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Herbert Stockinger, Manuela Krüger, Arthur Schüßler

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DNA barcoding of arbuscular mycorrhizal fungi

Kumulative Dissertation
zur Erlangung des Doktorgrades der Naturwissenschaften
an der Fakultät für Biologie der Ludwig-Maximilians-Universität München

vorgelegt von
Herbert Stockinger

München, Januar 2010

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List of abbreviations (SI units not included)

ac	acaulosporoid
AFLP	amplified fragment length polymorphism
AFTOL	Assembling the Fungal Tree of Life
AM	English: arbuscular mycorrhiza, Deutsch: Arbuskuläre Mykorrhiza
AMF	arbuscular mycorrhiza fungi
approx.	approximate(ly)
Att	attempt
BEG	International Bank for the *Glomeromycota*
BOLD	Barcode of Life Data Systems
bp	base pairs
BS	bootstrap support
CBOL	Consortium for the Barcode of Life
cf.	Latin: *confer* (English: compare)
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
COI	cytochrome c oxidase subunit I (also COXI)
comb. nov.	Latin: c*ombinatio nova* (English: new combination)
COX1	cytochrome oxidase subunit I (also COI)
CTAB	cetyltrimethylammonium bromide
DAOM	Agriculture and Agri-Food Canada National Mycological Herbarium
DIC	differential interference contrast
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
e.g.	Latin: *exempli gratia* (English: for example)
EnvGrA	Environmental Group A
fam. nov.	Latin: *familia nova* (English: new family)
gen. nov.	Latin: *genus novum* (English: new genus)
GINCO	*Glomeromycota* In Vitro Collection
GINCO-BEL	GINCO-Belgium
gl	glomoid
GlGrA	*Glomus* Group A
GlGrB	*Glomus* Group B

GlGrAa	*Glomus* Group Aa
GlGrAb	*Glomus* Group Ab
GTR	General Time Reversible
HPLC	high performance liquid chromatography
INVAM	International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi
ISSR	inter-simple sequence repeat
ITS	internal transcribed spacer
K2P	Kimura two-parameter
kb	kilo bases
LB	lysogeny broth
loc. cit.	Latin: *loco citato* (English: in the place cited)
LSU	large subunit
MAFF	Ministry of Agriculture, Forestry and Fisheries
matK	maturase K
max.	maximum
Mb	mega bases
ML	maximum likelihood
MLQP	maximum likelihood quartet puzzling
MOTU	molecular operational taxonomic unit
MP	maximum parsimony
ms	multispore
mt	mitochondrial
MUCL	Mycothèque de l'Universite Catholique de Louvain
Mya	million years ago
NJ	neighbour joining
no. or n	number
ns	not sequenced
nuc	nuclear
OSC	Oregon State University Herbarium
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PPA	pre-penetration apparatus
psbA	photosystem II protein D1

psbI	photosystem II reaction centre I protein I
psbK	photosystem II reaction centre protein K
PVLG	polyvinyl alcohol lacto-glycerol
QP	quartet puzzles, quartet puzzling
RAPD	random amplification of polymorphic DNA
rbcL	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
rDNA	ribosomal DNA
rf	root fragment
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROC	root organ culture
rpoB	RNA polymerase subunit beta
rpoC1	RNA polymerase subunit gamma
rRNA	ribosomal RNA
sc	spore cluster
s.d.	standard deviation
SI	Système International d'unités
SOC	super optimal broth with 'catabolite repression'
sp. nov.	Latin: *species nova* (English: new species)
ss	single spore
SSU	small subunit
Taq	*Thermus aquaticus*
TB	terrific broth
tRFLP	terminal restriction fragment length polymorphism
trnH	tRNA histidine
U	unit
wk	week

1 Abstract

Plant beneficial microorganisms, such as arbuscular mycorrhiza fungi (AMF), increasingly attract scientific and agronomic attention due to their capacity to increase nutrient accessibility for plants and to reduce inorganic fertilizer requirements. AMF are thought to form symbioses with most land plants, obtaining carbon from the autotrophic host whilst enhancing uptake of poorly available nutrients.

The species of AMF are mainly identified by spore morphology, which is time consuming, requires expertise and is rarely applicable to AMF identification in roots. Molecular tools such as analysis of standardized DNA fragment sequences may allow the recognition of species through a 'DNA barcode', which may partly overcome this problem. The focus of this study was to evaluate different regions of widely used rDNA repeats for their use as DNA barcodes for AMF including the small subunit rRNA gene (SSU), the internal transcribed spacer (ITS) and the large subunit rRNA gene (LSU). Closely related species in the genus *Ambispora,* members of which have dimorphic spores, could not be separated by analysis of the SSU region, but of the ITS region. Consequently, the SSU was not used for subsequent analysis, but a DNA fragment covering a small part of the SSU, the entire ITS region and about 800 bp of the LSU (SSUmCf-LSUmBr fragment) was analysed, providing phylogenetic resolution to species. New AMF specific primers for these potential barcoding regions were developed and can be applied, without amplification of non-target organisms, for AMF species determination, including identification from field and root samples.

Analyses based on the application of the SSUmCf-LSUmBr fragment showed that the widely used AMF model organism *Glomus* sp. DAOM197198 (formerly called *Glomus intraradices*) is not conspecific with *Gl. intraradices*. The SSUmCf-LSUmBr fragment clearly provides a much higher species resolution capacity when compared with the formerly preferred ITS and LSU regions.

Further study of several groups of AMF species using different regions of the SSUmCf-LSUmBr fragment revealed that only the complete SSUmCf-LSUmBr fragment allowed separation of all analysed species. Based on these results, an extended DNA barcode covering the ITS region and parts of the LSU region is suggested as a DNA barcode for AMF. The complete SSUmCf-LSUmBr fragment sequences can serve as a database backbone for also using smaller rDNA fragments as barcodes. Although the smallest fragment (approximately 400 bp) analysed in this study was not able to discriminate among AMF species completely, such short regions covering the ITS2 or LSU D2 regions, respectively, would most likely be suitable for community analyses with 454 GS-FLX Titanium sequencing, providing that the analyses is based on the longer DNA sequences.

2 Zusammenfassung

Arbuskuläre Mykorrhizapilze (AM-Pilze) bilden eine Symbiose mit den meisten Landpflanzen, in welcher sie der Pflanze Nährstoffe bieten und im Austausch Kohlenhydrate erhalten. Sie zählen zu den Mikroorganismen, die einen positiven Einfluss auf Pflanzen ausüben und erwecken zunehmend wissenschaftliche und agronomische Aufmerksamkeit, da sie die Nährstoffzugänglichkeit für Pflanzen erhöhen und damit zum Beispiel die benötigte Menge an Phosphordünger deutlich reduzieren können.

AM-Pilzarten wurden klassischerweise durch morphologische Analysen identifiziert, welche sowohl zeitaufwendig als auch schwierig sind und sich für Artidentifizierung in Pflanzenwurzeln nicht eignen. Die molekularbiologische Analyse eines standardisierten DNA-Fragments, welches für eine Art spezifisch ist und dadurch als „DNA Barcode" definiert wird, ist ein vielversprechendes Werkzeug für eine verlässliche Artidentifizierung.

Der Fokus dieser Arbeit lag in der Etablierung eines geeigneten DNA-Barcodes für arbuskuläre Mykorrhizapilze. Hierfür wurden verschiedene Abschnitte des in früheren Arbeiten bereits oft verwendeten „rDNA-Repeats" analysiert, darunter das „*small subunit rRNA gene*" (SSU), die „*internal transcribed spacer region*" (ITS) und das „*large subunit rRNA gene*" (LSU).

Nah verwandte Arten der Gattung *Ambispora,* welche zum Teil dimorphische Sporen aufweisen, konnten nicht durch Analysen der SSU Region aufgetrennt werden, was durch Analysen der ITS-Region aber möglich war. Die SSU-Region wurde folglich für weitere Analysen nicht weiter bearbeitet, sondern ein durch die Primer SSUmCf-LSUmBr amplifiziertes Fragment, das einen kleinen Teil der SSU, die ITS Region und ca. 800 bp der LSU umfasst. Mit Hilfe der SSUmCf-LSUmBr Fragmentes konnten alle untersuchten AM-Pilze bis hin zur Artenebene aufgelöst werden. Neue, AM-Pilz spezifische Primer für diese potentielle Barcode-Region wurden entwickelt und können für spezifische AM-Pilz Artbestimmung von Feld- und Wurzelproben benutzt werden.

Analysen basierend auf dem SSUmCf-LSUmBr Fragment ergaben, dass der weit verbreitete Modelorganismus *Glomus* sp. DAOM197198 (früher *Gl. intraradices* genannt) nicht zur Art *Gl. intraradices* gehört. Das SSUmCf-LSUmBr Fragment erlaubt eine deutlich besserer Artauflösung im Vergleich zu der früher oft benutzten ITS und LSU Regionen.

Detaillierte Analysen verschiedener Sequenzabschnitte des SSUmCf-LSUmBr Fragmentes ergaben, dass nur Analysen des kompletten Fragmentes alle analysierten AM-Pilzarten erfolgreich separieren kann. Basierend auf diesen Ergebnissen wird vorgeschlagen, das SSUmCf-LSUmBr Fragment als erweiterten DNA Barcode für AM Pilze zu nutzen. Das SSUmCf-LSUmBr Fragmente kann auch als ‚Rückgrat' für auf kürzeren Sequenzen basierende Analysen dienen. Die kürzesten hier

untersuchten rDNA Sequenzen (ca. 400bp) alleine können nicht alle AM-Pilzarten auflösen. Auf Basis von robusten, SSUmCf-LSUmBr Fragment basierten Phylogenien ist es aber wahrscheinlich möglich auch 400 bp Sequenzen verlässlich bestimmten Arten zuzuordnen. Dies wäre für 454 GS-FLX Titanium Pyrosequenzierungen zur Bestimmung von AM-Pilz-Gemeinschaften in Ökosystemen ein deutlicher Fortschritt.

3 Introduction

3.1 Arbuscular mycorrhizal fungi (AMF)

Arbuscular mycorrhizas (AM) are named from the treelike structures formed inside root cortical cells, called arbuscules ('little bushes'). They are symbioses between plants and a particular group of fungi, the so called arbuscular mycorrhizal fungi (AMF). Such symbioses are generally regarded as mutualistic, with a bidirectional transfer of nutrients (Smith & Read, 2008). The contact zones of mycorrhiza are in general on the surface or inside the roots of plants, which is reflected in the name mycorrhiza (ancient Greek: μυκης (mykes) = fungus and ριζα (rhiza) = root) (Frank, 2005). The fungal structures are always surrounded by the plant cell wall (intercellular) or the plant plasma membrane (intracellular). AMF provide the plant with nutrients such as phosphorus, nitrogen, zinc and copper. They receive carbon assimilate from the host plant. Whereas plants may be able to live without AMF, the fungi are thought to be obligate symbionts. It is said that approx. 70-90 % of all land plant species form AM (Brundrett, 2009). Potential host plants range from liverworts, hornworts, ferns and gymnosperms to angiosperms (Harley & Harley, 1987; Brundrett, 2009; Wang & Qiu, 2006).

Based on small subunit (SSU) rDNA sequences and their symbiotic lifestyle, the AMF were placed in the separate phylum *Glomeromycota* (Schüßler *et al.*, 2001). The origin of this phylum may be more than 1000 million years ago. The AM symbiosis is also ancient and AMF may have played an important role during land colonization by plants (Redecker *et al.*, 2000a; Remy *et al.*, 1994; Simon *et al.*, 1993;-Heckman *et al.*, 2001).

3.1.1 Effects of AMF on plants

It has long been recognised that AMF have an influence on plant nutrition and growth (Mosse, 1957; Gerdemann, 1965; Daft & Nicolson, 1966, 1969a, b; Hayman & Mosse, 1971, 1972; Mosse & Hayman, 1971). Much of the research on AMF is driven by the desire to improve plant production. Up to 20 % of plant photosynthates can be delivered to the AMF (Jakobsen & Rosendahl, 1990; Bago *et al.*, 2003). Some AM associations under some circumstances result in increased plant nutrient uptake and growth (Smith *et al.*, 2009), but this is not universal and there are examples in which there is no or even a negative mycorrhizal growth response (Tawaraya, 2003). Graham & Abbott (2000) noted growth depression by 10 different AMF cultures tested on

wheat in pot cultures. Different AMF cultured with the same plant gave different growth effects (van der Heijden *et al.*, 2003; Klironomos, 2003). Pringle & Bever (2008) showed that an early mortality of *Rumex* seedlings consistently occurred in the laboratory and in a field experiment, when inoculated with *Scutellospora pellucida*, but it is worth noting that *Rumex* spp. are considered generally to be non-mycorrhizal (Fransson *et al*. 2003). Barley showed a similar large growth depression in the presence of *Glomus geosporum* with 20 % colonization (6 weeks) or in the presence of a *Gl. intraradices*-like fungus with 72% colonization (6 weeks) (Grace *et al.*, 2009). The phenomenon of plant growth depression may indicate a potential occurrence of cheating within this symbiosis (Johnson *et al.*, 1997; Smith *et al.*, 2009). However, the plant might benefit from other characteristics of this symbiosis like tolerance against pathogens or drought (Newsham *et al.*, 1995).

The establishment of seedlings (van der Heijden, 2004) and the success of plant invasion (Klironomos, 2002) can be enhanced by AMF through their influence on plant productivity, plant diversity and plant community structure (Grime *et al.*, 1987; Gange *et al.*, 1990; van der Heijden *et al.*, 1998; Hartnett & Wilson, 1999; Klironomos *et al.*, 2000; Bever *et al.*, 2001; O'Connor *et al.*, 2002). The ability of plants to compete with each other may affect the structure of plant communities. For example, *Centauria maculosa* and *Festuca idahoensis* show no positive response to AM when cultivated in separate pots (Marler *et al.*, 1999). However, when cultivated together in the presence of AMF, *Centauria maculosa* responded more than *Festuca idahoensis* (Marler *et al.*, 1999). In this instance, no direct effect of AM on the individual plant species growth could be detected, but an indirect benefit from AM occurred when co-cultivating two different species.

The bacterial rhizosphere community also may be influenced by AMF (Marschner & Baumann, 2003) changing in reaction to different AMF species (Marschner & Timonen, 2005).

3.1.2 Plant nutrition and water relations effected by AMF

It is widely accepted that the main function of the AM symbiosis is the exchange of nutrients between partners. Phosphorus, which occurs in inorganic or organic forms in soil, in many ecosystems is the most important nutrient for which uptake is mediated by AMF. Inorganic phosphorous is held firmly within the soil in insoluble forms such as Al-, Ca-, and Fe-phosphates (Smith & Read, 2008), whereas organic phosphorous occurs as inositol phosphates (phytate), phospholipids and nucleic acids in the soil. Organic phosphorous is mineralised by desorption, precipitation and with the help of organisms (Smith & Read, 2008). The available phosphorous

around roots rapidly becomes exhausted and a depletion zone is formed. AMF can bridge this zone and make additional phosphorous accessible. Furthermore, the small diameter and rapid growth of the AMF hyphae compared with roots can reach smaller soil pores, facilitating phosphorous uptake. This may result in larger plants, which contain higher concentrations of phosphorous in their tissues (Smith & Read, 2008). Even when mycorrhizal plants do not grow larger, they can receive phosphorus from AMF (Grace *et al.*, 2009; Smith *et al.*, 2009). The increased inflow of phosphorus into mycorrhizal roots was first studied in *Allium cepa* by Sanders and Tinker (1973), followed by studies in other plants with varying responses and dependencies of the plants (Smith *et al.*, 2003, 2004).

Another main nutrient for plant growth is nitrogen. Nitrogen is a principal component of most fertilizers, on which modern day agriculture depends. Nitrogen production is very energy consuming. *Rhizobia* in root nodules can fix nitrogen in their natural environments. Additionally AMF hyphae can absorb nitrogen in form of ammonium and nitrate. The contribution of such absorption to plant growth is unclear. Some studies showed, that up to 30-42 % of total plant nitrogen can be taken up via AMF (Mäder *et al.*, 2000; Govindarajulu *et al.*, 2005), whereas Reynolds *et al.* (2005) showed no increase of total nitrogen uptake for some perennials plants.

Besides nitrogen and phosphorous, micronutrients, such as copper and zinc are also important for plant growth. AMF can increase copper concentration of different plant species (Mosse, 1957; Gildon & Tinker, 1983; Kucey & Janzen, 1987; Gnekow & Marschner, 1989; Li *et al.*, 1991) and may contribute up to 62% of total copper uptake of white clover (Li *et al.*, 1991). Zinc, which, like phosphorous, is poorly labile in the soil, can be transported via AMF hyphae to the plant over a distance of at least 14 cm (Jansa *et al.*, 2003) and therefore reduce zinc deficiency (Thompson, 1990; Bürkert & Robson, 1994). On the other hand, AMF colonization reduces zinc accumulation in plants growing on soils with high zinc content (Li & Christie, 2001; Zhu *et al.*, 2001; Burleigh *et al.*, 2003). Although AMF can affect copper and zinc uptake, little is known in respect of other micronutrients (Hart & Trevors, 2005).

Another fundamental factor for plant growth is the water availability. AMF has an effect on the plant water relations (Augé, 2001, 2004; Augé *et al.*, 2008). For example in the study of Mosse and Hayman (1971), mycorrhizal *Allium cepa* seedling did not wilt when tranplanted in contrast to non-mycorrhizal controls. Different AMF show different effects on plant water stress (Marulanda *et al.*, 2003; Aroca *et al.*, 2007), however, it remains unclear, if this effect results directly from, or is caused by, the increased phosphorous uptake and therefore by plant size or fitness. The mycelium in the soil changes soil moisture characteristics and improves soil structure by formation of soil aggregates (Rillig, 2004; van der Heijden *et al.*, 2006) and might therefore also effect water

availability (Augé, 2001, 2004). Augé (2004) showed that mycorrhiza deficient (non-mycorrhizal) mutant plants grown together with mycorrhizal plants in pots with mycorrhiza kept stomata longer open than in soil without AMF.

Although there is considerable evidence that AMF in general enhance the uptake of phosphorous, micronutrients and water, many questions about nutrient uptake of individual AMF species, both singly and in cohorts, remain to be solved.

3.1.3 AMF spore characteristics and morphology of root colonization

AMF form large spores, sometimes as big as 1.2 mm in diameter, containing many storage components and a numerous nuclei (576 to 35 000 in different species; Hosny *et al.*, 1998; Viera & Glenn, 1990), although the highest value is estimated rather than counted (Hosny *et al.*, 1998). It is suggested that nuclei of *Gl.* sp. DAOM197198, *Gl. etunicatum* and *Sc. castanea* are haploid (Bianciotto *et al.*, 1995; Hijri & Sanders, 2004). The DNA content of one nucleus is between 0.26 and 3.4 pg, depending on species and method of assessment (Viera & Glenn, 1990; Bianciotto & Bonfante, 1992; Hosny *et al.*, 1998). The genome size of *Gl.* sp. DAOM197198 was determined to be approximately 16.54 Mb (Hijri & Sanders, 2004), but preliminary results of the genome sequencing of a fungus of this name revealed the genome to be approx. five times larger than this (Martin *et al.*, 2008). The *Sc. pellucida* genome has been calculated to have a size of 1058 Mb (Bianciotto & Bonfante, 1992). It is still debated whether one spore contains several different nuclei (heterokaryotic; Hijri & Sanders, 2005) or identical nuclei (homokaryotic; Pawlowska & Taylor, 2004). This is interesting, in regard to the high genetic diversity for example within the ITS of a single species (Sanders *et al.*, 1995; Lloyd-Macglip *et al.*, 1996; Lanfranco *et al.*, 1999; Jansa *et al.*, 2002b). Variations have been also detected by AFLP analysis of a culture started with 30 spores (Rosendahl & Taylor, 1997) and in some genes involved in cellular function (Kuhn *et al.*, 2001; Sanders *et al.*, 2003; Pawlowska & Taylor, 2004). Kuhn *et al.* (2001) indicated that a nucleus of *Sc. castanea* contains different numbers of divergent ITS region sequences. This genetic variation within spores may have a significant impact on species definition and on phylogenetic identification. Whether the variation within a single organism influences the interactions of AMF and plants or the colonization of plants in various ecological habitats is still unclear.

AMF preferentially germinate from a spore even in the absence of roots. Plant derived signals such as strigolactones can induce hyphal branching (Akiyama *et al.*, 2005) and change fungal physiology and mitochondrial activity (Besserer *et al.*, 2006). Upon contact with the root, AMF form an

appressorium, also called hyphopodium (Genre *et al.*, 2008). The plant cell responds to this contact with the formation of a pre-penetration apparatus (PPA). The PPA then determines the path of the fungal growth and seems to form a tunnel. Additionally, the plant root cell triggers AMF growth (Genre *et al.*, 2005, 2008). According to Parniske (2008) the 'pre-infection thread' of legumes most likely evolved from the PPA. This relatedness is also reflected by common plant genes involved in rhizobial symbiosis and AM (Parniske, 2008).

The fungal colonization of the plant root is restricted to the cortical cells. Two distinct structural types of colonization were originally described (Gallaud, 1905): the *Arum*-type, in which hyphae grow intercellularly and form intracellular arbuscules, and the *Paris*-type, which exhibits intracellular growth and form hyphal coils cells from which small arbuscules can originate. More recent studies have shown that this differentiation is not so clear and several intermediate forms exist (Dickson, 2004). It remains unclear which factors control the colonization type and it seems it is influenced by both plant and fungus species (Cavagnaro *et al.*, 2001; Dickson, 2004; Kubota *et al.*, 2005; Dickson *et al.*, 2007).

3.1.4 AMF species communities in the field

Ecological studies on AMF have been widely performed based on spore identification and colonization intensity of plant. The surveys of different plant species for AM colonization have been summarized in the publications of Harley and Harley (1987), Wang and Qiu (2006), and Brundrett (2009) who gave an overview of the numerous plant hosts. There are many community studies based on spore morphology. In one, about 30 AMF species have been found in a field containing 50 plant species (Bever *et al.*, 2001). Different AMF spore communities were detected in different soil depths (Oehl *et al.*, 2005) and in different farming systems (Oehl *et al.*, 2003), both in cultivated and non-cultivated lands (Li *et al.*, 2007). Trap cultures were furthermore established to allow expression of species not sporulating at the time of sampling. These cultures are influenced by the plant host and age of the culture. Some species start their sporulation in such cultures only after 18 months (Oehl *et al.*, 2005). Furthermore, the spore community may not reflect the active AMF community colonizing roots and soil. Therefore, molecular methods were developed for the identification of AMF colonizing roots and soil. These molecular ecological studies have shown that many AMF phylotypes might correspond to undescribed species (Öpik *et al.*, 2008; Kottke *et al.*, 2008). In addition, Sýkorová *et al.* (2007a) have detected a bias between the analysis of roots from field, roots from trap cultures, and roots from bait plants. This bias and different methods and DNA

regions makes the comparison among publications very difficult or even impossible.

3.1.4.1 AMF-plant preferences

Early investigators stated that there is no specificity between AMF taxa and potential host plant taxa. No strict specificity can be expected, as the 214 known AMF species form AM with roughly 250 000 host plants species (Fitter, 2005). Some species like *Plantago lanceolata*, *Trifolium subterraneum* and *Sorghum sudanense* are considered to be generalists and therefore are used for cultivation of AMF species in trap cultures (Smith & Read, 2008). However, recent studies indicate that some preferences exist, e.g. for *Acer pseudoplatanus* which was only colonized by a fungus determined to be *Glomus hoi* (Helgason *et al.*, 2002). In addition, mycoheterotrophic plants such as *Arachnites*, *Voria* and *Voyriella* spp. are associated with a limited number of glomeromycotan symbionts possibly because of their narrow specificity to all kinds of fungal associates (Bidartondo *et al.*, 2002). Different plant species used in trap cultures, had also an effect on the sporulation of different AMF (Jansa *et al.*, 2002a). Bever *et al.* (2009) show that *Allium vineale* allocated more carbon to the AMF species with greater mutualistic tendencies. This kind of work on specific relationships among host plants and AMF is still in its infancy, and many questions remain unanswered providing opportunities for much future research.

3.1.5 Species concepts for AMF

The classical species concept for AMF is mainly based on spore morphology and development. Identifying species from spore morphology is not easy and even specialists do not agree on general characteristics of spores. About 214 AMF species have been described to date (www.amf-phylogeny.com), many of which are not in culture. Many, but still not all, new species descriptions include molecular evidence and provide reference cultures, which can be found in BEG (www.kent.ac.uk/bio/beg/), INVAM (http://invam.caf.wvu.edu/), GINCO (http://emma.agro.ucl.ac.be/ginco-bel/) or other available culture collections (e.g. Gamper *et al.*, 2009). Morphological identification of spores can sometimes be misleading. For example, single species with dimorphic spores (Sawaki *et al.*, 1998; Redecker *et al.*, 2000b) have been placed in two different taxa because of their morphological characteristics.

The molecular species concept is mainly based on rDNA sequences such as SSU, internal

transcribed spacer (ITS) region or partial large subunit (LSU) rDNA. Some new markers have also been used such as the mitochondrial LSU (mtLSU; Raab *et al.*, 2005) and tubulin genes (Corradi *et al.*, 2004a; Corradi *et al.*, 2004b; Msiska & Morton, 2009) and microsatellites (Croll *et al.*, 2008; Mathimaran *et al.*, 2008). Some of these can differentiate strains within species of AMF.

A biological species concept is not definable within AMF, because no sexual stage, indispensable for this concept, has yet been found. Therefore, it is assumed that AMF are asexual and clonal. Croll *et al.* (2008) introduced the idea of "non-self fusion compatibility groups" to define species based on anastomosis formation. Such analyses are time-consuming and only one species was analysed up to date. Application of such a concept is also very difficult, because the majority of species is not established in root organ cultures (ROC) or similarly controlled monoxenic systems. Another approach towards species identification utilizes a recombination test. Croll and Sanders (2009) have performed recombination test on 11 polymorphic markers, all showing recombination with 40 clonal isolates from a field, all of which were identified as *Gl. intraradices*, but which are probably more closely related to a different *Glomus* sp. DAOM197198, conspecific with, or closely related to *Gl. irregulare*.

3.1.6 Taxonomy and phylogeny of AMF

The most modern higher taxonomy within AMF is based on a combination of morphology and SSU rDNA sequences. The phylogenetic analysis of SSU rDNA sequences has placed the AMF in a separate phylum, the *Glomeromycota*. This phylum is divided into four orders (Figure 1 *Glomerales, Diversisporales, Archaeosporales, Paraglomerales*). Within this, 13 families have been defined (*Glomeraceae, Diversisporaceae, Acaulosporaceae, Entrophosporaceae, Scutellosporaceae, Gigasporaceae, Racocetraceae, Dentiscutataceae, Pacisporaceae, Geosiphonaceae, Ambisporaceae, Archaeosporaceae* and *Paraglomeraceae*), and 19 genera (*Glomus, Diversispora, Otospora, Acaulospora, Kuklospora, Entrophospora, Scutellospora, Gigaspora, Racocetra, Cetraspora, Dentiscutata, Fuscutata, Quatunica, Pacispora, Geosiphon, Ambispora, Archaeospora, Intraspora* and *Paraglomus*; see www.amf-phylogeny.com). Some of these taxa are paraphyletic, and some may by synonyms. In particular, regarding the separation of *Scutellospora* (personal communication Christopher Walker) many questions are still unanswered about taxonomy and phylogeny. Another example is the genus *Glomus* still appearing in two different orders. The order *Glomerales* is divided into at least two major clades, with both being referred to as *Glomeraceae.* Hence Schwarzott *et al.* (2001) divided them into two groups, *Glomus*

Group A and *Glomus* Group B. It is likely that these two groups will become separate families as soon as the type of *Glomus* could be assigned to one of these groups. To clearly understand the relationship within the *Glomeromycota* it is necessary to have comparable datasets for at least representative species for each clade. However, only one part of the rDNA sequence is available for some species, for example, only the LSU sequence is available for *Sc. nigra*, and therefore comparisons with other species are not possible. Some species are well covered for different rDNA regions, but the sequences may have originated from different cultures and despite their annotations may not be conspecific because of taxonomic difficulties. Such problems exist in particular when cultures have been lost over time and no voucher had been deposited. Even when taxonomic vouchers have been preserved, they often are not amenable to further DNA extraction. Inadequately prepared vouchers may even lack sufficient characters for verification of the species, or may contain more than one species or heavily parasitized spores (personal communication Christopher Walker). The introduction of an easily and quickly applicable method for species determination would be highly beneficial for reliable and comprehensive identification.

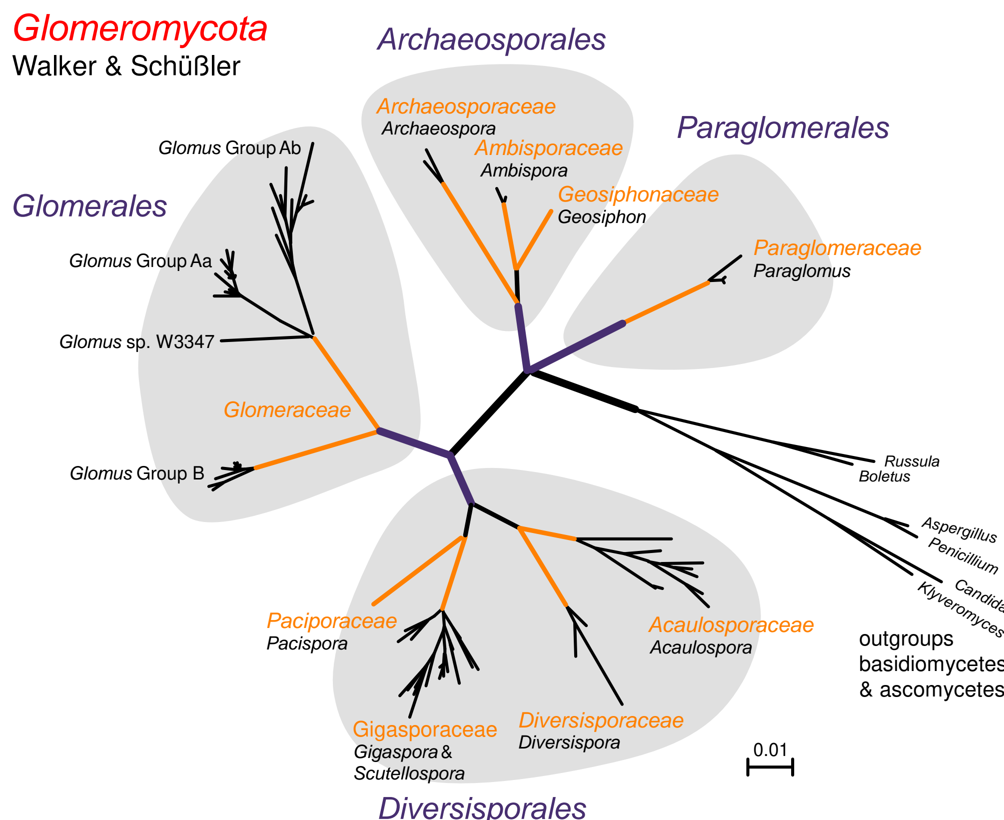

Figure 1: Phylogenetic tree of the AMF showing the organisation within the phylum *Glomeromycota*. After Schüßler *et al.* (2001).

3.2 DNA barcodes and molecular species identification

A DNA barcode is defined as a short and easily PCR amplifiable DNA fragment for identification of organisms to species level. DNA barcoding must ideally be feasible for non experts, accurate, rapid, cost-effective, culture-independent and universally accessible (Frézal & Leblois, 2008). The aim of a barcode system is to be applicable for all kingdoms of eukaryotic life and to simplify recognition of cryptic species. The identification of fragments from organisms or such from different life cycle stages will become possible, where morphological identification is not realizable (Gilmore *et al.*, 2009). This is also a general problem for microorganisms, also such living within other organisms and in the soil. In addition, a genetic barcode can be invaluable for controlling the trade in endangered species (see Convention on International Trade in Endangered Species of Wild Fauna and Flora, CITES) because of its practicality for non experts. Furthermore, it can facilitate the search for new species and may help to protect them before they are lost.

Morphological identification of fungi often requires cultivation of the target organism, which in many cases poses major problems (O'Brien *et al.*, 2005). Therefore molecular methods, such as DNA barcoding, may be the only way to identify such recalcitrant organisms. Of the estimated 1.5-3.5 million fungal species, about 100.000 are described (Hawksworth, 2001; O'Brien *et al.*, 2005; Kirk *et al.*, 2008). Currently no official DNA barcode is suitable for fungi, although many studies deal with the identification of fungi through molecular markers.

3.2.1 Official DNA barcodes

The first official barcode, that is a barcode accepted by the Consortium for the Barcode of Life (CBOL) consists of approx. 640 bp of the *cytochrome oxidase subunit I* (*COX1*) in the mitochondrial DNA, and it is mainly used for animal species identification (http://www.barcoding.si.edu/, Hebert *et al.*, 2003). Mitochondrial DNA of plants has a low substitution rate, and a two-locus barcode from the chloroplast region, using the *matK* and *rbcL* genes consequently was proposed by the CBOL plant working group (Hollingsworth *et al.*, 2009). These authors have observed a species separation capability of the two-locus barcode to a success rate of approx. 72 %, which did not increase significantly when taking in account all seven analysed markers (*matK, rbcL, rpoC1, rpoB, psbK-psbI, trnH-psbA, psbK-psbI*) together.

3.2.2 Molecular identification of fungi using *COX1*

COX1 works well for most animal species; however some drawbacks have been detected within fungi. In a case study of species in the genus *Penicillium*, *COX1* seems to be a likely candidate for species identification (Seifert *et al.*, 2007), although they have already shown considerable length variations possible within the fungi. The *COX1* gene of fungi varies between 1548 bp and 22 kb, similar to the barcoding region within *COX1* gene which ranges from 642 bp to 12.3 kb (Seifert *et al.*, 2007; Seifert, 2009). Only one *COX1* gene is published for just one glomeromycotan species with a length of 2200 bp (based on NC_12056, Lee & Young, 2009). However the length can vary as seen in other parts of the mitochondrial genome in *Glomeromycota*. The mitochondrial large subunit (mtLSU) for example has different sizes and introns even among closely related species (Raab *et al.*, 2005; Börstler *et al.*, 2008).

Multiple copies (paralogous) of the *COX1* gene have been found in the genus *Fusarium* and proved inadequate for species level identification (Gilmore *et al.*, 2009). A similar situation was observed within the complex group of *Aspergillus niger* (Geiser *et al.*, 2007). Lang and Hijri (2009) reported a *COX1* intron in *Glomus diaphanum* with a high sequence similarity to both a plant sequence and a *Rhizopus oryzae COX1* intron. This intron may result from a lateral gene transfer from fungi to plant (Vaughn *et al.*, 1995; Seif *et al.*, 2005; Lang & Hijri, 2009).

3.2.3 Molecular identification of fungi using rDNA sequences

Recent studies using molecular markers for species identification of fungi typically use nuclear ribosomal genes such as the small subunit (SSU), the ITS region (ITS1-5.8S-ITS2) or the partial large subunit (including two variable subregions called D1 and D2). For most fungi the ITS has become the default marker for species identification. This led Seifert (2009) to conclude, that "ITS [is] already functioning as *de facto* barcode". However, yeasts represent an exception, as the LSU is already widely used for species identification (Seifert, 2009). Most sequence data available for members of the *Glomeromycota* are still only from the SSU.

The fungal ITS is rich in insertions and deletions, rendering it useful for developing taxon-specific primers. The length of the ITS varies within fungi from 270 bp for yeasts to 973 bp for *Dothideomycetes*, but the indels and the length variations make the ITS difficult to align (Seifert, 2009).

3.2.3.1 Primer used for PCR amplification of AMF

Several primer combinations were published for the use of PCR amplification for AMF. The resulting DNA fragment varies in their discriminatory power. The most widely used primer combination for AMF is AM1 (Helgason *et al.*, 1998) - NS31 (Simon *et al.*, 1992) (~550 bp). NS31 amplifies all eukaryotic species, and although supposedly specific to AMF, AM1 excludes the ancestral lineages such as *Archaeosporales* and *Paraglomerales* (Redecker *et al.*, 2000b; Schüßler 2001). This primer combination is thus mainly useful for studying *Glomerales* and *Diversisporales*. Some of these studies reported additional amplifications even of non-AMF sequences (Helgason *et al.*, 2002; Douhan *et al.*, 2005; Rodríguez-Echeverría & Freitas, 2006; Santos-Gonzalez *et al.*, 2007). To solve this, new specific SSU primers known as AML1-AML2, covering the AM1-NS31 fragment, were designed that include species in all the major orders of AMF (Lee *et al.*, 2008; ~800 bp). Santos-Gonzalez *et al.* (2007) designed additional new primers (AM2, AM3) based on the AM1 to amplify additional groups, such as the *Glomus* Group B and *Diversisporaceae* species. Wubet *et al.* (2006) also published a new set of primers, specific for different families, amplifying an even longer fragment of about 1130 bp of the SSU (Figure 2).

Several group-specific primers were developed for the amplification of the ITS region (Redecker, 2000). In another approach, Renker *et al.* (2003) designed primers for both the amplification of the ITS region and partial LSU (SSU-Gom1/LSU-Glom1, Figure 2). They also reported that designing primers suitable for all AMF, but not amplifying other fungi is challenging. In addition, different primers were used on the LSU, wherein most of them cover the first 800 bp of the LSU or at least a part of it (Figure 2). These fragments were amplified using a nested PCR approach. All these different fragments make the comparison of results complicated and difficult.

Sequences of contaminated samples are a serious problem in AM research. Schüßler (1999) and Redecker *et al.* (1999) reported that some published rDNA sequences of *Scutellospora castanea* were from ascomycotan origin and even spores with a healthy appearance can contain non glomeromycotan fungi (Hijri *et al.*, 2002). Utilizing AMF specific primers would be an advantage for such contaminated material.

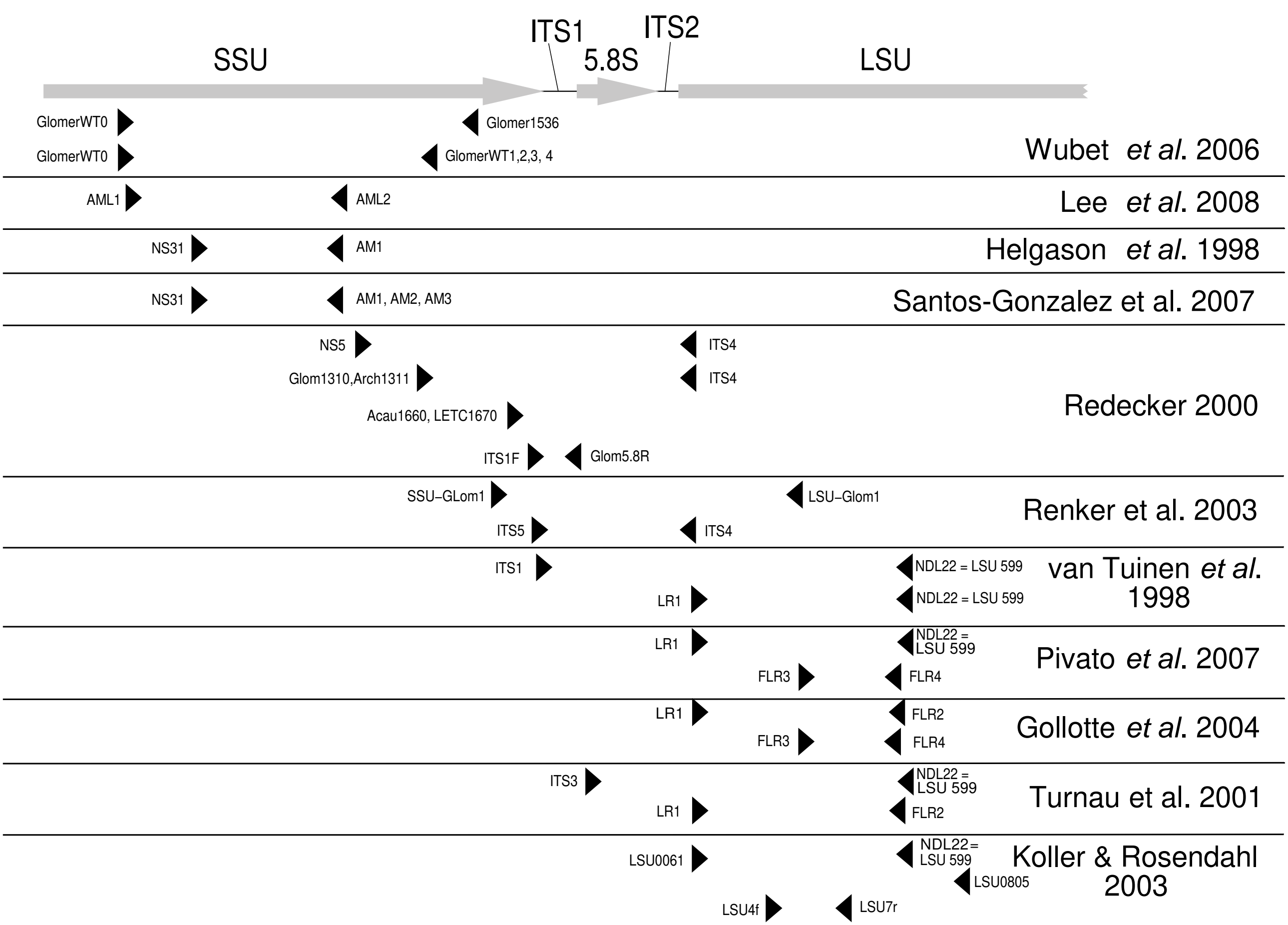

Figure 2: Schematic overview of different primer combinations used in AMF research citing the corresponding publications. A part of the rDNA repeats is illustrated on the top. Grey arrows indicate the genes; thin lines the internal transcribed spacers (ITS1& ITS2). For clarity, the LSU gene is not shown in full length. Triangles show priming site and direction. Publications showing several primer combinations indicate nested PCR approaches, except for Redecker (2000), who developed AMF subgroup-specific primers.

3.3 Aim of this study

The focus of this study was to develop a simple and robust system for the identification to species level of glomeromycotan fungi. The study aimed to contribute to the development of a DNA barcode for fungi and in particular for AMF such as that already established for animals. The identification system must ideally be based on parts of the widely used rDNA such as the SSU, the ITS or LSU region because the largest number of published sequences are available from these regions. In the course of the work, the ITS region was proposed as the possible DNA barcode for fungi. In addition to the ITS region, several other parts of the rDNA region were investigated for their potential DNA barcode suitability. The high variation of the rDNA within one AMF species or

even a single spore was critically checked for all fragments for successful species identification.

To amplify AMF in roots of plants and to overcome the problem with potential contaminations, new AMF specific primers were designed and tested in anticipation of them being used as AMF barcoding primers. In recognition of new sequencing technologies, such as the 454 GS-FLX Titanium system, approximate 400 bp long fragments were tested for its potential usage in such systems to identify species of AMF.

4 Molecular phylogeny and new taxa in the *Archaeosporales* (*Glomeromycota*): *Ambispora fennica* gen. sp. nov., *Ambisporaceae* fam. nov., and emendation of *Archaeospora* and *Archaeosporaceae*.

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Molecular phylogeny and new taxa in the *Archaeosporales* (*Glomeromycota*): *Ambispora fennica* gen. sp. nov., *Ambisporaceae* fam. nov., and emendation of *Archaeospora* and *Archaeosporaceae*

Christopher WALKER[a], *Mauritz* VESTBERG[b], *Filiz* DEMIRCIK[c], *Herbert* STOCKINGER[c], *Masanori* SAITO[d], *Hiromichi* SAWAKI[e], *Ibuki* NISHMURA[f], *Arthur* SCHÜßLER[c,*]

[a]Royal Botanic Garden Edinburgh, 21A Inverleith Row, Edinburgh EH3 5LR, UK
[b]MTT Plant Production Research Laukaa, FI-41330 Vihtavuori, Finland
[c]TU-Darmstadt, FB10 Botanik, Schnittspahnstr. 10, D-64287 Darmstadt, Germany
[d]National Institute for Agro-Environmental Sciences, Tsukuba, Ibaraki 305-8604, Japan
[e]National Institute of Livestock and Grassland Science, Nasu-Shiobara, 329-2793, Japan
[f]Ibaraki University, Ami, Ibaraki, 300-0393, Japan

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ABSTRACT

The AM fungal family *Archaeosporaceae* and the genus *Archaeospora* are rendered paraphyletic by the relationship with the *Geosiphonaceae*. This problem led to a more detailed study of the *Archaeosporales*. Members of the *Archaeosporaceae* were described as forming both glomoid and acaulosporoid spores, or solely acaulosporoid spores. However, we found that *Glomus callosum* fell into the same phylogenetic clade as *A. leptoticha* and *A. gerdemannii*, but exclusively formed glomoid spores. To resolve these inconsistencies, a genus, *Ambispora* gen. nov., typified by *Ambispora fennica* sp. nov., is erected based on morphological evidence and SSU and ITS region rDNA data. *Ambispora* contains three species known to produce both acaulosporoid and glomoid spores: *A. fennica*, *A. leptoticha* comb. nov. (basionym *G. leptotichum*), and *A. gerdemannii* comb. nov. (basionym *G. gerdemannii*). Another species, *A. callosa* comb. nov. (basionym *G. callosum*), is known only from glomoid spores. *Ambispora* is placed in a new family, the *Ambisporaceae* fam. nov. The *Archaeosporaceae* is maintained with the type species, *Archaeospora trappei* (basionym *Acaulospora trappei*), along with *Intraspora schenckii* (basionym *Entrophospora schenckii*). *Acaulospora nicolsonii*, known only from acaulosporoid spores, is discussed and is considered likely to belong in the *Ambisporaceae*, but is retained within its present genus because of inadequate morphological information and a lack of molecular data.

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Introduction

AMs are extremely common in terrestrial ecosystems (Smith & Read 1997), and their significance has been recognised in a broad range of studies of both basic and practical aspects. Fossils of presumed glomeralean fungal spores date back AM origins at least to the Ordovician, about 460 million years ago (Mya), and from molecular clock estimates,

* Corresponding author. Present address: LMU Munich, Genetics Institute, Maria-Ward-Strasse 1a, D-80638 Munich, Germany.
E-mail address: arthur.schuessler@lrz.uni-muenchen.de
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perhaps as far as 600 (Redecker *et al.* 2000a) or even >1000 Mya (Heckman *et al.* 2001), supporting the theory that the earliest land plants depended upon the symbiosis with AM fungi for their nutrient acquisition (Pirozynski & Malloch 1975; Schüßler 2002).

