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DNA-based species level detection of *Glomeromycota*: one PCR primer set for all arbuscular mycorrhizal fungi

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Summary

• At present, molecular ecological studies of arbuscular mycorrhizal fungi (AMF) are only possible above species level when targeting entire communities. To improve molecular species characterization and to allow species level community analyses in the field, a set of newly designed AMF specific PCR primers was successfully tested.
• Nuclear rDNA fragments from diverse phylogenetic AMF lineages were sequenced and analysed to design four primer mixtures, each targeting one binding site in the small subunit (SSU) or large subunit (LSU) rDNA. To allow species resolution, they span a fragment covering the partial SSU, whole internal transcribed spacer (ITS) rDNA region and partial LSU.
• The new primers are suitable for specifically amplifying AMF rDNA from material that may be contaminated by other organisms (e.g., samples from pot cultures or the field), characterizing the diversity of AMF species from field samples, and amplifying a SSU-ITS-LSU fragment that allows phylogenetic analyses with species level resolution.
• The PCR primers can be used to monitor entire AMF field communities, based on a single rDNA marker region. Their application will improve the base for deep sequencing approaches; moreover, they can be efficiently used as DNA barcoding primers.

Introduction

Arbuscular mycorrhizal fungi (AMF) are associated with 70–90% of land plants (Smith & Read, 2008) in a symbiosis called arbuscular mycorrhiza (AM), that has existed for > 400 million yr (Parniske, 2008; Schüßler *et al.*, 2009). The economic and ecological importance of these ancient biotrophic plant symbionts is therefore obvious. Arbuscular mycorrhizal fungi transfer inorganic nutrients and water to the plant and receive carbohydrates in exchange. By driving this bidirectional nutrient transport between soil and plants, they are highly relevant for global phosphorus (P), nitrogen (N) and CO_2 cycles. Moreover, they affect directly and indirectly the diversity and productivity of land-plant communities (van der Heijden *et al.*, 1998) by their central role at the soil–plant interface (van der Heijden *et al.*, 2008). They can also improve host plant pathogen resistance (Vigo *et al.*, 2000; de la Pena *et al.*, 2006) and drought stress tolerance (Michelson & Rosendahl, 1990; Aroca *et al.*, 2007).

Despite the enormous role of AMF in the entire terrestrial ecosystem, their biodiversity in relation to functional aspects is little understood. Most of the 214 currently described species (www.amf-phylogeny.com) are characterized only by spore morphology and the majority have not yet been cultured. Moreover, from molecular ecological studies we know that the species described represent only a small fraction of the existing AMF diversity (Kottke *et al.*, 2008; Öpik *et al.*, 2008). Problems with identification of AMF result from their hidden, biotrophic lifestyle in the soil, few morphological characters, and the potential formation of dimorphic spores. This led to many AMF species, phylogenetically belonging to different orders, being placed in one genus (*Glomus*) and, conversely, individual species forming different spore morphs being described as members of different orders.

Another drawback of morphologically monitoring AMF by their resting spores (Oehl *et al.*, 2005; Wang *et al.*, 2008) is that the presence of spores may not reflect a symbiotically active organism community. Furthermore, many species cannot be reliably identified at all from heterogeneous field samples, and when identifying described species (likely to represent less than 5% of the existing species diversity) similar morphotypes may be erroneously determined as a single species.

To reveal functional and ecological aspects of distinct AMF communities associated with different plants and/or under different environmental conditions it is essential to detect AMF communities in the field on the species level. However, there are as yet no unbiased methods for this purpose, not only for morphological identification but also for molecular methods. Principally, DNA sequence based methods are most useful for detecting organisms at different community levels, but for ecological work they also depend on reliable baseline databases and tools. For example, fingerprinting methods such as random amplification of polymorphic DNA (RAPD), inter-simple sequence repeat PCR (ISSR) and amplified fragment length polymorphism (AFLP) are expected to be error prone in uncharacterized environments because of too many 'unknowns' in the background, which hampers interpretation of specificity (Mathimaran *et al.*, 2008). A similar problem exists for DNA array techniques. Nevertheless, suitable molecular methods are crucial to overcome the limitations of morphological identification (Walker & Schüßler, 2004; Walker *et al.*, 2007; Gamper *et al.*, 2009; Stockinger *et al.*, 2009).

But how are DNA or RNA sequence data for community analyses obtained and how can the current limitations of molecular tools be overcome? Molecular characterization of AMF is in most cases achieved by PCR on DNA from roots of host plants, spores or soil samples. Several primers targeting the rDNA regions as molecular marker were claimed to be AMF specific. Most of these amplify only a restricted number of glomeromycotan taxa or DNA of nontarget organisms. The most comprehensive taxon sampling for the *Glomeromycota* covers the small subunit (SSU) rDNA region (Schüßler *et al.*, 2001a,b), for which a new, AMF specific primer pair was recently published (AML1 and AML2; Lee *et al.*, 2008). Unlike the often used AM1 primer (Helgason *et al.*, 1998) it is perhaps suitable to amplify sequences from all AMF taxa, but the SSU rDNA is inadequate for species resolution of AMF. Inclusion of the internal transcribed spacer (ITS) and the large subunit (LSU) rDNA region allows both robust phylogenetic analyses and species level resolution (Gamper *et al.*, 2009; Stockinger *et al.*, 2009).

The available public database sequences are scattered through SSU, ITS and LSU rDNA subsets with varying lengths, often only 500–800 bp. In most cases this does not allow species level analyses, and short sequences obtained with primers that have inaccurately defined specificity may result in errors. For example, some short database sequences labelled as *Gigaspora* (Jansa *et al.*, 2003) cluster with those of *Glomus versiforme* BEG47 (*Diversisporaceae*) (Gamper *et al.*, 2009). Because of the relatively few LSU sequences in the public databases, the design of improved primers is challenging or even impossible. We therefore sequenced the ITS region and the 5′ part of the LSU rDNA of a set of well-characterized, but phylogenetically diverse AMF, and designed new primers from the resulting database. These primers are suited to amplify DNA from members of all known glomeromycotan lineages and, by allowing elaboration of a more accurate baseline dataset, could be a breakthrough for molecular community analyses of AMF.

Materials and Methods

Fungal and plant material for primer tests

We first tested different samples as DNA templates for PCR to confirm the specificity of the newly designed primers. These included plasmid inserts (Table 1), DNA extractions from single AMF spores and root samples from the Andes (Ecuador) and the Spessart Mountains (Germany). Primers were tested for specificity by PCR with plasmids carrying rDNA fragments with known sequences. All these plasmids had been amplified from single spore DNA extracts with the SSU rDNA primer SSUmAf, described here, and the LSU rDNA primer LR4+2 (modified from LR4; www.aftol.org). The specificity of SSUmAf could therefore not be investigated directly.

DNA extraction for primer tests

All vials, tips, beads, solutions, and other equipment used were sterile and DNA free.

From cleaned, single AMF spores DNA was extracted with the Dynabead DNA DIRECT Universal Kit (Invitrogen, Karlsruhe, Germany) as described in Schwarzott & Schüßler (2001).

