A molecular approach to characterize the arbuscular mycorrhizal fungus, *Glomus* sp. AMykor isolate

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Dla Ukochanych Rodziców

SUMMARY

The arbuscular mycorrhizal fungi (AMF) interaction with plants has a major impact on the soil ecosystem. However, so far, only a few studies on AMF genetics have been performed and molecular information on the genetic diversity of AMF is limited. In this study a fundamental genetic characterization of the industrial isolate, *Glomus* sp. AMykor (AMykor GmbH, Bitterfeld, Germany) has been undertaken to increase the understanding of AMF genetic diversity.

Based on phylogenetic analysis of partial rDNA sequences, *Glomus* sp. AMykor isolate was proposed to belong to the *G. irregulare* species together with the reference isolate, DAOM197198. To investigate if both isolates differ in their ploidy level, fluorescence *in situ* hybridization (FISH) was performed and mainly one or two hybridization signals per nucleus were observed in both isolates. It is suggested that they harbour at least two major rDNA sites and possibly two minor sites. The DNA content was estimated by means of flow cytometry (FC) and confirmed by Feulgen densitometry (FD). The calculated average DNA content per nucleus is 153.0 ± 3.6 Mb for the *G. irregulare* AMykor isolate and 154.8 ± 6.2 Mb for the DAOM197198 isolate.

Since there are plenty criticisms coming recently of using rDNA sequence for fungal barcoding there is necessity of development other system for the identification to species level of Glomeromycotan fungi. The focus of this part of the study was the *GiFRD* gene encoding fumarate reductase enzyme for use as a potential candidate for AMP species determination.

Unfortunately, observed sequence variations do not allow the discrimination of Glomeromycotan species. However, further analysis of enzyme encoded by *GiFRD* showed a possible role of fumarate reductase in AMF redox balance maintaining under oxygen deficient conditions. Using a yeast expression system, it has been demonstrated that the protein encoded by *GiFRD* has fumarate reductase activity. The functional expression of *GiFRD* in the *S. cerevisiae* fumarate reductase deletion mutant restored the ability of growth under anaerobiosis which indicated that Gifrdp is able to functionally complement the *S. cerevisiae* missing genes. The fact that *GiFRD* expression was present only in the asymbiotic stage confirmed existence of at least one metabolic pathway involved in anaerobic metabolism and suggested that AMF behave as a facultative anaerobe in asymbiotic stage.

ZUSAMMENFASSUNG

Die Wechselwirkungen von arbuskulären Mykorrhizapilzen (AMPs) mit Pflanzen haben einen starken Einfluss auf das Bodenökosystem. Trotz ihrer Bedeutung gibt es vor allem für die Landwirtschaft bisher nur wenige Studien über genetische Prozesse bei AMPs und ihre genetischen Diversität. In der vorliegenden Arbeit wurde am Beispiel des AMP-Isolates *Glomus sp.* AMykor eine grundlegende genetische Charakterisierung vorgenommen, um den genetischen Hintergrund dieses Pilzes aufzuklären.

Basierend auf der phylogenetischen Analyse partieller rDNA-Sequenzen wurde *Glomus sp*. AMykor analog zum Referenzstamm DAOM197198 der Art *G. irregulare* zugeordnet. Zur Analyse des Ploidiegrades beider Stämme über rDNA-Hybridisierung wurde die Fluoreszenz-*in-situ*-Hybridisierung (FISH) genutzt. In beiden AMP-Stämmen konnten pro Zellkern ein bis zwei intensive Hybridisierungssignale und teilweise zwei weitere mit geringer Intensität detektiert werden. Damit ist die rDNA beider AMPs mindestens an zwei bevorzugten Orten innerhalb des Zellkernes nachweisbar. Der DNA-Gehalt der beiden zu analysierenden AMPs wurde mittels Durchflusszytometrie (FC) bestimmt und durch Feulgen Densitometrie (FD) bestätigt. Er betrug pro Zellkern 153.0 ± 3.6 Mb bei *G. irregulare* AMykor und 154.8 ± 6.2 Mb für den Referenzstamm DAOM197198.

Da vor allem in den letzten Jahren Kritik an der Verwendung von rDNA Sequenzen zur Bestimmung von pilzlichen Barcodes aufkam, sind neue Systeme zur Identifizierung der Arten von Glomeromycotapilzen zu etablieren. Aus diesem Grund wurde die Eignung von *Fumaratreduktase* (*GiFRD*) als Kandidatengen zur taxonomischen Klassifizierung der AMPs geprüft. In diesem Zusammenhang wurde das *GiFRD*-Gen isoliert und das kodierte Enzym charakterisiert.

Basierend auf der hohen Identität der Gensequenz zu *FRD*-Genen anderer AMPs, war das aus *G. irregulare* AMykor isolierte *GiFRD*-Gen nicht zur taxonomischen Klassifizierung von Glomeromycota-Arten geeignet. Biochemische Analysen des von *GiFRD* kodierten Enzyms, belegten dessen Rolle im AMP zur Aufrechterhaltung der Redox-Balance unter Sauerstoff-limitierenden Bedingungen. Das durch *GiFRD* kodierte Protein zeigte auch im Hefesystem Fumaratreduktaseaktivität. Die Komplementierung einer

S. cerevisiae Fumaratreduktasemutante mit deletierten *OSM1*- und *FRDS1*-Genen belegte, dass Gifrdp die Aktivität der beiden fehlenden Enzyme komplementieren kann. So sind die Hefen mit *GiFRD*-Gen auch zum Wachstum unter anaeroben Bedingungen befähigt. Das Auftreten der *GiFRD*-Expression nur im asymbiotischen Stadium von *G. irregulare* AMykor bestätigte außerdem die Existenz mindestens eines im anaeroben Metabolismus involvierten Stoffwechselweges, was auf ein fakultativ anaerobes Verhalten des Pilzes in diesem Stadium deutet.

LIST OF ABBREVIATIONS

A. adeninivorans	*Arxula adeninivorans*
AC	accession number
AFRD1	*A. adeninivorans fumarate reductase*
AM	arbuscular mycorrhizal
AMF	arbuscular mycorrhizal fungi
AP	alkaline phosphatase
approx.	approximate(ly)
A. thaliana	*Arabidopsis thaliana*
ATP	Adenosine-5'-triphosphate
BiP	gene encoding binding protein
bp	base pair
BS	bootstrap support
°C	Celsius degree
CBOL	Consortium for the Barcode of Life
COX1	cytochrome oxidase subunit I
DAOM	Agriculture and Agri-Food Canada National Mycological Herbarium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
FAD	flavin adenine dinucleotide
FC	flow cytometry
FD	Feulgen DNA image densitometry
FISH	fluorescence *in situ* hybridization
FMN	flavin mononucleotide
FRDS1	*S. cerevisiae fumarate reductase*
x g	times gravity speed
GFP	Green Fluorescence Protein
GiFRD	*G. irregulare fumarate reductase*
G. intraradices	*Glomus intraradices*

G. irregulare	*Glomus irregulare*
GlGrAa	*Glomus* group Aa
GlGrAb	*Glomus* group Ab
G. max	*Glycine max*
G. mosseae	*Glomus mosseae*
G. proliferum	*Glomus proliferum*
G. versiforme	*Glomus versiforme*
INVAM	International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi
ITS	internal transcribed spacer
kDa	kilo Dalton
LSU	large subunit of rDNA
Mb	mega bases
min	minute(s)
mM	millimolar
mtLSU	mitochondrial LSU
NAD	nicotinamide adenine dinucleotide
NJ	neighbour joining
OD	optical density
OSM1	*S. cerevisiae fumarate reductase*
PCR	polymerase chain reaction
rDNA	ribosomal DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
R. sativus	*Raphanus sativus*
RT	room temperature (20 - 22˚C)
sec	second(s)
S. cerevisiae	*Saccharomyces cerevisiae*
SSU	small subunit of rDNA

1. INTRODUCTION

1.1 Arbuscular mycorrhizal fungi

The symbiotic interaction refers to organisms that live in close and often long-term interaction with mutual benefits. The mycorrhiza is a symbiotic relationship between fungi and vascular plants; it is widespread in most of terrestrial ecosystems (Koide & Mosse, 2004). While only a small proportion of all species has been examined, 95% of those plant families are predominantly mycorrhizal (Trappe, 1987). The name of this correlation comes from Greek words: *myces* = fungus and *rhiza* = root. The term “mycorrhiza” was coined in 1885 by Bernhardt Frank. Arbuscular mycorrhizas (AM) were named according to the structures they form within plant cells, called arbuscules (from the Latin *arbusculum*, little tree) (Strack *et al*., 2003). Mycorrhiza is a unique interaction between two eukaryotes, an obligate biotrophic fungus and a plant; where both partners benefit from the reciprocal nutrient exchange (Bonfante & Genre, 2008; Kiers *et al*., 2011).

The mycorrhizae are divided in three major types: ectomycorrhizal fungi (belonging to the basidiomycota, ascomycota and zygomycota), ericoid mycorrhizal fungi (mostly from ascomycota order Helotiales) and endomycorrhizal fungi also called arbuscular mycorrhizal fungi (AMF) (formed only by Glomeromycota phylum) (Cairney, 2000; Schüßler *et al*., 2001) (Fig. 1).

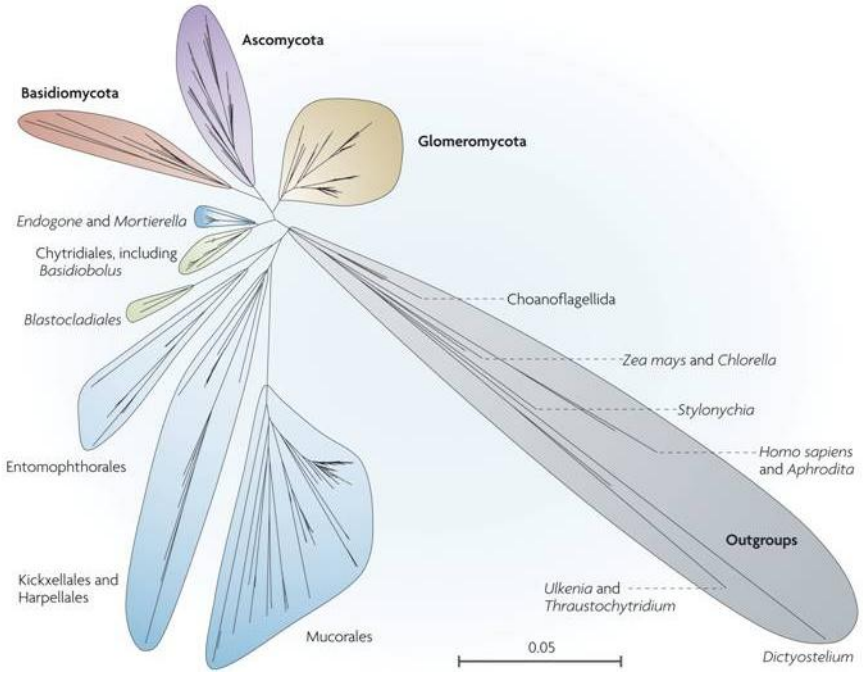

Figure 1 Phylogenetic relation among fungi based on SSU rRNA sequences (Parniske, 2008).

The Glomeromycota phylum has been divided into four orders (Glomerales, Paraglomerales, Archaeosporales and Diversisporales) based on the phylogenetic analysis of SSU rRNA gene sequences (Schüßler *et al*., 2001) (Fig. 2). Model AMF *Glomus intraradices* (recently reassigned to *Rhizophagus intraradices*) and closely related *Glomus irregulare* (recently reassigned to *Rhizophagus irregulare*) belong to Group Ab (GlGrAb) within the order Glomerales (Schüßler & Walker, 2010). This is important regarding the difficulties in species determination between isolates within *Glomus* group explained in Chapter 1.5.

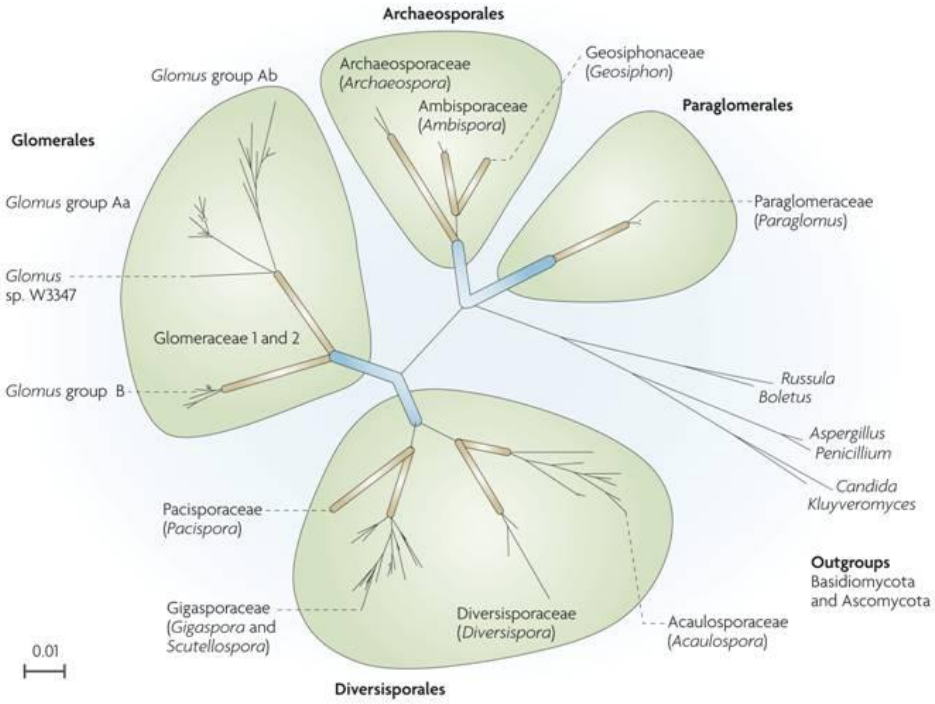

Figure 2 Phylogenetic relationships among members of the Glomeromycota phylum (Parniske, 2008).

The earliest hyphae and spore fossils, which resemble present of AMF morphological structures, have been found in the dolomite rocks of Wisconsin aged about 455 - 460 million years. A phylogenetic analyses of rDNA sequences of those fossils suggested that the origin of Glomeromycota fungi may be as early as 600 million years ago (Redecker *et al*., 2000). Because of the age of the symbiosis and clear benefits coming from AMF symbiosis, it is suggested that AMF may have played a crucial role for the adaptation of phototrophs to the terrestrial environment (Redecker *et al*., 2000; Remy *et al*., 1994).

1.2 The biology of AMF

1.2.1 The AMF structures

Arbuscular mycorrhizal fungi comprise intra- and extraradical structures. In Glomeromycota, intraradical hyphae can penetrate the outer cell wall of root and grow between or inside of the root cell wall and plasma membrane where they develop the intraradical structures, arbuscules and vesicles. The extraradical structures are hyphae and spores that develop outside of the roots in the soil (Fig. 3).

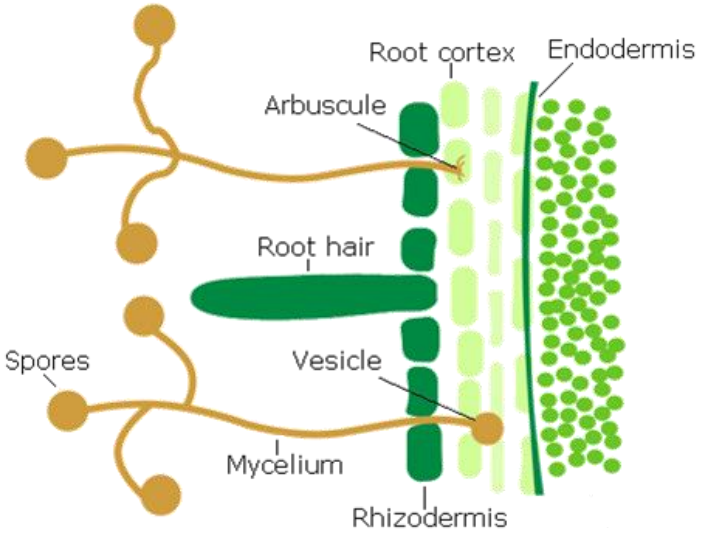

Figure 3 AMF structures during symbiosis with plant root (AMykor, GmbH).

Spores (greek: *spore* = germ) form as swellings on subtending hyphae in the soil or in roots (Fig. 4C). They contain cytoplasm, numerous nuclei (576 to 35000 in different species; Hosny *et al*., 1998; Viera & Glenn, 1990) and storage lipids as an energy source for presymbiotic growth. Spores function as storage structures, resting stages and propagules. They are the only plant-independent phase of the fungi. Their size varies strongly among the species between 20 - 100 µm (Dalpé *et al*., 2005). Spores usually develop thick walls with more than one layer but colour, morphology and the composition differ dependent on species (Błaszkowski *et al*., 2002). This was the reason why they were the most important structures considered so far in the classification of arbuscular fungi until rRNA sequencing methods appeared. The introduction of molecular methods resulted in the description of approx. 220 morphologically distinct species.

Extraradical hyphae radically increase the absorptive area of roots (Fig. 4A). They form hyphal bridges transferring nutrients from soil to co-occurring plants (Bücking & Shachar-Hill, 2005; Govindarajulu *et al*., 2005) and bind sand grains causing improvement of the soil aggregation (Rillig & Mummey, 2006). Hyphae can be single-walled, as in case of *Glomus versiforme* or double-walled, as with *Glomus fasciculatum* (Dalpé *et al*., 2005). **Inter- and intracellular hyphae** in roots contain storage material and take part in transport of the substances absorbed from the soil to plants and sugars from plants to the fungi (Solaiman & Saito, 1997).

