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Testing the role of arbuscular mycorrhizal fungi in plant adaptation to serpentine soil

by

Shannon Peters Schechter

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

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in the

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of the

University Of California, Berkeley

Committee in charge:

Professor Thomas D. Bruns, Chair

Professor John W. Taylor

Professor Bruce G. Baldwin

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Testing the role of arbuscular mycorrhizal fungi in plant adaptation to serpentine soil

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By Shannon Peters Schechter

## Abstract

Testing the role of arbuscular mycorrhizal fungi in plant adaptation to serpentine soil

by

Shannon Peters Schechter

Doctor of Philosophy in Microbiology

University of California, Berkeley

Professor Thomas D. Bruns, Chair

This dissertation explores a new theoretical and experimental framework in which plant edaphic adaptation is mediated through arbuscular mycorrhizal fungi (AMF). The first chapter describes the primary ecological relationship between adapted plants and AMF by examining the AMF assemblages associated with field populations of serpentine and non-serpentine adapted ecotypes of California native plant *Collinsia sparsiflora*. The second chapter tests for plant-fungal specificity between *C. sparsiflora* ecotypes and serpentine and non-serpentine AMF using a common garden greenhouse experiment. Chapter three tests if soil edaphic factors alone could shape distinct serpentine and non-serpentine AMF assemblages by sampling non-*C. sparsiflora* root AMF assemblages from adjacent serpentine and non-serpentine sites. The final chapter addresses the functional role of AMF in serpentine adaptation with a greenhouse experiment using serpentine and non-serpentine AMF and *C. sparsiflora* ecotypes grown in sterilized serpentine soil.

I found that serpentine and non-serpentine adapted ecotypes of *C. sparsiflora* associate with distinct AMF assemblages- an *Acaulospora* 1OTU- dominated serpentine, and a *Glomus* 1 OTU- dominated non-serpentine plant ecotype AMF assemblage (Chapter 1). However, I also found a relationship between plant ecotype AMF assemblage and soil nutrients. Thus, this distinction between plant ecotype AMF assemblages might be explained two ways: 1) the plant ecotypes have a high specificity for particular AM fungi within a ubiquitous soil assemblage or 2) the plant ecotypes were tapping non-specifically into AMF assemblages shaped by edaphic factors.

I tested the first scenario in Chapter 2 by growing *C. sparsiflora* serpentine and non-serpentine ecotypes in a common pool of serpentine and non-serpentine AMF and then identified the root AMF of each plant ecotype. I found that the mixing of serpentine and non-serpentine AMF soil inoculum as the source of the common garden resulted in a non-serpentine soil type.

Consequently, while the *C. sparsiflora* ecotypes associated with distinct AMF assemblages within the common garden, overall the ecotype AMF assemblages resembled that of a non-serpentine soil (i.e. *Glomus* 1 dominated). Therefore, I found no evidence of host-specificity between *C. sparsiflora* serpentine and non-serpentine ecotypes and serpentine and non-serpentine AMF. However, these results do indicate that the soil may select the AM fungi and

potential for host choice of AMF based on soil type is present.

I tested the second scenario in Chapter 3. I found that serpentine and non-serpentine AMF assemblages are distinct from each other. Variance partitioning analysis showed that both soil edaphic factors (33.5%), and plant assemblages (25.6%) drove the distinction between serpentine and non-serpentine AMF assemblages. This study confirms that there is a strong ecological relationship between AMF and plant tolerance to serpentine soil – plants growing in serpentine soil associate with serpentine-tolerant AMF taxa.

Finally, I tested for a functional difference between serpentine and non-serpentine AMF assemblages that directly impact *C. sparsiflora* growth and fitness on serpentine (Chapter 4). Only shoot dry weight showed a significant response to AMF source. I found that serpentine AMF significantly increased growth of hosts over non-serpentine AMF and AMF-free controls. This indicates that serpentine AMF have a specialized adaptation to serpentine conferring growth enhancement to hosts, but it is still unclear what this adaptation is or which function is contributing to growth enhancement. I also found trends that imply that *C. sparsiflora* serpentine adapted ecotypes have a greater response to AMF than non-serpentine ecotypes, but these trends were not significant.

**This dissertation is dedicated to my parents, who taught me the dedication and self-discipline needed to achieve my goals, my brother, who inspired the drive and competitive spirit I needed to be my best, and my husband Fred and son Benjamin, without whom none of this would matter.**

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## Introduction

Adaptation to edaphic factors referring to soil physical, chemical, and biological characteristics, has long been considered an important element in plant distribution, diversification, and speciation (Wallace, 1858). In fact, edaphic factors are viewed as second only to climate in their influence on plant distribution (Rajakaruna, 2004). Classic studies by Kruckeberg (1951; 1954; 1967) as well as more recent work (Macnair & Gardner, 1998; Rajakaruna *et al.*, 2003a; Rajakaruna *et al.*, 2003b; Wright *et al.*, 2006) have shown clear patterns of plant adaptation to edaphic factors. Despite several decades of work on this topic, however, the mechanisms of plant edaphic adaptation are not fully understood.

Approximately 85% of all plants interact with the soil environment through symbiosis with the arbuscular mycorrhizal fungi (AMF) (Wang & Qiu, 2006). These specialized fungi (Glomeromycota) are ubiquitous root symbionts that have co-evolved with plants for over 400 million years as an extension of the plants root system (Redecker, 2006; Schussler *et al.*, 2001). AMF have been shown to increase their plant hosts' establishment and growth in stressful environments by enhancing nutrient and water uptake and providing protection against toxic conditions (Allen *et al.*, 1981; Pairunan *et al.*, 1980; Smith & Read, 1997). Moreover, the more nutrient-stressed the environment, the more dependent plants are on their AMF associates for nutrient acquisition and growth (Habte & Manjunath, 1987; Johnson & Wedin, 1997; Yost & Fox, 1979). *From these facts it is clear that symbiosis with AMF may be a key evolutionary strategy for plants to overcome edaphic stress.*

Optimal performance of the symbiosis under environmental stress may require specific plant and fungal traits (Gonzalez-Chavez *et al.*, 2002; Meharg, 2003; Meharg & Cairney, 1999; Schultz *et al.*, 2001). AMF may mediate plant tolerance to environmental stress in two ways. AMF can be *tolerant* of edaphic stress, meaning species whose spores can germinate and colonize under stressful conditions, function “normally” in stressful sites by improving plant uptake of essential nutrients and water and, in so doing, stress tolerant AMF perform essential but “normal” functions for mycorrhizal plants growing in these stressful environments (Gonzalez-Chavez *et al.*, 2002; Meharg, 2003; Meharg & Cairney, 1999). Alternatively, a few AMF present in these stressful sites may have *specialized functions* that confer enhanced resistance to the host by modifying uptake and transport of specific nutrients or restricting transport of toxins to the plant host (Gonzalez-Chavez *et al.*, 2002; Meharg, 2003; Meharg & Cairney, 1999). *Thus adaptive AMF traits are important for mycorrhizal plant growth under edaphic stress.*

Plant traits involved in the establishment of and response to AMF may also be important for tolerance of stressful conditions. For example, plants have been shown to mediate adaptation to low nutrient soils by altering their relationship to AMF (Schultz *et al.*, 2001). Plant genotypes can differ in their “dependency” on (i.e. response to) AMF for normal growth and functioning (Smith & Read, 1997). Studying low and high P soil ecotypes of *Andropogon gerardii* (Poaceae), Schultz *et al.* (2001) found that the ecotypes differed in their dependence on AMF for P uptake and growth and hypothesized that low P ecotypes had adapted to low P soils by increasing their dependency on AMF. In fact, Kaeppeler and colleagues (2000) found quantitative trait loci (QTL) for responsiveness to AMF in maize that was also correlated to plant growth in low P. Indeed, in cultivated systems, selection of crop genotypes under high fertility conditions commonly results in decreased susceptibility and response to AMF, most likely due to decreased dependency on AMF (Hetrick *et al.*, 1992; Hetrick *et al.*, 1993; Hetrick *et al.*, 1996). *Therefore, plant edaphic ecotypes may differ in their requirement for and response to AMF.*

Serpentine as a Model System: Serpentine habitats provide a unique experimental system to explore the relationship between AMF and plant edaphic adaptation. Serpentine soils are naturally “stressful” soils found worldwide. Soils derived from serpentine, and ultramafic rock, are characterized by a low levels of essential nutrients, drought susceptibility, very low calcium:magnesium ratio, and high levels of heavy metals (Brady *et al.*, 2005; Kruckeberg, 1984; Rajakaruna & Bohm, 1999). Plant tolerance to serpentine is widespread both geographically and phylogenetically and involves tolerance to multiple edaphic stresses (Brady *et al.*, 2005). *Thus, serpentine soils provide an exceptional system to study the role of AMF in plant edaphic adaptation.*

This study was done at the Donald and Sylvia McLaughlin University of California Natural Reserve. I took advantage of two unique situations present at the reserve. First, Wright *et al.* (2006) have experimentally demonstrated that six populations of the California native plant *Collinsia sparsiflora* are locally adapted serpentine and non-serpentine ecotypes. These six populations are all within a close geographic range (75m to 1km). I used these populations to characterize and compare the AMF assemblages associated with serpentine and non-serpentine adapted plant ecotypes (Schechter & Bruns, 2008).

Second, UC Davis researchers established a 27.5 ha research grid at the reserve in which soil chemical analysis and vegetation surveys were done along grid points 50 meters apart across the entire grid. I used the fine scale mosaic of serpentine and non-serpentine soils found in the grid to examine the edaphic influence of serpentine on AMF assemblage structure and composition by comparing AMF assemblages associated with adjacent serpentine and non-serpentine soils. Additionally, I studied all AMF assemblages *in planta* via PCR, cloning, and sequencing techniques, thereby providing a more direct approach than spore-based identification methods (Helgason *et al.*, 1998; Husband *et al.*, 2002; Rosendahl & Stukenbrock, 2004).

*I used this model system to describe the primary ecological, evolutionary, and functional relationships between serpentine soil, AMF, and adapted plants.* I addressed four main questions in this dissertation:

1. Do serpentine and nonserpentine ecotypes of *Collinsia sparsiflora* associate with distinct AMF assemblages?
2. Do adapted ecotypes of *Collinsia sparsiflora* require specific AMF?
3. Do edaphic factors of serpentine soil shape AMF assemblages that are distinct from nonserpentine assemblages?
4. Do serpentine-derived AMF mediate plant adaptation by improving fitness on serpentine?

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## CHAPTER 1

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

## 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 analyzed the small subunit rDNA gene amplified from root DNA extracts using AMF-specific primers. A total of 1,952 clones from 24 root samples (four from each site) were sequenced. We used sequence similarity and phylogenetic analysis to determine operational taxonomic units (**OTUs**) resulting in 19 OTUs representing taxa from 6 AMF genera, including one serpentine-specific OTU. We used Bray-Curtis similarity, multidimensional scaling (**MDS**) and analysis of similarity (**ANOSIM**) 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.

## 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; Rajakaruna *et al.*, 2003b; 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 Ca:Mg ratio, low levels of essential nutrients (N,P,K), high to toxic levels of heavy metals (Fe, Cr, Co, Ni), and drought susceptibility (see reviews by Brady *et al.*, 2005; Brooks, 1987). 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). Indeed, the multifaceted nature of serpentine edaphic factors indicates that multiple traits are likely to be important in serpentine adaptation (Brady *et al.*, 2005; Rajakaruna *et al.*, 2003b).

The current thinking about serpentine adaptation is primarily based on aboveground 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 belowground. The ubiquity and impact of root symbionts on plant growth, distribution, and plant community dynamics are widely accepted (Benson & Dawson, 2007; Bever, 2003; Klironomos *et al.*, 2000; Reynolds *et al.*, 2003; Silvertown, 2004; Smith & Read, 1997; Wardle *et al.*, 2004). 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 non-mycorrhizal 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 (Allen *et al.*, 1981; Pairunan *et al.*, 1980; 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 (Habte & Manjunath, 1987; Johnson & Wedin, 1997; Yost & Fox, 1979). 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 (Gonzalez-Chavez *et al.*, 2002; Meharg, 2003; Meharg & Cairney, 1999; Schultz *et al.*, 2001). 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 CO<sub>2</sub>, N, P, and K additions (Chiariello *et al.*, 1982; Hopkins, 1987; Koide *et al.*, 1988; Koide & Mooney, 1987; Rillig *et al.*, 1999). Working with serpentine grassland plant and AMF communities, Castelli and 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, 2003; Bever *et al.*, 1996). 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 PCR, cloning, and sequencing techniques will provide a more direct approach than spore-based identification methods (Helgason *et al.*, 1998; Hijri *et al.*, 2006; Husband *et al.*, 2002; Rosendahl & Stukenbrock, 2004; Vandenkoornhuise *et al.*, 2002; Vandenkoornhuise *et al.*, 2003). 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 geographic 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>) (Figure 1). The geology of the McLaughlin reserve has resulted in a fine-scale mosaic of serpentine, volcanic, and valley sediment soil types occurring within meters of each other (Wright & Stanton, 2007; Wright *et al.*, 2006). 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. *C. 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 reciprocal transplant of populations among all six sites and measuring lifetime production of flowers and fruits, Wright (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 (Figure 1).

### *Sampling*

In March 2005, we sampled all six *C. sparsiflora* populations: three serpentine (S1, S2, S3) and three non-serpentine (NS1, NS2, NS3) (Figure 1) (Wright *et al.*, 2006). Whole plant and soil samples were taken from four patches of *C. sparsiflora* within each site, equaling twenty-four 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, labeled A – D, by taking a trowel slice 10 cm by 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 due to 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 hours of collection. We sent soil samples to A&L Western Agricultural Laboratories (Modesto, CA) within 24 hours of collection for chemical analysis (Wright *et al.*, 2006). All plant samples were processed within two 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 three days. We dried the roots in order to simplify processing and facilitate cell disruption via beadbeating for DNA extraction. These dried roots, representing ten to twenty-six *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 seconds or until a fine powder formed. The samples were then immediately placed on ice, and 1.5 ml of 2x 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 (PCR): We amplified a variable region of the 18S rDNA 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). Prior to PCR, we diluted the DNA extracts 1:10 or 1:100 in sterile double distilled water. Each 20 µl PCR reaction consisted of 12.4 µl of dH<sub>2</sub>O, 0.2 µl of 2.5 U *Pfu Turbo* DNA polymerase, 2 µl of manufacture's buffer (Stratagene), 2 µl of 10x dNTPs, and 0.2 µl of each 50µM primer. PCR conditions were the same as described by Helgason (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  $\mu$ l of nuclease free water. The purified products were concentrated by drying in a SpeedVac (Savant) and then resuspended in 10  $\mu$ 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 sec, 72° C for 1:30 min, 29 cycles of 95° C for 30 sec, 50° C for 30 sec, 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 done in one direction with AM1 using BigDye version 3.1 chemistry (Applied Biosystems). Sequences were determined with an ABI 3100 Genetic Analyzer (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: Prior to analysis, we compared our sequences to GeneBank 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 (OTU) 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 ClustalX (Thompson *et al.*, 1997) and then manually edited the sequences using MacClade v 4.08 (Maddison & Maddison, 2005). Two separate phylogenetic analyses were performed using *Oplidium brassica* as an outgroup: maximum likelihood (ML) was conducted using Garli (Genetic Algorithm for Rapid Likelihood Inference) v 0.95 (Zwickl, 2006), and Bayesian analysis was performed using MrBayes 3.1.1 (Ronquist & Huelsenbeck, 2003). Molecular evolutionary models for Bayesian analysis and maximum likelihood 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 (-LnL = 4056.8). Bayesian analysis was performed with two MCMC chains 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 GeneBank under accession numbers: 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, Plymouth, UK (Clarke & Warwick, 2001) to perform the AMF assemblages 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 non-metric Multidimensional Scaling (MDS). The MDS ordinations were used to represent the dissimilarities in assemblage composition among samples (Clarke & Warwick, 2001). We used the ANOSIM (analysis of similarities) 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 SIMPER (Similarity Percentages) routine to determine the relative contribution of individual OTUs toward 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 v. 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 was log transformed, and then the similarity matrix was produced using Euclidean distance (Clarke & Warwick, 2001). Non-metric 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 1,952 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 fifteen 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 (Figure 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 (Figure 2). All 98% sequence similarity groupings were present in phylogenetic analyses as well-supported clades (Figure 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 (Figure 2). These OTUs were used to construct the relative abundance matrix (Table 1). The OTU *Glomus* 6 was specific to serpentine ecotypes (Figure 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).

### *Assemblage Analysis*

**Comparing Assemblages:** Both of the rarefaction curves shows a clear leveling 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 (Figure 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 (Figure 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-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 (Figure S1). 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 (Figure 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, 4<sup>th</sup> 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 versus 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 avg. clones/site) and *Glomus* clones (102 avg. clones/site). However, non-serpentine ecotype samples show a strong bias toward *Glomus* (233 avg. clones/site) over *Acaulospora* (16 avg. 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 similarity percentage (**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 done in a similar fashion to Wright *et al.* (2006) except that only chemical analysis rather than both chemical and physical analysis was done on the samples (Table S1). 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 Ca:Mg 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 rank correlation score (N, P, K, Ca, Mg, Ca:Mg, Zn, Fe, Cu, and B) to include in further analyses. The non-metric 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 (Figure S2). 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 matrixes 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 (Figure 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 reaction, 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 prior to sequencing. Preliminary molecular work indicated that no single or combination of restriction enzymes would distinguish between all sequence groups (Schechter, unpublished). Therefore, screening clones with RFLP or T-RFLP prior to 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 done with caution. It is well known that PCR and cloning biases can alter clone abundances (Acinas *et al.*, 2005; Polz & Cavanaugh, 1998; Qiu *et al.*, 2001). Helgason (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 dominant 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 & Stanton, 2007; Wright *et al.*, 2006). 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 (Figure 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 (Carvalho *et al.*, 2003; Pringle & Bever, 2002; Wolfe *et al.*, 2007). The dominance and abundance of *Acaulospora* 1 and *Glomus* 1, however, contrasts this overall pattern of heterogeneity. Rosendahl and Stukenbrock (2004) attributed a similar pattern of dominance in



their system to a single AM fungus that covered a zone at least 10 meters in length. However, Rosendahl and 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 (Bever *et al.*, 1996; Bever *et al.*, 2002; Johnson *et al.*, 1992; Schultz *et al.*, 2001).

The design and results of this study opens 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 non-serpentine 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 non-specifically 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 and Stanton (2007) hypothesized that the plant traits under divergent selection are likely to be physiological and/or biochemical in nature and expressed belowground (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 toward identifying a role for AMF in serpentine adaptation.

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**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.

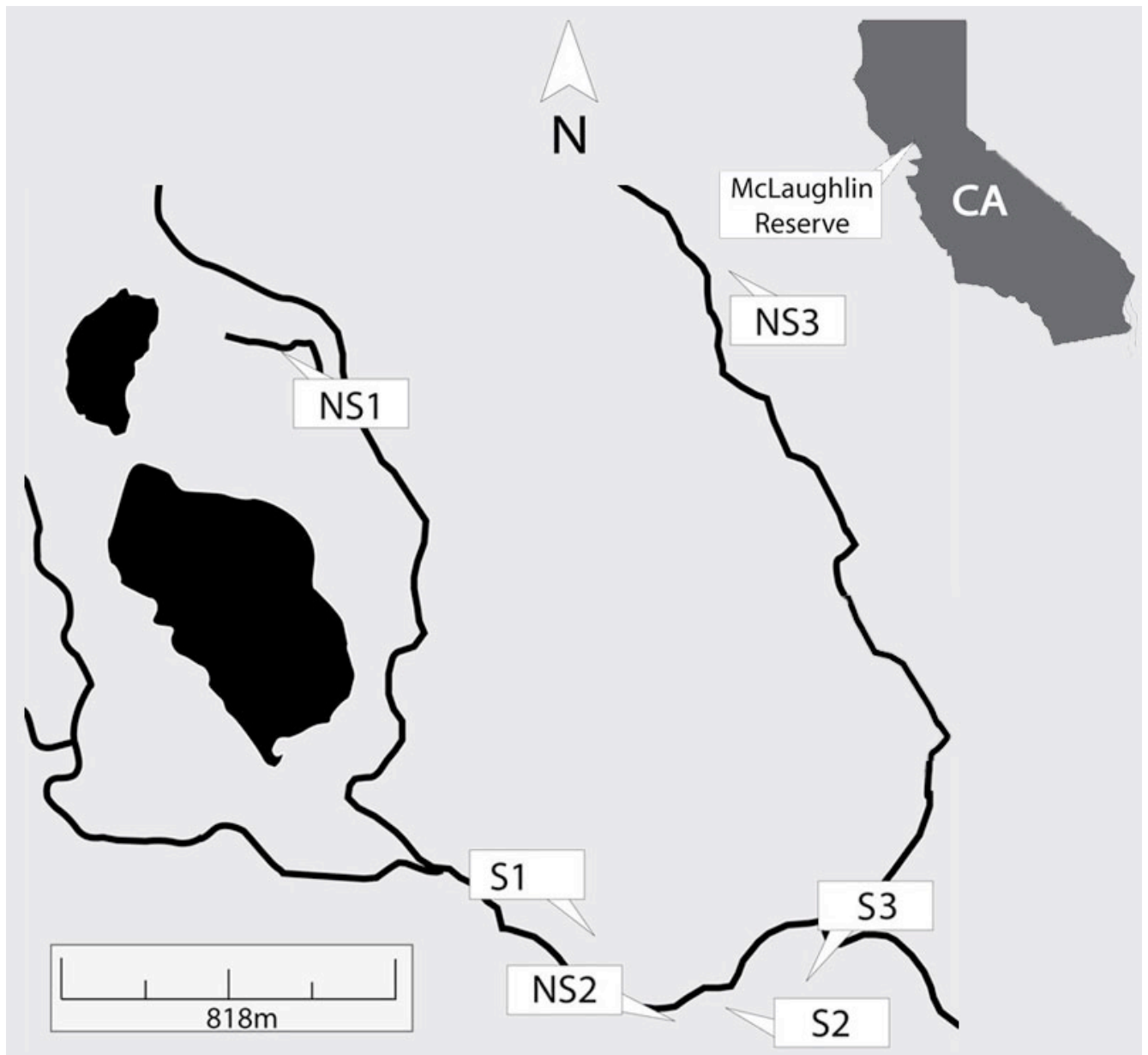
OTU	Site																			
	S1				S2				NS1				NS2				NS3			
	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
<b>Acaul 1</b>	6	15	80	83	20	47	43	47	0	0	6	2	0	0	5	2	12	1	4	0
Acaul 2	25	0	7	0	31	17	10	0	8	0	0	2	0	19	3	2	7	1	4	0
Acaul 3	3	0	0	0	1	4	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Acaul 4	0	0	2	0	0	0	0	0	0	0	0	0	0	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
<b>Glo 1</b>	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



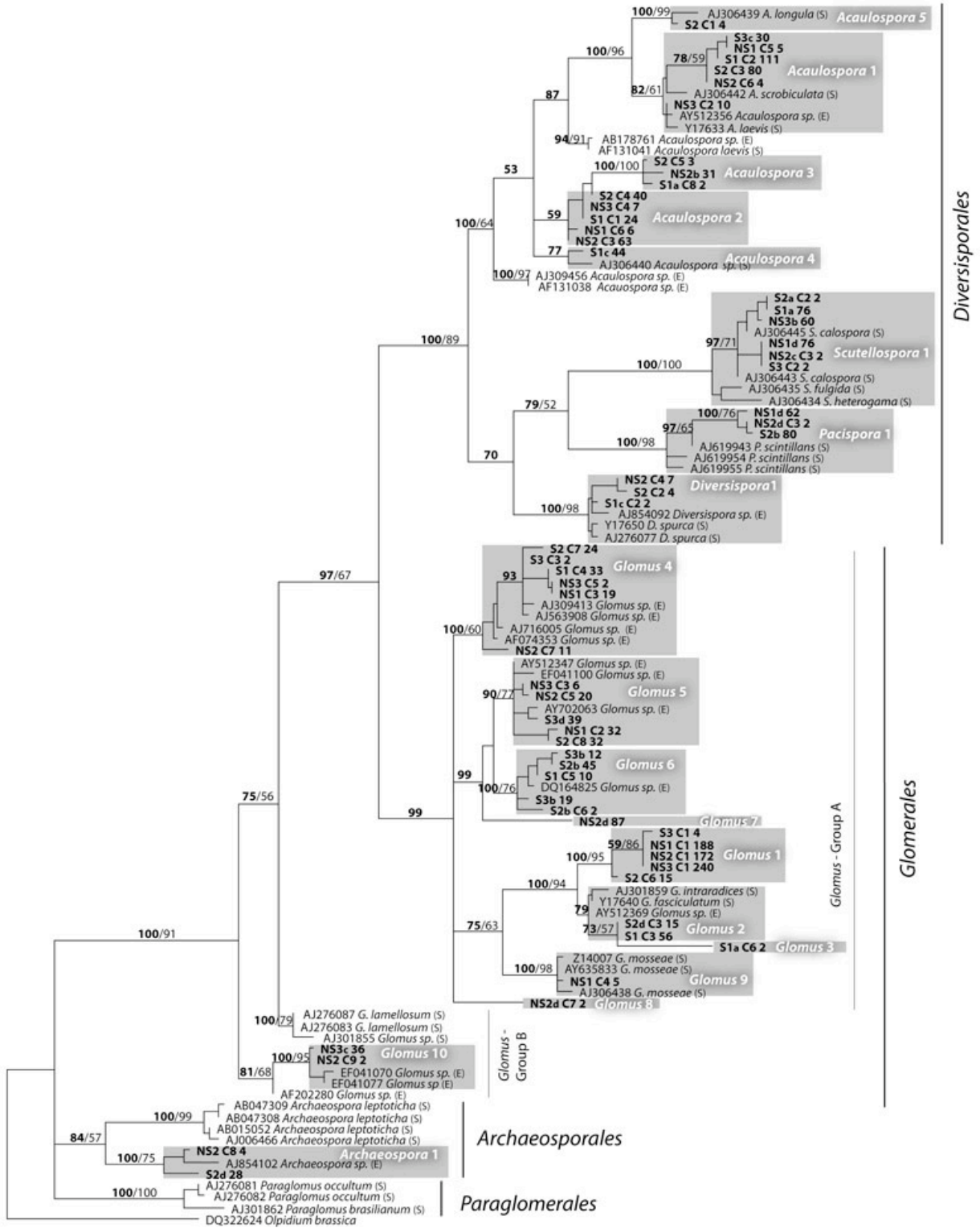
**Table 2.** Results of ANOSIM pairwise comparison of AMF assemblages associated with serpentine (S1 and S2) and non-serpentine (NS1, NS2 and NS3) ecotypes of *Collinisa sparsiflora*. Numbers are the  $R^a$  values associated with each pairwise comparison.

	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	-

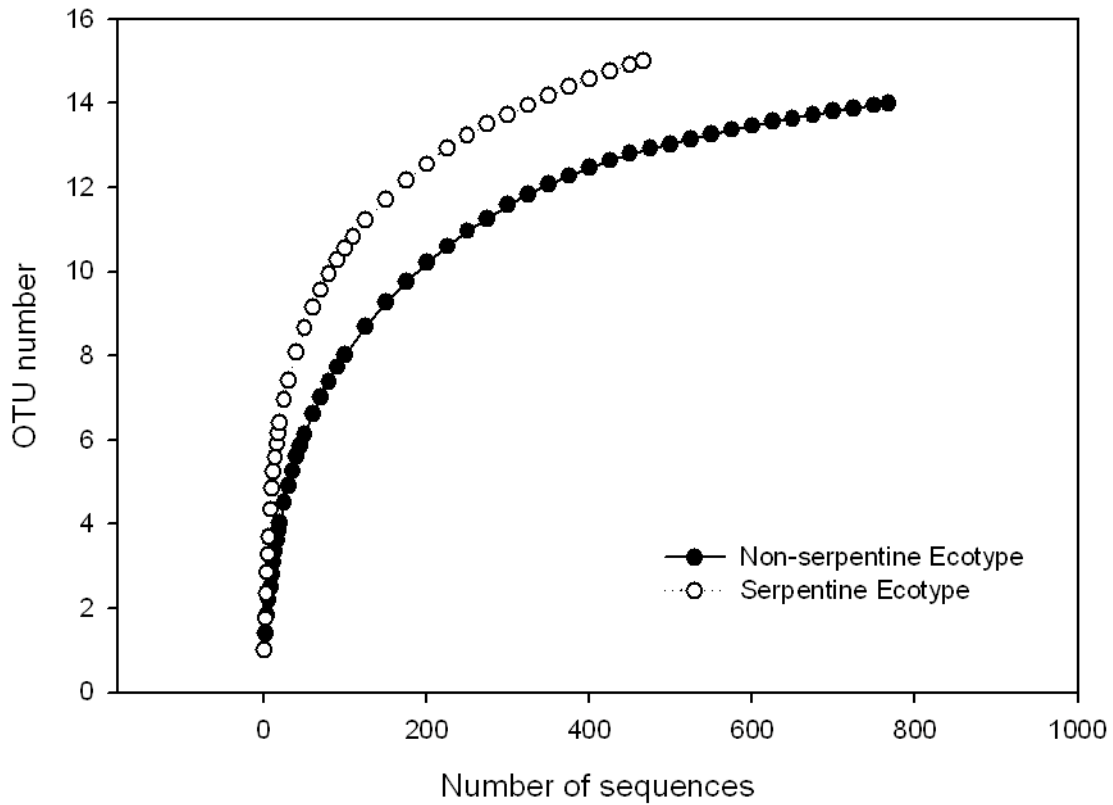
<sup>a</sup>  $R \approx 1$  if there is high differentiation of AMF assemblages;  $R \approx 0$  if AMF assemblages are indistinguishable. \* Indicates significant difference between samples ( $p < 0.03$ ).



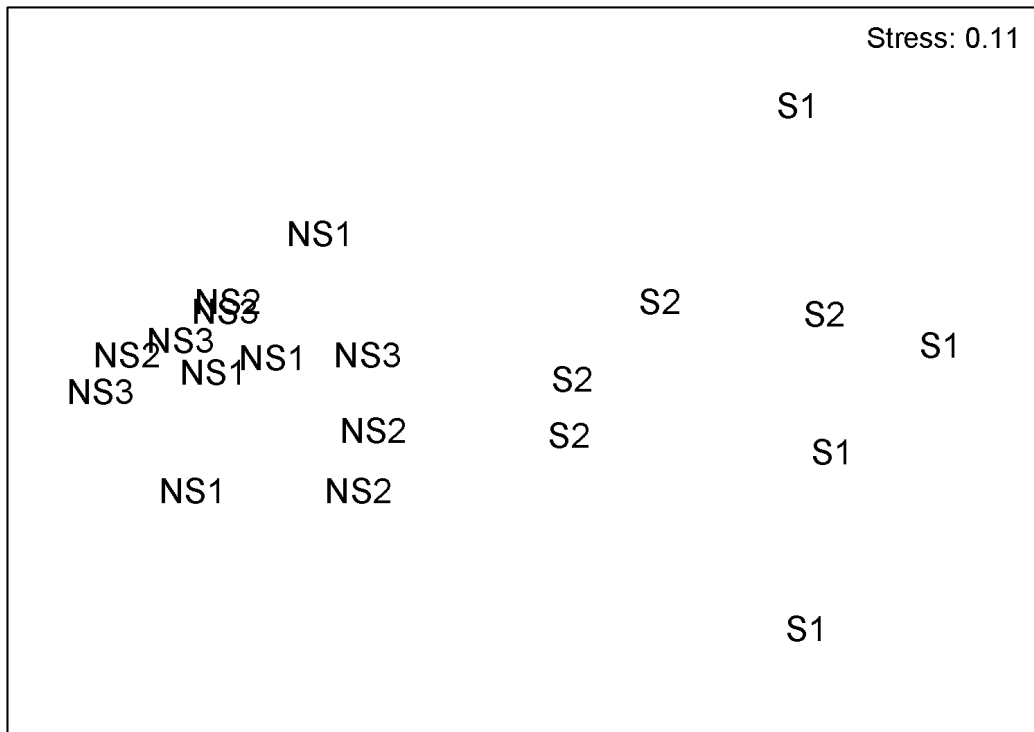
**Figure 1.** Study area at Donald and Sylvia McLaughlin Reserve, part of the UC Davis natural reserve system in northern California (CA). Labels indicate the location of serpentine (S1, S2, S3) and non-serpentine (NS1, NS2, NS3) ecotype populations of *Collinsia sparsiflora* sampled in this study. Roads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



**Figure 2.** Consensus tree (50% majority rule) from Mr. Bayes 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 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 out-group. Topology was similar between Bayesian and Garli analyses.



