Boxplot represent range with upper and lower quartiles and mean (black line), and outliers are provided as circles. Different letters correspond to statistically significant differences at $P < 0.05$ for Type-III analysis of variance, and n.s. non-significant differences at $P > 0.05$.

Significant differences were observed in herkogamy between floral morphs and cytotypes ($F_{4, 162}$ = 7.47, P < 0.001, $F_{5, 162}$ = 60.26, P < 0.001, respectively; Figure 4.7a, Table 4.2, Appendix 4.2). Herkogamy was always significantly higher in the long-styled morph than in the short-styled one. In the latter, anthers and stigmas are spatially nearer to each other than in the long-styled morph. Herkogamy in short-styled morph was similar in all cytotypes and the highest levels of herkogamy were found in the long-styled morph of tetraploids. No significant differences were observed in the reciprocity index from Sanchez et al. (2013) among cytotypes ($F_{4, 81}$ = 1.55, P > 0.05, Figure 4.7b, Appendix 4.2). Also, no differences were found in the total inaccuracy (index from Armbruster *et al*. 2017, $F_{4, 81}$ = 2.15, P > 0.05, Figure 4.7c, Appendix 4.2), with tetraploids showing the highest values and decaploids showing the lowest values, and with inaccuracy of high organs being higher than the inaccuracy of low organs in all cytotypes (Figure 4.7c, Table 4.2, Appendix 4.3).

Self- and morph-incompatibility

The number of developed pollen tubes differed significantly among treatments ($F_{2, 4156}$ = 2368.80, P < 0.001, $F_{2, 4149}$ = 1885.80, P < 0.001, $F_{2, 4040}$ = 978.14, P < 0.001, for top, middle, bottom of the style, respectively; Appendix 4.2, Figure 4.8a). The number of pollen tubes decreased along the style (from the top to the bottom of the style, *e.g*., Figure 4.4) in all three treatments, and inter-morph crosses had significantly more pollen tubes than self- and intra-morph crosses (P < 0.05). In self- and intra-morph crosses, pollen tube development was very low, with very few pollen tubes reaching the bottom of the style (Figure 4.8a).

Significant differences were also found among cytotypes within each treatment in the top ($F_{2, 4144}$ = 478.18, P < 0.001, $F_{12, 4144}$ = 17.98, P < 0.001, respectively), in the middle ($F_{2, 4137}$ = 392.45, P < 0.001, $F_{12, 4137}$ = 21.45, P < 0.001, respectively) and in the bottom of the style ($F_{2, 4028}$ = 186.73, P < 0.001, $F_{12, 4028}$ = 9.26, P < 0.001; respectively; Appendix 4.2, Figure 4.8b). In inter-morph crosses (legitimate pollinations), tetraploids had more pollen tubes developed than the other cytotypes at all levels of the style, while hexaploids and octoploids had less pollen tubes than the other cytotypes at the middle and bottom of the style (Figure 4.8b). In self and intra-morph crosses (illegitimate pollinations), the lowest pollen tube development was observed in hexaploids and decaploids (Figure 4.8b). When exploring differences between floral morphs across ploidy levels, significant differences were observed in the number of pollen tubes developed among cytotypes in all levels of the stigma for self-pollination, but only at the top and bottom levels of the style there were significant differences in both long- and short-styled morphs within cytotypes (top: $F_{4, 1456}$ = 3.32, P < 0.01, $F_{1, 1456}$ = 1.06, P > 0.05, $F_{4, 1456}$ = 3.85, P <

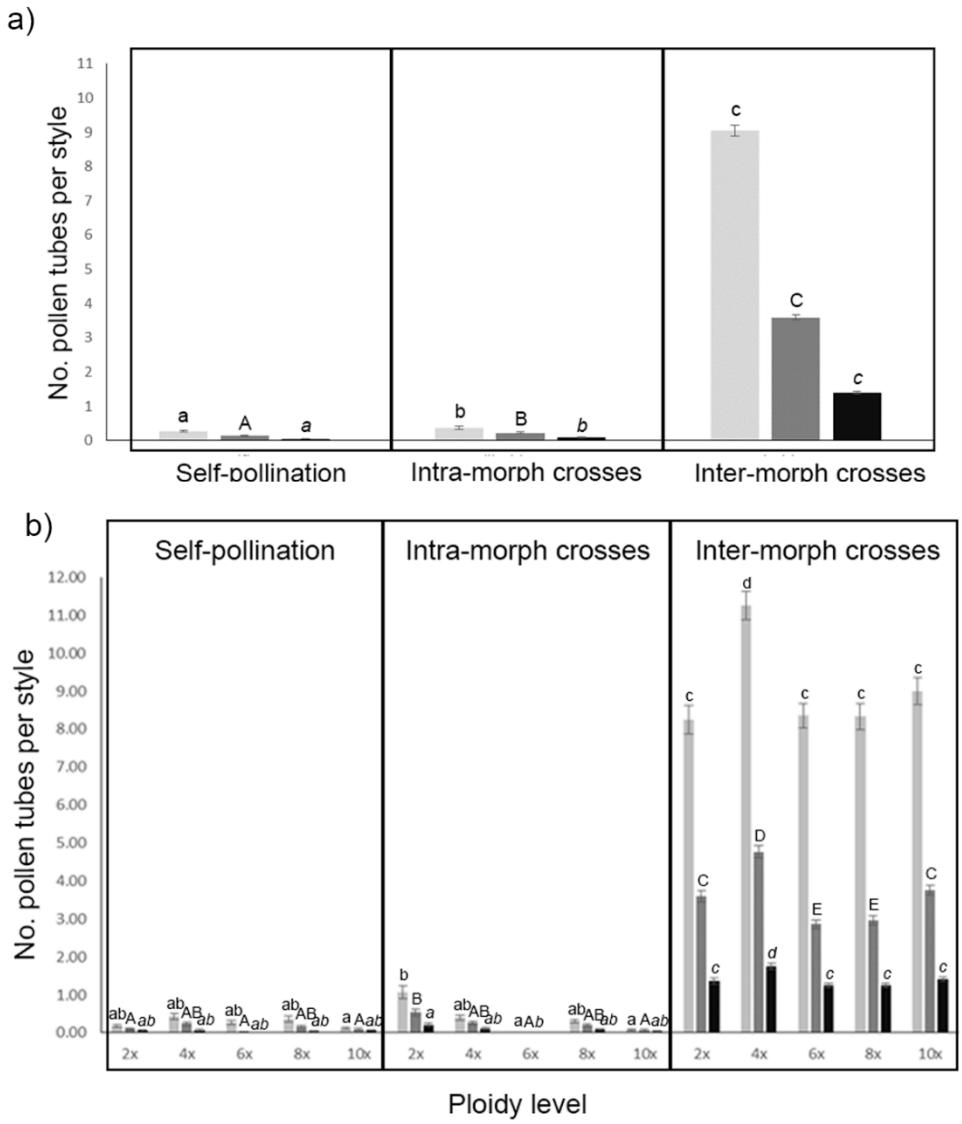

Figure 4.8. Average number of pollen tubes in the top (light grey), middle (dark grey) and bottom (black) level of the style in self-pollination, intra-morph crosses and inter-morph crosses (a), and in self-pollination, intra-morph crosses and inter-morph crosses of diploid (2*x*), tetraploid (4*x*), hexaploid (6*x*), octoploid (8*x*) and decaploid (10*x*) flowers (b) of *Linum suffruticosum s.l.*. Different letters correspond to statistically significant differences at $P < 0.05$ for Type-III analysis of variance: lowercase letters correspond to the top level of style, uppercase letters to the middle level, and letters in italic for the bottom level.

0.001, for cytotype, morph and the interaction of cytotype and morph, respectively; middle: $F_{4, 1456} = 6.17$, $P < 0.001$, $F_{1, 1456} = 1.81$, $P > 0.05$, $F_{4, 1456} = 2.26$, $P > 0.05$, for cytotype, morph and the interaction of cytotype and morph, respectively; bottom: $F_{4, 1449} = 5.36$, $P < 0.001$, $F_{1, 1449} = 3.27$, $P > 0.05$, $F_{4, 1449} = 4.55$, $P < 0.01$, for cytotype, morph and the interaction of cytotype and morph, respectively; Appendix 4.2, Figure 4.9a). In intra-morph crosses, significant differences were found in all levels of the stigma among cytotypes and for both morphs (top: $F_{4, 1382} = 47.78$, $P < 0.001$, $F_{1, 1382} = 73.03$, $P < 0.001$, $F_{4, 1382} = 2.89$ $P < 0.01$, for cytotype, morph and the interaction of cytotype and morph, respectively; middle: $F_{4, 1382} = 34.31$, $P < 0.001$, $F_{1, 1382} = 56.92$, $P < 0.001$,

$F_{4, 1382}$= 4.02, P < 0.001, for cytotype, morph and the interaction of cytotype and morph, respectively; bottom: $F_{4, 1380}$= 18.12, P < 0.001, $F_{1, 1380}$= 25.88, P < 0.001, $F_{4, 1380}$= 3.52, P < 0.001, for cytotype, morph and the interaction of cytotype and morph, respectively; Appendix 4.2, Figure 4.9b). In both illegitimate crosses, the number of pollen tubes were always higher in the long- than in the short-styled morph (Figure 4.9a and b).

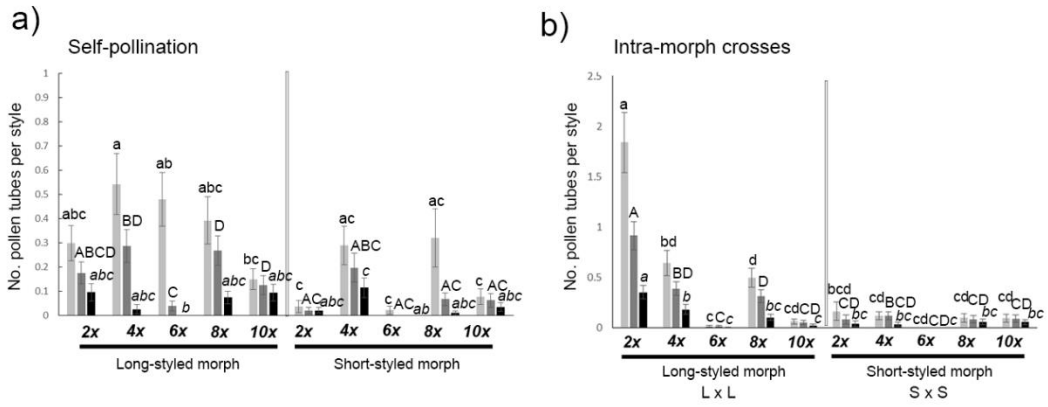

Figure 4.9. Average number of pollen tubes of diploid (2*x*), tetraploid (4*x*), hexaploid (6*x*), octoploid (8*x*) and decaploid (10*x*) flowers in the top (light grey), middle (dark grey) and bottom (black) level of the style in self-pollination (a) and intra-morph crosses (b) in short-styled and long-styled morphs of *Linum suffruticosum s.l.*. Different letters correspond to statistically significant differences at P < 0.05 for Type-III analysis of variance: lowercase letters for the top level of the style, uppercase letters for the middle and letters in italic for the bottom level.

Table 4.3. Incompatibility index (mean and standard deviation of the mean, SD) of each ploidy level (2*x*, diploid; 4*x*, tetraploid; 6*x*, hexaploid; 8*x*, octoploid; 10*x*, decaploid) following self-pollination and intra-morph crosses in *Linum suffruticosum s.l.*. Information about the number of styles observed (N styles), corresponding to the number of individuals is also provided. Different letters correspond to statistically significant differences at P < 0.05 for Type-III analysis of variance: lowercase letters for self-pollination, and uppercase letters for intra-morph crosses.

Treatment	Ploidy level	N styles	Incompatibility index (mean ± SD)
Self-pollination	2*x*	281	0.97 ± 0.17^{ab}
	4*x*	280	0.97 ± 0.17^{b}
	6*x*	283	1.00 ± 0.00^{a}
	8*x*	315	0.98 ± 0.14^{ab}
	10*x*	300	0.97 ± 0.17^{b}
Intra-morph crosses	2*x*	266	0.90 ± 0.29^{A}
	4*x*	281	0.94 ± 0.21^{AC}
	6*x*	265	1.00 ± 0.05^{B}
	8*x*	314	0.95 ± 0.21^{C}
	10*x*	266	0.98 ± 0.14^{BC}

All cytotypes were self- and morph-incompatible, but the strength of self- and morph-incompatibility differed significantly among cytotypes (self-incompatibility: $F_{4, 1454}$= 2.78, P < 0.05; morph-incompatibility: $F_{4,1385}$= 9.99, P < 0.001, Appendix 4.2). Hexaploid flowers were strongly self- and morph-incompatible. In contrast, diploid flowers were the less morph-incompatible, although not differing from tetraploids (Table 4.3).

Inter-cytotype cross ability

Pollen tube development was observed after inter-cytotype crosses (Figure 4.8). As observed for legitimate intra-cytotype crosses (see results above), the number of pollen tubes also decreased along the style in the inter-cytotype crosses. Significant differences in the number of pollen tubes were observed between treatments ($F_{2, 690}$ = 20.74, P < 0.001, $F_{2, 689}$ = 13.76 P < 0.001, $F_{2, 642}$ = 5.55, P < 0.001 for top, middle, bottom of the style, respectively, Appendix 4.2, Figure 4.10).

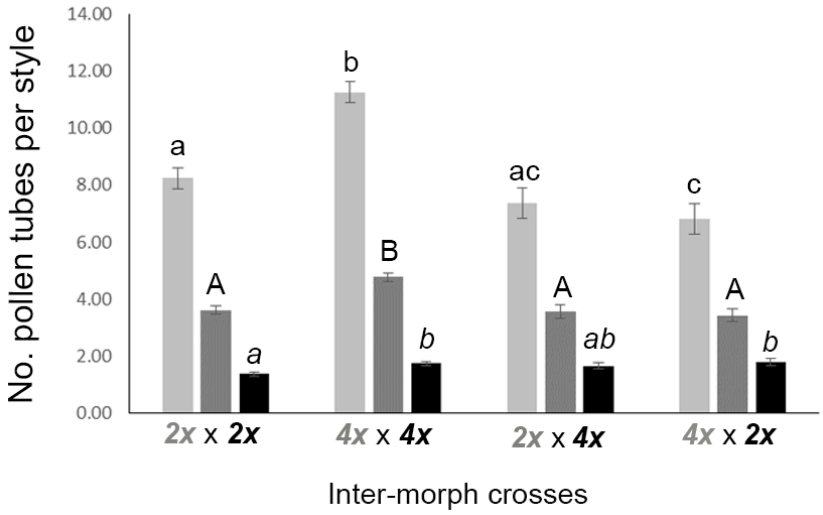

Figure 4.10. Average number of pollen tubes in the top (light grey), middle (dark grey) and bottom (black) level of the style in inter morph crosses within diploids (2*x* × 2*x*), within tetraploids (4*x* × 4*x*), and between diploids and tetraploids (2*x* × 4*x* and 4*x* × 2*x*) of *Linum suffruticosum s.l.*. Indication of the pollen receptor and donor are given in grey and black, respectively. Different letters correspond to statistically significant differences at P < 0.05 for Type-III analysis of variance: lowercase letters correspond to the top level of style, uppercase letters to the middle level, and letters in italic for the bottom level.

Intra-cytotype crosses with diploids (2*x* × 2*x*) had a lower number of pollen tubes than intra-cytotype crosses with tetraploids (4*x* × 4*x*) at all levels of the style (*i.e.*, top, middle and bottom). Both inter-cytotype crosses (*i.e.*, 2*x* × 4*x* and 4*x* × 2*x*) had a similar number of pollen tubes to intra-cytotype diploid crosses at the top and middle of the style. However, at the bottom of inter-cytotype crosses, no significant differences were observed among any type of cross (Figure 4.10). Indeed, the index of reproductive isolation was low and decreased along the style (2*x* ×

4*x* – top: 0.24, middle: 0.15, bottom: 0.00, 4*x* × 2*x* – top: 0.30, middle: 0.18, bottom: 0.00, Appendix 4.4). In intra-morph crosses, despite low pollen tube growth, significant differences in the number of pollen tubes were also observed between treatments ($F_{2, 629}$ = 19.81, P < 0.001, $F_{2, 629}$ = 17.09, P < 0.001, $F_{2, 627}$ = 10.37, P < 0.001 for top, middle, bottom of the style, respectively, Appendix 4.2, Appendix 4.5). The number of pollen tubes was always higher in the long- than in the short-styled morph (Appendix 4.5), as observed in intra-cytotype crosses (Figure 4.9, see results above).

Discussion

The study of the flower morphometry and incompatibility system of *L. suffruticosum s.l.* revealed several main findings: (1) the style polymorphism was maintained across the five ploidy levels, with most populations being isoplethic (Table 4.1) and there was no significant correlation between biased style morph proportions and population size (Figure 4.5 and Table 4.2); (2) sexual organs size tend to increase with increasing ploidy level but similar reciprocity levels were maintained across cytotypes and no morphological reproductive isolation between cytotypes was observed (Figure 4.6 and 4.7); (3) self- and morph-incompatibility was maintained after genome duplications (Figure 4.8 and Table 4.3); and (4) hand-pollination crosses confirm that there was no reproductive isolation between diploids and tetraploids (Figure 4.10 and Appendix 4.4 and 4.5). Below, I discuss the ecological and genetic mechanisms that could account for these results.

Maintenance of flower polymorphism after genome duplications

Here we observed that the 92 populations representing all five cytotypes reported in *L. suffruticosum s.l.* (2*x*, 4*x*, 6*x*, 8*x* and 10*x*) were all distylous with long- and short-styled morphs. Previous reports of polyploids of this species suggested that most likely distyly is maintained (Rogers *et al.* 1972; Zapatero *et al.* 1981; Elena Roselló *et al.* 1985; Nicholls 1985a, 1986a) whereas the sister species *L. tenuifolium* is monomorphic, self-compatible and diploid in all area of its distribution (Rogers *et al.* 1972; Nicholls 1986a; E. Olmedo-Vicente, A. Afonso and J. Arroyo unpublished data). There are some examples of heterostylous polyploid species (*e.g.*, *Oxalis tuberosa,* Trognitz and Hermann 2001; *Oxalis pes-caprae*, Costa *et al.* 2014) that maintain flower polymorphism across several ploidy levels. But this is not always the case and there are examples of other systems where the floral polymorphism changes after genome duplications. For example, in *Primula*, the species with the lower ploidy levels (2*x*, 4*x*) show distyly whereas species with higher ploidy levels (4x, 6x, 8x, 14x) show homostyly (Kelso 1992). Within some groups of Primulaceae and Rubiaceae, Naiki (2012) also found that individuals with a lower

ploidy level tended to have heterostyly and individuals with a higher ploidy level tended to have monomorphic flowers. In *Turnera*, the diploid and tetraploid species are distylous, whereas the hexaploid and octaploid species are monomorphic (Barrett and Shore 1987; Tamari *et al.* 2001). However, Shore and Barrett (1986) produced hexaploids using colchicine that showed distyly and self-incompatibility, suggesting that the breakdown of distylous to monomorphic flowers was not a necessary outcome of polyploidy. In early stages of neopolyploid formation, homostyly and its association with self-compatibility can be important to their successful establishment (Levin 1975). In some cases, heterostylous taxa can lose their self-incompatibility but retain the polymorphism, which in fact still means a breakdown of the linkage between style polymorphism and SI loci (Barrett *et al.* 1989, 2000; Richards and Koptur 1993). However, if polyploidization itself decreases inbreeding depression, self-compatible heterostylous flowers may be maintained.

Additionally, most of the populations were isoplethic (*i.e.*, long- and short-styled plants occurred at similar frequencies) and are likely in equilibrium. A ratio of 1:1 has been reported for *L. suffruticosum*, as well as the maintenance of the polymorphism, despite no information about ploidy level was available (Ruiz-Martín 2017). Theory predicts that morph ratios in heterostylous populations are governed by negative frequency-dependent selection typically resulting in equal morph ratios at equilibrium (Eckert *et al.* 1996) and for heterostyly to be maintained the frequency of disassortative mating must exceed the frequency of assortative mating in a population (Lloyd and Webb 1992a). For example, direct measurement of pollen transfer have demonstrated more frequent inter-morph pollination (*i.e.*, disassortative mating) than intra-morph crosses (*i.e.*, assortative mating) in style dimorphic populations with variation in style length but non-reciprocity of anther positions such as *Narcissus assoanus* (Cesaro and Thompson 2004) and *Narcissus papyraceus* (Simón-Porcar *et al.* 2015). In fact, the three-dimensional heterostyly observed in *L. suffruticosum* should be very effective in legitimate pollen transfer (Armbruster *et al.* 2006). Nicholls (1985a; c) also reported similar proportions of long-styled and short-styled pollen on both stigmas of this complex, but the long-style morph demonstrates a slightly higher proportion of legitimate pollen than short-styled morph. Contrarily to the most common distribution of anthers and stigmas in one dimension (*i.e.*, in height), in the case of *L. suffruticosum s.l.* anthers and stigmas show differences in the angle of divergence of the styles and stamens from the central axis of the flower and in the degree of rotation of the styles and filaments. As a result, the stigmas of short-styled morph may contact to the ventral side of the pollinator and the stigmas of long-styled morph contact the dorsal side. By opposition, the pollen from the short-styled morph is placed in the dorsal side of the pollinator, while the pollen from long-styled morph is placed in the ventral side. However,

empirical tests and observations in natural populations are lacking to test this functional hypothesis. As in other style polymorphic species (Simón-Porcar *et al.* 2015; Zhou *et al.* 2015b), in *Linum*, floral polymorphisms may be sufficient to promote disassortative mating and, together with heteromorphic incompatibility (see below), increase plant fitness through an efficient pollen delivering and receipt and maintain isoplethic populations. Studies of pollen transfer between and within morphs are needed to ascertain the amount of dissassortative and assortative pollination and mating in this species complex.

Nevertheless, several studies of distylous species have shown morph ratios that deviate significantly from theoretical expectations (*e.g.*, Eckert and Barrett 1992; Arroyo *et al.* 2002; Brys *et al.* 2008; Zhou *et al.* 2012, 2017; Castro *et al.* 2013; Ferrero *et al.* 2020). In this study, only six anisoplethic populations had higher frequency of long-styled plants, and three had higher frequency of short-styled plants. Like what we observe here, Nicholls (1985c) also reported some anisoplethic populations in this complex, most of them long-styled morph biased. Nicholls (1985c) suggests that populations biased towards the long-styled morph could be the reflection of limitations in pollen production and/or pollen transfer, since he found that short-styled morph receives a higher proportion of total pollen than long-styled morph, but long-styled morph receives a high proportion of legitimate pollen than short-styled morph. It has also been demonstrated that long-styled biased populations can occur when female fecundity of the long-styled morph is high (Brys *et al.* 2008) and/or when weak incompatibility may increase homozygous (long-styled) individuals in the populations (Lewis and Jones 1992). Additionally, high rates of pollen transfer from long-styled to short-styled plants can suggest short-styled may have a more female-biased gender than the long-styled morph and have a higher female fitness (Cesaro and Thompson 2004; Simón-Porcar *et al.* 2015). Theoretical models of pollen transfer indicate that biased morph ratios could result from differences in the mating system of the style-morphs (Barrett *et al.* 1996; Baker *et al.* 2000). Finally, random morph loss has been shown to result in skewed morph ratios, for example, in populations of several distylous species that have experienced strong reductions in population size (Arroyo *et al.* 2002; Endels *et al.* 2002; Jacquemyn *et al.* 2002; Brys *et al.* 2003; Costa, Castro, *et al.* 2016; Ferrero *et al.* 2020). However, in *L. suffruticosum s.l.* no correlation was found between morph ratio proportions and population size, and morph biases were found in intermediate populations. The lack of correlation between morph proportions and population size might be related with the characteristics of the species such as the long-lived perennial habit of *L. suffruticosum s.l.*, as plant populations of long-lived perennials exhibit high year-to-year survival, overlapping generations and usually do not exhibit dramatic population size fluctuations (Eckert *et al.* 1996). Consequently, the observed morph biases may be the result of stochastic events at the

population level that may have led to the loss of some plants and the populations are likely evolving to the equilibrium. Further measures and studies in anisoplethic populations are needed to understand the processes influencing morph ratios in this species complex.

Sexual organs size increase with increased ploidy level

In *L. suffruticosum s.l.* it was found that sexual organs increase with ploidy level increase. This is in line with the results obtained for several polyploids and has been proposed to results from having larger cells to accommodate bigger genome sizes that translates into larger tissues and organs (the so called 'gigas effect'; Müntzing 1936; Stebbins 1971), although exceptions to this directional effect have also been reported (Otto and Whitton 2000; Vamosi *et al.* 2007; Porturas *et al.* 2019). Additionally, in our morphometric analysis, style length has more variation among cytotypes and morphs than anther height, a feature already reported by Rogers (1979) in *L. suffruticosum*. This indicates a higher style dimorphism than anther dimorphism. This observation was not completely surprising since within dimorphic populations style length variation is usually more pronounced than anther height variation (Barrett 1992). Despite in *L. grandiflorum* the pattern is opposite (Lloyd and Webb 1992b), in most of distylous species the dimorphism in the style is higher than the dimorphism in anthers (*e.g.*, *Anchusa officinalis*, Philipp and Schou 1981, *Quinchamalium chilense*, Riveros *et al.* 1987, *Menyanthes trifoliata*, Olesen 1987). This is in agreement with Lloyd and Webb (1992a) in that the polymorphism in the style appeared first, and stamen dimorphism later. Style length contributes to both avoidance of physical interference with pollen from the same morph and contributing to the physiological control of incompatibility (Dulberger 1992). In contrast, the function of stamen length polymorphism is to create a reciprocity between the levels of anthers and stigmas between the different morphs. This higher dimorphism in the style length could be also correlated with the evolution and adaptation of the polymorphism in *L. suffruticosum s.l.* as the first step of heterostyly can be the appearance of mutant that differs discretely in style length and later the introduction of reciprocal herkogamy providing a more efficient transfer of outcrossed pollen Lloyd and Webb (1992a; b) suggested that the purpose of style polymorphisms, as of pollen and stigmas, are to reduce levels of self-pollination and self-interference, while reciprocal herkogamy actively promote cross-pollination with limited pollen discount. This could rather indicate a later introduction of reciprocal herkogamy and low anther dimorphism since other forces could be operating in maintained the outcrossing (*e.g.*, diallelic incompatibility). Another general expectation of polyploids is that they will likely exhibit greater variability in traits due to phenotypic and genomic instability in the generations following genome duplications (Soltis and Soltis 1995; Otto and Whitton 2000; Ramsey and Schemske

2002; Comai 2005). In *L. suffruticosum s.l.*, the range of style and anther heights was particularly high in hexaploid populations. Interestingly, this is the cytotype with highest variability in genome size and morphology (Afonso *et al*., 2020 in Chapter II) suggesting different evolutionary histories that could had led to high ranges in dimorphism of styles and anthers.

Interestingly, herkogamy (*i.e.*, the spatial separation of sex-organs within a flower) increased with ploidy level, being always higher in the long-styled morph. Increase of herkogamy with ploidy level has also been reported in *Primula* (Casazza *et al.* 2017). Moreover, herkogamy in the long-style morph is high in tetraploids and hexaploids and in short-styled morph is high only in decaploids. Herkogamy may evolve in response to selection on female function to avoid inbreeding depression (Charlesworth and Charlesworth 1974, 1987) (and/or interference between stamens and styles in the same flower (Lloyd and Webb 1992a; b; Harder and Barrett 2006). In the case of *L. suffruticosum s.l.* the long-styled morph exhibits higher herkogamy. This is the opposite of other study systems, for example in *Narcissus*, the short-styled morph exhibited high herkogamy to reduce interference and limiting assortative mating (Barrett *et al.* 1996). The higher herkogamy found in *L. suffruticosum s.l.* long-styled morph is mostly due to the high variation on the style length in the two morphs. In fact, variations in long-styled to short-styled length ratio is well documented (*e.g.*, *Linum flavum,* 1.8:1; *Limonium vulgare*, 2:1; *Primula auricular,* 4:1; *P. elatior,* 3:1; Dulberger 1992). Thus, other explanation could be the different cell elongation of the two morphs. It has been demonstrated in *Primula* that the initial length of stamens and styles are the same in the two morphs and later in the long-styled morph style and stamens are inducted to growth, while in the short-styled morph the style and stamens growth is repressed (reviewed in Dulberger 1992). Style length can also be associated with differential compatible pollen-tube growth or with differences in style structure and shape (Dulberger 1992). As it has been reported in other heterostylous species, styles from the long-styled morph frequently have larger stigmas and longer stigmatic papillae than styled from the short-styled morph, while stamens from the short-styled morph usually produce larger but fewer pollen grains than stamens from the long-styled morph (Dulberger 1975b). Indeed, dissimilar shape of the style and anther has been reported for *L. suffruticosum*: the long-styled morph has wide ribbon-like and usually white styles and sometimes brick-red anthers, whereas the short-style morph has fine thread-like and usually purple styles and cream or yellow anthers (Rogers 1979; Nicholls 1985b), although the significance of the color dimorphism is unknown. In addition, in *L. perenne*, (Nicholls 1986b) found that short-styled plant was a better pollen-donor (produced more pollen) and the long-styled plant was a better pollen-receiver (matured more seeds). These observations suggest a degree of sexual dimorphism in *L. perenne*, with the short-styled plants behaving functionally more as males and the long-style plant functionally more as

females, and a similar process might also occur in *L. suffruticosum s.l.*. In the case of short-styled morph herkogamy is high only in *L. suffruticosum s.l.* decaploids, suggesting a more efficient outcrossing in these larger flowers. In fact, intramorph variation in the length of the style and stamens occurs particularly in large flowers, as in *Plumbago capensis* and *Narcissus tazetta* (Dulberger 1992). However, overall sexual organs size and herkogamy increase with genome duplications and, since it has been proposed that populations with larger stigma–anther height separation have a higher outcrossing rate (Barrett and Shore 1987; Ganguly and Barua 2021), this could suggest that polyploids have a higher reproductive success. In fact, plants of polyploids tend to be bigger, more robust and have more flowers than diploids (Ana Afonso, pers. observations). In addition, if polyploidy leads to larger flowers and sexual organs, this may impact pollinators preferences and behavior, which could lead, in some cases, to assortative mating within the new neopolyploids (Segraves 2017) and allow its establishment and dispersal.

