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SHORT NOTE

Taxon-specific PCR primers to detect two inconspicuous arbuscular mycorrhizal fungi from temperate agricultural grassland

Hannes Gamper · Adrian Leuchtmann

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Abstract Taxon-specific polymerase chain reaction (PCR) primers enable detection of arbuscular mycorrhizal fungi (AMF, Glomeromycota) in plant roots where the fungi lack discriminative morphological and biochemical characters. We designed and validated pairs of new PCR primers targeted to the flanking regions of the variable domain 1 of the nuclear ribosomal large subunit RNA gene to specifically detect *Acaulospora paulinae* and an undescribed member of the Diversisporaceae. These two fungal taxa, sporulating late in soil-trap cultures and showing small, faintly coloured spores and weakly staining intraradical structures, were frequently found in roots of *Trifolium repens* from a high-input agricultural grassland. The newly developed PCR primers may thus enable studies on two inconspicuous AMF taxa that appear to have been overlooked in previous molecular AMF community analyses and for which no specific PCR primers have been published.

Keywords *Acaulospora paulinae* · Arbuscular mycorrhizal fungi · Diversisporaceae · Nuclear ribosomal large subunit (LSU, 28S) · *Trifolium repens* (white clover)

H. Gamper · A. Leuchtmann
Institute of Integrative Biology, ETH Zurich,
Universitätstrasse 16,
CH-8092 Zürich, Switzerland

Present address:
H. Gamper (✉)
Department of Biology, University of York,
P.O. Box 373, Heslington,
York YO10 5YW, UK
e-mail: hag500@york.ac.uk

Introduction

At present, molecular approaches are clearly superior to any morphological (Abbott and Robson 1979; Merryweather and Fitter 1998) or biochemical (Jansa et al. 1999; Sakamoto et al. 2004) methods for studying communities of arbuscular mycorrhizal fungi (AMF, Glomeromycota; Schüßler et al. 2001) in plant roots. This is due to a lack of distinctive features shown by AMF taxa below family level and in some cases the inability to stain certain species within host roots (Clapp et al. 2002; Redecker et al. 2003). Morphological species identification of AMF relies mainly on the characteristics of soil-borne spores and is also difficult in environmental samples due to developmental variation or parasitism of spores. Because factors controlling sporulation and morphological differentiation of AMF taxa are poorly understood, and occurrence of taxa in the spore community and in roots do not necessarily coincide, community studies based solely on spores appear to be of little ecological relevance (Clapp et al. 1995; Sanders 2004). This is why a focus on the fungal root colonisation patterns is crucial to expand knowledge of symbiotic interactions and environmental effects on the mycorrhizal symbiosis.

Molecular studies on AMF communities have repeatedly found new DNA sequence types (e.g. Vandenkoornhuyse et al. 2002; Rosendahl and Stukenbrock 2004; Wirsel 2004). These phylogenetically distinct sequences are either evidence for the occurrence of undescribed AMF taxa or highlight poor taxonomic sequence coverage. The present study addresses both of the above issues in describing new tools to specifically target two inconspicuous AMF taxa in environmental root samples. Polymerase chain reaction (PCR) amplification detection with taxon-specific primers is a simple, straightforward approach to assess the occurrence (presence/absence) of particular AMF taxa. It

contrasts with other more sophisticated molecular techniques to fingerprint whole communities, such as PCR-restriction fragment length polymorphism (Helgason et al. 1998; Vandenkoornhuyse et al. 2002) and PCR-denaturing gradient gel electrophoresis (Kowalchuk et al. 2002). Most previous PCR-based studies have targeted the nuclear ribosomal cistron to investigate AMF in environmental DNA samples (Clapp et al. 2002; Redecker et al. 2003), although recently a set of nested PCR primers for presumed single copy genes have been developed to analyse the spore community from natural soil (Stukenbrock and Rosendahl 2005a,b). The multicopy nature of ribosomal genes and available sets of universal primers for PCR amplification (e.g. White et al. 1990) have greatly facilitated earlier work aimed at directly detecting AMF species or species groups using either the nuclear ribosomal large subunit (nrLSU) RNA gene (van Tuinen et al. 1998a; Kjøller and Rosendahl 2000; Geue and Hock 2004) or the internal transcribed spacer (ITS) region (Redecker 2000; Millner et al. 2001; Renker et al. 2003).

