

Serpentine and non-serpentine ecotypes of *Collinsia sparsiflora* associate with distinct arbuscular mycorrhizal fungal assemblages

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Abstract

Although plant adaptation to serpentine soils has been studied for several decades, the mechanisms of plant adaptation to edaphic extremes are still poorly understood. Arbuscular mycorrhizal fungi (AMF) are common root symbionts that can increase the plant hosts' establishment and growth in stressful environments. However, little is known about the role plant–AMF interactions play in plant adaptation to serpentine. As a first step towards understanding this role, we examined the AMF assemblages associated with field populations of serpentine and non-serpentine ecotypes of California native plant *Collinsia sparsiflora*. We sampled roots of *C. sparsiflora* from three serpentine and three non-serpentine sites in close proximity (110 m to 1.94 km between sites) and analysed the small subunit ribosomal DNA gene amplified from root DNA extracts using AMF-specific primers. A total of 1952 clones from 24 root samples (four from each site) were sequenced. We used sequence similarity and phylogenetic analysis to determine operational taxonomic units (OTU) resulting in 19 OTUs representing taxa from six AMF genera, including one serpentine-specific OTU. We used Bray–Curtis similarity, multidimensional scaling and analysis of similarity to compare root sample AMF assemblages. These analyses clearly showed that plant ecotypes associated with distinct AMF assemblages; an *Acaulospora* OTU-dominated serpentine, and a *Glomus* OTU-dominated non-serpentine assemblages. Species diversity and evenness were significantly higher in serpentine assemblages. Finally, RELATE analysis showed a relationship between ecotype AMF assemblages and soil nutrients. This study reveals a strong relationship between AMF associates and plant adaptation to edaphic extremes.

Keywords: arbuscular mycorrhizal fungi (AMF), assemblages, *Collinsia sparsiflora*, ecotypes, edaphic adaptation, serpentine soil

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Introduction

Adaptation to edaphic factors (soil physical, chemical, and biological characteristics) has long been considered an important component in plant distribution, diversification, and speciation (Wallace 1858). While this topic has received attention for several decades (Kruckeberg 1951; Kruckeberg 1954; Kruckeberg 1967; Macnair & Gardner 1998; Rajakaruna *et al.* 2003a, b; Wright *et al.* 2006), the mechanisms of plant edaphic adaptation are not fully understood. Serpentine soils provide an exceptional system to study edaphic adaptation because plant adaptation to serpentine is

widespread both geographically and phylogenetically and involves similar tolerances to unique edaphic factors (see review by Brady *et al.* 2005).

Serpentine soils are generally characterized by a very low calcium : magnesium ratio, low levels of essential nutrients (nitrogen, phosphorus, potassium), high to toxic levels of heavy metals (iron, chromium, cobalt, nickel), and drought susceptibility (see reviews by Brooks 1987; Brady *et al.* 2005). Of these, low calcium and high magnesium levels are hypothesized to be the major edaphic factors involved in plant adaptation to serpentine (Brady *et al.* 2005). Although some studies have shown a clear physiological basis for serpentine tolerance, the actual tolerance mechanisms as well as the genetic components of serpentine adaptation are poorly understood (Brady *et al.* 2005).

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Indeed, the multifaceted nature of serpentine edaphic factors indicates that multiple traits are likely to be important in serpentine adaptation (Rajakaruna *et al.* 2003b; Brady *et al.* 2005).

The current thinking about serpentine adaptation is primarily based on above-ground reactions of plants to low calcium and high magnesium (Brady *et al.* 2005), yet the primary interface between these edaphic factors and the plant occurs below-ground. The ubiquity and impact of root symbionts on plant growth, distribution, and plant community dynamics are widely accepted (Smith & Read 1997; Klironomos *et al.* 2000; Bever 2003; Reynolds *et al.* 2003; Silvertown 2004; Wardle *et al.* 2004; Benson & Dawson 2007). However, the contribution of these important symbiotic relationships to plant adaptation to serpentine soil has yet to be thoroughly investigated (Kruckeberg 2002).

The vast majority of serpentine plants associate with arbuscular mycorrhizal fungi (AMF, Glomeromycota); this pattern even extends to plants belonging to families that are nonmycorrhizal in non-serpentine soils (Hopkins 1987). AMF have been generally shown to increase nutrient and water uptake as well as the root pathogen tolerance of their hosts (Pairunan *et al.* 1980; Allen *et al.* 1981; Smith & Read 1997). It has also been demonstrated that the more nutrient stressed the environment, the more dependent the plants are on their AMF associates for nutrient acquisition and growth (Yost & Fox 1979; Habte & Manjunath 1987; Johnson & Wedin 1997). Studies of plants growing in heavy metal and low-nutrient substrates show that AM fungal traits (e.g. AM fungal tolerance of or adaptation to heavy metals) and plant traits (e.g. ecotype-specific requirement for and response to AMF) are important for plant growth and survival in harsh environments (Meharg & Cairney 1999; Schultz *et al.* 2001; Gonzalez-Chavez *et al.* 2002; Meharg 2003). All of this work suggests that plant and/or fungal traits that affect symbiotic functioning may represent important traits for edaphic adaptation.

The limited studies about AMF in serpentine ecosystems have shown that AMF colonization is abundant in serpentine plants and can be differentially affected by carbon dioxide, nitrogen, phosphorus, and potassium additions (Chiariello *et al.* 1982; Hopkins 1987; Koide & Mooney 1987; Koide *et al.* 1988; Rillig *et al.* 1999). Working with serpentine grassland plant and AMF communities, Castelli & Casper (2003) used changes in AMF colonization and spore abundance to document feedback between plant and AM fungi similar to the feedback dynamics found in non-serpentine environments (Bever *et al.* 1996; Bever 2003). While giving important information on the general status and ecological dynamics of AMF in serpentine environments, none of these studies were designed to investigate whether an ecological connection exists between plants' AMF associates and adaptation to serpentine, which is a necessary first step towards understanding the role of AMF in edaphic adaptation.