Despite the overall increase in organ size with ploidy level, the changes in flower size do not affect reciprocity indexes (according with Sánchez *et al.* 2013) and similar reciprocity indexes were observed across ploidy levels. Additionally, besides the reciprocity index of Sánchez *et al*. (2013) I also used the adaptive inaccuracy index of Armbruster *et al*. (2017) to quantify reciprocity. Adaptive inaccuracy uses the mean and the variance to interpret the adaptive significance of the position of anthers and stigmas in relation to pollen pick-up and delivery (Armbruster *et al.* 2017) and this is important for the function of heterostyly (Armbruster *et al.* 2006). Our values of inaccuracy are lower than the values from previous studies (Armbruster *et al.* 2017; Jacquemyn *et al.* 2018; Matias *et al.* 2020) and suggest that reciprocity in *L. suffruticosum s.l.* is high, even without taking into account particular fine differences related with 3-D distyly. Long organs had larger inaccuracy values than short organs, a pattern that could be attributed to developmental variation, which is often greater in large organs (Brys *et al.* 2008; Armbruster *et al.* 2017; Jacquemyn *et al.* 2018). If the function of heterostyly relies on the close matching between reciprocal organs (Darwin 1877; Jacquemyn *et al.* 2018; Brys and Jacquemyn 2020), the measures of the reciprocal matching of sex organs provided a means of assessing the inter-morph pollen transfer and reproductive fitness (Sánchez *et al.* 2013; Brys and Jacquemyn 2015; Zhou *et al.* 2015b). This suggests that *L. suffruticosum s.l.* maintains efficient levels of reciprocity at all ploidy levels. In addition, the disassortative pollen transfer increase in flowers with greater reciprocity (*e.g.*, low inaccuracy – Jacquemyn *et al*., 2018; Brys and Jacquemyn, 2020). Indeed, heteromorphic incompatibility is strongly associated with reciprocal herkogamy in most heterostylous lineages, but there are also species with self-compatibility or morph-compatibility (Lewis 1943; Richards and Koptur 1993; Barrett *et al.* 2000; Arroyo *et al.* 2002; Pérez-Barrales *et al.* 2006; Ferrero *et al.* 2012; Costa *et al.* 2014; Simón-Porcar *et al.* 2015; Zhou

et al. 2015b; Yuan *et al.* 2019); in these, the amount of dissassortative mating could be sufficient to maintain the polymorphism (Simón-Porcar *et al.* 2015; Zhou *et al.* 2015b).

Maintenance of self- and morph-incompatibility after genome duplications

The results showed that self- and morph-incompatibility in *L. suffruticosum s.l.* is maintained across ploidy levels and that incompatibility occurs mainly in the stigmatic papillae, although pollen tube development is also selectively blocked across the style. Most heterostylous taxa are self-incompatible (Ganders 1979) and polyploid angiosperms have, on average, higher self-fertilization rates than their diploid relatives (Barringer 2007). Despite the lack of ploidy information, Rogers (1979) also had described *L. suffruticosum* as heterostylous and intra-morph incompatible in plants growing in eastern Spain. Nicholls (1985c) did legitimate and selfed experimental hand-pollinations in the two morphs of *L. suffruticosum*, in populations from the contact zone between the heterostylous race *L. suffruticosum* and the monomorphic race *L. tenuifolium*, and also found that the former species is strongly self-incompatible. However, it was described seed production after crosses between the short-styled morph and monomorphic race *L. tenuifolium* (Nicholls 1985b). Martín Ruiz (2017) also had reported low fruit production in self- and intra- morph crosses, whereas the closely related monomorphic *L. tenuifolium* has a very high fruit production after illegitimate pollinations. It was also reported that distylous species of *Linum* usually have an associated heteromorphic incompatibility system, whereas species with a single morph are often self-compatible (Dulberger 1992; Lewis and Jones 1992; Ruiz-Martín *et al.* 2018). Still, as mentioned above, studies of style polymorphic groups have reported self-compatibility (*e.g.*, *Eichhornia paniculata*, Barrett *et al.* 1989; *Guettarda scabra*, Richards and Koptur 1993; *Salvia brandegeei*, Barrett *et al.*, 2000) or morph-compatibility (*Glandora,* Ferrero et al., 2012*; Luculia pinceana*, Zhou *et al.*, 2015; *Narcissus*, Arroyo *et al*. 2002; Simón-Porcar *et al*., 2015; Pérez-Barrales *et al*. 2006) without the loss of the polymorphism. However, in polyploid species the loss of self-and morph-compatibility is normally accompained with the loss of the polymorphism (*e.g.*, Shore and Barrett 1986; Kelso 1992; Tamari *et al.* 2001), with very few exceptions (*Oxalis pes-caprae*, (Costa *et al.* 2014). For example, in *Primula* and *Turnera*, diploids and tetraplois are self–incompatible and distylous, while higher ploidy levels are self-compatible but homostylous (Shore and Barrett 1986; Kelso 1992; Tamari *et al.* 2001). Because the mechanism of heterostyly aims to prevent interference of sexual organs and promote outcrossing, the acquisition of self and morph-compatibility increases the number of mating partners and may allow the establishment of newly arisen cytotypes. In the case of *L. suffruticosum s.l.*, there was no breakdown of self- and morph-incompatibility although self-incompatibility is stronger than morph-incompatibility. Thus, more

likely, neither the breakdown of the incompatibility system nor the breakdown of the stylar polymorphism was involved in increasing mating availability at initial stages of new cytotype emergence in *L. suffruticosum* complex.

A characteristic of most heterostylous taxa in which pollen tube growth has been investigated is the difference in sites of inhibition between the floral morphs (Gibbs 1986). A variety of different inhibition sites for incompatible pollen tubes are evident in heterostylous plants (including the stigma, style and ovary; Dulberger, 1992). As pollen tube development along the style was observed in *L. suffruticosum s.l.*, the incompatibility system seems to be operating at several levels of the style, although most of the incompatibility reaction occur in the stigmatic papillae. Arrest of pollen tube growth in the stigmatic papillae or beginning of the style has also been reported for several other *Linum* species, such as, *L. narbonense* (Darwin 1877; Lewis 1942), *L. pubescens*, *L. mucronatum* (Dulberguer 1973), *L. austriacum*, *L. perenne* (Darwin 1877; Baker 1975) and *L. maritimum* (Dulberger 1987), as well as in other heterostylous species (reviewed in Dulberger 1992). Despite self- and morph-incompatibility, in both selfing and intra-morph crosses of long-styled morph showed higher number of pollen tubes reaching the bottom of the style of flowers than crosses of short-styled morph of *L. suffruticosum s.l.*. In the experiment of Nicholls (1985c), despite he reported a strongly self-incompatibility, he also found that the short-styled is more strongly incompatible than the long-styled morph in this complex. Thus, in short-styled flowers the inhibitory reaction of illegitimate pollinations is quick, while in long-styled flowers the inhibition seems to rely in part on the longer length of the style for incompatible reactions to occurs. Additionally, pollen morphology and size may impact the interactions between gametophyte and sporophyte, as well as male fitness. Morph-specific pollen size and morphology can act on pollen-pistil interactions (Dulberger 1992). Pollen grains can became incompatible to grow in one style morph as a result of adaptation to the other style morph (Darwin 1877). Moreover, the breakdown of heteromorphic self-incompatibility is also associated with a loss in pollen size polymorphism (reviewed in Dulberger 1992). In *L. suffruticosum*, pollen grains of the long-style morph had polymorphic surface excrescences with 1-4 conspicuous papillae and pollen grains of the short-styled morph have sub-monomorphic excrescences with 4-6 minute papillae (Nichols 1985c). In fact, in some heterostylous species of *Linum*, the pollen surface differs between morphs: in short-styled plants the pollen is composed apertures with similar sizes, while in long-styled plants the exine surface contains apertures with variable sizes (small and big) and papillae with variable shapes and sizes (Dulberger 1981). In *Primula* species, larger pollen grains produced by short-styled plants are adapted to grow faster in compatible long-style flowers but are inhibited at very earlier stages in the short-style flower. Since the short-style morph is shorter, there is a selective advantage to early inhibition of

incompatible short-styled pollen (Lewis 1942). On the other hand, in *Linum* species with equal sized pollen grains and contrary to which was observed in this study, in short-styled illegitimate pollinations, the pollen grains germinate, but the tubes are inhibited within the stigma and, in long-styled illegitimate pollinations, the pollen grains usually fail to adhere (Dulberger 1975a). On the other hand, Moreover, Lloyd and Webb (1992b) suggested that the occurrence of variable inhibition sites in heterostylous plants could be evidence that incompatibility reactions may have evolved separately in each floral morph. In the light of the available evidence, in the case of *L. suffruticosum s.l.*, the different size and shapes of pollen grains and stigmas could also play a role in maintaining self- and morph-incompatibility.

The hand-pollination experiments made here provide very interesting findings on the incompatibility reactions in the five cytotypes of *L. suffruticosum s.l.*. Yet, there are a couple of experimental drawbacks that need to be mentioned, namely: 1) the observation of pollen tube development only may hinder the detection of late-acting incompatibility reactions, and 2) the different flower lifespans among cytotypes might be related with different times for pollen tube growth and impact the results obtained here. First, a critical feature of self-incompatibility in some species is the occurrence of late acting incompatibility reactions, when self-pollen tubes grow until the ovary, but incompatibility reactions make ovules unavailable for fertilization (Sage *et al.* 1999). For example, in *N. papyraceus* (Arroyo *et al.* 2002), *N. tazetta* (Dulberger 1964) and also *N. triandrus* (Sage *et al.* 1999) pollen tube growth in the style is similar between self- and cross-pollination with self-rejection occurring in the ovary as a result of late-acting self-incompatibility. A late-acting self-incompatibility system has been described in others several species, such as, *Oxalis pes-caprae* (Costa *et al.* 2017), *Cyrtanthus breviflorus* (Vaughton *et al.* 2010), other *Narcissus* species (Dulberger 1964; Sage *et al.* 1999; Navarro *et al.* 2012), *Anchusa officinalis* (Philipp and Schou 1981), *Asclepias exaltata* (Lipow and Wyatt 2000) and *Spathodea campanulata* (Bittencourt *et al.* 2003). Here, because of logistic conditions we used pollen tube development as proxy of plant fitness and assumed that the number of pollen tubes that reach the end of the style would potentially fertilize the same number of ovules (Dulberguer 1992). We observe that pollen tube development was blocked at the stigmatic papillae and the number of pollen tubes in the style was low, particularly at the bottom of the style. However, considering the low number of ovules in *L. suffruticosum s.l.* (2 per carpel, maximum 10 ovules per flower), a low number of pollen tubes reaching the style may still represent a significant contribution to fitness and, in future studies, it would be ideal to run the experiment until the stage of seed development. Secondly, because flower senescence after pollination differed among cytotypes (lower ploidies senesce faster than higher ploidies), this could have affected the time available for pollen tube development across cytotypes. Yet, we would like to highlight that flower

lifespan is an attribute of each cytotype and, within cytotype, each treatment was left for pollen tube growth for the same amount of time, enabling comparisons across treatments. In future studies, it would be ideal to develop the experiment in complete living plants.

Reproductive isolation between cytotypes

Here, we investigated reproductive isolation between cytotypes 1) mediated by morphological mismatch between sexual organs of different cytotypes and 2) tested reproductive incompatibilities between diploid and tetraploid plants. Although a gigas effect has been observed, the high overlap in reciprocal sexual organs between different cytotypes was observed, which suggests that pollen flow between certain cytotypes in areas of sympatry could be possible. As described above, the occurrence of pollen flow has been proposed to be correlated with the close matching between reciprocal organs (Darwin 1877; Jacquemyn *et al.* 2018; Brys and Jacquemyn 2020) and reproduction between cytotypes has been documented in some polyploid systems (Hülber *et al.* 2015; Laport *et al.* 2016; Sonnleitner *et al.* 2016). When a neopolyploid occurs in nature, its establishment can be critical, taking into account that the pollen that will receive will be from individuals with a different ploidy level, which can lead to the production of inviable seeds or sterile hybrids (Levin 1975, 2002; Rodríguez 1996; Husband and Schemske 2000). 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 hybrids (Levin 1975, 2002; Rodríguez 1996; Husband and Schemske 2000). In *L. suffruticosum s.l.*, the morphological mismatch of sexual organs from different ploidy levels suggests that pollen flow between cytotypes can happen. However, only a few mixed-ploidy populations and minority cytotypes were found in natural populations (Afonso *et al.*, 2021 in Chapter II). Thus, in the absence of reproductive barriers, 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) like the ones observed in *L. suffruticosum s.l.*. In addition, different competitive abilities and/or ecological differentiation could also lead to a spatial separation of cytotypes (Rodríguez 1996; Rausch and Morgan 2005; Rieseberg and Willis 2007; Jersáková *et al.* 2010; Castro and Loureiro 2014; Thompson *et al.* 2014). In fact, polyploid plants of *L. suffruticosum s.l.* usually have a higher number of flowers than diploids and a high range of flowering time (Ana Afonso, pers. observations). However, most of *L. suffruticosum s.l.* cytotypes do not differ in suitable habitat (Chapter III). To fully understand the population dynamics that

led to the emergence of pure populations of five different cytotypes, testing pollen flow and reproductive success in mixed populations of *L. suffruticosum s.l.* will be necessary. Additionally, it is necessary to determine the effective pollinators of the cytotypes. Armbruster *et al.* (2009) reported *Usia* beeflies (Bombyliidae, Diptera) as the most frequent and efficient pollinators in southern populations of *L. suffruticosum s.l.*, though without known ploidy levels. This group of pollinators was observed in the northern populations studied in this chapter, but a specific study is necessary to determine the possible role of pollinators as promoters of reproductive barriers among *L. suffruticosum s.l.* cytotypes.

Supporting the lack of reproductive barriers are the hand pollinations between diploids and tetraploids. Crosses between diploids and tetraploids from the contact zone, suggest no reproductive isolation between these two cytotypes. Several mechanisms have been documented to regulate mating in populations, such as pre-zygotic and post-zygotic interactions (Harder and Barrett 2006). Thus, the results obtained here need to be discussed with caution as reproductive isolation is the product of several individual pre-zygotic and post-zygotic reproductive barriers (Ramsey and Schemske 1998) and, as mentioned above, we did not quantify seed production. However, Nicholls (1986a) also performed crosses, in this polyploid complex, between diploids and tetraploids with a mean seed set of only 13.9%., while crosses within the same cytotype showed 63-83 % of fertility.

There are other examples of polyploid species where post-pollination barriers significantly reduced the production of offspring from inter-cytotype crosses (Mráz 2003; Castro *et al.* 2011, 2018) and, sometimes, can mediate asymmetrical hybridization between cytotypes (Husband *et al.* 2002; Brock 2004; Buggs and Pannell 2007), which strongly influence the dynamics on contact zones. If there are differences in the mating systems of cytotypes this can result in different reproductive fitness and one of the cytotypes can have an advantage (*e.g.*, Husband *et al.* 2002; Brock 2004; Buggs and Pannell 2007). In the case of *L. suffruticosum s.l.*, no differences were detected in the number of pollen tubes reaching the bottom of the style between legitimate intra-cytotype crosses and inter-cytotype crosses suggesting that inter-cytotype offspring could be produced. Additionally, pollen tube growth in intercytotype crosses do not differ between diploids and tetraploids, suggesting a lack of asymmetrical hybridization between these two cytotypes. Yet, further gametic barriers may prevent hybridization between cytotypes and the ability of two cytotypes to create hybrids may not predict the probability that they will be formed under natural conditions. Indeed, hybrids are often expected to be sterile because of their meiotic irregularities and high frequency of aneuploid gametes, they are consequently excluded from the populations, being rare (Ramsey and Schemske 1998). In fact, a low number of triploids were found in the natural populations of *L. suffruticosum s.l.* (Afonso

et al., 2021 in Chapter II), which could be explained by their low fitness or competitive ability. Testing reproductive and competitive interactions between cytotypes are needed to understand if there is pollen flow between cytotypes and production of viable offspring.

Conclusions

Linum suffruticosum s.l. is a polyploidy complex with strong self- and morph-incompatibility, and most of the populations showed isoplethy with no correlation between population size and deviations in style-morph proportions. Thus, no breakdown of the distyly and incompatibility system was detected across the five cytotypes. Studies integrating genome size, breeding system and phylogenetic analyses are required to develop the evolutionary scenarios that have occurred in *L. suffruticosum s.l.*. In addition, pollen flow among cytotypes appears to be possible since there is overlap between reciprocal sexual organs and pollen tube reaching the bottom of the style in inter-cytotype crosses. However, further investigation on viable seeds and population genetic structure will be needed to confirm if actual gene flow is occurring. In addition, investigation on reproductive traits and on evolutionary paths will be important to understand the polyploid and heterostylous lineages of this complex.

Appendices

Appendix 4.1. Morph frequencies of the 92 populations of *Linum suffruticosum s.l.* with ploidy level (2*x*, diploid; 4*x*, tetraploid; 6*x*, hexaploid; 8*x*, octoploid; 10*x*, decaploid). Information about the taxa, population, country, number and frequency of short- and long-styled morph individuals, total number of flowers (and individuals) observed, range of population size (≤50, >50 and ≤100, >100 and ≤500, >500 and ≤1000, >1000 and ≤5000, and >5000 and ≤10000 individual plants), G test and *P* values (* denotes significant differences at $P < 0.05$) are also provided.

Entity	Population	Country	Ploidy level	N of short-styled morph	N of long-styled morph	Total no. Flowers	G value	*P* value	Frequency of short-styled morph	Frequency of long-styled morph	Range of population size (no. of individuals)
L. suffruticosum	AA15	Spain	2*x*	80	43	123	11.304	0.001*	0.65	0.35	>100 and <500
L. appressum-salsoloides	AA49	Spain	2*x*	36	64	100	7.946	0.005*	0.36	0.64	>100 and <500
L. suffruticosum	80JAM2016	Spain	2*x*	26	49	75	7.168	0.007*	0.35	0.65	>100 and <500
L. suffruticosum	81JAM2016	Spain	2*x*	25	44	69	5.300	0.021*	0.36	0.64	>100 and <500
L. salsoloides	85JAM	Spain	2*x*	46	69	115	4.631	0.031*	0.40	0.60	>100 and <500
L. suffruticosum	AC1	Spain	2*x*	28	21	49	1.003	0.316	0.57	0.43	>10 and <50
L. suffruticosum	AA33	Spain	2*x*	60	50	110	0.910	0.340	0.55	0.45	>100 and <500
L. suffruticosum	AA32	Spain	2*x*	11	7	18	0.896	0.344	0.61	0.39	≤50
L.suffruticosum	AA106	Morocco	2*x*	4	7	11	0.829	0.363	0.36	0.64	≤50
L. suffruticosum	AA78	Spain	2*x*	28	35	63	0.779	0.377	0.44	0.56	>50 and <100
L. suffruticosum	AA31	Spain	2*x*	18	14	32	0.501	0.479	0.56	0.44	≤50
L.suffruticosum	AA101	Morocco	2*x*	58	51	109	0.450	0.502	0.53	0.47	>100 and <500
L. appressum-salsoloides	AA45	Spain	2*x*	41	47	88	0.409	0.522	0.47	0.53	>100 and <500
L. suffruticosum	AA61	Spain	2*x*	50	44	94	0.383	0.536	0.53	0.47	>500 and <1 000
L. suffruticosum	AA75	Spain	2*x*	23	27	50	0.320	0.571	0.46	0.54	>500 and <1 000
L.suffruticosum	AA107	Morocco	2*x*	29	33	62	0.258	0.611	0.47	0.53	>1 000 and <5 000
L. suffruticosum	AA5	Spain	2*x*	33	30	63	0.143	0.705	0.52	0.48	>100 and <500
L. suffruticosum	AA77	Spain	2*x*	7	6	13	0.077	0.781	0.54	0.46	≤50
L. suffruticosum	AA29	Spain	2*x*	76	73	149	0.060	0.806	0.51	0.49	>100 and <500
L. suffruticosum	AA62	Spain	2*x*	33	32	65	0.015	0.901	0.51	0.49	>50 and <100

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L. appressum-salsoloides	AA52	Spain	2*x*	70	71	141	0.007	0.933	0.50	0.50	>500 and <1 000
L. suffruticosum	82JAM2016	Spain	4*x*	25	43	68	4.822	0.028*	0.37	0.63	>100 and <500
L. suffruticosum	84JAM	Spain	4*x*	52	34	86	3.795	0.051	0.60	0.40	>100 and <500
L. suffruticosum	AA4	Spain	4*x*	27	17	44	2.293	0.130	0.61	0.39	>100 and <500
L. suffruticosum	AA3	Spain	4*x*	14	22	36	1.793	0.181	0.39	0.61	≤50
L.suffruticosum	AA108	Morocco	4*x*	17	25	42	1.533	0.216	0.40	0.60	≤50
L. suffruticosum	AA65	Spain	4*x*	51	40	91	1.333	0.248	0.56	0.44	>5 000 and <10 000
L. suffruticosum	AA27	Spain	4*x*	32	24	56	1.147	0.284	0.57	0.43	>50 and <100
L. suffruticosum	83 JAM	Spain	4*x*	38	47	85	0.955	0.329	0.45	0.55	>100 and <500
L. suffruticosum	AA2	Spain	4*x*	28	35	63	0.779	0.377	0.44	0.56	>100 and <500
L. appressum-salsoloides	DP1995	Spain	4*x*	69	59	128	0.782	0.377	0.54	0.46	>5 000 and <10 000
L.suffruticosum	AA100	Morocco	4*x*	36	30	66	0.546	0.460	0.55	0.45	>1 000 and <5 000
L. appressum-salsoloides	AA81	Spain	4*x*	39	34	73	0.343	0.558	0.53	0.47	>50 and <100
L. suffruticosum	AA28	Spain	4*x*	19	16	35	0.257	0.612	0.54	0.46	≤50
L. suffruticosum	AA25	Spain	4*x*	57	62	119	0.210	0.647	0.48	0.52	>1 000 and <5 000
L. suffruticosum	AA26	Spain	4*x*	12	10	22	0.182	0.670	0.55	0.45	≤50
L. suffruticosum	AA1	Spain	4*x*	42	44	86	0.047	0.829	0.49	0.51	>50 and <100
L. appressum-salsoloides	AA86	Spain	4*x*	47	45	92	0.043	0.835	0.51	0.49	>100 and <500
L. appressum-salsoloides	AA46	Spain	4*x*	20	21	41	0.024	0.876	0.49	0.51	>100 and <500
L. suffruticosum	AA63	Spain	4*x*	105	106	211	0.005	0.945	0.50	0.50	>500 and <1 000
L. suffruticosum	MO6137	Spain	6*x*	7	19	26	5.754	0.016*	0.27	0.73	>50 and <100
L. suffruticosum	AA34	Spain	6*x*	93	64	157	5.388	0.020*	0.59	0.41	>100 and <500
L. suffruticosum	AA20	Spain	6*x*	70	46	116	5.002	0.025*	0.60	0.40	>100 and <500
L. suffruticosum	AA21	Spain	6*x*	67	49	116	2.804	0.094	0.58	0.42	>100 and <500
L. suffruticosum	AA22	Spain	6*x*	75	57	132	2.462	0.117	0.57	0.43	>500 and <1 000
L. suffruticosum	AA19	Spain	6*x*	73	57	130	1.974	0.160	0.56	0.44	>500 and <1 000
L.suffruticosum	AA111	Morocco	6*x*	13	21	34	1.900	0.168	0.38	0.62	>50 and <100
L. suffruticosum	AA35	Spain	6*x*	57	44	101	1.678	0.195	0.56	0.44	>500 and <1 000

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Intermediate individuals	AA14	Spain	6*x*	19	12	31	1.594	0.207	0.61	0.39	≤50
L. suffruticosum	AA24	Spain	6*x*	35	27	62	1.035	0.309	0.56	0.44	>100 and <500
L. appressum-salsoloides	AA50	Spain	6*x*	21	15	36	1.005	0.316	0.58	0.42	>50 and <100
L. suffruticosum	MO6136	Spain	6*x*	28	35	63	0.779	0.377	0.44	0.56	>50 and <100
L. suffruticosum	AA23	Spain	6*x*	92	81	173	0.700	0.403	0.53	0.47	>1 000 and <5 000
L. appressum-salsoloides	AA40	Spain	6*x*	42	36	78	0.462	0.497	0.54	0.46	>100 and <500
L. appressum-salsoloides	AA48	Spain	6*x*	18	22	40	0.401	0.527	0.45	0.55	>50 and <100
L. appressum-salsoloides	DP2020	Spain	6*x*	15	12	27	0.334	0.563	0.56	0.44	≤50
L. appressum-salsoloides	AA39	Spain	6*x*	66	61	127	0.197	0.657	0.52	0.48	>1 000 and <5 000
L. appressum-salsoloides	AA38	Spain	6*x*	22	25	47	0.192	0.662	0.47	0.53	>50 and <100
L. suffruticosum	AA84	Spain	6*x*	15	14	29	0.034	0.853	0.52	0.48	≤50
L. suffruticosum	AA83	Spain	6*x*	81	79	160	0.025	0.874	0.51	0.49	>100 and <500
L.suffruticosum Marocco	AA113	Morocco	6*x*	23	22	45	0.022	0.881	0.51	0.49	>50 and <100
L. appressum-salsoloides	AA51	Spain	6*x*	22	23	45	0.022	0.881	0.49	0.51	>100 and <500
Intermediate individuals	AA47	Spain	6*x*	15	15	30	0.000	1.000	0.50	0.50	>100 and <500
L. appressum-salsoloides	89 JAM	Spain	8*x*	34	52	86	3.795	0.051	0.40	0.60	>50 and <100
Intermediate individuals	AA53	Spain	8*x*	24	35	59	2.063	0.151	0.41	0.59	>500 and <1 000
L. suffruticosum	AA16	Spain	8*x*	59	45	104	1.890	0.169	0.57	0.43	>100 and <500
L. suffruticosum	AA68	Spain	8*x*	40	30	70	1.433	0.231	0.57	0.43	>500 and <1 000
L. suffruticosum	AA60	Spain	8*x*	30	22	52	1.236	0.266	0.58	0.42	≤50
L. appressum-salsoloides	AA80	Spain	8*x*	42	33	75	1.083	0.298	0.56	0.44	>100 and <500
L. suffruticosum	AA37	Spain	8*x*	17	12	29	0.866	0.352	0.59	0.41	>50 and <100
L. suffruticosum	AA73	Spain	8*x*	29	24	53	0.472	0.492	0.55	0.45	>50 and <100
L. suffruticosum	AA8	Spain	8*x*	53	47	100	0.360	0.548	0.53	0.47	>100 and <500
L. suffruticosum	AA69	Spain	8*x*	41	37	78	0.205	0.651	0.53	0.47	>1 000 and <5 000
Intermediate individuals	AA9	Spain	8*x*	43	39	82	0.195	0.659	0.52	0.48	>100 and <500
L. suffruticosum	AA94	Spain	8*x*	39	36	75	0.120	0.729	0.52	0.48	>500 and <1 000
L. suffruticosum	AA57	Spain	8*x*	18	16	34	0.118	0.732	0.53	0.47	>50 and <100

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Intermediate individuals	AA72	Spain	8*x*	22	20	42	0.095	0.758	0.52	0.48	>50 and <100
L. suffruticosum	AA67	Spain	8*x*	17	16	33	0.030	0.862	0.52	0.48	≤50
Intermediate individuals	86JAM	Spain	8*x*	59	59	118	0.000	1.000	0.50	0.50	>100 and <500
L. suffruticosum	AA7	Spain	8*x*	30	30	60	0.000	1.000	0.50	0.50	>100 and <500
L. suffruticosum	AA93	Spain	8*x*	40	40	80	0.000	1.000	0.50	0.50	>100 and <500
L. suffruticosum	AA13	Spain	10*x*	45	30	75	3.020	0.082	0.60	0.40	>50 and <100
L. suffruticosum	AA36	Spain	10*x*	38	25	63	2.702	0.100	0.60	0.40	>100 and <500
Intermediate individuals	AA11	Spain	10*x*	31	42	73	1.664	0.197	0.42	0.58	>50 and <100
Intermediate individuals	AA12	Spain	10*x*	13	9	22	0.731	0.392	0.59	0.41	>50 and <100
L. suffruticosum	AA58	Spain	10*x*	16	20	36	0.445	0.505	0.44	0.56	>10 and <50
L. suffruticosum	AA88	Spain	10*x*	33	28	61	0.410	0.522	0.54	0.46	>50 and <100
L. suffruticosum	AA91	Spain	10*x*	36	31	67	0.373	0.541	0.54	0.46	>50 and <100
Intermediate individuals	AA79	Spain	10*x*	62	56	118	0.305	0.581	0.53	0.47	>100 and <500
L. suffruticosum	AA90	Spain	10*x*	48	43	91	0.275	0.600	0.53	0.47	>1 000 and <5 000
L. suffruticosum	AA59	Spain	10*x*	7	9	16	0.251	0.617	0.44	0.56	≤50
L. suffruticosum	AA10	Spain	10*x*	51	52	103	0.010	0.922	0.50	0.50	>50 and <100

Appendix 4.2. Results of the Generalized linear models (GLMs) examining the effects of fixed factors in the response variables**.**

Response variable	Fixed factors	GLMs
Frequency of long-styled morph	Cytotype	$F_{4, 87} = 0.74$, $P < 0.05$
Frequency of short-styled morph	Cytotype	$F_{4, 87} = 0.74$, $P < 0.05$
Measure of sexual organ	Cytotype	$F_{4, 4837} = 314.04$, $P < 0.001$
	Morph	$F_{1, 4837} = 32.20$, $P < 0.001$
	Sexual organ (style/anther)	$F_{1, 4837} = 113.43$, $P < 0.001$
Herkogamy	Cytotype	$F_{5, 162} = 60.26$, $P < 0.001$
	Morph	$F_{4, 162} = 7.47$, $P < 0.001$
Reciprocity index (Sanchez *et al*. 2013)	Cytotype	$F_{4, 81} = 1.55$, $P > 0.05$
Total inaccuracy (Armbruster *et al*. 2017)	Cytotype	$F_{4, 81} = 2.15$, $P > 0.05$
Pollen tubes - top of the style	Treatment	$F_{2, 4156} = 2368.80$, $P < 0.001$,
Pollen tubes - middle of the style	Treatment	$F_{2, 4149} = 1885.80$, $P < 0.001$
Pollen tubes - bottom of the style	Treatment	$F_{2, 4040} = 978.14$, $P < 0.001$
Pollen tubes - top of the style	Cytotype	$F_{2, 4144} = 478.18$, $P < 0.001$
	Treatment	$F_{12, 4144} = 17.98$, $P < 0.001$
Pollen tubes - middle of the style	Cytotype	$F_{2, 4137} = 392.45$, $P < 0.001$
	Treatment	$F_{12, 4137} = 21.45$, $P < 0.001$
Pollen tubes - bottom of the style	Cytotype	$F_{2, 4028} = 186.73$, $P < 0.001$
	Treatment	$F_{12, 4028} = 9.26$, $P < 0.001$
Pollen tubes in self-pollination - top of the style	Cytotype	$F_{4, 1456} = 3.32$, $P < 0.01$
	Morph	$F_{1, 1456} = 1.06$, $P > 0.05$
	Cytotype x Morph	$F_{4, 1456} = 3.85$, $P < 0.001$
Pollen tubes in self-pollination - middle of the style	Cytotype	$F_{4, 1456} = 6.17$, $P < 0.001$
	Morph	$F_{1, 1456} = 1.81$, $P > 0.05$
	Cytotype x Morph	$F_{4, 1456} = 2.26$, $P > 0.05$
Pollen tubes in self-pollination - bottom of the style	Cytotype	$F_{4, 1449} = 5.36$, $P < 0.001$
	Morph	$F_{1, 1449} = 3.27$, $P > 0.05$
	Cytotype x Morph	$F_{4, 1449} = 4.55$, $P < 0.01$
Pollen tubes in intramorph crosses - top of the style	Cytotype	$F_{4, 1382} = 47.78$, $P < 0.001$
	Morph	$F_{1, 1382} = 73.03$, $P < 0.001$
	Cytotype x Morph	$F_{4, 1382} = 2.89$ $P < 0.01$
Pollen tubes in intramorph crosses - middle of the style	Cytotype	$F_{4, 1382} = 34.31$, $P < 0.001$
	Morph	$F_{1, 1382} = 56.92$, $P < 0.001$
	Cytotype x Morph	$F_{4, 1382} = 4.02$, $P < 0.001$
Pollen tubes in intramorph crosses - bottom of the style	Cytotype	$F_{4, 1380} = 18.12$, $P < 0.001$
	Morph	$F_{1, 1380} = 25.88$, $P < 0.001$
	Cytotype x Morph	$F_{4, 1380} = 3.52$, $P < 0.001$
Index of self-incompatibility	Cytotype	$F_{4, 1454} = 2.78$, $P < 0.05$
Index of morph-incompatibility	Cytotype	$F_{4,1385} = 9.99$, $P < 0.001$
Pollen tubes in the inter-cytotype crosses for inter-morph crosses - top of the style	Treatment	$F_{2, 690} = 20.74$, $P < 0.001$
Pollen tubes in the inter-cytotype crosses for inter-morph crosses- middle of the style	Treatment	$F_{2, 689} = 13.76$ $P < 0.001$
Pollen tubes in the inter-cytotype crosses for inter-morph crosses - bottom of the style	Treatment	$F_{2, 642} = 5.55$, $P < 0.001$
Pollen tubes in the inter-cytotype crosses for intra-morph crosses - top of the style	Treatment	$F_{2, 629} = 19.81$, $P < 0.001$
Pollen tubes in the inter-cytotype crosses for intra-morph crosses- middle of the style	Treatment	$F_{2, 629} = 17.09$, $P < 0.001$
Pollen tubes in the inter-cytotype crosses for intra-morph crosses - bottom of the style	Treatment	$F_{2, 627} = 10.37$, $P < 0.001$

Appendix 4.3. Measures of style length of long-style-morph (St) and short-styled morph (st), anther height of long-style-morph (a) and short-styled morph (A) and respective variances (Var), average organ measure (T), herkogamy (H) for both morphs, reciprocity index (IR), inaccuracy high organs (Ina-High), inaccuracy low organs (Ina-Low) and total inaccuracy (Total-Ina) in mm^2, standardized inaccuracy high organs (Ina-High), standardized inaccuracy low organs (Ina-Low) and standardized total inaccuracy (Total-Ina) in percentage (%) of the 86 populations of *Linum suffruticosum s.l.*. Entity, population code (Cod), ploidy level, number of long-styled morph individuals (N L) and short-styled morph individuals (N S) are given. Abbreviations: 2*x*, diploid; 4*x*, tetraploid; 6*x*, hexaploid; 8*x*, octoploid; 10*x*, decaploid.

