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Diverse mating consequences of the evolutionary breakdown of the sexual polymorphism heterostyly

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Reproductive systems of flowering plants are evolutionarily fluid, with mating patterns changing in response to shifts in abiotic conditions, pollination systems, and population characteristics. Changes in mating should be particularly evident in species with sexual polymorphisms that become ecologically destabilized, promoting transitions to alternative reproductive systems. Here, we decompose female mating portfolios (incidence of selfing, outcross mate number, and intermorph mating) in eight populations of *Primula oreodoxa,* a self-compatible insect-pollinated herb. This species is ancestrally distylous, with populations subdivided into two floral morphs that usually mate with each other (disassortative mating). Stages in the breakdown of polymorphism also occur, including "mixed" populations of distylous and homostylous (self-pollinating) morphs and purely homostylous populations. Population morph ratios vary with elevation in association with differences in pollinator availability, providing an unusual opportunity to investigate changes in mating patterns accompanying transitions in reproductive systems. Unexpectedly, individuals mostly outcrossed randomly, with substantial disassortative mating in at most two distylous populations. As predicted, mixed populations had higher selfing rates than distylous populations, within mixed populations, homostyles selfed almost twice as much as the distylous morphs, and homostylous populations exhibited the highest selfing rates. Populations with homostyles outcrossed with fewer mates and mate number varied negatively with population selfing rates. These differences indicate maintenance of distyly at low elevation, transition to monomorphic selfing at high elevation, and uncertain, possibly variable fates at intermediate elevation. By quantifying the earliest changes in mating that initiate reproductive transitions, our study highlights the key role of mating in promoting evolutionary divergence.

evolution | mating | pollination | sexual polymorphism | heterostyly

Significance

Reproductive systems of flowering plants often evolve in response to differences among populations in pollinator faunas. In addition to affecting fecundity, these differences should influence mating, with multigeneration consequences for genetic and phenotypic diversity in populations and speciation. We investigated mating in a primrose species undergoing the breakdown of distyly, a sexual polymorphism that usually enforces mating between floral morphs. This mating pattern was evident in populations with abundant pollinators, but not in less visited populations which contained a third floral morph capable of moderate to high rates of self-mating and fewer outcrossed mates. This study highlights the key role that the ecology of plant mating can play in floral divergence and potentially the evolution of reproductive isolation.

The unmatched diversity of reproductive systems among angiosperms reflects frequent evolutionary transitions within and among lineages as species adapt to altered pollination and mating environments (1). These changes can affect floral and inflorescence traits (2, 3), gender allocation (4, 5), and mating system characteristics (6, 7), and often occur in association with shifts in the presence and relative abundance of pollinators (8–10). Reproductive transitions have attracted considerable attention in flowering plants (11), but quantification of the spectrum of changes in mating patterns involved in specific transitions has been investigated less often (12, 13).

Heterostylous floral polymorphisms offer particularly revealing cases of the breadth of changes involved in reproductive transitions because the unique morphological and physiological features of heterostyly result in distinctive mating patterns within populations. This reproductive system typically involves two (distyly) or three (tristyly) floral morphs in a population which differ reciprocally from each other in stigma and anther heights within flowers (14, 15; Fig. 1, Classical heterostyly). The large physical separation of stigmas and anthers within flowers (herkogamy) limits self-pollination, and the reciprocal positioning of sex organs (reciprocal herkogamy) promotes pollinator-mediated cross-pollination between morphs. As detailed by Darwin (14), classical heterostyly couples this morphological polymorphism with a physiological incompatibility system (heteromorphic incompatibility) that prevents self and intramorph mating, thus enforcing strict disassortative (intermorph) mating (16, 17). Thus, heterostylous populations should exhibit distinctive mating portfolios (7), with male and female mates belonging to other floral morphs, but little to no selfing or outcrossing within morphs. Marker gene studies have confirmed disassortative mating in a few heterostylous species (18–20); however, mate number has not been investigated.

The distinctive disassortative mating of heterostylous species imposes negative frequency-dependent selection, which should adjust morph frequencies within populations over generations so that all morphs realize, on average, equal reproductive success (21, 22).

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Given these expected outcomes, Darwin (p. 138 in ref. 14) recognized heterostyly as “a most complex marriage-arrangement.” The polymorphism has evolved independently multiple times, occurring in at least 28 animal-pollinated angiosperm families, and is a striking example of convergent evolution in floral form and function (16, 23, 24).

Classical heterostyly can be maintained only if its mating benefits outweigh the costs; otherwise, it breaks down resulting in a range of derived reproductive systems (Fig.1 in ref. 24). Reciprocal herkogamy is beneficial as it promotes disassortative pollination and, by limiting self-pollination, increases pollen available for export. However, reciprocal herkogamy also involves costs, as it inhibits intramorph cross-pollination, thereby limiting the number of potential outcross mates in proportion to the fraction of the population represented by other plants of the same morph. From the female perspective, which is the focus of this study, the variance in genetic offspring diversity caused by mating with multiple partners can increase opportunities for zygote choice during seed development and the chance that some offspring will succeed, given variable establishment environments (25). Similarly, heteromorphic incompatibility is beneficial because by eliminating self-fertilization, it reduces inbreeding depression (26) among offspring and associated seed discounting (27). However, this type of incompatibility system precludes cross-fertilization by intramorph pollen, even though the resulting progeny may be of similar genetic quality to those resulting from intermorph mating. This feature of heterostyly perplexed Darwin (pp. 263 to 265 in ref. 14) who regarded heteromorphic incompatibility as an “incidental and purposeless” obstacle to outcrossing as any given plant in a population is unable to mate with “half its brethren.”

Given these benefits and costs, classical heterostyly should provide net mating benefits when pollinators capable of effectively mediating intermorph pollen transfer visit frequently and morphs are equally frequent within populations. In most heterostylous species, this process involves long-tongued pollinators (mostly bees and butterflies) capable of contacting stigmas and anthers in all floral morphs (Fig. 1 in ref. 28). These pollinators generally probe flowers for nectar in a stereotypical position and pick up pollen from the different anther levels, which becomes segregated on different parts of their bodies (29). Pollen is then deposited on stigmas that correspond in position to the compatible anther levels of the opposite morph (Fig. 1 in ref. 22). However, if pollinators capable of mediating disassortative pollen transfer visit populations infrequently, classical heterostyly may be difficult to maintain, favoring loss of heteromorphic incompatibility (Fig. 1). This change should have two significant mating consequences: it would expand the pool of potential outcross mates to all conspecifics, rather than just those of another morph; and it would allow a plant’s own pollen to self-fertilize its ovules. Given this increased variety of mating options, three different evolutionary transitions from classical heterostyly may occur as depicted in Fig. 1.

Once plants can potentially mate with all conspecifics, two alternative outcrossing paths become feasible if, despite reduced visitation by long-tongued pollinators, other pollinators that promote intermorph pollination visit frequently (Fig. 1). Reciprocal herkogamy may persist if the frequency of disassortative mating exceeds that of assortative mating (20, 22). This form of heterostyly (“compatible heterostyly”) occurs in various heterostylous lineages, including those in which most species possess heteromorphic incompatibility (17, 24).

