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

Joana Costa,[1,2] Sílvia Castro,[1,3] João Loureiro,[1] and Spencer C. H. Barrett[4]

[1]*Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas, 3000–456, Coimbra, Portugal*

[2]*E-mail: joana.costa@uc.pt*

[3]*Botanical Garden of the University of Coimbra, Calçada Martim de Freitas 3000–456, Coimbra, Portugal*

[4]*Department of Ecology and Evolutionary Biology, University of Toronto, 25 Willcocks Street, Toronto, Ontario M5S 3B2, Canada*

Most heterostylous plants possess a reciprocal arrangement of stigmas and anthers (reciprocal herkogamy), 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, we 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. We compared the amounts of compatible and incompatible pollen on stigmas in natural populations and calculated the proficiencies of pollen transfer for each mating type. We 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. Our results provided evidence that, while structural in nature, pollen-stigma dimorphisms are tightly associated with heteromorphic incompatibility and likely function to promote disassortative pollination, especially in the absence of reciprocal herkogamy.

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

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 1862; 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 1992; 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.

Heterostylous plant populations are characterized by the presence of two (distyly) or three (tristyly) floral morphs differing in stigma and anther heights, a condition known as reciprocal herkogamy (Webb and Lloyd 1986). 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 is 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 (Dulberger 1975a, 1992; Ganders 1979; Barrett 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 1975b; Ganders 1979; Barrett 1992), and its expression differs significantly between subfamilies. For example, in Plumbaginoideae distyly occurs without striking morphological differences in pollen and stigmas 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 1948, 1953, 1966; Dulberger 1975b). 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 1948, 1966; Dulberger 1975a, 1975b, 1992). Monomorphic species are either self-compatible (A/papillate, or less frequently, B/cob) or apomictic (A/cob or B/papillate) (Baker 1966; Dulberger 1975b). 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 (1975b), 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 5 hours 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 Mattson (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 1975a, 1992; Lloyd and Webb 1992a), but it has not been examined in natural populations of heteromorphic plants.

