Joana Filipa Martinho da Costa

The Function and Adaptive Significance of the Floral Polymorphism Heterostyly

Tese de Doutoramento em Biociências, especialização em Ecologia,
orientada pelo Professor Doutor João Carlos Mano Castro Loureiro, Doutora Sílvia Raquel Cardoso Castro e Professor Doutor Spencer Charles Hilton Barrett,
apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Universidade de Coimbra

The Function and Adaptive Significance of the Floral Polymorphism Heterostyly

Significado Funcional e Adaptativo do Polimorfismo Floral Heterostilia

Joana Filipa Martinho da Costa

Dissertation submitted to the Department of Life Sciences of the Faculty of Sciences and Technology of the University of Coimbra in partial fulfillment of the requirements for the degree Doctor of Philosophy 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 (Department of Life Sciences, University of Coimbra) and Professor Spencer Charles Hilton Barrett (Department of Ecology and Evolutionary Biology, University of Toronto).

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Departamento de Ciências da Vida
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UNIVERSIDADE DE COIMBRA

Aos meus Pais

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"Nothing in biology makes sense except in the light of evolution."

Theodosius Dobzhansky

Thesis cover image
Title: Charles Darwin's backyard
Media: Chinese ink & watercolour on paper
Author: Rui Gaspar

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ABBREVIATIONS

2C – two copies of the nuclear DNA content

1*x* – haploid

2*x* – diploid

2*n* – diploid number of chromosomes

3*x* – triploid

4*x* – tetraploid

5*x* – pentaploid

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

BF – Bayes Factor

BIC – Bayesian Information Criterion

BP – bootstrap percentage

ca. – approximately

cf. – (L. *confer*) compare with

cont. – to be continued

df – degrees of freedom

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

et al. – (L. *et alia*) and others

GLM – generalized linear model

GLMM – generalized mixed-effects model

GMT – Greenwich mean time

GTR – General Time Reversal model

i.e. – (L. *id est*) that is

L – long-styled morph

L-morph – long-styled morph

log – logarithm

M – mid-styled morph

MB – Mediterranean basin

MC – morph-compatibility

ML – maximum likelihood

M-morph – mid-styled morph

mya – million years ago

n – sample size

No. – number

n.s. – non-significant

OUT - outcrossing

P – probability

pers. observ. / comm. – personal observations/communication

pg – picograms

PHIIC – model selection criterion for molecular calibration with likelihood methods

PI – propidium iodide

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

Pop – population

PP – posterior probability

Refs – references

S – short-styled morph

SA – South Africa

SC – self-compatibility

SCM – stochastic character mapping

SD – standard deviation

SE – standard error

SEM – scanning electron microscopy

S-morph – short-styled morph

S/O – seed/ovule ratio

sp. – (L. *species*) species singular

spp. – (L. *species*) species plural

T_{ij} – pollen transfer proficiency

TSI – trimorphic incompatibility system

w/v – weight/volume

Note: all units used follow the SI (Système International d'Unités).

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ABSTRACT

The flowers of animal-pollinated plants display a remarkable diversity of structural adaptations that function to promote cross-pollen dispersal and reduce pollen wastage. Floral morphology determines the spectrum of suitable animal visitors, but also constrains the orientation of pollinators so that the site of contact between the animal's body and the sex organs of flowers results in effective cross-pollination. A noteworthy case of the functional fit between flowers and pollinators is heterostyly, a floral syndrome that has evolved in at least 28 angiosperm families. Heterostylous populations are characterized by the occurrence of two (distyly) or three (tristyly) floral morphs that differ in stigma and anther heights (reciprocal herkogamy). The stamen-style polymorphism is usually accompanied by a diallelic self-incompatibility system that prevents self- and intramorph mating, and ancillary polymorphisms of stigmas and pollen. Beginning with Darwin's seminal work on heterostylous plants, there has been sustained interest in the evolution and function of these polymorphisms, which have become model systems in ecology and evolutionary biology.

I investigated three main topics concerned with heterostyly in my PhD thesis: (1) the function and adaptive significance of the morphological traits that characterize the heterostylous syndrome using species from two unrelated families, (2) the evolutionary buildup of this floral polymorphism in a family with variability in heteromorphic character states, and (3) the demographic factors influencing the maintenance of heterostyly in two unrelated taxa, one native and one invasive in the Iberian Peninsula. Rather than focusing on a single species in my thesis, I have chosen particular taxa that are most appropriate for addressing the questions that I am interested in. I also used diverse approaches specific to the particular questions. To address (1) and (3), I combined sampling of variation in natural populations and pollination experiments under common garden conditions, and to investigate (2) I conducted an ancestral character state reconstruction on a molecular phylogeny of a family with heterostylous species.

To evaluate Darwin's cross-promotion hypothesis for the function of reciprocal herkogamy, I used tristylous *Lythrum salicaria* (Lythraceae), a species with a partially tubular corolla. For this, I quantified pollen transfer and capture in monomorphic and trimorphic experimental arrays composed of intact and emasculated flowers. My results provided experimental evidence supporting the Darwinian hypothesis, and also demonstrated the importance of floral design in governing compatible and incompatible

pollen capture. Compatible pollen capture varied in a predictable manner with sex-organ height. In this chapter, I also addressed methodological issues associated with the evaluation of the Darwinian hypothesis.

To address the functional significance of ancillary polymorphisms of pollen and stigmas, I investigated patterns of pollen transfer and capture in natural populations of three species of Plumbaginaceae (*Armeria maritima*, *A. pubigera* and *Limonium vulgare*), and studied pollen adherence and germination patterns in *A. maritima* after hand-pollinations. My results demonstrated for the first time that pollen-stigma dimorphisms serve to promote disassortative pollination in natural populations. The results from controlled pollinations were consistent with the hypothesis that the mechanisms limiting incompatible pollen deposition probably result from intimate interactions between the structural and physiological properties of pollen and stigmas.

To assess the evolutionary buildup of the heterostylous syndrome in Plumbaginaceae, I used molecular data to construct a phylogenetic tree for the family and an extensive survey of herbarium specimens to investigate variation in heteromorphic morphological traits. To determine the most likely order of establishment of components of the heterostylous syndrome, these were mapped onto phylogenetic trees. My comparative study provided evidence suggesting that the most recent common ancestor of the Plumbaginaceae was monomorphic in relation to sex-organ position, and was also most likely self-incompatible. The reconstructions were therefore consistent with a theoretical model of the evolution of the heterostylous syndrome in which the diallelic incompatibility system evolves before floral heteromorphism.

To explore the role of demographic and geographic factors in affecting morph ratios in tristylous populations, I investigated style morph frequencies in populations at the southwestern European native range limit of *Lythrum salicaria*. I measured morph composition, evenness, and size of 96 populations along a north to south latitudinal transect from Galicia to Andalucia, Iberian Peninsula, traversing a steep climatic gradient, particularly involving precipitation. I also examined reproductive traits in 19 populations. My study provided evidence that population size and evenness decreased from north to south. Nevertheless, there was no evidence that tristyly was destabilized at the southern margin of the range where populations were significantly smaller, thus indicating that the polymorphism is remarkably resilient to breakdown and to the factors causing deviations from isoplethy.

Finally, I investigated variation in the expression of trimorphic incompatibility in invasive populations from the western Mediterranean basin of *Oxalis pes-caprae* (Oxalidaceae). I tested the hypothesis that weakening of incompatibility may result from the benefits of self-compatibility arising from mate limitation following long-distance dispersal from the native South African range. I performed hand-pollinations and compared levels of self, intramorph and intermorph seed set in a common garden experiment using plants from native and invasive populations covering the entire distributional range in South Africa and the western Mediterranean basin, respectively. My results provided evidence of increased levels of self- and intramorph compatibility in introduced populations. The occurrence of weak self-incompatibility in Iberia may increase opportunities for sexual reproduction in the introduced range where most populations are either monomorphic or dimorphic for style morph composition.

In conclusion, the findings of my PhD thesis have demonstrated that both reciprocal herkogamy and ancillary polymorphisms promote disassortative pollination in heterostylous populations. These structural adaptations influence the pollen economy of populations by reducing pollen wastage on incompatible stigmas. Thus, heterostyly may function largely to improve plant fitness through male function. My investigations have also provided novel insights into the evolutionary assembly of the morphological and physiological components of this floral syndrome in a family of heterostylous species. And finally, they have also shed new light on the mechanisms involved in the maintenance of this complex polymorphism in natural populations.

KEY WORDS: ancillary characters; disassortative mating; geitonogamy; heterostyly; reciprocal herkogamy.

RESUMO

As plantas polinizadas por animais apresentam uma grande diversidade de adaptações estruturais para promover a polinização cruzada e diminuir perdas de pólen em estigmas incompatíveis. Entre elas, a grande diversidade de morfologias florais não só seleciona o espectro de polinizadores, como restringe os seus movimentos enquanto estes visitam as flores. Consequentemente, determinam o local específico de contacto entre o corpo do polinizador e os órgãos sexuais das flores. Um exemplo clássico de adaptação entre flores e polinizadores é a heterostilia, um polimorfismo floral que evoluiu em, pelo menos, 28 famílias de angiospérmicas. As populações de espécies heterostilas podem ser compostas por duas (distilia) ou três (tristilia) formas florais que diferem na altura a que se encontram o estigma e as anteras, *i.e.*, hercogamia recíproca. A hercogamia recíproca é normalmente acompanhada por um sistema dialélico de incompatibilidade que impede a fertilização após auto-polinizações e polinizações cruzadas entre plantas da mesma forma floral e, por fim, polimorfismos dos estigmas e do pólen. Desde a publicação de um dos mais influentes trabalhos sobre heterostilia por Charles Darwin, tem havido muito interesse no estudo da evolução e função destes polimorfismos florais, os quais se tornaram sistemas modelo em ecologia e biologia evolutivas.

Os três tópicos principais relacionados com vários aspectos da heterostilia que foram abordados na minha tese de Doutoramento são: (1) o significado funcional e adaptativo dos caracteres morfológicos que definem a heterostilia em duas famílias de plantas, (2) a evolução deste polimorfismo floral numa família com grande variabilidade de estados de caracteres heteromórficos, e (3) os factores demográficos que influenciam a manutenção da heterostilia em duas espécies diferentes, uma nativa e outra invasora na Pensínsula Ibérica. Em vez de me focar apenas num único sistema de estudo na minha tese, decidi escolher diferentes espécies de acordo com a questão a investigar. Para além disso, os métodos utilizados foram também específicos para cada questão e sistema de estudo. Para investigar os tópicos (1) e (3), amostrei populações naturais e realizei experiências de polinização em jardim experimental. Para explorar o tópico (2), fiz uma reconstrução filogenética de caracteres ancestrais.

Para testar a hipótese da polinização cruzada originalmente proposta por Charles Darwin para a função da hercogamia recíproca, usei a espécie tristila *Lythrum salicaria* (Lyhtraceae), cujas flores apresentam uma corola semi-tubular. Neste seguimento, quantifiquei a transferência e captura de pólen em parcelas experimentais monomórficas

e trimórficas, compostas por plantas com flores intactas e emasculadas. Os meus resultados apoiaram a hipótese proposta por Darwin, assim como demonstraram o papel da morfologia floral na determinação dos padrões de captura de pólen pelos estigmas. Mais especificamente, a captura de grãos de pólen compatíveis variou de forma esperada e de acordo com a posição dos órgãos sexuais na flor. Neste capítulo, também abordei detalhes metodológicos a ter em conta quando se pretende testar a hipótese Darwiniana para a função da hercogamia recíproca.

Para avaliar o significado funcional dos caracteres ancilares do pólen e estigma, investiguei os padrões de transferência e captura de pólen em populações naturais de três espécies de Plumbaginaceae (*Armeria maritima, A. pubigera* e *Limonium vulgare*), e estudei a aderência e germinação dos grãos de pólen após polinizações controladas em *A. maritima*. Os meus resultados demonstraram, pela primeira vez, que o dimorfismo polínico-estigmático funciona para promover a polinização cruzada entre formas florais em populações naturais. Os meus resultados das polinizações controladas foram consistentes com a hipótese de que os mecanismos que limitam a deposição de grãos de pólen incompatíveis resultam da acção conjunta de mecanismos estruturais e fisiológicos.

Para estudar a evolução da heterostilia nas Plumbaginaceae, usei dados moleculares para inferir uma árvore filogénetica para a família e amostrei um grande número de exemplares de herbário para obter informação acerca de caracteres morfológicos das flores. Com o objectivo de determinar a ordem pela qual ocorreu o estabelecimento dos vários caracteres que definem a heterostilia, os dados morfológicos foram mapeados numa árvore filogenética. O meu estudo comparativo sugeriu que o ancestral comum das Plumbaginaceae seria monomórfico para a posição relativa dos órgãos sexuais nas flores e também auto-incompatível. Assim, estes resultados são consistentes com um dos modelos teóricos para a evolução da heterostilia, o qual prevê que o sistema dialélico de incompatibilidade evoluíu antes do polimorfismo floral.

Com o objectivo de explorar os factores demográficos e geográficos que podem influenciar a composição em formas florais em populações tristilas, investiguei a frequência de morfos em populações de *Lythrum salicaria* no limite sudoeste da sua distribuição na Europa. Para cada população num total de 96, reúni dados de composição em formas florais e estimei o tamanho populacional, assim como medi variáveis reprodutivas em 19 locais. As populações amostradas localizaram-se ao longo de um transecto latitudinal desde a Galiza até à Andaluzia, e atravessando um gradiente

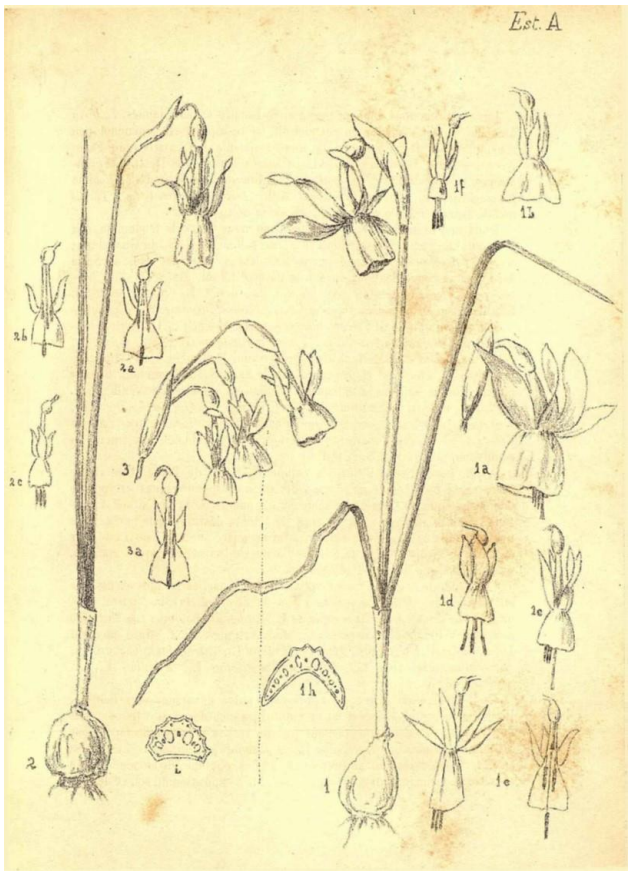
climático com variações sobretudo ao nível da precipitação. O meu estudo demonstrou que o tamanho das populações e o equilíbrio de formas florais em cada uma delas diminuía de norte para sul. De qualquer das formas, não encontrei evidência de que a manutenção da tristilia pudesse ser afectada no limite sul de distribuição da espécie, onde as populações são consideravelmente menores, o que indica que o polimorfismo floral é altamente resiliente a factores capazes de provocar desvios da situação de equilíbrio na Península Ibérica.

Por fim, testei a hipótese de que a variação no sistema de incompatibilidade de *Oxalis pes-caprae* (Oxalidaceae), em populações da área invadida na bacia do Mediterrâneo, possa ter resultado da limitação de parceiros sexuais após dispersão a longa distância a partir da área nativa, África do Sul. Para isto, realizei polinizações controladas e medi a produção de sementes em jardim experimental com plantas das áreas nativa e invadida, as quais eram representativas das áreas de distribuição da espécie na África do Sul e na zona sudoeste da bacia do Mediterrâneo, respectivamente. Os meus resultados mostraram que existe um enfraquecimento do sistema de incompatibilidade após auto-polinizações e polinizações cruzadas entre indivíduos da mesma forma floral em plantas da bacia do Mediterrâneo, o que pode criar novas oportunidades para reprodução sexual nesta área invadida.

Em conclusão, os resultados da minha tese de Doutoramento demostraram que a hercogamia recíproca e os caracteres ancilares promovem a polinização cruzada entre formas florais e, consequentemente, contribuem para reduzir o desperdício de pólen em estigmas incompatíveis em populações naturais. Assim sendo, a heterostilia funciona em grande parte para promover o êxito reprodutivo das plantas através da função masculina das flores. Para além do mais, os meus resultados apresentaram dados inovadores quanto à evolução deste polimorfismo floral tão complexo, assim como contribuíram para clarificar alguns dos mecanismos envolvidos na manutenção do mesmo em populações naturais.

PALAVRAS-CHAVE: caracteres ancilares; geitonogamia; hercogamia recíproca; heterostilia; polinização legítima.

Chapter 1 – General introduction



Narcissus draws by Júlio Henriques (Henriques, 1887). **1A-H.** *Narcissus calathinus*. **2A-I.** *N. triandrus*, **A.** S-morph, **B.** M-morph. **3A.** *N. triandrus concolor*.

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General features concerning heterostyly

Hermaphroditism is the most common sexual system among angiosperm species, with plants displaying both female and male sex organs in the same flower. Despite its advantages (Charnov *et al.*, 1976), hermaphroditism can cause sexual interference between female and male functions, the primary cost of which is self-fertilization and inbreeding depression, that is the reduced fitness of the selfed compared to the outcrossed offspring (Darwin, 1876; Charlesworth and Charlesworth, 1987). To limit the negative consequences from having both stigmas and stamens in close proximity, hermaphrodites have developed strategies for separating both functions at temporal (dichogamy) or spatial (herkogamy) scales (Lloyd and Webb, 1986; Barrett *et al.*, 2000). A remarkable example of herkogamy is heterostyly, a convergent floral polymorphism that has evolved numerous times in at least 28 angiosperm families (Ganders, 1979; Barrett and Shore, 2008). Heterostylous species have a reciprocal arrangement of anthers and stigmas, a condition referred to as reciprocal herkogamy (Webb and Lloyd, 1986). Heterostylous populations are commonly comprised of two (distyly; long- and short-styled morphs, that is L- and S-morphs, respectively; Fig. 1.1A) or three (tristyly; long-, mid- and short-styled morphs, that is L-, M- and S-morphs, respectively; Fig. 1.1B) style morphs, and the polymorphisms in stigma and anther heights are usually accompanied by a physiologically governed heteromorphic incompatibility system under sporophytic control and by ancillary polymorphisms of stigmatic papillae and pollen.

Since Darwin's pioneering work on heterostyly (Darwin, 1877), the floral polymorphisms have attracted much attention from evolutionary biologists interested in the selective forces underlying their origin, maintenance and breakdown. Heterostyly is a simply inherited genetic polymorphism controlled by a single genetic region, the *S*-locus, in distylous species, and by two diallelic loci in tristylous species, with the *S*-locus being epistatic to the *M*-locus, (Lewis and Jones, 1992). Apart from a few exceptions, the S-morph is dominant to the L-morph in distylous species; in tristylous species, the S-morph is generally dominant over the M- and the L-morphs, the last being homozygous recessive (Lewis and Jones, 1992). In natural populations, heterostyly is maintained by negative frequency-dependent selection resulting from disassortative mating between the style morphs (Barrett, 1993; Barrett and Shore, 2008). However, the breakdown of heterostyly has been reported in both distylous and tristylous taxa, and most commonly involves the loss of the incompatibility system and

a reduction in herkogamy so that anthers and stigmas are often in close contact. These morphological changes lead to the evolution of homostylous and semi-homostylous morphs from distylous and tristylous species, respectively, and these are usually autogamous (Ganders, 1979; Barrett and Shore, 2008). Homostylous forms of distylous species have the single anther level at the same height as the stigma, whereas semi-homostylous flowers have only one of the two anther levels in a flower at the same height as the stigma (Ganders, 1979; Barrett, 1989, 1993; Weller, 1992; Barrett *et al.*, 2009). Other evolutionary transitions in floral biology and mating patterns may occur in heterostylous taxa and these can involve a shift from distyly to dioecy [*e.g.*, *Mussaendra*, Rubiaceae (Baker, 1958; see Li *et al.*, 2010); *Nymphoides*, Menyanthaceae (Ornduff, 1966); *Cordia*, Boraginaceae (Opler *et al.*, 1975)] and from tristyly to distyly [*Oxalis*, Oxalidaceae (Weller *et al.*, 2007); *Pemphis*, Lythraceae (Lewis and Rao, 1971)].

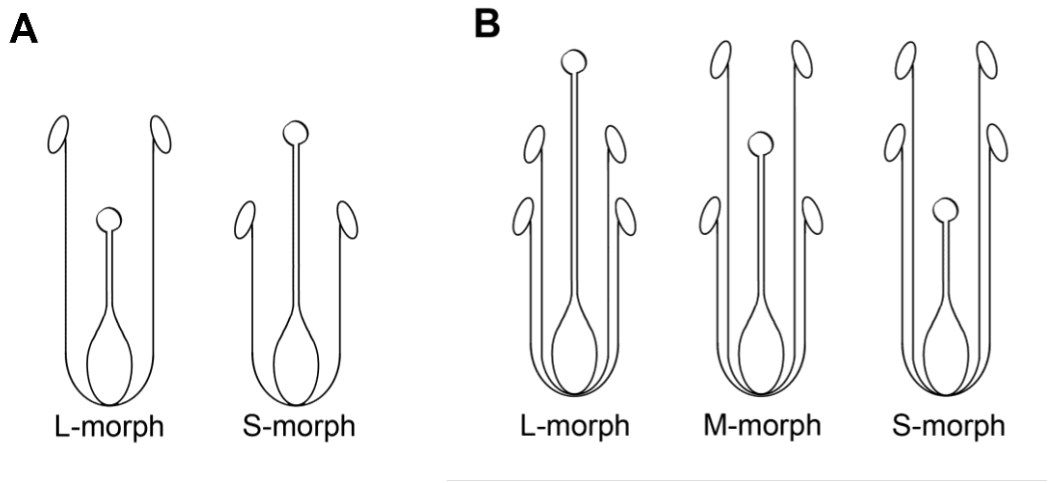


Figure 1.1. Schematic representation of heterostylous flowers. **A.** Distyly. **B.** Tristyly. L-, M- and S-morph represent the long-, mid- and short-styled morphs, respectively.

The occurrence of heterostyly in the Iberian Peninsula

The Iberian Peninsula is located in the southwestern Mediterranean region of continental Europe, and is characterized by a mosaic of heterogeneous landscapes that includes sea cliffs, saltmarshes, wetlands, mountains, deciduous forests, agricultural landscapes, shrublands commonly known as maquis, among others. Consequently, the Iberian Peninsula harbors a great diversity of animal and plant species, and a high level of endemism (Parga *et al.*, 1996; Médail and Quézel, 1999; Lozano *et al.*, 2000; García-Barros *et al.*, 2002) and thus, the entire Mediterranean basin is classified as a hotspot of

biodiversity for conservation purposes (Myers *et al.*, 2000). Several biogeographic, climatic, edaphic and geomorphologic factors account for the high species richness in the Iberian Peninsula. I will briefly focus on three of them. First, this area is bisected by two biogeographic zones, the Eurosiberian to the north and the Mediterranean from the centre to the south of the range (Rivas-Martinéz *et al*., 2004). Second, its orographic complexity generates different microclimates along altitudinal gradients, and the mountain systems act as a barrier to gene flow among isolated populations thus, increasing opportunities for speciation (Hewitt, 1996; Gómez and Lunt, 2006). Lastly, phylogeographic studies have provided evidence that the Iberian Peninsula was a glacial refugia during the Pleistocene, allowing the survival of numerous species during the glaciations (Thompson, 2005; Gómez and Lunt, 2006; Feliner, 2014). Altogether, these factors create the opportunity for lineage divergence and speciation, ultimately increasing genetic diversity and species richness (Hewitt, 1996; Turelli *et al.*, 2001; Thompson, 2005; Gómez and Lunt, 2006; Feliner, 2014).

Considering all this, the Iberian Peninsula offers the habitat complexity and diversity suitable to maintain the pollinator fauna of many flowering plants. Heterostylous species are mainly bee-pollinated (Barrett and Shore, 2008), and 1009 species of bees (Superfamily Apoidea) have been described in the Iberia Peninsula (Ascher and Pickering, 2016), which is a considerably higher species diversity than the observed in many other regions of the globe, given its land area (Table 1.1). Based on published work investigating the heterostylous syndrome and its taxonomic distribution, it can be estimated that 1,608 species possess these polymorphisms (reviewed by Naiki, 2012; Salter, 1944; Breteler, 1989; Marco and Arroyo, 1998; Pailler *et al*., 1998; Prather *et al.*, 2000; McDade *et al.*, 2008; Bräuchler *et al*., 2010; McDill and Simpson, 2011; Tokuoka, 2012; de Vos *et al*., 2014; Appendix 1.1). Assuming an approximate number of 352,000 angiosperm species (The Plant List, 2013), I estimate that ~0.46% of angiosperms are heterostylous. In the Iberian Peninsula, 41 out of the total number of 1,608 heterostylous species occur (Table 1.1; Figs. 1.2, 1.3; Appendix 1.1), which represent 2.5% of the total number of heterostylous species described so far. When comparing this result with data from other geographic locations with a Mediterranean climate (Table 1.1), three main conclusions emerge: (1) heterostylous species in Iberia belong to 11 families (Figs. 1.2, 1.3; Appendix 1.1), thus representing a large taxonomic and phylogenetic diversity; (2) only South Africa has a larger number of heterostylous species than Iberian Peninsula, but this is mainly due to hyperdiverse *Oxalis* in the Cape

region; (3) there is a weak positive correlation between the number of Apoidea species and number of heterostylous species in regions with Mediterranean climate (see Table 1.1; Pearson's product-moment correlation: r = 0.34). The diversity of heterostylous species in Iberian Peninsula indicates that this region provides many opportunities for studies addressing the ecology, evolution and genetics of heterostyly. Indeed, more than 30 studies on various aspects of heterostylous syndrome have been conducted using species that naturally occur in Portugal and/or Spain (Appendix 1.2). Below, I briefly review the main contributions of these studies to our current knowledge of the floral polymorphisms, that is distyly and tristyly.

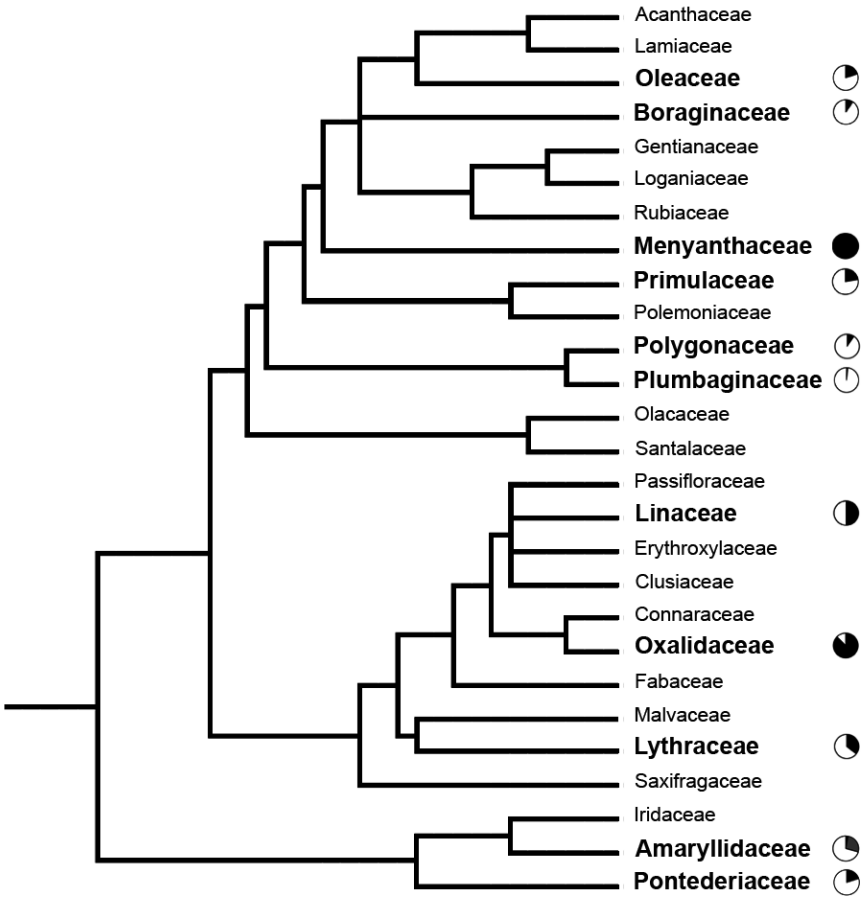


Figure 1.2. Phylogenetic tree of the families with heterostyly obtained from Phylomatic v.3 available online (Webb and Donoghue, 2004; http://phylodiversity.net/phylomatic/). In bold are represented the families that occur in the Iberian Peninsula. Pie diagrams show the proportion of species with floral polymorphisms: heterostylous species in black, stigma-height dimorphic species in dark grey and other floral polymorphisms in white.

Table 1.1. Estimated number of heterostylous species reported from different geographical locations. Data on land area (U.S. Census Bureau, 2010; The World Bank Group, 2016) and number of Apoidea species (Ascher and Pickering, 2016; UC Berkeley Urban Bee Lab, 2016) are also provided.

Geographic region	Area (km^2)	No. Apoidea species	No. Heterostylous species	References
Global	-	-	1,608	Reviewed in Naiki, 2012; Salter, 1944; Breteler, 1989; Marco and Arroyo, 1998; Pailler *et al.*, 1998; Prather *et al.*, 2000; McDade *et al.*, 2008; Bräuchler *et al.*, 2010; McDill and Simpson, 2011; Tokuoca, 2012; de Vos *et al.*, 2014
Chile	743,532.0	417	8	Reviewed in Naiki, 2012
Australia	7,682,300.0	1,626	10	Reviewed in Naiki, 2012; Tippery *et al.*, 2008
California	419,730.1	1,600	39	Reviewed in Naiki, 2012
Iberian Peninsula	591,280.0	1,009	41	Results herein
South Africa	1,213,090.0	1,145	243	Salter, 1944; Ornduff, 1974; Sánchez *et al.*, 2010; Turketti, 2010

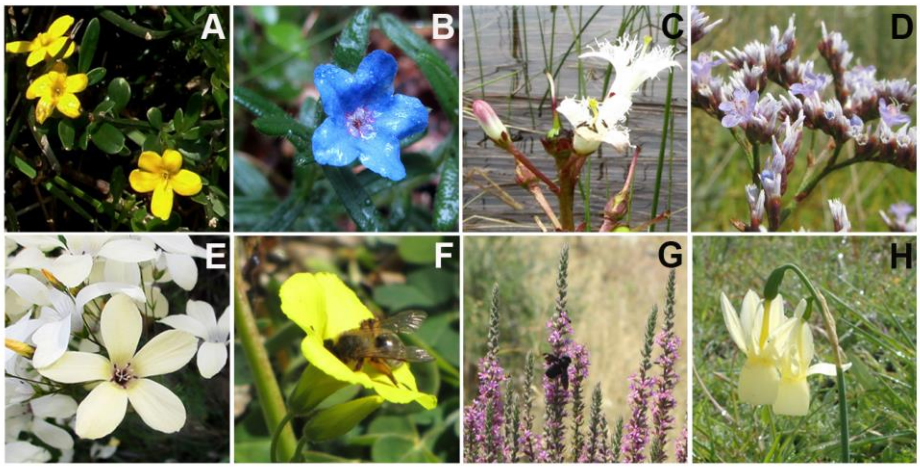


Figure 1.3. Floral diversity of heterostylous species in the Iberian Peninsula. **A.** *Jasminum fruticans* (Oleaceae). **B.** *Lithodora* sp. (Boraginaceae). **C.** *Menyanthes trifoliata* (Menyanthaceae). **D.** *Limonium vulgare* (Plumbaginaceae). **E.** *Linum suffruticosum* (Linaceae; image courtesy of Ana Afonso, CFE, University of Coimbra). **F.** *Oxalis pes-caprae* (Oxalidaceae) being visited by *Apis* sp. **G.** *Lythrum salicaria* (Lythraceae) being visited by *Xylocopa violacea*. **H.** *Narcissus triandrus* (Amaryllidaceae).

Floral biology: from the early discovery of heterostyly in *Narcissus* to the three-dimensional reciprocity of *Linum*

The first report of heterostyly in the Iberian Peninsula was made by the founder of the Herbarium of the University of Coimbra, the Portuguese botanist Júlio Henriques (1887, 1888), ten years after the publication of *The Different Forms of Flowers on Plants of the Same Specie*s by Charles Darwin (1877). Henriques (1887, 1888) described tristyly in *Narcissus triandrus* (Fig. 1.3H), and later studies by Professor Abílio Fernandes (University of Coimbra) provided a more detailed picture of stylar polymorphisms in the genus (Appendix 1.2). By performing intermorph, intramorph and self-pollinations, Fernandes (1964) concluded that *N. triandrus* was self-incompatible but not morph-incompatible, contrarily to most heterostylous species, since a large number of seeds were produced after intramorph crosses. However, Fernandes's claim of tristyly in *N. triandrus* was subsequently disputed by Bateman (1952) in an influential paper in *Nature*, and the occurrence of the floral polymorphism in this species became controversial. In the 1990s, Barrett and colleagues revisited this dispute and conducted extensive field sampling of populations in the Iberian Peninsula, as well as floral measurements and controlled pollinations (Barrett *et al*., 1997, 2004; Sage *et al*., 1999). They confirmed that *N. triandrus* was indeed tristylous, but the species possessed an unusual form of the polymorphism involving imperfect sex-organ reciprocity, biased morph ratios, and a novel self-recognition system. These studies confirmed that Júlio Henriques and Abílio Fernandes were correct.

The defining feature of heterostyly is reciprocal herkogamy, which involves morphological variation in the positioning of female and male sex organs among style morphs. Quantifying reciprocal herkogamy in natural populations is important for several reasons. For example, to confirm the heterostylous status of a species, or to investigate correlations between reciprocal herkogamy and male and female reproductive success (Sánchez *et al*., 2008). Hence, several indices have been proposed (Richards and Koptur, 1993; Eckert and Barrett, 1994; Sosenski *et al*., 2010); however, these measures failed to integrate both reciprocity and precision (*i.e*., data dispersion), which motivated Sánchez *et al*. (2008) to propose a new index to quantify reciprocity in distylous populations, and this was later extended to tristylous populations (Sánchez *et al*., 2013). This index has several advantages: it includes all stigma-anther height variation among plants in a sample rather than just the population average, and it integrates both reciprocity and precision into a single index. This index was used to

look at sex-organ variation in species of *Lithodora* (Fig. 1.3B) and of the related *Glandora* (Boraginaceae), Iberian taxa comprised of both distylous and stigma-height dimorphic species (Ferrero *et al*., 2011a, 2017). Additionally, Ferrero *et al*. (2011b) reported a correlation between pollinator efficiency and reciprocity in populations of *Lithodora* and *Glandora*, which might have implications for the stability of morph ratios and evolution of the floral polymorphisms.

The wild flax, *Linum suffruticosum* (Fig. 1.3E), displays a remarkable form of distyly not reported for other heterostylous groups. Armbruster *et al*. (2006) described three-dimensional reciprocity of sex organs in *L. suffruticosum*, which is achieved by twisting and bending of the styles and stamens during floral development. This causes L- and S-morph pollen to be placed on the ventral and dorsal regions of the pollinator's body, respectively, while stigmas of the L- and S-morph predominantly contact the dorsal and ventral parts of pollinator's body, thus assuring effective pollination between style morphs. Other studies that have focused on the floral and pollination biology of heterostylous species in the Iberian Peninsula include work on pollen dimorphism in *Linum* (Rogers, 1979, 2009), characterization of the distylous syndrome in *Jasminum* (Guitián *et al*., 1998; Thompson and Dommée, 2000; Olesen *et al*., 2003), experimental investigations on the expression of the incompatibility system in invasive tristylous *Oxalis pes-caprae* (Fig. 1.3F; Castro *et al.*, 2013; Costa *et al.*, 2014), studies on the response of pollinators to variation in floral design of *Jasminum fruticans* (Fig. 1.3A; Thompson, 2001), and studies of pollination networks (Ferrero *et al*., 2013) and intramorph pollen transfer and capture in natural populations of *O. pes-caprae* (Costa *et al.*, 2016).

Factors influencing style-morph ratios in populations

Heterostyly is maintained in populations by negative frequency-dependent selection, and populations at equilibrium are usually isoplethic (*i.e*., equal morph ratios) if there are no fitness differences among style morphs (Fisher, 1944; Heuch, 1979a). However, several stochastic and deterministic factors can cause deviations from isoplethy. Assessing morph ratios in populations of heterostylous species is straightforward, because style morphs are easily identified in the field. The first report of morph ratios in the Iberian Peninsula was made by Fernandes (1935a, 1965), who described L-morph biased populations of *Narcissus triandrus*. Later studies by Barrett and colleagues (*e.g*., Barrett *et al.*, 2004; Hodgins and Barrett, 2006a, 2008a; reviewed in Barrett and

Hodgins, 2006), confirmed the early observations of Fernandes and provided evidence that L-biased morph ratios were caused by asymmetrical mating among style morphs, especially due to assortative mating in the L-morph. Geographical patterns of style morph ratios in the Iberian Peninsula, including dimorphic populations lacking the M-morph in northern Iberia, were found to be associated with latitudinal variation in climatic conditions causing variation in plant and flower size (Hodgins and Barrett, 2008a). This variation was confirmed to be associated with a switch in the types of bees visiting flowers implicating a causal link among pollination, mating and morph ratios in the populations (Barrett *et al*., 2004; Hodgins and Barrett, 2008b).

Founder events associated with intercontinental migration and historical contingency can also be responsible for biased morph ratios, notably in species with clonal propagation and episodic sexual recruitment (*e.g.*, Barrett and Forno, 1982; Morgan and Barrett, 1988). Castro *et al*. (2007, 2013) documented style morph ratios in a large sample of invasive populations of tristylous *Oxalis pes-caprae* from the western Mediterranean basin. Populations were found to be predominantly monomorphic for the 5*x* S-morph, a pattern that has also been described for other Mediterranean regions (*e.g.*, Australia, California, Chile) where this species has been introduced and has become a noxious weed (Michael, 1964; Baker, 1965; Ornduff, 1987). Using common garden experiments, Castro *et al.* (2016) compared sexual and asexual reproductive traits in native and invasive plants from South Africa and the Mediterranean basin, respectively, and concluded that extensive clonal reproduction was the main factor responsible for the highly successful invasion.

Genetics: inheritance and mating patterns

The first steps in our understanding on the inheritance of heterostyly go back to pollination experiments carried out by Darwin (1877) with *Primula*, *Lythrum* and *Oxalis* species. Indeed, Darwin's work on primroses was close to repeating Mendel's classic work discovering the Laws of Inheritance (Charlesworth and Charlesworth, 2009) and anticipated the first demonstration of the inheritance of heterostyly by Bateson and Gregory (1905) in *Primula*. Early studies of the inheritance of tristyly were conducted by Fernandes (1935a, 1964, 1965). He analyzed the segregation of style morphs in the offspring obtained after intramorph and intermorph pollinations in *Narcissus triandrus*. As he himself recognized, species of *Narcissus* are not suitable for these kind of studies, since they take three to four years to flower after germination and

some plants fail to reach the reproductive stage of the life cycle (Fernandes, 1964). Consequently, the number of plants screened in the offspring was small and therefore, any conclusion from his studies should be treated with caution. Despite these constraints, Fernandes (1935a, 1964, 1965) proposed that the L-morph was homozygous recessive (*bbmm*), the M-morph was heterozygous recessive (*bbM*-) and the S-morph was heterozygous dominant (*Bb*--). However, his model of inheritance required several assumptions about lethality and thus, the work was later criticized since it was only able to explain a single generation of morph ratios (Bateman, 1968). Nevertheless, the model that Fernandes reported bears some resemblance to the typical inheritance pattern of tristyly found in the three most well-known tristylous families (reviewed in Lewis and Jones, 1992).

The increased use of neutral genetic markers has undoubtedly contributed to our knowledge of plant reproductive ecology. Over the past three decades, the application of markers to screen polymorphisms in open-pollinated families has enabled measurements of outcrossing and selfing rates, levels of inbreeding depression, gene flow, paternity and mate diversity, among others (Barrett and Harder, 1996). Genetic diversity within and among plant populations and differentiation among populations are largely determined by the relative amount of outcrossing versus selfing. Hodgins and Barrett (2006b, 2008c) used allozyme markers to estimate outcrossing rates in populations of *N. triandrus*, and concluded that strong inbreeding depression prevented inbred progeny from reaching the adult stage (Hodgins and Barrett, 2006b). Consequently, reproductive plants in populations mainly resulted from outcrossing. In addition, Hodgins and Barrett (2008b) used microsatellite markers to measure levels of assortative and disassortative mating in *N. triandrus*, and found asymmetrical mating in two natural populations. Microsatellite markers were also employed by Ferrero *et al*. (2015) in studies of the invasion genetics of *Oxalis pes-caprae*. They compared patterns of genetic diversity between native (South Africa) and invasive populations (Iberian Peninsula and Morocco) of *O. pes-caprae*, and concluded that populations in the introduced range were the result of multiple introductions from the native range. Moreover, they also showed that monomorphic populations of the 5*x* S-morph were not comprised of a single clone as previously thought, but instead contained a low level of genetic diversity implying that cryptic sex or other mechanisms, such as somatic mutations, are involved in generating diversity.

Evolution of heterostyly

The origin and evolution of heterostyly have intrigued evolutionary biologists for over a century, and two main models have been proposed to explain the evolutionary buildup of the heterostylous syndrome, namely the model by Charlesworth and Charlesworth (1979), and the model by Lloyd and Webb (1992a, b). These models focus on three main questions: (1) What is the selective force responsible for the buildup of heterostyly? (2) What are the ancestral and intermediate conditions involved in the transition? (3) What is the order (polarity) in which morphological and physiological traits are assembled? Charlesworth and Charlesworth (1979) proposed a "selfing avoidance" model for the evolution of distyly. They postulated that the ancestral condition to distylous species is a homostylous variant with a long style and long-level anthers. In this case, the selective force is the avoidance of inbreeding depression with the incompatibility system established before reciprocal herkogamy (Fig. 1.4A). In contrast, the "pollen transfer" model proposed by Lloyd and Webb (1992a, b) assumes that the promotion of cross-pollination is the principal selective force for the evolution of distyly. In contrast to the Charlesworth and Charlesworth (1979) model, they proposed that the ancestral condition was approach herkogamy, and reciprocal herkogamy established before the incompatibility system, which may or may not evolve depending on the importance of inbreeding avoidance in the population (Fig. 1.4B). In both models, stigma-height dimorphism is an intermediate and transitory stage. To distinguish between these two models for the evolution of heterostyly, it is necessary to determine the ancestral character state of heterostylous lineages and also the selective forces involved. To date, this has not been comprehensively undertaken. Nevertheless, some progress has been made, especially with regard to determining plausible ancestral and intermediate states using phylogeny reconstruction and character mapping of targeted groups.

Narcissus offers an opportunity to investigate the models for the evolution of heterostyly, because the genus includes monomorphic species with approach herkogamy, stigma-height dimorphism, distyly and tristyly (Graham and Barrett, 2004). Early studies of *Narcissus*, summarized in Fernandes (1975), led him to conclude that heterostyly was a derived condition in the genus. This was later confirmed by phylogenetic analysis and character mapping by Pérez *et al*. (2003) and Graham and Barrett (2004). The study by Graham and Barrett (2004) provided important insights into the evolutionary history of stylar polymorphisms in *Narcissus*. They showed that

the ancestral state was approach herkogamy with stigma-height dimorphism evolving at an intermediate stage, and that the two instances of heterostyly in the genus (distyly in *N. albomarginatus* and tristyly in *N. triandrus*) had separate origins (see Barrett and Harder, 2005: Fig. 2). Their study provided some support for the pollen transfer model of Lloyd and Webb (1992a, b). The stages leading to the evolutionary transition between stigma-height dimorphism and distyly in *Narcissus* appear to be associated with changes in pollinator fauna (Pérez-Barrales *et al.*, 2006) and with selection on floral design (Barrett and Harder, 2005; Santos-Gally *et al.*, 2013). Despite the separate origins of heterostyly in *Narcissus*, the two heterostylous species are distinctive in being the only members of the genus that possess a long floral tube and an extended tubular corolla. This pattern of convergence in floral design seems likely to be associated with depth-probed pollination and the requirement of a long lateral area for pollen segregation on the bodies of the long-tongued bees that visit these two species.

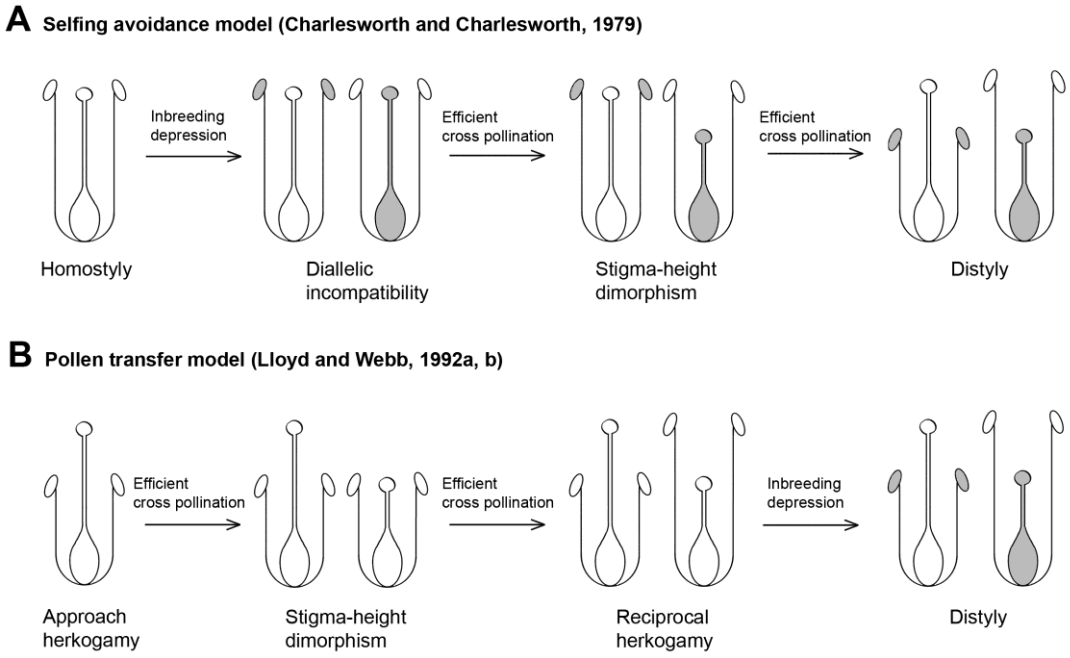


Figure 1.4. Models for the evolution of distyly. **A.** Selfing avoidance model proposed by Charlesworth and Charlesworth (1979). **B.** Pollen transfer model proposed by Lloyd and Webb (1992a, b). The selective forces are indicated above the arrows. Flowers with uniform anther and stigma heights are self-compatible; style morphs with shaded pollen or shaded stigmas are self-incompatible.

Ancestral character reconstructions in *Lithodora* and *Glandora* by Ferrero *et al.* (2009a, 2012) also provided evidence that stigma-height dimorphism was an

intermediate stage in the evolution of distyly and that the floral polymorphism and incompatibility system in species from these two genera have independent origins. Finally, Armbruster *et al.* (2006) used phylogenetic approaches to investigate the origins of distyly in *Linum*, and concluded that (1) three-dimensional distyly evolved in a lineage with "typical" distyly, (2) distyly evolved several times in the genus, and (3) at least one reversion from distyly to stylar monomorphism occurred. So far, phylogenetic reconstructions of the evolutionary history of heterostylous plants in the Iberian Peninsula have been restricted to a handful of groups and future work on other taxa (*e.g*., Plumbaginaceae) should provide novel insights into the evolutionary history of heterostyly.

Objectives and structure of the thesis

The main objective of my PhD thesis was to provide novel insights into the function and adaptive significance of the morphological traits that characterize the heterostylous syndrome, and to investigate the evolutionary history and demographic factors influencing the maintenance of the polymorphism. Here I combine literature surveys, sampling of natural populations, pollination experiments under common garden conditions and ancestral character reconstruction to address diverse questions on heterostyly. Apart from the *General introduction* and *General conclusions*, this PhD thesis is organized into three main sections, each with two chapters as follows:

Part I – The function of reciprocal herkogamy

Chapter 2 – Pollination biology of heterostyly – a comparative analysis

Here, I reviewed the literature on studies of pollen capture in heterostylous species and summarized the patterns that emerged. Data analysis was conducted taking into account the phylogenetic relations among species. My analysis demonstrated that these studies offer only limited support for Darwin's cross-promotion hypothesis, mainly because self-pollen was not excluded by emasculation, thus obscuring the real extent to which reciprocal herkogamy promotes disassortative pollination.

Chapter 3 – Experimental insights on Darwin's cross-promotion hypothesis in tristylous purple loosestrife (*Lythrum salicaria*)

In this chapter, I experimentally evaluated Darwin's cross-promotion hypothesis in tristylous *Lythrum salicaria,* a species with a partially tubular corolla. I examined the

extent to which the location of sex organs within a flower influence compatible and incompatible pollination. For this, I used experimental trimorphic and monomorphic arrays and emasculated flowers to quantify pollen transfer and capture among all sex-organ heights. I exploited the occurrence of pollen size heteromorphism in *L. salicaria* to determine the source of pollen that was transferred to stigmas by pollinators. My results provided experimental evidence in support of the Darwinian hypothesis and also demonstrate the important role of floral designs in governing compatible and incompatible pollen capture.

Part II – The function of ancillary characters and evolutionary history of heterostyly

Chapter 4 – Experimental insights on the function of ancillary pollen and stigma polymorphisms in plants with heteromorphic incompatibility

Here, I tested the topographical complementarity hypothesis for the function of ancillary characters by investigating patterns of pollen transfer and capture in natural populations of dimorphic *Armeria maritima* and *A. pubigera* and distylous *Limonium vulgare* (Plumbaginaceae), and by studying pollen adherence and germination patterns in *A. maritima* following controlled hand-pollinations. My observations provided partial evidence in support of the topographical complementary hypothesis for the adaptive significance of pollen and stigma polymorphisms.

Chapter 5 – Evolutionary history of the heterostylous syndrome in Plumbaginaceae

In this chapter, I investigated the evolutionary buildup of the heterostylous syndrome in the Plumbaginaceae by ancestral character state reconstruction. I used molecular data to construct a phylogenetic tree for the family and an extensive survey of herbarium specimens to investigate heteromorphic morphological traits. Components of the heterostylous syndrome were mapped onto phylogenetic trees to investigate their order of establishment. My comparative study provided evidence suggesting that the most recent common ancestor of the Plumbaginaceae was monomorphic in relation to sex-organ position and self-incompatible.

Part III – Maintenance and breakdown of the floral polymorphism

Chapter 6 – Variation in style morph frequencies in tristylous *Lythrum salicaria* in the Iberian Peninsula: the role of geographical and demographic factors

In this chapter, I investigated style morph frequencies in natural populations of *Lythrum salicaria* at the southwestern European range limit of the species, to explore the role of demographic and geographic factors in affecting morph ratios in its native range. I measured the morph composition, evenness, and size of 96 populations along a north to south latitudinal transect from Galicia to Andalucia, Iberian Peninsula, traversing a steep climatic gradient. I also examined reproductive traits in 19 populations. My study demonstrated that population size and evenness increased towards northern populations and that the floral trimorphism is highly resilient to factors causing deviations from isoplethy in Iberian Peninsula.

Chapter 7 – Variation in the incompatibility reactions in tristylous *Oxalis pes-caprae*: large-scale screening in South African native and Mediterranean basin invasive populations

In this chapter, I tested the hypothesis that there may be increased compatibility in invasive populations of *Oxalis pes-caprae* owing to mate limitation after long-distance dispersal from the native range. To do this, I investigated variation in the expression of trimorphic incompatibility in plants from 16 native populations covering the entire distributional range in South Africa, and 18 invasive populations from the western Mediterranean basin by performing controlled hand-pollinations in a common garden experiment. My controlled pollinations provided evidence of increased levels of self- and intramorph compatibility in introduced populations.

Appendix 1.1. Species growing in the Iberian Peninsula reported as distylous (D) or tristylous (T). "?" indicates that more information is needed. A non-exhaustive reference list is provided.

Family	Genus	Species	Distyly/Tristyly	Reference
Amaryllidaceae	*Narcissus*	*N. triandrus*	T	Fernandes, 1935a; Barrett *et al.*, 1997
Boraginaceae	*Amsinckia*	*A. calycina*	D ?	
		A. lycopsoides	D ?	
	Glandora	*G. diffusa*	D	Ferrero, 2009
		G. oleifolia	D	Ferrero, 2009
		G. nitida	D	Ferrero, 2009
	Pulmonaria	*P. affinis*	D	Richards and Mitchell, 1990
		P. longifolia	D	Fuentes and Blaise, 1988
Linaceae	*Linum*	*L. apressum*	D	Salinas, 2009
		L. austriacum	D	Salinas, 2009
		L. maritimum	D	Salinas, 2009
		L. narbonense	D	Salinas, 2009
		L. suffruticosum	D	Salinas, 2009
		L. tenue	D	Salinas, 2009
		L. viscosum	D	Salinas, 2009
Lythraceae	*Lythrum*	*L. acutangulum*	T	Velayos, 1997
		L. baeticum	T	Velayos, 1997
		L. flexuosum	T	Velayos, 1997
		L. junceum	T	Velayos, 1997
		L. salicaria	T	Darwin, 1877; Velayos, 1997
Menyanthaceae	*Menyanthes*	*M. trifoliata*	D	Lughada and Parnell, 1989
	Nymphoides	*N. peltata*	D	Ornduff, 1966
Oleaceae	*Jasminum*	*J. fruticans*	D	Guitián *et al*., 1998
		J. odoratissimum	D	Olesen *et al.*, 2003
Oxalidaceae	*Oxalis*	*O. articulata*	T	Personal observations
		O. bowiei	T	Salter, 1944
		O. debilis	T	Gardner *et al*., 2012
		O. latifolia	T	Gardner *et al*., 2012
		O. pes-caprae	T	Salter, 1944
		O. purpurea	T	Salter, 1944
Plumbaginaceae	*Limonium*	*L. vulgare*	D	Baker, 1953a, 1966
	Plumbago	*P. auriculata*	D	Ferrero *et al*., 2009b
		P. europea	D	Dulberger, 1975a
Polygonaceae	*Fagopyrum*	*F. esculentum*	D	Björkman, 1995
	Fallopia	*F. baldschuanica*	D ?	
		F. convolvulus	D ?	
		F. dumetorum	D ?	
Pontederiaceae	*Eichhornia*	*E. crassipes*	T	Barrett, 1977a

Cont.

Family	Genus	Species	Distyly/Tristyly	Reference
Primulaceae	*Androsace*	*A. vitaliana*	D	Dixon *et al*., 2009
	Primula	*P. acaulis*	D	Darwin, 1877
		P. elatior	D	Schou, 1983
		P. farinosa	D	Mast *et al*., 2006
		P. hirsuta	D	Mast *et al*., 2006
		P. integrifolia	D	Mast *et al*., 2001
		P. latifolia	D	Kress, 1997; not studied in detail
		P. pedemontana	D	Kress, 1997; not studied in detail
		P. veris	D	Mast *et al*., 2006

Appendix 1.2. Published research conducted on different aspects of heterostyly in the Iberian Peninsula.

Author(s), year	Topic	Study system	Significance
Henriques (1887, 1888)	Floral biology	*Narcissus*	Report the floral polymorphism
Fernandes (1935a, 1965)	Population structure and genetics	*N. triandrus*	L-biased morph ratios; novel proposal for the inheritance pattern of tristyly
Fernandes (1935b)	Cytogenetics	*N. triandrus*	No cytogenetic differences between style morphs
Fernandes (1964)	Floral biology and genetics	*N. triandrus*	Self-incompatibility system; inheritance pattern
Fernandes (1975)	Phylogenetic reconstruction	*Narcissus*	Heterostyly is a derived condition
Rogers (1979, 2009)	Palynology	*Linum*	Dimorphic pollen
Fuentes and Blaise (1988)	Morphological variation	*Pulmonaria longifolia*	Geographical patterns of morphological variation in vegetative and reproductive traits
Barrett *et al.* (1997)	Floral biology	*N. triandrus*	Tristylous species with a putatively multiallelic incompatibility system
Guitián *et al*. (1998)	Floral biology	*Jasminum fruticans*	Characterization of the distylous syndrome
Sage *et al.* (1999)	Floral biology	*N. triandrus*	Novel prezygotic self-recognition mechanism
Thompson and Dommé (2000)	Floral biology	*J. fruticans*	Continuous variation in herkogamy for the S-morph
Thompson (2001)	Pollination biology	*J. fruticans*	Pollinator-specific responses to variability in floral design and display
Pérez *et al.* (2003)	Ancestral character reconstruction	*Narcissus*	Convergent evolution of heterostyly
Olesen *et al*. (2003)	Floral biology	*J. odorantissimum*	Characterization of the distylous syndrome
Barrett *et al.* (2004)	Floral biology and population structure	*N. triandrus*	Correlation between variation in floral morphology and morph ratios over the species' distribution range
Graham and Barrett (2004)	Evolution of heterostyly	*Narcissus*	No evidence that stigma-height dimorphism preceded tristyly
Pérez-Barrales *et al.* (2006)	Ancestral character reconstruction	*Narcissus*	Evolutionary transitions between floral polymorphisms tightly associated to changes in pollinators
Armbruster *et al.* (2006)	Floral biology and ancestral character reconstruction	*Linum suffruticosum*	Novel floral polymorphism; several independent origins of heterostyly in the genus *Linum*
Hodgins and Barrett (2006a)	Population structure and female reproductive success	*N. triandrus*	Biased morph ratios possibly caused by asymmetrical mating and differences in female reproductive success among morphs
Hodgins and Barrett (2006b)	Mating patterns	*N. triandrus*	Largely outcrossing populations; strong inbreeding depression in progeny that do not reach the reproductive stage

Cont.

Author(s), year	Topic	Study system	Significance
Hodgins *et al*. (2007)	Genetics	*N. triandrus*	Development of eight microsatellite loci for mating pattern analyses
Hodgins and Barrett (2007)	Phylogeography and population genetics	*N. triandrus*	Genetic diversity and population structure likely reflect differences in demography and gene flow among populations
Castro *et al.* (2007, 2013)	Invasion and population structure	*Oxalis pes-caprae*	Dominant monomorphic populations of the S-morph; first report of fruit and seed production from natural invasive populations
Sanchéz *et al*. (2008, 2013)	Floral biology	Theoretical	Novel index to quantify reciprocal herkogamy in distylous and tristylous populations
Hodgins and Barrett (2008a)	Population structure	*N. triandrus*	Geographical patterns of style-morph ratios are likely influenced by climatic gradients
Hodgins and Barrett (2008b)	Mating patterns	*N. triandrus*	L-morph biased populations result from large intramorph mating in the L-morph
Hodgins and Barrett (2008c)	Selection on floral traits	*N. triandrus*	Stronger selection on floral design through male than female function
Ferrero *et al*. (2009a)	Evolution of distyly	*Lithodora*, *Glandora*	Partial support for Lloyd and Webb (1992a, b) model of evolution
Ferrero *et al*. (2011a)	Floral biology	*Lithodora*, *Glandora*	High phenotypic integration in floral traits
Ferrero *et al*. (2011b)	Pollinator efficiency	*Lithodora*, *Glandora*	Pollinator efficiency increases with increased reciprocal herkogamy
Ferrero *et al*. (2012)	Evolution of distyly	*Lithodora*, *Glandora*	Independent evolution of the floral polymorphism and the incompatibility system
Santos-Gally *et al*. (2013)	Evolution of heterostyly	*Narcissus*	Floral architecture played an important role in the evolution of heterostyly
Ferrero *et al*. (2013)	Invasion and pollination network	*O. pes-caprae*	Resilient pollination network; facilitative effects of the invasive on the reproductive success of native plants
Costa *et al.* (2014)	Maintenance of tristyly	*O. pes-caprae*	Weakening in the incompatibility system
Ferrero *et al*. (2015)	Invasion genetics	*O. pes-caprae*	Multiple introductions of the style morphs
Costa *et al.* (2016)	Pollination biology	*O. pes-caprae*	Intramorph pollen transfer and capture
Castro *et al.* (2016)	Invasion ecology	*O. pes-caprae*	Predominance of clonal reproduction in invasive compared with native populations
Ferrero *et al*. (2017)	Floral biology and population structure	*Lithodora*, *Glandora*	Variation in herkogamy associated with population style morph ratios

Part I – The function of reciprocal herkogamy

"The benefit which heterostyled dimorphic plants derive from the existence of the two forms is sufficiently obvious, namely, the intercrossing of distinct plants being thus ensured."

Darwin (1877 p. 30)

Chapter 2 – Pollination biology of heterostyly – a comparative analysis

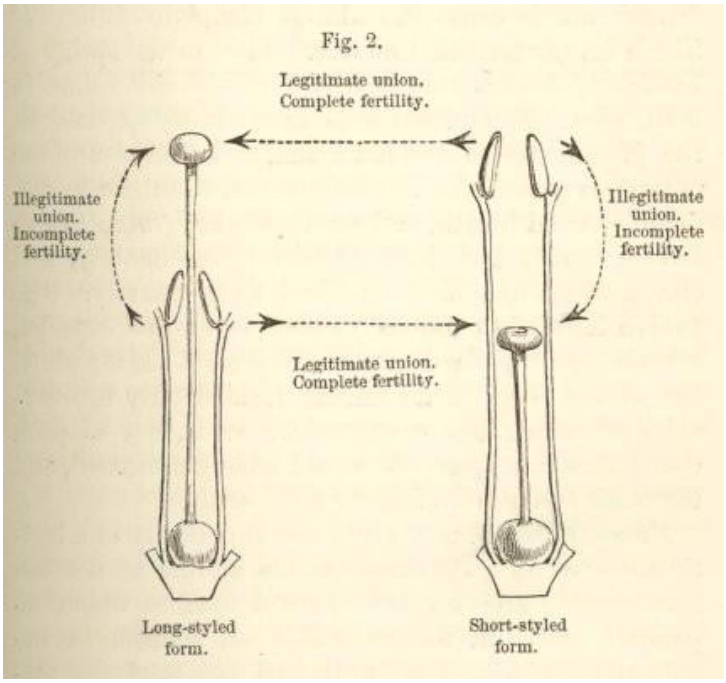


Illustration of legitimate and illegitimate "unions" in *Primula veris* (Darwin, 1877 p. 27).

ABSTRACT

Charles Darwin provided the first hypothesis on the adaptive significance of heterostyly. He proposed that this floral polymorphism functions to promote cross-pollination between style morphs. This hypothesis has been widely tested in populations of distylous species by the analysis of pollen captured by stigmas. Here, I review the literature on pollen capture studies in heterostylous species, accounting for the phylogenetic relatedness of the taxa analyzed, and provide a synthesis of the patterns that emerge. Since studies investigating pollen capture in tristylous populations were limited to a few species, they were excluded from the comparative analysis, which involved 26 species from 11 families. The data for intact flowers of distylous species indicate that: (1) incompatible pollen produced by both long- and short-level anthers has a higher probability than compatible pollen in being transferred to stigmas; (2) stigmas of the L-morph capture a larger number of pollen grains than stigmas of the S-morph, with the great majority of them being incompatible; and (3) subtle differences in pollinator's efficiency among the major floral visitors of distylous species (*i.e.*, bees, flies, butterflies and hummingbirds) were detected. Collectively, these results provide evidence for asymmetrical pollen transfer and capture in natural populations of distylous species, and more importantly, offer limited evidence supporting Darwin's cross-promotion hypothesis. However, this weak support reflects the failure by investigators to examine pollen loads of emasculated flowers, which enable comparisons of the critical components of the outcross pollen load, *i.e.*, intermorph versus intramorph, to be made. In the few cases where this was done, strong support for the Darwinian hypothesis was obtained.

KEY WORDS: convergent evolution; disassortative pollination; distyly; pollen capture; pollen heteromorphism; pollen transfer; reciprocal herkogamy.

INTRODUCTION

Heterostyly was first described in *Primula* during the 16th century by Clusius, but it was not until the 19th century that the first contributions clarifying the functional significance of heterostyly emerged with the works of Friedrich Hildebrand and Charles Darwin (reviewed in Ornduff, 1992). Hildebrand used the term "heterostyly" in a strict morphological sense, while Darwin also considered the incompatibility system (reviewed in Ganders, 1979; Ornduff, 1992). Darwin investigated the distylous syndrome in several species of *Primula* by performing hand-pollinations that he termed "legitimate and illegitimate unions" based on the identity of the style morphs involved in the experiment and the successful siring of seeds. He noticed that legitimate or compatible crosses, *i.e.*, crosses between reciprocal style morphs, produced significant amounts of seeds, whereas illegitimate or incompatible crosses, *i.e.*, crosses between plants of the same style morph, yielded much lower numbers of seeds (Darwin, 1877: Chapter I). Also, Darwin described pollen segregation along the body of a dead bumblebee after inserting its proboscis into the floral tube of *Primula veris* (Darwin, 1877: Chapter I p. 23). These observations led him to propose in *The Different Forms of Flowers on Plants of the Same Species* (Darwin, 1877) that reciprocal positioning of sex organs in heterostylous species functions to promote disassortative (intermorph) pollination between style morphs thus reducing pollen wastage on incompatible stigmas. This hypothesis is widely recognized as "Darwin's cross-promotion hypothesis" and has been evaluated in numerous heterostylous species.

The first investigations assessing Darwin's cross-promotion hypothesis were conducted during the 20th century, and were mainly based on studies of pollen loads on stigmas collected from intact distylous flowers in natural populations (reviewed in Ganders, 1979). The distinction between compatible (*i.e.*, intermorph) and incompatible (*i.e.*, intramorph) pollen grains in the total stigmatic pollen load is facilitated by the striking pollen size dimorphism that is a common feature of many heterostylous species. These studies usually compared the observed stigmatic pollen load with random expectations based on the total pollen production in the population taking into account style morph ratios (*e.g.*, Ornduff, 1970, 1971; Ganders, 1974). This form of analysis of compatible and incompatible pollen capture using stigmatic pollen load data allows a rough estimate of the extent of disassortative pollination in heterostylous species and consequently an evaluation of Darwin's cross-promotion hypothesis.

The most comprehensive early review on pollen capture in natural populations of distylous species was conducted by Ganders (1979). At that time, Ganders analyzed the studies on pollen capture for 13 distylous species and concluded that there was no strong support for Darwin's cross-promotion hypothesis. Most of the studies revealed random pollen capture for the L-morph (*e.g.*, *Amsinckia douglasiana*, Ganders, 1976) or the S-morph (*e.g.*, *Jepsonia parryi*, Ornduff, 1970) and/or high levels of incompatible pollen captured by both morphs (*e.g.*, *Pulmonaria obscura*, Olesen, 1979; *Primula vulgaris*, Ornduff, 1979). However, two issues need to be considered when evaluating whether these studies provide a rigorous evaluation of Darwin's cross-promotion hypothesis. First, as was originally pointed out by Ganders "Heterostyly, *per se*, does not influence the rate of intraflower selfing any more than would a monomorphic separation of stigmas and anthers. This is obvious because the reciprocal nature of the heterostylous polymorphism does not enter into the process of self-pollination within a single flower" (Ganders, 1979 p. 621; Fig. 2.1). Second, geitonogamous pollination, that is interflower self-pollination, will almost certainly occur in heterostylous populations of species with large floral displays, extensive clonal growth or in populations with clumped style morph distribution, because pollinators frequently move between flowers of the same plant or genet (Lloyd and Webb, 1992b). The distinction between the types of outcross pollen, *i.e.*, intermorph versus intramorph, is crucial for the evaluation of Darwin's cross-promotion hypothesis. Consequently, intraflower and geitonogamous components of the stigmatic pollen load should ideally be excluded by emasculation of flowers. This is because this procedure prevents the overestimation of incompatible pollen capture, allowing a more accurate measure of the effectiveness of reciprocal herkogamy in promoting disassortative pollination (Ganders, 1974, 1979; Lloyd and Webb, 1992b). Unfortunately, the emasculation of flowers can be technically challenging (Ganders, 1976; Schou, 1983) and therefore has rarely been undertaken in pollen flow studies.

More recently, Lloyd and Webb (1992b) reanalyzed two datasets on pollen capture in studies where emasculations were conducted by the authors. By comparing intact and emasculated flowers of a distylous (Ganders, 1974) and a tristylous species (Barrett and Glover, 1985) and calculating the transfer proficiency of compatible and incompatible pollen grains, Lloyd and Webb (1992b) were able to provide deeper insight into the functioning of heterostyly. Their reanalysis demonstrated that compatible pollen transfer to emasculated flowers of distylous *Jepsonia heterandra* was approximately

twice that of the incompatible transfer, while the opposite pattern was obtained for intact flowers. Similarly, in tristylous *Pontederia cordata*, compatible pollen transfer for emasculated flowers was larger than incompatible pollen transfer for each of the three style morphs. On the contrary, with the exception of the L-morph, incompatible pollen transfer was larger than compatible for intact flowers of the M- and S-morphs. Altogether, these studies provide evidence for Gander's claim (1974, 1979) that intraflower pollen transfer contributes a significant amount of incompatible pollen to the total stigmatic pollen load and if not excluded by emasculation, obscures the real extent to which reciprocal herkogamy promotes disassortative pollination.

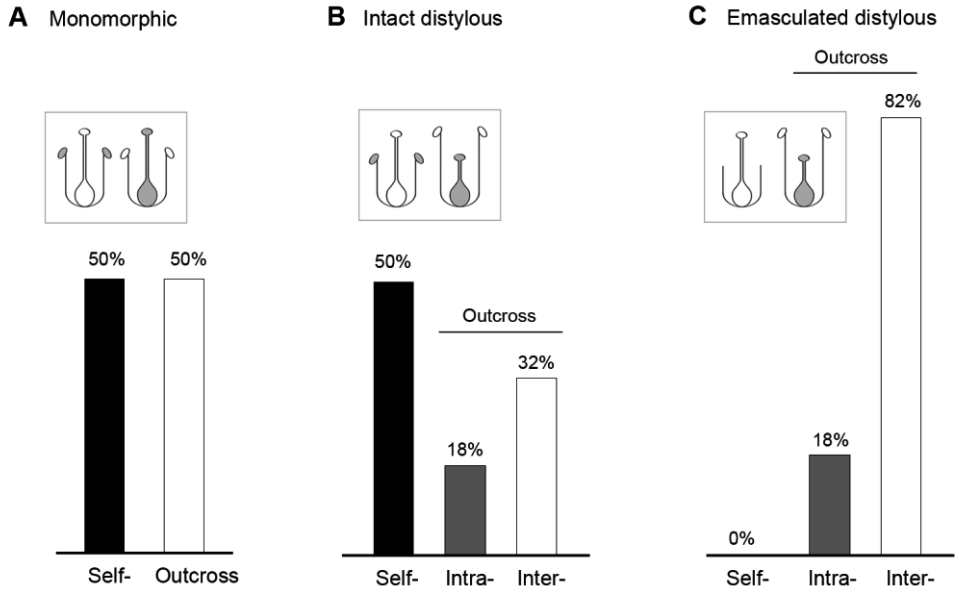


Figure 2.1. Decomposition of stigmatic pollen loads redrawn from Fig. 5 in Ganders (1979). **A.** Monomorphic population. **B.** Intact flowers from a distylous population. **C.** Emasculated flowers from a distylous population. Numbers above the bars are the hypothetic percentages of each component of pollen load on stigmas. Self-, intra- and inter- are self-pollen, intramorph and intermorph, respectively.

Empirical data from virtually all pollen flow studies indicates that the amount of compatible and incompatible pollen captured differs among the style morphs (*e.g.*, Ganders, 1974, 1976; Barrett and Glover, 1985; Piper and Charlesworth, 1986; Pailler *et al.*, 2002). Thus, asymmetrical pollen capture between style morphs is a general feature of pollen flow studies in distylous populations (*e.g.*, Ornduff, 1971, 1980a; Massinga *et al.*, 2005; reviewed in Barrett and Shore, 2008). This ubiquitous pattern was first interpreted as a consequence of the level of exertion/insertion of the stigmas and anthers within the floral tube (Ganders, 1974). Stone and Thomson (1994)

investigated how floral morphology influenced pollinator's positioning within a flower by recording bumblebee's visits to artificial flowers. These authors confirmed Lloyd and Webb's postulate (1992b p. 200: Fig. 1) that the available space for a probe to enter a flower dictates pollinator's positioning and consequently, the area of the pollinator's body that contacts anthers and stigmas, thus determining the patterns of pollen transfer and capture.

Over the past 40 years since Gander's (1979) review of pollen flow studies, many additional works investigating pollen capture in natural populations of heterostylous species have been conducted. Here, I conduct a comparative analysis of data in the literature on pollen flow studies and provide a synthesis of the general patterns that emerge. My study addressed the following specific questions: (1) What are the patterns of asymmetrical pollen transfer and capture in natural populations of heterostylous species? Based on Lloyd and Webb's (1992b p. 200: Fig. 1) view of the pollinator-flower interaction, I predicted that short-level organs would be more efficient in compatible pollen transfer and capture than long-level organs. The scarce data for tristylous species precluded their inclusion in this analysis, which is therefore restricted to distylous species. (2) How are the patterns of pollen transfer and capture distributed if phylogenetic relatedness among species is considered? Reciprocal herkogamy has evolved numerous times and in different families and therefore, it is possible that some phylogenetic signal is evident in patterns of pollen transfer and capture. (3) Do the main floral visitors of heterostylous species differ in their efficiency in pollen deposition? Animal pollinators display a wide variety in morphology and foraging behavior, often affecting the pollination process (*e.g.*, Herrera, 1987; Castellanos *et al.*, 2003). Since heterostylous flowers are visited by diverse animal species, I was interested to determine if they differed in pollination efficiency. By using comparative analysis, my study allowed me to summarize empirical evidence evaluating Darwin's cross-promotion hypothesis in a wide range of heterostylous species and to identify patterns in the pollination biology of heterostyly that are shared by species differing in their phylogenetic relatedness.

MATERIALS AND METHODS

Data collection

To compile data on the pollination biology of heterostylous species, I searched for published works using the ISI Web of Knowledge (time interval: 1900-2016; last time

accessed September 30, 2016) using the following key word combinations: "heterostyly", "disassortative", "legitimate pollination", "pollen flow", "pollen loads", "pollen transfer", "pollination". Only papers meeting the following criteria were analyzed: (1) the study was conducted in natural populations or in a common garden resembling the natural conditions where the species occurs; (2) emasculation was the only floral manipulation performed; (3) flowers were naturally pollinated (open pollination); (4) information on the style morph frequencies in the population or array was reported; and (5) data on the composition of stigmatic pollen loads was based on the occurrence of clear pollen size heteromorphism. In total, I identified 29 studies meeting these criteria (Appendix 2.1), representing 55 populations of 26 distylous species from 11 families. In addition, four studies of pollen capture in tristylous populations meeting these criteria were obtained, but these were not included given the small number of species involved (two species: *Pontederia cordata* and *P. sagittata*; Appendix 2.1). From each study, I recorded information on morph ratios, pollen production, the composition and size of stigmatic pollen loads for each style morph (Appendix 2.1), and floral visitors (Appendix 2.2). Because in the great majority (79.3%) of the studies emasculation was not performed, data on stigmatic pollen loads used in all statistical analyses was obtained from intact flowers only. In the cases where the stigmatic pollen load averages could not be obtained directly from tables or the text, I extracted the values from the graphs using the image analysis software ImageJ (Abràmoff *et al*., 2004). Floral visitors were grouped into four categories according to their taxonomical classification: bees, butterflies, flies and hummingbirds.