However, if instead, pollinators causing intramorph pollination visit frequently and assortative mating equals or exceeds disassortative mating, the limitation of the mating pool caused by reciprocal herkogamy renders it disadvantageous (22). This situation should promote the breakdown of heterostyly to monomorphic outcrossing (including mixed mating), like that of numerous non-heterostylous animal-pollinated angiosperms (6). Such species commonly exhibit more limited herkogamy than is typical of heterostylous species, which provides a compromise between promoting outcrossing and restricting self-pollination (30). Examples of this path of breakdown in heterostyly include some populations of *Turnera ulmifolia* var. *angustifolia* (12), and three independent transitions in the largely distylous genus *Primula* responsible for the evolution of mixed mating in *P. halleri* (31) and of two derived monomorphic, outcrossing clades recognized as section *Dodecatheon* and subsection *Cortusa* (32).

In contrast to the paths from heterostyly to alternative outcrossing and mixed mating systems, an evolutionary transition to predominant selfing (autogamy) is most likely if pollinators of any quality visit only rarely so that seed production within populations becomes pollen limited (Fig. 1). In such circumstances, plants confront the general costs of outcrossing, rather than just the costs

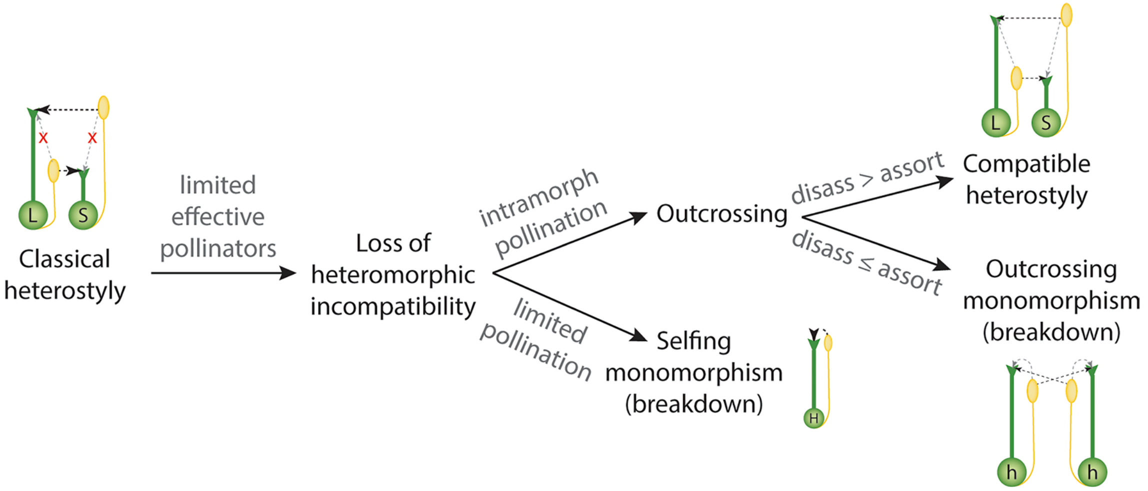

Fig. 1. Possible evolutionary trajectories if pollinators capable of effectively mediating disassortative mating in a heterostylous species become chronically infrequent visitors. Floral sketches (pistils green, stamens yellow) illustrate the morphological differences between major stages and the associated mating patterns (dashed lines). Letters on the ovaries identify floral morphs, including L, long-styled and S, short-styled morphs in distylous populations; H, smaller-flowered homostyles in selfing populations; h, plants with reduced herkogamy in monomorphic mixed mating populations.

of reciprocal herkogamy. As is generally the case when outcrossing fails and transitions to predominant selfing occur (33), this situation would favor floral variants with stigmas and anthers positioned close enough within flowers to facilitate autonomous self-pollination, a condition in heterostylous groups referred to as homostyly (14). The breakdown of heterostyly to selfing monomorphism via homostylous evolution is the most commonly studied mating-system transition and occurs widely among heterostylous families (16, 21, 24), including at least nine independent events in *Primula* (34).

The preceding scenarios imply that the loss of heteromorphic incompatibility in a classical heterostylous species initiates further mating-system changes, as populations transition to compatible heterostyly, monomorphic mixed mating, or predominant selfing (Fig. 1). For simplicity, we have outlined the possible expectations in the context of transitions from classical distyly, although they apply similarly to the breakdown of tristyly (35, 36). During these transitions, populations could differ in the proportions of the ancestral, highly herkogamous, heterostylous morphs as well as phenotypes with reduced herkogamy, as observed in some self-compatible *Primula* species (37), depending on their pollination environments. We predict that populations visited frequently by long-tongued pollinators should lack individuals with reduced herkogamy and predominately outcross with more disassortative than assortative mating and relatively high outcross-mate diversity. In these populations, the long- and short-styled morphs (hereafter L- and S-morphs) should be equally abundant owing to frequency-dependent selection caused by the prevalence of disassortative mating.

In contrast, transitional populations with abundant pollinators incapable of promoting disassortative mating should include mixtures of the mostly outcrossing distylous morphs and individuals with reduced herkogamy that may outcross less effectively. In such populations, disassortative mating should not exceed assortative mating. In addition, plants with reduced herkogamy should be able to self-fertilize more than the distylous morphs but could still outcross, as is the case in many monomorphic species (38, 39), although probably with fewer mates than the distylous morphs (40). Morph frequencies could vary extensively among these populations, given limited disassortative mating. Finally, in populations with few pollinators and hence few outcrossing opportunities, autonomously self-pollinating homostyles should become fixed in populations and have correspondingly limited outcross-mate diversity. Thus, the combination of floral forms in populations undergoing breakdown to monomorphic mixed mating versus homostylous selfing should exhibit complex but different mating patterns that signal the type of transition in which they are engaged.

Here, we infer the transition paths followed by populations of *Primula oreodoxa* Franchet based on these expectations. This species is well suited to such analysis for three reasons. First, unlike most distylous *Primula* species which possess heteromorphic incompatibility (41), *P. oreodoxa* is self-compatible and the highly herkogamous L- and S-morphs are intramorph compatible (42). Thus, the gamut of mating options (i.e. self, intramorph, and intermorph) is available, which should enable the reproductive transitions summarized in Fig. 1. Second, *P. oreodoxa* exhibits an unusual diversity of reproductive systems, suggesting that transitions are currently underway in contemporary populations. Specifically, populations range from dimorphic, with reciprocal herkogamy (i.e., L- and S-morphs: *SI Appendix*, Fig. S1 *A*–*C*); through mixed populations, with L- and S-morphs and a third morph (h-morph) with reduced herkogamy (*SI Appendix*, Fig. S1 *D*–*F*); to monomorphic populations with only homostylous plants (H-morph) with smaller flowers (42), a common feature of transitions to high selfing rates (2). These known features reflect the physiological and morphological shifts expected during transitions from classical distyly. Third, previous work (42) revealed the ecological context underlying the breakdown of distyly and spread of phenotypes with reduced herkogamy. Specifically, population type varies strongly with elevation, with a rapid shift from low-elevation dimorphic populations to high-elevation monomorphic populations between 1,600 and 2,000 m, in association with declines in pollinator abundance, especially that of long-tongued pollinators (Fig. 2). Therefore, the outstanding questions addressed here are whether mating patterns, inferred from parental and offspring microsatellite genotypes, differ among *P. oreodoxa* populations with contrasting morph structure, and which, if any, of the three pathways summarized in Fig. 1 are populations following? Our analyses of these questions considered three dimorphic populations, three mixed populations, and two monomorphic populations containing exclusively homostylous plants. The goal of our study was to characterize mating patterns during early stages of the breakdown of sexual polymorphism from classical distyly to mixed mating and/or predominant selfing.