The objective of this study was to develop and validate specific PCR primers targeted to the nrLSU RNA gene for direct detection of *Acaulospora paulinae* and an as yet undescribed member of the Diversisporaceae within roots of field-grown plants. We describe the design of primers, specificity tests using recombinant plasmids from single-spore PCR amplicons and multispore DNA extracts from pure fungal cultures, and successful application of the primers on a large number of environmental root-DNA extracts. Partial nrLSU sequences of indigenous AMF taxa from the experimental site served as reference information, besides sequences deposited in public databases.

Materials and methods

Arbuscular mycorrhizal fungi

A putatively undescribed *Glomus* sp. and five other AMF morpho-taxa belonging to the genus *Glomus* were recovered from trap cultures (Gilmore 1968; Brundrett et al. 1999) of soil from the Swiss-Free air CO_2 Enrichment (FACE) experiment [see Zanetti et al. (1996) for a detailed description of the field experiment] and successfully isolated into pure cultures, using either single spores (*Glomus* sp. isolates 83, 234 and 272) or a single apparently colonised spore of *Scutellospora calospora* (Nicol. & Gerd.) Walker & Sanders (*Glomus* sp. isolate 410). *A. paulinae* Blaszkowski was an additional species that abundantly sporulated in 5- to 8-month-old trap cultures and that might have been overlooked in the first spore screenings. Bulk inocula of all pure isolates have been stored in a 4°C room at the Institute of Plant Sciences of ETH in Eschikon (ZH) and reference slides with spores and samples of inoculum were sent to the living culture collections of the international culture collection of arbuscular and vesicular-arbuscular mycorrhizal fungi (INVAM) and the international bank of the Glomeromycota (BEG). In addition, samples of the isolates 83 and 272 of *Glomus* sp. were sent to the Glomeromycota *in vitro* collection in Belgium (GINCO-BEL). *Glomus* sp. is a member of the Diversisporaceae and will be formally described as a new species elsewhere. Spores of other indigenous AMF taxa from this field site were used to confirm the specificity of the newly designed oligonucleotide primers in cross-amplification tests. The INVAM isolate AZ420A of *G. eburneum* Kenn, Stutz & Morton (voucher W4729) served as reference.

DNA preparation

PCR templates from single spores were prepared following the procedure described by Jansa et al. (2003). DNA from multiple spores of monospore isolates was extracted using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany), which was also used to extract 20 mg of freeze-dried and ball-milled root samples of *T. repens*. Seven root patches of about 10 cm diameter were taken at least 15 cm apart from each of six permanent FACE plots and subdivided into four fractions, yielding 28 samples per plot and a total of 168 samples.

Nested PCR amplification from single-spore DNA extracts, vector-cloning and sequencing

In a nested PCR procedure, the universal eukaryote-specific primers ITS3 (White et al. 1990) and NDL22 (van Tuinen et al. 1998b), and then the eukaryote- LR1 (van Tuinen et al. 1998a) and fungus-specific primer FLR2 (Trouvelot et al. 1999) were used to obtain DNA fragments of approximately 700–760 bp length. Reactions were performed in final volumes of 25 and 60 µl, for the first and second amplification steps, respectively. Both PCR mixtures contained 1 × PCR buffer [supplied by the manufacturer, containing 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris–HCl (pH 9.0)], with additional 1 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM of each primer (HPLC purified, Microsynth GmbH, Balgach, Switzerland), 0.02 U/µl Taq DNA Polymerase [containing 0.2 mM Tris–HCl (pH 7.5), 0.02 mM dithiothreitol, 0.4 µM EDTA (Amersham Pharmacia Biotech, Piscataway, NJ, USA)], and 5 µl of single-spore DNA extract or 12 µl of diluted (1:10–1:1000) products from the first PCR. Thermocyclings used an initial denaturation cycle at 94°C (3 min) and 30 cycles with denaturation at 94°C (45 s), annealing at 60°C (55 s), and extension at 72°C (90 s), followed by a final extension at 72°C for 10 min and cooling down to 4°C.

