Factors limiting hybridization between *Penstemon spectabilis* and *Penstemon centranthifolius*

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Abstract: Speciation involves the formation of reproductive isolating mechanisms such as a difference in pollinators, incompatibility between pollen tubes and stylar tissue, hybrid seed abortion, or poor growth of hybrid seedlings. We studied reproductive isolating mechanisms in naturally sympatric populations of *Penstemon spectabilis* Thurber and *Penstemon centranthifolius* (Benth.) Benth. where F_1 hybrids occurred at very low frequency. We compared conspecific crosses, backcrosses, and heterospecific crosses in terms of pollen grain germination, pollen tube growth, fruit set, seed set, and offspring performance. We found several postpollination barriers to hybridization. When *P. spectabilis* was the ovule parent, the lack of natural hybridization was partially explained by the presence of two isolating factors: reduced pollen tube growth and reduced seed set. When *P. centranthifolius* was the recipient, the barrier to hybridization was nearly 100% effective and occurred primarily at the stages of pollen grain germination and fruit set. The success of backcrossing was generally intermediate between conspecific and heterospecific crossing. For these two species, it is likely that partial pollinator specificity in addition to strong postpollination reproductive isolating mechanisms contribute to maintaining the species boundary.

Key words: speciation, hybridization, introgression, *Penstemon*, reproductive isolating barriers.

Résumé : La spéciation implique la formation de mécanismes d'isolement de la reproduction, tels que des pollinisateurs différents, l'incompatibilité entre les tubes polliniques et les tissus du style, l'avortement des graines hybrides, ou encore une faible croissance des plantules hybrides. Les auteurs ont étudié les mécanismes d'isolement de la reproduction dans des populations naturellement sympatriques du *Penstemon spectabilis* Thurber et du *Penstemon centranthifolius* (Benth.) Benth. où on trouve une très faible fréquence d'hybrides F1. Ils ont comparé les croisement conspécifiques, les rétrocroisements et les croisements hétérospécifiques quant à la germination du pollen, la croissance du tube pollinique, la mise à fruit, la formation de la graine et la performance des descendants. Ils ont trouvé plusieurs barrières post-pollinisation à l'hybridation. Lorsque le *P. spectabilis* constitue le parent ovulaire, l'absence d'hybridation s'explique en partie par la présence de deux facteurs d'isolement : croissance pollinique réduite et formation de la graine réduite. Lorsque le *P. centranthifolius* est le récepteur, la barrière à l'hybridation est pratiquement efficace à 100% et survient surtout aux stades de la germination du pollen et de la mise à fruit. Le succès des rétrocroisements est généralement intermédiaire entre les croisements conspécifiques et hétérospécifiques. Chez ces deux espèces, il semble que la spécificité partielle des pollinisateurs, en plus de forts mécanismes post-pollinisation d'isolement de la reproduction, contribuent à maintenir les frontières entre les espèces.

Mots clés : spéciation, hybridation, introgression, *Penstemon*, barrières d'isolation de la reproduction.

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Introduction

Reproductive isolating barriers may operate at any stage in a plant's life cycle. A difference may arise in the ecological requirements of two species that keeps them from being able to grow microsympatrically ("ecological isolation", e.g., Clements et al. 1999; Goulson and Jerrim 1997; Hodges and Arnold 1994; Catling and Brown 1983). The two species may flower at different times of the year ("phenological isolation", e.g., Catling and Brown 1983). Populations may differ in the types of pollinators that visit them ("ethological isolation", e.g., Fulton and Hodges 1999; Grant 1994; cf. Waser 2000), or the pollen may not be transferred from the anthers of one species to the stigmas of the other ("mechanical isolation", e.g., Fulton and Hodges 1999; Grant and Grant 1964). The pollen may not germinate, or if it does, the pollen tubes may not grow down the style ("incompatibility", e.g., Van Rossum et al. 1996; Weiblen and Brehm 1996; Kress 1983). Even when there is fertilization, there may be abortion of hybrid seeds or the fruits that contain them, or the seeds may be small or unable to grow properly ("embryo failure", e.g., Weiblen and Brehm 1996). Perhaps viable seeds do form, but the hybrid offspring may be feebly endowed ("poor F_1 performance", e.g., Van Rossum et al. 1996). Or the F_1 plants may perform well, maybe even better than the parents, but they may be infertile due to failure of meiotic pairing ("hybrid sterility", e.g., Ohta 1999). Finally, hybridization may not be limited in the first generation, but it may be limited in subsequent genera-

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tions as genes are recombined and coadapted gene complexes are disrupted ("hybrid breakdown", e.g., Kalischuk et al. 1997; Keim et al. 1989). Whether one or more of these isolating mechanisms are responsible for maintaining a given species boundary is a question that can only be answered empirically.

Our report focuses on measuring the reproductive isolating mechanisms that separate *Penstemon spectabilis* Thurber and *Penstemon centranthifolius* (Benth.) Benth. (Scrophulariaceae). The two species often occur sympatrically and flower concurrently, yet hybrids in nature are rare (Wilson and Valenzuela 2002). Straw (1955) proposed that the near absence of naturally occurring hybrids is due to a combination of ethological and mechanical isolating barriers. He considered *P. spectabilis* to be visited and pollinated predominantly by the wasp *Pseudomasaris vespoides* and *P. centranthifolius* to be pollinated by hummingbirds (Straw 1956*a*). Straw (1956*b*) asserted that although pollinator specificity is not complete and hummingbirds may frequent *P. spectabilis* for nectar, the morphology of the flower mechanically prevents hummingbirds from being effective pollinators; therefore, hybridization is limited at the pollination stage. Additionally, Straw (1956*b*) maintained that when rare hybrids are formed, strong pollinator specificity favors the parental types such that backcrosses are eventually subsumed back into the two parental species.

