Newly discovered differences of reproductive traits between island and mainland plants support Baker's law

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Abstract

Background: Reproduction in angiosperms involves either one or two parents, through which selfed/cloned or outcrossed progeny is formed, respectively. Uniparental reproduction is advantageous when lack of mates and/or pollinators limits outcrossing opportunities. Baker's law predicts that the capacity for uniparental reproduction should be enriched in habitats colonized *via* long-distance dispersal, such as volcanic islands. To test Baker's law, we quantified variation of reproductive traits at multiple hierarchical levels and compared seed set after selfing and crossing experiments in both island and mainland populations of *Limonium lobatum*, a widespread species that Baker described as self-incompatible based on observations of stigma-pollen dimorphism in their flowers. In species with the type of pollen-stigma dimorphism that Baker detected in *L. lobatum,* pollen of one floral morph typically cannot fertilize ovules of the same floral morph.

Results: We discovered new variation and combinations of pollen-stigma traits never described before and determined that plants with such novel combinations were more common in island than mainland populations. We also documented, for the first time, a lack of correspondence between specific pollen-stigma combinations and pollen compatibility. Furthermore, the results of manual pollination experiments established that selfed seed-set was higher in island than mainland plants, while outcrossed seed-set was lower in island than mainland plants. Overall, more than 80% of all plants were self-compatible, while less than 20% were partially or entirely self-incompatible.

Conclusions: Contrary to previous descriptions by Baker, *L. lobatum*is a species that includes both self-compatible and self-incompatible plants characterized by both known and previously undescribed combinations of pollen-stigma traits. This type of variation was previously unknown in *Plumbaginaceae* and the lack of correspondence between pollen-stigma combinations was never described outside the highly variable species complex of *Armeria maritima*. Furthermore, island populations of *L. lobatum* harbor more plants with the newly discovered pollen-stigma combinations than mainland populations and are enriched in their capability for uniparental reproduction, corroborating Baker's law. Our study establishes a link between variability of reproductive traits and capability of uniparental reproduction on islands, connecting research on reproductive and island biology.

Background

Flowering plants display great diversity of reproductive systems involving either two parents, through which outcrossed progeny is formed, or only one parent, through which selfed and clonal progeny is formed. Sustained high levels of uniparental reproduction reduce genetic variation in populations, limiting their ability to adapt to environmental changes [1, 2]. Moreover, selfed progeny is frequently less fit than outcrossed progeny (i.e., inbreeding depression) due to the increased homozygosity of alleles at loci with recessive deleterious mutations and/or heterozygote advantage [3, 4]. As a result, a wide range of floral traits have evolved to promote outcrossing, including different types of floral heteromorphy [5, 6]. Conversely, uniparental reproduction provides reproductive assurance when mates and/or pollinators are

absent or scarce [7–9]. Uniparental reproduction can be advantageous when plants colonize distant, isolated habitats, including volcanic islands formed *de novo* through tectonic processes, *via* long distance-dispersal across oceanic barriers [10, 11]. Baker proposed that individuals capable of uniparental reproduction are more likely to found a new population and persist after long-distance dispersal to islands [12, 13], an idea termed "Baker's law" by Stebbins [8]. Baker's law thus provides a general hypothesis to test whether the ability for uniparental reproduction is indeed overrepresented in habitats colonized *via* long-distance dispersal.

Uniparental reproduction is common in plants and occurs either sexually *via* self-fertilization or asexually *via* vegetative propagation or apomixis, the latter term referring to clonal reproduction through seeds [14]. Plants can reproduce *via* self-fertilization if they are self-compatible (SC), that is, if pollen can germinate and fertilize the ovules of the same plant (i.e., facultative selfers). Conversely, fully self-incompatible (SI) plants, that is, plants where pollen of the same plant is rejected, can only reproduce *via* outcrossing (i.e., obligate outcrossers [15]). However, plant species are not always uniformly SC or SI. Indeed, some species exhibit intra-specific variation across their range in the strength and frequency of SI, either due to plastic environmental responses of SI or the spread of mutations that weaken or disable SI genes [16–21].

If Baker's law applies, then plants that can propagate uniparentally *via* selfing, apomixis, or vegetative propagation should be overrepresented in newly colonized, insular habitats over plants that are obligate outcrossers. Therefore, ancestrally SI species with populations in both mainland and volcanic islands are particularly well suited to test whether successful establishment on islands after long-distance dispersal is associated with an increased capability for uniparental reproduction. Such increase can be detected as increased production of selfed seed enabled by a shift to partial or total SC on islands vs. mainland. To our knowledge, controlled pollination experiments aimed at comparing levels of uniparental reproduction in mainland vs. island populations have been performed in only three species, all from the New World: *Lycium carolinianum* (*Solanaceae*: Hawaiian Islands and North America [22]), *Waltheria ovata* (*Malvaceae*: Galapagos Islands and Ecuador [23]), and the invasive *Oeceoclades maculata* (*Orchidaceae*: Puerto Rico and Brazil [24]). The intra-specific studies mentioned above found that the capacity for uniparental reproduction is enriched on islands, thus supporting Baker's law.

Baker developed the ideas behind his namesake law by studying the *Limonioideae* subfamily of *Plumbaginaceae*, where he analyzed intra- and interspecific variation of stigma and pollen morphologies and seed set after self- and cross-pollination experiments [12, 25–27]. *Limonioideae* include three different reproductive strategies that can vary both intra- and interspecifically [5, 25–28]: sexual reproduction with SI, sexual reproduction with SC, and apomixis. In *Limonioideae*, typical SI sexual reproduction is associated with stigma and pollen dimorphism, characterized by two genetically determined floral morphs: the so-called *cob*/*A* floral morph has stigmas with flat (*cob*) papillae and pollen with coarse exine sculpturing (*A* pollen); the so-called *pap*/*B* floral morph has stigmas with protruding (*pap*) papillae and pollen with fine exine sculpturing (*B* pollen; Fig. 1A; [25, 27, 29]). The combination of stigma and pollen dimorphism with SI prevents pollen from adhering and germinating on stigmas of both

the same individual and floral morph, favoring inter-morph mating and outcrossing [5, 30, 31]. Conversely, typical SC sexual reproduction is associated with stigma and pollen monomorphism: some taxa consist entirely of *pap*|*A* plants with *pap* stigmas and *A* pollen (Fig. 1B), while other taxa consist entirely of *cob*|*B* plants with *cob* stigmas and *B* pollen (Fig. 1C [5, 30, 31]). The mating system of monomorphic, SC *Limonioideae* has been investigated only in *Limonium carolinianum*, shown to be a mixed mating species [32]. Finally, apomictic *Limonioideae* produce either misshaped and irregularly sized *A* or *B* pollen grains of reduced viability or, rarely, no pollen [25, 33]. Apomicts reproduce *via* unfertilized seeds (obligate agamospermy [5]), or, occasionally, also sexually (facultative agamospermy). Thus, apomictic reproduction can be associated with stigma and pollen monomorphism for any stigma-pollen combination (*cob*|*A*, *cob*|*B, pap*|*A*, or *pap*|*B* and/or and pollen dimorphism (*cob*|*A* and *pap*|*B* [5, 25, 34–36]). To summarize, previous studies revealed a close relationship between pollen-stigma morphologies and reproductive strategies [25, 30, 31]. Therefore, combinations of stigma and pollen traits have been widely used to infer whether plants are SI sexual, SC sexual, or apomictic.

Species displaying intra-specific variation of reproductive traits and occurring on both mainland and volcanic islands are especially suitable to test Baker's law, as less divergence that could change the reproductive traits facilitating initial long-distance colonization is expected to occur within than between species. Intraspecific variation of reproductive traits and self-compatibility has been described in three sexual species of *Limonium* and *Armeria* [5, 26, 28, 35, 37–40]. However, none of these studies quantified whether stigma-pollen traits significantly differ between island and mainland populations of the same species or experimentally tested whether capacity for uniparental reproduction is overrepresented on islands, as predicted by Baker's law. This gap of knowledge is investigated here using *Limonium lobatum* as model system.

Limonium lobatum (L. fil.) Chaz. (synonym *L. thouinii* (Viv.) Kuntze; *L.* sect. *Pteroclados* subsect. *Odontolepideae*; Subfamily *Limonioideae*; *Plumbaginaceae* [41]) has a circum-Mediterranean distribution, occurring on both volcanic islands and mainland (see Baker's description of its distributional range in [25, 26, 42]). *Limonium lobatum* was described as stigma-pollen dimorphic and self/intra-morph incompatible by Baker [25, 26]. The observation that this widespread species appeared to have retained the floral dimorphism across its entire distributional range, including the large disjunction between mainland and Canary Islands, puzzled Baker: "*L. thouinii (Viv.) Kuntze is of particular interest because of its distribution from the Middle East throughout the Mediterranean lands (particularly on the North African side) to the Canary Isles. Despite the widespread distribution, including the large Atlantic disjunction, the species has kept its dimorphism of pollen and stigmata*" p. 616 [26]. However, recent field surveys of Canarian and Iberian plants of *L. lobatum* suggested that previously undiscovered variation of stigma-pollen traits might occur in this species (A. Jiménez, pers. obs.). Thus, *L. lobatum* warrants in-depth analyses of variation in pollen-stigma traits and production of selfed vs. outcrossed seeds between island and mainland populations to test whether the ability for uniparental reproduction is overrepresented on islands, as predicted by Baker's law.

