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Co-existing grass species have distinctive arbuscular mycorrhizal communities

P. VANDENKOORNHUYSE,* K. P. RIDGWAY, I. J. WATSON, A. H. FITTER and J. P. W. YOUNG
Department of Biology, University of York, PO Box 373, York YO10 5YW, UK

Abstract

Arbuscular mycorrhizal (AM) fungi are biotrophic symbionts colonizing the majority of land plants, and are of major importance in plant nutrient supply. Their diversity is suggested to be an important determinant of plant community structure, but the influence of host-plant and environmental factors on AM fungal community in plant roots is poorly documented. Using the terminal restriction fragment length polymorphism (T-RFLP) strategy, the diversity of AM fungi was assessed in 89 roots of three grass species (*Agrostis capillaris*, *Festuca rubra*, *Poa pratensis*) that co-occurred in the same plots of a field experiment. The impact of different soil amendments (nitrogen, lime, nitrogen and lime) and insecticide application on AM fungal community was also studied. The level of diversity found in AM fungal communities using the T-RFLP strategy was consistent with previous studies based on clone libraries. Our results clearly confirm that an AM fungal host-plant preference exists, even between different grass species. AM communities colonizing A. capillaris were statistically different from the others ($P < 0.05$). Although grass species evenness changed in amended soils, AM fungal community composition in roots of a given grass species remained stable. Conversely, in plots where insecticide was applied, we found higher AM fungal diversity and, in *F. rubra* roots, a statistically different AM fungal community.

Keywords: AM fungi, host-plant preference, soil treatment effect, SSU rRNA gene, T-RFLP, *trn*L intron

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Introduction

The arbuscular mycorrhiza (AM) symbiosis is the most widespread and abundant plant–microbe symbiosis, found in approximately two of three plant species (Fitter & Moyersoen 1996). The fungal partners in AM symbioses form a distinct phylum, the Glomeromycota (Schüßler *et al*. 2001a). AM fungi play a key role in plant phosphorus supply (Smith & Read 1997) and can promote plant performance in numerous other ways (Newsham *et al*. 1995). The AM fungal community colonizing plants can affect the composition of insect populations (Goverde *et al*. 2000; Gange *et al*. 2002). The composition of communities of AM fungi plays an important role in plant community structure and diversity (van der Heijden *et al*. 1998a, 1998b; O'Connor *et al*. 2002), so that changes to the AM fungal community could modify the plant community and hence ecosystem processes. To understand the importance for such processes of variation in AM fungal populations and AM fungal community composition requires knowledge of their distribution and abundance in plant roots from natural ecosystems; few such data are available.

Studies of AM fungal communities in environmental samples are difficult because the fungi are obligate symbionts that have not been cultured axenically and can be identified morphologically only in the spore stage. However, use of molecular markers such as the SSU rRNA gene avoids the need for culture: sequence variants can be classified into phylotypes (clades of closely related sequences with strong statistical support). The Glomeromycota is an ancient clade, so there is enough variation in the rather conserved SSU rRNA gene sequence to provide well-resolved phylogenies (e.g. Helgason *et al*. 1998, 1999; Schüßler *et al*. 2001b; Vandenkoornhuyse *et al*. 2002a, 2002b). There is a correlation between the clades identified by

Correspondence: J. Peter W. Young. Fax: + 44 (0) 1904 328505; E-mail: jpy1@york.ac.uk
*Present address: Université de Rennes 1 Campus de Beaulieu, CNRS UMR 6553 'Fonctionnement des écosystèmes et biologie de la conservation', Avenue du Général Leclerc, 35042 Rennes Cedex, France.

molecular phylogeny and the traditional taxonomy based on spore morphology, but it is far from perfect. Some highly divergent taxa were not distinguished by taxonomists relying on morphological evidence (Morton & Redecker 2001), while some species defined by morphological characters overlap at the sequence level, and some isolates in culture collections appear to be genetically heterogeneous (Lloyd-MacGilp *et al*. 1996; Helgason *et al*. 2002).

For these reasons, and because much of the molecular diversity discovered in the field is not available in culture, most sequence types cannot be assigned unambiguously to the traditional species. Nevertheless, there is now a substantial literature on AM fungal diversity based on molecular data (reviewed by Clapp *et al*. 2002; Redecker 2002). Using specific primers for the amplification of the AM fungal SSU rRNA gene (Helgason *et al*. 1998, 1999, 2002; Daniell *et al*. 2001; Vandenkoornhuyse *et al*. 2002a) or using general fungal SSU rRNA gene primers to analyse the broader fungal community in roots (Vandenkoornhuyse *et al*. 2002b; Vandenkoornhuyse 2002), the relationships between AM fungi and their host plant can now be studied in more detail. These studies have demonstrated the co-occurrence of several different AM fungi within a plant root system (Helgason *et al*. 1998, 1999, 2002; Daniell *et al*. 2001; Vandenkoornhuyse *et al*. 2002a, 2002b), and indications of AM fungal host preference (Helgason *et al*. 2002; Vandenkoornhuyse *et al*. 2002a). Along with this host preference, the fungi show distinct responses to environmental factors, such as soil disturbance (Helgason *et al*. 1998) or sewage sludge amendments (Vandenkoornhuyse *et al*. 2001): in the latter case, changes in the AM fungal populations were due apparently to enhanced recombination. Here, we have used a field experiment, in which soil communities were deliberately manipulated by the addition of limestone (lime) and nitrogen (N), to determine the composition of AM fungal populations in relation to changes in their biotic and abiotic environment.

