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Fern-associated arbuscular mycorrhizal fungi are represented by multiple *Glomus* spp.: do environmental factors influence partner identity?

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Abstract Symbioses involving arbuscular mycorrhizal fungi (AMF) are among the most important ecological associations for many plant species. The diversity of AMF associated with ferns, however, remains poorly studied. Using recently designed *Glomus*-specific primers, we surveyed the AMF community associated with ferns from deciduous, broad-leaved second-growth forest habitats at the eastern edge of the piedmont region of central Virginia, USA. Results indicate that this molecular approach may be a useful tool for detecting AMF in ferns compared to traditional techniques based on morphology. Over 30 potential fungal ribotypes were identified from eight fern species using denaturing gradient gel electrophoresis. Fungal ribotypes were found to differ widely in terms of (1) the number of fern partners with which they interact and (2) their relative frequency within each fern. Sequence analysis of fungal isolates from three species of fern indicated that the primers were generally highly specific for *Glomus* species but some non-target DNA was also amplified. Cloned polymerase chain reaction (PCR) products from *Polystichum acrostichoides* and *Osmunda regalis* revealed several phylogenetically distinct *Glomus* species. A single *Glomus* species was identified in the cloned PCR products from *Botrychium virginianum*. These findings challenge the hypothesis that the extent or degree of fern–fungal symbiosis is somehow tied to root complexity. Environmental factors appear to influence the suite of AMF that form partnerships with ferns. Some species of fern from similar habitats associated with dissimilar fungal partners (e.g., *P. acrostichoides* and *Athyrium filix-femina* var. *asplenioides*), whereas others harbored uniform fungal communities (e.g., *Asplenium platyneuron*). The significance of these data in terms of ecological and evolutionary dynamics of the AMF–fern symbiosis is discussed.

Keywords Symbiosis · Ferns · *Glomus* · Community structure

Introduction

Ecologists are broadly interested in identifying forces that generate natural patterns of organismal distribution. To this end, plant ecologists have identified a multitude of abiotic (e.g., light levels, precipitation levels, soil chemistry) and biotic (e.g., reproductive mode, competition, herbivory) factors that shape species distributions (see, e.g., Silvertown 2004; Flinn 2006). Of the biotic influences, mycorrhizae play an important role in helping to shape the host plant niche and represent an important part of the plant's extended phenotype. Evidence indicates that plants often benefit from the presence of mycorrhizal associates via a variety of mechanisms including the mobilization of essential minerals, enhancement of desiccation resistance, and protection from pathogens and herbivores (e.g., Smith and Read 1997).

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The influence of mycorrhizal symbioses extends beyond the individual host plant and shapes important aspects of community structure and function. The presence of arbuscular mycorrhizal fungi (AMF, Glomeromycota; Schuessler et al. 2001) reduces the number of plant species required to achieve equivalent levels of biomass production compared to communities that lack AMF (i.e., AMF increase the rate at which asymptotic relationships between plant species richness and community productivity are achieved—Klironomos et al. 2000). In a similar vein, AMF diversity appears to enhance overall community productivity (Maherali and Klironomos 2007). Püschel et al. (2007) recently found that species evenness among different habitats (and thus successional patterns) differed depending not only on the presence but also on the identity of AMF present on spoil banks (see also Leake et al. 2004).

This last point is significant because ecologists and evolutionary biologists are beginning to address the emerging view that many, if not most, mutualistic interactions involve guilds of interacting species (e.g., Stanton 2003). Due to the apparent low degree of specificity in AMF symbioses (Smith and Read 1997; Helgason et al. 1999; Kiers and van der Heijden 2006), it is probable that a single AMF species will interact with a variety of plant partners and vice versa (but see Sanders 2003; Vandenkoornhuyse et al. 2003). Partner identities become exceedingly important traits to describe when attempting to understand broad ecological patterns because meaningful differences likely exist among different host and symbiont species (e.g., van der Heijden et al. 2003; Helgason et al. 2007; Scheublin et al. 2007; Yao et al. 2008). The "conditional" aspect of symbiotic interactions involving AMF extends to the environmental milieu because partner quality also depends on local conditions (Gomulkiewicz et al. 2003).

As a step toward understanding AMF mutualisms, one must first identify the partners involved. Some of the technical problems associated with AMF identification, such as the paucity of taxonomically informative characters in the arbuscle, have been circumvented through judicious use of molecular techniques. Indeed, a growing literature has identified novel DNA sequence types in the Glomeromyota (e.g., Vandenkoornhuyse et al. 2002; Rosendahl and Stukenbrock 2004; Wirsel 2004; Sato et al. 2005; Hijri et al. 2006; Gamper and Leuchtmann 2007). Using AMF specific ribosomal (rDNA) primers, Helgason et al. (2002) found that some AMF species are (1) more selective in their association with particular plant hosts than other fungi, and (2) have different physiological roles in different plant backgrounds. Given the plethora of molecular approaches now available, it is possible to provide AMF species identifications from infected root tissues using polymerase chain reaction (PCR)-based strategies that employ highly specific primers.