Analyses of pollen transfer and capture in distylous populations

Based on clear pollen size dimorphism, the authors of papers were able to identify the source of pollen on naturally pollinated stigmas. Pollen grains were classified as "compatible" if from the reciprocal style morph, or "incompatible" if from the same style morph. Here, pollen capture is used to refer to the stigmatic pollen loads, while pollen transfer refers to the probability of a single pollen grain of a given anther level is deposited on the stigma of each style morph, *i.e.*, pollen transfer proficiency. I calculated pollen transfer proficiencies (T_{ij}) for each population following Lloyd and Webb (1992b):

$$T_{ij} = \frac{(\text{average stigma load})_{ij} \times (\text{number of flowers})_j}{(\text{pollen/flower})_i \times (\text{number of flowers})_i}$$

where *i* and *j* are the style morphs, and average stigma load$_{ij}$ is the average number of type *i* pollen grains on each type *j* stigma. This formula accounts for the variability in pollen and flower production between style morphs, and provides information on stigmatic pollen load data from the perspective of both female and male function (Lloyd and Webb, 1992b).

In cases where sampling of a given population was replicated in different years, I calculated average values of total, compatible and incompatible pollen loads for use in the statistical analyses. Flower production per style morph was not provided in the papers and thus, I assumed no differences between style morphs for this variable and used style morph ratio for the calculation of pollen transfer proficiencies.

I used *G*-tests for goodness-of-fit with Yates correction to test for deviations of the style morphs from isoplethy (1:1) in each population (Zar, 2010). I investigated differences in: (1) compatible and incompatible pollen transfer proficiency for each anther level and between anther levels; (2) total pollen capture for each style morph; (3) compatible and incompatible pollen capture between style morphs and for each style morph using generalized or linear mixed models (hereafter GLMM), the former with a Gamma distribution and a log link function. Anther level in (1) and style morph in (2) and (3) were used as fixed factors, and population nested within species was defined as a random factor in all analyses conducted. Prior to statistical analysis, stigmatic pollen load data was $\log_{10}(x+1)$ transformed (Zar, 2010).

Pollinator efficiency is often defined as the amount of conspecific pollen deposited on stigmas by pollinators (Inouye *et al.*, 1994; Ne'eman *et al.*, 2010). Here, I investigated differences in efficiency, measured as compatible and incompatible pollen capture, among the main pollinators of 19 distylous species by means of GLMMs with a Gamma distribution and a log link function. Pollinators were defined as fixed factor, and population nested within species was defined as a random factor. Post-hoc tests for multiple comparisons were conducted afterwards.

I performed all statistical analyses with R software version 3.0.1 (R Core Development Team, 2013) using the following packages: "car" for Type-III analysis of variance as an integrated part of the GLMMs (Fox and Weisberg, 2015), "lme4" for GLMMs (Bates *et al.*, 2014), "multcomp" for multiple comparisons after Type-III

analysis of variance (Hothorn *et al.*, 2015), "nlme" for linear mixed models (Pinheiro *et al*., 2015), and "stats" for Shapiro-Wilk normality test (R Core Development Team, 2013).

Comparative analysis

To analyze data on pollen transfer and capture taking into account phylogenetic relationships, I followed the supermatrix approach to infer a phylogenetic hypothesis for the species included in my data set (de Queiroz and Gatesy, 2007; Roquet *et al*., 2013). I downloaded sequences of the following genetic markers available online from GenBank database using Geneious v9.0.5 (Kearse *et al*., 2012): *ITS* of the nuclear genome, and *matK*, *Trna-Leu* and intergenic spacer *tnrL-trnF* of the plastid genome (Appendix 2.3). The sequences were aligned using MAFFT v7 available online (http://mafft.cbrc.jp/alignment/server/; Katoh and Standley, 2013), and all alignments were improved by removing poorly aligned or ambiguous regions by setting "automated 1" function on trimAl v1.3 software (Capella-Gutiérrez *et al*., 2009) available online at the Phylemon 2.0 server (http://phylemon.bioinfo.cipf.es/index.html; Sánchez *et al*., 2011). The trimmed alignments were then concatenated into a supermatrix of 3282 characters and 20 taxa using FASconCAT v1.0 (Kück and Meusemann, 2010). The obtained supermatrix was analyzed using a maximum likelihood approach (hereafter ML) following the GTR model as implemented in RaxML v8.2.8 (Stamatakis, 2014) available at the CIPRES server (http://phylo.org; Miller *et al*., 2010) by running 1000 bootstrap replicates. I kept the best ML phylogenetic tree (Appendix 2.4) and, when necessary, I used BioEdit v7.0.9.0 (Hall, 1999) to edit the sequences and the supermatrix, and FigTree v1.2 (Rambaut, 2008) to view and edit the phylogenetic tree.

Most comparative analyses require ultrametric trees, *i.e.*, trees in which branch lengths represent the time of divergence (Paradis, 2006; Garamszegi and Gonzalez-Voyer, 2014). Hence, I ultrametrized the final ML phylogenetic tree by molecular dating using likelihood methods as implemented in *chronos* function available from R package "ape" (Paradis *et al*., 2004). To calibrate the tree, it is necessary to use the values of minimum and maximum ages estimated for a family included in the analysis. Here, I used the Rubiaceae family and obtained the minimum (47 mya) and maximum (67 mya) ages from Bell *et al*. (2010). The values of the information criterion PHIIC for each model of substitution rate calculated are provided in Appendix 2.5.

Six species in my database did not have sequences available at GenBank (*i.e.*, *Amsinckia douglasiana*, *A. grandiflora*, *Lythrum californicum*, *Primula sieboldii*, *Palicourea demissa* and *Jepsonia heterandra*) and these species were grafted to their genus in the ultrametric tree by using *add.species.to.genus* function from R package "phytools" (Revell, 2012). As a consequence, this approach introduced some polytomies in the final tree (Appendix 2.6) that were randomly resolved by setting the function *multi2di* from R package "ape" (Paradis *et al*., 2004).

From the available methods to test for phylogenetic dependence in trait values, Pagel's λ is an evolutionary approach that assumes a Brownian motion model of trait evolution (Pagel, 1999) and uses a ML test to estimate the phylogenetical signal (Harmon *et al*., 2008; Münkemüller *et al*., 2012). This phylogenetic signal index is the least affected by phylogeny size and thus, performs reasonably well for small phylogenies (Münkemüller *et al*., 2012), as in this study. Pagel's λ varies between 0 and 1, with values close to 0 indicating no phylogenetic signal and values close to 1 indicating strong phylogenetic signal (Harmon *et al*., 2008; Münkemüller *et al*., 2012). Here, total, compatible and incompatible pollen transfer and capture were treated as continuous traits for each anther level and style morph, respectively, for each species. The calculation of Pagel's λ requires a single value for each trait per species. Thus, in case different populations of a single species were sampled, I calculated a weighted average for each trait per style morph. By doing this, I ended up with two values per species for each continuous trait, one for each style morph to be used for calculation of Pagel's λ. The phylogenetic index was calculated by setting *fitContinuous* function in R package "geiger" (Harmon *et al*., 2008) for continuous traits. Finally, for a better understanding of high values of phylogenetic signal and the species responsible, I plotted a projection of the phylogenetic tree defined by trait in the y-axis and time in the x-axis, *i.e.*, a traitgram, by using the function *phenogram* available from "phytools" R package (Revell, 2012).

RESULTS

Patterns of pollen transfer proficiency and capture

As predicted, compatible pollen transfer was significantly higher between short-level anthers of the L-morph and the stigma of the S-morph than between long-level anthers of the S-morph and the stigma of the L-morph ($\chi^2_1 = 100.40$, $P < 0.001$). Incompatible pollen transfer was significantly higher between short-level anthers of the L-morph and

stigmas of the L-morph than between long-level anthers of the S-morph and stigmas of the S-morph (χ^2_1 = 1.17E^{04}, $P < 0.001$). Pollen produced in the long-level anthers of the S-morph had a greater probability of being transferred to incompatible than to compatible stigmas (χ^2_1 = 1.86E^{05}, $P < 0.001$; Fig. 2.2), and a similar pattern was obtained for pollen produced by the short-level anthers (χ^2_1 = 41.14, $P < 0.001$; Fig. 2.2).

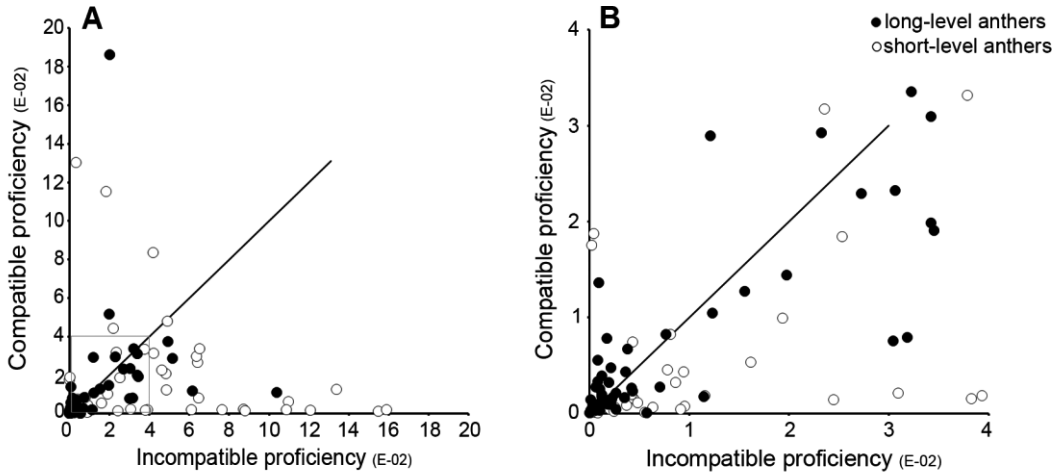


Figure 2.2. A. Pollen transfer proficiencies for long- and short-level anthers of the L- and S-morphs from the 55 distylous populations analyzed in this study. **B.** Close-up of (A) for pollen transfer proficiencies varying between 0.00 and 4.00E^{-02}. Equation for the line: $y = x$.

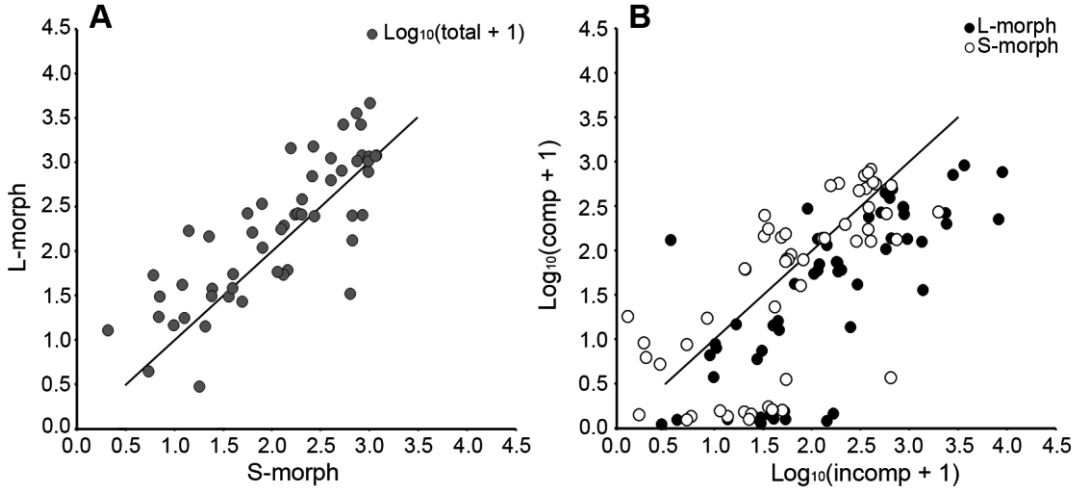


Figure 2.3. Pollen capture for L- and S-morphs in the 55 distylous populations analyzed in this study. **A.** Total pollen capture. **B.** Compatible (comp) and incompatible (incomp) pollen capture. Data are $\log_{10}(x+1)$ transformed. Equation for the line: $y = x$.

Total pollen capture was significantly larger for the L-morph than for the S-morph (χ^2_1 = 8.21, P = 0.004; Fig. 2.3A). The L-morph captured significantly more incompatible pollen than the S-morph (χ^2_1 = 21.48, P < 0.001), while the opposite pattern vas detected for compatible pollen capture (χ^2_1 = 5.54E^{03}, P < 0.001). The comparison between compatible and incompatible pollen captured by each style morph revealed that stigmas of the L-morph captured significantly more incompatible than compatible pollen (χ^2_1 = 25.03, P < 0.001; Fig. 2.3B), and a similar pattern was also detected for the S-morph (χ^2_1 = 5.03, P = 0.02; Fig. 2.3B).

Diversity of floral visitors

Distylous flowers are visited by a great diversity of pollinators. Of particular importance are species of bees (*e.g.*, *Bombus* spp., *Apis mellifera*, *Anthophora* spp., *Dialictus* spp.; Appendix 2.2), which were reported for 13 of the 19 distylous species from which data on floral visitors was available (Fig. 2.4). Other visitors include butterflies, flies and hummingbirds that were reported for six, four and two distylous species, respectively (Fig. 2.4; Appendix 2.2).

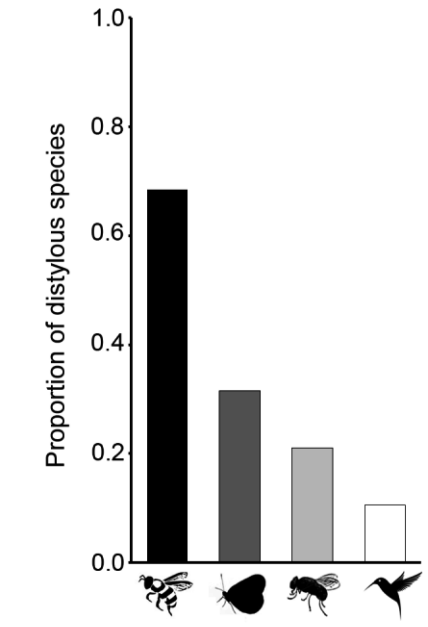


Figure 2.4. Proportion of distylous species (n = 19) visited by bees, butterflies, flies and hummingbirds.

Floral visitors differed significantly in their efficiency in promoting compatible pollination (χ^2_6 = 74.20, P < 0.001), with butterflies responsible for a lower total amount of compatible pollen deposition than bees (mean ± SE; butterflies: 1.05 ± 0.48; bees = 2.18 ± 0.09). Flies tended to be slightly more efficient (mean ± SE, 2.44 ± 0.14) and hummingbirds less efficient (mean ± SE, 0.38 ± 0.11) in compatible pollen transfer, although these trends were not significant. Concerning incompatible pollen deposition, I detected significant differences among floral visitors (χ^2_6 = 30.07, P < 0.001). Similar to what was found for compatible pollen deposition, butterflies transferred a lower amount of incompatible pollen than bees (mean ± SE; butterflies: 1.14 ± 0.37; bees = 2.54 ± 0.15). Distylous species visited by bees and flies tended to receive lower

numbers of incompatible grains (mean ± SE, 0.95 ± 0.21), whereas species visited by bees, butterflies and flies generally received large numbers of incompatible pollen grains (mean ± SE, 2.71 ± 0.11).

Phylogenetic signal for pollen transfer and capture

Values for the continuous traits investigated (Table 2.1) lack phylogenetic signal, and with two exceptions, were randomly distributed across the phylogenetic tree. The first exception was pollen transfer between the long-level anthers of the S-morph and stigmas of the L-morph (Pagel's λ = 0.93; Fig. 2.5A), and a closer examination of the corresponding traitgram revealed that compatible pollen transfer by the long-level anthers of *Menyanthes trifoliata* was much larger than of the remaining species. Exclusion of this outlier resulted in a value of zero for Pagel's λ. The second exception was incompatible pollen capture by the L-morph (Pagel's λ = 0.92; Fig. 2.5B), and I identified three groups of species in the corresponding traitgram that accounted for this pattern: (1) the four *Primula* species with high incompatible pollen capture, (2) the two *Pentanisia* species with very low values of incompatible pollen capture, and (3) the remaining species with intermediate values of incompatible pollen capture. Thus, apart from a few exceptions, there was no evidence that related species had similar patterns of pollen transfer and capture.

Table 2.1. Values of Pagel's λ calculated for each continuous trait. Phylogenetic signal for the traits investigated is given as "Yes" and "No". Only Pagel's λ values > 0.75 were considered to represent strong phylogenetic signal.

Trait	Pagel's λ	Phylogenetic signal
(a) L-morph		
Compatible pollen transfer	0.00	No
Incompatible pollen transfer	0.00	No
Total pollen capture	0.57	No
Compatible pollen capture	0.21	No
Incompatible pollen capture	0.92	Yes
(b) S-morph		
Compatible pollen transfer	0.93	Yes
Incompatible pollen transfer	0.00	No
Total pollen capture	0.30	No
Compatible pollen capture	0.00	No
Incompatible pollen capture	0.23	No

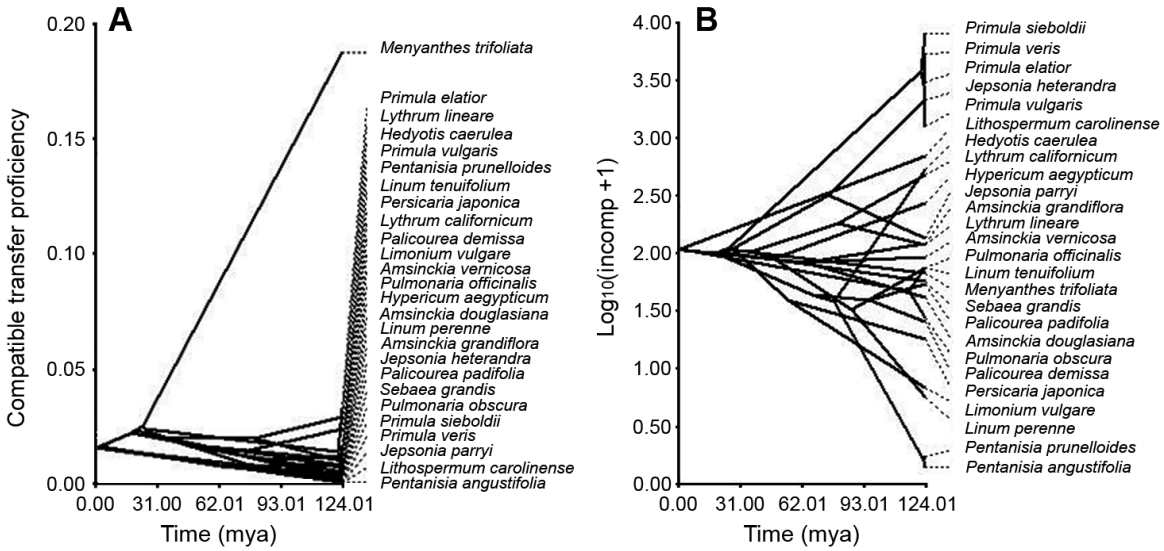


Figure 2.5. A. Traitgram of compatible pollen transfer proficiencies for long-level anthers. **B.** Traitgram of incompatible pollen capture by the L-morph. Data on pollen capture are $\log_{10}(x+1)$ transformed. Time is given in millions of years ago.

DISCUSSION

This comparative analysis allowed me to identify common patterns in the pollination biology of distylous species. The main findings were: (1) pollen is more likely to be involved in incompatible than compatible transfer, irrespective of the anther level in which it is produced (Fig. 2.1); (2) pollen capture is highly asymmetrical between style morphs (Fig. 2.2); and (3) floral visitors generally differ in their pollination efficiency. Collectively, these results offer limited evidence for Darwin's cross-promotion hypothesis. However, the fact that all studies I used in the comparative analysis investigated pollen loads on intact flowers represents a major drawback complicating a rigorous testing of the Darwin's cross-promotion hypothesis. Below, I first examine the use of pollen size in pollen flow studies, then consider the role of reciprocal herkogamy in promoting disassortative pollination in distylous species, and finally examine the factors confounding its detection in studies of pollen transfer and capture.

Pollen heteromorphism allows evaluation of Darwin's cross-promotion hypothesis

Pollen size heteromorphism is widespread among heterostylous species (Vuilleumier, 1967; Ganders, 1979; Dulberger, 1992) and allows the estimation of disassortative pollination through the analysis of stigmatic pollen load composition (references and results herein; Chapter 3). Nevertheless, the more restricted pollen heteromorphisms in color (*e.g.*, Massinga *et al.*, 2005) and exine sculpturing (*e.g.*,

Levin, 1968; Nicholls, 1985, 1986; Chapter 4) have also been used to distinguish between pollen types on stigmas. Only a few studies have investigated pollen capture in tristylous species. This is because of two main reasons. First, tristyly is far more restricted than distyly and is reported from only six families (*i.e*., Amaryllidaceae, Fernandes, 1935a; Connaraceae, Lemmens, 1989; Oxalidaceae, Linaceae, Lythraceae, and Pontederiaceae, Darwin, 1877). Second, discrete pollen trimorphism apparently only occurs in *Pontederia* (Barrett, 1977b; Price and Barrett, 1982; Glover and Barrett, 1983), with pollen produced by the mid- and short-level anthers usually overlapping in size in the remaining tristylous species that have been investigated (*e.g*., *Lythrum junceum*, Dulberger, 1970; *L. salicaria*, Mulcahy and Carporello, 1970, Chapter 3 Fig. 3.3; *Eichhornia* spp., Barrett, 1988). Consequently, the opportunities to investigate in detail the patterns of pollen transfer and capture in populations of tristylous species are limited.

A few distylous species also lack strong pollen size dimorphism with pollen produced by long- and short-level anthers exhibiting some degree of overlap (reviewed in Vuilleumier, 1967). To overcome this problem, a cut-off point either side of the overlap region has sometimes been used (*e.g*., Ganders, 1976; Stone, 1996; Ree, 1997) and the probability of transfer of pollen grains originated from different anther levels is considered to be equivalent in the region of overlap. However, this method is unsatisfactory and can introduce error in the estimation of disassortative pollen transfer and capture. Hence, Pailler *et al*. (2002) modified the pollen transfer proficiency formula developed by Lloyd and Webb (1992b), and used in this study, to include information on pollen-size frequency distributions. They applied the modified formula to the calculation of pollen transfer proficiencies for the tropical distylous *Gaertnera vaginata*, and found that pollen transfer was highly asymmetrical and that disassortative pollination was higher between long-level organs than between short-level organs. This result is the opposite to the overall patterns I identified in my comparative analysis. However, the main pollinator of *G. vaginata* was the introduced *Apis mellifera,* which largely collected pollen and most likely was responsible for mediating much of the pollen transfer between long-level organs.

The role of reciprocal herkogamy in promoting disassortative pollination

I found a consistent pattern of higher incompatible than compatible pollen transfer proficiency for long- and short-level anthers among the distylous species in my

analysis. Thus, from the male perspective, reciprocal herkogamy would appear to be ineffective in promoting disassortative pollen transfer. What factors account for this overall result? Certainly, pollen transfer will be affected by style morph ratios in a population, but this factor is accounted for in the calculation of pollen transfer proficiencies (Lloyd and Webb, 1992b), and the great majority of the populations included in the dataset exhibited 1:1 style morph ratios (83.6%). A similar pattern involving a higher probability of incompatible than compatible pollen transfer was previously reported by Lloyd and Webb (1992b) in their reanalysis of stigmatic pollen loads from intact as opposed to emasculated flowers of distylous *Jepsonia heterandra* (Ganders, 1974), and tristylous *Pontederia cordata* (Barrett and Glover, 1985). These findings therefore reinforce the early conclusions of Ganders (1974, 1979) that the emasculation of recipient flowers is essential for an accurate assessment of Darwin's cross-promotion hypothesis.

This literature analysis provided evidence supporting consistent differences in pollen capture between style morphs. Overall, the L-morph captured a larger amount of pollen than the S-morph, but a great proportion was incompatible. In contrast, the S-morph captured a smaller number of pollen grains than the L-morph, but the majority was compatible. This observed asymmetry in patterns of pollen capture is best explained by the influence of sex-organ height on the pollination process. By using glass-sided artificial flowers, Stone and Thomson (1994) found that bumblebees contacted the stigma of the L-morph with the head, thorax and abdomen, while the initial space available in S-morph flowers allowed them to enter perpendicularly and therefore mainly contacted the stigmas with their heads. In Chapter 3, I investigated the influence of sex-organs height on pollination in tristylous *Lythrum salicaria*. I found that disassortative pollination varied significantly with sex-organ height and that it was the highest for short-level organs and the lowest for long-level organs. These results indicate that the interaction between specific features of floral morphology and pollinator behavior is the most important factor determining the patterns of pollen transfer and capture in heterostylous species.

The pollen capture studies included in this review were mainly conducted in temperate regions from the Northern Hemisphere (24 studies out of 29), where bees are common pollinators. Consequently, species from this group were frequently reported as floral visitors of distylous species. On the contrary, bird pollination was mentioned for only two species, *Palicourea padifolia* and *P. demissa*, both Neotropical Rubiaceae. I

found some differences among floral visitors in pollination efficiency measured as compatible and incompatible pollen capture by stigmas. However, these results should be interpreted with caution regarding the efficiency of the four groups of floral visitors. A more reliable assessment of pollinator's efficiency requires the relation between conspecific pollen deposition and female reproductive success measured as seed set (Ne'eman *et al*., 2010), and also outcrossed siring success via pollen. Here, it was not possible to explore this association given that seed set data was generally not provided in the studies, nor data on seed paternity. Moreover, most of the data on floral visitors was not based on systematic and quantitative field observations, but instead from occasional observations while sampling flowers for stigmatic pollen loads analyses. Consequently, the pollinator observations are in most cased crude and may not reflect the relative importance of particular visitors that were recorded. Nonetheless, and despite these problems, it is clear from the limited data available in the literature that bees and hummingbirds have significantly different efficiencies with respect to compatible pollen deposition. Future investigations on the pollination biology of heterostylous species will benefit from a more detailed assessment of pollinator efficiency in which the quantity and quality of pollen transfer is compared for different pollinator groups visiting a given species.

Heterostyly – a convergent floral syndrome

Comparative analysis accounting for phylogenetic relatedness and the statistical non-independence among species traits has been increasingly used in a diverse range of topics, such as community ecology and macroecology (reviewed in Felsenstein, 1985; Paradis, 2006; Münkemüller *et al*., 2012). Here, with the exception of two continuous traits, patterns of pollen transfer and capture were consistent among the 26 distylous species investigated, as revealed by the absence of phylogenetic signal. This is not altogether surprising because heterostyly is a convergent floral syndrome that has evolved numerous times in at least 28 angiosperm families. However, a stronger comparative analysis would benefit from the inclusion of closely related non-heterostylous taxa. Nevertheless, in such a study it would only be possible to compare total stigma pollen loads because non-heterostylous lack floral polymorphisms and even if they were self-incompatible, it would be impossible to distinguish between incompatible and compatible pollen on stigmas.

I detected high phylogenetic signal in two out of ten tests made. First, compatible pollen transfer between the long-level anthers of the S-morph and the stigma of the L-morph of *Menyanthes trifoliata* was much larger than in the remaining species. This species is a clonal aquatic perennial and the population sampled was highly anisoplethic, with the L-morph representing almost 90% of the plants in the population (Lughadha and Parnell, 1989). Consequently, the probability of compatible pollen transfer after a pollinator visit to a clone of the S-morph was much larger. This result highlights the influence of morph ratios and clonality in determining patterns of pollen transfer in populations of distylous species. Second, I detected three well-defined groups concerning incompatible pollen capture by the L-morph: (1) the four *Primula* species with high incompatible pollen capture, (2) the two *Pentanisia* species with very low values of incompatible pollen capture, and (3) the remaining distylous species with intermediate values of incompatible pollen capture. High values of incompatible pollen capture by the L-morph of *Primula* species might be promoted by the interaction between low pollinator visitation rates (Ornduff, 1979, 1980a; Schou, 1983; Piper and Charlesworth, 1986; Washitani *et al*., 1994; Nishihiro *et al*., 2000) and the exerted position of the stigma. Concerning the two *Pentanisia* species, the morphological fit between butterflies' proboscises and the floral tube length of these species contributed to low incompatible pollen capture (Massinga *et al*., 2005). These findings suggest that in addition to the interaction between floral morphology and pollinator behavior in influencing pollination patterns (see above *The role of reciprocal herkogamy in promoting disassortative pollination*), pollinator identity and abundance are also likely to influence patterns of pollen transfer.

This comparative analysis supports earlier conclusions that pollen flow studies of intact flowers are not ideal for testing Darwin's cross-promotion hypothesis (Ganders, 1979; Lloyd and Webb, 1992b). However, these results do not entirely invalidate the previous pollen flow studies, some of which have contributed to our knowledge on the pollination biology of heterostyly. In some early studies, the authors concluded that reciprocal herkogamy was not very efficient in promoting disassortative pollination. For example, Ornduff concluded that "the overall pattern of pollen flow in the species does not fit the Darwinian ideal" after analyzing stigmatic pollen loads of distylous *Hypericum aegypticum* (Ornduff, 1975a). This conclusion is likely premature because without information on emasculated flowers a rigorous test of Darwin's cross-promotion hypothesis cannot be undertaken. The fact that so many studies (25) have

been conducted since Gander's original insight (Ganders, 1974) suggests that many researchers neglect the confounding influence of self-pollen deposition when attempting to evaluate the efficacy of heterostyly in the pollen economy of populations.

Appendix 2.1. Data on morph ratio, pollen production per flower, and stigmatic pollen load for each population of the distylous species included in the comparative analysis. The references (Refs) for each study are also provided. References in bold refer to the data on pollen production per flower. Values on pollen production per flower, total, compatible and incompatible pollen loads are given as mean. Measures of the spread of data distribution, SD and SE, are not included, because they were not available in the great majority of the original publications.

						Stigmatic pollen load						
		Morph ratio		Pollen production		Total		Compatible		Incompatible		
Species	**Pop**	**L**	**S**	**L**	**S**	**L**	**S**	**L**	**S**	**L**	**S**	**Refs**
Amsinckia douglasiana	1	0.60	0.40	17440.0	15380.0	107.3	80.7	40.4	61.0	66.9	19.7	4
Amsinckia grandiflora	1	0.55	0.44	32953.0	28650.0	255.3	175.3	71.0	143.7	184.3	31.7	18
Amsinckia vernicosa	1	0.44	0.56	16507.0	23947.0	256.3	205.5	112.8	152.4	143.5	53.1	4
Lithospermum caroliniense	1	0.38	0.62	72820.0	38280.0	31.9	652.7	4.9	2.7	27.0	650.0	6, **30**
	2	0.50	0.50	160800.0	64360.0	263.5	56.0	12.5	2.5	251.0	53.5	29
	3	0.50	0.50	140740.0	52111.0	683.5	265.0	101.5	136.0	582.0	129.0	29
	4	0.50	0.50	106000.0	37800.0	338.0	80.0	40.0	60.0	298.0	20.0	29
	5	0.43	0.57	72820.0	38280.0	1423.3	158.9	34.4	77.5	1388.9	81.4	30
Pulmonaria officinalis	1	0.59	0.41	26810.0	14363.0	189.3	134.3	68.6	77.7	120.7	56.6	1
	2	0.52	0.48	26810.0	14363.0	173.4	127.1	58.0	74.2	115.4	52.9	1
Pulmonaria obscura	1	0.50	0.50	10292.0	6681.0	57.2	115.6	11.6	39.0	45.6	76.6	14
Sebaea grandis	1	0.50	0.50	301187.0	208958.0	381.0	209.0	289.9	173.7	91.1	35.3	31
Hypericum aegypticum	1	0.50	0.50	48685.0	285575.0	621.6	413.8	235.4	194.4	386.2	219.4	17
Linum perenne	1	0.50	0.50	2640.0	2140.0	16.5	11.9	6.9	7.6	9.6	4.2	11
Linum tenuifolium	1	0.35	0.65	6200.0	6200.0	249.0	683.0	133.0	300.0	116.0	383.0	10
Lythrum californicum	1	0.70	0.30	26375.0	11765.5	793.7	530.5	134.7	125.0	659.0	405.5	19
Lythrum lineare	1	0.85	0.15	7099.0	14421.0	252.7	877.5	72.2	130.5	180.5	747.1	19
Menyanthes trifoliata	1	0.88	0.12	14421.0	7099.0	159.0	63.0	53.0	22.0	106.0	41.0	7
Persicaria japonica	1	0.50	0.50	616.0	607.0	36.3	23.8	6.3	16.3	30.0	7.5	12
Primula elatior	1	0.50	0.50	27742.0	17329.0	4604.0	1040.0	889.0	692.0	3715.0	348.0	25
Primula sieboldii	1	0.50	0.50	281198.0	149055.0	9895.0	2260.0	755.0	269.0	9139.5	1991.0	13, **9**
	2	0.50	0.50	281198.0	149055.0	143.9	22.1	0.2	0.3	143.7	21.9	28, **9**
Primula veris	1	0.50	0.50	211000.0	87000.0	1480.0	272.0	122.0	136.0	1358.0	136.0	22

Cont.

Species	Pop	Morph ratio		Pollen production		Stigmatic pollen load						Refs
						Total		Compatible		Incompatible		
		L	S	L	S	L	S	L	S	L	S	
Primula veris	2	0.50	0.50	211000.0	87000.0	8527.0	1128.0	222.0	750.0	8305.0	378.0	22
	3	0.50	0.50	211000.0	87000.0	2647.0	552.0	196.0	172.0	2451.0	380.0	22
Primula vulgaris	1	0.50	0.50	283000.0	89000.0	1103.4	411.2	132.5	124.8	970.9	286.3	20
	2	0.67	0.33	36620.0	17001.0	2635.4	839.1	258.8	256.1	2376.6	583.0	24
Hedyotis caerulea	1	0.60	0.40	13641.3	13148.8	1179.3	858.0	300.7	498.7	878.7	359.3	21
	2	0.50	0.50	13641.3	13148.8	1177.5	1212.0	305.0	808.5	872.5	403.5	21
	3	0.57	0.43	13641.3	13148.8	1019.5	773.5	384.5	466.5	635.0	307.0	21
	4	0.54	0.46	13641.3	13148.8	778.0	1009.5	261.0	558.0	517.0	451.5	21
	5	0.50	0.50	13641.3	13148.8	1142.0	1011.0	250.0	556.0	892.0	455.0	21
	6	0.50	0.50	13641.3	13148.8	1017.0	1008.0	440.0	583.0	577.0	425.0	21
	7	0.50	0.50	13641.3	13148.8	1161.5	1188.5	488.0	536.0	673.5	652.5	21
Palicourea demissa	1	0.50	0.50	1897.4	1886.9	59.6	148.2	14.9	88.0	44.7	60.2	26, **27**
	2	0.50	0.50	1897.4	1886.9	52.8	133.0	13.1	78.6	39.7	54.4	27
Palicourea padifolia	1	0.50	0.50	335.1	267.1	29.4	35.9	0.1	0.7	29.3	35.1	5, **23**
	2	0.50	0.50	335.1	267.1	13.1	20.0	0.2	0.5	12.8	19.5	5, **23**
	3	0.50	0.50	335.1	267.1	52.5	5.2	0.5	0.3	52.0	4.8	5, **23**
	4	0.50	0.50	335.1	267.1	29.9	23.4	0.3	0.4	29.6	23.0	5, **23**
	5	0.50	0.50	335.1	267.1	167.8	13.2	0.4	0.4	167.3	12.9	5, **23**
	6	0.50	0.50	335.1	267.1	40.7	11.2	0.3	0.6	40.4	10.6	5, **23**
	7	0.50	0.50	335.1	267.1	26.0	49.7	0.3	0.6	25.7	49.1	5, **23**
	8	0.50	0.50	335.1	267.1	53.6	39.7	0.3	0.6	53.4	39.1	5, **23**
	9	0.50	0.50	335.1	267.1	3.4	4.5	0.2	0.2	3.2	4.3	5, **23**
	10	0.50	0.50	335.1	267.1	36.9	39.1	0.4	0.6	36.6	38.5	5, **23**
Pentanisia angustifolia	1	0.52	0.48	2743.0	2674.0	2.0	17.3	0.1	17.0	1.9	0.3	8
Pentanisia prunelloides	1	0.52	0.48	8950.0	10050.0	130.3	689.9	127.7	533.6	2.6	156.3	8
Jepsonia heterandra	1	0.50	0.50	211000.0	91000.0	3522.0	756.0	700.0	567.0	2822.0	189.0	3, **16**
	2	0.50	0.50	211000.0	91000.0	244.8	279.9	56.3	247.9	188.5	32.0	16
Jepsonia parryi	1	0.50	0.50	78000.0	206000.0	262.5	187.8	58.8	139.9	203.6	47.9	15

Limonium vulgare	1	0.44	0.56	979.1	996.8	17.1	6.0	7.8	4.2	9.3	1.8	2
	2	0.53	0.47	979.1	996.8	13.6	9.0	5.5	8.1	8.1	0.9	2
	3	0.46	0.54	979.1	996.8	11.7	1.1	2.7	0.4	9.0	0.7	2
	4	0.44	0.56	979.1	996.8	29.5	6.2	13.6	5.2	15.9	1.0	2

List of references for the studies included in the comparative analysis. * studies on tristylous populations not included in this analysis.

*Barrett SCH, Glover DE. 1985. On the Darwinian hypothesis of the adaptative significance of tristyly. *Evolution* 39: 766-774.

[1]Brys R, Jacquemyn H, Hermy M, Beeckman T. 2008. Pollen deposition rates and the functioning of distyly in the perennial *Pulmonaria officinalis* (Boraginaceae). *Plant Syst Evol* 273: 1–12.

[2]Chapter 4 published as Costa J, Castro S, Loureiro J, Barrett SCH. 2016. Experimental insights on the function of ancillary pollen and stigma polymorphisms in plants with heteromorphic incompatibility. *Evolution* doi:10.1111/evo.13082.

[3]Ganders FR. 1974. Disassortative pollination in the distylous plant *Jepsonia heterandra*. *Can J Bot* 52: 2401–2406.

[4]Ganders FR. 1976. Pollen flow in distylous populations of *Amsinckia* (Boraginaceae). *Can J Bot* 54: 2530–2535.

*Glover DE, Barrett SCH. 1983. Trimorphic incompatibility in Mexican populations of *Pontederia sagittata* Presl. (Pontederiaceae). *New Phytol* 95: 439-455.

*Glover DE, Barrett SCH. 1986. Stigmatic pollen loads in populations of *Pontederia cordata* from the Southern U.S. *Am J Bot* 73: 1607-1612.

[5]Hernández A, Ornelas JF. 2007. Disassortative pollen transfer in distylous *Palicourea padifolia* (Rubiaceae), a hummingbird-pollinated shrub. *Ecoscience* 14: 8–16.

[6]Levin DA. 1968. The breeding system of *Lithospermum caroliniense*: adaptation and counteradaptation. *Am Nat* 102: 427–441.

[7]Lughadha EMN, Parnell JAN. 1989. Heterostyly and gene-flow in *Menyanthes trifoliata* L. (Menyanthaceae). *Biol J Linn Soc* 100: 337–354.

[8]Massinga PH, Johnson SD, Harder LD. 2005. Heteromorphic incompatibility and efficiency of pollination in two distylous *Pentanisia* species (Rubiaceae). *Ann Bot* 95: 389–99.

[9]Matsumara C, Washitani I. 1985. Heterostylous morph differences in pollen transfer and deposition patterns in *Primula sieboldii* on a visitation by a queen bumblebee, measured with a semi-natural experimental system. *Plant Species Biol* 17: 1-12.

[10]Nicholls MS. 1985. Pollen flow, population composition, and the adaptive significance of distyly in *Linum tenuifolium* L. (Linaceae). *Biol J Linn Soc* 25: 235–242.

[11]Nicholls MS. 1986. Population composition, gender specialization, and the adaptative significance of distyly in *Linum perenne* (Linaceae). *New Phytol* 102: 209–217.

[12]Nishihiro J, Washitani I. 1998. Patterns and consequences of self-pollen deposition on stigmas in heterosylous *Persicaria japonia* (Polygonaceae). *Am J Bot* 85: 352–359.

[13]Nishihiro J, Washitani I, Thomson JD, Thomson BA. 2000. Patterns and consequences of stigma height variation in a natural population of a distylous plant, *Primula sieboldii*. *Funct Ecol* 14: 502–512.

[14]Olesen JM. 1979. Floral morphology and pollen flow in the heterostylous species *Pulmonaria obscura* Dumort (Boraginaceae). *New Phytol* 82: 757–767.

[15]Ornduff R. 1970. Incompatibility and the pollen economy of *Jepsonia parryi*. *Am J Bot* 57: 1036–1041.

[16]Ornduff R. 1971. The reproductive system of *Jepsonia heterandra*. *Evolution* 25: 300–311.

[17]Ornduff R. 1975. Heterostyly and pollen flow in *Hypericum aegypticum* (Guttiferae). *Biol J Linn Soc* 71: 51-57.

[18]Ornduff R. 1976. The reproductive system of *Amsinckia grandiflora*, a distylous species. *Syst Bot* 1: 57–66.

[19]Ornduff R. 1978. Features of pollen flow in dimorphic species of *Lythrum* section *Euhyssopifolia*. *Am J Bot* 65: 1077–1083.

[20]Ornduff R. 1979. Pollen flow in a population of *Primula vulgaris* Huds. *Bot J Linn Soc* 78: 1–10.

[21]Ornduff R. 1980a. Heterostyly, population composition, and pollen flow in *Hedyotis caerulea*. *Am J Bot* 67: 95–103.

[22]Ornduff R. 1980b. Pollen flow in *Primula veris* (Primulaceae). *Plant Syst Evol* 135: 89–93.

[23]Ornelas JF, Jiménez L, González C, Hernández A. 2004. Reproductive ecology of distylous *Palicourea padifolia* (Rubiaceae) in a tropical montane cloud forest. I. Hummingbirds' effectiveness as pollen vectors. *Am J Bot* 91: 1052-1060.

[24]Piper J, Charlesworth B. 1986. The evolution of distyly in *Primula vulgaris*. *Biol J Linn Soc* 29: 123–137.

*Price SD, Barrett SCH. 1984. The function and adaptative significance of tristyly in *Pontederia cordata* L. (Pontederiaceae). *Biol J Linn Soc* 21: 315-329.

[25]Schou O. 1983. The distyly in *Primula elatior* (L.) Hill (Primulaceae), with a study of flowering phenology and pollen flow. *Bot J Linn Soc* 86: 261–274.

[26]Valois-Cuesta H, Soriano PJ, Ornelas JF. 2011. Asymmetrical legitimate pollination in distylous *Palicourea demissa* (Rubiaceae): the role of nectar production and pollinator visitation. *J Trop Ecol* 27: 393–404.

[27]Valois-Cuesta H, Soriano PJ, Ornelas JF. 2012. Gender specialization in *Palicourea demissa* (Rubiaceae), a distylous, hummingbird-pollinated treelet. *Plant Syst Evol* 298: 975-984.

[28]Washitani I, Osawa R, Namai H, Niwa M. 1994. Patterns of female fertility in heterostylous *Primula sieboldii* under severe pollinator limitation. *J Ecol* 82: 571–579.

[29]Weller SG. 1980. Pollen flow and fecundity in populations of *Lithospermum caroliniense*. *Am J Bot* 67: 1334–1341.

[30]Weller SG, Keeler KH, Thomson BA. 2000. Clonal growth of *Lithospermum caroliniense* (Boraginaceae) in contrasting sand dune habitats. *Am J Bot* 87: 237–242.

[31]Wolfe LM, Massinga PH, Johnson SD. 2009. A quantitative evaluation of the distylous syndrome in *Sebaea grandis* (Gentianaceae). *S Afr J Bot* 75: 785–790.

Appendix 2.2. Main floral visitors recorded in the original studies for each distylous species included in the comparative analysis. For a list of references (Refs), see Appendix 2.1. "-" no information was available in the paper.

Species	Main floral visitors	Floral visitor's species	Refs
Amsinckia douglasiana	-	-	4
Amsinckia grandiflora	Bees	*Anthophora edwardsii, Apis mellifera, Bombus edwardsii, Dialictus orthocarpi*	18
Amsinckia vernicosa	-	-	4
Lithospermum caroliniense	Bees	-	6, 30
	Butterflies	-	6
Pulmonaria officinalis	Bees	*Bombus pratorum, B. terrestris, B. pascuorum*	1
Pulmonaria obscura	Bees	*Bombus pascuorum, B. hortorum, B. hypnorum, B. lapidarius, B. pratorum, B. terrestris, P. bohemicus*	14
Sebaea grandis	-	-	31
Hypericum aegypticum	-	-	17
Linum perenne	-	-	11
Linum tenuifolium	-	-	10
Lythrum californicum	Bees	*Apis mellifera*	19
	Butterflies, Flies	-	19
Lythrum lineare	Bees, Butterflies	-	19
Menyanthes trifoliata	-	-	7
Persicaria japonica	Flies	*Eristalomya tenax*	12
	Wasps	*Campsomeriella annulata annulata, Polistes chinensis antennalis*	12
Primula elatior	Bees	*Bombus terrestris, B. lapidarius*	25
Primula sieboldii	Bees	*Bombus diversus tersatus, B. schrencki albidipleuralis, B. deuteronymus deuteronymus*	13
	Butterflies	*Polygonia c-aureum, Artogeia rapae crucivora*	28
Primula veris	Bees	-	22
Primula vulgaris	Bees	-	20
Hedyotis caerulea	Flies	-	21
Palicourea demissa	Hummingbirds	-	26, 27

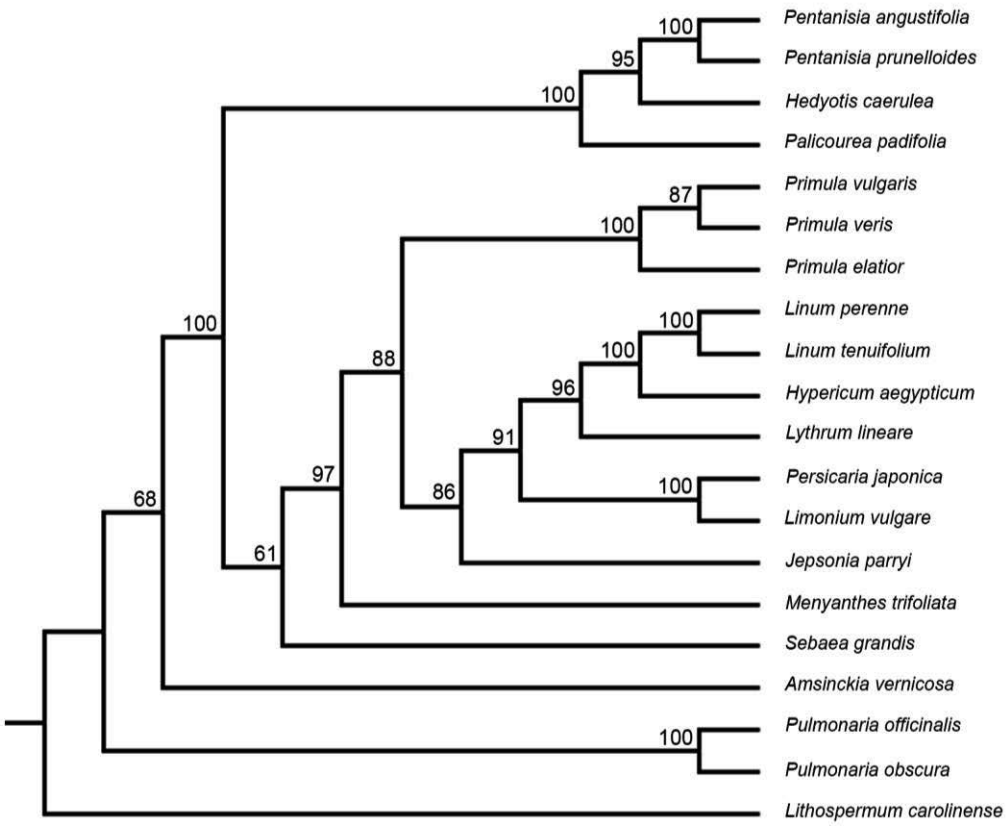
Cont.

Species	Main floral visitors	Floral visitor's species	Refs
Palicourea padifolia	Hummingbirds	-	5
Pentanisia angustifolia	Butterflies	*Papilio demodocus, Junonia archesia, Danaus chrysippus*	8
Pentanisia prunelloides	Butterflies	*Eurema brigitta*	8
Jepsonia heterandra	Bees	*Dialictus ornduffi*	3, 16
Jepsonia parryi	Bees, Flies	-	15
Limonium vulgare	Bees, Flies	-	2

Appendix 2.3. GenBank accession numbers of the species used to construct the phylogenetic tree. When no sequence was available for the target species, sequences from another species of the same genus were used instead and are denoted in square brackets. "-" no sequence available.

Species	***ITS***	***matK***	***tRNA-Leu***	***tnrL-trnF***
Amsinckia vernicosa	KP027090	-	KC542581	KC542581
Lithospermum caroliniense	FJ763231	FJ827260	FJ763288	FJ763288
Pulmonaria obscura	KP219537	-	FJ763264	FJ763264
Pulmonaria officinalis	-	HQ619799	FJ490773	FJ490773
Sebaea grandis	FJ666022	FJ014102	KC763533	KC763533
Hypericum aegypticum	KC709391	HQ331617	-	-
Linum perenne	FJ169524	AB038182	FJ160886	FJ160886
Linum tenuifolium	KU674813	HE966947	FJ160893	FJ160893
Lythrum lineare	AY910748	KJ772928 [*L. alatum*]	-	-
Menyanthes trifoliata	DQ276850	AJ429386	KM191908	KM191908
Persicaria japonica	EU196885	EU196938	EU197024	EU197024
Primula elatior	HM629085	DQ378361	JX231089	-
Primula veris	AY680704	AJ429293	AJ430883	-
Primula vulgaris	AJ427800	HQ619829	FJ490810	FJ490810
Hedyotis caerulea	AM939464	-	-	EU543109
Palicourea padifolia	AF072008	HM446727 [*P. croceoides*]	AF152615 [*P. guianensis*]	AF152615 [*P. guianensis*]
Pentanisia angustifolia	AM267025	-	AM266939	AM266939
Pentanisia prunelloides	AM267033	JQ024980	AF152617	AF152617
Jepsonia parryi	U51262	L34128	-	-
Limonium vulgare	AJ222839	JN895287	AJ391332	AJ391332

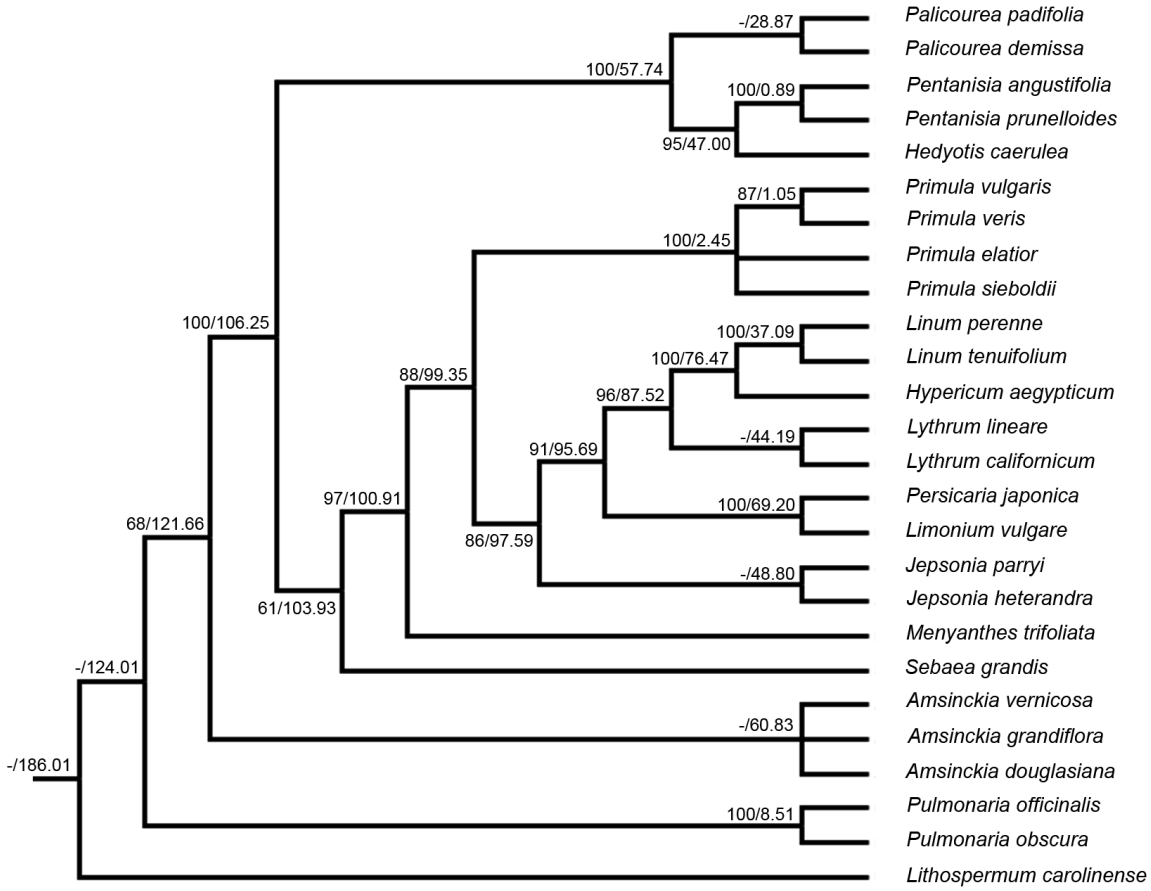
Appendix 2.4. Best ML phylogenetic tree (see *Materials and methods* for details). Values above the branches are BP calculated after 1000 bootstrap replicates.



Appendix 2.5. Models of substitution rate of variation among branches and values of the smoothing parameter, lambda, tested for creating an ultrametric tree. The final model and lambda value were chosen accordingly with the combination that resulted in the lowest value of PHIIC, *i.e.*, model selection criterion. In this case, no differences were obtained when changing the smoothing parameter for the model "strict" and thus, the simplest model was chosen (model "strict", lambda value "0").

Model	lambda	PHIIC
Relaxed	0	124.7950
Relaxed	0.1	125.0181
Relaxed	0.2	125.2845
Relaxed	1	127.3172
Correlated	0	124.7950
Correlated	0.1	124.7950
Correlated	0.2	124.7950
Correlated	1	124.7950
Strict	0	54.0346
Strict	0.1	54.0346
Strict	0.2	54.0346
Strict	1	54.0346

Appendix 2.6. Best ML phylogenetic tree after calibration (see *Materials and methods* for details). Values in the tree are BP followed by the node age given in millions of years. "-" no BP calculated.



Chapter 3 – Experimental insights on Darwin's cross-promotion hypothesis in tristylous purple loosestrife (*Lythrum salicaria*)

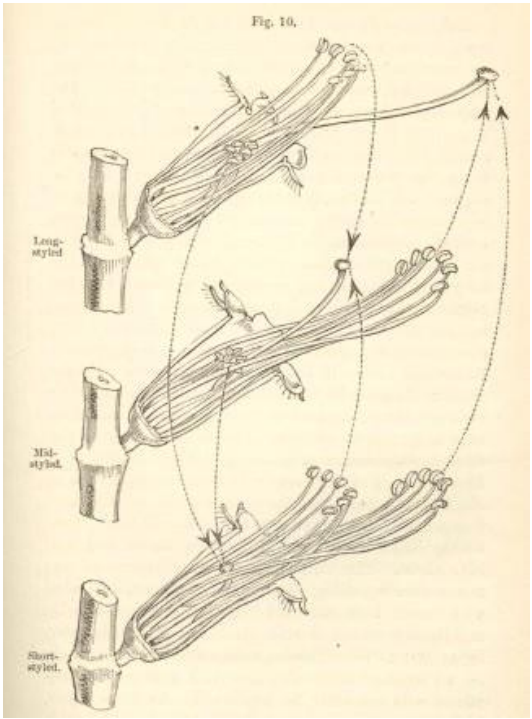


Diagram of three style morphs of *Lythrum salicaria*. Arrows represent compatible pollinations (Darwin, 1887 p.139).

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ABSTRACT

Darwin proposed that the reciprocal arrangement of anthers and stigmas in heterostylous plants promotes cross-pollination through pollen segregation on pollinators' bodies. The floral tube found in most heterostylous species constrains the feeding posture of pollinators determining the site of contact with sex organs located at different heights within a flower. Here, I evaluate Darwin's hypothesis in tristylous *Lythrum salicaria*, a species with a partially tubular corolla, and examine the extent to which the location of sex organs within a flower influence compatible and incompatible pollination. I predicted that the proficiency of cross-pollination would increase for more inserted sex organs due to the restrictions imposed by the floral tube on pollinator positioning. I used experimental trimorphic and monomorphic arrays and emasculated flowers to quantify intermorph pollen transfer and capture among all sex-organ heights, and estimated the contribution of intraflower self-pollination, geitonogamous self-pollination and intramorph outcross pollination to total intramorph pollination. As predicted, disassortative pollination varied significantly with sex-organ height and was highest for short-level organs and lowest for long-level organs. In monomorphic arrays, most intramorph pollination resulted from outcross pollination followed by intraflower and geitonogamous self-pollination. My results provide experimental evidence that reciprocal herkogamy promotes varying degrees of disassortative pollination among the floral morphs and that sex-organ heights play an important role in determining the composition of pollen loads.

KEY WORDS: cross-promotion hypothesis; disassortative pollination; floral design; heterostyly; pollen capture; pollen transfer proficiency.

INTRODUCTION

The remarkable diversity of floral traits among angiosperm species largely results from natural selection on the mechanisms promoting cross-pollination and limiting the costs of self-pollination. In many animal-pollinated species with tubular flowers, floral design determines the spectrum of suitable flower visitors by imposing restrictions on their posture while probing flowers for nectar (Darwin, 1862a; Faegri and Van der Pijl, 1971; Harder and Johnson, 2009). The floral polymorphism heterostyly provides a noteworthy example of the structural fit between flowers and pollinators. Darwin (1877) proposed the first adaptive explanation for the function of heterostyly. He interpreted reciprocal herkogamy as a floral mechanism promoting cross-pollination between anthers and stigmas positioned at equivalent heights, thus reducing pollen wastage on incompatible stigmas. He further suggested that disassortative pollination was achieved owing to segregated pollen deposition on the bodies of pollinators during their visits to flowers.

Empirical evaluation of "Darwin's cross-promotion hypothesis" has been conducted in numerous studies by examining pollen capture in natural populations of distylous species (*e.g.*, *Jepsonia heterandra*, Ganders, 1974; *Pulmonaria obscura*, Olesen, 1979; *Primula elatior*, Schou, 1983; *Linum perenne*, Nicholls, 1986; *Palicourea padifolia*, Ree, 1997; reviewed in Barrett and Shore, 2008; Chapter 2). The distinctive pollen size dimorphism of most distylous species allows the unambiguous identification of the source of pollen on stigmas, thus enabling estimates of the amounts of intermorph versus intramorph pollen transfer and capture. Studies of this type are more limited in tristylous species, not only due to their restricted taxonomical distribution, but also because pollen size trimorphism is less distinct in most species, which complicates identification of the three pollen types on stigmas of the floral morphs. In particular, tristylous species often exhibit considerable overlap in the size of pollen produced by mid- and short-level anthers (*e.g.*, *Eichhornia crassipes*, Barrett, 1977a; *E. paniculata*, Barrett, 1985; *Decodon verticillatus*, Eckert and Barrett, 1994; *Oxalis suksdorfii*, Ornduff, 1964; *O. alpina*, Weller, 1979). Nevertheless, studies of naturally occurring pollen loads in *Lythrum salicaria* (Mulcahy and Caporello, 1970) and *L. junceum* (Ornduff, 1975b) were conducted in an effort to assess Darwin's cross-promotion hypothesis. This was despite the fact that any conclusions reached could only concern long-level organs because of the inability to distinguish pollen from mid- and short-level stamens in these species. Only tristylous *Pontederia* species, which exhibit strong

pollen size trimorphism, provide an experimental system suitable for investigations of the effectiveness of reciprocal herkogamy in promoting disassortative pollination under field conditions. Indeed, several studies have examined pollen loads in natural populations of *P. cordata* (Price and Barrett, 1982; Barrett and Glover, 1985; Glover and Barrett, 1986) and *P. sagittata* (Glover and Barrett, 1983), the former providing good evidence for Darwin's cross-promotion hypothesis (reviewed in Lloyd and Webb, 1992b).

Besides the distinctive features of the heterostylous syndrome outlined in Chapter 1, families in which heterostyly has evolved share several other floral characters suggesting that particular floral traits are a prerequisite for heterostyly to evolve (Ganders, 1979; Lloyd and Webb, 1992a). Flowers are mostly insect or bird pollinated, hermaphroditic with the stigma in a central position within the flower and the stamens grouped into one (distyly) or two (tristyly) discrete levels (Ganders, 1979; Lloyd and Webb, 1992a). Corollas are predominantly actinomorphic, less frequently moderately zygomorphic (*e.g.*, *Pontederia*, Barrett, 2004; *Tylosema esculentum*, Hartley *et al*., 2002; *Salvia brandegeei*, Barrett *et al*., 2000), and usually form a distinct floral tube that conceals the floral reward (nectar) at its base. This floral design is described as stereomorphic, with pollinators inserting their proboscides deep within the floral tube (*sensu* depth-probed flowers; Lloyd and Webb, 1992a). However, some exceptions to these generalizations occur (Lloyd and Webb, 1992a p. 156). For example, *Hypericum aegypticum* has dish-shaped, nectarless flowers and numerous stamens (Ornduff, 1975a), and similar dish-shaped flowers are also present in *Fagopyrum esculentum* (Björkman, 1995) and *Turnera ulmifolia* (Barrett and Shore, 1987) raising the question on how the specific floral designs of heterostylous species function in the pollination process to promote disassortative pollination.

Here, I examine Darwin's cross-promotion hypothesis in tristylous purple loosestrife (*Lythrum salicaria*, Lythraceae) and assess the influence of sex-organ height on patterns of disassortative pollination. I was motivated to investigate this species for two reasons. First, the design of *L. salicaria* flowers suggests that patterns of pollen transfer and capture might differ among sex-organ levels. The species possesses weakly zygomorphic flowers in which the floral tube is only partially developed extending from the base of the ovary until approximately the height of mid-level organs. Consequently, short-level organs are completely concealed within the floral tube, mid-level organs are located at the mouth of the tube and long-level organs are exerted well beyond the floral

tube (Fig. 3.1). This variation seems likely to influence the nature of contacts between pollinators and sex organs with implications for the extent to which segregated pollen deposition (Darwin, 1877; Olesen, 1979; Lewis, 1982; Wolfe and Barrett, 1989; Brys *et al*., 2008) and disassortative pollination (Ganders, 1974; Barrett and Glover, 1985) occur among sex-organ heights. A second motivation for investigating *L. salicaria* was to improve on previous investigations of the species, which only considered pollen transfer between long-level organs because of the inability to distinguish pollination events involving mid- and short-level pollen (Mulcahy and Caporello, 1970). By using experimental arrays and the emasculation of flowers, I was able to distinguish pollen transfer and capture among the three sex-organ levels that characterize tristylous species, and thus more fully evaluate Darwin's cross-promotion hypothesis.

Figure 3.1. Flowers of *Lythrum salicaria*. **A.** long-styled morph. **B.** mid-styled morph. **C.** short-styled morph.

My study addressed the following specific questions: (1) How does variation in the degree of insertion of stamen and stigma levels affect intermorph pollen transfer and capture? I predicted that short-level organs borne deep within the floral tube would have the highest proficiency of compatible pollen transfer and capture, and that the long-level organs would have the lowest. This prediction is based on Lloyd and Webb's proposal that the floral tube in heterostylous species restricts insect feeding posture, and that the path followed by the pollinator's probe results in more precise pollen transfer between inserted than exerted sex organs (Lloyd and Webb, 1992b p. 200: Fig. 1; and see Stone and Thomson, 1994). (2) What are the relative components of intramorph pollination? By using monomorphic arrays and emasculated flowers, I compared the relative amounts of intramorph pollen that results from within-flower self-pollination, geitonogamous self-pollination and intramorph outcross pollination. This approach allowed me to investigate the conclusion of Mulcahy and Caporello (1970 p. 1030)

from their experimental studies that tristyly probably functions to "reduce selfing within flowers and between flowers on the same plant". My results allowed me to investigate Darwin's cross-promotion hypothesis in the only tristylous species that he investigated in detail (Darwin, 1864, 1868, 1877), and to evaluate the role of floral design in affecting the pollination process.

MATERIALS AND METHODS

Study species

Lythrum salicaria is a Eurasian wetland perennial plant that produces hundreds, often thousands, of purple-pinkish flowers (Mal *et al*., 1992) with a floral tube that is 5-6.5 mm long and 2-2.5 mm wide (Velayos, 1997). I collected seeds from two natural populations in Portugal (geographical coordinates, Coimbra: 40.20050, -8.42867; Penacova: 40.26967, -8.27467) in September 2013 and these were germinated in March 2014. Subsequently, individual plants were transferred singly to 2 L pots filled with a standard horticultural soil mix and fertilized every two weeks (Substral®) until used in the pollination experiments described below. I conducted the experiments in mid-July 2015 in an experimental garden located at the Botanical Garden of the University of Coimbra, Portugal. This corresponds to the natural flowering time of populations in this region of the native area. Plants from the two populations were near identical in phenotype, were of comparable stature and flowering phenology, and I therefore did not distinguish the source of plants in the experimental arrays.

I characterized pollen size by measuring the polar axis and the equatorial diameter of 20 pollen grains per anther level from five plants of each style morph under a light microscope (Leitz HM-LUX 3; 400× magnification) by using a calibrated ocular micrometer. To quantify pollen production per anther level for each style morph, I collected one flower from 10 plants of the three style morphs before anther dehiscence. One anther per level was later transferred to a microscope slide and dissected to release the pollen grains, which were then counted under a light microscope (Leitz HM-LUX 3; 100× magnification) as the slide was shifted back and forth in a linear fashion across the entire coverslip. I multiplied the value obtained from one anther by six to account for the number of anthers per stamen level within each flower.

Experimental trimorphic arrays

To investigate intermorph compatible and incompatible pollen transfer and capture, I set up four trimorphic arrays containing 12 plants, four of each style morph, placed approximately 20 cm apart in a six by two grid. Plants were trimmed to approximately the same number of flowers (between 15-20 open flowers per plant). In each array, one style morph was assigned as a "pollen donor", and its flowers were left intact, while the remaining two morphs were "pollen recipients" and their flowers were all emasculated (Fig. 3.2A). I emasculated flowers to exclude possibilities for intramorph pollination in each array and to unambiguously identify the source of intermorph pollen on recipient stigmas. I removed anthers 3h before anthesis by using fine forceps and both the anther filaments and corolla were left intact during this procedure.

Because of the overlap in size of pollen grains produced by mid- and short-level anthers of the L-morph (Fig. 3.3), two separate experimental arrays were required when using this morph as a pollen donor. In contrast, only a single array was necessary when using the M- and S-morphs as pollen donors. For the two arrays using the L-morph as a pollen donor, only a single anther level (either mid- or short-level) donated pollen to recipients in each array (Fig. 3.2A), with the alternate anther level being removed by emasculation.

Plants in the arrays were left to experience open pollination for 2h, after which time I removed stigmas from emasculated recipient flowers with fine forceps, and these were then squashed underneath a coverslip on a microscope slide using glycerin as a mounting medium. The experimental arrays received abundant pollinator visits and therefore 2h were sufficient to obtain pollinated stigmas from all plants. The sampled stigmas could potentially receive intermorph pollen from reciprocal and non-reciprocal anther levels. The four experimental arrays were randomly assigned to a given day and replicated twice, with one array during the morning (10-12 am, GMT) and the other during the afternoon (2-4 pm, GMT). The arrays involved different plants thus allowing to account for within-day variation in pollen transfer and capture. Following the completion of the first set of arrays, I performed a second set so that the four trimorphic arrays were replicated twice in a randomized block design with a two-day interval between the blocks.

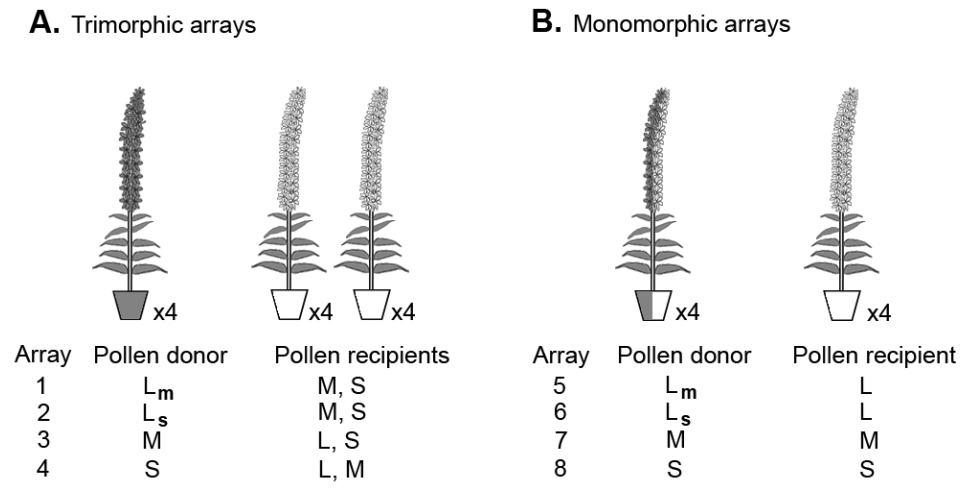

Figure 3.2. Experimental arrays of *Lythrum salicaria* used in this study. **A.** Trimorphic arrays. The shaded inflorescences represent the pollen donor with intact flowers, whereas unshaded inflorescences represent pollen recipients with all flowers emasculated. **B.** Monomorphic arrays. The half-shaded inflorescence represents the pollen donor with half of its flowers intact and half emasculated, whereas unshaded inflorescences indicate pollen recipients with all flowers emasculated. L_m and L_s indicate plants of L-morph with only mid-level anthers and L-morph plants with only short-level anthers as pollen donors, respectively. The number of pollen donors and recipient plants in an array is given next to each plant.

Experimental monomorphic arrays

To investigate intramorph pollination including self-pollen transfer and capture, I set up four monomorphic arrays containing eight plants of one style morph, placed approximately 20 cm apart in a four by two grid. Plants were trimmed to have approximately the same number of flowers, as in trimorphic arrays. In each array, four plants were assigned as "pollen donors" and half of their flowers were left intact and half were emasculated. This procedure was undertaken so that I could investigate intraflower and geitonogamous pollination separately. The remaining four plants were assigned as "pollen recipients" and their flowers were all emasculated (Fig. 3.2B). I emasculated recipient flowers so that intramorph pollination between plants was not confounded with intramorph pollination within plants. As for trimorphic arrays, two experimental arrays were required for the L-morph in which emasculation of one of the two anther levels was performed (Fig. 3.2B), and this allowed me to distinguish the pollen source in stigmatic pollen loads. I used the same emasculation procedure as for trimorphic arrays.

Plants were left for open pollination for 2h and then the stigmas were removed from pollen donors (intact and emasculated flowers) and recipient plants with fine forceps and treated in the same manner as described above. I used the same randomized block

design previously described for trimorphic arrays. On donor plants, intact flowers received intramorph outcross pollen, geitonogamous and intraflower self-pollen, whereas emasculated flowers of pollen donors captured intramorph outcross and geitonogamous pollen. Emasculated flowers of recipient plants captured only intramorph outcross pollen. By subtracting the average pollen load of emasculated flowers on recipient plants from the average pollen load of emasculated flowers from donor plants, I estimated the amount of geitonogamous pollination for each style morph. Similarly, by subtracting the average pollen load of emasculated flowers of donor plants from the average pollen load of intact flowers from donor plants, I estimated the amount of intraflower self-pollination for each style morph.

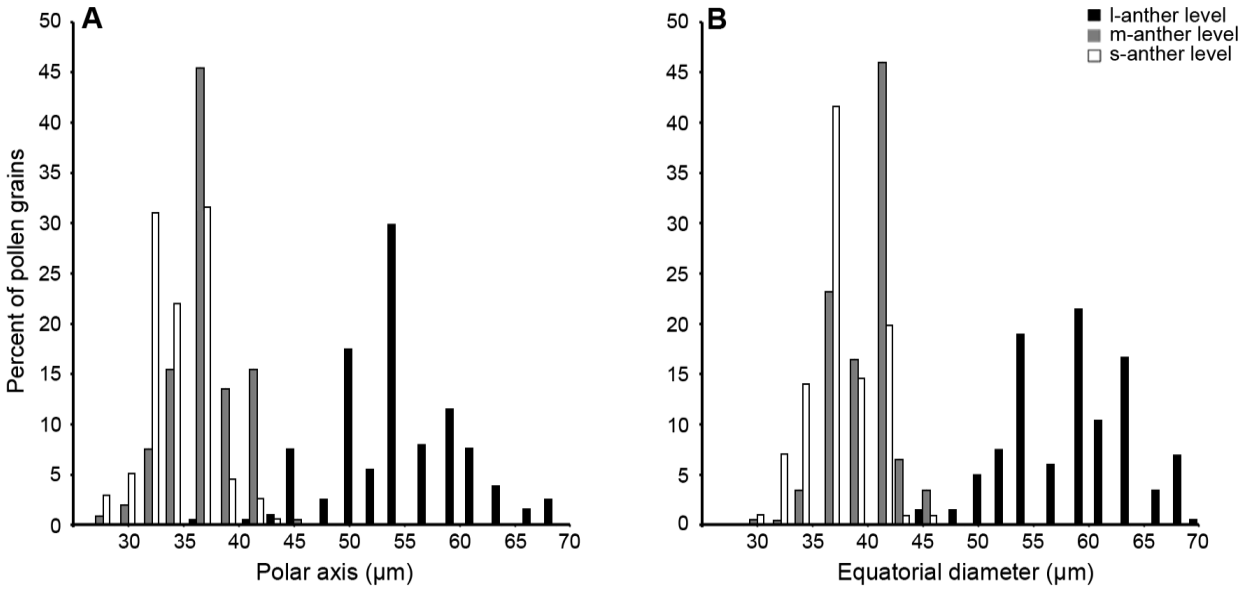

Figure 3.3. The frequency distribution of pollen size in *Lythrum salicaria* produced by each anther level (n = 200) used in experimental arrays. **A.** Polar axis of pollen. **B.** Equatorial diameter of pollen. Black, grey and white bars for l-, m- and s-anther levels, respectively.

Pollinator visitation

To determine if insects visiting *L. salicaria* flowers in experimental arrays showed a preference for intact compared to emasculated flowers, I conducted pollinator observations in trimorphic arrays. I recorded the identity of insect visitors to flowers and the number of intact versus emasculated flowers that were visited during 10 min intervals for a total of 180 min of observation across the two experimental blocks.

Statistical analyses

I assessed differences in pollen production between anther levels of each style morph and in total pollen production among style morphs either by ANOVA or the non-parametric Kruskal-Wallis test depending on data distribution. I investigated differences in pollen size (*e.g.*, polar axis and equatorial diameter) among anther levels and in pollinator visitation between intact and emasculated flowers by using GLMs with a Gamma distribution and a log link function.

To investigate pollen transfer in the experimental arrays, I calculated the pollen transfer proficiency (T_{ij}) following the equation provided in Chapter 2 p. 31 (Lloyd and Webb, 1992b). I investigated differences in pollen transfer proficiencies and capture among style morphs for: (1) compatible and incompatible intermorph pollinations, and (2) intramorph pollinations by GLMMs with a Gamma distribution and a log link function. In the intermorph comparisons, anther level and style morph were specified as fixed factors in a model either across anther levels or style morphs, for pollen transfer and capture, respectively. In the analyses conducted for assessing pollen transfer of each anther level and pollen capture by style morph, either style morph or anther level was treated as fixed factors, respectively. Intramorph pollen type (*i.e.*, intramorph outcross, geitonogamous and intraflower self-pollen) and style morph were designated as fixed factors in a model either across intramorph pollen types or style morphs for pollen transfer and capture, respectively. Style morph and intramorph pollen type were used as fixed factors in separate analysis of pollen transfer of each intramorph pollen type and capture by style morph, respectively. Here, and in the following GLMM analyses, time of day (*i.e.*, morning or afternoon) nested within day was treated as random factor. Prior to statistical analysis, stigmatic pollen load data was $\log_{10}(x+2)$ transformed (Zar, 2010).

I examined the proficiency of transfer of each component of the stigmatic load (*i.e.*, intermorph compatible, intermorph incompatible, intramorph outcross, geitonogamous and intraflower self-pollen) and their capture for each style morph separately. The component of the stigmatic pollen load was defined as a fixed factor in the GLMMs.

I performed all statistical analyses with R software version 3.0.1 (R Core Development Team, 2013) using the following packages: "car" for Type-III analysis of variance as an integrated part of the GLMs and GLMMs (Fox and Weisberg, 2015), "lme4" for GLMMs (Bates *et al.*, 2014), "multcomp" for multiple comparisons after Type-III analysis of variance (Hothorn *et al.*, 2015), "nlme" for linear mixed models

(Pinheiro *et al*., 2015), and "stats" for Shapiro-Wilk normality test, Kruskal-Wallis test, and GLMs (R Core Development Team, 2013).