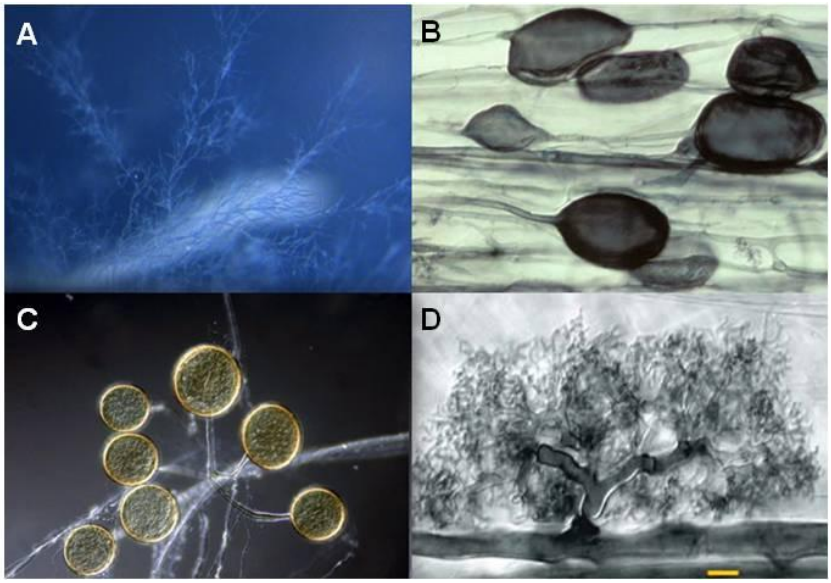

Figure 4 AMF extra- and intraradical structures: hyphae (A), vesicles (B), spores (C), arbuscules (D) (Brundrett, 2008).

Arbuscules (tree shape structures) are formed inside the host root cortical cell wall and are indicators of active mycorrhizae (Bago *et al*., 1998) (Fig. 4D). They are the main sites of nutrient, and perhaps signal exchange between the plant host and a fungus (Parniske, 2008). Arbuscules which are the key sites for nutrient exchange remain active for only 4 - 15 days (Goltapeh *et al*., 2008).

Vesicles originate from the swelling of intraradical hyphae and are localized inter- or intracellularly (Pawlowska *et al*., 1999) depending on the AMF species (Fig. 4B). They are filled with lipids, glycolipids and organelles required for autonomous growth (Dalpé *et al*., 2005).

1.2.2 AMF life cycle

There are three growth phases in AMF: asymbiotic, pre-symbiotic and symbiotic (Fig. 5). The asymbiotic mycelium stage includes spore germination and germ tube growth for about 2 - 3 weeks without physical contact with roots or root exudates under appropriate water and temperature conditions. During this phase, the fungus is living mostly from its triacylglyceride reserves. The presence of signals from plant root exudates (strigolactones) induces hyphal branching (Parniske, 2008). This phase is called pre-symbiotic. In takes one to several weeks to establish contact between the root and fungal hyphae (Declerck *et al*., 1998). Once the root-fungi contact is established, the fungal morphology and metabolism change radically which allows the fungus to enter into the symbiotic stage (Besserer *et al.,* 2006).

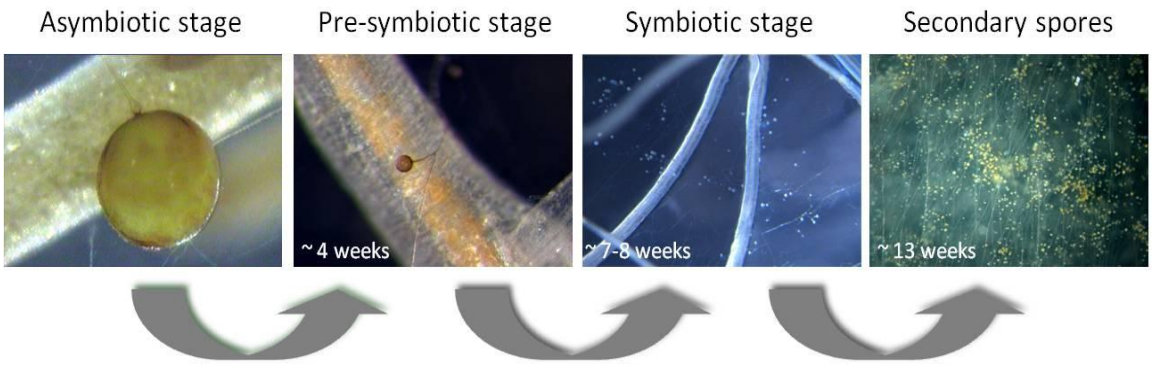

Figure 5 Life stages of an arbuscular mycorrhizal fungus belonging to *G. irregulare* species.

In the symbiotic stage, the fungus penetrates the surface of root and colonizes the root cortex. The plant cell response to this contact by developing a pre-penetration apparatus (PPA). The PPA is a subcellular membrane structure that predetermines the fungal growth pathway through the plant cell and seems to form a tunnel (Bonfante & Genre, 2008; Parniske, 2008). However, the signals that initiate the development of PPA are still unknown.

The fungus subsequently enters root cortical cells and branches to form an appressorium, also called hyphopodium. Two structural types of colonization may develop: the Arum-type, in which hyphae grow intercellularly and form intracellular arbuscules, and the Paris-type, which exhibits intracellular growth and forms hyphal coil cells from which small arbuscules can originate (Smith & Smith, 1997).

Current studies have shown that there also exists several intermediate colonization forms exist (Dickson, 2004). It is however still uncertain which factors influence the colonization type. With the exception of species from the genera Scutellospora and Gigaspora, all endomycorrhizal fungi that form arbuscules will later also form terminal or intercalary ellipsoid vesicles in the root apoplast (Hause & Fester, 2005).

Following the colonization of roots, extraradical hyphae are produced that grow out into the soil in search of mineral nutrients and they can also inhabit other available roots. In the soil, the AMF hyphal network may also connect plants of different species as a result of lack of host specificity (Goltapeh *et al*., 2008; Requena *et al*., 2007). New spores, called secondary spores, are typically synthesized outside of the plant root at the leading tip of individual fungal hyphae (Dalpé *et al*., 2005). The fungal life cycle is completed after formation of asexual spores on the external mycelium.

1.3 The mycorrhizal symbiosis

Glomeromycota fungi live in a permanent relationship with roots of the majority of all plants i.e. Angiosperms, Gymnosperms, Pteridophytes and some Bryophytes. AMF are able to establish a symbiotic relationship with at least 70 - 90% of known land plant species (Smith & Read, 2008). They play a crucial role in agricultural systems by increasing plant tolerance to abiotic and biotic stresses (Kiers *et al*., 2011). They increase plant growth (Smith & Read, 1997), improve salt and drought tolerance (Evelin *et al*., 2009; Marulanda *et al*., 2006, 2007; Smith & Read, 2008), increase the tolerance of host plant to pathogens (Hata *et al*., 2010; Liu *et al*., 2007; Newsham *et al*., 1994) and potentially improve heavy metal tolerance (Ricken & Hofner, 1996; Zhang *et al*., 2005). The symbiosis also plays a role in nutrient cycling in soil, in ecosystem productivity, and plant variety (Koch *et al*., 2006).

Zhang *et al*. (2005) proved that the presence of AMF increased the tolerance of rice plant to heavy metals in soil. In case of AMF colonized plants, the transport of lead (Pb) and cadmium (Cd) from plant roots to shoots is lower than in nonmycorrhizal controls. The mechanism of this effect is not yet known but there has been a suggestion, that AMF hyphae act as a filter by sequestering

heavy metals around the roots and possibly also within the root cortex (Joner & Leyval, 2008).

Experiments done by Liu *et al*. (2007) supported the hypothesis that AMF induce in the plant a functional defence response to pathogens. *Medicago truncatula* mycorrhized plants showed increased resistance to the virulent bacterial pathogen *Xanthomonas campestris*.

AMF develop an extraradical hyphal network with the plant root system which makes a significant improvement to the rhizosphere soil structure (Rillig & Mummey, 2006). Moreover, AMF can excrete glomalin, a hydrophobic glycoprotein into the soil. Glomalin has a positive influence on soil structure by increasing aggregate stability of soil, which correlates linearly with the amount of detected glomalin (Wright & Upadhayaya, 1998). Additionally, soil stability improves water filtration, aeration and the resistance to wind and water erosion.

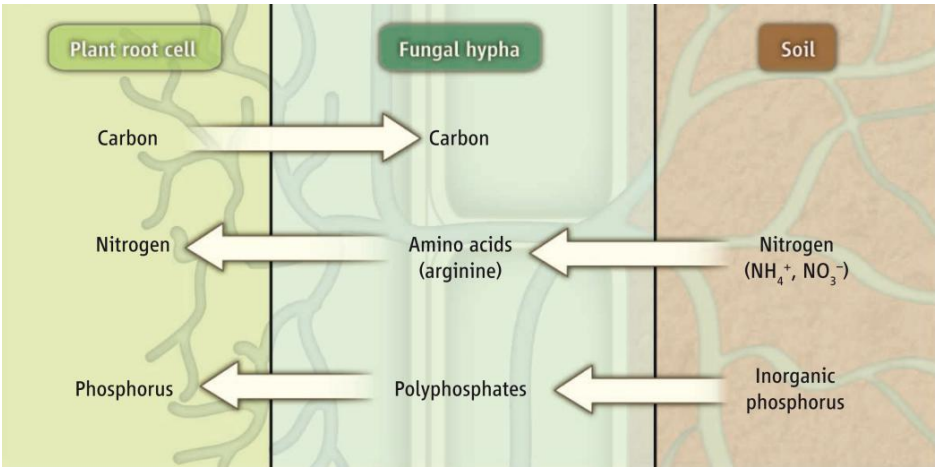

Figure 6 The benefits of plant-fungal symbiosis are stabilized by the constant of mutual nutrient supply (Selosse & Rousset, 2011).

The major benefit for plants when being mycorrhized, is an increase in plant nutrient uptake from the soil (Fig. 6). Inorganic and organic nutrients are absorbed by extraradical hyphae from the soil through specific transporters of phosphate (Bücking & Shachar-Hill, 2005; Maldonado-Mendoza *et al*., 2001), ammonium (Lopez-Pedrosa *et al*., 2006), nitrogen (Govindarajulu *et al*., 2005), amino acids (Cappellazzo *et al*., 2008), zinc (Gonzalez-Guerrero *et al*., 2005), and copper (Harrison, 1999). All of these are subsequently moved to the plant roots.

In soil, inorganic phosphorous is precipitated mostly in insoluble forms such as Al-, Ca-, and Fe-phosphates (Smith & Read, 2008) or organic forms such as inositol phosphates (phytate), phospholipids or nucleic acids (Smith & Read, 2008). The absorbed phosphate is then converted to polyphosphate and transported to intraradical hyphae and arbuscules were it is hydrolyzed to orthophosphate and moved to the host root (Bücking & Shachar-Hill, 2005).

After uptake of different nitrogen (N) forms by the AMF and N assimilation in the fungal hyphae, N is transported into the plant mainly in the form of arginine. Within the intraradical hyphae, nitrogen is released from arginine as urea and either transported to the plant immediately or decomposed to ammonium through the urea cycle (Govindarajulu *et al*., 2005; Jin *et al*., 2005). The contribution N uptake by AMF to plant is unclear. Some studies propose, that up to 30 - 42% of total plant nitrogen can be taken up by AMF (Govindarajulu *et al.*, 2005), whereas Reynolds *et al.* (2005) illustrated no increase of nitrogen uptake for some perennial plants.

AMF can increase copper uptake in different plant species, even up to 62% in case of white clover (Li *et al*., 1991). Zinc can be transported by hyphae to the plants over a distance of 14 cm (Jansa *et al.*, 2003).

In return to all of the benefits given to plants, AMF obtain photoassimilates. Up to 20% of plant assimilated carbon (C) can be transferred to AMF (Jakobsen & Rosendahl, 1990) (Fig. 6). AMF completely depend on the host plant for their carbon sources, since they are unable to take up carbohydrates. Sucrose, which is the most common photosynthate transported from shoots to hyphae, is cleaved either by sucrose synthases (Hohnjec *et al*., 2003) or invertases (Schaarschmidt *et al*., 2006). The resulting hexoses are imported into AMF through transport across the arbuscules fungal plasma membrane and subsequently converted, within the fungal cytoplasm, to lipids and glycogen for long-distance translocation and storage within the fungus (Bago *et al.,* 2002, 2003).

1.4 AMF species concept

Species determination in case of arbuscular mycorrhizal fungi is problematic which results from the fact that only a few morphological characters can be used for their identification. So far, characterization was mostly based on the formation

of dimorphic spores, spore morphology and development, the presence of hyphae structures, such as arbuscules or vesicles and reaction to Melzer's reagent (Bianciotto & Bonfante, 1999; Morton & Benny, 1990). To date about 200 AMF species have been described in this way (www.amf-phylogeny.com). However, morphological identification of spores can sometimes be misleading caused by the poor quality spore reference material from pot cultures and fact that single species can develop dimorphic spores, which can results in species being placing it in two different taxa (Redecker, 2000).

In vitro root organ cultures offer more appropriate material for species description. Monoxenic spore production provides the possibility to amplify for the first time ribosomal DNA from AMF, ensuring that the sequences were not from prokaryotes, other soil eukaryotes or fungi other than AMF present in pot system (Fortin *et al*., 2002). This growth system enabled it to be demonstrated that the model AM fungus, *G. intraradices* DAOM197198, through molecular analysis of its rDNA region, in fact belongs to a separate species, named *G. irregulare* (*Glomus* sp. 'irregulare-like' DAOM 197198) (Stockinger *et al*., 2009). Previously, however, only a small part of AMF species determination was confirmed by molecular data.

1.5 rDNA sequences - AMF molecular identification

The molecular species determination for AMF is mostly based on ribosomal DNA sequences such as from the small subunit (SSU), internal transcribed spacer (ITS) region or the large subunit (LSU). SSU and LSU are coding for 18S and 28S rRNA, which are the components of eukaryotic cytoplasmic ribosomes. Recently, a few other markers have been established for this purpose such as mitochondrial LSU (mtLSU; Raab *et al*., 2005), tubulin genes (Msiska & Morton, 2009) and microsatellites (Croll *et al*., 2008).

The internal transcribed spacer region (ITS) is the part of rDNA sequence which is most often used for species identification. The ITS region contains two variable non-coding sequences that are nested between the highly conserved small subunit (SSU), the 5.8S region, and the large subunit (LSU) of rDNA. The high copy number and small size (approx. 150 - 300 bp each) makes the ITS region easy to amplify by PCR and useful for determining evolutionary relationships, probably because of a relatively low evolutionary pressure acting

on such non-functional sequences (Baldwin *et al*., 1995). In contrast to many other fungi, in which rDNA sequences are similar in genotype of one species, in AMF there is a high polymorphism.

For most fungi, the rDNA sequence including the SSU - ITS – LSU region has become the typical marker for species identification. Recently, it has been considered to be a *de-facto* barcode for fungi (Seifert, 2009). A DNA barcode is described as a short and easily PCR-amplifiable DNA fragment used for species determination. The purpose of such barcode is to create an appropriate and simple system for species recognition in all eukaryotic kingdoms. The first official barcode, accepted by the Consortium for the Barcode of Life (CBOL) is composed of the *cytochrome oxidase subunit I* (*COX1*) in the mitochondrial DNA. It is mainly used for animal species identification (http://www.barcoding.si.edu/). In case of fungi there is a disadvantage when using this gene. Significant length variations were found within sequences of different fungal strains, between 1584 bp up to 22000 bp (Seifert, 2009). From the estimated 1.5 - 3.5 million fungal species, about 100.000 have been described (Kirk *et al.*, 2008). At present, there is no official fungal DNA barcode, although there are several groups dealing with this challenge (Krüger *et al*., 2009; Stockinger *et al*., 2010).

So far, several primers combinations for the amplification of different Glomeromycotan rDNA sequences have been published to allow species determination (Fig. 7) (Redecker *et al.,* 1997; Stockinger *et al.*, 2009, 2011; Tehler *et al*., 2000).

At first, the SSU rDNA region was analyzed for species differentiation of AMF. Unfortunately, it was shown that analyses of this region do not allow resolution of closely related species (Walker *et al*., 2007). For instance, two strongly related AMF species, *Glomus caledonium* and *Glomus geosporum*, show a difference of only 2 - 3 bp in this sequence region (Rosendahl, 2008). The phylogenetic analysis based on the SSU rDNA region of fungal species within Ascomycota and Zygomycota phylum showed only 52% success rate for species identification (Molitor *et al*., 2009).

The most common part of rDNA sequence used for Glomeromycota species identification is ITS region. It can be used to distinguish most of AMF species including the separation of AMF species with very similar spore morphology

(Lanfranco *et al*., 2001). Unfortunately, it was shown that ITS region alone is not sufficient to separate closely relates species, for example within a GlGrAa and GlGrAb (Krüger *et al*., 2009; Stockinger *et al*., 2009, 2010).

Another widely used part of DNA sequence for AMF species determination is the 5'-region of the LSU (Pivato *et al*., 2007). Using an approx. 800 bp LSU region resulted in more resolved species than using the ITS region. But classification for some members of the Gigasporaceae and GlGrAa was still not successful (Stockinger *et al*., 2010).

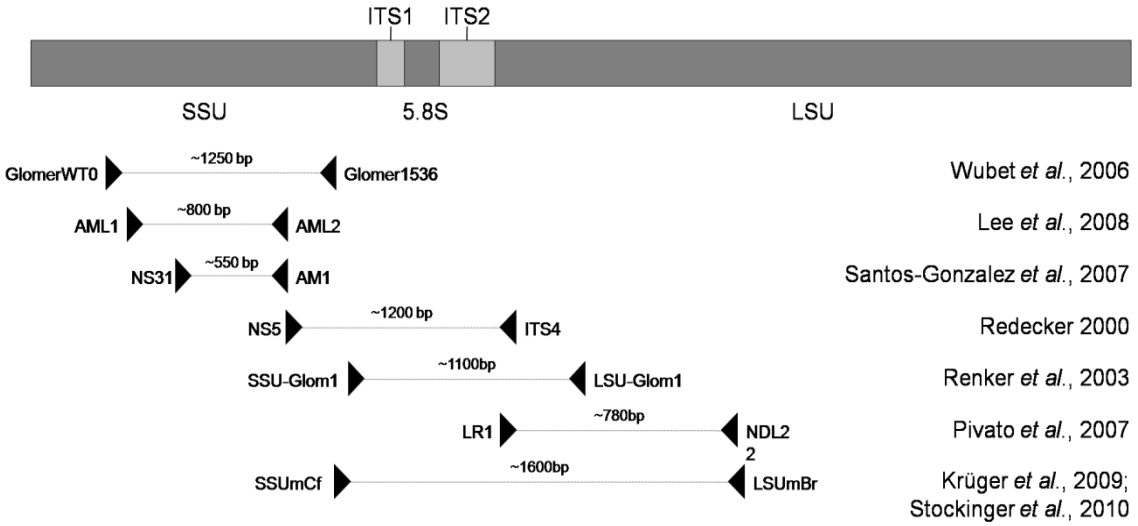

Figure 7 Schematic overview of rDNA primer pairs used for species determination in AMF research citing the corresponding publications (arrow heads indicate to primers, dotted lines to amplified rDNA fragments).

