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RESEARCH ARTICLE

Hybrid Sterility over Tens of Meters Between Ecotypes Adapted to Serpentine and Non-Serpentine Soils

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Abstract The development of hybrid sterility is an important step in the process of speciation, however the role of adaptive evolution in triggering these postzygotic barriers is poorly understood. We show that, in the California endemic plant *Collinsia sparsiflora*, ecotypic adaptation to two distinct soil types is associated with the expression of intrinsic F_1 hybrid sterility between ecotypes, over spatial scales of less than 1 km. First, we show that hybrids between soil-adapted ecotypes are less fertile than hybrids within soil ecotypes. Second, we show that between-ecotype postzygotic incompatibility is insensitive to soil growth environment, and can therefore operate under conditions relevant to both ecotypes in the wild. Third, we confirm there is little genetic differentiation between ecotypes using molecular markers, indicating that these postzygotic barriers are recently evolved. Finally, we explore specific soil attributes that might be the source of selective differentiation that confers hybrid sterility. Our results indicate that hybrid barriers are developing in response to strong adaptive differentiation between adjacent and very recently diverged lineages, despite likely ongoing gene exchange.

Keywords Adaptation · *Collinsia sparsiflora* · Natural selection · Fertility · Postzygotic barriers · Soil chemistry · Sympatry · Speciation

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Introduction

> "The crucial step differentiating an ecotype from an endemic is likely to be the acquisition of a partial or complete reduction in gene flow between normal, ancestral population and ecotype, allowing an independent gene pool to develop…How this occurs, in the absence of an extrinsic barrier to gene flow between two contiguous races, is, of course, the standard problem of all nonallopatric speciation models" (Macnair and Gardner 1998, p. 159).

Speciation requires the evolution of traits that act as barriers to gene flow between ancestrally compatible populations (Coyne and Orr 2004). While natural selection has often been tied to the evolution of traits that reduce the frequency of hybrid formation between diverging lineages (prezygotic barriers), the relative importance of selection in fixing loci that reduce the viability or fertility of hybrids (postzygotic barriers) is unknown (Coyne and Orr 2004). Hybrid inviability and sterility are generally thought to arise as a by-product of genetic differentiation between lineages, but identifying the specific evolutionary forces

responsible for this differentiation has proven to be challenging. Between well-diverged species, adaptive evolution has been implicated as major cause of hybrid inviability and sterility, based on patterns of molecular variation at genes contributing to hybrid failure (e.g., Barbash et al. 2003; Presgraves et al. 2003; Ting et al. 1998). However, the specific selective agents driving these patterns are unknown and, because these studies focus on well-differentiated species, the processes involved in the earliest stages of differentiation are obscured. To directly assess the role of adaptation in fixing the very first alleles that contribute to species barriers, the evolution of these barriers must be examined between lineages that are in the earliest stages of speciation. Ideally, these analyses would use taxon pairs that are very recently diverged and that show clear evidence for local adaptation (e.g. via reciprocal transplants) driven by known selective agents. In this study, we evaluate the strength of postzygotic barriers (hybrid sterility) between adjacent ecotypes of an annual plant species. These ecotypes are closely related, incompletely isolated through other potential barriers to gene flow, and strongly locally adapted to soil type.

Adaptation to soil conditions (edaphic adaptation) is arguably the exemplar of local adaptation in plants (Macnair and Gardner 1998; O'Dell and Rajakaruna 2011). Soil attributes—including nutrient status, water availability, and toxicity—provide especially rich conditions for divergent natural selection on obligately sessile plants, resulting in morphological and developmental divergence (Kruckeberg 1986), sometimes over very small spatial scales (Brady 2005; Macnair and Gardner 1998; Wyatt 1988). Soil-driven adaptation has repeatedly been implicated in the development of prezygotic barriers to gene flow, via changes in the location or timing of reproductive events (Antonovics 2006; Brady 2005; Macnair and Gardner 1998; reviewed in Moyle 2004; Kay et al. 2011). In comparison, evidence for a direct connection between adaptation to natural soil heterogeneity and the evolution of postzygotic hybrid incompatibility is rare (Kay et al. 2011). Additionally, very little is known about the environmental dependency (phenotypic plasticity) of these or other early-acting isolating barriers, and therefore their reliability as emerging barriers to gene flow among diverging populations (Campbell and Waser 2001).

To assess whether soil adaptation is accompanied by the development of postzygotic barriers to gene flow, we evaluated the strength and environmental dependence of F_1 hybrid sterility between adjacent, soil-adapted ecotypes of the annual plant *Collinsia sparsiflora* (Scrophulariaceae, *s.l.*; Olmstead et al. 2001). In our four study populations, reciprocal transplant experiments have demonstrated strong local adaptation to serpentine versus non-serpentine soils (Wright et al. 2006). Naturally-occurring serpentine soils display a unique combination of undeveloped soil structure, elevated heavy metals, unusually low calcium-to-magnesium ratios, nutrient poverty, and rapid water loss (Brady 2005; Brooks 1987; Kruckeberg 1986). These serpentine- and non-serpentine-adapted *C. sparsiflora* populations show genetically-based differences in morphological and phenotypic traits, including flower color and size (Wright et al. 2006, and see below), but can be readily crossed to produce hybrids that are viable when grown in the field and under benign greenhouse conditions (Wright and Stanton 2007; this paper).

