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Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities

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SUMMARY

Little information currently exists on species diversity in communities of arbuscular mycorrhizal fungi (AMF), mainly owing to difficulties in identification of field extracted spores on the basis of morphology. The possibility was explored to identify individual AMF spores from the field on the basis of a molecular marker, namely the nuclear ribosomal DNA encoding the highly conserved 5·8S rRNA with the two flanking internal transcribed spacers (ITS region), known to vary between species. A technique involving polymerase chain reaction followed by restriction fragment length polymorphism analysis (PCR–RFLP) was developed to amplify and characterize the ITS region from single AMF spores. PCR reactions with extracts from single spores of three AMF species, raised under glasshouse conditions, yielded reproducibly a single amplification product of the ITS region in sufficient amounts to allow cleavage with several restriction enzymes. The size of the ITS region, *c.* 600 base pairs, varied only slightly between species. Digestion of the PCR products with the restriction enzymes *Hin*fI and *Taq*I resulted in banding patterns that were reproducible for different individual spores of a given species, but showed clear differences between the three species tested. The sum of the fragment sizes was sometimes greater than the size of the original PCR product, e.g. in *Glomus mosseae*. Clones of the amplification product from a single spore of this fungus were obtained and sequenced. This yielded two closely related but different sequences, indicating that two different ITS regions co-existed in the spore. The RLFP pattern of the amplification product of the spore was a result of an amalgamation of these two sequences. The technique was applied to AMF spores collected from a species-rich grassland. Spores were sorted into morphological groups on the basis of their colour, size, and shape, and then subjected to PCR–RFLP analysis. In some morphological groups, a large percentage of spores failed to yield an amplification product, probably because they had lost their contents. A group of *Glomus* spores yielding amplification products in the majority of cases was further investigated: PCR–RFLP analysis on 10 individual spores from the field produced 10 different patterns. Similar results were obtained with other groups of spores. The results suggest that the diversity in natural AMF communities and the genetic diversity within individual spores might be much greater than previously thought.

Key words: Arbuscular mycorrhiza, biodiversity, internal transcribed spacer, PCR-RFLP, ribosomal DNA.

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are abundant in almost all plant communities (Harley & Smith, 1983). There are approx. 152 species of AMF which have been described on the basis of morphological characteristics of the spores (Walker & Trappe, 1993). Fungi with a similar appearance to AMF have been recorded in fossils of some of the first land plants (Pyrozynski & Dalpé, 1989), thought to be approx. 400 million years old. Despite this evolutionary old age, there has been little divergence of AMF over the last 400 million years (Law, 1988). Sequence analysis of the nuclear genes encoding the small-subunit rDNA of AMF support the evidence that divergence of the main genera of AMF occurred between 400 and 100 million years ago, followed by relatively little further divergence (Simon *et al.*,

1993). It is thought that mutualistic interactions are stable and that the inhabitant symbionts are not subject to strong selection for further speciation (Cook, 1985; Law, 1985).

The diversity of AMF in natural communities has not been investigated extensively and little information exists on which species, if any, are restricted to particular ecological niches. In natural plant communities the diversity of AMF populations is thought to be quite small, often with fewer than 10 species in grasslands. In agricultural soils the species diversity is even smaller than in natural communities (Johnson, 1993).

Investigations of AMF species diversity have been hindered by difficulties in species identification. Identification of AMF is based on characteristics of their spores. These tend to become degraded in the field and ideally identification should be made from pot-cultured material (each culture derived from a single spore) or from a large series of field-collected specimens (Walker, 1992). Producing pot cultures is time consuming. Moreover, obtaining single spore cultures from spores extracted from the field is unpredictable, partly owing to the small numbers of viable spores in the soil. Identification, whether from field-collected or pot-cultured material, is only useful to the species level.

Because of the difficulties in studying the morphology of AMF spores, molecular methods might be useful in species identification. The polymerase chain reaction (PCR) has the potential to amplify DNA from single spores, and PCR-based techniques have been used to amplify DNA from AMF. These techniques have been used to show that species identification is possible (Simon, Lalonde & Bruns, 1992; Wyss & Bonfante, 1994). The requirements of any molecular methods applied to investigations of AMF diversity and ecology are that they must be reproducible using DNA from single spores of AMF, involve rapid, uncomplicated and reliable extraction procedures, and be sufficiently cheap to allow the processing of many samples.

The nuclear ribosomal DNA encoding the highly conserved 5·8S rRNA with the two flanking internal transcribed spacers (ITS region) has been used for studying the phylogenetic relationships between fungal groups. Universal primers ITS1 and ITS4 amplify the 5·8S rRNA gene and the two internal transcribed spacers flanking it (White *et al.*, 1990). The 5·8S gene is highly conserved, although the internal transcribed spacers are more variable. Restriction fragment length polymorphism (RFLP) and sequence analyses of the ITS region have been successfully used for the identification and phylogenetic relationships of ectomycorrhizal fungi (Gardes *et al.*, 1991; Bruns & Gardes, 1993; Gardes & Bruns, 1993; Erland *et al.*, 1994), *Epichloë typhina* (Tsai *et al.*, 1994), the *Gaeumannomyces–Phialophora* complex (Ward & Akrofi, 1994) and *Penicillium* spp. (LoBuglio, Pitt & Taylor, 1994). Gardes & Bruns (1993) found that sequences of the ITS regions from different species of ectomycorrhizal fungi were characteristically different and could be used for identification purposes but that there was little variation between different isolates of the same species. In an investigation of variation of the ITS region in a natural population of the fungal endophyte *Epichloë typhina*, RFLP analysis did not reveal any differences between 48 isolates of the fungus, many of which had been shown previously to be genetically different according to random amplified polymorphic DNA analysis (K. Groppe, unpublished).