To examine the relationship between AMF and plant adaptation, a number of conditions need to be met. First, it is important to use a plant species in which serpentine adaptation has been experimentally shown through reciprocal transplant studies. Using proven serpentine and non-serpentine ecotypes of the same species will provide a comparison of AMF associates between plants of nearly identical genetic backgrounds except for the traits under edaphic selection. Second, the adapted ecotypes should be located within close proximity to each other in order to minimize distance effects. Finally, studying AMF assemblages *in planta* via polymerase chain reaction (PCR), cloning, and sequencing techniques will provide a more direct approach than spore-based identification methods (Helgason *et al.* 1998; Husband *et al.* 2002; Vandenkoornhuysen *et al.* 2002; Vandenkoornhuysen *et al.* 2003; Rosendahl & Stukenbrock 2004; Hijri *et al.* 2006). In this study, we took advantage of a unique experimental system in which populations of experimentally demonstrated serpentine and non-serpentine adapted ecotypes of the California native plant *Collinsia sparsiflora* (Wright *et al.* 2006) are found within a close geographical range (110 m to 1.94 km between sites). The goal of this study was to characterize and compare the AMF assemblages associated with serpentine and non-serpentine adapted ecotypes of *C. sparsiflora* using molecular techniques. We hypothesized that adapted plant ecotypes will associate with distinct AMF assemblages and that this distinction will be affected by the interrelationships between soils, plants, and AMF.

Materials and methods

Study system

This study was conducted at the Donald and Sylvia McLaughlin University of California Natural Reserve situated in Napa, Lake, and Yolo counties in northern California (<http://nrs.ucdavis.edu/McLaughlin.html>) (Fig. 1). The geology of the McLaughlin reserve has resulted in a fine-scale mosaic of serpentine, volcanic, and valley sediment soil types occurring within metres of each other (Wright *et al.* 2006; Wright & Stanton 2007). In 1999, Wright *et al.* (2006) established research sites in McLaughlin to study local adaptation of natural populations of *Collinsia sparsiflora* to serpentine and non-serpentine soil types. *Collinsia sparsiflora* (Plantaginaceae) is a California native annual that germinates with the first rains in October or November and sets seed and dies by the end of the rains in May or June (Wright *et al.* 2006). Wright *et al.* (2006) established six study populations of *C. sparsiflora*, three on serpentine soils and three on non-serpentine or 'normal' soils derived from volcanic or valley sediment materials. The sites are in close proximity to each other with distances between sites ranging from 110 m to 1.94 km. Using

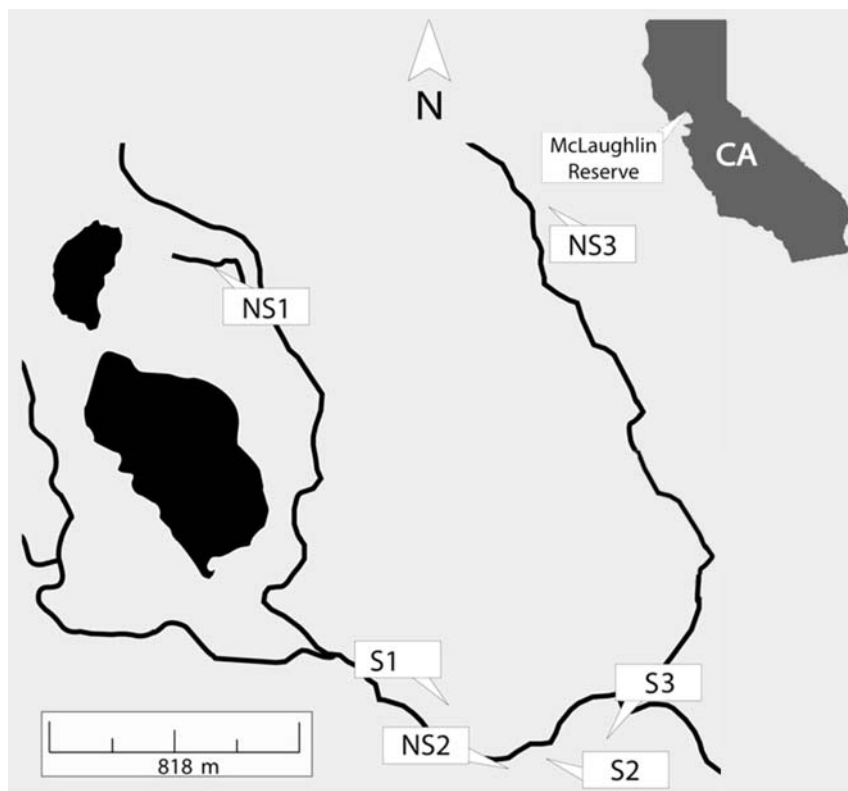


Fig. 1 Study area at Donald and Sylvia McLaughlin Reserve, part of the University of California Davis natural reserve system in northern California. Labels indicate the location of serpentine (S1, S2, S3) and non-serpentine (NS1, NS2, NS3) ecotype populations of *Collinsia sparsiflora* sampled in this study. Roads are outlined and lakes are indicated as filled-in areas. Distances between sites were determined by the 'ruler' function of Google Earth.

reciprocal transplant of populations among all six sites and measuring lifetime production of flowers and fruits, Wright *et al.* (2006) demonstrated the existence of serpentine- and non-serpentine-adapted ecotypes of *C. sparsiflora*. We used the same sites and ecotype populations of *C. sparsiflora* as defined by Wright *et al.* (2006) to compare AMF assemblages associated with the ecotypes (Fig. 1).

Sampling

In March 2005, we sampled all six *C. sparsiflora* populations: three serpentine (S1, S2, S3) and three non-serpentine (NS1, NS2, NS3) (Fig. 1) (Wright *et al.* 2006). Whole plant and soil samples were taken from four patches of *C. sparsiflora* within each site, equaling 24 samples in total. We were obligated to limit the number of *C. sparsiflora* patches sampled in order to minimize the ecological impact of whole plant sampling on the *C. sparsiflora* populations. We collected whole plants at each patch, labelled A–D, by taking a trowel slice 10 cm × 8 cm and 14 cm deep, which incorporated the entire root system of *C. sparsiflora* (personal observation). Sampling whole plants instead of only portions of roots from individuals was necessary because of the small size of *C. sparsiflora*. We also collected soil directly adjacent to the plant collection in each patch for soil analysis. All plant and soil samples were put directly into coolers and stored in a 4 °C cold room within 8 h of collection. We sent soil samples to A & L Western

Agricultural Laboratories within 24 h of collection for chemical analysis (Wright *et al.* 2006). All plant samples were processed within 2 weeks of collection.