Using plants raised under standard greenhouse conditions from seeds randomly collected in natural island and mainland populations of *L. lobatum* (Fig. 2; Table S1), we studied variation of stigma and pollen traits at different hierarchical levels (from intra-flower to inter-population) and tested Baker's law by comparing island and mainland populations for seed set after self- and cross-pollinations. Since colonizers often lack mates and/or pollinators after long-distance dispersal to volcanic islands, uniparental reproduction should be overrepresented on islands vs. mainland. Specifically, we tested whether: (i) *L. lobatum* harbors undescribed variation of stigma-pollen traits with differences between islands and mainland and (ii) island plants produce more seeds when selfed than mainland plants, hence are more SC. Contrary to previous reports by Baker [25, 26], we discovered that *L. lobatum* is not just pollen-stigma dimorphic. Rather, it also encompasses previously undescribed variation of stigma traits, especially in island plants, which produce more selfed progeny than mainland plants, conformant with the predictions of Baker's law. The results of our experiments support the conclusion that *L. lobatum* is largely SC, rather than fully SI, as previously described by Baker. The present study expands our knowledge of how variation in reproductive traits affects plant mating on islands vs. mainland, establishing new links between reproductive and island biology.

Results

(A) Stigma and pollen traits vary at different hierarchical levels in *Limonium lobatum*

By quantifying variation of stigma traits in 955 flowers from 208 experimental plants (specifically, 2-5 flowers per plant from 203 plants and one flower per plant from five plants), we discovered undescribed variation of female reproductive traits at four different hierarchical levels, from stigma papillae to stigmas to gynoecia to plants. First, we found variation at the level of stigma papillae, discovering a previously undescribed papillae shape that is intermediate between the typical flat (*cob*) and protruding (*pap*) shapes: we termed this novel papilla type '*int* papilla' (Fig. 3; Table 1). Secondly, we found variation at the level of stigmas (Figs 3-4). Specifically, out of 4748 stigmas, 3040 (or 64%) had uniformly flat papillae (i.e., typical *cob* stigmas), 1259 (or 26.5%) had uniformly protruding papillae (i.e., typical *pap* stigmas), and 449 (or 9.5%) had various combinations of *int*, *pap*, and *cob* papillae: we termed this third, novel stigma type '*var* stigmas' (Table 1). Thirdly, we found variation at the level of gynoecia. Specifically, out of 955 gynoecia, 591 (or 61.9%) were formed entirely by typical *cob* stigmas, 235 (or 24.6%) were formed entirely by typical *pap* stigmas, and 129 (or 13.5%) were formed by various combinations of *var*, *pap*, and *cob* stigmas: we termed this third, novel gynoecium type '*var* gynoecia'. Finally, we found variation at the level of plants. Specifically, out of 167 plants, 92 (or 55.1%) had flowers with typical *cob* gynoecia, 25 (or 15%) had flowers with typical *pap* gynoecia, and 50 plants (or 29.9%) had flowers with various combinations of *var*, *pap*, and *cob* gynoecia: we termed this fourth, novel plant type '*var* plants'. To summarize, previously undescribed variation of stigma traits occurred both within and among flowers of individual plants (Table 1).

Differently from stigma traits, we discovered no undescribed variation of pollen traits after analyzing 567 flowers in 167 experimental plants (specifically, 2-5 flowers per plant in 140 plants and one flower per

plant in 27 plants). Pollen grains from individual flowers were generally well developed, homogeneous in size, and had either coarse (*A* pollen: 164 out of 567 flowers, or 28.9%) or fine exine sculpturing (*B* pollen: 403 out of 567 flowers, or 71.1%; Figs 3, 4A). Moreover, out of the 140 plants from which multiple flowers were analyzed, 38 (or 27.1%) had flowers with typical *A* pollen and 102 (or 72.9%) had flowers with typical *B* pollen (Table S3). Thus, no variation of pollen traits was detected either within or among flowers (Table 1).

Finally, we discovered previously undescribed variation of stigma-pollen combinations within flowers, among flowers within plants, among plants in the same population, and among plants in different populations (Fig. 4; Table 1). At the intra-flower level ("Flower types" in Table 1), out of 770 flowers, 689 (or 89.5%) had typical stigma-pollen combinations (*cob*|*A*: 180 flowers; *cob*|*B*: 318 flowers; *pap*|*A*: 7 flowers; and *pap*|*B*: 184 flowers) and 81 (or 10.5%) had previously undescribed stigma-pollen combinations (*var*|*A*: 11 flowers; *var*|*B*: 70 flowers; Fig. 4C). At the intra-plant level ("Plant types" in Table 1), out of 167 plants, 117 (or 70.1%) had flowers with typical stigma-pollen combinations (*cob*|*A*: 37 plants; *cob*|*B*: 55 plants; and *pap*|*B*: 25 plants) and 50 (or 29.9%) had flowers with variable stigma-pollen combinations (variable combinations of *var*|*A*, *cob*|*A*, and *pap*|*A* flowers: 6 plants; variable combination of *var*|*B, cob*|*B*, and *pap*|*B* flowers: 44 plants). At the intra-population level ("Population types" in Table 1), out of 11 populations, nine (or 81.8%) had plants with previously undescribed stigma-pollen combinations: one population was monomorphic with all plants *var*|*B* [I-4], one was dimorphic with *var*|*B* and *pap*|*B* plants [I-5], four were dimorphic with *var*|*B* and *cob*|*B* plants [I-3, M-1, M-2, and DS], and three polymorphic with multiple combinations of plant types [I-1, I-2, and M-3]). The remaining two populations (or 18.2%) were typical, either monomorphic with all plants *cob*|*A* [M-4] or monomorphic with all plants *pap*|*B* [M-5]. To summarize, not all stigma-pollen combinations across the studied populations (Fig. 4D) conformed to combinations previously described in either SI sexual species (all populations consisting of *cob*|*A* and *pap*|*B* plants), SC sexual species (all populations monomorphic for either *pap*|*A* or *cob*|*B* plants), or apomictic species (populations monomorphic for *cob*|*A*, *pap*|*A*, *cob*|*B*, or *pap*|*B* plants and/or dimorphic with *cob*|*A* and *pap*|*B* plants). In conclusion, our results demonstrate that *L. lobatum* is characterized by previously undescribed variation of stigma traits and stigma-pollen combinations at all studied hierarchical levels. Therefore, *L. lobatum* represents a new type of polymorphic species with previously undescribed pollen-stigma variation.

(B) *Limonium lobatum* is a sexually reproducing species

The morphological results described above allowed the identification of three monomorphic populations: I-4 was monomorphic for *var*|*B*, M-4 for *cob*|*A*, and M-5 for *pap*|*B* (Fig. 4D). We then proceeded to experimentally determine whether such monomorphic populations consisted of SC, sexually reproducing plants or SI apomictic plants. We discovered that *A* pollen adhered, germinated, and formed pollen tubes that penetrated *cob* stigmas, and *B* pollen adhered, germinated, and formed pollen tubes that penetrated both *pap* and *var* stigmas (Fig. S1). Therefore, all 16 plants tested for pollen germinability from the three monomorphic populations were SC. Moreover, pollen grains were well developed and homogeneous in size in all 54 plants from the three monomorphic populations and, more generally, in all 167 experimental

plants (see results in section [A] above). Taken together, our results indicate that all three monomorphic populations with both previously described and novel pollen-stigma combinations are SC and sexually reproducing, whereas agamospermy does not appear to occur in *L. lobatum*.