We investigated the use of a method of PCR-RFLP of the ITS region for the identification of single spores of AMF and tested its use in a preliminary investigation of diversity in AMF populations from a grassland in Switzerland with high plant species-richness.

MATERIALS AND METHODS

Collection of AMF spores

Spores from established pot cultures of *Acaulospora laevis* Gerdemann & Trappe (BEG 13), *Gigaspora candida* Bhattacharjee, Mukerji, Tewari & Skoropad (BEG 17), *Glomus caledonium* (Nicol. & Gerd.) Trappe & Gerdemann (BEG 20), *Glomus geosporum* (Nicol. & Gerd.) Walker (BEG 18), *Glomus intraradices* Schenk & Smith (isolate Native Plants Inc.), *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe (BEG 12) and *Glomus* sp. (BEG 19) were extracted by a sucrose centrifugation and flotation technique (Walker, Mize & McNabb, 1982) and collected on a 125 μm sieve. Spores from the field were extracted from soil of a study site, Nenzlinger Weide, a calcareous grassland in the Jura Mountains, Switzerland (grid reference 255 609 of the Landeskarte der Schweiz, sheet 1067). The spores were also extracted by sucrose centrifugation and flotation and were collected on 250, 125, 63 and 32 μm sieves. The site is 450 m above sea level and supports a diverse flora, characteristic of calcareous grassland which fits to the phytosociological description of mesobrometum. The soil is a rendzina, with a clayey to silty clayey texture (pH 6·8–7·6).

Observations on morphology

Spores from the field site were sorted into groups, whereby the spores within one group appeared morphologically similar, based on the external features of the spore colour, size, shape, visible contents and shape of the subtending hypha. Spores of *Glomus* spp. were the most common although a few spores of *Acaulospora* spp. and sporocarps of *Sclerocystis* spp. were also found in small numbers. No fungi in the genera *Entrophosphora*, *Gigaspora*, or *Scutellospora* were found. Consequently, only spores

possessing the characteristic subtending hyphae of *Glomus* spp. or sporocarp forming *Glomus* spp. and *Sclerocystis* spp. were selected for this investigation.

Observations on the external morphology and colour of the spores from the field site were made using a dissecting microscope with reflected light. Colours were compared to a fungal colour chart (Anon., 1969). Spores and the colour chart were illuminated simultaneously by a split quartz-iodine fibre-optic light source at a colour temperature of 3200° K. Spores were sorted for size according to the mesh size of the sieve on which they were collected. Only spores with a globose form were selected. Spore contents were classified according to whether spores possessed visible vacuoles or not. This criterion was practical for the sorting of light coloured spores, but could not be used for spores of a dark colour, the contents of which were not easily visible.

Molecular analysis

In previous work, sonication of AMF spores, for 15 seconds, was used to remove surface debris and potential contaminating organisms (Wyss & Bonfante, 1994) followed by surface sterilization (Ravolanirina, Gianinazzi-Pearson & Gianinazzi, 1987). In our experience, this procedure was found not to change the resulting fragment sizes from the PCR and therefore, in routine work, spores were not surface sterilized before the DNA was extracted. Large concentrations of the sterilizing solution (Chloramine T and streptomycin) inhibited the PCR. DNA was extracted by crushing a single spore (or a sporocarp) with sterile mini pestles (Medipack, Switzerland) in tubes containing 20 μl 1 M Tris (Cl^-), pH 8·0, whereafter the samples were kept on ice until 20 μl Chelex 100 resin (20% w:v, 100–200 mesh sodium form, Bio-Rad Laboratories, CA) had been added. Samples were centrifuged for 5 min at 14000 rpm (Eppendorf 5415C centrifuge) and 5 μl of the supernatant was added to 20 μl of PCR mix, modified from Gardes & Bruns (1993), not more than 10 min following extraction. DNA extracts from the whole sporocarps were not diluted further before adding to the PCR reaction. The PCR mix contained a final concentration of 200 μM of each of dATP, dCTP, dGTP and dTTP, 50 mM KCl, 10 mM tris (Cl^-; pH 8·3), 1·5 mM $MgCl_2$, 0·1 mg ml^{-1} gelatin, 0·5 units of Supertaq polymerase (Stehelin, Basel) and 1 μM of each of the two primers ITS1 and ITS4. Each reaction was overlaid with 20 μl mineral oil (Perkin–Elmer Inc., CA). Reactions were carried out in a 96 V-shaped well microtitre plate and temperature cycling in a programmable thermal cycler (PTC-100, M. J. Research Inc., MA). Temperature cycling followed Gardes & Bruns (1993). Up to 12 replicate PCR reactions could be made from one fungal spore and PCR products from one spore were pooled before carrying out restriction digests. PCR products from field extracted spores were not pooled.

