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Seasonal Dynamics of Arbuscular Mycorrhizal Fungal Communities in Roots in a Seminatural Grassland[▽]†

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Symbiotic arbuscular mycorrhizal fungi (AMF) have been shown to influence both the diversity and productivity of grassland plant communities. These effects have been postulated to depend on the differential effects of individual mycorrhizal taxa on different plant species; however, so far there are few detailed studies of the dynamics of AMF colonization of different plant species. In this study, we characterized the communities of AMF colonizing the roots of two plant species, *Prunella vulgaris* and *Antennaria dioica*, in a Swedish seminatural grassland at different times of the year. The AMF small subunit rRNA genes were subjected to PCR, cloning, sequencing, and phylogenetic analysis. Nineteen discrete sequence types belonging to *Glomus* groups A and B and to the genus *Acaulospora* were distinguished. No significant seasonal changes in the species compositions of the AMF communities as a whole were observed. However, the two plant species hosted significantly different AMF communities. *P. vulgaris* hosted a rich AMF community throughout the entire growing season. The presence of AMF in *A. dioica* decreased dramatically in autumn, while an increased presence of *Ascomycetes* species was detected.

In temperate and boreal regions, seminatural grasslands are some of the most species-rich plant communities (37). Grasslands in Europe have been managed during the last millennia by grazing and haymaking. The total area and connectivity of grasslands have decreased dramatically during the last hundred years (77). This decline is one of the major threats to diversity for many groups of organisms (44).

Arbuscular mycorrhizal fungi (AMF), belonging to the phylum *Glomeromycota*, are obligate symbiotic fungi forming mutualistic associations with the roots of most land plants. Increased access to low-mobility soil mineral nutrients has been considered to be the main beneficial effect of AMF on their host plants (61). Experimental greenhouse studies have shown that AMF diversity can affect the diversity and productivity of the host plant communities and, therefore, the functioning of the whole ecosystem (21, 33, 74, 76, 78). Other benefits to host plants arising from the presence of AMF include a reduction in the occurrence of pathogenic infections (5, 41), the improvement of water relations (9), and the limitation of heavy-metal uptake (39). The effects of AMF diversity and species composition are thought to arise through differential effects of different AMF taxa on the growth of individual plant species (47), but descriptive ecological data on the structure of AMF communities are still few in comparison with the available information on the structure and dynamics of plant communities (52).

Investigations of the phenology, diversity, and functioning of natural AMF communities have traditionally been based on root colonization estimates and spore counts. The extension of the intraradical mycelia gives information on the mycorrhizal status of the plant but can provide hardly any information about the identities of the fungi. Soil spore analyses allow a taxonomical characterization of AMF communities, but sporulation is a seasonal phenomenon that can be highly related to the physiological status of the fungus. Moreover, many fungal taxa, known only from their DNA sequence signatures, do not seem to produce spores at any stage during their life cycles. The use of molecular tools to characterize the AMF colonizing the root systems of different plant species is not entirely problem free but presently provides new opportunities to improve our understanding of the role these fungi play in plant ecology. Both the percentage of root colonization (11, 15, 16, 19) and the compositions of AMF communities, as estimated from spore counts (3, 17–19, 66), have been shown to be highly seasonal in some cases. Less is known about the dynamics of the AMF colonization of root systems. The temporal changes in the AMF communities colonizing roots have been characterized by molecular methods in only a few studies. Interannual changes in the AMF community in a tropical forest in Panama have been reported previously (31), and temporal trends at the seasonal level have been analyzed in only two studies (50, 71). At the same site as that studied here, Santos et al. (54) detected a decreased occurrence of AMF, not only in relation to increasing levels of soil mineral N but also between June and September, by using denaturing gradient gel electrophoresis to analyze the AMF communities in two different plant species. To date, no study has used an intensive sampling schedule to determine whether there are major seasonal variations in the specific compositions of AMF communities across the growing season and how fast these possible shifts occur.

In the present study, two common plant species with different ecological requirements and wide distributions within the

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TABLE 1. Description of the group-specific primers used in the PCR targeting the 18S rRNA gene

Phylum or domain	Targeted group(s)	Primer sequence (5′–3′)	Primer name	Reference
Glomeromycota	*Glomus* group A, *Acaulosporaceae*, and *Gigasporaceae*	GTT TCC CGT AAG GCG CCG AA	AM1	26
	Glomus group B	GTT TCC CGT AAG GTG CCA AA	AM2	This study
	Diversisporaceae (*Glomus* group C)	GTT TCC CGT AAG GTG CCG AA	AM3	This study
Eucarya	All groups	TTG GAG GGC AAG TCT GGT GCC	NS31	60

same site studied previously by Santos et al. (54) were chosen. *Antennaria dioica* (L.) Gaertn. (*Asteraceae*) is a dioecious, perennial plant occurring in nutrient-poor, relatively dry, and often heavily grazed habitats (14). It reproduces often through stolons, which it uses to form monospecific patches. *Prunella vulgaris* L. (*Lamiaceae*) is a perennial clonal forb with a prostrate habit. It grows in soils with higher moisture and nitrogen levels than those in which *A. dioica* occurs, and ecologically, it is considered to be more of a generalist. It can occur in pastures, lawns, and open woods and also on disturbed land (57). *P. vulgaris* has been reported to be highly dependent on mycorrhiza during growth in lab experiments (73), and some traits of clonal reproduction seem to be affected by the identities of the glomalean fungi with which the plant forms mycorrhizal associations (62, 63).

The aim of the present study was to (i) describe the AMF colonizing the roots of two plant species in a seminatural grassland and (ii) determine whether there is a seasonal shift in the compositions of the AMF communities in the roots.

MATERIALS AND METHODS

Study site and sampling. Root samples were collected in a seminatural pasture located in central Sweden (Hönsgärde, Uppland County; 59°45′N, 17°57′W). The grassland is managed with cattle grazing. The understory vegetation is dominated by perennial forbs and grasses. Dispersed shrubs (*Juniperus communis* L.) are the dominant element in the overstory layer. The soil is a sandy loam with a pH of 5.1 to 6.2. More data on this site have been published previously (54).