Here, we 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 *A. maritima.* The two *Armeria* species possess striking morphological pollen-stigma dimorphisms (Figs. 1A–D, 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
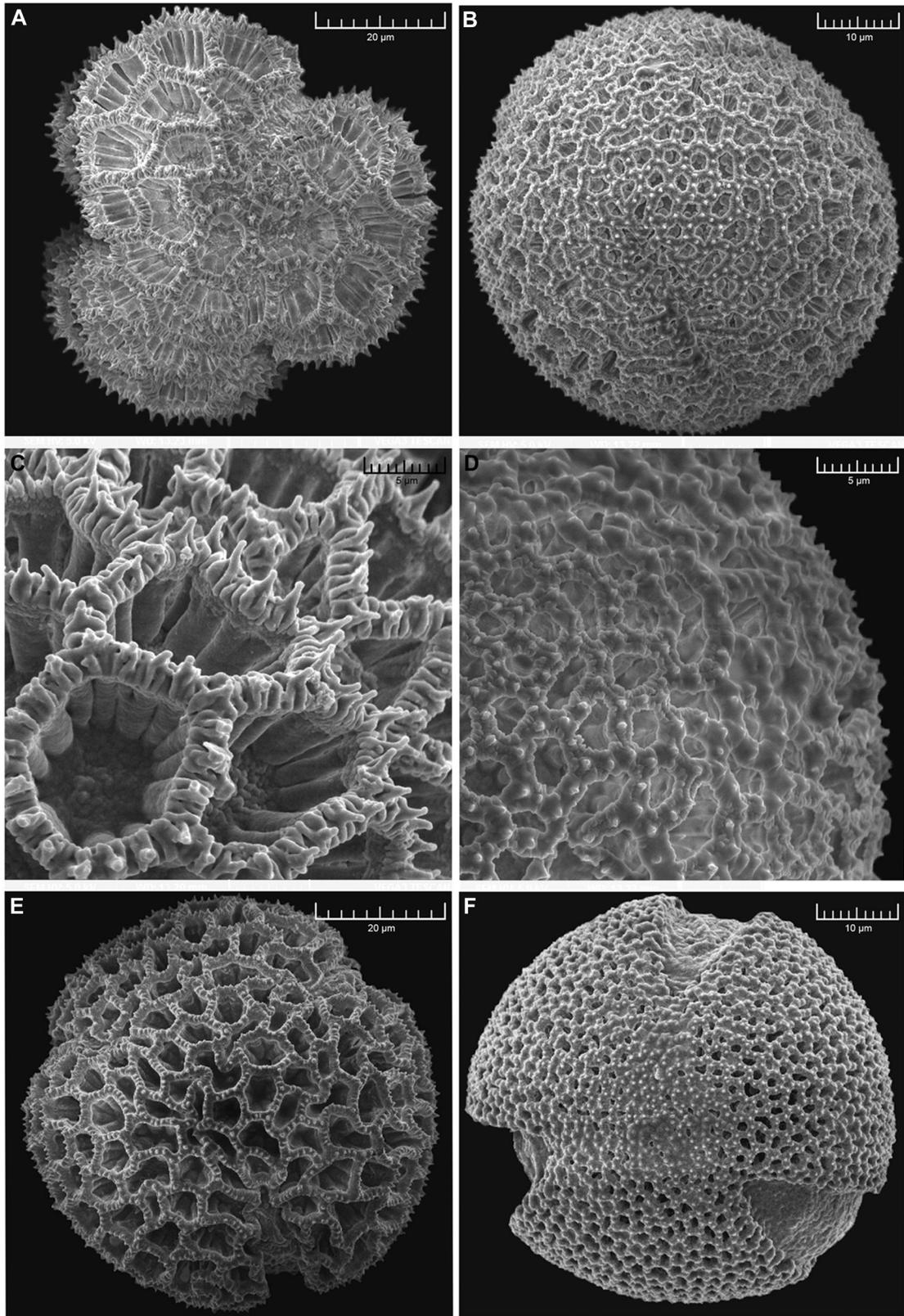
Figure 1. Scanning electron microscopy of pollen grains of *Armeria maritima* (A, B) from Fão, *A. pubigera* (C, D) from Areosa and *Limonium vulgare* (E, F) from Gafanha da Encarnação. A, C, E. Type A pollen grain; B, D, F. Type B pollen grain. For methods, see Appendix S1.
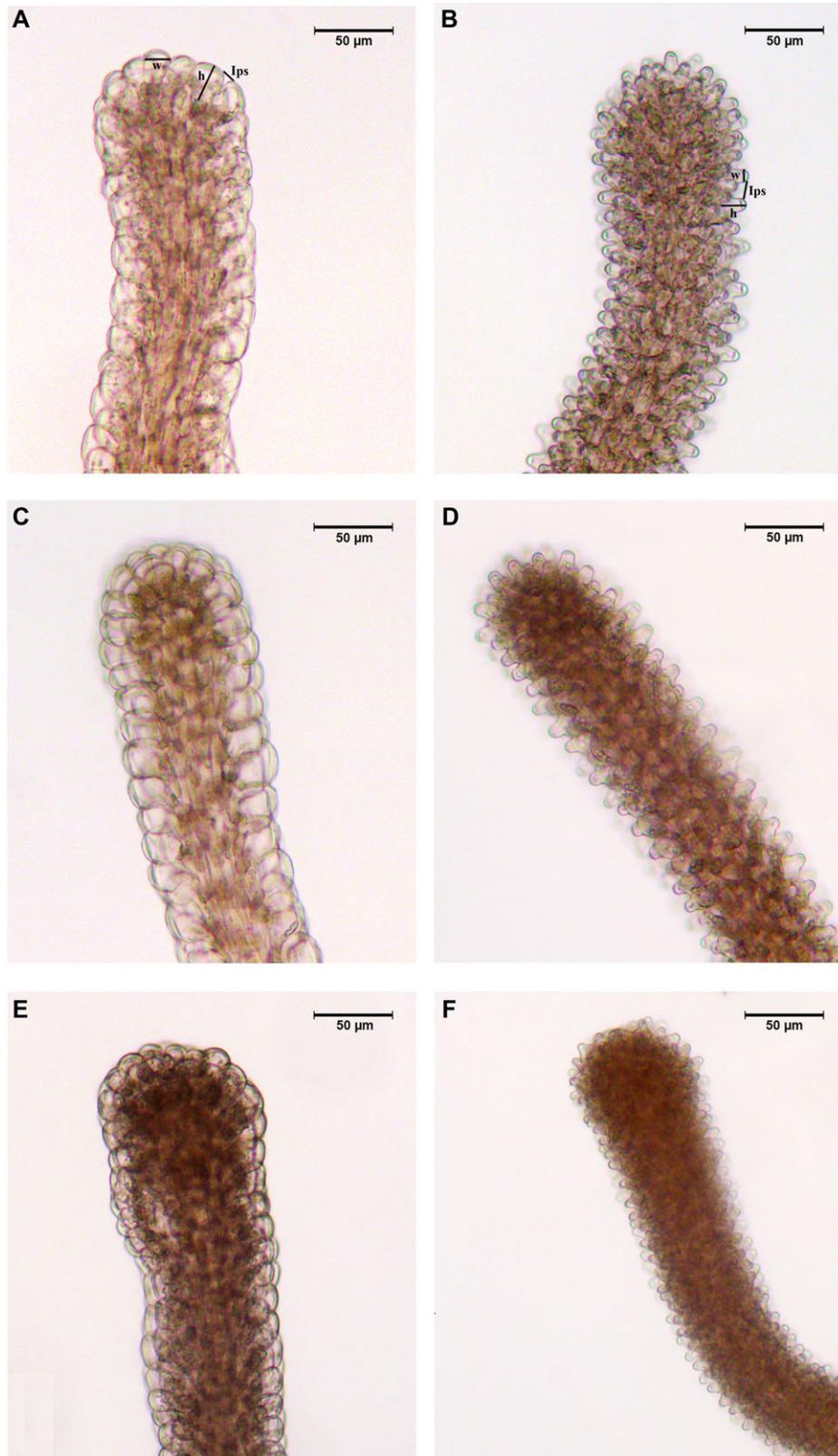
Figure 2. Light microscopy photographs of stigmas of *Armeria maritima* (A, B) from Fão, *A. pubigera* (C, D) from Areosa and *Limonium vulgare* (E, F) from Gafanha da Encarnação. A, C, E. cob stigma; B, D, F. papillate stigma. Morphological measurements: "h"—stigmatic papillae length, "w"—stigmatic papillae width and "Ips"—inter-papillae space between two adjacent stigmatic papillae. For methods, see Appendix S1.

