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Pollinator-mediated gene flow fosters genetic variability in a narrow alpine endemic, *Abronia alpina* (Nyctaginaceae)[1]

Meredith D. Jabis[2], Tina J. Ayers, and Gerard J. Allan

Environmental Genetics and Genomics Laboratory, and Department of Biological Sciences, Northern Arizona University, P.O. Box 5640, Flagstaff, Arizona 86011 USA

- *Premise of the study*: For rare and endemic plants that exist in small, isolated habitats, natural selection is expected to favor self-compatibility, which can result in low genetic diversity due to inbreeding and genetic drift. Using *Abronia alpina*, a rare alpine endemic of the California Floristic Province, we demonstrate that there are exceptions to these predictions.
- *Methods*: We present the results of both a pollination experiment and a genetic study using AFLPs (amplified fragment length polymorphisms). Using controlled hand-pollination and pollinator observations, we examined the breeding system, pollination ecology, and mechanism for self-incompatibility in *A. alpina*.
- *Key results*: *Abronia alpina* exhibits an allogamous mating system with probable self-incompatibility resulting from limited growth of pollen tubes originating from self-pollination. Only xenogamous crosses and open-pollinated controls produced seed, and only xenogamous crosses produced pollen tubes that reached the ovary. The molecular study shows that *A. alpina* has substantial genetic diversity for a rare, endemic species, evidenced by the high percentage of polymorphic loci and average expected heterozygosity. Gene flow among subpopulations, as inferred from AFLP markers, appears to be substantial, although the Kern River is an important physical barrier.
- *Conclusions:* Our results indicate that *A. alpina* is dependent on insects for both seed production and the maintenance of genetic diversity. This finding suggests that pollinators may be crucial to the long-term adaptive potential of rare, endemic plants and that conservation of rare endemics is, in part, dependent on community-level interactions such as plant–pollinator mutualisms.

Key words: *Abronia;* AFLP; allogamy; endemic; genetic diversity; outcrossing; pollination biology; population structure; rare plant; self-incompatibility.

Understanding genetic diversity within rare species is crucial to their conservation, as genetic diversity has been used as an indicator of extinction risk (Gaudeul et al., 2000; Allendorf and Ryman, 2002). Clarifying the life history traits that may contribute to or weaken levels of genetic variation in rare plants is an important, related goal (Gitzendanner and Soltis, 2000). Several studies suggest that rare species are more likely to be self-compatible, have lower reproductive investment, and lower genetic diversity when compared with closely related common species (Karron, 1987; Hamrick and Godt, 1989; Hamrick et al., 1991; Kunin and Gaston, 1993, 1997; Gaston and Kunin, 1997; Kunin and Shmida, 1997; Lavergne et al., 2004). However, other studies have found that narrowly endemic species can exhibit both outcrossing and have relatively high levels of genetic diversity (Tepedino et al., 1999; Williamson and Werth, 1999; Schmidt and Jensen, 2000; Zawko et al., 2001; Rottenberg and Parker, 2003). The mating system of rare plants is an important life history trait because it relates directly to gene flow and the maintenance of genetic diversity (Ellstrand and Elam, 1993). In general, allogamous breeding systems are expected to maintain high levels of genetic diversity in plants because they provide a mechanism for persistent gene exchange (Hamrick et al., 1991; Hamrick and Godt, 1996a, b). Conversely, natural selection resulting from pollinator limitation in small isolated or low-density populations is suggested to cause rare, isolated species to favor autogamy (Karron, 1987; Ellstrand and Elam, 1993; Holsinger, 1996; Kearns et al., 1998; Saunders and Sipes, 2006). In high-density populations, pollinators tend to be more species-constant and are more likely to carry appropriate pollen (Kunin, 1997). This combination of small population size and self-compatibility can further diminish genetic variability via reduced gene flow between individuals (Cole, 2003).

Endemic plants represent an important fraction of the native vascular plant species (2124 endemics out of 3488 total) in the California Floristic Province (CFP), a well-recognized center of global biodiversity (Shevock, 1996; Davis et al., 1997; Myers et al., 2000). Identifying mechanisms that promote genetic variability within biodiversity hotspots is crucial to preserving diversity worldwide, particularly in the context of global climate change. We used *Abronia alpina* to test whether its apparent allogamous breeding system, suggested by showy, fragrant flowers, promotes genetic diversity in this rare and narrowly endemic species. *Abronia alpina* is an especially good candidate for evaluating the relationship between reproductive ecology and genetic diversity in a rare endemic because its locally abundant population size permits experimental manipulation.

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[2]Author for correspondence (e-mail: Meredith.jabis@gmail.com)

[3]Inyo County Water Department, P.O. Box 337, 135 South Jackson St., Independence, California 93526 USA

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Abronia alpina is restricted to the Sierra Nevada Mountains of California, USA, and is a candidate species for listing under the Endangered Species Act. It is confined to sandy, arkosic gravel margins between sagebrush scrub and lodgepole pine forest on the edges of two meadows, Ramshaw and Templeton, at an elevation of approximately 8800–9000 ft (Fig. 1). Although its floral morphology suggests allogamy as a predominant mode of reproduction, little is known about its breeding system or genetic variability, both of which can have important consequences for its long-term survival. Many other species in the genus are obligate outcrossers, including *A. umbellata*, *A. maritima*, *A. latifolia*, and another narrow endemic, *A. macrocarpa* (Tillett, 1967; Williamson et al., 1994; Williamson and Bazeer, 1997). Some species in the family do exhibit self-compatibility (Spellenberg, 1986; McMullen, 1987; Kelso et al., 2003), and one endemic species of *Abronia, A. ammophila*, has a mixed mating system (Saunders and Sipes, 2006).

We established the following goals and objectives: (1) to determine the breeding system of *A. alpina* using a combination of experimental field- and laboratory-based studies; (2) to assess the potential for self-incompatibility; (3) to characterize the community of potential pollinators and their relative contribution to promoting gene flow via foraging behavior; and (4) to assess whether the genetic makeup of *A. alpina* is consistent with a predominantly allogamous or autogamous mode of reproduction and how genetic diversity was distributed both within and among subpopulations relative to other rare endemics and more common widespread species.

MATERIALS AND METHODS

Study species and sampling—*Abronia alpina* (Ramshaw Meadows sand verbena) is a short-lived, mat-forming, perennial species. Individuals have a primary taproot and do not exhibit asexual reproduction via rhizomes or propagative roots. The species is restricted to a single population of between 60 000 and 130 000 individuals distributed over 6 ha. The population is naturally fragmented into 34 subpopulations because of incursions of lodgepole pine forest or wet meadow into the sandy margin habitat (Fig. 1). These subpopulations are geographically separated by distances of between 500 and 3000 m, and they vary widely in their population numbers, ranging from under 100 individuals to 60 000 individuals in the largest subpopulation. The Kern River flows through the middle of Ramshaw Meadow, physically separating the northern from the southern half of the meadow. All the subpopulations exist in Ramshaw Meadow with the exception of a single Templeton Meadow subpopulation (Fig. 1). Extensive monitoring for approximately 20 yr by the U.S. Forest Service shows that the population experienced a decline between 2000 and 2009 (see Andre, 1990, for protocol). The wet spring of 2010 produced a substantial seedling crop, indicating that spring precipitation or runoff may be an important predictor of seedling success (Jabis, personal observation). Population counts vary from 10 000–60 000 individuals between census events, possibly due to differential survival of large seedling crops in subsequent years.