Five plots, each 2 m in diameter, were established. The plots were located between 50 and 100 m from one another. One *P. vulgaris* plant and one *A. dioica* plant from each plot were sampled on 21 July, 20 August, 23 September, and 25 October 2004 and 17 May and 22 June 2005. Plants, including parts of their root systems, were collected along with a 10-cm-deep soil core of 7 by 7 cm. Soil cores were placed in plastic bags and immediately transported to the laboratory, where they were kept frozen at −20°C until DNA was extracted.

DNA extraction from roots and PCR. Soil cores were thawed at room temperature. The roots of the targeted plant species were thoroughly rinsed in tap water and cut into 1-cm pieces. DNA was extracted using a DNeasy plant kit (QIAGEN, Crawley, United Kingdom). For each sample, a total length of 6 cm of root material was placed into a 2-ml screw-cap propylene tube half filled with 2.5-mm-diameter zirconia-silica beads (BioSpec Products, Bartlesville, OK). The tubes were filled with 700 μl of AP1 buffer from the DNeasy plant kit and shaken in a Mini-BeadBeater (BioSpec Products, Bartlesville, OK) at 5,500 rpm for 20 s, kept on ice for 1 min, and then shaken again with the same parameters.

PCR amplification procedures targeted a 550-bp-long region of the 18S rRNA gene. The universal eukaryotic NS31 primer (60) was used as the forward primer. A mixture of equal amounts of the AM1 (26), AM2, and AM3 primers was used as the reverse primer combination (Table 1). The AM1 primer, originally designed for the specific amplification of *Glomeromycota* DNA, presents some mismatches at the priming site with taxa belonging to *Glomus* group B and *Glomus* group C (*Diversisporaceae*) (59). The primers AM2 and AM3 are modifications of the primer AM1 and aim to amplify DNA from taxa belonging to *Glomus* group B and *Glomus* group C (*Diversisporaceae*), respectively. DNA from the divergent families *Archaeosporaceae* and *Paraglomeraceae* remain untargeted by the primer combination used in this study.

A PCR amplification kit (PuReTaq Ready-To-Go; Amersham Biosciences, Uppsala, Sweden) was used with a final reaction volume of 25 μl. As a template, 2 μl of extracted DNA was used in all reactions. The thermocycling program was as follows: 95°C for 3 min; 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min and 30 s; and 72°C for 8 min. Reaction mixtures were overlaid with a drop of mineral oil and run in a DNA thermal cycler (model 480; Perkin Elmer, Wellesley, MA). Reaction yields were estimated by using a 1.2% agarose gel containing ethidium bromide.

Cloning. PCR products were cloned into *Escherichia coli* plasmids by using the TOPO TA cloning kit according to the instructions of the manufacturer (Invitrogen, CA). The transformed bacterial colonies were picked with autoclaved wooden sticks and dipped into the PCR tubes with the rest of the PCR mixture. The reamplification procedure used the same primers and cycling program as the first PCR described above. Product quality and size were checked in agarose gels as described above. Of the clones, 96.3% had inserts of the correct size.

Sequencing. Before sequencing, PCR products were purified with the QiaQuick PCR purification kit (QIAGEN, Crawley, United Kingdom) with a final elution volume of 30 μl. Eight clones per library, i.e., root sample, were sequenced using NS31 as the sequencing primer. Sequencing reactions were carried out with the BigDye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA), in a GeneAmp PCR system 9700 (PE Applied Biosystems, Warrington, United Kingdom). The PCR products were cleaned using DyeEx 96 plates (QIAGEN, Crawley, United Kingdom) and sequenced in a 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

Phylogenetic analysis. A search for sequences similar to the ones from this study was done with the BLAST tool (2) provided in GenBank. The sequences identified by the search, together with sequences from cultured AMF taxa, including representatives of the major groups described by Schüssler et al. (58), were downloaded. A multiple alignment was assembled using the CLUSTAL_X program (69). The result was refined by eye with the sequence alignment editor Se-Al (46). *Endogone pisiformis* Link and *Mortierella polycephala* Coem, two species of *Zygomycota*, which is a sister group to *Glomeromycota* (67), were used as the out-group. The region with the most variation and, thus, with the most relevant phylogenetic information lies approximately 70 to 300 bp from the 5′ end of the amplicon. This region was used in the following steps of the phylogenetic analysis.

Phylogenetic relationships were analyzed using maximum parsimony (MP). The MP analysis was performed using PAUP* version 4.0b10 for Macintosh (65). A heuristic search with 1,000 replicates and the random addition of sequences was performed using tree bisection and reconnection as a swapping algorithm, with the MulTrees option in effect and no more than 20 trees saved for each replicate. Gaps were treated as missing data, uninformative characters were removed, and the rest were unordered and of equal weight. An MP bootstrap analysis (1,000 replicates) was performed with 10 random taxon additions per bootstrap replicate and the MulTrees option in effect to save no more than 10 trees for each replicate of random addition sequence.

Definition of sequence types. Among the *Glomeromycota*, the rRNA gene can have different variants within the same species and even within the same spore (53). It is therefore not always possible to assign a set of sequences to a particular species. Sequence types, equivalent to operational taxonomical units, were defined as groups of closely related sequences, usually with a high level of bootstrap support in the phylogenetic analysis and a level of pairwise similarity higher than 94.3%. This cutoff value, based on the ca. 230-bp-long region defined above, is approximately equivalent to a similarity of 97%, which in previous studies has been considered a threshold for separating possible AMF species (28) in analyses of sequence divergences along the whole 550-bp-long amplicon. The pairwise analysis within clusters was carried out using Lasergene MegaAlign 3.1.7 (DNAStar Inc., NY). The sequence types were named for the genera to which they belonged (Glo for *Glomus* and Aca for *Acaulospora*), followed by a number, e.g., Glo3 and Aca3. The number relates to previously described sequence types

that were found in GenBank and named according to the system used by others (27). This way of naming clones is the one most widely used in field studies of this type. In some cases, the names are followed by a letter indicating similarity to previously described groups, e.g., Glo50a and Glo9b. New AMF sequence groups of unknown identity are indicated by "unk" followed by a number, e.g., Glo unk1 and Aca unk1.