The majority of research in plant–AMF symbioses has focused on gymnosperms and angiosperms. Much less is known about AMF associations involving the pteridophytes (i.e., ferns and their allies). Morphological examination of fern roots has revealed interesting patterns of AMF colonization (e.g., Laferiére and Koske 1981; Iqbal et al. 1981; Berch and Kendrick 1982; Gemma et al. 1992; Schmid and Oberwinkler 1995). Some data indicate that the rate/extent of AMF colonization is lower in ferns with "primitive fleshy" (i.e., eusporangiate) roots than in ferns with "fine" (i.e., leptosporangiate) roots (Boullard 1979; Berch and Kendrick 1982; Gemma et al. 1992). Two recent studies employed molecular strategies for studying fern–fungal symbioses, and both focused on the genus *Botrychium* (Kovacs et al. 2007; Winther and Friedman 2007). Kovacs et al. (2007) found several AMF lineages (including both Gigasporaceae and Glomaceae) associated with the sporophytic portion of the life cycle of *B. virginianum*. Winther and Friedman (2007) examined mycoheterotrophic and autotrophic plants of *B. lanceolatum* and *B. crenulatum* and found that mycoheterotrophic plants appear to be more selective in the types of AMF symbionts with which they interact.

We had three objectives with the research reported here. First, we assessed the effectiveness of a new set of *Glomus*-specific primers (Sato et al. 2005) in the context of a natural setting involving a mixed AMF community. Attention was focused on ferns found in second-growth, deciduous broad-leaved, temperate forest habitats of central Virginia, USA. Second, we placed the identified fern-associated *Glomus* spp. in a phylogenetic context comparing eusporangiate and leptosporangiate species. Finally, we examined within-host fungal diversity and among-host patterns in specificity of AMF–fern associates. These findings are considered in the context of characteristics of the host habitat and morphology.

Methods and materials

Collection and DNA isolation

Eight species of fern were examined in this study including *Asplenium platyneuron*, *Athyrium filix-femina* var. *asplenioides*, *Botrychium virginianum*, *Onoclea sensibilis, Osmunda regalis*, *Polystichum acrostichoides*, *Pteridium aquilinum* var. *pseudocaudatum*, and *Woodwardia areolata*. Specimens came from three forested locations owned by the University of Richmond in the eastern portion of the piedmont region of central Virginia, USA. The "graveyard" (37°35′ N; 77°39′ W) and UR (37°34′ N; 77°32′ W) sites

are separated by just over 10 km. The "ballpark" site (37° 41′ N; 77°48′ W) is approximately 25 km from the UR site. General soil characteristics (e.g., moisture content, silt or sand content, amount of leaf litter), relative elevation, and lighting conditions were noted at the time of collection. In the field, tissue samples were removed from multiple locations in the root. Sections of roots collected from an individual fern were placed in a 1.5-ml tube and frozen in liquid nitrogen for transport back to the lab. All material was stored at −80°C until processing.

In the lab, excess soil was removed by hand and roots were washed with deionized water before DNA extraction. Root samples were placed on a cutting board and all samples were minced to approximately 1 mm lengths using a razor blade. All surfaces and razor blades were washed with deionized water and 70% ethanol between samples. To maximize the probability of encountering AMF symbionts, we combined the minced samples taken from all regions of the root. Genomic DNA from these samples was extracted using a 2× CTAB DNA extraction protocol (Hill et al. 2006) and visualized on 1% agarose gels.

Denaturing gradient gel electrophoresis-polymerase chain reaction

Approximately 260 bp of the 18S rDNA gene, corresponding to positions 577–834 of *Glomus intraradices*, was amplified using the primers of Sato et al. (2005; GC-AMV4.5 and AMDGR). Each 50 µl reaction contained GoTaq Green Master Mix (Promega, Madison, WI, USA), 0.5 µM of each primer, and 20 ng of DNA template. The cycling profile included a 5-min denaturing step at 95°C followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. A final extension for 9 min at 72°C was also included in the profile. All PCR products were visualized on 1% agarose gels.

Denaturing gradient gel electrophoresis (DGGE) was performed using Bio-Rad's Dcode™ Universal Mutation Detection System. AMF DGGE-PCR products were separated in a 10% polyacrylamide gel containing a 10–50% linear denaturing gradient (100% denaturing solution contains 40% formamide and 7 M urea). Electrophoresis was carried out at 60°C for 17 h at 100 V. The gel was stained with ethidium bromide (0.5 µg ml^{-1}) for 20 min and de-stained for an equal amount of time before image capture.

Sequencing and phylogenetic analysis

DGGE-PCR products from three species (the eusporangiate *B. virginianum*, and the leptosporangiate ferns *O. regalis* and *P. acrostichoides*) were selected for sequence analysis based on their relative position within the fern phylogeny. Gel-purified PCR products (Qiagen, MinElute Kit) were cloned using the TOPO® TA Cloning Kit following the manufacturer's protocol. Insert-positive colonies were selected from each fern species. Inserts of the appropriate size were identified via PCR using M13 forward and reverse primers. Plasmids isolated from colonies grown overnight in LB broth at 37°C (Qiagen, Miniprep) were sequenced at Virginia Commonwealth University's Nucleic Acid Research Facility.

