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Limited hybridization across an edaphic disjunction between the gabbro-endemic shrub *Ceanothus roderickii* (Rhamnaceae) and the soil-generalist *Ceanothus cuneatus*[1]

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- *Premise of the study:* Hybridization is thought to have played an important role in diversification of the speciose shrub genus *Ceanothus*; putative hybrid species have been described, and data suggest that intrinsic barriers may not exist among closely related species. However, the extent to which hybridization occurs in the wild is not known, and little is understood about how extrinsic factors such as soil chemistry may influence the process. The present research focuses on the gabbro-endemic *C. roderickii* and the closely related soil-generalist *C. cuneatus*. Though the species occur peripatrically, they remain distinct across an edaphic disjunction.
- *Methods:* AFLP was used to quantify hybridization and introgression. Biological data and experiments were used to test for prezygotic isolation. Growth trials were used to test for local adaptation and selection against hybrids.
- *Key results: Ceanothus cuneatus* and *C. roderickii* were strongly differentiated morphologically and genetically, despite a lack of evidence for prezygotic barriers. Hybrids and back-crosses were present but infrequent. Finally, there was selection against hybrids in nonnative soil.
- *Conclusions:* There is little genetic exchange between the focal species across an edaphic disjunction, despite the absence of prezygotic barriers. This result implies that soil conditions, as well as other extrinsic factors, should be considered as forces that may restrict hybridization and gene flow in *Ceanothus*, influencing local adaptation and speciation. Findings presented here are significant because they imply that exchange of genetic material between plants may be limited directly by the abiotic environment, rather than by the biology of the plants.

Key words: AFLP; adaptation; *Ceanothus*; ecology; genetic; hybridization; reproductive isolation; Rhamnaceae; soil.

The flowering plant genus *Ceanothus* L. has been cited as a model of diversification in the absence of intrinsic barriers to gene flow (Nobs, 1963; Raven and Axelrod, 1978; Ackerly et al., 2006; Wilken, 2006). This idea stems from anecdotal observation of hybridization (Parry, 1889; Nobs, 1963; Wilken, 2006) and is borne out by classic biosystematic studies carried out in a common-garden setting, in which reproductively viable hybrid progeny were produced in artificial cross pollinations between species of the same subgenus (McMinn, 1944; Nobs, 1963). However, past research has failed to find unequivocal evidence for hybridization in wild populations using morphological or genetic data (Nobs, 1963; Hardig et al., 2002), and there has been very limited field-based research into what factors—intrinsic vs. extrinsic, prezygotic vs. postzygotic—influence genetic exchange during adaptation and speciation in *Ceanothus*.

The present contribution focuses on the problem of genetic exchange across a disjunction in soil chemistry between two species of *Ceanothus*. The focal species are *Ceanothus roderickii* W. Knight and *Ceanothus cuneatus* Nutt. *Ceanothus roderickii* is endemic to soils derived from a single 100-km^2 outcrop of gabbro rock in the Sierra Nevada foothills of El Dorado County, California (Fig. 1). Gabbro soils are characterized by low levels of the important nutrients calcium and phosphorus and high concentrations of the potentially toxic elements iron and magnesium (Alexander, 1993; 2011; Burge and Manos, 2011). Gabbro soils, like serpentine, likely provide a stressful growing environment for plants (Burge and Manos, 2011). Over most of its extremely limited area of distribution (Fig. 1), *C. roderickii* occurs peripatrically with *C. cuneatus*, a soil-generalist species that is widespread in western North America. The species come into closest contact—less than 25 m apart in some places—on gabbro-derived soils. In these instances, the species appear to be separated by an edaphic disjunction, with *C. roderickii* on nutrient-poor soils and *C. cuneatus* on nutrient-rich soils (Burge and Manos, 2011). Although the species differ in morphology and life-history—*C. cuneatus* is erect with reproduction strictly from seed, while *C. roderickii* is decumbent

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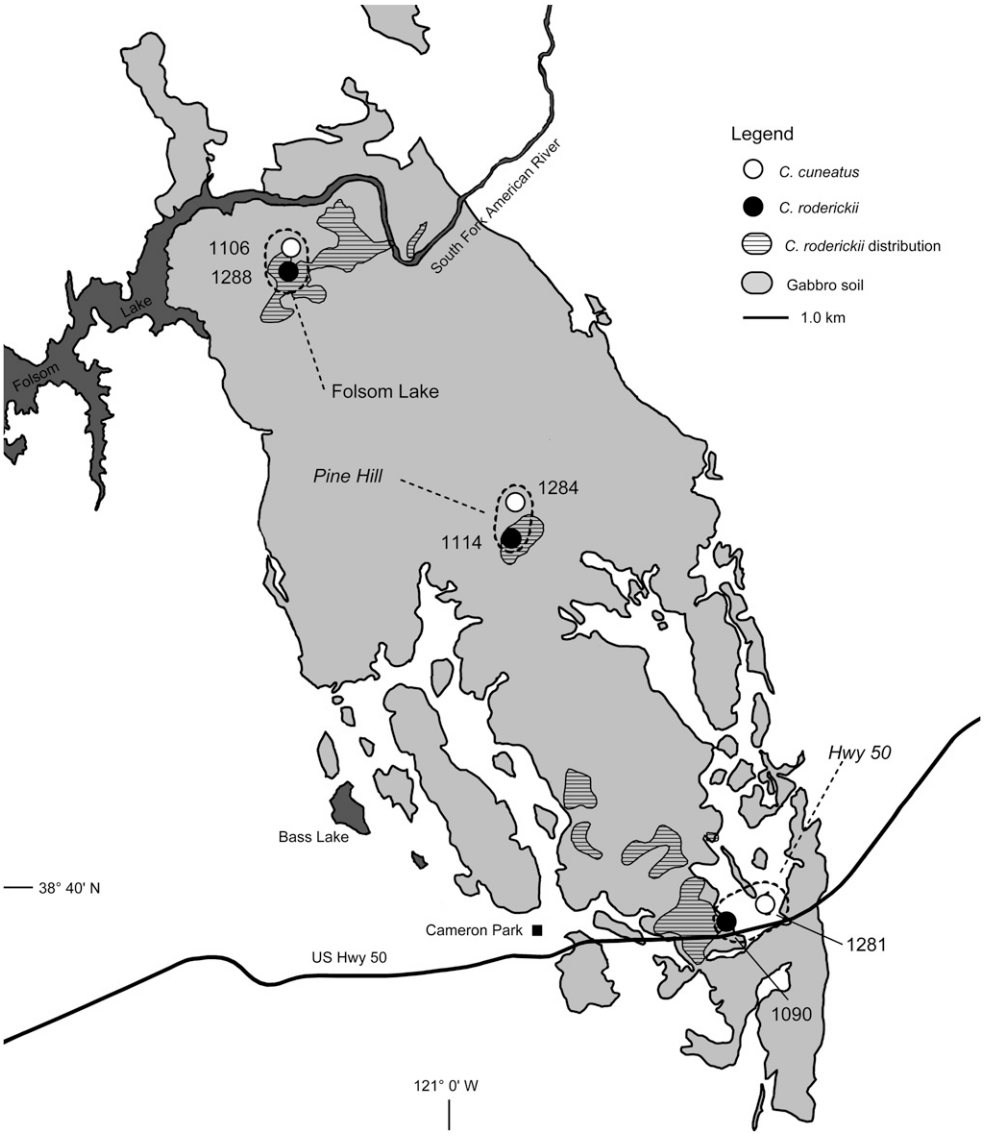

Fig. 1. Sampling and soil map for the Pine Hill region, El Dorado County, California, United States. Shaded polygon for gabbro-derived soils derived from GIS data layers in the Soil Survey Geographic (SSURGO) database for El Dorado Area, California (U.S. Department of Agriculture, Natural Resources Conservation Service). Shaded region comprises all gabbro-derived soils of the Pine Hill intrusive complex (Rogers, 1974). Global distribution of *C. roderickii* adapted from Hinshaw (2008, 2009). Named areas enclosed by dashed circles correspond to sites of peripatry between the focal taxa where samples were obtained for morphometric/AFLP analysis (Table 1).

and capable of reproduction via stem layering—they are very closely related; phylogenetic analyses suggest that *C. roderickii* is derived from within *C. cuneatus* (Burge and Manos, 2011). Because these species are closely related, apparently adapted to different soils, and maintain distinctness in areas of peripatry, they provide a favorable setting to determine how an edaphic disjunction may influence hybridization and introgression, with implications for speciation and subsequent maintenance of divergence in the many other habitat-specialized members of *Ceanothus*.

