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PATTERNS OF REPRODUCTIVE ISOLATION IN *NOLANA* (CHILEAN BELLFLOWER)

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We examined reproductive isolating barriers at four postmating stages among 11 species from the morphologically diverse genus *Nolana* (Solanaceae). At least one stage was positively correlated with both genetic and geographic distance between species. Postzygotic isolation was generally stronger and faster evolving than postmating prezygotic isolation. In addition, there was no evidence for mechanical isolation, or for reproductive character displacement in floral traits that can influence pollinator isolation. In general, among the potential isolating stages examined here, postzygotic barriers appear to be more effective contributors to reducing gene flow, including between sympatric species.

KEY WORDS: **Character displacement, hybrid, postzygotic, prezygotic, reinforcement, speciation.**

For sexually reproducing plants and animals, the origin of new species involves the evolution of reproductive isolating barriers between diverging lineages. Studying these isolating barriers therefore provides insight into the process of speciation (Coyne and Orr 2004). Several approaches have been used to examine the evolution of reproductive isolation within the same closely related group of species, including examining the relative strength of isolating barriers operating at different reproductive or developmental stages among different species pairs, across different degrees of evolutionary divergence, and/or among reciprocal crosses (Coyne and Orr 2004, and see below). These approaches aim to evaluate how rapidly barriers accumulate and which isolating barriers contribute most during initial divergence between lineages, among other questions. In combination, they can provide insight into the evolutionary forces and the genetic mechanisms responsible for the evolution of new, reproductively independent, lineages.

First, examining the reproductive compatibility of a single species pair at multiple developmental stages (e.g., premating, postmating prezygotic, and postzygotic) can be used to infer which stages are most effective at reducing current gene flow between these species, and therefore which stages might have been more or less influential during their initial divergence (e.g., Ramsey et al. 2003; Kay 2006; Martin and Willis 2007; Mendelson et al. 2007; Maroja et al. 2009; Dopman et al. 2010). For example, based on the estimated contributions of multiple pre- and postzygotic reproductive barriers between two monkeyflower sister species, *Mimulus lewisii* and *M. cardinalis* (Ramsey et al. 2003), factors acting prior to hybridization (specifically ecogeographic isolation and pollinator isolation) were inferred to be the primary isolating barriers in this system. Data such as these can also suggest the evolutionary forces that are most likely responsible for reducing gene flow between species. For example, estimates of pre- and postzygotic barriers between *M. guttatus* and *M. nasutus* (Martin and Willis 2007) revealed that prezygotic barriers contributed most to total isolation, likely as a result of adaptive divergence in mating systems (i.e., shift to self-pollination) and edaphic ecology (i.e., drought avoidance via phenological acceleration).

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Second, examining the relationship between genetic divergence and strength of isolation using multiple closely related species pairs can reveal the pattern of accumulation of reproductive isolation over evolutionary time (e.g., Coyne and Orr 1989; Moyle et al. 2004). This approach has been used to examine the accumulation of isolation barriers in many different animal groups (e.g., Coyne and Orr 1989; Sasa et al. 1998; Presgraves 2002; Mendelson 2003) with some similar studies in plants (e.g., Moyle et al. 2004; Archibald et al. 2005; Scopece et al. 2007). Most analyses detect a positive correlation between genetic distance and strength of reproductive isolation (Coyne and Orr 1989, 1997; Tilley et al. 1990; Sasa et al. 1998; Presgraves 2002; Mendelson 2003), suggesting an approximately "clocklike" accumulation of reproductive isolation. Because this approach involves an estimate of time since species pairs diverged (usually measured as neutral sequence divergence), it can also provide a rough assessment of the rates of evolution of different isolation stages and has been used to infer, for example, that rates of evolution of prezygotic isolation are typically higher than postzygotic isolation (at least in animals; e.g., Coyne and Orr 1989, 1997; Mendelson 2003). Similarly, rate comparisons can evaluate whether prezygotic isolation evolves faster among sympatric species than among allopatric species (e.g., Coyne and Orr 1989, 1997; Gleason and Ritchie 1998; Le Gac and Giraud 2008; Bradbury et al. 2010), a pattern consistent with the operation of speciation via "reinforcement" (Dobzhansky 1951; Ortiz-Barrientos et al. 2009).