Our goals in this study were fourfold. First, to evaluate whether postzygotic barriers to reproduction are associated with adaptation to serpentine and non-serpentine soils, we measured the fertility of F_1 hybrids derived from crosses made within and between populations (two serpentine, and two non-serpentine) from different soil ecotypes, grown under greenhouse conditions. If local adaptation to soil is associated with reduced hybrid fertility, then hybrids from population crosses between soil ecotypes (cross type 'BE') should be less fertile than hybrids from population crosses within each soil ecotype ('WE'). We also assessed the fertility of offspring from crosses among plants from within each population ('WP') to provide an additional baseline for fertility comparisons. Second, to evaluate whether postzygotic barriers are intrinsic (i.e. operate regardless of the soil environment experienced by hybrids), the fertility of all cross types was evaluated under alternative soil conditions, including field soil collected from native serpentine and non-serpentine sites. Hybrid sterility must be expressed under conditions relevant to the field if these barriers are to play an important role in limiting gene flow among wild populations. Third, to evaluate whether postzygotic barriers to gene flow could be actively contributing to ecotypic differentiation, we used microsatellite markers to assess evidence for recent and/or current gene flow between populations. Our study populations are separated by 130 to ~1,000 m (Wright and Stanton 2007; Wright et al. 2006)—distances that allow ample opportunity for gene flow via bee pollinators. Nonetheless, if gene flow is absent between ecotypes (for example, is completely prevented by prezygotic barriers), then postzygotic barriers will not play a major role in ongoing ecotypic differentiation. Finally, to identify which specific soil components are most strongly associated with potential reductions in hybrid pollen and seed fertility, we assessed the relationship between patterns of inter-population sterility and population differentiation with respect to 22 chemical and physical soil attributes previously measured in the study populations (Wright et al. 2006). The strength and prevalence of associations between sterility and specific soil characters can be used to generate hypotheses about the selective agents responsible for fixing alleles that contribute to reproductive isolation.

Methods

Study Populations

Study material and field treatment soils were collected from four populations at the University of California McLaughlin Natural Reserve—an open oak woodland with serpentine chaparral, serpentine meadows, and non-serpentine meadows and woodlands, located in the North Coast range of north-central California (reserve website: http://nrs.ucdavis.edu/mclaughlin.html). Soil types change across only meters in the North Coast range (Stebbins and Hrusa 1995), including at the McLaughlin Reserve. The native California annual plant, *Collinsia sparsiflora* (Scrophulariaceae *s.l.*) occurs on both serpentine and non-serpentine soils. A prior reciprocal transplant experiment performed in situ at the McLaughlin Reserve confirmed strong local adaptation to soil type among 6 populations (3 serpentine [S], 3 non-serpentine [NS]) growing within 1 km of one another (Wright et al. 2006). Four of these populations (NS1, NS2, S1, and S3) were used in the current study.

Experimental Design

We assessed the pollen and seed fertility of plants from three classes of population cross: Within Population (WP) crosses (between different maternal families drawn from the same field population); between population, Within Ecotype (WE) crosses (i.e. S1 × S3, and NS1 × NS2); and, between population, Between Ecotype (BE) crosses (i.e. S1 × NS1, S1 × NS2, S3 × NS1, and S3 × NS2). For each population pairing, we examined full-sib seeds from six independent mother-father combinations; i.e., six independent pairs within each of our four source population WP crosses ($6 \times 4 = 24$), six independent pairs for each of our two WE combinations ($6 \times 2 = 12$) and six independent pairs for our four between-soil BE population combinations ($6 \times 4 = 24$), for 60 'F_1' families in total. In each population combination, reciprocal cross directions were roughly equally represented, with the exception of one pair (NS1 × NS2), where appropriate seeds were not available in one direction. In very few cases ($\sim$5 % of families), some parental individuals were used as a maternal or paternal parent in more than one family. To take this into account, our statistical models included a maternal parent effect.

Experimental Material

To generate experimental plants, seeds were collected from individual plants growing at each of the four source field sites (NS1, NS2, S1, S3). Collected seeds were germinated, potted, and greenhouse-cultivated. The appropriate crosses (WP, WE, BE) were performed by hand, by emasculating flowers in the bud (i.e. prior to pollen release) and then pollinating with freshly-collected pollen from the appropriate sire plant. Resulting mature F_1 seeds were collected and stored until the initiation of the experiment. By using seeds generated under uniform greenhouse conditions, we minimized the potential influence of maternal environmental variation on fertility differences in the resulting plants, in order to focus specifically on genetic effects.

To initiate the experiment, F_1 seeds from the above crosses were germinated and cultivated as outlined below. For each experimental family, three full-sib replicates were grown on each of five soil types (standard U.C. Davis greenhouse "soil" mix (hereafter GH media), and soil derived from each of the four source sites) giving 60 families × 5 soils × 3 replicate plants = 900 plants in total. In each case, the soil was thoroughly mixed with sand (1:1 ratio) prior to potting. Because seed germination rates and seedling survival to flowering were less than 100 %, the final experimental population was 610 flowering adult plants; variation in germination and survivorship was distributed roughly equally across crosstypes (J. Wright, unpublished data). All plants were grown under uniform greenhouse conditions in individual 2.5 × 12 cm 'fir conetainers' (Stuewe & Sons, Inc., Corvallis, OR, USA) that were suspended in racks within large plastic trays placed on greenhouse benches; a total of 20 trays held the entire experiment. The design was completely randomized, and variation among trays was included in the statistical analyses as a block effect. Plants were sub-irrigated regularly with deionized water throughout the experiment.

Male and female fertility of experimental F_1 plants was estimated as the proportion of fertile pollen grains per flower and the number of seeds produced by a single manually self-pollinated fruit, respectively. Pollen fertility was estimated on the first (or—rarely—the second, when the first was unavailable) flower on each plant by dissecting the unopened bud and placing all undehisced anthers into lactophenol-aniline blue histochemical stain (Kearns and Inouye 1993). Anthers were gently agitated to release pollen into the dye solution. Samples were vortexed before a subsample was pipetted onto a hemacytometer for counting. Pollen inviability was indicated by the absence of a stained cytoplasm; this is a conservative measure of pollen infertility, as some grains that stain for cytoplasm might still be functionally inviable for other reasons (Kearns and Inouye 1993). Pollen fertility was scored as the proportion of stained pollen grains. The average number of pollen grains counted per sample was 162 (range 10–436).

As both ecotypes are fully self-fertile, seed fertility was determined indirectly by measuring the total seed number resulting from manual self-pollination of a single flower. On each plant, the second flower (or—rarely—the third)

was hand-selfed by physically transferring pollen from dehisced anthers onto the stigma of the same flower. Given the relatively small number of seeds matured per fruit in normal *C. sparsiflora* (a maximum of 10), this pollination method delivered a quantity of apparently viable pollen grains far in excess of that required to fully fertilize all available ovules. Nonetheless, note that because we used selfed pollen, our measure of seed sterility does not differentiate potential effects of pollen and ovule fertility. Pollinated flowers were tagged, capsules were individually collected at maturation, and filled seeds in each mature capsule were counted.