Restriction digests of the PCR products were made using a modified Gardes & Bruns (1993) method with 15 μl PCR product and 15 μl of double strength restriction enzyme buffer (as recommended by the vendors) and 5 units of restriction enzyme. For the enzymes, *Alu*I, *Hae*III, *Hin*fI, *Rsa*I, *Sau*3AI, the digestion was for 2 or 6 h at 37 °C in tubes. The restriction enzyme *Taq*I was incubated at 65 °C for 2 or 6 h.

DNA fragments from PCR were fractionated by electrophoresis through horizontal 8 cm × 6 cm × 0·5 cm agarose gels for up to 2 h (Gibco BRL, 1·5% w/v) in Tris-acetate-EDTA buffer (100 mM Tris, 12·5 mM sodium acetate, 1 mM EDTA, pH 8·0). Restriction digest products were separated in the same way except that Metaphor agarose (FMC, 2% w/v) was used to provide better resolution of small DNA fragments. Gels were stained with 1 μg ml^{-1} ethidium bromide for 15 min, destained in distilled water for 5 min and observed and photographed under UV light.

Negative controls (no template DNA) were used in every experiment. The PCR product from negative controls contained primer artefacts of > 100 base pairs in size. To avoid confusing small DNA fragments from the restriction digests with primer artefacts the negative control PCR product was also used as a control for restriction digests.

Cloning and sequencing of the ITS region from AMF DNA

Amplified ITS regions from the DNA of single AMF spores were inserted into the pCR II cloning vector (Invitrogen, CA) by TA cloning and transformed into *Escherichia coli* bacteria. Recombinant plasmid DNA was isolated by the alkaline method of Sambrook, Fritsch & Maniatis (1989). DNA preparations were sequenced using the Sequenase Version 1·0 sequencing kit (US Biochemicals, CA). Sequences of the ITS regions of *Glomus mosseae* (BEG 12) have been deposited in the EMBL database (accession numbers X84232 and X84233).

Searches were made in the EMBL sequence database for homologous sequences using the BLAST procedure. Searches included the whole sequence which also contains the highly conserved 5·8S ribosomal gene. They were also carried out separately on the ITS regions at both the ITS1 and ITS4 primer ends, after omitting the 5·8S gene sequence.

RESULTS

Testing the molecular analysis

Amplification could be achieved from single spore extractions of AMF DNA (Fig. 1*a*, *b*). The ITS