both reciprocal herkogamy, and ancillary pollen and stigma dimorphisms (Figs. 1E and F, 2E and 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).

Our 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). We were 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, we analyzed stigmatic pollen loads in natural populations of *A. maritima*, *A. pubigera,* and *L. vulgare* comparing compatible and incompatible pollen transfer and capture among species and between 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., salt-marshes, 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 (Nieto 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 (Nieto 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 our study area, both *Armeria* species flower in May, while *L. vulgare* flowers from June to August.

To provide a morphological context for our experiments on naturally and experimentally pollinated flowers described below, we conducted a detailed characterization of the structural features of pollen and stigma polymorphisms in the three taxa. To accomplish this, we undertook measurements of pollen size (i.e., polar axis and equatorial diameter) and lumina diameter, stigmatic papillae dimensions (i.e., height and width) and of the inter-papillae space between two adjacent stigmatic papillae. The methods and results are described in detail in the supporting information (Appendix S1; Tables S1 and S2).

POLLEN TRANSFER AND CAPTURE IN NATURAL POPULATIONS

To estimate the extent to which ancillary characters promote disassortative pollination, we investigated pollen loads on open-pollinated stigmas in 11 natural populations (Table S3). 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*, we 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, we routinely observed small bees, flies, and beetles foraging on flowers. In each population, we sampled flowers for analysis of stigmatic pollen loads every 2 m along transects to avoid resampling of genets. Where possible, we 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 Table S3). In populations of *A. maritima* and *A. pubigera*, we 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, we 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.

We 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 (intermorph or legitimate pollen) or incompatible (intramorph or illegitimate pollen) 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 Table S4. 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.

We assessed pollen transfer in natural populations by calculating the probability of a single pollen grain of a given type being deposited on the stigmas of each mating type, i.e., the estimation of pollen transfer proficiencies (T_{ij}) 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 mating types in a population, and (average stigma load)$_{ij}$ is the average number of type i pollen grains on each type j stigma. This measure accounts for variability in pollen and flower production between mating types, and provides information on stigmatic pollen load data from the perspective of both female and male flower functions (Lloyd and Webb 1992b).

We used the random sampling of flowers to estimate the frequency of mating types in each population (Table S3). We also estimated population size (individuals at reproductive stage only) by counting the number of flowering individuals in each population. Finally, in each population, we assessed floral display size and estimated the amount of pollen available for dispersal, i.e., the pollen pool, at the time of stigma collection. We 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, we 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. We 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.