Results

Self-Mating. Variation in the proportion of selfed seeds as a maternal parent (female selfing rate) was largely consistent with our expectations. Based on a six-population analysis of distylous and mixed populations, L- and S-morph plants primarily outcrossed, although the overall mean female selfing rate in mixed populations was twice that in dimorphic populations and mean selfing rates varied among populations of both types (Fig. 3*A* and *SI Appendix*, Table S1). Population ELS was noteworthy as the only polymorphic population in which more than 50% of progeny were self-fertilized. A separate analysis of the two monomorphic homostylous populations detected higher selfing overall than in the polymorphic populations (Fig. 3*A*) but no difference between the populations ($X^2_1 = 0.95$, $P > 0.3$). In contrast to the population differences, female selfing rates did not differ between the L- and S-morphs, and this equivalence did not vary between dimorphic and mixed populations or among populations within types (*SI Appendix*, Table S1).

Analyses of the mixed populations detected similar population differences to those of the six-population analyses but found large differences between morphs in their patterns of mating (*SI Appendix*, Table S2). Specifically, although plants with reduced herkogamy (h-morph) outcrossed to some extent, more than half of their offspring were self-fertilized (Fig. 3*B*). In addition, the mean female selfing rate of h-morph plants was twice that of the largely outcrossing herkogamous L- and S-morphs, which did not differ statistically (Fig. 3*B*).

Outcross Mate Number. As illustrated in *SI Appendix*, Fig. S2, seeds with known fathers often represented a minority of recognized outcrossed seeds in maternal families, especially for dimorphic populations. Recognition of known fathers generally varied positively with the proportion of plants of all morphs that had been genotyped, especially for mixed populations (*SI Appendix*, Fig. S2*B* and Table S3 for statistical details).

The mean number of other plants with which maternal plants were detected to have outcrossed varied statistically with per-plant genotyping effort and among populations, but mostly not among morphs (*SI Appendix*, Tables S1 and S2). Overall, male-mate number increased in a positive, decelerating manner with the number of genotyped outcrossed seeds per plant (e.g., Fig. 4*A* and *SI Appendix*, Tables S1 and S2 for partial regression

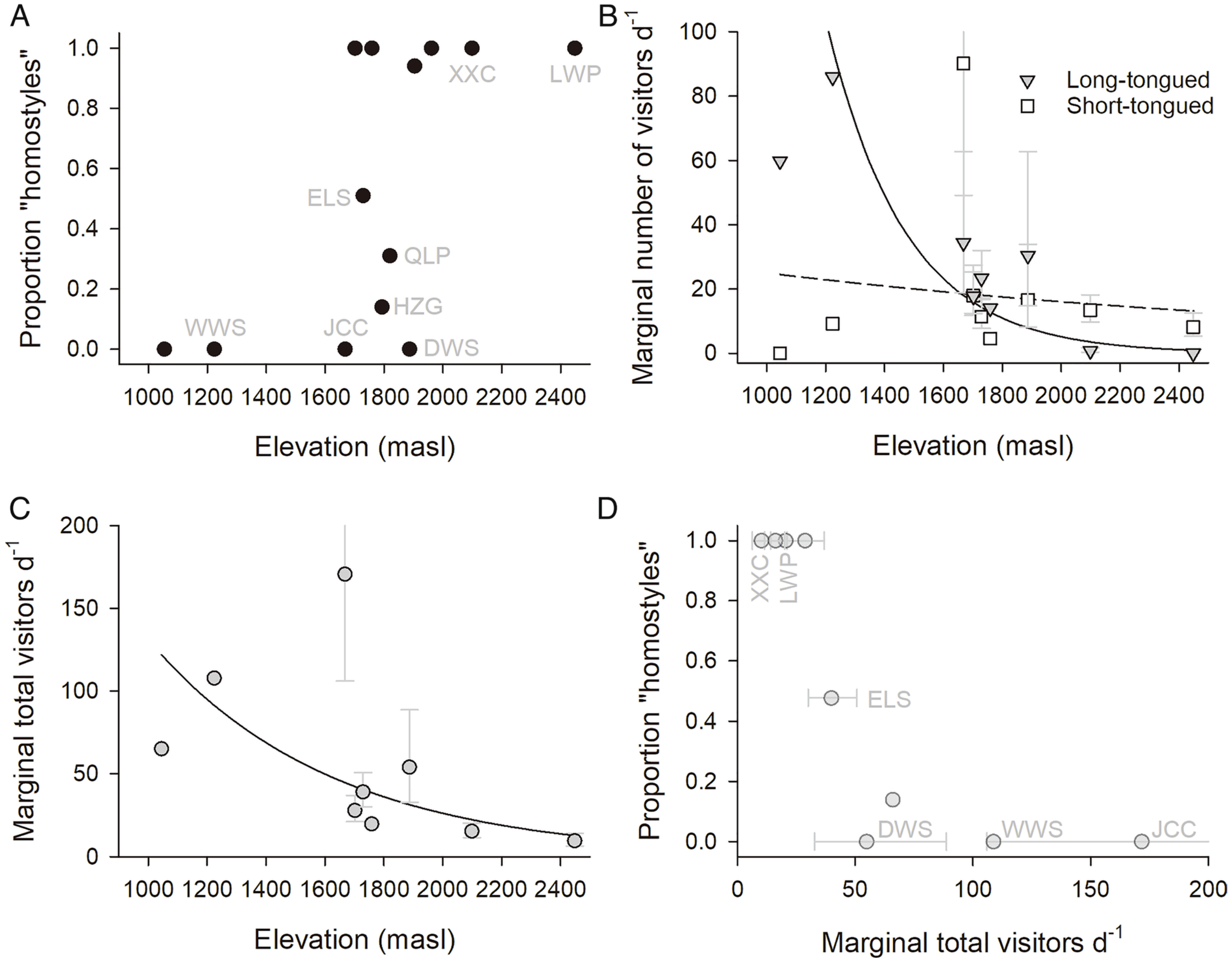

Fig. 2. Elevational variation among diploid populations of *Primula oreodoxa* in the abundances of (*A*) the proportion of "homostylous" plants, (*B*) flower visitation by long- and short-tongued insects, (*C*) flower visitation by all visitors, and (*D*) the association between the proportion of homostylous (reduced-herkogamy) plants with visitation by all visitors, as reported in *SI Appendix*, Table S5 for the labeled study populations and by Yuan et al. (42) for the remaining populations. Fewer populations were sampled for flower visitors than for the frequency of plants with reduced herkogamy, including homostyles in monomorphic populations. The observations were recorded in 2013 to 2015, and flower visits were counted from 900 to 1,700 h (a "day") in 3 × 5-m plots. The regression lines in (*B* and *C*) illustrate the results of generalized linear mixed models that accommodated negative binomial variation in visit number (ln link function) and included year and the ln(number of observed flowers) as additional independent variables (details are provided in *SI Appendix* A1). Visitation was recorded for only one day for the sites at 1,045, 1,224, and 1,779 m above sea level, for which SEs are not provided. In (*A*) and (*D*), the labeled symbols indicate populations considered in the present study.