Stockinger *et al*. (2010) suggested that the rDNA fragment which allows separation of all Glomeromycota species should include partial SSU, the complete ITS region and partial LSU sequences. This could allow identification of all closely related species in GlGrAb. This fragment fulfils the requirement of a DNA barcode by having high differentiation at the species level. Nevertheless, it would still not be as straightforward as barcoding in animals.

1.6 *Fumarate reductase* for species determination

Criticism has been made of using ITS region of the rDNA sequence for barcoding (Nilsson *et al*., 2008). To efficiently use ITS region for fungal barcode there are many prerequisites such as the wide taxonomic knowledge specific for each group of fungi, a large number of suitable samples from many populations

and geographic regions and creating a more tailored database for this purpose. It is then necessary to develop other systems for the identification to species level of Glomeromycotan fungi. Kuhn *et al*., (2001) showed high genetic diversity in AMF single copy gene encoding binding protein (*BiP*) which could be used for species determination. Unfortunately, the length of the sequence (approx. 300 bp) results in that the gene not meeting the conditions for barcoding. There is still a need to find a single molecular marker for species-level resolution in AMF. A focus of this study was a gene encoding fumarate reductase enzyme for use as a potential candidate for species determination.

Fumarate reductase irreversibly catalyses the reduction of fumarate to succinate, with fumarate being used as an electron sink in the absence of oxygen. Maintaining the redox balance is a crucial challenge for many organisms in hypoxic or even anoxic conditions. To achieve this aim, the cell has to oxidize reduced cofactors that are generated during substrate catabolism, using metabolic pathways that function in the absence of oxygen. For example, reoxidation of the cytosolic NADH created during glycolysis by glycerol-3-phosphate dehydrogenase (Gpd2) and alcohol dehydrogenase (Adh3) may serve as redox sink in many microorganisms, such as *S. cerevisiae* (Albertyn *et al*., 1994; Ansell *et al*., 1997; Bakker *et al*., 2000). In anaerobic respiration, an alternative respiratory terminal electron acceptor is required, such as nitrate, nitrite, fumarate, sulphite and carbon dioxide (Tielens & van Hellemond, 1998). Fumarate reductase is a key enzyme in fumarate respiration in both anaerobic and facultative anaerobic organisms such as the bacteria *Wolinella succinogenes*, *E. coli* and the yeast *S. cerevisiae* (Arikawa *et al*., 1998; Cecchini *et al*., 2002; Enomoto *et al*., 1996, 2002; Lauterbach *et al*., 1990; Lemire BD, 1986). This enzyme irreversibly catalyzes the reduction of fumarate to succinate and requires $FADH_2$, $FMNH_2$ or reduced riboflavin as electron donors. Several publications showed that anaerobic growth inhibition is caused by deficiency in the capacity to regenerate the intracellular pool of FAD^+, rather than an inability to regenerate NAD^+ (Arikawa *et al*., 1998; Camarasa *et al*., 2007, Enomoto *et al*., 2002).

Fumarate reductases can be divided into two classes, depending on the electron transfer mechanism. One class consists of the membrane-bound enzymes which are covalently linked to flavin cofactors (FAD or FMN).

They transfer electrons from quinol to fumarate and are involved in the production of ATP by oxidative phosphorylation in anaerobic bacteria (Dickie & Weiner, 1979) and lower eukaryotes (Tielens & van Hellemond, 1998). The second class of fumarate reductases has been identified in only two organisms; the yeast *S. cerevisiae* and the protozoan *Trypanosoma brucei* (Besteiro *et al*., 2002). In contrast to the first class, these enzymes are soluble and can catalyze the reduction of fumarate independent of the electron transport chain (Camarase *et al*., 2007).

In *S. cerevisiae*, two members of soluble fumarate reductase exist, one with mitochondrial and another with cytosolic localization, encoded by the *OSM1* and *FRDS1* genes, respectively (Enomoto *et al*., 1996, Muratsubaki & Enomoto, 1998). Camarasa *et al*. (2007) demonstrated the potential role of Frds1p as the main fumarate reductase in the regeneration of the FAD-prosthetic group of essential flavoproteins under anaerobic conditions. It can be functionally replaced by Osm1p, which contributes less than the cytoplasmic form to the total fumarate reductase activity.

Genomic sequencing projects have enabled the identification of homologues of *S. cerevisiae* Frds1p in other fungal species, such as *Laccaria bicolor*, *Neurospora crassa* and *Coprinus cinereus* (Galagan *et al*., 2003; Martin *et al*., 2008a; Stajich *et al*., 2010) but this class of enzymes has not been investigated in AMF.

AMF have to maintain the redox balance under the oxygen deficiency conditions which occur in the soil. Although atmospheric oxygen level is 20.9% of air, average oxygen concentration in a clay soil is below 10% (Smith & Dowdell, 2006). However, none of the genes, characterized in other organisms that are involved in redox balance preservation during absence of oxygen, have yet been identified in AMF. Knowledge of the AMF redox homeostasis system will be crucial for understanding influence of symbiosis with plants on fungi response to oxygen deficiency and their ability to maintain redox balance.

1.7 Genetics of Glomeromycota

Despite the obvious significance of AMF fungi in ecosystems, only a very few studies on AMF genetics have been performed. Basic information is lacking, such us a genome sequence, ploidy level and number of chromosomes

andthe origin of genetic material in spores. Currently it is unclear whether genetic exchange, recombination and segregation actually occur in AMF. However, this knowledge is crucial for understanding of basic genetics of these fungi.

There are number of reasons why so little is known about AMF genetics. They have never been grown successfully in the absence of host plants, so it's difficult to obtain clean and unlimited material for studies. Also, the generation time of 10 - 12 weeks for the fastest growing species, makes genetic experiments, involving several generations, time consuming. For many years, the only way to culture AMF was a pot system and even though sterilized soil and plant seedlings have been used, this system has a high risk of contamination with bacteria or fungi other than AMF (Hijri *et al*., 2002). Nevertheless, some basic investigations on AMF have been performed.

Hosny *el al.* (1997) estimated the average GC content in 10 Glomeromycota species to be approx. 30 - 35%. There are a few reports where the nuclear DNA content of AMF was determined using of flow cytometry (FC). Hosny *el al.* (1998) examined 11 Glomeromycota species belonging to four different genera. The nuclear DNA content seems to vary enormously from approx. 15 Mb in *G. irregulare* DAOM197198 (Hijri & Sanders, 2004) (previously *G. intraradices*, Stockinger *et al*., 2009) and 37 Mb in *Glomus etunicatum* (Hijri & Sanders, 2005), up to 250 Mb in *G. versiforme* (Bianciotto & Bonfante, 1992) and 1058 Mb in *Scutellospora gregaria* (Hosny *et al.,* 1998). However, some of those measurements may be imprecise because of the use of unsuitable or external standards. Also, there is only one report concerning the number of chromosomes for Glomeromycota species (*G. irregulare* DAOM197198; Hijri *et al*., 2007). Unfortunately, the maximum chromosome number could not be determined precisely in that study, however, the results estimated that there are at least four chromosomes.

G. irregulare DAOM197198 was also chosen for the first complete genome sequencing project on arbuscular mycorrhizal fungi. Although, the project started in 2004 the genome sequence has not yet been completed (Martin *et al*., 2008b; pers. comm.). Even when large amounts of sequencing data exist, there are difficulties in assembly caused by a high degree of polymorphism among the nuclei.

Many studies have shown high variation in AMF sequence, even within individual spores and single spore monoxenic cultures. Most of the studies confirmed the AMF polymorphism in the rDNA sequence (Börstler *et al*., 2008; Jansa *et al*., 2002; Lanfranco *et al*., 1999; Pawlowska & Taylor, 2004; Sanders, 1995) but there are also a few studies that show variations in genes encoding β-tubulin or ATPase (Corradi *et al*., 2004; Kuhn *et al*., 2001). Restriction fragment length polymorphisms (RFLP) on DNA fragments or cloning and sequencing of DNA have been used to look at the variation in AMF genome sequence (Sanders, 1995).

After the reports on AMF genetic variation, Sanders *et al*. (1996) suggest that this effect could occur by non purged mutations leads to genetically different nuclei in absence of recombination. The continuation of such state is probable by many nuclei moves into one spore on to the next generation. Unfortunately, it is not clear yet how nuclei transport is organized. Initially, two contrasting nuclei organization pattern were proposed (Fig. 8).

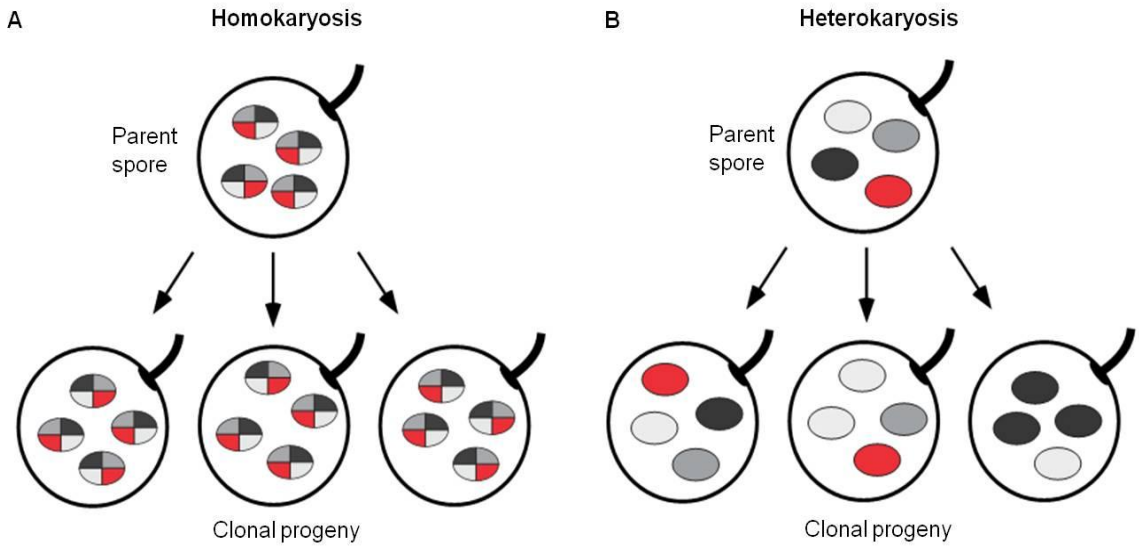

Figure 8 Models explaining how the nuclei genetic variation is organized in a single spore culture of *Glomus* (Pawlowska & Taylor, 2004).

One model, heterokaryosis, assumes that the genetic variation can be distributed between nuclei, with each cell containing multiple genomes (Fig. 8B) Kuhn *et al*. (2001) used fluorescence *in situ* hybridization (FISH) to show that two highly divergent internal transcribed spacer (ITS2) rDNA variants in *Scutelospora castanea* were segregated into different nuclei, and some nuclei contained both variants. The second model assumed AMF to be homokaryotic (Fig. 8A)

(Pawlowska & Taylor, 2004; Pawlowska, 2007). The ITS1 rDNA region from six individual nuclei of *Glomus etunicatum* representing three separate spores obtained by microdissection was sequences. Each of them contained all three previously detected ITS1 variants providing evidence for the homokaryosis model of genetic variation. This evidence supporting the second explanation of AMF genomic organization was much more convincing.

The multigenomic state observed in AMF was also explained by randomly accumulated mutations in functional genes (Sanders *et al*., 1996). This could lead to a partial lost of genome information in individual nuclei. Therefore, the single spores want to a complete functional genome by the presence of co-existing nuclei carrying partitioned genetic information with many nuclei entering a newly forming spore.

Because of having potentially many “backups” of each gene, AMF have fewer problems with deleterious mutations than other organisms. Based on this variability, AMF may increase their ecological diversity by adapting to a variety of environmental conditions. However, being multigenomic can also be a disadvantage. Replication of nuclei with lost functionality is unnecessary and energy consuming may cause problems for AMF fitness.

AMF are part of the most wide-spread symbiotic relationship on the Earth. Understanding the genetic processes of their reproduction is crucial for a number of reasons: i) to find mechanisms involved in AMF interactions with plants, ii) to verify the ancient asexual status of Glomeromycota and, iii) to develop high quality commercial mycorrhizal spores products.

2. AIM OF THE THESIS

Even though, AMF play an important role in ecosystems, little is known about their basic genetics. Therefore, this thesis aimed at the establishment of molecular methods for the characterization and species determination of the industrial *Glomus* sp. AMykor isolate and model AM fungus, *G. irregulare* DAOM197198.

The first aim of this work was to determine species affiliation of analyzed AM fungus and show the variation in AMF genome sequences within a single spore culture (one isolate) and within and among nuclei. Analysis of rDNA molecular variance was performed to investigate patterns of genetic variation in the single *G. irregulare* isolate. Additionally, the genome size and ploidy level was examined. Knowledge of the genome size of *G. irregulare* is useful for genome sequencing projects, allowing more accurate and faster recognition of genes involved in plant-fungal symbiosis, which may improve the use of this relationship in agriculture and industry.

The second aim of this thesis was focused on development of an identification system to species level of Glomeromycotan fungi based on single copy gene. Since rDNA sequence is not ideal for barcode, a better option is to base species determination on single copy gene, as in case of animals. It is indicated to develop other system for the identification to species level of Glomeromycotan fungi. Sequencing of the gene encoding fumarate reductase in *G. irregulare* was used as a potential candidate for species determination in AMF. Additionally, functional characterization of protein encoded by this gene was performed in heterologous expression system.

3. MATERIALS AND METHODS

3.1 Microorganisms

Table 1 Microorganisms and their genetic markers.

Line	*Genotype*	*Origin*
	Escherichia coli	
TOP10	F-mcrAΔ(mrr-hsdRMS-mcrBC)'80lacZ ΔM15ΔlacX74recA1araD139Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen- TOPO TA Cloning Kit for Sequencing
XL1 Blue	*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15 Amy Cm^R] hsdR17(r_K^- m_K^+)*	Stratagene
	Sacharomyces cerevisiae	
Y06849	Mat a, *his3D1; leu2D0; lys2D0; ura3D0; osm1::KanMX4*	Euroscarf
Y10288	Mat α, *met15D0; leu2D0; lys2D0; ura3D0; frds1::KanMX4*	Euroscarf
S0010	*his3D1; leu2D0; lys2D0; met15D0; ura3D0, osm1::kanMX4; frds1::kanMX4*	Created in our lab
Y15271	Mat a; *his3D1; leu2D0; lys2D0; ura3D0; YLR362w::kanMX4*	Euroscarf
S288c	Matα *SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6*	Euroscarf
	AMF cultures	
G. irregulare	AMykor 04Z	Germany, AMykor GmbH
G. irregulare	DAOM197198, Att690-23	Canadian Isolate
G. mosseae	L90	Germany, AMykor GmbH
	Plants and plant roots	
Daucus carota	Breeding line (500)	Germany, Gatersleben, IPK GeneBank AC: DAU 160
Arabidopsis thaliana	(L.) Heynh 'Col-0'	Germany, Gatersleben
Raphanus sativus	L. cv. 'Voran'	Germany, Gatersleben, IPK GeneBank AC: RA 34
Glycine max	L. cv. 'Idefix'	University of Vienna, Austria

3.2 Culture media

3.2.1 Bacteria media

Medium	Components	Concentration
Luria-Bertani (LB) pH 7.0	Tryptone	1%
	Yeast extract	0.5%
	Sodium chloride	1%
LB plates	Bacto-agar	2%
Dissolved in aqua dest., autoclaved, stored at 4°C not longer than 3 months		
SOB pH 7.5 adjust with HCl	Tryptone	2%
	Yeast extract	0.5%
	Sodium chloride	10 mM
	Potassium chloride	2.5 mM
	Magnesium sulfate (anhydrous)	10 mM
Dissolved in aqua dest., autoclaved, stored at 4°C not longer than 3 months		
SOC	SOB	
	Glucose	20 mM

3.2.2 Yeast media

SD Medium (minimal medium)	Yeast nitrogen base	0.67%
	Glucose / Galactose	1% / 2%
	Ammonium sulfate	0.5%
	Vitamin mix	0.5%
	Amino acids or nucleotides	0.5%
SD plates	Bacto-agar	2%
Dissolved in aqua dest., autoclaved, stored at 4°C not longer than 3 months		
Vitamin mix	Thiamine hydrochloride	1.3 mM
	Inositol	22.2 mM
	Niacin (Vit. B3)	0.81 mM
	Pantothenic acid	1.82 mM
	Pyridoxine	2.36 mM
	Biotin	0.016 mM
Dissolved in aqua dest., filtered sterile (0,1 µm), stored at 4°C not longer than 3 months		

YPD Medium (non-selective medium) pH 5,8 adjust with HCl	Yeast extract	1%
	Peptone	2%
	Glucose	1%
YPD plates	Bacto-agar	2%
Dissolved in aqua dest., autoclaved, stored at 4°C not longer than 3 months		
Sporulation Medium Plates McClary	Potassium acetate	1%
	Yeast extract	0.25%
	Glucose	0.1%
	Agar	2%
Dissolved in aqua dest., autoclaved, stored at 4°C not longer than 3 months		

3.2.3 Fungi medium

MSR (modified Strullu - Romand medium)	The composition of the medium and all solutions are documented in the book "*In Vitro* Culture of Mycorrhizas", Soil Biology, Vol.4 ed. by S. Declerck *et al*. (2005)

3.3 Plasmids and vectors

Table 2 List of plasmids/vectors used in this work.

Plasmid/Vector	Fenotype
pCR4-TOPO	Includes: promoter *lacI,* bacterial resistance Amp^R & Kan^R
pYES2	Includes: promoter *GAL1*, protein tags His (N&C), Xpress (N), V5 (C), bacterial resistance Amp^R, mammalian selection *URA3*

3.4 Oligonucleotides

Table 3 List of oligonucleotides used for PCR reactions.

