Development of new molecular markers for phylogeny and molecular identification of arbuscular mycorrhizal fungi (Glomeromycota)

INAUGURALDISSERTATION

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von
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Basel, April 2007

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

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Zusammenfassung

Aufgrund der hohen genetischen Variabilität der kerncodierten ribosomalen RNA-Gene in Einzelsporen von arbuskulären Mykorrhizapilzen (AMF, Glomeromycota) wurden alternative Markergene gesucht, um vor allem nah verwandte Taxa dieser Pilze besser auflösen zu können.

Basierend auf publizierten Nukleotidsequenzen der mitochondriellen großen Untereinheit der ribosomalen RNA (mt-LSU) wurden pilzspezifische Primer entwickelt und damit erstmals in grösserem Umfang Teilsequenzen der mt-LSU von arbuskulären Mykorrhizapilzen amplifiziert und sequenziert. Es wurde ein spezifischer Primer für *Glomus intraradices* und *Glomus proliferum* entwickelt und erfolgreich an Feldproben getestet.

Im Gegensatz zu den kerncodierten ribosomalen RNA-Genen zeigten die mt-LSU Sequenzen von *G. intraradices* und *G. proliferum* keinerlei Anzeichen von genetischer Variabilität innerhalb der Isolate. Desweiteren konnten sogar mt-LSU Sequenzen unterschiedlicher Isolate von *G. intraradices* klar voneinander abgetrennt werden.

Phylogenetische Untersuchungen der mt-LSU auf der Ebene der Pilzphyla ergaben keinen Aufschluss über die stammesgeschichtliche Stellung der Glomeromycota, da diese Sequenzen zwischen den Grossgruppen der Pilze zu variabel sind. Allerdings zeigten sich neben einer klaren Abgrenzung zwischen *G. intraradices* und *G. proliferum* zusätzlich vielversprechende spezifische Eigenschaften der mt-LSU Sequenzen in AMF. So wurden in den Teilsequenzen der mt-LSU diverser Isolate von *G. intraradices* Introns in verschiedener Anzahl identifiziert. Der unterschiedliche Introngehalt von Isolaten von *G. intraradices* und die Abwesenheit der Introns in *Glomus proliferum* weisen evolutionsgeschichtlich auf vor relativ kurzer Zeit eingetretene Ereignisse des Verlusts oder des Zugewinns dieser Introns hin. Die weitere Untersuchung der Verbreitung der Introns wird zeigen, in welchem zeitlichen Rahmen sie transferiert wurden. Innerhalb der Nukleotidsequenz eines aufgetretenen Introns in der mt-LSU einiger *G. intraradices*-Isolate wurde erstmalig in AMF ein offenes Leseraster (ORF) einer “Homing“-Endonuklease der LAGLIDADG2-Familie identifiziert. Phylogenetische Untersuchungen zur Verwandtschaft dieser Enzyme mit anderen Endonukleasen zeigten, dass die ähnlichsten bisher bekannten LAGLIDADG-Endonukleasen in Introns von

Ascomyceten gefunden wurden. Aufgrund des zu erwartenden gelegentlichen horizontalen Transfers der Introns stimmt die Phylogenie der Proteinsequenzen nicht mit der etablierten Phylogenie der Pilze überein. Dagegen eröffnen sich gerade aufgrund der enzymatischen Eigenschaften dieser Endonukleasen interessante Nutzungsmöglichkeiten für die Zukunft.
Insgesamt stellen die mt-LSU und ihre Introns vielversprechende molekulare Marker mit hoher Auflösung dar, um Artengemeinschaften und Populationen der Glomeromycota zu untersuchen.

Als weiteres potentielles Markergen für AMF wurde das kerncodierte RPB1-Gen analysiert, das für die grösste Untereinheit der RNA-Polymerase II codiert. Es wurden neue spezifische Primer für die *Glomus*-Gruppe A entwickelt und erfolgreich an diversen Mitgliedern dieser Gruppe getestet. Es zeigten sich auch hier keine Anzeichen von genetischer Variabilität innerhalb der Isolate. Basierend auf den neuen RPB1-Sequenzen wurden phylogenetische Analysen erstellt. Die Glomeromycota wurden als monophyletische Gruppe wiedergegeben, deren engste Verwandte die *Mortierellales* (Zygomycota) darstellen. Das Konzept einer monophyletischen Gruppe von Asco- Basidio- und Glomeromycota (“Symbiomycota“) fand in allen RPB1-basierenden Analysen keine Unterstützung, wohingegen die “Dikaryomycota“ (=Asco- und Basidiomycota) als Gruppe bestätigt wurden. Die kleinskaligere RPB1-Phylogenie der AMF wurde am besten über die Nukleotidsequenzen wiedergegeben. Dabei wurden Morphospezies der *Glomus*-Gruppe A gut aufgelöst. *Geosiphon pyriformis* wurde als basalstes Mitglied der Glomeromycota identifiziert. Für eine umfassende RPB1-Phylogenie der Glomeromycota fehlen derzeit jedoch noch Sequenzen verschiedener anderer Familien der AMF.
Bei allen Vertretern der Glomeromycota wurde ein Intron an derselben Stelle der RPB1 Sequenz entdeckt. Dieser Bereich scheint daher prädestiniert zu sein um zukünftig durch Restriktionsanalysen eine Artenbestimmung in Feldstudien zu ermöglichen.

Summary

Because of the high genetic variability of the nuclear-encoded ribosomal RNA genes within single spores of arbuscular mycorrhizal fungi (AMF, Glomeromycota), new alternative marker genes are needed to better resolve closely related taxa. Based on published nucleotide sequences of the mitochondrial large ribosomal subunit (mt-LSU), new fungal-specific primers were constructed and successfully used for the amplification and sequencing of a partial sequence of the mt-LSU from arbuscular mycorrhizal fungi. A specific primer for *Glomus intraradices* and *Glomus proliferum* was constructed and successfully tested on field samples.

In contrast to nuclear encoded ribosomal genes, there was no evidence for genetic variability of the mt-LSU sequences of *G. intraradices* and *G. proliferum* within fungal isolates. Furthermore, different isolates of *G. intraradices* could be clearly distinguished from each other by their mt-LSU sequences.

Phylum-level phylogenetic analyses of the mt-LSU in the true fungi did not provide compelling support for the relationships between Glomeromycota and other fungal lineages, because the sequences are too variable among phyla. Nevertheless, *G. intraradices* and *G. proliferum* were clearly distinguished. Different numbers of introns in the partial sequences of the mt-LSU were identified in several *G. intraradices* isolates. The varying number of introns among the different isolates of *G. intraradices* and the absence of introns in *G. proliferum*, suggest that the intron gain or removal events occurred relatively recently in evolutionary history. Further studies of the distribution of the introns will provide evidence of how frequently they are transferred.

An open reading frame (ORF) of a putative homing endonuclease from the LAGLIDADG2 family was identified in mt-LSU introns of several *G. intraradices* isolates. The phylogeny of the protein sequences showed that the closest known relatives of the LAGLIDADG endonucleases were found in mitochondrial introns of Ascomycetes. As occasional horizontal transfer of these introns is likely, it is not unexpected that the phylogeny obtained is in conflict with established fungal phylogenies based on other marker genes. However, peculiar properties of the homing endonucleases offer promising potential for future studies.

Overall, the mt-LSU and its introns are promising high-resolution molecular markers to analyze communities and populations of AMF.

The RPB1 gene was analyzed as a possible new nuclear-encoded molecular marker for the Glomeromycota. New *Glomus* group A-specific primers were designed and successfully tested on several members of this group. No evidence for genetic variability was found within the isolates. Based on the new RPB1 sequences, phylogenetic analyses were performed. The phylum-level phylogeny of the fungi was very well resolved by protein sequence analyses. The Glomeromycota were recovered as a monophyletic group, with the *Mortierellales* (Zygomycota) as closest relatives. A symbiomycotan clade (Asco-, Basidio and Glomeromycota) was not supported in the RPB1 phylogeny whereas the "Dicaryomycota" (Asco- and Basidiomycota) were supported in the trees. The morphospecies-level RPB1 phylogeny of *Glomus* group A performed best using nucleotide sequences. Interestingly, *Geosiphon pyriformis* was determined to be the most deeply-diverging lineage of the Glomeromycota. However, RPB1 sequences of representatives of the remaining families are needed for for a comprehensive phylogeny of glomeromycotan fungi. All members of the Glomeromycota contained an intron at the same location in their RPB1 gene. This sequence region seems to be ideal for molecular species identification using restriction analysis in community studies of the AMF in the future.

1. Introduction

1.1. The Glomeromycota

Arbuscular mycorrhizal fungi (AMF) colonize roots of the majority of land plants to form arbuscular mycorrhiza (AM). The evolutionary history of these fungi can be traced back to the Ordovician, 460 Myr BP (Redecker *et al.* 2000a). Although until today less than 200 morphospecies of the arbuscular mycorrhizal fungi have been described, AMF are the most widespread mycorrhiza-forming fungi worldwide. These fungi produce relatively large spores (40-800 µm) with layered walls, which contain several hundreds to thousands of nuclei (Becard & Pfeffer 1993). Their spores are formed singly, in clusters or can aggregate in sporocarps (Gerdemann & Trappe 1974). All AMF are placed in the fungal phylum Glomeromycota (Schüßler, Schwarzott & Walker 2001).

Arbuscular mycorrhiza is a symbiosis between plants and AMF. The fungi improve the plant mineral uptake and plant growth (Smith & Read 1997) and protect their host plants by suppressing plant diseases (Borowicz 2001). In turn, they receive carbohydrates from their plant host. Specific interactions among plant and fungal symbionts were shown to have an influence on the composition of plant communities (van der Heijden *et al.* 1998). Because of these attributes, AMF may play an important role in the current paradigm shift from conventional to sustainable land management practices (Ryan and Graham 2002).

1.1.1. Systematics of the Glomeromycota

For a long time the systematic position of the arbuscular mycorrhizal fungi within the fungi has not been clarified. Gerdemann & Trappe (1974) removed all AMF from *Endogone* and placed them in the four new genera *Glomus*, *Sclerocystis*, *Gigaspora* and *Acaulospora*. In 1990 Morton & Benny established a new order “Glomales” in the Zygomycota. After evidence accumulated that AMF are distinct from other Zygomycota (e.g. no zygospores are formed, obligate mutualistic symbiosis, rDNA

phylogeny) the arbuscular mycorrhizal fungi were grouped into an own phylum, the Glomeromycota (Schüßler *et al.* 2001).

The Glomeromycota are grouped into ten genera among which the genus *Glomus* with over 70 morphospecies is the largest.
The genus *Glomus* is divided into three groups (Schwarzott *et al.* 2001). *Glomus* groups A and B form a monophyletic clade. Their spores are formed by budding from a hyphal tip and typically have a layered wall structure. The sporogenic hyphae often remain attached to the mature spore. The glomoid mode of spore formation occurs not only in *Glomus* but also in *Paraglomus*, *Archaeospora*, *Pacispora*, *Diversispora* and *Geosiphon* (Redecker & Raab 2006). Based on molecular analysis some of these genera were separated from *Glomus*.

Members of the *Glomus* group A are the dominant and most diverse AMF in many field sites (Vandenkoornhuyse *et al.* 2002). They include the widespread representatives *Glomus intraradices* and *Glomus mosseae*. Some species forming *Glomus*-type spores in complex sporocarps were previously placed in the genus *Sclerocystis*. After Almeida and Schenk (1990) transferred all members except *Sclerocystis coremioides* into *Glomus* based on morphological considerations, Redecker *et al.* (2000c) could show through molecular phylogenetic analysis that the last remaining member grouped well within a clade of *Glomus* species and therefore was classified as *Glomus coremioides*.
The second group (*Glomus* group B) contains several AMF species like *Glomus etunicatum, Glomus claroideum* and *Glomus lamellosum* (Fig.1).
Glomus group C is more closely related to the Acaulosporaceae than to *Glomus* groups A and B based on phylogenies of nuclear-encoded ribosomal genes (n-rDNA) (Schwarzott *et al.* 2001). Based on ribosomal sequence signatures, Walker & Schüßler (2004) described one species in a new genus *Diversispora* as *Diversispora spurca*.

Acaulospora and *Entrophospora* are the two genera in the Acaulosporaceae. They both form spores next to “sporiferous saccules”, which collapse or completely disappear after spore maturation. The position of these sporiferous saccules is the

morphological criterion to distinguish *Acaulospora* and *Entrophospora*. It is produced laterally in *Acaulospora* and formed within the subtending hypha in *Entrophospora*.

The Gigasporaceae contain the two closely related genera *Scutellospora* and *Gigaspora*. *Scutellospora* possesses a “germination shield”, a membraneous structure that is used during the spore germination to penetrate the spore wall. Both can be distinguished morphologically by the absence of a flexible inner spore wall in the *Gigaspora*. The Gigasporaceae do not form vesicles within the roots and form so-called “auxiliary cells” on the extraradical mycelium.

Pacispora species form spores like *Glomus* but have flexible inner walls and a germination orb. This genus was recently established by Oehl & Sieverding (2004).

The Archaeosporaceae and Paraglomeraceae are thought to be the basal members of the Glomeromycota. This is based on phylogenetic studies of the n-rDNA by Redecker *et al.* (2000b) and the possession of unique fatty acids not found in other glomeromycotan fungi (Graham *et al.* 1995).

Geosiphon pyriformis is the only known member in the phylum forming a symbiosis with the cyanobacterium *Nostoc punctiforme.* Based on pylogenetic studies of the n-rDNA, it is closely related to the Archaeosporales (Schwarzott *et al.* 2001).

1.1.2. The arbuscular mycorrhizal symbiosis

The transfer of nutrients between the colonized cortical cells and the arbuscule is the most important feature of the mycorrhizal interaction. Plant carbohydrates (in form of sucrose) are exchanged for water and nutrients from the fungus (mainly phosphate and nitrogen). Arbuscular mycorrhizal fungi are obligate biotrophic. This can be explained through the fact that their extraradical hyphae are unable to take up carbohydrates (Pfeffer *et al.* 1999). In this symbiosis, there is a continuum from mutualistic to parasitic forms, in which the parasitic plants are able to invert the net flow of carbohydrates from the fungal network to them (Bidartondo *et al.* 2002).

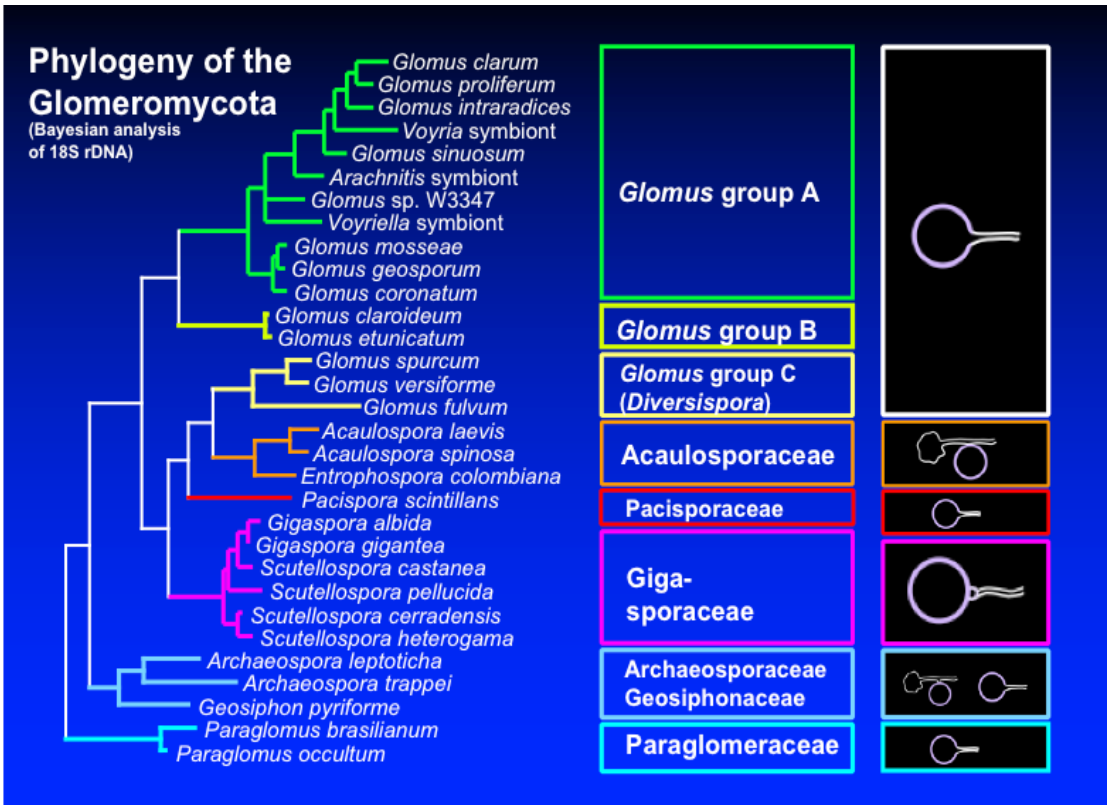

Fig.1

Phylogeny of the Glomeromycota based on Bayesian analysis of the 18S rDNA. Boxes on the right side depict the ways the spores are formed.

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Nutrients are actively transported across the periarbuscular membrane through membrane transporters that work with proton gradients across the membrane.

Guttenberger (2000) could show an acidic compartment in the periphery of arbuscules by staining mycorrhizal roots with Neutral Red and Lyso-Sensor Green. The existence of H^+-ATPases has been shown in both host plants (Murphy *et al.* 1997, Gianinazzi-Pearson *et al.* 2000, Krajinski *et al.* 2002) and AMF (Ferrol *et al.* 2000, Requena *et al.* 2003).

Govindarajulu *et al.* (2005) were able to show that ammonium from breakdown of fungal arginin by urease aminotransferase was transported to the host cell through ammonium channels. The responsible genes of these channels were highly expressed only in the intraradical mycelium (IRM). Arginine has the role of a carrier of nitrogen from the extraradical mycelium to the IRM. Anorganic nitrogen is converted to arginine in the ERM and then transported to the IRM. Here it is broken down to ornithine and ammonium.

Host plants were shown to rely on the phosphate uptake by the AMF even under conditions where this nutrient was not limited (Smith *et al.* 2003). Fungal phosphate transporters involved in the uptake of phosphate to the hyphae have been found in *Glomus versiforme* (Harrison and van Buuren 1995) and in *Glomus intraradices* (Maldonado-Mendoza *et al.* 2001). They are both similar to members of plant phosphate transporters from the family Pht1 (Rausch and Bucher 2002), which are specifically expressed in AM roots. Immunolocalization of MtPT4, a phosphate transporter found in *Medicago truncatula* (Harrison *et al.* 2002), suggests its location in the periarbuscular membrane. Therefore MtPT4 was assumed to use the pH gradient across the periarbuscular membrane to transport phosphate from the periarbuscular space to the host plant.

On the other hand the fungus obtains carbohydrates mainly in form of sucrose from the phloem broken down to glucose and fructose in the apoplast and taken up by fungal hexose transporters. The fungus transforms the hexoses into trehalose, which is then used in the pentose phosphate pathway or in the biosynthesis of glycogen and lipids (Pfeffer *et al.* 1999).

1.1.3. The life cycle of arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi are obligate biotrophic root symbionts that cannot be propagated in pure cultures. The fungal life cycle starts with the germination of hyphae from resting spores. The hyphae lack regular septation which is a characteristic that all glomeromycotan members have in common with the Zygomycota. The spores are able to germinate in the absence of host plants, but the growth of the hyphae is limited to a few days or weeks, depending on the fungus (Tamasloukht *et al.* 2003). These autors showed a strong increase in hyphal growth and branching only in the presence of root exudates. As fungal reaction, an activation of specific genes was reported (interestingly, several genes that are associated with mitochondrial activity) followed by subsequent physiological and morphological changes. On the other hand, germinating spores produce diffusible factors that lead to an expression of specific genes in the host plant root cells even in the absence of direct physical contact (Kosuta *et al.* 1998). The chemical nature of diffusible factors of plants and of fungi are not yet known. First insights in this field

were provided by Akiyama *et al.* (2005). They could show that strigolactones, i.e. sequiterpenes, induced hyphal branching in *Gigaspora margarita* at very low concentrations.

When host plant and fungus have their first physical contact between hyphae and plant root, the fungus forms an appressorium and subsequently penetrates the root surface and colonizes the intercellular space of the root cortex. Using a plant mutant of *Lotus japonicus* affected in the symbiosis genes SYM15 or SYMRK, Demchenko *et al.* (2004) could show that the plant actively allows the fungus to penetrate the rhizodermis. They identified three steps in the interaction that were differentially impaired in the mutants. First the surface opening, where the anticlinal cell walls of two adjacent epidermal cells separate from each other in the vicinity of fungal hyphae, second the intracellular passage of hyphae through an exodermal cell and an adjacent cell of the outermost cortical layer and third the arbuscule formation in cells of the two innermost cortical layers.