Roots potentially colonized by AMF were cut into ten 0.5 cm pieces and collected in a single 1.5 ml Eppendorf tube containing one tungsten carbide bead (diameter 3 mm; Qiagen, Hilden, Germany). They were immediately frozen in liquid N_2 within the closed tube, placed in liquid N_2 precooled Teflon holders, and ground to a fine powder in a MM2000 bead-mill (Retsch, Haan, Germany). Extraction was done by either an innuPREP Plant DNA Kit (Analytik Jena, Jena, Germany) following the instructions of the manufacturer, or a cetyltrimethylammonium bromide (CTAB) protocol modified from Allen *et al.* (2006). For the CTAB protocol, prewarmed extraction buffer (750 µl for 75 mg tissue) was added to each sample of frozen, ground tissue, followed by incubation at 60°C for 30 min. Next, one volume of a chloroform–isoamylalcohol mixture (24 : 1) was added. The samples were centrifuged for 5 min at 2570 ***g*** and the upper phase was transferred into a new tube. After addition of 2.5 µl RNase A (10 mg ml^{-1}) this was incubated at 37°C for 30 min. One volume chloroform–isoamylalcohol (24 : 1) was then added and the tube was centrifuged as above. The supernatant was collected and two-thirds volumes of isopropanol added. The samples were incubated at 4°C for 15 min. After centrifugation (10290 ***g*** for 10 min) the pellet was washed in 70% ethanol, air dried, and eluted in 100 µl of molecular biology grade H_2O. Volumes of 2–5 µl of each DNA extract were used as PCR template.

Table 1 Plasmids used to test primer specificity and their origin

Species (order)	Plasmid no.	Spore no.	Attempt number (culture code)	Voucher	Source (collector)	Origin
Glomus luteum (*Glomerales*)	pMK020.1	2	Att 676-5 (SA101)	W3184	INVAM	Saskatchewan, Canada
Glomus intraradices (*Glomerales*)	pHS051.14	283	Att 1102-12 (MUCL49410)	W5070	GINCO (Nemec)	Orlando, USA
Glomus sp. (*Glomerales*)	pMK010.1	11	Att 15-5 (WUM3)	W2940	Walker (Mercer)	Merredin, Australia
Acaulospora sp. (*Diversisporales*)	pMK005.1	19	Att 869-3 (WUM18)	W2941	Walker (Mercer)	Nedlands, Australia
Pacispora scintillans (*Diversisporales*)	pMK027.1	190	Field collected	W4545	Walker (Schüßler)	Griesheim, Germany
Gigaspora sp. (*Diversisporales*)	pMK003.1	14	Field collected	W2992	Walker (Cabello)	Tres Arroyos, Argentina
Scutellospora heterogama (*Diversisporales*)	pMK029.3	72	Att 334-16 (BEG35)	W3214	Walker (Miranda)	exact location unknown, North America
Glomus versiforme (*Diversisporales*)	pHS036.4	262	Att 475-45 (BEG47)	W5165	Walker (Bianciotto)	Corvallis, USA
Kuklospora kentinensis (*Diversisporales*)	pHS098.16	310	Att 1499-9 (TW111A)	W5346	INVAM	Tainan, Taiwan
Geosiphon pyriformis (*Archaeosporales*)	pMK044.1	8	GEO1	W3619	Schüßler	Bieber, Germany

Single spores from which the cloned amplicons (amplified with primers SSUmAf-LR4+2) originated and the geographic origin of the respective arbuscular mycorrhizal fungi (AMF) are shown.

PCR conditions

The Phusion High-Fidelity DNA polymerase 2× mastermix (Finnzymes, Espoo, Finland) was used for PCR with the SSUmAf–LSUmAr or SSUmCf–LSUmBr primer pairs. SSUmCf and LSUmBr were also applied as nested primers (see Fig. 1c). The final concentration of the reaction mix contained 0.02 U μl^{-1} Phusion polymerase, 1× Phusion HF Buffer with 1.5 mM $MgCl_2$, 200 μM of each dNTP and 0.5 μM of each primer. Thermal cycling was done in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) with the following conditions for the first PCR: 5 min initial denaturation at 99°C; 40 cycles of 10 s denaturation at 99°C, 30 s annealing at 60°C and 1 min elongation at 72°C; and a 10 min final elongation. The same conditions were used for the nested PCR primers except that the annealing temperature was 63°C and only 30 cycles were carried out. The PCR products were loaded on 1% agarose gels (Agarose NEEO; Carl Roth, Karlsruhe, Germany) with 1× sodium borate buffer (Brody & Kern, 2004) at 220 V, and visualized after ethidium bromide staining (1 μg ml^{-1}).

Cloning, restriction fragment length polymorphism (RFLP) and sequencing

Polymerase chain reaction products were cloned with the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) following the instructions of the manufacturer, except that to reduce costs only one-third of the specified volume of all components was used. Only SOC medium for initial bacterial growth after transformation was used in the volume as per the instructions. From each cloning we analysed up to 48 clones for correct length of plasmid inserts. In some instances fewer clones were available because of low cloning efficiency. Colony-PCR was performed with the GoTaq DNA Polymerase (5 U μl^{-1}; Promega, Mannheim, Germany) and modified M13F and M13R primers. To roughly detect intrasporal and intersporal sequence variability in the clones, RFLP was performed in 10 μl reaction volume, containing 5 μl colony-PCR product, one of the restriction enzymes HinfI (1 U), RsaI (1 U), or MboI (0.5 U) and the specific buffer. One or two clones for each restriction pattern were sequenced, using M13 primers, by the LMU Sequencing Service Unit on an ABI capillary sequencer with the BigDye v3.1 (Applied Biosystems, Foster City, CA, USA) sequencing chemistry. The sequences were assembled and edited in SEQASSEM (www.sequentix.de) and deposited in the EMBL/GenBank/DDBJ databases with the accession numbers FM876780 to FM876839.

Primer design

For the design of new AMF specific primers a sequence alignment was established with the programs ALIGN (www.sequentix.de) and ARB (Ludwig *et al.*, 2004). The alignments contained all AMF sequences present in the public databases and our new data. In total > 1000 AMF sequences, covering all known phylogenetic lineages, were analysed to design the SSU and LSU rDNA primers. To allow comparison to the existing SSU rDNA datasets the primers were designed to overlap (approx. 250 bp) with the SSU rDNA. We used BLAST against the public databases and the probe match tool in ARB to test the specificity of the newly designed primers *in silico*. For the alignment in the ARB database a combination of our new dataset and the 94th release version of the SILVA database (Pruesse *et al.*, 2007, www.arb-silva.de) was used. The oligonucleotides were then synthesized as standard primers (25 nmol, desalted) by Invitrogen.

