FEMS Microbiology Letters 241 (2004) 265–270

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Temporal temperature gradient gel electrophoresis (TTGE) as a tool for the characterization of arbuscular mycorrhizal fungi

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Received 5 May 2004; received in revised form 9 August 2004; accepted 17 October 2004

First published online 29 October 2004

Edited by H.B. Deising

Abstract

The aim of this study was to assess the feasibility of using temporal temperature gradient electrophoresis (TTGE) of PCR-amplified 18S rDNA fragments of different *Glomus* species for their detection and characterization. Screening of *Glomus clarum*, *Glomus constrictum*, *Glomus coronatum*, *Glomus intraradices*, *Glomus mosseae* and *Glomus viscosum* by PCR-TGGE revealed that the NS31-AM1 region of the 18S rRNA gene contained insufficient variation to discriminate between them. In contrast, TTGE analysis of the NS31-Glo1 region, which was obtained by nested PCR of the NS31-AM1 amplicon, showed that each species was characterized by a specific TTGE fingerprint. However, isolates of the same species could not be distinguished. The nested PCR-TTGE approach developed allowed identification of the *Glomus* species colonising the roots of different plant species.

Keywords: Arbuscular mycorrhizal fungi; 18S rDNA; TTGE

1. Introduction

Arbuscular mycorrhizal (AM) fungi are soil borne microorganisms known to establish a mutualistic symbiotic relationship with the roots of most land plants [1]. The most frequently described outcomes of the AM symbiosis include an enhanced mineral nutrient supply for the plant and a carbon supply for the fungus [1,2]. Establishment of the AM symbiosis protects plants against diverse abiotic and biotic stresses [3,4]. AM fungi occur in almost all habitats and climates [5], and there is increasing evidence that diversity of AM fungi has a significant impact on plant biodiversity, productivity and ecosystem stability [6,7]. In turn, plant communities can affect AM fungal diversity and community composition [8].

AM fungi are obligate biotrophs whose completion of life cycle depends on their ability to colonize the roots of a host plant [9]. AM fungi cannot readily be identified by their colonisation morphology and their identification has traditionally been based on the ontogeny and morphological characters of their large multinucleate spores [10]. To circumvent these problems of identification, an array of molecular techniques, such as amplified ribosomal DNA (rDNA) sequencing [11–13], amplified rDNA restriction analysis [14], random amplification of polymorphic DNA [15] and terminal-restriction fragment length polymorphism [16] have been employed.

The use of PCR targeting the 18S rDNA gene has provided a powerful mean of characterizing AM fungi, which has advanced our understanding of AM fungal

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0378-1097/$22.00
doi:10.1016/j.femsle.2004.10.030

diversity [17] and phylogeny [18]. Recently, molecular methods relying on sequence-specific separation of equal sized rDNA PCR amplified fragments using specific primers have been developed to identify AM fungi. These methods include single stranded conformation polymorphism (SSCP) [19] and denaturing gradient gel electrophoresis (DGGE) [20,21]. Temporal temperature gradient gel electrophoresis (TTGE), which has been used to characterize other types of microbial populations [22,23], has never been applied to profile and characterize AM fungi.

TTGE is a technique that works on the same basic principle than DGGE but without the requirement for a chemical denaturing gradient, thus producing more reproducible data [24]. The separation principle of TTGE is based on the melting behaviour of DNA molecules. In a denaturing acrylamide gel, double-stranded DNA is subjected to conditions that will melt it in discrete segments called melting domains, being the melting temperature (T_m) of these domains sequence specific. When the T_m of the lowest melting domain is reached, the DNA will become partially melted creating branched molecules. Partial melting of the DNA reduces its mobility in a polyacrylamide gel, making it possible to separate DNA fragments based on their melting behaviour. The aim of the present work was to evaluate the feasibility of using PCR-TTGE to differentiate between species within the genus *Glomus* based on sequence differences in a region of the 18S rDNA gene and to characterize the AM fungal species colonising a root.