QUANTIFICATION OF POLLEN ADHERENCE AND GERMINATION

The topographical complementarity hypothesis proposes that illegitimate pollen fails to adhere to incompatible stigmas preventing pollen germination and pollen-tube growth (Dulberger 1975b). To evaluate this hypothesis, we conducted controlled hand-pollinations on *A. maritima* during May 2016, using 16 plants, eight A/cob and eight B/papillate. We collected plants from the 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 pollination, here self-pollination, (b) legitimate pollination, pollination between mating types, and (c) mixed pollination, pollination with compatible and self-pollen applied simultaneously. Each day, we selected plants with six open flowers to be pollinated and randomly assigned a pollen donor from the opposite mating type. We 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, we emasculated flowers and removed the corolla to prevent self-pollen contamination and to facilitate precise pollen deposition. For each flower, we transferred a total of 50 grains, that is 10 pollen grains to each of the five stigmas. Mixed pollen loads consisted of 1:1 compatible to self-pollen grains, that is five compatible and five self-pollen grains per stigma. Under a dissecting microscope at 20× magnification (Leica Zoom 2000), we 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. We transferred pollen grains to recipient stigmas and used a hand lens to confirm that the requisite number of pollen grains was deposited. We collected stigmas 1 hour and 3 hours 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 8 M sodium hydroxide for 30 minutes, and placed overnight in 0.05% w/v aniline blue prepared in 0.1 M potassium phosphate (Dafni et al. 2005). We 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. We 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, we identified the mating type of adhered and germinated pollen grains on the stigma after the mixed pollination treatment.

STATISTICAL ANALYSIS

We 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, we used generalized linear models (hereafter GLM) with a Gamma distribution and a log link function. We assessed differences in pollen production between mating types for the three species using a linear model. Before statistical tests, we transformed total flower production with the $\log_{10} (x + 1)$ (Zar 2010).

For each species, we 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. We 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 nonrandom) 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, we used generalized-mixed models (hereafter GLMM) 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, we used GLMMs with a Poisson distribution and a log link function. Before analysis, we 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 a 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, we 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, we 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.

We 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, we 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.

We 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 GLM and GLMM models (Fox and Weisberg 2015). "lme4" for generalized mixed models (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, linear and generalized linear models (R Core Development Team 2013).

Results

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 ($P > 0.05$; Table S3). 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 $\times$ 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 $\pm$ 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*
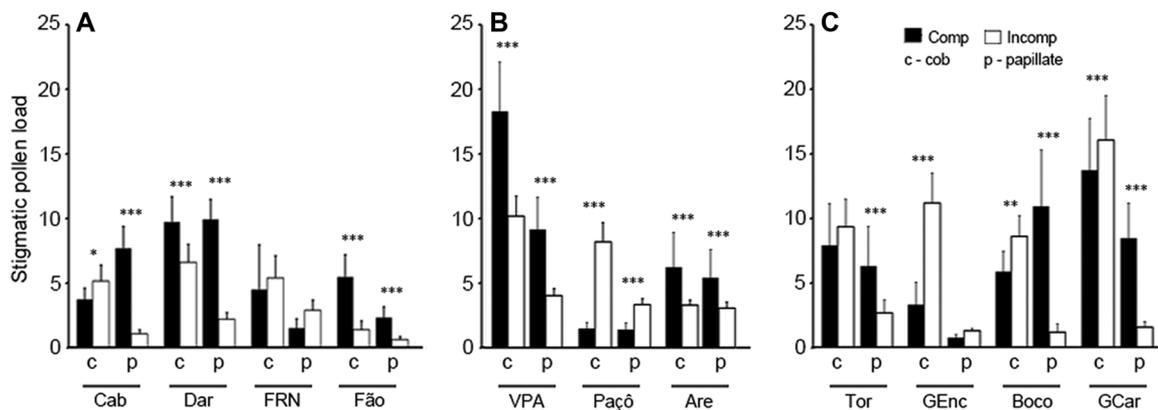
Figure 3. Compatible (Comp) and incompatible (Incomp) stigmatic pollen capture for *Armeria maritima*, *A. pubigera*, and *Limonium vulgare* grouped by population. (A) *Armeria maritima*: Cab – Cabedelo, Dar – Darque, FRN – Foz do Rio Neiva, Fão – Fão. (B) *Armeria pubigera*: VPA – Vila Praia de Âncora, Paçô – Paçô, Are – Areosa. (C) *Limonium vulgare*: Tor—Torreira, GEnc—Gafanha da Encarnação, Boco—Boco, GCar—Gafanha do Carmo. Values are mean and standard error 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; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