Basic Local Alignment Search Tool (BLAST) searches were conducted to find sequences that shared high identity with our fungal ribotypes. Highly similar sequences were aligned using ClustalX 2.0 (Thompson et al. 1997). We employed both maximum likelihood (ML)- and Bayesian-based approaches to our phylogenetic analyses. For both approaches, a model of sequence evolution was determined from aligned sequences using ModelTest (Vers. 3.1; Posada and Crandall 1998). PhyML (Guindon and Gascuel 2003) was used for ML analysis. We employed the GTR model of sequence evolution with 1,000 bootstrap replicates. All parameters were optimized based on empirical data. Bayesian analysis as implemented in Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist 2001) was performed until the average standard deviation of split frequencies was below 0.02 and the run achieved stationarity (n=500,000 generations). We used four independent chains in our analyses. The gamma distribution for among site substitution rates was uniform and approximated using four categories with a proportion of invariable sites. The first 25% of the samples were discarded as burn-in.

Community analyses

DGGE gel images were analyzed with ImageJ 1.40g (http://rsb.info.nih.gov/ij/). As in Nikolcheva and Bärlocher (2004), relative band intensity was estimated on a per lane basis to give proportional representation of each ribotype within a particular host. Interpretation of these data in a community context assumes that primer extension efficiencies are equivalent for all *Glomus* templates amplified, and that a strong correlation exists between the number of ribosomal operons present in the initial metagenomic mixture and cell biomass. By focusing only on *Glomus* AMF, we increase the probability of meeting the first assumption. Data indicate that DGGE band intensities are strongly correlated with initial fungal biomass (Muyzer et al. 1993; Nikolcheva et al. 2003) providing support for the second assumption. Thus, we used ribotype proportional representation from the DGGE gel to estimate *Glomus* species diversity and evenness within a given fern root system. Diversity was estimated using the Simpson $\left(D = 1/\sum p_i^2\right)$ and Shannon (H=-$\sum p_i$ ln p_i) indices (Magurran 1988). Evenness was estimated using E_D =

$1/\sum p_i^2 \times 1/S$ and Pielou's J ($J = H/S$) indices (Magurran 1988).

We used non-parametric, non-metric multidimensional scaling (nMDS), as implemented in Gingko (Bouxin 2005), to compare fungal community composition among fern roots. We used this ordination technique to determine whether patterns in the distribution of fungal ribotypes could be discerned (e.g., host specificity, environmental correlates). Analyses were performed on a presence–absence matrix generated from DGGE data. A Sorensen similarity matrix was built from the presence–absence data, and then converted to a dissimilarity matrix ($D = \sqrt{1 - S^2}$). A principal correspondence analysis was performed first to select the desired number of axes (n=6), which were used as starting coordinates for nMDS. We performed a final principal coordinates analysis to cumulate variance in the first axis. To avoid nMDS solutions that misrepresented sample relationships by converging to local minima in the two-dimensional plot, the nMDS was performed sequentially on five-, four-, three-, and two-dimensional configurations derived from an initial six-dimensional analysis with a final Kruskal's stress $-1<0.12$ (Legendre and Legendre 1998).

Results

Amplicons were not detected for six of the 20 ferns collected at each of the three sites (including representatives from the following species *P. acrostichoides*, *A. platyneuron*, and *A. filix-femina*). The lack of PCR product appeared to be due to a lack of fungal template DNA indicating that some roots that we collected were not colonized by AMF or at least had concentrations below the level needed to amplify under our conditions. For roots from which product was obtained, DGGE indicated that all of the species of fern examined appeared to harbor more than a single fungal type (Fig. 1). The AMF primers employed appeared to efficiently amplify AMF DNA from metagenomic pools of starting material.

Many species of *Glomus* were found to associate with ferns from our study sites (Fig. 2). Each of the three species of fern examined harbored unique *Glomus* species belonging to divergent clades. Each clade contained distinct assemblages of AMF from a variety of habitats around the world. Bayesian and ML analyses recovered well-supported, topographically similar trees that recovered relationships that generally match previously reported sequence diversity within the Glomeromycota (Fig. 2).