After subsequent penetration of the innermost cortical layers, the fungus forms tree-like fungal structures, called arbuscules, inside the root cortical cells. With some exceptions (e.g. the genera *Paraglomus*, *Scutellospora* and *Gigaspora*) all AMF then form intra-and intercellular storage organs (i.e.vesicles) in the late stage of the symbiosis (Smith and Read 1997, Morton and Redecker 2001).

These arbuscules are the central place of nutrient exchange in the symbiosis of the AM. After four to ten days, the arbuscules are degraded by the plant cells (Sanders *et al.*1977). The plant cells regain their original morphology (Jacquelinet-Jeanmougin *et al.* 1987) and enable a new colonization from AMF. The life cycle is completed by the formation of new spores by the AM fungus.

1.1.4. Current challenges in the genetics of AMF

For a long time the only molecular marker available for phylogenetic reconstruction and identification of AMF for all genera were the nuclear-coded ribosomal genes (n-rDNA). The n-rDNA comprises highly conserved as well as variable regions. Therefore, these genes are most useful from species identification up to phylum-level phylogeny.

However, the n-rDNA in AMF has the disadvantage that the variable regions show some variation within the organism (Lanfranco *et al.* 1999, Jansa *et al.* 2002). A similar phenomenon was reported for some protein genes but not others (Helgason *et al.* 2003, Corradi *et al.* 2004, Stukenbrock and Rosendahl 2005). From a single spore of an AMF a large number of slightly different variants of n-rDNA sequences can be recovered, causing problems in distinguishing and recognizing related AMF (Sanders *et al.* 1995, Jansa *et al.* 2002).

Kuhn *et al.* (1991) presented several indications for clonal reproduction through character incompartibility analysis of the ITS and 25S of several AMF. Additionally nuclear heterogeneity in AMF was predicted based on the results of fluorescent *in situ* hybridization analysis (FISH) from two ITS2 variants in *Scutellospora castanea* and based on analysis of the BIP gene in *Glomus intraradices*. Although the BIP gene is single copy and highly conserved in eukaryotes, 15 variants were identified in *Glomus intraradices.*

Based on studies of POL1- like sequences (PLS1 and PLS2) Pawlowska and Taylor (2004) favored the homokaryotic spore model with polyploid nuclei. They showed that all 13 variants of the PLS1 that existed in *Glomus etunicatum* were inherited from the mother spore to five daughter spores without the loss of one of these variants. A heterokaryotic model was excluded through several mathematical models assuming bottleneck events for nuclei transmission during spore gemination and spore formation. All calculated models predicted the loss of variants to the next generation in these scenarios using the single-copy PLS1 gene. Another simulation predicted a strictly clonal nuclear transmission from one generation to another.

Hijri and Sanders (2005) argued that these 13 variants of PLS1 would lead to a 13N polyploidy. After measuring the total nuclear DNA content of *Glomus etunicatum* to be 37.45 Mbp by flow cytometry, they calculated that these nuclei would contain only 2.88 Mbp in a 13N polyploidy which they considered improbable. Instead, they reported that *Glomus etunicatum* has a haploid genome and real-time PCR with PLS1 was used to determine that *Glomus etunicatum* has 1.88 copies of the gene per nucleus.

1.2. The mitochondrion and its genes

In addition to the nuclear genome, fungal cells possess a second genome separated from the nuclear genome in their mitochondria. The complete genetic information in the mitochondria is the chondriome. According to the endosymbiont theory its origin is the genome of phagocytized α-Proteobacteria (Dyall *et al.* 2004). The genomes of these bacteria differ from the mitochondrial genome in gene number by 1-3 orders of magnitude, which implies a loss of genetic information in the mitochondria (Adams and Palmer 2003). The sizes of chondriomes vary considerably among plants (200-2400 kbp), fungi (18-176 kbp), animals (14-42 kbp) and protists (5.7-76 kbp) (Backert *et al.* 1997, Gray 1998). Nonetheless mitochondria require several hundred proteins for proper function, the great majority of which (up to 99% in eukaryotes) are encoded by nuclear genes. Therefore, many mitochondrial genes have been transferred to the nucleus or have been replaced by pre-existing nuclear genes of similar function during mitochondrial evolution (Adams and Palmer 2003).

The mitochondrial genes can be divided into the three classes rRNA, tRNA and protein- coding genes with respect to their frequency and underlying biology of their loss from the mitochondrial genome (Adams and Palmer 2003). The small subunit rRNA (SSU) and the large subunit rRNA (LSU) genes are present in all mitochondrial genomes that have been analyzed.

In contrast to the universality of the SSU and LSU in all known mitochondrial genomes, the 5S rRNA gene is absent from most chondriomes and found only in land plants, some green algae, brown algae and red algae (Oudot-Le Secq *et al.* 2001) and the protozoan *Reclinomonas* (Bullerwell 2003b). The reason of this absence is not clear and still subject of speculations (Adams and Palmer 2003).

The number of distinct tRNA genes varies from none (Apicomplexa) to 27 tRNAs in many mitochondrial systems (Lang *et al.* 1999). Until now there is no evidence that these missing mitochondrial tRNA genes have been transferred to the nucleus. The loss has been explained by a double duty of the nuclear- encoded genes, whose tRNAs are now responsible for both mitochondrial and cytoplasmic protein synthesis.

The number of protein genes in mitochondrial genomes is highly variable across the eukaryotes and ranges from three in the Apicomplexa (Feargin 1994) to 67 protein genes in the protist *Reclinomonas americana* (Lang *et al.* 1997). No clear correlation between the number of tRNA genes and the protein genes in a chondriome could be

shown (Lang *et al.* 1999). For example, in fungi 13-14 protein genes and 24-25 tRNA genes are typically found in the chondriome. In yeast, only 7 protein genes were found but the full expected number of tRNA genes. On the other hand, the chytridiomycetes *Spizellomyces* and *Hyaloraphidium* possess the full number of protein genes, but only 7-8 tRNA genes were found (Forget *et al.* 2002, Lang *et al.* 1999).

1.2.1. Fungal inheritance of mitochondria

The fungal inheritance of mitochondria is more complex than in plants and animals and both uniparental and biparental inheritance are common (Taylor 1986). Many different modes of sexual reproduction and exclusion of paternal mitochondria exist in fungi. Some fusing haploid mating types of Basidiomycetes exchange their nuclei reciprocally. Additionally in some species like *Armillaria bulbosa* and *Armillaria ostoyae* mitochondria migrate across the fusion zone (Smith *et al.* 1990). The heteroplasmy in those heterokaryons rarely persists through later zygotic divisions by several mechanisms like selective elimination of the mitochondria of one strain postfertilisation (like in *Cryptococcus neoformans*; Yan & Xu 2003) or separated positions of the paternal mitochondria in the zygote (like in *Saccharomyces cerevisiae*; Nunnari *et al.* 1997). Additionally, persistence of mitochondrial DNA contributed from both mating types was reported in *Physarum polycephalum* (Kawano *et al.* 1987), *Armillaria sp.* (Smith et al. 1990) and in the anisogametous fungus *Neurospora* (Yang & Griffith 1993). Finally, evidence for recombination of mitochondrial DNA in fungi exists (Taylor 1986, Gillham 1994, Saville *et al.* 1998, Birky 2001) in particular in yeast (Dujon *et al.* 1974, Birky *et al.* 1982, Taylor 1986, MacAlpine *et al.* 1998).

The biological benefits of recombination events in fungi is that on one hand uniparental inheritance causes mitochondrial genomes to be vulnerable to the accumulation of deleterious mutants (Gabriel *et al.* 1993, Lynch 1996) and on the other hand it counters the spread of selfish organelles and parasites. Therefore a low amount of biparental mitochondrial transmission may allow organisms to achieve beneficial effects from both sides (Barr *et al.* 2005).

Furthermore, asexual mitochondrial genomes should be plagued with mutation accumulations because of the lack of recombination (Lynch 1996). This was supported by higher accumulation rates of non-synonymous mutations in mitochondrial genomes relative to their nuclear genomes (Lynch 1996). Nevertheless asexual fungal mitochondrial genomes remain functional and integral to fitness in nature (Burton *et al.* 1999, Rand 2001, Christie *et al.* 2004).

Several explanations for this divergence between theory and reality are currently discussed. The first explanation is that the low rate of recombination required to counter mutation accumulation may fall below the current threshold of detection and therefore mitochondrial DNA does undergo recombination, but in a manner or on a scale that it often escapes detection (Barr *et al.* 2005). A second explanation is that mechanisms apart from recombination exist that provide mutational clearance (Bergstrom & Pritchard 1998, Martin & Hermann 1998, Rispe & Moran 2000, Willett & Burton 2003).

Finally Lynch & Blanchard (1998) argued that the accumulation rate of mutations in the mitochondrial genome is slow enough that the fitness loss occurs on a large time scale of tens of million years and therefore mutation accumulation may not be a serious immediate problem for the integrity and function of mitochondrial genomes.

1.3. The RNA polymerase II and the RPB1 gene

The RNA polymerases are multi-subunit complexes. Three kinds of RNA polymerases have been found in eukaryotes. The RNA polymerase I (Pol I) transcribes the rRNA genes for the precursors of the 28S, 18S and 5.8S subunits, while RNA polymerase II (Pol II; also known as RNAP II) transcribes protein-encoding genes into mRNA as well as small nuclear RNAs (snRNA) U1, U2, U4 and U5 which act as ribonucleoparticles in the spliceosome. Finally, RNA polymerase III (Pol III) transcribes the 5S rRNA genes, many snRNAs like U6 and 7SL RNA, Alu-elements and all tRNA genes. The three RNA polymerases consist of 13 subunits in Pol I, twelve subunits in Pol II and 14 subunits in Pol III. RNA polymerase II usually comprises twelve subunits; interestingly, only ten subunits were found in analysis of the yeast Pol II structure (Cramer *et al.* 2001).

Pol II consists of four mobile modules and a clamp swinging over the active center. The clamp allows the entry of straight promoter DNA for the initiation of transcription. X-ray diffraction and phase determination analysis also identified three loops extending from this clamp, which may play roles in RNA unwinding and DNA rewinding during the transcription (Cramer *et al.* 2001). Additionally two metal ions were identified at the active site of Pol II, one of them persistently bound to the active center. In yeast Pol II an Mg^{2+} ion is bound by invariant aspartate sites in the D-region (Fig.2) D481, D483 and D485 of RPB1, which forms together with the RPB2 subunit, the active center in the RNA polymerase II.

The two largest subunits of the RNA polymerase II known as RPB1 (220 kDa) and RPB2 (150 kDa) offer excellent possibilities for molecular phylogenetic studies. They are strictly present as a single copy in fungi. So far paralogues have been found only in plants and trypanosomes. The RPB1 gene is coding for the largest subunit (B220) in the RNA polymerase II. The B220 belongs to a protein family that includes the largest subunit of all multisubunit RNA polymerases.

Mutations in RPB1 and RPB2 were shown to influence the accuracy of mRNA starting site selection (Hekmatpanah and Young 1991). Berroteran *et al.* (1994) could show that mutated SUA8 genes (which correspond to the RPB1 gene) work as suppressors for the CYC1 gene (cytochrom c). Other genes including ADH1 (alcohol dehydrogenase), CYC7 (cytochrom c) and HIS4 (histidinol dehydrogenase) were similarly affected. Therefore, RPB1 together with TFIIB, is an important determinant of transcription start site selection in *Saccharomyces cerevisiae*. Berroteran *et al.* (1994) suggested that this function might be conferred by interaction between these two proteins.

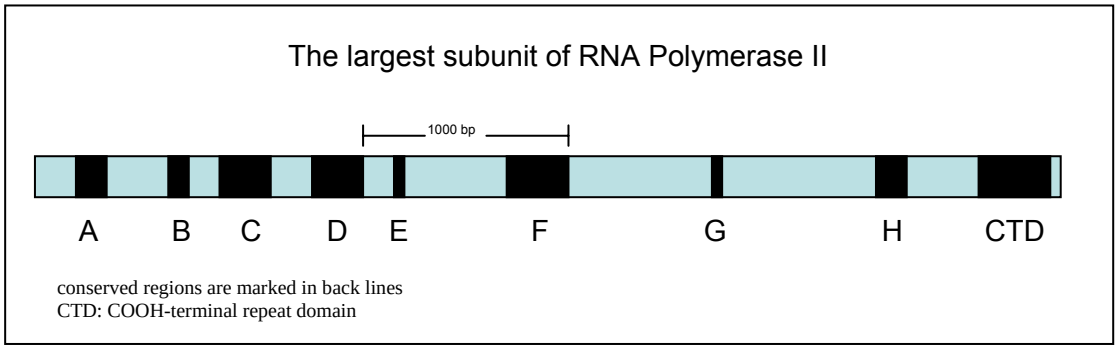


Fig.2
B220, product of the RPB1 gene, belongs to a protein family that includes the largest subunits of all multisubunit RNA polymerases. Note the conserved regions marked by heavy black lines. Region D contains the sequence NADFD which is the RNA polymerase active site. The two D residues are complexed to an Mg2+ ion.

1.4. Aim of the thesis

The only molecular markers for phylogenetic reconstruction and identification of AMF which are currently available for all genera are the nuclear encoded ribosomal genes (n-rDNA). These genes comprise highly conserved as well as variable regions. Therefore, they are useful for a wide range of purposes from phylum-level phylogeny to distinguishing species, and in particular for designing group-specific primers.

A peculiar disadvantage of n-rDNA in AMF is that the variable regions which are most useful for species identification also show some variation within the organism (Sanders *et al.* 1995, Jansa *et al.* 2002). From a single spore of an AM fungus, a large number of slightly different variants can be recovered, causing problems in distinguishing and recognizing closely related AM fungi.

Clapp *et al.* (1995) reported a high level of intra-isolate variation and inter-isolate variation in the nuclear encoded ribosomal large subunit, in the D2 region of *Glomus coronatum, Glomus mossae*, *Glomus constrictum* and *Glomus geosporum*.

This heterogeneity may or may not be linked with the proposed polygenomic structure of the glomeromycotan genome (Kuhn, Hijri & Sanders 2001, Hijri & Sanders 2005), but findings on that phenomenon appear to be in conflict so far (Pawlowska & Taylor 2004). In ecological studies using these sequence markers, slightly different sequences recovered from the environment do not necessarily mean that multiple species or even strains are present, which seriously complicates diversity estimates.

Many protein genes available from AMF that have been examined for heterogeneity within the organism also have been reported to be slightly polymorphic like the BIP gene, the H^+-ATPase gene, the actin gene or PLS genes (Kuhn et al. 2001, Corradi *et al.* 2004, Helgason *et al.* 2003, Pawlowska & Taylor 2004). On the other hand Stukenbrock & Rosendahl (2005) reported the absence of sequence heterogeneity of regions of the putative single copy genes GmFOX and GmGIN within spores of *Glomus mosseae*. The reason for this apparent discrepancy among genes will have to be addressed in the future. Generally, protein genes are less amenable to primer design due to their triplet codon structure and may cause additional complications in phylogenetic analyses caused by paralogues and gene families as found in the tubulin genes (Corradi *et al.* 2004).

In order to circumvent the problems related to heterogeneity of nuclear-coded genes and possible multiple genomes in AM fungi, sequences from an independent genetic system within the fungal organism, the mitochondria were analyzed in this work. The mitochondrial genomes of several fungi and related organisms have been sequenced completely and yielded new insights into their evolutionary biology (Paquin & Lang 1996; Bullerwell, Forget & Lang 2003). A region of the mitochondrial ribosomal large subunit (mt-LSU) has been used very successfully for phylogenetic analyses and the molecular identification of ectomycorrhizal fungi from colonized roots and a large dataset is available for comparison (Bruns *et al.* 1998).

The mitochondrial large ribosomal subunit was targeted as a potential novel sequence marker in AM fungi. The same *Glomus intraradices* isolate (JJ 291) that was used in the publication of Jansa *et al.* (2002) was analysed together with further *Glomus intraradices* isolates that were originated from the same field site in Tänikon, Switzerland. Additionally *Glomus intraradices* isolates originated from Switzerland, Canada and France were used in this work. For analysis of sequence variation among species the closely related AMF *Glomus proliferum* was analyzed and compared to the different isolates of *Glomus intraradices*.

Only a small part of the mt-LSU in *Glomus intraradices* strain DAOM 197198 was sequenced during the diploma work of Annemarie Brennwald (Ochsner 2002).

To verify the 210 bp long fragment of the mitochondrial large ribosomal subunit (mt-LSU) from *Glomus intraradices* isolate DAOM 197198 which was sequenced by A. Brennwald during her diploma work (Ochsner 2002) using the primers LAM3a and LAM4 (designed by A.Brennwald), further PCR reactions with the same primers should be performed with another strain of *Glomus intraradices* (isolate JJ 291) and the closely related species *Glomus proliferum*. Based on that work, the goal was to construct new primers to amplify a larger part of the mt-LSU in *Glomus intraradices* and *Glomus proliferum*.

DNA work with AMF is very prone to contamination by DNA of other microorganisms (Redecker *et al.* 1999; Corradi, Kuhn & Sanders, 2004) and other microorganisms can live inside some AMF (Hijri *et al.* 2002). In order to circumvent such contamination, monoxenic root organ culture plates from *Daucus carota* inoculated with several strains of *Glomus intraradices* and with *Glomus proliferum* were used in these studies.

Presence and absence of sequence variation in glomeromycotan protein genes (e.g. BIP gene, tubulin gene, and GmFOX2, GmTOR2, GmGIN1 genes) were used as arguments for their heterokaryotic and homokaryotic genetic models (Hijri & Sanders 2005, Pawlowska 2005).

The RPB1 gene was used in several phylogenetic analyses of eukarya including plants animals and fungi (Matheny *et al.* 2002, Tanabe *et al.* 2005, Stiller and Hall 1997, Stiller and Harrell 2005, Sidow *et al.* 1994). All works done with the fungal RPB1 gene so far have shown that the gene was single copy and no paralogues of the RPB1 gene occurred in fungi.

Tanabe *et al.* (2004) used RPB1 protein sequences for large-scale phylogenetic analysis to get further insights about the phylogenetic status of the Zygomycota which are thought to be the closest relatives to the Glomeromycota together with the Chytridiomycota (Corradi *et al.* 2004). Unfortunately, no glomeromycotan sequences were used in their analyses. Saturation analyses of fungal elongation factor 1 alpha sequences and the RPB1 gene indicated a low substitutional saturation rate in the RPB1 gene. Therefore this gene was suggested for further high-level phylogeny reconstructions in fungi (Tanabe *et al.* 2004). Together with the availability of many fungal RPB1 sequences through the public gene sequence databases and the AFTOL database (http://ocid.nacse.org/research/aftol/data.php) this gene is a promising candidate for further studies of intra-isolate protein gene variation in AMF and a useful tool to obtain better insights in the phylogenetic relationships among the members of Glomeromycota.

2. Material & Methods

2.1. Chemicals and Methods

All molecular biological standard methods used in this work were performed as described in “Molecular cloning” volume 1-3 (Sambrook *et al.* 1989) unless indicated otherwise.
All chemicals used in this work originated from Fluka Chemie, Buchs, Switzerland. Other manufacturers are mentioned in the text.

2.1.1. Instrumental equipment

ABI prism 310 Genetic Analyzer	PE Applied Biosystems	Foster City, USA
Autoclave AS-6	Schärer	Moosseedorf, Switzerland
Balance Sartorius 2004MP	Sartorius	Göttingen, Germany
Balance Mettler PM 4000	Mettler Instruments	Greifensee, Switzerland
Binocular	Carl Zeiss	Jena, Germany
Centrifuge Biofuge 17RS	Heraeus Sepatech	Zürich, Switzerland
Centrifuge Centra GP8R	Brouwer AG	Luzern, Switzerland
Centrifuge 5415D	Vaudaux-Eppendorf	Basel, Switzerland
Centrifuge 5810R	Vaudaux-Eppendorf	Basel, Switzerland
Gel electrophoresis chamber HORIZON 58	GIBCO BRL / Life Technologies	Gaithersburg, USA
Incubator	Salvis	Rotkreuz, Switzerland
Laminar flow hood PRETTL Laflow	Lufttrockner, Metallbau AG	Wettingen, Switzerland
Magnetic stirrer	Jahnke + Kunkel	Staufen, Germany
Microwave FM A935Q	Moulinex	Dübendorf, Switzerland
Microcentrifuge	Qualitron	Holland, USA
Milli-Q water Purification System	Millipore	Molsheim, France
Pipettes Pipetman P10-5000	Gilson	Middleton, USA
Shaking Incubator Multitron	Infors AG	Bottmingen, Switzerland
Thermal cycler PTC100	MJ Research	Watertown, USA
Thermal cycler Eppendorf Mastercycler	Vaudaux-Eppendorf	Basel, Switzerland
Termomixer Comfort	Vaudaux-Eppendorf	Basel, Switzerland
Vortex-Genie2	Scientific Industries	Bohemia, USA
Spectrophotometer UV-160	Shimadzu	Reinach, Switzerland
Stereomicroscope Olympus SZX12	Olympus	Hamburg, Germany

2.2. Monoxenic root organ cultures used in this study

The following monoxenic root organ cultures were provided by Dr. Jan Jansa, Dr. Natalia Requena and Kurt Ineichen.