In this study, we examined the strength of reproductive isolation at up to four postmating stages in 32 species pairs, using artificial crosses among 11 species from the plant genus *Nolana* (Solanaceae), commonly called Chilean bellflower (Freyre et al. 2005). The genus consists of 89 species found mainly in the coastal Peruvian desert and the Atacama desert of Chile (Dillon 2005). With respect to reproductive isolation, several features of the group are notable. First, many species have sympatric components to their ranges (e.g., Fig. 1), but documented natural hybrids are extremely rare (Dillon et al. 2009, see discussion), suggesting that species identity is actively preserved by reproductive isolation mechanisms. Second, the group has high floral diversity; between species, flowers vary up to fourfold in size, and corolla colors range from white to deep blue with white to yellow throats (Freyre et al. 2005; Tu et al. 2008; e.g., Fig. 1). Floral differentiation might contribute to reproductive isolation if different pollinators preferentially visit alternative floral types, thereby reducing the movement of pollen between heterospecific individuals (Kay and Sargent 2009). Floral differentiation can also influence the success of heterospecific crosses at postmating stages. For example, in some cases pollen from short-styled species is unable to sustain sufficient pollen tube growth to achieve fertilization in long-styled species—a form of postmating mechanical isolation due to species differentiation in style length (e.g., Williams and Rouse 1988; Kay and Schemske 2008). Third, in cases where this has been tested, most *Nolana* species appear to be genetically self-incompatible (SI) (Freyre et al. 2005; Douglas 2007; Freyre and Douglas 2008; Table S1). Genetic self-incompatibility is known to influence postmating prezygotic reproductive isolation between species. In particular, in some groups crosses between SI and self-compatible (SC) plant species show asymmetric reproductive isolation, such that SI pollen will grow in SC styles but the reciprocal cross is inhibited (the "SI $\times$ SC" rule; Lewis and Crowe 1958; de Nettancourt 1977; Brandvain and Haig 2005 and references therein).

We used our reproductive isolation data to assess the relative contributions of different isolation stages to overall reproductive isolation, and to examine the pattern and rate of accumulation of isolating barriers between *Nolana* species. Time since species divergence was estimated using DNA sequences from five loci. Geographical distance between species was estimated from collection data and species range maps. In addition, because *Nolana* species vary in their reproductive morphology in ways that might influence species barriers (see above), several floral traits were also measured and their relationship with genetic, geographic, and postmating reproductive isolation evaluated. Our primary goal was to examine the strength and patterns of reproductive isolation to make inferences about the evolutionary forces and genetic changes likely involved in *Nolana* speciation, including which factors maintain species identity in sympatry.

Materials and Methods

STUDY SYSTEM

The genus *Nolana* (Solanaceae) is distinguished from other relatives by its unique hard-coated fruits known as mericarps (Dillon et al. 2007, 2009). *Nolana* species prefer semi- to hyper-arid habitats; 70 of the 89 *Nolana* species live along the western coastline of Chile and Peru in South America (Dillon et al. 2007) in specialized fog-saturated habitats known as *lomas* formations. Within this distribution, most species are endemic with narrow geographic ranges and apparently specific ecological requirements, even though many have overlapping (sympatric) parts to their species ranges (Dillon et al. 2009). Molecular data indicate a deep biogeographical split between Peruvian and Chilean species (Tu et al. 2008); in addition, sequence analysis, biogeographic information, and morphological traits, have resolved several major clades in the genus, although within these clades a number of species relationships remain unresolved (Dillon et al. 2009; Fig. 1 lower right inset).

REPRODUCTIVE ISOLATION BARRIERS

Seeds from 11 *Nolana* species were collected from native Peruvian or Chilean populations (Fig. 1; Table S1), germinated, and

