Diversity of cultured isolates and field populations of the arbuscular mycorrhizal fungus *Glomus intraradices*:

Development and application of molecular detection methods for mitochondrial haplotypes

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Summary

Today's plant communities have evolved together with arbuscular mycorrhizal fungi (AMF, Glomeromycota) for millions of years. In "arbuscular mycorrhiza", a mutualistic symbiosis, plants provide carbohydrates to the fungi, which in turn make mineral nutrients like phosphate or nitrogen available to the plants. AMF species diversity is generally higher in natural sites than in agroecosystems, where it can be strongly reduced. The detection of AMF is either based on morphotyping of soil-borne spores or on molecular markers, which can be directly applied using colonized roots of the host plant.

Until recently, studies of AMF diversity on the population level were impossible, as no suitable marker genes were available. The first population studies on AMF had to rely on DNA from spores or root organ cultures (ROCs) and the molecular markers used could not be applied for the detection of AMF genotypes directly in colonized plant roots from the field. Previous work from our laboratory had shown that mitochondrial ribosomal RNA large subunit gene (mtLSU) sequences are homogeneous within several isolates of *Glomus* species and that the mitochondrial gene region is a promising marker for distinguishing strains of *G. intraradices*. The phylotype GLOM A-1 of this morphospecies which was defined in previous studies of our laboratory based on nuclear-encoded rDNA internal transcribed spacers (ITS) sequences seems to occur ubiquitously, showing a high ecological versatility. It is frequently used as model organism and its genome is being sequenced.

The aim of this thesis was to develop and apply detection methods based on the mtLSU in order to investigate the diversity of *G. intraradices* isolates and field populations. The main question was whether this marker is suitable to resolve the genetic structure of this morphospecies which might allow shedding light on the ecological role of strains within the species.

In the first part of this thesis, the diversity of the mtLSU was investigated in a set of 16 *G. intraradices* isolates originating from five continents, either obtained as soil inoculum or as ROC. Among these isolates, 12 different mtLSU haplotypes could be distinguished, whereas homogeneity of the marker within the isolates was confirmed. Several mtLSU haplotypes were already distinguishable by size differences of the PCR products, mainly based on the presence or absence of length-variable introns. The reliability of the marker is dependent on evolutionary intron stability, which was confirmed for some introns by comparisons of multiple culture lineages of the same isolate obtained from different culture collections. In phylogenetic analyses of mtLSU exon sequences from isolates and root-colonizing *G. intraradices*, several clades could be distinguished. Comparison with ITS sequences from the isolates showed a higher resolution of mtLSU exon sequences which was increased by intron sequences.

In order to increase the specificity for *G. intraradices* and to optimize amplification of the mtLSU fragment from colonized plant roots, a new nested PCR approach was developed and tested

using field root samples from a semi-natural grassland and a mine spoil in Hungary. A RFLP approach was developed to reduce time-consuming and expensive cloning and sequencing procedures.

In the second part of this thesis, the population structure of an AMF in roots from the environment was analyzed for the first time. Two agricultural field experiments in Switzerland, including different tillage treatments, and two semi-natural grasslands in Switzerland and France were chosen for the investigation of the genetic structure of *G. intraradices* phylotype GLOM A-1 using the PCR-RFLP approach. Each field site was dominated by one or two frequently found RFLP patterns of *G. intraradices* GLOM A-1, which were defined as Intra types. The composition of Intra types differed strongly between the agricultural sites and the semi-natural grasslands, but also between the two agricultural sites. In contrast to the situation often found in AMF species community studies, RFLP type richness was higher in the agricultural sites compared to the grasslands. Four Intra types, shared by different sites, were further resolved by sequence analyses, but only the two grasslands were found to share mtLSU sequence haplotypes. In phylogenetic analyses of completely sequenced examples of each Intra type, almost all haplotypes from the grassland sites fell within a separate "grassland clade".

If a single mtLSU haplotype could be specifically detected in a pool of others, such a molecular tool could be used for tracing single strains inoculated in a field site. Nested PCR primers were developed specifically for one single mtLSU haplotype, which dominated one of the agricultural sites and was known from previous studies analyzing ROCs. By applying this approach to all samples from the four study sites, it could be shown that the respective haplotype was only detected in samples previously tested positive for this type using the general approach. In other words, both methods confirmed each other.

Two further specific nested PCR approaches were developed for two mtLSU haplotypes representing the *G. intraradices* isolate BEG140. These approaches were designed to be applied for tracing this isolate inoculated in a field experiment performed in a mine spoil bank of the Czech Republic in the context of a reclamation project.

Besides the considerable genetic structure of this fungus among the isolates studied and in the roots of the field sites, evidence of specialized mtLSU haplotypes was reported, which might represent ecotypes or even different ("cryptic") species. It could be shown that world-wide mtLSU haplotype diversity of *G. intraradices* is considerably higher than previously assumed. More investigations of different ecosystems are required for the determination of adapted ecotypes.

The approaches developed here will be furthermore useful for instance in inoculation experiments and functional tests, e.g. in greenhouse experiments. By presenting first insights into the genetic structure of the most widespread species of arbuscular mycorrhizal fungi, the findings presented here will have major implications on our views of processes of adaptation and specialization in these plant ∕ fungus associations.

Chapter 1: General introduction

1.1 Mycorrhizas – origin, evolution and types

Diverse microbes and algae had evolved and developed in the oceans of our world at a time in which the land masses were still inhospitable for complex organisms. Approximately 2.7 billion years ago, photosynthetic organisms started to climb up the early continents and at least fringes of the land mass adjacent to the oceans or lakes became green (Buick 1992). Records for the origin of embryophytic land plants date back as early as the mid-Ordovician (early Llanvirn: ~ 476 Myr; Strother *et al.* 1996). Due to the activity of sea-born Cyanobacteria, the atmosphere had been enriched in oxygen. However, it is assumed that the crucial step of the colonization of the land by embryophyte lineages (Kenrick & Crane 1997) would have never taken place in such a successful way without the help of a partnership between plants and – fungi (Pirozynski & Malloch 1975). This association also may have been responsible for the enormous plant diversity in different environments like desert ecosystems, tropical rainforests or temperate habitats of the present time: fossil data have shown that typical structures (hyphae and spores) of today's arbuscular mycorrhizal fungi (AMF; see section 1.2) were already present 460 million years ago, a time, which was most likely dominated by liverwort-like plants on the bryophytic level as potential partners of the fungi (Redecker *et al.* 2000). This partnership between plants and fungi is known as "mycorrhiza" (Greek for "fungus root"). Fossils of *Aglaophyton* (a non-vascular plant; see in Edwards 1986), containing structures of mycorrhizal fungi, are dated back 400 million years ago (Remy *et al.* 1994; Taylor *et al.* 1995). However, today's members of the Glomeromycota (see section 1.2.1) do generally have partnerships with vascular plants and bryophytes, but one is also known to associate with cyanobacteria which is one of the reasons why it is assumed that these fungi were already associated with cyanobacteria or algae in semi-aquatic or humid habitats before they co-evolved with early land plants (Schüßler 2002).

In the scientific community it is well accepted that mycorrhizas are the chief organs for the nutrient uptake for the majority of higher plant species (Begon *et al.* 1998). Mycorrhizas are structures formed by the two different organisms, plant and fungus, which benefit from each other in a mutualistic symbiosis under ideal conditions, i.e. the plant provides carbohydrates for the fungus, which in turn makes nutrients available for the phytobiont. The term symbiosis also implies parasitism or neutral associations between different organisms (de Bary 1887), an aspect that also fits for the mycorrhiza itself. The proportion of benefits can shift towards one direction within a single association and bidirectional nutrient transfer, and therefore mutualism, becomes obviously doubtful in non-photosynthetic plants (Smith & Read 2008; see also section 1.2.4.2).

Despite the ubiquitous, important role of mycorrhizas, some plant families do not show any partnership with mycorrhizal fungi, but at least for descendants of arbuscular mycorrhizal ancestors

it is assumed that the loss of the symbiosis constitutes a derived condition (Sanders 2002). On the other hand, evolutionary transitions among parasitic, saprotrophic and mutualistic symbioses have been observed by phylogenetic analysis of basidiomycetes (Hibbett *et al.* 2000). This evolutionary instability was additionally reported for further taxa of the true fungi by e.g. James *et al.* (2006), who reconstructed the evolution of fungi using a six-gene phylogeny.

Over the time, different forms of mycorrhizas have evolved. So far, seven major important groups have been established, and more may be described in case that new morphological, anatomical or molecular observations will make it necessary (Peterson *et al.* 2004). The respective partners of (1) arbuscular mycorrhiza, (2) ectomycorrhiza, (3) ectendomycorrhiza, (4) arbutoid mycorrhiza, (5) monotropoid mycorrhiza, (6) ericoid mycorrhiza and (7) orchid mycorrhiza are listed in Table 1.

Table 1 The plant and fungal partner taxa and characteristics of the important mycorrhizal types. The structural characters given relate to the mature state, not the developing or senescent states. Entries in brackets indicate rare conditions. Modified from Smith & Read (2008).

Kinds of mycorrhiza	Arbuscular	Ecto	Ectendo	Arbutoid	Monotropoid	Ericoid	Orchid
Fungal taxa	Glomero	Basidio/ Asco (Glomero)	Basidio/ Asco	Basidio	Basidio	Asco	Basidio
Plant taxa	Bryo Pterido Gymno Angio	Gymno Angio	Gymno Angio	Ericales	Monotro- poidea	Ericales Bryo	Orchidales
Fungi septate	-	+	+	+	+	+	+
aseptate	+	-	-	-	-	-	-
Intracellular colonization	+	-	+	+	+	+	+
Fungal mantle	-	+	+ or -	+ or -	+	-	-
Hartig net	-	+	+	+	+	-	-
Achlorophylly	- (+)	-	-	-	+	-	+*

*, All orchids are achlorophyllus in the early seedling stages. Most orchid species are green as adults. The fungal taxa are abbreviated from Glomeromycota, Ascomycota and Basidiomycota; the plant taxa from Bryophyta, Pteridophyta, Gymnospermae and Angiospermae.

Several species of both plants and fungi are able to form different types of mycorrhiza, dependent on the respective partner, suggesting similarities or even structures that are in common among different types. All main characteristics of the single mycorrhiza types are summarized in Table. 1: the mantle consists of fungal hyphae, densely enclosing the root tips and replacing the root hairs and their function. Emanating from the mantle, hyphae penetrate the rhizodermis and grow into the root, between epidermal and cortical cells, forming a network – the Hartig net (Smith & Read 2008). All mycorrhiza types except ectomycorrhiza are characterized by intracellular structures, i.e. hyphae directly enter the root cells and do not only grow intercellularly. Intracellular structures can vary among the types: coils are produced in arbuscular –, arbutoid – and ericoid mycorrhizas, the coil-like structures in orchid mycorrhiza are called pelotons. The intracellular

proliferation structure of monotropoid mycorrhiza is named "fungal peg" (Smith & Read 2008). The most typical intracellular structure of arbuscular mycorrhiza is the arbuscule, which gave the name to the symbiosis. Detailed descriptions of the arbuscule and other possible structures of the AMF are following in section 1.2.3. A graphical overview of the most important structures of different mycorrhiza types is depicted in Fig. 1.
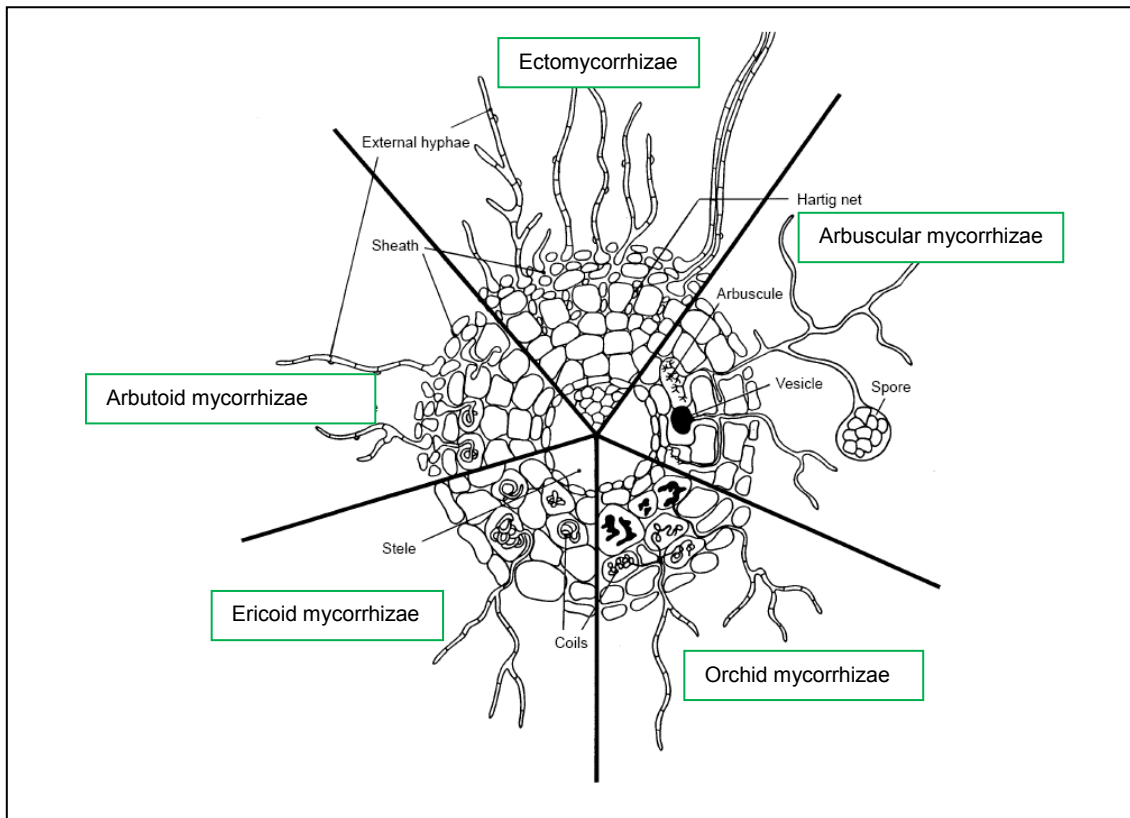
Fig. 1 Growth patterns of important mycorrhiza types. Modified from Selosse & Le Tacon (1998).

The different mycorrhiza types do not only differ in structural characteristics but also in global distribution, which is strongly correlated with the respective functional role. Read & Perez-Moreno (2003) illustrated major gradients consisting of e.g. pH, P/N ratio, increasing latitude or altitude, correlated with differing frequencies of occurrence of ericoid –, ecto- and arbuscular mycorrhizas (see Fig. 2). It is obvious that the different mycorrhiza types occur in concordance with the ecological preferences of the respective plant partners. The largest number of plant species forms arbuscular mycorrhiza, followed by the orchid – and ectomycorrhiza (see Fig. 3). In general, ectomycorrhiza is formed by trees, predominately in forest ecosystems (see e.g. Courty *et al.* 2010), and shrubs, but rarely by herbs, which are dominated by AMF associations in temperate areas. In the tropics, however, arbuscular mycorrhiza is also frequently found in the roots of trees (Smith & Read 2008; see also section 1.2.4.1).
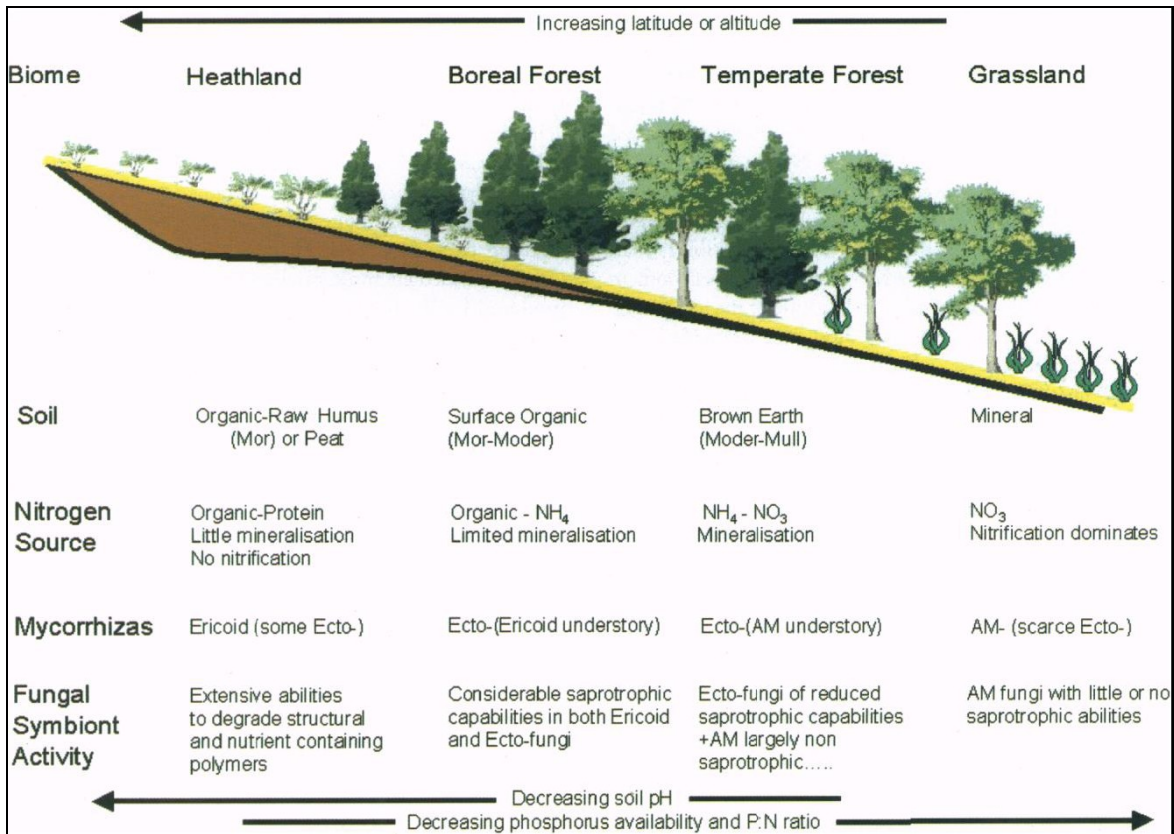
Fig. 2 The proposed relationship, on a northern hemisphere based global scale, between the distribution of biomes along environmental gradients and the roles of the prevailing mycorrhizal association in facilitation of N and P capture by the characteristic functional groups of plant. From Read & Perez-Moreno (2003).
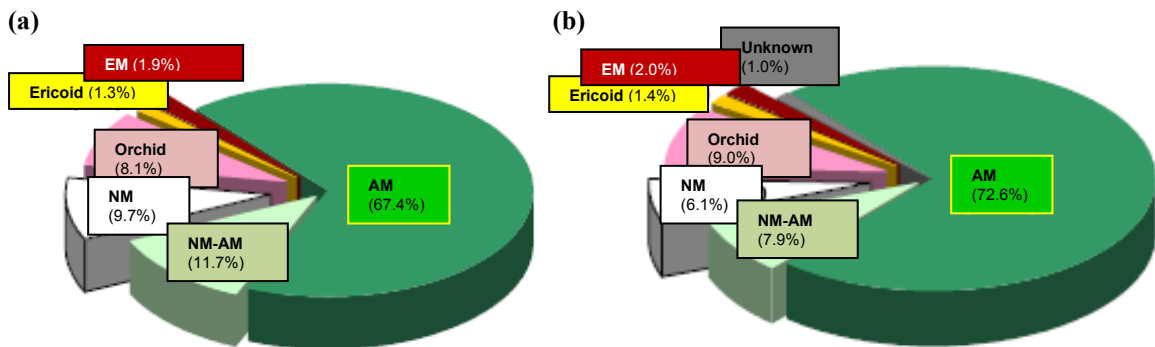
Fig. 3 The relative diversity of mycorrhizal or nonmycorrhizal plants (NM) for **(a)** all vascular plants and **(b)** for angiosperms. EM (ectomycorrhiza), AM (arbuscular mycorrhiza). Modified from Brundrett (2009).

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1.2 The arbuscular mycorrhiza (AM)

1.2.1 Phylogeny and taxonomy of the Glomeromycota

Arbuscular mycorrhizal fungi exclusively belong to the recently established phylum "Glomeromycota" (Schüßler *et al.* 2001), a fungal group that also comprises the Geosiphonaceae, whose member *Geosiphon pyriformis* associates with cyanobacteria in an endosymbiosis (Schüßler *et al.* 1994). Several revised classifications were established since AM fungi were originally placed within the Endogonaceae (Endogonales) of the Zygomycota, a fungal phylum which is now known not to be monophyletic. Cladistic analyses of spore characteristics (e.g. Morton & Benny 1990) and the taxonomy did not always correspond to a natural classification. AMF do not form any zygospores and furthermore represent strictly obligate symbionts, in contrast to other members of the Endogonales. After moving AMF species from Endogone into four new genera (Gerdemann & Trappe 1974) and the revised classification of Morton & Benny (1990), who separated a new order, Glomales, from the Endogonales, AMF were placed in a separate phylum by Schüßler *et al.* (2001):

These authors established a phylogenetic tree of higher fungal taxa, using the small ribosomal subunit (SSU) of ribosomal RNA genes (see Fig. 4). In this phylogeny the Glomeromycota form a well-supported monophyletic clade, whereas the polyphyletic Zygomycota are separate. A more comprehensive phylogeny of fungi was published by James *et al.* (2006). In this study, the Glomeromycota are forming a sister clade to the Basidio- and Ascomycota in accordance with Schüßler *et al.* (2001) (see Fig. 4) and also Tehler *et al.* (2003). However, phylogenies based on protein-encoding genes (e.g. alpha- and beta-tubulin, the RNA polymerase II subunits rpb1 and rpb2) do not support sister-group relationship to the Dikarya, but show the Glomeromycota closer to *Mortierella* (Helgason *et al.* 2003; Redecker & Raab 2006; Liu *et al.* 2009; see also section 1.3.2).

Progress of a natural systematics of AM fungi had started already before the new phylum Glomeromycota was established. Moreover, authors became aware that both morphological and phylogenetic analyses have to be considered for classification. In this context Morton & Redecker (2001) described two new families of AMF, the Archaeosporaceae and Paraglomeraceae. Some members of these families were previously assigned to the genus *Glomus* of the family Glomeraceae. Schwarzott *et al.* (2001) further analyzed this largest genus of the AMF and established a new family structure by dividing the remaining Glomeraceae into the Diversisporaceae and a family Glomeraceae containing the subgroups "*Glomus* group A" (includes e.g. *Glomus intraradices*, *G. proliferum*, *G. clarum* and *G. mosseae*; see Fig. 5) and "*Glomus* group B" (includes e.g. *G. claroideum*, *G. etunicatum*; see Fig. 5). Actually, these two subgroups are represented by two clades, which differ by phylogenetic distances equal to distances between other families (see Fig. 6). A further emendation was the establishment of the Ambisporaceae within the paraphyletic genus *Archaeospora*, whereas the family Archaeosporaceae was maintained with its

type species (Walker *et al.* 2007). Moreover, the new family Entrophosporaceae was established (Sieverding & Oehl 2006), but its phylogenetic position is unclear. It is not included in the phylogenetic tree implementing the most recent phylogenetic analysis of the Glomeromycota (see Fig. 6). Other just recently erected families and genera arising by splitting the Gigasporaceae (Oehl *et al.* 2008) are strongly discussed or disputed in the scientific community (Morton & Msiska in press) and not considered in Fig. 6.
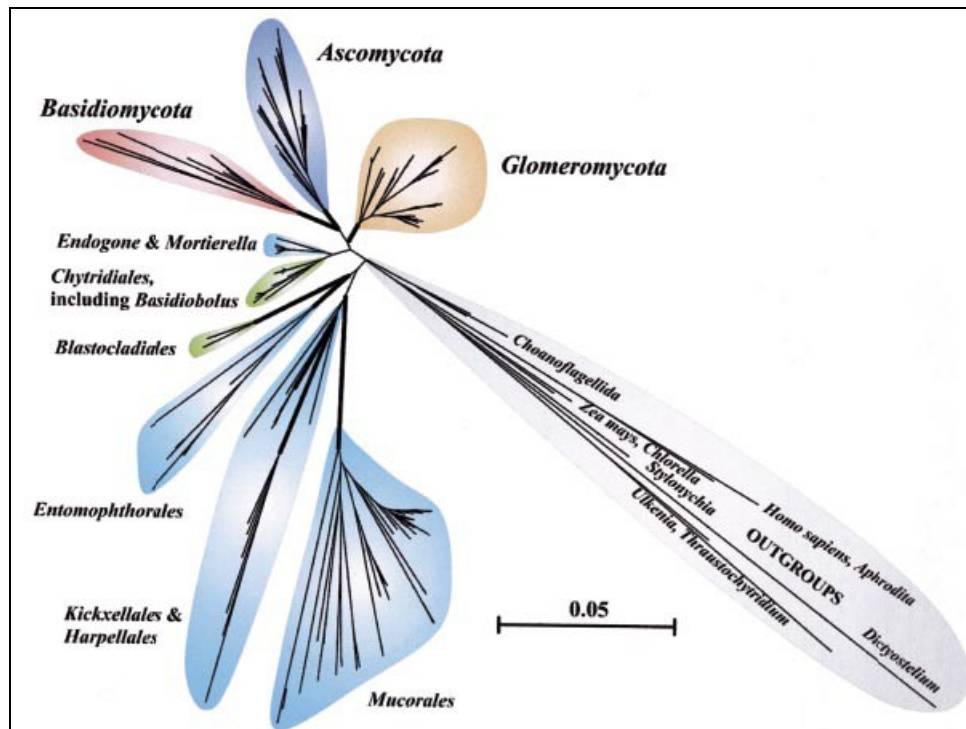
Fig. 4 Phylogeny of fungi based on SSU rRNA gene sequences. Thick lines delineate clades supported by bootstrap values above 90%. The Zygomycota and the Chytridiomycota do not form monophyletic clades and therefore are shown as the respective taxa representing the clade. From Schüßler *et al.* (2001).

The current classification of AMF is summarized in Table. 2. The history of new descriptions and emendations shows that the taxonomic concept is being adapted to a natural classification step by step. Before phylogenetic analyses of DNA sequences were established, lipid analyses were used and supported taxonomical assumptions (Sancholle & Dalpé 1993). Lipid profiles were also used for AMF identification later on (e.g. Jansa *et al.* 1999). However, the taxonomy was almost wholly reliant on the morphological and anatomical characteristics of spores and their development stages as the morphological diversification of other AMF structures is rather low (see section 1.2.3).

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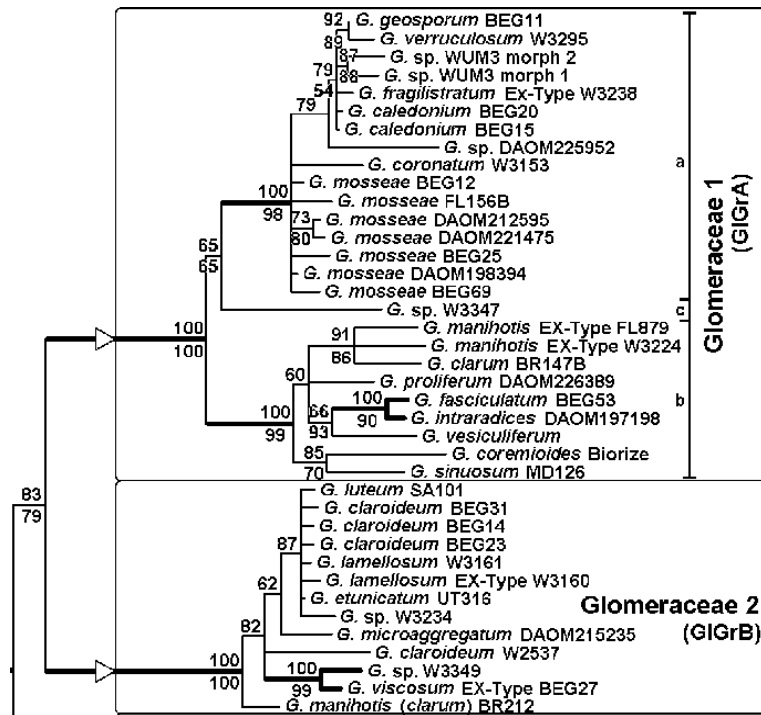
Fig. 5 Phylogenetic tree (based on SSU rDNA sequences) of the *Glomus* group A (GlGrA) and *Glomus* group B of the Glomeracecae. Modified from Schwarzott *et al.* (2001).

Several AMF genera share morphological spore features, which are easy to observe but might represent plesiomorphic characters (e.g. the mode of spore formation; Redecker & Raab 2006). Therefore, detailed microscopic analyses are required for the identification of anatomical subtleties for instance of spore wall layers. The homepage http://invam.caf.wvu.edu/fungi/fungindex.htm of the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) provides some links concerning classification, nomenclature, species descriptions and diagnosis (keys).

Spore formation has represented an important criterion for the identification of genera. Members of *Glomus* (Berch & Fortin 1984), *Pacispora* (Oehl & Sieverding 2004), *Diversispora* (Walker & Schüßler 2004), *Ambispora* (Walker *et al.* 2007) and *Paraglomus* (Morton & Redecker 2001) are developing spores by blastic expansion of a hyphal tip (glomoid spores). However, in *Ambispora* a second spore type occurs (even within the same – "dimorphic" – species) that is typical for *Acaulospora* (Gerdemann & Trappe 1974) and *Archaeospora* (*A. trappei*; Morton & Redecker 2001): a sporiferous saccule is blastically formed at the hyphal tip and the acaulosporoid spore is developing at the "saccule neck" (the side of the subtending hypha). In contrast, *Entrophospora* (Ames & Schneider 1979) develops spores directly from the saccule neck, thus not lateral but within the subtending hypha (entrophosporoid mode of spore formation). The fourth, gigasporoid, type of spore formation characterizes both *Gigaspora* (Gerdemann & Trappe 1974) and *Scutellospora* (Walker & Sanders 1986). Spores are formed on a "sporogenous cell" of a size of 25-50 μm.

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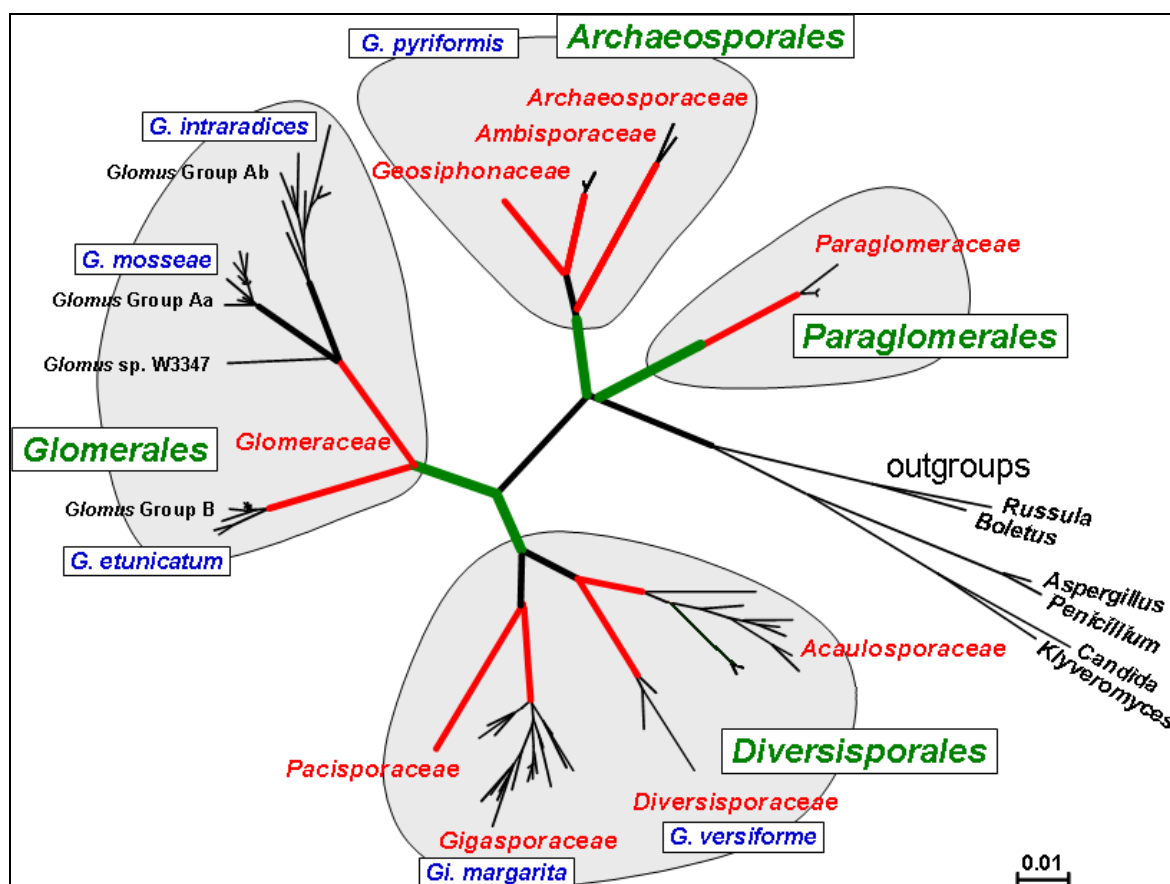
Fig. 6 Phylogenetic tree implementing the recent changes in the taxonomy of the Glomeromycota. The tree is based on SSU rDNA sequences. Some often used 'model species' are shown in blue. From http://www.lrz-muenchen.de/~schuessler/amphylo/.

Table 2 Classification of AM fungi (phylum/Glomeromycota, class/Glomeromycetes). This table was modified from http://www.lrz-muenchen.de/~schuessler/amphylo/ (Schüβler group; updated on April16, 2009). Details about the species (current names, synonyms and references) are available on the same webpage.

Orders	Families	Genera	Number of species
Glomerales	Glomeraceae	*Glomus*	105
Diversisporales	Gigasporaceae	*Gigaspora*	9
	Scutellosporaceae	*Scutellospora*	10
	Racocetraceae	*Racocetra* & *Cetraspora*	9 & 5
	Dentiscutataceae	*Dentiscutata* & *Fuscutata* & *Quatunica*	7 & 4 & 1
	Acaulosporaceae	*Acaulospora* & *Kuklospora*	34 & 2
	Entrophosporaceae	*Entrophospora* (unclear phylogenetic affiliation)	2
	Pacisporaceae	*Pacispora*	7
	Diversisporaceae	*Diversispora* & *Otospora* (unclear phylogenetic affiliation)	4 & 1
Paraglomerales	Paraglomeraceae	*Paraglomus*	3
Archaeosporales	Geosiphonaceae	*Geosiphon*	1
	Ambisporaceae	*Ambispora*	8
	Archaeosporaceae	*Archaeospora* & *Intraspora*	1 & 1

Other spore characteristics besides spore formation are for example presence or absence of flexible inner walls or the type of germination which occurs through the hyphal attachment (e.g. *Glomus*) or through the spore wall. For the latter germination type, germ tubes can arise from a germination orb (e.g. *Pacispora*), a germination shield (*Scutellospora*) or a warty layer, typical for *Gigaspora* (INVAM, see link above). However, these characteristics are only partly genus-specific and for the description or identification of species further detailed observations have to be performed. Most recent species descriptions or emendations are supplemented by sequence analyses. An example for a detailed investigation of spore structures is illustrated by different photographs from the species description of *Acaulospora alpina* (Oehl *et al.* 2006) in Fig. 7. The Schüβler group (see link in Table 2) provides an AMF taxa list that includes references for several species descriptions.

By comparing the current phylogeny and classification, it becomes obvious that a reexamination of the generic concept is necessary in particular for *Glomus*, which is not comparable with other genera regarding phylogenetic results. The Glomeraceae are definitely representing two different families (see above), if not even more, and other *Glomus* species are members of the Diversisporaceae. However, in the meantime, redescriptions are dependent on both structural observations and phylogenetic analyses and especially the first criterion is difficult for this morphotaxon, whose symplesiomorphic spore formation is also typical for *Ge. pyriformis*. Moreover, the type strain for *Glomus*, which was described in 1845 (Berch & Fortin 1984), needs to be re-cultivated and analyzed. With respect to these discrepancies on the genus level, ambiguities on the species level are comprehensible.
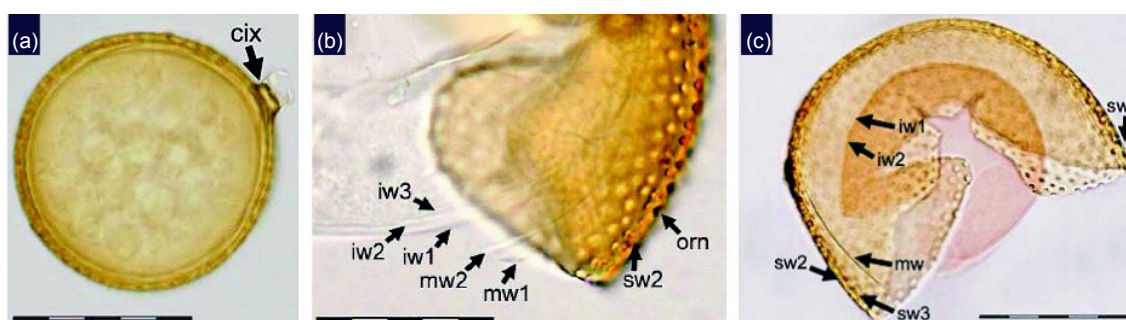
Fig. 7 *Acaulospora alpina* photographed from type specimen. **(a)** Spore with cylindric pedicel around cicatrix (cix); bar = 50 µm. **(b)** Cracked spore, with three walls (sw, mw, iw); outer spore wall three-layered (sw1–3) with pitted ornamentation (orn) on sw2; flexible middle wall (mw) with two usually adherent layers (mw1 and mw2; here separated) and inner wall (iw) with three tightly adherent layers (iw1–3); bar = 25 µm. **(c)** Inner wall (iw2) staining pale purple in Melzer's reagent; iw3 often difficult to observe even in broken spores; bar = 50 µm. Modified from Oehl *et al.* (2006).

1.2.2 Species concept and genetics of AM fungi (AMF)

In comparison to the taxonomy and classification of many plants and animals, which is well established to the species level, the species concept of AMF is difficult to handle. Based on the "Biological Species Concept" (Dobzhansky 1937), two organisms belong to the same species, when they produce fertile offspring. This definition is not easily applicable to AMF, which are currently thought to be asexual (but see also below), and thus the species concept is still mainly based on spore characteristics (Smith & Read 2008). To date about 200 AMF species have been described (Table 2). However, the growing number of sequences from environmental studies represents a plenitude of new phylotypes that could not be assigned to known species (e.g. Kottke *et al.* 2008; Opik *et al.* 2008). These phylotypes might display so far unrecognized cryptic species, suggesting that AMF species richness is far underestimated, and the high number further strongly underlines the necessity to supplement the species concept by sequence data, even though a pure molecular species concept might be problematic (see Redecker & Raab 2006; section 1.2.5.4). Indeed, it was shown that even the genetic diversity among isolates of the same phenetic species reflected diversity in development, function and symbiotic performance, thus on the phenotypic level (Koch *et al.* 2004; Munkvold *et al.* 2004).

It was assumed that anastomoses (hyphal cross-bridges) occur only within the same isolate as demonstrated for *G. mosseae* and *G. intraradices* (Giovannetti *et al.* 2004; Voets *et al.* 2006). However, recently, Croll *et al.* (2009) demonstrated anastomoses and genetic exchange among different isolates of one population of *G. intraradices*, and even recombination in *G. intraradices* was confirmed *in vitro* (Croll & Sanders 2009). These observations might be helpful for the currently discussed species concept in AMF and of *G. intraradices* in particular (see chapters 3 and 5). Anastomoses are definitely worth to further explore in other species and *in situ* (Purin & Morton 2009), too. Furthermore, these investigations might help to definitely solve the question of sexuality in AMF species, for which cryptic recombination events were already suggested by Gandolfi *et al.* (2003).

A species name is the basis for experimental studies of its ecology, evolution and phenotypical and functional role within natural ecosystems (Sanders *et al.* 1996; Smith & Read 2008). Therefore Smith & Read (2008) demanded: "A major challenge for the future is to provide a workable species concept for the Glomeromycota…".

It is also crucial to elucidate the genetics of AMF, which is still controversially discussed: based on data from flow cytometry, reassociation kinetics and genome reconstruction, Hijri & Sanders (2004, 2005) suggested that *G. intraradices* and *G. etunicatum* are haploid as other fungi, but that the coenocytic mycelium contains nuclei differing in their genomes (heterocaryosis). In contrast, Pawlowska & Taylor (2004) reported data, for instance, from microdissecting individual nuclei from *G. etunicatum*, supporting a polyploid organization and homokaryosis. There is no doubt that genetic variation occurs within the mycelium or spores of one single organism of AMF

as several studies of ribosomal DNA and protein-coding genes revealed (Kuhn *et al.* 2001; Jansa *et al.* 2002b; Helgason *et al.* 2003; Corradi *et al.* 2004). Besides the two models presented, a third possibility would suggest a homokaryotic spore with haploid nuclei but high copy numbers of certain genes explaining the heterogeneity (see Fig. 1 in Hijri & Sanders 2005). However, so far it cannot be excluded that nuclei are either heterogeneous or homogeneous in different AMF species or that even combinations of the possibilities exist within the same, single organism. Large differences of the genome sizes exist among different AMF taxa: ~ 16.54 Mb were calculated for *G. intraradices* (Hijri & Sanders 2004) but up to 1058.40 Mb in other AMF (Bianciotto & Bonfante 1992; Hosny *et al.* 1998). Currently the genome of the *G. intraradices* DAOM197198 isolate is being sequenced (Martin *et al.* 2008). About 350 Mb sequence material suggest that the effective "genome space" is probably ten times higher than the previously calculated 16 Mb (Young 2009a). The sequencing project turned out to be difficult to perform, which is probably due to the heterogeneous genetic system and the high content of transposons. However, once completed, the first genome of *G. intraradices* might help to solve several open questions concerning the genetics of AMF.

1.2.3 Structural features of the AM and life cycle of AMF

AM has three major components, which are depicted in Fig. 8: (i) the root of the plant, (ii) fungal structures within the root, and (iii) the extraradical mycelium (Smith & Read 2008).

Spores (see Figs 7 and 9 in this chapter and Fig. 2 in chapter 2) provide the only morphological and anatomical features that are useful for the classification of AMF (for details, see section 1.2.1). Some of them are visible by eye as they can reach up to 0.4 mm, however, the sizes vary not only among different species but also within a single species or strain (40-140 µm for *G. intraradices*; see INVAM link in section 1.2.1). Spores represent an important propagule of AMF that can be distributed by zoochory and can persist for many years in the soil (Smith & Read 2008). Besides abundant storage lipids and carbohydrates, spores were reported to contain a large number of nuclei, which can vary between 800 up to 35 000 in different species as estimated by Hosny *et al.* (1998). Germination of spores (for germination types see section 1.2.1) can also occur in absence of plants, but to complete their life cycle, the biotrophic AMF are strictly dependent on the photoautotrophic partner (Parniske 2008).
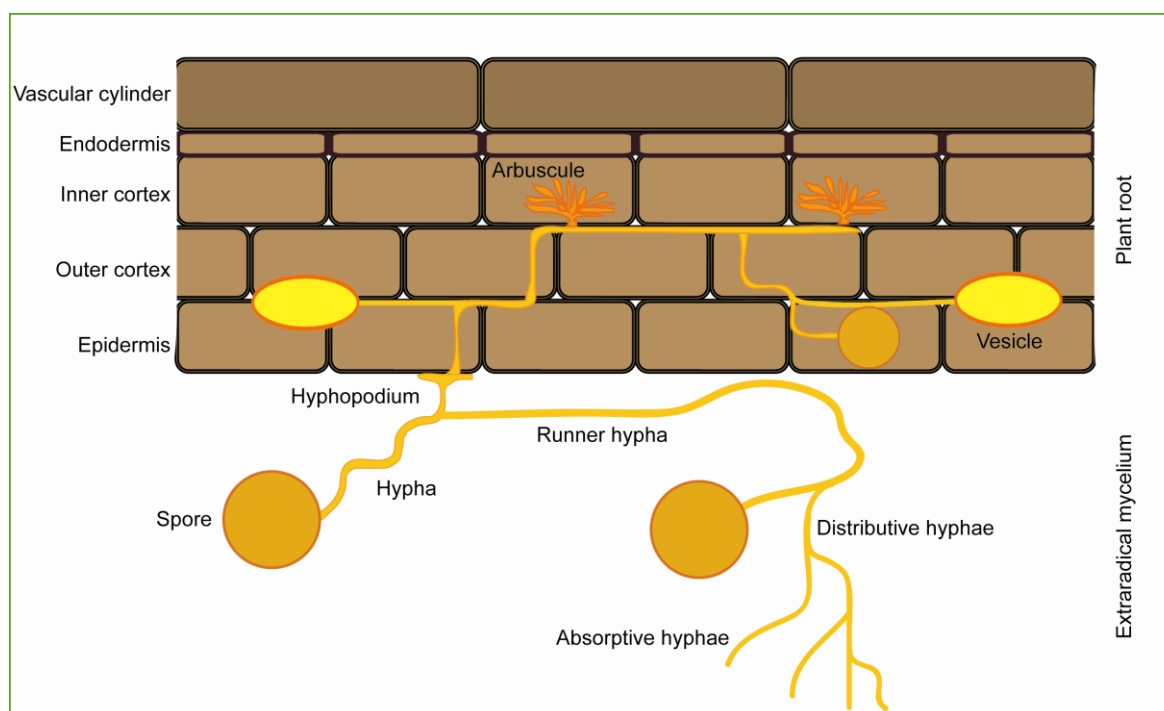
Fig. 8 Structures of arbuscular mycorrhizal fungi outside and within the root of the phytobiont.

A crucial step of any microbe/plant association is the recognition of the partner, which is well investigated for the symbiosis between rhizobia and legumes (Madigan *et al.* 2000). Similar to this partnership, root exudates act as initial factors for the establishment of AM. Only relatively recently, strigolactones were identified as one component of root exudates responsible for stimulating hyphal growth and branching but also for spore germination of AMF (Akiyama *et al.* 2005; Besserer *et al.* 2006). Despite developmental differences, rhizobial symbioses and AM utilize the same components (at least seven proteins) of a common signalling (Sym) pathway in legumes (Oldroyd & Downie 2004, 2006). Therefore, it is likely that different rhizobial and mycorrhizal signals (Nod factors and Myc factors) result into a common signalling pathway, whereas the output is unique in both symbioses, again (Oldroyd & Downie 2006; Kosuta *et al.* 2008). In AM, Myc factors are thought to induce calcium oscillations in root epidermal cells (Kosuta *et al.* 2008) and activate plant symbiosis-related genes (Kosuta *et al.* 2003).

The second step of the interaction is the attachment of the fungus at the host root. AMF form special types of appressoria (Fig. 9) to enter the epidermis, which are called hyphopodia and do not develop from germ tubes, as typical for appressoria of pathogenic fungi (e.g. *Magnaporthe grisea* [rice blast fungus]), but from mature hyphae (Bastmeyer *et al.* 2002). Subsequently, fungal hyphae penetrate the epidermal cell promoted by the plant itself as it was shown for *Medicago truncatula*: in a landmark study Genre *et al.* (2005) observed a prepenetration apparatus (PPA), a transient intracellular structure with a novel cytoskeletal organization, through which the hypha grows. In a subsequent study, Genre *et al.* (2008) showed that the intracellular growth of AMF in the outer cortex is also PPA dependent. Potential genes involved in the PPA formation were detected by

Siciliano *et al.* (2007). Further detailed or broader reviews dealing with the penetration of plant roots by AMF were published by Genre & Bonfante (2007), Bonfante & Genre (2008), Genre *et al.* (2009) and the supplementary material of Genre *et al.* (2005) provides an excellent video of the "AM hyphal penetration into a root epidermal cell following the path of the PPA".

In general, two different morphological growth patterns of AMF within the roots were already described by Gallaud (1905): (i) the *Arum* type is characterized by fast-growing hyphae spreading through intercellular air spaces and penetrating cortical cells by side branches, in which arbuscules (see below) are formed. This growth form is in particular typical for fast growing crops (Smith & Read 2008). (ii) The *Paris* type, on the other hand, is characterized by hyphae growing intracellularly from cell to cell, in which coils are formed. The *Paris* type can be found e.g. in the Gentianaceae (see Sýkorová *et al.* 2007b). While the *Arum* type can also develop few coils, the *Paris* type can additionally contain only "arbuscule-like" structures. Both, the plant and fungal identity are important for the determination of which type is formed (Smith & Read 2008).

The arbuscules are tree-shaped structures (see Fig. 9) formed by a branching hypha within the plasma membrane, which invaginates during hyphal penetration of the plant cell. Thus, the arbuscule remains within an apoplastic compartment and has no direct contact to the cytoplasm (Smith & Read 2008). In fact, the development of arbuscules needs more complex PPA-related mechanisms than the intracellular hyphal growth in the outer cortex as reported by Genre *et al.* (2008); see also above. Even though it is in continuum with the plasma membrane, the plant membrane surrounding the arbuscule is called periarbuscular membrane (PAM). The apoplastic interface between PAM and fungal membrane is referred to as the periarbuscular space (PAS). The arbuscules might represent the main structures for the nutrient exchange between the symbiotic partners (Parniske 2008), a functional role which is probably replaced by coils in the *Paris* type (see above; Karandashov *et al.* 2004; Glassop *et al.* 2005). The PAS of the arbuscules represents the interface for the nutrient transport and is characterized by the abundance of specialized molecules (e.g. Balestrini & Bonfante 2005). Especially the phosphate transport from the fungus to the plant is a main characteristic for AM (see section 1.2.4.2). Therefore, the detection of transporters involved is of high scientific interest. Even though some potential transporter genes have been cloned (see review by Karandashov & Bucher 2005), so far, only the MtPT4 transporter was localized on the PAM of the model plant *M. truncatula* (Harrison *et al.* 2002).

It is likely, that the plant-derived carbon is transported in form of sucrose via the PAS as shown in models by Pfeffer *et al.* (2001), Jin *et al.* (2005), Parniske (2008): the sucrose is further cleaved into hexose and released into the fungal cytoplasm by monosaccharide transporters located at the fungal plasma membrane. However, so far, a novel hexose transporter was only found in the symbiotic organs (fungal bladders) of *Ge. pyriformis* (see section 1.2.1; Schüβler *et al.* 2006) and not yet in the fungal membrane of the PAS in AMF. Furthermore, the intercellular hyphae could represent the location of carbon transfer to the fungus (Smith & Read 2008). These are long-lived

structures of AMF within the plant root, whereas arbuscules have a quick turnover (Smith & Read 2008). Even though arbuscules can be absent in many AM (see above), the name "arbuscular" is retained for this symbiosis, while the former term VAM (vesicular-arbuscular mycorrhiza) is not used anymore.

Vesicles (Fig. 9) represent a further characteristic structure of AMF in plant roots, but they are missing in some taxa (Smith & Read 2008), for example in the Gigasporaceae or Paraglomeraceae. Therefore, vesicles allow at least broad taxon discrimination. Like all other AMF structures they are easy to observe in colonized roots by microscopic investigations after staining procedures using e.g. lactophenol blue (see Fig. 9). Vesicles can vary in their shape (ovoid, irregularly lobed, box-like), which is dependent on the AMF species (Smith & Read 2008). However, as both the inter- or intracellular location, in which the vesicles are developed, has an influence on the shape, one should be cautious in interpreting it. Equal to spores, vesicles are thick-walled, contain high amounts of lipids and function as storage organs but also as important propagules (Smith & Read 2008).