Root organ cultures of *Daucus carota* transformed by *Agrobacterium rhizogenes* (Becard & Fortin 1988) were inoculated with the following *Glomus intraradices* and *Glomus proliferum* strains:

Species	**Strain**	**Origin**
Glomus intraradices	JJ 51	Tänikon (Switzerland)
Glomus intraradices	JJ 106	Tänikon (Switzerland)
Glomus intraradices	JJ 232	Tänikon (Switzerland)
Glomus intraradices	JJ 254	Tänikon (Switzerland)
Glomus intraradices	JJ 256	Tänikon (Switzerland)
Glomus intraradices	JJ 291 (=BEG 158)	Tänikon (Switzerland)
Glomus intraradices	JJ 323	Tänikon (Switzerland)
Glomus intraradices	JJ 351	Tänikon (Switzerland)
Glomus intraradices	JJ 746	Tänikon (Switzerland)
Glomus intraradices	DAOM 197198 (=MUCL 43194)	Quebec (Canada)
Glomus proliferum	DAOM 226389 (=MUCL 41827)	Guadeloupe (France)

The Tänikon isolates provided by Dr. Jan Jansa were harvested from a long-term experiment to study the impact of tillage, which started in 1987 (Anken *et al.* 1997). The field site had a size of 90 m x 110 m and was located at Hausweid, Tänikon, Switzerland. The whole field site was divided in plots of 6 m x 19 m under different soil treatments, but all with the same crop rotation of wheat- maize- wheat- rapeseed.

The isolates JJ 254 and JJ 256 originated from till field No.1, while isolates JJ 51 and JJ 106 were originated from non-tilled field No.29. Also JJ 232, JJ 291, JJ 323 and JJ 351 originated from non-tilled field No.40. Isolate JJ 746 originated from the chiselled plot No.12.

Soil from this field was used to inoculate trap cultures. Single spores from trap cultures were used to inoculate individual plants in order to produce single-spore isolates.

Spores from these single-spore isolates were then used to inoculate transformed carrot roots (Becard & Fortin 1988).

2.3. Preparation of the ROC medium culture plates

ROC medium culture plates were used for contaminant free inoculation of AMF spores with transformed *Daucus carota* roots.

Solutions A-E were set up according to table 1 and autoclaved. Afterwards the components G and H were added. The medium was filled with Milli-Q water up to a final volume of 1 liter and again autoclaved. Finally, component F was added and the medium was well mixed. The warm ROC medium (0.01 N citrate-acetate buffer) was then filled into Petri dishes under a lamina flow hood to ensure steril conditions. After the ROC medium was solidified over night, the plates were directly used for the inoculation or stored at 4°C.

Tab.1

ROC medium according to Becard & Fortin (1988), pH 5.5

Solution name	Ingredients	Stock solution (g/l)	Amount per 1l medium
A	$MgSO_4 \cdot 7H_2O$	73.10	10 ml
	KNO_3	8.00	
	KCl	6.50	
	KH_2PO_4	0.48	
B	$Ca(NO_3)_2 \cdot 4H_2O$	28.80	10ml
C	NaFe-EDTA	1.60	5ml
D	KI	0.75	1ml
E	$MnCl_2 \cdot H_2O$	6.00	1ml
	$ZnSO_4 \cdot H_2O$	2.65	
	H_3BO_3	1.50	
	$CuSO_4 \cdot H_2O$	0.13	
	$Na_2MoO_4 \cdot H_2O$ (2.4 g/l)	1.00 ml	
F	Glycin	0.30	10ml
	Thiamin·HCl	0.01	
	Pyridoxin·HCl	0.01	
	Nicotinic acid	0.05	
	Myoinositol	5.00	
G	Sucrose		10g
H	Gellan Gum		3g

2.4. Preparation of transformed root organ cultures of Daucus carota and colonization with spores

An approximately 5 cm long piece of transformed *Daucus carota* root organ culture (provided by Kurt Ineichen) was transferred to a new ROC medium plate and incubated for 1 week at 26°C.

The growth of new roots was checked by eye and a cluster of ca. 10-50 glomeromycotan spores from old root organ cultures was cut out and transferred to the new ROC medium plates with fresh roots. After 2-3 months of incubation at 26°C, the new sporulation could be observed in the ROC medium plates.

2.5. Isolation of spores from ROC medium plates (Doner & Bécard 1991)

Approximately 100 spores per ROC medium plate were cut out with a sterile scalpel and transferred to a 15 ml falcon tube, which was filled with 15 ml citrate buffer. The spores were then incubated for 40 minutes at 37°C with horizontal shaking at 250 U/min. Afterwards, the spores were left at room temperature for 5 minutes to sediment to the bottom of the tube. The supernatant was then discarded and the sediment was washed with autoclaved and filtered (pore size 22µm) Milli-Q water (PCR water). After 5 minutes the supernatant was again discarded and the sediment was washed with PCR water. After the sedimentation of the spores, they were pipetted to 1.5 ml reaction tubes and stored at -20°C.

The ingredients of the citrate stock solution contained 1.28g ascorbic acid in 200 ml Milli-Q water and 1.64g sodium acetate in 200 ml Milli-Q water for the Sodium acetate stock solution.

Citrate-buffer contained then 8.5 ml Citrate stock solution and 41.5 ml Sodium acetate filled to a final volume of 500 ml with Milli-Q water.

Citrate-buffer pH 6.0:
0.1N Citrate
0.1N Sodium acetate

2.6. Isolation of spores from pot cultures

Spores of several glomeromycotan cultures were wet-sieved from pot cultures (provided by Kurt Ineichen and Fritz Oehl).
About 5g of substrate from pot cultures were wet-sieved with a mesh size of 63 µm and transferred into Petri-dishes. Spores were then picked with a 20 µl pipette by eye under a dissecting microscope and transferred into 1.5 ml reaction tubes.

Species	**Isolate**
Glomus mosseae	ISCB 13
Glomus lamellosum	ISCB 48
Scutellospora castanea	BEG 01
Glomus geosporum	BEG 18
Glomus constrictum	BEG 19
Glomus etunicatum	BEG 34
Glomus intraradices	BEG 75

2.7. Spore extraction according to Redecker *et al.* (1997)

Single spores of AMF were cracked in a 1.5 ml reaction tube with a 10 µl pipette tip under a binocular. 2 µl of 0.25 M NaOH was added and the tube with the crushed spores was shortly centrifuged. The mixture was then incubated for 2 minutes at 95°C. 1 µl of Tris-HCl (0.5 M; pH 8.0) and then 2 µl HCl (0.25 M) was added and the mixture was again heated for 2 minutes at 95°C. Afterwards the reaction tube was shortly centrifuged and put on ice. The mixture was used directly as template DNA for PCR reactions.

2.8. DNA extraction from colonized plant roots and spores from monoxenic root cultures

The Qiagen D'Neasy Plant Kit (Hilden, Germany) was used for all DNA extractions of the colonized plant roots and the spores from the monoxenic root cultures.
Roots of *Festuca pratensis* colonized with *Glomus intraradices* (provided by Zuzana Sykorova) were rinsed with water, placed in 1.5 ml reaction tubes and stored at -20°C. The frozen samples of the colonized *Festuca pratensis* roots and the frozen samples of the AMF spores originated from the ROC medium plate cultures were crushed with a sterile micro-pestle in 1.5 ml reaction tubes and 400 µl of buffer AP1 was added. After this step 2µl of RNase A provided by the producer was added.
The following DNA extraction procedure was according to the protocol of the Qiagen D'Neasy Plant Kit (Hilden, Germany)**.** The total amount of extracted DNA was resuspended in 50 µl EB buffer and stored at -20°C.

2.9. The Polymerase Chain Reaction

The polymerase chain reaction (PCR) (Mullis & Faloona 1987) is a molecular biology technique for the expotential replication of DNA *in vitro*. DNA is enzymatically amplified by *Taq* polymerases. The PCR technique requires a DNA template, which contains the region of the DNA fragment to be amplified and two oligonucleotide primers binding in the flanking regions of the target DNA. In this work nested and semi-nested PCR reactions were performed, in which the amplified PCR product of one reaction was used again as template DNA for a second PCR using specific primers that anneal within the products of the first PCR. In a semi-nested PCR one of the primers that were used to amplify the first PCR product is again used in the second PCR. The (semi-) nested PCR has the advantage of a high sensitivity for the DNA from the targeted species and a higher robustness against inhibitors (Zani *et al.* 2000).

2.9.1. PCR primers

All new designed primers in this work were constructed, using the program Primer Designer v.3.0 (Scientific & Educational Software, Cary, NC, USA).

A 210 bp fragment of the mt-LSU of *Glomus intraradices* DAOM 197198 was successfully amplified and sequenced by A. Brennwald during diploma work with the primer sets LAM3A, LAM4 and LAM5. Partial mt-LSU sequences from *Glomus intraradices* strain JJ291 and *Glomus proliferum* were sequenced using the primers LAM3a, LAM4 and LAM5 in a nested PCR (results 3.1.1.). The DNA from the two *Glomus* isolates grown in monoxenic root cultures was extracted as described and used as template DNA in a semi-nested PCR with the fungal specific primers LAM3a and LAM5a under standard conditions. In the first reaction the PCR product was 1:100 diluted with PCR water and used as template DNA for the second PCR with the primer pairs LAM3a and LAM4. The PCR products were purified and cloned as described (material & methods). A colony PCR was performed and amplified products with a size of approximately 210bp were sequenced from both sides.

Based on these partial mt-LSU sequence a primer (RNL-5) was constructed for the specific binding to the mitochondrial large ribosomal subunit of *Glomus intraradices* and *Glomus proliferum* (Tab.2).

Four fungal specific primers RNL-1, RNL-2, RNL-3 and RNL-9 were constructed, based on fungal sequences of the mitochondrial large ribosomal subunit provided by Prof. B.F. Lang (University of Montreal) and from public databases.

New primers were constructed to amplify partial sequences (25%) of the RPB1 gene from AMF with a length of approximately 1700 bp. The primers RPB1-PR1F, RPB1-PR2F and RPB1-PR3R were constructed as specific primers for *Glomus* group A based on published fungal RPB1 sequences from the AFTOL database: (http://ocid.nacse.org/research/aftol/data.php).

For a specific amplification of Glomus group A members in the internal transcribed spacer region (ITS), the specific primer GLOM1310 was used (Redecker *et al.* 2000) together with the universal primers NS5, ITS4 (White *et al.* 1990) and ITS4i (Redecker *et al.* 2003).

Tab.2

List of primers used in PCR reactions

All primers were constructed with the software program Primer Designer v.3.0.

Target gene	Primer name	Sequence (5'→3')
mt-LSU	RNL-1	AGACCCGAARCCWRGTGATCT
mt-LSU	RNL-2	GGRAASAGCCCAGAAYA
mt-LSU	RNL-3	TGCATMATGGGTCAGCGAGT
mt-LSU	RNL-5	GAGCTTCCTTTGCCATCCTA
mt-LSU	RNL-9	CAGTAAAGCTGCATAGGGTCT
mt-LSU	RNL-10	AGAAGAAAGAGCTGGCTGTG
mt-LSU	RNL-10g	CTTGCTCTTCATCCCATAGTC
mt-LSU	RNL-11	AAGGCAACACGCCAGCACTT
mt-LSU	RNL-12	GATAGCGTAACAGCTCAGTG
mt-LSU	RNL-13	TGGTCGATGGACGACGGATA
mt-LSU	RNL-13b	ACACCCATAGCCTGCCTTGT
mt-LSU	RNL-14	AGGATAGGCCTGGAAACCAAGC
mt-LSU	RNL-15	CTGAGCTGTTACGCTATC
mt-LSU	RNL-16	ACCTGGAGATAGCTGGTCTT
mt-LSU	RNL-17	CCATAGAGTTGGCTCTAACA
mt-LSU	RNL-17f	GCTCGGAATTGAACCATAG
mt-LSU	LAM3a	GTAACTTCGGGATAAGGAG
mt-LSU	LAM4	TCGCTACCTTAGGACCCTCA
mt-LSU	LAM5a	GCTGCATAGGGTCTTCYCGTC
RPB1	RPB1-PR1F	TGTMCCTCCACCRCCTGTTCGTC
RPB1	RPB1-PR2F	CCTGTTCGTCCSAGTATTCAAATG
RPB1	RPB1-PR3R	CTCAGCACTACGACCAGCAGAGTCA
RPB1	RPB1-PR4F	GGTAAAGAAGGACGTCTTCGTGGA
RPB1	RPB1-PR5R	ACCTTGTTGTGCGGTRCCAACCGT
RPB1	RPB1-PR6F	TCGGACAATTTRCGTCATTCTACTC
RPB1	RPB1-PR7R	GGTAYRTGCATATTCATTTCATC
RPB1	RPB1-PR8R	CCATATAAGTTGCRCAATGA
RPB1	RPB1-PR9F	CAACTTTCCGTCTTAATCTCTCC
18S rDNA	NS5	AACTTAAAGGAATTGACGGAA
18S rDNA	GLOM1310	AGCTAGGYCTAACATTGTTA
28S rDNA	ITS4	TCCTCCGCTTATTGATATGC
28S rDNA	ITS4i	TTGATATGCTTAAGTTCAGCG

(R=A or G; W=A or T; S= C or G; Y=C or T; M=A or C)

2.9.2. PCR conditions

PCR reactions were performed with the Eppendorf Mastercycler® (Vaudaux-Eppendorf, Basel, Switzerland) and the *Taq* polymerase kit from Amersham Biosciences (Otelfingen, Switzerland) under the following ingredients and concentrations:

Tab.3
Standard PCR mastermix for a final volume of 25 µl

Reagents	**Volume (per 25 µl)**	**Concentration of stock solution**
10x PCR buffer	2.5 µl	
$MgCl_2$	2.0 µl	25 mM
dNTP	2.5 µl	10 mM
Primer 1	0.25 µl	50 pmol/µl
Primer 2	0.25 µl	50 pmol/µl
H_2O	16.25 µl	
Taq Polymerase	0.25 µl	5 U/ µl
Template DNA	1.0 µl	

2.10. Amplification of RPB1

The amplification of a partial sequence from the largest subunit of the RNA-polymerase II (RPB1) from different AMF species was performed in a semi-nested PCR with the new constructed primers RPB1-PR1F and RPB1-PR3R, followed by a second PCR with the primer pairs RPB1-PR2F and RPB1-PR3R (Fig.3). In both PCR reactions the standard reagent concentrations were used (Tab.3).
In the first and the second semi-nested PCR the following PCR conditions were used:

Step	Temperature (in °C)	Time (in minutes)
1	94	3
2	94	1
3	56	1
4	72	4
5	Repeat from step 2 for 29 more times	
6	72	5

The PCR product from the first nested PCR was diluted 1:10 with PCR water and used as a template in the second nested PCR.

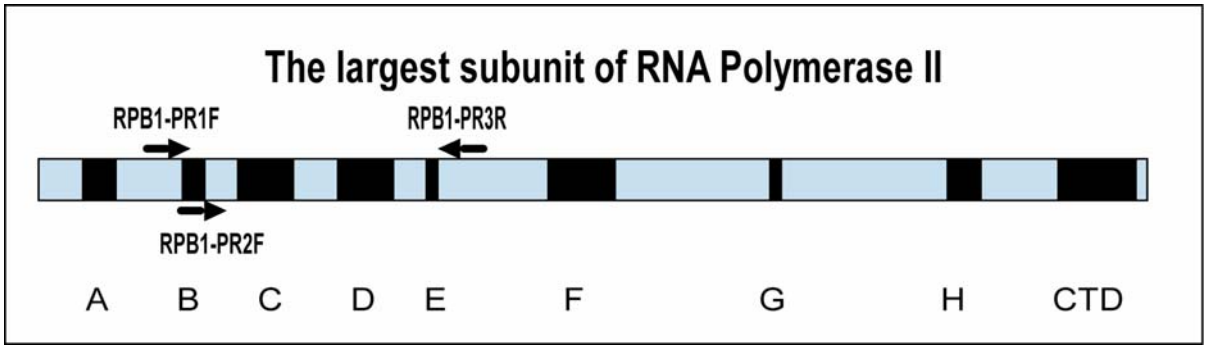


Fig.3

Binding sites of the constructed oligonucleotide primers in the RPB1 gene. Black blocks indicate the conserved regions (A-H) of the RPB1 gene and the carboxyl terminal repeat domain (CTD).

2.11. Amplification of the mitochondrial large ribosomal subunit

The primer pairs RNL-3 and LAM5a (or RNL-9) were used in the first nested PCR and the primer pairs RNL-1 (or RNL-2) and RNL-5 in the second nested PCR. Both PCR reactions contained the standard reagent concentrations (Tab.3).

The program of the first nested PCR had the following temperatures and times:

Step	Temperature (in °C)	Time (in minutes)
1	94	3
2	94	1
3	51	1
4	72	4
5	Repeat from step 2 for 34 more times	
6	72	5

The PCR products were diluted 1:100 and used as template DNA in the second step of the nested PCR which had the following parameters:

Step	Temperature (in °C)	Time (in minutes)
1	94	3
2	94	1
3	56	1
4	72	4
5	Repeat from step 2 for 29 more times	
6	72	5

2.12. Amplification of the ITS region

To verify the colonization of AMF in the root sample from *Festuca pratensis*, a nested PCR was performed to amplify the ribosomal internal transcribed spacer region (ITS) of AMF (Redecker *et al.* 2000) with the fungal specific primers NS5 and ITS4 in the first PCR. In a second nested PCR the primer ITS4i and the *Glomus* group A specific primer GLOM1310 were used.

In a first round of the nested PCR the program was conducted as follows:

Step	Temperature (in °C)	Time (in minutes)
1	94	3
2	94	0.5
3	51	0.5
4	72	2
5	Repeat from step 2 for 29 more times	
6	72	5

The PCR products were then diluted 1:100 and used as template DNA in the second PCR. A hot start PCR at 61°C was performed in the second nested PCR.

Step	Temperature (in °C)	Time (in minutes)
1	94	3
2	94	0.5
3	61	0.5
4	72	2
5	Repeat from step 2 for 29 more times	
6	72	5

2.13. Agarose gel electrophoresis

The PCR products were verified to be of the expected size by electrophoresis in 1x Tris-Acetate-EDTA buffer (Sambrook *et al.* 1989) gels containing 1% (w/v) Agarose (BMA, Rockland, USA). For PCR fragments with an expected size below 500 bp 2% (w/v) Nusieve GTG Agarose (BMA, Rockland, USA) was added. A 1 kb DNA ladder marker (MBI, Fermentas, St.Leon-Rot, Germany) was used to determine the fragment length.

Tris- acetic acid- EDTA (TAE)- buffer 50x
242 g Tris in 1l Milli Q water
57.1 ml acetic acid
100 ml 0.5M EDTA· 2 H_2O disodium salt (pH 8.0)

2.14. Purification of PCR products

PCR products of correct size were purified with High Pure PCR purification kit® (Roche, Basel, Switzerland) according to the manufacturer's instructions and then resuspended in 30 µl elution buffer provided by the manufacturer. The purified PCR products were stored at -20°C.

2.15. Cloning of PCR products

PCR products were ligated and cloned using with the Topo TA cloning kit for sequencing® (Invitrogen, Carlsbad, USA) containing Topo 10 chemically competent cells and Topo pcR4 vector (including topoisomerase I).

Ligation
0.33 µl salt solution
0.33 µl Topo pcR4 vector
1.33 µl purified PCR product

The ligations were incubated overnight at 12°C. 16 µl of the Topo 10 competent cells were added to a ligation mix and left on ice for 15 minutes. A heat shock at 42°C was performed for 30 seconds. The samples were directly put on ice for 5 min before 83 µl of SOC medium (provided by Invitrogen) was added. An incubation for 1h at 37°C with 250 rpm horizontal shaking followed. 100 µl of the medium was plated on Luria-Bertani (LB) plates containing 100 µg/l Ampicillin and incubated over night at 37°C.

Luria- Bertani medium
25 g LB Broth Miller (US-Biological, Swampscott, USA) 1l Milli Q water

SOC-medium
2% Tryptone 0.5% Yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM Glucose

2.16. Screening of clones by colony PCR

Transformed *Escherichia coli* colonies were picked with sterile toothpicks and used as template DNA for a PCR with the standard concentration conditions (Tab.3). Two primers with annealing sites within the Topo pCR4 vector (Invitrogen) were used for screening clones to inserts.

