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ECOLOGICAL REPRODUCTIVE ISOLATION OF COAST AND INLAND RACES OF *MIMULUS GUTTATUS*

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Adaptive divergence due to habitat differences is thought to play a major role in formation of new species. However it is rarely clear the extent to which individual reproductive isolating barriers related to habitat differentiation contribute to total isolation. Furthermore, it is often difficult to determine the specific environmental variables that drive the evolution of those ecological barriers, and the geographic scale at which habitat-mediated speciation occurs. Here, we address these questions through an analysis of the population structure and reproductive isolation between coastal perennial and inland annual forms of the yellow monkeyflower, *Mimulus guttatus*. We found substantial morphological and molecular genetic divergence among populations derived from coast and inland habitats. Reciprocal transplant experiments revealed nearly complete reproductive isolation between coast and inland populations mediated by selection against immigrants and flowering time differences, but not postzygotic isolation. Our results suggest that selection against immigrants is a function of adaptations to seasonal drought in inland habitat and to year round soil moisture and salt spray in coastal habitat. We conclude that the coast and inland populations collectively comprise distinct ecological races. Overall, this study suggests that adaptations to widespread habitats can lead to the formation of reproductively isolated species.

KEY WORDS: **Adaptation, drought, flowering time, population structure, salt tolerance, speciation.**

It is commonly observed that recently diverged sister species tend to inhabit environments with different ecological characteristics, and possess habitat-specific adaptations (Mayr 1947; Clausen 1951; Schemske 2000; Coyne and Orr 2004; Angert 2006; Nakazato et al. 2008). What is not always clear is the extent to which such habitat differentiation contributes to the process of speciation because adaptation to different habitats can influence several potential prezygotic and postzygotic reproductive isolating barriers (Via et al. 2000; Ogden and Thorpe 2002; Rundle 2002; McKinnon et al. 2004; Nosil 2007; for reviews see Schluter 2001; Coyne and Orr 2004; Lexer and Fay 2005; Rundle and Nosil 2005; Hendry et al. 2007). For example, if the different habitats exist only in disparate geographic regions, then the habitat-related adaptations may affect the overall distribution and range limits of sister species, such that the species rarely if ever come into contact with each other (Schemske 2000; Coyne and Orr 2004; Angert and Schemske 2005). Such environmental imposition of an allopatric distribution on sister species is often termed ecogeographic isolation (Mayr 1947; Clausen 1951; Schemske 2000; Ramsey et al. 2003; Husband and Sabara 2004; Kay 2006). If environmental variation exists on a finer spatial scale, then habitat adaptation can cause reproductive isolation between species if immigrants have low viability or fertility (e.g., Nosil et al. 2005). The strength of immigrant inviability and ecogeographic isolation can be tested

through reciprocal transplant experiments (Coyne and Orr 2004; Nosil et al. 2005). Indeed, numerous reciprocal transplant experiments conducted over the last 75 years support the role of local adaptation in restricting gene flow between species (Turresson 1922; Clausen and Heisey 1958; Linhart and Grant 1996; Wang et al. 1997; Nagy and Rice 1997; Angert and Schemske 2005; Hall and Willis 2006; Rieseberg and Willis 2007).

In addition to ecogeographic isolation and immigrant inviability, adaptation to alternative habitats may contribute to other components of reproductive isolation. It is well known, for example that in plants, sister species or even "ecotypes" adapted to different habitats often evolve differences in flowering time (Clausen 1951; Clausen and Heisey 1958; Grant 1981). These differences in the timing of reproduction can cause temporal isolation, potentially a strong premating barrier between species (Coyne and Orr 2004; Martin and Willis 2007; Martin et al. 2007; Pascarella 2007; Yang et al. 2007). Even if populations or species adapted to different habitats occasionally interbreed, hybrids may be maladapted to either parental habitat, resulting in extrinsic postzygotic isolation (Wang et al. 1997; Hatfield and Schluter 1999; Rundle and Whitlock 2001; Rundle 2002; Forister 2005; Campbell and Waser 2007). Because of these complex and multifaceted relationships between habitat adaptation and reproductive isolation, the importance of habitat adaptation relative to other potential prezygotic and postzygotic barriers has rarely been quantified (but see Nosil et al. 2005; Nosil 2007; Lowry et al. 2008). Furthermore, the specific ecological factors driving ecological reproductive isolation are often unknown. The geographic scale at which different stages of speciation occur has remained poorly understood, especially because geographically widespread population genetic analysis is rarely conducted in conjunction with the quantification of reproductive isolating barriers (Levin 1993; Coyne and Orr 2004; Rundle and Nosil 2005).

In this study, we address these issues by examining the extent of genetic divergence, habitat adaptations, population structure, and reproductive isolation between coastal perennial and inland annual forms of the wildflower *Mimulus guttatus*. *Mimulus guttatus* (yellow monkeyflower; Phrymaceae, historically Scrophulariaceae, order Lamiales) is a highly variable species within the even more diverse *M. guttatus* species complex (Vickery 1978), which is distributed over western North America. Different populations often appear adapted to a multitude of elevational, climatic, and edaphic habitats (Vickery 1978; Wu et al. 2008). Throughout the lower elevation inland meadows, seeps, and rocky outcrops, from the Pacific coastal mountain range of the United States and Canada east to the Rockies, *M. guttatus* populations are typically composed of spindly spring flowering annual plants (Vickery 1952; Clausen and Heisey 1958; Wu et al. 2008). In adjacent fog-bound cliffs, sand dunes, and coastal terrace that occur within a couple of kilometers of the Pacific Ocean, *M. guttatus* populations are composed of large, robust, late summer flowering perennial plants with compressed internodes (Vickery 1952; Clausen and Heisey 1958; Hitchcock and Cronquist 1973; Hall and Willis 2006). Based on field collections, the coastal perennial populations have frequently been assigned a separate taxonomic status: *M. guttatus var. grandis* Greene (Hitchcock and Cronquist 1973) and *M. guttatus ssp. litoralis* Pennell (Abrams and Ferris 1951). However, common garden experiments are needed to demonstrate that the morphological distinctness of coastal populations is not due to phenotypic plasticity. Inland perennial forms of *M. guttatus* are also found around permanently moist springs, creeks, and lakes throughout western North America (Vickery 1952; Clausen and Heisey 1958), but they are not included in the present study.

A recent reciprocal transplant between a pair of coast and inland *M. guttatus* populations from Oregon provides evidence of strong local adaptation to their respective habitats (Hall and Willis 2006). The inland habitat of the coast ranges of California and Oregon experiences a very long (~6 month), hot summer drought, in which moist areas (seeps, creeks, arroyos) that contain annual *M. guttatus* populations completely dry out. Coastal habitat also receives little or no rainfall during summer months, but persistent fog and cooler temperatures along the coast maintain year-round soil moisture and reduce the rate of plant transpiration (Corbin et al. 2005). However, coastal plants must contend with ocean salt spray, which is a major stress that is known to restrict the distribution of many plant species (Boyce 1954; Barbour 1978; Humphreys 1982; Wilson and Skyes 1999).

Here we report on a series of experiments to understand the evolution of reproductive isolation between coast and inland populations of *M. guttatus*. Using molecular genetic markers and common garden experiments, we ask whether multiple, broadly sampled coast and inland populations constitute two distinct morphological and genetically structured groups. Using additional reciprocal transplant experiments, we determine the extent to which coast and inland populations are locally adapted to their respective habitats. Finally, we combine results from field and greenhouse experiments to estimate the strength of multiple prezygotic and postzygotic barriers between coast and inland populations, and determine the role of adaptations to specific environmental factors in limiting hybridization and introgressive gene flow.

Materials and Methods

FIELD COLLECTIONS

In the spring of 2005, plants were collected from 14 latitudinal pairs of coast and inland populations (two extra inland populations, 30 populations total, 20–30 haphazardly collected plants per population) distributed from central California to northern Oregon (Fig. 1, Table 1). Inland populations used in this study

