The diversity of arbuscular mycorrhizal fungi amplified from grapevine roots (*Vitis vinifera* L.) in Oregon vineyards is seasonally stable and influenced by soil and vine age

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Abstract: The diversity of arbuscular mycorrhizal fungi (AMF) in 10 Oregon vineyards was assessed by examining spores in soil and amplifying mycorrhizal DNA from roots. Seventeen spore morphotypes were found in soil, including seven species in the Acaulosporaceae. Eighteen phylotypes were amplified from grape roots with AM1 and NS31 primers, and clones were dominated by *Glomus* spp. (> 99%). A few clones (< 1%) representing a single phylotype within Gigasporaceae, and a single clone within Archaeosporaceae were amplified from roots with AM1-NS31 primers. A separate experiment employing known proportions of grape roots colonized by *Glomus intraradices* or by *Gigaspora rosea* showed that fungi within Gigasporaceae might be underrepresented in clone abundance when *Glomus* spp. co-occur in roots. No clones representing fungi within the Acaulosporaceae were amplified from vineyards, although specific fungi within Acaulosporaceae were shown to colonize Pinot noir roots in sterilized soil and were amplified from these roots. Four *Glomus* phylotypes, including *G. intraradices*, were found in roots from all 10 vineyards, and these fungi accounted for 81% of clones. AMF phylotypes amplified from roots did not change during the growing season, although six phylotypes varied with soil type. The presence of three phylotypes was affected by vineyard age, and phylotype richness appeared to decline as vineyard age increased beyond 20 y. PCA analysis supported the hypothesis that the AMF community is different in red-hill soils than in valley soils and indicated certain phylotypes might be associated with lower soil and vine nutrient status. However, the changes in the AMF community in grape roots across vineyards were subtle because most root samples were dominated by the same three or four phylotypes. A separate analysis using primers to amplify AMF from the Archeasporaceae/Paraglomeraceae showed most root samples also were colonized by at least one *Paraglomus* or *Archaeospora* phylotype.

  Key words: AMF community, Glomeromycota, Pinot noir, ribosomal DNA, roots, spores

INTRODUCTION

Grapevines grown in red-hill soils (Ultisols) of Oregon are highly dependent on arbuscular mycorrhizal fungi (AMF) to obtain ample phosphorus (Schreiner 2007). A large proportion of fine roots are typically colonized by AMF and a high number of arbuscules are routinely found in grapevine roots in Oregon vineyards (Schreiner and Linderman 2005). Surveys of vineyards in other grape-growing regions also have found high levels of grapevine root colonization by AMF (Karagiannidis and Nikolaou 1999, Nappi et al 1985, Schubert and Cravero 1985), and a positive relationship between vine establishment and the presence of AMF in roots was found after fumigation of some vineyards in California (Menge et al 1983). Little is known however regarding the species composition of AMF colonizing grapevines in production vineyards. Spores of AMF isolated directly from vineyard soils or produced in trap cultures have been dominated by *Glomus* species. The most common isolates encountered in vineyard soils have been *Glomus intraradices*, *Glomus macrocarpum*, *Glomus mosseae* and *Paraglomus occultum* (Cheng and Baumgartner 2004, Karagiannidis et al 1997, Menge et al 1983, Nappi et al 1985, Oehl et al 2005, Schubert and Cravero 1985). It is not clear which of these fungi found as spores in soil actually colonize roots of grapevines. Some fungi might be associated with other plants in the vineyard, such as cover crops or weeds. Indeed these additional plants on the vineyard floor might play a role in maintaining AMF species diversity in the roots of grapevines.

Amplification of fungal DNA extracted from plant roots has made it possible to identify specific AMF that colonize plants in the field. Studies using this approach have shown that a number of host plant species, including those from agricultural and natural settings, harbor a large number of potential AMF species within their roots (Douhan et al 2005, Helgason et al 2002, Jansa et al 2003, Lekberg et al 2007, Öpik et al 2003, Stukenbrock and Rosendahl 2005, Wirsel 2004). Many of the sequences amplified

TABLE I. Characteristics of 10 Oregon vineyards where AMF were examined

	Vineyard									
	1	2	3	4	5	6	7	8	9	10
Vine age (years)	7	11	17	12	17	29	16	12	17	25
Soil series[a]	Bell.	Bell.	Jory	Jory	Jory	Jory	Will.	Sant.	Wood.	Jory
Soil order[b]	U	U	U	U	U	U	M	A	M	U
Soil pH	5.3	6.3	6.0	5.8	5.8	5.9	5.9	6.0	5.8	5.6
Elevation (m)	274	143	167	176	134	182	73	70	76	184
Vine density (ha^{-1})	2690	2445	1345	2150	1120	1795	1120	2240	1120	1995
Vine row orientation	N-S	N-S	E-W	N-S	E-W	E-W	E-W	N-S	E-W	N-S
In row cultivation	+	−	−	−	−	−	−	−	−	−

[a] Soil series abbreviations: Bell. = Bellpine, Will. = Willamette, Sant. = Santiam, Wood = Woodburn.

[b] Soil order abbreviations: U = Ultisol (red-hill soils), M = Mollisol (valley soils), A = Alfisol (valley soil).

from roots do not match those of known taxa, suggesting there is a considerable hidden diversity in AMF engaged in symbiosis with plants that is not apparent when diversity is assessed by identifying species based on spores in soil (Helgason et al 2007, Rosendahl 2008).

Understanding how the diversity of AMF within agricultural ecosystems relates to overall plant health or stress avoidance is a key component to developing sustainable production systems. Different species of AMF and even different isolates within a species cause divergent responses in plant growth or nutrient uptake (Bethlenfalvay et al 1989, Klironomos 2003, Munkvold et al 2004). Some fungi might be better suited to enhance uptake of certain nutrients or might impart a greater tolerance to drought than other fungi (Augé 2001). For example *Glomus mosseae*, isolated from an alluvial Mollisol, promoted greater Cu uptake by grapevines than the same species isolated from a nearby Ultisol whenever plants were grown in either soil type (Schreiner 2007). Most data regarding the variation in function of different AMF has come from similar kinds of experiments comparing single isolates. In some cases an additive effect of multiple AMF species (or complimentarity) can occur, but often one fungal species gives maximal plant performance that is not significantly enhanced by adding other AMF to the mix (Jansa et al 2008, Vogelsang et al 2006). Therefore, identity as well as diversity of the AMF community might play a significant role in enhancing plant growth or nutrient uptake, and this will depend on numerous variables encountered in a given production system.