Name	Sequence	Specification
NS5 (fwd)	5´-AAC TTA AAG GAA TTA CGG AA-3´	rDNA region
ITS4 (rev)	5´-TCC TCC GCT TAT TGA TAT GC-3´	rDNA region

Glom1310i (fwd)	5´-AGC TAG GCC TAA CAT TGT TA-3’	rDNA region
ITS4i (rev)	5´-TTG ATA TGC TTA AGT TCA GCG-3‘	rDNA region
SSUmAforw	5’-TGG GTA ATC TTT TGA AAC TTC-3’	rDNA region
LSUmArev	5’-TGC TCT TAC TCA AAT CTA TCA-3’	rDNA region
M13fwd	5’-GTA AAA CGA CGG CCA G-3’	sequencing
M13rev	5’-CAG GAA ACA GCT ATG AC-3’	sequencing
FRD1 (rev)	5’-CCA GTC GGA TGT ACT TGA AC-3’	*GiFRD* genome walking
FRD2 (rev)	5’-CGA AGT GCT TCA CCG GCC AAA A-3’	*GiFRD* genome walking
FRD3 (fwd)	5’-ATC GTC TTG GTG GAT CAT CT-3’	*GiFRD* genome walking
FRD4 (fwd)	5`-GGT GGT ATT TCT GTC ACA AAA-3´	*GiFRD* genome walking
FR_Not (rev)	5’-TTT TGT GAC AGA AAT ACC ACC C-3’	*GiFRD* amplification
FR_Eco (fwd)	5’-GAA TTC ATG GCT AGT CAA ATT ATT-3’	*GiFRD* amplification
GiFRD_EcoRI (fwd)	5’-GAA TTC GGT GAA GAA GTT ATT GGT GTA G -3’	Species determination
GiFRD_SalI (rev)	5’-GTC GAC ATC TTT TAC TTC AGA ATC AGG -3’	Species determination
RT_GiFRD_fwd	5’-GAA TTC GGT GAA GAA GTT ATT GGT GTA G-3’	RT-PCR (*GiFRD*)
RT_GiFRD_rev	5’-GTC GAC ATC TTT TAC TTC AGA ATC AGG-3’	RT-PCR (*GiFRD*)
RT_Tub_fwd	5’-TAC CAT GGA CTC CGT TCG T-3’	RT-PCR (*β-tubulin*)
RT_Tub_rev	5’-GAC GTG GAA AAG GCA CCA TA-3’	RT-PCR (*β-tubulin*)

RT_TEF_fwd	5'-GCC CTT ACT TGA TTT ACA AGT-3'	RT-PCR (*TEF*)
RT_TEF_rev	5'-TTG ACC GTC CTT GGA GAT A-3'	RT-PCR (*TEF*)
FRDS1_delet_fwd	5'-ATG TCT CTC TCT CCC GTT GTT GTT-3'	*S. cerevisiae FRDS1*
FRDS1_delet_rev	5'-TTA CTT GCG GTC ATT GGC AAT AG-3'	*S. cerevisiae FRDS1*
OSM1_delet_fwd	5'-ATG ATT AGA TCT GTG AGA AGG GTT TT-3'	*S. cerevisiae OSM1*
OSM1_delet_rev	5'-TCA GTA CAA TTT TGC TAT GTT ATC CG-3'	*S. cerevisiae OSM1*
Hind_GiFRD (fwd)	5'-AAG CTT ATG GCT AGT CAA ATT ATT GTC G-3'	*GiFRD* localization
Sac_linker_GiFRD (rev)	5'-CCG CGG **TTC TGG ACC TGG TAG TTC TGG ACC TGG TAG** TTT TGT GAC AGA AAT ACC ACC C-3'	*GiFRD* localization
SacI_GFP_(fwd)	5'-GAG CTC ATG GTG AGC AAG GGC GAG GAG-3'	*GiFRD* localization
NotI_GFP*_(rev)	5'-GCG GCC GCT TACT TGT ACA GCT CGT CCA TGC C-3'	*GiFRD* localization
Underlined letters - restriction enzymes site; **bold type letters** - - linker		

3.5 Enzymes and chemicals

- Restriction and DNA modification enzymes were obtained from the following companies: Restriction endonucleases (Thermo Scientific), AmpliTaq DNA-Polymerase (Applied Biosystems), T4 DNA Ligase (Thermo Scientific).
- Chemicals were obtained from the companies Roth, Sigma-Aldrich, Thermo Scientific, Qiagen, BioRad, Merck, Invitrogen, Serva and Steinheim.

3.6 Methods used for work with DNA

Methods used for work with DNA were base on techniques described in books "Molecular cloning: a laboratory manual" (Sambrook & Russell, 2001) and "Current Protocols in Molecular Biology" (Ausubel, 1991).

3.6.1 DNA fragment transformation to *E. coli* cells

DNA fragment were transformed into *E. coli* cells, using an Invitrogen “TOPO TA Cloning Kit for Sequencing". This kit provides chemically competent *E. coli* cells and a pCR4-TOPO vector. Transformation procedure was carried out as described in the user manual.

3.6.2 Plasmid DNA transformation to *E. coli* cells

10 - 20 µl ligation mixture was added to chemical-competent *E. coli* cells. Subsequently cells were incubated for 30 min on ice. Cells were placed at 42°C for 90 sec and then cooled on ice. 200 µl SOC medium was added and cells were incubated with shaking for 45 min at 37°C. The cells were then spread on selective LB plates containing the appropriate antibiotic and incubated overnight at 37°C.

3.6.3 Mini isolation of plasmid DNA from *E. coli* cells

Single colonies were selected and cultivated overnight in glass tubes with 2 ml liquid LB medium with ampicillin (100 µg ml^{-1}) at 37°C. Selected colonies were cultivated parallel on a “master LB – plate” for conservation. As a first transformation control a “quick test” was performed. 100 µl of *E. coli* cells from each overnight culture, were transferred to a 1.5 ml Eppendorf tube and 50 µl phenol-chloroform-isoamylalcohol (PCI, 25/24/1) and 10 µl DNA loading buffer were added. Tubes were vortexed for 10 sec and centrifuged for 3 min at 12 000 x g. From the upper phase, 15 µl was separated by agarose gel electrophoresis. The empty plasmid was added on the gel as a control. Successfully constructed plasmids should show a larger band (size depend on added DNA fragment) than the empty plasmid. Initially checked positive overnight cultures were transferred to 1.5 ml Eppendorf tubes and centrifuged for 3 min. The supernatant was removed completely. For the following steps three different buffers from “Qiagen” were used:

Buffer P1 - suspension buffer (RNase A included)

Buffer P2 - lysis buffer

Buffer P3 - neutralization buffer

The cell pellet was suspended in 200 µl buffer P1 (cold) by vortexing. Then, 200 µl of buffer P2 (RT) was added and incubated for 3 min in RT. After incubation, 200 µl buffer P3 (cold) was added and the solution was centrifuged for 20 min at 4°C. From the clear supernatant, 500 µl were transferred to a new 1.5 ml Eppendorf tube. The plasmid DNA was precipitated by adding 350 µl isopropanol, mixing and incubating for 2 min at RT. Centrifugation at 4°C for 25 min separated the pellet containing the plasmid DNA. The supernatant was removed and the pellet was washed with 70% ethanol (RT) and again centrifuged at 4°C for 3 min. The ethanol was removed and pellet was dried in a vacuum dryer for 10 min. The dried pellet was suspended in 50 µl 10 mM Tris / HCl pH 7.5. For all centrifugation steps, a force of 12000 x g was applied.

3.6.4 Polymerase Chain Reaction (PCR)

Depending on the further use of the PCR product, the PCR reaction was performed for 25 or 50 µl final volume. The PCR reaction was performed in an Eppendorf Mastercycler Gradient. The volumes of the components of the reaction are presented in Table 4. Standard program and calculation of annealing temperature is shown in Table 5.

Calculation of annealing temperature:

T_m = 64.9°C + 41°C x (number of G's and C's in the primer – 16.4) / N,

where N is the length of the primer (Sambrook & Russell, 2001)

Table 4 Composition of PCR reaction (for 50 µl).

Components	Basic concentration	Volume	Final concentration
Template DNA	5 - 100 ng μl^{-1}	1-2 µl	50 - 1000 pg μl^{-1}
5' primer	5 pM	5 µl	0.5 µM
3' primer	5 pM	5 µl	0.5 µM
Polymerase buffer	10 x	5 µl	1 x
dNTP's	10 mM	1 µl	200 µM
$MgCl_2$	25 mM	4 µl	
AmpliTag polymerase	1 - 5 u μl^{-1}	0.5 µl	0.01 - 0.02 u μl^{-1}
Water		to 50 µl	

Table 5 Standard programme for PCR reaction

	Step	Duration	Temperature
1.	Initial denaturation	5 min	94°C
2.	Denaturation	45 sec	94°C
3.	Annealing	30 sec	T_m°C*
4.	Extension	2 min	72°C
5.	Final extension	10 min	72°C
6.	Holding temperature	∞	4°C
Steps 2 till 4 were repeated 20 - 35 times			

3.6.5 Agarose gel electrophoresis

DNA was separated by agarose gel electrophoresis. Samples with 4 x DNA loading buffer were loaded onto the 0.8% agarose gel containing ethidium bromide (0.5 µg ml^{-1}). Electrophoresis was performed at RT in 1 x TB buffer with an applied voltage 8 - 10 V cm^{-1}. The size of separated DNA fragments was analyzed by comparison with simultaneously added DNA ladder.

4 x DNA loading buffer (100 ml)	Tris / HCl pH 7.6	10 mM
	Glycerol	60%
	EDTA	60 mM
	Bromophenol blue	0.03%
stored at RT not longer than 12 months		
10 x TB buffer	Tris	1 M
	EDTA	25 mM
	Boric acid	0.1 M
Dissolved in with aqua dest., stored at 4°C not longer than 3 months		

3.6.6 DNA fragment extraction from agarose gel

After electrophoresis the desired DNA fragment was cut out with a razor blade under UV light. For DNA extraction from the gel, a gel extraction kit from OLS (OMNI Life science) was used.

3.6.7 DNA restricted digestion

DNA digestion with restriction enzymes was performed as recommended by producer 1 unit of enzyme digests 1 µg of DNA in one hour in temperature optimal for specific enzyme.

3.6.8 DNA ligation

Ligation reaction was performed overnight at 14°C or for 2 - 3 hours at RT. The ratio of vector to insert was 1:5 or 1:3 depending on DNA concentration. 1 unit of T4 DNA ligase (5 u μl^{-1}, Thermo Scientific) was used in final mixture volume 30 µl. After the reaction, the enzyme was inactivated by heating for 15 min at 65°C and the DNA was precipitated by ethanol.

3.6.9 Ethanol precipitation of DNA

The DNA sample was mixed with 5 µl sodium acetate (0.1 M; pH 6.0) and 200 µl 96% cold (4°C) ethanol. After incubation for 1 hour at -20°C the sample was centrifuged for 10 min. Ethanol was removed and 500 µl of 70% cold ethanol were added and centrifuged again for 5 min. Ethanol was removed and 500 µl of 96% cold ethanol were added and centrifuged for 3 min. Ethanol was removed and the sample was additionally centrifuged for 1 min to remove any remaining ethanol. The pellet was dried at RT for 10 min and then dissolved in 20 - 30 µl 10 mM Tris / HCl pH 7.5. All centrifugation steps were conducted at 4°C and at maximum speed of 12000 x g.

3.7 Methods used for work with RNA

3.7.1 RNA isolation

For total RNA isolation the RNeasy Mini Kit from Qiagen was used. The procedure was performed as described by company. A maximum 100 mg wet weight spores and hyphal material was used and RLC precipitation buffer was added.

3.7.2 RNA sample preparation for electrophoresis

The RNA electrophoresis was performed on 1% agarose gel with ethidium bromide (0.5 μg ml^{-1}) in 1 x TB buffer. 1 volume of the RNA sample was mixed with 1 volume of the 2 x RNA loading dye (Thermo Scientific) and heated at 70°C for 10 min. Subsequently samples were chilled on ice for 3 min and loaded onto the gel.

3.7.3 Removal of genomic DNA from RNA preparations

Procedure was performed using Thermo Scientific DNase I, RNase free enzyme. Into the nuclease-free tubes on ice following reagents were added:

RNA	1 μg
10 x DNase I reaction buffer with $MgCl_2$	1 μl
DEPC-treated water	up to 9 μl
DNase I, RNase-free	1 μl

The mixture was incubated for 30 min at 37°C. 1 μl of 25 mM EDTA was then added and the sample was incubated for 10 min at 65°C to stop the reaction.

3.7.4 cDNA synthesis

The cDNA synthesis was performed using the First Strand cDNA Synthesis Kit (Thermo Scientific) and following the method described in the supplemented handbook. First Strand cDNA synthesis was accomplished using random hexamer primers.

3.8 Methods used for work with proteins

3.8.1 Determination of protein concentration

Protein concentration was determined by the method of Bradford (1976), using BioRad Protein Assay Kit and bovine serum albumin (BSA) as a standard.

3.8.2 Protein electrophoresis in SDS-PAGE gel

Proteins were separated by electrophoresis performed on polyacrylamide gel in denaturating conditions according to Laemmli (1970).

The polyacrylamide gel in size of 10 x 10 x 0.1 cm was placed in PerfectBlue Vertical Double Gel System (peQlab) and electrophoresis run at a constant current of 38 mA. Composition of both gels is presented in Table 6.

Table 6 Composition of SDS-PAGE gels.

Composition of separation gel (20ml)	
Compounds	10%
H_2O	7.5 ml
40% Acrylamid mixture	5.0 ml
1 M Tris / HCl, pH 8.8	7.5 ml
10% SDS	200 µl
10% TEMED	200 µl
10% APS	200 µl

Composition of stacking gel (4ml)	
Compounds	5%
H_2O	2.4 ml
40% Acrylamid mixture	0.5 ml
0.5 M Tris / HCl, pH 6.8	1 ml
10% SDS	40 µl
10% TEMED	40 µl
10% APS	40 µl

3.8.3 Protein sample preparation for SDS-PAGE gel

30 µl of protein sample was mixed with 10 µl 4 x SDS-PAGE loading buffer. Sample was boiled for 5 min and chilled on ice.

10 x SDS-PAGE running buffer	Tris / HCl	0.25 M
	SDS	35 mM
	Glycine	2 M
Dissolved in aqua dest., stored at 4°C not longer than 3 months		
4 x SDS-PAGE loading buffer	Tris / HCl pH 6.8	62.5 mM
	glycerol	10%
	SDS	2%
	ß-mercaptoethanol	2.5%
	bromophenol blue	0.01%
Dissolved in aqua dest., stored at -20°C not longer than 3 months		

3.8.4 Staining of SDS-PAGE gel

The SDS-PAGE gel was placed for 30 min in fixation solution and then moved to Coomassie Brilliant Dyes solution and incubated for one hour. The Coomassie solution was renewed once and gel was left overnight in the fridge. The gel was destained for 2 hours in decolourizer solution, enlarged in 5% glycerol solution and dried under vacuum for two hour.

Coomassie Brilliant Dye	Coomassie blue R250 Ethanol 96% Acetic acid	0.05% 50% 10%
filled with aqua dest., stored at RT not longer than 3 months		
Fixation solution	Acetic acid Ethanol 96%	10% 33%
filled with aqua dest., stored at RT not longer than 3 months		
Decolourizer solution	Ethanol 96% Acetic acid	50% 10%
filled with aqua dest., stored at RT not longer than 3 months		

3.8.5 Immunoblotting (Western blot analysis)

The polyvinylidene difluoride membrane (PVDF, Roth) was briefly washed in methanol and transferred to Western Blot transfer buffer together with 6 filter papers (Whatman 3MM, 6.5 cm x 8.5 cm) and 2 pieces of fabric per gel. The gel and membrane were placed between filter papers and fabric pieces and put to “wet” transfer apparatus. The proteins were transfer to nitrocellulose membrane for overnight at a constant current of 100 mA. Afterwards the membrane was washed 2 x 5 min in 1 x PBS buffer and incubated for one hour in blocking solution (1 x PBS, 0.05% Tween 20, 1% milk powder). Subsequently membrane was washed twice in 5 min in 1 x PBS - 0.05% Tween 20 buffer and once in 1 x PBS buffer and placed in first antibody solution (blocking solution) for one hour. The washing steps, twice in 1 x PBS - 0.05% Tween 20 buffer and once in 1 x PBS buffer, were repeated and membrane was placed in secondary antibody for one hour. Washing 4 x 5 min in 1 x PBS - 0.05% Tween 20 buffer was performed. Subsequently the membrane was developed in darkness by reaction with alkaline phosphatase conjugated to secondary antibodies (Western Blue Stabilized Substrate for Alkaline Phosphatase; Promega, Germany).

Western Blot transfer buffer pH 8.3 adjusted with HCl	Tris Glycine Methanol SDS	25 mM 192 mM 10% 0.1%
Dissolved in aqua dest., stored at 4°C not longer than 3 months		

10 x PBS pH 7.4 adjusted with NaOH	NaCl KCl Na_2HPO_4 x $2H_2O$ KH_2PO_4	1.48 M 26.8 mM 42.9 mM 13.9 mM
filled with aqua dest., autoclave, stored at 4°C not longer than 3 months		
Carbonate buffer pH 9.8 adjusted with NaOH	$NaHCO_3$ $MgCl_2$ x $6H_2O$	52.5 mM 0.5 mM
filled with aqua dest., autoclave, stored at 4°C not longer than 3 months		

Antibodies	Dilution	Origin
Anti rabbit IgG	1:3000	Promega
Anti GFP	1:2000	Invitrogen

3.9 Methods used for work with AMF

3.9.1 Cultivation of AMF spores

AMF *in-vitro* monoxenic cultures were established as described by Declerck *et al*. (2005). A carrot (*Daucus carota,* Gatersleben, Germany) Ri-T DNA transformed root organ culture was cultivated with single spore isolate of the AM fungus, *G. irregulare* (AMykor GmbH, Bitterfeld-Wolfen, Germany) (Tab. 1) in three-compartment Petri dishes which contained MRS medium gelled with 0.5% Phytagel. Host root fragment of 3 - 4 cm length was placed on one of the plate's compartment (Fig. 9). Afterwards, the Petri plates were sealed with plastic wrap and incubate at 26°C in a dark chamber. After three to four days, the freshly developed root tips (3 - 4) were inoculated with 20 - 30 sterile spores. The plates were again sealed with foil and stored in the dark at the optimal temperature for mycorrhizal development of 26°C. Spores were allowed to establish symbiosis and develop secondary spores between three and six months. Fungal hyphae, but not roots, were allowed to grow over the plastic barrier to the other compartments. The same system was used to culture *G. irregulare* DAOM197198 (Att1192-44) isolate (Sanders I. – Univ. Lausanne/Switzerland).