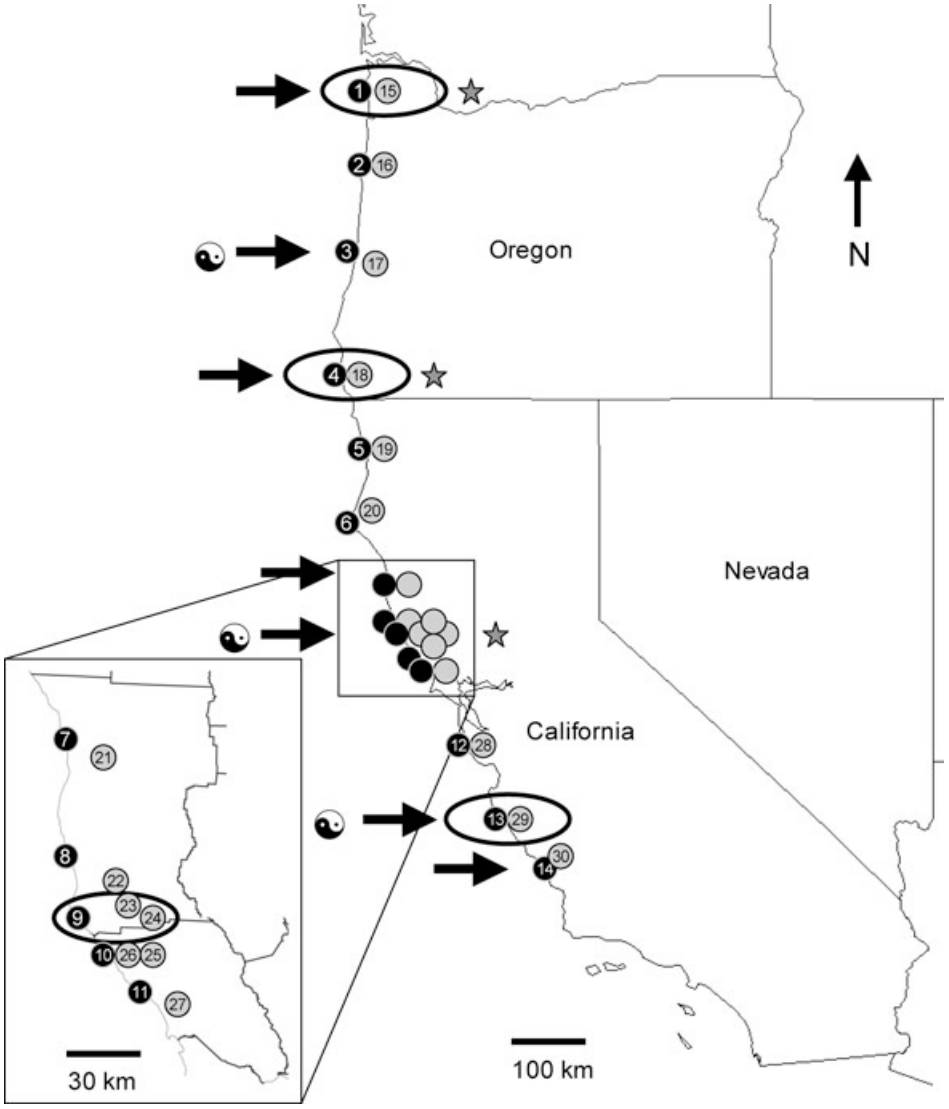

Figure 1. **Map of western United States showing locations of coast (black) and inland (gray) populations used in this study. Arrows indicate coast/inland population pairs used in common garden greenhouse experiment. Yin-yang symbol indicates locations of reciprocal transplant experiments in this study (California) and that of Hall and Willis (2006) in Oregon. Stars represent population pairs used to test for salt tolerance differences. Ovals denote population pairs used for analysis of intrinsic postzygotic isolation. Numbers correspond to populations listed in Table 1.**

were located 4.8–31.4 (mean = 16.7) kilometers from the Pacific Ocean. Coastal plants were all collected within 500 m of the ocean. Collected plants were shipped overnight to Durham, NC, where they were potted, raised to flowering, and self-pollinated in the Duke University greenhouses. Selfed seeds and tissue for DNA extraction were collected from these plants. Tissue was placed into 96-well Costar Plates and immediately deposited into an −80°C freezer.

MORPHOLOGICAL POPULATION STRUCTURE

To determine the extent of genetically based phenotypic divergence between coastal and inland populations; a common garden greenhouse experiment was conducted using six pairs of coast and inland populations and one extra coast population, for a total of 13 populations. Replicates within each population consisted of 12 selfed individuals, each descended from separately collected maternal plants. Some seeds failed to germinate, resulting in less than 12 replicates for some populations.

Seeds were sown individually in Fafard-4P soil in 4 in. square pots and were stratified in a dark room at 4°C for one week. Pots were then moved to the Duke University greenhouses for seed germination and subsequent growth. Greenhouse conditions consisted of 18-h days at 21°C with supplemental high-pressure sodium lights and 6-h nights at 16°C. Relative humidity was maintained at 30%. The location of all plants was fully randomized on the greenhouse bench.

Table 1. Coast and inland populations of *M. guttatus* used in this study. For each population longitude and latitude, the number of samples (*N*), the expected heterozygosity (H_S), mean number of alleles per locus (N_a), mean allelic richness (R_S), and mean inbreeding coefficient (F_{IS}) are listed. Ten codominant markers were used to calculate statistics in FSTAT 2.9.3.2 (Goudet 2001). "Num" corresponds to the label of populations in Figure 1.

Race	Pop ID	Num	Location	Latitude (N)	Longitude (W)	*N*	N_a	H_S	R_S	F_{IS}
M. guttatus	OSW	1	Oswald West SP, Tillamook Co., OR	45° 45′ 39″	123° 57′ 56″	20	2	0.263	1.80	0.236
(Coast)	CKI	2	Cape Kiwanda SP, Tillamook Co., OR	45° 14′ 31″	123° 58′ 07″	16	1.9	0.252	1.75	0.246
	HEC	3	Heceta Lighthouse, Lane Co., OR	44° 08′ 06″	124° 07′ 22″	14	2.1	0.297	1.88	0.308
	OPB	4	Otter Point SP, Curry Co., OR	42° 27′ 50″	124° 25′ 22″	20	2.6	0.227	2.05	0.056
	GBM	5	Gold Bluffs Marsh, Humbolt Co., CA	41° 22′ 43″	124° 04′ 10″	15	1.6	0.096	1.36	0.283
	CMD	6	Cape Mendocino, Humbolt Co., CA	40° 24′ 33″	124° 23′ 31″	16	2.6	0.350	2.16	0.382
	USB	7	Usal Beach, Mendocino Co., CA	39° 49′ 55″	123° 50′ 57″	20	2.6	0.320	2.14	0.275
	NAV	8	Navarro River, Mendocino Co., CA	39° 11′ 12″	123° 45′ 26″	16	2.3	0.277	2.04	0.008
	SWB	9	Irish Beach, Mendocino Co., CA	39° 02′ 09″	123° 41′ 25″	20	1.8	0.234	1.61	0.260
	SRN	10	Sea Ranch, Sonoma Co., CA	38° 44′ 07″	123° 29′ 23″	16	2.1	0.339	2.00	0.436
	MRR	11	Russian River, Sonoma Co., CA	38° 27′ 23″	123° 08′ 27″	14	2.6	0.353	2.28	0.283
	DAV	12	Davenport Beach, Santa Cruz Co., CA	37° 01′ 29″	122° 13′ 02″	20	2.6	0.374	2.20	0.155
	BCB	13	Big Creek Reserve, Monterey Co., CA	36° 03′ 46″	121° 35′ 31″	16	2.5	0.277	2.10	0.431
	ORO	14	Montana de Oro, San Luis Obisbo Co., CA	35° 16′ 24″	120° 53′ 20″	16	3	0.384	2.41	-0.028
M. guttatus	SAM	15	Saddle Mountain SP, Clatstop Co., OR	45° 57′ 33″	123° 40′ 46″	19	5.5	0.505	3.72	-0.016
(Inland)	LIN	16	Little Nestuca River, Tillamook Co., OR	45° 08′ 09″	123° 53′ 46″	16	5	0.534	3.60	0.300
	SWC	17	Mapleton, Lane Co., OR	43° 57′ 34″	123° 54′ 08″	11	4.2	0.655	3.77	0.169
	RGR	18	Rouge River, Curry Co., OR	42° 29′ 21″	124° 12′ 30″	16	4.3	0.561	3.30	0.377
	BHI	19	Bald Hills, Humbolt Co., CA	41° 09′ 17″	123° 53′ 22″	20	4	0.446	2.99	0.312
	BSR	20	Rio Dell, Humbolt Co., CA	40° 31′ 46″	124° 09′ 46″	16	4.1	0.482	3.07	0.165
	ANR	21	Angelo Reserve, Mendocino Co., CA	39° 44′ 12″	123° 37′ 51″	20	5.2	0.565	3.58	0.232
	SDA	22	Boonville, Mendocino Co, CA	39° 01′ 05″	123° 19′ 08″	14	4.9	0.672	3.81	0.359
	RNC	23	Rancheria Creek, Mendocino Co, CA	38° 54′ 40″	123° 14′ 42″	18	8.3	0.701	5.06	0.115
	LMC	24	Yorkville, Mendocino Co., CA	38° 51′ 50″	123° 05′ 02″	18	6.4	0.537	4.10	0.179
	USK	25	Skaggs-Springs, Sonoma Co., CA	38° 40′ 20″	123° 12′ 36″	16	6.3	0.668	4.47	0.114
	GUA	26	Gualala River, Sonoma Co., CA	38° 40′ 05″	123° 18′ 41″	12	4.3	0.536	3.43	0.008
	OAE	27	Occidental, Sonoma Co., CA	38° 24′ 40″	122° 57′ 34″	12	3.6	0.507	3.04	0.309
	LOR	28	Boulder Creek, Santa Cruz Co., CA	37° 06′ 30″	122° 07′ 04″	15	4.6	0.634	3.64	0.180
	CAN	29	Big Creek Reserve, Monterey Co., CA	36° 04′ 08″	121° 33′ 05″	16	1	0.000	1.00	-
	SLO	30	Morro Road, San Luis Obispo Co., CA	35° 27′ 38″	120° 44′ 24″	-	-	-	-	-

For each plant we recorded the number of days from seed germination to first flowering and at the same time measured 12 morphological traits using digital calipers (first and second internode length, first internode length, first and second leaf width and length, leaf thickness, maximal corolla width and length, plant height, and rosette diameter). We also counted the number of flowers 10 days after the first flower opened. To determine whether there is overall multivariate morphological divergence between coast and inland populations, a multivariate analysis of variance (MANOVA) was implemented with the 13 morphological traits and flowering time as the dependent variables and habitat (coast or inland) as the independent variable. To visually assess morphological divergence between coast and inland populations, principle components analysis was conducted using the 14 traits, and subsequently PC1 was plotted against PC2. To determine how morphological variation is partitioned between coast/inland habitats (fixed effect), among populations within habitats (random effect), and among individuals (error) within populations, a REML mixed-model nested analysis of variance (ANOVA) was conducted individually for each of the first two PCs and the 14 measured traits. To estimate the percent of variation partitioned among the hierarchical levels, the REML-nested ANOVA was rerun treating habitat as a random effect. All morphological analyses were implemented in JMP 6.0 (SAS Institute, 2005, Cary, NC).

MOLECULAR GENETIC POPULATION STRUCTURE

To determine if coast and inland populations are differentiated at loci located throughout the nuclear genome, molecular population genetic analysis was conducted with all 30 collected

populations. Genomic DNA was extracted from plant tissue using a modified hexadecyl trimethyl-ammonium bromide chloroform extraction protocol (Kelly and Willis 1998). We used a total of 10 codominant markers for population genetic analysis, including six microsatellites (Kelly and Willis 1998) and four markers that reveal length polymorphisms in the introns of single copy nuclear genes (as described in Fishman and Willis 2005; Sweigart et al. 2006). Primer sequences are available in the online Supplementary Table S1. All PCR products were subjected to capillary electrophoresis and fragment analysis on an ABI 3730×l DNA Analyzer. Size of the amplified fragments was scored automatically by the program GENEMARKER (SoftGenetics, 2005, State College, PA) and was confirmed by eye. The chromosomal location of all loci has been established by ongoing mapping studies. Although some markers were located on the same linkage group, they were never closer than 36.5 cM apart from each other (MgSTS423 and AAT230). Linkage disequilibrium is highly improbable at such large distances, but analysis with FSTAT 2.9.3.2 (Goudet 2001) was carried out to confirm that this is the case.