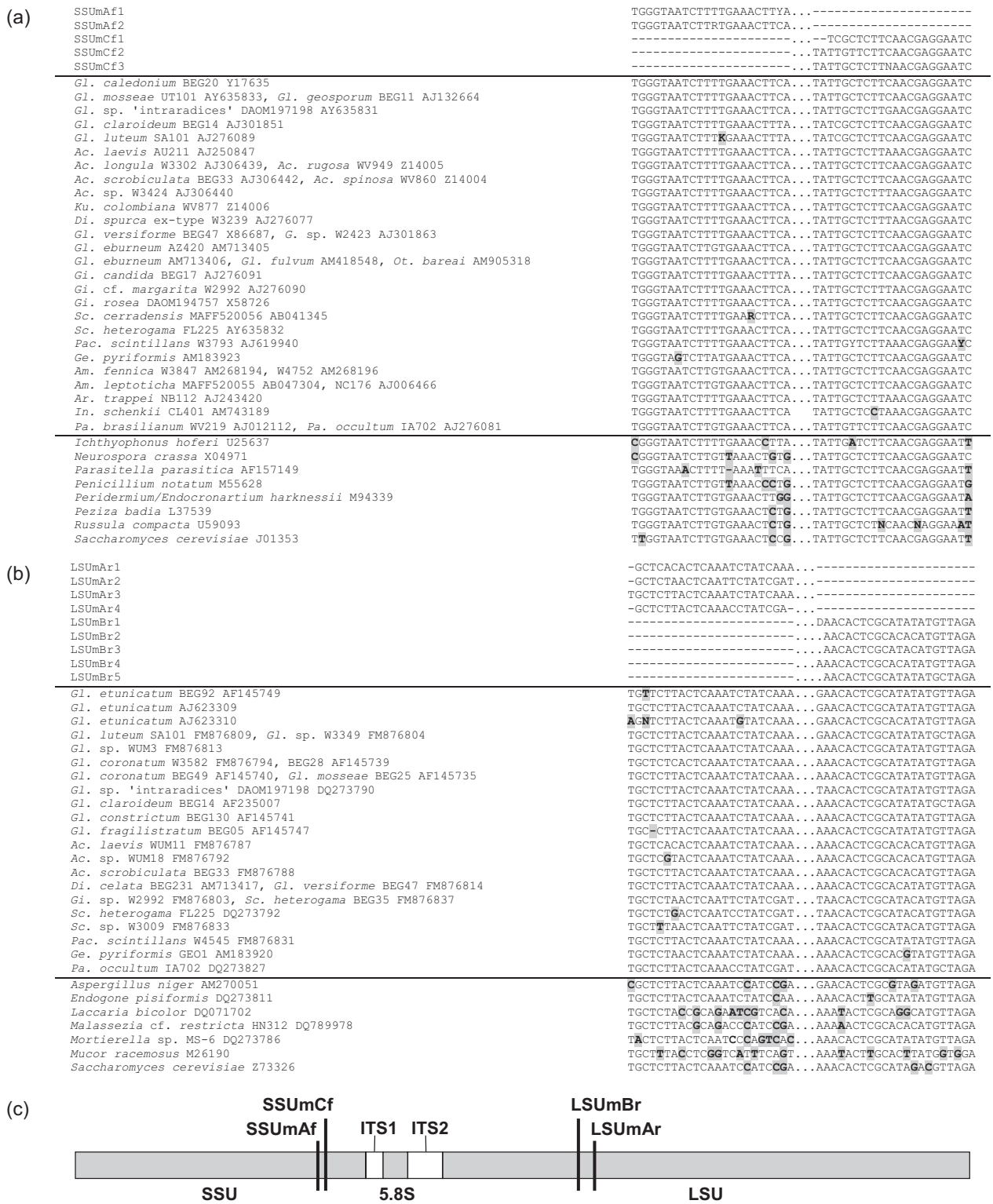

Fig. 1 Forward and reverse primers designed in this study (5′–3′ direction), compared with their annealing sites in sequences from representative members of all main AMF taxa and some non-AMF species. Variable sites not represented in any primer mixture are shaded. When no culture identifiers are known, voucher (W) numbers are given behind the species name. (a) Forward primers SSUmAf (mixture SSUmAf1-2) and SSUmCf (mixture SSUmCf1-3). (b) Reverse primers LSUmAr (mixture LSUmAr1-4) and LSUmBr (mixture LSUmBr1-5). (c) Small subunit (SSU) rDNA, internal transcribed spacer (ITS) region and large subunit (LSU) rDNA (5465 bp) of *Glomus* sp. 'intraradices' DAOM197198 (AFTOL-ID48, other culture/voucher identifiers: MUCL43194, DAOM181602; accession numbers: AY635831, AY997052, DQ273790) showing the binding sites of the newly designed forward and reverse primer mixtures.

Table 2 Polymerase chain reaction primer mixtures designed for amplification of arbuscular mycorrhizal fungi (AMF)

Primer	Nucleotide sequence (5′–3′)	nt	Target taxa (mainly)
SSUmAf1	TGG GTA ATC TTT TGA AAC TTY A	22	*Acaulosporaceae*, *Archaeosporaceae*, *Diversisporaceae*, *Geosiphonaceae*, *Gigasporaceae*, *Glomeraceae* (GlGrA & GlGrB), *Pacisporaceae*
SSUmAf2	TGG GTA ATC TTR TGA AAC TTC A	22	*Ambisporaceae*, *Diversisporaceae*, *Geosiphonaceae*, *Paraglomeraceae*
SSUmAf	Mix SSUmAf1-2 (equimolar)	22	All AMF lineages
SSUmCf1	T CGC TCT TCA ACG AGG AAT C	20	*Archaeosporaceae* (indirect evidence by amplification of *Ambispora fennica*), *Glomeraceae* (mainly GlGrB)
SSUmCf2	TAT TGT TCT TCA ACG AGG AAT C	22	*Paraglomeraceae*
SSUmCf3	TAT TGC TCT TNA ACG AGG AAT C	22	*Acaulosporaceae*, *Ambisporaceae*, *Archaeosporaceae*, *Diversisporaceae*, *Geosiphonaceae*, *Gigasporaceae*, *Glomeracea* (mainly GlGrA), *Pacisporaceae*
SSUmCf	Mix of SSUmCf1-3 (equimolar)	20–22	All AMF lineages
LSUmAr1	GCT CAC ACT CAA ATC TAT CAA A	22	*Acaulosporaceae*
LSUmAr2	GCT CTA ACT CAA TTC TAT CGA T	22	*Gigasporaceae*
LSUmAr3	T GCT CTT ACT CAA ATC TAT CAA A	23	*Acaulosporaceae*, *Diversisporaceae*, *Geosiphonaceae*, *Gigasporaceae*, *Glomeraceae* (GlGrA and GlGrB), *Pacisporaceae*
LSUmAr4	GCT CTT ACT CAA ACC TAT CGA	21	*Paraglomeraceae*
LSUmAr	Mix of LSUmAr1-4 (equimolar)	21–23	All AMF lineages
LSUmBr1	DAA CAC TCG CAT ATA TGT TAG A	22	*Acaulosporaceae*, *Archaeosporaceae*, *Glomeraceae* (GlGrA), *Pacisporaceae*
LSUmBr2	AA CAC TCG CAC ACA TGT TAG A	21	*Acaulosporaceae*
LSUmBr3	AA CAC TCG CAT ACA TGT TAG A	21	*Gigasporaceae*
LSUmBr4	AAA CAC TCG CAC ATA TGT TAG A	22	*Diversisporaceae*, *Geosiphonaceae*, *Glomeraceae*, *Paraglomeraceae*, (primer sequence was also found in amplicons from *Ambispora fennica* and an *Archaeospora* sp.)
LSUmBr5	AA CAC TCG CAT ATA TGC TAG A	21	*Gigasporaceae*, *Glomeraceae* (GlGrB)
LSUmBr	Mix of LSUmBr1-5 (equimolar)	21–22	All AMF lineages

Variable sites among primers of an individual mixture are shaded. Target taxa most likely amplified, according to known binding site sequences, are listed. Comments in parentheses indicate that the primer was successfully used to amplify the given taxon, although the binding site sequences were not known.