The research comprises four interlocking components. First, AFLP (amplified fragment length polymorphism) genome scans were used to investigate genetic exchange across the edaphic disjunction, with the expectation that gene flow should be extremely limited despite geographic proximity. Second, flowering time data, pollinator guild surveys, and field-based tests of interfertility were used to determine whether there is reproductive isolation between the species on the basis of these prezygotic, nonedaphic factors. Third, experimental greenhouse growth trials were conducted to determine whether the species are locally adapted to their respective soil types and whether there is natural selection against hybrid progeny on parental soils. In the absence of obvious prezygotic isolation, natural selection against hybrids would be consistent with edaphic factors maintaining differentiation between *C. roderickii* and *C. cuneatus*. The results of this research have implications for fundamental questions in plant evolutionary biology, including the ideas that new species may form despite ongoing hybridization with their closest relatives and that environmental factors like soil chemistry may provide a barrier to genetic exchange powerful enough to drive speciation.

MATERIALS AND METHODS

Sampling for morphometrics and AFLP—*Ceanothus roderickii* is known from only three major population centers (Fig. 1)—one at the southern extremity of the Pine Hill formation near U.S. Highway 50, a second on Pine Hill itself, and a third at the northern extremity of the formation, near Folsom Lake (James, 1996; Hinshaw 2008, 2009). At each of these population centers, *C. roderickii* occurs peripatrically with *C. cuneatus*, the two species being separated by the edaphic disjunction described in the introduction (Burge and Manos, 2011). In most cases, *C. cuneatus* occurs at the foot-slope of hills populated by *C. roderickii* (D. Burge, personal observation). At each major population center of *C. roderickii*, a representative site of peripatry with *C. cuneatus* was chosen. The sites of peripatry were informally dubbed "Highway 50", "Pine Hill", and "Folsom Lake" (Fig. 1; Table 1). At each site of peripatry, individuals of each species were selected from "locales" (Table 1). In each case, the selected locales were at most 400 m from one another (Fig. 1). In selecting locales, disturbed areas were actively avoided, including locations of recent fire, mechanical disruption of the soil (e.g., by earth-moving devices), or various human-associated influences (e.g., surface runoff associated with housing developments; see Discussion). Only three locales were selected to focus the research on the process of genetic exchange between the two species where they come into close contact at the edaphic disjunction. This strategy involves a trade-off between the number of sites sampled and the number of individuals sampled.

At each locale, 48 adult individuals were sampled using a randomly selected distance (0–50 m, in 1 m increments) and bearing (0–360° in 5° increments) from a central location (Table 1); randomly arranged lists of distance and bearing were prepared ahead of time, one for each locale, using a random number generator. Young leaf samples for DNA extraction were dried on silica gel. In total, 288 individual adult plants from six locales were sampled for AFLP work.

Naturally set seeds were also used for AFLP genotyping. Seeds as well as adult plants were genotyped to assess the degree to which genetic admixture differs between successive life history stages. For example, natural selection might eliminate hybrid seedlings before they reach adulthood, altering the proportions of hybrids present at each life history stage. An attempt was made to obtain seeds from 24 randomly selected individuals within each sampling locale using nylon mesh sacks placed over fruiting branches. Sacks were secured using plastic zip-ties. These sacks allowed the collection of seeds despite explosive dehiscence of the fruits. At one site (*C. roderickii* 1114, Pine Hill; Table 1), only 12 plants produced mature seeds. To maintain consistent sampling of seeds across sites for the AFLP genotyping, four seeds were taken from each of 12 parent plants per locale, for a total of 288 seeds. Seeds were selected randomly by scattering a portion of the bulk sample on a plastic Petri dish divided into four equal sections; from each section, the seed nearest the edge of the plate was selected.

Branch segments for measurement of morphometric variables were collected from each of the 288 plants sampled for AFLP. Fresh branch segments were selected haphazardly from individual plants, removed using garden shears, and stored on ice before returning to the laboratory. In addition, voucher specimens were collected at each site (Appendix 1).

Morphometric methods—Five semi-independent variables were used to assess morphological differentiation between *C. roderickii* and *C. cuneatus* using parts recovered from all 288 individual adult plants. Morphometric variables were measured in situ, or on fresh branch segments brought back to the laboratory. These included (1) plant height (H), measured as the distance in meters from ground level to the top of the highest stem on the plant, (2) mean leaf length (LL), calculated as the mean distance in millimeters from the base of the leaf lamina (excluding petiole) to the tip of the leaf on five haphazardly selected leaves, (3) mean leaf width (LW), calculated as the mean distance in millimeters across the widest portion of the leaf lamina, (4) mean tooth number (TN), calculated as the mean number of teeth per leaf for the same five leaves measured for LL and LW, and (5) mean internode length (IL), calculated as the mean length of the first five internodes from the previous year's growth along the primary branching axis. Measurements of H were taken to the nearest centimeter using a measuring tape; other measurements were taken to the nearest 0.5 mm using a ruler.

To test morphological differentiation between *C. cuneatus* and *C. roderickii*, we evaluated morphometric data with a combination of multivariate and univariate statistics. Overall divergence in morphology between the species was summarized using principal component analysis (PCA; Pearson, 1901), which accounts for variation at all variables. PCA was carried out in the program R, v 2.15.0 (R Development Core Team, 2012), using the ecodist package of Goslee and Urban (2007). The morphometric variables were transformed into *Z*-scores prior to analysis and PCA was carried out on the basis of a Euclidean distance matrix. The first two principal components were visualized, and the relative contribution of the morphometric variables to the components was

TABLE 1. Study sampling locales for *Ceanothus* species in California.

Locale[b]	Latitude	Longitude	Elev. (m)	Component[a]			
				Morpho/AFLP	Pollinator	Interfertility	Growth
C. cuneatus							
1011	38.9199	−120.8159	870			X	
1075	38.6789	−120.9148	430			X	
1106	38.7640	−121.0328	315	Folsom Lake			X
1112	38.6848	−120.9847	390		X		
1178	38.7640	−121.0328	315			X	X
1280	38.7552	−121.0276	350		X		X
1281	38.6624	−120.9455	455	Hwy 50			
1284	38.7249	−120.9912	476	Pine Hill			
C. roderickii							
1090	38.6603	−120.9518	440	Hwy 50			
1096	38.6826	−120.9812	440		X		
1102	38.6579	−120.9598	425			X	
1104	38.7603	−121.0309	350		X		X
1114	38.7191	−120.9914	600	Pine Hill			
1171	38.6600	−120.9566	450			X	
1278	38.6710	−120.9714	450			X	X
1288	38.7594	−121.0313	340	Folsom Lake			

Notes: Latitude and longitude given in the WGS84 datum; elevation from GPS at 5 m accuracy, 3D. Elev. = elevation above sea level.

[a] Research component described in Materials and Methods: Morpho/AFLP: sampling locales sampled for genetic and morphometric analysis indicated by one of three informal names corresponding to pairs of adjacent *C. cuneatus* and *C. roderickii* sampling locales; Pollinator: sampling locales used for pollinator guild survey; Interfertility: sampling locales used for tests of interfertility between species. Growth trial: sampling locales used for experimental growth trial.

[b] D. O. Burge collection code for sampling locale; vouchers listed in Appendix 1.

assessed based on vector loadings. The difference between the two species was tested using a Student's paired *t* test applied to scores from each component explaining more than 10% of variance.

***AFLP genotyping and analysis*—**AFLP was used to obtain estimates of genetic differentiation among the six locales, including both adult plants and their seeds. Genomic DNA was extracted from silica-dried leaf tissue or whole seeds using the CTAB method of Doyle and Doyle (1987), resulting in 576 unique extractions carried forward to the genotyping phase. For ensuring repeatability of AFLP genotyping, 48 extractions were genotyped twice using all primers. These samples were selected using a random number generator applied to the list of 576 extractions. Furthermore, each capillary electrophoresis gel included individuals from each of the six collecting locales. Individuals were ordered within gels using numbers from a random number generator applied to the complete list of PCR products (i.e., including unique samples, duplicates, and negative controls for each gel). AFLP genotyping (Vos et al., 1995) was carried out according to the protocol developed by Trybush et al. (2006). Capillary electrophoresis was carried out on an ABI Prism 3730 genetic analyzer (Applied Biosystems, Foster City, California) at the Georgia Genomics Facility.

AFLP fragment analysis was completed using GeneMarker v. 1.95 (SoftGenetics, LLC). Samples with weak or noisy signal were culled based on visual inspection of electropherograms. Loci were then sized according to DeWoody et al. (2004), excluding the 425-bp size marker due to its weak signal. A genotyping panel was created manually in GeneMarker v 1.95. Loci were then called automatically using this panel and default parameters of GeneMarker, with the following exceptions: (1) *Range* was restricted to between 60 and 600 bp, and (2) for Peak Detection Threshold, *Min Intensity* was set to 200. Repeatability of AFLP genotyping was estimated using the samples that were analyzed twice. For each locus, this was calculated as one minus the ratio of the total number of differences between repeated samples at that locus to the total number of repeated samples (Bonin et al., 2004; Pompanon et al., 2005). Loci less than 80% repeatable were excluded. The mean "per locus genotyping error rate" (total differences across loci/total number of comparisons across loci; Bonin et al., 2004) was 10.6%. Each AFLP locus was scored as 1 (present), 0 (absent), or ? (unknown; in the case of unscorable data).