During seed maturation, the developing infructescence reflexes and is eventually so weighted that fruits are overtopped by the foliage. Because the mother plant often touches or covers the ground, seed movement by wind or runoff is greatly inhibited. In her comparative study of nine species of *Abronia* in California, Wilson (1976) suggested dispersal is limited because *A. alpina* fruit lack winged appendages found in other members of the genus. Wilson further suggested that this trait might be an adaptive mechanism to keep the species in appropriate habitat in the sandy margins of the meadows. When in bloom, the plant is covered in pink, tubular, fragrant flowers (Fig. 2). Nectar is present at the base of the floral tube. Plants bloom from May to September (Jabis, personal observation).

Reproductive biology—*Floral morphology and phenology*—To determine the overall position of the floral parts for the breeding system experiment, flowers were field dissected and lengths of the floral tube, ovary, style, stigma, and distance between style and anthers were recorded for 47 flowers. To determine the number of days a single flower was open, a random flower was chosen from each of 54 random plants and was observed until it closed. Day of pollen dehiscence and changes in the stigma were observed.

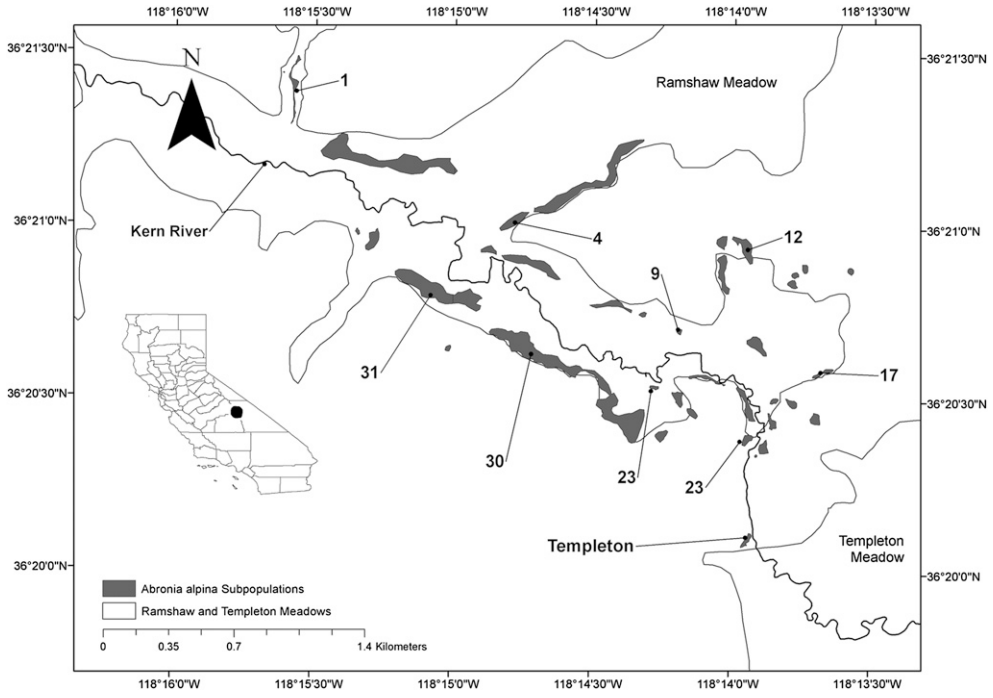

Fig. 1. Study site: Ramshaw and Templeton Meadows, Inyo National Forest, California, USA. *Abronia alpina* subpopulations are depicted by gray polygons. Numbered polygons indicate subpopulations sampled for the genetic diversity study. The black line depicts the south fork of the Kern River, which connects the two meadows, flowing southwest.

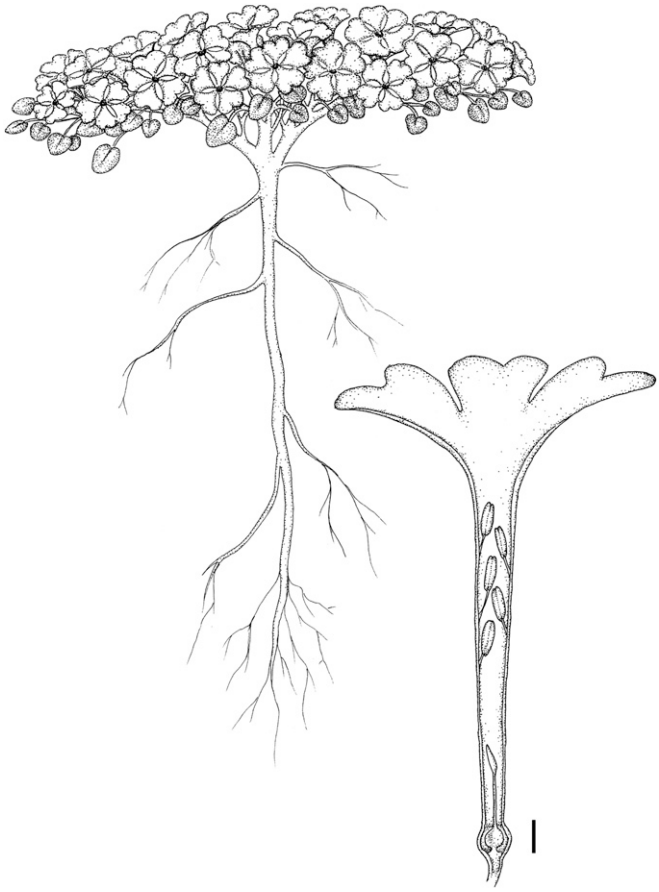

Fig. 2. *Abronia alpina* in flower. A large proportion of the plant is covered with fragrant flowers. The scale bar is 1 mm for the flower and 1 cm for the plant. Illustrator: Mara Trushell.

Breeding system—To determine the breeding system, 37 plants were randomly selected and isolated by using cages covered with nylon tulle to exclude potential insect pollinators. Plants were covered except when hand-pollinations were performed. Four pollination treatments were applied to each plant: (1) self-pollination—pollen from one flower was transferred to the stigma of the same flower; (2) self-pollination with emasculation—removal of male parts by cutting the top portion of the floral tube to control for emasculation in the two other treatments; (3) geitonogamy—transfer of pollen from a flower to the stigma of another flower on the same plant; and (4) xenogamy—transfer of pollen from a flower on one plant to the stigma of a flower on another plant. Each treatment was applied to one inflorescence per plant. All treatments were performed on day 1 and day 2 of anthesis to ensure pollen was transferred when the stigma was receptive. All geitonogamous and xenogamous crosses were emasculated before transference of foreign pollen. All xenogamous treatments were performed within 10 m of the donor plant. Pollen was transferred using #00 insect pins that were cleaned with 95% isopropyl alcohol between each flower. For each treatment plant, a neighboring plant was identified, and an inflorescence was marked as an open-pollinated control. Fruit set was recorded and seeds collected after 4 to 6 wk, once fruit was mature. Seeds were separated visually into two categories. Seeds that were extremely desiccated, with a dull seed coat, severely damaged, or underdeveloped were assumed to be nonviable. All other seeds were tested for viability by using a 1% solution of tetrazolium chloride following a modified protocol from the *Tetrazolium Testing Handbook* (Peters, 2000). Seeds were soaked for 18 hr in water, the seed coat was nicked with a dissecting probe, and the seeds then were soaked approximately 70 hr in tetrazolium chloride before we checked for viability using a 40× dissecting microscope. To assess the type of breeding system, the proportion of fruit set by treatment per plant was analyzed with a χ^2 test in JMP 8.0 (SAS Institute Inc., Cary, North Carolina, USA). To determine whether seed viability of experimental treatments differed from that of open-pollinated controls, we analyzed the percentage of viable seed by treatment using a χ^2 contingency table.