(C) Previously undescribed plant types of *Limonium lobatum* produce more selfed seed than outcrossed seeds

Selfed seed-set was generally high (Fig. 5A). Hence, the McMullen-index classified only 21 out of 164 plants (12.8%) as SI and the Ramírez & Nassar-index classified only 11 out of 57 plants (19.3%) as (partially) SI in *L. lobatum* (Fig. 6). Previous studies stated that *cob*/*A* and *pap*/*B* plants were SI, while *cob*/*B* and *pap*/*A* plants were SC in *Limonioideae* (Fig. 1 [5]). However, our results showed that selfed seed-set did not significantly differ between the stigma-pollen combinations traditionally regarded as SI (*cob*/*A* and *pap*/B) and those traditionally regarded as SC (*cob*/*B* and *pap*/*A*; all $P > 0.05$; Fig. S2; for detailed results of the generalized linear mixed effects model [GLMM] see Table S2A). In addition, flowers with previously undescribed stigma-pollen combinations had (significantly) higher seed set after manual selfing and significantly lower seed set after cross-pollination than flowers with stigma-pollen combinations traditionally regarded as SI ($P_{SELF\text{-}EITO} = 0.056$; $P_{CROSS} = 0.029$) and SC ($P_{SELF\text{-}GEITO} = 0.023$; $P_{CROSS} = 0.029$). Finally, significantly more plants with previously undescribed stigma traits were classified by the Ramírez & Nassar-index as partially or fully cross-incompatible than under random expectation (24.0 vs. 20.0, N = 55, Chi-square = 7.333, $P = 0.007$; Fig. 6B). Our results thus indicate that (i) previously undescribed plant types relied more on selfing than outcrossing and (ii) *L. lobatum* is a mostly SC species, contrary to the previous description by Baker that identified it as a self/intra-morph incompatible species.-

(D) Islands harbor more plants capable of producing selfed seeds and more previously undescribed plant types than the mainland

After showing that plants with previously undescribed pollen-stigma combinations relied more on selfing than outcrossing (Figs 6B, S2), we proceeded to compare seed set in insular vs. mainland populations of *L. lobatum*. According to Baker's law, selfed seed-set should be higher and outcrossed seed-set equal or lower on islands than mainland, respectively. Indeed, the results of our manual pollination experiments confirmed that island plants had (significantly) higher seed set after self-pollination ($P_{SELF\text{-}GEITO} = 0.010$; $P_{SELF\text{-}AUTO} = 0.068$) and significantly lower seed set after cross-pollination than mainland plants ($P_{CROSS} \leq 0.001$; Fig. 5A; for detailed GLMM results see Table S2B). Furthermore, significantly more SC plants occurred on islands than under random expectation (McMullen-index: 86 vs. 82, N = 164, Chi-square = 3.637, $P = 0.048$; Ramírez & Nassar-index: 28 *vs.* 22.6, N = 57, Chi-square = 13.160, $P \leq 0.001$; Fig. 6). Therefore, the capacity for uniparental reproduction was enriched on islands vs. mainland, supporting the predictions of Baker's law.

Given the results above (Figs 5A, 6, S2), we proceeded to test the hypothesis that plants with more variable stigma traits are overrepresented on islands. In agreement with this prediction, plants with

previously undescribed stigma-pollen combinations were significantly more frequent on the Canary Islands than the geographically close Iberian mainland (one-tailed *t*-test: $t = 6.481$, d.f. = 8, $P < 0.001$) and the geographically distant Dead Sea shore (one-tailed, one-sample *t*-test: $t = 5.621$, d.f. = 4, $P = 0.007$; Fig. 5B).

Discussion

The notion that individuals capable of uniparental reproduction are more successful long-distance colonizers than individuals dependent on biparental reproduction has fascinated evolutionary biologists since it was first postulated by Baker [12, 25-27] for plants and Longhurst [43] for animals. Experimental determination of reproductive characteristics of species occurring on both islands and the mainland is therefore crucial to test the hypothesis above. We discovered novel variation in the reproductive structures of the circum-Mediterranean species *Limonium lobatum* and experimentally demonstrated that most plants were SC, contrary to Baker's previous description of this species as self/intra-morph incompatible [25]. Moreover, we discovered that *A* pollen was compatible with *cob* stigmas and *B* pollen was compatible with *pap* stigmas, two stigma-pollen combinations typically assumed to be SI. Thus, our results imply that previously established correspondence between specific stigma-pollen combinations and SI or SC does not apply to *L. lobatum*. This is the first time that such a lack of correspondence has been described in *Limonioideae* outside the exceptionally variable species complex of *Armeria maritima* [44, 45], where it was observed in a single population on the Shetland Islands [5], and in a few populations growing on metal-contaminated soils [28, 46, 47]. Finally, plants with variable stigma traits relied more on self- than cross-pollination and were overrepresented on islands vs. mainland. Consequently, our results validate the main prediction of Baker's law, namely that the capacity for uniparental reproduction is enriched in habitats colonized *via* long-distance dispersal.

(A) *Limonium lobatum* harbors undescribed variation of reproductive traits and is largely self-compatible

Baker [25, 26] described *L. lobatum* as a stigma-pollen dimorphic and self/intra-morph incompatible species. Contrary to Baker's description, our morphological analyses revealed that *L. lobatum* consisted of mono-, di-, and polymorphic populations of previously described and/or undescribed floral morphs (Fig. 4D). Moreover, our pollination experiments revealed that most plants were SC (> 80%), while only 13%-19% were partially or fully SI (Fig. 6). Finally, we found that pollen grains of *L. lobatum* were consistently well developed and homogeneous in size (Figs 3, 4A), suggesting that this species reproduces sexually. We can thus conclude that *L. lobatum* is neither a typical SI sexual species characterized by dimorphic *pap*/*B*-*cob*/*A* populations, nor a typical SC sexual species characterized by monomorphic *pap*/*A* or *cob*/*B* populations, nor a typical apomictic species characterized by misshaped and irregularly sized pollen grains [36, 39, 43, 52]. Previous studies lend additional support to the conclusion that *L. lobatum* is not apomictic: (i) *L. lobatum* belongs to *L.* subg*. Pteroclados*, which is phylogenetically distinct from *L.* subg*. Limonium*, the clade comprising all described apomictic species [41, 48]; (ii) *L. lobatum* is a diploid annual, and apomixis is usually associated with polyploidy, while being extremely rare in diploid, annual plants [20]. Altogether, all lines of evidence suggest that *L.*

lobatum is a sexually reproducing, largely SC species characterized by uncommon variation in reproductive traits that deviates from previous species descriptions [25, 27, 29].

Taken together, the results above support the conclusion that we discovered a kind of variation of stigma traits never documented before in *Plumbaginaceae*. Indeed, deviations from typical surface morphologies of pollen (coarse vs. fine exine sculpturing) and stigmas (flat vs. protruding papillae cells) are rare in *Limonioideae*. Unusual surface morphologies of pollen and stigmas were noticed, to our knowledge, only in *L. humile* and *Armeria maritima* subsp. *sibirica* [31, 40, 49, 50]. Specifically, pollen grains with variable or intermediate exine sculpturing and stigmas with homogeneous intermediate papillae shapes were found in these two taxa, although the latter was attributed to premature termination of stigma development due to self-pollination in still-closed flowers of *A. maritima* subsp. *sibirica* [31, 49, 50]. Conversely, in *L. lobatum* we found no variation of male reproductive structure but variation of female reproductive structures not attributable to immature stage of floral development. Indeed, we detected well-developed stigmas characterized by papillae of flat (*cob*), protruding (*pap*) and/or intermediate (*int*) shapes, allowing us to describe a new stigma type in *Limonioideae* (i.e., *var* stigma; Figs 3-4, Table 1). Moreover, papillae shapes varied among stigmas of a gynoecium, and among flowers of a plant, revealing levels of variation never documented before. The discovery that female reproductive structures can be variable within plants, while male reproductive structures remain homogeneous, probably reflects the fact that flowers are developmental and functional mosaics comprising four organ whorls (calyx, corolla, androecium, and gynoecium), each with its own developmental genetic regulation [51, 52].

What are the potential causes of the unexpected variation of female reproductive structures we discovered in *L. lobatum*, especially in island populations? Variation of reproductive structures can be caused by developmental stage or position in an inflorescence, developmental plasticity, and/or developmental instability [51-54]. Developmental plasticity tends to produce consistent patterns of trait variation [53, 54], while developmental instability tends to produce more irregular patterns of trait variation [51-53]. The latter phenomenon might occur in plants of *L. lobatum* characterized by previously undescribed variation of female traits, where the shapes of stigmatic papillae varied randomly both among stigmas of single flowers and among flowers of single plants, particularly in island plants (Figs 4-6, Table 1). This inconsistent pattern of variation may be caused by homozygosity, which can introduce random errors during organ development [53]. Since selfing increases homozygosity, the floral organs of self-compatible plants may exhibit elevated developmental instability. Island plants of *L. lobatum* had both (i) more variable stigma traits and (ii) higher selfed and lower outcrossed seed set than mainland plants, implying that island plants self more frequently than mainland plants. Thus, our results are consistent with previous suggestions that developmental instability is particularly pronounced in isolated, peripheral, and self-compatible populations that are more likely to self [55].

(B) The capacity for uniparental reproduction is enriched in island populations of *Limonium lobatum*

The observation that *L. lobatum* appeared to have retained floral dimorphism and, presumably, SI across the large disjunction between mainland and Canary Islands puzzled Baker [26], as it contradicts the

predictions of his namesake law. Indeed, we discovered significant differences between island and mainland populations. Specifically, we found that island plants had more variable stigma traits and produced more selfed than outcrossed seeds, hence were more SC than mainland plants (Figs 5, 6), as discussed below.