A single ribotype was recovered from the five clones sequenced for *B. virginianum* (*Botrychium*_Isolate3E, Fig. 2). The *B. virginianum* (Accession FJ179559) isolate belongs to a well-supported clade that includes AMF from

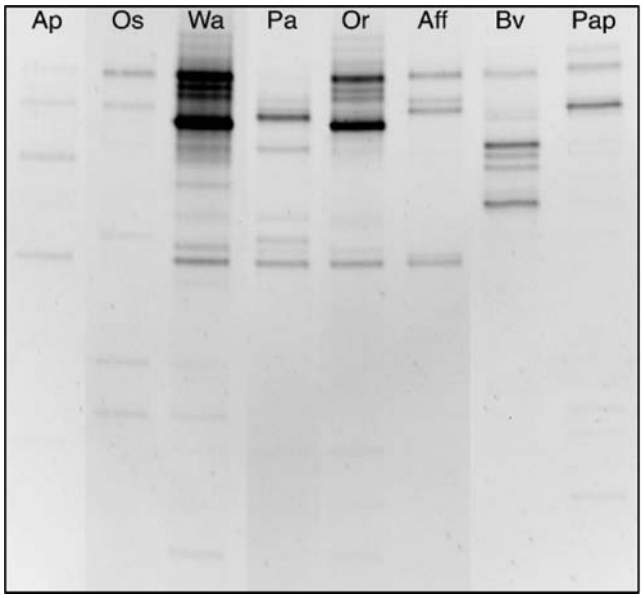

Fig. 1 Representative PCR-DGGE banding patterns for each fern species examined. *Letters above lanes* correspond with the following fern species: *Ap*, *Asplenium platyneuron*; *Os*, *Onoclea sensibilis*; *Wa*, *Woodwardia areolata*; *Pa*, *Polystichum acrostichoides*; *Or*, *Osmuda regalis*; *Aff*, *Athryium filix-femina* var. *asplenioides*; *Bv*, *Botrychium virginianum*; *Pap*, *Pteridium aquilinum var. pseudocaudatum*

Hyacinthoides non-scripta and *Chasmanthium sessiliforum* occurring in woodlands of England and Texas, respectively (Fig. 2). Four colonies were sequenced from the *O. regalis* library (Accession FJ179560–FJ179563), and each fell within distinct *Glomus* lineages (Fig. 2). Isolate NN fell within a clade that included AMF from a warm-temperate deciduous broad-leaved forest of Japan (Yamato and Iwase 2005). The BB isolate clustered with AMF isolated from alpine chalk gravel soils in Austria. The EE isolate was placed, with strong bootstrap support, in a clade that included AMF associated with Texas populations of *C. sessiliflorum* (distinct from isolate 3E identified in *Botrychium*, Fig. 2). Finally, *O. regalis* isolate FF belonged to a geographically diverse clade (Fig. 2) that included *Glomus* species obtained from plants in Estonia (e.g., *Hepatica nobilis*), England, Sweden, Ecuador, Costa Rica, and Ethiopia but none from North America.

Three clones were sequenced from the *P. acrostichoides* library (Accession FJ179564–FJ179566). Isolate QQ fell within a well-supported clade that included fungi from plants located in North America and Central Europe. However, one of the sequences within this clade (Glomus_3_AJ699064) was isolated from a liverwort found in New Zealand (Russell and Bulman 2005). Isolate PP did not fit unambiguously in any clade and BLAST searches continually pulled out *Glomus* sequences from Australian, European, and South American plants. One of the *P. acrostichoides* isolates was not a species of *Glomus*. Isolate H (Accession FJ179566) was found to belong to the Basidiomycota