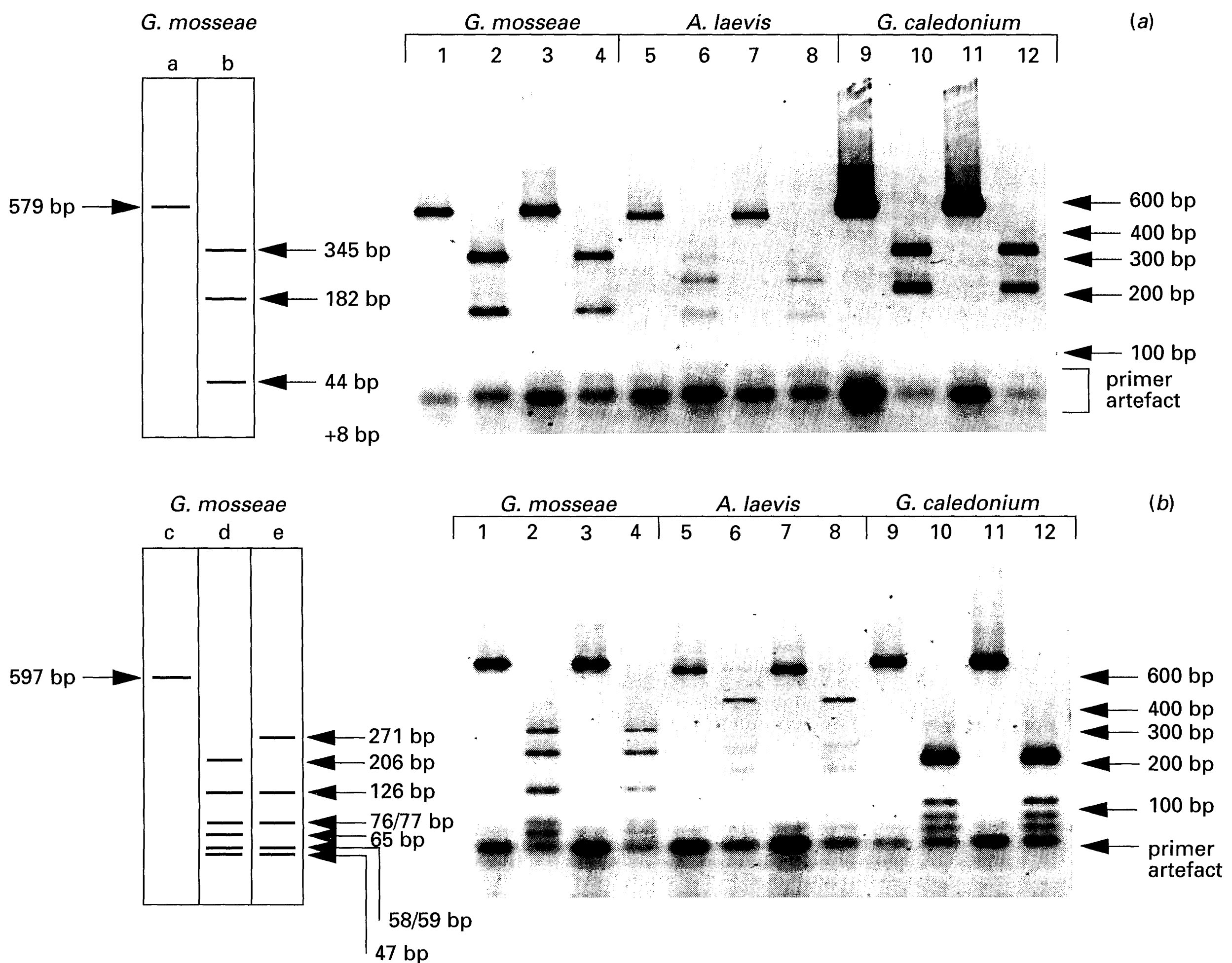

Figure 1. ITS fragments of three AMF and the patterns resulting from digests with restriction enzymes. (*a*) Lanes a and b, expected ITS fragment and banding pattern following digestion with *Hin*fI based on sequence information obtained for *G. mosseae*. Lanes 1 and 3, ITS fragments from individual *G. mosseae* (BEG 12) spores; lanes 2 and 4, ITS fragment from *G. mosseae* digested with *Hin*fI. Lanes 5 and 7, ITS fragment from individual spores of *A. laevis* (BEG 13); lanes 6 and 8, ITS fragment from *A. laevis* digested with *Hin*fI. Lanes 9 and 11, ITS fragment from single spores of *G. caledonium* (BEG 20); lanes 10 and 12, ITS fragment from *G. caledonium* digested with *Hin*fI. (*b*) Lanes c, d and e, expected ITS fragment and banding patterns with *Taq*I based on two different cloned DNA sequences of *G. mosseae* DNA. Lanes 1 and 3, ITS fragment from single spores of *G. mosseae*. Lanes 2 and 4, ITS fragment from *G. mosseae* digested with *Taq*I. Lanes 5 and 7, ITS fragment from individual spores of *A. laevis* (BEG 13); lanes 6 and 8, ITS fragment from *A. laevis* digested with *Taq*I. Lanes 9 and 11, ITS fragment from single spores of *G. caledonium* (BEG 20); lanes 10 and 12, ITS fragment from *G. caledonium* digested with *Taq*I. Fragment sizes determined on the basis of a 100 base pair marker (Gibco BRL).

fragment from *Acualospora laevis* was *c.* 530 bp in length whereas the fragments from *Glomus mosseae* and *Glomus caledonium* were *c.* 580 bp in length (Fig. 1*a*, *b*). ITS fragment sizes from *Glomus geosporum*, *Glomus intraradices*, *Glomus* sp. (BEG 19), *Gigaspora candida*, and another *Acaulospora* sp were *c.* 580 bp, 580 bp, 580 bp, 500 bp and 530 bp respectively (data not shown). Occasionally, more than one fragment was produced in the PCR. For the purposes of this analysis, PCR products were only considered as useful for further RFLP analysis if only one band was present. Any additional bands were assumed to be the result of contaminating DNA and this was verified in further investigations.

DNA fragments of various sizes were produced by cutting the amplified ITS fragment from different species of AMF with restriction enzymes (Fig. 1*a*, *b*). In all fungi that we used for these investigations no more than two enzymes were required to show differences between two species of AMF. The enzymes *Hin*fI, *Rsa*I, *Sau*3AI and *Taq*I were found to be the most useful for species identification, i.e. different sized DNA fragments were produced from the different fungal species. The enzymes *Alu*I and

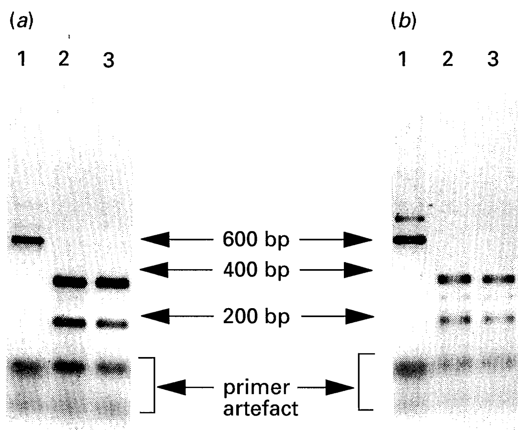

Figure 2. ITS fragments and restriction digests of *G. mosseae* (BEG 12) with *Hin*fI. Lane 1, ITS fragment; lanes 2 and 3; restriction digests. (*a*) *G. mosseae* spores; (*b*) *G. mosseae* sporocarp. Fragment sizes determined on the basis of a 100 base pair marker (Gibco BRL).

