Differential performance of reciprocal hybrids in multiple environments

Sarah Kimball[1]*, Diane R. Campbell[2] and Carrie Lessin[3]

[1]*Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, USA;* [2]*Department of Ecology and Evolutionary Biology, University of California, Irvine, CA, USA; and* [3]*Department of Biological Sciences, California State University, Sacramento, CA, USA*

Summary

1. Closely related taxa may be maintained as distinct species by a variety of reproductive isolating mechanisms. These include: inability to produce hybrid offspring, endogenous selection against hybrids in the form of genomic incompatibilities, and exogenous selection observable in genotype-by-environment interactions. To understand the relative importance of these three isolating mechanisms, we performed hand-pollination and reciprocal transplant experiments in a natural plant hybrid zone.

2. We measured reproductive isolation by making crosses between two parent species of *Penstemon* and naturally occurring hybrids. Inclusion of reciprocal hybrid crosses allowed us to determine whether fitness components differed depending on the identity of the mother.

3. Hybrid performance was evaluated in the greenhouse and in a reciprocal transplant experiment in the field. We measured fruit set, seed set, seed weight, time required for fruits to mature and seedling growth for potted plants. To test for exogenous isolation, we planted pure parents, reciprocal F_1 hybrids and later generation hybrids in a reciprocal transplant experiment, and measured survival.

4. On average, interspecific crosses produced as many seeds as conspecific crosses. Hybrid performance was also equal to or greater than parents in all environments, including the greenhouse and all field gardens, indicating a lack of endogenous isolation. Parent species and reciprocal F_1 hybrids differed in many traits measured. In each field garden, the hybrid with the native cytoplasm had a higher survival rate, suggesting local adaptation to different elevations.

5. *Synthesis*. Exogenous factors that differ along elevational gradients can be more important than intrinsic genetic incompatibilities in determining the fitness of plant hybrids. Our results illustrate the importance of studying hybrid performance in multiple environments and in generating reciprocal hybrids to test for isolating mechanisms in natural hybrid zones.

Key-words: cytoplasmic genes, elevational gradient, genotype-by-environment interactions, hybridization, hybrid zones, local adaptation, *Penstemon*, reciprocal transplant experiment, Sierra Nevada

Introduction

Naturally occurring plant hybrid zones are of ecological and evolutionary importance because the hybridizing species have diverse combinations of genetic material with the potential to influence entire communities of associated organisms (Whitham *et al*. 1999). The existence of large hybrid zones indicates incomplete reproductive isolating mechanisms, which can act prior to, or after, formation of the zygote. Post-zygotic isolation can be due to *endogenou*s selection (sensu Arnold 1997; Burke & Arnold 2001), meaning selection acting against hybrids regardless of the environment. Or it can be due to *exogenous* selection, meaning selection depending on environmental factors, such as habitat or pollinator type, that would show up as a genotype-by-environment interaction. The hybrid zone literature draws a distinction between these two types of selection because they reflect mechanisms that lead to formation of a hybrid zone and can help to determine whether the zone will be stable over time and where it will form (Barton & Hewitt 1989; Arnold 1997).

*Correspondence author. E-mail: kimballs@email.arizona.edu

Moreover, measurements of endogenous and exogenous selection clarify the relative role of particular ecological factors in the persistence of hybrid zones (Wang *et al*. 1997; Fritsche & Kaltz 2000; Campbell & Waser 2007).

In plants, pre-zygotic isolation can occur pre-mating, for example, due to differences in flowering time or reliance on different pollinators (Fulton & Hodges 1999; Schemske & Bradshaw 1999), or post-mating, due to reductions in heterospecific pollen germination, pollen tube growth and fertilization of ovules (Cruzan & Arnold 1994; Weiblen & Brehm 1996; Arnold 1997; Chari & Wilson 2001). Post-zygotic isolation can show up in abortion of hybrid embryos, low germination rates of hybrid seeds, reduced vegetative growth and reduced reproduction by hybrids (Arnold 1997 and references therein; Chari & Wilson 2001; Ramsey *et al*. 2003). Furthermore, reduced survival or seed set from hybrids beyond the F_1 stage can result from a breakdown in co-adapted gene complexes, such as the epistatic effects described by Dobzhansky–Muller incompatibilities (Dobzhansky 1936; Muller 1942; Hiesey *et al*. 1963; Kalischuk *et al*. 1997; Fishman & Willis 2001; Johansen-Morris & Latta 2006). Many studies of closely related plant species have reported some types of endogenous post-zygotic incompatibility, suggesting the frequent importance of such factors in maintaining species as distinct (review in Arnold 1997; Burke *et al*. 1998; Burgess & Husband 2004; Kirk *et al*. 2005). An important caveat, however, is that not all of these studies examined hybrid success in more than one environment, thereby demonstrating that the incompatibilities were endogenous.

A growing number of recent studies have suggested the importance of exogenous factors in determining the relative fitness of parents and hybrids (Emms & Arnold 1997; Fritsche & Kaltz 2000; Campbell & Waser 2007; Johansen-Morris & Latta 2008). Pollinator behaviour has some influence on hybrid fitness for species pairs of *Ipomopsis* (Campbell *et al*. 2002), *Iris* (Emms & Arnold 2000) and *Nicotiana* (Ippolito *et al*. 2004). Several studies of physiological traits have demonstrated differences in water-use-efficiency and nutrient use that correspond with distinct habitat use by parent species and their hybrids in *Artemisia* (McArthur *et al*. 1998), *Helianthus* (Schwarzbach *et al*. 2001) and *Ipomopsis* (Campbell *et al*. 2005).

The assessment of hybrid fitness and its dependence on the environment is complicated by the possibility of asymmetries involving which species is the ovule parent and which is the pollen parent. If endogenous isolation is asymmetrical, large hybrid zones may be formed with the cytoplasm of only one parent (Kirk *et al*. 2005). Cytoplasmic genes or the interaction of cytoplasmic and nuclear genes could influence local adaptation and the evolutionary outcome of the hybrid zone (Galloway & Fenster 1999). Reciprocal crosses are necessary to test for cytonuclear effects on fitness, as they create hybrid individuals with the same nuclear genes but different cytoplasmic genes.

Distinguishing between endogenous and exogenous reproductive isolation necessitates that we pay attention to ecological influences and estimate fitness in multiple environments. One direct method is a reciprocal transplant experiment in which fitness is assessed in multiple habitats including the habitats of the two parental species (Rieseberg & Carney 1998). Interaction between the effects of genes and the environment would be indicated by a difference in relative fitness (or fitness components) of hybrids that depends on the environment. In contrast, endogenous isolation would be indicated by low hybrid fitness in all environments. Several recent studies have performed such tests (Emms & Arnold 1997; Wang *et al*. 1997; Fritsche & Kaltz 2000; Rhode & Cruzan 2005), although very few have begun with seeds of known genetic background (Campbell & Waser 2001, 2007). Beginning with seeds is necessary to test for relatively early-acting genetic incompatibilities. Another approach is to compare fitness of hybrids in natural and controlled environments, as differences could be expected if exogenous isolation is important. While this approach has been advocated for some animals (Hatfield & Schluter 1999), very few plant studies have assessed hybrid fitness in the field and the greenhouse. One exception (Emms & Arnold 1997 and Burke *et al*. 1998 in combination) included reciprocal F_1s in the greenhouse but not in the field study. Planting reciprocal hybrids would allow testing the possibility that cytonuclear interactions depend on the environment.