George (1974) tested Straw's hypothesis. She found that while hummingbirds do prefer *P. centranthifolius*, they are also frequent visitors of *P. spectabilis*. She dusted hummingbirds with fluorescent powder and allowed them to visit hand-held flowers to determine whether they were mechanically able to pollinate *P. spectabilis*, and she found that in most cases, they contacted both anthers and stigmas. She did hummingbird exclusion tests and found that hummingbirds could be responsible for up to 15% of pollinations in populations of *P. spectabilis* as measured by fruit set and seed count. This might be an underestimate if by excluding birds, nectar accumulated and increased the visitation of insects to experimental plants. Using another line of evidence, George (1974) also found that *P. spectabilis* plants in hummingbird territories had significantly higher fruit set (51% versus 42%) and significantly higher seed set (49% versus 34%) than those outside hummingbird territories. In order to evaluate mechanical isolation directly, George (1974) watched hundreds of insects to see how they manipulated the flowers and where *Penstemon* pollen became lodged on their bodies. She concluded that many small bees can pollinate both species and that they may account for up to 15% of visits to *P. centranthifolius*. Therefore, since there are no ecological or phenological isolating barriers and only weak ethological and mechanical isolating barriers, we hypothesized that additional postpollination reproductive barriers may help to maintain the species boundary between *P. spectabilis* and *P. centranthifolius*.

In order to measure postpollination reproductive isolating mechanisms, we performed crosses between *P. spectabilis* and *P. centranthifolius*, and we backcrossed in both directions from naturally occurring presumptive F_1 hybrids (often called *Penstemon* ×*parishii* Gray). We then measured pollen grain adherence, pollen tube growth, fruit set, seed set, and offspring performance to determine intercompatibility between the various parental types.

Materials and methods

Species descriptions

Penstemon spectabilis and *P. centranthifolius* are both short-lived perennials. The flowers are protandrous with anthers dehiscing prior to stigma receptivity. In *P. spectabilis*, the stigma is generally receptive 3–5 days after the corolla begins opening. In *P. centranthifolius*, once the flower begins to open, the stigma is generally receptive within 1–2 days. Both species are often found in burned areas and otherwise unvegetated sites. Their geographic ranges are broadly overlapping in southern California and northern Baja California.

The corolla of *P. spectabilis* is vestibular with white on the inside and blue–violet to purple on the lips. The corolla of *P. centranthifolius* is tube shaped and uniformly scarlet. The hybrid is generally intermediate in floral and vegetative morphology between *P. spectabilis* and *P. centranthifolius* (Wilson and Valenzuela 2002). Its corolla is magenta. All three entities have pollen grains of similar size with no sign of poor development in the hybrid.

Study site

Our study began in 1999 at a location in the Santa Monica Mountains near Mulholland Highway, mile marker 24.57 (34°06′N, 118°42′W; 400 m elevation). The site had burned in the late fall of 1996. The soil was gravelly, and the dominant shrub before the burn was *Adenostema fasciculatum*. There were two patches of *P. centranthifolius* surrounded by many *P. spectabilis*. We found two presumptive F_1 hybrid individuals next to each other that were intermediate in flower color, leaf morphology, and many aspects of corolla shape. These two individuals resembled in all respects F_1 hybirds that we later produced artificially, and they were clearly distinguishable from artificial backcrosses. The rarity of these intermediate plants and the fact that they were surrounded by thousands of individuals identifiable as one or the other pure species argue for the likelihood of them being F_1 hybrids. At this site in 1999, *P. spectabilis*, *P. centranthifolius*, and the presumptive F_1s all began flowering in late April and tapered off in late May.

Frequency of hybrids

We collected one flower each from 101 plants and fixed them in formalin – acetic acid – alcohol. These included the two presumptive F_1 individuals that were identified initially by flower color. The rest of the collection consisted of roughly equal numbers from inside the patches of *P. centranthifolius* and amid the adjacent large population of *P. spectabilis*. The flowers were later measured in random order for the following dimensions: the circumference of the corolla at the mouth, the length of a rear stamen, and the length of the middle lobe of the lower lip. Bivariate plots were used to see if there were any intermediate individuals present aside from the two detected based on color.

Pollinator censuses

Although we did not redo George's (1974) extensive study of pollinators, we censused the floral visitors at *P. spectabilis* and *P. centranthifolius* at our study site about every other week throughout the 1999 flowering season (April–June) and on a few other occasions at the study site and elsewhere. Visitor censuses consisted of watching a patch of flowers for 30 min and observing the manner in which each animal handled the flowers and whether it was getting pollen on its body. We would usually census the two species in immediate succession or simultaneously. Insects were collected for later identification.

Design of crossing experiment

Our design involved hand pollinations where there were two possible recipients (either *P. spectabilis* or *P. centranthifolius*) and three possible pollen donor types (conspecifics, F_1s, or heterospecifics). In all hand pollinations, we blocked by recipient, i.e., we selected three similarly positioned flowers on each recipient individual to receive the three types of pollen. Most flowers within a block matured within a few days of one another. Only vigorous plants were chosen as parents. There were 40 blocks with *P. spectabilis* as recipient and 43 blocks with *P. centranthifolius* as recipient. In one case, an individual *P. spectabilis* recipient was used twice for all donor types, and the dependent variables involved were averaged before further analysis. As for donors, we did not keep track of individuals from our large donor pool. A few of them were probably reused. Thus, as regards conspecific and heterospecific crosses, there was a small amount of donor pseudoreplication. As for backcrossing, we had only two presumptive F_1 plants, so we were forced to pseudoreplicate from the point of view of donors, although we did replicate among individuals from the point of view of recipients.