Finally, by performing our sterility analysis under relatively benign conditions in the greenhouse (e.g. with ample water), we have potentially omitted important selection pressures that could operate in nature. However, because sterility is more likely to be exacerbated under stress (de Nettancourt 2001), our fertility measures most likely under- (rather than over-) estimate the strength of post-zygotic barriers that might operate between ecotypes under field conditions.

Fertility Data Analysis

All analyses were performed using SAS version 9.2 (2008, SAS Institute, Cary, NC, USA). Pollen fertility data were arcsine square-root-transformed prior to analysis to improve normality (giving a maximum value of 1.57 for 100 % fertile pollen); both pollen and seed fertility data met assumptions of normality and homoscedasticity required for parametric tests. To test specific hypotheses, we analyzed a series of related general mixed models (GMM; PROC MIXED in SAS), and used a posteriori Tukey–Kramer tests to test for pairwise differences between cross types or hybrid types.

In the first analysis (hereafter GMM1), we used the entire dataset to test for overall effects of cross type (WP, WE, BE) and treatment soil on offspring fertility (proportion pollen fertile, and seed number/capsule). The model included the fixed effects of cross type (3 levels: WP, WE, BE), treatment soil (3 levels: Serpentine, Non-serpentine, Greenhouse media), and maternal soil origin (2 levels: Serpentine, Non-serpentine), and all interactions between these three terms, in addition to three random effects: maternal site within maternal soil, maternal family (nested within maternal site and maternal soil) and block within the greenhouse. The sample size for this analysis ranged from 603 to 610. This model was used to generate the Least Squared Means (LSMs) presented in Figs. 1 and 2. A posteriori pairwise comparisons among cross type LSMs were performed with Tukey–Kramer tests adjusted for multiple testing (as in Fig. 1). To evaluate cross type effects within each treatment soil (as in Fig. 2), LSMs were compared within each treatment soil, using a posteriori pairwise comparisons corrected for multiple testing using a Bonferroni stepwise correction.

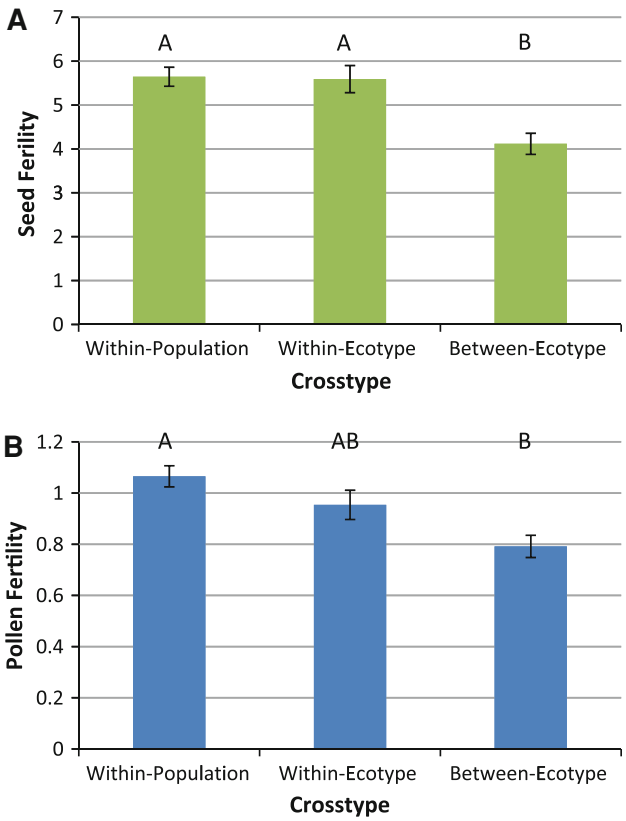

Fig. 1 Fertility in individuals from between-ecotype (*BE*), within-ecotype (*WE*), and within-population (*WP*) crosses. **a** Seed fertility was measured as seeds per self-pollinated fruit capsule. **b** Pollen fertility was measured as proportion of fertile pollen (arcsine square-root transformed; max. value = 1.57). Each panel shows LSMs ± SE across all soil types tested. Within each panel, *bars that share letters* are not significantly different, based on Tukey–Kramer a posteriori tests

In our second analysis (hereafter GMM2), we analyzed only the subset of experimental individuals that were the result of inter-population crosses; that is, we excluded WP individuals and instead assessed the relative fertility of BE individuals versus WE individuals. This is because, while within-population crosses can be a valuable reference point for fertility assessment, outcrossing among populations can have important effects on fertility and fitness, ranging from heterosis (when populations are inbred) through to outbreeding depression (when populations are highly differentiated) (Thornhill 1993). By comparing only between-population cross types, GMM2 specifically assesses the influence of ecotype hybridity on fertility, while accounting for other possible effects of between-population hybridity. In addition, GMM2 can also be used to compare the fertility of offspring of solely serpentine or non-serpentine parentage; in contrast, GMM1 does not distinguish WP and WE crosses representing pure serpentine or non-serpentine