For all populations, we calculated the observed number of alleles (N_a), private alleles restricted to coast or inland habitat (P_a), observed heterozygosity (H_O), expected heterozygosity (H_S), total gene diversity (H_T), allelic richness (R_S), level of inbreeding (F_{IS}), and overall genetic differentiation (F_{ST}). Genetic differentiation among all pairs of populations was also quantified as pairwise F_{ST} (Weir and Cockerham 1984). All summary statistics were calculated with FSTAT (Goudet 2001). In addition, FSTAT was implemented for 1000 permutations to test for significant differences in H_S, H_O, F_{ST}, and F_{IS} between coast and inland populations.

To test for population structure between coast and inland populations we employed three different methods. Analysis of molecular variation (AMOVA, Excoffier et al. 1992) was implemented to determine how genetic variation is partitioned between coast/inland habitats, among populations within habitats, and among individuals within populations. AMOVA was implemented in the program Arlequin 3.11 (Excoffier et al. 2005).

The program STRUCTURE is a multilocus model-based clustering method that assigns individuals to a predefined number of populations (K) and detects admixed/migrant individuals (Pritchard et al. 2000). STRUCTURE was run for three iterations for K-values from 2 to 35, using the admixture model, independent allele frequencies, $\lambda = 1$, with a 200,000 burnin and 200,000 MCMC. We reran STRUCTURE 50 times at $K = 2$ and combined the result of those runs with the program CLUMMP (Jakobsson and Rosenberg 2007) and visualized this combined data with DISTRUCT (Rosenberg 2004).

The program POPULATION GRAPH uses graph theory techniques to determine the topological relationship among populations that may currently be exchanging genes (Dyer and Nason 2004). This method is free of a priori assumptions about population geographic arrangements, unlike AMOVA, and works by simultaneously determining the high-dimensional covariance relationships among all populations using the genetic marker data. The program then determines the minimum set of edges (connections) that sufficiently explain the total among population covariance structure of all of the populations. The network of population connections can then be analyzed by various post hoc analyses. POPULATION GRAPH was implemented on the web (http://dyerlab.bio.vcu.edu/wiki/index.php/) using the population genetic dataset. A test for distinct clustering of coast and inland populations was conducted post hoc using the methods outlined by Dyer and Nason (2004).

RECIPROCAL TRANSPLANT EXPERIMENTS

To test for local adaptation and reproductive isolation between coast and inland populations, reciprocal transplant experiments were conducted in California. Two sets of reciprocal transplants experiments, at different latitudes, were used to test the generality of trends and to compare to the results of a previous transplant experiment in Oregon (Hall and Willis 2006). The field sites for the experiment were located in Mendocino County (Experiment 1) and on the University of California, Big Creek Reserve in Monterey County (Experiment 2). The Mendocino coastal field site was located on seepy cliffs, just north of Manchester Beach state park (coast site 1, N39°00′29″, W123°41′38″), whereas the Mendocino inland field site was located in hilly oak savanna habitat near Boonville, CA (inland site 1, N38°59′13″, W123°21′03″, 28.7 km from the ocean). The Big Creek coastal site was located near the reserve's bridge (coast site 2, N36°04′12″, W 121°36′00″), and the inland Big Creek site was located at Shakemaker Meadow in mix grassland/chaparral habitat (inland site 2, N36°03′41″, W121°33′16″, 3.5 km from the ocean). See online Supplementary Figure S1 for location of field sites and seed source populations.

Plants growing at reciprocal transplant field sites might be adapted to highly local environmental factors instead of common features of inland or coastal habitats. To control for highly local adaptation and eliminate the possibility of genetic contamination of source populations, field sites away from seed source populations were used for field experiments (online Supplementary Fig. S1). Seeds for experiment 1 were derived from the SWB (coast) and LMC (inland) populations, whereas the seeds for experiment 2 were derived from the BCB (coast) and CAN (inland) populations (see online Supplementary Fig. S1, Table 1 for location of these populations). Field sites in experiment 1 were located within existing *M. guttatus* populations. Field sites in experiment 2 were placed into habitat that appeared suitable for *M. guttatus* because UC Reserve restrictions forbid conducting transplant experiments within existing plant populations of the same species. All seeds

were outbred and were derived from lines that had been grown in the greenhouse at least one full generation to reduce maternal effects. Four to six independent outbred full-sibling families were planted from each population, with the intention to capture much of the genetic variation within each population. At each field site, 100 inland parental plants, 100 coast parentals, and 100 F1s were planted. F1s were derived from crosses between coast and inland parentals. In sum, 300 plants were planted at each field site, 600 plants per reciprocal transplant experiment, and 1200 plants total.

Because of concern that *Mimulus* seeds would be displaced in the field, plants were transplanted at the seedling stage. To try to capture variation of germination conditions mediated by site-specific environmental factors, seeds were germinated in the soil of their eventual destination in December 2005. This timing allowed for the transplantation of individuals at the four-leaf stage, which was comparable to the developmental stage of native plants found in situ. Plants were grown in soil-filled flats at the Bodega Marine Laboratory greenhouses until transplantation in January 2006. Rosette diameter, plant height, flowering time, leaf damage, and number of flowers produced were recorded for all plants every two to three weeks beginning on April 20, 2006 and continuing through December 27, 2006. Flowers and immature seedpods were removed from plants to reduce genetic contamination of local populations. This procedure prevented the collection of data on the number of seeds produced and thus, it was not possible to calculate full lifetime fitness.

ANALYSIS OF LOCAL ADAPTATION AND HYBRID PERFORMANCE

To test for local adaptation and to analyze the performance of hybrids relative to native parental plants, data were analyzed with the program ASTER (Geyer et al. 2007). ASTER is a maximum-likelihood method for analysis of multiple fitness components. This method represents an improvement over previous methods (e.g., ANOVA) for the analysis of reciprocal transplant experiments because it accounts for dependencies among different components of fitness and properly incorporates variables that have different probability distributions (Geyer et al. 2007).

For all analyses, ASTER was used to combine two dependent components of fitness, (1) survival to flowering and (2) number of flowers produced by plants that survived to flower. We fit multiple nested models to each set of data and compared these models using likelihood-ratio tests. The models listed here are the ones that best fit the data. A two-way analysis with ASTER was used to test for local adaptation of coast and inland populations. The model included genotype (coast or inland), family, and field site as independent variables. In this case, local adaptation is defined as a significant genotype $\times$ site interaction.

A separate analysis was conducted to assess extrinsic postzygotic isolation because this analysis involves the direct comparison of hybrids to local parentals. For the model that best fits this data, genotype (local parental or hybrid) was the independent variable and performance (survival to flowering combined with number of flowers produced) was the dependent variable.

SALT SPRAY TOLERANCE

Oceanic salt spray is known to cause leaf necrosis (Boyce 1954). To determine if coast, inland, and F1 hybrid plants differed in salt spray tolerance in the field, we assessed percentage leaf damage during each visit to coastal field sites. Percentage leaf damage from April 26, 2006 was used for analysis of salt tolerance at coast site 1 because enough plants were still alive to make this comparison. Data were analyzed with a one-way ANOVA, where type of plant was the independent variable and percent salt damage was the dependent variable. Tukey-Kramer HSD tests were used for post hoc comparisons of means (JMP 6.0).

Greenhouse experiments were used to more directly determine salt spray tolerance of coast and inland populations. Plants from three latitudinal pairs of inland/coast populations were used in this experiment (LMC/SWB-California, RGR/OPB-Southern Oregon, SAM/OSW-Northern Oregon, 25 individuals per population). In addition, salt spray tolerance was assessed for 25 F1 hybrids of a cross between the LMC and SWB populations, which are the same populations used in reciprocal transplant experiment 1. Plants were grown under greenhouse conditions as described in the common garden experiment (above). All plants were sprayed with $\sim$15 mL of 500 mM NaCl solution (approximately same concentration as ocean water) every other day starting four weeks after germination. Salt spray application leads to accumulation of salt directly on leaves as well as a build up in the soil, and in this way mimics conditions in the field at coastal sites. Percentage leaf damage and date of death (complete leaf necrosis) were recorded every other day. To compare the rate of accumulation of leaf damage between coast/inland habitats, among populations within habitats, and among individuals within populations, data were analyzed with a nested repeated-measures MANOVA. Here, damage at each time point was treated as a separate dependent variable. Time till death was compared with a nested ANOVA, where population was nested within coast or inland type (JMP 6.0). Tukey-Kramer HSD tests were used for post hoc comparisons of mean time till death of LMC (inland) and SWB (coast) plants, as well as F1 hybrids between these two populations.

INTRINSIC POSTZYGOTIC REPRODUCTIVE ISOLATION

To assess the level of intrinsic postzygotic isolation between coast and inland populations, crosses were made among four pairs of coast and inland populations from central California to northern Oregon (Fig. 1). Four to ten plants from each population were used in these crosses. Five different crosses were made for each

coast/inland population pair, for a total of 20 crosses. All F1 hybrids were then intercrossed to produce F2 hybrids. To screen for hybrid lethality/inviability, 86 F1 hybrids representing all 20 crosses, as well as 613 F2s hybrids, were grown under common garden greenhouse conditions (above). Hybrid lethality and inviability were scored for all crosses. For purposes of this study, hybrid lethality is defined as death of plants at or before the four-leaf stage. Hybrid inviability is defined as dwarfing, necrosis, and the inability to survive to flower under standard greenhouse conditions (as described above).