The number of SC individuals in a population should increase when the selective advantages of self-fertilization outweigh its costs [56-58]. Since the ability for self-fertilization may provide reproductive assurance when outcrossing opportunities are limited [7], selection should favor SC in annual plants and in plants colonizing distant, isolated habitats [16, 59-62]. Corroborating both predictions, most (> 80%) plants of the annual *L. lobatum* were SC and these SC plants were overrepresented on islands vs. mainland (Figs 5B, 6). Additionally, island plants produced more seeds after self- than cross-pollination, while seed set of mainland plants did not differ largely between the two pollination treatments (Fig. 5A). In summary, island plants displayed an increased ability for uniparental reproduction. Hence, our results fit the expectations of Baker's law that individuals with a higher capability for uniparental reproduction are more likely to become established and persist after long distance dispersal.

The transition from outcrossing to selfing is a two-step process [63]: (i) a shift from SI to SC at the individual level when mutations weaken/disable the SI system in individual plants; (ii) a shift to inbreeding at the population level when selection favours individuals with a weakened/disabled SI system, resulting in the spread and fixation of the selfing allele within a population. The latter usually occurs only when inbreeding depression is weak [10, 61, 64]. Selfed flowers of *L. lobatum* produced as many or more seeds than outcrossed flowers (Fig. 5A), suggesting that inbreeding depression is absent or negligible at the stage of seed production in *L. lobatum*. This result is consistent with findings in predominantly self-fertilizing populations [65] and species [64] reporting the absence of inbreeding depression at the seed production stage. Since inbreeding depression can be expressed at later stages of the life cycle [66], we cannot exclude the possibility that it could affect the fitness of selfed *L. lobatum* plants. Moreover, selfed and outcrossed seed-sets were similar on the mainland, whereas selfed seed-set was significantly higher than outcrossed seed-set on the islands (Fig. 5B). This finding suggests that in insular populations of *L. lobatum* genetic incompatibilities hinder the formation of outcrossed seeds (i.e., outbreeding depression [67-69]). Selfing increases the probability of fixation of genetic factors known to cause outbreeding depression (e.g., underdominant loci, chromosomal rearrangements, and negative epistatic interactions [1, 70-73]). Consequently, within-population outbreeding depression has only been reported in populations with considerable levels of selfing [67, 68, 70, 74-81]. It is therefore likely that island plants of *L. lobatum* self-fertilize more frequently than mainland plants and suffer from outbreeding depression. Future crossing experiments and/or population genomic analyses could elucidate the potential causes and genetic basis of outbreeding depression in island populations of *L. lobatum*.

Conclusions

Contrary to previously published accounts, we discovered that *L. lobatum* is a largely SC species characterized by previously undescribed variation of reproductive traits. Plants with variable stigma traits were overrepresented on islands, suggesting that the development of female reproductive organs is more error-prone on islands than on the mainland, perhaps as a consequence of elevated homozygosity caused by higher selfing on islands [53]. Indeed, we documented that selfed seed-set was significantly higher than outcrossed seed-set on the islands, even though outcrossed and self-seed set were similar on the mainland, suggesting that genetic incompatibilities might hinder the formation of outcrossed seeds of island plants. Conformant with the predictions of Baker's law, our study supports the notion that individuals with a higher capacity for uniparental reproduction are more likely to colonize distant, isolated habitats. In turn, elevated homozygosity stemming from selfing in isolated populations might introduce an element of randomness in the developmental program of female reproductive traits. In conclusion, our results confirm that comparative trait-based approaches of island and mainland populations are well suited to elucidate the eco-evolutionary dynamics of long-distance colonization and provide new links between reproductive and island biology.

Methods

(A) Study species

Limonium lobatum is a diploid (*2n* = 12), annual herb up to 50 cm tall with conspicuously winged, photosynthetic flowering stems bearing pentamerous flowers with pale-yellow corollas and pale-blue papery calyces [42, 82, 83]. The female reproductive structures (i.e., gynoecium) consist of one ovary with a single ovule and five styles (ca. 3 mm long) each subtending an elongated, linear stigma (ca. 2 mm long; Fig. 1). The male reproductive structures (i.e., androecium) comprise five anthers fused to the base of the corolla but free at the top [35, 42]. The architecture of the inflorescence is complex. Each inflorescence comprises several spikes, each spike comprises 6–12 spikelets, and each spikelet bears 2–3 flowers (Fig. S3). Within spikes and spikelets, flowers bloom from the proximal to the distal part. The corolla, stigma and anthers wither within less than 24 hours after flower opening, while the papery calyx persists throughout the growing season. The species typically grows on sandy or gravelly soils in dry areas along the coastlines of the Mediterranean Basin, extending also to Fuerteventura and Tenerife, two islands of the Canarian archipelago at the western end of its distributional range [84]. Plants usually occur in small patches with a maximum radius of 100 m in populations of about 100–200 individuals (A. Jiménez, pers. obs.).

(B) Seed collection, germination, and cultivation of experimental plants

Experimental plants were raised from seeds collected in autumn 2011 from at least 20 randomly selected natural plants from each of the ten studied populations: five in the Canary Islands (I; one in Tenerife and four in Fuerteventura) and five in the southern part of the Iberian mainland (M) (Fig. 2). One voucher specimen per population is kept at Herbarium Z (Zürich, Switzerland; Table S1). In addition, we raised

experimental plants from seeds obtained from a natural population on the shores of the Dead Sea (DS) *via* the Jerusalem Botanical Gardens (Israel; IL0Z-20150596). In spring 2012, we germinated 10 seeds per plant and randomly transplanted a total of 210 seedlings into individual pots: 20 seedlings from each of the Canarian and Iberian populations and 10 seedlings from the Dead Sea population (Fig. S4). Experimental plants thus represent a random subset of the studied natural populations.

Experimental plants were cultivated under standard conditions in the pollinator-free environment of a greenhouse at the Botanical Garden of the University of Zürich, Switzerland (temperature: 18–22°C; humidity: ~65%; photoperiod: 12h dark /12h light, minimum 30'000 LUX). All plants started blooming 10 to 12 weeks after germination.

(C) Stigma and pollen traits vary at different hierarchical levels in Limonium lobatum

Developmental stage (i.e., age) and/or position of flowers within an inflorescence can influence the expression of floral traits [51, 52]. To minimize the effects of developmental stage and position, we randomly harvested 2–5 freshly opened flowers from each of the 210 plants on the same day whenever possible (Table S3A; see also additional analyses in Methods & Results S1). Gynoecia of two harvested flowers were shriveled and the anthers of 392 harvested flowers shed all their pollen prior to the study, thus the total number of flowers analyzed for stigma and pollen traits was 955 from 208 plants and 567 from 167 plants, respectively (Fig. S4A).

For morphological analysis, stigmas and anthers were covered with a drop of water on a microscope slide and examined at 40x magnification using an optical light microscope (Olympus CH30; Olympus Corporation, Tokyo, Japan) equipped with a digital imaging system (AxioCam; Axio Vision Rel. 4.8; ZEISS, Oberkochen, Germany). For each flower, we recorded the variation of papillae shapes along the entire linear stigma for all five stigmas of each gynoecium and the coarseness of exine sculpturing in pollen grains from each anther. The observed stigma and pollen traits and/or their combination were then compared with previously described morphologies. Variation of floral traits can occur within female or male reproductive-structures of a flower, among flowers within plants, among plants of the same population, and among plants of different populations [51–54, 85]. Examination of 140 plants showed that pollen traits of *L. lobatum* do not vary within plants (Table S3A), thus it was possible to assign all flowers of each plant to a single pollen type as long as at least one flower in a plant had pollen-filled anthers. The final assignment of stigma-pollen traits to different types of stigma-pollen combinations was based on 770 flowers from 167 plants (Fig. S4A). In summary, we described variation of pollen and stigma traits, and/or their combination at the following hierarchical levels: intra-flower, intra-plant, intra-population, and inter-population.

(D) Limonium lobatum is a sexually reproducing species

Morphological analyses identified three monomorphic populations: I-4 was monomorphic for *var*|*B*, M-4 for *cob*|*A*, and M-5 for *pap*|*B* (see Results). The former stigma-pollen combination was previously undescribed, while the latter two stigma-pollen combinations were traditionally assumed to be SI (Fig. 1

[5]). However, for plants in such monomorphic populations to reproduce *via* seeds, they must be either SC sexuals or agamospermous apomicts. Since apomicts have misshaped *A* or *B* pollen grains, functional styles, and an intact SI system [31], they can be discerned from SC sexuals. To determine whether plants in monomorphic populations were SC or apomictic, we screened pollen grains for morphological irregularities (see section [C] above) and tested SI by manually selfing ten *cob*|*A* flowers of five plants from population M-4, 15 *pap*|*B* flowers of seven plants from population M-5, and six *var*|*B* flowers of four plants from population I-4. Flowers were harvested 24–48 hours after pollination, dissected, and stigmas stored in 70% ethanol. Pollen tubes were stained and microscope slides prepared following Martin's [86] method with minor adjustments. For each experimental flower, we visually checked whether any of the applied pollen grains germinated and any of the budding pollen tubes penetrated the stigmatic surface by direct illumination with UV-light using an optical microscope (Olympus Bx50; Olympus Corporation, Tokyo, Japan) equipped with a digital imaging system (AxioCam; Axio Vision Rel. 4.8; ZEISS, Oberkochen, Germany).