Results

Pollen size and production

There were significant differences in polar axis (mean ± SE, long-level: 54.05 ± 0.39; mid-level: 36.20 ± 0.20; short-level: 33.75 ± 0.20; $\chi^2_{2,597}$ = 3286.60, $P < 0.001$) and equatorial diameter (mean ± SE, long-level: 58.08 ± 0.37; mid-level: 39.06 ± 0.19; short-level: 36.67 ± 0.20; $\chi^2_{2,597}$ = 3908.70, $P < 0.001$) of pollen grains produced by the three anther levels of *L. salicaria*. However, there was considerable overlap in the overall size of pollen produced by mid- and short-level anthers, whereas pollen from long-level anthers was significantly larger (Fig. 3.3).

There was no significant difference in pollen production per flower between anther levels of the L-morph (mean ± SE, mid-level: 7,905.00 ± 529.34; short-level: 7,917.60 ± 711.88; $F_{1,18}$ = 0.002, P = 0.99). In contrast, pollen production per flower differed significantly between anther levels of the M-morph (mean ± SE, long-level: 5,566.80 ± 474.16; short-level: 8,614.30 ± 748.68; $F_{1,18}$ = 14.82, P = 0.001) and the S-morph (mean ± SE, long-level: 4,884.40 ± 402.48; mid-level: 7,671.30 ± 938.17; $H_{1,18}$ = 7.00, P = 0.008). In general, long-level anthers produced significantly less pollen than mid- and short-level anthers ($P < 0.05$), the latter two not differing in pollen production. I found no significant differences in total pollen production per flower among style morphs (mean ± SE, L-morph: 15,822.60 ± 1,066.00; M-morph: 14,181.10 ± 1,014.47; S-morph: 12,555.70 ± 1,251.63; $F_{2,27}$ = 2.14, P = 0.14).

Pollinator visitation

The primary visitor to flowers of *L. salicaria* during the experiment was the long-tongued bee *Anthidium manicatum* (Megachilidae) with individuals of this species accounting for 81.5% of the total insect visits. Less frequently, I observed *Apis mellifera* (Apidae), syrphid flies (Syrphidae) and *Xylocopa violacea* (Apidae) visiting flowers in the arrays. There was no significant difference in the level of visitation to intact and emasculated flowers (mean number of flowers visited during 10 min intervals ± SE, 12.02 ± 1.77 and 9.50 ± 1.20 for intact and emasculated flowers, respectively; $\chi^2_{1,106}$ = 3.75, P = 0.05).

Compatible and incompatible intermorph pollen transfer and capture

I detected a significant interaction between anther level and recipient style morph in pollen transfer proficiencies ($P < 0.001$; Table 3.1). Pollen produced by the exerted long-level anthers had a significantly higher probability of being transferred to stigmas of the L-morph than to stigmas of the M- or S-morphs ($\chi^2_2 = 34.49$, $P < 0.001$; Fig. 3.4A; Appendix 3.1). In contrast, pollen from mid-level anthers was transferred to stigmas of all three morphs equivalently ($\chi^2_2 = 1.51$, $P = 0.47$; Fig. 3.4A; Appendix 3.1). The most proficient transfer of pollen was evident from the inserted short-level anthers, which was preferentially transferred to stigmas of the S-morph ($\chi^2_2 = 34.83$, $P < 0.001$; Fig. 3.4A; Appendix 3.1).

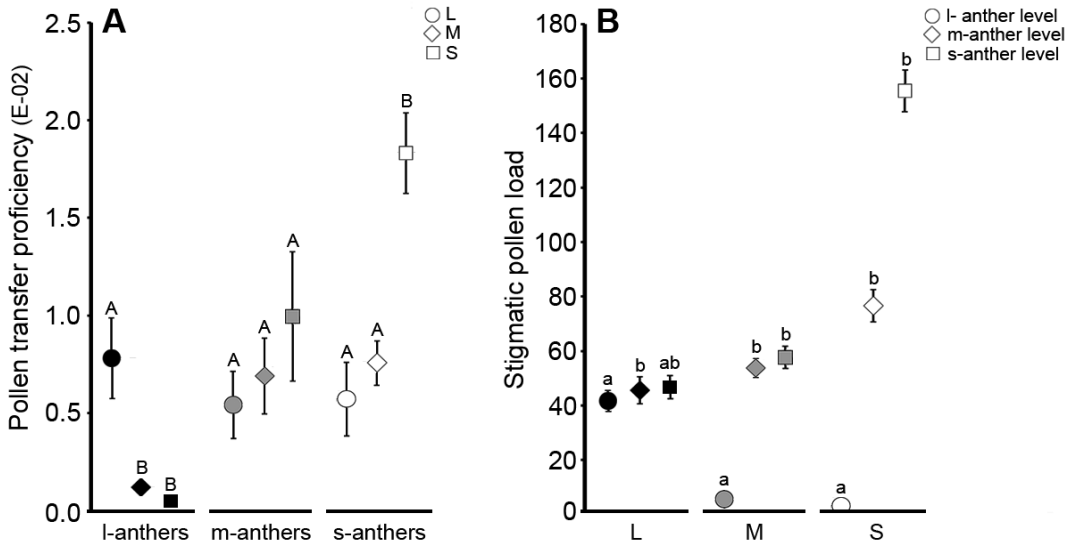

Figure 3.4. Pollen transfer and capture in trimorphic arrays of *Lythrum salicaria*. Values are mean and SE of pollen transfer proficiencies and number of pollen grains per stigma for pollen transfer and capture, respectively. **A.** Pollen transfer proficiency of each anther level to the stigma. See *Materials and methods* for the calculation of pollen transfer proficiencies. Different uppercase letters indicate statistically significant differences in pollen transfer proficiency. **B.** Pollen capture based on stigmatic pollen load for each style morph. Different lowercase letters represent statistically significant differences in pollen capture of style morphs.

Intermorph pollen capture was significantly different among the style morphs ($P < 0.001$; Table 3.1) and there was a significant interaction between style morph and anther level ($P < 0.001$; Table 3.1). Stigmas of the L-morph captured significantly more incompatible than compatible intermorph pollen ($\chi^2_2 = 31.31$, $P < 0.001$; Fig. 3.4B; Appendix 3.1), whereas stigmas of the S-morph captured significantly more compatible

than incompatible intermorph pollen (χ^2_2 = 897.44, P < 0.001; Fig. 3.4B; Appendix 3.1). Stigmas of the M-morph captured similarly large amounts of pollen from the mid and short-level anthers, but less pollen from long-level anthers (χ^2_2 = 345.39, P < 0.001; Fig. 3.4B; Appendix 3.1).

Table 3.1. Results of the GLMMs examining the pollen transfer and capture in (a-b) trimorphic and (c-d) monomorphic arrays of *Lythrum salicaria*. Values in bold represent statistically significant differences.

	df	χ^2	***P***
(a) Trimorphic arrays: pollen transfer			
Style morph	2	**69.02**	**< 0.001**
Anther level	2	0.20	0.905
Style morph × anther level	4	**68.95**	**< 0.001**
(b) Trimorphic arrays: pollen capture			
Style morph	2	**296.97**	**< 0.001**
Anther level	2	**52.42**	**< 0.001**
Style morph × anther level	4	**278.12**	**< 0.001**
(c) Monomorphic arrays: pollen transfer			
Style morph	2	3.84	0.146
Component of the pollen load	2	**11.74**	**0.003**
Style morph × component	4	2.78	0.595
(d) Monomorphic arrays: pollen capture			
Style morph	2	**13.26**	**0.001**
Component of the pollen load	2	**12.25**	**0.002**
Style morph × component	4	7.71	0.103

Intramorph pollen transfer and capture

The components of intramorph pollen differed significantly in their transfer to each style morph (P = 0.003; Table 3.1). The probability of intramorph outcross pollen transfer was significantly higher for the L-morph than for the M- and S-morphs (χ^2_2 = 23.42, P < 0.001; Fig. 3.5A; Appendix 3.2). In contrast, I detected no significant differences in geitonogamous pollen transfer (χ^2_2 = 2.11, P = 0.35; Fig. 3.5A; Appendix 3.2) or intraflower self-pollen transfer (χ^2_2 = 0.73, P = 0.70; Fig. 3.5A; Appendix 3.2) among style morphs.

There were significant differences in pollen capture among style morphs (P = 0.001; Table 3.1) and among the three components of the intramorph pollen load (P = 0.002; Table 3.1). Geitonogamous pollen capture was significantly lower than intramorph outcross and intraflower pollen capture in each of the three style morphs (L-morph, χ^2_2 = 8.64, P = 0.01; M-morph, χ^2_2 = 11.33, P = 0.003; S-morph, χ^2_2 = 16.91, P < 0.001; Fig. 3.5B; Appendix 3.2). There were small differences in capture of intramorph outcross pollen and intraflower self-pollen, but these were not statistically significant in each of the three style morphs (P > 0.05; Fig. 3.5B).

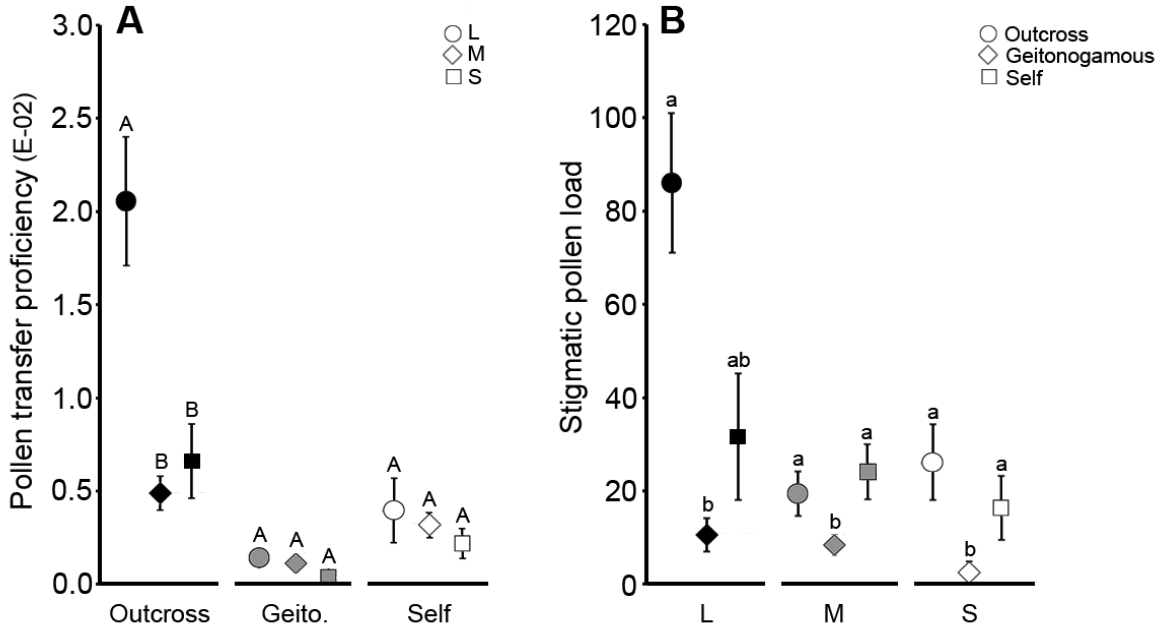

Figure 3.5. Pollen transfer and capture in monomorphic arrays of *Lythrum salicaria*. Values are mean and SE of pollen transfer proficiencies and number of pollen grains per stigma for pollen transfer and capture, respectively. **A.** Pollen transfer proficiency of each intramorph pollen type (*i.e.*, intramorph outcross, geitonogamous and intraflower self-pollen) to stigmas. See *Materials and methods* for the calculation of pollen transfer proficiencies. Different uppercase letters represent statistically significant differences in the proficiency of intramorph pollen transfer to the three style morphs. **B.** Pollen capture based on stigmatic pollen load for each style morph. Different lowercase letters represent statistically significant differences in pollen capture within style morph. "Geito." for geitonogamous pollen.

Evidence for Darwin's cross-promotion hypothesis

Darwin's cross-promotion hypothesis would be supported if values for compatible intermorph pollen transfer and capture were significantly higher than equivalent values from incompatible intermorph pollination. This was indeed the pattern for the M- and S-morph, with higher pollen transfer and capture between anthers and stigmas that were

reciprocally positioned (Fig. 3.6; Appendix 3.3). I found no differences between compatible and incompatible intermorph pollen transfer and capture for the L-morph (Fig. 3.6; Appendix 3.3).

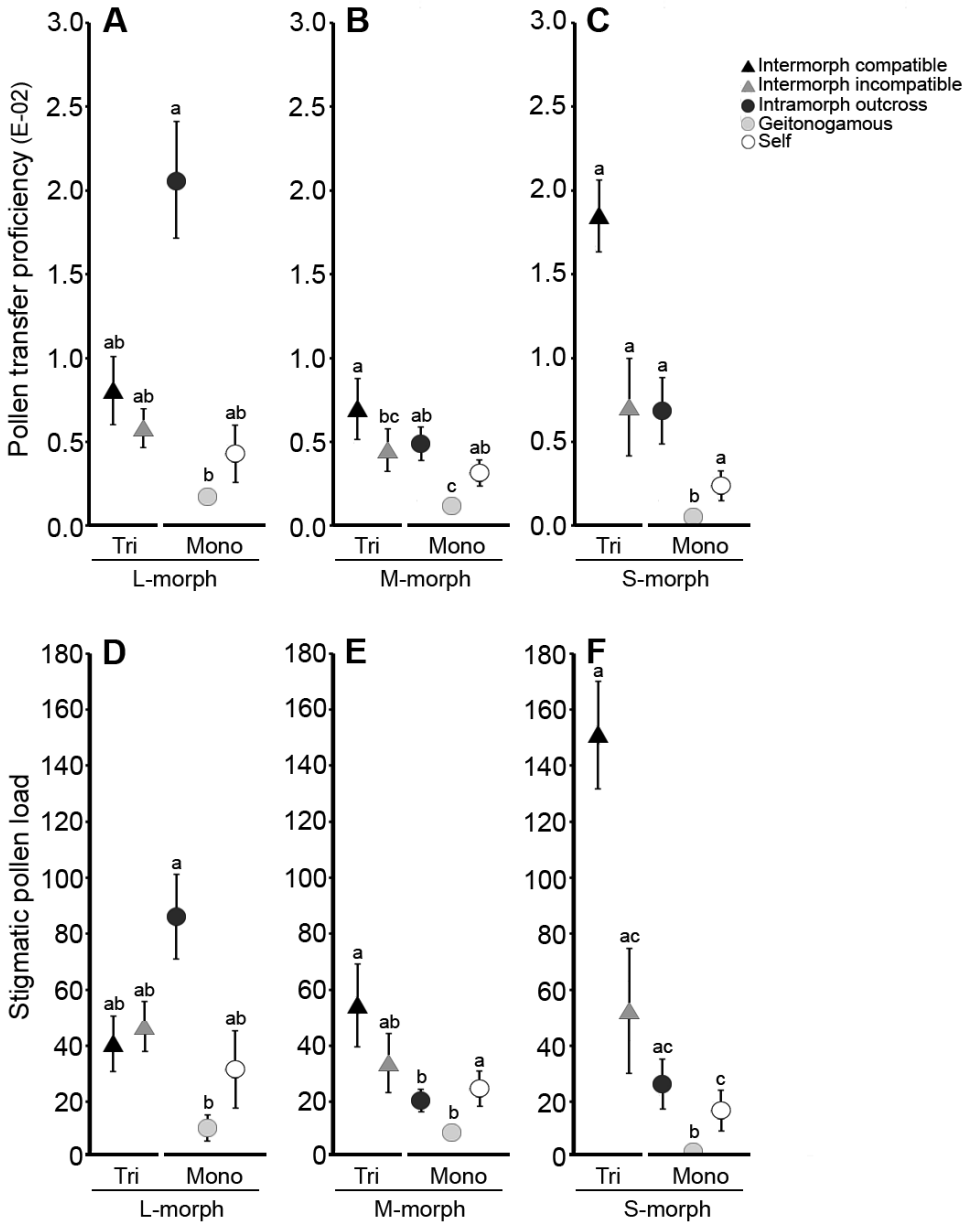

Figure 3.6. Pollen transfer and capture of each component of the stigmatic load in experimental arrays for *Lythrum salicaria*. Values are mean and SE. **A, B, C.** Pollen transfer proficiency of each pollen type (*i.e.*, compatible intermorph, incompatible intermorph, intramorph outcross, geitonogamous and intraflower self-pollen) to the stigma. See *Materials and methods* for the calculation of pollen transfer proficiencies. **D, E. F.** Pollen capture based on stigmatic pollen load for each style morph. Different lowercase letters represent statistically significant differences in pollen transfer and capture within style morph. "Tri" and "Mono" for trimorphic and monomorphic arrays, respectively.

DISCUSSION

My investigation of pollen transfer and capture in *Lythrum salicaria*, a tristylous species with partially tubular flowers, revealed several main findings: (1) as predicted, the amount of disassortative pollen transfer and capture was greatest for anthers and stigmas located deep within the floral tube and diminished with increased levels of sex-organ exertion (Fig. 3.4); (2) intramorph (incompatible) pollination occurred mainly between plants and was particularly high in the L-morph (Fig. 3.5); (3) most self-pollination resulted from intraflower pollen transfer and capture, with levels of geitonogamous pollination being relatively low in each of the three style morphs (Fig. 3.5). My results provide some support for Darwin's cross-promotion hypothesis (Fig. 3.6), but also indicate that floral design plays an important role in determining the amounts of disassortative pollen transfer among sex-organ levels. Below I consider the floral mechanisms governing the pollination process in *L. salicaria* and compare my findings with other studies of pollen capture in heterostylous species.

Evidence for disassortative pollination in purple loosestrife

The majority of the studies investigating Darwin's cross-promotion hypothesis have examined the composition of stigmatic pollen loads in natural populations of heterostylous species (reviewed in Lloyd and Webb, 1992b; Barrett and Shore, 2008). These studies have most commonly compared the relative frequency of compatible (legitimate) and incompatible (illegitimate) pollen captured by stigmas with random expectations based on the frequency of pollen types produced in the populations (*e.g.*, Ganders, 1974; Olesen, 1979; Glover and Barrett, 1983). In my study, I compared compatible and incompatible intermorph pollen capture in experimental arrays using emasculated flowers, so that I could distinguish pollen transfer among the three sex-organ levels. This was necessary because of the strong overlap in size of pollen produced by mid- and short-level anthers (Fig. 3.3). Following the identification of pollen types in stigmatic pollen loads, I used analysis of variance to compare the amounts of compatible and incompatible pollen capture, and quantified the proficiency of pollen transfer as the likelihood that a single pollen grain is involved in compatible or incompatible pollination. This method was first introduced by Lloyd and Webb (1992b) and provides a means of evaluating the effectiveness of heterostyly in promoting pollination success through male function. Since its introduction, this approach has been used to analyze patterns of pollen transfer in several distylous species (*Palicourea*

padifolia, Ree, 1997; *Persicaria japonica*, Nishihiro and Washitani, 1998; *Gaertnera vaginata*, Pailler *et al*., 2002; *Pulmonaria officinalis*, Brys *et al*., 2008; *Palicourea demissa*, Valois-Cuesta *et al*., 2012), and complements more traditional pollen flow studies examining stigmatic pollen loads, which provide a maternal perspective on the pollination process.

My results on intermorph pollen transfer were consistent with earlier studies in tristylous *L. salicaria* (Mulcahy and Caporello, 1970) and *L. junceum* (Ornduff, 1975b), both of which detected some degree of disassortative pollination of the L-morph. However, despite the high compatible pollen transfer proficiency by long-level anthers (Fig. 3.4A), I found that incompatible pollen accounted for approximately 50% of the intermorph pollen captured by stigmas of the L-morph. Earlier studies of pollen capture in distylous (*e.g*., Ornduff, 1979; Weller, 1980) and tristylous (Mulcahy and Caporello, 1970; Ornduff, 1975b) species have also reported high levels of incompatible pollination in the L-morph. In contrast, compatible pollen transfer was significantly higher for mid- and particularly for short-level anthers, and stigmas of the M- and S-morph captured mainly compatible intermorph pollen, particularly the S-morph. My results provide support for Darwin's cross-promotion hypothesis in the M- and S-morphs by demonstrating significant levels of disassortative pollination, although the amounts of compatible pollen transferred and captured varied between them. In the L-morph I found no strong evidence for disassortative pollination, although intermorph compatible pollen had a slightly larger transfer proficiency than intermorph incompatible pollen.

The morph-specific patterns of compatible intermorph pollination that I detected in *L. salicaria* are opposite to those previously reported in natural populations of tristylous *Pontederia cordata* (Barrett and Glover, 1985; Glover and Barrett, 1986) and *P. sagittata* (Glover and Barrett, 1983). In these species, compatible pollen capture occurred primarily in the L-morph, while the S-morph exhibited the lowest levels of compatible pollen capture. Unfortunately, it is not possible to fully compare my results with those previously obtained for *L. salicaria* (Mulcahy and Caporello, 1970). Despite the claim made by these authors that pollination was "predominantly legitimate" (Mulcahy and Caporello, 1970 p. 1027), this conclusion is not warranted based on the data presented in their article because of their inability to distinguish mid- and short-level pollen on stigmas. It is unclear what factors may be responsible for the contrasting patterns of pollen capture between the L- and S-morphs of *L. salicaria* and *Pontederia*

species, but differences in floral design, the spatial distribution of floral morphs and the pollinator fauna visiting flowers may contribute to the observed differences (see Glover and Barrett, 1983, 1986). It is also possible that my experimental treatments, including the removal of alternate anther levels in the L-morph, may have influenced pollen pickup and delivery in ways different from those that would occur in natural unmanipulated populations. However, I have no evidence from my data or observations of pollinators that this procedure affected the pollination events involving the L-morph as pollen donor. Therefore, my experiments go beyond the previous dichotomy involving "long pollen" versus "non-long pollen" in *Lythrum* pollen flow studies (Mulcahy and Caporello, 1970; Ornduff, 1975b) and provide for the first time unambiguous measures of intermorph pollination among the three sex-organ levels.

Influence of floral design on the pollination process

One of the main questions that I addressed in my study was to what extent the floral design of *L. salicaria* may play a role in influencing patterns of disassortative pollination. Specifically, I predicted that because the floral tube of *L. salicaria* is only partially developed with respect to the enclosure of sex organs, pollen transfer and capture would likely differ among organs located at different heights (Fig. 3.1). Because my experimental arrays were visited primarily by the solitary bee *Anthidium manicatum* (Pechuman, 1967), with 81.5% of flowers visited by this species, I can probably exclude pollinator identity as a source of variability in pollen transfer and capture, which allows me to isolate the influence of floral design on the pollination process. My experimental results revealed a consistent pattern in line with my predictions. There was a decrease in the precision of pollen transfer between anthers and stigmas from short- to mid- to long-level sex organs. The floral tube of *L. salicaria* restricts pollinator movements during contacts with short- and to a lesser extent mid-level organs. In contrast, the unspecialized dish-shaped terminal region of the flower, where the highly exerted long-level organs are located, allows a wide range of contact points between the sex organs and the pollinator's body.

Stereomorphic depth-probed flowers foster precise pollen transfer and capture promoting disassortative pollination in populations of heterostylous species. The height at which the centrally placed stigma is positioned in flowers of heterostylous species determines the initial space available for the pollinator while entering the flower (Lloyd and Webb, 1992b; Stone and Thomson, 1994). By using glass-sided artificial flowers,

Stone and Thomson (1994) confirmed that style length strongly affected the pollinator's posture, thus dictating its entry and exit paths. Flowers of *L. salicaria* are attached horizontally to the inflorescence axis forcing the contact between the lower part of the insect's body and the sex organs (Darwin, 1864), *i.e*., sternotribic pollination. I found that intermorph compatible pollination increased from mid- to short-level organs, owing to the combined effects of differences in the path taken by the pollinator's probe during entry into the M- and S-morph flowers, and the more concealed location of short-level organs (Lloyd and Webb, 1992b; Stone and Thomson, 1994). In contrast, the highly exerted long-level organs impose no constraint on the posture of pollinators, which can access the flower from virtually any angle. Studies of pollen capture in distylous *Fagopyrum esculentum* (Björkman, 1995) and *Hypericum aegypticum* (Ornduff, 1975a) revealed that intermorph pollen accounted for only 23% and less than 40%, respectively, of the stigmatic pollen load. The low level of intermorph pollen capture in these two species is most probably because they possess dish-shaped non-tubular flowers, thus limiting the precision of intermorph pollen transfer. Similarly, my results revealed that exerted long-level organs had the lowest values of intermorph pollen transfer and capture, and that stigmas of the L-morph also exhibited the highest values for intramorph outcross pollination, geitonogamous and intraflower self-pollination.

Decomposing the sources of intramorph pollination

I used experimental monomorphic arrays to provide insight into the sources of intramorph pollination in *L. salicaria*. Three potential types of intramorph pollen can be transferred to stigmas by pollinators – intraflower self-pollen, geitonogamous self-pollen and intramorph outcross pollen. I found that the main source of intramorph pollen on stigmas on each of the style morphs involved pollen transfer from other plants in the arrays and not self-pollination, which overall was much reduced. Values of intraflower self-pollen transfer were higher than geitonogamous pollen transfer, but there were no significant differences in these different forms of self-pollination among the three style morphs. Of particular interest was the finding that stigmas of the L-morph captured significantly more intramorph outcross pollen than stigmas of the M- and S-morphs, thus raising the question of what factors might account for this pattern.

Monomorphic arrays of L-morph plants resemble a non-heterostylous species with approach herkogamous flowers. This form of herkogamy is the most common type of intraflower spatial separation of anthers and stigmas in angiosperms (Webb and Lloyd,

1986). When pollinators visit approach herkogamous flowers, they usually contact the exerted stigma before touching the anthers, thus promoting some degree of outcrossing (Webb and Lloyd, 1986; Lloyd and Webb, 1992a; Barrett, 2003 p. 995: Fig. 3). Because the L-morph flowers of *L. salicaria* exhibit the approach herkogamous condition, the high level of intramorph outcross pollination that I detected for this style morph was probably caused by the initial contact between the pollinator's body and stigma while bees were entering the flower tube. Elsewhere, in the only other experimental study of a tristylous species that has used monomorphic arrays of each of the style morphs, Kohn and Barrett (1990) reported the highest outcrossing rates in arrays of the L-morph of *Eichhornia paniculata*. Collectively, these results support the hypothesis that the morphology of the L-morph is superior to those of the other style morphs in favoring pollen transfer between plants, including intramorph outcross pollination.

A criticism of the vast majority of studies investigating Darwin's cross-promotion hypothesis in natural populations of heterostylous species has been the failure to distinguish between self- and intramorph pollen on stigmas. This is necessary because reciprocal herkogamy is a population-level floral polymorphism, and therefore cannot reduce the amount of self-pollination any more than other monomorphic floral strategy (Ganders, 1979; Lloyd and Webb, 1992b; Chapter 2), contrary to what Mulcahy and Caporello (1970) concluded. A more rigorous assessment of the function of heterostyly requires the emasculation of flowers and the comparison of the relative amounts of intermorph and intramorph pollen on stigmas (*e.g.*, Ganders, 1974; Barrett and Glover, 1985). My estimates across the three style morphs of intraflower self-pollen transfer and capture were generally larger than equivalent values for between flower pollinations on the same plant. My results therefore provided evidence for a significant component of intraflower self-pollination that if not excluded by emasculation would lead to an overestimation of the extent of intramorph pollination. Indeed, when the confounding effects of self-pollination were excluded by emasculation in distylous *Jepsonia heterandra* (Ganders, 1974), *Palicourea padifolia* (Ree, 1997), *Persicaria japonica* (Nishihiro and Washitani, 1998) and tristylous *Pontederia cordata* (Barrett and Glover, 1985), reciprocal herkogamy was shown to be successful in promoting intermorph compatible pollination providing evidence in support of the Darwinian hypothesis.

Geitonogamous pollination is a unique mode of self-pollination because it involves the same mechanisms used for cross-pollination, but confers no reproductive assurance and results in complete pollen and seed discounting (Lloyd, 1992). Heterostyly

promotes cross-pollination through pollen segregation on the bodies of pollinators, and thus pollen grains remain on a pollinator's body until transferred to the stigma of a reciprocal style morph. In this way, heterostyly increases pollen carryover (Feinsinger and Busby, 1987; Harder and Barrett, 1996; Matsumura and Washitani, 2002) and in species with multiflowered inflorescences, it probably functions to limit geitonogamy and pollen discounting (Harder and Barrett, 1996). My study supports the hypothesis that heterostyly may function to limit geitonogamy. I found that levels of between flower self-pollination were the lowest of the three components of intramorph pollination in monomorphic arrays. The levels of geitonogamous pollination that I obtained for *L. salicaria* were roughly similar to previous estimates for distylous *Gaertnera vaginata* (Pailler *et al*., 2002), and were consistently low across each of the three style morphs indicating that their particular morphologies may limit self-pollination between flowers on an inflorescence. In the most comprehensive study investigating the relative contributions of intra- and between-flower selfing rates in a tristylous species, Eckert (2000) found that geitonogamy was the prevailing mode of selfing in *Decodon verticillatus*. However, in this highly clonal species floral display involve numerous ramets per clone, which together with the local foraging of pollinators and the mass flowering habit of the species may lead to particularly high levels of geitonogamy.

In conclusion, the manipulative nature of my experiment raises the question to what extent my findings apply to natural populations of *L. salicaria*. The pollination process in heterostylous species is affected by diverse factors including floral design (Harder and Barrett, 1993; Stone and Thomson, 1994), geographical variation in environmental factors (Hodgins and Barrett, 2008a; Cunha *et al*., 2014), pollinator diversity (Wolfe and Barrett, 1988; Stone, 1996), population size (Waites and Ågren, 2004), and the patterns of style morph distribution in populations (Levin, 1968; Lughadha and Parnell, 1989). In this study, I focused on the influence of floral design on pollen transfer and capture. Because most of the pollination in the experimental arrays was mediated by a single solitary bee, *Anthidium manicatum*, this allowed me to isolate the influence of floral morphology on the pollination process. The patterns that I observed among sex-organ levels seem likely to be amplified in natural populations of *L. salicaria*, because the diversity of insect visitors to flowers is considerably greater than in my experimental arrays. Bumblebees, solitary bees, honey bees, wasps, butterflies, and syrphid flies have all been observed foraging on flowers of *L. salicaria* in native

European populations (Waites and Ågren, 2004; J. Costa, pers. observ.) and introduced North American populations (Brown *et al*., 2002; King and Sargent, 2012). The patterns of pollen transfer and deposition that I report are therefore likely to be more complex in natural populations.

Appendix 3.1. Pollen transfer and capture in trimorphic arrays of *Lythrum salicaria.* Values are mean ± SE. Pollen transfer proficiencies calculated following the method of Lloyd and Webb (1992b) and given as E^{-02}. Results of the GLMMs investigating differences in (a) pollen transfer proficiencies for each anther level among style morphs and (b) pollen capture among anther levels for each style morph are given. Different uppercase and lowercase letters represent statistically significant differences among and within style morphs, respectively. *** $P < 0.001$, n.s. - non-significant at $P > 0.05$.

	Style morph			
Anther level	**L**	**M**	**S**	χ^2_2
(a) Pollen transfer				
Long	0.78 ± 0.21^{A}	0.12 ± 0.02^{B}	0.05 ± 0.02^{B}	34.49***
Mid	0.54 ± 0.18^{A}	0.69 ± 0.19^{A}	0.99 ± 0.34^{A}	$1.51^{n.s.}$
Short	0.57 ± 0.19^{A}	0.76 ± 0.11^{A}	1.83 ± 0.21^{B}	34.83***
(b) Pollen capture				
Long	41.54 ± 3.68^{a}	6.21 ± 0.56^{a}	3.20 ± 0.47^{a}	-
Mid	45.30 ± 5.18^{b}	53.84 ± 3.59^{b}	76.49 ± 6.11^{b}	-
Short	46.44 ± 3.52^{ab}	58.04 ± 3.96^{b}	155.41 ± 7.62^{b}	-
χ^2_2	31.31***	345.39***	897.44***	

Appendix 3.2. Pollen transfer and capture in monomorphic arrays of *Lythrum salicaria.* Values are mean ± SE. Pollen transfer proficiencies calculated following the method of Lloyd and Webb (1992a) and given as E^{-02}. Results of the GLMMs investigating differences in (a) pollen transfer proficiencies for each intramorph pollen component among style morphs and (b) pollen capture among intramorph pollen components for each style morph are given. Different uppercase and lowercase letters represent statistical significant differences among and within style morphs, respectively. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s. - non-significant at $P > 0.05$.

	Style morph			
	L	**M**	**S**	χ^2_2
(a) Pollen transfer				
Outcross	2.05 ± 0.35^{A}	0.49 ± 0.09^{B}	0.66 ± 0.20^{B}	23.42***
Geitonogamous	0.14 ± 0.04^{A}	0.12 ± 0.03^{A}	0.03 ± 0.03^{A}	$2.11^{n.s.}$
Self	0.40 ± 0.17^{A}	0.32 ± 0.06^{A}	0.21 ± 0.09^{A}	$0.73^{n.s.}$
(b) Pollen capture				
Outcross	86.09 ± 15.07^{a}	19.31 ± 4.63^{a}	25.77 ± 8.31^{a}	-
Geitonogamous	10.82 ± 3.50^{b}	8.44 ± 1.91^{b}	2.41 ± 2.06^{b}	-
Self	31.56 ± 13.78^{ab}	23.81 ± 6.01^{a}	16.06 ± 6.97^{a}	-
χ^2_2	8.64*	11.33**	16.91***	

Appendix 3.3. Pollen transfer and capture for *Lythrum salicaria*. Values are mean ± SE. Pollen transfer proficiencies calculated following the method of Lloyd and Webb (1992a) and given as E^{-02}. Results of the GLMMs investigating differences in (a) pollen transfer proficiencies and (b) pollen capture among stigmatic pollen load components for each style morph are given. Different letters represent statistical significant differences for each style morph. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s. - non-significant at $P > 0.05$.

	Style morph		
	L	**M**	**S**
(a) Pollen transfer			
Compatible intermorph	0.78 ± 0.21^{a}	0.69 ± 0.19^{a}	1.83 ± 0.21^{a}
Incompatible intermorph	0.55 ± 0.12^{ab}	0.44 ± 0.13^{bc}	0.68 ± 0.29^{a}
Intramorph outcross	2.05 ± 0.35^{a}	0.49 ± 0.09^{ab}	0.66 ± 0.20^{a}
Geitonogamous	0.14 ± 0.04^{b}	0.12 ± 0.03^{c}	0.03 ± 0.03^{b}
Self	0.40 ± 0.17^{ab}	0.32 ± 0.06^{ab}	0.21 ± 0.09^{a}
χ^2_4	12.62*	56.91***	25.64***
(b) Pollen capture			
Compatible intermorph	40.14 ± 10.80^{ab}	53.82 ± 15.23^{a}	151.12 ± 19.25^{a}
Incompatible intermorph	46.17 ± 9.74^{ab}	32.98 ± 10.77^{ab}	52.06 ± 22.65^{ac}
Intramorph outcross	86.09 ± 15.07^{a}	19.31 ± 4.63^{b}	25.77 ± 8.31^{ac}
Geitonogamous	10.82 ± 3.50^{b}	8.44 ± 1.91^{b}	2.41 ± 2.0^{b}
Self	31.56 ± 13.78^{ab}	23.81 ± 6.01^{a}	16.06 ± 6.97^{c}
χ^2_4	13.92**	25.60***	46.92***

Part II – The function of ancillary characters and evolutionary history of heterostyly

"After twenty-two hours these two stigmas were discoloured, slightly twisted, and penetrated by the tubes of numerous pollen-grains: the other three stigmas, covered with their own-form pollen, were fresh, and all the pollen-grains were loose; but I did not dissect the whole stigma."

Darwin (1877 p. 88)

Chapter 4 – Experimental insights on the function of ancillary pollen and stigma polymorphisms in plants with heteromorphic incompatibility

A. *Armeria maritima*. **B.** *Limonium vulgare*.
C. *Armeria pubigera*.

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ABSTRACT

Most heterostylous plants possess a reciprocal arrangement of stigmas and anthers, heteromorphic self-incompatibility and ancillary polymorphisms of pollen and stigmas. The topographical complementarity hypothesis proposes that ancillary polymorphisms function in the rejection of incompatible pollen thus promoting disassortative pollination. Here, I test this hypothesis by investigating patterns of pollen transfer and capture in populations of dimorphic *Armeria maritima* and *A. pubigera* and distylous *Limonium vulgare* (Plumbaginaceae), and by studying pollen adherence and germination patterns in *A. maritima* following controlled hand-pollinations. *Armeria* lacks reciprocal herkogamy allowing the evaluation of the extent to which ancillary polymorphisms affect the composition of pollen loads. I compared the amounts of compatible and incompatible pollen on stigmas in natural populations and calculated the proficiencies of pollen transfer for each mating type. I detected disassortative pollination in each species, and mating types did not differ in compatible pollen capture, although cob stigmas captured more incompatible pollen. Controlled hand-pollinations revealed the failure of incompatible pollen to adhere and germinate on stigmas. My results provided evidence that, while structural in nature, pollen-stigma dimorphisms are tightly associated with heteromorphic incompatibility and likely function to promote disassortative pollination in the absence of reciprocal herkogamy.

KEY WORDS: *Armeria*; disassortative pollination; floral function; heterostyly; *Limonium*; pollen-stigma dimorphism.

INTRODUCTION

Animal-pollinated plants display a multiplicity of structural adaptations that function to promote effective cross-pollen dispersal and reduce pollen wastage, especially on incompatible stigmas. These include different types of pollen aggregation (reviewed in Harder and Johnson, 2008), various pollen-dispensing mechanisms (*e.g.*, Harder and Barclay, 1994; Lebuhn and Anderson, 1994; Reith *et al*., 2007; Han *et al*., 2008), and diverse floral morphologies, which both filter animal visits to flowers and constrain the orientation of suitable pollinators, thus determining the site of contact between the animal's body and the sex organs of flowers (Darwin, 1862b; Faegri and Van der Pijl, 1971; Harder and Johnson, 2009). A particularly striking example of the functional fit between flowers and pollinators is heterostyly, a convergent floral syndrome that has evolved on numerous occasions in at least 28 angiosperm families (Darwin, 1877; Ganders, 1979; Barrett, 1992a; Lloyd and Webb, 1992a). Heterostylous species possess a reciprocal arrangement of sexual organs that promotes disassortative pollination by segregated pollen deposition on the bodies of animal pollinators (Darwin, 1877; Ganders, 1979; Kohn and Barrett, 1992; Lloyd and Webb, 1992b; Stone and Thomson, 1994; Barrett and Shore, 2008; Zhou *et al*., 2015). Heterostyly represents one of the classic structural adaptations in floral biology for which the function has been determined through experimental studies.

In the majority of heterostylous species, reciprocal herkogamy is associated with a heteromorphic diallelic incompatibility system that prevents self and intramorph mating and a suite of ancillary pollen (*e.g.*, differences in pollen production, size, shape and exine ornamentation) and stigma heteromorphisms (*e.g.*, papillae size and shape), which can vary widely in expression among heterostylous groups (Dulberger, 1992). Collectively, this association of traits is known as the heterostylous syndrome. While the adaptive significance of reciprocal herkogamy and heteromorphic incompatibility are well understood (reviewed by Barrett and Shore, 2008), little is known about the function of the ancillary characters of pollen and stigmas that often accompany these other traits. To experimentally investigate the components of the heterostylous syndrome, the polymorphic traits should ideally be isolated and studied independently (*e.g.*, Kohn and Barrett, 1992; Zhou *et al*., 2015). However, in the case of pollen and stigma polymorphisms this is a major challenge, because these traits are usually tightly associated with reciprocal herkogamy (Ganders, 1979; Barrett, 1992a; Dulberger,

1975b, 1992), thus limiting opportunities to separate their individual effects on the pollination process.

Plumbaginaceae is a cosmopolitan family consisting of 27 genera and approximately 650 species (Kubitzki, 1993). Distyly has been reported in several genera of the family, including *Ceratostigma*, *Dyerophytum*, *Limonium* and *Plumbago* (Baker, 1966; Dulberger, 1975a; Ganders, 1979; Barrett, 1992a), and its expression differs significantly between subfamilies. For example, in Plumbaginoideae distyly occurs without striking morphological differences in pollen and stigma between floral morphs, whereas ancillary characters are well developed in Staticoideae, where they occur both in the presence of reciprocal herkogamy in *Limonium,* or in its absence as in *Armeria* (Baker, 1948a, 1953a, 1966; Dulberger, 1975a). Both *Armeria* and *Limonium* are comprised of species that can be monomorphic or dimorphic for pollen exine sculpturing and stigmatic papillae morphology. In these genera, populations of dimorphic species usually possess dimorphic incompatibility and flowers of one mating morph produce pollen with a coarse reticulate sexine (pollen type A) and a cob-like stigmatic papillae, whereas the alternate morph has pollen with finely reticulated sexine (pollen type B) and papillate stigmas (Baker, 1948a, 1966; Dulberger, 1975a, b, 1992). Monomorphic species are either self-compatible (A/papillate, or less frequently, B/cob) or apomictic (A/cob or B/papillate) (Baker, 1966; Dulberger, 1975a). This variation in pollen-stigma combinations in *Armeria* and *Limonium* provides a valuable experimental system for investigating whether ancillary characters play a role in promoting disassortative pollen transfer and capture (hereafter disassortative pollination), and more generally to infer their functional significance in heterostylous species.

The first attempt to experimentally address the role of ancillary characters in heteromorphic plants was made by Dulberger (1975a), who investigated exine ornamentation and papillae shape in a series of self, intra- and intermorph controlled hand-pollinations in several species of Plumbaginaceae. She observed that no self or intramorph pollen adhered to stigmatic papillae 5h after pollinations, and proposed that the topographical complementarity between pollen exine sculpturing and stigmatic papillae shape was involved in the physiological incompatibility mechanism, because of the intimate contact between recognition sites of pollen and stigmatic papillae of the morphs. Later, studies on *Armeria maritima* by Mattsson (1983) extended these findings by demonstrating that morph-specific differences in the lipid composition of pollen play a role in the adhesion of pollen grains during their initial interaction with

stigmas. Collectively, these results indicate that there is an intimate functional relation between morphology and physiology, which is impossible to experimentally dissociate, with both structural and biochemical features of ancillary polymorphisms participating in the rejection of incompatible pollen from stigmas (reviewed by Heslop-Harrison and Heslop-Harrison, 1985). Thus, self and intramorph pollen grains are less likely to adhere to incompatible stigmas, either remaining on the pollinators' body or eventually falling off the stigma if deposited. This explanation has been referred to as the "topographical complementary hypothesis" for the function of ancillary characters (Dulberger, 1975b, 1992; Lloyd and Webb, 1992a), but it has not been examined in natural populations of heteromorphic plants.

Here, I investigate the topographical complementary hypothesis for the function of ancillary pollen and stigma polymorphisms by examining patterns of pollen transfer and capture in natural populations of three heteromorphic species of Plumbaginaceae, *Armeria maritima*, *A. pubigera* and *Limonium vulgare*, and by quantifying pollen adherence, germination and pollen-tube growth after controlled compatible and incompatible pollinations in *Armeria maritima.* The two *Armeria* species possess striking morphological pollen-stigma dimorphisms (Figs. 4.1A-D, 4.2A-D), but more importantly lack reciprocal herkogamy with the two morphs possessing anthers and styles of uniform height. In contrast, *L. vulgare* is a typical distylous species that exhibits both reciprocal herkogamy, and ancillary pollen and stigma dimorphisms (Figs. 4.1E-F, 4.2E-F). The long-styled morph has A/cob pollen-stigma combination and the short-styled morph has the B/papillate pollen-stigma combination. All three species possess dimorphic incompatibility and only pollinations between the floral morphs set seed, resulting in disassortative mating in populations (Iversen, 1940; Baker, 1966; reviewed by Dulberger, 1992).

My study addressed the following specific questions: (1) In the absence of reciprocal herkogamy, do the ancillary characters of pollen and stigma in *Armeria* species promote disassortative pollination, as predicted by the topographical complementarity hypothesis? (2) Are there differences between the floral morphs of each species in the amount of compatible and incompatible pollen that is captured by stigmas? Experimental studies of pollen loads in natural populations of heterostylous plants have routinely recorded asymmetrical patterns of pollen transfer with the long-styled morph capturing more total pollen than the short-styled morph, but with a higher fraction of this pollen being incompatible (reviewed in Ganders, 1979; Lloyd and Webb, 1992b;

Barrett and Shore, 2008; Chapter 2). I was therefore interested in testing to what extent species lacking reciprocal herkogamy might also exhibit similar asymmetrical patterns of pollen capture. (3) Are there differences between compatible and incompatible pollen in adhesion and germination once they are deposited on stigmas following experimental hand-pollinations? To address these questions, I analyzed stigmatic pollen loads in natural populations of *Armeria maritima, A. pubigera* and *Limonium vulgare* comparing compatible and incompatible pollen transfer and capture among species and morphs, and quantified pollen adherence and germination after controlled hand-pollinations in *A. maritima*.

MATERIALS AND METHODS

Study species

Armeria maritima (Mill.) Willd. is a widespread herbaceous perennial plant that occurs in coastal environments (*e.g.*, saltmarshes, coastal mud flats and sea cliffs) in the Northern Hemisphere, including Europe, Siberia and North America. It produces from one to several flowering stalks up to 30 cm in height, and has pale pink flowers, with a single-ovule, that are grouped in terminal compact spherical heads (Feliner, 1990; Kubitzi, 1993; Woodell and Dale, 1993). *Armeria pubigera* (Desf) Boiss. is a dwarf shrub restricted to rocky sea cliffs, endemic to the northwestern coast of the Iberian Peninsula. Flowering stalks grow up to 15 cm in height and bear terminal compact spherical heads of pale pink single-ovule flowers (Feliner, 1990; Kubitzi, 1993). *Limonium vulgare* Mill. is a distylous perennial herb that grows in mud flats and salt marshes in western and southern Europe and North Africa, frequently experiencing partial flooding (Boorman, 1967; Erben, 1993; Róis, 2014). Each plant produces from one to several flowering stalks up to 40 cm height, with numerous single-ovule flowers grouped into panicles (Erben, 1993; Kubitzi, 1993; Róis, 2014). In my study area, both *Armeria* species flower in May, while *L. vulgare* flowers from June to August.

Characterization of pollen and stigma dimorphisms

To provide a morphological context for my experiments on naturally and experimentally pollinated flowers described below, I conducted a detailed characterization of the structural features of pollen and stigma polymorphisms in the three taxa. To accomplish this, I undertook morphological measurements of pollen and stigmatic papillae under a light microscope (Leitz HM-LUX 3; 1000× magnification) by

using a calibrated ocular micrometer. I obtained pollen and stigma samples from dried material collected from one population per species (*A. maritima*: Darque; *A. pubigera*: Areosa; *L. vulgare*: Gafanha da Encarnação; Appendix 4.1). Before measurements, pollen grains were acetolised following Erdtman (1960) and mounted on microscope slides using glycerol as mounting medium. The following characters were measured for 10 pollen grains of three individuals per mating type and species: polar axis (P), equatorial diameter (E) and the diameter of three lumina (*i.e*., the space enclosed by the muri or ridges of the ornamentation in a reticulate pollen grain; Punt *et al*., 2007). I used the values of polar axis and equatorial diameter to calculate the P/E ratio. Stigmatic papillae characterization was performed on 10 stigmas per mating type and species; the stigmas were mounted in glycerol and three measurements of the following characters were made: stigmatic papillae length (h), stigmatic papillae width (w) and inter-papillae space (Ips) between two adjacent stigmatic papillae (Fig. 4.2 A-B).

To document pollen and stigma dimorphism in the two *Armeria* species and *L. vulgare*, I obtained images of the exine sculpturing of acetolised pollen using SEM (Tescan Vegan3) equipped with a direct image acquisition system (Fig. 4.1). I obtained images of the stigmas mounted in glycerol using a light microscope (Leica DM4000B), also equipped with a direct image acquisition system (Fig. 4.2).

Pollen transfer and capture in natural populations

To estimate the extent to which ancillary characters promote disassortative pollination, I investigated pollen loads on open-pollinated stigmas in 11 natural populations (Appendix 4.1). Sampling was conducted during peak flowering in 2015 (*A. maritima*: early May; *A. pubigera*: late May; *L. vulgare*: mid-July) and included the collection of open-pollinated flowers to determine: (1) stigmatic pollen load composition, (2) mating type frequencies, and (3) the characterization of the pollen-stigma dimorphism, and estimates of population size and floral display. In populations of *A. maritima* and *L. vulgare*, I started the collection of stigmas at least one hour after the lowest tide on sunny days. This procedure assured that flowers were exposed to pollinators before collection. Although pollinator activity in all populations was relatively low, owing to cool weather and/or persistent coastal winds, I routinely observed small bees, flies and beetles foraging on flowers. In each population, I sampled flowers for analysis of stigmatic pollen loads every 2 m along transects to avoid resampling of genets. Where possible, I sampled one flower per plant from 100

individuals, but because these species often occur in small populations, sample sizes are less than 100 individuals for half of the populations (see Appendix 4.1). In populations of *A. maritima* and *A. pubigera*, I dissected intact flowers directly in the field, removed the stigma with fine forceps, and squashed them underneath a coverslip on a microscope slide using glycerol as a mounting medium. Given the reduced size of *L. vulgare* flowers, a different approach was necessary. In this case, I collected labeled inflorescences, brought them back to the laboratory inserted on wet flower foam cushions, and removed stigmas using a dissection microscope at 10× magnification (Leica Zoom 2000), the same day of collection. This procedure was preferred over placing flowers directly into Eppendorf tubes containing 70% ethanol because it reduced the likelihood of pollen detachment from stigmatic papillae.

I counted pollen grains deposited on stigmas, *i.e.*, pollen capture, under a light microscope at 400× magnification (Leitz HM-LUX 3), and classified them as compatible or incompatible based on their exine ornamentation and on the identity of the recipient stigma. Sample sizes for each mating type in each population are provided in Appendix 4.2. Stigmas without pollen grains represented 12% (n_{total} = 209), 10% (n_{total} = 298) and 25% (n_{total} = 225) of the total stigmas observed for *A. maritima*, *A. pubigera*, and *L. vulgare*, respectively, and these were excluded from statistical analyses comparing pollen loads. I assessed pollen transfer in natural populations by calculating the pollen transfer proficiency (*Tij*) following the equation provided in Chapter 2 p. 31 (Lloyd and Webb, 1992b).

I used the random sampling of flowers to estimate the frequency of mating types in each population (Appendix 4.1). I also estimated population size (individuals at reproductive stage only) by counting the number of flowering individuals in each population. Finally, in each population, I assessed floral display size and estimated the amount of pollen available for dispersal, *i.e.*, the pollen pool, at the time of stigma collection. I estimated total flower production per sampled plant as the product of the average number of open flowers with dehiscent anthers, from three randomly selected inflorescences, by the total number of inflorescences with open flowers per individual. To estimate pollen production per flower, I collected one flower bud per plant from 10 individuals per mating type from three populations of each species and these were stored in 70% ethanol in Eppendorf tubes for later processing. I removed one anther from each flower bud and placed it in a drop of distilled water on a microscope slide. The anther was then opened with a fine needle and pollen grains released and counted

under a light microscope at 100× magnification (Leitz HM-LUX 3). Counts were multiplied by five, *i.e*., by the number of anthers per flower. The pollen pool was calculated as the product of the total pollen production per plant by the number of reproductive individuals at the time of sampling.

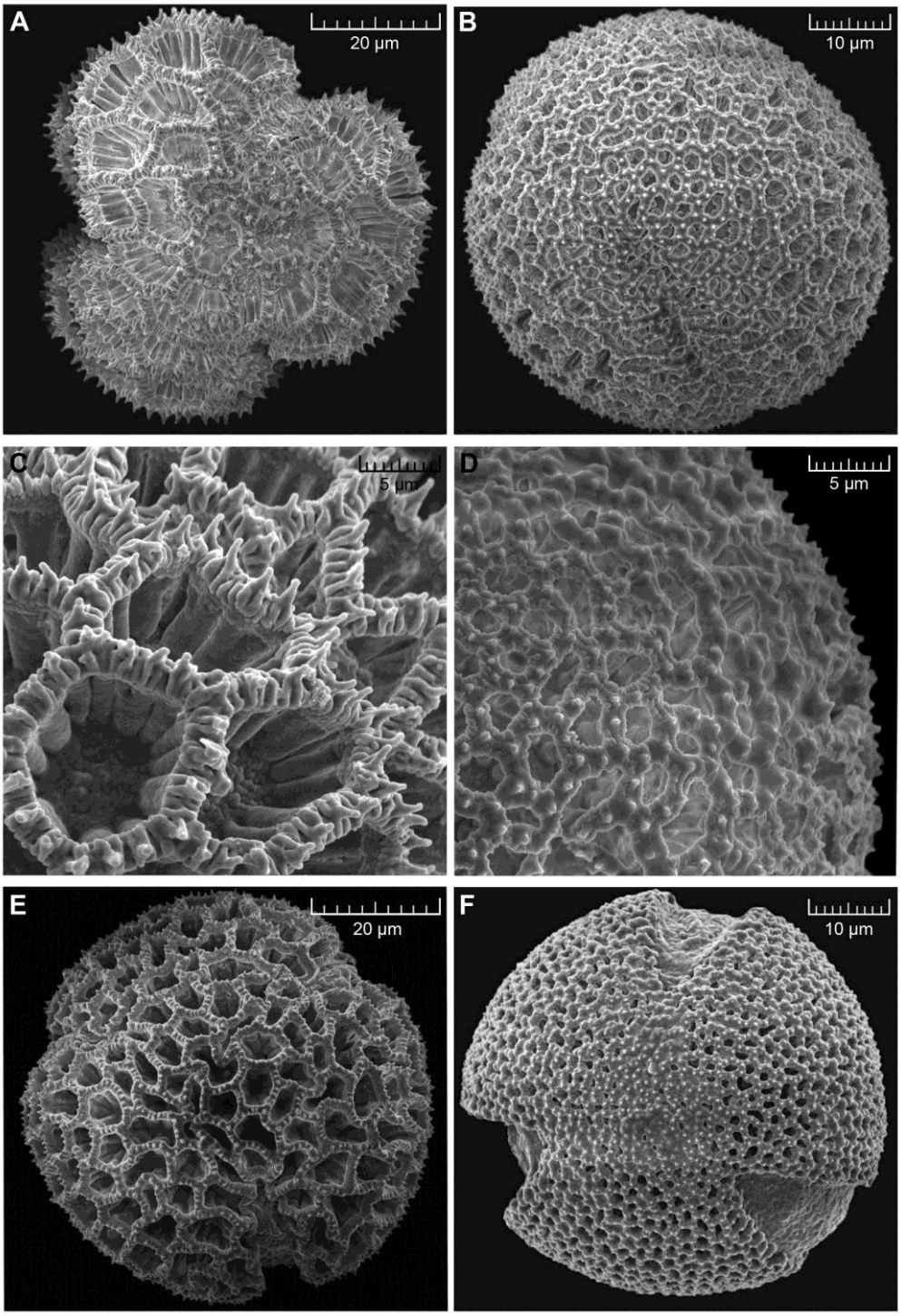


Figure 4.1. SEM images of pollen grains of *Armeria maritima* (A-B) from Fão, *A. pubigera* (C-D) from Areosa and *Limonium vulgare* (E-F) from Gafanha da Encarnação. **A, C, E.** Type A pollen grain; **B, D, F.** Type B pollen grain.

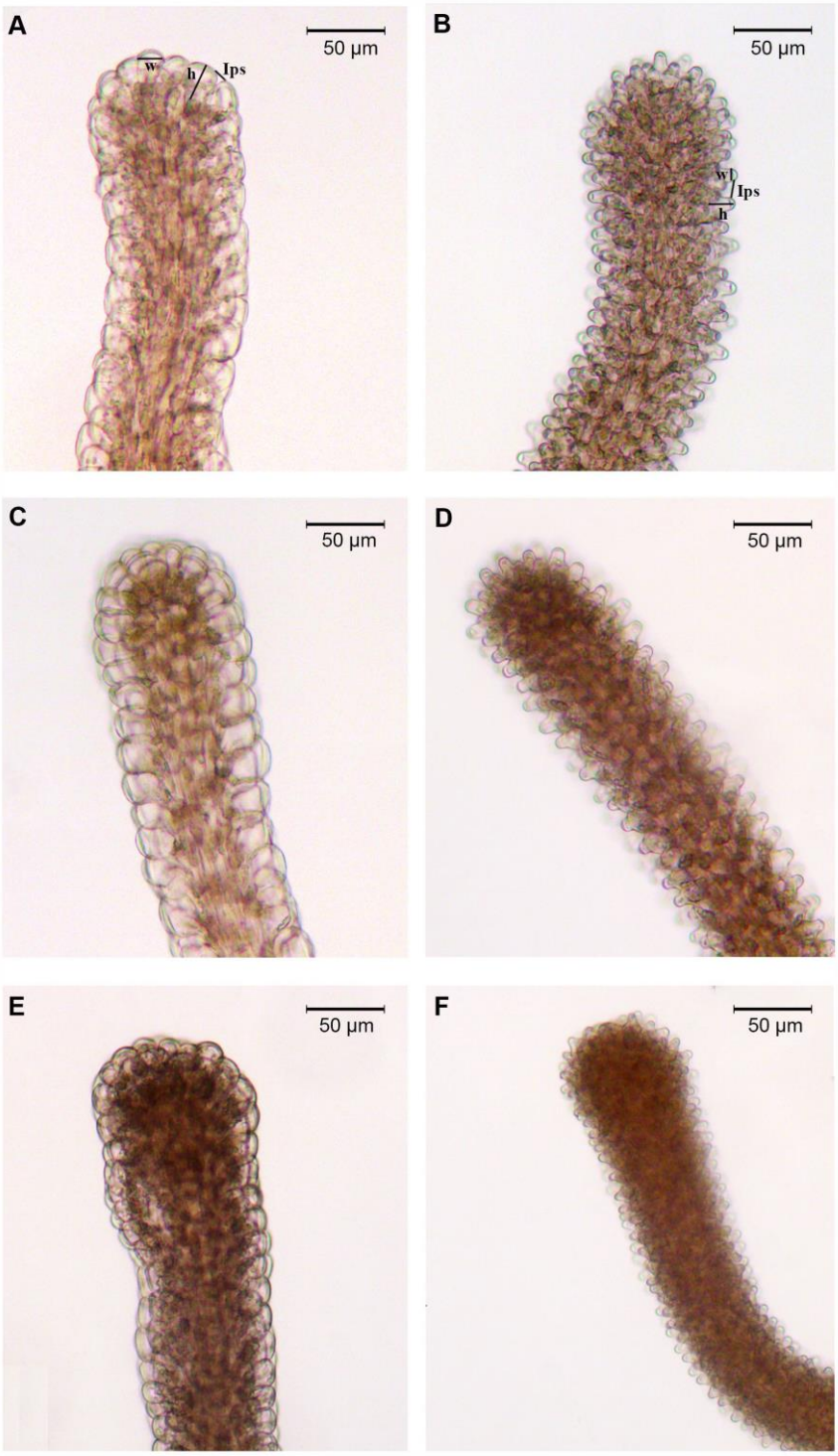


Figure 4.2. Light microscopy photographs of stigmas of *Armeria maritima* (A-B) from Fão, *A. pubigera* (C-D) from Areosa and *Limonium vulgare* (E-F) from Gafanha da Encarnação. **A, C, E.** cob stigma; **B, D, F.** papillate stigma. Morphological measurements: "h" - stigmatic papillae length, "w" - stigmatic papillae width (w) and "Ips" - inter-papillae space between two adjacent stigmatic papillae.

Quantification of pollen adherence and germination

The topographical complementarity hypothesis proposes that incompatible pollen fails to adhere to incompatible stigmas preventing pollen germination and pollen-tube

growth (Dulberger, 1975a). To evaluate this hypothesis, I conducted controlled hand-pollinations on *Armeria maritima* during May 2016, using 16 plants, eight A/cob and eight B/papillate. I collected plants from Darque population and brought them to the University of Coimbra in individual pots, where they were maintained in a pollinator-free glasshouse. Each plant received the following hand-pollination treatments: (a) illegitimate (incompatible) pollination, here self-pollination, (b) legitimate (compatible) pollination, pollination between mating types, and (c) mixed pollination, pollination with compatible and self-pollen applied simultaneously. Each day, I selected plants with six open flowers to be pollinated and randomly assigned a pollen donor from the opposite mating type. I used a randomized block design with each block of six pollinations performed twice per plant with a minimum of a 3-day interval between blocks. Before pollination, I emasculated flowers and removed the corolla to prevent self-pollen contamination and to facilitate precise pollen deposition. For each flower, I transferred a total of 50 grains, *i.e.*, 10 pollen grains to each of the five stigmas. Mixed pollen loads consisted of 1:1 compatible to self-pollen grains, *i.e.*, five compatible and five self-pollen grains per stigma. Under a dissecting microscope at 20× magnification (Leica Zoom 2000), I touched the anthers of the pollen donor with a fine needle and collected 10 pollen grains for treatments (a) and (b), and five compatible and five self-pollen grains for treatment (c), at a time. I transferred pollen grains to recipient stigmas and used a hand lens to confirm that the requisite number of pollen grains was deposited. I collected stigmas 1h and 3h after pollination, one stigma of each treatment per period of time, and these were transferred to a multiwell plate with a drop of glycerol. The collection times after hand-pollination were chosen to account for the possibility of delayed incompatible pollen hydration and germination (see Mattsson 1983).

By the end of the day, the stigmas were softened with 8M sodium hydroxide for 30 min, and placed overnight in 0.05% (w/v) aniline blue prepared in 0.1M potassium phosphate (Dafni *et al.*, 2005). I then transferred the stigmas to a microscope slide with a drop of glycerol, squashed them beneath a coverslip, and used a fluorescence microscope at 400× magnification (Leica DM4000B) to observe pollen grains and pollen tubes. I counted the number of pollen grains adhered to the stigma, the number of germinated pollen grains and the number of pollen tubes growing in the upper part of the style. Based on exine ornamentation, I identified the mating type of adhered and germinated pollen grains on the stigma after the mixed pollination treatment.

Statistical analyses

I used *t*-test for independent samples to compare pollen and stigmatic papillae measurements between mating types for each species, and the Mann-Whitney *U*-test to test for morphological complementarity between the average pollen lumen diameter of a given mating type and: (a) the average papillae width, and (b) the average inter-papillae distance from the opposite mating type for each species. Data on equatorial diameter for *A. pubigera* and the P/E ratio for *L. vulgare* were $\log_{10}(x)$ transformed to achieve normality (Zar, 2010); the non-parametric Mann-Whitney *U*-test was used when the normality assumption was not met.

I used *G*-tests for goodness-of-fit with Yates correction to test for deviations of the mating types from isoplethy (1:1) in each population (Zar, 2010). To investigate whether total daily flower production for each species differed significantly between mating types across populations, I used GLMs with a Gamma distribution and a log link function. I assessed differences in pollen production between mating types for the three species using a linear model. Before statistical tests, I transformed total flower production with the $\log_{10}(x+1)$ (Zar, 2010).

For each species, I used GLMs with a Poisson distribution and a log link function to investigate: (1) total pollen loads captured by stigmas of the mating types, and (2) the number of compatible and incompatible pollen grains captured by stigmas of each mating type within populations. I assessed the relations between population size and: (1) total and compatible pollen loads, and (2) random pollen capture by a GLM with a Poisson distribution and a log link function, and a GLM with a binomial distribution (1 for random versus 0 for non-random) and a logit link function, respectively. Prior to statistical analysis, population size was $\log_{10}(x)$ transformed (Zar, 2010). To explore whether the relative amount of compatible and incompatible pollen grains on stigmas differed among mating types and species, I used GLMMs with a binomial distribution and a logit link function. Species and mating type were specified as fixed factors, while population nested within species was defined as a random factor. The number of compatible and incompatible pollen grains was combined in the model as a matrix response variable. To investigate compatible and incompatible pollen capture in the presence versus absence of reciprocal herkogamy, I used GLMMs with a Poisson distribution and a log link function. Before analysis, I pooled data on pollen loads of the two non-reciprocal herkogamous *Armeria* species. Pollen load type, *i.e.*, compatible and

incompatible, and mating type were considered as fixed factors, whereas population was included as random factor.

To assess the role of: (1) ancillary characters alone (*A. maritima* and *A. pubigera*) and (2) ancillary characters with reciprocal herkogamy (*L. vulgare*) in promoting disassortative pollination, I tested for differences in compatible and incompatible pollen transfer proficiencies for each species across populations by means of a GLMM with a Gaussian distribution. Mating type and population were specified in the model as fixed and random factors, respectively. To investigate whether the probability of a single compatible pollen grain being deposited on stigmas of each mating type differed among species, I used GLMMs with a Gamma distribution and a log link function. Species and mating type were specified as fixed factors, and population nested within species was defined as a random factor.

I used GLMs with a quasipoisson distribution and a log link function to assess differences in pollen adherence between: (a) compatible and self-pollen in pure pollinations, and (b) compatible and self-pollen after mixed pollinations. Mating type, pollination block and time of collection nested within pollination treatment in pure pollinations, and time of collection nested within pollen type in mixed pollinations were specified as fixed factors. To investigate the germination success of compatible pollen grains after legitimate and mixed pollinations, I used GLMs with a quasipoisson distribution and a log link function to compare: (a) the number of adhered and germinated pollen grains on the stigma, and (b) the number of germinated pollen grains and pollen tubes growing in the upper part of the style. Mating type, pollination block and time of collection were specified as fixed factors.

I performed all statistical analyses with R software version 3.0.1 (R Core Development Team, 2013) using the following packages: "car" for Type-III analysis of variance as an integrated part of the GLMs and GLMMs (Fox and Weisberg, 2015), "lme4" for GLMMs (Bates *et al*., 2014), "multcomp" for multiple comparisons after Type-III analysis of variance (Hothorn *et al*., 2015), "nlme" for linear mixed models (Pinheiro *et al*., 2015), and "stats" for Shapiro-Wilk normality test, *t*-test for independent samples, Man-Whitney *U*-test, linear models and GLMs (R Core Development Team, 2013).

RESULTS

General characterization of the pollen-stigma dimorphism

The three species produced large pollen grains (50-100 µm; Appendix 4.3), except for the B/papillate mating type of *L. vulgare* that produced medium pollen grains (25-50 µm; Appendix 4.3) based on their longest axis, *i.e.*, the equatorial diameter. Concerning shape, pollen grains varied from suboblate (P/E: 0.75-0.88; Appendix 4.3) to oblate spheroidal (P/E: 0.88-1.00; Appendix 4.3) (following the classification of Erdtman, 1952). I found significant differences in polar axis, equatorial diameter and the P/E ratio between mating types for the three species ($P < 0.05$; Appendix 4.3), with pollen grains type A larger than pollen grains type B. Pollen type A had significantly larger lumina diameter than pollen type B ($P < 0.001$; Appendix 4.3).

Stigmatic papillae length varied between 10-23 µm (Appendix 4.4), and except for *A. pubigera* ($t = 3.24$, $P = 0.007$), with no differences between mating types in *A. maritima* and *L. vulgare* ($P > 0.05$; Appendix 4.4). Whereas average values of stigmatic papillae width were significantly larger for cob than papillate stigmas ($P < 0.001$; Appendix 4.4), the opposite pattern was evident for inter-papillae distance in each of the three species ($P < 0.001$; Appendix 4.4).

I found statistically significant differences between lumina diameter of pollen type A and papillate stigmas width for the three species (*A. maritima*, $U = 300.00$, $P < 0.001$; *A. pubigera*, $U = 297.00$, $P < 0.001$; *L. vulgare*, $U = 253.50$, $P = 0.001$). A similar pattern was detected for the comparison between lumina diameter of pollen type B and cob stigmas width (*A. maritima*, $U = 0.00$, $P < 0.001$; *A. pubigera*, $U = 0.00$, $P < 0.001$; *L. vulgare*, $U = 0.00$, $P < 0.001$). Inter-papillae distance in cob stigmas was similar to the average lumina diameter of pollen type B in the three species (*A. maritima*, $U = 164.00$, $P = 0.67$; *A. pubigera*, $U = 137.50$, $P = 0.71$; *L. vulgare*, $U = 89.50$, $P = 0.06$). For papillate stigmas, the inter-papillae distance was slightly larger than the lumina diameter of pollen type A, and was significantly different for *A. maritima* ($U = 29.50$, $P < 0.001$) and *A. pubigera* ($U = 68.50$, $P = 0.01$), but not for *L. vulgare* ($U = 87.00$, $P = 0.05$).

Contribution of mating types to the pollen pool

The frequencies of mating types within populations of each of the three species did not deviate significantly from the predicted 1:1 equilibrium expected from disassortative mating ($P > 0.05$; Appendix 4.1). Although there were differences in

average flower production among populations of *A. pubigera* ($\chi^2_{2,294}$ = 20.14, P < 0.001) and *L. vulgare* ($\chi^2_{3,221}$ = 15.29, P < 0.001), these were not dependent on mating type (mating type × population interaction: *A. pubigera* $\chi^2_{2,294}$ = 0.70, P = 0.71; *L. vulgare*, $\chi^2_{3,221}$ = 3.20, P = 0.36). Flower production was not significantly different between mating types for each of the three species (*A. maritima*: mean ± SE, A/cob: 33.67 ± 8.19, B/papillate: 33.58 ± 7.47; $\chi^2_{1,204}$ = 0.52, P = 0.47; *A. pubigera*: A/cob: 18.15 ± 3.96, B/papillate: 14.90 ± 2.49; $\chi^2_{1,294}$ = 0.98, P = 0.32; and *L. vulgare*: A/cob: 54.47 ± 8.04, B/papillate: 52.41 ± 4.42; $\chi^2_{1,221}$ = 0.49, P = 0.48). No significant differences between mating types in pollen production per flower among populations of *A. maritima* (mean ± SE, A/cob: 1001.17 ± 25.14, B/papillate: 972.24 ± 25.55; $F_{1,57}$ = 0.65, P = 0.42), *A. pubigera* (A/cob: 1048.62 ± 26.67, B/papillate: 992.66 ± 29.00; $F_{1,59}$ = 1.99, P = 0.16), and *L. vulgare* (A/cob: 979.14 ± 24.33, B/papillate: 996.83 ± 20.52; $F_{1,57}$ = 0.31, P = 0.58) were detected. Collectively, my results indicate that the mating types in populations of each species contribute equally to the pollen pool. Thus, significant differences in the relative amounts of compatible and incompatible pollen deposited on stigmas by pollinators would indicate deviations from random pollination in the populations.

Pollen capture in natural populations

Zero pollen loads were significantly more frequent for papillate than cob stigmas in *A. pubigera* ($\chi^2_{1,296}$ = 7.08, P < 0.01) and no differences were observed for *A. maritima* ($\chi^2_{1,207}$ = 0.57, P = 0.45). Total stigmatic pollen loads in populations of the two heteromorphic *Armeria* species were similar (*A. maritima*: 1 - 116 pollen grains; mean ± SE, 10.92 ± 1.01; *A. pubigera*: 1 - 135 pollen grains; mean ± SE, 13.02 ± 1.23; Appendix 4.2). However, cob stigmas captured significantly more pollen than papillate stigmas in both species (*A. maritima*: cob = 12.42 ± 1.63; papillate = 9.15 ± 1.04; $\chi^2_{1,181}$ = 44.13, P < 0.001; *A. pubigera*: cob = 16.60 ± 2.00; papillate = 8.93 ± 1.22; $\chi^2_{1,266}$ = 292.12, P < 0.001). Overall, populations of *A. maritima* exhibited significantly higher amounts of compatible pollen capture for both mating types than would be predicted from random pollination (Fig. 4.3A); the only exceptions were Foz do Rio Neiva, the smallest population sampled (n = 30 plants), where random pollen capture was detected for both mating types, and Cabedelo (Fig. 4.3A) in which cob stigmas had significantly

more incompatible pollen than would be expected from random pollination ($\chi^2_{1,46}$ = 5.40, P = 0.02; Fig. 4.3A). No case of random pollen capture was detected for any of the three sampled populations of *A. pubigera* (Fig. 4.3B). However, although two populations exhibited significant levels of disassortative pollen capture for both mating types, pollen capture was predominantly assortative in Paçô, with significantly higher numbers of incompatible than compatible pollen grains captured by stigmas of the two mating types.

In distylous *Limonium vulgare*, zero pollen loads were also significantly more frequent for papillate than cob stigmas ($\chi^2_{1,223}$ = 22.87, P < 0.001). Pollen loads on stigmas of the four populations investigated ranged between 1 to 76 pollen grains (mean ± SE, 13.36 ± 1.30; Appendix 4.2). Similar to *Armeria*, cob stigmas captured significantly more pollen than papillate stigmas (cob = 18.51 ± 1.92; papillate = 6.99 ± 1.37; $\chi^2_{1,166}$ = 381.12, P < 0.001). Overall, disassortative pollen capture was detected for the B/papillate mating type, while assortative pollen capture was observed for the A/cob mating type, except for cob stigmas from Torreira and papillate stigmas from Gafanha da Encarnação, where random pollen grain capture was detected (Fig. 4.3C).

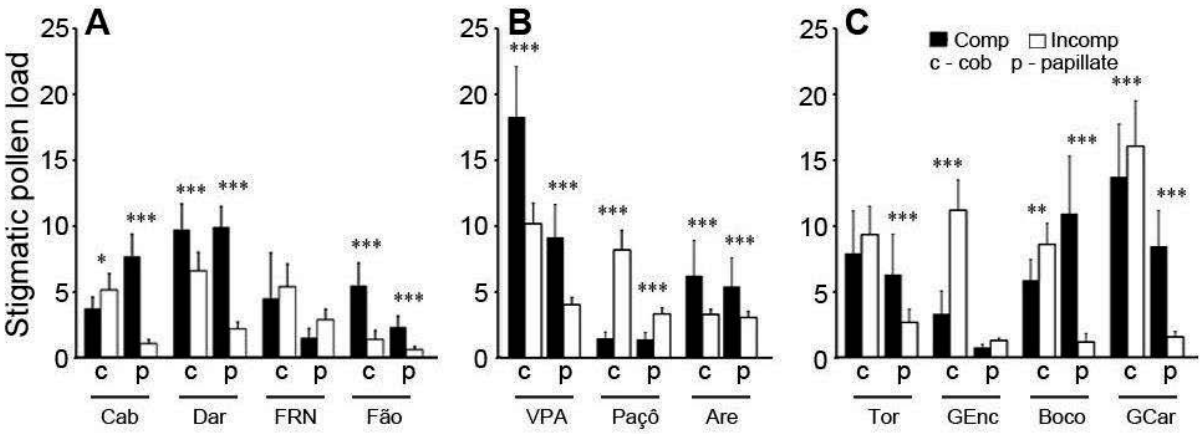


Figure 4.3. Compatible (Comp; black bars) and incompatible (Incomp; white bars) stigmatic pollen capture for *Armeria maritima*, *A. pubigera*, and *Limonium vulgare* grouped by population. **A.** *Armeria maritima*: Cab – Cabedelo, Dar – Darque, FRN – Foz do Rio Neiva, Fão – Fão. **B.** *Armeria pubigera*: VPA – Vila Praia de Âncora, Paçô – Paçô, Are – Areosa. **C.** *Limonium vulgare*: Tor – Torreira, GEnc – Gafanha da Encarnação, Boco – Boco, GCar – Gafanha do Carmo. Values are mean and SE for each mating type within populations. Mating types are represented by "c" – cob, and "p" – papillate. Statistically significant differences between compatible and incompatible stigmatic pollen loads for each mating type within populations are represented by asterisks; *** P < 0.001, ** P < 0.01, * P < 0.05.

Across the three species there were significant differences among populations in the average number of pollen grains captured by stigmas ($\chi^2_{1,617}$ = 13.44, P < 0.001). This variation in pollen load size was positively associated with the logarithm of population size (GLM: estimate = 0.05, SE = 0.01, z = 3.67, P < 0.001). Stigmas sampled from larger populations captured significantly more compatible pollen than stigmas from smaller populations ($\chi^2_{1,617}$ = 15.80, P < 0.001; GLM: estimate = 0.07, SE = 0.02, z = 3.98, P < 0.001). The occurrence of random pollen capture was negatively associated with the logarithm of population size ($\chi^2_{1,617}$ = 11.64, P < 0.001; GLM: estimate = -0.53, SE = 0.16, z = -3.38, P < 0.001). I found no significant differences in the relative amount of compatible and incompatible stigmatic pollen capture among species (χ^2_2 = 2.00, P = 0.37). However, there were significant differences between floral morphs, with higher disassortative pollen capture for the B/papillate than for the A/cob mating type across species (χ^2_2 = 116.10, P < 0.001; Table 4.1; Fig. 4.3). Overall, disassortative pollen capture was detected across populations of the two *Armeria* species ($\chi^2_{1,447}$ = 97.57, P < 0.001; Appendix 4.2), contrary to what was found for *Limonium vulgare* ($\chi^2_{1,332}$ = 5.89, P = 0.02; Appendix 4.2).