Flowers were chosen when they were showing signs of opening but before anther dehiscence. The corollas of chosen recipient flowers were torn lengthwise about one third of their length. The anthers were cut off the filaments and then the inflorescence was covered with a fine-mesh bag to keep pollinators out. All emasculated flowers were monitored daily to determine the degree of style bending and were pollinated 1 day after the style was fully bent. Donors were carefully picked so that anther dehiscence had just begun and fresh pollen was available. The entire flower was picked, and pollen was used no more than 2 h later. When *P. spectabilis* or a presumptive F_1 was the donor, pollination was done by gently squeezing the anther with forceps to expose fresh pollen and then brushing the anther with exposed pollen against the receptive stigma of the recipient. When *P. centranthifolius* was the donor, we found anthers that were only partially dehisced. In this case, the recipient stigma was pushed into the partially opened anther and rubbed into the pollen. Following pollination, the plants were rebagged for 1–8 days until it was clear that no further pollination could occur. We left the styles intact on about half the blocks, and on the other half, we removed the styles to examine pollen tube growth. Styles were monitored daily after pollination and were collected when they showed the first signs of withering. We tried to keep them attached as long as possible in case removal might affect fruit growth (which in retrospect we believe it did not). Styles were collected individually and fixed in 70% ethanol in microcentrifuge tubes.

Pollen tubes

The collected styles were scored for the number of pollen grains adhering to the stigma, the number of pollen tubes near the top part of the style, and the number of pollen tubes near the base of the style. In order to visualize pollen tubes, the styles were prepared for epifluorescence microscopy. Ethanol-preserved styles were rinsed in water and soaked in 1.0 M NaOH for 12 h when *P. spectabilis* was the recipient and for 18 h when *P. centranthifolius* was the recipient. Styles were then rinsed and soaked in 1% decolorized aniline blue in 0.01 M K_3PO_4 buffer for a minimum of 18 h. At this point, they had been shaken in three solutions, so nonadhering pollen grains should have been rinsed away. The tissue was then placed on a microscope slide, covered with a coverslip, and squashed with a pencil eraser. Additional aniline blue in phosphate buffer was added, and slides were placed in a humidity chamber for a minimum of 6 h. Pollen grains and tubes were viewed using an epifluorescence microscope with illumination at wavelengths of 450–490 nm. In scoring pollen grains and tubes, any count exceeding 100 was recorded as "101".

Fruit and seed set

We monitored fruit maturation in the field and collected fruits before any seeds dispersed. Four to 7 weeks after pollination, fruits were scored as plump and maturing or small and aborting. In order to eliminate any risk of parasitism, maturing fruits were then sprayed weekly with Safer® Yard and Garden Insect Killer (Safer Ltd. Scarborough, Ont.) containing 0.012% pyrethrins. Near the end of the maturation period, fruits were checked daily. At the first signs of dehiscence, a fruit was picked and placed in a glassine envelope to dry fully. When dry, the number of mature seeds was counted, and the seeds in a fruit were collectively weighed. Average seed mass was calculated as this mass divided by the number of seeds. Seeds were then stored in glassine envelopes and refrigerated.