Compared to AMF structures formed within the roots, the extraradical mycelium is far less diversely structured. The fungal structures outside the root differ strongly from other fungi, e.g. Basidiomycota or Ascomycota, forming ectomycorrhiza (see section 1.1). While the latter are characterized e.g. by fungal sheaths or fruit bodies, no complex mycelial strands, rhizomorphs or pseudoparenchymatous structures are formed by AMF in the soil (Peterson *et al.* 2004; Smith & Read 2008). Even though generally simply structured, the coenocytic (aseptate) hyphae fulfil important functions in the soil (Friese & Allen 1991; Peterson *et al.* 2004; Smith & Read 2008): so called runner hyphae are wide in diameter, fast-growing and explore the soil for new sources of organic C, i.e. new entry points of the same or another fungal partner, and soil derived nutrients. Hyphal bridges between two (or more) plants result in a common mycorrhizal network (CMN; van der Heijden & Horton 2009), which is further expanded by anastomoses that might also connect different "clones" of the same species. Other hyphae are narrow in diameter (2 μm), and thus capable to enter smaller soil pores than fine roots of the plant. Indeed, these highly branched and absorptive hyphae mobilize resources that may not be accessible by roots and function as their prolonged arms (see also section 1.2.4.2). External AMF hyphal lengths e.g. reached 111 m per cubic centimeter of soil from a Tallgrass Prairie (Miller *et al.* 1995). These expansive hyphal networks contribute considerably to soil stabilization by the production of glomalin (reported as a glycoprotein of AMF; Smith & Read 2008) agglomerating soil particles. Moreover, the mycelium in the soil represents a source of inoculum/propagule of AMF in addition to those mentioned before (Smith & Read 2008).
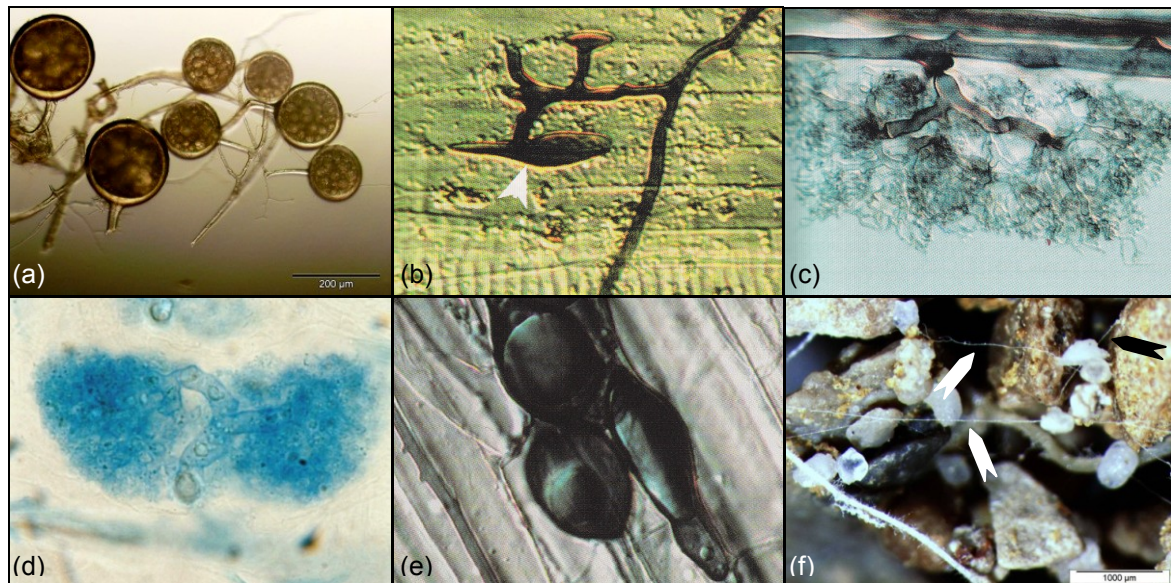
Fig. 9 (a) Spores of *Glomus clarum*. **(b)** AMF hyphopodium, see arrowhead; from Peterson *et al.* (2004). **(c)** Arbuscule; from Peterson *et al.* (2004). **(d)** Arbuscule, stained with lactophenol blue (photo by Verena Blanke). **(e)** Vesicles; from Peterson *et al.* (2004). **(f)** Hyphae, see arrowheads (photo by Florian Walder).

Besides other benefits, the mobilization of phosphate represents the major duty of the fungal partner in AM (see section 1.2.4.2). So far, fungal membrane P transporters, important for the P uptake by the extraradical mycelium, have been detected and described in three AMF species: *G. versiforme* (Harrison & Vanbuuren 1995), *G. intraradices* (Maldonado-Mendoza *et al.* 2001) and *G. mosseae* (Benedetto *et al.* 2005).

Spores are produced on the extraradical hyphae, but can also be developed within the colonized roots e.g. by *G. intraradices*, *G. clarum* or *G. fasciculatum* (Schenck & Smith 1982). Some AMF also form sporacarps or spore clusters (see e.g. Redecker *et al.* 2007), and *Gigaspora* spp. or *Scutellospora* spp. produce auxiliary cells in the extraradical mycelium (Peterson *et al.* 2004; Smith & Read 2008).

1.2.4 AM partnerships: benefits and ecology

1.2.4.1 Extent of plant – AMF associations in the plant kingdom

Arbuscular mycorrhizal fungi are associated with members of different plant taxa (see Table 1 in section 1.1), including sporophytes or even gametophytes of the Pteridophyta or those of the liverworts (Smith & Read 2008). Arbuscular mycorrhizas are typical for herbaceous plants in temperate areas, but are also found frequently in tropical trees (see section 1.1). Some tree genera like *Acer*, *Malus*, *Salix* or *Populus* are also colonized by AMF in temperate habitats (Smith & Read 2008). As mentioned in section 1.1, some plants are able to form several types of mycorrhiza:

Salix, for instance, can be colonized by ectomycorrhizal and arbuscular mycorrhizal fungi (Dhillion 1994; van der Heijden 2001; Becerra *et al.* 2009). The presence of two mycorrhizal types is also the norm e.g. in the roots of *Populus* (Walker & McNabb 1984).Without doubt, the AM is the most ancient and widespread mycorrhiza type (Brundrett 2009; see also Figs 2 and 3 in section 1.1). It can be assumed that perhaps 200 000 or 80-90% of terrestrial plant species form AM (Smith & Read 2008). One of the most detailed surveys of the incidence of mycorrhizas on the species level has been published by Harley & Harley (1987), already a classic in that field. In a recent survey of 3 617 plant species, Wang & Qiu (2006) showed that 92% of the families (80% of the species) potentially form at least one mycorrhiza type. A very detailed review about mycorrhizal associations was most recently published by Brundrett (2009).

Most species of the families Chenopodiaceae, Brassicaceae, Caryophyllaceae, Polygonaceae, Juncaceae and Proteaceae are thought to be non-mycorrhizal or just weakly and not consistently colonized (Smith & Read 2008; see also section 1.1). These generalizations should be interpreted cautiously as the mycorrhizal state of some species might be dependent on various factors (e.g. seasonal or geographic criteria), and thus the same plant species could occur in both the mycorrhizal or non-mycorrhizal state. Indeed, in biodiversity studies based on molecular marker genes (e.g. Börstler *et al.* 2006) or studies using microscopic analyses (e.g. Orlowska *et al.* 2002), AMF were detected in plant species which were thought to be typically non-mycorrhizal according to Harley & Harley (1987). On the other hand, the sensitivity of the PCR based methods might also detect adherent or weakly colonizing hyphae and not exclusively functional active AMF. Therefore, the mycorrhizal stage can only be definitely proved by microscopic identifications using staining methods (see section 1.2.3). Many plant species are underexplored and further detailed investigations of environments, which were thought to harbour mostly non-mycorrhizal plants (e.g. arid habitats, wetlands or salt marshes; see also next section), have already changed previous assumptions: "…the more we look the greater number of species turn out to be mycorrhizal" (Smith & Read 2008).

The assumption that probably 200 000 plant species are potential hosts of approximately 200 AMF species suggests a low fungus-plant specificity, in other words each fungal species must have many hosts (Smith & Read 2008). Indeed, molecular investigations revealed that a single plant can be colonized by as many as 20 different AMF species (Fitter 2005). Early conclusions that no absolute species specificity exists in AM (Stahl 1949; Gerdemann 1955) were supported in more recent studies (Smith & Read 2008), however, it cannot be excluded that some specificity exists in fungal/plant associations. Many conclusions about specificity were derived from greenhouse experiments, representing an artificial system that might support mostly AMF species adapted to these conditions. Moreover, a changed species concept (see section 1.2.2) would increase the possibility of species specific interactions (Smith & Read 2008). Recent studies, also including molecular identification approaches for AMF communities in both greenhouse experiments and

natural ecosystems, showed at least preferences for specific fungus/plant interactions or even evidence that host specificity may occur in few species among many non-specific species (e.g. Bever *et al.* 1996; Streitwolf-Engel *et al.* 1997; van der Heijden *et al.* 1998a; Eom *et al.* 2000; Helgason *et al.* 2002; Vandenkoornhuyse *et al.* 2002, 2003; Gollotte *et al.* 2004; Scheublin *et al.* 2004; Johnson *et al.* 2005; Sýkorová *et al.* 2007b). In contrast to green plant partners, specificity towards certain AMF groups is higher in mycoheterotrophic mycorrhizas (see in Bidartondo *et al.* 2002). In conclusion, so far no strict host specificity of AMF was observed in association with green plants.

1.2.4.2 Functional aspects of AM associations

Like other mycorrhizas, AM is generally thought to be mutualistic. The transfer of carbon (see section 1.2.3) represents the main benefit from the plant to the fungus, which it is unable to take up carbon via its extraradical mycelium (Pfeffer *et al.* 1999). However there is no evidence for mutualism in mycoheterotrophic mycorrhizas (see also section 1.1): the achlorophyllous plants receive the required carbon via a fungus which is also associated with neighboring autotrophs. Hence, the achlorophyllous plants act as epiparasites (for more details see Smith & Read 2008). In general, a continuum from parasitism to mutualism is found in AM associations, and the particular outcome in each case might depend on the identity of the respective plant and fungal partners as well as on certain environmental conditions. These aspects were reviewed and discussed by Jones & Smith (2004).

Besides their strict dependency on the plants' photosynthetic products, the biotrophic AMF also profit from the relatively constant environment within the root. It was even assumed that these homogenous conditions might explain the potential asexual habit of AMF (see section 1.2.2), because "...the selection pressures will be to maintain similarity to, rather than difference from the parents" (Smith & Read 2008). However, this suggestion excludes the extraradical phase of AMF, exposed to many environmental changes over time (Smith & Read 2008).

It cannot be excluded that the plant provides additional benefits for the fungus, for instance supplying vitamins or regulating hormones. The benefits the fungus provides to the plant may be much more diverse as demonstrated in the following paragraphs. Mineral nutrition has received much attention among AM benefits and it could be shown in many studies that this contribution of AMF leads to improved plant growth (Smith & Read 2008) and that plants give a healthier overall impression compared to e.g. non-colonized controls of the same species (see Fig. 10). The most relevant contribution of AMF is the mobilization of phosphate, corresponding to the broad occurrence of AM in mineral soils (see section 1.1). Even though highly demanded by the plant, P represents a limiting factor in many ecosystems and the network of hyphae (see section 1.2.3)

overcomes depletion zones of P developing around plant roots. Hyphae with their small diameter can reach soil pores that are inaccessible to roots (Smith & Read 2008). Details about the mechanisms of P uptake and translocation in AM are described in section 1.2.3.

Although it is generally assumed that there is a growth response of plants to AMF colonization, this is not always the case (e.g. Klironomos 2003; Tawaraya 2003). Smith *et al.* (2004) reported in a landmark study that the mycorrhizal P uptake pathway is not necessarily correlated with plant growth response. In other words, the uptake is present but does not become apparent. Furthermore, AM colonization can reduce or inactivate the direct P uptake by the plant (see also review by Smith *et al.* 2009). In that case, even weakly colonized plants can exhibit a growth depression which might have an impact on plant competition, especially between mycorrhizal and non-mycorrhizal plants (Facelli *et al.* 2010).

Fig. 10 Effects of mycorrhizal inoculation of a range of crop plants in fumigate soil. Right-hand block, inoculated with VA mycorrhizal fungi. Left-hand block, not inoculated. Crops (front to back): *Allium*, *Catalpa*, *Pisum*, *Vicia*, *Zea*. Photograph courtesy of V. Gianinazzi-Pearson. From Smith & Read (1997). VAM is the previously used term for AM (section 1.2.3).

The uptake and transport of organic or inorganic nitrogen is a typical feature of the ectomycorrhizal – or ericoid mycorrhizal fungi occurring in soils rich in organic matter (see section 1.1). However, this property was also observed in arbuscular mycorrhizas (e.g. Johansen *et al.* 1992, 1993, 1996; Hawkins *et al.* 2000; Hodge *et al.* 2001; Leigh *et al.* 2009), whereas the ability of AMF to decompose complex organic molecules is still discussed controversially. Nevertheless, Govindarajulu *et al.* (2005) and Jin *et al.* (2005) studied the uptake, metabolism, transport and transfer of ^{15}N-labelled inorganic nitrogen or arginine in AM and provided models for the inorganic nitrogen movement in this symbiosis: the extraradical mycelium takes up inorganic nitrogen, which is assimilated via nitrate reductase, followed by the glutamine synthetase-glutamate synthase cycle, and finally converted to arginine. This amino acid is then translocated to the intraradical mycelium, where it is broken down and ammonium is released to the host plant via ammonium channels.

The role of nitrogen in AM was also studied in field sites: Blanke *et al.* (2005), for instance, investigated a phosphate polluted site and found that mycorrhization rates were high in plots with low N availability, but low in plots with a higher N availability. Thus there was a very unbalanced N:P ratio. These authors concluded that N deficiency stimulated root colonization by AMF in this P-enriched field site. This study is one example showing that in addition to C both P and N budgets should be taken in account when studying interactive effects of resources on symbiotic outcome.

Early cost-benefit models only considered the exchange of C and P (Smith & Read 2008). Johnson (2010) reviewed potential resource stoichiometries that build upon these previous models: the authors presumed that the relative availability of C, P and N governs the AM symbiotic function. Different availabilities of these resources should determine the allocation of nutrients to plant or fungal structures in different dynamics as shown in Fig. 11.
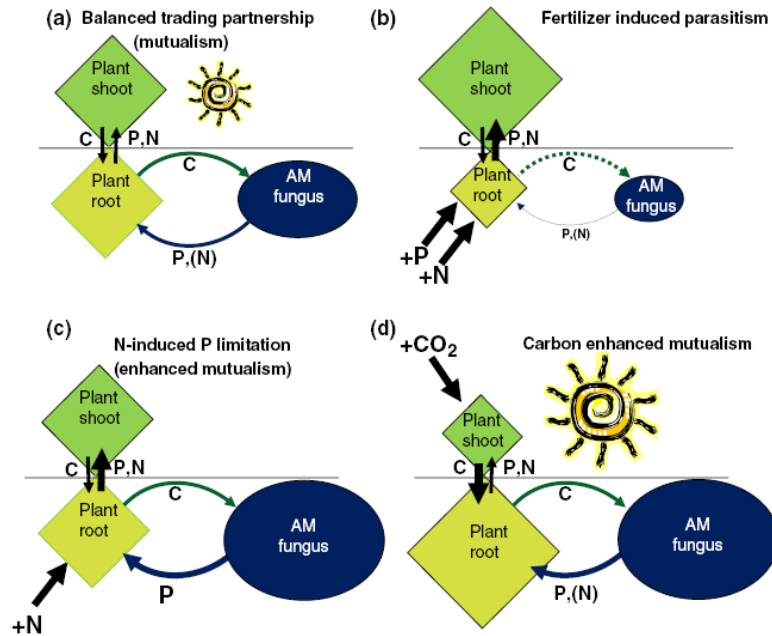
Fig. 11 The functional equilibrium model predicts that enrichment of above- and belowground resources will generate different arbuscular mycorrhizal (AM) dynamics. (a) A mutualistic AM symbiosis occurs when there is a balanced trading partnership between plant and fungus. The plant delivers carbon compounds to the fungus in return for mineral nutrients, most notably phosphorus, and in some circumstances nitrogen. (b) Nitrogen enrichment of a phosphorus-rich system generates a parasitic AM symbiosis because plants will reduce allocation to roots and mycorrhizas in favor of above ground structures when no belowground resources are limited. (c) Nitrogen enrichment of a phosphorus-limited system generates a mutualistic AM symbiosis because nitrogen-enriched plants require additional phosphorus to synthesize biomass and this increases the value of AM trading partnerships. (d) Increased light intensity or CO_2 enrichment of plants growing in nutrient-limited soils will increase plant demand for belowground resources and increase the value of AM trading partnerships. From Johnson (2010).

Equally to inorganic phosphate and ammonium, micronutrients like zinc or copper are poorly mobile in the soil solution. Even though less emphasized and observed, several studies have underlined the evidence that AMF also play an important role for the mobilization of micronutrients in AM (Smith & Read 2008). Considering that many of these elements become toxic in higher concentrations, interactions between them and AMF have been studied (Gadd 1993; Meharg 2003). The ability of AMF to accumulate toxic metals in the hyphae would represent a further advantage of colonized plants and several studies support this assumption (e.g. Christie *et al.* 2004; Chen *et al.* 2005). It could be shown that mycorrhizal plants are more tolerant to arsenic (e.g. Gonzalez-Chavez *et al.* 2002; Liu *et al.* 2005). Smith (2009) suggested that a decreased direct P uptake might increase arsenic tolerance in colonized plants as the mycorrhizal P uptake may have

a very high P/As selectivity. If this is the case, the "hidden" P uptake by AMF (see above) also provides an advantage compared to non-mycorrhizal plants. Effects of AMF might not only be important for plants in soils contaminated by heavy metals (Leyval *et al.* 1997), but also for plants occurring in saline soils: members of typical non-mycorrhizal plant families were colonized in dry salt marshes (Hildebrandt *et al.* 2001) and AMF decreased plant yield losses under high salt levels (Al-Karaki 2000).

It is not yet proven that AMF transport water via the hyphae to the host (George *et al.* 1992; Smith & Read 2008), assuming that they might have greater access to water reservoirs in small soil pores (see above and section 1.2.3) under drought conditions. Nonetheless, there is no doubt that plants exposed to water stress profit by AM (Augé *et al.* 2001). If not explained by direct water transfer, the effect of AMF on plant water relations could be due to changed soil properties by the extraradical mycelium that is generally stabilizing the soil texture (see section 1.2.3). Secondly, AM could have an indirect positive effect under dry conditions as the uptake of nutrients by AMF becomes even more important in dry soils where nutrients are difficult to access by the plant alone (Smith & Read 2008).

1.2.4.3 Multitrophic interactions

Arbuscular mycorrhizas are part of complex ecosystems and thus potential effects of AMF on interactions with biotic factors, e.g. herbivores or pathogens, were subject of many studies (see in Bennett *et al.* 2006). Kula *et al.* (2005) investigated the interactions among grasshoppers, plants and AMF in tallgrass prairie microcosms and showed that AM increased plant regrowth following defoliation in contrast to non-mycorrhizal plots and that above ground herbivory increased AMF colonization. Most recently, Kempel *et al.* (2009) studied tritrophic interactions among caterpillars, herbaceous plants and AMF. Based on their data, the authors suggested that the caterpillars' induction of plant resistance led to a translocation of resources provided by AMF from above ground growth towards herbivory defence. Hempel *et al.* (2009) reported that AMF may even affect a fourth trophic level: in a system using AMF, plants, aphids and parasitoid wasps, the rate of parasitism in aphids was increased when plants were colonized with *G. intraradices*. However, as reviewed by Gehring & Whitham (1994, 2002), in the majority of studies, above ground herbivory led to a decreased colonization of AMF, which might be attributed to a reduced support in organic C. In the case of underground pathogens, Dehne & Schonbeck (1979) and Davis & Menge (1980) reported that AM fungi increase the resistance to root-infecting fungi such as *Phytophthora parasitica* or the cosmopolitan *Fusarium oxysporum*. In contrast to above ground enemies, effects of AMF on soil pathogens could be simply explained by the presence of the mutualistic symbionts in the roots: AMF colonization might occupy the space needed for fungi or nematodes to infect and

the pathogens might be outcompeted. However, mobilization of plant defence mechanisms or improved nutrient supply, increasing overall plant fitness and resistance, might also represent crucial factors underground (Smith & Read 2008). Filion *et al.* (1999) reported direct interactions between *G. intraradices* and different rhizosphere microorganisms, which responded by different growth patterns in the presence of fungal substances. For more details, see also reviews by Newsham *et al.* (1995) and Azcon-Aguilar & Barea (1996).

1.2.4.4 Other ecological aspects: AMF and ecosystem functioning

The fact that the interactions of plants and fungi are not limited to the exchange of C and P might explain that several different AMF species can find a place in the same biotic niche due to a diversification of function: a single fungus might be good in one, but is most likely not the best in another benefit (Fitter 2005). The previous sections demonstrated that benefits of AMF or colonization per se can vary depending on the identity of the plant and fungal partners. Maherali & Klironomos (2007) outlined different functional roles of different AMF taxa: the authors showed that typical intraradical hyphal development of members of the Glomeraceae was, indeed, correlated with pathogen protection (see previous section) and that the high amount of extradical mycelium of members of the Gigasporaceae, in contrast, was correlated with enhanced P concentration in the hosts' shoots. Smith *et al.* (2004) showed that the transport of P to the host can vary depending on the AMF species, and that the response of the plant depends on the respective plant species, as well. Moreover, Facelli *et al.* (2010) showed that growth depressions of plants inoculated with *G. intraradices* could be mitigated by co-inoculating *Gigaspora margarita*. Differences in functional aspects are not restricted to morphospecies level but were also found among different isolates of AMF as already mentioned in section 1.2.2: Munkvold *et al.* (2004) observed high intraspecific variation within four *Glomus* spp. concerning plant growth response and improvement of P uptake in cucumber. Koch *et al.* (2006) showed that different isolates of a *G. intraradices* population differently affect plant growth.

Considering (i) that the AM of a single plant can consist of many different AMF species, (ii) that different isolates of the same species can contribute differently to the host, and (iii) that AM is just a component of a multitrophic system (see previous section), overall costs and benefits of the symbiosis and ecological relationships become more and more complex and are difficult to measure. Therefore, most of the results concerning function and ecology were obtained by using artificial systems. AMF cannot be grown axenically and currently the only way to obtain fungal material, free from contaminating microorganisms, are root organ cultures (ROCs; Tepfer & Tempe 1981; Bécard & Fortin 1988). However, the range of AMF cultivable in ROCs is restricted to few species (see e.g. http://emma.agro.ucl.ac.be/ginco-bel/collection.php). While ROCs are

helpful in answering some functional aspects (e.g. Elsen *et al.* 2003; Jaizme-Vega *et al.* 2003; Koch *et al.* 2006), more complex systems are needed to simulate natural systems. Most commonly used are pot cultures, moreover, these can be arranged in more complex forms as compartment systems, important to study complex interactions e.g. within one CMN (see section 1.2.3). In a compartment system experiment, Florian Walder (unpublished) showed that different plant species profit unequally from N and P uptake in the CMN. The plant which profited less even delivered more carbon to the CMN. Another landmark study, impressively illustrating the necessity to involve complex systems in the investigation of AM interactions, was performed by Grime *et al.* (1987). These authors were among the first to demonstrate the potential role of AMF to determine plants' community structure and diversity using microcosm experiments. More recently, van der Heijden *et al.* (1998a,b) also addressed the same question. While Grime *et al.* (1987) used only one type of natural inoculum, dominated by *G. constrictum*, van der Heijden *et al.* (1998a,b) used four different AMF taxa and combinations of them, showing that plant communities and their productivity were influenced differently depending on the type of the inoculum. Moreover, van der Heijden *et al.* (1998b) demonstrated in macrocosms that increasing AMF species richness increased plant diversity. Based on the results of these experiments, the authors concluded that both AMF community composition and species richness might be important factors in maintaining plant biodiversity and ecosystem functioning. *Vice versa*, the community structure and diversity of AMF might be regulated by plant community structures (e.g. Johnson *et al.* 2005).

In a recent study using a pot culture experiment, Johnson *et al.* (2010) determined the role of "home and away" combinations of soils and AMF communities on ecotypes of *Andropogon gerardii* from phosphorus-limited and nitrogen-limited grasslands. The authors observed that both AM fungi and plants were adapted to the respective conditions from the site of origin. Lekberg *et al.* (2007) set up a glasshouse experiment using *Sorghum bicolor* as host plant growing in sand, clay or a sand/clay mixture in order to detect niche restrictions of *Glomus* - and *Gigaspora* species inoculated alone or in combination. In absence of *Gigaspora*, root colonization by *Glomus* was always high, whereas colonization of singly inoculated *Gigaspora* was negatively correlated with clay content. As spore production of both *Glomus* and *Gigaspora* was significantly reduced only in sand, the authors suggested that competition could limit niches of both families in certain soil environments.

The role of niche restriction of members of the Glomeraceae and Gigasporaceae was also reported directly from natural sites (see section 1.2.5.5). The correlation of high plant and AMF diversity could be also shown in natural environments (see section 1.2.5.5). However, artificial systems will never reach the complexity of ecosystems in nature and the potential role of many other missing biotic or abiotic factors might bias the results. Sýkorová *et al.* (2007a) could demonstrate that the AMF community of trap cultures did not reflect the fungal diversity of the corresponding field site and the authors suggested that fungal succession under greenhouse

conditions might have led to these results. The authors also compared the AMF community of bait plants planted in the same field site and suggested that this bioassay might be a better option for functional tests concerning AMF communities compared to glasshouse approaches. Generally, glasshouse experiments cannot replace studies under natural conditions (see section 1.2.5.5), but they allow, on the other hand, observations impossible to conduct in the field, that can help to resolve complex ecological interactions. In other words, a combination of both types of approach, complementing each other, is necessary to understand the role and functioning of AM in diverse ecosystems.

1.2.5 Biodiversity studies of AMF communities and populations

1.2.5.1 Different strategies and methods for the detection of AMF in the field

In contrast to biodiversity studies of plants which are organisms usually visible above ground, the diversity of AMF in the field is more difficult to assess. Two main approaches can be applied: (i) the identification of AMF propagules in the soil by morphotyping spores or molecular tools, and (ii) the detection of AMF in the roots of the host plant by molecular tools. It depends on the aim of the study which approach should be applied as both have their advantages and disadvantages as it will become obvious in the following paragraphs.

Community analyses based on spore identification can be performed either by direct isolation of spores from the soil or alternatively by isolation after reproduction in trap cultures. In a study by Cousins *et al.* (2003), direct spore isolation underestimated species diversity by 3.2 species per sampling site relative to trap cultures, supporting the latter approach. When conducting community analyses based on soil-born AMF propagules it has to be considered that they might not reflect the community of functionally active AMF within the roots of their hosts, as reported in many studies (e.g. Clapp *et al.* 1995; Merryweather & Fitter 1998; Renker *et al.* 2005), but that they represent resting and dispersal stages. Recently, Hempel *et al.* (2007) evaluated AMF community composition using spores, extra- and intraradical mycelium and reported remarkable differences. Interestingly, these authors did not detect any *Paraglomus* sequences in root samples or spores and based on the high frequency of Paraglomeraceae in the extraradical mycelium, this AMF taxon was even suggested to reveal saprophytic habits. Nevertheless, *Paraglomus* was detected in the same field site in both spore and root samples by Börstler *et al.* (2006), who, moreover, also observed differences between AMF communities between spores and roots. *Gigaspora*, for instance, was detected only once in a single spore. This result is in congruence with a recent PhD study by Borriello (2010), who found *Gigaspora* exclusively in spores and not in roots. Generally, the occurrence and density of spores can vary in soils (Smith & Read 2008) and different AMF species

showed seasonality of sporulation (Bever *et al.* 2001). Agricultural field sites are generally characterized by high abundance of spores, which can be effected by site, season or crop species, and disturbance can influence AMF spore composition and diversity (e.g. Johnson *et al.* 1991, 1992; Jansa *et al.* 2002a).

Despite the disadvantages of monitoring AMF using spore morphology, this method might be less labor-intensive than molecular approaches (Oehl *et al.* 2010). However, spore-based microscopic methods require an excellent knowledge for the identification of each single spore (see section 1.2.1) and, therefore, molecular approaches represent a suitable alternative. As mentioned in section 1.2.2, molecular approaches revealed many phylotypes, i.e. sequence types, of species, which cannot be linked to any of the approximately 200 species described on the basis of spore morphology. Based on sequence comparisons of phylotypes, it was estimated that more than 2 000 AMF species might exist (Dirk Redecker, see in Brachmann 2006). These phylotypes might represent species undescribed so far, species, which are difficult to culture, or even cryptic species, which are not distinguishable by morphological characters.

Even though not necessarily representing active AM, community studies of soil borne AMF structures, especially spores, are generally highly needed as they represent an important part of the fungal life cycle (see section 1.2.3). Insights are needed about their geographical distribution and under which ecological conditions these propagules thrive. Ideally, spore-based results should be correlated with the molecular data on active root-colonizing AM fungi.

The detection of phylotypes can be conducted *directly* by the determination of AMF communities using plant root samples from the field, but also in another *indirect* community analysis by investigating plant roots of trap cultures. The latter approach, however, might include some bias compared to natural conditions (see section 1.2.4.4). By using the *direct* identification method, many AMF species can be detected, which might not sporulate during the time of investigation (Turnau *et al.* 2001). Nevertheless, authors preferring spore based methods have criticized that many primers used in molecular approaches (see next section) do not detect all AMF taxa (Oehl *et al.* 2010).

1.2.5.2 Marker genes for AMF community studies

Since Clapp *et al.* (1995) initiated field studies using molecular tools, several different molecular markers were developed and improvements are still required and in progress. Target genes are e.g. the nuclear-encoded small subunit (SSU) ribosomal RNA sequences (e.g. Helgason *et al.* 1998, 1999, 2002), or the large subunit (LSU) ribosomal RNA sequences (e.g. van Tuinen *et al.* 1998; Kjøller & Rosendahl 2000; Wu *et al.* 2007). The polymorphism of the latter is higher and, therefore, suitable to develop group-specific primers (Rosendahl 2008; Robinson-Boyer *et al.*

2009). In contrast, Lee *et al.* (2008) developed the primer pair AML1/AML2 targeting the SSU and claimed that this marker detects all published AMF sequences of this gene region except for *Archaeospora trappei*. Generally, the resolution of phylotypes from this gene region is lower than in approaches using internal transcribed spacers (ITS). Several markers targeting the ITS were developed and applied in field studies. For example, Renker *et al.* (2003) developed a nested PCR for the ITS with an intermediate restriction digest, in order to exclude the amplification of other fungi than AMF which contain the restriction site. However, in a later publication, this group clarified that the restriction digest causes to miss certain AMF species in the detection procedure (Hempel *et al.* 2007). Furthermore, the amplification of yeast species was still relatively prominent, which on the other hand allowed to investigate the biodiversity of basidiomycete yeasts inhabiting AMF-colonized roots or AMF spores (Renker *et al.* 2004). In order to reduce the risk of missing AMF species in plant roots, Redecker (2000) developed group-specific nested PCR approaches for the SSU-ITS covering all families of the Glomeromycota. Similar to the approach of Renker *et al.* (2003), this method was well-adapted in the community and applied in several studies (e.g. Wubet *et al.* 2003; Hijri *et al.* 2006; Sýkorová *et al.* 2007b; Appoloni *et al.* 2008). Öpik *et al.* (2006) and Robinson-Boyer *et al.* (2009) summarized several examples of studies using different target genes mentioned so far. It became obvious that all of these approaches have their advantages and disadvantages. It is rather dependent on the scientific aim, which approach might fit best for the respective study and field site, e.g. if a resolution on the genus or species level is required.

Recently, Krüger *et al.* (2009) developed a nested PCR approach to overcome some problematic issues: in order to avoid the need for several PCR reactions to detect all members of the Glomeromycota, mixed primer sets were used in the same reaction and successfully tested for several members of AMF families covering the whole phylum. The target fragment includes all ribosomal gene regions that have been broadly used in other studies (Fig. 12), resulting in approximately 1 800 bp in the first and 1 500 bp in the second PCR. Therefore, this approach allows an overall comparison with previous studies on the same taxonomic level. In particular, the SSU fragment allows broad phylogenetic analyses as this gene region is available for the majority of different AMF taxa (Rosendahl 2008), and the ITS region allows a resolution to species level. However, this approach has still to be tested intensively under field conditions as possible primer matches have been found in other fungi and plants, even though no misamplifications were observed in few initially tested environmental samples (Krüger *et al.* 2009).

The approach of Krüger *et al.* (2009) might lead to considerable sequence information of many unknown AMF taxa from various environments. Based on these data, the development of further primers for shorter species-specific amplicons might be possible that could be used for the new 454 FLX-titanium chemistry (Krüger *et al.* 2009). This new technique makes it possible to detect and analyze high amounts of species types in shorter time compared to the conventional

Sanger sequencing procedure. The 454 sequencing approach was already applied by Öpik *et al.* (2009); see section 1.2.5.5.
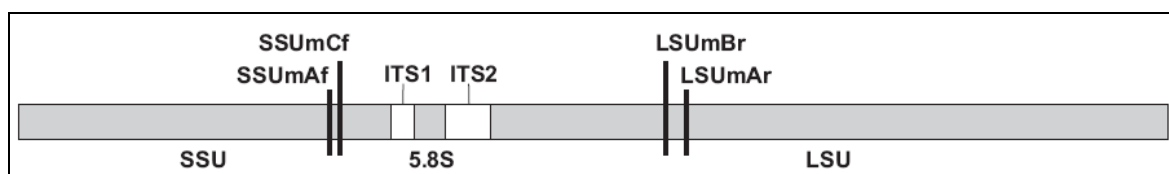
Fig. 12 Small subunit (SSU) rDNA, internal transcribed spacer (ITS) region and large subunit (LSU) rDNA (5465 bp) of *Glomus* sp. 'intraradices' DAOM197198 (AFTOL-ID48, other culture/voucher identifiers: MUCL43194, DAOM181602; accession numbers: AY635831, AY997052, DQ273790) showing the binding sites of the newly designed forward and reverse primer mixtures. From Krüger *et al.* (2009).

1.2.5.3 Fingerprinting methods and quantitative approaches

As the Sanger approach is not only time-consuming but also requires expensive cloning steps in AMF, DNA fingerprinting methods have often been used to characterize AMF communities in the field. Restriction fragment lengths polymorphism (RFLP) approaches were widely applied (e.g. Helgason *et al.* 1998; Sýkorová *et al.* 2007b; Appoloni *et al.* 2008), because it was shown to be a sufficient approach to minimize sequencing effort: usually, only representatives of RFLP types were sequenced. In the also widely applied T-RFLP approach (e.g. Vandenkoornhuyse *et al.* 2003; Johnson *et al.* 2004; Mummey & Rillig 2008), generally two restriction enzymes were used and only terminally labeled fragments were determined. In T-RFLP approaches, an additional confirmation via sequencing is usually not conducted. The community analyses strongly rely on the specificity of the PCR primers and biases due to primer mismatches might occur. Instead of sequencing, also denaturing gradient gel electrophoresis (DGGE) represents an alternative method for the identification of AMF species (e.g. de Souza *et al.* 2004). Other fingerprinting methods such as random amplification of polymorphic DNA (RAPD), inter-simple sequence repeat PCR (ISSR) and amplified fragment length polymorphism (AFLP) cannot be recommended in mixed DNA samples (Krüger *et al.* 2009).

Robinson-Boyer *et al.* (2009) reviewed the progress in shifting the balance from qualitative to quantitative analyses of AMF communities that might reveal the rates of root colonization and ecological significance of certain taxa. These authors reported the application of quantitative real-time PCR (qRT-PCR) in artificial systems using combinations of *G. intraradices* and *G. mosseae* (Alkan *et al.* 2004, 2006; Jansa *et al.* 2008). Pivato *et al.* (2007) compared the frequency of operational taxonomic units (OTUs; here: different phylotypes of *Glomus*) among four *Medicago* species, cultivated in containers, and the original soil taken from a fallow field. The authors observed preferential associations between AM fungal and plant genotypes. In contrast to these studies, Gamper *et al.* (2008) pointed out potential limitations with respect to the information

content of qRT-PCR results. Gamper *et al.* (2008) observed that AM fungal spores contribute much more DNA than hyphae to the pool of nuclear genomic DNA quantified via real-time PCR and that this over-dominance of the nucleic acid signal from spores cannot be alleviated by quantifying nuclear rRNA after reverse transcription. Moreover, in the same study it was observed that there may be considerable heterogeneity in the vitality of and nuclear distribution in extraradical fungal hyphae and nucleic acid contributions from different fragments of colonized roots. Hence, the quantitative abundance of nuclear rDNA or rRNA molecules, as measured by qRT-PCR, may be a poor indicator of functionally relevant hyphal abundance or fungal physiological activity (Gamper *et al.* 2008; Robinson-Boyer *et al.* 2009). To quantify mycorrhizal fungal contributions to ecological processes it may thus be better to use genes encoding proteins of a known function that, moreover, only occur in a single copy per genome (Gamper *et al.* 2010).

1.2.5.4 Marker genes available for the "species" or intraspecific level of AMF

To my knowledge, protein-encoding genes have not been yet applied in AMF species community studies, but were used for the detection of intraspecies groups (see below in this section). Therefore, so far, no alternatives to ribosomal gene markers exist on the species level. Nevertheless, the application of ribosomal markers also causes considerable problems due to the heterogeneity within AMF species (Sanders *et al.* 1995; Lloyd MacGilp *et al.* 1996), regardless of whether a heterokaryotic or a homokaryotic system is assumed (see section 1.2.2): sequences of the same single spore isolate *G. intraradices* JJ291 revealed a degree of polymorphism comparable to sequences from different other *G. intraradices* isolates (Jansa *et al.* 2002b). Gamper *et al.* (2010) reported that it might be difficult or even impossible to define species boundaries based on nuclear rDNA data due to the frequent overlap of sequence types among related species (de Souza *et al.* 2004; Rosendahl 2008). In conclusion, the problems born in ribosomal genes make it clear that these genes obviously cannot be applied for population studies of AMF in field settings.

On the other hand, de Souza *et al.* (2004) reported that PCR-DGGE patterns (based on SSU) could be used to differentiate geographic isolates of *Gigaspora* species. Koch *et al.* (2004) used AFLP (see previous section) to distinguish *G. intraradices* isolates in ROCs (see sections 1.2.2 and 1.2.4.4). Croll *et al.* (2008) and Mathimaran *et al.* (2008a) developed microsatellite simple sequence repeats (SSR) markers for the identification of different *G. intraradices* isolates established in ROCs. By comparing the studies of Croll *et al.* (2008) and Mathimaran *et al.* (2008a), it becomes clear that these authors obviously followed different strategies. Mathimaran *et al.* (2008a) investigated a relatively small set of eight isolates, but they originated from seven locations and four countries, and only two isolates shared the same genotype. In contrast, Croll *et al.* (2008) investigated a set of 48 isolates mainly originating from the same field site in Tänikon in

Switzerland, of which 18 distinct multilocus genotypes were distinguished. However, these codominant multilocus markers have not yet been applied in field settings. Population studies of AMF in field sites were initiated by Stukenbrock & Rosendahl (2005a). These authors used LSU rDNA markers that, indeed, are not as conserved as SSU markers (see section 1.2.5.2). However, they still display a degree of heterogeneity within the same culture, even though not as high as in ITS regions (Sanders *et al.* 1995; Lanfranco *et al.* 1999; Rosendahl *et al.* 2009). Thus, the authors additionally used the protein coding genes *GmFOX2* and *GmTOR2* in a multilocus co-dominant genetic marker approach previously developed by Stukenbrock & Rosendahl (2005b). The polymorphism of the single copy protein genes, showing no heterogeneity within the same organism (in contrast to other protein genes; see Kuhn *et al.* 2001), based on intron regions. Besides this study, the approach was applied in subsequent studies of *G. mosseae*, *G. caledonium* and *G. geosporum* populations (Rosendahl & Matzen 2008; Rosendahl *et al.* 2009). All of these studies were based on analyses of single spores, guaranteeing species identity (as the primers are not species-specific), and the correlation of the different loci for the corresponding genotype. The outcome of these studies is described in section 1.2.5.6.

1.2.5.5 AMF communities in different environments

Community studies of AMF were conducted in various environments, and since molecular markers were available, it was possible to analyze root samples (e.g. Helgason *et al.* 2002; Husband *et al.* 2002; Vandenkoornhuyse *et al.* 2002, 2003; Opik *et al.* 2003; Johnson *et al.* 2004; Rosendahl & Stukenbrock 2004). Fitter (2005) concluded that most of these studies share similar outcomes: (i) a high AMF species richness, even within the same root sample, (ii) many unknown phylotypes, whereas others seem to appear almost everywhere, and (iii) coexisting plants host different AMF species sets. Nonetheless, each of those studies revealed new insights into AMF species composition and ecology in different environments. Öpik *et al.* (2006) surveyed 26 publications addressing AMF occurrence in plant roots of different ecosystems (including few experimental studies in the glasshouse or field) around the globe. The authors showed that the number of AM fungal taxa per host plant differed among tropical forests (18.2), grasslands (8.3), temperate forests (5.6) and arable or polluted sites (5.2). Furthermore, the AMF community composition differed among tropical forests, temperate forests and anthropogenic influenced sites. By comparing the variation within the habitats "grassland", they harboured heterogeneous AMF communities around the world. These results show that different ecosystems characterized by different plant communities and other biotic or abiotic factors require most likely an adapted AMF community.

One of the most remarkable aspects in the study is the highly reduced AMF species richness in agriculturally used systems compared to plant species-rich natural sites (graphically depicted in

Fig. 13). This phenomenon was already observed in a landmark study by Helgason *et al.* (1998), who suggested that lower AMF diversity might be correlated with lower requirement for symbiotic function. A decreased AMF species richness was also observed in community studies based on spore identification: Oehl *et al.* (2003) reported that AMF diversity decreased from species-rich grasslands, sites representing low- to moderate-input farming, to sites representing high-input monocropping. Jansa *et al.* (2002a, 2003) showed that tillage affects the AMF community structure when investigating both AMF spores from the soil as well as root colonizing AMF. In an early spore based investigation Johnson (1993) reported that fertilization increased the abundance of *G. intraradices*, whereas other species like *Gi. gigantea*, *Gi. margarita*, *Scutellospora calospora* or *P. occultum* disappeared. Nevertheless, Hijri *et al.* (2006) observed that AMF species diversity does not have to be low in agricultural sites, and concluded that especially low-input agriculture, involving crop rotation, might maintain a relatively high AMF species richness.
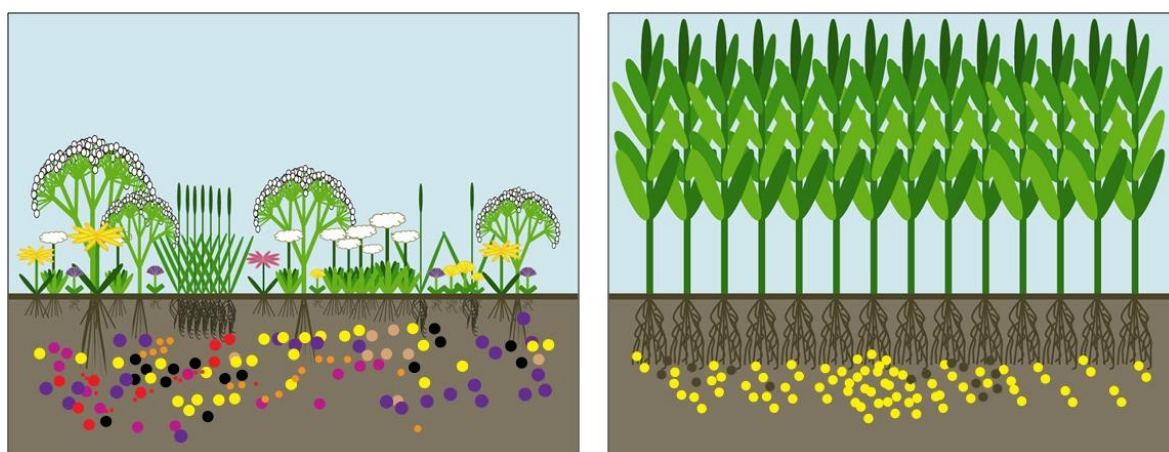
Fig. 13 Illustration of the putative relationship between AMF species diversity and plant communities. Diversity of AMF species indicated as spores of different colors in plant species rich natural sites (left) and arable monocropping sites (right).

Factors driving the AMF communities in semi-natural or natural environments are obviously equally complex. In contrast to the artificial experiments by e.g. van der Heijden *et al.* 1998b (see section 1.2.4.4), Börstler *et al.* (2006) reported that high AMF diversity is not necessarily correlated with high plant species richness: in an intensively farmed grassland (27 plant species) and in an extensively farmed grassland (43 plant species), 11 and 10 AMF species were found using molecular methods, respectively. However, the composition of AMF species varied considerably between both meadows. Besides different soil parameters or farming methods, the differing AMF composition could be explained by different host plant species: even though no strict host specificity in green plants could be proven to date, several studies observed at least host preferences (see section 1.2.4.1). Sýkorová *et al.* (2007b) showed that co-occurring *Gentiana* species harboured distinct AMF communities and that a member of the *Glomus* group B was almost exclusively detected in *Trifolium* species. Similar results were observed in many other field

studies as already mentioned above. Pivato *et al.* (2007) reported similar findings using soil from the field in containers (see section 1.2.5.3). In a recent investigation of a boreonemoral forest, Öpik *et al.* (2009) detected specialized AMF taxa in forest plants, whereas generalist plants occurring in the same plot harboured additional generalist AMF species. Based on the results, the authors suggested that specificity of fungal and plant partners might rather occur on the level of ecological groups than on species level. Lekberg *et al.* (2007) observed niche restrictions of AM fungi in natural sites, where members of the Glomeraceae occurred predominately in clay soils and members of the Gigasporaceae dominated in sand soils. Moreover, the occurrence of *Glomus* species was influenced by levels of soil organic carbon and nitrogen. Dumbrell *et al.* (2010b) reported that pH is a considerable abiotic factor for niche differentiation and structuring AMF communities. Oehl *et al.* (2010) demonstrated that the soil type was the driving force for AMF propagule composition in 16 sites (including grasslands and arable lands), whereas plant species composition only had a subordinate influence. Johnson *et al.* (2009) investigated spore communities from sites in Costa Rica, Mexico, Arizona, Utah, Colorado, and Minnesota USA, in various ecosystems, in order to address questions about factors affecting AMF diversity. Similar to Oehl *et al.* (2010), the authors also concluded that species richness of spores is not correlated with species richness of plant communities. Instead, AMF community composition was associated with latitude, temperature and precipitation, suggesting macro-scale distributions. Until recently, little has been known about whether patterns across AMF communities from different sites can be generalized. Therefore, Dumbrell *et al.* (2010a) compared their own data with the results of 25 published studies and found that AMF communities are typically dominated by a single taxon. Interestingly, this fungus was different in each site and did not correspond to the widespread generalists mentioned by Fitter (2005) (see above). However, Johnson *et al.* (1991) reported differing AMF community structures in two successional stages, where an abundant species became less abundant in the late succession and the community became more even. A shift in community composition over the time was observed by Husband *et al.* (2002): AMF species most frequently found in newly emerged seedlings of a tropical forest were replaced by previously rare species in the surviving seedlings of the following year.

1.2.5.6 The first studies of intraspecific gene structure of AMF species in field settings

Some members of AMF seem to lack any specificity for environmental conditions. Such species with a widespread and global distribution in different ecosystems are *G. intraradices* and *G. mosseae*, but also phylotypes of unknown taxonomic affiliation from the genera *Glomus* and *Scutellospora* (Öpik *et al.* 2006). The studies of generalists in population studies allowed to address whether environmental factors may have some influence on the intraspecific level. As mentioned in section 1.2.5.4, population studies of AMF species conducted directly in field settings have so far been limited to three *Glomus* species. Stukenbrock & Rosendahl (2005a) investigated the spore population structure of *G. mosseae*, *G. caledonium* and *G. geosporum* in an organically and a conventionally-cultured field using a hierarchical sampling design. The authors did not observe differences of the gene diversity of any target *Glomus* species between the field sites. On the other hand, these authors found "indications" for a subdivision between plots within the same field site. Subsequently, Rosendahl & Matzen (2008) analyzed the population structure of the same fungi in a cultivated and a fallow field in order to address whether fallowing has some influence on the distribution of genotypes. The results suggested a subdivision of *G. mosseae* haplotypes between the two fields that was not observed for the two other *Glomus* species. The authors concluded that agricultural practices differently affect the population structure of different AMF species. The most recent study (Rosendahl *et al.* 2009), using the same marker set, addressed the geographical distribution of *G. mosseae* and a set of 82 isolates of this species sampled worldwide was analyzed. The results showed that populations of this AMF species were not differentiated among different continents. Results of coalescence analyses suggested that diversification in the species occurred after the spread of continents. The authors concluded that human activity might have led to the distribution of *G. mosseae* and, therefore, that this activity had a major impact on the current population structure of this fungus. Like this study, investigations of the intraspecific structure of *G. intraradices* based on populations of isolates were not performed directly in the field (Koch *et al.* 2004; Croll *et al.* 2008; see section 1.2.5.4). The results of all population studies mentioned so far are further described and discussed in detail in the chapters 2, 3 and 5.

1.3 Mitochondria in AMF

1.3.1 Mitochondrial genome structure and inheritance

Mitochondria represent the "powerhouses" of eukaryotic cells as the processes of respiration and oxidative phosphorylation are localized in these organelles (Madigan *et al.* 2000). Mitochondria are bounded by a double-membrane (Henze & Martin 2003) and the theory of their origin by an endosymbiosis event > 1.5 billion years ago (Dyall *et al.* 2004; Keeling 2010) is well accepted: the genomic organization and its genes indicate that mitochondria are derived from an alpha-proteobacterium-like ancestor (Andersson *et al.* 1998; Gray *et al.* 1999). In the transition to organelles, a strong reduction of the genome took place, resulting in mitochondrial genomes (chondriomes), which can vary strongly in size among different eukaryotic organisms, ranging from < 6 kb in *Plasmodium falciparum* (Gray *et al.* 1999) up to 367 kb in *Arabidopsis thaliana* (Unseld *et al.* 1997). However, even the latter contains only one third of the genome from its eubacterial relative *Rickettsia prowazekii* (Gray *et al.* 1999). Some genes were lost (Gabaldón & Huynen 2003) others were transferred to the nucleus, which contributes up to 99% of the proteins required by mitochondria of extant eukaryotes (Adams & Palmer 2003). Due to various extents of loss or nucleus transfer, the presence or absence of certain genes and their frequency can vary among different eukaryotic lineages (Adams & Palmer 2003).