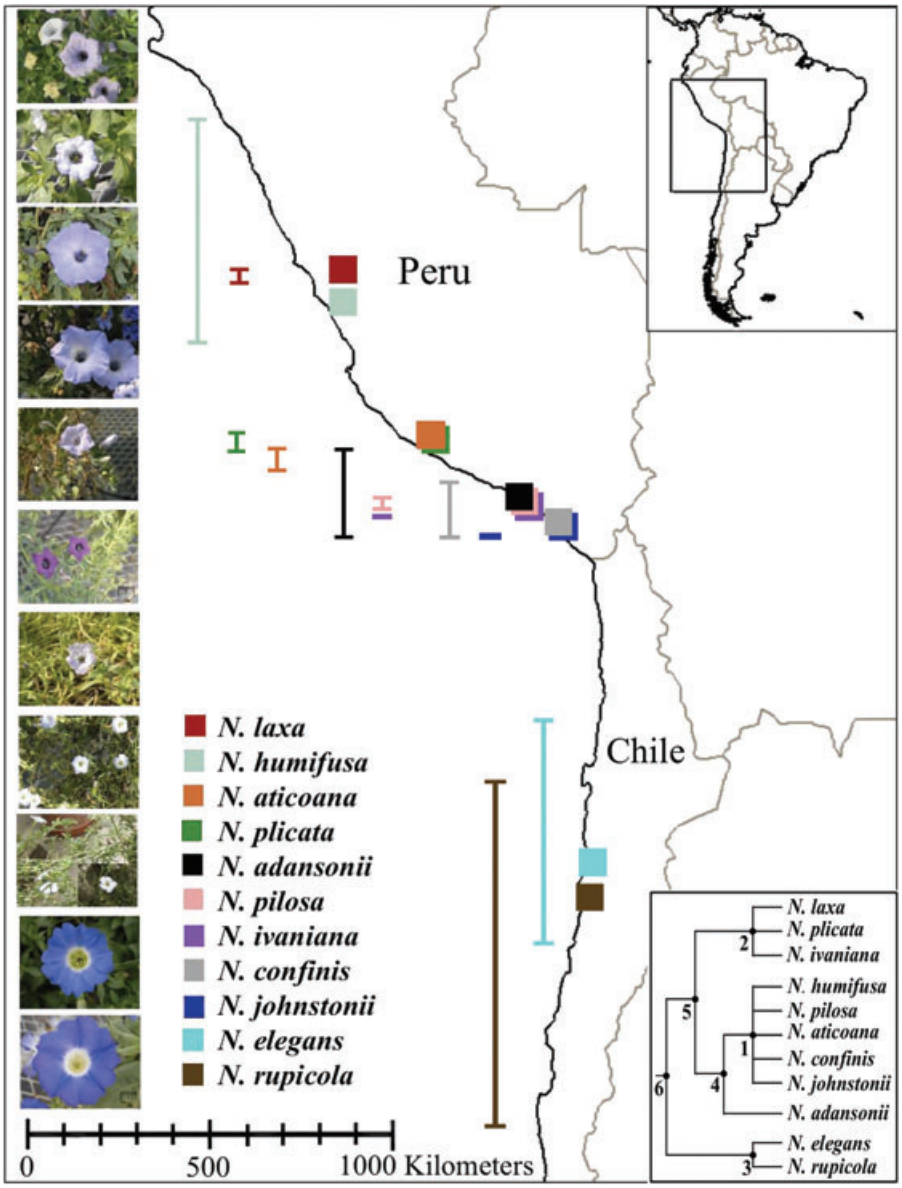

Figure 1. Collection sites (boxes) and latitudinal ranges (vertical bars) for 11 *Nolana* species. Left inset: floral variation in study species; species (north to south) order corresponds to legend order. Top right inset: Geographical location of study species in continental South America. Bottom right inset: Phylogenetic relationships between study species, based on Dillon et al. (2009); major clades are well resolved, however species resolution is limited within these groups. The cladogram shows nodes used in calculations of Phylogenetically Independent Contrasts (PICs) (see text).

resulting plants cultivated under standard greenhouse conditions (see Online Supporting Information). Artificial crosses were performed to assess reproductive isolating barriers between species at one postmating prezygotic (pollen–pistil compatibility) and three postzygotic (fertilization success, F_1 fruit (mericarp) size, and F_1 seed production) stages (described below). For each isolation stage, interspecific compatibility was assessed relative to intraspecific controls according to the following formula: Reproductive Isolation = 1 – (average success of interspecific crosses/average success of intraspecific crosses). All measures of compatibility are therefore reported as isolation indices that vary between zero (interspecific cross as compatible as intraspecific cross) and 1 (complete incompatibility in interspecific cross).