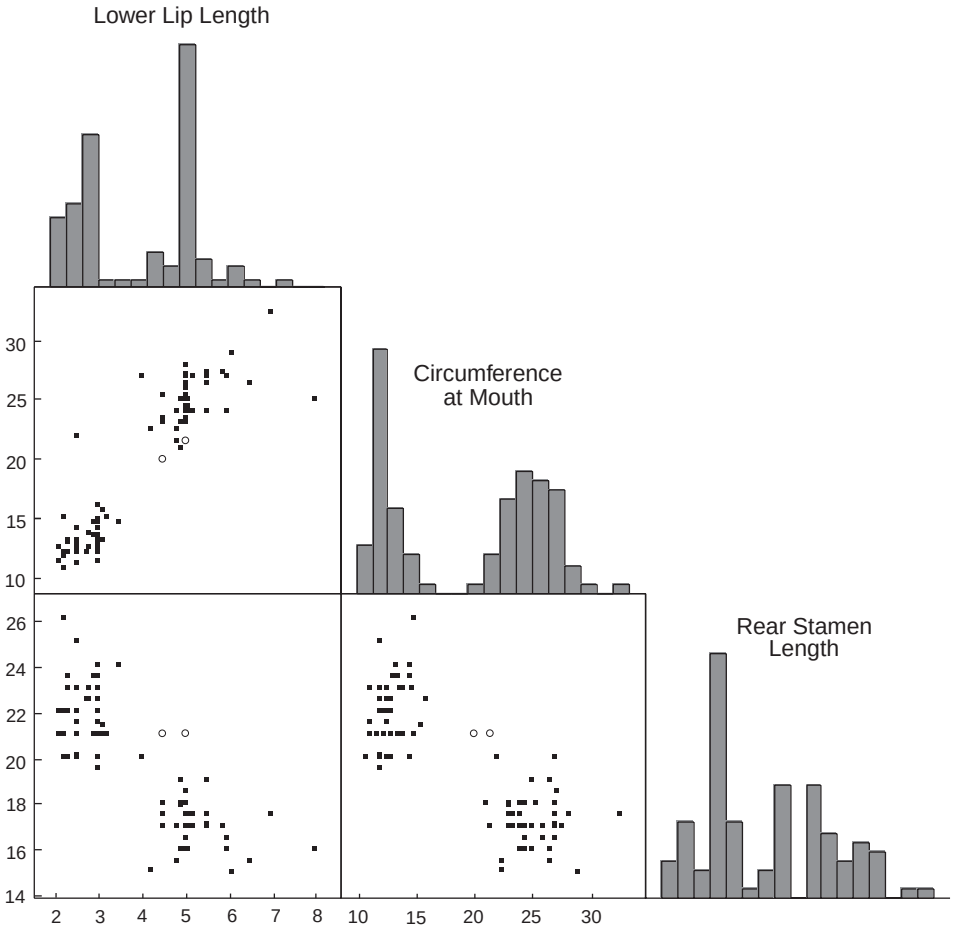
Offspring performance

For measuring offspring performance, we looked only at plants in which *P. spectabilis* was the ovule parent, since relatively few hybrid seeds were produced when *P. centranthifolius* was the ovule parent. Starting in mid-November, we planted five blocks weekly for 5 weeks, yielding a total of 25 blocks each with three donor treatments: conspecific, backcross, and heterospecific. Plantings were staggered because subsequent transplanting of all the seedlings at once would have been logistically impossible. Twenty seeds per fruit, when available, were planted for germination into pots with a mixture of two parts potting soil, two parts vermiculite, and one part perlite. In addition, we added approximately 0.5 g of powdered charcoal from the chaparral shrub *A. fasciculatum* to the soil surface for enhanced germination (Keeley 1991). *Penstemon* seeds were germinated in the greenhouse and kept moist. When the majority of seedlings had three sets of leaves, they were moved outside to acclimate for 3–7 days, and then individual seedlings were transplanted to 4-in. (1 in. = 2.54 cm) pots with a mixture of one part pumice to one part organic matter. Seven to 11 weeks later, when over two thirds of the plants within a block showed roots emerging at the bottom of the pots, plants were transplanted to 1-gal (1 gal = 4.546 L) pots in one part pumice to one part organic matter, and up to five plants from each fruit were grown to flowering. All plants had comparable watering and nutrient addition schedules. Five weeks after transplantation into 4-in. pots, the number of leaves and the length of the three longest leaves were recorded. This measurement was repeated a second time at 14 weeks (near the time when the first plants began to flower). We intended to measure vegetative size again at the start of flowering and reproductive effort in terms of flower number, but most of the plants did not flower anytime near the normal flowering season, and comparisons would have been greatly influenced by the length of time that plants had to grow. We were interested in growth of the overall plant, so we combined the changes in leaf length and leaf number from 5 to 14 weeks into one variable: this was done by averaging after each component variable was standardized by subtracting the minimum difference from each value and dividing by the range. Statistics were run on the combined variable.

Statistics

All crosses and the measurements that followed were done in a block design with pollen recipients as blocks. Since fruit set was a categorical variable, it was analyzed by McNemar's test for repeated measures of the same individual (Sokal and Rohlf 1995, box 17.16). If we had not done block tests (if we had used tests of independence), we would have been ignoring the fact that each recipient (block) was subjected to each of the three treatments as opposed to a recipient being subjected to only one treatment. The McNemar test removes the effect of block (recipient plant) and determines whether *on the same plant* heterospecific crosses are less likely to result in a mature fruit than conspecific crosses. Our anal-

Fig. 1. Histograms and bivariate scatterplots showing the bimodal distribution of *Penstemon* individuals into two species. The two individuals shown with open circles were identified as likely F_1s based on color. $N = 101$. Some points are underneath other points with exactly the same coordinates. Units are all millimetres.



yses of quantitative variables—pollen grains adhering, pollen tube ratio calculated as number of tubes at the base of the style divided by number at the top, seed number, seed mass, and offspring performance—were done as two-way mixed-model ANOVAs with recipient individual as the block factor and donor type as the fixed factor (Sokal and Rohlf 1995, box 11.3). Thus, in these analyses, the effect of recipient (block) was also removed, allowing us to contrast the effects of donor types on individual recipients.

When we found a significant effect of donor type, we did multiple comparisons between all combinations of donor types (conspecific, backcross, and heterospecific). Since these data were within a block design, we subtracted the block means from all numbers, i.e., we calculated the residuals from a one-way ANOVA among recipient plants, and then we did Tukey multiple comparisons on these residuals. All continuous variables were graphed as means ± SEs of residuals from blocks. These graphs show the relative (not absolute) performance of the three crosses within blocks. Unadjusted means are given in the text.

There were complications with the data on pollen grain adherence and pollen tube growth. Both of these variables yielded residuals that appeared normally distributed, but we were concerned about parametric assumptions given that a considerable number of the variates were scored as 101 (i.e., more than 100). Also, there were sometimes as many as three outlier data points out of 60; for these, we changed the value to the next most extreme value (Sokal and Rohlf 1995, p. 407). We will present parametric results from this modified data set; however, to assess the robustness of our results, we also did Kruskal–Wallis tests, and unless otherwise noted, they yielded similar conclusions. For nonparametric multiple comparisons on pollen grains adhering and pollen tube ratios, we did Mann–Whitney *U* tests adjusted by the simultaneous test procedure (Sokal and Rohlf 1995, box 13.8).

Results

Frequency of hybrids

Morphological measurements for *P. spectabilis* and *P. centranthifolius* were bimodally distributed, with nearly all individuals easily identified as one species or the other. The two individuals initially presumed to be hybrids were intermediate, as was one other individual (conceivably a backcross to *P. spectabilis*). This was clearly evident in bivariate scatterplots (Fig. 1). The frequency of intermediates was extremely low, even in this sample taken from where the two species came into contact and did hybridize. In fact, a few thousand additional individuals were observed without finding any more magenta flowers of the hybrid phenotype.

Pollinator censuses

During all of the 30-min visitor censuses, we observed animals at *Penstemon* flowers. Hummingbirds included Anna's, Costa's, and Black-chinned (*Calypte anna*, *Calypte costae*, and *Archilochus alexandri*), and usually they all had obvious

Table 1. Number of 30-min censuses at which hummingbirds and nectaring Hymenoptera were observed pollinating the two species of *Penstemon*.

	P. centranthifolius	*P. spectabilis*	Fisher's exact test *P*
Hummingbirds	13/13	5/9	0.017
Nectaring Hymenoptera	0/13	9/9	<0.001

Note: Ratios represent the number of times pollinators were present to the number of censuses.