Recently, the first complete mitochondrial genome of an AMF species became available: Lee & Young (2009) sequenced the whole chondriome of *G. intraradices* isolate #494. The set of mitochondrial genes was similar to other fungi, and the genes were encoded on the same strand. Genes of three ATP synthase (atp) subunits, three cytochrome oxidase (cox) subunits, seven subunits of the NADH dehydrogenase (nad), apocytochrome b (cob), the full set of 26 tRNAs, the ribosomal small subunit (rns) and large subunit (rnl) were characterized in a total of 70 606 bp mitochondrial genome (see Fig. 14). Only 26% of the chondriome are coding sequences, whereas 21% are occupied by a total of 26 introns, occurring most frequently in the cox1 gene followed by rnl (see Fig. 14). The majority could be identified as Group I introns. LAGLIDADG-type homing endonucleases (see also chapters 2, 3 and appendix) were mainly found in introns and based on phylogenetic analyses, Lee & Young (2009) suggested that they were gained by lateral transfer between species rather than by movement within the genome (see also Thiéry *et al.* 2010 in appendix). Recently, Formey & Roux (2009) presented first insights into the still unpublished mitochondrial genome of *G. intraradices* isolate DAOM197198 (Martin *et al.* 2008) and compared it with the chondriome of strain 494: the chondriome size is 70 783 bp, slightly longer than in isolate 494, and high sequence homology was observed in the coding regions, while non-coding regions displayed highly polymorphic islands.
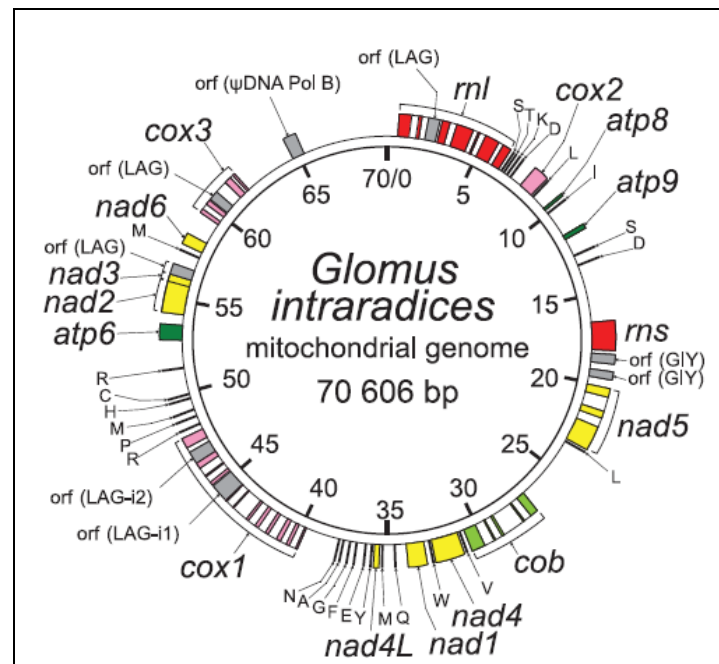
Fig. 14 The mitochondrial genome of *Glomus intraradices* isolate 494. The outer circle shows the location of genes (shaded) and introns (white); all genes are oriented clockwise. The scale is in kb. From Lee & Young (2009).

To date it is not resolved whether AMF are asexual as generally assumed, or whether cryptic sexual stages occur, e.g. via anastomosis (see section 1.2.2). Evidence for limited recombination among nuclear genes of *G. intraradices* isolates was so far only observed *in vitro* (Croll & Sanders 2009). No data about mitochondrial inheritance or recombination exist so far for the Glomeromycota. However, as Croll *et al.* (2009) observed the exchange of nuclei among different isolates, the exchange of mitochondria might most likely happen during the anastomosis process. In this case, the fate of different mitochondrial haplotypes or possible recombination remains to be investigated, but based on the available data, no heteroplasmy (i.e. the cell contains distinct mitochondrial genomes) has been observed to this date (for more details, see next section). Several types of mitochondrial inheritance appear possible, and they might differ among different AMF species. The majority of sexual eukaryotes inherit mitochondrial DNA (mtDNA) only from one "parent". However, exceptions were found in animals, plants and fungi. The latter exhibit the most complex modes: exclusion of paternal mitochondria occurs, biparental and uniparental inheritances are common and recombination e.g. occurs readily in yeast (reviewed in Taylor 1986; Barr *et al.* 2005). Yan *et al.* (2007) reported that mitochondrial inheritance of *Cryptococcus neoformans* (basidiomycete yeast) can be influenced by environmental factors. Based on the genetic exchange in *G. intraradices* (Croll *et al.* 2009) and the currently observed homoplasmy in this species, Young (2009b) concluded that in case of mitochondrial exchange, homoplasmy is restored relatively quickly. Lee & Young (2009) provided two scenarios for maintaining a single chondriome type: (i) segregation of mitochondria through a genetic bottleneck, and (ii) an active

mechanism of segregation, i.e. only homogeneous mtDNA is transmitted to descendants, as it was also observed in yeast (Barr *et al.* 2005).

Another complex aspect which is difficult to address is the inheritance and loss of introns that can be additionally characterized by homing endonucleases. General aspects and considerations about this topic are presented in detail by Thiéry *et al.* (2010); see appendix.

1.3.2 The use of mitochondrial genes in phylogenetic analyses or as molecular markers

Lee & Young (2009) performed phylogenetic analyses of mitochondrial protein sequences of *G. intraradices* and members of the Ascomycota, Basidiomycota, Blastocladomycota and Chytridiomycota, revealing several alternative topologies. *Glomus intraradices* either clustered together with *Mortierella verticillata*, but without significant support, or represented a sister taxon to either a *Mortierella-Rhizopus*-Dikarya clade or to *Smittium*. The latter genera are currently members of the paraphyletic Zygomycota (James *et al.* 2006). All topologies placing *G. intraradices* as a sister taxon to the Dikarya were rejected by statistical tests (see Figs 3 and 4 in Lee & Young 2009). This finding confirms other phylogenies based on nuclear-encoded protein-coding genes which also did not support the Dikarya as a direct sister clade of the Glomeromycota, but suggested a relationship to the Mortierellales (Helgason *et al.* 2003; Redecker & Raab 2006; Liu *et al.* 2009), in contrast to phylogenies based on nuclear rRNA genes (see section 1.2.1). Lee & Young (2009) suggested that the use of rRNA genes might introduce a bias due to fast evolutionary rates and skewed G + C nucleotide composition (Kumar & Rzhetsky 1996).

Besides further insights into the phylogeny of the Glomeromycota, mitochondrial genes provide other advantages: section 1.2.5.4 describes that heterogeneous nuclear-encoded rDNA is not a suitable marker for population studies, and that other markers had to be applied in within-species diversity studies targeting spores in the soil. Raab *et al.* (2005) provided the first mitochondrial markers for AMF. The authors reported the following findings: (i) in contrast to nuclear-encoded rDNA, the mitochondrial ribosomal RNA large subunit gene (mtLSU) sequences are homogeneous within the investigated isolates of *G. intraradices* and *G. proliferum*. (ii) Two different isolates of *G. intraradices* could be distinguished by different mtLSU haplotypes. (iii) The detection of the mtLSU from *G. intraradices* was possible in a field collected root sample. These results suggested mitochondrial markers as promising molecular tool for population studies of root colonizing AMF species under field conditions. The state of homoplasmy was largely confirmed by Lee & Young (2009), who sequenced total genomic DNA from altogether 24 spores of the isolate 454. Little evidence for sequence heterogeneity was observed, but the authors suggested that these minor variations in different reads might have been caused by nuclear copies of mtDNA, which are

widespread but usually nonfunctional (Bensasson *et al.* 2001). Therefore, the availability of the complete sequence material will allow the development of potential mitochondrial markers other than those developed by Raab *et al.* (2005) for diversity studies of AMF. These markers could be established similarly to other organisms: early studies using mitochondrial genes for phylogeny and diversity studies of animals were reviewed by Avise *et al.* (1987), Moritz *et al.* (1987) and in the meantime large datasets of mitochondrial sequences exist for other fungi, e.g. basidiomycetes (Bruns *et al.* 1998). The mtDNA has been furthermore pivotal for answering questions of conservation genetics (Robertson *et al.* 2007) or ancestry tracing (Cann *et al.* 1987). A fragment of the cox1 gene was even chosen as standardized tool for molecular identification ("barcoding") in animals (Ratnasingham & Hebert 2007). In a recent PhD study, Borriello (2010) amplified mitochondrial cox1 gene sequences from *Glomus* group A, *Gigaspora* and *Scutellospora* isolates. No heteroplasmy was observed and while intraspecific diversity of isolates from different geographic regions was always very low, it was higher among different species and genera: the lowest divergence was observed in *Gigaspora*, followed by *Scutellospora* and *Glomus* group A, in which the highest diversity was detected.

Besides almost ubiquitous homoplasmy, another reason for the potential broad application of mitochondrial markers is the easy amplification of these genes due to their multiple copies in cells of animals and plants, in which 50 to 100 000 fold more mitochondrial than nuclear genomes occur (Thorsness & Hanekamp 2001). A typical animal cell contains up to 1 000 mitochondria (Madigan *et al.* 2000). However, presenting an exact number might be misleading as it can vary depending on energy requirements (Krauss 2001), which could considerably differ among different cells and tissues. The yeast *Saccharomyces cerevisiae* dramatically regulates the number of mitochondria during cell growth (Jensen *et al.* 2000). Lang & Hijri (2009) visualized mitochondria in spores of *G. diaphanum* (see Fig. 15) and concluded that hundreds of mitochondria were present, as it is also true for nuclei in the Glomeromycota (see section 1.2.3).
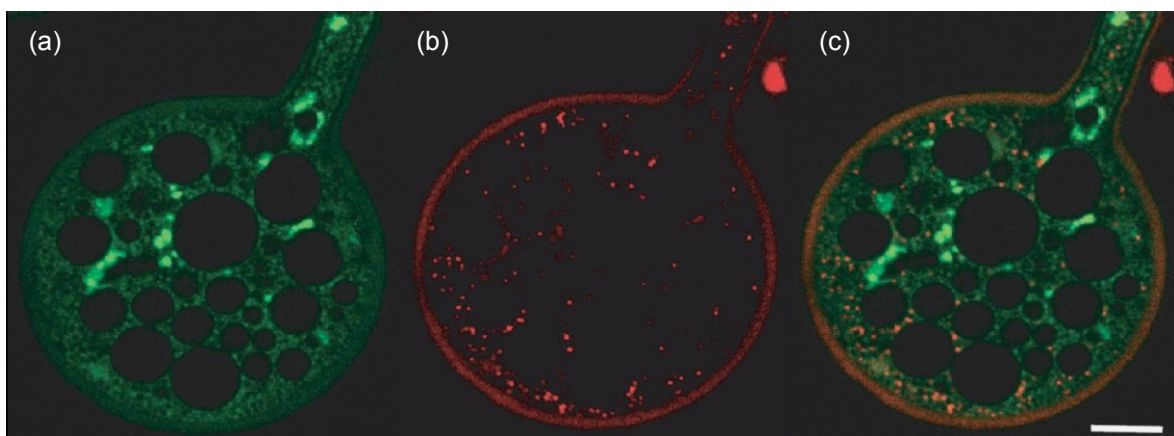
Fig. 15 Confocal microscopy of live *Glomus diaphanum.* (a) Nuclei stained with SytoGreen, green spots; (b) mitochondria stained with MitoTracker, small red spots; (c) merged image. Spore walls autofluoresce in both the green and red channels. From Lang & Hijri (2009).

1.4 Aims of this thesis

- The first major aim of this thesis (chapter 2) was to determine whether mtLSU haplotypes are polymorphic and stable enough to distinguish *G. intraradices* isolates, and whether these molecular markers are suitable for population studies, as suggested by Raab *et al.* (2005); see section 1.3.2.

  For this purpose, a set of 16 different *G. intraradices* isolates originating from five continents was chosen. Based on this world-wide sample, further questions were addressed: (i) is global diversity of different *G. intraradices* genotypes already present in one field site as assumed by Koch *et al.* (2004) and Croll *et al.* (2008), or is the world-wide intraspecies diversity higher than in one location? (ii) Does the mtLSU provide suitable sequence information for phylogenetic analyses and what is the resolution level compared to nuclear-encoded ribosomal marker genes?

  For the molecular investigations in this part of the thesis, the nested PCR approach developed for the mtLSU of *G. intraradices* and *G. proliferum* (Raab *et al.* 2005) was initially applied. However, based on additional sequence information from isolates, I developed a new nested PCR approach, in order to increase the specificity for only *G. intraradices* and to improve the amplification of the mtLSU out of colonized plant roots. Therefore, some environmental root samples were additionally considered, which were (also phylogenetically) compared with the isolates. Furthermore, I developed a RFLP system to facilitate the processing of a larger number of samples without the need to rely on laborious and expensive cloning and sequencing of all the PCR products. The newly developed PCR-RFLP identification method was also applied and tested in the next part of this thesis:

- The second major aim (chapter 3) was to address the diversity of *G. intraradices* directly within colonized roots from field settings to elucidate whether the populations are differentiated genetically among different arable or grassland sites and whether specialized ecotypes can be detected.

  In section 1.2.5.5, it was outlined that communities in agricultural sites are generally characterized by low AMF diversity, whereas species-rich AMF communities are typical for plant species-rich grasslands. Therefore, it was interesting to see whether these relationships hold also true on the intraspecific level. Another aspect was the investigation of potential effects of different tillage treatments on *G. intraradices* populations.

  For this project, two agricultural field experiments in Switzerland and two semi-natural grasslands in Switzerland and France were chosen. Factors like distance or host plants were additionally considered for the interpretation of the results.

    Besides these main goals in this part of the thesis, we addressed the question, whether it would be possible to specifically detect only one single mtLSU haplotype in these field sites. For this purpose, I developed a specific nested PCR approach for the mtLSU haplotype I, which was already known from different isolates sampled in different and distinct locations of the world (Raab *et al.* 2005; Croll *et al.* 2008). Such molecular tools might be helpful in tracing AMF strains after inoculation in field sites (see also below).

- Besides these two main aims of the thesis, I cooperated with Odile Thiéry on the amplification and characterization of mtLSU haplotypes of *G. clarum*. In her PhD thesis, O. Thiéry addressed the suitability of mtLSU as molecular marker for other *Glomus* species and the evolutionary dynamics of its introns (see appendix).

- The major task in a cooperation with Zuzana Sýkorová (Academy of Sciences of the Czech Republic, Průhonice) was the development of specific primers for two mtLSU haplotypes of the *G. intraradices* isolate BEG140 (chapter 4). The aim of Z. Sýkorová's project was the detection of BEG140 in plant roots after inoculation in a former brown-coal spoil bank.

Chapter 2: Genetic diversity of the arbuscular mycorrhizal fungus *Glomus intraradices* as determined by mitochondrial large subunit rRNA gene sequences is considerably higher than previously expected

Boris Börstler, Philipp A. Raab, Odile Thiéry, Joseph B. Morton and Dirk Redecker

Fig. 1 Root organ culture
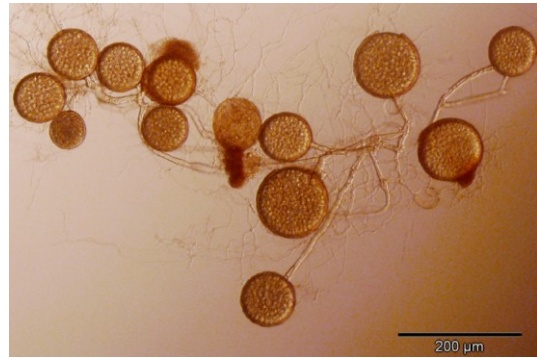
Fig. 2 Spores of *Glomus intraradices*

Fig. 3 Pot cultures
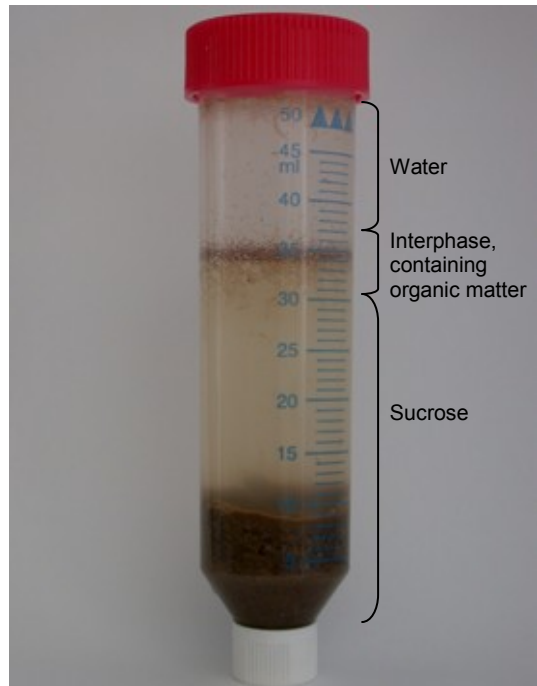
Fig. 4 Sucrose density gradient
(Photo by Kurt Ineichen)

2.1 Summary

- *Glomus intraradices* is a widespread arbuscular mycorrhizal fungus (AMF), which has been found in an extremely broad range of habitats, indicating a high tolerance for environmental factors and a generalist life history strategy. Despite this ecological versatility, not much is known about the genetic diversity of this fungal species across different habitats or over large geographic scales.

- A nested polymerase chain reaction (PCR) approach for the mitochondrial rRNA large subunit gene (mtLSU), distinguished different haplotypes among cultivated isolates of *G. intraradices* and within mycorrhizal root samples from the field.

- From analysis of 16 isolates of this species originating from five continents, 12 mitochondrial haplotypes were distinguished. Five additional mtLSU haplotypes were detected in field-collected mycorrhizal roots. Some introns in the mtLSU region appear to be stable over years of cultivation and are ancestral to the *G. intraradices* clade.

- Genetic diversity within *G. intraradices* is substantially higher than previously thought, although some mtLSU haplotypes are widespread. A restriction fragment length polymorphism approach also was developed to distinguish mtLSU haplotypes without sequencing. Using this molecular tool, intraspecific genetic variation of an AMF species can be studied directly in field plants.

Key words: arbuscular mycorrhiza, Glomeromycota, *Glomus intraradices*, intraspecific diversity, mitochondrial haplotypes, molecular markers.

2.2 Introduction

Arbuscular mycorrhizal fungi (AMF) are associated with the broad majority of plant species and play an important role in mineral nutrient uptake. In exchange for photosynthates provided by the plant symbionts, the fungal partners improve the plants' access to phosphate, nitrogen and other mineral nutrients. The diversity of AMF correlates with diversity of plant communities suggesting that AMF influence competitive interactions among plants (Streitwolf-Engel *et al.* 1997; van der Heijden *et al.* 1998b).

Molecular methods have been developed that allow identification of AMF within roots without the necessity of spore formation. In all studies to date addressing genetic diversity of AMF in roots in the field, only regions of nuclear-encoded ribosomal RNA genes have been used. Specific polymerase chain reaction (PCR) primers amplify diagnostic regions of these genes from colonized roots (Redecker 2006). The resulting PCR products are characterized by various

methods, including restriction fragment length polymorphism (RFLP) and DNA sequencing to identify the fungi. The applications of molecular identification methods in field settings have yielded novel insights into the ecology of these fungi (Öpik *et al.* 2006).

When using nuclear rRNA genes for phylogeny and identification of glomeromycotan fungi, the high variation among gene copies, present even within single spores of these organisms (Sanders *et al.* 1995; Lloyd MacGilp *et al.* 1996; Lanfranco *et al.* 1999) impairs not only the identification of closely related morphospecies, but also differentiation of isolates within a morphospecies. Variation is more acute in the internal transcribed spacers (ITS) than in the more conserved regions of rRNA genes. For example, ITS sequences within a single spore isolate of *Glomus intraradices* were as divergent as sequences from other isolates (Jansa *et al.* 2002b).

Intraspore rRNA gene variation could occur among rRNA gene copies in the genome of a single nucleus, as reported from other organisms (Buckler *et al.* 1997) or among nuclei inhabiting the same cell. The genetics of multiple nuclei in the glomeromycotan mycelia is conflicting, with some evidence suggesting nuclear populations are heterokaryotic (Kuhn *et al.* 2001; Hijri & Sanders 2005) and other data indicating they are homokaryotic (Pawlowska & Taylor 2004). A heterokaryotic genetic system implies absence of a fixed nuclear genotype for a fungal isolate, with populations of nuclei changing within a species. Rosendahl (2008) summarized recent progress in the budding field of AMF population biology.

Koch *et al.* (2004) characterized isolates of *G. intraradices* cultivated in root organ cultures using amplified fragment length polymorphism (AFLP), showing a high degree of genetic and phenotypic diversity among those isolates. With the exception of the Canadian isolate DAOM197198, all isolates originated from one field site in Tänikon, Switzerland (Jansa *et al.* 2002b).

Croll *et al.* (2008) used a larger set of root organ cultures of *G. intraradices* isolates from the same field site to elucidate local genetic diversity. They used 10 simple sequence repeat (SSR) loci as molecular markers as well as introns of the mitochondrial large subunit rRNA gene (mtLSU; Raab *et al.* 2005) and introns of a nuclear gene. Genetic diversity among fungal isolates was high, but isolates from other locations in Switzerland and Canada were not substantially different. These results complemented those of Koch *et al.* (2004) and indicated that much of the global genetic diversity of *G. intraradices* could be represented just within this field site. Multilocus genotypes also have been identified from individual fieldcollected spores of *G. mosseae* using markers from single copy nuclear genes *GmFOX2*, *GmTOR2*, and *GmGIN1* (Stukenbrock & Rosendahl 2005b).

Mathimaran *et al.* (2008a) used a set of 18 SSR markers to analyse genetic variation among eight isolates of *G. intraradices*. Only two isolates from this set appeared to be identical clones. Neither the SSR or AFLP markers listed earlier, nor the 'Single Nucleotide Polymorphisms' of Stukenbrock & Rosendahl (2005b) have been applied to mycorrhizal roots from the field so far.

Mitochondrial DNA has a long history as a molecular marker (especially for the Metazoa), which precedes the era in which PCR facilitated the access to its sequences from a broad range of organisms (Bruns *et al.* 1989). A region of the mtLSU was used so successfully for routine molecular identification of ectomycorrhizal fungal species from colonized roots that a large dataset is available for comparative study (Bruns *et al.* 1998).

Use of mitochondrial genes avoids possible complications from heterogeneous sequences encountered with nuclear genes. Raab *et al.* (2005) provided the first sequences from the mitochondrial genome of the Glomeromycota and documented the absence of any substantial variation in an mtLSU region within isolates of *G. intraradices* and *G. proliferum*. However, sequences were polymorphic among isolates of this species. Most notable was the presence/absence of introns and sequence variation within introns. These results suggest that mtLSU sequences provide useful information that distinguishes closely related *Glomus* species as well as intraspecific variation. A practical aspect of this approach is using mtLSU data to determine haplotypes of fungal symbionts directly amplified from mycorrhizal roots, an essential criterion for population studies of nonculturable organisms. Specific primers have been designed which directly amplify mtLSU sequences from mycorrhizal roots (Raab *et al.* 2005). Molecular analyses of field-collected mycorrhizal roots reveal high diversity and putative taxa that do not sporulate (Helgason *et al.* 2002). However, sequence types corresponding to a few well-known morphospecies were also detected in a broad range of habitats. *Glomus intraradices* has been the most common species detected in a range of studies and it is one of the most extensively studied species in Glomeromycota. This species was found in mycorrhizal roots in habitats as different as high-input and low-input agricultural field sites (Hijri *et al.* 2006) and species rich grasslands (Sýkorová *et al.* 2007b) in Switzerland, phosphate-polluted sites (Renker *et al.* 2005) and mountain meadows (Börstler *et al.* 2006) in Germany, and geothermal soils in Yellowstone National Park, USA (Appoloni *et al.* 2008). All of this ITS sequence variation clustered phylogenetically within a clade of sequences originating from a single spore of *G. intraradices* (Jansa *et al.* 2002b). Using more conserved rRNA gene regions *G. intraradices* was detected in grasslands of Estonia (Öpik *et al.* 2003) and even in tropical trees in Panama (Husband *et al.* 2002). This fungal species has been classified as a generalist because it is abundant across disturbed as well as more mature habitats (Sýkorová *et al.* 2007a). It is widespread geographically and tolerates a wide range of habitats (Öpik *et al.* 2006). It is also compatible with all culturing systems currently in use, from glasshouse pots to root-organ cultures (Jansa *et al.* 2002a), and thus is one of the most common fungal components in commercial inocula (Corkidi *et al.* 2004). Not surprisingly, therefore, it was chosen as the model AMF species for genome sequencing (Martin *et al.* 2004). Given the importance and ubiquity of this species, a detailed understanding of population structure is essential.

Defining boundary conditions for *G. intraradices* has been problematic because variation in morphological features intergrades within and between isolates. Spore wall organization and

structure is conserved and diagnostic, but number and color of layers are variable so that spore populations can vary considerably in size and color. Also, isolates can vary greatly in frequency and degree of aggregation in roots and/or soil.

This study addressed the following questions: Are mtLSU sequences polymorphic among isolates from different geographic locations? Do intron sequences provide stable markers that distinguish fungal haplotypes? An applied outcome of this work was an easy-to-use genotyping system based on mtLSU markers to study intraspecific genetic variation in the field.

2.3 Materials and Methods

2.3.1 Root organ cultures of *G. intraradices*

Isolate CC-4, originating from a fallow field in Clarence Creek, Ontario, Canada, was purchased from the Glomeromycota *in vitro* Collection (GINCO)/Belgium (ID codes MUCL43204, DAOM229456; for details see http://emma.agro.ucl.ac.be/ginco-bel/index.php). Isolate DAOM197198 originated from Pont Rouge, Québec, Canada, tree plantation/*Fraxinus americana* and was obtained independently from G. Bécard (University of Toulouse, France) in 1995 and from N. Requena (University of Karlsruhe, Germany) in 2005. This isolate also is known under the ID codes MUCL43194 and DAOM181602. Isolates JJ141, JJ145, and JJ183 originated from Hausweid, Tänikon, Switzerland (long-term field tillage experiment including crop rotation), and were obtained from J. Jansa (see Jansa *et al.* 2002b). All isolates were propagated in root organ cultures (ROCs; see Fig. 1) on transformed carrot roots as previously described by Bécard & Fortin (1988). For DNA extraction, spores (see Fig. 2) were dissolved in 10 mM sodium acetate–citrate buffer (pH 6.0) and washed in sterile water according to Doner & Bécard (1991). Croll *et al.* (2008) and Koch *et al.* (2004) used the isolate codes B7, C5, C2 and C3 for JJ291 (Raab *et al.* 2005), JJ141, JJ145 and JJ183, respectively.

2.3.2 Inocula and pot cultures of *G. intraradices*

Isolates of *G. intraradices* from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) were obtained as pot culture substrate-inoculum (see http://invam.caf.wvu.edu/index.html): AU212B (Australia), CA502 (California/USA), CR316A (Costa Rica), FL208A (Florida/USA), JA202 (Kitami Agricultural Station/Hokkaido/Japan from *Phaseolus vulgaris* crop), KE114 (Kenya), NB102C (Namibia; from a native bush in the Namib

desert), SW205 (Switzerland; same strain as JJ141 contributed to INVAM, for details see ROCs in section 2.3.1) and VA110 (Virginia/USA; soil from suburban home garden near Washington, DC). Isolate DD-4 (accession number DQ487216, nuclear-encoded small subunit rRNA gene) originated from a Dutch dry dune grassland (Provinciale Waterleidingduinen/Netherlands; 52°36′N, 4°38′E) and was obtained from M. G. A. van der Heijden (Vrije Universiteit Amsterdam) as pot culture substrate-inoculum. All pot culture substrate-inocula were stored at 4°C until DNA extraction. For isolate DD-4, a new pot culture (Fig. 3) was set up and cultured under the greenhouse conditions described in Tchabi *et al.* (2008). Substrate consisted of sterilized Terragreen (American aluminium oxide, Oil Dry US special, Type III R, < 0.125 mm; Lobbe Umwelttechnik, Iserlohn, Germany) and Loess from a local site mixed 9:1 (w : w). Approximately 1 g of contributed inoculum was layered under seeds of *Plantago lanceolata* and *Hieracium pilosella*. After 8 months of cultivation, a 10–15 ml sample from the original pot culture substrate-inocula and from the new culture of DD-4 was wet-sieved using a top sieve with 1 mm openings and a bottom sieve with 38 μm openings. The content of the bottom fraction was collected in 20 ml water, applied to a 70% (w : v) sucrose solution and centrifuged at 820 *g* for 2 min (Esch *et al.* 1994; see Fig. 4). All organic matter suspended in the supernatant was decanted, repeatedly rinsed in a 38 μm sieve, transferred to 1.5 ml tubes, and stored on ice until DNA extraction. Single spores were collected separately, washed thoroughly in distilled water and placed in 0.2 ml tubes.

2.3.3 Field-collected root samples

The DNA extracts of mycorrhizal roots collected from the 'Ramosch'-meadow in the Engadin region of Switzerland were provided by Z. Sýkorová (Sýkorová *et al.* 2007b). Samples S6 (*Trifolium* sp.) and S10 (*Trifolium* sp.) correspond to the samples 11-2v and 11-3a, respectively, of Sýkorová *et al.* (2007b) in the Supporting Information, Table S1. DNA extracts from samples of two *Plantago lanceolata* mycorrhizal root systems originating from the Gyöngyösoroszi mine spoil in the Matra mountains of Hungary (47°50′44″N, 19°53′05″E) were provided by I. Parádi (Eötvös Loránd University, Budapest, Hungary). These two samples were designated da2 and da4.

2.3.4 DNA extraction

The DNA of 1–15 spores was extracted according to Redecker *et al.* (1997): spores were crushed in 2 μl 0.25 N NaOH, heated to 95°C, and incubated for 2 min. One microliter of 0.5 M TrisHCl (pH 8.0) and 2 μl 0.25 N HCl were added to this crude extract, which was heated again for 2 min and then used directly as PCR template. DNA was extracted from: spore populations (> 15); 50–80 mg

(wet weight) of the organic fraction extracted from inocula/pot culture substrates and 50–80 mg (wet weight) of plant roots using the DNeasy plant mini kit (Qiagen, Hilden, Germany). Liquid nitrogen was used to grind frozen root samples. Depending on success of amplification, extracts were further diluted 1:10 and 1:100 in TE (Tris-ethylenediaminetetraacetic acid) buffer and used again as PCR template (Table 1).

2.3.5 PCR amplification of mtLSU

Isolates CC-4, DAOM197198 and JJ183 were amplified by nested PCR as described in Raab *et al.* (2005) with slight changes. For the first reaction the primer pair consisted of RNL-3 and RNL-9. Cycling parameters were 3 min at 95°C, 34 cycles of 1 min at 95°C, 1 min at 51°C, 4 min at 72°C, and finally 10 min at 72°C (parameter type 1). For the second reaction the primer pair was RNL-1 and RNL-5. Cycling parameters were 3 min at 95°C, 34 repeats of 1 min at 95°C, 1 min at 56°C, 4 min at 72°C and a final elongation of 5 min at 72°C (parameter type 2). The *Taq* polymerase from GE Healthcare (Otelfingen, Switzerland) included 2 mM $MgCl_2$, 0.5 μM of each primer and 0.25 mM of each desoxynucleotide in the master mix.

Based on these results and data from Raab *et al.* (2005), new primers with improved specificity were developed and applied in a nested PCR approach that was used for most other samples. Forward and reverse primers RNL-28a and RNL-5, respectively, were used in the first reaction and RNL-29 and RNL-30, respectively, in the second reaction (for primer sequences, see Table 2). *Taq* polymerase (master mix see above) or Phusion High-Fidelity DNA Polymerase from FINNZYMES (BioConcept, Allschwil, Switzerland) including 1x Phusion HF Buffer, 0.5 μM of each primer and 0.2 mM of each desoxynucleotide in the master mix, were used. Parameter type 1 (Table 1) was applied for both reactions when using *Taq* polymerase. Cycling parameters were changed when using Phusion polymerase: 30 s at 98°C, 33 cycles of 10 s at 98°C, 30 s at 55°C and 2 min at 72°C, followed by 10 min at 72°C (parameter type 3). Parameter type 4 (30 s at 98°C, 34 cycles of 10 s at 98°C, 30 s at 60°C, 2 min at 72°C, and 5 min at 72°C/Phusion polymerase) and parameter type 6 (see next paragraph) were used for exceptions (details summarized in Table 1). DNA was extracted at least twice from each isolate. If DNA was extracted from organic matter, results were confirmed by PCR products from spores in all isolates except JA202, NB102C and SW205.

Table 1 PCR conditions for isolates of *G. intraradices* and colonized plant roots (shaded in grey). Nested PCRs based on the same DNA extraction of one isolate are given in smaller case letters instead of numbers. Nested PCRs based on the same DNA extract like ITS sequences are shown in boldface. fwd (forward), iD (DNA of organic matter from inoculum), iS (spore from inoculum), nr (nested reaction), pD (DNA of organic matter from pot culture), rD (DNA of plant roots), rev (reverse), rS (spore from root organ culture).

Origin isolate or plant sample	nested PCR	DNA template origin/ dilution	RNL PCR primers 1. nr		RNL PCR primers 2. nr		Dilution of 2. nr template	parameter type 1. nr	parameter type 2. nr	Further application
			fwd	rev	fwd	rev				
AU212B	**1**	1xiS/no	28a	5	29	30	no	1	1	1x sequenced
	2	iD/1:10	28a	5	29	30	1:10	3	3	RFLP
CA502	**1**	1xiS/no	28a	5	29	30	no	1	1	1x sequenced
	2	iD/1:10	28a	5	29	30	1:100	3	3	RFLP
CC-4	**1**	>15rS/1:10	3	9	1	5	no	1	2	2x sequenced/RFLP
	2	15xrS/no	3	9	1	5	no	1	2	1x sequenced/RFLP
CR316A	1	1xiS/no	28a	5	29	30	no	1	1	1x sequenced
	2	iD/1:10	28a	5	29	30	no	3	3	RFLP
	3	iD/1:10	28a	5	29	30	no	1	1	RFLP
DAOM-197198	1	>15rS/no	3	9	1	5	1:100	1	2	5x sequenced
	2	>15rS/no	3	9	1	5	1:100	1	2	1x sequenced
DD-4	**1**	1xiS/no	28a	5	29	30	no	1	1	1x sequenced/RFLP
	2	pD/1:10	28a	5	29	30	no	1	1	RFLP
FL208A	1	iD/no	1	5	29	30	1:100	4	3	1x sequenced
	2	1xiS/no	1	5	29	30	no	4	3	RFLP
	3	1xiS/no	1	5	29	30	no	4	3	RFLP
	4	<15iS/no	1	5	29	30	no	4	3	RFLP
	5	>15iS/no	1	5	29	30	no	4	3	RFLP
JA202	**1**	iD/no	28a	5	29	30	no	1	1	1x sequenced
	2	iD/1:10	28a	5	29	30	no	3	3	RFLP
JJ141	**a**	5xrS/no	28a	5	28a	31	no	1	1	1x sequenced
	b	5xrS/no	28a	5	46	30	no	1	6	1x sequenced*
	c	5xrS/no	28a	5	46	5	no	1	6	1x sequenced*
JJ145	a	5xrS/no	28a	5	28a	31	no	1	1	1x sequenced
	b	5xrS/no	28a	5	46	30	no	1	6	1x sequenced*
	c	5xrS/no	28a	5	46	5	no	1	6	1x sequenced*
JJ183	1	4xrS/no	3	9	1	5	no	1	2	2x sequenced
	2	<15rS/no	3	9	1	5	no	1	2	1x sequenced/RFLP
KE114	**1**	1xiS/no	28a	5	29	30	no	1	1	1x sequenced
	2	iD/1:10	28a	5	29	30	no	3	3	RFLP
NB102C	**1**	iD/1:10	1	5	29	30	no	4	3	1x sequenced
	2	iD/1:10	28a	5	29	30	no	3	3	RFLP
SW205	1	iD/1:10	28a	5	29	30	no	3	3	RFLP
VA110	**1**	1xiS/no	28a	5	29	30	no	1	1	1x sequenced
	2	1xiS/no	28a	5	29	30	no	1	1	RFLP
	3	iD/1:10	28a	5	29	30	no	1	1	RFLP
Trifolium sp. S6	1	rD/1:100	28a	5	29	30	1:100	1	1	2x sequenced/RFLP
Trifolium sp. S10	1	rD/1:100	28a	5	29	30	1:100	1	1	2x sequenced/RFLP
Plantago lanceolata da2	1	rD/1:10	28a	5	29	30	no	1	1	2x sequenced/RFLP
Plantago lanceolata da4	1	rD/1:10	28a	5	29	30	no	1	1	1x sequenced/RFLP

* directly sequenced PCR product

Table 2 The sequences of RNL primers used to amplify regions of the mitochondrial rRNA large subunit gene (mtLSU) gene.

RNL primer	sequence	RNL primer	sequence
1*	AGACCCGAARCCWRGTGATCT	31	TTMGTGCCGCCACTTATTAG
2*	GGRAASAGCCCAGAAYA	33	CTGCCCTATAGAAGAGTTAC
2c	TCGTGATAAGGCGATTCGTC	35	TAACCCCTCAACGACCACAC
3*	TGCATMATGGGTCAGCGAGT	36	CACCTGTTCTGGGCTGTTGC
5*	GAGCTTCCTTTGCCATCCTA	36b	CACCAGTTCTGGGCTGTCGC
7*	CTTCTGCTTTCGGCGAAGAG	37	TAGCTGGGCTAAGAATGCTG
7b	CAGCTATGTCCACCGGCTCA	38	AGCTTGGACTAACCCACTAATG
9*	CAGTAAAGCTGCATAGGGTCT	39	CGGACCTATTGCCCAATACT
10*	AGAAGAAAGAGCTGGCTGTG	40	CCTAGAAGTGCTGGCGTGTT
10h	CCTAATAATCCTCCTACAAG	41	GGCTCTTTCCYGAACCTTAC
11*	AAGGCAACACGCCAGCACTT	42	TAGTACACTGCTAAGCTAGA
11b	AGGGCAACACGCCAGCACTT	43	CCCCTAACAGTTAATAACTC
12*	GATAGCGTAACAGCTCAGTG	44	CACTGCTAAGCTAGATTACT
12b	GATAGGGTAACAGCTCAGTG	45	ATACTCACCCTACCCTTACC
13*	TGGTCGATGGACGACGGATA	46	CCGACACAGGTCTGCAAGTA
13d	AGATCGMCGTAGTTCCTTCT	51	GATTCTGAAAGACGGAATAG
14*	AGGATAGGCCTGGAAACCAAGC	52	GGTTTTGCGTACGTAAGAGT
15*	CTGAGCTGTTACGCTATC	53	CATGGGTGTTGCTCTCAAATC
16*	ACCTGGAGATAGCTGGTCTT	54	AGCAACACCCATGTGCAAGT
17*	CCATAGAGTTGGCTCTAACA	55	ACTCTTACGTACGCAAAACC
17h	GCACGGAATTGAACCRTAGAG	57	CCTGTTAGGCGTACCTATGCC
24	GAGCATACTAAGGCGTAGAG	58	TAACCCATAATGCTCATCTA
25	ATCAGTGGGGAGAGGACAGT	59	GGAGATCATTGGGGTTATCC
26	CTTGTGCAAGTAGGCCTTCT	60	TAAGTAGGTGGACATAACTG
27	CCAACTATGCAACCGTAGG	61	GCCTAACCCTTCCCTATAGA
28a	CCATGGCCAAGTGCTATTTA	62	CCAGTGCCGTACCGTCTAGT
29	TAATAAGACTGAACGGGTGT	63	CATTATATGCTCCGGCGTAG
30	TAGCATCGGGCAGGTATCAG	64	AAGCACGGAATTGAACCATA

Shaded primers were used for the nested polymerase chain reactions; the others were used for sequencing.
* Primers designed by Raab *et al.* (2005).

2.3.6 PCR amplification of nuclear-encoded ITS rDNA

A nested PCR was performed according to Redecker (2000). The universal eukaryote primer pair NS5/ITS4 was used for the first PCR reaction (White *et al.* 1990). The *Glomus* group A-specific primer pair GLOM1310/ITS4i was used in the second PCR reaction. The *Taq* polymerase master mix contained following concentrations: 2 mM MgCl$_2$, 0.5 μM of each primer and 0.125 mM of each desoxynucleotide. Parameter type 5 cycling conditions for the first reaction were as follows: 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 51°C, 2 min at 72°C, and a final extension phase of 10 min at 72°C. The second nested step was performed under the same conditions, but with an annealing temperature of 61°C (parameter type 6). Depending on success of PCR amplification, 1 μl PCR product of the first reaction was used undiluted or at dilutions of 1:10 or 1:100 (in TE buffer) as template for the second reaction.

2.3.7 Cloning, sequencing and sequence analyses

The PCR products were purified using the High Pure Kit from Hoffmann LaRoche (Basel, Switzerland) and cloned into the pGEM-T vector (Promega/Catalys, Wallisellen, Switzerland) following the manufacturer's protocol. Before cloning, blunt-ended PCR products based on Phusion polymerase were incubated at 72°C for 13 min using *Taq* polymerase, 2 mM $MgCl_2$ and 0.125 mM dATP for adding 3′-adenines. Clones of mtLSU rDNA were amplified using the respective PCR primers of the second nested step or the vector primers M13fwd (GTA AAA CGA CGG CCA GTG) and M13rev (GGA AAC AGC TAT GAC CAT G). Products were purified and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit (ABI, Foster City, CA, USA) and an ABI 310 capillary sequencer. Sequencing primers for isolates differing from the sequencing set for JJ291 (Raab *et al*. 2005) are provided in Table 3. Complete sequences of the isolates JJ141 and JJ145 were composed by sequenced clones and directly sequenced PCR products (Tables 1 and 3). Clones of nuclear encoded ITS rDNA were sequenced in both directions using the primers of the second nested step or alternatively the universal forward primer ITS1F (CTT GGT CAT TTA GAG GAA GTA A) instead of GLOM1310. Sequences of mtLSU rDNA were aligned and corrected in BioEdit (Hall 1999), sequences of the ITS rDNA were edited in Sequence Navigator (version 1.0.1). Alignments were performed in BioEdit (Hall 1999) and in PAUP* 4.0b10 (Swofford 2001). DNA sequences were submitted to the European Molecular Biology Laboratory (EMBL) database under the accession numbers AM950203 to AM950227, and AM980833 to AM980863.

Isolates available as soil inoculum were extracted, amplified and analysed by RFLP or sequencing at least twice (Table 1).

2.3.8 Phylogenetic analyses

Phylogenetic trees were inferred using distance, parsimony or maximum likelihood criteria as implemented in PAUP*. Neighbor joining or heuristic search algorithms were applied for the respective criteria. Maximum likelihood models and parameters were estimated using Modeltest 3.5 (Posada 2004). In addition, Bayesian analyses were performed using MrBayes 3.1.1 for Macintosh (Ronquist & Huelsenbeck 2003).

Insertions and deletions in the introns were coded by appending binary characters (1 for deletion, 2 for insertion) to the sequence matrix. Each deletion of more than three bases was coded, resulting in 21 binary characters added to the whole dataset. Regions of exons and introns that could not be aligned unambiguously were excluded from the analyses. Phylogenetic networks were

obtained using SplitsTree 4.8 (Huson & Bryant 2006). The Neighbor Net option using uncorrected distances and equal angles was chosen.

Table 3 Sequencing primer sets used for *Glomus intraradices* isolates and root colonizing *G. intraradices* (shaded in grey) which are different from the sequencing set for JJ291 (Raab *et al.* 2005). F (M13fwd), R (M13rev).

Origin isolate or plant sample	Accession Number	Sequencing RNL-primers	
		fwd	rev
CC-4	AM950204	1, 16, 2, 12b, 24, 25	5, 26, 27, 7b, 15, 14
	AM950205		
	AM950206		
CR316A	AM950207	F, 16, 35, 12, 37, 24, 25	R, 26, 27, 7, 7b, 38, 36
AU212B	AM950214		
JJ141	AM950208*	F, 11, 13d, 16, 35, 37, 46, 44, 53, 55	R, 33, 15, 14, 39, 43, 40, 30, 52, 54, 27, 5
JJ145	AM950209*		
JJ183	AM950210	1, 11, 13d, 16, 2, 12	5, 7b, 15, 14, 17, 10
	AM950211		
	AM950212		5, 7b, 15, 14, 17h, 10h
DD-4	AM950213	F, 11b, 2, 12, 24, 44, 25	R, 26, 27, 41, 15, 36, 39
JA202	AM950215	F, 16, 35, 12, 37	R, 33, 15, 36
KE114	AM950216	F, 16, 12, 37	R, 33, 15
NB102C	AM950217	F, 11, 13, 16, 35, 37, 24, 25	R, 26, 27, 7, 38, 36, 17, 10h
CA502	AM950218	F, 11, 16, 35, 12, 37, 24, 63	R, 59, 27, 7, 15, 62, 64
VA110	AM950219		
FL208A	AM950220	F, 58, 16, 60	R, 57, 15, 61
Plantago lanceolata da4	AM950221	F, 11b, 2c, 12, 24, 25	R, 26, 27, 7, 15, 36, 39
Trifolium sp. S6	AM950223	F, 11b, 16, 35, 37	R, 33, 15, 36b, 39
	AM950222		R, 33, 15, 36, 39
Trifolium sp. S10	AM950224		
	AM950225		
Plantago lanceolata da2	AM950226	F, 2c, 12, 24, 42, 25	R, 26, 27, 41, 15, 36
	AM950227	F, 11, 13d, 16, 12, 24, 42, 25	R, 26, 27, 41, 15, 45, 51

* sequence was composed from cloned and directly sequenced PCR products (see Table 1)

2.3.9 RFLP analyses

Based on virtual restriction patterns of the sequence data of the mtLSU rDNA, a RFLP system was established in order to distinguish different sequence types. For RFLP analyses, 20 U *Dra*III, 2.5 U *Bsa*JI from New England Biolabs (BioConcept, Allschwil, Switzerland) and 2.5 U *Hin*dIII from MBI Fermentas (LabForce, Nunningen, Switzerland) were used per sample, respectively. For each reaction 8 μl of the final nested PCR products were digested overnight at 37°C (*Bsa*JI samples at 60°C) in a total volume of 15 μl. For visualization, 1.5% agarose gels (1% SeaKem-0.5% NuSieve; Cambrex Bio Science, Rockland, ME) were loaded with the total volume of the digestion products and run at 100 V for 1 h. Fragment lengths were determined using Quantity One (version 4.1.0).

The RFLP patterns were compared with the virtual patterns using a modified spreadsheet developed by Dickie *et al.* (2003).

2.4 Results

2.4.1 Diversity of ITS sequences and mtLSU haplotypes in *G. intraradices* isolates

Sixteen isolates of *G. intraradices* originating from geographic locations on five continents were analysed. These isolates included two strains (JJ291 and BEG75) previously studied by Raab *et al*. (2005). In ITS-based phylogenies (Fig. 5), most isolates grouped into a clade that included sequences originating from a single spore of isolate JJ291. This group is designated as '*G. intraradices* main clade' because members have been used to genetically define the species in field studies (Hijri *et al.* 2006; Sýkorová *et al.* 2007a). Isolate FL208A from Florida (USA), showed a closer relationship to *G. proliferum*, clustering with sequences from the same isolate previously obtained by another group (P. Sudarshana *et al*., unpublished), and with sequences from isolates VA110 and KS906 from Virginia and Kansas (USA), respectively. Isolate VA110 also was analysed in the present study, but the resulting sequences did not cluster with FL208A. Instead, it showed a close relationship with CA502 from California (USA). The cluster containing VA110 and CA502 showed a tendency to group outside the *G. intraradices* main clade in distance and parsimony analyses, but this was not supported by bootstrap values or in Bayesian analyses. Sequences AJ872051 and AJ872052 constitute a clade of environmental sequences (Hijri *et al.* 2006) which is a clearly separated sister group to the *G. intraradices* main clade. Generally, bootstrap and posterior probability values were relatively low, which may be caused by a very strict alignment, which left only 300 bp for analyses. Nevertheless, the topology of the tree was highly consistent between analyses. Omitting the VA110/CA502 sequences raised the bootstrap value of the *G. intraradices* main clade, indicating that unresolved sister clades may deteriorate the support for the main clade. To confirm that ITS and mtLSU sequence data originate from the same fungal genotype, sequences were amplified from the same spore for several isolates (Table 1).

Among the 16 cultivated isolates, 12 mitochondrial haplotypes were distinguished (Table 4). An additional five haplotypes were identified in five root samples. The exon–intron structure of the gene region among the isolates is graphically depicted in Fig. 6. The length of the analysed region of the mtLSU varied between 1070 bp and 3935 bp among isolates because of the presence/absence of introns at three locations, and considerable length variation within introns. One isolate, KE114 from Kenya, did not contain any of the three introns. Isolate DAOM197198 from Canada, which is used in the genome sequencing project, grouped with isolate JJ291 from Switzerland in haplotype I. Two additional haplotypes were found in isolates JJ183, JJ141 and JJ145, which originated from

the same field site as JJ291 in Switzerland. In addition to DAOM197198/JJ291, three pairs of isolates showed the same haplotypes, respectively (JJ141/JJ145, VA110/CA502, CC-4/CR316A).

Sequence polymorphism of the introns correlated with length polymorphism. In other words, variation in intron lengths mirrored differences in intron sequences (Fig. 6, Table 4).