Conventional taxonomy of glomeromycotan fungi was mainly dependent upon the relatively simple morphology of their asexual spores with consequent difficulties in finding suitable characters for species determination. For example, it was shown that '*Glomus*' lineages, as defined until recently, were clearly non-monophyletic (Schwarzott *et al.* 2001). Moreover, some AM fungi (AMF) were shown to produce two types of spores, each of which had been placed in a separate genus (*Glomus* and *Acaulospora*) in two different families, the *Glomeraceae* and *Acaulosporaceae*. This anomalous situation has been discussed several times (Walker 1992; Morton *et al.* 1997; Murakoshi *et al.* 1998).

From studies based on SSU ribosomal RNA (rRNA) gene sequences, Sawaki *et al.* (1998), were the first to show that a dimorphic spore forming fungus named as both *Acaulospora gerdemannii* and *Glomus leptotichum* did not belong phylogenetically to any of the AMF families defined at the time. As the latter spore type was said to be morphologically indistinguishable from those of *Glomus sensu lato*, it is likely that more species placed in *Glomus* are related to this lineage of AMF.

Further phylogenetic studies showed that several glomeromycotan species should be separated from the existing taxa, as they comprise independent phylogenetic lineages within the AMF (Redecker *et al.* 2000b). From molecular and morphological data, two new monogeneric families, *Archaeosporaceae* and *Paraglomeraceae*, were erected (Morton & Redecker 2001) to accommodate the new genera *Archaeospora* and *Paraglomus*. However, it had already been shown from studies of *A. leptoticha* (at the time named as *Acaulospora gerdemannii*) (Schüßler 1999; Kramadibrata *et al.* 2000), that the '*Archaeospora* lineage' also contains *Geosiphon pyriformis*, a fungus in the *Geosiphonaceae* that forms an 'AM-like' symbiosis with cyanobacteria. Recently, the new genus *Intraspora* was erected (Sieverding & Oehl 2006) to accommodate *I. schenckii* (basionym *Entrophospora schenckii*), placed in the *Archaeosporaceae* as a sister taxon to *A. trappei*.

There is now a taxonomic conundrum relating to the *Archaeosporaceae* in the *Glomeromycota* (Spain 2003; Morton & Redecker 2001). The AM species *A. trappei*, *A. gerdemannii*, and *A. leptoticha* are presently contained within the same genus, but whereas the last two are closely related, they have little in common with the first, except the production of spores in the neck of an initial, blastic, sporiferous saccule. Similarly, *I. schenckii*, shares much in common with *A. trappei* (Ames & Linderman 1976), but not with the other species presently placed in the family. Moreover, the SSU rDNA phylogeny shows that the *Archaeosporaceae* is paraphyletic (Schüßler *et al.* 2001).

We consequently undertook work on the systematics of these fungi, which are widespread in some grassland ecosystems (Murakoshi *et al.* 1998; Kojima *et al.* 2004), to understand the relationships among members of the *Archaeosporales*. We compared the morphology and the SSU and ITS rDNA analyses of new isolates of *Archaeospora leptoticha*, a fungus from western Finland similar in appearance to *A. gerdemannii*, and Japanese isolates of *Glomus callosum* that produced only glomoid spores.

Materials and methods

Parts of the work were performed in Finland, Japan, the UK, and Germany. These localities are noted in the following, as the methods used were not identical in each of the different laboratories.

Sampling and isolation

For the Finnish part of the work, samples of soil were taken on 20 Sep. 1989 from a ley (pasture) field in Kurikka, located in the Ostrobothnia region of western Finland (62° 30′ N; 22° 20′ E). The plant community consisted of mixed grasses and clover along with the volunteer plants from the seed bank in the soil, but it was not precisely determined because the crop had been harvested and identification of individual species from the remaining stubble was not practical. The soil was silty, with a pH in water of 5.3, available P 7 $mg\,l^{-1}$ (ammonium acetate extraction after Vuorinen & Mäkitie 1955), and conductivity 0.45 $mS\,cm^{-2}$. Other available ions were Ca^{2+} 423 $mg\,l^{-1}$, K^{+} 226 $mg\,l^{-1}$, and Mg^{2+} 49 $mg\,l^{-1}$. Subsamples of soil were used to establish open-pot cultures as soil traps, and from one of these, a single acaulosporoid spore was selected and introduced to the roots of a seedling of *Plantago lanceolata* (Walker 1999). This was then maintained in a Sunbag® (Sigma, Tufkirchen) to ensure that it was free from contamination by other AM fungi (Walker & Vestberg 1994). It then was maintained in both Sunbags and open-pot cultures through repeated subcultures in Finland and the UK.

In Japan, four cultures were established initially by soil traps from different parts of the country, followed by single-spore isolation as described in Murakoshi *et al.* (1998). The fifth culture was a multi-spore single species culture. Cultures were maintained in sealed Sunbag systems at the Soil Ecology Laboratory, National Institute of Livestock and Grassland Science. Examination of the resultant spores followed standard methods for morphological study of members of the *Glomeromycota* (e.g., Walker 1983, 1986; Walker *et al.* 1993; Morton 1988).

For molecular analyses, spores for DNA extraction were isolated by centrifugation and sugar-floatation (Walker *et al.* 1982) or by swirling with water and decanting through a fine sieve. For analyses done in Germany, spores were selected under a dissecting microscope and individually placed in Eppendorf® vials. They were carefully checked for fresh and undamaged appearance, and to verify that there was no obvious contamination from other fungi. For the Japanese work, one to ten spores were selected in a similar manner for later DNA extractions.

Morphological studies

Extracted spores were initially examined in water under a dissecting microscope, and then through a compound microscope after mounting in polyvinyl alcohol lacto-glycerol (PVLG) with or without the addition of Melzer's reagent

(Walker *et al.* 1993). Measurements were made with a calibrated eyepiece graticule. Records were kept in a database in which pot culture attempts are given attempt (Att) numbers and herbarium voucher collections given 'W numbers' (Walker & Vestberg 1998). For consistency, such numbers are given to all specimens examined, regardless of the collector (Table 1), including type material. Vouchers are deposited, where relevant, in the herbaria designated in their protologues or in the Walker collection lodged at the Herbarium at the Royal Botanic Garden Edinburgh (E).

Because of the different opinions regarding wall structure of spores in the *Glomeromycota*, we need to define the terminology used in the descriptions herein. There are no controlled developmental studies of the acaulosporoid propagules of members of the group of organisms under discussion, and the light microscope with pot-cultured specimens cannot provide the resolution needed to reconcile different layers and walls in these complex structures. We therefore applied the terminology of Walker (1983), modified by Walker & Vestberg (1998), in which the wall structure, as seen through a compound microscope, is described as having individual components which, when they remain adherent or close together on crushed specimens, are referred to as being in groups.

Samples of roots were stained in trypan blue or ink (Phillips & Hayman 1970; Walker 2005) to examine mycorrhizas, mounted in PVLG, and examined under a compound microscope.

Molecular studies

DNA extraction, cloning, and sequencing

Cultures, isolates, and spore morphs used, clone numbers, and sequence accession numbers are listed in Table 1. Whereas the morphological methods used were the same regardless of the origins of the fungi, the molecular studies were carried out at different times and locations. Consequently, the details of materials and methods are different for each laboratory. The 'Japanese fungi' were initially investigated in Japan, and later also in Germany, whereas the 'European fungi' were studied only in Germany, where the final analysis was carried out.

For the European AM fungi, DNA was extracted, PCR-amplified, and cloned from individual spores (stored at -80 °C) sampled from the cultures Att200-11 (sampled Jan. 2001; cloned May 2005 and Mar. 2006), Att200-21 (sampled Feb. 2002; cloned Mar. 2002), Att200-23 (sampled Sep. 2005, cloned Dec. 2005). Isolation of DNA from cleaned single spores and methods used for cloning and sequencing were as described in Schwarzott & Schüßler (2001). Primers and conditions for PCR of the SSU rDNA were also as described in that paper, except that in many of the PCR reactions the Phusion proofreading polymerase (Finnzymes, Espoo) was used. The ITS region rDNA was amplified using the primers SSU-Glom1 (Renker *et al.* 2003) and LSU-Glom1b (TCGTTTCCCTTTCAACAATTTC AC, designed for the present study). PCR was run with the Phusion proofreading polymerase with the following programme: 99 °C denaturation for 2 min; 35 cycles of 99 °C for 10 s, 65 °C for 30 s, 72 °C for 60 s; final elongation 72 °C for 5 min. After cloning and plasmid isolation, fragments were sequenced with vector primers and ITS4 (White *et al.* 1990), and sequences assembled with the program SeqAssem (http://science.do-mix.de/software.php).

For the Japanese cultures and isolates, DNA was extracted for amplification of rDNA as described in Sawaki *et al.* (1998). SSU rRNA gene fragments were PCR amplified using the primers EcoNS1 (GTAAAACGACGGCCAGTGAATTCGTAGTC ATATGATTGTCTC) and NS8KX (GTACCTCGAGGTACCTAATG ATCCTTCCGC), containing restriction sites for cloning, by a proofreading DNA polymerase (Takara, Kyoto, Japan). The PCR was performed with the following programme: 95 °C denaturation for 3 min; 41 cycles of 95 °C for 30 s, 53 °C for 45 s, 70 °C for 3 min; final elongation 72 °C for 10 min. DNA was purified (QIAquick; QIAGEN, Hilden) and digested with *Eco*RI and *Xho*I. After agarose gel electrophoresis (1 %) DNA was extracted from gels and fragments were ligated into Uni-ZAP XR (Stratagene, La Jolla, CA) and packaged *in vitro*. Several clones containing appropriate inserts were screened from each phage fraction with plaque-direct PCR, using P7 and P8 universal primers flanking the cloning sites. DNA of each clone was rescued with *in vivo* excision protocol. ITS rDNA was amplified from the spore DNA with ITS1 and ITS4 primers (White *et al.* 1990) by using a hot-start Taq DNA polymerase (Applied Biosystems, Foster City, CA). PCR was performed as follows: 95 °C denaturation for 10 min; 40 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 45 s; 72 °C for 10 min final elongation. DNA fragments were purified and cloned into pCR2.1 plasmid vector (Invitrogen, Carlsbad, CA). Templates for DNA sequencing were generated from phagemid plaque by PCR with Taq DNA polymerase (Nippon Gene, Tokyo) and universal primers (Sawaki & Saito 2001). The products were purified and used as templates for sequencing with T3 and T7 universal primers and the SSU rRNA gene specific primers NS2, NS3, NS4, and NS5 (White *et al.* 1990). Plasmid DNA was used as templates for sequence reaction with M13 primers. Sequences for SSU rDNA were compared and assembled with the program Sequencher (Hitachi, Tokyo, Japan).

Phylogenetic analyses

Sequence alignment was carried out manually, with the program Align (http://science.do-mix.de/software.php), taking secondary structure into account (Wuyts *et al.* 2000). The SSU rDNA alignment comprised all archaeosporalean near full-length sequences available in the databases (http://srs.ebi.ac.uk/); database sequences from the same isolate were composed to majority rule consensus sequences. The sequences from the two spore morphs from the Finnish fungus (Att200), described here as *Ambispora fennica* gen. sp. nov., were analysed as individual sequences or as consensus sequences, both, and sequences from members of the *Paraglomerales* were used as outgroups (Table 1). The ITS1, 5.8 S rDNA, ITS2 alignment consisted of 77 sequences. The new rDNA sequences derived from this study were deposited in the EMBL/GenBank/DDBJ databases (Table 1) with the accession numbers AM268192–AM268203, AB047302–AB047309, and AB259840–AB259846. The alignments are deposited at the EMBL database (http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-id+2Mjb01SgNM8+EMBLALIGN) under accession number ALIGN_001112 and ALIGN_001113.

From the aligned SSU rDNA dataset 1704 sites and for the ITS rDNA dataset 339 sites could be used for the phylogenetic

Table 1 – Sequences and cultures used in this study

Species and culture identity	Voucher/culture-no.	Clones	Sequences used
Outgroup SSU rDNA sequences from the database:			
Paraglomus brasilianum BR105	W3086/Att260-4		AJ301862
P. occultum CL700			AJ006798
P. occultum IA702-3			consensus AJ276081–82, DQ322629
P. occultum HA771			AJ006799
***Archaeosporales* SSU rDNA sequences**			
Ambispora callosa OK1 MAFF520057 (ss, gl)	W4769/Att1323-7	OK11-13	consensus AB047305–07
A. callosa V1 MAFF520058 (ms, gl)	W4771/Att321-10	V111,17	consensus AB047308–09
A. fennica (ms, ac)	W3569/Att200-11	pFD36-3-3	AM268192
A. fennica (ms, ac)	W3569/Att200-11	pFD98-1	AM268193
A. fennica (ss, ac)	W3847/Att200-21	pWD265-1-1	AM268194
A. fennica (ms, gl)	W3570/Att200-11	pFD35-6	AM268195
A. fennica (ss, gl)	W4752/Att200-23	pFD120-2	AM268196
A. gerdemannii AU215			AJ012202
A. leptoticha F3b MAFF520055 (ss, ac)	W4770/Att315-11	F3b4,21,23	consensus AB015052, AB047302–04
A. leptoticha NC176			consensus AJ301861, AJ006466, AJ006794–97
A. leptoticha FL130			AJ006793
A. leptoticha WL1			AB220172
Archaeospora trappei	W3179/Att186-1		Y17634
A. trappei AU219			AJ006801
A. trappei NB112			AJ006800
Geosiphon pyriformis GEO1	W3619/Att754-6		consensus X86686, Y17831, Y15904–05, AJ276074, AM183923
***Archaeosporales* ITS-region rDNA sequences**			
Ambispora callosa OK-m (ss, gl)	W4768/Att1321-4	OK-m18e	AB259840
A. callosa OK-m (ss, gl)	W4768/Att1321-4	OK-m18f	AB259841
A. callosa OK-m (ss, gl)	W4768/Att1321-4	OK-m18i	AB259842
A. callosa OK-m (ss, gl)	W4768/Att1321-4	OK-m8	AB259843
A. callosa HZ-6K (ss, gl)	W4772/Att1322-4	HZ-6K2	AB259844
A. callosa HZ-6K (ss, gl)	W4772/Att1322-4	HZ-6K7	AB259846
A. callosa HZ-6K (ss, gl)	W4772/Att1322-4	HZ-6K5	AB259845
A. fennica (ss, gl)	W4752/Att200-23	pFD120-6	AM268203
A. fennica (ms, gl)	W4752/Att200-23	pFD120-13	AM268198
A. fennica (ms, gl)	W3569/Att200-11	pFD120-7	AM268199
A. fennica (ms, gl)	W3569/Att200-11	pFD120-10	AM268197
A. fennica (ms, ac)	W3569/Att200-11	pFD114-12	AM268200
A. fennica (ms, ac)	W3569/Att200-11	pFD114-5	AM268201
A. fennica (ms, ac)	W3569/Att200-11	pFD114-6	AM268202
A. leptoticha F3b (ss, ac, gl)	W4770/Att315-11	F3bA15,A25-26,A32,A313, A411,A414,A43,A49,A51, G1,G8,G12-15,G17,A311+G16 (identical),A16+A36+A54 (identical),G18+G3 (identical), G7+G9 (identical)	AB048630, AB048632–33, AB048635–36, AB048638–42, AB048644–48, AB048650, AB048654, AB048634+AB048649 (identical), AB048631+AB048637+AB048643 (identical), AB048651–52 (identical), AB048653+ AB048655 (identical)
A. callosa OK1 MAFF520057 (ss, gl)	W4769/Att1323-7	OK113,17,33,222,313, 322+44 (identical), 38+411 (identical), 41,415,418,42, 320+321 (identical)	AB048656–59, AB048663, AB048662+ AB048670 (identical), AB048664+ AB048666 (identical), AB048665, AB048667, AB048668, AB048669, AB048660+AB048661 (identical)
A. callosa V1 MAFF520058 (ms, gl)	W4771/Att321-10	V113-15,21-23,32-34,422-24	AB048671–82
A. gerdemannii AU215			AJ012111
A. leptoticha NC176			AJ012109, AJ012110
A. leptoticha FL130			AJ012201
A. sp. from *Plantago lanceolata* roots		2v34.2	AJ567807
A. sp. from *Prunus africana* roots		Pa110+111+112 (identical)	AY236277–79 (identical)
A. sp. from *Taxus baccata* roots		201, 208, 210, 214, 224, 216+212+217 (identical)	AY174701–3, AY174707, AY174710, AY174705+AY174708–09 (identical)

Spore morphs are indicated as acaulosporoid (ac) or glomoid (gl) and cultures as single spore isolate (ss) or multi spore culture (ms).

analyses. PHYLIP 3.6a3 (Felsenstein 1989) was used for NJ, ML and MP analyses (Kumar & Gadkar 2000) and consensus tree computations. ML quartet puzzling (MLQP) analyses were performed with TREE-PUZZLE 5.2 (based on HKY, KN, as well as TN models; gamma distributed heterogeneous rates were estimated from the dataset). Although having a different base, QP analysis support values can be interpreted in much the same way as BS values. Transition:transversion ratios (1.8 for SSU; 1.65 for ITS) and nucleotide frequencies were estimated from the dataset with TREE-PUZZLE. The input order of species was always randomised. For the SSU rDNA and ITS region phylogenies, majority rule consensus trees were constructed from 1000-fold bootstrapped NJ (based on distances computed with F84 parameters) or MP analyses and 100-fold bootstrapped ML analyses. Trees were viewed, assembled and edited with the programs TREEME (http://science.do-mix.de/software.php), TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) and Microsoft PowerPoint.

Results

Molecular phylogenetic analyses

As expected due to the intraspecific sequence variability of AMF, many sequences of clones (derived from a single spore) were not identical but closely related (Figs 1–2). Clones that resulted in identical sequences were submitted as one sequence only, with the exception of SSU rDNA sequences AM268193 and AM268193, which were identical but derived from different spores with different morphs (but from the same culture) and were therefore submitted individually.

SSU rDNA analyses

NJ, MP, ML and MLQP analyses (Fig 1) together with the archaeosporalean near full-length sequences available in the databases revealed that the sequences from *Ambispora fennica*, derived from spores of both morphs, cluster within the *Archaeosporales* with high support. All phylogenetic methods applied gave the same result, showing that *A. fennica* belongs in a sister clade of the *Geosiphonaceae*, together with *Archaeospora gerdemannii*, *A. leptoticha*, and *Glomus callosum*. Based on the SSU rDNA, *Ambispora fennica* can be separated from the other species with good support. For the principal topology there was no difference among MLQP, NJ, ML, and MP trees. The distances shown in the phylogenetic trees were derived from a TREE-PUZZLE MLQP analysis.

ITS region and 5.8 S rDNA analyses

The ITS1/ITS4 primer set used in Japan amplified DNA fragments of approximately 570 bp in length. The region sequenced in Germany ranged from 800 bp (partially sequenced) up to 1100 bp. Phylogenetic analysis (Fig 2) of the new sequences (Table 1) together with sequences from *Archaeospora leptoticha* NC176, FL130, *A. gerdemannii* AU215, and from *Prunus africana*, *Taxus baccata* (Wubet *et al.* 2003a, 2003b) and *Plantago lanceolata* (Börstler *et al.* 2006) mycorrhizas showed that the related species could be divided into two subgroups with good support. The sequences from *A. leptoticha* FL130 (AJ012101), NC176 (AJ012109–AJ012110) and F3b (Japanese isolate) generated an individual clade. The two different spore morphs were sequenced separately for F3b, and neither morph could be separated into subclades in the analysis. The sequences of *Glomus callosum* HZ-6K, OK1, OK-m, and V1 formed another cluster, also not separating into subclades. The *A. gerdemannii* sequence (AJ012111) (Redecker *et al.*

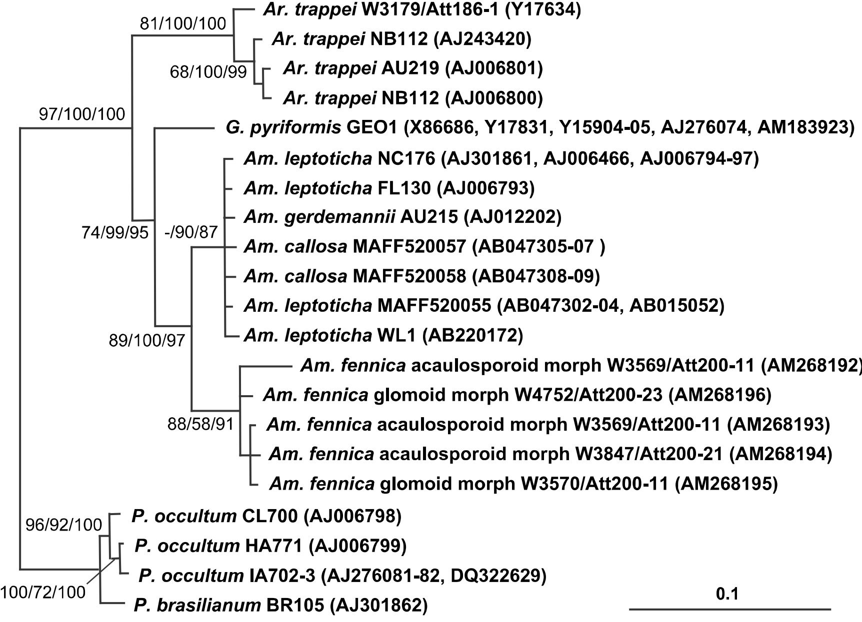


Fig 1 – Phylogenetic tree (SSU rDNA) of the *Archaeosporales* with *Paraglomerales* as outgroup. The support values shown at the branches are from MLQP/NJ (1000 BS)/ML (100 BS) analyses. Distances are derived from a MLQP analysis.

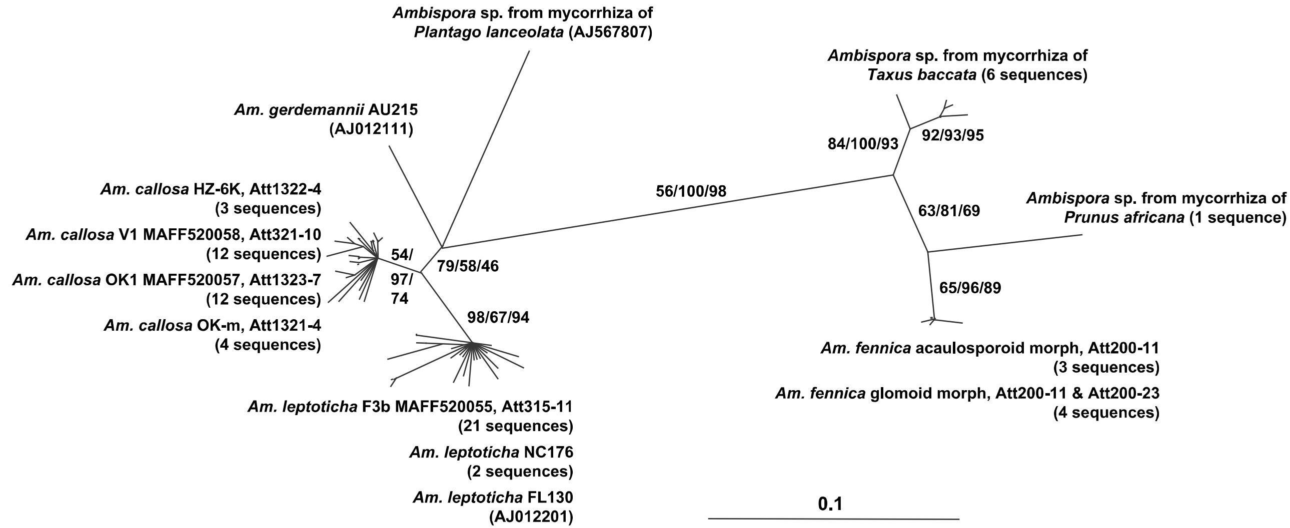


Fig 2 – Phylogenetic tree (ITS region rDNA, unrooted) of the sequences representing *Ambispora* gen. nov. (*Ambisporaceae*, *Archaeosporales*). The support values shown at the branches are from MLQP/NJ (1000 BS)/MP (100 BS) analyses. Distances are derived from a MLQP analysis. Sequence AJ567807 was originally assigned to the species *Archaeospora leptoticha* in the public database. The numbers of individual sequences included in the analysis (identical sequences were excluded) are shown.

2000b) is separated from both clades, but with relatively low BS support. The same is true for a sequence (AJ567807) from a *P. lanceolata* root that was annotated as *A. leptoticha* (Börstler *et al.* 2006). A better sequence sampling perhaps could resolve this situation of low support, but it seems that these sequences represent separate taxa, probably at the level of species.

Based on the ITS region phylogeny, *Ambispora fennica* is clearly separated from the defined *Archaeospora gerdemannii* (AU215) and *A. leptoticha* isolates, and also from *G. callosum*, but relatively closely related to sequences from *T. baccata* and *P. africana* roots. *Ambispora fennica* therefore belongs in a monophyletic clade together with *Archaeospora gerdemannii*, *A. leptoticha*, *G. callosum*, and *Geosiphon pyriformis* (*Geosiphonaceae*) to which the clade containing *A. trappei* represents a sister lineage (Fig 1). *A. trappei* is the type species of *Archaeospora* and the *Archaeosporaceae*. The genus *Archaeospora* and the family *Archaeosporaceae sensu* Morton and Redecker consequently are paraphyletic.

Morphological studies

Having established the need for new taxa to rationalise the systematic position of the organisms within the *Archaeosporales*, the morphological characteristics were assessed for comparison with existing species. The lengths and widths of spores measured, and similar information from published descriptions, are listed in Table 2. Our results are mainly incorporated into the new descriptions and new combinations in the taxonomic section below. We did not see germination of any of the spores examined, though specific germination studies were not undertaken.

Ambispora fennica *sp. nov. (Fig 3)*

We concluded, from the measurements, wall structure, and morphological details of the Finnish fungus (from Att200), that it was undescribed, and describe it herein as *Ambispora fennica* sp. nov.

The spores of *A. fennica* were either glomoid or acaulosporoid (Fig 3A). The latter were hyaline to white at first, becoming pale ochraceous with age (Fig 3, A–B). Each spore was formed on a large (199–248 μm diam) saccule (Fig 3C) that collapsed and became detached at maturity. Some spores were sessile (Fig 3E), but others had a persistent pedicel, similar to the subtending hypha of a *Glomus* spore (Fig 3D, I). The wall structure was complex, consisting of three groups (Fig 3F–K). Group 1 is formed by budding of the saccule neck, and consists of two components, a granular, evanescent outer component, often with adherent fine soil particles, overlaying a semi-flexible, hyaline to lightly pigmented (probably laminated) component. Component 1 reacts rapidly to become pink or red in Melzer's reagent (Fig 3I–K). Wall group 2 appears to develop separately and consists of a single, hyaline component that splits and fractures on crushing (Fig 3H–J), slightly yellowing in Melzer's reagent. Wall group 3 also develops independently and is of a single, relatively thick, semi-flexible component.

The glomoid spores were hyaline and varied in shape, from globose to subglobose to ellipsoid, with occasional obovoid or irregular specimens (Fig 3L–N). The wall structure was of two components in a single group. The outer component was thin, and often difficult to see, but with fine soil particles adherent (Fig 3L), indicating that it was sticky. The second component was finely laminated, and pliable, making it difficult to crush spores on microscope slides to see the detail of wall structure. Many of these spores were open-pored (lacking any occlusion), but where they were closed, it was by the formation of a septum from the laminated inner wall component (Fig 3N). They did not react to Melzer's reagent.

Mycorrhizas stained weakly in ink, producing arbuscules and hyphal coils and occasional vesicles (Fig 3O).

Table 2 – Lengths and widths of spores (μm) in the *Ambisporaceae* from new measurements (with voucher numbers) and published measurements

Identifier/voucher or source	Min length	Max length	Min width	Max width	Mean length	Mean width	S.D. length	S.D. width	No. measured
Glomoid spores									
Ambispora callosa OK-m/W4768	77	319	68	291	223	213	37.4	35.4	100
A. callosa OK1/W4769	87	245	95	257	174	172	29.2	30.0	100
A. callosa V1/W4771	84	316	84	270	172	170	40.4	36.9	100
A. callosa HZ6-K/W4772	93	270	93	248	192	186	32.7	28.4	100
Glomus callosum type/W4819	112	285	108	288	222	223	27.5	28.4	104
G. callosum/protologue[a]	84	319	84	300	–	–	–	–	–
G. leptotichum/protologue[b]	48	262	48	262	–	–	–	–	–
G. fecundisporum/protologue[b]	60	155	60	207	–	–	–	–	–
G. leptotichum type/W945	62	257	62	254	154	151	47.4	47.3	63
Ambispora leptoticha F3b/W4770	45	215	42	213	106	102	47.8	46.2	69
Archaeospora leptoticha/combination[c]	60	250	60	250	–	–	–	–	–
A. gerdemannii AU215A/combination[c]	40	120	40	120	80	80	–	–	142
Ambispora fennica sp. nov. glomoid/W4816	38	130	38	117	71	73	14.8	15.5	100
Acaulosporoid spores									
G. gerdemannii/protologue[d]	140	198	149	230	–	–	–	–	–
Archaeospora gerdemannii AU215A/ combination[c]	160	260	160	260	212	212	–	–	110
Acaulospora gerdemannii/protologue[e]	200	250	200	250	–	–	–	–	–
Archaeospora leptoticha/combination[c,g]	>160	>200	>160	>200	–	–	–	–	–
Acaulospora appendicula/protologue[f]	170	390	170	390	–	–	–	–	–
Ambispora leptoticha F3b/W4770	195	288	186	298	236	235	18.0	19.6	100
A. fennica sp. nov./W4815	124	201	134	201	168	166	14.0	13.5	100

a Sieverding (1988); b Schenck & Smith (1982); c Morton & Redecker (2001); d Rose *et al.* (1979); e Nicolson & Schenck (1979); f Schenck *et al.* (1984); g Only dimensions for spores after loss of outer wall components published. For types the basionym and for other published work the name used in the relevant papers are given.

Ambispora leptoticha *(Fig 4)*

Our Japanese isolate produced acaulosporoid and glomoid spores within the size range given for *Acaulospora appendicula* by Schenck *et al.* (1984) (Fig 4). From the molecular evidence, we move this species from *Archaeospora* to *Ambispora* as *A. leptoticha* comb. nov. (see below). Mycorrhizas stained weakly in trypan blue. They had arbuscules, hyphal coils, and occasional vesicles (Fig 4O).

Ambispora callosa *(Figs 5–6)*

All collections formed only glomoid spores, singly or in loose clusters in the soil, and there were neither sporocarps nor dense clusters of spores. There was no evidence of spores produced in roots. Spore dimensions from formaldehyde-preserved type material (Table 2) had a slightly narrower range than was given in the species description. Measurements of the Japanese material all were within the range for the species.

The wall structure of all the *Glomus callosum* material examined was interpreted differently from the protologue description. It consists of an outer, somewhat granular, evanescent component, overlaying a laminated component that has a tendency to be loosely adherent towards the interior of the spore Figs 5–6N–O). The Japanese fungi studied here could not be distinguished morphologically from *G. callosum*. The molecular evidence shows them to belong to *Ambispora*, and so we transfer the species as *A. callosa* comb. nov. Mycorrhizas stained weakly in trypan blue and ink, and no vesicles were observed.

Taxonomy

Archaeosporaceae J. B. Morton & Redecker 2001.

Differs from other families in the *Glomeromycota* by possession of small, hyaline spores formed laterally or centrally within the neck of a sporiferous saccule, that lack any distinctive reaction to Melzer's reagent. With rDNA phylogeny (Figs 1–2) and sequences differing from those of members of the *Geosiphonaceae* and *Ambisporaceae*.

Included genera:

Typus: *Archaeospora* J. B. Morton & Redecker 2001.

Intraspora Oehl & Sieverd (2006).

Archaeospora J. B. Morton & Redecker 2001.

Typus: *Archaeospora trappei* (R. N. Ames & Linderman) J. B. Morton & Redecker 2001.

Spores formed in the soil, rarely in roots, in the neck of a sporiferous saccule, hyaline, globose to subglobose to irregular, tending to remain suspended in water when extracted from substrate. Lacking amyloid or dextrinoid reaction to Melzer's reagent. Forming faintly staining arbuscular mycorrhizas.

Ambisporaceae C. Walker, Vestberg & Schuessler, **fam. nov.**
MycoBank no.: MB 510208

A familiis aliis in Archaeosporalibus ob sporas glomoideas, acaulosporoideas vel ambas formans et a characteribus rDNA differt.

Typus: *Ambispora* C. Walker, Vestberg & Schuessler 2007.

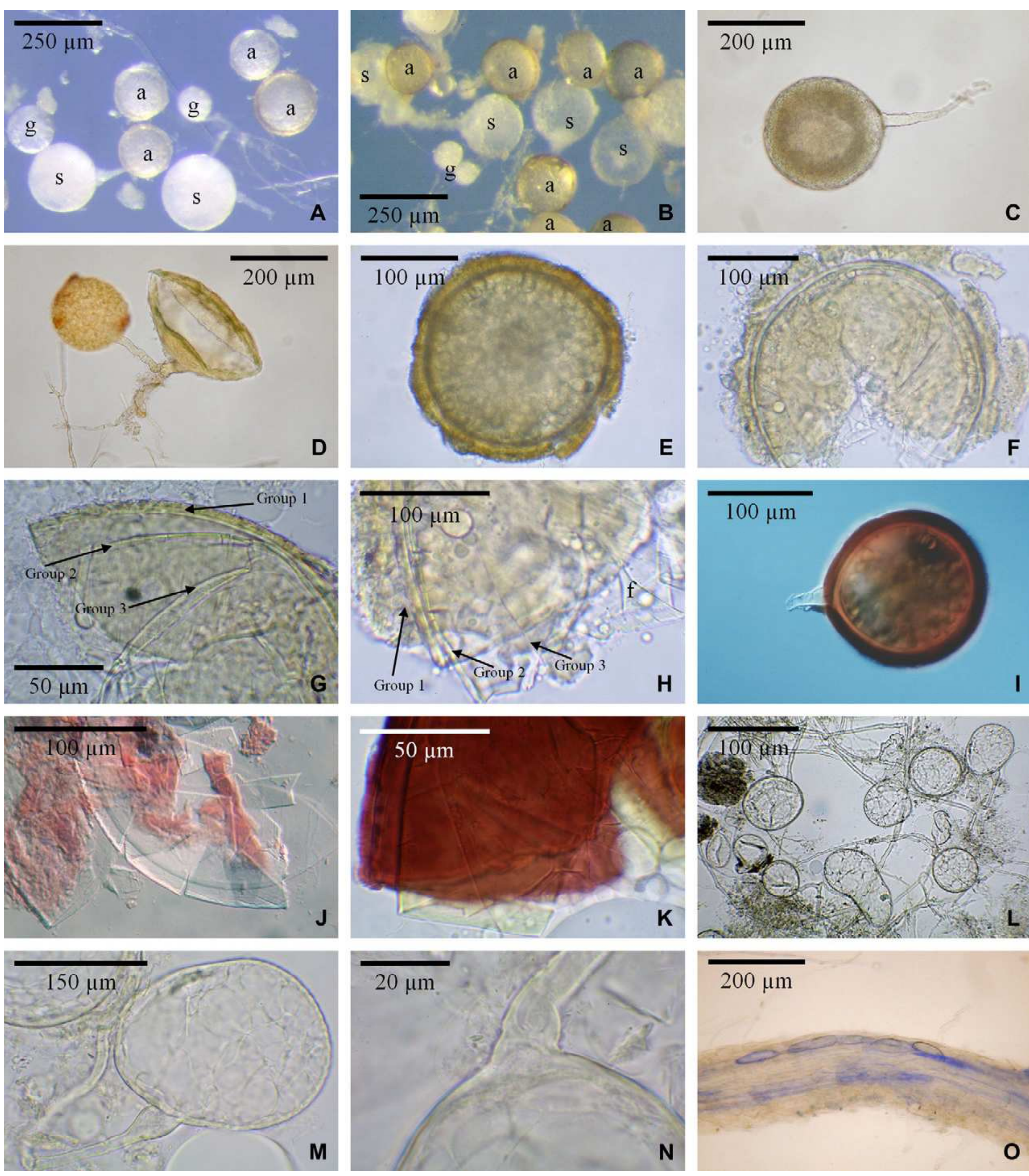


Fig 3 – Spores of *Ambispora fennica* from various subcultures of Att200 with single spore origin (brightfield microscopy except I and J, which are Nomarski DIC images). A–B. Spores in water. Saccules (s), acaulosporoid spores (a), and glomoid spores (g). A. The saccules still have their white contents, and one acaulosporoid spore is showing some pigmentation, though most are immature and lack pigmentation. B. Mature acaulosporoid spores are more pigmented and the saccules are empty. C. Saccule without development of an acaulosporoid spore. D. Collapsed saccule with the acaulosporoid spore attached by a long pedicel, giving the erroneous impression of a glomoid spore. E. Acaulosporoid spore detached from the saccule and lacking any pedicel. F. Acaulosporoid spore crushed in PVLG. The evanescent outer component has fragmented on crushing. G–H. Wall structure of acaulosporoid spores crushed in PVLG, showing the wall groups. G. Component 1 has either not yet developed or has disintegrated. H. Granular nature of component 1 (left), and the fractures (f) in component 4 (right). I–K. Reaction of acaulosporoid spores to PVLG with Melzer's reagent. Note pedicel in I, giving the spore a glomoid appearance. Component 1 reacts to become pink or red. The other components do not react, other than a slight yellowing in component 3. L–N. Glomoid spores in a loose cluster (L) showing different shapes, and singly showing funnel-shaped subtending hypha (M) and occlusion by formation of a septum from the laminated component (N). O, mycorrhiza stained with trypan blue showing vesicles.

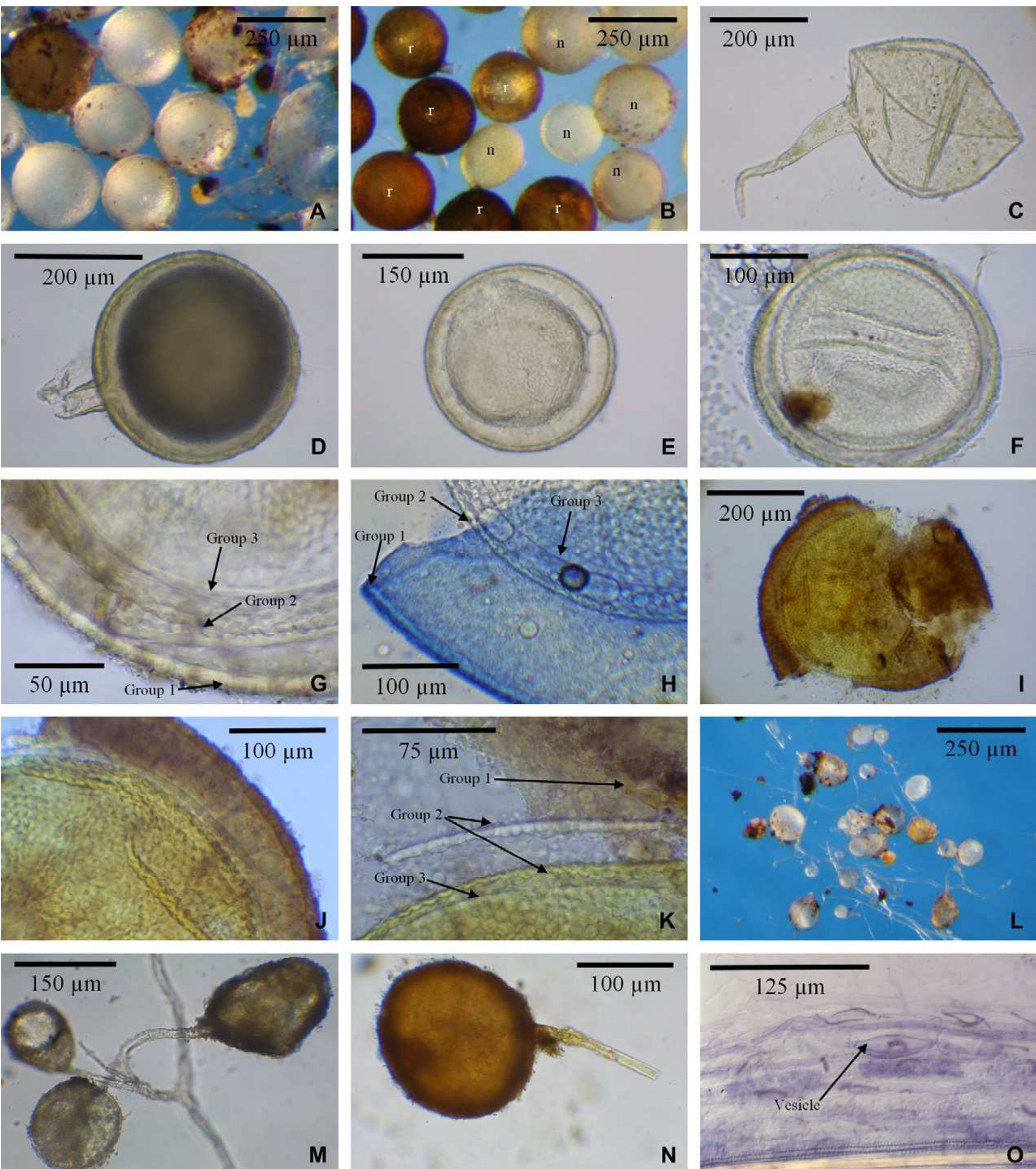
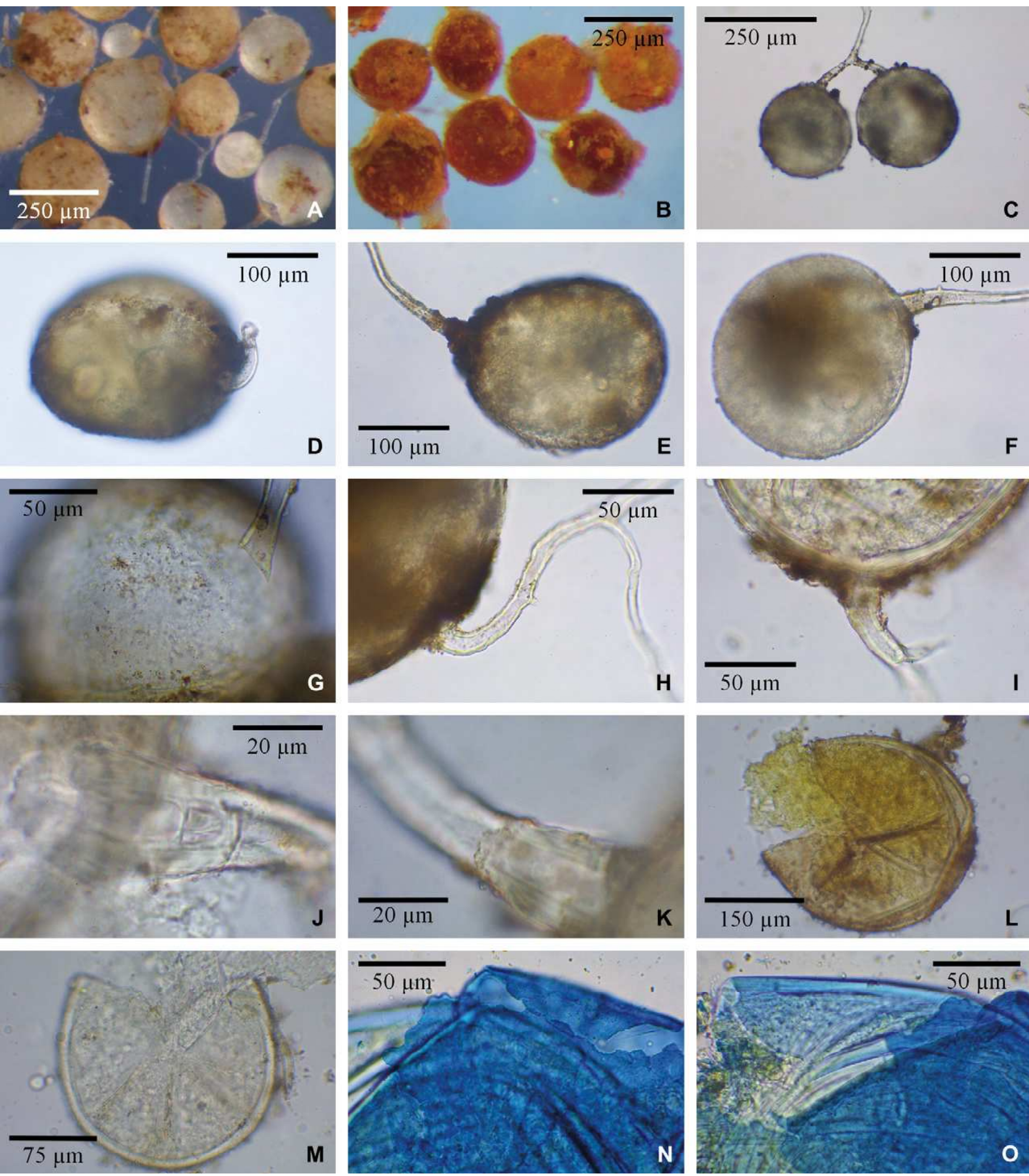
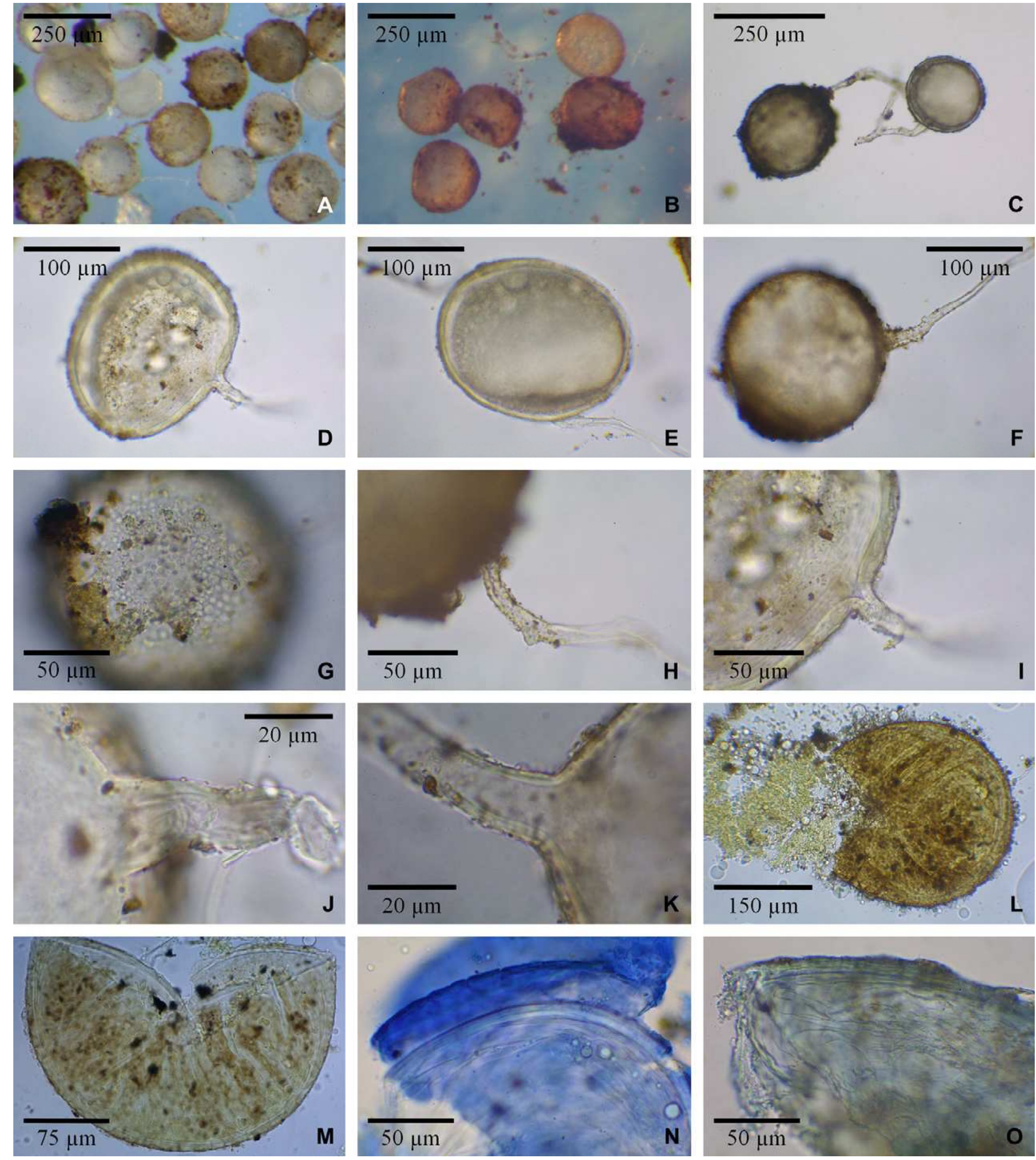


Fig 4 – Spores of *Ambispora leptoticha* from Att315-11, of single spore origin (brightfield microscopy). A–K. Acaulosporoid spores. L–O. Glomoid spores. A. Spores in water showing the range of pigmentation. The majority of spores are white to ivory, but some may be coloured due to age. B. Spores before (n) and after (r) immersion in Melzer's reagent. C. A collapsed, empty saccule after detachment of the spore. D–E. Intact spores with (D) and without (E) a pedicel. F. Wrinkling of a freshly squashed spore resulting from the pliable nature of the wall components. The scalloped nature of the components in group 2 appears as ornamentation. G. Lightly crushed spore showing wall groups. The two interlocking, scalloped components of group 2 have separated slightly at lower left. H. Spore crushed in PVLG with blue ink, showing the staining of the outer wall components. The components of wall groups 2 and 3 are only lightly stained. I. Reaction to Melzer's reagent of a lightly crushed spore, showing the rust-brown reaction of the outer wall group. J–K. Spores crushed in PVLG with Melzer's reagent, showing the brown reaction of outer components, and the yellow reaction of the innermost scalloped component of group 2. L. Glomoid spores, showing the large size range and the variation in colour. M. A cluster of three spores with adherent soil particles. N. A spore reacting to become brown after immersion in Melzer's reagent. O. Mycorrhiza stained with trypan blue showing a vesicle.