2. Materials and methods

2.1. Biological materials

The AM fungi used in this study were: *Glomus clarum* Nicolson & Schenk (BEG125), a *Glomus constrictum* Trappe isolate from a semiarid Mediterranean ecosystem in the province of Alicante (Spain), a *Glomus coronatum* Giovannetti, Avio & Salutini isolate from a coastal dune in Almería (Spain), two *Glomus intraradices* Schenck & Smith isolates (BEG121 and DAOM 197198), three *Glomus mosseae* Nicolson & Gerdemann isolates (BEG119, BEG122 and an additional one isolated from the Mediterranean ecosystem in Alicante, Spain) and two *Glomus viscosum* Walker, Giovannetti, Avio, Citernesi & Nicolson isolates (BEG126 and an additional one from Alicante, Spain). The different AM fungal isolates were maintained on a mixture of sterile soil/sepiolite/vermiculite (1/1/1, v/v/v) as growing substrate and *Trifolium repens* and *Sorghum vulgare* as host plants.

Lavandula latifolia Medik., *Thymus mastichina* L. and *Retama sphaerocarpa* (L.) Boiss. plantlets were inoculated with a mixture of the following AM fungi: *G. constrictum*, *G. intraradices* BEG121 and *G. mosseae* BEG122. Each plant received around 180 AM propagules consisting of infected root pieces, external mycelium and spores by mixing all the different AM inocula with the growing substrate (sterile soil/ sand, 3/1, v/v). Plants were grown in a greenhouse (16/8 h light/dark photoperiod, 25/18 °C day/night temperature and 60/80% day/night relative humidity), watered three times per week to 90% field capacity and harvested one year after inoculation.

2.2. Spore and root DNA extractions

Spores were isolated from the pot culture substrate by the wet sieving and decanting method, followed by sucrose centrifugation [25]. Spores were collected under a dissecting microscope and washed four times with 1 ml milli-Q water. Single spores were placed in 20 μl milli-Q water on a siliconized slide and crushed with a sterile needle to obtain a crude DNA extract. Multispore DNA extractions were performed using the DNeasy Plant Mini Extraction Kit (Qiagen) according to the manufacturer's specifications, except that the liquid nitrogen grinding step was substituted by crushing the spores in the lysis buffer using a micropestle.

Root DNA was extracted using the DNeasy Plant Mini Extraction kit (Qiagen) following manufacturer's instructions.

2.3. Selection of a discriminating DNA region used for TTGE analyses

To select a 18S rDNA region allowing discrimination of the different *Glomus* species in TTGE, a predictive analysis was performed by using the WinMelt software Version 2.0.13 (Bio-Rad Laboratories), which calculates the melting profile of any sequence. The NS31-AM1 sequences of the 18S rDNA gene of 20 different *Glomus* species were obtained from the GenBank database and used in the predictive analysis. The GenBank accession numbers of the AM fungi used are the following: *G. caledonium* BEG20: Y17635, *G. claroideum*: AJ276075, *G. clarum*: AJ505619, *G. coronatum*: AJ699076, *G. constrictum*: AJ506090, *G. etunicatum*: Y17639, *G. fasciculatum*: BEG53 Y17640, *G. fragilistratum*: AJ276085, *G. geosporum*: AJ245637, *G. intraradices* BEG121: AJ536822, *G. lamellosum*: AJ276087, *G. luteum*: AJ276079, *G. manihotis*: Y17648, *G. mosseae* BEG69: U96141, *G. mosseae* BEG122: AJ505616, *G. proliferum*: AF213462, *G. sinosum*: AJ133706, *G. versiforme*: X86687, *G. verruculosum*: AJ301858, *G. viscosum* BEG126: AJ505620.

2.4. PCR amplifications

All PCRs were performed in an automated thermal cycler (Mastercycler, Eppendorf). Amplifications were

carried out in a final volume of 25 μl using the pure-Taq™ Ready-To-Go PCR beads (Amersham Pharmacia Biotech) and 1 μM of each primer. PCRs on crude DNA extracts (20 μl) were performed using the universal primer NS31 with a GC-clamp at its 5′-end (GC-NS31) [20] and the AM fungal specific primer AM1 [12]. The GC clamp was added to one of the primers to stabilize the melting behaviour of the PCR product for TTGE analysis. The amplification program consisted of an initial denaturation for 10 min at 95 °C, followed by 35 cycles with denaturation for 45 s at 94 °C, annealing for 1 min 20 s at 58 °C and extension for 2 min at 72 °C, and a final extension step at 72 °C for 7 min.