In order to further confirm sequence homogeneity of the mtLSU rDNA within the same isolate (Raab *et al.* 2005), at least three cloned PCR products obtained from two different ROC plates were sequenced for each of the isolates CC-4, JJ183 and DAOM197198. The clones (see Tables 1 and 4) differed from the consensus sequence on average by 0.35% (CC-4) 0.24% (JJ183) and 0.28% (DAOM197198), which is within the range of the misincorporation error of *Taq* polymerase (Cline *et al.* 1996).
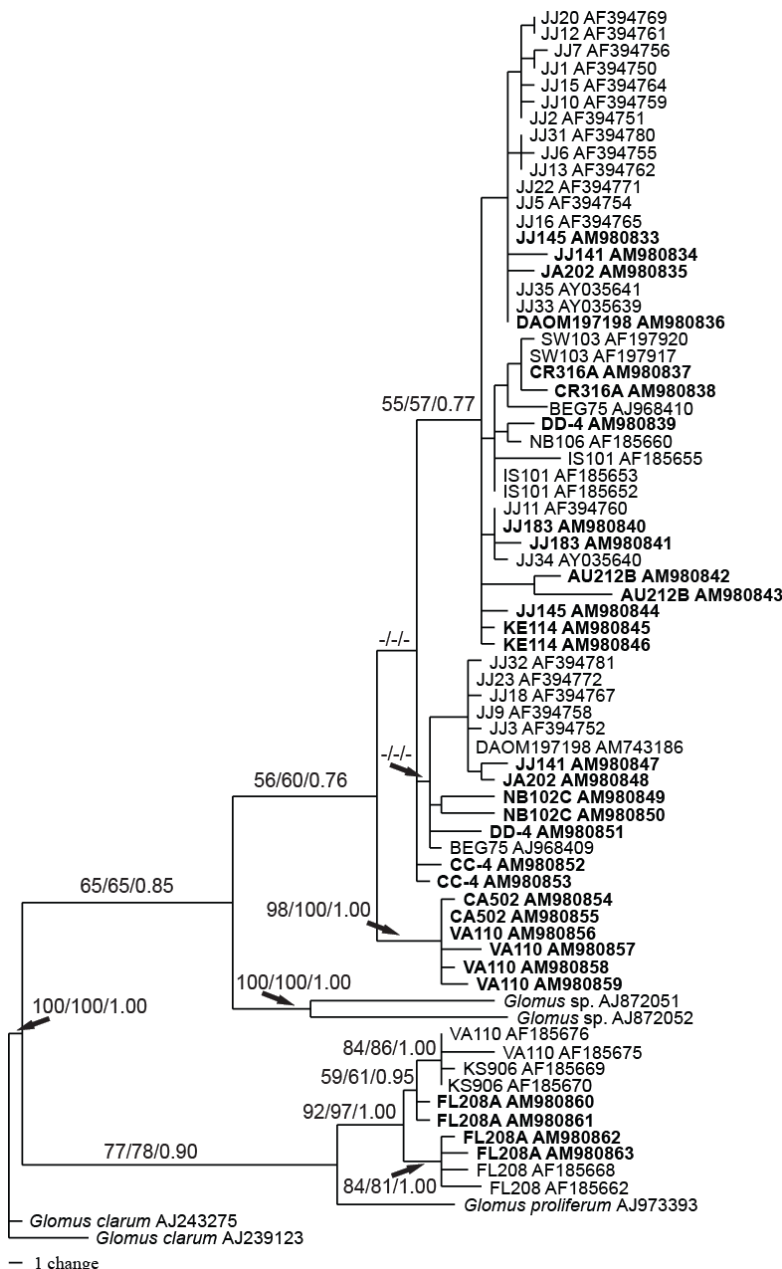
Fig. 5 Phylogenetic tree of *Glomus intraradices* isolates based on 5.8S rDNA and ITS2 sequences, with *Glomus clarum* as outgroup. The sequences JJ1 to JJ32 originated from a single-spore culture of *G. intraradices* JJ291 (Jansa *et al.* 2002b). The phylogenetic tree was generated following alignment of 300 characters and a heuristic search under the maximum parsimony criterion. Values at the nodes indicate: parsimony bootstrap values from 1000 replicates, neighbor-joining bootstrap values from 1000 replicates and Bayesian posterior probabilities. For clarity of the figure, only the values above 50% for the first five nodes from the root are provided. Sequences in bold type were unique to this study.

Table 4 Mitochondrial rRNA large subunit gene (mtLSU) rDNA sequence structure of *Glomus intraradices* isolates and root colonizing *G. intraradices* (shaded) within the priming sites of RNL-29/RNL-30.

Isolate/ plant sample (Origin)	Clones Accession number	Haplotype	Introns Pos. 1 (-type, bp)	Pos. 2 (-type, bp)	Pos. 3 (-type, bp)	Exon region Complete/parts between intron positions (bp)	Fragment length (bp)
DAOM-197198	AJ841804	I	1-1, 1057	2-1, 401	no	1073/363, 672, 38	2531
(Canada)	AJ841808**		1-1, 1058	2-1, 401	no	1063/363, 670, 30	2522
	AM950203		1-1, 1057	2-1, 401	no	1071/363, 670, 38	2529
JJ291* (Switzerland)	AJ973189		1-1, 1056	2-1, 401	no	1071/363, 670, 38	2528
	AJ973190		1-1, 1056	2-1, 401	no	1071/363, 670, 38	2528
	AJ973192		1-1, 1057	2-1, 401	no	1071/363, 670, 38	2529
CC-4 (Canada)	AM950204	II	no	2-2, 415	3-1, 809	1071/363, 670, 38	2295
	AM950205		no	2-2, 415	3-1, 808	1071/363, 670, 38	2294
	AM950206		no	2-2, 415	3-1, 809	1071/363, 670, 38	2295
CR316A (Costa Rica)	AM950207		no	2-2, 415	3-1, 809	1071/363, 670, 38	2295
BEG75* (Switzerland)	AJ938171	III	no	2-2, 414	no	1071/363, 670, 38	1485
	AJ938173		no	2-2, 414	no	1071/363, 670, 38	1485
	AM040984		no	2-2, 414	no	1071/363, 670, 38	1485
JJ141 (Switzerland)	AM950208	IV	1-2, 1107	2-1, 401	3-2, 1356	1071/363, 670, 38	3935
JJ145 (Switzerland)	AM950209		1-2, 1107	2-1, 401	3-2, 1356	1070/363, 669, 38	3934
JJ183 (Switzerland)	AM950210	V	1-3, 1094	2-3, 389	no	1071/363, 670, 38	2554
	AM950211		1-3, 1094	2-3, 389	no	1071/363, 670, 38	2554
	AM950212		1-3, 1094	2-3, 389	no	1071/363, 670, 38	2554
DD-4 (Netherlands)	AM950213	VI	1-4, 425	2-4, 303	3-3, 944	1067/363, 666, 38	2739
AU212B (Australia)	AM950214	VII	no	2-5, 430	3-4, 850	1071/363, 670, 38	2351
JA202 (Japan)	AM950215	VIII	no	2-1, 401	no	1071/363, 670, 38	1472
KE114 (Kenya)	AM950216	IX	no	no	no	1070/363, 670, 37	1070
NB102C (Namibia)	AM950217	X	1-1, 1057	2-6, 339	3-1, 809	1071/363, 670, 38	3276
CA502 (California)	AM950218	XI	1-5, 489	2-7, 233	3-5, 667	1087/377, 672, 38	2476
VA110 (Virginia)	AM950219		1-5, 489	2-7, 233	3-5, 667	1088/377, 673, 38	2477
FL208A (Florida)	AM950220	XII	1-6, 662	no	no	1124/358, 728, 38	1786
Plantago lanceolata da4 (Hungary)	AM950221	XIII	1-7, 444	2-4, 303	3-6, 739	1067/363, 666, 38	2553
Trifolium sp. S6 (Switzerland)	AM950222	XIV	1-7, 444	2-4, 303	no	1067/363, 666, 38	1814
	AM950223		1-7, 444	2-4, 303	no	1067/363, 666, 38	1814
Trifolium sp. S10 (Switzerland)	AM950224		1-7, 445	2-4, 303	no	1067/363, 666, 38	1815
	AM950225		1-7, 444	2-4, 303	no	1067/363, 666, 38	1814
Plantago lanceolata da2 (Hungary)	AM950226	XV	no	2-4, 303	3-3, 944	1068/363, 667, 38	2315
	AM950227	XVI	1-3, 1094	no	3-7, 938	1067/363, 666, 38	3099
*Festuca pratensis** (Switzerland)	AJ841288	XVII	1-8, 1108	no	no	1069/366, 665, 38	2177
	AJ841289		1-8, 1108	no	no	1068/366, 664, 38	2176

Haplotypes and intron types were distinguished by sequence differences. Introns containing putative open reading frames for LAGLIDADG are shaded.
* Raab *et al* (2005); ** sequence incomplete at 3′-end.

2.4.2 MtLSU exon and intron phylogeny

The MtLSU exon sequences clearly separated the FL208A isolate from all other *G. intraradices* isolates in the phylogenetic tree (Fig. 7), a pattern that was in agreement with ITS phylogeny. Isolate FL208A grouped closer to *G. proliferum*. Isolates CA502/VA110 and two sequences obtained from colonized roots of *Festuca pratensis* in a calcareous grassland (Raab *et al*. 2005)

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also grouped in clades with high bootstrap support. The exon and intron sequences of CA502 and VA110 did not differ by more substitutions than expected from *Taq* polymerase error.

All other *G. intraradices* isolates were quite similar in their mtLSU exon sequences (Fig. 7). A subclade containing mostly environmental sequences from grasslands received some bootstrap support in the exon tree (Fig. 7) and was also distinct in intron length (Fig. 6) and sequence (Table 4). Position 2 introns all were 303 bp in length and differed by only a few point mutations. All position 1 introns in this subclade were 425–444 bp long and lacked an open reading frame (ORF) for a homing endonuclease, which was detected in other isolates (see below).

Overall, sequences of the position 2 intron showed considerable similarity. This intron was present in 14 of 16 isolates and 6 of 9 environmental sequences. Phylogenetic analysis of the position 2 intron (Fig. 8) confirmed trends obtained from exon sequences. NeighborNet networks were used to provide more detailed visualization of any potential conflicts between intron phylogenies that might be caused by reticulate evolution. Four major groups of isolates were distinguished: the group of predominantly environmental sequences discussed above; a clade comprising all Tänikon isolates, DAOM197198 and JA202; a clade comprising BEG75, CC4 and CR316; and CA502/VA110. Some isolates did not fall into any of these groups, such as NB102C and AU212B. Isolate NB102C was not positioned on a distinct branch in the NeighborNet network in Fig. 8, possibly because the sequence region distinguishing the BEG75 and Tänikon isolate group was missing in this isolate.

A position 1 intron occurred in 10 of 16 isolates and 8 of 9 environmental sequences. A number of sequences containing this intron contained ORFs for homing endonucleases of the LAGLIDADG 2 type (Dalgaard *et al.* 1997). Furthermore, a LAGLIDADG type 1 ORF was found in isolates JJ141 and 145 in a position 3 intron. The position 2 intron did not contain any putative ORFs. Interestingly, some ORFs consisted of two regions separated by a putative noncoding sequence (Fig. 6). Phylogenetic analysis of position 1 intron sequences (Fig. 9) indicated that they were homologous. Some isolate groups described above were verified and the group comprising the Tänikon isolates and DAOM197198 was differentiated further into subgroups. Isolate NB102C associated closely with DAOM197198/JJ291, clarifying its ambiguous grouping in Fig. 8.

The position 3 intron was present in only 56% of the isolates. Although highly polymorphic in the central region, sequences of this intron were homologous in regions adjacent to the exons. In JJ141/JJ145, this intron contained an ORF for a type 1 homing endonuclease. Conflicts among exon and intron phylogenies were not detected, ruling out frequent transfer of these noncoding regions that could impair their use as intraspecific molecular markers.
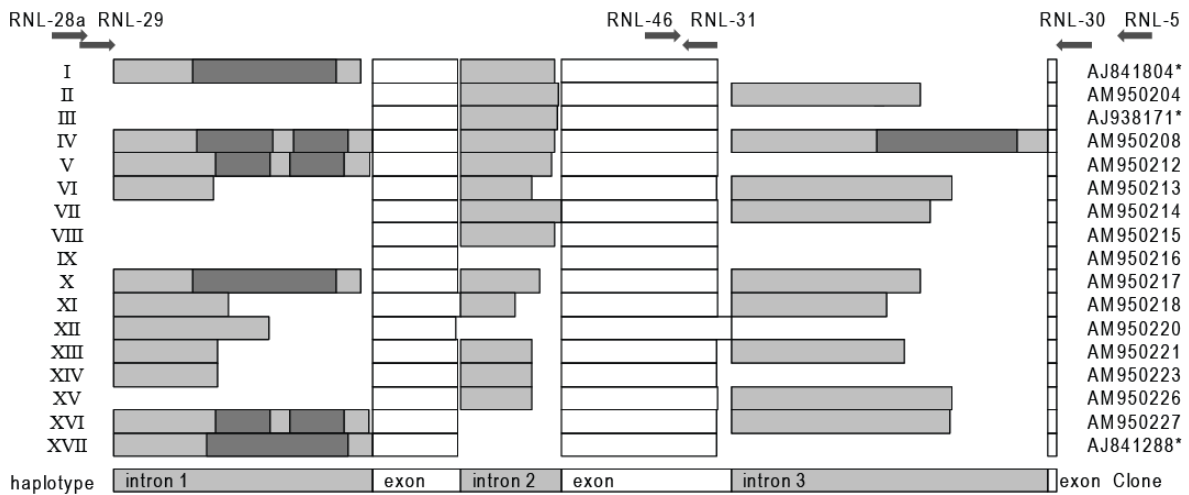
Fig. 6 Organization of the mitochondrial rRNA large subunit gene (mtLSU) region containing three exons and two to three introns for *Glomus intraradices* haplotypes I–XVII in 5′–3′ orientation. Introns are shaded in light grey and putative LAGLIDADG open reading frames (ORFs) in dark grey. Arrows show location and orientation of primers. *, Clones from Raab *et al.* (2005). Approximately to scale.
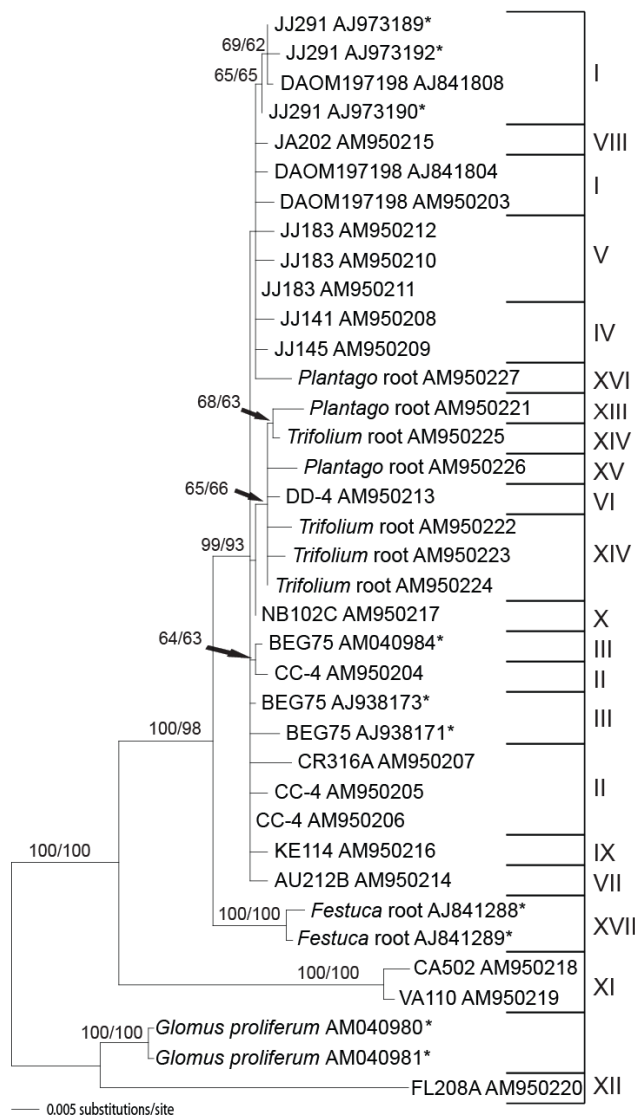
Fig. 7 Phylogeny of *Glomus intraradices* isolates and *Glomus proliferum* based on mitochondrial rRNA large subunit gene (mtLSU) exon sequences. The tree was rooted by midpoint rooting. Roman numerals indicate mtLSU haplotypes. The phylogenetic tree was obtained from 943 characters using a heuristic search under the maximum likelihood criterion. Values on the nodes indicate: neighbor-joining bootstrap values from 1000 replicates and maximum parsimony bootstrap values from 1000 replicates. Sequences from cultured isolates of *G. intraradices* are labeled with isolate codes and accession numbers. For sequences from roots, host species and accession number are indicated. *, Sequences from Raab *et al.* (2005).

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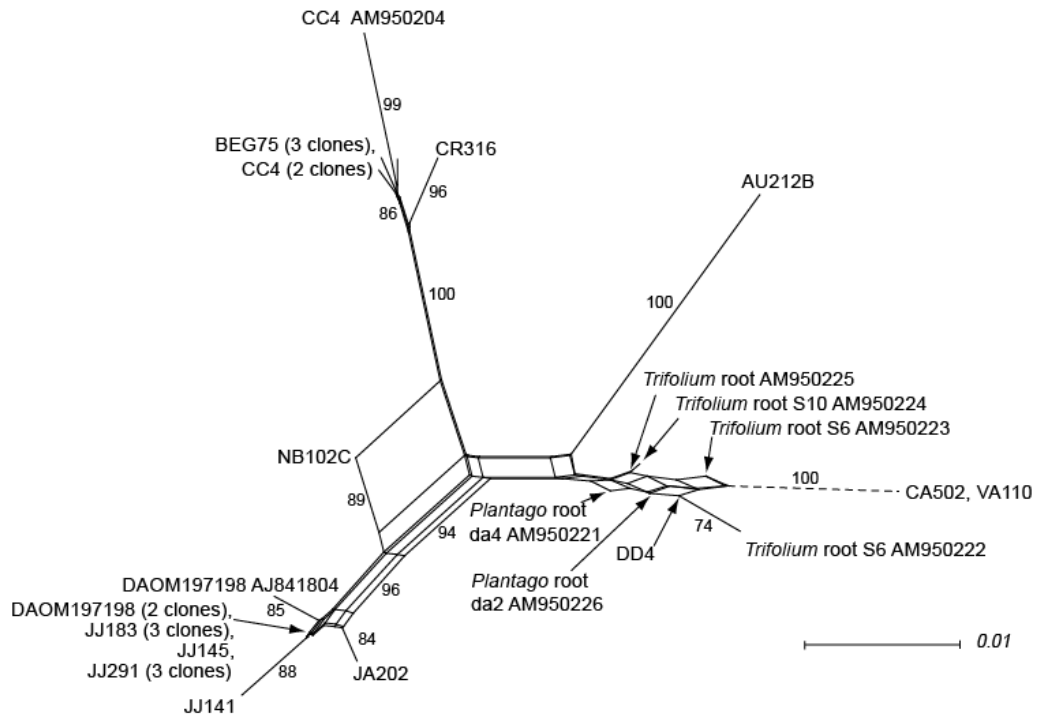
Fig. 8 NeighborNet network obtained from sequences of the position 2 intron. The dashed line indicates a branch that was reduced in length by a factor of 10 to improve readability of the figure. Numbers on the branches are bootstrap values from 1000 replications.
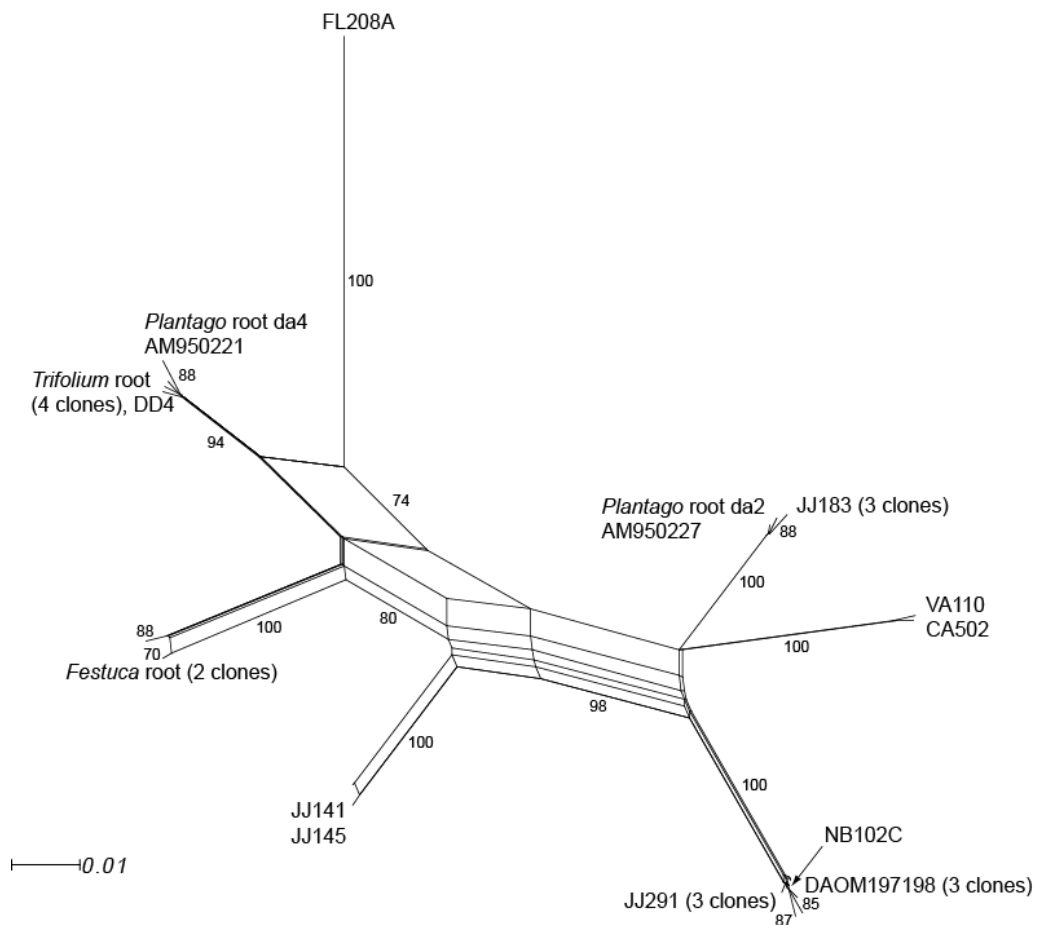
Fig. 9: NeighborNet network obtained from sequences of the position 1 intron. Numbers on the branches are bootstrap values from 1000 replications.

2.4.3 Intron stability in the mtLSU of *G. intraradices*

Comparisons of multiple culture lineages of the same isolates did not reveal any changes in the intron length or sequence of any isolate. For example, two lineages of isolate DAOM197198 were identical in both exon and intron sequences, even though one was obtained directly from G. Bécard (Toulouse, France) and has been propagated in the Botanical Institute in Basel since 1995 and the other was obtained from N. Requena (Karlsruhe, Germany) in 2005. The same result was obtained for two lineages of JJ291 cultivated independently over a 2-yr period. JJ141 and SW205 are two culture lineages originating from the same isolate and showed identical haplotypes. SW205 was pot cultured repeatedly at INVAM, whereas JJ141 was obtained as root organ culture. Identical haplotypes in populations from geographically distant locations (Switzerland/Canada, Virginia/California, Costa Rica/Canada) provided additional evidence for stability of markers.

2.4.4 Detection of haplotypes in field-collected roots

The mtLSU region could be amplified from field-collected roots using the improved primer combinations RNL-28a/RNL-5 and RNL-29/RNL-30. All amplified sequences clustered in the *G. intraradices* clade (Fig. 7). This result clearly demonstrated high primer specificity for the target clade. Interestingly, grassland isolate DD-4 was the only cultivated genotype clustering in the exon clade, which contained most environmental sequences from grassland communities. Among root samples, only one from *Plantago lanceolata* (sample code da2) yielded sequences of two different haplotypes (XV and XVI, Table 4).

2.4.5 RFLP analyses

From sequence data of the DNA region between primers RNL-29 and RNL-30, a combination of restriction enzyme sites were identified that unambiguously separated mtLSU haplotypes without sequencing (Table 5). A single conserved target site for *Dra*III was present in each mtLSU rDNA sequence. The restriction site was located in the exon region adjacent to the 5′-insertion site of position 2 intron. Restriction sites for *Bsa*JI were more abundantly distributed, but situated mainly nearer the 5′ ends of sequences. Restriction sites for *Hin*dIII are more abundant near the 3′ end of the DNA region.

Use of *Bsa*JI alone distinguished all haplotypes, whereas *Dra*III or *Hin*dIII clearly identified 59% and 71%, respectively, of these haplotypes. To avoid ambiguous identification as a result of

similar restriction patterns, using all three enzymes provided the most accurate assessment of haplotypes.

Table 5 Restriction fragments lengths of mtLSU rDNA sequences calculated for sequenced clones from *G. intraradices* isolates and mycorrhizal roots, based on the target sites of the restriction enzymes *Dra*III, *Bsa*JI and *Hin*dIII. The ends of all complete fragment lengths are defined by the priming sites for RNL-29 and RNL-30. Different RFLP-subtypes ("Intra-types") of the same haplotype are indicated by lower case letters. Subtypes b and c were most likely caused by minor sequencing errors, as they were only found in clones, but never detected in original PCR products.

Clones Accession number	Haplo-type	Intra-type	Complete fragment length (bp)	*Dra*III 5'...CACNNN▼GTG...3'	*Bsa*JI 5'...C▼CNNGG...3'	*Hin*dIII 5'...A▼AGCTT...3'
AJ841804	I	1	2571	1430, 1141	105, 1175, 1291	2069, 502
AJ841808**			2542	1431, 1111	105, 1176, 1261	2068, 474
AM950203			2569	1430, 1139	105, 1175, 1289	2067, 502
AJ973189*			2568	1429, 1139	105, 1174, 1289	2066, 502
AJ973190*			2568	1429, 1139	105, 1174, 1289	2066, 502
AJ973192*			2569	1430, 1139	105, 1175, 1289	2067, 502
AM950205	II	2a	2334	373, 1961	223, 438, 1673	1024, 508, 584, 218
AM950206			2335	373, 1962	223, 438, 1674	1024, 508, 585, 218
AM950204		2b	2335	373, 1962	44, 179, 438, 1674	1024, 508, 585, 218
AM950207		2c	2335	373, 1962	223, 438, 1674	1532, 585, 218
AJ938171*	III	3a	1525	373, 1152	223, 438, 864	1023, 502
AJ938173*			1525	373, 1152	223, 438, 864	1023, 502
AM040984*		3b	1525	373, 1152	44, 179, 438, 864	1023, 502
AM950208	IV	4	3975	1480, 2495	106, 1224, 2645	2117, 508, 459, 686, 205
AM950209			3974	1480, 2494	106, 1224, 2644	2116, 508, 459, 686, 205
AM950210	V	5a	2594	1467, 1127	106, 1211, 448, 829	2092, 502
AM950211			2594	1467, 1127	106, 1211, 448, 829	2092, 502
AM950212		5b	2594	1467, 1127	106, 1659, 829	2092, 502
AM950213	VI	6	2779	798, 1981	107, 541, 1224, 907	1337, 709, 733
AM950214	VII	7	2391	373, 2018	223, 2168	1039, 508, 626, 218
AM950215	VIII	8	1512	373, 1139	223, 1289	1010, 502
AM950216	IX	9	1110	373, 737	223, 887	609, 501
AM950217	X	10	3316	1430, 1886	105, 1175, 2036	2005, 508, 585, 218
AM950218	XI	11a	2516	877, 1639	106, 621, 1789	75, 1282, 498, 661
AM950219		11b	2517	877, 1640	106, 621, 1790	75, 1283, 1159
AM950220	XII	12	1826	1030, 796	105, 1721	433, 100, 710, 204, 379
AM950221	XIII	13	2593	817, 1776	107, 560, 1926	1356, 504, 733
AM950222	XIV	14	1854	817, 1037	107, 560, 1187	1356, 498
AM950223			1854	817, 1037	107, 560, 1187	1356, 498
AM950224			1855	818, 1037	107, 561, 1187	1357, 498
AM950225			1854	817, 1037	107, 560, 1187	1356, 498
AM950226	XV	15	2355	373, 1982	223, 1225, 907	913, 709, 733
AM950227	XVI	16	3139	1467, 1672	106, 1211, 921, 901	1703, 703, 733
AJ841288*	XVII	17	2217	1484, 733	106, 210, 1018, 883	609, 1111, 497
AJ841289*			2216	1484, 732	106, 210, 1018, 882	609, 1111, 496

* Raab *et al*. (2005), ** Sequence incomplete at 3'-end.

This RFLP approach was applied successfully to all isolates from INVAM, isolate DD-4 and all of the other sample types in this study (see Table 1, selected examples in Fig. 10). RFLP patterns were diagnostic for all of the haplotypes present in this study.
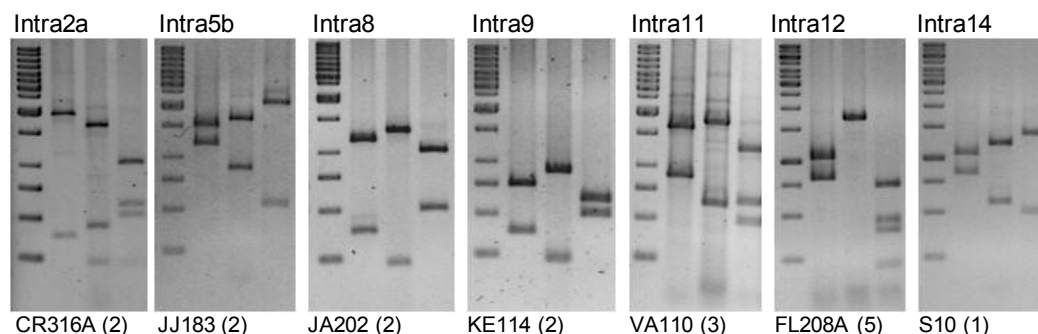
Fig. 10 Banding patterns of selected *Glomus intraradices* restriction fragment length polymorphism (RFLP) types. Respective isolate/root sample and polymerase chain reaction (PCR) product ID (in brackets) are shown below the pictures (for details see Table 1). The PCR products of the mitochondrial rRNA large subunit gene (mtLSU) rDNA generally were amplified using the primer pair RNL-29/30 in the second PCR step. RNL-1/5 was used for isolate JJ183, the only example of a cloned PCR product. DNA was digested with restriction enzymes *Dra*III, *Bsa*JI, *Hin*dIII and loaded in the same order onto gels. DNA ladder in left-most lane of each gel (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000.

2.5 Discussion

We showed in this study that intraspecific groups within *G. intraradices* were resolvable by variation and organization in a region of the mtLSU gene. Both exons and introns of this region provided stable molecular markers to identify haplotypes of this species from spores, colonized roots from field sites and even root fragments and mycelium mixed with organic matter obtained by sucrose extraction from pot cultures.

The AFLP markers were used initially to genetically differentiate intraspecific groups in *G. intraradices* (Koch *et al.* 2004), but they had limitations in that markers were nonspecific and could be analysed only using pure DNA from the target organism. DNA from microorganisms present associated with potcultured or field-collected AMF spores could confound AFLP results, so root organ cultures (Bécard & Fortin 1988) provided the means to obtain contamination-free DNA. *Glomus intraradices* was one of a limited range of AMF species compatible with this culture environment.

Recently, microsatellite simple sequence repeats (SSR) were developed for *G. intraradices* (Croll *et al.* 2008; Mathimaran *et al.* 2008a). Five of the *G. intraradices* isolates resolved as five mtLSU haplotypes in this study were also resolved as distinct genotypes by SSRs (Mathimaran *et al.* 2008a). The *G. intraradices* isolates from the Tänikon field site used in the present study allow the comparison with the multilocus genotypes of Croll *et al.* (2008), because some isolates were

used in both studies. Based on the introns in positions 1 and 2, Croll *et al.* (2008) distinguished the same three mitochondrial haplotypes (JJ291, JJ145/141 and JJ183). In addition, JJ145 and JJ141 could be distinguished by a single base pair length difference of one SSR locus. JJ291 and DAOM197198 were also differentiated by SSR data. These authors, like Raab *et al.* (2005), were not aware of the position 3 intron present in JJ141 and JJ145, which offers additional resolving power (e.g. among BEG75 and CC-4).

It is not yet clear if microsatellite SSR variation (Croll *et al.* 2008) will elucidate the whole range of intraspecific diversity characterized by the mtLSU locus, because primers specific to *G. intraradices* genotypes were designed exclusively from isolates in root organ cultures, most of them originating from the Tänikon field site. Generally, multilocus population analyses from environmental samples face the problem that genotypes from different loci cannot be linked to each other if more than one target organism occurs in a sample. Mathimaran *et al.* (2008a) showed that SSR markers could be applied to measuring fungal diversity in colonized roots, but not yet under field conditions. Specificity must be tested exhaustively to exclude amplified products from plants and other associated microorganisms. Despite these potential problems, comparisons of both genotyping systems in more detail in future studies will offer new and intriguing insights.

Based on a greater breadth of isolate sampling, mtLSU data clearly show that intraspecific diversity within *G. intraradices* is considerably higher than previously reported. Croll *et al.* (2008) reported that genotypic diversity in isolates from the Tänikon site was higher than that characterized in other isolates from Switzerland and one isolate from Canada (DAOM197198). Based on these comparisons, they concluded that intraspecific variation in *G. intraradices* is more diverse locally than globally, a hypothesis also put forward by Koch *et al.* (2004).

The finding that almost every *G. intraradices* isolate we sampled from a broad geographical range constitutes a different mtLSU haplotype certainly is surprising. Among 16 cultured fungal isolates, 75% comprised distinct haplotypes. Moreover, unique haplotypes were identified in most of the root samples analysed. Conversely, evidence also suggests that some haplotypes are distributed over a very broad geographical distribution, the most striking of them being haplotype I. Considerable effort, including sampling of multiple isolates from each of a number of field sites around the world, will be needed to obtain a comprehensive overview of local versus pandemically distributed haplotypes in *G. intraradices*.

In contrast to SSR or AFLP fingerprinting methods, mtLSU sequences can be analysed phylogenetically, providing insights into the evolutionary relationships of the isolates and allowing to confirm the origin of the sequences from the target taxon.

Phylogenetic resolution within the *G. intraradices* clade was higher with mtLSU exon than ITS sequence data. The clade comprising sequences JJ1–JJ32 in the ITS trees has been used as a molecular grouping criterion for *G. intraradices* in field studies (Sýkorová *et al.* 2007a). Moreover, this clade also contains the isolate currently being sequenced to represent the genome of

G. intraradices (Martin *et al.* 2004). MtLSU exon data clearly distinguish well-separated lineages within this group, among them the CA502/VA110 lineage, which is present in the ITS tree but its separation from the 'main clade' is not supported by bootstrap analysis.

A second clade of *G. intraradices* isolates can be distinguished in the ITS-5.8S phylogeny constructed in this study. It comprises isolates FL208, VA110 and KS906 grouping with *G. proliferum*. Some of these sequences have been present in the public database for years (e.g. AF185662, AF185668, AF185669, AF185670, AF185675 and AF185676 in Fig. 5). We confirmed that isolate FL208 groups in this clade, but even after repeated sequencing of multiple clones, ITS sequences from VA110 spores we obtained did not group with FL208A. Instead, its ITS and mtLSU sequences consistently grouped with CA502. The reason for this remains unclear. In any case, the mtLSU exon phylogeny confirms the genetic distance of FL208A from the other *G. intraradices* isolates. Based on unequivocal evidence from the ITS phylogeny, where a true outgroup could be used, the root of the tree is located between FL208/*G. proliferum* and the remaining taxa, which is consistent with the mtLSU exon phylogeny rooted by midpoint rooting (Fig. 7).

With its taxonomic resolution superior to ITS, the mtLSU exon region will be a useful molecular marker to contribute to a taxonomic consolidation of *G. intraradices*. Data from this and other gene regions will expand discovery of other isolates and clarify their interrelationships and distribution.

Fungal lineages generally are thought to lose introns more quickly rather than to gain them (Goddard & Burt 1999). The complete absence of mtLSU introns in the KE114 isolate of *G. intraradice*s, and in *G. proliferum* may therefore represent a derived condition. The hypothesis that the introns were inserted as independent events in all other isolates is not parsimonious. Introns in similar positions (1 and 2) have also been found in the more distantly related isolates of *G. mosseae* (Thiéry *et al.* 2010; see appendix). Possibly, these introns evolved in an ancestor common to both lineages. As in the study by Raab *et al*. (2005), ORFs coding for putative homing endonucleases were detected in some of the introns. Most of them were present in position 1 introns but an endonuclease ORF was also found in the position 3 intron. Homing endonucleases catalyse the spread of the intron-containing allele that encodes them to other intron-less alleles (Dalgaard *et al.* 1997).

Evidence has been accumulating that not all symbiotically active AMF necessarily sporulate in field settings (Hempel *et al.* 2007). The frequently used 'trap culturing' approach to propagate AMF from field samples in order to obtain spores for morphological analyses may bias the range of species-level taxa detected (Sýkorová *et al.* 2007a). A similar bias may be expected among isolates of a species within a population. Thus, it is highly desirable to develop specific molecular tools as culture-independent techniques to analyse intraspecific genetic diversity of glomeromycotan fungi directly within mycorrhizal roots. The possibility of amplifying and then characterizing

glomeromycotan fungi that do not show evidence of sporulation in a field setting (Rosendahl & Stukenbrock 2004) and therefore are not culturable is a significant asset of mtLSU primers. MtLSU markers targeting higher-level phylogenetic taxa can be developed to easily obtain sequence data from other species. By contrast, design of primers to characterize SSR loci in isolates of other species will require considerably more genomic information.

As the RFLP method to detect haplotype variation within *G. intraradices* was tested using a worldwide sampling of isolates, it can be expected to be applicable to the whole range of diversity found in this species. No laborious cloning steps are necessary to analyse PCR products, and direct sequencing is possible unless several haplotypes are present in the same root. In some cases sequencing may still be useful to obtain additional information about the haplotypes present or to confirm the RFLP results. Such an approach will facilitate sampling over a broader geographic range and more diverse habitats. Biogeographic patterns can be elucidated and the role of human intervention can be examined in such studies, and the hypothesis that *G. intraradices* is a true generalist can be tested. Moreover, it will be interesting to determine whether some of the mtLSU haplotypes or haplotype groups correspond to ecotypes and how these correlate with degree of culturability.

2.6 Acknowledgements

Chapter 3: Diversity of mitochondrial large subunit rDNA haplotypes of *Glomus intraradices* in two agricultural field experiments and two semi-natural grasslands

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Fig. 1 Tänikon, field experiment
(Photo by Dirk Redecker)

Fig. 2 Frick, field experiment
(Photo by Thomas Alföldi)

Fig. 3 Landskron, grassland

Fig. 4 Ramosch, grassland
(Photo by Dirk Redecker)

3.1 Abstract

Glomus intraradices, an arbuscular mycorrhizal fungus (AMF), is frequently found in a surprisingly wide range of ecosystems all over the world. It is used as model organism for AMF and its genome is being sequenced. Despite the ecological importance of AMF, little has been known about their population structure, because no adequate molecular markers have been available. In the present study we analyse for the first time the intraspecific genetic structure of an AMF directly from colonized roots in the field. A recently developed PCR-RFLP approach for the mitochondrial rRNA large subunit gene (mtLSU) of these obligate symbionts was used and complemented by sequencing and primers specific for a particularly frequent mtLSU haplotype. We analysed root samples from two agricultural field experiments in Switzerland and two semi-natural grasslands in France and Switzerland. RFLP type composition of *G. intraradices* (phylogroup GLOM A-1) differed strongly between agricultural and semi-natural sites and the *G. intraradices* populations of the two agricultural sites were significantly differentiated. RFLP type richness was higher in the agricultural sites compared with the grasslands. Detailed sequence analyses which resolved multiple sequence haplotypes within some RFLP types even revealed that there was no overlap of haplotypes among any of the study sites except between the two grasslands. Our results demonstrate a surprisingly high differentiation among semi-natural and agricultural field sites for *G. intraradices*. These findings will have major implications on our views of processes of adaptation and specialization in these plant ⁄ fungus associations.

Keywords: agriculture, genetic differentiation, *Glomus intraradices*, mutualism, mycorrhiza, population structure

3.2 Introduction

Arbuscular mycorrhizal fungi (AMF) form a mutualistic symbiosis with the majority of land plants. AMF are placed in the Glomeromycota, a fungal phylum established by Schüßler *et al.* (2001). In this symbiosis, the plant provides carbohydrates for the fungus, which in turn makes nutrients such as phosphate or nitrogen available to the phytobiont (Smith & Read 2008). It has been shown that underground diversity and species composition of AMF affect plant diversity and plant community composition above ground (van der Heijden *et al.* 1998b). One of the most widespread species of AMF is *Glomus intraradices*, which has been detected in almost all studies of AMF diversity in field settings.

A recent study by Stockinger *et al.* (2009) has highlighted the need to redefine this species based on molecular phylogeny and morphology, because the type strain from Florida (FL208), which has apparently not been detected in any environmental study so far, and the ubiquitous

fungal clade appear to be distinct. Until this dilemma is resolved in nomenclature, we refer to this widely-known clade as "*G. intraradices* GLOM A-1", in concordance with our own field studies based on nuclear-encoded rRNA genes (e.g. Hijri *et al.* 2006; Sýkorová *et al.* 2007b). Furthermore, we use the term '*G. intraradices* sensu lato' to comprise all clades previously identified as *G. intraradices* based on spore morphology (see chapter 2).

Glomus intraradices GLOM A-1 has been detected in an amazing variety of habitats, like mountain meadows (Börstler *et al.* 2006), calcareous grasslands (Sýkorová *et al.* 2007a), alpine meadows (Sýkorová *et al.* 2007b), high-input and low-input agricultural field sites (Hijri *et al.* 2006), and geothermal sites in Yellowstone (Appoloni *et al.* 2008). It was shown to be a generalist with regard to its host preferences (Sýkorová *et al.* 2007b) and its life history strategy (Sýkorová *et al.* 2007a). *Glomus intraradices* GLOM A-1 is the most frequently-used model organism in AMF research (e.g. Helber & Requena 2008), and the isolate DAOM197198 is currently the subject of the first genome sequencing project of an AMF species (Martin *et al.* 2008). For the isolate FACE#494 the complete mitochondrial genome is available (Lee & Young 2009).

In contrast to phytopathogenic fungi (e.g. Hovmøller *et al.* 2008) or ectomycorrhizal mutualists (e.g. Kretzer *et al.* 2005), the study of the diversity of AMF populations has only been possible since relatively recently when suitable molecular markers became available (Rosendahl 2008). In particular, the polymorphism of nuclear-encoded rRNA genes within the organism and within spores (Sanders *et al.* 1995) had been an obstacle to resolve intraspecies genetic diversity. Stukenbrock & Rosendahl (2005a) conducted a pioneering study on *Glomus* species, widespread in disturbed settings. The same markers (i.e. the nuclear encoded genes *GmFOX2* and *GmTOR2* and a region of nuclear large subunit rDNA) were applied in subsequent studies (Rosendahl & Matzen 2008; Rosendahl *et al.* 2009) of *G. mosseae* and its close relatives *G. geosporum* and *G. caledonium*, but in all of these studies spores of these fungi had to be used. The possible discrepancy between symbiotically active AMF and the presence of their propagules has been known for a long time from molecular studies of AMF species communities (e.g. Clapp *et al.* 1995), and a similar bias may be inevitable when using spores for population studies.

Genotyping methods used in recent studies of a field population of *G. intraradices* even required fungal biomass from root organ cultures (ROCs), which is the only currently known approach to obtain spores and mycelium free of contaminating microorganisms from these obligate biotrophs (Koch *et al.* 2004; Croll *et al.* 2008). Only a few AMF species can be grown in this culture system, demonstrating a strong bias during the cultivation procedure, which may also exclude *G. intraradices* genotypes not well adapted to the artificial culture conditions. Koch *et al.* (2004) used amplified fragment length polymorphism (AFLP) to characterize isolates from tillage and no-tillage treatments of a field experiment in Tänikon (Switzerland). The same set of isolates was analysed by Croll *et al.* (2008) using co-dominant multilocus markers. In this landmark study, the authors demonstrated considerable genetic structure within the population as well as genotype-

level host preferences. By comparing the Tänikon isolates to a genetically similar isolate from Canada, and some isolates from other locations in Switzerland, the authors concluded that the global diversity of *G. intraradices* was low compared with local diversity. Another set of multilocus markers was presented by Mathimaran *et al.* (2008a). Importantly, Croll *et al.* (2009) demonstrated that some of the isolates they could differentiate genetically formed anastomoses (hyphal cross-bridges) and exchanged genetic markers *in vitro*. The application of these multilocus genetic markers in field-collected roots would be possible after developing nested PCR primers based on flanking sequences of the repeat motifs as suggested by Croll *et al.* (2008). However, the flanking sequences are often polymorphic themselves (Mathimaran *et al.* 2008b) and even if primers are successfully designed, the principal problem remains how to correlate the different loci from environmental samples which may contain several genotypes.

Raab *et al.* (2005) and Börstler *et al.* (2008) (see chapter 2) demonstrated that the mitochondrial rRNA large subunit gene (mtLSU) and its introns can be used to distinguish isolates of *G. intraradices*. The mtLSU markers apparently have a resolution lower than multilocus markers (Croll *et al.* 2008). Nevertheless, 12 haplotypes were distinguished among 16 isolates of *G. intraradices* collected worldwide (Börstler *et al.* 2008; see chapter 2). In contrast to the previously mentioned multilocus sequence markers, the mtLSU of *G. intraradices* can be amplified specifically from colonized root samples, offering a less biased view of fungal populations. Furthermore, phylogenetic analysis of the mtLSU exon sequences allows to test whether the organism detected belongs to the target clade. Börstler *et al.* (2008) developed a RFLP approach that simplifies the identification of mtLSU haplotypes, allowing larger numbers of samples to be analysed by avoiding the laborious task of screening libraries of cloned sequences and reducing the amount of sequencing required (see chapter 2).

On the species level, it has been shown that agricultural practice alters AMF community composition in the soil. Many arable soils, in particular in monocropping systems exhibit a reduced AMF diversity compared with natural ecosystems (Helgason *et al.* 1998; Oehl *et al.* 2003; Hijri *et al.* 2006). Nevertheless, it is still not well understood how AMF communities adapt to environmental conditions, in particular due to the striking ubiquity of some species, especially *G. intraradices* GLOM A-1.

Within morphospecies, differences in the production of external hyphae and hyphal phosphorus uptake of *G. mosseae* isolates were reported by Munkvold *et al.* (2004). Distinct growth parameters and effects on plant growth of different isolates of *G. intraradices* originating from Tänikon (see above) were observed by Koch *et al.* (2006). These findings suggest considerable functional differentiation within species, implying the need to study the genetic foundation of these differences and the spatial and temporal dynamics of population diversity.

In this study, we used the mtLSU PCR-RFLP-sequencing approach developed by Börstler *et al.* (2008) (see chapter 2) to investigate intraspecific genetic diversity of *G. intraradices* GLOM A-

1 in field-collected roots. The efficiency of the RFLP approach was verified using a PCR primer set specifically designed for the haplotype I of *G. intraradices*, which comprises most isolates from Tänikon (Croll *et al.* 2008), as well the isolate DAOM197198 from Canada, which is used for genome sequencing.

By analysing the mtLSU haplotype diversity of *G. intraradices* GLOM A-1 in two arable and two grassland field sites in Switzerland and France, we wanted to address the following questions:

- is population-level diversity decreased in arable soils?
- does *G. intraradices* show genetic differentiation between field sites or between different types of agricultural management?
- may the surprising ubiquity of *G. intraradices* be due to previously unresolved intraspecific groups, for example, ecotypes, as preliminary results on a 'grassland clade' had suggested (Börstler *et al.* 2008; see chapter 2)?

The resulting data can be expected to provide a better understanding of how genetic diversity is partitioned in this ecologically important phylum of mutualists, in particular the model species *G. intraradices*, and better hints toward the processes by which this diversity is generated and maintained.

3.3 Materials and methods

3.3.1 Field sites and sampling

The agricultural study sites were the long-term tillage experiment in Tänikon (Switzerland; see Fig. 1), which was established in 1987, and a field experiment in Frick (Switzerland; see Fig. 2), which was established in 2002. For soil properties and other details in Tänikon see Jansa *et al.* (2002a, 2003), for the details in Frick see Berner *et al.* (2008). Both experiments included different treatment plots (six treatments in Tänikon, eight treatments in Frick) organized in a randomized block design in Tänikon or a split plot design in Frick. In both field experiments, all different treatments were replicated four times. Both sites were subject to crop rotation, from which *Zea mays* was sampled. Samples in Tänikon were taken in June 2007 and sampling in Frick was performed in July 2008. In Tänikon, complete root systems of a total of 48 plants were removed using a spade from the following treatment plots: no-tillage, chisel (loosening soil with a wing share chisel to the depth of 25 cm without turning soil upside-down), tillage (ploughed to the depth of 25 cm). In Frick, a total of 32 root cores were taken adjacent to the plants using a soil corer (diameter of 4 cm) to a depth of 10 cm. The treatment plots were: chisel [chisel plough (15 cm)

including a stubble cleaner (5 cm)] and conventional tillage (mouldboard plough, operating at 15-cm depth). Crop residues were not removed in the sampled plots of Tänikon, in which mineral fertilization was applied. The sampled plots in Frick were fertilized with slurry, additional preparations were not applied. A similar sampling design was used in both experiments: four samples were taken at equal distance to each other along a transect crossing the middle of each treatment plot (19 m transects of 19 x 6 m plots in Tänikon, 17 m diagonal transects of 12 x 12 m plots in Frick), resulting in 16 samples per treatment. Samples covered with soil were stored at 4°C immediately after transport. Within the next 4 days, the root systems of each plant sample were rinsed in tap water, fine root pieces were randomly placed in Petri dishes and washed again in distilled water. Finally, aliquots of 50–80 mg (fresh weight) root fragments per sample were frozen in liquid nitrogen and stored at −80°C.

The semi-natural sites were one calcareous grassland next to the Landskron castle (near the village Leymen, Alsace, France; see Fig. 3), and two adjacent sample areas from the alpine region near the village of Ramosch, Engadin region, Switzerland (see Fig. 4). Both semi-natural study sites were mown regularly and were species-rich grasslands. However, plant communities and soil properties differed between Landskron and Ramosch, for details see Sýkorová *et al.* (2007a,b), respectively. Both study sites were previously investigated with regard to their AMF diversity using ITS regions as marker genes by the latter authors. In these studies, sampling was performed in July 2002 and July 2005 in Landskron, in May 2003 in Ramosch. All semi-natural plant samples were removed randomly in soil cores due to a random distribution of different plant species. Sizes of the collection areas were approximately 15 m^2 in Landskron and approximately 40 m^2 and 30 m^2 in Ramosch. Roots were treated using the same procedures as described for the agricultural study sites. In this study, we have chosen DNA extracts that were screened positive for *G. intraradices* GLOM A-1 by Sýkorová *et al.* (2007a,b). In Landskron, *G. intraradices* was detected in the roots of 21 plant samples. Additionally, one *Medicago sativa* root sample originating from a compartment system established with inoculum from Landskron (Sýkorová *et al.* 2007a) was analysed for comparison. In Ramosch, 26 root samples were tested positive for *G. intraradices*, two samples were already analysed for mtLSU haplotypes by Börstler *et al.* (2008) (see chapter 2). The samples from the two Ramosch sampling areas were pooled as Sýkorová *et al.* (2007b) could not observe significant differences of AMF communities between both collecting areas. This approach was confirmed by initial analyses of *G. intraradices* mtLSU haplotypes in the present study. Plant species of the grassland samples are listed in Table 1.

Table 1 Plant species and samples tested positive for *Glomus intraradices* GLOM A-1 by Sýkorová *et al.* (2007a,b) in the grasslands of Landskron and Ramosch, respectively.

Landskron		Ramosch	
Plant species	**Number of root samples**	**Plant species**	**Number of root samples**
Bromus erectus	5	*Gentiana acaulis*	3
Festuca pratensis*	3	*Gentiana verna*	4
Inula salicina	3	*Hieracium hoppeanum*	2
Medicago sativa	5	*Leontodon hispidus*	2
Origanum vulgare	5	Poaceae sp.	2
		Ranunculus montanus	1
		Thymus pulegioides	1
		Trifolium sp.**	11

* not published before, ** two samples previously analyzed for mtLSU haplotypes by Börstler *et al.* (2008); see chapter 2.

An isolate of *G. clarum* (ID code MUCL46238), originating from La Palma (Pinar del Rio, Cuba), was cultivated as ROC and used as outgroup for phylogenetic analyses in this study. For DNA extraction, the medium was dissolved in 10 mM sodium acetate–citrate buffer (pH 6.0) and spores were washed in sterile water according to Doner & Bécard (1991).

3.3.2 DNA extraction

DNA extractions of all plant samples were performed using the DNeasy plant mini kit (Qiagen, Hilden, Germany): the complete root aliquots were ground in liquid nitrogen, followed by DNA extraction according to the manufacturer's instructions. Finally, DNA was eluted in two steps using 50 µl each. DNA extraction of spores from *G. clarum* was performed as described in Redecker *et al.* (1997).