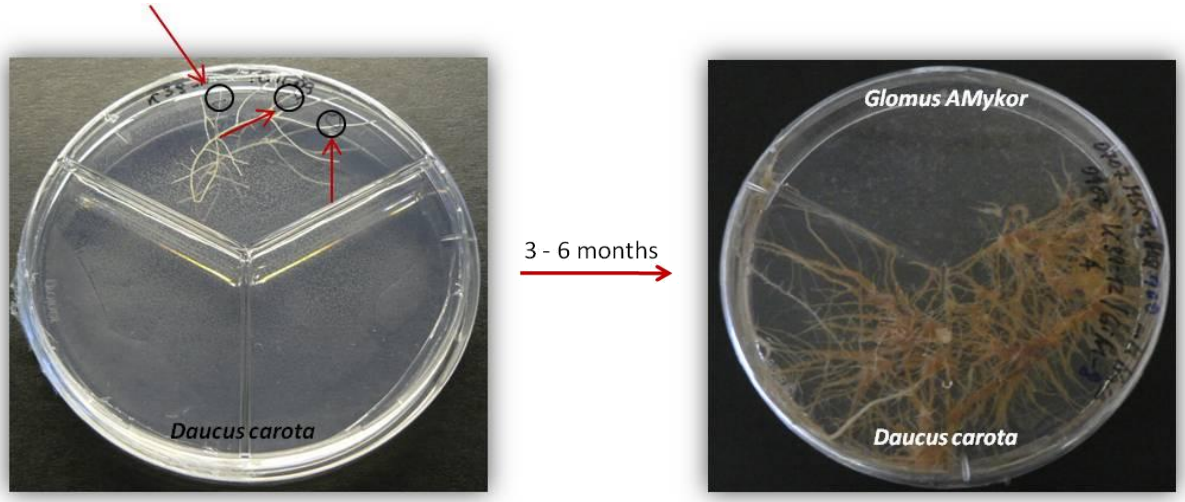

Figure 9 AMF *in vitro* culture on MRS medium three-compartment plates.

3.9.2 Extraradical mycelia recovery

Extraradical mycelia and secondary spores were recovered from the root-free plate compartments by dissolving the culture medium in sterile 10 mM citrate buffer (Declerck *et al*., 2005). The fungal material was collected with a pipette tip under sterile conditions (Bago *et al.*, 1999) and stored at -20°C or -80°C until used.

10 mM Citric buffer pH 6.0 adjusted with NaOH	100 mM sodium citrate 100 mM citric acid Aqua dest.	82 ml 18 ml 900 ml
autoclaved, stored at RT		

3.9.3 DNA extraction from AMF single spores

DNA from a single spore was obtained by crushing a spore with a pipette tip in 10 µl water. Then 20 µl 20% Chelex-Resin solution was added (Bio-Rad, Hercules, CA; solved in water) and mixture was boiled for 1 min at 95°C, incubated on ice for 5 min and then centrifuged. 2 µl of this dissolved DNA was used for the PCR reaction (Jansa *et al*., 2002).

3.9.4 DNA extraction from a group of AMF spores

DNA was extracted from 100 mg of spores and hyphae using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

3.9.5 Phylogenetic analysis based on AMF rDNA sequences

After sequencing, the forward and reverse sequences from each template (clones) were manually edited and combined in single consensus sequences using CHROMASPRO 1.41. Alignment of the sequences obtained from AMykor and DAOM197198 isolates and sequences downloaded from NCBI (Tab. 8) was done by using ClustalX 2.0.4 (Larkin *et al.*, 2007) and controlled manually. *Glomus proliferum* MUCL41827 was defined as an out-group in all of these analyses.

MODELTEST 3.7 (Posada & Crandall, 1998) was used to test different models of sequence evolution. The GTR + I + Γ model was chosen as the best fitting using the Akaike information criterion (Akaike, 1974). Pairwise genetic distances were calculated in PAUP* v4.0b10 (Swofford, 2002) on the basis of the GTR + I + Γ model and the resulting distance matrix was analyzed with the neighbour joining (NJ) algorithm. Branch support (bs in %) was tested with 1000 bootstrap resamples (NJ). Bayesian phylogenetic analyses were performed in MRBAYES 3.1.2p (Hülsenbeck & Ronquist, 2001). For Bayesian inference (BI), two simultaneous runs were done, each with eight chains for 12 x 10^6 generations under the GTR + I + Γ model for sequence evolution with a temperature of 0.1 and tree sampling every 1000 generations. The initial 25% of generated trees were discarded as burn-in and posterior probabilities (pp) were calculated from the remaining 18002 trees. Results were visualized using FigTree v1.3.1 (Shapiro *et al*., 2006)

3.9.6 Flow cytometry

Freshly collected spores and hyphae of both *Glomus* isolates (AMykor and DAOM197198) were fixed with 4% formaldehyde in 10 mM Tris buffer (pH 7.5) for 10 min on ice, washed twice in 10 mM Tris buffer and collected by centrifugation at 4˚C for 3 min at 3000 x g. Nuclei were released by crushing the fungi material with a micro-pestle in 1 ml 10 mM Tris buffer supplemented with 50 µg ml^{-1} propidium iodide (PI) and 50 µg ml^{-1} DNase free RNase. The suspensions were filtered through a 30 µm mesh (CellTrics, PARTEC, Görlitz, Germany) and subsequently mixed with nuclei suspensions from one of the reference standards, either *A. thaliana* (L.) Heynh 'Col-0' (0.32 pg / 2C; Bennett *et al*., 2003) or *R. sativus* L. cv. 'Voran' (IPK gene bank accession

number: RA 34; 2C = 1.11 pg; Schmidt-Lebuhn *et al*., 2010) (Tab. 1). For this purpose, approx. 0.5 cm^2 of young leaf tissue was fixed and washed as described above. Nuclei were isolated by chopping the leaf material with a sharp razor blade in 1 ml 10 mM Tris buffer supplemented with PI and RNase. After filtration and mixing with the nuclei isolated from *Glomus*, suspensions were measured using a FACSAria (BD Biosciences, San José, CA, USA) with a 488 nm laser or a FACSStarPLUS (BD Biosciences, San José, CA, USA) equipped with an INNOVA 90C Argon Ion Laser (COHERENT, Santa Clara, CA, USA) adjusted at 514 nm. Usually 10,000 nuclei per sample were analyzed and the absolute DNA amounts of the samples were calculated based on the values of the G1 peak means.

3.9.7 Feulgen DNA image densitometry

Freshly collected spores and hyphae of both *Glomus* isolates (AMykor and DAOM197198) were fixed in a 3:1 mixture of ethanol and acetic acid and stored in 96% ethanol before measurement. The nuclear DNA content was determined from Feulgen-stained material using DNA image densitometry with CIRES (Cell Image Retrieval and Evaluation System, version 3.1; KONTRON ELEKTRONIK, Munich, Germany) according to Greilhuber and Temsch (2001). Root tip meristem of *G. max* cv. ´Idefix´ (2C = 2.56 pg; Dolezel *et al*., 1998) were co-processed as standard material for internal calibration. Measurements were performed under a 100 × immersion objective in the green monochromatic light (green channel of the CCD camera and green interference filter).

3.9.8 Labelling of the DNA sample via Nick translation

The prepared assay solution (Tab. 7) was mixed gently, centrifuged briefly and incubate for 90 - 120 min at 15°C. The reaction was interrupted by cooling on ice and 1 / 10 of the total volume was loaded on 1% agarose gel electrophoresis. If most labelled fragments were of 400 - 500 bp in size, the reaction was stopped by adding 1 µl 0.5 M EDTA, pH 8.0 and incubating for 10 min at 65°C. Briefly, 4 µl of the labelled probe was precipitated with 3 M Na-acetate, pH 5.2 and 96% cold ethanol at -80°C for 30 min. After centrifugation the pellet was dried under vacuum, and then dissolved in hybridization solution DS20.

Table 7 Assay used for Nick translation.

Components	Volume
PCR product (500 ng / µl)	10 µl
dNTP-Mix	5 µl
1 mM Cy3-dUTP	4 µl
NT buffer	5 µl
0.1 M β-mercaptoethanol	5 µl
DNase I (1:250 dilution of 1 mg/ml stock in 0.15 M NaCl, 50% Glycerol)	2.5 µl
DNA polymerase I (10 U/µl, MBI Thermo Scientific)	1 µl
H_2O	17.4 µl

dNTP-Mix	dATP dCTP dGTP dTTP	250 µM 250 µM 250 µM 50 µM
NT buffer	Tris $MgCl_2$ BSA	0.5 M 50 mM 0.05%
Dissolved in aqua dest., stored at 4°C not longer than 3 months		
DS20	Dextransulfate Formamid In 2 x SSC	20% 50%
2 x SSC **pH 7.0 adjust with HCl**	Sodium chloride (NaCl) Sodium citrate ($Na_3C_6H_5O_7$)	300 mM 30 mM
Dissolved in aqua dest., stored at 4°C not longer than 3 months		

3.9.9 AMF nuclei preparation for FISH analysis

To isolate nuclei suitable for FISH, spores and hyphae were fixed, washed, crushed with a micro-pestle, filtered through a mesh and suspended in 10 mM Tris buffer with 1 µg ml^{-1} DAPI. The nuclei were isolated via flow sorting on a FACSAria (BD Biosciences, San José, CA, USA) using a 375 nm laser. Spores were collected in microfuge tubes and subsequently, 15 µl of sorted nuclei were dropped into 15 µl of sucrose buffer (100 mM Tris, 50 mM KCl, 2 mM $MgCl_2$, 0.05% Tween 20 and 5% sucrose) on a microscopic slide. Slides were air-dried overnight and either used immediately or stored at -20°C.

3.9.10 Fluorescence *in situ* hybridization on AMF nuclei

Fluorescence *in situ* hybridization was performed according to Lysak *et al.* (2006) with minor modifications. Labelled probe was incubated for 60 min at 37°C, denatured for 5 min at 95°C and then kept on ice. Slides were pre-treated for 30 min at 60°C, fixed in 4% paraformaldehyde in 1 x PBS (10 min) and washed in 1 x PBS. Denatured probe was dropped onto the slides; both were denaturated at 80°C for 2 min and hybridized from 12 - 36 hours in a moisture chamber at 37°C. A longer hybridization time usually resulted in slightly more intense signals. The post-hybridization washes were made three times with 50% formamide in 2 x SSC (42°C, 5 min), three times with 2 x SSC (42°C, 5 min each), then slides were dehydrated in an ethanol series (70%, 90%, 96%) and dried on air. A drop of 10 µl DAPI solution (0.5 µg ml^{-1} in Vectashiel; Vector Laboratories, USA) was added and each slide was covered with a cover slip and stored at 4°C. Slides were examined using a Zeiss Axioskop Epifluorescence microscope (Carl Zeiss, Germany) equipped with an ORCA-ER camera system (Hamamatsu, Shizuoka, Japan). Images of nuclei and signals were captured separately with appropriate filter combinations in black and white using Simple PCI software, pseudo-coloured and then merged using Adobe Photoshop.

3.9.11 Subcellular localization

To obtain the pYES2-GiFRD::GFP construct, a variant of *GiFRD* was fused to the N-terminus of green fluorescence protein gene (*GFP*) in pYES2. The *GiFRD* was PCR amplified from genomic DNA using the primers Hind_GiFRD (fwd) and a Sac_linker_GiFRD with the 30 bp linker (rev) (Tab. 3). The full length of *GiFRD* with the 30 bp linker, flanked by *Hin*dIII and *Sac*I sequences, was then subcloned into a pCR4 vector in *E. coli* TOP10 cells, released from the vector by digestion and cloned into linearized pYES2 vector in *E. coli* BL21 cells (Fig. 10). Next, *GFP* was PCR amplified using the primers SacI_GFP (fwd) and NotI_GFP* (rev) on available vector with *GFP* (Tab. 3). The *GFP,* flanked by *Sac*I and *Not*I restriction sites was subcloned into the pCR4 vector in *E. coli* TOP10 cells, released by digestion and cloned into the *Sac*I / *Not*I linearized vector pYES2-GiFRD in *E. coli* BL21 cells (Fig. 11). The control vector pYES2-GFP was constructed in analogous way (Fig. 12).

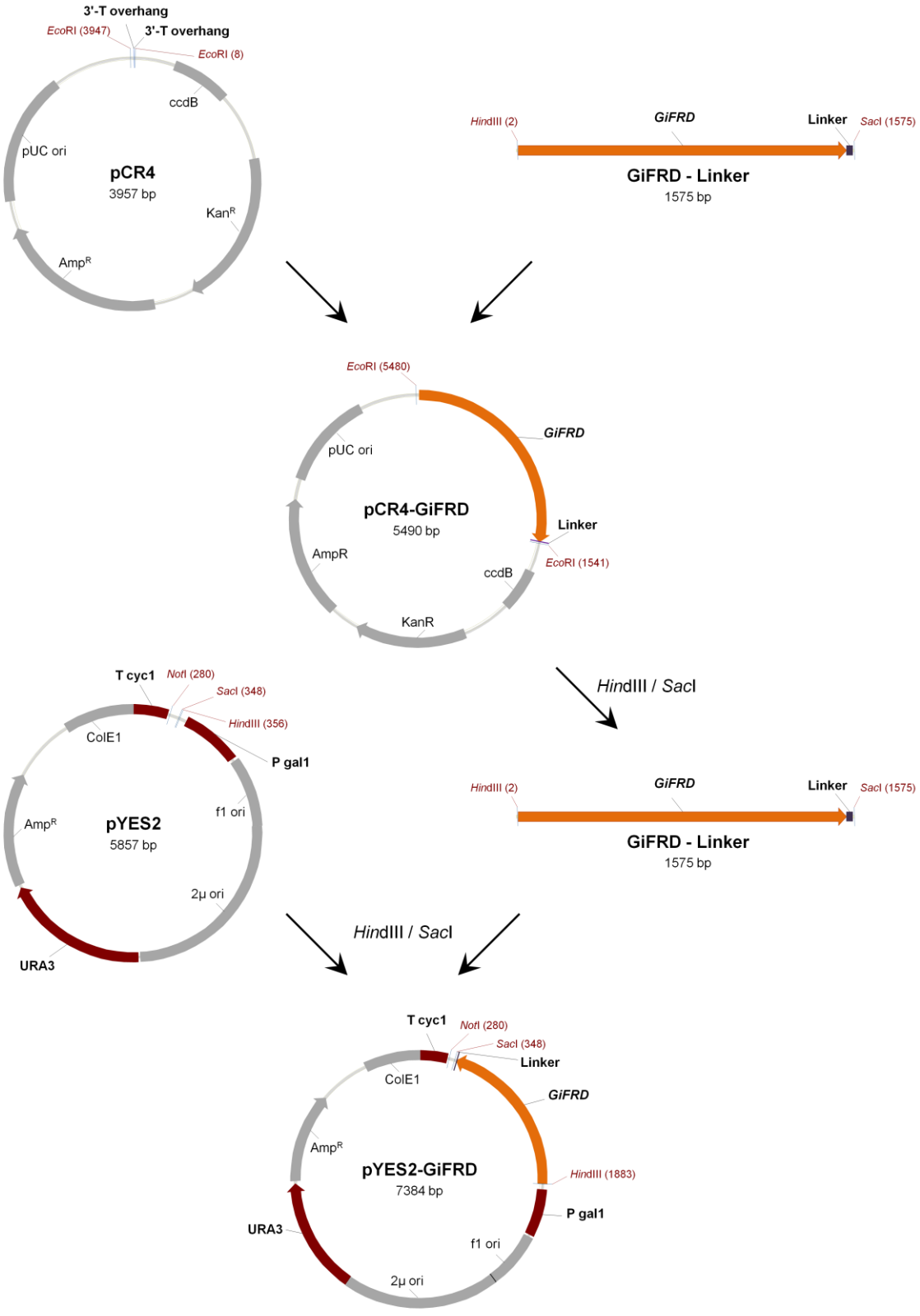

Figure 10 Design and physical map of the expression vector pYES2-GiFRD used in this study. The final vector contains all *E. coli* elements for propagation in the *E. coli* system (grey) as well as yeast elements, *URA3* gene as selection marker, *GAL1* promoter, and *CYC1* terminator (red) and *GiFRD* gene from *G. irregulare* encoding fumarate reductase (orange).

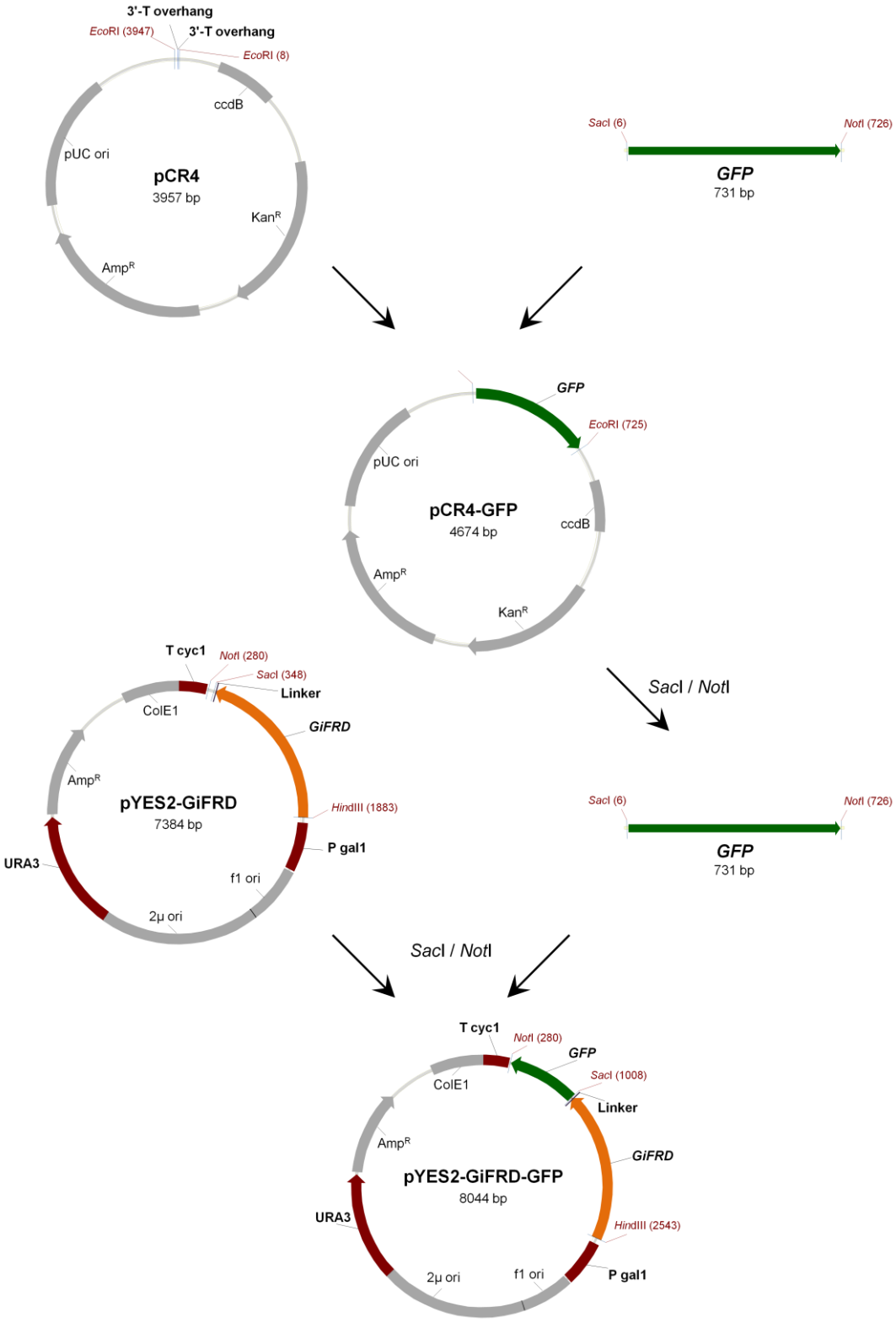

Figure 11 Design and physical map of the expression vector pYES2-GiFRD-GFP used in this study. The final vector contains all *E. coli* elements for propagation in the *E. coli* system (grey) as well as yeast elements, *URA3* gene as selection marker, *GAL1* promoter, and *CYC1* terminator (red), *GiFRD* gene from *G. irregulare* encoding fumarate reductase (orange) and *GFP* gene encoding green fluorescence protein (green).