A nested PCR was performed on the NS31/AM1 PCR products using the GC-NS31 and the Glo1 (5′-GCCTGCTTTAAACACTCTA-3′) primers. Conditions for the nested PCR were as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles with denaturation for 45 s at 94 °C, annealing for 45 s at 52 °C and extension for 1 min at 72 °C, and a final extension step at 72 °C for 7 min. PCR products were separated electrophoretically on 1.6% agarose, stained with ethidium bromide, visualized by UV illumination and, when necessary, the expected band was excised with a scalpel and DNA isolated from the gel using the QIAEX gel extraction kit (Qiagen) following the manufacturer's protocols.

2.5. TTGE analysis

TTGE was performed by using the DCode universal mutation detection kit (BioRad Laboratories) and gels were 16 cm × 16 cm × 1 mm. For the separation of the GC-NS31/AM1 PCR fragments, electrophoresis was performed in a polyacrylamide:bisacrylamide (37.5:1) 6% (w/v) gel and 8 M urea with 1.5× TAE buffer (1× TAE = 40 mM Tris acetate, 20 mM acetic acid, 1 mM EDTA, pH 8.3) at a constant voltage of 130 V. A temperature gradient from 54 to 61 °C and a ramp temperature of 1 °C/h was applied. For the separation of the GC-NS31/Glo1 PCR products, 8% polyacrylamide gels containing 8 M urea were prepared and a temperature gradient from 52 to 59 °C with a ramp temperature of 1 °C/h was applied. Three-microlitre samples of PCR products were deposited in the wells and the temperature was adjusted to the gradient lowest temperature before the temperature gradient was built up. After runs, gels were stained for 20 min with ethidium bromide (1 mg/L in 1× TAE), rinsed for 10 min in ddH_2O, and photographed under UV light (Gel Doc 2000, BioRad Laboratories).

2.6. Cloning and DNA sequencing analysis

To characterize the different bands obtained after TTGE analysis of the NS31-Glo1 PCR fragments, bands were excised from the TTGE gels and DNA was isolated from the gel using the QIAEX gel extraction kit (Qiagen). The eluted DNA was reamplified with primers NS31 and Glo1. PCR products were then purified as previously described, cloned into the pGEM-T vector (Promega) and sequenced by using *Taq* polymerase cycle sequencing and an automated DNA sequencer (Perkin–Elmer ABI Prism 373). All DNA fragments were sequenced in both strands. The sequences obtained were compared to database sequences by using BLAST-N [26]. Alignments were performed using the CLUSTALW software [27].

3. Results and discussion

3.1. TTGE analysis of different Glomus species

The NS31-AM1 region of the 18S rDNA subunit, which allows discrimination of AM fungi at the species level [12,13,28], was initially targeted to analyse the potential of the TTGE technique to characterize AM fungi. PCR amplification of spore DNA from six *Glomus* species using the AM specific primer (AM1) and the universal one GC-NS31 yielded a single band of about 550 bp in all the species tested (data not shown). TTGE analysis of the NS31-AM1 amplicons showed slight differences in the TTGE patterns of some species; however other species such as *G. coronatum* and *G. mosseae* as well as *G. contrictum* and *G. viscosum* yielded indistinguishable TTGE patterns (Fig. 1). Moreover, when a mixture of the NS31-AM1 amplicons from all the AM fungal species studied was loaded on the gel a smear was obtained not allowing resolution of the bands corresponding to the different AM fungi (Fig. 1, lane 8). In spite of an extensive TTGE optimisation protocol (varying gradient steepness, running time and voltage), resolution could not be improved when analysing this ribosomal region. Recently, Öpic et al. [21] also observed that DGGE analysis of the NS31-AM1 ribosomal region allowed discrimination between AM fungal genera, but not always between species.

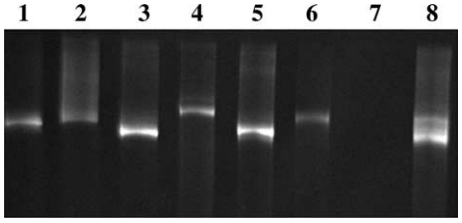

Fig. 1. TTGE analysis of the 18S rDNA NS31-AM1 regions of *G. clarum* (lane 1), *G. constrictum* (lane 2), *G. coronatum* (lane 3), *G. intraradices* BEG 121 (lane 4), *G. mosseae* BEG 122 (lane 5) and *G. viscosum* BEG 126 (lane 6). Lane 7 is a negative PCR control and lane 8 represents a mixture of the PCR products of each *Glomus* species.