The following specific questions are addressed in our study. (i) What is the AM fungal diversity in roots of grasses in a permanent grassland? (ii) Do co-occurring grass species share a similar AM fungal community in their roots? To address these questions, three grass species (*Agrostis capillaris*, *Poa pratensis* and *Festuca rubra*) that co-occurred at the same grassland site were sampled. (iii) Is the fungal community robust to environmental change? This was tested in an experimental field site that had been divided into plots that received different treatments (control and plots amended with nitrogen, lime, nitrogen and lime, or a biocide).

To deal with these aims, a large number of roots must be analysed. Therefore, rather than using a conventional environmental polymerase chain reaction (PCR)/cloning/sequencing strategy the AM fungal community in more than 100 root samples was analysed using the terminal-restriction fragment length polymorphism (T-RFLP) approach (Liu *et al*. 1997). The T-RFLP method is based on the detection of terminal restriction fragments on a sequencing gel directly after digestion of end-labelled PCR products obtained from the environmental sample DNA. T-RFLP is independent of cloning, so can be applied to a larger number of environmental samples using less time and money. It generates a reproducible community fingerprint or 'diversity signature' (Clement *et al*. 1998; Osborn *et al*. 2000; Scala & Kerkhof 2000) with a level of resolution similar to that of denaturing gel electrophoresis (Moeseneder *et al*. 1999). One possible bias when using T-RFLP is a greater susceptibility to heteroduplex formation and a possible 'PCR mutagenesis' effect relative to 'traditional' PCR amplification, cloning and sequencing (Qiu *et al*. 2001). Nevertheless, T-RFLP has been used successfully to analyse communities of bacteria and archaea from various samples (e.g. Lüdeman *et al*. 2000; Braker *et al*. 2001; Fish *et al*. 2002; Kuske *et al*. 2002) and also to analyse an ectomycorrhizal fungi community (Zhou & Hogetsu 2002). In our study, the T-RFLP method was optimized to analyse the pattern of communities of AM fungi colonizing roots.

Materials and methods

The field site

This study is part of the NERC 'Soil Biodiversity' field experiment at the Macaulay Institute's Sourhope field station near Kelso (Scotland, UK). A 1.05-ha area of seminatural hill pasture was divided into plots that, from May 1999, received different treatments: control plot (C), nitrogen amendment (N), lime amendment (L), both nitrogen and lime amendments (NL), biocide (B). Each treatment was applied to five replicate plots in a randomized design. Nitrogen was applied as NH_4NO_3 at 17.2% N of both ammoniacal N and nitric N at an annual rate of 24 g/m^2 (i.e. 240 kg/ha) in two doses. Limestone was applied as $CaCO_3$ at a final concentration of 600 g/m^2 (i.e. 6000 kg/ha) in one dose. For biocide treatment, the insecticide Dursban (chlorpyrifos) was applied at the annual rate of 0.15 mL/m^2 (i.e. 1.5 L/ha) after each mowing as soon as weather permitted. A map of the field experiment is available at http://www.nmw.ac.uk/soilbio/Sourhope_Design.htm. Two years after treatments began, a botanical survey of the different plots showed a drift in plant community composition (Burt-Smith 2002). *F. rubra* and *P. pratensis* became more abundant in plots amended with nitrogen, lime and especially in nitrogen-and-lime plots, but the abundance of *A. capillaris* remained unchanged (Burt-Smith 2002). In the biocide plots, the plant species composition remained comparable to that of the control plots. The above-ground biomass increased in both nitrogen and nitrogen-and-lime amended plots, but not in biocide and

control plots. The main direct effect of the amendments on the soil was a significant increase in the soil pH related to the application of calcium carbonate (pH ≈ 4.8 in control and pH ≈ 6.2 in lime and nitrogen-and-lime).

Sampling and total DNA extraction from roots

Core samples (6.3 cm diameter) were collected to a depth of 20 cm from all plots and subplots (two per plot) on 31 May 2001, giving a total of 10 core samples for each of the five soil treatments. Few roots penetrate below 20 cm in this soil. On average, one core contained 3.2 plant species. As in our previous study (Vandenkoornhuyse *et al*. 2002a), plants were identified, separated, thoroughly washed from each core sample, and then roots were collected and frozen. Total DNA was extracted from 100 mg (wet weight) of roots (Edwards *et al*. 1997) followed by an additional step of DNA purification using the concert rapid purification kit (Gibco BRL Life Technologies) (Vandenkoornhuyse *et al*. 2002a).