(E) Previously undescribed plant types of Limonium lobatum produce more selfed seeds than outcrossed seeds

Data collection

To quantify variation of uniparental vs. biparental reproduction in typical vs. previously undescribed plant types and test Bakers law (see section [F] below), we performed the following pollination experiments. In freshly opened, intact flowers of each plant, we executed three treatments: (i) no pollen was manually transferred onto stigmas, allowing only for autonomous fertilization within the same flower ($SELF_{AUTO}$ treatment); (ii) stigmas were manually pollinated with pollen from two flowers of the same plant, allowing for both geitonogamous and autonomous self-fertilization ($SELF_{GEITO}$ treatment); and (iii) stigmas were cross-pollinated with pollen from two flowers of two randomly selected plants from the same population, allowing for both cross-fertilization and autonomous self-fertilization (CROSS treatment). The $SELF_{AUTO}$ treatment was repeated on five flowers per plant, while the $SELF_{GEITO}$ and CROSS treatments were each repeated on ten flowers per plant. Fruits were collected when ripe. Each experimental flower was checked for presence or absence of a seed (binary trait: each flower can produce only one seed). Due to the death of some of the plants initially included in the pollination experiments, seed set was tallied in a total of 2881 flowers from 174 experimental plants (1633 flowers from 99 island plants [25 typical, 65 unusual, and nine undetermined plant types] and 1248 flowers from 75 mainland plants [63 typical, five previously undescribed, and seven undetermined plant types]; Fig. S4B and Table S4). Seed set was not appreciably altered by a time lag between the $SELF_{GEITO}$ and CROSS treatments (details in Methods & Results S2). Furthermore, we did not record the pollen type of the pollen donors in the CROSS treatment. It is therefore possible that we have pollinated flowers with *A* pollen, *B* pollen, or a mixture of both pollen types in pollen-dimorphic populations. If the two types of pollen trigger incompatibility reactions of different strengths, it could affect the success of outcrossed seed-set in pollen-dimorphic vs. pollen-monomorphic populations. However, outcrossed seed-set was not appreciably altered by the presence of two pollen types vs. one pollen type (details in Methods & Results S2).

To further assess the ability for uniparental reproduction, we estimated for each plant, if applicable, two SI indexes from seed-set data after cross-pollination (CROSS) and/or self-pollination ($SELF_{AUTO}$ or $SELF_{GEITO}$, if seed set was higher in the latter). First, following McMullen [87], we used 20% of seed set after self-pollination as threshold value to designate a plant as SC ($SI_{index\ M}$). The values of the $SI_{index\ M}$ were calculated from 1465 self-pollinated flowers of 164 experimental plants (81 typical, 67 previously undescribed, and 16 undetermined plant-types) from nine populations (Table S4). Second, following Ramírez and Nassar [88], we estimated a widely used incompatibility index per plant based on a relative measure of reproductive output following self- and cross-pollinations with the modified equation

$$SI_{indexR-N} = \frac{(selfedseedset + x)}{(outcrossedseedset + x)}$$

1

,

where x is a very small number (i.e., 0.00001). Values indicate: $SI_{index\ R\text{-}N} = 0$, full SI; $0 < SI_{index\ R\text{-}N} < 1$, partial SI (pSI); $SI_{index\ R\text{-}N} = 1$, SC; $SI_{index} > 1$ or $0 < (1/\ SI_{index\ R\text{-}N}) < 1$, partial cross-incompatibility (pCI); and $SI_{index\ R\text{-}N} \sim \infty$ or $(1/\ SI_{index} \sim 0)$, full cross-incompatibility (CI); the latter two categories indicate presence of outbreeding depression. To summarize, $SI_{index\ R\text{-}N}$ values < 1 indicate (partial) SI, while $SI_{index\ R\text{-}N}$ values ≥ 1 indicate full SC. The $SI_{index\ R\text{-}N}$ can be estimated only for those plants that received cross- and self-pollination treatments. Since the CROSS treatment was performed on a subset of plants and populations, the values of $SI_{index\ R\text{-}N}$ were calculated from 497 cross- and 833 self-pollinated flowers of 57 experimental plants (30 typical, 25 previously undescribed, and two undetermined plant types) from six populations (Table S4). These numbers are well within the range used to estimate SI indexes in other systems [e.g., 22, 89, 90, 91], and well above the minimum number of 20 flowers recommended by Ramírez & Nassar [88].

Statistical analyses

Previous studies concluded that specific combinations of stigma and pollen traits tend to predict whether a species is SI or SC in *Limonioideae* (Fig. 1 [5, 25, 30, 31, 35]), with SI leakiness suggested in *Armeria maritima* [92]. Thus, one could expect crosses between *A* pollen and *pap* stigmas and between *B* pollen and *cob* stigmas to produce higher seed set, while crosses between *A* pollen and *cob* stigmas and between *B* pollen and *pap* stigmas to produce very low seed set. To test these predictions and determine whether previously undescribed plant types are SI or SC, we compared seed set of selfed and cross-pollinated flowers using a GLMM (binomial error distribution and logit link function) with contrasts and *pollination treatment* ($SELF_{AUTO}$, $SELF_{GEITO}$, and CROSS), *plant type* (previously undescribed and typical; the latter subdivided into plant previously described as SI and SC), and their interaction as fixed effects. We accounted both for (i) hierarchical data structure by using *plant* nested within *population* as random effects and (ii) unbalanced data set by using Satterthwaite's method to determine the approximate denominator degree of freedom (see Fig. S4B).

Selfed seed-set was (significantly) higher than outcrossed seed-set in previously undescribed plant types, implying that genetic incompatibilities hinder the formation of their outcrossed seeds. To test this prediction, we assessed whether significantly more previously undescribed plant types were classified as (p)CI by the Ramírez & Nassar index than under random expectation using a Pearson's chi-squared test.

(F) Islands harbor more plants capable of producing selfed seeds and more previously undescribed plant types than the mainland

If Baker's law applies to *L. lobatum*, we would expect seed set from self-pollination treatments ($SELF_{AUTO}$, $SELF_{GEITO}$, or both) to be higher on islands than mainland and seed set from cross-pollination treatment (CROSS) to be equal or lower on islands than mainland. Hence, more SC plants should occur on islands than under random expectation. To test these predictions, we compared the following variables between islands and mainland: (i) seed set of selfed and cross-pollinated flowers using a GLMM (binomial error distribution and logit link function) with contrasts and *pollination treatment* ($SELF_{AUTO}$, $SELF_{GEITO}$, and CROSS), *population locality* (mainland and island), and their interaction as fixed effects; (ii) numbers of SC plants (estimated by McMullen-index for SC plants and Ramírez & Nassar-index for SC, pCI, and CI plants) using Pearson's Chi-squared tests.

Finally, to test whether previously undescribed plant types that relied more on self- than cross-pollination (see Results) were significantly more frequent on Canary Islands than Iberian mainland and Dead Sea we used a one-tailed, two-sample *t*-test and a one-tailed, one-sample *t*-test, respectively. All statistical analyses were performed in SPSS version 27.0.0 (IBM Corp., Armonk, NY, USA) and sequential Bonferroni corrections were used to adjust significance levels for multiple tests, if applicable.

Declarations

- Ethics approval and consent to participate

Not applicable

- Consent for publication

Not applicable

- Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files (Tables **S3, S4**).

- Competing interests

The authors declare that they have no competing interests

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- Authors' contributions

AJ, BK, and EC conceived the idea and designed the project; AJ, BA, and BK performed the study; BK analysed the data; BK wrote the paper with input from AJ, BA, EM-C, KK, and EC.

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Table

Table 1 is available in the Supplementary Files section.