Table 4.1. Results of the GLMMs examining the effects of mating type and species on: (a) the relative amount of compatible and incompatible pollen on stigmas, and (b) compatible pollen transfer proficiency. Values in bold represent statistically significant differences.

	df	χ^2	***P***
(a) compatible : incompatible stigmatic pollen loads			
mating type	1	**116.19**	**<0.001**
species	2	2.00	0.37
mating type × species	2	**102.11**	**<0.001**
(b) compatible pollen transfer proficiency			
mating type	1	0.37	0.55
species	2	0.05	0.97
mating type × species	2	0.16	0.92

Pollen transfer proficiencies in natural populations

There were no significant differences between mating types in the probability of compatible pollen transfer to the stigmas of the three species (*A. maritima*: χ^2_1 = 0.24, P = 0.62; *A. pubigera*: χ^2_1 = 2.40, P = 0.12; *L. vulgare*: χ^2_1 = 1.64, P = 0.20). However,

incompatible pollen transfer proficiency was larger for cob than papillate stigmas in each of the three species (*A. maritima*: $\chi^2_1 = 14.40$, $P < 0.001$; *A. pubigera*: $\chi^2_1 = 4.75$, $P = 0.03$; *L. vulgare*: $\chi^2_1 = 58.38$, $P < 0.001$; Fig. 4.4; Appendix 4.5). Finally, there were no significant differences in the overall proficiencies of compatible pollen transfer among the three species ($\chi^2_2 = 0.05$, $P = 0.97$; Table 4.1).

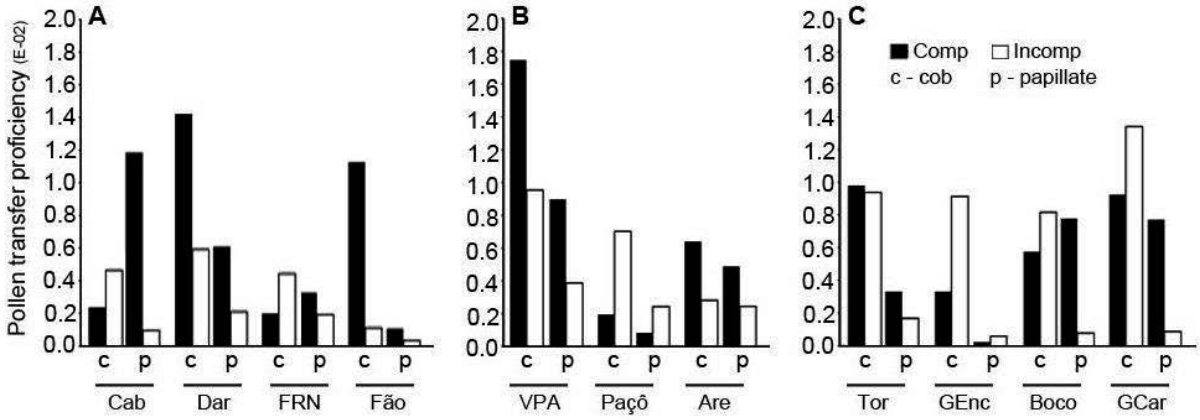


Figure 4.4. Compatible (Comp) and incompatible (Incomp) pollen transfer proficiencies calculated for *Armeria maritima*, *A. pubigera*, and *Limonium vulgare* grouped by population. See *Materials and methods* for the calculation of pollen transfer proficiencies. **A.** *Armeria maritima*: Cab – Cabedelo, Dar – Darque, FRN – Foz do Rio Neiva, Fão – Fão. **B.** *Armeria pubigera*: VPA – Vila Praia de Âncora, Paçô – Paçô, Are –Areosa. **C.** *Limonium vulgare*: Tor – Torreira, GEnc – Gafanha da Encarnação, Boco – Boco, GCar – Gafanha do Carmo. Mating types are represented by "c" – cob, and "p" – papillate.

Pollen adherence and germination

Preliminary studies revealed that it was considerably more challenging to get self-pollen to adhere to stigmas in comparison with compatible pollen in controlled pollinations. For each self-pollination treatment, several attempts were required before the exact number of pollen grains was deposited on stigmas. Self-pollen grains failed to attach to stigmatic papillae and dropped from flowers. As a result, overall pollen adherence after 1h and 3h was much lower in self-pollinations when compared with legitimate pollinations ($\chi^2_{1,122} = 13.18$, $P < 0.001$; Appendix 4.6). In the very few cases in which self-pollen adhered to the stigmatic papillae, the pollen grains failed to germinate (Appendix 4.7). In contrast, more than 95% of compatible pollen grains that adhered to stigmas germinated ($\chi^2_{1,123} = 0.04$, $P = 0.85$; Appendix 4.6), and there were no differences between blocks ($\chi^2_{1,123} = 0.09$, $P = 0.76$), mating types ($\chi^2_{1,123} = 0.54$, $P = 0.46$) or time of stigma collection ($\chi^2_{1,123} = 0.50$, $P = 0.48$). The number of pollen tubes

growing in the upper part of the style was significantly lower than the number of germinated pollen grains on the stigmatic papillae after legitimate pollination ($\chi^2_{1,123}$ = 58.68, $P < 0.001$; Appendix 4.6), and no differences between blocks ($\chi^2_{1,123}$ = 0.28, P = 0.60), mating types ($\chi^2_{1,123}$ = 0.03, P = 0.87) or time of stigma collection ($\chi^2_{1,123}$ = 0.95, P = 0.33) were found.

In mixed pollinations, self-pollen adherence was once again significantly lower than compatible pollen adherence ($\chi^2_{1,122}$ = 25.01, $P < 0.001$; Appendix 4.6) with no differences between blocks ($\chi^2_{1,122}$ = 0.11, P = 0.75), mating types ($\chi^2_{1,122}$ = 1.59, P = 0.21) or time of stigma collection ($\chi^2_{1,122}$ = 0.82, P = 0.67). The very few self-pollen grains that adhered to stigmas (n = 3) failed to germinate. Large numbers of compatible pollen grains adhered to the stigma and germinated (> 90%; $\chi^2_{1,123}$ = 0.09, P = 0.77; Appendix 4.6), with no differences between blocks ($\chi^2_{1,123}$ = 0.49, P = 0.48), mating types ($\chi^2_{1,123}$ = 2.28, P = 0.13) or time of stigma collection ($\chi^2_{1,123}$ = 0.77, P = 0.38). I detected a significant decrease in the number of pollen tubes growing in the upper part of the style when compared with the number of germinated pollen grains on the stigmatic papillae ($\chi^2_{1,123}$ = 33.45, $P < 0.001$; Appendix 4.6), with no differences between blocks ($\chi^2_{1,123}$ = 0.64, P = 0.42), mating types ($\chi^2_{1,123}$ = 0.18, P = 0.67) or time of stigma collection ($\chi^2_{1,123}$ = 0.73, P = 0.39).

DISCUSSION

My investigation of pollen transfer and capture in three species of Plumbaginaceae, and the quantification of pollen adherence in *Armeria maritima*, represents the first attempt to evaluate the role of ancillary characters in promoting disassortative pollination in natural populations of heteromorphic plants. My analyses of natural stigmatic pollen loads revealed several novel findings: (1) ancillary characters in *Armeria* species promoted disassortative pollination in the absence of reciprocal herkogamy, although the levels of compatible pollen transfer and capture varied considerably among populations; (2) the overall amounts of compatible pollen captured by the two mating types in each species were roughly similar, but pollen loads contained significantly more incompatible grains on cob than papillate stigmas; (3) controlled pollination experiments with *A. maritima* resulted in a near total failure of

self-pollen to adhere to stigmas and germinate in contrast to compatible pollen. Finally, I also obtained quantitative evidence, based on structural measurements, for morphological complementarity between the pollen sexine and stigmatic papillae of the mating morphs, as predicted by Dulberger's hypothesis. Below, I consider the role of pollen-stigma dimorphisms in promoting disassortative pollination in heterostylous species and discuss the relevance of my results to the topographical complementarity hypothesis.

Disassortative pollination in the absence of reciprocal herkogamy

Floral morph frequencies in each of the 11 populations that I investigated were not significantly different from the 1:1 ratio expected in populations of heteromorphic species experiencing high levels of disassortative mating. This mating pattern is driven by negative frequency-dependent selection, which rapidly drives floral morph ratios to equality (Ganders, 1979; Barrett, 1992a), even in the relatively small-sized populations that I encountered in this study. In common with most heteromorphic species (reviewed by Barrett and Cruzan, 1994), disassortative mating in Plumbaginaceae is guaranteed by the occurrence of a strong diallelic incompatibility system (Baker, 1948a, 1966), which prevents opportunities for intramorph and self mating to cause deviations from isoplethy. Although pollinator activity and stigmatic pollen loads in populations were relatively low, and some stigmas failed to capture pollen (≤ 25%), my analysis revealed significant levels of disassortative pollen capture in nine of the 14 morph × population combinations in the *Armeria* species (Fig. 4.3) in the absence of reciprocal herkogamy. This finding supports my prediction that the pollen and stigma polymorphisms function in the rejection of incompatible pollen and thus play a role in promoting disassortative pollen capture.

Interpretations of the function of heterostyly usually involve a distinction between the morphological and physiological features of the syndrome, with the different components viewed as serving complementary functions (Yeo, 1975; Ganders, 1979; Kohn and Barrett, 1992; Lloyd and Webb, 1992a; Barrett, 2002). Reciprocal herkogamy functions primarily during the pollination process by causing pollen segregation on the pollinators' body and promoting disassortative pollen transfer and reducing pollen wastage (Darwin, 1877; Ganders, 1979; Kohn and Barrett, 1992; Lloyd and Webb, 1992b; Stone and Thomson, 1994; Barrett and Shore, 2008; Zhou *et al*., 2015). In contrast, heteromorphic incompatibility is generally viewed as a post-pollination

mechanism governing mating patterns by ensuring disassortative mating and limiting the harmful effects of self-fertilization (Barrett and Cruzan, 1994). Ancillary characters have largely been ignored in these discussions, most probably because it has been unclear what specific function(s) they serve, and also because in heterostylous groups that do not possess heteromorphic incompatibility they are absent or not well developed (Dulberger, 1992). Additionally, separating the morphological and physiological functions of ancillary characters is technically challenging. Indeed, as discussed below, attempting to do so may not be worthwhile because the incompatibility responses after incompatible pollination involve both structural and chemical components working in concert with neither subordinate to the other (Dulberger, 1975a, b). In contrast to homomorphic incompatibility, the mating types in heterostylous species differ structurally with heteromorphic characters of stigmas and pollen grains participating in the physiological mechanism of incompatibility (Dulberger, 1975b p. 407; Mattsson, 1983; Heslop-Harrison and Heslop-Harrison, 1985). It is therefore not unexpected that these polymorphic differences play an important role in the pollination process in a manner completely different from the self-rejection mechanisms evident in species with homomorphic incompatibility.

My observations and measurements of pollen and stigmas provided partial evidence for the "lock and key" mechanism originally proposed by Dulberger (1975a). I found that pollen lumen diameter of a given pollen type and inter-papillae distance of the opposite mating type matched one another (Appendices 4.3, 4.4). Adherence and recognition of compatible pollen appears to require intimate contact between at least two lumina of a given pollen type and two stigmatic papillae of the opposite mating type. Although this seems to be the case for papillate stigmas, it is not as clear for cob stigmas (Dulberger, 1975a, 1992; Baker, 1966). In this latter case, large papillae width combined with a small inter-papillae distance probably contributes to some adherence of incompatible pollen type A to cob stigmas, given the larger lumina diameter of this pollen type. However, since incompatibility responses in Plumbaginaceae occur at the stigmatic surface (Dulberger, 1975a, 1992 p. 59: Table 5), incompatible pollen fails to germinate, as was also revealed by my controlled pollination experiment.

Polymorphisms in pollen size and exine sculpturing, and stigmatic papillae length and shape, are involved in controlling the adherence and germination of pollen on stigmas after deposition by pollinators (Iversen, 1940; Dulberger, 1975a, b, 1992; Mattsson, 1983; Heslop-Harrison and Heslop-Harrison, 1985). Because the precise

physiological processes operating during the incompatibility response are not well understood, the experimental dissociation of morphological and physiological features of the polymorphism is impossible at this stage. A study of the physical and chemical aspects of early events in pollen adhesion, hydration and germination in *A. maritima* reported that exine lipids act as a lipophilic adhesive in compatible pollinations, with this process mediated by the morph-specific differences in the structural characteristic of the pollen exine cavities and the stigmatic papillae (Mattsson, 1983). Compatible pollen grains became firmly 'glued' to the stigma within a few seconds and by 30-60s had become hydrated. In contrast, incompatible pollen grains remaining on the stigma failed to hydrate or hydration was greatly delayed (> 2h). These experimental results are consistent with morphological observations and controlled pollination studies herein indicating that structural features of the pollen and stigma polymorphisms play a role in mediating the rejection of incompatible pollen. Because of the absence of morphological differences in height between the styles and stamens of the mating types in *Armeria,* pollen transfer by insects may be essentially random and thus pollen loads will be composed of a substantial component of incompatible pollen. However, during the pollination process, the combined effects of morphology and physiology function in 'sorting' pollen loads so that compatible pollen is overrepresented when compared with what would have occurred under random pollination.

My data demonstrating significant disassortative pollen capture in *A. maritima* and *A. pubigera* are consistent with this idea. An early observation of Iversen (1940), who reported that 95% of the pollen deposited on open-pollinated stigmas of *Armeria maritima* originated from the opposite floral morph is also in accord with this interpretation. Unlike most heterostylous species, in *Armeria* the sites of inhibition of incompatible pollen in both floral morphs occur on the stigmatic surface (reviewed in Dulberger, 1975a, 1992 p. 47: Table 5; Barrett and Cruzan, 1994). This feature of self-rejection in concert with the absence of reciprocal herkogamy probably explains why intermorph structural differences between stigmatic papillae and pollen grains are especially well developed in *Armeria* compared with most other heterostylous species.

Although I detected significant amounts of disassortative pollen capture in more than half of the populations sampled, 10 of the 22 comparisons across each of the three species involved random or assortative pollen capture. Several factors may account for this result. First, the examination of intact flowers, as was done in my study, did not allow me to distinguish between the incompatible components of the pollen load, in

particular whether incompatible pollen resulted from self-pollination, including intra-flower and geitonogamous self-pollination, or from intramorph cross-pollination. Elimination of the self-pollen component of pollen loads by emasculation allows a more accurate quantitative assessment of the role of morphological traits in promoting disassortative pollen capture (Ganders, 1974; Barrett and Glover, 1985; Lloyd and Webb, 1992b; Chapter 2). However, flowers of each of the three species I investigated are of small size (5.5-8.5 mm; Feliner, 1990; Erben, 1993), which precluded the possibility of emasculation without significant levels of self-pollen contamination.

Pollen loads in populations exhibiting random pollination may have contained a significant component of self-pollen because of small population size and low pollinator activity. Indeed, I detected a positive relation between population size and both total and compatible pollen load size, and a negative relation between population size and random pollination. Large floral displays generally result in higher attractiveness to pollinators and a larger pollen pool available for export (*e.g.*, Eckhart, 1991; Ågren, 1996; Fausto *et al*., 2001). Thus, it seems probable that my measures of the proficiency of compatible pollen transfer and disassortative pollen capture are conservative and would likely be greater if I had been able to use emasculated flowers to study pollen loads, particularly in populations of distylous *L. vulgare*.

Assortative pollination was found to be consistently higher for cob than papillate stigmas in each of three species. This finding is in accord with previous results of intramorph pollinations performed in four *Limonium* species (Dulberger, 1975a). Pollen grains of type A adhered to both stigma types, whereas pollen type B adhered to cob but not papillate stigmas. Similar differential adhesion between dimorphic pollen and stigmatic papillae has also been reported after intramorph pollinations in distylous *Linum* species (Dulberger, 1974, 1981; Ghosh and Shivanna, 1980). Therefore, it seems likely that in each of the three species that I investigated, the asymmetry I observed in incompatible pollen capture arises because of differences in pollen adherence to stigmas of the mating types. In distylous *Limonium vulgare*, however, stylar dimorphism may also have contributed towards differences in pollen capture between the morphs. Asymmetrical pollen capture is widely reported in distylous species, with the L-morph capturing more incompatible pollen than the S-morph (reviewed in Ganders, 1979; Barrett and Shore, 2008; Chapter 2). Morph-specific differences in pollen load composition have been attributed to the contrasting stigma positions of the morphs and the influence that this has on pollinator contacts (Ganders, 1974). Whereas contact

between the pollinator's body and short stigmas is highly restricted, because of their insertion in the floral tube, exerted long stigmas are more accessible to pollinators, which can assume various orientations in the flower resulting in a greater probability of incompatible pollen transfer (Ganders, 1974; Lloyd and Webb, 1992b; Stone and Thomson, 1994). However, differences in stigma height do not occur in *Armeria* species, and therefore this potential cause of asymmetrical pollen capture cannot occur.

Ancillary characters and incompatibility responses in heterostylous species

The morphological complementarity between exine sculpturing and stigmatic papillae shape in dimorphic species of Plumbaginaceae involves the establishment of a very close physical contact between the pollen exine and stigma cuticle involving chemical (Dulberger, 1975b; Mattsson, 1983) and possibly electrostatic interactions (Vaknin *et al*., 2000). The initial physical contact between the pollinator's body and the stigmatic surface may diminish the likelihood of incompatible pollen detachment and deposition, thus reducing pollen wastage on incompatible stigmas (Dulberger, 1975b, 1992). Thus, by creating the opportunity for preferential adhesion of compatible pollen grains, pollen-stigma dimorphisms serve as a mechanism promoting disassortative pollination.

During my controlled hand pollination experiment, I noticed sharp differences when transferring compatible versus self-pollen to stigmas. When I touched the stigmas with the pollinating needle, compatible pollen always adhered on first contact. On the contrary, self-pollen fell off the stigma the majority of times requiring many more attempts to assure that the required number of self-pollen grains were deposited. However, after one hour virtually all self-pollen grains were absent from stigmas and had fallen off. Under field conditions, a larger amount of incompatible pollen was present on stigmas of all three species indicating that differential adhesion is not an absolute barrier and with sufficient quantities of assortative pollen transferred by insects some remains on stigmas and is wasted, owing to its failure to hydrate and germinate (cf. Mattsson, 1983). Heteromorphic incompatibility thus guarantees disassortative mating even when pollen loads contain a substantial fraction of incompatible pollen.

My results on natural pollen loads on papillate stigmas provide partial support for the topographical complementarity hypothesis. However, this mechanism does not seem to be a feature of both floral morphs as both pollen types adhered to cob stigmas (Dulberger, 1975a, 1992; Baker, 1966). Indeed, my study revealed a significant

asymmetry in incompatible pollen capture in each of the three species with cob stigmas capturing significantly more incompatible pollen than papillate stigmas. On the contrary, a clear pattern of self-pollen failure to adhere and germinate emerged from my hand pollination experiment, regardless of which mating morph was self-pollinated, and it is unclear why self-pollen was not evident on cob stigmas. A combination of factors might help to interpret the differences in incompatible pollen adherence in natural versus hand-pollinated stigmas. First, it is likely that my field sample of stigmas involved flowers of mixed ages and incompatible pollen transfer to flowers probably increased with flower age due to a longer exposure to pollinators. Also, whereas in natural populations the stigmas I sampled were directly transferred to a microscope slide, in the controlled pollination experiment stigmas were placed in a liquid fixative, required for subsequent fluorescence microscopy, and this could have resulted in dislodging of self-pollen from stigmas. Regardless of the causes involved, earlier research indicates that only pollen grains type B succeed in germinating on cob stigmas, (Dulberger, 1975a, 1992), and this was also confirmed by the results of my controlled pollination experiment. Ancillary polymorphisms therefore likely serve in promoting disassortative pollination and in reducing pollen wastage. However, in addition, as Lloyd and Webb (1992a p. 171) suggested, by participating in the mechanisms of self-incompatibility they also restrict the success of self-pollinations.

Experimental studies of pollen loads in emasculated flowers (Ganders, 1974; Barrett and Glover, 1985; reviewed by Lloyd and Webb, 1992b), and studies of mating patterns using genetic markers (Kohn and Barrett, 1992; Zhou *et al*., 2015), have provided convincing evidence that reciprocal herkogamy functions to promote disassortative pollen transfer in heterostylous populations. Because the majority of heterostylous species also possess ancillary pollen and stigma polymorphisms their function may appear redundant. However, ancillary polymorphisms may aid in reinforcing the effectiveness of the stamen-style polymorphism in promoting compatible pollen transfer, especially since most pollen flow studies indicate that substantial amounts of incompatible pollen are deposited on stigmas by pollinators (reviewed in Ganders, 1979; Lloyd and Webb, 1992b; Chapter 2). Interestingly, there was no evidence from my study that the combined influence of both sets of polymorphisms in distylous *Limonium* was any more effective in promoting disassortative pollen capture than when pollen and stigma polymorphisms acted alone, as in *Armeria*. Thus, whereas reciprocal herkogamy may function mainly to promote intermorph pollen transfer, pollen-stigma

dimorphisms may additionally serve to limit self-pollen deposition by both structural and chemical mechanisms. The combined effects of this suite of morphological polymorphisms results in the promotion of disassortative pollination and a reduction in pollen wastage in heterostylous plants.

Appendix 4.1. Mating type frequencies, population size (*n*), total number of plants sampled in each population (in parentheses), and *G-test* values for goodness-of-fit (df = 1) for sampled populations of *Armeria maritima*, *A. pubigera* and *Limonium vulgare* (n.s. - non-significant at $P > 0.05$). Geographical coordinates for each population sampled are provided.

Population	**Geographical coordinates**		***n***	**Mating type frequency**		***G-test***
				A/cob	**B/papillate**	
Armeria maritima						
Cabedelo	41.68902	-8.81777	95 (48)	0.54	0.46	$0.34^{n.s.}$
Darque	41.68860	-8.80214	472 (100)	0.55	0.45	$1.01^{n.s.}$
Foz do Rio Neiva	41.60811	-8.80559	30 (24)	0.50	0.50	$0.00^{n.s.}$
Fão	41.51428	-8.77310	54 (40)	0.55	0.45	$0.41^{n.s.}$
Armeria pubigera						
V Praia de Âncora	41.82889	-8.87494	213 (100)	0.54	0.46	$0.65^{n.s.}$
Paçô	41.75641	-8.87751	156 (100)	0.48	0.52	$0.16^{n.s.}$
Areosa	41.71768	-8.86611	152 (100)	0.50	0.50	$0.00^{n.s.}$
Limonium vulgare						
Torreira	40.75103	-8.70105	50 (43)	0.44	0.56	$0.60^{n.s.}$
Gafanha Encarnação	40.62375	-8.73566	400 (100)	0.46	0.54	$0.65^{n.s.}$
Boco	40.58854	-8.68783	34 (34)	0.53	0.47	$0.12^{n.s.}$
Gafanha Carmo	40.58765	-8.74699	52 (52)	0.44	0.56	$0.71^{n.s.}$

Appendix 4.2. Total number of pollen grains and number of compatible and incompatible pollen grains on stigmas of the mating types in populations of *Armeria maritima*, *A. pubigera* and *Limonium vulgare*. Results of the GLMs investigating differences between compatible and incompatible pollen loads per mating type are presented. The results of GLMMs using pooled data for all populations of the two *Armeria* species, and for populations of *Limonium vulgare* are given in bold. Values are mean ± SE. *n* = total number of stigmas per mating type per population. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s. - non-significant at $P > 0.05$.

	Stigma type	*n*	Pollen type			χ^2_1
			Total	Compatible	Incompatible	
Armeria maritima						
Cabedelo	cob	24	8.8 ± 1.3	3.7 ± 0.9	5.1 ± 1.3	5.4*
	papillate	20	8.8 ± 1.7	7.7 ± 1.7	1.1 ± 0.3	73.4***
Darque	cob	50	16.3 ± 2.9	8.7 ± 1.9	6.6 ± 1.4	29.5***
	papillate	43	12.1 ± 1.7	9.9 ± 1.6	2.2 ± 0.5	174.9***
Foz do Neiva	cob	10	9.9 ± 4.4	4.5 ± 3.5	5.4 ± 1.7	0.8$^{n.s.}$
	papillate	8	4.4 ± 0.7	1.5 ± 0.7	2.9 ± 0.7	3.3$^{n.s.}$
Fão	cob	15	6.9 ± 1.6	5.4 ± 1.7	1.4 ± 0.6	31.0***
	papillate	13	2.9 ± 0.7	2.3 ± 0.8	0.6 ± 0.2	11.0***
Armeria pubigera						
V Praia Âncora	cob	53	28.5 ± 4.3	18.3 ± 3.8	10.2 ± 1.5	118.6***
	papillate	44	13.2 ± 2.6	9.1 ± 2.5	4.0 ± 0.6	82.7***
Paçô	cob	44	9.7 ± 1.5	1.5 ± 0.5	8.2 ± 1.5	161.1***
	papillate	40	4.7 ± 0.7	1.4 ± 0.5	3.3 ± 0.5	29.5***
Areosa	cob	46	9.5 ± 2.7	6.2 ± 2.7	3.3 ± 0.4	40.8***
	papillate	41	8.5 ± 2.1	5.4 ± 2.2	3.0 ± 0.5	26.8***
Pooled data for *Armeria* species				**7.38 ± 0.72**	**4.79 ± 1.80**	**97.57*** **
Limonium vulgare						
Torreira	cob	19	17.1 ± 4.5	7.8 ± 3.2	9.3 ± 2.1	2.2$^{n.s.}$
	papillate	16	8.9 ± 3.9	4.2 ± 2.1	1.8 ± 0.7	22.3***
Gafanha Encarnação	cob	35	14.4 ± 2.8	2.7 ± 1.4	9.0 ± 2.0	131.2***
	papillate	29	2.0 ± 0.3	0.4 ± 0.1	0.7 ± 0.1	3.7$^{n.s.}$
Boco	cob	17	14.4 ± 2.5	5.5 ± 1.5	8.1 ± 1.5	8.6**
	papillate	12	12.0 ± 4.6	8.1 ± 3.4	0.9 ± 0.5	62.8***
Gafanha Carmo	cob	22	29.5 ± 4.7	13.6 ± 4.0	15.9 ± 3.4	0.04*
	papillate	18	9.9 ± 3.0	5.2 ± 1.8	1.0 ± 0.3	67.1***
Pooled data for *L. vulgare* populations				**6.34 ± 1.38**	**7.02 ± 3.24**	**5.89***

Appendix 4.3. Pollen polar axis (P), equatorial diameter (E), P/E and lumen diameter measures for 30 pollen grains from each mating type of *Armeria maritima*, *A. pubigera* and *Limonium vulgare*. Results of the *t*-test for independent samples (*t*) and Mann-Whitney *U*-test (*U*) comparisons of pollen measurements between mating types are also given. Values are mean ± SE (µm). For more details, see *Materials and methods*. *** $P < 0.001$, * $P < 0.05$.

	Mating type		
	A/cob	**B/papillate**	**Statistical test**
Armeria maritima			
Polar axis (P)	59.23 ± 0.89	52.68 ± 0.70	$t = 9.15$***
Equatorial diameter (E)	63.00 ± 0.94	59.30 ± 0.71	$t = 5.36$***
P/E	0.94 ± 0.01	0.89 ± 0.01	$t = 4.66$***
Lumen diameter	9.87 ± 0.31	3.36 ± 0.09	$U = 900.00$***
Armeria pubigera			
Polar axis (P)	58.30 ± 0.94	47.55 ± 0.71	$t = 5.79$***
Equatorial diameter (E)	63.43 ± 1.20	55.57 ± 0.91	$t = 3.13$*
P/E	0.92 ± 0.01	0.86 ± 0.01	$t = 3.97$***
Lumen diameter	9.76 ± 0.24	3.43 ± 0.10	$U = 900.00$***
Limonium vulgare			
Polar axis (P)	46.65 ± 0.51	39.10 ± 0.37	$t = 11.95$***
Equatorial diameter (E)	53.62 ± 0.52	48.17 ± 0.30	$t = 9.14$***
P/E	0.87 ± 0.01	0.81 ± 0.01	$t = 4.42$***
Lumen diameter	6.40 ± 0.29	2.64 ± 0.06	$U = 900.00$***

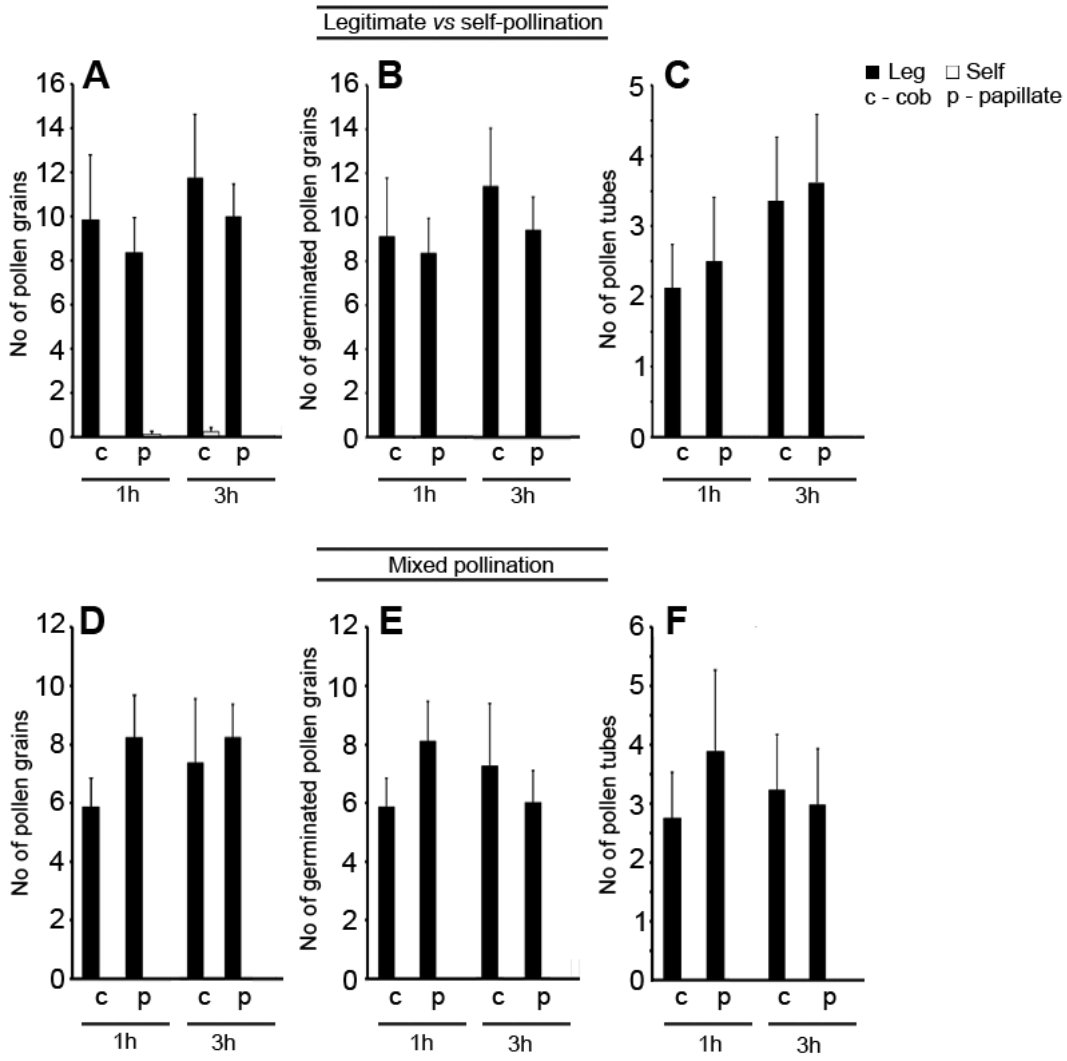
Appendix 4.4. Measurements of stigmatic papilla length, width and inter-papillae distance for 10 stigmas from each mating type of *Armeria maritima*, *A. pubigera* and *Limonium vulgare*. Results of the *t*-test for independent samples (*t*) and Mann-Whitney *U*-test (*U*) comparisons of stigmatic papillae measurements between mating types are also given. Values are mean ± SE (µm). For more details, see *Materials and methods*. *** $P < 0.001$, * $P < 0.05$, n.s. - non-significant at $P > 0.05$.

	Mating type		
	A/cob	**B/papillate**	**Statistical test**
Armeria maritima			
Papillae length	18.13 ± 0.87	16.30 ± 0.33	$t = 1.93$ n.s.
Papillae width	23.03 ± 0.85	6.18 ± 0.10	$U = 100.00$***
Inter-papillae distance	3.30 ± 0.17	13.83 ± 0.84	$U = 0.00$***
Armeria pubigera			
Papillae length	18.13 ± 0.84	15.13 ± 0.38	$t = 3.24$*
Papillae width	19.97 ± 0.63	6.87 ± 0.25	$U = 100.00$***
Inter-papillae distance	3.57 ± 0.20	11.20 ± 0.62	$U = 0.00$***
Limonium vulgare			
Papillae length	13.60 ± 0.39	12.83 ± 0.37	$t = 1.46$ n.s.
Papillae width	13.50 ± 0.63	4.80 ± 0.16	$U = 100.00$***
Inter-papillae distance	2.90 ± 0.10	7.37 ± 0.39	$U = 0.00$***

Appendix 4.5. Pollen transfer proficiencies for *Armeria maritima*, *A. pubigera* and *Limonium vulgare* calculated following the method of Lloyd and Webb (1992b). Values are given as E^{-02}.

	Stigma type	Pollen transfer proficiency	
		Compatible	Incompatible
Armeria maritima			
Cabedelo	cob	0.25	0.47
	papillate	1.19	0.10
Darque	cob	1.44	0.60
	papillate	0.62	0.22
Foz do Neiva	cob	0.21	0.45
	papillate	0.33	0.20
Fão	cob	0.11	0.12
	papillate	1.44	0.004
Armeria pubigera			
V Praia Âncora	cob	1.76	0.97
	papillate	0.91	0.40
Paçô	cob	0.21	0.72
	papillate	0.10	0.26
Areosa	cob	0.65	0.29
	papillate	0.50	0.25
Limonium vulgare			
Torreira	cob	0.99	0.95
	papillate	0.34	0.18
Gafanha Encarnação	cob	0.34	0.92
	papillate	0.003	0.07
Boco	cob	0.58	0.82
	papillate	0.79	0.09
Gafanha Carmo	cob	0.93	1.35
	papillate	0.78	0.10

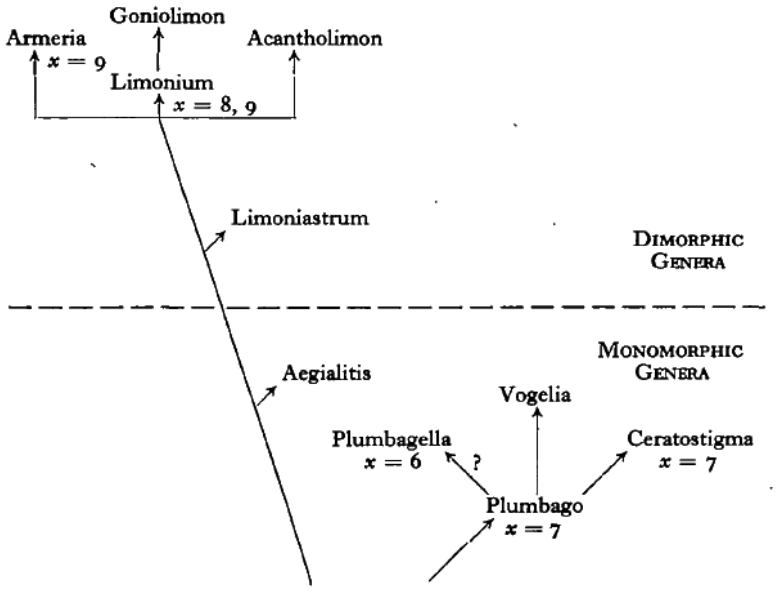
Appendix 4.6. A, D. Pollen adherence; **B, E.** pollen germination; and **C, F.** number of pollen tubes growing in the upper part of the style after controlled hand self-pollinations and legitimate pollinations of *Armeria maritima*. Only data from pollination block 1 is presented; data from block 2 is given in Appendix 4.7. **A, B, C.** Legitimate and self-pollinations. **D, E, F.** Mixed pollinations. Values given are the mean and SE for each mating type. Mating types are represented by "c" – cob, and "p" – papillate. "1h" and "3h" represent time of stigma collection after pollination.



Appendix 4.7. Total number of pollen grains adherent to the stigma, total number of germinated pollen grains, and total number of pollen tubes in the upper part of the style after controlled hand pollinations of *Armeria maritima*. For mixed pollinations, data is given separately for self- and legitimate (leg.) pollen. For details see *Materials and methods*. Values are the mean ± SE.

Pollination treatment	Stigma type	Pollen type	Adherent pollen grains		Germinated pollen grains		Pollen tubes in the upper part of the style	
			1h	3h	1h	3h	1h	3h
Block 1								
Legitimate	cob		9.88 ± 2.91	11.75 ± 2.88	9.13 ± 2.66	11.5 ± 2.64	2.13 ± 0.61	3.38 ± 0.91
	papillate		8.38 ± 1.58	10.00 ± 1.46	8.25 ± 1.63	9.50 ± 1.49	2.50 ± 0.91	3.63 ± 0.98
Self	cob		0.00 ± 0.00	0.25 ± 0.16	-	0.00 ± 0.00	-	-
	papillate		0.13 ± 0.13	0.00 ± 0.00	0.00 ± 0.00	-	-	-
Mixed	cob	Leg.	5.88 ± 0.97	7.38 ± 2.19	5.86 ± 0.97	7.25 ± 2.12	2.75 ± 0.77	3.25 ± 0.94
		Self-	0.00 ± 0.00	0.00 ± 0.00	-	-	-	-
	papillate	Leg.	8.25 ± 1.44	6.13 ± 1.13	8.13 ± 1.34	5.88 ± 1.08	3.88 ± 1.38	3.00 ± 0.94
		Self-	0.00 ± 0.00	0.00 ± 0.00	-	-	-	-
Block 2								
Legitimate	cob		9.25 ± 2.19	9.88 ± 2.41	9.13 ± 2.22	9.50 ± 2.50	3.38 ± 1.27	3.75 ± 0.96
	papillate		10.00 ± 2.62	8.88 ± 1.71	10.0 ± 2.62	8.63 ± 1.70	4.13 ± 0.95	4.25 ± 0.82
Mixed	cob	Leg.	8.88 ± 1.54	7.13 ± 0.93	7.88 ± 1.22	6.88 ± 1.01	2.88 ± 0.58	2.50 ± 0.46
		Self-	0.00 ± 0.00	0.00 ± 0.00	-	-	-	-
	papillate	Leg.	5.25 ± 1.52	4.88 ± 1.30	5.25 ± 1.52	4.88 ± 1.30	3.63 ± 1.44	2.50 ± 0.87
		Self-	0.25 ± 0.16	0.13 ± 0.13	0.00 ± 0.00	0.00 ± 0.00	-	-
Self	cob		0.00 ± 0.00	0.13 ± 0.13	-	0.00 ± 0.00	-	-
	papillate		0.00 ± 0.00	0.00 ± 0.00	-	-	-	-

Chapter 5 – Evolutionary history of the heterostylous syndrome in Plumbaginaceae



Phylogenetic scheme for the family Plumbaginaceae (Baker, 1948a: Fig. 13).

Chapter section in preparation to submission as an original article to SCI journal:
Costa J, Torices R, Castro S, Loureiro J, Barrett SCH. Evolutionary history of the heterostylous syndrome in Plumbaginaceae.

ABSTRACT

Two main theoretical models have been developed to explain the evolution of distyly. The models differ from one another in terms of the selective forces, ancestral traits and sequence of characters involved in the evolutionary assembly of the polymorphism. The "selfing avoidance" model assumes a long homostylous ancestor, and that diallelic incompatibility evolves prior to the establishment of reciprocal herkogamy as a selfing avoidance mechanism, owing to the cost of inbreeding depression. The "pollen transfer" model assumes an approach herkogamous ancestor, involves the reverse pattern of character state buildup, and is largely based on selection for the promotion of proficient cross-pollination. Although these models were developed several decades ago, comparative evidence supporting them is rather limited, although what little data are available are more consistent with the pollen transfer model. Here, I investigate the evolutionary buildup of the heterostylous syndrome in Plumbaginaceae by stochastic character mapping and Bayesian analyses of a well resolved molecular phylogeny of 123 species in the family based of five nuclear and plastid gene regions. I conducted an extensive sampling of herbarium material to characterize three morphological characters (reciprocal herkogamy, pollen exine sculpturing and stigmatic papillae shape) associated with distyly, and in combination with information from the literature I inferred the compatibility status of species. My results indicate that the most likely common ancestor of Plumbaginaceae was self-incompatible and monomorphic for sex-organ arrangement and pollen and stigma characters. Character state reconstructions indicated that reciprocal herkogamy may have evolved at least three independent times, and that reversions from self-incompatibility to self-compatibility, and from sexual reproduction to apomixis, occurred multiple times. My results contribute novel insights into the evolutionary pathway involved in the evolution of distyly in Plumbaginaceae, and provide evidence supporting the selfing avoidance model for the evolution of the floral polymorphism.

KEY WORDS: ancestral condition; ancillary characters; apomixis; homostyly; incompatibility system; monomorphism; reciprocal herkogamy.

INTRODUCTION

The heterostylous syndrome is a notable case of convergent evolution in floral form, with multiple independent origins among the angiosperms. Other features of floral biology to those typical of heterostyly are commonly found in families in which this floral polymorphism has evolved, indicating that there are probably morphological preconditions for heterostyly to arise (Ganders, 1979; Lloyd and Webb, 1992a). Heterostylous flowers are mainly actinomorphic (but see Barrett *et al*., 2000, 2004; Hartley *et al*., 2002), with a depth-probed floral tube concealing nectar at the base, a relatively limited number of stamens (but see Ornduff, 1975a), a stigma occupying a central position within the flower, and plants are exclusively animal pollinated. Collectively, these features suggest that similar selective forces acting on the floral biology of populations were responsible for the evolution of heterostyly in the ~28 families in which the polymorphisms have been reliably reported (Ganders, 1979; Lloyd and Webb, 1992a; Barrett and Shore, 2008).

The origin and evolution of heterostyly has received sustained interest since Darwin's (1877) classic book on the topic. However, it was not until the latter part of the last century that evolutionary biologists began to investigate theoretically the selective forces responsible for the evolutionary buildup of this convergent floral syndrome. The models focused in particular on the sequence in which morphological characters and the physiological incompatibility system became established (Charlesworth, 1979; Charlesworth and Charlesworth, 1979; Lloyd and Webb, 1990a, b). Although the models are widely cited in the literature, explicit testing of their contrasting predictions has been limited.

The selective forces, ancestral states, and evolutionary pathways involved in the two models for distyly (Charlesworth and Charlesworth, 1979: Chapter 1 Fig. 1.4A; Lloyd and Webb, 1992a, b: Chapter 1 Fig. 1.4B) are not consensual (reviewed in Chapter 1; Barrett, 1992a). In the "selfing avoidance" model by Charlesworth and Charlesworth (1979), diallelic incompatibility precedes the establishment of reciprocal herkogamy, whereas the "pollen transfer" model assumes the opposite order of establishment of morphological and physiological characters (Lloyd and Webb, 1992a, b). A comprehensive test of these models requires that the ancestral character state of heterostylous lineages be determined, as well as the selective forces involved. Comparative data available from phylogenetic reconstructions and character mapping provide some support in favor of Lloyd and Webb's model (*Narcissus*, Graham and

Barrett, 2004; *Lithodora* and related *Glandora*, Ferrero *et al*., 2009a, 2012; *Exochaenium*, Kissling and Barrett, 2013), thus suggesting that selection for improved cross-pollination may have been the selective force driving the evolution of heterostyly. However, these analyses are by no means conclusive, and because the polymorphism has clearly evolved on numerous occasions, it is possible that both main models for the evolution of distyly will be supported in different lineages once more data becomes available (Barrett and Shore, 2008).

Plumbaginaceae is of worldwide distribution and comprised of 27 genera and ~650 species (Kubitzki, 1993). The family exhibits a great diversity of morphological characters associated with the heterostylous syndrome, thus providing an opportunity to investigate the evolutionary buildup of the floral polymorphism. This fact was first recognized by Herbert G. Baker (1948a, b, 1966), who pointed out that species from subfamily Staticoideae displayed significant morphological differences in pollen and stigma characters when compared with species from subfamily Plumbaginoideae. Taxa in Plumbaginoideae are largely distylous and display relatively uniform patterns of pollen exine ornamentation and stigmatic papillae shape between style morphs (Baker, 1948a, 1966; Dulberger, 1975a; Erdtman, 1986; Ghobary, 1986; Ferrero *et al*., 2009b), and hereafter I refer to these taxa as monomorphic for pollen exine ornamentation and stigmatic papillae shape (Fig. 5.1). In contrast, patterns of variation in subfamily Staticoideae exhibit considerable morphological variation. The most distinctive feature of many species in Staticoideae is striking pollen-stigma dimorphism (*i.e*., ancillary pollen and stigma characters). Generally referred to as *Armeria*-type pollen as opposed to *Plumbago*-type pollen from the Plumbaginoideae (Erdtman, 1986), dimorphic pollen commonly has a coarse or a finely reticulate sexine, named pollen type A or B, respectively (Fig. 5.1; Chapter 4: Fig. 4.1). Plants producing pollen type A have a cob-like stigmatic papillae, whereas plants with pollen type B have papillate stigmas (Baker, 1948a, b, 1966; Dulberger, 1975a, b, 1992; Fig. 5.1). As discussed in Chapter 4, pollen-stigma dimorphisms are tightly associated with a diallelic physiological self-incompatibility system, and likely function to promote disassortative pollination because of the absence of reciprocal herkogamy in Staticoideae (see Dulberger, 1975a, 1992; Mattsson, 1983).

The first phylogenetic hypothesis for the buildup of the heterostylous syndrome in Plumbaginaceae was proposed by Baker (1948a, 1966), based on patterns of geographical distribution of species and studies of morphological characters,

particularly of pollen and stigmas. It is important to note, this hypothesis was not based on today's perspective of a "phylogenetic hypothesis" because no explicit phylogenetic methods were involved. Baker simply ordered species and characters in a particular sequence based on his own ideas on the most plausible evolutionary scenario (see Fig. 5 in Baker, 1966; and image in the cover page of this Chapter). He postulated that the heterostylous syndrome evolved from a common ancestor with diallelic incompatibility and monomorphic pollen and stigmas, but by different pathways in the two subfamilies (Baker, 1948a: Fig. 13, 1966: Fig. 5). He considered that reciprocal herkogamy was the only morphological addition to the ancestral incompatibility system in Plumbaginoideae, whereas in Staticoideae, the floral polymorphisms evolved in three stages. Specifically, he proposed that pollen dimorphism established first in species with monomorphic capitate stigmas of *Goniolimon*, *Acantholimon* and some *Limonium*. Subsequently, filiform dimorphic stigmas and dimorphic pollen evolved in species of *Armeria*, *Limoniastrum* and *Limonium*. Finally, reciprocal herkogamy evolved in a single species of *Limonium* (pollen-stigma dimorphic *Limonium vulgare*). Baker assessed the compatibility status of a large number of *Limonium* by performing controlled hand-pollinations (Baker, 1953a, b). He inferred that self-incompatibility was widespread in the family, apart from a few self-compatible or apomictic species of *Limonium* with "secondary monomorphism" of pollen and stigmas. He considered this secondary monomorphism as derived from pollen-stigma dimorphism as a result of either the evolution of homostyly [small flowered selfing forms generally derived from heterostylous ancestors (Darwin, 1877; Ganders, 1979)] or through the replacement of sexual reproduction by apomixis (Baker, 1966). Baker's hypothesis (1948a, 1966) shares some features with the Charlesworth and Charlesworth model (1979) for the evolution of distyly as both involve the evolution of self-incompatibility prior to the establishment of reciprocal herkogamy.

Here, I investigate Baker's ideas on the evolutionary buildup of the heterostylous syndrome in Plumbaginaceae using more rigorous comparative methods. I combine molecular data to construct a phylogenetic tree for the family and use an extensive survey of herbarium specimens and the literature to investigate heteromorphic morphological traits. My study addressed the following specific questions: (1) Was the most likely common ancestor of Plumbaginaceae self-incompatible with monomorphic pollen and stigmas? (2) How many times has reciprocal herkogamy evolved in the family and was this prior to or after the establishment of self-incompatibility and pollen

and stigma dimorphisms? (3) Is there evidence of reversions from pollen-stigma dimorphism to monomorphism? (4) Is there evidence for the breakdown of distyly to homostyly and for the transition from sexual to asexual reproduction as a result of the origin of apomixis? Following the presentation of my results, I consider the extent to which my findings provide support for competing models of the evolution of distyly, and also of Baker's original ideas on the evolutionary history of the heterostylous syndrome in Plumbaginaceae.

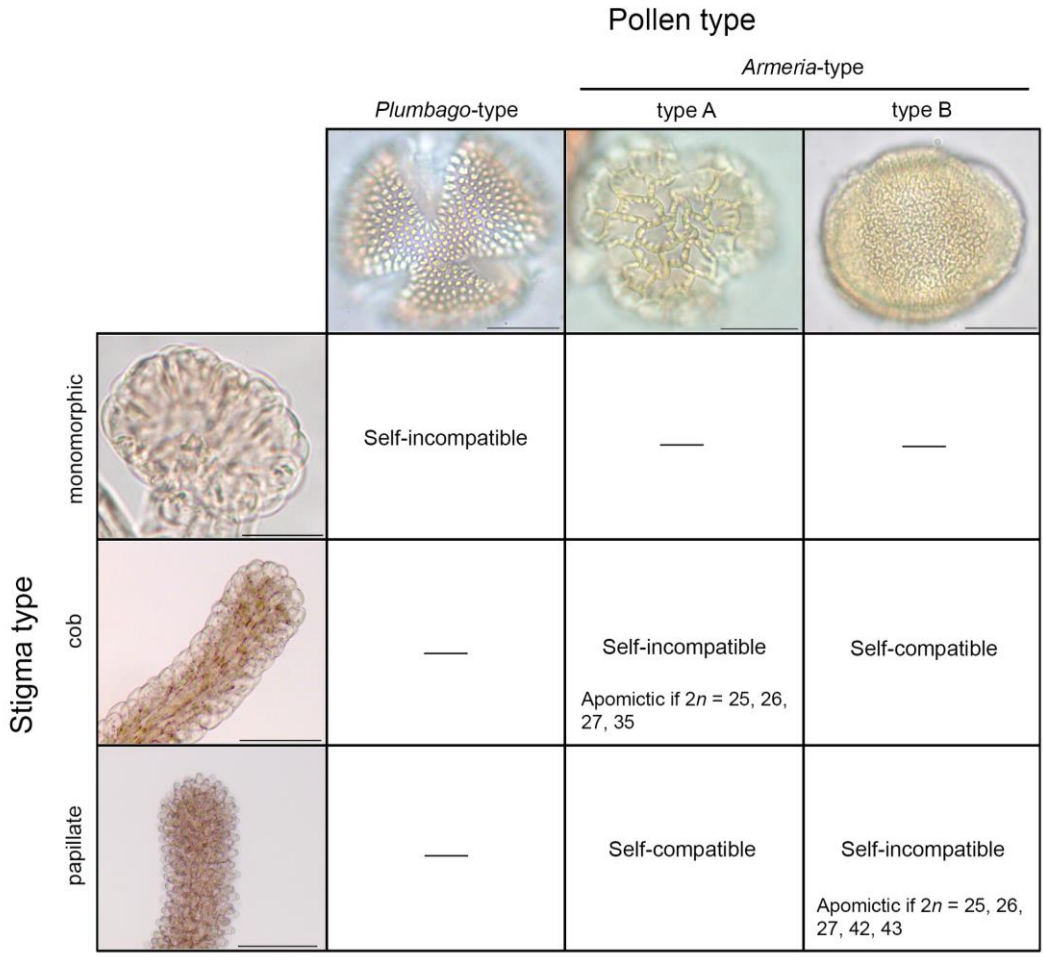


Figure 5.1. Pollen and stigma combinations found in Plumbaginaceae based on Baker (1948a, 1966) and Erben (1979). Light microscopy photographs of pollen grains and stigmas obtained at 1000× and 200× magnification (Leitz HM-LUX 3), respectively. Scale bars: pollen grains, 20 µm; monomorphic stigma, 25 µm; cob and papillate stigmas, 100 µm.

MATERIALS AND METHODS

Characterization of the floral polymorphism and associated characters

To characterize the distribution of the heterostylous syndrome among taxa of Plumbaginaceae, I combined two approaches: (1) data collection from the literature, and (2) sampling of herbarium specimens. I obtained morphological data on reciprocal

herkogamy, anther and stigma dimorphism available from species descriptions in floras and published peer reviewed papers after web searches using the species name as key word in Google Scholar (last time accessed October 15, 2016). However, most (99 out of 129 species) of the morphological data was obtained after sampling herbarium specimens at Coimbra (COI) and Edinburgh (E) herbaria. COI has the largest collection of Portuguese plants, whereas E has a very complete collection from the Middle East (F. Sales, pers. comm.), and the herbaria combined cover the two main centers of diversification of the family (Erben, 1993; Kubitzki, 1993). I sampled one flower per specimen for a maximum of three specimens per species from each herbarium, for a total of 317 specimens sampled (Appendix 5.1). Under a dissecting microscope, I carefully removed one flower from each specimen and transferred it to a paper bag properly identified with the species name and herbarium code. To re-hydrate flowers, I transferred each one to a labeled Eppendorf tube with an aqueous solution of soap for 24h. Later, I dissected the flower under a dissecting microscope to: (1) record the relative position of anthers and stigmas within the flower, (2) transfer one anther to a microscope slide with a drop of distilled water to open it and release the pollen grains to investigate exine ornamentation, and (3) remove the stigma to a microscope slide with a drop of distilled water for characterization of stigma morphology and stigmatic papillae shape. I performed pollen and stigma characterization under a light microscope at 400× magnification (Leitz HM-LUX 3).

I used the morphological data to construct a matrix with six characters each with two alternate states, except for stigma morphology: reciprocal herkogamy versus monomorphic arrangement of sex organs; self-incompatible versus self-compatible; *Armeria*-type pollen versus monomorphic pollen; stigma dimorphism versus stigma monomorphism; fimbriate/lanceolate/pear-shaped stigma versus capitate stigma versus filiform stigma; and sexual reproduction versus apomixis (Appendix 5.2). To construct the character matrix, I established several criteria as follows: (1) the observation of the relative position of anthers and stigmas within a flower was sometimes complicated by the use of flower buds or senescent flowers and if no information was available from the literature, the species was scored as "no information available" for reciprocal herkogamy. (2) Since I did not experimentally test for self-incompatibility in the species included in my analysis, I combined two approaches to infer the compatibility status (*i.e.*, self-incompatible versus self-compatible). For species in Plumbaginoideae, I used only information available from the literature as observations of pollen and stigmas,

which are monomorphic, were uninformative. For species in Staticoideae, I combined information from the literature if available (*e.g.*, Baker 1966: Table 1) with my own observation of pollen exine sculpturing and stigmatic papillae shape. I scored species as self-incompatible based on the possession of A/cob and B/papillate pollen-stigma combinations, and as self-compatible for A/papillate or B/cob pollen-stigma combinations. This approach follows criteria established by Baker (1948a, 1966: Fig. 3) and Erben (1979: Fig. 3) as illustrated in Fig. 5.1. (3) To refer to the patterns of pollen exine sculpturing, I used the terminology introduced by Erdtman (1986), that is *Armeria*-type and *Plumbago*-type. As *Plumbago*-type pollen is monomorphic for exine sculpturing, with very slight differences between style morphs (*e.g.*, Ghobary, 1986; Ferrero *et al.*, 2009b), this condition was considered monomorphic in all analyses. (4) Four species of *Acantholimon* (*A. albocalycinum*, *A. flabellum*, *A. latifolium*, and *A. zaefii*) included in the phylogenetic tree (see *Phylogenetic analysis*) had no information on exine sculpturing and stigmatic papillae shape. However, I scored these four species as pollen and stigma dimorphic because several pieces of indirect evidence suggested that this is the most likely state in all species of *Acantholimon*. In particular, Bokhari (1972) reported that all Turkish *Acantholimon* were pollen and stigma dimorphic, and I confirmed that all herbarium specimens of *Acantholimon* (not including the four species listed above) that I observed were pollen and stigma dimorphic. (5) Finally, to categorize species of *Limonium* as sexual or apomictic, I combined my own observations of pollen exine sculpturing and stigmatic papillae shape with information on chromosome counts available from the literature. Dimorphic species with A/cob and B/papillate pollen-stigma combinations and chromosome counts $2n$ = 12, 14, 16, 18, 32, 34, 36, 54 were scored as sexual, whereas monomorphic species for pollen and stigmas (A/cob or B/papillate) mainly with odd chromosome counts were scored as apomictic. I followed the scheme proposed by Erben (1979: Fig. 4) and data available from Baker (1966: Table 1) to infer sexual versus apomictic species as illustrated in Fig. 5.1.

Finally, all names of species included in the character matrix were confirmed and assigned following *The Plant List* (2013) and *Species 2000 & ITIS Catalogue of Life* (Roskov *et al.*, 2016) available online. This character matrix (Appendix 5.2) was used for analyses of ancestral character state reconstructions.

Phylogenetic analysis

I used the supermatrix approach (de Queiroz and Gatesy, 2007; Roquet *et al*., 2013) to construct a phylogenetic hypothesis for the evolutionary relationships of the species included in my dataset. I used the five most common markers in previous phylogenetic studies of Plumbaginaceae: *ITS* of the nuclear genome, *rbcL*, *matK*, intergenic spacers *tnrL-trnF* and *trnT-trnY* of the plastid genome (Appendix 5.3). I downloaded the available sequences of these markers using Geneious v9.0.5 (Kearse *et al*., 2012). Finally, I selected six outgroup species from three families considered to be the closest relatives to Plumbaginaceae: *Polygonum amphibium*, *P. capitatum*, *Rumex obtusifolius* and *Triplaris americana* of the Polygonaceae, *Tamarix aphylla* of the Tamaricaceae and *Frankenia pulverulenta* of the Frankeniaceae (Soltis *et al*., 2000).

I aligned sequences using MAFFT v7 available online (http://mafft.cbrc.jp/alignment/server/; Katoh and Standley, 2013). All alignments were improved by removing poorly aligned or ambiguous regions by setting "automated 1" function on trimAl v1.3 software (Capella-Gutiérrez *et al*., 2009) available online at the Phylemon 2.0 server (http://phylemon.bioinfo.cipf.es/index.html; Sánchez *et al*., 2011). To construct the supermatrix, I concatenated the trimmed alignments using FASconCAT v1.0 (Kück and Meusemann, 2010), which resulted in a supermatrix of 4480 characters and 94 taxa. The supermatrix obtained was analyzed using a maximum likelihood (hereafter ML) approach following the GTR model as implemented in RAxML v8.2.8 (Stamatakis, 2014) available at the CIPRES server (http://phylo.org; Miller *et al*., 2010) by running 1000 bootstrap replicates. I kept the best ML phylogenetic tree and I collapsed nodes with a bootstrap value lower than 50% using TreeGraph 2 (Stöver and Müller, 2010; Appendix 5.4). When necessary, I used BioEdit v7.0.9.0 (Hall, 1999) to edit the sequence files and supermatrix, and FigTree v1.2 (Rambaut, 2008) to view and edit the phylogenetic trees.

To obtain an ultrametric phylogenetic tree, I used molecular dating by likelihood methods as implemented in *chronos* function available from R package "ape" (Paradis *et al*., 2004; Paradis, 2013) on the ML tree previously obtained. Minimum and maximum ages for the Plumbaginaceae family were obtained from Bell *et al*. (2010) and used for calibration purposes (minimum = 27 mya, maximum = 57 mya). The selection of the model of substitution rate was based on the information criterion PHIIC, and the model with the lowest PHIIC was selected (Paradis, 2013). PHIIC values for the tested models are available in Appendix 5.5. Here, no differences were obtained when

changing the smoothing parameter for the model "strict" and thus, the simplest model was chosen (model "strict", lambda value "0").

For 35 species included in my database that did not have sequences available at GenBank, I searched in the literature for information regarding monophyly of the genus. If the genus was monophyletic, I grafted the species onto their respective genera in the ultrametric tree by using *add.species.to.genus* function from R package "phytools" (Revell, 2012). When no information regarding monophyly was available, I searched the literature for the putatively closest relative and added the species to a specific node by setting *bind.tip* function from R package "phytools" (Revell, 2012). For one species, *Limonium minutiflorum*, the closest relatives (*i.e.*, species from the same section according to Boissier, 1848) were in two different positions in the tree, and to avoid errors in character reconstruction associated with the grafting of this species, I created two trees, each with *L. minutiflorum* in a different position. The grafting of species introduced some polytomies in the final trees (Appendix 5.6) that were randomly resolved by creating two sets of 100 trees using Mesquite v3.04 (Maddison and Maddison, 2015).

Character mapping and inferences on ancestral states

To infer the evolutionary history of floral characters in Plumbaginaceae, I combined stochastic character mapping and evolutionary models of character evolution. First, I mapped transitions between states for the six morphological characters by means of stochastic character mapping (hereafter SCM; Huelsenbeck *et al*., 2003) in the two sets of 100 trees using *make.simmap* function from R package "phytools" (Revell, 2012). This function simulates stochastic character maps on a phylogenetic tree or sets of phylogenetic trees. The most likely value of the transition matrix was used to fit the prior distribution, and I ran Markov chains for 100 simulations in each tree, that is 10 000 simulations for each set of trees for each character. My dataset had some species with missing data and their character states were considered uncertain. SCM using *make.simmap* function allows estimation of the posterior probability (hereafter PP) of all possible states for a character that is considered uncertain. To do this, it is necessary to input a binary matrix, that is a matrix of probabilities ranging from 0 to 1, with states of a given character as columns and species as rows. For example, *Plumbago auriculata* is distylous, and thus has 100% probability of having reciprocal herkogamy and 0% probability of not having reciprocal herkogamy. On the other hand, no information was

available for *Saharanthus ifniensis* and thus, it has equal prior probability of being distylous and not being distylous. In this case, the value "0.5" was added to both columns in the binary matrix with data regarding reciprocal herkogamy. The character mapping allowed me to estimate the PP of each state in the empirical ancestor of each node, and to calculate the average number of changes between states, as well as the time spent in each state. I calculated the relative rate of transition between states by dividing the frequency of each transition (*i.e.*, the ratio between one specific change and the total number of changes) by the frequency of time spent in each state (Torices and Anderberg, 2009).

Second, to assess the ancestral states of the six characters investigated, I conducted Bayesian analyses (hereafter BA) using the two sets of 100 trees (see *Phylogenetic analysis*) to account for phylogenetic uncertainty. I used the MultiState continuous-time Markov model of character evolution for discrete data as implemented in BayesTraits V2 (Pagel *et al*., 2004). BA require the use of priors, that is a probability distribution that shows some background knowledge about the studied parameters (Ronquist, 2004). However, setting the prior distribution is a hard task, given its subjective nature and the association between the prior distribution and all parameters to be estimated (Huelsenbeck *et al*., 2002). To reduce the uncertainty and randomness associated with this procedure, I used hyperpriors as recommended (Pagel *et al*., 2004). The hyperprior is a uniform distribution that is used to seed the values of the gamma or exponential priors (Pagel *et al*., 2004).

The BA were implemented by running Markov chains for one million iterations that were sampled each 1000 steps, with a burn-in of 10 000, and by setting the stepping stone sampling to use 100 stones and run each for 10 000 iterations. I conducted the analyses by assuming two scenarios: (1) the transition rates between states were the same, and the model was "restricted", and (2) the transition rates between states were different, and the model was "unrestricted". I compared restricted and unrestricted models by calculating the Bayes Factor (hereafter BF) as follows:

Log BF = 2(log marginal likelihood of the unrestricted model - log marginal likelihood of the restricted model)

by using both the marginal likelihood from the harmonic mean and from the stepping stone sampler. If Log BF > 2, there is positive evidence favoring the restricted model

(Gilks *et al*., 1996). Values of BF comparing restricted and unrestricted models are provided in Appendix 5.7. The calculation using the marginal log likelihood of the harmonic mean and of the stepping stone were in agreement in favoring restricted models in all cases. To test for the most likely state of each character at the root of the family, I fixed the possible states of a given character at the most recent common ancestor of the family, that is the node containing all Plumbaginaceae species. In this way, it is possible to test which character state at that node provides the best fit given the dataset and the phylogenetic hypothesis used. Thus, with the exception of stigma morphology, two different models were fitted for each character, one for each state, and the models were compared by means of Bayesian Information Criterion (hereafter BIC) calculation as follows:

$$BIC = -2 \times loglikelihood + d \times \log(N)$$

where the *loglikelihood* is the loglikelihood of the model, *d* is the number of parameters estimated and *N* is the sample size. I obtained the values of the parameters estimated after BA using Tracer V1.6 (Rambaut *et al*., 2013).

RESULTS

Phylogenetic distribution of character states

Reciprocal herkogamy occurs in the two subfamilies of Plumbaginaceae, but its overall frequency differed between the subfamilies. All species of Plumbaginoideae possess reciprocal herkogamy and are therefore distylous (*n* = 7 species; Fig. 5.2A; Appendix 5.2). In the Staticoideae, distyly occurs in *Goniolimon,* some *Acantholimon* species and *Limonium vulgare* for a total of 12 species of the 116 investigated (Fig. 5.2A; Appendix 5.2). In contrast to reciprocal herkogamy, self-incompatibility, as inferred by information from the literature and pollen-stigma character state combinations (see Fig. 5.1), was widespread in the family, whereas only four *Limonium* species were inferred to be self-compatible based on these criteria (Fig. 5.2B; Appendix 5.2).

Regarding the ancillary polymorphisms of pollen and stigma, dimorphic exine sculpturing of the *Armeria*-type was only found in the Staticoideae (Fig. 5.3), whereas monomorphic exine of the *Plumbago*-type appeared to be restricted to the Plumbaginoideae (Fig. 5.2C; Appendix 5.2). However, eight species of *Limonium*

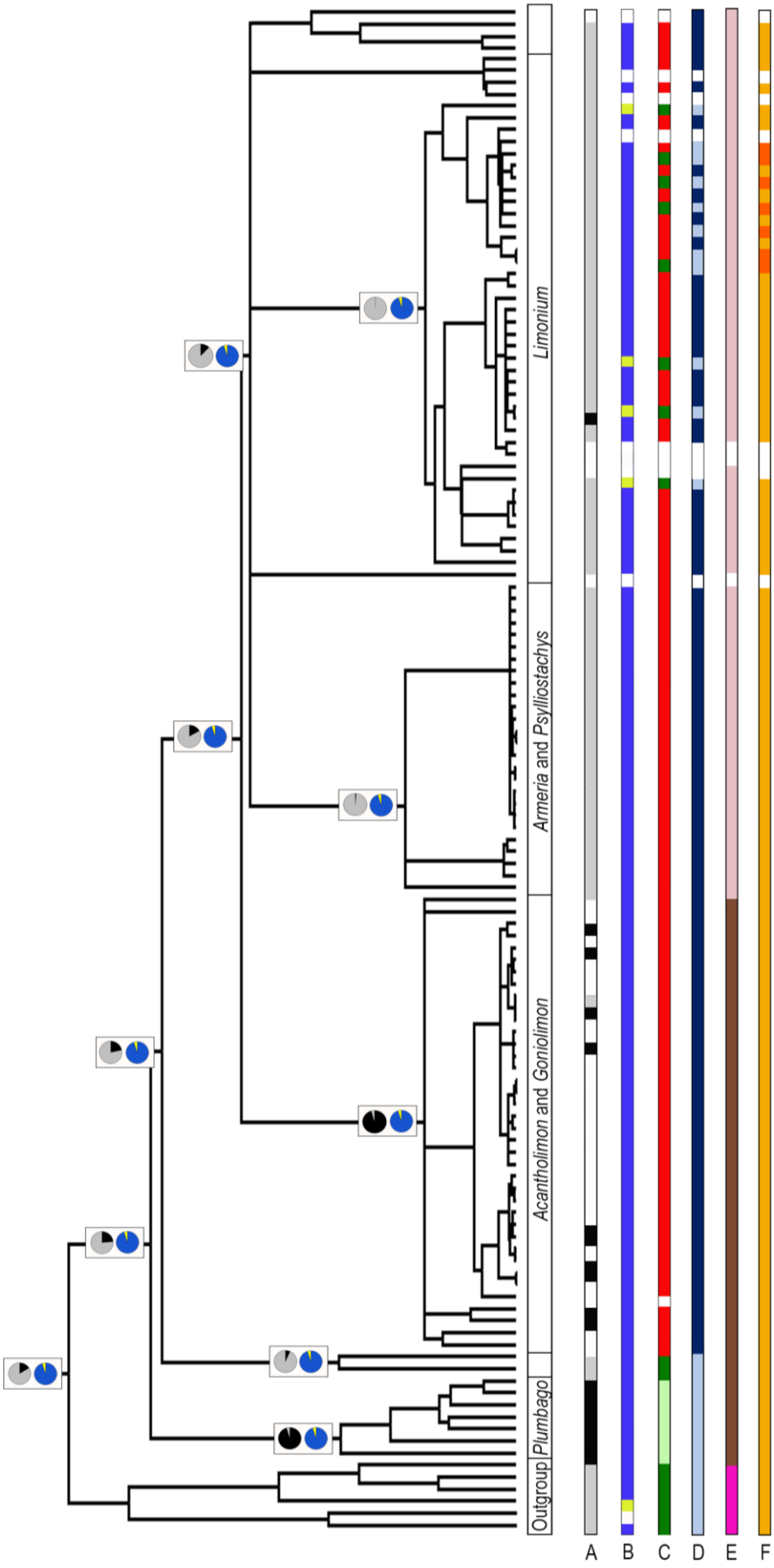

Limonium
Armeria and Psylliostachys
Acantholimon and Goniolimon
Plumbago
Outgroup
A
B
C
D
E
F

Figure 5.2. Phylogenetic distribution of the six characters investigated. **A.** Reciprocal herkogamy. Black – reciprocal herkogamous, grey – monomorphic or homostylous. **B.** Incompatibility status. Blue – self-incompatible, yellow – self-compatible. **C.** Exine ornamentation. Dark green – monomorphic, light green – *Plumbago*-type, red - *Armeria*-type. **D.** Stigma dimorphism. Dark blue – dimorphic, light blue – monomorphic. **E.** Stigma morphology. Brown – capitate, light pink – filiform, dark pink – fimbriate/pear-shaped/ lanceolate. **F.** Apomixis. Yellow – sexual, orange – apomictic. In all cases, white represents missing information for a given species. Pie diagrams show the PP of reciprocal herkogamy (black) versus stigma monomorphism/homostyly (grey), and of self-incompatibility (blue) versus self-compatibility (yellow) for a given node.

produced only one pollen type, mainly pollen type A (*L. echioides* produced pollen type B), and therefore each species was inferred to be monomorphic. Similarly, stigma dimorphism only occurred in species of the subfamily Staticoideae (Fig. 5.4), with 11 species of *Limonium* with monomorphic stigmas, which were either cob or papillate (Fig. 5.2D; Appendix 5.2).

Concerning stigma morphology, capitate stigmas were characteristic of all species from the Plumbaginoideae included in this study, and also species of *Acantholimon* (n = 38; Fig. 5.4A-B), *Cephalorrhizum* (n = 1; Fig. 5.4C-D), *Dictyolimon* (n = 1), and *Goniolimon* (n = 3), all in the Staticoideae (Fig. 5.2E; Appendix 5.2). Finally, almost all species investigated were inferred to be sexual based on data from the literature, and the combination of pollen-stigma dimorphism with chromosome counts as illustrated in Fig. 5.1, whereas a few *Limonium* were inferred to be apomictic owing to their possession of monomorphic pollen and stigma, and odd chromosome numbers (Fig. 5.1; Fig. 5.2F; Appendix 5.2). Species of *Aegialitis* shared few of the character states with other species of Staticoideae, the subfamily in which they are usually included. *Aegialitis annulata* and *A. rotundifolia* were inferred to be sexual and produced monomorphic pollen and monomorphic capitate stigmas (Fig. 5.2C-E; Appendix 5.2). In addition, these species lacked reciprocal herkogamy and were inferred to be self-incompatible as reported in the literature (Fig. 5.2A-B; Appendix 5.2).

Character evolution

The patterns obtained from SCM and BA, using the two sets of 100 trees differing in the position of *Limonium minutiflorum*, were very similar with respect to the most likely ancestral condition of Plumbaginaceae, and the number of transitions between character states. Therefore, only results from the first set of trees are described below. Results for the second set of trees are provided in Appendices 5.7-5.10.