To increase the resolution of the TTGE approach a different ribosomal region was targeted. To select a discriminating region within the AM1-NS31 region, the melting profile of the sequences of the 20 different species under study was obtained (data not shown) and a new primer (Glo1) was designed which in combination with the NS31 primer amplifies a DNA region of about 230 bp showing differences on the lowest melting temperature for the different *Glomus* species. Since the Glo1 primer is not specific of AM fungi, a nested PCR using the purified AM1-NS31 band as target was performed to avoid DNA amplification of contaminating organisms. The NS31-Glo1 fragments of the six analysed species migrated to different positions in the TTGE gel (Fig. 2). While a single specific band was obtained for *G. clarum*, *G. constrictum*, *G. coronatum*, *G. intraradices* and *G. mosseae*, four were detected for *G. viscosum*. Sequence analysis of the different bands yielded by *G. viscosum* revealed that all of them showed the highest homology (98–99%) to the 18S rDNA sequences of *G. viscosum* present in the database. These data suggest that the different bands produced by *G. viscosum* might be due to the genetic variability among rRNA gene copies described in certain AM fungi [14]. In spite of AM fungi are clonal, they have evolved to contain a surprisingly high within-species genetic variability [29], and genetically different nuclei can coexist within individual spores [30]. Although this genetic variability may complicate interpretation of TTGE results, each species is characterized by a specific TTGE fingerprint, which might be used to generate species-specific rDNA fingerprints for AM fungal detection.

To assess whether TTGE allows discrimination of different fungi in a complex mixture 2 μl of the NS31-Glo1 amplicons obtained for the different species were mixed and loaded on the gel. Bands of the different *Glomus* species were clearly distinguishable (Fig. 2, lane 8), which indicates the potential of PCR-TTGE to analyse complex AM fungal communities. The nested PCR strategy described in this paper combines the specificity for AM fungi of the AM1 primer with the resolving power on the denaturing gels of the NS31-Glo1 primer set. However, it should be kept in mind that although the AM1 primer allows discrimination against non AM fungi [12], sequences of several AM fungi, such as *Acaulospora gerdemannii*, *Geosiphon* and *Paraglomus occultum*, are not amplified with this primer [28]. Consequently, the approach presented in this paper may not be useful for the identification of these particular AM fungi.

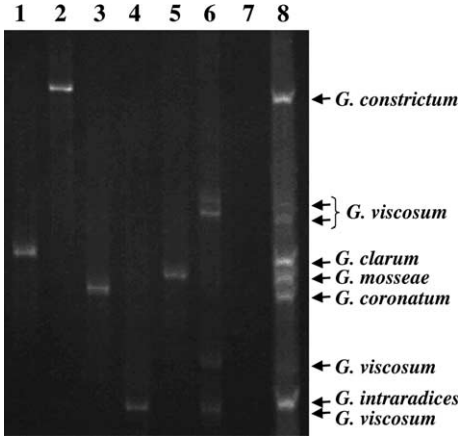

Fig. 2. TTGE analysis of the 18S rDNA NS31-Glo1 regions of *G. clarum* (lane 1), *G. constrictum* (lane 2), *G. coronatum* (lane 3), *G. intraradices* BEG 121 (lane 4), *G. mosseae* BEG 122 (lane 5) and *G. viscosum* BEG 126 (lane 6). Lane 7 is a PCR negative control and lane 8 represents a mixture of the PCR products from each *Glomus* species. The position of the bands for the different species is indicated on the right.

3.2. Determination of TTGE sensitivity

The sensitivity of detection of minority AM fungal populations via DNA extraction, nested PCR amplification and TTGE analysis was tested by mixing different number of spores of *G. clarum* and *G. coronatum*. DNA was extracted from mixtures containing 1 spore of *G. clarum* plus 1 of *G. coronatum*, 1 of *G. clarum* plus 10 of *G. coronatum*, and 10 spores of *G. clarum* plus 1 of *G. coronatum*. In the three cases both species were identified in the TTGE gel, although the *G. clarum* DNA was preferentially amplified when DNA was extracted from one spore of each species (Fig. 3(a)). Since the success in obtaining PCR products from single spores is variable and unpredictable, sensitivity of the TTGE approach was also tested by combining DNAs extracted from *G. clarum* and *G. coronatum* in different proportions. As shown in Fig. 3(b) a clear limit of detection was observed when the minority species accounted for 1:1000 or less of the total DNA concentration.