Entity	Cod	PL	Country	N L	N S	H -Long - styled morph	H-short-styled morph	IR	St	A	st	a	T	Var (St)	Var (A)	Var (st)	Var (a)	Ina-High (mm^2)	Ina-High (%)	Ina-Low (mm^2)	Ina-Low (%)	Total-Ina (mm^2)	Total-Ina (%)
Intermediate individuals	86JAM2016	8*x*	Spain	15	15	1.64	1.46	0.68	8.95	7.32	6.10	6.82	7.30	0.22	0.26	0.31	0.18	3.13	5.88	1.01	1.89	4.14	7.77
Intermediate individuals	AA11	10*x*	Spain	15	15	2.46	1.38	0.51	11.50	10.50	8.52	8.61	9.78	0.19	0.55	0.43	0.24	1.74	1.82	0.67	0.70	2.41	2.52
Intermediate individuals	AA14	6*x*	Spain	14	17	2.77	1.48	0.42	8.55	7.50	6.37	5.94	7.09	0.47	0.44	0.29	0.33	2.00	3.97	0.81	1.61	2.80	5.58
Intermediate individuals	AA53	8*x*	Spain	15	15	2.27	1.47	0.66	9.70	8.95	7.41	7.27	8.33	0.31	0.27	0.43	0.30	1.16	1.67	0.75	1.09	1.91	2.75
Intermediate individuals	AA72	8*x*	Spain	15	15	2.28	1.74	0.66	10.40	8.78	7.44	7.81	8.61	1.43	0.97	0.61	0.98	5.04	6.81	1.72	2.33	6.77	9.13
Intermediate individuals	AA79	10*x*	Spain	15	15	2.19	1.65	0.69	10.11	8.82	7.44	7.57	8.49	0.25	0.54	0.42	0.29	2.47	3.42	0.73	1.01	3.19	4.43
Intermediate individuals	AA9	8*x*	Spain	15	15	1.98	1.75	0.80	9.70	8.51	7.33	7.17	8.18	0.62	0.29	0.18	0.25	2.34	3.50	0.46	0.69	2.80	4.18
L. appressum-salsoloides	89JAM	8*x*	Spain	15	15	2.02	1.32	0.54	9.23	7.98	6.90	6.52	7.66	0.51	0.19	0.31	0.21	2.26	3.86	0.66	1.13	2.92	4.98
L. appressum-salsoloides	AA38	6*x*	Spain	17	15	2.55	1.13	0.64	10.16	9.42	7.93	7.36	8.71	0.56	0.41	0.26	0.14	1.52	2.01	0.72	0.94	2.24	2.95
L. appressum-salsoloides	AA40	6*x*	Spain	14	14	2.24	1.26	0.58	9.29	8.17	6.77	7.08	7.83	0.37	0.40	0.49	0.21	2.04	3.33	0.81	1.32	2.84	4.64
L. appressum-salsoloides	AA45	2*x*	Spain	15	15	2.41	1.09	0.68	7.25	6.98	5.17	5.50	6.22	0.39	0.15	0.27	0.16	0.61	1.58	0.53	1.37	1.14	2.95
L. appressum-salsoloides	AA46	4*x*	Spain	15	15	2.72	1.36	0.63	8.58	7.52	6.52	6.46	7.27	0.49	0.44	0.38	0.17	2.05	3.88	0.55	1.04	2.60	4.92
L. appressum-salsoloides	AA48	6*x*	Spain	15	14	2.36	1.53	0.64	9.36	8.40	6.85	7.13	7.93	0.20	0.13	0.54	0.14	1.26	2.00	0.76	1.21	2.02	3.21
L. appressum-salsoloides	AA49	2*x*	Spain	16	13	2.27	1.36	0.72	7.81	7.00	5.28	5.95	6.51	0.10	0.38	0.33	0.16	1.14	2.68	0.95	2.25	2.09	4.93
L. appressum-salsoloides	AA50	6*x*	Spain	15	7	2.80	1.49	0.67	9.44	8.48	7.00	7.17	8.02	0.57	0.37	0.29	0.26	1.87	2.90	0.59	0.91	2.46	3.81

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L. appressum-salsoloides	AA51	6*x*	Spain	15	15	2.21	1.40	0.66	10.26	8.64	7.01	7.71	8.41	0.43	0.24	0.21	0.28	3.31	4.68	0.98	1.38	4.29	6.07
L. appressum-salsoloides	AA52	2*x*	Spain	15	15	2.23	1.54	0.71	7.99	7.35	5.66	5.82	6.70	0.32	0.23	0.11	0.09	0.96	2.15	0.23	0.51	1.19	2.65
L. appressum-salsoloides	AA80	8*x*	Spain	15	15	2.32	1.96	0.62	9.52	8.58	6.94	7.20	8.06	0.32	0.28	0.42	0.13	1.48	2.27	0.62	0.95	2.09	3.22
L. appressum-salsoloides	AA81	4*x*	Spain	15	15	2.56	1.22	0.71	8.96	8.22	6.53	6.97	7.67	0.41	0.17	0.18	0.16	1.13	1.93	0.53	0.90	1.67	2.83
L. appressum-salsoloides	AA86	4*x*	Spain	15	15	2.38	2.19	0.60	9.59	8.93	7.10	7.29	8.23	0.59	0.35	0.48	0.18	1.37	2.03	0.70	1.03	2.07	3.06
L. appressum-salsoloides	DP1995	4*x*	Spain	15	15	2.54	1.38	0.69	8.51	7.65	6.16	6.52	7.21	0.45	0.16	0.13	0.22	1.36	2.61	0.48	0.92	1.83	3.53
L. appressum-salsoloides	DP2020	6*x*	Spain	15	15	2.22	1.91	0.78	9.31	8.37	6.93	6.80	7.85	1.40	0.46	0.37	0.48	2.75	4.47	0.86	1.39	3.61	5.86
L. salsoloides	85JAM2016	2*x*	Spain	15	15	1.80	1.07	0.79	7.11	6.44	4.77	5.19	5.88	0.26	0.23	0.51	0.13	0.94	2.73	0.82	2.36	1.76	5.09
L. suffruticosum	80JAM2016	2*x*	Spain	15	15	2.13	1.57	0.61	7.81	6.96	5.39	5.68	6.46	0.37	0.20	0.15	0.09	1.29	3.09	0.32	0.77	1.61	3.85
L. suffruticosum	81JAM2016	2*x*	Spain	15	15	2.23	1.50	0.58	8.04	6.74	5.25	5.81	6.46	0.29	0.27	0.21	0.15	2.24	5.37	0.68	1.64	2.93	7.01
L. suffruticosum	82JAM2016	4*x*	Spain	15	16	1.92	1.66	0.48	8.97	7.73	6.18	5.95	7.21	0.52	0.25	0.37	0.29	2.30	4.42	0.71	1.37	3.01	5.79
L. suffruticosum	83JAM2016	4*x*	Spain	15	15	1.76	1.49	0.69	8.61	7.06	5.59	5.83	6.77	0.62	0.38	0.31	0.26	3.38	7.37	0.63	1.38	4.01	8.75
L. suffruticosum	84JAM2016	4*x*	Spain	15	15	1.59	1.04	0.77	9.16	7.28	6.08	6.23	7.19	0.26	0.49	0.42	0.18	4.27	8.26	0.63	1.21	4.90	9.48
L. suffruticosum	AA1	4*x*	Spain	12	12	2.04	1.42	0.61	8.10	6.20	5.45	5.97	6.43	0.75	0.18	0.23	0.28	4.55	10.99	0.78	1.88	5.33	12.87
L. suffruticosum	AA10	10*x*	Spain	15	15	2.44	1.30	0.57	11.22	10.15	8.40	9.24	9.75	0.38	0.22	0.20	0.23	1.75	1.84	1.13	1.18	2.87	3.02
L. suffruticosum	AA13	10*x*	Spain	15	14	3.02	1.54	0.47	9.49	8.64	6.76	7.13	8.00	0.79	0.56	0.32	0.34	2.07	3.24	0.80	1.25	2.87	4.48
L. suffruticosum	AA16	8*x*	Spain	15	15	2.93	1.20	0.55	9.71	7.89	6.80	7.29	7.92	0.28	0.25	0.38	0.27	3.85	6.14	0.90	1.43	4.75	7.57
L. suffruticosum	AA19	6*x*	Spain	15	16	2.13	0.75	0.59	9.51	8.51	7.39	6.96	8.09	0.65	0.29	0.30	0.40	1.95	2.97	0.88	1.35	2.83	4.32
L. suffruticosum	AA2	4*x*	Spain	15	15	2.34	1.84	0.56	9.71	7.81	6.46	6.85	7.71	0.30	0.30	0.19	0.23	4.19	7.05	0.57	0.95	4.76	8.00
L. suffruticosum	AA20	6*x*	Spain	15	15	2.36	2.01	0.70	10.13	8.61	7.06	7.28	8.27	0.39	0.45	0.43	0.65	3.14	4.59	1.13	1.65	4.26	6.23
L. suffruticosum	AA21	6*x*	Spain	15	15	2.86	1.35	0.64	9.09	8.11	6.85	6.85	7.73	1.11	0.43	0.24	0.61	2.50	4.18	0.85	1.42	3.35	5.61
L. suffruticosum	AA22	6*x*	Spain	15	15	3.17	1.97	0.38	9.23	7.89	6.80	6.83	7.69	0.36	0.56	0.32	0.08	2.74	4.63	0.40	0.68	3.14	5.31
L. suffruticosum	AA23	6*x*	Spain	15	15	2.65	1.50	0.60	10.07	8.70	7.34	7.34	8.36	0.68	0.43	0.27	0.32	2.97	4.25	0.59	0.84	3.56	5.09
L. suffruticosum	AA24	6*x*	Spain	13	15	2.69	1.89	0.72	9.29	8.17	6.64	6.93	7.76	0.46	0.32	0.20	0.29	2.04	3.39	0.57	0.95	2.61	4.34
L. suffruticosum	AA25	4*x*	Spain	15	13	2.21	1.10	0.58	8.89	7.64	5.67	5.71	6.98	0.61	0.51	0.23	0.12	2.70	5.54	0.35	0.72	3.05	6.27
L. suffruticosum	AA26	4*x*	Spain	12	8	2.59	1.92	0.46	9.06	6.89	5.39	6.40	6.94	0.24	0.21	0.24	0.20	5.13	10.67	1.46	3.04	6.59	13.71

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L. suffruticosum	AA27	4*x*	Spain	3	7	1.97	1.32	0.48	9.44	8.04	6.16	6.75	7.60	0.19	0.35	0.80	0.02	2.48	4.30	1.16	2.02	3.65	6.32
L. suffruticosum	AA28	4*x*	Spain	13	8	2.12	0.99	0.66	9.03	6.80	5.71	6.81	7.09	0.44	0.47	0.64	0.27	5.87	11.69	2.13	4.25	8.00	15.93
L. suffruticosum	AA3	4*x*	Spain	16	14	2.50	1.85	0.65	8.61	7.82	5.90	6.02	7.09	0.36	0.33	0.36	0.40	1.32	2.62	0.78	1.55	2.10	4.17
L. suffruticosum	AA31	2*x*	Spain	10	9	3.27	1.41	0.82	7.72	6.76	5.44	5.70	6.40	0.35	0.31	0.38	0.43	1.58	3.86	0.88	2.14	2.46	6.00
L. suffruticosum	AA32	2*x*	Spain	8	8	2.00	1.69	0.71	8.25	6.73	5.31	6.21	6.62	0.35	0.21	0.38	0.13	2.88	6.56	1.34	3.05	4.22	9.61
L. suffruticosum	AA33	2*x*	Spain	15	15	2.29	1.83	0.62	8.40	6.78	5.48	5.96	6.66	0.21	0.50	0.21	0.23	3.31	7.48	0.66	1.49	3.97	8.97
L. suffruticosum	AA34	6*x*	Spain	15	13	1.99	1.49	0.69	9.50	8.56	7.21	7.23	8.12	0.41	0.65	0.21	0.19	1.94	2.94	0.40	0.60	2.34	3.54
L. suffruticosum	AA35	6*x*	Spain	15	14	2.62	2.11	0.68	9.78	8.60	7.08	7.37	8.20	0.35	0.23	0.30	0.23	1.97	2.93	0.61	0.91	2.58	3.84
L. suffruticosum	AA36	10*x*	Spain	11	14	3.48	2.80	0.59	8.90	7.92	6.72	6.50	7.51	0.79	0.28	0.28	0.22	2.02	3.59	0.54	0.96	2.57	4.55
L. suffruticosum	AA37	8*x*	Spain	11	15	2.61	1.14	0.52	8.21	7.84	6.38	6.35	7.20	0.45	0.97	0.46	0.16	1.55	2.99	0.61	1.18	2.16	4.17
L. suffruticosum	AA4	4*x*	Spain	15	15	2.85	1.55	0.57	7.75	7.06	5.74	5.79	6.58	0.53	0.59	0.36	0.69	1.60	3.68	1.05	2.43	2.65	6.11
L. suffruticosum	AA5	2*x*	Spain	15	15	2.41	1.52	0.69	8.22	7.51	5.74	5.94	6.85	0.32	0.33	0.45	0.17	1.15	2.45	0.67	1.42	1.82	3.87
L. suffruticosum	AA57	8*x*	Spain	15	15	2.56	1.63	0.69	9.64	8.97	7.23	7.36	8.30	0.45	0.37	0.38	0.22	1.27	1.85	0.62	0.89	1.89	2.74
L. suffruticosum	AA58	10*x*	Spain	15	10	2.00	1.61	0.58	10.67	9.24	7.04	8.37	8.83	0.18	1.62	2.01	0.18	3.83	4.92	3.96	5.08	7.79	9.99
L. suffruticosum	AA59	10*x*	Spain	4	13	2.44	1.16	0.57	9.55	8.18	6.97	7.44	8.03	0.07	0.51	0.70	0.30	2.45	3.79	1.22	1.89	3.66	5.68
L. suffruticosum	AA60	8*x*	Spain	15	15	2.51	1.44	0.56	10.38	9.37	7.28	8.48	8.88	1.03	0.31	0.27	0.28	2.37	3.01	2.00	2.53	4.37	5.54
L. suffruticosum	AA61	2*x*	Spain	15	15	2.69	1.54	0.60	8.35	7.09	5.85	6.01	6.82	0.28	0.18	0.40	0.12	2.05	4.40	0.55	1.19	2.60	5.59
L. suffruticosum	AA62	2*x*	Spain	15	15	2.64	1.26	0.65	8.20	6.75	5.37	5.75	6.52	0.17	0.44	0.48	0.27	2.73	6.42	0.89	2.10	3.62	8.52
L. suffruticosum	AA63	4*x*	Spain	15	15	2.13	1.22	0.70	9.09	7.87	6.02	6.59	7.39	0.14	0.30	0.23	0.16	1.93	3.54	0.71	1.30	2.65	4.84
L. suffruticosum	AA65	4*x*	Spain	15	15	2.71	1.09	0.63	8.74	7.32	5.91	5.47	6.86	0.38	0.10	0.19	0.17	2.52	5.35	0.55	1.17	3.07	6.52
L. suffruticosum	AA67	8*x*	Spain	16	14	2.42	1.09	0.69	10.43	9.88	8.43	8.14	9.22	1.20	0.35	0.31	0.54	1.84	2.17	0.93	1.10	2.78	3.26
L. suffruticosum	AA68	8*x*	Spain	15	15	1.86	1.46	0.55	9.38	8.07	6.93	6.78	7.79	0.62	0.27	0.34	0.32	2.60	4.28	0.68	1.12	3.28	5.40
L. suffruticosum	AA69	8*x*	Spain	15	15	2.44	1.53	0.67	9.57	8.51	6.86	7.38	8.08	0.36	0.36	0.39	0.09	1.86	2.85	0.76	1.17	2.62	4.01
L. suffruticosum	AA73	8*x*	Spain	15	15	1.90	2.09	0.67	9.86	9.20	7.25	7.55	8.47	0.55	0.68	0.46	0.15	1.66	2.32	0.70	0.98	2.37	3.30
L. suffruticosum	AA75	2*x*	Spain	15	15	2.28	1.45	0.66	8.12	7.84	6.15	6.32	7.11	0.15	0.34	0.28	0.14	0.57	1.13	0.45	0.90	1.03	2.03
L. suffruticosum	AA78	2*x*	Spain	15	15	2.60	1.14	0.61	8.61	8.12	6.44	6.60	7.44	0.22	0.22	0.16	0.19	0.67	1.21	0.38	0.69	1.05	1.90
L. suffruticosum	AA8	8*x*	Spain	15	15	2.59	1.34	0.49	9.92	8.62	7.40	7.36	8.32	0.35	0.23	0.55	0.11	2.28	3.29	0.66	0.95	2.93	4.24
L. suffruticosum	AA83	6*x*	Spain	15	15	2.32	1.64	0.69	8.61	7.80	6.19	6.61	7.30	0.70	0.31	0.23	0.48	1.67	3.14	0.88	1.66	2.55	4.79

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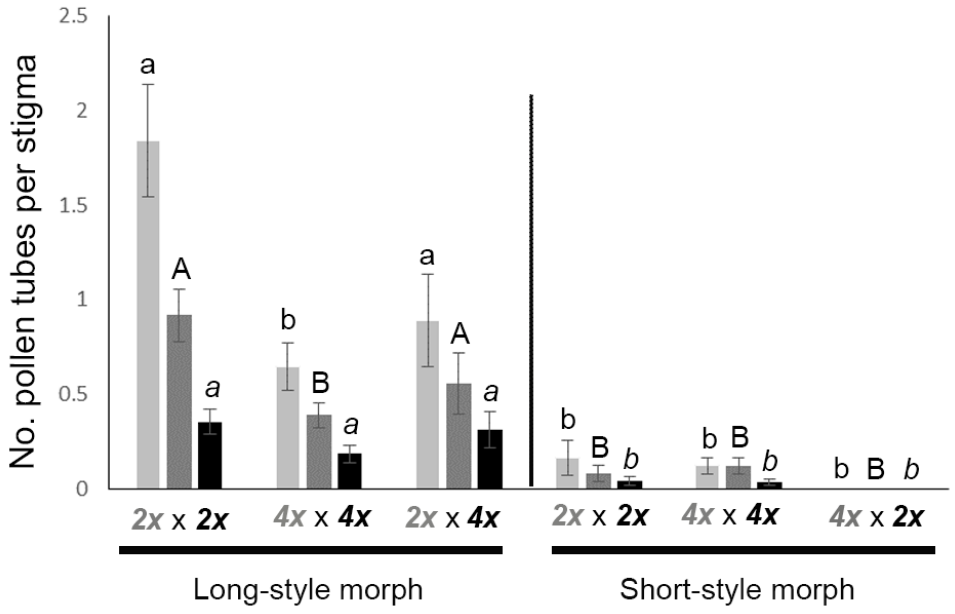
L. suffruticosum	AA84	6*x*	Spain	15	15	2.53	1.18	0.67	9.06	7.88	6.72	6.62	7.57	0.54	0.76	0.36	0.26	2.69	4.70	0.63	1.10	3.32	5.80
L. suffruticosum	AA88	10*x*	Spain	15	15	2.43	1.00	0.55	11.06	10.28	8.37	8.84	9.64	0.22	0.40	0.24	0.23	1.23	1.33	0.69	0.74	1.92	2.07
L. suffruticosum	AA90	10*x*	Spain	16	14	2.89	1.98	0.72	10.56	9.93	7.83	8.12	9.11	0.74	0.41	0.52	0.12	1.55	1.86	0.72	0.87	2.27	2.73
L. suffruticosum	AA91	10*x*	Spain	15	16	2.36	1.88	0.56	10.04	9.44	7.87	7.61	8.74	0.64	0.68	0.25	0.41	1.69	2.21	0.73	0.96	2.42	3.17
L. suffruticosum	AA93	8*x*	Spain	15	15	2.41	1.21	0.59	9.93	9.37	7.18	7.56	8.51	0.54	0.42	0.34	0.39	1.28	1.77	0.87	1.20	2.15	2.97
L. suffruticosum	AA94	8*x*	Spain	15	15	2.29	2.20	0.53	8.92	7.56	6.56	6.49	7.38	0.68	0.51	0.63	0.41	3.03	5.57	1.04	1.91	4.07	7.48
L. suffruticosum	AC1	2*x*	Spain	15	15	2.11	1.21	0.73	7.90	6.22	5.17	5.68	6.24	0.44	0.30	0.23	0.27	3.57	9.15	0.76	1.94	4.33	11.10
L. suffruticosum	MO6136	6*x*	Spain	15	15	2.44	2.09	0.63	9.70	8.17	7.17	7.02	8.01	0.25	0.73	0.64	0.25	3.33	5.19	0.91	1.42	4.24	6.61
L. suffruticosum	MO6137	6*x*	Spain	15	7	2.44	1.57	0.65	9.90	8.96	7.70	7.25	8.45	0.53	0.30	0.16	0.11	1.71	2.39	0.47	0.66	2.18	3.05
L. suffruticosum	AA100	4*x*	Morocco	15	15	1.75	1.81	0.56	9.55	9.39	7.55	7.21	8.42	1.38	0.41	0.34	0.36	1.81	2.55	0.83	1.16	2.64	3.72
L. suffruticosum	AA101	2*x*	Morocco	15	15	1.85	1.72	0.61	8.36	7.98	6.48	6.60	7.36	0.41	0.47	0.39	0.19	1.03	1.90	0.60	1.11	1.63	3.01
L. suffruticosum	AA106	2*x*	Morocco	6	4	2.28	1.77	0.53	7.86	7.03	5.99	6.27	6.79	0.14	0.32	0.20	0.04	1.14	2.49	0.32	0.69	1.46	3.18
L. suffruticosum	AA107	2*x*	Morocco	15	15	2.16	1.69	0.64	7.94	6.88	5.81	6.14	6.69	0.14	0.14	0.10	0.05	1.40	3.11	0.26	0.59	1.66	3.70
L. suffruticosum	AA108	4*x*	Morocco	15	15	2.34	1.24	0.62	10.20	9.71	7.69	7.84	8.86	0.41	0.31	0.23	0.30	0.97	1.23	0.55	0.70	1.52	1.93
L. suffruticosum	AA111	6*x*	Morocco	15	15	1.80	1.69	0.69	11.68	11.42	9.31	9.06	10.37	0.50	0.99	0.79	0.11	1.56	1.45	0.96	0.90	2.53	2.35
L. suffruticosum	AA112	2*x*	Morocco	9	4	2.01	1.68	0.71	9.33	8.60	7.14	7.69	8.19	0.53	0.85	0.08	0.29	1.91	2.85	0.68	1.02	2.59	3.87
L. suffruticosum	AA113	6*x*	Morocco	15	15	2.22	1.05	0.53	12.90	12.18	9.38	9.42	10.97	0.89	1.25	0.28	0.21	2.65	2.20	0.49	0.41	3.14	2.61

Appendix 4.4. Number of pollen tubes in each style level (top, middle, bottom) for intra-cytotype and (2*x* × 2*x* and 4*x* × 4*x*), and inter-cytotype (2*x* × 4*x* and 4*x* × 2*x*) crosses and index of reproductive isolation (IRI) for inter-cytotype crosses at each style level (top, middle, bottom) of *Linum suffruticosum s.l.*.

Treatment	Top (mean ± SD)	IRI	Middle (mean ± SD)	IRI	Bottom (mean ± SD)	IRI
Intra-cytotype crosses (2*x* × 2*x* and 4*x* × 4*x*)	9.76 ± 6.08	-	4.19 ± 2.47	-	1.55 ± 1.22	-
Inter-cytotype crosses (2*x* × 4*x*)	7.37 ± 4.89	0.24	3.56 ± 2.22	0.15	1.65 ± 1.04	0
Inter-cytotype crosses (4*x* × 2*x*)	6.81 ± 4.72	0.30	3.43 ± 2.09	0.18	1.79 ± 1.03	0

Appendix 4.5. Average number of pollen tubes in the top (light grey), middle (dark grey) and bottom (black) level of the style in intra-morph crosses within diploids (2*x* × 2*x*), within tetraploids (4*x* × 4*x*), and between diploids and tetraploids (2*x* × 4*x* and 4*x* × 2*x*) of *Linum suffruticosum s.l.*. Indication of the pollen receptor and donor are given in grey and black, respectively. Different letters correspond to statistically significant differences at $P < 0.05$ for Type-III analysis of variance: lowercase letters correspond to the top level of style, uppercase letters to the middle level, and letters in italic for the bottom level.



Chapter V - Multiple events of genome duplications in the differentiation of the polyploid complex *L. suffruticosum s.l.*

Abstract

The abundance of biodiversity in the Mediterranean region is correlated with the complex and diverse climatic and geological history. Polyploidization is another factor that may have led to a high diversity of species in this region as it can facilitate reproductive isolation and adaptation to new environments. Although an increased number of polyploid complexes have been described in the Mediterranean region, few have been studied using a phylogenetic or phylogeographic approach. This study aimed to reconstruct the historical setting of the *Linum suffruticosum s.l.* polyploid complex throughout its distribution range in the Western Mediterranean Basin. For that, DNA extractions from populations of all cytotypes of *L. suffruticosum s.l.* were made. Two plastid makers and one nuclear marker were used to estimate an haplotype-ribotype network and infer the phylogenetic relationships among the ploidy levels of this species. Results showed a much higher variability of ribotypes and haplotypes in diploid populations from the east Pyrenees, southern France and northern Italy than in diploid and polyploid populations from Spain and Morocco, and some ribotypes and haplotypes in polyploids were shared between the Iberian Peninsula and northern Morocco. The results suggest that several genome duplications and differentiation events occurred along the distribution area and recent evolution of this polyploid complex. The differentiation and evolution of *L. suffruticosum s.l.* seem to be linked with the geographic and climatic history of the Mediterranean zone. This study provides valuable data for future phylogeny and phylogeography studies.

Key words: genome duplications, haplotype network, *Linum*, Mediterranean Basin, rybotype network.

Introduction

The Mediterranean region is considered a biodiversity hotspot, and it contains about 25,000 species and 10% of the total plant diversity in the world (Quézel 1978; Cowling *et al.* 1996). This richness in biodiversity is correlated with its complex and diverse climatic and geological history. The climate in this region is characterised by mild, rainy winters and hot, dry summers. Still, the evolution and oscillation of its climate significantly impacted the emergence and survival of species and the dynamics of species ranges (Thompson 2020). The Mediterranean Basin has served as a refugium for many species. Throughout its paleogeographic history, it often isolated some taxa or led to the contact of related taxa, contributing to the emergence of new species (Thompson 2020). Another factor that may have led to a high diversity of species in the Mediterranean basin is polyploidy, which can facilitate reproductive isolation and adaptation to new environments (Tate *et al.*; Levin 1975, 2002; Glennon *et al.* 2014). Polyploidization has been considered to play a significant role in the evolution and diversification of flowering plants and is recognised as an important mechanism of speciation (Ramsey and Schemske 1998; Soltis and Soltis 1999). Indeed, 36.5% of polyploids have been reported in the Mediterranean flora (48.0% for the Iberian Peninsula; Marques *et al.* 2018). Many polyploid complexes have been described in the Mediterranean region (*e.g.*, Lumaret *et al.* 1997; Segraves *et al.* 1999; Mansion *et al.* 2005; Buggs and Pannell 2007; Kolář *et al.* 2009; Winterfeld *et al.* 2009; Eilam *et al.* 2010; Marques *et al.* 2018; López-Jurado *et al.* 2019; Bougoutaia *et al.* 2021). However, only a reduced percentage of those polyploid complexes have been studied using a phylogenetic or phylogeographic approach (*e.g.*, Koch *et al.* 1998; Gielly *et al.* 2001; Fiz *et al.* 2002; Valcárcel *et al.* 2003; Escudero *et al.* 2018; López-González *et al.* 2021; Maguilla *et al.* 2021a; b).