We processed each plant sample individually in a fume hood, which was cleaned thoroughly between samples. Whole plant samples allowed for easy identification of *C. sparsiflora* roots from the roots of other plant species included in the trowel slice. We carefully dissected *C. sparsiflora* roots from those of surrounding plants found within the sample. We then grouped whole root systems from all *C. sparsiflora* individuals found within that sample together as a collective root sample. Roots were then thoroughly washed to remove as much soil as possible. We took a small portion of the washed roots to visually examine AMF colonization (Peters & Habte 2001), and the rest were put into coin envelopes and dried in a 37 °C oven for 3 days. We dried the roots in order to simplify processing and facilitate cell disruption via beadbeating for DNA extraction. These dried roots, representing 10–26 *C. sparsiflora* individuals per sample, were then placed into a 2-mL cryotube and stored in a –80 °C freezer until DNA extraction.

Molecular analysis

DNA extraction. We extracted DNA from each *C. sparsiflora* root sample (24 total). We crushed the dried and frozen roots by beadbeating (Mini-Beadbeater, Biospec Products)

with sterile glass beads for 30 s or until a fine powder formed. The samples were then immediately placed on ice, and 1.5 mL of 2× cetyltrimethyl ammonium bromide (CTAB) buffer (2% CTAB, 1% PVP, 0.1 M Tris pH 8.0, 1.4 M NaCl, 0.02 M EDTA) was added to the cryotube. We used a chloroform–isoamyl alcohol extraction method (Kennedy *et al.* 2003) to extract DNA from these samples, and extracts were purified using the DNeasy Tissue Kit (QIAGEN).

Polymerase chain reaction. We amplified a variable region of the 18S ribosomal DNA using Pfu Turbo DNA polymerase (a proof-reading enzyme that creates a blunt-ended fragment) (Stratagene) and universal eukaryotic primer NS31 (Simon *et al.* 1992) paired with AM1, an AMF primer designed to avoid plant sequences (Helgason *et al.* 1998) but thought to exclude taxa from the Paraglomeraceae and Archaeosporaceae (Redecker *et al.* 2000). Before PCR, we diluted the DNA extracts 1:10 or 1:100 in sterile double-distilled water. Each 20- μ L PCR consisted of 12.4 μ L of dH₂O, 0.2 μ L of 2.5 U Pfu Turbo DNA polymerase, 2 μ L of manufacturer's buffer (Stratagene), 2 μ L of 10× dNTPs, and 0.2 μ L of each 50- μ M primer. PCR conditions were the same as described by Helgason *et al.* (2002).

Cloning and sequencing. We gel purified and concentrated the PCR products before cloning because using straight PCR resulted in low cloning efficiency. PCR products (approximately 550 bp) from each sample were gel purified according to the manufacturer's instructions (QIAquick Gel Extraction Kit, QIAGEN) and eluted in 30 μ L of nuclease free water. The purified products were concentrated by drying in a SpeedVac (Savant) and then resuspended in 10 μ L of nuclease free water. We then cloned the purified and concentrated PCR products into pPCR-Script Amp SK(+) and transformed into *Escherichia coli* XL10-Gold Kan Ultracompetent cells (Stratagene). We picked 192 putative positive transformants per sample. Transformants were screened for correctly sized inserts using plasmid primers T3/T7 and the following PCR conditions (94 °C for 10 min, 95 °C for 2 min, 50 °C for 45 s, 72 °C for 1:30 min, 29 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1:30 min, final 72 °C for 7 min). Then, we selected 96 gel-confirmed positive transformants per sample for cleaning and sequencing. We cleaned these PCR products with ExoSAP-IT using the manufacturer's instructions (USB), and sequencing reactions were carried out in one direction with AM1 using BigDye version 3.1 chemistry (Applied Biosystems). Sequences were determined with an ABI 3100 Genetic Analyser (Applied Biosystems). We edited the sequences using SEQUENCHER 4.2.2 (Gene Codes) and eliminated vector sequences using VECSCREEN (<http://www.ncbi.nlm.nih.gov/VecScreen/>).

Chimera detection. Before analysis, we compared our sequences to GenBank sequences using BLAST (version 2.21, Altschul *et al.* 1997), and those with low bit scores and high E-values were suspected as chimeras. We also used the CHIMERA CHECK program in RDPII (version 2.7, Cole *et al.* 2003) to check for chimeras. However, we observed that this program often gave false-positives when we checked sequences in only one direction. As a result, we required that a sequence be indicated as a chimera in both directions before designating it as a chimera. In addition, oddities in global alignments and changes in phylogenetic position (see below for description of methods) were also used to indicate chimeric sequences. Suspect sequences identified under any criteria were eliminated from the data set.

Data analysis

Operational taxonomic unit determination. We determined OTUs in this experiment by using a combination of grouping by sequence similarity and phylogenetic analysis. We first combined sequences from each site at 98% similarity using SEQUENCHER 4.2.2 to create site contigs and singletons in order to keep information about site origin intact. Then we compared all site contigs and singletons together at 98% to determine 98% sequence similarity groupings for the entire data set; these groupings were used to define putative OTUs.

We aligned these sequences, as well as close BLAST matches and additional GenBank AMF sequences that filled out all major Glomeromycota clades (Schussler *et al.* 2001) using CLUSTAL_X (Thompson *et al.* 1997) and then manually edited the sequences using MACCLADE version 4.08 (Maddison & Maddison 2005). Two separate phylogenetic analyses were performed using *Oplidium brassica* as an outgroup: maximum likelihood (ML) was conducted using GARLI version 0.95 (genetic algorithm for rapid likelihood inference, Zwickl 2006), and Bayesian analysis was performed using MRBAYES 3.1.1 (Ronquist & Huelsenbeck 2003). Molecular evolutionary models for Bayesian analysis and ML analysis were estimated with MRMODELTEST (Nylander 2004) and MODELTEST 3.7 (Posada & Crandall 1998), respectively. The best-fit model in both cases was GTR + I + G (–Ln L = 4056.8). Bayesian analysis was performed with two Markov chain Monte Carlo over 100 000 generations with trees sampled every 100 generations for two runs. A 50% consensus tree was constructed after the exclusion of the first 10% of trees (burn-in), and posterior probabilities were estimated for the remaining sampled generations. Reliability of clades in the ML analysis was assessed using nonparametric bootstrapping in GARLI (100 replicates; 10 000 generations).

We used the results of the phylogenetic analyses to confirm OTUs. We looked for consistency in topology between

analyses and > 50% bootstrap or Bayesian posterior probability branch support for clades that included the putative OTU sequences (98% sequence similarity groupings). These OTUs were then used to determine the assemblages of AM fungi associated with each *C. sparsiflora* root sample. One representative sequence from each OTU was deposited into GenBank under accession nos EU573716–EU573773.