Figures

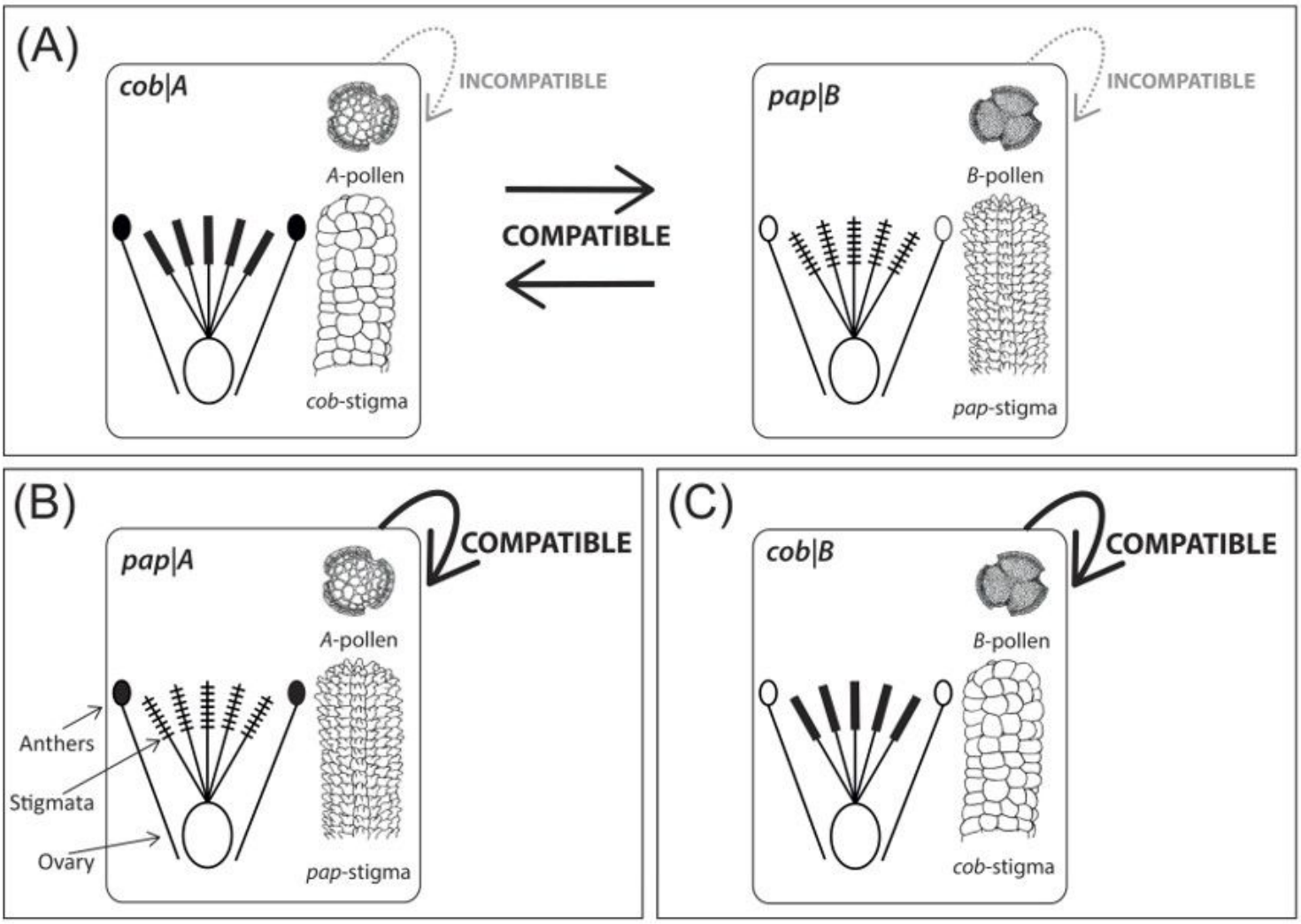

Figure 1

Schematic drawings of previously described combinations of female and male organs in sexually reproducing *Limonioideae*. **(A)** dimorphic species comprising inter-morph compatible (but self- and intra-morph incompatible) *cob*|*A* (with *cob* stigmas and *A* pollen) and *pap*|*B* (with *pap* stigmas and *A* pollen) flowers on separate individuals; **(B)** monomorphic species comprising self- and intra-morph-compatible *pap*|*A* (with *pap* stigmas, *A* pollen) flowers; and **(C)** monomorphic species comprising self- and intra-morph compatible *cob*|*B* (with *cob* stigmas, *B* pollen) flowers. Solid cross lines on stigmas indicate

protruding (*pap*) papillae, absence of cross lines indicates flat (*cob*) papillae. Filled and empty anthers represent *A* and *B* pollen, respectively. Drawings of pollen grains and stigmas were modified from Baker [31].

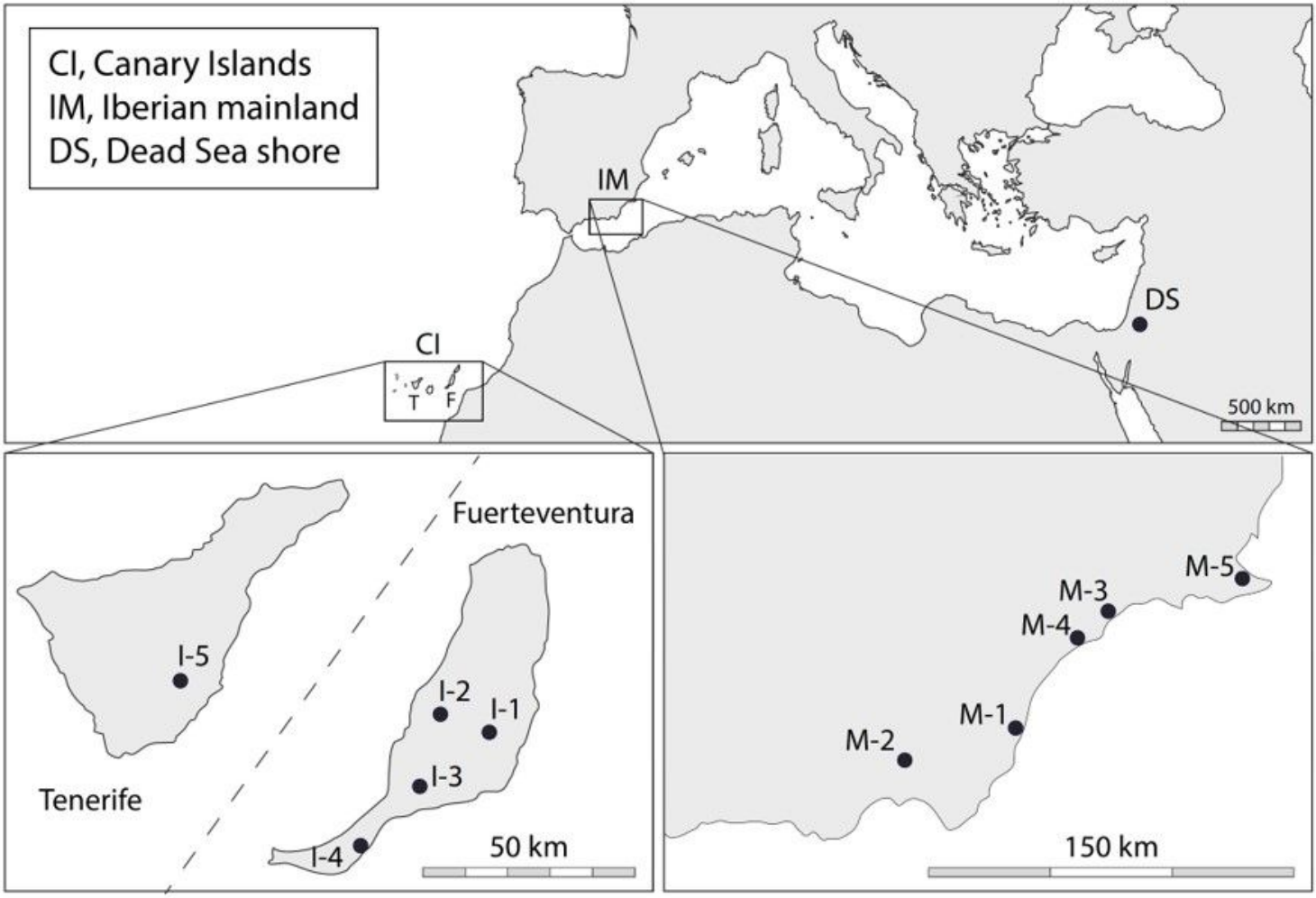

Figure 2

Locations of the eleven populations of *Limonium lobatum* sampled for this study. Locations of the five populations from the Canary Islands (Antigua: I-1; Betancuria: I-2; Tarajalejo: I-3; Esquinzo: I-4; and El Viso: I-5), the five populations from the Iberian mainland (Castillo de Macenas: M-1; Tabernas: M-2; Cala Blanca: M-3; Los Cocedores: M-4; and Los Nietos: M-5), and the single population from the Dead Sea shore. Maps modified from https://d-maps.com/.