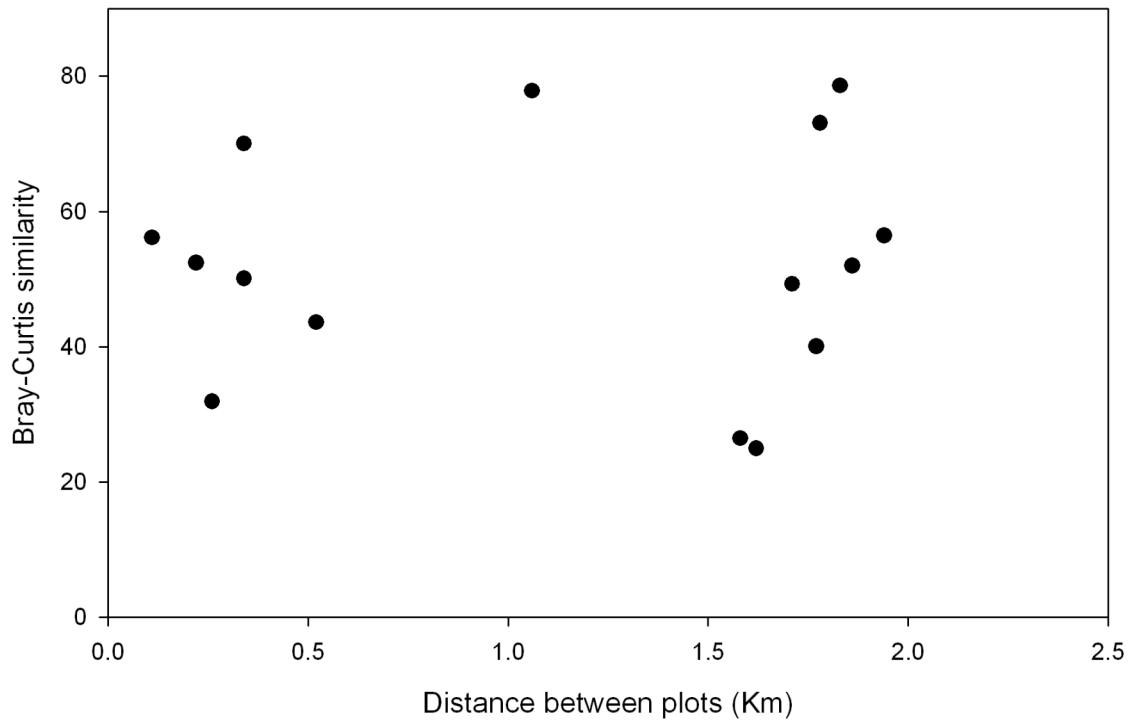
**Figure 3.** Rarefaction curve of the total number of sequences sampled in non-serpentine (NS1, NS2, NS3) ecotype (solid circles) and serpentine (S1, S2) ecotype (open circles) populations of *Collinsia sparsiflora*. Rarefaction curve was produced with the Species-Area Plot routine using 999 permutations.



**Figure 4.** Non-metric multi-dimensional 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 non-metric scale of the ordination does not assign values to the axes.

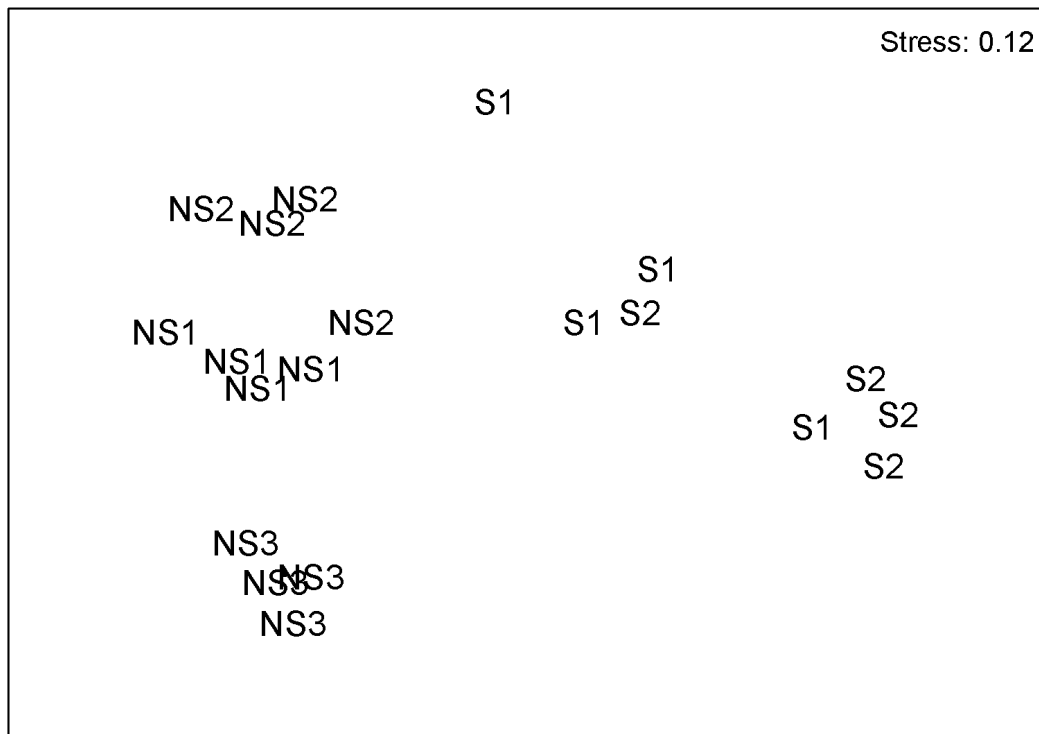
**Supplemental Table S1.** Site soil chemical variables (S = serpentine and NS = non-serpentine). Values are means with standard deviation below in parentheses. Nitrogen (as NO<sub>3</sub>) phosphorus (P, Weak Bray), potassium (K), magnesium (Mg), calcium (Ca), zinc (Zn), iron (Fe), copper (Cu), and 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 Ca:Mg 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.

Site	N	P	K	Mg	Ca	Ca:Mg	Zn	Fe	Cu	B	pH	CEC
S1	2.50 <sub>b</sub> (1.29)	3.75 <sub>c</sub> (0.96)	111.75 <sub>b</sub> (40.55)	1435.00 <sub>b</sub> (292.55)	343.00 <sub>c</sub> (86.15)	0.24 (0.03)	1.05 <sub>ab</sub> (0.79)	33.00 <sub>ab</sub> (4.00)	0.98 <sub>bcd</sub> (0.59)	0.22 <sub>b</sub> (0.05)	7.00 <sub>b</sub> (0.08)	13.90 <sub>bcd</sub> (2.94)
S2	1.50 <sub>b</sub> (0.58)	2.00 <sub>c</sub> (0.00)	65.75 <sub>b</sub> (23.77)	1021.25 <sub>bc</sub> (197.56)	292.50 <sub>c</sub> (41.44)	0.30 (0.11)	0.65 <sub>ab</sub> (0.24)	22.25 <sub>c</sub> (4.72)	0.35 <sub>d</sub> (0.10)	0.32 <sub>b</sub> (0.13)	6.98 <sub>b</sub> (0.22)	10.20 <sub>d</sub> (1.51)
S3	1.50 <sub>b</sub> (0.58)	5.25 <sub>c</sub> (2.06)	248.00 <sub>a</sub> (48.15)	3209.75 <sub>a</sub> (659.5)	870.25 <sub>b</sub> (82.10)	0.28 (0.05)	0.50 <sub>b</sub> (0.08)	21.75 <sub>c</sub> (1.50)	1.75 <sub>a</sub> (0.13)	0.45 <sub>b</sub> (0.17)	7.35 <sub>a</sub> (0.13)	31.48 <sub>a</sub> (5.80)
NS1	2.50 <sub>b</sub> (0.58)	16.00 <sub>b</sub> (4.16)	278.25 <sub>a</sub> (28.89)	860.75 <sub>bc</sub> (162.60)	2143.50 <sub>a</sub> (162.60)	2.58 (0.69)	1.42 <sub>a</sub> (0.19)	37.00 <sub>a</sub> (4.69)	1.22 <sub>ab</sub> (0.22)	1.12 <sub>a</sub> (0.32)	6.52 <sub>c</sub> (0.05)	19.98 <sub>b</sub> (1.39)
NS2	7.25 <sub>a</sub> (2.36)	7.00 <sub>c</sub> (2.31)	246.50 <sub>a</sub> (40.50)	801.25 <sub>bc</sub> (251.71)	869.50 <sub>b</sub> (49.10)	1.18 (0.43)	0.98 <sub>ab</sub> (0.13)	35.50 <sub>ab</sub> (6.95)	0.55 <sub>cd</sub> (0.21)	1.65 <sub>a</sub> (0.50)	6.28 <sub>cd</sub> (0.22)	13.00 <sub>cd</sub> (1.85)
NS3	3.25 <sub>b</sub> (1.89)	43.75 <sub>a</sub> (5.32)	224.50 <sub>a</sub> (19.94)	307.25 <sub>c</sub> (48.08)	2287.25 <sub>a</sub> (47.42)	7.57 (1.06)	0.75 <sub>ab</sub> (0.06)	26.25 <sub>bc</sub> (2.63)	1.18 <sub>abc</sub> (0.10)	0.30 <sub>b</sub> (0.08)	6.12 <sub>d</sub> (0.10)	16.82 <sub>bc</sub> (0.78)



**Supplemental Figure S1.** Relationship between similarity of AMF assemblages and distance between samples. No trend was found.





**Figure S2.** Non-metric multi-dimensional scaling (MDS) ordination of soil nutrients (N, P, K, Mg, Ca, Mg:Ca, Zn, Mn, Fe, Cu, and B) 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 BIOENV 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 non-metric scale of the ordination does not assign values to the axes.

## CHAPTER 2

Is there host-fungal specificity between *Collinsia sparsiflora* serpentine and non-serpentine ecotypes and serpentine and non-serpentine arbuscular mycorrhizal fungi?  
A common garden experiment

## Abstract

As common root symbionts that can increase the plant hosts' establishment and growth in stressful environments, arbuscular mycorrhizal fungi (AMF) may play an important role in plant adaptation to serpentine soil. As a first step toward identifying this role, a previous study found that serpentine and non-serpentine adapted ecotypes of *Collinsia sparsiflora* associated with distinct AMF assemblages – an *Acaulospora* 1OTU- dominated serpentine, and a *Glomus* 1 OTU-dominated non-serpentine plant ecotype AMF assemblage. Two possibilities could account for this distinction: 1) plant ecotype AMF specificity or 2) soil type selection of distinct AMF. This study tests if the distinction between plant ecotype AMF assemblages was due to a high specificity of plant ecotypes for particular AMF – specifically if serpentine plant ecotypes have specificity for *Acaulospora* 1 and if non-serpentine plant ecotype have specificity for *Glomus* 1. I conducted a common garden experiment in which I grew *C. sparsiflora* serpentine and non-serpentine ecotypes in a common pool of serpentine and non-serpentine AMF, and then identified the root AMF of each plant ecotype through amplification of the small subunit rDNA using AMF specific primers, cloning, and sequencing. The ecotype AMF assemblages found in the common garden were compared to serpentine-only and non-serpentine-only AMF controls. The mixing of serpentine and non-serpentine AMF soil inoculum as the source of the common garden resulted in a non-serpentine soil type. Consequently, while the *C. sparsiflora* ecotypes associated with distinct AMF assemblages within the common garden, overall the ecotype AMF assemblages resembled that of a non-serpentine soil (i.e. *Glomus* 1 dominated). Thus, there was no evidence of host-specificity between *C. sparsiflora* serpentine and non-serpentine ecotypes and serpentine and non-serpentine AMF. However, these results do indicate that the soil may select the AM fungi and potential for host choice of AMF based on soil type is present.

## Introduction

Serpentine soils are well known edaphic extremes that create locally adapted plant ecotypes (Brady *et al.*, 2005). Although the process of plant adaptation to serpentine has been studied for several decades, the mechanisms of plant adaptation to serpentine are still poorly understood (Brady *et al.*, 2005; Kruckeberg, 1984; Rajakaruna, 2004). Arbuscular mycorrhizal fungi (AMF), as common root symbionts that can increase the plant hosts' establishment and growth in stressful environments, have the potential of playing an important role in plant adaptation to serpentine soil (Schechter & Bruns, 2008).

As a first step toward identifying the role of AMF in plant adaptation to serpentine, Schechter and Bruns (2008) characterized and compared the AMF assemblages associated with serpentine and non-serpentine adapted ecotypes of *Collinsia sparsiflora*. The authors found that serpentine and non-serpentine *C. sparsiflora* ecotypes associated with distinct AMF assemblages; *Acaulospora* 1-dominated serpentine, and a *Glomus* 1-dominated the non-serpentine assemblage along with other less abundant AMF taxa that also showed a potential plant ecotype bias. However, they also found a relationship between plant ecotype AMF assemblages and soil nutrients (Schechter & Bruns, 2008), thus opening up the possibility that soil or host or both factors could be responsible for the distinction between plant ecotype AMF assemblages. The authors considered two main scenarios that might explain the distinction between plant ecotype AMF assemblages: 1) the plant ecotypes have high specificity for particular AM fungi within a ubiquitous soil assemblage or 2) the plant ecotypes were tapping nonspecifically into AMF assemblages that had been shaped by edaphic factors (Schechter & Bruns, 2008). This study tests the first scenario in a common garden experiment.

AMF have been generally described as ubiquitous symbionts with very low host-specificity (Sanders, 2002; Smith & Read, 1997). However, recent evidence has revealed plant and AMF genotypic variation in mycorrhizal symbiotic effectiveness (Croll *et al.*, 2008; Ehinger *et al.*, 2009; Engqvist *et al.*, 2006; Singh *et al.*, 2002), and plant preference of particular AMF taxa (Hausmann & Hawkes, 2009; Helgason *et al.*, 2002; Pivato *et al.*, 2007; van der Heijden *et al.*, 2004; Vandenkoornhuyse *et al.*, 2002; Vandenkoornhuyse *et al.*, 2003). Therefore, strong edaphic selection on both fungi and plants may create host-fungi specificity in that environment. Strong host-fungal specificity may facilitate local adaptation of both plants and fungi to serpentine and account for the distinction of plant ecotype AMF assemblages found by Schechter and Bruns (2008). If found, this would be the first indication of host-fungi specificity in photosynthetic plants (Helgason & Fitter, 2009; Kiers & van der Heijden, 2006) as the only known examples of specificity and co-evolution in the AMF symbiosis are restricted to myco-heterotrophic plants (Bidartondo *et al.*, 2002; Merckx & Bidartondo, 2008).

One way to determine host-fungi specificity between serpentine and non-serpentine *C. sparsiflora* ecotypes and AMF assemblages is to conduct a common garden experiment. Conducting a "common garden" experiment requires growing environmentally diverse individuals together in a common environment. Historically, these types of experiments have been used by plant ecologists as a way to tease apart environmental and genetic effects from observed phenotypes (Reed & Martiny, 2007). Common garden experiments have also been used in microbial communities to test for an effect of community composition on functioning within a standard environment (Reed & Martiny, 2007). However, few studies combine both plant and microbial communities in common gardens. Existing plant common gardens have been used to detect differences in associated microbes between plant genotypes (Schweitzer *et al.*, 2008), and to determine factors that influence plant host switching between ectomycorrhizal and arbuscular

mycorrhizal fungi (Gehring *et al.*, 2006), but in both cases the microbes analyzed were those native to the garden environment. Only one study transplanted soils along with plant genotypes in a common garden experiment (Miglia *et al.*, 2007) but they did not evaluate if there was an interaction between soil microbe composition and plant genotype.

The goal of this study is to determine if the distinction between AMF assemblages associated with serpentine and non-serpentine *C. sparsiflora* ecotypes in the field (Schechter and Bruns, 2008) was due to host-fungi specificity. To do this, I conducted a common garden experiment in which *C. sparsiflora* serpentine and non-serpentine ecotypes were grown with a common pool of serpentine and non-serpentine AMF under greenhouse conditions. In order to determine if the plant ecotypes select specific AMF taxa from the common garden, I identified the root AMF associates of each plant ecotype via molecular methods. I hypothesized that if the *C. sparsiflora* ecotypes show a similar pattern of associated taxa when grown in a common AMF pool as found in the field, this would suggest host-specificity for particular AMF taxa. Specifically, I would expect *Acualospora* 1 to be the dominant AMF associate in the *C. sparsiflora* serpentine ecotypes and *Glomus* 1 to be dominant in the non-serpentine ecotypes.

## Materials and Methods

### *Study System*

Seeds, soil, and AMF for this study were collected at the Donald and Sylvia McLaughlin University of California Natural Reserve situated in Napa, Lake, and Yolo counties in northern California. I collected from the same serpentine and non-serpentine *Collinsia sparsiflora* ecotypes populations as described in Schechter and Bruns (2008).

### *Soil Collection*

In March 2007, I collected field rhizosphere soil and roots of *C. sparsiflora* from four populations: two serpentine (S1 and S2) and two non-serpentine (NS1 and NS3) (Schechter and Bruns, 2008). These four field collections served as the sources of AMF inoculum for the common garden. I chose these four *C. sparsiflora* ecotype populations because they were the best representatives of serpentine and non-serpentine ecotype populations and AMF (Schechter and Bruns, 2008). I collected the *C. sparsiflora* rhizosphere soil and roots in the field adjacent to the same patches sampled by Schechter and Bruns (2008). All root and soil samples were put directly into coolers and stored in a 4°C cold room within 8 hours of collection.

All soil collections were processed in a fume hood. I first removed all above-ground plant material, crushed soil by hands to release roots, cut the roots into 1 cm segments, and finally, passed the rhizosphere soil and cut roots through a #4 sieve. I then dried each collection for 24 hours in the fume hood at room temperature. The dried collections were stored at 4°C up to one week until used for planting.

### *Experimental Design*

In order to test if *C. sparsiflora* ecotypes selected specific AMF from a “common garden” of serpentine and non-serpentine AMF, I grew individual seedlings of *C. sparsiflora* ecotypes in a mix of serpentine and non-serpentine AMF soil inoculum. The common garden of serpentine and non-serpentine AMF was produced by thoroughly mixing equal amounts of the field collected *C.*

*sparsiflora* rhizosphere soil and roots (S1, S2, NS1, and NS3) and then diluting this mixture 1:1 (volume to volume) with sterilized sand.

### *Planting*

I collected *C. sparsiflora* seeds from the four field populations (S1, S2, NS1, and NS3) in May 2006. These seeds were pregerminated in 1% water agar and transplanted into individual “stubby cell” cone-tainers (Stuewe and Sons) filled with the soil:sand (AMF common garden) mixture. Twenty replicates of each ecotype population were transplanted for a total of 80 seedlings for the experiment. Seedlings were arranged in a completely randomized design (CRD) and grown in a greenhouse (UC Berkeley, Berkeley, CA) from April until flowering in June 2007. The seedlings were sub-irrigated by placing the cone-tainer trays in a tub of water as needed. J. Wright recommended sub-irrigation of *C. sparsiflora* seedlings as the best watering method for this plant species. No fertilizer was used in the experiment.

*Controls.* Serpentine and non-serpentine AMF controls were also planted for this experiment. For the serpentine-only AMF control, I planted the four *C. sparsiflora* ecotype populations (S1, S2, NS1, and NS3) in an equal mix of rhizosphere soil and roots from S1 and S2 *C. sparsiflora* serpentine populations combined 1:1 with sterile sand as above. For the non-serpentine-only AMF control, the four ecotype populations were grown in an equal mix of rhizosphere soil and roots from NS1 and NS3 *C. sparsiflora* serpentine populations combined 1:1 with sterile sand as above. These controls were harvested and AMF associates identified in the same manner as the common garden experiment seedlings (see below).

### *Harvest*

All seedlings were harvested after flowering. However, only ten of the twenty seedlings from the S2 population survived to flowering stage. I measured plant height, the number of flowers, shoot and root dry weight, AMF colonization, and identified root associated AMF taxa via molecular methods (see below). Soil was sampled from harvested seedlings and sent to A & L Western Agricultural Laboratories for chemical analysis. I dried the shoots in a 37°C oven for three days before being weighed. Roots of individual seedlings were thoroughly washed to remove as much soil as possible. I took a small portion (1 mg wet weight) 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 three days. The dried roots were weighed and then placed into a 2 ml cryotube and stored in a -80°C freezer until DNA extraction.

### *Molecular Analysis*

DNA extraction: I extracted DNA from each *C. sparsiflora* root sample. I crushed the dried and frozen roots by beadbeating (Mini-Beadbeater, Biospec Products) with sterile glass beads for 30 seconds or until a fine powder formed. The samples were then immediately placed on ice, and 1.5 ml of 2x 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. I extracted DNA from each root sample as described in Schechter and Bruns (2008).

Polymerase chain reaction (PCR): Due to the fact that only ten seedlings of the S2 population survived to harvest, I equalized the number of seedlings analyzed for AMF associates by randomly choosing ten root-DNA extracts from each of the other *C. sparsiflora* ecotype populations for PCR amplification. This resulted in a total of 40 PCR reactions (ten from each ecotype population). I amplified a variable region of the 18S rDNA using *Pfu Turbo* DNA

polymerase (Stratagene) and universal eukaryotic primer NS31 (Simon *et al.*, 1992) paired with AM1 (Helgason *et al.*, 1998) as described in Schechter and Bruns (2008). Each 20  $\mu$ l PCR reaction consisted of 12.4  $\mu$ l of dH<sub>2</sub>O, 0.2  $\mu$ l of 2.5 U *Pfu Turbo* DNA polymerase, 2  $\mu$ l of manufacture's buffer (Stratagene), 2  $\mu$ l of 10x dNTPs, and 0.2  $\mu$ l of each 50 $\mu$ M primer. PCR conditions were the same as described by Helgason (2002) with the exception that the annealing temperature was increased to 64° C (optimized through gradient PCR analysis) in order to decrease amplification of non-AMF sequences.

Cloning and Sequencing: I pooled the PCR products from two replicates of the same ecotype together for cloning to equal five cloning reactions per ecotype population. This was done to increase the amount of AMF DNA for cloning and to reduce cloning costs. I first gel purified and concentrated the pooled PCR products before cloning as described by Schechter and Bruns (2008). I then cloned the pooled PCR products into pPCR-Script Amp SK(+) and transformed into *Escherichia coli* XL10-Gold Kan Ultracompetent cells (Stratagene). I picked 192 putative positive transformants per cloning reaction. I screened transformants for correctly sized inserts using plasmid primers T3/T7 under the same PCR conditions as described by Schechter and Bruns (2008). Then, I selected 72 gel confirmed positive transformants per cloning reaction for cleaning and sequencing. I cleaned these PCR products with ExoSAP-IT using the manufacturer's instructions (USB), and sent the clean PCR products to the UC Berkeley Sequencing Facility (Berkeley, CA) for sequencing in one direction with AM1. I 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: Prior to analysis, I checked for possible chimeric sequences by comparing my sequences to GeneBank sequences using BLAST (version 2.21, Altschul *et al.*, 1997), and checking my sequences in the Chimera Check program in RDPII (version 2.7, Cole *et al.*, 2003) as described by Schechter and Bruns (2008). 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 (OTU) determination: I determined AMF OTUs in this experiment using the same combination of sequence similarity and phylogenetic analysis methods described by Schechter and Bruns (2008). I first combined sequences obtained from each cloning reaction (pooled PCR products from two seedling replicates of the same ecotype population) at 98% similarity using Sequencher 4.2.2 to create AMF sequence contigs and singletons associated with each pair of seedling replicates (referred to here on out as "paired-seedlings"). This process resulted in one AMF OTU sequence database for each of the paired-seedlings (i.e. five OTU sequence databases for each *C. sparsiflora* ecotype population). Then I compared all contigs and singletons together at 98% to determine 98% sequence similarity groupings for the entire data set; these groupings were used to define OTUs.

I aligned these sequences along with those sequences used in Schechter and Bruns (2008) using ClustalX (Thompson *et al.*, 1997) and then manually edited the alignment using MacClade v 4.08 (Maddison & Maddison, 2005). Two separate phylogenetic analyses were performed using *Olpidium brassica* as an outgroup: maximum likelihood (ML) was conducted using Garli (Genetic Algorithm for Rapid Likelihood Inference) v 0.95 (Zwickl, 2006), and Bayesian

analysis was performed using MrBayes 3.1.1 (Ronquist & Huelsenbeck, 2003). These analyses were conducted using the same methods described by Schechter and Bruns (2008).

I used the results of the phylogenetic analyses to confirm OTUs as in Schechter and Bruns (2008). This included looking 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 of the *C. sparsiflora* paired-seedlings.

Assemblage Analyses: AMF assemblages were analyzed in the same manner described by Schechter and Bruns (2008). I used the PRIMER 5 software (Plymouth Routines in Multivariate Ecological Research) (Clarke & Warwick, 2001) to perform the AMF assemblage analyses. I prepared a relative abundance matrix of OTUs present in each of the paired-seedlings root samples based on the number of clones representing those OTUs. I then produced a similarity matrix using the Bray-Curtis similarity measure after performing a square-root transformation. I used non-metric Multidimensional Scaling (**MDS**) ordinations to represent the dissimilarities in assemblage composition among samples and the **ANOSIM** (analysis of similarities) routine to perform statistical analysis of assemblage data (Clarke & Warwick, 2001). I also used the **SIMPER** (Similarity Percentages) routine to determine the relative contribution of individual OTUs toward dissimilarity between *C. sparsiflora* ecotype population AMF assemblages.

I produced a rarefaction curve to determine if clone sampling effort saturated the number of OTUs using the EstimateS 8.0 Mao Tau estimator (Colwell *et al.*, 2004). I also used PRIMER 5 to compute Shannon-Wiener diversity ( $H'$ ), richness, and evenness for each ecotype population, and tested for differences between ecotype populations in the univariate indices using one-way ANOVA (JMP v. 5). Tukey HSD tests were used for all *a posteriori* comparison of means.

Plant Harvest: I used one-way ANOVA (JMP v. 5) to test for differences between ecotype populations in the plant height, number of flowers, root and shoot dry weight, and colonization (arcsine transformed). Tukey HSD tests were used for all *a posteriori* comparison of means.

Post-hoc Analysis (Field vs. Common Garden): Analysis of the common garden experiment led to a decision to look back at data presented by Schechter and Bruns (2008) as a means to understand the results. Using AMF assemblage and soil chemical data from Schechter and Bruns (2008), I looked for a relationship between the relative abundance of Acaulospora 1 and Glomus 1 found in individual *C. sparsiflora* root samples and concentrations of individual soil chemical variables associated with the same root samples. This regression analysis was done using JMP (v.5).

I also used the PRIMER 5 software to compare differences in soil chemical characteristics between common garden soil and field soil collected from the S1, S2, NS1 and NS3 ecotype population sites (Schechter and Bruns, 2008). Soil chemical data was log transformed, and then the similarity matrix was produced using Euclidean distance (Clarke & Warwick, 2001). Non-metric MDS was used to demonstrate differences in soil chemical characteristics between samples. One-way ANOVA (JMP v. 5) was also used to compare soil chemical data (log transformed) between common garden experiment soil and field soil collected from the S1, S2, NS1 and NS3 ecotype population sites (Schechter and Bruns, 2008). Tukey HSD tests were used for all *a posteriori* comparison of means.



## Results

### *Plant Harvest Data*

There were no significant differences between *C. sparsiflora* ecotype populations in any growth parameter (Table S1). Plant height ( $F = 0.89$ ,  $P < 0.89$ ), number of flowers ( $F = 1.18$ ,  $P < 0.34$ ), shoot dry weight ( $F = 0.51$ ,  $P < 0.68$ ), root dry weight ( $F = 0.45$ ,  $P < 0.72$ ), shoot + root dry weight ( $F = 0.42$ ,  $P < 0.74$ ), and AMF colonization ( $F = 0.13$ ,  $P < 0.94$ ) were all similar across ecotype populations.

### *Assemblage Identification*

*General.* A total of 1,543 clones were sequenced in the common garden study. Of these, 96% were AMF sequences, 1.2% were of bacterial origin, 0.4% were ascomycete fungi, 0.1% were of plant origin, and 0.4% were chimeric sequences; the rest were poor quality and discarded. Each *C. sparsiflora* ecotype population was represented by similar numbers of AMF sequences (S1 = 340, S2 = 427, NS1 = 367, NS3 = 367).

*Phylogenetic analysis – Common Garden.* I detected only three AMF genera in this study (Figure 1). *Glomus* species were the most abundant by far, representing 99.8% of the sequences. The two other genera are both in the Archaeosporales: *Archaeospora* (0.1%), and a newly described genus *Ambispora* (0.1%) (Walker *et al.*, 2007). Using the combined sequence similarity and phylogenetic criteria for OTU determination, I established 11 AMF OTU (Table 1), seven of which matched OTUs found in Schechter and Bruns (2008) (Figure 1). Of these, two OTU were the most abundant sequence types: *Glomus* 1 (47%) and *Glomus* 4 (20%).

*Phylogenetic analysis – Controls.* AMF assemblages of the *C. sparsiflora* ecotypes populations growing in the serpentine-only control were different from those growing in the non-serpentine-only control (Table 2). AMF assemblages of ecotype populations growing in the serpentine-only control were dominated by taxa in the *Glomus* genus (98% of sequences) especially by the *Glomus* 4 OTU (53%), but also included sequences belonging to the *Acaulospora* (*Acaul* 1, 1%) and *Scutellospora* (*Scut* 1, 1%) genera. In contrast, AMF assemblages of the ecotype populations growing in the non-serpentine-only control contained only *Glomus* taxa sequences, dominated by sequences of the *Glomus* 1 OTU (36%). All OTUs but one (*Glomus* C) matched the OTUs found in Schechter and Bruns (2008) (Figure 1.).

### *Assemblage Analysis*

*Comparing assemblages.* The rarefaction analysis shows saturation in the curves representing *C. sparsiflora* ecotype populations S1, S2, and NS1, while the curve for NS3 shows a clear leveling off (Figure 2). This indicates that the sequence sampling effort was sufficient for a comparison of AMF assemblages associated with each ecotype population. The MDS showed a separation between AMF assemblages associated with serpentine ecotype populations (S1 and S2) and those associated with non-serpentine ecotype populations (NS1 and NS3) (Figure 3). This slight distinction was significant in the ANOSIM analysis ( $R = 0.132$ ,  $P < 0.034$ ). However, ANOSIM analysis in which AMF assemblages are compared between individual ecotypes populations, showed that the S1 and S2 ecotype population AMF assemblages were both significantly different from the NS1 ecotype population but neither was significantly different from the NS3 ecotype population AMF assemblage (Table 3). In fact, the S1 and S2 ecotype population AMF assemblages were significantly different from each other, while the NS1 and NS3 ecotype

populations had similar AMF assemblages (Table 3). But all significant differences between ecotype AMF assemblages disappeared when analysis was switched from relative abundance to presence/absence of AMF OTUs ( $R = 0.026$ ,  $P < 0.196$ ).

When comparing AMF assemblages found in the common garden with those found in the serpentine-only and non-serpentine-only controls, it is obvious that the common garden AMF assemblages are much more similar to those found in the non-serpentine-only controls than the AMF assemblages of the serpentine-only control (Figure 4). The ANOSIM analysis confirmed this with the common garden AMF assemblages significantly different from those in the serpentine-only controls ( $R = 0.643$ ,  $P < 0.001$ ), but not different from the non-serpentine-only control AMF assemblages ( $R = 0.081$ ,  $P < 0.31$ ). The serpentine-only control AMF assemblages were significantly different from those of the non-serpentine-only controls ( $R = 0.944$ ,  $P < 0.03$ ), but no differences were detected between the ecotype populations ( $R = -0.704$ ,  $P < 0.97$ ). Taken together, this data suggests that while in a common garden setting *C. sparsiflora* ecotype populations associate with distinct AMF assemblages, but these assemblages overall resemble that of a non-serpentine soil.

*OTU contribution to assemblage differences.* SIMPER analysis showed that three AMF OTU contributed the most to the distinction between AMF assemblages associated with serpentine *C. sparsiflora* ecotype populations (S1 and S2) and non-serpentine ecotype populations (NS1 and NS3) in the common garden experiment: *Glomus* 1 (31% contribution of the total dissimilarity between ecotype assemblages), *Glomus* 9 (19%), and *Glomus* 4 (17%) (Table 1). These three OTU also contributed to the distinction between AMF assemblages of the S1 and S2 ecotype populations and that of NS1: *Glomus* 1 (39%), *Glomus* 4 (19%), and *Glomus* 9 (18%). However, the set of AMF OTU that contributed to the distinction between the S1 and the S2 ecotype population AMF assemblages included a new OTU: *Glomus* 1 (38%), *Glomus* 4 (17%), and *Glomus* 7 (16%) (Table 1). Species diversity ( $F = 2.85$ ,  $P < 0.07$ ), evenness ( $F = 3.40$ ,  $P < 0.05$ ), and richness ( $F = 0.61$ ,  $P < 0.62$ ) were not significantly different between *C. sparsiflora* ecotype population AMF assemblages.

*Comparison of Field versus Common Garden.* Comparing the average OTU relative abundance of AMF associated with the *C. sparsiflora* ecotype populations when grown in common garden with those when growing in the field shows a clear change in AMF OTU dominance (Figure 5). In the field, *Acaulospora* 1 was the dominant OTU associated with the serpentine ecotypes populations (S1 and S2), while *Glomus* 1 was the dominant OTU associated with the non-serpentine ecotype populations (NS1 and NS3) (Figure 5a). However, in the common garden experiment, *Glomus* 1 now dominated both serpentine and non-serpentine ecotype populations, while *Acaulospora* 1 was completely absent (Figure 5b). Thus, the common garden AMF assemblages were much more like those found in the non-serpentine soil sampled in the field experiment. This is supported by the similarity between the common garden and non-serpentine-only control AMF assemblages and distinction from the serpentine-only control AMF assemblages (Figure 4).

The MDS ordination comparing soil nutrient concentrations between growth medium sampled from the common garden experiment and those found in serpentine and non-serpentine soil sampled from the field (Schechter and Bruns, 2008), clearly show that the common garden soil is clustered within the non-serpentine soils (Figure S1). The similarity between the common garden soil and non-serpentine soil is also clear when comparing individual nutrients (Table S2).