Verification of plant identification and purity of root samples

To check that root samples were not contaminated by roots of another plant species, the plastid-encoded *trn*L (UAA) intron was amplified from each root DNA extract. PCR-RFLP of the *trn*L (UAA) intron as a molecular tool for plant identification has been developed recently by Brunner *et al*. (2001) for tree roots. Here, the method has been adapted to identify grasses. A sequence database of the *trn*L intron was developed for the three species under investigation. DNA was isolated from the cotyledons of germinated certified seed of *A. capillaris*, *P. pratensis* and *F. rubra* (Herbiseed, Twyford, UK) using the cetyltrimethyl ammonium bromide (CTAB) method (Gardes & Bruns 1993). The optimized method has been validated for a wide range of grass species. The *trn*L intron was amplified from plant DNA using a primer pair that targets conserved plastid-encoded exons (Taberlet *et al*. 1991). Amplifications were performed in PCR buffer (Invitrogen), 1 mM $MgCl_2$, 0.2 mM each dNTP, 10 pmol of each primer and 1.25 U of *Taq* DNA polymerase (Invitrogen) in a final volume of 50 μL. The cycling regime was: 3 min denaturation at 94 °C, then 35 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 1 min, DNA extension at 72 °C for 1 min, then a final extension step at 72 °C for 5 min. PCR products obtained from each grass were checked on a 1% agarose gel and purified using the Concert Rapid Purification kit (Gibco BRL Life Technologies). Sequencing of both strands was performed with the BigDye Terminator Cycle Sequencing kit utilizing the forward or reverse amplification primer, and reactions were resolved on an ABI 377 automated sequencer according to the manufacturer's protocol (Applied Biosystems, Foster City, USA). Nucleotide sequences were edited using DNAStar Lasergene and theoretical digests were performed on edited sequences to determine an appropriate RFLP strategy for discriminating among the three taxa. It was assumed that agarose gel electrophoresis could discriminate reliably fragments of 50 bp or more that differed in size by at least 20 bp. The PCR product of each grass was digested to confirm theoretical results. Digests were performed in a volume of 10 μL containing 5 μL of *trn*L PCR product, 1 × buffer and 5 units of restriction enzyme (Promega) and incubated at 37 °C for 1.5 h. Digests were resolved on 2% agarose:metaphor agarose (BioWhittaker Molecular Applications, Rockland ME, USA) gels in 0.5 × TBE, stained with ethidium bromide and photographed under UV light to visualize bands. The *trn*L (UAA) intron was then amplified from each root DNA extract as above and digested with the appropriate restriction enzyme for discrimination. RFLP patterns were compared to those obtained from reference species for the purpose of root identification.

PCR amplification of fluorescent PCR products

Part of the SSU rRNA gene (550 bp) was amplified using a universal eukaryotic primer NS31 (Simon *et al*. 1992) and an AM fungal-specific primer AM1 (Helgason *et al*. 1998). The choice of this set of primers was driven by the fact that nothing other than AM fungi is amplified in our stringent PCR conditions. The limitation of this primer set is that some clades of AM fungi are not or are poorly amplifiable (Paraglomaceae and Archeosporaceae according to Schüßler *et al*. 2001a). These clades are very distant and, so far, no primer has been described that will amplify all known AM fungi without also amplifying sequences from other fungi. The consequence is that we cannot assume that all the AM fungi in a community are analysed when NS31 and AM1 are used. Nevertheless, this does not invalidate our data on changes of AM fungal diversity in roots.

Primers NS31 and AM1 were labelled at their 5′ ends with fluorescent dyes HEX and 6-FAM, respectively. PCR amplifications were run in a PTC100 thermocycler (MJ Research Inc., USA). The typical reaction mixture to synthesize the labelled amplicons was 10 pmol of NS31-HEX, 10 pmol of AM1-6-FAM, 0.725 units of proof-reading *Pfu* DNA polymerase (Promega) in the manufacturer's reaction buffer, and 0.2 mM of each dNTP (Invitrogen Ltd, Paisley, UK). The cycling regime followed Helgason *et al*. (1999). Then excess primers were removed with the concert rapid purification kit (Invitrogen Ltd, Paisley, UK) and purified PCR amplicons were eluted in one volume of TE buffer.

T-RFLPs

As a first step, the restriction maps of available AM fungal SSU rRNA gene fragment sequences were computed (using

DNA-Strider) to determine the ability of 134 different restriction endonuclease enzymes to generate high polymorphism for the terminal restriction fragments (TRFs). The sequences used were all those available in GenBank plus unpublished sequences from our own laboratory. On the basis of these known sequences two different restriction endonuclease enzymes, *Hin*fI and *Hsp*92II, were chosen because they showed the highest polymorphism of cleavage sites at the extremities of the amplified DNA fragment. Each digestion was performed separately in a sterile and siliconized 1.5 mL microtube using 2 U of enzyme (*Hin*fI or *Hsp*92II) in the manufacturer's buffer (Promega) and 15 μL of the purified fluorescent SSU rRNA PCR product, incubated for 4 h at 37 °C.

Digestion products were precipitated with 2 μL of a 3-M sodium acetate buffer (pH 5.6) and 50 μL of absolute ethanol. After centrifugation, the pellet was washed in 70% ethanol and dried in an oven at 42 °C for 20 min Pellets were suspended in 3 μL of formamide/EDTA (50 mM, pH 8.0). Then, 0.5 μL of loading buffer and 0.5 μL of internal lane size standard GENESCAN-400HD-ROX (Perkin Elmer, Wellesley, USA) were added. Samples were denatured for 3 min at 90 °C and run on a standard sequencing gel at 52 °C on an ABI377 automated sequencer (Applied Biosystems, Foster City, USA). After electrophoresis, labelled fragment sizes were calculated by comparison to the internal standard using GENESCAN® software (Applied Biosystems, Foster City, USA).