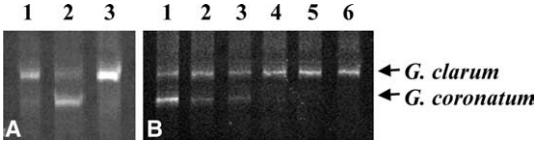

Fig. 3. TTGE sensitivity analysis. 18S DNA fragments were obtained by nested PCR amplification of: (a) genomic DNA extracted from mixtures containing different number of spores of *G. clarum* and *G. coronatum*. Lane 1, 1 *G. clarum* spore plus 1 *G. coronatum* spore; lane 2, 1 *G. clarum* spore plus 10 *G. coronatum* spores; lane 3, 10 *G. clarum* spores plus 1 *G. coronatum* spore; or (b) a mixture containing different amounts of genomic DNA from each species. Lane 1, 100 ng *G. clarum* DNA plus 100 ng *G. coronatum* DNA; lane 2, 100 ng *G. clarum* DNA plus 10 ng *G. coronatum* DNA; lane 3, 100 ng *G. clarum* DNA plus 1 ng *G. coronatum* DNA; lane 4, 100 ng *G. clarum* DNA plus 0.1 ng *G. coronatum* DNA; lane 5, 100 ng *G. clarum* DNA plus 0.01 ng *G. coronatum* DNA; lane 6, 100 ng *G. clarum* DNA plus 0.001 ng *G. coronatum* DNA. The position of the bands for the two species is indicated on the right.

3.3. TTGE analysis of different isolates from the same species

The TTGE profiles of two ecotypes of *G. intraradices*, three of *G. mosseae* and two of *G. viscosum* were compared to test if isolates from the same species could be distinguished in the denaturing gel. The different isolates of *G. intraradices* and those of *G. mosseae* always migrated at the same position on the TTGE gel, and could therefore not be distinguished from each other (Fig. 4). However, the two isolates of *G. viscosum* produced two bands that migrated at the same position on the TTGE gel, but additional bands were observed in one of the isolates, making it possible to distinguish between them. Resolution of different isolates might require, as discussed by Kowalchuk et al. [20], analysis of more variable DNA regions, such as a different region within the 18S rDNA [31] or regions within the 25S rDNA [32].

3.4. Application of TTGE to mycorrhizal roots

To test the potential of the technique to detect the AM fungal species colonising a root, DNA was extracted from the roots of different shrub plants inoculated with three of the AM fungal species characterized in this paper and a nested PCR was performed using the primer sets described above. Mycorrhizal colonization degrees of the *L. latifolia*, *T. mastichina* and *R. sphaerocarpa* test plants were 34, 35 and 60% respectively. In all the root samples analysed, the AM fungal species colonising the roots were clearly identified by TTGE analysis (Fig. 5). Comparison of the TTGE bands obtained from the roots with the TTGE fingerprint of each of the AM fungal species allowed identification of *G. constrictum*, *G. intraradices* and *G. mosseae* in the three analyzed root systems. These data clearly reveal the feasibility of the nested PCR-TTGE approach described in this paper to identify the AM fungal species colonizing a root.

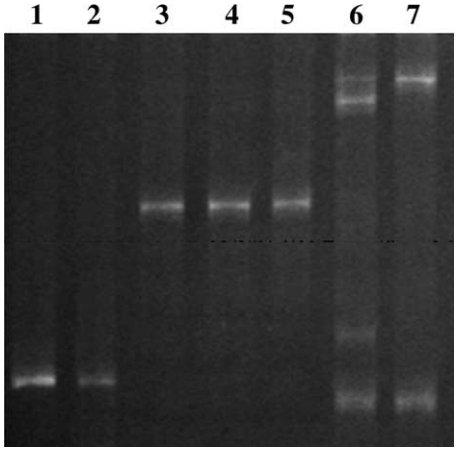

Fig. 4. TTGE analysis of the 18S rDNA NS31-Glo1 region of different ecotypes of *G. intraradices* (lanes 1 and 2), *G. mosseae* (lanes 3, 4 and 5), *G. viscosum* BEG126 (lanes 6 and 7) and *G. viscosum* isolated from Alicante (lane 7).