Self-incompatibility—To test for a potential mechanism for self-incompatibility, 15 plants were subjected to three treatments described in the breeding system section: self-pollination (n = 89 flowers), geitonogamy (n = 87 flowers), and xenogamy (n = 86 flowers). All treatment flowers were emasculated. Hand-pollinations occurred during two separate pollination treatments in June and July. Following hand-pollinations, the pollen tube was allowed to grow for 6, 10, 12, 24, or 48 hr. The entire emasculated flower then was collected and fixed in either 95% ethanol or in a 3 : 1 solution of 95% ethanol and acetic acid. Fixed tissues then were treated with 1 N NaOH for approximately 1 hr to soften and clear the styles (Williamson and Bazeer, 1997). The tissue then was rinsed with deionized water and soaked in a 0.1% decolorized analine blue solution for 12 hr, the perianth was removed, and the ovary, stigma, and style were mounted on a slide with a drop of a decolorized analine blue glycerol solution (1 : 1). Tissues were viewed under a Leitz Aristoplan fluorescent microscope using the 10× objective lens (Leica Microsystems, Wetzlar, Germany). The distance of the longest pollen tube for each treatment, and each time interval, were recorded in increments (0, 0.1, 0.5, 1.0, 1.5, 2.5 mm). Pollen tube length was averaged by treatment and analyzed using PERMANOVA (Anderson, 2001, 2005).

Pollinator observations—To determine which potential insect pollinators visited *A. alpina* and whether pollination was likely effective, insects were observed and collected during the months of June, July, and August 2008. All pollination experiments and observations were conducted in the largest subpopulation (subpopulation 30, see Table 1 and Fig. 1) to limit any potential harm to individuals in smaller subpopulations. Eight 10-m^2 sampling locations were designated for 45 observation sessions that lasted 30 min. During each session, the identity of all insects that visited *A. alpina* plants and their actions and behaviors were recorded. A single observer recorded the number of visits to both flowers and plants by each insect. Visitors were not identified to the species level, and only potentially effective pollinators were identified. Potentially effective pollination required a proboscis of substantial length, approximately 10 mm, to reach the stigma. Observation sessions were conducted at dawn (0500–0700, n = 2), in the morning (0700–1200, n = 17), in the afternoon (1200–1700, n = 20), in the evening (1700–1900, n = 1), at dusk (1900–2030, n = 3), and after dark (2030–2200, n = 2). Between each observation session, insects were collected and proboscis lengths recorded. All insects were identified by the Bogart Museum of Entomology at University of California, Davis, and vouchers were deposited at NAU Colorado Plateau Museum of Arthropod Diversity.

To determine which potential pollinators were the most frequent visitors to *A. alpina*, we analyzed differences in overall visitation rate by species per hr and pairwise differences between species using PERMANOVA. The potential pollinator with the greatest visitation rate when present was found by dividing the total number of flowers it was observed visiting by the amount of time it was actually present in hr. Pollinator activity also was analyzed by month using PERMANOVA and by time of day using a Kruskal-Wallis test. Nonparametric tests were chosen because the data did not conform to assumptions of normality or equal variance because of the absence of some pollinator species during certain months. PERMANOVA was used because it allows partitioning of variance of the distance matrix while preserving the distribution-free qualities of nonparametric tests. We calculated the test statistic directly from the distance matrix and the *P* values using random permutations of the data (Anderson, 2001).

TABLE 1. Genetic diversity within subpopulations in *Abronia alpina*. *N* is the estimated subpopulation size, *N2* is the number of individuals that produced profiles, *P* is the percentage of polymorphic loci, and H_e is the average expected heterozygosity assuming Hardy-Weinberg equilibrium.

Subpopulation	*N*	*N2*	*%P*	H_e
1	950	26	80.17	0.278
4	513	29	82.64	0.311
9	85	30	83.47	0.317
12	1435	26	73.55	0.266
17	1395	29	75.21	0.271
Templeton	405	29	74.38	0.267
23	325	30	80.99	0.310
26	225	30	81.82	0.313
30	59933	24	72.73	0.278
31	1875	24	76.03	0.300

Genetic variability—*Population sampling and DNA extraction*—Leaf material was collected from a single stem from each plant. Young leaf tissue was collected from 30 individuals in 10 subpopulations for a total of 300 individuals. Locations of sampled subpopulations are shown in Fig. 1, and average estimated population size of each subpopulation from the USFS 2009 Census is summarized in Table 1. The sampling design ensured representation of the entire subpopulation. To test for genetic-based geographic correlation, we sampled from both neighboring and geographically distant subpopulations.

Fresh leaf tissue was collected in the field and dried by using either silica gel or Drierite (W. A. Hammond, Xenia, Ohio, USA). Dried material was weighed and then pulverized in a Geno/Grinder (SPEX Sample Prep, Metuchen, New Mexico, USA) before extraction. We extracted DNA using a Qiagen DNeasy 96 Plant Kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol with slight modifications. Quality and quantity of DNA was checked by gel electrophoresis with a 2% agarose gel. DNA also was quantified by using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA).

Genetic analyses—The amplified fragment length polymorphism (AFLP) method (Vos et al., 1995) was used to assess genetic diversity and structure in *A. alpina*. This method is especially useful for rare plants because it produces markers that are randomly distributed throughout the genome, allowing small genetic differences to be detected with relatively little amounts of DNA (Mariette et al., 2001; Rottenberg and Parker, 2003). In addition, it is particularly effective for detecting genetic variation, structure, and differentiation below the species level (Mueller and Wolfenbarger, 1999; Althoff et al., 2007). We used a modified protocol adapted from Hersch-Green and Cronn (2009) to generate AFLP markers. A total of 5–50 ng of genomic DNA was cut with restriction enzymes *EcoRI* and *MseI* to generate fragments. These fragments then were ligated with 5 and 50 pmol of the adapters *EcoRI* and *MseI*, respectively, which contain complementary sequences, for 16 h at 22°C. We performed a preselective amplification using the *EcoRI*-A (Applied Biosystems, Foster City, California, USA) and *MseI*-C primers (Integrated DNA Technologies, San Diego, California, USA).