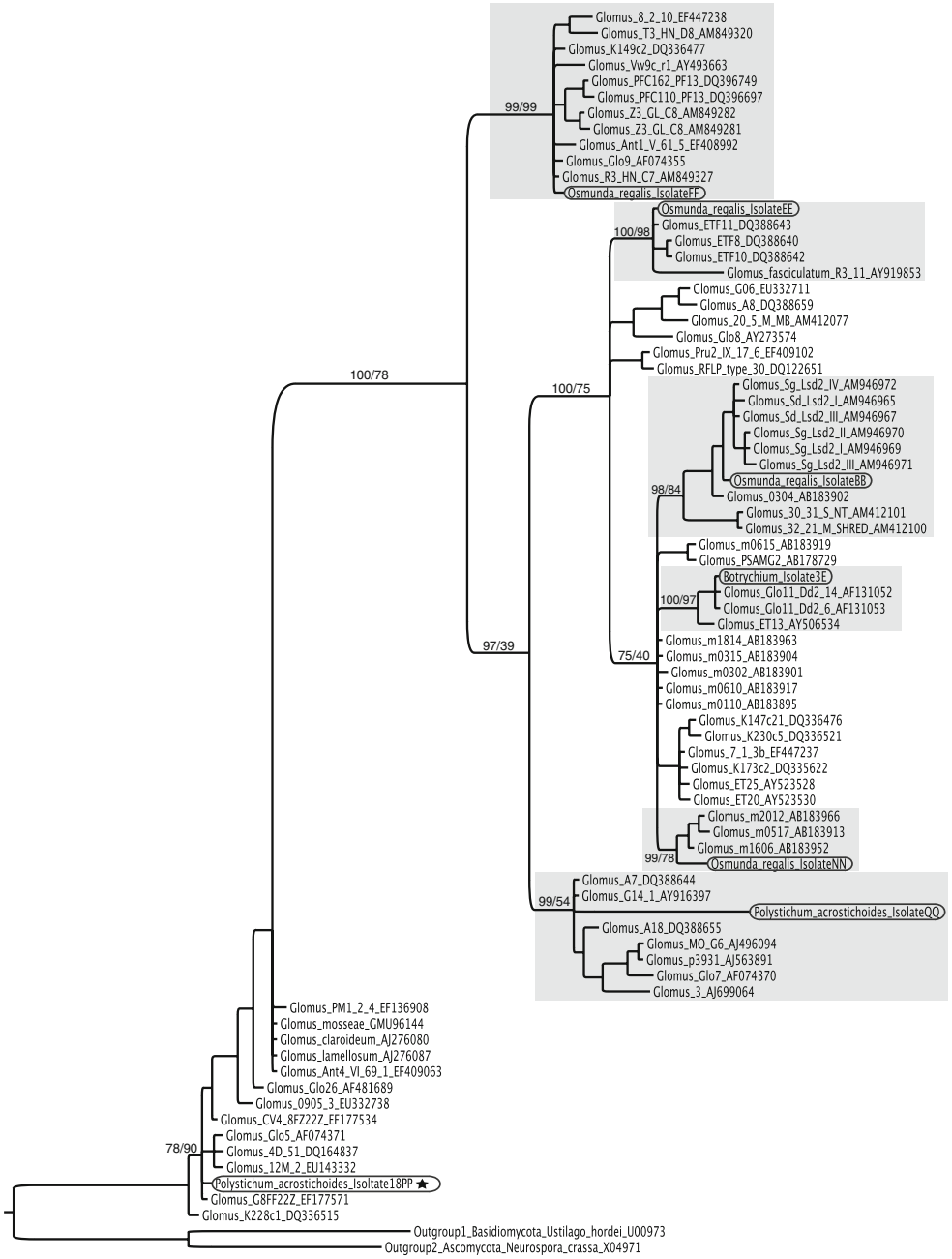

Fig. 2 Phylogeny of *Glomus* species isolated from *Botrychium virginianum*, *Polystichum acrostichoides*, and *Osmunda regalis*. Clades containing fern-derived sequences (*capsules*) are highlighted. Isolate PP from *P. acrostichoides* (see *asterisk*) failed to cluster within any clade. Bayesian posterior probabilities (numerator) and ML bootstrap support values (denominator) are shown above the branches of major lineages. Accession numbers are shown next to all *Glomus* species except for those identified in this study, which are listed in the text

(Agaricomycetes) based on identities from BLAST searches. This indicates that a small percentage of the DGGE bands in Fig. 1 might be non-*Glomus*.

In all, 34 different phylotypes were identified from DGGE and the number of fungal ribotypes recovered from a single individual root ranged from 4 to 19 (Fig. 3). The overall average number of fungal ribotypes per host plant species was 8.36 with interesting, but not significant ($F_{2,\ 13}$=2.52, p=0.13), average differences in fungal ribotypes per host plant species among sites (UR=8 (±1.9), "graveyard"=11.75 (±1.9), and "ballpark"=5.5 (±1.6)). When the one outlier (*W. areolata* from UR) was removed, ferns from the "graveyard" site harbored significantly more fungal ribotypes per host plant than either of the other sites. The relationship between species diversity and ribotypic richness differed depending on the index used. H showed a significant, but slow, rate of increase in diversity as a function of ribotypic richness, whereas no correlation was detected between D and number of ribotypes (Fig. 3). Both measures of evenness (J and E_D) showed a significant decrease as a function of ribotypic richness (Fig. 3). Fungal communities harbored within a single root were generally dominated by one or two fungal ribotypes. Only three ferns (Pa1, Pa3, and Bv1) had relative abundances that were equal for each fungal ribotype.