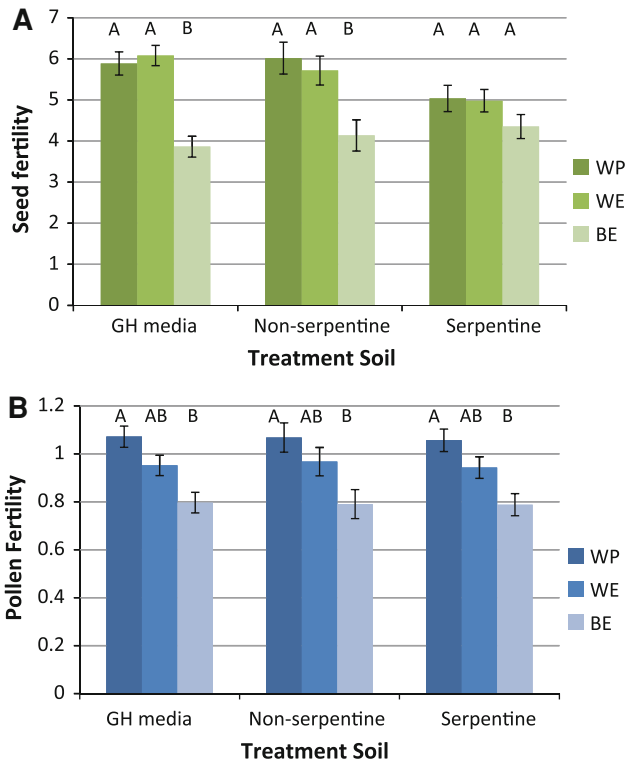

Fig. 2 Treatment soil effects on fertility in individuals from between-ecotype (*BE*), within-ecotype (*WE*), and within-population (*WP*) crosses. **a** Seed fertility was measured as seeds per selfed capsule. **b** Pollen fertility was measured as proportion of fertile pollen (arcsine square root-transformed; max. value = 1.57). Each panel shows LSMs ± SE within each treatment soil type. *Letters above each bar* show contrasts between LS means for each cross within each treatment soil; *bars that share letters* are not significantly different based on Tukey–Kramer a posteriori tests. Note that the marginal differences in seed fertility of *BE*, *WE*, and *WP* individuals grown on serpentine soil are due to the poor performance of non-serpentine genotypes on serpentine soils (see text; Fig. 3)

parentage. GMM2 included the fixed effects of treatment soil (3 levels: Serpentine, Non-serpentine, Greenhouse media) and 'hybrid type' (3 levels: WE non-serpentine, WE serpentine, or between-ecotype), and two random effects (block and maternal parent within hybrid type), and had a sample size of 331–334. This model was used to produce the LSMs presented in Fig. 3 (and Suppl. Figure 1). A posteriori pairwise comparisons among hybrid-type LSMs were performed with Tukey–Kramer tests adjusted for multiple testing (as in Suppl. Figure 1). To evaluate effects of hybrid type within each treatment soil (as in Fig. 3), LSMs were compared within each treatment soil, using a posteriori pairwise comparisons corrected for multiple testing with a Bonferroni stepwise correction.

Gene Flow Assessment Using Microsatellite Markers

Flowering plants from all four populations were collected in the field during the spring of 2006 (sample sizes:

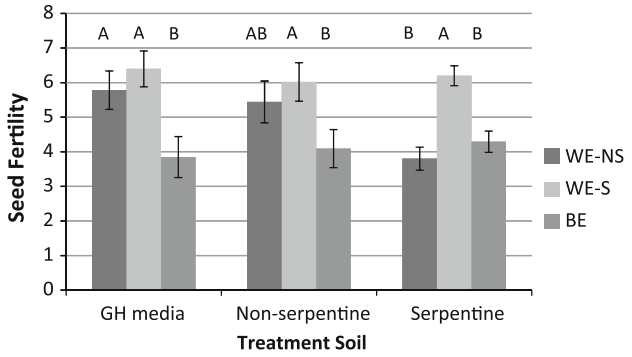

Fig. 3 Soil growth environment and seed fertility. *Letters* above each bar show LSM contrasts between hybrid types, evaluated separately within each soil treatment [GH (greenhouse) media, non-serpentine soil, serpentine soil]; *bars* that share *letters* are not significantly different within each soil treatment. Note that non-serpentine hybrids (*WE* crosses, made between populations occupying the same soil type) showed reduced seed fertility when grown on soils collected from serpentine population sites. The reduced performance of non-serpentine plants on serpentine soils is also revealed as a significant interaction between treatment soil (GH (greenhouse) media, serpentine, and non-serpentine) and hybrid type (i.e. 'pure' serpentine, 'pure' non-serpentine or a hybrid mixture of the two) ($F_{4,299} = 4.19$, $P = 0.0026$)

NS1 = 29, NS2 = 20, S1 = 27, S3 = 29). Plants were placed on ice and returned to the lab for DNA extraction following the manufacturer's directions for the PureGene DNA extraction kit (Gentra). Five microsatellite primer pairs were scored using an ABI 3730 XL capillary sequencer at the University of California College of Agricultural and Environmental Sciences Genomics Sequencing Facility. Details of PCR reactions, and primer and microsatellite sequences can be found in Online Supplemental Table 1. All loci were found to be variable within all populations (Online Supplemental Table 2). Genotype frequencies were analyzed in Arlequin 3.01 (Excoffier et al. 2005), using the "standard" data type. (The "microsatellite" data type was not used because we were unable to unambiguously assign repeat numbers to one of the five loci.) An Analysis of Molecular Variation (AMOVA) was performed using genotypic data from all five microsatellite markers to partition observed genetic variance into within population, within ecotype, and between ecotype components, to assess the degree of genetic differentiation between ecotypes and to evaluate evidence for recent/ongoing gene flow.

Consequences of Variation in Soil-of-Origin Attributes for Hybrid Sterility

If adaptation to specific soil attributes is a direct driver of postzygotic isolation, then we expect to find that crosses between populations occupying the most dramatically different soils also show the lowest fertility. Although soils in

Table 1 Responses of *C. sparsiflora* from different lineages to three different soil types in the greenhouse

Fertility metric	Effect	Num. DF	Denom. DF	F value	Pr > F
Seed fertility	Cross type (Cross)	2	45.0	12.74	**<0.0001**
	Treatment soil (Tsoil)	2	548.0	5.56	**0.0041**
	Maternal soil (Msoil)	1	45.1	5.48	**0.0237**
	Cross × Tsoil	4	547.0	4.64	**0.0011**
	Cross × Msoil	2	45.0	0.67	0.5166
	Tsoil × Msoil	2	548.0	11.93	**<0.0001**
	Cross × Tsoil × Msoil	4	547.0	2.67	**0.0313**
Pollen fertility	Cross type (Cross)	2	46.6	10.47	**0.0002**
	Treatment soil (Tsoil)	2	546.0	0.43	0.6493
	Maternal soil (Msoil)	1	46.6	0.57	0.4535
	Cross × Tsoil	4	546.0	0.12	0.9754
	Cross × Msoil	2	46.6	0.92	0.4050
	Tsoil × Msoil	2	546.0	0.06	0.9396
	Cross × Tsoil × Msoil	4	546.0	1.43	0.2218