Looking at AMF assemblage and soil chemical data from Schechter and Bruns (2008), I found strong relationships between the relative abundance of the *Acaulospora* 1 OTU and the

*Glomus* 1 OTU and the concentration of soil potassium (K) (Figure 6) and the calcium:magnesium (Ca:Mg) ratio (Figure 7). The relative abundance of *Acaulospora* 1 decreases from 80% to zero as soil K increases (adjusted  $R^2 = 0.58$ ,  $F = 28.0$ ,  $P < 0.0001$ ) (Figure 6a). In contrast, the relative abundance of *Glomus* 1 increases from zero to almost 100% as soil K increases (adjusted  $R^2 = 0.63$ ,  $F = 33.2$ ,  $P < 0.0001$ ) (Figure 6b). Similar trends were also found for soil Ca:Mg. The relative abundance of *Acaulospora* 1 decreases as soil Ca:Mg increases (adjusted  $R^2 = 0.40$ ,  $F = 13.8$ ,  $P < 0.0016$ ) (Fig 7a), while the relative abundance of *Glomus* 1 increases as soil Ca:Mg increases (adjusted  $R^2 = 0.52$ ,  $F = 21.2$ ,  $P < 0.0002$ ) (Figure 7b). It is interesting to note that concentration of soil K found in the common garden (K = 206 ppm, Table S2) is at the concentration in these regressions where the relative abundance of *Acaulospora* 1 is almost zero and *Glomus* 1 is at 80% (Figure 6). The same for the Ca:Mg of the common garden soil (Ca:Mg = 1.71, Table S2): *Acaulospora* 1 relative abundance is almost zero and *Glomus* 1 relative abundance is near maximum (Figure 7).

## Discussion

### *Experimental Approach*

To my knowledge, this study is the first AMF common garden study. This was made possible for two reasons, first, knowledge of edaphically distinct AMF assemblages, and second, the ability to identify changes in root associated AMF taxa through molecular methods. However, the use of soil inoculum for this experiment limited my ability to test for host-fungal specificity in a “neutral” environment. Prior to execution of this experiment, I made several attempts at producing single-species cultures of field AMF taxa from single spores without success. One reason for these failures was that spore extractions from field soil yielded very few spores, indicating that soil mycelium and infected root pieces may be the primary source of AMF inoculum in field conditions. In addition, it is highly unlikely that all field AMF can be cultured under typical AMF culturing conditions (J. Morton, *pers. comm.*). Therefore, while not allowing for a completely “neutral” growth environment, using soil inoculum was the only way to ensure that all native serpentine and non-serpentine AMF found in the field had the chance to colonize *C. sparsiflora* ecotype populations in this experiment.

Like Schechter and Bruns (2008), direct amplification of root AMF with NS31/AM1, cloning, and sequencing was an effective way to identify and compare AMF assemblages in the common garden experiment as well as with those found in the controls. In fact, ten of the fifteen OTUs found in the common garden experiment and controls were the same as those found in the field study (Schechter and Bruns, 2008) giving great confidence in this method of AMF OTU detection and determination. The detection of five “new” OTU was either due to the saturation of sequence sampling (Figure 2) and the improvement in the efficiency of AMF amplification in this experiment or to the unique greenhouse – single plant environment. Increasing the annealing temperature improved the success of AMF amplification from 64% (Schechter and Bruns, 2008) to 96% of sequences. One fear often mentioned with the use of AM1 is that it does not amplify AMF from the Archaeosporales (Redecker *et al.*, 2000), however, even under more restrictive PCR conditions, sequences from *Archaeospora* were once again amplified with this primer set along with the newly described genus in this family *Ambispora*. While this does not show that this primer pair can be used to amplify all taxa in the Archaeosporales (which is probably not the case), it does show that it is not as restricted as once believed.

As was found in Schechter and Bruns (2008), clone relative abundance of AMF OTUs played an important role in the distinction of AMF assemblages between ecotype populations. I practiced the same caution discussed in Schechter and Bruns (2008) in using clone relative abundance as a proxy for the relative abundance of AMF associates. Once again, I saw no evidence of primer bias for a single sequence group causing false dominance of that sequence group in those samples (Table 1 and Table 2). Thus, I am again confident that clone relative abundance provided a reasonable estimation of the relative abundance of dominate AMF taxa.

#### *The Greenhouse Affect on AMF Assemblages*

The use of the serpentine-only and non-serpentine-only AMF controls showed how greenhouse conditions affected the diversity and abundance of AMF colonizing *C. sparsiflora*. The serpentine AMF assemblages associating with the ecotype populations under greenhouse conditions were very different from those found in the field. The first obvious difference is that the field dominant *Acaulospora* 1 OTU was only present as one sequence under greenhouse conditions. This may be due to its lack of competitive ability under greenhouse growth conditions, or the possibility that interaction with one small *C. sparsiflora* may not have provided enough carbon to facilitate the same level of colonization as was found in the field. *Acaulospora* species typically have larger spore size than *Glomus* species, which may indicate higher carbon demand in this genus (Powell *et al.*, 2009; Smith & Read, 1997). In the field, *C. sparsiflora* typically grows in patches of several individuals along with other plant species, which could represent an interconnected mycorrhizal network (Helgason & Fitter, 2009) that could provide much more carbon in concert than an individual *C. sparsiflora* in a small pot.

One encouraging similarity between serpentine AMF found in greenhouse and those in the field is the pattern of presence and abundance of the *Glomus* 1 OTU. *Glomus* 1 was once again found in a “hit and miss” pattern across ecotypes and at low abundance in the serpentine-only control (Table 2). This may indicate that *Glomus* 1 does not function as well in serpentine soil, or it may just reflect its low abundance in the field. However, the drastic increase in abundance of *Glomus* 7 and *Glomus* 9 (Table 2), which were not found associated with any ecotypes growing in serpentine in the field (Schechter and Bruns, 2008), indicates that low abundance in the field does not necessarily hamper colonization under greenhouse conditions. The greenhouse conditions seemed to also favor increased abundance of *Glomus* 4, which was the second most abundant AMF OTU associated with ecotypes growing in serpentine soil in the field (Schechter and Bruns, 2008). This change of *Glomus* 4 to the overwhelming dominant OTU in the serpentine-only controls may be due to release from competition with *Acaulospora* 1 (Table 2).

The AMF associated with *C. sparsiflora* ecotypes in the non-serpentine-only control was much more like those found in the field. *Glomus* 1 was once again the dominant OTU associated with ecotypes growing in non-serpentine soil (Table 2) (Schechter and Bruns, 2008). *Glomus* 4 showed an increase from 5% in the field to over 20% under greenhouse conditions, again indicating that greenhouse conditions favor its increase in abundance, however in this case, *Glomus* 1 was still the dominant taxa. The same pattern of increased abundance under greenhouse conditions was seen in *Glomus* 7 and *Glomus* 9, which were only found in very low abundance in field non-serpentine soil (Schechter and Bruns, 2008). The most surprising addition to the AMF OTUs found in the non-serpentine-only control was *Glomus* 6. In the field, *Glomus* 6 was restricted to serpentine soil and thus dubbed a “serpentine-specific” OTU, however, it was detected in the non-serpentine-only control albeit only associated with the S2

serpentine ecotype (Schechter and Bruns, 2008) (Table 2). In contrast, *Glomus* 6 was found in the serpentine-only control with every ecotype (Table 2). While I have to obviously drop the “serpentine-specific” label for *Glomus* 6, this difference in pattern of presence between serpentine-only and non-serpentine-only controls may still imply “preference” for serpentine soil.

#### *Comparison of AMF Assemblages*

This study found that *C. sparsiflora* ecotype populations associate with distinct AMF assemblages when exposed to a common garden of serpentine and non-serpentine AMF, but overall, these assemblages resemble that of a non-serpentine soil. Finding that plant genotypes show preference for particular AMF either as spores or root colonization is not new. While showing differences in AMF preferences between distinct host species is a common result (Bever, 2002; Bever, 2003; Castelli & Casper, 2003; Hausmann & Hawkes, 2009; Helgason *et al.*, 2002; Opik *et al.*, 2009; Pivato *et al.*, 2007; van der Heijden *et al.*, 2004; Vandenkoornhuyse *et al.*, 2002; Vandenkoornhuyse *et al.*, 2003), the same level of preference has also been seen at the finer host genotypic scale (Douds *et al.*, 1998; Eason *et al.*, 2001; Ehinger *et al.*, 2009; Graham *et al.*, 1997; Linderman & Davis, 2004; Singh *et al.*, 2002). However, unlike many of those studies, I found no difference in growth response associated with “preference” for particular AMF assemblages (Table S1). The lack of association between growth response and differences in AMF assemblage may be due to the fact that essentially all ecotypes were growing in a non-serpentine soil type (Figure S1) and associating with non-serpentine AMF assemblages (Figure 4).

#### *No Host-fungi Specificity*

None of the results of this study show evidence of host-fungal specificity between *C. sparsiflora* serpentine and non-serpentine ecotypes and serpentine and non-serpentine AMF. Host-fungal specificity would be manifest as an extreme fidelity for specific AMF taxa (Bidartondo *et al.*, 2002; Merckx & Bidartondo, 2008). In the most extreme examples of host-fungi specificity, monotrope host specialization to specific ectomycorrhizal fungal lineages appeared to be a fixed trait, which was phylogenetically correlated within monotrope lineages (Bidartondo & Bruns, 2005). I expected that in the *C. sparsiflora* system, host-fungi specificity would be manifested as fidelity between serpentine plant ecotypes and *Acaulospora* 1, and non-serpentine plant ecotypes and *Glomus* 1 when exposed to a common garden of AMF in a “neutral” environment. However, even though propagules of *Acaulospora* 1 were known to be present (as seen in serpentine-only control), it was not detected in the roots of serpentine plant ecotypes in either the serpentine-only control or the common garden (Tables 1 and 2).

Non-serpentine plant ecotypes did associate with *Glomus* 1 in the non-serpentine-only control and in the common garden but so did the serpentine plant ecotypes and in both cases, the ecotypes were growing in non-serpentine soil. The only potential test of fidelity between non-serpentine ecotypes and *Glomus* 1 in a non-serpentine soil was in the serpentine-only control. But even there, only one of the two non-serpentine ecotypes associated with *Glomus* 1 in low abundance (Table 2). These results are more indicative of a soil-type effect on selection of AMF associates rather host-fungi specificity.

#### *Host Choice based on Soil-type?*

Looking back the field AMF assemblage and soil chemical data from Schechter and Bruns (2008) provided some insight into the possible soil-type effect on ecotype AMF assemblages found in the common garden experiment. Regression analysis showed a strong correlation between the relative abundance of *Acaulospora* 1 and *Glomus* 1 found in *C. sparsiflora* roots samples and associated concentrations of soil K and Ca:Mg those plants were experiencing (Fig 6 and 7). In the field, plants decreased the abundance of *Acaulospora* 1 and increased the abundance *Glomus* 1 in roots as soil K and Ca:Mg increased. This type of pattern is suggestive of a “partner choice” relationship in which the host is choosing a better symbiont as soil nutrient conditions change. Kiers and van der Heijden (2006) defined partner choice as “the ability to discriminate partners based on symbiotic functioning”. According to the authors, partner choice requires first, an evaluation of the effectiveness of the partner and second, a “decision” as to whether or not to enter (or remain) in the interaction (Kiers & van der Heijden, 2006). The authors further suggest that while host selection at the time of infection is unlikely, “on-going” partner choice in which the partner can modify the amount of resources exchanged (host-sanctions) or the duration of the interaction is feasible in the AMF symbiosis. In fact, Bever *et al.* (2009) found that plant hosts preferentially allocate carbon to the most beneficial AMF symbiont. In this way, partner choice is fundamentally different that host-fungi specificity which requires identification of and high fidelity to specific partners, presumably irrespective of edaphic conditions.

If the pattern found in the field is indeed “partner choice”, then one could speculate that *Acaulospora* 1 is a better symbiont than *Glomus* 1 at low K and Ca:Mg, and therefore worth the higher C demands, while *Glomus* 1 is a better symbiont at higher K and Ca:Mg or perhaps providing a new service under better growth conditions as well as requiring a lower C demand. Thus, the plant either decreases C allocation to *Acaulospora* 1 and/or allocates more C to *Glomus* 1 as soil nutrients increase resulting in a shift in abundance from *Acaulospora* 1 to *Glomus* 1, as found in Schechter and Bruns (2008). Using these assumptions, the finding in the common garden experiment where all *C. sparsiflora* ecotype populations associated with a non-serpentine AMF assemblage when growing in a non-serpentine soil-type could also be explained as partner or more specifically “host choice” of the better symbionts in a non-serpentine soil type.

Regardless of the explanation, the results of the common garden experiment confirm that the distinction between AMF assemblages associated with serpentine and non-serpentine *C. sparsiflora* ecotypes in the field (Schechter and Bruns, 2008) was *not* due to host-fungi specificity. This leaves the second scenario, in which the distinction is due to AMF assemblages shaped by edaphic factors as the most likely situation. The experiment that tests this scenario will be described in the next Chapter.

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**Table 1.** Relative abundance matrix of operational taxonomic units (OTU) of AM fungi associated with *Collinsia sparsiflora* seedling replicates (a, b, c, and d) of two serpentine ecotype populations (S1 and S2) and two non-serpentine ecotype populations (NS1 and NS3) grown in a common garden of serpentine and non-serpentine AM fungi. Italicized OTUs show ecotype affects.

OTU	S1					S2					NS1					NS3				
	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
<i>Glo 1</i>	61	25	24	23	49	45	81	63	91	60	57	34	79	10	51	20	20	23	55	36
Glo 2	0	0	3	39	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Glo 4</i>	14	43	15	17	16	30	2	10	4	7	33	25	10	30	20	20	51	19	22	22
Glo 5	3	14	0	0	0	8	5	1	0	4	1	0	2	22	4	7	16	10	9	25
Glo 6	3	2	8	13	6	5	0	15	1	1	3	3	2	4	6	0	3	0	3	3
<i>Glo 7</i>	16	11	44	9	6	8	5	1	2	28	3	3	2	4	6	10	1	0	9	9
<i>Glo 9</i>	2	6	6	0	22	4	7	8	1	0	4	35	6	29	14	43	9	45	3	3
Glo A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	1
Arch 2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Amb 1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

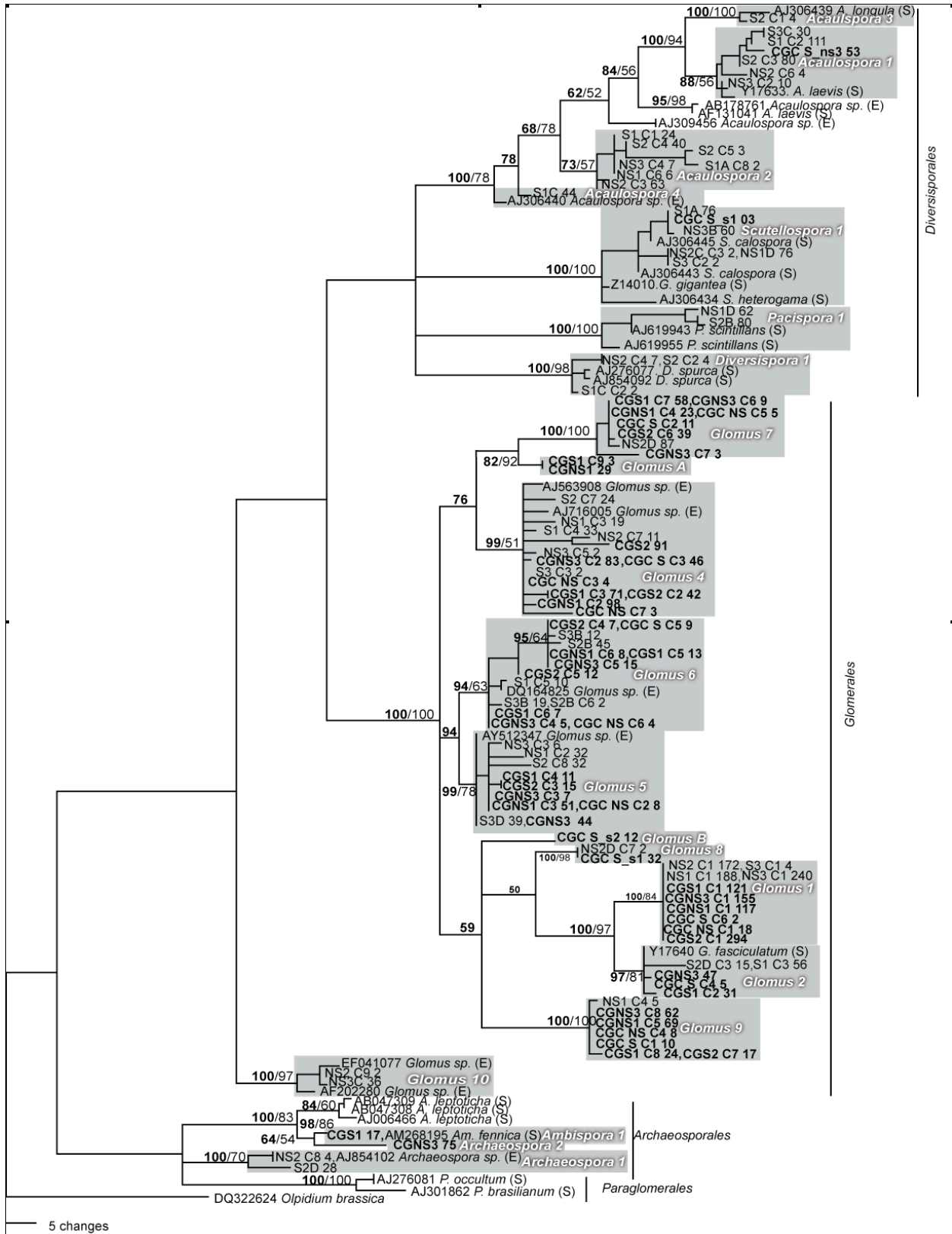
**Table 2.** Relative abundance matrix of operational taxonomic units (OTU) of AM fungi associated with two serpentine ecotype populations (S1 and S2) and two non-serpentine ecotype populations (NS1 and NS3) of *Collinsia sparsiflora* seedlings grown in either serpentine (serpentine-only control) and non-serpentine (non-serpentine only control) soil that contain native AM fungi found in those soils as a control for the common garden experiment. None of the *C. sparsiflora* S1 ecotype seedlings survived in non-serpentine-only control to flower, and therefore was not included in the analysis. Italicized OTUs show soil type affects.

OTU	SOIL TYPE						
	Serpentine				Non-Serpentine		
	ECOTYPE						
	S1	S2	NS1	NS3	S2	NS1	NS3
<b>Glo 1</b>	<i>4</i>	<i>0</i>	<i>0</i>	<i>8</i>	<i>36</i>	<i>31</i>	<i>42</i>
Glo 2	12	0	8	0	0	0	0
<b>Glo 4</b>	<i>42</i>	<i>41</i>	<i>73</i>	<i>54</i>	<i>14</i>	<i>6</i>	<i>25</i>
<b>Glo 5</b>	<i>0</i>	<i>0</i>	<i>0</i>	<i>0</i>	<i>23</i>	<i>19</i>	<i>0</i>
Glo 6	8	5	15	15	18	0	0
Glo 7	12	27	4	8	5	25	0
Glo 9	15	23	0	8	5	19	33
Glo 8	4	0	0	0	0	0	0
Glo B	0	5	0	0	0	0	0
Acaul 1	0	0	0	8	0	0	0
Scut 1	4	0	0	0	0	0	0

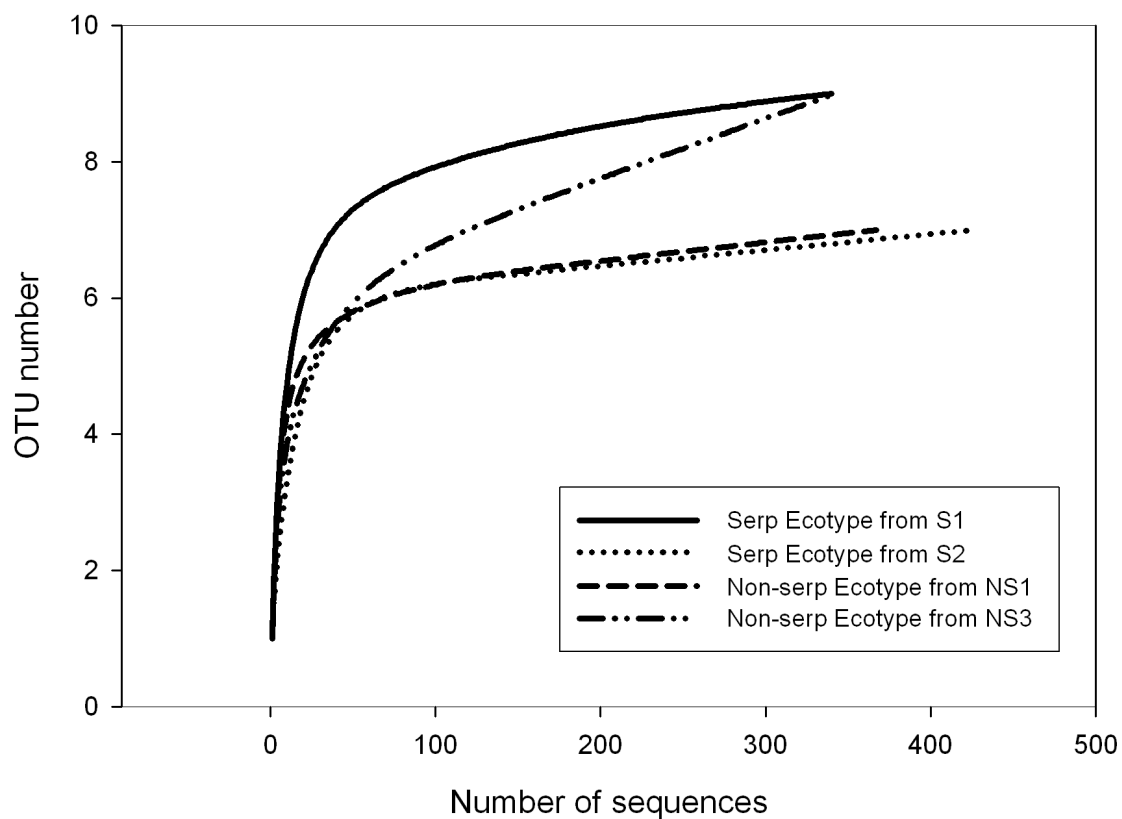
**Table 3.** Results of ANOSIM pairwise comparison of AMF assemblages associated with serpentine (S1 and S2) and nonserpentine (NS1 and NS3) ecotypes of *Collinisa sparsiflora* grown in a common garden of serpentine and non-serpentine AMF. Numbers are the  $R^a$  values associated with each pairwise comparison.

	S1	S2	NS1	NS3
S1	-			
S2	0.30*	-		
NS1	0.32*	0.46*	-	
NS3	0.16	-0.04	0.20	-

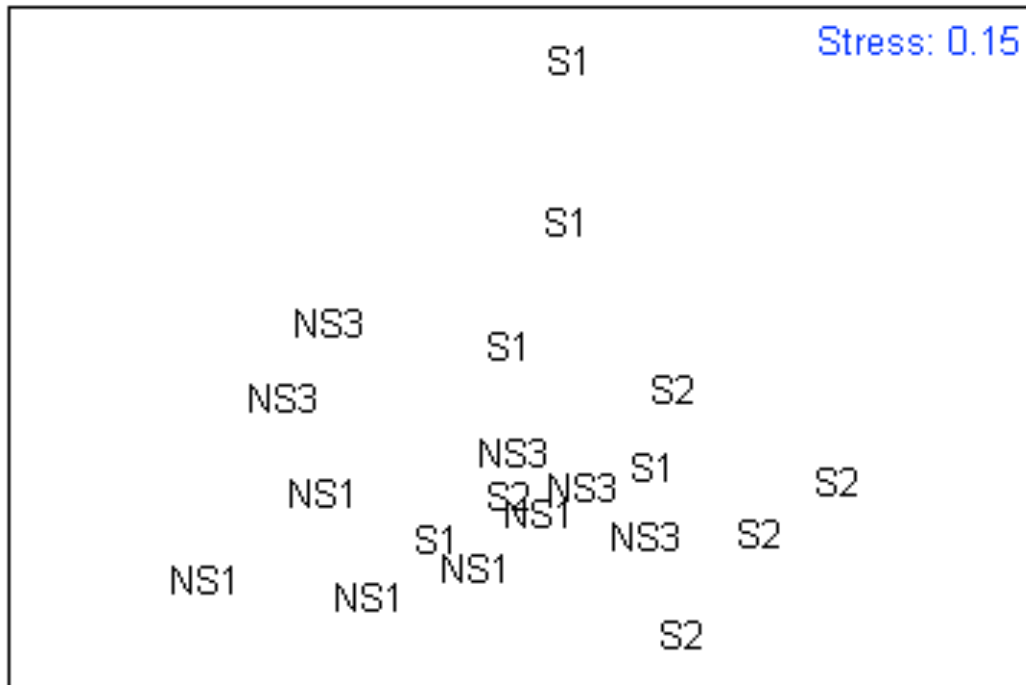
<sup>a</sup>  $R \approx 1$  if there is high differentiation of AMF assemblages;  $R \approx 0$  if AMF assemblages are indistinguishable. \* Indicates significant difference between samples ( $p < 0.05$ ).



**Figure 1.** Consensus tree (50% majority rule) from Mr. Bayes analysis showing the phylogenetic relationship of the AMF sequences obtained from roots sampled from common garden experiment from two serpentine (CGS1 and CGS2) and two nonserpentine (CGNS1 and CGNS3) ecotype populations and from the serpentine-only control samples (CGC\_S) as well as the non-serpentine only controls (CGC\_NS) of *Collinsia sparsiflora*, in bold. Additional sequences from roots sampled from three serpentine (S1, S2, S3) and three nonserpentine (NS1, NS2, NS3) ecotype populations of *Collinsia sparsiflora* field experiment were included (Schechter and Bruns, 2008). Letters directly behind site designation refers to an individual sample; no letter means that the sequence is a representative from a 98% consensus of sequences found in multiple samples within that site. 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 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. *Oplidium brassica* was used as an out-group. Topology was similar between Bayesian and Garli analyses.

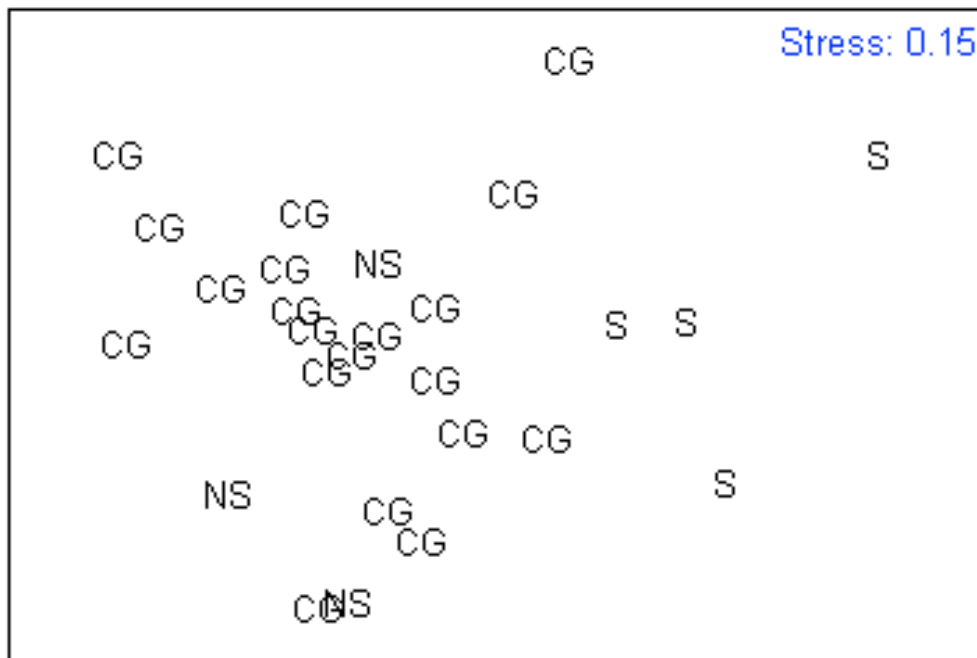


**Figure 2.** Rarefaction curve of the total number of sequences sampled from serpentine ecotype (S1, S2) and non-serpentine ecotype (NS, NS3) populations of *Collinsia Sparsiflora* grown in a common garden of serpentine and non-serpentine AMF. Rarefaction curves were produced by the EstimateS version 8.0 Mao Tau estimator (Colwell et al. 2004).

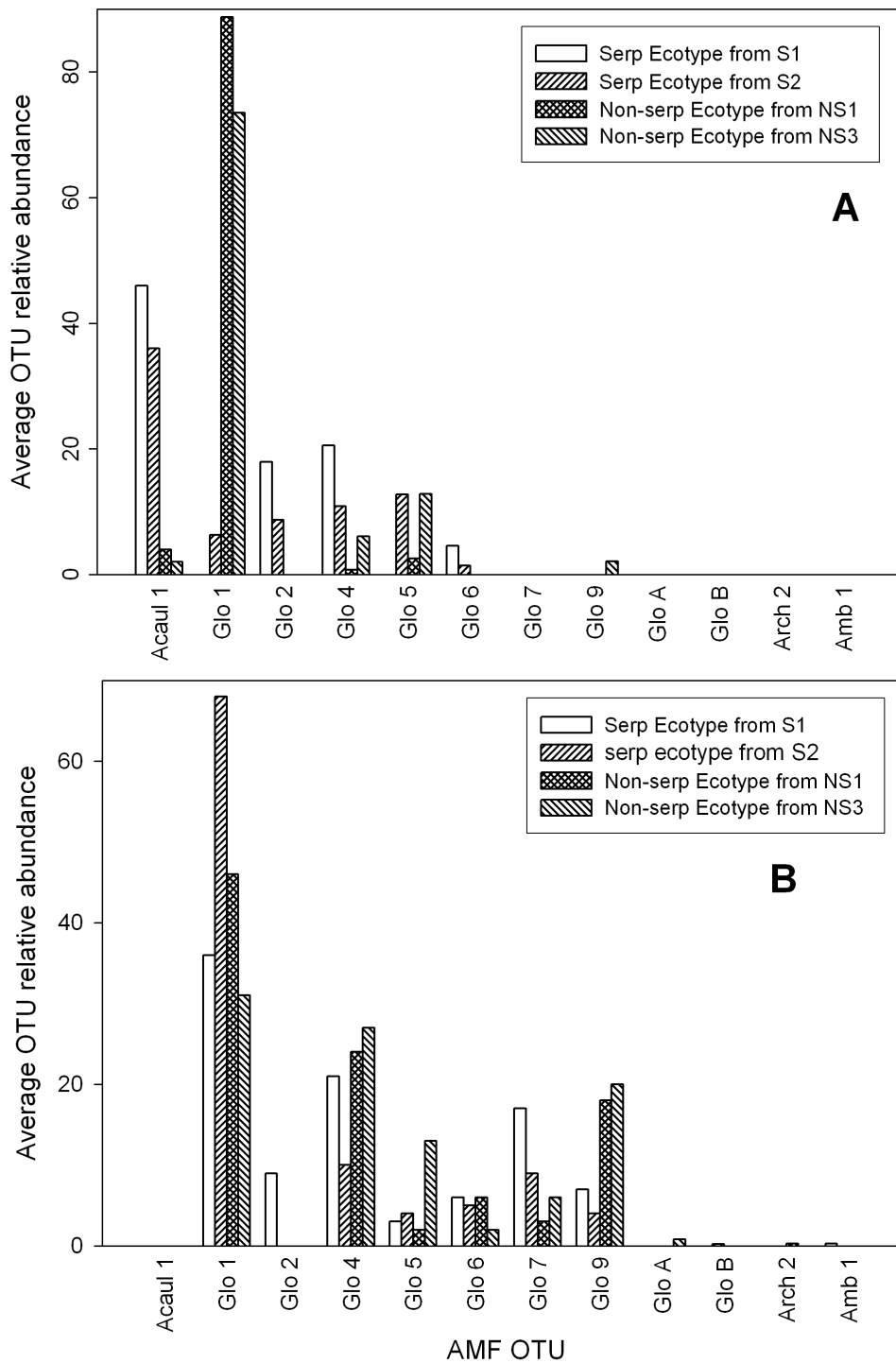


**Figure 3.** Non-metric multi-dimensional scaling (MDS) ordination of AMF assemblages associated with serpentine ecotype (S1, S2) and non-serpentine ecotype (NS1, NS3) populations of *Collinsia sparsiflora* grown in a common garden of serpentine and non-serpentine AMF. The MDS ordination is a configuration 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.