The preselective reaction contained 2.5 μL of 1 : 5 diluted ligation product added to a final volume of 25 μL of the reaction mixture, which contained 1× reaction buffer (BioRad, Hercules, California, USA), 1 mg/mL BSA (New England BioLabs, Ipswich, Massachusetts, USA), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 0.05 U/μL *Taq* DNA polymerase (BioRad), and 0.375 uM and 1 uM of *EcoRI*-A and *MseI*-C primers, respectively. The amplicons of this preselective PCR were amplified again with selective primer pairs.

Eight selective primer pairs were tested on 10 individuals to determine those producing the most informative and robust profiles. Three were chosen: *EcoRI*-ACT *MseI*-CGT, *EcoRI*-AGC *MseI*-CAG, *EcoRI*-ACC *seI*-CGT. The selective reaction contained 1.25 μL of 1 : 2 diluted preselective amplicon added to a final volume of 10 μL of the reaction mixture, which contained 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 0.05 U/μL *Taq* DNA polymerase, and 0.375 uM and 1uM of *EcoRI*- and *MseI*-selective primers, respectively. The *EcoRI*-selective primers were labeled with either FAM or NED fluorescent dyes.

The selective amplicons were diluted 1 : 8, and 1.5 μL was added to 10 μL MapMarker1000 and 10 μL of formamide solution for a total injection volume of 11.5 μL. This mixture was injected on an AB 3730XL genetic analyzer (Applied Biosystems) by using capillary electrophoresis to separate the fragments. We analyzed the AFLP fragments using GeneMapper v.3.7 (Applied Biosystems).

Data analysis—*Within-population genetic diversity*—AFLP fragments were scored as present or absent for each individual. Only repeatable bands with peak heights ≥300 relative fluorescence units (rfu) were scored. Loci that were 95–100% monomorphic were removed from the data set, and only polymorphic markers were used for further analyses. Estimation of allele frequencies with dominant markers such as AFLPs presents statistical challenges (Lynch and Milligan, 1994; Zhivotovsky, 1999; Tero et al., 2003). However, it has been shown that highly polymorphic data sets with approximately 30 individuals per population sampled give accurate estimates (Krauss, 2000; Bonin et al., 2007). Given that *A. alpina* exhibits a floral morphology that suggests outcrossing as a predominant mode of reproduction, we assumed that populations were in Hardy-Weinberg equilibrium, as demonstrated in studies of other outcrossing species (Krauss, 2000; Kremer et al., 2005; Bonin et al., 2007; Meudt and Clarke, 2007). The resulting data matrix was analyzed for population genetic diversity using Popgen 1.31 (Yeh and Boyle, 1997) and GenAlEx 6.2 (Peakall and Smouse, 2006). Percentage polymorphic loci and average expected heterozygosity (H_e) were calculated for each subpopulation and for the entire population, whereas Nei's genetic diversity (1987) was averaged across loci for all individuals for a single value for the entire population. Because genetic diversity is expected to decrease as population size decreases (Ellstrand and Elam, 1993), we also generated a McDonald's (2008) Spearman rank correlation coefficient to examine the potential correlation between subpopulation size, N, and genetic diversity, H_e.

Among-population genetic structure and geographic correlation—We estimated gene flow between subpopulations using two indirect measures. First, using Popgen 1.31, we calculated Nei's (1987) measure of subpopulation differentiation, G_{ST}, and Nm, an estimate of gene flow from G_{ST}. Second, we used analysis of molecular variance (AMOVA; Excoffier et al., 1992) in GenAlEx 6.2 to calculate the percentage of genetic variation that resides within, between, and among areas of subpopulations using 9999 permutations. Pairwise Φ_{PT} values also were generated from the AMOVA using 9999 permutations.

We tested geographic correlation using the unweighted pair group method with arithmetic mean (UPGMA), created using Nei's (1972) genetic distance as implemented in *MEGA 4* (Tamura et al., 2007). A two-dimensional scatter plot using a nonmetric multidimensional Scaling (NMDS) ordination was generated in PCORD5 (McCune and Mefford, 2006). Jaccard distance was chosen because it accounts for only shared presence of bands, not shared absence, thus reducing the risk of absence homoplasy (Bonin et al., 2007). We also performed a Mantel test to check for correlation between genetic (Nei, 1972) and geographic distances (km) using GenAlEx 6.2. A PERMANOVA was used to test for pairwise genetic differences between populations on the basis of shared fragment presence using 4999 permutations.

Population structure also was analyzed with the program Structure 2.2 (Pritchard et al., 2000; Falush et al., 2003, 2007) to determine the most likely number of true populations from our data set on the basis of probability of assignment. We conducted a series of 10 independent runs for each possible value of K (the number of subpopulations) between 2 and 10, using a burn-in period of 5000 iterations and an MCMC (Markov chain Monte Carlo) of 100 000 iterations. Admixture between subpopulations and correlated allele frequencies was assumed. We used the average ln likelihood for each value of K after the 10 independent runs to determine the optimum value of K; the smallest number of subpopulations (K) with the highest ln likelihood was assumed to be the true number of subpopulations. In addition, we selected the smallest number of subpopulations (K) with the greatest rate of change in log probability for all potential values of K. In addition, this value of K had the highest ΔK or rate of change of the likelihood function (Evanno et al., 2005). The program CLUMPP 1.1.1 (Jakobsson and Rosenberg, 2007) then was used to combine the independent runs to produce a single probability of membership for each individual for the optimal value of K. The program *DISTRUCT* 1.1 (Rosenberg, 2004) was used to graph individual membership probabilities.

RESULTS

Reproductive biology—*Floral morphology/phenology*—Each inflorescence had an average of 3.4 flowers, and each flower was open for approximately 2.85 d. Anther sacs were open on the first day of anthesis concurrent with pollen dehiscence. The stigmatic surface appeared receptive on the first day of anthesis; the only change was a slight drying in the stigma observed over time. Flowers were open diurnally and nocturnally, and they remained open throughout anthesis. A small amount of nectar was present, likely less than 1 mm in depth, at the base of the floral tube. The perianth had an average floral tube length of 13 mm, range 8–17 mm. Flowers were noticeably fragrant.

Breeding system—Only xenogamous crosses and open-pollinated controls produced fruit (n = 130, 120, 113, 121, and 102 flowers manipulated for self-pollination, self-pollination with emasculation, geitonogamous, xenogamous, and open-pollinated controls, respectively). Fifty-eight percent of xenogamous

crosses and 73% of open-pollinated controls produced anthocarps. No significant difference existed between the proportion of seeds produced per plant for the xenogamous treatments and for the open-pollinated controls ($\chi^2 = 2.39$, df = 1, $P = 0.122$). The stigma was receptive on days 1 and 2 of anthesis on the basis of observation of the surface and xenogamous seed set.

Self-incompatibility—Consistent with our breeding system study, we found only xenogamous crosses produced pollen tubes that reached the ovary (Figs. 3A, B). This finding was statistically significant by treatment, with the xenogamous pollen tubes growing significantly longer than both forms of selfing ($F_{2, 254} = 14.34$, $P = 0.0002$). Mean xenogamous pollen tube growth was 0.76 mm (n = 88), whereas autogamous and geitonogamous treatments rarely extended beyond the stigma (mean = 0.23 mm, n = 85; and 0.27 mm, n = 84; respectively).