coefficients). Holding this effect constant revealed that, on average, maternal plants in dimorphic populations had more male mates than those in mixed populations (Fig. 4*B*: only L- and S-morph plants included). Mean male-mate number also varied among populations within population type, being comparatively low in JCC among dimorphic populations and high in HZG among mixed populations. Based on a separate analysis, mean male-mate number was lowest in the two monomorphic populations, but it did not differ statistically among them ($X^2_1 = 0.12$, $P > 0.7$: Fig. 4*B*). In addition, male-mate number varied negatively with selfing rates among populations (test of linear trend for polymorphic populations; $t_{112} = 2.41$, $P < 0.025$), although mixed population QLP had low mean mate number compared to other polymorphic populations despite being primarily outcrossing (Fig. 4*C*). In the six-population analysis for the L- and S-morphs, male-mate number did not differ statistically among floral morphs, overall, between population types or among populations within types (*SI Appendix*, Table S1). For the mixed populations, male-mate number did not differ statistically between h-morph plants and either of the distylous morphs (*SI Appendix*, Fig. S3). However, this analysis revealed more male mates for maternal S-morph plants than L-morph plants in population HZG (*SI Appendix*, Fig. S3).

Relative Intramorph and Intermorph Outcrossing. The genotyped families of 112 L- and S-morph maternal plants from the six polymorphic populations included 516 outcrossed offspring sired by a father with known morph (median 4 offspring per mother; see *SI Appendix*, Fig. S2*A*). Based on this sample, individual maternal plants commonly outcrossed with either plants of a different morph (including h-morph individuals: intermorph mating) or of the same morph (intramorph mating), rather than with a mixture of plants of the same and different morphs (Fig. 5*A*). Indeed, 43.8% of the 89 maternal plants from which we collected multiple seeds with known fathers mated exclusively within or among morphs. Thus, the mean proportions of intermorph mating for individual populations or morphs obscure considerable heterogeneity in mating patterns among maternal plants.

The mean proportion of outcrossed progeny in maternal families resulting from intermorph mating generally varied among

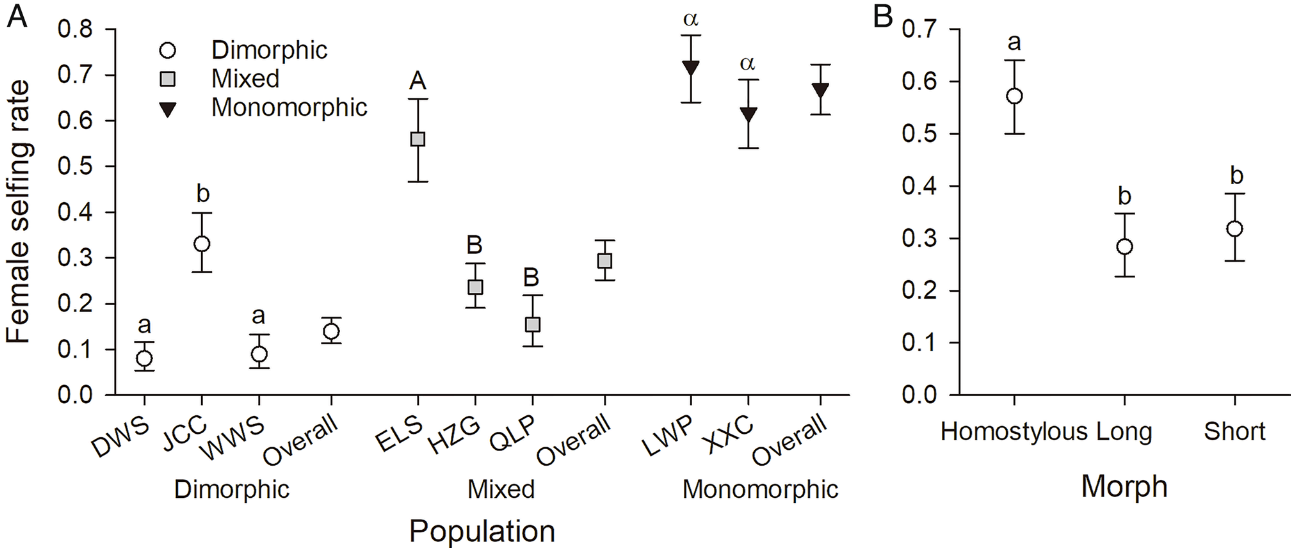

Fig. 3. Variation in mean (±SE) female selfing rates (*A*) among three dimorphic, three mixed populations (L- and S-morph only) and two monomorphic populations of *Primula oreodoxa*, and (*B*) among the three morphs in mixed populations (averaged over populations). Means associated with different letters differ statistically (*A*, Dunn–Šidák procedure; *B*, Tukey's test). In (*A*), means were compared within each population type, as indicated by the lowercase and uppercase letters.

populations and morphs but not overall between dimorphic and mixed populations (*SI Appendix*, Tables S1, S4). In a six-population analysis (L- and S-morphs only), differences were evident only among dimorphic populations (specifically, DWS > WWS; Fig. 5*B*). Intermorph mating statistically exceeded intramorph mating (i.e., proportion > 0.5) for both the L and S morphs only in population DWS, whereas in populations JCC (dimorphic) and ELS (mixed), only L-morph plants engaged in excess intermorph mating (Fig. 5*B*). Proportional intermorph mating by the L-morph exceeded that by the S-morph only in the mixed population ELS (morph x population within type: Fig. 5*B*). Comparison of all morphs for the mixed populations detected more intermorph than intramorph mating only in h-morph plants, which also engaged in more intermorph mating than S-morph plants (Fig. 5*E*). Overall, the mean proportion of intermorph mating did not vary statistically with the mean female selfing rate among populations (linear trend; $t_{99} = 0.65$, $P > 0.5$); however, when dimorphic population DWS was excluded, an unexpected positive relation was evident ($t_{99} = 2.10$, $P < 0.05$: Fig. 5*C*). In contrast, the relative incidence of intermorph mating did not vary consistently with male-mate number among populations ($t_{99} = 1.26$, $P > 0.2$: Fig. 5*D*).

Consideration of the extent to which the incidence of intermorph mating differed from the relative frequency of other morphs in populations (Eq. **1**) indicated that, on average, plants generally outcrossed randomly with their own and other morphs, with a few exceptions (Fig. 6). In dimorphic population DWS, and perhaps JCC, plants mostly mated disassortatively, as predicted by the Darwinian hypothesis. In contrast, in the mixed population, QLP plants of the distylous, morphs mostly mated assortatively, whereas h-morph plants mated randomly.