Although variation among ploidy levels may reflect their evolutionary relationships, more profound knowledge is only achieved with appropriate molecular markers and their phylogenetic and phylogeographic analyses, particularly within ploidy levels. The recent molecular techniques developed have increased the knowledge about polyploids, not only about their evolutionary significance, mechanisms of polyploid formation, and their establishment (Felber 1991; Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Otto and Whitton 2000; Husband *et al.* 2013), but also about the genomic evolution of polyploids (Soltis and Soltis 2000; Adams *et al.* 2004; Lim *et al.* 2004). Furthermore, in some cases, this allowed us to distinguish between auto- and allopolyploids (Leitch and Bennett 1997; Baumel *et al.* 2002; Barker *et al.* 2016). Autopolyploids result within a single species without hybridisation, while allopolyploidy involves a hybridisation process between genetically distinct taxa or lineages (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Soltis and Soltis 1999;

Levin 2002). They mainly differ by the degree of genetic differentiation between parental species (Stebbins 1971; Grant 1981; Soltis and Soltis 2000). Additionally, a range of processes can subtly rearrange single chromosomes, altering chromosome numbers or the nuclear DNA content (Vimala *et al.* 2021). Thus, the origin of a polyploid and its divergence is not always easy or even possible to date, especially in the case of sympatric speciation (Doyle and Egan 2010).

Linum suffruticosum s.l. (Linaceae) is distributed through the western Mediterranean basin and bears a high complexity and morphological variability. In addition, the species complex presents geographic overlap and high cytogenetic diversity (ranging from diploid to decaploid populations) in both margins of the western Mediterranean basin (Afonso *et al*. 2021 in Chapter II). All of this might indicate multiple origins of the polyploids from the same and/or different progenitors (Nicholls 1986a; Ruiz-Martín 2017; Afonso *et al.* 2021). *Linum suffruticosum* complex is considered very recent, having originated probably at the beginning of the Pleistocene, while the genus originated and began to diversify in the early Oligocene to late Miocene (Ruiz-Martín *et al.* 2018; Maguilla *et al*. 2021a). The dispersal and differentiation of *L. suffruticosum s.l.* can be related to the Mediterranean Basin's geological and paleoclimatic history, and different possible origins of polyploids could have occurred in Europe and NW Africa. Although phylogenetic relationships have been recently studied in the whole genus *Linum* (McDill *et al.* 2009; Schneider *et al.* 2016; Ruiz-Martín *et al.* 2018), little is known about the phylogenetic relationships in the white flax group and, in particular, within L*. suffruticosum s.l.* In this context, *L. suffruticosum s.l.* polyploid complex represents an excellent study system for testing evolutionary hypotheses and correlations with polyploidy.

Ultimately, the main objective of this study was to reconstruct the historical setting of the polyploid complex throughout its range in the Western Mediterranean Basin and its biogeographic and climatic transitions (*i.e.*, from the arid Mediterranean to temperate Europe), taking into consideration shifts in ploidy level. The specific aims of this study were: (1) to estimate haplotype-ribotype networks of *L. suffruticosum s.l*. cytotypes; (2) to assess the significance of correlated evolution of polyploidy across the haplotype-ribotype networks; and (3) to integrate all these results in a geographical and ecological context to infer the conditions under which polyploidy most likely evolved.

Materials and methods

Study system and sampling populations

Linum suffruticosum s.l. is a distylous polyploid complex distributed through the western Mediterranean basin (Rogers 1979; Nicholls 1985b; c, 1986a; Armbruster *et al.* 2006; McDill *et al.* 2009). Recent detailed studies have shown that *L. suffruticosum s.l.* has a high cytogenetic

diversity [diploids (2*x*), tetraploids (4*x*), hexaploids (6*x*), octoploids (8*x*), and decaploids (10*x*)] with a complex mosaic distribution distributed along the western Mediterranean basin. Cytotypes are distributed parapatrically and geographically structured in several contact zones (Afonso *et al*. 2021 in Chapter II). Most of the cytogenetic diversity was found in the Iberian Peninsula, with the remaining areas of the species distribution in Europe being characterised by homogeneously diploid populations (Afonso *et al.* 2021 in Chapter II). The current study uses populations with known ploidy levels following the sampling made in Chapter II. Furthermore, all sampled populations from the eastern Pyrenees, southern France, and northern Italy are composed of diploid individuals (E. Olmedo-Vicente, A. Afonso & J. Arroyo, unpublished data). This complex also included *L. tenuifolium,* a sister species of *L. suffruticosum* complex often treated in the same taxon (Rogers 1979; Nicholls 1985a; b, 1986). *Linum tenuifolium* is style monomorphic and diploid in all its distribution (Rogers 1979; Nicholls 1985a; b, Ruíz-Martín *et al*., 2017; E. Olmedo-Vicente, A. Afonso & J. Arroyo, unpublished data). Both species co-occur in north-east Spain, south-east France, north of Italy, and a second contact zone might occur in north-east Algeria (Quézel and Santa 1962; Ockendon and Walters 1968; Ozenda 1977; López González 1979; Martínez-Labarga and Garmendia 2015). Most taxonomic treatments currently agree on recognising *L. tenuifolium* as a single species(Ockendon and Walters 1968; López González 1979; Martínez-Labarga and Garmendia 2015), and most of the taxonomic disagreement and morphological and cytogenetic diversity, is found in *L. suffruticosum* complex. The taxonomic treatment followed by Afonso *et al.* (2021), in Chapter II, which considers a high morphological diversity in association with cytogenetic variability of this complex, was used. Therefore, the populations used in this study were those treated as *L. suffruticosum*, *L. appressum-salsoloides*, *L. salsoloides,* and *L. suffruticosum* var*. milletii* (Afonso *et al*., 2021 in Chapter II). Populations with a very high degree of identification uncertainty were excluded (those treated as *Intermediate taxon* - Afonso *et al*., 2021 in Chapter II).

DNA extraction, amplification, and sequencing

In total, 61 populations were used for the DNA extractions. Specifically, we used 33 diploid, 9 tetraploid, 11 hexaploid, 6 octoploid, and 2 decaploid populations of *L. suffruticosum s.l.* (Appendix 5.1). A large number of diploid populations reflects their vast geographic area of distribution. DNA from *L. tenuifolium* and *L. strictum* was extracted from natural populations (see Ruiz-Martín 2017) to act as the closest related species to *L. suffruticosum s.l.*. DNA was

extracted from 15-20 g of dried leaves per sample using the DNEasy Plant Minikit extraction kit (QIAGEN Inc.), following the manufacturer's instructions, and stored at -20 °C.

One nuclear DNA region, ITS (internal transcribed spacer), and two plastid DNA regions, *ndh*F-*rpL*32 spacer and *ndh*A intron were amplified, purified, and sequenced (Appendix 5.2). These two plastid DNA regions were selected because they were the most variable for the study species (a total of 17 plastid DNA regions were tested; see Taberlet *et al.* 1991; Weisburg *et al.* 1991; Graham and Olmstead 2000; Shaw *et al.* 2005, 2007). The amplification of ITS and the two plastid regions was carried out by PCR using Ecogen polymerase, whose commercial name is EcoTaq 500 U, with a concentration of 5 U/µl. The mixture for the PCR reaction consisted of 2.5 µl 10 x BioTherm reaction buffer, 200 µM dNTPs (2.5 mM), 10 µM for each marker, 2 mM $MgCl_2$, 0.025U/µl polymerase and 2 µl of extracted DNA. The final PCR volume was 25 µl. The amplified DNA was purified using the Exo SAP-IT® for PCR Product Clean-Up enzyme from the USB laboratories. Each PCR cycle comprises 5 min at 93 °C, 32 cycles at 93 °C for 1 min, 51–52 °C (ITS: 51 °C, *ndh*F-*rpL*32: 52 °C, *ndh*A: 52 °C) for 1–2 min, and 72 °C for 2 min. The amplification products were tested on 1.5% agarose gels in TAE (Tris-acetate buffer) and labelled with SYBR Green. The PCR products were sent to Macrogen Inc. (http://www.macrogen.co.kr) for sequencing. The DNA extraction and amplification of the regions were carried out at the Molecular Systematics Laboratory of the Madrid Botanical Garden and at the Department of Ecology and Evolution, University of Seville, while the sequencing was carried out by Macrogen Inc. (Seoul, South Korea).

Sequence alignment, haplotype, and ribotype network

All forward and reverse sequences were checked and aligned manually using Geneious Pro v.3.6.1 (http://www.geneious.com). Then, a matrix was constructed for each region. Incongruence between plastid DNA regions was discarded, and the two DNA plastid regions were combined in a single matrix, including *L. tenuifolium* and *L. strictum*. Next, a matrix was built for the ITS region, including *L. tenuifolium*. Finally, three closed related species were added to the matrix of ITS regions, *L. flos-carmini*, *L. strictum*, and *L. setaceum* (McDill *et al.* 2009; Ruiz-Martín *et al.* 2018). These ITS sequences for the latter three species were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank).

The combined matrix of two plastid DNA regions (*ndh*F-*rpl*32 and *ndh*A, 956 sites) was used to generate a haplotype network. This network included 26 diploid, 6 tetraploid, 5 hexaploid, 3 octoploid, and 1 decaploid population of *L. suffruticosum s.l.* (Appendix 5.1). Amplification of the *ndh*A intron was difficult; therefore, the available data for the *ndh*F-*rpL*32

spacer was higher and comprised more populations and ploidy levels than for the *ndh*A. For this reason, the matrix of *ndh*F-*rpL*32 was used to generate another haplotype network. This network included 32 diploid populations, 9 tetraploid populations, 11 hexaploid populations, 5 octoploid populations, and 2 decaploid populations (Appendix 5.1).

Haplotypes were defined using the software TCS v.1.21 (Clement *et al.* 2000), providing a 95% plausible set for all haplotype linkages of *L. suffruticosum s.l.* cytotypes, *L. strictum* and *L. tenuifolium*. The ribotype network was generated using the SplitsTree4 software package (http://www.splitstree.org; Huson et al., 2006) with the ITS matrix. The analysis with *L. tenuifolium* comprised 24 diploid, 7 tetraploid, 8 hexaploid, 3 octoploid, and 2 decaploid populations (Appendix 5.1).

Phylogenetic analyses

Phylogenetic analyses were performed using Maximum Likelihood (ML) with Mega X software (Kumar *et al.* 2018). The following matrices were used: (1) consensus matrix of two plastid sequences and (2) ITS. The best fit model of nucleotide substitution was based on the highest Akaike Information Criterion corrected (AICc) and highest (less negative) log-likelihood (lnL). The General Time Reversible model with a gamma-distributed rate variation among sites and an extent of static, unchanging sites in a dataset (GTR+G+I) was selected for the matrices of the consensus matrix of two plastid sequence types (*ndh*F-*rpL*32 and *ndh*A) and ITS (Appendix 5.3). The tree with the highest log-likelihood of the chosen model was inferred from 1000 bootstraps to estimate clade support for each analysis (Felsenstein 1985).

Results

Haplotype network

When analysing the matrix of the combined plastid DNA regions, 20 haplotypes were found for *L. suffruticosum s.l.* in two main sub-networks (Appendix 5.1, Figures 5.1 and 5.2). Although variability was found within polyploids, most of the variability was found in diploid populations, particularly in the homogeneously diploid zone (east of the Pyrenees, southern France, and northern Italy). In addition to being found in diploid populations of France and Italy, haplotype A was also common in *L. tenuifolium*. In the first sub-network, haplotypes B, D, I, J, K, and L belong to diploid populations from the eastern Pyrenees, southern France, and northern Italy, identified in most cases as *L. appressum-salsoloides* (except for the L haplotype, identified as *L. suffruticosum*; Appendix 5.1, Figures 5.1 and 5.2). On the other hand, haplotype E (represented by *L. appressum-salsoloides* and *L. suffruticosum*) was found in northern and

central Spain diploid populations. Later, this haplotype gave rise to haplotype F (represented by *L. suffruticosum*), present in diploid and tetraploid populations further south. Finally, haplotypes G and H (also represented by *L. suffruticosum*) detected in diploid populations in the south of Spain emerged from haplotype F. In this part of the network, haplotype C, which originated from haplotype A, was found in a hexaploid population of *L. suffruticosum* in northern Morocco (Appendix 5.1, Figures 5.1 and 5.2).

Concerning the second sub-network derived from haplotype A, the haplotype M comprised tetraploid populations from southern Spain and Morocco. Still, it was also found in a hexaploid population in the western Pyrenees. From this haplotype, we can verify a first set comprising the N haplotype that was only identified in diploid populations of an *L. suffruticosum* variety growing in Catalonia (var. *milletii*) and the O haplotype identified in decaploid populations of *L. suffruticosum*. From haplotype M, we also detected the emergence of the P (diploid and octoploid), Q (octoploid), and R (hexaploid and octoploid) haplotypes present in the north of Spain. The haplotype T also emerged from haplotype M and comprised tetraploid populations in Central Spain. This haplotype is linked with the hexaploid haplotype S, which also emerged from haplotype R. The *L. strictum* outgroup was not connected with the haplotype network (Appendix 5.1, Figures 5.1 and 5.2). When analysing the matrix of *the ndh*F-*rpL*32 spacer, 22 haplotypes were found for *L. suffruticosum s.l.* with two main sub-networks (Appendices 5.4, 5.5 and 5.6). This network with a higher population sampling but only one plastid DNA region was congruent with the previous network based on the two haplotypes. The main differences were: 1) the haplotype network starts by differentiating haplotypes A and B, which comprise diploid populations from *L. suffruticosum var. milletii*, from haplotype D, a diploid population identified as *L. salsoloides*; and 2) most haplotypes from Morocco were separated (haplotype N bears hexaploid and diploid populations, O is a tetraploid population, P comprises hexaploid and diploid populations, and Q is a diploid population (Appendices 5.4, 5.5 and 5.6).

Ribotype network

In the ribotype network, most of the variability was found in diploid populations in the homogeneously diploid zone (east of the Pyrenees, southern France and northern Italy; Appendix 5.1, Figures 5.3 and 5.4). Ribotypes A to E represents diploid populations in France and Italy (identified as *L. appressum-salsoloides*). Ribotypes G, H and F represent populations in the Pyrenees of *L. appressum-salsoloides* and *L. suffruticosum* var. *milletii*, and one population *of L.*

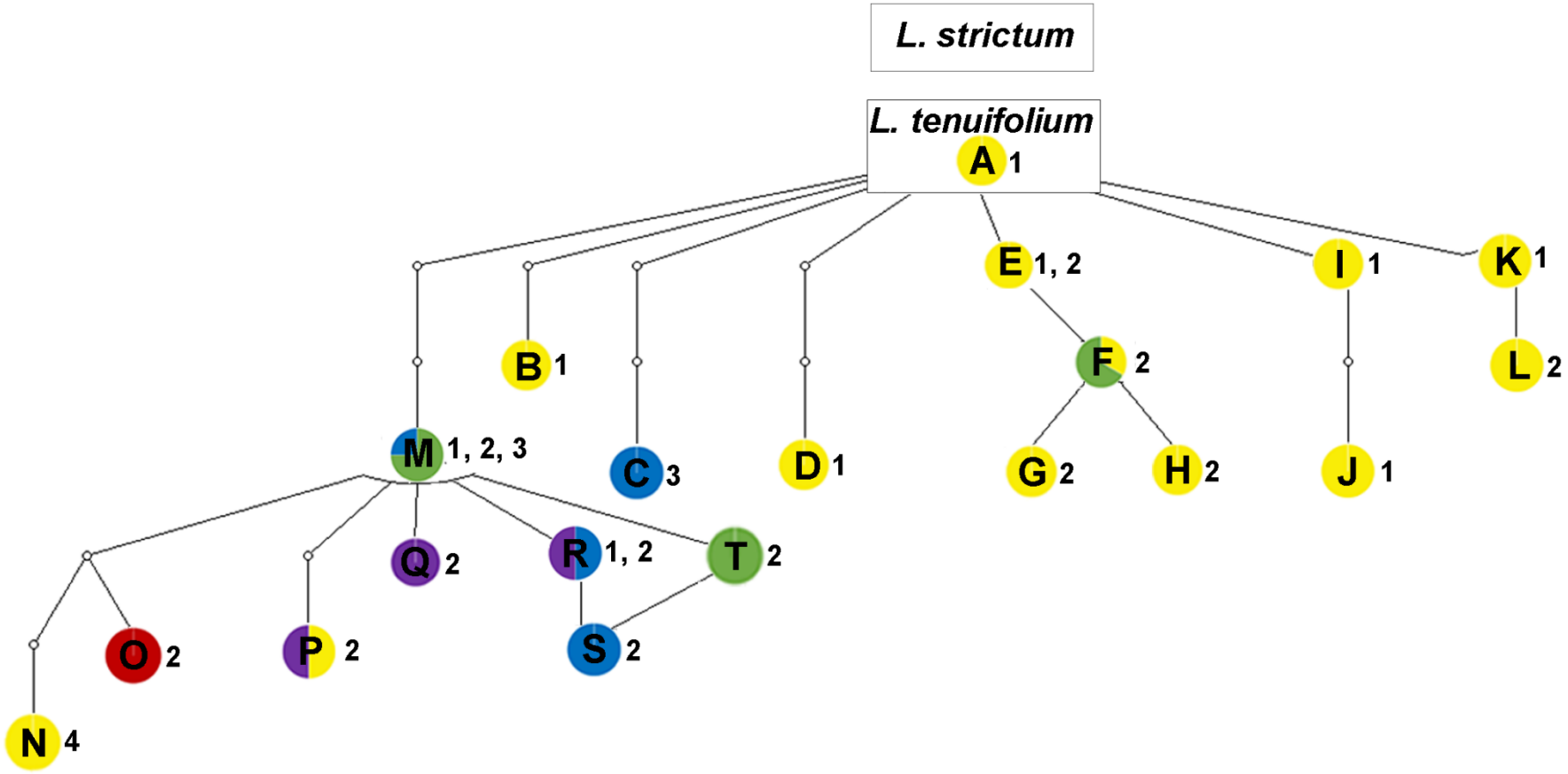


Figure 5.1. Haplotype network of ploidy levels of *L. suffruticosum s.l.* (diploid – yellow; tetraploid – green; hexaploid – blue; octoploid – purple; decaploid – red), based on the consensus matrix of the two plastid regions (*ndh*F-*rpL*32 spacer and *ndh*A intron) combined with the outgroups *L. tenuifolium* and *L. strictum*. Taxa of *L. suffruticosum s.l.* are indicated on the side of each haplotype (1 - *L. appressum-salsoloides;* 2 - *L. suffruticosum* from Iberian Peninsula*;* 3 - *L. suffruticosum* from Morocco; and 4 - *L. suffruticosum* var. *milletii*).

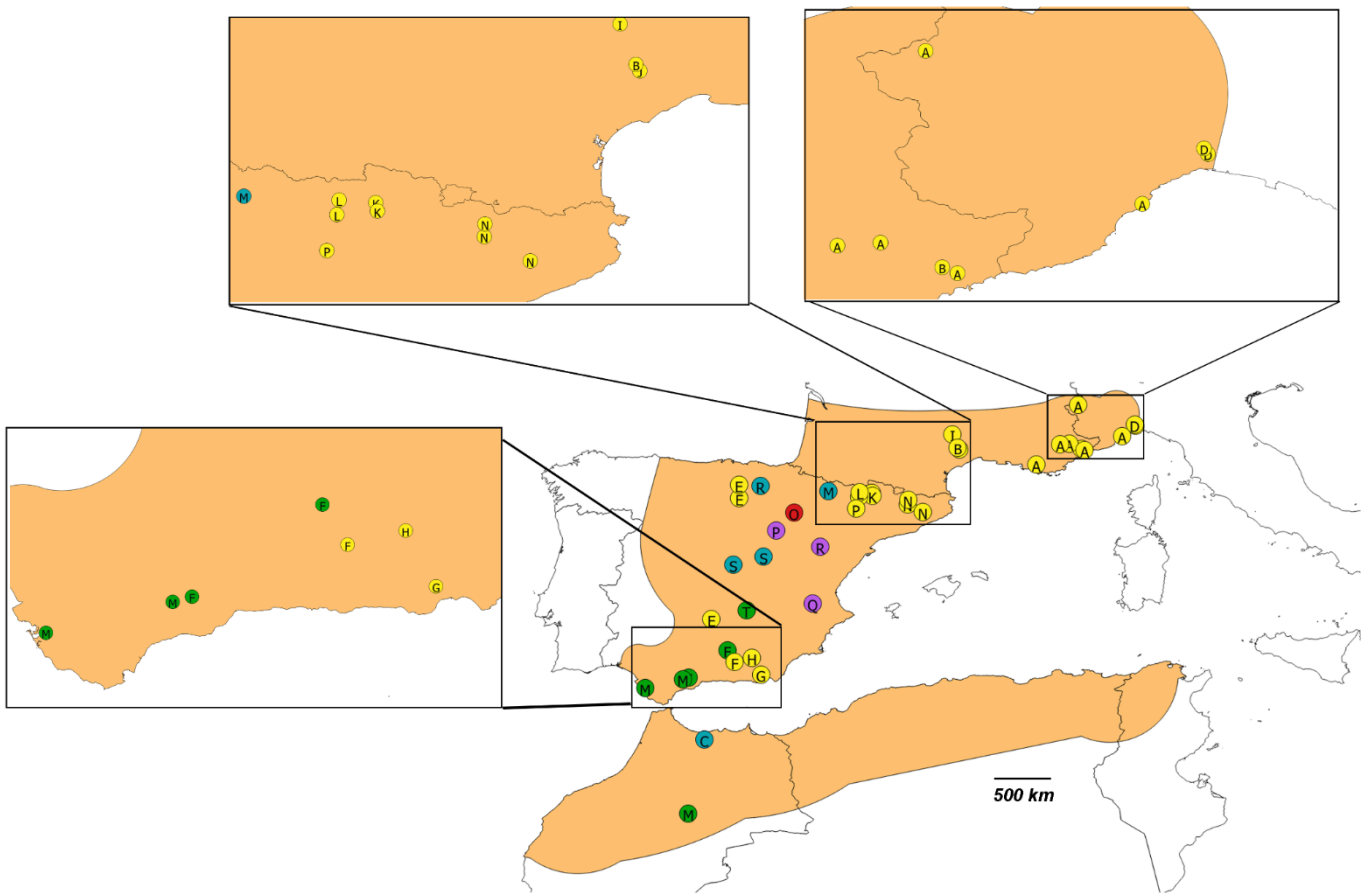


Figure 5.2. Geographic locations of the haplotype network of ploidy levels of *L. suffruticosum s.l.* (diploid – yellow; tetraploid – green; hexaploid – blue; octoploid – purple; decaploid – red) based on the consensus matrix of the two plastid regions (*ndhF-rpL32* spacer and *ndhA* intron) combined with the outgroups *L. tenuifolium* and *L. strictum.* The base map was downloaded from https://www.diva-gis.org/gdata.

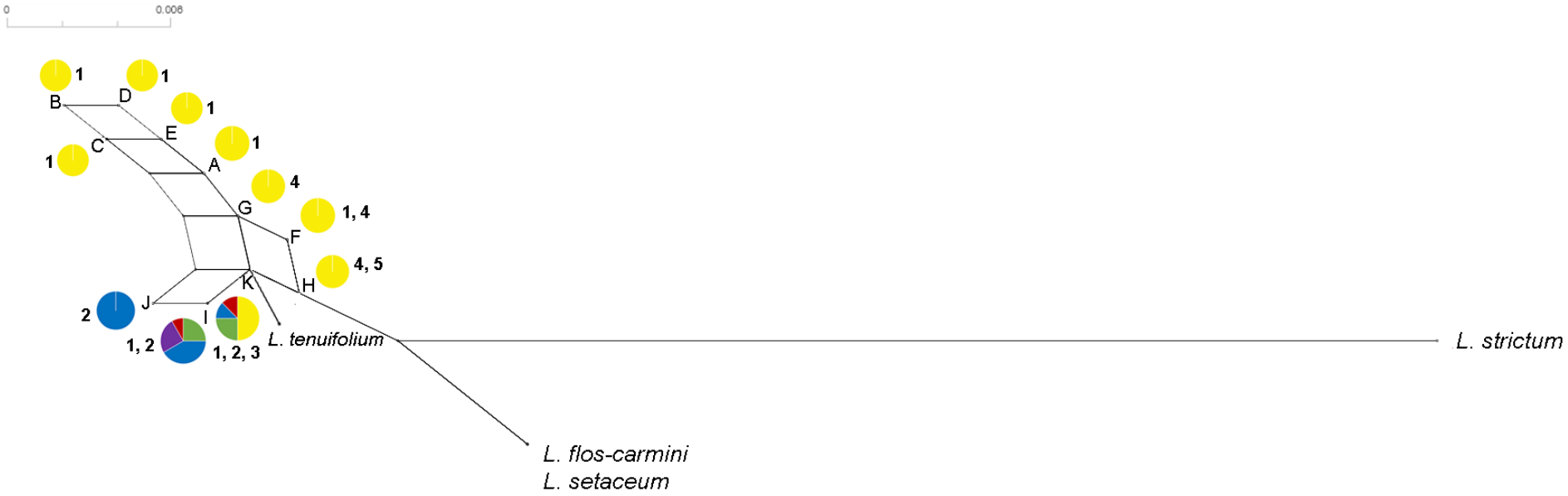


Figure 5.3. Rybotype network of ploidy levels of *L. suffruticosum s.l.* (diploid – yellow; tetraploid – green; hexaploid – blue; octoploid – purple; decaploid – red) based on the matrix of ITS (internal transcribed spacer) combined with the outgroups *L. tenuifolium*, *L. flos-carmini, L. setaceum* and *L. strictum.* Taxa of *L. suffruticosum s.l.* are indicated on the side of each haplotype (1 - *L. appressum-salsoloides;* 2 - *L. suffruticosum* from Iberian Peninsula*;* 3 - *L. suffruticosum* from Morocco; 4 - *L. suffruticosum* var. *milletii*; and 5 - *L. salsoloides*).

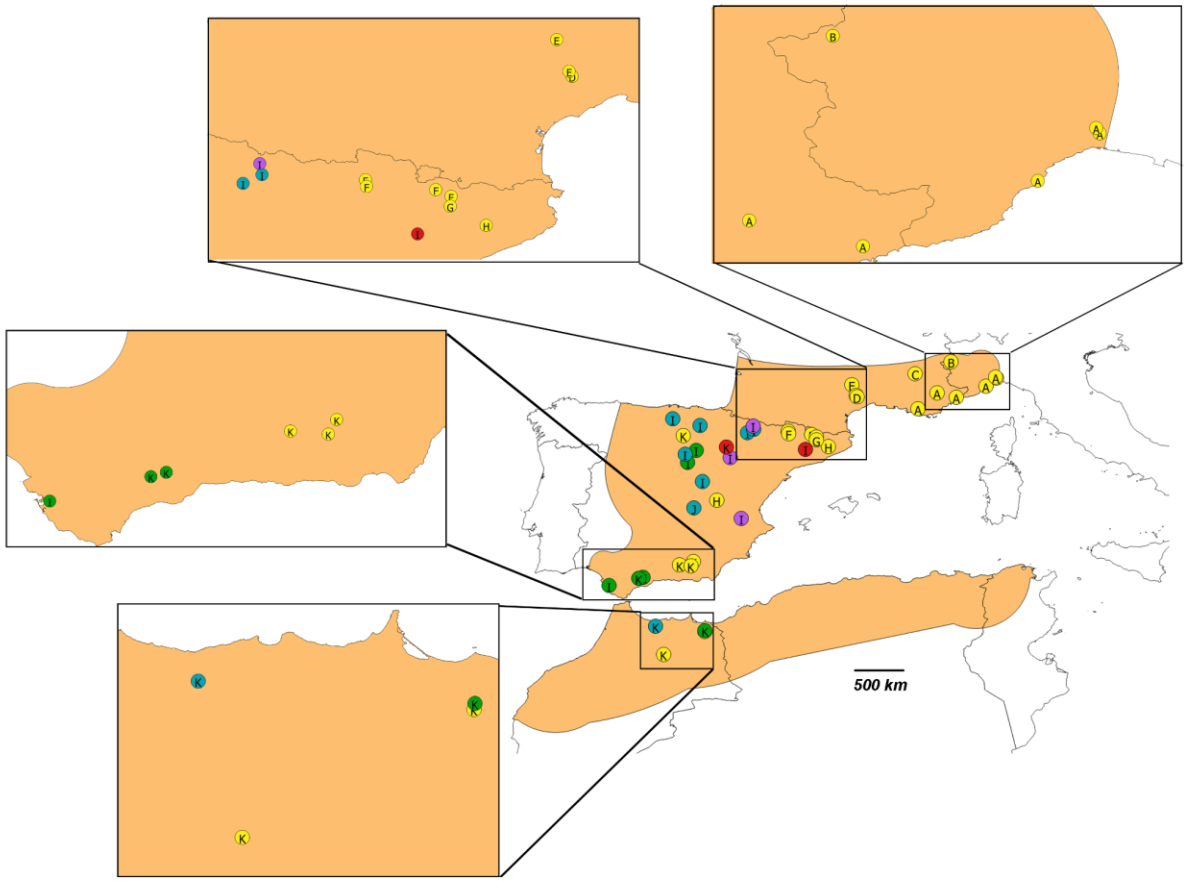


Figure 5.4. Geographic locations of the rybotype network of ploidy levels of *L. suffruticosum s.l.* (diploid – yellow; tetraploid – green; hexaploid – blue; octoploid – purple; decaploid – red) based on the matrix of ITS (internal transcribed spacer) combined with the outgroups *L. tenuifolium*, *L. flos-carmini, L. setaceum*, and *L. strictum.* The base map was downloaded from https://www.diva-gis.org/gdata.

salsoloides from the centre of Spain. The ribotype network of polyploids appears to be somewhat displaced from the rest of the ribotype network. Ribotype K is the one that represents populations of all ploidy levels throughout the Iberian Peninsula and Morocco (it is the only ribotype found in Morocco). Ribotype I represents all polyploids (tetraploids, hexaploids, octoploids and decaploids) in the Iberian Peninsula. These ribotypes correspond to *L. appressum-salsoloides* and *L. suffruticosum*. A hexaploid population in the centre of Spain was composed of ribotype J. The outgroup species, including *L. tenuifolium*, were connected to the network through ribotype K, between the diploid ribotypes in the homogeneous diploid zone and diploid and polyploid ribotypes from the Iberian Peninsula and Morocco (Appendix 5.1, Figures 5.3 and 5.4).

Phylogenetic shifts among ploidy levels

The phylogeny of the plastid DNA regions (Figure 5.5) corroborated the results of the haplotype network. Several clades were detected, one mainly bearing diploid populations (haplotypes K, L, B and D, with the exception of haplotype C, a hexaploid population from Morocco), in which populations from the contact zone between *L. suffruticosum s.l.* and *L. tenuifolium* were more closely related (haplotypes A, I and J), and the other clade bearing the polyploids detected in the Iberian Peninsula and Morocco. Furthermore, another clade bearing diploid and tetraploid populations from the south of Spain (haplotypes E, F, G and H; Figure 5.5) was also found. As for the analysis with the nuclear DNA region, it was possible to verify that most of the diploids from France and Italy (ribotypes A to E) and one from the Pyrenees (ribotype G) were in a separate clade from the polyploids (ribotypes I, J and K). Additionally, diploids from Spain and the Pyrenees (ribotypes H and F) were separated and more closely related to the outgroups *Linum flos-carmini*, *L. strictum*, and *L. setaceum* (Figure 5.6).