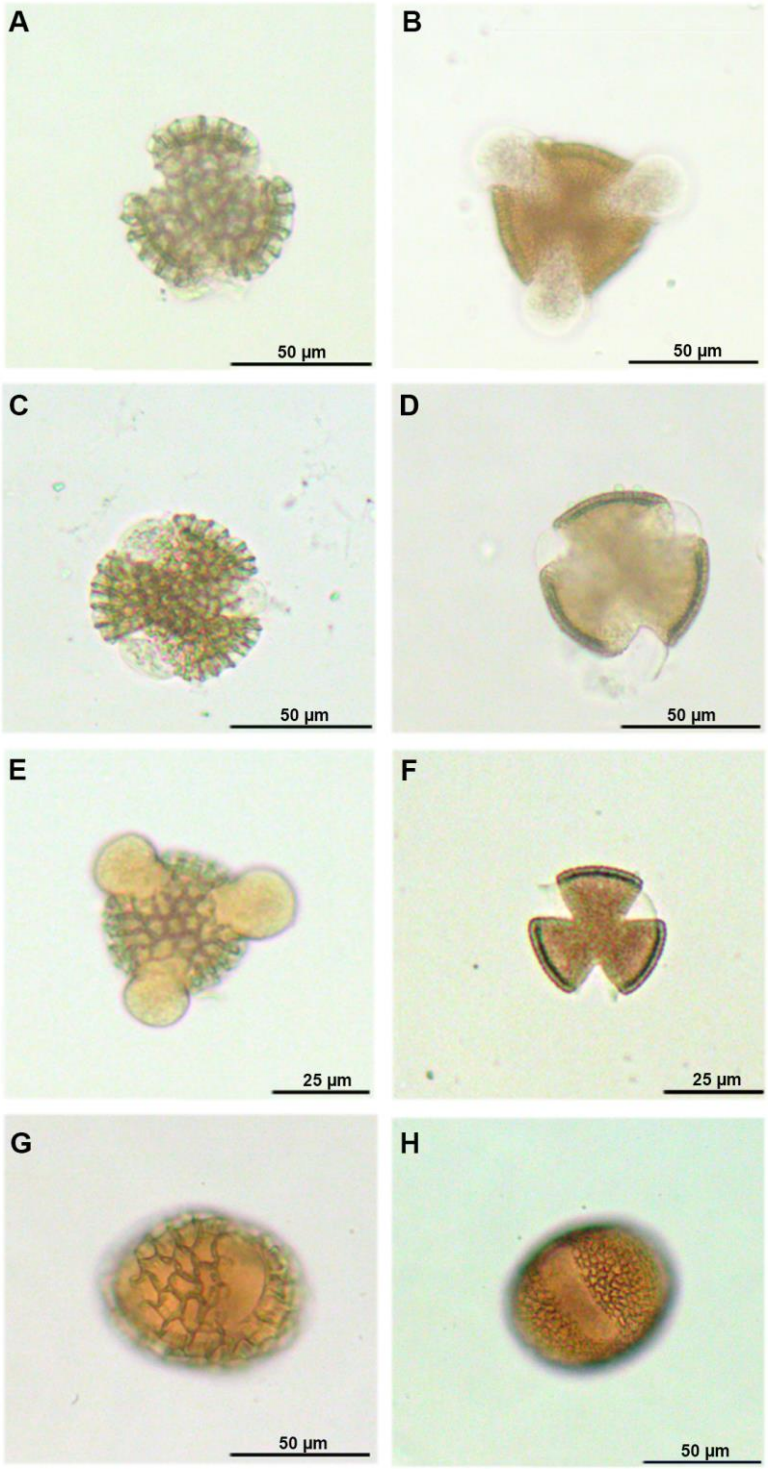


Figure 5.3. Light microscopy photographs of pollen grains type A (A, C, E, G) and type B (B, D, F, H) obtained at 200× magnification (Leitz HM-LUX 3). Except for G and H in equatorial view, the remaining are in polar view. **A, B.** *Acantholimon pterostegium.* **C, D.** C*ephalorrhizum coelicolor*. **E, F.** *Psylliostachys suworowii.* **G, H.** *Limoniastrum guyonianum.*

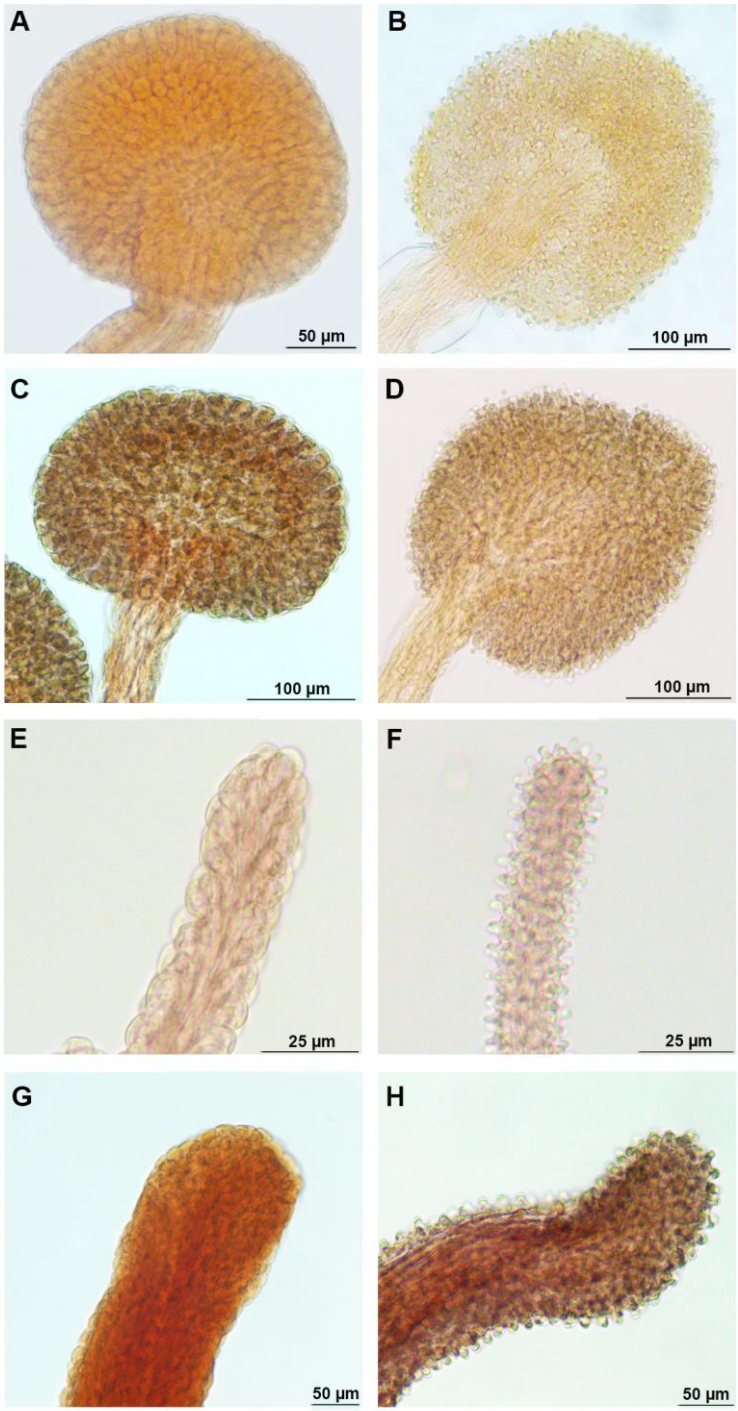


Figure 5.4. Light microscopy photographs of capitate cob (A, C) and papillate (B, D), and filiform cob (E, G) and papillate (F, H) obtained at 200× magnification (Leitz HM-LUX 3). **A, B.** *Acantholimon pterostegium.* **C, D.** C*ephalorrhizum coelicolor*. **E, F.** *Psylliostachys suworowii*. **G, H.** *Limoniastrum guyonianum.*

The most likely common ancestor of the Plumbaginaceae was monomorphic for sex-organs position (SCM, PP = 0.763) and self-incompatible (SCM, PP = 0.952), as revealed by character mapping (Fig. 5.2) and BA (Table 5.1). The average number of transitions (± SE) from the ancestral monomorphic condition to reciprocal herkogamy

was 3.88 ± 0.01, whereas the transition from self-incompatibility to self-compatibility was inferred to be exceptionally high, 74.53 ± 0.15. In addition, SCM estimated that reversions from reciprocal herkogamy to homostyly (5.23 ± 0.03), and from self-compatibility to self-incompatibility (73.94 ± 0.15) also occurred. The proportion of time spent in each state was larger for monomorphic sex-organ positions than for reciprocal herkogamy (monomorphic/homostylous = 0.74; reciprocal herkogamy = 0.26), and for self-incompatibility than for self-compatibility (SI = 0.95; SC = 0.05). In addition, the most likely change in sex-organ arrangement was the reversion from reciprocal herkogamy to homostyly (Table 5.2), whereas changes in incompatibility were inferred to be most likely from self-compatibility to self-incompatibility (Table 5.2), despite some losses of self-incompatibility in *Limonium* (Fig. 5.2B).

Table 5.1. Restricted models investigating the character state at the most recent common ancestor of Plumbaginaceae (see *Character mapping and inferences on ancestral states*). Values in bold are the best evolutionary models according to the BIC for the first set of 100 trees. "SI" - self-incompatible, "SC" - self-compatible. "Others" - fimbriate/lanceolate/ pear-shaped stigma.

Model	**BIC**
(a) Sex-organs arrangement	
1- Monomorphic	**57.078**
2- Reciprocal herkogamous	62.796
(b) Incompatibility status	
1- SI	**50.968**
2- SC	61.296
(c) Exine sculpturing	
1- Monomorphic	**94.184**
2- *Armeria*-type	96.596
(d) Stigma dimorphism	
1- Monomorphic	**88.986**
2- Dimorphic	89.394
(e) Stigma morphology	
1- Filiform	43.632
2- Capitate	**39.380**
3- Others	46.158
(f) Apomixis	
1- Sexual	**52.230**
2- Apomictic	65.332

Table 5.2. Rate of change between states for the six characters investigated for the first set of 100 trees. Values are mean ± SE. "RH" – reciprocal herkogamy, "SI" – self-incompatible, "SC" – self-compatible. For details on the calculation of frequency of transition and relative rate of transition see *Character mapping and inferences on ancestral states.*

Character state	No. of transitions	Frequency of transition	Relative rate of transition
(a) Sex-organs arrangement			
Monomorphic to RH	3.88 ± 0.01	0.33	0.45
RH to homostylous	5.23 ± 0.03	0.67	2.55
(b) Incompatibility status			
SI to SC	74.53 ± 0.15	0.50	0.53
SC to SI	73.94 ± 0.15	0.50	10.04
(c) Exine sculpturing			
Armeria-type to monomorphic	9.23 ± 0.02	0.90	1.65
Monomorphic to *Armeria*-type	0.97 ± 0.01	0.10	0.21
(d) Stigma dimorphism			
Dimorphic to monomorphic	12.39 ± 0.03	0.71	1.26
Monomorphic to dimorphic	4.98 ± 0.04	0.29	0.66
(e) Stigma morphology			
Filiform to capitate	0.04 ± 0.002	0.01	0.02
Filiform to others	0.00 ± 0.00	0.00	0.00
Capitate to filiform	0.85 ± 0.01	0.16	0.43
Capitate to others	0.00 ± 0.00	0.00	0.00
Others to filiform	1.31 ± 0.01	0.25	1.23
Others to capitate	3.00 ± 0.01	0.58	2.81
(d) Apomixis			
Apomictic to sexual	8.86 ± 0.03	0.67	16.67
Sexual to apomictic	4.32 ± 0.02	0.33	0.34

Concerning ancillary characters of pollen and stigmas, both results from SCM (Fig. 5.2C-E) and BA (Table 5.1) were consistent with a common ancestor with monomorphic pollen (SCM, PP = 0.712) and monomorphic (SCM, PP = 0.602) capitate stigmas (SCM, PP = 0.612). The average number of transitions (± SE) from the ancestral monomorphic to dimorphic pollen and stigmas was 0.97 ± 0.01 and 4.98 ± 0.04, respectively. The opposite pattern, involving reversion from dimorphism to monomorphism for pollen and stigmas was estimated to be 9.23 ± 0.02 and 12.39 ± 0.03, respectively. Although the proportion of time spent with monomorphic pollen (0.45) is similar to the time spent with a dimorphic state of pollen exine (0.55), the transition from dimorphic pollen of the *Armeria*-type, to monomorphic pollen in the Staticoideae was by far more likely to occur (Table 5.2). A similar pattern was evident for dimorphism of the stigmatic papillae, with the proportion of time spent in each state

being similar (monomorphism = 0.43; dimorphism = 0.57), but the loss of stigma dimorphism was more likely than the opposite transition in the Staticoideae (Table 5.2). With regards to stigma morphology, the transition from capitate to filiform stigmas occurred frequently (0.85 ± 0.01), whereas the reversion was found to be very unlikely, as estimated by SCM (Table 5.2). Again, time spent with capitate (0.38) and filiform (0.42) states, as occur in the Plumbaginaceae, was not very different. Species from the outgroup had capitate, fimbriate, pear-shaped and lanceolate stigmas (Appendix 5.2). When considering the diversity of stigma morphologies in the outgroup, the most likely transition was to capitate stigmas (Table 5.2).

Finally, the common ancestor of Plumbaginaceae species was most likely sexual (SCM, PP = 0.981), as was the case for the great majority of species included in these analyses. The average number of transitions (± SE) from sexual to asexual reproduction by apomixis was 4.32 ± 0.02. The proportion of time spent by a species in the sexual state (0.96) was very different from the time spent in the apomictic state (0.04). The loss of the capacity for sexual reproduction and development of apomictic asexual strategies was restricted to *Limonium* species (Table 5.2; Fig. 5.32F).

DISCUSSION

My comparative analysis of the evolutionary history of the heterostylous syndrome in Plumbaginaceae revealed several main findings: (1) the most likely common ancestor of the family was inferred to be self-incompatible, and had a monomorphic arrangement of sex organs with monomorphic pollen and stigmas (Fig. 5.2A-B; Table 5.1); (2) reciprocal herkogamy was inferred to have evolved on at least three different occasions from an ancestor with monomorphic style and stamen lengths (Fig. 5.2A; Table 5.2); (3) pollen-stigma dimorphism is likely to have evolved before the establishment of reciprocal herkogamy in Staticoideae (Fig. 5.2); (4) reversions from pollen-stigma dimorphism to monomorphism associated with transitions to self-compatibility and apomixis occurred on several occasions in *Limonium* (Fig. 5.2; Table 5.2). These character reconstructions are the first comprehensive re-evaluation of Baker's hypotheses for the evolutionary buildup of the heterostyly syndrome in Plumbaginaceae. They provide some support for Baker's proposal, and more broadly, to Charlesworth and Charlesworth "selfing avoidance model" for the evolution of distyly (Charlesworth and Charlesworth, 1979). Below I discuss these findings and provide

several refinements to the evolutionary pathways originally proposed by Baker (1948a, 1966).

Evolution of reciprocal herkogamy

Stochastic character mapping suggests that in Plumbaginaceae reciprocal herkogamy evolved on at least three different occasions from a common ancestor with a monomorphic arrangement of sex organs; once in the Plumbaginoideae and twice in the Staticoideae. Previous phylogenetic reconstructions of heterostylous groups have provided evidence for multiple origins of reciprocal herkogamy in Boraginaceae (Ferrero *et al*., 2009a; Cohen, 2013), *Linum* (Armbruster *et al*., 2006; McDill *et al*., 2009), *Nymphoides* (Tippery and Les, 2011) and *Narcissus* (Graham and Barrett, 2004). Concerning Plumbaginoideae, my sampling included species from three of the four currently accepted genera – *Plumbago*, *Dyerophytum* and *Ceratostigma* – and all investigated species in these genera were distylous. Evidence from character mapping suggested that the ancestral condition in this subfamily is reciprocal herkogamy. The fourth genus currently included in Plumbaginoideae is *Plumbagella*, which is Asian in distribution and is comprised of one species, *P. micrantha* (Kubitzi, 1993). This species is annual, the corolla is very reduced in size (ca. 4-5 mm; eFloras, 2008) and its monomorphic arrangement of sex organs is most probably a derived condition involving the breakdown of distyly to homostyly (see Baker, 1948a, 1966), rather than an ancestral monomorphic condition. In common with other homostylous taxa in heterostylous groups (reviewed in Darwin, 1877; Ganders, 1979; Barrett, 1989), *P. micrantha* is probably autogamous and the facility for autonomous self-pollination may have enabled establishment following long-distance dispersal to Asia.

In contrast to subfamily Plumbaginoideae, the common ancestor of all Staticoideae most likely possessed a monomorphic arrangement of sex organs although this does not imply that they were selfing, as seems likely for derived homostylous in the family. Reciprocal herkogamy appears to have evolved at least twice from stylar monomorphism in Staticoideae. *Limonium vulgare* was early described as distylous and this was thought to be the only case of reciprocal herkogamy in Staticoideae for a long time (Baker, 1948a, 1966). My results support the hypothesis that reciprocal herkogamy is a derived condition in this species (see Baker, 1948a, 1966). However, later studies on *Acantholimon* (Bokhari, 1972), *Goniolimon tataricum* (Schill *et al.*, 1985) and *G. italicum* (Morretti *et al*., 2015), as well as my own sampling of herbarium specimens,

demonstrate the occurrence of distylous species in both genera. *Acantholimon* and *Goniolimon* form a clade (Fig. 5.2, Appendices 5.4, 5.6; and see Lledó *et al*., 1998, 2001, 2005) for which reciprocal herkogamy is most likely the basal condition, as revealed by stochastic character mapping. Consequently, the lack of reciprocal herkogamy in some species of *Acantholimon* is most likely a derived condition, and these taxa are probably homostylous resulting from the breakdown of distyly, as inferred for *Plumbagella*.

Pollen-stigma dimorphism and the incompatibility system

My results suggest that the most likely common ancestor of Plumbaginaceae had monomorphic pollen and monomorphic capitate stigmas, and was self-incompatible. To infer the compatibility status of species in Plumbaginaceae, I combined direct evidence available from controlled hand pollinations (*e.g*., Baker, 1953b; Dulberger, 1975a; Ferrero *et al*., 2009b; reviewed in Dulberger, 1992) and assumptions based on pollen exine ornamentation and stigmatic papillae shape in Staticoideae (Fig. 5.1). Species of Plumbaginoideae resemble the basal condition in the family in having no evident signs of pollen-stigma dimorphism and capitate stigmas (Fig. 5.2; Appendix 5.2; Baker, 1948a, b, 1966). Although there are slight differences in exine sculpturing, as revealed by scanning electron microscopy of pollen from long- and short-styled morphs of *Dyerophyton africanum* and *D. indicum* (Ghobary, 1986), these are not sufficiently developed to classify *Plumbago*-type pollen as dimorphic (Erdtman, 1986). Experimental tests for the presence of self-incompatibility have been conducted in some species of Plumbaginoideae (*e.g*., *Plumbago capensis* and *P. europaea*, Dulberger, 1975a; *P. auriculata*, Ferrero *et al*., 2009b; *Ceratostigma willmottianum*, Dulberger, 1975a), and with the exception of *Plumbagella micrantha* (Dahlgren, 1918), they were all found to be self-incompatible setting no or few seeds upon self-pollination. As mentioned above, self-compatibility in *P. micrantha* has been interpreted as a derived condition allowing reproduction by seed in this annual likely homostylous species (see Baker, 1948a).

In contrast, pollen-stigma dimorphism is highly developed in Staticoideae, except for *Aegialitis*. The only two species in this genus, *A. annulata* and *A. rotundifolia*, are distinct from the remaining Staticoideae in having *Plumbago*-type pollen (Baker, 1966; Ghobary, 1984) and monomorphic capitate stigmas (Baker, 1966; Lledó *et al*., 2001). Indeed, *Aegialitis* resembles the most likely common ancestor of Plumbaginaceae in all

character states, which suggests that this genus is probably most basal in Staticoideae (Baker, 1948a, b, 1966). More recently, the phylogenetic position of *Aegialitis* has been investigated by parsimony analyses, and the main conclusion reached was that this genus constitutes a distinct lineage in Staticoideae (Lledó *et al*., 2001), which has diverged from the most likely common ancestor before pollen-stigma dimorphism evolved in the subfamily (see Baker, 1948a, b, 1966).

The stochastic character mapping I conducted suggested only one origin of pollen-stigma dimorphism in Plumbaginaceae, which was later "superimposed" on a previously established self-incompatibility system. Early investigations in *Limonium* (Baker, 1953a; Dulberger, 1975a) and *Armeria maritima* (Iversen, 1940; Dulberger, 1975a; Mattsson, 1983; Chapter 4) confirmed that species with pollen-stigma dimorphism were self-incompatible. In addition to marked differences in exine sculpturing, pollen type A and B also differ in their lipid composition (Mattsson, 1983), and these biomolecules have been suggested as playing a key role in the initial interaction between pollen and stigmas, during the adhesion phase (Mattsson, 1983; Heslop-Harrison and Heslop-Harrison, 1985). Consequently, polymorphisms in pollen and stigmas are tightly associated with the diallelic physiological incompatibility system found in this subfamily (Dulberger, 1975a, b; Chapter 4).

A large number of transitions from self-incompatibility to self-compatibility was inferred by stochastic character mapping. The shift from outcrossing to selfing is the most common reproductive transition in angiosperms (Stebbins, 1974), with selfing commonly selected when it confers reproductive assurance (Lloyd, 1992). This shift in mating system is frequently associated with long-distance dispersal and colonization (Baker, 1955, 1959, 1967; Pannell *et al*., 2015) so that the patterns I observed are probably associated with migration of the family to novel environments. However, stochastic character mapping also inferred a high number of reversions from self-compatibility to self-incompatibility, which is biologically very unlikely (reviewed in Barrett, 2013). According to "Dollo's Law", the loss of a complex character such as self-incompatibility is generally irreversible (Dollo, 1893; Bull and Charnov, 1985; Goldberg and Igic, 2008), and studies of the evolutionary history of mating systems in Solanaceae, a family in which self-incompatibility has been repeatedly lost but gained only once, have provided convincing evidence for the generally irreversible nature of self-incompatibility loss (Igic *et al*., 2006, 2008). The high estimate for the number of gains of self-incompatibility that I obtained is therefore likely to be a spurious result

associated with stochastic mapping procedure. Only four *Limonium* species in the character matrix were self-compatible, and the prior used in the stochastic character mapping was most likely insufficient to account for this. Consequently, a large number of transitions between states was inferred along the branches in the phylogenetic tree, but the character state at the tips (self-incompatible) was the same as it was in the most likely common ancestor (self-incompatible), except for the four species. Further character mapping with stronger priors are desirable to rigorously evaluate evolutionary transitions in compatibility status in Plumbaginaceae.

Analysis of the distribution of characters on phylogenetic trees suggests that the transition from pollen-stigma dimorphism and self-incompatibility to monomorphism and self-compatibility most likely occurred four independent times in *Limonium* (Fig. 5.2). In addition, pollen-stigma monomorphism and self-compatibility are also reported for *Armeria maritima* subsp. *sibirica* (Baker, 1948c, 1966) and some *Limonium* species from North America and Australia (Baker, 1953a, b), but these were not included in my character mapping. Monomorphic self-compatible species most frequently produce pollen type A and have papillate stigmas, whereas the combination B/cob has only been found in the annual Mediterranean species *L. echioides.* It seems likely that self-compatibility has arisen in the genus in association with dispersal events from Europe (Baker, 1953a, b, 1959), especially from the western Mediterranean, which is the main center of diversification of *Limonium* (Erben, 1993; Kubitzki, 1993), to America and Australia, where species with pollen-stigma monomorphism and self-compatibility are most frequent (Baker, 1953a, b). However, these hypotheses concerning migration need to be substantiated by phylogeographical analyses, which would likely provide valuable insights on the biogeography of mating systems in this group.

In addition to sexual reproductive strategies involving outcrossing or selfing, asexual reproduction via apomixis has also been described for some *Limonium* species (*e.g.*, D'Amato, 1940, 1949; Baker, 1953a, 1966; Erben, 1979; Ingrouille and Stace, 1985; Cowan *et al*., 1998; Róis *et al*., 2016). In angiosperms, apomictics are most commonly polyploid (Asker and Jerling, 1992; Carman, 1997), which is also the case for apomictic *Limonium* (*e.g.*, D'Amato, 1949; Erben, 1979; Cowan *et al*., 1998; Caperta *et al*., 2016; Róis *et al*., 2016). Apomixis is often associated with hybridization between species and can overcome the sexual sterility that is a feature of many interspecific hybrids (Asker and Jerling, 1992). Indeed, reproductive barriers between species of *Limonium* are often weak and hybridization is frequent (Erben, 1993). *Limonium* apomictics have

monomorphic pollen and stigmas with combinations A/cob or B/papillate, which would normally produce an incompatible pollen-stigma combination (Fig. 5.1). In addition, pollen production per flower is very low and pollen grains are mainly aborted (Baker, 1966; Erben, 1979; Cowan *et al*., 1998). As previously reported in other groups (*e.g*., Souza *et al*., 2012; Aliyu *et al*., 2013), the character mapping in this study revealed multiple independent origins of apomixis in lineages composed of mainly sexual species. It seems probable that this mixture of sexual and asexual reproductive strategies in *Limonium* may have contributed to the radiation of this genus in the western Mediterranean (Lledó *et al*., 2005).

The evolutionary buildup of the heterostylous syndrome

My results are generally in accord with Baker's hypothesis (Baker, 1948a, 1966) on the evolutionary buildup and breakdown of the heterostylous syndrome. My more in-depth comparative study has provided insights into the evolutionary history of heteromorphic traits, especially in the subfamily Staticoideae (Fig. 5.5). Hybridization between species, polyploidy and apomixis are probably the main causes of the complex and unresolved systematics and phylogenetic problems of this family, especially in Staticoideae (Erben, 1993; Cowan *et al*., 1998; Lledó *et al*., 2005). These issues have posed problems for the taxonomy of the family (reviewed in Lledó *et al*., 2005), and also the phylogenetic relations among species (Lledó *et al*., 1998, 2000, 2001, 2005; Palacios *et al*., 2000; Moharrek *et al*., 2014). In addition, new species and microspecies (species with very narrow distributions) have been described, especially *Limonium* (*e.g*., Llorens and Tébar, 1988; Gil and Llorens, 1991; Saéz and Rosselló, 1996; Crespo, 2009). Current knowledge of the systematics of the family is certainly stronger than it was when Baker attempted to understand the distribution of characters associated with heterostyly (Baker, 1953a, b) and proposed a narrative model for the evolution of the floral syndrome (Baker, 1948a, 1966).

My results support two distinct pathways for the buildup of characters associated with the heterostylous syndrome; a relatively simple one for Plumbaginoideae and a more complex pathway for Staticoideae (Fig. 5.5). Concerning Plumbaginoideae, my results are in general agreement with Baker's hypothesis (1948a, 1966) that reciprocal herkogamy evolved in an ancestor that was self-incompatible (Fig. 5.5). In contrast, in Staticoideae the first step in the evolutionary buildup of heterostyly was inferred to be the acquisition of pollen-stigma dimorphism in a self-incompatible lineage. My results

suggest that pollen and stigma dimorphism may have evolved together in concert rather than at different times. This finding is in disagreement with Baker (1948a, 1966), who proposed that pollen dimorphism evolved first in self-incompatible species with monomorphic stigmas (*e.g.*, as in *Goniolimon*, *Acantholimon* and some *Limonium*). However, this order seems unlikely as later investigations of *Goniolimon* (Schill *et al.*, 1985; Morretti *et al.*, 2015) and *Acantholimon* (Bokhari, 1972), and my own observations of herbarium specimens, have demonstrated that taxa in these genera exhibit pollen-stigma dimorphisms. In fact, pollen-stigma dimorphisms are widespread in Staticoideae, and apart from *Aegialitis*, monomorphic pollen and stigmas are most likely derived and associated with the transition from outcrossing to selfing or apomixis in some *Limonium* species (see above *Pollen-stigma dimorphism and the incompatibility system*). My results further suggest that reciprocal herkogamy most likely resulted from two independent origins after the establishment of pollen-stigma dimorphism in the clade formed by *Acantholimon* and *Goniolimon*, and separately in *Limonium*. Additional analyses of character evolution involving broader taxon sampling should provide further insights on the most probable order of establishment of heteromorphic characters in Plumbaginaceae.

Finally, my results have general relevance to competing models on the evolution of distyly. One of the main findings of my reconstructions is that reciprocal herkogamy evolved after the establishment of diallelic self-incompatibility in Plumbaginaceae. This is the order of establishment proposed in the inbreeding avoidance model of Charlesworth and Charlesworth (1979), and opposite to the polarity predicted in the pollen transfer model of Lloyd and Webb (1992a, b). My results therefore represent the first comparative evidence supporting the sequence proposed in the Charlesworth and Charlesworth (1979) model. Despite the differences in the selective forces and polarities implicated in the two models, both involve a transient stage of stigma-height dimorphism on the pathway from stylar monomorphism to distyly. However, unlike *Narcissus* (Graham and Barrett, 2004), *Lithodora* and *Glandora* (Ferrero *et al.*, 2009a, 2012), in which species with this form of polymorphism occur, there is no evidence as that any species of Plumbaginaceae possesses this form of stylar polymorphism. Stigma-height dimorphism rarely occurs in distylous families and it is quite possible that this putative intermediate stage in theoretical models is ephemeral and rapidly replaced once distyly has evolved in a lineage (reviewed in Barrett *et al.*, 2000). Future comparative analyses on other heterostylous taxa are needed to determine which of the

two models for the evolution of distyly is most plausible. Given the numerous independent origins of this convergent floral syndrome across angiosperm families, it is not unlikely that features of both models for the evolution of heterostyly may be correct.

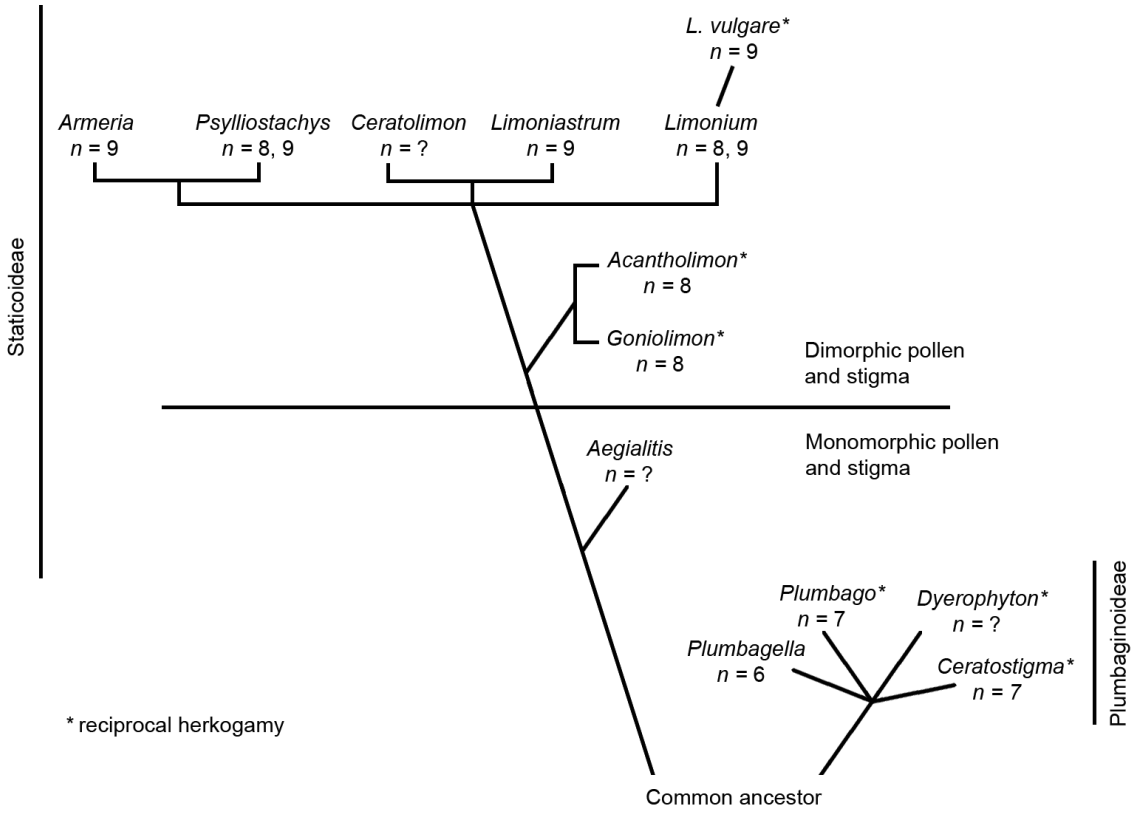


Figure 5.5. Scheme of the evolutionary buildup of the heterostylous syndrome in Plumbaginaceae modified from Baker (1948a, 1966). *n* - haploid number of chromossomes, "?" – no information.

Appendix 5.1. Detailed information of each herbarium specimen sampled for data on sex-organs arrangement and ancillary characters. Herbarium acronyms: COI – Herbarium of the University of Coimbra, E – Royal Botanic Garden of Edinburgh Herbarium. Date of collection is given as day-month-year. "NA" – no barcode; "*s. col.*", "*s. dat.*" and "unknown" for collector's name, date of collection and collecting locality not provided in the herbarium specimen label; "?" – doubt about the information provided; "Is" – island; "Mt" – mountain; "NP" – national park.

Taxon	Herbarium	Barcode	Collector(s)	Date of collection	Collecting country/locality
Acantholimon acerosum	E	E00175326	P. H. Davis	18-VIII-1949	Turkey
Acantholimon acerosum	E	E00457075	E. K. Balls	17-VII-1934	Turkey, Gumushane
Acantholimon acerosum	E	E00457077	P. H. Davis, I. Hedge	26-VII-1957	Turkey, Erzincan
Acantholimon acmostegium	E	E00457009	B. S. Barris	22-IV-1975	Iran, Semnan
Acantholimon acmostegium	E	E00457012	F. Schmid	15-VI-1956	Iran
Acantholimon acmostegium	E	E00457015	K. H. Rechinger, F. Rechinger, P. Allen, E. Esfandiari	13/14-VI-1948	Iran
Acantholimon androsaceum	E	NA	T. H. H. Von Heldreich	VII-1870	unknown
Acantholimon androsaceum	E	NA	*s. col.*	25-VII-1938	Crete
Acantholimon androsaceum	E	NA	E. Reverchon	18-VII-1883	Crete, Drakona
Acantholimon araxanum	E	E00457095	A. A. Grossheim, I. Iljinskaja, M. E. Kirpichnikov	08-VI-1947	Azerbaijan, Norashen
Acantholimon aspadanum	E	E00457021	J. C. Archibald	03-V-1966	Iran, Arak
Acantholimon bracteatum	E	E00453425	P. H. Davis, O. V. Polunin	31-VII-1954	Turkey, Van
Acantholimon bracteatum	E	E00453432	P. H. Davis	03-VII-1966	Turkey, Van
Acantholimon bracteatum	E	E00453435	K. H. Rechinger	04-VII-1974	Iran, Chalil Kuh
Acantholimon bromifolium	E	E00453109	J. C. Archibald	15-VIII-1966	Kurdistan, Sanandaj
Acantholimon bromifolium	E	E00453110	J. C. Archibald	20-VII-1966	Iran, Malayer
Acantholimon collare	E	E00453172	K. H. Rechinger	01-VI-1977	Iran, Qaleh Ahangaran
Acantholimon curviflorum	E	E00453143	P. H. Davis	18-VII-1966	Turkey, Kars
Acantholimon demavendicum	E	E00453455	K. H. Rechinger, F. Rechinger	26/27-VII-1948	Iran, Gorgan
Acantholimon demavendicum	E	E00205682	J. C. Klein	08-VIII-1971	Iran
Acantholimon erinaceum	E	E00453263	K. H. Rechinger, F. Rechinger	08-VIII-1948	Iran, Mazandaran
Acantholimon erinaceum	E	E00200931	J. C. Klein	21-VIII-1972	Iran
Acantholimon erinaceum	E	E00190582	J. C. Klein	21-VIII-1972	Iran

Acantholimon festucaceum	E	E00457025	P. E. B. Wendelbo, H. Foroughi	30-V-1975	Iran
Acantholimon festucaceum	E	E00457023	P. H. Davis	23-VII-1939	Iran
Acantholimon gorganense	E	E00453321	K. H. Rechinger, F. Rechinger, P. Aellen	03-VI-1948	Iran, Khorasan
Acantholimon gulistanum	E	E00457005	L. Ekberg, P. E. B. Wendelbo	19-VII-1969	Afghanistan, Bamian
Acantholimon gulistanum	E	E00457004	L. Ekberg, P. E. B. Wendelbo	18-VII-1969	Afghanistan, Parvan
Acantholimon heratense	E	E00453146	P. E. B. Wendelbo, I. Hedge, L. Ekberg	12-V-1969	Afghanistan, Herat
Acantholimon hohenackeri	E	E00453324	N. Jardine	14-VIII-1963	Iran, Mazandaran
Acantholimon hohenackeri	E	E00453326	J. M. Lamond	10-VI-1971	Iran, Qotur
Acantholimon hohenackeri	E	E00453327	D. Ferguson	19-VII-1970	Iran, Herowabad
Acantholimon karelinii	E	E00453152	A. A. Grossheim, I. Iljinskaja, M. E. Kirpichnikov	06-V-1947	Azerbaijan, Nakhichevan
Acantholimon karelinii	E	E00665370	V. Ernst	15-VI-2010	Armenia
Acantholimon karelinii	E	E00453155	K. H. Rechinger	17-VII-1974	Iran
Acantholimon leucacanthum	E	E00453178	K. H. Rechinger	31-V-1974	Iran
Acantholimon lycopodioides	E	E00453120	L. Ekberg, P. E. B. Wendelbo	22-VII-1969	Afghanistan, Baghlan
Acantholimon lycopodioides	E	E00453119	L. Ekberg, P. E. B. Wendelbo	14-VII-1969	Afghanistan, Laghman
Acantholimon lycopodioides	E	E00453121	I. Hedge, P. E. B. Wendelbo	17-VII-1962	Afghanistan, Parvan
Acantholimon pterostegium	E	E00457018	F. Schmid	15-VI-1956	Iran
Acantholimon pterostegium	E	E00457019	K. H. Rechinger, F. Rechinger, P. Aellen	30-V-1948	Iran, Khorasan
Acantholimon pterostegium	E	E00457020	K. H. Rechinger, F. Rechinger, P. Aellen	07/10-VI-1948	Iran, Khorasan
Acantholimon quinquelobum	E	E00453164	J. C. Archibald	11-VII-1986	Turkey, Kars
Acantholimon quinquelobum	E	E00453163	N. Jardine	02-VII-1963	Turkey, Kars
Acantholimon quinquelobum	COI	NA	A. Qommmes (?), A. Fomir (?)	1903	Armenia
Acantholimon raddeanum	E	E00457049	K. H. Rechinger	07-VI-1975	Iran
Acantholimon raddeanum	E	E00457048	J. R. Edmondson	28-VI-1973	Iran, Khorasan
Acantholimon raddeanum	E	E00457050	P. E. B. Wendelbo, G. Cobham	27-VII-1974	Iran, Gorgan
Acantholimon restiaceum	E	E00453127	K. H. Rechinger	27-V-1977	Iran, Khorasan
Acantholimon sahendicum	E	E00453402	N. Jardine	21-VII-1963	Iran, Darband
Acantholimon sahendicum	E	E00453397	J. Bornmüller, A. Bornmüller	14-VII-1902	Iran, Elburs

Cont.

Taxon	Herbarium	Barcode	Collector(s)	Date of collection	Collecting country/locality
Acantholimon scorpius	E	E00453183	J. F. N. Bornmüller	01-V-1892	Iran, Kerman
Acantholimon scorpius	E	E00453187	M. Assadi, J. R. Edmondson, A. G. Miller	13-VI-1977	Iran, Kerman
Acantholimon scorpius	E	E00453186	B. S. Parris	04-V-1975	Iran, Kerman
Acantholimon senganense	E	E00453223	K. H. Rechinger	02-VI-1974	Iran
Acantholimon senganense	E	E00453226	R. Petrovitz	30-VI-1968	Iran, Hamadan
Acantholimon senganense	E	E00453227	M. Jacobs	17-VI-1963	Kordestan, Sanandaj
Acantholimon tragacanthinum	E	E00453188	J. M. Lamond	07-VI-1971	Azerbaijan
Acantholimon tragacanthinum	E	E00453189	H. Foroughi, Sanii, Moayed, H. Amini	03-VI-1974	Iran, Tehran
Acantholimon tragacanthinum	E	E00453190	P. H. Davis, M. H. Bokhari	31-III-1974	Iran
Aegialitis rotundifolia	E	NA	J. W. Helfer	1838	India, Calculatta
Aegialitis rotundifolia	E	NA	J. M. Cowan	*s. dat.*	India
Aegialitis rotundifolia	E	NA	J. M. Cowan	*s. dat.*	India
Armeria alliacea	E	E00104714	M. F. Gardner, S. G. Gardner	22-VII-1983	Spain
Armeria alliacea	E	E00104715	Reading Uni. Botany Dept. Expedition	13-VII-1979	Spain
Armeria alliacea	E	E00104720	M. F. Gardner, S. G. Gardner	01-VIII-1979	Andorra
Armeria alliacea	COI	NA	A. R. da Cunha	VI-1885	Portugal, Rio de Mouro
Armeria alliacea	COI	NA	Oberwinkler	VI-1963	France, Alpes Maritimes
Armeria alliacea	COI	NA	F. Sennen	VI/VIII-1915	Spain, Cerdagne
Armeria alpina	E	NA	P. H. Davis	15-VII-1951	Italy, Apennine Mt.
Armeria alpina	E	NA	B. Kitanov	23-VII-1952	Bulgary
Armeria alpina	E	NA	M. Eysn	*s. dat.*	Austria, Salzburg
Armeria alpina	COI	NA	B. Kitanov	23-VII-1952	Bulgaria, Mt. Rila
Armeria alpina	COI	NA	G. A. Poscharsky	19-VI-1884	Slovene, Bohinj
Armeria alpina	COI	NA	R. Nègre	06-XI-1965	France, Montégru
Armeria baetica	E	NA	E. Reverchon	16-V-1887	Spain, Algeciras
Armeria baetica	E	NA	V. H. Heywood, D. M. Moore, et al.	27-III-1969	Spain, Cadiz
Armeria berlengensis	E	NA	J. Daveau	V/VI-1884	Portugal, Berlenga Is.

Armeria berlengensis	COI	NA	A. Mendonça (?)	26-VI-1924	Portugal, Berlenga Is.
Armeria canescens	E	E00104725	J. R. Akeroyd, S. L. Jury, C. J. Milles, F. J. Rumsey	25-VII-1983	Italy
Armeria canescens	E	E00315309	R. D. Reeves, S. Massoura	23-VII-2002	Greece
Armeria canescens	E	E00104724	S. L. Jury, M. F. Watson, D. A. Webb, M. B. Wyse Jackson	18-VII-1985	Italy
Armeria canescens	COI	NA	E. Levier	25-VI-1887	Italy
Armeria cantabrica	E	NA	D. W. Dresser	21-VIII-1956	Spain, Peña Vieja
Armeria cantabrica	E	NA	E. Boissier, L. Leresche, E. Levier	1879	Spain, Santader
Armeria cantabrica	E	NA	E. Boissier, G. F. Reuter	VII-1858	Spain, Peña Redonda
Armeria cantabrica	COI	NA	M. Pérez	17-VI-1973	Spain, Léon
Armeria fasciculata	E	NA	E. Reverchon	20-V-1880	Corsica, Bonifacio
Armeria fasciculata	E	NA	A. Fiori, A. Béguinot, R. Pampanini	*s. dat.*	Italy
Armeria fasciculata	E	NA	A. Fiori, A. Béguinot, R. Pampanini, A. Vaccari	V-1904	Italy, Porlo Pollo
Armeria filicaulis	E	E00104710	Reading Uni. Botany Dept. Expedition	23-VI-1979	Spain
Armeria filicaulis	E	NA	E. Reverchon	VIII-1895	Spain, Teruel
Armeria filicaulis	E	NA	F. Sennen	06-IX-1913	Spain, Catalonia
Armeria gaditana	E	NA	B. Valdés	02-V-1982	Spain, Huelva
Armeria gaditana	E	NA	E. Galiano, P. E. Gibbs, S. Silvestre, B. Valdés	03-V-1969	Spain, Cadiz
Armeria gaditana	COI	NA	A. Mendonça	03-V-1936	Portugal, Faro
Armeria gaditana	COI	NA	M. Costa, E. Valdés-Bermejo	20-V-1977	Spain, Huelva
Armeria gaditana	COI	NA	S. Castroviejo, E. Valdés-Bermejo	01-V-1978	Spain, Huelva
Armeria langei	E	E00315496	R. R. Brooks	11-V-1990	Spain
Armeria langei	E	E00267605	R. R. Brooks	09-V-1990	Portugal
Armeria langei	COI	NA	J. de Maria	VI-1888	Portugal, Miranda do Douro
Armeria langei	COI	NA	A. Fernandes, R. Fernandes, J. Neto	27-VI-1968	Portugal, Bragança
Armeria langei	COI	NA	J. Andrés, F. Llamas	16-VII-1982	Spain, Léon
Armeria leucocephala	E	NA	E. Reverchon	04-VII-1882	Sardinia, Monte Limbardo
Armeria leucocephala	E	NA	E. Reverchon	04-VII-1882	Sardinia, Monte Limbardo

Cont.

Taxon	Herbarium	Barcode	Collector(s)	Date of collection	Collecting country/locality
Armeria leucocephala	E	NA	E. Reverchon	09-VII-1878	Corsica, Bastelica
Armeria leucocephala	COI	NA	E. Reverchon	*s. dat.*	Corsica, Mt. Coscione
Armeria littoralis	E	NA	A. Moller	V-1888	Portugal, Portimão
Armeria littoralis	E	NA	Welwitschii	V-1840	Portugal, Extremadura
Armeria macrophylla	COI	NA	Casaseca, Ladero, F. Navarro	22-IV-1981	Portugal, Quarteira
Armeria majellensis	E	NA	T. De Heldreich	19/20-VII-1885	Spain, Monte Pindo
Armeria majellensis	E	NA	R. Huter, P. Porta, G. Rigo	09-VIII-1877	Italy, Majella
Armeria majellensis	E	NA	E. Levier	29-VII-1876	Italy, Majella
Armeria multiceps	E	NA	E. Reverchon	23-VI-1885	Corsica
Armeria multiceps	E	NA	E. Reverchon	03-VII-1879	Corsica, Mont Coscione
Armeria nebrodensis	E	NA	A. Fiori, A. Béguinot, R. Pampanini	08-VI-1905	Italy, Sicily
Armeria nebrodensis	E	NA	M. Lojacono	12-VI-1880	unknown
Armeria nebrodensis	E	NA	A. Fiori, A. Béguinot	14-VII-1904	Italy, Aprutino
Armeria plantaginea	E	NA	E. Reverchon, A. Derbez	19-VI-1888	France, Fugeret
Armeria plantaginea	E	NA	A. Fiori, A. Béguinot, R. Pampanini, P. Bolzon	20-V-1905	Italy, Parma
Armeria plantaginea	E	NA	A. Mermot	19-VII-1886	France, Rémy
Armeria rouyana	COI	NA	*s. col.*	*s. dat.*	Portugal, Baixo Alentejo
Armeria rouyana	COI	NA	G. Felgueiras	V-1919	Portugal, Leiria
Armeria rouyana	COI	NA	S. Castroviejo, E. Valdés-Bermejo	23-V-1979	Portugal, Alentejo
Armeria rumelica	E	E00104727	M. F. Gardner, S. G. Gardner	28-VII-1985	Bulgaria
Armeria rumelica	E	NA	E. K. Balls, W. B. Gourlay	09-VI-1937	Greece, Qeta
Armeria rumelica	E	NA	W. Greuter, A. Charpin, L. Bernardi, et al.	27-VII-1977	Macedonia, Vrondous Mt.
Armeria rumelica	COI	NA	Z. Hinkova	10-VI-1951	Bulgaria, Mt. Vitosha
Armeria splendens	E	E00104713	M. F. Gardner, S. G. Gardner	15-VII-1983	Spain
Armeria splendens	E	NA	V. H. Heywood, P. H. Davis	12-VII-1948	Spain, Sierra Nevada
Armeria splendens	E	E00104712	M. F. Gardner, S. G. Gardner	21-IV-1982	Spain
Armeria splendens	COI	NA	*s. col.*	27-VII-1900	unknown

Armeria trachyphylla	E	NA	A. G. Huxley	VI-1966	Spain, Tragacete
Armeria velutina	COI	NA	F. A. Mendonça	20-V-1924	Portugal, Faro
Cephalorhizum coelicolor	E	E00453774	I. Hedge, P. E. B. Wendelbo	11-V-1962	Afghanistan, Baghlan
Cephalorhizum coelicolor	E	E00453773	D. Podlech	20-VI-1965	Afghanistan, Takhar
Cephalorhizum coelicolor	E	E00253306	I. Hedge, P. E. B. Wendelbo	29-VI-1969	Afghanistan, Takhar
Ceratolimon feei	E	NA	A. Faure	25-IV-1938	Algeria, Sud-Oranais
Ceratolimon feei	E	NA	P. H. Davis, J. Davis	05-IV-1969	Morocco
Ceratostigma minus	E	NA	G. Forrest	VIII-1917	China, Yunnan
Ceratostigma minus	E	NA	G. Forrest	X-1906	China, NW Yunnan
Ceratostigma minus	E	NA	G. Forrest	VII-1910	China, Yunnan
Dictyolimon macrorrhabdos	E	NA	J. D. A. Stainton	15-V-1958	Pakistan, Chitral
Dictyolimon macrorrhabdos	E	NA	J. D. A. Stainton	05-V-1958	Pakistan, Chitral
Dyerophytum africanum	COI	NA	R. Seydel	29-IV-1959	Namibia, Namibrand
Dyerophytum africanum	COI	NA	*s. col.*	15-IX-1897	Euffel Rivier (?)
Goniolimon speciosum	E	NA	H. Krascheninnikov	VII-1937	Bashikiria
Goniolimon speciosum	E	E00714961	L. R. Phillipe, J. B. Taft, C. H. Dietrich, E. Warren, G. A. Lazkov	14-VII-1999	Kyrgyzstan
Goniolimon speciosum	E	NA	T. Leonova	10-VII-1968	Kazachstania
Goniolimon tataricum	E	E00453788	J. Leonard	28-VII-1978	Russia
Goniolimon tataricum	COI	NA	K. Rechinger	*s. dat.*	Romania, Transylvania
Goniolimon tataricum	COI	NA	J. Wolff	VII-1888	Romania, Turda
Goniolimon tataricum	COI	NA	*s. col.* (?)	09-VI-1873	Romania, Transylvania
Limoniastrum guyonianum	E	NA	J. Ball	II-1880	Algeria
Limoniastrum guyonianum	E	NA	P. H. Davis	16-V-1971	Algeria
Limoniastrum guyonianum	E	NA	C. J. Pitard	III-1908	Tunisia, Gafsa
Limoniastrum monopetalum	E	NA	P. A. C. Endress	VI/VIII-1829	Spain (?)
Limoniastrum monopetalum	E	NA	F. Rugel	VIII-1835	unknown
Limoniastrum monopetalum	COI	NA	E. J. Mendes	09-IX-1968	Portugal, Loulé
Limoniastrum monopetalum	COI	NA	A. Guimarães	IX-1880	Portugal, Faro

Cont.

Taxon	Herbarium	Barcode	Collector(s)	Date of collection	Collecting country/locality
Limoniastrum monopetalum	COI	NA	A. R. Moura	13-VI-1985	Portugal, Manta Rota
Limonium anatolicum	E	E00184690	G. Akaydin	22-IX-2002	Turkey
Limonium articulatum	E	NA	E. Reverchon	02-VIII-1881	Sardinia, S. Teresa Gallura
Limonium articulatum	E	NA	E. Reverchon	02-VIII-1881	Sardinia, S. Teresa Gallura
Limonium articulatum	E	NA	Req. [Requien]	VII-1848	Corsica, Ajaccio
Limonium aureum	E	NA	W. Arnott	*s. dat.*	Liberia
Limonium aureum	E	NA	*s. col.*	*s. dat.*	unknown
Limonium australe	E	NA	R. Brown	1802-1805	Australia
Limonium australe	E	NA	R. Brown	1802-1805	Australia
Limonium australe	E	NA	R. Brown	1802-1805	Australia
Limonium axillare	E	E00068876	I. S. Collenette	09-II-1980	Saudi Arabia, Jeddah
Limonium axillare	E	E00068875	A. G. Miller	22-IX-1979	Oman, Dhofar
Limonium axillare	E	E00181755	A. G. Miller	15-IX-1984	Oman
Limonium bicolor	E	NA	R. C. Ching	4/5-VII-1923	China
Limonium bicolor	E	NA	M. S. Clemens	16-VI-1913	China, Tientsin
Limonium bicolor	E	NA	P^{r}. Chanet	15-VI-1905	China
Limonium bicolor	COI	NA	P^{r}. Chanet	15-VI-1905	China
Limonium caesium	E	NA	Herb. Acad. Rheno-Trai	02-VI-1962	Spain, Alicante
Limonium caesium	E	NA	E. Bourgeau	26-V-1850	Spain
Limonium caesium	E	NA	C. Bicknell	V-1900	Spain, Alicante
Limonium caprariense	E	NA	H. Bianor	05-VIII-1913	Spain, Palma
Limonium caprariense	E	NA	P. Porta, G. Rigo	16-VII-1885	Spain, Menorca
Limonium caprariense	E	NA	H. Bianor	05-VIII-1913	Spain, Palma
Limonium carnosum	E	E00453713	A. Rawi, H. El-Kholy	02-XII-1982	Kuwait, Al'Asimah
Limonium carnosum	E	E00068911	R. A. Western	03-II-1983	United Arab Emirates
Limonium carnosum	E	E00068908	I. S. Collenette	31-III-1987	Saudi Arabia, Tarut Is.
Limonium carolinianum	E	E00770656	*s. col.*	04-VIII-1968	Canada, Mahone Bay

Limonium carolinianum	E	NA	S. M. Tracy	09-IV-1899	unknwon
Limonium carolinianum	E	NA	A. E. Radford, J. R. Boxeman, S. W. Leonard	17-IX-1967	USA, South Carolina
Limonium caspium	E	NA	V. Bogdan	09-VI-1900	Russia, Saratov
Limonium caspium	E	NA	Sofinski	16-VIII-1905	Russia, Kujbyschev
Limonium caspium	E	NA	Meyer (?)	*s. dat.*	Caspium Sea
Limonium delicatulum	E	E00104872	M. F. Gardner, S. G. Gardner	24-VII-1981	Spain
Limonium delicatulum	E	E0010487	S. G. Gardner	X-1975	Spain
Limonium delicatulum	E	E00104873	Reading Uni. Botany Dept. Expedition	09-VII-1979	Spain
Limonium delicatulum	COI	NA	P. Ferrer	14-IX-1949	Spain, Mallorca
Limonium duriusculum	E	NA	F. Sennen	01-VII-1909	Spain, Valencia
Limonium duriusculum	E	NA	A. Autheman	VIII-1876	France
Limonium duriusculum	E	NA	F. Sennen	24-VI-1909	Spain, Valencia
Limonium echioides	E	NA	J. Ball	*s. dat.*	France, Marseille
Limonium echioides	E	NA	H. Bianor	05-VI-1913	Spain, Palma
Limonium echioides	COI	NA	Welwitschii	1848	Portugal, Cabo S. Vicente
Limonium echioides	COI	NA	S. Castroviejo, C. Prada	27-VI-1978	Spain, Madrid
Limonium echioides	COI	NA	I. Haesler	14/18-V-1967	Greece
Limonium fruticans	E	NA	D. Bramwell	14-IV-1969	Canary Islands
Limonium fruticans	E	NA	G. Perez	04-I-1903	Canary Islands, Tenerife
Limonium fruticans	E	NA	J. Ball	*s. dat.*	Canary Islands, Tenerife
Limonium girardianum	E	NA	J. W. White	13-VIII-1903	France, Aude
Limonium girardianum	E	NA	A. Autherman	VIII-1890	France, Bouches-du-Rhône
Limonium girardianum	E	NA	A. Autherman	VIII-1890	France, Bouches-du-Rhône
Limonium gmelinii	E	NA	T. G. Orphanides	1850	unknown
Limonium gmelinii	E	NA	Woloszezak	*s. dat.*	Hungary
Limonium gmelinii	E	NA	Dörfler	18-VIII-1888	unknown
Limonium gmelinii	COI	NA	F. Weber	15-VIII-1935	Slovakia
Limonium gougetianum	E	NA	P. Porta, G. Rigo	10-VII-1885	Spain, Minorca

Cont.

Taxon	Herbarium	Barcode	Collector(s)	Date of collection	Collecting country/locality
Limonium gougetianum	E	NA	E. Reverchon	VII-1896	Algeria, Gouraya
Limonium gougetianum	E	NA	E. Reverchon	1896	Algeria
Limonium iconicum	E	E00453622	G. Wagenitz	05-X-1957	Turkey, Nidge
Limonium iconicum	E	E00453621	J. McNeill	28-VII-1956	Turkey, Ankara
Limonium iconicum	E	E00453617	C. R. Frase-Jenkins	20-VIII-1970	Turkey, Nidge
Limonium inarimense	E	NA	A. Fiori, A. Béguinot	VI-1907	Italy, Campania
Limonium inarimense	E	NA	*s. col.*	*s. dat.*	unknown
Limonium iranicum	E	E00453663	J. R. Edmondson	02-VIII-1971	Iran, Mooteh
Limonium iranicum	E	E00453664	I. Hedge, P. E. B. Wendelbo, H. Foroughi	20-IX-1974	Iran, Tehran
Limonium iranicum	E	E00453661	R. W. Haines	03-X-1961	Iraq, Karbala
Limonium latifolium	E	NA	F. C. Crawford	1908	Caspian steppe
Limonium latifolium	E	NA	A. Jakuschev	31-VII-1911	Russia, Novocherkassk
Limonium latifolium	E	E00453521	R. F. Hohenacker	*s. dat.*	Caucasus
Limonium lilacinum	E	E00453627	P. H. Davis, I. Hedge	29-VIII-1957	Turkey, Kayseri
Limonium lilacinum	E	E00453625	G. Wagenitz, H.-J. Beug	25-VII-1969	Turkey, Ankara
Limonium lilacinum	E	E00453624	P. H. Davis, J. G. Dodds, R. Çetik	26-VI-1952	Turkey, Kayseri
Limonium minutiflorum	E	NA	Dr. Nicotra	VII-1888	Italy, Trapani
Limonium minutiflorum	E	NA	P. Porta, G. Rigo	VI/VII-1885	Spain, Menorca
Limonium minutiflorum	E	NA	E. Bourgeau	04-VI-1869	Spain, Mallorca
Limonium minutum	E	NA	E. Reverchon	VIII-1895	Spain, Valencia
Limonium minutum	E	NA	A. Autheman	02-VII-1840	France, Bouches-du-Rhône
Limonium minutum	E	NA	S. R. Lenormand	1883	France, Marseille
Limonium mouretti	E	E00328911	E. S. Salmon	01-IX-1999	Morocco, Bekrit
Limonium otolepis	E	NA	N. Androssov, M. Kelov	19-V-1901	Turkmenistan, Czardzhou
Limonium otolepis	E	NA	N. Androssov	05-VIII-1901	Uzbekistan, Buchara
Limonium otolepis	E	NA	D. Litwinow	23-V-1899	Turkmenistan, Ashgabat
Limonium perfoliatum	E	NA	J. Bornmüller	VI/VIII-1900	Turkmenistan, Ashgabat

Limonium perfoliatum	E	NA	J. Bornmüller	06-VIII-1900	unknown
Limonium pseudebusitanum	E	E00240957	D. Bramwell, Z. I. Bramwell	17-IX-1973	Spain, Mallorca
Limonium reniforme	E	E00453639	I. Hedge, P. E. B. Wendelbo	17-VI-1962	Afghanistan, Kobul
Limonium reniforme	E	E00453648	T. F. Hewer	13-X-1977	Iran, Golestan
Limonium reniforme	E	E00453642	D. Podlech	21-VII-1965	Afghanistan, Takhar
Limonium saxicola	E	NA	D. Porta, G. Rigo	13-VII-1885	Spain, Minorca
Limonium scabrum	E	NA	*s. col.*	*s. dat.*	South Africa, Franskraal
Limonium scabrum	E	NA	D. J. Clarkson	09-I-1948	South Africa, Cape Town
Limonium scabrum	E	NA	O. M. Hilliard, B. L. Burtt	06-XII-1977	South Africa, Alexandria
Limonium sinense	E	NA	C. Y. Chiao	11-VI-1930	China, Shantung
Limonium sinense	E	NA	I. B. Balfour	1910	China
Limonium sinense	E	NA	T. Ying	17-IV-1928	China, Hong Kong
Limonium sinuatum	E	NA	P. E. Gibbs, S. Silvestre, B. Valdés	12-IV-1969	Spain, Almeria
Limonium sinuatum	E	E00104701	M. F. Gardner, S. G. Gardner	12-IV-1982	Spain
Limonium sinuatum	E	NA	E. Reverchon	08-VI-1987	Spain, Algeciras
Limonium sinuatum	COI	NA	B. Cabezudo, et al.	14-V-1971	Spain, Malaga
Limonium sinuatum	COI	NA	J. M. Laza	15-V-1972	Spain, Malaga
Limonium sinuatum	COI	NA	C. O. Carvalho	VIII-1920	Portugal, Sintra
Limonium suffruticosum	E	E00453658	G. M. Proskuriakova	18-IX-1969	Azerbaijan
Limonium suffruticosum	E	NA	M. Korotky, Z. Lebedeva	07-VIII-1913	Kazakhstan, Kustanaj
Limonium suffruticosum	E	NA	L. Smirnov, I. Maskil	20-VIII-1934	Kazakhstan, Dshangalinsky
Limonium suffruticosum	COI	NA	G. M. Proskuriakova	18-IX-1969	Azerbaijan, Schemakha
Limonium tetragonum	E	NA	The Yokohama Nursery Co., LTD.	VIII-1909	Japan, Yokohama
Limonium tetragonum	E	NA	E. J. Taquet	XII-1907	South Korea
Limonium tetragonum	E	NA	U. J. Faurie	VIII-1906	Korea
Limonium tetragonum	COI	NA	*s.col.* (?)	20-XI-1968	Japan, Tokushima
Limonium tetragonum	COI	NA	M. Togasi	26-VIII-1958	Japan, Hikari
Limonium thouinii	E	NA	M. Mairlot	04-VIII-1908	Belgium

Cont.

Taxon	Herbarium	Barcode	Collector(s)	Date of collection	Collecting country/locality
Limonium thouinii	E	E00104865	M. F. Gardner, S. G. Gardner	13-IV-1982	Spain
Limonium thouinii	E	E00104866	M. F. Gardner, S. G. Gardner	10-IV-1981	Spain
Limonium thouinii	COI	NA	J. B. Peris, G. Stubing	20-IV-1984	Spain, Murcia
Limonium thouinii	COI	NA	L. Chevalier	IV-1892	Algeria
Limonium thouinii	COI	NA	Mall	IV-1871	Morocco
Limonium virgatum	E	E00453608	P. H. Davis	19-X-1981	Greece, Tingakion
Limonium virgatum	E	E00453601	V. Janka	VIII-1871	Turkey
Limonium virgatum	E	E00453596	P. H. Davis	06-X-1981	Greece, Samos
Limonium wrightii	E	E00770639	H. Izumi, M. Fujimoto	19-X-1969	Japan, Kyushu
Limonium wrightii	E	NA	E. H. Walker, S. Tawada	06-IX-1951	Japan, Ishigaki Is.
Limonium wrightii	E	NA	R. Moran	24-XII-1955	Japan, Yakushima
Myriolimon ferulaceum	E	E00194448	F. Sales, I. Hedge	21-VI-2004	Portugal, Algarve
Myriolimon ferulaceum	E	NA	R. Coll	01-VII-1905	Italy, Trapani
Myriolimon ferulaceum	E	NA	D. Bramwell, B. Valdés	08-X-1968	Spain, Huelva
Myriolimon ferulaceum	COI	NA	G. D. Santos	VI-1897	Portugal, Algarve
Myriolimon ferulaceum	COI	NA	A. Fernandes, R. Fernandes, J. Matos	18-VI-1960	Portugal, Faro
Myriolimon ferulaceum	COI	NA	*s. col.* (?)	*s. dat.* (?)	Portugal
Plumbago auriculata	E	NA	E. S. Pooley	27-XI-1969	South Africa, Ubombo
Plumbago auriculata	E	NA	J. B. Allan	1836	South Africa, C. Good Hope
Plumbago capensis	E	NA	M. Mackee	09-XI-1972	New Caledonia
Plumbago capensis	COI	NA	P. O. Schallert	01-V-1963	USA, Florida
Plumbago europaea	E	NA	P. Uotila	14-IX-1972	Croacia, Hrvatska
Plumbago europaea	E	E00470474	P. H. Davis	29-VII-1966	Turkey, Erzurum
Plumbago europaea	E	E00470484	L. B. Hewitt	16-VIII-1970	Turkey, Mt. Ararat
Plumbago europaea	COI	NA	S. I. Laínz	19-IX-1970	Spain, Orense
Plumbago europaea	COI	NA	M. L. Duma (?)	25-VI-1890	unknown (?)
Plumbago europaea	COI	NA	*s. col.* (?)	05-X-1957	unknown (?)

Plumbago indica	E	E00351367	D. J. Middleton	19-I-2004	Thailand, Kaeng Krachan NP
Plumbago indica	E	NA	H. B. G. Garrett	13-I-1941	unknown
Plumbago indica	E	NA	A. F. C. Kerr	*s. dat.*	Thailand, Hue Sai
Plumbago zeylanica	E	E00068929	A. G. Miller	13-X-1978	Yemen, Wadi Hijan
Plumbago zeylanica	E	E00068932	A. G. Miller	23-IX-1979	Oman, Dhofar
Plumbago zeylanica	E	E00068928	K. Muller-Hohenstein, U. Deil	20-X-1982	Yemen
Plumbago zeylanica	COI	NA	P. V. Konnel	24-I-1953	Liberia, Massambolahun
Psylliostachys beludshistanicus	E	E00453724	K. H. Rechinger	22/23-V-1967	Afghanistan, Kandahar
Psylliostachys beludshistanicus	E	E00453723	I. Hedge, P. E. B. Wendelbo, L. Ekberg	08-V-1969	Afghanistan, Herat
Psylliostachys leptostachya	E	E00392690	Peace Project	15-IV-2009	Afghanistan
Psylliostachys leptostachya	E	E00453729	J. M. Lamond	15-VI-1971	Iran, Mahabad
Psylliostachys leptostachya	E	E00453727	K. H. Rechinger	11-V-1967	Afghanistan, Kataghan
Psylliostachys spicatus	E	E00453759	P. H. Davis, M. H. Bokhari	11-IV-1974	Iran, Bushehr
Psylliostachys spicatus	E	E00428311	R. Brown, A. Prestage	14-V-2010	Georgia, Kiziki
Psylliostachys spicatus	E	E00453758	Hunting Aero Survey	23-IV-1955	Jordan, Azraq
Psylliostachys spicatus	COI	NA	*s. col.* (?)	30-V-1958	unknwon (?)
Psylliostachys spicatus	COI	NA	Dept. Botany	19-III-1957	Iraq
Psylliostachys suworowii	E	E00453734	T. F. Hewer	15-V-1969	Afghanistan, Balkh
Psylliostachys suworowii	E	E00453735	P. E. B. Wendelbo, I. Hedge, L. Ekberg	27-V-1969	Afghanistan, Faryab
Psylliostachys suworowii	E	E00453741	K. H. Rechinger	11-V-1967	Afghanistan, Kataghan

Appendix 5.2. Character states used in the ancestral character state reconstruction. Species with an asterisk were not included in character reconstruction analyses. Sex-organs arrangement: a – reciprocal herkogamy, b – monomorphic; compatibility status: a - self-incompatible, b – self-compatible; exine sculpturing: a – monomorphic, b – *Armeria*-type, c- *Plumbago*-type; stigma morphology: capitate – a, filiform – b, fimbriate/pear-shaped/lanceolate – c; stigma dimorphism: monomorphism – 0, dimorphism – 1; apomixis – 0, sexual - 1. "-" no information.

Taxon	Sex-organs arrangement	Compatibility status	Exine sculpturing	Stigma morphology	Stigma dimorphism	Apomixis	Refs
Outgroup							
Frankenia pulverulenta	b	a	a	b	0	0	Brightmore, 1979; Guerra, 1993
Polygonum amphibium	b	a	a	a	0	0	Partridge, 2001; Villar, 1990
Polygonum capitatum	b	a	a	a	0	0	Villar, 1990
Rumex obtusifolius	b	a	a	c	0	0	Allen and Hiscock, 2008; González, 1990
Tamarix aphyyla	b	-	a	c	0	0	Gaskin, 2003
Triplaris americana	b	b	a	c	0	0	Lledó *et al*., 2001; Melampy and Howe, 1977
Plumbaginaceae							
Acantholimon acerosum	-	a	b	a	1	0	this study
Acantholimon acmostegium	b	a	b	a	1	0	this study
Acantholimon alavae	-	a	b	a	1	0	Rechinger, 1974
Acantholimon albocalycinum	-	a	b	a	1	0	-
Acantholimon androsaceum	-	a	b	a	1	0	this study
Acantholimon araxanum	a	a	b	a	1	0	this study
*Acantholimon aspadanum**	-	a	b	a	1	0	this study
Acantholimon blandum	-	a	b	a	1	0	Rechinger, 1974
Acantholimon bodeanum	-	a	b	a	1	0	Rechinger, 1974
Acantholimon bracteatum	a	a	b	a	1	0	this study
Acantholimon bromifolium	-	a	b	a	1	0	this study

Acantholimon cephalotoides	-	a	b	a	1	0	Rechinger, 1974
Acantholimon collare	b	a	b	a	1	0	this study
Acantholimon curviflorum	-	a	b	a	1	0	this study
Acantholimon cymosum	-	a	b	a	1	0	Rechinger, 1974
Acantholimon demavendicum	a	a	b	a	1	0	this study
Acantholimon erinaceum	b	a	b	a	1	0	this study
Acantholimon festucaceum	-	a	b	a	1	0	this study
Acantholimon flabellum	-	a	b	a	1	0	-
*Acantholimon gorganense**	-	a	b	a	1	0	this study
*Acantholimon gulistanum**	-	a	b	a	1	0	this study
Acantholimon heratense	-	a	b	a	1	0	this study
Acantholimon hohenackeri	a	a	b	a	1	0	this study
Acantholimon karelinii	b	a	b	a	1	0	this study
Acantholimon latifolium	-	a	b	a	1	0	-
*Acantholimon lycopodioides**	-	a	b	a	1	0	this study
Acantholimon oliganthum	-	a	b	a	1	0	Rechinger, 1974
Acantholimon pterostegium	a	a	b	a	1	0	this study
Acantholimon quinquelobum	-	a	b	a	1	0	this study
Acantholimon raddeanum	a	a	b	a	1	0	this study
Acantholimon restiaceum	a	a	b	a	1	0	this study
Acantholimon sahendicum	a	a	b	a	1	0	this study
Acantholimon scorpius	b	a	b	a	1	0	this study
Acantholimon senganense	-	a	b	a	1	0	this study
Acantholimon spinicalyx	-	a	b	a	1	0	Rechinger, 1974
Acantholimon tomentellum	-	a	b	a	1	0	Rechinger, 1974
Acantholimon tragacanthinum	-	a	b	a	1	0	this study
Acantholimon zaeifii	-	a	b	a	1	0	-

Cont.

Taxon	Sex-organs arrangement	Compatibility status	Exine sculpturing	Stigma morphology	Stigma dimorphism	Apomixis	Refs
Aegialitis annulata	b	a	a	a	0	0	Baker, 1966; Ghobary, 1984
Aegialitis rotundifolia	b	a	a	a	0	0	this study
Afrolimon peregrinum	-	-	-	-	-	-	-
Afrolimon purpuratum	-	-	-	-	-	-	-
Armeria alliacea	b	a	b	b	1	0	this study
Armeria alpina	b	a	b	b	1	0	this study
Armeria berlengensis	b	a	b	b	1	0	this study
Armeria canescens	b	a	b	b	1	0	this study
Armeria cantabrica	b	a	b	b	1	0	this study
Armeria gaditana	b	a	b	b	1	0	this study
Armeria hirta	b	a	b	b	1	0	this study
Armeria langei	b	a	b	b	1	0	this study
Armeria leucocephala	b	a	b	b	1	0	this study
*Armeria linkiana**	b	a	b	b	1	0	this study
Armeria macrophylla	b	a	b	b	1	0	this study
Armeria maritima	b	a	b	b	1	0	this study; Chapter 4
Armeria multiceps	b	a	b	b	1	0	this study
Armeria nebrodensis	b	a	b	b	1	0	this study
Armeria pubigera	b	a	b	b	1	0	this study; Chapter 4
Armeria pungens	b	a	b	b	1	0	this study
Armeria rouyana	b	a	b	b	1	0	this study
Armeria rumelica	b	a	b	b	1	0	this study
Armeria soleirolii	b	a	b	b	1	0	this study
Armeria splendes	b	a	b	b	1	0	this study
*Armeria trachyphylla**	b	a	b	b	1	0	this study
Armeria velutina	b	a	b	b	1	0	this study

Armeria welwitschii	b	a	b	b	1	0	this study
Cephalorhizum coelicolor	-	a	b	a	1	0	this study
Ceratolimon feei	b	a	b	b	1	0	this study
Ceratolimon rechingeri	-	-	-	b	-	-	-
Ceratostigma minus	a	a	c	a	0	0	this study
Dictyolimon macrorrhabdos	-	a	b	a	1	0	this study
Dyerophytum africanum	a	a	c	a	0	0	this study; Ghobary, 1986
Dyerophytum indicum	a	a	c	a	0	0	Ghobary, 1986
Eremolimon sogdianum	-	-	-	b	-	-	-
Goniolimon italicum	a	a	b	a	1	0	Morretti *et al*., 2015
Goniolimon speciosum	a	a	b	a	1	0	this study; Baker, 1953a
*Goniolimon tataricum**	a	a	b	a	1	0	this study; Baker, 1953a; Schill *et al*., 1985
Limoniastrum guyonianum	b	a	b	b	1	0	this study
Limoniastrum monopetalum	b	a	b	a	1	0	this study
Limonium aureum	b	a	b	b	1	0	this study; Baker, 1953a
Limonium axillare	b	a	b	b	1	0	this study; Baker, 1953a
Limonium bellidifolium	b	a	b	b	1	0	Baker, 1953a; Ingrouille, 1984
Limonium bicolor	b	a	b	b	1	0	this study; Baker, 1953a, b
Limonium caesium	b	a	b	b	1	0	this study; Baker, 1953b
Limonium camposanum	b	a	a	b	0	1	Erben, 1979; Castro and Rosselló, 2007
*Limonium caprariense**	b	a	b	b	1	0	this study
Limonium carnosum	b	a	b	b	1	0	this study
Limonium carolinianum	b	b	a	b	0	0	this study; Baker, 1953a, b
Limonium caspium	b	a	b	b	1	0	this study
Limonium cossonianum	b	a	b	b	1	0	this study; Ingrouille, 1984
Limonium delicatulum	b	a	b	b	0	1	this study; Ingrouille, 1984

Cont.

Taxon	Sex-organs arrangement	Compatibility status	Exine sculpturing	Stigma morphology	Stigma dimorphism	Apomixis	Refs
Limonium dufourii	b	a	a	b	0	1	Ingrouille, 1984; Palacios and González-Candelas, 1997; Palob-Esteban *et al.*, 2007
Limonium duriusculum	b	a	b	b	0	1	this study; Ingrouille, 1984
Limonium echioides	b	b	a	b	0	0	this study; Baker, 1953a, b; Ingrouille, 1984
Limonium furfuraceum	b	a	b	b	1	0	Ingrouille, 1984
Limonium girardianum	b	a	a	b	0	1	this study; Ingrouille, 1984
Limonium gmelinii	b	a	b	b	1	0	this study; Baker, 1953a
Limonium gymnesicum	b	a	a	b	0	1	Erben, 1979; Rosato *et al.*, 2012
Limonium humile	b	b	a	b	0	0	Baker, 1953a, b; Dawson and Ingrouille, 1995
Limonium iconicum	b	a	b	b	1	0	this study
Limonium iranicum	b	a	b	b	1	0	this study
Limonium latifolium	b	a	b	b	1	0	this study; Baker, 1953a
Limonium lilacinum	b	a	b	b	1	0	this study
Limonium meyeri	b	a	b	b	1	0	Dulberger, 1975a
Limonium minutiflorum	b	a	b	b	0	1	this study; Brullo and Pavone, 1981
Limonium minutum	b	a	b	b	1	0	this study; Baker, 1953a; Ingrouille, 1984
Limonium mouretii	b	-	-	b	-	-	this study
Limonium narbonense	b	a	b	b	1	0	Palob-Esteban *et al.*, 2011
Limonium otolepis	b	a	b	b	1	0	this study
Limonium reniform	b	a	b	b	1	0	this study
Limonium rigualii	-	-	-	b	-	-	-
Limonium scabrum	b	-	a	b	1	-	this study; Baker, 1953a
Limonium sinense	b	a	b	b	1	0	this study; Baker, 1953a, b
Limonium sinuatum	b	a	b	b	1	0	this study; Baker, 1953a

Limonium spectabile	-	-	-	b	-	-	-
Limonium suffruticosum	b	a	b	b	1	0	this study; Baker, 1953a
Limonium tenellum	b	a	b	b	1	0	Baker, 1953a
Limonium tetragonum	b	b	a	b	0	0	this study; Baker, 1953a, b
Limonium thouinii	b	a	b	b	1	0	this study; Baker, 1953a
Limonium virgatum	b	a	b	b	1	0	this study; Baker, 1953a
Limonium vulgare	a	a	b	b	1	0	this study; Baker, 1953a, b Chapter 4
Myriolimon ferulaceum	b	a	b	b	1	0	this study; Baker, 1953a, b
Plumbago auriculata	a	a	c	a	0	0	this study; Ferrero *et al.*, 2009b
Plumbago europaea	a	a	c	a	0	0	this study; Dulberger, 1975a
Plumbago indica	a	a	c	a	0	0	this study
Plumbago zeylanica	a	a	c	a	0	0	this study
Psylliostachys beludshistanicus	b	a	b	b	1	0	this study; Baker, 1953a
Psylliostachys leptostachya	b	a	b	b	1	0	this study; Baker, 1953a
Psylliostachys spicatus	b	a	b	b	1	0	this study; Baker, 1953a
Psylliostachys suworowii	b	a	b	b	1	0	this study; Baker, 1953a
Saharanthus ifniensis	-	-	-	-	-	-	-

Appendix 5.3. GenBank accession number for all taxa included in the supermatrix for maximum likelihood analysis. When no sequence was available for the target species, sequences from another species of the same genus were used instead and are denoted in square brackets. "-" no sequence available.