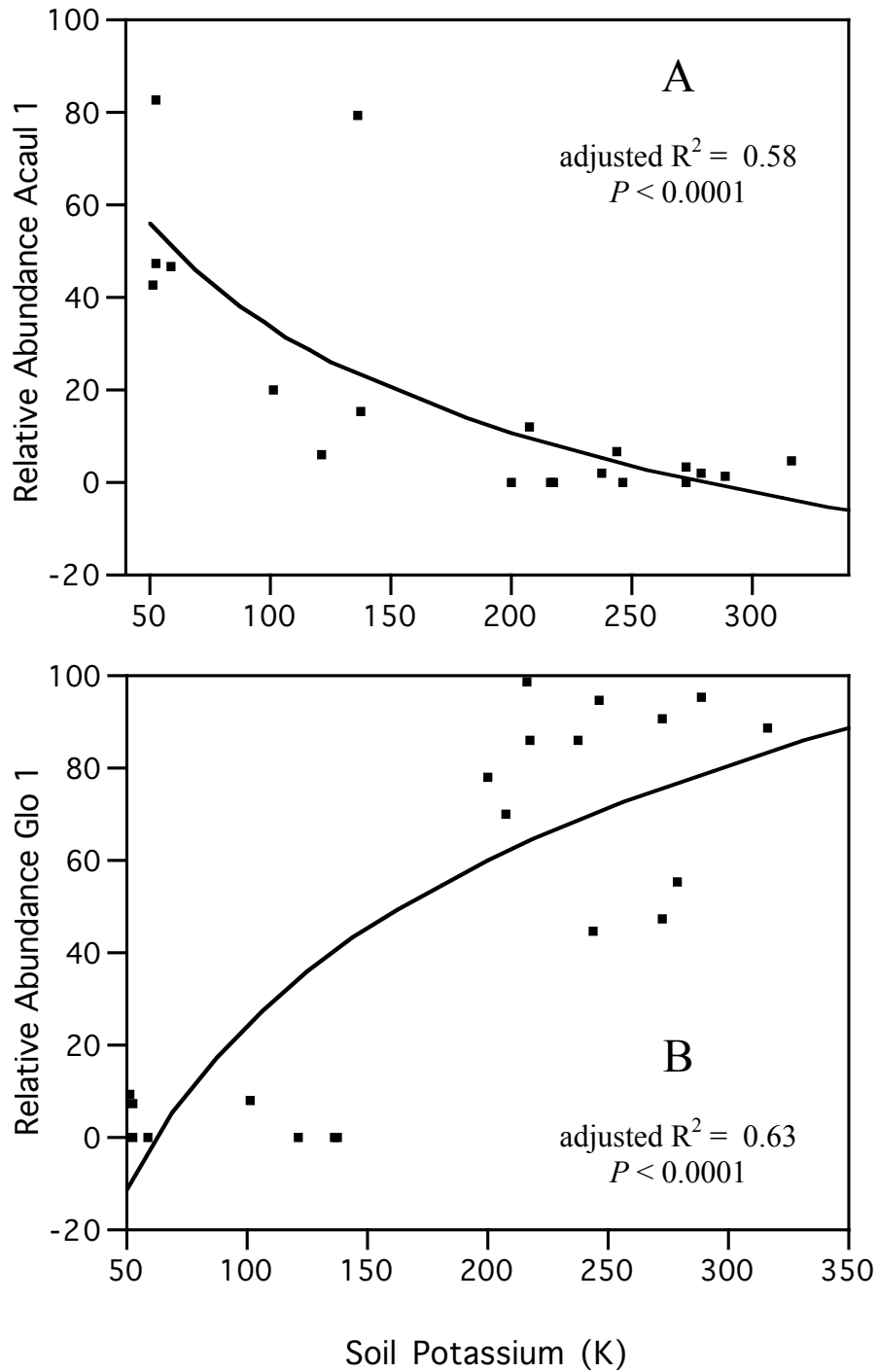




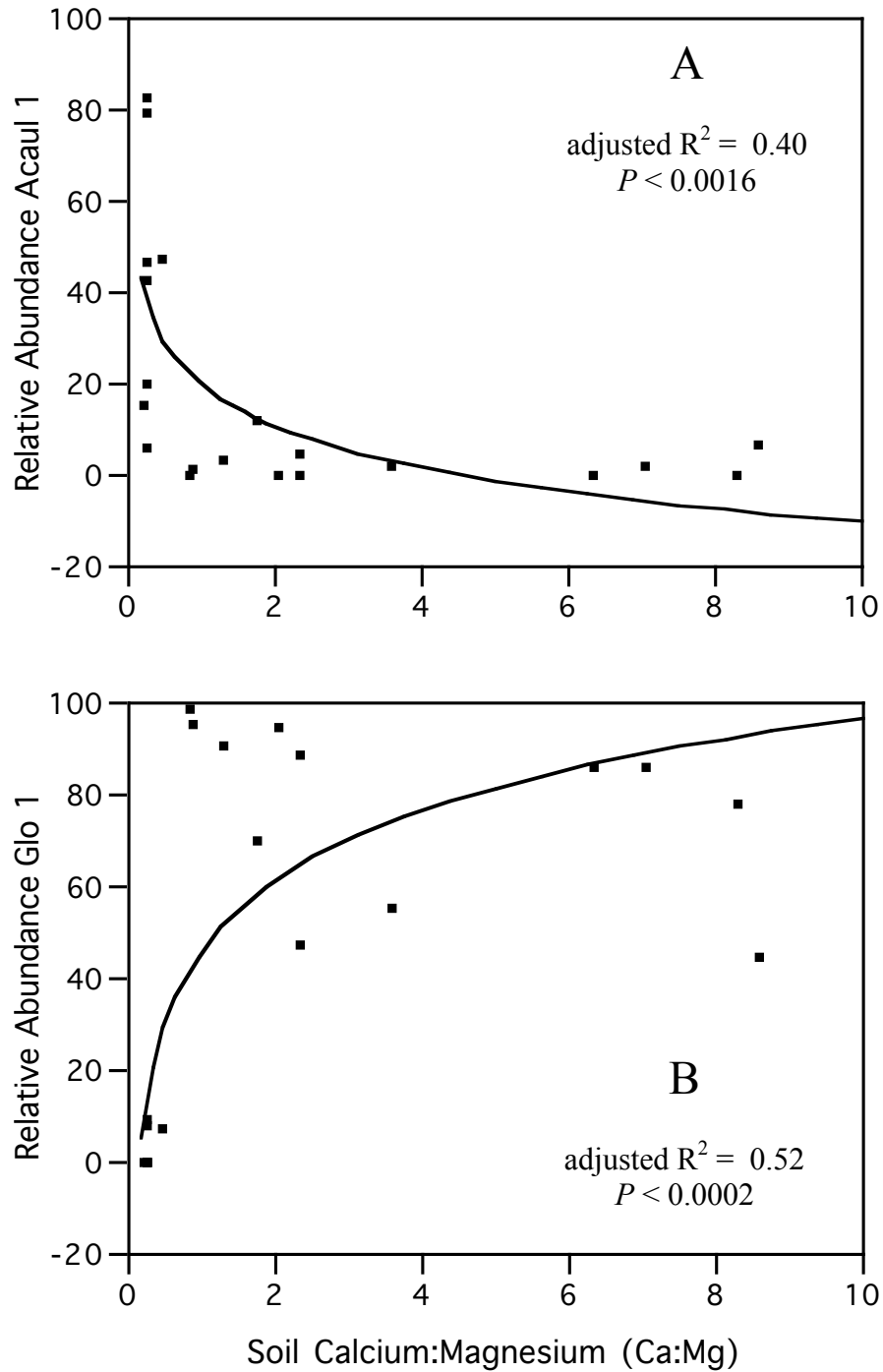
**Figure 4.** Non-metric multi-dimensional scaling (MDS) ordination of AMF assemblages associated with serpentine and non-serpentine ecotype populations of *Collinsia sparsiflora* grown in a common garden of serpentine and non-serpentine AMF (CG), serpentine soil control (S) or non-serpentine soil control (NS) AMF. 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.



**Figure 5.** Comparison of the average OTU relative abundance of AMF associated with the *Collinsia sparsiflora* ecotype populations (S1, S2, NS1, and NS3) when grown in A) in the field (Schechter and Bruns (2008) or when grown in B) a common garden of mixed serpentine and non-serpentine AMF in the greenhouse.



**Figure 6.** Relationship between the relative abundance of the AMF OTU *Acaulospora 1* (*Acaul 1*, A) and *Glomus 1* (*Glo 1*, B) found in individual *Collinsia sparsiflora* roots samples and soil K concentrations associated with the same root sample (Schechter and Bruns, 2008).



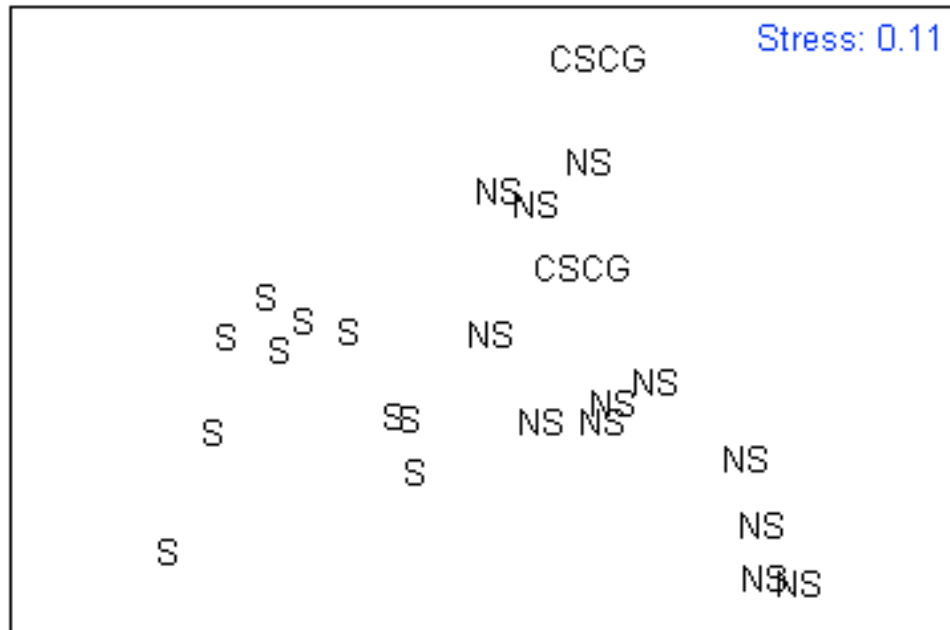
**Figure 7.** Relationship between the relative abundance of the AMF OTU *Acaulospora 1* (*Acaul 1*, A) and *Glomus 1* (*Glo 1*, B) found in individual *Collinsia sparsiflora* roots samples and soil Ca:Mg associated with the same root sample (Schechter and Bruns, 2008).

**Supplemental Table S1.** Results of the harvest of *Collinsia sparsiflora* ecotype populations (S1, S2, NS1, and NS3) after being grown in a common garden of serpentine and non-serpentine AM fungi. Values are means (N= 10) with standard deviation below in parentheses. Letters indicate significant differences at  $P < 0.05$ .

Ecotype	Height (cm)	# of Flowers	Shoot dry weight (g)	Root dry weight (g)	Shoot+Root dry weight (g)	Colonization (%)
S1	14.15 <sup>a</sup> (2.43)	8.10 <sup>a</sup> (3.17)	0.10 <sup>a</sup> (0.03)	0.016 <sup>a</sup> (0.007)	0.12 (0.04)	55.50 <sup>a</sup> (9.89)
S2	13.80 <sup>a</sup> (1.78)	11.40 <sup>a</sup> (5.17)	0.09 <sup>a</sup> (0.03)	0.015 <sup>a</sup> (0.004)	0.10 <sup>a</sup> (0.03)	52.00 <sup>a</sup> (11.51)
NS1	12.85 <sup>a</sup> (1.95)	9.90 <sup>a</sup> (3.26)	0.11 <sup>a</sup> (0.04)	0.019 <sup>a</sup> (0.010)	0.13 <sup>a</sup> (0.04)	54.20 <sup>a</sup> (5.33)
NS3	14.82 <sup>a</sup> (1.50)	13.10 <sup>a</sup> (5.42)	0.11 <sup>a</sup> (0.04)	0.014 <sup>a</sup> (0.005)	0.13 <sup>a</sup> (0.04)	52.60 <sup>a</sup> (9.97)

**Table S2.** Soil chemical variables (S = serpentine, NS = non-serpentine, and CSCG = *Collinsia sparsiflora* common garden soil). Values are means with standard deviation below in parentheses. Nitrogen (as NO<sub>3</sub>) 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 Ca:Mg 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.

Site	N	P	K	Mg	Ca	Ca:Mg	Zn	Fe	pH
S1	2.50 <sup>b</sup> (1.29)	3.75 <sup>c</sup> (0.96)	111.75 <sup>b</sup> (40.55)	1435.00 <sup>a</sup> (292.55)	343.00 <sup>d</sup> (86.15)	0.24 (0.03)	1.05 <sup>b</sup> (0.79)	33.00 <sup>ab</sup> (4.00)	7.00 <sup>a</sup> (0.08)
S2	1.50 <sup>b</sup> (0.58)	2.00 <sup>c</sup> (0.00)	65.75 <sup>b</sup> (23.77)	1021.25 <sup>ab</sup> (197.56)	292.50 <sup>d</sup> (41.44)	0.30 (0.11)	0.65 <sup>b</sup> (0.24)	22.25 <sup>c</sup> (4.72)	6.98 <sup>a</sup> (0.22)
CSCG	8.50 <sup>a</sup> (3.53)	16.50 <sup>b</sup> (3.53)	206.00 <sup>a</sup> (0.00)	668.00 <sup>bc</sup> (2.83)	1140.50 <sup>b</sup> (17.68)	1.71 (0.04)	10.05 <sup>a</sup> (1.34)	31.00 <sup>abc</sup> (1.50)	6.55 <sup>b</sup> (0.07)
NS1	2.50 <sup>b</sup> (0.58)	16.00 <sup>b</sup> (4.16)	278.25 <sup>a</sup> (28.89)	860.75 <sup>b</sup> (162.60)	2143.50 <sup>a</sup> (162.60)	2.58 (0.69)	1.42 <sup>b</sup> (0.19)	37.00 <sup>a</sup> (4.69)	6.52 <sup>b</sup> (0.05)
NS2	7.25 <sup>a</sup> (2.36)	7.00 <sup>c</sup> (2.31)	246.50 <sup>a</sup> (40.50)	801.25 <sup>b</sup> (251.71)	869.50 <sup>c</sup> (49.10)	1.18 (0.43)	0.98 <sup>b</sup> (0.13)	35.50 <sup>ab</sup> (6.95)	6.28 <sup>bc</sup> (0.22)
NS3	3.25 <sup>b</sup> (1.89)	43.75 <sup>a</sup> (5.32)	224.50 <sup>a</sup> (19.94)	307.25 <sup>c</sup> (48.08)	2287.25 <sup>a</sup> (47.42)	7.57 (1.06)	0.75 <sup>b</sup> (0.06)	26.25 <sup>bc</sup> (2.63)	6.12 <sup>c</sup> (0.10)



**Supplemental Figure S1.** Non-metric multi-dimensional scaling (MDS) ordination of soil nutrients (N, P, K, Mg, Ca, Mg:Ca, Zn, Mn, and Fe) associated with serpentine (S) and non-serpentine (NS) ecotype field populations of *Collinsia sparsiflora*, and soil sampled from the common garden soil (CSCG). Soil nutrients included were chosen by the BIOENV routine.

## CHAPTER 3

Serpentine and non-serpentine arbuscular mycorrhizal fungal assemblages  
are distinct from each other



## Abstract

The mechanisms involved in plant adaptation to serpentine soil are still poorly understood. A previous study was the first to test if plant symbiotic association with arbuscular mycorrhizal fungi (AMF) could play a role in plant adaptation to serpentine soil. They found that serpentine and non-serpentine adapted ecotypes of *Collinisa sparsiflora* associated with distinct AMF assemblages. A related study showed that this distinction was not due to host-fungi specificity between serpentine and non-serpentine *C. sparsiflora* ecotypes and AMF. This study tests if the distinction between plant ecotype AMF assemblages was due to edaphic factors shaping distinct serpentine and non-serpentine AMF assemblages by comparing AMF assemblages between adjacent serpentine and non-serpentine soil types. I sampled non-*C. sparsiflora* plant roots from five serpentine and five non-serpentine sites in close proximity (50 m – 150 m between sites) and identified AMF associated with the root samples by amplification of rDNA using AMF specific primers, cloning, and sequencing. I also identified the plant species associated with the root samples by amplification of intergenic spacer region between the *trnL* (UAA) 3' exon and *trnF* (GAA) gene, cloning, and sequencing to use as a covariant for statistical analysis. I sequenced a total of 1,071 AMF clones, and 664 plant clones from 40 root samples (4 from each site), and used a combination of sequence similarity and phylogenetic analysis to determine AMF and plant operational taxonomic units (OTUs). I used Bray-Curtis similarity, multidimensional scaling, and analysis of similarity to compare root sample AMF assemblages. I used multivariate variance partitioning analysis to determine how much of the AMF assemblage variation could be accounted for by soil chemical variables alone, plant assemblages alone, and how much was shared between the two. Assemblage analysis clearly showed that serpentine and non-serpentine AMF assemblages are distinct from each other. Variance partitioning analysis showed that soil nutrients (33.5%), and plant assemblages (25.6%) drove the distinction between serpentine and non-serpentine AMF assemblages. This study confirms that there is a strong ecological relationship between AMF and plant tolerance to serpentine soil.

## Introduction

Plant adaptation to serpentine soil has held the attention of plant evolutionary ecologists for several decades without full resolution of the mechanisms that drive plant adaptation to these edaphic extremes (Brady *et al.*, 2005; Kruckeberg, 1984; Rajakaruna, 2004). Serpentine soils are generally characterized by a very low Ca:Mg ratio, low levels of essential nutrients (N,P,K), high to toxic levels of heavy metals (Fe, Cr, Co, Ni), and drought susceptibility (Brady *et al.*, 2005; Brooks, 1987). Of these, low calcium and very high to toxic levels of magnesium are believed to be the primary edaphic factors involved in plant adaptation to serpentine soils (Brady *et al.*, 2005). However, the multifaceted nature of serpentine edaphic features indicates that multiple plant traits may be important in serpentine adaptation.

Schechter and Bruns (2008) were the first to test if plant symbiotic association with arbuscular mycorrhizal fungi (AMF, Glomeromycota) could play a role in plant adaptation to serpentine soil. As common root symbionts known to increase plant hosts' establishment and growth in stressful environments, AMF are potentially important belowground players in plant adaptation to edaphic extremes (Schechter and Bruns, 2008). In fact, Schechter and Bruns (2008) found that serpentine and non-serpentine adapted plant ecotypes of *Collinsia sparsiflora* associate with distinct AMF assemblages in the field. The authors proposed two scenarios that could account for this distinction between plant ecotype AMF assemblages: 1) the plant ecotypes have high specificity for particular AM fungi within a ubiquitous soil assemblage or 2) the plant ecotypes were tapping nonspecifically into AMF assemblages that had been shaped by edaphic factors (Schechter and Bruns, 2008). Previous work tested for *C. sparsiflora* ecotype specificity for particular AM fungi in a common garden experiment and found no evidence for host-fungal specificity (Chapter 2). This study tests the second scenario as a comparative AMF assemblage analysis of adjacent serpentine and non-serpentine soils.

Read (2002) suggested that edaphic selection may create AMF populations unique to a given soil type. This idea has been supported by research that has shown relationships between soil factors and AMF assemblage composition (Allen *et al.*, 1995; Egerton-Warburton *et al.*, 2007; Fitzsimons *et al.*, 2008; Johnson *et al.*, 1992; Klironomos, 1995; Lekberg *et al.*, 2007; Schreiner & Mihara, 2009). While these studies indicate that differences in assemblage composition are due to variation between AMF species in resource-based fundamental or realized niches (Fitzsimons *et al.*, 2008; Lekberg *et al.*, 2007), factors that generate dissimilarity in AMF composition may be different in extreme edaphic environments. For instance, in metal contaminated sites, variation between AMF species in tolerance of and/or adaptation to a specific metal contaminant is likely to generate differences in assemblage composition (Gonzalez-Chavez *et al.*, 2002; Meharg, 2003; Meharg & Cairney, 1999). Serpentine environments are more comparable to metal contaminated sites than studies in which soil types only vary slightly in soil chemistry or texture. Thus, strong edaphic selection of serpentine adapted/tolerant AMF taxa may be the main mechanism structuring AMF assemblages in serpentine environments.

Serpentine soils provide an excellent system to study the effect of edaphic selection on AMF assemblage structure and composition. AMF assemblages on serpentine have been under constant selection pressure from 10,000 to 10 million years depending on location (Kruckeberg, 1984). This persistent edaphic stress may be enough to cause distinct changes in AMF assemblage structure and composition relative to non-serpentine assemblages as well as generate unique serpentine AMF. Distinct serpentine adapted AMF assemblages may provide specific services to plants in this harsh environment and facilitate plant adaptation to serpentine. However, it is difficult to isolate edaphic selection from the influence of plant community

differences on AMF assemblage structure and composition (Bever *et al.*, 1996; Bever *et al.*, 2002; Johnson *et al.*, 1992; Schultz *et al.*, 2001). We know that edaphic selection on serpentine soils does generate distinct serpentine floras that contain higher numbers of native and endemic species (Brady *et al.*, 2005; Kruckeberg, 1984; Kruckeberg, 2002). Therefore, any comparison of serpentine and non-serpentine AMF assemblages must also account for differences in associated plant communities.

The goal of this study is to determine if the distinction between AMF assemblages associated with serpentine and non-serpentine *C. sparsiflora* ecotypes in the field (Schechter and Bruns, 2008) was due to edaphic factors shaping distinct serpentine and non-serpentine AMF assemblages. To do this, I compared AMF assemblages associated with non-*C. sparsiflora* roots sampled from plants found in adjacent serpentine and non-serpentine soil types using molecular methods. I took advantage of the fine-scale mosaic of serpentine and non-serpentine soils associated with the McLaughlin Reserve Research Grid to chose serpentine and non-serpentine sites within a close geographical range (50 m to 150 m between sites). I also identified the plant species associated with each root sample using molecular methods to use as a covariant for statistical analyses. I hypothesized that AMF assemblages would be distinct between serpentine and non-serpentine soil-types and that this would also be associated with differences in serpentine and non-serpentine plant communities sampled.

## Materials and Methods

### *Study System*

This study was done at the Donald and Sylvia McLaughlin University of California Natural Reserve located in Napa, Lake, and Yolo counties in northern California (Figure 1). The McLaughlin reserve is situated over a minor fault line that has produced a fine-scale mosaic of serpentine, volcanic, and valley sediment soil types occurring within meters of each other (Wright & Stanton, 2007; Wright *et al.*, 2006). In 2001, researchers from the University of California Davis used the unique geology of the research to establish a 27.5 ha research grid that encompassed this fine-scale mosaic of soil types. They established grid points 50 meters apart across the entire grid area in which they did soil physical and chemical analyses as well as vegetation surveys at each 50 meter grid point (<http://nrs.ucdavis.edu/mcl/visitor/facilities.html>) (Figure 1). Therefore, the grid data could be used to classify and establish serpentine and non-serpentine soil type sites in close proximity to each other. I used the McLaughlin research grid to establish five serpentine and five non-serpentine soil type sites for this study (Figure 1).

### *Sampling*

In March 2007, after using the McLaughlin research grid soil analysis data as a guide, I sampled soil at putative serpentine and putative non-serpentine grid points for soil analysis. These soil samples were sent off to A & L Western Agricultural Laboratories (Modesto, CA) to confirm “serpentine” (Ca:Mg < 1) and “non-serpentine” (Ca:Mg > 1) soil type classification at each site (Wright *et al.*, 2006). Following confirmation of serpentine and non-serpentine soil type classification of grid points, I chose five serpentine (average Ca:Mg = 0.36) and five non-serpentine (average Ca:Mg = 6.35) grid points from which to compare soil type AMF assemblages. This allowed for a comparison of “very” serpentine to “very” non-serpentine soil types. In May 2007, I collected four root samples at cardinal direction points along a 1.0 m

diameter circle placed around each grid point (S1, S2, S3, S4, S5 and NS1, NS2, NS3, NS4, NS5) (Figure 1). I collected roots at each cardinal direction, labeled A – D, by taking a trowel slice 10 cm by 8 cm and 14 cm deep, at each grid point for a total of 40 root samples for the entire study (twenty serpentine and twenty non-serpentine). I also collected soil directly adjacent to the plant root collection in each sample point, but to keep down costs, I combined equal amounts of soil from each sample point to equal one soil sample per grid point for soil analysis (10 soil samples for the entire study). All plant and soil samples were put directly into coolers and stored in a 4°C cold room within 8 hours of collection. I sent soil samples to A&L Western Agricultural Laboratories (Modesto, CA) within 24 hours of collection for chemical analysis. All plant samples were processed within two weeks of collection.

I processed each grid point plant root sample individually in a fume hood, which was cleaned thoroughly between samples. I collected all roots within each sample and then thoroughly washed them to remove as much soil as possible. I took a small portion (0.5 g wet weight) 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 three days. These dried roots were then placed into a 2 ml cryotube and stored in a -80°C freezer until DNA extraction as described below.

### *Molecular Analysis*

DNA extraction: I extracted DNA from each grid point root sample (40 total). I crushed the dried and frozen roots by beadbeating (Mini-Beadbeater, Biospec Products) with sterile glass beads for 30 seconds or until a fine powder formed. The samples were then immediately placed on ice, and 1.5 ml of 2x 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. I extracted DNA from each root sample as described in Schechter and Bruns (2008).

Polymerase chain reaction (PCR) - AMF: I amplified a variable region of the 18S rDNA using *Pfu Turbo* DNA polymerase (Stratagene) and universal eukaryotic primer NS31 (Simon *et al.*, 1992) paired with AM1 (Helgason *et al.*, 1998) as described in Schechter and Bruns (2008). Each 20 µl PCR reaction consisted of 12.4 µl of dH<sub>2</sub>O, 0.2 µl of 2.5 U *Pfu Turbo* DNA polymerase, 2 µl of manufacture's buffer (Stratagene), 2 µl of 10x dNTPs, and 0.2 µl of each 50µM primer. PCR conditions were the same as described by Helgason (2002) with the exception that the annealing temperature was increased to 64° C (optimized through gradient PCR analysis) in order to decrease amplification of non-AMF sequences.

Polymerase chain reaction (PCR) - Plants: I amplified the intergenic spacer region between the *trnL* (UAA) 3' exon and the *trnF* (GAA) gene using the primer pair *trnL*-e and *trnL*-f (Taberlet *et al.*, 1991) and *Pfu Turbo* DNA polymerase (Stratagene). Each 20 µl PCR reaction consisted of 14.4 µl of dH<sub>2</sub>O, 0.2 µl of 2.5 U *Pfu Turbo* DNA polymerase, 2 µl of manufacture's buffer (Stratagene), 2 µl of 10x dNTPs, and 0.2 µl of each 50µM primer and 1 µl of DNA. PCR conditions were the same as described by Taberlet *et al.* (1991).

Cloning and Sequencing: I gel purified and concentrated the AMF PCR products before cloning because using straight PCR resulted in low cloning efficiency. It was not necessary to purify and concentrate plant PCR products. AMF PCR products from each sample were gel purified and concentrated as described by Schechter and Bruns (2008) I then cloned AMF and plant PCR products (40 AMF and 40 plant PCR cloning reactions total) into pPCR-Script Amp SK(+) and transformed into *Escherichia coli* XL10-Gold Kan Ultracompetent cells (Stratagene). I picked 48 putative positive transformants per sample. Transformants were screened for

correctly sized inserts using plasmid primers T3/T7 under the same PCR conditions as described in Schechter and Bruns (2008). Then, I selected 24 gel confirmed positive AMF PCR transformants and 16 positive plant PCR transformants per sample for cleaning and sequencing. I cleaned these PCR products with ExoSAP-IT using the manufacturer's instructions (USB), and sent the clean PCR products to the UC Berkeley Sequencing Facility (Berkeley, CA) for sequencing. Finally, I 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: Prior to analysis, I compared the AMF and plant sequences to GeneBank sequences using BLAST (version 2.21, Altschul *et al.*, 1997), and those with low bit scores and high E-values were suspected as chimeras. I also used the Chimera Check program in RDP-II (version 2.7, Cole *et al.*, 2003) to check for chimeras in AMF sequences as described by Schechter and Bruns (2008). In addition, I also looked for oddities in global alignments and changes in phylogenetic position (see below for description of methods) to indicate chimeric sequences. I eliminated any suspect sequence, identified under any of these criteria, from the data set.

### *Data Analysis*

Operational Taxonomic Unit (OTU) determination- AMF: I determined AMF OTUs in this experiment using the same combination of grouping by sequence similarity and phylogenetic analysis as described by Schechter and Bruns (2008). I first combined sequences from each grid point root sample at 98% similarity using Sequencher 4.2.2 to create grid point contigs and singletons in order to keep information about grid point origin intact. Then I compared all grid point 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.

I aligned these sequences, as well as all AMF sequences included in Schechter and Bruns (2008) using ClustalX (Thompson *et al.*, 1997) and then manually edited the sequences using MacClade v 4.08 (Maddison & Maddison, 2005). Like Schechter and Bruns (2008) two separate phylogenetic analyses were performed using *Olpidium brassica* as an outgroup: maximum likelihood (ML) was conducted using Garli (Genetic Algorithm for Rapid Likelihood Inference) v 0.95 (Zwickl, 2006), and Bayesian analysis was performed using MrBayes 3.1.1 (Ronquist & Huelsenbeck, 2003). I ran the phylogenetic analysis using the same methods as described in Schechter and Bruns (2008).

I used the results of the phylogenetic analyses to confirm OTUs. I looked for consistency in topology between analyses and > 50% bootstrap or Bayesian posterior probability branch support for terminal clades that included the putative OTU sequences (98% sequence similarity groupings). I used these OTUs to determine the assemblages of AM fungi associated with each grid point root sample (40 total).

Operational Taxonomic Unit (OTU) determination- Plant: I determined plant OTUs in this experiment by using a combination of grouping by sequence similarity and phylogenetic analysis. I first combined plant sequences from each grid point root sample at 97% similarity using Sequencher 4.2.2 to create grid point contigs and singletons in order to keep information about grid point origin intact. Then I compared all grid point contigs and singletons together at 97% to determine 97% sequence similarity groupings for the entire data set; these groupings were used to define putative plant OTUs.

I aligned these sequences along with close BLAST matches and additional GenBank plant sequences of congeners known to be present at the research grid using ClustalX (Thompson

*et al.*, 1997) and then manually edited the sequences using MacClade v 4.08 (Maddison & Maddison, 2005). Because of the difficulty aligning these plant sequences across all families, I used four separate alignments for phylogenetic analysis: Poaceae (outgroup: *Streptochoeta sodiroa*), Asterids (outgroup: *Brassica nigra*), Rosids/Asterids (outgroup: *Nicotiana attenuata*), and Onagraceae (outgroup: *Rotala indica*). For each alignment I performed two separate phylogenetic analyses: maximum likelihood (ML) was conducted using Garli (Genetic Algorithm for Rapid Likelihood Inference) v 0.95 (Zwickl, 2006), and Bayesian analysis was performed using MrBayes 3.1.1 (Ronquist & Huelsenbeck, 2003). Molecular evolutionary models for Bayesian analysis and maximum likelihood analysis were estimated with MrModeltest (Nylander, 2004) and Modeltest 3.7 (Posada & Crandall, 1998) respectively. Bayesian analysis was performed with two MCMC chains 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).

I used the results of the phylogenetic analyses to confirm plant OTUs. Once again, I looked for consistency in topology between analyses and > 50% bootstrap or Bayesian posterior probability branch support for terminal clades that included the putative OTU sequences (97% sequence similarity groupings). As above, I used these OTUs to determine the communities of plants associated with each grid point root sample (40 total).

Assemblage Analyses: I analyzed the AMF and Plant assemblages using the PRIMER 5 software (Plymouth Routines in Multivariate Ecological Research) (Clarke & Warwick, 2001). I prepared a separate relative abundance matrix of OTUs present in each grid root sample based on the number of clones representing those OTUs within each sample for AMF and plant assemblages. Therefore, each relative abundance matrix represents the AMF and plant assemblages associated with each grid point root sample. I then produced a separate similarity matrix for each relative abundance matrix using the Bray-Curtis similarity measure after performing a square-root transformation (Clarke & Warwick, 2001). I used non-metric Multidimensional Scaling (**MDS**) ordinations to represent the dissimilarities in assemblage composition among grid point samples and the **ANOSIM** (analysis of similarities) routine to perform statistical analysis of assemblage data (Clarke & Warwick, 2001) separately for AMF and plant assemblages. In addition, I used the **SIMPER** (Similarity Percentages) routine to determine the relative contribution of individual AMF and plant OTUs toward dissimilarity between grid points.

I used the EstimateS 8.0 Mao Tau estimator (Colwell *et al.*, 2004) to produce a rarefaction curve for both AMF and plant assemblages to determine in clone sampling effort saturated the number of OTUs. I also computed Shannon-Wiener diversity ( $H'$ ), richness, and evenness for each grid point AMF assemblage using PRIMER 5, and used one-way ANOVA (JMP v. 5) to test for differences between soil types 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.