Results

Primer design

Potentially suited binding sites for primers that match AMF sequences but discriminate against plant and non-AM fungal (non-AMF) sequences were identified for the SSU rDNA and LSU rDNA. They were located at positions 1484 and 1532 on the SSU, and at positions 827 and 928 on the LSU rDNA (based on *Glomus* sp. 'intraradices' DAOM197198 sequence; Fig. 1c). Sequence variation made it impossible to derive individual primer sequences that specifically amplify all *Glomeromycota*. Thus, a set of four primer mixtures was designed, each targeting one binding site (Table 2, Fig. 1). Certain non-3′ located mismatches that only slightly altered melting temperature and some mismatches (*Glomus etunicatum*) that were perhaps caused by low sequence quality were accepted for primer design (Fig. 1). To discriminate against nontarget organisms mismatches at the 3′ end of the primers were included. BLAST searches indicated high specificity of the new primer pairs for AMF.

Glomeromycota sequences that represent the known variability at the primer binding sites are shown in Fig. 1. We aimed to include as many main phylogenetic lineages (Fig. 2) for primer design as possible. However, the following taxa could not be included for LSU rDNA binding sites analyses: *Entrophosporaceae*, containing only two species lacking sequence data; *Archaeosporaceae*, because available sequences did not cover the LSU rDNA binding sites; *Otospora* for which only two nonoverlapping partial SSU rDNA sequences are known; *Intraspora*, represented by only one SSU rDNA database sequence.

Primer specificity – discrimination against plants

The discrimination of primer SSUmAf1 against 'lower' plants is weak and exemplified by only one mismatch to database sequences from mosses (*Polytrichastrum*, *Leptodontium* and *Pogonatum*), a liverwort (*Trichocoleopsis*), a hornwort (*Phaeoceros*) and a clubmoss (*Selaginella*). *Burmannia*, one *Phaseoleae* sp. and some other plant sequences also showed only one mismatch. All other plant sequences had a minimum

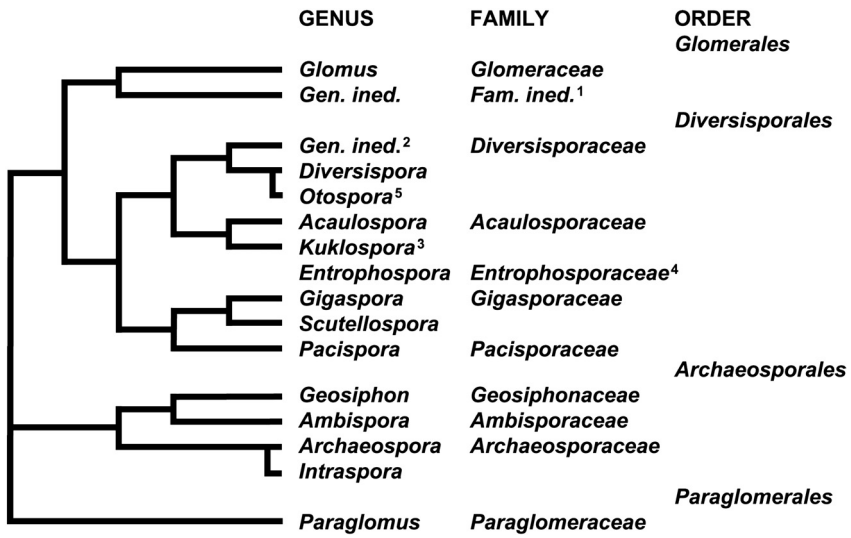

Fig. 2 Phylogenetic relationships of taxa in the *Glomeromycota* (Schüßler *et al*., 2001b; Walker *et al*., 2007). [1]Species currently named *Glomus*. One of the main *Glomus* clades (GlGrA or GlGrB) will represent the *Glomeraceae*, once the phylogenetic affiliation of the type species of *Glomus* is known; [2]contains *Glomus fulvum, Gl. megalocarpum, Gl. pulvinatum*; [3]contains *Kuklospora colombiana* and *Ku. kentinensis* (formerly *Entrophospora*) (Sieverding & Oehl, 2006); [4]contains one genus with two species, *Entrophospora infrequens* and *En. baltica* (Sieverding & Oehl, 2006), neither of which is phylogenetically characterized; [5]*Otospora* (Palenzuela *et al*., 2008) contains one species, *Otospora bareai*. Based on small subunit (SSU) rDNA sequences and from a phylogenetic viewpoint this genus is congeneric with *Diversispora*.

of two mismatches, mainly at the 3′ end of the primer. For SSUmAf2 there were at least two mismatches to all plant sequences, except for a moss (*Archidium*) with only one mismatch. For the nested forward primer SSUmCf1 a minimum of three mismatches for all plants, except for one environmental *Phaseoleae* sequence with two mismatches, were observed. SSUmCf2 mismatched at one site to the same *Phaseoleae* sequence and to liverworts (*Radula*, *Ptilidium* and *Porella*), a hornwort (*Anthoceros*) and a *Taxus* species. Other plant sequences displayed a minimum of two mismatches, at least one at the 3′ end. For SSUmCf3 the above mentioned sequence of *Phaseoleae* showed no mismatch, but all other environmental *Phaseoleae* sequences had at least one mismatch at the 3′ region of the primer. SSUmCf3 also showed only one mismatch for sequences of liverworts (*Radula*, *Ptilidium* and *Porella*), a hornwort (*Anthoceros*) and for one *Liliopsida* and *Taxus* sequence. The remaining BLAST hits displayed two mismatches (several *Taxus* spp., *Pinus* and the liverwort *Haplomitrium*) or more. These results show that for primer mixtures SSUmAf and SSUmCf the discrimination against 'lower' plants is less than for vascular plants.

The LSU rDNA primers had at least two mismatches to plant sequences. The minimum for LSUmAr1 was four mismatches to a *Brassica* sequence. LSUmAr2 and LSUmAr3 showed four mismatches for a *Medicago* sequence, in the case of LSUmAr2 this holds also true for *Vitis vinifera* and *Oryza sativa*. All other plant sequences showed more mismatches to LSUmAr1, LSUmAr2 and LSUmAr3. For LSUmAr4, which was designed to target *Paraglomeraceae*, two mismatches were found for *Solanum lycopersicum* followed by at least three for all other plant sequences. The LSUmBr primer set had a minimum of three mismatches to plant sequences. LSUmBr1 shows more than three mismatches to a *Lotus* and a *Brassica* sequence. At least three mismatches (to *Ephedra* and *Larix*) occurred for LSUmBr2. There were three mismatches for LSUmBr3 to *Selaginella*, followed by a liverwort (*Trichocoleopsis*) and a moss (*Bryum*) species with four. LSUmBr4 had three mismatches for *V. vinifera* and at least five for all other plant sequences. LSUmBr5 displayed more than four mismatches to any plant sequence.