	GenBank accession number				
Taxon	***ITS***	***rbcL***	***matK***	***trnL-trnF***	***trnT-trnY***
Outgroup					
Polygonum amphibium	EF653699	EF653776	JN895115	EF653803	-
Polygonum capitatum	FJ648807	HM850243	-	-	-
Rumex obtusifolius	GQ340059	AF297126	EF438023	-	-
Triplaris americana	KP271195	Y16910	AY042668	AJ312251	-
Tamarix aphylla	AF484767	AY099903	JX495763	-	-
Frankenia pulverulenta	-	Z97638	HM851067	-	-
Plumbaginaceae					
Acantholimon acerosum	-	Z97639	-	AJ391314	-
Acantholimon alavae	AB979533	-	-	-	AB979601
Acantholimon albocalycinum	AB979534	-	-	-	AB979602
Acantholimon araxanum	AB979535	-	-	-	AB979603
Acantholimon blandum	AB979540	-	-	-	AB979606
Acantholimon bodeanum	AB979541 [subsp. *bodeanum*]	-	-	-	AB979607 [subsp. *bodeanum*]
Acantholimon bracteatum	AB979544	-	-	-	AB979609
Acantholimon cephalotoides	AB979546	-	-	-	AB979611
Acantholimon curviflorum	AB979549	-	-	-	AB979614
Acantholimon cymosum	AB979550	-	-	-	AB979615
Acantholimon demavendicum	AB979551	-	-	-	AB979616
Acantholimon flabellum	AB979556	-	-	-	AB979618
Acantholimon heratense	AB979561	-	-	-	AB979621
Acantholimon hohenackeri	AB979563	-	-	-	AB979623

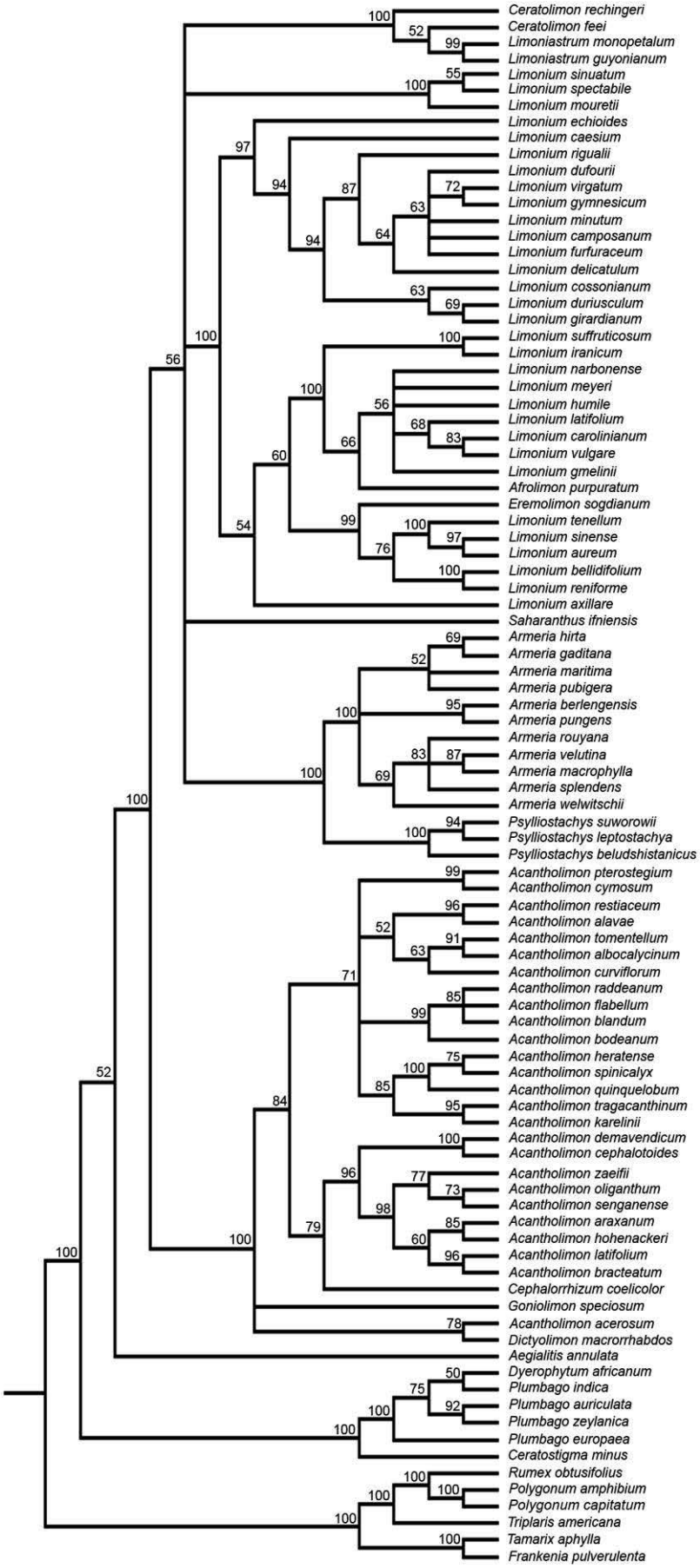
Acantholimon karelinii	AB979565	-	-	-	AB979625
Acantholimon latifolium	AB979567	-	FN597642 [*A. lycopodioides*]	-	AB979627
Acantholimon oliganthum	AB979570	-	-	-	AB979630
Acantholimon pterostegium	AB979571	-	-	-	AB979631
Acantholimon quinquelobum	AB979572	-	-	-	AB979632
Acantholimon raddeanum	AB979573	-	-	-	AB979633
Acantholimon restiaceum	AB979574	-	-	-	AB979634
Acantholimon senganense	AB979580 [subsp. *senganense*]	-	-	-	AB979639 [subsp. *senganense*]
Acantholimon spinicalyx	AB979583	-	-	-	AB979641
Acantholimon tomentellum	AB979584	-	-	-	AB979643
Acantholimon tragacanthinum	AB979585	-	-	-	AB979644
Acantholimon zaeifii	AB979587	-	-	-	AB979646
Aegialitis annulata	-	AJ312252	-	AJ312245	-
Afrolimon spp.	-	JQ412383 [*A. peregrinum*]	AY042537 [*A. purpuratum*]	-	-
Armeria berlengensis	AY179763	-	GQ901426	GQ901200	-
Armeria gaditana	AJ225589	-	GQ901424	GQ901195	-
Armeria hirta	AJ225568	-	GQ901414	GQ901180	-
Armeria macrophylla	AY179790	-	GQ901418	GQ901186	-
Armeria maritima	GQ901329	HM849792	GQ901431	GQ901205	AB979647
Armeria pubigera	AY179807	-	GQ901430	GQ901202	-
Armeria pungens	AY179810	-	GQ901439	GQ901214	-
Armeria rouyana	AJ225567	-	GQ901411	GQ901176	-
Armeria splendens	AY179817	Y16908	-	AJ391316	-
Armeria velutina	AJ225564	-	GQ901422	GQ901193	-
Armeria welwitschii	AY179832	-	GQ901403	GQ901168	AB979648

Cont.

Taxon	*ITS*	*rbcL*	*matK*	*trnL-trnF*	*trnT-trnY*
Cephalorhizum coelicolor	JX983658 [*C. turcomanicum*]	-	-	-	AB979649 [*C. turcomanicum*]
Ceratolimon feei	HE602420	AJ286357	EU531681	AJ391318	-
Ceratolimon rechingeri	-	AJ286360	-	AJ391322	-
Dictyolimon macrorrhabdos	-	Y16909	-	AJ391317	-
Eremolimon sogdianum	JX983723	-	-	-	AB979654
Goniolimon tataricum	-	AJ312254 [*G. speciosum*]	AF204855 [*G. tataricum*]	AJ312247 [*G. speciosum*]	-
Limoniastrum guyonianum	HE602418	AJ286358	-	AJ391319	-
Limoniastrum monopetalum	HE602419	Z97642	AY042609	AJ391321	-
Limonium aureum	KF866379	JN187124	-	-	-
Limonium axillare	JX983660	AJ286362	-	AJ391323	-
Limonium bellidifolium	JX983722	KF997474	-	-	-
Limonium caesium	AJ222859	Z97643	-	-	-
Limonium camposanum	AJ222841	KJ608041	-	-	-
Limonium carolinianum	-	KJ773631	KJ772894	-	-
Limonium cossonianum	AJ132331	KJ608017	-	-	-
Limonium delicatulum	AJ222850	Y16903	-	AJ391324	-
Limonium dufourii	AJ222840	AJ286363	-	AJ391326	-
Limonium duriusculum	AJ222852 [subsp. *cavanillesii*]	GQ248629 [subsp. *thiniense*]	AY042610 [subsp. *cavanillesii*]	-	-
Limonium echioides	AJ222861	KJ608037	-	-	-
Limonium furfuraceum	AJ222856	Y16902	-	-	-
Limonium girardianum	AJ222845	KJ608013 [subsp. *grosii*]	-	-	-
Limonium gmelinii	AB979591	-	-	-	AB979650
Limonium gymnesicum	AJ222842	KJ608045	-	-	-
Limonium humile	-	JN891805	JN894792	-	-

Limonium iranicum	AB979592	-	-	-	AB979651
Limonium latifolium	JX983694	-	AY514861	-	-
Limonium meyeri	AB979593	-	-	-	AB979652
Limonium minutum	AJ132332	KJ608027	-	-	-
Limonium mouretii	AJ132333 [*L. lobatum*]	Y16901	AF204854	-	-
Limonium narbonense	AJ222838	AJ286364	AF204853	AJ391327	-
Limonium reniforme	AB979594	-	-	-	AB979653
Limonium rigualii	AJ222854	Z97645	AM889717	AJ391328	-
Limonium sinense	AB190852 & AB190853	FJ872106	JQ946307	FJ872102	-
Limonium sinuatum	AJ222860	Y16900	-	AJ391329	-
Limonium spectabile	-	Z97646	-	AJ391330	-
Limonium suffruticosum	JX983666	-	-	-	AB979655
Limonium tenellum	AB190856 & AB190857 [*L. tetragonum*]	AJ286365	-	AJ391331	-
Limonium virgatum	AJ222855	KJ608053	-	-	-
Limonium vulgare	AJ222839	Y16904	JN895287	AJ391332	-
Psylliostachys beludshistanicus	AB979596	-	-	-	AB979656
Psylliostachys leptostachya	AB979597	-	-	-	AB979657
Psylliostachys suworowii	AJ132446	Y16907	AY042639	AJ391335	-
Saharanthus ifniensis	-	AJ286359	-	AJ391320	-
Ceratostigma minus	-	Z97641	AY042566	AJ391333	-
Dyerophytum africanum	-	AJ312253	AY042581	AJ312246	-
Plumbago auriculata	JF831220	EU002283	EU002187	JF831319	-
Plumbago europaea	AB979599	-	AY042634	AJ391334	AB979659
Plumbago indica	LC029454	KF261598	AF204857	-	-
Plumbago zeylanica	KM887374	Y16905	-	AJ312248	-

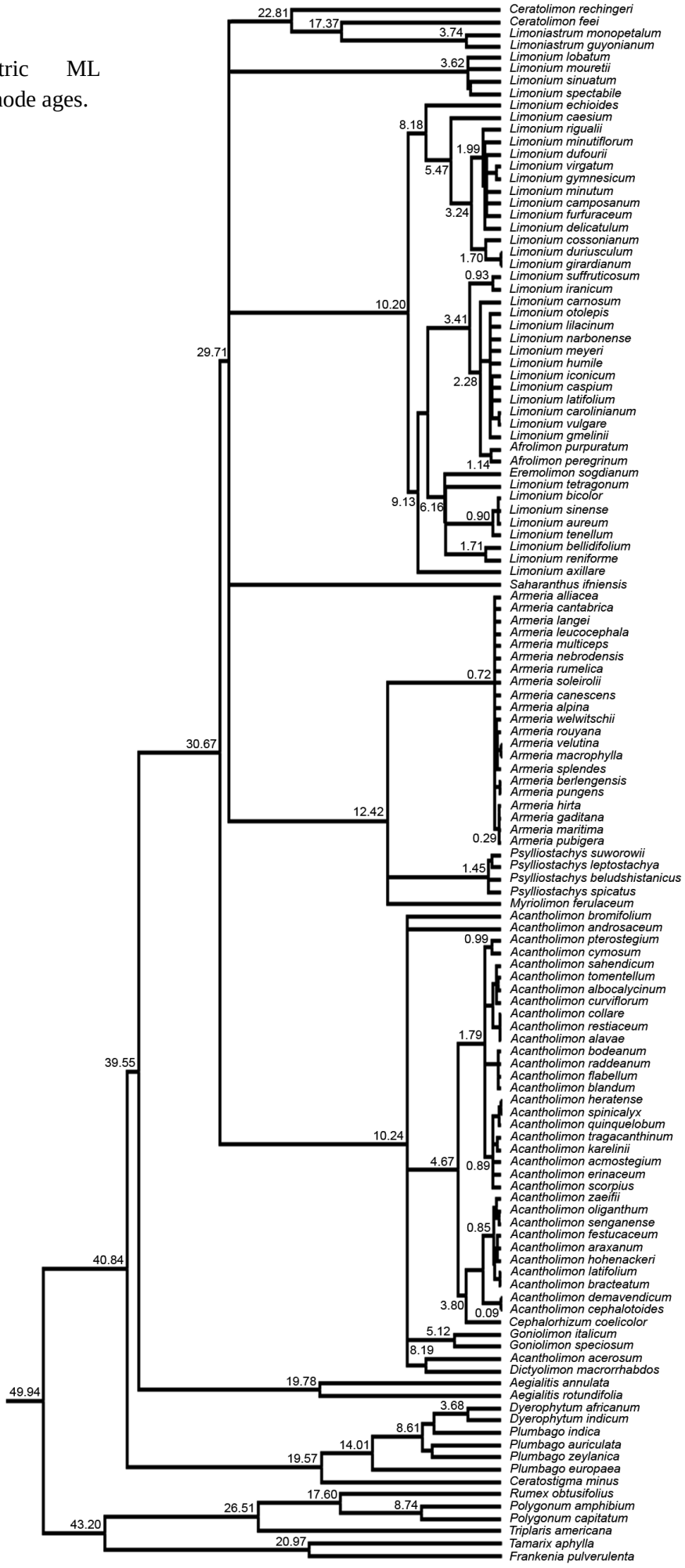
Appendix 5.4. Best ML phylogenetic tree after collapsing the nodes with a BP lower than 50%. Values above branches are BP.



Appendix 5.5. Models of substitution rate of variation among branches and values of the smoothing parameter, lambda, tested for creating an ultrametric tree. The final model and lambda value were chosen accordingly with the combination that resulted in the lowest value of PHIIC. In this case, no differences were obtained when changing the smoothing parameter for the model "strict" and thus, the simplest model was chosen (model "strict", lambda value "0").

Model	lambda	PHIIC
Relaxed	0	511.62
Relaxed	0.1	512.21
Relaxed	0.2	512.85
Relaxed	1	517.44
Correlated	0	511.63
Correlated	0.1	511.63
Correlated	0.2	511.63
Correlated	1	511.63
Strict	0	175.08
Strict	0.1	175.08
Strict	0.2	175.08
Strict	1	175.08

Appendix 5.6. Ultrametric ML phylogenetic tree. Values are node ages.



Appendix 5.7. BF values comparing restricted and unrestricted models testing the character state at the most recent common ancestor of the Plumbaginaceae (see *Character mapping and inferences on ancestral states*). BF were calculated using the marginal likelihood values of the harmonic mean and the stepping stone. Set 1 and Set 2 refer to the two sets of 100 phylogenetic trees used. "SI" – self-incompatible, "SC" – self-compatible. "Others" - fimbriate/pear-shaped/lanceolate.

Model	**Bayes Factor (BF)**			
	Set 1		**Set 2**	
	harmonic mean	**stepping stone**	**harmonic mean**	**stepping stone**
(a) Sex-organs arrangement				
1- Monomorphic	1.06	7.33	3.10	5.13
2- Reciprocal herkogamous	1.22	2.36	1.40	1.53
(b) Incompatibility status				
1- SI	8.87	14.49	8.53	15.23
2- SC	14.03	17.38	9.90	18.30
(c) Exine sculpturing				
1- Monomorphic	0.50	6.09	0.50	6.45
2- *Armeria*-type	8.70	3.71	2.52	4.05
(d) Stigma dimorphism				
1- Monomorphic	22.45	8.88	3.35	7.86
2- Dimorphic	5.43	7.70	6.64	6.64
(e) Stigma morphology				
1- Filiform	25.05	39.13	32.58	36.46
2- Capitate	29.11	43.87	28.58	39.02
3- Others	28.45	37.82	35.45	37.30
(f) Apomixis				
1- Sexual	18.81	22.22	18.13	22.62
2- Apomictic	25.22	26.92	24.27	26.91

Appendix 5.8. Rate of change between states for the six characters investigated for the second set of 100 trees. Values are mean ± SE. "RH" – reciprocal herkogamy, "SI" – self-incompatible, "SC" – self-compatible. "Others" - fimbriate/pear-shaped/lanceolate. For details on the calculation of frequency of transition and relative rate of transition see *Character mapping and inferences on ancestral states*.

Character state	No. of transitions	Frequency of transition	Relative rate of transition
(a) Reciprocal herkogamy			
Monomorphic to RH	3.93 ± 0.01	0.43	0.58
RH to homostylous	5.21 ± 0.03	0.57	2.16
(b) Incompatibility system			
SI to SC	77.42 ± 0.15	0.50	0.53
SC to SI	76.84 ± 0.15	0.50	10.13
(c) Exine sculpturing			
Dimorphic to monomorphic	9.15 ± 0.02	0.90	1.64
Monomorphic to dimorphic	0.97 ± 0.01	0.10	0.21
(d) Stigma dimorphism			
Dimorphic to monomorphic	15.25 ± 0.15	0.98	1.64
Monomorphic to dimorphic	0.34 ± 0.003	0.02	0.05
(e) Stigma morphology			
Filiform to capitate	0.05 ± 0.002	0.01	0.02
Filiform to others	0.00 ± 0.00	0.00	0.00
Capitate to filiform	0.84 ± 0.01	0.16	0.43
Filiform to others	0.00 ± 0.00	0.00	0.00
Others to filiform	1.32 ± 0.01	0.25	1.22
Others to capitate	3.03 ± 0.01	0.58	2.81
(d) Apomixis			
Apomictic to sexual	14.93 ± 0.04	0.59	11.12
Sexual to apomictic	10.40 ± 0.03	0.41	0.43

Appendix 5.9. Restricted models investigating the character state at the most recent common ancestor of the Plumbaginaceae (see *Character mapping and inferences on ancestral states*). Values in bold are the best evolutionary models according to the BIC for the second set of 100 trees. "SI" – self-incompatible, "SC" – self-compatible. "Others" - fimbriate/pear-shaped/lanceolate.

Model	BIC
(a) Sex-organs arrangement	
1- Monomorphic	**60.692**
2- Reciprocal herkogamous	65.574
(b) Incompatibility status	
1- SI	**52.392**
2- SC	62.396
(c) Exine sculpturing	
1- Monomorphic	**94.220**
2- *Armeria*-type	96.504
(d) Stigma dimorphism	
1- Monomorphic	**96.324**
2- Dimorphic	96.578
(e) Stigma morphology	
1- Filiform	39.490
2- Capitate	**43.570**
3- Others	40.014
(f) Apomixis	
1- Sexual	**61.138**
2- Apomictic	72.422

Appendix 5.10. Character state of the most common recent ancestor of the Plumbaginaceae with PP. Results obtained for the second set of 100 trees.

Character state	PP
Monomorphic for sex-organs arrangement	0.764
Self-incompatible	0.950
Monomorphic pollen	0.712
Monomorphic stigma	0.937
Capitate stigma	0.607
Sexual	0.996

Part III – Maintenance and breakdown of the floral polymorphism

"According to Vaucher and Wirgten, the three forms coexist in all parts of Europe. Some friends gathered for me in North Wales a number of twigs from separate plants growing near one another, and classified them. My son did the same in Hampshire, (…). If twice or thrice the number had been collected, the three forms would probably have been found nearly equal (...)".

Darwin (1877 p. 144)

Chapter 6 – Variation in style morph frequencies in tristylous *Lythrum salicaria* in the Iberian Peninsula: the role of geographical and demographic factors

A. *Lythrum salicaria* being visited by *Xylocopa violacea*. **B.** *L. salicaria* growing in the riverbed near Coimbra (Image courtesy of Sílvia Castro, CFE, University of Coimbra). **C.** Natural population in Foz do Neiva, Portugal.

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ABSTRACT

The balance between stochastic forces and negative frequency-dependent selection largely determines style morph frequencies in heterostylous populations. Investigation of morph frequencies at geographical range limits can provide insights on the forces maintaining the floral polymorphism, and the factors causing biased morph ratios. Here, I investigate style morph frequencies in populations at the southwestern European range limit of tristylous *Lythrum salicaria*, to explore the role of demographic and geographic factors influencing morph ratios in its native range. I measured morph composition and evenness, and the size of 96 populations, along a north to south latitudinal transect from Galicia to Andalucia, Iberian Peninsula, traversing a steep climatic gradient. To examine the potential influence of morph-specific fitness components on morph ratios, I examined reproductive traits in 19 populations. Most populations of *L. salicaria* were trimorphic (94.79%), the majority exhibiting 1:1:1 morph ratios (68.75%). Populations with biased morph ratios had a deficiency of the short-styled morph. Population size and morph evenness were positively associated with latitude, with smaller populations and those with less even morph ratios occurring towards the south. Greater variance in morph evenness was evident at the southern range margin. There were no consistent differences in components of reproductive fitness among style morphs, but southern populations produced less fruit and seed than more northerly populations. My results demonstrate the influence of finite population size on morph frequencies in *L. salicaria*. However, they also illustrate the resilience of Iberian populations to the factors causing deviations from isoplethy and morph loss, especially at the southern range limit where populations are smaller. The maintenance of tristyly in small populations of *L. salicaria* may be aided by the genetic connectivity of populations in agricultural landscapes resulting from gene flow through pollen and seed dispersal.

KEY WORDS: frequency-dependent selection; genetic drift; Iberian Peninsula; isoplethy; *Lythrum salicaria*; population size; range limits; stochastic forces; tristyly.

INTRODUCTION

Populations of tristylous plants are typically composed of three style morphs that differ in the reciprocal positioning of stigma and anther heights within a flower. The floral forms are referred to as the long-, mid- and short-styled morphs (hereafter L-, M-, and S-morphs; Chapter 1: Fig. 1.1B), because of their discrete variation in style length. Associated with these differences in sex-organ position is a sporophytically controlled trimorphic incompatibility system that prevents self- and intramorph mating. Compatible mating in most tristylous species involves pollinations between anthers and stigmas of equivalent height (Darwin, 1877; Barrett and Cruzan, 1994). Thus, trimorphic incompatibility enforces phenotypic disassortative mating in populations (Barrett *et al*., 1987) and, as a result of negative frequency-dependent selection (Eckert *et al*., 1996a), a 1:1:1 style morph ratio (*i.e*., isoplethy) is expected in populations at equilibrium, when there are no fitness differences among the style morphs (Fisher, 1944; Heuch, 1979a). The tristylous genetic polymorphism is governed by two diallelic loci (*S*, *M*) with the *S* locus epistatic to the *M* locus (reviewed in Lewis and Jones, 1992). Although tristyly is only known from six angiosperm families, beginning with Darwin's (1877) early work on the polymorphism, it has been used as a model system for investigating a range of questions concerning the ecology, genetics and evolution of populations (reviewed in Barrett, 1993; Chapter 1). Because the style morphs in tristylous populations are easily identified under field conditions, a particular focus of research has involved surveys of their frequencies to determine if they occur at the expected isoplethic equilibrium and, if not, what factors might cause biased morph ratios (*i.e*., anisoplethy).

A variety of stochastic and deterministic factors can cause biased morph ratios in tristylous populations. Founder events and genetic drift in small populations are a common cause of anisoplethy and morph loss (reviewed in Barrett, 1993). Because of the genetic control of tristyly and differences in the relative frequencies of alleles at the *S* and *M* loci at equilibrium, the style morphs are differentially susceptible to stochastic loss from populations through genetic drift. Theoretical studies indicate that the S-morph should be lost more often and the L-morph least often (order of loss S>M>L; Heuch, 1980; Barrett *et al*., 1989), and field surveys of several tristylous species have provided empirical support for this pattern of asymmetrical morph loss (Eckert and Barrett, 1992; Husband and Barrett, 1992). Founder events and historical contingency can also cause biased morph ratios in tristylous populations, especially in species with

extensive clonal propagation (Ornduff, 1972; Barrett and Forno, 1982; Morgan and Barrett, 1988; Castro *et al*., 2013; Cunha *et al*., 2014). Although less common, morph-specific fitness differences in reproductive traits affecting pollen transfer and mating can also result in consistent deviations from isoplethy (Barrett *et al*., 1983, 2004; Weller, 1986; Weber *et al*., 2013). Identifying the mechanisms causing biased morph ratios in tristylous populations requires studies of the demographic characteristics of populations and the variation in reproductive fitness of style morphs.

The study of variation in style morph ratios along environmental gradients, especially those encompassing geographical range limits, has the potential to provide insights into the factors maintaining tristyly and those causing its evolutionary breakdown. According to predictions of the "abundant-centre distribution theory" (see Wulff, 1950; Hengeveld, 1990; Abeli *et al*., 2014), populations at a species' range limit should be smaller, more isolated, and have lower reproductive success than those at the core of the distribution (see Brussard, 1984; Vucetich and Waite, 2003; Sexton *et al*., 2009). Under these circumstances, stochastic forces are more likely to play a role in the demography of range margin populations than for populations at the centre of the distribution. Edge populations are predicted to have lower genetic diversity and to be more genetically differentiated than core populations (Lesica and Allendorf, 1995; Eckert *et al*., 2008). Support for the abundant-centre distribution theory is mixed (Yakimowski and Eckert, 2007; Sexton *et al*., 2009; Abeli *et al*., 2014), and geographically marginal populations do not always show reduced genetic diversity in comparisons with central populations (Eckert *et al*., 2008; Simón-Porcar *et al*., 2015); however, there is some evidence that the demographic and genetic characteristics of range edge populations of the style-dimorphic *Narcissus papyraceus* (Arroyo *et al*., 2012; Santos-Gally *et al*., 2013; Simón-Porcar *et al*., 2015) and several tristylous species (Barrett *et al*., 1989, 2004; Eckert and Barrett, 1993; Ness *et al*., 2010) differ from those at the centre of the range. If populations of tristylous species at range edges are smaller than more centrally located populations, they may be more vulnerable to destabilization of the polymorphism by stochastic processes.

Here, I investigate variation in style morph frequencies along a climatic gradient at the southwestern European range limit of purple loosestrife (*Lythrum salicaria* L., Lythraceae) to assess the role of geographical and demographic factors in the maintenance of floral trimorphism. *Lythrum salicaria* is perhaps the most well-known tristylous species and has been studied extensively since Darwin established the general

features of tristyly in the species using controlled crosses (Darwin, 1864; 1877), and Fisher and Mather (1943) worked out the genetic basis of the polymorphism. The species is native to wetland habitats in Europe and Asia, but has been introduced to various parts of the world, where it has spread extensively and become an aggressive invader, especially in eastern North America (Stuckey, 1980; Thompson *et al*., 1987; Mal *et al*., 1992; Colautti and Barrett, 2013). Early surveys of style morph ratios in the European range revealed that most populations were tristylous, although some deviations from isoplethy and occasional dimorphic and monomorphic populations were reported (*e.g*., Haldane, 1936; Schoch-Bodmer, 1938; Halkka and Halkka, 1974; Andersson, 1994; Ågren and Ericson, 1996; Eckert, *et al*., 1996b). In contrast, an extensive survey of 102 introduced populations in Ontario, Canada, revealed that 23% of populations were missing style morphs, and the patterns observed were consistent with those predicted by theoretical models of asymmetical morph loss in small populations (Eckert and Barrett, 1992). Morph loss consistent with stochastic processes was also reported from colonizing populations of *L. salicaria* in Minnesota, U.S.A. (Anderson and Ascher, 1995). With the exception of the survey of morph ratios in France by Eckert *et al*. (1996b), all surveys in the native range of *L. salicaria* have been performed in central and northern Europe. Little is known about the reproductive biology of *L. salicaria* populations at the drier southern margins of the European range.

The Iberian Peninsula is the continental southwestern range limit for many native plant species in Europe, and is also a region of transition between the Eurosiberian and Mediterranean climates (Rivas-Martinéz *et al*., 2004). I therefore focused my sampling of morph ratios in *L. salicaria* on a north to south transect on the western side of the Iberian Peninsula, where the species is mostly abundant and distributed along a rainfall gradient. Because of the aquatic habit of *L. salicaria*, I predicted that this climatic gradient might influence the demography and distribution of populations with potential influences on variation in style morph ratios. My study addressed the following specific questions: (1) Are *L. salicaria* populations generally isoplethic and, if not, is there evidence of a consistent bias in morph frequencies or pattern of morph loss? I was interested in testing the hypothesis that deviations from isoplethy may be more common at the southern range limit. (2) What is the relation between population size and morph evenness? I hypothesized that stochastic forces would likely contribute towards greater variance in morph ratios in smaller than larger populations. (3) Is there geographical variation in population size and evenness? I predicted that because of deteriorating

conditions for a wetland plant along the climatic gradient from north to south, populations size and evenness would be positively correlated with latitude. (4) Are deviations from isoplethy associated with variation in the reproductive success of style morphs? Morph-specific differences in fruit and seed set have the potential to cause biased morph ratios in tristylous species. To address these questions, I measured style morph composition, evenness, and the size of 96 populations, and estimated reproductive fitness components of the style morphs in 19 populations distributed along the climatic gradient. My study is the first investigation of variation in style morph frequencies at the southern margin of the native range of *L. salicaria*. It therefore provides an opportunity to compare my results with earlier surveys in the native and introduced ranges, most of which focused on sampling populations in cooler and wetter climatic regimes.

MATERIALS AND METHODS

Study species

Lythrum salicaria is an insect-pollinated perennial herb that produces from one to several flowering shoots. Plants form easily identified clumps up to 1.0 m in diameter, but there is no evidence of extensive clonal propagation in the species (Velayos, 1997); thus, colonization and establishment occurs exclusively by seed (Yakimowski *et al*., 2005). Plants vary considerably in size throughout the species' geographical range, but in the region I sampled, they generally grow to 2.5 m in height and can produce hundreds (often thousands) of purple-pinkish flowers arranged in whorl-like cymes forming a terminal spike (Velayos, 1997). The species occurs in a wide range of wetland habitats including marshes, ditches, flooded fields and the edges of rivers and streams. It is distributed throughout much of Europe, from Fennoscandia to the Mediterranean, but also occurs in China and Japan, and has been introduced to various parts of the world, including New Zealand, South Africa and North America (reviewed in Mal *et al*., 1992).

Population surveys

To investigate variation in style morph frequencies I sampled 96 populations along a latitudinal transect from Galicia to Andalucia, extending through Spain and Portugal, spanning 7.61 degrees of latitude (43.68ºN – 36.08ºN; Fig. 6.1). The transect bisected two biogeographic zones in the Iberian Peninsula, the Eurosiberian to the north and the

Mediterranean from the center to the south of the region (Rivas-Martinéz *et al*., 2004). The zones exhibit distinct climates; for example, annual mean precipitation ranges from 996 mm $year^{-1}$ in La Coruña, Galicia, to 572 mm $year^{-1}$ in Sevilla, Andalucia (Rodriguez-Puebla *et al*., 1998). My sampling was conducted at peak flowering (July-August) in 2014. Populations occurred in a variety of habitats, including irrigation and roadside ditches, riverbanks, creek beds and freshwater marshes. For the purpose of my study, a population was considered to be a group of individuals bounded by anthropogenic or natural barriers and separated from the nearest other population by a minimum of 1 km, although this distance was much larger for the vast majority of surveyed populations.

Style morph frequencies were easily estimated by inspection of flowering ramets, *i.e.*, flowering shoots originating from the same rootstock. Flowering ramets were sampled every 2 m along transects across the population to avoid resampling of genets (Haldane, 1936). Where possible, at least 100 flowering individuals were surveyed, where populations were smaller all individuals were scored. I estimated population size by counts of the number of flowering and non-flowering individuals (not including seedlings) in each population. The vast majority of plants in populations were flowering at the time of sampling.

Fruit and seed production

During peak flowering, I tagged ~10 plants per style morph in 19 populations of *L. salicaria* distributed across the entire sampling area, and these were given a plastic label with a unique identification number. Later in the season, I returned to the population and randomly collected one infructescence on each marked plant for measurements of the following traits: number of scars on the infructescence (representing flowers that did not develop fruits), number of capsules, number of filled seeds for three randomly chosen indehiscent fruits per infructescence (hereafter seed production per fruit), and the presence/absence of fruit mining insects on each sampled plant. I later calculated fruit set per infructescence as the proportion of flowers developing into fruits by dividing the total number of capsules by the total number of flowers per inflorescence (*i.e.*, the sum of scars and capsules). I estimated the seed production per infructescence of each sampled plant by multiplying fruit set by average seed production per fruit.

Statistical analyses

To test for deviations from isoplethy, I used *G*-tests for goodness-of-fit and Yates correction for populations lacking a floral morph, *i.e.*, dimorphic populations (Zar, 2010). I used a De Finetti diagram to graphically illustrate the variation in style morph frequencies in my sample (see Barrett, 1993).

I calculated an index of evenness for each population as follows:

$$E = \frac{1 - (f(L)^2 + f(M)^2 + f(S)^2)}{0.6667}$$

where f (X) represents the frequency of the X-morph (L, M and S, for long-, mid- and short-styled morph, respectively). This index varies between 0 (monomorphic populations) and 1 (trimorphic populations with 1:1:1 morph ratios); for further details see Barrett *et al*. (1989).

To test whether there was a consistent bias in style morph frequencies among trimorphic populations across the sampled area, and to investigate the relation between the evenness index and: (1) population size, and (2) latitude for dimorphic and trimorphic populations, I used a GLM with a Gamma distribution and a log link function. To examine population size variation across the sampled area, I used two different approaches. First, I tested the relation between population size and latitude using a Pearson correlation. Second, I explored whether population size could be predicted by latitude using a linear model with a Gaussian distribution. Prior to analysis, I arcsine transformed the style morph frequency data, whereas population size was transformed with the $\log_{10}(x)$ (Zar, 2010).

I examined the relation between latitudinal variation and individual measures of reproductive fitness by means of GLMs with a Gamma distribution and a log link function. To investigate whether there were significant differences among populations and style morphs in fruit set and seed production per fruit and infructescence, I used GLMs with a Gamma distribution and a log link function. I tested for differences among style morphs within populations for all response variables using a Type-III analysis of variance or a Kruskal-Wallis test for Gaussian and non-Gaussian distributions, respectively, followed by post-hoc tests for multiple comparisons. I investigated latitudinal variation of fruit mining insects by means of a GLM with a

binomial distribution and a logit link function. Before statistical tests, I transformed fruit set with the asin(x) and seed production with the $\log_{10}(x+2)$ (Zar, 2010).

I performed all analyses with R software version 3.0.1 (R Core Development Team, 2013) using the following packages: "car" for Type-III analysis of variance (Fox and Weisberg, 2015), "effects" for evaluating each explanatory variable effect in the selected model (Fox, 2003), "multcomp" for multiple comparisons after Type-III analysis of variance (Hothorn *et al*., 2015), "pgirmess" for post-hoc tests after Kruskal-Wallis tests (Giraudoux, 2014), and "stats" for linear models and GLMs, Kruskal-Wallis tests and Pearson correlation (R Core Development Team, 2013).

RESULTS

Variation in style morph frequencies

The 96 populations of *L. salicaria* that I sampled varied in size from 2 to 1209 plants (mean ± SE, 144.48 ± 20.10; median 67). The frequencies of style morphs for all populations and their locality, size and evenness are given in Appendix 6.1. I also found isolated single individuals at five locations, but they are not considered further. The majority of populations that I sampled were trimorphic (94.79%, n = 91), but populations lacking one (4.17%, n = 4) or two (1.04%, n = 1) style morphs were also found (Figs. 6.1, 6.2). The mean frequencies (± SE) of the L-, M- and S-morphs across all 96 populations sampled were 0.35 (± 0.01), 0.34 (± 0.01), and 0.32 (± 0.01), respectively (Table 6.1); these ratios deviated significantly from the expected 1:1:1 equilibrium (G_{total} = 502.38, df = 186; G_{pooled} = 165.10, df = 2; both $P < 0.001$). I also calculated morph frequencies weighted by population size, but this did not change overall average morph frequencies (Table 6.1). Of the 96 populations sampled, 68.75% (n = 66) were isoplethic according to separate G-tests ($P > 0.05$) (Figs. 6.1, 6.2), but there was significant heterogeneity in morph frequencies among the total sample of populations (G_{het} = 337.29, df = 184, $P < 0.001$), with a consistent deficiency of the S-morph in anisoplethic trimorphic populations ($\chi^2_{2,73}$ = 12.51, P = 0.002) and also among all tristylous populations ($\chi^2_{2,270}$ = 7.78, P = 0.02).

The index of morph evenness (E) ranged from 0 to 1 among populations of *L. salicaria*, averaging 0.89 ± 0.02 (± SE). There was a positive relation between morph evenness and the logarithm of population size (GLM: estimate = 0.05, SE = 0.01, t =

4.11, $P < 0.05$), with greater variation in morph structure detected among smaller than larger populations (Fig. 6.3A; $\chi^2_{1,93} = 16.91$, $P < 0.05$).

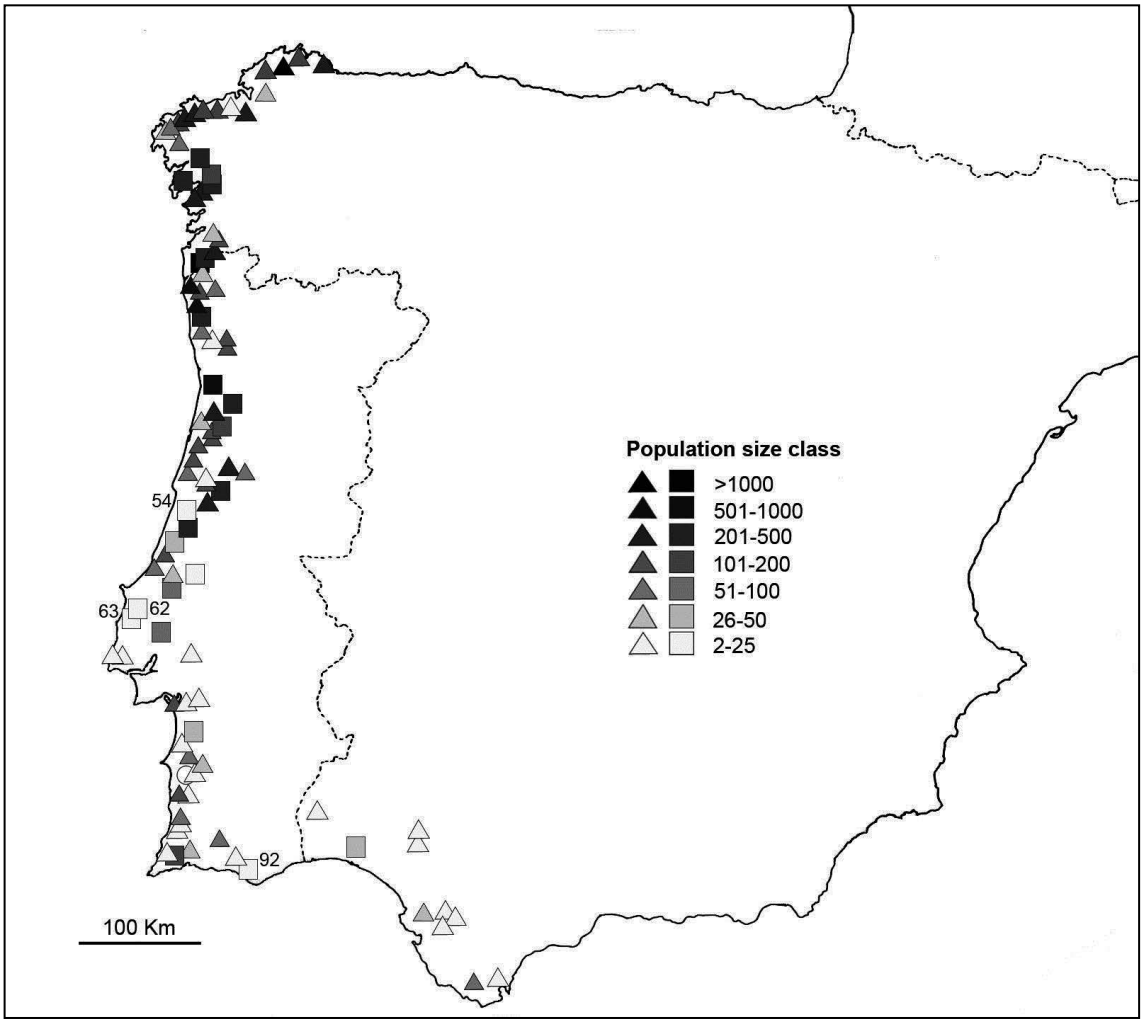


Figure 6.1. The geographical distribution of the 96 populations of *Lythrum salicaria* sampled in the Iberian Peninsula for this study. Triangles, squares and circles represent trimorphic, dimorphic and monomorphic populations, respectively. Shading of symbols indicates different population size classes (see key). The border between Spain and Portugal is indicated.

Geographical patterns

There was a positive correlation between the logarithm of population size and latitude ($r = 0.65$, $P < 0.001$; Fig. 6.4), with smaller populations more frequently occurring at the southern border of the range in the Iberian Peninsula. This effect was supported by the results of the linear model (GLM: estimate = 0.18, SE = 0.02, $t = 8.24$, $P < 0.05$), which detected a latitudinal effect on population size across the sampled area (latitude: $F_{1,94} = 67.94$, $P < 0.05$). Similarly, I found a positive relation between the

evenness index and latitude (Fig. 6.3B; GLM: estimate = 0.007, SE = 0.004, t = 1.981, P = 0.05), indicating that northern populations tended to approach isoplethy more frequently than southern populations ($F_{1,93}$ = 3.95, P = 0.049).

Table 6.1. Average style morph frequencies (± SE) for *Lythrum salicaria* populations sampled in the Iberian Peninsula. Population size was used to calculate average morph frequencies for populations weighted by their size.

	Style morph frequency (average ± SE)		
	L	**M**	**S**
(a) Average frequencies			
All populations	0.35 ± 0.01	0.34 ± 0.01	0.32 ± 0.01
Trimorphic populations	0.35 ± 0.01	0.34 ± 0.01	0.31 ± 0.01
(b) Weighted frequencies			
All populations	0.38 ± 5.24E^{-4}	0.33 ± 5.29E^{-4}	0.29 ± 6.95E^{-4}
Trimorphic populations	0.38 ± 5.15E^{-4}	0.33 ± 6.30E^{-4}	0.29 ± 6.76E^{-4}

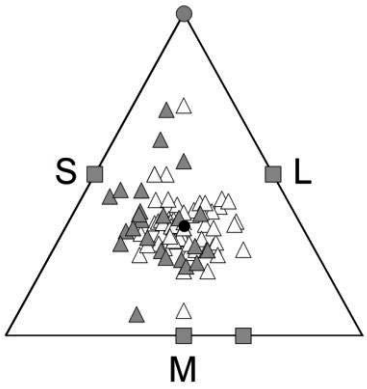


Figure 6.2. De Finetti diagram of style morph frequencies in 96 *Lythrum salicaria* populations in the Iberian Peninsula. Triangles, squares and circles represent trimorphic, dimorphic and monomorphic populations, respectively. White and grey symbols correspond to isoplethic (n = 66) and anisoplethic populations (n = 30), respectively, based on G-tests (see *Materials and methods*). Each side of the triangle represents a style morph and each point in the triangle represents a sampled population. The distance from a given point to each side is proportional to the frequency of style morphs in the population, and an equidistant point to the three sides of the triangle represents the isoplethic equilibrium, which is indicated by the filled circle.

I found a positive relation between three fitness components and latitude (GLM: fruit set, estimate = 0.027, SE = 0.009, t = 2.973, P < 0.01; seed production per fruit, estimate = 0.028, SE = 0.003, t = 8.116, P < 0.001; seed production per infructescence, estimate = 0.051, SE = 0.006, t = 8.354, P < 0.001). Fruit set ($\chi^2_{1,353}$ = 8.84, P < 0.01), seed production per fruit ($\chi^2_{1,1063}$ = 65.88, P < 0.001) and seed production per infructescence ($\chi^2_{1,1063}$ = 69.78, P < 0.001) each declined significantly in more southerly

populations. The opposite pattern was evident for the presence of fruit mining insects (GLM: estimate = -0.138, SE = 0.031, t = -4.389, P < 0.001), with capsules being attacked more commonly in southern than northern populations ($\chi^2_{1,1063}$ = 19.26, P < 0.001).

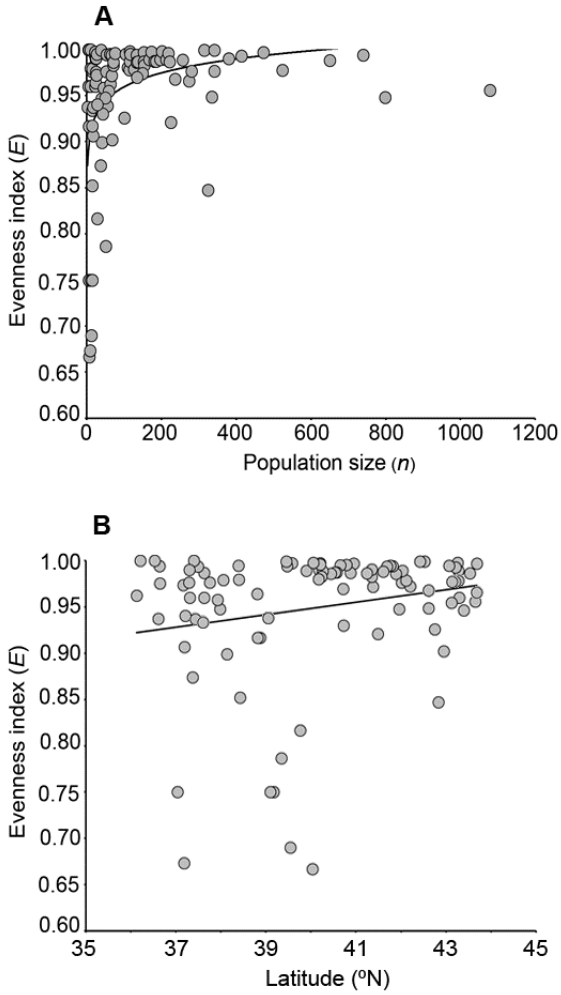


Figure 6.3. A. Relation between style morph evenness (E) and population size (n) among dimorphic and trimorphic populations of *Lythrum salicaria* in the Iberian Peninsula. The equation for this relation is: $E = 0.8589 + 0.0218 * \log_{10}(n)$. **B.** Relation between style morph evenness and latitude for the same sample of populations. The equation for this relation is: $E = 0.6801 + 0.0067 *$ *latitude*; $r^2_{adj} = 0.04$.

Comparisons of fitness components among populations and style morphs

There was a significant variation among the 19 *L. salicaria* populations in fruit set ($\chi^2_{18,336}$ = 111.24, P < 0.05), seed production per fruit ($\chi^2_{18,1046}$ = 106.83, P < 0.05), and seed set per infructescence ($\chi^2_{18,1046}$ = 286.82, P < 0.05). However, I detected no significant differences among style morphs in these traits (fruit set: $\chi^2_{2,352}$ = 0.07, P = 0.97; seed production per fruit: $\chi^2_{2,1062}$ = 2.12, P = 0.35; and seed production per

infructescence: $\chi^2_{2,1062} = 0.87$, $P = 0.65$; Fig. 6.5). Within populations (hereafter Pop), differences among style morphs in fruit set (Pop 28, $F_{2,27} = 6.76$, $P < 0.01$) and seed production per fruit (Pop 12, $H_2 = 8.30$, $P < 0.05$; Pop 34, $H_2 = 15.51$, $P < 0.001$; Pop 89, $H_2 = 7.94$, $P < 0.05$; Pop 100, $H_2 = 12.75$, $P < 0.01$) were occasionally found, but there was no consistent association with style morph across the populations sampled.

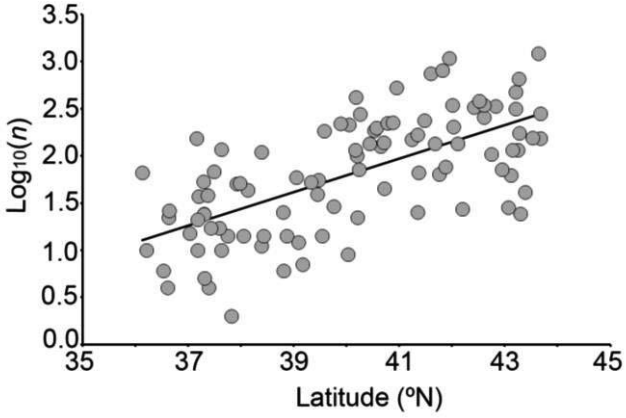


Figure 6.4. Relation between the logarithm of population size [$\log_{10}(n)$] and latitude for all populations of *Lythrum salicaria* in the Iberian Peninsula that were sampled in this study. The equation for this relation is: $\log_{10}(n) = -5.3005 + 0.1773^*$ *latitude*; $r^2_{adj} = 0.41$.

DISCUSSION

My survey of style morph ratios in populations of *L. salicaria* from the Iberian Peninsula revealed several main findings: (1) most populations were trimorphic and isoplethic, with a deficiency of the S-morph in trimorphic populations (Fig. 6.2); (2) there was a positive relation between population size and style morph evenness, with greater variation in morph ratios among smaller populations (Fig. 6.3A); (3) throughout the region I sampled, both population size and style morph evenness decreased from north to south (Figs. 6.3B, 6.4); (4) despite significant variation among populations in reproductive fitness components, there were no consistent differences among style morphs within populations (Fig. 6.5). Below I discuss the ecological and genetic mechanisms that could account for these patterns and compare my results with previous surveys of style morph ratios in native and introduced populations.

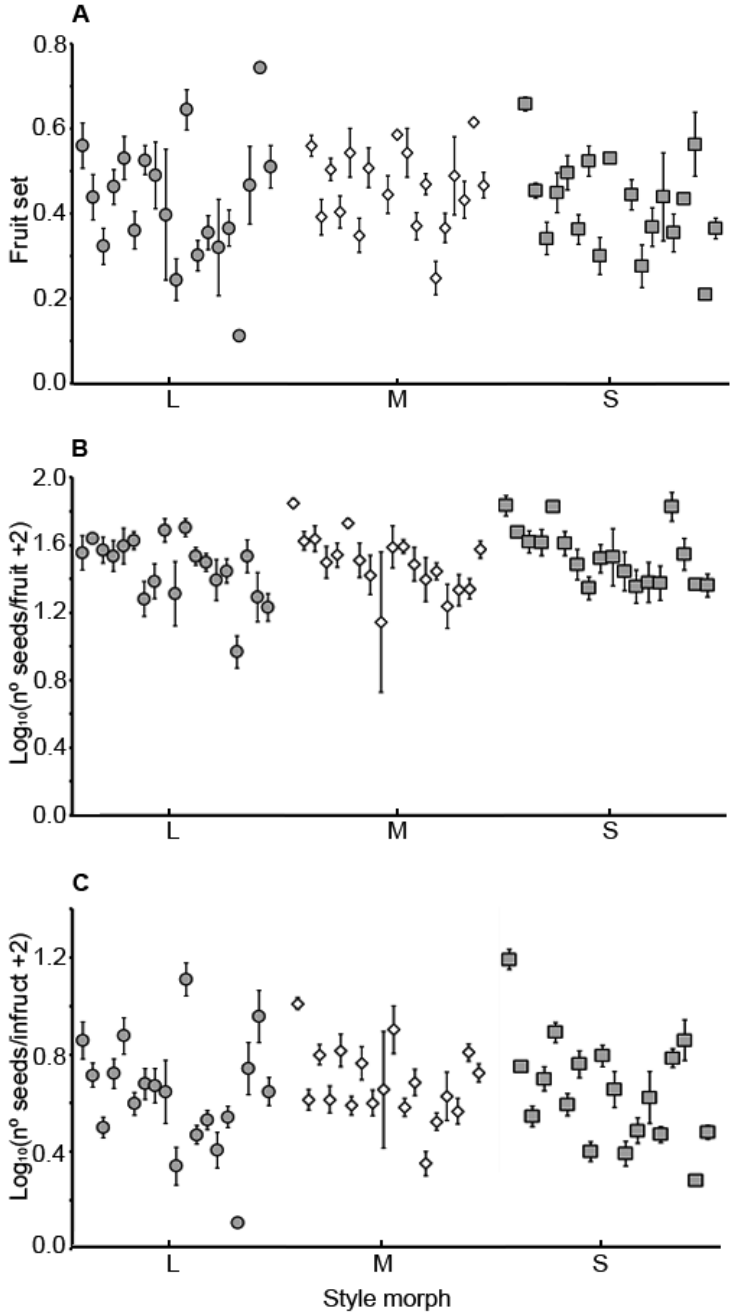


Figure 6.5. Reproductive fitness components in 19 populations of *Lythrum salicaria* in the Iberian Peninsula grouped by style morph. **A.** Fruit set. **B.** Seed production per fruit; **C.** Seed production per infructescence. For details of data transformation see *Materials and methods*. Data presented is the mean and SE for each population. Closed circles, open diamonds and closed squares represent the L, M, and S-morphs, respectively.

Maintenance of stylar trimorphism in the Iberian Peninsula

The results of my survey suggest that tristyly is likely to be maintained in most populations of *L. salicaria* that occur in the Iberian Peninsula, as long as they are of sufficient size. Despite the frequent occurrence of small populations with lower

evenness values at the southern margin of the species' range, only five of the 96 populations I sampled were missing style morphs. All dimorphic and monomorphic populations contained less than 15 individuals. In a survey of style morph ratios of *L. salicaria* populations in France, only five of 102 populations were missing style morphs and all contained fewer that eight plants (Eckert *et al*., 1996b). These associations between small population size and style morph absence are consistent with the role of genetic drift in causing morph loss.

Several features of *L. salicaria* may contribute to limiting morph loss from populations in comparison with several other tristylous species. Populations of this relatively long-lived perennial plant exhibit high year-to-year survival, overlapping generations and usually do not exhibit dramatic population size fluctuations (Eckert *et al*., 1996a). This contrasts with annual *Eichhornia paniculata*, in which population size fluctuations are commonly associated with stochastic morph loss (Husband and Barrett, 1992, 1998), and with clonal *Decodon verticillatus*, *Eichhornia crassipes* and *Oxalis* species, in which founder events are a common cause of biased morph frequencies and non-trimorphic population structure (Ornduff, 1972; Barrett and Forno, 1982; Eckert and Barrett, 1992; Castro *et al*., 2013). Frequent gene flow via pollen among neighbouring populations of *L. salicaria* may be mediated by butterflies and bumblebees, which are common pollinators of the species and capable of long flight distances (*e.g*., up to 2.2 km reported for bumblebees; Kreyer *et al*., 2004). Also, *L. salicaria* produces copious amounts of tiny seed (~1 mm; Velayos, 1997) that are easily dispersed in water, or by human agents because populations frequently occur in anthropogenically disturbed habitats, such as roadside ditches. Finally, computer simulations and empirical surveys of tristylous species indicate that the susceptibility to morph loss of populations is strongly influenced by whether a tristylous species is able to self-fertilize (reviewed in Barrett, 1993). For example, the loss of style morphs commonly occurs in the self-compatible *E. paniculata* (Husband and Barrett, 1992), a pattern consistent with models allowing for selfing in tristylous populations (Barrett *et al*., 1989; Eckert and Barrett, 1992). In contrast, trimorphic incompatibility in *L. salicaria* serves to stabilize tristyly by enforcing outcrossing through disassortative mating. Thus, in general, tristylous species that possess trimorphic incompatibility should be more resilient to stochastic morph loss than those that are self-compatible.

The extent to which gene flow is important in maintaining tristyly in small populations of *L. salicaria* remains unclear. Frequent gene flow was invoked to account

for the maintenance of tristyly in small island (Halkka and Halkka, 1974) and lake edge (Andersson, 1994) populations in Scandinavia. Similarly, based on a metapopulation model with gene flow, and a dataset showing a high frequency of tristyly in French populations (22 of 27 populations, $n \leq 25$ plants), Eckert *et al*. (1996b) suggested that gene flow was probably sufficient to maintain tristyly in small populations. The agricultural landscapes of the region of France they sampled seem likely to have promoted genetic connectivity among populations. Similar arguments could be applied to the populations I sampled in the Iberian Peninsula, as the majority occurred in agricultural landscapes traversed by roads, ditches, and drainage canals contributing to connectivity among populations. However, it is important to note that theoretical studies of the influence of finite population size on the maintenance of tristyly in the absence of gene flow indicate that the tristyly can remain stable for up to 150 generations if population sizes are above 20 (Heuch, 1980). Therefore, even without recurrent gene flow, tristyly can be maintained for many generations in small populations, although for those that I sampled with less than 15 individuals (n = 18 populations) future stochastic morph loss is likely, unless population growth and/or gene flow with neighbouring populations occurs.

Stochastic morph loss from tristylous populations should give rise to a characteristic signature of style morph representation in dimorphic populations. This is because the alleles governing tristyly differ in their frequency in equilibrium populations (S = 0.085, M = 0.151, m = 0.849; Heuch, 1980), and are therefore differentially vulnerable to loss through drift and founder events. Populations missing the S-morph (L-M dimorphic) should occur more commonly than L-S and M-S populations. However, the number of dimorphic populations in my sample was too small (n = 4) to discern any pattern. This result differs from the patterns of style morph variation in Ontario, Canada. Two independent surveys conducted 25 years apart indicate a much higher frequency of morph absence among populations (Eckert and Barrett, 1992 – 23%, n = 102; Balogh and Barrett, 2016 – 26%, n = 114), with the pattern of stylar dimorphism predicted by genetic drift and frequent founder events. These contrasting results point to fundamental differences in the intensity and type of stochastic processes operating in native versus introduced populations of *L. salicaria*.

The S-morph was significantly under-represented in my sample of trimorphic populations from the Iberian Peninsula (mean frequency: L-morph = 0.35, M-morph = 0.34, S-morph = 0.31; n = 91 populations). Other European surveys have often,

although not exclusively, found a similar pattern of S-morph deficiency in tristylous populations (*e.g.*, France: Eckert *et al.*, 1996b; Sweden: Andersson, 1994; Ågren and Erickson, 1996; other examples reviewed in Heuch, 1979a) raising the question of what mechanism(s) are responsible for this small but significant bias. The two most likely hypotheses to account for the lower frequency of the S-morph in trimorphic populations are morph-specific fitness differences and stochastic processes operating in sub-structured populations.

Compatible crosses among the style morphs of *L. salicaria* have demonstrated reduced seed set in the S- compared to the L- and M-morphs (*e.g.*, Darwin, 1877; Barlow, 1913; Anderson and Ascher, 2000). However, there is no evidence that this pattern translates into consistent differences in fertility among style morphs under field conditions. Indeed, my comparison of reproductive traits in 19 populations of *L. salicaria* failed to detect any consistent differences among the morphs in fitness components. Elsewhere, Ågren and Ericson (1996) found that the L-morph had the lowest fertility in Swedish populations, but they concluded that inherent differences among style morphs in reproductive success were unlikely to cause the anisoplethic morph ratios they reported. Therefore, given my failure to demonstrate differences among the morphs in fruit and seed set in my survey, I am doubtful whether the deficiency of the S-morph in comparison with isoplethic expectations is associated with variation in maternal fertility among the morphs. However, other fitness components (*e.g.*, low germination of seeds produced by the S-morph; Nicholls, 1987) cannot be entirely ruled out. Finally, variation in the expression of trimorphic incompatibility has been reported in *L. salicaria* (reviewed in Colautti *et al.*, 2010) and could conceivably influence morph ratios. Yet, Heuch (1979b) investigated this problem theoretically and concluded that the observed frequencies in European populations were unlikely to be explained by unequal rates of self-fertilization among the style morphs owing to variation in the expression of trimorphic incompatibility.

Another potential cause of S-morph deficiency in tristylous populations concerns stochastic processes and population structure. Although, genetic drift in spatially homogeneous, finite populations should not cause a lower average frequency of any particular style morph (see Fig. 1 in Eckert and Barrett, 1992), if populations are spatially structured into demes the same processes that operate in finite populations could occur at a local spatial scale resulting in the loss of the S-morph in some demes and not others (Heuch, 1980). Averaging across all demes would then result in a

deficiency of the S-morph at the population level, especially where seed and pollen flow are spatially restricted. For reasons discussed earlier, it seems unlikely that seed and pollen flow would be sufficiently restricted within most *L. salicaria* populations I sampled to foster the type of population structure required for this process to operate. However, in very large populations this mechanism could potentially occur. Based on the range of population sizes encountered in my sample, I am doubtful that the S-morph deficiency I report has arisen in this manner.

Geographical patterns of population size and evenness

As predicted, I detected geographical gradients in both the size and style morph evenness of *L. salicaria* populations in the Iberian Peninsula. *Lythrum salicaria* is a wetland plant and water availability is therefore expected to strongly influence its distribution and population size. The Mediterranean climate in the south of the Iberian Peninsula is characterized by strong seasonality in rainfall and hot, dry summers. This contrasts with the Atlantic influence in the north, where cooler temperatures and higher summer rainfall occurs (Rodriguez-Puebla *et al*., 1998; Gasith and Resh, 1999; Kottek *et al*., 2006). This climatic gradient influences the availability, distribution and size of wetland habitats suitable for the persistence of *L. salicaria.* The more stressful growing conditions at the southwestern range limit had demographic consequences in terms of population size and spatial isolation. Smaller populations of *L. salicaria* also displayed greater variation in style morph evenness than larger populations (Fig. 6.3A), a pattern reported in several other tristylous species (*e.g*., Weller, 1986; Husband and Barrett, 1992; Barrett and Arroyo, 2012; Cunha *et al*., 2014). A variety of ecological and demographic factors affecting sexual reproduction in geographically marginal populations of *L. salicaria* have the potential to slow progress to the isoplethic equilibrium. The greater variance in style morph frequencies may therefore, in part, reflect historical contingency associated with founding genotypes and non-equilibrium conditions.

Several of my findings are consistent with predictions of the abundant-centre distribution theory (Sagarin *et al*., 2006). Populations at the southwestern range margin tended to be smaller in size, produced fewer fruits and seeds, and were more likely to be attacked by fruit mining insects. These effects may influence the reproductive rate of populations. My study cannot be considered a formal test of the abundant-centre distribution theory, because my population samples were restricted to the Iberian

Peninsula and did not include populations from the central and northern portions of the European range. Population sizes in the northern Iberian Peninsula are roughly comparable (population sizes, Northern Iberia > 40 ºN: mean = 273, median = 160; *n* = 36 populations) to those reported from the French survey (France: mean = 266, median = 80; *n* = 102 populations) conducted by Eckert *et al*. (1996b). In contrast, populations in Sweden (Ågren and Ericson, 1996) and Ontario, Canada (Eckert and Barrett, 1992) appear to be on average larger, probably reflecting the greater availability of wetland habitats for colonization and more suitable climatic conditions for population growth in these regions.

In conclusion, virtually all studies investigating patterns of genetic variation across species' ranges have measured polymorphism at marker genes experiencing little or no selection (reviewed in Eckert *et al*., 2008). In contrast, my study of tristyly examined geographical patterns of adaptive phenotypic variation maintained by negative frequency-dependent selection. In *L. salicaria*, both stochastic processes and features of life history affect the strength of selection on tristyly and therefore influence the time populations take to reach the isoplethic equilibrium (Eckert *et al*., 1996a). Notwithstanding the common occurrence of anisoplethy among the populations of *L. salicaria* I sampled, my data demonstrate the strong resilience of tristyly to the various forces that can cause dissolution of the polymorphism in other species. The association of tristyly with trimorphic incompatibility plays a key role in maintaining the polymorphism in most populations of the species.

Appendix 6.1. Style morph frequencies, population size (*n*), index of evenness (*E*) and *G-test* values for goodness-of-fit for sampled populations of *Lythrum salicaria* in the Iberian Peninsula, including five sites with isolated plants. * indicates populations with anisoplethic style morph ratios**.** Country: SP, Spain; PT: Portugal. # indicates that a Yates correction was applied (Zar, 2010) to dimorphic populations and a deviation from 1:1 was tested instead (see *Materials and methods*).

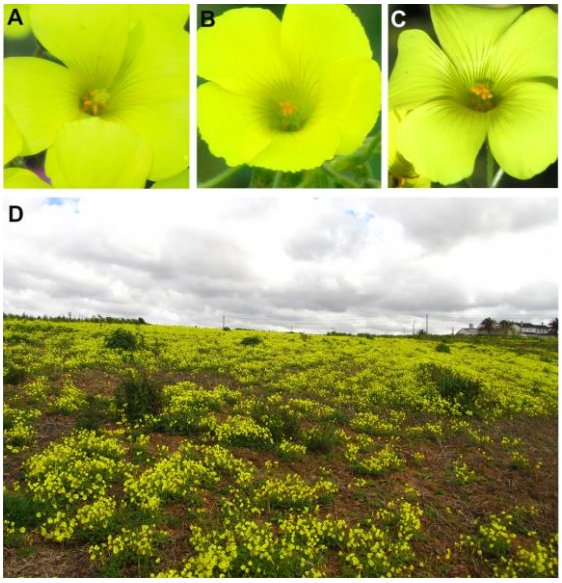
	Location	GPS coordinates		Morph frequency			Population size	Evenness index (*E*)	*G*
				L	M	S			
1	SP: Xove	43.68362	-7.50449	0.45	0.30	0.25	280	0.966	13.383 *
2	SP: As Naveiras	43.68175	-7.90164	0.36	0.30	0.35	153	0.997	2.614
3	SP: Casaldegonce	43.64389	-7.99294	0.44	0.36	0.20	1209	0.956	18.644 *
4	SP: Leiras	43.53273	-8.21420	0.35	0.26	0.39	154	0.987	4.069
5	SP: O Castelo	43.39537	-8.12260	0.33	0.20	0.47	41	0.947	3.894
6	SP: Igrexario	43.29637	-8.47444	0.20	0.40	0.40	24	0.960	1.612
7	SP: Buño	43.27735	-8.77726	0.40	0.29	0.30	173	0.989	4.165
8	SP: Brexo	43.26837	-8.31700	0.39	0.23	0.38	650	0.978	12.911 *
9	SP: A Laracha	43.25560	-8.58779	0.36	0.33	0.31	115	0.998	1.781
10	SP: A Caguenla	43.21034	-8.88350	0.43	0.26	0.31	314	0.977	7.730 *
11	SP: O Pinheiro	43.20725	-8.99782	0.36	0.36	0.28	473	0.993	4.126
12	SP: Magro	43.14995	-9.05804	0.31	0.26	0.43	115	0.978	7.660 *
13	SP: Mouzo	43.12179	-9.11702	0.46	0.32	0.22	62	0.955	4.525
14	SP: Anobres	43.06639	-9.18990	0.38	0.29	0.33	28	0.995	0.733
15	SP: Colúns	42.95143	-9.03213	0.23	0.54	0.23	71	0.902	9.725 *
16	SP: Brión	42.82859	-8.91565	0.49	0.43	0.08	335	0.847	59.145 *
17	SP: Pousada	42.75143	-8.60334	0.39	0.45	0.15	104	0.926	13.294 *
18	SP: Lixó	42.61176	-9.01056	0.43	0.39	0.18	341	0.948	15.199 *
19	SP: Caldas de Reis	42.61092	-8.64616	0.39	0.21	0.40	256	0.968	8.729 *
20	SP: Cabanelas	42.52223	-8.76380	0.35	0.33	0.33	380	0.999	3.471
21	SP: Pontes	42.41788	-8.85403	0.35	0.33	0.32	324	0.999	3.458
22	SP: A Gándara	42.20432	-8.60377	0.28	0.28	0.44	27	0.972	1.318
23	SP: O Barreiro	42.11425	-8.54670	0.41	0.35	0.24	133	0.979	4.240
24	PT: Valença do Minho	42.03419	-8.64481	0.27	0.34	0.39	201	0.989	3.912
25	SP: O Bouzón	42.01463	-8.70383	0.30	0.27	0.43	341	0.976	7.431 *
26	SP: Góian	41.95397	-8.75939	0.44	0.38	0.19	1079	0.948	29.575 *
27	PT: Vilar de Mouros	41.88560	-8.79078	0.36	0.26	0.38	76	0.986	1.905
28	PT: Vila Praia de Âncora	41.81686	-8.86865	0.38	0.30	0.32	798	0.994	3.786
29	PT: Barros	41.75671	-8.60294	0.29	0.38	0.33	63	0.995	1.466
30	PT: Mazarefes	41.68522	-8.76818	0.37	0.35	0.28	133	0.994	3.005

Cont.

	Location	GPS coordinates		L	M	S	*n*	*E*	*G*
31	PT: Foz do Neiva	41.60627	-8.79628	0.37	0.37	0.26	740	0.988	5.157
32	PT: Apúlia	41.48495	-8.75996	0.50	0.32	0.18	236	0.921	20.867 *
33	PT. Sobreposta	41.37368	-8.70234	0.27	0.29	0.44	66	0.972	3.336
34	PT: Lousado	41.34808	-8.52017	0.40	0.35	0.25	167	0.983	3.776
35	PT: Bragadela	41.34780	-8.56668	0.28	0.39	0.33	25	0.991	0.697
36	PT: Alfena	41.23981	-8.52496	0.33	0.27	0.40	148	0.987	3.521
37	PT: Água Longa	41.23134	-8.49175	0.00	0.00	1.00	1	0.000	-
38	PT: Esmoriz	40.95384	-8.63714	0.33	0.37	0.30	524	0.997	6.246 *
39	PT: S. Roque	40.87954	-8.47230	0.37	0.37	0.26	225	0.987	7.059 *
40	PT: Pardilhó	40.78546	-8.60724	0.35	0.36	0.29	221	0.996	3.348
41	PT: Torreira	40.72208	-8.69936	0.30	0.50	0.20	45	0.930	4.740
42	PT: Fermelã	40.71159	-8.56837	0.35	0.23	0.42	137	0.970	9.223 *
43	PT: Vale de Mouro	40.64976	-8.55454	0.36	0.35	0.29	126	0.995	2.826
44	PT: Mamodeiro	40.57132	-8.56459	0.32	0.40	0.28	197	0.987	5.671
45	PT: Boco	40.53392	-8.66669	0.41	0.32	0.28	185	0.987	4.989
46	PT: Lagoa de Mira	40.44324	-8.75238	0.38	0.36	0.26	134	0.986	3.809
47	PT: S. João do Campo	40.25869	-8.49936	0.38	0.35	0.26	274	0.989	5.098
48	PT: Camarção	40.23775	-8.79078	0.42	0.27	0.31	71	0.983	2.950
49	PT: Gatões	40.20882	-8.70991	0.35	0.30	0.35	22	0.997	0.504
50	PT: Casal da Misarela	40.20595	-8.35882	0.37	0.34	0.29	100	0.996	1.931
51	PT: Arzila	40.17887	-8.55530	0.33	0.28	0.39	414	0.990	6.955 *
52	PT: Monte Sta Olaia	40.17140	-8.71452	0.25	0.41	0.34	114	0.980	4.091
53	PT: Soure	40.05077	-8.63152	0.36	0.34	0.30	213	0.998	3.345
54	PT: Marinha das Ondas	40.03589	-8.84136	0.33	0.00	0.67	9	0.667	0.823 #
55	PT: Aroeira	39.89347	-8.84775	0.26	0.38	0.36	218	0.989	6.426 *
56	PT: S. Pedro de Moel	39.76444	-8.99804	0.26	0.61	0.13	29	0.817	8.752 *
57	PT: Casal Mota	39.57980	-9.06426	0.36	0.30	0.34	181	0.997	3.017
58	PT: Chiqueda	39.54350	-8.95338	0.20	0.70	0.10	14	0.690	6.137 *
59	PT: Salir do Porto	39.47370	-9.14264	0.34	0.29	0.37	55	0.994	1.110
60	PT: Santana	39.45541	-9.06273	0.32	0.35	0.32	39	0.999	0.797
61	PT: A-Da-Gorda	39.34590	-9.17526	0.60	0.07	0.33	52	0.787	22.988 *
62	PT: Porto Novo	39.16950	-9.35742	0.50	0.50	0.00	7	0.750	0.000 #
63	PT: Casalinhos de Alfaiata	39.09509	-9.34577	0.00	0.50	0.50	12	0.750	0.000 #
64	PT: Casais Novos	39.05306	-8.96424	0.48	0.33	0.19	59	0.939	6.157 *
65	PT: Sto Estevão	38.87167	-8.68338	0.33	0.50	0.17	14	0.917	2.334

66	PT: Rodízio	38.81753	-9.46598	0.33	0.50	0.17	6	0.917	1.167
67	PT: Ribeira de Sintra	38.80755	-9.40075	0.37	0.42	0.21	25	0.964	1.844
68	PT: Alberge	38.43062	-8.52295	0.46	0.08	0.46	14	0.852	5.139
69	PT: Alcácer do Sal	38.39703	-8.60674	0.43	0.29	0.29	11	0.980	0.415
70	PT: Comporta	38.39473	-8.75344	0.31	0.38	0.31	109	0.995	1.675
71	PT: Grândola	38.13791	-8.59138	0.54	0.29	0.18	43	0.899	6.089 *
72	PT: Santa Cruz	38.05311	-8.70583	0.25	0.33	0.42	14	0.979	0.747
73	PT: Santiago do Cacém	37.98586	-8.65215	0.18	0.42	0.39	51	0.948	4.486
74	PT: S. Domingos	37.92848	-8.54241	0.28	0.25	0.47	50	0.958	3.210
75	PT: Cercal	37.81877	-8.68327	0.00	1.00	0.00	2	0.000	-
76	PT: Trajanitos	37.75052	-8.65944	0.23	0.38	0.38	14	0.976	0.917
77	PT: V. N. Mil Fontes	37.63163	-8.74681	0.26	0.38	0.36	116	0.987	3.783
78	PT: Rio Mira	37.62901	-8.72304	0.40	0.20	0.40	10	0.960	1.075
79	SP: Rivera de la Viguera	37.59227	-7.30377	0.18	0.35	0.47	17	0.934	2.729
80	PT: S. Teotónio	37.49387	-8.72130	0.38	0.29	0.32	68	0.994	1.091
81	PT: Ribeira de Seixe	37.42916	-8.75599	0.17	0.42	0.42	17	0.937	1.931
82	SP: Arroyo Ardachón	37.39657	-6.25620	0.33	0.33	0.33	4	1.000	0.060
83	SP: Gibraléon	37.37230	-6.96457	0.45	0.45	0.10	38	0.874	10.209 *
84	PT: Ribeira da Cerca	37.31025	-8.78585	0.40	0.20	0.40	5	0.960	0.537
85	SP: Río Guadiamar	37.30492	-6.26505	0.30	0.30	0.40	24	0.990	0.395
86	PT: Ribeira de Arade	37.29046	-8.28890	0.33	0.42	0.24	53	0.976	3.064
87	PT: Ribeira de Odelouca	37.20855	-8.51233	0.19	0.34	0.47	37	0.940	4.644
88	PT: Ribeira da Carrapateira	37.18872	-8.89083	0.20	0.27	0.53	21	0.907	2.971
89	PT: Ribeira de Algibre	37.18677	-8.08476	0.14	0.71	0.14	10	0.673	4.373
90	PT: Cotifo	37.17235	-8.69471	0.00	1.00	0.00	1	0.000	-
91	PT: Vilarinha	37.16876	-8.85485	0.43	0.24	0.33	153	0.974	7.799 *
92	PT: Faro	37.03501	-7.98030	0.50	0.00	0.50	15	0.750	0.000 #
93	SP: Río Guadalete	36.64366	-6.05143	0.24	0.41	0.35	26	0.976	1.199
94	SP: Arroyo de la Suara	36.63637	-5.92085	0.38	0.31	0.31	22	0.994	0.4116
95	SP: El Portal	36.63280	-6.12936	1.00	0.00	0.00	1	0.000	-
96	SP: Arroyo de los Toreros	36.61046	-5.80230	0.50	0.25	0.25	4	0.937	0.551
97	SP: Arroyo del Perdiguero	36.53082	-5.91323	0.33	0.33	0.33	6	1.000	0.060
98	SP: Río Barbate	36.26448	-5.95848	0.00	0.00	1.00	1	0.000	-
99	SP: Taraguilla	36.21598	-5.44509	0.33	0.33	0.33	10	1.000	0.060
100	SP: Facinas	36.13174	-5.69678	0.46	0.28	0.26	66	0.962	3.577
101	SP: Río de los Molinos	36.07621	-5.63196	1.00	0.00	0.00	1	0.000	-

Chapter 7 – Variation in the incompatibility reactions in tristylous *Oxalis pes-caprae*: large-scale screening in South African native and Mediterranean basin invasive populations



Oxalis pes-caprae. **A.** L-morph. **B.** M-morph. **C.** S-morph. **D.** Invasive population in Portugal.