Fixed effect results from a general mixed model (GMM1) comparing fertility among all experimental individuals resulting from three types of experimental cross (*WP* within-population, *WE* between-population but within-ecotype, and *BE* between ecotypes), derived from maternal parents of either the serpentine or non-serpentine soil ecotype (MSoil), and grown on one of three soil types (TSoil). Seed fertility was measured as number of fertile seeds per capsule; Pollen fertility is the proportion fertile pollen/flower (arcsine square root-transformed). GMM1 also included three random effects: maternal site within maternal soil type, maternal family (nested within maternal site and maternal soil), and block within the greenhouse (see text). Significant *P* values are in *bold*

our study area can generally be characterized as "serpentine" or "non-serpentine" based on calcium-magnesium ratio, there can be substantial variation within these types for other soil characteristics. We took advantage of that variation to identify which axes of soil variation were associated with hybrid fertility variation. To accomplish this, we used soil attribute data from the reciprocal transplant experiment (Wright et al. 2006) in which 23 physical and chemical soil attributes were assayed from 5 soil samples taken from each population site, then averaged to give a population mean value for each attribute. For each pairwise combination of our four populations (NS1, NS2, S1 and S3), we estimated the pair-wise population "soil distance" as the absolute value of each soil attribute for the maternal site of origin minus the paternal site of origin; therefore for each soil attribute, we had 6 pair-wise estimates of inter-population 'soil distance'. For each population pair we also generated an average fertility value, across all soil treatments. Using these pairwise interpopulation fertility and 'soil distance' estimates, for each soil attribute pairwise fertility values were regressed on the pair-wise soil distances, to generate a coefficient of determination (R^2 value). Two fertility measures (pollen and seed), and 22 soil attributes, gave 44 comparisons in total. Because of the large number of statistical tests involved, we performed sequential Bonferroni (Dunn-Sidak) corrections to make a conservative correction for the overall experiment-wise error rate. Observed *P* values and those that remain significant after correction are reported in the "Results".

Results

Five main findings emerge from our analyses within and among *C. sparsiflora* ecotypes. First, offspring from crosses between ecotypes show reduced fertility in comparison to offspring from crosses within ecotype. Second, this reduced fertility in BE individuals is observed regardless of soil growth environment. In both cases, reduced fertility in between-ecotype hybrids is most evident in seeds produced per capsule. These two findings suggest that partial intrinsic (environment-independent) hybrid seed sterility is accompanying edaphic adaptation between serpentine and non-serpentine ecotypes. Third, our two soil ecotypes have different intrinsic fertility and different responses to soil growth environment, consistent with local adaptation (in at least one direction) to soil. Fourth, based on molecular genetic variation at anonymous markers, there is little evidence for general genetic differentiation between populations and ecotypes, a result consistent with recent and potentially ongoing gene flow between the four study populations. Finally, pairwise population differences in fertility are most strongly associated with differences in soil chemistry rather than other soil attributes. Specific analyses supporting each finding are detailed below.

Hybrids Between Ecotypes have Reduced Fertility

Both our analysis of all cross types, and of crosses only between populations, indicated that between-ecotype hybrid individuals have reduced fertility. In particular, seed fertility varied strongly among cross types (GMM1) and between hybrid types (GMM2) (Table 1, Suppl. Table 3), and a posteriori comparisons within each model confirmed that between-ecotype individuals had the lowest seed fertility (Fig. 1a; Suppl. Fig 1A). For example, seed fertility in hand-selfed fruits was reduced 26 % in between-ecotype hybrids compared to within-ecotype hybrids (adj. $P = 0.0014$; Fig 1a).

For pollen fertility, we also detected a strong but more incremental effect of cross type (GMM1; Table 1 (Fig. 1b); hybrids between ecotypes were clearly less fertile than offspring from within-population crosses (adj. P value = 0.0001) but had marginally lower pollen fertility than within-ecotype hybrids (adj. P value = 0.0715; Fig. 1b). As expected from this result, hybrid type was not significant when we compared only within-ecotype and between-ecotype hybrid individuals (Adj. $P = 0.116$; Suppl. Table 3; Suppl. Fig 1B).

Between-Ecotype Hybrid Fertility is Relatively Insensitive to Soil Environment, Unlike Within-Ecotype Fertility Which Reflects Edaphic Adaptation

The same GMMs that showed strong effects of cross type or hybrid type indicate that the fertility differences among these groups occur across all soil treatments. Indeed, compared with hybrids within ecotypes, reduced seed fertility in between-ecotype hybrids was evident in all soil conditions, although the disadvantage was not statistically significant when F_1 plants were grown on serpentine soil (Fig. 2a; compare the relative fertility of each cross type on each different treatment soil). The reduced pollen fertility of hybrids between ecotypes was consistent across all soils tested (Fig. 2b).

In general, the effects of soil growth environment on seed fertility were much stronger than those on pollen fertility. Unlike seed fertility, we detected no main effect and no significant interactions of treatment soil on pollen fertility (Table 1; Suppl. Table 3). In contrast, the average number of seeds per capsule was greater on greenhouse and non-serpentine soils than on serpentine soil (Suppl. Table 4), which is consistent with the high toxicity of serpentine soils. Moreover, different cross types or hybrid types responded differently to soil growth conditions. In particular, while the seed fertility of between-ecotype hybrids was much less sensitive to soil growth conditions, both within-ecotype hybrids and individuals from within-population crosses performed particularly poorly on serpentine soils (GMM1; Fig. 2a). These differences in response to soil type generated a significant interaction between cross type and treatment soil for seed fertility (GMM1; Table 1). It is likely that this finding is due to strong ecotype-specific responses to soil conditions, which we address next.