The goal of this research was to identify specific AMF colonizing grapevines in production vineyards in the Willamette Valley of Oregon and to gain an understanding of how the diversity of AMF symbionts in grapevine roots is influenced by time of sampling,

vine age and soil type. AMF were identified by amplifying DNA extracted from roots with AM1 and NS31 primers, followed by cloning, RFLP analysis and sequencing of unique RFLP phenotypes. Spores were isolated from soil samples from the same vineyards to determine whether the community assessed via spores would be similar to that amplified from roots with a PCR approach. A nested PCR designed to amplify the ancestral groups of AMF also was used to examine whether grape roots were colonized by AMF from the Archaeosporaceae or Paraglomeraceae because these fungi are not usually amplified with AM1 and NS31 primers (Redecker 2000).

MATERIALS AND METHODS

Plant and soil samples.—A total of 10 self-rooted Pinot noir vineyards were selected from Schreiner and Linderman (2005) to examine AMF fungal diversity (TABLE I). Root and soil samples were collected from beneath the vines (in the planting row) in each vineyard at bloom (flowering Jun 28–Jul 2) and at véraison (onset of ripening Sep 13–17) with a 3 cm diam soil core 50 cm deep. Four replicate samples were collected at each vineyard along a transect running diagonally across each vineyard. The location of each sample was noted, and identical vines were sampled at bloom and véraison. Five soil cores from adjacent vines were pooled to comprise each replicate sample. Fine grape roots (primary roots with an intact cortex) were hand-collected from samples, sonicated in a water bath 30 s and rinsed with distilled water. Subsamples (0.1 g) of fine roots were frozen in liquid N and stored at −80 C. Leaves were collected at bloom from the same vines as soil samples, pooled from five plants per replicate (n = 4 per vineyard), oven dried (70 C) and finely ground for mineral nutrient analysis (N by combustion; P, K, Ca, Mg, Fe, Mn, B, Cu and Zn by ICP-OES) (Schreiner and Linderman 2005). Available soil nutrients also were determined at bloom after pooling equal volumes of air-dried soil from each replicate sample (n = 1 per vineyard). Soil nutrient analysis was conducted

by the Oregon State University, Central Analytical Lab, using procedures for western Oregon soils (Schreiner and Linderman 2005).

Characterizing AMF spores in soil.—AMF spores were extracted from 100 g (fresh weight) soil at bloom with the wet-sieving/sucrose centrifugation method of Daniels and Skipper (1982). The number of spores obtained per sample was low (< 15 in most samples). Therefore all spores retrieved from the four replicates per vineyard were pooled into a single sample (n = 1 per vineyard). All spores were mounted on slides in PVLG (Koske and Tessier 1983), examined under a compound microscope at up to 400-fold magnification and identified based on current species descriptions and identification manuals (Schenck and Pérez 1988, http://invam.caf.wvu.edu/fungi/taxonomy/speciesID. htm, http://www.lrz-muenchen.de/~schuessler/amphylo/ amphylo_species.html).

Amplifying and characterizing AMF DNA in roots.—Genomic DNA was extracted from frozen root samples with the QIAGEN (Valencia, California) DNEasy Plant kit, according to the manufacturer's instructions, except 0.1% (w/v, final concentration) polyvinylpyrrolidone (PVP-40, Sigma Chemical Co., St Louis, Missouri) was added to the initial extraction buffer (AP1) to precipitate phenolic compounds in grape roots known to interfere with *Taq* polymerase. Arbuscular mycorrhizal, 18S rDNA was PCR-amplified in 50–100 µL reactions with AM1 and NS31 primers (Helgason et al 1999, Simon et al 1992). A total of 2–5 µL extracted template DNA was used directly or diluted up to 100-fold as needed. The final reaction mixture contained 0.5–1.0 U Platimun *Taq* ploymerase (Invitrogen, Carlsbad, California), 2.0 mM MgCl2, 0.2 mM each dNTP (Invitrogen) and 7.5 pmol of each primer. PCR thermo-cycle parameters were the same as those described by Helgason et al (1999). Positive (grape root DNA extract from vineyard 10) and negative (no template) controls were included in every PCR amplification. All transfers of PCR reagents were conducted in a laminar flow hood.

After confirming PCR products were of the right size (~ 550 bp) by electrophoresis on 2% agarose gels, products were cloned with the TOPO TA sequencing cloning kit (Invitrogen) according to the manufacturer's instructions (except the ligation reaction carried out at room temperature was allowed to proceed 30 min instead of 5 min). Typically 30 clones from each root sample (representing one of four replicate root extracts per vineyard) were sampled randomly, streaked for isolation and cultured in LB broth with ampicillin (50 µg/mL) overnight at 37 C. The presence of the target insert was confirmed by PCR amplification in a 50 µL reaction with 2 µL from the broth culture as template, as described above. Those clones giving correct size products were cleaned with the QIAquick PCR purification kit (QIAGEN) and characterized by RFLP analysis after digesting with *Hinf* I + *Alu*I, and also with *Rsa*I + *Mae*II for certain unresolved *Hinf* I + *Alu*I types. RFLP fragments were analyzed after electrophoresis on 2% metaphor agarose gels (Cambrex BioScience, Rockland, Maine).