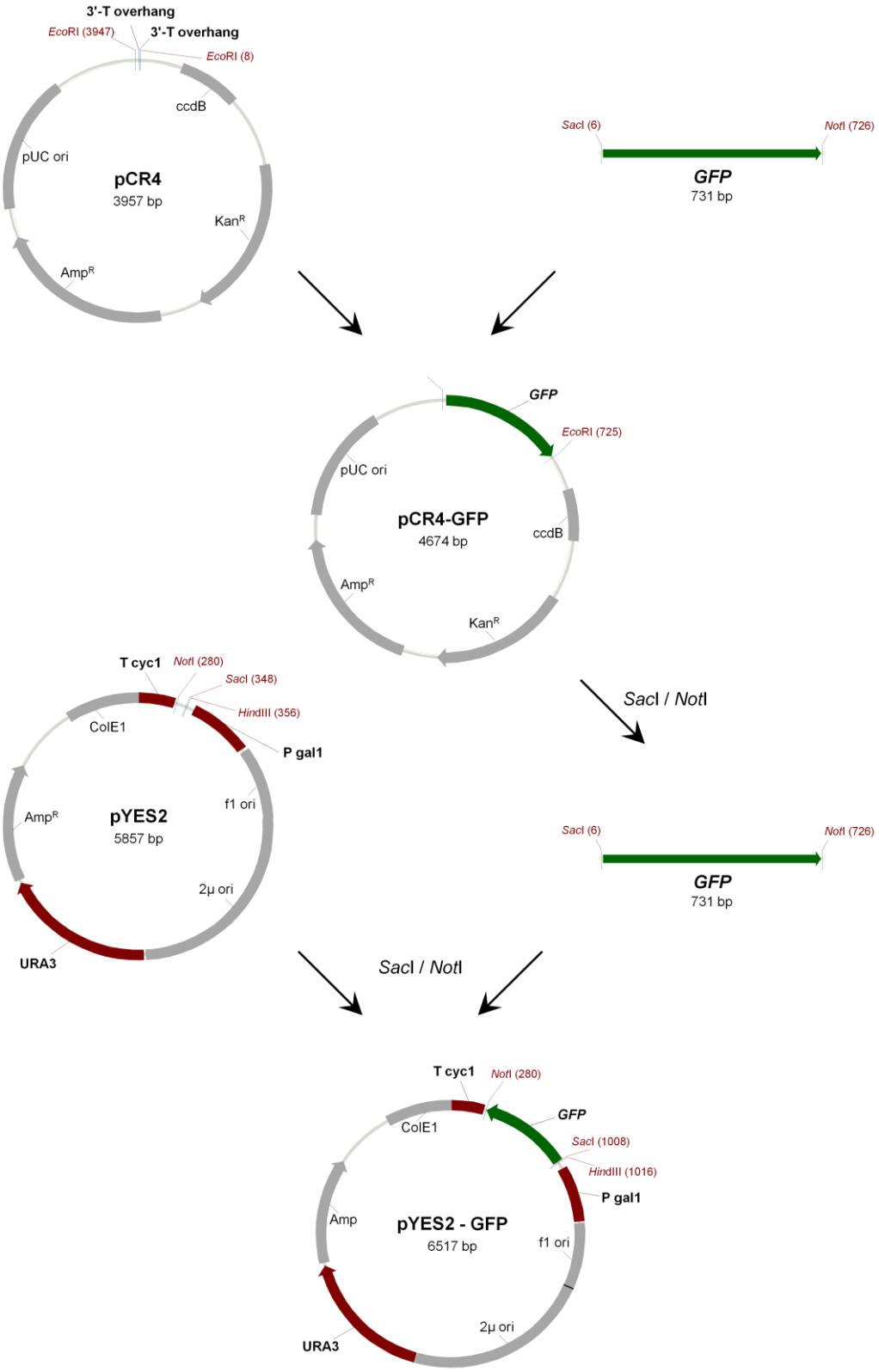

Figure 12 Design and physical map of the expression vector pYES2-GFP used in this study. The final vector contains all *E. coli* elements for propagation in the *E. coli* system (grey) as well as yeast elements, *URA3* gene as selection marker, *GAL1* promoter, and *CYC1* terminator (red) and *GFP* gene encoding green fluorescence protein (green).

3.10 Methods used for work with yeast

3.10.1 Preparation of competent cells (Dohmen *et al*., 1991)

Yeast cells were cultured in YPD medium at 30°C for 3 - 4 hours to ~OD_{600nm} 1.5. Subsequently cells were collected by centrifugation at RT for 5 min at 3500 x g. Pellet was washed with 0.4 vol. of solution A and centrifuged again in the same conditions. Cells were suspended in 0.125 vol. of 0.15 M DTT and 0.125 vol. of 0.1 M $CaCl_2$ and incubate for 20 min at 37°C. Centrifugation was repeated and cells were suspended in 0.02 vol. solution A and 0.07 vol. DMSO and incubate for 5 min at 37°C. Suspension was divided to sterile Eppendorf tubes and froze at -80°C.

Solution A	Sorbitol	1 M
	Bicine-NaOH pH 8.35	10 mM
	Ethylene glycol	3%
Dissolved in aqua dest., stored at 4°C not longer than 3 months		

3.10.2 DNA transformation to competent cells (Dohmen *et al*., 1991)

5 - 10 µg of DNA was added on the surface of competent cells yeast without touching them. The cells were incubated at 37°C for 5 min in thermo mixer with shaking (over 9600 x g), then 1 ml of sterile 40% PEG 1000 + 0.2 M bicine pH 8.35 solution was added. The tubes were inverted once and incubated for one hour at 30°C without shaking. Afterwards, centrifugation for 5 min at 2000 x g in RT was performed. Supernatant was discarded using a pipette and pellet was suspended in 1 ml sterile 0.15 M NaCl + 10 mM bicine, pH 8.35 solution. Cells were centrifuged for 5 min at 2000 x g in RT and supernatant was again removed. Cells were suspended in 500 µl sterile 0.15 M NaCl + 10 mM bicine, pH 8.35 solution and spread on plates with selective medium (100 µl per plate) according to Rösel and Kunze (1998).

3.10.3 Isolation of yeast chromosomal DNA

Overnight culture of yeast in 5 ml YPD was centrifuged at 1500 x g for 5 min at RT. The cell pellet was suspended in 1 ml water, vortexed and again centrifuged at 1700 x g for 5 min. Cells were incubated for one hour at 37°C in 500 µl fresh prepared lysis solution and centrifuged at 1750 x g for 10 min.

Afterwards, pellet was suspended in 500 µl 50 mM EDTA pH 7.5 and 50 µl 10% SDS. Incubation for 30 min at 65°C was performed and pellet was chilled on ice. Subsequently, 200 µl 5 M potassium acetate were added and incubated for one hour on ice. After centrifugation at 12000 x g for 5 min at 4°C the clear supernatant was transferred to a new tube and 750 µl of cold isopropanol were added. The mixture was incubated for 5 min in RT and centrifuged at 12000 x g for 15 min at 4°C. The pellet was washed in 10 µl 70% ethanol and centrifuged at 12000 x g for 10 min at RT. The supernatant was discarded, the pellet dried and then suspended in 40 - 50 µl water.

Lysis solution	Lyticase from *Arthrobacter luteus* (2000 u / mg)	50 mg
	50 mM Na-P-buffer pH 7.5	20 ml
	14.3 M ß-mercaptoethanol	20 µl

3.10.4 Protein extraction from yeast cells

Cells were grown to exponential phase in 2% galactose SD medium at 30°C, and collected by centrifugation at 4°C for 10 min at 3500 x g. Cells were suspended in 0.5 ml 10 mM Na-Phosphate buffer, pH 7.5 and disrupted with silica beads for 3 min at 30 Hz frequency in mixer mill (Retsch, MM 400, Germany). Cell homogenates were centrifuged at 4°C for 10 min at 23000 x g and the supernatants were used directly as cell extracts for analysis.

4. MOLECULAR ANALYSIS OF TWO *GLOMUS* ISOLATES: AMYKOR AND DAOM197198

4.1 Species determination by phylogenetic analysis

To obtain the 3' partial SSU rDNA, the ITS region, and the 5' partial LSU rDNA sequence for further analysis, PCR was performed on DNA isolated from both *Glomus* isolates (AMykor and DAOM197198). The universal eukaryote primer pair NS5 / ITS4 (Fig. 13; Tab. 3) was used for the first amplification step (Redecker, 2000). Depending on the success of PCR amplification, PCR product of the first reaction was used at dilutions of 1:10, 1:50 or 1:100 (in water) as a template for the second amplification. The *Glomus* group A specific primer set, GLOM1310i / ITS4i (Fig. 13; Tab. 3), was used in a nested PCR reaction (Redecker, 2000) (Tab. 4, 5).

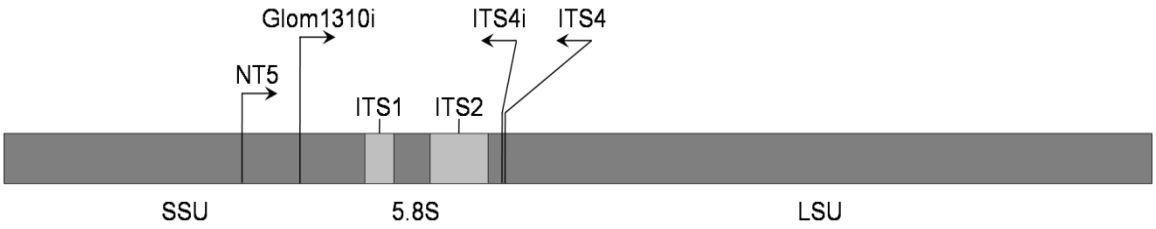

Figure 13 Physical map of the rDNA unit including positions of the forward and reverse primers used in this study for 3' partial nuclear small subunit (SSU), the ITS region and the 5' partial nuclear large subunit LSU rDNA amplification (drawing to scale).

The partial SSU, entire ITS region and the partial LSU rDNA sequence from 10 spores of each of AMykor and DAOM197198 isolates were amplified, cloned into a bacterial plasmid (Tab. 2) by the TOPO TA Cloning Kit for Sequencing (Invitrogen) and the subcloned sequences from 10 *E. coli* clones per spore were sequenced with primers M13forw and M13rev included in the kit (Tab. 3). The lack of proof reading activity by AmpliTaq polymerase could cause 0.1% errors during sequences analysis.

The neighbour joining (NJ) and Bayesian interference (BI) phylogenetic analyses were performed on obtained sequences. Both, NJ and BI phylogenetic analysis algorithms resulted in similar tree topologies (Fig. 14).

Table 8 *Glomus* isolates analysed in this study.

Identified as	Origin	Isolate code / Voucher number
Glomus irregulare (Stockinger)	Germany	BEG195 / Att1485-12
Glomus sp. 'irregulare-like' (Stockinger, Sanders)	Canada	DAOM197198 / Att690-23
Glomus irregulare (Piche)	Canada	DAOM197198 / Att1192-53
Glomus irregulare (Błaszkowski)	Poland	Błaszkowski
Glomus cf. clarum (Redecker)	Iceland	Att894-7
Glomus proliferum (Declerck)	Guadeloupe	MUCL41827
Glomus intraradices (Stockinger)	Florida, USA	INVAM FL208 / Att4-64
Glomus intraradices (Stockinger)	Florida, USA	MUCL49410 / Att1102-12

The *G. proliferum* MUCL41827 (defined as an out-group) and *Glomus clarum* Att894-7 were placed in well-defined clades and the two *G. intraradices* isolates INVAM FL208 and MUCL49410 clustered together in a clade for their own. All of these separations are statistically supported by a posterior probability (pp) of 1 and NJ bootstrap support of 100% (both maximum values).

The largest clade in the tree (1 pp / 100 bs) included sequences obtained from the database and was classified as *G. irregulare* (BEG195 / Att1485-12, DAOM197198 / Att690-23, and DAOM197198 / Att1192-53). The Błaszkowski isolate is placed on a relatively long branch as sister to the remainder of the *G. irregulare* clade. Both, AMykor and DAOM197198 isolates clearly fell into this *G. irregulare* clade.

All cloned sequences of the DAOM197198 isolate are placed on a subclade of their own (0.85 pp) and form a clade together with DAOM197198 sequences downloaded from the database. Thus, the DAOM197198 isolate represents a monophyletic lineage within the *G. irregulare* clade. Despite, the AMykor isolate not appearing as a monophyletic lineage in this analysis, it belongs to the *G. irregulare* clade as well.

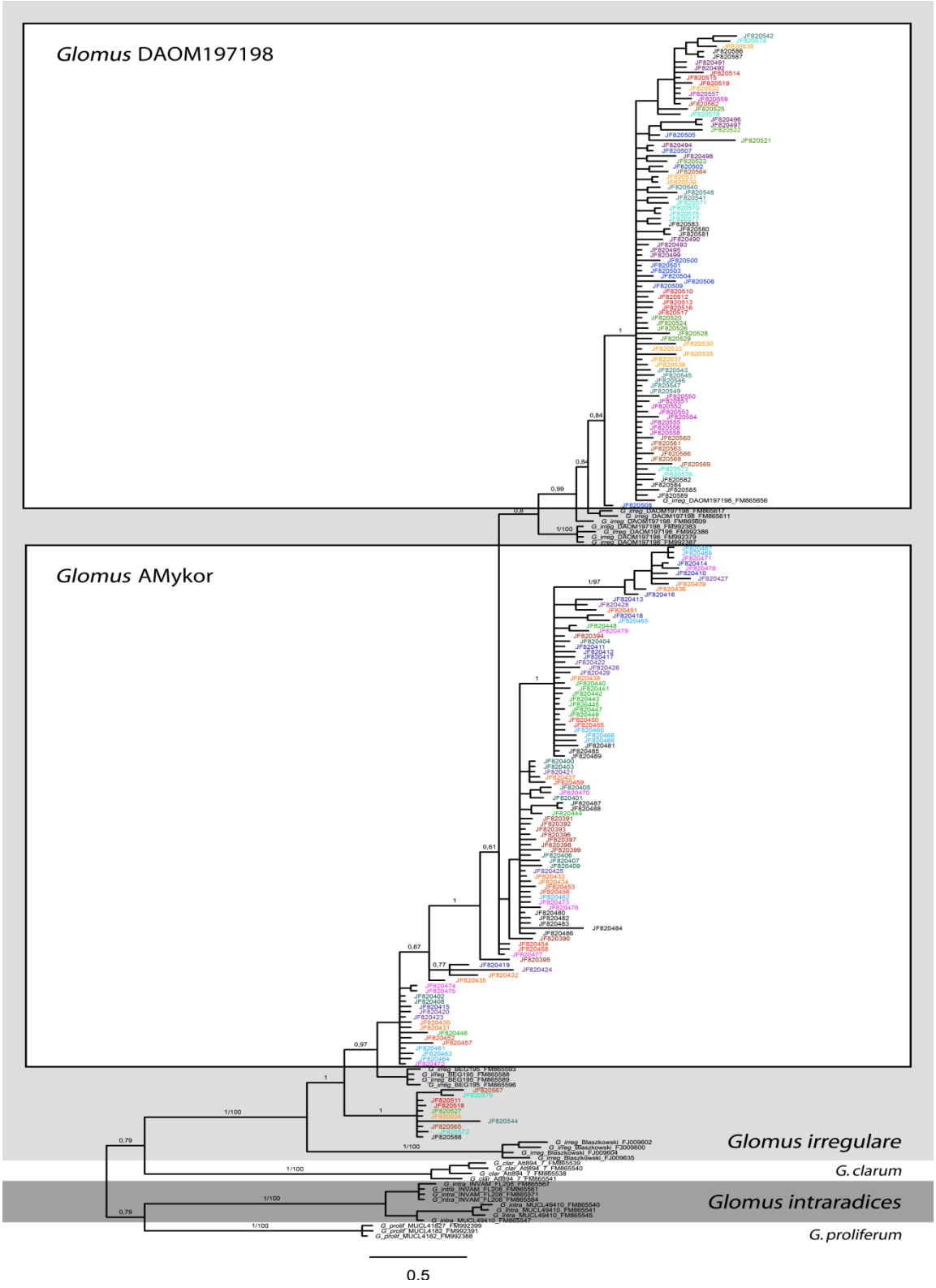

Figure 14 Phylogenetic tree (BI) (partial SSU, ITS1, 5.8S, ITS2, partial LSU rDNA sequences of approx. 1000 bp length) of *Glomus* isolates analysed with *G. proliferum* (MUCL41827) sequences used as an out-group. Both *Glomus* isolates (AMykor and DAOM197198) are placed into two separate groups, but clearly fall into the clade containing isolates identified as *G. irregulare*. Sequences derived from an individual spores are marked in the same colour. The values above the branches correspond to support from Bayesian/NJ analyses (pp / bs in %).