Ecotypes Differ in Intrinsic Fertility and Seed Production on Serpentine Soils

We detected a moderate effect of maternal soil of origin (serpentine versus non-serpentine) on seeds per capsule (GMM1; Table 1) such that, across all cross types and treatment soils, serpentine mothers produce more seeds on average than non-serpentine mothers (5.47 vs. 4.78 seeds/capsule for serpentine versus non-serpentine mothers, respectively; Adj. P value = 0.0236). Effects of maternal soil of origin were not observed for pollen fertility (data not shown). More interesting with respect to mechanisms of local adaptation, when we compared population hybrids from within-serpentine, within-non serpentine, and between-ecotype crosses (GMM2), our seed data showed a significant interaction between treatment soil and hybrid type ($F_{4,299} = 4.19$, $P < 0.0026$; Suppl. Table 3); in particular, seed fertility of WE non-serpentine F_1 individuals was reduced specifically on serpentine soils (Fig. 3). This finding is consistent with previous reciprocal transplant experiments in the field that have shown other viability or fertility reductions in non-serpentine derived plants that were transplanted to serpentine soils (Wright et al. 2006). In the current study, any reduced fertility must be due to chemical or physical properties of serpentine soil, rather than other environmental variables that were not replicated in our greenhouse experiment, including abiotic factors like soil temperature and water availability, and biotic factors such as competition. Interestingly, unlike previous field transplant studies that show serpentine lineages were disadvantaged when moved into non-serpentine sites (Wright et al. 2006), in our experiment the seed fertility of WE serpentine lineages was insensitive to soil type under greenhouse conditions (Fig. 3). This suggests that reduced fitness observed in field studies of serpentine transplants on non-serpentine sites is due to factors other than soil chemistry or physical properties. For pollen fertility, GMM1 and GMM2 revealed no interactions between experimental soil and either cross type or hybrid type (Table 1; Suppl. Table 3).

Ecotypes are not Genetically Differentiated at Marker Loci

Despite high levels of allelic variation detected at five microsatellite loci, our genetic analysis indicated no significant genetic differentiation between serpentine and

non-serpentine ecotypes at these anonymous markers (Table 2). Negligible genetic variance was detected between ecotypes, whereas we detected significant variation between populations within ecotype and within populations (Table 2). An earlier analysis of four allozyme loci similarly found negligible (<1 %) and non-significant differentiation between ecotypes when examining these same populations, plus one additional serpentine and non-serpentine population from the McLaughlin Reserve (Wright and Stanton 2011). These findings are consistent with very close relationships and recent, relatively unrestricted, gene flow between serpentine and non-serpentine populations at the loci analyzed.

Fertility Differences Between Populations are Most Strongly Associated with Soil Chemistry Differences

To identify specific soil attributes that might be causally related to hybrid sterility, we examined the association between the fertility of F_1 hybrids between each pair of study populations and soil differences between those same population site pairs for 22 soil attributes (Wright et al. 2006). Strong ($R^2 > 0.50$) associations between hybrid fertility and site-to-site soil attribute differences were all negative, as expected if reduced hybrid fertility results from greater adaptive differentiation with respect to soil conditions (Table 3). Note that these relationships remain negative even when only the four BE population pairs are analyzed, although the strength (R^2) of the relationships is reduced, especially for seed sterility measures (data not shown). However, because of the large number of statistical tests involved, none of our detected associations withstood statistical correction for multiple testing; this is unsurprising, given the low power to detect even highly significant associations based on six site-to-site comparisons. Nonetheless, we note that the strongest of our observed associations with sterility involved differences in soil chemical attributes, specifically calcium-to-magnesium ratio, and zinc and boron content (Table 3; Fig. 4). In contrast, we found no evidence for associations between fertility and differentiation in soil physical attributes, including sand, clay, and silt content (Suppl. Table 5).

Table 3 Coefficients of determination (R^2 values) measuring the strength of associations between pairwise population differences in soil factors and population inter-fertility

Soil attribute	Interpopulation pollen fertility	Interpopulation seed fertility
Ca/Mg	0.853**	0.692*
B	0.269	0.607^{+}
Zn	0.803*	0.564^{+}
Mn	0.549^{+}	0.530

Regressions only include data from crosses made between populations (WE and BE; N = 6 population pairs), and only show results for which R^2 exceeded 0.50 for either seed or pollen hybrid fertility

Also tested, but with $R^2 < 0.50$, were the following soil attributes: Ca, Co, Fe, H, K, Mg, Na, Ni, NO_3, P, SO_4, Cation Exchange Capacity (CEC), pH, organic content, percent sand, salts, and clay. Soil attributes are ranked according to their strength of association (R^2) with interpopulation seed fertility. Analysis results for all soil attributes are in Supplementary Table 5. As this is an exploratory analysis, *P* values shown have not been adjusted for multiple comparisons

$^{+}$ $P < 0.1$, * $P < 0.05$, ** $P < 0.01$

Discussion

Using ecotypes that are closely related, incompletely isolated through other potential barriers to gene flow, and strongly locally adapted to two chemically distinct natural soil types (Wright and Stanton 2007; Wright et al. 2006), we have shown that ecotypic differentiation is associated with the expression of partial, intrinsic hybrid sterility among nearby populations of the California endemic plant *Collinsia sparsiflora*. These findings are relevant to our understanding of speciation in two important respects. First, they demonstrate a clear, apparently direct connection between environmental adaptation and the origin of postzygotic reproductive isolation. Second, because the alleles underlying partial reproductive isolation appear to persist among populations that continue to exchange genes,

Table 2 Analysis of molecular variance (AMOVA) for five microsatellite loci across the four study populations

	df	Sum of squares	Variance components	Percentage of variation
Between ecotypes (serp vs. non-serp)	1	19.599	−0.01355	−0.6
Between populations within ecotypes	2	40.922	0.35934	15.96*
Within populations	206	392.498	1.90533	84.64*
Total	209	453.019	2.25112	

The analysis indicates no significant genetic differentiation among ecotypes. Summary data for variation at each locus is provided in Supplementary Table 2. Note that the marginally negative value for the BE variance component is due to the effects of finite allelic sampling within an AMOVA (Excoffier et al. 2005), and indicates the true value is not different from zero