Figs 5–6 – Comparison of spores of the type material (Fig 5) of *Ambispora callosa* (basionym *Glomus callosum*) and Japanese isolate from Att1323-7 of single spore origin (Fig 6) (brightfield microscopy except J, which is a Nomarski DIC image). A. Spores in water showing the attached soil particles on some spores giving an erroneous impression of pigmentation. B. Spores in water after immersion in Melzer's reagent. Note the change in colour. C. Pair of attached spores, uncrushed in PVLG. D–F. Individual spores, uncrushed in PVLG, showing differences in shape. G. Surface view of a spore in PVLG, showing the false impression of a warty ornamentation due to the granular nature of the outer wall component and attached fine soil particles. H–K. Subtending hyphae, showing shape differences (cylindrical to funnel-shaped) and occlusion by formation of a septum. L. Spore crushed in PVLG with Melzer's reagent, showing the rust-coloured reaction of the outer wall component, the yellow reaction of the contents exuded through the break, and the creasing resulting from the pliable nature of the wall components. M. Spore crushed in PVLG, also showing creasing. N–O. Wall structure of spores in PVLG with blue ink, showing the outer component staining more heavily than the laminated inner component. O. The loose, laminated nature of component 2 is evident, giving an erroneous impression of individual inner flexible components.



Figs 5–6 (*continued*)

Arbuscular mycorrhizal fungi forming glomoid spores, acaulosporoid spores, or both glomoid and acaulosporoid spores. *Glomoid spores* almost lacking in pigmentation, and with a soft, pliable nature resulting in wrinkling and resistance to fracturing when crushed. *Acaulosporoid spores* formed in the neck of a hyaline to subhyaline to whitish sporiferous saccule, often but not exclusively on a short pedicel resulting in a glomoid appearance once detached from the collapsed saccule. Separated from other families in the *Archaeosporales* by its rDNA characteristics (Figs 1–2).

Ambispora C. Walker, Vestberg & Schuessler, **gen. nov.**
MycoBank no.: MB 510209

Etym.: Latin '*ambispora*' referring to the capability of some organisms in the genus to produce two different kinds of propagules, acaulosporoid and glomoid.

Sporae alterutrae glomoideae, acauosporoideae vel ambae. A generibus aliis in Archaeosporalibus differt a ordinatione SSU rDNA sequenti: CAAAACCAATCTCGTCTTCGGGC.

Typus: *Ambispora fennica* C. Walker, Vestberg & Schuessler, 2007.

Spores formed in the soil, either as glomoid spores only, or both acaulosporoid and glomoid spores. *Glomoid spores* formed singly in the soil or in loose clusters, pliable, differing from the normally brittle nature of such spores in the genus *Glomus*; open-pored or sealed by a septum formed from the inner wall component. *Sporiferous saccule* initially similar in appearance to the glomoid spores, with a soft, pliable nature, formed blastically from a hyphal tip, and becoming sealed by a septum. *Acaulosporoid spores* formed, usually laterally, in the neck of the sporiferous saccule; spore wall structure complex is of two or three groups. Outermost wall group continuous with the saccule wall, enclosing a distinct and separate entity with a wall structure of more than one flexible component in two groups. Forming arbuscular mycorrhizas. Separated from other genera in the *Archaeosporales* by its rDNA characteristics (Figs 1–2), e.g. the SSU rDNA sequence motif CAAAACCAATCT CGTCTTCGGGC.

Ambispora fennica C. Walker, Vestberg & Schuessler, **sp. nov**. (Fig 3)

MycoBank no.: MB 510210

Etym.: *'fennica'* (of Finland) referring to the country in which the species was first isolated.

Sporae acaulosporoideae et glomoideae ambae formantes. Sporae acaulosporoideae 124–201 × 134–201 μm, hyalinae vel pallide ochaceae, turmis tribus parietum: turma prima partibus duabus, pars exterior asperula; turma secunda parte una, in solutione Melzeri lutescenti; turma tertia usque ad 3 μm crassa. Sporae glomoideae 38–130 × 38–117 μm, turma una partibus duabus: paries primus <1 μm crassus; paries secundus usque ad 3 μm crassus, laminatus.

Typus: **Finland**: *Kurikka*, 62° 30′ N; 22° 20′ E, from a pot culture on *Plantago lanceolata*, 7 Jun. 2002, *C. Walker* W3887 (E–holotypus)

Acaulosporoid spores formed singly in the soil, laterally in the neck of a sporiferous saccule that collapses and may detach at maturity; hyaline to pale ochraceous, globose to subglobose to pyriform, 124–201 × 134–201 μm ($n = 100$), attached to the saccule [199–248 μm diam ($n = 15$)] by a slightly raised collar or by a thickened stalk. *Wall* components in three groups. Wall group 1 with two adherent components, with a roughened, granular outer component (up to 4 μm thick) that tends to disintegrate with time, overlaying a semi-rigid, inner structural component up to 4 μm thick. Outermost component expanding to up to 15 μm thick on crushing in PVLG, reacting to Melzer's reagent to become rust-brown or red; wall group 2 apparently with a single component, relatively thick (up to 4 μm) and more or less rigid, fracturing and splitting on crushing, becoming yellow in Melzer's reagent. Wall group 3 thin, semi-flexible, up to 3 μm thick. *Glomoid spores* 38–130 × 38–117 μm ($n = 100$), with a thin, wall of two components in a single group. Outer component evanescent, <1 μm thick, adherent to an inner component that is up to 3 μm thick and laminated. Subtending hypha rarely cylindrical, normally funnel-shaped, often with a slight constriction at the spore base. Pore open or occluded by a distal septum formed by the laminated wall component. Separated from other species in the *Archaeosporales* by its rDNA characteristics (Figs 1–2), e.g. the SSU rDNA sequence motif GGAGAGTCGGCATGTCCTTTGTTGGGTGTGCC.

Additional specimens examined: **Finland**: *Kurikka*, from a pot culture with *Plantago lanceolata* established with several glomoid spores taken from a trap pot culture with *Trifolium repens* started with soil from the root zone of pasture grasses and possibly *T. pratense*, 1 Nov. 1991, *M. Vestberg* (Att200-0, W1586) (E); *loc. cit.* from a pot culture with *P. lanceolata* of single acaulosporoid spore origin, 6 Feb. 2002, *M. Vestberg* [Att200-8, W3011 (acaulosporoid morph) and W3012 (glomoid morph), 6 Feb. 1998] (E); *loc. cit.* from a pot culture with *P. lanceolata* of single acaulosporoid spore origin, 14 Jan. 2001, *C. Walker* [Att200-11, W3570 (acaulosporoid morph) and W3569 (glomoid morph)] (E); *loc. cit.* from a pot culture with *P. lanceolata* of single acaulosporoid spore origin, 27 Feb. 2002, *C. Walker* [Att200-21, W3847 (both morphs)] (E);; *loc. cit.* from a pot culture with *P. lanceolata* of single acaulosporoid spore origin, 28 Sep. 2005, C. Walker [Att200-23, W4752 (both morphs), 7 Jun. 2002] (E).

Ambispora gerdemannii (S. L. Rose, B. A. Daniels & Trappe) C. Walker, Vestberg & Schuessler, **comb. nov.**

MycoBank no.: MB 510211

Basionym: *Glomus gerdemannii* S. L. Rose, B. A. Daniels & Trappe, *Mycotaxon* **8**: 297 (1979).

Synonym: *Archaeospora gerdemannii* (S.L. Rose, B.A. Daniels & Trappe) J. B. Morton & Redecker, *Mycologia* **93**: 186 (2001).

A species description can be found in Morton & Redecker (2001). Separated from *Archaeospora trappei* (*Archaeosporaceae*) and other species in the genus *Ambispora* by its rDNA characteristics (Figs 1–2).

Ambispora leptoticha (N.C. Schenck & G.S. Sm.) C. Walker, Vestberg & Schuessler, **comb. nov.** Fig 4

MycoBank no.: MB 510212

Basionym: *Glomus leptotichum* N. C. Schenck & G. S. Sm., *Mycologia* **74**: 82–83 (1982).

Synonyms: *Acaulospora gerdemannii* N. C. Schenck & T.H. Nicolson, *Mycologia* **71**: 193 (1979).

Acaulospora appendicula Spain, Sieverd. & N. C. Schenck, *Mycologia* **76**: 686 (1984).

Glomus fecundisporum N.C. Schenck & G.S. Sm. *Mycologia* **74**: 81 (1982).

Archaeospora leptoticha (N.C. Schenck & G.S. Sm.) J. B. Morton & Redecker, *Mycologia* **93**: 184 (2001).

A species description can be found in Morton & Redecker (2001). Separated from *Archaeospora trappei* (*Archaeosporaceae*) and other species in the genus *Ambispora* by its rDNA characteristics (Figs 1–2).

Specimens examined: **Japan**: *Tochigi*, Nishinasumo, Fujinitayama, from a pot culture with *Sorghum bicolor, T. repens, M. sativa* and *Dactylis glomerata*, established with soil sievings containing roots of *D. glomerata* and *Zoysia sinensis*, 1 Nov. 1994, *T. Murakoshi* (Att315-0, W1974) (E); *loc. cit.* from a multi-spore pot culture established with *S. sudanense*, *T. repens* and *Miscanthus sinensis* from a pot culture with Att315-0 in its ancestry, 1 Dec. 1996, *M. Saito*, culture F3b, MAFF520055, (Att315-4, W2875) (E) and from a subsequent pot culture, 31 Jan. 2005, *M. Saito* (Att315-11, W4770) (E).

Ambispora callosa (Sieverd.) C. Walker, Vestberg & Schuessler, **comb. nov.** Figs 5–6

MycoBank no.: MB 510213

Basionym: *Glomus callosum* Sieverd., *Angew. Bot.* **62**, 374 (1988).

Sporocarps unknown. *Spores* sub-hyaline to white to ivory, yellowing somewhat with age or storage in preservative, borne singly or in loose clusters in the soil. Acaulosporoid spores unknown. *Glomoid spores* globose to subglobose to broadly

ellipsoid, sometimes ovoid or obovoid, sometimes eccentrically produced from the subtending hypha; very variable in size, 84–319 × 84–300 μm, often with immature (open-pored) spores closely attached to mature (occluded) spores. Subtending hypha straight to funnel-shaped, open-pored or occluded by a septum formed from the laminated wall component. Wall structure continuous with that of the sporogenous mycelium, consisting of two components, an inner, laminated structural component and an outer, unit component, roughened on the surface, which may disintegrate with time. Spores sometimes covered by a sticky mucilaginous coat, appearing as a separate wall component. Adherent fine soil particles giving the impression of pigmentation. Lacking brittle or rigid wall components. Components pliable and thus creasing and rather difficult to break open on crushing. In Melzer's reagent, or PVLG with Melzer's reagent, spores rapidly becoming deep yellow to orange, the reaction being mostly in the roughened outer wall component and the mucilaginous component. Melzer's reaction fading within a few days of preparation of PVLG–Melzer's slide mounts. Separated from the *Glomerales* but not the *Archaeosporales* at the ordinal level, and from *Archaeospora trappei* at the family and genus levels by rDNA nucleotide sequence data (Figs 1–2). Differing from other described members of the *Ambisporaceae* by production only of glomoid spores.

Specimens examined: **Japan**: *Sakurajima Island*, Kagoshima, from a subculture with *T. repens* with a single spore isolate (Att1323-5, host unknown) in its lineage, 3 Jan. 2006, *M. Saito*, isolate OK1, MAFF520057, (Att1323-7, W4769) (E); Morioko, Iwate, Tohoku National Agricultural Experiment Station, from a subculture with a multi spore culture (Att321-0, on *S. bicolor*) in its lineage, 31 Jan. 2005, *M. Saito* culture V1, MAFF520058 (Att321-1 on *T. repens*, W1980 and Att321-9 with *P. notatum* and *T. repens*, W4771) (E); *Hokkaido*, Sapporo, from a subculture with *P. notatum* with a single spore isolate (Att1322-2, host unknown) in its lineage, 7 Dec. 2004, *M. Saito*, isolate HZ-6K (Att1322-4, W4772) (E); Miyakejima Island, grassland (predominantly *M. sinensis*) on volcanic soil, from a subculture with *P. notatum*, with a single spore isolate (Att1321-2, host unknown) isolate OK-m in its lineage, 7 Dec. 2004, *M. Saito*, (Att1321-4, W4768) (E) — **Zaire**: *South Kivu Province*, near Bukavu, Agricultural School Mushweshwe, from a multi-spore subculture with *Sorghum bicolor*, 15 May 1988, *E. Sieverding*, culture GTZ-21, (Att1324-0, W4819; Holotype; GOET).

Discussion

Molecular phylogenetic analyses

Our molecular and morphological data and the sequences from roots indicate a substantial diversity within this deeply branched glomeromycotan lineage, the *Archaeosporales*, providing further phylogenetic and taxonomic insights into this poorly studied group of AM fungi. The formation of spores within the neck of a sporiferous saccule is shared by members of the genus *Acaulospora*, which is genetically very distant from the *Archaeosporales*. Analysis of SSU rDNA phylogeny showed the *Archaeosporaceae sensu* Morton & Redecker (2001) to be composed of three clades, one containing *Archaeospora trappei* and the second containing *A. leptoticha* and *A. gerdemannii*. The latter forms a sister clade with *Geosiphon pyriformis* (*Geosiphonaceae*), rendering the *Archaeosporaceae* paraphyletic (Schüßler *et al.* 2001). No molecular analysis of *Intraspora* (Sieverding & Oehl 2006) is available, but its morphological and developmental characteristics suggest that *I. schenckii* is likely to belong in the same clade as *A. trappei*, and therefore can remain in the *Archaeosporaceae*.

The analysis of SSU rDNA shows that three main clades, resolved at the taxonomic level of family, can be defined within the *Archaeosporales*: the *Geosiphonaceae*, the *Archaeosporaceae*, and the *Ambisporaceae*. *Ambispora gerdemannii* clearly is related to *A. fennica* (Fig 1) and is non-monophyletic (family level) with *Archaeospora trappei* (*Archaeosporaceae*). An alignment of ITS region sequences between the *Ambisporaceae*, the *Geosiphonaceae*, and the *Archaeosporaceae* is not possible due to the large molecular distances. Based on the ITS region, the species within the *Ambisporaceae* can clearly be separated (Fig 2). Among the main clades in the *Archaeosporales*, two (typified by *Archaeospora trappei* and *G. pyriformis*, respectively) already were defined as the families *Archaeosporaceae* and *Geosiphonaceae*. The third clade comprises the *Ambispora fennica* lineage. The situation of paraphyly for the *Archaeosporaceae*, which contained *A. leptoticha* and *A. gerdemannii*, could have been resolved by placing this clade into the *Geosiphonaceae*, or by erecting a new family. In our opinion, the latter is most appropriate and this clade is herein described as a new family, the *Ambisporaceae*. Two putative species sequenced from *Taxus* (from Germany) and *Prunus* (from Africa) roots (Wubet *et al.* 2003a, 2003b) also belong to this clade.

The resolution of the SSU rDNA analysis within the *Ambisporaceae* is not sufficiently discerning to distinguish the species investigated unless used in concert with the ITS region. With respect to the ITS region, *A. leptoticha* strain FL130 and NC176 are well characterized, and from the ITS data, isolate F3b from Japan can be considered to belong to the same species (Fig 2). Similarly, *A. callosa* HZ-6K, OK-m, OK1, and V1 are clearly separated from the other species and sufficiently close to each other to be considered conspecific. *A. fennica* is well separated from all other species in the clade and unequivocally separated from *Archaeospora gerdemannii* AU215 (AJ012202). Although the latter is very closely related to *A. leptoticha* based on the SSU rDNA data (Fig 1), the single ITS sequence published does not give sufficient resolution for a complete phylogenetic analysis. However, it is well separated from the other species in the clade.

The sequence AJ567807, derived from *Plantago* roots, is labelled in the public database as *A. leptoticha*, but it is more probably another, undescribed, species in *Ambispora*. This shows that care should be taken when annotating environmental sequences with species names, as in many instances, the database is not yet sufficiently large to be discerning at this level.

Morphology

Archaeospora trappei *and* Intraspora schenckii

The original species description of *Archaeospora trappei* was modified first by Morton & Redecker (2001) and later by Spain (2003). The differences in these descriptions cannot be reconciled without more detailed study, but whichever author is followed, the species is different in most respects from other species in the genus. The wall structure, when examined in

PVLG, appears simple (Morton & Redecker 2001), consisting of an outer component (probably a continuation of the wall of the sporiferous saccule) enclosing two adherent components making up a separate wall group. The spores are very small (40–48 × 50–72 μm), and have neither pigmentation nor a reaction to Melzer's reagent. The emendation by Spain (2003) described a much more complex wall structure, and indicated that the species is capable of forming glomoid spores. This work was carried out with a completely different approach from the established methods. Without further examination and comparison of all other species with the same methods, it is not possible to make the necessary morphological comparisons. Our own examination of pot cultured material did not reveal a glomoid phase as described in Spain (2003), and thus we suggest there is a need for independent verification of this aspect.

The molecular data show some differences among different cultures and origins of this fungus, which has few characteristics that can be used to distinguish species. We have noted differences among SSU signatures in organisms determined as *A. trappei* (Fig 1). It is possible that cryptic species exist that can be defined only or mainly through molecular analysis. The combination of morphological and molecular characteristics set *A. trappei* apart from the other organisms included by Morton & Redecker (2001) within the *Archaeosporaceae*. For *Intraspora schenckii*, there is no molecular evidence to show how closely it is related to *A. trappei*, but the two organisms are certainly very similar morphologically, differing only in the topological position of the spore within the neck of the sporiferous saccule, and it is retained within our concept of the family *Archaeosporaceae*.

Ambispora fennica *and* A. gerdemannii

These are very close morphologically, but the rDNA phylogenies clearly set them apart and show that they cannot be the same species (Fig 1). The type material of the former *Glomus gerdemannii* was unavailable for us to examine. Consequently, the comparison has to be made from the detailed illustrations and descriptions in the literature.

Ambispora gerdemannii has cream to orange–brown to dark orange–brown acaulosporoid spores that are much darker in colour than the hyaline to pale ochraceous spores (Fig 3A–B) of *A. fennica* They are described as having a 'pedicel' with a minimum length of 50 μm that gives a somewhat glomoid appearance. A similar pedicel is present in some, but not all, spores of *A. fennica*. The spores and saccules of the former are generally larger than those of the latter (Table 2). The acaulosporoid spores have an outer group that is probably continuous with the saccule wall, consisting of a more or less evanescent mucilaginous component that reacts to become pink to red in Melzer's reagent (often with adherent particles from the soil) overlaying a 'semi-flexible' component. The outermost component of *A. gerdemannii* fractures into angular fragments, whereas that of *A. fennica* disintegrates into fine granules. For both species, within this outer envelope, a second wall group develops which has an apparently brittle nature, fracturing when crushed to give the impression of 'polygonal shards' (Fig 3N, J). A third wall group of a single somewhat flexible component finally develops.

The glomoid spores of *A. gerdemannii* are white to pale cream, and those of *A. fennica* colourless, both are similar in size (Table 2). Their subtending hyphae differ somewhat. For *A. gerdemannii*, they are cylindrical to slightly flared and 6–12 μm wide at the spore base, and occluded by spore wall thickening. For *A. fennica*, they are cylindrical to distinctly funnel-shaped, often a little constricted at the base, up to 20 μm wide at the widest point, and open pored, or sealed by a distal septum formed by the inner wall component. The glomoid spore wall structures are similar, consisting of an evanescent outer component overlaying a somewhat flexible, finely laminated structural component. In contrast to *A. leptoticha*, glomoid spores of *A. fennica* have no reaction to Melzer's reagent.

There is some confusion in the redescription of *A. gerdemannii*, with regard to its origins. It is described as having been collected from 'Tasmania, Kakadu National Park' in Australia by C. Gazey (Morton & Redecker 2001). Kakadu National Park is in the Northern Territory, not Tasmania, and Chris Gazey (personal communication) has not collected organisms from the Northern Territory or Tasmania. The Australian collection at UWA is all carefully catalogued and numbered, but in the re-description, only an INVAM number is given, and there is no detail in the website (as of Jan. 2006), either as an accession, or as an available culture of AU215, at http://invam.caf.wvu.edu. It has proved impossible to obtain further information by direct contact with INVAM. Consequently, there must remain some doubt as to the precise origins of the fungus, when it was isolated, and by whom.

Ambispora leptoticha

This discussion refers to the interpretation of the former *Acaulospora appendicula*, *A. gerdemannii*, *Glomus leptotichum* and *G. fecundisporum* combined as conspecific. The morphologies of the organisms concerned are described in Morton & Redecker (2001). Although there can be some debate as to the correctness of combining *A. gerdemannii* and *A. appendicula* into a single species, their acaulosporoid spores are clearly different from those of *Ambispora fennica* because of their scalloped interlocked inner wall components. The acaulosporoid spores of the isolate F3b (*A. leptoticha* from Japan) correspond well in all aspects with the description of *Acaulospora appendicula*, and therefore with the concept of *Archaeopora leptoticha sensu* Morton & Redecker.

Similarly, the combination of heterotypic synonyms for the glomoid morphs, represented by *G. leptotichum* and *G. fecundisporum*, into *A. leptoticha* may be debated, but the glomoid spores of *Ambispora fennica*, are smaller, have a much smaller size range (38–130 × 38–117 compared with 48–262 × 48–262 μm, respectively), and show no reaction to Melzer's reagent.

There are still some fundamental questions that must be addressed eventually with regard to the synonymisation of four organisms and the choice of the epithet to represent them. For example, the sample of the type material of *G. leptotichum* examined during this study appeared to be very close morphologically to *G. callosum*, and showed no evidence of production of acaulosporoid spores as mentioned by Morton *et al.* 1997. Further examination of living material re-isolated from type localities might provide molecular evidence, and may be the only way to address this matter fully. Until then, the *status quo* is best maintained.

Ambispora callosa

Except for minor size differences, the type material of *G. callosum* is indistinguishable from the Japanese fungi OK-m, HZ-6K, OK1 and V1 (Figs 5–6). We are confident of our morphological species determination and the molecular evidence (Fig 1) in transferring this species to *Ambispora*. Spores of *A. callosa* are similar to the glomoid spores of *A. leptoticha sensu* Morton & Redecker (2001), but are generally larger.

The outer mucilaginous coating on spores of this species can give the impression of ornamentation. This was referred to in the species protologue as ornamentation formed of crowded minute warts. Examination of the type material shows this to be a misinterpretation (Figs 5G, 6G) of soil particles adherent to the sticky outer wall component. A similar effect was reported from *G. viscosum* (Walker *et al.* 1995).

In the protologue, the species in *Archaeosporaceae* are described as forming dimorphic or monomorphic spores (Morton & Redecker 2001). In the description, it is made clear that when spores produced are monomorphic, only the acaulosporoid form is found. *A. callosa* Att321 was established in 1989 (Saito & Vargas 1991), and the same species from Att1323 was isolated in 1993. For more than a decade these isolates, repeatedly sub-cultured with several different host plants and various soil conditions, have not produced acaulosporoid spores. It is impossible to prove that such spores do not exist, but Morton *et al.* (1997) themselves also described some cultures (from FL184) that exclusively form glomoid spores. It can thus be concluded that organisms with only glomoid spores do exist in the genus *Archaeospora sensu* Morton & Redecker (2001). The species description of *G. callosum* is of a fungus, from pot culture, that forms only glomoid spores. Incidentally, the same is true of *G. leptotichum* and *G. fecundisporum*, and questions might still be raised as to whether or not the evidence that these organisms should be synonymised (Morton & Redecker 2001) with the acaulosporoid-producing species was correct.

Acaulospora nicolsonii

The type material of *Acaulospora nicolsonii* was re-examined with a view to revising its systematic position. Re-examination of the type material suggests that there are three wall groups, the first with two components resolvable by light microscopy, and the second and third with one component each, rather than that shown in the protologue's murograph as four distinct walls in three groups (Walker *et al.* 1984). The outer component has a roughened appearance due to gradual disintegration, and can be considered as evanescent, in that some spores appear to have lost it completely. This is similar to the outer component of acaulosporoid spores of *Ambispora fennica*, but the outer wall of the *A. fennica* reacts with Melzer's reagent, whereas for *Acaulospora nicolsonii*, there is no such reaction. Although no fresh material of the latter species was available to check this, specimens were soaked from a polyvinyl alcohol lactophenol slide and re-tested, and no Melzer's reaction occurred. A similar exercise was carried out for spores of *Ambispora fennica* from a PVLG mount in which the reaction had faded, and the reaction was refreshed. The second component, which is inseparable by crushing from the first, is laminated and relatively rigid. Contained within this outer envelope is a separate and slightly flexible component, and within this a third group consisting of a very thin, flexible component. The appearance and structure of these inner wall components is similar to those of *A. fennica* and *A. gerdemannii*, though lacking the fracturing and splitting nature of those species.

No glomoid morph was recognised in the original species description of *A. nicolsonii*, though because the cultures from which it was derived were not pure, such spores at the time would have been recognised as belonging to the genus *Glomus*, and would not have been associated as belonging to the same organism. It is likely that *Acaulospora nicolsonii* belongs in *Ambispora*, but there is insufficient evidence to merit moving it at this time. The fungus was originally obtained in mixed pot culture from a farm owned and operated by the University of Leeds. Consequently, the prospects of re-isolating the organism in due course must be relatively good. It would then be possible to confirm the correct taxonomic position based on both morphological and molecular evidence.

It is increasingly clear that similar spore morphologies may not always be sufficient evidence for conspecificity (or sometimes even a close phylogenetic relationship at any level), and that molecular data are becoming a requirement for determining the correct phylogenetic position of members of the *Glomeromycota*. We clearly have cryptic species in this phylum, implying that defining species solely on morphological grounds is risky, though not impossible if the characteristics used to define them are sufficiently different from those of existing species. In some circumstances, it may not be possible to obtain molecular evidence, but where possible, the combination of molecular and morphological evidence is needed to be confident of the existence of new genera or species. For example, here *Ambispora fennica* and *A. gerdemannii* have similar morphologies, but are clearly well separated by molecular evidence, and cannot be conspecific. This raises some questions about the validity of using the Australian organism to re-define the North American *G. gerdemannii*. These doubts can only be reconciled if the latter is re-isolated from its type locality and examined for both molecular and morphological characteristics. We are in a similar dilemma with the Japanese species that we have determined as *A. callosa*. Morphologically, the characteristics of the Japanese isolates agree with those of the type material from Zaire, but until new living material is obtained from somewhere close to the type locality, there must always remain an element of doubt. However, the type material of *G. callosum* is in excellent condition and shows all the characteristics described in the protologue, in contrast to the type of *G. gerdemannii*, which was described by Morton & Redecker (2001) as being in poor condition.

According to the previous descriptions of mycorrhizas formed by members of the more ancient clades in the *Glomeromycota*, these do not form vesicles. This characteristic was considered to have important phylogenetic implications. However, staining of mycorrhizas of both *A. fennica* and *A. leptoticha* proved the existence of vesicles. It seems that there must be some doubt as to the value of this as a taxonomic character, and further research is needed to assess its value.

Therefore we remove the emphasis on mycorrhizal morphology from our formal descriptions.

The combination of near full-length SSU rDNA and ITS region analyses is proving to be a valuable strategy for resolving taxonomic and evolutionary questions about AM fungal species. SSU rDNA sequences often do not allow the separation of species in the *Glomeromycota*. The ITS region sequences help to uncover the diversity hidden within such SSU rDNA sequences, which in biodiversity studies often are grouped as 'sequence types'. It is likely that the real species diversity is much higher than that revealed by studies based on SSU rDNA sequence types, as here shown for the *A. leptoticha*–*A. gerdemannii*–*A. callosa* clade. The addition of data from LSU rDNA, and perhaps also other genes, may further enhance resolution, but the combination of the SSU and ITS rDNA regions seems to be well suited to define species in the *Glomeromycota* at the molecular level.

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

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

Manuela Krüger, Herbert Stockinger, Claudia Krüger and Arthur Schüßler

Ludwig-Maximilians-University Munich, Dept Biology I, Genetics, Großhaderner Strasse 4, D–82152 Planegg-Martinsried, Germany

Author for correspondence:
Manuela Krüger
Tel: +49 89 2180 74714
Email: manuela.krueger@lrz.uni-muenchen.de

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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.

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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.

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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 $\mu g\ ml^{-1}$).

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.

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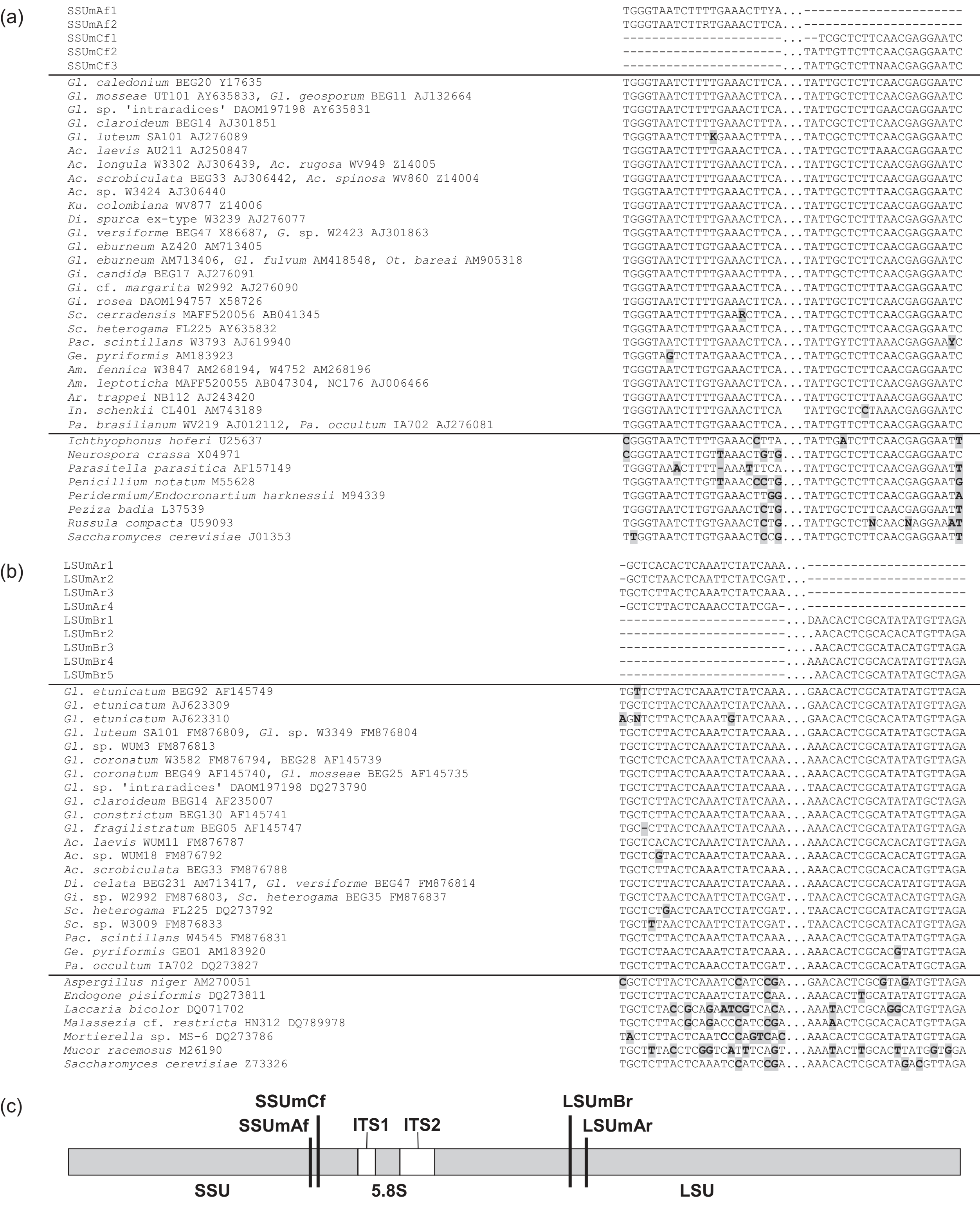

Fig. 1 Forward and reverse primers designed in this study (5′–3′ direction), compared with their annealing sites in sequences from representative members of all main AMF taxa and some non-AMF species. Variable sites not represented in any primer mixture are shaded. When no culture identifiers are known, 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.

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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

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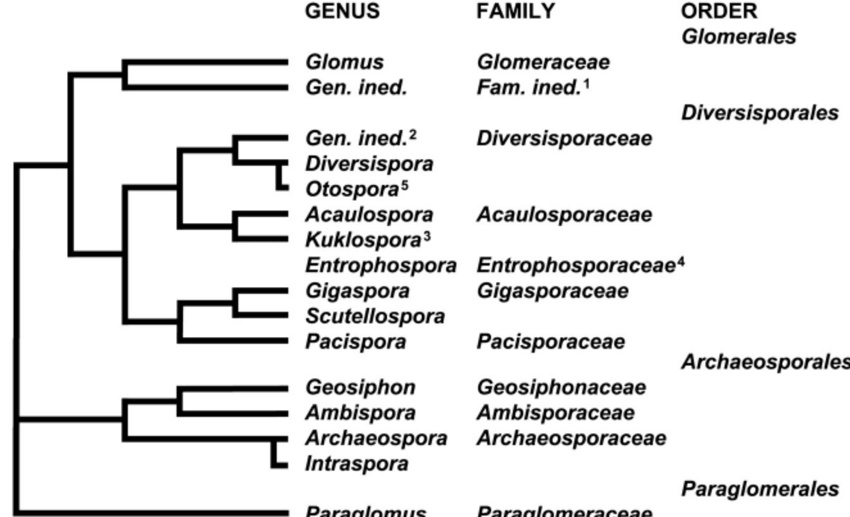

Fig. 2 Phylogenetic relationships of taxa in the *Glomeromycota* (Schüßler *et al*., 2001b; Walker *et al*., 2007). [1]Species currently named *Glomus*. One of the main *Glomus* clades (GlGrA or GlGrB) will represent the *Glomeraceae*, once the phylogenetic affiliation of the type species of *Glomus* 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.

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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.

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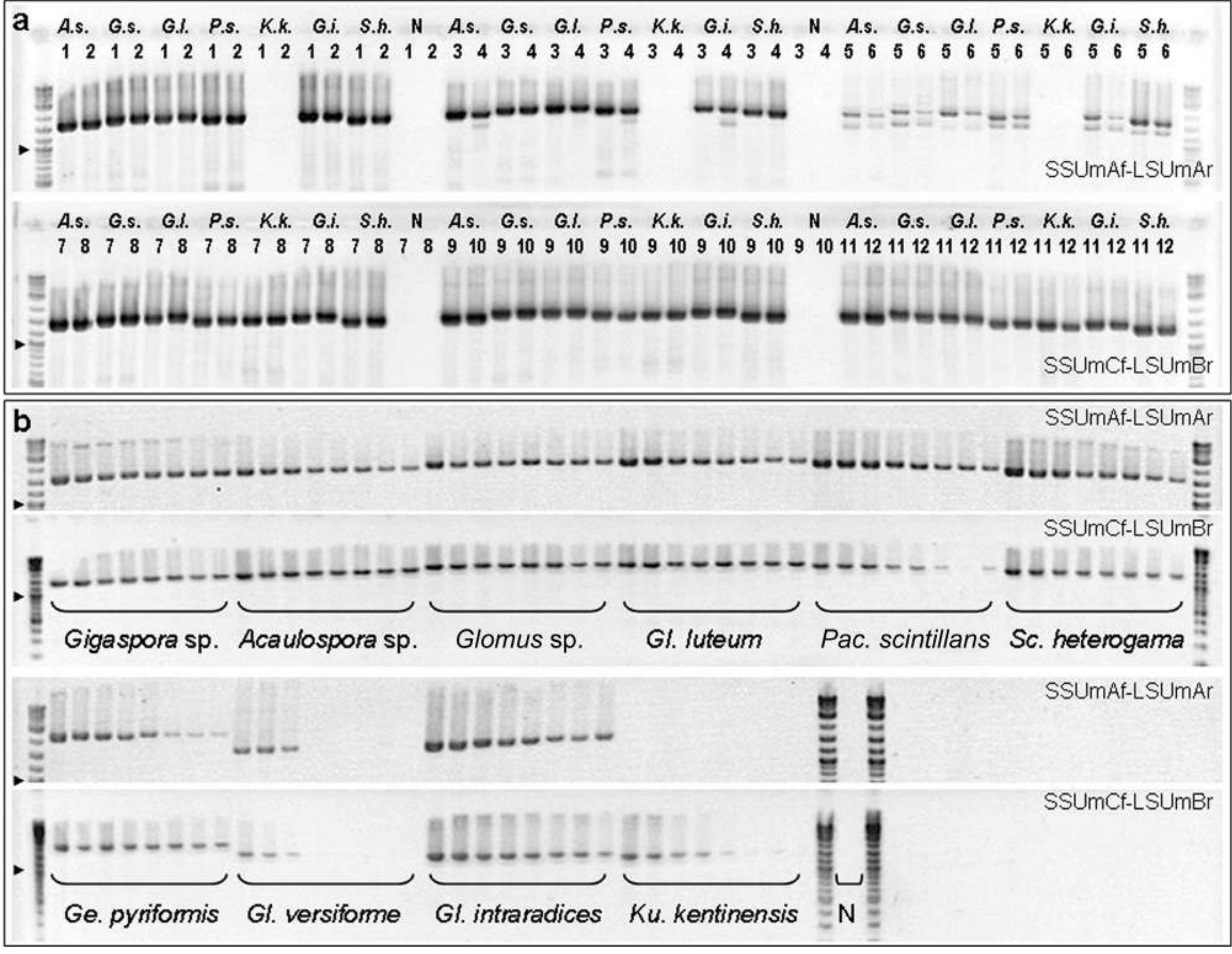

Fig. 3 Polymerase chain reaction amplification with primers SSUmAf–LSUmAr (approx. 1800 bp amplicons) and SSUmCf–LSUmBr (approx. 1500 bp amplicons). (a) PCR on cloned DNA fragments, using different annealing temperatures and a template concentration of 1 ng µl^{-1}. *A.s.*, *Acaulospora* sp.; *G.s.*, *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 proofreading DNA polymerase used could hamper discrimination

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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.

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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

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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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6 '*Glomus intraradices* DAOM197198', a model fungus in arbuscular mycorrhiza research, is not *Glomus intraradices*

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'*Glomus intraradices* DAOM197198', a model fungus in arbuscular mycorrhiza research, is not *Glomus intraradices*

Herbert Stockinger[1], Christopher Walker[2] and Arthur Schüßler[1]

[1]LMU Munich, Dept. Biology I, Genetics, Großhaderner Str. 4, D–82152 Planegg-Martinsried, Germany; [2]Royal Botanic Garden Edinburgh, 20A Inverleith Row, Edinburgh EH3 5LR, UK

Author for correspondence:
Arthur Schüßler
Tel: +49 2180 74730
Email: arthur.schuessler@lmu.de

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Summary

- *Glomus intraradices*-like fungi are the most intensely studied arbuscular mycorrhizal (AM) fungi. However, there are several AM fungi named as *G. intraradices* that may not be conspecific. Therefore, the hypothesis was tested that DAOM197198 and similar AM fungi, such as BEG195, correspond to the type of *G. intraradices*.
- The *G. intraradices* isotype material, a descendant (INVAM FL208) of the type culture, and a morphologically corresponding AM fungus (MUCL49410) isolated from the type locality were studied and compared with several cultures of DAOM197198 and BEG195.
- Phylogenetic analyses of the partial small subunit (SSU), complete internal transcribed spacer (ITS) and partial large subunit (LSU) nuclear rDNA regions revealed two clades, one including *G. intraradices* FL208 and MUCL49410, the other containing DAOM197198 and BEG195.
- The two clades were clearly separated by sequence analyses, despite the high intraspecific and intrasporal ITS region sequence divergence of up to > 23%. We conclude that the AM fungi with the identifiers DAOM197198 and BEG195 are not *G. intraradices*, but fall in a clade that contains the recently described species *G. irregulare.*

Introduction

About 70–90% of land plant species form arbuscular mycorrhiza (Smith & Read, 2008), so it is obvious that the interaction of plants and the obligate symbiotic arbuscular mycorrhizal (AM) fungi of the *Glomeromycota* (Schüßler *et al.*, 2001) is of major importance for the entire terrestrial ecosystem. In research on AM fungi (hereafter AMF), a fungus named *Glomus intraradices* is the most frequently used member of the *Glomeromycota*. To date, > 1200 publications refer to this species, > 130 of which have the name in the title. This wide use resulted from the first AMF established in *in vitro* root organ culture (ROC) being determined as *G. intraradices* (Chabot *et al.*, 1992). The descendants of this ROC established in Canada, often referred to as DAOM197198 (or DAOM181602, another voucher number for the same fungus), are extensively used in basic research (e.g. for a genome sequencing project; Martin *et al.*, 2008) and to demonstrate transient genetic transformation (Helber & Requena, 2008). It also is a component of some commercial plant growth-enhancing products (Corkidi *et al.*, 2004; http://www.pro-mixbas.com).

However, a very basic question still remains to be resolved: does the DAOM197198 fungus indeed correspond to *Glomus intraradices*? It is possible that more than one species may have been identified with this name.

Despite the large body of published work, including recent publications dealing with genetic recombination and anastomoses compatibility between isolates of AMF that are closely related to DAOM197198 (Croll & Sanders, 2009; Croll *et al.*, 2009), the definition of '*G. intraradices*' is far from clear. The species was described by Schenck & Smith (1982) from a citrus orchard in Florida. The type specimens came from a pot culture established from root fragments of a *Citrus* sp., a descendant of which was donated to the INVAM collection (http://www.invam.caf.wvu.edu) where it was catalogued as FL208. Since then, many cultures and isolates have been determined to be *G. intraradices.* Analyses of their rDNA region showed that they belong within the *Glomus* Group Ab (GlGrAb) (Schwarzott *et al.*, 2001; Jansa *et al.*, 2002b; Börstler *et al.*, 2008). Both DAOM197198 and BEG195 have been identified as *G. intraradices*, but there appears to be no published work comparing them with the original description, the type material, or FL208. Therefore, these

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cultures might represent one species, but they may instead also belong to a cohort of related species. This element of doubt stimulated a re-examination of the molecular evidence in relation to the species *G. intraradices*.

A fungal species is defined by its nomenclatural type, although as such it is a preserved sample and thus not available for study as a living entity. However, being its descendant, the culture *G. intraradices* FL208 can provide 'living evidence' of the true nature of the species. Subculture of FL208, herein termed 'ex-type' or 'type-culture', are available for such comparative study, so it is possible to investigate whether other AMF in the *Glomus* Group Ab (Schwarzott *et al.*, 2001) may be other species rather than *G. intraradices*. Strictly speaking, the Botanical Code defines an 'ex-type culture' as being obtained from type material permanently preserved in a metabolically inactive state, but for convenience we extend this here to include cultures such as FL208, derived from the 'type culture' through a series of living subcultures.

We compared the *G. intraradices* isotype with ex-type specimens from pot cultures of FL208. Ex-type material was then compared by partial nuclear small subunit (SSU), internal transcribed spacer (ITS) and partial nuclear large subunit (LSU) rDNA region sequencing with three other cultures: a new isolate from the type locality corresponding morphologically to *G. intraradices* (now cultured as ROC in the GINCO collection, http://emma.agro.ucl.ac.be/ginco-bel, as MUCL49410), DAOM197198 (from Pont Rouge, Canada); and BEG195 (from Germany). The aim of this research was to determine if these organisms indeed all correspond to *G. intraradices*.