Initially three clones were sequenced for each unique RFLP phenotype per replicate root sample (when this was

possible) to characterize RFLP phenotypes that produced consistent sequences (> 9 9.0% identity). A three-clone consensus sequence representing these RFLP phenotypes (49 of 59 total RFLP phenotypes eventually encountered) was determined and retained in our dataset to reduce errors that could be due to *Taq* infidelity. Subsequent clones representing these 49 phenotypes rarely differed from this three-clone consensus sequence by more than two bases (> 99.6% identity). However different clones from 10 RFLP phenotypes from grape roots produced inconsistent sequences (or phylotypes) and these RFLP phenotypes were always sequenced thereafter to identify AMF. In addition at least one clone of all RFLP phenotypes from each vineyard was sequenced to confirm that consistent sequences were obtained from the 49 "good" RFLP phenotypes across vineyards. Sequencing was conducted by the Oregon State University Center for Biotechnology with fluorescent dideoxyterminators on an automated ABI Prism 3700 DNA analyzer with M13 primers.

A total of 947 clones from 40 root samples (10 vineyards) were analyzed at bloom, and 330 clones from 12 root samples (six vineyards) were analyzed at véraison. An average 24.6 clones was characterized per individual root sample. Of the 1277 clones generated with AM1 and NS31 primers 14% (177 clones) were sequenced. Forward and reverse sequences were aligned and edited with Seqlab (GCG 10, Wisconsin Package, Accelrys, San Diego, California) or BioEdit 7.0.5.3 (Hall 1999), and multiple sequences were aligned with CLUSTAL X 1.81 (Thompson et al 1997). Phylogenetic analysis using parsimony (PAUP 4.0b10) (Swofford 2002) was used to initially characterize aligned sequences. New sequences matching an existing three-clone consensus sequence by greater than 99% identity were considered to be that phylotype and were excluded from the final sequence dataset. BLAST analyses were conducted to find the closest known sequences to each of our grape root phylotypes using nucleotide BLASTt with no restrictions (Altschul et al 1997, http://www.ncbi.nlm.nih.gov/ blast/Blast.cgi). The final nucleotide sequence dataset containing the sequences from Pinot noir roots (15 three-clone consensus sequences and three single-clone sequences) and 55 sequences from GenBank was analyzed with maximum likelihood (see below).

The presence of ancestral AMF (Archaeosporaceae/ Paraglomeraceae) in our root samples was examined with the nested PCR procedure and primers (NS5 and ITS4 in the first PCR, ARCH1311 and ITS4 in the second PCR) described by Redecker (2000). Template DNA was serially diluted up to 1000-fold for use in the first PCR, and all reactions regardless of the presence of bands on gels were diluted up to 10 000-fold and used as template for the second PCR, as described by Redecker (2000). Samples giving positive products of the correct size (~ 1000 bp) in the second PCR were cloned with the TOPO TA kit, as described above. Two clones per root sample were chosen randomly for sequencing after confirming clones had the right size inserts, as described above. BLAST analyses were conducted on the sequences to find the closest matches in GenBank. We examined nine individual root samples from matched replicates at each sampling time (bloom and

véraison) with root extracts from vineyards 1, 2, 6, 7 and 10 (see TABLE I). A total of 32 clones were sequenced from 16 positive, nested PCR reactions. A root extract from a pot culture of *Archaeospora trappei* grown with *Sorghum* was used as a positive control for PCR.

Phylogenetic analysis.—Final AMF sequences amplified from Pinot noir roots with AM1 and NS31 primers (GenBank accession numbers FJ194498–FJ194515) and sequences from GenBank, including the closest reported sequence to each Pinot noir root phylotype, were aligned in 510 positions with CLUSTAL X. Taxonomic relations among fungal sequences were inferred by performing a maximum likelihood analysis with RaxML-VI-HPC 2.0 (Stamatakis 2006) with a GTR-MIX model of evolution. The analyses were run for 100 iterations and a total of 200 independent bootstrap analyses were performed to provide nodal support (Felsenstein 1985).

Assessing biases amplifying AMF from roots.—*Glomus* spp. are known to form abundant vesicles or even spores in roots, while fungi from the Gigasporaceae do not form vesicles in roots (Smith and Read 1997). The production of vesicles in roots might provide a more easily extracted source of AMF DNA than that provided by AMF, which produce only hyphae and arbuscules in roots. Thus, the high proportion of *Glomus* clones in our samples relative to clones from fungi in the Gigasporaceae might reflect the presence of vesicles in roots not the presence of different fungi per se. A greenhouse experiment was conducted in sterilized soil to test whether a greater proportion clones would be derived from *Glomus*-colonized roots than *Gigaspora*-colonized roots when roots are mixed in various known proportions. Fine roots of Pinot noir grafted onto 3309 C roots colonized either by *Glomus intraradices* (INVAM No. UT126) or by *Gigaspora rosea* (INVAM No. FL103) were mixed in different ratios, providing root samples with 0, 25, 50, 75 or 100% of root fresh mass derived from roots colonized by *G. intraradices* (the remaining portion of roots were colonized by *Gi. rosea*). The original root samples colonized by *G. intraradices* had 84% of root length colonized, while those with *Gi. rosea* were colonized at 75% of root length (colonization was not significantly different). DNA was extracted from the mixed root samples and AMF clones were produced and characterized by RFLP analysis in the same manner as the vineyard samples. The experiment was repeated with a new mixture of root extracts in the same ratios ∼ 11 mo later. A chi-squared (χ^2) analysis was used to test the significance of the *Glomus* versus *Gigaspora* DNA extraction experiments (using only the data from those mixes with both fungi present).