The automated sequencer detects all fluorescent DNA fragments. If there is a strong secondary structure, or partial digestion, a signal that does not correspond to a true TRF can be detected. To guard against such artefacts, two replicates (i.e. independent PCRs and independent digestions) were performed. In addition, all T-RFLP gels were duplicated. Besides eliminating inconsistent peaks, these replicates led to an increase in the number of observed TRFs per T-RFLP profile by allowing us to retain small peaks that were present consistently. Therefore, it was not necessary to apply a minimal cut-off peak height because by comparing replicated profiles it was easy to determine background noise from peaks.

The adequacy of the T-RFLP method was checked using other root DNA extracts for which an extensive analysis of cloned PCR products and sequencing has already been published (Vandenkoornhuyse *et al*. 2002a). T-RFLP detected all or almost all terminal restriction fragments predicted from the cloned sequences.

Diversity comparisons

The total number of TRFs from one root sample, including both enzymes for both forward and reverse DNA strands, was used as a measure of AM community diversity. Two different statistical analyses were performed on this data set. First, a two-way ANOVA was performed for partitioning the variations in the response variable (number of TRFs). Here, the null hypotheses tested were no effects of host-plant or soil treatment on mycorrhizal community (number of TRFs) and no effects of soil treatment nested within host plant. Second, the data set was analysed more deeply by testing differences for all possible pairs of group means using an unplanned pairwise comparisons procedure. For comparison of the three host species, *A. capillaris*, *P. pratensis* and *F. rubra* samples from all soil treatments were combined; similarly, samples from all hosts were combined for host-plant comparisons. To determine which groups are different, statistical significance is determined by an ANOVA *F*-test. Various tests have been used for this but, according to Quinn & Keough (2002: 199–201), Tukey's honestly significant difference (HSD) test, based on the studentized range statistics, is better than Fisher's LSD test, Duncan's multiple range test and the Student–Neuman–Keuls test because it controls for the familywise Type I error rate. The nominal level used here was 0.05. Unplanned pairwise comparisons and Tukey's HSD test were implemented in STATISTICA.

Patterns of AM fungal community analysis

Because the peak size or peak area of a given TRF was somewhat variable among replicates, each TRF was coded just as a discrete character (0 for absent or 1 for present). To compare the mycorrhizal communities (i.e. T-RFLP patterns) to each other, we computed a parsimony tree as described in Vandenkoornhuyse *et al*. (2002a). The advantage of this method is that only informative TRFs are taken into account. To combine the information provided by the different TRFs, a multivariate approach based on principal component analysis (PCA) was performed. The principle of the PCA is to transform the data set by calculating new variables (principal component factors). This method is based on extraction of eigenvectors and eigenvalues from matrices of associations between variables (TRFs) and object (mycorrhizal community in root). To deal with our data set, covariance PCAs were computed. Non-centred PCAs were calculated, as these keep the differences of means among variables (i.e. the frequency of observation of each TRF). The projected variance accounted for 52–63% of the total variance. From these PCAs it is possible to determine the statistical reliability of the data by computing confidence ellipses. These ellipses are defined by a centre of gravity that corresponds to the mean of observations and by two axes reflecting the deviation from the mean (Sokal & Rohlf 1995). Here, confidence ellipses group the mycorrhizal communities by plants and, for a given plant, by soil treatment. All PCAs and confidence ellipses were implemented with the ADE-4 software package (Thioulouse *et al*. 1997).

Results

Grass root identification by PCR-RFLP

When nucleotide sequences of the *trn*L (UAA) intron from the three grasses were compared to the GenBank database they showed greatest similarity to *Poa* spp. and other members of the subtribe Poaceae. The three grasses yielded amplified fragments that varied in size. *A. capillaris* (AY177347) had a *trn*L (UAA) amplicon of 496 bp while the size of *F. rubra* (AY177345) and *P. pratensis* (AY177349) amplicons were 609 bp and 626 bp, respectively. This length heterogeneity provided a useful character for separating *A. capillaris* from the other two grasses without the aid of RFLP. The *trn*L (UAA) intron sequences of *P. pratensis* and *F. rubra* were examined for differences in restriction sites that would allow discrimination by RFLP. A theoretical digest of the PCR product with *Dde*I was predicted to yield three fragments from *F. rubra* (53 bp, 57 bp, 499 bp) and four fragments from *P. pratensis* (53 bp, 57 bp, 187 bp, 329 bp). Theoretical patterns were confirmed by agarose gel electrophoresis and thus a combination of length heterogeneity (LH) PCR and PCR-RFLP enabled us to identify the three taxa from sampled root fragments.

Overall, DNA was extracted from 123 root samples. Among these, 16 did not show the expected *trn*L (UAA) intron RFLP pattern: seven grass samples were apparently misidentified and nine showed additional bands which may have resulted from contamination with rootlets from different plant species. Therefore, these 16 samples were not analysed further.

T-RFLP analysis of AM fungal diversity

T-RFLP analysis using the labelled NS31-AM1 primers was performed on the 107 root samples for which the plant identity was confirmed unambiguously. To check that all the fragments detected are true TRFs (i.e. not artefactual signal related to a possible partial digestion or to a strong secondary structure), two independent PCRs, restriction endonuclease digestions and electrophoresis runs were performed for all samples. For 17% of the samples (i.e. 18 of 107 root DNA samples), the replicate T-RFLPs did not display the same qualitative diversity signature (data not shown). Because it was impossible to interpret these particular T-RFLP patterns without speculation, these 18 samples were removed from the initial data set. The other 83% of the environmental samples provided identical T-RFLP replicates that allowed to conclude with confidence that all peaks taken into account were true TRFs even when they were of low intensity. However, in this study, the use of peak height or peak height-based indices to reflect the evenness of particular types in a DNA sample was inappropriate because relative peak height varied among replicates. We therefore analysed the qualitative composition of the T-RFLP profiles as a description of the community complexity and hence the species richness of the 89 valid samples.