I also used the PRIMER 5 software to compare differences in soil chemical characteristics between grid points. I produced a similarity matrix of soil chemical data (log transformed) using Euclidean distance (Clarke & Warwick, 2001). I then used non-metric MDS and ANOSIM to explore differences in soil chemical characteristics between grid points. The BIO-ENV routine was used to determine which of the soil chemical variables best explained the

differences between samples. I 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), between the AMF assemblage similarity matrix and the plant assemblage similarity matrix, and between the plant assemblage similarity matrix and the soil chemical similarity matrix (using only the BIO-ENV soil variables). Finally, I used multivariate variance partitioning to determine how much of the AMF assemblage variation could be accounted for by soil chemical variables alone, plant assemblage variation alone, and how much was shared by the two (Borcard *et al.*, 1992) by means of the CANOCO program for Windows 4.5 (Ter Braak, 1988)

## Results

### *Assemblage Identification*

*General – AMF.* All 40 grid point root samples were highly colonized (48- 57% root length) by AMF with no significant differences in colonization between samples ( $F = 1.02$ ,  $P < 0.41$ ). A total of 1,071 clones were sequenced to determine grid point AMF assemblages. Of these, a total of 91% were AMF sequences, 2% were ascomycete fungi, 0.3% were of bacterial origin, and 0.2% were chimeric. The rest were of too poor of quality to give reliable data. Each grid point sampled was represented by similar numbers of AMF sequences (NS1 = 137, NS2 = 92, NS3 = 94, NS4 = 87, NS5 = 88, S1 = 140, S2 = 81, S3 = 89, S4 = 90, S5 = 80).

*Phylogenetic analysis – AMF.* I detected only two AMF genera in this study (Table 1). *Glomus* species were the overwhelming dominants, representing 99.8% of the sequences. The only other AMF genus detected was *Archaeospora* (0.2% of the sequences). I established 12 AMF OTU (Table 1), 8 of which matched OTUs found in Schechter and Bruns (2008) and the common garden study (Chapter 2). Two OTUs were most dominant in these samples, *Glomus* 1 (34% of sequences) and *Glomus* 5 (29%).

*General – Plants.* A total of 664 clones were sequenced to determine plant assemblages associated with grid point root samples. Of these, 93% were plant sequences, 0.4% were chimeric, and the rest were of too poor of quality to give reliable data. Each grid point sampled was represented by similar numbers of plant sequences (NS1 = 60, NS2 = 61, NS3 = 80, NS4 = 57, NS5 = 64, S1 = 61, S2 = 59, S3 = 61, S4 = 59, S5 = 56).

*Phylogenetic analysis – Plants.* I detected 13 plant families within the grid point root samples. The most abundant family was Poaceae (66% of sequences), followed by Asteraceae (17%), Scrophulariaceae (4%), Onagraceae (3%), Geraniaceae (2%), Juglandaceae (2%), Solanaceae (2%), Polemoniaceae (1%), Rosaceae (1%), Apiaceae (0.8%), Caryophyllaceae (0.5%), Linaceae (0.4%), and Lamiaceae (0.3%). I established 31 plant OTUs using the combined sequence similarity and phylogenetic criteria for OTU determination (Table 2). Of these, four OTUs were most dominant in these samples: *Avena* 1 (33% of sequences), *Aster* 2 (11%), *Bromus* 1 (8%), and *Vulpia* 1 (6%).

### *Assemblage Analysis*

*Comparing AMF assemblages.* The rarefaction analysis shows that the curves for most grid point samples are clearly leveling off and all contained relatively few OTUs (< 5, Figure 3). This indicates that the sequence sampling effort was sufficient for a comparison of AMF assemblages associated with the grid point root samples. The MDS ordination shows that the AMF assemblages found in root samples associated with the serpentine soil type were much

more similar to each other than those associated with the non-serpentine soil type and vice versa (Figure 4). This distinction was supported by ANOSIM analysis ( $R = 0.513$ ,  $P < 0.001$ ). The distinction between soil type AMF assemblages was still highly significant after presence/absence transformation of the relative abundance matrix data ( $R = 0.339$ ,  $P < 0.001$ ). These results clearly show that AMF assemblages of plant roots associated with serpentine and non-serpentine soil types are distinct from each other.

*OTU contribution to AMF assemblage differences.* SIMPER analysis shows that two AMF OTUs contributed the most to the distinction between soil type AMF assemblages: *Glomus* 1 (36%) and *Glomus* 5 (23%). *Glomus* 1 showed the most conspicuous pattern in the relative abundance matrix, being completely absent in the root samples from the serpentine soil type but highly abundant in root samples from the non-serpentine soil type with the exception of the NS3 grid point (Table 1). In contrast, *Glomus* 5 was found in every grid point root sample but was much more abundant in those sampled from the serpentine soil type (Table 1). Interestingly, *Glomus* 6, which had been believed to be a serpentine-only OTU (Schechter and Bruns, 2008), was also found in root samples from the non-serpentine soil type although the highest levels of abundance were sampled from serpentine grid points. Species diversity ( $F = 0.04$ ,  $P < 0.84$ ), evenness ( $F = 1.10$ ,  $P < 0.30$ ), and richness ( $F = 0.13$ ,  $P < 0.73$ ), were not significantly different between serpentine and non-serpentine soil types.

*Comparing plant assemblages.* The rarefaction analysis shows that the curves for all grid point samples show a clear leveling off except for NS2 and NS4, which seem to still be increasing (Figure 5). This indicates that for all but NS2 and NS4, I identified the majority of the plant taxa associated with the grid point root samples. The MDS ordination of all the grid point root samples showed some clear outlier plant assemblages (S1a, S1d, NS5b, NS5c, and NS5d, Figure S1, supplementary materials), which created a poor ordination. Redoing the MDS analysis after eliminating the outliers showed a separation between plant assemblages associated serpentine and non-serpentine soil types (Figure 6). This slight separation was significant (including the outliers) in the ANOSIM analysis ( $R = 0.145$ ,  $P < 0.001$ ), even after the presence/absence transformation ( $R = 0.138$ ,  $P < 0.003$ ). This data shows that the plant assemblages associated with the root samples were different between soil types.

*OTU contribution to plant assemblage differences.* Three plant OTUs contributed the most to the soil type distinction between plant assemblages associated with the root samples (SIMPER analysis): *Avena* 1 (19%), *Aster* 2 (12%), and *Bromus* 1 (7%). *Avena* 1 was found in each non-serpentine grid point root sample at relatively high abundance, it was also found in three of the five serpentine grid point root samples but at lower abundance (Table 2). *Aster* 2 was absent from the non-serpentine grid point root samples but present in three of five serpentine grid point root samples. *Bromus* 1 was found in four of the five non-serpentine root samples but was only found in two of the five serpentine root samples (Table 2). Species diversity ( $F = 1.52$ ,  $P < 0.23$ ), evenness ( $F = 1.27$ ,  $P < 0.27$ ), and richness ( $F = 4.11$ ,  $P < 0.06$ ), were not significantly different between plant assemblages associated with root samples from serpentine and non-serpentine soil types.

*Contribution of soil nutrients and plant assemblages to AMF assemblage differences.* The MDS of the soil nutrients showed that serpentine and non-serpentine soil types are clearly distinct from each other ( $R = 0.972$ ,  $P < 0.008$ ) (Figure S2). However, when comparing individual nutrients across soil type, only Ca ( $F = 30.95$ ,  $P < 0.0008$ ) Mg ( $F = 56.49$ ,  $P < 0.0001$ ), and Ca:Mg ( $F = 15.91$ ,  $P < 0.005$ ) levels were significantly different between soil types (Table 3). Using the BIO-ENV routine, soil K, Ca, Mg, Ca:Mg, and B had the highest



Spearman's rank correlation score, and therefore were used to test for a relationship between these soil nutrients and both AMF and plant assemblages. The RELATE test between soil nutrients (K, Ca, Mg, Ca:Mg, and B) and AMF assemblages was significant ( $Rho = 0.398$ ,  $P < 0.02$ ). However, the RELATE test between the same soil nutrients and plant assemblages was *not* significant ( $Rho = 0.025$ ,  $P < 0.37$ ). Therefore, while there is a relationship between soil nutrients and AMF assemblages, the plant assemblages did not show any association to soil nutrients.

The RELATE test was also used to test for a relationship between plant assemblages and AMF assemblages. This test was significant ( $Rho = 0.186$ ,  $P < 0.007$ ), indicating that there is a relationship between plant and AMF assemblages. Finally, variance partitioning analysis showed that 33.5% of the variance between AMF assemblages could be uniquely described by soil nutrients alone, 25.6% could be described by plant assemblage alone, 28.9% shared between soil nutrients and plant assemblage, and that 12% of the variance was unexplained.

## Discussion

### *Molecular Approach*

Using the same molecular approach, this study found a much lower diversity of AMF genera than was found in the *C. sparsiflora* ecotype field study (Schechter and Bruns, 2008). Sampling only *C. sparsiflora* roots of six ecotype populations from the McLaughlin Reserve in March 2005, Schechter and Bruns (2008) found 6 AMF genera represented mainly by *Glomus* (72% of sequences) and *Acaulospora* (25%) species. In contrast, this study sampled a great diversity of plant roots (representing 13 plant families) from ten sites within the same area in May 2007, and detected only two AMF genera, predominantly *Glomus* species (99.8% of sequences). This huge shift in AMF genera diversity could be explained by differences between the studies in three main factors: plant types sampled, year/season, and soil factors. The plants sampled differed greatly between the two studies. *C. sparsiflora* (Plantaginaceae) is a short-lived winter annual that germinates in October or November and sets seeds and dies in May or June (Wright *et al.*, 2006), while the plants sampled in this study were a mixture of later season annuals and perennials mainly represented by species within the Poaceae. Other AMF molecular studies of similar California grassland species were also dominated by *Glomus* species (Hausmann & Hawkes, 2009; Hawkes *et al.*, 2006), possibly indicating a preference for California grassland species to associate with *Glomus* species.

Another possible explanation for the change in AMF genera diversity between the two studies is a shift in AMF taxa composition between 2005 and 2007. Rainfall records for the McLaughlin Reserve shows that 2005 was a much wetter year than 2007 with ten more inches of rainfall in 2005 than 2007 (<http://cdec.water.ca.gov/>). Seasonal and yearly changes in AMF taxa composition has been found in other studies (Bever *et al.*, 2001; Pringle & Bever, 2002; Santos-Gonzalez *et al.*, 2007; Vandenkoornhuyse *et al.*, 2002) and may offer a feasible explanation to the differences in AMF assemblage diversity between studies. However, the biggest change in AMF genera diversity between studies was found between serpentine *C. sparsiflora* ecotype and the serpentine soil grid point root samples, shifting from an *Acaulospora* dominant assemblage (57% of sequences) in the serpentine *C. sparsiflora* (Schechter and Bruns, 2008) to a complete absence of *Acaulospora* species in the serpentine grid point samples (Table 1). This may be due to the fact that, while Ca:Mg was similar between studies, the serpentine soils sampled in the *C.*

*sparsiflora* field study were much lower in soil K (serpentine K average = 88 ppm) (Schechter and Bruns, 2008), than those sampled in this study (serpentine K average = 279 ppm) (Table 3). In fact, the concentrations of soil K found in the serpentine soils of this study are at the concentration where, in the *C. sparsiflora* field study, the relative abundance of the dominant *Acaulospora* species was almost zero and the relative abundance of the dominant *Glomus* species was near 100 (Figure 6, Chapter 2). This may signify that the change in AMF genera diversity between the studies, especially between AMF associated with serpentine soil, is due to “preference” for or increased competitive ability of *Acaulospora* taxa over *Glomus* taxa in lower soil K concentrations. Nevertheless, perhaps the best explanation for the difference in AMF diversity between the studies is a combination of the three main factors (plant type, year/season, and soil factors).

The molecular identification of plants associated with root samples is a brand new method for AMF studies. Other AMF studies that have associated AMF assemblage with plant assemblages have only used aboveground plant identification as the basis for plant assemblage determination (Gai *et al.*, 2009; Hawkes *et al.*, 2006; Husband *et al.*, 2002; Landis *et al.*, 2004; van der Heijden *et al.*, 1998). The choice of the intergenic spacer region between *trnL* and *trnF* to determine plant assemblages associated with root samples had its advantages and disadvantages. The advantages were that it is a shorter and much more variable marker than *rbcL*, which made it much easier to amplify and clone, and allowed for good resolution of plant taxa at lower taxonomic levels (genus and species levels) than possible with the more conserved *rbcL* (mainly limited to family level), in addition, the primers are highly conserved across all plant lineages, and the marker has a large representation in GenBank (Borsch & Quandt, 2009; Taberlet *et al.*, 2007; Taberlet *et al.*, 1991).

The major disadvantages of using the intergenic spacer region between *trnL* and *trnF* is that, while common in phylogenetic studies of known plant taxa, it is rarely if ever used to identify unknown plant taxa. Therefore, the fact that little is known about the sequence divergence between or within plant genera for this marker poses problems for straightforward species identification (Borsch & Quandt, 2009; Gielly & Taberlet, 1994). This problem is compounded by the fact that it is impossible to align sequences from this marker between dicots and monocots, and even within the same family in some cases (Gielly & Taberlet, 1994). The best models for plant OTU determination of unknowns are plant DNA barcoding studies using the *trnL* intron, another non-coding cpDNA marker. Soininen *et al.* (2009) used the *trnL* intron in an attempt to identify plant taxa in the feces of small herbivores, and identified the unknown plant sequences by comparing them to a sequence database of known plant taxa and identifying unknowns based on 98% sequence similarity to known taxa. In some cases, the unknown sequence matched two or more known taxa and therefore identification was assigned the higher taxonomic level that included all matches (genus or family) (Soininen *et al.*, 2009). I started my OTU determination of unknown plant sequences in a similar manner, but when comparing 98% groupings to phylogenetic trees, I found that grouping sequences at 97% sequence similarity produced better supported phylogenetic terminal clades. Therefore, the main criteria I used to determine plant OTUs was 97% sequence similarity. It is unclear whether these groupings give species level determination, especially since so many different plant families were represented in this study, so I assigned plant OTU names based on genus level in most cases, or to the family level in cases where it was not clear from phylogenetic analysis that genus could be assigned. However, phylogenetic analyses were *only* used to help assign names and determine appropriate

sequence groupings of plant sequences, *not* as a means to determine phylogenetic history or relationships between plant taxa.

Another major unknown in this study was using clone relative abundance as a proxy for relative abundance of plant OTUs found in each root sample. I have used clone abundance as a proxy for the relative abundance of AMF OTUs cautiously in prior studies as well as this one, and consistency in results across studies has given me confidence that clone relative abundance provided a reasonable estimation of the relative abundance of dominate AMF taxa. I have no reason to believe that this would not also be the case for the plant sequence data, but it has, to my knowledge, never been tested for this marker. In most cases, sequence dominance matched estimated aboveground dominance of plant taxa, but in some cases, aboveground plant taxa did not match sequences found in the roots (data not shown). However, the question of whether clone sequence abundance relates to plant OTU abundance is moot for this study since the difference between plant assemblages associated with serpentine and non-serpentine root samples was found to be significant even after the presence/absence transformation. But, if this marker is to be used to determine plant taxa in future AMF studies, this question should be addressed experimentally before clone abundance is used as a proxy for plant OTU abundance.

#### *Distinction between soil type AMF assemblages*

I found that AMF assemblages associated with serpentine and non-serpentine soil types are distinct from each other. This distinction was even clear after presence/absence transformation of the AMF OTU relative abundance matrix. This shows that highly abundant OTUs were not as important for the distinction between AMF assemblages as was found in the *C. sparsiflora* field study (Schechter and Bruns, 2008). But this statement is slightly misleading, since it was the complete absence of the most highly abundant non-serpentine AMF OTU – *Glomus* 1, from serpentine sites that contributed the most to the distinction between assemblages. Therefore, both relative abundance and presence/absence information together give the clearest representation of how these AMF OTUs are interacting with soil type.

Soil type had a large effect on presence and abundance of certain AMF OTUs. Once again, *Glomus* 1 was the overwhelming dominant AMF OTU in non-serpentine soil (Schechter and Bruns, 2008) (Table 1). The fact that *Glomus* 1 is still the non-serpentine dominant at different sites, on different plant types, and two years after the *C. sparsiflora* sampling, implies that *Glomus* 1 is the best AMF competitor on non-serpentine soils at McLaughlin Reserve. In contrast, the complete absence of *Glomus* 1 on serpentine soils in this study and its low abundance in serpentine *C. sparsiflora* roots (only 3% of *Glomus* 1 sequences were found in serpentine; Schechter and Bruns, 2008), indicate that serpentine has a strong negative effect on *Glomus* 1. This may mean that it is poorly adapted to serpentine soil and/or a bad competitor with better-adapted AMF taxa in serpentine, namely *Acaulospora* 1 in the *C. sparsiflora* study, and *Glomus* 5 and *Glomus* 4 in this study. This is also consistent the results of the Common Garden study (Chapter 2). In the Common Garden study, *Glomus* 1 was dominant in every non-serpentine soil type, but found at very low presence and abundance in serpentine soil (Chapter 2, Table 2). The potential negative effect on serpentine soil on *Glomus* 1 becomes more intriguing if one considers the presence of a new OTU in this study – *Glomus* 1A. This very closely related OTU was only found in serpentine soil at high abundance at two serpentine grid points (Figure 2). If serpentine soil does indeed have a strong negative effect on *Glomus* 1, the appearance of *Glomus* 1A may represent a serpentine-evolved relative of *Glomus* 1.

The effect of soil type on AMF assemblage composition found in this study was not restricted to serpentine versus non-serpentine soil types. The AMF assemblages associated with the non-serpentine grid point NS3 were outliers even to the rest of the non-serpentine AMF assemblages (Table 1). While NS3 was chosen as a “non-serpentine” soil type based on Ca:Mg, it varies considerably from the other non-serpentine soils in both pH and soil K (Table 3). This difference in soil type is also apparent in the grid point map as a white alluvial fan, potentially representing a completely different parent material than the other non-serpentine soil types (Figure 1). This was also reflected in the detection of three new AMF OTUs in this grid point: *Glomus* F, *Glomus* D, and *Glomus* G (Table 1). Of these, *Glomus* F and *Glomus* D were highly abundant, and the other three OTUs detected at this grid point were in low abundance including the only “previously found” OTUs, *Glomus* 4 and *Glomus* 5. Soil pH is known to alter AMF composition, and therefore may be the soil factor driving this unique AMF assemblage. However, it is interesting to note that while the AMF assemblages differed at this grid point, the plant assemblages were similar to those found in other non-serpentine grid points (Table 2).

#### *Distinction between soil type plant assemblages*

I also found that plant assemblages associated with the root samples differed between soil types. This result is only for those roots sampled at the different grid points, and does not necessarily reflect aboveground plant assemblage composition differences. Even though the difference in plant root sample assemblages between soil types was significant, the R value was very low, indicating very little distinction between assemblages. This may be reflective of the high number of rare OTUs found in the root samples (26 of 31 plant OTUs were only found once; Table 2). Another factor contributing to the poor distinction between soil type plant root sample assemblages is the presence of invasive species in both serpentine and non-serpentine grid points, which is known from plant community studies at McLaughlin (Davies *et al.*, 2005). For example, sequences of *Avena* 1 matched both *Avena barbata* and *Avena fatua* GenBank sequences at 99% and 98% similarity respectively, which are invasive species known to occupy both serpentine and non-serpentine soils (Harrison *et al.*, 2001).

The level of detection of plant OTUs using these molecular methods was satisfactory. All plant families found are known to be at the McLaughlin Reserve, even Juglandaceae (<http://herbarium.ucdavis.edu/flora/mclaughlin.htm>). Two plant OTUs found only on serpentine were good matches to serpentine plants - *Navarretia* 1 is a 99% match to *Navarretia jepsonii* a native and rare serpentine endemic, and *Hesperolinon* 1 is a 99% match to *Hesperolinon californicum* a native strong serpentine indicator species (<http://www.calflora.org>). The most interesting discovery when looking at plant identity from the root perspective is that the aboveground identification did not always match belowground identification. For example, *Plantago erecta* was the aboveground plant dominant at S5, but no sequences were found in root samples and instead sequences from a unknown aster was the most dominant plant sequence (Table 2). This shows that care must be taken when trying to link aboveground plants to AMF, as identification of aboveground plants at or around the sampling site may not be reflective of the identity of the roots.

#### *Relationship between soil type, plant and AMF assemblages*

To my knowledge this is the first study that has attempted to directly quantify the relative contributions of both soil and plant factors in AMF assemble structure and composition through the molecular identification of both plant and AMF taxa associated with root samples. Even

though plant root sample assemblages differed between soil types, there was only a weak association between plant assemblages and AMF assemblages. This was most clearly seen in the S3 and S4 grid point samples. These grid point root samples were both dominated by the *Avena* 1 plant OTU, but they were not associated with *Glomus* 1 as was found in the other root samples dominated by *Avena* 1 (Table 1 and 2). This is of particular interest since several studies have shown that invasive plants (like *Avena barbata* and *A. fatua*) typically have an effect on microbial community composition including AMF (Batten *et al.*, 2006; Batten *et al.*, 2008; Hausmann & Hawkes, 2009; Hawkes *et al.*, 2006). This shows that soil type has a stronger effect than plant assemblage composition on AMF assemblage structure and composition in this study. This was confirmed by the variance partitioning analysis. Soil nutrients alone explained 33.5% of the AMF assemblage variance, while plant assemblage alone explained 25.6% of the AMF assemblage. However, 28.9% of the variance was shared between soil nutrients and plant assemblage, leaving only 12% of the variance unexplained. So, while soil type had a stronger effect than plant assemblage composition on AMF assemblage structure and composition, there is clearly a combined effect of both interacting together to shape AMF assemblages and that these two variables (alone and together) accounted for 88% of the variance between AMF assemblages.

When Lekberg *et al.* (2007) measured the relative contribution of soil type (sand vs. clay) and spatial variables to AMF assemblage composition associated with maize roots across a 25 km distance, these variables accounted for 38.6% and 23.5% of the variation respectively with very little interaction between the two factors (2%). Lekberg *et al.* (2007) attributed this finding to the role of AMF taxa traits (niche restriction to specific soil texture), local environmental factors, and regional dispersal dynamics in structuring AMF assemblages. While this study corroborates the potential role of AMF taxa traits and local environmental factors, it adds plant assemblage and soil and plant interactions to the list of mechanisms that generate differences in AMF assemblage composition. However, the close geographical range between serpentine and non-serpentine sites sampled in this study (50m – 150m) also indicate strong edaphic selection of serpentine tolerant/adapted AMF taxa as an additional factor shaping AMF assemblages in this study.

### *Conclusion*

The primary goal of this study is to determine if the distinction between AMF assemblages associated with serpentine and non-serpentine *C. sparsiflora* ecotypes in the field (Schechter and Bruns, 2008) was due to edaphic factors shaping distinct serpentine and non-serpentine AMF assemblages. This study clearly shows that serpentine and non-serpentine AMF assemblages are indeed distinct from each other even on a fairly fine spatial scale. Soil nutrients, plant assemblages, and the combined effect of both, drove this distinction between serpentine and non-serpentine AMF assemblages. Thus, this study confirms that there is a strong ecological relationship between AMF and plant tolerance to serpentine as was indicated by Schechter and Bruns (2008)– plants growing in serpentine soil associate with serpentine-tolerant AMF taxa. Moreover, this study was able to clearly identify plant assemblage, and plant and soil type interactions as additional mechanisms that generate differences in AMF assemblages.

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**Table 1.** Relative abundance matrix of operational taxonomic units (OTU) of AM fungi associated with plants roots collected from five serpentine (S) and five non-serpentine grid points. Highlighted OTUs show soil type affects.

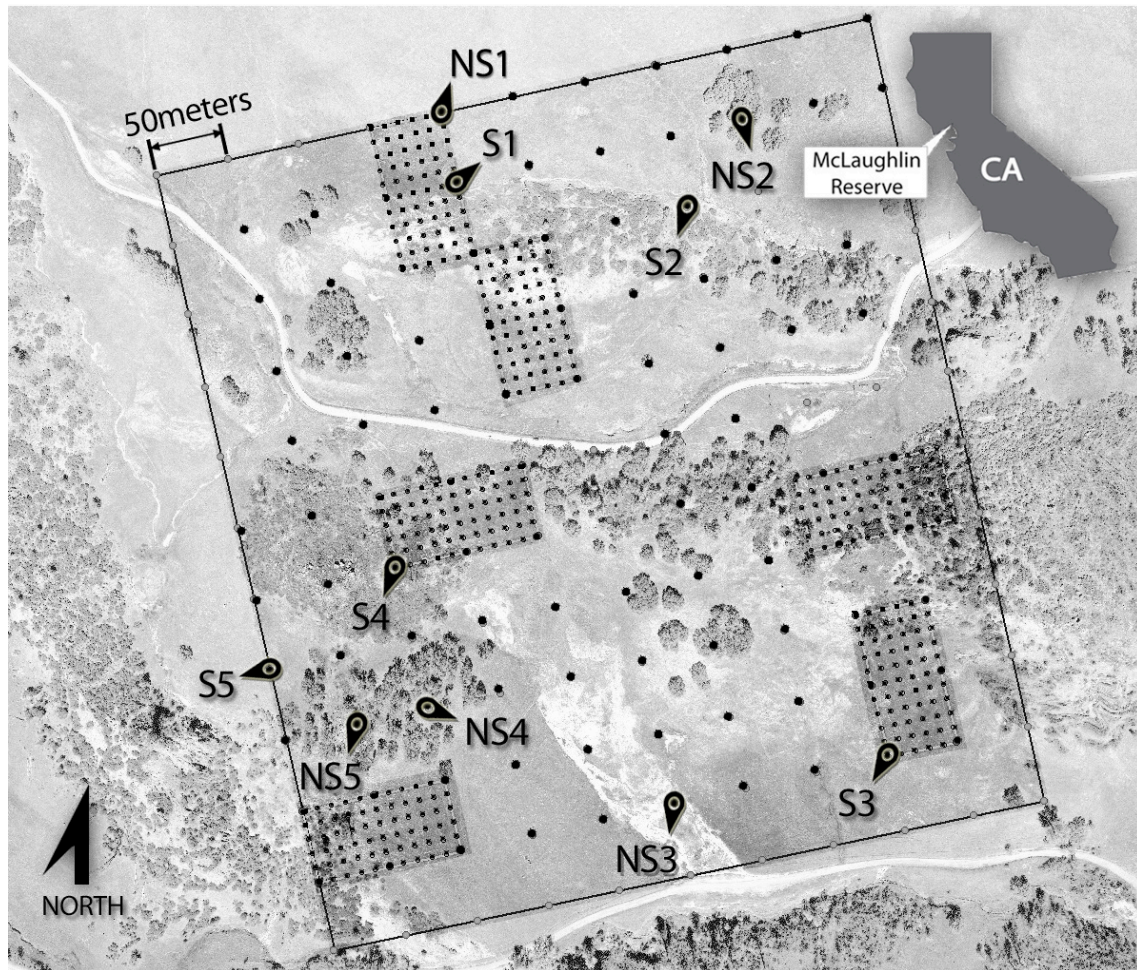
OTU	Grid Points									
	S1	S2	S3	S4	S5	NS1	NS2	NS3	NS4	NS5
GLO 1	0	0	0	0	0	89	84	0	82	68
GLO 1A	0	0	0	30	65	0	0	0	0	0
GLO F	0	0	0	0	0	0	0	54	0	0
GLO 2	5	0	19	0	22	0	0	0	0	2
GLO 4	29	7	7	10	1	0	8	2	1	14
GLO 5	64	43	70	58	3	1	5	6	17	15
GLO 6	2	44	3	0	10	8	2	0	0	1
GLO 9	0	0	1	2	0	1	1	0	0	0
GLO G	0	0	0	0	0	0	0	5	0	0
GLO C	0	2	0	0	0	1	0	0	0	0
GLO D	0	0	0	0	0	0	0	32	0	0
ARCH 1	0	2	0	0	0	0	0	0	0	0

**Table 2.** Relative abundance matrix of operational taxonomic units (OTU) of plant assemblages associated with plants roots collected from five serpentine (S) and five non-serpentine grid points. Highlighted OTUs show soil type affects

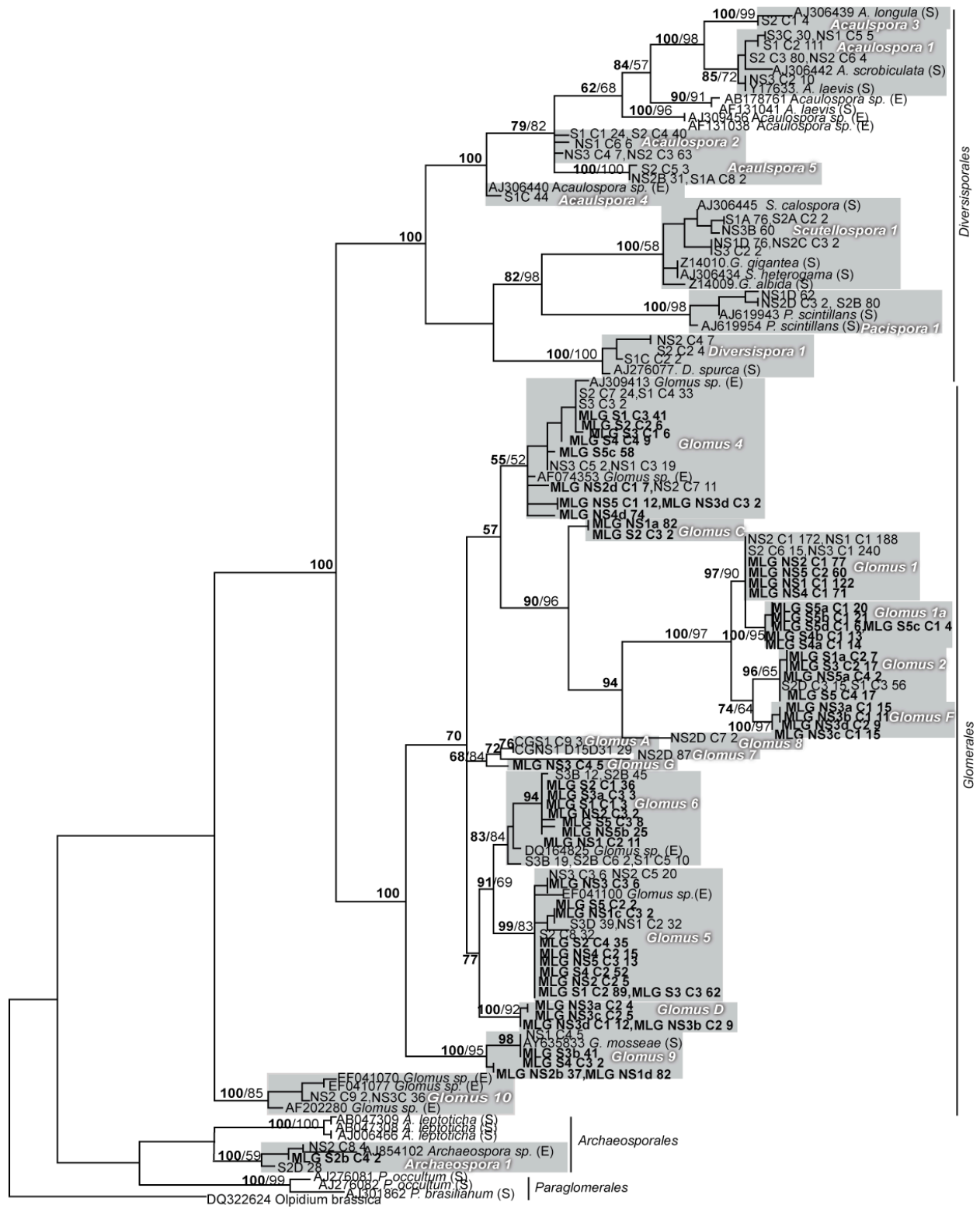
OTU	Grid Points									
	S1	S2	S3	S4	S5	NS1	NS2	NS3	NS4	NS5
<i>Hordeum 1</i>	0	0	0	0	0	0	2	0	0	0
<i>Triticum 1</i>	0	0	0	5	0	7	0	0	0	0
<i>Poa 1</i>	52	0	0	0	0	0	0	0	0	0
<i>Nassella 1</i>	0	15	0	0	0	0	0	0	0	0
<i>Avena 1</i>	0	2	57	37	0	88	38	18	75	27
<i>Avena 2</i>	0	0	0	0	0	0	2	0	0	2
<i>Avena 5</i>	0	0	0	0	0	0	2	0	0	0
<i>Avena 6</i>	0	0	0	0	0	0	0	0	2	0
<i>Bromus 1</i>	11	0	0	0	2	0	49	5	11	2
<i>Bromus 2</i>	0	0	0	0	0	0	0	1	0	0
<i>Lolium 1</i>	0	0	41	0	0	2	0	0	2	0
<i>Vulpia 1</i>	11	2	0	22	0	0	0	18	4	0
<i>Poaceae 1</i>	0	32	2	3	5	3	7	0	4	0
<i>Microseris 1</i>	0	22	0	0	9	0	0	0	0	0
<i>Centaurea 1</i>	0	0	0	0	0	0	0	23	0	0
<i>Aster 1</i>	0	2	0	0	0	0	0	0	0	0
<i>Aster 2</i>	0	25	0	27	66	0	0	0	0	0
<i>Adenostoma 1</i>	0	0	0	0	0	0	0	0	0	9
<i>Navarretia 1</i>	0	0	0	0	13	0	0	0	0	0
<i>Erodium 1</i>	0	0	0	0	0	0	0	15	0	0
<i>Asterales 1</i>	15	0	0	0	0	0	0	0	0	0
<i>Epilobium 1</i>	10	0	0	0	0	0	0	0	0	0
<i>Clarkia 1</i>	0	0	0	5	0	0	0	0	0	0
<i>Hesperolinon 1</i>	0	0	0	0	5	0	0	0	0	0
<i>Monardella 1</i>	0	0	0	0	0	0	0	0	4	0
<i>Mimulus 1</i>	0	0	0	0	0	0	0	0	0	38
<i>Caryophyllaceae 1</i>	0	0	0	0	0	0	0	4	0	0
<i>Apiaceae 1</i>	0	0	0	0	0	0	0	0	2	0
<i>Apiaceae 2</i>	0	0	0	0	0	0	0	0	0	6
<i>Solanum 1</i>	0	0	0	0	0	0	0	0	0	17
<i>Juglans 1</i>	0	0	0	0	0	0	0	18	0	0

**Table 3.** Soil chemical variables (S = serpentine, NS = non-serpentine) associated with McLaughlin Reserve grid points. Nitrogen (as NO<sub>3</sub>) 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 Ca:Mg ratio; serpentine soils have a ratio much less than one and non-serpentine soils have ratios greater than one. \* Indicates significant differences between soil types at P < 0.05.