This study addresses the strength of several forms of reproductive isolation, including endogenous and exogenous isolation, in a *Penstemon* hybrid zone by combining studies of potted plants with a reciprocal transplant experiment. In addition to including a rare assessment of hybrid performance in the field and the greenhouse, this study is novel in the use of plants taken originally from two contact sites, in this case from elevational transects in the Sierra Nevada, CA, USA. That feature allows us to test the generality of results over different geographical locations. Furthermore, we used advanced hybrids as well as reciprocal F_1s, the latter of which are rarely included in tests of hybrid fitness (Burke *et al*. 1998; Campbell & Waser 2001; Levin 2003; Burgess & Husband 2004). We addressed three specific questions: (i) How do hybrid crosses compare with pure parental crosses in seed set, seed weight, seed germination and seedling growth? (ii) Do these aspects of cross performance depend on the direction of the cross? (iii) Do parental and hybrid genotypes survive and grow equally well throughout the natural hybrid zone, and in both the greenhouse and the field, or is relative fitness dependent on the environment? In addition, we tested for self-compatibility (see Appendix S1 in Supplementary material for methods and results), as the ability to self can influence the degree of hybridization in nature (Johansen-Morris & Latta 2006) and appears to vary within the *Penstemon* genus (Lange & Scott 1999; Lange *et al*. 2000).

Methods

STUDY SYSTEM

Penstemon davidsonii is a perennial wildflower that occurs in the alpine zone of the Sierra Nevada and Cascade mountain ranges in

Fig. 1. Photographs of *Penstemon newberryi* (left), *P. davidsonii* (right), and a natural hybrid (center). Note the differences in flower colour and shape. The two species also differ in inflorescence height, presence of leaf serrations and leaf size. In nature, hybrids exhibit all intermediate forms.

the western USA. Its close relative, *P. newberryi*, is found in the montane zone of the Sierra Nevada (Fig. 1). Wherever their ranges overlap, at intermediate elevations, hybrids are formed (Clausen *et al.* 1940; Chabot & Billings 1972; Datwyler & Wolfe 2004). Hybrids are more abundant than either parent at intermediate elevations, and morphological characters change gradually along elevational gradients in a clinal pattern, suggesting hybridization beyond the F_1 generation (Kimball 2008). Our study sites were two elevational transects in the eastern Sierra Nevada of California. One transect (hereafter 'Piute') is along the Piute Pass trail above Bishop, from 2400 to 3800 m elevation (37°14′22″ N 118°36′24″ W to 37°14′44″ N 118°40′36″ W). The second transect (hereafter 'Lee Vining') is located 110 km to the north in Lee Vining Canyon, just east of Yosemite National Park (from 37°56′08″ N 119°10′42″ W to 37°58′15″ N 119°11′27″ W, 2200–3600 m elevation).

CROSSES AND SEED PRODUCTION

Cuttings were taken from pure *P. newberryi*, pure *P. davidsonii*, and natural hybrids at low, middle and high elevation sites along the two transects. Collections of the parent species were made at the extreme elevational ends of their ranges to minimize any introgression (Kimball 2008). Plants from the Piute Transect were taken from multiple populations, with no more than three plants from each patch, which ensured that crosses within a species were outbred. The cuttings were rooted in water and then transplanted into 1-gallon pots with a 1 : 1 mixture of potting soil mix and pumice. Plants were grown outside at University of California, Irvine, CA near sea level, for 1 year until they flowered.

During the spring of 2004, we hand-pollinated plants of both species and hybrids. For each study transect, we made nine outcrosses representing all factorial combinations of the three types (*P. davidsonii*, *P. newberryi* and natural hybrids) used as the mother crossed with the three types used as the father. These outcrosses represent pure parents, reciprocal F_1 hybrids, reciprocal backcrosses and crosses between natural hybrids. Over 1400 such crosses were made on more than 150 plants. For each cross type, each of 41 to 58 different maternal plants received pollen from one to six fathers. There were a total of 18 to 50 different individual fathers for each cross type.

Corollas with anthers attached were removed on unopened flowers to prevent self-pollination and to prevent animal pollinators from visiting the flowers. When the stigma was mature (style bent over and stigma sticky), pollen from a recently opened flower on a different plant was placed on the stigma. The name of the father was placed on a label on the pedicel of the recipient flower, and the date of the cross was recorded. When fruits were mature (hard, brown and just beginning to dehisce), they were collected, and the date was recorded. Seeds were separated from each capsule and weighed in a batch to determine the average weight of seeds in each fruit. Flowers not used in experimental crosses were allowed to set fruit naturally. Means of seed set, seed weight, the probability of setting fruit, and the number of days to maturity for a particular maternal plant and cross type were analyzed by three-way ANOVA with study transect, type of mother and type of father as fixed effects. For this and all other ANOVAs, we also performed two planned comparisons: (i) conspecific crosses vs. heterospecific crosses to compare the parentals with the hybrids; and (ii) F_1 hybrids with *P. davidsonii* as the mother vs. F_1 hybrids with *P. newberryi* as the mother to test for reciprocal effects. Mean seed set was calculated based only on fruits that set seed. Fruits were scored with a 1 or 0 to indicate whether they set seed or aborted. The average value was calculated for each maternal plant within each cross type to determine whether the probability of setting fruit differed depending on the cross type. Any reduction in production of fruit or seeds from crosses between the two species would constitute pre-zygotic isolation, whereas crossing success of natural hybrids is an aspect of hybrid performance and thus reflects post-zygotic isolation.

GERMINATION AND VEGETATIVE PERFORMANCE IN POTS

Lack of germination success or vegetative growths of hybrids are aspects of post-zygotic isolation. To assess these aspects of early performance, for each cross type, seeds from one to six fathers resulting from each mother plant were mixed together and one to eight batches of 25 seeds each were taken at random from the seed pool. Seeds were stored in a refrigerator for 9 months. Each batch of 25 seeds was placed in a Petri dish on filter paper in a growth chamber

at 4 °C with a 12-h light–dark cycle for 2 weeks and then soaked in 500 p.p.m. gibberellic acid for 24 h to break seed dormancy. Seeds were planted in 4-inch pots in a 1 : 1 potting soil : pumice mixture. The number of seedlings was counted every 2 weeks for 20 weeks. The total number of seedlings in each pot was then divided by 25 to determine the germination rate. We counted the number of seedlings every 2 weeks, so that a seed that germinated and later died was still included in the germination rate. For each individual mother within every cross type, we determined the average germination rate and the average number of days between planting and first germination. Seeds from the Lee Vining transect were germinated 1 month prior to seeds from the Piute transect, so data for each transect were analyzed separately from germination through seedling performance. Germination was analyzed by two-way ANOVA with type of mother and type of father as fixed effects. For each transect, we also analyzed with two-way ANOVA a composite index of cross success reflecting the number of seedlings resulting from each hand-pollination: seedlings/flower = fruits/flower × seeds/fruit × seedlings/seed.