STRENGTH OF REPRODUCTIVE ISOLATING BARRIERS

The strength of reproductive isolating barriers between coastal and inland population was calculated from the reciprocal transplant experiments using methods modified from previous studies (Ramsey et al. 2003; Kay 2006; Martin and Willis 2007; Lowry et al. 2008). Potential for gene flow into inland habitat was calculated separately from potential gene flow into coastal habitat for reproductive isolating barriers that act asymmetrically. Data from both inland field sites were combined for these analyses, but not for coastal field sites (see Results).

To assess the potential for hybridization via pollen dispersal between habitats, we determined the approximate flowering phenologies of experimental plants growing in their native habitat by conducting censuses every 2–3 weeks and recording the total number of flowers produced since the previous census. To calculate a crude, conservative measure of reproductive isolation due to less than complete flowering time overlap, we assumed that pollinators disperse pollen at random among flowers within and between the two habitats despite allopatry, that the abundances of native flowers at the two habitats are equal during any flowering overlap period, and that all flowers produce equal seeds and pollen grains. With these admittedly unrealistic assumptions, the frequency of hybrids expected with complete flowering overlap is 0.5 at each site. Let the proportion of coastal flowers that were open during the overlap period be F_C and that of inland flowers be F_I. Given the above assumptions, the expected frequency of hybrids produced as seeds at the coastal site, q_C, is equal to $F_C/2$, and that for seeds produced at the inland site is $q_I = F_I/2$. Following the reasoning outlined in Martin and Willis (2007), the reproductive isolation at the coastal site due solely to flowering phenology differences between plants in allopatric habitats is $RI_{FA,C} = 1 - [q_C/(1 - q_C)]$, with an analogous formula for isolation at the inland site.

Gene flow can also occur between species through seed dispersal. However, seeds that disperse between habitats must germinate, survive, and flower in the nonnative habitat in order for hybridization to potentially occur. Thus, habitat-mediated selection against immigrants, which may prevent survival and limit reproduction, can act as an impediment to gene flow (Nosil et al. 2005). We calculated the strength of reproductive isolation due to selection against immigrants as $RI_I = 1 - \bar{w}_i/\bar{w}_n$, where $\bar{w}_i$ is the mean number of flowers produced by immigrant individuals, and $\bar{w}_n$ is the mean number of flowers produced by individual plants native to the habitat of the field site.

For immigrants that survive to flower, hybridization cannot occur if immigrants flower at different times than native plants. To determine if flowering time differences restricts hybridization between immigrants and native plants in artificial sympatry, we calculated the probability that an individual immigrant flowered at the same time as any members of the native population and estimated this form of temporal reproductive isolation between immigrants and natives in sympatry, say at the coastal site, as $RI_{FS,C} = 1 - F_{M,I}$ where $F_{M,I}$ is the probability that a migrant from the inland population flowers whereas any native plants are flowering at the coastal site.

Postzygotic isolation can occur through intrinsic genetic incompatibilities. To determine the strength of intrinsic postzygotic isolation, the mean seedling to adulthood viability of F1 hybrids, $\bar{v}_{F1}$, was compared to the mean viability of parental populations. We did not include data from the F2 generation in our calculation as it is unclear what the relative frequency of F2 versus backcross hybrids would be in a natural situation. Because we are interested here in intrinsic barriers, we focused on viability in the relatively benign environment of our greenhouse experiments. Because all parental individuals survived from seedling to adulthood in these experiments, we calculated intrinsic postzygotic reproductive isolation as $RI_{IP} = 1 - \bar{v}_{F1}/\bar{v}_P$.

Postzygotic isolation could also occur, even where there is no intrinsic postzygotic isolation, if the hybrids are less fit than native parental plants due to extrinsic ecologically based inviability or sterility (Hatfield and Schluter 1999). The strength of extrinsic postzygotic reproductive isolation was calculated as $RI_{EP} = 1 - \bar{w}_h/\bar{w}_n$, where $\bar{w}_h$ is the mean lifetime number of flowers produced by F1 hybrids in the field, and $\bar{w}_n$ is the mean lifetime number of flowers produced by native parental plants.

Results

MORPHOLOGICAL POPULATION STRUCTURE

Striking morphological differences were found between coast and inland populations in the common garden greenhouse experiment. Joint analysis of 13 morphological traits and flowering time showed substantial quantitative genetic divergence between coast and inland habitats (MANOVA, $F_{13,83} = 61.20$, $P < 0.0001$). In general, the coastal populations flowered later, had thicker stems, shorter internodes, and larger flowers than inland populations (Fig. 2). Plotting of PC1 and PC2 showed clear differences between coast and inland individuals (Fig. 2C; see online Supplementary Table S2 for trait loadings on PC1 and PC2). Most of

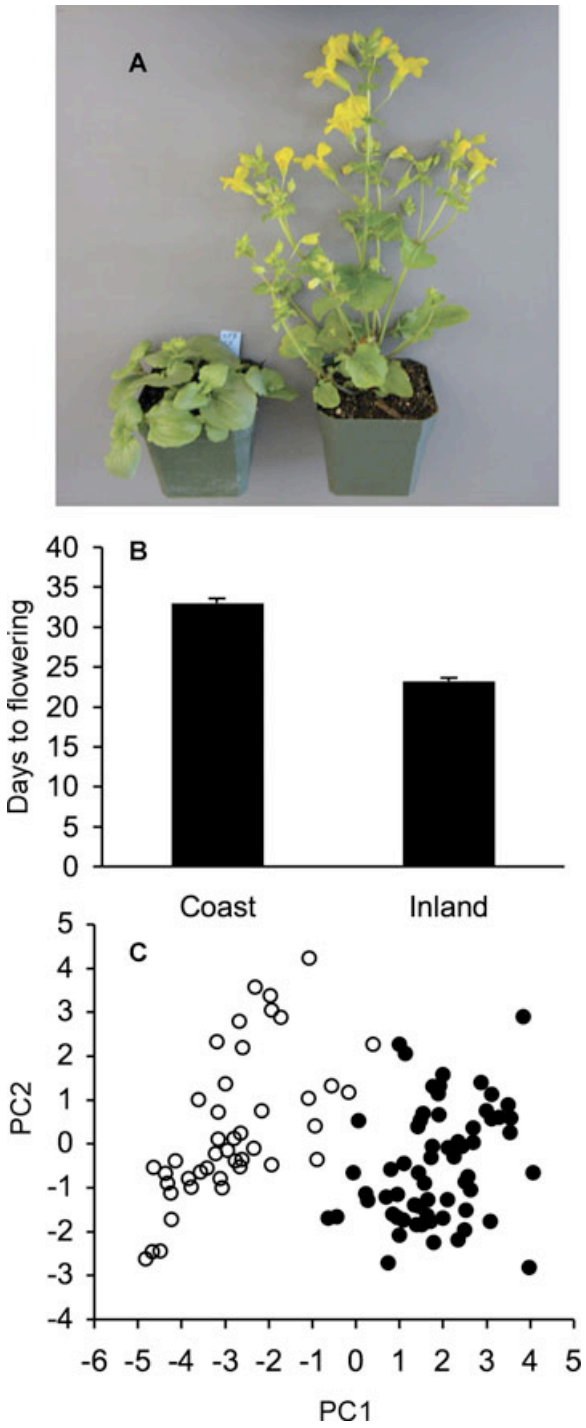

Figure 2. Morphological divergence of coast and inland populations. (A) Morphological differences between coast (left) and inland (right) populations grown in a common garden greenhouse environment. (B) Flowering time was significantly different between coast and inland races ($F = 49.55$, $P < 0.0001$). See Table 2 for analysis of all traits. Error bars denote one standard error. (C) Principle components analysis (PC1 plotted against PC2) of individuals from coast (closed circle) and inland populations (open circle) using morphological data (14 traits, $N = 12$ populations, 107 individuals). Data for tetraploid SLO population removed from this analysis.

the variation in flowering time (71.9%) and PC1 (88.6%), but not PC2 (6.1%), was partitioned between groups (coast vs. inland) in the REML-nested ANOVA (see Table 2 for other traits). It should be noted that we excluded the tetraploid (see below) inland SLO population for all morphological analysis.

MOLECULAR GENETIC POPULATION STRUCTURE

All 10 codominant markers were highly polymorphic and successfully amplified alleles in all 30 populations. No linkage disequilibrium was detected among any of the 10 loci using FSTAT 2.9.3.2 (Goudet 2001). Two populations (SLO, CAN) had aberrant molecular signatures and were removed from the subsequent analyses. All individuals ($N = 16$) of the CAN population were completely homozygous for one allele at each of the 10 loci. Plants of the SLO population had more than two alleles at multiple loci. Follow up analysis with flow cytometry revealed that plants of the SLO population are tetraploid (J. Modliszewski, pers. comm.).

Genetic divergence was high in all pair-wise population comparisons (mean pairwise $F_{ST} = 0.48$, online Supplementary Table S3). Genetic variation was greater within inland populations than within coastal populations of *M. guttatus*. For all 10 loci, observed heterozygosity (H_O) and expected heterozygosity (H_S) were significantly greater for the inland populations than the coastal populations, whereas genetic divergence (F_{ST}) was significantly greater for coastal populations than inland populations (FSTAT: $N = 28$ populations, 1000 permutations, $P < 0.0001$ for all tests, online Supplementary Fig. S2A; see online Supplementary Table S4 for locus-specific summary statistics). In contrast, the level of inbreeding (F_{IS}) did not differ ($P = 0.486$) between coast and inland populations (online Supplementary Fig. S2A). The inland populations collectively harbored far more habitat-restricted private alleles than coastal populations (online Supplementary Fig. S2B and Table S4). Across all loci, 64.13% of the alleles found in inland populations were restricted to the inland habitat, whereas only 17.57% of the alleles found in coastal populations were restricted to the coast habitat (online Supplementary Fig. S2B, $N = 28$ populations, 2 habitats, 10 loci, $F = 39.34$, $P < 0.0001$).

We found strong evidence for genetic divergence between coast and inland populations with all three analyses employed. Analysis of molecular variation (AMOVA) detected structure (1023 permutations, $F_{CT} = 0.0845$, $P < 0.001$) between the coast and inland habitats. Even so, only 8% of the genetic variation was partitioned between habitats, whereas 39% of the variation was among populations within habitats, and the remaining 52% was among individuals within habitats (Table 3).