*Hae*III were able to cut the ITS fragment but always cut the fragment at the same place. This resulted in fragments of similar size from different species of fungi (data not shown). Patterns of DNA fragments after restriction digestion of the amplified ITS fragment were reproducible within a species, i.e. RFLP patterns obtained from different single spore samples of the same species always gave the same banding pattern (Fig. 1*a*, *b*). This was consistent with both 2 and 6 h digestions (data not shown). DNA fragments of approx. 50 base pairs were difficult to observe as they were obscured by the primer artefacts.

In the case of digestion with the enzyme *Hin*fI, no unusual RFLP patterns were observed (Fig. 1*a*,). In particular, the RFLP pattern obtained for *G. mosseae* matched exactly the expected RFLP pattern (Fig. 1*a*, lanes a and b) based on the two homologous but different sequences obtained from clones representing the ITS region of this fungus (see below), which both have identical *Hin*fI sites. In the case of digestion with the enzyme *Taq*I, the sums of the fragment sizes generated from the amplification product of either *Glomus mosseae* or *Acaulospora laevis* were larger than expected (Fig. 1*b*). For *Glomus mosseae*, this could be explained by the fact that one of the two ITS sequences of this fungus (see below) has an extra *Taq*I restriction site in addition to the four common ones, giving rise to two fragments of 206 and 65 base pairs, respectively, instead of a 271 bp fragment (Fig. 1*b*, lanes d and e). The RFLP pattern obtained with *Taq*I for single spores *G. mosseae* with *Taq*I (Fig. 1*b*, lanes 2 and 4) reproducibly contained an amalgamation of the two RFLP patterns expected from the sequenced DNA.

PCR using DNA from sporocarp-forming AMF was also tried. The amplified ITS region from single spores of *Glomus mosseae* were digested with *Hin*fI and produced reproducible fragment sizes (Figs. 1*a* and 2*a*). Using whole sporocarps of *Glomus mosseae* (containing approximately four to eight spores per sporocarp) from pot cultures amplification was achieved with an additional fragment in the PCR product which presumably originates from contaminants in the material surrounding the individual spores (Fig. 2*b*). The ITS fragment of AMF origin from sporocarps produced the same sized DNA fragments as from single spores, following restriction digestion with *Hin*fI. However, PCR of extracts from whole sporocarps never produced bands which were as intensely stained as using single spores which had been removed from sporocarps.