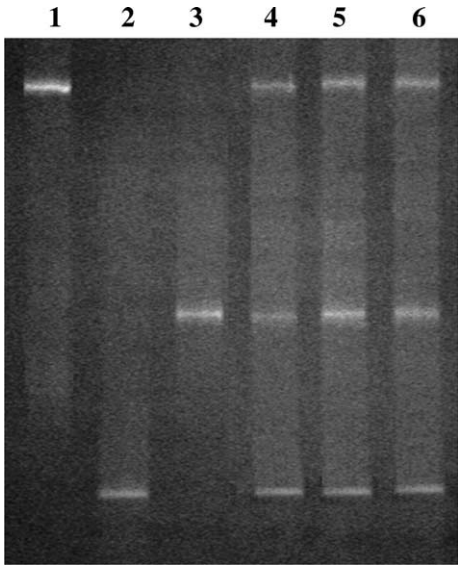

Fig. 5. Detection of the AM fungi colonising the roots of different plants by TTGE analysis of their 18S rDNA NS31-Glo1 regions. Lanes 1–3 are nested PCR fragments from spores of *G. constrictum* (lane 1), *G. intraradices* (lane 2) and *G. mosseae* (lane 3). Lanes 4–6 are nested PCR fragments from roots of *Lavandula latifolia* (lane 4) *Thymus mastichina* (lane 5) and *Retama sphaerocarpa* (lane 6) inoculated with a mixture of the AM fungi indicated above.

4. Conclusions

We have shown that it is feasible to differentiate species within the genus *Glomus* by TTGE analysis of the NS31-Glo1 region of their 18S rDNA. Moreover, this approach allows characterization of the AM fungal species actually colonising a root. Even though more research is needed to assess whether this technology allows discrimination between other AM fungal species, the experimental approach here described seems to be appropriate for ecological studies involving AM fungi. Ongoing research is being performed to determine the potential of this method when applied to field roots with unknown AM symbionts.

Acknowledgement

This research was supported by the Spanish CICYT-EU co-financed programme (Projects REN2000-1506 GLO and REN2003-00968/GLO), and by the EU project (CONSIDER EVK2-2001, 00254). Pablo Cornejo was supported by a grant from the Spanish Agency for International Cooperation (AECI).

References

[1] Smith, S.E. and Read, D.J. (1997) Mycorrhizal Symbiosis, 2nd edn. Academic Press, London, UK.

[2] Ferrol, N., Barea, J.M. and Azcón-Aguilar, C. (2002) Mechanisms of nutrient transport across interfaces in arbuscular mycorrhizas. Plant Soil 244, 231–237.

[3] Azcón-Aguilar, C. and Barea, J.M. (1996) Arbuscular mycorrhizas and biological control of soil-borne plant pathogens - An overview of the mechanisms involved. Mycorrhiza 6, 457–464.

[4] Leyval, C., Joner, E.J., del Val, C. and Haselwandter, K. (2002) Potential of Arbuscular Mycorrhizal Fungi for Bioremediation In: Mycorrhizal Technology in Agriculture: From Genes to Bioproducts (Gianinazzi, S., Schüepp, H., Barea, J.M. and Haselwandter, K., Eds.), pp. 175–186. Birkhäuser, Basel, Boston, Berlin.

[5] Barea, J.M., Azcón, R. and Azcón-Aguilar, C. (2002) Mycorrhizosphere interactions to improve plant fitness and soil quality. Antony van Leeuwenhoek 81, 343–351.

[6] van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A. and Sanders, I.R. (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396, 72–75.

[7] Klironomos, J.N., McCune, J., Hart, M. and Neville, J. (2000) The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. Ecol. Lett. 3, 137–141.

[8] Johnson, D., Vandenkoornhuyse, P.J., Leake, J.R., Gilbert, L., Booth, R.E., Grime, J.P., Young, J.P.W. and Read, D.J. (2003) Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosm. New Phytol. 161, 503–515.

[9] Bago, B. and Bécard, G. (2002) Bases of the Obligate Biotrophy of Arbuscular Mycorrhizal Fungi In: Mycorrhizal Technology in Agriculture: From Genes to Bioproducts (Gianinazzi, S., Schüepp, H., Barea, J.M. and Haselwandter, K., Eds.), pp. 33–48. Birkhäuser, Basel, Boston, Berlin.

[10] Morton, J. and Benny, J. (1990) Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glominae and Gigasporinae, and two new families, Acaulosporaceae and Gigasporaceae with an emendation of Glomaceae. Mycotaxon 37, 471–491.