AM fungal community diversity

Each restriction enzyme that generates a T-RFLP pattern is likely to reveal variation in a different part of the sequence, so the resolution of the T-RFLP strategy can be improved by selecting more than one restriction enzyme that generates a good polymorphism for the TRFs for the two labelled ends. We have grouped the data generated for the two ends with *Hsp*92II and with *Hin*fI. The total number of TRFs is treated as an estimate of the AM fungal community complexity or diversity. From our data, it is possible to estimate that on average the number of AM fungi colonizing the grass roots is 24.5/4, i.e. 6.1, where the numerator is the mean number of TRFs (Table 1) and the denominator is two enzymes plus two ends. This value is consistent with a previous study using PCR-derived clone libraries (Vandenkoornhuyse *et al*. 2002a) and the co-occurrence of various AM fungi in roots of a single plant confirms earlier findings (Helgason *et al*. 1998, 1999; Daniell *et al*. 2001).

Host-plant effect

The mean of the total number of TRFs varied significantly among grass species, being lowest in *P. pratensis* (15.76) and highest in *A. capillaris* (29.80) (Table 1a). To examine the data in more detail, the T-RFLP profiles were compared by parsimony analysis. The main advantage of this method is that it uses informative TRFs to group related patterns. This analysis indicates that AM fungal communities differ among grass species (Fig. 1A). In addition to that, the AM fungal communities (i.e. T-RFLP patterns) were also compared using noncentred PCA, a second independent data analysis that plots the AM fungal community from each root sample in a multidimensional space. This analysis demonstrates that the AM fungal community colonizing *A. capillaris* is significantly different ($P = 0.05$) from the one colonizing *F. rubra* or *P. pratensis* (Fig. 2). Using the more conventional-centred PCA instead of noncentred PCA, no overlapping of the three ellipses of confidence was found (not shown), but this method is biased slightly in that the frequency of observation of each TRF is not taken into account. In agreement with the noncentred PCA, the two-way ANOVA shows that plant effect on AM community (number of TRFs) is significant ($P = 0.001$) (Table 2).

Soil treatment effect

The two-way ANOVA does not reject the null hypothesis of

Table 1 AM fungal species richness comparisons using unplanned pairwise analysis to test possible significant differences for all possible pairs of group means (i.e. AM community diversity estimated by the number of TRFs) using unequal *n* Tukey's honestly significant difference (HSD) test. (A) Group means compared are the number of TRFs in *F. rubra*, *P. pratensis*, and *A. capillaris* whatever the soil treatment. (B) Group means compared are the number of TRFs in roots sampled in control plots 'C', plots amended with nitrogen 'N', lime 'L', nitrogen and lime 'NL' and soil treated with biocide 'B' whatever the plant species

				P-values using unequal *n* HSD test		
(A) Host-plant	No. of root samples	Mean no. of TRFs	SD	*F. rubra*	*P. pratensis*	*A. capillaris*
F. rubra	24	25.34	5.69	—	0.0001	0.0095
P. pratensis	26	15.76	4.68	0.0001	—	0.0001
A. capillaris	39	29.80	5.68	0.0095	0.0001	—
All groups	89	24.46	8.11			

				P-values using unequal *n* HSD test				
(B) Soil treatment	No. of root samples	Mean no. of TRFs	SD	C	N	L	NL	B
C	20	24.50	7.49	—	0.2153	0.7194	0.9192	0.4204
N	19	20.97	8.69	0.2153	—	0.9542	0.7912	0.0026
L	16	22.24	8.83	0.7194	0.9542	—	0.9933	0.0395
NL	16	23.00	8.57	0.9192	0.7912	0.9933	—	0.1093
B	18	27.45	5.55	0.4204	0.0026	0.0395	0.1093	—
All groups	89	24.46	8.11					

Table 2 Partition of the total variance using a two-way anova to test the null hypotheses of no host-plant effect or soil treatment effect on AM fungal community (number of TRFs) and no interaction phenomenon (host plant × soil treatment)

Effect	d.f. effect	MS effect	d.f. error	MS error	*F*	*P*
Host plant	2	7.6494	8	0.2038	37.5273	< 0.001
Soil treatment	4	0.6298	8	0.2038	3.0900	0.0817
Interaction	8	0.2038	74	0.1457	1.3993	0.2111

no effect of soil treatment on mycorrhizal community in plant roots ($P = 0.082$) (Table 2). However, compared to the control plots, the biocide treatment tended to show higher AM community diversity colonizing grasses, while a lower diversity was observed when soils were amended with nitrogen or lime (Table 1b), although none of these was significantly different from the control plots. When the AM fungal communities are compared by parsimony analysis, no clear evidence of a soil effect is found (Fig. 1B). The noncentred PCA suggests that there is no soil treatment effect on AM communities colonizing *A. capillaris*, as all the confidence ellipses overlap (Fig. 3A). For communities colonizing *P. pratensis* (Fig. 3B), however, the confidence ellipse for biocide-treated communities has little overlap with the others, although the difference remains nonsignificant (Fig. 3B). The large size of the biocide ellipse is due mainly to the low number of *P. pratensis* samples from the plots of this soil treatment. For *F. rubra*, the noncentred PCA shows a striking and significant difference between AM fungal community diversity in root samples from biocide plots and in all the samples from the other soil treatments ($P < 0.05$) (Fig. 3C). The *F. rubra* root samples from control plots and those from lime, nitrogen, and nitrogen-and-lime amended plots share comparable AM fungal communities, whereas the root communities in biocide-treated soils are much more diverse. The results of standard (centred) PCA are comparable.