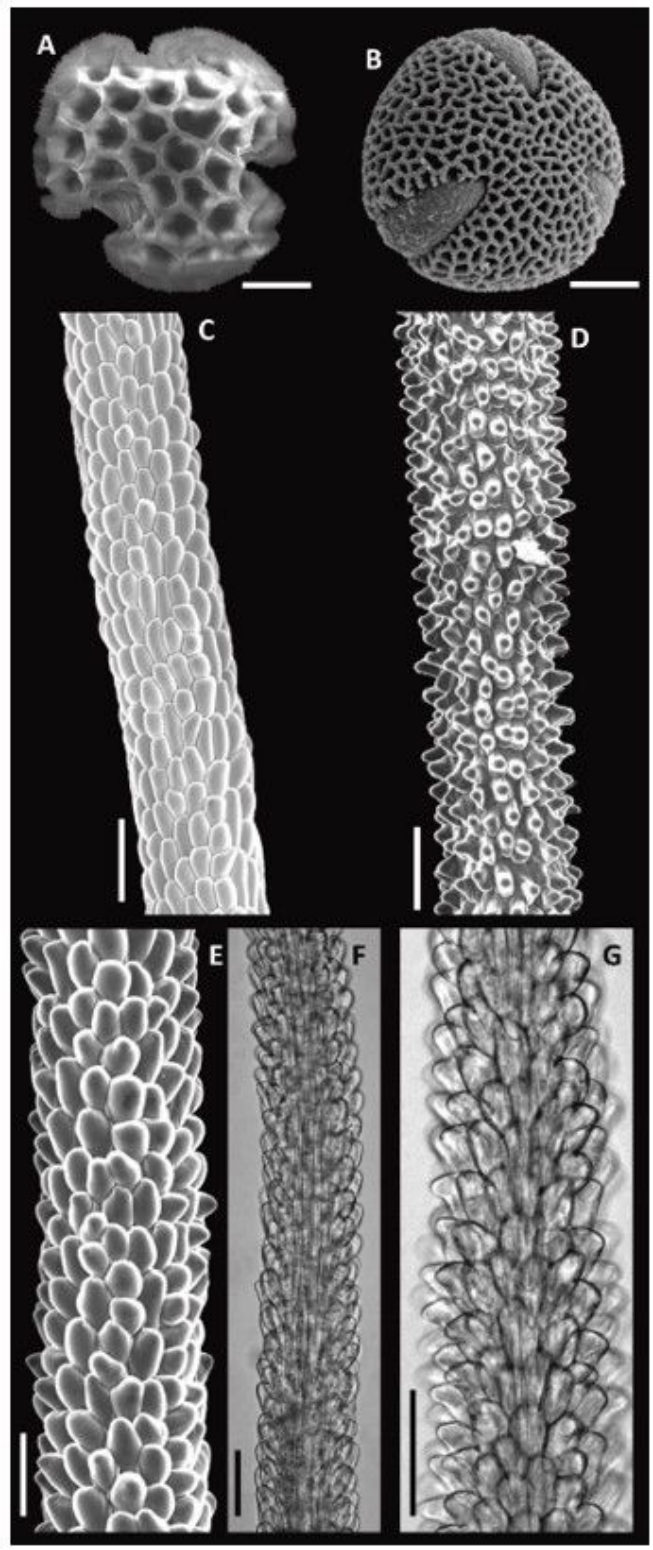

Figure 3

Variation of pollen exine sculpturing and shapes of stigmatic papillae discovered in *Limonium lobatum*. Pollen types: **(A)** *A* pollen with coarse exine sculpturing and **(B)** *B* pollen with fine exine sculpturing. Previously described (typical) stigmatypes: **(C)** stigma with uniformly flat (*cob*) papillae and **(D)** stigma with uniformly protruding (*pap*) papillae. Previously undescribed stigma types: **(E-G)** stigmas with various combinations (*var* stigmas) of *cob*, *pap*, and papillae of intermediate (*int*) shape in the same stigma.

Scale bars for A and B indicate 10 µm; scale bars for C-G indicate 50 µm. Photographs A-E were taken with a scanning electron microscope (JEOL 6360 VL; JEOL USA, Inc., Peabody, MA, USA); photographs F and G were taken with an optical microscope (Olympus CH30; Olympus Corporation, Tokyo, Japan) equipped with a digital imaging system (AxioCam; Axio Vision Rel. 4.8; ZEISS, Oberkochen, Germany). For definitions of pollen and stigma traits, see Table 1.

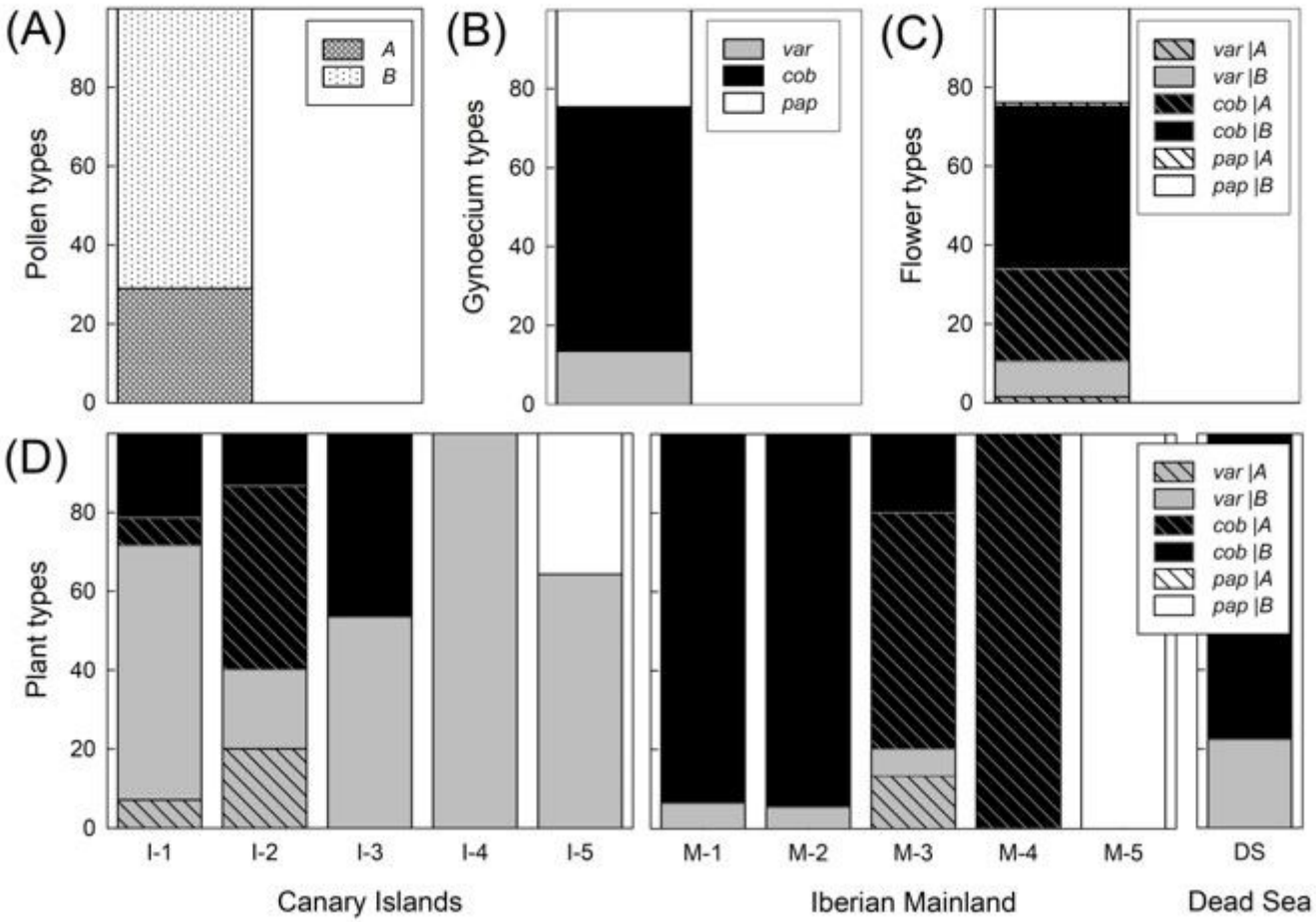

Figure 4

Islands harbour more plants of *Limonium lobatum* with previously undescribed pollen-stigma variation than the mainland. Upper panels: Proportions of different **(A)** pollen types, **(B)**gynoecium types, and **(C)** flower types across all studied plants. **Lower panels**: Proportion of different plant types **(D)**, subdivided into five insular (I-1 to I-5), five mainland (M-1 to M-5), and one Dead Sea (DS) populations. Location abbreviations as reported in Fig. 2. **(B-D)** Colour symbolism: black, typical morphologies with *cob* papillae; white, typical morphologies with *pap*papillae; grey, previously undescribed morphologies; hatched, *A* pollen; non-hatched, *B* pollen. For definitions of pollen and stigma traits, see Table 1.