[11] Simon, L., Lalonde, M. and Bruns, T.D. (1992) Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Appl. Environ. Microbiol. 58, 291–295.

[12] Helgason, T., Daniell, T.J., Husband, R., Fitter, A.H. and Young, J.P.W. (1998) Ploughing up the wood-wide web?. Nature 384, 431.

[13] Ferrol, N., Calvente, R., Cano, C., Barea, J.M. and Azcón-Aguilar, C. (2004) Analysing arbuscular mycorrhizal fungal diversity in shrub-associated resource-islands from a desertification-threatened semiarid mediterranean ecosystem. App. Soil Ecol. 25, 123–133.

[14] Sanders, I.R., Alt, M., Groppe, K., Boller, T. and Wiemken, A. (1995) Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol. 130, 419–427.

[15] Lanfranco, L., Wyss, P., Marzachi, C. and Bonfante, P. (1995) Generation of RAPD-PCR primers for the identification of isolates of *Glomus mosseae*, an arbuscular mycorrhizal fungus. Mol. Ecol. 4, 61–68.

[16] Vandenkoornhuyse, P., Ridgeway, K., Watson, I.J., Fitter, A.H. and Young, J.P.W. (2003) Grass plants, soil treatments, and mycorrhizal communities. Mol. Ecol. 12, 3085–3095.

[17] Helgason, T., Fitter, A.H. and Young, J.P.W. (1999) Molecular diversity of arbuscular mycorrhizal fungi colonising *Hyacinthoides non-scripta* (bluebell) in a seminatural woodland. Mol. Ecol. 8, 659–666.

[18] Schüßler, A., Schwarzott, D. and Walker, C. (2001) A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. Mycol. Res. 103, 1413–1421.

[19] Kjøller, R. and Rosendahl, S. (2000) Detection of arbuscular mycorrhizal fungi (Glomales) in roots by nested PCR and SSCP (single stranded conformation polymorphism). Plant Soil 226, 189–196.

[20] Kowalchuk, G.A., de Souza, F.A. and van Veen, J.A. (2002) Community analysis of arbuscular mycorrhizal fungi associated with *Ammophila arenaria* in Dutch coastal sand dunes. Mol. Ecol. 11, 571–581.

[21] Öpik, M., Moora, M., Liira, J., Koljalg, U., Zobel, M. and Sen, R. (2003) Divergent arbuscular mycorrhizal fungal communities colonize roots of *Pulsatilla* spp. in boreal Scots pine forest and grassland soils. New Phytol. 160, 581–593.

[22] Bosshard, P., Santini, Y., Grüter, D., Stettler, R. and Bachofen, R. (2000) Bacterial diversity and community composition in the chemocline of the meromictic alpine Lake Cadagno as revealed by 16S rDNA analysis. FEMS Microbiol. Ecol. 31, 173–182.

[23] Ogier, J.-C., Son, O., Gruss, A., Tailliez, P. and Delacroix-Buchet, A. (2002) Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. Appl. Environ. Microbiol. 68, 3691–3701.

[24] Yoshino, K., Nishigaki, K. and Husimi, Y. (1991) Temperature sweep gel electrophoresis: a simple method to detect point mutations. Nucl. Acids Res. 19, 3153.

[25] Sieverding, E. (1991) Vesicular-Arbuscular Mycorrhiza Management in Tropical Agrosystems. GTZ. Eschborn, Deutschland.

[26] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

[27] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.

[28] Schüßler, A., Gehrig, H., Schwarzott, D. and Walker, C. (2001) Analysis of partial *Glomales* SSU rRNA gene sequences: implications for primer design and phylogeny. Mycol. Res. 105, 5–15.

[29] Hijri, M. and Sanders, I.R. (2001) Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. Nature 414, 745–748.

[30] Trouvelot, D., van Tuinen, D., Hijri, M. and Gianinazzi-Pearson, V. (1999) Visualization of ribosomal DNA loci in spore interphasic nuclei of glomalean fungi by fluorescence in situ hybridization. Mycorrhiza 8, 203–206.

[31] Kodner, R. and Graham, L.E. (2000) Glomalean fungi from the Ordovician. Science 289, 1920–1921.

[32] van Tuinen, D., Jacquot, E., Zhao, B., Gollote, A. and Gianinazzi-Pearson, V. (1998) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol. Ecol. 7, 879–887.