For quantifying and testing genetic differentiation between *C. cuneatus* and *C. roderickii*, the AFLP data was statistically analyzed for the successfully genotyped samples, partitioning the samples into adult (283 individuals) and seed (286 individuals) groups. AFLP data were visualized using principal coordinates analysis (PCO). Presence–absence data for each of the loci were used to create a Nei–Li genetic distance matrix in the program PAUP* v 4.01b10 (Swofford, 2000). The PCO was then carried out in R v 2.10.1 (R Development Core Team, 2012) on the basis of this matrix.

AFLP data were also used to calculate indices of genetic diversity, partitioning variation at the levels of species, generation (seed vs. adult), and locale. Nei's average genetic diversity per locus (Nei, 1973) and percentage polymorphic loci (where p, the frequency of the presence allele, obeys $95 \leq 100 \pm p \leq 5$; Bonin et al., 2007) were calculated using the program AFLP-SURV 1.0 (Vekemans et al., 2002). These calculations were based on both square-root (Stewart and Excoffier, 1996) and Bayesian (Zhivotovsky, 1999) methods for estimating the frequency of alleles, the latter relying on default priors of AFLP-SURV 1.0 (Vekemans et al., 2002). This follows the recommendations of Bonin et al. (2007) according to the advantageous assumptions and properties of allele frequency-based methods when used in the context of dominant markers such as AFLP. Genetic differentiation was quantified using Wright's *F*-statistic (F_{ST}; Weir, 1996), with pairwise calculations (1) between species for adult plants, (2) between species for seeds, (3) among sampling locales of adults, (4) among sampling locales of seeds, and (5) between adults and seeds within each sampling locale. Calculations were made in AFLP-SURV using both square-root and Bayesian methods of allele frequency estimation. This method was selected based on Bonin et al. (2007), in which these methods yielded the most accurate results for simulated AFLP data. 1000 permutations were used for all F_{ST} tests.

The Bayesian clustering program STRUCTURE v 2.3 (Pritchard et al., 2000; Falush et al., 2003, 2007; Hubisz et al., 2009) was used to test for genetic admixture that might stem from hybridization between *C. cuneatus* and *C. roderickii*. The program is particularly well suited to these goals in the context of AFLP data as it allows for explicit treatment of AFLP loci as dominant markers and does not rely on prior designation of "pure" individuals to estimate genetic admixture (Bonin et al., 2007; Falush et al., 2007). Following a burn-in of 10 000 generations, 100 000 Markov chain Monte Carlo (MCMC) replicates were used, applying an admixture ancestry model with the allele frequencies independent. AFLP loci were explicitly modeled as dominant by designating the absence allele (0) as recessive at all 235 loci and setting the RECESSIVEALLELES parameter to 1. This analysis was repeated 10 times, varying *K* (the number of ancestral sampling locales) from 1 to 10. Optimal *K* was defined by the model giving the highest peak in Δ K according to Evanno et al. (2005). As $K = 2$ was optimal under these criteria (Appendix S1; see Supplemental Data with the online version of this article), subsequent analyses were run using $K = 2$.

The program STRUCTURE was used to assess statistical support for hybridization (USEPOPINFO option; Pritchard et al., 2000). For the ancestry model, GENSBACK = 3 was used to allow for misclassification of the individuals; MIGRPRIOR = 0.05 was used for stringency (Falush et al., 2007). As described above, *K* was set to 2. In this way, individuals were classified as (1) "pure" individuals of either species, (2) the product of first-generation genetic admixture (product of hybridization between a "pure" individual of each species), or (3) second-generation admixture (the product of a cross between first-generation hybrids, or a back-cross between a first generation hybrid and a "pure" individual). Analyses were run at two significance thresholds ($P \geq 0.05$ and $P \geq 0.01$).

***Tests for prezygotic isolation*—**Intrinsic barriers to gene flow among members of each *Ceanothus* subgenus are thought to be weak or nonexistent (Nobs, 1963; Hardig et al., 2002; Wilken, 2006), an idea based on anecdotal field observations, as well as garden-based experiments (McMinn, 1944; Nobs, 1963). Nevertheless, only very limited tests of interfertility among *Ceanothus* species have been attempted in the field (Nobs, 1963), and none have been tried for *C. roderickii* and *C. cuneatus*. The existence and strength of prezygotic barriers between *C. roderickii* and *C. cuneatus* was assessed with an analysis of flowering time overlap, pollinator guild overlap, and interfertility of the species in the wild.

To determine whether the species overlap significantly in flowering time, herbarium records (Appendix S2; see Supplemental Data with the online article) were examined. A total of 30 relevant herbarium specimens were located at CAS, DH, UC, JEPS, and DAV. These were supplemented with 54 collections taken by DOB between spring 2007 and summer 2010. Each specimen represents a separate occurrence of one of the focal species. Each specimen was classified according to the Julian day number on which it was collected, and the significance of flowering time overlap was assessed using a Student's paired *t* test. Field observations of flowering time overlap were also made, noting the period over which plants flowered simultaneously in areas of peripatry included in this research (Fig. 1). Overall, these observations encompass every known occurrence of *C. roderickii*, and a large proportion of the known occurrences of *C. cuneatus* in the study area (Fig. 1). Combined with multiyear observations of overlap between 2007 and 2010, these data provide a portrait of flowering time overlap between the species, at present and in recent decades. However, it is important to note that these data do not confirm that simultaneous flowering has been a feature of the study system over evolutionary timescales. Climate change, for instance, would be expected to alter the patterns of overlap, especially in a topographically diverse setting such as the Sierra Nevada foothills.

To estimate the potential for insect-mediated cross pollination between *C. roderickii* and *C. cuneatus*, we studied pollinator guilds in adjacent occurrences of the focal species. All *Ceanothus* species possess an open system of pollination; small, fragile, bisexual flowers are produced in abundance, and cross pollination is generally effected by swarms of small insects (Wilken, 2006). Previous work indicates that a variety of pollinators visit *C. roderickii* (James, 1996). Although the generalist, open pollination system of *Ceanothus* suggests that *C. roderickii* should share pollinators with nearby individuals of *C. cuneatus*, the pollinator guild of *C. cuneatus* has never been investigated in comparison to *C. roderickii*. Four locales were visited for pollinator observations, representing two cases of close peripatry (Table 1; Fig. 1). At each locale, pollinators were obtained during four 15-min bouts of collection, alternating between the two species at each site of peripatry. All insect collections were obtained between 1200 and 1600 hours on 28 March 2010. Only insects that were observed to visit flowers were collected. Insects were stored in 70% ethanol for transport to Duke University where specimens were sorted to morphospecies, counted, and identified to order by the first author. Pollinator species that were shared between locales were identified to family by DOB. Ants, including workers of the genera *Camponotus*, *Formica*, and *Monomorium*, were found to visit flowers of both *Ceanothus* species. However, these were not included in the analysis because it was unlikely that wingless insects such as ants could transfer pollen between locales up to 400 m apart.

As a means of assessing the extent to which *C. cuneatus* and *C. roderickii* are interfertile under field conditions, experimental cross pollinations were

carried out in April 2009. Pollinations were performed within and among four *Ceanothus* locales, two each of *C. cuneatus* and *C. roderickii* (Table 1). A single adult plant served as the receptive individual in each locale. In addition to attempted interspecific pollination, each receptive plant received pollen from itself (selfing) and a different pollen-donor plant from the same locale, both as a control for the cross-specific pollinations, and as a test for self-incompatibility (Castric and Vekemans, 2004). The work of Nobs (1963) showed that emasculation of *Ceanothus* flowers, which are small and very delicate, resulted in flower decline. Because of this, experimental flowers were not emasculated after cross pollination. However, the work of Nobs (1963) suggests that many species of *Ceanothus* are self-incompatible, including *C. cuneatus*, and so emasculation is probably not required to obtain pure crosses. The inclusion of a "self" treatment in the cross pollination experiment also allows for an independent test of self-incompatibility.

On each plant, between 18 and 254 flowers on three branches were used for each attempted combination of parents. Flowers were protected from nonexperimental pollination using fine-mesh nylon drawstring bags (Nanoseeum Netting; Thru-Hiker.com). Bags were affixed to branches before anthesis and removed following fruit set. Seed capturing sacks, as described above, were applied before dehiscence to capture seeds. Pollen was transferred directly from anthers to stamens using a water-moistened camel-hair paintbrush treated with absolute ethanol between individuals. Cross pollinations were carried out each week until all experimental flowers had opened and received at least one pollen transfer. Fruits resulting from cross pollinations were counted in May of 2009, and seeds were collected and counted in July 2009 following dehiscence of the fruits.