There was a striking dearth of admixture among populations in our analyses with STRUCTURE, such that all individuals within a given population were assigned to the same cluster

Table 2. Results of REML mixed-model nested ANOVA of first two principle components, flowering time, and 13 measured morphological traits for 132 individual plants from coast (*N*=7) and inland (*N*=5) populations. Plants were grown in a common garden greenhouse environment so observed differences should be genetically based. Estimates for the percent of variation (%var) distributed between habitats, among populations within habitats, and among individuals within populations (error) estimated by treating all hierarchical levels as random effects. **P*<0.05, ***P*<0.01, ****P*<0.001.

Trait	Coast Mean (SE)	Inland Mean (SE)	Source of variation: Habitats *F*-ratio	Habitats %var	Populations χ^2	Populations Var comp	Populations %var	Individuals (Error) Var comp	Individuals (Error) %var
PC1	1.86 (0.13)	−2.87 (0.20)	181.24***	88.6	4.7*	0.22	1.7	1.23	9.7
PC2	−0.14 (0.16)	0.36 (0.25)	1.58	6.1	63.6***	1.42	57.6	0.89	36.3
Flowering time (days)	33.00 (0.57)	23.25 (0.40)	49.55***	71.9	15.0***	4.31	6.5	14.27	21.6
Stem thickness 1 (mm)[1]	6.23 (0.13)	2.99 (0.17)	63.11***	78.2	18.5***	0.40	5.8	1.08	15.9
Internode length (mm)	23.28 (2.10)	59.49 (4.21)	9.88*	48.4	70.8***	355.38	29.7	262.34	21.9
Leaf length 1 (mm)[3]	57.12 (1.60)	39.00 (1.86)	14.32**	45.1	20.3***	56.27	15.9	138.07	39.0
Leaf width 1 (mm)[3]	33.01 (0.98)	27.50 (1.36)	3.24	11.6	18.5***	22.74	23.7	61.86	64.7
Leaf thickness (mm)	0.439 (0.01)	0.401 (0.02)	0.55	0.0	53.5***	0.01	49.9	0.01	50.1
Corolla length (mm)	37.17 (0.59)	25.80 (0.69)	80.27***	71.1	3.3*	2.45	2.7	23.91	26.2
Corolla width (mm)	31.38 (0.51)	20.55 (0.62)	67.46***	73.3	7.7**	3.31	4.2	17.5	22.4
Number of flowers	27.37 (1.60)	33.40 (2.96)	0.75	0.0	24.5***	98.53	31.8	27.33	68.2
Stem thickness 2 (mm)[2]	6.25 (0.14)	1.99 (0.11)	137.53***	88.0	16.1***	0.299	2.9	0.946	9.1
Leaf length 2 (mm)[4]	71.47 (2.14)	25.43 (1.83)	85.30***	79.4	8.8**	50.52	3.8	225.09	16.8
Leaf width 2 (mm)[4]	45.88 (0.94)	24.06 (1.14)	127.08***	78.5	2.1	4.90	1.6	59.91	19.9
Rosette diameter (mm)	100.3 (6.62)	29.11 (2.88)	14.31**	50.1	36.5***	913.01	19.0	1488.2	31.0
Height (mm)	326.40 (14.0)	279.21 (15.8)	0.83	0.0	67.3***	8334.27	55.9	6451.20	44.1

[1]Thickness of the first internode.
[2]Thickness of the second internode.
[3]First true leaf.
[4]Second true leaf.

regardless of *K*-value. Most populations were correctly assigned (at $K = 2$) to the habitat from which they were collected (Fig. 3A). However, three populations (ORO, BSR, and ANR) were misassigned on different runs. BSR was consistently misassigned on all runs, whereas ORO was misassigned in 68% of the runs and ANR in 10% of the runs (50 total runs). Interestingly, ANR and BSR were both collected from along the Eel River in Northern California (see Discussion). At greater *K* values ($K = 3$–35), the partitioning of populations by STRUCTURE was much more complicated and population assignments were much less consistent among runs (online Supplementary Fig. S3). Out of all of the models tested with STRUCTURE, the model with $K = 29$ had the highest likelihood. In addition, we observed little evidence of isolation by distance, except for densely sampled populations in central California (analysis not shown, but see online Supplementary Fig. S3).

Our analysis with POPULATION GRAPH found that the coast and inland populations cluster as two distinct groups. There

Table 3. Results of analysis of molecular variation (AMOVA) preformed in Arlequin 3.11. The nested model included habitats (coast/inland), populations within habitats, and individuals within populations (**P*<0.001).**

Source of variation	df	Sum of squares	Variance components	% of variation
Among coast and inland habitats	1	195.29	0.312	8.45***
Among populations within habitats	26	1299.71	1.456	39.48***
Within populations	898	1724.93	1.921	52.08***
Total	925	3219.93	3.689	

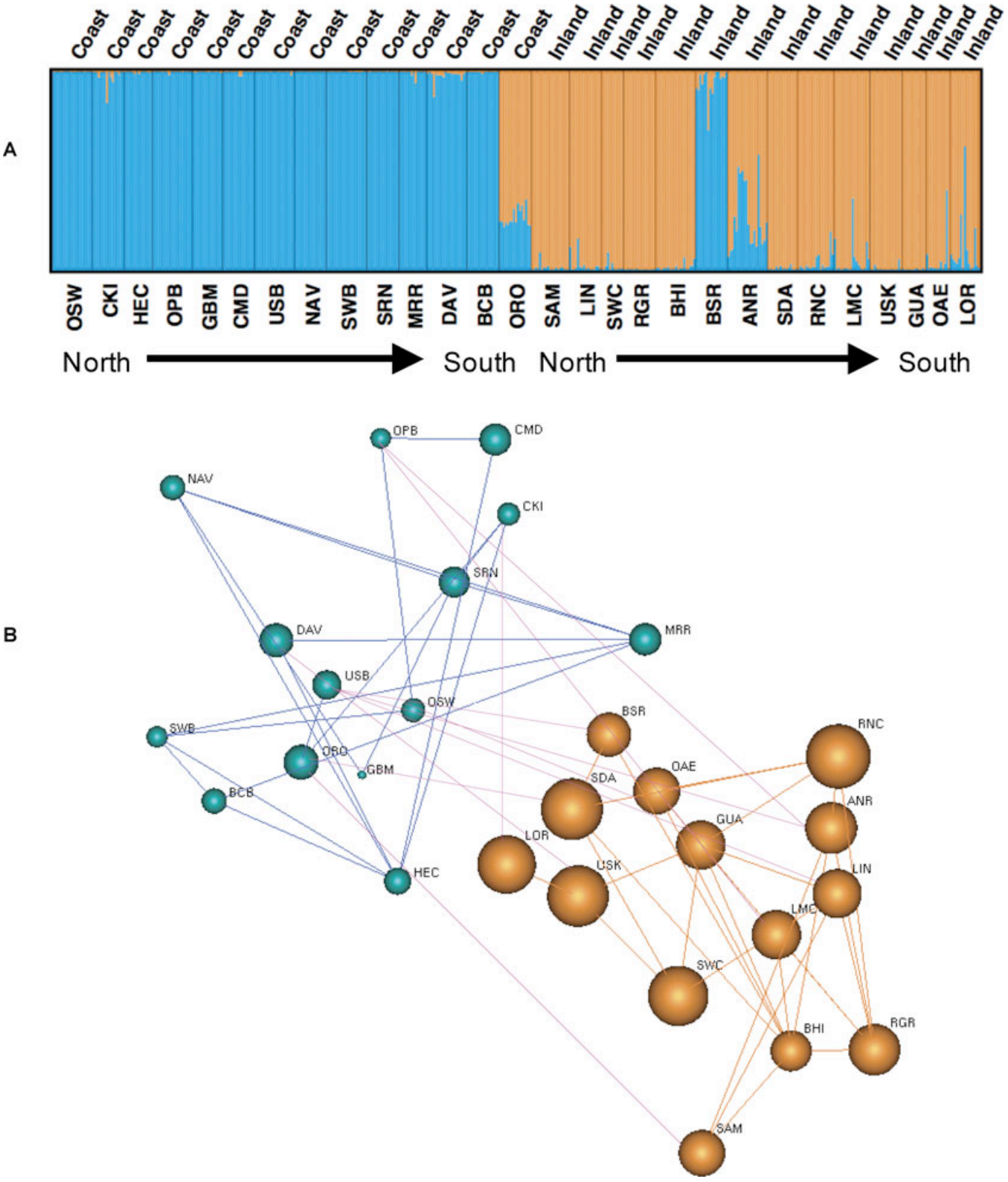

Figure 3. Genetic population structure of coast and inland populations. (A) Analysis with the program STRUCTURE (Pritchard et al. 2000) run 50 times at $K = 2$. Results of multiple runs combined with CLUMMP (Jakobsson and Rosenberg 2007) and visualized with DISTRUCT (Rosenberg 2004). (B) Analysis with the program POPULATION GRAPH (Dyer and Nason 2004). There were significantly more edges within coast (blue lines) and inland (orange lines) groups than between (pink lines) groups ($P < 0.0001$). The diameter of each sphere is equal to the expected heterozygosity (H_S) of the population, which was calculated in FSTAT (Goudet 2001). For both analyses: $N = 14$ coast populations, 14 inland populations, 479 individuals, 10 codominant loci.

were 29 edges among inland populations, 21 edges among coast populations, but only 10 edges between coast and inland populations (Fig. 3B). This represents highly significant genetic differentiation between coast and inland groups ($N = 28$ populations, 10 loci, $P < 0.0001$).

Two marker loci (AAT217 and MgSTS278) are located on linkage group 8 near two previously identified quantitative trait loci (QTLs), which are partly responsible for the phenotypic divergence between a pair of coastal and inland populations (Hall et al. 2006). Because selection at those QTLs may result in divergence of linked markers, we reran our analyses of population structure without those loci. Similar levels of clustering were observed using the eight remaining loci for both STRUCTURE (data not shown) and POPULATION GRAPH (23 edges among inland pops, 23 edges among coast pops, and 13 edges between coast and inland pops, $P < 0.0001$).