(mean $\pm$ SE, A/cob: 1001.17 $\pm$ 25.14, B/papillate: 972.24 $\pm$ 25.55; $F_{1,57} = 0.65$, $P = 0.42$), *A. pubigera* (A/cob: 1048.62 $\pm$ 26.67, B/papillate: 992.66 $\pm$ 29.00; $F_{1,59} = 1.99$, $P = 0.16$), and *L. vulgare* (A/cob: 979.14 $\pm$ 24.33, B/papillate: 996.83 $\pm$ 20.52; $F_{1,57} = 0.31$, $P = 0.58$) were detected. Collectively, our 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 $\pm$ SE, 10.92 $\pm$ 1.01; *A. pubigera*: 1–135 pollen grains; mean $\pm$ SE, 13.02 $\pm$ 1.23; Table S4). However, cob stigmas captured significantly more pollen than papillate stigmas in both species (*A. maritima*: cob = 12.42 $\pm$ 1.63; papillate = 9.15 $\pm$ 1.04; $\chi^2_{1,181} = 44.13$, $P < 0.001$; *A. pubigera*: cob = 16.60 $\pm$ 2.00; papillate = 8.93 $\pm$ 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. 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. 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. 3A). No case of random pollen capture was detected for any of the three sampled populations of *A. pubigera* (Fig. 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 *L. 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 $\pm$ SE, 13.36 $\pm$ 1.30; Table S4). Similar to *Armeria*, cob stigmas captured significantly more pollen than papillate stigmas (cob = 18.51 $\pm$ 1.92; papillate = 6.99 $\pm$ 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. 3C).

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 model: 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 model: 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 model: estimate = -0.53, SE = 0.16, $z = -3.38$, $P < 0.001$). We found

Table 1. Results of the generalized-mixed models (GLMM) 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	Wald 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

df = degrees of freedom.

no significant differences in the relative amount of compatible and incompatible stigmatic pollen capture among species ($\chi^2_2 = 2.00$, $P = 0.37$). 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 ($\chi^2_2 = 116.10$, $P < 0.001$; Table 1; Fig. 3). Overall, disassortative pollen capture was detected across populations of the two *Armeria* species ($\chi^2_{1,447} = 97.57$, $P < 0.001$; Table S4), contrary to what was found for *L. vulgare* ($\chi^2_{1,332} = 5.89$, $P = 0.02$; Table S4).

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*: $\chi^2_1 = 0.24$, $P = 0.62$; *A. pubigera*: $\chi^2_1 = 2.40$, $P = 0.12$; *L. vulgare*: $\chi^2_1 = 1.64$, $P = 0.20$). 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; Table S5). 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 1).

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 1 hour

and 3 hours was much lower in self-pollinations when compared with legitimate pollinations ($\chi^2_{1,122} = 13.18$, $P < 0.001$; Fig. S1A). In the very few cases in which self-pollen adhered to the stigmatic papillae, the pollen grains failed to germinate (Table S6). In contrast, more than 95% of compatible pollen grains that adhered to stigmas germinated ($\chi^2_{1,123} = 0.04$, $P = 0.85$; Fig. S1B), 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 ($\chi^2_{1,123} = 58.68$, $P < 0.001$; Fig. S1C), 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 ($\chi^2_{1,122} = 25.01$, $P < 0.001$; Fig. S1D) 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%; $\chi^2_{1,123} = 0.09$, $P = 0.77$; Fig. S1E), 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 ($\chi^2_{1,123} = 0.77$, $P = 0.38$). We 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 ($\chi^2_{1,123} = 33.45$, $P < 0.001$; Fig. S1E and F), 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