One year following germination, we measured plant height, width, number of nodes on the main branch and number of branches to determine the vegetative performance of the seedlings. The averages of these variables were calculated for every mother within a cross type and analyzed by MANOVA with type of mother and type of father as fixed factors. We used separate canonical discriminant analyses to determine which vegetative variables were responsible for the significant main effects of mother and father.

RECIPROCAL TRANSPLANT EXPERIMENT IN THE FIELD

To examine hybrid survival in the field, we established common gardens at three different elevations (2382, 2888 and 3505 m) along the Lee Vining transect, representing sites with naturally occurring populations of the three different plant types (*P. newberryi*, hybrids and *P. davidsonii*, respectively). The montane (*P. newberryi*) site was dominated by *Artemisia tridentata* (sagebrush) and *Chrysothamnus nauseosus* (rabbit brush), surrounded by large *Pinus jeffreyi* (Jeffrey pine) in mixed granite and metamorphic gravelly soil. The hybrid site consisted of *Leptodactylon pungens* and *Erigeron compositus* with scattered pines (*Pinus contorta murryana* and *Pinus flexilis*) in mostly granitic gravel. The alpine (*P. davidsonii*) site was above tree line, with *Eriogonum ovalifolium*, *Erigeron pygmaeus*, *Castilleja nana* and *Phlox condensata* in granitic gravel. Five different cross types from Lee Vining transect plants were planted into each garden. The five cross types represented pure parents, reciprocal F₁ hybrids, and later generation hybrids (resulting from natural hybrid-by-natural hybrid crosses). We used 50 different genetic individuals of each cross type, and the experimental design called for two replicate individuals of each of these clones to be planted into each of the three gardens.

Cuttings for the low garden plants were taken from potted plants on 24 March 2006, for the middle garden on 4 April, and for the high garden on 19 April. The cuttings were placed in tubes of water and planted when they had roots that were more than 4 cm long, between 11 April and 16 May. Each garden was planted as early as possible during the growing season, after the snow had melted off the garden and naturally occurring plants were green and exhibited new growth. The low garden was planted on 9–12 June, the middle garden on 27–30 June, and the high garden on 5–9 July. Plants were placed approximately 50 cm apart, and were planted in random order. They were watered immediately after planting. In addition, we planted Rainbird Irrigation Supplement with each plant to provide supplemental water during establishment. As much as possible,

we avoided disturbing existing vegetation during planting. Survival of each plant was noted after 1–7 days, on several subsequent dates during the main summer season of 2006 (23 June, 19–22 July, 13–14 August), on 26–27 September and in the summer of 2007 (June and 28–29 August).

We used a GLM (Proc Genmod in SAS with logit link) to test whether survival through the peak growing season (live or dead in mid-August 2006) depended on plant type, garden elevation or the interaction between the two. This model is analogous to a two-way ANOVA except that residuals were assumed to be binomially distributed (as survival can take on only two values) rather than normally distributed. Four types of planned comparisons were also done: *P. newberryi* vs. *P. davidsonii*, F₁ hybrids vs. pure parents (to test for heterosis), later generation hybrids resulting from natural hybrid-by-natural hybrid crosses vs. all other cross types (lower fitness could be due to hybrid breakdown in later generations), and F₁ hybrids with *P. newberryi* mothers vs. F₁ hybrids with *P. davidsonii* mothers (to determine whether the direction of the cross mattered). Because many clones were represented by two plants that may not behave as independent data points, we also used the macro GLIMMIX in SAS to test a mixed model that included the random effect of clone nested within plant type, in addition to the fixed factors of plant type and garden elevation. The clones did not differ significantly from one another. Also, patterns in which other effects were significant were the same for the Proc Genmod and GLIMMIX analyses, so for simplicity we report only the former. To analyze patterns in survival over time, we also performed a lifetable analysis (Proc Lifereg in SAS) to determine whether the number of days until death differed depending on the type of plant, the garden or the interaction between the two.

Results

SEED PRODUCTION

The probability of fruit set was higher for crosses with *P. davidsonii* as the mother than for crosses with *P. newberryi* as the mother (Fig. 2, Table 1), with natural hybrids falling in between. In contrast, the type of father did not significantly affect fruit set. An average of 87% of Lee Vining crosses resulted in fruit set compared to 82% of Piute crosses ($P = 0.054$ for effect of transect, Table 1). Fruits resulting from crosses to make F₁ hybrids were just as likely to set seed as fruits from pure parental crosses ($F_{1,427} = 0.182$, $P = 0.670$). Crosses generating F₁ hybrids with *P. davidsonii* as the mother, however, produced higher fruit set than F₁ hybrid crosses with *P. newberryi* as the mother (means = 92% vs. 76%, $F_{1,427} = 9.788$, $P = 0.002$).

For those fruits that set, there was no significant difference between seed set resulting from pure parental crosses and hybrid crosses (Fig. 2, $F_{1,403} = 0.017$, $P = 0.897$). *Penstemon davidsonii* mothers produced significantly more seeds than *P. newberryi* mothers, and natural hybrid mothers produced intermediate numbers of seeds (Table 1). Surprisingly, the effect of father was reversed, such that *P. newberryi* and hybrid fathers produced significantly more seeds per fruit than *P. davidsonii* fathers (Table 1). Plants from the Lee Vining transect produced fruits with more seeds than plants from the Piute transect, although the effect of transect was not significant ($P = 0.078$). F₁ heterospecific crosses with *P.*

Table 1. Results of three-way ANOVAs that tested whether fruit set, seeds/fruit, seed weight and the number of days required for fruits to mature varied depending on the type of mother, type of father or study transect

Source	Fruit set				Seeds/Fruit			
	df	MS	*F*	*P*	df	MS	*F*	*P*
Mother	2	0..678	10.033	< 0.001	2	14504.869	26.697	< 0.001
Father	2	0.07572	1.121	0.327	2	2711.595	4.991	0.007
Transect	1	0.252	3.726	0.054	1	1694.774	3.119	0.078
Mother × father	4	0.02183	0.323	0.862	4	401.474	0.739	0.566
Mother × transect	2	0.135	2.004	0.136	2	1307.034	2.406	0.092
Father × transect	2	0.101	1.496	0.225	2	1231.985	2.268	0.105
Mother × father × transect	4	0.07453	1.103	0.355	4	1182.834	2.177	0.071
Error	427	0.06757			403	543.316		
Total	445				421			

Source	Seed weight				Days to maturity			
	df	MS	*F*	*P*	df	MS	*F*	*P*
Mother	2	1162.606	18.679	< 0.001	2	1116.84	94.971	< 0.001
Father	2	411.408	6.61	0.001	2	19.989	1.7	0.184
Transect	1	35.581	0.572	0.45	1	2.749	0.234	0.629
Mother × father	4	26.04	0.418	0.795	4	3.047	0.259	0.904
Mother × transect	2	181.823	2.921	0.055	2	21.506	10829	0.162
Father × transect	2	79.773	1.282	0.279	2	8.014	0.681	0.506
Mother × father × transect	4	87.798	1.411	0.23	4	1.724	0.147	0.964
Error	404	62.241			397	11.76		
Total	422				415			

davidsonii mothers produced significantly more seeds per fruit than the reciprocal F_1 crosses with *P. newberryi* mothers (Fig. 2, $F_{1,403} = 49.435$, $P < 0.001$).