RECIPROCAL TRANSPLANT EXPERIMENTS

Early season survival was high at three of the four field sites whereas all experimental plants at coast site 2 died early in the season (online Supplementary Fig. S1, Table 4). As a result, we restricted our analyses to the three remaining field sites. Landslides destroyed three of the ten blocks at coast site 1. Therefore, the sample size at coast site 1 is lower than at the two inland sites. At the three field sites with survivors, local plants consistently outperformed immigrant plants (Fig. 4, Table 4). In addition, there was very little overlap in flowering time between coast and inland plants (Fig. 4A).

ANALYSIS OF LOCAL ADAPTATION AND HYBRID PERFORMANCE

Selection was very strong against immigrants between habitats in both directions of migration. At the two inland sites, only one coastal plant of 183 (0.5%) survived to flower and this plant only produced one flower. In contrast, 41% of inland plants at the two inland field sites survived to flower, and survivors produced an average of over three flowers. At coast site 1, over twice as many coast plants survived to flower than inland plants. In addition, coast plants that survived to flower at coast site 1 produced ~3.5 times as many flowers as inland plants at coast site 1 (Table 4). Overall, the population × site interaction (Fig. 4B,C) in the reciprocal transplant between coast site 1 and inland site 1 was highly significant (ASTER, df = 11, $z = -4.170$, $P < 0.0001$). Because all plants died prematurely at coast site 2, analysis of a site × genotype interaction was not possible for experiment 2. Therefore, we restricted our analysis to a one-way comparison of the performance of coast and inland plants within inland site 2. Due to the fact that none of the coast (BCB) plants survived to flower at inland site 2, performance of inland plants (CAN) was significantly greater than the performance of coast plants at this site (Fig. 4D,E; ASTER, df = 3, $z = 2.459$, $P = 0.0139$).

At the inland site 1, the native inland (LMC) plants survived to flower at ~1.25 times the rate of F1 hybrids, and these inland plants produced ~1.25 times as many flowers as the F1 hybrids (Table 4, ASTER, df = 3, $z = 2.568$, $P = 0.0102$). At inland site 2, there was no difference in fitness between hybrids and local inland (CAN) plants (ASTER, df = 3, $z = -0.10$, $P = 0.92$). At coast site 1, hybrids greatly outperformed native coast (SWB) plants (ASTER, df = 3, $z = 5.259$, $P < 0.0001$) as ~2.5 times more hybrids survived to flower and these survivors produced ~1.5 times more flowers (Table 4).

SALT SPRAY TOLERANCE

In the field, leaf damage (presumably due to salt spray) significantly differed among coast, inland, and F1 hybrids at coast site

Table 4. Summary of results from reciprocal transplant field experiments. The percentage of plants surviving (Early Survival) and rosette diameter (mm) in late April are listed for inland, coast, and F1 hybrid plants at the three field sites. Survival to flowering is the amount of plants that were planted in the field as seedlings that survived to flower. Number of flowers is the number of flowers produced by plants that survived to flower. Means ± standard errors are list with sample sizes in parentheses.

Site	Type	Early season traits		Fitness components	
		Early survival	Rosette diameter	Survival to flowering	Number of flowers
Mendocino	Inland (LMC)	68% (100)	36.00±2.06 (68)	57% (100)	3.53±0.90 (57)
Inland site 1	Coast (SWB)	62% (99)	14.48±2.17 (61)	1% (99)	1.00 (1)
	F1 Hybrid	85% (98)	41.36±1.86 (83)	45% (98)	2.755±0.90 (44)
Mendocino	Inland (LMC)	49% (69)	31.56±4.54 (34)	17% (69)	2.83±5.85 (12)
Coast site 1	Coast (SWB)	90% (69)	42.77±3.36 (62)	38% (68)	10.23±3.98 (26)
	F1 Hybrid	87% (68)	45.44±3.44 (59)	72% (68)	15.20±2.90 (49)
Big Creek	Inland (CAN)	52% (85)	18.07±2.21 (40)	22% (85)	3.37±2.08 (19)
Inland site 2	Coast (BCB)	51% (84)	15.36±2.24 (39)	0% (84)	0.00 (0)
	F1 Hybrid	49% (79)	26.24±2.27 (38)	13% (79)	7.00±1.51 (10)

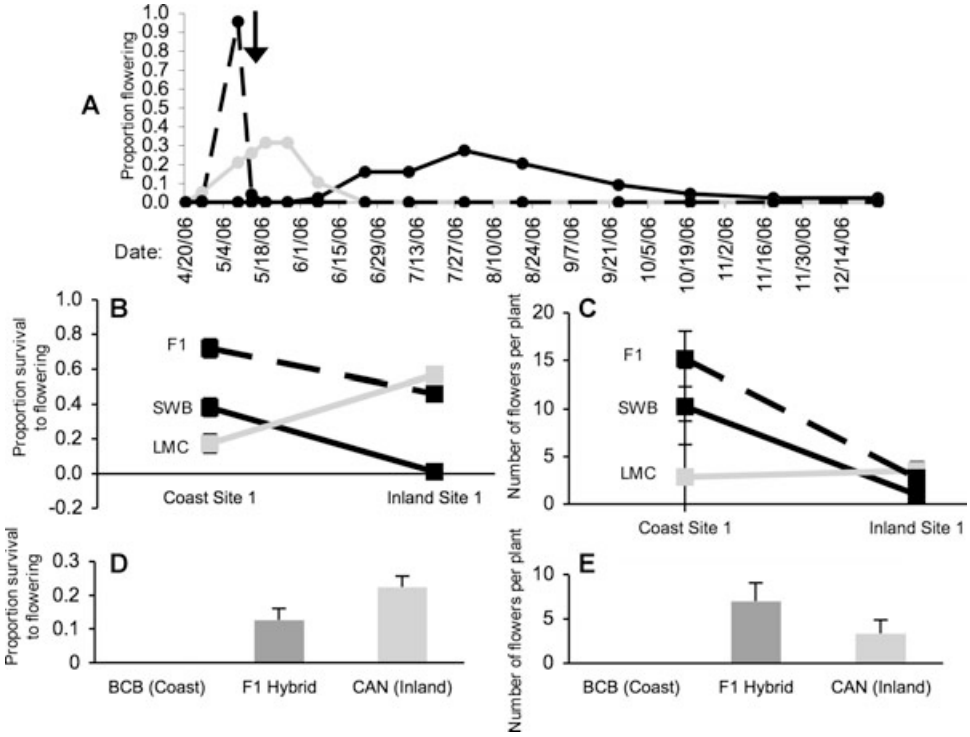

Figure 4. **Results of reciprocal transplant experiment in Northern California. (A) Flowering time differences between coast and inland populations was assessed through a reciprocal transplant experiment between coast site 1 and inland site 1. There was no overlap in flowering time between inland (LMC) plants at inland site 1 (dashed line) and coast (SWB) plants at coast site 1 (black line). When placed into artificial sympatry, there was a slight overlap between inland immigrants (LMC) at coast site 1 (gray line) with coast plants at this site (black line). Only one coast (SWB) immigrant survived to flower at inland site 1 and it overlapped in flowering with 4% of the inland plants at this site (dashed line). (B) Survival to flowering of coast (black), inland (gray), F1 hybrids (dashed) between sites in experiment 1. (C) Number of flowers produced by plants that survived to flower between sites in experiment 1. (D) Survival to flowering of coast (BCB, black), inland (CAN, light gray), and F1 hybrids (dark gray) at inland site 2. (E) Number of flowers produced by plants that survived to flower at inland site 2. All error bars denote one standard error.**

1 in early spring (April 26, 2006, $F_{2,148} = 23.08$, $P < 0.0001$, Fig. 5A). Leaf damage was an order of magnitude greater for inland (LMC) plants than coast (SWB) plants. Both coast and F1 hybrids had significantly less leaf damage than inland (LMC) plants in the post hoc analysis ($P < 0.05$, Fig. 5A). Further, 36% (13 out of 36) of the inland (LMC) plants alive in late April were subsequently killed by leaf damage. Leaves and flowers were severely wilted on all of the inland that survived to flower ($N = 12$) at coast site 1.

The greenhouse salt tolerance experiment confirmed that coastal populations are more tolerant to salt spray than inland populations. Inland plants accumulated salt spray damage at nearly three times the rate of coast plants ($F_{1,148} = 244.69$, $P < 0.0001$, Fig. 5C). Further, coastal plants survived salt spray treatment about twice as long as inland plants ($F_{1,149} = 155.65$, $P < 0.0001$, Fig. 5D). There was also significant variation in leaf damage ($F_{4,148} = 19.71$, $P < 0.0001$) and time to mortality ($F_{4,149} = 12.46$, $P < 0.0001$) among populations within coast and inland habitats. F1 hybrids between coast (SWB) and inland (LMC) populations were significantly more tolerant than LMC, but just as salt tolerant as SWB in a Tukey-Kramer post hoc analysis ($P < 0.05$, Fig. 5C,D).