Penstemon pollen on their foreheads. Nectaring Hymenoptera included *Bombus*, *Anthophora*, *Osmia*, *Centris*, *Xylocopa*, *Apis*, and the wasp *Pseudomasaris vespoides*; these nectaring insects often had pollen on their heads and the backs of their thoracies. We also saw a number of small pollen-collecting bees that would turn upside down, manipulating the anthers with their mouth parts and legs; however, we believe that when visitation by nectaring animals is high, pollen collectors are probably parasitic "pollen thieves" (Thomson et al. 2000; Wilson and Thomson 1991). Pollen collectors visited both *P. spectabilis* and *P. centranthifolius*, and since the animals are small, there are no physical barriers preventing them from reaching the anthers (Mitchell 1989). Considering only nectar collectors, there were significant differences between the plant species in whether they were pollinated by hummingbirds or Hymenoptera (Table 1). For hummingbirds, this preference was incomplete; although hummingbirds visited *P. spectabilis* less than *P. centranthifolius*, visits to both species were recorded and could well be responsible for some level of interspecific pollination. Nectaring insects were only seen visiting *P. spectabilis*, and it appears that the floral tube of *P. centranthifolius* is too narrow to admit a bee or a wasp as large or larger than a honeybee.

Pollen grains and tubes

Conspecific crosses were generally more successful in terms of pollen adherence and pollen tube growth than were backcrosses or heterospecific crosses, although not all multiple comparisons were significant. When *P. spectabilis* was the recipient plant, there were no significant differences among donor types in the number of pollen grains adhering to the stigma (unadjusted means: 96.8 for conspecifics, 92.0 for backcrosses, 94.7 for heterospecifics; Fig. 2A). However, the ratio of pollen tubes that reached the bottom of the style differed significantly among donor types, with conspecific crosses having a greater proportion of pollen tubes reaching the bottom than either backcrosses or heterospecific crosses (respective unadjusted means: 1.177, 0.827, 0.789; Fig. 3A). For *P. centranthifolius* recipients, Tukey multiple comparisons indicated that significantly more pollen grains adhered to *P. centranthifolius* stigmas when pollen was conspecific than when it was heterospecific; the backcross treatment was intermediate and not significantly different from either conspecific or heterospecific treatment (unadjusted means: 93.2 for conspecifics, 80.6 for backcrosses, 65.2 for heterospecifics; Fig. 2B). When these data were analyzed nonparametrically, the overall Kruskal–Wallis test indicated significant heterogeneity among donor types, but the simultaneous test procedure failed to identify any significant contrasts. Regarding pollen tubes in *P. centranthifolius*, there was no significant difference among donor types (respective unadjusted mean ratios: 0.534, 0.452, 0.516; Fig. 3B).

Fruit set, seed set, and seed mass

Fruit set, seed set, and seed mass generally tended to be higher in conspecific crosses than in backcrosses or heterospecific crosses. On *P. spectabilis* plants, fruit set (the ratio of the number of mature fruit to the number of flowers) was significantly greater in conspecific crosses (38/40, not taking into account the blocking of recipients) than in heterospecific crosses (29/40), although backcrosses (33/40) were not significantly different from either (Fig. 4A). For flowers that set fruit on *P. spectabilis*, seed number was significantly greater in conspecific crosses and backcrosses than in heterospecific crosses (respective unadjusted means: 55.3, 50.8, 34.5; Fig. 5A). There was a similar pattern for seed mass (0.748, 0.737, 0.682 mg; Fig. 6A). On *P. centranthifolius* plants, fruit set systematically decreased with conspecifics having the greatest fruit set (27/43), backcrosses having intermediate fruit set (10/43), and heterospecifics having the least (1/43); all treatments were significantly different (Fig. 4B). For those *P. centranthifolius* fruits that set, seed number was significantly greater in conspecific crosses than in backcrosses (respective unadjusted means: 36.9 and 17.3; Fig. 5B), and seed weight was significantly greater in conspecifics than in backcrosses (respective unadjusted means: 1.548 and 1.209 mg; Fig. 6B). There was only one heterospecific cross that set fruit, so we could not test for differences between it and other types of crosses for seed set and seed mass.

Offspring performance

Hybrid seed (although not germinating at anywhere near 100%) had much greater germination rates than conspecific seed. It may be that there is some mechanism of dormancy in *P. spectabilis* that is less prominent in the hybrids. For those seeds that did germinate, we were very successful at growing plants to maturity, but they did not all mature at the same time. Maturation varied by block and dramatically by treatment, with the F_1s flowering in early summer and the offspring of conspecific crosses in the late summer. Most of the backcrosses did not flower until the following spring. Offspring performance, as measured by our composite variable indexing the amount of leaf growth from week 5 to week 14, did not differ much between treatments (respective unadjusted means: 0.521, 0.537, 0.504). F_1 offspring were larger by about 1% of the range than conspecifics, and backcrosses were about 2% smaller. Neither of these differences was significant, although F_1s were signficantly larger than backcrossed offspring (Fig. 7).

Discussion

Multiple reproductive isolating barriers?