Seeds from *P. davidsonii* mothers weighed more than seeds from *P. newberryi* mothers, and seeds from natural hybrid mothers were intermediate (Fig. 2, Table 1). Once again, the effect of father was reversed, and *P. newberryi* fathers produced heavier seeds than *P. davidsonii* fathers (Table 1). Seeds from both transects weighed similar amounts. There was no difference in seed weight of parents and F_1 hybrids ($F_{1,404} = 1.589$, $P = 0.208$). F_1 hybrid seeds with *P. davidsonii* as the mother were heavier than those with *P. newberryi* as the mother ($F_{1,404} = 400.147$, $P < 0.001$).

The number of days from pollination to fruit dehiscence was significantly shorter when *P. davidsonii* was the mother than when *P. newberryi* was the mother (Fig. 2, Table 1). There was no significant effect of transect of origin or of father (Table 1). The time required for seeds from pure parental crosses to reach maturity did not differ from that required for F_1 hybrids to reach maturity ($F_{1,397} = 0.180$, $P = 0.672$). F_1 hybrid fruits with *P. davidsonii* as the mother matured faster than F_1 hybrid fruits with *P. newberryi* as the mother ($F_{1,397} = 44.399$, $P < 0.001$).

In summary, crosses between species produced as many fruits and as many seeds per fruit as crosses within a species. The type of plant used as the mother in crosses had strong effects on all aspects of seed production, with natural hybrids intermediate to the two parental species. The type of father had strong effects on seeds per fruit and seed weight. For all aspects of seed production, patterns were similar for plants originating from the Lee Vining and Piute transects. We detected no interactions between transect and other factors (Table 1).

GERMINATION AND VEGETATIVE PERFORMANCE IN POTS

Only about 6% of the seeds germinated (Fig. 3). For the Lee Vining transect, seeds with *P. davidsonii* or *P. newberryi* as the mother had significantly higher germination rates than those with natural hybrids as the mother (Table 2). Seeds with *P. newberryi* as the father had higher germination rates than seeds with *P. davidsonii* as the father. Hybrid fathers had intermediate germination rates (Table 2). For the Piute transect, seeds with *P. davidsonii* as the mother had significantly higher germination rates than seeds with *P. newberryi* as the mother (Table 2). Seeds with natural hybrid mothers were intermediate. The effect of father was reversed from the mother effect, such that seeds with *P. newberryi* as the father had higher germination rates than those with *P. davidsonii* as the father (Table 2). For seeds from plants of both transects, F_1 hybrids with *P. davidsonii* as the father had about a 12% germination rate. Lee Vining pure *P. newberryi* seeds also had high germination rates, although pure *P. newberryi* seeds from the Piute transect plants only had a 4% germination rate. F_1 hybrids with *P. davidsonii* as the mother had higher germination rates than hybrids with *P. newberryi* as the mother (Lee Vining $F_{1,167} = 13.784$, $P < 0.001$, Piute $F_{1,228} = 37.584$, $P < 0.001$). On average, F_1 hybrid seeds germinated as well as or better than seeds of the two parent species (Lee Vining
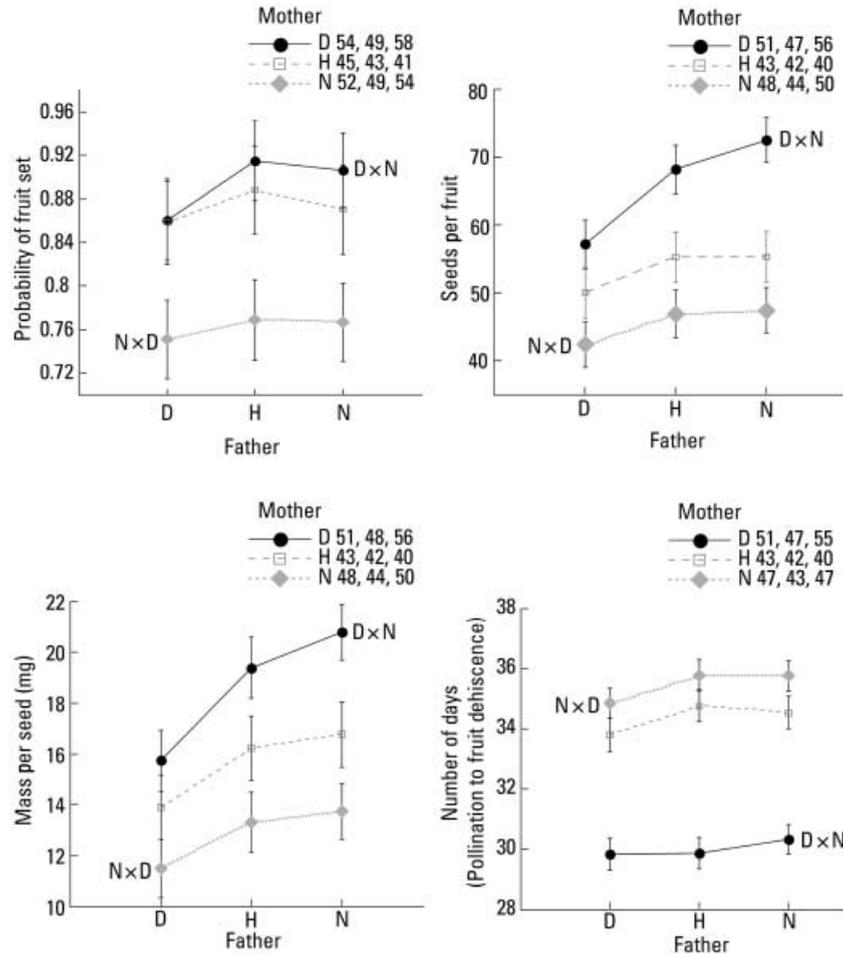
Fig. 2. Probability of fruit set, number of seeds per fruit, seed mass, and number of days between pollination and fruit dehiscence for different crosses. The identity of the mother is given in the legend and the x-axis indicates the identity of the father where D = *P. davidsonii*, N = *P. newberryi*, and H = natural hybrid. Lines connect crosses with the same type of mother. Values are means ± SD. Reciprocal F₁ hybrids are labelled. The first letter indicates the identity of the mother and the second letter indicates the identity of the father. Sample sizes are listed after each mother in the same order as the data are presented on the *x*-axis. Results for the two transects were pooled following the finding that transect differences were non-significant for these variables.