Glomus mosseae

```
                 10                    30                    50                    70                    90
                                                        1  2
Clone 1  TCCGTAGGTG AACCTGCGGA AGGATCATTA ATGATTTTAA AGCGAGTCG. AGCGTTAAGC GAGGCTTGCG AAAATATTTA AAACCCCACT CTTTATAACT
Clone 2  ---------- ---------- ---------- ---------- ---------A ---------- ---------- ---------- ---------- -C--T-----

                110                   130                   150                   170                   190
                                                                                         2
Clone 1  TTAAGAAAAT AAATCATGAT ACATGAATTT AAAAAAAAGA TCACTTTCAA CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC GAAATGCGAT
Clone 2  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                210                   230                   250                   270                   290
                                     1      2 1
Clone 1  AAGTAGTGTG AATTGCATAA TTTTGTGAAT CATCGAATCT TTGAACGCAA ATTGCACTCC CTGGTATTCC GGGGAGTATG CCTGTTTGAG GGTCGTTAGA
Clone 2  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                310                   330                   350                   370                   390
                 2
Clone 1  ATAAAAAATC GAGCGTGTCG CTCTTTTTTT TAAGGGTGAT CGCGTCGGAA TTGCAGCCGC TTTCAAATGT TAATTCATGT CAAAGTGGCT TGAAATTCAT
Clone 2  ---------- ---A-C---- ---------- ---------- ---------G ---AGC-GT- ---------- ---------- ---------- -A--------

                410                   430                   450                   470                   490

Clone 1  CCATCCGGTA CGGTTAAAGC GTATTTAAGA TCAATTTTGA TTAAGAACGC GCGATGACGT ACCATCTCAT GTAGTACG.T TGACCTGGGT CGTCAGGTTC
Clone 2  ---------- --A------- ---------- ---------T -C-------- ---------- ---------- --------T- ---------- -A--------

                510                   530                   550                   570
                         2
Clone 1  ACTCGTATAC TAAACTCGAA CTTTTGACCT CAAATCAGGT AAGAATACCC GCTGAACTTA AGCATATCAA TAAGCGGAGG A
Clone 2  -T-------- G-T---TA-- ---------- ---------- ---------- ---------- ---------- ---------- -
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Figure 3. Alignment of the two ITS sequences obtained from a single spore of *Glomus mosseae* (BEG 12). The full sequence is indicated for clone 1 and the sequence for clone 2 is identical except where indicated. The positions of the ITS1 and ITS4 primers are shown in italics and the restriction sites for *Hin*fI and *Taq*I are indicated by 1 and 2 respectively.

Table 1. *Mean percentages of field extracted* Glomus *and* Sclerocystis *spores which gave reliable PCR products with primers ITS1 & ITS 4. Numbers in parentheses indicate the range*

Spore group	Mean % of reliable PCR products*
Glomus, fuscous-black, 125–250 μm (fitting the description of *G. constrictum*)	21 (0–44)
Glomus, fuscous-black, 63–125 μm (fitting the description of *G. constrictum*)	27 (0–90)
Glomus, rusty tawny-dark brick, 125–250 μm	72 (66–100)
Glomus, rusty tawny-dark brick, 63–125 μm	47 (44–50)
Glomus aggregatum	< 10 (0–10)
Glomus invermaium	< 10 (0–10)
Sclerocystis rubiformis	< 10 (0–10)

* Percentage of DNA samples that yielded consistently reliable single PCR products from three replicate assays of the same DNA sample.

Sequences of ITS regions from AMF

Amplified DNA of the ITS region of *G. mosseae* was successfully cloned. Three clones obtained from the PCR product of a single spore were sequenced. Each sequence was 579 base pairs in length. Two of them were identical whereas the third one differed at 22 positions, indicating that a single spore contained two homologous but different ITS regions (Fig. 3). Comparing the whole sequence to entries in the EMBL database, high homology was found to the 5·8S gene of other fungi. Highest scores were obtained with the 5·8S gene of *Saccharomyces cerevisiae*, *Neurospora crassa* and *Cladosporium sphaerosperum* (with a smallest Poisson probability of $1{\cdot}3^{-45}$ to $1{\cdot}8^{-45}$). This indicates strongly that fungal ITS regions containing the 5·8S gene have been sequenced. Searches for homology of the ITS regions resulted in no matches at the ITS4 primer end for either sequence. The ITS1 primer ends exhibited no homology to ITS regions of any other organism in the EMBL database.