Experimental germination and growth trials—The performance of hybrid individuals relative to their nonhybrid half-siblings was tested using the parental soils of *C. cuneatus* and *C. roderickii*. Seeds for this greenhouse experiment were obtained in 2010. Interspecific hybrid seeds were generated by controlled cross pollination between individual *C. roderickii* and *C. cuneatus* plants from four sampling locales (Table 1), as described already. Flowers used to make crosses were protected from unintended cross pollination as described. Naturally set seeds (i.e., from open, natural pollination) were obtained from the same individuals that served as receptive plants for the generation of hybrid seeds. These seeds served as the "nonhybrid" half siblings for use in germination and growth trials. While it would have been preferable to know the paternity of "nonhybrid" seeds, the very low rate of seed set obtained from intraspecific crosses (see Results) made this impossible.

Two experimental soils were prepared: (1) a composite of gabbro soils collected from the *C. roderickii* sampling locales (CRG), (2) a composite of gabbro soils collected from the focal sampling locales of *C. cuneatus* (CCG; Table 1). Soils were collected and seeds treated as described earlier. Seeds were planted in $10 \times 10 \times 12$ cm pots filled with a single soil each. Pots were divided into six cells using clear plastic strips, and 1–5 seeds were planted per cell. Seeds were randomly assigned to individual cells within each treatment (CCG vs. CRG), planting seeds from a single mother plant in each cell. Pots were randomly assigned to greenhouse trays. All randomization was done using pregenerated lists, ordered against numbers from a random number generator. In addition to experimental soils, a small number of hybrid and nonhybrid seeds were planted in Fafard 4P Mix (Conrad Fafard, Inc., Agawam, Massachusetts, USA) as a control (M). On 26 January 2011, pots were moved from the cold room to the greenhouse and germination, survival, and presence of true leaves recorded daily. Pots were bottom-watered every 48 h. The experiment continued for 14 d, ending 8 February 2011. Experimental design is further detailed in Appendix S3 (see online Supplemental Data).

Because all experimental seeds that germinated did so within the first 4 d, seed germination was analyzed as a binary character, germination (1) vs. nongermination (0), using logistic regression. Tests were carried out to determine whether the probability of germination differed with respect to seed parentage (*C. roderickii*, *C. cuneatus*, or hybrid), treatment (CCG or CRG), or interaction between parentage and treatment. Logistic regression was carried out in SAS software v 9.1 (SAS Institute, Cary, North Carolina, USA) using a 5% significance threshold. Number of days to first true leaf pair was analyzed using an ANOVA model with parentage, treatment, and an interaction between parentage and treatment. Greenhouse tray was included as a random effect in the model. Seed germination and leaf-out data were selected rather than seedling mortality for the analyses described above due to the very high survivorship of the seedlings once they germinated; less than 8% of seedlings died during the course of the experiment.

RESULTS

Morphological differentiation—*Ceanothus roderickii* and *C. cuneatus* are strongly divergent morphologically. In comparison to *C. cuneatus*, *C. roderickii* has a lower stature (H); shorter, narrower leaves (LL and LW); more teeth (TN); and shorter internodes (IL; online Appendix S4. Differences are significant for all five traits (Student's paired *t* tests, two tailed, $P < 0.01$ [H: $t = 45.35$, df = 255, $P << 0.01$; LL: $t = 23.21$, df = 374, $P << 0.01$; LW: $t = 27.73$, df = 373, $P << 0.01$; TN: $t = -20.16$, df = 299, $P << 0.01$; IL: $t = 10.07$, df = 402, $P << 0.01$]). Principal component analysis summarizes the morphological differentiation. The first three principal components account for 91% of total variance, with 64% on the first principal component, 18% on the second, and 10% on the third. The first principal component is positively correlated with H, LL, LW, and IL (vector loadings = 0.50, 0.50, 0.51, and 0.33, respectively), and negatively correlated with TN (vector loading = −0.37). A biplot of the first two principal components shows these relationships, and the strong differentiation between *C. cuneatus* and *C. roderickii* on the basis of the TN, LL, LW, and H (Fig. 2). Student's paired *t* tests (two tailed) allow for rejection of the null hypothesis of no difference between mean PCA scores for the two species on the first component ($t = 43.90$, df = 366, $P << 0.01$) and the second component ($t = -3.89$, df = 475, $P = 0.0001$), but not the third ($t = 0.79$, df = 465, $P = 0.43$). In general, *C. roderickii* appears to be most strongly differentiated from *C. cuneatus* on the basis of tooth number (Fig. 2), though all measured traits contribute strongly to the differentiation of the species.

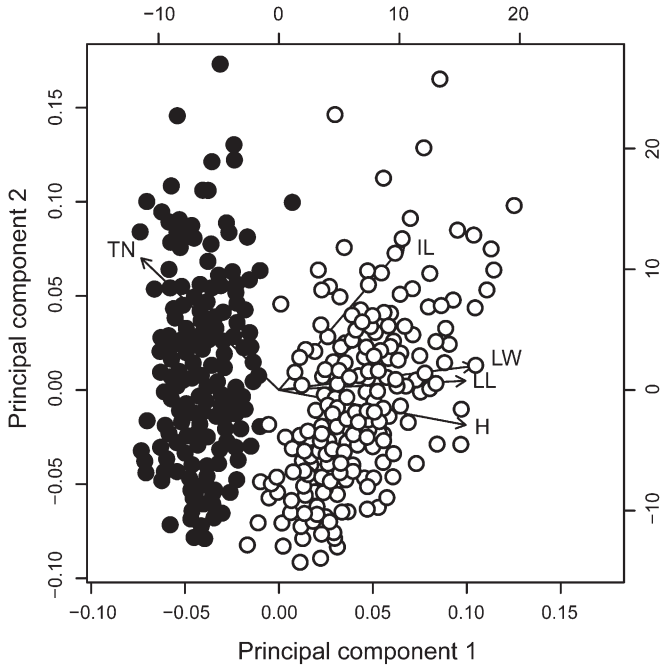

Fig. 2. Biplot from principal component analysis of morphological data. Plot based on analysis of five variables for 288 plants representing three sampling locales each of *C. cuneatus* and *C. roderickii* (Table 1); arrows show direction and magnitude of loading on PC axes; bottom and left axes apply to loading; top and right to scores.

Genetic diversity and differentiation—*Ceanothus roderickii* was generally more genetically diverse than *C. cuneatus* based on the indices of diversity that were calculated (Table 2). This pattern applies to both the adult and seed life stages. The seeds of *C. cuneatus* were much less diverse than adults, while seeds of *C. roderickii* contained a nearly equal amount of diversity as the adults.

Genetic differentiation was strongest between the two species, but was also strong among sampling locales, even in pairwise comparisons between locales representing the same species (Table 3); all estimates of F_{ST} between locales differed significantly from zero based on 1000 permutations ($P < 0.01$; see Table 3 for details). Furthermore, differentiation was also strong between adult and seed life stages at individual locales (online Appendix S5), with all estimates of F_{ST} between adults and seeds significantly different from zero based on 1000 permutations ($P < 0.01$; see Table 3 for details). Plots of PCO axes from analyses of genetic distances for adult plants and seeds provide a visual representation of genetic differentiation between species and among sampling locales (Fig. 3), emphasizing the overall pattern of strong differentiation between the species at the level of both seed and adult life stages, with lesser differentiation among locales of the same species.

Bayesian clustering provided evidence for genetic admixture between *C. roderickii* and *C. cuneatus* (Fig. 4). Analyses utilizing prior information on species membership provide support ($P \geq 0.05$) for first-generation genetic admixture in six adults (4 *C. cuneatus*; 2 *C. roderickii*) and six seeds (3 *C. cuneatus*; 3 *C. roderickii*); first-generation admixed individuals are the product of hybridization between a "pure" individual of each species, while second-generation admixed individuals are those stemming from crosses among first-generation hybrids or from back-crosses between first generation hybrids and "pure" individuals. Second-generation admixture was supported in 11 adults (8 *C. cuneatus*; 3 *C. roderickii*) and 20 seeds (6 *C. cuneatus*; 14 *C. roderickii*). A binomial exact test showed that fewer first and second generation hybrids were identified among adults than among seeds of *C. roderickii* ($x = 5$, $n = 141$, $H_{0,p} = 22/285$, $P = 0.035$). By contrast, there was no significant difference in the number of hybrids in different life stages of *C. cuneatus* ($x = 12$, $n = 142$, $H_{0,p} = 21/284$, $P = 0.748$), or in the full complement of samples for this species ($x = 17$, $n = 283$, $H_{0,p} = 43/569$, $P = 0.193$).