PCR amplification products were purified, vector-cloned and sequenced from plasmids following standard procedures (Sambrook et al. 1989) using ABI Prism® BigDye Terminator Cycle Sequencing chemistry (version 1.1) on an automated capillary ABI Prism® 310 sequencer (Applied Biosystems, Forster City, CA, USA). Electropherograms were checked and ambiguities corrected using Sequence Navigator PPC (version 1.0.2b3, Applied Biosystems, Forster City, CA, USA) before comparing the sequences with the public database entries via basic local alignment (BLASTn, Altschul et al. 1997) to confirm glomalean origin.

Taxon-specifc nested PCR amplification from crude DNA extracts of environmental root samples

Primer pairs for *A. paulinae* (f6 5′- TAA ATC TCC GAG GTT TCC TTG GC -3′, r1 5′- TCA TCT TTC CCT CAC GGT ACT TG -3′) and *Glomus* sp. (f4 5′- TAA ATC TAC CTG GTT CCC AGG TC -3′, r2 5′- TGA ACC CAA AAC CCA CCA AAC TG -3′) were used in nested PCR amplifications on 168 crude DNA extracts from roots of *T. repens* plants from the field. The nested PCR amplification comprised a first round of amplification with the universal fungal primer pair LR1/FLR2 in a final volume of 25 µl as described above and a second, nested amplification with the newly designed specific primer pairs. Mixtures of the second PCR each contained 1× PCR buffer (specified above), 1 mM $MgCl_2$, 0.12 mM of each dNTP, 0.6 µM of each specific primer (HPLC purified, Microsynth GmbH, Balgach, Switzerland), 0.02 U/µl Taq DNA polymerase (specified above), and 2 µl of a 1:10 to 1:10,000 dilution of the first PCR products, depending on the band intensity of the first amplicons on 1.5% (w/v) agarose gels. Annealing temperatures were 55 and 65°C and cycle numbers 30 and 35 for the PCR of *A. paulinae* and *Glomus* sp., respectively. All other thermocycling parameters were as in the first round of amplification, except that time for annealing was only 40 s and time for extension only 70 s.

The specificity of the newly designed primers was verified by vector-cloning the specific PCR products of four root samples per AMF taxon and sequencing at least ten vector-inserts from re-amplification products obtained with the universal vector primers M13f/r following standard procedures.

Phylogenetic positioning of the two target taxa

The newly derived partial nrLSU RNA gene sequences of *A. paulinae* and *Glomus* sp. were aligned with reference sequences from public databases of taxonomically identified fungal isolates of all major clades of AMF, using Clustal X (version 1.83, Thompson et al. 1997). Fine adjustments to the alignment were made manually in MacClade (version 4.08, Sinauer Associates, Sunderland, MA, USA) after visual inspection. Ambiguously aligned regions were excluded before evaluation of the appropriate evolutionary model and phylogenetic analyses. The analysed data set comprised 87 sequence taxa and 503 sites of which 205 were parsimony-informative and 238 constant.

Appropriate models of sequence evolution were determined using ModelTest (version 3.7, Posada and Crandall 1998). A variable base and transition, but equal transversion frequency model with variable substitution rates among sites and a proportion of invariable sites (TrN + G) was selected by hierarchical likelihood ratio tests. In contrast, by use of the Akaike Information Criterion (AIC) a general time reversible sequence evolution model with variable substitution rates among sites (GTR + G) was favoured. Both sets of best-fit evolutionary model parameters were used for distance neighbour-joining analyses (objective function set to minimum evolution, 10,000 bootstrap pseudo-replicates) using the program Paup*4b10 (Swofford 2003) and Bayesian analyses using the Metropolis-coupled Markov chain Monte Carlo search algorithm as implemented in the program MrBayes (version 3.1, Ronquist and Huelsenbeck 2003). Four Markov chains were run in parallel for 300,000 generations and trees sampled every 100th generation. Tree samples taken before the 25% burn-in were discarded before calculating posterior probabilities for tree branches. Log-likelihood values and consensus trees from stationary samples of each of two replicate runs were compared to verify convergence to congruent phylogenetic trees. The trees were rooted with an outgroup of three reference sequences of *Archaeospora gerdemannii* (Rose, Daniels & Trappe) Morton & Redecker, which is a species belonging to an ancestral lineage of AMF (Redecker et al. 2000).