Discussion

This study showed that polyploids of *L. suffruticosum s.l.* could have multiple origins, probably influenced by climatic changes during Pleistocene glaciations and later glacial warming (Thompson 2020; Maguilla *et al.* 2021a). Two main results were found: 1) a higher variability of haplotypes and ribotypes in diploid populations of the homogeneously diploid zone than in diploid and polyploid populations from Spain and Morocco; 2) some haplotypes and ribotypes in polyploids were shared between the Iberian Peninsula and northern Morocco. These results are in agreement with the assumption that despite the diversification of the genus had begun

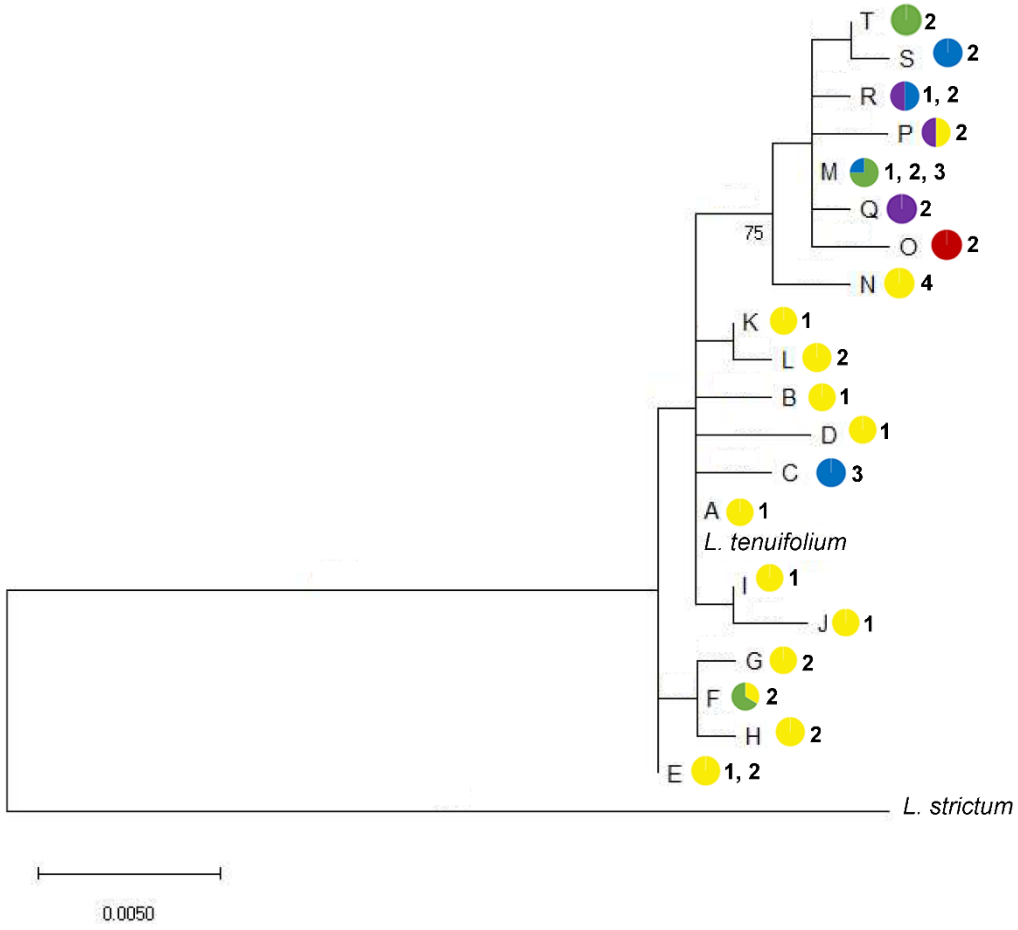


Figure 5.5. Evolutionary history inferred using the Maximum Likelihood method and General Time Reversible model (Nei and Kumar 2000), based on consensus matrix *ndhf-rpl*32 and ndha introns combined with the outgroups *L. tenuifolium* and *L. strictum*. The tree with the highest log likelihood (-1660.86) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.1000)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 47.07% sites). The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. Bootstrap values lower than 70% are not shown (bellow the branches). This analysis involved 22 nucleotide sequences and a total of 956 positions in the final dataset. Ploidy levels are also provided: diploid – yellow; tetraploid – green; hexaploid – blue; octoploid – purple; decaploid – red. Taxa of *L. suffruticosum s.l.* are indicated on the side of each haplotype (1 - *L. appressum-salsoloides;* 2 - *L. suffruticosum* from Iberian Peninsula*;* 3 - *L. suffruticosum* from Morocco; and 4 - *L. suffruticosum* var. *milletii*).

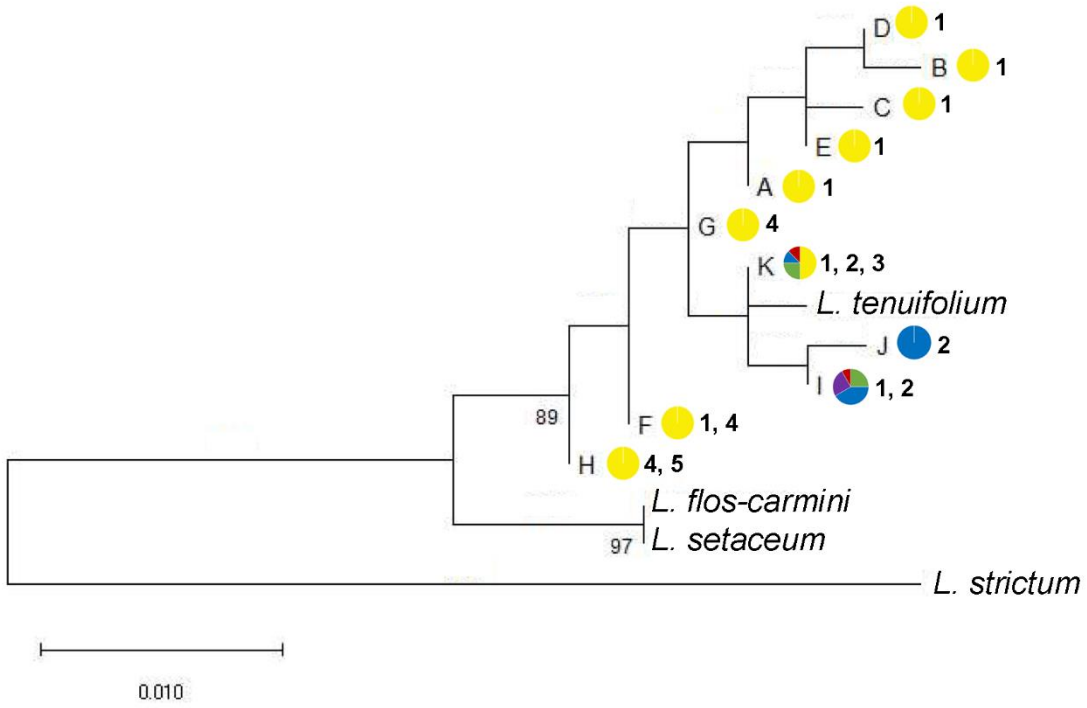


Figure 5.6. Evolutionary history inferred using the Maximum Likelihood method and General Time Reversible model (Nei and Kumar, 2000). based on the matrix of ITS (internal transcribed spacer) combined with the outgroups *L. tenuifolium*, *L. flos-carmini, L. setaceum* and *L. strictum*. The tree with the highest log likelihood (-79.05) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.1201)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*],], 46.24% sites). The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. Bootstrap values lower than 70% are not shown (bellow the branches). This analysis involved 15 nucleotide sequences and a total of 426 positions in the final dataset. Ploidy levels are also provided: diploid – yellow; tetraploid – green; hexaploid – blue; octoploid – purple; decaploid – red. Taxa of *L. suffruticosum s.l.* are indicated on the side of each haplotype (1 - *L. appressum-salsoloides;* 2 - *L. suffruticosum* from Iberian Peninsula*;* 3 - *L. suffruticosum* from Morocco; 4 - *L. suffruticosum* var. *milletii*; and 5 - *L. salsoloides*).

in the early Oligocene to late Miocene, this polyploid complex is relatively recent, and its dispersal and evolution started at a time when both sides of the Mediterranean were long time separated (Steininger and Rögl 1984; Krijgsman 2002; Meulenkamp and Sissingh 2003; Maguilla *et al.* 2021a).

The high haplotype-ribotype variability found in the homogeneously diploid zone (the east Pyrenees, southern France and northern Italy) and the lack of common haplotypes with individuals from the Iberian Peninsula and north Morroco (where the polyploids were found) suggest a divergent evolution of these populations. *Linum tenuifolium*, a sister species of *L. suffruticosum s.l.* considered in the past to be part of the same complex (Rogers *et al.* 1972; Rogers 1979; Nicholls 1985a; b, 1986), is closely related to *L. suffruticosum s.l.* populations of

this zone. *Linum tenuifolium* is style monomorphic, self-compatible (Rogers *et al.* 1972; Rogers 1979; Nicholls 1985a; b, 1986) and distributed from the east of the Pyrenees, extending from southern and central France through Europe to northern Turkey. The two species are in contact in north-east Spain and south-east France (Ockendon and Walters 1968). They seem to hybridise at least in three studied localities (J. Arroyo, E. Olmedo and A. Afonso, field observations). A second contact zone might occur in north-east Algeria, where African *L. suffruticosum* populations came into contact with *L. tenuifolium* populations, probably with an Italy and Sicily origin (Quézel and Santa 1962; Ozenda 1977). However, it was not possible to sample in these regions. Additionally, *L. tenuifolium* has the same base chromosome number as *L. suffruticosum s.l.* populations at the contact zone (Rogers *et al.* 1972; Afonso *et al.* 2021 in Chapter II). This suggests a close evolutionary history between these two species in the contact zone, which is separated through the Pyrenes from the diploid-polyploid mosaic distribution observed in Spain.

The haplotype-ribotype variability found in polyploids could result from a very recent evolution and recent genome duplications events. The few haplotypes found in the Iberian Peninsula were also found in northern Morocco. However, there were haplotypes restricted to each area, indicating a complex history of migration and isolation between the two sides of the Strait of Gibraltar. These results might suggest a different and separated evolutionary history in both areas of the Mediterranean. Some authors have suggested that some populations identified as *L. suffruticosum* in northern Morocco may be another taxon (Martínez Labarga and Ferrer-Gallego 2020). Nevertheless, as reported in other polyploid complexes, these results suggest that *L. suffruticosum s.l.* polyploids originated several times from diploid populations in both parts of the Mediterranean basin (Bougoutaia *et al.* 2021) over the complex's evolutionary history.

Furthermore, the haplotype-ribotype diversity may reflect waves of migration and differentiation, probably at very recent times, due to the weak morphological and niche differentiation. This study shows different centres of differentiation and diversity: 1) the homogeneously diploid zone of *L. suffruticosum s.l.* (the east Pyrenees, south of France and north of Italy) in contact with *L. tenuifolium* bearing most of the haplotype and ribotype diversity; 2) the Iberian Peninsula that comprises all the cytogenetic diversity; and 3) the north of Morocco with differences in the haplotype network. It has been suggested that diploid lineages in the Mediterranean basin remain in the area of origin, and recent and ancestral polyploidisation facilitates colonisation and establishment in other regions (Maguilla *et al.* 2021a). This could explain the distribution of ploidy levels *of L. suffruticosum s.l.,* and concurs with the ecological attributes of *L. suffruticosum s.l.*, in which there was an absence of environmental niche differences among most of the polyploids, but with the niche of the

diploids differing significantly from that of the polyploids, being the widest among all cytotypes (Chapter III). These diploid populations from east Pyrenees to Italy had been also considered morphological different from the diploid-polyploid populations of Spain and Morocco (Ockendon and Walters 1968; Martínez-Labarga and Garmendia 2015; Afonso *et al.* 2021 in Chapter II). Additionally, this high haplotype-rybotype diversity in diploids can help to identify some varieties within this complex, for example populations identified as *L. salsoloides* and *L. suffruticosum* var. *miletii* appear to be more genetically separated from the rest of the complex.

The lack of morphological and niche differentiation among polyploids could indicate that cytotype distribution might be driven, at least to some degree, by reproductive isolation among cytotypes. Thus, shared haplotypes might have originated in many cases before cytogenetic differences. Reproductive isolation also seems to be favoured by the maintenance of the heteromorphic self-incompatibility throughout the ranges of cytotypes (Chapter IV). Besides, on the two sides of the Mediterranean basin (SW Europe and NW Africa), the morphology and ecological niche of *L. suffruticosum s.l.* are different as well as the niche of diploids and polyploids in each area (Chapter III). This could suggest that different differentiation patterns occur at each area of the distribution of *L. suffruticosum s.l.* and that polyploids originated several times from diploid populations in both parts of the Mediterranean.

Conclusions

Although the results failed to ascertain, unambiguously, the ancestral condition of the polyploids, the differentiation and evolution of *L. suffruticosum s.l.* polyploid complex is correlated with the geographic and climatic history of the Mediterranean zone. The climate and topography and the geological and historical variations are the main factors for these regions' rapid diversification and speciation (Rundel *et al.* 2016). Nevertheless, dating polyploidy events and their role in creating new taxa has been limited to the timing of the emergence of major clades. Also, sometimes, there is a lack of sufficient detail to compare particular species (Wood *et al.* 2009). Although this study could not disentangle the phylogenetic relationships within the polyploid complex, it does help to understand that several genome duplications and differentiation events occurred along the distribution area. Future and more detailed studies of phylogeny and phylogeography with more appropriate markers on this complex may be instrumental in understanding the complex's phylogenetic relationships and support future taxonomic studies.

Appendices

Appendix 5.1. Sampled populations of *L. suffruticosum s.l.* with population code (COD), country, coordinates, ploidy level, taxon haplotypes for the matrix of two plastid sequence types, *ndhF-rpl32* and *ndhA* intron with the outgroups *L. tenuifolium* and *L. strictum* (Haplotype) and ribotypes for the matrix of ITS (internal transcribed spacer, Rybotype) combined with the outgroups *L. tenuifolium*, *L. flos-carmini*, *L. setaceum* and *L. strictum* .

COD	Country	Locality	Coordinates	Ploidy level	Taxon	Haplotype	Ribotype
JRM09-50	France	Provence-Alpes-Côte d'Azur	43.9691, 6.7787	2*x*	*L. appressum-salsoloides*	A	-
JRM09-47	Italy	Savona	44.2082, 8.3923	2*x*	*L. appressum-salsoloides*	A	A
JRM09-51	France	Provence-Alpes-Côte d'Azur	43.9502, 6.5115	2*x*	*L. appressum-salsoloides*	A	A
40JML09	France	Provence-Alpes-Côte d'Azur	43.78156, 7.2522	2*x*	*L. appressum-salsoloides*	A	A
22RPB10	France	Provence-Alpes-Côte d'Azur	43.3380, 5.7767	2*x*	*L. appressum-salsoloides*	A	A
34JML09	Italy	Turim	45.1520, 7.0563	2*x*	*L. appressum-salsoloides*	A	B
24RPB10	France	Provence-Alpes-Côte d'Azur	43.8181, 7.1585	2*x*	*L. appressum-salsoloides*	B	-
19RPB10	France	Provence-Alpes-Côte d'Azur	43.8574, 3.3871	2*x*	*L. appressum-salsoloides*	B	E
AA113	Morocco	Targuist	34.9497, -4.3342	6*x*	*L. suffruticosum*	C	K
JRM09-45	Italy	Genova	44.51626, 8.7968	2*x*	*L. appressum-salsoloides*	D	A
30RPB10	Italy	Alessandria	44.5510, 8.7735	2*x*	*L. appressum-salsoloides*	D	A
AA52	Spain	Burgos	42.7131, -3.2808	2*x*	*L. appressum-salsoloides*	E	-
AA64	Spain	Ciudad Real	38.6182, -4.1142	2*x*	*L. suffruticosum*	E	-
AA49	Spain	Burgos	42.3079, -3.2669	2*x*	*L. appressum-salsoloides*	E	K
AA27	Spain	Jaen	37.6754, -3.6350	4*x*	*L. suffruticosum*	F	-
AA2	Spain	Malaga	36.8430, -4.8168	4*x*	*L. suffruticosum*	F	K
AA32	Spain	Almeiria	37.3123, -3.4077	2*x*	*L. suffruticosum*	F	K
AA5	Spain	Almeiria	36.9363, -2.6063	2*x*	*L. suffruticosum*	G	-
AA31	Spain	Granada	37.4401, -2.8829	2*x*	*L. suffruticosum*	H	K
JRM09-53	France	Occitanie	44.2636, 3.2260	2*x*	*L. appressum-salsoloides*	I	E

⇩Cont.

17RPB10	France	Provence-Alpes-Côte d'Azur	43.7915, 3.4256	2*x*	*L. appressum-salsoloides*	J	D
JRM09-56	Spain	Lérida	42.4667, 0.7740	2*x*	*L. appressum-salsoloides*	K	F
06RPB10	Spain	Lérida	42.3831, 0.7903	2*x*	*L. appressum-salsoloides*	K	F
AA75	Spain	Huesca	42.3469, 0.3849	2*x*	*L. suffruticosum*	L	-
AA76	Spain	Huesca	42.4957, 0.4049	2*x*	*L. suffruticosum*	L	-
AA105	Morocco	Midelt	32.6974, -4.8238	4*x*	*L. suffruticosum*	M	-
AA1	Spain	Cadiz	36.5162, -6.1382	4*x*	*L. suffruticosum*	M	I
AA39	Spain	Huesca	42.5327, -0.5495	6*x*	*L. appressum-salsoloides*	M	I
AA28	Spain	Malaga	36.7941, -4.9901	4*x*	*L. suffruticosum*	M	K
AA41	Spain	Burgos	42.2540, 1.87157	2*x*	*L. suffruticosum* var. *milletii*	N	F
AA42	Spain	Burgos	42.1280, 1.8634	2*x*	*L. suffruticosum* var. *milletii*	N	G
AA43	Spain	Burgos	41.8829, 2.3250	2*x*	*L. suffruticosum* var. *milletii*	N	H
AA79	Spain	Zaragoza	41.8674, -1.6022	10*x*	*L. suffruticosum*	O	K
AA15	Spain	Huesca	41.9878, 0.2838	2*x*	*L. suffruticosum*	P	-
AA17	Spain	Zaragozsa	41.3233, -2.1516	8*x*	*L. suffruticosum*	P	-
AA7	Spain	Valencia	39.1046, -1.0323	8*x*	*L. suffruticosum*	Q	I
AA93	Spain	Terruel	40.8298, -0.7981	8*x*	*L. suffruticosum*	R	-
AA40	Spain	Burgos	42.6858, -2.6215	6*x*	*L. appressum-salsoloides*	R	I
AA22	Spain	Malaga	40.2862, -3.4506	6*x*	*L. suffruticosum*	S	-
AA20	Spain	Gualadajara	40.5262, -2.5271	6*x*	*L. suffruticosum*	S	I
AA25	Spain	Ciudad Real	38.8870, -3.0530	4*x*	*L. suffruticosum*	T	-
AA111	Spain	Driouch	34.8755, -3.1488	6*x*	*L. suffruticosum*	-	-
AA112	Spain	Driouch	34.9008, -3.5469	2*x*	*L. suffruticosum*	-	-
AA93	Spain	Terruel	40.8272, -0.7936	8*x*	*L. suffruticosum*	-	-
MO6136	Spain	Valladolid	41.3940, -5.2677	6*x*	*L. suffruticosum*	-	-
44RPB10	France	Rhône-Alpes	44.6918, 5.6726	2*x*	*L. appressum-salsoloides*	-	C
13RP09	Spain	Lérida	42.3408, 1.6773	2*x*	*L. suffruticosum* var. *milletii*	-	F

⇩Cont.

85JAM	Spain	Cuenca	39.8175, -1.9813	2*x*	*L. salsoloides*	-	H
89JAM2016	Spain	Huesca	42.6728, 0.5798	8*x*	*L. appressum-salsoloides*	-	I
AA16	Spain	Zaragosa	41.4546, -1.4665	8*x*	*L. suffruticosum*	-	I
AA36	Spain	Barcelona	41.77531, 1.4433	10*x*	*L. suffruticosum*	-	I
AA38	Spain	Huesca	42.4191, -0.7923	6*x*	*L. appressum-salsoloides*	-	I
AA46	Spain	Guadalajara	41.2620, -3.0931	4*x*	*L. appressum-salsoloides*	-	I
AA51	Spain	Burgos	42.9428, -3.6785	6*x*	*L. appressum-salsoloides*	-	I
DP1980	Spain	Soria	41.5834, -3.2035	6*x*	*L. appressum-salsoloides*	-	I
DP1995	Spain	Soria	41.7376, -2.7759	4*x*	*L. appressum-salsoloides*	-	I
AA24	Spain	Cuenca	39.5083, -2.8583	6*x*	*L. suffruticosum*	-	J
AA101	Spain	Berkane	34.7533, -2.4357	2*x*	*L. suffruticosum*	-	K
AA106	Morocco	Taza	33.8722, -4.0300	2*x*	*L. suffruticosum*	-	K
AA108	Spain	Berkane	34.7952, -2.4291	4*x*	*L. suffruticosum*	-	K
AA29	Spain	Granada	37.2769, -2.9774	2*x*	*L. suffruticosum*	-	K

Appendix 5.2. DNA regions and primers used: nuclear DNA region, ITS (internal transcribed spacer), and two plastid DNA regions, *ndh*F-*rpl*32 and *ndh*A.

DNA Region	Primer	Sequence (5'-3')	Reference
ITS	P1A	GGA AGG AGA AGT CGT AAC AAG G	White *et al.* 1990
	P4	TCC TCC GCT TAT TGA TAT GC	White et al. 1990
*ndh*F-*rpl*32	ndhF	GAA AGG TAT KAT CCA YGM ATA TT CCA	Shaw, 2007
	rpL32-R	CCA ATA TCC CTT YYT TTT CCA A GCY	Shaw, 2007
*ndh*A intron	ndhA x1	GCY CAA TCW ATT AGT TAT GAA ATA CC	Shaw, 2007
	ndhA x2	ATTTGAACTGGTGACACGAG	Shaw, 2007

Appendix 5.3. Akaike Information Criterion corrected (AICc), Maximum log likelihood (lnL) and Bayesian Information Criterion (BIC) for each model in the four phylogenetic analysis for *L. suffruticosum s.l.* with the matrices of ITS (internal transcribed spacer) combined with the outgroups *L. tenuifolium*, *L. flos-carmini, L. setaceum* and *L. strictum* (ITS), two plastid sequence types, *ndhF*-rpl32 and *ndhA* intron with the outgroups *L. tenuifolium* and *L. strictum* (Plastid markers) and *ndhF-rpL*32 spacer combined with the outgroups *L. tenuifolium* and *L. strictum* (ndhF). Selected models for each analysis were in bold.

Plastids markers				ITS			
Model	BIC	AICc	lnL	Model	BIC	AICc	lnL
GTR+G+I	**3746.53**	**3342.017**	**-1619.88**	**GTR+G+I**	**1913.863**	**1664.364**	**-794.959**
GTR+G	3739.86	3343.274	-1621.51	T92+G	1867.803	1665.44	-802.572
GTR	3731.728	3343.068	-1622.42	GTR+G	1909.417	1666.65	-797.114
GTR+I	3741.665	3345.078	-1622.42	T92+I	1869.334	1666.971	-803.338
TN93+G	3750.749	3377.944	-1641.86	T92+G+I	1876.412	1667.313	-802.5
HKY+G	3740.944	3376.067	-1641.93	T92	1864.08	1668.453	-805.089
TN93+G+I	3761.133	3380.4	-1642.09	HKY+G	1884.417	1668.584	-802.124
HKY+G+I	3751.325	3378.52	-1642.15	GTR+I	1912.015	1669.248	-798.413
T92+G	3721.675	3372.653	-1642.23	TN93+G	1892.304	1669.736	-801.69
T92+G+I	3732.066	3375.116	-1642.46	HKY+I	1885.945	1670.111	-802.888
TN93+I	3756.161	3383.355	-1644.57	GTR	1906.482	1670.447	-800.024
TN93	3746.224	3381.346	-1644.57	HKY+G+I	1893.027	1670.458	-802.051
HKY	3736.409	3379.459	-1644.63	HKY	1879.735	1670.636	-804.161
HKY+I	3746.346	3381.468	-1644.63	TN93+I	1893.618	1671.049	-802.347
T92	3717.14	3376.046	-1644.93	TN93	1887.293	1671.459	-803.562
T92+I	3727.076	3378.054	-1644.93	TN93+G+I	1900.945	1671.643	-801.633
K2+G	3874.849	3533.755	-1723.79	K2+G	1870.753	1675.126	-808.425
JC+G	3864.924	3531.759	-1723.79	K2+I	1872.178	1676.551	-809.138
K2+G+I	3885.068	3536.046	-1723.93	K2	1865.903	1677.013	-810.378
JC+G+I	3875.144	3534.05	-1723.93	K2+G+I	1879.377	1677.014	-808.36
K2	3871.257	3538.091	-1726.96	JC+G	1871.245	1682.355	-813.049
K2+I	3881.194	3540.1	-1726.96	JC+I	1871.793	1682.903	-813.323
JC	3861.329	3536.092	-1726.96	JC	1865.488	1683.335	-814.548
JC+I	3871.266	3538.1	-1726.96	JC+G+I	1879.049	1683.422	-812.573

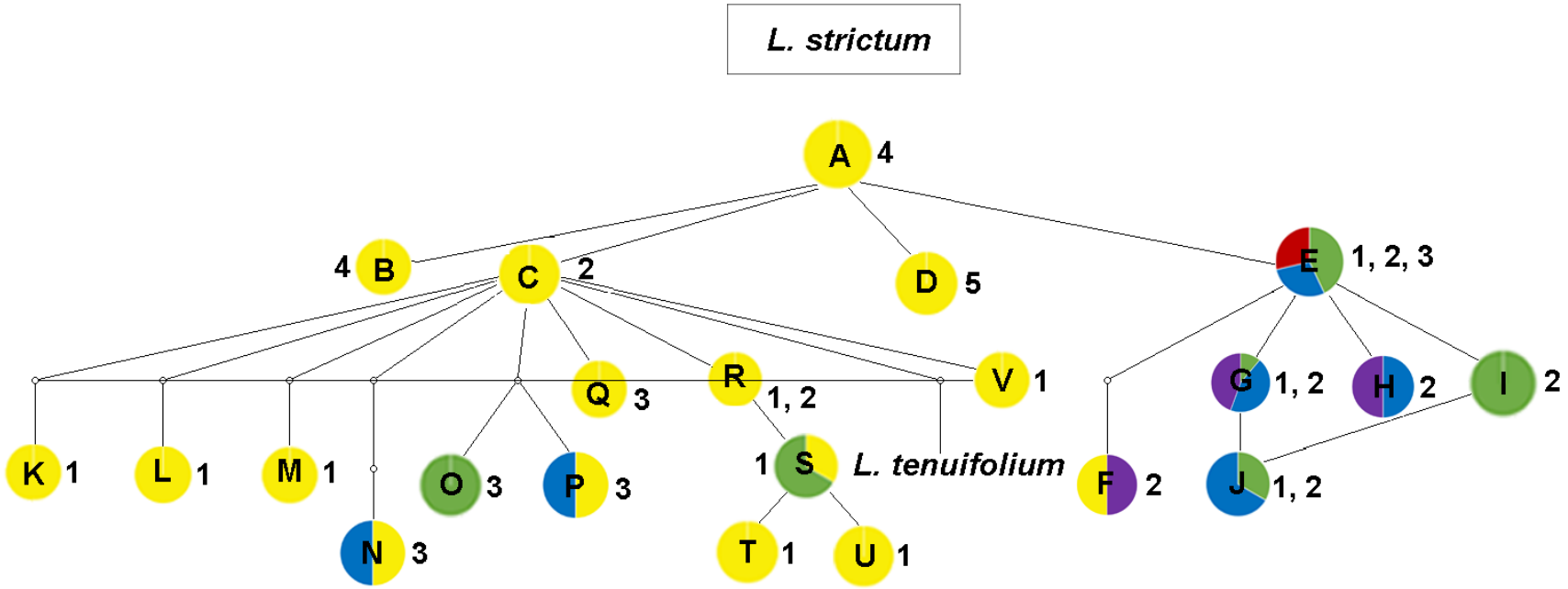
Appendix 5.4. Sampled populations of *L. suffruticosum s.l.* with population code (COD), country, coordinates, ploidy level, taxon, haplotypes for the matrix of *ndhF-rpL32* spacer combined with the outgroups *L. tenuifolium* and *L. strictum* (Haplotype).