Prezygotic isolating barriers—Consideration of herbarium material from the study region (Fig. 1) indicates that over the past 78 yr the focal species have overlapped broadly in terms of flowering time (Appendix S6). However, *C. cuneatus* has a longer period during which it is known to flower (earliest recorded 27 February; latest recorded 3 June) compared with *C. roderickii* (20 March to 19 May). The average flowering time of *C. cuneatus* does not differ significantly from that of *C. roderickii* based on a Student's paired *t* test (two-tailed; $t = -1.52$, df = 64, $P = 0.13$). In addition, the pairs of sampling locales selected for assessment of morphological and genetic differentiation (Table 1) flowered simultaneously during large portions of spring 2007–2010 (Appendix 1; online Appendix S7).

From the collections of pollinators, 41 species from three orders of insects were identified (Appendix S8). Diversity was strongly variable among the four sites at which it was surveyed. Between three and 10 pollinators were unique to each site. Total

TABLE 2. AFLP diversity summary statistics for *Ceanothus* species.

Sampling locale(s)[a]	Percent polymorphic loci[b]	Gene diversity (H_j)[c]
C. cuneatus		
Adult		
All (n = 142)	33.6; 37.0	0.094; 0.098
1106 (n = 48)	34.0; 37.9	0.098; 0.107
1281 (n = 47)	25.5; 30.2	0.084; 0.094
1284 (n = 47)	29.8; 34.9	0.091; 0.102
Seed		
All (n = 142)	28.5; 28.9	0.085; 0.087
1106 (n = 48)	24.7; 26.0	0.079; 0.083
1281 (n = 47)	27.7; 30.2	0.087; 0.094
1284 (n = 47)	28.1; 30.6	0.079; 0.085
C. roderickii		
Adult		
All (n = 141)	36.6; 37.9	0.117; 0.120
1090 (n = 47)	35.3; 37.4	0.115; 0.122
1114 (n = 48)	37.4; 42.1	0.111; 0.122
1288 (n = 46)	34.5; 37.4	0.106; 0.114
Seed		
All (n = 144)	36.2; 38.7	0.114; 0.117
1090 (n = 48)	31.9; 35.3	0.099; 0.108
1114 (n = 48)	31.9; 35.3	0.102; 0.111
1288 (n = 48)	41.3; 44.3	0.125; 0.134

Notes: All estimates were obtained using AFLP-SURV 1.0 (Vekemans et al., 2002).

[a] Refer to Table 1 for more information on sampling locales.

[b] Number of polymorphic loci at the 5% level based on square-root (Stewart and Excoffier, 1996; Bonin et al., 2007) and Bayesian (Zhivotovsky, 1999) methods of allele frequency estimation, respectively.

[c] Nei's average genetic diversity per locus based on square-root and Bayesian methods of allele frequency estimation.

TABLE 3. Genetic differentiation between sampling locales for *Ceanothus* species.

	F_{ST}					
Sampling locale[a] (Locale code)	1106	1288	1284	1114	1281	1090
Adult						
Folsom Lake						
C. cuneatus (1106)	–	0.138	0.011	0.179	0.026	0.144
C. roderickii (1288)	0.162	–	0.137	0.060	0.142	0.018
Pine Hill						
C. cuneatus (1284)	0.016	0.165	–	0.184	0.029	0.140
C. roderickii (1114)	0.209	0.084	0.224	–	0.189	0.047
Highway 50						
C. cuneatus (1281)	0.032	0.168	0.040	0.222	–	0.148
C. roderickii (1090)	0.166	0.024	0.165	0.066	0.172	–
Seed						
Folsom Lake						
C. cuneatus (1106)	–	0.123	0.014	0.143	0.031	0.130
C. roderickii (1288)	0.142	–	0.127	0.060	0.117	0.036
Pine Hill						
C. cuneatus (1284)	0.018	0.144	–	0.157	0.022	0.143
C. roderickii (1114)	0.164	0.070	0.178	–	0.143	0.057
Highway 50						
C. cuneatus (1281)	0.043	0.135	0.034	0.165	–	0.131
C. roderickii (1090)	0.150	0.044	0.072	0.072	0.156	–

Notes: Within each generation (adult or seed), upper diagonal values are based on the Bayesian method of allelic frequency estimation (Zhivotovsky, 1999); lower diagonal values are based on the square-root method (Stewart and Excoffier, 1996; Bonin et al., 2007). All estimates, obtained using AFLP-SURV 1.0 (Vekemans et al., 2002), differed significantly from zero based on 1000 permutations ($P < 0.001$).

[a] Refer to Table 1 for more information on sampling locales.

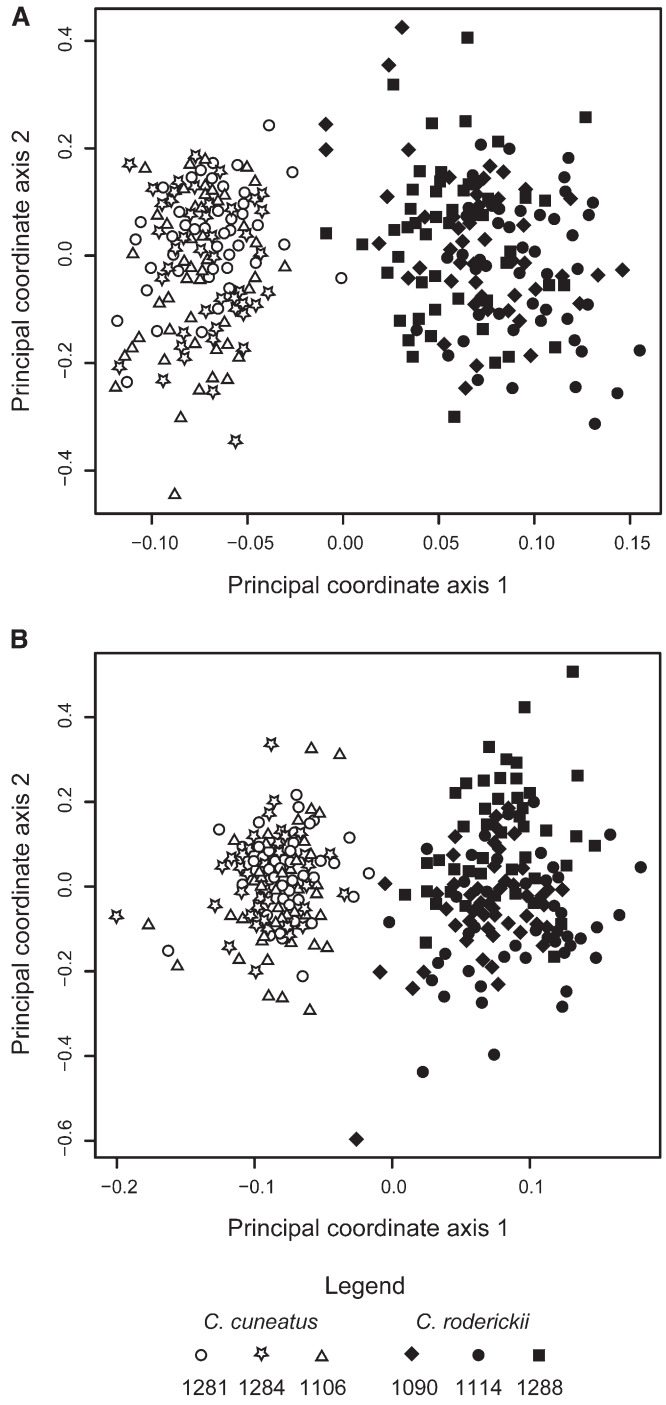

Fig. 3. Results of principal coordinate analysis of Nei–Li genetic distance based on AFLP data for a total of 283 adult plants (A) and 286 seeds (B) collected from three peripatric pairs of *C. roderickii* and *C. cuneatus* sampling locales (Table 1): 1281 and 1090, Highway 50; 1284 and 1114, Pine Hill; 1106 and 1288, Folsom Lake (Fig. 1).

pollinator guild diversity was higher for *C. cuneatus* (30 species) than for *C. roderickii* (21 species). In addition, a higher proportion of pollinators were unique to *C. cuneatus* (19/30) than to *C. roderickii* (10/21). In both comparisons between adjacent sites, guild diversity was also higher for *C. cuneatus* than for *C. roderickii*. Of the 12 pollinators shared between two or more sites, 10 were shared between peripatric locales for the two species.