Postmating prezygotic isolation

To assess the strength of incompatibilities expressed after pollination but before fertilization, we evaluated pollen germination success and pollen tube growth following artificial hybridizations within and between seven species (Table S2), using epifluorescence microscopy on fixed and stained pollinated styles (see Online Supporting Information). Pollen tube growth in each pistil was evaluated on a four point scale: 0 = unsuccessful

germination/no pollen tube growth; 1 = tube growth halted in stigma; 2 = pollen tubes extend to midway down style; 3 = successful/pollen tubes extend to the base of the style. Crosses were allowed up to 48 h after pollination, which is ample time to evaluate relative pollen growth/performance (e.g., normal conspecific fertilization is completed less than 8 h postpollination; Douglas 2007, and see Online Supporting Information). Crossability of each species pair was calculated using the mean of all scores standardized by the mean intraspecific pollen tube performance in the seed parent species.

Postzygotic isolation

Using eight *Nolana* species (Tables S3–S5), artificial crosses were performed to assess compatibility at three stages: fruit set (the number of developing fruits that resulted for each pollination that was made); mericarp size (the average mass [mg] of the matured fruit resulting from crosses); and seed set (the number of fully formed seeds within each matured mericarp, evaluated from x-ray images). These three stages can be considered sequentially acting stages of reproductive compatibility (see Online Supporting Information). Intra- and interspecific crosses were performed at different times on the same individuals, to prevent differential resource allocation from influencing the probability of relative cross success. Total postzygotic isolation was determined for all interspecific crosses that had a reproductive isolation (RI) index value for each of these three postzygotic stages, using the absolute contribution and total reproductive isolation equations described in Ramsey et al. (2003) (see Online Supporting Information).

MORPHOLOGICAL DIFFERENTIATION

When flowers were available, up to four floral traits were measured on each plant: corolla diameter, corolla depth, style length, and stigma diameter (all in mm). Style length and stigma diameter were only measured on those species used to assess postmating prezygotic isolation. At least five flowers per plant were measured to calculate plant means and, with these, species mean trait values.

GEOGRAPHIC ISOLATION

Geographical coordinates of population collection sites (Table S1) were used to map the sample location of each species using ArcGlobe 10 (ESRI 2011) (Fig. 1). Pairwise geographic distances between species collection sites were calculated using the online program Movable Type Scripts (Veness 2002–2010). Species latitudinal ranges (from Dillon 2011) were used to classify species pairs, and specific collection sites, as allopatric or sympatric. A species pair was considered sympatric if the ranges and/or collection sites were identical or if one species range was completely within another species range. Allopatric species (the majority of pairs examined here) had nonoverlapping ranges and collection sites.

GENETIC DISTANCE

Sequences for two loci (*ADH2*, *atpB*) were generated *de novo* from each species, using standard protocols and previously developed primer sets (Hoot et al. 1995; Nesbitt and Tanksley 2002) (see Online Supporting Information). Sequences from three additional loci (*ndhF, psbA-trnH, rps16*) were obtained from GenBank (Table S1). Aligned sequences from the five total loci were analyzed in DnaSP version 4.9 (Rozas et al. 2003). For each locus, genetic divergence between each species pair was estimated as *K*s (synonymous substitutions per synonymous site) (see Online Supporting Information); mean *K*s values of all five loci were then calculated for each species pair, to estimate average genetic distance.

STATISTICAL ANALYSES

To evaluate the magnitude of correlations between pairwise reproductive isolation and genetic and geographic distance for each isolation stage, we used both standard parametric correlations and Mantel tests of matrix correlations; relationships between reproductive isolation and genetic distance were also evaluated with phylogenetically independent contrasts (PICs; Felsenstein 1985). Mantel tests are regularly used to analyze pairwise data in which species are involved in more than 1 comparison (Harmon and Glor 2010), but are known to be difficult to interpret, have reduced power overall, and may have elevated type-1 error (Legendre and Legendre 1998; Castellano and Balletto 2002). Similarly, by reducing the size of datasets, PICs also often have reduced power to detect significant relationships. Nonetheless, there are few alternative approaches for taking into account nonindependence among data points in such pairwise matrix data (Harmon and Glor 2010). Partial Mantel tests were used to assess the strength of association between genetic and geographic distance and pairwise reproductive isolation, for each isolation stage. Significance for each Mantel test was evaluated with 10,000 permutations. For PICs, values for genetic distance and for each isolation stage were averaged across each independent contrast (Fig. 1 lower right inset) as described in Fitzpatrick (2002) to generate a set of phylogenetically "corrected" taxon pairs (as per Coyne and Orr 1989; Moyle et al. 2004). Because of limited resolution (i.e., polytomies) in the consensus tree for *Nolana*, and missing reproductive isolation data for some independent contrasts, each isolation stage produced only four PICs for correlation analyses (See Results).