* $P < 0.0001$

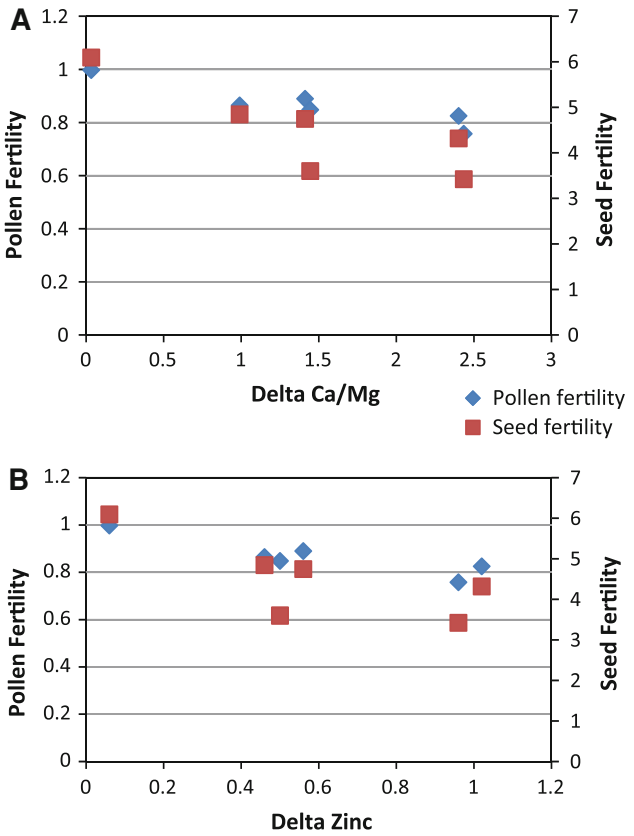

Fig. 4 Associations between population soil differentiation and between-population fertility. Relationship between population inter-fertility for pollen viability (*diamond*; *left axis*) and seeds per fruit (*square*; *right axis*), as associated with pairwise population site differences in **a** soil calcium-to-magnesium ratio (Pollen: $R^2 = 0.853$, $P = 0.0085$; Seed: $R^2 = 0.691$, $P = 0.0402$), and **b** soil zinc content (Pollen: $R^2 = 0.803$, $P = 0.0156$; Seed: $R^2 = 0.564$, $P = 0.085$). All associations are negative, as expected if reduced hybrid fertility among populations is associated with increased population differentiation in soil attributes. Pollen fertility was arcsine-transformed for analysis and plotting (max. value = 1.57)

they provide insight into specific ecological conditions that allow populations to diverge even while experiencing gene flow.

Edaphic Adaptation and the Evolution of Postzygotic Barriers

We found evidence that postzygotic reproductive barriers are uniquely associated with adaptation to different soil types in *C. sparsiflora*. Although natural selection has long been implicated in the process of speciation (Coyne and Orr 2004), the role and importance of adaptive diversification in triggering hybrid sterility is still poorly understood. This is partly because of the dual difficulties of capturing diverging populations during the initial development of postzygotic barriers, and of unambiguously identifying the relevant selective agent involved in adaptive diversification. One solution to this dilemma is to experimentally evolve populations under different environmental conditions and then assess the consequences of resulting adaptation for the development of hybrid sterility (e.g. Dettman et al. 2007). Alternatively, here we have used 'natural experiments'—the adaptation of wild plant populations to natural soil heterogeneity—to show that local adaptation to soil chemistry is accompanied by the recent development of intrinsic partial sterility barriers in the form of F_1 pollen and seed sterility. Given the sparse literature connecting the evolution of reproductive isolation to a specific selective context, this case provides a new compelling example that ecological adaptation has been accompanied by the early development of postzygotic isolation.

While divergent edaphic conditions have long been thought to provide strong stimuli for population differentiation and speciation (Kruckeberg 1986; Macnair and Gardner 1998; Rajakaruna 2004), soil adaptation has most often been associated with the development of prezygotically-acting barriers, especially changes in the location and timing of reproductive activity (Macnair and Gardner 1998; Brady 2005; reviewed in Kay et al. 2011). One well-known exception has been observed between races of *Mimulus guttatus*, where a single locus responsible for soil copper tolerance is tightly linked to, or pleiotropic with, a locus that produces interracial F_1 inviability (Macnair and Christie 1983). However, the consequences of this locus for natural barriers among wild ecotypes is unclear because the selective conditions in this case are anthropogenic (copper-contaminated mine sites), and hybrid inviability was measured in crosses between populations on different continents. In perhaps the clearest example involving divergence associated with natural soil variation, serpentine-adapted *Mimulus nudatus* is isolated from sympatric, non-serpentine populations of its sister species *Mimulus guttatus* by very low F_1 seed viability, a barrier that might have arisen during the fixation of alleles conferring adaptive differences (Macnair and Gardner 1998). In this case, fitness differences on contrasting soil types are thought to be primarily due to different drought tolerances between serpentine and non-serpentine types, rather than to differential tolerance of soil chemical factors (Macnair and Gardner 1998; Gardner and Macnair 2000).

In contrast, *C. sparsiflora* ecotypic differences clearly include differential responses to natural soil chemistry. For example, our non-serpentine plants had reduced seed production/capsule when grown on serpentine soils (Fig. 3), despite being liberally watered. This indicates that selection against non-serpentine immigrant genotypes is mediated, at least in part, by soil chemistry and possibly fertility, and not simply by factors such as soil water-holding capacity. Additionally, we found that the magnitude of inter-population sterility was most strongly

associated with inter-population soil differences in several specific features of soil chemistry that are characteristic of serpentine soils, especially low Ca:Mg ratios (including both depleted Ca and elevated Mg), and heavy metals (Brady 2005; O'Dell and Rajakaruna 2011). Because these detected associations are subject to statistical and biological limitations (e.g., non-independence among soil factors and population pairs, corrections for multiple testing), they are most useful as an exploratory heuristic to generate working hypotheses for further studies. Future analyses can more directly assess the mechanistic connection between divergence at genes associated with serpentine adaptation (e.g. Turner et al. 2010) and the expression of postzygotic isolation among these soil ecotypes. Regardless, on the basis of our current data, this is one of very few studies that support a connection between adaptation to naturally heterogenous soil types and the early stages of development of postzygotic hybrid incompatibility in the wild.