A secondary aim was to contribute to strategies that might be used for species determination based on DNA sequences. Such identification, termed 'DNA barcoding', must be accurate, rapid, cost-effective, culture-independent, universally accessible, and usable by nonexperts (Frézal & Leblois, 2008). For animal DNA barcoding, the mitochondrial cytochrome *c* oxidase I (*COI*) gene is widely used. In fungi, *COI* possesses length variation (0.64–12.3 kb; Seifert *et al.*, 2007) too large to fulfil barcoding requirements. Because the molecular identification of fungi has been based mainly on the ITS rDNA region (Nilsson *et al.*, 2008; http://unite.ut.ee) the ITS region will most likely become the primary barcode for fungi. The Barcoding of Life Database (BOLD, http://www.barcodinglife.org) already supports the storage and analysis of ITS sequences, and we therefore intended also to study, using GlGrAb sequences from public databases, whether the ITS region alone can be used to resolve species in the *Glomeromycota*.

Materials and Methods

Fungal type material

The isotype of *G. intraradices*, voucher OSC40255, was borrowed from Oregon State University herbarium (OSC). It consisted of spores and stained roots on dried-out microscope slides, and spores and blue-stained roots preserved in lactophenol. It was examined microscopically by accepted methods (Walker *et al.*, 2007).

Cultures

All pot cultures were established as closed systems in Sunbags (Sigma-Aldrich, Germany) (Walker & Vestberg, 1994).

Ex-type cultures Pot culture substrate of FL208 was obtained on 23 March 2007 from INVAM (http://www.invam.caf.wvu.edu, where in the 'accessions details page' it is described as 'subculture of the original isotype'). A subculture attempt (Att) number (Att4-36) was assigned to this material on receipt (Att4-0 is the identifier in our database for the original, root fragment, open pot culture of S. Nemec, established in autumn 1974). Replicate ex-type pot cultures were established in Germany (Att4-37, Att4-39–Att4-43), England (Att4-38) and Belgium (Att4-44). Moreover, a ROC (Declerck *et al.*, 1998) was established in Belgium (Att4-45) on transformed carrot (*Daucus carota*) roots (from one root fragment of Att4-44). From this, one spore cluster on a single hypha was taken to establish Att4-46 (MUCL49413 in GINCO-BEL) on transformed chicory (*Chichorium intybus*) root (Fontaine *et al.*, 2004). Material from a subsequent chicory ROC (Att4-64) was used for DNA extraction in Germany.

New isolate from the type locality A sample was provided by S. Nemec, collected in Florida, USA, from the type locality of *G. intraradices* (a citrus plantation between Clermont and Minneola close to Highway 27). From the description of the locality, an approximate latitude and longitude was estimated (28°33′41″N, 81°44′40″W) using Google Earth. A trap culture with roots and soil was established with *Plantago lanceolata* as host (Att1102-0, 14 October 2001) in disinfested 3 : 1 (v : v) horticultural sand-expanded attapulgite clay (Oil-Dri Corp., Chicago, IL, USA). Voucher samples determined as *G. intraradices* growing in the culture were made on 9 August 2002 (W4064), 17 March 2003 (W4344), and 3 March 2004 (W4598). From the 2004 sampling, a single spore was germinated on a filter fragment (Brundrett & Juniper, 1995) and successfully used to establish Att1102-7 with *P. lanceolata*. Spores formed in this culture corresponded broadly to the type material of *G. intraradices*. They were found abundantly singly in the substrate, in loose clusters of 2 to > 100, attached to fine, hyaline mycelium around roots. The fungus also sporulated heavily in the root cortex. On 6 June 2006, a single spore ROC was established (Att1102-9, MUCL49410) and subsequently subcultured as part of the GINCO collection.

Descendants of DAOM197198 Several cultures corresponding to DAOM197198 (originally collected in Pont Rouge, Canada) were examined. DAOM181602 is an earlier voucher

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number for a sample from an ancestral pot culture taken in 1981, before the fungus was transferred to the company Premier Tech Ltée. (Québec, Canada). The ROC widely used in AM research (Chabot *et al.*, 1992) was initiated from a pot culture vouchered as DAOM197198 in 1987. Details about the culturing history of this fungus will be provided in a subsequent publication. As is common in many studies we use DAOM197198 as organism identifier, but stress that it is actually a voucher number of the herbarium in Ottawa and thus defines what was present in the culture at the time of sampling. We obtained ROC cultures of this fungus from several sources. (1) Att1192-44 originated from a ROC culture traced back to the laboratory of G. Becard (France), from where it was sent to the laboratory of I. Sanders (Switzerland), then to the laboratory of U. Paszkowski (Switzerland). In 2007 it was sent to the laboratory of M. Parniske (Germany) and from there to our laboratory. (2) Att1192-27 was obtained from the laboratory of P. Bonfante (Italy) in 2007, via the laboratory of P. Lammers (USA), where it was established from material produced by Premier Tech for the genome sequencing project (http://www.jgi.doe.gov/genome-projects/). (3) Att1192-53 was sent to us in 1996 by Y. Piché and spores were stored at −80°C. (4) Att690-23 was obtained via the University of Western Australia by C. Walker in November 2006 and established as a closed pot culture in Munich (March 2007). All this material purportedly stems from the same ROC established in the early 1990s (Chabot *et al.*, 1992).

BEG195 This fungus originally was sampled from an agricultural field with winter cereals near Hannover (Germany). It was cultured at the University of Marburg, passed to the Sainsbury Laboratory (Norwich, UK) and then to the laboratory of M. Parniske (Munich, Germany), and thence, from Att1485-12, to our subculture (Att1485-13) used for DNA extraction.

***Glomus proliferum* (MUCL41827)** This AMF was cultured from banana plantation in Guadeloupe. It was described by Declerck *et al.*, (2000) and is available as ROC from GINCO.

Identifiers used in this publication To distinguish the different cultures and isolates studied here, we refer to them by using the most common descriptors. It should be borne in mind that these correspond either to vouchers (DAOM197198), organisms (INVAM FL208, MUCL49410, BEG195), or to individual subculture attempts (Att). DAOM197198 is used for the Canadian fungus from Pont Rouge (the fungus used in the AMF genome sequencing project), FL208 for the ex-type cultures from Florida, and MUCL49410 (Att1102-12 and descendants) for the new isolate from the type locality. BEG195 (as Att1485-13) was included in the analysis to represent a *G. intraradices*-like fungus from Europe.

DNA extraction, PCR amplification, cloning and sequencing

DNA extraction and PCR amplification Spores were cleaned and DNA extracted as described in Schwarzott & Schüßler (2001). In a first PCR, an amplicon containing a part of the SSU, the whole ITS1–5.8S–ITS2 region, and a part of the LSU rDNA were amplified with two different primer pairs (Table 1), using the Phusion High-Fidelity PCR Mastermix (Finnzymes, Espoo, Finland). As template 5 µl of DNA extract (except for Att1192-27 and *G. proliferum*, where 2 µl were used) were used in 20-µl final reaction volumes. The final primer concentration was 0.5 µM of each primer. For primers SSUmAf (Krüger *et al.*, 2009) and LR4+2 (ACCAGAGTTTCC-TCTGGCT; modified LR4 primer, http://www.aftol.org) the PCR parameters were: 5 min initial denaturation at 99°C; 40 cycles of: 10 s at 99°C, 30 s at 59°C, 1 min at 72°C; final elongation 10 min at 72°C. The cycling parameters for the SSUmAf and LSUmAr (Krüger *et al.*, 2009) PCR mix were identical, except for the annealing (15 s at 60°C) and elongation (45 s at 72°C) parameters.

When the first PCR did not result in visible bands after gel electrophoresis of 6 µl of PCR product, a second (nested) PCR was performed. The PCR reactions were initiated as for the first PCR with 0.1 µl, 0.2 µl, 0.5 µl or 1 µl from the first PCR used as template. Either the primer combination SSU-Glom1 (Renker *et al.*, 2003) and NDL22 (van Tuinen *et al.*, 1998) or the AMF specific primers SSUmCf and LSUmBr (Krüger *et al.*, 2009) were used for the nested PCR. The cycling regime for SSUGlom1-NDL22 was: 5 min at 98°C; 30 cycles of: 10 s at 98°C, 30 s at 65°C and 1 min at 72°C; final elongation 10 min at 72°C. For SSUmCf-LSUmBr it was: 5 min at 99°C; 30 cycles of 10 s at 99°C, 15 s at 63°C and 45 s at 72°C; final elongation 10 min at 72°C. Nested PCR amplifications for Att1192-44 (pHS059) were performed with *Taq* DNA polymerase (Peqlab, Erlangen, Germany) and for Att4-38 (pHS080) with Top-*Taq* polymerase (Qiagen, Hilden, Germany), using the primer pair SSUGlom1-NDL22. In these cases the PCR program was 5 min at 94°C; 30 cycles of: 30 s at 94°C, 30 s at 58°C and 2 min at 72°C; final elongation of 10 min at 72°C.

Cloning and sequencing The PCR products were cloned with the TOPO TA or the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) according to manufacturer's protocol, except that all components were used as 1/3 volume (except SOC medium for initial bacterial growth, which was used as full volume). The pHS113 clones (*G. proliferum*) were obtained using the StrataClone Blunt PCR Cloning Kit (Stratagene Agilent Technologies, La Jolla, CA, USA) according to the manual. Clones were analysed using colony PCR and products showing correct fragment size were used for RFLP with MboI, HinfI and RsaI. Selected clones were grown in liquid Terrific Broth media and plasmids isolated with the NucleoSpin

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Table 1 Cultures used in this study

Culture/voucher	Identifier	Cloning no. (no. of clones sequenced)	Primers used	Spore(s) for DNA extract
Att4-41 (rf, pot)/W5413	FL208 (ex-type)	pHS096 (16)	SSUmCf/LSUmBr (n)	ss
Att4-38 (rf, pot)/W5166	FL208 (ex-type)	pHS089 (8), pHS086 (3); pHS080 (2)	SSUmCf/LSUmBr (n); SSU Glom1/NDL22 (n)	ss
Att4-64 (sc, ROC)/W5507	FL208 (ex-type)	pHS099 (11)	SSUmCf/LSUmBr (n)	ss
Att1102-12 (ss, ROC)/W5070	MUCL49410 (re-isolate)	pHS051 (5)	SSUmAf/LR4+2	sc
Att 1192-44 (rf, ROC)/W5533	DAOM197198 ('Paszkowski')	pHS059 (9)	SSUGlom1/NDL22 (n)	ss
Att1192-27 (rf, ROC)/W5495	DAOM197198 ('Naumann')	pMK041 (7)	SSUmAf/LR4+2	ms (3)
Att1192-53(rf, ROC)/W3182	DAOM197198 ('Piché')	pMK009 (3)	SSUGlom1/NDL22 (n)	ss
Att690-23 (rf, pot)/W5499	DAOM197198	pHS111 (11)	SSUmC+LSUmB	ss
Att1485-12 (ss, pot)/W5272	BEG195	pHS060 (9)	SSUGlom1/NDL22 (n)	ss
Att894-7 (ss, pot)/W3776	*Glomus* cf. *clarum*	pHS029 (9)	SSUGlom1/NDL22 (n)	ss
MUCL41827 (ROC)	*Glomus proliferum*	pHS113 (12)	SSUmCf/LSUmBr (n)	ss
MUCL41827 (ROC)	*Glomus proliferum*	pHS116 (2), pHS117 (1)	SSUmAf/LSUmAr	ms (10)

Information about culture numbers, vouchers, clones, PCR-primers and DNA extraction are shown. ss, Single spore; ms, multispore; rf, root fragment; sc, individual spore-cluster; n, nested PCR.

Multi-8 Plasmid kit (Macherey & Nagel, Düren, Germany). Alternatively, a 'quick and easy' method modified after Ganguly *et al.* (2005) was used. Sanger sequencing was performed by the LMU Sequencing Service Unit on an ABI capillary sequencer using BIGDYE v3.1 sequencing chemistry. The new rDNA sequences derived from this study were deposited in the EMBL/GenBank/DDBJ databases with the accession numbers FM865536–FM865617 and FM992377-FM992402.

Phylogenetic analysis

The 3′ partial SSU rDNA, the ITS region, and the 5′ partial LSU rDNA were either analysed for the sequences derived from this study together with some selected shorter sequences of characterized AMF from the database, or the ITS region alone was used for comparison with public database sequences. Sequences were assembled and proof-read with the program SEQASSEM and aligned with ALIGN (both from http://www.sequentix.de). Sequence divergences in per cent (uncorrected pairwise distances) were calculated by using BIOEDIT (Hall, 1999) and based on alignments containing either sequences of *G. intraradices*-related species or of *Ambispora* spp., including all positions. The alignment of the maximal common length (representing the SSUGlom1-NDL22 fragment) sequences comprised 1555 sites, 1387 of which could be unambiguously aligned and were used for the analyses.

When sequences derived from the same PCR reaction were identical after excluding ambiguous sites from the alignment, only one was included in phylogenetic analyses and accession numbers of the other sequences were later annotated to the corresponding clade. The analyses of database sequences included identical ones, because it is difficult to interpret whether they came from the same or different spores, PCR reactions or even cultures. Phylogenetic analysis was performed with PHYLIP (Felsenstein, 1989), RAXML (Stamatakis & Hoover, 2008), TREE-PUZZLE (Schmidt *et al.*, 2002) and MRBAYES (Ronquist & Huelsenbeck, 2003). Consensus trees were constructed after 1000-fold bootstrapped neighbour-joining (NJ, based on Kimura-2-parameter model with PHYLIP 3.8; Felsenstein, 1989) analyses. RAXML was set to a maximum likelihood (ML) search for best-scoring tree after 1000 bootstraps and the proportion of invariable sites was estimated by the program. The ML quartet puzzling (MLQP) analyses were performed with TREE-PUZZLE 5.2 (based on GTR model), estimating nucleotide frequencies and gamma distributed heterogeneous rates from the dataset. As an alternative approach, the sequences (SSUGlom1-NDL22 fragment) were aligned automatically using the MAFFT online server (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/), for comparison with the results from the manual alignment. The iterative refinement option of MAFFT was set to FFT-NS-i (Katoh *et al.*, 2002). Phylogenetic analysis was performed by RAXML with settings as above.

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For the ITS region analyses, public database sequences labelled as *G. intraradices* and such of closely related species were included. Analyses were based on a manually made alignment. Identical ITS region sequences were excluded and afterwards annotated to the appropriate clade. In total 395 sites could be unambiguously aligned. Phylogenetic analyses were performed with PHYLIP (NJ) and RAXML (ML). The two sequence alignments (SSU + ITS + LSU rDNA sequences from this study and ITS region including database sequences) are available from http://www.amf-phylogeny.com.

Results

The isotype material of *G. intraradices* is in relatively poor condition. It appears heavily parasitized and the spore wall structure was difficult to determine. It was also much darkened in colour because of storage in lactophenol. However, it was possible to see characteristics used by the original authorities to describe the species, along with other details that were not published in the protologue, and to compare them with those of DAOM197198. We do not show the detailed morphological comparisons here, since a detailed re-description including designation of an epitype of *G. intraradices* is currently in preparation for publication elsewhere.

To characterize the 'model AMF' DAOM197198 at the molecular level, we studied several cultures, including some from single-spore isolates. The phylogenetic analyses comprising the partial SSU, entire ITS region, and the partial LSU rDNA sequences show a clear separation into two clades (Fig. 1). The first clade includes the ex-type culture of *G. intraradices*, FL208 (Att4-38, Att4-41, Att4-64) and the new isolate from the type locality, MUCL49410 (Att1102-12). The second clade contains DAOM197198 (Att690-23, Att1192-27, Att1192-44, Att1192-50) and BEG195 (Att1485-12). Shorter sequences of *G. irregulare*, a *G. intraradices*-like species (Błaszkowski *et al.*, 2008), and isolates from Switzerland were included in the analyses and also cluster in the latter clade. When using a fully automated MAFFT alignment as a base for the phylogenetic analyses, the same, distinct clades were resolved. Later, we named the clade containing FL208 and MUCL49410 as the '*G. intraradices* clade' and the clade containing DAOM197198, BEG195, *G. irregulare* and the Swiss isolates as the '*G. irregulare* clade'. A separation of these clades also existed when using only the ITS1 region, the partial LSU sequences, or the combined partial SSU + 5.8S + partial LSU (without ITS1 + ITS2) fragment (RAXML bootstrap support > 70%, for all three options; data not shown). However, analyses of only the ITS2, or the ITS1 + 5.8S + ITS2 region resulted in a monophyletic grouping for the *G. irregulare* clade sequences, but the *G. intraradices* clade appeared paraphyletic. This indicated that the ITS2 region alone carries conflicting phylogenetic signal or too much noise, hindering resolution. Further ITS region analyses including database sequences (see below) also show that this region is not suitable to resolve species in GlGrAb.

The maximal pairwise uncorrected distance values (p-distances) within the *G. irregulare* clade were 9.3% for the newly obtained SSU + ITS + LSU rDNA sequences. The divergence in the G. *intraradices* clade was up to 14.1%. An overlap of highest intraspecific p-distances in the *G. intraradices* clade with the lowest interspecific (relating to the *G. irregulare* clade) p-distance values was observed for the full-length sequences and ITS region, indicating the lack of a so called 'barcode gap' (http://www.barcoding.si.edu/). The variation in the partial LSU sequences only was 7.8% for the *G. irregulare* clade and 11.8% for the *G. intraradices* clade.

The most variable ITS region showed p-distances of up to 16.3% for the *G. irregulare* clade and up to 23.1% for the *G. intraradices* clade. This enormous ITS region variability was found within one FL208 ex-type culture spore from Att4-41 (Fig. 1) and is the highest ever recorded for an individual AMF spore. Within this spore the maximal p-distance for the SSU + ITS + LSU rDNA sequence is 13.9%.

Analysing the ITS region including database sequences revealed several clades. Most ITS-region sequences designated as *G. intraradices* fell within the *G. irregulare* clade, including those of BEG158, BEG195 and the known ITS sequences of the genotypes (II, VI, XII, XV, XVII, XVIII; Croll *et al.*, 2008; Croll & Sanders, 2009) of isolates from a field site in Switzerland (Jansa *et al.*, 2002a; Koch *et al.*, 2004). The FL208 sequences from the public database (Börstler *et al.*, 2008, Sudarshana P. *et al.*, unpublished), as well as KS906 (AF185669-73) cluster together with the FL208 sequences from our analysis. Three new sequences (FM865546 from pHS051-20, FM865599 from pHS099-16 and FM865604 from pHS099-41) that stem from ROC of either a *G. intraradices* FL208 descendant or the new isolate (MUCL49410) cluster distantly from the main clade (Fig. 2). Sequences from the recently described *G. irregulare*, which was studied together with a fungus identified as *G. intraradices* isolated from the same trap culture (Błaszkowski *et al.*, 2008), clearly fall into subclades containing DAOM197198 + BEG195 sequences (Figs 1, 2).

The analyses of database sequences solely from the ITS region indicated that some that were annotated as *G. intraradices* are resolved to belong to separated, highly supported clades. One of these clades is represented by EY118 + INVAM GR104 (AF185684, AF185686 and AF185651) and another one by INVAM VA110 + INVAM CA502 (AM980854–59) (see also Börstler *et al.*, 2008). However, further VA110 sequences (AF185674-6) group within the *G. intraradices* FL208 + MUCL49410 clade. Another closely related clade is represented by sequences of *Glomus* cf. *clarum* Att894-7 and the *G. clarum* (CL883A) database sequences AJ243275 and AJ239123. A very distinct cluster of sequences (AJ517450-61; EnvGrA in Fig. 2) annotated as *G. intraradices* (Renker *et al.*, 2005) is clearly separated from both, the *G. intraradices* and *G. irregulare* clades.

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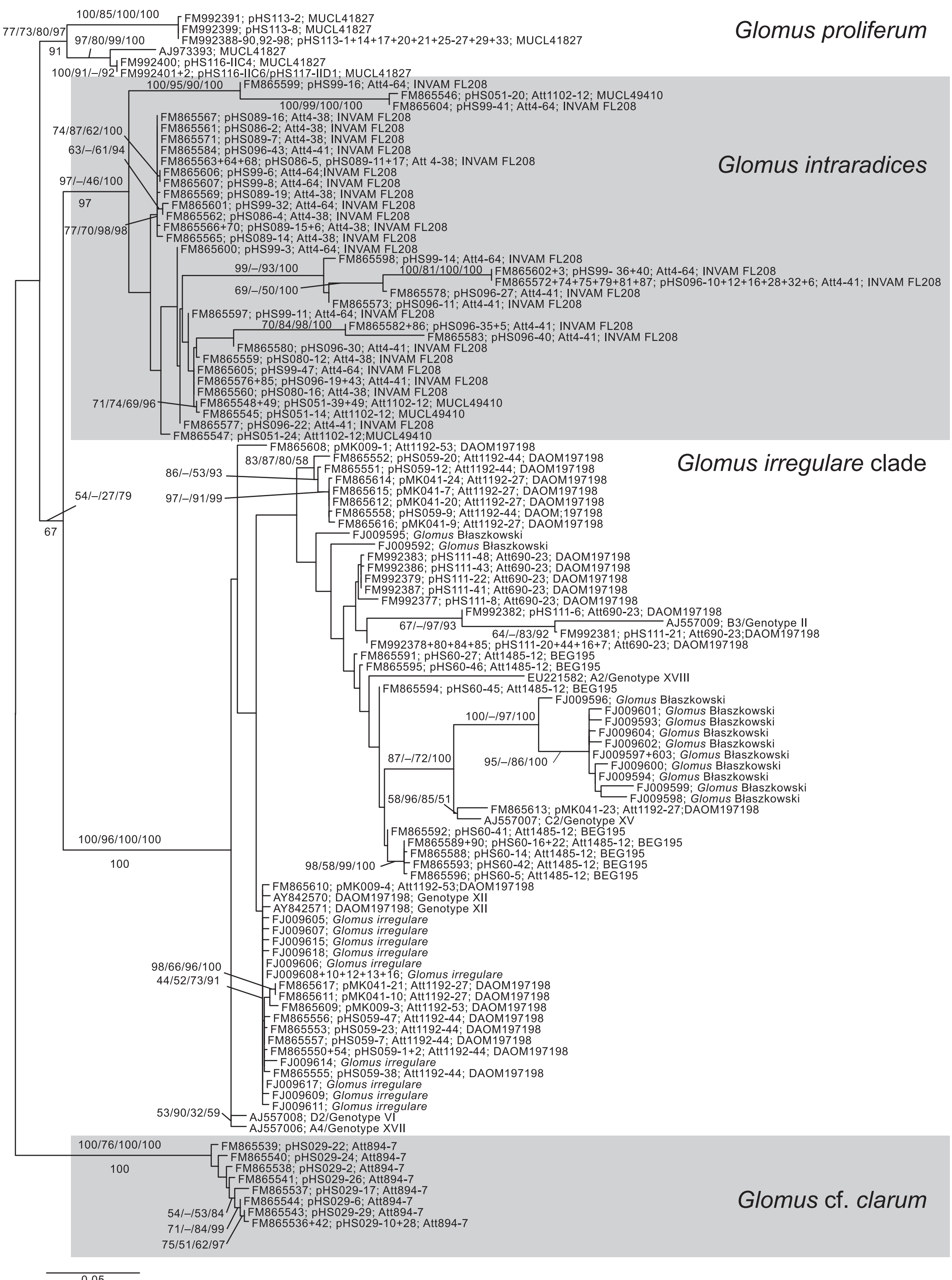

Fig. 1 Phylogenetic tree (partial small subunit (SSU), internal transcribed spacer (ITS) region, partial large subunit (LSU) rDNA sequences of approx. 1.5 kb) of *Glomus* cultures analysed, with *Glomus* cf. *clarum* (Att894-7) sequences as outgroup. DAOM197198, BEG195 and *Glomus irregulare* cluster together. The ex-type cultures of *Glomus intraradices* (FL208 and descendants) and the new isolate from the type location (MUCL49410) form a clearly separated clade. Distances of the phylogenetic tree are derived from a RAXML analysis. The values above the branches correspond to supports from RAXML/MLQP/NJ/Bayesian analyses. Support values are not shown if lower than 50% in more than one analysis, except for some main clades. Performing RAXML analysis based on a MAFFT alignment using the complete sequence length resulted in the same main topology, with even more strongly supported main clades (values below branches).

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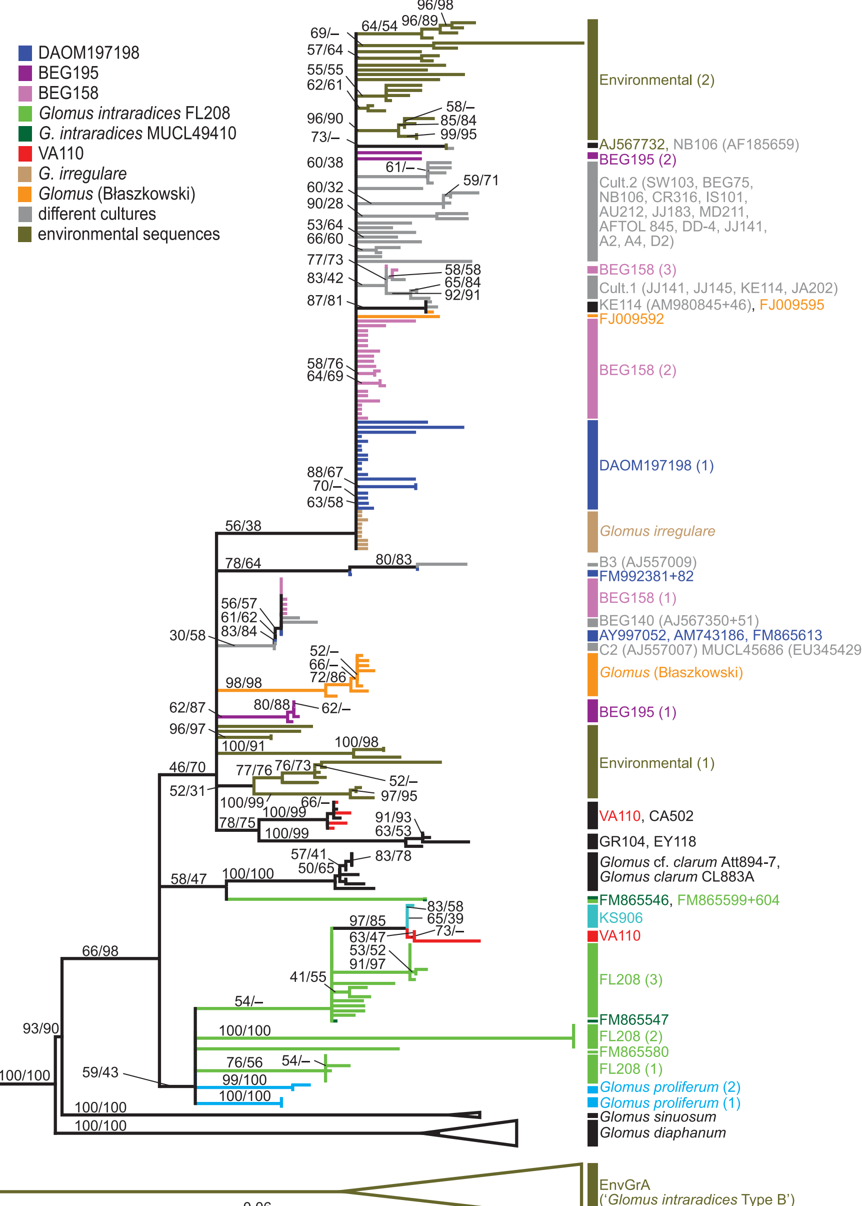

Fig. 2 Simplified phylogenetic tree of a RAXML analysis of 285 internal transcribed spacer (ITS) region sequences that are annotated as *Glomus intraradices*, and related species. Clades with support values < 50 in both analyses were collapsed to polytomies. For simplicity, some well supported clades were collapsed to triangles whose sizes correspond to the respective sequence number contained. The values above the branches correspond to supports from RAXML and neighbour-joining (NJ) analyses. Accession numbers of clades or groups (code for the clade or group underlined, additional information written in brackets) are as follows: Environmental Group A (EnvGrA), AJ517450–61; *Glomus diaphanum*, AJ972457–63; *Glomus sinuosum*, AJ437105–6; *Glomus proliferum* (1), FM992388–99; *G. proliferum* (2), FM992400–2, AJ973393; FL208 (1), FM865545, 548–549;559–560, FM865573, 576, 577, 585, 597–598, 600, 605, AM980862–63; FL208 (2), FM865572, 574, 575, 579, 581, 583, 586–87, 602–3; FL208 (3), FM865547, 61–71, 78, 84, 601, 606–7, AF185661–64, AF185667–68; VA110, AF185674–76; KS906, AF185669–73; *Glomus* cf. *clarum* Att894–7, FM865536–44; CL883A, AJ239123, AJ243275; GR104, AF185651; EY118, AF185684–86; VA110, CA502, AM980854–59; Environmental (1), EF989103–05, AJ567352, EF989109–12; EF989106, 108; AJ567773, AJ968411; AJ567769–70; BEG195 (1), FM865588–90, 92–94, 96; *Glomus* Błaszkowski, FJ009593–94, FJ009596–604; BEG158 (1), AF394752–53, 58, 67, 72–73, 78, 81; *Glomus irregulare*, FJ009605–18; DAOM197197 (1), FM992377–80, 83–87, FM865550–58, FM865608–12, FM865614–17, AM980836, AY842570–71; BEG158 (2), AF394750–51, 54, 56, 57, 59–64, 69–71, 74, 77, 79, 80, AY035641; Cultures 1 (Cult.1), AF197917, 20 (SW103) AF197919 (MD211), AJ968410 (BEG75), AM980834 (JJ141), 37, 38 (CR316), 39 (DD–4), 40, 41 (JJ183), 42, 43 (AU212), AF185652, 53, 55, 56 (IS101), 60 (NB106), AY997054 (AFTOL 845), AJ557006 (A4,XVI),8 (D2,V), EU221582 (A2, XVIII); BEG158 (3), AF394765, 68, 76; Cult. 2, AM980835 (JA202), AM980833, AY035639 (JJ141), AM980844 (JJ145), AM980846 (KE114); BEG195 (2), FM865591, 95; Environmental (2), AJ517773–75, 77, 79–80, AJ504622, 28–29, AJ416417, AJ968411–12, AJ567733, 35–39, 61–62, 64, 66–68.

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Discussion

The species *G. intraradices* is defined from its type material and the accompanying protologue. Unfortunately, the latter, while perhaps being adequate at the time it was written, does not describe characteristics in the detail required by recent species descriptions for glomeromycotan organisms. Indeed, defining morphospecies within the clade in which *G. intraradices* is placed by molecular analysis (GlGrAb, Schwarzott *et al.*, 2001) seems to be difficult. *Glomus intraradices* produces spores both in the roots and in the substrate. The presence of intraradical spores itself is not a species-specific character but a symplesiomorphy shared with other AMF species. These intraradical spores have considerable variation in size, shape, subtending hyphal characteristics and reaction to Melzer's reagent, and generally a similar wall structure. The extraradical spores are predominantly globose to subglobose. A detailed description of the morphological characteristics of the species is currently in preparation for publication elsewhere, based on a re-examination of the type and study of both ex-type material and a new isolate obtained in pot culture and ROC from the type locality.

Organisms described as *G. intraradices* encompass more than one species

Our results revealed that many cultures or isolates frequently used in AM research and named *G. intraradices* very likely do not correspond to that species. In particular, the model fungus DAOM197198 cannot be phylogenetically resolved as *G. intraradices* (Fig. 1). This is in agreement with recently published studies dealing with mtLSU and nuc5.8S-ITS2 sequence data (Börstler *et al.*, 2008) and analyses of nucITS2 sequences (Jansa *et al.*, 2002b), although these studies did not focus on the species concept of *G. intraradices*. Based on our analyses, the name *G. intraradices* should only be applied for the INVAM FL208 descendants (ex-type), MUCL49410 (new isolate from the type locality) and other AMF that share the same phylogenetic and morphological characteristics. Because the recently described species *G. irregulare* clearly clusters with DAOM197198, although this was not shown in the original publication (Błaszkowski *et al.*, 2008), we use the label '*G. irregulare* clade' for this group also containing sequences from BEG195 and well investigated isolates from a field site in Switzerland. The published *G. irregulare* sequences form a weakly supported cluster within that clade (Fig. 1). This is most likely caused by sequencing only one main ITS rDNA variant, because the ITS variability in the published sequences of *G. irregulare* is only *c.* 1% and exceptionally low for GlGrAb. This raises questions of how to define species in such a complex clade. The answer to this question will require extensive further detailed analyses.

It can, however, be deduced that the *G. irregulare* clade (DAOM197198 + BEG195 + *G. irregulare* + Swiss isolates) and the *G. intraradices* clade (FL208 + MUCL49410) represent distinct AMF species, because:

- despite the large intraspecific sequence variability, DAOM197198 sequences from AMF cultures that were widely separated in space and time all cluster together in the *G. irregulare* clade, clearly separated from the *G. intraradices* clade showing even larger intraspecific (and intrasporal) variability;
- BEG195 (from Europe) sequences are embedded within the DAOM197198 (from North America) sequences, and thus within the *G. irregulare* clade;
- no isolate gave rise to any sequence (including those from the database) that cluster with members of the other clade, indicating that no rRNA gene flow takes place between the two clades. If the *G. irregulare* and the *G. intraradices* clades would be conspecific, we would expect at least some sequences crossing the borders of the phylogenetic clades. This expectation is supported by recently published evidence for genetic recombination between *G. irregulare* clade members (Croll & Sanders, 2009);
- the *G. intraradices* and the *G. irregulare* clades separate clearly from each other and from those representing closely related species (*G. proliferum*, *G. clarum*).

Conclusively, the new isolate (MUCL49410) from the type-locality corresponds phylogenetically to the *G. intraradices* FL208 ex-type culture. This is an indication that FL208 had not been contaminated over the years and should be accepted as indeed corresponding with the type. From these results, the earlier morphological identification of DAOM197198 and BEG195 as *G. intraradices*, while perhaps satisfying earlier morphological criteria, is shown to be incorrect by our molecular evidence.

Morphological, phylogenetic and biological species concepts

A morphological species concept is historically used for AMF, but a biological concept is preferable if it can be defined. A phylogenetic concept based on nonparalogous molecular markers may be congruent with a biological concept. It might be characterized by using just a single marker but only if such a marker coincides with the species boundaries.

With these concepts in mind, we have investigated some closely related AMF morphospecies in the past, to find out if they can be distinguished by their rDNA sequences. For example, morphological identification was difficult in the family *Ambisporaceae* in which some species form both acaulosporoid and glomoid spores. Molecular analyses proved that they were well separated from either the *Acaulosporaceae* or the *Glomeraceae* and ITS region analyses allowed separation of what were interpreted as different species, improving the morphological concept (Sawaki *et al.*, 1998; Redecker *et al.*, 2000; Walker *et al.*, 2007). Nevertheless, the resolution of the ITS region seems to be limited, and in GlGrAb the situation

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is complicated. On one hand, isolates in this group were named as *G. intraradices* (e.g. DAOM197198, BEG195 and isolates from Switzerland) but are phylogenetically clearly separated from the clade that actually includes *G. intraradices* (FL208). On the other hand, *G. irregulare* seems morphologically different from both DAOM197198 and BEG195 (Błaszkowski *et al.*, 2008), although from the molecular evidence presented here, they might be interpreted as being conspecific.

How can we explain such a situation? One simple reason could be morphological plasticity making it difficult to distinguish species microscopically in this group of AMF. *Glomus irregulare* is described from supposedly consistent morphological characteristics, which are not shared by DAOM197198. However, the DAOM197198 descendent Att690-23 is also morphologically different from other DAOM197198 cultures, but supposedly shares the same ancestry. There is, moreover, some preliminary evidence that plasticity may relate to host plant species or culture conditions (Walker, 2008). It is thus possible that morphological differences in this group do not consistently correlate with phylogeny, but we cannot yet draw final conclusions about these aspects.

Possible plasticity may be correlated with the theory of conspecificity of different genotypes of Swiss AMF from one field site belonging to the *G. irregulare* clade. Some of these showed different growth characteristic phenotypes (Koch *et al.*, 2004) and analyses indicated recombination events, at least for the studied genotypes II (isolate B3) and VI (isolate D2), between isolates (Croll & Sanders, 2009). Anastomosis compatibility experiments using five isolates from that field site indicated that isolates with different genotypes (A4 = XVIII, B3 = II, C2 = XV, C3 = XVII, D1 = VIII) can anastomose and that some progenies of C2 and C3 were genetically recombinant (Croll *et al.*, 2009). It is possible that *G. irregulare* and DAOM197198-like fungi may be in one anastomosis compatibility group. To answer such questions, AMF must be cultured as isolates.

Regarding a phylogenetic concept for GlGrAb, species cannot reliably be separated by analyses of the ITS region (Fig. 2). For *G. intraradices*, a phylogenetic signal in the ITS2 region, which was not found in earlier works analysing either 5.8S + ITS2 or ITS2 only (Jansa *et al.*, 2002b; Börstler *et al.*, 2008), hindered phylogenetic resolution. However, when using the full-length (including the 3′ SSU and 5′ LSU rDNA region) fragments, the *G. intraradices* and *G. irregulare* clades were clearly separated. Our species concept is also in line with mitochondrial marker analyses (Börstler *et al.*, 2008). We cannot yet conclude whether sequences clustering as more ancestral in a subclade may represent pseudogenes, but if this were to be the case they evolved after speciation because the full-length fragment carries the phylogenetic signal separating the clades. However, it is evident that concerted evolution of the rDNA repeats is extremely relaxed in members of GlGrAb.

Sequence variability and DNA barcoding

For the SSU + ITS + LSU rDNA fragment sequences the phylogenetic analyses resulted in a well-supported tree topology separating *G. intraradices* and the *G. irregulare* clade. We obtained the same results with a manual alignment and after fully automated alignment with MAFFT (Fig. 1), showing that a relatively simple, automated phylogenetic approach could resolve these two AMF subclades, interpreted as containing different species. We cannot yet conclude whether the fungi in the *G. irregulare* clade indeed are all conspecific, but from the molecular evidence this may well be the case.

The current discussion about fungal DNA barcoding (species identification) focuses on the ITS region, because of its historical use for identification of fungi (Nilsson *et al.*, 2008). An important question is: 'how can AMF species be distinguished and identified by potential DNA barcoding methods?' This question is directly related to a species concept and the enormous intraspecific rDNA variability in AMF. In their recent publication about suitability of different rDNA regions for fungal DNA barcoding Nilsson *et al.* (2008) calculated an average glomeromycotan ITS variability of 7.5%. However, most data on *Glomeromycota* published therein will require thorough reinterpretation because of inaccurate species definition. For example, from 36 ITS sequences used to calculate 7.6% intraspecific ITS variability for *G. versiforme*, three are from a well-defined culture (BEG47), three are most likely from the same organism (although without identifiers in the public database) and the remaining 30 (> 83%) sequences analysed stem from environmental roots or spores without any reliable species affiliation. There is even evidence that the different *G. versiforme* sequences encompass distinct species (Gamper *et al.*, 2009). Another dataset is composed of 12 sequences from six different *Paraglomus occultum* cultures, but because the species concept among *Paraglomus* is not yet well defined it is unclear whether these cultures indeed are conspecific. For the members of the *G. irregulare* clade, Nilsson *et al.* (2008) report 8.7%, intraspecific ITS variability and Jansa *et al.* (2002b) up to 18% for *G.* sp. BEG158 (Fig. 2) intrasporal ITS variability. For one *G. intraradices* FL208 spore we could show > 23% variability. Some further values reported for AMF are 9% for *Gigaspora margarita* (Lanfranco *et al.*, 1999), 6% for *Glomus mosseae* (Lloyd-Macgilp *et al.*, 1996), and 13.4% (when calculated based on the alignment of Walker *et al.*, 2007) to 9.8% (median of absolute uncorrected (Hamming) distances calculated after automated pairwise alignments; Nilsson *et al.*, 2008) for *Ambispora leptoticha*.

Although these values cannot yet be conclusively compared because of the different of clones or variants, general sampling densities, alignments and calculation methods, it appears that *G. intraradices*-related AMF in the clade GlGrAb show considerable sequence variability within the *Glomeromycota*. The LSU variability reported here is also higher than in most

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other studies, especially in the *G. intraradices* FL208 type culture (11.8%). The uncorrected p-distances for the diversisporacean *Glomus aurantium*, *Glomus eburneum* and *Diversispora celata* are 6.6, 1.4 and 2.5%, respectively, in the partial LSU region sequences analysed (Gamper *et al.*, 2009). Generally, our results show that it will be necessary to include the 5′ LSU region in addition to the ITS region for DNA barcoding of AMF.

How many sequence variants in one species?

Assuming that *Saccharomyces*, *Aspergillus* and *Neurospora* spp. have 45–200 rDNA repeats (Kobayashi, 1998; Simon & Weiss, 2008) and the AMF *Scutellospora castanea* 75 (Hosny *et al.*, 1999), fewer than 200 rDNA repeats would be expected within a nucleus of the AMF investigated here. It is still debated whether AMF are homokaryotic (Pawlowska & Taylor, 2004) or heterokaryotic (Hijri & Sanders, 2005), but the data presented here cannot resolve this question (see later). There are at least 149 ITS sequence variants from the different cultures and isolates in the *G. irregulare* clade (Fig. 2). For DAOM197198, we found 23 different ITS variants in the 30 sequences published here. From the public database, five additional sequences were identified for the complete ITS region, and further four covering ITS1 only. This makes 32 variants for DAOM197198, but the total number will be higher. This variability is derived from different cultures, which means that it potentially includes variants derived from recombination in different culturing lineages and may be higher than the number present in one spore. The variability found is not too high to be encoded within one nucleus. An interesting question is whether the very high variability in the GlGrAb rDNA is also reflected in other parts of the genome, which might be a problem when using members of this AMF lineage as genetic model systems.

Other subclades comprising *G. intraradices*-like AMF species

There are several other species known in the GlGrAb clade, and it was recently indicated that the only published *G. proliferum* sequence might cluster as a sister lineage to *G. intraradices* FL208 (Börstler *et al.*, 2008). However, the longer sequences of *G. proliferum* published here form a clade separated from the *G. intraradices* and *G. irregulare* clades, although not with high bootstrap support. Based on the analyses of the ITS region alone further, well-separated clades could be considered as likely to represent distinct species. The cluster EnvGrA, named as '*G. intraradices* Type B' (Renker *et al.*, 2005), represents a distinct species, being more distant from *G. intraradices* than from species such as *Glomus diaphanum* and *Glomus sinuosum*.

For some of the AMF investigated, phylogenetic relationships cannot be satisfactory interpreted, such as for INVAM VA110. Some VA110 sequenced cluster with FL208, as already indicated by Börstler *et al.* (2008). These were submitted to the database in 1999 as part of a *G. intraradices* dataset that obviously included many contaminant sequences, as shown by their phylogenetic placement in different AMF orders. On the INVAM website it is noted that VA110 was derived from a mixed culture containing several species. VA110 is listed there as *Glomus* sp. on the 'Accessions Culture Information' pages and it is likely that sequences appearing in distinct phylogenetic clades actually represent different organisms. In general, sequence data from mixed cultures should be interpreted with caution.

In this study, we showed that *G. intraradices* (FL208 ex-type culture and MUCL49410 isolated from the type locality) clearly separates from the AMF in the *G. irregulare*-clade (DAOM197198 + BEG195 + Swiss isolates). Further, even more distant clades (e.g. EnvGrA) that were annotated as *G. intraradices* represent different, possibly undescribed AMF species. The model fungus used in AM research, DAOM197198, does not represent *G. intraradices* and is closely related to or perhaps even conspecific with *G. irregulare.*

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7 DNA barcoding of arbuscular mycorrhizal fungi

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7.1 Abstract

- Currently, no official DNA barcode region is defined for the *Fungi*. The genes COX1 and mtLSU turned out to be difficult to apply, and the ITS region, the primary candidate for a fungal DNA barcode, was shown not to resolve closely related species of arbuscular mycorrhizal fungi (AMF).
- DNA barcoding analyses were performed with datasets from several phylogenetic lineages of the *Glomeromycota*. We tested an approx. 1500 bp fragment of the nuclear ribosomal DNA, covering approx. 240 bp of the SSU and 800 bp of the LSU rRNA genes and the complete ITS region, for species resolving power. Moreover, the complete ITS region, the 800 bp LSU rDNA, as well as three shorter fragments, spanning the ITS2 and 5.8S rRNA gene, the LSU-D1 rDNA domain, or the LSU-D2 domain, were analysed.
- The results show, that only the longest fragment resolves all analysed species. All other, shorter fragments failed to distinguish some closely related species.
- We recommend using the 1500 bp fragment as a base for phylogenetic DNA barcoding of AMF. This will also allow future identification of AMF at species level using shorter DNA amplicons, e.g. from deep sequencing approaches.

7.2 Introduction

The aim of the present study was the definition of a DNA barcoding region for arbuscular mycorrhizal fungi (AMF), which is also useful for molecular in-field community studies. Despite the fact, that AMF are perhaps the most important fungi in terrestrial ecosystems, forming intimate, mutualistic symbioses with approximately 80% of land plants (Brundrett, 2009), much of their biology still is enigmatic. The asexual, obligate symbiotic and below ground lifestyle makes AMF difficult to study and, e.g., the nutrients transport capabilities and efficiencies in different AMF-plant associations, formed by individual species or species combinations, are little understood. Different functional traits and mechanisms regarding plant nutrition by AMF are expected to exist, but it is mostly unknown which AMF (communities) preferentially associate with which plants, and under which environmental conditions. As long as AMF species cannot be reliably identified and monitored in the field, such questions are difficult to answer and the lack of such basic data will hinder the exploration of functional and causal aspects for differential AMF-plant associations. Moreover, a better understanding of AMF-plant associations and preferences may also directly

impact human life, e.g. by improvement of sustainable management practices in agriculture and forestry, making use of efficient AMF-plant combinations.

7.2.1 Identification of AM fungal species from the field

All AMF belong to the phylum *Glomeromycota* (Schüßler *et al.*, 2001). Historically, species recognition was mainly through spore morphology, but modern molecular methods revealed cryptic species and, on the other hand, spore types that were affiliated to distinct families turned out to be of conspecific origin. A well known example are the spore morphs of *Ambispora leptoticha*, which were thought to represent different species but in fact are an example of spore dimorphism (Sawaki *et al.*, 1998; Redecker *et al.*, 2000; Walker *et al.*, 2007). Many AMF community analyses are based on morphologically monitoring AMF spore occurrences in the soil (e.g., Oehl *et al.*, 2009; Robinson-Boyer *et al.*, 2009). However, such assays use resting stages and consequently do not necessarily reflect the AMF that are physiologically active in the soil and plant roots (Sanders, 2004). Also, relatively little is known about the AMF sporulation behaviour, which may depend on season, environment, or host plant, and change over both space and time (Walker *et al.* 1982).
To overcome such drawbacks, molecular methods were developed to detect AMF directly within roots. The most frequently used markers are one or more of the nuclear rRNA genes, e.g. the widely used SSU rRNA gene (Helgason *et al.*, 1999; Wubet *et al.*, 2006; Lee *et al.*, 2008; Öpik *et al.*, 2009), the ITS rDNA region including the 5.8S rRNA gene (Wubet *et al.*, 2004; Hempel *et al.*, 2007; Sýkorová *et al.*, 2007), and a part of the LSU rRNA gene (Turnau *et al.*, 2001; Gollotte *et al.*, 2004; Gamper & Leuchtmann, 2007; Pivato *et al.*, 2007). However, also many molecular analyses are biased, as often only parts of the AMF community are detected with the used primers and the taxon-resolution level is uncertain. Moreover, neither a single molecular marker suitable for species level resolution of all AMF, nor a comprehensive and validated sequence database is yet available. Nevertheless, recent research shows that species level community analyses should be feasible based on rDNA regions (Gamper *et al.*, 2009; Krüger *et al.*, 2009; Stockinger *et al.*, 2009).