	Grid Points											
	S1	S2	S3	S4	S5	S avg	NS1	NS2	NS3	NS4	NS5	NS avg
N	6	10	6	6	5	<b>7</b>	3	9	8	9	3	<b>6</b>
P	13	11	11	12	11	<b>12</b>	15	20	16	49	16	<b>23</b>
K	276	223	233	290	373	<b>279</b>	568	348	135	448	299	<b>360</b>
Mg	2284	2678	2613	2229	2679	<b>2497*</b>	1405	876	584	394	350	<b>722*</b>
Ca	731	843	694	1256	887	<b>882*</b>	5611	2640	3645	3951	2971	<b>3764*</b>
Ca:Mg	0.32	0.31	0.27	0.56	0.33	<b>0.36*</b>	3.99	3.01	6.24	10.03	8.49	<b>6.35*</b>
pH	7	7	7.4	6.8	6.8	<b>7</b>	7	6.5	8	7	6.6	<b>7</b>
Zn	1.2	0.7	0.6	1.1	1.2	<b>1.0</b>	2	5	0.6	3.8	1.6	<b>2.6</b>
Mn	13	16	20	45	43	<b>27</b>	16	18	14	47	70	<b>33</b>
Fe	27	26	18	28	24	<b>25</b>	19	38	12	26	21	<b>23</b>
B	0.70	0.70	0.60	0.50	0.50	<b>0.60</b>	0.70	5.80	1.60	1.60	0.70	<b>2.08</b>



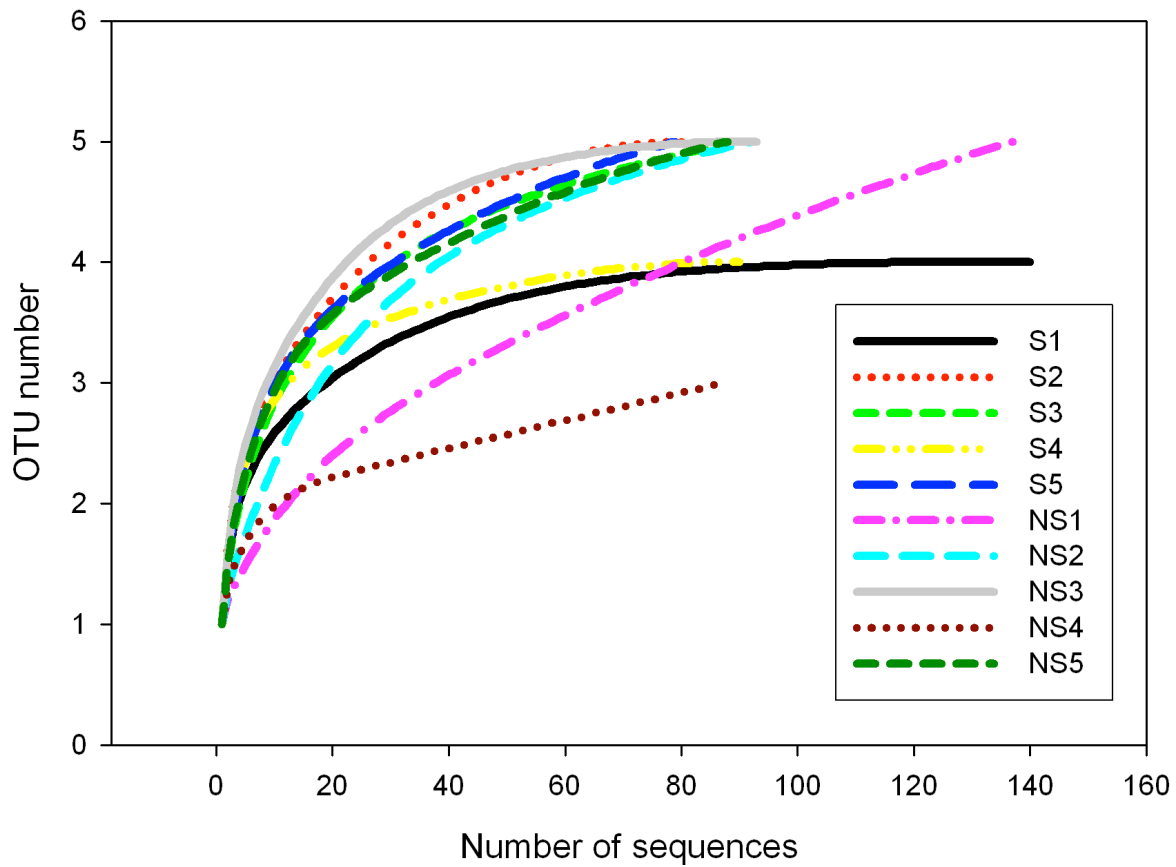
**Figure 1.** Study area at Donald and Sylvia McLaughlin Reserve, part of the University of California Davis natural reserve system in Northern California. Dots represent the research grid, labels indicate the location of serpentine (S1, S2, S3, S4, and S5) and non-serpentine (NS1, NS2, NS3, NS4, NS5) grid points sampled in this study.



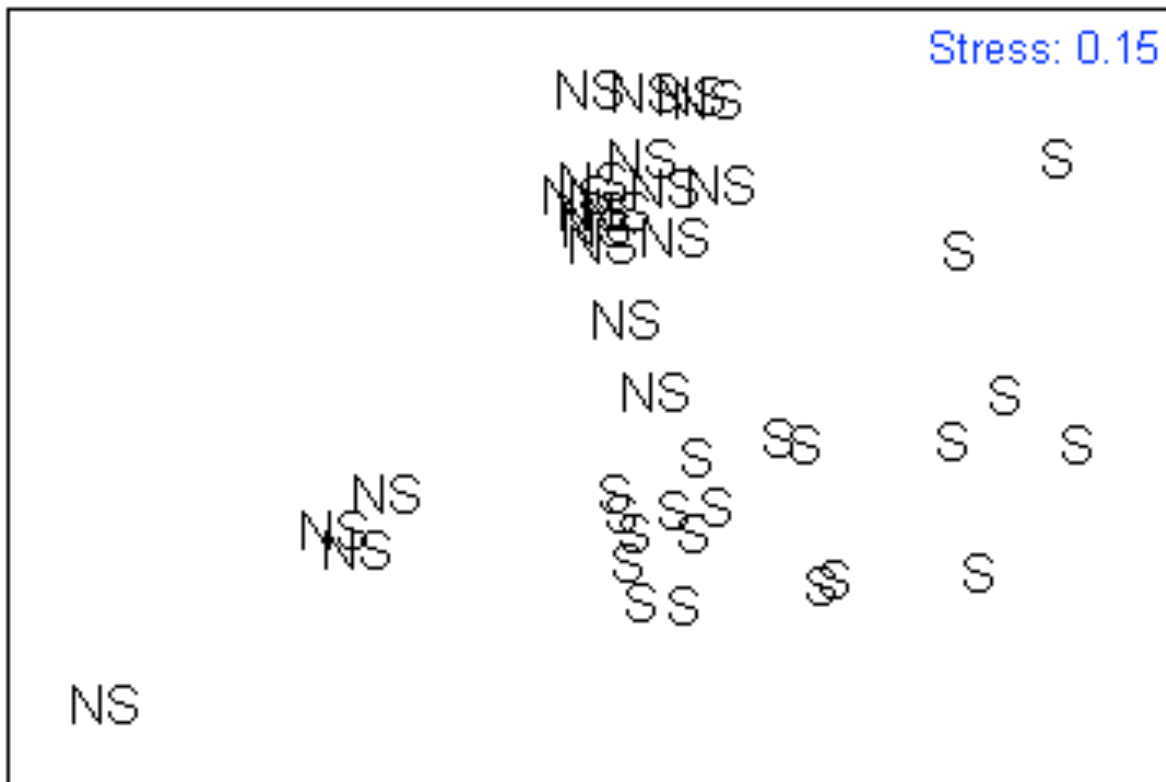
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**Figure 2.** Consensus tree (50% majority rule) from Mr. Bayes analysis showing the phylogenetic relationship of the AMF sequences obtained from roots sampled from McLaughlin research grid from five serpentine (MLG\_S1, MLG\_S2, MLG\_S3, MLG\_S4, MLG\_S5) and five non-serpentine (MLG\_NS1, MLG\_NS2, MLG\_NS3, MLG\_NS4, MLG\_NS5) grid points, in bold. Additional sequences from roots sampled from three serpentine (S1, S2, S3) and three nonserpentine (NS1, NS2, NS3) ecotype populations of *Collinsia sparsiflora* field experiment were included (Schechter and Bruns, 2008). Letters directly behind site designation refers to an individual sample; no letter means that the sequence is a representative from a 98% consensus of sequences found in multiple samples within that site. 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 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 out-group. Topology was similar between Bayesian and Garli analyses and did not affect OTU delimitation.

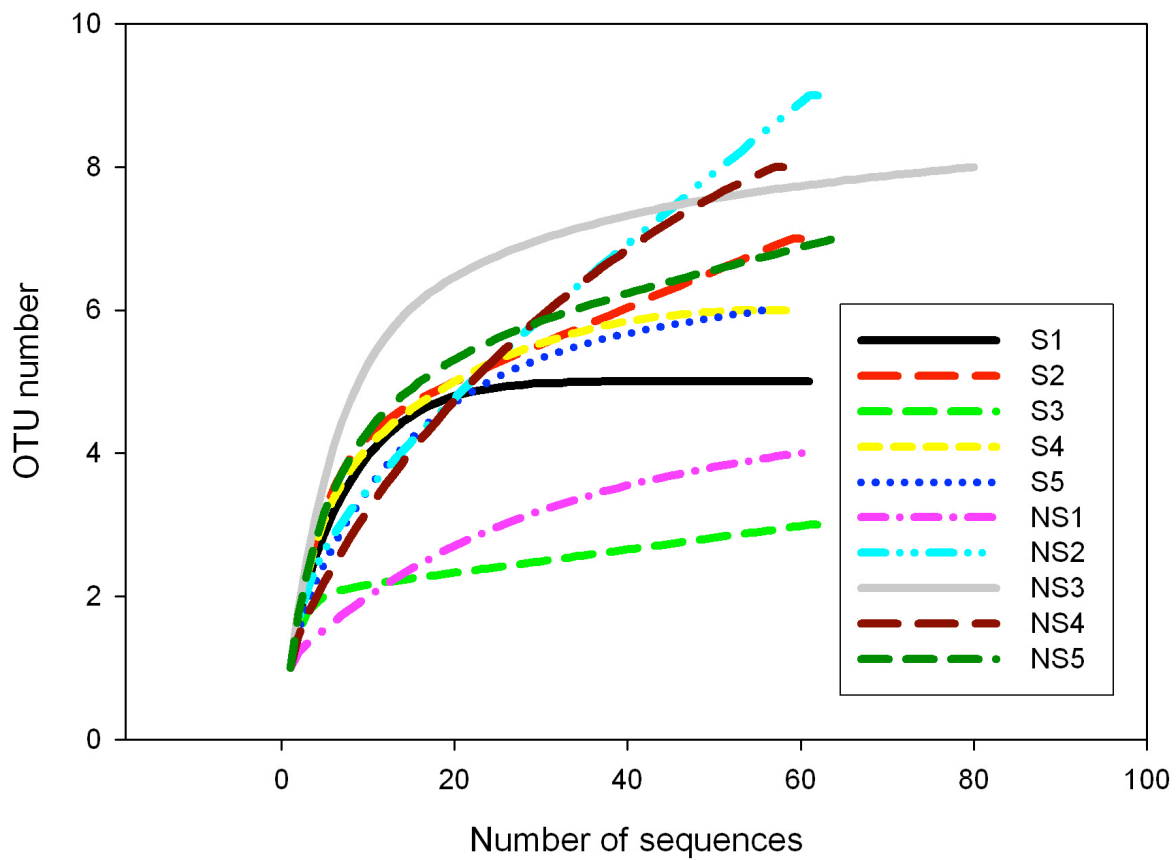




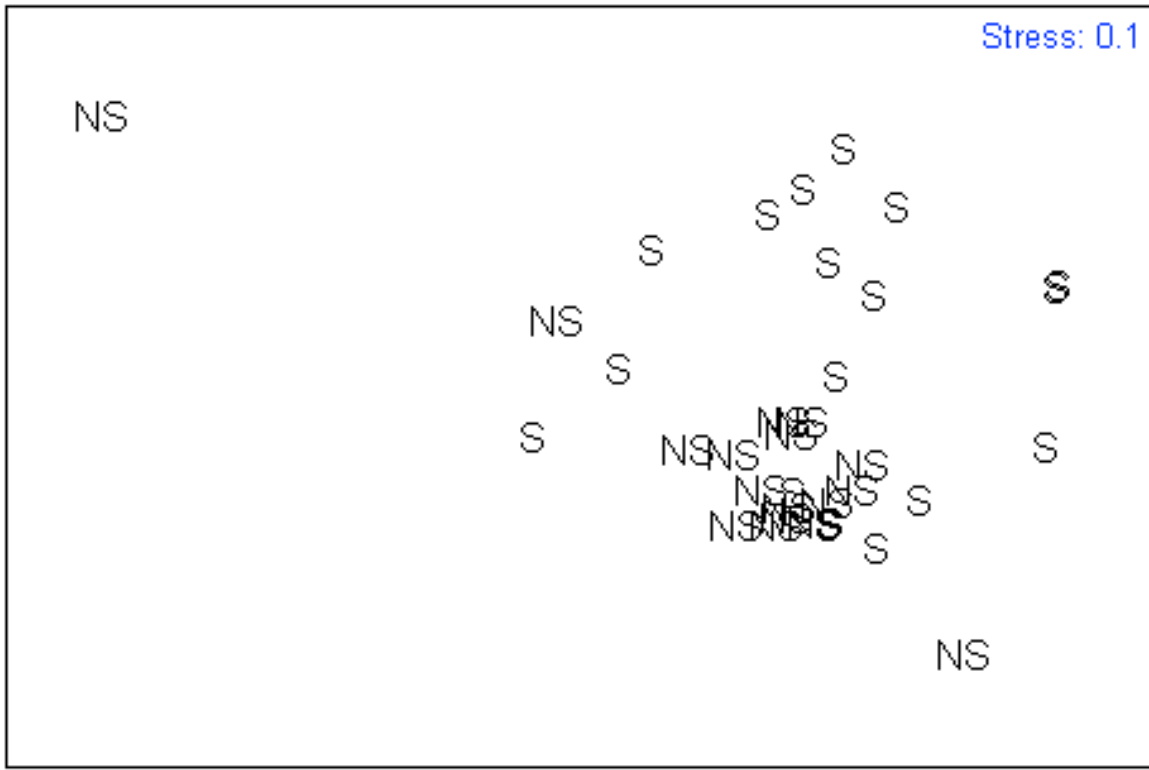
**Figure 3.** Rarefaction curve of the total number of AMF sequences sampled from plant roots from serpentine (S) and non-serpentine (NS) grid points. Rarefaction curves were produced by the EstimateS version 8.0 Mao Tau estimator (Colwell et al. 2004).



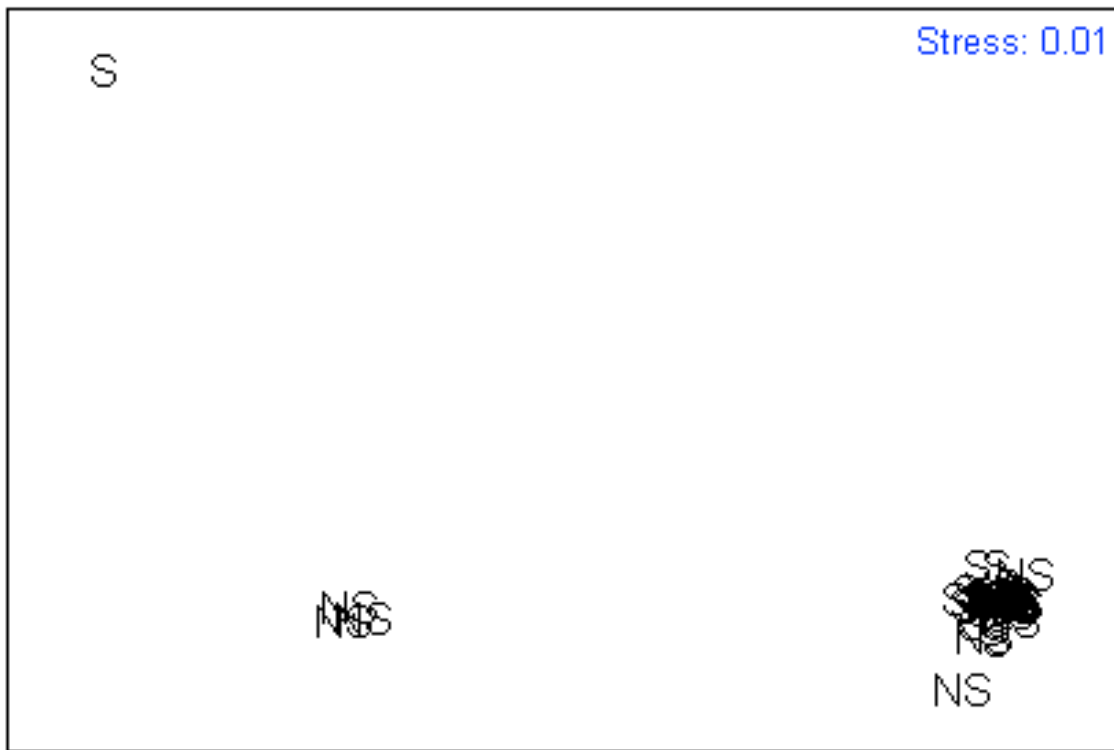
**Figure 4.** Non-metric multi-dimensional scaling (MDS) ordination of AMF assemblages associated with plant roots found in serpentine (S) and non-serpentine (NS) grid points. The MDS ordination is a configuration of the samples in which relative positions are assigned based on the Bray–Curtis similarity 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.



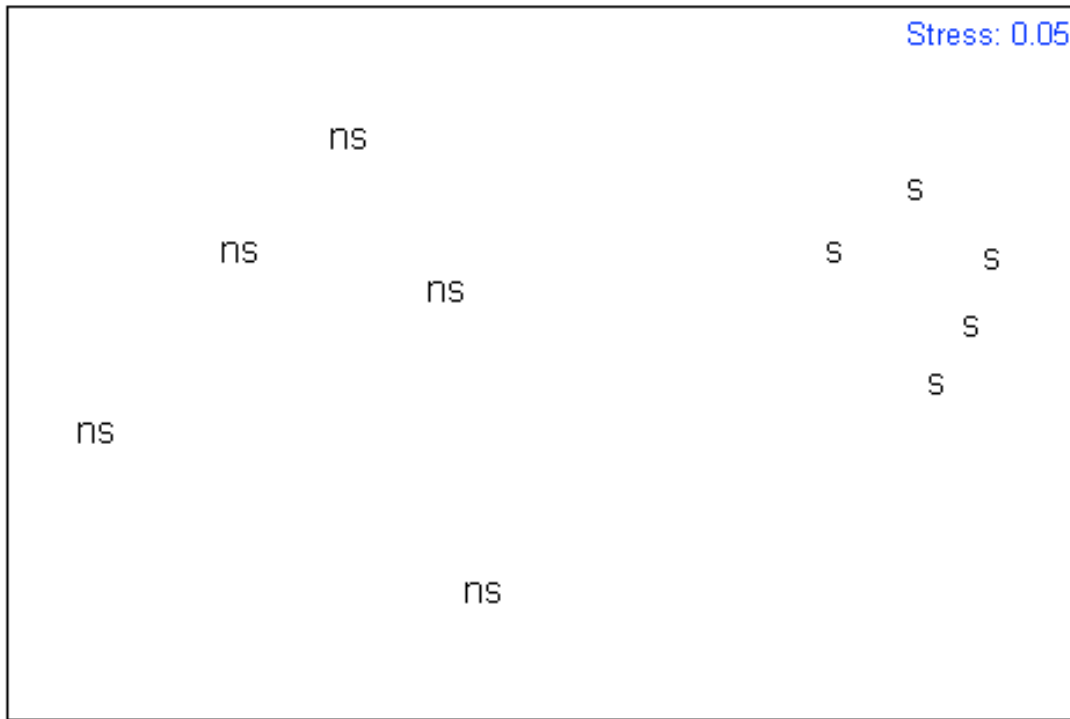
**Figure 5.** Rarefaction curve of the total number of plant sequences from plant roots sampled from serpentine (S) and non-serpentine (NS) grid points. Rarefaction curves were produced by the EstimateS version 8.0 Mao Tau estimator (Colwell et al. 2004).



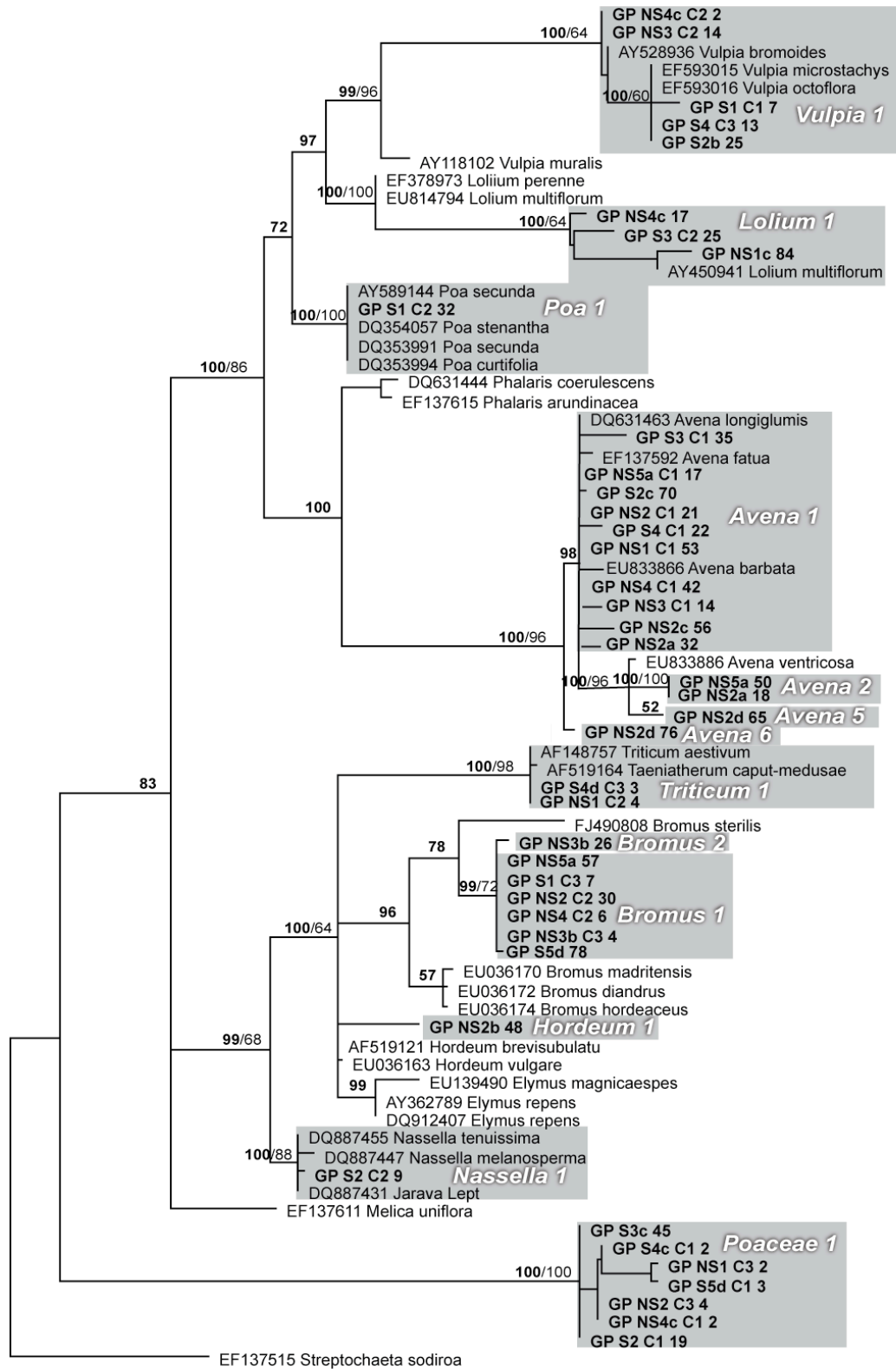
**Figure 6.** Non-metric multi-dimensional scaling (MDS) ordination of plant assemblages associated with plant roots sampled from serpentine (S) and non-serpentine (NS) grid points. This excludes samples S1a, S1d, NS5b, NS5c, and NS5d. The MDS ordination is a configuration of the samples in which relative 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.



**Supplemental Figure S1.** Non-metric multi-dimensional scaling (MDS) ordination of plant assemblages associated with plant roots sampled from serpentine (S) and non-serpentine (NS) grid points. 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.



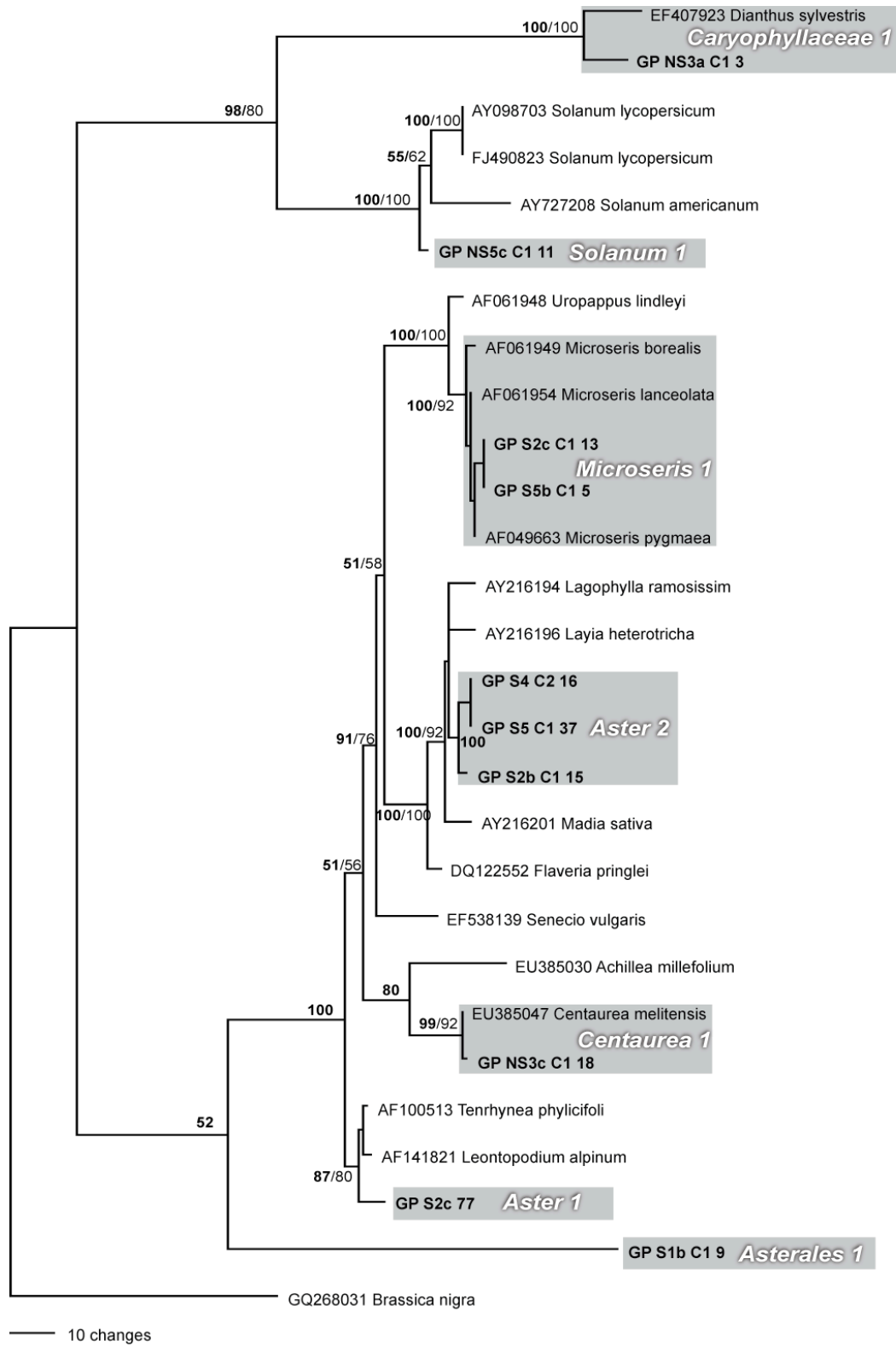
**Figure S2.** Non-metric multi-dimensional scaling (MDS) ordination of soil nutrients (N, P, K, Mg, Ca, Mg:Ca, Zn, Mn, B, Fe, and pH) associated with soil collected from serpentine (S) and non-serpentine (NS) grid points.



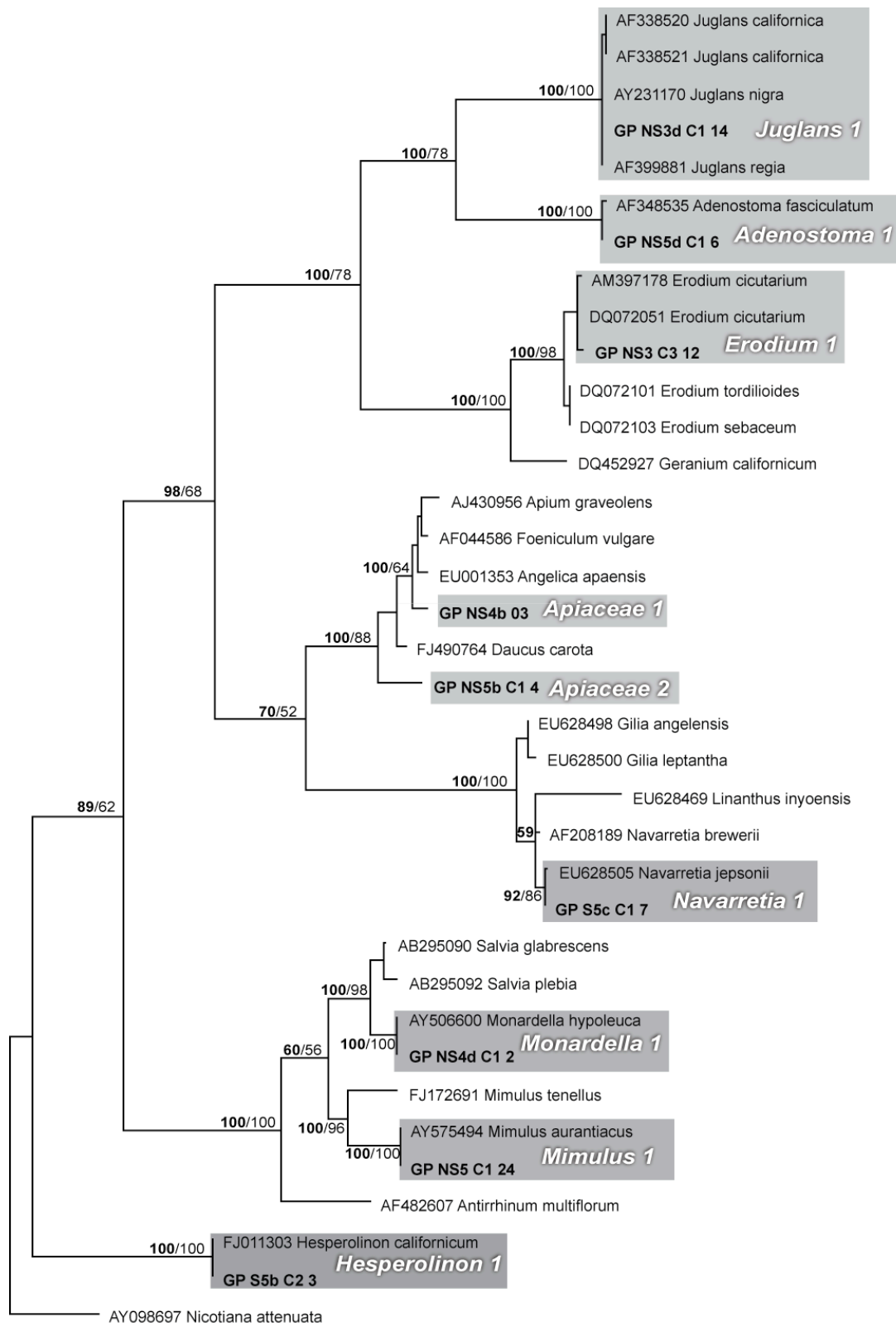
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**Figure S3.** Consensus tree (50% majority rule) from Mr. Bayes analysis showing the phylogenetic relationship of the Poaceae plant sequences obtained from roots sampled from McLaughlin research grid from five serpentine (GP\_S1, GP\_S2, GP\_S3, GP\_S4, GP\_S5) and five non-serpentine (GP\_NS1, GP\_NS2, GP\_NS3, GP\_NS4, GP\_NS5) grid points, in bold. Letters directly behind site designation refers to an individual sample; no letter means that the sequence is a representative from a 98% consensus of sequences found in multiple samples within that site. 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. 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. *Streptochaeta sodiroa* was used as an out-group. Topology was similar between Bayesian and Garli analyses.