Assemblage analyses. We are using the term 'assemblage' rather than 'community' to describe the AM fungal taxa in accordance with the distinction made by Fauth et al. (1996) in which they define an assemblage as a 'phylogenetically related group within a community'. We used the PRIMER 5 software (Plymouth Routines in Multivariate Ecological Research) from the Plymouth Marine Laboratory (Clarke & Warwick 2001) to perform the AMF assemblage analyses. We prepared a relative abundance matrix of OTUs present in each sample based on the number of clones representing those OTUs within each sample. The relative abundance matrix represents the AMF assemblage associated with each *C. sparsiflora* root sample. A similarity matrix was then produced using the Bray–Curtis similarity measure after performing a square-root transformation on the data to down-weight the importance of highly abundant OTUs (Clarke & Warwick 2001). The similarity matrix was the basis of further data representation and analysis.

We represented the similarity matrix data using nonmetric multidimensional scaling (MDS). The MDS ordinations were used to represent the dissimilarities in assemblage composition among samples (Clarke & Warwick 2001). We used the analysis of similarities (ANOSIM) routine to perform statistical analysis of assemblage data. One-way ANOSIM tests were performed to test for significant differences in assemblage composition over all sites (global test) and to detect significant differences in AMF assemblages between sites (pairwise test). In addition, we used the similarity percentages (SIMPER) routine to determine the relative contribution of individual OTUs towards dissimilarity between sites. The species-area plot routine was used to determine if clone sampling effort saturated the number of OTUs (e.g. rarefaction curve). We also computed Shannon-Wiener diversity (H'), richness, and evenness for each site, and one-way ANOVA (JMP version 5) was used to test for differences between soil types and sites in the univariate indices, soil chemical data (log transformed) and colonization (arcsine transformed). Tukey HSD tests were used for all a posteriori comparison of means.

We also used the PRIMER 5 software to compare differences in soil chemical characteristics between samples. Soil chemical data were log transformed, and then the similarity matrix was produced using Euclidean distance (Clarke &

Warwick 2001). Nonmetric MDS and one-way ANOSIM were used to demonstrate differences in soil chemical characteristics between samples. The BIO-ENV routine was used to determine which of the soil chemical variables best explained the differences between samples. Finally, we used the RELATE procedure to test for a relationship between AMF assemblage similarity matrix and the soil chemical similarity matrix (using only the BIO-ENV soil variables).

Results

Assemblage identification

General. All six ecotype populations of *Collinsia sparsiflora* were highly colonized (44–57% root length) by AMF with no significant differences in colonization between samples ($F_5 = 1.22$, $P < 0.31$). All root samples resulted in positive PCR product except for those from S3. Only three root samples from S3 yielded PCR product, and these products resulted in very few clones. We suspect this was due to the high tissue magnesium found in this *C. sparsiflora* population possibly inhibiting PCR (data not shown). A total of 1952 clones were sequenced in this study. Of these, 64% were AMF sequences, 3% were of plant origin, 1% were ascomycete and basidiomycete fungi, and 0.6% were chimeric sequences; the rest of the sequences were of too poor quality to give reliable data. Each site produced similar numbers of AMF sequences from root samples (S1 = 242, S2 = 224, NS1 = 257, NS2 = 244, NS3 = 267) except for those from S3, which generated only 15 AMF sequences. Due to the paucity of sequences, S3 was excluded from assemblage analysis but included in the phylogenetic analysis.

Phylogenetic analysis. We detected six AMF genera in this study (Fig. 2). *Glomus* species were the most abundant representing 72% of the sequences, followed by *Acaulospora* (25%), *Diversispora* (1%), *Scutellospora* (0.7%) *Archaeospora* (0.4%), and *Pacispora* (0.3%). The majority of *Glomus* species were in the *Glomus* 'A' group (Fig. 2). All 98% sequence similarity groupings were present in phylogenetic analyses as well-supported clades (Fig. 2). This match between sequence similarity and phylogenetic support clearly defined the AMF OTUs associated with the *C. sparsiflora* ecotypes. Using this criterion, we established 19 OTUs identified by their genus affiliation (Fig. 2). These OTUs were used to construct the relative abundance matrix (Table 1). The OTU *Glomus* 6 was specific to serpentine ecotypes (Fig. 2). Unlike other OTUs, *Glomus* 6 was found in all three serpentine ecotype populations, and the well-supported *Glomus* 6 clade included a BLAST match to an AMF clone isolated from a heavy metal-polluted soil in Italy (Vallino *et al.* 2006).