INTRINSIC POSTZYGOTIC REPRODUCTIVE ISOLATION

Seeds germinated in all 20 of the interpopulation crosses, and 84 of 86 of the F1 progeny were fully viable. Only one cross of 20 resulted in inviable F1 hybrids, and none of the crosses led to hybrid lethality. The cross leading to inviability was conducted between the OSW (coast) and the SAM (inland) populations. The inviability appears to be a form of hybrid necrosis (Bomblies and Weigel 2007) as plants were dwarfed with most of the leaves turning brown. Overall, the affected F1 family contained two inviable hybrids and three fully viable hybrids. A cross between viable members of this F1 family and another independent F1 family also resulted in inviable plants (3 out of 20) in the F2 generation. The inviability was more severe in the F2 generation

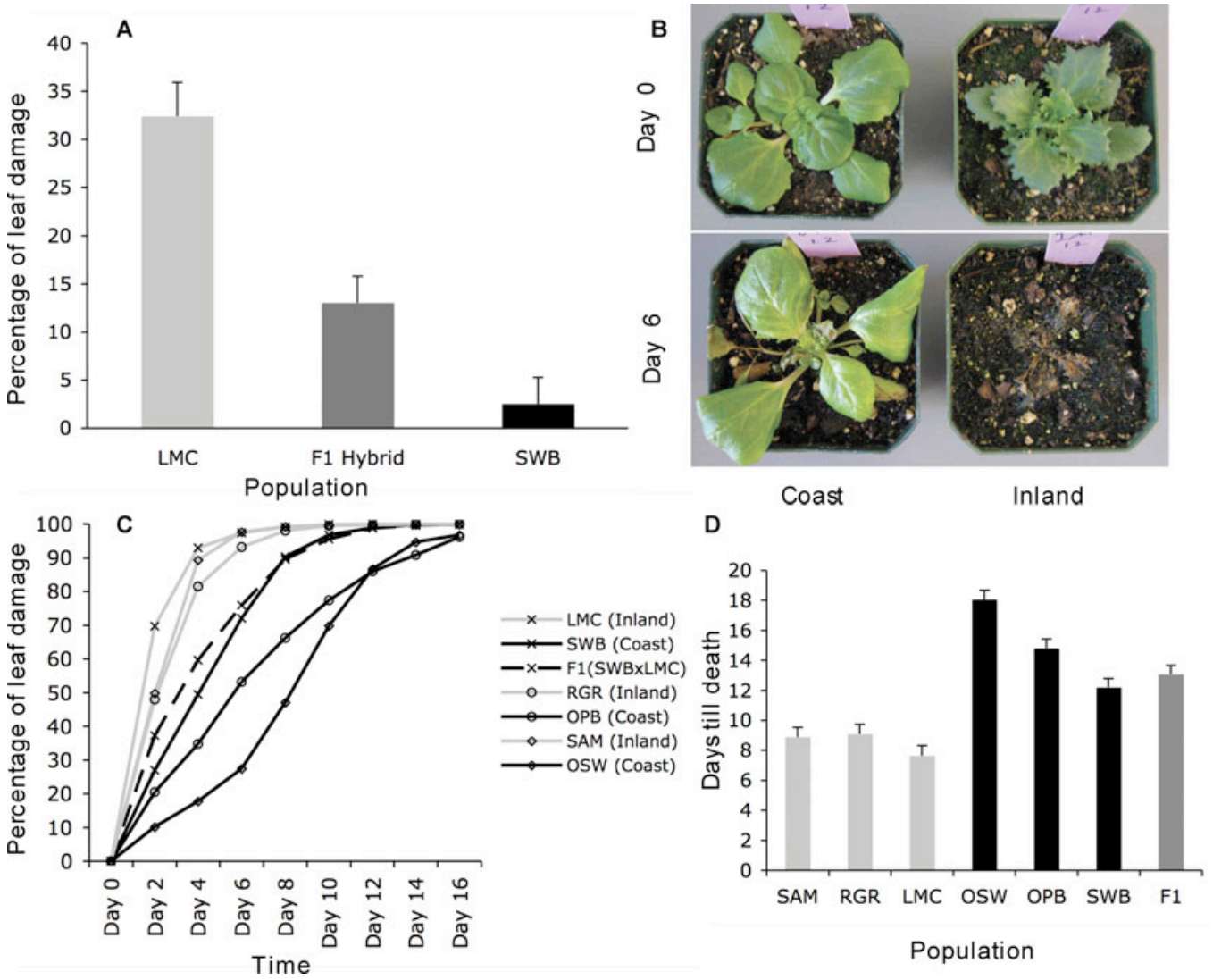

Figure 5. Salt spray tolerance differed among coast (black), inland (light gray), and F1 hybrids (dark gray, cross between LMC and SWB). (A) In the field, leaf damage was significantly greater for inland plants than coastal plants and F1 hybrids at coast site 1 ($F_{2,148} = 23.08$, $P < 0.0001$, Tukey-Kramer comparison of means with $\alpha = 0.05$). In the greenhouse: (B) Inland plants (right) had lower tolerance to 500 mM NaCl solution than coast plants (left). (C) Accumulation of leaf damage, from application of 500 mM NaCl solution, was significantly different between coast and inland populations ($F_{1,148} = 244.69$, $P < 0.0001$). (D) Days until mortality was also significantly different between coast and inland populations as well ($F_{1,149} = 155.65$, $P < 0.0001$). All error bars denote one standard error.

than the F1 generation, and F2 plants were severely dwarfed. We did not observe any additional inviable hybrids in the F2 generation of other population crosses.

THE STRENGTH OF REPRODUCTIVE ISOLATING BARRIERS

The level of reproductive isolation between coast and inland populations was near complete, with prezygotic barriers much stronger than postzygotic barriers in their contribution to overall reproductive isolation. For each barrier listed in Table 5, reproductive isolation ranged from ≤ 0 (unrestricted gene flow) to 1 (complete reproductive isolation). Because there was no overlap in flowering time between coast plants in coastal habitat and inland plants in inland habitat, there is complete ($RI_{F,A} = 1.000$) temporal flowering isolation between habitats. However, it should be kept in mind that this barrier only applies to gene flow through pollen movement, not seed dispersal. Selection against immigrants was near complete ($RI_{I,I} = 0.999$) for coastal immigrants moving into inland habitat, and was also strong for inland immigrants moving into coastal habitat ($RI_{I,C} = 0.874$). The one immigrant coast (SWB) plant that survived to flower in inland habitat flowered during the last 4% of the flowering period of the native inland plants. However, because this immigrant's only flower was opened while native plants were still flowering we calculated that there was no

Table 5. The strength of reproductive isolating barriers between coast and inland populations. Data calculated from results of reciprocal transplant studies and controlled crossing experiments. Barriers range from negative values (unrestricted gene flow) to 1 (complete reproductive isolation). The first column is the strength of the barrier reducing gene flow into coastal populations, whereas the second column is the restriction on gene flow into inland populations.

Isolating barrier	Strength of barrier	
	Coast	Inland
Temporal flowering isolation among habitats (RI_{FA})	1.000	1.000
Selection against immigrants (RI_I)	0.874	0.999
Temporal flowering isolation in sympatry (RI_{FS})	0.895	0.00[1]
Intrinsic postzygotic isolation (RI_{IP})	0.023	0.023
Extrinsic postzygotic isolation (RI_{EP})	−1.801	0.233

[1]This was calculated for one surviving coast plant in inland habitat.

reproductive isolation ($RI_{FS,I} = 0.000$). At coast site 1, 12 inland plants survived to flower, but only 10.5% of the total immigrant (LMC) flowers were open at the same time as native coast (SWB) plants ($RI_{FS,C} = 0.895$). Extrinsic postzygotic isolation or ecological selection against hybrids provided a small barrier to gene flow into inland habitat ($RI_{EP,I} = 0.233$), but was nonexistent in coastal habitat ($RI_{EP,C} = -1.801$), where hybrids outperformed local plants. The strength of intrinsic postzygotic isolation between coast and inland populations is very low ($RI_{IP} = 0.023$).

Discussion

Our results indicate that coastal perennial and inland annual populations of *M. guttatus* comprise two distinct morphologically and molecular genetically diverged groups. Nearly complete prezygotic isolation through a combination of geography, selection against immigrants, and flowering time isolation likely maintains the genetic differentiation of these coast and inland groups. In the inland habitat, the onset of the summer drought prevents the successful immigration of late flowering coastal perennial plants, whereas early flowering annual life-history (Hall and Willis 2006) and low salt tolerance of inland plants inhibit their immigration into coastal habitat. Overall, these results are consistent with the role of habitat-dependent natural selection in the formation of widespread reproductively isolated species.

PATTERNS OF MORPHOLOGICAL AND MOLECULAR GENETIC DIFFERENTIATION

Our analysis of morphological data clearly demonstrates that *M. guttatus* populations derived from coast and inland habitats are genetically distinct (Fig. 2; Table 2). This pattern is consistent with the findings of other botanists, who have long recognized a common suite of morphological differences between coast and inland plant populations of a variety of species (Turresson 1922; Stebbins 1950; Clausen 1951; Clausen and Heisey 1958; Grant 1981). Clausen (1951) argued that the consistent distinctness of coastal populations in species such as *Layia platyglossa*, *Potentilla glandulosa*, and *Achillea borealis* suggest that morphologically distinct coastal populations should be collectively classified as ecological races. Ecological races of plants are somewhat analogous to host races in insects (Berlocher and Feder 2002; Dres and Mallet 2002; Funk et al. 2002; Nosil 2007), and are defined as a large set of populations that are restricted to a particular type of habitat by abiotic and/or biotic factors (Clausen 1951; Clausen and Heisey 1958).

Our analyses of the highly variable molecular genetic markers, using STRUCTURE and POPULATION GRAPH, are also consistent with the hypothesis that coast and inland populations of *M. guttatus* constitute distinct ecological races. Our analysis implies that geographically distant coastal populations (>1000 km apart from each other) are more closely related to each other than they are to adjacent inland populations, which are often only a few kilometers away. Despite the clear overall divergence between coast and inland races, only a relatively small proportion of the total molecular variation is partitioned between races in the AMOVA. Interestingly, far more of the variation in morphology (PC1), flowering time, and nine other traits (Table 2) is partitioned between coast and inland groups than genetic variation (Table 3). A high level of quantitative trait divergence coupled with modest levels of genetic divergence is consistent with habitat-mediated selection driving morphological evolution (Spitze 1993; McKay and Latta 2002). It should be noted that our common garden experiment was performed after only one generation in a common environment and thus, does not properly control for maternal effects. However, individual lines grown for multiple generations under common growth conditions maintain morphological distinctness between coast and inland habitats (data not shown).

Average pairwise F_{ST} for inland populations is high in both this study (0.32) and a previous study (0.32, Awadalla and Ritland 1997). Even greater F_{ST} was observed among coastal populations (0.55). Because F_{ST} depends inversely on within population diversity (Nei 1973, 1987; Charlesworth et al. 1997), the elevated among coast population F_{ST} is likely the result of consistently low within coast population heterozygosity. It should be noted that rare long-distance migration likely occurs among *M. guttatus* populations through dispersal by water (over 4.5 km in a single season, Levine 2001), deer (over a kilometer in a season, Vickery 1986), and birds (Lindsay 1964). Even so, restricted migration among all population of *M. guttatus* may increase the partitioning of molecular genetic variation among populations and individuals

while diminishing the between group (coast vs. inland) partition of genetic variation in the AMOVA analysis.