Primer specificity – discrimination against nontarget fungi

The primer mixture SSUmAf should partly exclude amplification of nontarget fungi, whereas SSUmCf poorly discriminates non-AMF (Fig. 1a). Therefore, the highly specific amplification of AMF rDNA results mainly from the LSU primers. The primer mixture LSUmAr discriminates well against most non-AMF. An exception is LSUmAr1 with only one mismatch to a group of sequences from uncultured soil fungi (*Basidiomycota* related) from a Canadian forestry centre. For all other known non-AMF sequences more than four mismatches to LSUmAr1 and three to LSUmAr2 were observed. The primer LSUmAr3 shows only one mismatch with several chytrid sequences. For all other non-AMF LSUmAr3 as well as LSUmAr4 mismatched with at least two sites, mainly at the 3′ end.

For the (nested) LSUmBr primer mixture the specificity is lower; for example, LSUmBr1 showed no mismatch to some fungi in the more ancestral lineages, namely *Endogone lactiflua* and *Mortierellaceae* species, chytrids (*Rhizophlyctis* and *Gonapodya*), an uncultured alpine tundra soil fungus and matched one ascomycete sequence (*Catenulostroma*). For LSUmBr2, no mismatches occurred for sequences of some basidiomycetes (*Bulleribasidium*, *Paullicorticium* and *Russula*) and a zygomycete (*Spiromyces minutus*). Only one mismatch was observed for sequences including basidiomycetes (*Calocera*, *Calostoma* and *Ramaria*) and ascomycetes (*Pyxidiophora*, *Eremithallus* and *Phaeococcus*), and some other fungi. LSUmBr3 discriminates well against other fungi with at least three mismatches, except for one uncultured soil fungus sequence (*Cryptococcus* related) that matched completely. The primer LSUmBr4 showed no mismatch to *Clavulina griseohumicola* and only one to some fungal sequences including ascomycetes (*Pyxidiophora* and *Phaeococcus*) and basidiomycetes (*Cryptococcus* spp.). LSUmBr5 showed only one mismatch to fungal sequences of *Mortierella* spp., a chytrid (*Rhizophlyctis rosea*), and some ascomycetes (*Schizosaccharomyces*, *Verrucocladosporium*, *Passalora* and *Catenulostroma*). In general the LSUmAr primers discriminate better against non-AMF than the nested primers LSUmBr.

Primer efficiency – tests on plasmids and DNA extracts from single spores

The new primer pairs were designed to amplify fragments of approx. 1800 bp (SSUmAf–LSUmAr) and 1500 bp (SSUmCf–LSUmBr). In a first PCR amplification test, samples were chosen to encompass divergent phylogenetic lineages of the *Glomeromycota*. Cloned rDNA of the AMF species *Acaulospora* sp. and *Kuklospora kentinensis* (*Acaulosporaceae*), *Glomus luteum*, *Gl. intraradices* and a *Glomus* sp. (*Glomeraceae*), *Pacispora scintillans* (*Pacisporaceae*), and *Scutellospora heterogama* (*Gigasporaceae*) were used (Table 1, Fig. 3a). In addition, rDNA fragments were amplified from single spore DNA extracts from *Geosiphon pyriformis* (*Geosiphonaceae*), *Gl. mosseae* (*Glomeraceae*), *Gl. eburneum* and *Gl. versiforme* (*Diversisporaceae*), a *Paraglomus* sp. (*Paraglomeraceae*), and a *Gigaspora* sp. (*Gigasporaceae*) (not shown). All tested AMF species were successfully amplified with the new primer set.

To test the potential sensitivity of the new primers, the same plasmids as in the first PCR test and additional plasmids carrying inserts of a *Gigaspora* sp., *Gl. versiforme* and *Ge. pyriformis* (Table 1, Fig. 3b) were used. They were diluted over several magnitudes to contain 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg and 0.01 fg DNA μl^{-1}. One microlitre was used as template for PCR, whereas the four lowest concentrations correspond with 5000, 500, 50 and 5 plasmid molecules in the 20 µl PCR reaction volume. Both primer sets were tested independently. Differences between specificity of the first and nested primer sets were observed for *Pacispora*, *Kuklospora*, and *Geosiphon*. For *Pacispora* the PCR with SSUmAf and LSUmAr yielded, even with the lowest DNA concentration, a clearly visible band, whereas PCR with SSUmCf and LSUmBr yielded weaker bands, indicating lower specificity. Weaker bands were also observed for the rDNA amplification of *Ku. kentinesis* with the primers SSUmCf-LSUmBr and for *Ge. pyriformis* with SSUmAf-LSUmAr. However, these differences may be within the error-range of photometric DNA concentration measurement of the plasmid stock-solutions. Only slight or no differences occurred between the other plasmid templates, when comparing the intensity of the bands, except for *Gl. versiforme*. Here, clearly visible bands were only found for the higher DNA concentrations, but with the same pattern for both primer pairs. However, this was an artefact caused by low template DNA integrity. Later dilution series with fresh plasmid preparations (also from other *Diversisporaceae*) were indistinguishable from those obtained with the other species shown in Fig. 3(b). For *Ku. kentinensis* no amplicon could be observed after PCR with the primers SSUmAf–LSUmAr, because the cloned fragment was originally amplified with the nested primers. The plasmid therefore serves only as a negative control in the first PCR and as positive control for the PCR with the nested primers.

Primer efficiency – tests on field and nursery sampled roots and spores

To test whether the newly designed primers discriminate against nonglomeromycotan fungi and plants, we used them on DNA extracted from single spores from pot cultures, environmental root samples, and root samples from a tree nursery, in nested PCR approaches. We observed not a single non-AMF contaminant sequence in the 12 environmental root and 40 single spore samples processed. The discrimination against plants was tested with DNA extracts from roots of potential AMF hosts. The species collected comprised *Poa* cf. *annua*, *Ranunculus* cf. *repens*, and *Rumex acetosella* from a field site in Germany, and *Podocarpus* cf. *macrostaqui*, *Heliocarpus americanus* and *Cedrela montana* tree seedlings from a tree nursery in Ecuador. From a large number of nested PCR approaches, on just one occasion, three identical clones carrying a plant sequence (*R. acetosella*) were obtained. The *Rumex* related database sequence (AF189730, 630 bp) covers the ITS region, but not the binding sites for the nested primers. The new primers were also used successfully on DNA extractions from single AMF spores from pot cultures and a root organ culture (ROC). This demonstrates PCR amplification with a broad phylogenetic coverage of AMF, while efficiently discriminating against non-AMF and plants (Table 3).

The results show that the new primers are suitable to amplify DNA from members of the whole *Glomeromycota* and can be used for species level analyses of AMF communities in the field.