Results

nrLSU sequences and location of taxon-specific primers for *A. paulinae* and *Glomus* sp.

Two specific primer pairs f6/r1 for *A. paulinae* and f4/r2 for *Glomus* sp. (see Materials and methods) were deduced based on a multiple alignment of newly generated sequences of AMF taxa indigenous to the field site and selected reference sequences of other taxa from public databases. The priming sites were chosen within distinct regions where the majority of sequence haplotypes of the targeted taxon showed identical nucleotide sequences. Primability and stability of priming sites were confirmed as high in PCR simulations using Amplify (version 1.2, Engels 1993) and specificity with respect to all publicly available

sequences was checked with BLASTn searches (http://www.ncbi.nlm.nih.gov/). The forward primer f6 was unique for *A. paulinae* and the reverse primer r2 was unique for *Glomus* sp., which enables specific PCR amplification of these two taxa. The annealing sites for the forward primers f4 and f6 lie immediately upstream of the variable domain 1 (D1) and the reverse primers r1 and r2 in the region between D1 and D2, nested within the DNA fragment covered by the primer pair LR1/FLR2 (Fig. 1). Therefore, both pairs of newly designed specific primers cover D1, giving rise to PCR amplification products of 255 bp in the case of *A. paulinae* and 358 bp in the case of *Glomus* sp. The newly generated partial sequences of the nrLSU RNA gene (LR1-FLR2 fragment) were deposited in GenBank under the accession numbers: AY639263-AY639265, AY639327-AY639328 (*A. paulinae*), AY639225-AY639241, AY639306, DQ350448-DQ350453 (*Glomus* sp.), and EF067886-EF067888 (*G. eburneum*, AZ420A, W4729).

Primer specificity, sensitivity and applicability under field conditions

A three-step approach was used to test amplification ability of primers and to check their specificity. First, recombinant plasmids of the five most common sequence types of each of the two AMF taxa were used to optimise PCR conditions for new primer pairs, then plasmids of other AMF species from the field site were used to test specificity in the presence of a target and nontarget template of known sequence. Secondly, PCR was performed on multispore DNA extracts prepared from single-spore isolates, using a nested (see Materials and methods) and direct PCR amplification approach. Finally, the broader specificity of the new primers was evaluated on crude root DNA extracts from *T. repens* plants sampled in the field.

Amplification tests using recombinant plasmid templates yielded DNA fragments of the expected size for the target taxa only. Testing against nontarget plasmid templates confirmed the absolute specificity of the primer pairs by the absence of any cross-amplification products (data not shown). Additional PCR amplification tests, using multi-spore-DNA extracts of single spore cultures, confirmed specificity by the absence of cross-amplification with the tested four AMF taxa (data not shown). The primer pair f4/r2 yielded strong amplification in direct PCR on multispore genomic DNA extracts of the four pure cultures of *Glomus* sp., making a nested PCR approach superfluous and demonstrating high sensitivity of this primer pair.

A high specificity and sensitivity of the two new primer pairs was confirmed on a large number of DNA extracts from roots sampled in the field. One hundred fifty-five out of 168 DNA extracts (92.3%) gave visible PCR amplicons on agarose gels after the first fungus-specific amplification with the primer pair LR1/FLR2. Fifty-seven DNA extracts (33.9%) gave nested amplification products with the primer pair f6/r1 specific for *A. paulinae* and 39 extracts (23.2%) with the primer pair f4/r2 specific for *Glomus* sp. Figure 2 shows the amplicons from nested PCR amplification of a DNA extract from roots which were apparently simulta-