7.2.2 DNA barcoding for species definition and identification

DNA barcoding is presently defined as the analysis of an easily amplifiable PCR fragment for sequence based identification of species. Identifications must be accurate, rapid, cost-effective, culture-independent, universally accessible, and usable by non-experts (Frézal & Leblois, 2008). In addition, cryptic species could be recognized, and organisms can be identified in life cycle stages

not suited for morphological identification (Gilmore *et al.*, 2009).

In DNA barcoding, species are separated by a 'barcode gap' analysis or by fast phylogenetic analysis. A barcode gap is based on the difference between the maximum intraspecific sequence variation of a species and the minimum interspecific variation. If the minimum interspecific variation is bigger than the maximum intraspecific variation, a barcode gap exists. Alternatively, phylogenetic neighbour joining analysis based on Kimura two-parameter (K2P = K80) distances are often used. Though these are the currently preferred methods, in future more sophisticated phylogenetic methods will probably be developed.

For animals, a part of the mitochondrial *cytochrome c oxidase 1* (*COX1*) gene has become the first official DNA barcode (Hebert *et al.*, 2004) (http://www.barcoding.si.edu/) and a barcoding system for plants was established recently, based on the *rbcL* and *matK* loci (Hollingsworth *et al.*, 2009).

7.2.3 DNA barcode(s) for fungi

There is not yet an officially agreed DNA barcoding method for fungi. Such a DNA based species identification system would be very useful, as most fungi cannot be identified in their vegetative, hyphal growth phase. There are approximately 100000 named fungal species (Kirk *et al.*, 2008), and estimates suggest there may be as many as 1.5-3.5 million species in existence (Hawksworth, 2001; O’Brien *et al.*, 2005), many of them perhaps will only be accessible by molecular methods, in future.

The nuclear ITS rDNA region is commonly used for fungi since long (White *et al.*, 1990; Gardes & Bruns, 1993) and will probably be proposed to the Consortium for the Barcode of Life (CBOL, www.barcoding.si.edu) as a fungal barcode (Seifert *et al.*, 2009). Unfortunately, quality problems exist for fungal barcoding, as sequence data are often derived from inaccurately identified material (Ryberg *et al.*, 2008), the lack of vouchers precludes verification (Agerer *et al.*, 2000), and third party annotations in sequence database as GenBank are not possible (Bidartondo *et al.,* 2008). Initiatives like UNITE (http://unite.ut.ee) were established to provide validated and curated data, but such data are still lacking for most fungi, AMF inclusive.

7.2.4 *COX1* is not suited as general fungal barcode

A prerequisite for not using the *COX1* region, according to the CBOL standards, should be the demonstration that it is unsuitable for easy species identification. Although this region showed promise for *Penicillium* spp. (Seifert *et al.*, 2007), the length of fungal *COX1* is highly variable

(from 1.6 to 22 kb). The shortest potential barcoding region varies in length from 642 bp to >12 kb (Seifert, 2009), presenting difficulties for PCR-amplification and sequencing. Moreover fungal species level discrimination with *COX1* genes is inaccurate (Chase & Fay, 2009) and in *Fusarium* and the *Aspergillus niger* complex multiple paralogous copies hinder species level resolution (Geiser *et al.*, 2007; Gilmore *et al.*, 2009). For AMF, the barcoding region of *COX1* in *Glomus* sp. FACE#494 spans 2200 bp and contains introns (Lee & Young, 2009). Land and Hijri (2009) stated that the mtDNA of *Glomus diaphanum* contains a *COX1* intron with high sequences similarity to a corresponding *COX1* intron detected in plants and *Rhizopus oryzae*. The plant intron probably originated by horizontal gene transfer (HGT) from fungi (Vaughn *et al.*, 1995; Lang & Hijri, 2009), further questioning the general usability of *COX1* as a barcode for fungi, and also plants.

As *COX1* seems unsuited for *Glomeromycota*, the mitochondrial large subunit (mtLSU) rRNA gene was considered as an alternative. Unfortunately it also seems inapplicable because introns create very variable fragment sizes even in closely related species (Börstler *et al.*, 2008).

7.2.5 Defining a DNA barcoding region for AMF

The goal of the present study was to compare different nuclear rRNA gene regions with the ITS rDNA region for use as a general DNA barcode for *Glomeromycota*, and to make recommendations based on the analyses of new rDNA sequence datasets. This directly relates to the applicability of environmental deep sequencing approaches using the 454 GS-FLX Titanium system, currently allowing approx. 400 bp average read lengths (www.454.com). It was already demonstrated, that the highly variable ITS rDNA region cannot discriminate some closely related species, e.g. in *Glomus* Group Ab (‘*Gl. intraradices* group’), which includes the model AMF *Glomus* sp. DAOM197198 (Stockinger *et al.*, 2009).

For convenience, we further on abbreviate the nuc SSU rRNA gene, as SSU, the nuc LSU rRNA gene, as LSU, and the 5.8S rRNA gene, as 5.8S; the term ITS region is used for the complete ITS1-5.8S-ITS2 rDNA (Fig. 3). In this study, a DNA fragment of approx. 1500 bp was sequenced from species in divergent AMF clades, covering approx. 240 bp of the SSU, the complete ITS region, and approx. 800 bp of the LSU. We compared the complete fragment (1420-1602 bp), the ITS-region (400-526 bp), the LSU-region (776-852 bp), and three 400-500 bp fragments, covering the 5.8S+ITS2, LSU-D1, or LSU-D2, respectively, for species resolving power and suitability as DNA barcodes.

7.3 Material & Methods

7.3.1 Taxa and public sequences used for analyses

The 'core dataset' sequences investigated in this study (Table 1) cover the partial SSU, the ITS-region, and the partial LSU, at least corresponding to a fragment spanning the region amplified with primers SSU-Glom1 (Renker *et al.*, 2003) and NDL22 (van Tuinen *et al.*, 1998). For all AMF analysed, a culture identifier or a voucher deposited in a herbarium (W-numbers) are known, for most both information is available. The attempt (Att) numbers refer to the collection of Christopher Walker, BEG identifiers to the ‘International bank for the *Glomeromycota*’ (http://www.kent.ac.uk/bio/beg), INVAM to the international culture collection of (vesicular) arbuscular mycorrhizal fungi (http://invam.caf.wvu.edu) and MUCL to the *Glomeromycota* in vitro collection (GINCO; http://emma.agro.ucl.ac.be/ginco-bel/). In addition some other identifiers are listed for certain AMF species (Table 1).

For analysis of the five AMF species analysed in the AFTOL project (James *et al.*, 2006), the individual SSU, ITS and LSU sequences of each species were assembled to a consensus sequence. For the ‘extended dataset’ analyses of the *Ambisporaceae, Diversisporaceae* and *Glomus* Group Aa, additional public database sequences were included (Table S1-S5) Sequences indicated in Schüßler *et al.* (2003) as probably derived from contaminants were not used.

Table 1: Sequences used to assemble the core dataset. Number of spores used for DNA extraction is shown, if known (ss, single spore; ms, multi-spore), as well as cloning numbers (in parentheses, following the number of sequences) and the primers used for the new sequences published here (in parentheses, following the accession numbers; [n], amplified by nested PCR).

Identifier, culture/voucher	**Species name**	**No. of sequences**	**DNA extraction**	**Acc Nos.**
BEG12, Att109-20/W5147	*Glomus mosseae*	8 (pHS101), 7 (pHS110)	1 x ss	FN547474-6,82-93 (SSUmCf-LSUmBr [n])
WUM3, Att15-5/W2939	*Glomus* sp. WUM3	5 (pMK23)	ss	FN547477-81 (SSUGlom1-NDL22 [n])
MUCL41827, -/-	*Glomus proliferum*	2 (pHS113)	ss	FN547500-1(SSUmCf-LSUmBr [n])
BEG13, -/W5258	*Acaulospora laevis*	7 (pHS054)	ss	FN547507-12, 16 (SSUmAf-LR4+2)
Att423-4/W3077	*Acaulospora* cf. *laevis*	6 (pHS032)	ss	FN547502-6,17 (SSUmAf-LR4+2)
BEG26, -/-	*Acaulospora* cf. *laevis*	5 (pHS030)	ss	FN547513-5,18,19 (SSUGlom1-NDL22[n])
INVAM TW111, Att1499-9/W5346	*Kuklospora kentinensis*	4 (pHS098)	ss	FN547520-3 (SSUmCf-LSUmBr [n])
Att1235-2/W5156	*Ambispora appendicula*	11 (pMK096)	ms (3 spores)	FN547524-34 (SSUmAf-LSUmAr)
Att200-23/W4752	*Ambispora fennica*	12 (pMK094)	ss	FN547535-46 (SSUmCf-LSUmBr [n])
BEG34, -/-	*Gigaspora margarita*	24 (pHS108)	ss	FN547547-70 (SSUmAf-LSUmAr)
DAOM194757, Att1509-20/W5384	*Gigaspora rosea*	6 (pHS106), 18 (pHS105), 3 (pHS104)	1 x ss	FN547571-97 (SSUmCf-LSUmBr [n])
FCPC1145, Att590-16/W5342	*Scutellospora gilmorei*	21 (pHS107), 5 (pHS103)	1 x ss	FN547598-622 (SSUmCf-LSUmBr [n])
Att1505-8/W5347	*Glomus etunicatum*	12 (pHS112)	ss	FN547623-34 (SSUGlom1-NDL22 [n])
BEG20, Att263-15/W3294	*Glomus caledonium*	6 (pHS031)	ss	FN547494-9 (SSUGlom1-NDL22 [n])
BEG47, Att475-45/W5165	*Glomus versiforme*	2 (pHS034)	ss	FN547635-6 (SSUGlom1-NDL22 [n])
BEG47, Att475-22/W3180	*Glomus versiforme*	10 (pMK73), 6 (pMK72)	2 x ss	FN547666-81 (SSUmAf-LR4+2)
Att1296-0/W4728	*Glomus aurantium*	11 (pHS109)	ss	FN547655-65 (SSUmCf-LSUmBr [n])
Att246-18/W4119	*Diversispora spurca*	18 (pHS100)	ss	FN547637-54 (SSUmCf-LSUmBr [n])
WUM18, Att869-3/-	*Acaulospora* sp. WUM18	2 [1]	ss	FM876792-3
BEG33, Att209-37/-	*Acaulospora scrobiculata*	4 [1]	ss	FM876788-91
BEG231, FACE#234	*Diversispora celata*	3 [2]	ms	AM713402-4
INVAM AZ420A, Att1290-5/W4729	*Glomus eburneum*	12 [2]	ms	AM713405-16
BEG28, Att108-7/-	*Glomus coronatum*	5 [1]	ss	FM876794-8
WUM3, Att15-5/W2940	*Glomus* sp. WUM3	1 [1]	ss	FM876813
INVAM SA101, Att676-5/-	*Glomus luteum*	5 [1]	ss	FM876808-12
Att565-11/W3349	*Glomus* sp. W3349	4 [1]	ss	FM876804-7
WUM11, Att862-7/W2928	*Acaulospora laevis*	8 [1]	ss	FM876780-7
Att894-7/-	*Glomus* cf. *clarum*	9 [3]	ss	FM865536-44
DAOM197198 related, -/W5533, W5495, W3182, W5499; BEG195, -/W5272	*Glomus* sp. 'irregulare-like'	39 [3]	4 x ss, 1 x ms (3 spores)	FM865550-8, FM865588-96, FM865608-17, FM992377-87
INVAM FL208, -/W5413, W5166, W5507; MUCL49410, -/W5070	*Glomus intraradices*	45 [3]	4 x ss	FM865545-49, FM865559-87, FM865597-607
-/W4545	*Pacispora scintillans*	2 [1]	ss	FM876831-2
INVAM TW111, Att1499-9/W5346	*Kuklospora kentinensis*	10 [1]	ss	FM876821-30
MUCL41827, -/-	*Glomus proliferum*	15 [3]	1 x ss, 1 x ms	FM992388-402
-/W3009	*Scutellospora spinosissima*	3 [1]	ss	FM876834-6

BEG35, Att334-16/-	*Scutellospora heterogama*	3 [1]	ss	FM876837-9
BEG47, Att475-45/W5165	*Glomus versiforme*	7 [1]	ss	FM876814-20
AFTOL-139/ INVAM UT101	*Glomus mosseae*	1 [4]	unknown	Consensus AY635833 + AY997053 + DQ273793
AFTOL-845/ 4695rac-11G2	*Glomus* sp. 'irregulare-like'	1 [4]	unknown	Consensus DQ273828 + DQ322630 + AY997054
AFTOL-48/ DAOM181602	*Glomus* sp. 'irregulare-like'	1 [4]	ms	Consensus AY635831 + AY997052 + DQ273790
AFTOL-138/ INVAM FL225	*Scutellospora heterogama*	1 [4]	unknown	Consensus AY635832 + AY997088 + DQ273792
AFTOL-844/ INVAM IA702	*Paraglomus occultum*	1 [4]	unknown	Consensus DQ322629 + DQ273827 + AY997069

[1] Krüger *et al.* 2009, [2] Gamper *et al.* 2009, [3] Stockinger *et al.* 2009, [4] James *et al.* 2006

7.3.2 DNA extraction, PCR amplification, cloning and sequencing

Spores were cleaned and DNA was extracted as described in Schwarzott & Schüßler (2001). Some spores were crushed in 5x PCR buffer according to Lumini *et al.* (2007). As PCR template, 5 µl of the DNA extract were used in 20 µl final reaction volume. In the early phase of the study, PCR was performed using the primers SSU-Glom1 and NDL22 or LR4+2 (Stockinger *et al.*, 2009). Most AMF cultures studied later were characterised by the nested PCR approach with AMF specific primers (Krüger *et al.*, 2009; see Table 1). PCR using the Phusion High Fidelity DNA polymerase (Finnzymes, Espoo, Finland), cloning, RFLP analyses and sequencing were performed as described in Krüger *et al.* (2009), with exception of *Gl. caledonium* BEG20 which was amplified using a Taq DNA polymerase (Peqlab, Erlangen, Germany) and some clones which were obtained using the StrataClone Blunt PCR Cloning Kit (Stratagene Agilent Technologies, La Jolla, CA, USA), according to the manual. Sequences were assembled and proofread with SeqAssem (http://www.sequentix.de) and deposited in the EMBL/GenBank/DDBJ databases with the accession numbers FN547474-FN547681.

7.3.3 Phylogenetic and sequence divergence analyses

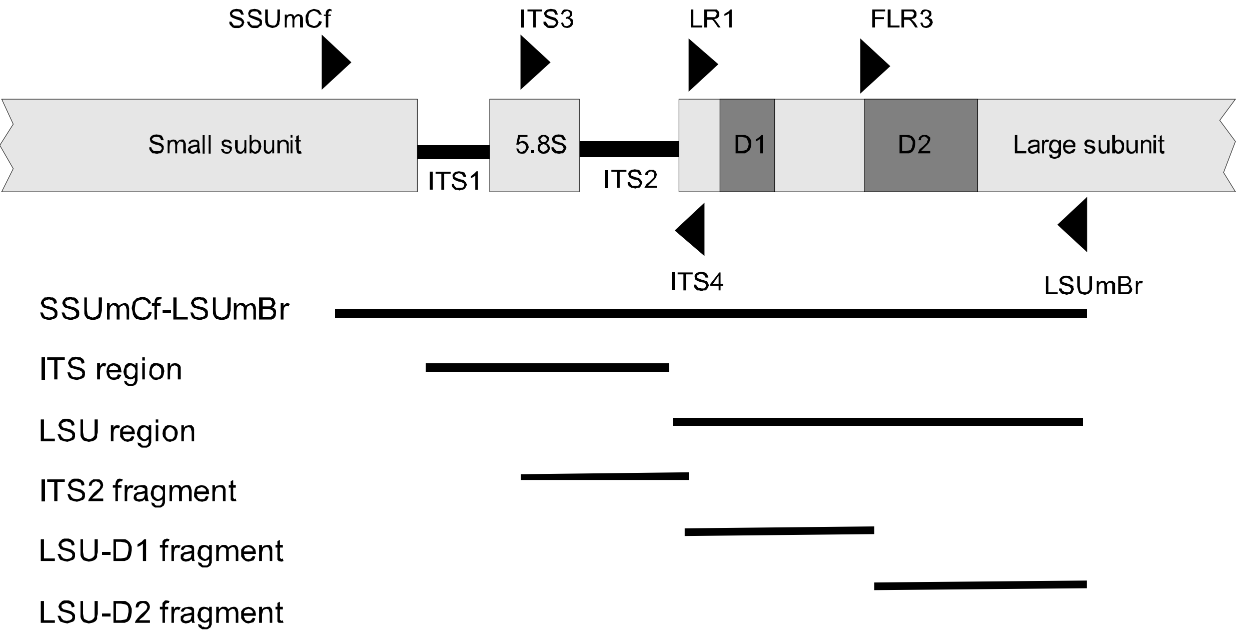
The 3′ partial SSU, the ITS region, and the 5′ partial LSU sequences from this study and public database sequences covering the same regions were analysed for their species resolving power (Table 1). Data were mainly from characterized AMF species, from single spore DNA extractions or single spore isolates. Regions were separated either by the gene borders, or by frequently used primer binding sites. The ITS region (400-526 bp) including the 5.8S was cut at the border to SSU and LSU rRNA genes; the LSU fragment (776-852 bp) covering the 5' LSU rRNA gene region until the binding site of primer LSUmBr (Krüger *et al.*, 2009); the ITS2 fragment (352-430 bp) corresponds to an ITS3-ITS4 (White *et al.*, 1990) amplicon and includes most of the 5.8S rRNA gene and the complete ITS2 region; the LSU-D1 fragment (281-394 bp) corresponds to a LR1-FLR3reverse amplicon (van Tuinen *et al.*, 1998), whereas FLR3 was designed as a forward primer (Gollotte *et al.*, 2004); the LSU-D2 fragment (370-436 bp) corresponds to an FLR3-LSUmBr amplicon (Fig. 3).

For three AMF families, the ITS region or a part of the LSU was analysed after adding shorter or less well defined sequences from the database. Those were aligned to the core dataset with Align (http://www.sequentix.de) or ARB (Ludwig *et al.*, 2004; http://www.arb-home.de). The resulting

dataset is referred to as ‘extended dataset’. Sequence divergences were calculated based on the K2P model (Kimura, 1980) with pairwise deletion of gaps, using the APE package of R (Paradis *et al.*, 2004). To illustrate the sequence divergences within and between species, TaxonGap2.3 (Slabbinck *et al.*, 2008) was used.

The analyses of database sequences included identical sequences, when it was unclear if these originated from the same or different spores or even cultures. Phylogenetic analyses were performed with PHYLIP3.6 (Felsenstein, 2004), using the neighbour joining method (based on K2P model). A consensus tree was calculated from 1000-fold bootstrapped analyses with SumTrees (Sukumaran & Holder, 2008) and the bootstrap (BS) values were mapped on the neighbour joining tree. As an alternative approach, the sequences were aligned automatically using the MAFFT online server (MAFFT version 6; http://align.bmr.kyushu-u.ac.jp/mafft/online/server/), for comparison with the results from the manual alignment. The iterative refinement option of MAFFT was set to FFT-NS-i (Katoh *et al.*, 2002). Phylogenetic trees were processed with TreeGraph2 (treegraph.bioinfweb.info), TreeViewJ (Peterson & Colosimo, 2007), and Treedyn (Chevenet *et al.*, 2006) and refined with Adobe Illustrator CS3.

Figure 3: Schematic representation of the nuclear ribosomal DNA regions studied. Positions of priming sites that were used as borders for in silico analyses of the fragments are shown as black triangles. Black lines indicate the fragments analysed.



7.4 Results

7.4.1 Intraspecific rDNA sequence variation

No general intraspecific percentage of sequence variation (K2P distance) could be defined as a threshold to separate AMF species, even within individual families in the *Glomeromycota*. For the longest DNA fragment studied, SSUmCf-LSUmBr (approx. 1500 bp, see Table S6, corresponding to the core dataset), the variation ranged from 0.47-10.8 %. When taking only the seven species into account for which at least 24 sequence variants are available (*Ac. laevis*, *Gi. margarita, Gi. rosea, Sc. gilmorei, Gl. intraradices*. *Glomus* sp. 'irregulare-like' and *Gl. versiforme*) the minimum intraspecific variation was 1.55 %. The highest value of 10.8 % was found in *Gl. intraradices* (cultures FL208 and MUCL49410).

The ITS region revealed a variation of 0.23-14.6 %, or 2.96-14.6 % when only analysing the seven species with at least 24 variants of the SSUmCf-LSUmBr fragment available, respectively. *Glomus intraradices* (FL208 and MUCL49410) again showed the highest intraspecific variation with 14.6 %. Interestingly, the highest maximum intraspecific variation of 15.7 %, for *Gl. intraradices*, was found in the LSU-D2 fragment (FLR3-LSUmBr). The range of variation in this region was 0-15.7 % (2.8-15.7 % for species with at least 24 variants known).

For the LSU-D1 fragment (LR1-FLR3), five species lacked intraspecific variation (number of distinct sequences in parentheses): *Glomus* sp. WUM3 (6), *Gl. caledonium* (3), *Acaulospora scrobiculata* (4), *Gl. luteum* (5), *Diversispora celata* (3). In general, this region showed the lowest intraspecific variation for most species analysed, but with exceptions. For *Kuklospora kentinensis* (14) a variation of 0.5 % was found in the LSU-D1 fragment but the ITS2 fragment (ITS3-ITS4) showed no variation at all for this species.

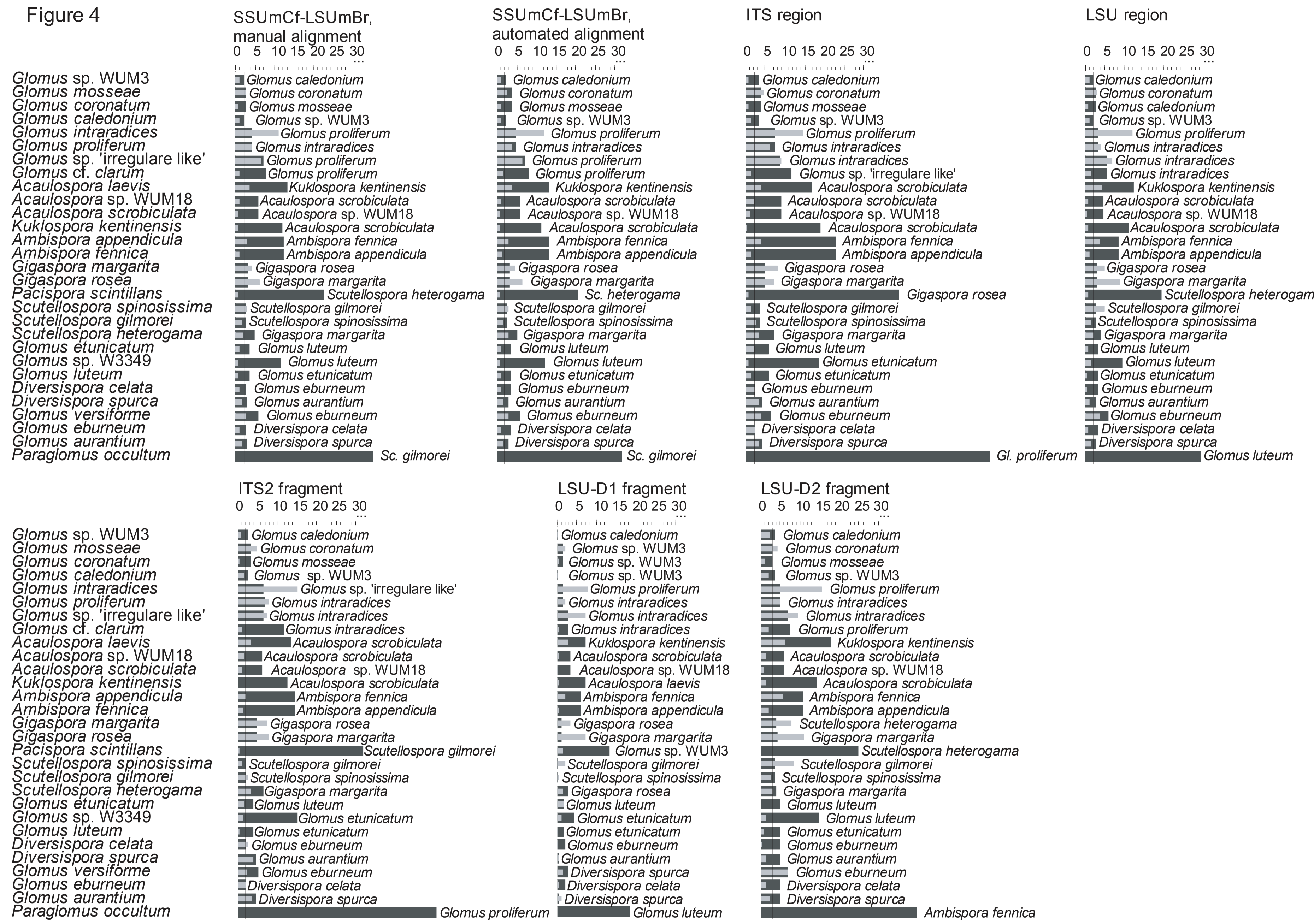
7.4.2 Barcode gap analyses

A barcode gap is not a prerequisite for DNA barcoding, but may be helpful to distinguish species without the need of phylogenetic analyses (e.g., Hebert *et al.*, 2004). Comparison of the different regions, regardless of the alignment method used (Table S6, Figure 4), showed that the complete fragment (SSUmCf-LSUmBr) had the lowest number (4) of species without a barcode gap, followed by the complete ITS region with 5 species and the LSU region with 7. Analysis of the LSU-D2 fragment resulted in 7 species lacking a barcode gap, whereas the less variable LSU-D1 fragment revealed 12 species without a barcode gap. The ITS2 fragment (covering most of the 5.8S)

resulted in 8 species without a barcode gap. For the complete fragment, the size of the existing barcode gaps varied from only 0.1 % to 22 %.

Figure 4: Barcode gap analyses of the rDNA regions studied. The SSUmCf-LSUmBr fragment was aligned either manually or automated (MAFFT). Light bar: maximum intraspecific variation, dark bar: minimum interspecific variation; to the right of the bar the closest species is given, respectively. Scale on top is % variation based on K2P distances. Vertical line indicates the minimal interspecific variation.

Figure 4



7.4.3 Phylogenetic analyses of the core dataset

The *Gigasporaceae, Acaulosporaceae, Diversisporaceae*, *Ambisporaceae*, *Glomus* Group B, *Glomus* Group Aa and *Glomus* Group Ab were analysed separately, as the high variation in the ITS region made it impossible to align across family level groups. For each group, five defined regions covered by the SSUmCf-LSUmBr fragment were analysed (Figure 3). All positions in the alignment were included in the phylogenetic analyses (Figure 5, Supplementary Figs S1-S6), as summarized in Table 2 for the core dataset (Figure 5 and Supplementary Figs S1-S6).
Using the complete fragment (SSUmCf-LSUmBr) resulted in the best discriminatory power. Each of the known species was resolved with bootstrap support of at least 72 %, for most species of >90 %. Almost all species studied could be separated using the complete ITS region, except *Gl. intraradices* and its close relatives. Similarly, maximum likelihood analyses of this region did not resolve sequences of *Gl. intraradices* (FL208 and MUCL49410) and related species as monophyletic clades (Stockinger *et al.*, 2009).
Analyses of the LSU region resulted in the similar problems regarding lacking resolution of *Gl. intraradices* and its close relatives. Two other species, *Scutellospora spinosissima* (3 sequences) and *Gl. proliferum* (15 sequences), were not resolved as monophyletic. In addition, the *Gigaspora rosea* clade (27 sequences) had bootstrap support below 50 %; the other species were separated with support values of at least 55 %. When the three shorter ITS2, LSU-D1 and LSU-D2 fragments were analysed separately, the LSU-D1 fragment performed worst. Sequences from 11 of the 25 species did not form monophyletic clades. The ITS2 and LSU-D2 fragments performed better, but still could not resolve two species (*Gl. proliferum*, 15 sequences; *Gl. intraradices* FL208 and MUCL49410, together 47 sequences), respectively. *Gigaspora margarita* BEG34 did not form a well supported clade for either fragment. The third species not resolved as monophyletic in the LSU-D2 analysis was *Sc. spinosissima* (3 sequences). The AFTOL sequences of *Gl. mosseae* and *Sc. heterogama* cluster with other sequences of the corresponding species, whereas the AFTOL *Glomus* sp. ‘intraradices’ sequences, derived either from culture MUCL43194 (=DAOM197198, =DAOM181602; used for the *Glomus* genome sequencing project) or from “GINCO #4695rac-11G2”, clearly cluster with *Glomus irregulare* in the '*Gl. irregulare*-clade', confirming the evidence of Stockinger *et al.* (2009).

Table 2: Neighbour joining analyses (based on K2P distances, 1000 bootstraps) of six different regions (complete SSUmCf-LSUmBr fragment, complete ITS region, ITS2, LSU, LSU-D1 and LSU-D2 fragments). Respective bootstrap values supporting species as monophyletic are shown.

	SSUmCf-LSUmBr	ITS region	LSU	ITS2 (ITS3-ITS4)	LSU-D1 (LR1-FLR3)	LSU-D2 (FLR3-LSUmBr)
Gigaspora margarita	**88**	**75**	**55**	**47**		**34**
Gigaspora rosea	**100**	**90**	**48**	**90**		**59**
Scutellospora gilmorei	**100**	**99**	**88**	**100**		**69**
Scutellospora spinosissima	**92**	**96**		**95**		
Scutellospora heterogama	**100**	**100**	**100**	**100**	**97**	**98**
Length of alignment (positions)	*1505*	*468*	*795*	*394*	*398*	*376*
Acaulospora laevis	**100**	**100**	**100**	**100**	**100**	**100**
Acaulospora scrobiculata	**100**	**100**	**100**	**100**	**100**	**100**
***Acaulospora* sp. WUM18**	**100**	**100**	**100**	**100**	**100**	**100**
Kuklospora kentinensis	**100**	**100**	**100**	**100**	**100**	**100**
Length of alignment (positions)	*1591*	*525*	*826*	*436*	*403*	*401*
Diversispora celata	**100**	**95**	**100**	**70**	**99**	**100**
Diversispora spurca	**100**	**96**	**100**	**97**		**100**
Glomus aurantium	**100**	**100**	**94**	**95**		**94**
Glomus eburneum	**100**	**75**	**100**	**72**	**99**	**93**
Glomus versiforme	**100**	**100**	**100**	**100**	**100**	**100**
Length of alignment (positions)	*1600*	*497*	*860*	*407*	*398*	*440*
Glomus* cf. *clarum	**100**	**100**	**100**	**100**	**100**	**100**
Glomus intraradices	**72**					
***Glomus* sp. 'irregulare-like'**	**100**	**96**	**99**	**53**		**95**
Glomus proliferum	**94**	**80**				
Length of alignment (positions)	*1644*	*540*	*863*	*437*	*400*	*440*
Glomus mosseae	**100**	**97**	**100**	**93**	**98**	**99**
***Glomus* sp. WUM3**	**100**	**97**	**100**	**98**		**100**
Glomus caledonium	**100**	**100**	**96**	**99**		**97**
Glomus coronatum	**100**	**100**	**96**	**100**	**99**	**99**
Length of alignment (positions)	*1664*	*565*	*862*	*448*	*397*	*442*
Glomus etunicatum	**100**	**99**	**100**	**90**	**96**	**100**
***Glomus* sp. W3349**	**100**	**100**	**100**	**100**	**100**	**100**
Glomus luteum	**100**	**100**	**100**	**100**	**96**	**93**
Length of alignment (positions)	*1624*	*539*	*843*	*433*	*392*	*430*

Figure 5: Phylogenetic tree computed from all approx. 1500 bp SSUmCf-LSUmBr fragment sequences analysed (core dataset), demonstrating species level resolution. Neighbour joining analyses (1000 BS) with BS support displayed down to the level of species. Note that the BS support values differ from those given in Table 2, because an unambiguous alignment of ITS1 and ITS2 sequences between families, as computed here, in fact is impossible. Therefore, the BS values shown here are biased by ambiguously aligned sites in the highly variable regions and for species level comparison the values from Table 2 should be referred to. Left of each cluster the corresponding species is written. For better readability every second cluster is highlighted in grey

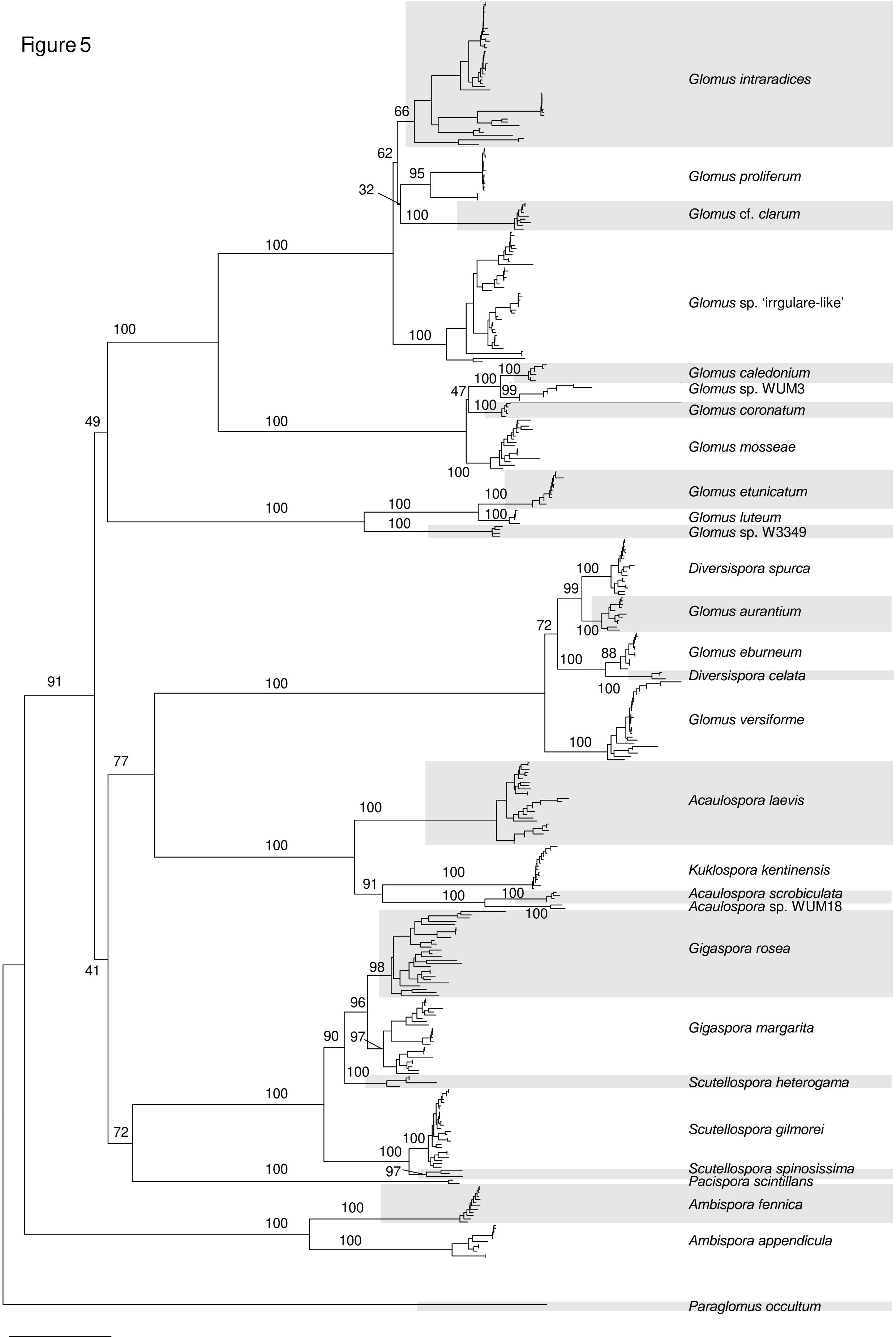


Figure 5
Glomus intraradices
Glomus proliferum
Glomus cf. clarum
Glomus sp. 'irrgulare-like'
Glomus caledonium
Glomus sp. WUM3
Glomus coronatum
Glomus mosseae
Glomus etunicatum
Glomus luteum
Glomus sp. W3349
Diversispora spurca
Glomus aurantium
Glomus eburneum
Diversispora celata
Glomus versiforme
Acaulospora laevis
Kuklospora kentinensis
Acaulospora scrobiculata
Acaulospora sp. WUM18
Gigaspora rosea
Gigaspora margarita
Scutellospora heterogama
Scutellospora gilmorei
Scutellospora spinosissima
Pacispora scintillans
Ambispora fennica
Ambispora appendicula
Paraglomus occultum
0.04

7.4.4 Phylogenetic analyses of the extended dataset

Shorter sequences from the public database were included in some analyses, selected according to their assigned name or culture identifier. Additionally, some environmental sequences were included, predominantly from the *Ambisporaceae, Diversisporaceae* and *Glomus* Group Aa.

7.4.4.1 Analyses of Ambisporaceae

Whereas only for two *Ambisporaceae* species the SSUmCf-LSUmBr fragment is available (Table S6, Figure 4), for the ITS region five *Ambispora* species and several environmental sequences could be analysed. All were phylogenetically well separated, with bootstrap support of at least 82 % (Figure S8). The ITS2 fragment analysis showed a very similar result, with environmental sequences not matching any of the characterized *Ambispora* species. The environmental sequences (number in parentheses) from *Taxus baccata* (6), *Prunus africana* (1) or *Plantago lanceolata* (1) roots form well separated branches, as already shown before (Walker *et al.,* 2007). For all of them the distance to the closest related species or clade is two to three-times higher than the minimal intraspecific distances within *Am. appendicula* (11 sequences)*, Am. leptoticha* (26), and *Am. callosa* (34). The intraspecific variation in the ITS region was 2.3-7.3 %. The barcode gap analysis showed that *Am. leptoticha*, with 7.3% intraspecific variation, lacked a barcode gap to *Am. appendicula* (Figure S7)*,* but not *vice versa*. The phylogenetic analysis clearly separated both species from each other.

7.4.4.2 Analyses of Diversisporaceae

The ITS analyses of the *Diversisporaceae* using the extended dataset (Figure S9) did not reveal any fundamental differences to the analyses of the core dataset (Figure S5). It was already known, that *Gl. fulvum* (5 sequences), *Gl. megalocarpum* (2), and *Gl. pulvinatum* (2) form a clade much apart from the other *Diversisporaceae* species and together probably represent a distinct taxon at the genus level (Redecker *et al.*, 2007). Some additional ITS database sequences formed separated clades, one of them containing sequences from the INVAM cultures AZ237B from Arizona (4 sequences) together with NB101 from Namibia (4 sequences). These eight sequences are most likely conspecific and represent the closest known relative to *Di. celata* (3 sequences), as already stated by Gamper *et al.* (2009). At this point it should be mentioned that several *Glomus* species have not yet been formally transferred to the genus *Diversispora* and therefore carry the ‘wrong’

genus name. The naming of sequences also is misleading for a set of 30 environmental sequences that are annotated as *Gl. versiforme* but separate from *Gl. versiforme* BEG47 (31 sequences) in several sub clades. From our analyses, they should be annotated as unknown *Diversispora* species.
The ITS region of *Gl. fulvum* (5 sequences) showed the highest intraspecific variation in the *Diversisporaceae*, with about 15 %. This is caused by sequence AM818544, which originated from a field collected specimen different from the other sequences. Perhaps AM818544 is a different species. Excluding AM818544, *Gl. fulvum* showed an intraspecific variation of 9.8 %. All other characterized species in the *Diversisporaceae* had a variation below 7 %.
For the LSU analyses (Figure S10), the four database sequences (AM947664,65, AY842573,74) from *Gl. versiforme* BEG47 clustered with the sequences of the core dataset of *Gl. versiforme* BEG47 (25 sequences). The sequence EU346868 from a '*Gl. versiforme*' culture HDAM-4 and that of BEG47 were widely separated, and they are unlikely to have originated from a conspecific fungus. However, further sequences from such cultures are needed for more detailed analyses. The database sequences EF067886-88 clustered with the *Gl. eburneum* sequences, consistent with the fact that all sequences refer to the same culture identifier, INVAM AZ420A. The LSU sequences of *Di. celata* (Gamper *et al.,* 2009) clustered with those of our core dataset. There are three *Gl. aurantium* LSU database sequences (EF581860,62,63), derived from trap cultures, which clustered with the *Gl. aurantium* sequences from our core dataset. However, two additional *Gl. aurantium* sequences (EF581861,64) form a distinct clade. All five sequences, linked to voucher W4728, originate from the same trap culture setup with material collected near Tel Aviv in Israel (J. Błaszkowski, personal communication 21.9.2009). As trap cultures usually contain several species, it is not certain that the sequences were indeed derived from conspecific organisms. Without cultures established as isolates such questions can hardly be answered. The inclusion of the public sequences increased the intraspecific variation of *Di. celata* to 2.6 % (26 sequences), of *Gl. versiforme* to 4.1 % (29 sequences), and of *Gl. aurantium* to 1.9 % (14 sequences). When including the outliers (EU346868, isolate HDAM-4) for *Gl. versiforme* the variation raised to 9.1 % and in the case of *Gl. aurantium* (EF581861,64) to 8.1 %, whereas both species lost the barcode gap to their neighbours (Figure S7).

7.4.4.3 Analyses of Glomus *Group Aa ('*Gl. mosseae *group')*

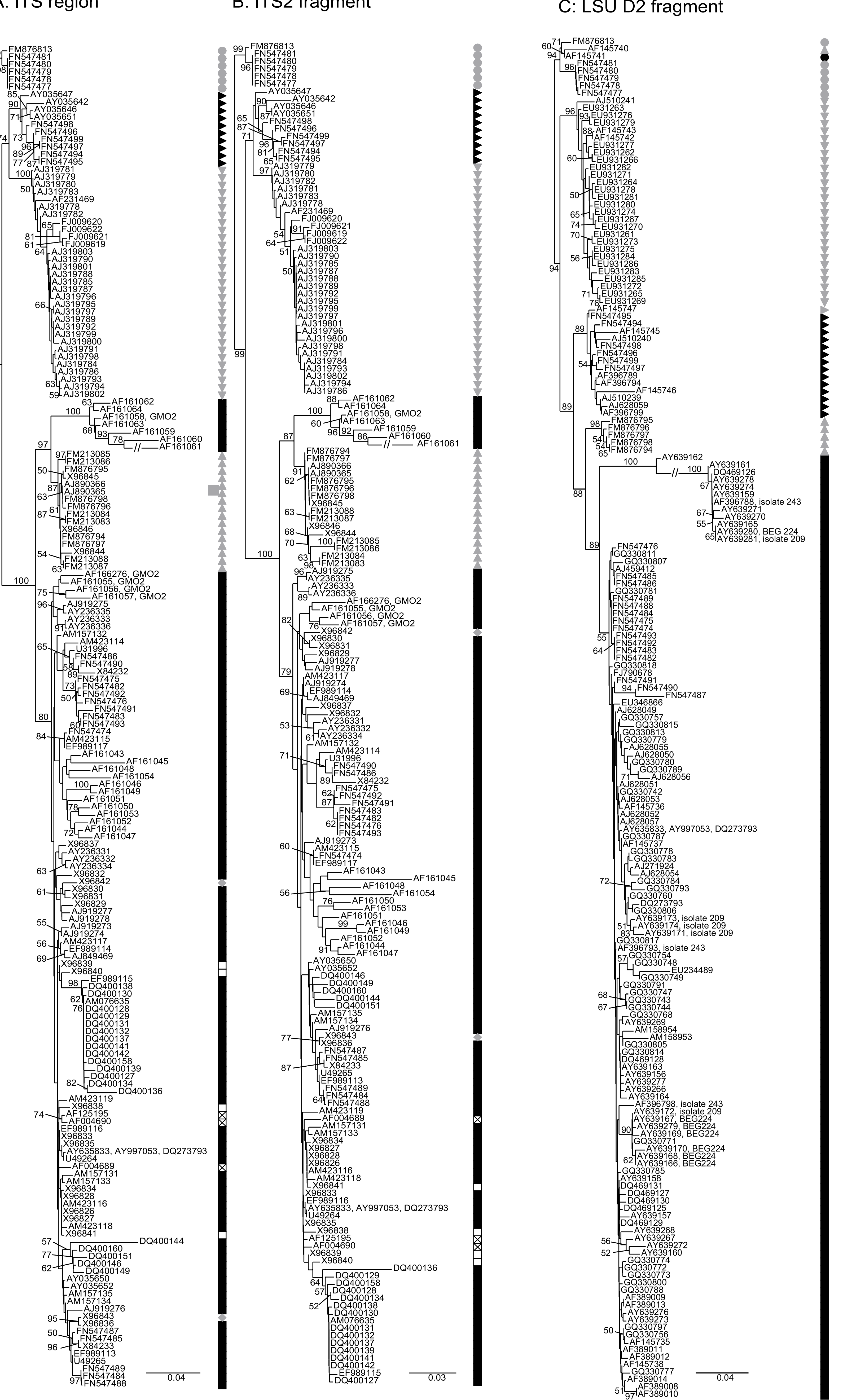
Analysis of our core dataset of this group showed clear separation of species with the ITS region, the ITS2 fragment, and the LSU fragments analysed. However, the situation changed considerably when including additional database sequences for the ‘extended dataset’.