Although most populations were correctly assigned to coastal or inland habitats using STRUCTURE, three of the populations (ANR, BSR, and ORO) did not cluster with other populations from their respective habitats. One possible explanation is that these populations are derived from the admixture of coast and inland populations. The two misassigned inland populations (ANR and BSR) are located along the Eel River in Northern California. ANR appears to be admixed based on STRUCTURE results and BSR may have been colonized by coastal plants from the nearby tidal estuary region of the Eel River. Further, BSR contained two different sized morphotypes, which may indicate that it is a mixed coast and inland population. Interestingly, the morphology of the ANR population is much like coastal plants in that these plants have many lateral branches and adventitious roots. More detailed sampling along the Eel River will be necessary to determine the evolutionary history of populations in this region. The misassignment of the ORO (coast) population may simply be the result of being located at the very southern end of our sampling area.

HABITAT ADAPTATION AND REPRODUCTIVE ISOLATION BETWEEN COAST AND INLAND ECOLOGICAL RACES

The results of this study and a previous reciprocal transplant experiment (Hall and Willis 2006) clearly demonstrate that total reproductive isolation between coast and inland populations of *M. guttatus* is nearly complete as a result of local adaptation. The allopatric distribution of coast and inland populations may be a byproduct of ecological range limits of these two races and suggests that ecogeographic isolation (as defined by Schemske 2000) may be the key barrier to gene flow. Even so, as explained above, rare dispersal of pollen or seed probably occurs between coast and inland populations because they are often in about as close proximity to each other as are different populations within habitats. Even if rare dispersal occurs, the high estimates of reproductive isolation due to immigrant inviability and flowering time differences will sharply limit the opportunity for gene flow.

Intrinsic postzygotic isolation between coast and inland populations appears to be insignificant. One caveat of our analysis of intrinsic postzygotic isolation is that we did not assess the strength of crossing barriers, hybrid sterility, or levels of transmission ratio distortion (but see Hall and Willis 2005). However, we were able to successfully generate F2 hybrids in all intercrosses among F1 progeny. Therefore, the rate of hybrid sterility is likely to have limited effect on gene flow.

Extrinsic postzygotic isolation is thought to be a common byproduct of local adaptation of different species (Schluter 2001; Rundle and Whitlock 2001; Nosil et al. 2005). However, heterosis is known to offset the effect of intrinsic incompatibilities in early generation hybrids (Rhode and Cruzan 2005), and may offset extrinsic effects as well (Rundle and Whitlock 2001; Lowry et al. 2008). Indeed, the high levels of coast/inland F1 hybrid performance in the field indicate that hybrids could actually facilitate gene flow into coastal habitats, while at most it would act as a weak barrier to gene flow into inland habitats. The elevated fitness of F1 hybrids in coastal habitat may be a product of heterosis combined with high F1 salt tolerance (Fig. 5). The effects of heterosis may be mitigated in inland habitat, where flowering time is key to fitness, because F1s flower later than inland parentals (Fig. 2B; Table 5).

Although extrinsic postzygotic isolation is insignificant for coast/inland F1 hybrids, extrinsic postzygotic isolation may act on advance generation hybrids, where the effect of heterosis will be diminished (Burke and Arnold 2001; Rundle and Whitlock 2001). Even so, the patchy distribution of *M. guttatus* populations means that a large number of hybrids would be backcrosses to local individuals. Such backcrosses would be composed of genetic segregrants that are 50% homozygous for locally adapted alleles and heterozygous at the rest of their loci. Backcrosses to locally adapted populations typically perform as well as parent species under field conditions (Burke and Arnold 2001; Johnston et al. 2001; Rundle 2002; Lexer et al. 2003a,b,c). This also appears to be the case in a previous reciprocal transplant between coast and inland *M. guttatus* populations, where backcrosses to locally adapted populations performed just as well as locally adapted parentals (Hall and Wills 2006). Therefore, it appears unlikely that extrinsic postzygotic isolation plays a major role in restricting gene flow between coast and inland populations.

Even though extrinsic postzygotic isolation may not be strong overall, particular genomic regions may not be able to introgress between habitats due to selection in advanced generation hybrids, whereas neutral loci introgress more readily (Wu 2001; Turner et al. 2005). Although it appears from our marker data that most genomic regions show at least some divergence, alternative alleles at loci involved in flowering time or salt tolerance may show even more restriction between habitats. We are currently in the process of creating near-isogenic lines with flowering time and salt tolerance QTLs to determine if habitat-dependent selection can restrict the spread of adaptive loci.

Although it is clear that prezygotic barriers result in essentially complete reproductive isolation between these ecological races, we did not calculate cumulative total isolation or proportional contribution of each barrier to total isolation, unlike several previous studies that have quantified reproductive isolating barriers (Ramsey et al. 2003; Coyne and Orr 2004; Nosil et al. 2005; Kay 2006). Such calculations, which are based on multiplicative functions, are not appropriate in most cases because sequential barriers are often not independent (Martin and Willis 2007). Further, our analysis of reproductive isolation was only based on a few

field sites and reproductive isolation may be weaker in other geographic locations. Such context-dependent reproductive isolation could explain the apparently admixed populations (BSR, ANR, ORO) in the STRUCTURE analysis. We also did not measure all possible reproductive isolating barriers, which is important for a comprehensive understanding reproductive isolation (Lowry et al. 2008). Finally, it is not clear for this system how much weight should be given to ecogeographic isolation versus temporal isolation and isolation due to immigrant inviability because the frequency of rare dispersal events is notoriously difficult to study.

THE ORIGIN, MAINTENANCE, AND REPRODUCTIVE ISOLATION OF ECOLOGICAL RACES

Ecological races have long been thought of as an intermediate stage in the process of plant speciation (Clausen 1951; Clausen and Heisey 1958; Grant 1981; Barrett 2001). Although our results demonstrate essentially complete prezygotic isolation and suggest that the coastal perennial and inland annual races of *M. guttatus* are in fact distinct biological species, the process by which most ecological races form, maintain their genetic distinctness, and accumulate further reproductive isolating barriers remains poorly understood.

Ecological races may be the product of a single evolutionary event or races may be derived from multiple geographically disjunct parallel evolution (speciation) events driven by repeated evolution of the same reproductive isolation mechanisms (Schluter and Nagel 1995; Rundle et al. 2000; Nosil et al. 2002; Rajakaruna et al. 2003). Our molecular genetic results suggest that the coastal populations of *M. guttatus* may be the result of a single evolutionary origin because coastal populations consistently had a lower allelic diversity than inland populations. Further, populations throughout the coastal range appear to have a subset of the alleles of the inland race (online Supplementary Fig. S2 and Table S4). Of course the hypothesis of parallel origins of ecological races is difficult to reject because gene flow among parallel lineages can wipe out the molecular signal of such a history. Further, the low diversity of coastal populations of *M. guttatus* may also be the result of a lower effective population size due to the narrow band of suitable habitats along the Pacific coast, and their perennial, potentially clonal life history.

Ecological races are thought to maintain their genetic integrity as a result of habitat-mediated natural selection (Clausen 1951; Clausen and Heisey 1958; Schemske 2000). Local adaptation can directly reduce gene flow through selection against immigrants between individual populations (Nosil et al. 2005; Rundle and Nosil 2005; Nosil 2007) or through species-wide ecogeographic reproductive isolation as a result of the evolution of range limits of species (Mayr 1947; Clausen 1951; Schemske 2000; Ramsey et al. 2003; Husband and Sabara 2004; Kay 2006). However, studies of other ecological races will be necessary to draw any general conclusions about the relative importance of different types of reproductive isolating barriers.

Although the coastal and inland races of *M. guttatus* appear to show approximately complete reproductive isolation, the process by which ecological races become good species remains unclear. If ecological races actually are intermediates in the process of speciation, then there must be a mechanism by which additional reproductive isolating alleles spread between races to complete the speciation process. Levin (1993, 1995) argues that most intrinsic incompatibility alleles are at best mildly deleterious, such as those derived from underdominant chromosomal rearrangements, and will only be fixed in local populations through drift (Lande 1979, 1985). Therefore, Levin (1993, 1995) concludes that plant speciation must be initiated and completed in local populations. However, recent studies suggest that genic incompatibilities are frequently involved in intrinsic postzygotic isolation between plant species (Fishman and Willis 2001; Sweigart et al. 2006; Bomblies et al. 2007; Moyle 2007; Sweigart et al. 2007; Case and Willis 2008; reviewed in Bomblies and Weigel 2007 and Lowry et al. 2008) and may be driven by natural selection or genomic conflict (Macnair and Christie 1983; Ting et al. 1998; Presgraves et al. 2003; Orr et al. 2007). If incompatibilities in plants are indeed driven by natural selection or genomic conflict, then incompatibility alleles may readily spread between widespread ecological races (Kane and Rieseberg 2007) and facilitate the conversion of ecological races into good species. Future research is clearly needed to resolve this issue.

Over a half century ago, Clausen (1951) envisioned that future studies and comparative analysis from the local population through ecological races to good species would facilitate a general understanding of how ecology and geography interact to create new species. The rapid development of modern molecular techniques and expansion of genomic resources to many taxa make these prospects only brighter.

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Supporting Information

The following supporting information is available for this article:

Table S1. Primers used in this study for population genetic analysis.

Table S2. Individual eigenvector trait loadings on the first two principle component axes.

Table S3. Pair-wise F_{ST} among all populations.

Table S4. Populations genetic summary statistics for SSR and intron markers.

Figure S1. Map of location of field sites (square) and seed source populations (circle) used in the reciprocal transplant experiments.

Figure S2. Comparison of population genetic summary statistics and habitat restricted alleles.

Figure S3. The program STRUCTURE run on *K*-values from 2 to 6.

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

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