Cross pollination experiments indicate that *C. roderickii* and *C. cuneatus* are interfertile. Of the six attempted interspecific cross pollinations, three (*C. cuneatus* 1075 × *C. roderickii* 1102, both directions; *C. cuneatus* 1075 × *C. roderickii* 1171) resulted in the production of fruits and seeds (Appendix S9). Neither selfing nor cross pollination among different individuals of the same species at a locale resulted in the production of fruits or seeds. The majority of the seeds obtained from the interspecific crosses were viable, as indicated by the results of the experimental growth trials (see below). However, a large number of hybrid propagules floated during hot water treatment (1.9–30.6%; Appendix S10). Dissection of these seeds revealed that the embryo and endosperm were deflated and dry. These seeds were assumed to be inviable and were not planted. Naturally set groups of seeds from the same mother plants, by comparison, contained only a few seeds of this kind (0–1.2%; Appendix S10).

***Experimental growth trials*—**Results of the experimental growth trial indicated a complicated relationship between genotype and germination/survival on parental soil types. Germination data indicate very strong differences among the three parentage categories (*C. roderickii*, *C. cuneatus*, and hybrid; Appendix S11A;). Although naturally set seeds from *C. cuneatus* had a high germination rate on both CRG and CCG soils (95.2% and 100%, respectively), *C. roderickii* had a much lower germination rate on CCG soil (50%) compared with CRG (83.3%). Finally, hybrid seeds showed consistently low germination rates (69.6% on CRG and 40% on CCG; Table 2). These patterns are summarized by the logistic regression on germination, which showed a significant overall interaction between parentage and treatment (Appendix S11B).

The date of the appearance of the first true leaves was also recorded for each plant on each soil type. These data indicate that for successful germinants there was only slight variation in growth rate. ANOVA for leaf-out data shows a significant effect of parentage, and the interaction between parentage and treatment (Appendix S11C). These results are also seen in plots of output from the ANOVA model, in which *C. roderickii* consistently lags behind *C. cuneatus* for leaf-out date, while seedlings of hybrid parentage leaf out at rates that match those of the species whose native soil type they were grown on (Fig. 5).

DISCUSSION

***Summary*—**The present work deals with the evolutionary biology of *Ceanothus*, a group of shrubs that is widespread, ecologically important, and horticulturally significant (Wilken, 2006). In the past, *Ceanothus* has been cited as a classic example of a plant group in which diversification has taken place in the absence of barriers to hybridization (Raven and Axelrod, 1978; Ackerly et al., 2006; Wilken, 2006), an idea that was inspired by observations of hybridization in the wild (Parry, 1889; Nobs, 1963; Wilken, 2006), as well as studies showing that hybrid offspring survive and are able to produce offspring themselves (Nobs, 1963; McMinn, 1944). In fact, the ease of crossing in *Ceanothus* has led to the development of many hybrid cultivars, including some of great economic value and cultural significance (Wilken, 2006). However, the existence of rampant genetic exchange has not been confirmed in the wild. The aim

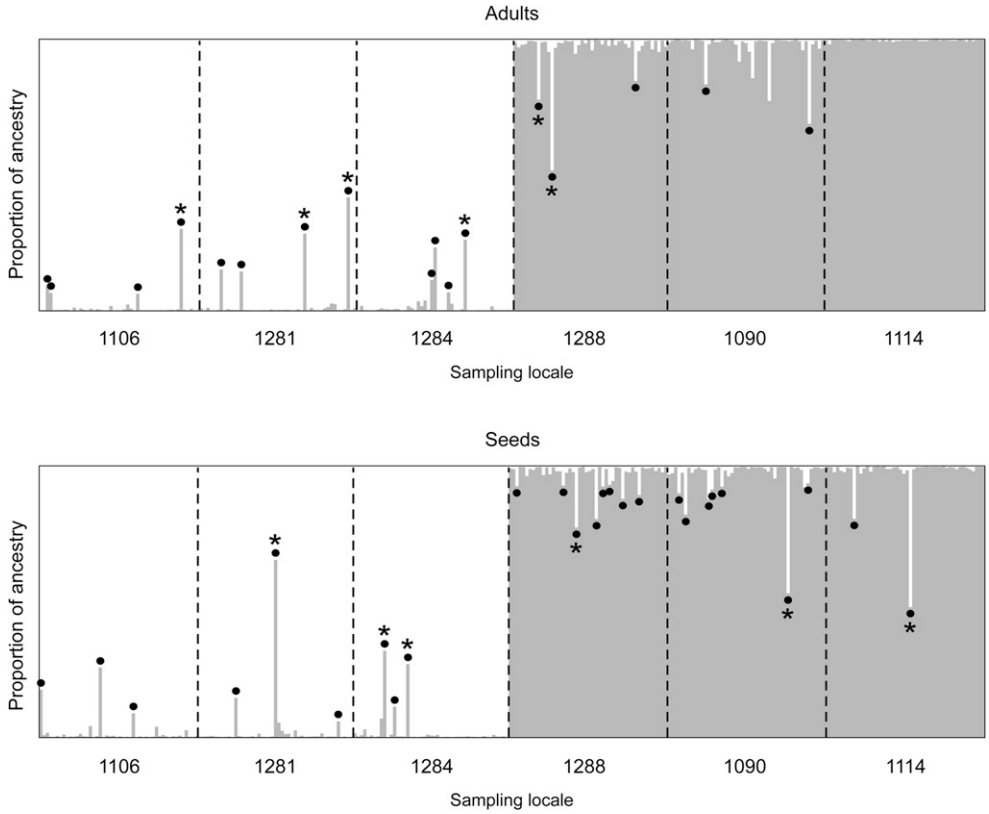

Fig. 4. Ancestry of *Ceanothus* individuals estimated using the program STRUCTURE based on AFLP data for a total of 283 adult plants and 286 seeds collected from three peripatric pairs of *C. roderickii* and *C. cuneatus* sampling locales (Table 1; Fig. 1). See Materials and Methods for details of the analysis. Each vertical bar represents an individual plant or seed; gray portions indicate inferred *C. roderickii* ancestry; white portions indicate *C. cuneatus* ancestry. Asterisks indicate individuals of first generation hybrid ancestry based on separate runs of STRUCTURE using species-identity information in the ancestry model; bullets indicate second-generation hybrids.

of the present research was to quantify hybridization between two species of *Ceanothus*—*C. roderickii* and its close relative *C. cuneatus*. An additional goal was to determine how a difference in soil chemistry association between the two species might be related to a reduction in hybridization, with implications for adaptation and the formation of species in *Ceanothus*.

Results suggest that there is very little hybridization between the focal species, despite the close proximity of sampling locales across a disjunction in soil chemistry—*C. roderickii* on nutrient-poor forms of gabbro-derived soil, and *C. cuneatus* on nearby patches of nutrient-rich gabbro-derived soil. Furthermore, barriers to fertilization between the species (also known as "prezygotic" barriers) were found to be weak, if they exist at all. Finally, there was evidence for natural selection against hybrid offspring in the soils of the parent species. The results imply that hybridization in *Ceanothus* may not be as rampant as researchers generally assume—at least in the field and in the absence of disturbance. The results further suggest that extrinsic factors, such as soil and climate, should be considered among factors that may limit gene flow and thereby contribute to adaptation and speciation in the genus.

Morphological and genetic patterns—Results presented here confirm the strong morphological differentiation between *C. roderickii* and *C. cuneatus* that was the basis for the original description of *C. roderickii*, following its discovery on the gabbro-derived soils of western El Dorado County 56 yr ago (Knight, 1968). Morphological differentiation between *C. roderickii* and *C. cuneatus* is maintained in all three major areas of peripatry between the species (Fig. 2). The work presented here provides evidence that strong morphological differentiation between *C. cuneatus* and *C. roderickii* is matched by strong genetic differentiation, indicated by AFLP genome scans (Table 3; Fig. 3). These results agree with the findings of Burge and Manos (2011) based on a low-copy nuclear gene, in which *C. roderickii* formed a cohesive group nested among *C. cuneatus* populations.

The pervasive genetic divergence between *C. cuneatus* and *C. roderickii* is consistent with the status of *C. roderickii* as a young species undergoing differentiation from its probable parent taxon, *C. cuneatus* (Burge and Manos, 2011). This situation is comparable with that of another Californian plant, the serpentine-endemic *Layia discoidea* Keck, which evolved from within the more widespread soil-generalist *Layia glandulosa* (Hook.) Hook. & Arn. (Baldwin, 2005).