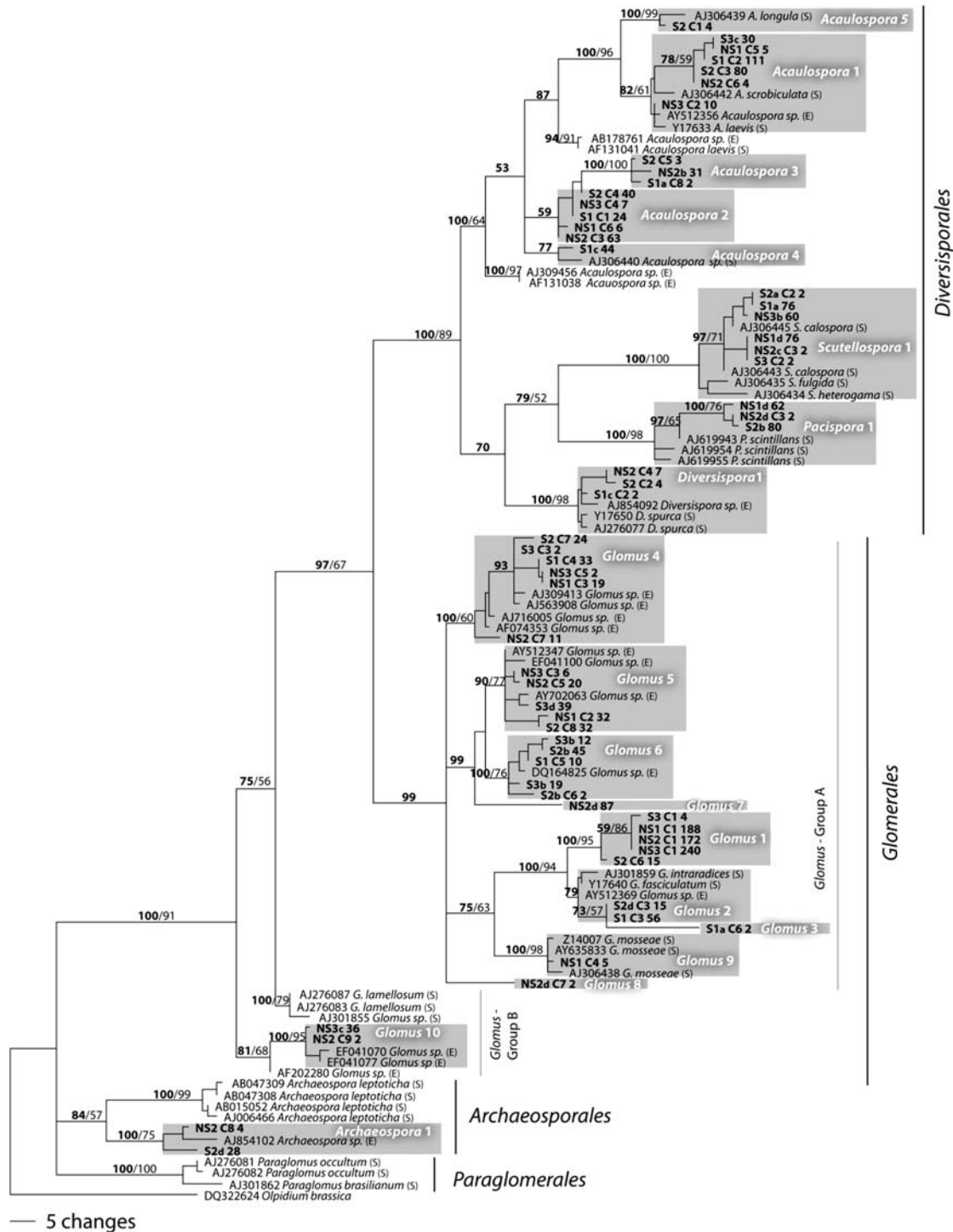


Fig. 2 Consensus tree (50% majority rule) from MRBAYES analysis showing the phylogenetic relationship of the AMF sequences obtained from roots sampled from three serpentine (S1, S2, S3) and three non-serpentine (NS1, NS2, NS3) ecotype populations of *Collinsia sparsiflora*, in bold. Letters directly behind site designation indicates an individual clone sequence; no letter indicates that the clone sequence used is a representative member of a 98% sequence identity contig and the last number of the label is the number of constituent clone sequences. Grey blocks encompass groups of sequences that are 98% similar and designate experiment OTUs (in white by genus affiliation). Other sequences are GenBank Accessions of closely related BLAST matches as well as Glomeromycota voucher sequences (Schussler *et al.* 2001). Letters behind GenBank Accessions refer to origin of the sequence (S, spore; E, environmental). The values above the branches are Bayesian posterior probabilities (bold) followed by bootstrap values (100 replicates in GARLI maximum-likelihood analysis), only support greater than 50 is shown. *Olpidium brassica* was used as an outgroup. Topology was similar between Bayesian and GARLI analyses.

Table 1 Relative abundance matrix of operational taxonomic units (OTU) of AM fungi associated with *Collinsia sparsiflora* sampled from four patches (a, b, c, and d) taken within two serpentine ecotype populations (S1 and S2) and three non-serpentine ecotype populations (NS1, NS2 and NS3). Highlighted OTUs show strong ecotype affects

| OTU | Site | | | | | | | | | | | | | | | | | | | |
|----------------|------|----|----|----|----|----|----|----|-----|----|----|----|-----|----|----|----|-----|----|----|----|
| | S1 | | | | S2 | | | | NS1 | | | | NS2 | | | | NS3 | | | |
| | a | b | c | d | a | b | c | d | a | b | c | d | a | b | c | d | a | b | c | d |
| Acaul 1 | 6 | 15 | 80 | 83 | 20 | 47 | 43 | 47 | 0 | 0 | 6 | 2 | 0 | 0 | 5 | 2 | 12 | 1 | 4 | 0 |
| Acaul 2 | 25 | 0 | 7 | 0 | 31 | 17 | 10 | 0 | 8 | 0 | 0 | 2 | 0 | 19 | 3 | 2 | 7 | 1 | 4 | 0 |
| Acaul 3 | 3 | 0 | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Acaul 4 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Acaul 5 | 0 | 0 | 0 | 0 | 1 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Arch 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 2 | 0 | 0 | 0 | 0 |
| Diver 1 | 0 | 0 | 4 | 0 | 1 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 0 | 3 | 0 | 0 | 0 | 0 |
| Glo 1 | 0 | 0 | 0 | 0 | 8 | 8 | 10 | 0 | 86 | 78 | 44 | 86 | 95 | 47 | 89 | 55 | 70 | 95 | 91 | 99 |
| Glo 2 | 60 | 0 | 0 | 12 | 0 | 0 | 0 | 35 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Glo 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Glo 4 | 0 | 77 | 4 | 1 | 13 | 0 | 21 | 9 | 0 | 22 | 0 | 2 | 0 | 12 | 0 | 5 | 2 | 0 | 0 | 1 |
| Glo 5 | 0 | 0 | 0 | 0 | 22 | 8 | 14 | 7 | 6 | 0 | 43 | 2 | 5 | 7 | 0 | 21 | 9 | 1 | 0 | 0 |
| Glo 6 | 3 | 8 | 4 | 4 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Glo 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Glo 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 |
| Glo 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Glo 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 0 | 0 | 2 | 0 |
| Paci 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 |
| Scut 1 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 3 | 0 | 0 | 1 | 0 | 0 |

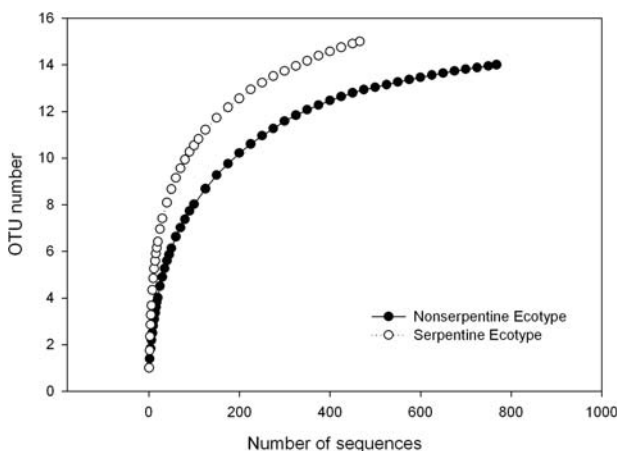


Fig. 3 Rarefaction curve of the total number of sequences sampled in non-serpentine (NS1, NS2, NS3) ecotype (solid circles) and serpentine (S1, S2) ecotype (open circles) populations of *Collinsia sparsiflora*. Rarefaction curve was produced with the species-area plot routine using 999 permutations.