Pollinator observations—Consistent with an allogamous breeding system, 157 insects per flower per hr and 48 insects per plant per hr were observed visiting the flowers of *A. alpina* during active pollinator sessions. Potential pollinators were observed only during morning and afternoon sessions, with no pollinators observed at dawn, during the evening, at dusk, or after dark. Sixteen species of insects visited the plants; these were seen either landing on or collecting pollen from the plants, whereas only seven species were observed as displaying potentially "effective" pollinator behavior, feeding on nectar from the base of the ovary with a proboscis of substantial length. These represented five families of insects: Hesperiidae (skippers), Papilionidae (swallowtails), Nymphalidae (brush-footed butterflies), Sphingidae (sphinx moths or hawk moths), and Bombyliidae (bee flies). One species of Apidae (bees) also was observed visiting the flowers and crawling into the floral tube; however, the proboscis of this species is too short to affect pollination. Two species of thrips (*Frankliniella occidentalis* and *F. minuta*) were found in the flowers, apparently feeding on the pollen. All insects considered potentially effective pollinators had proboscises of at least 10 mm (Table 2), with the exception of the *Bombylius*. Although the proboscis of the *Bombylius* appears too short to effect pollination (6.4 mm), this species primarily feeds on nectar (Hull, 1973); so it seems likely that its proboscis can reach the stigmatic surface if it can reach the bottom of the floral tube to collect nectar. The four most frequent visitors were *Thorybes mexicanus* (Mexican cloudywing), *Bombylius lancifer* (bee fly), *Hyles lineata* (white-lined sphinx), and *Polites sonora* (Sonora skipper) (Fig. 4A). Pairwise tests using PERMANOVA indicated that *B. lancifer*, *T. mexicanus*, and *P. sonora* visit significantly more times than all others ($F_{6, 224} = 10.23$, $P = 0.0002$, pairwise Bonferroni corrected $\alpha = 0.0024$, $t > 3.03$, $P < 0.0024$).

The most effective potential pollinator appeared to be *Hyles lineata* (Fig. 4B), a hawk moth in the Sphingidae. This species was observed during only two sessions, but it visited on average 600 flowers during the single hr it was present. It was observed visiting many flowers per plant and many plants in rapid succession. Insect visitors were observed in June, July, and August, but June was statistically the most important month for pollination ($F_{2, 30} = 5.9144$, $P = 0.0002$; $t = 3.1$ and 2.6, and $P = 0.0002$, respectively, for pairwise comparisons), this seems to be driven by two important likely pollinators: *Thorybes mexicanus* and *Bombylius lancifer*, which were observed only in June and were not present in July or August. No difference in

Fig. 3. Pollen tube growth following experimental pollination treatments. (A) Pollen tube of a xenogamous cross reaches ovary. (B) Geitonogamous cross; pollen tube does not grow past stigma. Scale bar = 1 mm.

pollinator activity was found between the morning and afternoon sessions overall ($\chi^2 = 1.29$, df = 1, $P = 0.2549$).

Genetic variability—*Within-population and subpopulation genetic diversity*—With the use of three primer pairs, 150 scored fragments ranging in size from 50–600 bp were generated, 121 (80.7%) of which were polymorphic. Of the 300 individuals assayed, 277 showed robust amplification profiles across all three primer combinations. On average, each individual produced 82 bands. Subpopulation size estimates, number of individuals that produced profiles, and genetic diversity values for each subpopulation are listed in Table 1. Percent polymorphic loci ranged from a high of 83.5 to a low of 72.7, average expected heterozygosity ranged from 0.313 (±0.017) to 0.267 (±0.018), and the Shannon's information index ranged from 0.395–0.468. Population-level average expected heterozygosity was 0.291, and gene diversity, H_T (Nei, 1987), was 0.351. All diversity statistics gave similar information: *A. alpina* maintains substantial genetic diversity, and this variation appears to be evenly distributed across subpopulations. No correlation between subpopulation size and genetic diversity was found (Spearman's $\rho = -0.612$, $P = 0.06$). The largest subpopulation showed moderate levels of genetic diversity when compared with smaller subpopulations with fewer individuals; the smaller subpopulations showed no evidence of reduced genetic variability.

Among-population genetic structure—The allogamous reproductive strategy of *A. alpina* appears to permit substantial gene flow between subpopulations. This is documented with four separate analyses. Nei's measure of subpopulation differentiation indicates medium to low overall population structuring ($G_{ST} = 0.172$) and relatively high estimated gene flow (Nm = 2.42). The AMOVA also indicates little among subpopulation variation (13%, Table 3, $P = 0.0001$), with most of the variation, 78%, found within subpopulations ($P = 0.0001$), which is similar to the among-population differentiation calculated by G_{ST} (17.2% among, 82.8% within). An analysis of the two sections of the meadow, separated by the south fork of the Kern River, indicates a low level (9%) of subpopulation structuring by section ($P = 0.0001$). Average pairwise Φ_{PT} was 0.182, with a low of 0.058 between subpopulations 9 and 12 and a high of 0.369 between subpopulations 30 and Templeton. Some subpopulation pairs, particularly within regions, show very low Φ_{PT} values, indicating subpopulation similarity. This structured result is corroborated by UPGMA, AMOVA, and NMDS analyses.

The UPGMA tree indicates two groups (Fig. 5) separated by the Kern River. The Templeton Meadow subpopulation, though located on the south side of the river, weakly clusters with the northern group. This overall geographic structuring indicates some amount of topographic isolation created by the south fork of the Kern River, which agrees with the AMOVA findings and with lower Φ_{PT} differentiation values for the subpopulations within each clade in the UPGMA tree.

The NMDS ordination concurs with low overall differentiation. The final ordination represents 54% of the original distance matrix. All individuals are relatively close in ordination space; there are no extremely distant clusters of individuals (Fig. 6). Clustering of individuals by the northern or southern side of Ramshaw Meadow is significant ($F_{1, 275} = 38.09$, $P = 0.0002$, Fig. 6), confirming some amount of genetic isolation by the south fork of the Kern River.

TABLE 2. Pollinator species collected with proboscis lengths.

Scientific name	No. collected	Proboscis length (mm)
Hesperiidae (skippers)		
Gaeides editha editha	2	—
Polites sonora sonora	6	11.5
Thorybes mexicanus nevada	3	12.0
Papilionidae (swallowtails)		
Papilio zelicaon	1	18.0
Nymphalidae (brush-footed butterflies)		
Euphydryas editha aurilacus	2	8.5
Sphingidae (sphinx moths/hawk moths)		
Proserpinus clarkiae	1	15.0
Hyles lineata	0	25.0 – 48.0
Bombyliidae (bee flies)		
Bombylius lancifer	6	6.4

Geographic correlation/isolation—As was indicated by the AMOVA, the UPGMA tree, and the NMDS ordination, some amount of partitioning of genetic variation exists on either side of Ramshaw Meadow. The Mantel test revealed no genetic by geographic distance correlation ($R^2 = 0.039$, $P = 0.125$). Isolation by distance was not found. This indicates that linear distance is not as important as the physical isolation created by the south fork of the Kern River.