T3: 5'-ATTAACCCTCACTAAAGGGA-3'
T7: 5'-TAATACGACTCACTATAGGG-3'

The PCR was conducted under the following conditions:

Step	**Temperature (in °C)**	**Time (in minutes)**
1	94	3
2	94	1
3	55	1
4	72	2
5	Repeat from step 2 for 27 more times	
6	72	5

The PCR products were checked by agarose gel electrophoresis. PCR products with correct insert size were purified with the High Pure PCR purification kit® and used as template DNA for the sequencing PCR.

2.17. The sequencing PCR

The sequencing PCR was performed based on to the method of Sanger *et al.* (1977). In addition to the common PCR reagents, fluorescence- labelled didesoxyribonucleotides (ddNTPs) were added to the PCR mastermix. The inserted ddNTPs cause a stop of the amplification by the DNA-polymerase. The final product of the PCR is a mixture of amplified DNA fragments, which all possess a ddNTP at the 3'-strain. The fragments are separated by size by electrophoresis and the fluorescence is measured with a laser.
The sequencing PCR was performed using the ABI PRISM Big Dye Terminator v.3.1 Cycle Sequencing Ready Reaction Kit® (Applied Biosystems, Foster City, USA). 4 µl of the sequencing mix was added to 5 µl purified colony PCR product and 1 µl of a 5 µM primer.

The PCR conditions were the following with a constant ramping time of 1°C per second:

Step	**Temperature (in °C)**	**Time (in seconds)**
1	96	30
2	50	15
3	60	240
4	Repeat from step 1 for 26 more times	

For purification, DNA was precipitated with ethanol. The PCR reactions were added to 25 µl ethanol (abs.) and 1 µl of 3M Sodium acetate (pH 5.2), vortexed and incubated at room temperature for 15 minutes. Afterwards, the mixture was centrifuged at 16100 g for 20 min at room temperature. The supernatant was

discarded and 250 µl of 70% ice-cold ethanol was added. The samples were centrifuged for 5 minutes at 16100 *g* by room temperature and the supernatant was discarded. Again 250 µl of 70% ice-cooled ethanol was added to the samples and centrifuged for 5 min. The supernatant was carefully removed and the samples were air dried for 30 minutes under a laminar flow hood and resuspended in 30 µl of Template Suppressing Reagent (Applied Biosystems). The samples were denaturated for 1 minute at 90°C and stored at 4°C. The fluorescence measurements were performed by a capillary sequencer(ABI PRISM 310 Genetic Analyzer, Applied Biosystems) and the fluorescent signals were analyzed using the software program DNA Sequencing Analysis (Applied Biosystems). The sequences were proof read and aligned with the software package BioEdit v.7.0.4.1. (Hall 1999).

2.18. Phylogenetic analysis

Phylogenetic analysis trees were calculated using the PAUP 4 software package (Swofford 2001).

Bayesian analysis as performed using MrBayes 3.11 for Macintosh (Ronquist & Huelsenbeck 2003). The appropriate substitution model was chosen using Mr.Modeltest 2.0 (Nylander 2004). In this work the program always calculated the general time reversible+gamma+proportion of invariants (GTR+I+G) model as best fit model for Bayesian trees.

GTR models calculate the probabilities of each six substitution types with its specific values. Additionally the gamma distribution was included. The gamma distribution takes into account that the sites in an alignment have different evolutionary rates.

The gamma distribution depicts the heterogeneity of those substitution rates by the parameter α. This parameter is distinguished through the shape of the curve in a graph that includes the variation length and the different substitution rates. If a certain amount of sites are distinguished as non-variable an additional parameter “I” is included. In this case only the variable sites are used for the calculations of the gamma distribution.

2.18.1. The phylogenetic analysis criteria of the mitochondrial large ribosomal subunit

To determine the phylogenetic position of the the Glomeromycota among other fungi a Bayesian tree based on 550 characters from conserved regions of the mitochondrial large ribosomal subunit was calculated. The sequences of the mitochondrial large ribosomal subunit amplified from the *Glomus intraradices* isolates and *Glomus proliferum* were divided into three sections Intron I, Intron II and Exon region and a maximum parsimony tree was calculated for each section. The parsimony method was chosen because it depicts most directly the single changes between the sequences. The trees were calculated under the standard maximum parsimony criteria in PAUP with 1000 replicates of bootstrap analysis. In all maximum parsimony trees the gaps were counted as alternative substitution (i.e. counted as fifth character state).

2.18.2. Phylogenetic analysis criteria of the RPB1 gene

Bayesian phylogenetic analyses of the new RPB1 sequences were expected to give new insights about the phylogenetic relationships within the true fungi and among the members of the arbuscular mycorrhizal fungi. Therefore several bayesian analyses with different data sets and parameters were used.

Because of unalignable substitution sites within the intronic regions only exon regions were analyzed. All characters in nucleotide alignments were partitioned by their codon position. Only the first and second codon positions were used for calculations. Using MrModeltest (Nylander 2004), the GTR+I+G model was chosen to be the best-fit model for all RPB1 nucleotide alignments. The dirichlet distribution was determined as equal valued between all nucleotides (1,1,1,1). MrBayes was performed using two chains over two million generations retaining every 100^{th} tree. A 50% consensus tree was calculated after discarding the first 10% of the constructed trees (‘burn-in’).

For the protein alignment only alignable sites were chosen to be analyzed with a final dataset of 444 amino acids. Occurring stop codons, most likely the result of sequencing errors, were treated as missing data.
The PAM100 substitution matrix was chosen for this alignment because of the moderate length of the alignment with 444 amino acids and the moderate sequence similarities between the the taxa. As one of the first protein substitution matrices, the PAM (percent of accepted mutations) matrix was developed by Margret O. Dayhoff in 1978. It provides the likelihood of different amino acids exchanges through evolutionary time. The matrix is calculated by observing the differences among closely related proteins.
Bayesian analyses over one million chains counting every 100^{th} tree were caluclated. The logarithmic likelihood value of the cold chain was determined with 0.0046 and 10% of the first trees were discarded.

2.19. Storage of clones

For long time storage of clones, *E.coli* colonies were picked with a sterile pipette tip and transferred to 15 ml falcon reaction tubes containing 3 ml of LB-medium. The cultures were then incubated overnight at 37°C with 250 rpm horizontal shaking. The next day 500 µl of a culture was transferred in a 1.5 ml reaction tube and 500 µl 60% glycerol was added. The media were well mixed and shock-frozen in liquid nitrogen. The frozen cultures were then stored at -80°C.

3. Results

3.1. Part A: Amplification and phylogenetic analyses of partial mt-LSU sequences from *Glomus intraradices* and *Glomus proliferum*

3.1.1. Primer design for the mt- LSU

Using the primers designed by Annemarie Brennwald (Ochsner 2002), a 210 bp fragment of the mt-LSU was amplified and sequenced from *Glomus intraradices* JJ291 and *Glomus proliferum*. The resulting mt-LSU sequence from *Glomus intraradices* JJ291 were identical to those obtained from DAOM 197891 by A. Brennwald. The mt-LSU sequence from *Glomus proliferum* showed strong similarity (86,8%) to *Glomus intraradices*.

In order to confirm that JJ291 and DAOM 197891 have the same sequence of the partial mt-LSU, additional independent subcultures of these isolates were analyzed. A ROC-medium culture of DAOM 197891 was provided by Dr. N. Requena, which had been independently obtained from Dr. G. Bécard, who established this isolate in 1988. Likewise, another batch of JJ291 was obtained again from Dr. J. Jansa. All of these cultures yielded the same mt-LSU sequence.

Based on these sequences and on the alignment of database sequences, additional primers were designed to obtain a larger PCR fragment. Other primers were designed as sequencing primers (Tab.2). The binding sites are shown in Figure 4.

In order to be able to amplify the target region from non-sterile fungal material (i.e. colonized roots and spores from pot cultures), some of the new primers were designed to exclusively amplify glomeromycotan mt-LSU fragments.

A new specific primer RNL-5 was constructed based on the known sequences of *Glomus intraradices* and *Glomus proliferum*. Additionally four fungal specific primers RNL-1, RNL-2, RNL-3 and RNL-9 were constructed to obtain PCR fragments of larger size (Fig.4).

3.1.2. Sequences obtained from *Glomus proliferum* and different isolates of *Glomus intraradices*

Based on published fungal mt-LSU sequences in the alignment the expected fragment size for RNL-1 and RNL-5 was assumed to be approximately 1200 bp corresponding to *Saccharomyces cerevisiae* S288C (NC 001224) position 578 to 1795 which is approximately 40% of the total mt-LSU. A 750 bp long product was expected for the PCR reactions performed with the primers pairs RNL-2 and RNL-5. Surprisingly, the total size of the PCR products amplified with the primers RNL-1 and RNL-5 of the *Glomus intraradices* isolates was determined to be 2900 bp by gel electrophoresis. *Glomus intraradices* PCR products amplified with the primers RNL-2 and RNL-5 had a size of 1900 bp, while all the amplified PCR products from *Glomus proliferum* had the expected sizes.
The PCR products were purified and cloned as described and the clones with correct insert sizes were sequenced and phylogenetically analysed.

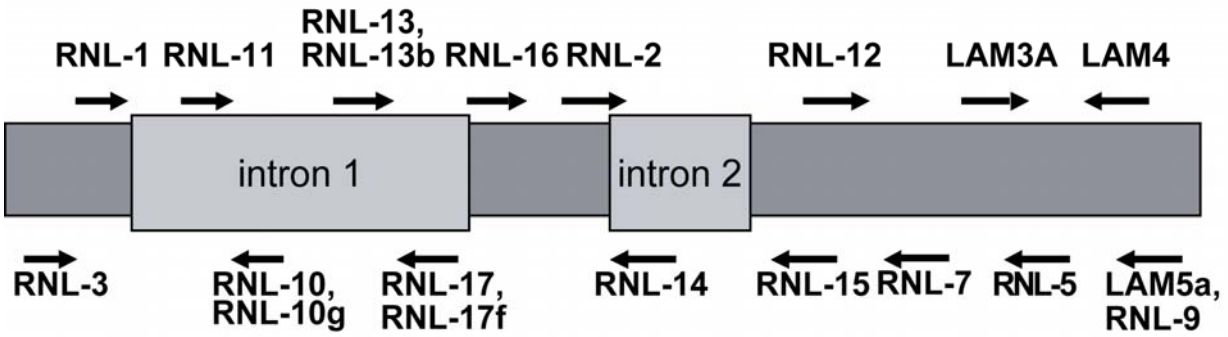


Fig.4

Arrows show the location and orientation of the primers in the 2900 bp fragment of the mitochondrial large ribosomal subunit from *Glomus intraradices*. Approximately to scale.

Moreover eight *Glomus intraradices* isolates cultivated in root organ culture (provided by Dr. Jan Jansa) were tested with the new constructed mitochondrial primers (Tab.4). They originated from the same field side as JJ 291 (i.e. Tänikon, Switzerland). The purified PCR products of these isolates JJ 51, JJ 106, JJ 232, JJ 254, JJ 256, JJ 323, JJ 351 and JJ 746 were directly sequenced using the new designed mt-LSU primers (Fig.6).

The fungal DNA from spores of a *Glomus intraradices* BEG 75 pot culture was extracted as described by Redecker *et al.* (1997). A nested PCR was performed and the products of the second nested PCR with the primer pairs RNL-1 and RNL-5 were cloned and screened by colony PCR. Five clones were sequenced and phylogenetically analysed (Tab.4).

Fig.5

The nuclear-encoded ITS rDNA are polymorphic within single spores and AMF isolates. The sequences JJ1 to JJ32 all originate from a single-spore culture *of Glomus intraradices* JJ 291 (Jansa et al. 2002). The sequences of the isolates BEG 75, DAOM 197898 as well as the environmental sequences obtained from *Festuca* roots group within this range. Sequences newly obtained are marked by asterisks (*). The phylogenetic tree was obtained by neighbor-joining from 5.8S rDNA subunit and ITS2. Numbers denote bootstrap values from 1000 replicates. Only the values above 50% for the first five nodes from the root are provided.

DNA originating from a root sample of *Festuca pratensis* (provided by Zuzana Sykorova) harvested from a seminatural grassland (Mesobrometum) in the Basel region (Landskrone, France) was extracted. The roots were colonized exclusively by *Glomus intraradices*, which was demonstrated by amplification of the ITS region by nested PCR reactions with several glomeromycotan specific primers (Redecker 2000) including the *Glomus* group A specific primer GLOM1310 (Redecker 2000). Only PCR reactions using the GLOM1310 primer resulted in amplification of a PCR

product. The purified PCR products were cloned and sequenced as described. Phylogenetic analysis showed that the amplified PCR products were from *Glomus intraradices* (Fig.5). The ITS obtained from *Glomus proliferum* DAOM 226389 and the *Glomus intraradices* isolates BEG 75 and DAOM 197198 were also included in the tree.

Tab.4

List of successfully amplified AMF isolates and clones obtained. Numbered PCR indicate different PCR reaction within the same species and the used primer pairs. Multiple PCR's from the same isolate are shown as PCR1, PCR2, etc.

Species/Strain	**PCR/Primer**	**Clone No**	**Accession No**
Glomus proliferum	PCR1 / RNL1-RNL5	783/4	AJ841801
Glomus proliferum	PCR2 / RNL1-RNL5	784/1	AJ841802
Glomus proliferum	PCR3 / RNL1-RNL5	1155/1	AM040980
Glomus proliferum	PCR3 / RNL1-RNL5	1155/8	AM040981
Glomus proliferum	PCR3 / RNL1-RNL5	1155/12	AM040982
Glomus proliferum	PCR4 / RNL2-RNL5	813/3	AJ841290
Glomus proliferum	PCR4 / RNL2-RNL5	813/4	AJ841797
Glomus proliferum	PCR4 / RNL2-RNL5	813/5	AJ841798
Glomus proliferum	PCR4 / RNL2-RNL5	813/6	AJ841799
Glomus proliferum	PCR4 / RNL2-RNL5	813/7	AJ841800
Glomus proliferum	PCR5 / RNL2-RNL5	785/2	AJ841803
Glomus intraradices DAOM197198	PCR1 / RNL1-RNL5	814/1	AJ841805
Glomus intraradices DAOM197198	PCR1 / RNL1-RNL5	814/2	AJ841804
Glomus intraradices DAOM197198	PCR1 / RNL1-RNL5	814/9	AJ841806
Glomus intraradices DAOM197198	PCR1 / RNL1-RNL5	814/10	AJ841807
Glomus intraradices DAOM197198	PCR1 / RNL1-RNL5	814/11	AJ841808
Glomus intraradices DAOM197198	PCR2 / RNL1-RNL5	1164/5	----------
Glomus intraradices BEG 75	PCR1 / RNL1-RNL5	1023/1	AJ938171
Glomus intraradices BEG 75	PCR1 / RNL1-RNL5	1023/4	AJ938172
Glomus intraradices BEG 75	PCR1 / RNL1-RNL5	1023/8	AJ938173
Glomus intraradices BEG 75	PCR2 / RNL1-RNL5	1154/1	AM040983
Glomus intraradices BEG 75	PCR2 / RNL1-RNL5	1154/12	AM040984
Glomus intraradices field sample	PCR1 / RNL1-RNL5	868/15	AJ841288
Glomus intraradices field sample	PCR1 / RNL1-RNL5	868/25	AJ841289
Glomus intraradices JJ 291	PCR1 / RNL1-RNL5	867/1	AJ973189
Glomus intraradices JJ 291	PCR1 / RNL1-RNL5	867/4	AJ973190
Glomus intraradices JJ 291	PCR1 / RNL1-RNL5	867/5	AJ973192
Glomus intraradices JJ 291	PCR1 / RNL1-RNL5	867/6	AJ973193
Glomus intraradices JJ 291	PCR2 / RNL2-RNL5	839/4	AJ973191
Glomus intraradices JJ 51	PCR1 / RNL1-RNL5	seq. directly	----------
Glomus intraradices JJ 106	PCR1 / RNL1-RNL5	seq. directly	----------
Glomus intraradices JJ 232	PCR1 / RNL1-RNL5	seq. directly	----------
Glomus intraradices JJ 254	PCR1 / RNL1-RNL5	seq. directly	----------
Glomus intraradices JJ 256	PCR1 / RNL1-RNL5	seq. directly	----------
Glomus intraradices JJ 323	PCR1 / RNL1-RNL5	seq. directly	----------
Glomus intraradices JJ 351	PCR1 / RNL1-RNL5	seq. directly	----------
Glomus intraradices JJ 746	PCR1 / RNL1-RNL5	seq. directly	seq. directly

In order to test whether the newly designed mt-LSU primers RNL-1, RNL-2 and RNL-5 can be used for further field studies in the future, nested PCR reactions with the mitochondrial primer pairs RNL-1/RNL-5 and RNL-2/RNL-5 were successfully performed using the extracted root sample DNA as template. The purified PCR product originated from the RNL-1/RNL-5 reaction was cloned and two clones were sequenced and phylogenetically analysed (Tab.4).

3.1.3. Mitochondrial large ribosomal subunit sequences are homogeneous within *Glomus* isolates

In order to determine intra-isolate sequence variation, a region of the mt-LSU from *Glomus proliferum* comprising approximately 790bp (RNL-2/RNL-5) was sequenced from six different clones in parallel. An additional five clones were sequenced from a PCR product using the RNL-1 and RNL-5 primers that comprised approximately 1120bp within this region. The 11 clones differed from a consensus sequence on average by 0.26 %. This deviation was within the range of the misincorporation error of *Taq* polymerase (Cline, Braman & Hogrefe 1996).

Using the same approach, four clones from *Glomus intraradices* JJ 291 were sequenced, each comprising 2500bp (RNL-1/RNL-5) plus one additional clone comprising 1130bp (RNL-2/RNL-5). The full-length clones, a large proportion of which were putative intron sequences, on average differed from the consensus by 0.12 % in the exonic regions, and 0.24 % (with a standard deviation of 0.04%) in the introns.

Comparing the mt-LSU sequences of *Glomus intraradices* JJ 291 and *Glomus proliferum*, there was no increased occurrence of deviations in the variable exon regions of the mt-LSU where *Glomus intraradices* and *Glomus proliferum* differed from each other. Seven substitutions were found in the conserved regions, and two in the variable regions, respectively, which corresponds roughly to the proportion of variable and conserved regions. Indels were relatively rare among sequences from one isolate, and occurred in only one position in *Glomus proliferum*, and in two positions in JJ 291.

```
JJ291
Gi839_4       GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi867_1       GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi867_4       GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi867_5       GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi867_6       GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
------------
DAOM197891
Gi814_1       GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi814_2       GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi814_9       GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGGGGGAGTGGCAGTT
Gi814_10      GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi814_11      GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi1164_5      GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
------------
BEG75
Gi1023_1      GTATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi1023_4      GTATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi1023_8      GTATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi1154_1      GTATATTACCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi1154_12     GTATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
------------
G.intra.root
Gi868_15      GTATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTATTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
Gi868_25      GTATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTATTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
------------
G.proliferum
Gp783_4       GAATATAGCCTGGTGAGGGCACCAAGAATGTAACGGGTCTATAGTAGTTGTCTCTCT------------AAATGGAGAGAGTGGCAGTT
Gp784_1       GAATATAGCCTGGTGAGGGCACCAGGAATGTAACGGGTCTATAGTAGTTGTCTCTCT------------AAATGGAGAGAGTGGCAGTT
Gp785 2       GAATATAGCCTGGTGAGGGCACCAAGAATGTAACGGGTCTATAGTAGTTGTCTCTCT------------AAATGGAGAGAGTGGCAGTT
Gp813_3       GAATATAGCCTGGTGAGGGCACCAAGAATGTAACGGGTCTATAGTAGTTGTCTCTCT------------AAATGGAGAGAGTGGCAGTT
Gp813_4       GAATATAGCCTGGTGAGGGCACCAAGAATGTAACGGGTCTATAGTAGTTGTCTCTCT------------AAATGGAGAGAGTGGCAGTT
Gp813_5       GAATATAGCCTGGTGAGGGCACCAAGAATGTAACGGGTCTATAGTAGTTGTCTCTCT------------AAATGGAGAGAGTGGCAGTT
Gp813_6       GAATATAGCCTGGTGAGGGCACCAAGAATGTAACGGGTCTATAGTAGTTGTCTCTCT------------AAATGGAGAGAGTGGCAGTT
Gp813_7       GAATATAGCCTGGTGAGGGCACCAAGAATGTAACGGGTCTATAGTAGTTGTCTCTCT------------AAATGGAGAGAGTGGCAGTT
Gp1155 1      GAATATAGCCTGGTGAGGGCACCAAGAATGTAACGGGTCTATAGTAGTTGTCTCTCT------------AAATGGAGAGAGTGGCAGTT
Gp1155 8      GAATATAGCCTGGTGAGGGCACCAAGAATGTAACGGGTCTATAGTAGTTGTCTCTCT------------AAATGGAGAGAGTGGCAGTT
Gp1155 12     GAATATAGCCTGGTGAGGGCACCAAGAATGTAACGGGTCTATAGTAGTTGTCTCTCT------------AAATGGAGAGAGTGGCAGTT
------------
JJ51          GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
JJ106         GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
JJ232         GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
JJ254         GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
JJ256         GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
JJ323         GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
JJ351         GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
JJ746         GAATATTGCCTGGTGAGGGCGCCAAAAATGTAACGGGTCTATAGTAGTTGTCTCTCTGTTCCAGCTTTGGATATGGAGGGAGTGGCAGTT
```

Fig.6

Representative region of the nucleotide alignment of the mt-LSU from different strains of *G. intraradices* and *G. proliferum*. Coloured letters indicate variable sites.