Assemblage analysis

Comparing assemblages. Both of the rarefaction curves show a clear levelling off starting after approximately 400 sequences in the non-serpentine samples and approximately 300 sequences in the serpentine samples, with only

two more OTUs included following additional sampling of sequences in both cases (Fig. 3). This indicates that our sequence sampling effort obtained a large proportion of the diversity of AMF associated with both of the *C. sparsiflora* ecotypes. Thus, the sequence sampling effort was sufficient in both ecotypes to compare AMF assemblages. Comparing similarities between AMF assemblages associated with each *C. sparsiflora* root sample with MDS showed that assemblages associated with serpentine ecotypes were much more similar to each other than those associated with non-serpentine ecotypes and vice versa (Fig. 4). The low stress level of the ordination (0.11) indicates a good representation of the data (Clarke & Warwick 2001), and thus supports this result.

Statistical analysis supported the distinction between ecotype AMF assemblages. The ANOSIM global test was significant ($R = 0.49$, $P < 0.001$) indicating differences in assemblages, and pairwise tests showed specific differences between sites (Table 2). Serpentine ecotype assemblages (S1 and S2) were not significantly different from each other, but both were significantly different from the non-serpentine ecotype assemblages (NS1, NS2, and NS3) (Table 2). The pattern was the same for the non-serpentine ecotype assemblages, which were not different from each other but were distinct from the serpentine assemblages (Table 2). These results, coupled with those from MDS, clearly show that AMF assemblages associated with serpentine and non-

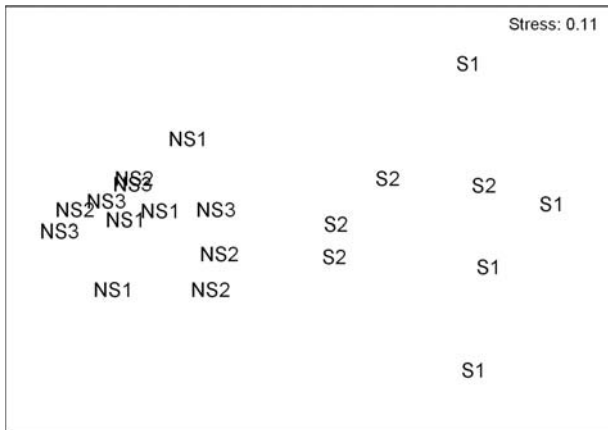


Fig. 4 Nonmetric multidimensional scaling (MDS) ordination of AMF assemblages associated with two serpentine (S1, S2) and three non-serpentine (NS1, NS2, NS3) ecotype populations of *Collinsia sparsiflora*. The MDS ordination is a configuration of the samples in which relative positions are assigned based on the Bray–Curtis similarity matrix of the data so that samples closer together have a higher similarity of component taxa than samples farther apart and overlapping samples are highly similar. The nonmetric scale of the ordination does not assign values to the axes.

Table 2 Results of ANOSIM pairwise comparison of AMF assemblages associated with serpentine (S1 and S2) and non-serpentine (NS1, NS2 and NS3) ecotypes of *Collinsia sparsiflora*. Numbers are the R^+ values associated with each pairwise comparison

| | S1 | S2 | NS1 | NS2 | NS3 |
|-----|-------|-------|-------|-------|-----|
| S1 | — | | | | |
| S2 | 0.35 | — | | | |
| NS1 | 0.99* | 0.84* | — | | |
| NS2 | 0.95* | 0.68* | −0.23 | — | |
| NS3 | 0.94* | 0.90* | −0.12 | −0.07 | — |

*Indicates significant difference between samples ($P < 0.03$); $+R \approx 1$ if there is high differentiation of AMF assemblages; $R \approx 0$ if AMF assemblages are indistinguishable.

serpentine ecotypes of *C. sparsiflora* are distinct from each other.

To examine whether this difference in ecotype AMF assemblages could have been caused by distance, we plotted similarity by distance (Fig. S1, Supplementary material). If distance was a factor, then this plot should show a trend in which similarity between sites decreased as distance between sites increased. However, no trend was found in the similarity by distance plot of the data (Fig. S1), showing distance was not a dominant factor in shaping ecotype assemblages.

We also tested the relative contribution of common and rare species to the differences in assemblages by comparing

them after performing a range of data transformations (square root, 4th root, and presence/absence) that progressively down-weight the importance of highly abundant OTUs (Clarke & Warwick 2001). Increasing the severity of the data transformation progressively increased the spread of the samples in the MDS ordination (including the stress values) and decreased the clustering within and the distance between a subset of serpentine and non-serpentine samples (data not shown). However, only the most extreme transformation of the data changed the outcome of the ANOSIM pairwise tests. After presence/absence transformation, S1 assemblages were significantly different from all sites except for S2, but S2 assemblages did not differ significantly from the non-serpentine site assemblages (data not shown). These results indicate that the strength of the distinction between ecotype assemblages was driven by the most abundant OTUs.

OTU contribution to assemblage differences. Increased relative abundance of *Acaulospora* clones in serpentine vs. non-serpentine ecotype assemblages is apparent (Table 1). Based on the average clone number per site (data not shown), serpentine ecotype root samples have similar abundances of *Acaulospora* (133 average clones/site) and *Glomus* clones (102 average clones/site). However, non-serpentine ecotype samples show a strong bias towards *Glomus* (233 average clones/site) over *Acaulospora* (16 average clones/site).

The relative abundance matrix showed a more specific pattern of OTU contribution to dissimilarities in ecotype assemblages and differences in abundance between *Glomus* and *Acaulospora* OTUs in serpentine and non-serpentine samples (Table 1). Overall, the presence of OTUs was patchy across samples and within sites and included several rare taxa (*Acaulospora* 4 and 5, *Glomus* 3, 7, 8, and 9) (Table 1). However, there are two major exceptions to this overall pattern of patchiness: *Acaulospora* 1 and *Glomus* 1 (Table 1). *Acaulospora* 1 was found in every serpentine ecotype root sample in high abundance. This OTU was also found in non-serpentine ecotypes but had a patchy distribution and low abundance. In contrast, *Glomus* 1 was the dominant OTU in non-serpentine ecotype samples being found in every root sample at very high abundance (Table 1). *Glomus* 1 was also present in serpentine ecotypes but only in a few samples at low abundance. Species SIMPER analysis confirmed the large contributions of these OTUs to the dissimilarities of the ecotype assemblages. *Glomus* 1 and *Acaulospora* 1 contributed 47% of the total dissimilarity between ecotype assemblages, each contributing 28% and 19%, respectively.