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ABSTRACT

Establishment and spread of organisms after long-distance dispersal often involve changes to the mating systems. Here, I test for increased compatibility under a mate limitation scenario after long-distance dispersal of the tristylous *Oxalis pes-caprae*, a South African geophyte invasive in regions with Mediterranean climate. I investigated variation in the expression of the trimorphic incompatibility system in plants from 16 native populations covering the entire distribution range in South Africa, and 18 invasive populations from the western Mediterranean basin by performing controlled hand-pollinations. After compatible pollinations, native plants had higher fruit and seed set than invasives, while after incompatible pollinations (self- and intramorph pollinations), invasives were more successful than natives. I detected morph-specific differences in the weakening of the incompatibility system and within-population variability in the expression of the incompatibility reactions for invasive plants. Additionally, the offspring obtained after incompatible pollinations was able to germinate, survive and flower under glasshouse conditions. These results provide experimental evidence for differences in the expression of the incompatibility system between native and invasive populations of *O. pes-caprae* after introduction and under strong compatible mate's limitation. The weakening in the incompatibility system reported here for the L- and M-morphs might possibly constitute an additional strategy for reproductive assurance in the invaded range of the Mediterranean basin.

KEY WORDS: biological invasions; geographical patterns; mate limitation; reproductive assurance; trimorphic incompatibility; tristyly.

INTRODUCTION

Colonization after long-distance dispersal often involves the successful establishment of a new population founded by a limited number of individuals. After introduction into an unoccupied habitat by that species, colonizer individuals are subjected to a demographic sieve, *i.e*., the interplay between a reduced population size, mate limitation and reproductive mode, that will determine if single individuals or small colonies are able to successfully establish in the new environment (Baker, 1955, 1967; Stebbins, 1957; reviewed by Pannell *et al*., 2015). Species with mating systems that confer some level of reproductive assurance during colonization of novel habitats (*e.g*., Sutherland, 2004; Pyšek and Richardson, 2007; van Kleunen *et al*., 2010; Moravcová *et al*., 2015) are in advantage, as they will be able to persist and establish a new colony (Baker, 1955, 1967; Stebbins, 1957). Since the reproductive strategy of a species, among others, affects the genetic diversity and differentiation of its populations (Fryxell, 1957; Schoen and Brown, 1991; Hamrick and Godt, 1996), it strongly influences the capacity of colonizers to respond to novel and unpredictable environmental fluctuations, as well as, the opportunities for local adaptation after establishment (García-Ramos and Rodríguez, 2002; Barrett, 2011).

Reproductive assurance under mate limitation during colonization is guaranteed by the predominance of asexual over sexual strategies via apomixis (Amsellem *et al*., 2001) or clonality (*e.g*., Hollingsworth and Bailey, 2000; Wang *et al*., 2005; Ferrero *et al*., 2015), and by the transition from outcrossing to selfing (Petanidou *et al*., 2012; Ward *et al*., 2012). For example, some successful invaders have prolific vegetative reproduction (*e.g.*, *Carpobrotus edulis*, Roiloa *et al*., 2010; *Nymphoides peltata*, Wang *et al*., 2005; *Salvinia molesta*, Oliver, 1993), which has been involved in their successful establishment and spread in the non-native ranges. Also, several studies describing the mating system of invasive plant species in their non-native ranges provided evidence for the dominance of self-compatibility over self-incompatibility when invasive species were compared with species from the communities being invaded (Rambuda and Johnson, 2004; Sutherland, 2004; van Kleunen and Johnson, 2007; Harmon-Threatt *et al*., 2009; Hao *et al*., 2011). Increased self-compatibility was also shown to be higher in introduced naturalized species when compared with related non-naturalized ones (van Kleunen *et al*., 2008; but see Sutherland, 2004; Burns *et al*., 2011). To evaluate shifts in the mating systems during colonization, it is necessary to simultaneously compare the reproductive biology of a species in its native and

introduced areas (Barrett, 2011). However, this approach has been seldom explored and only a few studies have gathered data from both ranges (Petanidou *et al*., 2012; Ward *et al*., 2012).

The Bermuda buttercup (*Oxalis pes-caprae* L., Oxalidaceae) is a polyploid, highly clonal South-African geophyte (2*x*, 4*x* and 5*x* cytotypes; Ornduff, 1987; Castro *et al*., 2007, 2013; Turketti, 2010) that was introduced to regions with Mediterranean climate, where it became a widespread invasive (Michael, 1964; Ornduff, 1987; Vilà *et al*., 2006; Castro *et al*., 2007, 2013; Signorini *et al*., 2013). *Oxalis pes-caprae* is tristylous, and native populations are composed by the three style morphs (see Chapter 1: Fig. 1.1B) associated with a trimorphic self-incompatibility system (hereafter TSI; Ornduff, 1987; Turketti, 2010) that prevents self- and intramorph fertilizations (Barrett, 1993; Barrett and Shore, 2008). Contrarily, most invaded areas worldwide were colonized by the 5*x* S-morph, which successfully spread through asexual reproduction (Michael, 1964; Baker, 1965; Ornduff, 1987; Castro *et al*., 2007, 2013). In the Mediterranean basin, most populations are composed by the 5*x* S-morph only, and the occurrence of 4*x* plants of the three style morphs is quite restricted, particularly the 4*x* S-morph (Castro *et al*., 2013). The patterns of style morph composition of the populations in this area resulted from multiple introductions from the native range of all three style morphs (Ferrero *et al*., 2015). However, the timing of introduction of each style morph is unknown (Ferrero *et al*., 2015), thus raising several questions about the selective pressures that each style morph has been subjected to after its introduction and the mechanisms involved in the current structure of the populations.

The opportunities for sexual reproduction of *O. pes-caprae* in the Mediterranean basin are reduced because of compatible mate's limitation, which is caused by the under-representation of reciprocal style morphs (L- and M-morphs), and by the dominance of a predominantly asexual pentaploid cytotype across the entire region (Castro *et al*., 2007, 2013). Notwithstanding, *O. pes-caprae* effectively integrated the pollination networks (Ferrero *et al*., 2013) with successful pollen flow in invasive populations of the western Mediterranean, regardless of morph composition (Costa *et al*., 2016). In addition, preliminary observations pointed to a weakening in the incompatibility system under experimental conditions (Castro *et al*., 2013; Costa *et al*., 2014), and some seed production was reported in several invasive populations (Castro *et al*., 2013; Costa *et al*., 2016), although it remains unclear how frequent is the

recruitment from seed in this area. Altogether, the available data suggests the ocurrence of changes in the TSI of *O. pes-caprae* in the invaded area of the Mediterranean basin.

Here, I investigate geographical differences in the TSI between native and invasive populations of *O. pes-caprae*, which might suggest evolutionary transitions in the mating system during invasion. I addressed the following specific questions: (1) Do native and invasive populations differ in the strength of the TSI? I predicted that native populations would have a strong TSI, while invasive populations would show a weakening in the strength of the TSI. This prediction is based on preliminary observations of fruit and seed production after incompatible hand-pollinations (Castro *et al*., 2013; Costa *et al*., 2014), and of natural seed production under a scenario of strong compatible mate limitation (Castro *et al*., 2013; Costa *et al*., 2016). (2) Is there evidence of a style morph specific incompatibility weakening in the invaded area? Given the high sterility of the 5*x* S-morph, I hypothesized that some level of compatibility would be particularly advantageous for the 4*x* morphs as an additional mechanism for reproductive assurance. (3) Is the strength of the TSI variable among individual plants within native and invaded areas? I expected to detect natural variation in the strength of the incompatibility system. (4) Does the offspring sired after incompatible pollinations (*i.e.*, intramorph and self-pollinations) reach the adult stage? I hypothesized that the offspring sired after incompatible pollinations should reach the adult stage and flower in order to be able to contribute to the recruitment from seed in natural populations (even if its frequency is unknown). To answer these questions, I compared the strength of the TSI between native and invasive populations of *O. pes-caprae* by setting up a controlled hand-pollination experiment under common garden conditions with plants from 16 native and 18 invasive populations covering the entire native distribution range and the western Mediterranean region, respectively.

MATERIALS AND METHODS

Study species and populations

Oxalis pes-caprae is a geophyte that can grow up to 40 cm high (Pedraja, 2015), and has a great capacity of asexual reproduction via the production of numerous bulbs associated with the contractile capacities of its roots (Pütz, 1994). Every year, *O. pes-caprae* plants produce a basal rosette of leaves and yellow tristylous flowers arranged in terminal umbellate cymes (Pedraja, 2015). In the native range, it flowers from May to

August (Dreyer *et al*., 2006), while in the invaded area of the Mediterranean basin, flowering occurs from December to April (Castro *et al*., 2007).

Plants used in the controlled hand-pollinations were obtained by growing bulbs previously collected in South Africa (hereafter SA) and in the Mediterranean basin (hereafter MB) between 2010 and 2012. In the field, style morph composition was assessed in each population by recording the floral morph of at least 100 plants along 2-3 longitudinal transects across the entire population. Sampled plants were at least 5 m apart to avoid resampling the same genet (Castro *et al*., 2007, 2013). Data on style morph composition is provided in Table 7.1. Natural fruit production was assessed by sampling one infructescence from 10 plants per style morph in the populations (Table 7.1). Bulbs from 16 native populations (including 4*x* L-, 4*x* M- and 4*x* S-morphs) and 18 invasive populations (including 4*x* L-, 4*x* M- and 4*x* S-morphs and the 5*x* S-morph; Table 7.1) were planted in 2 L plastic pots (11 × 11 cm wide, 21 cm depth) filled with common garden substrate during the autumn 2010-2012. Plants were maintained outdoors under uniform conditions at the Botanical Garden of the University of Coimbra and were protected from pollinators with a nylon mesh.

Ploidy level assessment

The ploidy level of each plant used in the pollination experiment was confirmed by flow cytometric analyses of fresh leaves. Nuclei were isolated following the procedure of Galbraith *et al*. (1993) by chopping 1 cm^2 of leaf tissue of *O. pes-caprae* and 1 cm^2 of leaf tissue of *Solanum lycopersicum* 'Stupické' (internal reference standard with 2C = 1.96 pg; Doležel *et al*., 1992) in 1 mL of WPB buffer (Loureiro *et al*., 2007). The nuclear suspension was filtered using a 50 µm nylon mesh and 50 $\mu g.ml^{-1}$ of propidium iodide (PI, Fluka, Buchs, Switzerland) was added to stain the DNA. To avoid staining of double stranded RNA, 50 $\mu g.ml^{-1}$ of RNAse (Fluka, Buchs, Switzerland) was also added. Samples were analysed in a Partec CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW; Partec GmbH., Görlitz, Germany) for PI excitation. I followed the pooled sample strategy, and leaflets from 5 individuals were analysed simultaneously (Kolář *et al*., 2009); when several peaks were obtained, individual samples were prepared to assign the ploidy levels to each sampled plant. As a quality standard, I only considered histograms with a coefficient of variation below 5%. By dividing the *O. pes-caprae* G_1 peak mean by that of *S. lycopersicum*, I obtained the DNA index. For DNA index values (mean ± SD) of 0.70 ± 0.03, plants were scored as

tetraploids, while for values of 0.86 ± 0.02, plants were scored as pentaploids (Castro *et al*., 2007).

Table 7.1. *Oxalis pes-caprae* invasive and native populations studied, number of plants involved in the controlled pollination experiment, number of crosses performed, style morph frequency in natural populations, and natural fruit set. Detailed information on cytotype composition and fruit set for each sampled population from the invaded area is given in Castro *et al.* (2013). Data on fruit set from native range available from Ferrero *et al*. (2015), except the ones marked with an asterisk that correspond to unpublished data.

Population		Geographical coordinates		*n*		Style morph (%)				Fruit set (%)
				Plants	Crosses	L	M	S (4*x*)	S (5*x*)	
Native range: South Africa										
P1	ZA: Yzerfontein	-33.60528	18.23389	5	16	32	22	46	0	85.0
P4	ZA: Lamberts Bay	-32.32694	18.57333	10	27	28	26	46	0	84.0
P17	ZA: Worcester	-33.73639	19.92000	17	37	40	37	22	0	83.0
P19	ZA: Suurbraak	-34.18028	20.72944	5	18	53	16	32	0	47.5*
P21	ZA: Barrydale	-33.85194	21.31444	12	34	37	23	40	0	60.0
P22	ZA: Oudtshoorn	-33.76306	22.00333	8	18	39	56	5	0	84.0
P23	ZA: Mossel Bay	-34.26806	22.16111	11	26	20	51	29	0	83.8*
P24	ZA: Gouritsmond	-34.47861	21.91556	11	28	30	38	32	0	73.0
P25	ZA: Stilbaai	-34.41694	21.41750	4	16	21	41	38	0	57.1*
P26	ZA: Witsand	-34.28278	21.13778	11	33	24	41	35	0	50.5*
P27	ZA: Bredasdorp	-34.33583	20.25917	9	29	33	21	46	0	57.9*
P28	ZA: L'Agulhas	-34.79194	20.07167	11	49	24	24	52	0	61.0
P30	ZA: Standford	-34.55306	19.59611	5	18	36	3	60	0	77.3*
P31	ZA: Caledon	-34.43361	19.44417	13	48	8	11	81	0	40.9*
P32	ZA: Botrivier	-34.32778	-19.45944	9	35	26	70	4	0	63.0*
P33	ZA: Cape Point	-34.26472	18.51111	15	38	15	76	8	0	54.0
				156	**470**					

Invaded range: Western Mediterranean basin										
4.	SP: Baiona	42.30750	-8.99333	12	47	0	0	0	100	0.0
6.	PT: Belinhos	41.77889	-8.819444	8	23	100	0	0	0	0.4
7.	PT: Lavras	41.24060	-8.71452	8	26	0	0	0	100	0.0
8.	PT: São Pedro da Maceda	40.99000	-8.82361	17	39	22	0	0	78	9.9
9.	PT: Praia de Mira	40.52139	-8.97668	8	23	0	0	0	100	0.4
10.	PT: Coimbra	40.30083	-8.53639	13	57	0	0	0	100	7.2
11.	PT: Vieirinhos	40.04528	-8.89472	9	26	0	0	0	100	0.0
12.	PT: Marinha Grande	39.84528	-8.95083	15	40	9	0	0	91	15.8
16.	PT: Casais da Areia	39.23083	-9.64278	15	49	31	0	0	69	13.3
20.	PT: Colares I	38.80417	-9.48361	81	254	50	22	3	25	47.0
21.	PT: Colares II	39.02306	-9.62694	52	166	63	18	4	15	47.8
19.	PT: Colares III	39.00889	-9.57611	38	133	39	13	9	39	NA
26.	PT: Troia	38.62083	-9.00722	10	30	5	0	0	95	9.1
27.	PT: Melides	38.35083	-9.03361	12	45	0	0	0	69	7.4
28.	PT: Almograve	37.87917	-8.87222	13	39	0	0	0	90	10.4
31.	PT: Armação de Pêra	37.30444	-8.33917	15	48	0	0	0	81	4.3
32.	MA: Moulay-Bousselham	32.01722	-6.51417	13	36	31	0	0	69	0.2
39.	MA: Essaouira	31.68389	-9.92722	3	14	4	0	0	96	0.0
				342	**1095**					

Incompatibility system assessment

Controlled hand-pollinations were carried out between 2011 and 2014, during the winter months corresponding to the species flowering period (December-April) at the latitude of the experimental garden. Before conducting the pollination experiment, plants were grown for one generation to avoid potential maternal effects and, despite some differences in flowering phenology between ranges were detected, the flowering period of native and invasive plants largely overlapped. Details on the number of plants used per population and the total number of pollinations performed are given in Table 7.1. The following pollination treatments were performed (Fig. 7.1): intermorph pollinations (*i.e.*, compatible pollinations), intramorph and self-pollinations (*i.e.*, incompatible pollinations). An additional non-manipulated flower per plant was used as control for pollen contamination, and no fruit and seed production were obtained in this treatment. Each plant involved in the study received all pollination treatments; in some cases, with several replicates, while in other plants it was not possible to completely follow this approach due to limited pollen availability. Except for the selfing treatment, all recipient flowers were emasculated before hand-pollinations to avoid stigma contamination with self-pollen. Pollinations were done by gently rubbing anthers against the recipient stigmas using fine forceps. Intermorph and intramorph pollinations were performed using anthers from three distinct individuals from the same population. The pollination experiment is illustrated in detail in Fig. 7.1. However, due to mate limitation in monomorphic invasive populations, intermorph pollinations of the 5*x* S-morph were performed using reciprocal pollen donors (4*x* morphs) from other invasive populations. Compatible pollinations always involved 4*x* reciprocal floral morphs as pollen donors. Specifically, I cross-pollinated 4*x* L-morph flowers with pollen from long-level anthers of the 4*x* S- and 4*x* M-morphs, 4*x* M-morph flowers with pollen from mid-level anthers of the 4*x* L- and 4*x* S-morphs, and both 4*x* and 5*x* S-morph flowers with pollen from short-level anthers of the 4*x* L- and 4*x* M-morphs (Fig. 7.1A). For incompatible pollinations of the L- and S-morphs, I used pollen from the closest anther level, *i.e.*, the mid-level, while for the M-morph, pollen from long-level anthers was used (Fig. 7.1B). Pollination treatments were labeled with cotton lines of different colors laced around the flower pedicel. Since *O. pes-caprae* capsules are dehiscent, fruits were bagged to prevent seed losses, and fruit and seed production were recorded when mature. Fruit set per pollination treatment was calculated as the percentage of pollinated flowers that developed into fruits. Seed set per pollination treatment was

calculated as the mean number of seeds produced per fruit. A measure of reproductive success was calculated per individual and pollination treatment by multiplying fruit set by seed production.

Ten flowers from distinct individuals per floral morph and ploidy level were used to estimate the mean number of ovules, for native and invaded areas. Estimations were made under fluorescence microscopy following the procedure described in Dafni *et al*. (2005). The obtained values were then used to calculate several reproductive indices described below.

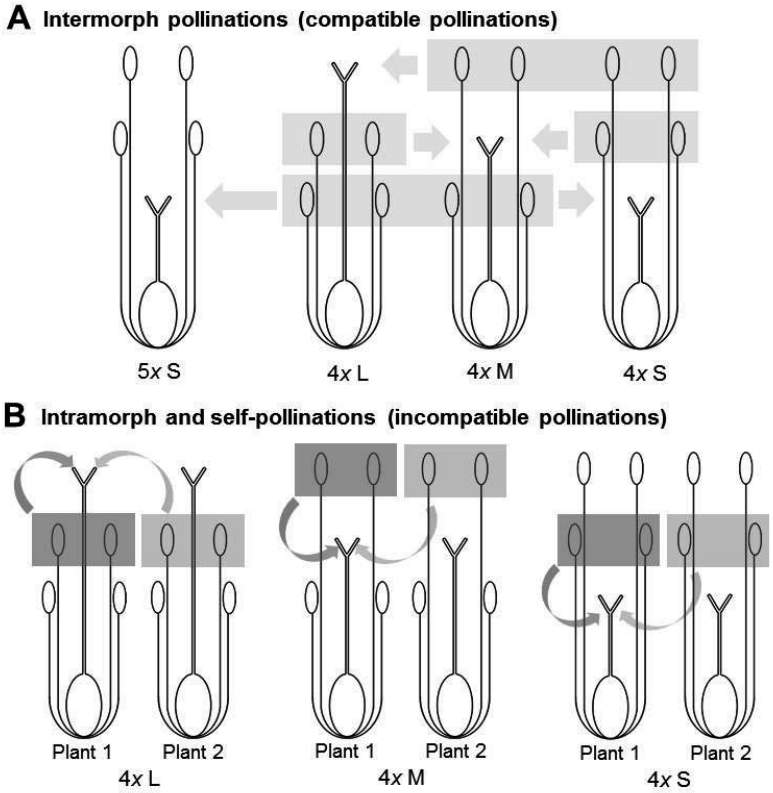


Fig. 7.1. Illustration of the experimental design. **A.** Intermorph pollinations, *i.e*., compatible pollinations, involved 4*x* reciprocal floral morphs as pollen donors with 4*x* L-morph flowers crossed with pollen from long-level anthers of the 4*x* S- and 4*x* M-morphs; 4*x* M-morph flowers crossed with pollen from mid-level anthers of the 4*x* L- and 4*x* S-morphs; and both 4*x* and 5*x* S-morph flowers crossed with pollen from short-level anthers of the 4*x* L- and 4*x* M-morphs. **B.** Intramorph and self-pollinations, *i.e*., incompatible pollinations, L- and S-morphs were pollinated with pollen from the closest anther level, *i.e*., the mid-level, while for the M-morph, pollen from long-level anthers was used (here exemplified for the 4*x* morphs, but the pollinations of the 5*x* S-morph followed the same methodology).

Reproductive indices

Outcrossing (OUT), morph-compatibility (MC) and self-compatibility (SC) measures were calculated. An outcrossing measure was obtained for each plant by calculating the

seed/ovule (S/O) ratio after compatible pollinations. Morph- and self-compatibility indices were obtained for each plant by dividing the S/O ratio of intramorph and self-pollinations, respectively, by the S/O ratio after intermorph pollinations (modified from Lloyd and Schoen, 1992). The compatibility indices varied between 0 (incompatible) and 1 (full compatible). Three criteria were established before the calculation of the indices: first, for individuals in which no compatible pollination was made, the average outcrossing S/O ratio for the population was used to calculate the compatibility indices; second, if the S/O ratio of self- and/or intramorph pollinations were larger than the S/O of compatible pollinations (resulting in indices larger than 1), the plant was scored as full compatible (*i.e.*, 1); lastly, when there were several replicates of a given treatment in one plant, average outcrossing, morph-compatibility and/or self-compatibility measures were calculated per individual and these averaged values were used in subsequent statistical analysis.

Fitness of illegitimate offspring

To evaluate if seeds obtained after incompatible crosses (*i.e.*, intramorph and self-pollinations) were able to germinate and produce viable plants, I studied the fitness of the offspring obtained after compatible and incompatible crosses of the invasive populations. Seeds obtained in the first season (2011-2012) were sown in the following season (2012-2013) in 1 L plastic pots (8.6 × 8.6 cm wide, 21 cm depth) filled with standard soil, one seed per pot. Germination was assessed weekly during the first two months and plant survival and flowering assessed in the peak flowering. The 4*x* S-morph plants did not sire any seeds after incompatible crosses and thus, these were not included in this experiment.

Statistical analyses

Since two ploidy levels are described for the S-morph in the invaded area of the Mediterranean basin (Castro *et al.*, 2007, 2013), style morph and cytotype were combined and designated as "form", as follows: 4*x* L-morph, 4*x* M-morph, 4*x* S-morph and 5*x* S-morph. Differences in fruit set, seed set and reproductive success among forms, pollination treatments and area (*i.e.*, native and invaded) and the interaction between the latter two were investigated by means of GLMMs or GLMs. In both cases, a binomial distribution with a logit link function was used for fruit set and a Poisson distribution with a log link function was used for seed set and reproductive success. In

all statistical analyses, individual and population variability were included in GLMMs as random factors. However, when the estimated variance for these factors was smaller than the residuals, they were removed from the analysis and GLMs were used instead (Bolker *et al*., 2009). Form, pollination treatment and area were specified as fixed effects in the models. Because the interaction between pollination treatment and area was significant, I explored the differences between areas for each pollination treatment and the differences within areas among pollination treatments separately, accounting for form in both, and following the procedure described above. When significant differences were obtained, post-hoc tests for multiple comparisons were conducted afterwards.

Before calculation of the reproductive measures, I assessed differences in ovule number between regions and among style morphs using one-way ANOVA Type-III analysis of variance. Since no differences in mean ovule number (species mean ± SE, 39.85 ± 0.87; invaded range: 39.60 ± 0.98, native range: 41.35 ± 1.72) between regions ($F_{1,135}$ = 0.500, P = 0.481), among style morphs ($F_{2,134}$ = 0.015, P = 0.985) and between cytotypes in the invaded area ($F_{2,114}$ = 0.528, P = 0.591) were found, I used the average value for the calculations. The values of the outcrossing measure and of the compatibility indices were transformed with the $\log_{10}(x+2)$ before all statistical analyses (Zar, 2010). Differences in the outcrossing measure and compatibility indices between areas and among forms within areas were investigated using GLMMs or GLMs, with a gamma distribution and a log link function, followed by a Type-III analysis of variance.

Differences in seed germination, plant survival and flowering among forms and between pollination treatments (*i.e*., compatible and incompatible pollinations), and the interaction between the two were investigated with GLMs. I also tested for (a) fitness differences among forms after each pollination treatment separately, and (b) compared offspring fitness for each form between pollination treatments. In all cases, a binomial distribution with a logit link function was used for the three response variables. Form and pollination treatment were specified as fixed factors. When significant differences were obtained, post-hoc tests for multiple comparisons were made afterwards.

All analyses were performed in R software version 3.0.1 (R Core Development Team, 2013), using the packages "car" for Type-III analysis of variance (Fox and Weisberg, 2015), "lme4" for GLMMs (Bates *et al*., 2014), "multcomp" for multiple

comparisons after Type-III analysis of variance (Hothorn *et al*., 2015), and "stats" for GLMs (R Core Development Team, 2013).

RESULTS

Effect of the pollination treatment in the reproductive success

Most pollination treatments produced fruits and seeds, even if in low amounts (Fig. 7.2). Overall, GLMMs analyses revealed a significant effect of pollination treatment in all reproductive variables measured (Fig. 7.2; Appendix 7.1). A more detailed analysis within area of origin equally showed a significant effect of pollination treatment in fruit production, seed set and reproductive success (Table 7.2). Native and invasive plants significantly produced more fruits and seeds after compatible than after incompatible pollinations ($P < 0.05$; Fig. 7.2A-D). Accordingly, reproductive success after intermorph crosses was significantly higher than after intramorph crosses and selfing for plants from both areas of origin ($P < 0.05$; Fig. 7.2E-F).

Strength of the incompatibility system in native and invaded ranges

Plants from South Africa produced significantly more fruits after compatible pollinations than plants from the Mediterranean basin (SA: 0.97 ± 0.01; MB: 0.82 ± 0.02; Table 7.3; Fig. 7.2A-B). The opposite pattern was found for intramorph pollinations (SA: 0.03 ± 0.02; MB: 0.19 ± 0.02) and for self-pollinations (SA: 0.04 ± 0.02; MB: 0.21 ± 0.03; Table 7.3; Fig. 7.2A-B), with invasive plants having higher fruit set than natives after incompatible pollinations. South African plants yielded, on average, more than twice the number of seeds produced by invasive plants after intermorph pollinations (SA: 18.73 ± 0.79; MB: 4*x* plants, 11.13 ± 0.52 and 5*x* S-morph, 3.95 ± 0.24). No statistically significant differences in seed production were detected between areas for intramorph and self-pollinations (Table 7.3; Fig. 7.2C-D).

Overall, native plants had a significantly higher reproductive success after compatible pollinations than invasive plants (SA: 18.14 ± 0.81; MB: 6.60 ± 0.33; Table 7.3; Fig. 7.2E-F). Contrarily, plants from the Mediterranean basin tended to have a higher reproductive success after intramorph (SA: 0.05 ± 0.02; MB: 0.71 ± 0.13) and self-pollinations than natives (SA: 0.48 ± 0.27; MB: 0.56 ± 0.121; Table 7.3; Fig. 7.2E-F).

The patterns of reproductive success described above led to a significant effect of area of origin in all reproductive measures calculated (Table 7.4). Plants from the native area had a significantly higher OUT measure than plants from the invaded area (SA: 0.58 ± 0.02; MB: 0.11 ± $1E^{-2}$; Fig. 7.3A), while the MC (SA: 0.002 ± $1E^{-2}$; MB: 0.09 ± 0.01; Fig. 7.3B) and SC (SA: 0.02 ± $1E^{-3}$; MB: 0.08 ± 0.01; Fig. 7.3C) indices were found to be significantly higher in plants from invasive populations than from native ones (Table 7.4).

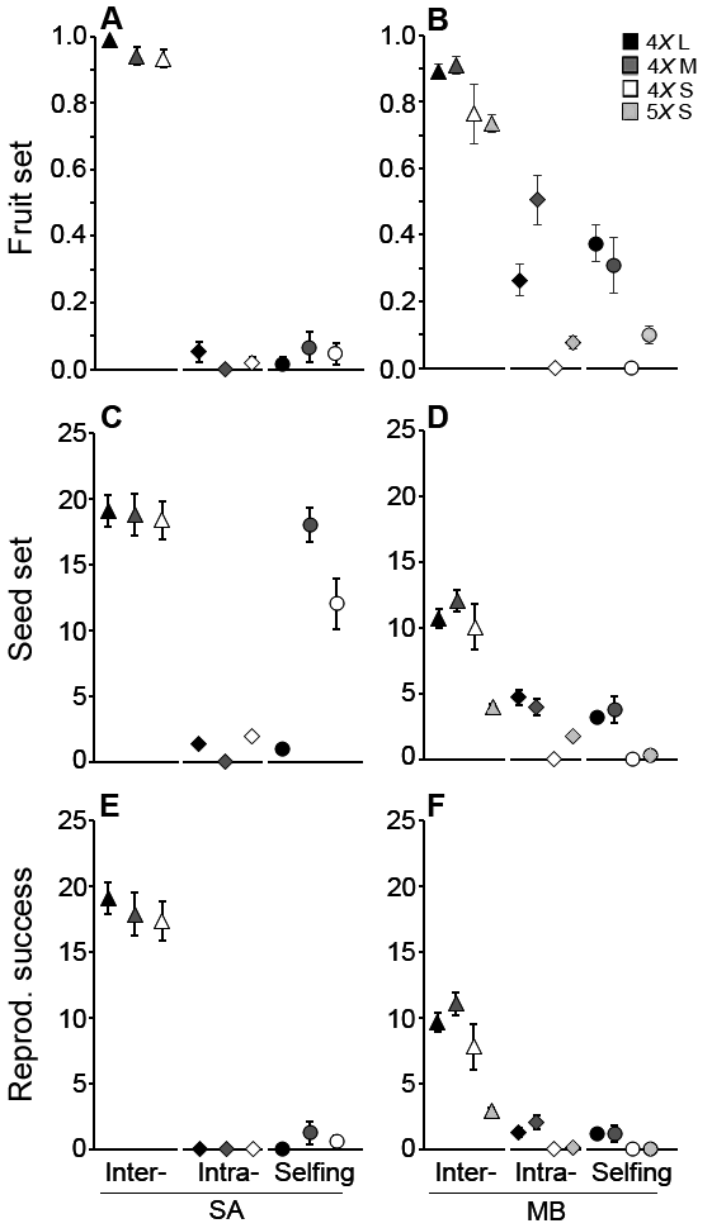


Figure. 7.2. Reproductive success of *Oxalis pes-caprae* plants from SA and the MB after hand-pollination treatments. **A-B.** Fruit set. **C-D.** Seed set. **E-F.** Reproductive success. Results are mean and SE. Triangles, diamonds and circles represent intermorph (inter-) pollinations, intramorph (intra-) pollinations and self-pollinations (selfing), respectively.

Table 7.2. GLM analyses of the effect of pollination treatment, *i.e*., intermorph, intramorph and self-pollination on the reproductive variables of *Oxalis pes-caprae* for each area separately. Fruit set was analyzed using GLMMs. Statistically significant differences are highlighted in bold.

		Native area			Invaded area	
Factors	**df**	χ^2	***P***	**df**	χ^2	***P***
(a) Fruit set						
Pollination treatment	2	11.56	**0.003**	2	320.68	**<0.001**
Form	2	0.01	0.996	3	32.21	**<0.001**
(b) Seed production						
Pollination treatment	2	74.36	**<0.001**	2	154.04	**<0.001**
Form	2	2.40	0.302	3	53.186	**<0.001**
(c) Total reproductive success						
Pollination treatment	2	573.40	**<0.001**	2	1059.64	**<0.001**
Form	2	0.97	0.616	3	52.74	**<0.001**

Table 7.3. GLMM analyses of the effect of area of origin and form on the reproductive variables of *Oxalis pes-caprae* measured after each pollination treatment separately, *i.e*., intermorph, intramorph and self-pollinations. '*' – a GLM was used instead. Statistically significant differences are highlighted in bold.

		Intermorph		Intramorph		Self	
Factors	**df**	χ^2	***P***	χ^2	***P***	χ^2	***P***
(a) Fruit set							
Area	1	12.563	**<0.001**	11.452	**<0.001**	20.650*	**<0.001**
Form	3	11.104	**0.010**	25.140	**<0.001**	21.124*	**<0.001**
(b) Seed production							
Area	1	420.390*	**<0.001**	2.037	0.153	3.596*	0.060
Form	3	633.150*	**<0.001**	7.922	**0.048**	14.161*	**<0.010**
(c) Total reproductive success							
Area	1	577.500*	**<0.001**	1.022	0.312	0.700	0.403
Form	3	890.360*	**<0.001**	2.163	0.539	2.609	0.456

Morph-specific differences

A significant effect of form, *i.e*., the combination of style morph and cytotype as 4*x* L-, 4*x* M-, 4*x* S- and 5*x* S-morph, was detected in almost all analyses conducted (Tables 7.2-7.5). While this factor had no effect in the comparisons within the native range for any of the response variables studied (Tables 7.3, 7.5; Figs 7.2, 7.3), differences among invasive forms were detected in reproductive variables across all pollination treatments (Table 7.2; Fig. 7.2) and reproductive indices (Table 7.4; Fig. 7.3), which were mostly driven by the reduced reproductive success of the 5*x* S- and 4*x* S-morph when compared with the other forms (Figs. 7.2, 7.3).

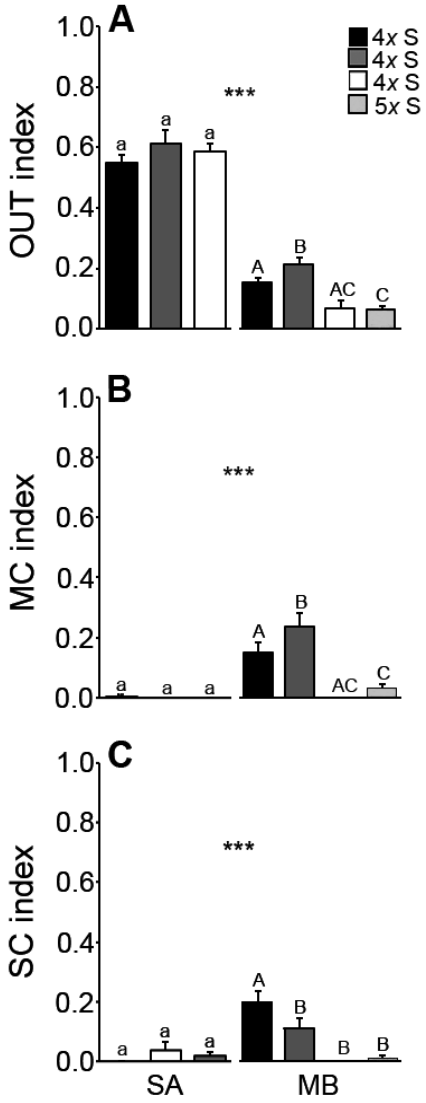


Figure 7.3. Compatibility indices of *Oxalis pes-caprae* plants from SA and the MB. **A.** Outcross (OUT) measure. **B.** Morph-compatibility (MC) index. **C.** Self-compatibility (SC) index. Results are mean and SE. Different upper and lower case reveal statistically significant differences among forms within native and invasive populations, respectively, at $P < 0.05$. Asterisks refer to statistically significant differences between areas at $P < 0.001$.

Individual variation in the expression of incompatibility

By plotting the frequency of the individuals with different levels of MC and SC indices, I could observe the variation in morph- and self-incompatibility within native and invasive populations (Fig. 7.4; Appendix 7.2). Within native populations, most individuals were self- and morph-incompatible with a few individuals presenting low levels of morph-compatibility (MC < 0.2) being occasionally detected. Surprisingly, two individuals exhibited considerably high values of self-compatibility (SC > 0.7) when compared with the population's SC averages, in all cases not exceeding 0.15.

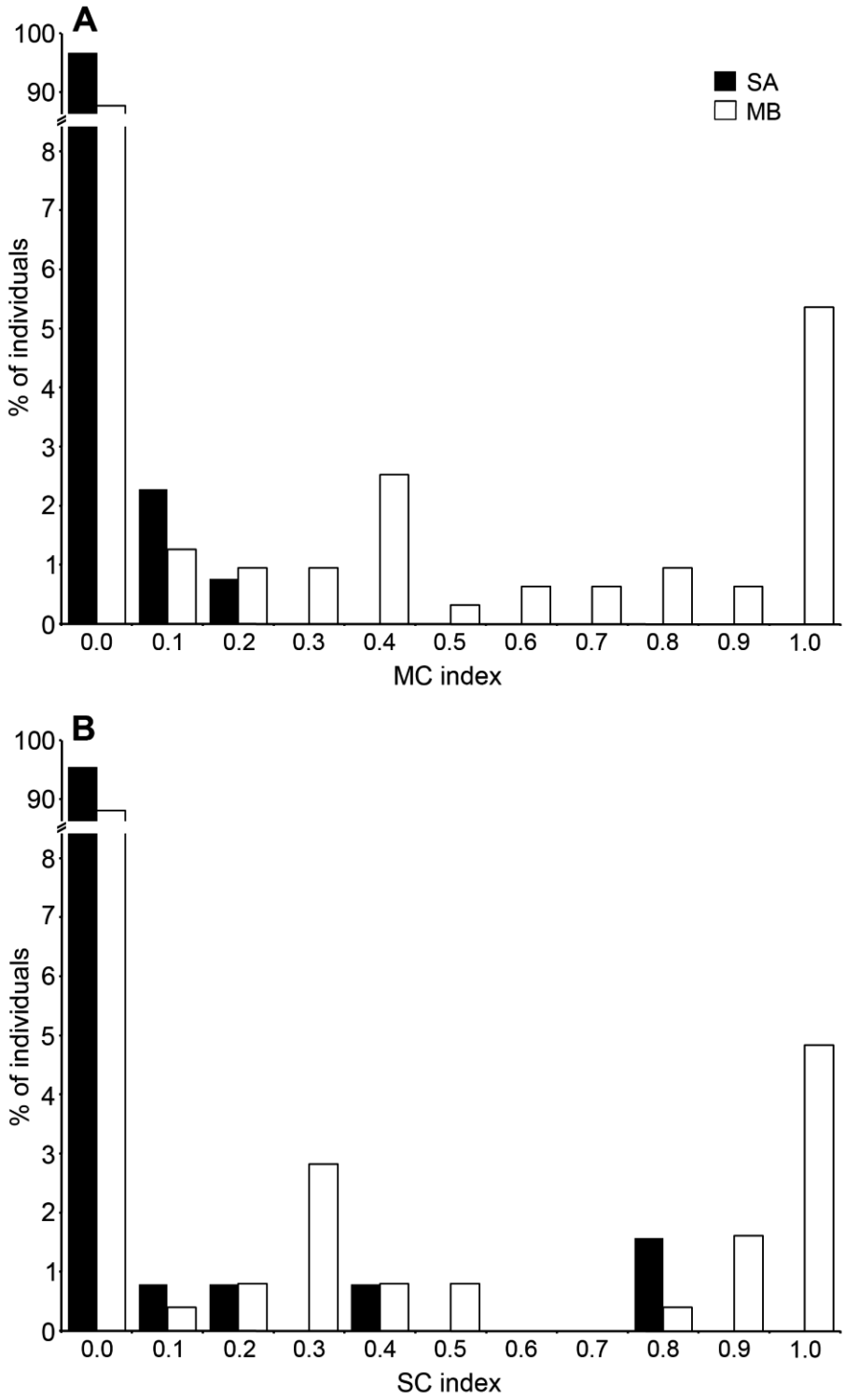


Figure 7.4. Frequency of individuals of *Oxalis pes-caprae* given as percentage (%) from SA and the MB with variable levels of trimorphic incompatibility. **A.** Morph-compatibility index. **B.** Self-compatibility index.

A higher variability in the expression of TSI was observed among invasive plants in comparison with natives. Despite most of the invasive individuals were self- and morph-incompatible, with population averages of MC and SC indices being always below 0.3, individuals spanning the full range of possible values were detected (Fig.

7.4; Appendix 7.2). Plants from the Mediterranean basin ranged from complete incompatible (MC and SC = 0) to full compatible (MC and SC = 1).

Table 7.4. GLMM analyses of the effect of area of origin and form on the reproductive indices calculated for *Oxalis pes-caprae* native (SA) and invasive plants (MB) after controlled hand-pollinations. '*' – a GLM was used instead. Statistically significant differences are highlighted in bold.

		Outcrossing		**Morph-compatibility**		**Self-compatibility**	
Factors	**df**	χ^2	***P***	χ^2	***P***	χ^2	***P***
(a) SA and MB							
Area	1	389.980	<**0.001**	24.913	<**0.001**	16.759	<**0.001**
Form	3	46.100	<**0.001**	29.671	<**0.001**	25.797	**0.010**
(b) SA							
Form	2	1.139	0.566	2.729 *	0.256	1.771	0.413
(c) MB							
Form	3	80.522	<**0.001**	25.939	<**0.001**	36.482	<**0.001**

Fitness of illegitimate offspring

The seeds obtained after compatible pollinations had a significantly higher germination rate than the seeds obtained after incompatible pollinations (Table 7.5; Fig. 7.5A). While form had no significant effect on the germination of seeds obtained after incompatible pollinations (4*x* L-, 28.57 ± 4.93 %; 4*x* M-, 17.46 ± 4.78 %; 5*x* S-, 11.11 ± 7.41 %; Fig. 7.5A), differences among forms were detected after compatible pollinations (Table 7.5), with the 4*x* M-morph having the lowest germination rate (4*x* L-, 45.14 ± 4.15 %; 4*x* M-, 30.33 ± 4.16 %; 5*x* S-, 52.76 ± 3.59 %; Fig. 7.5A). When comparing pollination treatments within form, seeds illegitimately sired by the 4*x* L- and 5*x* S-morph had a significantly lower germination rate than the ones legitimately sired, while no differences between treatments were observed for the 4*x* M-morph (Table 7.5; Fig. 7.5A).

Plant survival was always above 80% (Fig. 7.5B), and no differences were detected between pollination treatments and among forms, nor the interaction of the two (Table 7.5). Also, pollination treatment had no significant effect on the survival of plants sired by each of the forms considered (Table 7.5).

Except for the offspring illegitimately sired by the 5*x* S-morph that did not flower in the first year, between 40% and 70% of the plants that survived were able to produce inflorescences already in the first year (Fig. 7.5C), while the remaining stayed

vegetative. No effect of pollination treatment, form and the interaction of the two were found for plant flowering (Table 7.5). Also, pollination treatment had no significant effect on the flowering of plants sired by each of the forms considered (Table 7.5).

Table 7.5. GLM analyses of (a) the offspring fitness obtained after pollination treatments, *i.e.*, compatible and incompatible pollinations, of *Oxalis pes-caprae* plants from the invaded area, (b) the effect of form, *i.e.*, 4*x* L-, 4*x* M- and 5*x* S-morph, on the offspring fitness of *O. pes-caprae* for each pollination treatment separately, and (c) the effect of pollination treatment, *i.e.*, incompatible and incompatible pollinations, on the offspring fitness of *O. pes-caprae* of each form considered separately. Statistically significant differences are highlighted in bold. "-" not tested.

		Germination		**Survival**		**Flowering**	
Factors	**df**	χ^2	***P***	χ^2	***P***	χ^2	***P***
(a) Offspring fitness							
Pollination treatment	1	6.022	**0.014**	3.101	0.078	0.789	0.163
Form	2	3.888	0.143	0.343	0.842	1.946	0.874
Pollination treatment × Form	2	3.385	0.184	1.280	0.527	0.787	0.675
(b) Testing the effect of "form"							
Compatible pollination	2	15.071	**<0.001**	1.341	0.511	4.351	0.114
Incompatible pollination	2	3.888	0.143	0.343	0.842	0.270	0.874
(c) Testing the effect of "pollination treatment"							
4*x* L-morph	1	6.022	**0.014**	3.101	0.078	1.946	0.163
4*x* M-morph	1	3.496	0.062	0.027	0.870	0.007	0.933
5*x* S-morph	1	8.233	**0.004**	$1E^{-4}$	0.993	-	-

DISCUSSION

My large scale comparison of the TSI between native and invasive populations of *O. pes-caprae* revealed several main findings: (1) native and invasive populations significantly differed in the strength of the TSI: plants from SA maintain a strong TSI, while invasives have a significantly higher reproductive success following incompatible crosses than natives; (2) total reproductive success after intermorph pollinations was significantly higher for 4*x* natives than for 4*x* invasive plants; (3) morph-specific differences in the weakening of TSI were detected among invasive plants, with the L- and M-morphs being more self- and morph-compatible than the S-morph; (4) I detected variation in the expression of incompatibility among invasive plants, thus suggesting high standing genetic variation that might provide opportunities for natural selection on the strength of TSI; and finally, (5) seeds produced in incompatible crosses were able to germinate and survive to adult stage, with some plants remaining vegetative, while most of them were able to flower in the first year. Despite of the differences detected in the

TSI between ranges and the increase in compatibility in the invaded area, reproductive assurance is most probably guaranteed through asexual means. Notwithstanding, I detected some morph- and self-compatibility that might become selectively advantageous in the invaded area. Below I discuss these results in detail and focus their relevance for understanding the evolution of complex mating systems, such as heterostyly, during invasion.

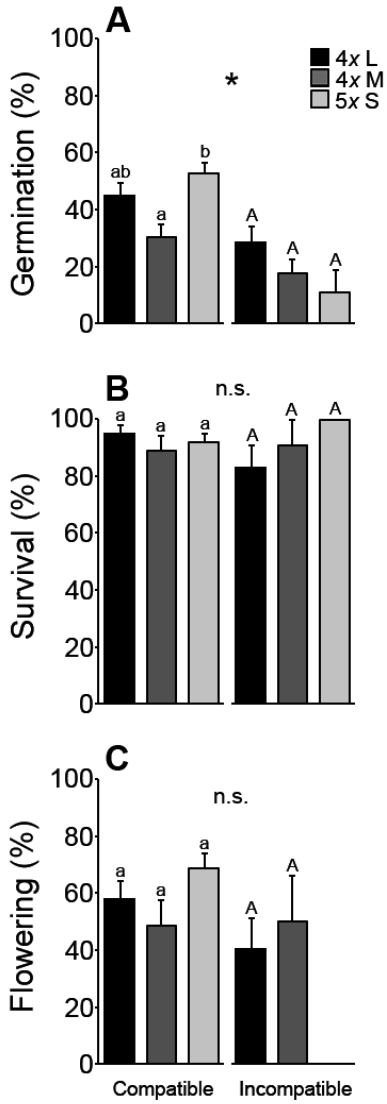


Figure 7.5. Fitness of the offspring of invasive plants of *Oxalis pes-caprae* given as mean and SE (%) after compatible and incompatible pollinations. **A.** Seed germination. **B.** Plant survival. **C.** Plant flowering. Different uppercase and lowercase letters reveal statistically significant differences among forms within pollination treatments, respectively, at $P < 0.05$. Asterisks refer to statistically significant differences between pollination treatments at $P < 0.05$; n.s. – no statistically significant differences were found at $P > 0.05$.

Trimorphic incompatibility system in the native area

My results from controlled pollinations of South African plants confirmed that *O. pes-caprae* is strongly morph- and self-incompatible, which is in accord with earlier investigations (Ornduff, 1987). The maintenance of TSI in native populations is expected for several reasons. First, negative frequency-dependent selection operating in these populations maintains the three style morphs, usually in isoplethy (Ornduff, 1987; Turketti, 2010; Ferrero *et al*., 2015). Second, these are homogeneously tetraploid and thus, compatible cytotypes co-occur (Krejčiková *et al*., 2013; Ferrero *et al*., 2015). Nonetheless, incompatible crosses yielded small amounts of seeds, similar to what was also previously obtained by Ornduff (1987). Natural variation in the strength of the incompatibility system within and/or among populations of self-incompatible taxa (*e.g*., O'Neil, 1992; Barrett and Cruzan, 1994; López-García and Maillet, 2005; Mable *et al*., 2005; Arroyo *et al.*, 2012) has already been reported. Variability in the incompatibility reactions in sporophytic incompatibility systems has been attributed to modifiers of the *S* gene activity that would allow the production of variable amounts of fruits and seeds after incompatible pollinations of obligate outcrossers (Levin, 1996; Brennan *et al*., 2011). Such mechanism might explain the sporadic production of seeds by native plants after incompatible pollinations, and in particular the high values of seed set obtained after selfing of two individuals from the native range included in this study.

Increased compatibility during colonization

Native 4*x* plants performed better after compatible pollinations than 4*x* individuals from the MB that yielded approximately half the number of seeds produced by natives. These differences were expected based on different historical processes in the two ranges. A recent comparison of patterns of genetic diversity between the two areas found that native populations of *O. pes-caprae* are genotypically more diverse than invasives, but the latter are not genetically uniform (Ferrero *et al*., 2015). Genetic drift during introduction was suggested to decrease genetic diversity, thus having strong negative impacts in reproductive success. However, some recombination by sexual means might explain why invasive populations are not genetically uniform. An additional factor might, however, contribute to the low genetic diversity reported: the presence in invasive populations of the 5*x* form, which has an even lower reproductive success. Plants with odd ploidy levels, *e.g*., 3*x* and 5*x*, show meiotic irregularities, resulting in a high production of aneuploids, and less frequently 1*x*, 2*x*, 3*x*, 4*x* and/or 5*x*

gametes (Vignoli, 1937; Ramsey and Schemske, 1998; Risso-Pascotto *et al.*, 2003). Thus, reproductive success of 5*x* invasives is expected to be strongly affected by ploidy and to be significantly lower than the 4*x* morphs as it was observed. Still, it is worth noticing that these plants are not entirely sterile and are able to produce some viable gametes and offspring (*e.g.*, Brandham, 1982; Burton and Husband, 2000). Indeed, the results obtained here as well as from previous investigations in *O. pes-caprae*, show that the 5*x* S-morph is able to produce small amounts of fruits and seeds after incompatible (Castro *et al.*, 2013; Costa *et al*., 2014) and compatible pollinations, and seeds are able to germinate and seedlings reach the adult stage.

My large-scale comparison of the TSI between native and invaded areas gives further support to the preliminary findings of Castro *et al*. (2013) and Costa *et al*. (2014), who reported a weakening in the incompatibility system of *O. pes-caprae* based on experiments carried with a few populations from the MB only. Here, I detected differences in the mating system between native and invaded regions with increased self- and morph-compatibility in the later. A similar pattern was found for a few invasive species, as *Echium plantagineum* (Petanidou *et al*., 2012) and *Gomphocarpus physocarpus* (Ward *et al.*, 2012). Theoretical models indicate that selection for self-compatibility depends on several factors, including the extent of the reproductive assurance provided by selfed progeny (Lloyd, 1992). This is particularly important after long-distance dispersal, because it allows the establishment and spread of a species in a new range under low compatible mates' density (Baker's law; Baker, 1955, 1967; Stebbins, 1957; Barrett, 2011; Pannell *et al*., 2015). For species with heteromorphic incompatibility systems (*i.e*., self- and morph-incompatibility), this situation should not only include single colonizers, but also small colonies formed exclusively by the same morph. Invasive individuals of *O. pes-caprae* are under strong mate limitation, especially the 4*x* L- and 4*x* M-morphs, and although asexual reproduction is the main mechanism of spread (Castro *et al*., 2016), the ability to produce some self- and intramorph offspring might be selectively advantageous. Despite the plants raised from seed are able to germinate, survive and flower in the first year under glasshouse conditions, it is not clear how frequent the recruitment from seed occurs in natural populations. Thus, studies addressing these questions in natural conditions are desirable before further conclusions on selection of reproductive traits are reached.

Different reproductive strategies among style morphs during colonization

Different compatibility levels were observed among invasive forms. These patterns were surprising, but not unexpected. Populations of *O. pes-caprae* in the MB are predominantly monomorphic of the 5*x* S-morph with low sexual reproduction (Castro *et al*., 2007, 2013), being genetically depauperate (Ferrero *et al.*, 2015). Thus, the opportunities for selection of compatibility in these individuals are expected to be limited. On the contrary, for the 4*x* L- and 4*x* M-morphs, still growing under strong mate limitation in populations dominated by the 5*x* S-morph, recombination opens the possibility for increased variability in the TSI. Indeed, my results revealed that the 4*x* L- and 4*x* M-morphs are more self- and morph-compatible, respectively, and this is in accordance with previous findings (Castro *et al.*, 2013).

Strong mate limitation in the MB regardless of this species abundance might be driving the changes in the incompatibility reactions in the 4*x* L- and 4*x* M-morphs. First, the low availability of viable compatible gametes in mixed populations caused by the occurrence of 5*x* S-morph plants and a deficiency/lack of 4*x* M-morph plants (Castro *et al*., 2007, 2013) might cause a selective environment driving the observed changes in the incompatibility system. Second, 5*x* S-morph plants may act as strong competitors via clonal propagation, investing more resources in producing large bulbs (Castro *et al.*, 2016) overcoming the inability to produce the maximum/optimum number of viable gametes. Strong mate limitation also results from the rarity of the 4*x* S-morph in the MB (Castro *et al*., 2007, 2013) and its apparent inability to reproduce sexually in the absence of compatible mates (results herein). Altogether, these observations suggest that founder events after introduction or some selection against the *S* allele may be occurring and could be responsible for the rarity of the 4*x* S-morph in the invaded area.

Despite a few exceptions (Lewis and Jones, 1992), the S-morph (*Ss*--) is dominant over the M- (*ssM*-) and the L-morphs (*ssmm*), as it carries the *S* allele, which is dominant over the *M* allele (Heuch and Lie, 1985; Lewis and Jones, 1992). Theoretical simulations have shown that alleles governing tristyly differ in their frequency in populations at equilibrium (S = 0.085, M = 0.151, m = 0.849; Heuch, 1980). Consequently, style morphs differ in the probability of loss through drift and founder events, with the S-morph being more susceptible to loss than the other morphs (*e.g.*, Eckert and Barrett, 1992). Additionally, some selection against the *S* allele has already been reported for other heterostylous species (Weller, 1992), and might be responsible for the low frequency of the 4*x* S-morph observed in this study. In *O. pes-caprae*, the 4*x*

S-morph is thus expected to be more susceptible to loss, while populations with the 4*x* L- and 4*x* M-morphs are expected to be maintained. Under both scenarios, the 5*x* S-morph seems in disadvantage, depending mostly on its asexual reproduction capacity, which also revealed to be significantly lower in comparison with the 4*x* floral morphs (Castro *et al*., 2016). Based on the available information, the dominance of the 5*x* S-morph in the western Mediterranean basin can be explained by the introduction of the three style morphs at different timings. Thus, it is highly probable that the introduction of the currently widespread 5*x* S-morph preceded a more recent set of introductions of the other two style morphs (4*x* L- and 4*x* M-morphs), for which the available data suggests higher sexual and asexual fitness than for the 5*x* S-morph [results herein for sexual reproduction under mate limitation; Castro *et al.*, (2016) for sexual and asexual reproductive traits].

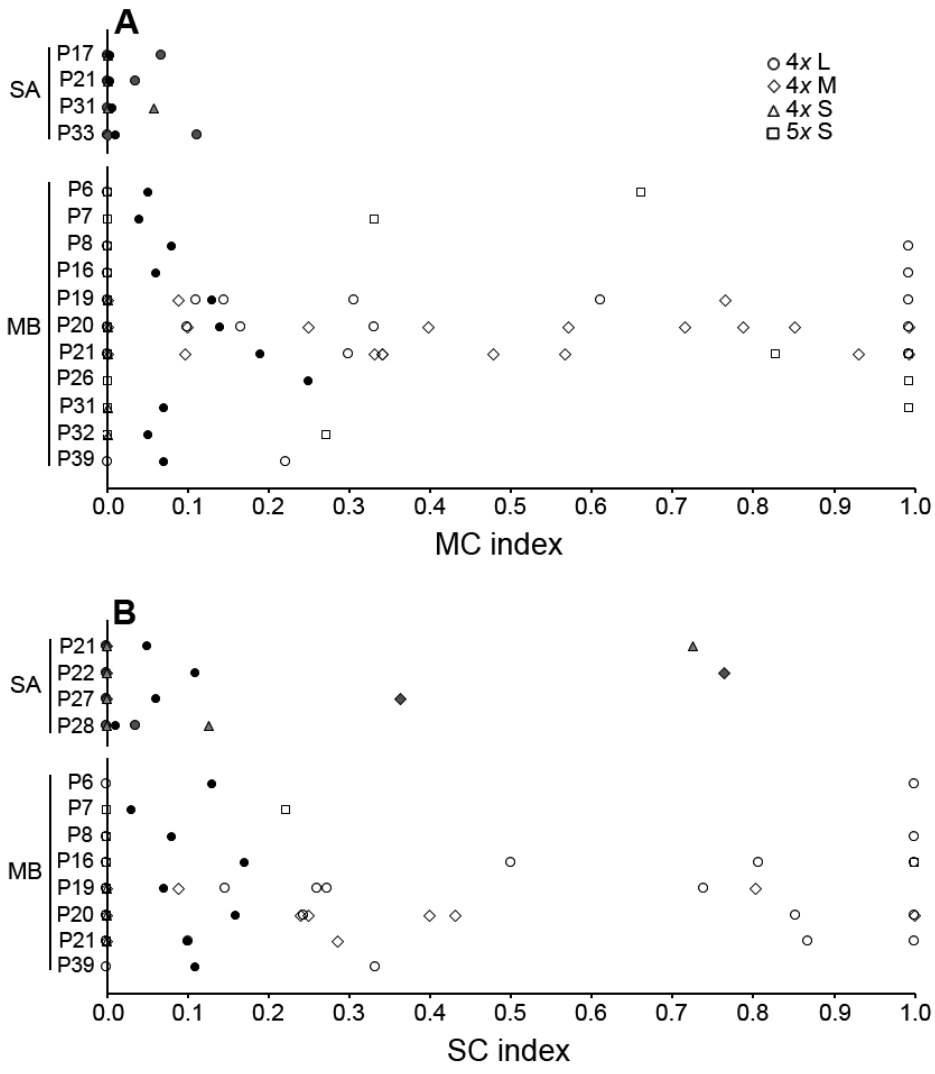
Changes to the mating system in the Mediterranean basin

Several features contribute for the undoubted success of *O. pes-caprae* as an invader of Mediterranean climate regions. Notably, this species has a profuse ability to reproduce vegetatively (Pütz, 1994), showing higher clonal reproduction in invasive than in native populations (Castro *et al.*, 2016); it has successfully integrated the novel pollination networks in the MB (Ferrero *et al*., 2013); it inhabits disturbed and anthropogenized places (Gimeno *et al.*, 2006); and finally, to my knowledge, no natural enemies have been reported so far in the MB. Additionally, my study demonstrated increased ability for reproduction under a strong mate limitation scenario via incompatibility weakening in invasive populations. I detected variability in the expression of TSI among invasive plants; individuals ranged from morph- and self-incompatible to full morph- and self-compatible with the self- and intramorph offspring being able to achieve sexual maturity in the first year after germination under glasshouse conditions. These findings might suggest a basis for natural selection acting on the mating system during invasion. Altogether, these characteristics make *O. pes-caprae* a successful invader difficult to eradicate. Moreover, if the ongoing changes in the incompatibility system allow the production of viable seeds after incompatible pollination that are able to germinate, survive and flower in natural conditions, this might contribute to increase the genetic diversity in the populations (Ferrero *et al*., 2015), thus favoring the establishment and spread of more competitive and fitter genotypes.

Appendix 7.1. GLM analyses of the sexual reproductive variables after pollination treatments, *i.e.*, intermorph, intramorph and self-pollinations, of *Oxalis pes-caprae* plants from native and invaded areas. Fruit set was analyzed using GLMMs. Statistically significant differences are highlighted in bold.

		Fruit set		Seed production		Total reproductive success	
Factors	**df**	χ^2	***P***	χ^2	***P***	χ^2	***P***
Pollination treatment	2	324.669	**<0.001**	155.178	**<0.001**	1061.169	**<0.001**
Area	1	6.955	**0.008**	17.242	**<0.001**	23.245	**<0.001**
Form	3	28.773	**<0.001**	54.964	**<0.001**	54.146	**<0.001**
Pollination treatment × Area	2	29.275	**<0.001**	20.045	**<0.001**	173.678	**<0.001**

Appendix 7.2. A. Morph-compatibility (MC) index; **B.** Self-compatibility (SC) index. Values are population average (closed circles) and individual plant value of compatibility (open symbols). SA and MB for South Africa and Mediterranean basin, respectively.



Chapter 8 – General conclusions

"From this fact we may infer that most of the genera have acquired their heterostyled structure independently of one another; that is, they have not inherited this structure from some one or even two or three progenitors in common."

Darwin (1887 p. 135)

Heterostyly – where does our knowledge stand?

Since the publication of *The Different Forms of Flowers on Plants of the Same Species* by Charles Darwin (1877), there has been a long-standing interest in the floral polymorphism heterostyly. This is undoubtedly the most influential work on the study of floral polymorphisms, and it has motivated numerous studies in plant reproductive biology since then. The contributions to our current knowledge on this floral polymorphism can be grouped into four periods, which also relied on methodological advances and the availability of different techniques and tools. The first period took place during the 19th century and the main contributor was Charles Darwin (1864, 1868, 1877). This was mainly "a time when the morphological nature of heterostyly was described, its functional significance suggested, its occurrence documented, and some field studies conducted" (Ornduff, 1992 p. 36).

Several important findings on heterostyly came to light during the 20th century. During the first half of the 20th century, the valuable contributions of Bateson and Gregory (1905), Barlow (1923), East (1927), Mather and De Winton (1941), Fisher and Mather (1943) and Mather (1950), among others, elucidated the genetics and inheritance of the polymorphism. The second half of the 20th century saw the birth of evolutionary ecology and was a very productive time for studies on reproductive biology and pollen capture in natural populations of heterostylous plants (*e.g.*, Levin, 1968; Ornduff, 1970, 1979, 1980b, 1982; Ganders, 1974, 1976; Weller, 1980; Glover and Barrett, 1983, 1986; Nicholls, 1985), reports of new heterostylous species (*e.g.*, Devi, 1964; Barrett *et al*., 1997; Pailler and Thompson, 1997), investigations on the factors responsible for the maintenance and breakdown of the polymorphism (*e.g.*, Ornduff, 1972; Barrett, 1979; Barrett *et al*., 1983, 1989; Weller, 1986; Eckert and Barrett, 1992, 1995; Eckert *et al.*, 1996b), development of the two main models for the evolution of distyly (Charlesworth and Charlesworth, 1979; Lloyd and Webb, 1992a, b), and the only model that tries to explain the evolution of tristyly (Charlesworth, 1979). Towards the end of the century, it was published the first volume since Darwin's book in 1877 that contained a synthesis of what was known about heterostyly, and it was written by leading experts in the field (Barrett, 1992b). By this time, heterostyly was the most well-studied of the floral polymorphisms described in Darwin's original volume.

Finally, over the past two decades we have witnessed the advent of molecular tools, which allowed the quantitative study of mating patterns in natural populations and

efforts to investigate their genetic and genomic consequences (*e.g*., Hodgins and Barrett 2006b, 2008b; Ness *et al*., 2010, 2012; Weber *et al*., 2013; Arunkumar *et al*., 2015; Zhou *et al*., 2015), the testing of models on the evolution of heterostyly (Graham and Barrett, 2004; Ferrero *et al*., 2009a, 2012; Kissling and Barrett, 2013), and significant advances in the understanding of the molecular basis of distyly (*e.g*., McCubbin *et al*., 2006; Li *et al*., 2007, 2016; Nowak *et al*., 2015; Huu *et al*., 2016) and tristyly (Arunkumar *et al*., 2017). Despite the enormous amount of information available on heterostyly only briefly summarized above, various questions remained unanswered at the commencement of my own studies. My PhD thesis aimed to address some of these questions and provide novel insights into the function and adaptive significance of reciprocal herkogamy and ancillary characters of pollen and stigma, and also to investigate the evolutionary history and demographic factors involved in the maintenance of the polymorphism. Below I briefly discuss the main findings of my PhD thesis and how these contribute to advancing our knowledge on heterostyly.

Evidence for Darwin's cross-promotion hypothesis

Data presented in Chapter 2 confirmed that although Darwin's cross-promotion hypothesis for the functional significance of reciprocal herkogamy has been experimentally evaluated numerous times in unrelated taxa, problems still remain. My literature review identified that most studies failed to detect strong support for Darwin's cross-promotion hypothesis, confirming Gander's (1979) earlier review of a much smaller sample of species. My review found that asymmetrical pollen transfer and capture in natural populations of distylous species was frequent. To precisely investigate the extent to which reciprocal herkogamy promotes disassortative pollination, it is essential to exclude the confounding influence of self-pollen deposition, and this can only be achieved by the emasculation of flowers (Ganders, 1974, 1979; Lloyd and Webb, 1992b). In the few studies that have compared intermorph versus intramorph outcross components of the stigmatic pollen load, strong support for the Darwinian hypothesis was obtained (Ganders, 1974, 1976; Schou, 1983; Nicholls, 1986; Piper and Charlesworth, 1986; Nishihiro and Washitani, 1998; reviewed in Lloyd and Webb, 1992b). Unfortunately, for most species that have been investigated it is not known whether heterostyly promotes significant disassortative pollen transfer because of the confounding influence of self-pollen deposition and the inability of investigators to distinguish this component of the pollen loads from intramorph outcross

pollen deposition. Thus, the great majority of pollen flow studies available have not rigorously evaluated the effectiveness of heterostyly in the pollen economy of populations, and consequently their conclusions cannot be used to satisfactorily evaluate the Darwinian hypothesis.

A few studies of pollen capture have been conducted in tristylous species (Price and Barrett, 1982; Glover and Barrett, 1983, 1986; Barrett and Glover, 1985; Mulcahy and Caporello, 1970; Ornduff 1975b). Two studies on *Lythrum* (Mulcahy and Caporello 1970; Ornduff 1975b) were unconvincing with regards to the role of reciprocal herkogamy in promoting disassortative pollination among style morphs, because of the inability of investigators in distinguishing pollen produced by short- and mid-level anthers. I was therefore interested in investigating this problem further and my results from Chapter 3 go beyond the previous dichotomy involving "long pollen" versus "non-long pollen" in *Lythrum* pollen flow studies, and extended our knowledge to an unrelated species of Pontederiaceae, the only tristylous family for which similar studies have been successfully conducted. My estimates of intraflower pollen deposition provided convincing evidence that this constitutes the main source of intramorph pollen, highlighting why the examination of pollen loads in stigmas from intact flowers can provide misleading information on the efficacy of heterostyly in promoting disassortative pollen transfer (Chapter 2). Harder and Barrett (1996) suggested that heterostyly might also limit geitonogamy and pollen discounting by increasing pollen carryover, but this has never been experimentally tested. Although my results support this hypothesis by showing low levels of geitonogamous pollination in monomorphic arrays of *Lythrum salicaria*, a more rigorous experimental test should be conducted in the future. Finally, disassortative pollen transfer varied in a predictable way according to sex-organs height, with increased precision of compatible pollen transfer with decreased organ heights from long- to mid- to short-level anthers and stigmas. This finding supports the general inference that floral design plays a key role in determining patterns of pollen transfer and capture in heterostylous species.

Disassortative pollination in the absence of reciprocal herkogamy

The ancillary characters of pollen and stigmas have been the least investigated feature of the heterostylous syndrome from a functional viewpoint. Early investigations of exine ornamentation and papillae shape, as well as stigmatic pollen loads after controlled hand-pollinations in Plumbaginaceae led Dulberger (1975a) to propose the

topographical complementary hypothesis for the function of ancillary characters. Stimulated by Dulberger's hypothesis, Mattsson (1983) experimentally investigated the role of morph-specific differences in exine sculpturing in the adhesion of pollen grains during their initial interaction with stigmas. Until very recently, these were the only two attempts to understand the functional role of heteromorphic pollen and stigmas in the heterostylous syndrome. In Chapter 4, I revisited the topographical complementarity hypothesis and found partial evidence supporting it. Mating types of the three species I investigated did not differ in compatible pollen capture, although cob stigmas captured more incompatible pollen in natural populations. In addition, I provided novel evidence showing that pollen-stigma dimorphisms serve to promote disassortative pollination in natural populations of heteromorphic species in the absence of reciprocal herkogamy. Although structural in nature, pollen-stigma dimorphisms are tightly associated with the physiological incompatibility system and function in concert to limit incompatible pollen deposition on stigmas.

The selfing-avoidance model likely explains the evolution of heterostyly in Plumbaginaceae

While a fascinating topic of research that goes back to Darwin's early ideas, the evolution of heterostyly remains poorly understood. The few studies available from *Narcissus* (Graham and Barrett, 2004; Pérez-Barrales *et al*., 2006), Boraginaceae (Ferrero *et al.*, 2009a, 2012) and *Exochaenium* (Kissling and Barrett, 2013) provided some support for Lloyd and Webb's (1992 a, b) model for the evolution of heterostyly. According to this model, reciprocal herkogamy evolves first, via a stage of stigma-height dimorphism, to promote more effective cross-pollination in a hypothetical population with approach herkogamy. Following the establishment of the stamen-style polymorphism, diallelic incompatibility may then evolve in lineages in which inbreeding depression is strong. Contrarily, the competing model for the evolution of distyly by Charlesworth and Charlesworth (1979) involves the reverse polarity, and commences with the establishment of diallelic incompatibility owing to strong inbreeding avoidance. The ancestral character mapping and Bayesian analyses for the evolution of the heterostylous syndrome in Plumbaginaceae provided in Chapter 5 supported the model proposed by Charlesworth and Charlesworth (1979). This result fits the early expectations of Baker (1948a, 1966), who without the benefits of modern phylogenetic inference presented a scheme for the evolution of distyly in the family

(Baker, 1948a: Fig. 13; Baker, 1966: Fig. 5) that is consistent with my own results. Much of what is known about Plumbaginaceae, especially *Limonium* and *Armeria*, concerning pollen and stigma polymorphisms, the incompatibility system, the occurrence of reciprocal herkogamy and the geographical distribution of species resulted from serious investigation of this family by Baker (1948a, b, c, 1953a, b, 1966). In fact, he was the first to hypothesize that pollen-stigma dimorphism and reciprocal herkogamy were "superimposed" on a common ancestor with a monomorphic arrangement of sex organs and self-incompatibility (Baker, 1948a, 1966). My results support this and also provide evidence that both self-compatibility and apomixis are likely to be derived character states, which have evolved multiple independent times in *Limonium*. However, and as discussed in Chapter 5, additional character and taxa sampling should be conducted in future efforts to reconstruct the evolutionary history of the heterostylous syndrome in Plumbaginaceae.

Maintenance of tristyly in populations of *Lythrum salicaria* from Iberian Peninsula

Tristyly is maintained in populations by a balance between negative frequency-dependent selection and stochastic forces (*e.g.*, genetic drift). Data on morph ratios is available for several tristylous species, and both the maintenance of trimorphism (*e.g.*, Haldane, 1936; Ågren and Ericson, 1996; Eckert *et al*., 1996b) and stochastic morph loss have been described (*e.g.*, Barrett and Forno, 1982; Castro *et al*., 2013; Cunha *et al*., 2014). My extensive survey of morph ratios in Iberian populations of tristylous *Lythrum salicaria* was the first to be conducted in the southwestern European range of the species. At the range limit in SW Iberian Peninsula, populations were smaller, and with lower evenness values compared to populations further north. However, despite the ecological and demographic stressful conditions associated with the habitats that this wetland plant experiences at the range limit caused by strong seasonality in rainfall and hot, dry summers, I detected no morph loss from populations. These results illustrate the strong resilience of tristyly to the various forces capable of causing the dissolution of the polymorphism, and demonstrate that the association of tristyly with trimorphic incompatibility plays a key role in maintaining the polymorphism. Maintenance of tristyly in European populations contrasts strongly with the situation in invasive populations in eastern North America, where on average ~25% of populations are dimorphic (Eckert and Barrett, 1992; Balogh and Barrett, 2016). This difference probably reflects the greater genetic connectivity of populations in Europe, owing to

residing in more agricultural landscapes. These differences in spatial ecology probably provide greater opportunities for gene flow through pollen and seed in restoring missing morphs from populations.

Variability in trimorphic incompatibility among invasive plants of *Oxalis pes-caprae*

The successful establishment and spread of invasive plant species can be promoted by diverse sexual and asexual reproductive strategies. For example, transitions from sexual to asexual reproduction (*e.g.*, Amsellem *et al*., 2001; Wang *et al*., 2005; Ferrero *et al*., 2015) and from outcrossing to selfing (Petanidou *et al*., 2012; Ward *et al*., 2012) are often reported in association with plant invasions (reviewed in Barrett, 2011). To detect differences in the reproductive strategy of individuals involved in plant invasions, it is necessary to investigate reproductive traits in individuals from both native and introduced regions, and this can be done by measuring the traits under common garden conditions or through analyses of genetic variation in natural populations. In Chapter 7, I compared the incompatibility system of native South African and Mediterranean basin invasive plants of *Oxalis pes-caprae*, a successful clonal invader that now occupies all Mediterranean regions of the globe. I found evidence of higher levels of self- and intramorph compatibility in invasive plants compared to those from the native range. This finding is consistent with the hypothesis that self-compatibility may be adaptive in situations where mate limitation prevails, as in the case of *O. pes-caprae*, owing to the dominance of the 5*x* S-morph in western Mediterranean. In this case, the lack of compatible style morphs and cytotypes may create the opportunity for selection for intramorph compatibility with consequences for the mating system. However, before this hypothesis is fully accepted, it is important to point out that the vast majority of invasive populations in Iberia, and much likely elsewhere in the invasive range, reproduce through clonal propagation (Ferrero *et al*., 2015). Thus, at this stage, it is premature to conclude that sexual reproduction is playing an important role in invasion success. Future studies investigating mating patterns in natural populations are desirable before further conclusions on the selection and adaptive value of increased levels of compatibility in invasive populations are made.

Chapter 9 – Literature cited

Abeli T, Gentili R, Mondoni A, Orsenigo S, Rossi G. 2014. Effects of marginality on plant population performance. *J Biogeogr* 41: 239–249.

Abràmoff MD, Hospitals I, Magalhães PJ. 2004. Image processing with ImageJ. *Biophotonics Int* 11: 36–42.

Ågren J. 1996. Population size, pollinator limitation, and seed set in the self-incompatible herb *Lythrum salicaria*. *Ecology* 77: 1779–1790.

Ågren J, Ericson L. 1996. Population structure and morph-specific fitness differences in tristylous *Lythrum salicaria*. *Evolution* 50: 126–136.

Aliyu OM, Seifert M, Corral JM, Fuchs J, Sharbel TF. 2013. Copy number variation in transcriptionally active regions of sexual and apomictic *Boechera* demonstrates independently derived apomictic lineages. *Plant Cell* 25: 3808–23.

Allen AM, Hiscock SJ. 2008. Evolution and phylogeny of self-incompatibility systems in angiosperms. Pp. 73–101 *in* Franklin-Tong VE, ed. *Self-Incompatibility in Flowering Plants – Evolution, Diversity, and Mechanisms*. Springer-Verlag, Berlin, Germany.

Amsellem L, Noyer J-L, Hossaert-McKey M. 2001. Evidence for a switch in the reproductive biology of *Rubus alceifolius* (Rosaceae) towards apomixis, between its native range and its area of introduction. *Am J Bot* 88: 2243–2251.

Anderson NO, Ascher PD. 1995. Style morph frequencies in Minnesota populations of *Lythrum* (Lythraceae) II. Tristylous *L. salicaria* L. *Sex Plant Reprod* 8: 105–112.

Anderson NO, Ascher PD. 2000. Fecundity and fitness in cross-compatible pollinations of tristylous North American *Lythrum salicaria* populations. *Theor Appl Genet* 101: 830–843.

Andersson S. 1994. Unequal morph frequencies in populations of tristylous *Lythrum salicaria* (Lythraceae) from southern Sweden. *Heredity* 72: 81–85.

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 Phytol* 171: 581–590.

Arroyo J, Barrett SCH, Hidalgo R, Cole WW. 2012. Evolutionary maintenance of stigma-height dimorphism in *Narcissus papyraceus* (Amaryllidaceae). *Am J Bot* 89: 1242-1249.

Arroyo MTK, Humaña AM, Domínguez D, Jespersen G. 2012. Incomplete trimorphic incompatibility expression in *Oxalis compacta* Gill. ex Hook. et Arn. subsp. *compacta* in the central Chilean Andes. *Gayana Bot* 69: 88–99.

Arunkumar R, Ness RW, Wright SI, Barrett SCH. 2015. The evolution of selfing is accompanied by reduced efficacy of selection and purging of deleterious mutations. *Genetics* 199: 817-829.

Arunkumar R, Wang W, Wright SI, Barrett SCH. 2017. The genetic architecture of tristyly and its breakdown to self-fertilization. *Mol Ecol* doi:10.1111/mec.13946.