COD	Country	Locality	Coordinates	Ploidy level	Taxon	Haplotype
13RP09	Spain	Lérida	42.3408, 1.6773	2*x*	*L. suffruticosum* var. *milletii*	A
AA41	Spain	Burgos	42.2541, 1.8716	2*x*	*L. suffruticosum* var. *milletii*	B
AA42	Spain	Burgos	42.1281, 1.8634	2*x*	*L. suffruticosum* var. *milletii*	B
AA43	Spain	Burgos	41.8829, 2.3251	2*x*	*L. suffruticosum* var. *milletii*	B
JRM09-50	France	Provence-Alpes-Côte d'Azur	43.9691, 6.7787	2*x*	*L. appressum-salsoloides*	C
JRM09-47	Italy	Savona	44.2083, 8.3924	2*x*	*L. appressum-salsoloides*	C
JRM09-51	France	Provence-Alpes-Côte d'Azur	43.9502, 6.5115	2*x*	*L. appressum-salsoloides*	C
40JML09	France	Provence-Alpes-Côte d'Azur	43.7816, 7.2523	2*x*	*L. appressum-salsoloides*	C
22RPB10	France	Provence-Alpes-Côte d'Azur	43.3381, 5.7768	2*x*	*L. appressum-salsoloides*	C
34JML09	Italy	Turim	45.1520, 7.0564	2*x*	*L. appressum-salsoloides*	C
44RPB10	France	Rhône-Alpes	44.6919, 5.6726	2*x*	*L. appressum-salsoloides*	C
JRM09-53	France	Occitanie	44.2637, 3.2260	2*x*	*L. appressum-salsoloides*	C
JRM09-56	Spain	Lérida	42.4667, 0.7741	2*x*	*L. appressum-salsoloides*	C
06RPB10	Spain	Lérida	42.3832, 0.7904	2*x*	*L. appressum-salsoloides*	C
85JAM	Spain	Cuenca	39.8175, -1.9813	2*x*	*L. salsoloides*	D
AA105	Morocco	Midelt	32.6974, -4.8239	4*x*	*L. suffruticosum Maroc*	E
AA1	Spain	Cádiz	36.5163, -6.1383	4*x*	*L. suffruticosum*	E
AA36	Spain	Barcelona	41.7753, 1.4433	10*x*	*L. suffruticosum*	E
AA38	Spain	Huesca	42.4192, -0.7924	6*x*b	*L. appressum-salsoloides*	E
AA39	Spain	Huesca	42.5327, -0.5496	6*x*b	*L. appressum-salsoloides*	E
AA28	Spain	Malaga	36.7942, -4.9901	4*x*	*L. suffruticosum*	E
AA79	Spain	Zaragoza	41.8674, -1.6022	10*x*	*L. suffruticosum*	E
AA15	Spain	Huesca	41.9879, 0.2838	2*x*	*L. suffruticosum*	F
AA17	Spain	Zaragozsa	41.3233, -2.1516	8*x*	*L. suffruticosum*	F
AA93	Spain	Terruel	40.8273, -0.7937	8*x*	*L. suffruticosum*	G
89JAM2016	Spain	Huesca	42.6729, 0.5799	8*x*	*L. appressum-salsoloides*	G
AA16	Spain	Zaragosa	41.4547, -1.4666	8*x*	*L. suffruticosum*	G
AA40	Spain	Burgos	42.6859, -2.6216	6*x*	*L. appressum-salsoloides*	G
AA46	Spain	Guadalajara	41.2620, -3.0931	4*x*	*L. appressum-salsoloides*	G
AA51	Spain	Burgos	42.9428, -3.6785	6*x*	*L. appressum-salsoloides*	G
DP1980	Spain	Soria	41.5834, -3.2036	6*x*	*L. appressum-salsoloides*	G
AA24	Spain	Cuenca	39.5084, -2.8584	6*x*	*L. suffruticosum*	G
MO6136	Spain	Valladolid	41.3940, -5.2677	6*x*	*L. suffruticosum*	H

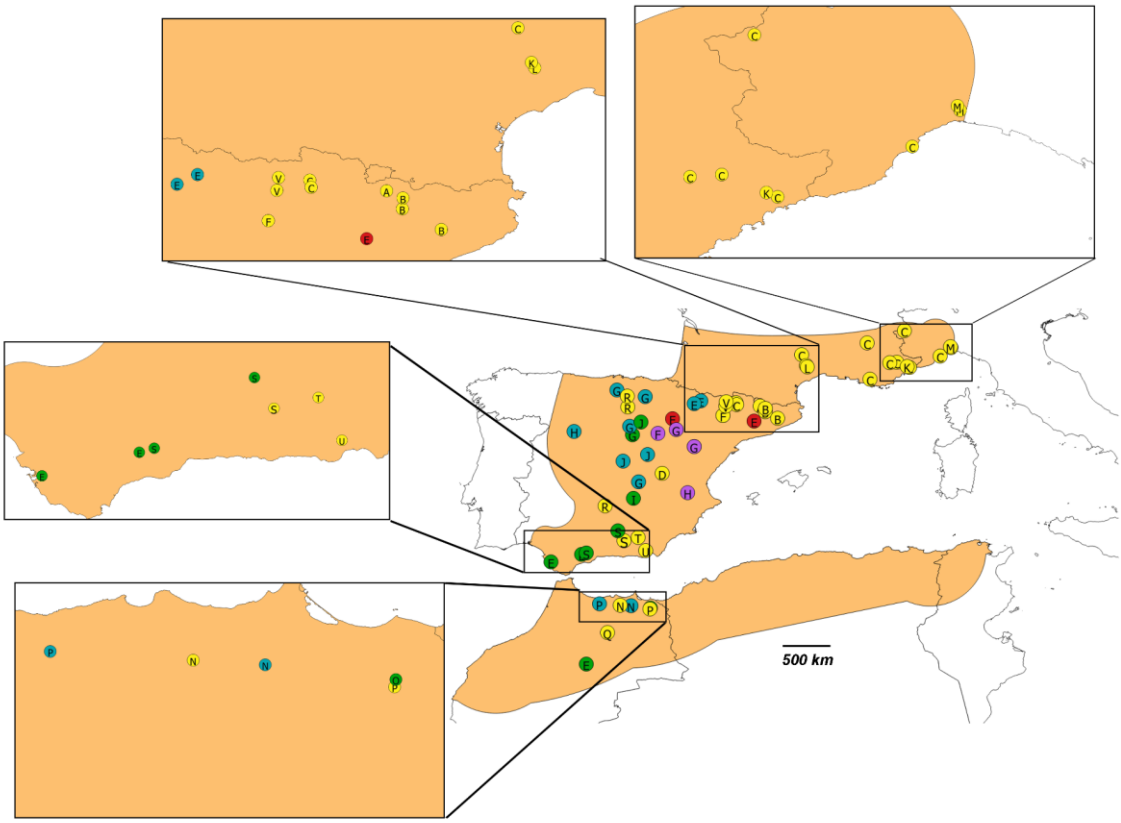
⇩Cont.

AA7	Spain	Valencia	39.1047, -1.0323	8*x*	*L. suffruticosum*	H
AA25	Spain	Ciudad Real	38.8871, -3.0531	4*x*	*L. suffruticosum*	I
AA22	Spain	Malaga	40.2863, -3.4507	6*x*	*L. suffruticosum*	J
AA20	Spain	Gualadajara	40.5263, -2.5271	6*x*	*L. suffruticosum*	J
DP1995	Spain	Soria	41.7377, -2.7759	4*x*	*L. appressum-salsoloides*	J
24RPB10	France	Provence-Alpes-Côte d'Azur	43.8182, 7.1585	2*x*	*L. appressum-salsoloides*	K
19RPB10	France	Provence-Alpes-Côte d'Azur	43.8574, 3.3872	2*x*	*L. appressum-salsoloides*	K
17RPB10	France	Provence-Alpes-Côte d'Azur	43.7915, 3.4256	2*x*	*L. appressum-salsoloides*	L
JRM09-45	Italy	Genova	44.5163, 8.7968	2*x*	*L. appressum-salsoloides*	M
30RPB10	Italy	Alessandria	44.5510, 8.7735	2*x*	*L. appressum-salsoloides*	M
AA111	Spain	Driouch	34.8756, -3.1489	6*x*	*L. suffruticosum*	N
AA112	Spain	Driouch	34.9008, -3.5469	2*x*	*L. suffruticosum*	N
AA108	Spain	Berkane	34.7953, -2.4292	4*x*	*L. suffruticosum*	O
AA101	Spain	Berkane	34.7534, -2.4358	2*x*	*L. suffruticosum*	P
AA113	Morocco	Targuist	34.9498, -4.3343	6*x*	*L. suffruticosum*	P
AA106	Morocco	Taza	33.8722, -4.0300	2*x*	*L. suffruticosum*	Q
AA52	Spain	Burgos	42.7132, -3.2809	2*X*	*L. appressum-salsoloides*	R
AA64	Spain	Ciudad Real	38.6182, -4.1142	2*x*	*L. suffruticosum*	R
AA49	Spain	Burgos	42.3079, -3.2669	2*x*	*L. appressum-salsoloides*	R
AA27	Spain	Jaen	37.6755, -3.6350	4*x*	*L. suffruticosum*	S
AA2	Spain	Malaga	36.8430, -4.8169	4*x*	*L. suffruticosum*	S
AA32	Spain	Almeiria	37.3124, -3.4077	2*x*	*L. suffruticosum*	S
AA31	Spain	Granada	37.4401, -2.8830	2*x*	*L. suffruticosum*	T
AA5	Spain	Almeiria	36.9364, -2.6063	2*x*	*L. suffruticosum*	U
AA75	Spain	Huesca	42.3469, 0.3850	2*x*	*L. suffruticosum*	V
AA76	Spain	Huesca	42.4958, 0.4050	2*x*	*L. suffruticosum*	V

Appendix 5.5. Haplotype network of ploidy levels of *L. suffruticosum s.l.* (diploid – yellow; tetraploid – green; hexaploid – blue; octoploid – purple; decaploid – red) based on the matrix of the *ndhF-rpL32* spacer combined with the outgroups *L. tenuifolium* and *L. strictum.* Taxon of *L. suffruticosum s.l.* is indicated on the side of each haplotype (1 - *L. appressum-salsoloides;* 2 - *L. suffruticosum* from Iberian Peninsula*;* 3 - *L. suffruticosum* from Morocco*,* 4 - *L. suffruticosum* var*. milletii* and 5 - *L. salsoloides*).



Appendix 5.6. Geographic locations of the haplotype network of ploidy levels of *L. suffruticosum s.l.* (diploid – yellow; tetraploid – green; hexaploid – blue; octoploid – purple; decaploid – red) based on the matrix of the *ndhF-rpL32* spacer combined with the outgroup *L. tenuifolium* and *L. strictum. The base map was downloaded from https://www.diva-gis.org/gdata.*



Chapter VI – General Conclusions

General Conclusions

The Mediterranean region has been considered a biodiversity hotspot with a complex geological and climatic history (Jansson and Dynesius 2002; Thompson 2020). The ecology and evolution of the reproductive traits of flowering plants impacted the rapid diversification and speciation in this region (Thompson 2020). Moreover, polyploidization has long been acknowledged as one of the major mechanisms responsible for flowering plants speciation (Ramsey and Schemske 1998; Soltis and Soltis 1999). Polyploidization might be particularly relevant in species with complex breeding systems, such as heterostylous species, as it may pose different challenges for neopolyploid establishment. Distyly is widespread and very common in the genus *Linum* (Rogers 1979). Polyploidy has also been reported in some *Linum* species (*e.g.* Nilsson and Lassen 1971; Rogers *et al.* 1972; Chennaveeraiah and Joshi 1983). Still, to date, the great majority of the studies have focused on economically relevant groups of *Linum* (Ockendon 1968; Chennaveeraiah and Joshi 1983; Bolsheva *et al.* 2015), while little was known about the ecological processes involved with the emergence and successful establishment and spread of polyploids in other sections and its impact for the maintenance of complex breeding systems, as observed in general in many other polyploid complexes (Soltis *et al.* 2010). *Linopsis*, in which *L. suffruticosum s.l.* is included, is a clear example of a section that has received less attention, and its diversity was still largely unknown.

The studies carried out in this PhD thesis increased the current knowledge about the role of polyploidization in plant evolution and diversification by studying a Mediterranean polyploid complex, *L. suffruticosum s.l.* This species complex bears a high cytogenetic, morphological and genetic variability and a complex breeding system with several taxonomical treatments in the last decades. 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.

Cytogenetic diversity and environmental requirements

This study revealed an outstanding cytogenetic diversity with five main cytotypes found in *L. suffruticosum s.l.*, namely diploids, tetraploids, hexaploids, octoploids and decaploids. Some minor cytotypes were also described, with triploids, hexaploids, and decaploids being described here for the first time for this complex (Chapter II). In other species of *Linum*, multiple ploidy levels have been reported (*e.g.*, Nilsson and Lassen 1971; Rogers *et al.* 1972; Chennaveeraiah and Joshi 1983), but in about ¼ of the taxa with available data, only the diploid and tetraploid levels were reported (Ruiz-Martín *et al.* 2018; Appendix 1.1). Furthermore, the study reveals

wider cytogenetic variability within the complex than previously thought. Variations in chromosome number, ploidy level and genome size were also detected. First, I observed two basic chromosome numbers (n = 8 and 9; with the former being reported here for the first time for *L. suffruticosum s.l.*) and consequently, different chromosome numbers within the same cytotype (namely for diploids, tetraploids and hexaploids). Second, variation in genome sizes within ploidy levels were observed and, consequently, heterogeneity in 1Cx values (*e.g.*, for hexaploids and diploids or between *L. suffruticosum* individuals from Spain and Morocco). This could suggest that whole genome duplications (alone or together with hybridization events) are one of the key mechanisms in the diversification of *L. suffruticosum s.l.* and support different evolutionary histories (Chapter II).

The different ploidy levels were distributed parapatrically, thus having a geographic structure and several contact zones, and mixed-ploidy populations were rarely found (Chapter II). Spatial structuring of cytotypes (Husband and Schemske 2000; Hülber *et al.* 2015) can contribute to cytotype isolation. Indeed, ecological differentiation is among the most important mechanisms of reproductive isolation among cytotypes (Levin 1975; Buggs and Pannell 2007; Glennon *et al.* 2014; Thompson *et al.* 2014; Muñoz-Pajares *et al.* 2018; López-Jurado *et al.* 2019). The ecological niche modelling (climatic and soil characteristics) of *L. suffruticosum s.l.* suggested that the parapatric distribution of the cytotypes can be partly explained by differences in the ecological niche (Chapter III). A strong association between the spatial distribution of cytotypes and their environmental requirements has been explored using niche modelling tools in several polyploid complexes (Glennon *et al.* 2014; Thompson *et al.* 2014; Visger *et al.* 2016; Muñoz-Pajares *et al.* 2018). In this study differences in the ecological attributes of *L. suffruticosum* s.l. cytotypes were found, with polyploids being associated with habitats with increased drought, temperature ranges, higher soil pH, and decreased soil water and cation exchange capacities. These results could be explained as an adaptation of polyploids to dry and harsh environments (Chapter III). Polyploidization has been shown to have consequences on the ability of polyploids to grow in habitats that differ from their progenitors, enabling polyploids to expand to new areas (Levin 1975; Buggs and Pannell 2007; Ramsey 2011; Hao *et al.* 2013). Despite the absence of environmental niche differences among most polyploids, the niche of the diploids differed significantly from that of the polyploids, being the widest among all cytotypes. Polyploids may have spread to environments less suitable for the diploids to escape competition. The capacity to disperse and colonize new niches escaping competition with the progenitor individuals may increase the probability of establishment by reducing the minority cytotype disadvantage (Levin 1975; Ramsey 2011; Hao *et al.* 2013).

In summary, the ecological requirements of cytotypes can support in part the distribution, with diploids having a broader environmental niche and polyploids occupying marginal areas of the diploid niche in harsh environments (Chapter III). However, in young polyploid complexes (as *L. suffruticosum s.l.* seems to be, Ruiz-Martín *et al.* 2018; Maguilla *et al.* 2021a), polyploids may partially occupy the niche of their progenitors, thus growing in climatic conditions of diploids as they did not had time yet to disperse further, specialize and/or completely diverge in their niche (Felber 1991; Kim *et al.* 2012b; Glennon *et al.* 2014). Still, I obtained important data on cytogenetic patterns of the polyploid complex and relevant data about each cytotype's niche requirements for future competition and reciprocal transplant experiments.

Impacts of genome duplications and maintenance of cytogeographical patterns

Linum suffruticosum s.l. is a polyploid complex with strong self- and morph-incompatibility, and most of the populations showed isoplethy with no correlation between population size and the rare deviations from isoplethy in morph proportions. Thus, no breakdown of the distyly and the incompatibility system were detected across the five cytotypes. Despite this, pollen flow among cytotypes appears possible since there is overlap between their reciprocal sexual organs (Chapter IV). Thus, in contact areas and mixed-ploidy populations, no mechanical barriers are expected to prevent inter-cytotype pollen flow. Contact zones are frequent in most polyploid complexes and enable cytotype interaction; still, mixed-ploidy populations are considered a transitory stage and are expected to be rare because positive frequency-dependent selection will exclude the cytotype in lower frequency (Levin 1975). Indeed, here I observed that mixed-ploidy populations were rare (15%), being found sporadically in contact zones between cytotypes, as well as in areas with a dominant cytotype (Chapter II). The lack of reproductive barriers among cytotypes was thus supported by reciprocal positioning of sexual organs (Chapter IV), by the growth of pollen tubes after experimental intercytotype crosses (Chapter IV) and by detecting some minor cytotypes (including a few triploids and aneuploids) in the field (Chapter II).

Yet, the observation of rare cytotypes such as triploids may also suggest the possible production of unreduced gametes (Husband 2004). The production of unreduced gametes is an important mechanism for the emergence of new polyploid entities (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Mason *et al.* 2011; Mason and Pires 2015; Marques *et al.* 2018). Indeed, in outcrossing plants, such as the case of *L. suffruticosum s.l.,* polyploids tend to be formed mostly through a triploid intermediate (Ramsey and Schemske 1998), and it is known

that hybrids produce high amounts of unreduced gametes (on average, hybrids produced 27.5% of unreduced gametes (Levin 2002), potentially enabling the emergence of a higher and stable ploidy level. However, hybrids are often expected to be sterile because of their meiotic irregularities and high frequency of aneuploid gametes, and thus, they are consequently excluded from the populations, being rare (Ramsey and Schemske 1998). Interestingly, a few triploids were found in the natural populations of *L. suffruticosum s.l.* (ChapterII). In the homogeneous diploid zones most likely triploids originated by unreduced gametes. Whereas in contact zones may be also involve intercytotype crosses, since pollen tube development occurred in intercrosses between diploid and tetraploids (Chapter IV). The detection of minority cytotypes in natural populations are also indicative that polyploidization is a dynamic process in this complex and may be continuously contributing for the genetic diversity.

Altogether, the obtained results reflect dynamic contact zones. Neopolyploids can be formed and may disperse to other localities (see previous section). It has been suggested that *Linum* seeds could potencial be transported over long distances, depending on chemical composition and hydration of seeds (Kreitschitz *et al.* 2015), however little information in known about *L. suffruticosum s.l.*. Hybridization between cytotypes can occur since pollen flow among cytotypes appears to be possible due to overlap between reciprocal sexual organs and pollen tube development reaching the bottom of the style in inter-cytotype crosses (Chapter IV). If the incompatibility system is heteromorphic sporophyte SI, legitimated inter-morph crosses should terminate as viable seed (Dulberger 1975a, 1992; Allen and Hiscock 2008), although specific studies on reproductive barriers should be performed to fully ascertain it. Since ecological preferences, mechanical barriers and gametic interactions do not constitute strong barriers in *L. suffruticosum s.l.* polyploids, the rare minor cytotypes and mixed-ploidy populations detected in nature may be transitory stages towards a dominant cytotype. Besides, a decline in the frequency of a once-dominant cytotype seems to be ongoing. Mixed-ploidy populations such as tetraploid-hexaploid, hexaploid-octoploid and octoploid-decaploid, where the lower ploidy occurs in low frequency, suggest a successful expansion of higher ploidies over lower ones. Differences in other attributes, such as in fitness or competitive ability enable their maintenance. Polyploid plants of *L. suffruticosum s.l.* usually have larger flowers in higher numbers than diploids and an increased range of flowering time (Ana Afonso, pers. observations). Differences in phenology, flower morphology and physiology have been documented to impact the establishment of the neopolyploid since they can promote assortative mating (*e.g.*, Segraves and Thompson 1999; Husband and Sabara 2004; Jersáková *et al.* 2010; Castro *et al.* 2011). In addition, an increase in the overall size of the organs (Segraves and Thompson 1999; Levin 2002) as a result of WGDs ("giga effect") although not impacting the

structure of the sexual organs in *Linum suffruticosum s.l.*, may still potentially affecting the interactions with pollinators (Segraves and Thompson 1999; Marques *et al.* 2007) and, consequently, the reproductive success (Husband and Schemske 2000). Further research on reproductive traits and competitive interactions among cytotypes, including pollination, in contact zones are needed to confirm this. This can be particularly informative since it has been argued that pollinator achieving legitimate pollen transfer between morphs are quite specific (*Usia* sp. bombyliid flies), as in other style dimorphic *Linum* species (Johnson and Dafni 1998; Bigio *et al.* 2017; Lebel *et al.* 2018).

Multiple origins of *L. suffruticosum s.l.* cytotypes

The differentiation and evolution of *L. suffruticosum s.l.* polyploid complex is clearly correlated with the geographic and climatic history of the western Mediterranean basin. Higher variability of haplotypes and ribotypes in diploid populations were found in the homogeneously diploid zone than in the diploid and polyploid populations from Spain and Morocco, with some haplotypes and ribotypes in polyploids shared between the Iberian Peninsula and northern Morocco (Chapter V). This haplotype-ribotype diversity found in *L. suffruticosum s.l.*, along with cytogenetic patterns, suggests multiple origins and divergent evolution in different areas. Polyploids were found in the Iberian Peninsula and Northern Africa, with the remaining areas of the species distribution in Europe being characterized by homogenously diploid populations only. In Northern Africa, diploid, tetraploid and hexaploid populations were found, with the species being less abundant there than in Europe. Additionally, on the two sides of the Mediterranean basin (SW Europe and NW Africa), the ecological niche of *L. suffruticosum s.l.* was different (Chapter III). Spatial segregation and isolation might drive evolutionary divergence, promoting the accumulation of differences among the cytotypes (Otto and Whitton 2000; Soltis *et al.* 2010). Nevertheless, this polyploid complex is very recent (Ruiz-Martín *et al.* 2018; Maguilla *et al.* 2021a). The lack of morphological and niche differentiation among polyploids could indicate that cytotype distribution might be driven by reproductive isolation among cytotypes. Thus, shared haplotypes might have originated through recurrent polyploid formation and/or gene flow between different cytogenetic entities. The diversity found may reflect waves of migration and differentiation, probably at very recent times, due to the weak morphological, crossability and niche differentiation. In fact, this complex has not been found in western Mediterranean islands, despite their large size, weak isolation in terms of timing and distance and similar Mediterranean climate and limestone edaphic conditions.

Overall, the results indicated multiple origins of cytotypes. Still, it was impossible to properly disentangle polyploids' origin from the same and/or different progenitors, most likely due to a very recent origin of the species complex. Moreover, the cytotypes may have arisen by auto- or allopolyploidy, and given the variety of diploid chromosomal numbers, it may have generated different lineages. It has been hypothesized that heterostylous polyploids could be restricted to those having autotetraploid origins, and allopolyploids are self-compatible (Barrett and Shore 1987; Shore *et al.* 2006). Indeed, it is surprising that the polyploid formation has not been paralleled by a loss of the polymorphism and/or the associated heteromorphic self-incompatibility (Chapter IV), as predicted (Barrett and Shore 1987; Shore *et al.* 2006), particularly for allopolyploid taxa. I hypothesize that multiple origins of polyploids from different diploid parent individuals may have contributed to the diversity in this complex, with a prevalence of autopolyploidy in the south and allopolyploidy occurrence in the north, where the different taxonomic entities co-occur. Future studies integrating genome size, breeding system and detailed phylogenetic analyses are needed to develop the evolutionary scenarios.

Broader future perspectives

This PhD thesis reveals the need for further ecological studies at different levels and contact zones of ploidy levels of *L. suffruticosum s.l.* The large-scale cytogenetic screening revealed a much higher cytogenetic and morphological diversity than expected, and genome size and/or chromosome counts might be valuable tools for identifying individuals of *L. suffruticosum s.l.* However, the complex variability of the group requires additional taxonomic studies accounting for the diversity found here. Niche modelling analyses were revealed to be helpful 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. However, interactions at contact zones are still poorly understood and require further reciprocal transplant experimental studies in the field. This study also found no breakdown of the distyly and incompatibility system across the five cytotypes. However, pollen flow among cytotypes appears possible since there was overlap between reciprocal sexual organs and pollen tube reaching the bottom of the style in inter-cytotype crosses. Studies on viable seeds and population genetic structure, as well as pollinator studies, will be needed to confirm this hypothesis. Furthermore, future phylogenetic and phylogeographic studies with more robust genetic tools coupled with niche modelling analyses are required to understand the relationships among *L. suffruticosum s.l.* entities, and to disentangle the ecological requirements that might explain the success of polyploids and their current distribution patterns. In addition,

information about distribution patterns in all the distribution areas of North Africa is required to understand the adaptations and evolution of the different entities of the complex.

Chapter VII – Literature cited

Adams KL, Percifield R, Wendel JF. 2004. Organ-specific silencing of duplicated genes in a newly synthesized cotton allotetraploid. *Genetics* 168: 2217–2226.

Afonso A, Loureiro J, Arroyo J, Olmedo-Vicente E, Castro S. 2021. Cytogenetic diversity in the polyploid complex *Linum suffruticosum s.l.* (Linaceae). *Botanical Journal of the Linnean Society* 195: 216–232.

Ahuja MR. 2005. Polyploidy in gymnosperms: Revisited. *Silvae Genetica* 54: 59–69.

Allen A, Hiscock S. 2008. Evolution and phylogeny of self-incompatibility systems in angiosperms In: Allen A, Hiscock S (eds.), Self-incompatibility in flowering plants. Berlin, Heidelberg: Springer, pp 73-101.

Araújo M, New M. 2007. Ensemble forecasting of species distributions. *Trends in Ecology & Evolution* 22: 42–47.

Armbruster WS, Pérez-Barrales R, Arroyo J, Edwards ME, Vargas P. 2006. Three-dimensional reciprocity of floral morphs in wild flax (*Linum suffruticosum*): A new twist on heterostyly. *New Phytologist* 171: 581–590.

Armbruster WS, Bolstad GH, Hansen TF, Keller B, Conti E, Pélabon C. 2017. The measure and mismeasure of reciprocity in heterostylous flowers. *New Phytologist* 215: 906–917.

Arroyo J, Barrett SCH, Hidalgo R, Cole WW. 2002. Evolutionary maintenance of stigma-height dimorphism in *Narcissus papyraceus* (Amaryllidaceae). *American Journal of Botany* 89: 1242–1249.

Baack EJ. 2004. Cytotype segregation on regional and microgeographic scales in snow buttercups (*Ranunculus adoneus*: Ranunculaceae). *American Journal of Botany* 91: 1783–1788.

Baack EJ. 2005. To succeed globally, disperse locally: Effects of local pollen and seed dispersal on tetraploid establishment. *Heredity* 94: 538–546.

Baack EJ, Stanton ML. 2005. Ecological factors influencing tetraploid establishment in snow buttercups (*Ranunculus adoneus*): Niche differentiation and tetraploid establishment. *Evolution* 59: 1936–1944.

Baker H. 1975. Sporophyte-garmetophyte interactions in *Linum* and other genera with heteromorphic self-incompatibility In: Mulcahy DL (ed.), Gamete competition in plants and animals. North Holland, Amsterdam, pp 191-199.

Baker AM, Barrett SCH, Thompson JD. 2000. Variation of pollen limitation in the early flowering Mediterranean geophyte *Narcissus assoanus* (Amaryllidaceae). *Oecologia* 124: 529–535.

Balao F, Casimiro-Soriguer R, Talavera M, Herrera J, Talavera S. 2009. Distribution and diversity of cytotypes in *Dianthus broteri* as evidenced by genome size variations. *Annals of Botany* 104: 965–973.

Barker MS, Arrigo N, Baniaga AE, Li Z, Levin DA. 2016. On the relative abundance of autopolyploids and allopolyploids. *New Phytologist* 210: 391–398.

Barrett SCH, Shore JS. 1987. Variation and evolution of breeding systems in the *Turnera ulmifolia* L. complex (Turneraceae). *Evolution* 41: 340–354.

Barrett SCH. 1992. Heterostylous genetic polymorphisms: Model systems for evolutionary analysis In: Barrett SCH (Ed), Evolution and function of heterostyly. Springer, Berlin, Germany, pp 1-29.

Barrett SCH, Lloyd DG, Arroyo J. 1996. Stylar polymorphisms and the evolution of heterostyly in *Narcissus* (Amaryllidaceae) In: Harder LD and Barret SHC (eds.), Floral biology. Springer, Boston, pp, 339-376.

Barrett SCH, Wilken DH, Cole WW. 2000. Heterostyly in the Lamiaceae: The case of *Salvia brandegeei*. *Plant Systematics and Evolution* 223: 211–219.

Barrett SCH. 2002a. Sexual interference of the floral kind. *Heredity* 88: 154–159.

Barrett SCH. 2002b. The evolution of plant sexual diversity. *Nature Reviews Genetics* 3: 274–284.

Barrett SCH, Shore JS. 2008. New insights on heterostyly: Comparative biology, ecology and genetics. In: Allen A, Hiscock S (eds.), Self-Incompatibility in Flowering Plants: Evolution, Diversity, and Mechanisms. Springer, Berlin, Heidelberg, pp, 3-32.

Barrett SCH. 2013. The evolution of plant reproductive systems: How often are transitions irreversible? *Proceedings of the Royal Society B: Biological Sciences* doi.org/10.1098/rspb.2013.0913

Barrett SCH. 2019. 'A most complex marriage arrangement': recent advances on heterostyly and unresolved questions. *New Phytologist* 224: 1051–1067.

Barringer BC. 2007. Polyploidy and self-fertilization in flowering plants. *American Journal of Botany* 94: 1527–1533.

Baumel A, Ainouche ML, Bayer RJ, Ainouche AK, Misset MT. 2002. Molecular phylogeny of hybridizing species from the genus *Spartina* Schreb. (Poaceae). *Molecular Phylogenetics and Evolution* 22: 303–314.

Best DJ, Roberts DE. 1975. Algorithm AS 89: The Upper Tail Probabilities of Spearman's Rho. *Applied Statistics* 24: 377.

Bigio L, Lebel M, Yuval S. 2017. Do different measures of maternal fitness affect estimation of natural selection on floral traits? A lesson from *Linum pubescens* (Linaceae). *Journal of Plant Ecology* 10: 406–413.

Bittencourt NS, Gibbs PE, Semir J. 2003. Histological study of post-pollination events in *Spathodea campanulata* beauv. (Bignoniaceae), a species with late-acting self-incompatibility. *Annals of Botany* 91: 827–834.

Blaine Marchant D, Soltis DE, Soltis PS. 2016. Patterns of abiotic niche shifts in allopolyploids relative to their progenitors. *New Phytologist* 212: 708–718.

Blanc G, Wolfe KH. 2004. Widespread Paleopolyploidy in Model Plant Species Inferred from Age Distributions of Duplicate Genes. *The Plant Cell* 16: 1667–1678.

Blondel J, Aronson J, Bodiou J-Y, Boeuf G. 2010. The Mediterranean Basin – Biological Diversity in Space and Time. Oxford: Oxford University Press.

Bolker BM, Brooks ME, Clark CJ, Geange SW, Poulsen JR, Stevens MHH, White JSS. 2009. Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in Ecology and Evolution* 24: 127–135.

Bolsheva NL, Melnikova NV, Kirov IV, Speranskaya AS, Krinitsina AA, Dmitriev AA, Belenikin MS, Krasnov GS, Lakunina VA, Snezhkina AV, Rozhmina TA, Samatadze TE, Yurkevich OY, Zoshchuk SA, Amosova AV, Kudryavtseva AV, Muravenko OV. 2010. Evolution of blue-flowered species of genus *Linum* based on high-throughput sequencing of ribosomal RNA genes. *BMC Evolutionary Biology* 17: 253

Bolsheva NL, Zelenin AV, Nosova IV, Amosova AV, Samatadze TE, Yurkevich OY, Melnikova NV, Zelenina DA, Volkov AA, Muravenko OV. 2015. The diversity of karyotypes and genomes within section *Syllinum* of the genus *Linum* (Linaceae) revealed by molecular cytogenetic markers and RAPD analysis. *PLoS ONE*. doi.org/10.1371/journal.pone.0122015

Borges LA, Souza LGR, Guerra M, Machado IC, Lewis GP, Lopes AV. 2012. Reproductive isolation between diploid and tetraploid cytotypes of *Libidibia ferrea* (= *Caesalpinia ferrea*)

(Leguminosae): Ecological and taxonomic implications. *Plant Systematics and Evolution* 298: 1371–1381.

Bougoutaia Y, Garnatje T, Vallès J, Kaid-Harche M, Ouhammou A, Dahia M, Tlili A, Vitales D. 2021. Phylogeographical and cytogeographical history of *Artemisia herba-Alba* (Asteraceae) in the Iberian Peninsula and North Africa: Mirrored intricate patterns on both sides of the Mediterranean Sea. *Botanical Journal of the Linnean Society* 195: 588–605.