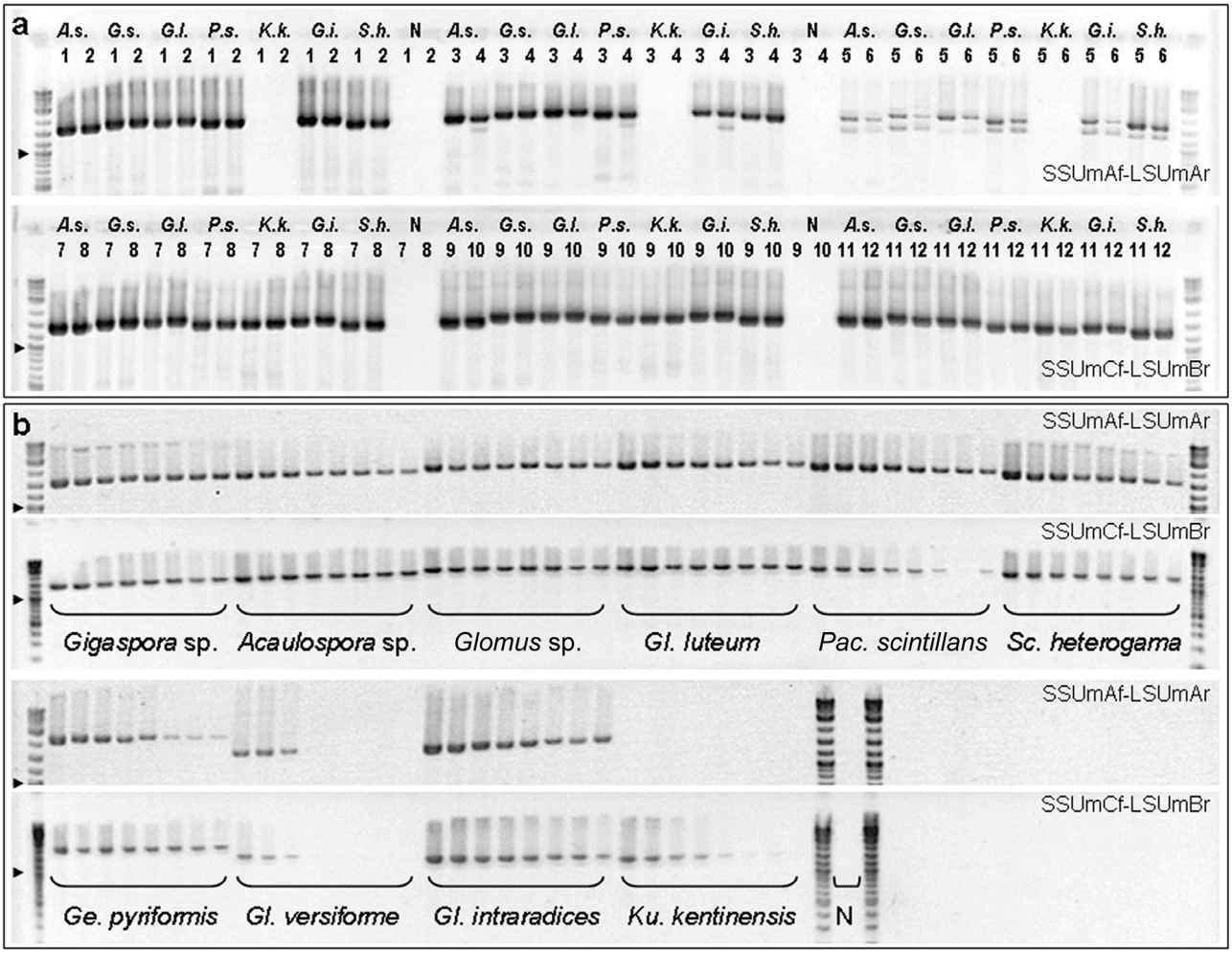

Fig. 3 Polymerase chain reaction amplification with primers SSUmAf–LSUmAr (approx. 1800 bp amplicons) and SSUmCf–LSUmBr (approx. 1500 bp amplicons). (a) PCR on cloned DNA fragments, using different annealing temperatures and a template concentration of 1 ng μl^{-1}. *A.s.*, *Acaulospora* sp.; *G.s.*, *Glomus* sp.; *G.l.*, *Glomus luteum*; *P.s.*, *Pacispora scintillans*; *K.k.*, *Kuklospora kentinensis*; *G.i.*, *Glomus intraradices*; *S.h.*, *Scutellospora heterogama*; N, negative control. Annealing temperatures: 1, 55°C; 2, 55.7°C; 3, 57.8°C; 4, 60.5°C; 5, 63.1°C; 6, 65°C; 7, 55.2°C; 8, 56.6°C; 9, 59.1°C; 10, 61.8°C; 11, 64.2°C; 12, 65.5°C. (b) PCR using 1 µl of a 10-fold plasmid dilution (100 pg – 0.01 fg μl^{-1}) as template, corresponding to 5×10^7 to 5 plasmid molecules in 20 µl PCR reaction volume. Annealing temperatures: SSUmAf–LSUmAr 60°C; SSUmCf-LSUmBr 63°C. N, negative control; Marker, NEB 2-Log DNA Ladder (bp: 10 000, 8000, 6000, 5000, 4000, 3000, 2000, 1500, 1200, 1000 (arrowhead), 900, 800, 700, 600, 500, 400, 300, 200, 100).

Discussion

There have been numerous efforts to design PCR primers generally applicable for detection of the whole group of AMF (Simon *et al.*, 1992; Helgason *et al.*, 1998), but later studies showed that they do not amplify DNA of all *Glomeromycota* or they also amplify ascomycetes, basidiomycetes or plant DNA (Clapp *et al.*, 1995, 1999; Helgason *et al.*, 1999). Other primers were successfully used for certain groups of the *Glomeromycota* (Kjøller & Rosendahl, 2000; Redecker, 2000; Turnau *et al.*, 2001; Wubet *et al.*, 2003, 2006; Gamper & Leuchtmann, 2007).

Many of the approaches require different primer pairs and independent PCR attempts for distinct target taxa. Comparison of such studies can be difficult since the distinct primer binding sites may behave very different in PCR and do not allow semiquantitative approaches. A single primer set for PCR amplification that covers all groups of the *Glomeromycota* and allows the identification of AMF at the species level was not available.

We have chosen the strategy of mixed primer sets to cover the defined sequence variability, instead of using fully degenerated primers. This reduces the degree of degeneration and results in a higher ratio of efficiently binding primers. The approach also allows adjustment of the concentrations of individual primers in future attempts. At the beginning of the study we speculated that the exonuclease activity of the proof-reading DNA polymerase used could hamper discrimination

Table 3 PCR amplification with the new primer pairs; DNA extracted from roots or spores