Discussion

T-RFLP as a tool for the study of AM fungal populations

Until now, most studies of AM fungal communities in roots have resolved the diversity within each root by cloning the PCR product and characterizing a number of individual clones (Helgason *et al*. 1998, 1999, 2002; Daniell *et al*. 2001; Vandenkoornhuyse *et al*. 2002a). While this approach is very informative, and can allow fungi to be identified by sequencing, each root sample requires considerable time and resources and the precision with which the root community can be described depends on the number of clones analysed. T-RFLP provides, much more rapidly, a direct description of each community so that it is feasible to compare a large number of single root communities (over 100 in this study). We have shown that the presence or absence of specific TRF bands is generally reproducible, as duplicate PCRs from the same root

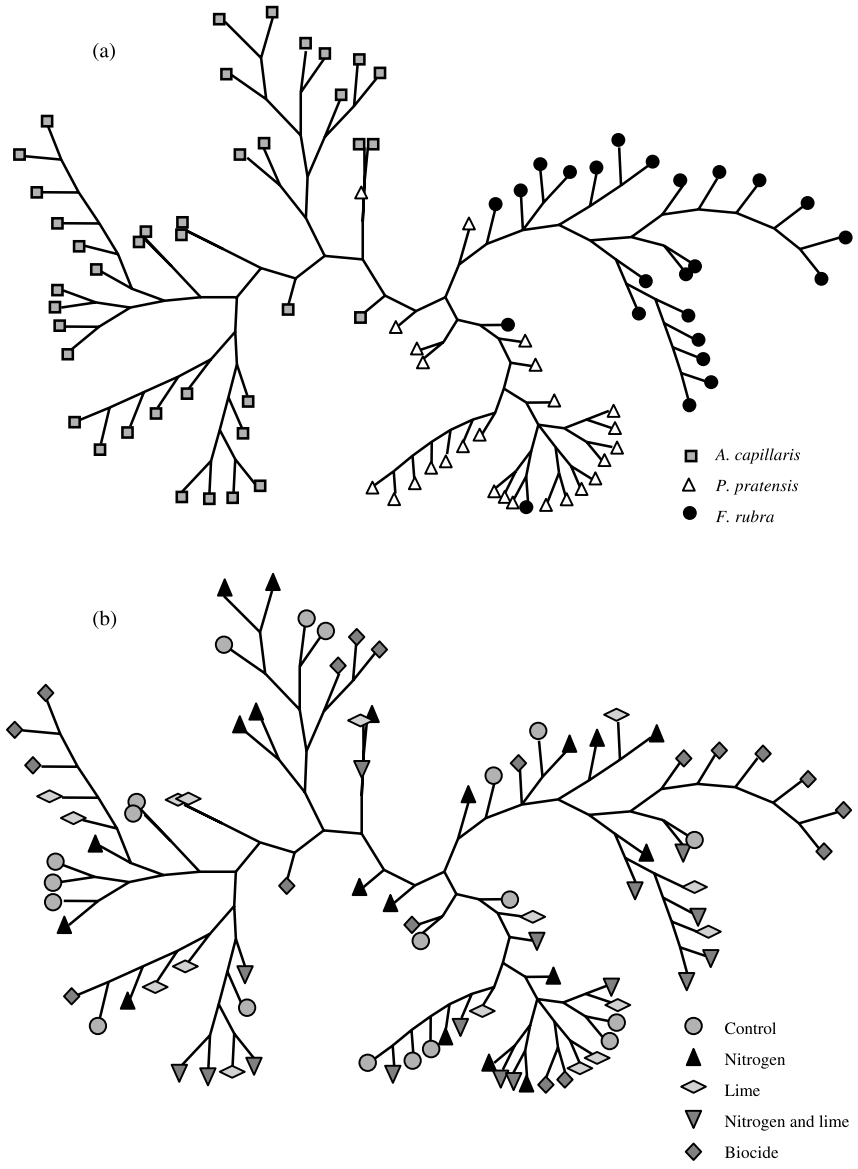

Fig. 1 The similarity among AM fungal communities displayed as the shortest unrooted maximum parsimony tree. The shortest topology found was calculated using a heuristic tree search with 100 replicates of random addition and using the TBR swapping algorithm with reweighted characters according to consistency index. Each terminal corresponds to the AM fungal community in a single root; the closer they are the most similar is their profile of T-RFLP fragments. The same tree is shown labelled (A) by host species and (B) by soil treatment.

sample led to exact concordance in the list of bands present in 83% of cases. This approach demonstrated very clear host-plant effects, and the large number of independent samples provides strong support for the conclusions. The T-RFLP approach should be valuable in future studies that aim to look for differences between AM fungal communities. If interesting differences are seen, cloning and sequencing can then be targeted on a small number of representative samples in order to define exactly which fungi are involved.