Discussion

The mating characteristics of *P. oreodoxa* populations revealed by our analyses contrast with the classic textbook depiction of heterostylous species engaging in symmetrical disassortative mating between morphs (e.g., figure 21.16A in ref. 43, figure 2.8B in ref. 44). Individuals in most populations mated randomly among morphs and the expected pattern of significant disassortative mating for a distylous species was evident in at most two dimorphic populations (Fig. 6). Overall selfing rates in mixed populations were significantly higher than in dimorphic populations (Fig. 3*A*), and within mixed populations, homostylous individuals selfed almost twice as much as the distylous morphs (Fig. 3*B*). In monomorphic populations, homostylous plants were predominantly but not exclusively selfing. In addition, except for population HZG which contained a low proportion (0.14) of h-morph individuals, plants in mixed and monomorphic populations generally outcrossed with fewer mates, even after accounting for variation in the number of outcrossed seeds (Fig. 4*B*). We interpret these results as evidence of the evolutionary breakdown of distyly, primarily in populations above 1,600 masl and that initial stages of breakdown are characterized by increased mating complexity as floral morphs are released from strict intermorph mating.

Fig. 1 illustrates possible evolutionary trajectories that classical heterostylous populations might follow when long-tongued pollinators capable of mediating high rates of disassortative mating become infrequent visitors. The paths involve two distinct routes to floral monomorphism with contrasting mating systems: either maintenance of mixed mating in populations visited by short-tongued pollinators, with assortative mating equalling or exceeding disassortative mating, or the transition to autogamy when pollinators visit rarely and selection for reproductive assurance favors homostyles capable of autonomous self-pollination. Below, we evaluate the extent to which data and observations from *P. oreodoxa* populations are consistent with these contrasting scenarios.

Reproductive Mechanisms Governing Mating. Physiological incompatibility limits the range of mating opportunities in most heterostylous species by preventing selfing and intramorph mating (16, 17). In contrast, in self-compatible heterostylous species deviations from strict disassortative outcrossing may occur when ecological conditions limit intermorph pollen transfer. Our findings for *P. oreodoxa* are consistent with this expectation, as plants in all populations selfed to some extent (Fig. 3) and at least one morph mated randomly or assortatively (Fig. 6). Heteromorphic incompatibility occurs widely in *Primula* (41) and self-compatibility in *P. oreodoxa* is likely a derived condition, perhaps in response to limited pollinator service, as depicted in Fig. 1.

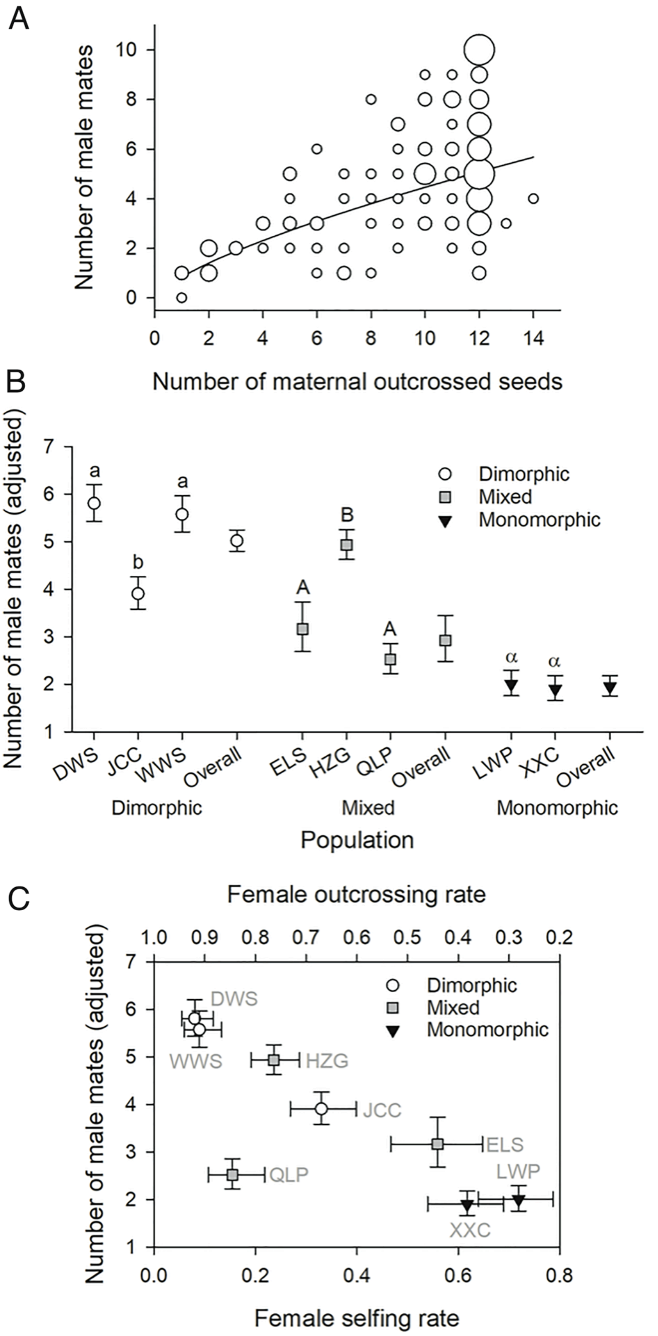

Fig. 4. Aspects of variation in the number of male mates among the outcrossed seeds genotyped for maternal *Primula oreodoxa* plants in three dimorphic, three mixed (L- and S-morph only), and two monomorphic populations, including the influences of (*A*) the number of genotyped seeds, (*B*) population, and (*C*) the association with the population mean female selfing rate. In (*A*), symbol size varies positively with the number of plants represented by a specific combination of seed sampling and mate number. (*B*) and (*C*) depict means ± SE that have been adjusted for among-plant variation in seed sampling. In (*B*), population means within a specific population type with different letters differ statistically (Dunn–Šidák procedure).

A second key reproductive trait that directly influences mating in self-compatible populations is the extent of herkogamy (30). Selfing rates generally increase as the distance between stigmas and anthers diminishes, in part because of greater opportunities for autonomous self-pollination (45–47). Similarly, *P. oreodoxa* plants in mixed populations with reduced herkogamy selfed almost twice as much as the distylous morphs (Fig. 3*B*). Nevertheless, h-morph plants commonly also outcrossed, especially in HZG and QLP. This mixture of selfing and outcrossing suggests that pollinators mediated self-pollination to some extent, rather than selfing occurring only autonomously. Such mixed mating differs from the predominant selfing that characterizes homostyles in the well-studied *P. vulgaris* (21, 48, 49) and other heterostylous lineages (13, 36, 50). In the two monomorphic *P. oreodoxa* populations, most offspring from H plants resulted from self-mating, but with appreciable (30 to 37%) outcrossing.

Ecological Mechanisms Governing Mating. In *Primula*, ecological conditions unfavorable for reliable cross-pollination are commonly associated with the breakdown of distyly to selfing monomorphism (51–54). The striking transition in *P. oreodoxa* from dimorphic populations at low elevations to monomorphic populations at high elevations with mixed populations occurring only between 1,600 and 2,000 masl (Fig. 2*A*) in association with declines in pollinator abundance indicates such ecological influences on the morph structure of populations and their mating patterns. If insufficient or inferior pollinator service persists or becomes exacerbated, plants with reduced herkogamy should experience an overall fertility advantage, leading to in situ replacement of the distylous morphs by homostyles (21), although this may not be an inevitable outcome (48). The incidence of h-morph plants in *P. oreodoxa* populations is consistent with this scenario. Significantly, pollinators, especially long-tongued insects, visited substantially less frequently in populations with h-plants than in distylous populations (Fig. 2 *B* and *C*). Such reduced visitation coupled with the functional subdivision of a population by distyly should favor reduced herkogamy, allowing plants to mate more generally (Fig. 1). Replacement of distylous morphs by homostyles likely explains much of the prevalence of purely monomorphic populations of *P. oreodoxa* at high elevation, where pollinators are infrequent (Fig. 2*B*). An additional relevant feature that may contribute to the predominance of monomorphic populations at high elevation involves the capacity of a single, autonomously selfing plant to establish a new population (Baker's Law: ref. 55). Indeed, haplotype network analysis indicates that two homostylous populations (CSQ and XXC) are likely derived from mixed population QLP, probably as the result of separate colonizing events, as they do not share haplotypes (42).