In terms of genetic diversity, it is notable that *C. roderickii* harbors more genetic variation than *C. cuneatus* at the scale of the study area (Table 2). Though seemingly paradoxical given the narrow geographic range of *C. roderickii* and its capability for clonal reproduction, this result is consistent with findings from studies on other flowering plants, in which high levels of genetic diversity were discovered in rare or narrowly endemic species (Stebbins, 1980; Ranker, 1994; Young and Brown, 1996; Xue et al., 2004; Ellis et al., 2006). In a review of this

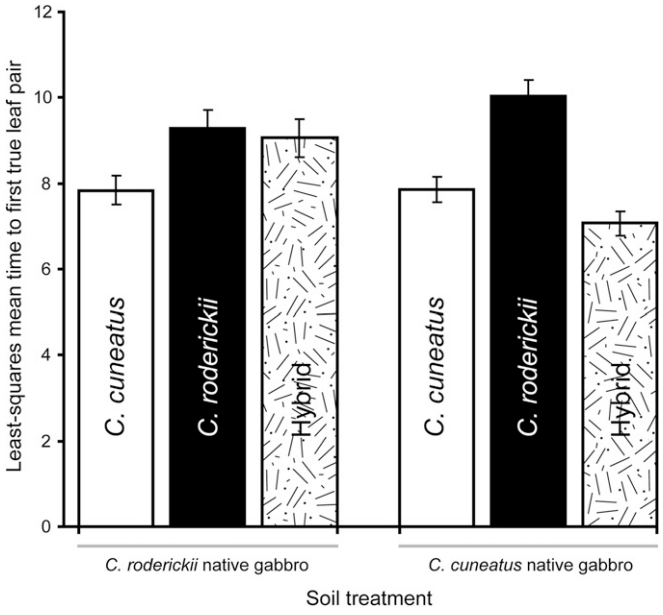

Fig. 5. ANOVA on leaf-out rates for hybrid vs. nonhybrid seedlings during greenhouse growth trial (Appendix S11). Bars represent least-squares mean of time to emergence of first true leaf pair for individual seedlings, with standard error bars. Seedlings from each parentage category (*Ceanothus cuneatus*, *C. roderickii*, and hybrid) plotted separately by treatment (*C. cuneatus* native gabbro soil [CCG], vs. *C. roderickii* native gabbro soil [CRG]).

problem, Gitzendanner and Soltis (2000) concluded that, in general, rare plant species are no less diverse than their more common congeners, suggesting that there may be no basis for the long-held idea of a link between rarity and low genetic diversity. In fact, Ellstrand and Elam (1993) predicted high genetic diversity in rare species if they have large populations. *Ceanothus roderickii* is restricted to three major occurrences, totaling 828 acres of the Pine Hill formation, with a current estimated population of about 33 000 individuals (Hinshaw, 2008, 2009; Fig. 1). *Ceanothus cuneatus*, by contrast, tends to occur in smaller patches within the study area, rarely attaining population sizes comparable to those in *C. roderickii* (D. Burge, personal observation), although detailed data on the demographics and distribution of *C. cuneatus* in the study area are lacking.

In contrast to the results presented here, a population genetic study targeting another gabbro-endemic plant of the Pine Hill formation, *Wyethia reticulata* E. Greene (Asteraceae), found slightly less genetic diversity in populations of this species than in its widespread and partly sympatric congener *W. bolanderi* (A. Gray) W.A. (Ayres and Ryan, 1999). Nevertheless, *W. reticulata* is more strongly clonal than *C. roderickii*, forming extensive genets that regenerate from deeply buried rhizomes following fire (Ayres and Ryan, 1999). *Ceanothus roderickii*, by comparison, is killed by fire and recruits from the soil seed bank (Boyd, 2007).

AFLP data also suggest that in *C. cuneatus*, genetic diversity may be higher in adult generations than in the seed generations, though not at all locales (Table 2). In *C. roderickii*, by contrast, the generations did not differ strongly in terms of genetic diversity. In *C. cuneatus*, decreasing genetic diversity with later generations is unexpected, given that DNA extractions for seeds were obtained from whole seeds, and therefore contained genetic material from the embryo—a combination of maternal and paternal genomes—as well as the endosperm and seed coat, which derive from the maternal plant genome (Kigel, 1995). Higher genetic diversity observed in adults than in seeds could be an artifact of sampling. Another possible explanation could be a high rate of inbreeding (Tonsor et al., 1993). However, there was no direct evidence of preferential within-locale breeding in *C. cuneatus*. Instead, experimental data suggest that the plants may be strongly outcrossing (see below).

Tests of genetic differentiation reveal significant genetic structure between sampling locales of the same species (Table 3). This was not expected, as such a result is not consistent with the outcrossing biology that seems to prevail in this system (discussed later). This finding is particularly unusual given that many of these sampling locales are only a few kilometers from one another (Fig. 1). On the other hand, the geographic distribution of both species is highly fragmented in the study area. If this fragmentation is a consequence of range contraction from a formerly larger distribution, then the observed high rates of differentiation among locales could be the result of genetic drift driven by a population bottleneck (reviewed by Levin, 1988). Even in a limited geographic area like the Pine Hill Region, such differentiation could be maintained by rugged topography, which probably severely limits the migration of pollen and seeds.

Prezygotic isolating barriers—No evidence was found for prezygotic reproductive isolation between the two species. Hybridization between *C. cuneatus* and *C. roderickii* is not precluded by flowering time differences, pollinator specificity, or sexual incompatibility. The results show that *C. cuneatus* and *C. roderickii* flowered simultaneously in El Dorado County during 2008–2010 (Appendix S7), a pattern that has probably played out for at least the past 78 yr (Appendix S6). This strong overlap suggests that hybridization is probably not prevented by differences in flowering time. However, it is also important to note that these observations account for just a small window in evolutionary time and do not indicate whether simultaneous flowering has occurred continuously as long as the species have occupied the Pine Hill region. For instance, past changes in climatic conditions could have altered the relative timing of the flowering period to prevent hybridization. It would be desirable to obtain additional data on flowering time, including population variation and total receptive period of the flowers, neither of which were obtained for the current study.

During periods of simultaneous flowering, transfer of pollen between the species would be achieved by shared pollinators. Results presented here show that the focal species are pollinated by a great variety of small insects from three orders, several of which were common to both species in areas of peripatry (Appendix S8). Though the overlap in pollinator guild indicates that pollen transfer between *C. cuneatus* and *C. roderickii* is possible, particularly in areas of peripatry where the species occur as close as 25 m from one another, it is also possible that pollen transfer is limited by the foraging habits of the shared pollinators. Nevertheless, at least two of the shared insects, a bee fly (genus *Bombylius*) and a bumblebee (genus *Bombus*), are powerful fliers that are widely appreciated as effective and ecologically important pollinators (Toft, 1983; Osbourne et al., 1999; Darvill et al., 2004; Boesi et al., 2009). In the genus *Bombus*, for example, individual foraging ranges more than 1200 m in diameter have been recorded (Osbourne et al., 1999). On the other hand, it is possible that the foraging habits of even the most vigorous and strong flying pollinators limit genetic exchange in

this study system. Though the small size of most *Ceanothus* pollinators precludes observation of foraging habits, it would be helpful to compare visitation rates at flowers of each species in a controlled setting. This was attempted during the course of the research (D. Burge, unpublished data), but was not successful due to a lack of pollinator visitation on experimental flowers.

Opportunities for cross pollination between the focal species beg the question, are they interfertile? Results presented here show that controlled cross pollination between *C. roderickii* and nearby *C. cuneatus* results in fruits and seeds (Appendix S9). In fact, crosses between *C. cuneatus* and *C. roderickii* represent the bulk of successful cross pollinations, indicating that in the event of natural pollen transfer between the species, hybrid seeds may result. These findings are consistent with long-noted anecdotal evidence that species of *Ceanothus* from the same subgenus readily hybridize when they come into contact (McMinn, 1944; Wilken, 2006), as well as classic biosystematic work in which experimental cross pollinations among members of subgenus *Cerastes* produced viable seeds as well as reproductively viable F_1 and F_2 generations (Nobs, 1963; McMinn, 1944).

In contrast to the interspecific crosses, within-locale, conspecific pollen transfers and self-pollinations produced no fruits or seeds (Appendix S9). Although it was not quantified at the time, overall fruit set on nonexperimental branches of the mother plants was moderate during 2009 (D. Burge, personal observation), indicating that conditions were suitable for the formation of fruits and seeds. Interestingly, results from field experiments conducted by Nobs (1963)—using several *Cerastes* species from the Bay Area of California—are consistent with the findings presented here and indicate that mating between conspecific individuals from separate sampling locales may be favored over mating between individuals from the same locale, possibly via self-incompatibility (Castric and Vekemans, 2004). On the other hand, estimates of genetic differentiation among sampling locales (Table 3) suggest very little genetic exchange among sampling locales of the same species, which is the opposite of what one would expect given a predilection for outcrossing by the focal species. Overall, further work will be required to paint a satisfying portrait of prezygotic barriers in the *C. roderickii–C. cuneatus* system, especially replication of interfertility results using larger sample sizes, possibly in a more controlled setting as well as in the wild.