Statistical analysis. Multivariate analyses were used in order to analyze the AMF community distribution patterns and to test whether AMF community composition was related to the environmental variables of sampling time and plant host. Firstly, an indirect gradient analysis was carried out in order to discern the differences in AMF communities among the different samples. Detrended canonical analysis (DCA) as implemented in CANOCO 4.52 (Biometrics-Plant Research International, Wagenigen, The Netherlands) was used instead of canonical analysis in order to avoid the arch effect (68). Sequence type abundances in the species data set were coded as relative abundances in relation to the total number of glomalean clones detected in the clone library. This approach assumes that all DNA behaves in a similar way during the extraction, amplification, and cloning processes (27). Sequences of nonglomalean origins were excluded from the analysis. The seven samples that either did not give PCR products or produced only nonglomalean sequences were excluded from the DCA.

A constrained ordination model was constructed in order to analyze and test the effects of sampling time and host plant upon the whole AMF community. Canonical correspondence analysis (CCA) was used since the DCA indicated (length of gradient, >3) that at least some species had a unimodal distribution (68). The environmental variable of plant host was coded as a dummy variable, i.e., 0 and 1. The sampling time was coded as a quantitative variable in order to detect linear trends in the data (38). Plot identity was included as a covariable. The analysis focused on intersample distances and used Hill's scaling. The statistical significance of the environmental variables was evaluated by manual forward selection using a Monte Carlo permutation test with 499 permutations. Permutations were restricted by using the restrictions available in the CANOCO software for experimental designs of the split-plot type. Permutations with cyclic shifts were applied for all the analyzed samples from the same plot in different months. In order to have homogeneous sizes among the different sample levels, the seven samples with missing values were interpolated. In these cases, species frequencies were calculated as the average of those occurring in the rest of the samples.

Sample-effort curves for the observed richness and for the nonparametric richness estimator ACE (7) were obtained using the software EstimateS 7.50 (8). The sample order was randomized by 1,000 replications. The species abundance matrix was previously transformed into an incidence matrix (presence-absence data).

Nucleotide sequence accession numbers. Sequences generated in this study were registered in GenBank under the accession numbers EF408992 to EF409238.

RESULTS

Identities of the sequences in the clone libraries. DNA extraction from 60 root samples was conducted. Only five samples did not give any PCR product. From the 55 clone libraries, a total of 427 sequences were obtained. The BLAST search revealed that 354 sequences (82.9%) had a high degree of similarity to sequences from taxa belonging to the phylum *Glomeromycota*, 71 sequences (16.6%) were similar to sequences from the *Ascomycota*, and 2 sequences (0.5%) were similar to sequences from the *Zygomycota*. Sequences belonging to the phylum *Ascomycota* were detected in 24 clone libraries, and the two identical sequences belonging to the phylum *Zygomycota* (with 97% similarity to sequences from *E. pisiformis*, a mycorrhizal fungus) were detected in the same library. The portion of the small subunit rRNA gene analyzed did not have enough variation to support taxonomic affiliation at the species level with known sequences of *Ascomycetes*.

Phylogenetic analysis of AMF sequences. The constructed alignment included 95 sequences that were downloaded from GenBank and the 173 different glomalean sequences that were recovered in this study. Some clones produced the same sequence and are represented just once in the alignment (see the supplemental material for a detailed description of the clone groups with identical sequences). The alignment had 278 characters, 138 of which were parsimony informative. The MP search found 2,880 equally parsimonious trees of 916 steps (Fig. 1). The sequences obtained in this study were assigned to 19 discrete groups. The sequences within each group had pairwise similarities ranging from 94.3 to 100%.

The families *Paraglomeraceae*, *Gigasporaceae*, and *Diversisporaceae* were well supported in the analysis (bootstrap value, 100%). The families *Acaulosporaceae* and *Archaeosporaceae* were unsupported. *Glomus* group A and group B, representing two putative families within the order *Glomerales* (59), were relatively well supported (bootstrap values, 84 and 77%, respectively) but appeared to be paraphyletic in the analysis.

Overall, 19 sequence types were detected. *Glomus* group A was the most frequently represented group, with 14 sequence types belonging to this group found among 310 clones. *Glomus* group B corresponded to two types, and *Acaulospora* was represented by three types. Only one sequence may be related to a previously named species: Glo8 clustered together with the species complex *Glomus intraradices-G. fasciculatum*. Glo3 clustered together with the taxonomically undescribed *Glomus* sp. isolate UY1225. The sequences assigned to Aca2 showed high degrees of variation, nesting together with several previously named and spore-derived sequences, but an unequivocal affiliation with any of these known sequences could not be established. The same was true for Glo unk6 within *Glomus* group B.

Most of the sequence types were related to root-derived sequences in GenBank and are therefore not taxonomically characterized. Three AMF sequence types, Glo unk5, Glo unk4, and Glo unk2, did not seem to be related to any previously reported sequences.

AMF community structure. *P. vulgaris* had a richer AMF community than *A. dioica*, hosting all of the 19 types found in this study (Fig. 2A). The sequence type Glo50a was the most widespread; it was present in 61% of the samples and accounted for 21% of the AMF sequences found in the clone libraries. Overall, the four most common sequence types (Glo50a, Glo2, Glo3, and Glo8) accounted for 65% of the AMF sequences. These types were relatively equally distributed in both plant hosts, with the exception of Glo8, which corresponded to a species that colonized *P. vulgaris* more frequently. Three of the more uncommon types (Glo unk2, Glo9b, and Aca2) were also found more frequently in samples from *P. vulgaris*, while Glo17 seemed to be more frequent in *A. dioica*. Four rare sequence types (Glo unk7, Glo10, Glo unk4, and Glo unk5) appeared exclusively in *P. vulgaris* roots. No sequence types occurring exclusively in *A. dioica* were found in this study.