The phylogenetic tree that was constructed included 100 ITS sequences from each of the AMykor and DAOM197198 isolates. All clones originating from one individual spore are marked in the same colour (Fig. 14). Not all of the sequences from a single spore fell in the same clade. Figure 14 shows that the colour assigned to a single spore is randomly distributed within the clades or is, in some cases, in polytomies, i.e. regions in the tree where phylogenetic relationships are not resolved. According to those observations, a single spore does not appear as a genetic entity within the fungi; rather it shows a high genetic diversity within a single spore that may cause the high genetic diversity within the organism.

Stockinger *et al*. (2010) claimed that only longer (approx. 1500 bp) nuclear rDNA fragment (spanning SSU, ITS region and LSU) can be successfully applied to species delineation within the GlGrAa and GlGrAb. In view of this idea, additional phylogenetic analyses were performed with longer fragment (approx. 1700 bp) of rDNA sequence from two spores of both isolates and from 5 - 6 clones of each. The SSUmAforw / LSUmArev primer pair (Tab. 3), based on primers obtained for all AMF lineages by Krüger *et al*. (2009) was used for the amplification. The distance matrix was analyzed with a maximum likelihood (MLE) algorithm and subsequently with Bayesian phylogenetic analyses. Posterior probabilities (pp) were calculated from the remaining 9002 trees.

The data obtained did not differ from the previous analyses with shorter rDNA fragment which also spans the partial SSU, entire ITS and partial LSU region, not only ITS region. From this result, it can be concluded that rDNA fragments of ~1000 bp as used previously, can be used for species determination in these isolates. Also, closely related isolates of AMykor and DAOM197198 were similarly resolved with statistically good support of 0.75 pp in second analyzes and 0.80 pp in first analyses (Fig. 15).

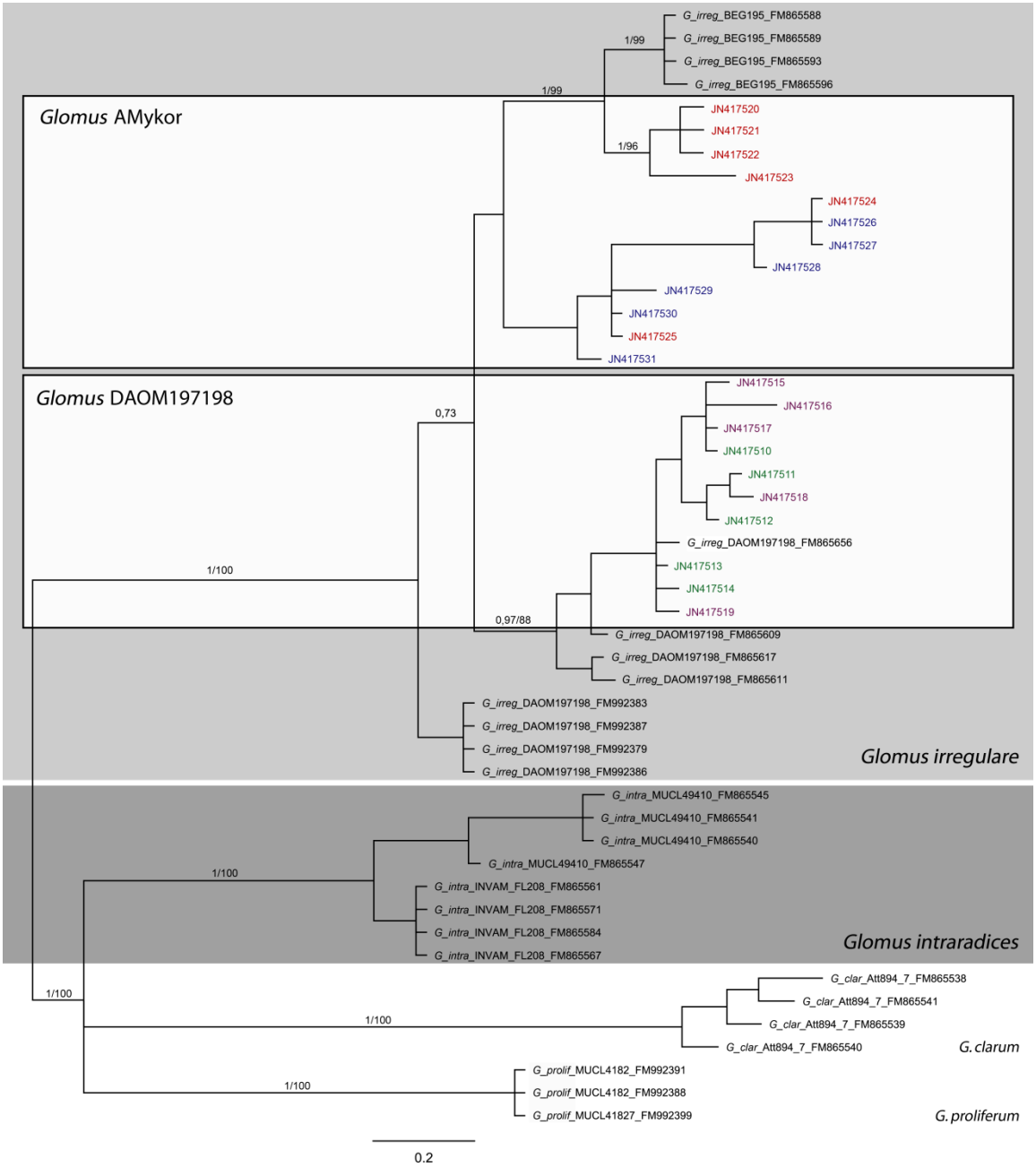

Figure 15 Phylogenetic tree (BI) (partial SSU, ITS1, 5.8S, ITS2, partial LSU rDNA sequences of approx. 1700 bp length) of *Glomus* isolates analysed with *G. proliferum* (MUCL41827) sequences used as an out-group. Sequences derived from an individual spore are marked in the same colour. The values above the branches correspond to support from Bayesian/MLE analyses (pp / bs in %).

4.2 Variants of 5.8S rDNA sequence

Additionally, 5.8S rDNA sequences were compared to establish the number of variants within single *Glomus* isolate. Only the 5.8S sequences were included because the sequences of the entire ITS region were too variable to make a reasonable alignment.

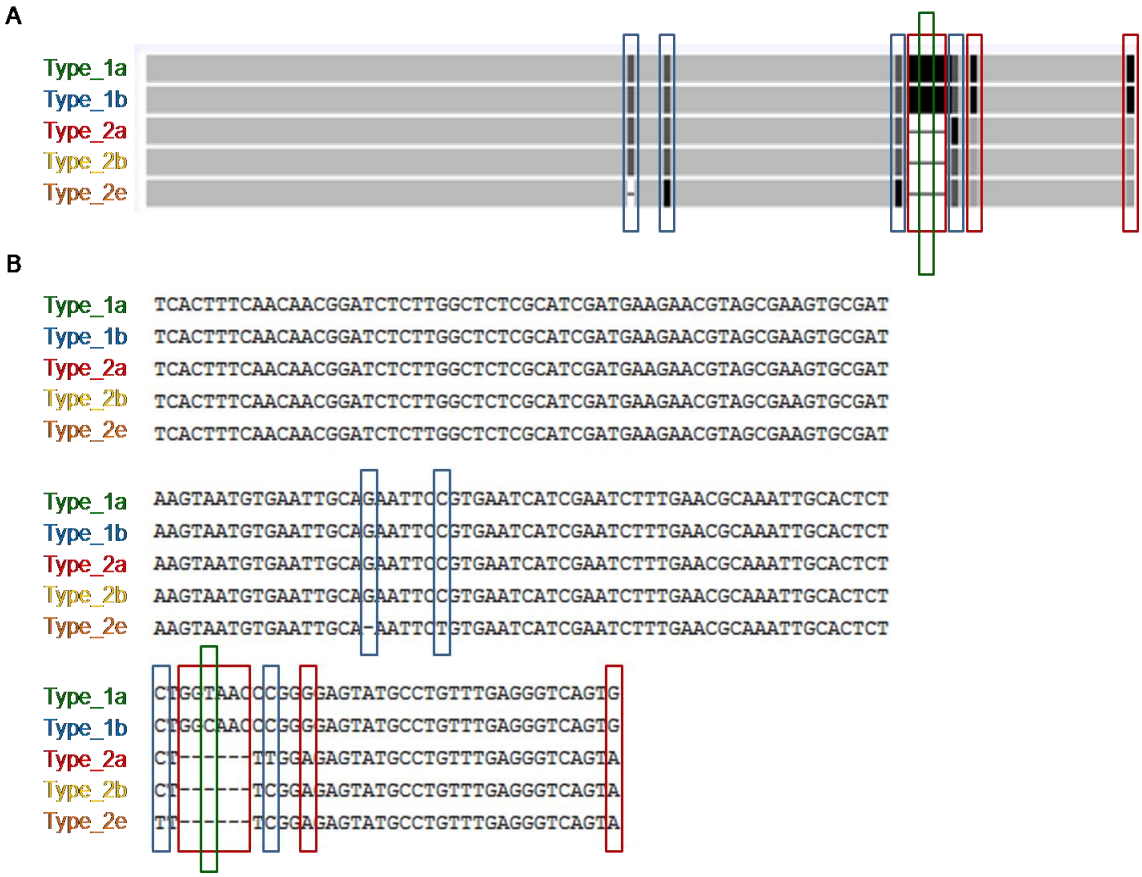

Figure 16. Multiple sequence alignment for 5.8S rDNA variants in *G. irregulare* DAOM197198 and AMykor isolates (Geneious 5.5).

Two distinct sequences types, called type 1a and type 2a, were identified within the 5.8S rDNA sequences from a single spore isolate of *G. irregulare* DAOM197198 (Fig. 16 A, Fig. 17). The major difference between these two sequence types was deletion of the six-nucleotide fragment GGNAAC in type 2a called “shorter” (Fig. 16 B, red boxes). The ration of clones belonging to type 1a and 2a was approx. 9 : 1 (76 clones : 10 clones). Phylogenetic analysis based on fragment of rDNA sequence showed a high support value (1 pp / 100 bs) for the separation of the sequences types 1a and 2a (Fig. 17).

In contrast, within the 5.8S rDNA sequences from *G. irregulare* AMykor isolate, four types of sequences were found. Normal-size type 1a also occurred in this isolate, as well as second normal-size type 1b with the single position substitution in the block of additional six nucleotides (Fig. 16 B, green box). The “shorter” type was represented by two type 2b and 2e which differed in two single substitutions and length, 152 bp and 151 bp, respectively (Fig. 16 B, blue boxes). In *G. irregulare* AMykor isolate ration between normal-size and “shorter” variants was approx. 3 : 1 (58 clones : 22 clones).

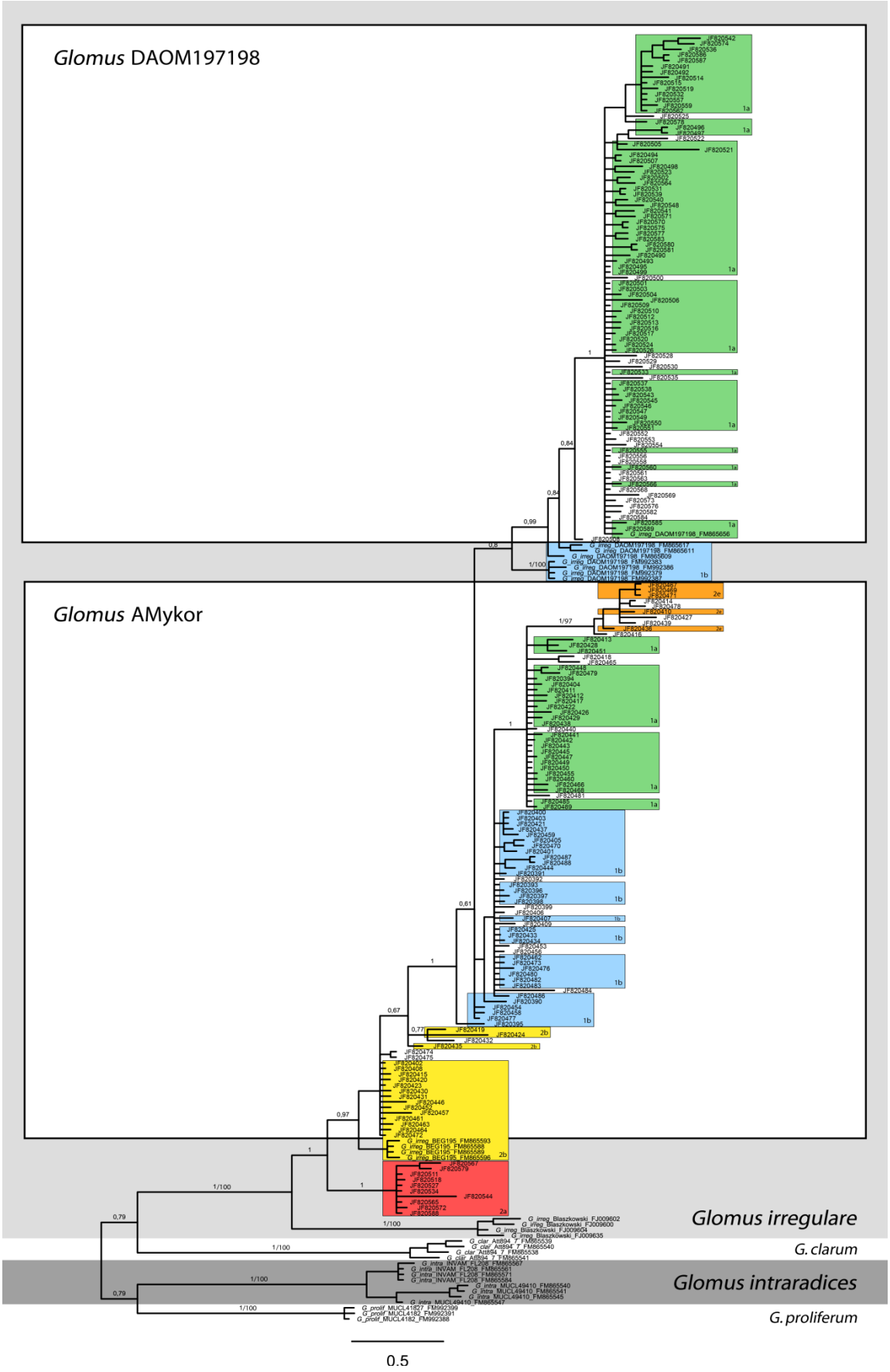

Figure 17 Phylogenetic tree (BI) based on rDNA sequences of *Glomus* isolates. The colour boxes represent the different 5.8S rDNA variants described in subsection 4.2.

4.3 Determination of rDNA loci number by FISH

Phylogenetic analyses revealed that both *Glomus* isolates correspond to *G. irregulare* although they cluster into different subclades. To investigate if both isolates also differ with respect to their number of rDNA sites, FISH was performed on flow-sorted nuclei using a rDNA-specific probe amplified from genomic DNA of the DAOM197198 isolate.

Using flow-sorted nuclei for the FISH procedure has the advantage of providing nuclear material free of cytoplasmatic contamination, which decreases non-specific binding of the hybridization probe and increases accessibility to the nuclear DNA. Furthermore, since nuclei sorted according to similar fluorescence intensity have a similar DNA content, a mixture of replicated and un-replicated nuclei, which would negatively influence the enumeration of signals, can be avoided.

To isolate nuclei suitable for FISH, spores and hyphae were fixed, washed, collected and released as described in Materials and methods.

DNA probes of partial SSU, the internal transcribed spacer (ITS1 and ITS2) region and partial LSU rDNA sequence from genomic DNA of the *G. irregulare* DAOM197198 isolate were amplified by PCR with the *Glomus* group A specific primer set (ITS4i / Glom1310i). The product was labelled by nick translation (Tab. 7) (Ward, 2002) with Cy3-dUTP as described in Materials and Methods section (Fig. 18).

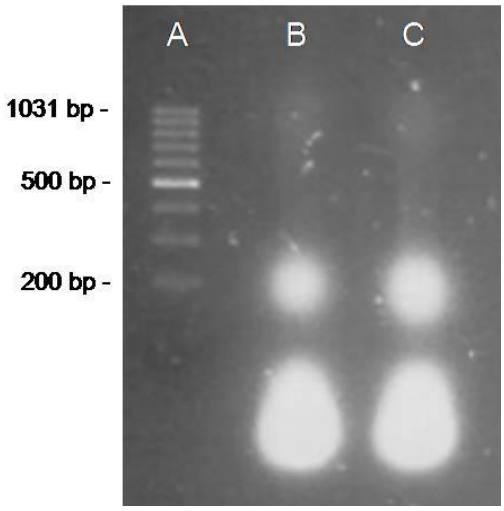

Figure 18 *G. irregulare* rDNA labelled probes by the Nick translation (A) GeneRulerTM 100 bp DNA ladder; (B, C) rDNA sequence from genomic DNA of the *G. irregulare* DAOM197198 amplified by PCR with primer set ITS4i / Glom1310i.

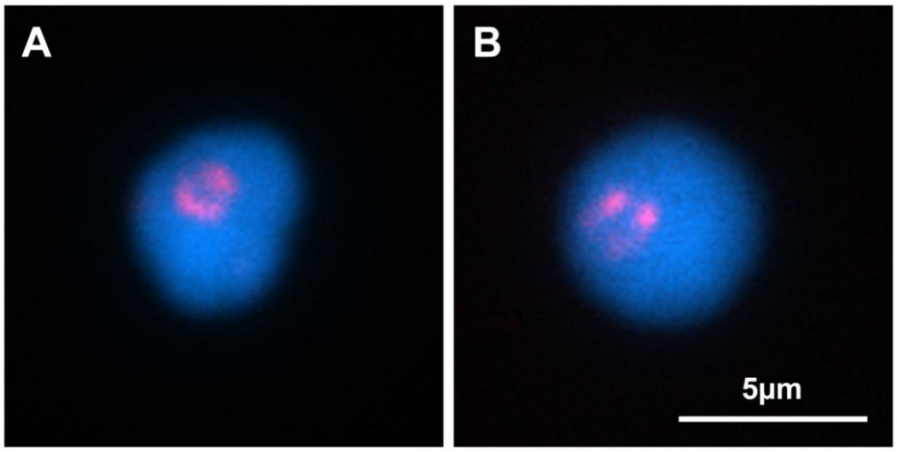

Figure 19 Fluorescence *in situ* hybridization of partial rDNA sequence in nuclei of *G. irregulare* DAOM197198 (A) and AMykor (B) isolates using Cy3-labeled probes. The double hybridization Cy3 signals are visualized as fluorescent red spots which are localized in a blue-stained background (DAPI) of whole nuclei.