Species diversity and evenness differed between serpentine and non-serpentine ecotype assemblages. Serpentine ecotype assemblages had significantly higher species diversity ($H' = 1.17$, $F_1 = 5.2$, $P = 0.03$) and evenness ($J' = 0.68$,

$F_1 = 4.48$, $P = 0.048$) than the non-serpentine ecotype assemblages ($H' = 0.68$, $J' = 0.45$). However, there was not a significant difference in species richness between ecotype assemblages ($F_1 = 1.18$, $P = 0.29$). Sites varied in species evenness ($F_4 = 3.08$, $P = 0.023$) and diversity ($F_4 = 4.04$, $P = 0.02$). Both evenness and diversity were significantly larger in S2 ($H' = 1.53$, $J' = 0.79$) than NS3 ($H' = 0.42$, $J' = 0.29$), but S2 was not significantly different than any other site (S1, NS1 and NS2). Species richness did not differ significantly between sites ($F_4 = 1.16$, $P = 0.37$).

Soil type relationship to assemblage differences. Since the adaptive differentiation between ecotype populations of *C. sparsiflora* was driven by soil type, it is imperative to investigate the potential relationship between ecotype AMF assemblages and soil type. Characterization of soils within sites was carried out in a similar fashion to Wright *et al.* (2006) except that only chemical analysis rather than both chemical and physical analysis was carried out on the samples (Table S1, Supplementary material). Not surprisingly, the soil chemical analysis results from this study are very similar to Wright *et al.* (2006). Serpentine and non-serpentine sites were clearly defined by their calcium : magnesium ratio, serpentine soils having a ratio much less than 1 and non-serpentine soils have ratios greater than 1 (Table S1). Like Wright *et al.* (2006), we found high variability in soil chemical characters within sites (Table S1).

Using the BIO-ENV routine, we selected soil variables that had the highest Spearman's rank correlation score (nitrogen, phosphorus, potassium, calcium, magnesium, calcium : magnesium, zinc, iron, copper, and boron) to include in further analyses. The nonmetric MDS ordination of the soil nutrients showed that serpentine and non-serpentine soil types are distinct from each other in the ordination, but non-serpentine samples also have discrete groupings within sites (Fig. S2, Supplementary material). ANOSIM analysis confirmed that there are differences in soil nutrients between sites ($R = 0.733$, $P < 0.001$). We used the RELATE routine to test for a relationship between soil nutrients and ecotype AMF assemblages. This routine compares similarity matrices using Spearman's rank correlation to test if two patterns are significantly matched (Clarke & Warwick 2001). The RELATE test was significant ($Rho = 0.507$, $P < 0.001$) indicating that there is a relationship between ecotype assemblages and soil nutrients.

Discussion

Molecular approach

In this study, direct amplification using NS31/AM1 captured the majority of AMF diversity associated with the *C. sparsiflora* ecotypes (Fig. 3). The NS31/AM1 primer set

revealed a surprising diversity of AMF genera including *Archaeospora*, which has been generally absent from other studies using this primer pair. Although AM1 is known to exclude the Paraglomeraceae, NS31/AM1 is the only primer set that is used without a nested PCR, a process that can compound PCR bias and lower sequence diversity (Stach *et al.* 2001). Unlike most AMF molecular studies, we did not use a clone-screening technique before sequencing. Preliminary molecular work indicated that no single or combination of restriction enzymes would distinguish between all sequence groups (S.P. Schechter, unpublished data). Therefore, screening clones with restriction fragment length polymorphism (RFLP) or terminal RFLP before sequencing would have lowered the diversity of AM sequences.

We used the relative abundance of clones as a proxy for the relative abundance of AMF associates which we readily acknowledge should be carried out with caution. It is well known that PCR and cloning biases can alter clone abundances (Polz & Cavanaugh 1998; Qiu *et al.* 2001; Acinas *et al.* 2005). Helgason *et al.* (1999) discussed this potential problem specifically for AM1/NS31 PCR products in detail and concluded that relative abundance of dominant sequence groups was a reasonable estimate of species abundance. One concern in this study was the potential for primer bias of a single sequence group causing false dominance of that sequence group in those samples. However, a primer bias would show up in every sample in which that group was present. The two most abundant species and largest contributors to ecotype dissimilarity, *Glomus* 1 and *Acaulospora* 1, showed no such bias even though half of the samples had both sequence groups present in the DNA extraction (S2a,b,c; NS1c,d; NS2cd; NS3a,b,c; Table 1). In addition, the rarefaction curves showed that clone sampling nearly saturated the diversity of AM fungi associated with the *C. sparsiflora* ecotypes. Thus, we are confident that clone-relative abundances provided a reasonable estimation of the relative abundance of dominate species. Moreover, the use of these data in the PRIMER 5 software simplified multivariate analysis and allowed us to ask very specific questions about the AMF assemblages.

Distinction between ecotype AMF assemblages

We found that serpentine and non-serpentine ecotypes of *C. sparsiflora* associate with distinct AM fungal assemblages. To our knowledge, this is the first study to compare the AMF assemblages associated with edaphically adapted ecotypes. However, it is not the first study to compare serpentine and non-serpentine mycorrhizal assemblages. Moser *et al.* (2005) compared the ectomycorrhizal (ECM) assemblages associated with *Quercus garryana* growing on serpentine and non-serpentine soil and found dramatically different results from those presented in this study. They

found no significant differences in similarity, species diversity, richness or evenness between ECM fungal assemblages, although they had expected serpentine soils to have lower ECM fungal diversity. They proposed that the diverse edaphic challenges of serpentine soils might promote or support greater diversity of mycorrhizal fungi in order to counter-balance the effects of these soils on plant growth (Moser *et al.* 2005). Our finding of greater diversity and evenness in serpentine ecotype AMF assemblages may support Moser *et al.*'s view.

While the differences in the results between the two studies may be due in part to the distribution and dispersal differences between ECM fungi (Basidiomycota and Ascomycota) and AM fungi (Glomeromycota), another possibility is that the comparison between serpentine and non-serpentine *Q. garryana* ECM may not have been as distinct as expected. Moser *et al.* (2005) suggested that *Q. garryana* may grow on serpentine soils in alluvial areas that have intrusions of non-serpentine materials, allowing for broad phenotypic/genotypic tolerance to a minimal serpentine influence (Kruckeberg 1984) instead of signifying a distinct ecotype adapted to serpentine.