We seemed to find reproductive isolating barriers at several stages for one or both crossing directions: pollinator visitation, pollen germination and tube growth, fruit set and seed set, and backcrossed offspring performance. In actuality, our design did not always show whether each stage truly represented a separate incompatibility or was an effect of a

Figs. 2–7. Various barriers to interbreeding. S, *P. spectabilis*; X, presumptive F_1s; C, *P. centranthifolius*. In all cases except Fig. 4, these are two-way mixed-model ANOVAs with recipient individuals as blocks and donor type as a fixed factor. Values are means ± SEs of the residuals from blocks; donor types with the same letter were not significantly different ($P > 0.05$) by Tukey multiple comparisons. Fig. 2. Pollen grain germination on *P. spectabilis* stigmas ($MS_{error} = 300.6$, df = 38; $MS_{recipient} = 479.0$, df = 19; $MS_{donor} = 115.8$, df = 2; $P = 0.68$) and on *P. centranthifolius* stigmas ($MS_{error} = 980$, df = 40; $MS_{recipient} = 1336$, df = 20; $MS_{donor} = 4130$, df = 2; $P < 0.022$). Fig. 3. Pollen tube growth in *P. spectabilis* styles ($MS_{error} = 0.177$, df = 38; $MS_{recipient} = 0.175$, df = 19; $MS_{donor} = 0.915$, df = 2; $P < 0.010$) and in *P. centranthifolius* styles ($MS_{error} = 0.129$, df = 34; $MS_{recipient} = 0.136$, df = 17; $MS_{donor} = 0.034$, df = 2; $P = 0.77$). Fig. 4. Fruit set. Values are number of blocks, i.e., recipient plants. Thus, the 29 in the top left corner is the number of plants on which both the flower that received *P. spectabilis* pollen and the flower that received *P. centranthifolius* pollen set fruit. The comparison of interest in each small table is between the upper right cell [–, +] and the lower left cell [+, –]: 0 versus 9 in the top table shows that conspecific failure with heterospecific fruit set never happened, whereas heterospecific failure with conspecific fruit set occurred on nine plants. These comparisons of the upper right with lower left counts are binomial sign tests with a Bonferroni adjustment for three comparisons using the same data; a significant difference is indicated when $P <= 0.017$, as denoted by an asterisk. Fig. 5. Seed set in *P. spectabilis* fruits ($MS_{error} = 226$, df = 52; $MS_{recipient} = 887$, df = 26; $MS_{donor} = 3239$, df = 2; $P < 0.0001$) and in *P. centranthifolius* fruits ($MS_{error} = 148$, df = 6; $MS_{recipient} = 194$, df = 6; $MS_{donor} = 1340$, df = 1; $P = 0.024$). Fig. 6. Average mass of seeds $\times 10^{-4}$ g in *P. spectabilis* fruits ($MS_{error} = 0.978$, df = 52; $MS_{recipient} = 6.723$, df = 26; $MS_{donor} = 3.427$, df = 2; $P = 0.037$) and in *P. centranthifolius* fruits ($MS_{error} = 1.642$, df = 6; $MS_{recipient} = 8.854$, df = 6; $MS_{donor} = 40.34$, df = 1; $P = 0.003$). Fig. 7. Offspring performance from 5 to 14 weeks after transplanting ($MS_{error} = 0.00823$, df = 278; $MS_{recipient} = 0.14232$, df = 22; $MS_{donor} = 0.02826$, df = 2; $P = 0.03366$).

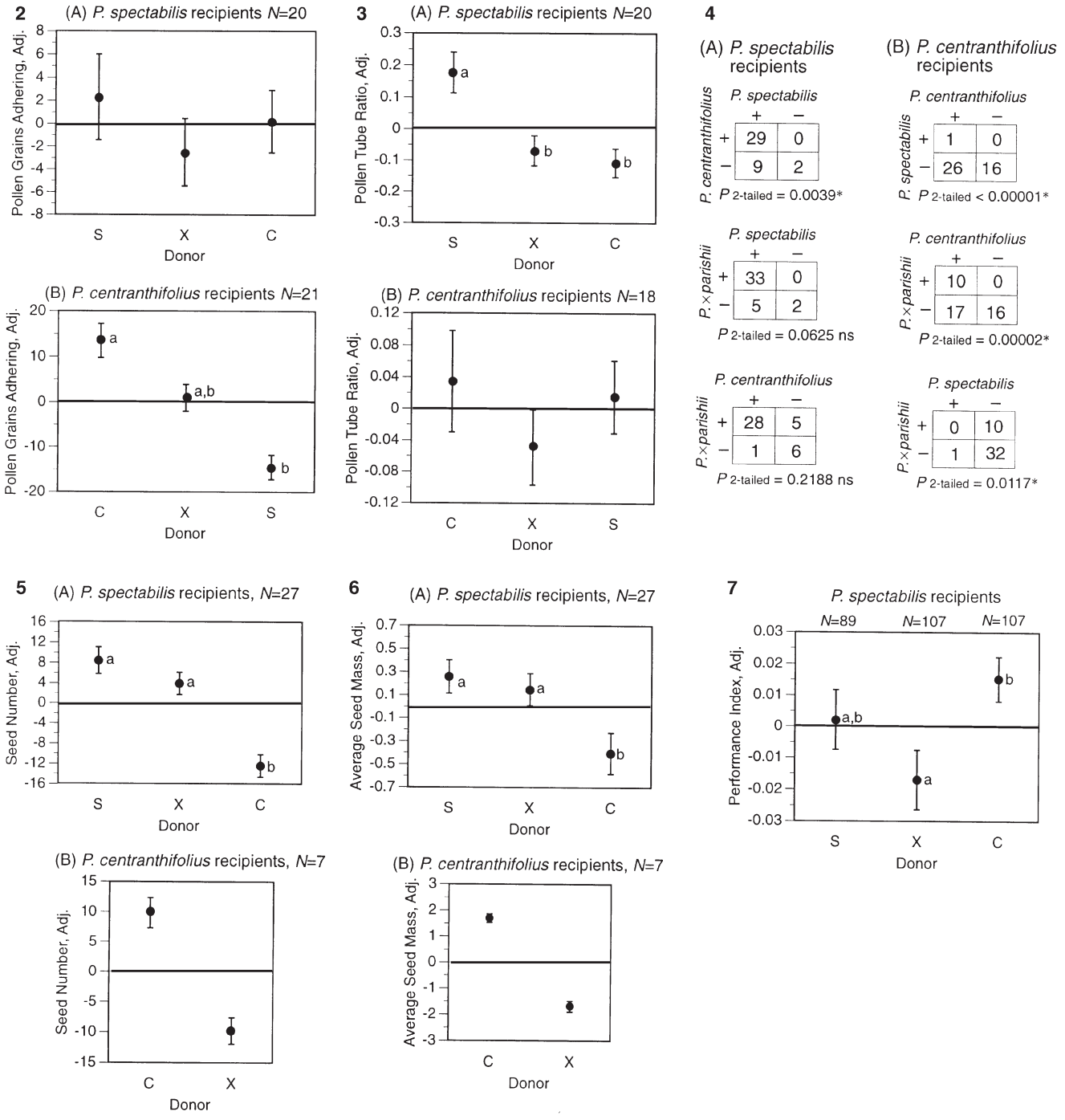
Table 2. Summary of hybridizing success expressed as proportions of conspecific reproductive success at each stage.