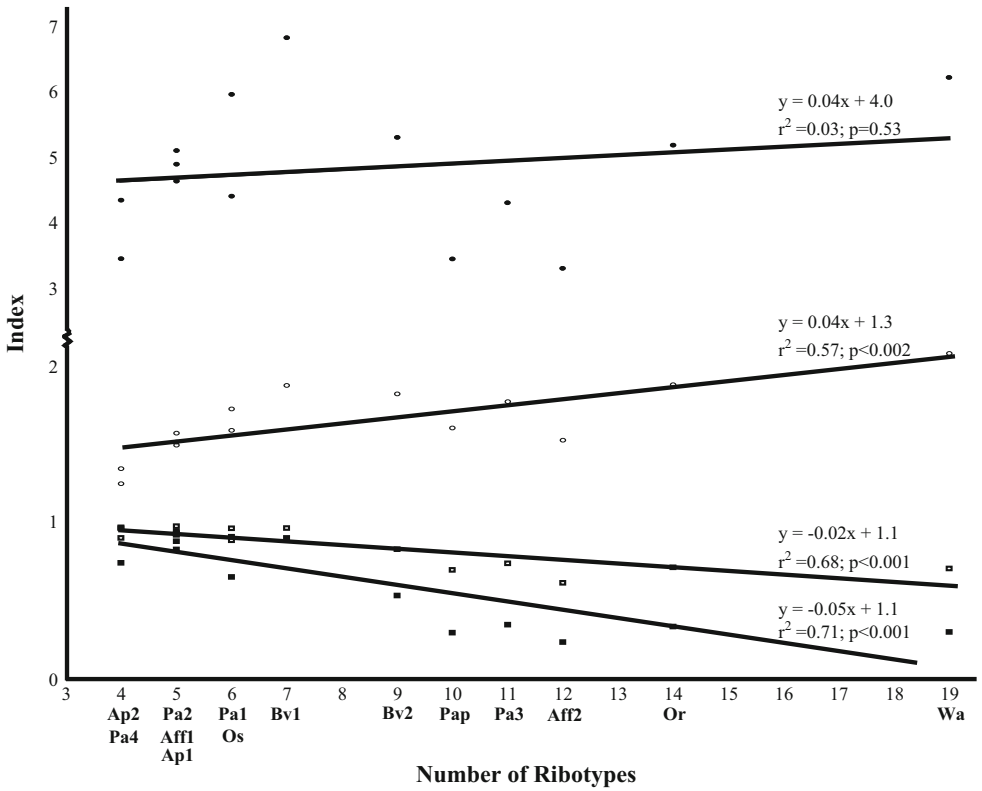

Fig. 3 Diversity and evenness indices as a function of number of ribotypes present within fern roots. *Closed circles* = Simpson's index (*D*), *open circles* = Shannon's index (*H*), *open squares* = Pielou's evenness (*J*), and *closed squares* = E_D. Note change in *y*-axis scale to accommodate the greater variation in *D*. Equations for regressions are provided *above the lines* along with r^2 and *p* values. The *x*-axis includes the fern source of ribotypic richness (abbreviations as in Fig. 1). When more than one fern is included at a point, their vertical position corresponds to the indices in the figure

Analysis of patterns in fern–fungal associations via nMDS indicated that environmental and geographic factors may shape the symbiotic partnership (Fig. 4). Four species of fern were represented by root samples collected from more than one individual plant. Two samples of *A. platyneuron* taken from the "ballpark" site had nearly identical DGGE banding patterns and both individuals were tightly clustered in multidimensional space (Fig. 4). Two individuals from another species (*A. filix-femina* var. *asplenioides*) also occupied similar space in our nMDS analysis despite the fact that they were collected at two different sites ("graveyard" and UR). The other species with multiple root samples (*B. virginianum* and *P. acrostichoides*) shared few DGGE bands and occupied distinct points within the multidimensional space.

Analysis of fern host associations within a three-dimensional projection of the first three axes clearly identified two major groupings of fungi. The two-dimensional projection is shown in Fig. 4. The first group included nearly all the ferns collected in habitats with wet soils (e.g., creek banks/beds (Wa, Pa2, and Pa3), lake shore (Aff1), or low-lying swamp (Aff2, and Or)). This group is comprised only of ferns from the "graveyard" and UR sites. *O. sensibilis* from banks of a creek on the UR site did not cluster with this group. The second group was dominated by ferns collected from the "ballpark" site (Ap1&2, Bv2, and Pa4) as well as one fern from the UR site (Pa1). The *A. platyneuron* ferns were collected along a trail at the base of a hill approximately 10 m from a creek. The other ferns in this group (Pa1&4 and Bv2) were all collected from well-drained soils on the sides of hills. Two of the major outliers, *P. aquilinum var. pseudocaudatum* and *B. virginianum* (Bv2), were collected from hills with well-drained soils at the "graveyard" and UR sites, respectively.

Discussion

The first objective of our study was to assess the effectiveness of a new set of *Glomus*-specific primers in the context of a natural setting involving a mixed AMF community. The Sato et al. (2005) primers appear to efficiently amplify DNA from all major lineages of the Glomerales even when presented with complex metagenomic DNA extracted from soil environments. However, some level of non-specificity was detected (e.g., isolate H (Accession FJ179566)), and caution should be exercised interpreting results if these primers are used for community analysis without sequence analysis. Nonetheless, the molecular approach adopted here might be a good complement to surveys of colonization rates/percentages that are based on visual surveys of root tissue, which suffer from a variety of technical problems including the limited number of taxonomically informative characters present in the arbus-

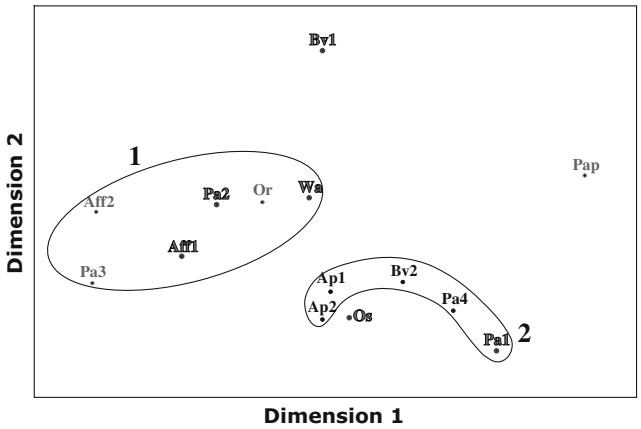

Fig. 4 nMDS plot of fungal ribotypic presence/absence data. Species abbreviations are as in Fig. 1. Ferns collected at the UR site are shown in black, "graveyard" in *gray*, and "ballpark" in *outline form*. The first grouping is composed of ferns collected from wet habitats. The second grouping is composed of ferns from drier soils

cule. For example, our results indicated that *Glomus* colonization involves multiple species in *O. regalis*, whereas Berch and Kendrick (1982) found no morphological evidence of AMF colonization in the royal fern.