Clone sequences originate from *G. intraradices* JJ 291, *G. intraradices* DAOM 197198, *G. intraradices* BEG 75, the *Festuca pratensis* root sample and from *G. proliferum*. Sequences from *G. intraradices* JJ 51-JJ 746 were determined directly without cloning.

Five clones of *Glomus intraradices* DAOM 197198 had identical sequences to *Glomus intraradices* JJ 291 (Fig.4). They differed from the consensus sequence in the intron regions by 0.8% and in the exon regions by 0.32%.
Five sequenced clones originating from BEG 75 differed from a consensus sequence on average by 0.25% in the exons and 0.05% in the introns.
Therefore, contrary to the nuclear-encoded ribosomal genes (Jansa *et al.* 2002) no evidence was found in the mitochondrial large ribosomal subunit for intra-isolate sequence heterogeneity (Fig.6).

3.1.4. PCR fragments obtained from *Glomus proliferum* and *Glomus intraradices* isolates differ in their intron content

Nested PCR products obtained from several strains of *Glomus intraradices* with the primer pairs RNL-1 and RNL-5 in the second nested PCR showed clear differences in their sizes. The sequence from isolates JJ 51, JJ 106, JJ 232, JJ 254, JJ 256, JJ 291, JJ 323, JJ 351, JJ 746 and DAOM 197198 had a size of approximately 2700 bp in the agarose gels, while the amplified PCR product of isolate BEG 75 was 1600 bp long and the sample of *Festuca pratensis* roots colonized with *Glomus intraradices* had a length of approximately 2300 bp. The smallest fragment was amplified from *Glomus proliferum* with a band length of 1200 bp (Fig.7).

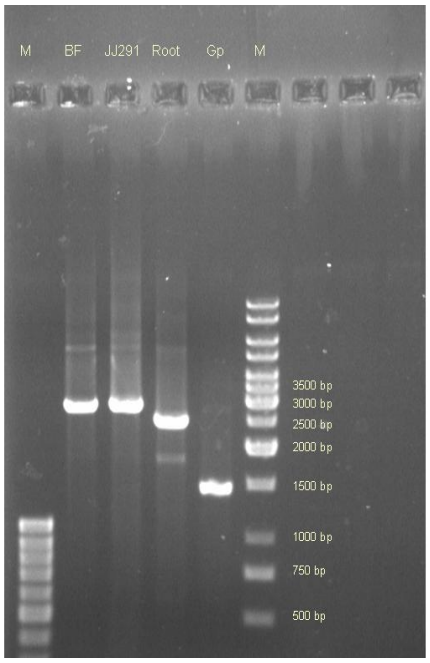


Fig. 7
Clones amplified with Topo pCR4 vector primers T3 and T7 from Canadian *Glomus intraradices* strain DAOM 197198 (BF), *Glomus intraradices* strain JJ 291, the *Festuca pratensis* root sample from Landskrone meadow (Root) and *Glomus proliferum* (Gp)

The nucleotide sequences obtained from those species gave new insights. Based on an alignment of mt-LSU sequences from the obtained *Glomus intraradices* and *Glomus proliferum* sequences and from mt-LSU sequences of other fungi, introns in the partial mt-LSU sequence of *Glomus intraradices* were identified. Two introns were identified in all sequences of *Glomus intraradices* strains JJ 51, JJ 106, JJ 232, JJ 254, JJ 256, JJ 291, JJ 323, JJ 351, JJ 746 and DAOM 197198 with a length of approximately 1000 bp in the intron 1 and 400 bp in the intron 2 (Fig.9). These introns were exclusively occurring in isolates of *Glomus intraradices*. While all these *Glomus intraradices* strains had identical sequences, the isolate JJ 106 differed by indels in intron 1 and the intron 2 from the other *Glomus intraradices* strains. Additionally *Glomus intraradices* BEG 75 lacked the intron 1, whereas the *Glomus intraradices* strain obtained from the *Festuca* root sample lacked the intron 2 (Fig.8).
Interestingly all *Glomus intraradices* strains that contained both introns showed no variation in the exon and intron regions. However, BEG 75 and the *Festuca* root sample showed clear differences to other *Glomus intraradices* strains in their intronic sequences by indels.
In addition, the exon regions of the root sample showed 3.2% difference to the other *Glomus intraradices* strains. In the variable regions where *Glomus intraradices* and *Glomus proliferum* species differed, the root symbiont sequence showed a much higher similarity to the *Glomus intraradices* nucleotide sequences than to *Glomus proliferum*.

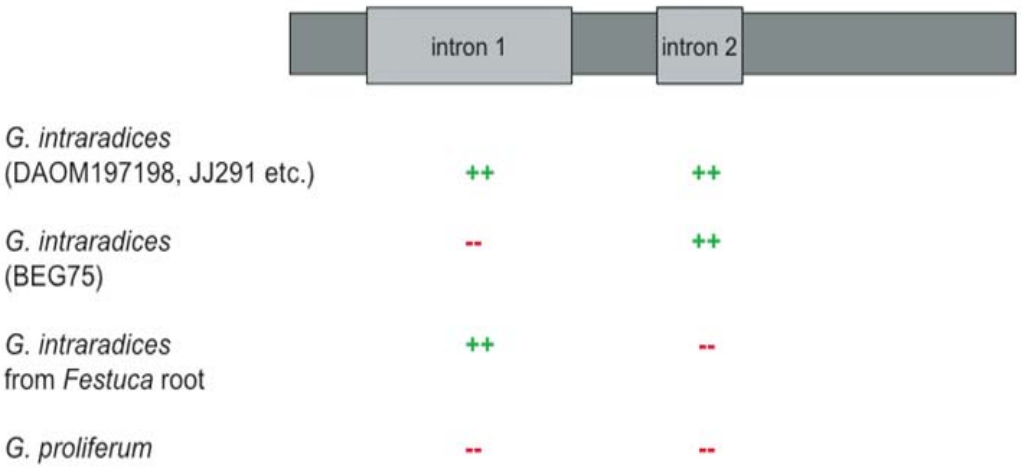


Fig.8

Intron absence (--) or presence (++) in the amplified partial mt-LSU of different *Glomus intraradices* strains. © by Dirk Redecker

3.1.5. A putative homing endonuclease of the LAGLIDADG type identified in intron 1 of *Glomus intraradices* strains

An open reading frame (ORF) of a putative site- specific homing endonuclease from the LAGLIDADG 2 family (Dalgaard *et al.* 1997) was identified within intron 1 of *Glomus intraradices.* All *Glomus intraradices* isolates that possessed the intron 1 (Fig.8) also hosted the homing endonuclease ORF in intron 1. The putative ORF corresponded to 202 amino acids (Fig.9). The closest matches in the databases were found to be mitochondrial intron-encoded homing endonucleases in the cytochrome B gene of *Podospora sp.* (E48326, CAA38776 and CAB72448) (Fig.14). For phylogenetic analysis of the homing endonuclease found in *Glomus intraradices* isolates see chapter 3.1.10.

Interestingly, almost all *Glomus intraradices* strains that possessed the intron 1 had identical sequences. However one of the cloned sequences originating from *Glomus intraradices* DAOM 197198 showed an increased amount of variable positions in the intron 1, causing the average variability in intron 1 to rise to 0.8 %, which is slightly higher than in the other isolates and gene regions. The point mutations found in the intron 1 of clone 814/2 with an apparently increased mutation rate resulted in five exchanged amino acids in the homing endonuclease sequence, four of which were located in last 21 amino acids of the homing endonuclease. Nevertheless, the amino acid sequence of clone 814/2 showed clearly the highest homology to the endonucleases of the LAGLIDADG2 type found in the intron 1 of other *Glomus intraradices* strains.

The intron sequence of *Glomus intraradices* strain JJ 106 diverged from the other *Glomus intraradices* isolates not only by several SNP's (single nucleotide point mutations) in the intron 1 but also by an insertion in the coding region of the homing endonuclease.

The highest divergence in the intron 1 of *Glomus intraradices* mt-LSU nucleotide sequences as found in the root sample from *Festuca pratensis*. Within the coding region of the LAGLIDADG homing endonuclease in the intron 1 the difference was 14.1%, while the intronic sequences upstream of the ORF of the homing endonuclease differed by 27.6%. Downstream from the sequence coding for the homing endonuclease the intronic sequence differed by 17.6%. Interestingly, the

exon region of the root sample showed only 3.2% difference to other *Glomus intraradices* strains.

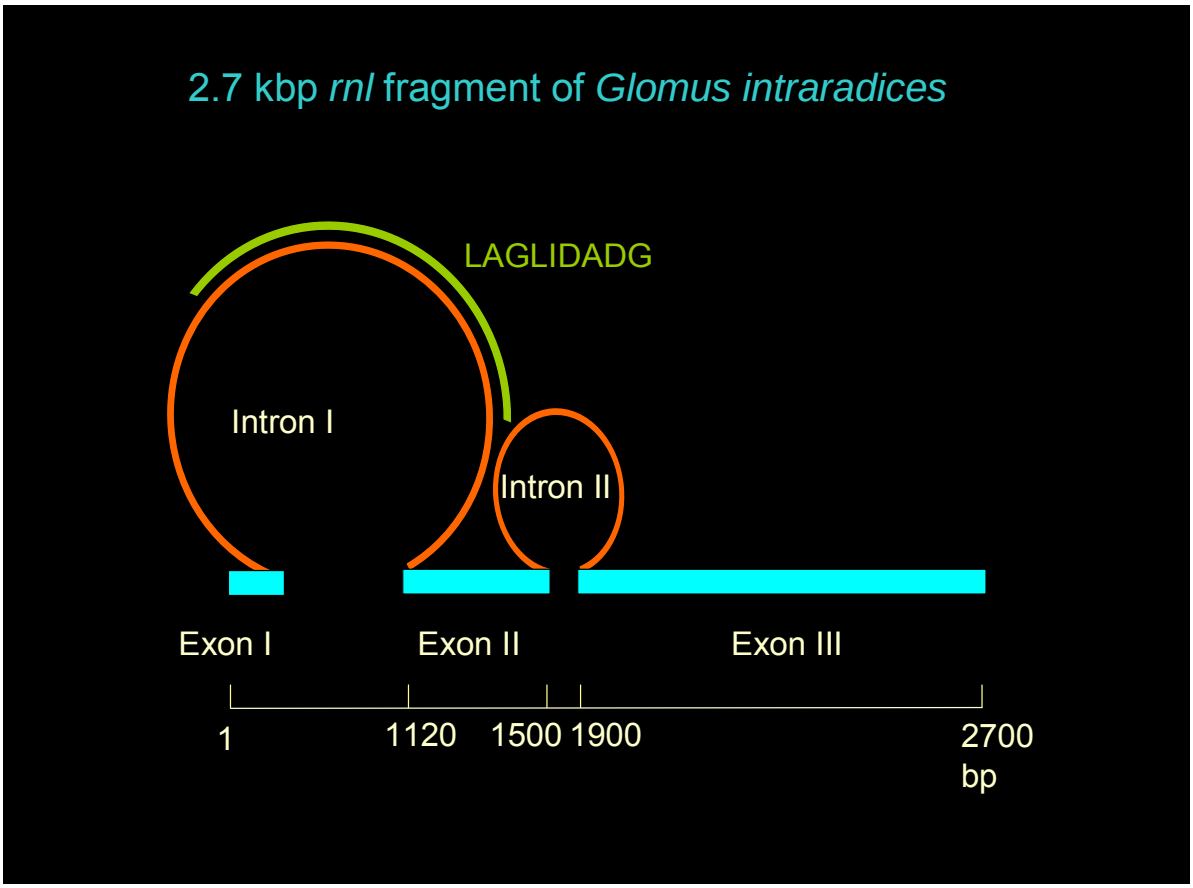


Fig.9

Diagram of the partial mitochondrial large ribosomal subunit in *Glomus intraradices* amplified using the RNl-1 and RNL-5 primers. The partial sequence includes two introns (Intron I and Intron II). Intron I includes a putative ORF of a homing endonuclease (LAGLIDADG). Diagram not to scale.

3.1.6. Fungal phylogeny of the mitochondrial large ribosomal subunit

A phylogenetic tree of the true fungi was constructed using the software package MrBayes (Ronquist & Huelsenbeck 2003) (Fig.10). The region of the sequenced mt-LSU was highly variable among fungal taxa. At the level of fungal phyla, only a small

proportion of the sequences were alignable to each other, corresponding to the most- conserved regions. The analysis was based on the 550 most conserved characters from the nucleotide sequence alignment of the mitochondrial large ribosomal subunit. A consensus mt-LSU sequence from *Glomus proliferum* was used as representative of the Glomeromycota to determine the phylogenetic position of the Glomeromycota within the true fungi. The mt-LSU sequences of *Zea mays* and the choanoflagellate *Monosiga brevicollis* were used as outgroups in the tree. The best nucleotide evolution model was determined using MrModeltest (Nylander 2004). The program determined the general time reversible (GTR+I+G) model as best fit model (see 2.16.).
A 50% consensus tree was constructed after the exclusion of 10% of the trees from the first stage of the run. The numbers on the branches represent posterior probabilities of the clades (Fig.10).

The mt-LSU sequence of *Glomus proliferum* grouped within the true fungi, but not within any of the fungal phyla represented in the dataset. Instead, *Glomus proliferum* was placed as an independent branch among other fungal phyla in the bayesian analysis, as this would be expected for members of the Glomeromycota. Therefore, potential contamination by the ubiquitous airborne spores of Ascomycota can be excluded, as well as yeast, bacteria and plant pollen. However the posterior probability support for the separation of the *Glomus proliferum* sequence was low with 0,61 (Fig.10).
Neither a symbiomycotan clade (Tehler 2003 *et al.*) was supported, in which the Glomeromycota are grouped as a sister group to the Dicaryomycota, nor the well established dicaryomycotan clade (Schaffner 1975) itself was supported in this tree. Instead the Basidiomycota and the Ascomycota grouped together with the Chytridiomycota (Fig.10). Overall support for the basal branches, i.e. relationships of fungal phyla, was low. Therefore, the relation of the Glomeromycota to other major fungal lineages cannot be easily addressed using the region of this gene we sequenced.

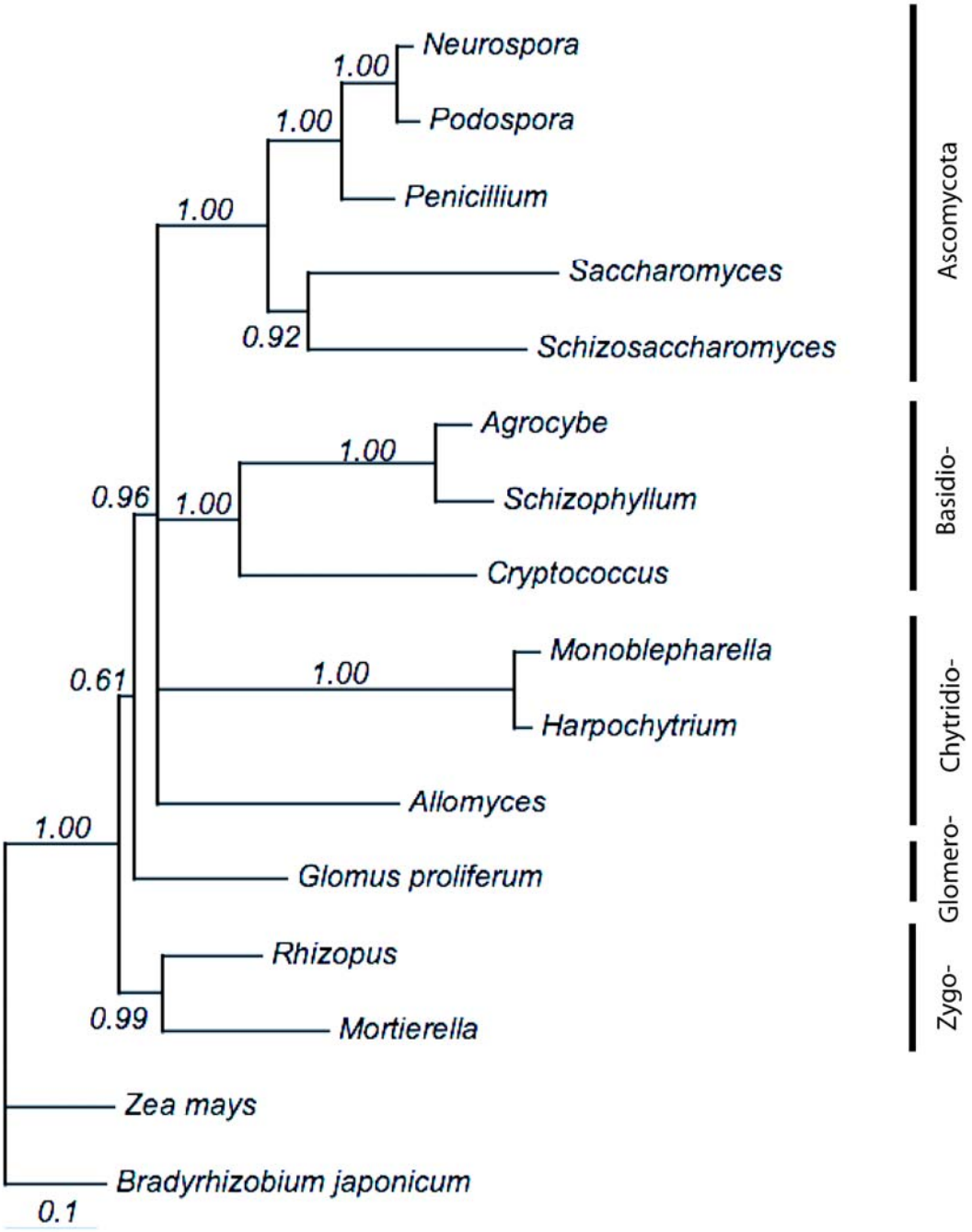


Fig.10

Bayesian tree obtained from fungal nucleotide sequences of the partial mt-LSU. Numbers on the nodes indicate Bayesian posterior probabilities.

3.1.7. Phylogeny based on the exon region of the mt-LSU of *Glomus intraradices* and *Glomus proliferum*

Sequences of *Glomus proliferum* could be separated from all other *Glomus intraradices* sequences in the exon regions with a bootstrap support of 100% (Fig.11). Indeed the *Glomus proliferum* sequences diverged strongly from *Glomus intraradices* strains sequences in the alignment through a large number of indels, variable sites and SNP's. The clone sequences originating from the *Festuca pratensis* root sample could also be clearly separated from the other *Glomus intraradices* strains by a 12 bp long variable region between intron 1 and intron 2, a deletion of 5 bp after intron 2 and many SNP's in the alignment. This caused a separation from other *Glomus intraradices* sequences in the maximum parsimony tree with a bootstrap support of 100% (Fig.11). The sequences originating from the BEG 75 strain could be separated from all other *Glomus intraradices* strains by a C→T transition which all clones originating from strain BEG 75 had in common. A transversion from T→A was located directly behind the intron 2, which all clone sequences of isolate BEG 75 had in common together with the two clone sequences originated from the root sample. Therefore the BEG 75 clone sequences and the clone sequences from the *Festuca pratenisis* root sample were separated from the other *Glomus intraradices* sequences in the tree (Fig 11).

3.1.8. Phylogeny of intron 1

Three different clades of isolates of *Glomus intraradices* could be separated from each other in the maximum parsimony tree based on the intron 1 (Fig.12). The Canadian strain DAOM 197198 clustered together with the *Glomus intraradices* strains originating from Tänikon (JJ strains) in the maximum parsimony tree (Fig.12). The sequence of *Glomus intraradices* stain JJ 106 was clearly separated from the other *Glomus intraradices* isolates, although strain JJ 106 originated from the same field in Tänikon as all other Jansa strains (JJ) (Fig.12). Finally, the two sequences originating from the *Festuca pratensis* root sample (Gi 868-15 and Gi 868-25) grouped in an independent clade with a bootstrap support of 100%.