The lack of *Acaulospora* clones in the Pinot noir roots suggests grapevines are not colonized by fungi in the Acaulosporaceae or that our method did not amplify fungi in the Acaulosporaceae from grape roots. A greenhouse experiment was conducted in sterilized soil to test whether fungi from the Acaulosporaceae colonize grape roots and whether these fungi are amplified from roots. Self-rooted Pinot noir cuttings with two different *Acaulospora* spp. (*Acaulospora laevis*, INVAM No. OR217, *Acaulospora*
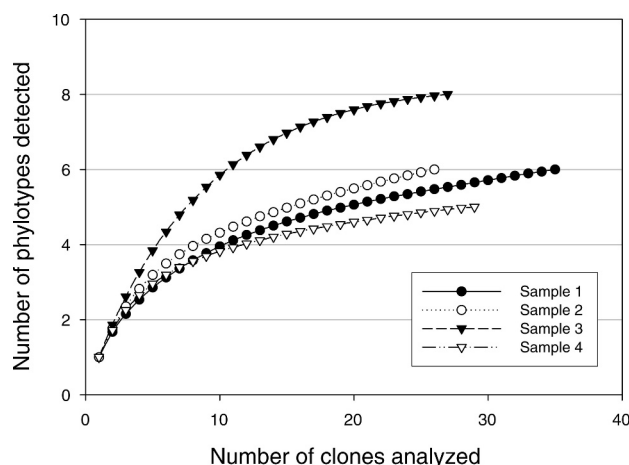

FIG. 1. Sampling effort curves for individual (replicate) root samples in vineyard 2, computed analytically in EstimateS 8.0 (Colwell 2005).

morrowiae, INVAM No. CL735) were grown 15 wk in a low P, sandy-loam soil, and fine root samples were collected and stored at −80 C. After confirming roots were colonized by both fungi (clearing and staining with trypan blue) DNA was extracted from root samples, amplified with AM1 and NS31 primers, and cloned and sequenced with the same methods as above.

Statistical analysis.—Phylotype accumulation curves (sampling effort curves) to estimate richness and associated error terms were computed with EstimateS 8.0 (Colwell 2005). This analysis indicated that ∼ 20 clones was a reasonable number to assess diversity of AMF amplicons with the AM1-NS31 primer pair (FIG. 1). χ^2 was used to test whether different AMF phylotypes were present in roots based on sampling time (bloom versus véraison), soil group (valley versus red-hill), or vine age (11–12 y, 17 y, 24–29 y). Subsets of matched root samples from the overall dataset were used for these analyses as follows: (i) temporal changes in AMF diversity from roots over a single growing season (bloom versus véraison) were compared with data from 12 root samples collected from the same vines at each sampling time from six vineyards (two replicates per vineyard); (ii) differences in AMF diversity in different soil types were assessed by comparing data with 13 root samples taken from three valley soil sites (vineyards 7, 8 and 9) to data from three similarly aged red-hill sites (vineyards 3, 4 and 5); (iii) changes in AMF diversity due to vine age was determined in vines from red-hill soils only by comparing data from nine root samples from 11–12 y old vines (vineyards 2 and 4), 17 y old vines (vineyards 3 and 5) and 24–29 y old vines (vineyards 6 and 10).

A principal component analysis (PCA) was used to better understand how relative abundance of different AMF phylotypes commonly found in Pinot noir roots were related to each other and to other variables measured. The PCA was specified with eight AMF phylotypes that exceeded 1% abundance of all clones at bloom and were normally distributed (Kolgorov-Smirnov). Data for all

TABLE II. AMF spores present in soil from 10 Oregon vineyards

AM fungus	Vineyard									
	1	2	3	4	5	6	7	8	9	10
Archaeospora trappeii		+					+			
Scutellospora calospora	+	+	+	+	+	+		+	+	+
Entrophospora infrequens	+						+			
Acaulospora elegans	+	+	+		+	+			+	+
Acaulospora lucanosa	+			+		+		+	+	
Acaulospora laevis				+						+
Acaulospora rehmii		+								
Acaulospora sp. 1					+	+			+	
Acaulospora sp. 2				+			+			
Glomus intraradices		+		+	+	+	+	+	+	+
Glomus mosseae	+	+	+	+	+		+	+		+
Glomus rubiformus	+				+				+	+
Glomus clariodeum	+						+	+	+	
Glomus sp. 1	+		+	+		+		+	+	+
Glomus sp. 2		+					+		+	+
Glomus sp. 3	+		+			+				+
Glomus sp. 4			+					+		+
Total number of species	9	7	6	7	6	7	7	7	9	10

variables used in PCA was centered and standardized (Ludwig and Reynolds 1988). Plant and soil nutrients (leaf concentrations and soil extract concentrations of N, P, K, Ca, Mg, Fe, Mn, B, Cu and Zn), soil pH and soil moisture content (reported by Schreiner and Linderman 2005) were passively projected into the ordination space. Only those leaf or soil variables showing a relatively strong relationship to the first two PCA axes (vector length greater than 0.5 units) are shown. Statistical analysis was conducted with Statistica 8.0 software (Statsoft Inc., Tulsa, Oklahoma).

RESULTS

AMF spores in soil.—The number of AMF spores retrieved from the vine row soil samples was low, with most samples having fewer than 15 spores per 100 g (fresh weight) of soil. The individual replicate samples from each vineyard therefore were pooled, mounted on a single slide, and the presence or absence of different described and unknown species was determined. Each vineyard had 6–10 species of AMF spores in soil at bloom (TABLE II). *Scutellospora calospora* was the most common species isolated (present in nine of 10 vineyards) and the only species present from the Gigasporaceae. *Glomus intraradices* and *Glomus mosseae* each were found in eight vineyards, and *Acaulospora elegans* was found in seven vineyards. A total of six *Acaulospora* spp. and eight *Glomus* spp. were found across all sites. It is possible that *Glomus* sp. 1 and *Glomus* sp. 2 are the same fungus because we could distinguish these morphotypes based only on color (clear versus light brown).

AMF amplified from roots.—All individual root samples used to amplify AMF DNA were well colonized by AMF as confirmed via clearing and staining separate subsamples of roots. The average colonization by AMF in the 10 vineyards examined at bloom was 70% of root length (42–90%), with arbuscular colonization averaging 29% of root length (data not shown). All root samples produced positive PCR reactions with the AM1-NS31 primer pair, giving products of ∼ 550 bp, and abundant clones were obtained for all samples. In all, 59 unique RFLP phenotypes using a combination of two double digests were identified (data not shown). Of these, 10 gave inconsistent sequence phylotypes and could not be relied on to place the given clone in the correct sequence-based phylotype.