The Structure analysis indicated a value of $K = 2$, which was the lowest and most likely number of subpopulations from our data (ln likelihood = –21050.28, ln probability = –22537.97). In addition, this value of K had the highest ΔK (Evanno et al., 2005) or rate of change of the likelihood function. This corroborates well with overall estimates of gene flow between subpopulations and the topographic effect of the Kern River found in the AMOVA, UPGMA, and NMDS ordination. The Structure graph of individual membership assuming two subpopulations is shown in Fig. 7A. Templeton Meadow is placed with the northern group by Structure, which agrees with the UPGMA tree. Subpopulation 23, on the south side of the river, shows some individuals who share membership with the northern group. This subpopulation is located in a severe constriction, where the Kern River exits Ramshaw Meadow, a distance of ~35 m separates the two sides of the meadow at this juncture; here the river would not present as much of a physical barrier to gene flow. The graph assuming $K = 3$ clearly distinguishes the Templeton subpopulation from both the northern and southern groups (Fig. 7B). Assuming $K = 10$, a greater number of individuals showing high levels of admixture is depicted (Fig. 7C). The Structure analysis confirms substantial gene flow between subpopulations and is consistent with the NMDS ordination, AMOVA, and UPGMA results showing substructure created by the Kern River.

DISCUSSION

Floral morphology, breeding system, and self-incompatibility—Although rare plants are expected to be self-compatible (Karron, 1987), our study demonstrates that *A. alpina* is dependent on pollination for fruit and seed set. The floral morphology of *A. alpina* readily allows for self-pollination; however, only xenogamous crosses produced seed or pollen tubes that successfully reached the ovary. These results are consistent with the large effort the plant allocates for pollinator attraction,

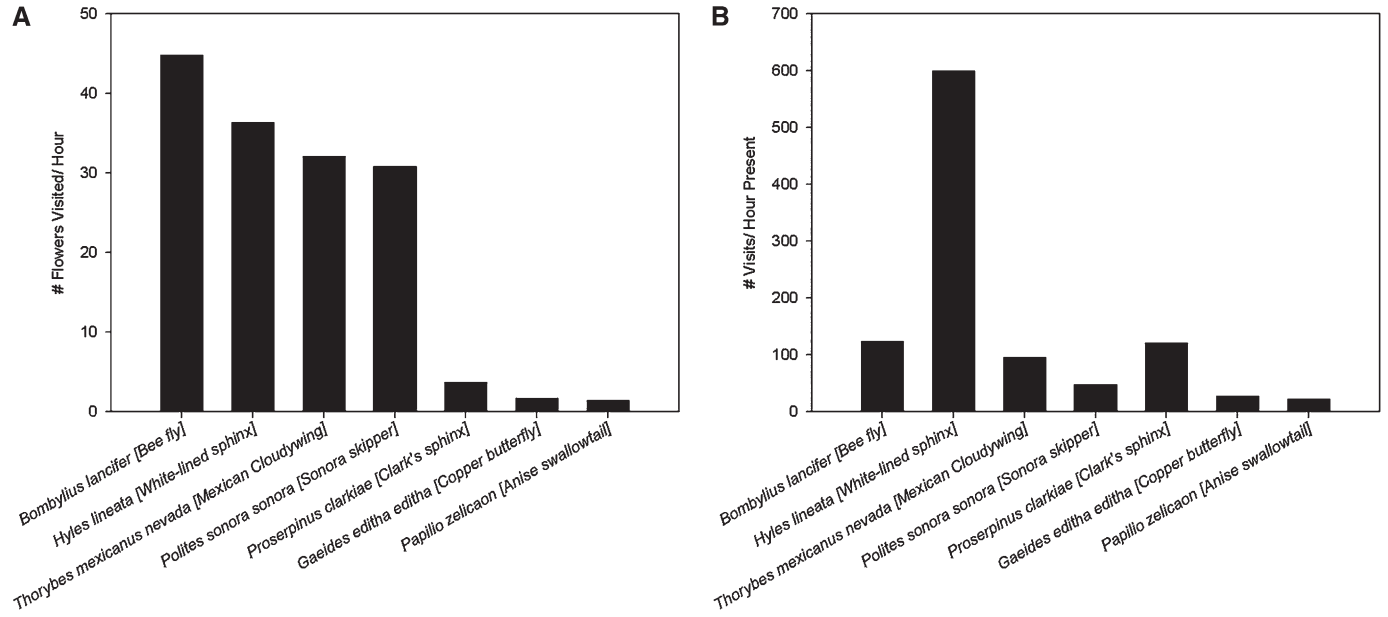

Fig. 4. (A) Visitation rate by hour overall by potential pollinator species using total observation time during all morning and afternoon sessions. Pairwise tests indicate that the *Bombylius* and the *Polites* pollinate significantly more times than all others except *Thorybes mexicanus* (pairwise $t > 3.03$, $P < 0.0024$). (B) Visitation rate by hour when present. *Hyles lineata* was present only during two observation sessions, but it visited on average 600 flowers per hour when present.

exemplified by a showy form and strong fragrance. Because of its apparent reliance on pollinators for seed set, the abundance of likely pollinators in any given year may be one factor contributing to the species' erratic population fluctuations. Thirteen species in the Nyctaginaceae have been documented to be sphingophilous, pollinated by hawk moths, nocturnal butterflies or moths. Noctuid moths were important primary pollinators for another endemic species, *Abronia ammophila* (Saunders and Sipes, 2006). The floral morphology of *A. alpina* is consistent with the hawk moth floral syndrome: pale pink to white, fragrant flowers with a long, slender nectar tube (Grant, 1983; Grant and Grant, 1983; Hodges 1995). In our study, the mechanism of self-incompatibility appears to be the result of slow growth or no growth of pollen tubes derived from self-pollination. This is consistent with other studies that have demonstrated that self-pollen grows slower than outcrossed pollen (Aizen et al., 1990; Williamson and Bazeer, 1997).

Pollinators—Rare plants in isolated habitats are expected to experience pollinator limitation due to reduced visitation (Lamont et al., 1993; Fischer and Matthies, 1998; Groom, 1998; Hackney and McGraw, 2001; Mustajavari et al., 2001), transfer of incompatible pollen (Rathcke, 1983; Jennersten, 1988; Aizen and Feinsinger, 1994), or low fidelity (Sih and Baltus, 1987; Kunin, 1992; Kunin, 1993; Kunin, 1997; Tepedino et al., 1999; Tepedino et al., 2007). The largest subpopulation of *Abronia alpina* received relatively high visitation, at an average of 48 insect visits per plant per hr during times of the day when pollinators were active. However, it is possible that visitation is lower in smaller subpopulations.