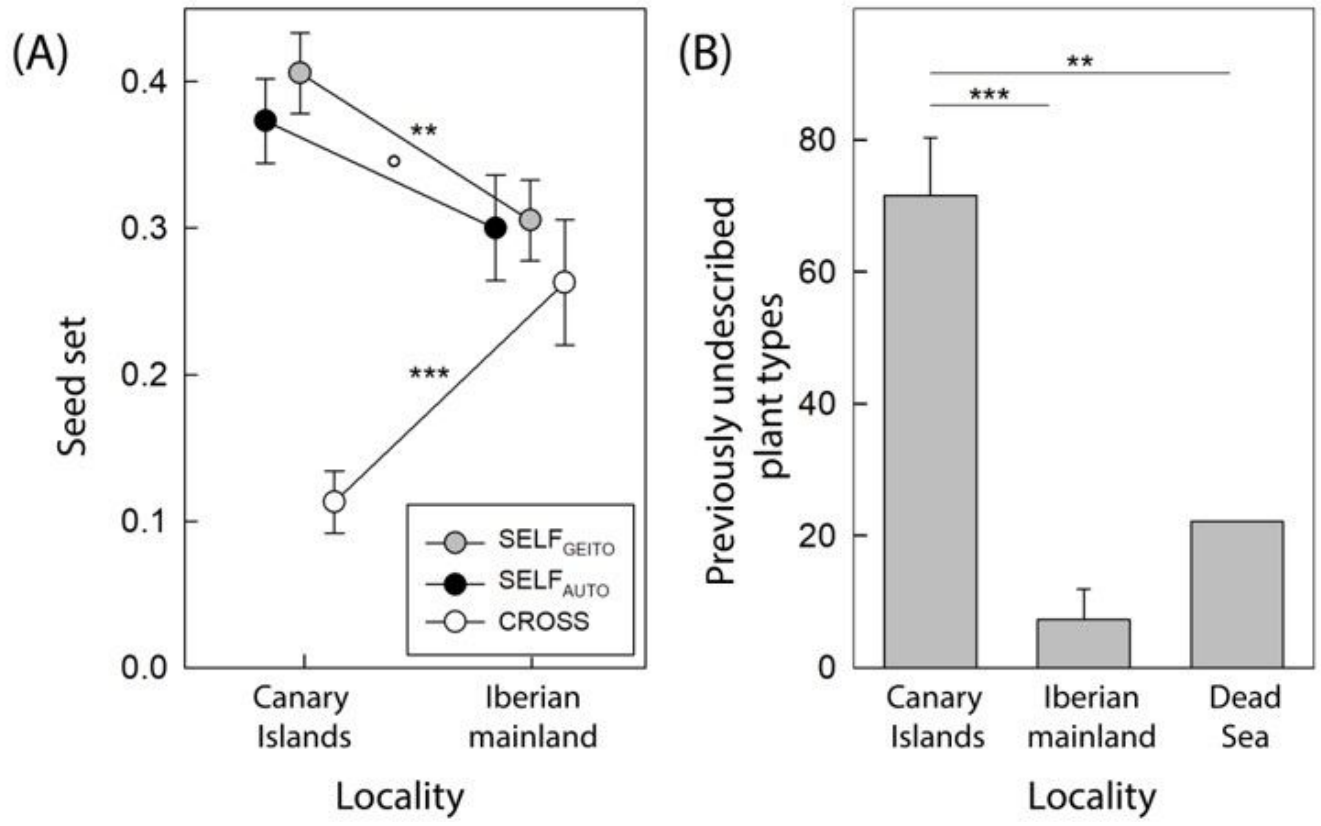

Figure 5

Island-mainland comparisons. (A) Seed set after self- and cross-pollinations: $SELF_{AUTO}$, seed set after autonomous selfing; $SELF_{GEITO}$, seed set from manual pollinations between flowers of the same plant (geitonogamous selfing); CROSS, seed set from manual pollinations between flowers of different plants (cross-pollination). **(B)** Relative frequencies of previously undescribed plant types (*var/A* and *var/B*). Significance levels: ***$P \leq 0.001$, **$P \leq 0.01$, °$P = 0.068$. Error bars denote standard errors SE.

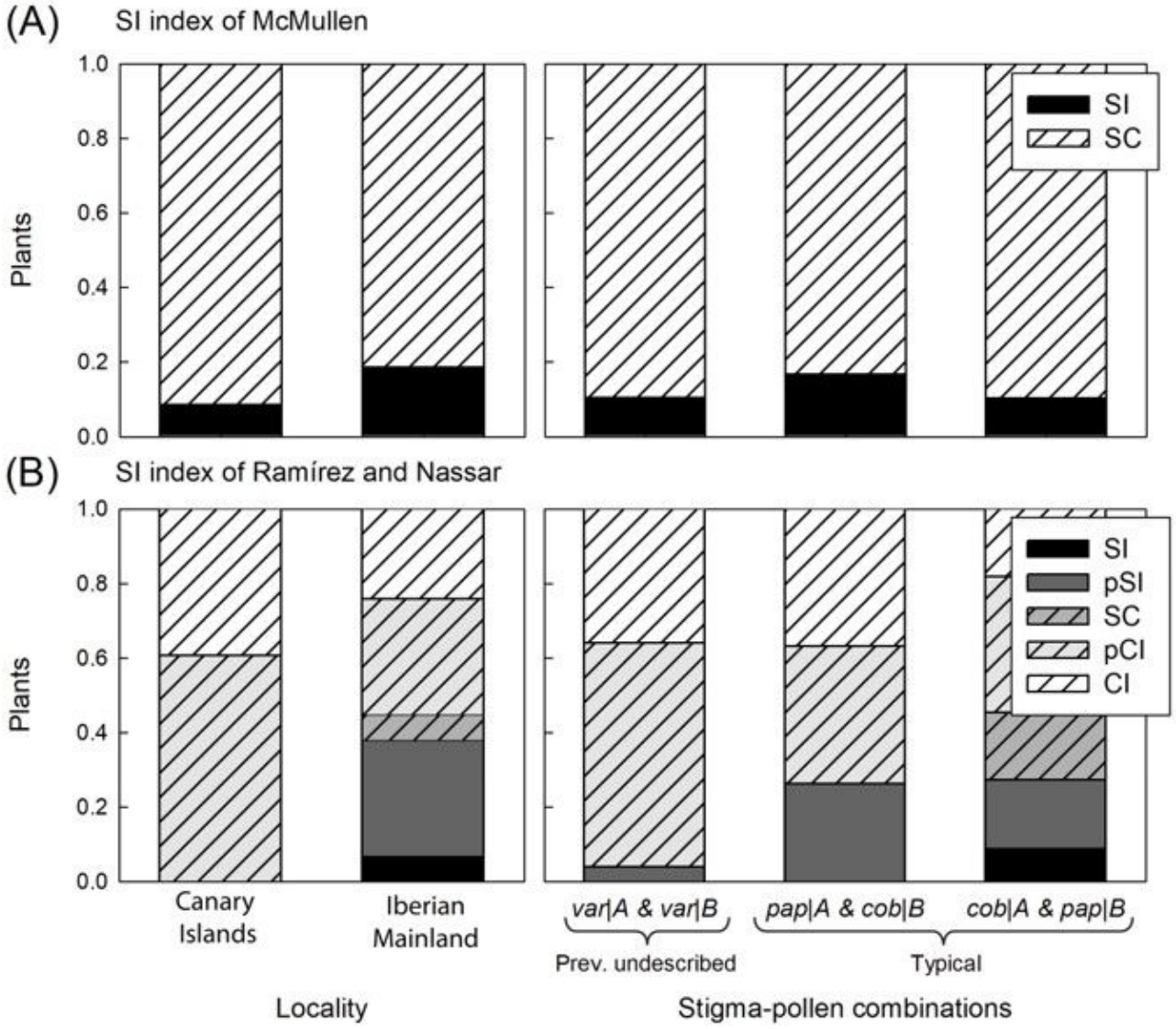

Figure 6

Occurrence of self-compatible plants (shown hatched) in *Limonium lobatum*. Left panels: island vs. mainland populations. **Right panels**: plants with previously undescribed (*var|A* and *var|B*) vs. typical stigma-pollen combinations subdivided into combinations previously described as SC (*pap|A* & *cob|B*) and SI (*cob|A* & *pap|B*). **(A)** index of McMullen ($SI_{index\ M}$ [87]) using 20% of seed set after manual ($SELF_{GEITO}$) or autonomous selfing ($SELF_{AUTO}$) as threshold value to indicate that a plant is self-compatible; **(B)** index of Ramírez and Nassar ($SI_{index\ R\text{-}N}$ [88]) based on the relative measure of reproductive output following self- ($SELF_{AUTO}$ or $SELF_{GEITO}$, if seed set was higher in the latter) and cross-pollinations (CROSS). **Abbreviations**: **(A)** SI, self-incompatibility (seed set after self-pollination is < 20%; SC, self-compatibility (seed set after selfing is ≥ 20%); **(B)** SI, full self-incompatibility ($SI_{index\ R\text{-}N} = 0$); pSI, partial self-incompatibility ($0 < SI_{index\ R\text{-}N} < 1$); SC, self-fertility ($SI_{index\ R\text{-}N} = 1$); pCI, partial cross-incompatibility ($SI_{index\ R\text{-}N} > 1$ or $0 < [1/\ SI_{index\ R\text{-}N}] < 1$); and CI, full cross-incompatibility ($SI_{index\ R\text{-}N} \sim \infty$ or

(1/ $SI_{index\ R\text{-}N}$) ~ 0); the latter two categories indicate presence of outbreeding depression; $SI_{index\ R\text{-}N}$ values < 1 indicate (partial) SI; and $SI_{index\ R\text{-}N}$ values ≥ 1 indicate full SC.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx
- KellerSupportingInformationKellerTablesS3S4.xlsx
- KellerSupportingInformation20231010.docx