The sequence variations between the strains were several insertions, deletions, SNP's and larger variable regions found the intron 1 of the mt-LSU from *Glomus intraradices*. While the flanking regions of the intron 1 were conserved in nearly all *Glomus intraradices* strains, variations before the start codon of the homing endonuclease were found in the strain JJ 106 and the *Festuca* root sample. The coding sequence of the LAGLIDADG homing in *Glomus intraradices* JJ 106 and the root sample also differed significantly from the other *Glomus intraradices* strains by indels and variable sites.

3.1.9. Phylogeny of intron 2

The parsimony phylogeny based on the nucleotide sequence alignment of the intron 2 again divided the sequences of the *Glomus intraradices* isolates into three linages (Fig.13). With a bootstrap support of 100% the clone sequences of *Glomus intraradices* BEG 75 were separated in an independent clade and also the sequence of *Glomus intraradices* JJ 106 was separated from the remaining strains with a bootstrap support of 91% (Fig.13). This was mainly caused by a 130 bp long variable region within the intron 2 in which sequences of BEG 75 had a 42 bp insertion followed by a short conserved region of 19 bp length and a second variable region that included a deletion. Interestingly the strain JJ 106 also had a deletion of 12 bp length in this region.

Other *Glomus intraradices* strains except isolate JJ 106 and BEG 75 showed no variations in their sequences above the expected *Taq* error level (Cline, Braman & Hogrefe 1996) and therefore clustered together in one common clade (Fig.13).

Fig.11

Midpoint rooted maximum parsimony tree of the exon regions from the partial sequence of the mitochondrial large ribosomal subunit based on 923 characters. Numbers at the nodes designated the bootstrap support above 50%. Sequences labeled DAOM and the clone number originate from the Canadian *Glomus intraradices* strain DAOM 197198. Clone sequences G.prol. were amplified from *Glomus proliferum*. Clone sequences originating from *Glomus intraradices* strains BEG 75 and JJ 291 are labeled with their clone number. Root samples from Festuca pratensis mycorrhizal symbiont are labeled as "Root" and the clone number.

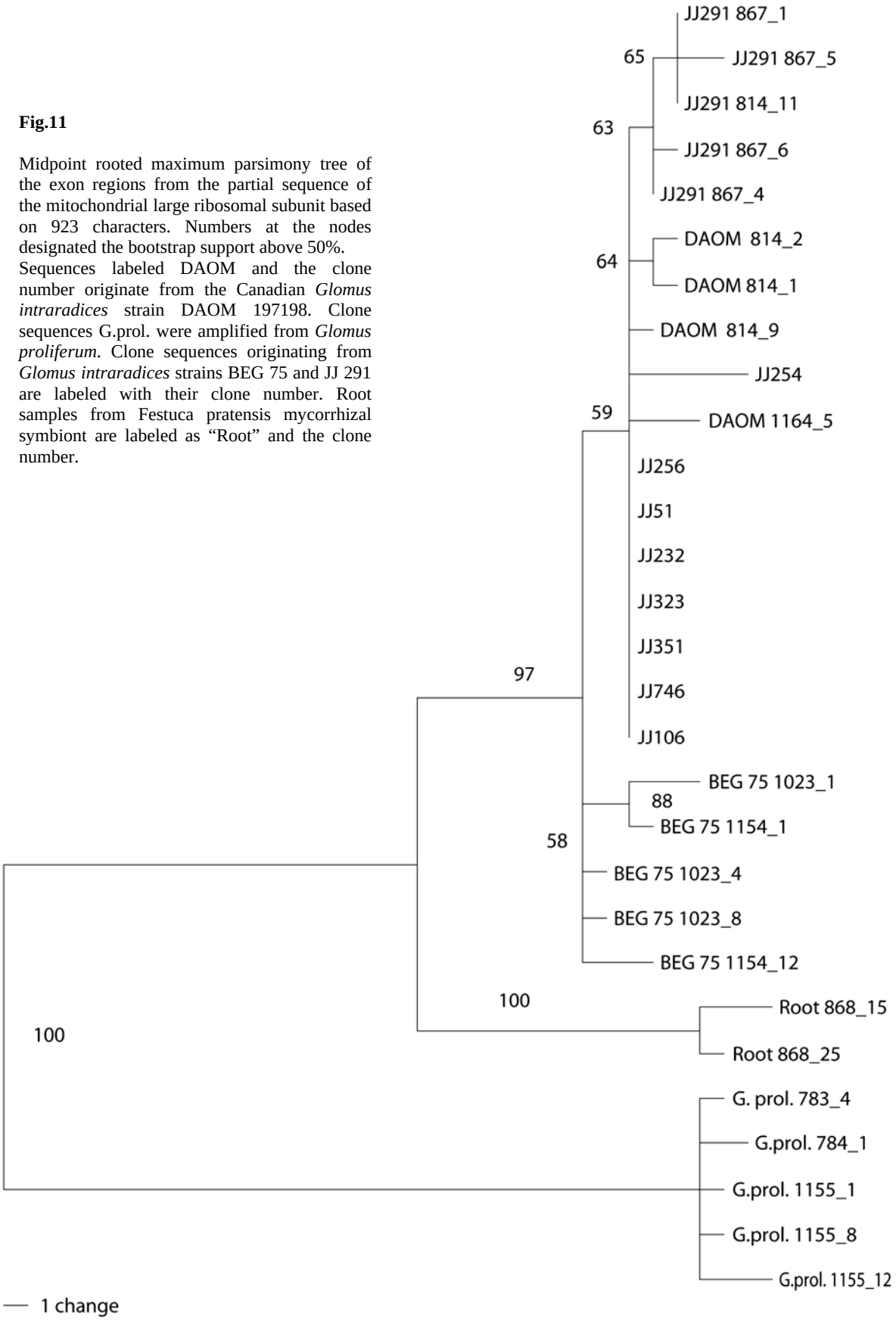


Fig.12

Midpoint rooted maximum parsimony tree from the partial sequence of the mitochondrial large ribosomal subunit based on 1101 bp of intron 1 sequences. Numbers at the nodes indicate the bootstrap support above 50%.
Sequences labeled DAOM and the clone number originate from the Canadian *Glomus intraradices* strain DAOM 197198. Clone sequences originating from *Glomus intraradices* strain JJ 291 are labeled with their clone number. Root samples from *Festuca pratensis* mycorrhizal symbiont are shown as "Root" followed by the clone number.

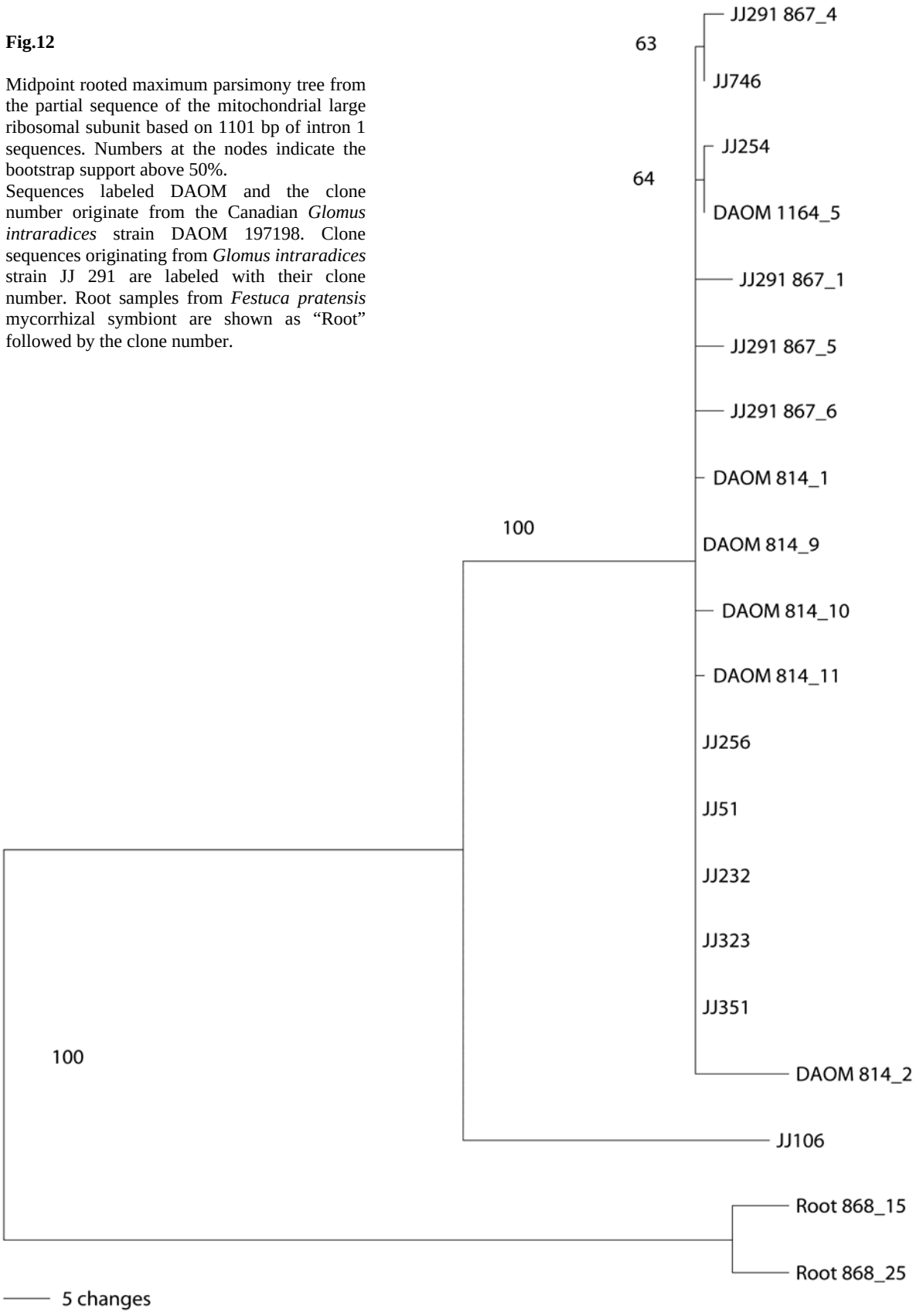
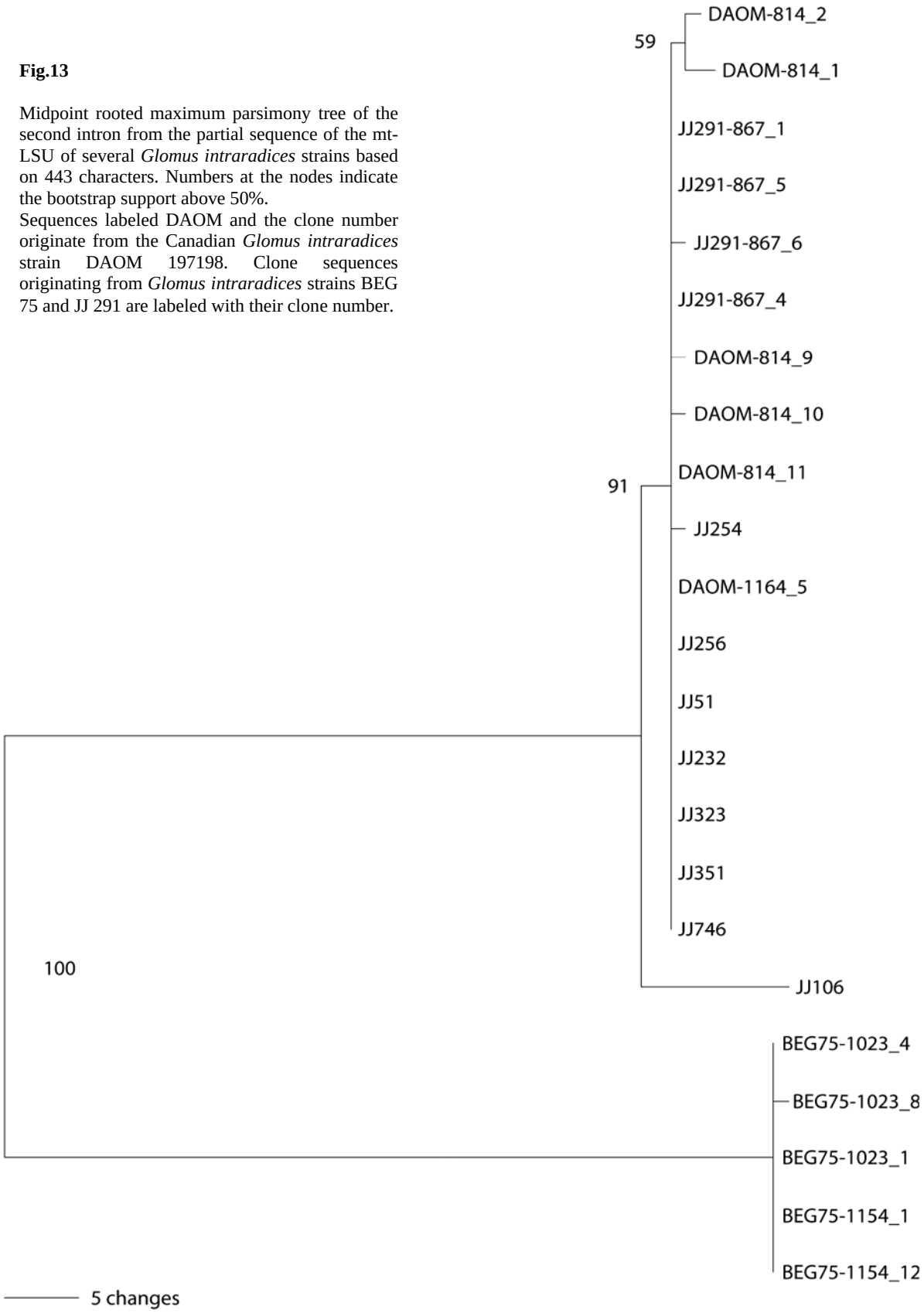


Fig.13

Midpoint rooted maximum parsimony tree of the second intron from the partial sequence of the mt-LSU of several *Glomus intraradices* strains based on 443 characters. Numbers at the nodes indicate the bootstrap support above 50%.
Sequences labeled DAOM and the clone number originate from the Canadian *Glomus intraradices* strain DAOM 197198. Clone sequences originating from *Glomus intraradices* strains BEG 75 and JJ 291 are labeled with their clone number.



3.1.10. Phylogenetic analyses of LAGLIDAG homing endonucleases

In all the intron 1 sequences of the *Glomus intraradices* strains JJ 51, JJ 106, JJ 232, JJ 254, JJ 256, JJ 291, JJ 323, JJ 351, JJ 746, DAOM 197198 and the *Festuca pratensis* field sample from Landskrone, a putative ORF of a LAGLIDADG homing endonuclease was identified. The protein sequences of the homing endonucleases were identical in all *Glomus intraradices* isolates, except the field sample and isolate JJ 106. Therefore only one sequence from these *Glomus intraradices* strains (JJ 291) was included in the alignment (Fig.14).

A phylogenetic tree was calculated using the three different *Glomus intraradices* homing endonucleases protein sequences and sequences from other closely related homing endonucleases found in public databases (www.ncbi.nlm.nih.gov). The tree was calculated under the maximum parsimony criterion using PAUP 4.

All LAGLIDADG sequences found in different strains of *Glomus intraradices* grouped as an independent clade with a high bootstrap support (Fig.14).

The protein sequence from the root sample, *Glomus intraradices* 291 and *Glomus intraradices* JJ 106 were distinct from other LAGLIDADG sequences.

According to the parsimony tree, two groups of mitochondrial intron-encoded endonucleases in Ascomycetes were found to be the closest relatives to the LAGLIDADG homing endonucleases identified in *Glomus intraradices*. The *Glomus intraradices* homing endonuclease sequences consistently grouped with a clade of homing endonucleases from cytochrome b introns of *Podospora* sp. (Fig.14). Several intron sequences from yeast formed a sister group to this clade. However, the bootstrap support was low for the deep branches.

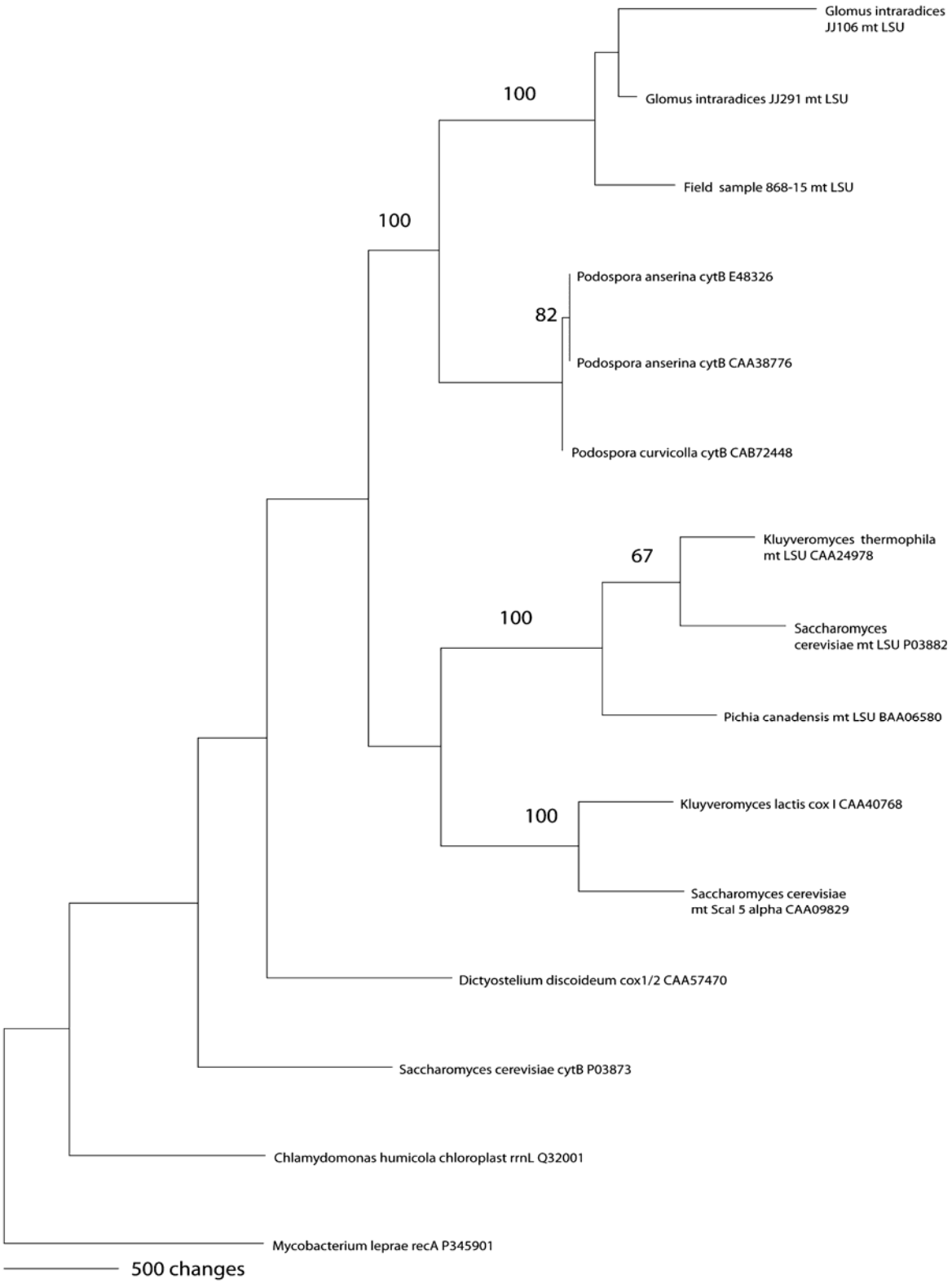


Fig.14

Maximum parsimony tree based on protein sequences of the closest relative homing endonucleases from the identified LAGLIDADG homing endonuclease found in the intron 1 of the partial mt-LSU of *Glomus intraradices.* Numbers on the nodes indicate the bootstrap support.

3.2. Part B: Amplification and phylogenetic analysis of the largest subunit of RNA Polymerase II (RPB1)

3.2.1. Construction of new specific primers for Glomus group A

Based on partial RPB1 sequences from closely-related AMF fungi published in AFTOL (http://ocid.nacse.org/research/aftol/data.php), new specific primers RPB1-PR1F, RPB1-PR2F, RPB1-PR3R for *Glomus* group A were constructed and tested with *Glomus intraradices* strains DAOM 197198 and JJ 291, *Glomus proliferum*, *Glomus mossae* ISCB13 and *Glomus geosporum*.