To assess if morphological differences were associated with genetic and geographical isolation between species samples, for each species pair we calculated the absolute value of the difference in each floral trait (e.g., pairwise differences in size of corolla diameter). For these morphological difference indices, we evaluated their strength of correlation with genetic and geographic distance using standard parametric correlations and Mantel tests

Table 1. Absolute and relative contributions to reproductive isolation of four sequential isolating stages among *Nolana* species.

	Stage	*N* (pairs)	Mean absolute contribution	Mean relative contribution
Prezygotic	Pollen-pistil	15	0.0717	NA
Postzygotic	Fruit set	22	0.460	0.567
	Mericarp size	21	0.191	0.278
	Seed set	21	0.113	0.155
	Total postzygotic	20	0.763	1.00

on uncorrected data, and using PICs calculated from morphological data. For postmating prezygotic isolation, we also assessed the relationship between differences in style length and this isolating barrier.

Relative "rates" of evolution were estimated for each species pair by dividing their pairwise isolation estimate (for each reproductive stage) by their genetic distance. These values were used in one-way ANOVAs to compare average "rates" between each isolating stage and between species pairs that are sympatric or allopatric. Statistical analyses were performed in R.

Results

ESTIMATES OF REPRODUCTIVE ISOLATION

For postmating prezygotic isolation (pollen-pistil interactions; $N = 15$ species pairs), pollen germination and complete pollen tube growth was the most common outcome (Table S2). In contrast, for the three postzygotic isolation stages ($N = 20$–22 species pairs), barrier strength varied from 0 to 1 depending on the species pair and stage examined (Tables S3–S5). Of the three postzygotic stages assessed, fruit set (the first acting stage) had the highest absolute contribution to overall postzygotic isolation, and therefore the greatest relative contribution to the total postzygotic isolation (Table 1). Overall, we estimate that these three postzygotic stages can cumulatively act to prevent, on average, 76.3% (range 39.3–100%) of gene flow between species.

COMPARISON OF PREZYGOTIC AND POSTZYGOTIC STAGES

Not all species pairs examined for prezygotic isolation had matching data for postzygotic isolation and vice versa (see Methods), precluding paired comparisons between these stages. In addition, the average genetic relatedness was lower for species pairs used to estimate prezygotic compared to postzygotic isolation (Fig. 2). Therefore, we standardized each incompatibility index by genetic distance for each species pair to calculate and compare the mean "rates" of evolution for each reproductive isolating stage. A one-way ANOVA found that stage of reproductive isolation significantly influenced the mean "rate" of evolution (ANOVA $F = 7.7$, $P = 0.0001$). All pairwise comparisons, evaluated using post-hoc tests (Tukey's HSD), indicated that prezygotic pollen–pistil isolation evolves significantly slower (mean "rate" = 0.0063) than all postzygotic stages (mean "rates" = 0.0268–0.0326;Table S6). "Rates" of evolution for each postzygotic stage were not different from one another. No differences were found between the rates of isolation within any stage when comparing species pairs classified as having overlapping ranges (sympatric) or nonoverlapping ranges (allopatric) (Table S7) (ANOVA $F = 0.2$, $P = 0.90$).