$F_{1,167} = 0.000$, $P = 0.986$, Piute $F_{1,228} = 7.928$, $P = 0.005$). The effects on germination were not attributable to differences in seed mass. When average seed mass for the maternal family was added as a covariate into the analyses, it had no detectable influence on germination success ($P > 0.2$ for both transects), and it did not change the significance of the effects due to type of mother and father.

The only early performance character in which any hybrid type performed significantly less well than the parents was time to germination. Seeds with hybrid mothers took longer to germinate than seeds with *P. davidsonii* or *P. newberryi* mothers for both transects (Fig. 3, Table 2). However, there was no difference in the time required for pure parents and F₁ hybrid seeds to germinate (Lee Vining $F_{2,141} = 0.301$, $P = 0.584$, Piute $F_{2,172} = 0.310$, $P = 0.579$,). Seeds with *P. davidsonii* as the father took significantly more time to germinate than seeds with *P. newberryi* as the father (Table 2). We saw a strong reciprocal effect in which the number of days required for germination was longer for F₁ hybrid seeds with

P. davidsonii as the mother than for those with *P. newberryi* as the mother (Lee Vining $F_{1,141} = 10.023$, $P = 0.002$, Piute $F_{1,172} = 28.534$, $P < 0.001$).

The overall index of cross success (fruit set × seeds/fruit × seedlings/seed) differed significantly depending on the types of mother and father along both transects (Fig. 4, Table 2). Consistent with the effects for most of the individual components, crosses with *P. davidsonii* mothers and *P. newberryi* fathers resulted in the highest success index (Lee Vining $F_{1,157} = 23.857$, $P < 0.001$, Piute $F_{1,223} = 52.957$, $P < 0.001$). For plants from the Piute transect, this particular F₁ cross had strikingly higher performance than any of the other eight cross types (Fig. 4). Although there was no significant difference between success of the pure parents compared to the average of F₁ hybrid crosses for Lee Vining plants ($F_{1,157} = 1.059$, $P = 0.305$), the high success of F₁ hybrids with *P. davidsonii* mothers led to a significant difference between average F₁ hybrid success and average pure parental success for Piute transect plants ($F_{1,223} = 10.453$, $P = 0.001$).

Table 2. Comparisons of germination rates and fitness indices for crosses from the Lee Vining and Piute study transects. Two-way ANOVAs with type of mother and type of father as fixed factors

	Lee Vining				Piute			
Source	df	MS	*F*	*P*	df	MS	*F*	*P*
Germination rate								
Mother	2	0.03182	8.79	< 0.001	2	0.107	30.486	< 0.001
Father	2	0.04692	12.963	< 0.001	2	0.02547	7.256	0.001
Mother × father	4	0.01643	0.454	0.769	4	0.01094	3.116	0.016
Error	167	0.0362			228	0.00351		
Total	176				237			
Days to germination								
Mother	2	3494.73	5.25	0.006	2	8094.74	11.99	< 0.001
Father	2	4330.76	6.506	0.002	2	6361.22	9.422	< 0.001
Mother × father	4	973.213	1.462	0.217	4	713.695	1.057	0.379
Error	141	665.619			172	675.113		
Total	150				181			
Cross success index								
Mother	2	136.244	5.25	0.006	2	614.132	11.99	< 0.001
Father	2	159.353	6.506	0.002	2	74.395	9.422	< 0.001
Mother × father	4	9.145	1.462	0.217	4	61.743	1.057	0.379
Error	157	15.398			214	14.119		
Total	166				223			

After 1 year of growth, the multivariate response of seedling height, width, branch number and node number differed significantly depending on the type of mother and the type of father (see Table S1 in Supporting Information). Pure *P. newberryi* were larger than pure *P. davidsonii*, and F_1 hybrids were intermediate. The canonical discriminant analysis comparing mothers suggested that the effect of mother was due to the larger overall size of plants with *P. newberryi* mothers (Table 3). For both transects, the first canonical discriminant function correlated positively with all size measures and accounted for 88–90% of the variation ($P < 0.0001$). For the Lee Vining transect, the effect of father was similar, such that seedlings with *P. newberryi* fathers were larger than seedlings with hybrid or *P. davidsonii* fathers. For plants from the Piute transect, the first canonical discriminant function instead correlated negatively with the number of branches and positively with the other size characters, reflecting a relatively greater number of branches in plants with *P. davidsonii* fathers ($P < 0.0001$).

RECIPROCAL TRANSPLANT EXPERIMENT

In the field experiment, the probability of surviving to mid-August 2006 (as analyzed with a GLM) differed depending on the type of plant and the garden (Table 4). Survival was much higher in the middle garden than in the two parental gardens (Fig. 5). F_1 hybrids had high survival in all gardens (Fig. 5), exceeding the survival on average of the two parental species ($P < 0.0001$ for heterosis contrast in Table 5). In the low garden, F_1 hybrids with *P. newberryi* mothers had higher survival than those with *P. davidsonii* mothers (contrast $P < 0.05$). In the middle garden, both types of F_1 hybrids had higher survival than all other plant types, but the direction of the cross did not matter. In the high garden, F_1 hybrids with

P. davidsonii mothers had higher survival than those with *P. newberryi* mothers (Table 5). Thus, survival of F_1 hybrids to mid-August 2006 depended on a match between the maternal type and the garden where planted. Survival over time differed significantly depending on the type of plant, the garden, and the type-by-garden interaction (Table 4). By August 2007, all survivors in the low garden were F_1 hybrids with a *newberryi* maternal background. Not a single *P. newberryi* plant survived to that point in the high garden, and the only survivors had a *P. davidsonii* maternal background. In the middle garden, F_1 hybrids had the highest survival to August 2007. Progeny from crosses between natural hybrids performed similarly to the average for the parentals and F_1s, so there was no evidence for hybrid breakdown in survival of later generation hybrids during the peak growing season (contrast $P > 0.05$ in Table 5).

Discussion

In this study, we investigated aspects of reproductive isolation in a *Penstemon* hybrid zone. Crosses between species generated as many seeds as crosses within a species. Furthermore, all types of hybrid offspring performed as well or better than the parental types in pots in a greenhouse setting and when averaged over the three field environments, so there was no evidence for endogenous post-zygotic isolation. Instead, in our field transplant experiment, we found exogenous isolation in the form of differences in the survival of reciprocal F_1 hybrids that depended on the elevation of the garden.