For the ITS region, *Glomus* sp. WUM3 (6 sequences), *Gl. caledonium* (10 sequences) and *Gl. geosporum* (31 sequences) were well separated, but the ex-type of *Gl. coronatum* BEG28 (16 sequences) clade clustered in-between the two *Gl. mosseae* clades (Figure 6). Both *Gl. mosseae* clades were well supported by 80 % and 100 % bootstrap (BS) values, respectively. All new *Gl. mosseae* sequences from the present study, those from Avio *et al.* (2009), and from the databases being annotated as *Gl. mosseae* cluster together in the major clade (109 sequences). The smaller clade consists of seven sequences derived from field sampled spores with identifiers GMO2 and GMO3. One sequence (AF161058) characterised from spore GMO2 clusters in this minor clade while all the other ones (AF161055-57, AF166276) cluster within the major *Gl. mosseae* clade.
The ITS sequences in *Glomus* Group Aa reveal more discrepancies. *Glomus monosporum* (IT102: AF004689; FR115: AF004690, AF125195), *Gl. dimorphicum* (BEG59: X96838-41), and '*Gl. fasciculatum'* BEG58 (X96842,43, but see below) sequences cluster in the major *Gl. mosseae* clade. Another species that has two sequences in the *Gl. mosseae* clade is *Gl. fasciculatum* BEG58. This species' morphology is very distinct from *Gl. mosseae*, as already discussed in Lloyd-Macglip *et al.* (1996), and in contrast to the BEG58 ITS sequences a SSU sequence from *Gl. fasciculatum* BEG53 clusters in the *Glomus* Group Ab (Schwarzott *et al.*, 2001), which is consistent with morphological data. The phylogenetic analysis of the ITS2 fragment revealed a similar separation of the analysed species, but, e.g., the support value for the clade of *Gl. caledonium* decreased from 90 % to insignificant 41 %.
For the *Gl. mosseae* major clade, when taking only the sequences into account that are annotated under this species name, the intraspecific variation of the complete ITS region is 12.1 % (100 sequences). When assuming that, although annotated with distinct names (*Gl. monosporum, Gl. fasciculatum* BEG58*, Gl. dimorphicum*), all sequences clustering within the major *Gl. mosseae* clade are conspecific, but excluding the single outlier sequence from GMO2 and all sequences of GMO3 (minor clade), the variation only marginally increased to 12.2 % (109 sequences). The variation rose to 20.0 % (140 sequences) when adding the GMO2 and GMO3 'outlier' sequences. The intraspecific variation of all other well characterized and supported species within *Glomus* Group Aa varied between 0.8 and 2.8 %.
The LSU-D2 fragment analysis resulted in clear separation into several well supported clades (Figure 6). However, also here, some clades contain sequences from more than one species. One *Gl. fragilistratum* sequence clusters within the *Gl. caledonium* clade. One *Gl. coronatum* BEG49 sequence is far apart from those of ex-type *Gl. coronatum* BEG28 (=Att108). *Glomus coronatum* BEG49 clusters with *Glomus* sp. WUM3, but also a *Gl. constrictum* BEG130 sequence falls in this clade. Within the *Gl. mosseae* clade, 12 sequences form a separated subclade (AY639159,61,62,65,

AY639270,71,74,78,80,81, DQ469126, AF396788; upper subclade in Fig. 6). Interestingly, LSU-D2 sequences from single spore isolates (H. Gamper isolates 209 and BEG224; J. Jansa isolate 243) can be found in both of the *Gl. mosseae* subclades. The major clade (lower subclade in Fig. 6) also harbours all sequences of *Gl. mosseae* analysed by Rosendahl *et al.* (2009), representing cultures from six continents. For all *Gl. mosseae* sequences together, the intraspecific variation of the LSU-D2 fragment is 19.4 % (170 sequences). The major clade had a variation of 15.8 % (158 sequences) and the smaller clade of 11.2 % (12 sequences). All other species in the clade showed an intraspecific variation between 1.2-5.0 % (5-28 sequences).

DNA barcoding of arbuscular mycorrhizal fungi

Figure 6: ITS region (A), ITS2 fragment (B) and the LSU-D2 fragment (C) neighbour joining analyses (1000 BS) of *Glomus* Group Aa. Analysis C is performed with a different dataset than A and B (for details see Table S4, S5). Some long branches were reduced in length to 50 % (//). 'AY635833, AY997053, DQ273793' represents the consensus sequences of these sequences. *Glomus mosseae* (■) *Glomus* sp. WUM3 (●), *Gl. coronatum* (▲), *Gl. caledonium* (▶), *Gl. monosporum* (☒), Gl. fasciculatum (♦), *Gl. geosporum* (▼), *Gl. dimorphicum* (□), *Gl. constrictum* (●), *Gl. fragilistratum* (▶).



DNA barcoding of arbuscular mycorrhizal fungi

7.5 Discussion

We analysed several regions of the nuclear rDNA region as possible candidates for DNA barcoding of AMF, including the widely used ITS region, which probably will become an official barcode for fungi (Seifert, 2009). However, Stockinger *et al.* (2009) already demonstrated that the ITS region is unsuitable to resolve some closely related AMF species, which could be resolved analysing a longer fragment, covering a part of the SSU, the ITS region and part of the LSU. Therefore, we used this approx. 1500 bp long and easily PCR amplifiable PCR fragment (Krüger *et al.,* 2009) as a baseline for the present study. Based on this, shorter DNA fragments were studied for their power for species resolution (DNA barcoding). This was also done with respect to potential species monitoring by 454 GS-FLX Titanium pyrosequencing (www.454.com; Valentini *et al.*, 2009).

7.5.1 Intraspecific rDNA variation

Analyses presented in earlier publications (e.g., Lloyd-Macglip *et al.*, 1996; Jansa *et al.*, 2002; Walker *et al.*, 2007) showed variable nuclear rDNA sequences to exist within one AMF spore, which holds true for all nuclear rDNA regions studied, but at different levels. One individual spore of *Gl. intraradices* (FL208) contains approximately the same extent of variation within the ITS region as found in two *Gl. intraradices* isolates from the same field site, but with 20 years in-between (Stockinger *et al.*, 2009). Based on that result, a single spore isolate or single spore DNA extractions could be interpreted as being valid to roughly define the intraspecific variability, but this will have to be proven in future, also with respect to differences in different phylogenetic lineages.
The intraspecific and intrasporal variation varied considerably between the studied AMF species, for all regions analysed. This may only partly caused by different sampling density, as also when only AMF species with at least 24 known sequence variants were taken into account, the extent of intraspecific variation was diverse. In the present study, we followed the CBOL barcoding rules (www.barcoding.si.edu). This has to be mentioned, as the numbers for sequence variation differ significantly, depending on the method used for estimation. As an example, for *Gl. intraradices* (cultures FL208 and MUCL49410, together 47 sequences) the variation based on K2P distances is 14.6 % in the ITS region, whereas a calculation based on uncorrected distances including gaps as a fifth character (Stockinger *et al.*, 2009) raises the value to more than 23 %. Similarly high K2P distances were found, for the ITS region, of *Gl. mosseae* (12.2 %, 109 sequences) and *Gl. fulvum* (15 %, 5 sequences), including database sequences. The *Gl. mosseae* variation increased to

enormous 20.3 % when outlier sequences were included, but it is unlikely that these are from the same species. In comparison, the intrasporal ITS variation of *Gl. mosseae* analysed in this study was 4.6 % (16 sequences) and only slightly increased to 5.3 % when adding 45 sequences from *Gl. mosseae* cultures with geographically widespread origin (Avio *et al.* 2009). Based on these data, there is some doubt whether all public database sequences annotated as *Gl. mosseae* are from the same species.

The intraspecific variation of the complete LSU region was intermediate between that of the LSU-D1 and the LSU-D2 fragment, with the highest variation (in the LSU-D2 core dataset) in *Gl. intraradices* (15.7 %; FL208 and MUCL49410). When including public database and outlier sequences, *Gl. mosseae* reached an intraspecific variation of 19.4 % (170 sequences in total), whereas without outliers the variation is 15.8 %. As for the ITS data, also here it is impossible to be sure about the conspecific origin of the database sequences annotated as *Gl. mosseae*.

In general, the simple use of a % variation value as threshold to define and cluster molecular operational taxonomic units (MOTUs) for species identification must be considered inapplicable.

7.5.2 Barcode gap analyses

The comparison of the maximum intraspecific and the minimum interspecific variation revealed that none of the studied DNA fragments allowed absolute AMF species separation from a simple barcode gap analysis. Barcode gaps may often be an artefact of insufficient taxon sampling (Wiemers & Fiedler, 2007) and the likely existence of a large number of undescribed and uncharacterised species (Sýkorová *et al.*, 2007; Kottke *et al.*, 2008; Öpik *et al.*, 2009) adds further complexity to the topic. Evidently, this method cannot be applied to AMF, when based on the rDNA regions.

7.5.3 Phylogenetic analyses

A barcode gap is not necessarily needed for a barcode-based identification of species, which can also be derived from phylogenetic inference. The phylogenetic analysis of the complete fragment (SSUmCf-LSUmBr) resulted in the strongest support for species. All species, where sequence data were available, could be separated by using simple neighbour joining analysis based on K2P distance. The about 1500 bp long sequences resulted in a clear distinction between *Gl. proliferum*, *Gl.* cf. *clarum* (Att894-7), *Gl. intraradices* (FL208, MUCL49410) and the closely related species or species complex represented by the *Gl. irregulare*-clade, in *Glomus* Group Ab. Maximum

likelihood analyses resulted in the same separation, with even higher support of the topology (Stockinger *et al.*, 2009). The species concept is indirectly supported by the fact that also the mitochondrial LSU rDNA as a marker distinguishes between *Gl. intraradices* and sequences from the *Gl. irregulare*-clade (Börstler *et al.*, 2008). It should be mentioned that the species description of *Gl. irregulare* did not compare all morphologically similar species and *Gl. irregulare* therefore may be a synonym of earlier described species (Walker, 2009).

7.5.4 The ITS region

The ITS region resolved many of the known species, but not the closely related members within *Glomus* Groups Ab and Aa, respectively. The intraspecific ITS variation is extremely high in these species and it is unclear how such highly divergent sequences can persist in an organism, even under relaxed concerted evolution. On the other hand, the ITS region resolved relatively closely related species in the *Ambisporaceae* (Walker *et al.,* 2007*)*, including *Am. appendicula*, which was earlier discussed as possibly being conspecific with *Am. gerdemannii* and *Am. leptoticha* (Morton & Redecker, 2001). Another example are the environmental ITS sequences labelled as *Gl. versiforme*, which do not cluster with the *Gl. versiforme* BEG47 and most likely represent distinct species, exemplifying that assigning environmental sequences to species should be done with care.

For *Glomus* Group Aa, sequences with uncertain assignment to species are, e.g., from *Gl. dimorphicum* and *Gl. monosporum*, which were, on morphological grounds, discussed as possibly conspecific with *Gl. mosseae* (Walker, 1992). Further difficulties result from sequences originated from mixed cultures. For example, the fungus identified as *Gl. monosporum* INVAM FR115 was in a mixed culture that additionally contained spores of *Gl. mosseae* and *Paraglomus occultum.* The culture *Gl. monosporum* INVAM IT102 additionally contained *Gl. mosseae* and *Gl. etunicatum* spores (http://invam.caf.wvu.edu, 24.11.2009). It can neither be ruled out that the spores identified as *Gl. mosseae* and *Gl. monosporum* are conspecific, nor that contaminant sequences (Schüßler, 1999) gave rise to wrong affiliations. In general, the *Gl. mosseae* sequences form two distinct clades, whereas the minor clade consists of sequences from two field sampled spores (GMO2 and GMO3). All GMO3 sequences (AF161059-64) and one GMO2 sequence (AF161058) fall in the minor clade, the remaining GMO2 sequences all fall in the major clade. Sequence AF161058 might be interpreted as a contaminant originating from spore GMO3 and sequences AF161058 and AF161059-64, consequently, might be interpreted as derived from an unidentified species, as discussed by Antoniolli *et al.* (2000). However, also here it is not possible to draw final conclusions, as the existing intrasporal ITS variation is unknown. In particular when including the database ITS

sequences it seems impossible to state whether the *Gl. mosseae* clade consists of one species or several species that cannot be separated. Analysing the complete fragment (SSUmCf-LSUmBr) for more cultures might solve this question.

7.5.5 The LSU region

Using the 800 bp LSU region resulted in more unresolved species in our neighbour joining analyses than using the ITS region. The reason could be 'noise' carried by the LSU-D1 fragment, which behaved worst with regard to resolving species, also for the core dataset. This could explains that the LSU-D2 fragment alone resolved species better than the complete 800 bp LSU region, with approximately the same species resolution power as the ITS region. Sequences of the cultures analysed by Rosendahl (2009), with geographically widespread origin, all fell into the main *Gl. mosseae* subclade (Fig. 6, lower clade). The authors suggested, based on the genetic variability found in the LSU and in two *FOX2* and *TOR* gene introns that the geographical widespread isolates are closely related and the panglobal distribution likely is caused by anthropogenic dispersal. Our analyses may support this interpretation, but it should also be mentioned that three single spore isolates (HG isolate 209, BEG224, JJ isolate 243) gave rise to sequence variants in both, the major and the minor *Gl. mosseae* subclades. This indicates that the LSU variation reported in some studies may be an underestimate, caused by a lack of perhaps rare sequence types (represented by the upper LSU-D2 subclade in Figure 6).

7.5.6 Database sequences

It is clear that there are many inaccurate species determinations in the public sequence databases. For example, some ITS and SSU sequences annotated as of glomeromycotan origin were demonstrated to be from an ascomycete (Redecker *et al.* 1999; Schüßler, 1999). But also AMF contaminants sequences cannot be ruled when using spores from mixed species cultures. Some database sequences seem to be assigned to the wrong species. For example, *Glomus fasciculatum* sequences of two cultures (BEG53 and BEG58) cluster in two different groups, BEG53 in *Glomus* Group Ab and BEG58 in *Glomus* Group Aa. From a morphological viewpoint, it is very unlikely that BEG58 sequences X96842 and X96843 indeed belong to *Gl. fasciculatum*. A third party annotation facility in GenBank (as proposed by many mycologists, e.g., Bidartondo *et al.*, 2008) or well defined and curated databases like UNITE would help to resolve sequence annotation problems in the *Glomeromycota.*

7.5.7 DNA fragments for 454 GS-FLX Titanium pyrosequencing technology

The 454 GS-FLX Titanium pyrosequencing technology currently allows an average read length of approx. 350-450 bp and offers great potential for ecological studies. Our data indicate that a read length of 400 bp will not be sufficient to identify all AMF species with certainty, based on neighbour joining analyses using such a short fragment only. However, there are alternative phylogenetic approaches that may overcome this lack of resolution when taking an alignment based on longer sequences as a 'backbone' for the phylogenetic inference. For example, the program RAxML 7.2.2 (http://wwwkramer.in.tum.de/exelixis/software.html) includes a new algorithm that offers likelihood values for phylogenetic positions of short sequences within a robust tree topology computed from longer sequences. We show the LSU-D2 and ITS2 fragments to be good candidates for species identification by 454 pyrosequencing. The LSU-D2 region may be preferred if AMF should be specifically amplified from roots or soil and it may have a slightly superior species resolution power. In studies where other groups of fungi are investigated together with the AMF diversity, the ITS2 fragment is a good alternative and can be amplified with established primers. We did not test the AM1-NS31 SSU fragment, used in many environmental studies including a recent 454 GS-FLX sequencing approach, because the AM1 primer evidently discriminates many AMF taxa and this region clearly lacks species resolution power.

7.5.8 Conclusion

We show that, based on the rDNA regions studied, there is no barcode gap for many AMF species. The intraspecific variation is heterogeneous and exceptionally high in some phylogenetic groups. Neighbour joining analyses of the approx. 1500 bp SSUmCf-LSUmBr rDNA fragment distinguished all investigated species, whereas shorter rDNA fragments did not allow a separation of very closely related species. Regarding high throughput 454 GS-FLX Titanium pyrosequencing technology, the LSU-D2 and ITS2 fragments appear most suitable. However, beside purely methodological aspects, species recognition is mainly hampered by the lack of a comprehensive and accurate baseline dataset. For future analyses, a 'quantitative world of community analysis' beyond 454 GS-FLX Titanium amplicon sequencing may soon become feasible by affordable high throughput sequencing of even longer DNA fragments (Pacific Biosciences www.pacificbiosciences.com; Eid *et al.*, 2009). This may be taken as another argument in favour of using longer DNA barcodes for easier species resolution.

We propose the sequencing of the easily amplifiable SSUmCf-LSUmBr 1.5 kb fragment variants, covering the partial SSU, the ITS region and a part of the LSU, as a DNA barcoding region for *Glomeromycota*. We also recommend that such a molecular characterisation should be included as a prerequisite to AMF species description whenever possible. Such validated sequence data will be important for comprehensive molecular studies of AMF-plant associations in the field and they will help to uncover and study preferential associations, which are still mostly hidden.

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8 General discussion

The aim of my thesis was to develop and establish tools for the molecular characterisation and 'molecular tracing' of AMF species in ecosystems. As a base, different genes and DNA regions had to be characterised and analysed for their potential use as DNA barcodes for AMF. DNA barcoding uses relatively short and standardized DNA marker sequences to determine species accurately, rapidly and cost efficiently (Frézal & Leblois, 2008). Ideally, DNA barcodes must be applicable for non-experts, culture independent and universally accessible. For AMF, DNA barcode identification would contribute significantly to biological research and agronomic field analyses as AMF are hidden in the soil, but known to increase plant biomass in varying degrees, depending on the AMF-plant species combinations (van der Heijden *et al.*, 2003; Klironomos, 2003; Smith & Read, 2008). A suitable AMF DNA barcode would help to determine the effects of particular AMF species to plants, in the field. The present work focused on the characterisation of a potential DNA barcode by using parts of widely studied rDNA sequences. The SSU rDNA region is largely giving the current molecular baseline for the higher AMF taxonomy (Schüßler *et al.*, 2001), but often does not allow resolution at species level. Different regions of the rDNA were therefore analysed and evaluated for species resolution and identification power on several exemplary datasets. A summary of these analyses and results is shown in Figure 7. This included the *in silico* analysis of approximately 400 bp fragments with regard to 454 GS-FLX Titanium sequencing. This new technology produces up to approx. 400 000 sequences with read lengths of approx. 400 bp in one run, which makes it suitable for environmental barcoding studies (Valentini *et al.*, 2009).

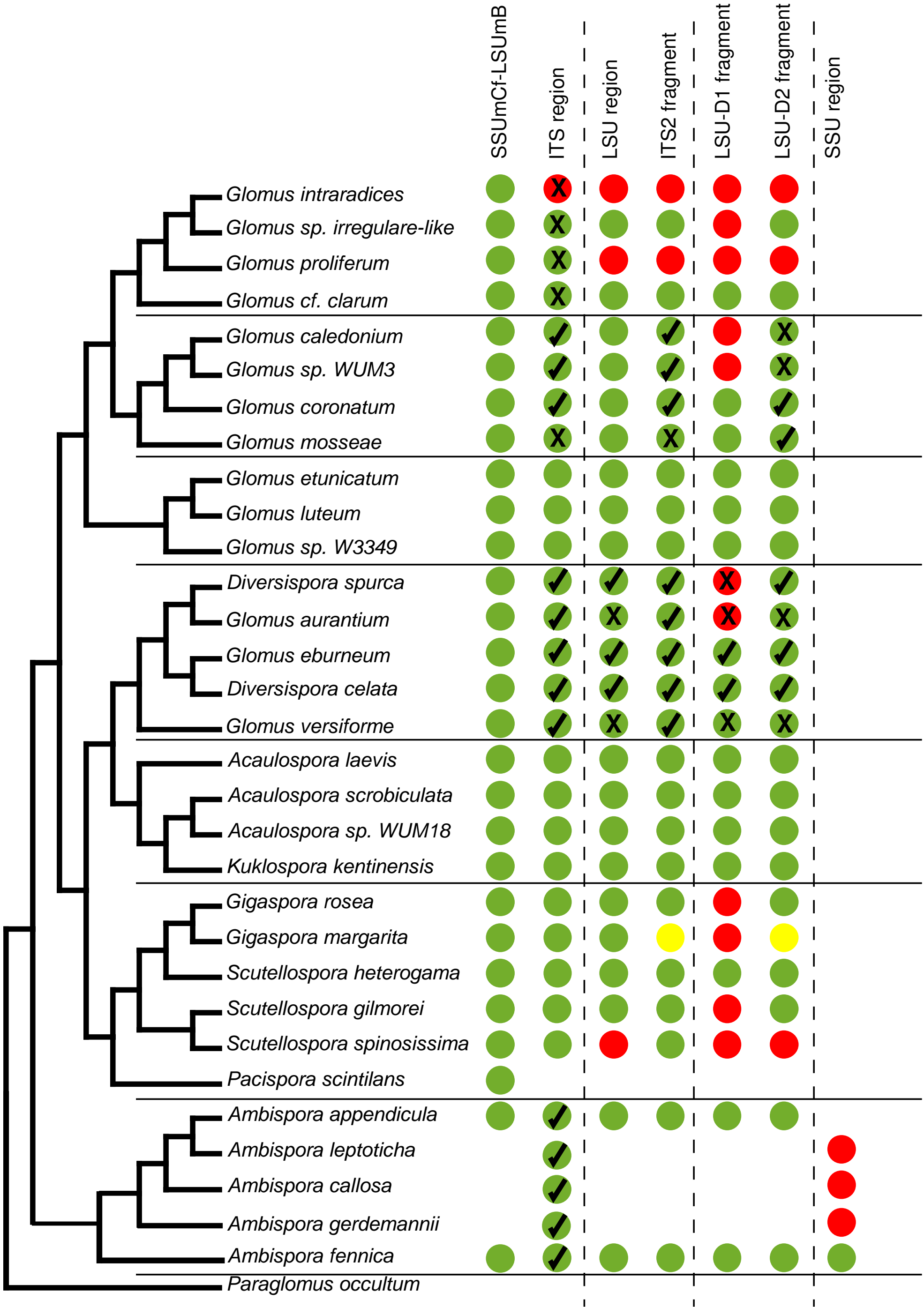

Figure 7: Overview summarizing the main results of this thesis. A schematic phylogenetic tree is shown to the left. Analyses from sequences generated within this thesis are marked as coloured circles. The rDNA fragments analysed are displayed at the top. In relation to species resolution using neighbour joining analyses, sequences from one species form a monophyletic clade with a bootstrap support higher than 50 are indicated in green, sequences from one species form a monophyletic clade with a bootstrap support below 50 in yellow and sequences from one species, which do not form a monophyletic clade are shown in red. When analyses used additional sequences taken from databases the circles are marked with √ or X. √ marks successful species resolution after including additional database sequences, X marks lacking species resolution resulting after addition of database sequences for the given species.

8.1 AMF species resolution using the SSU rDNA

In a first approach, the SSU rDNA was analysed for species resolution of AMF from the *Ambisporaceae* (chapter 4). Many glomeromycotan species have corresponding sequences of the SSU rDNA in the public databases, but not all are correctly identified. In the meanwhile, most AMF sequences in the databases are unidentified and of environmental origin. The results in chapter 4 showed that analyses of the SSU rDNA do not allow resolving all *Ambispora* species. Members of this genus are known to produce dimorphic spores (both glomoid and acaulosporoid), which makes identification difficult when using purely morphological methods, particularly as some are thought to produce only glomoid spores. The limited resolution of the SSU rDNA is in agreement with a later study of Gamper *et al.* (2009) in which it proved impossible for members of the *Diversisporaceae* to separate all species. Another example are two closely related species, *Gl. caledonium* and *Gl. geosporum*, only showing a difference of 2-3 bp in this marker region (Rosendahl, 2008), which may be problematic when the quality of sequences is insufficient. Phylogenetic analyses of selected soil fungal species within *Ascomycota* and *Zygomycota* using partial SSU sequences only showed 52 % success rate for species identification (Molitor *et al.*, 2009). Bruns *et al.* (1991) already indicated that the SSU rDNA may not be suitable to separate fungi to species level, although at this time fewer sequences were available.

Nevertheless, SSU or partial SSU fragments are widely used in characterizing AMF communities (Helgason *et al.*, 1999; Wubet *et al.*, 2006; Öpik *et al.*, 2009). These analyses cannot be interpreted at species level and the comparison of such studies is difficult, a situation not changing principally with the introduction of operational taxonomic units (OTUs), molecular OTUs (MOTUs) or 'virtual taxa'. The concept of OTUs follows that of bacteria, where OTUs are widely used based on a 3 % threshold of the SSU rDNA divergence to separate at species level (Quince *et al.*, 2008). A similar transfer of the 3% threshold to AMF SSU analyses would, for instance, combine *Gl. caledonium* and *Gl. geosporum* to one species.

Many unidentified AMF sequence types and clusters have been found in various ecological studies using parts of the SSU rDNA (e.g. Kottke *et al.*, 2008; Öpik *et al.*, 2009) highlighting the possible existence of many unknown AMF species. However, such sequence types or clusters (or OTUs) may contain more than one species, as exemplified in chapter 4 for *Am. callosa*, *Am. gerdemannii* and *Am. leptoticha.* On the other hand, several sequence clusters interpreted as distinct OTUs may correspond to one species. Due to the lack of species resolution, the SSU rDNA and the application of OTUs based on the SSU clearly are unsuitable for DNA barcoding.

8.2 AMF species resolution using the ITS region

The ITS region is one of the most widely used markers for identification of fungi and has recently been considered to be a *de-facto* barcode for fungi (Seifert 2009). The ITS region was therefore chosen as the consequent promising marker sequence for AMF DNA barcoding. The first study (chapter 4) with a phylogenetic approach using the ITS region appeared promising. It was not only suited to resolve all well defined *Ambisporaceae* species tested, but also cryptic species. In addition, the ITS region separated species with very similar spore morphology. The ITS region was also used to separate AMF species in other families (Redecker *et al.*, 2007; Gamper *et al.*, 2009). Our subsequent research then focused on separating closely related species within the *Glomus* Group Ab, which contains the major model organism for AMF research, *Glomus* sp. DAOM197198. Although this organism was called *Gl. intraradices*, our studies (chapter 6) have shown it to be a different species more closely related to or perhaps conspecific with *Gl. irregulare*. However, in this case the ITS region alone was not suited to separate the closely related species, irrespective whether a simple neighbour joining or a more sophisticated maximum likelihood phylogenetic analysis was used. This partly is caused by the high intraspecific ITS region variation of up to 14.6 % K2P distance. High intraspecific ITS variation, such as uncorrected distances, within the *Glomus* Group Ab was already reported by Jansa *et al.* (2002b). The ITS region was also unsuited to distinguish all analysed species in some non-AMF fungal groups, e.g. the genus *Cladosporium* (Molitor *et al.* 2009). Although for non-AMF the intraspecific variation can also reach relatively high levels, e.g. in *Xylaria hypoxylon* (*Ascomycota*, 24.2 %) and *Rhizoctonia bataticola* (*Basidiomycota*, 17.3 %), but is generally much lower than in glomeromycotan species (Nilsson *et al*., 2008). However, it should be noted that these values cannot be directly compared to the intraspecific variation reported in our and many other studies, because of methodological differences in pairwise distance calculation (see chapter 7).

An analysis within this thesis (chapter 6) revealed that for *Gl. intraradices* FL208 a single AMF spore may contain approx. the same sequence variation as found within the two isolates available for this fungus (descendants from the FL208 'type culture' and from a 20 years later re-isolation from the type locality). This high variation within a species and within an individual spore is remarkable and exceptional. Some studies argue that one AMF coenocyte contain genetically different nuclei (are heterokaryotic; Kuhn *et al.*, 2001), though this is still moot (Pawlowska & Taylor, 2004). If the highly variable ITS sequences are located within one nucleus, a highly relaxed concerted evolution, which usually homogenizes the different repeats of rDNA, is indicated. Concerted evolution was demonstrated to be very efficient in some fungal species (Ganley &

Kobayashi, 2007) but e.g. relaxed in some species of cacti (Harpke & Peterson, 2006) and the grasshopper *Podisma pedestris* (Keller *et al.*, 2006). This study (chapter 7) demonstrates, that not only AMF from *Glomus* group Ab, but also several species from other clades show a very high intraspecific ITS region (and also the LSU) variation. Therefore, relaxed concerted evolution seems widespread in *Glomeromycota*.

The functional relevance of the numerous variants is still not clear, but some of them may represent pseudogenes. Such rDNA pseudogenes were detected in other organisms, for example in a fish (Xu *et al.*, 2009), a coral (Marquez *et al.*, 2003) and a cactus species (Hartmann *et al.*, 2001).

The high intraspecific variation makes DNA barcoding for AMF even more complex because for analyses it is desirable to have highly similar sequences within a species (Valentini *et al.*, 2009). The distance between the maximum intraspecific and the minimum interspecific variation (K2P distances), defined as barcode gap (Hollingsworth *et al.*, 2009; www.barcoding.si.edu), is very low in the ITS region of AMF species and overlaps in some closely related species (chapter 7). Species identification based on this simple method was therefore not successful for AMF. For rapid detection of species, Hebert *et al.* (2004) proposed that the minimum interspecific variation should be 10 times of the maximum intraspecific variation analysed with *COXI* for animals. The ITS region of AMF clearly does not fulfil this criterion, but the ITS region could be used to distinguish most, but not all, of the AMF species analysed here by phylogenetic methods.

8.3 AMF species resolution using the LSU region

Another widely used rDNA part for AMF species identification is the LSU region. Like the ITS region, this region has also been used for AMF species identification and community analyses (van Tuinen *et al.*, 1998; Kjøller & Rosendahl, 2000; Gollotte *et al.*, 2004; Pivato *et al.*, 2007; Gamper *et al.*, 2009). Most studies used the first 800 bp or even smaller parts of the 5' region of the LSU rRNA gene. Two variable regions, D1 and D2, exist within the first 800 bp of the LSU, which are of great interest for species identification. Our study therefore focused on this fragment, and on two sub-fragments containing either the D1 or the D2 region. Gamper *et al.* (2009) demonstrated successful separation of species in the *Diversisporaceae* using a partial LSU fragment. These analyses are in agreement with our results, except for some potentially misannotated sequences from the database. The species identification using the 800 bp part of the LSU was not successful for some members of *Glomus* Group Ab and the *Gigasporaceae*. In some other fungal groups, for example in the *Lecanoromycetes* (*Ascomycota*; Hofstetter *et al.*, 2007) and rusts (Vialle *et al.*,

2009), the LSU also was not suited to resolve all species, whereas the LSU species resolution was better than that of four studied mitochondrial genes, for the rusts. For their studies on AMF, Gollotte *et al.* (2004) excluded the D1 by using PCR primers amplifying only the D2 domain. This seems very reasonable, as in the present work the LSU-D1 fragment analyses could not correctly separate about half of the species studied and, moreover, the analysis of the D2 performed as good as the analyses of the D1 and D2 regions together, indicating a high amount of 'phylogenetic noise' in the D1 domain. Analysing a LSU fragment covering the D2 domain, Rosendahl *et al.* (2009) showed very little sequence differences of max. 6 bp for *Gl. mosseae* isolates from several continents. The variation within their analysed *Gl. mosseae* isolates is smaller, compared to the one *Gl. mosseae* isolate Att109-20/BEG12 analysed here (chapter 7). Rosendahl *et al.* (2009) argue that the small variation of the LSU and two other genes analysed indicate an anthropogenic dispersal of the geographically very widespread isolates.

8.4 Species resolution with ITS region and partial LSU sequences

The longest fragment (SSUmCf-LSUmBr, approx. 1500 bp) used for our analyses covers approx. 240 bp of the SSU, the complete ITS region, and approx. 800 bp of LSU. This fragment allowed separating all species analysed by a simple phylogenetic method (chapter 7). In addition, our analyses showed that the model organism *Glomus* sp. 'irregulare-like' DAOM197198 (formerly called *Gl. intraradices*) is not conspecific with *Gl. intraradices*, which is in agreement with mtLSU analyses of sequences of *Glomus* Group Ab (Börstler *et al.*, 2008).

The successful resolution by using the longer SSUmCf-LSUmBr fragment highlights the often ignored aspect that longer sequences usually have better phylogenetic resolution (Nei *et al.*, 1998; Rokas & Carroll, 2005), if carrying additional phylogenetically informative regions. The conserved regions in the LSU can help to affiliate unknown sequences, because they provide higher-level taxonomic information and are also easier to align unambiguously. Because our study was the first using both the ITS and the LSU region for detailed phylogenetic analyses of AMF, a comparison of the longest fragment (SSUmCf-LSUmBr) for a wide range of AMF species is not yet possible. Some ectomycorrhizal community studies were carried out on the ITS and partial LSU region together to identify species (Tedersoo *et al.*, 2008), whereas the additional use of the ITS region enhanced species resolution (Smith *et al*., 2007).

The phylogenetic analyses of the longest fragment (SSUmCf-LSUmBr) clearly separated all species analysed in this study, but such separation could not be revealed with a simple barcode gap analysis.

Generally, barcode gaps may often represent an artefact caused by insufficient taxon sampling, as discussed by Wiemers and Fiedler (2007). Our results clearly indicate that simple barcode gap analyses are impossible for AMF, when based on the rDNA fragments, and that phylogenetic methods are needed for species resolution.

8.5 Evaluation of short rDNA fragments for new sequencing technologies

New sequencing technologies such as 454 sequencing (www.454.com) allow the generation of up to 400 000 sequences per run with an average read length of approx. 400 bp (Valentini *et al.*, 2009) and are promising tools to overcome the labour and cost intensive cloning approach for analyses of organism communities. When testing the power of such relatively short fragments to separate AMF species, it turned out that in particular the LSU-D1 (LR1-FLR3) fragment was the least useful of the tested regions (chapter 7). The ITS2 fragment and the LSU-D2 (FLR3-LSUmBr) fragment performed better. In some 454 studies the ITS1 was used as target region for fungal community analyses (Buée *et al.*, 2009; Jumpponen & Jones, 2009), but Nilsson *et al.* (2009) have shown that the results of BLAST search based on the ITS1 or ITS2 disagree in 40 % over the taxonomic affiliation of the query sequence, which is a fundamental problem. Hence the right selection of the fragment and the use of high quality annotate sequences will be crucial for comparability of 454 analyses.

In the 454 sequencing approach of Öpik *et al.* (2009), a part of the SSU rDNA was sequenced with approx. 250 bp average read lengths. However, the maximum resolution of such studies, after 97 % clustering to ‘virtual taxa’, clearly is above species level. One virtual taxon defined and detected by the criteria published may in fact represent a number of different species. In the *Ambisporaceae,* the full length SSU is not suited to distinguish species (chapter 4). Care has to be taken to avoid over-interpretation of data, and using the term ‘virtual taxa’ for MOTUs is misleading, as the taxonomic or even the phylogenetic level is not defined.

Other next-generation sequencing systems, such as Genetic Analyzer/Solexa (Illumina), SOLiD DNA Sequencer (Applied Biosystems) and Heliscope (Helicos) produce even more sequences, but with maximal read length of 75 bases (Valentini *et al.*, 2009; www.illumina.com). Such short read lengths seem to be insufficient for AMF determination, because even the 400 bp analysed here failed to identify all species. However, species identification based on 400 bp sequences could be considerably improved by phylogenetic classification using a ‘backbone’ based on the analysis of

longer fragments. Such tools are already available, as e.g. implementation in the RAxML software (http://wwwkramer.in.tum.de/exelixis/software.html).

A recently published paper describes another promising next-generation sequencing technology (Pacific Bioscience www.pacificbiosciences.com; Eid *et al.*, 2009). With this method sequencing of long DNA fragments from environmental DNA samples may become feasible without prior PCR amplification, and the longest fragment (SSUmCf-LSUmBr) analysed here could be used for community analyses of AMF.

8.6 Arbuscular mycorrhizal fungi DNA barcoding - a conclusion

DNA barcodes, by definition, should be applicable to identify species. The long fragment (approx. 1500 bp) analysed here fulfils this requirement, as shown by phylogenetic analyses (chapter 6 and 7). The fragment contains the ITS region, which is a proposed DNA barcode region for fungi (Seifert, 2009). Even closely related species could be separated, which was also supported by independent mitochondrial LSU marker analyses (Börstler *et al.*, 2008). The minimal length of DNA barcodes was proposed to be 500 bp (Frézal & Leblois, 2008; Seifert, 2009). On the other hand, for analysing herbarium specimens DNA barcodes should be short enough to allow the amplification of degraded DNA, which is difficult for more than 150 bp (Valentini *et al.*, 2009). In this case, the maximum barcode length is limited by technical considerations. When DNA barcoding was first attempted, Sanger sequencing technology allowed read lengths of approx. 700 bp, which perhaps was one of the reasons for the size of the first official barcode (*COX1*) of about 650 bp. However, Hajibabaei *et al.* (2006) had to increase the barcode length of *COX1* to 1500 bp to resolve two chimpanzee species.

The ITS region as a common fungal barcode did work for all, except some AMF species examined here for our datasets (chapter 7). However, this must be seen on the background that only a part of the known AMF was analysed. Environmental studies done with the ITS region or the SSU region reveal many new AMF sequence types, which at least partly will turn out to be uncharacterised species (Renker *et al.*, 2005; Sýkorová *et al.*, 2007b; Öpik *et al.*, 2009; Wubet *et al.*, 2009). Ryberg *et al.* (2009) reported that the genus *Glomus* had a high proportion of insufficiently identified sequences. Such unknown species may reduce the resolution of the ITS barcode. Incorporating sequences from the database in the ITS region analyses resulted in reduced species resolution, although this could also be an effect of misannotated sequences. Nilsson *et al.* (2006) showed that up to 20 % of fungal sequences in the public databases may be incorrectly identified at species

level. Feau *et al.* (2009) also highlighted misidentified *Melampsora* rust species in herbarium and sequence databases. Such problem sequences emphasize the necessity of curated databases, as for example BOLD (Ratnasingham & Hebert, 2007) or UNITE (Kõljalg *et al.*, 2005). Alternatively, a third party annotation in the public databases (GenBank, EMBL, DDBJ) would help to overcome this problem (Bidartondo *et al.*, 2008), but currently this is not allowed.

All AMF species analysed in the present thesis could be separated if the proposed, relatively long SSUmCf-LSUmBr fragment was used. In general, DNA regions of AMF analysed in this work showed a very high intraspecific variation. In birds, the *COX1* intraspecific variation is about 2 % (Hebert *et al.*, 2003), whereas in AMF the ITS region variation may be up to 14.6 %. Interestingly, one AMF spore can harbour approx. the entire degree of variability of two independent isolates, as shown for *Gl. intraradices* (chapter 6).

Regarding the DNA barcode region for AMF proposed here, new primers (SSUmCf-LSUmBr; SSUmAf-LSUmAr) were designed and tested for both AMF spores and environmental root samples (chapter 5). DNA barcoding primers should optimally be universal to higher taxa, for example, the entire fungal kingdom. However, this kingdom is huge and contains estimated 1.5–3.5 million species (Hawksworth, 2001; O'Brien *et al.*, 2005). For many gene regions it will perhaps not be possible to design primers covering all fungi. The new primers described in chapter 5 are designed specifically for AMF and therefore allow analyses from contaminated and root material. In some former studies, ascomycotan sequences were assigned as of glomeromycotan origin, as demonstrated in Schüßler (1999) and Redecker *et al.* (1999). It later turned out that even healthy looking and surface-sterilized spores were colonized and therefore contaminated by other fungi (Hijri *et al.*, 2002). An important application for AMF specific primers is the PCR-amplification of AMF DNA from plant roots. Such roots are not only colonized by AMF, but also by other fungi such as pathogens and many endophytes. The DNA amplified with these new primers includes the priming sites for the widely used primer pair ITS4 and ITS5, which is often used to identify fungal species and may be used as a general fungal DNA barcoding primer pair (Seifert, 2009). Therefore, the DNA barcode proposed here for AMF also covers and is compatible with the one suggested as a general fungal barcode. Beside the better species separation power of the long fragment, it will also allow to affiliate LSU sequences to their species if the ITS region fails to match to a sequence in the database. In AMF, use of the combined ITS region and partial LSU regions currently seems unavoidable for species separation, when based on rDNA.

8.7 Outlook

AMF DNA barcodes provide a powerful and useful tool to identify species and provide a complementary method to classical morphological analyses. In the near future it will be desirable to generate more DNA barcodes for a greater coverage of AMF species. Morphologically well characterized AMF species and cultures are urgently needed to build up such a database, and AMF cultures should also be made available to the scientific community. Additionally, the DNA barcoding region should be analysed for all newly described species and the sequences should be deposited in a public database, similar to the classical need of conserving vouchers in a herbarium. For AMF several divergent sequences from a given species should be included. As indicated in chapter 6 for *Gl. intraradices* FL208, the analysis of a single spore may already cover a significant part of the intraspecific variability.

The combined application of plant and AMF DNA barcodes will create an opportunity for analysing AMF-plant associations from root samples. The identification of the host and the symbiont could simultaneously be established from the same sample. Information about AMF species preferentially associated with certain plants may, e.g., be important for restoration and reforestation success (Wubet *et al.*, 2009; Urgiles *et al.*, 2009). This minimal destructive sampling method would also allow sampling of rare and endangered species.

In phylogenetic analyses of AMF with molecular biological tools, individual sequences are often used to determine the identity. For short sequences, this in many cases may be insufficient for the species level, which is partly due to limited information content, partly to the high intraspecific variation (especially when analysing closely related species). On the other hand, a characterisation of intraspecific sequence variation of the longer fragment (SSUmCf-LSUmBr) proposed here as barcode will facilitate the separation of species based on shorter fragments (ITS or LSU) in future, at least to some extent.

DNA barcode identification for AMF appears to offer a significant contribution to biological research and agronomic field analyses in the near future. In general, AMF supply phosphorus and other nutrients to plants, influence the plant and soil water relations and can stabilise soil through their hyphal network and secreted glycoproteins. These multifunctional beneficial effects on plant are gaining more interest because of the increasing demand for food and other plant materials, and for sustainable agricultural systems. Traditionally, plant production is promoted in high input systems by high amounts of fertilizers, but rock phosphate resources are depleting (Cordell *et al.*, 2009) and the increases in energy prices raise costs for nitrogen fertilizers (Huang, 2007). The application of AMF can help to reduce the fertilizer requirements for plant production (Sharma &

Adholeya, 2004). Therefore it is imporant to identify the most ‘suitable’ AMF species in the field or in nurseries. Molecular tools, such as DNA barcoding, are needed to characterise the efficient plant-fungus combinations. Already a decade ago, van der Heijden *et al.* (1998) showed that plant species respond differently to various AMF species combinations. In addition, AMF species identification will play a role in approaches to breed plant cultivars with higher responsiveness to AM (Boomsma & Vyn, 2008). Another applied aspect is the fact that a standardized species classification system (DNA barcodes) will help inoculum producers to improve their quality control systems and to define their used AMF species. In addition, the use of a general fungal barcode allows the screening of inoculum for pathogenic fungi. DNA barcoding would eventually also reduce costs and time when compared to microscopical examination (von Alten *et al.*, 2002; Gianinazzi & Vosátka, 2004). The importance of quality control in AMF production was emphasized at the formation of a working group within the COST action 870, which focuses on this topic (www.cost870.eu/cost.htm). Besides of being useful in laboratory research and for inoculum producers, a DNA barcode will facilitate AMF identification for quarantine and control agencies. An accurate AMF DNA barcode will moreover, at least in certain cases, allow tracing of introduced AMF in the field and the verification of the successful establishment and survival of applied AMF inoculum.

Molecular tools are the only possibility to analyse the AMF species colonizing plant roots and to answer related ecological questions in AMF research. Several studies have been done on biodiversity and community analyses of AMF with molecular tools (Renker *et al.*, 2005; Wubet *et al.*, 2006; Hempel *et al.*, 2007; Sýkorová *et al.*, 2007b; Öpik *et al.*, 2009). Most of the used DNA regions, however, lack species resolution. Furthermore, the primers used were often selective for subgroups within AMF and not covering the complete community (Schüßler, 2001). With both the newly designed primers presented in this work (chapter 5), targeting all *Glomeromycota,* and the knowledge of the intra- and interspecific sequence variation, species identification should be possible, also for AMF species communities. Although differences between two different types of AMF communities could be shown working with 'virtual taxa' (Öpik *et al.*, 2009), such types of analyses would be much improved if performed at species or individual organismic level.

It has been shown here that DNA barcoding in AMF is possible (chapter 7), but it is by far not as straightforward as barcoding in animals (e.g. Hebert *et al.*, 2004). It is desirable that such a barcode is compatible with that used for other fungi. Although it would be a great improvement to use a uniform DNA barcode, such as the ITS region, for all fungi, the region on its own is not sufficiently accurate for robust species delineation and should be extended for *Glomeromycota*. This thesis contributes significantly to the discussion and evaluation of DNA barcodes for fungi and offers

improvements with regard to selected primers and detailed analyses of barcoding regions for AMF. The approx. 1500 bp SSUmCf-LSUmBr fragment is proposed as an extended DNA barcode for AMF, whereas the ITS2 and the LSU-D2 regions both are proposed as shorter barcodes for analyses making use of 454GS-FLX Titanium sequencing approaches. This and other deep sequencing methods will offer the potential of a rapid screening and monitoring of AMF communities in close future. However, robust deep sequencing data interpretation will strongly depend on well characterised and annotated 'hand made' sequences as a fundamental baseline, which in turn depends on the availability of diverse and well characterised AMF isolates.

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11 Appendix

11.1 Supplementary data

The following data are supplementary material of the submitted publication "DNA barcoding of arbuscular mycorrhiza fungi" (chapter 7).

Table S1: Sequences used for analysis of the *Ambisporaceae* ITS region and ITS2 fragment (see Figure S8).