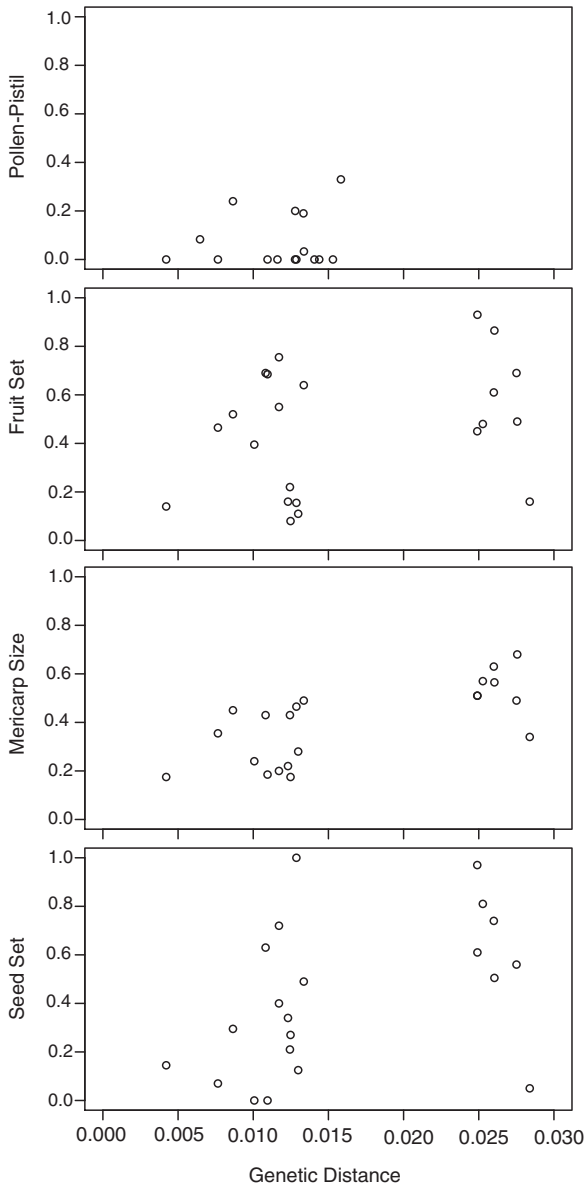

Figure 2. Relationship between pairwise genetic distance and each of (A) postmating prezygotic isolation; (B) postmating isolation estimated from fruit set/pollination; (C) postmating isolation estimated from mericarp size; (D) postmating isolation estimated from F_1 seed set. Statistics for each relationship are shown in Table 2.

Table 2. Relationship between genetic and geographic distance, and reproductive isolation, for four isolating stages. *P*-values less than 0.05 are in bold.

			Pairwise genetic distance					Pairwise geographical distance		
			All data			PICs		All data		
		N (pairs)	Cor.	*P*-value (Mantel)	*P*-value (Pearson's)	Cor.	*P*-value (Pearson's)	Cor.	*P*-value (Mantel)	*P*-value (Pearson's)
Prezygotic	Pollen-pistil	15	0.164	0.651	0.559	0.965	0.168	0.136	0.668	0.630
Postzygotic	Fruit set	22	0.317	0.256	0.150	0.425	0.401	0.372	0.148	0.087
	Mericarp size	21	0.696	**0.014**	**<0.0005**	0.518	0.292	0.711	**0.010**	**<0.0005**
	Seed set	21	0.439	0.122	**0.046**	0.383	0.454	0.509	0.0589	**0.018**
	Total post	20	0.463	0.101	**0.040**	0.332	0.521	0.573	**0.034***	**<0.010**

CHANGE IN REPRODUCTIVE ISOLATION OVER GEOGRAPHIC AND GENETIC DISTANCE

For each stage of isolation, we individually evaluated the strength of relationship between genetic and geographic distance, and pairwise isolation, between species pairs (Table 2, Fig. 2, Figure S1). Based on Mantel tests, reduced relative mericarp size was the only reproductive isolation metric significantly correlated with both genetic and geographic distances (Table 2, Fig. 2, Figure S1); parametric tests of these relationships showed significant correlations for this stage as well as for seed set and total postzygotic isolation (Table 2). Comparisons using PICs did not have sufficient power to detect significant relationships, however these analyses reveal correlations that are consistent in magnitude and direction with those detected with the other approaches (Table 2). Generally, with increased geographic and genetic distance between populations, reproductive isolation increased (Fig. 2, Figure S1) even where these relationships were not significant. Because genetic and geographic distances are themselves highly correlated ($F = 203.2$; df $= 1, 77$; $R^2 = 0.72$; $P < 0.0001$), it is difficult to distinguish which of these two factors is more causally associated with increased reproductive isolation between species. Using partial Mantel tests, we found no significant correlations between isolation (at any stage) and genetic distance or geographic distance, once the alternative metric of distance was controlled for (Table S8), suggesting we have insufficient power to distinguish the relative influence of these two factors on our measures of isolation.