Our investigation of pollen transfer and capture in three species of Plumbaginaceae, and the quantification of pollen adherence in *A. maritima*, represents the first attempt to evaluate the role of ancillary characters in promoting disassortative pollination in natural populations of heteromorphic plants. Our 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
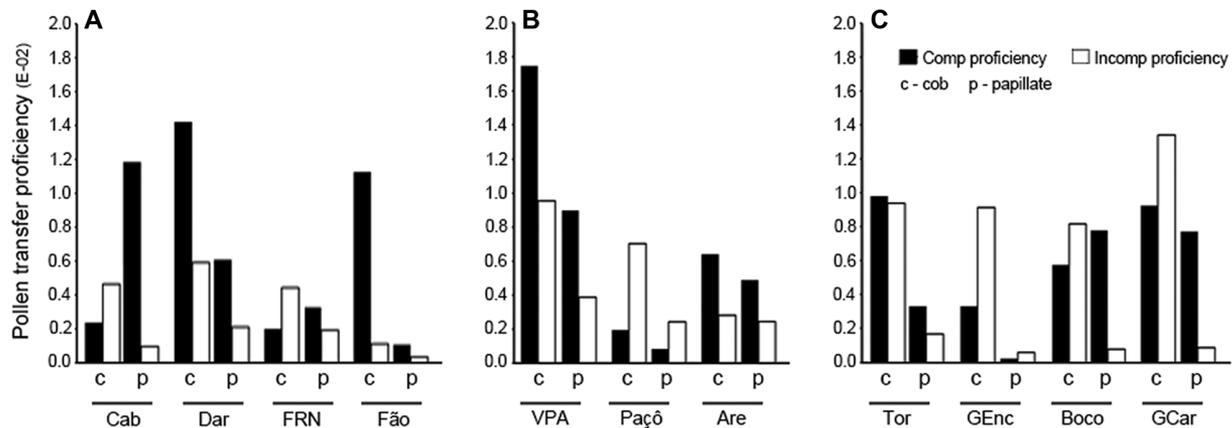
Figure 4. Compatible (Comp) and incompatible (Incomp) pollen transfer proficiencies calculated for *Armeria maritima*, *A. pubigera*, and *Limonium vulgare* grouped by population. See Materials and Methods for the calculation of pollen transfer proficiencies. (A) *Armeria maritima*: Cab – Cabedelo, Dar – 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.

contrast to compatible pollen. Finally, we 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, we consider the role of pollen-stigma dimorphisms in promoting disassortative pollination in heterostylous species and discuss the relevance of our results to the topographical complementarity hypothesis.

DISASSORTATIVE POLLINATION IN THE ABSENCE OF RECIPROCAL HERKOGAMY

Floral morph frequencies in each of the 11 populations we 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 1992), even in the relatively small-sized populations that we encountered in our study. In common with most heteromorphic species (reviewed in Barrett and Cruzan 1994), disassortative mating in Plumbaginaceae is guaranteed by the occurrence of a strong diallelic incompatibility system (Baker 1948, 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 ($\leq 25\%$), our analysis revealed significant levels of disassortative pollen capture in nine of the 14 morph $\times$ population combinations in the *Armeria* species (Fig. 3) in the absence of reciprocal herkogamy. This finding supports our 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 illegitimate pollination involve both structural and chemical components working in concert with neither subordinate to the other (Dulberger 1975a, 1975b). 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 1975a, 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 ho-momorphic incompatibility.

Our observations and measurements of pollen and stigmas provided partial evidence for the "lock and key" mechanism originally proposed by Dulberger (1975b). We found that pollen lumen diameter of a given pollen type and inter-papillae distance of the opposite mating type matched one another (Tables S1 and S2). 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 1975b, 1992; Baker 1966). In this latter case, large papillae width combined with a small interpapillae distance probably contributes to some adher-ence of incompatible pollen type A to cob stigmas, given the larger lumina diameter of this pollen type. However, since in-compatibility responses in Plumbaginaceae occur at the stigmatic surface (Dulberger 1975b, 1992, p. 59), incompatible pollen fails to germinate, as was also revealed by our 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, 1975b, 1992; Mattson 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 (Mattson 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 (> 2 hours). These experimental results are consistent with our morphological observations and controlled pollination studies 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.

Our data demonstrating significant disassortative pollen cap-ture 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 *A. maritima* originated from the opposite floral morph is also in accord with this interpretation. Unlike most heterostylous species, in *Arme-ria* the sites of inhibition of incompatible pollen in both floral morphs occur on the stigmatic surface (reviewed in Dulberger 1975b, 1992, p. 59; 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 heterostylous species.