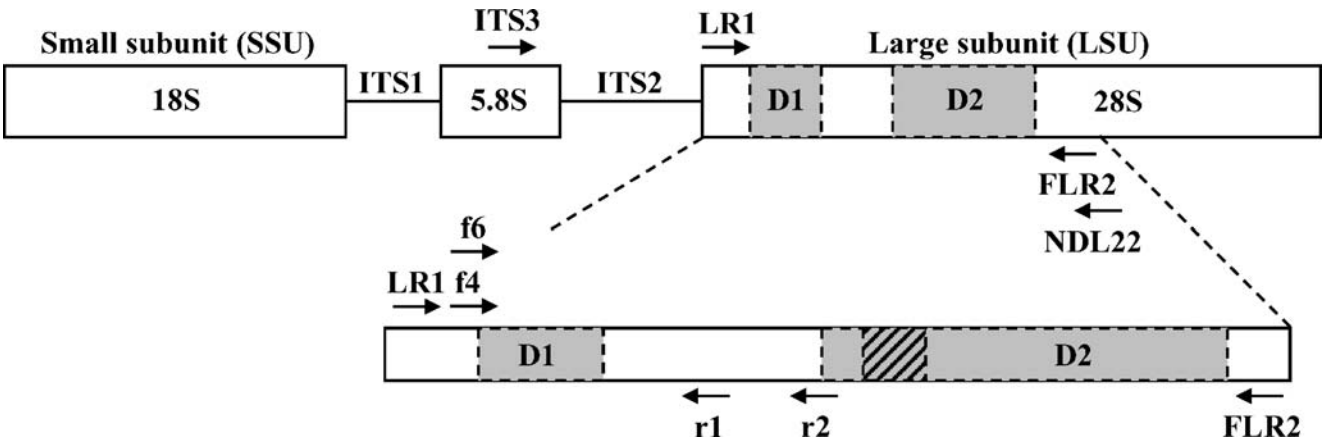

Fig. 1 Schematic representation of the nuclear ribosomal RNA cistron: positions of priming sites at the 5′ end of the nuclear ribosomal large subunit (LSU, 28S) RNA gene. Forward PCR primers are shown *above*, reverse primers *below* the gene *boxes*; *arrows* indicate the 5′–3′orientation of primers. Fragments of approximately 700–760 bp are obtained using a nested PCR approaches with primer pairs ITS3/NDL22 (universal eukaryote-specific primers; White et al. 1990; van Tuinen et al. 1998b), and LR1/FLR2 (universal eukaryote- and fungus-specific primer, respectively; van Tuinen et al. 1998a; Trouvelot et al. 1999) on single-spore DNA extracts. Nested PCR approaches with crude genomic DNA extracts from environmental root samples and the primer pairs LR1/FLR2 and f4/r2 for *Glomus* sp. and f6/r1 for *Acaulospora paulinae* Blaszkowskid yield DNA fragments of 358 and 255 bp, respectively. The variable domains D1 and D2 are indicated in *grey*, and the indel, distinguishing members of Glomerales and Diversisporales, is highlighted with *black hatching*. The diagram is not to scale