Analysis of field populations of AMF

The spores which were extracted from the field study site and sorted into different morphological groups were also used for PCR–RFLP analysis. Seven groups of spores were sorted. There were other morphologically distinct spores in the soil which were so infrequent that groups of spores could not be made from these species. Reliable amplification products were variable from the different groups (Table 1). The groups of rusty tawny (14) to dark brick (2) coloured 63–125 μm and 125–250 μm *Glomus* spores gave the most reliable amplification products. Subsequent observations under the compound microscope revealed that spores of the sporocarp-forming species, fitting the descriptions of *Glomus aggregatum* Schenk & Smith emend. Koske, *Glomus invermaium* Hall and *Sclerocystis rubiformis* Gerdemann & Trappe, were almost always empty. In addition, many of the 63–125 and 125–250 fuscous-black (36) *Glomus* spores, fitting the description of *Glomus constrictum* Trappe, also often had no contents and therefore produced no PCR product. Adequate numbers of viable spores of this group could, however, be found and reliable PCR products could be obtained from them.

RFLP analysis is only presented here for the group of rusty tawny (14) to dark brick (20) *Glomus* spores, > 125 μm in size. In this investigation, 15 spores were picked at random from a group of approx. 100 spores, all of which had been sorted on the basis of their similar external appearance. The ITS region of each individual spore was amplified and single banded ITS fragments were produced from 10 of the spores (Fig. 4). The other five PCR products were discarded as either having contaminants or because no detectable amplification had occurred. Subsequent restriction digests of the PCR products with only two restriction enzymes, *Taq*I (Fig. 5*a*) and *Hin*fI (Fig. 5*b*), revealed that five different banding patterns were seen with each enzyme. However, comparing the banding patterns of the fragments with the two enzymes indicated that no two banding patterns from different spores were alike (Fig. 5*a*, *b*), i.e. that 10 spores gave 10 different banding patterns. By contrast, the group of spores which fitted the description of *Glomus constrictum* produced the same sized DNA fragments after digestion of the ITS fragments using several restriction enzymes, in all cases where single fragment sized PCR products were used (data not shown).

DISCUSSION

Using a rapid and simple DNA extraction technique amplification of the ITS region from AMF was possible from single spores. The resulting DNA fragment could be cut using some of the low cost restriction enzymes. Thus, the technique is particularly useful for ecological studies. The technique was also useful for sporocarp-forming species but only if initial investigations were made with single spores of the sporocarp in order to know which PCR products were of AMF origin and which might have been the result of contaminant DNA (as determined in Fig. 2*a*, *b*). This, however, seems difficult to apply to field-collected sporocarps from our study site as many of the spores from sporocarps appeared to be unviable.

In some cases the sum of the DNA fragment sizes produced from the restriction digestion is larger than the original size of the ITS fragment (Fig. 1*b*).

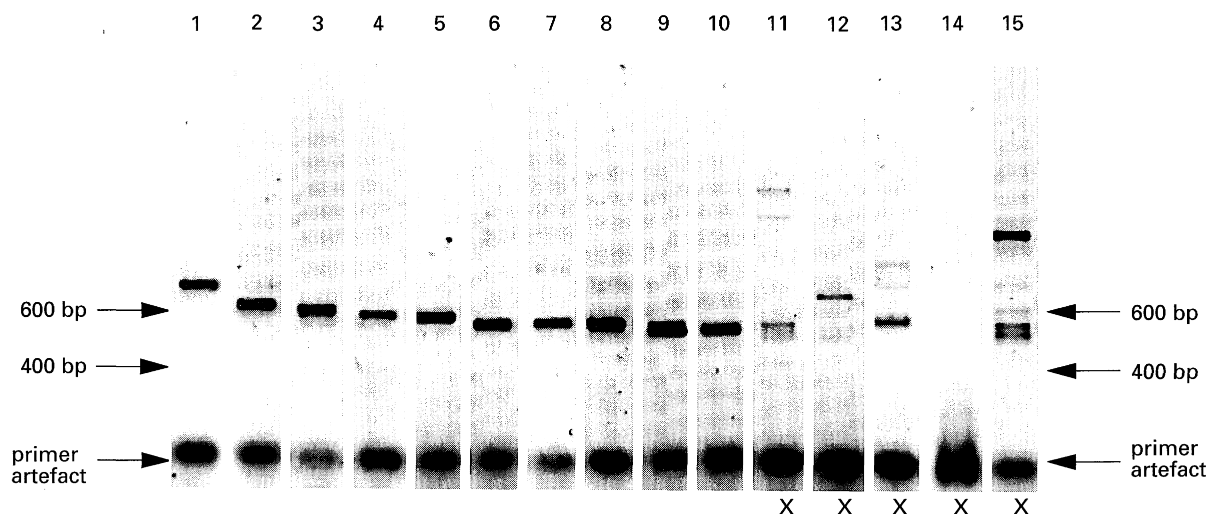

Figure 4. ITS fragments from 15 *Glomus* spores from a field population, all of which exhibited a similar external morphology. Samples have been ordered from left to right on the basis of decreasing fragment size. Three replicate PCR products were amplified from each spore. Only one replicate PCR product from each spore is shown. X indicates PCR products which were not further analysed. Fragment sizes determined on the basis of a 100 base pair marker (Gibco BRL).

Before obtaining the sequence information for the ITS region of *G. mosseae* we tested whether this could be owing to incomplete digestion of the PCR product. It seems unlikely that incomplete digestion occurred, in this case, as faint bands corresponding to the same size as the original PCR fragment should be visible after restriction digestion. The restriction enzymes were checked by digestion with known sequences of DNA from the fungus *Epichloë typhina* and the expected RFLP patterns were obtained consistently. Mixing known sequences of cleaned DNA with the uncleaned PCR products from the ITS of AMF did not inhibit enzyme action, thereby demonstrating that there are no inhibitors to restriction enzyme activity in the PCR product. Another possibility is that contaminant DNA could have given the same size ITS fragment with a different sequence from that of the AMF. This is also highly unlikely as known contaminants from the sporocarps of *G. mosseae* produced a different sized ITS fragment from that of the AMF (Fig. 2).