The primer target area of the RPB1 gene included the conserved domains C, D, E and a region of the domain B corresponding to *Saccharomyces cerevisiae* RPB1 amino acid position 250-720 (Fig.3). This region contained the active center of the enzyme in the area of the conserved domain D (Cramer *et al.* 2001).

Additionally, representatives of other AMF groups were tested with these primers. *Scutellospora castanea*, *Glomus lamellosum* and *Glomus etunicatum* showed no amplification, while a fragment of approximately 1.6 kb was amplified from all *Glomus* group A members using the newly constructed primers.

The PCR products of *Glomus intraradices* strains DAOM 197198 and JJ 291, *Glomus proliferum*, *Glomus mosseae* ISCB13 and *Glomus geosporum* were cloned and sequenced as previously described. New primers for the sequencing reaction were constructed. These new primers are shown in table 2.

3.2.2. Homogenous sequences of RPB1 within isolates

In order to screen for sequence variations within isolates of Glomeromycota, four clones originating from a PCR product from *Glomus proliferum* were sequenced. The cloned sequences differed from the consensus sequence by 0.23 % which is within the range of the misincorporation error of *Taq* polymerase (Cline, Braman and Hogrefe 1996). The calculated mean *Taq* error rate for the four clonal sequences

from *Glomus proliferum* was 3,8 x 10^{-5} (average mutationrate per bp) which is in agreement to the reported value of *Taq* error (Bracho *et al.* 1998).

To test variation between strains of members of the Glomeromycota two clone sequences from *Glomus mosseae* ISCB 13 were sequenced and compared with a published sequence of *Glomus mosseae* strain UT 101 (AFTOL project). The sequences showed no variations and could not be distinguished from each other. PCR products amplified from *Glomus intraradices* strains JJ 291 and DAOM 197198 were directly sequenced and no differences among these strains JJ 291 and DAOM 197198 or the published *Glomus intraradices* strain GINCO 4695 rac-11G2 from the AFTOL project were detected. In conclusion, no sequence variations in RPB1 were found within isolates of Glomeromycota or between strains of the same species.

Interestingly, all members of the Glomeromycota possessed a putative intron at the same position. This intron was situated between the conserved domains B and C in the RPB1 gene sequence corresponding to *Saccharomyces cerevisiae* S288c RPB1 gene position 889 (Fig.15).

Fig. 15

RPB1 nucleotide sequence alignment in the region of the glomeromycotan intron. Sequences cloned during the presented work are highlighted. Sequences from the following organisms were used:

Geosiphon pyriformis, Paraglomus occultum, Glomus proliferum (Gp), *Glomus intraradices* (Gi), *Glomus mosseae* (Gm), *Glomus geosporum* (Gg), *Rhizopus stolonifer, Mortierella verticillata, Basidiobolus ranarum, Coemansia reversa, Smittium culisetae, Entomophthora muscae, Endogone pisiformis, Saccharomyces cerevisiae, Aspergillus fumigatus, Tilletiaria anomala, Phanerochaete chrysosporium, Filobasidiella neoformans, Chytriomyces angularis, Nowakovskiella sp., Spizellomyces punctatus, Rhizophydium macroporosum, Allomyces arbusculus* and *Monosiga brevicollis*

3.2.3. Phylogeny of the True Fungi based on RPB1 protein sequences

The amino acid RPB1 phylogeny (Fig.16) presents strong support for a monophyletic phylum Glomeromycota. The closest relatives of the phylum were lineages of the Zygomycota (Mortierellales, Mucorales, Endogonales). Ascomycota and Basidiomycota formed a clade distant from the Glomeromycota. The members of the *Entomophthorales* were split into two groups. While *Basidiobolus ranarum* was grouping among other *Zygomycetes,* the sequence of *Entomophthora muscae* was closer to *Allomyces arbusculus* (Chytridiomycota).

Glomus geosporum and *Glomus mosseae* sequences were sister groups in one clade of the *Glomus* group A, *Glomus intraradices* and *Glomus proliferum* were forming a second clade (Fig.16). *Glomus intraradices* and *Glomus proliferum* were not resolved as separate clades, because their amino acid sequences were too similar. The basal glomeromycotan lineage in the RPB1 amino acid tree was *Geosiphon pyriformis*.

3.2.4. Phylogeny of the True Fungi based on RPB1 nucleotide sequences

The RPB1 phylogeny based on the nucleotide sequences gave insights into the different characteristics of nucleotide and amino acid sequence phylogenies. While the amino acid tree was unable to resolve closely related fungal members like *Glomus proliferum* and *Glomus intraradices,* the nucleotide-based tree could clearly distinguish sequences from these species since synonymous substitutions were distinguished in the analysis (Fig.17).

Ascomycota and Basidiomycota grouped together with a weak posterior probability support. As in the RPB1 amino acid phylogeny, Zygomycota were found to be the closest relatives to the Glomeromycota (Fig.16 and Fig.17), but there was only weak support among the closest lineages (*Mortierella, Basidiobolus, Rhizopus and Endogone*).

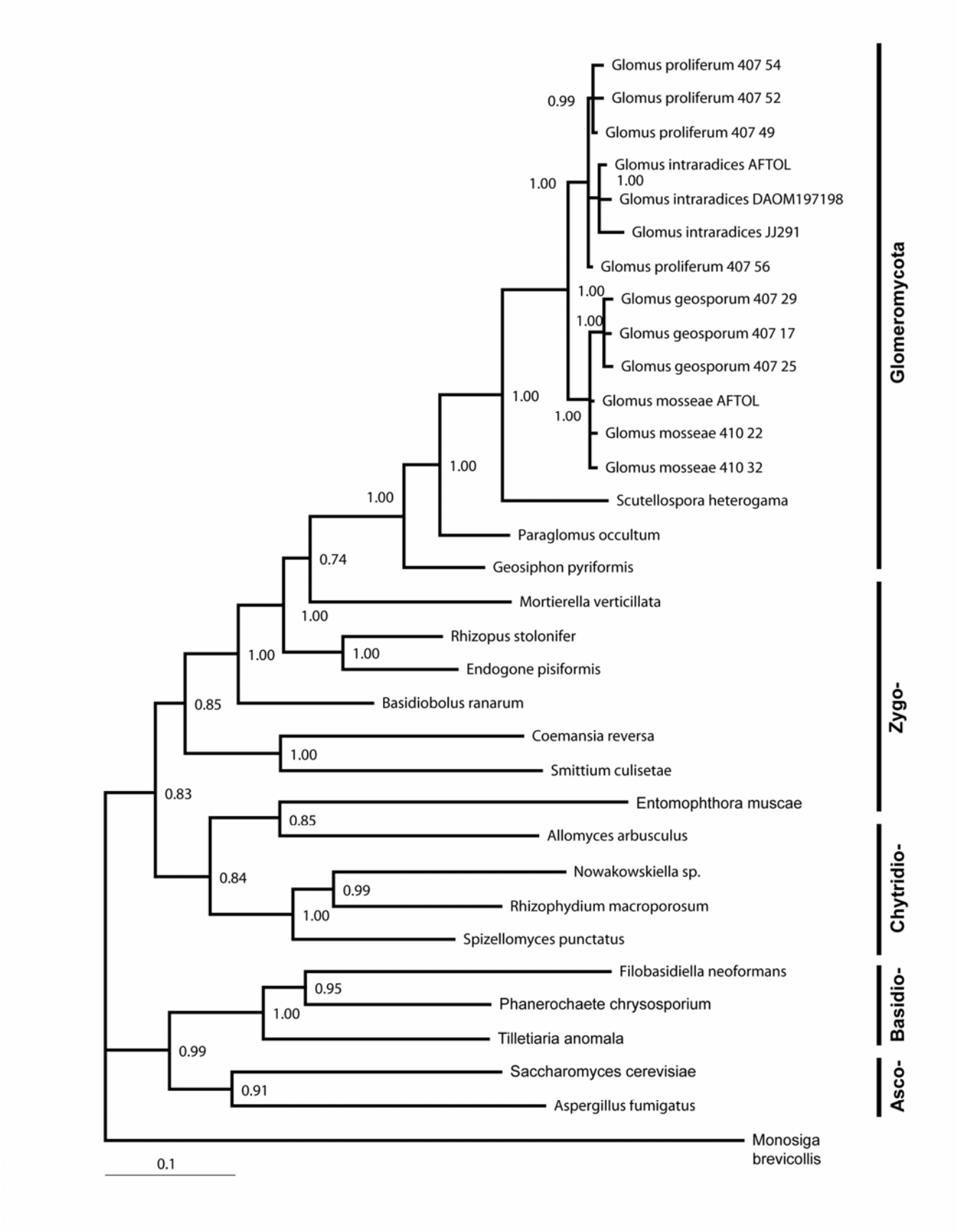


Fig. 16

Fungal phylogeny of RPB1. Bayesian tree obtained from protein sequences of RPB1. Numbers on the nodes indicate Bayesian posterior probabilities. *Monosiga brevicollis* was chosen as outgroup.

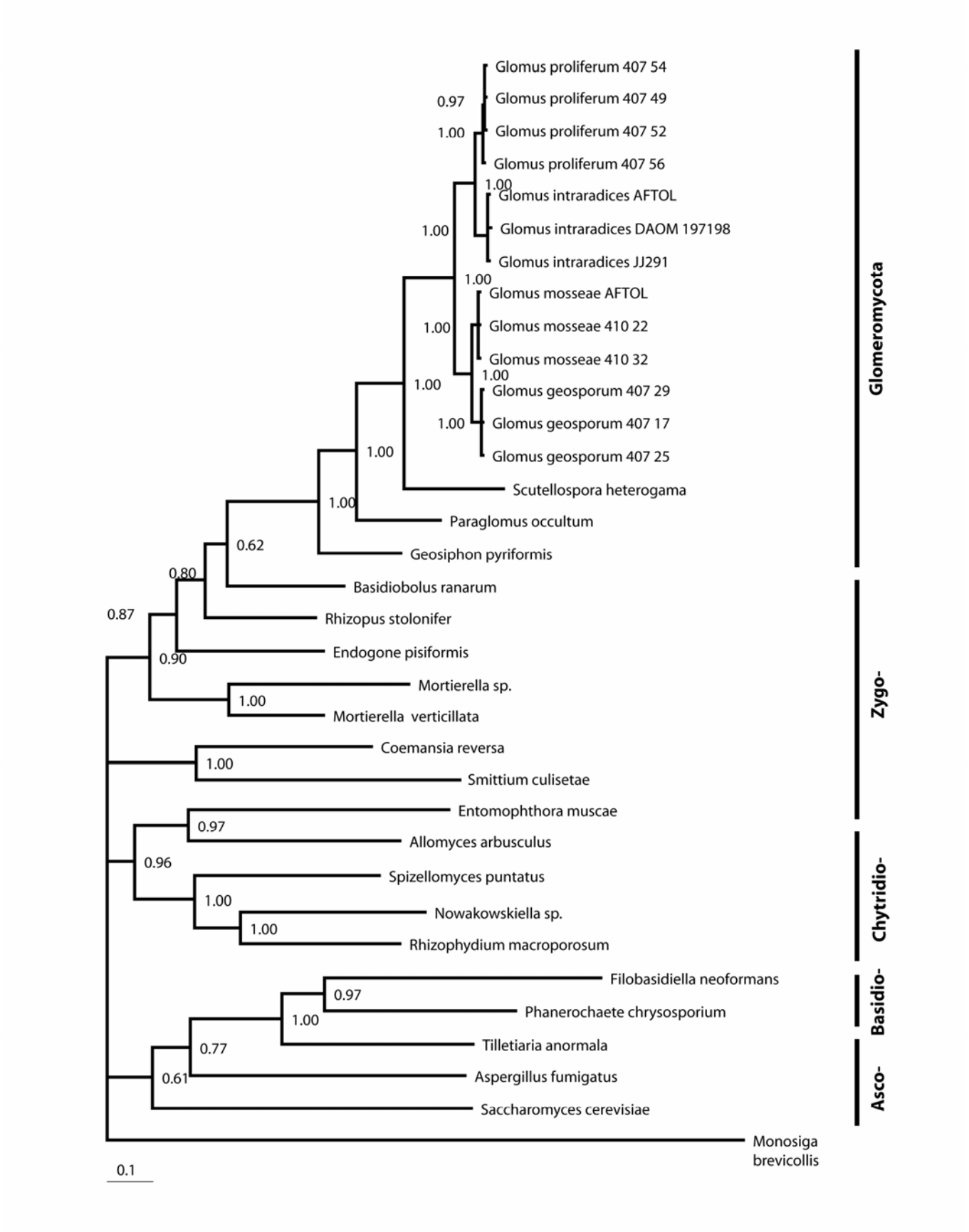


Fig. 17

Fungal phylogeny of RPB1. Bayesian tree obtained from nucleotide sequences of RPB1. Numbers on the nodes indicate Bayesian posterior probabilities. *Monosiga brevicollis* was chosen as outgroup.

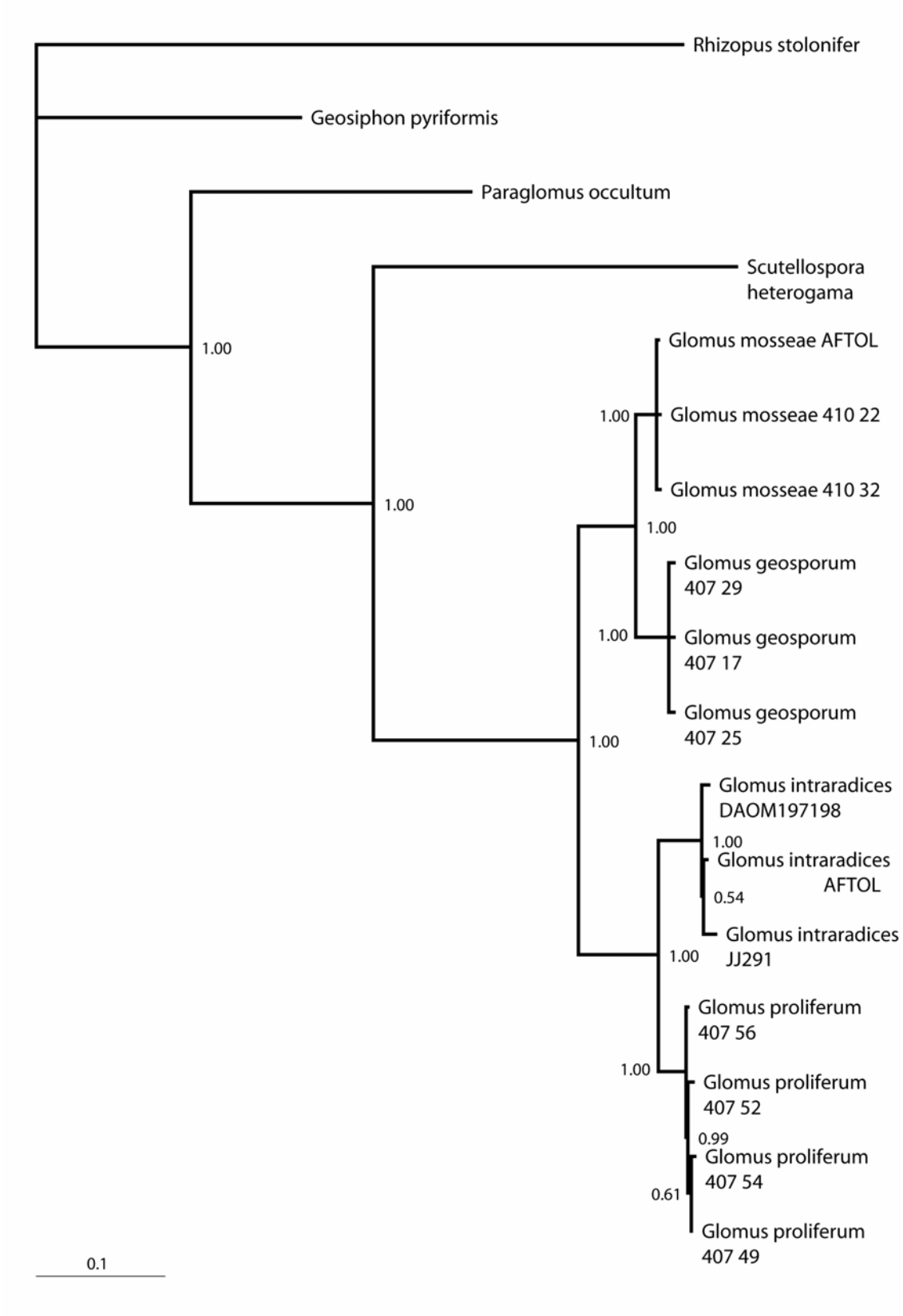


Fig.18

Glomeromycotan phylogeny of RPB1. Bayesian tree obtained from nucleotide sequences of RPB1. Numbers on the nodes indicate Bayesian posterior probabilities. The RPB1 nucleotide sequence of the zygomycote *Rhizopus stolonifer* was used as outgroup.

3.2.5. Phylogeny of the Glomeromycota based on RPB1 nucleotide sequences

In the analysis of glomeromycotan RPB1 nucleotide sequences, *Glomus* group A was supported as a clade with high posterior probability support. Sequences originating from *Glomus mosseae* ISCB13 grouped together with *Glomus mosseae* isolate UT101 analyzed by AFTOL. All *Glomus intraradices* strains (DAOM197898, JJ291 and GINCO 4695 rac-11G2 from the AFTOL project) also grouped as a clade (Fig.18).The clone sequences originating from *Glomus proliferum* formed an independent clade. *Glomus geosporum* sequences originating from two separate single-spore PCR reactions (sequence 407/17 from the first PCR and the sequences 407/25 and 407/29 from a second PCR) grouped together with strong support (Fig.18). Similar to the nucleotide and amino acid phylogenies of the True Fungi (Fig.16 and Fig.17), *Geosiphon pyriformis* was placed as the basal member of the Glomeromycota in this tree.

4. Discussion

4.1. Homogeneous RPB1 sequences in AMF

In the current discussion about the nature of nuclear genomes in spores of the Glomeromycota, protein genes showing sequence heterogeneity within single spores (BIP, Actin, EF-1 alpha, tubulin genes, H^+-ATPase) and other genes apparently not present in variants in the spores (FOX, TOR, GIN genes) were used as arguments by proponents of the heterokaryotic or the homokaryotic spore models (Kuhn, Hijri & Sanders 2001, Hijri & Sanders 2005, Pawlowska & Taylor 2004) The RPB1 gene was found to be strictly present as a single copy in other fungi (Matheny *et al.* 2002, Tanabe *et al.* 2004, James *et al.* 2006). In the present work, no sequence variation among sequences cloned from single spores, nor between different strains of the same *Glomus* species was detected. In contrast, another putative single copy gene, the BIP gene was found to be highly variable in *Glomus intraradices* (Kuhn et al., 2001). While in 16 BIP gene variant sequences 59 variable sites were identified in a total length of 680 bp, the four RPB1 clone sequences originating from *Glomus proliferum* had only 11 variable sites in a total length of 1520 bp. However only a partial region of the RPB1 gene was sequenced and analyzed, which included the coding region for the active center of the enzyme. Therefore, the amplified region of the gene possibly was too conserved. Some sequence variation between two clone sequences amplified from *Glomus mosseae* of RPB1 were found in the AFTOL sequence. On the other hand, both cloned sequences from *Glomus mosseae* ISCB13 in this work showed no differences from each other. Therefore, *Taq* polymerase errors should be considered as the source of these reported variations.
Although only about a third of the whole RPB1 gene was sequenced in this work, including the active center region, the nucleotide sequence variation between the glomeromycotan species was high enough to distinguish even closely-related morphospecies. No intra-isolate variations were reported until now in any fungal RPB1 gene sequences. Additional PCR products identified in one study were found to originate from subunits of the DNA-dependent RNA polymerases I and III and could be easily discriminated through BLAST searches (Tanabe *et al.* 2004).

The reason why some proteins show variants within single spores in the Glomeromycota and others do not is still unclear and needs to be addressed in further research.

4.2. The RPB1 phylogeny

The RPB1 phylogeny based on nucleotide and protein sequences always presented strong support for a monophyletic clade of the AMF. This is in accordance with other AMF phylogeny studies and supports the assumption of an independent phylum for AMF, the Glomeromycota (Schüßler *et al.* 2001).

In all constructed RPB1 trees *Geosiphon pyriformis* was placed as the most basal member of the Glomeromycota, branching off earlier than *Paraglomus occultum*. Recent studies placed *Geosiphon* close to the Archaeosporaceae (Schwarzott *et al.* 2001), but as no RPB1 sequences are available from *Archaeospora* the exact phylogenetic relationships among the basal glomeromycotan lineages remain to be elucidated.