Local Adaptation and the Evolution of Reproductive Isolation in the Face of Gene Flow

The question of whether, and under what conditions, lineages are able to speciate while still exchanging genes, continues to excite vigorous debate (see Fitzpatrick et al. 2008 for a recent review). Models indicate that the conditions for divergence in the face of gene flow are stringent, whether among parapatric (physically adjacent) or sympatric (randomly interbreeding) populations. This is especially true of alleles causing postzygotic hybrid inviability and sterility; unless these genes have other advantages that outweigh the disadvantage of producing low fitness offspring in between-type matings, they are never expected to persist (Coyne and Orr 2004, p. 85).

We found evidence that postzygotic reproductive barriers operate between local ecotypes of *C. sparsiflora* that occupy contrasting soils but are likely still exchanging genes. Our study cannot evaluate whether the genetic changes underlying postzygotic isolation initially arose during sympatry between edaphic ecotypes. Locally soil-adapted ecotypes are thought to have evolved repeatedly from adjacent non-serpentine types in several other plant species (e.g. Vekemans and Lefebvre 1997; Brady 2005), however a phylogeographic analysis—assessing relationships among edaphic ecotypes throughout the species range—would be necessary to determine the historical context of initial ecotypic divergence in *C. sparsiflora* (Coyne and Orr 2004). Nonetheless, our analysis indicates that the barriers we have detected currently *persist* among adjacent ecotypes, despite likely gene flow. Very recent and/or ongoing gene exchange is indicated by the lack of detectable differentiation between ecotypic populations at five anonymous molecular markers (and at four allozyme loci in a previous study; Wright and Stanton 2011), and consistent with the fact that these populations are separated by only 130 to ~1,000 m—distances that allow ample opportunity for gene flow via bee pollinators. Because postzygotic isolation alleles are not expected to persist in the face of gene flow, our results imply that these alleles are actively being maintained by strong countervailing forces. Although the current study cannot disentangle which specific agents are involved, our observations suggest that factors associated with edaphic adaptation might be responsible for enabling such alleles to persist. Moreover, these detected postzygotic barriers could themselves play a role in the ongoing maintenance or enhancement of ecotypic differentiation. High levels of gene flow can reduce or even eliminate local adaptation (Holt and Gomulkiewicz 1997; Kirkpatrick and Barton 1997; Kisdi 2002; Kawecki and Ebert 2004), so a direct association between mechanisms of local adaptation and barriers to gene flow could be particularly important for preserving and potentially amplifying ecotypic differences. The potential for such barriers to preserve ecotypic differences is further suggested by our finding that these partial sterility barriers between soil ecotypes are relatively insensitive to soil environmental context. Therefore, these barriers could operate under soil conditions relevant to both ecotypes in the field. The frequency with which selection against hybrids is environment-independent is very poorly understood (Campbell and Waser 2001); a limited number of reciprocal transplant studies of F_1 hybrids suggest that components of hybrid fitness, including survivorship, can be environment-dependent (e.g. Emms and Arnold 1997; Campbell and Waser 2001), unlike the barriers described here. Nonetheless, barriers that are environmentally-insensitive might be expected to contribute proportionately more to reproductive isolation, because they can act in both parental environments and under novel conditions. Overall, our data indicate that the partial postzygotic barriers we detected could both be the product of adaptive differentiation between soil types, and could further contribute to the maintenance of these ecotypic differences under both non-serpentine and serpentine soil conditions.

The Transition from Ecotype to Species

Our results suggest that *C. sparsiflora* ecotypes might be in an early phase of lineage differentiation (i.e. speciation) that has been driven by natural selection in one of the exemplar contexts for plant adaptation—adaptation to natural variation in soils. Nonetheless, the transition from ecotype to species requires more than the evolution of alleles for local adaptation or even partial isolation; populations must eventually reduce inter-ecotype gene flow to negligible levels. In addition to our findings, prior work indicates that these populations are differentiated for other traits that could also limit gene movement, suggesting that

further reductions in gene flow between adjacent ecotypes is a plausible future trajectory for these *C. sparsiflora* ecotypes. For example, our study populations are known to be differentiated genetically for floral traits (non-serpentine plants have smaller, white flowers in comparison to the larger purple flowers of serpentine plants; Wright and Stanton 2007) that could influence the frequency of gene flow via pollinators (Kearns and Inouye 1993, and references therein) and therefore prezygotic isolation. In addition, in situ reciprocal transplant experiments indicate there is natural selection against inter-ecotype immigrants, including reduced viability of non-serpentine individuals on serpentine sites, and reduced competitive ability of serpentine plants on non-serpentine sites (Wright and Stanton 2007, 2011). Overall, this combination of selection against immigrants, potential prezygotic barriers, and genetically-based postzygotic barriers to gene flow, might play an important future role in facilitating the transition from soil ecotype to new species in *C. sparsiflora*.

Still, of all these factors, postzygotic barriers have two features that might allow them a unique role in further precipitating divergence. First, partial postzygotic isolating barriers can themselves act as the selective impetus for further strengthening prezygotic isolation mechanisms (in order to avoid the formation of partially sterile hybrid offspring between ecotypes), a process that can eventually lead to completion of speciation via reinforcement (Coyne and Orr 2004). Second, because barriers that confer hybrid inviability and sterility are unlikely to be reversible (Muller 1939; Coyne and Orr 2004), the postzygotic sterility we detected might cause permanent barriers to gene flow between these ecotypes. Nonetheless, not all postzygotic isolating mechanisms are expected to be equally effective in generating new species. The F_1 sterility we observed in *C. sparsiflora* is equally consistent with negative epistasis between partially dominant genes from the two ecotypic backgrounds (i.e. Dobzhansky-Muller incompatibilities; Coyne and Orr 2004) or with the presence of chromosomal inversions or translocations between the two ecotypes (King 1993). In the face of ongoing gene flow, chromosomal inversions that cause sterility might better be able to maintain ecotypic differences (King 1993), especially if these inversions contain loci involved in local adaptation (Kirkpatrick and Barton 2006). Patterns of sterility in the F_2 offspring of our F_1 hybrids (Moyle et al. unpublished data) will allow us to differentiate these genetic alternatives in the future. Overall, like several other emerging model systems for examining the genetics of edaphic divergence (reviewed in O'Dell and Rajakaruna 2011), *C. sparsiflora* could substantially contribute to our understanding of the forces and mechanisms that underlie the critical transition between divergent ecotype and fully-fledged species.

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