Accession	Species	Culture/voucher
FN547524	*Ambispora appendicula*	Att1235-2/W5156
FN547525	*Ambispora appendicula*	Att1235-2/W5156
FN547526	*Ambispora appendicula*	Att1235-2/W5156
FN547527	*Ambispora appendicula*	Att1235-2/W5156
FN547528	*Ambispora appendicula*	Att1235-2/W5156
FN547529	*Ambispora appendicula*	Att1235-2/W5156
FN547530	*Ambispora appendicula*	Att1235-2/W5156
FN547531	*Ambispora appendicula*	Att1235-2/W5156
FN547532	*Ambispora appendicula*	Att1235-2/W5156
FN547533	*Ambispora appendicula*	Att1235-2/W5156
FN547534	*Ambispora appendicula*	Att1235-2/W5156
AB048656	*Ambispora callosa*	MAFF520057/W4769
AB048657	*Ambispora callosa*	MAFF520057/W4769
AB048658	*Ambispora callosa*	MAFF520057/W4769
AB048659	*Ambispora callosa*	MAFF520057/W4769
AB048660	*Ambispora callosa*	MAFF520057/W4769
AB048661	*Ambispora callosa*	MAFF520057/W4769
AB048662	*Ambispora callosa*	MAFF520057/W4769
AB048663	*Ambispora callosa*	MAFF520057/W4769
AB048664	*Ambispora callosa*	MAFF520057/W4769
AB048665	*Ambispora callosa*	MAFF520057/W4769
AB048666	*Ambispora callosa*	MAFF520057/W4769
AB048667	*Ambispora callosa*	MAFF520057/W4769
AB048668	*Ambispora callosa*	MAFF520057/W4769
AB048669	*Ambispora callosa*	MAFF520057/W4769
AB048670	*Ambispora callosa*	MAFF520057/W4769
AB048671	*Ambispora callosa*	MAFF520058/W4771
AB048672	*Ambispora callosa*	MAFF520058/W4771
AB048673	*Ambispora callosa*	MAFF520058/W4771
AB048674	*Ambispora callosa*	MAFF520058/W4771
AB048675	*Ambispora callosa*	MAFF520058/W4771
AB048676	*Ambispora callosa*	MAFF520058/W4771
AB048677	*Ambispora callosa*	MAFF520058/W4771
AB048678	*Ambispora callosa*	MAFF520058/W4771
AB048679	*Ambispora callosa*	MAFF520058/W4771
AB048680	*Ambispora callosa*	MAFF520058/W4771
AB048681	*Ambispora callosa*	MAFF520058/W4771

AB048682 *Ambispora callosa* MAFF520058/W4771
AB259840 *Ambispora callosa* OK-m1/W4768
AB259841 *Ambispora callosa* OK-m1/W4768
AB259842 *Ambispora callosa* OK-m1/W4768
AB259843 *Ambispora callosa* OK-m1/W4768
AB259844 *Ambispora callosa* MAFF520073/W4752
AB259845 *Ambispora callosa* MAFF520073/W4752
AB259846 *Ambispora callosa* MAFF520073/W4752
AM268197 *Ambispora fennica* Att200-11/W3569
AM268198 *Ambispora fennica* Att200-23/W4752
AM268199 *Ambispora fennica* Att200-11/W3569
AM268200 *Ambispora fennica* Att200-11/W3569
AM268201 *Ambispora fennica* Att200-11/W3569
AM268202 *Ambispora fennica* Att200-11/W3569
AM268203 *Ambispora fennica* Att200-23/W4752
FN547535 *Ambispora fennica* Att200-23/W4752
FN547536 *Ambispora fennica* Att200-23/W4752
FN547537 *Ambispora fennica* Att200-23/W4752
FN547538 *Ambispora fennica* Att200-23/W4752
FN547539 *Ambispora fennica* Att200-23/W4752
FN547540 *Ambispora fennica* Att200-23/W4752
FN547541 *Ambispora fennica* Att200-23/W4752
FN547542 *Ambispora fennica* Att200-23/W4752
FN547543 *Ambispora fennica* Att200-23/W4752
FN547544 *Ambispora fennica* Att200-23/W4752
FN547545 *Ambispora fennica* Att200-23/W4752
FN547546 *Ambispora fennica* Att200-23/W4752
AM743187 *Ambispora gerdemannii* INVAM AU215
AB048630 *Ambispora leptoticha* MAFF520055/W4770
AB048631 *Ambispora leptoticha* MAFF520055/W4770
AB048632 *Ambispora leptoticha* MAFF520055/W4770
AB048633 *Ambispora leptoticha* MAFF520055/W4770
AB048634 *Ambispora leptoticha* MAFF520055/W4770
AB048635 *Ambispora leptoticha* MAFF520055/W4770
AB048636 *Ambispora leptoticha* MAFF520055/W4770
AB048637 *Ambispora leptoticha* MAFF520055/W4770
AB048638 *Ambispora leptoticha* MAFF520055/W4770
AB048639 *Ambispora leptoticha* MAFF520055/W4770
AB048640 *Ambispora leptoticha* MAFF520055/W4770
AB048641 *Ambispora leptoticha* MAFF520055/W4770
AB048642 *Ambispora leptoticha* MAFF520055/W4770
AB048643 *Ambispora leptoticha* MAFF520055/W4770
AB048644 *Ambispora leptoticha* MAFF520055/W4770
AB048645 *Ambispora leptoticha* MAFF520055/W4770
AB048646 *Ambispora leptoticha* MAFF520055/W4770
AB048647 *Ambispora leptoticha* MAFF520055/W4770
AB048648 *Ambispora leptoticha* MAFF520055/W4770
AB048649 *Ambispora leptoticha* MAFF520055/W4770
AB048650 *Ambispora leptoticha* MAFF520055/W4770
AB048651 *Ambispora leptoticha* MAFF520055/W4770

AB048652	*Ambispora leptoticha*	MAFF520055/W4770
AB048653	*Ambispora leptoticha*	MAFF520055/W4770
AB048654	*Ambispora leptoticha*	MAFF520055/W4770
AB048655	*Ambispora leptoticha*	MAFF520055/W4770
AJ567807	*Am*. sp. from *Plantago lanceolata* roots	environmental
AY236277	*Am*. sp. from *Prunus africana*	environmental
AY174701	*Am.* sp. from *Taxus baccata*	environmental
AY174702	*Am.* sp. from *Taxus baccata*	environmental
AY174703	*Am.* sp. from *Taxus baccata*	environmental
AY174707	*Am.* sp. from *Taxus baccata*	environmental
AY174708	*Am.* sp. from *Taxus baccata*	environmental
AY174710	*Am.* sp. from *Taxus baccata*	environmental

Table S2: Sequences used for analyses of the *Diversisporaceae* ITS region and ITS2 fragment (see Figure S9).

Accession	Species	Culture/voucher
AM713402	*Diversispora celata*	FACE234; BEG231
AM713403	*Diversispora celata*	FACE234; BEG231
AM713404	*Diversispora celata*	FACE234; BEG231
FN547637	*Diversispora spurca*	Att246-18/W4119
FN547638	*Diversispora spurca*	Att246-18/W4119
FN547639	*Diversispora spurca*	Att246-18/W4119
FN547640	*Diversispora spurca*	Att246-18/W4119
FN547641	*Diversispora spurca*	Att246-18/W4119
FN547642	*Diversispora spurca*	Att246-18/W4119
FN547643	*Diversispora spurca*	Att246-18/W4119
FN547644	*Diversispora spurca*	Att246-18/W4119
FN547645	*Diversispora spurca*	Att246-18/W4119
FN547646	*Diversispora spurca*	Att246-18/W4119
FN547647	*Diversispora spurca*	Att246-18/W4119
FN547648	*Diversispora spurca*	Att246-18/W4119
FN547649	*Diversispora spurca*	Att246-18/W4119
FN547650	*Diversispora spurca*	Att246-18/W4119
FN547651	*Diversispora spurca*	Att246-18/W4119
FN547652	*Diversispora spurca*	Att246-18/W4119
FN547653	*Diversispora spurca*	Att246-18/W4119
FN547654	*Diversispora spurca*	Att246-18/W4119
AM418549	*Glomus pulvinatum*	environmental
AM418550	*Glomus pulvinatum*	environmental
AJ849468	*Glomus aurantium*	Holotype. Błaszkowski J., 2444 (DPP)
FN547655	*Glomus aurantium*	Att1296-0/W4728
FN547656	*Glomus aurantium*	Att1296-0/W4728
FN547657	*Glomus aurantium*	Att1296-0/W4728
FN547658	*Glomus aurantium*	Att1296-0/W4728
FN547659	*Glomus aurantium*	Att1296-0/W4728
FN547660	*Glomus aurantium*	Att1296-0/W4728
FN547661	*Glomus aurantium*	Att1296-0/W4728
FN547662	*Glomus aurantium*	Att1296-0/W4728
FN547663	*Glomus aurantium*	Att1296-0/W4728
FN547664	*Glomus aurantium*	Att1296-0/W4728
FN547665	*Glomus aurantium*	Att1296-0/W4728
AM713405	*Glomus eburneum*	AZ420A/W4729
AM713406	*Glomus eburneum*	AZ420A/W4729
AM713407	*Glomus eburneum*	AZ420A/W4729
AM713408	*Glomus eburneum*	AZ420A/W4729
AM713409	*Glomus eburneum*	AZ420A/W4729
AM713410	*Glomus eburneum*	AZ420A/W4729
AM713411	*Glomus eburneum*	AZ420A/W4729
AM713412	*Glomus eburneum*	AZ420A/W4729
AM713413	*Glomus eburneum*	AZ420A/W4729
AM713414	*Glomus eburneum*	AZ420A/W4729
AM713415	*Glomus eburneum*	AZ420A/W4729

AM713416	*Glomus eburneum*	AZ420A/W4729
AM418544	*Glomus fulvum*	environmental
AM418545	*Glomus fulvum*	environmental
AM418546	*Glomus fulvum*	environmental
AM418547	*Glomus fulvum*	environmental
AM418548	*Glomus fulvum*	environmental
AM418551	*Glomus megalocarpum*	environmental
AM418552	*Glomus megalocarpum*	environmental
AF185677	*Glomus* sp.	INVAM AZ237B
AF185679	*Glomus* sp.	INVAM AZ237B
AF185680	*Glomus* sp.	INVAM AZ237B
AF185681	*Glomus* sp.	INVAM AZ237B
AF185682	*Glomus* sp.	INVAM NB101
AF185690	*Glomus* sp.	INVAM NB101
AF185693	*Glomus* sp.	INVAM NB101
AF185694	*Glomus* sp.	INVAM NB101
AJ504642	*Glomus* sp. 'versiforme'	environmental
AJ504643	*Glomus* sp. 'versiforme'	environmental
AJ504644	*Glomus* sp. 'versiforme'	environmental
AJ516922	*Glomus* sp. 'versiforme'	environmental
AJ516923	*Glomus* sp. 'versiforme'	environmental
AJ516924	*Glomus* sp. 'versiforme'	environmental
AJ516925	*Glomus* sp. 'versiforme'	environmental
AJ516926	*Glomus* sp. 'versiforme'	environmental
AJ516927	*Glomus* sp. 'versiforme'	environmental
AJ516928	*Glomus* sp. 'versiforme'	environmental
AJ516929	*Glomus* sp. 'versiforme'	environmental
AJ516930	*Glomus* sp. 'versiforme'	environmental
AJ516931	*Glomus* sp. 'versiforme'	environmental
AJ516932	*Glomus* sp. 'versiforme'	environmental
AJ516933	*Glomus* sp. 'versiforme'	environmental
AJ516934	*Glomus* sp. 'versiforme'	environmental
AJ516935	*Glomus* sp. 'versiforme'	environmental
AJ517781	*Glomus* sp. 'versiforme'	environmental
AM076636	*Glomus* sp. 'versiforme'	environmental
AM076637	*Glomus* sp. 'versiforme'	environmental
AM076638	*Glomus* sp. 'versiforme'	environmental
DQ400187	*Glomus* sp. 'versiforme'	environmental
DQ400194	*Glomus* sp. 'versiforme'	environmental
DQ400197	*Glomus* sp. 'versiforme'	environmental
DQ400198	*Glomus* sp. 'versiforme'	environmental
DQ400212	*Glomus* sp. 'versiforme'	environmental
DQ400223	*Glomus* sp. 'versiforme'	environmental
DQ400225	*Glomus* sp. 'versiforme'	environmental
DQ400227	*Glomus* sp. 'versiforme'	environmental
DQ400229	*Glomus* sp. 'versiforme'	environmental
AF246141	*Glomus versiforme*	BEG47
AF246142	*Glomus versiforme*	BEG47
AF246143	*Glomus versiforme*	BEG47
AY842567	*Glomus versiforme*	BEG47

AY842568	*Glomus versiforme*	BEG47
AY842569	*Glomus versiforme*	BEG47
FM876814	*Glomus versiforme*	BEG47/W5165
FM876815	*Glomus versiforme*	BEG47/W5165
FM876816	*Glomus versiforme*	BEG47/W5165
FM876817	*Glomus versiforme*	BEG47/W5165
FM876818	*Glomus versiforme*	BEG47/W5165
FM876819	*Glomus versiforme*	BEG47/W5165
FM876820	*Glomus versiforme*	BEG47/W5165
FN547635	*Glomus versiforme*	BEG47/W5165
FN547636	*Glomus versiforme*	BEG47/W5165
FN547666	*Glomus versiforme*	BEG47/W3180
FN547667	*Glomus versiforme*	BEG47/W3180
FN547668	*Glomus versiforme*	BEG47/W3180
FN547669	*Glomus versiforme*	BEG47/W3180
FN547670	*Glomus versiforme*	BEG47/W3180
FN547671	*Glomus versiforme*	BEG47/W3180
FN547672	*Glomus versiforme*	BEG47/W3180
FN547673	*Glomus versiforme*	BEG47/W3180
FN547674	*Glomus versiforme*	BEG47/W3180
FN547675	*Glomus versiforme*	BEG47/W3180
FN547676	*Glomus versiforme*	BEG47/W3180
FN547677	*Glomus versiforme*	BEG47/W3180
FN547678	*Glomus versiforme*	BEG47/W3180
FN547679	*Glomus versiforme*	BEG47/W3180
FN547680	*Glomus versiforme*	BEG47/W3180
FN547681	*Glomus versiforme*	BEG47/W3180

Table S3: Sequences used for analyses of the *Diversisporaceae* LSU region, LSU-D1 and LSU-D2 fragment (see Figure S10).

Accession	Species	Culture/voucher
AM713402	*Diversispora celata*	BEG231 (FACE234)
AM713403	*Diversispora celata*	BEG231 (FACE234)
AM713404	*Diversispora celata*	BEG231 (FACE234)
AM713405	*Glomus eburneum*	AZ420A/W4729
AM713406	*Glomus eburneum*	AZ420A/W4729
AM713407	*Glomus eburneum*	AZ420A/W4729
AM713408	*Glomus eburneum*	AZ420A/W4729
AM713409	*Glomus eburneum*	AZ420A/W4729
AM713410	*Glomus eburneum*	AZ420A/W4729
AM713411	*Glomus eburneum*	AZ420A/W4729
AM713412	*Glomus eburneum*	AZ420A/W4729
AM713413	*Glomus eburneum*	AZ420A/W4729
AM713414	*Glomus eburneum*	AZ420A/W4729
AM713415	*Glomus eburneum*	AZ420A/W4729
AM713416	*Glomus eburneum*	AZ420A/W4729
FN547635	*Glomus versiforme*	BEG47/W5165
FN547636	*Glomus versiforme*	BEG47/W5165
FM876814	*Glomus versiforme*	BEG47/W5165
FM876815	*Glomus versiforme*	BEG47/W5165
FM876816	*Glomus versiforme*	BEG47/W5165
FM876817	*Glomus versiforme*	BEG47/W5165
FM876818	*Glomus versiforme*	BEG47/W5165
FM876819	*Glomus versiforme*	BEG47/W5165
FM876820	*Glomus versiforme*	BEG47/W5165
FN547637	*Diversispora spurca*	Att246-18/W4119
FN547638	*Diversispora spurca*	Att246-18/W4119
FN547639	*Diversispora spurca*	Att246-18/W4119
FN547640	*Diversispora spurca*	Att246-18/W4119
FN547641	*Diversispora spurca*	Att246-18/W4119
FN547642	*Diversispora spurca*	Att246-18/W4119
FN547643	*Diversispora spurca*	Att246-18/W4119
FN547644	*Diversispora spurca*	Att246-18/W4119
FN547645	*Diversispora spurca*	Att246-18/W4119
FN547646	*Diversispora spurca*	Att246-18/W4119
FN547647	*Diversispora spurca*	Att246-18/W4119
FN547648	*Diversispora spurca*	Att246-18/W4119
FN547649	*Diversispora spurca*	Att246-18/W4119
FN547650	*Diversispora spurca*	Att246-18/W4119
FN547651	*Diversispora spurca*	Att246-18/W4119
FN547652	*Diversispora spurca*	Att246-18/W4119
FN547653	*Diversispora spurca*	Att246-18/W4119
FN547654	*Diversispora spurca*	Att246-18/W4119
FN547655	*Glomus aurantium*	Att1296-0/W4728
FN547656	*Glomus aurantium*	Att1296-0/W4728
FN547657	*Glomus aurantium*	Att1296-0/W4728
FN547658	*Glomus aurantium*	Att1296-0/W4728

FN547659	*Glomus aurantium*	Att1296-0/W4728
FN547660	*Glomus aurantium*	Att1296-0/W4728
FN547661	*Glomus aurantium*	Att1296-0/W4728
FN547662	*Glomus aurantium*	Att1296-0/W4728
FN547663	*Glomus aurantium*	Att1296-0/W4728
FN547664	*Glomus aurantium*	Att1296-0/W4728
FN547665	*Glomus aurantium*	Att1296-0/W4728
FN547666	*Glomus versiforme*	BEG47/W3180
FN547667	*Glomus versiforme*	BEG47/W3180
FN547668	*Glomus versiforme*	BEG47/W3180
FN547669	*Glomus versiforme*	BEG47/W3180
FN547670	*Glomus versiforme*	BEG47/W3180
FN547671	*Glomus versiforme*	BEG47/W3180
FN547672	*Glomus versiforme*	BEG47/W3180
FN547673	*Glomus versiforme*	BEG47/W3180
FN547674	*Glomus versiforme*	BEG47/W3180
FN547675	*Glomus versiforme*	BEG47/W3180
FN547676	*Glomus versiforme*	BEG47/W3180
FN547677	*Glomus versiforme*	BEG47/W3180
FN547678	*Glomus versiforme*	BEG47/W3180
FN547679	*Glomus versiforme*	BEG47/W3180
FN547680	*Glomus versiforme*	BEG47/W3180
FN547681	*Glomus versiforme*	BEG47/W3180
AY842574	*Glomus versiforme*	BEG47/W3180
AY842573	*Glomus versiforme*	BEG47/W3180
EF067888	*Glomus eburneum*	INVAM AZ420A
EF067887	*Glomus eburneum*	INVAM AZ420A
EF067886	*Glomus eburneum*	INVAM AZ420A
AM947665	*Glomus versiforme*	BEG47
AM947664	*Glomus versiforme*	BEG47
EU346868	*Glomus versiforme*	HDAM-4
AY639306	*Diversispora celata*	BEG231 (FACE234)
AY639235	*Diversispora celata*	BEG231 (FACE234)
AY639234	*Diversispora celata*	BEG231 (FACE234)
AY639233	*Diversispora celata*	BEG231 (FACE234)
AY639241	*Diversispora celata*	BEG232 (FACE272)
AY639240	*Diversispora celata*	BEG232 (FACE272)
AY639239	*Diversispora celata*	BEG232 (FACE272)
AY639238	*Diversispora celata*	BEG232 (FACE272)
AY639237	*Diversispora celata*	BEG232 (FACE272)
AY639236	*Diversispora celata*	BEG232 (FACE272)
DQ350448	*Diversispora celata*	BEG233 (FACE410)
DQ350449	*Diversispora celata*	BEG233 (FACE410)
DQ350450	*Diversispora celata*	BEG233 (FACE410)
DQ350451	*Diversispora celata*	BEG233 (FACE410)
DQ350452	*Diversispora celata*	BEG233 (FACE410)
DQ350453	*Diversispora celata*	BEG233 (FACE410)
AY639232	*Diversispora celata*	BEG230 (FACE83)
AY639231	*Diversispora celata*	BEG230 (FACE83)
AY639230	*Diversispora celata*	BEG230 (FACE83)

AY639229	*Diversispora celata*	BEG230 (FACE83)
AY639228	*Diversispora celata*	BEG230 (FACE83)
AY639227	*Diversispora celata*	BEG230 (FACE83)
AY639226	*Diversispora celata*	BEG230 (FACE83)
EF581864	*Glomus aurantium*	Att1296-0/W4728
EF581863	*Glomus aurantium*	Att1296-0/W4728
EF581862	*Glomus aurantium*	Att1296-0/W4728
EF581861	*Glomus aurantium*	Att1296-0/W4728
EF581860	*Glomus aurantium*	Att1296-0/W4728

Table S4: Sequences used for analysis of the *Glomus* Group Aa ITS region and ITS2 fragment (see Figure 6).

Accession	Species	Culture/voucher
X96842	*Glomus* cf. *fasciculatum*	BEG58
X96843	*Glomus* cf. *fasciculatum*	BEG58
AY035642	*Glomus caledonium*	JJ36
AY035646	*Glomus caledonium*	JJ40
AY035647	*Glomus caledonium*	JJ41
AY035651	*Glomus caledonium*	BEG161
FN547494	*Glomus caledonium*	BEG20/W3294
FN547495	*Glomus caledonium*	BEG20/W3294
FN547496	*Glomus caledonium*	BEG20/W3294
FN547497	*Glomus caledonium*	BEG20/W3294
FN547498	*Glomus caledonium*	BEG20/W3294
FN547499	*Glomus caledonium*	BEG20/W3294
AJ890365	*Glomus coronatum*	IMA3
AJ890366	*Glomus coronatum*	IMA3
FM213083	*Glomus coronatum*	environmental
FM213084	*Glomus coronatum*	environmental
FM213085	*Glomus coronatum*	environmental
FM213086	*Glomus coronatum*	environmental
FM213087	*Glomus coronatum*	environmental
FM213088	*Glomus coronatum*	environmental
FM876794	*Glomus coronatum*	BEG28 (Att108-7)
FM876795	*Glomus coronatum*	BEG28 (Att108-7)
FM876796	*Glomus coronatum*	BEG28 (Att108-7)
FM876797	*Glomus coronatum*	BEG28 (Att108-7)
FM876798	*Glomus coronatum*	BEG28 (Att108-7)
X96844	*Glomus coronatum*	BEG28
X96845	*Glomus coronatum*	BEG28
X96846	*Glomus coronatum*	BEG28
X96838	*Glomus dimorphicum*	BEG59
X96839	*Glomus dimorphicum*	BEG59
X96840	*Glomus dimorphicum*	BEG59
X96841	*Glomus dimorphicum*	BEG59
AF231469	*Glomus geosporum*	unknown
AJ319778	*Glomus geosporum*	unknown
AJ319779	*Glomus geosporum*	unknown
AJ319780	*Glomus geosporum*	unknown
AJ319781	*Glomus geosporum*	unknown
AJ319782	*Glomus geosporum*	unknown
AJ319783	*Glomus geosporum*	unknown
AJ319784	*Glomus geosporum*	unknown
AJ319785	*Glomus geosporum*	unknown
AJ319786	*Glomus geosporum*	unknown
AJ319787	*Glomus geosporum*	unknown
AJ319788	*Glomus geosporum*	unknown
AJ319789	*Glomus geosporum*	unknown
AJ319790	*Glomus geosporum*	unknown

AJ319791	*Glomus geosporum*	unknown
AJ319792	*Glomus geosporum*	unknown
AJ319793	*Glomus geosporum*	unknown
AJ319794	*Glomus geosporum*	unknown
AJ319795	*Glomus geosporum*	unknown
AJ319796	*Glomus geosporum*	unknown
AJ319797	*Glomus geosporum*	unknown
AJ319798	*Glomus geosporum*	unknown
AJ319799	*Glomus geosporum*	unknown
AJ319800	*Glomus geosporum*	unknown
AJ319801	*Glomus geosporum*	unknown
AJ319802	*Glomus geosporum*	unknown
AJ319803	*Glomus geosporum*	unknown
FJ009619	*Glomus geosporum*	unknown
FJ009620	*Glomus geosporum*	unknown
FJ009621	*Glomus geosporum*	unknown
FJ009622	*Glomus geosporum*	unknown
AF004689	*Glomus monosporum*	INVAM IT102
AF004690	*Glomus monosporum*	INVAM FR115
AF125195	*Glomus monosporum*	INVAM FR115
AF161043	*Glomus mosseae*	environmental (GMO1a)
AF161044	*Glomus mosseae*	environmental (GMO1b)
AF161045	*Glomus mosseae*	environmental (GM01c)
AF161046	*Glomus mosseae*	environmental (GMO1d)
AF161047	*Glomus mosseae*	environmental (GMO1e)
AF161048	*Glomus mosseae*	environmental (GMO1f)
AF161049	*Glomus mosseae*	environmental (GMO1g)
AF161050	*Glomus mosseae*	environmental (GMO1h)
AF161051	*Glomus mosseae*	environmental (GMO1i)
AF161052	*Glomus mosseae*	environmental (GMO1j)
AF161053	*Glomus mosseae*	environmental (GMO1l)
AF161054	*Glomus mosseae*	environmental (GMO1)
AF161055	*Glomus mosseae*	environmental (GMO2a)
AF161056	*Glomus mosseae*	environmental (GMO2b)
AF161057	*Glomus mosseae*	environmental (GMO2c)
AF161058	*Glomus mosseae*	environmental (GMO2e)
AF161059	*Glomus mosseae*	environmental (GMO3a)
AF161060	*Glomus mosseae*	environmental (GMO3b)
AF161061	*Glomus mosseae*	environmental (GMO3c)
AF161062	*Glomus mosseae*	environmental (GM03d)
AF161063	*Glomus mosseae*	environmental (GM03e)
AF161064	*Glomus mosseae*	environmental (GM03f)
AF166276	*Glomus mosseae*	environmental (GMO2d)
AJ849469	*Glomus mosseae*	unknown
AJ919273	*Glomus mosseae*	INVAM AZ225C
AJ919274	*Glomus mosseae*	INVAM AZ225C
AJ919275	*Glomus mosseae*	INVAM NB114
AJ919276	*Glomus mosseae*	INVAM IN101C
AJ919277	*Glomus mosseae*	INVAM FL156
AJ919278	*Glomus mosseae*	INVAM FL156

AM076635	*Glomus mosseae*	environmental
AM157131	*Glomus mosseae*	ISCB13
AM157132	*Glomus mosseae*	ISCB17
AM157133	*Glomus mosseae*	ISCB22
AM157134	*Glomus mosseae*	ISCB19
AM157135	*Glomus mosseae*	ISCB20
AM423114	*Glomus mosseae*	IMA1
AM423115	*Glomus mosseae*	IMA1
AM423116	*Glomus mosseae*	BEG25
AM423117	*Glomus mosseae*	BEG25
AM423118	*Glomus mosseae*	BEG25
AM423119	*Glomus mosseae*	BEG25
AY035650	*Glomus mosseae*	BEG160
AY035652	*Glomus mosseae*	BEG161
AY236331	*Glomus mosseae*	SP301
AY236332	*Glomus mosseae*	SP302
AY236333	*Glomus mosseae*	SP303
AY236334	*Glomus mosseae*	SP304
AY236335	*Glomus mosseae*	SP305
AY236336	*Glomus mosseae*	SP306
AY997053	*Glomus mosseae*	INVAM UT101 (AFTOL-ID 139)
DQ400127	*Glomus mosseae*	environmental
DQ400128	*Glomus mosseae*	environmental
DQ400129	*Glomus mosseae*	environmental
DQ400130	*Glomus mosseae*	environmental
DQ400131	*Glomus mosseae*	environmental
DQ400132	*Glomus mosseae*	environmental
DQ400134	*Glomus mosseae*	environmental
DQ400136	*Glomus mosseae*	environmental
DQ400137	*Glomus mosseae*	environmental
DQ400138	*Glomus mosseae*	environmental
DQ400139	*Glomus mosseae*	environmental
DQ400141	*Glomus mosseae*	environmental
DQ400142	*Glomus mosseae*	environmental
DQ400144	*Glomus mosseae*	environmental
DQ400146	*Glomus mosseae*	environmental
DQ400149	*Glomus mosseae*	environmental
DQ400151	*Glomus mosseae*	environmental
DQ400158	*Glomus mosseae*	environmental
DQ400160	*Glomus mosseae*	environmental
EF989113	*Glomus mosseae*	environmental
EF989114	*Glomus mosseae*	environmental
EF989115	*Glomus mosseae*	environmental
EF989116	*Glomus mosseae*	environmental
EF989117	*Glomus mosseae*	environmental
FN547474	*Glomus mosseae*	BEG12
FN547475	*Glomus mosseae*	BEG12
FN547476	*Glomus mosseae*	BEG12
FN547482	*Glomus mosseae*	BEG12
FN547483	*Glomus mosseae*	BEG12

FN547484	*Glomus mosseae*	BEG12
FN547485	*Glomus mosseae*	BEG12
FN547486	*Glomus mosseae*	BEG12
FN547487	*Glomus mosseae*	BEG12
FN547488	*Glomus mosseae*	BEG12
FN547489	*Glomus mosseae*	BEG12
FN547490	*Glomus mosseae*	BEG12
FN547491	*Glomus mosseae*	BEG12
FN547492	*Glomus mosseae*	BEG12
FN547493	*Glomus mosseae*	BEG12
U31996	*Glomus mosseae*	BEG 12
U49264	*Glomus mosseae*	UKJII8
U49265	*Glomus mosseae*	INVAM FL156
X84232	*Glomus mosseae*	BEG12
X84233	*Glomus mosseae*	BEG12
X96826	*Glomus mosseae*	BEG25
X96827	*Glomus mosseae*	BEG25
X96828	*Glomus mosseae*	BEG25
X96829	*Glomus mosseae*	BEG55
X96830	*Glomus mosseae*	BEG54
X96831	*Glomus mosseae*	BEG54
X96832	*Glomus mosseae*	BEG54
X96833	*Glomus mosseae*	BEG57
X96834	*Glomus mosseae*	BEG57
X96835	*Glomus mosseae*	BEG57
X96836	*Glomus mosseae*	BEG61
X96837	*Glomus mosseae*	BEG61
FM876813	*Glomus* sp. WUM3	WUM3/W2940
FN547477	*Glomus* sp. WUM3	WUM3/W2939
FN547478	*Glomus* sp. WUM3	WUM3/W2939
FN547479	*Glomus* sp. WUM3	WUM3/W2939
FN547480	*Glomus* sp. WUM3	WUM3/W2939
FN547481	*Glomus* sp. WUM3	WUM3/W2939

Table S5: Sequences used for analysis of the *Glomus* Group Aa LSU-D2 fragment (see Figure 6).

Accession	Species	Culture/voucher
FN547474	*Glomus mosseae*	BEG12/W5147
FN547475	*Glomus mosseae*	BEG12/W5147
FN547476	*Glomus mosseae*	BEG12/W5147
FN547477	*Glomus* sp.	WUM3/W2939
FN547478	*Glomus* sp.	WUM3/W2939
FN547479	*Glomus* sp.	WUM3/W2939
FN547480	*Glomus* sp.	WUM3/W2939
FN547481	*Glomus* sp.	WUM3/W2939
FN547482	*Glomus mosseae*	BEG12/W5147
FN547483	*Glomus mosseae*	BEG12/W5147
FN547484	*Glomus mosseae*	BEG12/W5147
FN547485	*Glomus mosseae*	BEG12/W5147
FN547486	*Glomus mosseae*	BEG12/W5147
FN547487	*Glomus mosseae*	BEG12/W5147
FN547488	*Glomus mosseae*	BEG12/W5147
FN547489	*Glomus mosseae*	BEG12/W5147
FN547490	*Glomus mosseae*	BEG12/W5147
FN547491	*Glomus mosseae*	BEG12/W5147
FM876813	*Glomus* sp.	WUM3/W2940
FN547492	*Glomus mosseae*	BEG12/W5147
FN547493	*Glomus mosseae*	BEG12/W5147
FM876798	*Glomus coronatum*	BEG28 (Att108-7)
FM876796	*Glomus coronatum*	BEG28 (Att108-7)
FM876797	*Glomus coronatum*	BEG28 (Att108-7)
FM876794	*Glomus coronatum*	BEG28 (Att108-7)
FM876795	*Glomus coronatum*	BEG28 (Att108-7)
FN547494	*Glomus caledonium*	BEG20/W3294
FN547495	*Glomus caledonium*	BEG20/W3294
FN547496	*Glomus caledonium*	BEG20/W3294
FN547497	*Glomus caledonium*	BEG20/W3294
FN547498	*Glomus caledonium*	BEG20/W3294
FN547499	*Glomus caledonium*	BEG20/W3294
AF145741	*Glomus constrictum*	BEG130
AF145747	*Glomus fragilistratum*	BEG05
AF145735	*Glomus mosseae*	BEG25
AF145745	*Glomus caledonium*	BEG20
AF145740	*Glomus coronatum*	BEG49
AF145742	*Glomus geosporum*	BEG90
AF396789	*Glomus caledonium*	RMC658
AF396794	*Glomus caledonium*	RWC658

AF145736	*Glomus mosseae*	BEG85
AF396799	*Glomus caledonium*	SC_658
AJ510239	*Glomus caledonium*	BEG86
AF396788	*Glomus mosseae*	243
AF396793	*Glomus mosseae*	243
AF396798	*Glomus mosseae*	243
AY639156	*Glomus mosseae*	8
AY639157	*Glomus mosseae*	8
AY639158	*Glomus mosseae*	8
AY639160	*Glomus mosseae*	environmental
AY639162	*Glomus mosseae*	environmental
AY639163	*Glomus mosseae*	environmental
AY639164	*Glomus mosseae*	101
AY639270	*Glomus mosseae*	environmental
AY639159	*Glomus mosseae*	environmental
AY639274	*Glomus mosseae*	environmental
AY639281	*Glomus mosseae*	209
AY639271	*Glomus mosseae*	environmental
AY639278	*Glomus mosseae*	102
AY639280	*Glomus mosseae*	BEG224 (FACE 130)
AY639161	*Glomus mosseae*	environmental
AY639165	*Glomus mosseae*	BEG224 (FACE 130)
AY639166	*Glomus mosseae*	BEG224 (FACE 130)
AY639167	*Glomus mosseae*	BEG224 (FACE 130)
AY639168	*Glomus mosseae*	BEG224 (FACE 130)
AY639169	*Glomus mosseae*	BEG224 (FACE 130)
AY639170	*Glomus mosseae*	BEG224 (FACE 130)
AY639171	*Glomus mosseae*	209
AY639172	*Glomus mosseae*	209
AY639173	*Glomus mosseae*	209
AY639174	*Glomus mosseae*	209
AY639266	*Glomus mosseae*	8
AY639267	*Glomus mosseae*	8
AY639268	*Glomus mosseae*	8
AY639269	*Glomus mosseae*	8
AY639272	*Glomus mosseae*	environmental
AY639273	*Glomus mosseae*	environmental
AY639276	*Glomus mosseae*	environmental
AY639277	*Glomus mosseae*	101
DQ469128	*Glomus mosseae*	505
AJ628059	*Glomus caledonium*	BEG86
AJ510241	*Glomus geosporum*	BEG11
DQ273793	*Glomus mosseae*	INVAM UT101 (AFTOL-ID 139)
AY639279	*Glomus mosseae*	BEG224 (FACE 130)

FJ790678	*Glomus mosseae*	DDAM
EU931286	*Glomus geosporum*	BEG199
EU931285	*Glomus geosporum*	BEG199
EU931284	*Glomus geosporum*	BEG199
EU931283	*Glomus geosporum*	BEG199
EU931282	*Glomus geosporum*	BEG199
EU931281	*Glomus geosporum*	BEG199
EU931280	*Glomus geosporum*	BEG199
EU931279	*Glomus geosporum*	BEG199
EU931278	*Glomus geosporum*	BEG199
EU931277	*Glomus geosporum*	BEG199
EU931276	*Glomus geosporum*	BEG199
EU931275	*Glomus geosporum*	BEG199
EU931274	*Glomus geosporum*	BEG199
EU931273	*Glomus geosporum*	BEG211
EU931272	*Glomus geosporum*	BEG211
EU931271	*Glomus geosporum*	BEG211
EU931270	*Glomus geosporum*	BEG211
EU931269	*Glomus geosporum*	BEG211
EU931267	*Glomus geosporum*	BEG211
EU931266	*Glomus geosporum*	BEG211
EU931265	*Glomus geosporum*	BEG211
EU931264	*Glomus geosporum*	BEG211
EU931263	*Glomus geosporum*	BEG211
EU931262	*Glomus geosporum*	BEG211
EU931261	*Glomus geosporum*	BEG211
EU346866	*Glomus mosseae*	HDAM-2
EU234489	*Glomus mosseae*	BEG116
AM158954	*Glomus mosseae*	BEG167
AM158953	*Glomus mosseae*	BEG167
DQ469131	*Glomus mosseae*	505
DQ469130	*Glomus mosseae*	505
DQ469129	*Glomus mosseae*	505
DQ469127	*Glomus mosseae*	505
DQ469126	*Glomus mosseae*	505
DQ469125	*Glomus mosseae*	505
AJ459412	*Glomus mosseae*	environmental
AJ628057	*Glomus mosseae*	BEG29
AJ628056	*Glomus mosseae*	BOL3
AJ628055	*Glomus mosseae*	BOL1
AJ628054	*Glomus mosseae*	V150
AJ628053	*Glomus mosseae*	V249
AJ628052	*Glomus mosseae*	V293
AJ628051	*Glomus mosseae*	V91

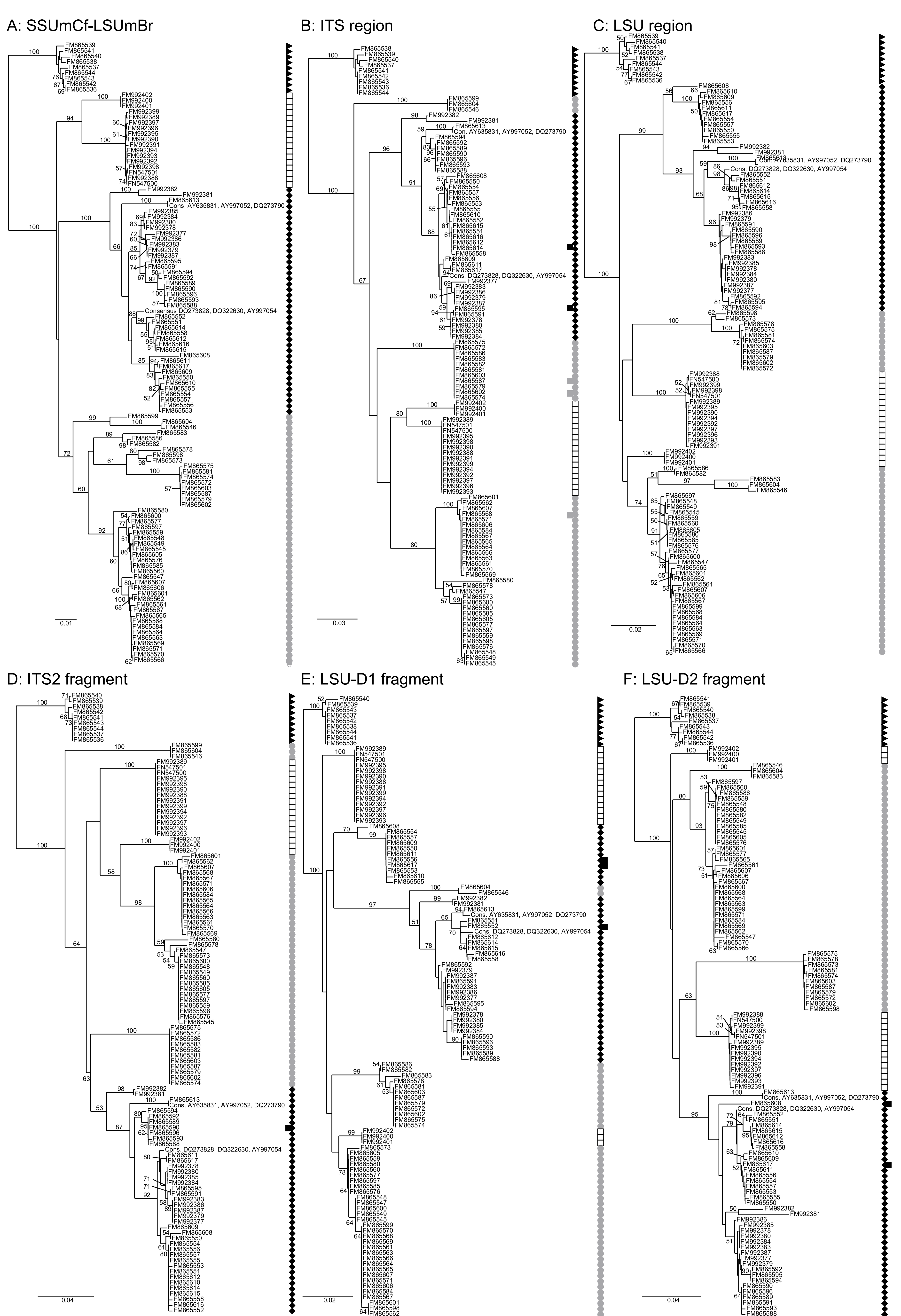
AJ628050	*Glomus mosseae*	V296
AJ628049	*Glomus mosseae*	V296
AF145746	*Glomus caledonium*	BEG86
AF145743	*Glomus geosporum*	BEG106
AF145738	*Glomus mosseae*	BEG84
AF145737	*Glomus mosseae*	BEG83
AJ271924	*Glomus mosseae*	HM-CL1
AJ510240	*Glomus caledonium*	BEG20
AF389014	*Glomus mosseae*	BEG68
AF389013	*Glomus mosseae*	BEG68
AF389012	*Glomus mosseae*	BEG68
AF389011	*Glomus mosseae*	BEG68
AF389010	*Glomus mosseae*	BEG68
AF389009	*Glomus mosseae*	BEG68
AF389008	*Glomus mosseae*	BEG68
GQ330818	*Glomus mosseae*	AU34
GQ330817	*Glomus mosseae*	AU33
GQ330815	*Glomus mosseae*	AU8
GQ330814	*Glomus mosseae*	AU2
GQ330813	*Glomus mosseae*	WUM16
GQ330811	*Glomus mosseae*	Narrabii
GQ330807	*Glomus mosseae*	Bur11
GQ330806	*Glomus mosseae*	INVAM JA205c
GQ330805	*Glomus mosseae*	BEG229
GQ330800	*Glomus mosseae*	BEG55
GQ330797	*Glomus mosseae*	INVAM NB103c
GQ330793	*Glomus mosseae*	INVM SF1171
GQ330791	*Glomus mosseae*	INVAM CU134a
GQ330789	*Glomus mosseae*	DKB01D4
GQ330788	*Glomus mosseae*	DKK04D22
GQ330787	*Glomus mosseae*	DKGm1
GQ330785	*Glomus mosseae*	Sp813
GQ330784	*Glomus mosseae*	Sp6314
GQ330783	*Glomus mosseae*	Sp4318
GQ330781	*Glomus mosseae*	Sp2735
GQ330780	*Glomus mosseae*	Sp1841
GQ330779	*Glomus mosseae*	BEG128
GQ330778	*Glomus mosseae*	BEG124
GQ330777	*Glomus mosseae*	BEG85
GQ330774	*Glomus mosseae*	Dk11107
GQ330773	*Glomus mosseae*	Dk21107
GQ330772	*Glomus mosseae*	Dk17107
GQ330771	*Glomus mosseae*	BEG230
GQ330768	*Glomus mosseae*	Dk23135

GQ330760 *Glomus mosseae* INVAM WY111
GQ330757 *Glomus mosseae* INVAM MT107
GQ330756 *Glomus mosseae* INVAM OR229
GQ330754 *Glomus mosseae* INVAM SC226
GQ330749 *Glomus mosseae* INVAM MN101
GQ330748 *Glomus mosseae* INVAM MI210
GQ330747 *Glomus mosseae* INVAM ON201
GQ330744 *Glomus mosseae* INVAM WI101
GQ330743 *Glomus mosseae* INVAM NV106
GQ330742 *Glomus mosseae* INVAM IN101

Table S6: Barcode gap analyses with TaxonGap 2.3 based on pairwise comparison of K2P distances based on a manual or automated alignment (MAFFT). Variation is given in % K2P distances. The closest species, presence or absence of a barcode gap were identical for the manual and MAFFT alignments, respectively. Seq, number of sequences; CS, closest species; BG, barcode gap; Max. ISV, maximum intraspecific variation; Min. ISV, minimum intraspecific variation; ?, unknown.

					manual alignment		**MAFFT alignment**	
Family	**Species**	**Seq**	**CS**	**BG**	**Max. ISV**	**Min. ISV**	**Max. ISV**	**Min. ISV**
	Glomus mosseae	16	*Gl. coronatum*	Yes	2.52	2.66	2.58	3.78
Glomeraceae	*Gl.* sp. WUM3	6	*Gl. caledonium*	Yes	0.85	2.1	0.85	2.09
(*Glomus*	*Gl. coronatum*	5	*Gl. mosseae*	Yes	0.5	2.66	1.01	3.78
Group Aa)	*Gl. caledonium*	3	*Gl.* sp. WUM3	Yes	0.8	2.1	0.8	2.09
	Gl. intraradices	47	*Gl. proliferum*	No	10.77	4.29	11.75	4.7
Glomeraceae	*Gl. proliferum*	15	*Gl. intraradices*	Yes	4.02	4.29	3.89	4.7
(*Glomus*	*Gl.* sp. 'irregulare-like’	39	*Gl. proliferum*	Yes	6.43	7	6.29	6.94
Group Ab)	*Gl. clarum*	9	*Gl. proliferum*	Yes	1.09	7.58	1.59	7.96
	Acaulospora laevis	26	*Ku. Kentinensis & Ac. scrobiculata*	Yes	3.42	13.07	3.99	13.16
	Acaulospora sp. WUM18	2	*Ac. scrobiculata*	Yes	1.02	5.66	1.02	5.66
Acaulo-	*Ac. scrobiculata*	4	*Ac.* sp. WUM18	Yes	0.47	5.66	0.47	5.66
sporaceae	*Kuklospora kentinensis*	14	*Ac. scrobiculata*	Yes	0.54	11.98	0.54	11.38
Ambi-	*Ambispora appendicula*	11	*Am. fennica*	Yes	2.87	12.11	2.87	13.26
sporaceae	*Am. fennica*	12	*Am. appendicula*	Yes	1	12.11	1.14	13.26
	Gigaspora margarita	24	*Gi. rosea*	No	4.15	3.26	4.42	3.34
	Gi. rosea	27	*Gi. margarita*	No	6.17	3.26	6.53	3.34
	Sc. gilmorei	25	*Sc. spinosissima*	Yes	1.55	2.64	1.62	2.5
Giga-	*Sc. spinosissima*	3	*Sc. gilmorei*	No	2.84	2.64	2.84	2.5
sporaceae	*Sc. heterogama*	4	*Gi. margarita*	Yes	1.95	4.69	2.74	5.07
Glomeraceae	*Glomus* sp. W3349	4	*Gl. luteum*	Yes	0.77	11.54	0.71	12.27
(*Glomus*	*Gl. etunicatum*	12	*Gl. luteum*	Yes	0.93	3.64	0.94	3.63
Group B)	*Gl. luteum*	5	*Gl. etunicatum*	Yes	0.64	3.64	0.96	3.63
	Diversispora celata	3	*Gl. eburneum*	Yes	0.9	2.61	0.83	3.39
	Gl. eburneum	12	*Di. celata*	Yes	0.92	2.61	0.92	3.39
	Gl. versiforme	25	*Gl. eburneum*	Yes	2.52	5.81	2.79	5.64
Diversi-	*Diversispora spurca*	18	*Gl. aurantium*	Yes	1.59	2.73	1.66	2.87
sporaceae	*Gl. aurantium*	11	*Di. spurca*	Yes	1.71	2.73	1.71	2.87
Para-glomeraceae	*Paraglomus occultum*	1	*Sc. gilmorei*	?	-	34.93	-	31.7
Paci-sporaceae	*Pacispora scintillans*	2	*Sc. heterogama*	Yes	0.62	22.59	0.55	20.55

Figure S1: SSUmCf-LSUmBr (A), ITS region (B), LSU region (C), ITS2 fragment (D), LSU-D1 fragment (E), LSU-D2 fragment (F) neighbour joining analyses (1000 BS) of *Glomus* Group Ab from the core dataset. *Glomus* cf. *clarum* (►) *Gl. intraradices* (●), *Gl. proliferum* (□), *Glomus* sp. 'irregulare-like' (◆).