Measured effect	Pure effect	Cumulative effect
When *P. spectabilis* was the maternal parent and F_1s were backcrossed to *P. spectabilis*		
Pollen adhering, $a = 0.978$	$a = 0.978$	0.978
Tubes at top of style, $b = 1.043$	$b/a = 1.066$	1.043
Tubes at bottom of style, $c = 0.779$	$c/b = 0.747$	0.779
Seeds produced, $d = 0.457$	$d/c = 0.587$	0.457
F_1 performance, $e = 1.030$	$e = 1.030$	0.471
F_1-sired pollen adhering, $f = 0.950$	$f = 0.950$	0.447
F_1 tubes at top, $g = 0.854$	$g/f = 0.899$	0.402
F_1 tubes at bottom, $h = 0.702$	$h/g = 0.822$	0.330
F_1-sired seeds, $i = 0.729$	$i/h = 1.038$	0.343
Backcross performance, $j = 0.968$	$j = 0.968$	0.332
When *P. centranthifolius* was the maternal parent and F_1s were backcrossed to *P. centranthifolius*		
Pollen adhering, $a = 0.700$	$a = 0.700$	0.700
Tubes at top of style, $b = 0.591$	$b/a = 0.845$	0.591
Tubes at bottom of style, $c = 0.593$	$c/b = 1.003$	0.593
Seeds produced, $d = 0.024$	$d/c = 0.041$	0.024
F_1 performance	—	—
F_1-sired pollen adhering, $f = 0.865$	$f = 0.865$	0.021
F_1 tubes at top, $g = 0.879$	$g/f = 1.016$	0.025
F_1 tubes at bottom, $h = 0.812$	$h/g = 0.925$	0.022
F_1-sired seeds, $i = 0.173$	$i/h = 0.213$	0.004
Backcross performance	—	—

Note: Pollen tubes at top and bottom of style were accounted for separately here (unlike in Fig. 3), and seeds produced here was calculated including flowers that produced zero seeds (unlike in Fig. 5).

barrier at a previous stage. For example, if pollen grains did not germinate well and pollen tubes did not grow well, these incompatibilites could have caused fruit not to set well or seed counts to be low. In the cases of fruit and seed set, we measured cumulative effects. In other cases, such as offspring performance, we measured the effect of a stage by itself, what might be termed a "pure" effect.

Here we attempt to parse out the pure effect of each of a series of stages in the life cycle (for a similar approach, see Campbell et al. 1998). We do this by calculating the pure fitness component of heterospecific crosses at a stage relative to that of conspecific crosses (Table 2). Keeping track of statistical error would be difficult, but the exercise is at least useful as a way of structuring our conclusions. First, we express the interbreeding success as a proportion of the conspecific success at each stage that we measured. For example, to calculate the relative success of pollen tubes reaching the bottom of the style, we divide the average number of pollen tubes at the bottom of the style in heterospecific crosses by the average number found at the bottom in conspecific crosses. This calculation gives us our "measured effect" c. To calculate the pure effect, we assume that between stages the fitness compo-



nents are multiplicative. Therefore, the pure effect of the tubes reaching the bottom of the style is the measured effect *c* divided by the measured effect of the previous stage, i.e., of relative tube success at the top of the style *b*. The cumulative effect is the product of all pure effects since the start of heterospecific pollination and is equal to the measured effect for each stage up through the production of seeds *d*. The next stage, F_1 performance *e*, is conditional on seeds having set, so the cumulative effect here is $d \times e$. We then proceed to imagine that the F_1s would backcross to their maternal parent species, and we continue to calculate cumulative effects by multiplication as the species introgress. Details of the calculations are given in Chari (2000).

When *P. spectabilis* was the ovule parent (Table 2), there was essentially no reduction in hybridization success as pollen grains were germinating and beginning to grow tubes, but at the bottom of the style, there were fewer tubes. Then at the stage of seed production, there was another relatively large drop in the cumulative crossing success, and the pure effect on seed production seems substantial at 0.587. Those F_1 seeds that were produced, however, went on to do well, and pollen from F_1 plants germinated well. There was then a slight decrease in the success of F_1 pollen tubes reaching the bottom of the style. Subsequent seed set was high, and the backcrossed offspring performance was only slightly worse than that of conspecific plants. The cumulative effect of hy-

bridization when *P. spectabilis* was the ovule parent and F_1 pollen was backcrossed to *P. spectabilis* stigmas yielded offspring leaf issue at a rate of 0.332 compared with conspecifically bred *P. spectabilis*.

When *P. centranthifolius* was the ovule parent (Table 2), there appeared to be some barrier to heterospecific pollen germination, and there were additional strong limits to hybridization at the stage of seed production. Then, in the F_1 generation, the backcrossing success once more decreased significantly, again at the stage of seed production. Cumulatively, there was a nearly complete barrier to introgression with backcrossed seeds being produced at a rate of 0.004 per conspecific seed.