Although we 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 ac-count for this result. First, the examination of intact flowers, as was done in our study, did not allow us to distinguish between the incompatible components of the pollen load, in particular whether incompatible pollen resulted from self-pollination, in-cluding intraflower and geitonogamous self-pollination, or from intramorph cross-pollination. Elimination of the self-pollen com-ponent of pollen loads by emasculation allows a more accurate quantitative assessment of the role of morphological traits in pro-moting disassortative pollen capture (Ganders 1974; Barrett and Glover 1985; Lloyd and Webb 1992b). However, flowers of each of the three species we investigated are of small size (5.5–8.5 mm; Nieto Feliner 1990; Erben 1993), which precluded the possi-bility 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 be-cause of small population size and low pollinator activity. Indeed, we 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 dis-plays 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 our mea-sures of the proficiency of compatible pollen grain transfer and disassortative pollen capture are conservative and would likely be greater if we 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 find-ing is in accord with previous results of intramorph pollinations

performed in four *Limonium* species (Dulberger 1975b). 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 we investigated, the asymmetry we observed in incompatible pollen capture arises because of differences in pollen adherence to stigmas of the mating types. In distylous *L. vulgare*, however, stylar dimorphism may have also contributed towards differences in pollen capture between morphs. Asymmetrical pollen capture is widely reported in distylous species (Ganders 1979; Barrett and Shore 2008), and 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 greater 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 1975a; Mattson 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 1975a, 1992). Thus, by creating the opportunity for preferential adhesion of compatible pollen grains, pollen-stigma dimorphisms serve as a mechanism promoting disassortative pollination.

During our controlled hand pollination experiment, we noticed sharp differences when transferring compatible versus self-pollen to stigmas. When we 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.

Our 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 (Baker 1966; Dulberger 1975b, 1992). Indeed, our 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 our 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 our 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 we 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 of type B succeed in germinating on cob stigmas, (Dulberger 1975b, 1992), and this was also confirmed by the results of our 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; 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). Interestingly, there

was no evidence from our 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.

DATA ARCHIVING
The doi for our data is 10.5061/dryad.r48vj.

LITERATURE CITED

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

Baker, H. G. 1948. Dimorphism and monomorphism in the Plumbaginaceae I. Survey of the family. Ann. Bot. 12:207–219.

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

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

Barrett, S. C. H. 1992. Evolution and function of heterostyly. Springer-Verlag, Berlin.

———. 2002. The evolution of plant sexual diversity. Nat Rev Genet. 3:274–284.

Barrett, S. C. H., and M. B. Cruzan. 1994. Incompatibility in heterostylous plants. *In* E. G. Williams, A. E. Clarke, and R. B. Knox, eds. Genetic control of self-incompatibility and reproductive development in flowering plants. Kluwer Academic Publishers, Boston.

Barrett, S. C. H., and J. S. Shore. 2008. New insights on heterostyly: comparative biology, ecology and genetics. Pp. 3–32 in V. E. Franklin-Tong, ed. Self-incompatibility in flowering plants - evolution, diversity, and mechanisms. Springer-Verlag, Berlin.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2014. "lme4" — linear mixed-effects models using Eigen and S4. R package version 1.0.5. Available at https://cran.r-project.org/web/packages/multcomp/index.html.

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

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

Darwin, C. 1862. On the various contrivances by which British and foreign orchids are fertilised by insects, and on the good effects of intercrossing. John Murray, London.

———. 1877. The different forms of flowers on plants of the same species. John Murray, London.

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

———. 1975a. S-gene action and the significance of characters in the heterostylous syndrome. Heredity 35:407–415.

———. 1975b. Intermorph structural differences between stigmatic papillae and pollen grains in relation to incompatibility in Plumbaginaceae. Proc. R Soc. L. B 188:257–274.

———. 1981. Dimorphic exine sculpturing in three distylous species of *Linum* (Linaceae). Plant Syst. Evol. 139:113–119.

———. 1992. Floral polymorphisms and their functional significance in the heterostylous syndrome. Pp. 41–84 in S. C. H. Barrett, ed. Evolution and function of heterostyly. Springer-Verlag, Berlin.

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

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

Faegri, K., and Van der Pijl, L. 1971. The principles of pollination ecology. Pergamon Press, New York.

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

Fox, J., and S. Weisberg. 2015. Car: companion to applied regression. R package version 2.0.19. Available at http://cran.r-project.org/web/packages/car/index.html

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

———. 1979. The biology of heterostyly. New Zeal. J. Bot. 17:607–635.

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

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

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

Harder, L. D., and S. D. Johnson. 2008. Function and evolution of aggregated pollen in Angiosperms. Int. J. Plant Sci. 169:59–78.

———. 2009. Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. New Phytol. 183:530–545.

Heslop-Harrison, J., and Y. Heslop-Harrison. 1985. Surfaces and secretions in the pollen-stigma interaction — a brief review. J. Cell Sci. Suppl. 2:287–300.