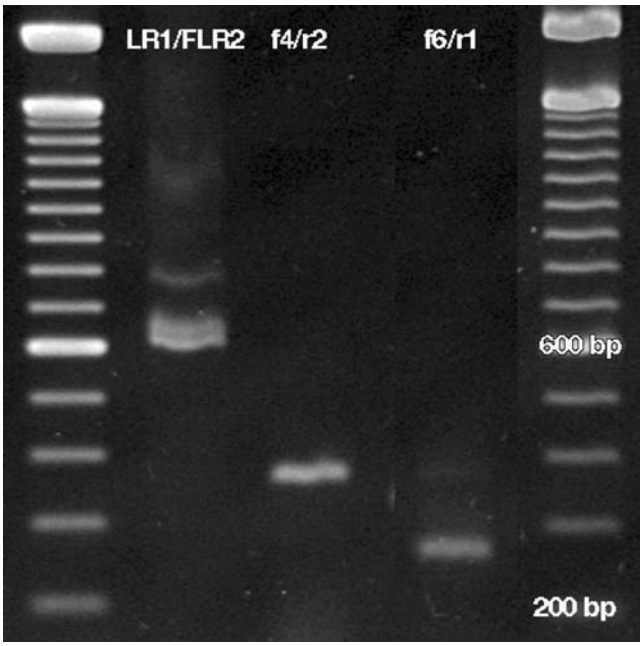

Fig. 2 Example of primer use with a crude genomic DNA extract from environmental root samples of *Trifolium repens* L.: ethidium-bromide-stained agarose gel (2% w/v in 1 × TBE) showing PCR products of nested amplifications by the two newly designed taxon-specific PCR primers (indicated *above lanes*). Lane 1 (primers: *LR1/FLR2*) contains the products from the first, universal fungus-specific amplification, showing multiple fragments of different length due to the presence of different fungal species. In lane 2 (primers: *f4/r2*, 358 bp) the specific PCR amplicons from *Glomus* sp. and in lane 3 (primers: *f6/r1*, 255 bp) those from *Acaulospora paulinae* Blaszkowski are resolved. The negative controls of both amplification reactions contained no products (data not shown)

neously colonised by both AMF taxa. The mean relative frequency of occurrence in the seven sampled root patches per field plot (see Materials and methods) varied between 7.1 and 67.9% [mean (SEM): 33.9 (10.0)%] for *A. paulinae* and 0.0 and 46.4% [mean (SEM): 23.2 (6.9)%] for *Glomus* sp. among the six FACE plots.

Sequencing of selected, vector-cloned PCR products confirmed the high specificity of the new PCR primer pairs under field conditions, and showed the presence of sequence variability within the short PCR amplified DNA fragments covering D1 of the nrLSU RNA gene. At least one recovered sequence haplotype was found several times among the sequenced PCR products of the two AMF taxa. Whereas the primer pair f4/r2 targeted to *Glomus* sp. showed absolute specificity, the primer pair f6/r1 targeted to *A. paulinae* yielded three nonspecific PCR amplicons with an additional band on agarose gels from the 168 analysed root-DNA extracts (1.8%). Vector-cloning of two of these PCR amplicons revealed that 18 out of 38 determined sequences (47.4%) were of nontarget origin and affiliated with basidiomycetous yeasts.

Phylogenetic position and distinctiveness of *A. paulinae* and *Glomus* sp.

The phylogenetic position of the two AMF taxa targeted by the newly developed specific primer pairs was evaluated by Bayesian and neighbour joining analyses. Both types of analyses yielded similar results. All sequences derived from single spores of *A. paulinae* formed a clearly distinct sister clade to *A. longula* Spain & Schenck within the family Acaulosporaceae, whereas those from the four single-spore isolates of *Glomus* sp. clustered in a distinct clade (posterior probability > 0.95), sister to a clade of sequences from the INVAM isolate AZ420A (*G. eburneum*) both of which are in sister position to the isolate BEG 47 (*G. versiforme* Daniels & Trappe), belonging to the Diversisporaceae (Fig. 3, Schüßler et al. 2001).

The clade of *Glomus* sp. was further structured into four subclades containing sequences derived from up to three different single-spore isolates, pointing at intra-individual polymorphism at the nrLSU RNA gene loci of this fungal taxon. Median pairwise sequence similarity among the 24 *Glomus* sp. sequences was high (99.1%) compared with similarities of these sequences to the two public database sequences of *G. versiforme* BEG 47 (92.4%) and to three sequences of INVAM isolate AZ420A of *G. eburneum* (95.9%). Moreover, 21 nucleotide sites discriminate between *Glomus* sp. and *G. eburneum* (AZ420A), which is good evidence for the genetic distinctiveness of these two members of the Diversisporaceae.

All major clades formed by taxa of other AMF families as inferred from reference sequences from public databases were highly resolved. Branch support by posterior probabilities from Bayesian analyses (Fig. 3) was similar to that from nonparametric bootstrap resampling in neighbour joining analyses (data not shown). Moreover, tree topologies were nearly identical in both analyses and remained unchanged with different selected best-fit evolutionary models in Bayesian analyses (TrN + G and GTR + G; see Materials and methods), suggesting that phylogenetic trees based on these partial nrLSU RNA gene sequences were sufficiently robust.