The identity of fungi amplified from grape root extracts was quite different from those AMF identified as spores in soil. A total of 18 phylotypes were found from Pinot noir roots using the AM1-NS31 primer pair (FIG. 2). Three phylotypes (ARCH 1, GLO 4, GLO 6) were encountered only once as a single clone and may be considered rare. The remaining 15 phylotypes were encountered a sufficient number of times to obtain a three-clone consensus sequences for our analysis. The maximum likelihood tree (FIG. 2) is nearly identical to earlier trees we used to initially characterize sequences using maximum parsimony (PAUP) and this tree is generally consistent with works using the same PCR primers (Husband et al 2002a, Öpik et al 2003, Jumpponen 2005).

FIG. 2. Phylogenetic tree of arbuscular mycorrhizal fungi obtained by maximum likelihood analysis of partial 18S rDNA sequences (~ 510 bp). The tree was rooted with *Geosiphon pyriforme* and sequences from Oregon Pinot noir roots are shown in boldface and preceded by the prefix ORVIN. The most closely related sequence(s) from GenBank to each Pinot noir sequence were included in our analysis.

TABLE III. Relative abundance of AMF phylotypes in roots of Pinot noir grapevines from 10 Oregon vineyards at bloom (using AM1-NS31 primers)

Phylotype	Percentage of clones in each vineyard									
	1	2	3	4	5	6	7	8	9	10
ARCH 1	1.1									
SCUT 1		2.6				1.1		0.9		1.1
GLO 1A[a]	1.1	26.5	5.9	7.7	3.2	18.0	22.47	1.8	11.9	2.2
GLO 1B[a]	7.7	6.0	7.9	1.3	1.1	15.7		3.6	3.0	11.1
GLO 1C		3.4	2.0				2.6			
GLO 1D[a]	1.1	1.7	1.0					11.8		
GLO 1E		1.7							5.9	
GLO 1F									5.9	
GLO 2[a]	27.5	8.5	18.8	23.1	13.8	13.5	7.9	5.5	6.9	31.1
GLO 3A[a]	40.7	24.8	17.8	38.5	47.9	22.5	38.2	34.5	25.7	12.2
GLO 3B[a]	11.0	0.9	4.0	1.3						1.1
GLO 3C[a]	5.5	23.1	36.6	26.9	27.7	29.2	21.1	10.9	37.6	38.9
GLO 3D	1.1		4.9							
GLO 3E[a]			1.0	1.3	6.4		7.9	12.7	2.0	2.2
GLO 3F								16.7		
GLO 4		0.9								
GLO 5	3.3									
GLO 6									1.0	
Total clones	91	117	101	78	94	89	76	110	101	90
Total number of Phylotypes	10	11	10	7	6	6	6	9	9	8

[a] Phylotypes used for PCA analysis (FIG. 4).

Phylotype richness at each vineyard was 6–11, with a minimum number of unique phylotypes in any one root sample of three and a maximum of eight (TABLE III). AMF amplified from Pinot noir roots were clearly dominated by *Glomus* spp. (GLO). Not a single clone was found representing fungi in the Acaulosporaceae, and only a small fraction (< 1%) of clones were found from the Gigasporaceae. Nine phylotypes (all within *Glomus*) occurred above 1% of clone abundance, and four of these (GLO 1A, GLO 2, GLO 3A, GLO 3C) accounted for 81% of all clones. Six of the 18 phylotypes appeared in only a single vineyard.

Assessing biases amplifying AMF from roots.—Data from the mixed root sample experiment, using known quantities of roots colonized by two fungi, *G. intraradices* or *Gi. rosea*, indicated that the proportion of *Glomus* clones amplified from mixed root samples were more numerous than expected based on the percentage of root mass colonized by *G. intraradices* (TABLE IV). The bias favoring greater representation of *Glomus* clones differed most from expected values when *Glomus* roots accounted for only 25% of root mass. However some *Gi. rosea* clones were encountered in all the mixtures, even when *Glomus*-colonized roots accounted for 75% of the root sample. These results support our hypothesis that

Glomus DNA is more easily extracted or more readily amplified from roots than is DNA from fungi within the Gigasporaceae and indicate fungi from the Gigasporaceae might be under-represented in clone abundance data whenever *Glomus* spp. co-occur in root samples.

Data from the *Acaulospora* inoculation trial showed that fungi within the genus can colonize Pinot noir roots and are easily amplified from grape root DNA extracts. *A. laevis* and *A. morrowiae* colonized 10% and 59% of fine root length in Pinot noir cuttings. Sequences of clones obtained after PCR with AM1 and NS31 primers placed both samples within the Acaulosporaceae. The closest described species to the grape clones from *A. laevis*-colonized roots was *A. laevis* (GenBank accession number Yl7633), while clones from *A. morrowiae*-colonized grape roots were closely related to *A. rugosa* (GenBank accession number AM214005).

Factors influencing AMF phylotypes in roots.—Sampling time had no influence on phylotype richness (FIG. 3A) or on the relative abundance of unique phylotypes amplified from Pinot noir roots, except a single phylotype (GLO 3E), which was most abundant at véraison (TABLE V). Phylotype richness was lower in roots from red-hill soils (11) compared to valley soils (14), but a greater sampling effort would be required

TABLE IV. Predicted and observed frequency of *Glomus* clones in fine root mixtures with known proportions of *Glomus intraradices* and *Gigaspora rosea* (using AM1-NS31 primers)

| Percent *Glomus* roots in sample | Number of Clones | | | | | |
| | Experiment 1 | | | Experiment 2 | | |
	Observed	Expected	Total	Observed	Expected	Total
100	26	26	26	26	26	26
75	19	18	24	21	18	24
50	22	13.5	27	15	12.5	25
25	9	5	20	14	6.25	25
0	0	0	24	0	0	22
χ^2 significance[a]		0.013			0.005	

[a] Chi-square analysis included only those mixtures with both fungi present (df = 2).