Hothorn, T., F. Bretz, and P. Westfall. 2015. multcomp: simultaneous inference in general parametric models. R package version 1.3.1. Available at http://cran.r-project.org/web/packages/multcomp/index.html

Iversen, J. 1940. Blütenbiologische Studien. I. Dimorphie und monomorphie bei *Armeria*. K. Danse Vidensk. Selsk. Skr. 15:1–39.

Kohn, J. R., and S. C. H. Barrett. 1992. Experimental studies on the functional significance of heterostyly. Evolution 46:43–55.

Kubitzki, K. 1993. Plumbaginaceae. Pp. 523–530 in K. Kubitzki, J. G. Rohwer, and V. Bittrich, eds. The families and genera of vascular plants. Springer-Verlag, Berlin.

Lebuhn, G., and G. J. Anderson. 1994. Anther tripping and pollen dispensing in *Berberis thunbergii*. Am. Midl. Nat. 131:257–265.

Lloyd, D. G., and C. J. Webb. 1992a. The evolution of heterostyly. Pp. 151–178 *in* S. C. H. Barrett, ed. Evolution and function of heterostyly. Springer-Verlag, Berlin.

———. 1992b. The selection of heterostyly. Pp. 179–208 *in* S. C. H. Barrett, ed. Evolution and function of heterostyly. Springer-Verlag, Berlin.

Mattson, O. 1983. The significance of exine oils in the initial interaction between pollen and stigma in *Armeria maritima*. Pp. 257–264 *in* D. L. Mulcahy and E. Ottaviano, eds. Pollen biology and implications for plant breeding. Elsevier Science Publishing, Amsterdam.

Nieto Feliner, G. 1990. *Armeria* Willd. Pp. 642–721 *in* S. C. Bolibar and R. M. Valverde, eds. Flora Iberica Vol. II. Real Jardín Botánico, C. S. I. C., Madrid, Spain.

Pinheiro, J., D. Bates, S. DebRoy, D. Sarkay, and R Core Development Team. 2013. nlme: linear and nonlinear mixed effects models. R package version 3.1-122. Available at http://cran.r-project.org/web/packages/nlme/index.html

R Core Development Team. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at http://www.R-project.org/.

Reith, M., G. Baumann, R. Classen-Bockhoff, and T. Speck. 2007. New insights into the functional morphology of the lever mechanism of *Salvia pratensis* (Lamiaceae). Ann. Bot. 100:393–400.

Róis, A. S. 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). Ph.D. thesis, University of Lisbon, Portugal.

Stone, J. L., and J. D. Thomson. 1994. The evolution of distyly: pollen transfer in artificial flowers. Evolution 48:1595–1606.

Vaknin, Y., S. Gan-Mor, A. Bechar, B. Ronen, and D. Eisikowitch. 2000. The role of electrostatic forces in pollination. Plant Syst. Evol. 222:133–142.

Webb, C. J., and D. G. Lloyd. 1986. The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. New Zeal. J. Bot. 24:163–178.

Woodell, S. R. J., and A. Dale. 1993. Biological flora of the British Isles: *Armeria maritima* (Mill.) Willd. (*Statice armeria* L.; *S. maritima* Mill.). J. Ecol. 81:573–588.

Yeo, P. F. 1975. Some aspects of heterostyly. New Phytol. 75:147–153.

Zar, J. H. 2010. Biostatistical analysis, 5th ed. Prentice-Hall, Inc., New Jersey.

Zhou, W., S. C. H. Barrett, H. Wang, and D.-Z. Li. 2015. Reciprocal herkogamy promotes disassortative mating in a distylous species with intramorph compatibility. New Phytol. 206:1503–1512.

Associate Editor: J. Busch
Handling Editor: P. Tiffin

Supporting Information

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

APPENDIX S1. Characterization of pollen and stigma dimorphisms

Figure S1. A, D. Pollen adherence; **B, E.** pollen germination; and **C, F.** the 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 Table S6. **A, B, C.** Legitimate and self-pollinations. **D, E, F.** Mixed pollinations.

Table S1. Pollen polar axis (P), equatorial diameter (E), ratio between polar axis and equatorial diameter (P/E) and lumen diameter measures for 30 pollen grains from each mating type of *Armeria maritima*, *A. pubigera* and *Limonium vulgare*.

Table S2. 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*.

Table S3. 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*.

Table S4. 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*.

Table S5. Pollen transfer proficiencies for *Armeria maritima*, *A. pubigera* and *Limonium vulgare* calculated following the method of Lloyd and Webb (1992b).

Table S6. 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*.