CROSS SUCCESS IN POTS

Performance of plants in pots was just as high or higher for F_1 hybrids as for the pure parents on average. The lack
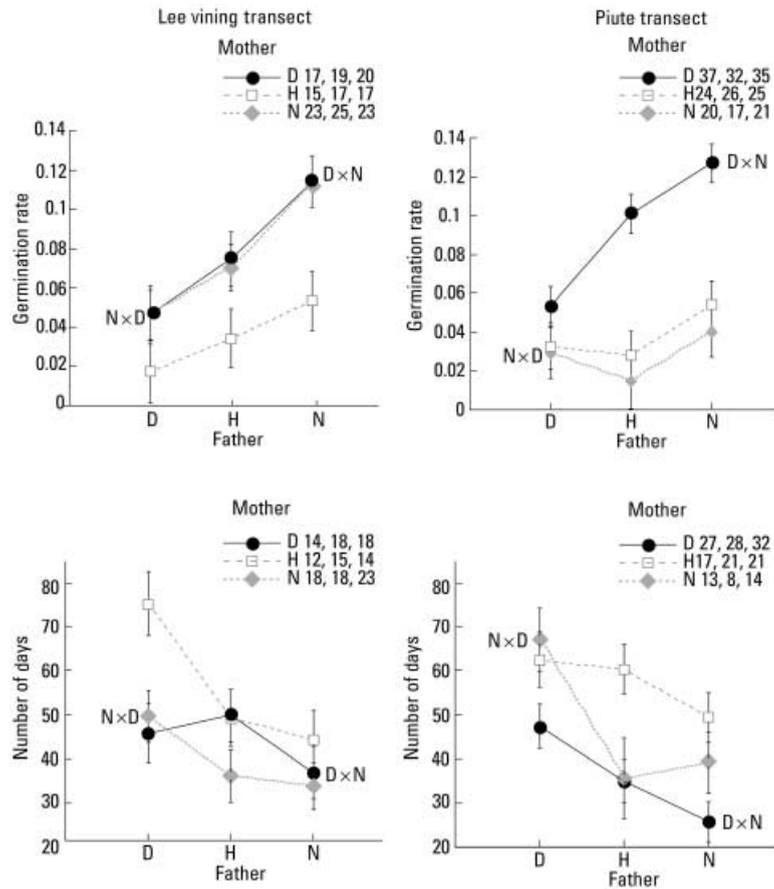
Fig. 3. Germination rate and number of days between planting seeds and germination for crosses between three plant types from two study transects. The identity of the mother is given in the legend, and the *x*-axis indicates the identity of the father where D = *P. davidsonii*, N = *P. newberryi*, and H = natural hybrid. Sample sizes are listed after each mother in the same order as the data are presented on the x-axis. Lines connect crosses with the same type of mother. Values are means ± SD.

of reduction in formation of F_1 seeds suggests absence of post-mating, pre-zygotic isolation, but based only on single-species crosses. We did not test success of pollen in mixed species loads on the stigma, and in some cases, the success of heterospecific pollen is reduced in the presence of conspecific pollen even though single-donor heterospecific crosses generate seeds (Carney *et al*. 1996; Williams *et al*. 1999).

Early offspring performance, assessed as germination, was also as high for F_1 hybrids as for the pure parents. Furthermore, the composite measure of cross success (fruit set × seeds per fruit × seedlings per seed) was actually higher for F_1 crosses than for the parents. Crosses with natural hybrids as the mother or father were generally intermediate to the two parental types. These results suggest that endogenous isolation is absent, contrasting with results for many other hybrid zones (Weiblen & Brehm 1996; Arnold 1997; Burke *et al*. 1998; Wolf *et al*. 2001; Schweitzer *et al*. 2002; Ramsey *et al*. 2003; Burgess & Husband 2004; Kirk *et al*. 2005; Lexer *et al*. 2005; Fritz *et al*. 2006), although it is still possible that fitness reduction of hybrids would show up at a later stage. In some cases, F_1 hybrids outperform parental crosses due to

release from inbreeding depression (Rhode & Cruzan 2005). In our experiment, however, the high success of F_1 crosses cannot be explained in this fashion, because the parental crosses from Piute were made between plants from many different populations and so are outbred. Furthermore, high pollinator visitation by an extremely generalized set of pollinators (Kimball 2008) and decreased seed set with selfing (Appendix S1) make it unlikely that most individuals even in the same population are closely related.

The success of F_1 hybrids depended heavily on which species was used as the maternal parent. Asymmetrical performance of reciprocal hybrids also occurs in some other plant systems (Burke *et al*. 1998; Campbell & Waser 2001; Tiffin *et al*. 2001; Rhode & Cruzan 2005). Reciprocal effects are theoretically consistent with expression of cytoplasmic genes inherited through the mother, cytonuclear interactions or maternal environmental effects, any of which can cause additional variation among progeny of different mothers (Roach & Wulff 1987; Levin 2003; Burgess & Husband 2004). In our case, two factors weigh against the explanation of maternal environmental effects, although we cannot rule them out. First, all plants used as parents had been grown

Table 3. Results of canonical discriminant analyses. Values are the correlations between the canonical discriminant variable and the original variables

Variable	Piute mothers		Piute fathers		Lee Vining mothers		Lee Vining fathers	
	Can 1	Can 2	Can 1	Can 2	Can 1	Can 2	Can 1	Can 2
Branches	0.320497	−0.700555	−0.57329	0.208131	0.816319	0.125392	0.73979	−0.574309
Height	0.999369	0.029832	0.562822	0.401095	0.923684	−0.023872	0.954847	0.237645
Width	0.577212	0.361402	0.376126	−0.432717	0.481906	0.564413	0.772054	−0.057625
Nodes	0.479527	0.377512	0.06742	0.585227	0.410179	0.658455	0.276472	0.449579
P-value	< 0.0001	0.0495	< 0.0001	0.711	< 0.0001	0.215	< 0.0001	0.0091
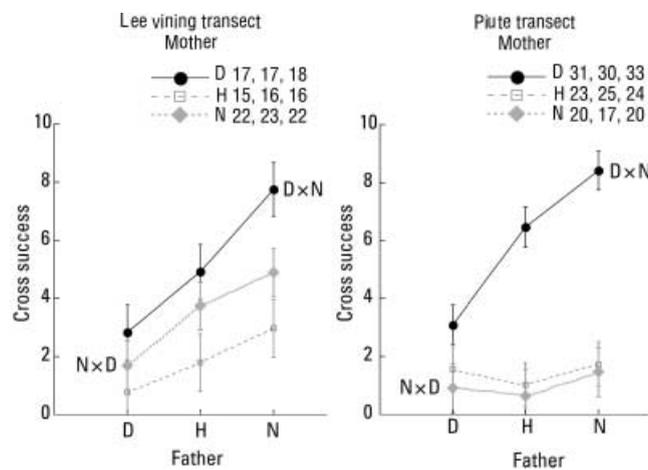
Fig. 4. An index of cross success calculated as fruit set × seeds/fruit × germination rate for crosses between three plant types from two study transects. The identity of the mother is given in the legend, and the *x*-axis indicates the identity of the father where D = *P. davidsonii*, N = *P. newberryi*, and H = natural hybrid. Sample sizes are listed after each mother in the same order as the data are presented on the x-axis. Lines connect crosses with the same type of mother. Values are means ± SD.

from cuttings in a common environment for 2 years prior to this experiment. Second, maternal environmental effects are often propagated through effects of seed mass (Roach & Wulff 1987), but seed mass did not explain the observed differences in germination.