We based our analysis on the presence or absence of TRF bands, rather than on their relative intensity, because an examination of duplicate traces showed considerable variation in relative intensities. Some previous studies have not reported such variations (e.g. Lueders & Friedrich 2000; Kuske *et al*. 2002) but did not compare replicate PCRs. Conversely, Osborn *et al*. (2000) found that relative peak height varied substantially even among replicate tracks of the same digest on a single gel. Advances in technology may improve the quantification of bands, but there are a number of factors likely to affect the relationship between the abundance of a fungal type and the intensity of the corresponding band, and these will need to be addressed if this information is to be useful for community studies.

Host-plant preference of AM fungi and its consequences

In a previous study at the same field site (Vandenkoornhuyse *et al*. 2002a) we demonstrated that *Trifolium repens* (Fabaceae) and *Agrostis tenuis* (Poaceae) had different AM fungal communities. These hosts, a

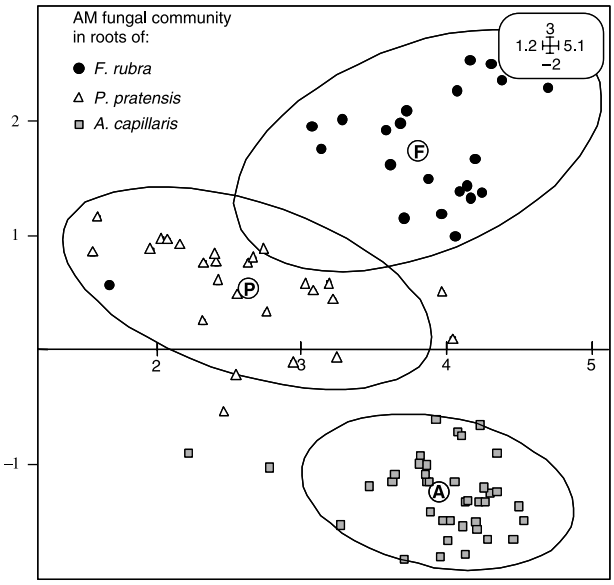

Fig. 2 Noncentred PCA analysis showing differentiation among AM fungal communities according to host plant. Each point represents the AM fungal community in a single root, characterized by T-RFLP profile. For each host–plant species, confidence ellipses ($P < 0.05$) are shown and the labelled circles indicate the centre of gravity (mean value): *A. capillaris* (A), *F. rubra* (F) and *P. pratensis* (P).

legume and a grass, belong to very different plant families, but in the present study we have shown differences among three species that are related much more closely. Taking this together with evidence from other studies (Johnson *et al*. 1991; Bever *et al*. 1996; Eom *et al*. 2000; Bidartondo *et al*. 2002; Helgason *et al*. 2002), it seems clear that plants and AM fungi recognize the diversity in their potential symbiotic partners and the resulting interactions are far from indiscriminate.

It is striking that the AM fungi colonizing *A. capillaris* were almost twice as diverse as those on *P. pratensis* (a mean of 29.8 TRF bands per root vs. 15.76, Table 1). In addition, each of the three grass species had a distinctive combination of AM types (Figs 1 and 2). The observation that a single host species supports only part of the available AM fungal diversity leads to the prediction that the diversity should be reduced in a monospecific plant population or crop monoculture. Indeed, the AM fungal diversity in arable crops does seem to be much lower than in grassland