Environmental samples	Sample or culture	First PCR	Nested PCR	Clones sequenced, most likely genus (BLAST hits for full length and partial sequences)
Cedrela montana roots (tree nursery pot)	N1	–	+	pCK011.1-7 *Ambispora* (uncultured *Archaeospora* LSU)
Cedrela montana roots (tree nursery pot)	N3	+	+	first PCR: pCK009.1-3 *Glomus* (mycorrhizal symbiont of *Marchantia foliacea* SSU, ITS, LSU; *Glomus* sp. MUCL43206 LSU); nested PCR: pCK016.1-3, pCK017.1 *Glomus* (uncultured AMF clone Glom3524.1 SSU; symbiont of *M. foliacea* SSU, ITS, LSU; *Glomus* sp. MUCL43206 LSU, MUCL43194, LSU; *Glomus* sp. 'intraradices' AFTOL-ID845 LSU)
Cedrela montana roots (tree nursery pot)	N8	+	+ (ns)	pCK010.1,2 *Gigaspora* and/or *Scutellospora* (uncultured *Gigasporaceae* clone S2R2 SSU, ITS, LSU; *Gi. rosea* SSU, ITS, LSU; *Sc. heterogama* AFTOL-ID138 LSU)
Heliocarpus americanus roots (tree nursery pot)	N2	–	+	pCK012.2-4 *Archaeospora* and *Glomus* (*Ar. trappei* NB112 SSU, ITS, LSU; *Glomus* sp. 'intraradices' AFTOL-ID845 LSU)
Podocarpus cf. *macrostaqui* root without nodules (seedling from forest)	P0	+	+ (ns)	pCK018.1 *Acaulospora* (*Ac. alpina* clone 1060/33 SSU, ITS; uncultured *Acaulospora* clone: A3-68-c LSU)
Podocarpus cf. *macrostaqui* root with nodules (seedling from forest)	P1	+	+ (ns)	pCK020.1-13 *Acaulospora* (*Ac. alpina* clone 1060/33 SSU, ITS; *Acaulospora* clone: A3-68-c LSU)
Podocarpus cf. *macrostaqui* root nodules only (seedling from forest)	P2	–	+	pCK006.1,2 *Glomus* (*Gl. diaphanum* clone 3.3 SSU, ITS, LSU; *Gl. coronatum* BEG28 LSU; symbiont of *M. foliacea* SSU, ITS1; uncultured *Glomus* LSU)
Podocarpus cf. *macrostaqui* root nodules only (seedling from forest)	P3	–	+	pCK007.1,3,4 *Glomus* (*Glomus* sp. 0171 SSU, ITS; uncultured *Glomus* clone K7-10 SSU, ITS; *Glomus* clone K31-1 LSU; uncultured *Glomus* clone 1298-21 SSU, ITS, LSU; uncultured glomeromycete 2-09 LSU); pCK007.5,6 pCK008.1,3-7 *Glomus* (uncultured *Glomus* clone S1R2 + S2R1/2 SSU, ITS, LSU; *Glomus* sp. MUCL43206 LSU, MUCL43207 LSU; symbiont of *M. foliacea* SSU, ITS1; uncultured *Glomus* clone: A10-28 LSU)
Ranunculus repens roots (field sample)	1A	–	+	pMK078.1-3 *Acaulospora* (uncultured *Acaulospora* SSU; LSU)
Ranunculus repens roots (field sample)	3A	–	+	pMK083.2,3,5 *Acaulospora* (*Acaulospora* sp. ZS2005 SSU, ITS; *Ac. paulinae* clone 2.2 LSU)
Ranunculus repens roots (field sample)	5A	–	+	pMK077.1-5 *Glomus* (uncultured *Glomus* clones S1R2 + 850-23 SSU, ITS; uncultured *Glomus* clone H5-2 LSU)
Ranunculus repens roots (field sample)	7A	–	+	pMK080.1-5 *Diversispora* (*Gl. aurantium* SSU, ITS, LSU; *Gl. versiforme* BEG47 LSU, uncultured *Glomus* LSU); pMK080.6,7 *Glomus* (uncultured *Glomus* clone S1R2 SSU, ITS; uncultured *Glomus* LSU)
Poa annua roots (field sample)	1C	–	+	pMK082.1,4,6,9-17 *Acaulospora* (uncultured *Acaulospora* SSU, ITS, LSU; uncultured *Acaulospora* LSU)
Poa annua roots (field sample)	2C	–	+	pMK081.1,3-5 *Acaulospora* (uncultured *Acaulospora* SSU, ITS, LSU; *Ac. laevis* BEG13 LSU)
Plantago lanceolata roots (pot culture, inoculated with *C. montana* roots)	Att 1451-8	+	+ (ns)	pCK024.1,3,4 *Glomus* (uncultured *Glomus* clone S2R2 SSU, ITS, LSU; uncultured *Glomus* clone S1R2 SSU, ITS, LSU; *Glomus* sp. 'intraradices' AFTOL-ID845 LSU, *Glomus* sp. MUCL43206 LSU; *Glomus* sp. MUCL43203 LSU)
Plantago lanceolata roots (pot culture, inoculated with *H. americanus* roots)	Att 1456-1	–	+	pCK025.1-4 *Glomus* (uncultured *Glomus* clone S1R2 SSU, ITS, LSU; *Glomus* sp. MUCL43203 LSU)
AMF ss (ss pot culture)	Att 1449-5	–	+	pCK022.1-3 *Diversispora* (*Gl. aurantium* SSU, LSU; *Gl. versiforme* BEG47 LSU)
AMF ss (ss pot culture)	Att 1450-1	–	+	pCK023.1-4 *Acaulospora* (*Ac. colossica* clones 15.1+15.4 SSU, ITS, LSU; uncultured *Acaulospora* clone H1-1 LSU)
AMF ss (ss pot culture)	Att 1456-7	–	+	pCK026.1,2-6 *Archaeospora* (uncultured *Archaeospora* clone 1400-71 SSU, ITS, clone R8-37 LSU; *Ar. trappei* SSU, ITS, LSU)
AMF ss (ss pot culture)	Att 1456-11	–	+	pCK027.1-3 *Glomus* (*Gl. claroideum* clone 57.10 SSU, ITS, LSU)
AMF ss (ss pot culture)	Att 1449-10	–	+	pCK028.2-5,7-12 *Glomus* (*Gl. claroideum* clone 57.10 SSU, ITS, LSU)
AMF ss morphotype 1 (ms pot culture)	Att 1451-6	+	+	first PCR: pCK029.1 *Glomus* (*Gl. claroideum* clone 57.10 SSU, ITS, LSU); nested PCR: pCK030.1-6 *Glomus* (uncultured *Glomus* clone Pa127 SSU, ITS, LSU; uncultured *Glomus* clone S1R2 SSU, ITS, LSU; *Gl. etunicatum* LSU; *Glomus* sp. MUCL43203 LSU)
AMF ss morphotype 2 (ms pot culture)	Att 1451-6	–	+	pCK031.1,2 *Gigaspora* (*Gi. rosea* clone Gr8.2 SSU, ITS, LSU; *Sc. heterogama* AFTOL-ID138 LSU)
Glomus intraradices spore cluster (ROC)	Att 4-64 (from FL208)	–	+	pHS099.3,6,8,11,14,16,25,32,36,40,41,47 *Glomus* (uncultured *Glomus* clone S2R2 SSU, ITS, LSU; *Glomus* sp. MUCL43203 LSU, *Glomus* sp. MUCL43206 LSU, MUCL43207 LSU, *Glomus* sp. 'intraradices' AFTOL-ID845 LSU)

First PCR, SSUmAf–LSUmAr; nested PCR, SSUmCf–LSUmBr. PCR reactions are given as positive when a PCR product of the expected size was visible. The closest BLAST hits are shown for the first and/or nested PCR derived sequences. Att, culture attempt; ITS, internal transcribed spacer; LSU, large subunit; ms, multi spore; ns, not sequenced; ROC, root organ culture; ss, single spore; SSU, small subunit.

by terminal 3′ primer mismatches, but no such problems were detected.