Butterflies and large moths are expected to travel between plants and between subpopulations more than other insect pollinators, affecting long-distance pollen dispersal (Schmitt, 1980; Courtney et al., 1982; Miyake and Yahara, 1998; Barthelmess et al., 2006). Two species of Sphingidae capable of this type of dispersal were observed visiting the flowers of *A. alpina*: *Proserpinus clarkiae* (Clark's sphinx), and *Hyles lineata* (white-lined sphinx). *Hyles lineata* was observed infrequently but visited over 600 flowers per hr and could account for substantial pollen transfer within subpopulations and promote gene flow among subpopulations.

The highest insect visitation rate was observed in June and was primarily driven by two consistent and loyal insects: *Thorybes mexicanus nevada* and *Bombylius lancifer*. This finding is consistent with the life cycle of *Thorybes mexicanus*, which has only one brood in June (Scott, 1986). Less important pollinator species, *Proserpinus clarkiae* and *Papilio zelicaon* (anise swallowtail), also have only one brood ending in June. All other potentially effective pollinators have one brood from approximately June to August, with the exception of *Hyles lineata*,

TABLE 3. Analysis of molecular variance (AMOVA) for 277 *Abronia alpina* individuals with geographic (by side of the Kern River) structure.

Source of variation	df	Sum of squares	Mean squares	Variance	Percentage of variation	*P*
Among regions	1	314.813	314.813	1.775	9%	0.0001
Among subpopulations	8	646.863	80.858	2.391	13%	0.0001
Within subpopulations	267	3922.187	14.690	14.690	78%	0.0001
Total	276	4883.863		18.856	100%	

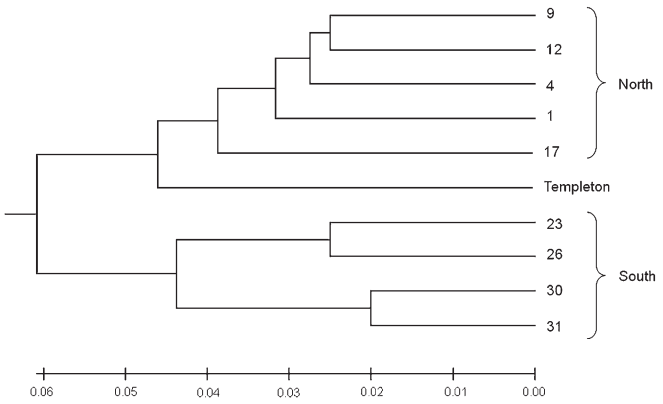

Fig. 5. UPGMA tree. Two main clades are apparent, separated by the Kern River into the northern and southern sides of Ramshaw Meadow.

which has two broods from February to November; this species is likely important for late-season pollination.

Overall genetic diversity, comparisons across life history, and population size—*Abronia alpina* exhibits a relatively high level of genetic diversity despite its restricted range. Nybom (2004) compiled 187 RAPD or AFLP dominant marker studies using a statistic that is equivalent to average expected heterozygosity; H_{pop}. When compared with other short-lived perennial species ($H_{pop} = 0.2$), other outcrossing species (0.27), narrowly endemic species (0.2 and 0.28 respectively), or even widespread species (0.22), *A. alpina* exhibits high genetic diversity (H_e = 0.291; Nei's gene diversity, $H_T = 0.351$). In the absence of a widespread congener with which to compare genetic resources (Karron, 1987), other endemics in the genus can be used on the basis of allozyme and intersimple sequence repeat (ISSR; Bornet and Branchard, 2001) markers. *Abronia macrocarpa* (allozyme-based $H_e = 0.197$, Williamson and Werth, 1999), an endangered species, and *A. umbellata* subsp. *breviflora* (ISSR-based H_e = 0.140, McGlaughlin et al., 2002) also maintain substantial levels of genetic diversity. Members of this genus appear to sustain ample genetic variability despite their small populations and restricted ranges.

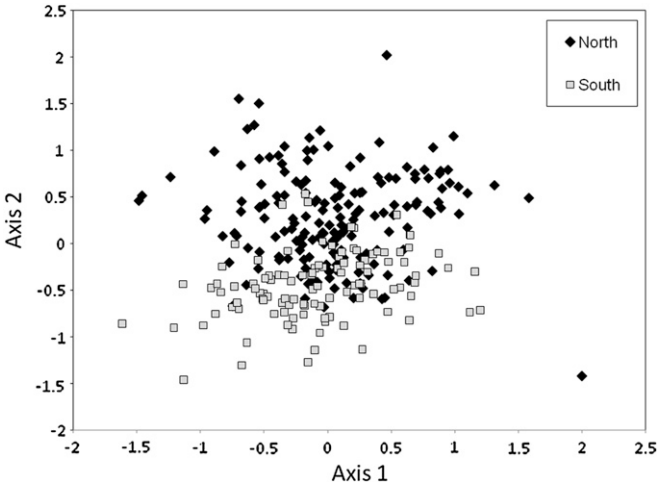

Fig. 6. Nonmetric multidimensional scaling ordination of 277 individuals color-coded by area. Black diamonds depict individuals on the north side of the Kern River, whereas squares depict individuals on the south side of the river. Individuals from both sections cluster together, indicating greater genetic similarity within groups and some physical isolation caused by the Kern River. However, some individuals from each side of the meadow are close in ordination space, suggesting there is still some amount of genetic similarity and likely some gene exchange across these distinct sections of the meadow.

Other studies that have used AFLPs to detect genetic diversity in rare, endemic or endangered species have also found high levels of polymorphism. Gaudeul et al. (2000) found an endangered alpine plant to have 90.9% polymorphic loci and an average expected heterozygosity (H_e) of 0.198. *Abronia alpina*, with 80.7% polymorphism, and other endemics in the genus; *Abronia macrocarpa* (Williamson and Werth, 1999), at 53.2% polymorphism; and *A. umbellata* subsp. *breviflora* (McGlaughlin et al., 2002), with 63.6% polymorphism continue to show substantial genetic variation. Rare, endemic plants can therefore maintain substantial genetic diversity, especially if they are neoendemics adapted to small population size (Ellstrand and Elam, 1993).

Gene flow maintains genetic diversity among subpopulations—Although our measure of gene flow is based on G_{ST} and is therefore indirectly estimated, our results, combined with strong evidence for a xenogamous mating system, suggest that *A. alpina* maintains substantial levels of genetic connectivity between subpopulations. When compared with 10 selfing species, which exhibit a high G_{ST} of 0.59, *A. alpina* exhibits lower levels of population structuring (0.172) consistent with 38 other outcrossing species exhibiting an average G_{ST} of 0.22 (Nybom, 2004).

Minimal population structuring is expected to be associated with an allogamous breeding system (Coppi et al., 2008), and our data concur with this prediction. Our AMOVA results also show low population differentiation (13%, $P = 0.0001$), suggesting high gene flow between subpopulations and a majority of the genetic variation found within subpopulations (78%, $P = 0.0001$, Table 3). Although overall subpopulation differentiation is minimal, a proportion of the variation indicates that there is some structuring due to the Kern River (9%, $P = 0.0001$). This result may be due to limited potential for insects to cross the river.