Additional research is needed to determine the utility of this PCR-based approach in AMF survey work given that 30% of ferns that were initially screened were PCR-negative for *Glomus*. While fungal symbionts may have been lacking in those ferns, it is also possible that technical problems that go beyond the absence of fungal DNA in the extraction could have been involved (e.g., inhibitors to PCR in the DNA extraction, non-optimal fern/fungal DNA ratio, etc.). Pairing the molecular approach employed here with a morphological examination of fern roots would help establish the rate at which fungal symbionts are missed in both strategies.

Our estimates of *Glomus* diversity (and evenness) present in fern roots must be interpreted with some caution because the data rest on several assumptions. First is that roots were sampled thoroughly enough to ensure detection of symbionts that are distributed patchily throughout the root. Our sampling scheme maximized that probability but future work should include a root sampling scheme that relates fungal diversity as a function of the amount of root sampled. Second, it is assumed that the primers we employed were equally efficient during amplification of a mixed-species environmental sample (i.e., no bias existed in amplification stages). By focusing our efforts on a small section of 18S specific to *Glomus*, we increased the likelihood that annealing and amplification were equally efficient. However, significant phylogenetic diversity is represented in this genus and we do not know how much this influences PCR. Third, we assumed that initial ribosomal operon concentration matched biomass in the sampled roots, which is supported by previous work showing that DGGE band intensities are correlated with initial fungal biomass (e.g., Muyzer et al. 1993; Nikolcheva et al. 2003). Fourth, heteroduplexes may elevate estimates of ribotype diversity, and we did not sequence DGGE bands to estimate the frequency of this artifact. Finally, we assumed that the majority of bands were *Glomus* species, but we know of at least one case where a species belonging to the Basidiomycota (*P. acrostichoides* isolate H) was recovered. To resolve fully estimates of the number of *Glomus* species that associate with different species of fern, it is vital to determine the percentage of non-*Glomus* sequences amplified with these primers.

Our second major objective was to place any identified fern-associated *Glomus* spp. in a phylogenetic context. Our results complement and extend two recent studies that used molecular approaches to study AMF symbioses in *Botrychium* ferns (Kovacs et al. 2007; Winther and Friedman 2007). The focus on members of the Ophioglossaceae is understandable due to their reliance on mycoheterotrophy at various life history stages, which might to lead to a greater degree of specificity in the association (e.g., Winther and Friedman 2007). However, we extended the molecular examination of fern–AMF symbioses by including leptosporangiate ferns in our surveys because much less is known about the nature of the host–symbiont association for these ferns (e.g., Boullard 1979; Iqbal et al. 1981; Berch and Kendrick 1982).We chose to sequence and phylogenetically analyze AMF from *O. regalis* because the Osmundaceae represent an early branch off the leptosporangiate lineage (Schuettpelz and Pryer 2007; Metzgar et al. 2008). *P. acrostichoides*, on the other hand, belongs to the more derived leptosporangiate family Dryopteridaceae.

One of our key findings was that the ferns we examined associate with multiple fungal species belonging to the Glomeraceae within the Glomerales. Over half of our isolates likely belong to the *Glomus* Ab clade identified in Schwarzott et al. (2001). Evidence for this comes from the fact that *G. fasciculatum* (a conspicuous member of the Ab clade) is a member of the well-supported lineage that includes three *O. regalis* isolates and the *Botrychium* isolate. Isolate 18PP from *P. acrostichoides* is another probable member of the Ab clade. It seems likely that our *Botrychium* isolate may belong to the MH2 clade described by Winther and Friedman (2007). Isolate 3E may even belong to their K group given that our glomalean fungal sequences both came from photosynthetic sporophytes. The *O. regalis* isolate FF likely belongs to the *Glomus* Ac clade (Schwarzott et al. 2001) due to its high identity (>98%) with sequences that are identical to the diagnostic sequence for this group (*Glomus* sp. W3357: AJ301857). Isolate 18PP from *P. acrostichoides* defied placement in a well-resolved

group, but evidence from BLAST searches with other sequences in the phylogeny indicated that this is likely a member of the *Glomus* B clade (Schwarzott et al. 2001).