Bretagnolle F, Lumaret R. 1995. Bilateral polyploidization in *Dactylis glomerata* L. subsp. *lusitanica*: occurrence, morphological and genetic characteristics of first polyploids. *Euphytica* 84: 197–207.

Bretagnolle F, Thompson JD. 1995. Gametes with the somatic chromosome number: mechanisms of their formation and role in the evolution of autopolyploid plants. *New Phytologist* 129: 1–22.

Brochmann C, Brysting AK, Alsos IG, Borgen L, Grundt HH, Scheen AC, Elven R. 2004. Polyploidy in arctic plants. *Biological Journal of the Linnean Society* 82: 521–536.

Brock MT. 2004. The potential for genetic assimilation of a native dandelion species, *Taraxacum ceratophorum* (Asteraceae), by the exotic congener *T. officinale*. *American Journal of Botany* 91: 656–663.

Broennimann O, Fitzpatrick M, Pearman P, Petitpierre B, Pellissier L, Yoccoz NG, Thuiller W, Fortin MJ, Randin C, Zimmermann NE, Graham CH. 2012. Measuring Ecological Niche Overlap From Occurrence. *Global Ecology and Biogeography* 21: 481–497.

Brys R, Jacquemyn H, Beeckman T. 2008. Morph-ratio variation, population size and female reproductive success in distylous *Pulmonaria officinalis* (Boraginaceae). *Journal of Evolutionary Biology* 21: 1281–1289.

Brys R, Jacquemyn H, Endels P, Hermy M, De Blust G. 2003. The relationship between reproductive success and demographic structure in remnant populations of Primula veris. *Acta Oecologica* 24: 247–253.

Brys R, Jacquemyn H. 2015. Disruption of the distylous syndrome in *Primula veris*. *Annals of Botany* 115: 27–39.

Brys R, Jacquemyn H. 2020. The impact of individual inaccuracy of reciprocal herkogamy on legitimate pollen deposition and seed set in a distylous self-incompatible herb. *Journal of Ecology* 108: 81–93.

Buggs RJA, Pannell JR. 2007. Ecological differentiation and diploid superiority across a moving ploidy contact zone. *Evolution* 61: 125–140.

Casazza G, Boucher FC, Minuto L, Randin CF, Conti E. 2017. Do floral and niche shifts favour the establishment and persistence of newly arisen polyploids? A case study in an *Alpine primrose*. *Annals of Botany* 119: 81–93.

Castro S, Münzbergová Z, Raabová J, Loureiro J. 2011. Breeding barriers at a diploid-hexaploid contact zone in *Aster amellus*. *Evolutionary Ecology* 25: 795–814.

Castro M, Castro S, Loureiro J. 2012a. Genome size variation and incidence of polyploidy in Scrophulariaceae sensu lato from the Iberian Peninsula. *AoB PLANTS*. doi.org/10.1093/aobpla/pls037

Castro S, Loureiro J, Procházka T, Münzbergová Z. 2012b. Cytotype distribution at a diploidhexaploid contact zone in *Aster amellus* (Asteraceae). *Annals of Botany* 110: 1047–1055.

Castro S, Ferrero V, Costa J, Sousa AJ, Castro M, Navarro L, Loureiro J. 2013. Reproductive strategy of the invasive *Oxalis pes-caprae*: Distribution patterns of floral morphs, ploidy levels and sexual reproduction. *Biological Invasions* 15: 1863–1875.

Castro S, Loureiro J. 2014. The role of reproduction in the emergence and evolution of polyploid plants. *Ecosistemas* 23: 67–77.

Castro M, Castro S, Figueiredo A, Husband B, Loureiro J. 2018. Complex cytogeographical patterns reveal a dynamic tetraploid-octoploid contact zone. *AoB PLANTS* 10: 1–18.

Castro M, Loureiro J, Serrano M, Tavares D, Husband BC, Siopa C, Castro S. 2019. Mosaic distribution of cytotypes in a mixed-ploidy plant species, *Jasione montana*: nested environmental niches but low geographical overlap. *Botanical Journal of the Linnean Society* 190: 51–66.

Castro M, Loureiro J, Figueiredo A, Serrano M, Husband BC, Castro S. 2020. Different patterns of ecological divergence between two tetraploids and their diploid counterpart in a parapatric linear coastal distribution polyploid complex. *Frontiers in Plant Science* 11: 1–16.

Cesaro AC, Thompson JD. 2004. Darwin's cross-promotion hypothesis and the evolution of stylar polymorphism. *Ecology Letters* 7: 1209–1215.

Charlesworth D, Charlesworth B. 1974. A model for the evolution of distyly. *The American Naturalist* 114: 467–498.

Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annual review of ecology and systematics* 18: 237–268.

Chelaifa H, Monnier A, Ainouche M. 2010. Transcriptomic changes following recent natural hybridization and allopolyploidy in the salt marsh species *Spartina* × *townsendii* and *Spartina anglica* (Poaceae). *New Phytologist* 186: 161–174.

Chennaveeraiah MS, Joshi KK. 1983. Karyotypes in Cultivated and Wild Species of *Linum*. *Cytologia* 48: 833–841.

Clement M, Posada D, Crandall K. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9: 1657–1659.

Clo J, Kolář F. 2021. Short- and long-term consequences of genome doubling: a meta-analysis. *American Journal of Botany* 108: 2315–2322.

Cocker JM, Wright J, Li J, Swarbreck D, Dyer S, Caccamo M, Gilmartin PM. 2018. *Primula vulgaris* (primrose) genome assembly, annotation and gene expression, with comparative genomics on the heterostyly supergene. *Scientific Reports* 8: 1–13.

Comai L. 2005. The advantages and disadvantages of being polyploid. *Nature Reviews Genetics* 6: 836–846.

Cook BI, Anchukaitis KJ, Touchan R, Meko DM, Cook ER. 2016. Spatiotemporal drought variability in the Mediterranean over the last 900 years. *Journal of Geophysical Research* 121: 2060–2074.

Costa J, Ferrero V, Loureiro J, Castro M, Navarro L, Castro S. 2014. Sexual reproduction of the pentaploid, short-styled *Oxalis pes-caprae* allows the production of viable offspring. *Plant Biology* 16: 208–214.

Costa J, Ferrero V, Castro M, Jorge A, Afonso A, Loureiro J, Castro S. 2016. Pollen flow between flowers of the same morph in invasive populations of *Oxalis pes-caprae* L. in the western Mediterranean region. *Plant Biosystems* 150: 923–931.

Costa J, Castro S, Loureiro J, Barrett SCH. 2016. Variation in style morph frequencies in tristylous *Lythrum salicaria* in the Iberian Peninsula: The role of geographical and demographic factors. *Annals of Botany* 117: 331–340.

Costa J, Castro S, Loureiro J, Barrett SCH. 2017. Experimental insights on darwin's cross-promotion hypothesis in tristylous purple loosestrife (*Lythrum salicaria*). *American Journal of Botany* 104: 616–626.

Costa J. 2017. *The function and adaptive significance of the floral polymorphism heterostyly*. PhD Thesis. Universidade de Coimbra, Portugal. Angewandte Chemie International Edition, 6(11), 951–952.

Cowling RM, Rundel PW, Lamont BB, Arroyo MK, Arianoutsou M. 1996. Plant diversity in mediterranean-climate regions. *Trends in Ecology and Evolution* 11: 362–366.

Cui L, Wall PK, Leebens-Mack JH, Lindsay BG, Soltis E, Doyle JJ, Soltis PS, Carlson JE, Arumuganathan K, Barakat A, Albert VA, Ma H, dePamphilis CW. 2006. Widespread genome duplications throughout the history of flowering plants*. Genome Research* 16: 738–749.

Dafni A, Pacini E, Nepi M. 2005. Pollen and stigma biology. In Dafni A, Kevan P, and Husband B, eds. Pratical Pollination Biology. Enviroquest, Ontario, Canada.

Dai X, Li X, Huang Y, Liu X. 2020. The speciation and adaptation of the polyploids: A case study of the *Chinese Isoetes* L. diploid-polyploid complex. *BMC Evolutionary Biology* 20: 1–13.

Darlington CD, Ammal J, AMMAL EJ, WYLIE AP. 1955. Chromosome Atlas of Cultivated Plants In: Darlington CD and Wylie AP (eds.), Chromosome Atlas of Flowering Plants. George Allen & Unwin, United Kingdom.

Darwin C. 1863. On the existence of two forms in species of Linum. *The natural history review: a quarterly journal of biological science* 476

Darwin C. 1877. The Different Forms of Flowers on Plants of the Same Species. John Murray, London, UK.

De Haan A, Maceira N, Lumaret R, Delay J. 1992. Production of 2n gametes in diploid subspecies of *Dactylis glomerata* L. 2. Occurrence and frequency of 2n eggs. *Annals of Botany* 69: 345–350.

DeWet J. 1980. Origins of polyploids In: Lewis WH (ed.), Polyploidy. Springer, Boston, MA, pp 3-15.

Di Cola V, Broennimann O, Petitpierre B, Breiner FT, D'Amen M, Randin C, Engler R, Pottier J, Pio D, Pio A, Pellissier L, Mateo RG, Hordijk W, Salamin N, Guisan A. 2017. Ecospat: an R package to support spatial analyses and modeling of species niches and distributions. *Ecography* 40: 774–787.

Diederichsen A, Richards K. 2003. Cultivated flax and the genus *Linum* L. Taxonomy and germoplasm conservation In: Muir A and Westcott N (eds.), Flax: the genus *Linum*. Taylor & Francis Ltd, London, pp 22–54.

Dimitrova D, Ebert I, Greilhuber J, Kozhuharov S. 1999. Karyotype constancy and genome size variation in Bulgarian *Crepis foetida s. l.* (Asteraceae). *Plant Systematics and Evolution* 217: 245–257.

Doležel J, Sgorbati S, Lucretti S. 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. *Physiologia Plantarum* 85: 625–631.

Doležel J, Greilhuber J, Suda J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* 2: 2233–2244.

Doyle JJ, Doyle JL, Rauscher JT, Brown AHD. 2004. Evolution of the perennial soybean polyploid complex (*Glycine* subgenus *Glycine*): A study of contrasts. *Biological Journal of the Linnean Society* 82: 583–597.

Doyle JJ, Egan AN. 2010. Dating the origins of polyploidy events. *New Phytologist* 186: 73–85.

Dray S, Dufour A. 2007. The ade4 Package: Implementing the Duality Diagram for Ecologists. *Journal of Statistical Software* 22: 1–20.

Dressler S, Repplinger M, Bayer C. 2014. Linaceae In: Kubitzki K. (ed.), The families and genera of vascular plants. Vol. XI. Flowering plants, Eudicots: Malpighiales. Springer, Berlin, pp, 237–246.

Dulberger R. 1964. Flower dimorphism and self-incompatibility in Narcissus tazetta L. *Evolution* 18: 361–363.

Dulberguer R. 1973. Distyly in *Linum pubescens* and *L. mucronatum*. *Botanical Journal of the Linnean Society* 66: 117–126.

Dulberger R. 1975a. Intermorph structural differences between stigmatic papillae and pollen grains in relation to incompatibility in Plumbaginaceae. P*roceedings of the Royal Society of London. Series B. Biological Sciences* 188: 257–274.

Dulberger R. 1975b. S-gene action and the significance of characters in the heterostylous syndrome. *Heredity* 35: 407–415.

Dulberger R. 1981. Dimorphic exine sculpturing in three distylous species of *Linum* (Linaceae). *Plant Systematics and Evolution* 139: 113–119.

Dulberger R. 1987. Fine structure and cytochemistry of the stigma surface and incompatibility in some distylous *Linum* species. *Annals of Botany* 59: 203–217.

Dulberger R. 1992. Floral polymorphisms and their functional significance in the heterostylous syndrome. In: Barrett SCH (ed), Evolution and function of heterostyly. Springer, Berlin, Germany, pp 41–84.

Eckert CG, Barrett SCH. 1992. Stochastic loss of style morphs from populations of tristylous *Lythrum salicaria* and *Decodon verticillatus* (Lythraceae). *Evolution* 46: 1014–1029.

Eckert CG, Manicacci D, Barrett SCH. 1996. Frequency-dependent selection on morph ratios in tristylous *Lythrum salicaria* (Lythraceae). *Heredity* 77: 581–588.

Eilam T, Anikster Y, Millet E, Manisterski J, Feldman M. 2010. Genome Size in Diploids, Allopolyploids, and Autopolyploids of Mediterranean Triticeae. *Journal of Botany* doi:10.115.

Elena Rosselló JA, González Zapatero MA, Navarro Andrés F. 1985. Sobre los niveles de ploidía y otras particularidades cromosómicas de algunos vegetales castellano-leoneses de preferencias calcícolas. *Studia Botanica Universidad de Salamanca* 4: 109-115

Emberger L, Maire R. 1941. Catalogue des plantes du Maroc. Vol. IV. Alger: Minerva.

Endels P, Jacquemyn H, Brys R, Hermy M, De Blust G. 2002. Temporal changes (1986-1999) in populations of primrose (*Primula vulgaris* Huds.) in an agricultural landscape and implications for conservation*. Biological Conservation* 105: 11–25.

Escudero M, Balao F, Martín-Bravo S, Valente L, Valcárcel V. 2018. Is the diversification of Mediterranean Basin plant lineages coupled to karyotypic changes? *Plant Biology* 20: 166–175.

Felber F. 1991. Establishment of a tetraploid cytotype in a diploid population: Effect of relative fitness of the cytotypes. *Journal of Evolutionary Biology* 4: 195–207.

Nieto-Feliner G. 2014. Patterns and processes in plant phylogeography in the Mediterranean Basin. A review. *Perspectives in Plant Ecology, Evolution and Systematics* 16: 265–278.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 29: 783–791.

Fennane M, Tattou MI, Mathez J, Ouyahya A, El Oualidi J. 2007. Flore pratique du Maroc, Manuel de détermination des plantes vasculaires. Angiospermae (Leguminosae-Lentibulariaceae). Rabat: Institut Scientifique de Rabat.

Ferrero V, Arroyo J, Castro S, Navarro L. 2012. Unusual heterostyly: Style dimorphism and self-incompatibility are not tightly associated in Lithodora and Glandora (Boraginaceae). *Annals of Botany* 109: 655–665.

Ferrero V, Navarro L, Castro S, Loureiro J, Sánchez JM, Carvallo GO, Barrett SCH. 2020. Global patterns of reproductive and cytotype diversity in an invasive clonal plant. Biological Invasions 22: 1691–1703.

Fiz O, Valcárcel V, Vargas P. 2002. Phylogenetic position of Mediterranean Astereae and character evolution of daisies (*Bellis*, Asteraceae) inferred from nrDNA its sequences. *Molecular Phylogenetics and Evolution* 25: 157–171.

Fowler N, Levin D. 1984. Ecological constraints on the establishment of a novel polyploid in competition with its diploid progenitor. *American Naturalist* 124: 703–711.

Fox J, Weisberg S, Adler D, Bates D, Baud-Bovy G, S. E. 2005. car: Companion to applied regression. Available at http://CRAN.R-project.org/package=car.

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220: 1049–1051.

Ganders FR. 1979. The biology of heterostyly. *New Zealand Journal of Botany* 17: 607–635.

Ganguly S, Barua D. 2021. Inter-morph pollen flow and reproductive success in a self-compatible species with stigma-height dimorphism: the influence of herkogamy and reciprocity. *Plant Biology* 23: 939–946.

Gibbs PE. 1986. Do homomorphic and heteromorphic self-incompatibility systems have the same sporophytic mechanism? *Plant Systematics and Evolution* 154: 285–323.

Gielly L, Debussche M, Thompson JD. 2001. Geographic isolation and evolution of Mediterranean endemic Cyclamen: Insights from chloroplast trnL (UAA) intron sequence variation. *Plant Systematics and Evolution* 230: 75–88.

Gilmartin PM. 2015. On the origins of observations of heterostyly in *Primula*. *New Phytologist* 208: 39–51.

Glennon KL, Rissler LJ, Church SA. 2012. Ecogeographic isolation: A reproductive barrier between species and between cytotypes in *Houstonia* (Rubiaceae). *Evolutionary Ecology* 26: 909–926.

Glennon KL, Ritchie ME, Segraves KA. 2014. Evidence for shared broad-scale climatic niches of diploid and polyploid plants. *Ecology Letters* 17: 574–582.

Godsoe W, Larson MA, Glennon KL, Segraves KA. 2013. Polyploidization in *Heuchera cylindrica* (Saxifragaceae) did not result in a shift in climatic requirements. *American Journal of Botany* 100: 496–508.

Graham SW, Olmstead RG. 2000. Utility of 17 chloroplast genes for inferring the phylogeny of the basal angiosperms. *American journal of botany* 87: 1712–1730.

Grant V. 1956. The Influence of Breeding Habit on the Outcome of Natural Hybridization in Plants. *The American Naturalist* 90: 319–322.

Grant V. 1981. Plant Speciation. New York, NY, USA: Columbia University Press.

Greilhuber J, Doležel J, Lysák MA, Bennett MD. 2005. The origin, evolution and proposed stabilization of the terms “genome size” and “C-value” to describe nuclear DNA contents. *Annals of Botany* 95: 255–260.

Greilhuber J, Temsch EM, Loureiro J. 2007. Nuclear DNA content measurement. In: Dolezel J, Greilhuber J, Suda J (eds.), Flow cytometry with plant cells: analysis of genes, chromosomes and genomes. Weinheim: Wiley-VCH, pp 67-101.

Gross K, Schiestl FP. 2015. Are tetraploids more successful? Floral signals, reproductive success and floral isolation in mixed-ploidy populations of a terrestrial orchid. *Annals of Botany* 115: 263–273.

Guggisberg A, Mansion G, Kelso S, Conti E. 2006. Evolution of biogeographic patterns, ploidy levels, and breeding systems in a diploid-polyploid species complex of *Primula*. *New Phytologist* 171: 617–632.

Habibi F, Vít P, Rahiminejad M, Mandák B. 2018. Towards a better understanding of the *Chenopodium album* aggregate (Amaranthaceae) in the Middle East: A karyological, cytometric and morphometric investigation. *Journal of Systematics and Evolution* 56: 231–242.

Haldane JBS. 1926. A mathematical theory of natural and artificial selection. Mathematical Proceedings of the Cambridge *Philosophical Society* 23: 363–372.

Hao GY, Lucero ME, Sanderson SC, Zacharias EH, Holbrook NM. 2013. Polyploidy enhances the occupation of heterogeneous environments through hydraulic related trade-offs in *Atriplex canescens* (Chenopodiaceae). *New Phytologist* 197: 970–978.

Harder L, Barrett SCH. 2006. Ecology and evolution of flowers. Oxford: Oxford University Press.

Hastie TJ, Pregibon D. 1992. Generalized linear models. In: Chambers JM, Hastie TJ (eds.), Statistical Models. S. Chapman & Hall/CRC, pp, 195-246

Hengl T, De Jesus JM, Heuvelink GBM, Gonzalez MR, Kilibarda M, Blagotić A, Shangguan W, Wright MN, Geng X, Bauer-Marschallinger B, Guevara MA, Vargas R, MacMillan RA, Batjes NH,

Leenaars JGB, Ribeiro E, Wheeler I, Mantel S, Kempen B. 2017. SoilGrids250m: Global gridded soil information based on machine learning. *PLoS ONE* 12: 1–40.

Hewitt G. 2011. Mediterranean peninsulas: the evolution of hotspots. In: Zachos FE, Habel JC. (eds.), Biodiversity Hotspots. Springer, Berlin, Heidelberg, pp 123–147.

Heywood VH. 1993. Flowering plants of the world. Oxford: Oxford University Press.

Hickey M. 1988. 100 Families of flowering plants. Cambridge: Cambridge University Press.

Hijmans R, van Etten J, Cheng J, Mattiuzz M, Sumner M, Greenberg JA, Lamigueiro OP, Bevan A, Racine EB, Shortridge A, Ghosh A, 2017. Package 'raster'. Available at: https://cran.r-project.org/package=raster.

Hildebrand F. 1863. Dimorphismus von *Primula sinensis. Verh Naturh Vereins Rheinl Westf Sitzungber* 20: 183–184.

Hildebrand F. 1864. Experimente uber Dimorphismus von Linum perenne und *Primula sinensis. Botanische Zeitung* 22: 1–5.

Hohmann N, Schmickl R, Chiang TY, Lučanová M, Kolář F, Marhold K, Koch MA. 2014. Taming the wild: Resolving the gene pools of non-model Arabidopsis lineages. *BMC Evolutionary Biology* 14: 1–21.

Hothorn T, Bretz F, Westfall P, Heiberger RM. 2017. Multcomp: simultaneous inference for general linear hypotheses. Available at http://CRAN.Rproject.org/package=multcomp.

Hülber K, Sonnleitner M, Suda J, Krejčíková J, Schönswetter P, Schneeweiss GM, Winkler M. 2015. Ecological differentiation, lack of hybrids involving diploids, and asymmetric gene flow between polyploids in narrow contact zones of *Senecio carniolicus* (syn. *Jacobaea carniolica*, Asteraceae). *Ecology and Evolution* 5: 1224–1234.

Husband BC, Schemske DW. 1998. Cytotype distribution at a diploid-tetraploid contact zone in *Chamerion* (Epilobium) *angustifolium* (Onagraceae). *American Journal of Botany* 85: 1688–1694.

Husband BC. 2000. Constraints on polyploid evolution: a test of the minority cytotype exclusion principle. *Proceedings of the Royal Society B: Biological Sciences* 267: 217–223.

Husband BC, Schemske DW. 2000. Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. *Journal of Ecology* 88: 689–701.

Husband BC, Schemske DW, Burton TL, Goodwillie C. 2002. Pollen competition as a unilateral reproductive barrier between sympatric diploid and tetraploid *Chamerion angustifolium*. *Proceedings of the Royal Society B: Biological Sciences* 269: 2565–2571.

Husband BC. 2004. Chromosomal variation in plant evolution. *American Journal of Botany* 91: 621–625.

Husband BC, Sabara HA. 2004. Reproductive isolation between autotetraploids and their diploid progenitors in fireweed, *Chamerion angustifolium* (Onagraceae). *New Phytologist* 161: 703–713.

Husband BC, Ozimec B, Martin SL, Pollock L. 2008. Mating consequences of polyploid evolution in flowering plants: Current trends and insights from synthetic polyploids. *International Journal of Plant Sciences* 169: 195–206.

Husband BC, Baldwin SJ, Suda J. 2013. The Incidence of Polyploidy in Natural Plant Populations: Major Patterns and Evolutionary Processes In: Greilhuber J, Dolezel J, Wendel JF (eds.), Plant genome diversity. vol. 2, SpringerVerlag, Vienna, pp, 255-276.

Husband BC, Baldwin SJ, Sabara HA. 2016. Direct vs. Indirect effects of whole-genome duplication on prezygotic isolation in *Chamerion angustifolium*: Implications for rapid speciation. *American Journal of Botany* 103: 1259–1271.

Huu CN, Keller B, Conti E, Kappel C, Lenhard M. 2020. Supergene evolution via stepwise duplications and neofunctionalization of a floral-organ identity gene. *Proceedings of the National Academy of Sciences* 117: 23148–23157.

Jacquemyn H, Brys R, Hermy M. 2002. Patch occupancy, population size and reproductive success of a forest herb (*Primula elatior*) in a fragmented landscape. *Oecologia* 130: 617–625.

Jacquemyn H, Gielen M, Brys R. 2018. Is sexual organ reciprocity related to legitimate pollen deposition in distylous *Pulmonaria* (Boraginaceae)? *OIkos* 8: 1216–1224.

Jahandiez E, Maire R. 1932. Catalogue des plantes du Maroc (Spermatophytes et Pteridophytes). Vol II. Algiers: Imprimerie Minerva.

Jansson R, Dynesius M. 2002. The fate of clades in a world of recurrent climatic change: Milankovitch oscillations and evolution. *Annual Review of Ecology and Systematics* 33: 741–777.

Jersáková J, Castro S, Sonk N, Milchreit, K, Schödelbauerová I, Tolasch T, Dötterl S. 2010. Absence of pollinator-mediated premating barriers in mixed-ploidy populations of *Gymnadenia conopsea s.l.* (Orchidaceae*). Evolutionary Ecology* 24: 1199–1218.

Johnson SD, Dafni A. 1998. Response of bee-flies to the shape and pattern of model flowers: implications for floral evolution in a Mediterranean herb. *Funcional Ecology* 12: 289–297.

Kappel C, Huu CN, Lenhard M. 2017. A short story gets longer: recent insights into the molecular basis of heterostyly. *Journal of Experimental Botany* 68: 5719–5730.

Keller B, Thomson JD, Conti E. 2014. Heterostyly promotes disassortative pollination and reduces sexual interference in Darwin's primroses: Evidence from experimental studies. *Functional Ecology* 28: 1413–1425.

Kelso S. 1992. The genus *Primula* as a model for evolution in the Alaskan flora. Arctic & Alpine Research 24: 82–87.

Kennedy BF, Sabara HA, Haydon D, Husband BC. 2006. Pollinator-mediated assortative mating in mixed ploidy populations of *Chamerion angustifolium* (Onagraceae). *Oecologia* 150: 398–408.

Kim S, Rayburn AL, Parrish A, Lee DK. 2012a. Cytogeographic Distribution and Genome Size Variation in Prairie Cordgrass (*Spartina pectinata* Bosc ex Link). *Plant Molecular Biology Reporter* 30: 1073–1079.

Kim S, Rayburn AL, Boe A, Lee DK. 2012b. Neopolyploidy in *Spartina pectinata* Link: 1. Morphological analysis of tetraploid and hexaploid plants in a mixed natural population. *Plant Systematics and Evolution* 298: 1073–1083.

Knouft JH, Losos JB, Glor RE, Kolbe, JJ, Losos B Glor, E Kolbe J. 2012. Phylogenetic Analysis of the Evolution of the Niche in Lizards of the *Anolis sagrei* Group. *Ecology* 87: S29-S38.

Koch M, Mummenhoff K, Hurka H. 1998. Molecular biogeography and evolution of the *Microthlaspi perfoliatum s.l.* polyploid complex (Brassicaceae): Chloroplast DNA and nuclear ribosomal DNA restriction site variation. *Canadian Journal of Botany* 76: 382–396.

Kolář F, Čertner M, Suda J, Schönswetter P, Husband BC. 2017. Mixed-Ploidy Species: Progress and Opportunities in Polyploid Research. *Trends in Plant Science* 22: 1041–1055.

Kolář F, Tech M, Trávníek P, Rauchová J, Urfus T, Vít P, Kubesová M, Suda J. 2009. Towards resolving the *Knautia arvensis* agg. (Dipsacaceae) puzzle: Primary and secondary contact zones and ploidy segregation at landscape and microgeographic scales. *Annals of Botany* 103: 963–974.

Krahulcová A, Trávníček P, Krahulec F, Rejmánek M. 2017. Small genomes and large seeds: Chromosome numbers, genome size and seed mass in diploid *Aesculus* species (Sapindaceae). *Annals of Botany* 119: 957–964.

Kreitschitz A, Kovalev A, Gorb SN. 2015. Slipping vs sticking: Water-dependent adhesive and frictional properties of *Linum usitatissimum* L. seed mucilaginous envelope and its biological significance. *Acta Biomaterialia* 17: 152–159.

Krijgsman W. 2002. The Mediterranean: Mare Nostrum of Earth sciences. *Earth and Planetary Science Letters* 205: 1–12.

Kron P, Suda J, Husband BC. 2007. Applications of Flow Cytometry to Evolutionary and Population Biology. *Annual Review of Ecology, Evolution, and Systematics* 38: 847–876.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* 35: 1547–1549.

Laport RG, Hatem L, Minckley RL, Ramsey J. 2013. Ecological niche modeling implicates climatic adaptation, competitive exclusion, and niche conservatism among *Larrea tridentata* cytotypes in North American deserts. *The Journal of the Torrey Botanical Society* 140: 349–363.

Laport RG, Minckley RL, Ramsey J. 2016. Ecological distributions, phenological isolation, and genetic structure in sympatric and parapatric populations of the *Larrea tridentata* polyploid complex. *American Journal of Botany* 103: 1358–1374.

Lebel M, Obolski U, Hadany L, Sapir Y. 2018. Pollinator-mediated selection on floral size and tube color in *Linum pubescens*: Can differential behavior and preference in different times of the day maintain dimorphism? *Ecology and Evolution* 8: 1096–1106.

Leitch IJ, Bennett MD. 1997. Polyploidy in angiosperms. *Trends in Plant Science* 2: 470–476.

Levin DA. 1975. Minority cytotype exclusion in local plant populations. *Taxon* 24: 35–43.

Levin DA. 2002. The role of chromosomal change in plant evolution. Oxford: University Press.

Lewis D. 1942. The physiology of incompatibility in plants - I. The effect of temperature. Proceedings of the Royal Society of London. *Series B - Biological Sciences* 131: 13–26.

Lewis D. 1943. The Physiology of Incompatibility in Plants: II. *Linum grandiflorum*. *Annals of Botany* 7: 115–122.

Lewis D, Jones D. 1992. The genetics of heterostyly In: Barrett SCH (ed), Evolution and function of heterostyly. Springer, Berlin, Germany, pp 129-150.

Lexer C, van Loo M. 2006. Contact zones: Natural labs for studying evolutionary transitions. *Current Biology* 16: 407–409.

Li BH, Xu X-M, Ridout M. 2004. Modelling the establishment and spread of autotetraploid plants in a spatially heterogeneous environment. *Journal of Evolutionary Biology* 17: 562–573.

Li J, Cocker JM, Wright J, Webster M.A, McMullan M, Dyer S, Swarbreck D, Caccamo M, Oosterhout Cv, Gilmartin PM. 2016. Genetic architecture and evolution of the S locus supergene in *Primula vulgaris*. *Nature plants* 2: 1–7.

Lim KY, Matyasek R, Kovarik A, Leitch AR. 2004. Genome evolution in allotetraploid *Nicotiana*. *Biological Journal of the Linnean Society* 82: 521–536.

Lipow SR, Wyatt R. 2000. Single gene control of postzygotic self-incompatibility in poke milkweed, *Asclepias exaltata* L.. *Genetics* 154: 893–907.

Lloyd DG, Webb CJ. 1992a. The selection of heterostyly In: Barrett SCH (ed), Evolution and function of heterostyly. Springer, Berlin, Germany, pp 179-208.

Lloyd DG, Webb CJ. 1992b. The evolution of heterostyly In: Barrett SCH (ed), Evolution and function of heterostyly. Springer, Berlin, Germany, pp 151-178.

Loginova DB, Silkova OG. 2017. Mechanisms of unreduced gamete formation in flowering plants. *Russian Journal of Genetics* 53: 741–756.

López González G. 1979. Algunas consideraciones sobre los linos del grupo *Linum tenuifolium* L. en España. *Memoires de la Société Botanique de Genéve* 1: 99–109.