Ecological factors associated with pollinator abundance and quality probably also played an important role in the relaxation of disassortative mating. Darwin (14) proposed that reciprocal positioning of sexual organs in heterostylous populations promotes intermorph cross-pollination, resulting in disassortative mating. In contrast to demonstrations of disassortative pollen transfer in several heteromorphic species (22, 56, 57), including *Primula* (58), our findings do not support Darwin's cross-pollen transfer hypothesis for most *P. oreodoxa* populations. After adjusting for morph frequencies, individuals of the distylous morphs mostly mated randomly, with few exceptions (e.g., DWS, mostly disassortative mating; QLP, mostly assortative mating: Fig. 6). Furthermore, among all six polymorphic populations, almost half of maternal plants with known male mates mated exclusively either within or among morphs, rather than outcrossing with a mixture of morphs. Particularly puzzling is the dominance of assortative, rather than disassortative, outcrossing in QLP and WWS despite their low selfing rates and presence of distylous morphs (Figs. 5*C* and 6). The pollinator conditions at QLP are unknown, but plants at WWS were visited frequently, mostly by long-tongued pollinators several years before the current study (Fig. 2 *B* and *C*, population at 1,224 m). These results indicate broader influences on mating outcomes than floral morphology and pollinator abundance, such as the possible spatial aggregation of morphs, which await future exploration.

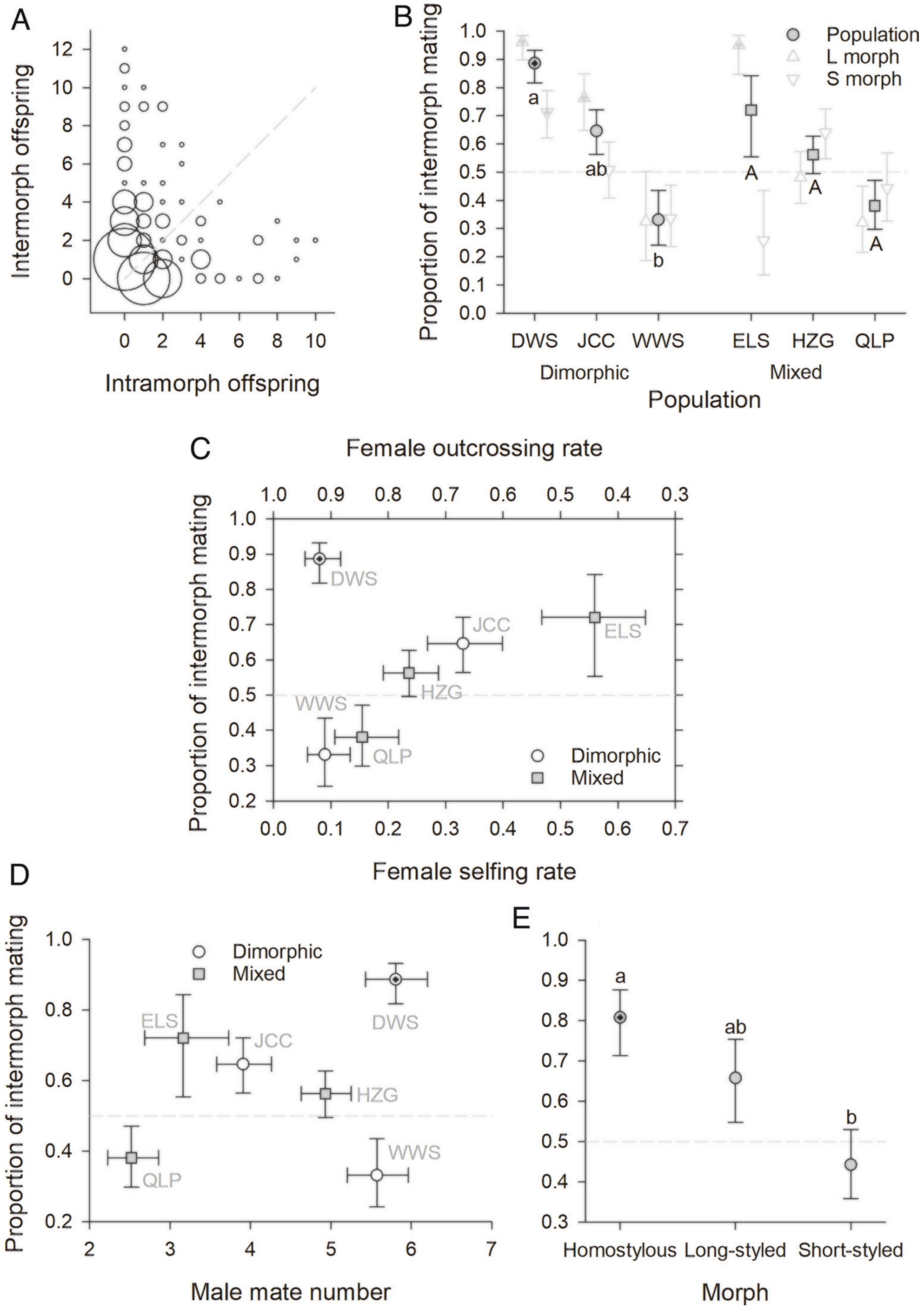

Fig. 5. (*A*) The association of the numbers of seeds sampled from individual maternal *Primula oreodoxa* plants that resulted from intermorph or intramorph mating (including homostyles) and (*B*–*E*) effects on variation in the mean (±SE) proportion of seeds resulting from intermorph mating. Illustrated influences on proportional intermorph mating include (*B*) an interaction of maternal morph (L- and S-morph only) among dimorphic and mixed populations; the relations of population mean intermorph mating to population mean (±SE) (*C*) female selfing rate and (*D*) male-mate number in all six populations (L- and S-morph only); and (*E*) overall morph differences for the three mixed populations. The dashed line in each panel indicates equivalent intramorph and intermorph mating. In (*B*–*E*), symbols with a central dot indicate cases in which intermorph mating exceeded intramorph mating statistically (i.e., proportion of intermorph mating > 0.5). Means associated with different letters differ statistically (*B*, Dunn–Šidák procedure; *E*, Tukey's test).

Mate Number. Mate diversity and specifically mate number is an important, if poorly studied (7, 59), component of a plant's mating portfolio (but see refs. 60 and 61). In particular, a plant's male-mate number affects the genetic relatedness of siblings (62) and their competition for maternal resources (63), bet-hedging (64), and opportunities for female mate choice (65). In *P. oreodoxa*, male-mate number differed among populations and between population types. As mate number varies positively with a plant's number of outcrossed seeds (Fig. 4*A*), it should correspondingly vary negatively with the fraction of seeds that are self-fertilized. This latter expectation was evident among populations, with one exception (Fig. 4*C*). The cause of the low mate number in population QLP, despite is low selfing rate is unclear, although it was unusual for its low density, which could reduce pollinator visitation but lengthen visits by attracted pollinators and their pollen deposition (66, 67).