Our final goal with this work was to examine within-host fungal diversity and among-host patterns in specificity of AMF–fern associates. We were interested in comparing eusporangiate and leptosporangiate species in terms of *Glomus* species richness found within the roots in part because AMF colonization has been proposed to be higher in the fleshier roots of the former, and lower in the finer roots of the latter (Baylis 1975; Boullard 1979). Earlier work showed that leptosporangiate ferns experience much greater variability (0–100%) in the percentage of roots infected with AMF than do eusporangiate ferns, which experience colonization rates near 100% (Berch and Kendrick 1982). What is not known is whether fungal species richness is correlated with the level of root colonization. We found fewer *Glomus* species within the roots of *B. virginianum* than either *P. acrostichoides* and *O. regalis*. Because we did not measure the percent colonization, we cannot directly examine Boullard's (1979) hypothesis, but our data indicate that there may be no correlation between the level of colonization and *Glomus* species richness. These observations raise several interesting questions about host and symbiont selectivity (i.e., ecological promiscuity) in ferns. For example, do leptosporangiate ferns exert stronger control over intraradical *Glomus* density (hence the greater variability in colonization rate) than eusporangiate ferns? Are eusporangiate hosts capable of supporting larger *Glomus* densities? Do *Glomus* species find leptospongiate ferns to be sub-optimal hosts? The hypotheses derived from these questions would be fairly easy to test.

The level of intimacy among partners in AMF symbioses has been presumed to be low though the application of molecular techniques has challenged some of these assumptions (e.g., Bruns et al. 2002; Sanders 2002; Vandenkoornhuyse et al. 2003; Santos-Gonzales et al. 2007). The broad geographic and host distributions for some of the *Glomus* species we identified may indicate minimal host/habitat fidelity. For example, the *O. regalis* isolate FF clade belonged to a lineage including fungi from plant hosts found in Europe, South America, Central America, and Africa, and habitats ranging from coniferous/montane forests (AM849281, AM849282, AM849320, AM849327, DQ396697, DQ 396749, DQ336477, and EF447238) to high latitude grasslands (EF408992) and canopy bromeliads (AY493663). Whether this reflects a low level of intimacy in interacting partners remains to be tested, but it does indicate some broad environmental, and potential host, tolerances for these *Glomus* species. This finding contrasts, to some extent, with work indicating that the diversity and composition of intraradical AMF communities vary among habitat types (e.g., Öpik et al. 2006). However, our *O. regalis* isolate NN fell within a clade that included AMF from a warm-temperate deciduous broad-leaved forest of Japan (Yamato and Iwase 2005). This habitat type is much like the one sampled in the present study and might indicate that there is a unique fungal community common to this habitat type (as suggested by Öpik et al. 2006).

Our nMDS data indicate that environmental factors may play a considerable role in shaping fern–fungal associations even though some fern species may experience a greater degree of host specificity with their fungal partners than other species (e.g., *A. filix-femina* var. *asplenioides*). A fern with narrow habitat requirements may encounter only a subset of all the fungal symbionts with which it could otherwise associate. Conversely, fern species with broad environmental tolerances might find themselves interacting with many different fungal partners over their entire ecological distribution. For a species like *P. acrostichoides* that was found in several different habitat types, association with different fungal partners might lead to significant differences in a variety of host performance characteristics, and some combinations might be "better" than others at conferring selective benefits. This is significant from an ecological perspective because mycorrhizae have been shown to influence important fitness parameters (e.g., Turnau et al. 2005), and varying combinations of fungal partner associations can lead to differential host plant performance (e.g., Gustafson and Casper 2006). Thus, the colonization behavior of *Glomus* species could have significant effects on host plant performance and ultimately community structure.

The role AMF may play in the ecological distribution of ferns has been largely ignored. Fern distributions are undoubtedly influenced by a number of abiotic and biotic factors, but to date, little work has focused on the role AMF may play in these distributions. For example, Flinn (2006) proposed that distributional differences among three species of fern could be related to their reproductive strategy. She hypothesized that diminished selfing capabilities for some species (e.g., *P. acrostichoides*) reduced the ability to colonize post-agricultural fields due to low reproductive success. However, soil microflora are known to be influenced by a variety of factors including disturbance history, plant monocultures growing in a given area, and past agricultural practice (e.g., Janos 1980; Lemanceau et al. 1995; Helgason et al. 1998; Oehl et al. 2003; Viebahn et al. 2005). Post-agricultural fields might have substantially depleted AMF communities that could influence distributional patterns of some ferns (e.g., Flinn 2006).

In conclusion, the Sato et al. (2005) primers appear to be very efficient at picking up AMF DNA from mixed

pools of fungal DNA (despite some level of non-specificity). As in other studies, ferns appear to associate with a variety of fungal symbionts and the level of host–symbiont fidelity appears to be low. Along these lines, fern–fungal symbiont patterns appear to be shaped by environmental and geographic factors, but additional work is needed to determine how flexible the fern–AMF symbiosis can be. Nonetheless, this study uncovered an impressive diversity of *Glomus* species and extends work on AMF symbioses to include an interesting taxonomic group of plant hosts.

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