3.3.3 PCR amplification of mtLSU

The nested PCR described in Börstler *et al.* (2008) (see chapter 2) was optimized for amplification from plant root DNA extracts on the Eppendorf Mastercycler epgradient S (Vaudaux-Eppendorf, Schönenbuch, Switzerland). For the first reaction the primers were RNL-28a and RNL-5. Phusion High-Fidelity DNA Polymerase from FINNZYMES (BioConcept, Allschwil, Switzerland) including 1 µl genomic DNA, 1x Phusion HF Buffer, 0.5 µM of each primer, 0.2 mM of dNTPs, 4x BSA and 3% DMSO in a volume of 25 µl were used. Cycling parameters were 30 s at 98°C, 37 cycles of 10 s at 98°C, 20 s at 57°C and 1 min 40 s at 72°C, followed by 10 min at 72°C. Products of the first PCR were diluted 1:100 in distilled water and used as template for the second reaction consisting of the primer pair RNL-29 and RNL- 30. Phusion polymerase including 1 µl of diluted

PCR product from the first reaction, 1x Phusion HF Buffer, 0.4 μM of each primer, 0.15 mM of dNTPs, 4x BSA and 3% DMSO in a volume of 50 μl were used. Cycling parameters were 30 s at 98°C, 36 cycles of 10 s at 98°C, 20 s at 60°C and 1 min 40 s at 72°C, followed by 10 min at 72°C. These improved PCR conditions were additionally tested on different isolates of *G. intraradices* used in Börstler *et al.* (2008) (see chapter 2), with PCR products ranging from 1110 to 3975 bp in length. Examples for PCR products from isolates and field collected roots are shown in Fig. 5. Reproducibility of the nested PCR approach was confirmed by testing all Tänikon samples twice. For samples from all study sites that revealed no product, PCRs were repeated at least once using positive controls. Besides samples already analysed by Börstler *et al.* (2008) (see chapter 2), mtLSU products of four further root samples from Landskron and the compartment system were amplified using *Taq* polymerase as described by Börstler *et al.* (2008) (see chapter 2). The mtLSU of *G. clarum* was amplified using the primers RNL-1 and RNL-5 (Raab *et al.* 2005). The PCR master mix was the same as described for the first nested reaction used for the root samples (see above). Cycling parameters were 30 s at 98°C, 37 cycles of 10 s at 98°C, 20 s at 58°C and 2 min at 72°C, followed by 10 min at 72°C.
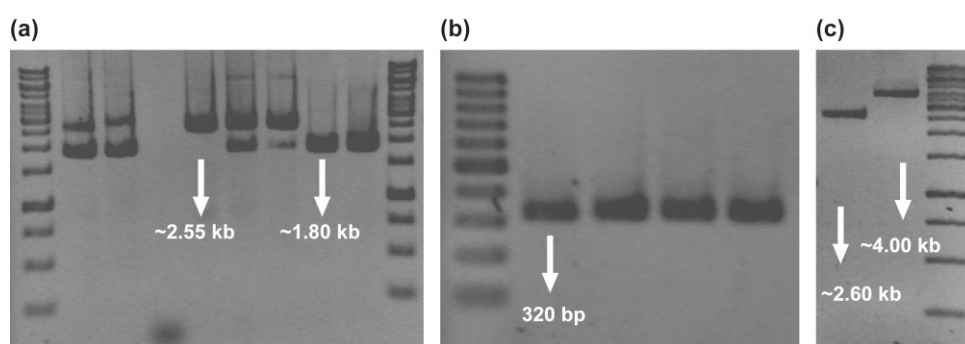
Fig. 5 (a) MtLSU PCR products from colonized root samples (Landskron) of the general PCR approach for *Glomus intraradices* (Börstler *et al.* 2008) (see chapter 2), **(b)** mtLSU PCR products from colonized root samples (Tänikon) of the specific approach for haplotype I, and **(c)** mtLSU PCR products of the isolates JJ183 (left lane) and JJ141 (middle lane) amplified with the adapted PCR conditions of the nested PCR approach developed by Börstler *et al.* (2008) (see chapter 2) for plant root DNA. For the general mtLSU PCR approach a 1 kb ladder (#SM0311) was used, for the haplotype specific approach a 100 bp ladder (#SM0242) was used (Fermentas Life Science; www.fermentas.com).

3.3.4 Specific PCR amplification of mtLSU haplotype I

A specific nested PCR approach was developed for the detection of mtLSU haplotype I of *G. intraradices*. For the first reaction the forward primer RNL-47 (GTT GAG GGG TGA CCT TCA AT) and the reverse primer RNL-49 (GCT ACC TAT GCC GGG TTT TC) were designed. *Taq* polymerase from GE Healthcare (Otelfingen, Switzerland) was used with 2 mM MgCl₂, 0.5 μM of each primer and 0.25 mM of each desoxynucleotide in a volume of 25 μl per sample. Cycling parameters were 3 min at 95°C, 32 cycles of 30 s at 95°C, 30 s at 64.5°C and 2 min 30 s at

72°C, followed by 10 min at 72°C. Products of the first PCR were diluted 1:100 in distilled water and used as template for the next step. For the second reaction, the forward primer RNL-48 (AGC CTA AGG TCT TAG AGA CT) was newly designed. The primer RNL-10 (Raab *et al.* 2005) was chosen as reverse primer. The *Taq* polymerase master mix contained following concentrations: 2 mM $MgCl_2$, 0.5 μM of each primer and 0.125 mM of each desoxynucleotide. Cycling parameters were 3 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 64.5°C and 2 min at 72°C, followed by 10 min at 72°C.

3.3.5 RFLP analyses and definition of RFLP types

In order to distinguish different haplotypes, all mtLSU PCR products were analysed using the RFLP approach developed and described in detail by Börstler *et al.* (2008) (see chapter 2). For each sample, digestion products of the restriction enzymes were always loaded in the same order (*Dra*III, *Bsa*JI and *Hin*dIII) onto the agarose gels. The combined pattern of the three digestion products was defined as RFLP type 'Intra type' when sequence analyses (see below) of the respective PCR product matched with *G. intraradices* GLOM A-1. Different Intra types were distinguished by Arabic numerals corresponding to the Roman numeral of the mtLSU haplotype determined by complete sequence data (see next section), either already defined by Börstler *et al.* (2008) (see chapter 2) or in the present study (e.g. Intra8 of haplotype VIII). Occasionally, the same Intra type can comprise several mtLSU haplotypes which are not distinguishable with the RFLP approach. In that case the Arabic numeral corresponds to the Roman numeral of the mtLSU haplotype detected first (e.g. Intra3 of the haplotypes III and XIX). Different RFLP subtypes of the same mtLSU haplotype, indicated by lower case letters, were defined by Börstler *et al.* (2008) (see chapter 2). They were most probably caused by *Taq* polymerase errors and did not occur in this study using Phusion polymerase (Table 2). Different RFLP types of non-target taxa were distinguished by Arabic numerals.

Table 2 Restriction fragment lengths of mtLSU rDNA sequences calculated *in silico* for sequenced clones (except for the directly-sequenced product labeled by *) from root colonizing *Glomus intraradices* and the isolate MUCL46238 of *Glomus clarum*, based on the target sites of the restriction enzymes *Dra*III, *Bsa*JI and *Hin*dIII. The ends of all complete fragment lengths are defined by the priming sites for RNL-29 and RNL-30. RFLP types from *G. intraradices* are indicated as "Intra types". They were defined after previously detected Intra types by Börstler *et al.* (2008) (see chapter 2) or, if detected for the first time, after the number of the corresponding haplotype. A RFLP type from *G. clarum* ("Clar type") is added for comparison.

Clones/PCR product (Accession Number)	RFLP type	Complete fragment length (bp)	*Dra*III Restriction site: 5'...CACNNN▼GTG...3'	*Bsa*JI Restriction site: 5'...C▼CNNGG...3'	*Hin*dIII Restriction site: 5'...A▼AGCTT...3'
FN377581	Intra1	2569	1430, 1139	105, 1175, 1289	2067, 502
FN377583		2569	1430, 1139	105, 1175, 1289	2067, 502
FN377580	Intra2a	2302	373, 1929	223, 448, 1631	998, 508, 578, 218
FN377578	Intra3a	1500	373, 1127	223, 448, 829	998, 502
FN377587		1525	373, 1152	223, 438, 864	1023, 502
FN377582	Intra5a	2594	1467, 1127	106, 1211, 448, 829	2092, 502
FN377576	Intra6	2807	826, 1981	107, 569, 1224, 907	1365, 709, 733
FN377589		2801	820, 1981	107, 563, 1224, 907	1359, 709, 733
FN377592		2798	817, 1981	107, 560, 1224, 907	1356, 709, 733
FN377577	Intra8	1512	373, 1139	223, 1289	1010, 502
FN377593	Intra13	2596	820, 1776	107, 563, 1926	1359, 504, 733
FN377597		2581	807, 1774	107, 550, 1924	1346, 502, 733
FN377598		2572	798, 1774	107, 541, 1924	1337, 502, 733
FN377599		2581	807, 1774	107, 550, 1924	1346, 502, 733
FN377600		2596	820, 1776	107, 563, 1926	1359, 504, 733
FN377591	Intra14	1854	817, 1037	107, 560, 1187	1356, 498
FN377594		1857	820, 1037	107, 563, 1187	1359, 498
FN377595		1848	811, 1037	107, 554, 1187	1350, 498
FN377596		1848	811, 1037	107, 554, 1187	1350, 498
AM950222**		1854	817, 1037	107, 560, 1187	1356, 498
AM950223**		1854	817, 1037	107, 560, 1187	1356, 498
AM950224**		1855	818, 1037	107, 561, 1187	1357, 498
AM950225**		1854	817, 1037	107, 560, 1187	1356, 498
FN377579	Intra20	2314	373, 1941	223, 2091	1010, 508, 578, 218
FN377584	Intra22	1904	1171, 733	103, 30, 11, 210, 667, 883	1407, 497
FN377585	Intra23	2713	1171, 1542	103, 30, 11, 210, 667, 1692	1407, 503, 585, 218
FN377586	Intra24	2259	373, 1886	223, 2036	948, 508, 585, 218
FN377588*	Intra25	3293	1171, 817, 1305	103, 30, 11, 210, 667, 2272	1407, 1631, 32, 223
FN377590	Intra27	3210	817, 2393	107, 560, 2543	1356, 504, 1145, 205
FN377601	Clar1	2217	1427, 790	106, 1171, 940	1677, 540

** Börstler *et al.* (2008); see chapter 2

3.3.6 Sequencing, cloning and sequence analyses

In addition to the RFLP approach, sequencing and cloning reactions were performed for different purposes: (i) confirmation that PCR products originated from *G. intraradices* GLOM A-1, (ii) facilitation of banding pattern interpretation in mixed samples, and (iii) increased resolution of RFLP patterns shared between different sites.

In order to confirm the identity of *G. intraradices* for every unknown RFLP pattern, exon regions from mtLSU PCR products were sequenced. At least one representative sample of each RFLP type of *G. intraradices* (Intra type) in every study site was sequenced completely for the detailed determination of the respective sequence type, which was defined as mtLSU haplotype according to Börstler *et al.* (2008) (see chapter 2). Based on these results, precise restriction fragment sizes were calculated *in silico* for each Intra type and are listed in Table 2. PCR products containing more than one mtLSU haplotype yielded mixed RFLP patterns. Therefore, these products were cloned in order to separate them for RFLP analyses and ∕ or sequence analyses. For all single products, cloning was not necessary before sequencing, but it was also performed in order to receive enough template for complete sequencing reactions of mtLSU PCR products and to store clones as reference material. Furthermore, RFLP types shared by more than one study site were analysed at least in diagnostic sequence regions (see section 3.4) in order to see, if the same RFLP type is also represented by the same sequence type (see section 3.3.5). Additionally, all PCR products of the haplotype I-specific approach were sequenced.

For cloning and sequencing reactions, PCR products were purified using the High Pure Kit from Hoffmann LaRoche (Basel, Switzerland). Cloning was performed using the pGEM-T vector (Promega ∕ Catalys, Wallisellen, Switzerland) as described in the manufacturer's instructions. Clones were amplified using the respective PCR primers of the second nested PCR reactions or the vector primers M13fwd (GTA AAA CGA CGG CCA GTG) and M13rev (GGA AAC AGC TAT GAC CAT G). For sequencing of PCR products or amplified clones the BigDye Terminator Cycle Sequencing Kit (ABI, Foster City, USA) and either the ABI 310 capillary sequencer or the AB3130xl genetic analyser were used. Sequencing primers for the respective samples are listed in Table 3. Sequences of the primers not published in Raab *et al.* (2005) and Börstler *et al.* (2008) (see chapter 2) are provided in Table 4. Sequences were initially edited in Sequence Navigator (version 1.0.1) and corrected and aligned in BioEdit (Hall 1999). Complete sequences were submitted to the European Molecular Biology Laboratory (EMBL) database under the accession numbers FN377576–FN377601.

Table 3 Sequencing primer sets used for completely sequenced mtLSU PCR products of *Glomus intraradices* and sequencing primers used for diagnostic sequence regions of PCR products corresponding to RFLP types of *G. intraradices* (Intra types) shared by more than one study site (shaded). Additionally, sequencing primers used for the determination of RFLP types belonging to other fungi than *G. intraradices* ("Fung types") are given. F (M13fwd), R (M13rev).

RFLP type	Haplotypes of completely sequenced Intra types	Forward sequencing RNL-primers	Reverse sequencing RNL-primers
Intra1	I	F, 29, 11, 13, 16, 2, 12, 37	R, 30, 7, 7b, 15, 14, 17, 10
Intra2a	XXI	F, 16, 35, 12, 37, 24, 25	R, 26, 27, 7, 7b, 38, 36
Intra3a	XIX	F, 16, 2, 35, 12, 37	R, 33, 15, 36
	III	F, 16, 2, 35, 12, 37	R, 33, 7, 15, 36
Intra5a	V	F, 11, 13d, 16, 2, 12, 37	R, 30, 7, 15, 14, 17, 10
Intra6	XVIII	F, 11b, 16, 2, 12, 24, 44, 25	R, 26, 27, 41, 15, 36, 39
	XXVI	F, 29, 11b, 16, 2, 12, 24, 44, 25	R, 26, 27, 41, 15, 36, 39
	XXVIII	F, 29, 11b, 16, 2, 12, 24, 44, 25	R, 26, 27, 41, 15, 36, 39
Intra8	VIII	F, 16, 35, 12, 37	R, 33, 7, 15, 36
Intra13	XXIX	F, 11b, 16, 35, 37, 24, 25	R, 26, 27, 7b, 38, 36, 40b
	XXXII	F, 29, 11b, 16, 35, 35b, 12, 37, 24, 25	R, 26, 26b, 27, 7b, 38, 36, 40b
	XXXIII	F, 29, 11b, 16, 35, 12, 24, 25	R, 26, 27, 7b, 38, 36, 40b
Intra14	XIV, XXX	F, 29, 11b, 16, 35, 37	R, 33, 15, 36, 39
	XXXI	F, 29, 11b, 16, 35, 37, 37b	R, 33, 15, 36, 39
Intra20	XX	F, 16, 35, 12, 37, 24, 25	R, 26, 27, 7, 7b, 38, 36
Intra22	XXII	F, 29, 11, 76, 16, 12, 37	R, 30, 7b, 15, 75, 74
Intra23	XXIII	F, 29, 11, 76, 16, 12, 24, 25	R, 30, 26, 27, 7b, 15, 75, 74
Intra24	XXIV	F, 16, 35, 12, 37, 24, 25	R, 26, 27, 7, 7b, 38, 36
Intra25	XXV	29, 11, 76, 16, 12, 37, 24, 25, 83	30, 84, 26, 31, 7, 15, 75, 74
Intra27	XXVII	F, 29, 11b, 16, 35, 12, 37, 24, 44, 53, 55	R, 52, 54, 27, 7b, 38, 36, 40b
Intra3a		2	
Intra13		29	
Intra14		29	
Fung1		29, 11, 16, 12	81, 80
Fung2		F, 29, 11, 67	
Fung3		11, 16, 12	81, 79
Fung4		29, 11, 16, 12	81, 80
Fung5		29, 11, 68	30
Fung6		29, 11, 68	30
Fung7		16, 12	81, 80
Fung8		29, 11, 68	
Fung9		11, 68	
Fung10		11, 68	
Fung11		29, 16, 12	81, 15
Fung12		16, 12	81, 15

Table 4 Sequencing primers used for the mitochondrial rRNA large subunit gene (mtLSU), which were newly designed in this study. Other sequencing primers were published by Börstler *et al.* (2008) (see chapter 2) and Raab *et al.* (2005).

RNL primer	Sequence
26b	CTAGTGCAAGTAGGCCTTCT
35b	TAACCCCTTAACGACCACAC
37b	CAGCTGGGCTAAGAATGCTG
40b	CCTGCTTTGTAAGTCTACCT
67	AGATTTAACACCCGCAGCCT
68	GTCTGCCTTGTTTCAATTAC
74	CTTGCTCTTCATCTCATAGTC
75	AGATAATCTCCTTACCTTAC
76	TCTTCCTTCCGAGATAGGAG
79	GACCATCTAACATCTTACAG
80	CCTGCTGATTGTCCCTATGT
81	TGCCTTAGAGGCCGCTGCTT
83	CCCTAACAACATGTCTGTAT
84	GGCGAGAATATACTCCACCT

3.3.7 Phylogenetic analyses

Complete mtLSU sequences were analysed phylogenetically using distance, parsimony and maximum likelihood criteria as implemented in PAUP* 4.0b10 (Swofford 2001). Neighbour joining or heuristic search algorithms were applied, respectively. Maximum likelihood models and parameters were estimated using Modeltest 3.5 (Posada 2004).

3.3.8 Population genetics

Hierarchical F statistics was calculated using the 'hierfstat' package (Goudet 2005) in the program package 'R' (http://www.R-project.org). P-values were obtained by 1000 permutations.

3.3.9 Statistical analyses

The sampling effort curve was calculated for each study site and all treatments of the agricultural sites in the program EstimateS 8.0.0 (Colwell 2005), sample order was randomized in 100 replications.

The mtLSU haplotype diversity determined by RFLP was estimated for all study sites and within different treatments of the field experiments using the (i) Simpson's index of diversity, (ii) Shannon index, and (iii) Evenness index.

In order to investigate the influence of the environmental variables (field sites and different treatments of the agricultural sites) on the distribution of mtLSU haplotypes (RFLP or sequence data) of *G. intraradices*, canonical correspondence analyses (CCA) were conducted in CANOCO for Windows version 4.5 (ter Braak & Smilauer 2004) using the haplotype presence or absence data for each root sample. Additionally, Monte-Carlo permutation tests were conducted using 499 random permutations in order to determine the statistical significance of the relation between the whole set of environmental variables and the mtLSU haplotypes. Forward selection was used for ranking the environmental variables in importance for determining the haplotype distribution in case of more than two variables.

Relationships between geographical distances and similarities of RFLP types of *G. intraradices* of the experimental plots in Frick and Tänikon were examined using the Mantel test with 99 999 permutations. The software PASSaGE (ver 1.1; Rosenberg 2001) was used to conduct these analyses.

3.4 Results

3.4.1 Amplification success

MtLSU PCR products of *G. intraradices* (sensu GLOM A-1) were detected in 99 out of 127 root samples analysed in this study (see Table 5). PCR product lengths ranged from 1 500 to 3 293 bp for *G. intraradices* (Table 6), sizes above 4 000 bp were exclusively reached by non-target fungi (not shown). Twenty-one samples contained mixtures of up to three different haplotypes of *G. intraradices*. MtLSU PCR products from other fungi were found in 20 samples. Combinations of both products from other species and from *G. intraradices* occurred in four samples. Although all samples from Landskron and Ramosch were expected to be positive for *G. intraradices* due to positive ITS results, no mtLSU PCR product could be obtained from one sample from Ramosch.

Table 5 Number and distribution of restriction fragment length polymorphism (RFLP) patterns of *Glomus intraradices* ("Intra types") and other fungi ("Fung types") detected in the Tänikon field site (three treatments: no-tillage, chisel and tillage), the Frick field site (two treatments: chisel and tillage) and the semi-natural grasslands of Landskron and Ramosch.

Intra type	Tänikon				Frick			Landskron	Ramosch
	No-tillage	Chisel	Tillage	∑	Chisel	Tillage	∑		
Intra1	3	11	12	26					
Intra2a		2		2					
Intra3a		2	1	3	7	5	12		
Intra5a			1	1					
Intra6	1			1				2	
Intra8		2		2					
Intra13								13	16
Intra14								14	11
Intra20		1		1					
Intra22					1		1		
Intra23						1	1		
Intra24					4	1	5		
Intra25					3	8	11		
Intra27								2	
Total number of RFLP patterns of all Intra types	4	18	14	36	15	15	30	31	27
∑ different Intra types	2	5	3	7	4	4	5	4	2
Positive Intra type-root samples/root sample number (%)	4/16 (25)	13/16 (81)	12/16 (75)	29/48 (60)	11/16 (69)	13/16 (81)	24/32 (75)	21/21 (100)	25/26 (96)
Fung type									
Fung1	1			1					
Fung2	1			1					
Fung3	5	3	3	11					
Fung4	3			3					
Fung5			1	1					
Fung6			1	1					
Fung7			1	1					
Fung8						1	1		
Fung9						1	1		
Fung10						1	1		
Fung11									1
Fung12									1
Total number of RFLP patterns of all Fung types	10	3	6	19	0	3	3	0	2
∑ different Fung types	4	1	4	7	0	3	3	0	2
Positive Fung type-root samples/root sample number (%)	8/16 (50)	3/16 (19)	5/16 (31)	16/48 (33)	0/16 (0)	2/16 (13)	2/32 (6)	0/21 (0)	2/26 (8)
Root samples containing Intra - and Fung types/root sample number (%)	0/16 (0)	0/16 (0)	1/16 (6)	1/48 (2)	0/16 (0)	1/16 (6)	1/32 (3)	0/21 (0)	2/26 (8)

Table 6 Sequence structure of the mitochondrial rRNA large subunit gene (mtLSU) rDNA from root-colonizing *Glomus intraradices* obtained using primers RNL-29 ⁄ RNL-30 (excluding primer annealing sites)

Origin and plant samples	Clones (accession number)	Intra type	Haplotype	Introns Pos. 1 (-type,bp)	Pos. 2 (-type, bp)	Pos. 3 (-type, bp)	Exon region Complete/parts between intron positions (bp)	Fragment length (bp)
Tänikon (Switzerland)								
15/1 (*Zea mays*)	FN377576	6	XVIII	1-9, 453	2-4, 303	3-3, 944	1067/363, 666, 38	2767
12/2 (*Zea mays*)	FN377577	8	VIII	No	2-1, 401	No	1071/363, 670, 38	1472
12/4 (*Zea mays*)	FN377578	3a	XIX	No	2-3, 389	No	1071/363, 670, 38	1460
18/2 (*Zea mays*)	FN377579	20	XX	No	2-1, 401	3-8, 802	1071/363, 670, 38	2274
18/4 (*Zea mays*)	FN377580	2a	XXI	No	2-3, 389	3-8, 802	1071/363, 670, 38	2262
24/1 (*Zea mays*)	FN377581	1	I	1-1, 1057	2-1, 401	No	1071/363, 670, 38	2529
24/2 (*Zea mays*)	FN377582	5a	V	1-3, 1094	2-3, 389	No	1071/363, 670, 38	2554
24/3 (*Zea mays*)	FN377583	1	I	1-1, 1057	2-1, 401	No	1071/363, 670, 38	2529
Frick (Switzerland)								
W7/4 (*Zea mays*)	FN377584	22	XXII	1-10, 795	No	No	1069/366, 665, 38	1864
O4/4 (*Zea mays*)	FN377585	23	XXIII	1-10, 795	No	3-1, 809	1069/366, 665, 38	2673
	FN377586	24	XXIV	No	2-6, 339	3-1, 809	1071/363, 670, 38	2219
O7/3 (*Zea mays*)	FN377587	3a	III	No	2-2, 414	No	1071/363, 670, 38	1485
W9/1 (*Zea mays*)	FN377588†	25	XXV	1-10, 795	No	3-9, 1389	1069/366, 665, 38	3253
Landskron (France)								
L5 (*Bromus erectus*)	FN377589	6	XXVI	1-11, 447	2-4, 303	3-3, 944	1067/363, 666, 38	2761
L8 (*Festuca pratensis*)	FN377590	27	XXVII	1-7, 444	2-4, 303	3-2, 1356	1067/363, 666, 38	3170
L9 (*Festuca pratensis*)	FN377591	14	XIV	1-7, 444	2-4, 303	No	1067/363, 666, 38	1814
L10 (*Festuca pratensis*)	FN377592	6	XXVIII	1-7, 444	2-4, 303	3-3, 944	1067/363, 666, 38	2758
L14 (*Medicago sativa*)	FN377593	13	XXIX	1-11, 447	2-4, 303	3-6, 739	1067/363, 666, 38	2556
	FN377594	14	XXX	1-11, 447	2-4, 303	No	1067/363, 666, 38	1817
L15 (*Medicago sativa*)	FN377595	14	XXXI	1-12, 438	2-4, 303	No	1067/363, 666, 38	1808
	FN377596	14	XXXI	1-12, 438	2-4, 303	No	1067/363, 666, 38	1808
L21 (*Inula salicina*)	FN377597	13	XXXII	1-13, 434	2-4, 303	3-6, 737	1067/363, 666, 38	2541
Ramosch (Switzerland)								
R4 (*Gentiana verna*)	FN377598	13	XXXIII	1-4, 425	2-4, 303	3-6, 737	1067/363, 666, 38	2532
R11 (*Trifolium* sp.)*S6	AM950222	14	XIV	1-7, 444	2-4, 303	No	1067/363, 666, 38	1814
	AM950223	14	XIV	1-7, 444	2-4, 303	No	1067/363, 666, 38	1814
R12 (*Trifolium* sp.)*S10	AM950224	14	XIV	1-7, 445	2-4, 303	No	1067/363, 666, 38	1815
	AM950225	14	XIV	1-7, 444	2-4, 303	No	1067/363, 666, 38	1814
R23 (Poaceae sp.)	FN377599	13	XXXII	1-13, 434	2-4, 303	3-6, 737	1067/363, 666, 38	2541
Compartment system								
C1 (*Medicago sativa*)	FN377600	13	XXIX	1-11, 447	2-4, 303	3-6, 739	1067/363, 666, 38	2556

Haplotypes and intron types were distinguished by sequence differences. Introns containing putative open reading frames for LAGLIDADG homing endonucleases are shaded. The corresponding RFLP patterns defined as 'Intra types' are listed for comparison. Inoculum for the 'compartment system' originated from the Landskron field site (Sýkorová *et al.* 2007a). For three samples, two clones of the same Intra type were sequenced. * Börstler *et al.* (2008); see chapter 2. † PCR-product.

3.4.2 RFLP analyses

Fourteen different RFLP patterns of *G. intraradices* GLOM A-1 (referred to as Intra types in this study) could be distinguished. In addition, 12 different patterns of other fungi were found (Table 5). The sampling effort curves clearly reach saturation for both semi-natural grasslands (Fig. 6). This is not as clearly the case for the agricultural sites, although the curves begin to level off and finding an additional RFLP type would require the analysis of a considerable amount of samples. Intra type richness was highest in Tänikon followed by the other agricultural site in Frick (Table 7, Fig. 7).

The latter site also revealed higher diversity indices than the grasslands, whereas the distribution of Intra types in Tänikon was uneven, which is expressed in lower Simpson's and Shannon indices compared to the grassland in Landskron (Table 7).

(a)

(b)
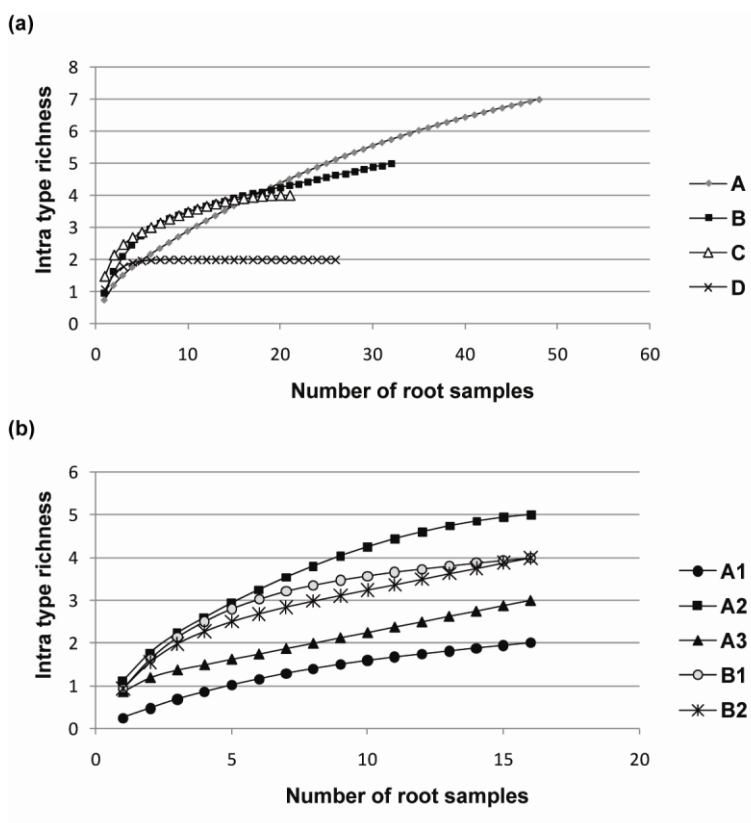
Fig. 6 (a) Sampling effort curves based on RFLP types of *Glomus intraradices* (Intra types) for the study sites Tänikon (A), Frick (B), Landskron (C) and Ramosch (D). **(b)** Sampling effort curves based on Intra types for the Tänikon treatments, no-tillage (A1), chisel (A2), tillage (A3) and the Frick treatments chisel (B1) and tillage (B2). Sample orders were randomized in 100 replications using EstimateS, version 8.0.0 (Colwell 2005).

Table 7 Indices of diversity, richness and evenness of RFLP types (Intra types) of *Glomus intraradices* in all study sites and different treatments. F (Frick), T (Tänikon).

Fiel site or treatment	Intra type richness (S)	Simpson's index of diversity (D)	Shannon index (H)	Evenness index (E)
Tänikon	7	0.48	1.06	0.55
Frick	5	0.70	1.26	0.78
Landskron	4	0.63	1.08	0.78
Ramosch	2	0.50	0.68	0.98
T/no-tillage	2	0.50	0.56	0.81
T/chisel	5	0.62	1.19	0.74
T/tillage	3	0.27	0.51	0.46
F/chisel	4	0.71	1.21	0.87
F/tillage	4	0.64	1.06	0.77
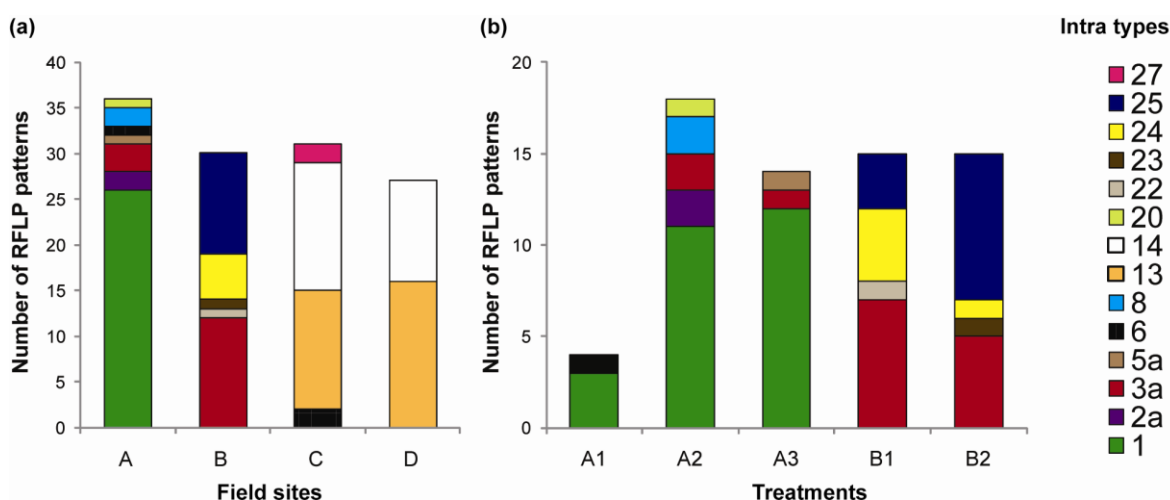
Fig. 7 (a) Total number of restriction fragment length polymorphism (RFLP) patterns of *Glomus intraradices* and different RFLP types ('Intra types') found in the Tänikon field site (A), the Frick field site (B), the Landskron grassland (C) and the Ramosch grassland (D). **(b)** Number of RFLP patterns and different Intra types found in different treatments of the Tänikon field site (A1 ∕ no-tillage, A2 ∕ chisel, A3 ∕ tillage) and the Frick field site (B1 ∕ chisel, B2 ∕ tillage).

All study sites were dominated by one or two RFLP types (see Fig. 7). RFLP patterns of these most frequently found RFLP types are shown in Fig. 8. RFLP type composition differed strongly among all study sites, except between the two grasslands: Intra13 and Intra14 were the only Intra types found frequently in Ramosch and the same types were also dominating the other semi-natural site in Landskron. Intra13 was also found in the sample from the 'compartment system', a greenhouse culture derived from inoculum from the Landskron site (Sýkorová *et al.* 2007a). Only two further Intra types were shared by more than one site: Intra3a was detected in both agricultural sites, though less frequently found in Tänikon, and Intra6 was detected in Tänikon as well as in Landskron.
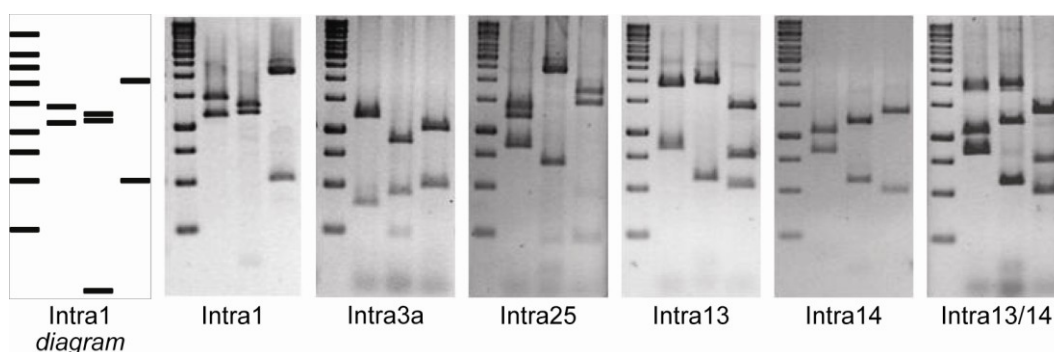
Fig. 8 Banding patterns of the most frequent *Glomus intraradices* restriction fragment length polymorphism (RFLP) patterns ("Intra types") from the Tänikon field site (Intra1), the Frick field site (Intra3a/Intra25), the grasslands Landskron and Ramosch (Intra13/Intra14). An example for a diagram made *in silico* from the sequence data is shown for Intra1. A mixed pattern is shown for Intra13/Intra14. PCR products were amplified using the primer pair RNL-29/30 for the mitochondrial rRNA large subunit gene (mtLSU) rDNA. DNA was digested using the restriction enzymes *Dra*III, *Bsa*JI, *Hin*dIII according to Börstler *et al.* (2008) (see chapter 2) and loaded in the same order per sample onto the gels. DNA ladder in left-most lane of each gel (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10 000. Fragment standard in left-most lane of the diagram (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 4000.

The highest Intra type diversity was found in the chisel treatments within each experiment in Tänikon and Frick (Table 7, Fig. 7). Interestingly, only four samples were tested positive for *G. intraradices* in the no-tillage treatment of Tänikon, which, on the contrary, revealed the highest frequency of non-*G. intraradices* RFLP type patterns (Table 5). CCA revealed that all canonical axes of Tänikon explained 11.4% of the whole variability of the data. According to the Monte-Carlo permutation test, the influence of the environmental factors was significant (P = 0.002 including and P = 0.042 excluding samples without *G. intraradices*). After ranking the factors using forward-selection, only the no-tillage treatment, characterized by many samples negative for *G. intraradices* (included as 'NoIntra' in the analyses), differed significantly from the other treatments (P = 0.002), followed by chisel (P = 0.094). If 'NoIntra' samples were excluded, none of these factors was significant (P = 0.134). The different treatments in Frick had no significant influence on the distribution of RFLP types based on the Monte-Carlo permutation test (P = 0.116). Nevertheless, the results of the CCA analyses, shown as a biplot in Fig. 9, indicate some preferences of the Intra types for the respective tillage treatment.
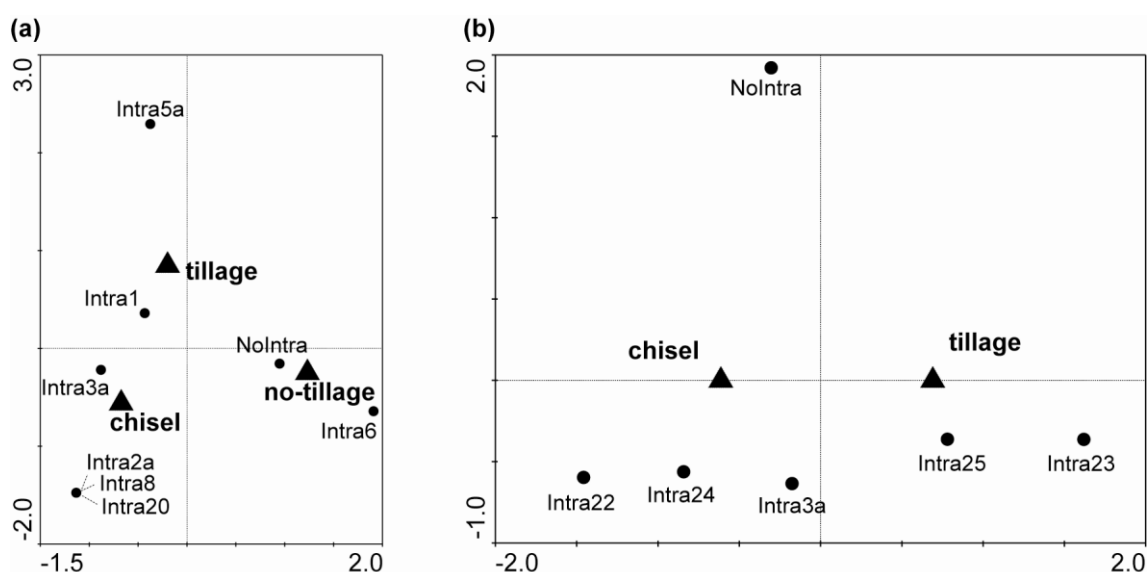
Fig. 9 CCA-biplots of RFLP type data obtained from mtLSU PCR products (using biplot scaling on symmetric distances) from **(a)** Tänikon samples and **(b)** Frick samples. The different treatments no-tillage, chisel and tillage represent the environmental variables and are shown as triangles. Filled circles indicate the position of different RFLP types of *Glomus intraradices* (Intra types) in the ordination space. Samples tested negative for *G. intraradices* were included as 'NoIntra'.

Mantel tests comparing the possible correlation of RFLP types of *G. intraradices* to spatial distance among the plots revealed no relationship between these variables for Tänikon (r = 0.03556; P = 0.51566) as well as for Frick (r = 0.02781; P = 0.57692). These results indicate that the plots located close to each other did not preferentially harbour the same *G. intraradices* RFLP types.

Hierarchical F statistics revealed significant genetic differentiation between the two arable sites which was even slightly stronger when phylogenetic outliers (see Fig. 10; sister group

containing haplotypes XVII, XXII, XXIII, XXV) were excluded (F_{ST} = 0.4631, P = 0.001). Treatments within these sites were not significantly different ($F_{(site/treatment)}$ = 0.0627, P = 0.142). In contrast, the two grasslands did not show significant differences in their RFLP types (F_{ST} = −0.00361, P = 0.35), neither were host preferences detectable ($F_{(site/host)}$ = 0.04519, P = 0.124).
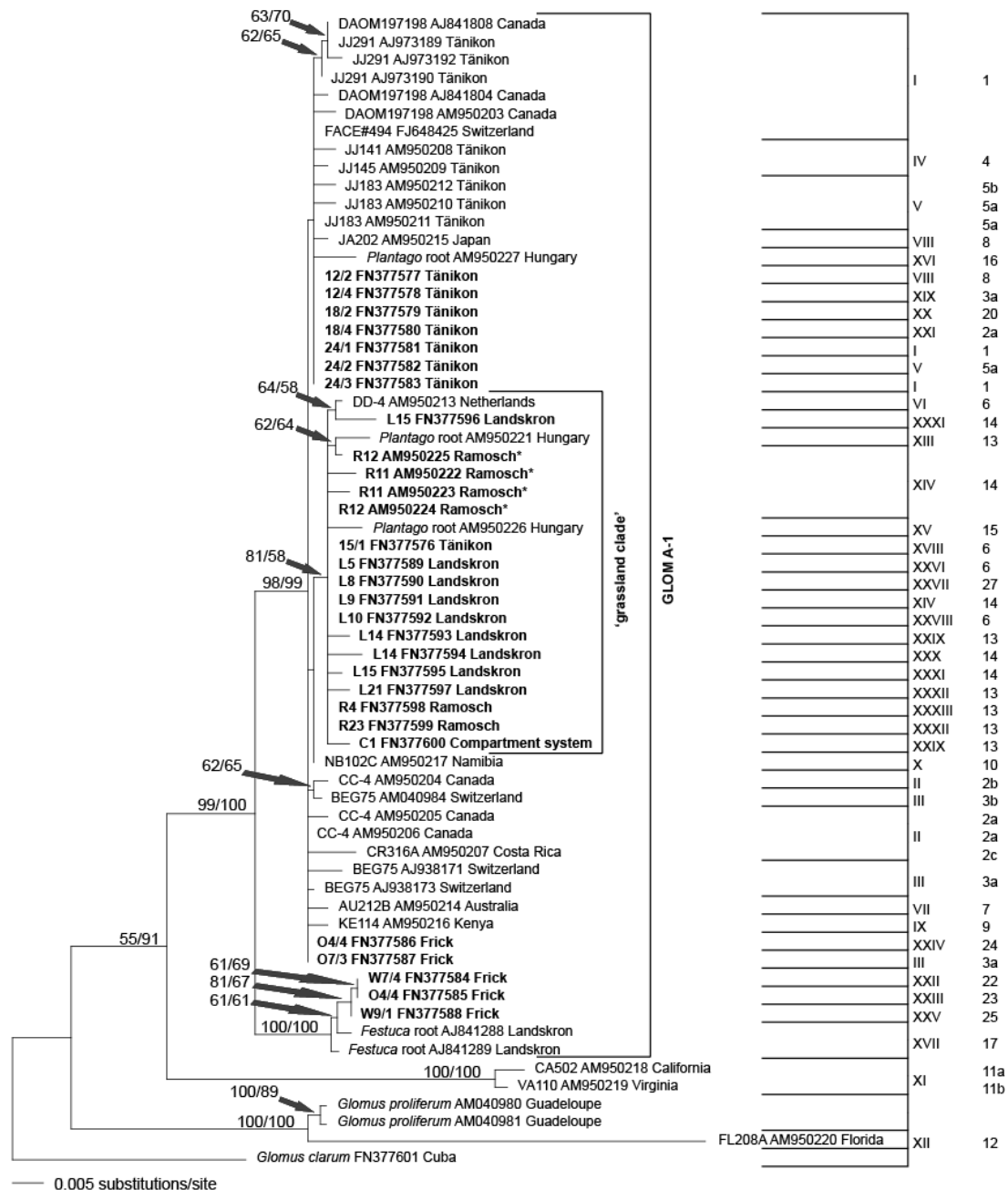
Fig. 10 Phylogenetic tree of *Glomus intraradices*, *Glomus proliferum* and *Glomus clarum*, which was used as outgroup. The phylogeny was obtained using 931 characters of mitochondrial rRNA large subunit gene (mtLSU) exon sequences by a heuristic search under the maximum likelihood criterion. Maximum parsimony bootstrap values from 1000 replicates and Neighbour-joining bootstrap values from 1000 replicates are shown at the nodes. Sample code, accession number and origin are shown in boldface for sequences from root samples originating from the arable field sites and grasslands of the present study. *G. intraradices* isolates are labelled with their identification code. Roman numerals indicate mtLSU haplotypes, Arabic numerals indicate RFLP types ('Intra types'). *Sequences from Ramosch previously analysed by Börstler *et al.* (2008); see chapter 2.

Because of a different sampling structure and the possibility that grassland phylotypes might represent different species (see below), therefore precluding an overall population analysis, grassland and arable site samples were not analysed together. On the contrary, the fact that only the rarely found RFLP type Intra6 was shared between arable and semi-natural sites is obvious evidence supporting genetic differentiation (Fig. 7).

3.4.3 Sequence-based analyses

Although RFLP types were shared by some sites, detailed sequence analyses revealed that there was no overlap of mtLSU haplotypes among any of the study sites except between the two grasslands. All PCR products of Intra6, found in Tänikon and Landskron, were sequenced completely and differed within and between the sites in the intron at position 1 (Table 6). Intra3a which was shared by the two agricultural sites contained only an intron at position 2 (Table 6), which was sequenced for all PCR products as the only sequence region where polymorphism would be expected. Sequencing revealed that the mtLSU sequence type detected within Intra3a was the same within each field site, but they differed between the sites (Fig. 11).

Fig. 11 Alignment of the diagnostic sequence region in the intron at position 2 of mtLSU haplotypes represented by the same RFLP type "Intra3a" from Tänikon and Frick (samples from Frick are shaded). Gaps are shown as hyphens. An unequivocal alignment of the region between positions 71 until 114 is not possible. Sample identification codes are provided in addition to the origin of sequences from clones (C) or PCR products (PCR).

Completely sequenced PCR products of Intra13 and Intra14 from five root samples each confirmed that the only variable sequence regions in these PCR products were located in the intron at position 1 (Table 6), which was expected from previous analyses of an isolate from the Netherlands and a root sample from Hungary (Börstler *et al.* 2008; see chapter 2). Therefore, this gene region was sequenced from all PCR products assigned to these RFLP types. Based on these partial sequences, the two Intra types could be further separated into nine different sequence haplotypes (Figs 12 and 13). The differences consisted mainly of short indels and not of more substantial sequence differences as they were typical for different introns of the highly diverse agricultural field sites (Table 6).


```
Sample      Sequence position 1            Sequence position 2                            Intron-type  Haplotype
L1/C-b      ACCACCTT---------AAAGATAC       ATTAAGTTTCATTTTTAAAAACAAGTTCCATGCTA            1-7          XIII
L6/C-a      .......---------........        .........A.........................            1-7          XIII
L8/C-b      .......---------........        .........A.........................            1-7          XIII
L9/C-b      .......---------........        .........A.........................            1-7          XIII
L18/C-a     .......---------........        .........A.........................            1-7          XIII
L3/C-a      .......CTTAGAGAT........        .........------....................            1-11         XXIX
L4/PCR      .......CTTAGAGAT........        .........------....................            1-11         XXIX
L5/C-a      .......CTTAGAGAT........        .........------....................            1-11         XXIX
L7/PCR      .......CTTAGAGAT........        .........------....................            1-11         XXIX
L12/PCR     .......CTTAGAGAT........        .........------....................            1-11         XXIX
L13/C-b     .......CTTAGAGAT........        .........------....................            1-11         XXIX
L14/C-a     .......CTTAGAGAT........        .........------....................            1-11         XXIX
L21/C-a     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R3/PCR      .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R6/PCR      .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R8/PCR      .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R9/PCR      .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R10/PCR     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R15/PCR     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R16/PCR     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R18/PCR     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R19/C-a     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R20/C-a     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R21/PCR     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R22/PCR     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R23/C-a     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R25/C-a     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R26/C-a     .......CTTAGAGAT........        .........------------------........            1-13         XXXII
R4/C-b      .......---------........        .........------------------........            1-4          XXXIII
C1/C-a      .......CTTAGAGAT........        .........------..........C........            1-11         XXIX
AM950221    ......C.---------........        ...................................            1-7          XIII
```

Fig. 12 Alignment of two diagnostic sequence regions in the intron at position 1 of mtLSU PCR products represented by the same RFLP type "Intra13" from Landskron and Ramosch. Conservation is viewed by plotting identities to a standard as dot, gaps are given as shaded hyphens. Samples from the Ramosch grassland are shaded. Two additional samples from the compartment system based on inoculum from Landskron (C1) and from Hungary (AM950221; Börstler *et al.* 2008; see chapter 2) are given for comparison. Sample identification codes are given in addition to the origin of sequences from clones (C) or PCR products (PCR). Three changes of a single base occurred for samples amplified with *Taq* polymerase (C1, AM950221), only one change of a single base occurred for sample L1 amplified with Phusion polymerase. All these single changes are most probably caused by polymerase or sequencing errors.

```
Sample      Sequence position 1            Sequence position 2                            Intron-type   Haplotype
L1/C-a      ACCACCTT---------AAAGATAC       ATTAAGTTTAATTTTTAAAAACAAGTTCCATGCTA            1-7           XIV
L2/PCR      .......---------........        ...................................            1-7           XIV
L6/C-b      .......---------........        ...................................            1-7           XIV
L9/C-a      .......---------........        ...................................            1-7           XIV
L10/C-a     .......---------........        ...................................            1-7           XIV
L11/PCR     .......---------........        ...................................            1-7           XIV
L17/PCR     .......---------........        ...................................            1-7           XIV
L18/C-b     .......---------........        ...................................            1-7           XIV
L20/PCR     .......---------........        ...................................            1-7           XIV
L13/C-a     ........CTTAGAGAT........       ...........------...................           1-11          XXX
L14/C-b     ........CTTAGAGAT........       ...........------...................           1-11          XXX
L15/PCR     .......---------........        ...........------...................           1-12          XXXI
L16/PCR     .......CTTAGAGAT........        .........------------------........            1-13          XXXIV
L19/PCR     .......CTTAGAGAT........        .........------------------........            1-13          XXXIV
R4/C-a      .......---------........        .........------------------........            1-4           XXXV
R1/PCR      .......---------........        ...................................            1-7           XIV
R2/PCR      .......---------........        ...................................            1-7           XIV
R7/C-a      .......---------........        ...................................            1-7           XIV
R11/C-b     .......---------........        ...........G.......................            1-7           XIV
R12/C-b     .......---------........        ...................................            1-7           XIV
R13/PCR     .......---------........        ...................................            1-7           XIV
R14/PCR     .......---------........        ...................................            1-7           XIV
R17/PCR     .......---------........        ...................................            1-7           XIV
R24/PCR     .......---------........        ...................................            1-7           XIV
R20/C-b     ........CTTAGAGAT........       .........------------------........            1-13          XXXIV
```

Fig. 13 Alignment of two diagnostic sequence regions in the intron at position 1 of mtLSU PCR products represented by the same RFLP type "Intra14" from Landskron and Ramosch. Conservation is viewed by plotting identities to a standard as dot, gaps are given as shaded hyphens. Samples from the Ramosch grassland are shaded. Sample identification codes are given in addition to the origin of sequences from clones (C) or PCR products (PCR). Only one change of a single base occurred for sample R11 previously amplified with *Taq* polymerase by Börstler *et al.* (2008) (see chapter 2) and is most probably due to a polymerase or sequencing error.