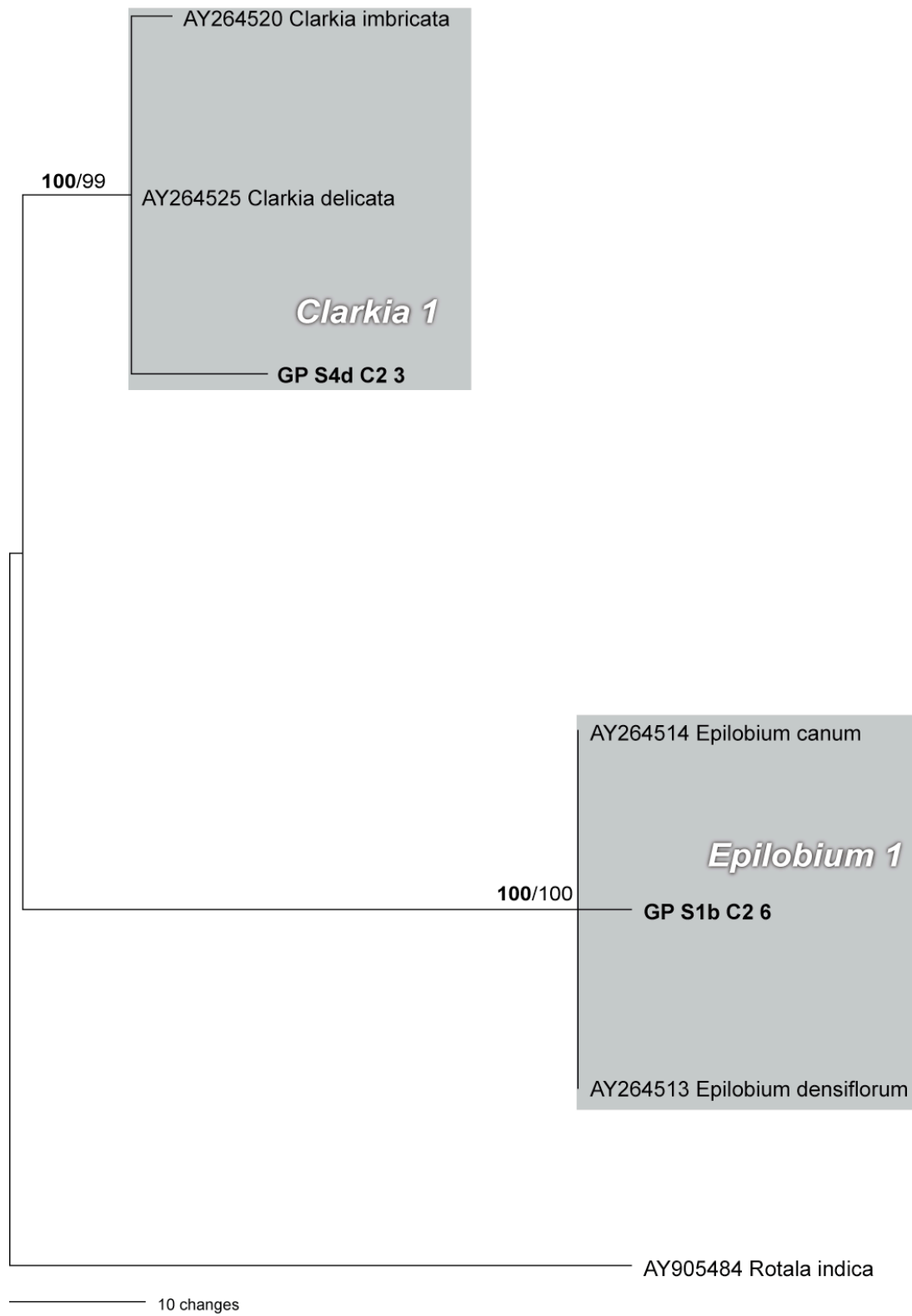


**Figure S4.** Consensus tree (50% majority rule) from Mr. Bayes analysis showing the phylogenetic relationship of the Asterids plant sequences obtained from roots sampled from McLaughlin research grid from five serpentine (GP\_S1, GP\_S2, GP\_S3, GP\_S4, GP\_S5) and five non-serpentine (GP\_NS1, GP\_NS2, GP\_NS3, GP\_NS4, GP\_NS5) grid points, in bold. Letters directly behind site designation refers to an individual sample; no letter means that the sequence is a representative from a 98% consensus of sequences found in multiple samples within that site. 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. 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. *Brassica nigra* was used as an out-group. Topology was similar between Bayesian and Garli analyses.



— 10 changes

**Figure S4.** Consensus tree (50% majority rule) from Mr. Bayes analysis showing the phylogenetic relationship of the Rosids/Asterids plant sequences obtained from roots sampled from McLaughlin research grid from five serpentine (GP\_S1, GP\_S2, GP\_S3, GP\_S4, GP\_S5) and five non-serpentine (GP\_NS1, GP\_NS2, GP\_NS3, GP\_NS4, GP\_NS5) grid points, in bold. Letters directly behind site designation refers to an individual sample; no letter means that the sequence is a representative from a 98% consensus of sequences found in multiple samples within that site. 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. 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. *Nicotiana attenuata* was used as an out-group. Topology was similar between Bayesian and Garli analyses.



**Figure S5.** Consensus tree (50% majority rule) from Mr. Bayes analysis showing the phylogenetic relationship of the Onagraceae plant sequences obtained from roots sampled from McLaughlin research grid from five serpentine (GP\_S1, GP\_S2, GP\_S3, GP\_S4, GP\_S5) and five non-serpentine (GP\_NS1, GP\_NS2, GP\_NS3, GP\_NS4, GP\_NS5) grid points, in bold. Letters directly behind site designation refers to an individual sample; no letter means that the sequence is a representative from a 98% consensus of sequences found in multiple samples within that site. 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. 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. *Rotala indica* was used as an out-group. Topology was similar between Bayesian and Garli analyses.

## CHAPTER 4

Differential effects of arbuscular mycorrhizal fungal source on growth of *Collinsia sparsiflora* serpentine and non-serpentine adapted ecotypes in serpentine soil

## Abstract

Plants adapted to serpentine soil are expected to show physiological tolerance to serpentine. However, plant responses to the complex edaphic stresses of serpentine are not solely dictated by plant physiological or morphological traits. Symbiosis with arbuscular mycorrhizal fungi (AMF) may be an important mechanism for plant serpentine tolerance. Previous studies have shown that serpentine and non-serpentine AMF assemblages are distinct, implying that it is necessary for adapted plants to associate with serpentine tolerant AMF taxa in serpentine soil. But this ecological relationship does not address functional differences between assemblages that directly impact plant growth and fitness on serpentine. The goal of this experiment is to determine the functional role of AMF in serpentine adaptation with regards to both fungal and plant symbiotic traits. I conducted a fully factorial greenhouse experiment to compare the effects of serpentine and non-serpentine AMF cultures as well as a non-mycorrhizal control on the relative fitness, growth response, and nutrient uptake of serpentine and non-serpentine adapted ecotypes of *Collinsia sparsiflora* grown in sterilized serpentine soil. Only shoot dry weight showed a significant response to AMF source. Serpentine AMF significantly increased growth of hosts over non-serpentine AMF and AMF-free controls. This indicates that serpentine AMF have a specialized adaptation to serpentine conferring growth enhancement to hosts, but it is still unclear what this adaptation is or which function is contributing to growth enhancement. There were also trends that imply that *C. sparsiflora* serpentine adapted ecotypes have a greater response to AMF than non-serpentine ecotypes, but these trends were not significant.



## Introduction

It has been long recognized that plants adapt to different soil types; yet, little is known about the mechanisms of plant edaphic adaptation (Brady *et al.*, 2005). Perhaps one of the best studied and understood examples of plant edaphic adaptation is the phylogenetically and geographically widespread occurrence of plant adaptation to serpentine soils. Soils derived from serpentine, an ultramafic rock, are characterized by a low levels of essential nutrients, drought susceptibility, very low calcium:magnesium ratio, and high levels of heavy metals (Brady *et al.*, 2005; Kruckeberg, 1984; Rajakaruna & Bohm, 1999). It is believed that there are three main physiological mechanisms that could be responsible for plant tolerance to serpentine soils: tolerance of the low calcium:magnesium ratio, magnesium toxicity evasion, and/or increased magnesium requirement (Brady *et al.*, 2005). However, since plant responses to the complex edaphic stresses of serpentine are not solely dictated by plant physiological or morphological traits, the actual tolerance mechanisms and the specific genetic components of serpentine adaptation are poorly understood.

Symbiosis with arbuscular mycorrhizal fungi (AMF) has been proposed as one of mechanisms of plant serpentine tolerance (Schechter & Bruns, 2008). Approximately 85% of all plants interact with the soil environment through symbiosis with AMF (Wang & Qiu, 2006). These specialized fungi (Glomeromycota) are ubiquitous root symbionts that have co-evolved with plants for over 400 million years as an extension of the plants root system (Redecker, 2006; Schussler *et al.*, 2001). AMF have been shown to increase their plant hosts' establishment and growth in stressful environments by enhancing nutrient and water uptake and providing protection against toxic conditions (Allen *et al.*, 1981; Pairunan *et al.*, 1980; Smith & Read, 1997). Moreover, the more nutrient-stressed the environment, the more dependent plants are on their AMF associates for nutrient acquisition and growth (Habte & Manjunath, 1987; Johnson & Wedin, 1997; Yost & Fox, 1979). From these facts it is clear that symbiosis with AMF may be a key evolutionary strategy for plants to overcome serpentine edaphic stress.

The possibility that AMF play an important role in plant adaptation to serpentine has been explored from an ecological perspective. Analysis of AMF assemblages associated with field populations of serpentine and non-serpentine adapted ecotypes of *Collinisa sparsiflora* clearly showed that adapted plant ecotypes associate with distinct AMF assemblages (Schechter and Bruns 2008). This distinction was driven by two dominant AMF taxa – *Acaulospora* 1 with serpentine ecotypes and *Glomus* 1 with non-serpentine ecotypes, indicating differences in tolerance to serpentine between these two taxa. The effect of serpentine edaphic factors on shaping AMF assemblages was further illustrated when looking at AMF assemblages associated with non-*C. sparsiflora* root communities on adjacent serpentine and non-serpentine soils (Chapter 3). *Glomus* 1 was once again the dominant AMF taxa in non-serpentine plant roots, but this study had a new dominant AMF taxon in serpentine plant roots – *Glomus* 5 (Chapter 3).

These studies showed that serpentine and non-serpentine AMF assemblages are distinct regardless of plant roots sampled, but plant identity may determine AMF taxa dominants within the edaphically shaped AMF assemblages. This implies that it is necessary for adapted plants to associate with serpentine tolerant AMF taxa in serpentine soil. However this ecological relationship does not address functional differences between assemblages that directly impact plant growth and fitness on that substrate or differences in plant symbiotic traits between serpentine and non-serpentine adapted plants.

Optimal performance of the symbiosis under edaphic stress may require specific plant and fungal traits (Gonzalez-Chavez *et al.*, 2002; Meharg & Cairney, 1999; Schultz *et al.*, 2001). AMF may mediate plant tolerance to environmental stress in two ways. AMF can be *tolerant* of edaphic stress, meaning species whose spores can germinate and colonize under stressful conditions, function “normally” in stressful sites by improving plant uptake of essential nutrients and water and, in so doing, stress tolerant AMF perform essential but “normal” functions for mycorrhizal plants growing in these stressful environments (Gonzalez-Chavez *et al.*, 2002; Meharg, 2003a; Meharg & Cairney, 1999). Alternatively, a few AMF present in these stressful sites may have *specialized functions* that confer enhanced resistance to the host by modifying uptake and transport of specific nutrients or restricting transport of toxins to the plant host (Gonzalez-Chavez *et al.*, 2002; Meharg, 2003a; Meharg & Cairney, 1999). Thus adaptive AMF traits are important for mycorrhizal plant growth under edaphic stress.

Plant traits involved in the establishment of and response to AMF may also be important for tolerance of stressful conditions. For example, plants have been shown to mediate adaptation to low nutrient soils by altering their relationship to AMF (Schultz *et al.*, 2001). Plant genotypes can differ in their “dependency” on (i.e. response to) AMF for normal growth and functioning (Smith & Read, 1997). Studying low and high P soil ecotypes of *Andropogon gerardii* (Poaceae), Schultz *et al.* (2001) found that the ecotypes differed in their dependence on AMF for P uptake and growth and hypothesized that low P ecotypes had adapted to low P soils by increasing their dependency on AMF. In fact, Kaeppler and colleagues (2000) found quantitative trait loci (QTL) for responsiveness to AMF in maize that were also correlated to plant growth in low P. Indeed, in cultivated systems, selection of crop genotypes under high fertility conditions commonly results in decreased susceptibility and response to AMF, most likely due to decreased dependency on AMF (Hetrick *et al.*, 1992; Hetrick *et al.*, 1993; Hetrick *et al.*, 1996). Therefore, plant edaphic ecotypes may differ in their requirement for and response to AMF.

Thus, synergistic combinations of plant and AMF symbiotic traits (high requirement for and large response to AMF along with association with stress adapted and specialized AMF) may play a significant role in plant adaptation to serpentine. In order to determine if plant-AMF symbiotic traits play a role in edaphic adaptation, it is imperative to relate assemblage structure with function. The goal of this experiment is to determine the functional role AMF play in serpentine adaptation with regards to both fungal and plant symbiotic traits. To do this, I conducted a manipulative fully factorial greenhouse experiment to compare the effects of serpentine and non-serpentine AMF cultures of field isolates as well as a non-mycorrhizal control on the relative fitness, growth response, and nutrient uptake of serpentine and non-serpentine *C. sparsiflora* grown in autoclaved serpentine soil. I hypothesized that the serpentine plant ecotypes would have lower fitness and growth without serpentine AMF.

## **Materials and Methods**

### *Study System*

Seeds and soil for this study were collected at the Donald and Sylvia McLaughlin University of California Natural Reserve situated in Napa, Lake, and Yolo counties in northern California. I collected from the same serpentine and non-serpentine *Collinsia sparsiflora* ecotypes populations as described in Schechter and Bruns (2008).

### *Soil Collection*

In March 2009, I collected field rhizosphere soil and roots of *C. sparsiflora* from four populations: two serpentine (S1 and S2) and two non-serpentine (NS1 and NS3) (Schechter and Bruns, 2008). These four field collections served as the sources of AMF inoculum for the production of serpentine and non-serpentine AMF cultures. I chose these four *C. sparsiflora* ecotype populations because they were the best representatives of serpentine and non-serpentine ecotype populations and AMF (Schechter and Bruns, 2008) and were used for the common garden experiment (Chapter 2). I collected the *C. sparsiflora* rhizosphere soil and roots in the field adjacent to the same patches sampled by Schechter and Bruns (2008). All root and soil samples were put directly into coolers and stored in a 4°C cold room within 8 hours of collection.

All soil collections were processed in a fume hood. I first removed all above-ground plant material, crushed soil by hands to release roots, cut the roots into 1 cm segments, and finally, passed the rhizosphere soil and cut roots through a #4 sieve. I then dried each collection for 24 hours in the fume hood at room temperature. The dried collections were stored at 4°C until used for serpentine and non-serpentine AMF cultures.

In May 2009, I collected serpentine soil from the field rhizosphere and roots of *C. sparsiflora* of the S1 population to serve as the sterile serpentine soil used as the basis of the greenhouse experiment. The soil from this population was chosen as the model serpentine soil due to its low Ca:Mg ratio. The soil was processed as described above.

### *AMF Culture Production*

Serpentine and non-serpentine AMF cultures were produced by mixing soil from individual collections 1:1 (volume:volume) with Turface (<http://turface.com>) and seeded with sudan grass, marigold, or corn. I harvested the cultures after four months and then sampled the roots to evaluate AMF colonization (Peters & Habte, 2001). The cultures with the highest AMF colonization were combined per soil type (serpentine and non-serpentine), and then used in a mean infection percentage (MIP) assay (<http://invam.caf.wvu.edu/methods/assays/MIP.html>). This assay measures the percentage mycorrhizal colonization of a common host plant grown in a series of inoculum (AMF cultures) dilutions, which allowed me to select concentration of each AMF culture that resulted in similar colonization levels. I did this so that I could equalize the inoculum potential of the AMF cultures added to each pot and therefore eliminate culture differences as a source of error.

### *Experimental Design*

In order to test for a functional role of AMF in plant adaptation to serpentine soil, I conducted a greenhouse experiment to compare presence and source of AMF inoculum on plant growth and relative fitness between serpentine and non-serpentine *C. sparsiflora* ecotypes when grown in sterilized serpentine soil. This experiment consisted of 3 AMF inoculum treatments (serpentine, non-serpentine, and no AMF control) X 2 *C. sparsiflora* serpentine ecotypes populations (S1 and S2) X 2 *C. sparsiflora* non-serpentine populations (NS1 and NS3) as a fully crossed factorial experiment with 10 replicates per treatment combination for a total of 120 plants. All plants and AMF inoculum treatments were interacting in a sterilized serpentine soil.

### *Planting*

I prepared the growth medium by first sterilizing the serpentine soil through autoclaving three times (250°F for 1 hour) with one day rest in between treatments after first saturating the soil with deionized water prior to autoclave treatment. Then, in order to improve drainage, I then mixed the sterilized serpentine soil 4:1 (soil volume:sand volume) with sterilized sand. Prior to planting, I sent both field serpentine and sterilized serpentine soil:sand mixture samples to A & L Western Laboratories (Modesto, CA) in order to compare soil nutrient status before and after sterilization. The sterilized serpentine soil and sand mixture was then inoculated with the appropriate AMF culture (serpentine or non-serpentine) or control (Turface alone) as well as with a fine filtered leachate of the field serpentine soil to reestablish the resident bacterial community in the sterilized serpentine soil as described by Gustafson and Casper (2006). I then transplanted *C. sparsiflora* seeds from the four field populations (S1, S2, NS1, and NS3) that had been pregerminated in 1% water agar into individual “stubby cell” cone-tainers (Stuewe and Sons) filled with the appropriate growth medium-inoculum treatment mixture. Seedlings of the same inoculum treatment were put into separate trays and then arranged in a completely randomized design (CRD) and grown in a greenhouse (UC Berkeley, Berkeley, CA) from June until flowering in August 2009. Trays were rotated every day on the greenhouse bench. The seedlings were sub-irrigated by placing each of the cone-tainer trays in a separate tub of water as needed. This was done to eliminate cross-contamination between inoculum treatments. No fertilizer was used in the experiment.

### *Harvest*

All seedlings were harvested after flowering. I measured plant height, relative fitness (number of flowers + number of fruits), shoot and root dry weight, AMF colonization, and identified root associated AMF taxa via molecular methods (see below). I dried the shoots in a 37°C oven for three days before being weighed. Shoots were then grouped by inoculum treatment (due to extremely small dry weights of individual treatments) and sent to A & L Western Agricultural Laboratories for plant tissue nutrient analysis. Roots of individual seedlings were thoroughly washed to remove as much soil as possible. I took a small portion (1 mg wet weight) 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 three days. The dried roots were weighed and then placed into a 2 ml cryotube and stored in a -80°C freezer until DNA extraction.

### *Molecular Analysis*

DNA extraction: I extracted DNA from each *C. sparsiflora* root sample. I crushed the dried and frozen roots by beadbeating (Mini-Beadbeater, Biospec Products) with sterile glass beads for 30 seconds or until a fine powder formed. The samples were then immediately placed on ice, and 1.5 ml of 2x 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. I extracted DNA from each root sample as described in Schechter and Bruns (2008).

Polymerase chain reaction (PCR): I amplified a variable region of the 18S rDNA using *Pfu Turbo* DNA polymerase (Stratagene) and universal eukaryotic primer NS31 (Simon *et al.*, 1992) paired with AM1 (Helgason *et al.*, 1998) as described in Schechter and Bruns (2008). Each 20 µl PCR reaction consisted of 12.4 µl of dH<sub>2</sub>O, 0.2 µl of 2.5 U *Pfu Turbo* DNA polymerase, 2 µl of manufacture’s buffer (Stratagene), 2 µl of 10x dNTPs, and 0.2 µl of each 50µM primer. PCR conditions were the same as described by Helgason (2002) with the

exception that the annealing temperature was increased to 64° C (optimized through gradient PCR analysis) in order to decrease amplification of non-AMF sequences.

Cloning and Sequencing: I pooled the PCR products from each treatment combination together for cloning to equal 12 cloning reactions. I first gel purified and concentrated the pooled PCR products before cloning as described by Schechter and Bruns (2008). I then cloned the pooled PCR products into pPCR-Script Amp SK(+) and transformed into *Escherichia coli* XL10-Gold Kan Ultracompetent cells (Stratagene). I picked 48 putative positive transformants per cloning reaction. I screened transformants for correctly sized inserts using plasmid primers T3/T7 under the same PCR conditions as described by Schechter and Bruns (2008). Then, I selected 16 gel confirmed positive transformants per cloning reaction for cleaning and sequencing. I cleaned these PCR products with ExoSAP-IT using the manufacturer's instructions (USB), and sent the clean PCR products to the UC Berkeley Sequencing Facility (Berkeley, CA) for sequencing. I 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: Prior to analysis, I checked for possible chimeric sequences by comparing my sequences to GeneBank sequences using BLAST (version 2.21, Altschul *et al.*, 1997), and checking my sequences in the Chimera Check program in RDPII (version 2.7, Cole *et al.*, 2003) as described by Schechter and Bruns (2008). 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 (OTU) determination: I determined AMF OTUs in this experiment using the same combination of sequence similarity and phylogenetic analysis methods described by Schechter and Bruns (2008). I first combined sequences obtained from each cloning reaction at 98% similarity using Sequencher 4.2.2 to create contigs and singletons associated with each treatment combination. This process resulted in one OTU sequence database for each treatment combination. Then I compared all 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.

I aligned these sequences along with those sequences used in Schechter and Bruns (2008) using ClustalX (Thompson *et al.*, 1997) and then manually edited the sequences using MacClade v 4.08 (Maddison & Maddison, 2005). Two separate phylogenetic analyses were performed using *Olpidium brassica* as an outgroup: maximum likelihood (ML) was conducted using Garli (Genetic Algorithm for Rapid Likelihood Inference) v 0.95 (Zwickl, 2006), and Bayesian analysis was performed using MrBayes 3.1.1 (Ronquist & Huelsenbeck, 2003). These analyses were conducted using the same methods described by Schechter and Bruns (2008).

After Schechter and Bruns (2008), I used the results of the phylogenetic analyses to confirm OTUs. This included looking 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 treatment combination.

Assemblage Analyses: AMF assemblages were analyzed in the same manner described by Schechter and Bruns (2008). I used the PRIMER 5 software (Plymouth Routines in Multivariate Ecological Research) (Clarke & Warwick, 2001) to perform the AMF assemblage

analyses. I prepared a relative abundance matrix of OTUs present in each treatment combination root samples based on the number of clones representing those OTUs. I then produced a similarity matrix using the Bray-Curtis similarity measure after performing a square-root and presence/absence transformations. I used the ANOSIM (analysis of similarities) routine to perform statistical analysis of assemblage data (Clarke & Warwick, 2001). I produced a rarefaction curve to determine if clone sampling effort saturated the number of OTUs using the EstimateS 8.0 Mao Tau estimator (Colwell *et al.*, 2004).

Plant Harvest: I used ANOVA (JMP v. 5) to test for differences between inoculum treatments, *C. sparsiflora* ecotypes, and individual populations as well as an interaction between inoculum treatments and ecotypes in the plant height, number of flowers, root and shoot dry weight, and colonization (arcsine transformed). Tukey HSD tests were used for all *a posteriori* comparison of means.

## Results

### *Identification of root associated AMF*

*General.* I detected no AMF associated with the AMF-free controls through either visual examination of stained roots or through the amplification and sequencing of root PCR products. Roots of *C. sparsiflora* ecotype populations associated with serpentine and non-serpentine AMF treatments were overall poorly colonized (1-11% of root length) with no significant difference in AMF colonization between treatment combinations ( $F = 0.344$ ,  $P < 0.56$ ). The poor colonization resulted in lower AMF sequences detection efficiency: 76% AMF sequences, 8% sequences of bacterial origin, 6% chimeric sequences, 2% of ascomycete origin, and the rest were of too poor of quality to give reliable data. Notably, I detected no AMF sequences from the NS3 *C. sparsiflora* ecotype grown with non-serpentine AMF even though AMF colonization was visually observed although low (3% root length).

I detected only three AMF OTUs associated with the serpentine AMF treatment: *Glomus* 4, *Glomus* 6, and *Glomus* 7 (Table 1). These OTUs matched those found in Schechter and Bruns (2008) (Figure 1). I detected six AMF OTUs associated with the non-serpentine AMF treatment: *Glomus* 1, *Glomus* 4, *Glomus* 4a, *Glomus* 4b, *Glomus* 6, and *Glomus* 7 (Table 1). Only two (*Glomus* 4a and 4b) of the non-serpentine AMF OTUs were newly detected, yet very closely related to previously detected *Glomus* 4 OTU (Figure 1). The *Glomus* 4 OTU was the most abundance AMF sequence found in both serpentine and non-serpentine AMF treatments (72% of all AMF sequences).

*Comparing inoculum treatment AMF assemblages.* The rarefaction analysis shows saturation in the curve representing the serpentine AMF treatment and a leveling off in the curve representing the non-serpentine AMF treatment (Figure 2). This indicates that the sequence sampling effort was sufficient to compare the AMF assemblages associated with serpentine and non-serpentine AMF treatments. ANOSIM analysis comparing AMF assemblages associated with the serpentine AMF treatment to those associated with the non-serpentine AMF treatment did not show a significant difference using the square root transformation ( $R = 0.167$ ,  $P < 0.29$ ), which gives greater importance to highly abundant OTUs. However, after the presence/absence transformation, ANOSIM analysis showed a marginally significant difference between serpentine AMF and non-serpentine AMF treatment assemblages ( $R=0.444$ ,  $P = 0.057$ ). ANOSIM analysis comparing AMF assemblages associated with individual ecotype populations

across inoculum treatments were not significantly different from each other under any transformation (square root:  $R = 0.185$ ,  $P < 0.29$ ; presence/absence:  $R = 0.167$ ,  $P < 0.41$ ), showing that there was no selectivity for particular AMF OTUs between ecotype populations. Overall, this data suggests that serpentine and non-serpentine AMF treatments differed in AMF composition, but the dominance of the *Glomus* 4 OTU was similar across all treatments.

### Harvest

*Soil analysis.* Soil analysis showed that autoclaving serpentine soil did not result in large changes in soil nutrients, except for soil manganese (Table S1, supplementary materials). Autoclaving serpentine soil appears to have released manganese resulting in a large increase after autoclave treatment (Table S1). However, soil analysis data represents only one sample per treatment and therefore, no significant differences in soil nutrient concentrations between treatments could be determined.

*Plant growth.* There were no significant differences between inoculum treatments, *C. sparsiflora* ecotypes, individual populations or any interaction between inoculum treatments and ecotypes in any growth parameter except shoot dry weight and shoot + root dry weight (Table S2). Plant height ( $F_{\text{trt}} = 1.49$ ,  $P < 0.23$ ;  $F_{\text{ecotype}} = 1.57$ ,  $P < 0.21$ ;  $F_{\text{pop}} = 1.11$ ,  $P < 0.34$ ;  $F_{\text{trt*ecotype}} = 1.25$ ,  $P < 0.29$ ); number of flowers ( $F_{\text{trt}} = 1.82$ ,  $P < 0.17$ ;  $F_{\text{ecotype}} = 0.01$ ,  $P < 0.93$ ;  $F_{\text{pop}} = 0.05$ ,  $P < 0.98$ ;  $F_{\text{trt*ecotype}} = 1.56$ ,  $P < 0.21$ ); and root dry weight ( $F_{\text{trt}} = 0.92$ ,  $P < 0.40$ ;  $F_{\text{ecotype}} = 0.14$ ,  $P < 0.71$ ;  $F_{\text{pop}} = 0.22$ ,  $P < 0.88$ ;  $F_{\text{trt*ecotype}} = 0.06$ ,  $P < 0.94$ ) were all similar across treatments, ecotypes, and populations.

In contrast, both shoot dry weight ( $F_{\text{trt}} = 6.13$ ,  $P < 0.003$ ;  $F_{\text{ecotype}} = 8.89$ ,  $P < 0.003$ ;  $F_{\text{pop}} = 2.98$ ,  $P < 0.03$ ;  $F_{\text{trt*ecotype}} = 1.22$ ,  $P < 0.30$ ) and shoot + root dry weight ( $F_{\text{trt}} = 5.75$ ,  $P < 0.004$ ;  $F_{\text{ecotype}} = 6.57$ ,  $P < 0.01$ ;  $F_{\text{pop}} = 2.37$ ,  $P < 0.07$ ;  $F_{\text{trt*ecotype}} = 1.15$ ,  $P < 0.32$ ) showed significant differences between inoculum treatments and *C. sparsiflora* ecotypes, but only shoot dry weight had significant differences between populations. Neither parameter had a significant interaction between inoculum and ecotypes. However, shoot + root dry weight most likely reflects the shoot dry weight differences as there were no differences in root dry weight (Table S2), therefore, I will only focus on the shoot dry weight differences.

Shoot dry weight of *C. sparsiflora* growing in the serpentine AMF treatment was significantly higher than both non-serpentine AMF and the AMF-free control treatments, which were not significantly different from each other (Figure 3). Overall, non-serpentine ecotypes had higher shoot dry weight than serpentine ecotypes, but serpentine and non-serpentine *C. sparsiflora* ecotypes showed different responses to inoculum treatments (Figure 4). Serpentine ecotypes responded the most to serpentine AMF inoculum relative to the controls (+64%) versus non-serpentine ecotypes (+13%) (Figure 4). In contrast, serpentine ecotypes responded only slightly to the non-serpentine AMF inoculum relative to the controls (+13%) but non-serpentine ecotypes actually showed a growth reduction relative to the controls when associating with non-serpentine AMF inoculum (-18%).

The individual *C. sparsiflora* ecotype populations showed similar but more specific trends in shoot dry weight responses to inoculum treatments. Both serpentine ecotype populations showed greatest response to serpentine AMF inoculum relative to the controls, but differed in their response to the non-serpentine AMF inoculum treatment – S1 showed a non-significant positive growth response, while S2 had a non-significant negative growth response to the non-serpentine AMF treatment (Figure 5). The non-serpentine ecotypes varied greatly in their response to inoculum treatments. The NS1 ecotype population showed a non-significant

negative growth response to both serpentine and non-serpentine AMF treatments, while the NS3 ecotype had a strong significant positive response to the serpentine AMF treatment and non-significant negative growth response to the non-serpentine AMF treatment relative to the control (Figure 5).

Plant tissue nutrients were also analyzed in this experiment. However, the shoots had to be grouped according to inoculum treatment (serpentine AMF, non-serpentine AMF, and AMF-free control) for analysis because the shoot dry weight was too low for tissue nutrient analysis at any other level (pers. comm., A & L Western Laboratories). Therefore, there is only one data point for each nutrient concentration per inoculum treatment making statistical analysis of the plant tissue data impossible. I will only be reporting general trends between inoculum treatments. There is very little difference between inoculum treatments in plant uptake of N, P, and Mg but both serpentine and non-serpentine AMF treatments showed an increase in K uptake relative to the control (Figure 6a). The most obvious difference in plant uptake was in shoot Ca concentration. The AMF-free control plants had nearly double the concentration of Ca than those growing with either serpentine and non-serpentine AMF treatments, but shoot Ca concentration was higher in plants growing with the serpentine AMF treatment than those growing in the non-serpentine AMF treatment (Figure 6a). All shoot micronutrient concentrations showed the same trend – higher concentrations of all micronutrients measured in the plants growing in the AMF-free control treatment with very little difference in shoot concentrations between serpentine and non-serpentine AMF treatments (Figure 6b).

## Discussion

### *Experimental Approach*

This is the first study to directly test the role of AMF in plant adaptation to serpentine using experimentally confirmed serpentine and non-serpentine adapted plant ecotypes and distinct serpentine and non-serpentine AMF assemblages. The approach was to use a mixed AMF culture for each serpentine and non-serpentine AMF treatment that better represented the whole serpentine and non-serpentine AMF assemblages. This was done, not only because it is a more realistic approximation of the field environment, but also because past studies that used individual AMF isolates to determine the role of AMF under metal stress proved inconclusive due to the strong individual host – AMF isolate genotypic effects (Meharg, 2003b; Redon *et al.*, 2009; Shetty *et al.*, 1994; Sudova *et al.*, 2008; Weissenhorn *et al.*, 1995). In fact, Joe Morton, curator of the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM), who has vast experience in the production and use of single species cultures, personally recommended the production and use of mixed AMF cultures over single species for the same reason.