Subpopulation segregation and geographic correlation—If isolation were created by the Kern River, we would expect other analyses to show the same result. Three other analyses corroborate this pattern. First, the UPGMA tree shows two main clusters representing the same two groups as the AMOVA analysis (Fig. 5). Second, the NMDS ordination corroborates some degree of isolation on either side of the river because two distinct clusters are apparent when individuals are coded by side of the river (Fig. 6). Finally, the Mantel test supports a physical barrier to gene flow, indicating that isolation by distance (km) was not significant. For this species, the physical isolation of the Kern River is a more important barrier to gene flow than is linear distance.

While little is known about the foraging behavior of the insects in this study, we speculate that the Kern River presents some barrier to pollinator movement. This could be due to two factors. First, the distance between two adjacent subpopulations on the same side of the river is rarely as great as the distance a pollinator would have to move to reach the same habitat on the other side of the Kern River (Fig. 1). Second, moving from subpopulation to subpopulation on the same side of the meadow

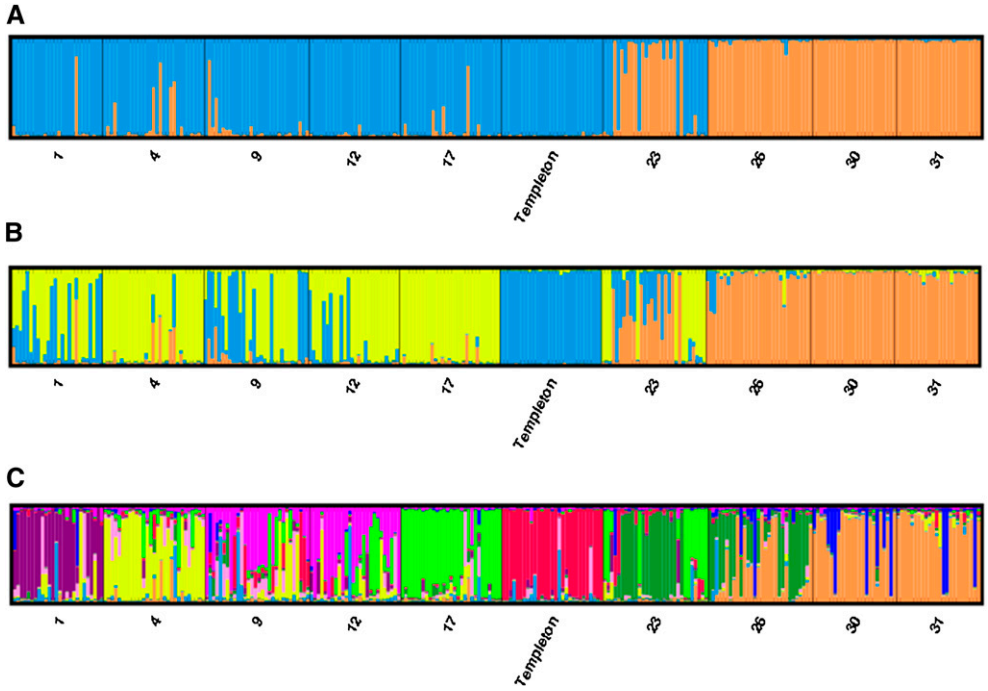

Fig. 7. Graph of three potential subpopulation values. Each individual plant is represented by a single vertical line broken into *K*-colored segments. Line length is proportional to each of the *K*-inferred clusters. The numbers (1 to 31) on the *x*-axis correspond to the predetermined populations: (A) with two subpopulations assumed, some genetic isolation is suggested, but individuals with admixture (represented by lines with more than one color) reveal the presence of gene flow; (B) with three subpopulations assumed, the Templeton subpopulation clearly segregates; and (C) with 10 subpopulations assumed, gene flow is apparent; migrants are present in each of the subpopulations.

would allow potential pollinators to stay within suitable habitat during foraging and to maximize foraging effort.

Three main patterns emerge from the model-based Bayesian population structure analysis consistent with both overall gene flow in the entire population and some level of geographic isolation on either side of the river. First, the optimal value obtained by the program Structure of two subpopulations suggests some amount of genetic isolation; however, some gene flow is occurring, evidenced by individuals that show admixture in each of the two regions (Fig. 7A). The plot assuming three subpopulations indicates some amount of geographic isolation of the Templeton Meadow subpopulation because it clearly segregates from the other two groupings (Fig. 7B). Finally, the plot that assumes 10 subpopulations clearly shows genetic exchange among all subpopulations sampled, indicated by admixed individuals in each subpopulation (Fig. 7C).

Reproductive system influences genetic variability—Gene flow by pollen is suggested to be more important than gene flow by seed in the maintenance of genetic viability in many plants (Ellstrand, 1992; Govindaraju, 1988). For outcrossing species, studies have shown that populations isolated by thousands of meters were not found to be genetically isolated because of gene flow via pollen (Hamrick and Godt, 1989; Delvin and Ellstrand, 1990; Ellstrand, 1992). We argue that of all the potential life history characteristics, outcrossing mating systems may be the most crucial to maintaining genetic diversity in rare, endemic plants (Hamrick and Godt, 1989; Hamrick and Godt, 1996a).

Genetic variability is directly related to the ability of a species to respond to environmental changes, and gene flow is known to affect the adaptive potential of species (Barrett and Kohn, 1991; Ellstrand, 1992, and references therein). In the case of alpine endemics particularly threatened by climate change (Parmesan and Yohe, 2003; Thomas et al., 2004), genetic variability may be crucial for allowing rapid response to changing environmental conditions. This is particularly important because in the California Floristic Province, up to 66% of species are expected to experience 80% reductions in range size (Loarie et al., 2008) due to climate change.

Implications for management—Our data identify several potential threats to the survival of this candidate species. First, because the species is dependent on insect pollination for seed set, loss of these likely pollinators could result in rapid population decline, eventually leading to local or global extinction. Second, in smaller subpopulations, biparental mating of siblings or close relatives could lead to lower genetic diversity and inbreeding depression. Third, the loss of butterflies and large moths could lead to greater subpopulation differentiation and the fixation of alleles in small subpopulations. Our data suggest that some number of subpopulations on both sides of the Kern River should be maintained to retain as much genetic diversity as possible and to promote pollinator activity, thereby preserving the metapopulation as a whole.

Conclusions—*Abronia alpina* is a rare, endemic plant that exhibits an obligate outcrossing mating system. This reproductive strategy appears to maintain high levels of genetic diversity in the species and may contribute to its survival and persistence under changing environmental conditions. However, while pollinator-based allogamy is the most genetically viable reproductive system, it leaves the species dependent on insects for survival. If there truly are only seven species of likely insect pollinators, and only two of them are responsible for long-distance

pollen transfer, these pollinators are critical to the long-term persistence of *A. alpina*. Although these pollinator species are not currently declining, environmental change can have unknown impacts on pollinator foraging, and ranges may move upward in elevation or northward in latitude (Parmesan, 2006). Thus, monitoring of these potential pollinator populations is recommended if the current rates of population decline continue. Our study highlights the need for increased awareness of the role of insect communities in rare plant conservation, particularly the importance of plant–pollinator mutualism (Kearns et al., 1998). Future research into the potential effect of pollinator limitation in small subpopulations of *A. alpina* may give insight into their recently documented decline.

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