The species-effort curves (Fig. 2B) showed a decreasing rate of accumulation of sequence types but did not reach an asymptote. This pattern indicates that the real richness of the AMF communities was higher than that measured. The estimated richness curves increased steeply with small sample sizes, producing a hump-like profile. With increasing numbers of analyzed samples, the species accumulation curves flattened and the trend in the estimated richness stabilized, following the observed richness more closely. The final predicted richness of

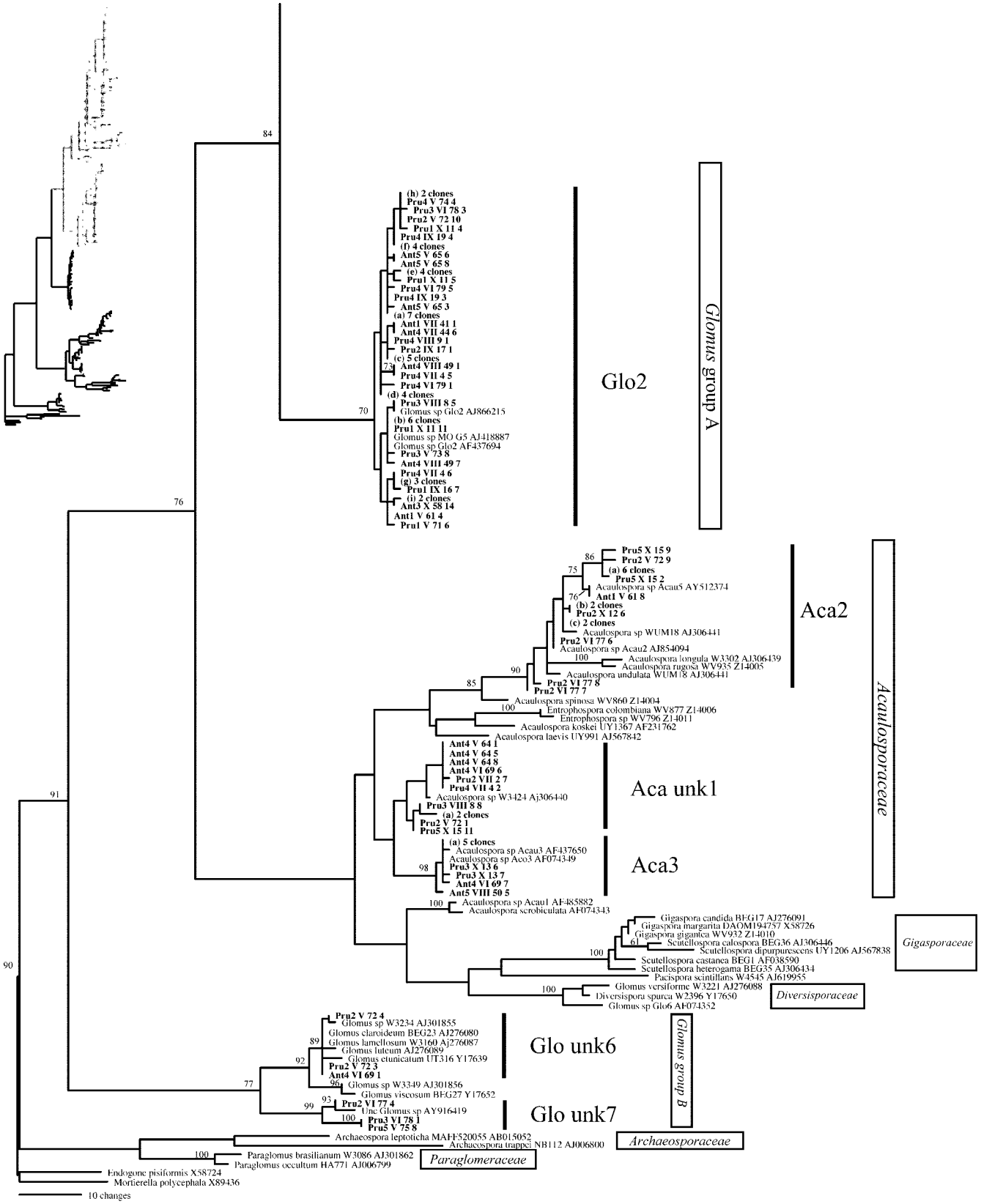

FIG. 1. One of the most parsimonious trees obtained in the MP analysis showing the phylogenetic affiliations of the sequences of AMF obtained from the roots of *P. vulgaris* and *A. dioica* at different times of the growing season. *E. pisiformis* and *M. polycephala* were used as out-groups. The values above the branches are bootstrap values (1,000 replicates) for an MP analysis; only values of ≥70% are shown. Sequence identifiers from this study are in bold type, indicate the host (Pru, *P. vulgaris*; Ant, *A. dioica*) and the time of collection (V, May; VI, June; VII, July; VIII, August; IX, September; X, October), and include a clone identity number. Sequence group identifiers on the right (e.g., Glo2 and Aca2) identify clusters with pairwise sequence similarities of ≥94.3%. Identical sequences are grouped and represented by letters, followed by the number of clones having that particular sequence. See the supplemental material for a detailed description of the clone identifiers included in each group. White boxes to the right indicate different AMF families and groups (58).