to prove this (FIG. 3B). The abundance of individual phylotypes was affected by soil type, with six of the 16 fungal phylotypes significantly different in red-hill versus valley soils (TABLE V). The most striking
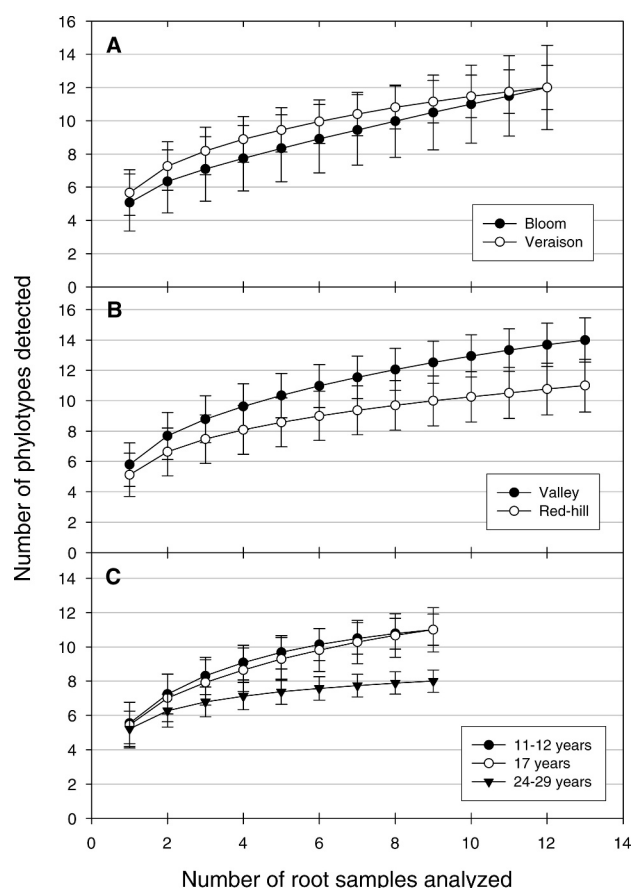

FIG. 3. Sampling effort curves for matched root samples used to assess the affect of (A) sampling time, (B) soil type and (C) vine age on AMF diversity. Curves were computed analytically in EstimateS 8.0 (Colwell 2005), and points are shown ± standard deviation. See MATERIALS AND METHODS for explanation of matched samples.

differences were the absence of GLO 3F in red-hill soils, the higher abundance GLO 3E in valley soils and the higher abundance of GLO 2 in red-hill soils. Vine age influenced the total number of phylotypes amplified from roots. The older vines in this study, representing some of the older vineyards in the region, had significantly fewer phylotypes in roots than the younger vines (FIG. 3C). The abundance of GLO 1A decreased while GLO 1B increased as vines aged, and GLO 3A decreased in abundance in the oldest vines (TABLE V).

Results from PCA indicated the abundance of AMF phylotypes GLO 3E, GLO 1D and GLO 3A were associated in different vineyards (more abundant in valley soils) and their co-occurrence might be linked to higher levels of soil nutrients (Ca, Fe, Mg, Mn and NO_3) along axis 1 (38% of variation in AMF phylotype abundance) (FIG. 4). The five remaining phylotypes were not closely associated, although GLO 1A and GLO 3C diverge from GLO 2 and GLO 3B along with differences in some leaf nutrients (C, N, K, Ca and Mg) along axis 2 (27% of variation in AMF phylotype abundance). These results support the differences we observed in AMF diversity based on χ^2 analysis. Valley soil vineyards formed a group generally located in the upper right quadrant of the PCA plot, while red-hill soil vineyards formed a loose group primarily located in the lower left quadrant. This separation coincides with greater abundance of GLO 2 (lower left quadrant) in red-hill sites and greater abundance of GLO 3E, GLO 1D and GLO 1A (upper right quadrant) in valley sites, as determined by χ^2 analysis.

Presence of Archaeospora/Paraglomus *in Pinot noir roots.*—All Pinot noir root extracts from the bloom sampling and 78% (seven of nine) of the extracts from véraison produced detectable products in the nested PCR using Archaeosporaceae primers (TABLE VI). The vast majority of clones sequenced closely matched either *Paraglomus* (40% of clones) or

TABLE V. Influence of sampling time (phenology), soil type and vine age on the abundance of AMF in Pinot noir roots

| | Number of clones in matched samples[a] | | | | | | |
| | Sample date | | Soil type | | Vine age (years) | | |
Phylotype	Bloom	Véraison	Valley	Red-hill	11–12	17	24–29
ARCH 1	1	0	0	0	0	0	0
SCUT 1	1	2	1	1	4	1	2
GLO 1A	60	64	44*	28*	55*	21*	31*
GLO 1B	17	21	8	13	10*	12*	25*
GLO 1C	2	2	3	2	4	2	0
GLO 1D	0	0	13*	1*	2	1	0
GLO 1E	1	0	6	0	2	0	0
GLO 1F	0	1	6	0	0	0	0
GLO 2	45	53	36*	70*	31	39	43
GLO 3A	91	75	98	106	69*	76*	36*
GLO 3B	3	3	0	6	4	4	1
GLO 3C	70	84	74*	110*	62	77	72
GLO 3D	0	3	0	8	0	8	0
GLO 3E	5*	19*	28*	8*	3	7	9
GLO 3F	0	0	18*	0*	0	0	0
GLO 5	2	3	1	0	0	0	0
GLO 6	0	0	1	0	0	0	0
Total number of clones	298	330	337	353	246	248	219
Total number of phylotypes	12	12	14	11	11	11	8
Number of vineyards (root samples) analyzed	6 (12)	6 (12)	3 (13)	3 (13)	2 (9)	2 (9)	2 (9)

[a] See MATERIALS AND METHODS for explanation of matched samples.