Fruits from *P. davidsonii* mothers had significantly more seeds that were significantly heavier and germinated more frequently than fruits with other mothers. Surprisingly, the effect of father was significant in the opposite direction, such that fruits with *P. newberryi* fathers had the greatest number and weight of seeds, with highest germination. Strong maternal effects exist in other systems (Campbell & Waser 2001; Burgess & Husband 2004; Kirk *et al*. 2005), but we do not know of other studies that have also found a significant father effect in the opposite direction. One possible explanation for such a reversal is that the nuclear genes of *P. newberryi* and the cytoplasmic genes of *P. davidsonii* interacted to produce seeds with high germination. This response was unidirectional, as crosses using *P. newberryi* mothers and *P. davidsonii* fathers had lower crossing success than all other cross types, suggesting cytonuclear interactions rather than nuclear heterozygosity as the reason for high *davidsonii* × *newberryi* hybrid fitness. Increased maternal provisioning by *P. davidsonii* cannot

completely explain high *davidsonii* × *newberryi* fitness either, because *davidsonii* × *davidsonii* crosses had lower success indices than crosses with *P. newberryi* nuclear genes (Fig. 3).

The alpine *P. davidsonii* mothers produced more seeds per fruit and heavier seeds than did *P. newberryi* mothers. This result may at first seem to contradict the well-documented trade-off between seed number and seed weight (Sawada *et al*. 1994; Stocklin & Favre 1994; Leishman *et al*. 1995). However, the two species have different reproductive strategies, with *P. davidsonii* producing fewer flowers per plant than *P. newberryi*. Thus, on a per-plant basis, there was still a negative correlation between seed number and seed weight. Alpine plants sometimes produce larger seeds than their lower elevation relatives (Sawada *et al*. 1994). However, the opposite pattern occurred in two perennial *Epilobium* species, where the alpine species produced a larger number of lighter seeds per fruit than its montane relative (Stocklin & Favre 1994).

Germination rates in pots were low for all cross types, probably due to high seed dormancy rather than low seed viability. Despite our use of cold stratification and gibberellic acid, seeds took an average of 46 days to germinate. Infestations of fungus gnats and snails also contributed to mortality for all types of seedlings, with the number of seedlings that
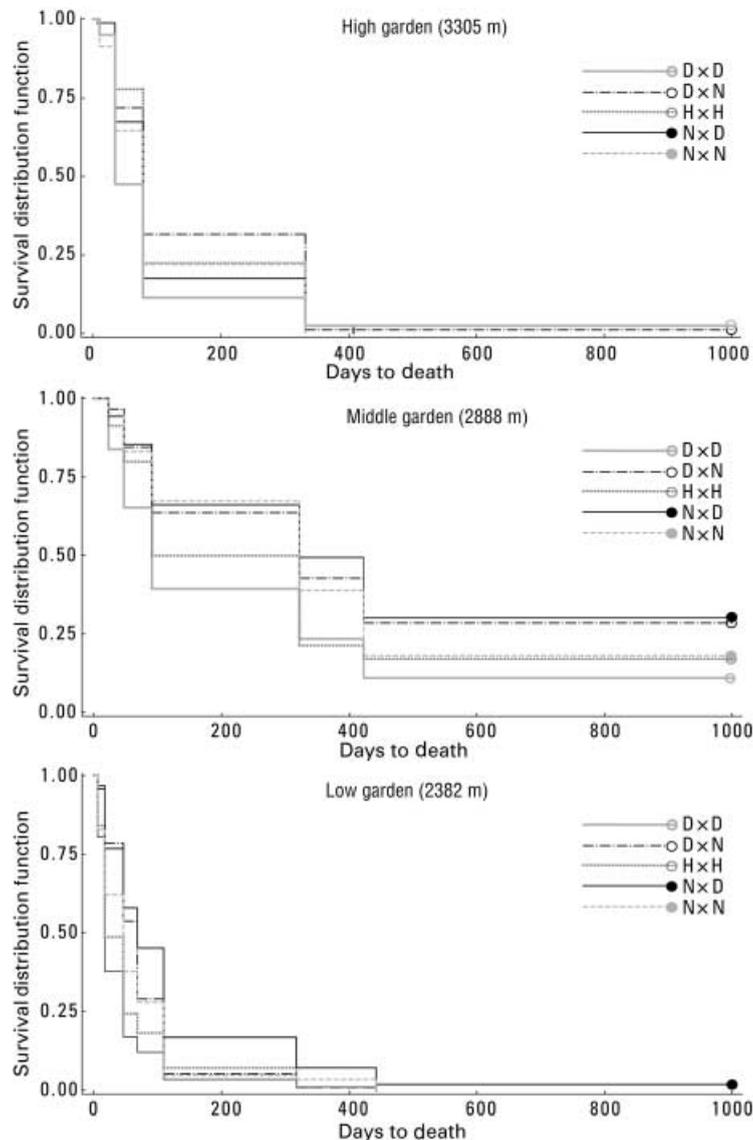
Fig. 5. Survival distribution functions for five cross types in the three gardens. The lines are coded according to the type of cross. The first letter indicates the identity of the mother and the second letter the identity of the father where D = *P. davidsonii*, N = *P. newberryi*, and H = natural hybrid.

died during this period similar regardless of mother, father or the interaction ($P > 0.10$ for all tests). Most species of *Penstemon* do have prolonged dormancy that is fairly difficult to break, even with cold stratification and gibberellic acid (Baskin & Baskin 1998), and alpine *Penstemon* can require longer periods of cold stratification than *Penstemon* from lower elevations (Meyer *et al.* 1995). These results are consistent with our finding that seeds with *P. davidsonii* fathers took longer to germinate than those with hybrid or *P. newberryi* fathers.

Penstemon davidsonii fruits took less time to mature than *P. newberryi* fruits. Fast fruit maturity may be an adaptation to life in the alpine zone, where the growing season is short. The formation of seeds and fruits can slow or stop completely in cold temperatures (Korner 2003), so alpine species must develop quickly before early fall temperatures prevent seed formation. This difference in the timing of fruit maturation could be of great importance in this system if *P. newberryi* is unable to complete its life cycle at high elevations because it takes too long to develop mature fruits from flowers. In a study comparing high-elevation *Mimulus lewisii* to low-elevation *M. cardinalis*, the length of the growing season limited the upper elevational range of *M. cardinalis* for this reason (Angert & Schemske 2005).