López-González N, Bobo-Pinilla J, Padilla-García N, Loureiro J, Castro, S, Rojas-Andrés BM, Martínez-Ortega MM. 2021. Genetic similarities versus morphological resemblance: Unraveling a polyploid complex in a Mediterranean biodiversity hotspot. *Molecular Phylogenetics and Evolution* 155.

López-Jurado J, Mateos-Naranjo E, Balao F. 2019. Niche divergence and limits to expansion in the high polyploid *Dianthus broteri* complex. *New Phytologist* 222: 1076–1087.

Loureiro J, Rodriguez E, Dolezel J, Santos C. 2007. Two New Nuclear Isolation Buffers for Plant DNA Flow Cytometry: A Test with 37 Species. *Annals of Botany* 100: 875–888.

Loureiro JCM. 2007. *Flow cytometic approaches to study plant genomes*. PhD Thesis. Universidade de Aveiro, Portugal.

Lumaret R, Guillerm JL, Delay J, Ait Lhaj Loutfi A, Izco J, Jay M. 1987. Polyploidy and habitat differentiation in *Dactylis glomerata* L. from Galicia (Spain). *Oecologia* 73: 436–446.

Lumaret R, Guillerm JL, Maillet J, Verlaque R. 1997. Plant species diversity and polyploidy in islands of natural vegetation isolated in extensive cultivated lands. *Biodiversity and Conservation* 6: 591–613.

Mable BK. 2004. Polyploidy and self-compatibility: Is there an association? *New Phytologist* 162: 803–811.

Maceira N, Jacquard P, Lumaret R. 1993. Competition between diploid and derivative autotetraploid *Dactylis glomerata* L. from Galicia. Implications for the establishment of novel polyploid populations. *New Phytologist* 124: 321–328.

Madlung A. 2013. Polyploidy and its effect on evolutionary success: Old questions revisited with new tools. *Heredity* 110: 99–104.

Maguilla E, Escudero M, Ruíz- Martín J, Arroyo J. 2021a. Origin and diversification of flax and their relationship with heterostyly across the range. *Journal of Biogeography* 48: 1994–2007.

Maguilla E, Escudero M, Jiménez-Lobato V, Díaz-Lifante Z, Andrés-Camacho C, Arroyo J. 2021b. Polyploidy Expands the Range of *Centaurium* (Gentianaceae). *Frontiers in Plant Science* 12.

Mahelka V, Suda J, Travnicek P, Krahulec F. 2005. Genome size discriminates between closely related taxa *Elytrigia repens* and *E. intermedia* (Poaceae: Triticeae) and their hybrid. *Folia Geobotanica* 40: 367–384.

Maherali H, Walden AE, Husband BC. 2009. Genome duplication and water stress. *New Phytologist* 184: 721–731.

Mandák B, Vít P, Krak K, Trávníček P, Drašnarová A, Hadincová V, Zákravský P, Jarolímová V, Bacles CFE, Douda J 2016. Flow cytometry, microsatellites and niche models reveal the origins and geographical structure of *Alnus glutinosa* populations in Europe. *Annals of Botany* 117: 107–120.

Mansion G, Zeltner L, Bretagnolle F. 2005. Phylogenetic patterns and polyploid evolution within the Mediterranean genus *Centaurium* (Gentianaceae - Chironieae). *Taxon* 54: 931–950.

Marques I, Loureiro J, Draper D, Castro M, Castro S. 2018. How much do we know about the frequency of hybridisation and polyploidy in the Mediterranean region? *Plant Biology* 20: 21–37.

Marques I, Rosselló-Graell A, Draper D, Iriondo JM. 2007. Pollination patterns limit hybridization between two sympatric species of *Narcissus* (Amaryllidaceae). *American Journal of Botany* 94: 1352–1359.

Martin SL, Husband BC. 2012. Whole Genome Duplication Affects Evolvability of Flowering Time in an Autotetraploid. *Plant. PLoS ONE* doi.org/10.1371/journal.pone.0044784.

Martínez-Labarga JM, Garmendia FM. 2015. *Linum* L. In: Castroviejo S, Muñoz Garmendia F, Navarro C, Quintanar A, Buira A (eds.), Flora Iberica. Rhamnaceae-Polygalaceae. Real Jardín Botánico, CSIC, Madrid. 9: 172-259.

Martínez Labarga JM, Ferrer-Gallego PP. 2020. Taxonomic and Nomenclatural Notes on African *Linum Squarrosum Sensu Lato* (Linaceae). *Phytotaxa* 439: 127–135.

Mason AS, Nelson MN, Yan G, Cowling WA. 2011. Production of viable male unreduced gametes in *Brassica* interspecific hybrids is genotype specific and stimulated by cold temperatures. *BMC Plant Biology* 11.

Mason AS, Pires JC. 2015. Unreduced gametes: Meiotic mishap or evolutionary mechanism? *Trends in Genetics* 31: 5–10.

Mast AR, Kelso S, Conti E. 2006. Are any primroses (*Primula*) primitively monomorphic? *New Phytologist* 171: 605–616.

Masterson J. 1994. Stomatal Size in Fossil Plants: Evidence for give very different estimates for families Polyploidy in Majority of Angiosperms. *Science* 264: 421–424.

Matias R, Pérez-Barrales R, Consolaro H. 2020. Patterns of variation in distylous traits and reproductive consequences in *Erythroxylum* species and populations. *American Journal of Botany* 107: 910–922.

McDill J, Repplinger M, Simpson BB, Kadereit JW. 2009. The Phylogeny of *Linum* and Linaceae Subfamily Linoideae, with Implications for Their Systematics, Biogeography, and Evolution of Heterostyly. *Systematic Botany* 34: 386–405.

McDill JR, Simpson BB. 2011. Molecular phylogenetics of Linaceae with complete generic sampling and data from two plastid genes. *Botanical Journal of the Linnean Society* 165: 64–83.

McIntyre PJ. 2012. Polyploidy associated with altered and broader ecological niches in the *Claytonia perfoliata* (Portulacaceae) species complex. *American Journal of Botany* 99: 655–662.

Meulenkamp JE, Sissingh W. 2003. Tertiary palaeogeography and tectonostratigraphic evolution of the Northern and Southern Peri-Tethys platforms and the intermediate domains of the African-Eurasian convergent plate boundary zone. Palaeogeography, Palaeoclimatology, *Palaeoecology* 196: 209–228.

Miller JS, Venable DL. 2000. Polyploidy and the Evolution of Gender Dimorphism in Plants. *Science, New Series* 289: 2335–2338.

Morgan EJ, Čertner M, Lučanová M, Kubíková K, Marhold K, Kolář F. 2020. Niche similarity in diploid-autotetraploid contact zones of Arabidopsis arenosa across spatial scales. *American Journal of Botany* 107: 1375–1388.

Mráz P. 2003. Mentor effects in the genus *Hieracium S.str*. (Compositae, Lactuceae). *Folia Geobotanica* 38: 345–350.

Muñoz-Pajares AJ, Perfectti F, Loureiro J, Abdelaziz M, Biella P, Castro M, Castro S, Gómez JM. 2018. Niche differences may explain the geographic distribution of cytotypes in *Erysimum mediohispanicum*. *Plant Biology* 20: 139–147.

Müntzing A. 1936. The Evolutionary Significance of Autopolyploidy. *Hereditas* 21: 363–378.

Muravenko O V., Lemesh VA, Samatadze TE, Amosova AV, Grushetskaya ZE, Popov KV, Semenova OY, Khotyuleva LV, Zelenin AV. 2003. Genome comparisons with chromosomal and molecular markers for three closely related flax species and their hybrids. *Russian Journal of Genetics* 39: 414–421.

Muravenko OV., Bolsheva NL, Yurkevich OY, Nosova IV, Rachinskaya OA, Samatadze TE, Zelenin AV. 2010. Karyogenomics of species of the genus *Linum* L. *Genetika* 46: 1339–1342.

Murray B. 2013. Karyotype variation and evolution in gymnosperms In: Greilhuber J, Dolezel J, Wendel JF (eds), Plant genomic diversity, Vol II. Springer, Vienna, Austria, pp 231– 243.

Murray BG. 2005. When does intraspecific C-value variation become taxonomically significant? *Annals of Botany* 95: 119–125.

Myers B, Mittermeier R, Mittermeier C, Da Fonseca G, Kent J. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853–858.

Naiki A, Nagamasu H. 2004. Correlation between distyly and ploidy level in *Damnacanthus* (Rubiaceae). *American Journal of Botany* 91: 664–671.

Naiki A. 2012. Heterostyly and the possibility of its breakdown by polyploidization. *Plant Species Biology* 27: 3–29.

Nakamura K, Denda T, Kameshima O, Yokota M. 2007. Breakdown of distyly in a tetraploid variety of *Ophiorrhiza japonica* (Rubiaceae) and its phylogenetic analysis. *Journal of Plant Research* 120: 501–509.

Navarro L, Ayensa G, Ferrero V, Sánchez JM. 2012. The avoidance of self-interference in the endemic daffodil *Narcissus cyclamineus* (Amaryllidaceae). *Plant Ecology* 213: 1813–1822.

Nei M, Kumar S. 2000. Molecular evolution and phylogenetics. Oxford University Press, USA.

Nicholls MS. 1985a. A systematic study of the *Linum tenuifolium* group (Linaceae). *Botanical Journal of the Linnean Society* 91: 473–490.

Nicholls MS. 1985b. The Evolutionary Breakdown of Distyly in *Linum tenuifolium* (Linaceae). *Plant Systematics and Evolution* 150: 291–301.

Nicholls MS. 1985c. Pollen flow, population composition, and the adaptive significance of distyly in *Linum tenuifolium* L. (Linaceae). *Biological Journal of the Linnean Society* 25: 235–242.

Nicholls MS. 1986a. Variation and evolution in Linum tenuifolium (Linaceae). Plant Systematics and Evolution 153: 243–258.

Nicholls MS. 1986b. Population Composition, Gender Specialization, and the Adaptive Significance of Distyly in *Linum Perenne* (Linaceae). *New Phytologist* 102: 209–217.

Nilsson O, Lassen P. 1971. Chromosome numbers of vascular plants from Austria, Mallorca and Yugoslavia. *Botaniska notiser* 124: 270–276.

Ockendon DJ, Walters SM. 1968. *Linum* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA (eds.), Flora Europaea. Cambridge: University Press. 2: 206-211

Ockendon DJ. 1968. Biosystematic Studies in the *Linum Perenne* Group. *New Phytologist* 67: 787–813.

Ockendon DJ. 1971. Taxonomy of the *Linum perenne* group in Europe. *Watsonia* 8: 205–235.

Olesen JM. 1987. Heterostyly, homostyly, and long-distance dispersal of *Menyanthes trifoliata* to Greenland. *Canadian Journal of Botany* 65: 1509–1513.

Ornduff R. 1969. Reproductive biology in relation to systematics. *Taxon* 18: 121–133.

Otto SP, Whitton J. 2000. Polyploid incidence and evolution. *Annual Review of genetics* 34: 401–437.

Ozenda P. 1977. Flora of the Sahara. PARIS: CNRS.

Ozimec B, Husband BC. 2011. Effect of recurrent selfing on inbreeding depression and mating system evolution in an autopolyploid plant. *Evolution* 65: 2038–2049.

Padilla FM, Pugnaire FI. 2007. Rooting depth and soil moisture control Mediterranean woody seedling survival during drought. *Functional Ecology* 21: 489–495.

Pannell JR, Dorken ME, Eppley SM. 2005. "Haldane's Sieve" in a metapopulation: Sifting through plant reproductive polymorphisms. *Trends in Ecology and Evolution* 20: 374–379.

Parisod C, Broennimann O. 2016. Towards unified hypotheses of the impact of polyploidy on ecological niches. *New Phytologist* 212: 540–542.

Parrott WA, Smith RR. 1986. Evidence for the existence of endosperm balance number in the true clovers (*Trifolium* spp.) . *Canadian Journal of Genetics and Cytology* 28: 581–586.

Pérez-Barrales R, Vargas P, Arroyo J. 2006. New evidence for the Darwinian hypothesis of heterostyly: Breeding systems and pollinators in *Narcissus* sect. *Apodanthi*. *New Phytologist* 171: 553–567.

Petit C, Lesbros P, Ge X, Thompson JD. 1997. Variation in flowering phenology and selfing rate across a contact zone between diploid and tetraploid *Arrhenatherum elatius* (Poaceae). *Heredity* 79: 31–40.

Philipp M, Schou O. 1981. An unusual heteromorphic incompatibility system: distyly, self-incompatibility, pollen load and fecundity in *Anchusa officinalis* (Boraginaceae). *New Phytologist* 89: 693–703.

Phillips S, Anderson R, Schapire R. 2006. Maximum entropy modeling of species geographic distributions. *Ecological Modelling* 190: 231–259.

Phillips S. 2008. Transferability, sample selection bias and background data in presence-only modelling: a response to Peterson et al. (2007). *Ecography* 31: 272–278.

Porturas LD, Anneberg TJ, Curé AE, Wang S, Althoff DM, Segraves KA. 2019. A meta-analysis of whole genome duplication and the effects on flowering traits in plants. *American Journal of Botany* 106: 469–476.

Prančl J, Koutecky P, Travniček P, Jarolímová V, Lučanová M, Koutecká E, Kaplan Z. 2018. Cytotype variation, cryptic diversity and hybridization in *Ranunculus* sect. *Batrachium* revealed by flow cytometry and chromosome numbers. *Preslia* 90: 195–223.

Quézel P, Santa S. 1962. Nouvelle flore de l'Algérie et des régions désertiques méridionales. PARIS: CNRS.

Quézel P. 1978. Analysis of the Flora of Mediterranean and Saharan Africa. *Annals of the Missouri Botanical Garden* 65: 479–534.

R Core Development Team. 2019. A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Available at http://www.R-project.org/.

Ramsey J, Schemske DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics* 29: 467–501.

Ramsey J, Schemske DW. 2002. Neopolyploidy in flowering plants. *Annual Review of Ecology and Systematics* 33: 589–639.

Ramsey J. 2011. Polyploidy and ecological adaptation in wild yarrow. *Proceedings of the National Academy of Sciences of the United States of America* 108: 7096–7101.

Rasband WS. 2008. ImageJ 1997–2007. US National Institutes of Health: Bethesda, Maryland, USA.

Rausch JH, Morgan MT. 2005. Effect of Self-Fertilization, Inbreeding Depression, and Population Size on Autopolyploid Establishment. *Evolution* 59: 1867.

Richards J, Barrett SCH. 1992. The development of heterostyly In: Barrett SCH (ed), Evolution and function of heterostyly. Springer, Berlin, Germany, pp 85-127.

Richards JH, Koptur S. 1993. Floral variation and distyly in *Guettarda scabra* (Rubiaceae). *American Journal of Botany* 80: 31–40.

Rieseberg LH, Willis JH. 2007. Plant speciation. *Science* 317: 910–914.

Riveros M, Arroyo MTK, Humaña AM. 1987. An unusual kind of distyly in *Quinchamalium chilense* (Santalaceae) on Volcan Casablanca, Southern Chile. *American Journal of Botany* 74: 313–320.

Rodríguez DJ. 1996. A model for the establishment of polyploidy in plants. *American Naturalist* 147: 33–46.

Rodríguez-Sánchez F, Pérez-Barrales R, Ojeda F, Vargas P, Arroyo J. 2008. The Strait of Gibraltar as a melting pot for plant biodiversity. *Quaternary Science Reviews* 27: 2100-2117.

Rogers CM, Mildner R, Harris BD. 1972. Some additional chromosome numbers in the Linaceae. *Brittonia* 24: 313–316.

Rogers CM. 1979. Distyly and pollen dimorphism in *Linum* suffruticosum (Linaceae). *Plant Systematics and Evolution* 131: 127–132.

Rogers C. 1982. The Systematics of *Linum* sect . *Linopsis* (Linaceae). *Plant Systematics* 140: 225–234.

Rosenbaum G, Lister GS, Duboz C. 2002. Reconstruction of the tectonic evolution of the western Mediterranean since the Oligocene. *Journal of the Virtual Explorer* 8.

Ruiz-Martín J, Jiménez-Mejiás P, Martínez-Labarga JM, Pérez-Barrales R. 2015. *Linum flos-carmini* (Linaceae), a New Species from Northern Morocco. *Annales Botanici Fennici* 52: 383–395.

Ruiz-Martín J. 2017. Evolución y función de la heterostilia en el género *Linum* (Linaceae). PhD Thesis. Universidad de Seville, Espana.

Ruiz-Martín J, Santos-Gally R, Escudero M, Midgley J, Pérez-Barrales R, Arroyo J. 2018. Style polymorphismin Linum (Linaceae): a case of Mediterranean parallel evolution? *Plant Biology* 20(Suppl 1):100–111.

Rundel WP, Arroyo MTK, Cowling RM, Keeley JE, Lamont BB, Vargas P. 2016. Mediterranean Biomes: Evolution of Their Vegetation, Floras, and Climate. *Annual Review of Ecology, Evolution, and Systematics* 47: 383–407.

Sage TL, Strumas F, Cole WW, Barrett SCH. 1999. Differential Ovule Development Following Self- and Cross-Pollination : The Basis of Self-Sterility in *Narcissus triandrus* (Amaryllidaceae). *American Journal of Botany* 86: 855–870.

Sampson JF, Byrne M. 2012. Genetic diversity and multiple origins of polyploid *Atriplex nummularia* Lindl. (Chenopodiaceae). *Biological Journal of the Linnean Society* 105: 218–230.

Sánchez JM, Ferrero V, Navarro L. 2013. Quantifying reciprocity in distylous and tristylous plant populations. *Plant Biology* 15: 616–620.

Saxton KE, Rawls WJ. 2006. Soil water characteristic estimates by texture and organic matter for hydrologic solutions. *Soil Science Society of America Journal* 70: 1569–1578.

Schemske DW, Horvitz CC. 1984. Variation among floral visitors in pollination ability: A precondition for mutualism specialization. *Science* 225: 519–521.

Schneider AC, Freyman WA, Matt Guilliams C, Springer YP, Baldwin BG. 2016. Pleistocene radiation of the serpentine-adapted genus *Hesperolinon* and other divergence times in Linaceae (Malpighiales). *American Journal of Botany* 103: 221–232.

Schoener T. 1970. Nonsynchronous spatial overlap of lizards in patchy habitats. *Ecology* 51: 408–418.

Segraves KA, Thompson JN, Soltis PS, Soltis DE. 1999. Multiple origins of polyploidy and the geographic structure of *Heuchera grossulariifolia*. *Molecular Ecology* 8: 253–262.

Segraves KA, Thompson JN. 1999. Plant polyploidy and pollination: Floral traits and insect visits to diploid and tetraploid *Heuchera grossulariifolia*. *Evolution* 53: 1114–1127.

Segraves KA. 2017. The effects of genome duplications in a community context. *New Phytologist* 215: 57–69.

Shaw J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J, Siripun KC, Winder CT, Schilling EE, Small RL. 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92: 142–166.

Shaw J, Lickey EB, Schilling EE, Small RL. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The Tortoise and the hare III. *American Journal of Botany* 94: 275–288.

Shore JS, Arbo MM, Fernández A. 2006. Breeding system variation, genetics and evolution in the Turneraceae. *New Phytologist* 171: 539–551.

Shore JS, Barrett SCH. 1986. Genetic modifications of dimorphic incompatibility in the *Turnera ulmifolia* L. complex (Turneraceae). *Canadian Journal of Genetics and Cytology* 28: 796–807.

Simón-Porcar VI, Meagher TR, Arroyo J. 2015. Disassortative mating prevails in style-dimorphic *Narcissus papyraceus* despite low reciprocity and compatibility of morphs. *Evolution* 69: 2276–2288.

Siopa C, Dias MC, Castro M, Loureiro J, Castro S. 2020. Is selfing a reproductive assurance promoting polyploid establishment? Reduced fitness, leaky self-incompatibility and lower inbreeding depression in neotetraploids. *American Journal of Botany* 107: 526–538.

Smith S, Donoghue M. 2010. Combining historical biogeography with niche modeling in the *Caprifolium* clade of *Lonicera* (Caprifoliaceae, Dipsacales). *Systematic Biology* 59: 322–344.

Soberón J. 2007. Grinnellian and Eltonian niches and geographic distributions of species. *Ecology* Letters 10: 1115–1123.

Soltis PS, Doyle JJ, Soltis DE. 1992. Molecular Data and Polyploid Evolution in Plants In: Soltis PS, Soltis DE, Doyle JJ (eds.), Molecular Systematics of Plants. Boston: Springer, MA.177–201.

Soltis DE, Soltis PS. 1995. The dynamic nature of polyploid genomes. *Proceedings of the National Academy of Sciences of the United States of America* 92: 8089–8091.

Soltis DE, Soltis PS. 1999. Polyploidy: Recurrent formation and genome evolution. *Trends in Ecology and Evolution* 14: 348–352.

Soltis PS, Soltis DE. 2000. The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences of the United States of America* 97: 7051–7057.

Soltis PS. 2005. Ancient and recent polyploidy in angiosperms. *New Phytologist* 166: 1–5.

Soltis DE, Albert VA, Leebens-Mack J, Bell CD, Paterson AH, Zheng C, Sankoff D, Depamphilis CW, Wall PK, Soltis PS. 2009. Polyploidy and angiosperm diversification. *American Journal of Botany* 96: 336–348.

Soltis DE, Buggs RJA, Doyle JJ, Soltis PS. 2010. What we still don't know about polyploidy. *Taxon* 59: 1387–1403.

Sonnleitner M, Flatscher R, Escobar García P, Rauchová J, Suda J, Schneeweiss GM, Hülber K, Schönswetter P. 2010. Distribution and habitat segregation on different spatial scales among diploid, tetraploid and hexaploid cytotypes of *Senecio carniolicus* (Asteraceae) in the Eastern Alps. *Annals of Botany* 106: 967–977.

Sonnleitner M, Hülber K, Flatscher R, García PE, Winkler M, Suda J, Schönswetter P, Schneeweiss GM, 2016. Ecological differentiation of diploid and polyploid cytotypes of *Senecio carniolicus sensu lato* (Asteraceae) is stronger in areas of sympatry. *Annals of Botany* 117: 269–276.

Spoelhof JP, Soltis PS, Soltis DE. 2017. Pure polyploidy: Closing the gaps in autopolyploid research. *Journal of Systematics and Evolution* 55: 340–352

Ståhlberg D, Hedrén M. 2009. Habitat differentiation, hybridization and gene flow patterns in mixed populations of diploid and autotetraploid *Dactylorhiza maculata s.l*. (Orchidaceae). *Evolutionary Ecology* 23: 489.

Stebbins GL. 1971. Chromosomal evolution in higher plants. London, UK: Edward Arnold.

Steininger FF, Rögl F. 1984. Paleogeography and palinspastic reconstruction of the Neogene of the Mediterranean and Paratethys. *Geological Society Special Publication* 17: 659–668.

Suda J, Kron P, Husband BC, Trávníček P. 2007. Flow cytometry and ploidy: applications in plant systematics, ecology and evolutionary biology In: In Doležel J, Greilhuber J, Suda J (eds.), Flow cytometry with plant cells: analysis of genes, chromosomes and genomes. Weinheim: Wiley-VCH. 103-130.

Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.

Talebi MS, Masoud S, Morteza A, Fariba S, Zahra N. 2012. Genome size, morphological and palynological variations, and heterostyly in some species of the genus *Linum* L. (Linaceae) in Iran. *African Journal of Biotechnology* 11: 16040–16054.

Talebi SM, Sheidai M, Atri M, Sharifnia F, Noormohammadi Z. 2015. Infraspecific morphological and genome size variations in *Linum glaucum* in Iran. *Biodiversitas* 16: 69–78.

Tamari F, Athanasiou A, Shore JS. 2001. Pollen tube growth and inhibition in distylous and homostylous *Turnera* and *Piriqueta* (Turneraceae). *Canadian Journal of Botany* 79: 578–591.

Tate JA, Soltis DE, Soltis PS. 2005. Polyploidy in plants In: Ryan GT (ed.), The evolution of the genome. Academic Press, pp 371-426.

Te Beest M, Le Roux JJ, Richardson DM, Brysting AK, Suda J, Kubešová M, Pyšek P. 2012. The more the better? The role of polyploidy in facilitating plant invasions. *Annals of Botany* 109: 19–45.

Theodoridis S, Randin C, Broennimann O, Patsiou T, Conti E. 2013. Divergent and narrower climatic niches characterize polyploid species of European Primroses in *primula* sect. *aleuritia*. *Journal of Biogeography* 40: 1278–1289.

Thompson JD. 2020. Plant Evolution in the Mediterranean: Insights for conservation. USA: Oxford University Press.

Thompson KA, Husband BC, Maherali H. 2014. Climatic niche differences between diploid and tetraploid cytotypes of *Chamerion angustifolium* (Onagraceae). *American Journal of Botany* 101: 1868–1875.

Thuiller W, Georges D, Engler R, Breiner F, Georges M, Thuiller C. 2016. Package 'biomod2'. ftp://ftp2.de.freebsd.org/pub/ misc/cran/web/packages/biomod2/biomod2.pdf.

Trognitz BR, Hermann M. 2001. Inheritance of tristyly in *Oxalis tuberosa* (Oxalidaceae). *Heredity* 86: 564–573.

Ushijima K, Nakano R, Bando M, Shigezane Y, Ikeda K, Namba Y, Kume S, Kitabata T, Mori H, Kubo Y. 2012. Isolation of the floral morph-related genes in heterostylous flax (Linum grandiflorum): The genetic polymorphism and the transcriptional and post-transcriptional regulations of the S locus. *Plant Journal* 69: 317–331.

Valcárcel V, Fiz O, Vargas P. 2003. Chloroplast and nuclear evidence for multiple origins of polyploids and diploids of *Hedera* (Araliaceae) in the Mediterranean basin. *Molecular Phylogenetics and Evolution* 27: 1–20.

Valdés B, Rejdali M, Kadmiri AA El, Jury SL, Montserrat JM. 2007. Catalogue des plantes vasculaires du Nord du Maroc, incluant des clés d'identification / Checklist of vascular plants of N Morocco with identification keys. Madrid: Consejo Superior de Investigaciones Cientificas.

Vamosi JC, Goring JS, Kennedy NF, Mayberry RJ, Moray CM; Neame LA, Tunbridge ND, Elle E. 2007. Pollination, Floral Display, and the Ecological Correlates of Polyploidy. *Functional Ecosystems and Communities* 1: 1-9

Vamosi JC, Scott Armbruster W, Scott Armbruster W, Scott Armbruster W, Renner SS. 2014. Evolutionary ecology of specialization: Insights from phylogenetic analysis*. Proceedings of the Royal Society B: Biological Sciences* 281.

Van Dijk P, Bijlsma R. 1994. Simulations of flowering time displacement between two cytotypes that form inviable hybrids. *Heredity* 72: 522–535.

Van Dijk W. 1943. La découverte de l'hétérostylie chez *Primula* par Ch. de l'Écluse et P. Reneaulme. *Nederlandsch kruidkundig archief* 53: 81–85.

Vaughton G, Ramsey M, Johnson SD. 2010. Pollination and late-acting self-incompatibility in *Cyrtanthus breviflorus* (Amaryllidaceae): Implications for seed production. *Annals of Botany* 106: 547–555.

Vimala Y, Lavania S, Lavania UC. 2021. Chromosome change and karyotype differentiation–implications in speciation and plant systematics. *Nucleus (India)* 64: 33–54.

Visger CJ, Germain-Aubrey CC, Patel M, Sessa EB, Soltis PS, Soltis DE. 2016. Niche divergence between diploid and autotetraploid *Tolmiea*. *American Journal of Botany* 103: 1396–1406.

Wagner V, Chytrý M, Zelený D, von Wehrden H, Brinkert A, Danihelka J, Hölzel N, Jansen F, Kamp J, Lustyk P, Merunková K, Palpurina S, Preislerová Z, Wesche K. 2017. Regional differences in soil pH niche among dry grassland plants in Eurasia. *Oikos* 126: 660–670.

Wagner W. 1983. Reticulistics: the recognition of hybrids and their role in cladistics and classification. *Advances in cladistics* 2: 63–79.

Wan J-N, Guo Y-P, Rao G-Y. 2019. Unraveling independent origins of two tetraploid *Achillea* species by amplicon sequencing. *Journal of Systematics and Evolution* doi: 10.1111/jse.12544

Warren DL, Glor RE, Turelli M. 2008. Environmental niche equivalency versus conservatism: Quantitative approaches to niche evolution. *Evolution* 62: 2868–2883.

Webb CJ, Lloyd DG. 1986. The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. herkogamy. *New Zealand Journal of Botany* 24: 163–178.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173: 697–703.

White TJW, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications* 18: 315–322.

Winterfeld G, Schneider J, Röser M. 2009. Allopolyploid origin of Mediterranean species in *Helictotrichon* (Poaceae) and its consequences for karyotype repatterning and homogenisation of rDNA repeat units. *Systematics and Biodiversity* 7: 277–295.

Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB, Rieseberg LH. 2009. The frequency of polyploid speciation in vascular plants. *Proceedings of the National Academy of Sciences of the United States of America* 106: 13875–13879.

Wu LY, Chang FF, Liu SJ, Scott Armbruster W, Huang SQ. 2018. Heterostyly promotes compatible pollination in buckwheats: Comparisons of intraflower, intraplant, and interplant pollen flow in distylous and homostylous *Fagopyrum*. *American Journal of Botany* 105: 108–116.

Xavier KS, Mildner RA, Rogers CM. 1980. Pollen morphology of *Linum*, sect. *Linastrum* (linaceae). *Grana* 19: 183–188.

Yuan S, Barrett SCH, Li C, Li X, Xie K, Zhang D. 2019. Genetics of distyly and homostyly in a self-compatible *Primula*. *Heredity* 122: 110–119.

Zachos JC, Dickens GR, Zeebe RE. 2008. An early Cenozoic perspective on greenhouse warming and carbon-cycle dynamics. *Nature* 451: 279–283.

Zapatero ÁG, Elena-Rosselló JA, Andrés FN. 1981. Números cromosomáticos de plantas occidentales, 527-532. *Anales del Jardín Botánico de Madrid* 38: 269–272.

Zar J. 2010. Biostatistical Analysis. 5th ed. Prentice-Hall, Inc., New Jersey, USA.

Zhou W, Barrett SCH, Li HD, Wu ZK, Wang XJ, Wang H, Li DZ. 2017. Phylogeographic insights on the evolutionary breakdown of heterostyly. *New Phytologist* 214: 1368–1380.

Zhou W, Barrett SCH, Wang H, Li DZ. 2012. Loss of floral polymorphism in heterostylous *Luculia pinceana* (rubiaceae): A molecular phylogeographic perspective. *Molecular Ecology* 21: 4631–4645.

Zhou W, Barrett SCH, Wang H, Li DZ. 2015a. Reciprocal herkogamy promotes disassortative mating in a distylous species with intramorph compatibility. *New Phytologist* 206: 1503–1512.

Zhou X, Mo X, Gui M, Wu X, Jiang Y, Ma L, Shi Z, Luo Y, Tang W. 2015b. Cytological, molecular mechanisms and temperature stress regulating production of diploid male gametes in Dianthus caryophyllus L. *Plant Physiology and Biochemistry* 97: 255–263.