* Indicates significance of sample date, soil type, or vine age on the frequency of a given AMF phylotype (χ^2 analysis at 95% confidence).
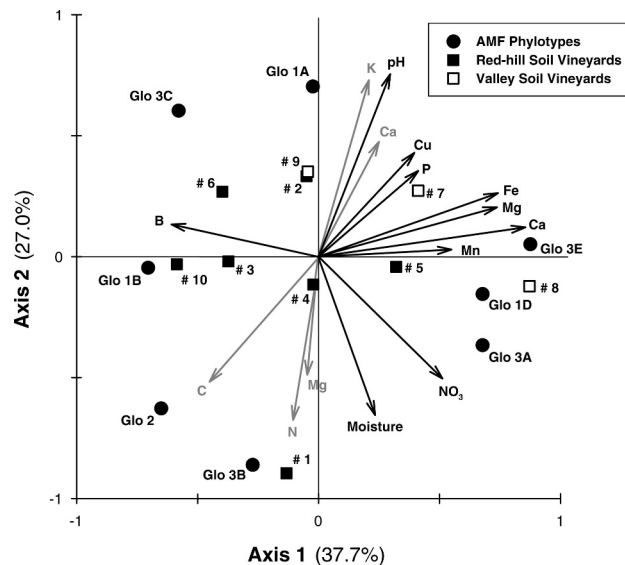

FIG. 4. Correlation bi-plot (PCA) of eight normally distributed AMF phylotypes with > 1% abundance in 10 Oregon vineyards. Vectors for soil (black lines) and leaf (gray lines) variables were passively projected into the ordination space (only those variables with vectors > 0.5 units long are shown). Vineyard coordinates were multiplied by 0.2 to fit the coordinate system.

Archaeospora (44% of clones) sequences in GenBank. Four clones were related to *Glomus* sequences, and a single clone was a non-AMF fungus (*Cladosporium elatum*). The *Paraglomus* clones in Pinot noir roots were most closely related to *Paraglomus* symbionts amplified from maize roots from loess in Switzerland (Hijri et al 2006; GenBank accession numbers 872024, 872025), while *Archaeospora* clones were most closely related to a sequence obtained from specialized, AMF-containing root nodules of a New Zealand rainforest tree (Russell et al 2002, GenBank accession number 452634) or to a sequence obtained directly from soil (unpubl, GenBank accession number 421303).

DISCUSSION

Similar estimates of AMF richness in Oregon Pinot noir vineyards were obtained based on the analysis of spores in soil and the analysis of DNA phylotypes amplified from grape roots. Seventeen unique spore morphotypes were identified from soil (TABLE II), while 18 unique sequence phylotypes were amplified from grape root extracts (TABLE III). However, there was a large difference in the identity of fungi detected

TABLE VI. Presence of *Paraglomus* and *Archaeospora* phylotypes in 'Pinot noir' roots in matching samples at bloom and véraison (n = 9)

Sampling time	# of + PCR reactions/total	# clones sequenced	*Paraglomus* clones	*Archaeospora* clones	*Glomus* clones	Other fungi
Bloom	9/9	18	8	7	2	1
Véraison	7/9	14	5	7	2	0
Total		32	13	14	4	1

by either approach. For example, six species within genus *Acaulospora* were found as spores in soil but no *Acaulospora* phylotypes were amplified from roots. The lack of agreement between AMF spores in soil and those amplified from grape roots contradicts Hijri et al (2006), who found fairly good agreement between spores isolated from soil and phylotypes amplified from roots in a survey of five agricultural fields. However, others have reported a poor match between AMF spores and AMF in roots of grassland plants (Börstler et al 2006, Hempel et al 2007). Indeed Hempel et al (2007) also detected *Acaulospora* phylotypes as spores in soils but did not find *Acaulospora* phylotypes in roots, similar to our results in vineyards.

We suspected that fungi within the Acaulosporaceae might not colonize grapevine roots because studies using AM1 and NS31 primers amplified fungi from the Acaulosporaceae from a variety of host plants (Helgason et al 1999, 2002, Husband et al 2002a, Öpik et al 2003). Results from our greenhouse study showed that Pinot noir can be colonized by *Acaulospora* spp. in sterilized soil and appropriate *Acaulospora* sequences are readily amplified from these roots. Therefore the lack of Acaulosporaceae species in roots from Oregon vineyards is apparently not a result of a procedural bias. Fungi within the family appear to be poor competitors (or colonizers) of grape roots in the vineyards sampled, which is supported by the relatively weak colonization of roots by both *Acaulospora* isolates in our greenhouse experiment. *A. laevis* had colonized only 10% of the fine root length of Pinot noir vines, but even *A. morrowiae* had notably less intense colonization of roots than typically observed in grape roots with other AMF (Schreiner 2007). The lack of *Acaulospora* clones having been amplified from 52 different root samples (> 1200 clones) in this study clearly shows AMF within the Acaulosporaceae are poor competitors in grape roots in the field. Therefore, it is likely that the six species of *Acaulospora* spores found in the vine row soils are associated with other host plant species (cover crops or weeds) present in our sites. Spores of *Glomus mosseae, Glomus clariodeum* and *Entrophospora infrequens* also were found in the

vine row soils but were never amplified from grape roots.

It is not surprising that most of the spores retrieved from the vineyards were described species (∼ 65%) while most phylotypes amplified from grape roots were not closely related to any described species (∼ 17% were related to known species). Root-amplified AMF are dominated by undescribed taxa in nearly all molecular studies of AMF diversity (Rosendahl 2008). Of the 15 *Glomus* phylotypes identified from grape roots, only GLO 3C is a described species, *G. intraradices*. This phylotype was the second most abundant across all vineyards, supporting its designation as a cosmopolitan fungus in many ecosystems (Sýkorová et al 2007). *G. intraradices* has dominated both soil and root trap cultures in our efforts to isolate and culture the phylotypes common in Pinot noir roots. Sequences of *G. intraradices* isolated from vineyard 4 were a perfect match (100% identity) of the GLO 3C sequence in our dataset, and this isolate has been deposited in INVAM (No. OR216). The other 14 *Glomus* phylotypes found in Pinot noir roots often were closely related to other root-derived sequences from various ecosystems around the world, but none were closely related to any described (known) AMF species in GenBank (FIG. 2).