Although seeds with *P. davidsonii* mothers were the heaviest, they did not have greater seedling growth than *P. newberryi* or hybrid mothers in the experimental garden near sea level. After 1 year of growth, the largest seedlings were from the type of cross with the lightest seeds. Similarly, in a study of *Senecio* hybrids, one parent species produced many heavy hybrid seeds, while the other species produced hybrids with greater vegetative growth (Kirk *et al.* 2005). Our result

Table 4. The effects of garden elevation, plant type, and the interaction between the two on survival during the peak of the 2006 growing season as measured with a GLM (proc Genmod), and over time with a life table analysis (proc lifereg)

Test	Source	DF	χ^2	P
Proc Genmod	Garden	2	212.97	< 0.0001
	Type	4	21.45	0.0003
	Garden-by-type	8	15.23	0.0549
Proc Lifereg	Garden	2	588.5389	< 0.0001
	Type	4	60.6719	< 0.0001
	Garden-by-type	8	28.2014	0.0004

Table 5. Results of specific contrasts from Proc Genmod on survival during the peak of the 2006 growing season. Heterosis: comparison of mid-parental value of the two species and the F1 average. Natural hybrid: comparison of crosses between natural hybrids and the average of the mid-parent and F1 hybrids. Direction of F1: comparison of the two reciprocal F1 crosses

Garden	Contrast	df	χ^2	P
All gardens	Species	1	0.87	0.3501
	Heterosis	1	18.28	< 0.0001
	Natural hybrid	1	0.13	0.7227
	Direction of F1	1	0.59	0.4435
Low	Species	1	0.15	0.6988
	Heterosis	1	3.37	0.0665
	Natural hybrid	1	0.11	0.7454
	Direction of F1	1	5.71	0.0169
Middle	Species	1	0.18	0.6714
	Heterosis	1	24.99	< 0.0001
	Natural hybrid	1	0.13	0.7177
	Direction of F1	1	0.02	0.8782
High	Species	1	2.79	0.0947
	Heterosis	1	3.92	0.0478
	Hybrid breakdown	1	0.27	0.603
	Direction of F1	1	4.04	0.0445

that *P. davidsonii* yearlings were smaller than *P. newberryi* and hybrid yearlings was not surprising, because alpine plants usually grow more slowly than their montane relatives (Korner 2003; Von Arx *et al*. 2006).

Fruit and seed set in pots did not vary by transect. The greatest difference between plants from different transects was for germination rates, which may reflect the differences in growth conditions that we imposed. In all cases, the relative performance of hybrids in pots was similar for plants taken from the two localities, suggesting that these results are generalizable across transects.

COMPARISONS ACROSS ENVIRONMENTS AND EXOGENOUS ISOLATION

We can test for environmental effects in two ways: most directly by comparing survival across gardens in the reciprocal transplant experiment, and also by comparing the general results from the greenhouse and the field studies. Survival in the reciprocal transplant experiment differed strongly depending on the type of plant, the elevation of the garden

and the type-by-garden interaction, the latter indicating the importance of exogenous isolation or isolation caused by environmental factors. Survival was low in all gardens, likely due to drought stress, especially in the low garden where maximum daily temperatures during summer regularly exceeded 30 °C and vapour pressure deficits were often above 5 kPa (Kimball 2007). Conditions following planting in summer 2006 were relatively dry compared to normal. The closest continuously operational rain gauge, in Bishop, CA, recorded total precipitation across July, August and September 2006 at 0.81 cm less than average, with none falling after the latter part of July <http://cdo.ncdc.noaa.gov/ancsum/ACS?stnid=20002386>. Soil measurements taken with a dielectric aquameter probe (part #S-SMA-M003, Onset Computer Corp.), indicated that the soil in all three gardens was unusually dry in the months following planting, with an average of < 0.024 m^3/m^3 volumetric water content in all three gardens. So our results for relative survival rates are probably most applicable to relatively dry years. Under the conditions in 2006, we found a significantly higher survival rate for F_1 hybrids. Just as in the greenhouse study on early fitness components, F_1 hybrids performed at least as well as the parental types in all of the field gardens. The average results for F_1s do not differ greatly across environments. While we were unable to measure all components of fitness in this study, the consistent finding of high performance for hybrids in nearly all measures in the greenhouse and the field suggests that markedly different results, at least for the survival component of fitness, would be unlikely.

However, the two types of reciprocal F_1 hybrids differed at elevational extremes, such that those with the native mother had highest survival rates in the low and high gardens (Tables 4 and 5, Figure 5). This difference in the survival of reciprocal hybrids across environments was the strongest form of exogenous isolation that we observed, and would not have been possible to detect with just a greenhouse study. Indeed, in the experiment with potted plants, F_1s with *P. davidsonii* as the mother performed consistently better than the reciprocal cross. In most flowering plants, mitochondrial and chloroplast DNA are maternally inherited, and these cytoplasmic genes have the potential to influence fitness (Mazer & Gorchov 1996; Galloway & Fenster 1999; Tiffin *et al*. 2001; Levin 2003). The higher survival of F_1 hybrids with *P. newberryi* mothers in the low elevation garden and of F_1 hybrids with *P. davidsonii* mothers in the high elevation garden could be due to local adaptation of cytoplasmic genes (Galloway & Fenster 1999), although as noted earlier we cannot rule out long-lasting environmental maternal effects. The lack of F_1 hybrids at elevational extremes in nature combined with their high survival rates in the transplant experiment suggests that F_1 hybrids may not form often at elevational extremes, possibly due to phenological differences in flower production.

Many other reciprocal transplant experiments have documented that plants do best in their native environment, indicating local adaptation (Clausen *et al*. 1940; Brassard & Schoen 1990; Wang *et al*. 1997; Fritsche & Kaltz 2000; Campbell

& Waser 2007). In this transplant experiment, however, survival at the parental sites was not higher for the pure parent that originated there. It is possible that differences in overall fitness would have been observed if a sufficient sample of plants had survived long enough to measure reproductive success. There was also no significant difference in the performance of later generation hybrids, indicating either absence of hybrid breakdown in later generations, or positive selection of a subset of recombinant genotypes sufficient to overcome hybrid breakdown.

Although this study only tested post-pollination reproductive isolation, the two parent species and natural hybrids are visited by many of the same species of pollinators (Kimball 2008), so it is unlikely that events during pollination can completely prevent hybridization. Furthermore, as outcrossed flowers make more seeds than selfed flowers (Appendix S1), and pollinator visitation rates are relatively high, hybridization is unlikely to be prevented by a reliance on selfing. Instead, the abundance of hybrids at intermediate elevations, the absence of evidence for endogenous isolation, and the significant plant type-by-garden interaction for survival lead us to conclude that the hybrid zone is likely structured by exogenous isolation.

Conclusion

Our study demonstrates that pre-zygotic and post-zygotic endogenous isolating mechanisms may not be necessary in order to maintain a stable hybrid zone. We found no evidence of genomic incompatibilities preventing hybridization between *P. newberryi* and *P. davidsonii* or between later generation hybrids. Instead, we found differences in plant survival depending on the type of plant and the elevation of the garden, suggesting that exogenous factors, through genotype-by-environment interactions, are more important in the maintenance of these species. Reciprocal F_1 hybrid crosses differed significantly in fruit set, seed number, seed weight, number of days to fruit maturity, vegetative growth and performance in field gardens, suggesting also the importance to hybrid fitness of effects other than those due to nuclear genes. Our results lend support to the growing number of studies documenting the importance of exogenous factors in structuring hybrid zones (Emms & Arnold 1997; Fritsche & Kaltz 2000; Campbell & Waser 2007; Johansen-Morris & Latta 2008).

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Supporting Information

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

Appendix S1 Self-compatibility crosses

Table S1 Vegetative performance in pots

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