Glomus group A was supported in all RPB1 trees as independent clade with a strong posterior probability support. As the protein sequences of *Glomus intraradices* and *Glomus proliferum* differed by only one substitution, no clear resolution between those species was obtained. The phylogenetic analysis based on the nucleotides of RPB1 considered synonymous as well as non-synonymous substitutions and was therefore able to better resolve the phylogeny of closely-related members of the Glomeromycota. On the other hand, the phylogeny of the fungal phyla was resolved better in the RPB1 phylogeny based on protein sequences. The fungal phylogeny trees (Fig.16, Fig.17) show a paraphyletic assemblage of zygomycotan and chytridiomycotan lineages. One zygomycotan group including the Mortierellales, *Basidiobolus ranarum*, *Rhizopus stolonifer* and *Endogone pisiformis* contained the apparently closest relatives to the Glomeromycota which suggests a close relationship of the Glomeromycota and zygomycotan lineages. The two representatives of the Entomophthorales*, Basidiobolus ranarum* and *Entomophthora muscae* were grouping separately in the trees, which has also been reported in other phylogenetic studies (Tanabe *et al.* 2004, Keeling *et al.* 2003, Lutzoni *et al.* 2004). *Basidiobolus ranarum* consistently groups with the Chytridiomycota in 18S rRNA

phylogenies (James *et al.* 2000) but recent analyses suggest that *Basidiobolus ranarum* is a zygomycete-like fungus which is only distantly related to other Entomophthorales (Keeling *et al.* 2003, Tanabe *et al.* 2004). This was supported in both fungal RPB1 phylogenies in this work. *Entomophthora muscae* grouped together with *Allomyces* which is consistent with a previous study (Tanabe *et al.* 2004). The monophyletic clade comprising the Dimargitales, Harpellales and Kickxellales reported in the study of Tanabe *et al.* (2004) was also recovered in both fungal RPB1 phylogenies.

Ascomycota and Basidiomycota are generally resolved as monophyletic sister groups (Bruns *et al.* 1992, James *et al.* 2006). Both feature the production of dicaryotic stages (binucleate, functionally diploid) in the life cycle. Schaffer (1975) therefore referred to the clade containing these groups as the "Dicaryomycota".

Based on analysis of nuclear-encoded ribosomal RNA placing the Glomeromycota as a sister group to the "Dicaryomycota", Tehler *et al.* (2003) named the clade comprising Asco-, Basidio- and Glomeromycota the “Symbiomycota”. Their main characteristic feature is the ability of many members to form mutualistic symbioses with plants and algae. Additionally, these fungi have the ability to form anastomoses, a trait which is rare or absent in Zygomycota and Chytridiomycota (Redecker and Raab 2006). In multilocus analysis, the symbiomycotan clade was well-supported but conflicting signals among the single gene partitions were also detected (James *et al.* 2006). The fungal RPB1 phylogeny based on nucleotide and amino acid sequences did not support a symbiomycotan clade which is in agreement with other phylogenetic studies, like the elongation factor and actin genes (Helgason *et al.*, 2003).

The analysis of glomeromycotan RPB1 nucleotide sequences supported *Glomus* group A as a clade with high posterior probability support. Sequences originating from different strains of *Glomus mosseae* (ISCB13, UT101) grouped together in one clade with high posterior probability support. The same was true for the different *Glomus intraradices* strains (DAOM197898, JJ291, GINCO 4695 rac-11G2). The relative phylogenetic position of the *Glomus* group A species is in accordance with other studies of AMF phylogeny based on rDNA (Schwarzott *et al.* 2001, Hijri *et al.* 2006). The high potential of the RPB1 gene as new maker to resolve the species of glomeromycotan fungi was well demonstrated in the RPB1 nucleotide sequence phylogeny. However, RPB1 sequences of representatives from Acaulosporaceae,

Archaeospora and *Glomus* groups B and C are needed to obtain a better picture of the phylogenetic relationships within glomeromycotan fungi.

No conserved intron locations in the RPB1 from representatives of different eukaryotic lineages have been found so far. Therefore, Stiller and Hall (1997) suggested that most introns were inserted subsequently to the divergence of the protist ancestors of those taxa. Stiller and Hall (1997) argued that the number and positions of RPB1 introns would not be useful for the determination of the relationships among the major eukaryotic groups. In this work a common intron between the conserved regions B and C was found in all glomeromycotan sequences and suggests an insertion event of this intron in the common ancestor of the Glomeromycota. However, some members of the Zygomycota, the Homobasidiomycete *Phanerochaete chrysosporium* and the Choanoflagellate *Monosiga brevicollis* contain an intron at the same position. Therefore, additional losses and/or gains of this intron must have had occurred outside the glomeromycotan lineage. Nevertheless this region is ideal for constructing new gene markers for AMF species identification in the future because the intron length variations found among all AMF species so far can be used for restriction analysis methods.

4.3. The mt-LSU as a molecular marker in the Glomeromycota

In this work new fungal- specific primers were constructed and successfully used for the amplification and sequencing of a partial sequence of the mt-LSU from arbuscular mycorrhizal fungi. A specific primer (RNL-5) for *Glomus intraradices* and *Glomus proliferum* was constructed and successfully tested on field samples.
For the first time, a considerable amount of sequences from a mitochondrial gene, mitochondrial introns, and a putative LAGLIDADG homing endonuclease from the Glomeromycota were presented in this work. The mt-LSU sequences originating from several strains of *Glomus intraradices* and from *Glomus proliferum* were sequenced and analysed phylogenetically.

Fungal mt-LSUs typically contain short, conserved areas frequently interrupted by a large amount of introns. Only a small subset of characters could be aligned and used for phylogenetic analyses. Therefore, mt-LSU turned out to be less useful for phylogenetic analyses on the phylum level. Nevertheless, the glomeromycotan sequences could be differentiated from other phyla in the phylum-level fungal phylogeny (Fig. 10). Basidiomycota and Ascomycota grouped together with the Chytridiomycota and neither a symbiomycotan clade (Tehler 2003 *et al.*) was supported nor the well established dicaryomycotan clade (Schaffner 1975) itself was supported in the fungal phylogeny tree. Therefore, the relationship of the Glomeromycota to other major fungal lineages cannot be easily addressed using the region of this gene. The weak posterior probability support for the basal branches in the fungal mt-LSU tree could be explained by the low number of alignable characters, but even the more conserved regions may have acquired some homoplasy over the long periods of the evolution of kingdom fungi.

The mt-LSU showed high potential to distinguish closely-related species or even different isolates of the same species. The mt-LSU sequence of the two closely-related species *Glomus intraradices* and *Glomus proliferum* diverged strongly in several regions and by 9.6 % over the whole sequence. For comparison, the n-rDNA small subunit sequences of the two species diverge only by 2.2 % in the region used for identification by Helgason *et al.* (2003). The highest pairwise variation among ten ITS/5.8S subunit sequences from a single spore of *Glomus intraradices* JJ291 (Jansa *et al.* 2002) was 5.2 %, not counting indels, whereas *Glomus proliferum* differed by 12.1-13.2 % from these sequences. By selecting variable regions of the mt-LSU, the latter value can be easily exceeded without the problem of intra-spore and intra-isolate variation. The data show the potential to use the mt-LSU to destinguish different isolates of the same species, but more isolates of *Glomus intraradices* from distinct areas of the world and from other glomeromycotan species have to be analyzed to explore this possibility. Once more taxa and sequence data are available, the mt-LSU and other mitochondrial genes may become excellent molecular markers for future ecological studies.

Several *Glomus intraradices* isolates were identified to contain introns in the partial sequences of the mt-LSU. The different number of introns among the different

isolates of *Glomus intraradices* and the absence of introns in *Glomus proliferum*, suggest that the intron gain or removal events have occurred relatively recently in evolutionary history. Future studies on the distribution of introns among *G. intraradices* isolates will show whether the transfer of introns is an ongoing process and whether the frequency of transfer may limit their use as molecular markers. Nevertheless, the intron sequences have a the potential to be highly sensitive molecular markers for future studies of population diversity and phylogeography of *G. intraradices*. The presence/absence patterns may open up new approaches for evolutionary biology of the Glomeromycota as it has been demonstrated with other organisms (Neuveglise *et al.* 1997, Gonzalez *et al.* 1999, Qiu *et al.* 1998).

Contrary to the nuclear encoded ribosomal genes (Jansa *et al.* 2002), no evidence was found in the mitochondrial large ribosomal subunit of *Glomus intraradices* and *Glomus proliferum* for intra-isolate sequence heterogeneity. Their deviation of the clone sequences of the mt-LSU from different *Glomus intraradices* isolates and from *Glomus proliferum* were within the range of misincorporation error of *Taq* polymerase. A model which could explain this phenomenon will be discussed in the chapter below (Fig.20 in chapter 4.4.).

Different *Glomus intraradices* isolates originating from the same field site as the isolates used in this study (i.e. Tänikon, Switzerland) showed a high genetic variability (Koch *et al.* 2006, Corradi *et al.* 2007). In the study of Corradi *et al.* (2007) all four analysed genes (18S, 5.8S, 25S and BIP genes) showed gene copy number polymorphisms among these *Glomus intraradices* isolates. In case of the nuclear-encoded ribosomal genes a variation in the number of rDNA genes from two to four-fold among the *Glomus intraradices* isolates has been reported (Corradi *et al.* 2007). Interestingly, the relative copy numbers of the three ribosomal genes among isolates had not been different from one another, which did not support a theory for independent duplication event of these genes. Because all the rDNA genes showed the same relative differences among isolates, it was speculated that the whole rDNA tandem array had been subject to deletion and duplication events in the genome of the three isolates (Corradi *et al.* 2007).
These duplications and deletions could be the result of “unequal crossing over” events in the *Glomus intraradices* isolates. Two mechanisms i.e. unequal crossing

over and biased gene conversion have been the principal explanations of concerted evolution, the process that is thought to keep copies of the ribosomal RNA genes identical (Hillis *et al.* 1991). Both mechanisms are active during mitose and meiose events.

Since no sexual reproduction has been reported in Glomeromycotan fungi, the nuclear encoded ribosomal sequence homogenisaton could be the result of a "limited" concerted evolution during mitose events. During mitose such unequal crossing over events between sister chromatids could have been the cause of duplication or deletion found in the nuclear ribosomal genes of the *Glomus intraradices* isolates. The deletion or inversion could also be the cause of of intra-chromatid-recombination between direct repeats (which would result in deletions) or inverted repeats (which would result in insertions).

Mitochondrial inheritance may impose different restrictions on intra-isolate gene variation than in the nuclear genome in the Glomeromycota (see chapter 4.4. and Fig. 20). The mt-LSU is most likely present in single copy per mitochondrial genome and does not show the genetic mechanisms described above. The nine analysed *Glomus intraradices* isolates originating from the field site in Tänikon did not show this high genetic variability in their mt-LSU sequences. Only one isolate (JJ106) differed from the other isolates by indels in intron 1 and intron 2.

Interestingly, the Canadian *Glomus intraradices* strain analysed in the study of Koch *et al.* (2004) was reported to be not genetically distant to the Tänikon isolates. The authors speculated that these results suggest a potential gene flow between the Canadian isolate and some of the Swiss population or that the total diversity comprised within a small scale may already account for most diversity on a much larger scale" (Koch *et al.* 2004). In agreement with these findings, the mt-LSU of the Canadian strain of *Glomus intraradices* was found to be identical to most of the Tänikon isolates. In contrast, the uncultured *Glomus intraradices* field sample was clearly distinct from the Tänikon isolates. These findings raise interesting questions about the possible influence of different field management practices on genetic diversity of *Glomus intraradices* isolates. Now that the first glomeromycotan mt-LSU sequences are available, this needs to be tested in the future with more *Glomus intraradices* isolates and together with further population studies and studies on the phylogeography of the Glomeromycota.

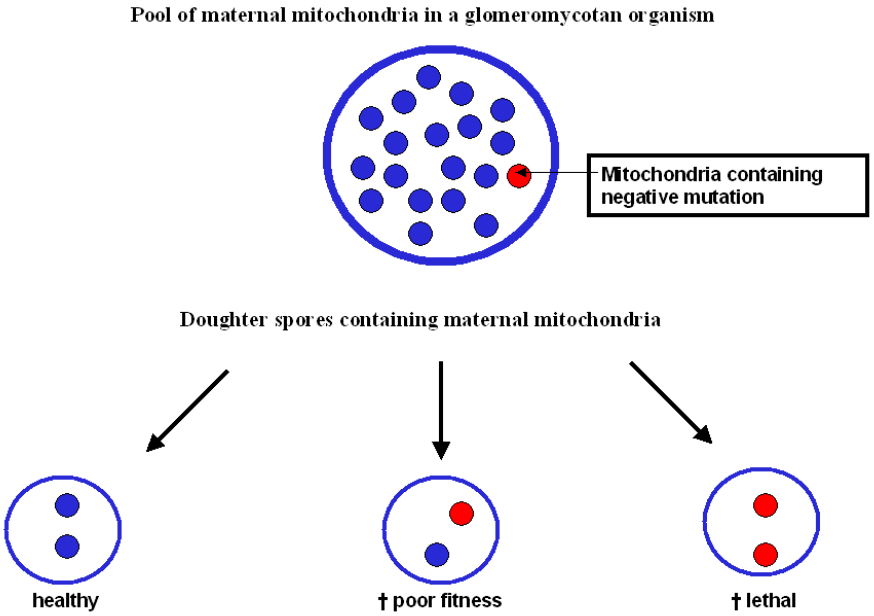
4.4. Inheritance of mitochondrial genomes in AMF

The lack of sequence variation of the mitochondrial large ribosomal subunit within spores of *Glomus intraradices* and *Glomus proliferum* raises the question about the mechanism that -in contrast to the nuclear genome- conserves the mitochondrial genome in the Glomeromycota.

The lack of sequence variations found in the mitochondrial large ribosomal subunit of *Glomus intraradices* and *Glomus proliferum* could be explained by the "bottleneck" due to drastic reduction of the number of mitochondria in glomeromycotan spores (Tamasloukht *et al.* 2003) and the asexual life cycle in AMF that causes an increased selection pressure on deleterious mutations in the mitochondrial genome. Two factors should be responsible for this increased selection pressure (Fig.20). First, deleterious mutations in the mitochondrial genome of AMF would result in the reduced fitness or lethality of those individuals especially in regions coding for the respiratory metabolism. AMF can exclusively metabolize via respiration and therefore a shift to fermentation as in the case of the petite mutants in yeast (Williamson 2002) is not possible and will result in reduced fitness or lethality. Second, the selection effect is increased through the drastic reduction of the number of mitochondria in the glomeromycotan spore state (Tamasloukht *et al.* 2003), which causes a bottleneck effect on the mitochondrial genomes contrary to the nuclear genomes. In contrast to many other fungi, the Glomeromycota do not form spores containing a single nucleus at any stage of their lifecycle. In fact, it was shown microscopically that several nuclei migrate into spores of *G. intraradices* during spore formation. (J.L. Jany, T. E. Pawlowska, ICOM 5 presentation, Granada 2006).

The expected accumulation of neutral mutations in the mitochondrial genome of asexual organisms (Lynch 1996, 1997) is not valid for large ribosomal subunits because of their special characteristics. A mutation would influence the secondary structure of the ribosomal RNA and therefore influence the fitness of the ribosome itself. The selection pressure in the population of *G. intraradices* should therefore suppress any genetic drift in their mitochondrial genome. This theory should be verified by analyses of further mitochondrial genes in AMF in the future.

Fig.20 Model for the inheritance of mitochondrial genomes in the Glomeromycota



Interestingly, no sequence variations were found within single strains of *Glomus intraradices* even within the intron sequences of the mt-LSU although accumulation of mutations should be expected in these regions (Lynch 1996, 1997). While in a single spore of *Glomus intraradices* the highest pairwise variation among ten ITS/5.8S subunit sequences was 5.2%, no significant sequence variations were found in any intron or exon sequences in the mt-LSU of *Glomus intraradices* and *Glomus proliferum*. This lack of sequence variation is remarkable considering the strong sequence differences between *Glomus intraradices* and *Glomus proliferum* and even between different strains of *Glomus intraradices*, which indicates a high average substitution rate in the mt-LSU of AMF.

4.5. The LAGLIDADG homing endonucleases

A putative ORF of a LAGLIDADG homing endonucleases has been identified in all *Glomus intraradices* strains containing the intron 1. Homing endonucleases are divided into four families, the LAGLIDADG, GIY-YIG, HNH and His-Cys-box

enzymes. These proteins are encoded by an open reading frame in genetically mobile, selfsplicing introns (group I and II) and inteins (Toor & Zimmerly 2002).

Homing endonucleases are site-specific enzymes, which can recognize and cleave alleles that lack introns or intein sequences. They promote the lateral transfer of their encoding intron or intein by a targeted transposition mechanism called “homing” (Dujon *et al.*1989).

Homing endonucleases have evolved to cleave their target sequence without being overly deleterious to the host organism and to avoid disrupting host gene function by self-splicing at the RNA level in introns or at the protein level in inteins. These enzymes tend to be of relatively small size of <40 kDa, a property due to the length limitations of the mobile sequences in which they reside (Chevalier and Stoddard 2001).

Homing endonucleases are tolerant to some nucleotide exchanges at their homing site, probably a characteristic that ensures their propagation despite the occurrence of genetic drift in their target sequences.

Homing endonucleases and their “extra-Mendelian genetic phenomenon” (Coen *et al*. 1970) were first described in 1970 in a group I intron of yeast. The genetic marker “ω” in *Saccharomyces cerevisiae* was found to transfer to strains lacking the marker when crossed to ω^+ stains (Coen *et al*. 1970). Further analysis indicated that the gene duplication event required a double-strand break at the target side and the expression of an intron-encoded ORF. This ORF was shown to encode a site-specific endonuclease initiating the “homing” event. This protein named I-SceI was the first of over 250 known homing endonucleases found since then.

Interestingly, this homing endonuclease was found in a 1.1 kbp group I intron from the mitochondrial large ribosomal RNA gene of *Saccharomyces cerevisiae,* similar to the homing endonuclease in *Glomus intraradices.* While the I-SceI LAGLIDADG2 homing endonuclease (P03882) s encoded at the positions 61022-61729 of the mitochondrial genome sequence of *Saccharomyces cerevisiae* S288c (NC 001224), the LAGLIDAG motif of *Glomus intraradices* is encoded in the intron 1, at a position corresponding to the positions 58644- 58645 in *Saccharomyces cerevisiae* S288c.

Homing endonucleases are widespread in nature (Belfort and Roberts (1997), Dujon (1989), Belfort and Pearlman (1995), Lambowitz and Belfort (1993). They have been found in group I and group II introns, archaeal introns and inteins in all domains from Archaea over Eubacteria to Eucaryota. About 30% of all group I introns are

estimated to contain ORFs and a significant number of them appear to be mobile (Chevalier and Stoddard 2001). In the eucaryotes, homing endonucleases were found in nuclear, mitochondrial and chloroplast genomes.

The different mechanisms in recognition and cleavage of group I and group II introns and the existence of multiple distinct families of homing endonucleases are evidence that the “homing” has evolved multiple times in nature (Chevalier and Stoddard 2001).

The phylogeny of the putative LAGLIDADG homing endonucleases found in several *Glomus intraradices* strains was in conflict with fungal phylogenies obtained using other marker genes. This finding is not surprising as these introns are not exclusively transferred from parent to offspring but most likely are occasionally transferred horizontally, also between unrelated organisms. In fact, these homing endonucleases could be transferred between strains of *Glomus intraradices*, e.g. by anastomosis. Hyphal fusions among AMF of the same species have already been observed (Giovannetti *et al.*1999). This possibility opens up interesting perspectives for an application of these genes as molecular markers, e.g. by pairing strains with and without homing endonuclease and monitoring possible transfers.

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Acknowledgements

First of all, I would like to thank PD Dr. Dirk Redecker for providing me with the opportunity to work in the field of molecular ecology of arbuscular mycorrhiza, the freedom to explore my ideas and the guidance to consolidate my findings.

I would also like to express my gratitude to Prof. Thomas Boller and Prof. Andres Wiemken for their critical appraisal of my work and for their constructive suggestions.

I would like to thank Kurt Ineichen, Dr. Jan Jansa and Dr. Natalia Requena for their constant help and support during this work.

I wish to thank all present and past members of Dirk's lab for these wonderful and enjoyable years: Zuzana Sýkorová, Isabelle Hijri, Boris Börstler, Odile Thiéry, Annemarie Brennwald-Ochsner, Susann Appoloni, Saskia Agten, Noelle Kuonen, Daniela Serio, Janine Dörig and Chris Morris. A special thanks to Zuzana Sýkorová, Boris Börstler and Odile Thiéry for correcting my thesis.

Many thanks to all other people at Hebelstrasse not mentioned by name for their support.

Finally, warmest thanks must go to my family. Without their sacrifices and constant support, this thesis would not have been written.

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