Several other studies have found *G. intraradices* (or closely related fungi) dominates the community of AMF amplified from roots, with few if any fungal clones representing fungi from the Acaulosporaceae or Gigasporaceae clades (Hijri et al 2006, Husband et al 2002a, Lekberg et al 2007, Öpik et al 2003, Jumpponen et al 2005, Sýkorová et al 2007, Wirsel 2004). Fungi within the Gigasporaceae might be more prevalent in winter (Helgason et al 1999), which could explain the scarcity of our SCUT 1 phylotype in Pinot noir roots. However, fungi from the Gigasporaceae also might be under-represented in comparison to other AMF when amplifying root DNA extracts, as was demonstrated by the results from our mixed root sample experiment with *G. intraradices* and *Gi. rosea* (TABLE IV). The lower than expected abundance of *Gi. rosea* in our mixed root samples was probably due to the absence of vesicles (Smith and Read 1997) in *Gi. rosea*-colonized roots. Vesicles (produced by most

fungi in the Glomeraceae and Acaulosporaceae) obviously would provide a greater quantity of easily extracted AMF DNA as compared to much smaller hyphae and arbuscule structures produced in roots by all AMF. While the SCUT 1 phylotype might be more prevalent in grape roots than our clone numbers indicate, it is still unlikely to be a major root colonizer in the field because its total abundance was less than 1% (TABLE III). The SCUT 1 phylotype amplified from Pinot noir roots is most likely *Scutellospora calospora*, which was found as spores in 90% of our vineyards (TABLE II) and which is believed to be synonymous with *Scutellospora dipurpurescens* (C. Walker pers comm), the known fungus that SCUT 1 was closely related to in our likelihood analysis (FIG. 2). It appears that *S. calospora* also might be a poor competitor in colonizing grape roots in Oregon vineyards or that the host plant favors fungi from Glomerales.

The common occurrence of *G. mosseae* spores in our vineyards, but the absence of this fungus in root amplicons also was reported from peas in an agricultural field (Kjøller and Rosendahl 2001) and from a mixed grassland community (Hempel et al 2007). Fungi within the *G. mosseae* clade are considered to be ruderal species because they have been common early colonizers in tree seedlings (Husband et al 2002a) and in greenhouse trap plants (Sýkorová et al 2007) and later are replaced by other phylotypes after 10–12 mo. *G. mosseae* was one of the most common spores in the vineyard soils, and this fungus colonizes grape roots intensely and improves P uptake and plant growth substantially (Schreiner 2007). *G intraradices* also might be a ruderal species, being a common early colonizer and later replaced by other phylotypes in tree seedlings (Husband et al 2002a). However our data from grape roots do not support this hypothesis. We found *G. intraradices* was a dominant colonizer of Pinot noir in nearly every vineyard and actually had the lowest abundance from roots at the tilled site in our dataset (vineyard 1). We also detected no change in the abundance of *G. intraradices* with vineyard age (TABLE V).

We found no temporal change in AMF diversity in grape roots within a single growing season (TABLE V). Indeed the striking similarity of phylotype abundance and diversity between bloom and véraison after we analyzed the first 12 root samples from the latter time point precluded any further analysis. Others have found significant changes in AMF diversity in roots over similar short intervals (Helgason et al 1999, Husband et al 2002a, b, Sýkorová et al 2007). While other studies also have reported no change in AMF diversity in roots over a single growing season (Rosendahl and Stukenbruck 2004, Wirsel 2004).

This lack of change over a growing season in our vineyards is perhaps not surprising when one considers that the soil profile in the vine row (where we sampled) is a stable environment (no tillage, except vineyard 1). Although a shift in the AMF community in grape roots did occur as vines aged from about 12 y to more than 24 y. The abundance of GLO 1A and GLO 3A declined in roots as vineyard age increased, and these phylotypes were partially replaced by greater abundance of GLO 1B (TABLE V). Our data also suggest the AMF diversity in the roots of grapevines decreases as plantings aged beyond ~ 24 y (FIG. 3), but this finding needs to be rigorously tested.

Different AMF communities were found in Pinot noir roots growing in different soil types (TABLE V). GLO 2 and GLO 3C (*G. intraradices*) were more abundant in roots from vineyards with red-hill soils, while four phylotypes were more abundant in vineyards with valley soils. These differences also were observed by PCA ordination of common AMF phylotypes and indicate that the diversity of fungi in grapevine roots is related to soil fertility and plant nutrient status (FIG. 4). The AMF community in valley soils diverged from red-hill soils (albeit weakly) along with higher levels of many soil nutrients and pH, suggesting PCA is a meaningful tool for understanding AMF communities in Pinot noir. Valley soils generally are more fertile (particularly for Ca, Mg and P) and have higher pH than red-hill soils (Schreiner and Linderman 2005). Indeed young Pinot noir vines do not grow appreciably without AMF in red-hill soils but can achieve similar growth rates with or without AMF in valley soils (Schreiner 2007).

More than 90% of the root samples we examined with the nested PCR and primers for the ancestral AMF produced clones with sequences closely related to either *Paraglomus* or *Archaeospora*. All clones we sequenced generated with these primers (excluding four *Glomus* clones and one Ascomycete clone) showed strong homology to the same, few accessions in GenBank. The *Paraglomus* clones we amplified from Pinot noir are likely to be *Paraglomus occultum* because this fungus has been found commonly in soils from numerous vineyards around the world (Cheng and Baumgartner 2004, Oehl et al 2005, Schubert and Cravero 1985). The *Archaeospora* clones from Pinot noir roots are most likely *Archaeospora trappeii*, which was identified from spores in soil at two of our vineyards (TABLE II).

CONCLUSIONS

Similar estimates of AMF species richness were found in Oregon vineyards based on spores in soil or AMF

DNA amplified from roots, although different fungi were identified by each method. We relied more heavily on the results from PCR because we are most interested in AMF that colonize grape roots in the field. However, while PCR can amplify only fungi that are present in (or on) the roots that are extracted, we do not know what fungi might be missed (or at least under-represented) with this approach. On the other hand examining spores in soil might be unreliable in identifying fungi that colonize a given host plant because those fungi identified as spores might associate with another host plant in the system. By using both approaches and by conducting additional controlled experiments we have shown that grapevines associate more readily with certain AMF (*Glomus* spp.) and exclude other fungi in the field (Acaulosporaceae). What ultimately drives host plant selectivity for specific AMF by grapevines and the resulting community structure of AMF in grape roots is largely unknown but appears to involve soil fertility in different soil types and vineyard age.

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