However, the production of these cultures proved extremely difficult. After four years of attempts and failures, I received NSF Doctoral Dissertation Improvement Grant (DDIG) funds to fly Joe Morton to UC Berkeley for a direct consultation on the production of these cultures. While his recommendations resulted in the best AMF cultures I had produced to date, the inoculum potential (i.e. the ability of the cultures to colonize plant host roots) of the cultures was very low – as illustrated by the low colonization of the *C. sparsiflora* ecotypes in this experiment (1-11% root length) compared to the common garden experiment (52 – 55% root length) which used whole field soil and roots as the AMF inoculum source. While I cannot say whether the



results of this experiment would have been different with better serpentine and non-serpentine AMF inoculum, it is likely that the low inoculum potential may be the source of the great variation in plant host growth responses found in this experiment. Longer storage time and/or repeated culturing of the first cultures may have improved the inoculum potential of these cultures (<http://invam.caf.wvu.edu/methods/cultures/trapcultures.htm>), and these methods will be attempted in future experiments.

Another approach used in this experiment was the use of an autoclave treatment as the method of soil sterilization. This was successful as it created an AMF-free soil without drastically changing the soil nutrient status, which is a common fear with this method of soil sterilization. Abou-Shanab (2003) also found only minor changes in nutrient status after autoclave treatment of serpentine soil, which is encouraging because gamma-irradiation is cost prohibitive and is also known to release soil nutrients (Alphei & Scheu, 1993).

#### *Serpentine and Non-serpentine AMF Culture Treatments.*

Despite problems with low inoculum potential, the dominant AMF OTUs found in each culture treatment were similar to those found in the common garden greenhouse study (Chapter 2, Table 2). The serpentine AMF culture treatment was dominated by *Glomus* 4, *Glomus* 6, and *Glomus* 7 – which are the same AMF OTUs that were dominant in the serpentine only controls (*C. sparsiflora* ecotypes grown in only field collected serpentine soil) of the common garden experiment. This confirms that *Glomus* 4 is the dominant serpentine AMF OTU under greenhouse conditions. It is known that the trap culturing method used in this experiment commonly results in the reduction of the diversity of AMF (Liu & Wang, 2003), so it is reassuring that these dominant AMF OTUs persisted through the culturing process and reasonable that the more rare AMF taxa were eliminated in this culturing process.

The non-serpentine AMF culture treatment showed the same pattern of dominant AMF OTUs persistence and the elimination of rare taxa when compared to the common garden experiment, with one main exception - change in dominance from *Glomus* 1 to *Glomus* 4. In the non-serpentine only control (*C. sparsiflora* ecotypes grown in only field collected non-serpentine soil) of the common garden experiment, *Glomus* 1 was found in every *C. sparsiflora* ecotype population root samples at a higher relative abundance than *Glomus* 4 (Chapter 2, Table 2). However, in the present study, *Glomus* 1 was only found associated with the serpentine ecotype populations root samples while *Glomus* 4 was found in every ecotype root sample at high relative abundance (Table 1). However, this pattern is consistent with the behavior of *Glomus* 1 in serpentine soil seen in previous studies (Chapter 1, 2, and 3), giving more credence to the hypothesis that *Glomus* 1 is a poor competitor in/ intolerant of serpentine soil. However, the only way to be sure would be to compare AMF assemblages associated with the non-serpentine AMF culture before and after serpentine soil exposure, which was not done.

#### *Plant Response to Inoculum Treatments*

I hypothesized that the *C. sparsiflora* serpentine ecotype would have lower fitness without serpentine AMF. However, there was no significant difference between inoculum treatments or ecotypes in relative fitness (number of flowers + number of fruit). This may be due to the high variability found in this experiment or the nature of the greenhouse environment. Wright *et al.* (2006) found a significant difference in relative fitness between *C. sparsiflora* ecotypes growing in “home” or “away” soil in her reciprocal transplant field experiment but found no difference in relative fitness when she repeated the experiment in the greenhouse. Thus it may be difficult to

detect relative fitness differences of *C. sparsiflora* ecotypes under standard greenhouse growth conditions.

This experiment showed an AMF source effect on plant growth under edaphic stress. Overall, *C. sparsiflora* in the serpentine AMF treatment had significantly higher shoot dry weight than both non-serpentine AMF and AMF-free control treatments (Figure 3). This is a unique result. Similar studies looking at AMF source and plant source effects on plant growth in metal contaminated soil (Redon *et al.*, 2009; Shetty *et al.*, 1994; Sudova *et al.*, 2008) and under thermal stress (Bunn *et al.*, 2009) showed no AMF source effect on plant growth. However, in all but one study (Bunn *et al.*, 2009), investigators only tested individual AMF isolates not mixed AMF assemblages. This approach was only able to detect specific host-AMF isolate genotypic effects. The use of a mixed AMF inoculum in this study provided a better approximation of the field environment, and therefore may be more relevant for and reflective of soil assemblage effects.

An unexpected result was that, overall, *C. sparsiflora* non-serpentine ecotypes had significantly higher shoot dry weight than serpentine ecotypes growing in serpentine soil. This is opposite to what Wright *et al.* (2006) found in their reciprocal transplant field experiment using selfed F1 seeds. For this experiment, I used transplants germinated from field-collected seeds, allowing possible carry over maternal effects of field conditions (e.g. larger non-serpentine seeds due to better soil conditions). As a result, I observed that the non-serpentine ecotype seedlings were larger when transplanted than the serpentine ecotype seedlings (data not shown), which is the most likely explanation as to why the non-serpentine ecotypes grew larger than the serpentine ecotypes in this experiment. Using selfed F1 seeds for this experiment would have most likely eliminated these maternal effects. However, despite these carry over maternal effects, serpentine and non-serpentine ecotypes still showed differential responses to inoculum treatments (Figure 4).

### Conclusion

This study provided a good foundation about the plant and AMF symbiotic traits important in plant adaptation to serpentine. This experiment clearly showed that serpentine AMF have a greater growth effect on host plants under edaphic stress than non-serpentine AMF or non-mycorrhizal hosts. This indicates that serpentine AMF have a specialized adaptation to serpentine conferring growth enhancement of hosts. However, it is still unclear what this adaptation might be or which function is contributing to the growth enhancement. This study also indicated that differences in serpentine and non-serpentine AMF are at the population level, as AMF OTU *Glomus* 4 was dominant in both serpentine and non-serpentine inocula.

This experiment also implied that serpentine adapted ecotypes of *C. sparsiflora* have a greater response to AMF than non-serpentine adapted ecotypes. This supports the hypothesis founded by other studies that increased response to AMF may be one mechanism of plant edaphic adaptation (Hetrick *et al.*, 1992; Hetrick *et al.*, 1993; Schultz *et al.*, 2001). The results of this study also confirm the finding in the common garden experiment that there is no selectivity of *C. sparsiflora* ecotypes for particular AMF. Therefore, it appears that host-AMF selectivity has no role in serpentine adaptation, at least not for this particular host.

However, the low inoculum potential, the use of field-collected seeds, as well as standard greenhouse conditions all contributed to the variability and therefore the inability to conclusively accept or reject the main hypothesis that serpentine ecotypes have lower fitness and growth without serpentine AMF. But the lessons learned in this experiment have not been for naught. I

have been given the opportunity to repeat and expand this experiment as an USDA post-doctoral fellow. Given this opportunity, I am confident that I will be able to clearly define the role AMF play in plant adaptation to serpentine.

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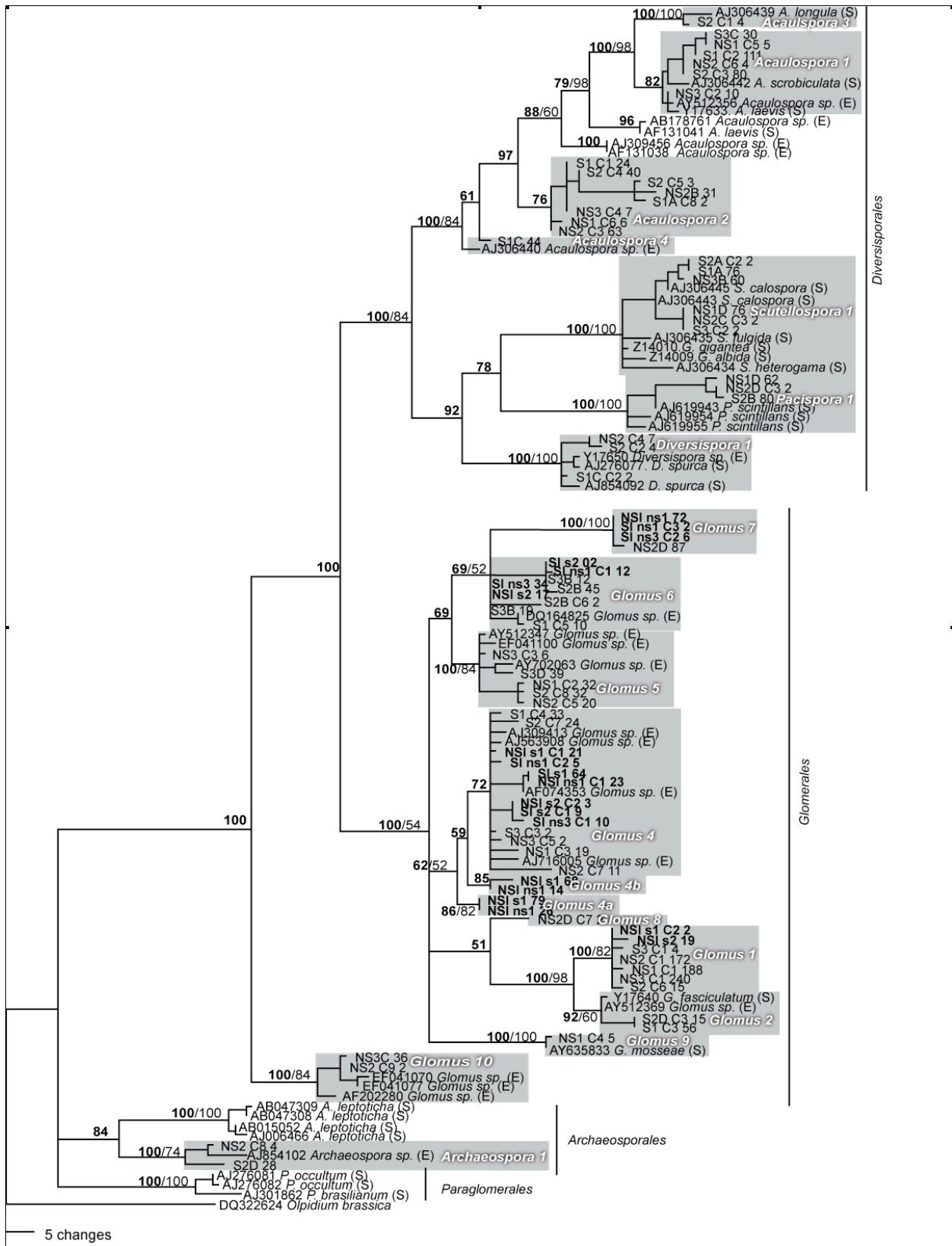
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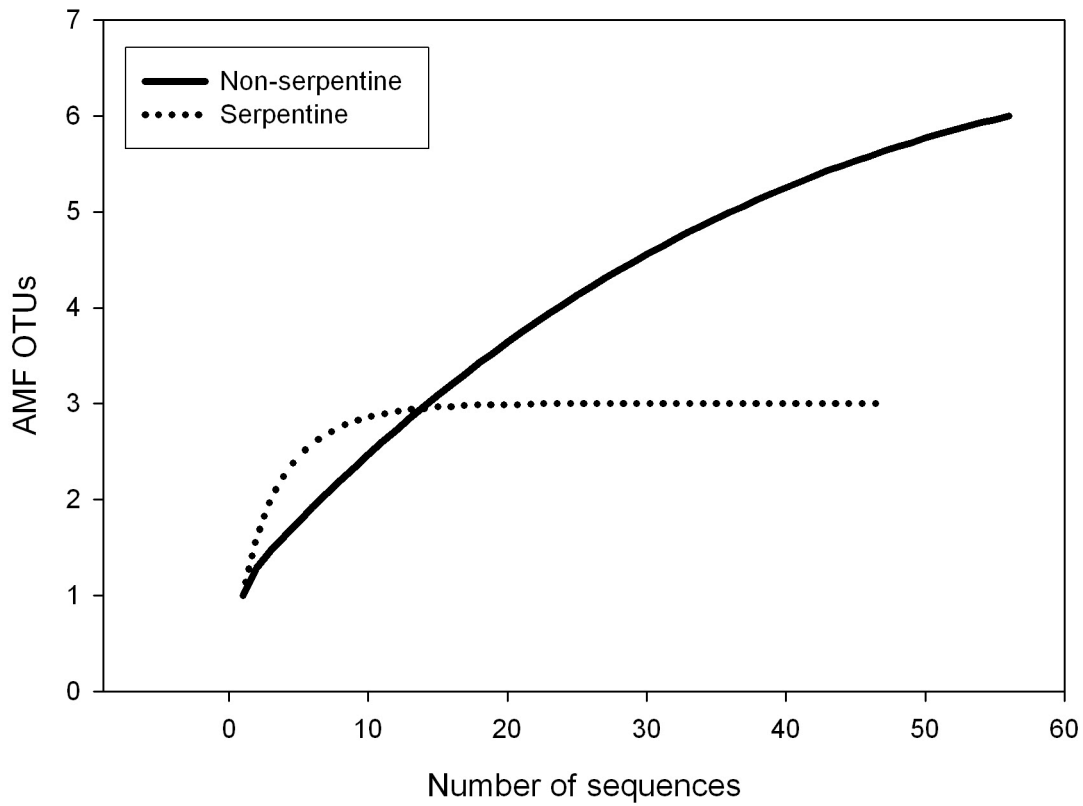
**Table 1.** Relative abundance matrix of operational taxonomic units (OTU) of AM fungi associated with two serpentine ecotype populations (S1 and S2) and two non-serpentine ecotype populations (NS1 and NS3) of *Collinsia sparsiflora* seedlings grown in either serpentine and non-serpentine AMF inoculum. No AMF sequences were detected in the *C. sparsiflora* NS3 ecotype seedlings when inoculated with non-serpentine AMF although they were colonized by AMF. There was no AMF colonization and no AMF sequences detected in the non-AMF controls.

OTU	INOCULUM							
	Serpentine				Non-Serpentine			
	S1	S2	NS1	NS3	S1	S2	NS1	NS3
Glo 1	0	0	0	0	8	20	0	0
Glo 4a	0	0	0	0	4	0	4	0
Glo 4b	0	0	0	0	4	0	4	0
Glo 4	100	90	26	59	84	60	88	0
Glo 6	0	10	63	6	0	20	0	0
Glo 7	0	0	11	35	0	0	4	0

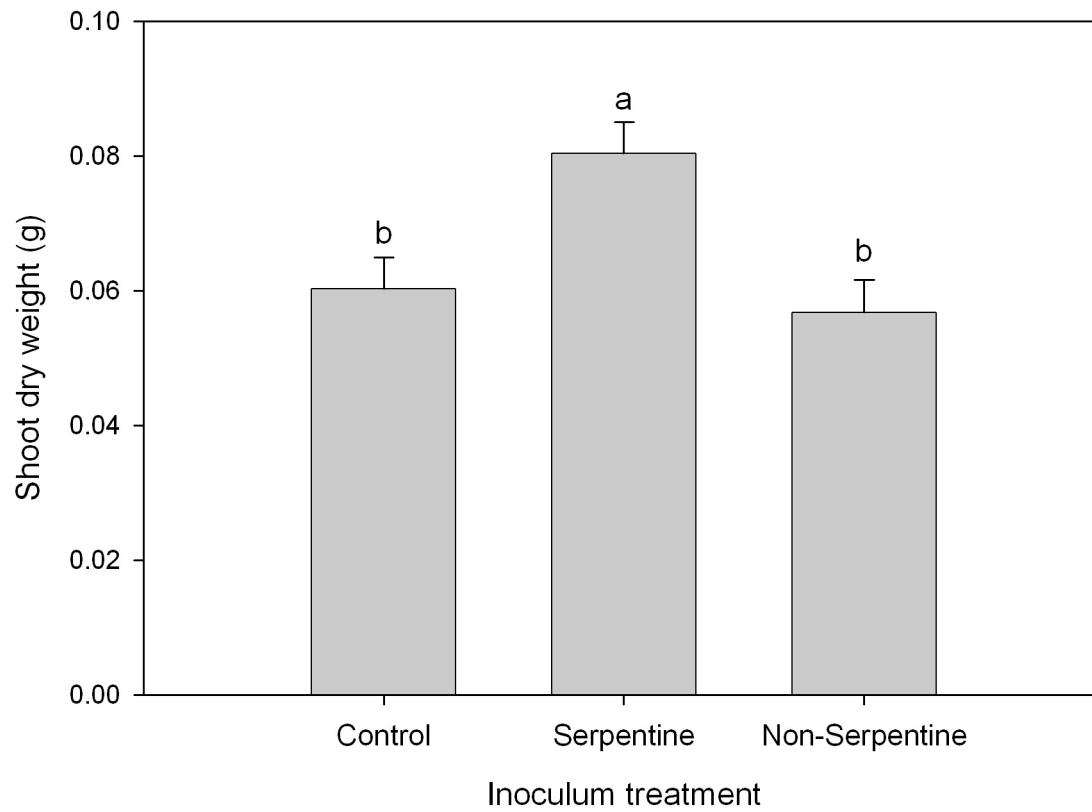




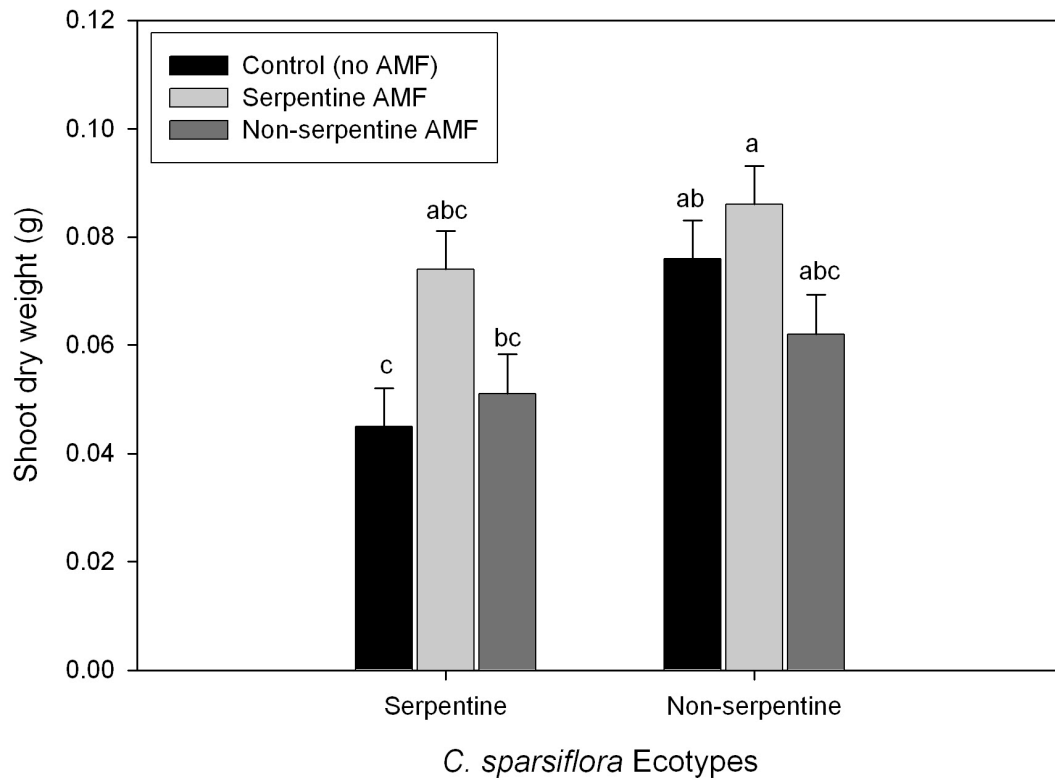
**Figure 1.** Consensus tree (50% majority rule) from Mr. Bayes analysis showing the phylogenetic relationship of the AMF sequences obtained from roots sampled from a greenhouse experiment with *Collinsia sparsiflora* serpentine ecotypes associating with a serpentine AMF treatment (SI\_s1 and SI\_s2) and a non-serpentine AMF treatment (NSI\_s1 and NS1\_s2) and with *Collinsia sparsiflora* non-serpentine ecotypes associating with a serpentine AMF treatment (SI\_ns1 and SI\_ns3) and a non-serpentine AMF treatment (NSI\_ns1 and NS1\_ns3), in bold. Additional sequences from roots sampled from three serpentine (S1, S2, S3) and three nonserpentine (NS1, NS2, NS3) ecotype populations of *Collinsia sparsiflora* field experiment were included (Schechter and Bruns, 2008). Letters directly behind site designation refers to an individual sample; no letter means that the sequence is a representative from a 98% consensus of sequences found in multiple samples within that site. 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 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 out-group. Topology was similar between Bayesian and Garli analyses.



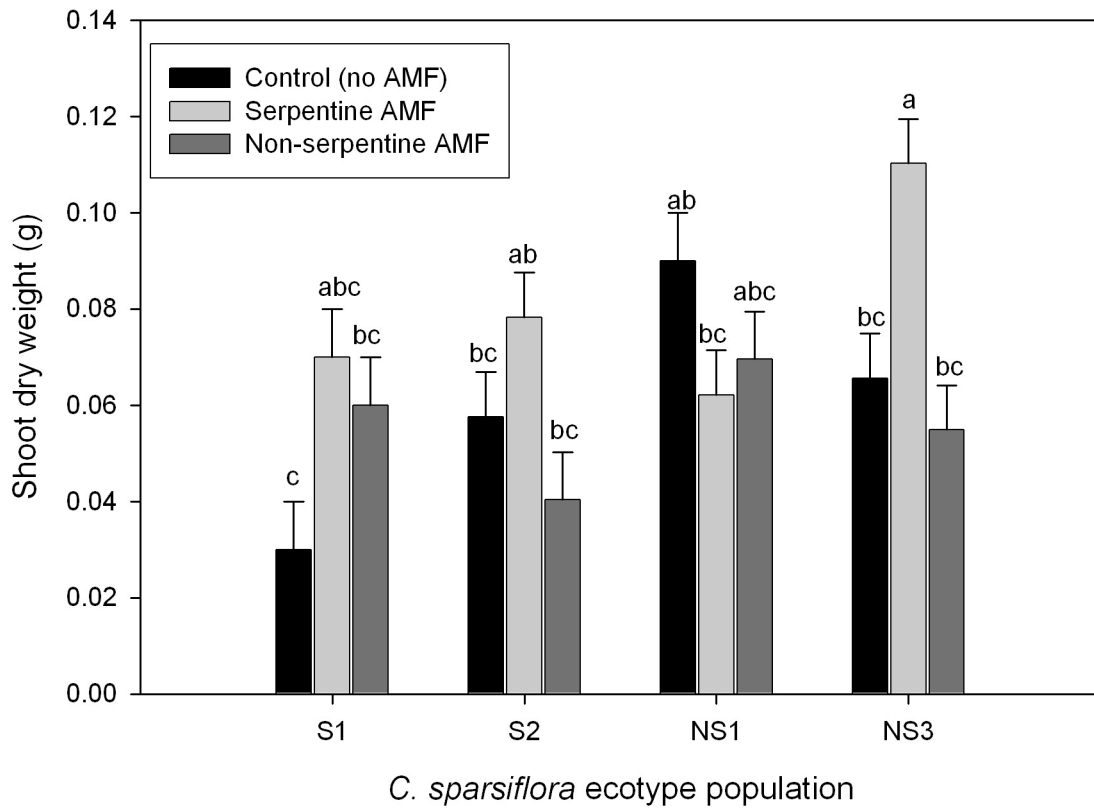
**Figure 2.** Rarefaction curve of the total number of sequences sampled from combined *Collinsia sparsiflora* ecotype populations (S1, S2, NS1, and NS3) grown in serpentine soil associated with either serpentine AMF or non-serpentine AMF. Rarefaction curves were produced by the EstimateS version 8.0 Mao Tau estimator (Colwell et al. 2004).



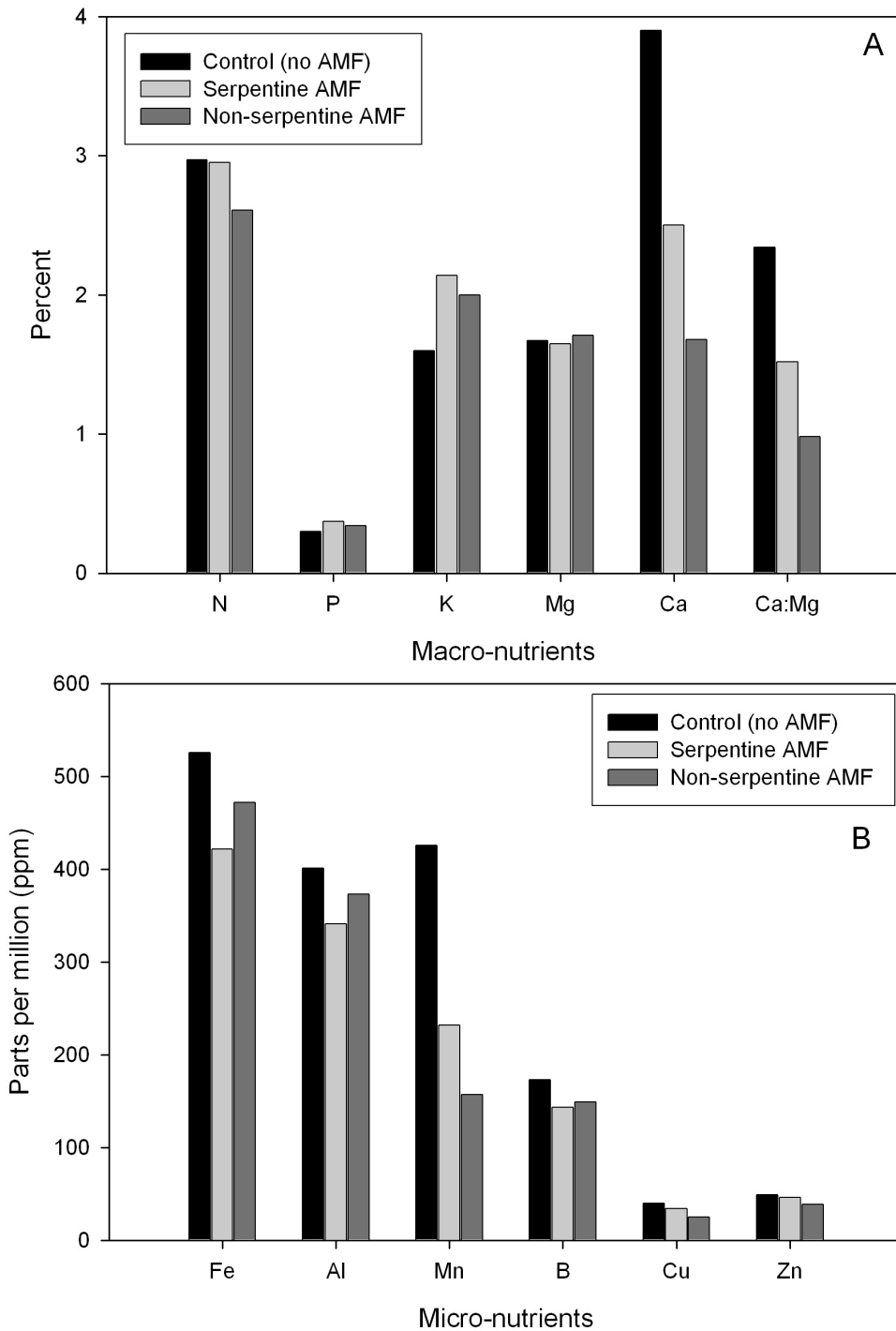
**Figure 3.** Comparison of shoot dry weight from combined *Collinsia sparsiflora* ecotype populations (S1, S2, NS1, and NS3) grown in serpentine soil associated with either no AMF (control), serpentine AMF, or non-serpentine AMF. Letters indicate significant differences at  $P < 0.05$ .



**Figure 4.** Comparison of shoot dry weight from combined *Collinsia sparsiflora* serpentine ecotype populations (S1 and S2) and non-serpentine ecotype populations (NS1 and NS3) grown in serpentine soil associated with either no AMF (control), serpentine AMF, or non-serpentine AMF. Letters indicate significant differences at  $P < 0.05$ .



**Figure 5.** Comparison of shoot dry weight between *Collinsia sparsiflora* ecotype populations (S1, S2, NS1, and NS3) grown in serpentine soil associated with either no AMF (control), serpentine AMF, or non-serpentine AMF. Letters indicate significant differences at  $P < 0.05$ .



**Figure 6.** Comparison of plant tissue nutrient analysis results from combined *Collinsia sparsiflora* ecotype populations (S1, S2, NS1, and NS3) grown in serpentine soil associated with either no AMF (control), serpentine AMF, or non-serpentine AMF. A) Macronutrients: nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca) and Ca:Mg, and B) Micronutrients: iron (Fe), aluminum (Al), manganese (Mn), boron (B), copper (Cu), and zinc (Zn).

**Supplemental Table S1.** Comparison of soil chemical variables of serpentine soil before and after autoclave treatment. Values are single measurements. Nitrogen (NO<sub>3</sub>), phosphorus (P, Weak Bray), potassium (K), magnesium (Mg), calcium (Ca), sulfur (S), zinc (Zn), manganese (Mn), 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 Ca:Mg ratio; serpentine soils have a ratio much less than one and non-serpentine soils have ratios greater than one.

Growth medium	N	P	K	Mg	Ca	Ca:Mg	S	Zn	Mn	Fe	Cu	B	C.E.C.	pH
Serpentine only	3	4	111	1359	379	0.28	5	0.3	12	25	0.5	0.3	13.4	7.5
Autoclaved Serpentine	3	5	99	1206	397	0.33	5	0.3	58	13	0.3	0.5	12.6	6.8

**Supplemental Table S2.** Results of the harvest of *Collinsia sparsiflora* ecotype populations (S1, S2, NS1, and NS3) after being grown without AMF (control) or with serpentine and non-serpentine AMF inoculum. Values are means (N= 10) with standard deviation below in parentheses. Letters indicate significant differences at  $P < 0.05$ .

Inoculum	Ecotype	Height (cm)	# of Flowers	Shoot dry weight (g)	Root dry weight (g)	Shoot+Root dry weight (g)
CONTROL	S1	8.92 <sup>a</sup> (1.83)	4.30 <sup>a</sup> (3.30)	0.032 <sup>c</sup> (0.015)	0.0059 <sup>a</sup> (0.0009)	0.034 <sup>c</sup> (0.018)
	S2	10.75 <sup>a</sup> (3.18)	7.70 <sup>a</sup> (5.48)	0.060 <sup>bc</sup> (0.027)	0.0131 <sup>a</sup> (0.0090)	0.068 <sup>bc</sup> (0.034)
	NS1	11.05 <sup>a</sup> (2.33)	8.20 <sup>a</sup> (4.34)	0.086 <sup>ab</sup> (0.033)	0.0122 <sup>a</sup> (0.0048)	0.093 <sup>ab</sup> (0.037)
	NS3	9.52 <sup>a</sup> (3.58)	7.20 <sup>a</sup> (5.39)	0.066 <sup>bc</sup> (0.043)	0.0115 <sup>a</sup> (0.0027)	0.071 <sup>abc</sup> (0.051)
SERPENTINE	S1	10.89 <sup>a</sup> (2.96)	7.78 <sup>a</sup> (5.14)	0.070 <sup>abc</sup> (0.034)	0.0099 <sup>a</sup> (0.0024)	0.079 <sup>abc</sup> (0.035)
	S2	12.25 <sup>a</sup> (2.20)	9.20 <sup>a</sup> (5.13)	0.078 <sup>ab</sup> (0.022)	0.0095 <sup>a</sup> (0.0025)	0.087 <sup>ab</sup> (0.026)
	NS1	8.15 <sup>a</sup> (2.09)	5.40 <sup>a</sup> (2.72)	0.062 <sup>bc</sup> (0.030)	0.0088 <sup>a</sup> (0.0048)	0.069 <sup>a</sup> (0.033)
	NS3	11.95 <sup>a</sup> (1.52)	8.40 <sup>a</sup> (2.99)	0.11 <sup>a</sup> (0.042)	0.0115 <sup>a</sup> (0.0041)	0.119 <sup>a</sup> (0.044)
NON-SERPENTINE	S1	11.94 <sup>a</sup> (2.50)	8.33 <sup>a</sup> (3.87)	0.062 <sup>bc</sup> (0.028)	0.0144 <sup>a</sup> (0.0035)	0.071 <sup>abc</sup> (0.030)
	S2	11.28 <sup>a</sup> (3.01)	3.67 <sup>a</sup> (2.12)	0.040 <sup>bc</sup> (0.010)	0.0066 <sup>a</sup> (0.0025)	0.047 <sup>bc</sup> (0.012)
	NS1	11.11 <sup>a</sup> (3.09)	6.22 <sup>a</sup> (3.99)	0.070 <sup>abc</sup> (0.031)	0.0093 <sup>a</sup> (0.0030)	0.074 <sup>abc</sup> (0.036)
	NS3	10.35 <sup>a</sup> (3.87)	5.30 <sup>a</sup> (2.31)	0.055 <sup>bc</sup> (0.020)	0.0010 <sup>a</sup> (0.0033)	0.062 <sup>bc</sup> (0.023)