Discussion

PCR primers were designed to specifically detect for the first time *A. paulinae* and a currently undescribed AMF taxon *Glomus* sp. (member of the Diversisporaceae) and validated on DNA extracts from roots of field-grown plants. The primers target the 5′ end of the nrLSU RNA gene, where they flank the variable domain D1. Control PCR confirmed the lack of any cross-amplification in the presence of recombinant plasmids and multispore DNA

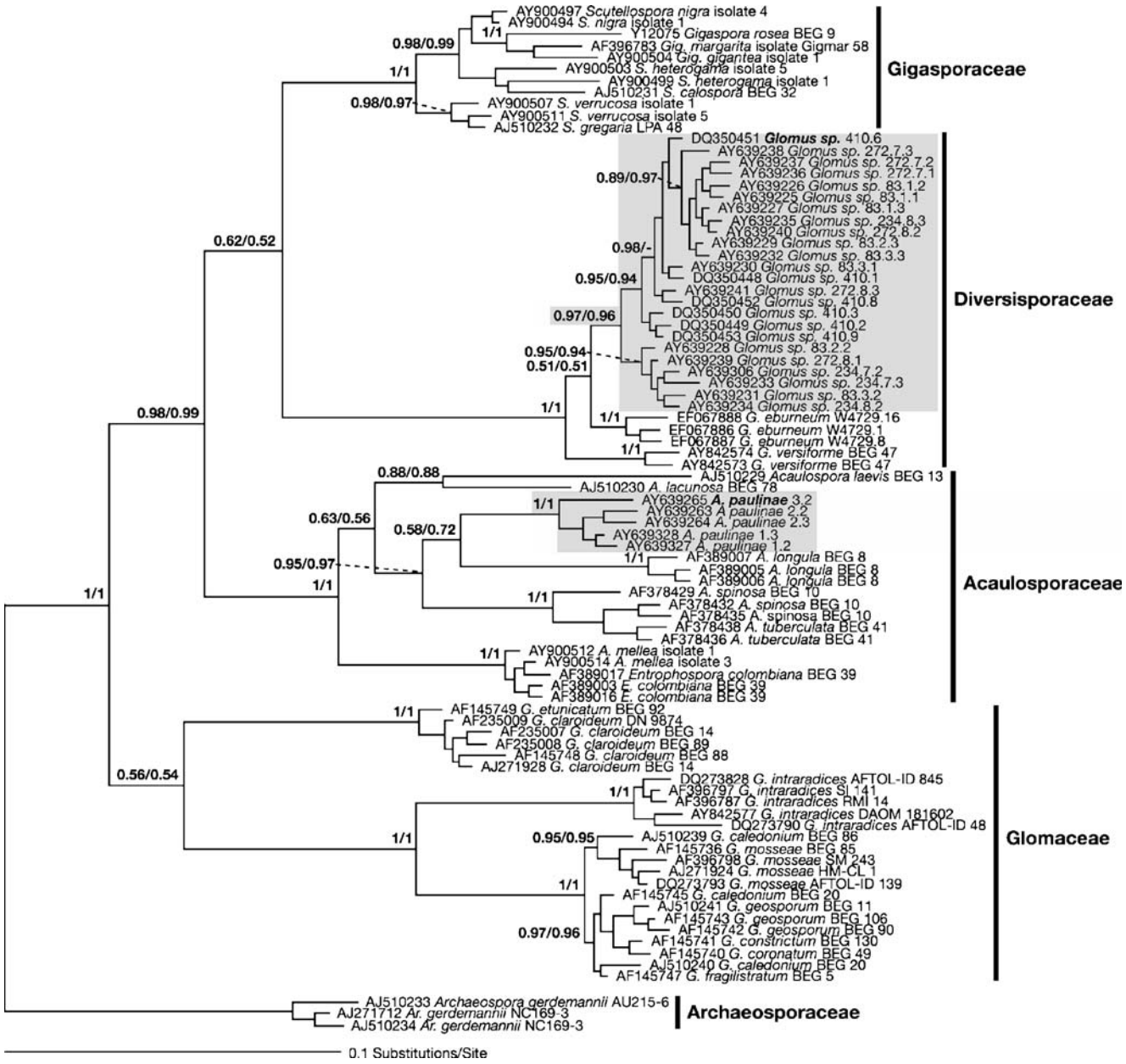

Fig. 3 Rooted phylogenetic consensus tree inferred by Bayesian analysis and based on partial sequences of the nuclear ribosomal large subunit RNA gene (503 unambiguously aligned sites). The clades formed by the two arbuscular mycorrhizal fungi *Acaulospora paulinae* Blaszkowski and *Glomus* sp. (Glomeromycota), targeted by the newly developed pairs of specific PCR primers, are highlighted with *grey boxes*. Sequences were obtained by nested PCR amplification and vector-cloning from single spores of *A. paulinae* and multispore DNA extracts of four single-spore cultures of *Glomus* sp. Accession numbers of new sequences obtained in this study are suffixed with the spore (or isolate) and clone identifiers. Reference sequences were obtained from public sequence databases and are given with their accession numbers, species names and isolate or strain identifiers. Reference sequences of the basal glomeromycotan taxon *Archaeospora gerdemannii* (Rose, Daniels & Trappe) Morton & Redecker were used as the outgroup. Branch support values of major clades are indicated *above branch nodes* as the posterior probabilities under the two best-fit evolutionary models TrN + G and GTR + G

extracts of a set of other AMF species present at the same field site. Even when applied to a large number of environmental root-DNA extracts the new primers proved to be highly specific with a negligible number of samples (1.8%) compromising the specificity of the primer pair f6/r1 for *A. paulinae*.

Although previous studies have already successfully used the 5′ end of the nrLSU RNA gene (van Tuinen et al. 1998a; Kjøller and Rosendahl 2000), discrimination with specific primers in earlier field studies relied on three consecutive PCR reactions employing two pairs of nested primers for a second fungus-specific, and then a third AMF

taxon-specific, PCR amplification after a first PCR with universal eukaryote-specific primers (Turnau et al. 2001; Jansa et al. 2003). Such double-nested PCR approaches are laborious and bear a high risk of PCR errors and formation of artefacts (Wagner et al. 1994; von Wintzingerode et al. 1997; Speksnijder et al. 2001). The newly developed specific primers described here reliably yielded PCR products from environmental samples in a single nested amplification round reducing, though not completely eliminating, the risk of artefacts.