In contrast to the arable sites, the grassland samples were thus exhaustively analysed by sequencing (see also below for the remaining RFLP type Intra27), and the sequence types could therefore be included into statistical analyses. The distribution of all 12 different mtLSU haplotypes based on sequence data across the grasslands is shown in Fig. 14: haplotype XIV was found frequently in both sites. Two further haplotypes were shared: haplotype XXXII was most frequently found in Ramosch, but only rarely detected in Landskron and haplotype XXXIV was found rarely in both grasslands. The CCA revealed that the environmental variables explained 8.5% of the total variance and according to the Monte-Carlo permutation test the influence of the field sites on haplotype distribution was significant (P = 0.002). The results of the CCA analysis are shown as a biplot in Fig. 15.

Contrary to the RFLP analysis, the two grasslands sites were significantly differentiated based on sequence haplotype data (F_{ST} = 0.17448, P = 0.01). Host preference, however, again only had a weak influence on population structure ($F_{(site/host)}$ = 0.04519, P = 0.124).

Generally, at least one example of each different RFLP type of *G. intraradices* in every study site was sequenced completely for the determination of the respective mtLSU haplotype based on sequence data (Table 6).
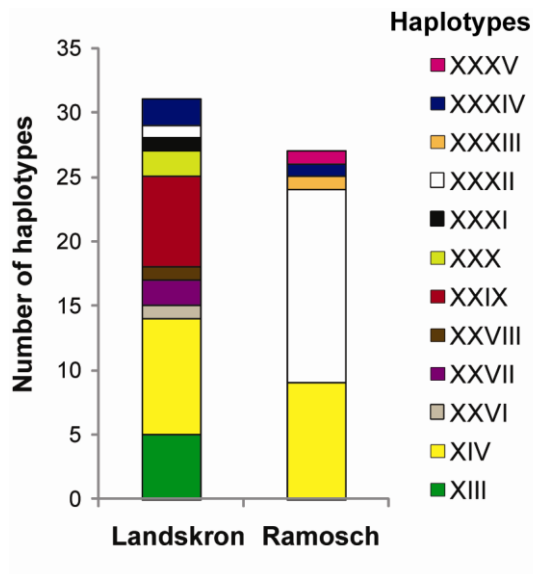
Fig. 14 Number and distribution of different mtLSU haplotypes of *Glomus intraradices* found in the grasslands in Landskron and Ramosch.
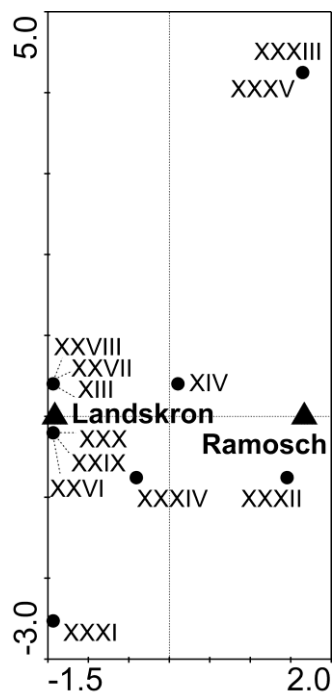

Fig. 15 CCA-biplot of mtLSU haplotype data (using Hill's scaling focused on inter-species distances) from Landskron and Ramosch. The field sites represent the environmental variables and are shown as triangles. Filled circles indicate the position of 12 different haplotypes of *Glomus intraradices* in the ordination space, which are given in Roman numerals. Only one sample from Ramosch was tested negative for *G. intraradices* and was excluded from the analysis.

3.4.4 Phylogenetic analyses

All completely sequenced mtLSU PCR products were compared phylogenetically with sequences from isolates of *G. intraradices* and previously analysed root samples generated by Raab *et al.* (2005) and Börstler *et al.* (2008) (see chapter 2). A phylogenetic tree was inferred (Fig. 10). All haplotypes from the two grasslands and the 'compartment system' derived from the Landskron site were falling into one subclade, which also comprises two haplotypes from two root samples from Hungary and only one haplotype of an isolate from a Dutch dry dune grassland. Only one haplotype from the agricultural sites was found in this group. Interestingly, this unique haplotype was found in the no-tillage treatment of the Tänikon site. Because of a small number of unique sequence positions, this 'grassland subclade' was consistently recovered throughout all phylogenetic analyses although it only received moderate bootstrap support, and was not recovered as sister group to the remaining isolates including DAOM197198 (which is subject of the genome sequencing project of *G. intraradices*) and FACE#494, of which the complete mitochondrial genome was sequenced by Lee & Young (2009).

A clearly distinct sister clade to these two groups comprised sequences found in the Landskron grassland by Raab *et al.* (2005), using different primers, and the Frick arable site. It is currently impossible to decide whether this lineage or even the grassland clade constitute different biological species, but to avoid confounding analyses of intraspecific genetic structure variation we excluded these groups or analysed them separately in some analyses to assess whether this would affect our main conclusions.

3.4.5 The specific approach to detect haplotype I

A nested PCR approach designed to specifically detect mtLSU haplotype I uses characteristic sequence regions of the introns at position 1 and 2, and results in a 320-bp DNA fragment of the first intron, which is easy to amplify (see Fig. 5). The PCR product can be sequenced directly without any cloning steps. This approach was applied on the DNA extracts from all study sites. Positive PCR products were only obtained in Tänikon samples also containing the RFLP type Intra1, which corresponds to haplotype I. In other words, the specific approach for haplotype I confirmed the results of the general PCR-RFLP approach for *G. intraradices* in each case and vice versa. All PCR products were additionally confirmed by sequencing. So far, only the haplotype X of the isolate NB102C (Börstler *et al.* 2008; see chapter 2) shows the same sequence for the final PCR product, but an amplification of this haplotype would not be possible because of a missing primer annealing site.

3.5 Discussion

By resolving intraspecies mtLSU haplotypes of *G. intraradices*, this study is the first one to address the genetic structure of AMF populations directly in the symbiotic state, that is, without the need to rely on spores or fungal isolates obtained from the field. The method we used here was shown to work reliably: the two different approaches (i) using group-specific primers and RFLP, and (ii) using haplotype-specific primers, respectively, provided concordant results. The specificity of the group-specific primers was high enough to assure that 84% of the amplified sequences were expected targets. Although some mtLSU RFLP types or sequence haplotypes might be further resolved by multilocus markers (Croll *et al.* 2008), our RFLP data demonstrate considerable genetic structure among field sites, which was refined by sequencing of diagnostic regions in a subset of samples. In contrast to our previous studies (Raab *et al.* 2005; Börstler *et al.* 2008; see chapter 2), we used mainly a proofreading DNA polymerase which considerably reduced sequencing noise in our datasets. In samples, containing several RFLP types, minority types were visible as faint bands, demonstrating that the approach is suitable for diagnosing multiple types without the need for cloning / sequencing approaches which may bear the high possibility of overseeing minority types.

When assigning mtLSU haplotypes to the strains used by Koch *et al.* (2004) and Croll *et al.* (2008), it becomes clear that many of the significant effects of AMF genotypes on symbiotic properties and growth parameters in these studies are due to different mtLSU haplotypes, indicating that these haplotypes are functionally relevant categories. On the contrary, mtLSU types do not necessarily represent separate biological species, because Croll *et al.* (2009) demonstrated that representative strains of all three mtLSU haplotypes found by these authors in the Tänikon field site formed anastomoses with each other and exchanged genetic markers.

We analysed samples from the same field site in Tänikon that was assessed by Croll *et al.* (2008) with a different approach and the comparison of the datasets offers interesting insights. In contrast to these authors, who characterized isolates of ROCs originally obtained in trap cultures from this site in 1999 (Jansa *et al.* 2002a), we directly used the colonized roots from the same plots, although 8 years later. Croll *et al.* (2008) found three mitochondrial haplotypes, whereas we recovered two of those (I, V) and five additional ones (VIII, XVIII, XIX, XX, XXI). Haplotype VIII was identical to an isolate JA202 originating from Japan (Börstler *et al.* 2008; see chapter 2), further supporting a wide geographical distribution of certain haplotypes.

Interestingly, haplotype I, which already dominated among the isolates from this site in 1999, was also the most frequently found haplotype within the roots in 2007. The genome sequencing isolate from Canada (DAOM197198) also belongs to this haplotype, as well as isolates from other sites (Eschikon and Changins) in Switzerland (Croll *et al.* 2008). Overall, this would indicate that haplotype I is particularly well adapted to both arable soils and root organ cultures and geographically widespread. Therefore, it came as a surprise that this haplotype was not at all

present in the second arable field site in Frick we analysed. These results were confirmed with a primer specifically designed for haplotype I, ruling out any possibility that the haplotype was merely concealed by other, more abundant ones, which may happen in approaches using general primers.

Our findings are in contrast to the studies by Koch *et al.* (2004) and Croll *et al.* (2008), which suggested that the global genetic diversity of *G. intraradices* is not higher than the diversity within the Swiss field site these authors analysed. As already shown by Börstler *et al.* (2008) (see chapter 2) based on isolates in open pot culture, the mtLSU haplotype diversity worldwide is considerably higher. Now we also demonstrate that direct detection from roots allows to detect a higher haplotype richness than cultivation procedures, which involve several bottlenecks and biases such as the initial propagation in pot cultures using different host plants ('trap culturing') and establishment of ROCs from single spores. As our samples and those of Croll *et al.* (2008) were not taken in the same year, we cannot completely exclude that temporal fluctuations among the haplotypes occurred, but there is no reason to assume that the RFLP type richness in Tänikon has more than doubled only due to the different sampling time. The sampling effort curve (Fig. 6) suggests that the richness may in fact even be higher.

Although we could not find statistically-significant differences among the tillage treatments of the arable sites, obvious tendencies of the treatments to alter the community structure of *G. intraradices* were observed. Similarly, Rosendahl & Matzen (2008) reported that agricultural practice affects both the abundance and the population structure of different AMF species. The strongly reduced frequency of *G. intraradices* in the no-tillage treatment of Tänikon, also expressed by a lower haplotype richness, might be explained by a replacement of *G. intraradices* by other species better adapted to these environmental condition. On the AMF species level Jansa *et al.* (2003) already showed that tillage affected the community structure in the field experiment of Tänikon.

An unexpected finding of our study was the genetic differentiation between the two arable field sites, which were situated at a distance of 67 km, indicating that soil chemistry and geographic factors may have a stronger influence on population structuring in agriculturally used soils than previously recognized. It will be intriguing to specifically address these possible relations in future studies. Nevertheless, the data suggest that even in arable soils, only a subset of the genotypes of *G. intraradices* may be easily recovered by culturing.

In contrast to the arable sites, the grasslands contained a completely different set of mtLSU haplotypes. Only one haplotype XIII from the semi-natural sites was previously detected in a root sample from a mine spoil in Hungary (Börstler *et al.* 2008; see chapter 2). In fact, the 'grassland types' could consistently be phylogenetically distinguished from the arable soil types, although only by a few different base pairs, which was impossible by nuclear-encoded rDNA (Sýkorová *et al.* 2007a,b). This may either mean that the 'grassland types' constitute a genetically different

ecotype, or that they even are a previously unrecognized ('cryptic') species. Interestingly, the only 'grassland type' detected in an arable site (haplotype XVIII) was found in the no-tillage treatment, which is the least disturbed treatment of the two field trials. In contrast to some haplotypes from arable fields, which were reported to form hyphal cross-links (anastomoses; Croll *et al.* 2009), and therefore can be safely assumed to belong to one biological species, the grassland types have not been analysed yet. This is complicated by the fact that they are apparently difficult to cultivate, even in conventional open pot culture, and therefore are strongly under-represented among cultivated isolates. In any case, the strong differentiation between arable and semi-natural sites is a finding that will have considerable impact, for example, on conservation biology, because it renders it unlikely that semi-natural grasslands act as diversity reservoirs for arable sites for *G. intraradices*. Detailed studies of genotypes in arable soils and neighbouring grasslands, including intensively used farmland meadows, will be necessary to answer this question conclusively. However, the occurrence of a 'grassland type' in a greenhouse culture obtained from a grassland site ('compartment system'), demonstrates that at least some of these haplotypes are in principle capable of colonizing roots under high-disturbance conditions. The detection of members of this clade in degraded but non-arable ecosystems from Hungary supports this view. Further analyses of other habitats will elucidate under which range of environmental conditions these fungi thrive.

The example of the sister group of haplotypes outside the clade containing arable land and grassland haplotypes in Fig. 10, which we consider neither to belong to the arable land or grassland groups, shows that even similar haplotypes may occur in very different environments. The 'grassland types' are relatively similar to each other in their mtLSU intron sequences and are mainly distinguished only by short indels not easily picked up by RFLP. Therefore, the two grassland sites were not differentiated based on mtLSU RFLP data. Using sequences of diagnostic regions, however, we detected significant differentiation. Still, it seems remarkable that the two grasslands were the only sites in our study to share sequence haplotypes, considering that they are 230 km apart on different sides of the Alps and strongly differing with regard to edaphic conditions and plant communities.

Although it was shown that species-level richness is not necessarily reduced to one or two species in all arable soils (Hijri *et al.* 2006), it is generally expected to be lower than in semi-natural soils (Helgason *et al.* 1998; Oehl *et al.* 2003). In contrast, the data presented here show that *G. intraradices* mtLSU RFLP types are in fact more diverse in arable sites and allow to hypothesize that disturbance promotes genotypic diversity in some adapted AMF species, rather than reducing it. Higher intraspecific diversity could also be correlated with higher abundance of the species in a given setting. Testing this would in addition require assessing species-level diversity qualitatively and quantitatively. In this context it is interesting that Munkvold *et al.* (2004)

proposed that even in systems of low species diversity, considerable functional diversity is still maintained.

Studies of populations of ectomycorrhizal fungi (Kretzer *et al.* 2005; Geml *et al.* 2006; Carriconde *et al.* 2008) have frequently revealed unexpectedly high diversity and 'cryptic' species, but few generalizations seem to be possible about their biogeography, which seems to be related to specific ecological strategies of the respective species (Bruns & Kennedy 2009). On the contrary, studying populations of phytopathogenic fungi has provided a better understanding of their epidemiology and the evolutionary and ecological processes involved (e.g. Hovmøller *et al.* 2008).

By providing the first direct insights into the genetic structure of field populations of the Glomeromycota, an ecologically important group of mutualistic symbionts, our study opens up a new range of questions that can be addressed now. It demonstrates that *G. intraradices* GLOM A-1, possibly one of the most frequently occurring fungal species or species complexes in the world, is strongly structured genetically with regard to habitats and environmental conditions. It is a first step towards a better understanding of the processes generating and maintaining diversity in AMF and their reciprocal interaction with plants.

3.6 Acknowledgements

Chapter 4: Development of specific mitochondral markers for the *Glomus intraradices* isolate BEG140

4.1 Introduction

The *Glomus intraradices* isolate BEG140 was used in an inoculation experiment performed by the Department of Mycorrhizal Symbioses, Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice. The study site was situated in a coal mine spoil bank Merkur near Chomutov, North Bohemia, Czech Republic. The scope of this experiment was to test whether organic matter or AMF inoculation have an effect on the growth of a technical crop (*Phalaris arundinacea* var. ´Palaton S´) in the major context of reclamation of this mine spoil bank. In order to trace BEG140 directly in the symbiotic state, that is, in the roots of *P. arundinacea* and to address the questions of inoculation success and the persistence of this strain over time, the development of specific molecular markers was required. In chapter 3, it was reported that the mitochondrial rRNA large subunit gene (mtLSU) is a useful molecular marker for the detection of genetically different *G. intraradices* strains directly in colonized plant roots from field settings. Moreover, the specific detection of mtLSU haplotype I in roots was demonstrated by using a specific nested PCR approach (see chapter 3). A cooperation project with Zuzana Sýkorová at the Academy of Sciences of the Czech Republic was initiated in order to develop a similar approach for BEG140.

AMF-inoculation as a bio-fertilization tool for agriculture and horticulture has been increasingly applied (Gianinazzi & Vosatka 2004). However, it has not been possible to prove the efficiency of this treatment in terms of inoculum establishment so far. Therefore, the results of this project would be of major importance for basic research on the ecology of AMF on the within-species level, as well as for the commercial use of mycorrhizal inoculation.

4.2 Characterization of mitochondrial haplotypes of BEG140

4.2.1 Materials and Methods

The isolate BEG140 was collected in 1998 in the Chvaletice region of the Czech Republic by Jana Rydlová and later on cultivated and stored as multi-spore culture at the Department of Mycorrhizal Symbioses (Průhonice, see section 4.1). These cultures were established in open pots with *Zea mays, Trifolium pratense* or *Plantago lanceolata* as host plants, and either sterilized substrate from the collection site (7 pots) or inert substrate (a mixture of sterilized zeolite and sand 1:1 or 1:2, in 18 pots) were used. Furthermore, the isolate was deposited in the International Bank for the

Glomeromycota (BEG; see http://www.kent.ac.uk/bio/beg/englishhomepage.htm) and inoculum of this isolate is being continuously produced by the company Symbiom, Lanškroun, Czech Republic (see http://www.Symbiom.cz/).

Three main sources were used for the characterization of the isolate BEG140: (i) one sample of ca 35 g of the original inoculum (zeolite-liapor-mixture, containing maize root fragments and spores; produced by Symbiom) used for the field experiment in 2006, (ii) twenty-five open pot cultures established from 1999 to 2007 in Průhonice, and (iii) eight cultures obtained from Symbiom in 2009.

The characterization of the isolate BEG140 was performed as described in chapter 3: (i) DNA extraction out of spores or plant roots. (ii) Amplification of the mtLSU between the priming sites RNL-29 and RNL-30, whereas the improved nested PCR approach for colonized plant roots was used. (iii) Identification of the RFLP profile: PCR products were digested using the enzymes *Dra*III, *Bsa*JI and *Hin*dIII, and loaded in this order onto agarose gels. (iv) PCR products representing different RFLP types were cloned and sequenced for the determination of the corresponding mtLSU sequence type.

These approaches were mainly performed in Průhonice, but one DNA extract from one root sample of the open pot cultures was completely analyzed in Basel. The ABI 310 capillary sequencer was used for sequencing. Sequencing forward primers were M13fwd, RNL-11, 13, 16, 35, 37, 24, 25 and sequencing reverse primers were M13rev, RNL-26, 27, 7, 38, 36, 70, 71. For primer sequences see Raab *et al.* (2005) and chapter 2. Newly designed were RNL-70 (AGC TCG GAA TTG AAC CAT AG) and RNL-71 (TCC TCC TAC GAG GAT TTC AC). For this sample, methods, chemicals and kits were exactly the same as used in chapter 3, after DNA extraction. DNA extractions out of spores (2-11 sub-samples per sample mentioned above; 1-3 spores per sub-sample) were mainly performed in Průhonice (8 sub-samples from one open pot culture were analyzed in Basel), using the quick method developed by Redecker *et al.* (1997) as in chapter 2. The DNA extraction from a plant root sample later analyzed in Basel was performed in Průhonice, using the Ultra Clean Soil DNA kit (produced by MO BIO; supplied by Elisabeth Pharmacon, Brno, Czech Republic). The DNA extraction of a root sample from the original inoculum was performed using the DNeasy plant mini kit (Qiagen, Hilden, Germany). The following procedures applied in Průhonice differed from those described in chapter 3: PCR products of the second nested reaction were purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA), cloning was performed using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), and re-amplified clones were purified using the QIAquick PCR Purification Kit (Quiagen, Hilden, Germany). For sequencing, one amplified and purified clone from the root sample analyzed in Průhonice was sent to the company GATC (www.gatc-biotech.com; Germany). For sequencing the forward primers M13fwd, RNL-29, 16, 12, 24, 25 and the reverse primers M13rev, RNL-30, 26, 27, 7, 7b and 36 were chosen. For primer sequences see Raab *et al.* (2005) and chapter 2.

4.2.2 Results and Discussion

MtLSU PCR products could be amplified out of 17 from a total of 34 cultures. Three different RFLP types could be distinguished and were named Intra A, B and C. Intra C corresponds to Intra1 (see Fig. 8 in chapter 3), which is widely occurring in different field locations (see chapters 2 and 3). This RFLP type was found in two spores from only one open pot culture established in Průhonice and was therefore considered as greenhouse contamination. The RFLP type Intra A (see Fig. 1) was detected in the original inoculum, two open pot cultures and five cultures from Symbiom purchased in 2009. Intra B (see Fig. 1) was found in six open pots and four cultures from Symbiom from 2009. Based on these results, it could be confirmed that the isolate BEG140 is represented by two different RFLP types.

One PCR product of both was sequenced completely for the determination of the corresponding mtLSU sequence type (see section 4.2.1: haplotype A was analyzed in Průhonice, haplotype B was sequenced in Basel). None of the two mtLSU haplotypes has been detected in previous studies (Raab *et al.* 2005; Croll *et al.* 2008; Lee & Young 2009; chapters 2 and 3). The sequence structure of haplotype A and B is shown in comparison to known mtLSU haplotypes of *G. intraradices* in Fig. 2. Haplotype A contains a unique intron "position 2", that lacks a sequence region of 42 bp, which is present in the furthermore identical intron at this position found in the haplotypes II and III (see chapters 2 and 3). An intron "position 1" is missing in haplotype A and the intron "position 3" is identical to those found in the haplotypes II, X, XXIII, XXIV (see chapters 2 and 3) and haplotype B. The latter contains an intron "position 2", which is identical to those of haplotypes X and XXIV (see chapters 2 and 3), but also revealed a unique intron at position 1. This intron is most similar to intron "position 1" found in haplotype XVII (see chapter 2) detected by Raab *et al.* (2005), but lacks a sequence region of 39 bp and differs furthermore in four base pair changes.

The mtLSU haplotypes A and B were analyzed phylogenetically as described in chapter 3, using the same data set and alignment. Both haplotypes grouped in the same clade, comprising a number of isolates, including DAOM197198 (which is subject of the genome sequencing project of *G. intraradices* (Martin *et al.* 2008) and FACE#494, of which the complete mitochondrial genome was sequenced (Lee & Young 2009; not shown, but see chapter 3).

Based on the sequence results, precise restriction fragment sizes of both haplotypes were calculated *in silico*: haplotype A is characterized by 373, 1920 (*Dra*III), 223, 2070 (*Bsa*JI) and 982, 508, 585, 218 bp fragments (*Hin*dIII) (see Fig. 1). Haplotype B is characterized by 1442, 1886 (*Dra*III), 106, 1186, 2036 (*Bsa*JI) and 570, 1447, 508, 585, 218 bp fragments (*Hin*dIII) (see Fig. 1). Despite the overall similarity between the haplotypes A and II, the corresponding RFLP patterns (Intra A and Intra2a) are easily to distinguish as the additional sequence region of the latter (see above) reveals an additional restriction site for the enzyme *Bsa*JI (see Fig. 1). However, by comparing the patterns in the modified spreadsheet developed by Dickie *et al.* (2003) (see chapter

2), containing the fragment data of all known mtLSU haplotypes of *G. intraradices*, *G. proliferum* and *G. clarum*, the RFLP pattern of haplotype A revealed high similarity to the RFLP patterns Intra20 and Intra24 of the haplotypes XX and XXIV. The latter types could be distinguished by Börstler *et al.* (2010) (see chapter 3) as one contains a RFLP fragment clearly above 1 000 bp, whereas the other shows a fragment below 1 000 bp in the *Hin*dIII restriction digest. The corresponding fragment of haplotype A falls between these two sizes. The patterns might therefore be difficult to discern on some gels (see Fig. 1). In contrast, Intra B is so far unique and easy to distinguish from the remaining RFLP types currently known.
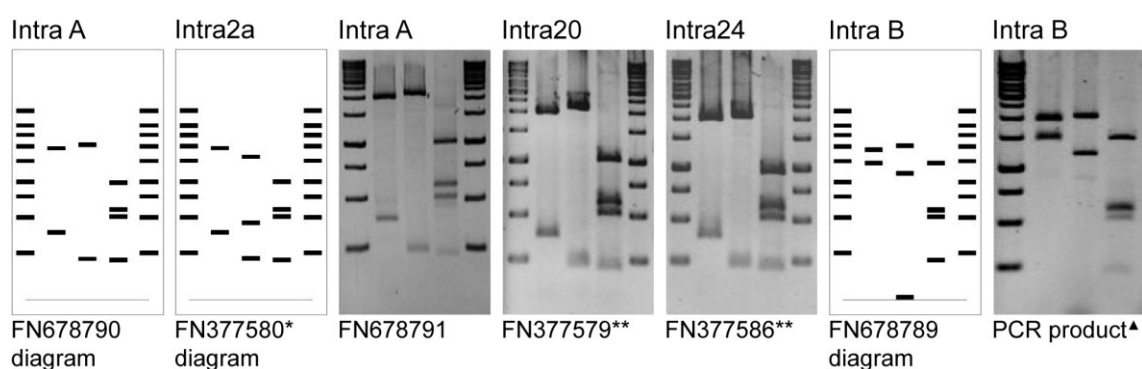

Fig. 1 Banding patterns of RFLP types Intra A and Intra B in comparison with the respective patterns of Intra2a, Intra20 and Intra24. PCR products were amplified using the primer pair RNL-29/RNL-30 for the mitochondrial rRNA large subunit gene (mtLSU; see chapter 3). DNA was digested using the restriction enzymes *Dra*III, *Bsa*JI, *Hin*dIII as described in chapter 2 and loaded in the same order per sample onto the gels. DNA ladder in left-most lane of each gel (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000. Size standard in the left-most lane of the diagrams produced *in silico* (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 4000. Accession numbers of the respective clones are shown beneath the patterns. ▲ Original PCR product from which the clone FN678789 was derived. * Analyzed by Börstler *et al.* (2008); see chapter 1. ** Analyzed by Börstler *et al.* (2010); see chapter 2. The restriction digests of Intra A and B were performed by Z. Sýkorová.

Fig. 2 Mitochondrial rRNA large subunit gene (mtLSU) region containing two exons and up to three introns (positions 1, 2, 3) for *Glomus intraradices* haplotypes A, B and I–XXXIII in 5′–3′ orientation. The corresponding clones used as representatives for the haplotypes are shown in Figs 5 and 6 and Table 1. Exons are shaded in grey. Different introns can be recognized by the type code defined by Börstler *et al.* (2008) and Börstler *et al.* (2010); see Table 4 in chapter 2 and Table 6 in chapter 3. Intron regions identical to those found in the haplotypes A and B are shown in the same color. In case of minor sequence differences of 4 single base pairs compared to the corresponding intron region of haplotype B, this region was highlighted in dark orange for haplotype XVII. RNL-primers (2c, 16, 38, 85, 87, 88, 89, 90, 142, 145) used for the specific detection of the haplotypes A and B are shown as arrows at the respective annealing sites. Drawn to scale.

4.3 Development of specific nested PCRs for the mitochondrial haplotypes A and B of BEG140

4.3.1 Materials and Methods

The mtLSU sequences of the haplotypes A and B were screened for sequence regions of general (universal in all so far known haplotypes), characteristic (specific for more than one haplotype) or unique primers, suitable for an application in a potentially specific nested PCR approach for each type. In order to optimize specificity, the position of potential primers was considered: certain haplotypes can simply be excluded from amplification when the primer is localized in an intron which is missing in these types. The combination of four primers in a nested PCR approach is evidently instrumental to increase specificity: if just one fails to fit the template, amplification success is most unlikely. Moreover, the nesting of the primers creates smaller amplicons, which are not only easy to amplify but also less problematic to sequence, as demonstrated in the specific PCR approach for haplotype I (see chapter 3). The quality of potential primers was improved using the program Primer Designer (version 3.0; Scientific & Educational Software, Cary, NC, USA).

For haplotype A, the primer pair RNL-16 (forward)/RNL-89 (reverse) was chosen for the first PCR reaction of the nested PCR approach, and the primer pairs RNL-2c/RNL-90 and RNL-2c/RNL-145 were suggested as two alternative approaches for the final PCR reaction. For haplotype B, the primer pair RNL-85/RNL-38 was chosen for the first PCR, and the alternative primer pairs RNL-87/RNL-88 and RNL-87/RNL-142 for the final PCR. The following primers were newly designed: RNL-85 (CAG CCA ATT ATG GTG TTA GT), RNL-87 (AAG CAG GAA GAA GGA AGA CT), RNL-88 (GCA GCA GAA CGT CAT CTC TA), RNL-89 (CTA TGC AAC CGT AGG TAA GC), RNL- 90 (CTA AGA GGG TCA GGA GAC AC), RNL-142 (GTT GCC TCT GTC CTT ATA GC) and RNL-145 (ATA GGT ACG CCT AAG AGG GT). For sequences of the other primers see Raab *et al.* (2005) and chapter 2. Fig. 2 shows which part of the mtLSU sequence region is targeted by the particular primer.

The PCR conditions and dilutions of the templates were chosen according to the specific nested PCR approach for the mtLSU haplotype I (see chapter 3): the PCR master mixes of all first and second reactions for the haplotypes A and B were the same as the respective reactions for haplotype I. The cycling parameters were also the same with following minor exceptions and further adjustments: for all PCR reactions only 30 cycles were applied. The elongation time of the second PCR reactions was decreased to 1 min 30 s. In Průhonice, all PCRs of the second reaction were performed with a denaturing temperature of 94°C in instead of 95°C like in Basel or for haplotype I. Furthermore, suitable annealing temperatures, increasing the specificity of the primers, were determined by gradient PCRs mainly performed in Průhonice (examples from Basel are shown in Fig. 3): the temperature for all first PCR reactions was 61°C. Possible higher

temperatures were avoided in order to facilitate amplification of low template amounts. However, the temperatures were considerably increased for the second PCR reactions: RNL-2c/90 (66°C), RNL-2c/145 (64.5°C), RNL-87/88 (64°C) and RNL-87/142 (64°C). The expected product lengths of the first PCR reactions are 1 440 bp (haplotype A) and 1 441 bp (haplotype B).
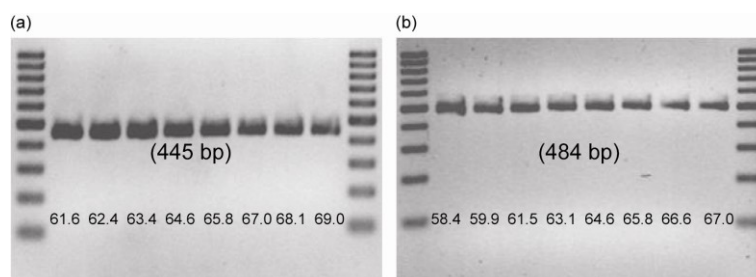

Fig. 3 PCR products of gradient PCRs for the second reaction of the nested PCR approaches using **(a)** RNL-2c/RNL-145 (developed for haplotype A) and **(b)** RNL-87/RNL-142 (developed for haplotype B). The respective temperatures (°C) are shown beneath the bands, the PCR product length is shown in brackets. The template for (a) was a PCR product of a first PCR reaction using the primer pair RNL-16/RNL-89 (see Fig. 4), diluted 1:100 in distilled water. For this reaction a DNA extract of haplotype II (isolate CC-4; see chapter 2) was used, which has an identical sequence structure compared to haplotype A, with the exception of a 42 bp fragment within the amplicon of the second PCR reaction. The template for (b) was a PCR product of a first PCR reaction using the primer pair RNL-85/RNL-38. For this reaction the DNA extract of BEG140 was used, originating from a pot culture from Průhonice and analyzed in Basel (see section 4.2). A 100 bp ladder was used (#SM0242; Fermentas Life Science; www.fermentas.com)

4.3.2 Results and Conclusions

The product lengths of the second PCR reactions are listed in Table. 1. The success of the amplification of the haplotypes A and B was confirmed by gradient PCRs (examples are shown in Fig. 3). Moreover, the amplification success was additionally tested *in silico* (see Table 1), using the program Amplify, version 3, © Bill Engels, 2005, University of Wisconsin (http://engels.genetics.wisc.edu/amplify/). In this approach, one representative of all known mtLSU haplotypes of *G. intraradices*, *G. proliferum* and *G. clarum* was also tested for potential amplification success. Furthermore, PCR reactions were applied practically for selected examples of available templates, which revealed positive or questionable results in the *in silico* analysis (see Fig. 4). All samples used in this application were initially tested with the general PCR-RFLP approach for mtLSU haplotypes of *G. intraradices* (see section 4.2.1), in order to confirm the template quality and identity (not shown).

The analyses revealed that the primer pairs RNL-2c/145 (haplotype A) and RNL-87/142 (haplotype B) of the second PCR reactions showed the most promising results for a specific nested PCR approach. The respective annealing sites of these primers are shown in comparison to sequences of all known mtLSU haplotypes of *G. intraradices*, *G. proliferum* and *G. clarum* in Fig. 5 (haplotype A) and Fig. 6 (haplotype B). Only few additional haplotypes besides the targets

possibly would amplify using these primers, namely haplotype II using the nested PCR for haplotype A, and haplotype XVII using the nested PCR for haplotype B. In both cases, amplicons of the additional haplotypes can be easily distinguished from the expected targets by sequencing, as both haplotype A and B so far reveal unique sequences in the final PCR products (see section 4.2.2). However, only PCR products of the same product size must be verified by sequencing. For example, it would not be necessary for PCR products of haplotype II, which differ in product size from haplotype A by 42 bp (see Table 1). Besides length polymorphism and sequencing confirmation, samples with positive PCR products of the specific approach can be further verified by additionally testing the DNA extracts with the general PCR-RFLP approach for mtLSU haplotypes, as it was demonstrated for haplotype I (see chapter 3). All specific PCR approaches developed in this thesis are compared and generally discussed on an ecological background in chapter 5.

Table 1 Results of *in silico* amplifications for all PCR reactions developed for the specific detection of the mtLSU haplotypes A and B of the *Glomus intraradices* isolate BEG140. The program Amplify, version 3, © Bill Engels, 2005, University of Wisconsin (http://engels.genetics.wisc.edu/amplify/) was used. In addition to the haplotypes A and B, one representative of all mtLSU haplotypes sequenced so far between the priming sites RNL-29/RNL-30 of *G. intraradices* (I-XXXIII), *G. clarum* (clar) and *G. proliferum* (prol) (see Raab *et al.* 2005; chapters 2 and 3), were tested for successful amplification based on all available sequences. Possible products caused by primer mismatches are shown in brackets. For all results of the second PCR reactions, the size of the amplicons is shown. These results are marked by an asterisk if amplification would most probably fail in a nested PCR reaction, because of missing priming sites in the products of the first PCR reaction. In addition, products (including those of primer mismatches) are shaded when they are part of a complete nested PCR. Some reactions revealed additional misamplifications (▲). In these cases, only the most likely product is shown. However, in the practical PCR test using the primers RNL-87/RNL-142 for haplotype B, only single bands were observed (Fig. 3).

Accession number	Haplotype	1. reaction/ haplotype A RNL-16/ RNL-89	2. reaction/ haplotype A RNL-2c/ RNL-90	2. reaction/ haplotype A RNL-2c/ RNL-145	1. reaction/ haplotype B RNL-85/ RNL-38	2. reaction/ haplotype B RNL-87/ RNL-88	2. reaction/ haplotype B RNL-87/ RNL-142
FN678790	A	yes	yes, 393 bp	yes, 403 bp	(yes)	no	no
FN678789	B	yes	(yes, 393 bp)	no	yes▲	yes, 530 bp▲	yes, 484 bp▲
FN377598	XXXIII	yes	no	no	no	(yes, 1543 bp)	no
FN377597	XXXII	yes	no	no	no	(yes, 1543 bp)	no
FN377595	XXXI	no	no	no	no	(yes, 1423 bp)▲	(yes, 1423 bp)
FN377594	XXX	no	no	no	no	no	no
FN377593	XXIX	yes	no	no	no	(yes, 1556 bp)	no
FN377592	XXVIII	yes	no	no	no	(yes, 1562 bp)	no
FN377590	XXVII	yes	no	no	no	(yes, 1562 bp)	no
FN377589	XXVI	yes	no	no	no	(yes, 1556 bp)	no
FN377588	XXV	(yes)▲	no	no	no	(yes, 1385 bp)▲	(yes, 1601 bp)▲
FN377586	XXIV	yes	(yes, 393 bp)	no	(yes)	no	no
FN377585	XXIII	yes	no	no	(yes)	(yes, 1385 bp)▲	no
FN377584	XXII	no	no	no	no	(yes, 1385 bp)▲	no
FN377580	XXI	yes	(yes, 393 bp)	no	(yes)	no	no
FN377579	XX	yes	(yes, 393 bp)	no	(yes)	no	no
FN377578	XIX	no	(yes, 393 bp)	no	no	no	no
FN377576	XVIII	yes	no	no	no	(yes, 1562 bp)	no
AJ841288	XVII	no	no	no	(yes)	yes, 530 bp▲	yes, 484 bp▲
AM950227	XVI	yes	no	no	no	(yes, 564 bp)▲	(yes, 359 bp)
AM950226	XV	yes	no	no	no	no	no
AM950222	XIV	no	no	no	no	no	no
AM950221	XIII	yes	no	no	no	(yes, 1562 bp)	no
AM950220	XII	no	no	no	no	no	no
AM950218	XI	yes	no	no	no	no	no
AM950217	X	yes	(yes, 393 bp)	no	(yes)	(yes, 358 bp)*▲	(yes, 358 bp)*
AM950216	IX	no	no	no	no	no	no
AM950215	VIII	no	no	no	no	no	no
AM950214	VII	yes	no	no	(yes)	no	no
AM950213	VI	yes	no	no	no	(yes, 1543 bp)	no
AM950210	V	no	(yes, 393 bp)	no	no	(yes, 564 bp)▲	(yes, 359 bp)
AM950208	IV	yes	(yes, 393 bp)▲	(yes, 952 bp)*	no	(yes, 366 bp)▲	(yes, 525 bp)▲
AJ938171	III	no	yes, 435 bp	yes, 445 bp	no	no	no
AM950204	II	yes	yes, 435 bp	yes, 445 bp	(yes)	no	no
AJ973192	I	no	(yes, 393 bp)	no	no	(yes, 358 bp)▲	(yes, 358 bp)
AM040980	prol	no	no	no	no	no	no
FN377601	clar	no	no	no	no	(yes, 527 bp)▲	(yes, 360 bp)

(a)　　　　　　　　　　　(b)　　　　　　　　　　　(c)

1 500 bp　　　　　　　　　　　400 bp　　　　　　　　　　　400 bp

X XXIV I II IV III VIII　　　X XXIV I II IV III VIII　　　X XXIV I II IV III VIII
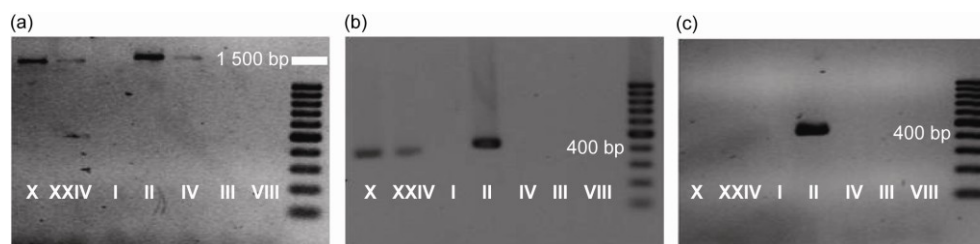
Fig. 4 Verification of the specificity of the PCR approach developed for haplotype A, using **(a)** the primer pair RNL-16/RNL-89 **(b)** RNL-2c/RNL-90 or **(c)** RNL-2c/RNL-145: DNA extracts of the mtLSU haplotypes X (isolate NB102c), XXIV (field root sample), I (isolate JJ291), II (isolate CC-4), IV (isolate SW205), III (field root sample) and VIII (isolate JA202) were tested for potential amplifications. The PCR products of the first reaction (a) were used as template (diluted 1:100 in distilled water) for the second PCR reaction (b, c). For the origin of isolates and field root samples, see chapters 2 and 3. A 100 bp ladder was used (#SM0242; Fermentas Life Science; www.fermentas.com)

```
                  RNL-16                RNL-89                RNL-2c                RNL-145

                  Exon part 1           Intron pos. 3         Exon part 1           Intron pos. 2

FN678790_A        ACCTGGAGATAGCTGGTCTT  CTATGCAACCGTAGGTAAGC  TCGTGATAAGGCGATTCGTC  ATAGGTACGCCTAAGAGGGT
FN678789_B        ....................  ....................  ....................  [shaded hyphens].
FN377598_XXXIII   ....................  ....................  ....................  [shaded hyphens]
FN377597_XXXII    ....................  ....................  ....................  [shaded hyphens]
FN377595_XXXI     ....................  [grey]                ....................  [grey]
FN377594_XXX      ....................  [grey]                ....................  [grey]
FN377593_XXIX     ....................  ....................  ....................  [shaded hyphens]
FN377592_XXVIII   ....................  ....................  ....................  [shaded hyphens]
FN377590_XXVII    ....................  ....................  ....................  [shaded hyphens]
FN377589_XXVI     ....................  ....................  ....................  [shaded hyphens]
FN377588_XXV      ....................  .......CTT.......G..  ...............T...   [grey]
FN377586_XXIV     ....................  ....................  ...............T...   [shaded hyphens].
FN377585_XXIII    ....................  ....................  ...............T...   [shaded hyphens]
FN377584_XXII     ....................  [grey]                ...............T...   [shaded hyphens]
FN377580_XXI      ....................  ....................  ....................  TA.TTCC.TTAGG.A.ACC.
FN377579_XX       ....................  ....................  ....................  TA.TTCC.TTAGG.A.ACC.
FN377578_XIX      ....................  [grey]                ....................  TA.TTCC.TTAGG.A.ACC.
FN377576_XVIII    ....................  ....................  ....................  [grey]
AJ841288_XVII     ....................  [grey]                ...............T...   [grey]
AM950227_XVI      ....................  ....................  ....................  [grey]
AM950226_XV       ....................  ....................  ....................  [grey]
AM950222_XIV      ....................  [grey]                ....................  [shaded hyphens]
AM950221_XIII     ....................  ....................  ....................  [grey]
AM950220_XII      ....A...............  [grey]                .T.........A...T...   [grey]
AM950218_XI       ....A...............  ....................  ..A.............T...  [grey]
AM950217_X        ....................  ....................  ....................  [shaded hyphens].
AM950216_IX       ....................  [grey]                ....................  [grey]
AM950215_VIII     ....................  ....................  ....................  TA.TT.C.TTAGG.A.ACC.
AM950214_VII      ....................  ....................  ....................  .ATCAA.TAT.A.CA.TT.A
AM950213_VI       ....................  ....................  ...C................  [shaded hyphens]
AM950210_V        ....................  [grey]                ....................  TA.TTCC.TTAGG.A.ACC.
AM950208_IV       ....................  ....................  ....................  TA.TTCC.TTAGG.A.ACC.
AJ938171_III      ....................  [grey]                ....................  ....................
AM950204_II       ....................  ....................  ....................  ....................
AJ973192_I        ....................  [grey]                ....................  TA.TTCC.TTAGG.A.ACC.
AM040980_prol     ....A...............  [grey]                .T.........A...T...   [grey]
FN377601_clar     .................T..  ....................  ...............T...   ....................
```

Fig. 5 Sequences of RNL-16/RNL-89 (first PCR reaction) and RNL-2c/RNL-145 (second PCR reaction) of the nested PCR approach developed for mtLSU haplotype A in comparison to sequences of all other available mtLSU haplotypes at the respective annealing site. The alignment is always shown in 5′–3′ orientation of the primers. Accession numbers are provided for the representatives of the mtLSU haplotypes A, B, I-XXXIII of *Glomus intraradices*, *G. proliferum* (prol) and *G. clarum* (clar). Identical sequences to haplotype A are shown as dots in the respective positions. Sequences which do not contain the respective intron at the primer annealing site are shown in dark grey. Gaps at the primer locations are shown as shaded hyphens.

```
                        RNL-85                  RNL-38                    RNL-87                  RNL-142
                        Intron pos. 1           Intron pos. 2             Intron pos. 1           Intron pos. 1

FN678789_B       CAGCCAATTATGGTGTTAGT    AGCTTGGACTAACCCACTAATG    AAGCAGGAAGAAGGAAGACT    GTTGCCTCTGTCCTTATAGC
FN678790_A       [grey]                  ....................      [grey]                  [grey]
FN377598_XXXIII  .................C      ....................      ........GA.TAAC.TTGC    [grey]
FN377597_XXXII   .................C      ....................      ........GA.TAAC.TTGC    [grey]
FN377595_XXXI    .................C      ....................      ........GA.TAAC.TTGC    [grey]
FN377594_XXX     .................C      ....................      ........GA.TAAC.TTGC    [grey]
FN377593_XXIX    .................C      ....................      ........GA.TAAC.TTGC    [grey]
FN377592_XXVIII  .................C      ....................      ........GA.TAAC.TTGC    [grey]
FN377590_XXVII   .................C      ....................      ........GA.TAAC.TTGC    [grey]
FN377589_XXVI    .................C      ....................      ........GA.TAAC.TTGC    [grey]
FN377588_XXV     ...................      [grey]                    ....................      [grey]
FN377586_XXIV    ...................      [grey]                    ....................      [grey]
FN377585_XXIII   ...................      ....................      ....................      [grey]
FN377584_XXII    ...................      ....................      ....................      [grey]
FN377580_XXI     [grey]                  ....................      [grey]                  [grey]
FN377579_XX      [grey]                  ....................      [grey]                  [grey]
FN377578_XIX     [grey]                  ....................      [grey]                  [grey]
FN377576_XVIII   .................C      ....................      ........GA.TAAC.TTGC    [grey]
AJ841288_XVII    ...................      ....................      ....................      ....................
AM950227_XVI     ...........AA....C      ....................      ..........CTATGA.GA    ...........T....TC.
AM950226_XV      [grey]                  ....................      [grey]                  [grey]
AM950222_XIV     .................C      ....................      ........GA.TAAC.TTGC    [grey]
AM950221_XIII    .................C      ....................      ........GA.TAAC.TTGC    [grey]
AM950220_XII     ...................      [grey]                    [grey hyphen]            [grey]
AM950218_XI      .......A....AA....C      [grey hyphen]             ..........C..TCTT.A.    [grey]
AM950217_X       ...........AA....C      ....................      ..........CTATGA.GA    ...........T....T.
AM950216_IX      [grey]                  [grey]                    [grey]                  [grey]
AM950215_VIII    [grey]                  ....................      [grey]                  [grey]
AM950214_VII     [grey]                  ....................      [grey]                  [grey]
AM950213_VI      .................C      ....................      ........GA.TAAC.TTGC    [grey]
AM950210_V       ...........AA....C      ....................      ..........CTATGA.GA    ...........T....TC.
AM950208_IV      .................C      ....................      ..........CTAT.A.GA    ...A.............T.
AJ938171_III     [grey]                  ....................      [grey]                  [grey]
AM950204_II      [grey]                  ....................      [grey]                  [grey]
AJ973192_I       ...........AA....C      ....................      ..........CTATGA.GA    ...........T....T.
AM040980_prol    [grey]                  [grey]                    [grey]                  [grey]
FN377601_clar    ...........AA....C      [grey]                    ..........CTATGA.GA    ...........T....TC.
```

Fig. 6 The sequences of RNL-85/RNL-38 (first PCR reaction) and RNL-87/RNL-142 (second PCR reaction) of the nested PCR approach developed for the mtLSU haplotype B in comparison to sequences of all other available mtLSU haplotypes at the respective annealing site. The alignment is always shown in 5′–3′ orientation of the primers. Accession numbers are provided for the representatives of the mtLSU haplotypes A, B, I-XXXIII of *Glomus intraradices*, *G. proliferum* (prol) and *G. clarum* (clar). Identical sequences to haplotype B are shown as dots in the respective positions. Sequences which do not contain the respective intron at the primer annealing site are shown in dark grey. Gaps at the primer locations are shown as shaded hyphens.

Chapter 5: General discussion

5.1 General context and progress using mitochondrial rRNA large subunit gene (mtLSU) markers

The MSc thesis of Anna Ochsner (Ochsner 2002) and the PhD research of Philipp Raab (Raab 2007) represent the basis for the studies of this thesis as well as the PhD project of Odile Thiéry: Raab *et al.* (2005) provided the first primers and sequence data of the mitochondrial rRNA large subunit gene (mtLSU) from two *Glomus intraradices* isolates, one *G. proliferum* isolate and a root-colonizing AMF (see section 1.3.2), which were helpful for the development of new PCR approaches for *G. intraradices* (chapters 2, 3 and 4) and also for other AMF species (appendix). These two subsequent studies also built on each other and provided helpful information and tools that could be exchanged. For example, mtLSU sequences of AMF other than *G. intraradices* generated by Odile Thiéry provided helpful information for a first classification of unknown "Fung types" described in chapter 3.

The mtLSU markers and molecular methods for their detection have been adopted by other research groups: the practical application of the PCR-RFLP approach described in section 2 was taught in the COST (European Cooperation in the Field of Scientific and Technical Research) Action 870 workshop, held at INRA, Dijon, in May 2009. Moreover, the data published by Raab *et al.* (2005) allowed the investigation of mitochondrial haplotypes by Croll *et al.* (2008), who developed another PCR approach used for *G. intraradices* isolates cultivated on root organ cultures (ROCs).

The results of this thesis were discussed in detail in the respective chapters. In the following the main discussion points of the previous sections are taken up again and set in the broader context, outlined in the general introduction (chapter 1) to this thesis. Finally, some newest literature reports and further results are mentioned that open directions for possible future research, based on mitochondrial gene markers.

5.2 Suitability and reliability of the general PCR-RFLP approach for the detection of mitochondrial haplotypes of *Glomus intraradices*

The PCR-RFLP approach for the mtLSU of *G. intraradices* was developed on the basis of a set of 16 different isolates originating from different parts of the world and different ecosystems. It was shown that several mtLSU sequence haplotypes could already be distinguished by the size of the PCR products. This is because the length of mtLSU gene sequences varies considerably among

individual *G. intraradices* strains (size range: 1 110 – 3 975 bp; chapter 2). Presence and absence of length-variable introns explains these pronounced differences in *G. intraradices* mtLSU haplotypes (chapter 2). The retrieval of mtLSU haplotypes known from fungal isolates in field-grown roots nearly one decade after these isolates had been collected, is evidence for the great potential of our mtLSU marker system and intron stability (chapter 3), which was also shown by comparing different lineages of the same isolate (chapter 2). Despite their considerable length, the mtLSU fragments amplified with our PCR primer sets do not impose major difficulties in amplification, as PCR chemistry has improved markedly in recent years. Moreover, the quality of PCR products was confirmed by directly sequencing a 3 293 bp mtLSU fragment. In fact, the recently published nuclear rDNA marker system by Krüger *et al.* (2009) relies on similarly sized PCR amplicons as those from the mtLSU, although less variable in length. In contrast, the intra-strain genetic homogeneity of the mitochondrion and the inter-strain sequence length polymorphism are considerable advantages of the mtLSU approach, which permit more straight forward taxon discrimination. All of these features of mtLSU markers may help to reduce cloning and sequencing efforts, once an inventory of the different mtLSU haplotypes from a field site has been established.