Though results presented here suggest that "traditional" prezygotic barriers between the focal species are weak or nonexistent, it is possible that a different type of isolating barrier is at work in the system—namely, geography (Sobel et al., 2010). According to Sobel et al. (2010), geographic isolation between species or populations (whether due to adaptation to different habitats or historical processes) is a frequently overlooked source of strong prezygotic isolation. In the *C. roderickii–C. cuneatus* system, the species never co-occur; though individual plants of the two species may be separated by distances of less than 25 m, they are generally well separated in space based on their association with divergent types of soil. It could be argued that such spatial isolation, though seemingly minimal, may provide the basis for the pervasive genetic divergence and ecological distinctness of the species despite close peripatry.

Intrinsic postzygotic isolation— While cross pollinations between the focal species resulted in the production of fruits and seeds, as much as 30% of the hybrid seeds resulting from individual cross pollinations was inviable, containing necrotic or dried, deflated embryos and endosperm (Appendix S10). In contrast, very few such inviable seeds were present in naturally set groups of seeds included in the experimental growth trial. These results suggest the action of an intrinsic postzygotic isolating mechanism that acts at the level of seed formation, preventing some hybrid seeds from developing properly. A similar pattern was found by Gardner and MacNair (2000). Such a mechanism might provide at least a partial basis for genetic differentiation between *C. cuneatus* and *C. roderickii*. However, further crosses must be performed in a more controlled setting with higher replication to accurately quantify the extent to which hybrid seed inviability may contribute to isolation, particularly in comparison to potential extrinsic factors, as discussed next.

Extrinsic postzygotic isolation— Results from experimental growth trials and AFLP genome scans provide evidence for natural selection against hybrids, indicating a possible mechanism for reproductive isolation and speciation in the *C. cuneatus–C. roderickii* system. First, selection against hybrids was detected on both the nutrient-rich soil of *C. cuneatus* (40% germination; Appendix S11A) and the nutrient-poor soils of *C. roderickii* (70% germination). Once germinated, the hybrid individuals performed poorly on the nutrient-rich soils of *C. cuneatus* (Fig. 5). However, there was not a reciprocal effect on the nutrient-poor gabbro soils of *C. roderickii*; in this setting, the hybrids performed nearly as well as *C. roderickii*.

Experimental evidence for selection against hybrids is corroborated by AFLP-based estimates of hybridization rates in naturally set seed vs. adult plant generations (Fig. 4), where significantly fewer first and second-generation hybrids—F1 progeny, progeny that result from crosses between F1 hybrids, and back-crosses between F1 hybrids and parental plants (Falush et al., 2007)—were identified among adults than among seeds of *C. roderickii* (Binomial exact test $x = 5$, $n = 141$, $H_{0,p} = 22/285$, $P = 0.035$). This suggests that reproductive isolation might be achieved—at least in part—via selection against genetically admixed seeds. Nevertheless, the results should be interpreted with caution, as they are based on a small number of seeds, especially in the case of hybrids.

Diverse studies have demonstrated a link between edaphic specialization and the evolution of prezygotic and postzygotic isolating barriers (reviewed in Rajakaruna, 2004; O'Dell and Rajakaruna, 2011; Kay et al., 2011), which suggests that edaphic conditions alone may be capable of driving speciation. However, previous studies have concluded that such barriers are intrinsic, including increased selfing (Ornduff, 1966; McNeilly and Antonovics, 1968), flowering time divergence (McNeilly and Antonovics, 1968; MacNair and Christie, 1983; Christie and MacNair, 1987; Rajakaruna and Bohm, 1999), prezygotic genetic incompatibilities (Searcy and MacNair, 1990; MacNair and Christie, 1983; Rajakaruna and Whitton, 2004), and postzygotic incompatibilities (Gardner and MacNair, 2000). Nevertheless, it has also been hypothesized that the substrate itself may provide a potent isolating factor, acting extrinsically via selection against hybrids in one or both parental environments (Rajakaruna, 2004). Although such extrinsic prezygotic reproductive isolation has been observed in animals, including both vertebrates (Grant and Grant, 1993; Hatfield and Schluter, 1999; Vamosi et al., 2000; Pfennig and Rice, 2007) and invertebrates (Craig et al., 1997; Giokas et al., 2000; Egan and Funk, 2009), it has rarely been observed in flowering plants (Wang et al., 1997; Campbell and Waser, 2007). Studies in plants typically find hybrid vigor instead (reviewed by Lowry et al., 2008); it is common for plant populations to harbor high levels of inbreeding depression, and thus hybridization leads to heterosis

(Rundle and Whitlock, 2001; Rhode and Cruzan, 2005) rather than the reduction in fitness observed in the present research.

Overall, the results indicate that selection against hybrid offspring in the contrasting edaphic environments of the parents provides a mechanism for genetic isolation of *C. roderickii* from *C. cuneatus*. However, the present research also provides evidence for genetic incompatibilities (Appendix S10), suggesting that the edaphically driven extrinsic mechanism proposed here may not be the exclusive isolating factor. Nevertheless, an expanded research program is clearly needed to confirm some results of the present study, particularly with respect to the extent of local edaphic specialization by each species, and the relative strength of isolating factors, including natural selection against hybrids.

***Future directions*—**Additional work is needed to confirm some of the results presented here, particularly with respect to flowering time, pollinator sharing, and natural selection under field conditions. Future research on the *C. roderickii–C. cuneatus* system should also aim to directly address the problem of disturbance. As mentioned in the introduction, apparent hybrids between *C. roderickii* and *C. cuneatus* are sometimes encountered in the field, recognized by their intermediate morphology and tendency to form what appear to be small hybrid swarms (D. Burge, personal observation; D. O. Burge collections 1022, 1081, 1091, 1097, and 1099; Appendix 1). However, these plants are always confined to areas that have been subjected to recent substrate disturbance, for instance, by bulldozers (D. Burge, personal observation). These areas were avoided for the present research to focus on patterns of genetic exchange in the absence of disturbance. Results presented here show that under natural conditions, there is little genetic exchange between the species, despite close peripatry, and that this lack of genetic exchange may be maintained by the edaphic disjunction between the species. These results imply that disturbance of the edaphic regime in areas of peripatry, such as during the construction of fire roads, could lead to increased genetic exchange. Research comparing disturbed and undisturbed conditions should be carried out to determine whether this is occurring in the wild. Such research will improve understanding of adaptive divergence and speciation in the *C. roderickii–C. cuneatus* system, and possibly point toward conservation applications to prevent genetic swamping of *C. roderickii* by *C. cuneatus*.

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APPENDIX 1. New voucher specimens. All collections are at DUKE (Duke University Herbarium). Some collections are also represented at RSA (Rancho Santa Ana Botanic Garden) and CAS (California Academy of Sciences). For a list of voucher specimens used in the analysis of flowering time overlap, see Appendix S2 (see Supplemental Data with the online version of this article). For use of locales in research, see Table 1.

Ceanothus cuneatus Nutt.—*Burge 1011*, Wentworth Springs Road, El Dorado Co., CA. *Burge 1075*, Tennessee Creek watershed, roadside on Shingle Springs Road, El Dorado Co., CA. *Burge 1106*, South Fork American River watershed, NW of Mormon Hill, El Dorado Co., CA. *Burge 1112*, City of Cameron Park, El Dorado Co., CA. *Burge 1178*, South Fork American River watershed, NW of Mormon Hill, El Dorado Co., CA. *Burge 1280*, South Fork American River watershed, NW of Mormon Hill, El Dorado Co., CA. *Burge 1281*, Wild Chaparral Drive, El Dorado Co., CA. *Burge 1284*, Pine Hill, El Dorado Co., CA.

Ceanothus roderickii W. Knight—*Burge 1090*, Wild Chaparral Drive, El Dorado Co., CA. *Burge 1096*, East of Cameron Airpark, El Dorado Co., CA. *Burge 1102*, Between Durock Road and Hwy 50, El Dorado Co., CA. *Burge 1104*, South Fork American River watershed, NW of Mormon Hill, El Dorado Co., CA. *Burge 1114*, Pine Hill, El Dorado Co., CA. *Burge 1171*, Wild Chaparral Drive, El Dorado Co., CA. *Burge 1278*, City of Cameron Park, Borica Road, El Dorado Co., CA. *Burge 1288*, South Fork American River watershed, NW of Mormon Hill, El Dorado Co., CA.

Ceanothus roderickii W. Knight × ***Ceanothus cuneatus*** Nutt.—*Burge 1022*, South of US Hwy 50, between Durock Road and Hwy 50, El Dorado Co., CA. *Burge 1081*, Pine Hill, just east of summit, El Dorado Co., CA. *Burge 1091*, City of Cameron Park, north side of US Hwy 50, El Dorado Co., CA. *Burge 1097*, City of Cameron Park, east of Cameron Airpark, El Dorado Co., CA. *Burge 1099*, City of Cameron Park, Bureau of Land Management Pine Hill Preserve, east of Cameron Park Drive, El Dorado Co., CA.