Other micro-organisms are reported to live on or inside some AMF spores (Scannerini & Bonfante, 1991). It seems likely that in cases where PCR fragments of more than one size had been amplified, the extra fragments could be the result of contaminating DNA from such organisms. Estimations of numbers of nuclei in single AMF spores vary from 9000 in *Glomus caledonium* to 35000 in *Gigaspora decipiens* (Burggraaf & Beringer, 1989; Viera & Glenn, 1990). It is, therefore, improbable that non-AMF DNA is being amplified in each reaction as contaminant DNA is likely to be a small part of the total DNA contained in a spore.

The two different sequences obtained from cloned DNA from a single spore and the matching of the RFLP patterns explain exactly why the sum of the fragment sizes is larger than the original size of the ITS fragment. This strongly indicates that individual spores of this AMF contain at least two different ITS sequences. It seems likely that they occur in similar frequency in the *G. mosseae* genome as the RFLP bands are all of similar intensity. The question remains as to whether the different sequences are the result of different ITS repeats in the same nucleus or whether different families of nuclei exist with different ITS sequences within single AMF spores.

The PCR–RFLP method was useful for spores collected from the field study site though the success in obtaining PCR products was variable and unpredictable. For some groups of spores, reliable products were obtained in a minority of cases and it was more usual to find no PCR product other than multiple fragments. This indicates that many spores extracted from the field soil were either empty (as was observed on close examination), contained insufficient DNA for amplication or contained substances which inhibited the PCR reaction. Probably, greater success was achieved with the groups of rusty tawny to dark brick *Glomus* spores because the spore contents were easily visible and this increased the chances of selecting spores which contained only AMF DNA. It will be important in the future to devise methods for selecting viable spores which will give amplification products from the PCR.

The high diversity of *Glomus* (10 genetically different spores in a sample size of 10 spores) based on the results of the PCR-RFLP indicates that diversity of AMF in natural communities might be much greater than expected from species determination based on morphology. At this stage one can only speculate whether this observed level of diversity is a result of inter- or intra-specific diversity.

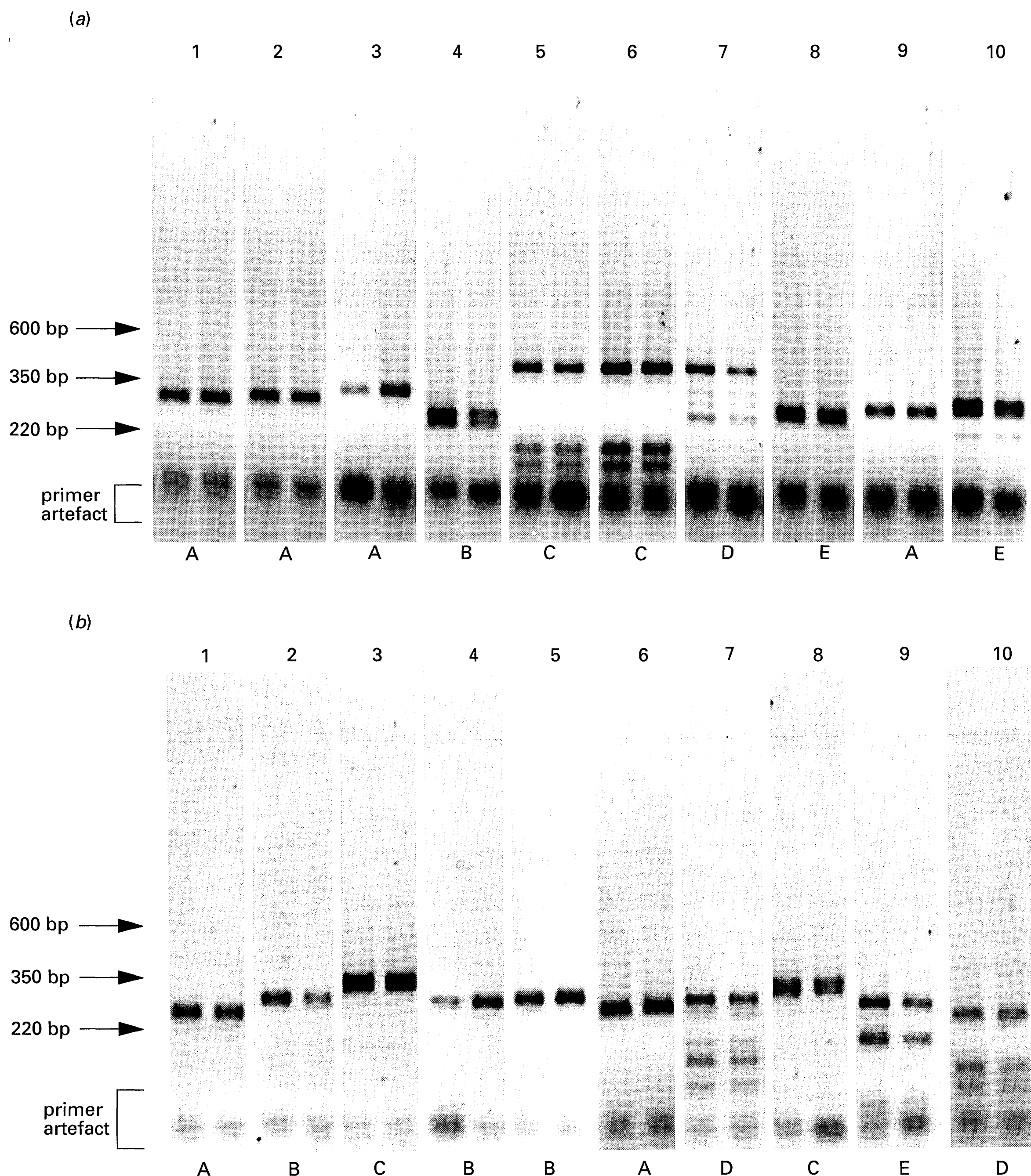

Figure 5. Restriction digests of the ITS fragments from 10 *Glomus* spores, shown in Figure 4, with (*a*) *Taq*I and (*b*) *Hin*fI. Numbers above lanes indicate digests from one PCR product (numbers correspond to lane numbers in Figure 4) and are shown in duplicate for each PCR product. Same letters below lanes indicate the same banding pattern, following digestion with one restriction enzyme. Fragment sizes determined on the basis of a 100 base pair marker (Gibco BRL).

However, information from other fungal groups indicates that the ITS region tends to be polymorphic between species but only to a small extent within species. Thus we think that the diversity in the ITS region in AMF might be representative for species diversity.

This investigation was concentrated on the genus *Glomus* but further investigations in the other genera of AMF from the study site, e.g. *Acaulospora* and *Sclerocystis* should help to identify levels of AMF diversity in this community. The results presented here, using this method, will be extended in the AMF groups in which the technique has already worked, to groups of other AMF where the technique was initially less successful, and to AMF in roots.

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