Ascher JS, Pickering J. 2016. *Discover Life: Bee Species Guide and World Checklist (Hymenoptera: Apoidea: Anthophila).*

Available online: http://www.discoverlife.org/mp/20q?guide=Apoidea_species [Last time accessed November 15, 2016).

Asker SE, Jerling L. 1992. *Apomixis in plants*. CR Press, Boca Raton, USA.

Baker HG. 1948a. Dimorphism and monomorphism in the Plumbaginaceae I. Survey of the family. *Ann Bot* 12: 207–219.

Baker HG. 1948b. Relationships in the Plumbaginaceae. *Nature* 161: 400.

Baker HG. 1948c. Significance of pollen dimorphism in Late-Glacial *Armeria*. *Nature* 161: 770–771.

Baker HG. 1953a. Dimorphism and monomorphism in the Plumbaginaceae II. Pollen and stigmata in the genus *Limonium*. *Ann Bot* 17: 433–445.

Baker HG. 1953b. Dimorphism and monomorphism in the Plumbaginaceae III. Correlation of geographical distribution patterns with dimorphism and monomorphism in *Limonium*. *Ann Bot* 17: 615–627.

Baker HG. 1955. Self-compability and establishment after "long-distance" dispersal. *Evolution* 9: 347–348.

Baker HG. 1958. Studies in the reproductive biology of West African Rubiaceae. *J West Afr Sci Assoc* 4: 9-24.

Baker HG. 1959. The contribution of autecological and genecological studies to our knowledge of the past migrations of plants. *Am Nat* 93: 255-272.

Baker HG. 1965. Characteristics and modes of origin of weeds. Pp. 147–168 *in* Baker HG, and Stebbins GL, eds. *The Genetics of Colonizing Species*. Academic Press, New York, USA.

Baker HG. 1966. The evolution, functioning and breakdown of heteromorphic incompatibility systems. I. The Plumbaginaceae. *Evolution* 20: 349–368.

Baker HG. 1967. Support for Baker's Law – as a rule. *Evolution* 21: 853–856.

Balogh CM, Barrett SCH. 2016. Stochastic processes during invasion: the influence of population size on style-morph frequency variation in *Lythrum salicaria* (purple

loosestrife). *Int J Plant Sci* 177: 409-418.

Barlow N. 1913. Preliminary note on heterostylism in *Oxalis* and *Lythrum*. *J Genet* 3: 53–65.

Barrett SCH. 1977a. Tristyly in *Eichhornia crassipes* (Mart.) Solms (water hyacinth). *Biotropica* 9: 203-238.

Barrett SCH. 1977b. The breeding system of *Pontederia rotundifolia* L., a tristylous species. *New Phytol* 78: 209–220.

Barrett SCH. 1979. The evolutionary breakdown of tristyly in *Eichhornia crassipes* (Mart.) Solms (water hyacinth). *Evolution* 33: 499–510.

Barrett SCH. 1985. Floral trimophism and monomorphism in continental and island populations of *Eichhornia paniculata* (Spreng.) Solms. (Pontederiaceae). *Biol J Linn Soc* 25: 21–40.

Barrett SCH. 1988. Evolution of breeding systems in *Eichhornia* (Pontederiaceae): a review. *Ann Mo Bot Gard* 75: 741–760.

Barrett SCH. 1989. The evolutionary breakdown of heterostyly. Pp. 151–169 *in* Bock JH, and Linhart YB, eds. *The Evolutionary Ecology of Plants*. Westview Press, Bolder, USA.

Barrett SCH. 1992a. Heterostylous genetic polymorphisms: model systems for evolutionary analysis. Pp. 1-29 *in* Barret SCH, ed. *Evolution and Function of Heterostyly*. Springer-Verlag, Berlin, Germany.

Barrett SCH, ed. 1992b. *Evolution and Function of Heterostyly*. Springer-Verlag, Berlin, Germany.

Barrett SCH. 1993. The evolutionary biology of tristyly. Pp. 283–326 *in* Futuyma D, and Antonovics J, eds. *Oxford Surveys in Evolutionary Biology*. Oxford University Press, Oxford, UK.

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

Barrett SCH. 2003. Mating strategies in flowering plants: the outcrossing-selfing paradigm and beyond. *Philos T Roy Soc B* 358: 991–1004.

Barrett SCH. 2004. Pontederiaceae (water hyacinth family). Pp. 474–476 *in* Smith N, Mori SA, Henderson A, Stevenson DW, and Heald SV, eds. *Flowering Plants of the Neotropics*. Princeton University Press, Princeton, USA.

Barrett SCH. 2011. Why reproductive systems matter for the invasion biology of plants. Pp. 195-210 *in* Richardson DM, ed. *Fifty Years of Invasion Ecology: The Legacy of Charles Elton*. Oxford University Press, Oxford, UK.

Barrett SCH. 2013. The evolution of plant reproductive systems: how often are transitions irreversible? *Proc R Soc Lond B* 280: 20130913.

Barrett SCH, Arroyo MTK. 2012. Variation in floral morph ratios in tristylous *Oxalis squamata* (Oxalidaceae): an Andean alpine endemic. *Botany* 90: 1180–1185.

Barrett SCH, Brown AHD, Shore JS. 1987. Disassortative mating in tristylous *Eichhornia paniculata* (Pontederiaceae). *Heredity* 58: 49–55.

Barrett SCH, Cole WW, Arroyo J, Cruzan MB, Lloyd DG. 1997. Sexual polymorphisms in *Narcissus triandrus* (Amaryllidaceae): is this species tristylous? *Heredity* 78: 135–145.

Barrett SCH, Cruzan MB. 1994. Incompatibility in heterostylous plants. Pp. 188-219 *in* Williams EG, Clarke AE, and Knox RB, eds. *Genetic Control of Self-Incompatibility and Reproductive Development in Flowering Plants*. Kluwer Academic Publishers, Boston, USA.

Barrett SCH, Forno IW. 1982. Style morph distribution in New World populations of *Eichhornia crassipes* (Mart.) Solms-Laubach (water hyacinth). *Aquat Bot* 13: 299–306.

Barrett SCH, Glover DE. 1985. On the Darwinian hypothesis of the adaptative significance of tristyly. *Evolution* 39: 766–774.

Barrett SCH, Harder LD. 1996. Ecology and evolution of plant mating. *Trends Ecol Evol* 11: 73–79.

Barrett SCH, Harder LD. 2005. The evolution of polymorphic sexual systems in daffodils (*Narcissus*). *New Phytol* 165: 45–53.

Barrett SCH, Harder LD, Cole WW. 2004. Correlated evolution of floral morphology and mating-type frequencies in a sexually polymorphic plant. *Evolution* 58: 964-975.

Barrett SCH, Hodgins KA. 2006. Floral design and the evolution of asymmetrical mating. Pp. 239-255 *in* Harder LD, and Barrett SCH, eds. *The Ecology and Evolution of Flowers*. Oxford University Press, Oxford, UK.

Barrett SCH, Jesson LK, Baker AM. 2000. The evolution and function of stylar polymorphisms in flowering plants. *Ann Bot* 85: 253–265.

Barrett SCH, Morgan MT, Husband BC. 1989. The dissolution of a complex genetic polymorphism: the evolution of self-fertilization in tristylous *Eichhornia paniculata* (Pontederiaceae). *Evolution* 43: 1398–1416.

Barrett SCH, Ness RW, Vallejo-Marín M. 2009. Evolutionary pathways to self-fertilization in a tristylous species. *New Phytol* 183: 546–556.

Barrett SCH, Price SD, Shore JS. 1983. Male fertility and anisoplethic population structure in tristylous *Pontederia cordata* (Pontederiaceae). *Evolution* 37: 745–759.

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

Barrett SCH, Shore JS. 2008. New insights on heterostyly: comparative biology, ecology and genetics. Pp. 3–32 *in* Franklin-Tong VE, ed. *Self-Incompatibility in Flowering Plants – Evolution, Diversity, and Mechanisms*. Springer-Verlag, Berlin, Germany.

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

Bateman AJ. 1952. Trimorphism and self-incompatibility in *Narcissus*. *Nature* 170: 496–497.

Bateman AJ. 1968. The role of heterostyly in *Narcissus* and *Mirabilis*. *Evolution* 22: 645-646.

Bates D, Maechler M, Bolker B, Walker S, Christensen RHB, Singmann H, Dai B. 2014. *lme4 - Linear mixed-effects models using Eigen and S4*. R package version 1.0.5. URL https://cran.r-project.org/web/packages/lme4/index.html

Bateson W, Gregory RP. 1905. On the inheritance of heterostylism in *Primula*. *Proc R Soc Lond B* 76: 581-586.

Bell CD, Soltis DE, Soltis PS. 2010. The age and diversification of the angiosperms re-revisited. *Am J Bot* 97: 1296–1303.

Björkman T. 1995. The effectiveness of heterostyly in preventing illegitimate pollination in dish-shaped flowers. *Sex Plant Reprod* 8:143–146.

Boissier E. 1848. Plumbaginales. Pp. 617-696 *in* de Candolle AP, ed. *Prodromus systematis naturalis regni vegetabilis - Vol. 12*. Treuttel et Wurz, Paris, France.

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 Ecol Evol* 24: 127–135.

Boorman LA. 1967. Biological flora of the British Isles: *Limonium vulgare* Mill. and *L. humile* Mill. *J Ecol* 55: 221–232.

Bokhari MH. 1972. A brief review of stigma and pollen types in *Acantholimon* and *Limonium*. *Notes Roy Bot Gard Edinburgh* 32: 79–84.

Brandham PE. 1982. Inter-embryo competition in the progeny of autotriploid *Aloineae* (Liliaceae). *Genetica* 59: 29–42.

Bräuchler C, Meimberg H, Heubl G. 2010. Molecular phylogeny of Menthinae (Lamiaceae, Nepetoideae, Mentheae) – taxonomy, biogeography and conflicts. *Mol Phylogenet Evol* 55: 501–523.

Brennan AC, Tabah DA, Harris SA, Hiscock SJ. 2011. Sporophytic self-incompatibility in *Senecio squalidus* (Asteraceae): *S* allele dominance interactions and modifiers of cross-compatibility and selfing rates. *Heredity* 106: 113–123.

Breteler F. 1989. *The Connaraceae: a Taxonomic Study with Emphasis on Africa*. Agricultural University Wageningen, Wageningen, The Netherlands.

Brightmore D. 1979. Biological flora of the British Isles: *Frankenia laevis* L. *J Ecol* 67: 1097–1107.

Brown BJ, Mitchell RJ, Graham SA. 2002. Competition for pollination between an invasive species (purple loosestrife) and a native congener. *Ecology* 83: 2328–2336.

Brullo S, Pavone P. 1981. Chromosome numbers in the Sicilian species of *Limonium* Miller (Plumbaginaceae). *Anales Jard Bot Madrid* 37: 535-555.

Brussard PF. 1984. Geographic patterns and environmental gradients: the central-marginal model in *Drosophila* revisited. *Annu Rev Ecol Syst* 15: 25–64.

Brys R, Jacquemyn H, Hermy M, Beeckman T. 2008. Pollen deposition rates and the functioning of distyly in the perennial *Pulmonaria officinalis* (Boraginaceae). *Plant Syst Evol* 273: 1–12.

Bull JJ, Charnov LL. 1985. On irreversible evolution. *Evolution* 39: 1149-1155.

Burns JH, Ashman TL, Steets JA, Harmon-Threatt A, Knight TM. 2011. A phylogenetically controlled analysis of the roles of reproductive traits in plant invasions. *Oecologia* 166: 1009–1017.

Burton TL, Husband BC. 2000. Fitness differences among diploids, tetraploids, and their triploid progeny in *Chamerion angustifolium*: mechanisms of inviability and implications for polyploid evolution. *Evolution* 54: 1182–1191.

Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 25: 1972–1973.

Caperta AD, Castro S, Loureiro J, Róis AS, Conceição S, Costa J, Rhazi L, Espírito-Santo D, Arsénio P. 2016. Biogeographical, ecological and ploidy variation in related asexual and sexual *Limonium* taxa (Plumbaginaceae). *Bot J Linn Soc* doi:10.1111/boj.12498.

Carman JG. 1997. Asynchronous expression of duplicate genes in angiosperms may

cause apomixis, bispory, tetraspory, and polyembryony. *Bot J Linn Soc* 61: 51–94.

Castellanos C, Wilson P, Thompson JD. 2003. Pollen transfer by hummingbirds and bumblebees, and the divergence of pollination modes in *Penstemon*. *Evolution* 57: 2742–2752.

Castro S, Castro M, Ferrero V, Costa J, Tavares D, Navarro L, Loureiro J. 2016. Invasion fosters change: independent evolutionary shifts in reproductive traits after *Oxalis pes-caprae* L. introduction. *Front Plant Sci* 7: 1–13.

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

Castro S, Loureiro J, Santos C, Ater M, Ayensa G, Navarro L. 2007. Distribution of flower morphs, ploidy level and sexual reproduction of the invasive weed *Oxalis pes-caprae* in the western area of the Mediterranean region. *Ann Bot* 99: 507–517.

Castro M, Rosseló JA. 2007. Karyology of *Limonium* (Plumbaginaceae) species from the Balearic Islands and the western Iberian Peninsula. *Bot J Linn Soc* 155: 257-272.

Charlesworth D. 1979. The evolution and breakdown of tristyly. *Evolution* 33: 486-498.

Charlesworth B, Charlesworth D. 1979. A model for the evolution of distyly. *Am Nat* 114: 467–498.

Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annu Rev Ecol Syst* 18: 237–268.

Charlesworth B, Charlesworth D. 2009. Darwin and genetics. *Genetics* 183: 757-766.

Charnov EL, Smith JM, Bull JJ. 1976. Why be an hermaphrodite? *Nature* 263: 125–126.

Cohen JI. 2013. A phylogenetic analysis of morphological and molecular characters of Boraginaceae: evolutionary relationships, taxonomy, and patterns of character evolution. *Cladistics* 30: 1-31.

Colautti RI, Barrett SCH. 2013. Rapid adaptation to climate facilitates range expansion of an invasive plant. *Science* 342: 364–366.

Colautti RI, White NA, Barrett SCH. 2010. Variation of self-incompatibility within invasive populations of purple loosestrife (*Lythrum salicaria* L.) from Eastern North America. *Int J Plant Sci* 171: 158–166.

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 Biosyst* 150: 923–931.

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

Cowan R, Ingrouille MJ, Lledó MD. 1998. The taxonomic treatment of agamosperms in the genus *Limonium* Mill. (Plumbaginaceae). *Folia Geobot* 33: 353–366.

Crespo MB. 2009. A new coastal species of *Limonium* (Plumbaginaceae) from Southeastern Spain. *Folia Geobot* 44: 177–190.

Cunha NL, Fischer E, Lorenz-Lemke AP, Barrett SCH. 2014. Floral variation and environmental heterogeneity in a tristylous clonal aquatic of the Pantanal wetlands of Brazil. *Ann Bot* 114: 1637–1649.

Dafni A, Pacini E, Nepi M. 2005. Pollen and stigma biology. Pp. 83-142 *in* Dafni A, Kevan P, and Husband B, eds. *Pratical Pollination Biology*. Enviroquest, Ontario, Canada.

Dahlgren KVO. 1918. Heterostylie innerhalb der Gattung *Plumbago*. *Svensk Botanisk Tidskrift* 12: 362–372.

D'Amato F. 1940. Contributo all'embriologia delle Plumbaginaceae. *Nuovo Giorn Bot Ital* 47: 349–382.

D'Amato F. 1949. Triploidia e apomissia in *Statice oleafolia* Scop. var. *confusa* Godr. *Caryologia* 401: 157–161.

Darwin C. 1862a. *Fertilization of Orchids by Insects*. John Murray, London, UK.

Darwin C. 1862b. *On the Various Contrivances by Which British and Foreign Orchids Are Fertilised by Insects, and on the Good Effects of Intercrossing*. John Murray, London, UK.

Darwin C. 1864. On the sexual relations of the three forms of *Lythrum salicaria*. *Bot J Linn Soc* 169–196.

Darwin C. 1868. On the character and hybrid-like nature of offspring from the illegitimate unions of dimorphic and trimorphic plants. *Bot J Linn Soc* 393–437.

Darwin C. 1876. *The Effects of Self and Cross Fertilization in the Vegetable Kingdom*. John Murray, London, UK.

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

Dawson HJ, Ingrouille MJ. 1995. A biometric survey of *Limonium vulgare* Miller and *L. humile* Miller in the British Isles. *Watsonia* 20: 239-254.

de Queiroz A, Gatesy J. 2007. The supermatrix approach to systematics. *Trends Ecol*

Evol 22: 34–41.

Devi MP. 1964. Heterostyly in *Biophytum sensitivum* DC. *J Genet* 59: 41–48.

de Vos JM, Hughes CE, Schneeweiss GM, Moore BR, Conti E. 2014. Heterostyly accelerates diversification via reduced extinction in primroses. *Proc R Soc Lond B* 281: 20140075.

Dixon CJ, Schönswetter P, Vargas P, Siegrun E, Schneeweiss GM. 2009. Bayesian hypothesis testing supports long-distance Pleistocene migrations in a European high mountain plant (*Androsace vitaliana*, Primulaceae). *Mol Phylogenet Evol* 53: 580-591.

Doležel J, Sgorbati S, Lucretti S. 1992. Comparison of 3 DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. *Physiol Plantarum* 85: 625-631.

Dollo L. 1893. Les lois de l'évolution. *Bull Soc Belge Géol Paléont Hydrol* 7: 164-166.

Dreyer LL, Esler KJ, Zietsman J. 2006. Flowering phenology of South African *Oxalis* - possible indicator of climate change? *S Afr J Bot* 72: 150–156.

Dulberger R. 1970. Tristyly in *Lythrum junceum*. *New Phytol* 69: 751–759.

Dulberger R. 1974. Structural dimorphism of stigmatic papillae in distylous *Linum* species. *Am J Bot* 61: 238–243.

Dulberger R. 1975a. Intermorph structural differences between stigmatic papillae and pollen grains in relation to incompatibility in Plumbaginaceae. *Proc R Soc Lond B* 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 Syst Evol* 139: 113–119.

Dulberger R. 1992. Floral polymorphisms and their functional significance in the heterostylous syndrome. Pp. 41–84 *in* Barrett SCH, ed. *Evolution and Function of Heterostyly*. Springer-Verlag, Berlin, Germany.

East EM. 1927. The inheritance of heterostyly in *Lythrum salicaria*. *Genetics* 12: 393–414.

Eckert CG. 2000. Contributions of autogamy and geitonogamy to self-fertilization in a mass-flowering, clonal plant. *Ecology* 81: 532–542.

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, Barrett SCH. 1993. Clonal reproduction and patterns of genotypic diversity in *Decodon verticillatus* (Lythraceae). *Am J Bot* 80: 1175–1182.

Eckert CG, Barrett SCH. 1994. Tristyly, self-compatibility and floral variation in *Decodon verticillatus* (Lythraceae). *Biol J Linn Soc* 53: 1–30.

Eckert CG, Barrett SCH. 1995. Style morph ratios in tristylous *Decodon verticillatus* (Lythraceae): selection vs. historical contingency. *Ecology* 76: 1051–1066.

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

Eckert CG, Manicacci D, Barrett SCH. 1996b. Genetic drift and founder effect in native versus introduced populations of an invading plant, *Lythrum salicaria* (Lythraceae). *Evolution* 50: 1512–1519.

Eckert CG, Samis KE, Lougheed SC. 2008. Genetic variation across species' geographical ranges: the central-marginal hypothesis and beyond. *Mol Ecol* 17: 1170–1188.

Eckhart VM. 1991. The effects of floral display on pollinator visitation vary among populations of *Phacelia linearis* (Hydrophyllaceae). *Evol Ecol* 5: 370–384.

eFloras. 2008. Published online: http://www.efloras.org [accessed December 30, 2016] Missouri Botanical Garden, St. Louis, MO & Harvard University Herbaria, Cambridge, MA, USA.

Erben M. 1979. Karyotype differentiation and its consequences in Mediterranean *Limonium*. *Webbia* 34: 409–417.

Erben M. 1993. *Limonium* Mill. Pp. 2–143 *in* Castroviejo S, Laínz M, González G, Montserrat P, Garmendia F, Paiva J, and Villar L, eds. *Flora Iberica - Vol. III*. Real Jardín Botánico, C. S. I.C., Madrid, Spain.

Erdtman G. 1952. *Pollen Morphology and Plant Taxonomy. Angiosperms*. Almquist and Wiksell, Stockholm, Sweden.

Erdtman G. 1960. The acetolysis method. A revised description. *Sven Bot Tidskr* 54: 561-564.

Erdtman G. 1986. *Pollen Morphology and Plant Taxonomy: Angiosperms*. *An introduction to the study of pollen grains and spores.* E. J. Brill, Leiden, The Netherlands.

Faegri K, Van der Pijl L. 1971. *The Principles of Pollination Ecology*. Pergamon Press, New York, USA.

Fausto JA, Eckhart VM, Geber MA. 2001. Reproductive assurance and the evolutionary ecology of self-pollination in *Clarkia xantiana* (Onagraceae). *Am J Bot* 88: 1794–1800.

Feinsinger P, Busby WH. 1987. Pollen carryover: experimental comparisons between morphs of *Palicourea lasiorrachis* (Rubiaceae), a distylous, bird-pollinated, tropical treelet. *Oecologia* 73: 231–235.

Feliner GN. 1990. *Armeria* Willd. Pp. 642–721 *in* Castroviejo S, Laínz M, González G, Montserrat P, Garmendia F, Paiva J, and Villar L, eds. *Flora Iberica – Vol. II*. Real Jardín Botánico, C. S. I.C., Madrid, Spain.

Feliner GN. 2014. Patterns and processes in plant phylogeography in the Mediterranean basin. A review. *PPEES* 16: 265-278.

Felsenstein J. 1985. Phylogenies and the comparative method. *Am Nat* 125: 1–15.

Fernandes A. 1935a. Remarque sur l'hétérostylie de *Narcissus triandrus* L. et de *N. reflexus* Brot. *Bol Soc Brot* Série 2, 10: 5–15.

Fernandes A. 1935b. Les satellites chez *Narcissus reflexus* Brot. et *N. triandrus* L. *Bol Soc Brot* Série 2, 10: 249–277.

Fernandes A. 1964. Contribution à la connaissance de la génétique de l'hétérostylie chez le genre *Narcissus* L. I. Résultats de quelques croisements. *Bol Soc Brot* Série 2, 38: 81–96.

Fernandes A. 1965. Contribution à la connaissance de la génétique de l'hétérostylie chez le genre *Narcissus* L. II. L'hétérostylie chez quelques populations de *N. triandrus* var. *cernuus* et *N. triandrus* var. *concolor*. *Genet Iber* 17: 215–239.

Fernandes A. 1975. L'evolution chez le genre *Narcissus* L. *An Inst Botánico A J Cavanilles* 32: 843–872.

Ferrero V. 2009. Ecología y evolutión del polimorfismo floral en *Lithodora* (Boraginaceae). Doctoral thesis, Universidad de Vigo, Spain.

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). *Ann Bot* 109: 655–665.

Ferrero V, Arroyo J, Vargas P, Thompson JD, Navarro L. 2009a. Evolutionary transitions of style polymorphisms in *Lithodora* (Boraginaceae). *PPEES* 11: 111–125.

Ferrero V, Barrett SCH, Castro S, Caldeirinha P, Navarro L, Loureiro J, Rodríguez-Echeverría S. 2015. Invasion genetics of the Bermuda buttercup (*Oxalis pes-caprae*):

complex intercontinental patterns of genetic diversity, polyploidy and heterostyly characterize both native and introduced populations. *Mol Ecol* 24: 1927–1941.

Ferrero V, Barrett SCH, Rojas D, Arroyo J, Navarro L. 2017. Associations between sex-organ deployment and morph bias in related heterostylous taxa with different stylar polymorphisms. *Am J Bot* in press.

Ferrero V, Castro S, Costa J, Acuña P, Navarro L, Loureiro J. 2013. Effect of invader removal: pollinators stay but some native plants miss their new friend. *Biol Invasions* 15: 2347–2358.

Ferrero V, Castro S, Sánchez JM, Navarro L. 2011b. Stigma–anther reciprocity, pollinators, and pollen transfer efficiency in populations of heterostylous species of *Lithodora* and *Glandora* (Boraginaceae). *Plant Syst Evol* 291: 267–276.

Ferrero V, Chapela I, Arroyo J, Navarro L. 2011a. Reciprocal style polymorphisms are not easly categorised: the case of heterostyly in *Lithodora* and *Glandora* (Boraginaceae). *Plant Biol* 13: 7–18.

Ferrero V, de Vega C, Stafford GI, Van Staden J, Johnson SD. 2009b. Heterostyly and pollinators in *Plumbago auriculata* (Plumbaginaceae). *S Afr J Bot* 75: 778-784.

Fisher RA. 1944. Allowance for double reduction in the calculation of genotype frequencies with polysomic inheritance. *Ann Eugen* 12: 169–171.

Fisher RA, Mather K. 1943. The inheritance of style length in *Lythrum salicaria*. *Ann Eugen* 12: 1–23.

Fox J. 2003. Effect displays in R for generalised linear models. *J Stat Softw* 8: 1-27.

Fox J, Weisberg S. 2015. *car - Companion to applied regression*. R package version 2.0.19. URL http://cran.r-project.org/web/packages/car/index.html

Fryxell PA. 1957. Mode of reproduction of higher plants. *Bot Rev* 23: 135–233.

Fuentes E, Blaise S. 1988. Variabilité morphologique chez *Pulmonaria longifolia* (Bast.) Boreau subsp. *longifolia*. Relations avec la distribution géographique et l'hétérostylie. *Bull Soc Bot Fr - Lettres Bot* 135: 181–190.

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E. 1993. Rapid flow cytometric analysis of the cell-cycle in intact plant-tissues. *Science* 220: 1049-1051.

Ganders FR. 1974. Disassortative pollination in the distylous plant *Jepsonia heterandra*. *Can J Bot* 52: 2401–2406.

Ganders FR. 1976. Pollen flow in distylous populations of *Amsinckia* (Boraginaceae). *Can J Bot* 54: 2530–2535.

Ganders FR. 1979. The biology of heterostyly. *New Zeal J Bot* 17: 607–635.

Garamszegi LZ, Gonzalez-Voyer A. 2014. Working with the three of life in comparative studies: how to build and tailor phylogenies to interspecific datasets. Pp. 19-48 *in* Garamszegi LZ, ed. *Modern Phylogenetic Comparative Methods and their Application in Evolutionary Biology*. Springer-Verlag, Berlin, Germany.

García-Barros E, Gurrea P, Luciáñez MJ, Cano JM, Munguira ML, Moreno JC, Sainz H, Sanz MJ, Simón JC. 2002. Parsimony analysis of endemicity and its application to animal and plant geographical distribtuion in the Ibero-Balearic region (western Mediterranean). *J Biogeogr* 29: 109–124.

García-Ramos G, Rodríguez D. 2002. Evolutionary speed of species invasions. *Evolution* 56: 661–668.

Gasith A, Resh VH. 1999. Streams in Mediterranean climate regions: abiotic influences and biotic responses to predictable seasonal events. *Annu Rev Ecol Syst* 30: 51–81.

Gaskin JF. Tamaricaceae. Pp. 363–368 *in* Kubitzki K, Rohwer JG, and Bittrich V, eds. *The Families and Genera of Vascular Plants*. Springer-Verlag, Berlin, Germany.

Ghobary MOW-E. 1984. The systematic relationships of *Aegialitis* (Plumbaginaceae) as revealed by pollen morphology. *Plant Syst Evol* 144: 53–58.

Ghobary MOW-E. 1986. Dimorphic exine sculpturing in two distylous species of *Dyerophytum* (Plumbaginaceae). *Plant Syst Evol* 152: 267–276.

Ghosh S, Shivanna KR. 1980. Pollen-pistil interaction in *Linum grandiflorum*: scanning electron microscopic observations and proteins of the stigma surface. *Planta* 149: 257–261.

Gil L, Llorens L. 1991. *Limonium barceloi* y *L. bolosii* Gil & Llorens, nuevas especies de la isla de Mallorca (Baleares). *Anales Jard Bot Madrid* 49: 51–56.

Gilks WR, Richardson S, Spiegelhalter DJ. 1996. *Markov Chain Monte Carlo in Practice*. Chapman & Hall/CRC, New York, USA.

Gimeno I, Vilà M, Hulme PE. 2006. Are islands more susceptible to plant invasion than continents? A test using *Oxalis pes-caprae* L. in the western Mediterranean. *J Biogeogr* 33: 1559–1565.

Giraudoux AP. 2014. *pgirmess: Data analysis in ecology*. R package version 1.5.8. URL http://cran.r-project.org/web/packages/pgirmess/index.html

Glover DE, Barrett SCH. 1983. Trimorphic incompatibility in Mexican populations of *Pontederia sagittata* Presl. (Pontederiaceae). *New Phytol* 95: 439–455.

Glover DE, Barrett SCH. 1986. Stigmatic pollen loads in populations of *Pontederia*

cordata from the Southern U.S. *Am J Bot* 73: 1607–1612.

Goldberg EE, Igic B. 2008. On phylogenetic tests of irreversible evolution. *Evolution* 62: 2727-2741.

Gómez A, Lunt DH. 2006. Refugia within refugia: patterns of phylogeographic concordance in Iberian Peninsula. Pp. 155–188 *in* Weiss S, and Ferrand N, eds. *Phylogeography of Southern European Refugia*. Springer-Verlag, Berlin, Germany.

González GL. 1990. *Rumex* L. Pp. 595–634 *in* Castroviejo S, Laínz M, González G, Montserrat P, Garmendia F, Paiva J, and Villar L, eds. *Flora Iberica - Vol. II*. Real Jardín Botánico, C. S. I.C., Madrid, Spain.

Graham SW, Barrett SCH. 2004. Phylogenetic reconstruction of the evolution of stylar polymorphisms in *Narcissus* (Amaryllidaceae). *Am J Bot* 91: 1007–1021.

Guerra AS. *Frankenia* L. 1993. Pp. 446–453 *in* Castroviejo S, Laínz M, González G, Montserrat P, Garmendia F, Paiva J, and Villar L, eds. *Flora Iberica - Vol. III*. Real Jardín Botánico, C. S. I.C., Madrid, Spain.

Guitián J, Guitián P, Medrano M. 1998. Floral biology of the distylous Mediterranean shrub *Jasminum fruticans* (Oleaceae). *Nord J Bot* 18: 195–201.

Haldane J. 1936. Some natural populations of *Lythrum salicaria*. *J Genet* 32: 393–397.

Halkka O, Halkka L. 1974. Polymorphic balance in small island populations of *Lythrum salicaria*. *Ann Bot Fenn* 11: 267–270.

Hall T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41: 95–98.

Hamrick JL, Godt MJW. 1996. Effects of life history traits on genetic diversity in plant species. *Philos T Roy Soc B* 351: 1291–1298.

Han Y, Dai C, Yang C-F, Wang Q-F, Motley TJ. 2008. Anther appendages of *Incarvillea* trigger a pollen-dispensing mechanism. *Ann Bot* 102: 473–479.

Hao JH, Qiang S, Chrobock T, van Kleunen M, Liu QQ. 2011. A test of Baker's law: breeding systems of invasive species of Asteraceae in China. *Biol Invasions* 13: 571–580.

Harder LD, Barclay RMR. 1994. The functional significance of poricidal anthers and buzz pollination: controlled pollen removal from *Dodecatheon*. *Funct Ecol* 8: 509–517.

Harder LD, Barrett SCH. 1993. Pollen removal from tristylous *Pontederia cordata*: effects of anther position and pollinator specialization. *Ecology* 74: 1059–1072.

Harder LD, Barrett SCH. 1996. Pollen dispersal and mating patterns in animal-

pollinated plants. Pp. 140–190 *in* Lloyd DG, and Barrett SCH, eds. *Floral Biology: Studies on Floral Evolution in Animal-Pollinated Plants*. Chapman and Hall, New York, USA.

Harder LD, Johnson SD. 2008. Function and evolution of aggregated pollen in Angiosperms. *Int J Plant Sci* 169: 59–78.

Harder LD, Johnson SD. 2009. Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. *New Phytol* 183: 530–545.

Harmon-Threatt AN, Burns JH, Shemyakina LA, Knight TM. 2009. Breeding system and pollination ecology of introduced plants compared to their native relatives. *Am J Bot* 96: 1544–1550.

Harmon LJ, Weir JT, Brock CD, Glor RE, Challenger W. 2008. GEIGER: investigating evolutionary radiations. *Bioinformatics* 24: 129–131.

Hartley ML, Mekeng ET, Thomas SM. 2002. Functional heterostyly in *Tylosema esculentum* (Caesalpinoideae). *Ann Bot* 89: 67–76.

Hengeveld R. 1990. *Dynamic Biogeography*. Cambridge University Press, Cambridge, UK.

Henriques J. 1887. Observações sobre algumas espécies de *Narcissus*, encontrados em Portugal. *Bol Soc Brot* 5: 168–174.

Henriques J. 1888. Additamento ao catálogo das Amaryllideas de Portugal. *Bol Soc Brot* 6: 45–47.

Herrera CM. 1987. Components of pollinator ''quality'': comparative analysis of a diverse insect assemblage. *Oikos* 50: 79–90.

Heslop-Harrison J, Heslop-Harrison Y. 1985. Surfaces and secretions in the pollen-stigma interaction – a brief review. *J Cell Sci* Suppl 2: 287-300.

Heuch I. 1979a. Equilibrium populations of heterostylous plants. *Theor Popul Biol* 15: 43–57.

Heuch I. 1979b. The effect of partial self-fertilization on type frequencies in heterostylous plants. *Ann Bot* 44: 611–616.

Heuch I. 1980. Loss of incompatibility types in finite populations of the heterostylous plant *Lythrum salicaria*. *Hereditas* 92: 53–57.

Heuch I, Lie RT. 1985. Genotype frequencies associated with incompatibility systems in tristylous plants. *Theor Popul Biol* 27: 318–336.

Hewitt GM. 1996. Some genetic consequences of ice ages, and their role, in divergence and speciation. *Biol J Linn Soc* 58: 247–276.

Hodgins KA, Barrett SCH. 2006a. Female reproductive success and the evolution of mating-type frequencies in tristylous populations. *New Phytol* 171: 569–580.

Hodgins KA, Barrett SCH. 2006b. Mating patterns and demography in the tristylous daffodil *Narcissus triandrus*. *Heredity* 96: 262–270.

Hodgins KA, Barrett SCH. 2007. Population structure and genetic diversity in tristylous *Narcissus triandrus*: insights from microsatellite and chloroplast DNA variation. *Mol Ecol* 16: 2317–2332.

Hodgins KA, Barrett SCH. 2008a. Geographic variation in floral morphology and style-morph ratios in a sexually polymorphic daffodil. *Am J Bot* 95: 185–195.

Hodgins KA, Barrett SCH. 2008b. Asymmetrical mating patterns and the evolution of biased style-morph ratios in a tristylous daffodil. *Genet Res* 90: 3–15.

Hodgins KA, Barrett SCH. 2008c. Natural selection on floral traits through male and female function in wild populations of the heterostylous daffofil *Narcissus triandrus*. *Evolution* 62: 1751–1763.

Hodgins KA, Stehlik I, Wang P, Barrett SCH. 2007. The development of eight microsatellite loci in the wild daffodil *Narcissus triandrus* (Amaryllidaceae). *Mol Ecol Notes* 7: 510-512.

Hollingsworth ML, Bailey JP. 2000. Evidence for massive clonal growth in the invasive weed *Fallopia japonica* (Japanese knotweed). *Bot J Linn Soc* 133: 463–472.

Hothorn T, Bretz F, Westfall P, Heiberger RM, Schuetzenmeister A, Scheibe S. 2015. *multcomp: Simultaneous inference in general parametric models*. R package version 1.3.1. URL http://cran.r-project.org/web/packages/multcomp/index.html

Huelsenbeck JP, Larget B, Miller RE, Ronquist F. 2002. Potential applications and pitfalls of Bayesian inference of phylogeny. *Syst Biol* 51: 673–688.

Huelsenbeck JP, Nielsen R, Bollback JP. 2003. Stochastic mapping of morphological characters. *Syst Biol* 52: 131–158.

Husband BC, Barrett SCH. 1992. Genetic drift and the maintenance of the style length polymorphism in tristylous populations of *Eichhornia paniculata* (Pontederiaceae). *Heredity* 69: 440–449.

Husband BC, Barrett SCH. 1998. Spatial and temporal variation in population size of *Eichhornia paniculata* in ephemeral habitats: implications for metapopulation dynamics. *J Ecol* 86: 1021–1031.

Huu CN, Kappel C, Keller B, Sicard A, Takebayashi Y, Breuninger H, Nowak MD, Bäurle I, Himmelbach A, Burkart M, Ebbing-Lohaus T, Sakakibara H, Altschmied

L, Conti E, Lenhard M. 2016. Presence versus absence of CVP734A50 underlies the style length dimorphism in primroses. *eLife* 5: e17956.

Igic B, Bohs L, Kohn JR. 2006. Ancient polymorphism reveals unidirectional breeding system shifts. *P Natl Acad Sci USA* 103: 1359-1363.

Igic B, Lande R, Kohn JR. 2008. Loss of self-incompatibility and its evolutionary consequences. *Int J Plant Sci* 169: 93-104.

Ingrouille MJ. 1984. A taxometric analysis of *Limonium* (Plumbaginaceae) in Western Europe. *Plant Syst Evol* 147: 103-118.

Ingrouille MJ, Stace CA. 1985. Pattern of variation of agamospermous *Limonium* (Plumbaginaceae) in the British Isles. *Nord J Bot* 5: 113–125.

Inouye DW, Gill DE, Dudash MR, Fenster CB. 1994. A model and lexicon for pollen fate. *Am J Bot* 81: 1517–1530.

Iversen J. 1940. Blütenbiologische Studien. I. Dimorphie und monomorphie bei *Armeria*. *K Danse Vidensk Selsk Skr* 15: 1–39.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 30: 772–780.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28: 1647–1649.

King VM, Sargent RD. 2012. Presence of an invasive plant species alters pollinator visitation to a native. *Biol Invasions* 14: 1809–1818.

Kissling J, Barrett SCH. 2013. Variation and evolution of herkogamy in *Exochaenium* (Gentianaceae): implications for the evolution of distyly. *Ann Bot* 112: 95–102.

Kohn JR, Barrett SCH. 1992. Experimental studies on the functional significance of heterostyly. *Evolution* 46: 43–55.

Kolář F, Štech M, Trávníček P, Rauchová J, Urfus T, Vít P, Kubešová 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. *Ann Bot* 103: 963-974.

Kottek M, Grieser J, Beck C, Rudolf B, Rubel F. 2006. World map of the Köppen-Geiger climate classification updated. *Meteorol Z* 15: 259–263.

Krejčiková J, Sudová R, Oberlander KC, Dreyer LL, Suda J. 2013. The ploidy enigma of *Oxalis pes-caprae*: where do the invasive pentaploids come from? *Biol Invasions*

15: 1189-1194.

Kress A. 1997. *Primula*. Pp 10-21 *in* Castroviejo S, Laínz M, González G, Montserrat P, Garmendia F, Paiva J, and Villar L, eds. *Flora Iberica - Vol V*. Real Jardín Botánico, C. S. I.C., Madrid, Spain.

Kreyer D, Oed A, Walther-Hellwig K, Frankl R. 2004. Are forests potential landscape barriers for foraging bumblebees? Landscape scale experiments with *Bombus terrestris* agg. and *Bombus pascuorum* (Hymenoptera, Apidae). *Biol Conserv* 116: 111–118.

Kubitzki K. 1993. Plumbaginaceae. Pp. 523–530 *in* Kubitzki K, Rohwer JG, and Bittrich V, eds. *The Families and Genera of Vascular Plants*. Springer-Verlag, Berlin, Germany.

Kück P, Meusemann K. 2010. FASconCAT. Forschungsmuseum A. Koenig, Germany.

Lebuhn G, Anderson GJ. 1994. Anther tripping and pollen dispensing in *Berberis thunbergii*. *Am Midl Nat* 131: 257–265.

Lemmens R. 1989. Heterostyly. Pp. 56–69 *in* Jongkind C, and Lemmens R, eds. *The Connaraceae - A Taxonomic Study with Special Emphasis on Africa*. University of Wageningen, Wageningen, The Netherlands.

Lesica P, Allendorf FW. 1995. When are peripheral populations valuable for conservation? *Conserv Biol* 9: 753–760.

Levin DA. 1968. The breeding system of *Lithospermum caroliniense*: adaptation and counteradaptation. *Am Nat* 102: 427–441.

Levin DA. 1996. The evolutionary significance of pseudo-self-fertility. *Am J Bot* 148: 321–332.

Lewis D. 1982. Incompatibility, stamen movement and pollen economy in a heterostyled tropical forest tree, *Cratoxylum formosum* (Guttiferae). *Proc R Soc Lond B* 214: 273–283.

Lewis D, Jones DA. 1992. The genetics of heterostyly. Pp. 129–150 *in* Barrett SCH, ed. *Evolution and Function of Heterostyly*. Springer-Verlag, Berlin, Germany.

Lewis D, Rao AN. 1971. Evolution of dimorphism and population polymorphism in *Pemphis acidula* Forst. *Proc R Soc Lond B* 178: 79-94.

Li J, Cocker JM, Wright J, Webster MA, McMullan M, Dyer S, Swarbreck D, Caccamo M, van Oosterhout C, Gilmartin PM. 2016. Genetic architecture and evolution of the *S* locus supergene in *Primula vulgaris*. *Nat Plants* 2: 16188.

Li J, Webster M, Furuya M, Gilmartin PM. 2007. Identification and characterization of

pin and thrum alleles of two genes that co-segregate with the *Primula S* locus. *Plant J* 51: 18–31.

Li A-M, Wu X-Q, Zhang D-X, Barrett SCH. 2010. Cryptic dioecy in *Mussaendra pubescens* (Rubiaceae): a species with stigma-height dimorphism. *Ann Bot* 106: 521-531.

Lledó MD, Crespo MB, Cameron KM, Fay MF, Chase W. 1998. Systematics of Plumbaginaceae based upon cladistic analysis of *rbcL* sequence data. *Syst Bot* 23: 21–29.

Lledó MD, Crespo MB, Cox AV, Fay MF, Chase MW. 2000. Polyphyly of *Limoniastrum* (Plumbaginaceae): evidence from DNA sequences of plastid *rbcL*, *trnL* intron and *trnL-F* intergene spacer. *Bot J Linn Soc* 132: 175–191.

Lledó MD, Crespo MB, Fay MF, Chase MW. 2005. Molecular phylogenetics of *Limonium* and related genera (Plumbaginaceae): biogeographical and systematic implications. *Am J Bot* 92: 1189–1198.

Lledó MD, Karis PO, Crespo MB, Fay MF, Chase MW. 2001. Phylogenetic position and taxonomic status of the genus *Aegialitis* and subfamilies Staticoideae and Plumbaginoideae (Plumbaginaceae): evidence from plastid DNA sequences and morphology. *Plant Syst Evol* 229: 107–124.

Llorens L, Tébar J. 1988. *Limonium escarrei* y *Limonium boirae* Llorens & Tébar, dos nuevas especies de la Isla de Mallorca (Baleares). *Anales Jard Bot Madrid* 45: 173–180.

Lloyd DG. 1992. Self- and cross-fertilization in plants. II. The selection of self-fertilization. *Int J Plant Sci* 153: 370–380.

Lloyd DG, Schoen DJ. 1992. Self- and cross-fertilization in plants I. Functional dimensions. *Int J Plant Sci* 153: 358–369.

Lloyd DG, Webb CJ. 1986. The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. *New Zeal J Bot* 24: 135–162.

Lloyd DG, Webb CJ. 1992a. The evolution of heterostyly. Pp. 151–178 *in* Barrett SCH, ed. *Evolution and Function of Heterostyly*. Springer-Verlag, Berlin, Germany.

Lloyd DG, Webb CJ. 1992b. The selection of heterostyly. Pp. 179–208 *in* Barrett SCH, ed. *Evolution and Function of Heterostyly*. Springer-Verlag, Berlin, Germany.

López-García M, Maillet J. 2005. Biological characteristics of an invasive South African species. *Biol Invasions* 7: 181–194.

Loureiro J, Rodriguez E, Doležel J, Santos C. 2007. Two new nuclear isolation buffers

for plant DNA flow cytometry: a test with 37 species. *Ann Bot* 100: 875-888.

Lozano FD, Herbada DG, Rivero LM, Saiz JCM, Ollero HS. 2000. Areas of high floristic endemism in Iberia and the Balearic islands: an approach to biodiversity conservation using narrow endemics. *Belgian J Entomol* 2: 171–185.

Lughadha EMN, Parnell JAN. 1989. Heterostyly and gene-flow in *Menyanthes trifoliata* L. (Menyanthaceae). *Biol J Linn Soc* 100: 337–354.

Mable BK, Robertson AV, Dart S, Di Berardo C, Witham L. 2005. Breakdown of self-incompatibility in the perennial *Arabidopsis lyrata* (Brassicaceae) and its genetic consequences. *Evolution* 59: 1437–1448.

Maddison WP, Maddison DR. 2015. Mesquite: a modular system for evolutionary analysis. Version 3.04. Available online: http://mesquiteproject.org

Mal TK, Doust JL, Doust LL, Mulligan GA. 1992. The biology of Canadian weeds. 100. *Lythrum salicaria*. *Can J Bot* 72: 1305–1330.

Marco DE, Arroyo MTK. 1998. The breeding system of *Oxalis squamata*, a tristylous South American species. *Bot Acta* 111: 497–504.

Massinga PH, Johnson SD, Harder LD. 2005. Heteromorphic incompatibility and efficiency of pollination in two distylous *Pentanisia* species (Rubiaceae). *Ann Bot* 95: 389–399.

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

Mast AR, Kelso S, Richards AJ, Lang DJ, Feller DMS, Conti E. 2001. Phylogenetic relationships in *Primula* L. and related genera (Primulaceae) based on noncoding chloroplast DNA. *Int J Plant Sci* 162: 1381-1400.

Mather K. 1950. The genetic architecture of heterostyly in *Primula sinensis*. *Evolution* 4: 340–352.

Mather K, De Winton D. 1941. Adaptation and counter-adaptation of the breeding system in *Primula*. *Ann Bot* 5: 297–311.

Mattsson O. 1983. The significance of exine oils in the initial interaction between pollen and stigma in *Armeria maritima*. Pp. 257–264 *in* Mulcahy DL, and Ottaviano E, eds. *Pollen Biology and Implications for Plant Breeding*. Elsevier Science Publishing, Amsterdam, The Netherlands.

Matsumura C, Washitani I. 2002. Heterostylous morph differences in pollen transfer and deposition patterns in *Primula sieboldii* on a visitation by a queen bumblebee, measured with a semi-natural experimental system. *Plant Species Biol* 17: 1–12.

McCubbin AG, Lee C, Hetrick A. 2006. Identification of genes showing differential expression between morphs in developing flowers of *Primula vulgaris*. *Sex Plant Reprod* 19: 63–72.

McDade LA, Daniel TF, Kiel CA. 2008. Toward a comprehensive understanding of phylogenetic relationships among lineages of Acanthaceae *s.l.* (Lamiales). *Am J Bot* 95: 1136–1152.

McDill JR, 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. *Syst Bot* 34: 386-405.

McDill JR, Simpson BB. 2011. Molecular phylogenetics of Linaceae with complete generic sampling and data from two plastid genes. *Bot J Linn Soc* 165: 64–83.

Médail F, Quézel P. 1999. Biodiversity hotspots in the Mediterranean basin: setting global conservation priorities. *Conserv Biol* 13: 1510-1513.

Melampy MN, Howe HF. 1977. Sex ratio in the tropical tree *Triplaris americana* (Polygonaceae). *Evolution* 31: 867-872.

Michael PW. 1964. The identity and origin of varieties of *Oxalis pes-caprae* L. naturalizad in Australia. *Trans Roy Soc S Aust* 88: 167–173.

Miller MA, Pfeiffer W, Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Pp. 1–8 *in Proceedings of the Gateway Computing Environments Workshop* (GCE). New Orleans, USA.

Moharrek F, Kazempour S, Assadi M. 2014. Molecular phylogeny of Plumbaginaceae with emphasis on *Acantholimon* Boiss. based on nuclear and plastid DNA sequences in Iran. *Biochem Syst Ecol* 57: 117–127.

Moravcová L, Pyšek P, Jarošík V, Pergl J. 2015. Getting the right traits: reproductive and dispersal characteristics predict the invasiveness of herbaceous plant species. *PLoS One* 10: e0123634.

Morgan MT, Barrett SCH. 1988. Historical factors and anisoplethic populations structure in tristylous *Pontederia cordata*: a reassessment. *Evolution* 42: 496–504.

Morretti F, Puppi G, Giuliani C, Conti F. 2015. Heterostyly in *Goniolimon italicum* (Plumbaginaceae), endemic to Abruzzo (central Apennines, Italy). *Anales Jard Bot Madrid* 72: 1–5.

Myers N, Mittermeier RA, Mittermeier CG, Fonseca GAB, Kent J. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853–858.

Mulcahy DL, Caporello D. 1970. Pollen flow within a tristylous species: *Lythrum*

salicaria. *Am J Bot* 57: 1027–1030.

Münkemüller T, Lavergne S, Bzeznik B, Dray S, Jombart T, Schiffers K, Thuiller W. 2012. How to measure and test phylogenetic signal. *Methods Ecol Evol* 3: 743–756.

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

Ne'eman G, Andreas J, Newstrom-Lloyd L, Potts SG, Dafni A. 2010. A framework for comparing pollinator performance: effectiveness and efficiency. *Biol Rev* 85: 435-451.

Ness RW, Siol M, Barrett SCH. 2012. Genomic consequences of transitions from cross- to self-fertilization on the efficacy of selection in three independently derived selfing plants. *BMC Genom* 13: 611.

Ness RW, Wright SI, Barrett SCH. 2010. Mating-system variation, demographic history and patterns of nucleotide diversity in the tristylous plant *Eichhornia paniculata*. *Genetics* 184: 381–392.

Nicholls MS. 1985. Pollen flow, population composition, and the adaptive significance of distyly in *Linum tenuifolium* L. (Linaceae). *Biol J Linn Soc* 25: 235–242.

Nicholls MS. 1986. Population composition, gender specialization, and the adaptative significance of distyly in *Linum perenne* (Linaceae). *New Phytol* 102: 209–217.

Nicholls MS. 1987. Pollen flow, self-pollination and gender specialization: factors affecting seed-set in the tristylous species *Lythrum salicaria* (Lythraceae). *Plant Syst Evol* 156: 151–157.

Nishihiro J, Washitani I. 1998. Patterns and consequences of self-pollen deposition on stigmas in heterostylous *Persicaria japonia* (Polygonaceae). *Am J Bot* 85: 352–359.

Nishihiro J, Washitani I, Thomson JD, Thomson BA. 2000. Patterns and consequences of stigma height variation in a natural population of a distylous plant, *Primula sieboldii*. *Funct Ecol* 14: 502–512.

Nowak MD, Russo G, Schlapbach R, Huu CN, Lenhard M, Conti E. 2015. The draft genome of *Primula veris* yields insights into the molecular basis of heterostyly. *Genome Biol* 16: 12.

Olesen JM. 1979. Floral morphology and pollen flow in the heterostylous species *Pulmonaria obscura* Dumort (Boraginaceae). *New Phytol* 82: 757–767.

Olesen JM, Dupont YL, Ehlers BK, Valido A, Hansen DM. 2003. Heterostyly in the Canarian endemic *Jasminum odoratissimum* (Oleaceae). *Nord J Bot* 23: 537–539.

Oliver JD. 1993. A review of the biology of giant Salvinia (*Salvinia molesta* Mitchell).

J Aquat Plant Manage 31: 227–231.

O'Neil P. 1992. Variation in male and female reproductive success among floral morphs in the tristylous plant *Lythrum salicaria* (Lythraceae). *Am J Bot* 79: 1024–1030.

Opler PA, Baker HG, Frankie GW. 1975. Reproductive biology of some Costa Rican *Cordia* species (Boraginaceae). *Biotropica* 7: 234-247.

Ornduff R. 1964. The breeding system of *Oxalis suksdorfii*. *Am J Bot* 51: 307–314.

Ornduff R. 1966. The origin of dioecism from heterostyly in *Nymphoides* (Menyanthaceae). *Evolution* 20: 309–314.

Ornduff R. 1970. Incompatibility and the pollen economy of *Jepsonia parryi*. *Am J Bot* 57: 1036–1041.

Ornduff R. 1971. The reproductive system of *Jepsonia heterandra*. *Evolution* 25: 300–311.

Ornduff R. 1972. The breakdown of trimorphic incompatibility in *Oxalis* section *Corniculatae*. *Evolution* 26: 52–65.

Ornduff R. 1974. Heterostyly in South African flowering plants: a conspectus. *J S Afr Bot* 40: 169–187.

Ornduff R. 1975a. Heterostyly and pollen flow in *Hypericum aegypticum* (Guttiferae). *Biol J Linn Soc* 71: 51-57.

Ornduff R. 1975b. Pollen flow in *Lythrum junceum*, a tristylous species. *New Phytol* 75: 161–166.

Ornduff R. 1979. Pollen flow in a population of *Primula vulgaris* Huds. *Bot J Linn Soc* 78: 1–10.

Ornduff R. 1980a. Pollen flow in *Primula veris* (Primulaceae). *Plant Syst Evol* 135: 89–93.

Ornduff R. 1980b. Heterostyly, population composition, and pollen flow in *Hedyotis caerulea*. *Am J Bot* 67: 95–103.

Ornduff R. 1987. Reproductive systems and chromosome races of *Oxalis pes-caprae* L. and their bearing on the genesis of a noxious weed. *Ann Mo Bot Gard* 74: 79–84.

Ornduff R. 1992. Historical perspective on heterostyly. Pp. 31–39 *in* Barrett SCH, ed. *Evolution and Function of Heterostyly*. Springer-Verlag, Berlin, Germany.

Pagel M. 1999. Inferring the historical patterns of biological evolution. *Nature* 401: 877–884.

Pagel M, Meade A, Barker D. 2004. Bayesian estimation of ancestral character states on

phylogenies. *Syst Biol* 53: 673–684.

Pailler T, Humeau L, Thompson JD. 1998. Distyly and heteromorphic incompatibility in oceanic island species of *Erythroxylum* (Erythroxylaceae). *Plant Syst Evol* 213: 187–198.

Pailler T, Thompson JD. 1997. Distyly and variation in heteromorphic incompatibility in *Gaertnera vaginata* (Rubiaceae) endemic to La Reunion Island. *Am J Bot* 84: 315–327.

Pailler T, Maurice S, Thompson JD. 2002. Pollen transfer patterns in a distylous plant with overlapping pollen-size distributions. *Oikos* 99: 308–316.

Palacios C, González-Candelas F. 1997. Analysis of population genetic structure and variability using RAPD markers in the endemic and endangered *Limonium dufourii* (Plumbaginaceae). *Mol Ecol* 6: 1107-1121.

Palacios C, Rosselló JA, González-Candelas F. 2000. Study of the evolutionary relationships among *Limonium* species (Plumbaginaceae) using nuclear and cytoplasmic molecular markers. *Mol Phylogenet Evol* 14: 232–249.

Palob-Esteban M, Segarra-Moragues JG, González-Candelas F. 2007. Historical and biological determinants of genetic diversity in the highly endemic triploid sea lavender *Limonium dufourii* (Plumbaginaceae). *Mol Ecol* 16: 3814-3827.

Palob-Esteban M, Segarra-Moragues JG, González-Candelas F. 2011. Polyploid origin, genetic diversity and population structure in the tetraploid sea lavender *Limonium narbonense* Miller (Plumbaginaceae) from eastern Spain. *Genetica* 139: 1309-1322.

Pannell JR, Auld JR, Brandvain Y, Burd M, Busch JW, Cheptou P, Conner JK, Goldberg EE, Grant A, Grossenbacher DL, Hovick SM, Igic B, Kalisz S, Petanidou T, Randle AM, de Casas RR, Pauw A, Vamosi JC. 2015. The scope of Baker ’s law. *New Phytol* 208: 656–667.

Paradis E. 2006. *Analysis of Phylogenetics and Evolution with R*. Springer, New York, USA.

Paradis E. 2013. Molecular dating of phylogenies by likelihood methods: a comparison of models and a new information criterion. *Mol Phylogenet Evol* 67: 436–444.

Paradis E, Claude J, Strimmer K. 2004. APE: analysis of phylogenetics and evolution in R language. *Bioinformatics* 20: 289–290.

Parga IC, Saiz JCM, Humphries CJ, Williams PH. 1996. Strengthening the Natural and National Park system of Iberia to conserve vascular plants. *Bot J Linn Soc* 121: 189–205.

Partridge JW. 2001. Biological flora of the British Isles: *Persicaria amphibia* (L.) Gray (*Polygonum amphibium* L.). *J Ecol* 89: 487-501.

Pechuman LL. 1967. Observations on the behavior of the bee *Anthidium manicatum* (L.). *J New York Entomol S* 75: 68–73.

Pedraja OS. 2015. *Oxalis* L. Pp. 383–405 *in* Castroviejo S, Laínz M, González G, Montserrat P, Garmendia F, Paiva J, and Villar L, eds. *Flora Iberica - Vol. IX*. Real Jardín Botánico, C. S. I.C., Madrid, Spain.

Pérez R, Vargas P, Arroyo J. 2003. Convergent evolution of flower polymorphism in *Narcissus* (Amaryllidaceae). *New Phytol* 161: 235–252.

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 Phytol* 171: 553–67.

Petanidou T, Godfree RC, Song DS, Kantsa A, Dupont YL, Waser NM. 2012. Self-compatibility and plant invasiveness: comparing species in native and invasive ranges. *PPEES* 14: 3–12.

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Development Team. 2015. *nlme: Linear and nonlinear mixed effects models*. R package version 3.1-122. URL http://cran.r-project.org/web/packages/nlme/index.html

Piper J, Charlesworth B. 1986. The evolution of distyly in *Primula vulgaris*. *Biol J Linn Soc* 29: 123–137.

Prather LA, Ferguson CJ, Jansen RK. 2000. Polemoniaceae phylogeny and classification: implications of sequence data from the chloroplast gene *ndhF*. *Am J Bot* 87: 1300–1308.

Price SD, Barrett SCH. 1982. Tristyly in *Pontederia cordata* (Pontederiaceae). *Can J Bot* 60: 897–905.

Punt W, Hoen PP, Blackmore S, Nilsson S, Le Thomas A. 2007. Glossary of pollen and spore terminology. *Rev Paleobot Palyno* 143: 1-81.

Pütz N. 1994. Vegetative spreading of *Oxalis pes-caprae* (Oxalidaceae). *Plant Syst Evol* 191: 57–67.

Pyšek P, Richardson DM. 2007. Traits associated with invasiveness in alien plants: where do we stand? Pp. 99-126 *in* Nentwig W, ed. *Biological Invasions, Ecological Studies*. Springer-Verlag: Berlin, Germany.

R Core Development Team. 2013. *A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rambaut A. 2008. *FigTree - Tree Figure Drawing Tool*. Edinburgh. Version 1.2. Available online: http://tree.bio.ed.ac.uk/software/figtree/

Rambaut A, Suchard MA, Xie W, Drummond AJ. 2013. *Tracer - MCMC Trace Analysis Tool*. Edinburgh. Version 1.6. Available online: http://tree.bio.ed.ac.uk/software/tracer/

Rambuda TD, Johnson SD. 2004. Breeding systems of invasive alien plants in South Africa: does Baker's rule apply? *Divers Distrib* 10: 409-416.

Ramsey J, Schemske DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annu Rev Ecol Syst* 29: 467–501.

Rechinger KH. 1974. Plumbaginaceae. *in Flora des Iranischen Hochlandes und der Umrahmenden Gebirge – Vol. 108.* Akademische Druck-u. Verlagsanstalt, Graz, Austria.

Ree RH. 1997. Pollen flow, fecundity, and the adaptive significance of heterostyly in *Palicourea padifolia* (Rubiaceae). *Biotropica* 29: 298–308.

Reith M, Baumann G, Classen-Bockhoff R, Speck T. 2007. New insights into the functional morphology of the lever mechanism of *Salvia pratensis* (Lamiaceae). *Ann Bot* 100: 393–400.

Revell LJ. 2012. phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol Evol* 3: 217–223.

Richards A, Mitchell J. 1990. The control of incompatibility in distylous *Pulmonaria affinis* Jordan (Boraginaceae). *Bot J Linn Soc* 104: 369–380.

Richards JH, Koptur S. 1993. Floral variation and distyly in *Guettarda scabra* (Rubiaceae). *Am J Bot* 80: 31–40.

Risso-Pascotto C, Pagliarini MS, Do Valle CB, Mendes-Bonato AB. 2003. Chromosome number and microsporogenesis in a pentaploid accession of *Brachiaria brizantha* (Gramineae). *Plant Breed* 122: 136–140.

Rivas-Martinéz S, Penas A, Díaz TE. 2004. *Biogeographic Map of Europe*. Cartographic Service, University of Léon, Spain. Available online: http://www.globalbioclimatics.org/form/bg_med.htm [Last time accessed September 30, 2016].

Rodriguez-Puebla C, Encinas A, Nieto S, Garmendia J. 1998. Spatial and temporal patterns of annual precipitation variability over the Iberian Peninsula. *Int J Climatol* 18: 299–316.

Rogers CM. 1979. Distyly and pollen dimorphism in *Linum suffruticosum* (Linaceae).

Plant Syst Evol 131: 127–132.

Rogers CM. 2009. Pollen dimorphism in distylous species of *Linum* sect. *Linastrum* (Linaceae). *Grana* 19: 19–20.

Roiloa SR, Rodríguez-Echeverría S, de la Peña E, Freitas H. 2010. Physiological integration increases the survival and growth of the clonal invader *Carpobrotus edulis*. *Biol Invasions* 12: 1815–1823.

Róis AS. 2014. Strategies for conservation of rare and endemic species: characterization of genetic and epigenetic variation and unusual reproductive biology of coastal species from *Limonium ovalifolium* and *Limonium binervosum* complexes (Plumbaginaceae). Doctoral thesis, University of Lisbon, Portugal.

Róis AS, Sádio F, Paulo OS, Teixeira G, Paes AP, Espírito-Santo D, Sharbel TF, Caperta AD. 2016. Phylogeography and modes of reproduction in diploid and tetraploid halophytes of *Limonium* species (Plumbaginaceae): evidence for a pattern of geographical parthenogenesis. *Ann Bot* 117: 37–50.

Ronquist F. 2004. Bayesian inference of character evolution. *Trends Ecol Evol* 19: 475–481.

Roquet C, Thuiller W, Lavergne S. 2013. Building megaphylogenies for macroecology: taking up the challenge. *Ecography* 36: 13–26.

Rosato M, Rosseló JA, Fraga P, Ferrer P, Laguna E. 2012. IAPT/IOPB chromosome data 14 – Plumbaginaceae. *Taxon* 61: 1336-1345.

Roskov Y, Abucay L, Orrell T, Nicolson D, Flann C, Bailly N, Kirk P, Bourgoin T, DeWalt RE, Decock W, and De Wever A, eds. 2016. *Species 2000 & ITIS Catalogue of Life, 2016 Annual Checklist*. Digital resource at www.catalogueoflife.org/annual-checklist/2016 Species 2000: Naturalis, Leiden, the Netherlands. ISSN 2405-884X.

Sagarin RD, Gaines SD, Gaylord B. 2006. Moving beyond assumptions to understand abundance distributions across the ranges of species. *Trends Ecol Evol* 21: 524–530.

Sage TL, Strumas F, Cole WW, Barret SCH. 1999. Differential ovule development following self- and cross-pollination: the basis of self-sterility in *Narcissus triandrus* (Amaryllidaceae). *Am J Bot* 86: 855–870.

Sáez L, Rosselló JA. 1996. *Limonium inexpectans* (Plumbaginaceae), a new apomictic species from Mallorca (Balearic Islands). *Anales Jard Bot Madrid* 54: 285–289.

Salinas MJ. 2009. *Linum.* Pp. 262–268 *in* Blanca G, Cabezudo B, Cueto M, López CF, and Torres CM, eds. *Flora Vascular de Andalucía Oriental – Vol. II*. Consejería de Medio Ambiente, Andalucía, Spain.

Salter TM. 1944. The genus *Oxalis* in South Africa – a taxonomic revision. *J S Afr Bot* Suppl 1: 355.

Sánchez JM, Ferrero V, Arroyo J, Navarro L. 2010. Patterns of style polymorphism in five species of the South African genus *Nivenia* (Iridaceae). *Ann Bot* 106: 321–331.

Sánchez JM, Ferrero V, Navarro L. 2008. A new approach to the quantification of degree of reciprocity in distylous (*sensu lato*) plant populations. *Ann Bot* 102: 463–472.

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

Sánchez R, Serra F, Tárraga J, Medina I, Carbonell J, Pulido L, De María A, Capella-Gutíerrez S, Huerta-Cepas J, Gabaldón T, Dopazo J, Dopazo H. 2011. Phylemon 2.0: a suite of web-tools for molecular evolution, phylogenetics, phylogenomics and hypotheses testing. *Nucleic Acids Res* 39: 1–5.

Santos-Gally R, Pérez-Barrales R, Simón VI, Arroyo J. 2013. The role of short-tongued insects in floral variation across the range of a style-dimorphic plant. *Ann Bot* 111: 317–28.

Schill R, Baumm A, Wolter M. 1985. Comparative micromorphology of stigma surfaces in angiosperms; relations to pollen grain surfaces in heterostylous taxa. *Plant Syst Evol* 148: 185–214.

Schoch-Bodmer H. 1938. The proportion of long-, mid- and short-styled plants in natural populations of *Lythrum salicaria* L. *J Genet* 36: 39–46.

Schoen DJ, Brown AHD. 1991. Intraspecific variation in population gene diversity and effective population size correlates with the mating system in plants. *P Natl Acad Sci USA* 88: 4494–4497.

Schou O. 1983. The distyly in *Primula elatior* (L.) Hill (Primulaceae), with a study of flowering phenology and pollen flow. *Bot J Linn Soc* 86: 261-274.

Sexton JP, McIntyre PJ, Angert AL, Rice KJ. 2009. Evolution and ecology of species range limits. *Annu Rev Ecol Evol Syst* 40: 415–436.

Signorini MA, Giovampaola ED, Bruschi P, Foggi B, Tani C. 2013. Karyological investigations on the South African invasive *Oxalis pes-caprae* L. (Oxalidaceae) in native and invaded areas, with special focus on Italy. *Plant Biosyst* 147: 298–305.

Simón-Porcar VI, Picó FX, Arroyo J. 2015. Range-wide population genetics and variation in morph ratio in style-dimorphic *Narcissus papyraceus*. *Am J Bot* 102: 449-456.

Soltis DE, Soltis PS, Chase MW, Mort ME, Albach DC, Zanis M, Savolainen V, Hahn WH, Hoot SB, Fay MF, Axterll M, Swensen SM, Prince LM, Kress WJ, Nixon KC, Farris JS. 2000. Angiosperm phylogeny inferred from 18S rDNA, *rbcL,* and *atpB* sequences. *Bot J Linn Soc* 133: 381–461.

Sosenski P, Fornoni J, Molina-Freaner FE, Weller SG, Domínguez CA. 2010. Changes in sexual organ reciprocity and phenotypic floral integration during the tristyly–distyly transition in *Oxalis alpina*. *New Phytol* 185: 829–840.

Souza LGR, Crosa O, Speranza P, Guerra M. 2012. Cytogenetic and molecular evidence suggest multiple origins and geographical parthenogenesis in *Nothoscordum gracile* (Alliaceae). *Ann Bot* 109: 987–999.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* doi 10.1093/bioinformatics/btu033.

Stebbins GL. 1957. Self fertilization and population variability in the higher plants. *Am Nat* 91: 337–354.

Stebbins GL. 1974. *Flowering Plants: Evolution Above the Species Level*. Harvard University Press, Cambridge, USA.

Stone JL. 1996. Components of pollination effectiveness in *Psychotria suerrensis*, a tropical distylous shrub. *Oecologia* 107: 504–512.

Stone JL, Thomson JD. 1994. The evolution of distyly: pollen transfer in artificial flowers. *Evolution* 48: 1595–1606.

Stöver BC, Müller KF. 2010. TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics* 11: 7.

Stuckey R. 1980. Distributional history of *Lythrum salicaria* (purple loosestrife) in North America. *Bartonia* 100: 3–30.

Sutherland S. 2004. What makes a weed a weed: life history traits of native and exotic plants in the USA. *Oecologia* 141: 24–39.

The Plant List 2013. *The Plant List - Version 1.1*. Available online: http://www.theplantlist.org/ [Last time accessed November 28, 2016].

The World Bank Group 2016. *Land area (sq. km)*. Available online: http://data.worldbank.org/indicator/AG.LND.TOTL.K2 [Last time accessed November 28, 2016].

Thompson JD. 2001. How do visitation patterns vary among pollinators in relation to floral display and floral design in a generalist pollination system? *Oecologia* 126: 386–394.

Thompson JD. 2005. *Plant Evolution in the Mediterranean*. Oxford University Press, Oxford, UK.

Thompson JD, Dommée B. 2000. Morph-specific patterns of variation in stigma height in natural populations of distylous *Jasminum fruticans*. *New Phytol* 148: 303–314.

Thompson DQ, Stuckey RL, Thompson EB. 1987. *Spread, impact, and control of purple loosestrife* (Lythrum salicaria) *in North American wetlands*. U.S. Fish and Wildlife Service, Jamestown, USA.

Tippery NP, Les DH. 2011 Phylogenetic relationships and morphological evolution in *Nymphoides* (Menyanthaceae). *Syst Bot* 36: 1101–1113.

Tippery NP, Les DH, Padgett DJ, Jacobs SWL. 2008. Generic circumscription in Menyanthaceae: a phylogenetic evaluation. *Syst Bot* 33: 598–612.

Tokuoka T. 2012. Molecular phylogenetic analysis of Passifloraceae *sensu lato* (Malpighiales) based on plastid and nuclear DNA sequences. *J Plant Res* 125: 489–497.

Torices R, Anderberg AA. 2009. Phylogenetic analysis of sexual systems in Inuleae (Asteraceae). *Am J Bot* 96: 1011–1019.

Turelli M, Barton NH, Coyne JA. 2001. Theory and speciation. *Trends Ecol Evol* 16: 330–343.

Turketti SS. 2010. A study of tristyly in South African *Oxalis*. Doctoral thesis, Stellenbosch University, South Africa.

UC Berkeley Urban Bee Lab. 2016. *Common Bee Groups of California*. Available online: http://www.helpabee.org/common-bee-groups-of-ca.html?lang=en [Last time accessed December 10, 2016].

U.S. Census Bureau. 2016. *American Fact Finder*. Available online: http://factfinder.census.gov/faces/tableservices/jsf/pages/productview.xhtml?src=bkmk [Last time accessed December 10, 2016].

Vaknin Y, Gan-Mor S, Bechar A, Ronen B, Eisikowitch D. 2000. The role of electrostatic forces in pollination. *Plant Syst Evol* 222: 133–142.

Valois-Cuesta H, Soriano PJ, Ornelas JF. 2012. Gender specialization in *Palicourea demissa* (Rubiaceae), a distylous, hummingbird-pollinated treelet. *Plant Syst Evol* 298: 975–984.

van Kleunen M, Johnson SD. 2007. Effects of self-compatibility on the distribution range of invasive European plants in North America. *Conserv Biol* 21: 1537–1544.

van Kleunen M, Manning JC, Pasqualetto V, Johnson SD. 2008. Phylogenetically

independent associations between autonomous self-fertilization and plant invasiveness. *Am Nat* 171: 195–201.

van Kleunen M, Weber E, Fischer M. 2010. A meta-analysis of trait differences between invasive and non-invasive plant species. *Ecol Lett* 13: 235–245.

Velayos M. 1997. *Lythrum* L. Pp. 15-25 *in* Castroviejo S, Laínz M, González G, Montserrat P, Garmendia F, Paiva J, and Villar L, eds. *Flora Iberica - Vol VIII*. Real Jardín Botánico, C. S. I.C., Madrid, Spain.

Vignoli L. 1937. Fenomeni reproduttivi di *Oxalis cernua* Thunb. *Lavori Inst Bot Palermo* 8: 5–30.

Vilà M, Bartolomeus I, Gimeno I, Traveset A, Moragues E. 2006. Demography of the invasive geophyte *Oxalis pes-caprae* across a Mediterranean island. *Ann Bot* 97: 1055–1062.

Villar L. 1990. *Polygonum* L. Pp. 571-586 *in* Castroviejo S, Laínz M, González G, Montserrat P, Garmendia F, Paiva J, and Villar L, eds. *Flora Iberica - Vol II*. Real Jardín Botánico, C. S. I.C., Madrid, Spain.

Vucetich JA, Waite TA. 2003. Spatial patterns of demography and genetic processes across the species' range: null hypotheses for landscape conservation genetics. *Conserv Genet* 4: 639–645.

Vuilleumier B. 1967. The origin and evolutionary development of heterostyly in the angiosperms. *Evolution* 21: 210–226.

Waites AR, Ågren J. 2004. Pollinator visitation, stigmatic pollen loads and amog-population variation in seed set in *Lythrum salicaria*. *J Ecol* 92: 512–526.

Wang Y, Wang Q-F, Guo Y-H, Barrett SCH. 2005. Reproductive consequences of interactions between clonal growth and sexual reproduction in *Nymphoides peltata*: a distylous aquatic plant. *New Phytol* 165: 329–336.

Ward M, Johnson SD, Zalucki MP. 2012. Modes of reproduction in three invasive milkweeds are consistent with Baker's rule. *Biol Invasions* 14: 1237–1250.

Washitani I, Osawa R, Namai H, Niwa M. 1994. Patterns of female fertility in heterostylous *Primula sieboldii* under severe pollinator limitation. *J Ecol* 82: 571–579.

Webb CO, Donoghue MJ. 2004. Phylomatic: tree assembly for applied phylogenetics. *Mol Ecol Notes* 5: 181–183.

Webb CJ, Lloyd DG. 1986. The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *New Zeal J Bot* 24: 163–178.

Weber JJ, Weller SG, Sakai AK, Nguyen L-Q, Khuu D. 2013. Breeding system evolution in *Oxalis alpina*: asymmetrical expression of tristylous incompatibility. *Int J Plant Sci* 174: 179–188.

Weller SG. 1979. Variation in heterostylous reproductive systems among populations of *Oxalis alpina* in Southeastern Arizona. *Syst Bot* 4: 57-71.

Weller SG. 1980. Pollen flow and fecundity in populations of *Lithospermum caroliniense*. *Am J Bot* 67: 1334–1341.

Weller SG. 1986. Factors influencing frequency of the mid-styled morph in tristylous populations of *Oxalis alpina*. *Evolution* 40: 279–289.

Weller SG. 1992. Evolutionary modifications of tristylous breeding systems. Pp. 247–270 *in* Barrett SCH, ed. *Evolution and Function of Heterostyly*. Springer-Verlag, Berlin, Germany.

Weller SG, Domínguez CA, Molina-Freaner FE, Fornoni J, LeBuhn G. 2007. The evolution of distyly from tristyly in populations of *Oxalis alpina* (Oxalidaceae) in the Sky Islands of the Sonoran Desert. *Am J Bot* 94: 972–985.

Wolfe LM, Barrett SCH. 1988. Temporal changes in the pollinator fauna of tristylous *Pontederia cordata*, an aquatic plant. *Can J Zool* 66: 1421–1424.

Wolfe LM, Barrett SCH. 1989. Patterns of pollen removal and deposition in tristylous *Pontederia cordata* L. (Pontederiaceae). *Biol J Linn Soc* 36: 317–329.

Woodell SRJ, Dale A. 1993. Biological flora of the British Isles: *Armeria maritima* (Mill.) Willd. (*Statice armeria* L.; *S. maritima* Mill.). *J Ecol* 81: 573–588.

Wulff EV. 1950. *An Introduction to Historical Plant Geography*. Chronica Botanica Company, Waltham, USA.

Yakimowski SB, Eckert CG. 2007. Threatened peripheral populations in context: geographical variation in population frequency and size and sexual reproduction in a clonal woody shrub. *Conserv Biol* 21: 811–822.

Yakimowski SB, Hager HA, Eckert CG. 2005. Limits and effects of invasion by the nonindigenous wetland plant *Lythrum salicaria* (purple loosestrife): a seed bank analysis. *Biol Invasions* 7: 687–698.

Yeo PF. 1975. Some aspects of heterostyly. *New Phytol* 75: 147–153.

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

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