The banding patterns of the RFLP types already indicate whether the corresponding PCR product belongs to *G. intraradices* or another fungus: 96 % of all RFLP types of *G. intraradices* (Intra types) described in this thesis, thus all except one, contain a single restriction site for *Dra*III. In contrast, only 25% of RFLP types of unknown affiliation (Fung types) share this characteristic. In addition, only 16% of amplified sequences from the field samples did not belong to the expected target (chapter 3). Target taxon specificity of the PCR primers developed for the mtLSU of the GLOM A-1 group of *G. intraradices* is, in fact, higher than that of several PCR primers targeted to nuclear rDNA sequences, which frequently pick-up 25-40% of non-target templates (Börstler *et al.* 2006; Sýkorová 2007a,b). On the other hand, some co-amplification of closely related non-target sequences can be taken as evidence for comprehensive sequence sampling.

Assay development on the basis of information from a world-wide representative sample of 16 different *G. intraradices* isolates proved successful in the application at selected test sites, not requiring the use of additional restriction endonucleases to increase resolution (chapter 3). Correspondences between RFLP types that have been found repeatedly at several sites could be verified via confirmatory re-sequencing of short diagnostic regions, since minor sequence differences, which are only difficult to assess by RFLP, could have remained undetected. Minor sequence variations were typically found within the RFLP types Intra13 and Intra14, which co-occurred also in the same field site. Interestingly, no further variation was observed by sequencing more than one complete PCR product or at least characteristic parts of the same RFLP type from the same agricultural field experiment: besides the two completely sequenced products of Intra1 (in addition to all sequenced products of the specific approach) and the diagnostic regions of Intra3a

(see chapter 3), a diagnostic sequence region of Intra24 was sequenced for all five corresponding PCR products (unpublished data), and no single base pair differences were observed. These results indicate that the RFLP approach was largely sufficient to resolve mtLSU haplotypes within the same agricultural site. However, to strictly address this question, all mtLSU PCR products would have to be sequenced completely. Moreover, it cannot be excluded that an additional restriction enzyme might be useful in other field sites, including agricultural ones. In that case, an additional restriction enzyme could be introduced after initial analyses of samples. In conclusion, the application of this PCR-RFLP approach, in the two agricultural field experiments and two semi-natural grasslands of the present study, has shown that the marker was suitable to resolve considerable genetic structure of *G. intraradices* among and within the field sites. Nevertheless, detailed sequence analyses showed a higher resolution of mtLSU sequence types than RFLP analyses.

5.3 Alternative approaches for detecting intraspecific genetic variation in AMF species and progress in using mitochondrial marker genes

Markers based on microsatellite or simple sequence repeat (SSR) loci may reveal a higher genetic diversity in *G. intraradices* (Croll *et al.* 2008) than the mtLSU marker system. Therefore, to further increase the resolution, mtLSU markers may be combined with other co-dominant marker systems. However, all other available markers, i.e. microsatellites (Croll *et al.* 2008; Mathimaran *et al.* 2008a) or such based on protein-encoding genes (Stukenbrock & Rosendahl 2005b) have not been tested or applied directly on environmental samples. The marker system by Stukenbrock & Rosendahl (2005b) has only been applied on morphotyped spores from the field or from isolates in pot culture (Stukenbrock & Rosendahl 2005a; Rosendahl & Matzen 2008; Rosendahl *et al.* 2009) as the PCRs are not sufficiently taxon-specific (see section 1.2.5.4). Applying microsatellite marker systems to environmental samples in which several closely related non-target taxa may co-occur is problematic, since specificity can usually not be verified. Microsatellite flanking regions are intentionally kept short and thus often do not contain sufficient nucleotide information for taxon discrimination. Moreover, any multilocus typing approach on mixed-taxon samples faces the problem of not being able to link the obtained information among loci for the different taxa. Furthermore, taxon sampling is generally poor, with *G. intraradices* being the only comprehensively sequenced taxon at the moment, that any AMF sequence will turn out to show highest similarity to the database entry of *G. intraradices*, whether it originates from a member of the target species *G. intraradices* or not. Finally, it should not be assumed that closely related AMF species have diverged sufficiently to make all microsatellite loci species-specific. Intron-localized microsatellites may further be prone to horizontal transfer among taxa. Evidence for horizontal

transfer of an intron between *G. clarum* and *G. intraradices* was reported by Thiéry *et al.* (2010) (see appendix). In short, any approach involving microsatellite markers will have to be thoroughly tested against closely and more distantly related non-target taxa, before one can have a certain degree of confidence that, indeed, the target organism is detected when applied to taxonomically complex field samples.

Besides the above-mentioned alternative nuclear gene-based approaches, alternative mitochondrial genes may be evaluated as well for their marker potential. One such gene may be cox1, encoding the cytochrome c oxidase subunit 1. This gene is used for molecular "barcoding" in animals (Ratnasingham & Hebert 2007; http://www.barcoding.si.edu/). Unfortunately, however, length variation and frequently also total length exceed those acceptable for a reliable molecular genetic marker system (Stockinger *et al.* 2010). Borriello (2010) showed that the cox1 gene displayed almost no heterogeneity among different isolates of the same *Gigaspora*, *Scutellospora* and *Glomus* group A species, including two isolates of *G. intraradices*, which is in agreement with the lack of sequence variation in the mtLSU reported for non-*G. intraradices* AMF isolates by Thiéry *et al.* (2010) (see appendix). It has been consistent among all studies on mitochondrial sequences of AMF that no intra-individual sequence variation has yet been found (Raab *et al.* 2005). This homoplasmy of the mitochondrial genome is a favorable feature, since mitochondrial markers may develop into a valuable alternative to nuclear gene markers, which may show unfavorably high intra-individual and shared interspecific sequence diversity (section 1.2.5.4). Despite the fact that technological development in sequencing may soon allow an easy recovery of much longer sequence reads, nuclear markers might be no viable long-term alternative to mitochondrial gene markers. Although we have now a much clearer idea about the power of the various nuclear rDNA regions to delimitate AMF taxa (Stockinger *et al.* 2010), nuclear sequence diversity still imposes a major hindrance for straightforward interpretations of data from field studies on AMF populations and communities.

Given the availability of a whole mitochondrial genome (Lee & Young 2009) and the unfavorably high intra-individual nuclear gene diversity, alternative mitochondrial gene markers (listed in section 1.3.1) to those of the mtLSU and cox 1 may be worthwhile to explore, even though protein-encoding genes may be too conserved to be able to serve as markers at lower taxonomic levels (Borriello 2010).

Intron sequences, whether nuclear *GmFOX2* and *GmTOR2* (Stukenbrock & Rosendahl 2005a), or mitochondrial (this study) seem helpful for discriminating taxa in AMF, even when the exon regions of genes are highly conserved. Moreover, intergenic spacers might display enough variability for the differentiation of AMF species or even strains of the same species.

Overall, molecular genetic detection methods based on mitochondrial genes bear great potential, since they may not have the difficulties of nuclear gene markers in the discrimination of intra- versus inter-organism sequence diversity. Allelic information from multilocus genotyping of

taxonomically complex environmental samples will, however, not be assignable to individual organisms. Thus, the establishment of multilocus genotypes from the analysis of mixed template samples will remain an impossible task when using conventional PCR approaches. More novel emulsion PCR-based analyses (Williams *et al.* 2006) of mixed nucleic acid templates may provide an avenue to soon overcome this analytical bottleneck.

It would be interesting, to correlate different mitochondrial gene markers of different taxonomic resolution to possible host preferences or responses to other biotic or abiotic factors using ROCs, greenhouse or field experiments. A higher resolution of the genetic structure does not necessarily have to better mirror functionality (see section 3.5).

Different (mitochondrial) marker genes of differing resolution might be also helpful to solve problematic cases concerning cryptic species: phylogenies from more than one gene might better represent the history of the species than a possible history of a single gene, which might evolve at different rates compared to other genes (Rosendahl 2008).

5.4 Detection of single mitochondrial haplotypes of *G. intraradices* by specific nested PCR approaches

Three specific nested PCR approaches for the detection of the mtLSU haplotypes I (chapter 3), A and B (chapter 4) have been developed in the course of this PhD study. These three approaches differ in the specificity with which the different newly designed PCR primers detect either one single or two sequence haplotypes. The PCR assay to detect haplotype I showed absolute specificity, since no other sequence haplotype was ever detected, as confirmed by *in silico* amplification analyses using the software Amplify, and actual PCR amplification tests in the laboratory (not shown). The nested PCR assays for the haplotypes A and B, each yielded only one single additional haplotype, which can, however, be easily distinguished from the target type by sequencing analyses, showing also these PCR assays' rather high specificity.

Nevertheless, our field surveys, described in chapter 3, show that global mtLSU haplotype richness in *G. intraradices* must be considerably higher than in the set of 16 reference strains of members of the morphospecies *G. intraradices* (chapter 2). This means that the information about the specificity of our PCR assays should be taken only relative to our test material and that absolute specificity with respect to global mtLSU sequence richness may be considerably lower. Therefore, it is likely, that other so far unknown mtLSU types might be additionally detectable with the specific nested PCR approaches. However, in the present study using the specific approach for haplotype I, all positive samples were additionally and successfully confirmed with the general PCR-RFLP approach (see chapter 3). Nevertheless, if the target strain is only weakly colonizing the roots, its RFLP pattern might not become visible in the general approach.

All in all, when individual mtLSU types are to be traced in the field after application as inocula, prior pilot studies will be indispensable to make inventories of the natural mtLSU types before manipulation. With such initial investigations one could also identify the PCR assay, most appropriate to detect the mtLSU strain newly introduced to the given native field assemblage. Of course, inoculation schemes aiming at the tracing of fungal strains should ensure that the introduced strain is unique enough so that it can be reliably distinguished from native strains. The mtLSU approach may not provide for all AMF strains sufficient resolution, which means that also alternative tracing tools such as the generally more variable microsatellites (Croll *et al.* 2008; Mathimaran *et al.* 2008a) should be considered.

5.5 First evidence of population-level diversity in *G. intraradices*

5.5.1 Taxonomic resolution of the mitochondrial LSU marker

Using the newly developed mtLSU approach, we were the first to determine the population structure of an AMF directly within roots from the field (chapter 3). This was a considerable step forward in the study of individual strains in the symbiotic stage of a single AMF species. It was found that all mtLSU sequence haplotypes from two distantly located grasslands fall in one phylogenetic clade, which is distinct from a clade with haplotypes from arable fields and one in which both haplotypes from grasslands as well as arable fields group (Fig. 10 in chapter 3). However, the exact taxonomic resolution of the mtLSU exon remains to be determined, although it is clear that the analyzed mtLSU fragment has a higher resolution than the ITS fragment that was used to define phylotype *G. intraradices* GLOM A-1. Three mtLSU clades belong to this phylotype (Fig. 10 in chapter 3). Future studies may elucidate the taxonomic resolution of mtLSU exon sequences when the genus *Glomus* will have been taxonomically revised (see in the sections 1.2.1 and 1.2.2). As outlined in chapter 3, in the meantime Stockinger *et al.* (2009) verified that most environmental sequences, including those falling in phylotype GLOM A-1 and a considerable number of fungal isolates grown in living cultures do not actually belong to *G. intraradices*, and assigned it to *Glomus irregulare* (Blaszkowski *et al.* 2008). The separation of the type strain FL208 of *G. intraradices* from isolates belonging to the phylotype GLOM A-1 is also evident in Figs 5 and 7 in chapter 2. The exact taxonomic affiliation of phylotype *G. intraradices* GLOM A-1 and thus also our mtLSU exon sequence clades will have to await a morphological and phylogenetic re-description of *G. intraradices*. Unfortunately, the Botanical Code still demands a formal morphotaxonomic species description, even in species of fungi that are obviously difficult to classify morphologically (see sections 1.2.1 and 1.2.2).

Other mitochondrial genes, richer in phylogenetic information, may help to further resolve taxonomy in *G. intraradices sensu lato* as already discussed in section 5.3. An anastomosis test (vegetative compatibility test) using representatives from phylogroup *G. intraradices* GLOM A-1 (corresponding to *G. irregulare*) and phylogenetic relatives of *G. intraradices* (strain FL208) would be advantageous, because such a test could contribute to a biological species delimitation in *G. intraradices sensu lato* and simultaneously clarify the resolution of the mtLSU markers.

5.5.2 Ecological interpretations of the distribution of mitochondrial haplotypes in *G. intraradices*

The Biological Species Concept (Dobzhansky 1937), based on mating, is difficult to apply to AMF for which genetic recombination was only observed in one study under artificial conditions, so far (Croll *et al.* 2009). In contrast, the ecological definition of a population as "a group of organisms of the same species occupying a particular space at a particular time" (Krebs 1994), whose members "have an opportunity to interact with each other" (Waples & Gaggiotti 2006) is, indeed, also applicable to AMF (Rosendahl 2008). Further phylogeny-based circumscriptions of AMF population and species could profit from information of mtLSU haplotypes.

In fact, the geographical distribution of mtLSU haplotypes of the *G. intraradices* GLOM A-1 taxon can be interpreted ecologically, independent of a thorough definition of populations. The GLOM A-1 phylotype of *G. intraradices sensu lato* was detected in almost all previous environmental AMF studies, employing nuclear-encoded rDNA markers. Interestingly, it was missing in a study about AMF communities in Oman that investigated the AMF communities in date palm plantations and at close-by natural arid sites (Al-Yahya'ei *et al.* in press). Nevertheless, it seems fair to assume that *G. intraradices* GLOM A-1 occurs almost everywhere and that it thus may represent a generalist species (Sýkorová *et al.* 2007a), appearing in most field root samples, roots of bait plants planted to the field, and also greenhouse cultures (see section 1.2.4.4).

In considerable contrast, however, the resolution provided by the mtLSU sequence markers has already shown for the global set of 16 different isolates mainly belonging to *G. intraradices* GLOM A-1 that there is considerable genetic variation in the associated mitochondrial genome.

AMF species communities are generally characterized by high species richness in natural habitats, whereas species richness is decreased in agriculturally used sites (e.g. Oehl *et al.* 2003; see section 1.2.5.5). Based on the mtLSU RFLP data of this thesis, however, we must conclude the opposite for genotypic diversity within the phylotype *G. intraradices* GLOM A-1. Explanations for the increased RFLP type richness in the agricultural sites, compared to the grasslands, were already discussed in section 3.5. Therein, it was argued, that higher intraspecific diversity could be correlated with disturbance or with higher abundance of the species in a given setting. Faster

sporulation cycles (= shorter generation times) and less severe competition for soil-habitat space in arable fields as compared to grasslands, owing to periodic disturbance by soil tillage, may favor the evolution of more genotypic variation at a low taxonomic level and co-existence of more similar organisms. Assuming functional differentiation among taxa with distinct mtLSU types, one may suggest that it is strains of a population that perform different functions under intensive agriculture, but different species under no-till agriculture or in semi-natural grassland ecosystems.

Many unknown AMF phylotypes are generally found in each species community study (Fitter 2005), which is in common with the results of the present study (see chapter 3). However, Fitter (2005) further outlined that in AMF community investigations some phylotypes, so called generalists, seem to occur everywhere, an aspect, which could not be confirmed on population level in the present study. Even mtLSU haplotype I, dominating in the Tänikon field site, was not detected in Frick. This came as a surprise, since we expected a wide distribution, given that it corresponds to the isolate DAOM197198 from Canada (see chapter 2), and isolates from Eschikon and Changins in Switzerland (Croll *et al.* 2008). Most recently, the corresponding RFLP type Intra1 of haplotype I was also found in a coal mine spoil bank at Merkur near Chomutov (Czech Republic) by Zuzana Sýkorová (unpublished data). The same mtLSU sequence haplotype I was recorded in thermal soils of Iceland by Odile Thiéry (unpublished data). The RFLP type of sequence haplotype I was probably also detected in a root sample from Portugal, analyzed during the COST 879 workshop in Dijon, 2009 (Armelle Gollotte; personal communication). Even though it cannot be excluded that Intra1 comprises other mtLSU sequence haplotypes, current evidence suggests that mtLSU haplotype I is not only widespread, but also an ecological generalist. A similarly widespread occurrence and ecological amplitude may characterize haplotypes III (Frick) and XIX (Tänikon), which both yield RFLP type Intra3a (see chapter 3). Haplotype III is represented by the isolate BEG75 from Wädenswil, Switzerland (Raab *et al.* 2005), and haplotype XIX has in the meantime been detected in a root sample of a non-thermal site in Yellowstone National Park, USA, by Odile Thiéry (unpublished data). Moreover, the banding patterns of Intra3a occurred in mixed RFLP patterns from samples of the coal mine spoil bank Merkur in Czech Republic (Zuzana Sýkorová; unpublished data). Since cloning and sequencing was not done it remains unclear whether the RFLP patterns originated from sequence haplotype III, XIX or a so far unknown haplotype. Any RFLP data from new sites should, however, be treated cautiously, as resolution by RFLP can be considerably lower than by sequencing (see chapter 3).

Surprisingly, in spite of their broad occurrence, the RFLP types Intra1 and Intra3a, known from arable field sites, were never found in semi-natural grasslands. Instead, in grasslands it is other mtLSU haplotypes that dominate, namely represented by the RFLP types Intra13 and Intra14. RFLP type Intra13 was detected in a root sample from a mine spoil in the Matra mountains of Hungary (see chapter 2) and most recently in the coal mine spoil bank Merkur (Zuzana Sýkorová;

unpublished data). Identity of the corresponding mtLSU sequence types was only confirmed for samples from Landskron and the root sample from Hungary.

Possible evidence for resilience in taxon distribution under changing environmental conditions comes from the finding that one "grassland hyplotype" could be recovered from the no-tillage treatment at Tänikon. Moreover, the sampling site of the mine spoil bank Merkur in the Czech Republic is subject of reclamation and, therefore, represents still a transition state, at which both haplotypes of disturbed and undisturbed sites may co-occur. When grassland ecosystems become older stable populations of *G. intraradices* may develop, while disturbance-adapted, less competitive strains of *G. intraradices*, get outcompeted.

In essence, growing evidence for a disjunct distribution of many mtLSU haplotypes may be explained either by different ecological requirements of the organisms giving rise to them or else by limited dispersal. A few mtLSU haplotypes, such as haplotype I or isolates of *G. mosseae,* as reported by Rosendahl *et al.* (2009), show that there may, nevertheless, be generalist AMF with high dispersal abilities. Some AMF strains and species may be globally distributed as a consequence of human traveling and trading activity. Given human activity and the fact that the populations in the arable rather than grasslands sites were those that differed most in composition of mtLSU sequence haplotypes, it appears that it is the environmental conditions that select which ecologically specialized mtLSU members of the *G. intraradices* GLOM A-1 nuclear phylotype can persist in a certain ecosystem.

5.6 Perspectives for future use of the mtLSU detection methods for *G. intraradices*

The progress in community studies of AMF species was outlined in chapter 1. However, it became obvious that such studies are falling short in possibly not looking at the right level of genetic variation (Munkvold *et al.* 2004; Koch *et al.* 2006). Particularly, in agroecosystems, characterized by physical and other disturbances, intraspecific genetic differentiation may play a crucial role (Rosendahl 2008).

Glomus intraradices GLOM A-1, being a ruderal, wide-spread taxon (Sýkorová *et al.* 2007a,b), should receive particular attention. Therefore, I strongly defend future studies on the population structure of this fungal taxon in different ecosystems. Contrasting the fungal population structures in extreme environments, such as those at extreme temperatures or soil moisture contents should be favored. Molecular genetic detection in concert with functional tests may help to identify specialized ecotypes. However, the biogeographic study of more common agroecosystems, such as maize fields in North America, Africa or Asia could also provide interesting information about the distribution and speciation of AMF. In fact, the same field sites in Tänikon and Frick could be

further analyzed with respect to whether crop rotation and thus host plant identity has an influence on the populations of *G. intraradices* GLOM A-1. In this context, the existence of possible host preferences or specificity of mtLSU haplotypes should be explored e.g. in plant species-rich sites, although no evidence for such a phenomenon was found here (chapter 3). Tracking populations of *G. intraradices* at different stages of plant community succession may be another possible future research direction. Furthermore, co-existence patterns of different mtLSU haplotypes needs definitively further attention. Colonized root systems should be dissected and analyzed separately for the occurrence of different mtLSU haplotypes. Some of the root samples analyzed here were found to have been occupied by several different mtLSU haplotypes. An analysis on the spatial distribution of mtLSU haplotypes in a root system, may, in fact, contribute to a much more informed root sampling strategy for future AMF population studies. Comparing the *G. intraradices* GLOM A-1 population members present as spores and as root colonizers could be a further topic of interest, revealing much about AMF autecologies.

Study on the dispersal of AMF may also benefit from investigations on populations of *G. intraradices* in different regions of the world. It remains to be proven, whether AMF can, indeed, occur everywhere, as it is suggested by the "ubiquitous dispersal hypothesis" (Finlay 2002; Fenchel & Finlay 2004) and assumed after human intervention into AMF dispersal (Rosendahl *et al.* 2009). So far, all obtained results would suggest, that at least some mtLSU haplotypes have a great potential to disperse all over the world, but the success of establishment in a given habitat could be strongly dependent on the biotic and abiotic environment. This may be relevant when considering the application of AMF-inocula, since not each AMF strain may manage to establish and survive at each site.

Pot culturing may be necessary for spore-based morphological identification of otherwise poorly sporulating grassland mtLSU haplotypes. Controlled experiments in pots will be necessary to learn about functional or ecotypic differences among strains belonging to different mtLSU haplotypes, especially from the grasslands. Therefore, mtLSU assays may be of future use both in greenhouse experiments as well as field surveys.

First evidence for the existence of ecotypic differentiation (Sýkorová *et al.* 2007a) in the *G. intraradices* GLOM A-1 phyloclade (Koch *et al.* 2006), could be verified in common garden experiments after inoculum transfer, whole soil mixing. The relative importance of different environmental factors could be studied in experiments similar to that with the "home versus away" approach (Johnson *et al.* 2010), described in section 1.2.4.4.

The mtLSU approach developed here will make it now possible to distinguish different strains of *G. intraradices* so that functional experimentation at the sub-species level could progress and go beyond single strain inoculation experiments, as performed by Munkvold *et al.* (2004) and Koch *et al.* (2006). The possibility to reliably and unequivocally trace and soon also quantify individual mtLSU strains in taxonomically complex mixtures opens up tremendously many new perspectives

for future ecological research. Overall, fungal physiological activity could be estimated via quantification of the numbers of mitochondria, using real-time PCR approaches (see section 1.2.5.3) targeted to a mtLSU sequence. Since mitochondrial replication dependents on the physiological activity (see section 1.3.2), even the quantification of DNA, rather than RNA, may be functionally informative (Gamper *et al.* 2010; see also section 1.2.5.3).

The newly developed mtLSU approach bears also considerable potential for elucidating how mitochondrial genomes are inherited after the anastomosing of genetically different fungal strains (Croll *et al.* 2009). Investigations are now possible on how fast mitochondrial homoplasmy is reached again after the mixing of genetically different cytoplasm upon anastomosing. In fact, much could be contributed by studies on the inheritance and compatibility of mitochondrial genomes that may also inform research on vegetative incompatibility and the apparent selective maintenance of heterogeneity among nuclear genomes (Young 2009).

5.7 Epilog

Since the Convention on Biological Diversity was signed by 156 governments in Rio de Janeiro in June 1992, many countries recognized that it is necessary to protect biodiversity as an insurance for proper ecosystem functioning. For example, greater biodiversity leads to increased productivity in plant communities, greater nutrient retention and greater stability of ecosystems (Tilman 2000), likely strongly mediated by AMF (van der Heijden *et al.* 1998b). Given this recognized importance of biodiversity, it is alerting that anthropogenic influences have triggered the sixth major extinction event in the history of life (Raup 1986; Chapin *et al.* 2000). At a time of intensive land use (e.g. Noble & Dirzo 1997), global warming and desertification (Reynolds *et al.* 2007), it appears of utmost importance to preserve biodiversity as one of the driving factors for ecosystem stability. It is well known, that every little component of an ecosystem is important for the functioning of the whole network, as small as it might be. Therefore, an inconspicuous fungus, such as *G. intraradices*, may be as relevant as a chain in the system as a big elephant in the same ecosystem. Going one level of biological integration lower, intraspecific variation as tiny as it may be, could still play a critical role for the health of an ecosystem. Future studies are expected to reveal how this intraspecific genetic diversity will affect productivity of agroecosystems and the integrity of natural ecosystems. Being able to track neutral genetic variation, as enabled by the PCR-RFLP approach, may provide a first tool for the study of the distribution of root colonizing AMF ecotypes, such as strains of *G. intraradices*, adapted to arid conditions.

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Appendix: Evolutionary dynamics of introns and homing endonuclease ORFs in a region of the large subunit of the mitochondrial rRNA in *Glomus* species (arbuscular mycorrhizal fungi, Glomeromycota)

Odile Thiéry, Boris Börstler, Kurt Ineichen and Dirk Redecker

A.1 Abstract

The large subunit of the mitochondrial ribosomal RNA genes (mtLSU) has previously been identified as a highly sensitive molecular marker for intraspecies diversity in the arbuscular mycorrhizal fungus *Glomus intraradices*. In this study, the respective region was analyzed in five species of *Glomus* (*G. mosseae*, *G. geosporum*, *G. caledonium*, *G. clarum*, *G. coronatum*) from the same major clade (*Glomus* group A), *Glomus* sp. ISCB 34 from the related *Glomus* group B and two species of *Scutellospora*. Results show low level of genetic polymorphism between related morphospecies. Introns homologous to those found in *G. intraradices* were detected as well as new ones, some of them containing putative ORFs for homing endonucleases (HEs). Introns without ORFs for HEs seem to have been inherited strictly vertically from the ancestors of *Glomus* groups A and B while other introns indicate occasional horizontal transfer and possibly maintenance, degeneration and loss together with their associated HE ORFs. Overall, we provide first insights into the evolutionary dynamics of introns and HEs in this ecologically important group of fungi, which was previously not analyzed in this respect.

Keywords: mitochondrial large subunit RNA genes, molecular markers, homing endonucleases, group 1 introns, *Glomus* group A, Glomeromycota

A.2 Introduction

Arbuscular mycorrhiza is a ubiquitous symbiosis between the large majority of land plants and fungi from the phylum Glomeromycota. The fungal partners are obligate symbionts and their genetics has been the subject of a lively debate, with some authors claiming genetic heterogeneity between the nuclei in the coenocytic mycelium (Kuhn *et al.* 2001; Koch *et al.* 2004; Hijri & Sanders 2005), which was disputed by others (Pawlowska & Taylor 2004).

In contrast to rDNA sequences and other genes from the nuclear genome (Sanders *et al.* 1995; Lloyd MacGilp *et al.* 1996), mitochondrial large subunit rDNA sequences (mtLSU) of the arbuscular mycorrhizal fungi (AMF) *Glomus intraradices* and *Glomus proliferum* were demonstrated to lack polymorphism within the same strain (Raab *et al.* 2005; Börstler *et al.* 2008). The recently sequenced mitochondrial genome of *G. intraradices* confirmed this homogeneity for the whole genome (Lee & Young 2009).

The mtLSU and in particular its introns were shown to be highly sensitive molecular markers to genotype different isolates of *G. intraradices* (*sensu lato*) and it was used to differentiate mtLSU haplotypes directly from colonized field-collected roots (Börstler *et al.* 2008), which is a promising approach to obtain a better understanding of the diversity and dynamics of field communities and populations of AMF. The exon phylogeny of a region of the mtLSU showed superior resolution among subclades of *G. intraradices* compared to nuclear-encoded rDNA internal transcribed spacers (Börstler *et al.* 2008).

Mitochondrial DNA has a long history as a molecular marker that extends into the era before PCR facilitated the access to its sequences from a broad range of organisms (e.g. Bruns *et al.* 1989). In metazoan population studies, mitochondrial genes have played a prominent role due to the variability of the mitochondrial control region (Zischler *et al.* 1995). Their maternal inheritance and almost complete absence of recombination make the organelle genomes a unique tool for population biology. An interesting exception to the rule is the occasional recombination reported for fungal mitochondria (Saville *et al.* 1998). Several modes of inheritance have been reported from different groups of fungi (Yan & Xu 2005), but it is currently not known whether one of them occurs in the Glomeromycota.

Group I introns are widespread genetic elements mostly characterized in the mitochondrial genome of fungi including the one of *G. intraradices* (Lee & Young 2009). They are also found in chloroplasts and mitochondria of plants, algae and in nuclei of ciliates, slime molds, algae and fungi (Jurica & Stoddard 1999). These introns can fold into a secondary structure with a conserved core region. Stem-loops can be found, formed by extra nucleotides. The introns are capable of splicing by two sequential ester-transfer reactions (Cech 1990) and many of them encode homing endonucleases (HEs). These enzymes are generally known for their role in proliferation of the introns they reside in. This "homing mechanism" involves double-strand cleavage of specific recognition sites (15–35 bp) in the exons. The double-strand breaks are catalyzed by an endonuclease encoded by an ORF within the intron. The process is followed with subsequent double-strand repair and insertion of intron-containing allele at the respective site via homologous recombination (reviewed by Chevalier & Stoddard 2001). Several families of homing endonucleases are known based on conserved motifs, the most widespread being the LAGLIDADG family. The ORFs were thought to go through a "life cycle" consisting of fixation within a population, degeneration, intron loss and possible "re-colonization" (Goddard & Burt 1999).

Invasion of HEs in introns ensure their propagation and horizontal transfer of group I introns was reported to occur frequently between species of the same or of different kingdoms (Haugen *et al.* 2007). Alternatively, intron transfer can be promoted by reverse splicing. Contrary to homing, this process does not require a long recognition sequence but only few nucleotides (4–6 nt) that can pair bases with the internal guide sequence (IGS) of the intron (Cech 1985; Woodson & Cech 1989). Therefore, reverse splicing could give rise to transposition of introns into new genes.

Introns have been used as sensitive molecular markers in population studies (Neuveglise *et al.* 1997), because they tend to change very fast in evolution due to low selection pressure. On the other side, introns have been used to elucidate events of early evolution of land plants (Cho *et al.* 1998; Qiu *et al.* 1998). The view that some introns have been transferred from fungi to angiosperms very frequently (Vaughan *et al.* 1995; Cho *et al.* 1998; Sanchez-Puerta *et al.* 2008) was recently challenged and instead a history of ancient origin and frequent losses was suggested (Cusimano *et al.* 2008). Some introns are thought to be ancient and appear to have remained in the same position for millions of years (Qiu *et al.* 1998), some have apparently been transferred horizontally a relatively short time ago. In the light of these findings it would be interesting to better understand the evolution of mtLSU introns in the Glomeromycota, which have turned out to be efficient molecular markers.

Previously available mtLSU sequences in the Glomeromycota were limited to the two relatively closely related morphospecies *G. intraradices* (*sensu lato*; see section A.3 for details) and *G. proliferum*. The aim of the present study was to assess the evolutionary dynamics of the respective gene region from other glomeromycotan lineages in addition to their previously demonstrated discriminative power. In the focus was *Glomus* group A (Schwarzott *et al.* 2001), a monophyletic group which also contains *G. intraradices* and *G. proliferum*. Members of this clade represent a large part of glomeromycotan diversity and dominate almost all ecosystems studied so far using molecular identification methods. *Glomus mosseae*, *Glomus geosporum*, *Glomus caledonium*, *Glomus clarum* and *Glomus coronatum* were used as representatives of the second major clade within *Glomus* group A besides the *G. intraradices* clade. A species from *Glomus* group B, which constitutes the sister clade to *Glomus* group A, and two *Scutellospora* species from the more distantly related Gigasporaceae were used as outgroups. Using data from these species our objectives were to address the following questions:

- Does the lack of polymorphism in the mtLSU within fungal isolates also hold true for other species?
- Does the mtLSU represent a strain specific marker for other species?
- Do introns procure evidence about their evolutionary history and their potential spreading strategy possibly involving HEs?

A.3 Material and methods

A.3.1 Biological material

Spores of *G. coronatum*, *G. mosseae*, *G. caledonium*, *G. geosporum*, *G. clarum*, *Glomus* sp., *Scutellospora verrucosa* and *Scutellospora castanea* (Table 1) were harvested either from root organ cultures (ROCs) of *Daucus carota* (Bécard & Fortin 1988) or from pot cultures (Table 1). Spores from ROCs were retrieved using 10 mM sodium acetate-citrate buffer (pH 6.0) and washed in water (Doner & Bécard 1991). Substrate from pot cultures (about 10 ml) was wet-sieved using a sieve cascade with openings of 1 mm combined with either 80 or 32 μm. Organic matter from the sieves was suspended in 20 ml water, applied to a 70% (w : v) sucrose solution and centrifuged for 2 min at 820 g (Esch *et al.* 1994). The layer containing spores was rinsed in the bottom sieve transferred into Petri dishes before spores were placed in 1.5 ml tubes for DNA extraction.

Table 1 Arbuscular mycorrhizal fungi used in this study.

Species	Isolate code	Host(s)	Location of origin
Glomus coronatum	ZTL	*Allium porrum*	Ghaziabad, India
Glomus mosseae	ISCB 18	*Allium porrum*	Therwil, Switzerland
Glomus mosseae	ISCB 13	*Allium porrum*	Biengen, Germany
Glomus mosseae	ISCB 14	*Allium porrum*	Binningen, Switzerland
Glomus mosseae	BEG 12	*Fragaria vesca*	Rothamsted, England
Glomus caledonium	BEG 20	*Allium porrum, Hieracium pilosella, Plantago lanceolata*	Bedforshire, England
Glomus geosporum	BEG 18	*Allium porrum, Hieracium pilosella, Plantago lanceolata*	Nenzlingen, Switzerland
Glomus clarum	BEG 142	*Allium porrum, Hieracium pilosella, Plantago lanceolata*	Brazil
Glomus clarum	MUCL 46238	*Daucus carota* (ROC)	Pinar del Rio, Cuba
Glomus sp.	ISCB 34	*Hieracium pilosella, Plantago lanceolata*	Therwil, Switzerland
Scutellospora verrucosa	MN 186	*Daucus carota* (ROC)	Central Kisa, Kenya
Scutellospora castanea	BEG 01	*Allium porrum*	France

A.3.2 DNA extraction

DNA extracts were obtained by three different approaches (i) DNA of *S. castanea* and all *Glomus* species was extracted from at least 20 spores using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). (ii) Extracts of *Glomus* group A species were obtained from single spores as described by Redecker *et al.* (1997): each spore was crushed in 2 μl of NaOH (0.25 N). The extract was heated for 2 min at 95°C before adding 1 μl of TrisHCl (0.5 M, pH 8.0) and 2 μl of HCl (0.25 N) were added. DNA was incubated for 2 more min at 95°C and stored at −20°C. (iii) DNA of

S. verrucosa and *S. castanea* was extracted from single spores and from hyphae using the approach of Redecker *et al.* (1997) and amplified with the GenomiPhi™ Amplification Kit (GE Healthcare).

A.3.3 Amplification of mtLSU

For the amplification of mtLSU DNA, primers were selected either from the literature or newly designed using the software Primer Designer (version 3.0; Scientific & Educational Software, Cary, NC, USA). Based on sequences obtained from preliminary approaches (see Box 1, Fig. 1, Table 2), a general improved nested PCR method was developed to amplify DNA from single or multiple spores of all species using the primers RNL-1/RNL-117 in the first step of the nested PCR and the primers RNL-29/RNL-118 in the second step of the nested PCR (Table 3, Fig 2). DNA from all species was successfully amplified. PCR reactions were carried out with a Phusion High-Fidelity DNA polymerase from Finnzymes (Bioconcept, Allschwil, Switzerland) in a total volume of 25 µl containing 1 µl of genomic DNA or 1 µl of water for the negative control, 1x Phusion HF buffer, 0.2 mM of dNTPs, 0.5 µM of each primer, 4x BSA, 3% DMSO, 0.02 U/µl of Phusion DNA polymerase. PCR products of the first step of the nested PCR were diluted 1:100 in water and used as template in the second step of the nested PCR. Cycling parameters were 30 s at 98°C, then 29 cycles of 10 s at 98°C, 20 s at 58.4°C, 2 min at 72°C and a final elongation of 10 min at 72°C. This approach was also tested on DNA extracted from roots colonized by *G. coronatum* using the DNeasy Plant Mini Kit (Fig. 3).

Box 1 Nested PCR approaches

The mt-LSU rRNA gene was first amplified through nested PCR with the *Taq* DNA Polymerase Kit (GE Healthcare, Otelfingen, Switzerland) using previously published or newly designed primers (Table 1, Table 2). PCR reactions were performed in a total volume of 25 µl containing 1 µl of genomic DNA or 1 µl of water for the negative control, 1x PCR buffer, 2 mM of MgCl$_2$, 0.25 mM of dNTPs, 0.5 µM of each primer, 0.05 U/µl of *Taq* polymerase. The PCR program consisted of a denaturation step of 3 min at 95°C, followed by 34 cycles of 1 min at 95°C, 1 min at 51 or 56°C for the first and the second step of the nested PCR respectively, and 4 min at 72°C. The PCR ended with a final elongation of 10 or 5 min, for the first and the second step of the nested PCR respectively, at 72°C. The polymerase used and the PCR conditions are referred to approach 1 (Table 4).

In order to increase PCR yield and fidelity, PCR reactions were carried out with a Phusion High-Fidelity DNA polymerase from Finnzymes (Bioconcept, Allschwil, Switzerland) in a total volume of 25 µl containing 1 µl of genomic DNA or 1 µl of water for the negative control, 1x Phusion HF buffer, 0.2 mM of dNTPs, 0.5 µM of each primer, 4x BSA, 3% DMSO, 0.02 U/µl of Phusion DNA polymerase. New primers were designed (Table 2). Cycling conditions were changed as following: denaturation of 30 s at 98°C, then 34 cycles of 10 s at 98°C, 30 s at 51°C, 2 min at 72°C. The PCR ended with a final elongation at 72°C of 10 min. In the case of *G. clarum*, the PCR conditions were 30 s at 98°C, then 37 cycles of 10 s at 98°C, 20 s at 58°C, 2 min at 72°C. The PCR ended with a final elongation at 72°C of 10 min. This is referred to approach 2 (Table 4).

At this stage, DNA was amplified with the primers (i) RNL3/RNL15 for Gigasporaceae (ii) RNL29/RNL15 for *G. geosporum* (iii) RNL1/RNL5 for *G. clarum* (see also Börstler *et al.* 2010) (iv) RNL1/RNL98 – RNL96/RNL116 – RNL129/ RNL139 or RNL128/RNL141* for *G. caledonium*, *G. mosseae* and *G. coronatum**.

The final approach described in Material and Methods section is referred to approach 3.
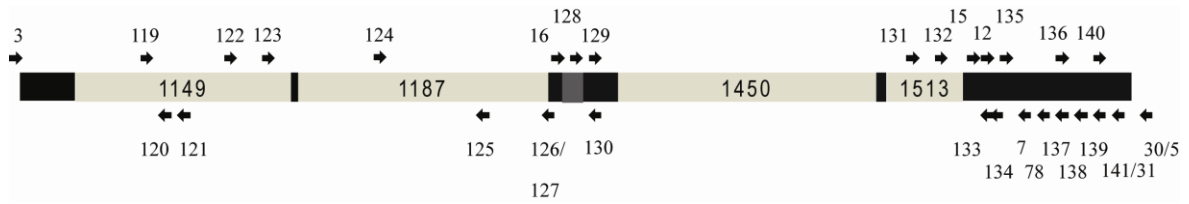
Fig. 1 Location and orientation of additional RNL primers used in approaches 1 and 2 and not required in approach 3. RNL126 was specific to Gigasporaceae and RNL-12/7/78/5 were only used to sequence *G. clarum*.

Table 2 Additional primers used in the two first approaches, which are not required in approach 3. *Primers from Raab *et al.* (2005); **Primers from Börstler *et al.* (2008).

Primer name	Sequence (5'-3')	Primer name	Sequence (5'-3')
RNL- 3*	TGCATMATGGGTCAGCGAGT	RNL-15*	CTGAGCTGTTACGCTATC
RNL-119	TCCTCGTAGGAGGAATGT	RNL-12*	GATAGCGTAACAGCTCAGTG
RNL-120	TGTTGCACGTATAGACGAGC	RNL-133	CAGACCGCTGAGCTGTTACG
RNL-121	CAGACGACACGTTGCAGCTG	RNL-134	GCGGAAGACCAGCTAATC
RNL-122	TTGGGCCGAGGGGGGTATAA	RNL-135	AGGCGAACCTTCGCTTGTAA
RNL-123	GTAAGGTCTATCGGGTAGGG	RNL-7*	CAGCTATGTCCACCGGCTCA
RNL-124	GGAGATTCTGCCTCTGTTTG	RNL-78**	AAGCAACGGCCTCTAAGACA
RNL-125	GCAGGAAGACTCCTGAAT	RNL-136	GCCGATGAGTAACTCTTCTG
RNL-16*	ACCTGGAGATAGCTGGTCTT	RNL-137	GAGTTACTCATCGGCACTCC
RNL-126	GTAGGTGGGAGCCTACTGAT	RNL-138	GCTGTAGTTCCTGCTAAG
RNL-127	TCAGACCACTGAGCTGTTAC	RNL-139	GGTCATCTTGCCGAGTTCCT
RNL-128	CCACACGGTACTACGTACCT	RNL-140	TGAAGGAACTCGGCAAGATG
RNL-129	AGACGGTACGGCACTGGAAG	RNL-31**	TTMGTGCCGCCACTTATTAG
RNL-130	TGCCGTACCGTCTAGTAA	RNL-141	CTTATTAGTGGCACCCCTTC
RNL-131	AGCCTTTGGTTCGAGTCT	RNL-30**	TAGCATCGGGCAGGTATCAG
RNL-132	CACACGCCGAAACTAAAC	RNL-5*	GAGCTTCCTTTGCCATCCTA

Table 3 Primer sequences. The primers used in the general optimized nested PCR approach are shaded in grey. *Primers from Raab *et al.* (2005); **Primers from Börstler *et al.* (2008).

Primer name	Sequence (5'-3')	Primer name	Sequence (5'-3')
RNL- 1*	AGACCCGAARCCWRGTGATCT	RNL-103	CTGATTGCCCACCTTTGT
RNL-29**	TAATAAGACTGAACGGGTGT	RNL-104	CACACGGTACTACGTACCTA
RNL-11*	AAGGCAACACGCCAGCACTT	RNL-73	GCCTTATCACGAATCGTCTG
RNL-91	GCTCGTCTATACGTGCAACA	RNL-105	TGACGAATCGCCTTATCACG
RNL-92	AAGTGCTGGCGTGTTGCCTT	RNL-106	CAAGGCAGTCCTCTCCAGTG
RNL-93	TGCAGCTGGAAAAAGGAGTC	RNL-107	GCCACAGGTTGAATCCACTA
RNL-94	GCTGCTCCTTGAGGTCCTTA	RNL-108	ACGGAAGCTGAAGCTAGT
RNL-77	AGCCAACTCTATGGTTCAAT	RNL-109	CTCGGATCCTCAGAGACTAA
RNL-17*	CCATAGAGTTGGCTCTAACA	RNL-110	TGCTGAGTGACGTGCATTCT
RNL-95	ACGGACCTATTGCCCAATAC	RNL-111	AGCCGATCGAATGAGATACC
RNL-96	CGACTCATAACGGTGAATCC	RNL-112	CAGTCTAGGCACAAGGACTC
RNL-97	ATCGTAACACGTCGACACTC	RNL-113	GTGCCTAGACTGGAAGAGTA
RNL-98	GAGTGTCGACGTGTTACGAT	RNL-114	GCCTTGTKWTCACTCAGTAG
RNL-99	GGAGATTCTGCCTCTGTTTG	RNL-115	TTGGTTCCAGGCCTATCCTT
RNL-100	GCTAATCCTTCYGCTGTTAG	RNL-116	CCTGCTGATTGTCCAATCCT
RNL-101	AACTGTGGGGCAGTGTATGA	RNL-117	CTTCTGCTTYCGGCGAAGAG
RNL-102	GTCGACCTTTGCCCTCTTTG	RNL-118	TCARACCACTGAGCTGTTAC
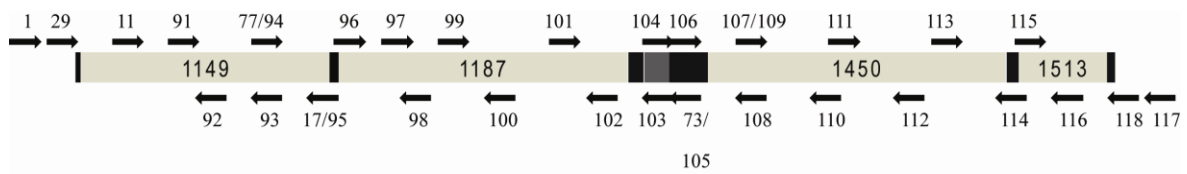
Fig. 2 Location and orientation of RNL primers. RNL-77, 17 and 73 were only used to sequence *G. clarum*. RNL-109–113 and RNL-107–108 were specific to *G. coronatum* and *Glomus* sp., respectively. Additional primers are shown in Fig. 1.

A.3.4 Amplification of nuclear rDNA

Nuclear rDNA amplification of each DNA extract was performed according to Redecker (2000) using a *Taq* DNA Polymerase Kit from GE Healthcare (Otelfingen, Switzerland), the universal eukaryote primers NS5/ITS4 for the first step of the nested PCRs and GLOM1310/ITS4i or GIGA1313/GIGA5.8R for the *Glomus* spp. and the *Scutellospora* spp., respectively for the second step of the nested PCRs.

A.3.5 Cloning of the PCR products and DNA sequencing

Blunt-ended PCR products obtained with Phusion polymerase were modified by an addition of an A overhang to the 3'-end by an incubation of 13 min at 72°C with *Taq* polymerase, 2 mM MgCl$_2$ and 0.125 mM dATP. All PCR products were purified using the High Pure Kit (Hoffmann LaRoche, Basel, Switzerland). Cloning was performed according to the manufacturer's instructions of the pGEM® Vector System I Kit (Promega/Catalys, Wallisellen, Switzerland). Cloned PCR products were re-amplified with the vector primers M13fwd (GTA AAA CGA CGG CCA GTG) and M13rev (GGA AAC AGC TAT GAC CAT G). After purification of the PCR products, DNA was resuspended in 30 µl of elution buffer and stored at −20°C until use.

The ABI PRISM Big Dye Terminator v.3.1 Cycle Sequencing Ready Reaction Kit® (Applied Biosystems, Foster City, CA) was used to perform the sequencing PCR in both directions. Samples were run on an ABI prism 310 capillary sequencer (Applied Biosystems) or on an ABI3130xl capillary sequencer.

Table 4 Numbers of spores and clones analyzed to obtain partial or complete sequences from DNA extract of a single spore (* or of several spores) by directly sequencing or by cloning. When two approaches were used, samples directly sequenced were from approach 3.

AMF	Mt-LSU			Nuclear rRNA
	Approach	Direct sequencing	Cloning	
Glomus coronatum (ZTL)	2	3 spores	1 spore (3 clones)	2 spores (1 clone each)
Glomus mosseae (ISCB 18)	2	3 spores	2 spores	2 spores (1 clone each)
Glomus mosseae (ISCB 13)	2	2 spores	2 spores	2 spores (1 clone each)
Glomus mosseae (ISCB 14)	2	4 spores	3 spores	2 spores (1 clone each)
Glomus mosseae (BEG 12)	2	2 spores	1 spore	2 spores (1 clone each)
Glomus caledonium (BEG 120)	2	2 spores	1 spore	2 spores (1 clone each)
Glomus geosporum (BEG 18)	2-3	1 spore	1 spore	1 spore (2 clones)
Glomus clarum (BEG 142)	2	-	1 spore	1 spore (2 clones)
Glomus clarum (MUCL 46238)	2	-	1 spore	1 spore (2 clones)
Glomus sp. (ISCB 34)	3	-	2 spores	2 spores (1 clone each)
Scutellospora verrucosa (MN 186)	1 -3	1 clone (DNA from hyphae)	1 spore (3 clones)	1 spore (2 clones)
Scutellospora castanea (BEG 01)	1-3	1 clone	spores (4 clones)	Spores (1 clone) 2 spores (1 clone each)

A.3.6 Sequence and phylogenetic analyses

The sequences obtained were edited and compiled using Sequence Navigator software (version 1.0.1, Applied Biosystems). In case of sequences obtained from the same DNA extract but with different primer sets, a concatenated sequence was created based on evidence of mtLSU homogeneity and only when a significant overlap (≥ 190 bp) allowed excluding ambiguity. Sequences were manually aligned to previously published sequences (Raab *et al.* 2005; Börstler *et al.* 2008) with BioEdit (Hall 1999) or PAUP* 4.0b10 (Swofford 2001). Sequences were deposited in the EMBL database under the accession numbers FN377857 to FN377867 and FN377601 for mitochondrial sequences and FN423686 to FN423707 and FN423502 to FN423503 for nuclear sequences. Phylogenetic trees were generated by distance, parsimony and maximum likelihood criteria using PAUP* 4.0b10 (Swofford 2001). Modeltest 3.5 (Posada 2004) was used to estimate maximum likelihood models and parameters and MrBayes 3.1.1 (Ronquist & Huelsenbeck 2003) to perform Bayesian analyses. For parsimony analyses each gap of three bases or more was coded by inserting a binary character (1) in introns.

Unless indicated otherwise, Bayesian analyses were run for 1,000,000 generations with a "burnin" of 10%. The stationarity of the Markov chains was verified using the command "sump". For protein phylogenies, a mixed model was used that integrates different protein sequence evolution models.

Introns were identified by the software RNAweasel (Lang *et al.* 2007). Search of ORFs was performed by means of the programme NEB Cutter (version 2.0; Vincze *et al.* 2003) with the "Mold, Protozoan, and Coelenterate Mitochondrial Code and the Mycoplasma/Spiroplasma Code" (transl_table = 4). Detected ORFs were translated, submitted to the BLASTp network server (NCBI; www.ncbi.nlm.nih.gov) and conserved domains were detected using the Conserved Domain Database (CDD) (Marchler-Bauer *et al.* 2009). Statistical modeling of LAGLIDADG allowed the observation of single versus double-motif HEs as well as intact versus disrupted conserved domains (Belfort & Roberts 1997; Dalgaard *et al.* 1997; Heath *et al.* 1997). Additionally, intronic sequences were translated in six reading frames (http://www.expasy.ch/tools/dna.html) to observe any putative LAGLIDADG domains that were not encoded by ORFs. The rates of synonymous (dS) and non-synonymous (dN) substitutions were computed using the Synonymous Non-synonymous Analysis Program (Korber, 2000).

For simplicity, we use the species name *G. intraradices* in the broad sense throughout this study for isolates morphologically identified as this species. It has been shown that within this definition several distinct divergent lineages can be distinguished by nuclear-encoded rDNA sequences (e.g. Hijri *et al.* 2006) and mtLSU (Börstler *et al.* 2008). Recently, it was shown that the lineage comprising most isolates, environmental sequences, and in particular the strain currently used for sequencing the genome of *G. intraradices*, is distinct from the type strain of the species (FL208; Stockinger *et al.* 2009). Consequently, the majority of isolates and environmental sequences will have to be renamed in the future. Until a generally accepted epithet for this enormously widespread fungal clade is at hand, we will refer to this clade as *G. intraradices* GLOM A-1 in concordance with the phylotype nomenclature of our previous field studies (e.g. Hijri *et al.* 2006).