Primer specificity

The primers designed show some mismatches to AMF sequences at the 5′ end (Fig. 1), which do not hinder PCR amplification (Bru *et al.*, 2008). Primer mismatches such as C–T, T–C and T–G do not impair amplification strongly even when situated at the 3′ end of the primer (Kwok *et al.*, 1990). The forward primers SSUmAf as well as the reverse primers LSUmBr mismatched once with *Ge. pyriformis*, but did not hamper amplification. The LSU rDNA primers show sufficient sequence similarity to the target organisms, as the mismatches are either in the middle or at the 5′ end. LSUmAr primers displayed individual mismatches to sequences of *Scutellospora* spp., *Gl. etunicatum*, and one *Acaulospora* sp. (Fig. 1). Nevertheless, DNA of these species was successfully amplified from environmental samples and in the primer efficiency test (Fig. 3). *Ambisporaceae* and *Archaeosporaceae* species could not be included in the design of the LSU primers, but *Ambispora fennica* DNA from a single spore extraction (not shown) and *Archaeospora* sp. from single spores and roots of an Ecuadorian tree seedling (Table 3) could be amplified with the new primers, indicating well matching binding sites. Sequences from *Otospora* (*Diversisporaceae*; Palenzuela *et al.*, 2008; matching the SSU primers), *Intraspora* (closely related to *Archaeospora*), and *Entrophospora* (sensu Oehl & Sieverd.; with two species only) are either not or only partly characterized and therefore could not be included in several aspects of primer design. *Otospora* and *Intraspora* are very closely related to their sister genera (maybe congeneric), so the lack of LSU rDNA sequences was therefore interpreted as a minor limitation.

We could successfully amplify all AMF tested with the new primers, but because of the lower number of LSU rDNA sequences available for AMF an optimization of the LSU primers might be reasonable in future. The discrimination against non-AMF and plant DNA is excellent, as shown on DNA extracts from environmental samples and spores from pot cultures. To discriminate against non-AMF, LSUmAr works much better than the nested primers LSUmBr. The cloned plant (*Rumex*) rDNA fragment that originated from root material can be interpreted as an 'outlier'. The primer binding sites could not be investigated for *Rumex*, because of lacking sequence coverage. It should be indicated in this context that we did not use HPLC-purified primers. This means a certain fraction of primers may not be fully synthesized and could result in less specific amplification. All plasmids used in the plasmid test carried inserts that were originally amplified with SSUmAf. Therefore, the efficiency of this primer could not be validated, but because of the high number of SSU rDNA sequences known, it can be stated that the binding sites in the cloned fragments correspond to a realistic situation. The efficient amplification from spore DNA extracts was, moreover, confirmed in numerous former PCR.

Advantages over previously used PCR primer sets

In most former field studies SSU rDNA phylotypes were analysed for molecular detection of AMF. However, this region does not allow species resolution and each defined phylotype, irrespective of the used distance threshold value or phylogenetic analysis method, may hide a number of species (Walker *et al.*, 2007). In general, the LSU rDNA region allows species resolution, and thus the LSU primer pair FLR3–FLR4 (Gollotte *et al.*, 2004) was used for species-level community analyses. However, in particular, FLR4 is not phylogenetically inclusive (Gamper *et al.*, 2009) and discriminates many lineages, including *Diversisporales*, *Archaeosporales* and *Paraglomerales*, which results in a strong bias in community analyses towards the *Glomeraceae*. The primer FLR3 binds to DNA of many nontarget fungi as it shows no mismatch to > 1300 basidiomycete sequences and some ascomycete sequences in the public databases. Such problems obviously may bias tRFLP community analyses (Mummey & Rillig, 2008) and seminested PCR approaches (Pivato *et al.*, 2007) using FLR3 and/or FLR4. The primer pair SSUGlom1–LSUGlom1 (Renker *et al.*, 2003) amplifies many non-AMF and plants. Combined with the primers ITS5–ITS4 in a nested PCR (Hempel *et al.*, 2007) this resulted in a 5.8S rDNA phylogenetic analysis, which resolved only the genus level. Even the ITS region does not always resolve species for AMF (Stockinger *et al.*, 2009).

In some cases, species-specific detection tools are available for individual species or certain well-defined and closely related species. The three closely related AM fungi *Gl. mosseae*, *Gl. caledonium* and *Gl. geosporum* were detected by using LSU primers in field studies (Stukenbrock & Rosendahl, 2005; Rosendahl & Matzen, 2008), but these primers were designed to only amplify subgroups or certain taxa in the *Glomeromycota*. For the well-studied *Gl. intraradices* related AMF (e.g. DAOM197198), which are, however, not conspecific with *Gl. intraradices* (Stockinger *et al.* 2009), microsatellite markers are available for their detection in the field (Croll *et al.*, 2008; Mathimaran *et al.*, 2008). Some mtLSU region markers were also studied (Börstler *et al.*, 2008), but because of the high length variation observed (1070–3935 bp) and the difficulty in amplifying this region it is not very promising for community analyses. Thus, such markers cannot be used for general AMF community analyses.

The new primers described in the present study were used to amplify efficiently and specifically target rDNA from environmental samples of the main phylogenetic groups in the *Glomeromycota*. For the first time, this will allow molecular ecological studies covering all AMF lineages to be carried out with only one primer set. Furthermore, the long sequences allow robust phylogenetic analyses and species level resolution

by inclusion of the variable ITS and LSU rDNA region (Walker *et al.*, 2007; Gamper *et al.*, 2009; Stockinger *et al.* 2009), whereas formerly used primers mainly amplified rDNA fragments of up to 800 bp (Helgason *et al.*, 1999; Redecker, 2000; Lee *et al.*, 2008).

Potential application as DNA barcoding primers

The new primers are suited to amplify the most likely primary DNA barcode region for fungi, the ITS region (already online at the Barcode of Life Data Systems (BOLD) website; www.barcodinglife.org). In general 'barcode primers' should amplify short fragments and for the ITS region the amplicons generated by our primers are in fact too long. However, the main criterion for DNA barcodes is the resolution at species level. Since for *Glomeromycota* this is difficult or impossible to achieve with the ITS region only (Stockinger *et al.*, 2009), the inclusion of the 5′ LSU rDNA fragment is strongly recommended. Our new primer set (SSUmAf, SSUmCf, LSUmAr and LSUmBr) appears to be well suited as barcoding primers for *Glomeromycota*. The primers will be helpful for the molecular characterization of AMF, including species descriptions (Gamper *et al.*, 2009), resulting in a sequence database that allows the design of further primers for the detection of AMF from field samples. LSUmAr and LSUmBr, located approximately at positions 930–950 and 830–850 on the LSU rRNA gene, may be used in combination with new forward LSU primers for amplification of fragments within the variable D1/D2 LSU regions. Based on such amplicons, deep sequencing approaches with the now feasible longer reads of the new 454 FLX-titanium chemistry will allow species level detection of the 'unknown' AMF community, in future molecular ecological studies.

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