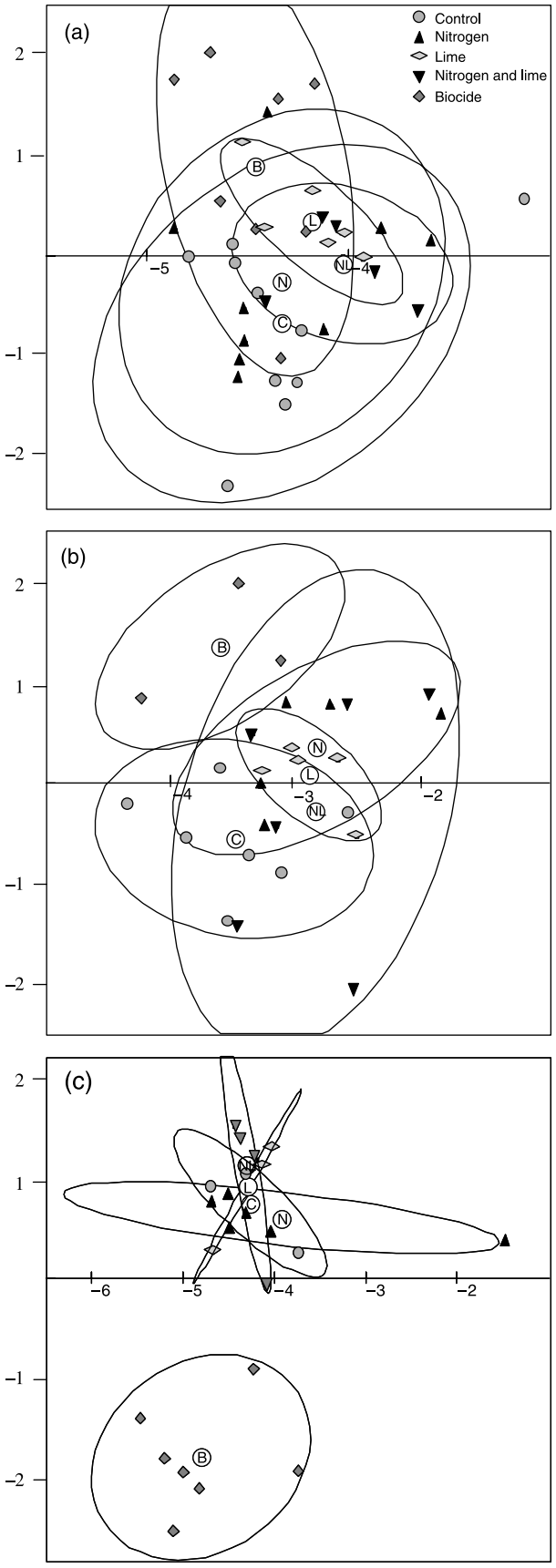

Fig. 3 Noncentred PCA analysis showing differentiation among AM fungal communities according to soil treatment for root samples of the grass species (a) *A. capillaris* (b) *P. pratensis* and (c) *F. rubra*. For each soil treatment, confidence ellipses ($P < 0.05$) are shown and the labelled circles indicate the centre of gravity (mean value): control plots (C), plots amended with nitrogen (N), lime (L), nitrogen and lime (NL), and soil treated with biocide (B).

or woodland (Helgason *et al*. 1998; Daniell *et al*. 2001). In a typical arable cropping system, monocultures of different plant species will typically be grown in a rotation over several years. Although this does increase the diversity of potential host species to which the AM fungal community is exposed, the expected effect will be to reduce the diversity still further because only those fungi that thrive in more than one host species will persist. AM fungal host-plant preference also explains directly the positive correlation found between AM fungal diversity and plant diversity in a grassland ecosystem (van der Heijden *et al*. 1998a).

In typical grassland ecosystems most plants are perennial, reproducing vegetatively and/or by seed, but most seeds disperse over a short distance. This results in a 'pseudo-vertical' transmission of the fungal consortium (Wilkinson 1997), which may allow a given AM fungus to become adapted specifically to colonize a given plant and its progeny in a local environment. Because AM fungi are obligate biotrophs they are competitors for the same ecological niche, the plant roots. This competition among AM fungi could be primarily a competition for resources that arises from limited shared resources (carbon assimilates provided by the host plant). It has been proposed repeatedly that resource competition is the main interspecific interaction and motor of adaptive radiation and divergent natural selection (e.g. Mayr 1942; Lack 1947; Schluter 2000). We suggest that competition for resources may be driving the preference of partners in the AM symbiosis.

The effect of soil treatments on AM fungal diversity

Treatment of the soil with nitrogen, lime, or both had no effect on the composition of the AM fungal communities in the roots of the three grass species. This is a surprising observation because the treatments had a marked effect on the composition of the plant community, with increased abundance of *F. rubra* and *P. pratensis*, two species often associated with improved pasture. Furthermore, lime treatment increased the pH from ≈ 4.8 to ≈ 6.2, and studies based on spore data have indicated that soil pH has a significant influence on the AM fungal community (e.g. Johnson *et al*. 1991; Wang *et al*. 1993). One interpretation of our results is that the soil treatments will affect the AM community via their effect on the plant community. Because plant species have different fungal communities, a change in the plant community composition will lead to a change in the overall fungal community even if there is no change in the fungi colonizing each plant host.

The second surprise is that insecticide treatment, which does not alter the plant community composition, does affect the AM fungal community. This is strikingly evident on the roots of *F. rubra* (Fig. 3C); a similar trend is apparent in *P. pratensis* but it is too weak to be significant (Fig. 3B). The effect of insecticide is to increase the fungal diversity. There is no clear effect in *A. capillaris* roots, but this species harboured the greatest fungal diversity in the first place. We can only speculate as to the link between insecticide and AM fungi, but it might result from a lower predation pressure related to a depletion of mesofaunal populations, especially collembolan species (springtails) that are known to be mycophagous (e.g. Petersen & Luxton 1982; Moore *et al*. 1985). Reduced predation might lead to the increased growth of AM fungal types that were kept in check previously at undetectable levels.

Towards an understanding of AM fungal communities

This study provides further evidence that, far from being an undifferentiated swarm of interchangeable types, AM fungi are heterogeneous. The fact that different plant species growing in the same soil become colonized by different fungi implies that the partners have recognition mechanisms that confer specificity. It also implies a linkage between the diversity of hosts and of mycosymbionts. The effect of insecticide in changing AM diversity illustrates the point, perhaps an obvious one, that AM fungi will differ in their sensitivity to biotic and abiotic stresses (Fitter 2001). Finally, and perhaps most importantly, the diversity of AM fungi must presumably also extend to diversity in their function within ecosystem processes, such as their processing of organic carbon from the plant. In all these aspects, we still have no more than a glimpse of the diversity of AM fungi, but a method such as T-RFLP that can rapidly survey diversity and chart changes in diversity will be a valuable tool in the studies that lie ahead.

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Philippe Vandenkoornhuyse worked as postdoctoral research fellow within the NERC Soil Biodiversity thematic programme that focused on soil biota diversity and functions at a common field site. His main areas of research are community ecology, genetics and evolution. Karyn Ridgway is a postdoctoral research fellow working on spatial diversity of AM fungi. Irene Watson is a technician in molecular ecology. Professors Alastair Fitter and Peter Young lead research groups in plant functional ecology and microbial molecular ecology, respectively.