MORPHOLOGICAL CHARACTERISTICS AND GEOGRAPHIC, GENETIC AND REPRODUCTIVE ISOLATION

Species pairwise differences in flower size (corolla diameter) were significantly positively associated with genetic distance (Cor = 0.513; Mantel test $P = 0.004$; Pearson's $P = 0.0003$) and with geographic distance (Cor = 0.322; Mantel test $P = 0.057$; Pearson's $P = 0.031$) (Figure S2). These relationships suggest that differentiation in flower size accumulates with increasing evolutionary divergence and that spatial proximity is not associated with elevated floral differences, as would be expected if there were selection for reproductive character displacement and/or if species coexistence were permitted by this displacement. There were no correlations between the remaining floral traits and geographic and genetic distance (Table S10). In addition, we found that prezygotic isolation was not correlated with pairwise style length differences between species pairs (Figure S3), (Cor = −0.085; Mantel test $P = 0.808$; Pearson's $P = 0.765$), indicating no evidence that species crossability is determined by simple mechanical isolation between species. To further evaluate this inference, we performed additional crosses specifically between long- and short-styled species pairs and found no association between pollination success and the direction of these crosses (data not shown). Finally, in species pairs evaluated for postmating prezygotic isolation, we used Student's *t*-tests to evaluate whether crossing success differed significantly between reciprocal pairings. Only one species pair showed a significant reciprocal difference in pollen-pistil compatibility: when *N. pilosa* served as the seed parent and *N. plicata* as the pollen parent, the cross was highly successful, but the reciprocal cross failed ($t = 4.6$, $P = 0.003$). However, this species pair does not differ significantly for style length (Table S9); additionally, both these species are SI (Table S1), so the "SI × SC" rule (i.e., mating system differences) does not explain this asymmetric barrier either. Conversely, we also found no evidence that crosses between SC and SI species followed this rule for pollen-pistil barriers (Tables S1 and S2).

Discussion

To assess the strength and patterns of reproductive isolation within *Nolana*, we combined two approaches that have been used to evaluate the evolutionary forces and genetic changes likely contributing to speciation in groups of closely related species (Coyne and Orr 2004). Two general conclusions emerge from our analyses.

First, like other analyses (Coyne and Orr 1989, 1997; Tilley et al. 1990; Gleason and Ritchie 1998; Sasa et al. 1998; Presgraves 2002; Mendelson 2003), we found clear evidence for interspecific reproductive isolation expressed at both pre- and postzygotic stages, and positive relationships between the strength of reproductive isolation and the degree of genetic differentiation between species. Although all isolation stages showed a trend toward positive associations, reduced relative F_1 fruit (mericarp) size was the only stage consistently correlated with genetic and geographic distance. Regardless, because of the sequential action of postzygotic isolating barriers, the postzygotic factor with the greatest influence on isolation was the earliest acting postzygotic stage examined: the ability of species' pairs to produce fruit (i.e., fruit set per pollination). In comparison, the final stage examined (seed set), added relatively little to total postzygotic isolation. Nonetheless, the estimated rates of evolution for these postzygotic stages were not statistically different; in addition, their strengths did not vary when species pairs are classified as sympatric or allopatric.

Second, in comparison to our postzygotic isolation stages, we found little evidence that the prezygotic mechanisms we examined are strong contributors to isolating barriers among these species. This conclusion is directly supported by our measures of postmating prezygotic isolation, but also suggested by our assessment of floral divergence among species, and its relationship with genetic and geographic divergence. In general, isolation expressed postpollination and prior to fertilization was weak. In addition, the change in prezygotic isolation over genetic distance appears to be slower than that of the postzygotic isolating barriers we examined. In other systems, prezygotic isolation has been found to evolve more rapidly than postzygotic isolation, possibly because traits mediating prezygotic isolation are subject to strong divergent sexual selection in different lineages (Panhuis et al. 2001; van Doorn et al. 2009). Also, if reinforcement (selection for prezygotic traits that prevent hybridization) is occurring, prezygotic factors should evolve faster in sympatry (Coyne and Orr 1989); this pattern has been observed in several animal groups (e.g., Coyne and Orr 1989; Bradbury et al. 2010). In comparison, we found no evidence that these factors are strongly affecting postmating prezygotic isolation operating during pollen–pistil interactions among our species. Importantly, our analysis did not examine competitive pollen interactions (i.e., where both homo- and heterospecific pollen is "competed" on the same style). It is possible that pollen–pistil mediated isolation might be manifest only under these competitive conditions (Waser 1978); some studies have detected substantial "conspecific pollen precedence" among species (e.g., Carney et al. 1996; Montgomery et al. 2010) although others have not (reviewed in Howard 1999). At present, our data cannot evaluate the influence of pollen competitive interactions on prezygotic isolation in *Nolana*.