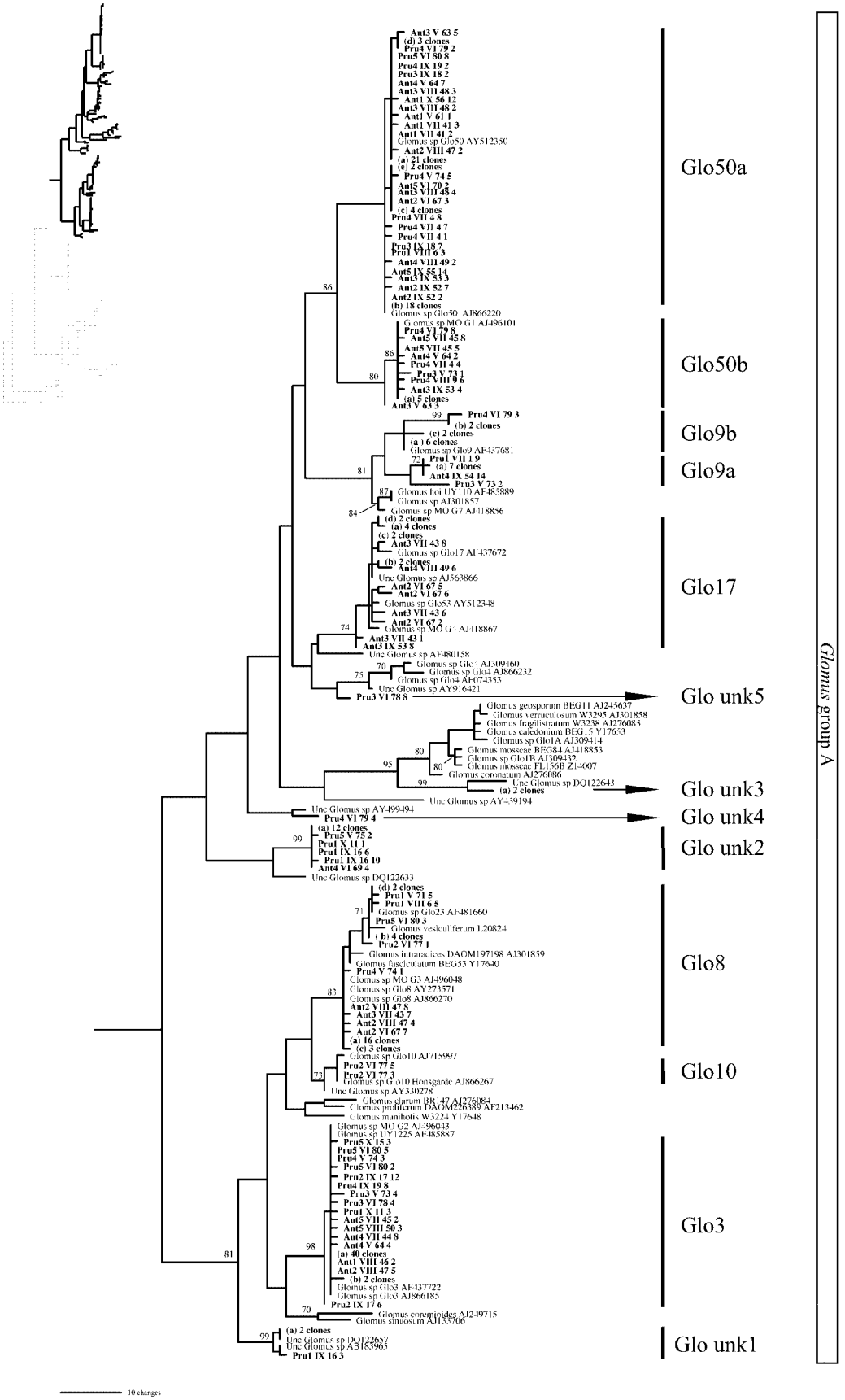

FIG. 1—*Continued.*

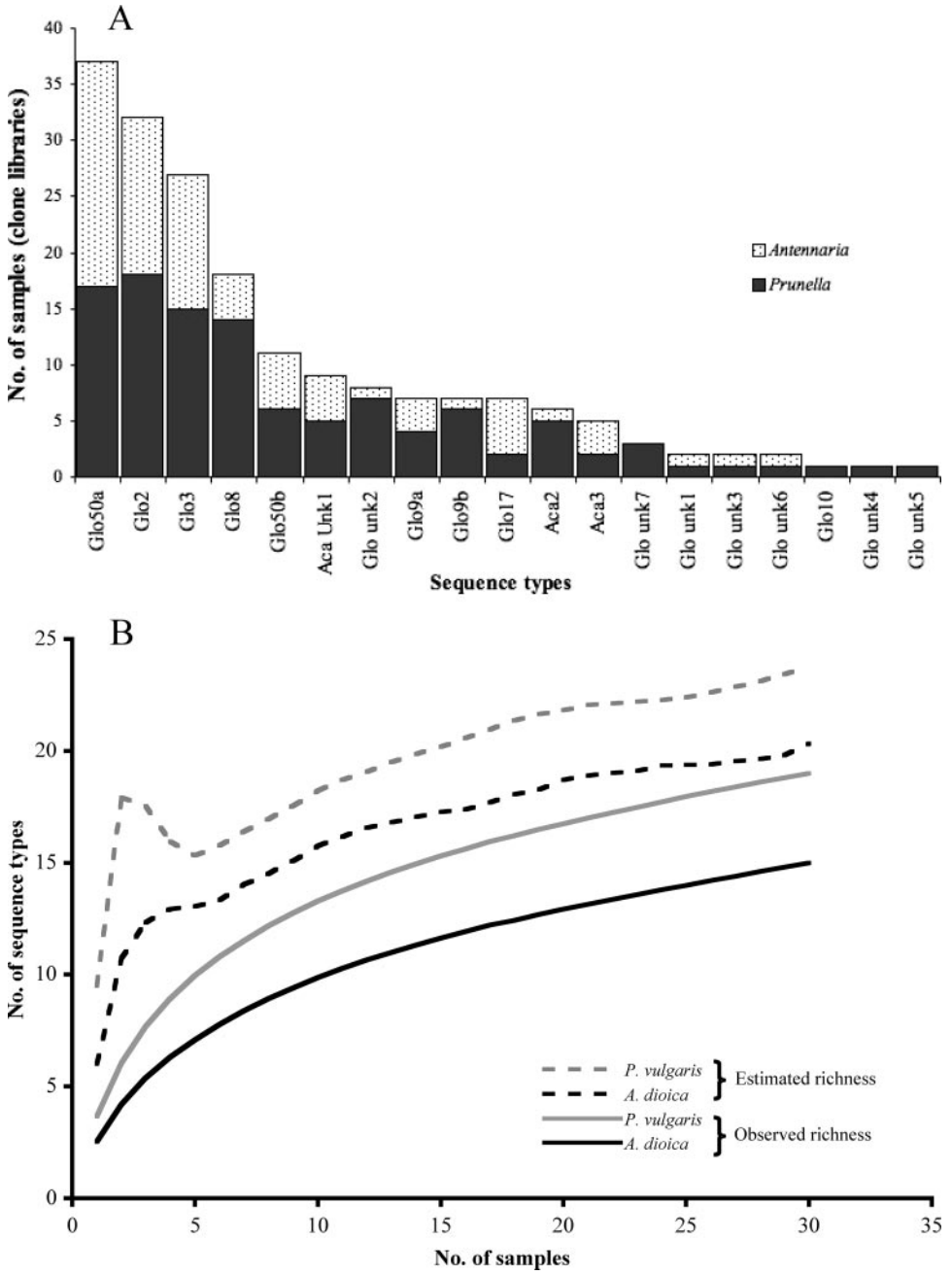

FIG. 2. (A) Rank frequency diagram of AMF sequence types present in the roots of *P. vulgaris* and *A. dioica*. (B) Sample-effort curves for the AMF community representing the observed and the estimated richness. The sample order was randomized by 1,000 replications. Both diagrams are based only on presence-absence data for each sequence type in the clone libraries.

sequence types using the ACE estimator for *P. vulgaris* or *A. dioica* was 23.8 or 20.3, respectively.

Seasonal variation in the compositions of the AMF communities was evaluated by comparing the relative abundances of the sequence types in the clone libraries (Fig. 3). The degree of AMF richness was highest in the May and June samples from both plant hosts. *P. vulgaris* showed a relatively high number of AMF sequence types in both summer and autumn. In the September and October samples from *A. dioica*, the level of AMF richness decreased. This decrease was accompanied by a dramatic increase in the proportion of *Ascomycetes* sequences. Among the most common AMF sequence types, only Glo8 showed any obvious seasonal trend. This sequence type was hardly present in September and absent in October.

The significance of the sampling time and the host for the AMF community composition was tested using multivariate analysis. This analysis did not consider the presence of *Ascomycetes* in the libraries since the focus of the study was on the seasonal trends of AMF. The two main axes of the DCA explained 24.1% of the total variance in the species data

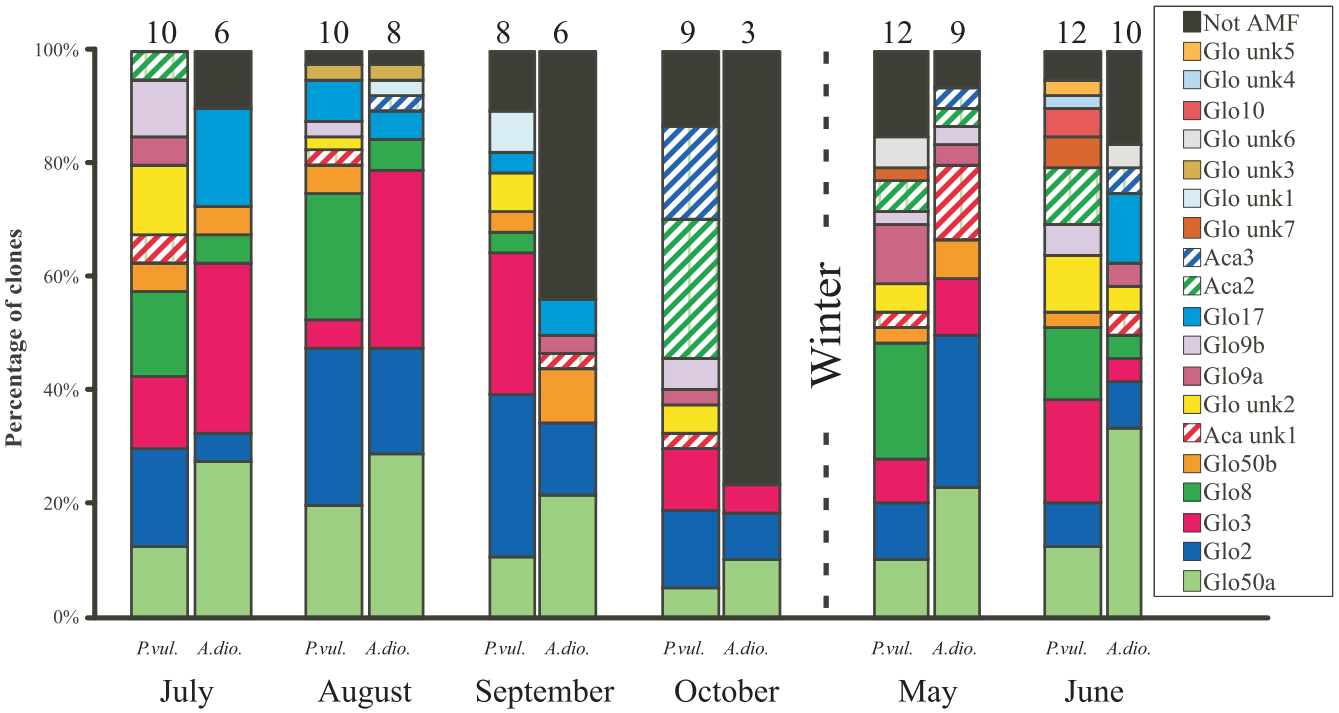

FIG. 3. Proportional distribution of the AMF sequence types in the roots of *P. vulgaris* (*P.vul.*) and *A. dioica* (*A.dio.*) at different times during the growing season. The *y* axis indicates the proportion of clones assigned to each particular sequence type. Proportions were calculated by pooling samples from all the plots at the same time points for each host. The bar widths are proportional to the numbers of studied clone libraries. The richness of AMF sequence types is indicated on the tops of the respective bars.