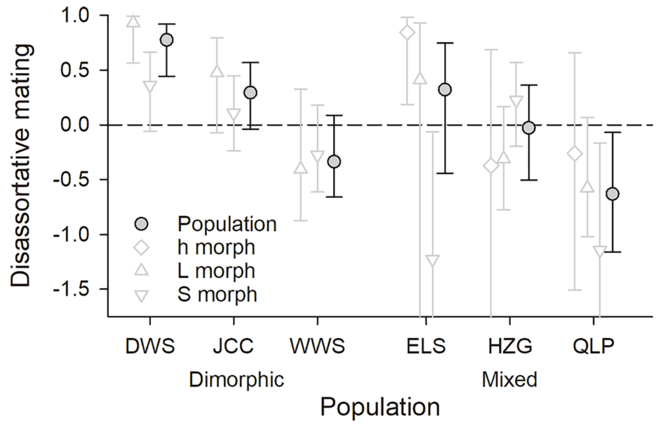

Fig. 6. Variation in the mean (±95% CI) incidence of disassortative mating (Eq. **1**) among six populations and floral morphs of *Primula oreodoxa*. The dashed line indicates no excess intermorph mating compared to the expectation for random mating based on the relative morph frequencies in a population. Cases with CIs completely above the dashed line involved excess disassortative mating, whereas those completely below the dashed line involved excess assortative mating.

In contrast to the differences among populations, male-mate number did not differ statistically between morphs. This equivalence suggests that the breakdown of distyly in *P. oreodoxa* does not involve gender differentiation, with one morph increasingly specialized for male function and the other morph for female function. Such specialization has been implicated for transitions from heterostyly to alternative dimorphic, outcrossing sexual systems, such as gynodioecy and dioecy (68–70).

Stages and Mating Consequences of the Dissolution of Heterostyly. In *Primula*, distyly has repeatedly become destabilized causing breakdown of the floral polymorphism (21, 34, 37, 48, 71–73). In this genus, breakdown events are frequently associated with speciation and the origin of selfing homostylous taxa (34, 72). The most important functional changes associated with this process are the loss of self-incompatibility followed by the origin and spread of variants with reduced herkogamy, both of which have important mating consequences (Fig. 1).

The presence of distylous morphs and those with reduced herkogamy within mixed populations allowed comparison of the influence of contrasting floral phenotypes on mating under similar ecological conditions. Among the three dimorphic populations, plants in DWS and JCC outcrossed disassortatively (Fig. 6), indicating that they exhibit compatible heterostyly (Fig. 1). By comparison, the persistence of dimorphism in the third population, WWS, is enigmatic as its plants outcross randomly (Fig. 6). Perhaps variants with reduced herkogamy have yet to arise in WWS. At the other extreme, the higher-elevation small-flowered homostylous populations (LWP, XXC) also studied by Yuan et al. (42) are cases of the breakdown of distyly to monomorphism and high selfing rates (Fig. 1). Molecular evidence indicates that the very small-flowered, highly selfing homostylous species *P. dumicola*, which occurs from 2,400 to 3,000 masl (74), is likely derived from *P. oreodoxa*, or at least the two sister taxa share a recent distylous ancestor (72), thus potentially implicating the transition to selfing via homostyle evolution in speciation.

The outstanding question is what is the fate of mixed populations of *P. oreodoxa*? One option is that they will follow the same pathway to monomorphic selfing as occurs in high-elevation populations and that likely occurred in *P. dumicola*. But the transition to autogamy may be slower for these mixed populations because of weaker selection owing to somewhat more frequent pollinator visitation. The alternative option is transition to mixed mating in monomorphic populations promoted by larger flowers and some herkogamy. Given that mating in mixed populations is not disassortative (Fig. 6), the distylous morphs may be disadvantaged compared to plants with reduced herkogamy as they both self-fertilize more readily (Fig. 3*B*) and outcross more with other morphs (Fig. 5*E*). Time will tell whether mixed mating is maintained as a stable strategy in mixed populations, and in monomorphic populations with some degree of herkogamy. However, if sufficient pollinator service to maintain fertility characterizes these populations, this scenario seems plausible, at least for monomorphic populations, and appears to have occurred in some *Primula* species (31, 37) and other heterostylous taxa (47, 75).

Morph ratios were highly heterogeneous in mixed populations of *P. oreodoxa,* with h-morph frequencies ranging from 0.14 to 0.94 (*SI Appendix*, Table S5; Table 1 in ref. 42). This variation probably reflects, in part, differences among morphs in mating patterns, relative gamete transmission, and progeny fitness associated with the characteristics of the local pollinator fauna, including its temporal variation. Our analysis of mating patterns revealed that the h-morph sired an appreciable fraction of seeds with known fathers produced by distylous morphs. The most extreme case occurred in population ELS, where h-morph plants sired 46% of outcrossed seeds on S-morph plants and 90% of those on L-morph plants. Because h-morph plants also self-fertilize more of their own seeds than the distylous morphs (Fig. 3*B*), they should realize a genetic-transmission advantage favoring their spread and fixation in populations. This advantage would be amplified by the higher fruit set of h-morph plants than by the distylous morphs in mixed populations, as expected given their ability to self-pollinate autonomously when seed production is pollinator limited (42). We have no experimental data on the severity of inbreeding depression in progeny of the floral morphs to assess the likelihood that homostyles will replace the distylous morphs in mixed populations (also see ref. 48).

In conclusion, analysis of mating patterns in eight populations of *P. oreodoxa* uncovered mating complexity not previously observed in heterostylous populations and certainly more complex than the marriage arrangements envisioned by Darwin (14). Mating data on variation among individuals provide opportunities to expose the proximate mechanisms governing reproductive success, and sexual polymorphisms such as heterostyly are especially informative in this regard. Unlike monomorphic species, polymorphic populations are reproductively subdivided into morphologically discrete phenotypes maintained by negative frequency-dependent selection. Intrinsic and extrinsic ecological factors that disturb morph-ratio equilibrium can cause deviations from disassortative mating and frequently set in train processes leading to the breakdown of floral polymorphism and the evolution of alternative floral strategies. By quantifying the earliest changes in mating behavior accompanying these transitions, our study highlights the key role that the mating biology of populations can play in floral divergence and the evolution of reproductive isolation.

Materials and Methods

Study System. *Primula oreodoxa* is an insect-pollinated, herbaceous perennial restricted to western Sichuan province, China (102 to 104°E, 28 to 31°N). Populations grow along streams and margins of woodlands between 1,050 and 2,450 m and flower from March to April, with fruits maturing from late May to June. Our studies of mating patterns involved three distylous (DWS, JCC, and WWS; hereafter "dimorphic"), three mixed populations (ELS, HZG, and QLP), and two monomorphic, homostylous populations (LWP and XXC). All studied populations were diploid ($2n = 24$ chromosomes), although occasional tetraploid populations occur (42). Detailed information on the reproductive biology of *P. oreodoxa* and the characteristics of each population, including their morph frequencies, is presented in ref. 42 and *SI Appendix*, Table S5 and *Methods*.