A.4 Results

A.4.1 Primer design and PCR

So far, primers of Raab *et al.* (2005) and Börstler *et al.* (2008) were designed to amplify *G. proliferum* and *G. intraradices* but no set of primers was available allowing the detection of other taxa. Primers reported previously and newly designed primers (Table 2) were used to amplify parts of the mtLSU from five species of *Glomus* group A (*G. clarum*, *G. geosporum*, *G. mosseae*,

G. caledonium, *G. coronatum*), one species from *Glomus* group B and two species of the more distantly related Gigasporaceae. From the obtained sequences, a new set of primers (RNL-1/RNL-117 and RNL-29/RNL-118) was designed allowing detection of all samples. The primer pairs RNL-1/RNL-117 and RNL-29/RNL-118 were successfully used in a nested PCR approach, yielding products of sizes ranging from 538 to 4003 bp (Fig. 4, Table 5).

All PCR products could in principle be directly sequenced after PCR, but most amplicons were sequenced after cloning because this proved to be more efficient as numerous sequencing reactions were required to cover the long DNA fragments. Nuclear-encoded rRNA was amplified from the same biological samples and analyzed phylogenetically as a control for species identity (Figs 5 and 6).

The general mtLSU primer set was specific enough to be used for amplification of fungal DNA from colonized roots, as shown by successful amplification of the expected fragment from *Allium porrum* colonized roots (Fig. 3).
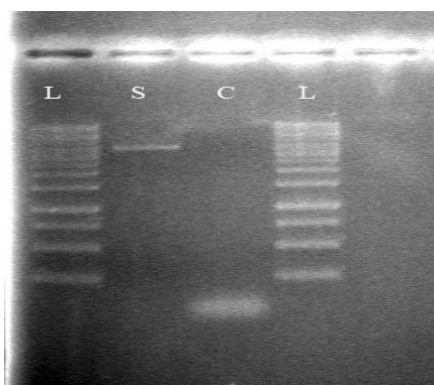
Fig. 3 Agarose gel electrophoresis showing the PCR products of *Allium porrum* roots colonized by *G. coronatum* (S). C indicates the negative control and L DNA ladder (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000.
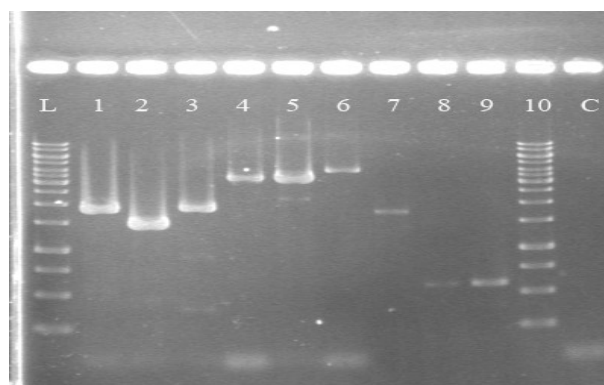
Fig. 4 Agarose gel electrophoresis showing the length of the PCR products obtained using the primers 29/Ar5 for the following species: 1, *G. intraradices*; 2, *G. clarum*; 3, *G. geosporum*; 4, *G. mosseae*; 5, *G. caledonium* BEG20; 6, *G. coronatum*; 7, *G. etunicatum*-like; 8, *S. verrucosa*; 9, *S. castanea*. L indicates DNA ladder (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000. C shows the negative control.

Table 5 Exon/intron structure of the mtLSU sequences between the priming sites RNL-29/RNL-118. *G. intraradices* JJ291 and *G. proliferum* DAOM226389 were included as references. *Numbers denote positions in the *G. intraradices* exon FACE#494 (Lee & Young 2009). Each table cell lists intron length and intron type.

Species – Accession numbers – (Isolates)	Introns				Exons	
	Position 1149* (Length, bp) – intron group	Position 1187* (Length, bp) – intron group	Position 1450* (Length, bp) – intron group	Position 1513* (Length, bp) – intron group	Complete length/parts separated by introns (bp)	Total length (bp)
G. proliferum (DAOM226389)	No	No	No	No	386/39, 261, 62, 24	386
G. intraradices (JJ291)	1056 – IA3	No	No	401 – IB	386/39, 261, 62, 24	1843
G. clarum FN377859 (BEG142) FN377601(MUCL46238)	1057 – IA3	No	No	No	384/39, 259, 62, 24	1441
G. geosporum FN377861 (BEG18)	1133 – IA3	No	No	272 – IB	497/39, 373, 61, 24	1902
G. mosseae FN377862 (ISCB18) FN377863 (BEG12) FN377864 (ISCB13) FN377865 (ISCB14)	1136 – IA3	1378 (1379 for ISCB 18) – IA3	No	272 – IB	496/39, 372, 61, 24	3282
G. caledonium FN377857 (BEG20)	1101 – IA3	1374 – IA3	No	272 – IB	496/ 39, 372, 61, 24	3243
G. coronatum FN377860 (ZTL)	1181 – IA3	606 – IA3	1448 – I derived	272 – IB	496/39, 372, 61, 24	4003
Glomus sp. FN377858 (ISCB34)	No	No	1045 – IB	215 – IB	417/39, 293, 61, 24	1677
S. verrucosa FN377866 (MN186)	No	No	No	No	538/39, 414, 61, 24	538
S. castanea FN377867 (BEG01)	No	No	No	No	545/39, 421, 61, 24	545

A.4.2 Exon/intron structure

The broad size range of the amplicons was mainly caused by introns inserted in four locations (Fig. 7, Table 5). The two *Scutellospora* spp. did not contain any intron, whereas *G. coronatum* on the other extreme contained all of them. All introns were identified to be group 1 introns except the *G. coronatum* intron position 1450 which was characterized as "group I derived". Several species contain short insertions (44–165 bp) absent in others, which in the absence of evidence defining them as introns are, however, regarded as "expansion segments". The occurrence of such expansion segments or nucleotide extensions in the mtLSU was firstly reported in Basidiomycota (*Agrocybe aegerita*), Ascomycota (*S. cerevisiae*) and also in algae (*Prototheca whickerhamii*), (Sor & Fukuhara 1983; Wolff *et al.* 1993; Gonzalez *et al.* 1999) possibly constituting parts of the exon highly variable in length. The evolution of these fragments is discussed by Hancock & Dover

(1988) and Yokoyama & Suzuki (2008). They were excluded from phylogenetic analyses of the exon because they were difficult to align and decreased the support of the phylogenetic trees.
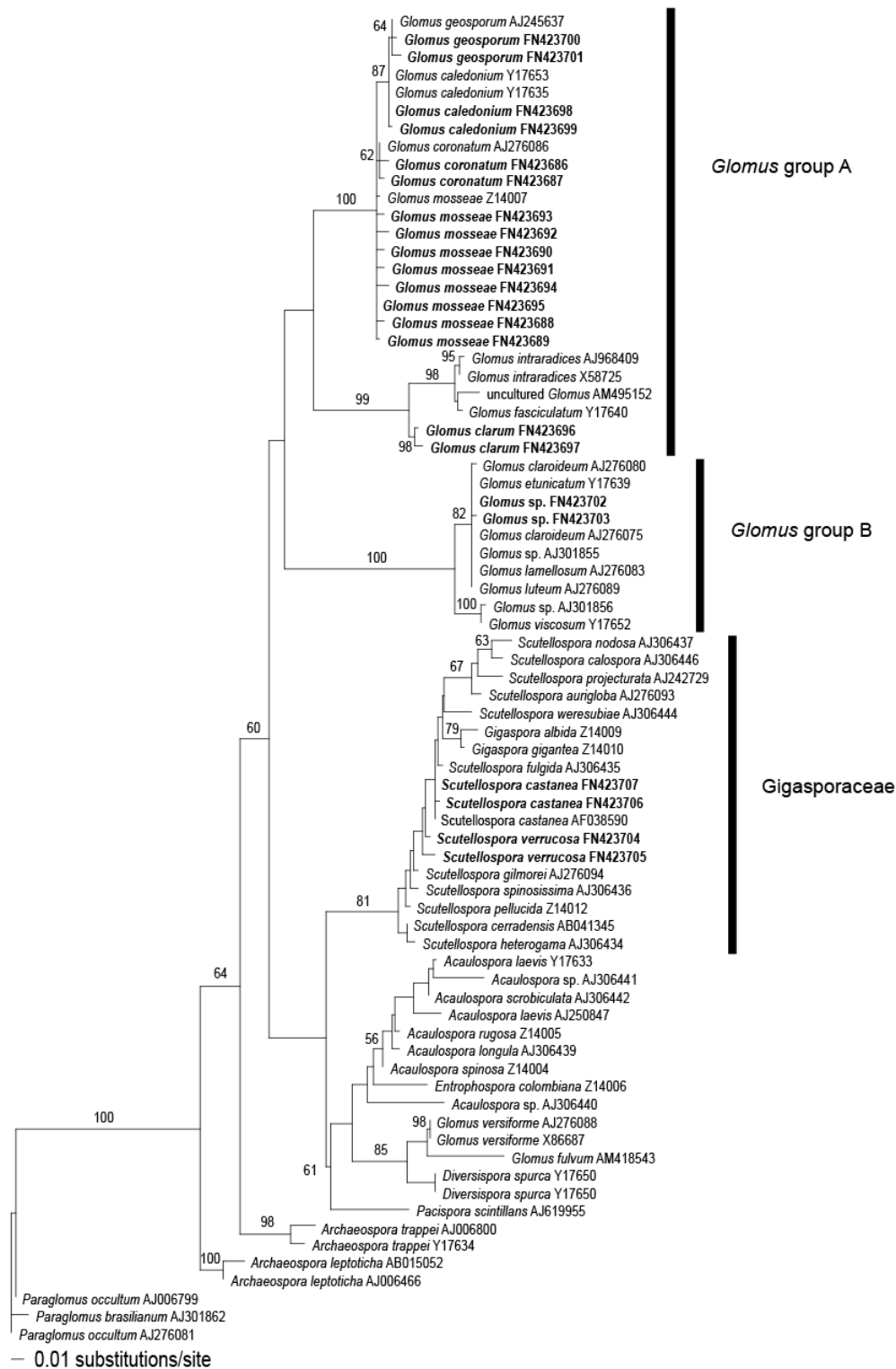
Fig. 5 Phylogenetic tree of Glomeromycota based on 369 bp of 18S n-rDNA sequences. *Paraglomus brasilianum* was used as outgroup. The tree was generated by a heuristic search under the maximum likelihood criterion. Numbers on the nodes indicate neighbor-joining bootstrap values from 1000 replicates. Labels include species name, accession numbers and isolate codes (in brackets). Sequences obtained in the present study are shown in boldface.
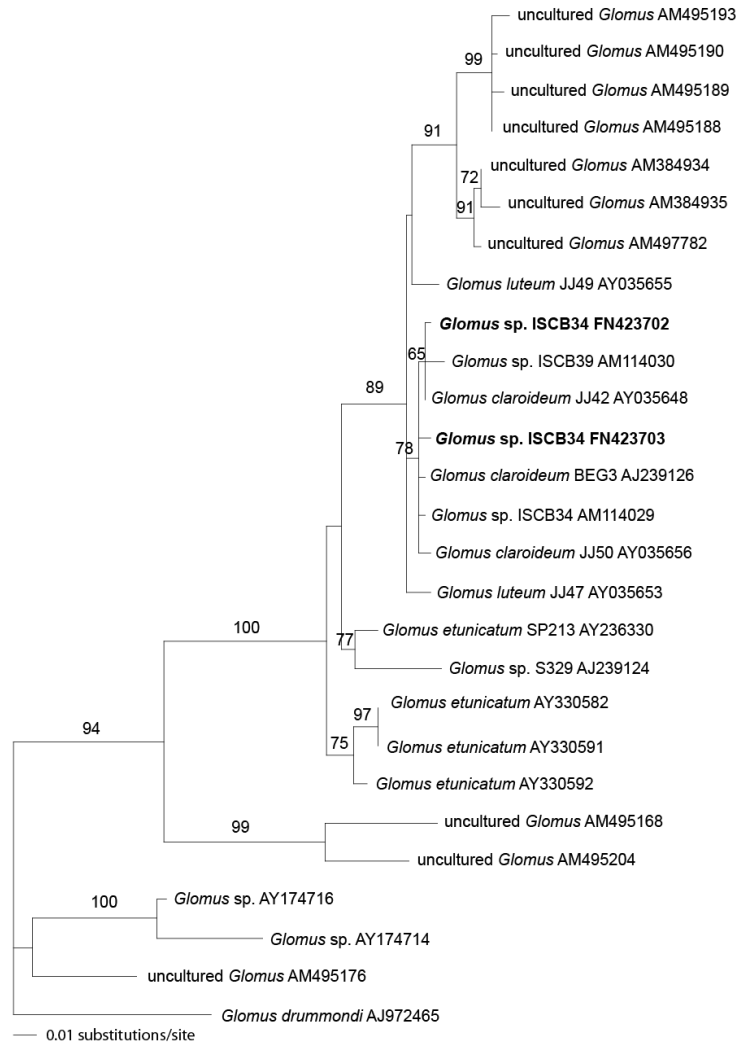
Fig. 6 Phylogenetic tree of *Glomus* group B based on 410 bp of 5.8S rRNA and ITS2 sequences. *Glomus drummondi* was used as outgroup. The tree was generated from a heuristic search under the maximum likelihood criterion. Numbers at the node indicate neighbor-joining bootstrap values from 1000 replicates. Labels include species name, accession numbers and isolate codes (in brackets). Sequences obtained in the present study are shown in boldface.
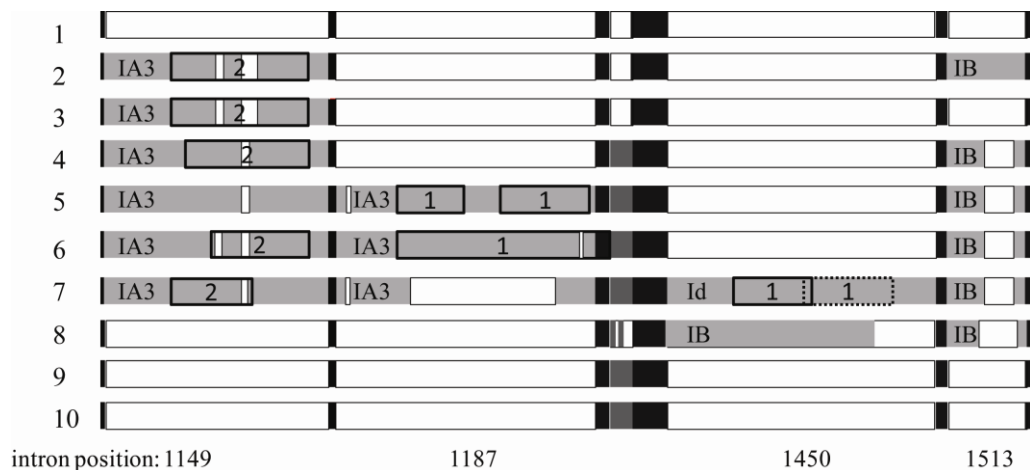
Fig. 7 Schematic sequence alignment of the mitochondrial rRNA large subunit (mtLSU) gene region for the following isolates of different species: 1, *G. proliferum* DAOM226389 – 2, *G. intraradices* JJ291 – 3, *G. clarum* BEG142/MUCL46238 – 4, *G. geosporum* BEG18 – 5, *G. mosseae* ISCB18 – 6, *G. caledonium* BEG20 – 7, *G. coronatum* ZTL – 8, *Glomus* sp. ISCB34 – 9, *S. verrucosa* MN186 – 10, *S. castanea* BEG01. Exons are represented in black (■), introns in medium grey (■), gaps in white (□) and insertions in dark grey (■). The intron type is indicated by IA3, Id for group I derived and IB. Open reading frames appear in rectangular boxes with the number corresponding of the putative LAGLIDADG type. Diagram approximately to scale.

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A.4.3 Intraspecies sequence homogeneity

Four isolates of *G. mosseae* originating from Switzerland (two different sites), Germany and United Kingdom were analyzed including the strain BEG12 widely used in previous studies (e.g. Gamalero *et al.* 2004; Repetto *et al.* 2007; Pivato *et al.* 2009). To further verify sequence polymorphism of the mtLSU within and between isolates of *G. mosseae*, sequences were amplified and sequenced from 3 up to 7 spores for each isolate. No sequence variation was detected between the clones due to the fidelity of the proof-reading DNA polymerase used: the error rate of Phusion is determined to be 4.4 x 10^{-7} according to the manufacturer. Sequences of *G. mosseae* ISCB13 and BEG12 originating from Germany and United Kingdom, respectively, were identical. Sequences of *G. mosseae* ISCB18 and ISCB14 both originating from Switzerland but at different sites were 99.8% similar. Sequences of *G. mosseae* ISCB18 and *G. mosseae* ISCB14 were 99.9% and 99.9%, respectively, similar to *G. mosseae* ISCB13 and BEG12. On average, the percentage of similarity between these four isolates was 99.9%. Two isolates of *G. clarum* BEG142 and MUCL46238 originating from Brazil and Cuba, respectively, were also analyzed. They also showed a remarkably high similarity of 99.9%. In consequence, mtLSU sequences were considered as being identical between different isolates of *G. mosseae* and *G. clarum*.

A.4.4 Exon phylogeny

The relatively short exon regions yielded a phylogeny largely consistent with the one obtained using n-rDNA (Fig. 8). Gigasporaceae, *Glomus* group A and *Glomus* group B grouped as expected and while they could be clearly assigned to the Glomeromycota, they showed considerable genetic distance from each other. The well-established sister group relationship between the *G. mosseae* and the *G. intraradices* clades was recovered to different extents by different phylogenetic analyses. In parsimony- and maximum likelihood-based and Bayesian analyses the *G. mosseae* clade was not resolved as monophyletic group, which is most likely due to the low phylogenetic signal (in particular parsimony-informative characters) differentiating this clade. The isolate CA502 showed a closer affinity to *G. clarum* than in ITS-based phylogenies, where it groups within the GLOM A-1 clade.

Overall, the polymorphism in the exon region studied was relatively low between closely related species, with no differences at all between *G. mosseae/G. coronatum* and 99.2% similarity between *S. verrucosa/S. castanea*. In the 18S n-rDNA based phylogeny these two pairs of species are very closely related sister taxa (Fig. 5).
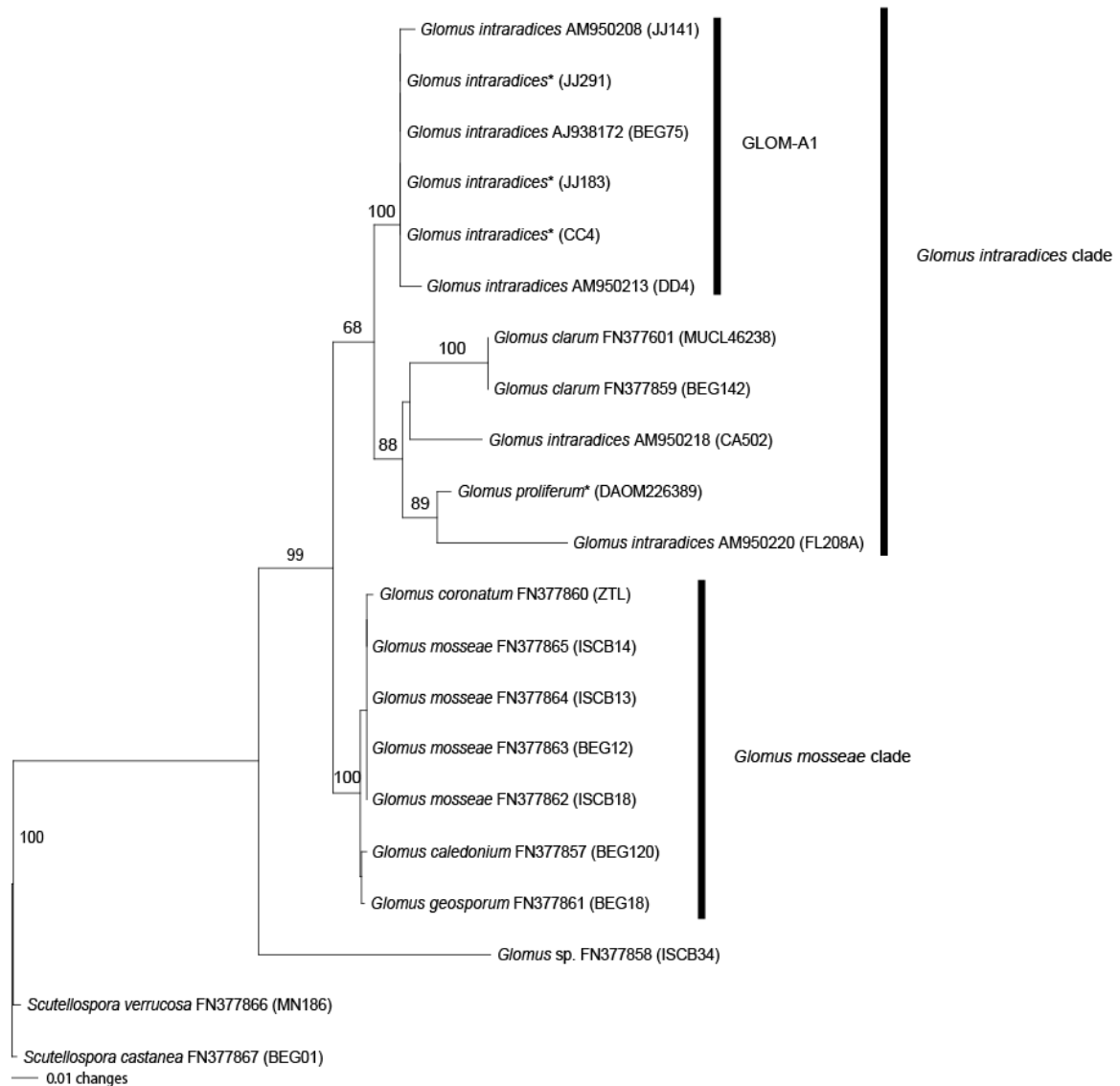
Fig. 8 Neighbor-joining tree of Glomeromycota based on 361 bp of exon mtLSU, with *Scutellospora* spp. used as outgroup. Values on the nodes indicate neighbor-joining bootstrap values from 1000 replicates. After species name are accession numbers and isolates (in brackets). *Consensus sequences obtained from AM950210, AM950211 and AM950212 for *G. intraradices* JJ183; AJ973189, AJ973190 and AJ973191 for *G. intraradices* JJ291; AM950204, AM950205 and AM950206 for *G. intraradices* CC4; AM040980, AM040981 and AM040982 for *G. proliferum*.

A.4.5 Evolution of ORFs and conserved domains of the LAGLIDADG in introns

A.4.5.1 Intron 1149: evidence for intraphylum horizontal transfer

Within intron 1149, *G. geosporum* and *G. clarum* displayed ORFs for LAGLIDADG HEs with one conserved domain, as for *G. intraradices* isolate JJ291 even though in the latter, the conserved domain was disrupted. In *G. intraradices* isolates JJ141 and JJ183, the entire conserved domain was encoded in two separate putative ORFs (Börstler *et al.* 2008). In *G. coronatum* and

G. caledonium the ORFs were shorter compared to *G. geosporum* and *G. clarum*, because some parts of the domain were shifted in frame, but the overall intron length was similar (Fig. 7). In *G. mosseae*, no ORF was detected, but interestingly some parts of the conserved motif were still present in the respective region. In three isolates of *G. intraradices* (DD-4, CA502, FL208A), neither ORFs nor conserved domains were present and the size of intron 1149 in these isolates (425–662 bp) was substantially shorter, because the sequences coding for the ORFs in the other isolates were lacking.

The ratio of synonymous (dS) to non-synonymous (dN) changes (Table 6) showed an overall dS/dN value of 1.6217, indicating a negative selection on the HE ORFs to maintain the protein activity. The dS/dN ratio was higher in functional (2.0119) compared to disrupted (1.3913) endonuclease domains. This can be interpreted as a faster degeneration of HE ORFs non-encoding putative active LAGLIDADG. The shortened *G. coronatum* HE ORF even showed stronger signs of degeneration (dS/dN = 0.8287).

Table 6 Functional domains were from *G. geosporum* (1) and *G. clarum* isolates MUCL46238 (2) and BEG142 (3); disrupted domains from *G. coronatum* (4), *G. caledonium* (5), and *G. intraradices* isolates JJ291 (6), JJ141 (7) and JJ183 (8). N.A. means not applicable. Average of all pairwise comparisons: dS/dN = 1.6217.

dS/dN value within functional domain	dS/dN value within disrupted domain	dS/dN value in *G. coronatum*
2.0119 (1-2)	1.2433 (4-5)	0.6994 (4-1)
2.0119 (1-3)	0.5547 (4-6)	0.7600 (4-2)
N.A. (2-3)	1.0238 (4-7)	0.8938 (4-3)
Mean value : 2.0119	0.6259 (4-8)	1.2433 (4-5)
	2.1563 (5-6)	0.5547 (4-6)
	2.4253 (5-7)	1.0238 (4-7)
	1.7540 (5-8)	0.6259 (4-8)
	1.0724 (6-7)	Mean value : 0.8287
	1.9714 (6-8)	
	1.0861 (7-8)	
	Mean value : 1.3913	

Therefore, within the *G. mosseae* clade, there appears to be a progression from a large ORF containing a LAGLIDADG domain towards an apparent partial or entire loss of the ORF by losing start codons and/or introducing stop codons. Interestingly, even in *G. mosseae*, which does not possess an ORF, the degradation of the sequence is limited.

The phylogeny of the LAGLIDADG ORFs and the remaining parts of the intron was in congruence which favors the hypothesis of transfer of both endonuclease and intron 1149 as a unit (Figs 9 and 10). Some isolates of *G. intraradices* grouped differently from the exon phylogeny. In particular, the intron of JJ183 showed a striking similarity to the *Glomus clarum* isolates, sharing several characteristic motifs. This is evidence for horizontal transfer of the intron, either from *G. intraradices* JJ183 to *G. clarum* or vice versa. Similarly, *G. intraradices* JJ141 groups with the *G. mosseae* clade.
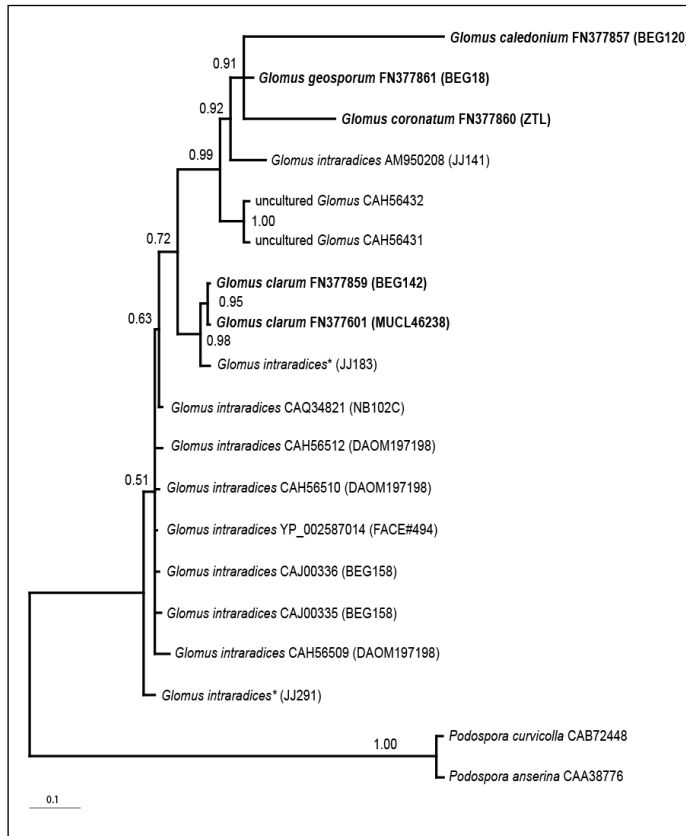
Fig. 9 Phylogeny of the putative LAGLIDADG type 2 proteins encoded by intron 1149 ORFs in the Glomeromycota, with Ascomycota as outgroup. The phylogenetic tree was generated based on an alignment of 267 amino acids. Values on the nodes indicate Bayesian posterior probabilities. Labels include species names, accession numbers and isolate codes (in brackets). * Consensus sequences obtained from AM950210, AM950211 and AM950212 for *G. intraradices* JJ183; AJ973189, AJ973190 and AJ973191 for *G. intraradices* JJ291.
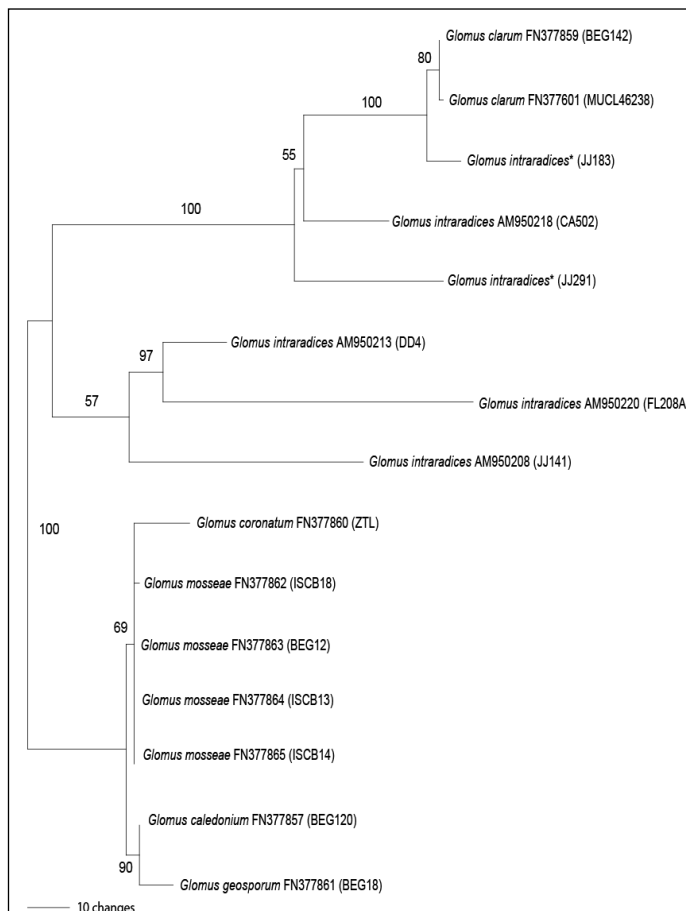
Fig. 10 Phylogeny of Glomeromycota based on 1239 bp of intron 1149 sequences. Single most parsimonious obtained by a heuristic search under the parsimony criterion. Numbers on the nodes indicate bootstrap values from 1000 replicates. Labels include species name, accession numbers and isolate codes (in brackets). *Consensus sequences obtained from AM950210, AM950211 and AM950212 for *G. intraradices* JJ183; AJ973189, AJ973190 and AJ973191 for *G. intraradices* JJ291.

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A.4.5.2 Intron 1187: secondary split of double-motif LAGLIDAG ORFs

Intron 1187 was only found in three species, therefore no phylogenetic analysis was conducted. Two species contained putative LAGLIDADG type 1 ORFs in this intron: *G. caledonium* possessed two copies of the conserved amino acid motif LAGLIDADG whereas *G. mosseae* had two distinct ORFs for each conserved LAGLIDADG motif. This split was due to a stop codon arising from a deletion of 5 bp. In *G. coronatum* this intron was present but its size was reduced from 1378 to 606 bp. Here, the start codon and some other remaining sequences from the LAGLIDADG ORF were still present, indicating a large part of the ORF was lost, and providing conclusive evidence for the direction of this evolutionary process. In *G. geosporum*, which is closely related to *G. caledonium*, the intron was completely absent.

In order to elucidate the phylogenetic relationships of HEGs (HE genes) of Glomeromycota and other taxa with single or double conserved LAGLIDADG domains, the phylogenetic analysis of the HEGs (Fig. 11) was conducted on each conserved domain: for that purpose the N-terminal and C-terminal domains of the two conserved domains of double-motif LAGLIDADG were split, aligned to each other and to the two ORFs from *G. mosseae*, which clearly represent homologs of the two domains in *G. caledonium*. Overlapping ORFs of HEs found in *G. coronatum* in intron 1450 were also included in this analysis.
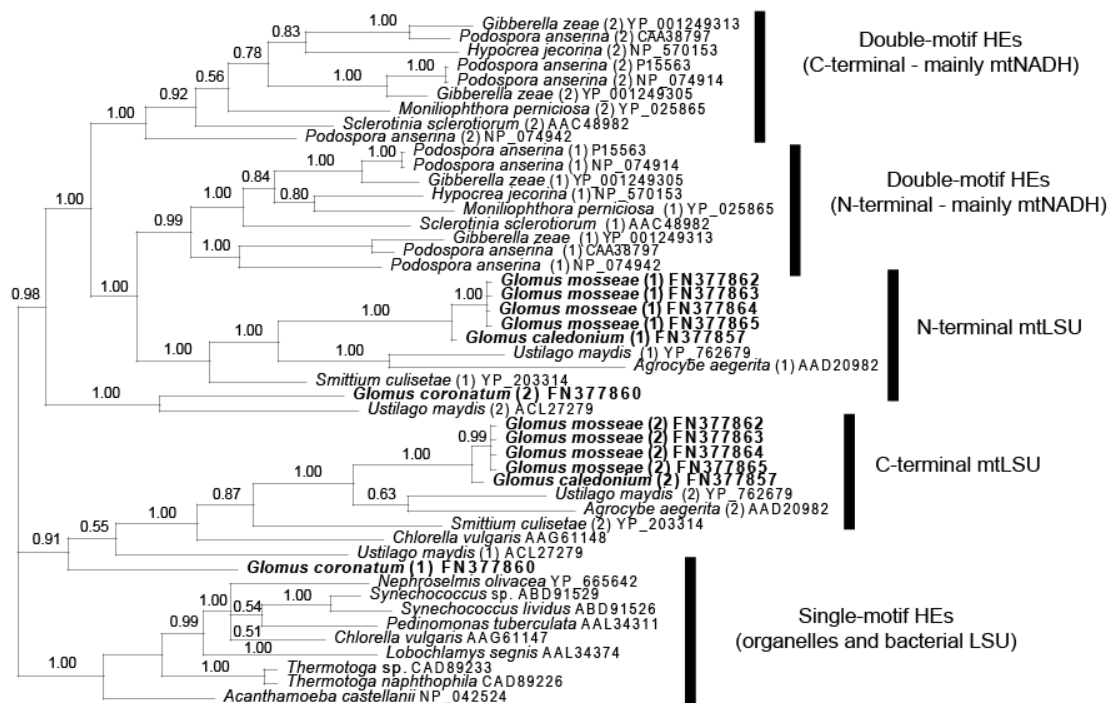
Fig. 11 Phylogenetic tree of putative LAGLIDADG type 1 proteins, based on an alignment of 143 amino acids. Values on the nodes indicate Bayesian posterior probabilities. LAGLIDADG conserved domains were treated separately in the alignment. Numbers in brackets designate the N-terminal domain (1) or the C-terminal domain (2) for doublemotif HEs, or in the case of *G. mosseae* and *G. coronatum* for distinct ORFs in the same intron.

Single-domain LAGLIDADG from organelles of algae and other organisms were used as outgroup since it has been shown that double-motif LAGLIDADGs have evolved by duplication of single-motif proteins (Belfort & Roberts 1997). A monophyletic group almost exclusively containing fungal two-domain LAGLIDADG was recovered, which was well-supported in Bayesian analysis (posterior probability = 1.00). Single-domain LAGLIDADG ORFs from *G. mosseae* and *G. coronatum* as well as from the green alga *Chlorella* in this clade were the only exception we detected. The sequence from the alga grouped rather basally. Interestingly, two overlapping single-domain ORFs from intron 1450 of *G. coronatum* also grouped basally in this clade. Their origin from single or double-domain LAGLIDADG remains unclear.

Besides two clades formed by mtLSU-hosted N-terminal and C-terminal domains of fungal HEGs, respectively, two more monophyletic groups were found, representing the domains of LAGLIDADG inserted in other fungal mitochondrial genes, mainly NADH. The N-terminal domains from mtLSU and NADH formed a clade highly supported by posterior probabilities, indicating that they share a common ancestor, whereas a clade of the C-terminal domains was not supported, but neither could be rejected. The tree indicates that the two single motifs of *G. mosseae* are clearly derived from double-motif ancestors.

This phylogenetic analysis indicates that mtLSU glomeromycotan HEGs spread to other mitochondrial target genes like NADH of other fungi, mainly Ascomycetes. Interestingly, the clade appears to be restricted to fungi, suggesting that the HEGs spread within the true fungi or even coevolved with them.

A.4.5.3 Intron 1450: two different intron types in the same insertion site

As mentioned above, *G. coronatum* displayed overlapping genes of LAGLIDADG type 1 HEs, orientated in the same direction, which could be attributed to a duplication event. This process may have been the same as the one originally leading to the domain duplication in LAGLIDADG type 1 gene of *G. caledonium* by duplication of overlapping sequence regions and their elimination (Keese & Gibbs 1992).

Interestingly, the *Glomus* sp. ISCB 34 intron 1450 was not related to the *G. coronatum* intron in the same position, they did not even belong to the same subtypes (Table 5). No conserved domains or ORFs were recognizable in any of the six frames translated from the intronic sequence.

A.4.5.4 Intron 1513: vertically inherited from the ancestor of *Glomus* groups A and B

Translation of the intronic sequence in six reading frames did not show any conserved domains of endonucleases similarly to the *Glomus* sp. ISCB34 intron 1450, which belongs to the same subtype (IB). The phylogeny of this intron (Fig. 12) showed a close similarity to the exon phylogeny (Fig. 8). Together with the fact that it was also present in a member of *Glomus* group B, this suggests that it was present in the common ancestor of *Glomus* groups A and B and was inherited in a rather conservative manner throughout the evolutionary history of these groups with occasional losses.

There is some evidence that reverse splicing (Bhattacharya *et al.* 2005) could play a role in the spread of introns of this group, which did not show hints for endonuclease ORFs: a conserved motif (5'-TCAAAT-3') was found at the insertion site of the intron 1450 and was also present just downstream of the 50 splice site of the intron 1513.
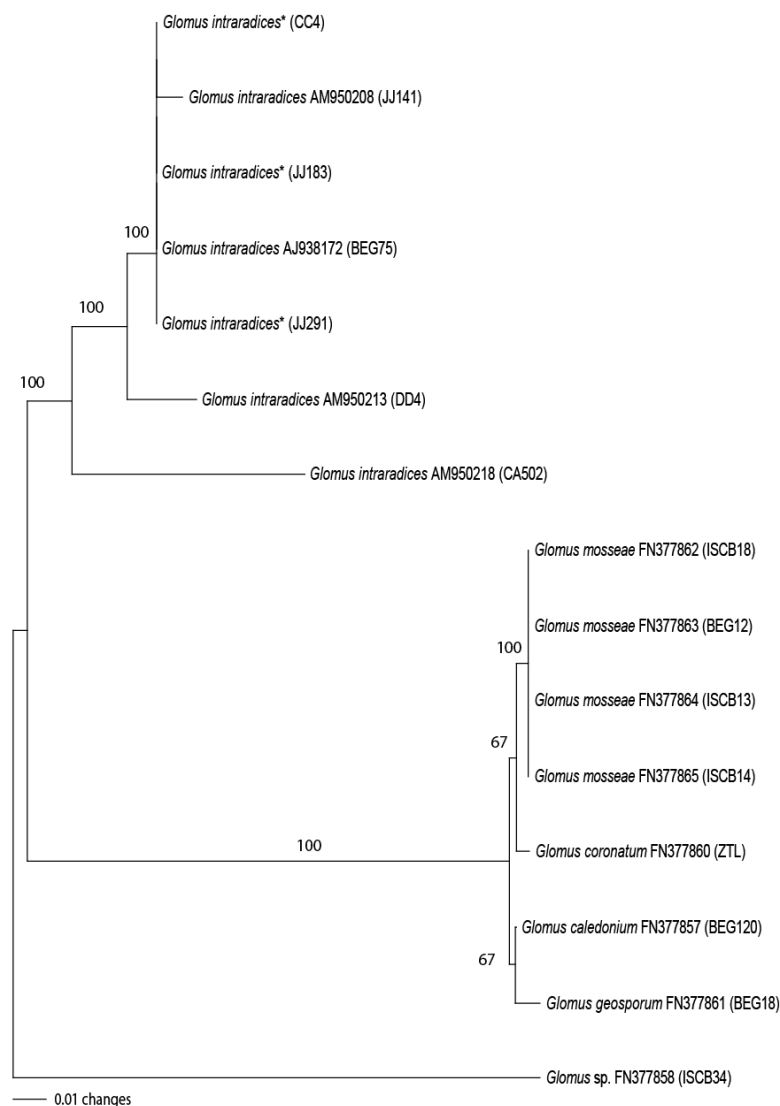
Fig. 12 Phylogeny of Glomeromycota based on 261 bp of intron 1513 sequences. The phylogenetic tree was generated by a heuristic search under the parsimony criterion. Numbers at the node indicate bootstrap values for 1000 replicates. Labels include species name, accession numbers and isolate codes (in brackets). *Consensus sequences made from AM950210, AM950211 and AM950212 for *G. intraradices* JJ183; AJ973189, AJ973190 and AJ973191 for *G. intraradices* JJ291; AM950204, AM950205 and AM950206 for *G. intraradices* CC4.

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A.5 Discussion

Raab *et al.* (2005) showed that in strong contrast to many nuclear-encoded genes, in particular rDNA, mtLSU in the two glomeromycotan species *G. intraradices* and *G. proliferum* was homogeneous within the same strain. Lee & Young (2009) confirmed this finding for the complete mitochondrial genome of *G. intraradices*. Here we show that this genetic homogeneity of mtLSU is also a general feature of other taxa of arbuscular mycorrhizal fungi.

While not showing within-strain mtLSU polymorphisms, many isolates of *G. intraradices* differed strongly with regard to their mtLSU introns (Börstler *et al.* 2008), allowing insights into the population structure of this ubiquitous species, which has also gained importance as model organism because its genome is being sequenced (Martin *et al.* 2008). Species definition of *G. intraradices* is problematic and within the current morphology-based circumscription, several genetic lineages were identified that may eventually correspond to species. In particular, it was demonstrated that the ubiquitous lineage containing the strain used for genome sequencing (GLOM A-1) is distinct from the type strain (Stockinger *et al.* 2009). However, as some of these isolates of different mtLSU haplotypes were shown to fuse hyphae and exchange genetic markers, these haplotypes do not necessarily correspond to cryptic species (Croll *et al.* 2009).

In contrast to the high level of polymorphism among *G. intraradices* isolates, we show that two *G. clarum* isolates and four *G. mosseae* isolates from different locations did not differ at all in the respective sequence region. We cannot exclude that by analyzing a larger number of isolates some polymorphisms would be eventually obtained, but the low level of divergence even of the related morphospecies *G. coronatum*, *G. geosporum* and *G. caledonium* does not make this kind of search promising. The low level of genetic polymorphism in *G. mosseae* is in agreement with other authors' findings (Rosendahl & Matzen 2008), that this species does not show geographical structure across continents (Giovannetti *et al.* 2003). The biological reason for this apparent discrepancy between the *G. intraradices* and the *G. mosseae* groups currently remains unclear but it will be interesting to address this on a broader scale by analyzing additional families of the Glomeromycota.

As the species in the *G. mosseae* clade, the two *Scutellospora* species are relatively closely related, as judged by their partial 18S n-rDNA sequences. However, in their mtLSU exon short but distinct sequence signatures were present distinguishing them.

The introns found in the different *Glomus* spp. provide evidence for a variety of processes of intron and HEG evolution. This is the first time that these processes are addressed in the phylum Glomeromycota.

Among the introns, there is a wide gamut of the different degrees of conservation. Intron 1513 has apparently been inherited strictly vertically from the ancestors of *Glomus* groups A and B, and its phylogeny correlates well to the exon phylogeny. This group IB intron was already shown to be relatively stable in *G. intraradices* (Börstler *et al.* 2008).

The phylogeny of intron 1149 was not completely concordant with what would be expected from the exon and n-rDNA. In particular the striking similarity of the corresponding sequences from *G. intraradices* JJ183 and *G. clarum* indicates occasional horizontal transfer. However, all evidence was in favor of a transfer of the intron together with its HEG, which is in agreement with some findings of Haugen & Bhattacharya (2004) even though these authors also reported intron-independent mobility of HEGs (Haugen *et al.* 2004). In this intron, we can see a gradient of purifying selection between putatively active endonucleases and putatively non-functional sequences. The overall negative selection in intron 1149 may have played a role in maintaining LAGLIDADG genes to ensure their spreading to intron-less individuals. Evolutionary pressure still exists in disrupted endonuclease domains of *G. intraradices* isolates JJ141 and JJ183 as well as in *G. caledonium* but is relatively low in the putative non-functional ORF of *G. coronatum*, suggesting that when homing activity ceases, the degeneration of HE accelerates until its total suppression. The presence, maintenance and degeneration of LAGLIDADG ORFs observed in this study are highly compatible to the stages in theory of "life cycle" described by Goddard & Burt (1999). Interestingly, even in *G. mosseae*, which does not seem to possess an intact ORF, the degradation of the sequence is limited. We can speculate that purifying selection might occur on parts of introns required for self-splicing.

In the *G. mosseae* clade, we found different stages of the degradation of the HEG-containing intron 1187: intact ORF in *G. caledonium*, the ORF split into two parts in *G. mosseae* and the ORF lost from the intron in *G. coronatum*. *G. geosporum* which lacks this intron may represent an ancestral state before the intron was inserted. However, considering the established n-rDNA phylogeny of these species which places *G. geosporum*/*G. caledonium* as sister group to *G. mosseae* and *G. coronatum* (Schwarzott *et al.* 2001), it appears more likely that the common ancestor of the clade possessed the intron and *G. geosporum* lost it completely. It will be intriguing to address in future studies whether different stages of the "homing cycle" can be detected within populations of each of these species as demonstrated for other fungi by Reeb *et al.* (2007). In *G. intraradices* isolates, different stages of degradation of HEG ORFs are known, in *G. mosseae* on the other hand we could not find any polymorphisms so far in the introns.

Haugen & Bhattacharya (2004) analyzed the evolutionary relationships of HEGs and postulated gene duplication through which double-motif HEGs arose from single-motif HEGs. Interestingly, these authors found exclusively fungal sequences in the monophyletic clade of double-motif HEs, a fact that they did not discuss further. This "clade 1" corresponds to the clade of double-motif HEs in Fig. 11. The strict predominance of fungal sequences in this clade indicates that these HEs have evolved and may even have originated within the true fungi.

In contrast to Haugen & Bhattacharya (2004), we also included HEGs in the dataset which are hosted in other mitochondrial genes than LSU, for instance NADH3. The phylogenetic analyses (Fig. 11) suggest that the spread towards other genes occurred after the domain duplication. The

fact that only the N-terminal domains in mtLSU and other genes are supported as a monophyletic group may indicate that this target change occurred relatively quickly after the duplication. This interpretation would be compatible with the hypothesis that duplication facilitates the spread of the HE to a broader range of target sequences, because single domain HEs assembling into homodimers are mainly limited to palindromic target sites (reviewed by Gimble, 2000).

Interestingly, the two overlapping single-motif ORFs in intron 1450 of *G. coronatum* also belong to this major clade, but evolved separately. It is intriguing to speculate that these ORFs are the remnants of another lineage of double-motif HEGs that by a frameshift fragmented in a way reminiscent of the HEG in intron 1187 in *G. mosseae*, but with non-coding sequences between the ORFs. Alternatively, the observed overlapping could represent an early stage of the fusion of two HEGs into a two-domain ORF.

In contrast to other studies claiming rampant interkingdom intron transfer (Cho & Palmer 1999), which, however, were challenged by other authors (Cusimano *et al.* 2008), most introns in the DNA region we studied appear to be predominantly spreading among the true fungi. This is in agreement with the results of Haugen & Bhattacharya (2004), who demonstrated extensive change of target sites within the same target gene (LSU), but exclusively within true fungi. However, intron transposition between mycorrhizal fungi and plant hosts seems possible as suggested by Lang & Hijri (2009).

HEs have recently received considerable attention in biotechnology as model systems for the engineering of enzyme activity (Silva *et al.* 2006). This is possible because active domains and DNA target sites are well-characterized and can be manipulated. Our data provide insights into the in situ dynamics of HEGs in a natural system, demonstrating major stages of the "Homing Cycle" (Chevalier & Stoddard 2001). By providing an evolutionary snapshot of LAGLIDADG HEGs in a group of "lower fungi", which have been strongly underrepresented in the databases, this study will contribute to a better understanding of the evolution of these ubiquitous genes.

A.6 Acknowledgments

A.7 References

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May 99 – Jun 99	**Jatun Sacha Foundation**, Guandera Biological Reserve, Ecuador Management of tree nursery, reforestation, assistance in scientific research
Apr 96 – Jun 96	**Gudhorst Permaculture Farm**, Rottorf, Germany Farm work with different low-input management and permaculture methods
Jun 95 – Sep 95	**Kibbutz Hukok**, Israel Planting, nursing/cultivating and harvesting in banana and date palm plantations

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Apr 95 – Mar 96	**Bicycle-Tour** around the Mediterranean, multicultural experiences in 15 countries

PUBLICATIONS – only ISI-listed journals

Thiéry O, **Börstler B**, Ineichen K, Redecker D (2010) Evolutionary dynamics of introns and homing endonuclease ORFs in a region of the large subunit of the mitochondrial rRNA in *Glomus* species (arbuscular mycorrhizal fungi, Glomeromycota). *Molecular Phylogenetics and Evolution* **55:** 599-610.

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SYMPOSIA & CONFERENCES, I have participated as first author during my PhD time

Symposium of the Zurich-Basel Plant Science Center (PSC): Plant-Microbe Interaction, 2009 in Basel (Switzerland). **Poster presentation**: Diversity of mitochondrial large subunit rDNA (mtLSU) haplotypes of *Glomus intraradices* in different environemts. Authored by: Börstler B, Thiéry O, Sýkorová Z & Redecker D.

ICOM6: 6th International Conference On Mycorrhiza ´Beyond the roots´, 2009 in Belo Horizonte (Brazil). **Contributed oral presentation:** Diversity of mitochondrial large subunit rDNA (mtLSU) haplotypes of *Glomus intraradices* in different environemts. Authored by: Börstler B, Thiéry O, Sýkorová Z & Redecker D.

EURECO-GfÖ: 11th European Ecological Conference of the European Ecological Federation jointly with the 38th Annual Conference of the Ecological Society of Germany, Austria and Switzerland, 2008 in Leipzig (Germany). **Contributed oral presentation**: Mitochondrial large ribosomal subunit sequences as potential marker for population studies of *Glomus intraradices*. Authored by: Börstler B, Thiéry O, Raab P & Redecker D.

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Mycological Society of America (MSA) Annual Meeting, 2007 in Baton Rouge (LA, USA). **Contributed oral presentation**: Mitochondrial large ribosomal subunit sequences as potential marker for population studies of *Glomus intraradices*. Authored by: Börstler B, Raab P & Redecker D.

AWARDS

Symposium of the Zurich-Basel PSC: Plant-Microbe Interaction, 2009 in Basel (Switzerland):
Poster Award, 3rd place

International Conference: ICOM6, 2009 in Belo Horizonte (Brazil):
Laval University Prize for first runner-up for an oral presentation by a graduate student

International Conference: Plant-Microbial Interactions, 2008 in Kraków (Poland):
Award for the best young scientist oral presentation