(Table 2). In the DCA ordination diagram (Fig. 4), the symbols representing samples from *P. vulgaris* are spread throughout and intermingle to a certain extent with those representing samples from *A. dioica*, which cluster in the upper right part of the diagram.

In the CCA model (Table 2), the two main axes explained 16.7% of the variance in the species data matrix. Only the variable of plant host was found to have a significant effect on the AMF community in the forward analysis ($F = 2.337$; $P = 0.022$). The variable of sampling time was found to be nonsignificant, both in the consideration of all the samples together ($F = 1.148$; $P = 0.272$) and in analyses of the *A. dioica* samples ($F = 1.795$; $P = 0.168$) and the *P. vulgaris* samples ($F = 0.811$; $P = 0.740$) separately.

In a parallel analysis in which the identities of the plots (coded as dummy variables) were used as a predictor instead of covariables, the Monte Carlo permutation test did not find any plot to have a significant effect upon the AMF species composition ($F = 2.375$; $P = 0.116$).

DISCUSSION

Composition of the AMF community. The AMF communities colonizing the roots of *P. vulgaris* and *A. dioica* were

TABLE 2. Results from the ordination analysis

Model and parameter	Result for:				
	All axes	Axis 1	Axis 2	Axis 3	Axis 4
DCA					
Eigenvalue (inertia)	4.806	0.703	0.458	0.292	0.216
% of variance of species data explained	34.7	14.6	9.5	6.1	4.5
CCA					
Eigenvalue (inertia)	3.855	0.16	0.482	0.399	0.379
% of variance of species data explained	36.8	4.1	12.6	10.3	9.8
Correlation with host (*Antennaria*)[a]		−0.5887	−0.2126	0	0
Correlation with host (*Prunella*)[a]		0.5887	0.2126	0	0
Correlation with sampling time		0.3257	−0.3842	0	0

[a] Significative variable in forward selection ($P < 0.05$) using Monte Carlo permutation tests ($n = 499$).

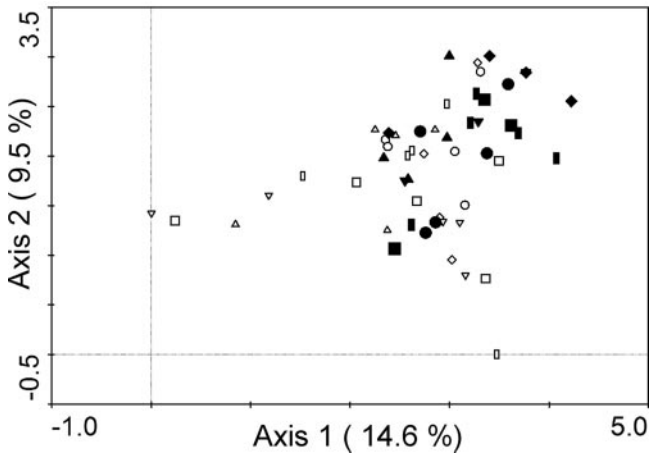

FIG. 4. DCA diagram. Open symbols represent AMF communities in *P. vulgaris* and closed symbols represent AMF communities in *A. dioica*. Sampling months are represented as right-side-up triangles for May, squares for June, rectangles for July, circles for August, diamonds for September, and inverted triangles for October.

dominated by a few sequence types belonging to *Glomus* group A. Three types belonging to *Acaulospora* and two types corresponding to *Glomus* group B were found among the more infrequent sequence types. No sequences from *Scutellospora* and *Diversispora* were detected, although in a previous study (54), we found sequences belonging to these two genera (two types and one type, respectively) at the same locality; nor were sequences belonging to the previously observed sequence type Glo4 (*Glomus* group A) detected. Between that previous study and the present study, 23 sequence types have been found at this site. This level of richness is similar to that demonstrated by the 24 types found previously both in an upland grassland in Scotland (71) and in a grassland in Japan (51). However, those richness values may not be directly comparable, since slightly different criteria were used to define sequence types. The common sequence types Glo3, Glo8, and Glo2 in our study are dominant types in grasslands in different parts of the world (43), but they also occur in other ecosystems. The most common sequence type we detected was Glo50a, which was more common in *A. dioica* than in *P. vulgaris* (Fig. 2A and 3). This sequence type has previously been found in *Pulsatilla* species roots in boreal forest and dry meadows in Estonia (42) and in different plant species from a coastal grassland in The Netherlands (56). In the latter study, Glo50a seemed to be dominant only in *Hieracium pilosella*, a plant that often grows together with *A. dioica* at our study site and that has similar ecological requirements (14). These data suggest that Glo50 typically occurs in dry and nutrient-poor soils in grasslands. On the other hand, the species corresponding to Glo8, identified as *G. intraradices*, seldom colonized *A. dioica*. The increased presence of *G. intraradices* was previously found to be related to nitrogen-enriched soils (34, 36), and this species is considered to be a nitrophilic taxon. In a study of different plant species (56), *G. intraradices* was also more frequent in legumes, which had higher N contents than nonlegumes. Among the legumes, *G. intraradices* was more frequent in nodules, where atmospheric nitrogen fixation takes place, than in roots. In a previous study using only the AM1 primer (54), we failed to detect sequences from *Glomus* group B and found only a single *Diversisporaceae* sequence, potentially due to incompatibilities between the AM1 primer and the corresponding targets of these AMF groups. The multiple-primer strategy employed here would be expected to detect these groups, but we detected only two *Glomus* group B sequence types and no *Diversisporaceae* sequences.