Sampling of the polymorphic populations occurred during March and April 2016, whereas that of the monomorphic populations occurred during spring 2020. Within each population, we searched for flowering plants, identified their floral morph, and marked them for later sampling of open-pollinated seed families. We collected fresh leaf material from these plants, which was dried in silica gel for subsequent genotyping. During late May, we collected two or three mature open-pollinated fruits from each marked plant. Seeds from two fruits per plant were mixed and sown on soil-filled flats at the Biological Resources Research Station at E'mei Mountain (altitude 800 m), Sichuan Province. The resulting seedlings were cultivated in a glasshouse for about two months before being moved outside to a common garden under seminatural conditions. During the February after seed collection, we randomly selected 8 to 12 seedlings (average 11.9) from each family and collected three to five fresh leaves from each seedling, which were dried in silica gel. In total, we sampled 64/762, 66/790, and 28/326 families/progeny from the L-, S-, and h-morphs, respectively, from the polymorphic populations and 36/426 families/progeny from the monomorphic populations (details are provided in *SI Appendix*, Table S5).

DNA Extraction and Genotyping. We used standard procedures to characterize microsatellite variation. DNA extraction from dried leaf tissue for all parental plants and progeny involved a modified cetyl trimethyl ammonium bromide protocol (76). Multiplex PCR was used to amplify eleven SSR markers per individual. Because of contrasting amplification between dimorphic and mixed populations, the marker sets used for the two population types included only three markers in common (*SI Appendix*, *Methods*). SSR markers were divided into two (mixed populations) or three groups (dimorphic populations) for PCR amplification. We assayed PCR products using an ABI PRISM 3100 Genetic Analyser (Invitrogen) with an internal size standard (GeneScan™ 500 LIZ). Allele binning and calling involved GeneMarker version 2.4.0 (SoftGenetics LLC, State College).

Paternity Assignment and Estimation of Mating-System Components. We used COLONY 2.0.6.5 (77, 78) to identify maximum-likelihood two-generation pedigrees for each population sample given the multilocus genotypes of the sampled maternal plants and seedlings. Based on the inferred pedigree for a sample, COLONY estimates the probabilities of whether each offspring is selfed (same maternal and paternal parent) or outcrossed (different parents) and of the most likely paternal parent. Identified seed fathers could be included in the genotyped sample of paternal plants ("known" fathers) or be unsampled plants for which the genotype was inferred ("unknown" fathers). COLONY analyses involved the following conditions: polygamy for both female and male parents with inbreeding, no clonality, full-likelihood method, medium length run, medium precision, and allele frequencies were not updated, as recommended in Wang et al. (78). We allowed locus-specific error rates per population from the genotypic data and used 0.01 as the average genotype error rate.

Subsequent statistical analyses involved various subsets of the 2304 genotyped seeds. The analysis of the female selfing rate considered the 99.0% of genotyped seeds for which the probability of selfing was either <0.05 (i.e., outcrossed) or >0.95 (i.e., selfed). Similarly, analysis of the number of outcross male mates for maternal plants involved the 96.1% of the 1473 outcrossed seeds for which the genotype of the known or unknown father was inferred with probability >0.95. We did not assess the corresponding male parameters as recognized male outcrossing was underestimated to the extent that seeds sired on unknown maternal plants were unidentified. Estimation of the incidence of intramorph versus intermorph mating required identification of the floral morphs of a seed's maternal and paternal parents and so involved only the 44.6% of outcrossed seeds sired by known fathers. As this subsample of seeds was further divided into intramorph versus intermorph classes, we consider these mating outcomes only from the perspective of maternal plants.

Statistical Methods. Most statistical analyses involved generalized linear models (79) of mating outcomes for maternal families. These analyses considered distributions and link functions appropriate for the characteristics of the dependent variables (*SI Appendix*, Tables S1 and S2) and were conducted with R version 4.1.1 (https://cran.rproject.org/) using the glmmTMB package version 1.1.2.2 (80). Distribution assumptions were checked with the testDispersion function of the DHARMa package version 0.4.3. Because dimorphic populations differed from mixed populations by lacking homostylous plants, we conducted two sets of analyses. One set involved only the L- and S-morphs for all six populations and included population type (dimorphic, mixed) and morph as crossed factors, as well as population nested within population type and its interaction with morph. The other set involved the L-, S-, and h-morphs for the three mixed populations and included morph and population as crossed factors. Some analyses also considered continuous covariates to account for sampling variation (e.g., number of genotyped seeds in analyses of mate number; a discussion of the merits of this approach and implications for study design are provided in *SI Appendix*, *Methods*). Comparisons among marginal factor means involved Tukey's test for main effects and the Dunn–Šidák procedure for nested effects and interactions (81) and were conducted using the R emmeans package version 1.6.3. We also assessed whether population mean male-mate number and the relative frequency of inter-morph mating varied with mean female outcrossing rates using contrasts to test linear trends (81). To illustrate statistical results, we present back-transformed marginal means and their (usually asymmetrical) SEs or 95% CIs.

The analysis of intermorph mating required slight modifications of the data to overcome fitting problems associated with invariant mating outcomes among L-morph plants in population ELS. Specifically, the numbers of intramorph and intermorph matings for the plant with the most (11) seeds with known fathers were respectively increased and decreased by 0.1.

Heterostyly is expected to promote disassortative mating (14), so that the frequency of intermorph mating should exceed that expected if plants mated randomly. Estimation of the extent of disassortative mating by known maternal plants of morph j involved the morph's estimated (mean and CI) proportion of intermorph mating (I_j) and their relative frequency in the population (f_j). For a plant of morph j, the expected frequency of random intermorph mating should vary directly with the relative frequency of plants of all other morphs, i.e., $E_j = 1 - f_j$. Therefore, the observed extent of nonrandom disassortative mating is $I_j - E_j$. In general, the maximum possible extent of disassortative mating is $1 - E_j$, so a standardized measure that facilitates comparison among morphs or populations is (18):

$$D_j = \frac{I_j - E_j}{1 - E_j}.$$

Estimates of f_j, mean $\bar{I}_j$, and its (perhaps asymmetrical) CI, allow calculation of

$$\begin{aligned} \bar{D}_j &= \frac{\bar{I}_j - E_j}{1 - E_j}, \\ \text{lower } CI &= \bar{D}_j - \frac{\bar{I}_j - CI - E_j}{1 - E_j}, \\ \text{upper } CI &= \frac{\bar{I}_j + CI - E_j}{1 - E_j} - \bar{D}_j. \end{aligned} \tag{1}$$

We conducted these calculations using the population morph frequencies presented in *SI Appendix*, Table S5 and the marginal morph or population means estimated from analyses of the proportion of intermorph matings. As the analyses of the proportion of intermorph mating involved the logit link function, means and their confidence intervals were back-transformed to proportions before conducting these calculations. The population marginal mean is a simple average of the morph means, so we used the simple average of morph-specific E_j to calculate the population-specific E_j.

Data, Materials, and Software Availability. SSR marker data have been deposited in Dryad (https://doi.org/10.5061/dryad.fj6q573zn). All study data are included in the article and/or *SI Appendix*.

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