The comparative weakness of examined prezygotic barriers is also suggested by our observations of reproductive and floral variation. We found no evidence for the consistent involvement of mechanical (postmating) isolation or mating system differences in the expression of postmating prezygotic barriers, and no evidence of character displacement in the four floral traits we examined. Similarly, although not examined quantitatively, there is little indication that flower color is strongly divergent between cooccuring species (Fig. 1). This is intriguing in a group with high reproductive and floral diversity among species. Traditionally, floral characters have been hypothesized to play a strong role in mediating isolating barriers between species, especially via their effects on pollinator behavior (Grant 1949). This expectation is supported in some studies (e.g., Schemske and Bradshaw 1999; Hodges et al. 2002; Cozzolino and Scopece 2008; Martin et al. 2008), but has also been challenged on the basis that pollinators often show insufficient fidelity to be anything but very weak barriers to gene flow (Waser 2001). Indeed, some studies fail to find pollinator discrimination between cooccuring species, even when there are evident differences in floral morphology (e.g., Cooley et al. 2008).

Clearly, we did not examine pollinator behavior in our study; while the floral characters we assessed are unlikely to contribute to pollinator isolation among cooccurring *Nolana*, other unexamined traits (including, for example, floral scent or temporal differences) might still be important in effecting pollinator-mediated prezygotic isolation. Our study therefore narrows the suite of traits most likely to contribute to reducing gene flow between these species. Many *Nolana* species live in sympatry and have overlapping ranges. Nonetheless, documented natural hybrids are very rare—we know of only one case that involves *N. pilosa* and *N. thinophila* (R. Freyre, pers. obs.), despite decades of field collections and observations (Freyre et al. 2005; Dillon et al. 2009)—indicating that reproductive isolating barriers are likely maintaining species identity, even in close spatial proximity. Among the factors considered here, our analyses suggest that the pollen–pistil interactions and several floral characters are likely weak contributors to this species isolation, whereas postzygotic barriers contribute more strongly. In particular, isolation that acts shortly after fertilization but before substantial investment in F_1 hybrid offspring could contribute strongly to maintaining species differences. Factors other than these might also be involved in preventing hybridization among *Nolana*, including other unexamined phenological and morphological traits that might affect the likelihood of mating via prezygtoic mechanisms.

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Supporting Information

The following supplementary information is available for this article:

Table S1. Accession information for 11 *Nolana* study species.

Table S2. Prezygotic (pollen-pistil) isolation indices for all species pairs.

Table S3. Postzygotic isolation (fruit set/pollination) indices for all species pairs.

Table S4. Postzygotic isolation (mericarp size) indices for all species pairs.

Table S5. Postzygotic isolation (seed set) indices for all species pairs.

Table S6. Estimated "rates" of evolution of different stages of reproductive isolation.

Table S7. Estimated "rates" of evolution of allopatric (nonoverlapping) versus sympatric (overlapping) pairs, at four different stages of reproductive isolation.

Table S8. Results of partial Mantel tests of the relationship between reproductive isolation and both genetic and geographical distance, for each of four isolation stages.

Table S9. Species means for floral traits.

Table S10. Relationship between genetic and geographic distance, and floral trait differences.

Figure S1. Relationship between geographical distance and each of (A) postmating prezygotic isolation; (B) postmating isolation estimated from fruit set/pollination; (C) postmating isolation estimated from mericarp size; (D) postmating isolation estimated from F1 seed set. Statistics for each relationship are shown in Table 2.

Figure S2. Relationship between pairwise species differences in flower size (corolla diameter) and (A) genetic distance (Cor = 0.513, Mantel test $P = 0.0039$; Pearson's $P = 0.000314$), and (B) geographic distance (Cor = 0.322, Mantel test $P = 0.0567$; Pearson's $P = 0.0309$).

Figure S3. Relationship between pairwise species differences in style length and postmating prezygotic isolation index (Cor = −0.0845, Mantel test $P = 0.808$; Pearson's $P = 0.765$).

Supporting Information may be found in the online version of this article.

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