Besides the ability to directly detect and discriminate among otherwise indistinguishable fungal taxa in roots, a strength of the specific primers is to increase PCR amplification sensitivity compared with amplifications using taxonomically more inclusive primers. A nested PCR approach involving two sets of primers in two steps of amplification is commonly used in AMF research to overcome PCR inhibition and to increase sensitivity for rare DNA templates in the presence of an overwhelming background of nontarget DNA (van Tuinen et al. 1998a). The nested PCR approach developed here involved initial amplification of nrLSU sequence fragments of the whole fungal community and subsequent amplification on the diluted products from the first PCR with taxon-discriminating primers (f6/r1, f4/r2).

The primers introduced here were developed on the basis of the most abundant sequence haplotypes of two AMF taxa found at one geographic location. They may, therefore, not cover the entire sequence diversity of these morpho-species present elsewhere. Given the high intra-individual and intra-specific sequence diversity of ribosomal genes in AMF (e.g. Kuhn et al. 2001; Pawlowska and Taylor 2004), the present approach designing taxon-specific primers based on the most frequent sequences appears to be justified and should enable sensitive detection of the most frequent and thus biologically relevant individuals. However, it remains possible that at other field sites the populations of the two AMF taxa consist of different abundant sequence haplotypes, making it necessary to adjust the specific primers. Moreover, the poor taxonomic coverage of the Diversisporaceae by nrLSU sequences in public databases calls for caution regarding the specificity of the primer pair f4/r2. A closer inspection of the binding site of the reverse primer r2 showed only two nucleotide mismatches with those of *G. eburneum* and *G. versiforme*, suggesting that together with primer f4 PCR amplification of sequences of these species may also be permitted. However, the use of stringent PCR conditions may avoid co-amplification, as demonstrated here in the intensive screening of environmental root samples.

Using new discriminative PCR primers, it has been possible to track the fate of two fungal taxa, *A. paulinae* and *Glomus* sp., that have never been targeted with PCR before and may have been overlooked in previous community studies. The PCR detection of *A. paulinae* and *Glomus* sp. in replicate root samples of *T. repens* from a temperate, agricultural grassland in Switzerland indicates that these species occur in high but variable frequency.

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