The 0.004 represents a near complete barrier to hybridization, but the 0.332 potential for introgression toward *P. spectabilis* does not seem sufficient to explain why backcrossed plants are not evident in the field. Having grown backcrosses to flowering, we believe that we would notice them most but not all of the time (a few individuals are very like *P. spectabilis*). It may be that since hummingbirds are only responsible for 15% of the pollinations in *P. spectabilis* (George 1974), and only a limited percentage of these hummingbirds will carry heterospecific pollen, the cumulative effect is lessened further. And it is conceivable that the hybrids may be undervisited by both birds and bees (Straw 1955). Finally, pollen competition may be occurring whereby, if both heterospecific and conspecific pollen is received on a stigma, the conspecific pollen may outcompete the heterospecific pollen.

Generally, isolating barriers that happen before seed development waste less of the mother plant's energy than producing seed that is inviable or hybrid offspring that are sterile. In our species pair, it appears that the F_1 individuals (at least those with *P. spectabilis* as the ovule parent) if anything are more robust than the parental species, and the bulk of reproductive isolation is indeed before seed development. F_1 pollen fertility is generally high, with backcrossed pollen tubes and seed production generally intermediate between heterospecific and conspecific success rates. Backcrossed offspring perform nearly as well as conspecific offspring with only very weak hybrid breakdown (Fig. 7). It should be noted, however, that we grew our plants with abundant water and nutrients. Natural, harsher conditions might have selected more strongly against backcrosses. Such has been found in *Artemesia* (Wang et al. 1997). However, habitat does not always influence hybrid survivability, as found in *Iris* (Emms and Arnold 1997) and in *Phlox* (Levin and Schmidt 1985).

Biases in the direction of crossing

In addition to a downward trend in compatibility from conspecific crosses to backcrosses to heterospecific crosses, the success of a cross is also greatly affected by which species is the ovule parent and which is the pollen parent. Reproductive isolating barriers are strongly asymmetrical. When *P. centranthifolius* was the maternal parent receiving heterospecific pollen, only one in 43 fruit set, whereas when *P. spectabilis* received heterospecific pollen, fruits set in 29 of the 40 trials (Fig. 4). Since pollen grain germination is significantly inhibited when *P. centranthifolius* receives *P. spectabilis* pollen, the actual mechanism may occur at this early stage, at fertilization, or postzygotically (Figs. 2–5). Regardless of the mechanism, pollen transfer from *P. centranthifolius* to *P. spectabilis* is much more likely to produce hybrid individuals than pollen transfer in the other direction. There are also probably biases in the direction of pollen movement. George (1974) found that hummingbirds often visit *P. spectabilis* flowers after extensive foraging bouts on *P. centranthifolius*. The directional asymmetry continues in the backcrossing stage. When *P. centranthifolius* is the maternal parent, both seed number and seed mass are significantly less in backcrosses than in conspecifics (Figs. 5 and 6). This difference between backcrosses and conspecifics is not present when *P. spectabilis* is the maternal parent.

Biases in directionality have been found in many recent studies on pollen tube competition (Wolf et al. 2001; Howard 1999). When pollen tubes are competing in the same style, heterospecific tubes may grow at a slower rate or show a greater attrition than conspecific tubes, with the degree of isolation dependent upon the stylar tissue. This has been found in *Prunus* (Perez and Moore 1985), *Iris* (Carney and Arnold 1997; Carney et al. 1996), *Helianthus* (Rieseberg et al. 1995), and *Hibiscus* (Klips 1999). Therefore, in natural sympatric populations, where pollen loads are likely to be mixed, hybridization may be prevented by pollen tube competition. Variable attrition rates may offer an explanation for the asymmetry in hybridization abilities. If the styles of the two species vary in length, then when the species with a longer style acts as the pollen recipient, hybridization may be limited to a greater extent than the reciprocal cross because heterospecific pollen tubes are equipped to travel shorter distances (Howard 1999). A similar argument could be envisioned based on chemical encouragement of pollen tubes by stylar tissue rather than style length per se. This may explain the asymmetry, and it could be a further reason why hybrids are so rare. Our study did not explore pollen tube competition, but in the future, it will be of interest to ascertain whether this prezygotic isolating mechanism is at work in *Penstemon*.

Further confirmation of biases in directionality are found in molecular studies of our *Penstemon* complex. When looking at chloroplast geneologies, Wolfe and Elisens (1995) found that the two collections of naturally occurring presumptive F_1s (*Penstemon* ×*parishii*) that they studied both had *P. spectabilis* haplotypes, indicating that *P. spectabilis* was the maternal parent. In Wolfe and colleagues' research using allozymes (Wolfe and Elisens 1993), nuclear rDNA restriction sites (Wolfe and Elisens 1994), and ISSR markers (Wolfe et al. 1998*a*, 1998*b*), there was more successful transfer of markers from *P. centranthifolius* to *P. spectabilis* than in the reverse direction. Wolfe et al. (1998*a*, 1998*b*) concluded that there is likely pollen-mediated gene flow via hummingbirds occurring from *P. centranthifolius* to *P. spectabilis*.

Conclusion

In summary, a series of reproductive isolating mechanisms exist between *P. spectabilis* and *P. centranthifolius* occurring at various stages of the life cycle. These include both pre- and postzygotic barriers to hybridization. Initially, there is weak pollinator specialization in that bees and wasps tend to visit *P. spectabilis*, while hummingbirds tend to prefer *P. centranthifolius*. Following pollination, with both

P. spectabilis and *P. centranthifolius* recipients, there is partial pollen–style incompatibility. In instances where pollen tubes do grow successfully, there is another barrier present in that fruits do not set as successfully in heterospecifics, and when fruits do set, seeds are fewer in number and may be smaller. Finally, weak hybrid breakdown may limit hybrid swarming.

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