As in several other studies (27, 56, 70), we found an AMF community dominated by taxa belonging to *Glomus* group A. This dominance would occur within the roots if taxa belonging to *Glomus* group A have more extensive intraradical mycelia than other AMF groups. Different specific colonization patterns were observed previously when isolates belonging to several AMF families were grown on different plant species (22). The previous study showed, for example, that diverse *Glomus* species, particularly several isolates of *G. intraradices*, exhibit high levels of root colonization with poor hyphal extension in the soil while *Acaulospora* has low levels of both root and soil colonization. The quantification of the AMF community based on extramatrical mycelia and that based on intraradical mycelia may thus give different results. In any case, AMF community analysis based on the estimation of gene frequency by using clone libraries generated from mixtures of 18S rRNA must be done cautiously. Potential sources of bias, like PCR drift and selection, have been reported previously (45, 64). Moreover, the AMF genome size and the number of copies of the rRNA gene can vary greatly among different AMF species (4, 30). On the other hand, Edwards et al. (13) found that competitive PCR can provide a good estimate of root colonization percentages.

Dynamics of the AMF community. Analyses of the seasonality of AMF communities colonizing roots have been carried out in only a few studies. A study of AMF communities in roots in a coastal grassland in Denmark (50), with four sampling times during the same season, was unable to reveal any seasonal variation. In another study of grasslands in Scotland (71), a change in the AMF community over two consecutive growth seasons was found, but the effect of a change in land management during the study could not be ruled out. To our knowledge, the present study is the most extensive seasonal analysis of an AMF community by molecular methods. We did not find a significant seasonal change in the patterns of the AMF community composition as a whole, but there were interesting differences between the two plant species. The most dominant sequence types, except for Glo8, did not exhibit any clear seasonal trend. The weak presence of Glo8 in September and October is difficult to explain but may be related to seasonal fluctuations in N mineralization. This mineralization seems to reach the highest levels at the beginning of the growth season in spring, the lowest levels in summer, and relatively high levels in autumn (32, 40), but it is not known how *G. intraradices* responds in relation to these seasonal flushes. The genus *Acaulospora* has been shown to occur more frequently in spring in arable fields (29). This trend is not reflected by our data. The three *Acaulospora* sequence types detected in our study did not show a consistent temporal trend. They occurred at low frequencies throughout the entire sampling period; they were nearly absent in September and dominant in the case of *P. vulgaris* in October.

It has to be borne in mind, as discussed above, that the present data refer only to the AMF distribution in planta and

that the extraradical mycelia in the soil may have very different dynamics. The results presented here do not, therefore, necessarily imply a lack of seasonal changes at the community level. Very little is known about the dynamics of the extraradical mycelium and the ratio between the intraradical and the extraradical mycelia (22). For example, the intraradical mycelia of some perennial plants, like *P. vulgaris*, may undergo a few changes during the year, while only the extension of the extraradical mycelia changes over time. The intraradical mycelium may play an important role, not only as a nutrient exchange structure, but also as a wintering stage for many AMF species. Plant species like *P. vulgaris* with a rich AMF community in the roots all year round can be useful in the restoration of degraded grasslands by supporting a perennial pool of diverse AMF species able to facilitate the integration of other plant species into a rich fungal network. It has been shown that AMF promote seedling establishment and survivorship in perennial grassland communities (21, 75), where the recruitment of new seedlings is fundamental for maintaining plant diversity.

The results from this study also indicate that AMF community compositions in different plant species can vary. Host specificity has been reported in several previous studies (20, 28, 71, 72). The plant host effect upon AMF species composition reported here may be due not strictly to species-specific relationships between the fungal partner and the host but also to patterns of spatial heterogeneity in soil and biotic parameters that affect the distributions of the two plant species on a small scale.

Presence of *Ascomycetes*. The presence of nonglomalean sequences in our study is not surprising. This lack of specificity of the AM1 primer has been widely reported in the literature (10, 12, 28, 48, 49, 54). In our study, we used two additional primers to target a wider range of AMF taxonomical groups, but these primers can also yield amplified *Ascomycetes* sequences. The proportion of nonglomalean sequences amplified with the AM1 primer can vary for different plant hosts (12) and different ecosystems. In a previous study of maize roots in arable land, over half of the sequences were found to be those of *Ascomycetes* (J. C. Santos-González, unpublished data). Most of these *Ascomycetes* are usually referred to as dark septate (DSE) fungi in the literature, although many of them may not have darkly pigmented hyphae (1). The DSE fungi comprise a miscellaneous assemblage of root endophytic fungi with disparate phylogenetic and life histories. The nature of the relationships between these microfungal endophytes and their hosts remains obscure in most cases (35), ranging from pathogenic to beneficial (23). We did not quantify root colonization by AMF and DSE fungi, but we observed the conspicuous presence of DSE hyphae and microsclerotia in root mounts (data not shown). In our case, we detected a change in the pattern of the fungal community colonizing the roots of *A. dioica* in September and October, when *Ascomycetes* sequences became dominant in the clone libraries. In May, AMF were dominant again in the clone libraries. A major change in the fungal community also occurred during winter or after the spring snow melting, when microbial activity has been shown to be higher than previously thought (6, 55). We did not sample sites in April after the spring snow melting because no signs of plant growth aboveground were obvious.

The *Ascomycetes* recorded in the present study may be saprotrophs that take over the roots when they die. We have limited knowledge about the life span and turnover of *A. dioica* roots, but the analyzed root pieces looked healthy and lacked symptoms of necrosis. Another possibility is that *Ascomycetes* displace glomalean fungi during the autumn, when plant growth and mineral nutrient demand are low and AMF cease to obtain carbohydrates from their host. Reductions in the light level and, thus, photosynthesis have been related to decreased mycorrhization (24, 25); however, we can only speculate about the role of these *Ascomycetes* in the roots.

In summary, this study shows that AMF communities can be very different depending on their host plants, even within the same ecosystem. These differences are significant not only in terms of species composition, but also in their seasonal dynamics. Further studies such as the one presented here will help to provide fundamental insights into the basic ecology of glomalean fungi. This information needs to be taken into account in experimental studies if we are to obtain a better understanding of the function of AMF and their interactions with plant communities (52). Experimental ecological studies are now revealing more about the mechanisms of interaction between AMF and plants (76), but a remaining challenge in the future will be to integrate this information with better knowledge about other groups of root-colonizing fungi and how these groups influence patterns of colonization by AMF.

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