A strength of this study is the *C. sparsiflora* research system. The use of a reciprocal transplant experiment clearly distinguished between broad tolerance and local adaptation within plant species found on contrasting environments (Wright *et al.* 2006; Wright & Stanton 2007). Establishing this difference is key for the investigation of an AM fungal role in serpentine adaptation. In addition, the close proximity between populations helped to eliminate distance as a factor in ecotype assemblage dissimilarities (Fig. S1). Distance is an important consideration when comparing AMF assemblages, as other studies have shown a strong distance effect on AMF assemblage composition when comparing sites beyond 1 km apart (Husband *et al.* 2002; Lekberg *et al.* 2007).

The relative abundance matrix showed an intriguing pattern of AMF composition and abundance. The majority of OTUs showed a patchy distribution, which may be a consequence of the small-scale heterogeneity of AMF abundance and composition reported in several studies (Pringle & Bever 2002; Carvalho *et al.* 2003; Wolfe *et al.* 2007). The dominance and abundance of *Acaulospora* 1 and *Glomus* 1, however, contrasts this overall pattern of heterogeneity. Rosendahl & Stukenbrock (2004) attributed a similar pattern of dominance in their system to a single AM fungus that covered a zone at least 10 m in length. However, Rosendahl & Stukenbrock (2004) studied AMF associated with *Hieracium pilosella* along transects within a continuous area, while the ecotype populations sampled in our study are on distinct and discontinuous sites. Thus, the dominance of *Acaulospora* 1 in the serpentine and *Glomus* 1 in the non-serpentine ecotype samples most likely reflects the influence of specific soil and/or host factors and may represent

differences in tolerance to serpentine soil, rather than a spatially dominant individual. Serpentine soil in particular is known to exert strong edaphic selection on inhabitants (Brady *et al.* 2005). Moser *et al.* (2005) found several serpentine-specific ECM taxa. The presence of the serpentine only taxa of *Glomus* 6 supports a strong soil-type factor in assemblage composition. The inclusion of an AMF clone isolated from a polluted soil in this serpentine-specific clade gives additional credence to this hypothesis.

Relationship between soil and AMF assemblages

The significant relationship between soil nutrients and *C. sparsiflora* ecotype AMF assemblages found using RELATE was not surprising because it is likely that complex interactions between soil, plant ecotypes and AMF combine to shape the outcome of ecotype AMF assemblage composition. It has previously been shown that both soil (Johnson *et al.* 1992; Landis *et al.* 2004; Lekberg *et al.* 2007) and host specificity (Helgason *et al.* 2002; Vandenkoornhuyse *et al.* 2002; Vandenkoornhuyse *et al.* 2003) can have a strong influence on AMF assemblage composition. However, it is difficult to isolate edaphic selection from host-specific or plant-community influences on AMF assemblage structure and composition (Johnson *et al.* 1992; Bever *et al.* 1996; Schultz *et al.* 2001; Bever *et al.* 2002).

The design and results of this study open up the possibility that soil or host or both factors are responsible for the distinction between ecotype AMF assemblages. One way to ascertain *C. sparsiflora* ecotype choice is to conduct a common garden experiment, allowing the plant to choose among a collective serpentine and non-serpentine AM fungal inoculum. Sampling non-*Collinsia* AMF assemblages across several serpentine and nonserpentine sites can test the general influence of soil type on AMF assemblages. Manipulative experiments with collections of these AMF taxa and *C. sparsiflora* ecotypes will further clarify the function of these fungi in serpentine adaptation and identify possible serpentine-tolerant AMF ecotypes. These studies are presently underway and will hopefully help illuminate the interrelationship between soil and/or host factors in ecotype AMF assemblage composition and the role of AMF in serpentine adaptation.

Implications of distinct C. sparsiflora ecotype AMF assemblages

The distinction in *C. sparsiflora* ecotype assemblage indicates a strong relationship between AMF associates and plant adaptation to serpentine; this is true whether the ecotypes are choosing specific AMF fungi within a ubiquitous soil assemblage or just tapping nonspecifically into an assemblage that has been shaped by edaphic factors. Both scenarios imply that it is necessary for adapted plants to

associate with serpentine-tolerant AMF taxa in serpentine soil. Studying the role of selection in the ecotypic differentiation of *C. sparsiflora*, Wright & Stanton (2007) hypothesized that the plant traits under divergent selection are likely to be physiological and/or biochemical in nature and expressed below-ground (Wright & Stanton 2007). The differential associations between *C. sparsiflora* ecotypes and AMF imply that these unknown traits may involve symbiotic interactions. There is a growing body of evidence from metal contaminant (Meharg & Cairney 1999), low nutrient (Schultz *et al.* 2001), and thermotolerance (Redman *et al.* 2002) research that fungal symbionts are important contributors to plant edaphic adaptation; our work is a first step towards identifying a role for AMF in serpentine adaptation.

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Shannon Schechter is interested in plant-fungal interactions, ecology, and evolution. Tom Bruns is broadly interested in fungal ecology and evolution.

Supplementary materials

The following supplementary material is available for this article:

Fig. S1 Relationship between similarity of AMF assemblages and distance between samples. No trend was found.

Fig. S2 Nonmetric multidimensional scaling (MDS) ordination of soil nutrients (nitrogen, phosphorus, potassium, magnesium, calcium, magnesium : calcium, zinc, manganese, iron, copper, and boron) associated with two serpentine (S1, S2) and three non-serpentine (NS1, NS2, NS3) ecotype populations of *Collinsia sparsiflora*. Soil nutrients included were chosen by the BIO-ENV routine. The MDS ordination is a configuration of the samples in which relative positions are assigned based on the Euclidean distance similarity matrix of the data so that samples closer together have a higher similarity of soil nutrients than samples farther apart and overlapping samples are highly similar. The nonmetric scale of the ordination does not assign values to the axes.

Table S1 Site soil chemical variables (S, serpentine and NS, non-serpentine). Values are means with standard deviation below in parentheses. Nitrogen (as NO₃), phosphorus (P, Weak Bray), potassium (K), magnesium (Mg), calcium (Ca), zinc (Zn), iron (Fe), copper (Cu), and boron (B) are reported in parts per million (ppm). Cation exchange capacity (CEC) is reported as milliequivalents per 100 grams of soil. Highlighted numbers indicate calcium : magnesium ratio; serpentine soils have a ratio much less than one and non-serpentine soils have ratios greater than one. Letters indicate significant differences at $P < 0.05$

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