Appendix

Appendix

Figure S2: SSUmCf-LSUmBr (A), ITS region (B), LSU region (C), ITS2 fragment (D), LSU-D1 fragment (E), LSU-D2 fragment (F) neighbour joining analyses (1000 BS) of *Glomus* Group Aa from the core dataset. *Glomus mosseae* (■) *Glomus* sp. WUM3 (●), *Gl. coronatum* (▲), *Gl. caledonium* (►).

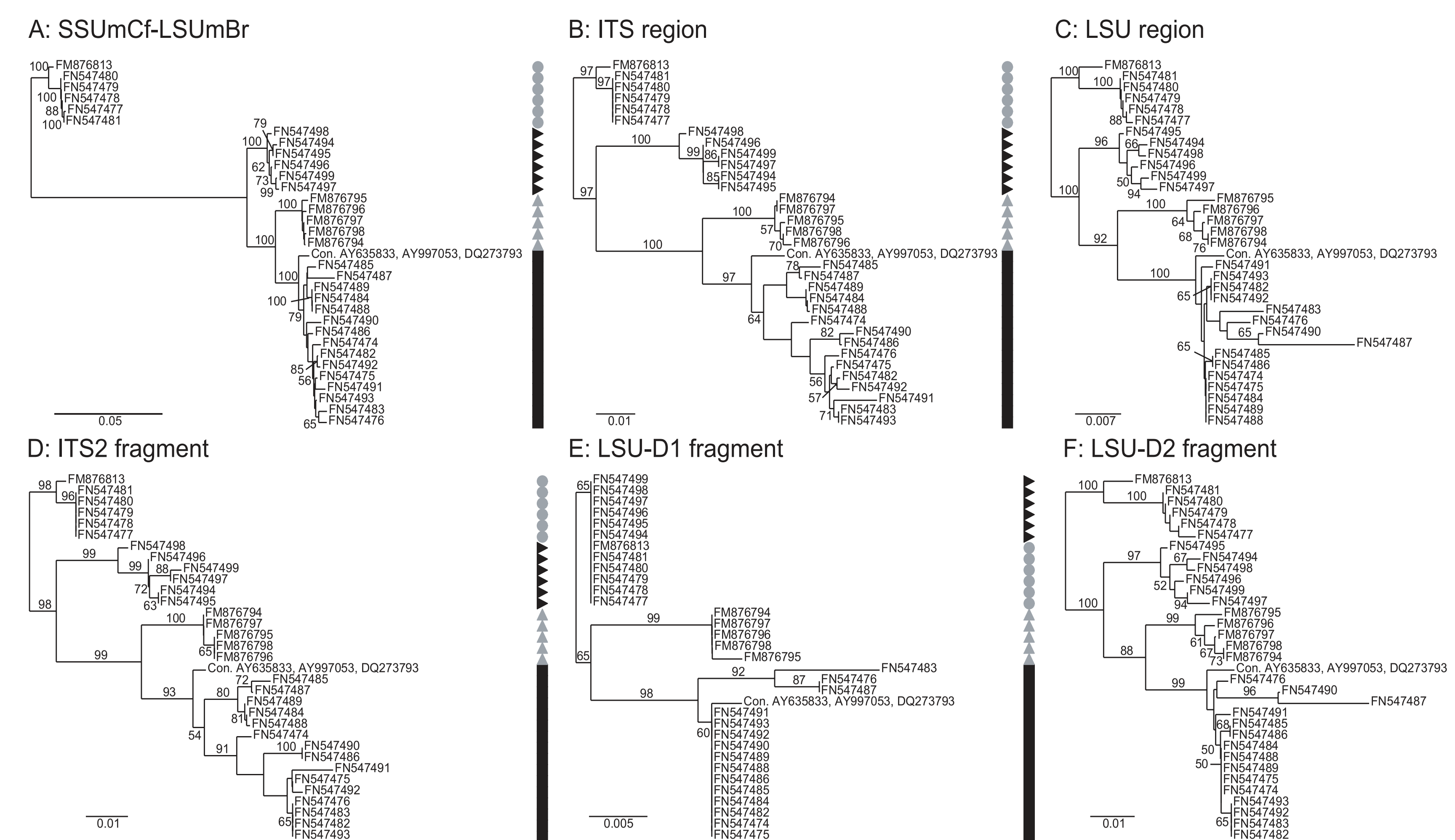


Figure S3: SSUmCf-LSUmBr (A), ITS region (B), LSU region (C), ITS2 fragment (D), LSU-D1 fragment (E), LSU-D2 fragment (F) neighbour joining analyses (1000 BS) of *Acaulosporaceae* from the core dataset. *Kuklospora kentinensis* (◆), *Acaulospora* sp. WUM18 (◄), *Ac. scrobiculata* (□), *Ac. laevis* (●).

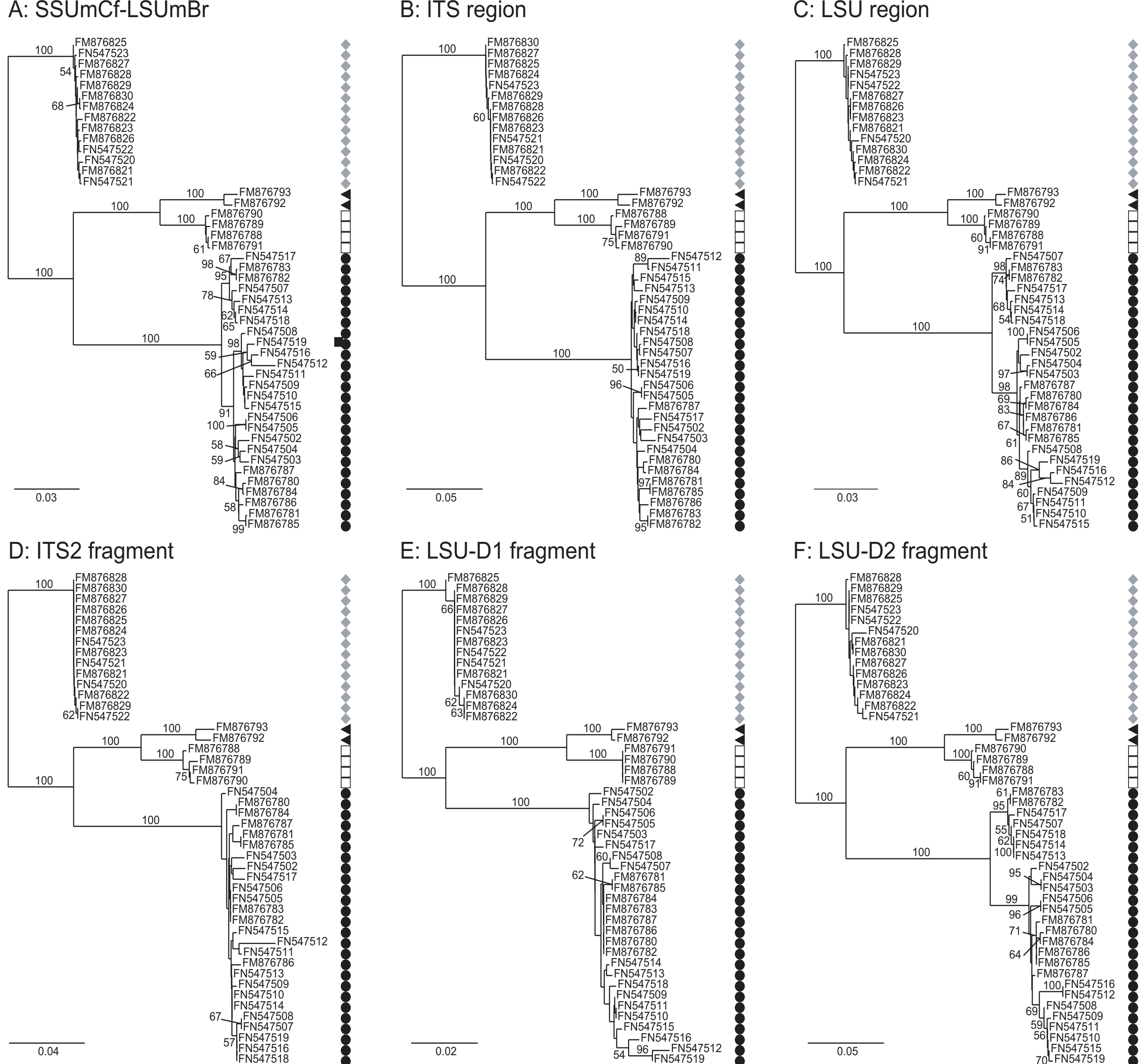


Figure S4: SSUmCf-LSUmBr (A), ITS region (B), LSU region (C), ITS2 fragment (D), LSU-D1 fragment (E), LSU-D2 fragment (F) neighbour joining analyses (1000 BS) of *Glomus* Group B from the core dataset. *Glomus* sp. W3349 (◆), *Glomus luteum* (□), *Gl. etunicatum* (●).

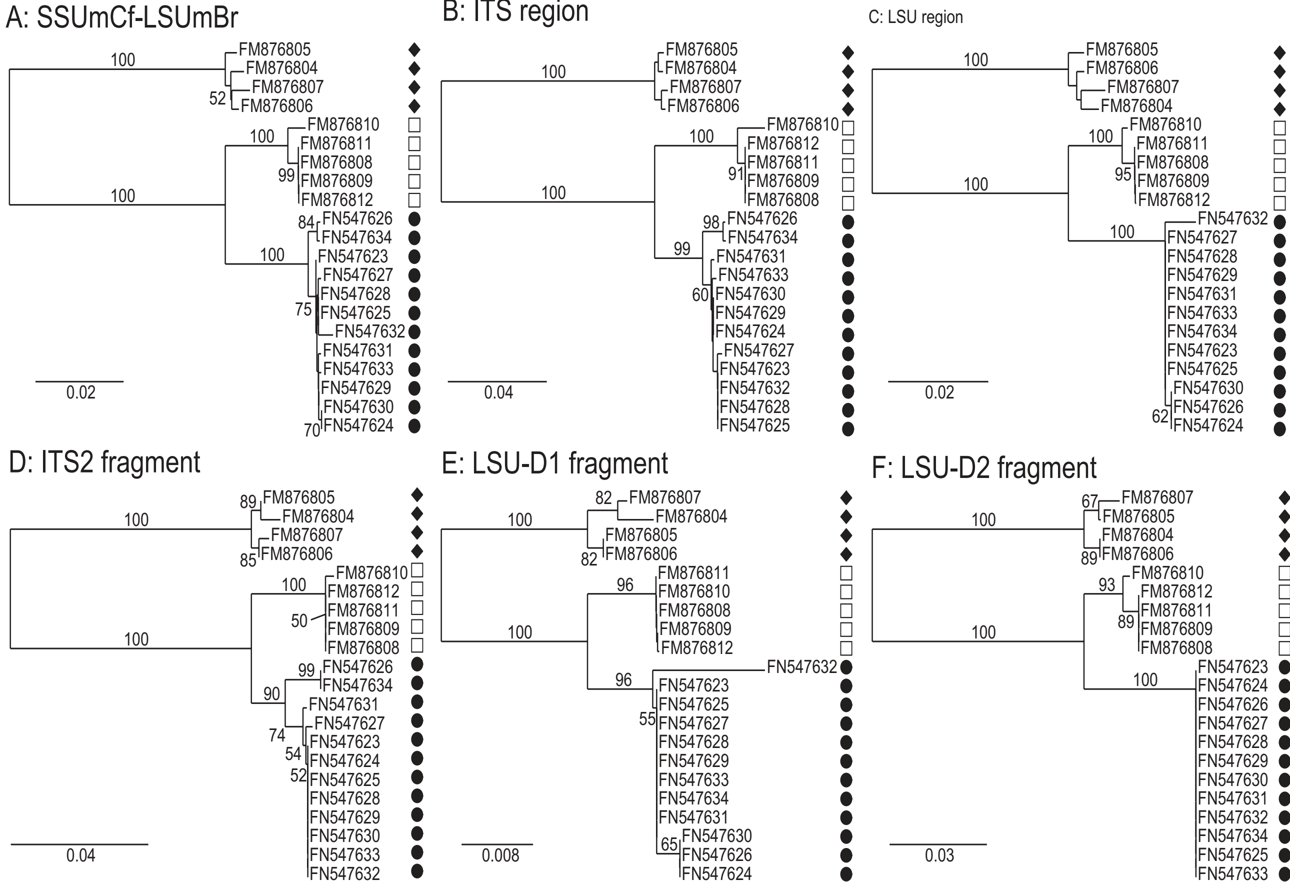


Figure S5: SSUmCf-LSUmBr (A), ITS region (B), LSU region (C), ITS2 fragment (D), LSU-D1 fragment (E), LSU-D2 fragment (F) neighbour joining analyses (1000 BS) of *Diversisporaceae* from the core dataset. *Glomus eburneum* (▼), *Gl. aurantium* (◄), *Gl. versiforme* (□), *Diversispora celata* (◄), *Di. spurca* (▼).

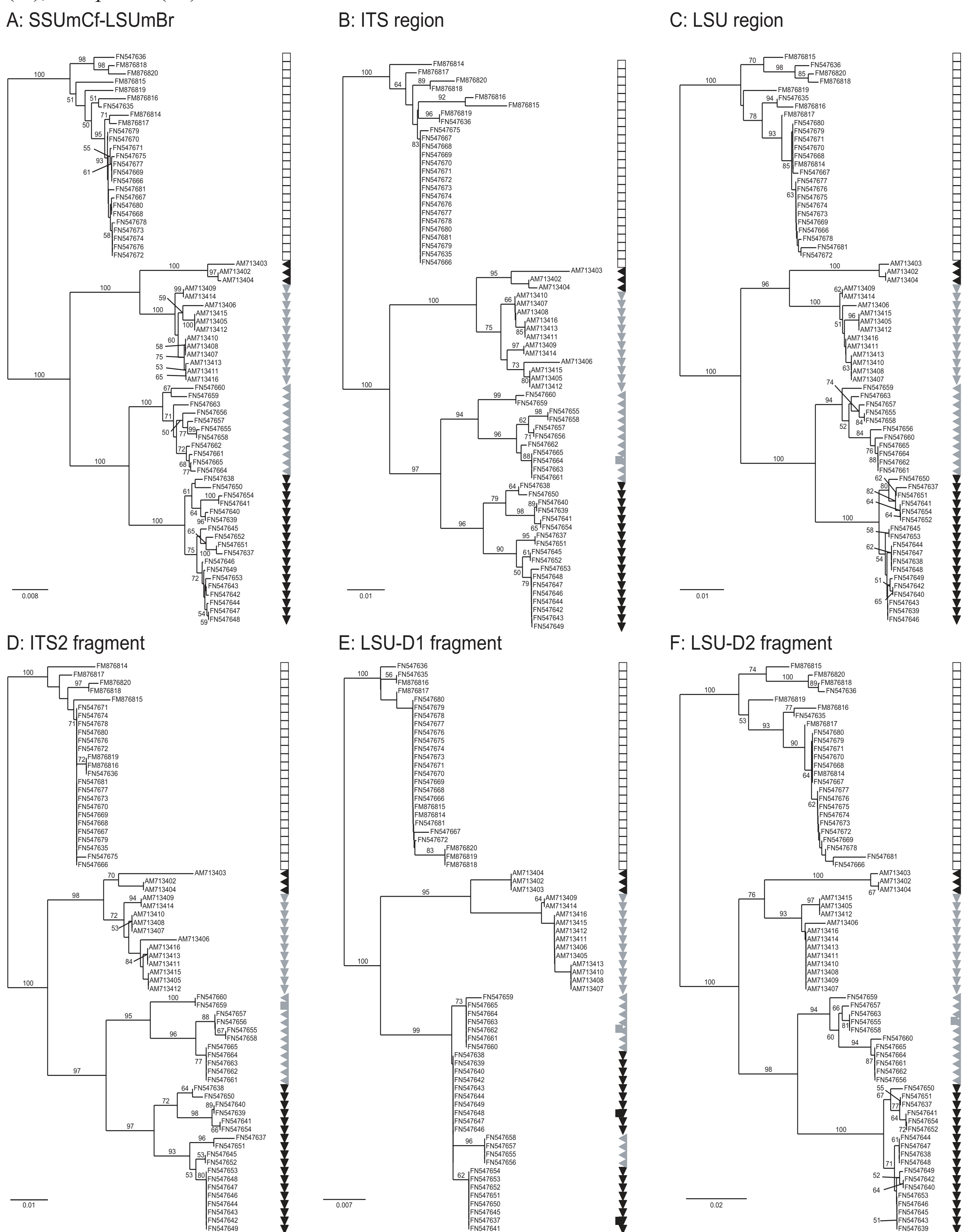


Figure S6: SSUmCf-LSUmBr (A), ITS region (B), LSU region (C), ITS2 fragment (D), LSU-D1 fragment (E), LSU-D2 fragment (F) neighbour joining analyses (1000 BS) of *Gigasporaceae* from the core dataset. *Scutellospora spinosissima* (■), *Sc. heterogama* (□), *Gigaspora rosea* (▼), *Sc. gilmorei* (◆), *Gi. margarita* (●).

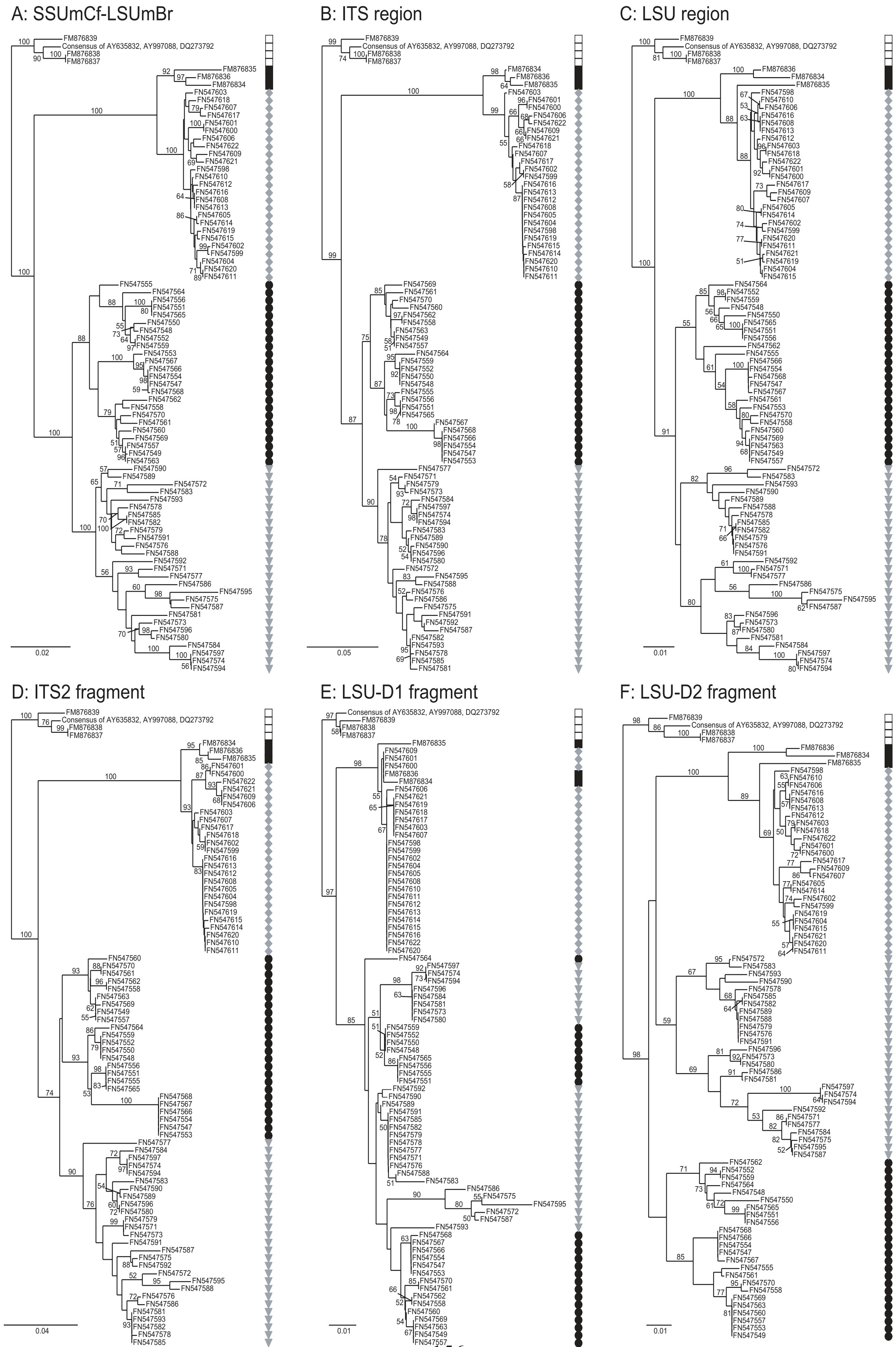

A: SSUmCf-LSUmBr
B: ITS region
C: LSU region
D: ITS2 fragment
E: LSU-D1 fragment
F: LSU-D2 fragment
0.02
0.05
0.01
0.04
0.01
0.01

Figure S7: A: Barcode gap analyses of the *Ambisporaceae* including database sequences of the complete ITS region and the ITS2 fragment. B: Barcode gap analyses of the *Diversisporaceae*, including database sequences. Analyses at the right were performed including outlier sequences that are likely not corresponding to the respective species. Light grey bar: max. intraspecific variation, dark grey bar: min. interspecific variation, to the right of the bar the closest species is given, respectively. Scale on top is % variation based on K2P distances. Vertical line indicates minimal interspecific variation.

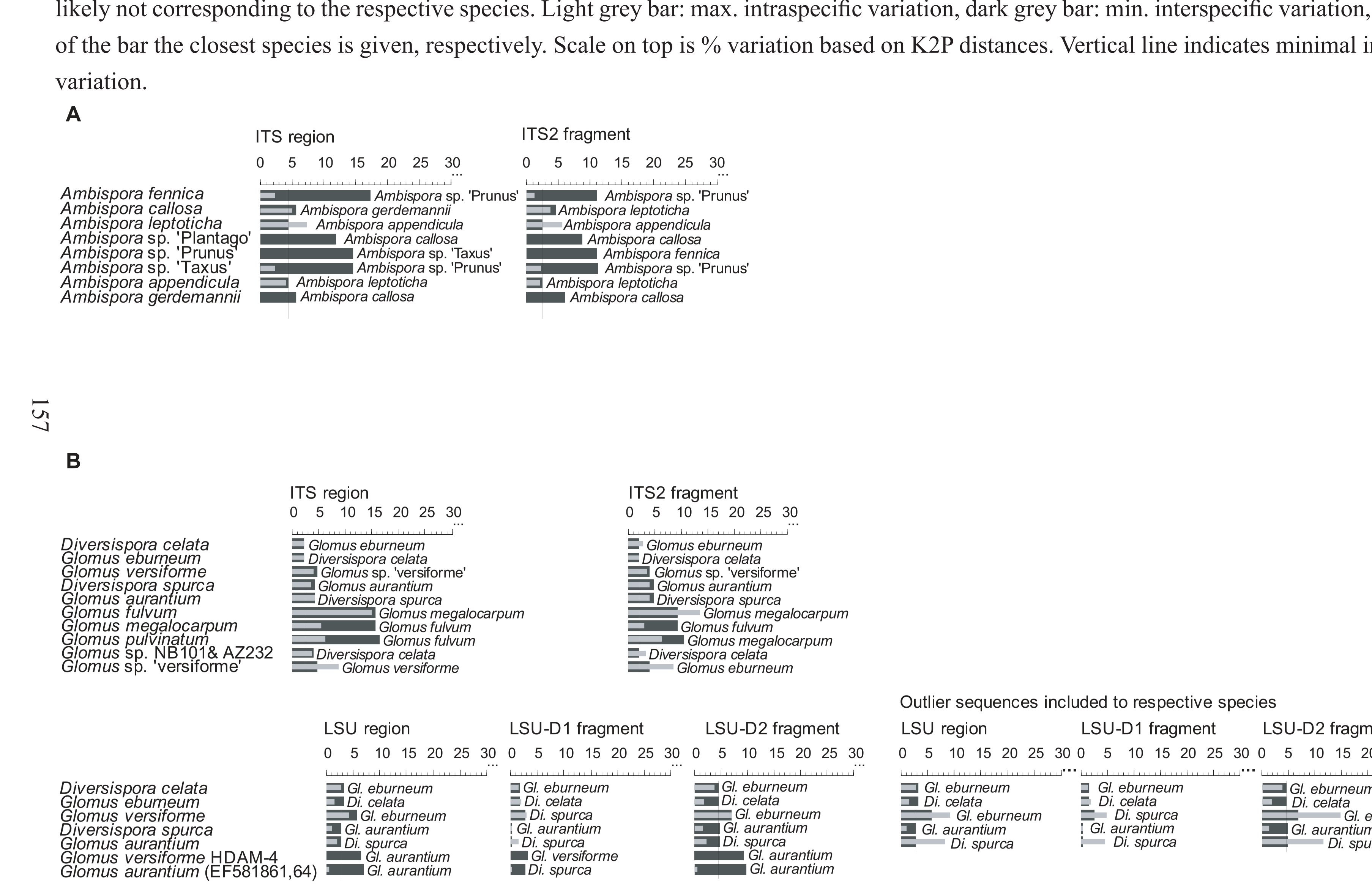


Figure S8: ITS region (A) and ITS2 fragment (B) neighbour joining analyses (1000 BS) of the *Ambisporaceae*. *Ambispora gerdemanii* (▼), *Am. leptoticha* (⊠), *Am. callosa* (◄), *Am. fennica* (▲), *Am. appendicula* (◆), *Ambispora* sp. from *Plantago* (■), *Ambispora* sp. from *Prunus* (□), *Ambispora* sp. from *Taxus* (►).

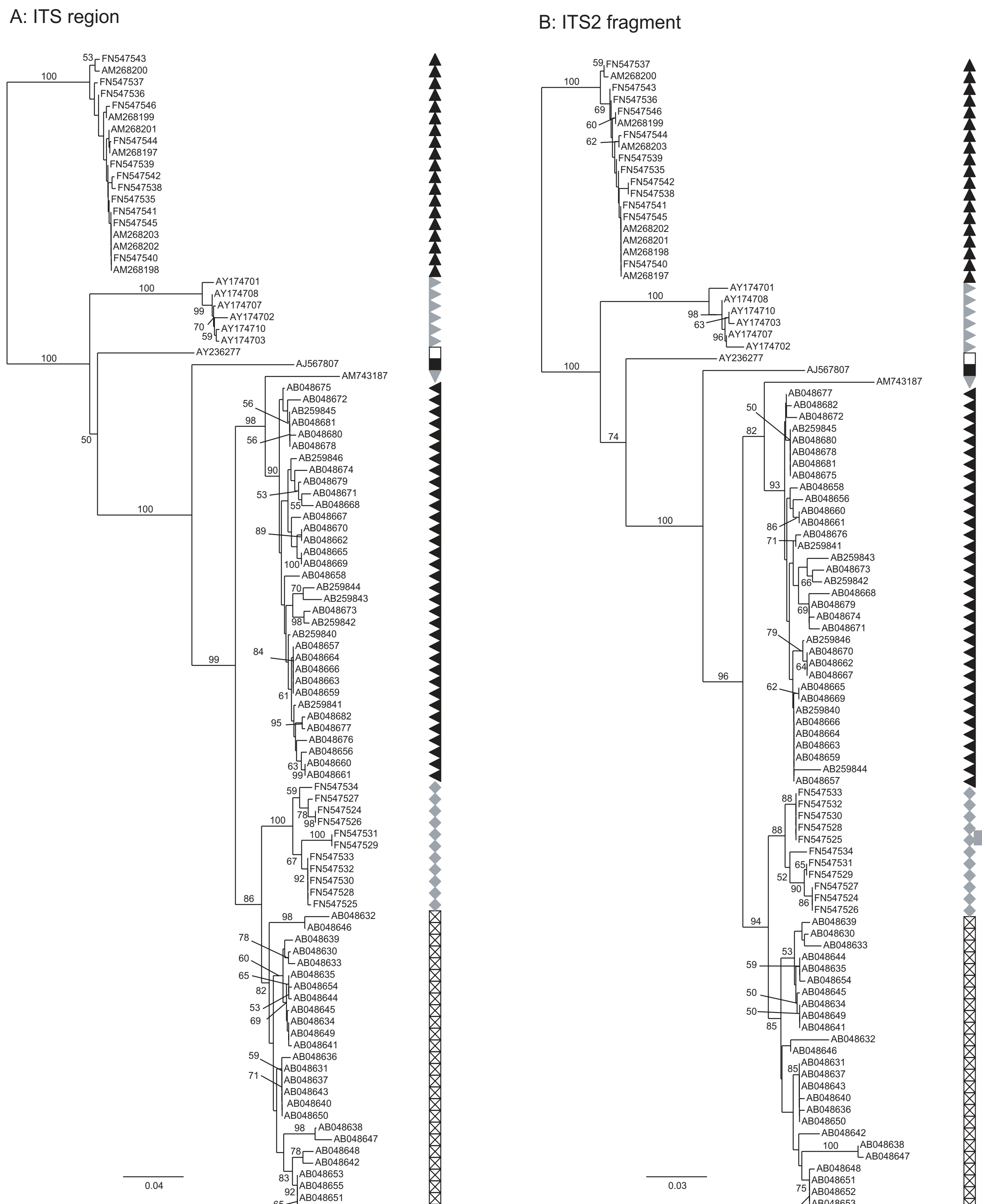


Figure S9: ITS region (A) and ITS2 fragment (B) neighbour joining analyses (1000 BS) of the *Diversisporaceae*. *Glomus eburneum* (▼), *Gl. aurantium* (◄), *Gl. versiforme* (□), Diversispora *celata* (◄), *Di. spurca* (▼), *Gl. megalocarpum* (●), *Gl. fulvum* (●), *Gl. pulvinatum* (►), *Glomus* sp. NB101 (▲), *Glomus* sp. AZ37B (▲), *Glomus* sp.'versiforme' environmental (⊠).

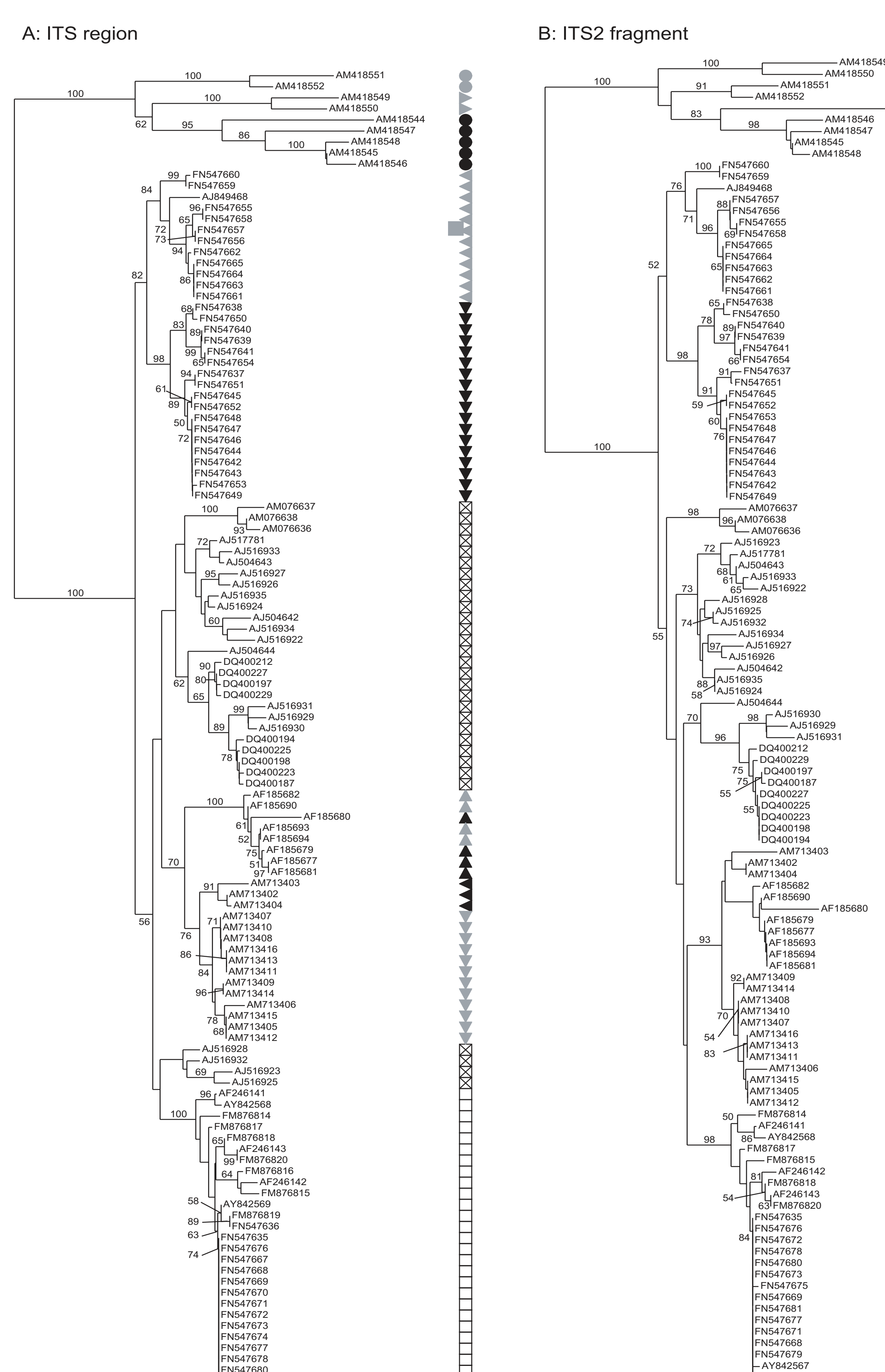
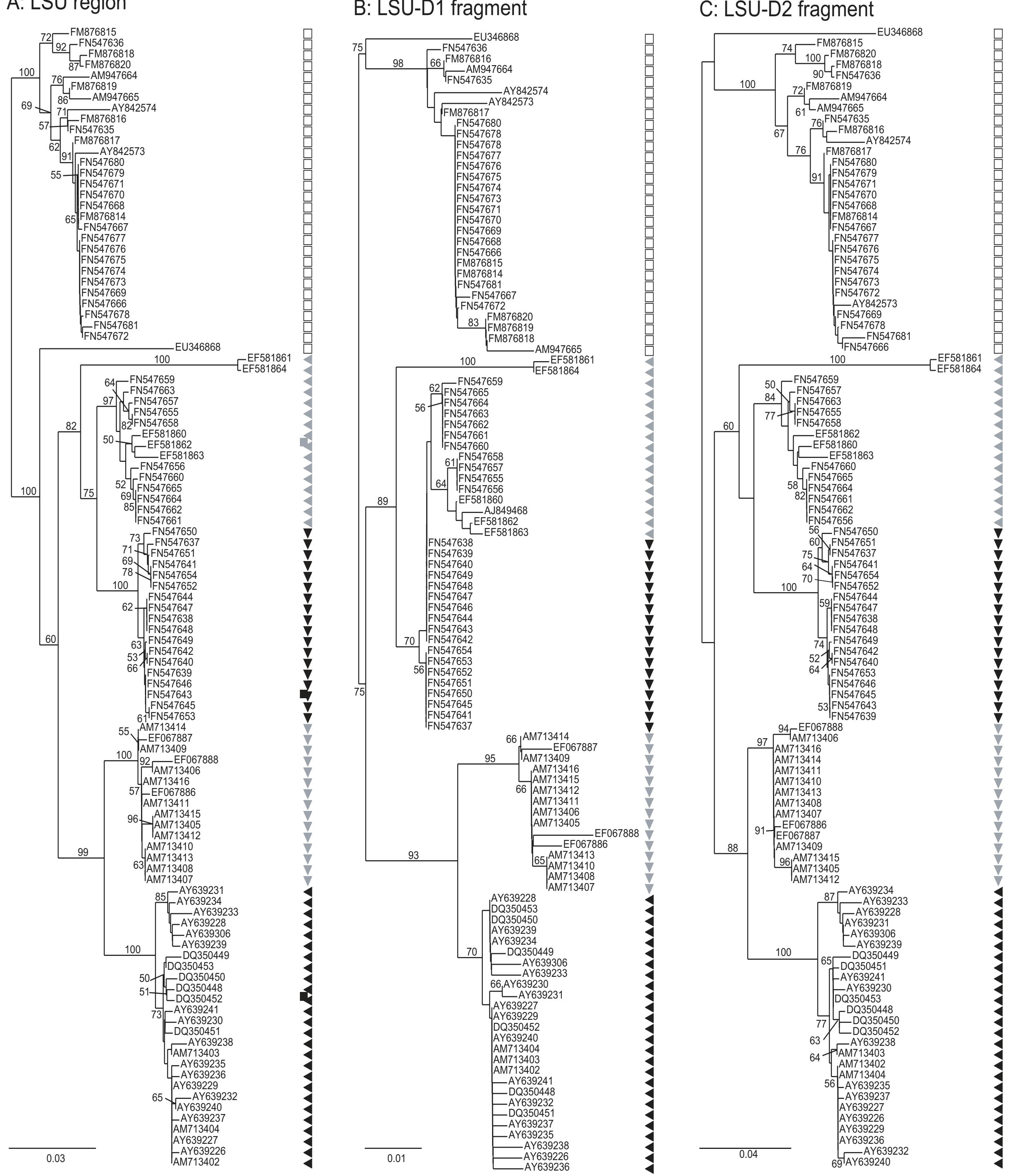

A: ITS region
AM418551
AM418552
AM418549
AM418550
AM418544
AM418547
AM418548
AM418545
AM418546
FN547660
FN547659
AJ849468
FN547655
FN547658
FN547657
FN547656
FN547662
FN547665
FN547664
FN547663
FN547661
FN547638
FN547650
FN547640
FN547639
FN547641
FN547654
FN547637
FN547651
FN547645
FN547652
FN547648
FN547647
FN547646
FN547644
FN547642
FN547643
FN547653
FN547649
AM076637
AM076638
AM076636
AJ517781
AJ516933
AJ504643
AJ516927
AJ516926
AJ516935
AJ516924
AJ504642
AJ516934
AJ516922
AJ504644
DQ400212
DQ400227
DQ400197
DQ400229
AJ516931
AJ516929
AJ516930
DQ400194
DQ400225
DQ400198
DQ400223
DQ400187
AF185682
AF185690
AF185680
AF185693
AF185694
AF185679
AF185677
AF185681
AM713403
AM713402
AM713404
AM713407
AM713410
AM713408
AM713416
AM713413
AM713411
AM713409
AM713414
AM713406
AM713415
AM713405
AM713412
AJ516928
AJ516932
AJ516923
AJ516925
AF246141
AY842568
FM876814
FM876817
FM876818
AF246143
FM876820
FM876816
AF246142
FM876815
AY842569
FM876819
FN547636
FN547635
FN547676
FN547667
FN547668
FN547669
FN547670
FN547671
FN547672
FN547673
FN547674
FN547677
FN547678
FN547680
FN547681
FN547679
FN547666
AY842567
FN547675
0.04
B: ITS2 fragment
AM418549
AM418550
AM418551
AM418552
AM418544
AM418546
AM418547
AM418545
AM418548
FN547660
FN547659
AJ849468
FN547657
FN547656
FN547655
FN547658
FN547665
FN547664
FN547663
FN547662
FN547661
FN547638
FN547650
FN547640
FN547639
FN547641
FN547654
FN547637
FN547651
FN547645
FN547652
FN547653
FN547648
FN547647
FN547646
FN547644
FN547643
FN547642
FN547649
AM076637
AM076638
AM076636
AJ516923
AJ517781
AJ504643
AJ516933
AJ516922
AJ516928
AJ516925
AJ516932
AJ516934
AJ516927
AJ516926
AJ504642
AJ516935
AJ516924
AJ504644
AJ516930
AJ516929
AJ516931
DQ400212
DQ400229
DQ400197
DQ400187
DQ400227
DQ400225
DQ400223
DQ400198
DQ400194
AM713403
AM713402
AM713404
AF185682
AF185690
AF185680
AF185679
AF185677
AF185693
AF185694
AF185681
AM713409
AM713414
AM713408
AM713410
AM713407
AM713416
AM713413
AM713411
AM713406
AM713415
AM713405
AM713412
FM876814
AF246141
AY842568
FM876817
FM876815
AF246142
FM876818
AF246143
FM876820
FN547635
FN547676
FN547672
FN547678
FN547680
FN547673
FN547675
FN547669
FN547681
FN547677
FN547671
FN547668
FN547679
AY842567
FN547666
FN547674
FN547670
FN547667
FM876819
FM876816
FN547636
AY842569
0.04

Figure S10: LSU region (A), LSU-D1 fragment (B) and LSU-D2 fragment (C) neighbour joining analyses (1000 BS) of the *Diversisporaceae*. *Glomus eburneum* (▼), *Gl. aurantium* (◄), *Gl. versiforme* (□), *Diversispora celata* (◄), *Di. spurca* (▼).



11.2 Contribution of the author

Walker C, Vestberg M, Demircik F, Stockinger H, Saito M, Sawaki H, Nishmura I, Schüßler A. 2007. Molecular phylogeny and new taxa in the *Archaeosporales* (*Glomeromycota*): *Ambispora fennica* gen. sp. nov., *Ambisporaceae* fam. nov., and emendation of *Archaeospora* and *Archaeosporaceae*. *Mycological Research* **111**: 137-153.

Parts worked out by Herbert Stockinger: He contributed a part of the rDNA sequences for this analysis.

Krüger M, Stockinger H, Krüger C, Schüßler A. 2009. DNA-based species level detection of *Glomeromycota*: one PCR primer set for all arbuscular mycorrhizal fungi. *New Phytologist* **183**: 212-223.

Parts worked out by Herbert Stockinger: He participated in the alignment of new and database sequences and in the design of the primers. In addition, a part of the newly published sequences were generated by him.

Stockinger H, Walker C, Schüßler A. 2009. '*Glomus intraradices* DAOM197198', a model fungus in arbuscular mycorrhiza research, is not *Glomus intraradices*. *New Phytologist* **183**: 1176-1187.

Parts worked out by Herbert Stockinger: He cultured many of the AMF isolates used in this study and generated the vast majority of the new sequences published in this study and he performed the phylogenetic analyses. Together with C. Walker and A. Schüßler, he wrote the manuscript.

Stockinger H, Krüger M, Schüßler A: DNA barcoding for arbuscular mycorrhiza fungi

Parts worked out by Herbert Stockinger: He generated the majority of the sequences, aligned them to the existing database. He performed all DNA barcoding analyses. A Schüßler and he wrote the manuscript.

I hereby confirm the above statements:

Herbert Stockinger PD Dr. Arthur Schüßler

11.3 Curriculum vitae

Herbert Stockinger

Personal data

Date of birth:	1 September 1977
Place of birth:	Braunau am Inn, Austria

Education

2006 –	PhD about "DNA barcode of arbuscular mycorrhizal fungi" in the framework of the European project TRACEAM (TRACEability of Arbuscular Mycorrhizal fungi as plant-beneficial micro-organisms in agro-environments, MEST-CT-2005-021016; Technical University of Darmstadt and Ludwig Maximilian University Munich, Germany) →
2005	Master study in biology passed with distinction, final examination on 28 November 2005 Master Thesis: "Mycorrhizal status of endangered plant species: *Crocus albiflorus, Euphorbia verrucosa* and *Hypochoeris maculata"* Supervisors: Prof. Dr. Kurt Haselwandter, University of Innsbruck, & Prof. Dr. Thomas Peer, University of Salzburg
2003 – 2005	Master Student of Botany at the University of Salzburg
1999 – 2003	Bachelor Student of Environmental Biology at the University of Salzburg
1992 – 1997	College for agriculture in Ursprung in the county of Salzburg
1988 – 1992	Secondary School in Munderfing in the county Upper Austria
1984 – 1988	Elementary School Lochen in the county Upper Austria

Congress Contributions

2009 Apr 7th – 9th	Summer School of the Project TRACEAM with the topics: Molecular ecology, phylogeny, DNA barcoding, bioinformatics tools, AMF taxonomy, Tracing and evolution of AMF endobacteria, Production of AMF *in vitro,* Phylochips and 454FLX sequencing - detection of AMF from the field, Genome structure of AMF, LMU, Munich, Germany, oral presentation

2008 Aug 2nd – 8th	93rd Ecological Society of America Annual Meeting, Milwaukee, Wisconsin, USA. Participation on two workshops of the Fungal Environmental Sampling and Informatics Network (FESIN) – poster presentation
2008 Aug 9th – 13th	Mycological Society of America (MSA) 2008 Annual Meeting, Penn State University, State College, Pennsylvania, USA – poster presentation
2008 Jan 17th – 19th	Winterschool with the topic AM fungi: soil ecology, molecular identification, and endobacteria (BLOs); information exchange with the FUNGIMYC project. University of Turin (UNITO), Turin, Italy, oral presentation.
2007 Oct 3rd – 5th	DNA Barcoding in Europe, Leiden, The Netherlands, poster presentation
2007 May 17th – 19th	EMBO Workshop “Molecular Biodiversity and DNA Barcode”, Roma Italy, poster presentation
2006 Nov 23rd – 25th	Mycorrhizal snapshots, Turino, Italy
2006 Sep 18th – 20th	IXth International Fungal Biology Conference & 16th New Phytologist Symposium: “Impact of genomics on fungal biology“. Nancy/France, poster presentation
2006 Jul 23rd – 27th	5th International Conference on Mycorrhiza “Mycorrhiza for Science and Society“ Granada/Spain
2005 July 17th – 23rd	XVII International Botanical Congress, Vienna/Austria, poster presentation
2005 June 2nd – 4th	Management Committee and Final meeting of COST Action 8.38, Dijon/France, poster presentation
2004 Oct 21st – 23rd	8th Management Committee Meeting of COST Action 8.38, Granada/Spain, poster presentation

Professional experiences

2009 Apr	Scientific Employee Ludwig Maximilian University (LMU) Munich, Germany
2005 Aug – Dec	Technical assistant in the project “Phylogenetic and geographical analyses of the orchid genus *Bulbophyllum* on Madagascar and the Mascarens”, Department of Organismic Biology, University of Salzburg, funded by the Austrian Science Fund (FWF), Austria
2002 – 2005	Teaching Assistant: Bioindication, Soil ecology, Native habitats, Phytology II: *Poaceae*, *Juncaceae* & *Cyperaceae* and ecosystems & nutrient cycles, University of Salzburg, Austria
2001 Jul – Sep	Practical at a farm in Dalwigksthal Hesse, Germany
1999 Apr – 2000 Oct	Deputy Managing Director of Maschinenring Oberes Mattigtal / Upper Austria
1998	Civilian service at the Red Cross Salzburg, Austria