For both *G. irregulare* AMykor and DAOM197198 isolates, clear, reproducible signals, closely associated with the nucleoli in 70 - 80% of all investigated nuclei were obtained (Fig. 19). In the *G. irregulare* DAOM197198 isolate roughly equal proportions of one or two foci per nucleus was observed, whereas about 62% of the AMykor isolate nuclei possessed two foci and 28% a single signal. In 10% of all nuclei from both isolates three or four weaker signals were found (Tab. 9). Considering that rDNA loci tend to fuse within interphase nuclei, it can be speculated that the maximum detectable number of signals may reflect the number of rDNA loci within the genome. Therefore, it can be concluded that both isolates do not differ in the number of rDNA loci and both genomes harbour at least two major sites and possibly two minor sites.

Table 9 Percent of nuclei showing 1, 2, 3 or 4 rDNA hybridization signals detected by FISH analysis. N is the number of evaluated nuclei

G. irregulare	Number of hybridization signals			N
	1	2	3 or 4	
DAOM197198	48%	42%	10%	70
AMykor	28%	62%	10%	76

In the FISH approach, no RNase treatment was performed which might lead to the conclusion that some of the hybridization signal could be due to the hybridization of ribosomal RNA. Comprehensive experiences with FISH on flow-sorted plant nuclei (*A. thaliana, R. sativus*) demonstrated that an RNase treatment can be omitted without diminishing the value of the results. Moreover, the presence of ribosomal RNA would most likely result in a diffuse background signal throughout the nucleolus and not distinct signals. Nevertheless, additional experiments were performed with an RNase treatment (40 min incubation at 37˚C with 0.1 mg ml^{-1} concentrated RNase) and similar data to that presented above was obtained (Tab. 10).

Table 10 Percent of nuclei showing 1, 2, 3 or 4 rDNA hybridization signals detected by FISH analysis with additional RNase treatment.

G. irregulare	Number of hybridization signals		
	1	2	3 or 4
DAOM197198	75%	6%	19%
DAOM197198 + RNase treatment	64%	28%	8%
AMykor	80%	16%	4%
AMykor + RNase treatment	60%	35%	5%

4.4 Estimation of the nuclear DNA content

The nuclear DNA content of both *G. irregulare* isolates (AMykor and DAOM197198) was determined by flow cytometry on six independent nuclei isolations using *A. thaliana* as an internal reference standard.

The nuclei of *A. thaliana* were isolated from differentiated leaf tissue, which is characterized by distinct endopolyploidization resulting in a mixture of polyploidy nuclei (Galbraith *et al*., 1991). Replication of chromosomes is taking place without the division of the cell nucleus resulting in cells that harbour nuclei with different DNA content (2C, 4C, 8C and so on). Accordingly, flow cytometry histograms representing the relative fluorescence intensities of stained nuclei comprised the 2C peak used for the genome size estimation and additional peaks of higher ploidy level (Fig. 20).

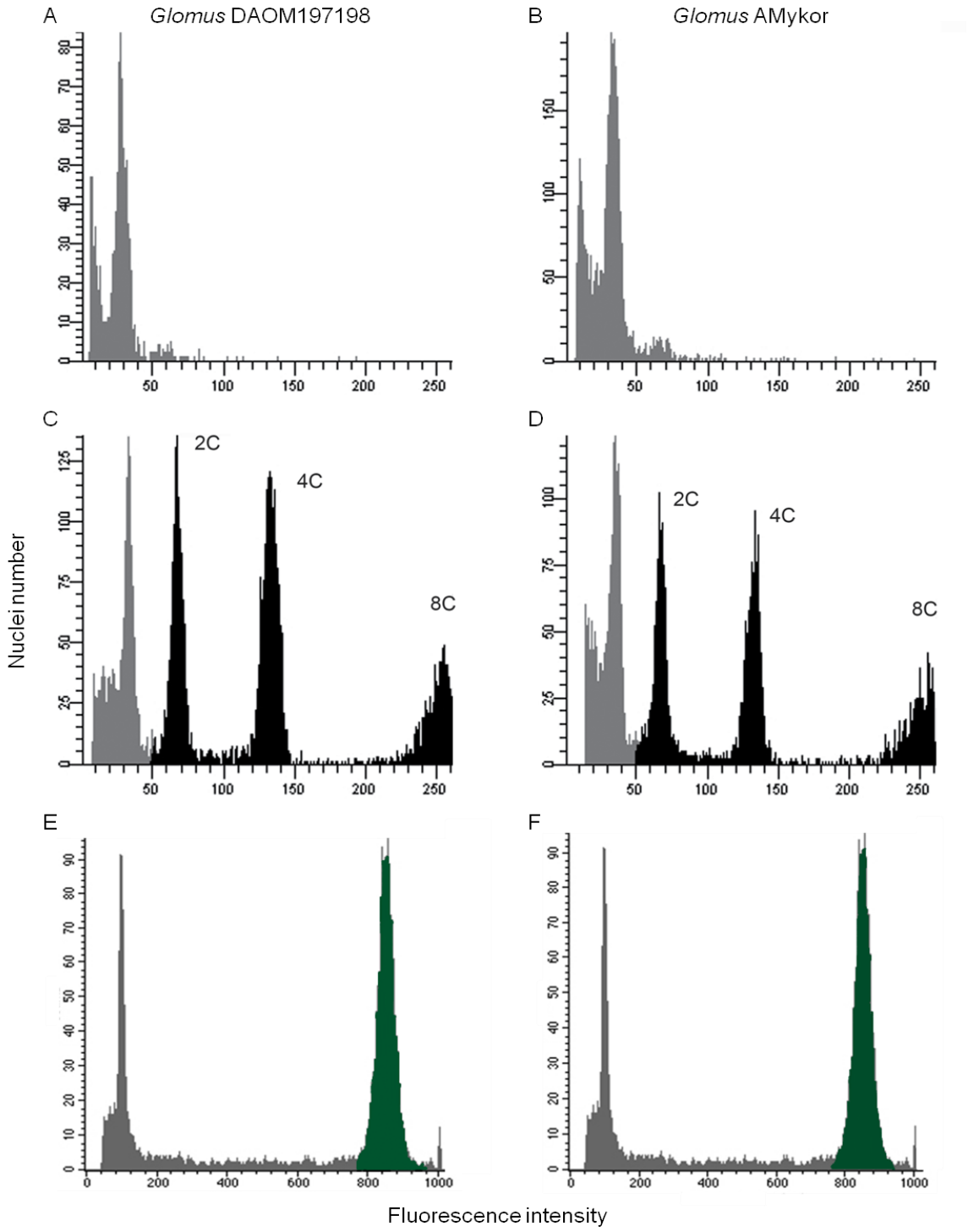

Figure 20 Histograms of relative fluorescence intensities obtained after flow cytometry analysis of propidium iodide-stained nuclei of (A) *G. irregulare* DAOM197198, (B) *G. irregulare* AMykor, (C) *G. irregulare* DAOM197198 (grey) with internal reference standard *A. thaliana* 'Col-0' (black), (D) *G. irregulare* AMykor (grey) with internal reference standard *A. thaliana* 'Col-0' (black), (E) *G. irregulare* DAOM197198 (grey) with internal reference standard *R. sativus* (green), (F) *G. irregulare* AMykor (grey) with internal reference standard *R. sativus* (green). Due to endoreduplication occurring in *A. thaliana* leaf tissue nuclei (Galbraith *et al*., 1991), different ploidy levels are detectable. The first peak represents 2C nuclei with a DNA content of 0.32 pg. Fluorescence intensity of nuclei is expressed in arbitrary units (linear scale).

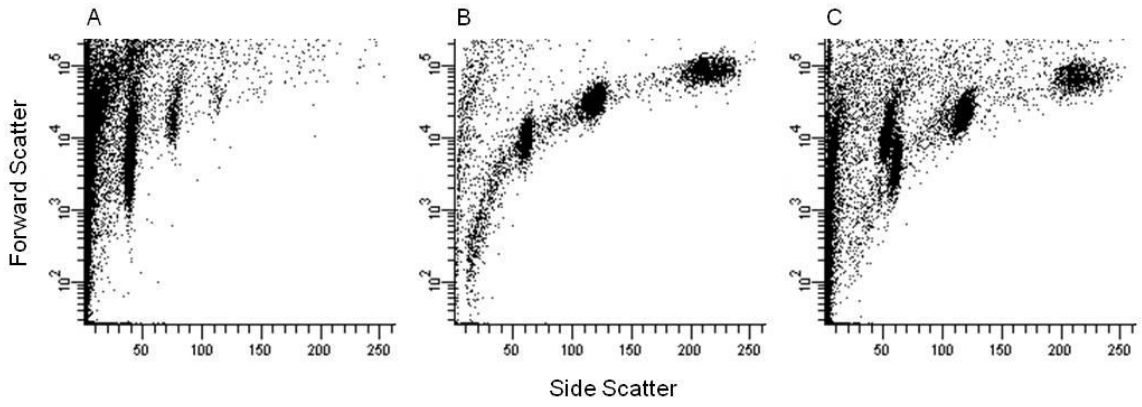

Figure 21 Dot plot assays for *G. irregulare* AMykor (A), *A. thaliana* (B) and mixed *G. irregulare* AMykor and *A. thaliana* nuclei (C).

Due to different nuclei isolation protocols used for fungi and plants, nuclei were isolated separately and subsequently mixed for flow cytometry (FC) measurements. The same fixation procedure and staining buffers were used for both nuclei to minimize inaccuracies caused by the different treatments of the sample and the standard. The only difference in the procedure was in the mechanical disruption of the cells (Fig. 21).

Using this procedure, the DNA content per nuclei was determined to be 0.156 pg for the *G. irregulare* AMykor isolate and 0.157 pg for the *G. irregulare* DAOM197198 isolate (Tab. 11) (Sędzielewska *et al*., 2011). According to Hijri and Sanders (2004), and the Glomus Genome Consortium (Francis Martin, pers. comm.), *G. irregulare* is a haploid organism. Thus, the genome size of both isolates can be estimated to be approx. 150 Mb indicating a similar genome size as *A. thaliana* (157 Mb / 1C; Bennett *et al*., 2003).

The reliability of the present data was confirmed by additional flow cytometry measurements using a second internal reference standard, as well as by Feulgen densitometry measurements (FD). FC measurements of two *G. irregulare* isolates using *R. sativus* (543 Mb / 1C; Dolezel *et al*., 1998) as a reference revealed a nuclear DNA content of 0.146 pg for the *G. irregulare* AMykor isolate and 0.147 pg for the *G. irregulare* DAOM197198 isolate (Tab. 11). Although, the values obtained from measurements with two different reference standards are quite similar, they deviate from each other by 1.07. Since it is generally accepted that the genome size of the reference standard should be in the same range of magnitude as the unknown sample to avoid

instrumental problems with the linearity (Vindeløv *et al*., 1983), the values estimated with *A. thaliana* as reference were considered as more precise.

Feulgen densitometry analyses of 20 nuclei per isolate in comparison with 10 telophase nuclei of *Glycine max* as a reference standard (Dolezel *et al*., 1998) showed the estimated DNA content of *G. irregulare* AMykor 0.185 pg and 0.170 pg for the DAOM197198 isolate (Tab. 11). These values are in agreement with the flow cytometry data and confirm that the genome of *G. irregulare* is ten times bigger than previously postulated (Hijri & Sanders, 2004).

Table 11 Absolute DNA content values obtained by Flow cytometry (FC) and Feulgen DNA image densitometry (FD). N is the number of an individual isolations or preparations / number of nuclei used for analysis**.**

G. irregulare	Method	Reference standard	DNA content (pg)	N
AMykor	FC	*A. thaliana*	0.156	6
DAOM197198	FC	*A. thaliana*	0.157	6
AMykor	FC	*R. sativus*	0.146	2
DAOM197198	FC	*R. sativus*	0.147	2
AMykor	FD	*G. max*	0.185	1/20
DAOM197198	FD	*G. max*	0.170	1/20

Contamination of the nuclear suspension by nuclei of the plant host is very unlikely given the fungal isolation procedure that was used. Even if contamination occasionally occurred it would not have devalued the present results because the genome size of the host plant *Daucus carota* is 473 Mb (Arumuganathan & Earle, 1991) i.e. much larger than that of *G. irregulare* or *A. thaliana* (Fig. 22). In *Glomus* measurements, no fluorescence peak possibly coming from *D. carota* nuclei was observed at the corresponding position.

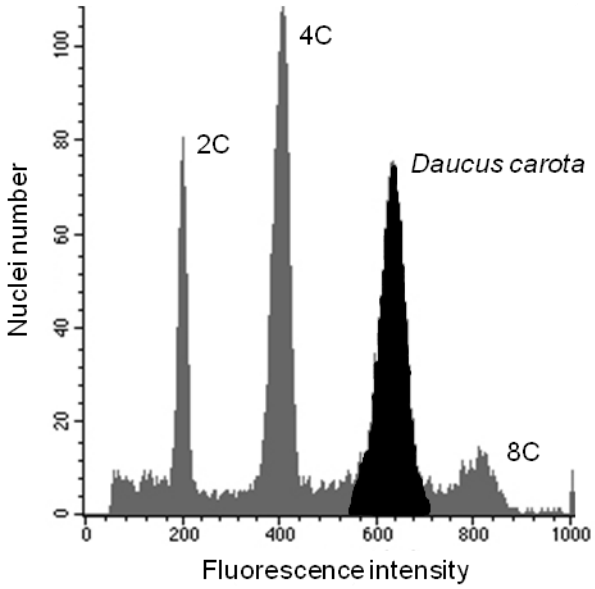

Figure 22 Histograms of relative fluorescence intensities obtained after flow cytometry analysis of propidium iodide-stained nuclei of *A. thaliana* (grey) and *D. carota* (black) Fluorescence intensity of nuclei is expressed in arbitrary units (linear scale).

5. *GiFRD* ENCODES A PROTEIN INVOLVED IN ANAEROBIC GROWTH IN ARBUSCULAR MYCORRHIZAL FUNGUS *GLOMUS IRREGULARE*

5.1 Isolation and characterization of *GiFRD*

A 743 bp cDNA fragment corresponding to a gene with homology to the fumarate reductase family members was identified in a *G. irregulare* AMykor cDNA library. The full-length cDNA, named *GiFRD* was obtained by Genome Walking method.

```
   1    ATGgctagtc  aaattattgt  cgttggtggt  ggtctttctg  gactttctgc  cgctcataca
   1     M  A  S    Q  I  I  V    V  G  G    G  L  S    G  L  S  A    A  H  T

  61    gtcttggaac  atggtgctaa  tgttcttgtt  attgataaga  actctttctt  tggtggtaat
  21     V  L  E    H  G  A  N    V  L  V    I  D  K    N  S  F  F    G  G  N

 121    tctaccaagg  ctacctctgg  tatcaacggt  gctctaacca  aaactcaaat  agctcttggt
  41     S  T  K    A  T  S  G    I  N  G    A  L  T    K  T  Q  I    A  L  G

 181    attaaagact  ctgccgcttt  atttcaggag  gatacaacgc  gctcagctcg  ggatcttgct
  61     I  K  D    S  A  A  L    F  Q  E    D  T  T    R  S  A  R    D  L  A

 241    cgacctgacc  ttattaaagt  tcttgcagga  aattctgctt  ctgccgtcga  atggctccaa
  81     R  P  D    L  I  K  V    L  A  G    N  S  A    S  A  V  E    W  L  Q

 301    gataaattca  atttggacct  ttctcttgtt  tcacggttag  gtggacactc  tcaacctcgt
 101     D  K  F    N  L  D  L    S  L  V    S  R  L    G  G  H  S    Q  P  R

 361    acacatcgtg  gaaaggagat  gtttcctggt  atgacaatta  catatgcttt  gatggaacgt
 121     T  H  R    G  K  E  M    F  P  G    M  T  I    T  Y  A  L    M  E  R

 421    ctcgaagata  ttgctgaaaa  ccagccaaat  cgtgcacgta  tcatcaagaa  agcgcgtgtg
 141     L  E  D    I  A  E  N    Q  P  N    R  A  R    I  I  K  K    A  R  V

 481    acaaatttga  tcaaagaagg  tgaagaagtt  attggtgtag  aatacgaaaa  ggacggtcaa
 141     T  N  L    I  K  E  G    E  E  V    I  G  V    E  Y  E  K    D  G  Q

 541    acattaaaag  aatacggtcc  cgtgattttg  gctactggcg  gttatgcagc  tgatttcacg
 161     T  L  K    E  Y  G  P    V  I  L    A  T  G    G  Y  A  A    D  F  T

 601    gaaaattcgt  tacttaaaaa  atatcgacca  gatatttatg  atttacctac  cactaatgga
 181     E  N  S    L  L  K  K    Y  R  P    D  I  Y    D  L  P  T    T  N  G

 661    gatcattgta  ccggagatgg  gcataaaatg  gttttggcta  ttggcggcaa  agctattgat
 201     D  H  C    T  G  D  G    H  K  M    V  L  A    I  G  G  K    A  I  D

 721    cttgaaaaag  ttcaagtaca  tccgactggt  cttgttgatc  ctaaagatcc  agattctaaa
 221     L  E  K    V  Q  V  H    P  T  G    L  V  D    P  K  D  P    D  S  K

 781    attaaatttt  tggccgctga  agcacttcga  ggtgttggag  gtttgttaat  aaacgcagaa
 241     I  K  F    L  A  A  E    A  L  R    G  V  G    G  L  L  I    N  A  E

 841    ggaaaacgat  tctgtgatga  attaggacat  cgtgactatg  ttaccggtga  aatttggaaa
 261     G  K  R    F  C  D  E    L  G  H    R  D  Y    V  T  G  E    I  W  K

 901    acaaaaggcc  cagttagact  tgtgctcaat  agtaaagcct  caaaagagat  tgaatggcat
 281     T  K  G    P  V  R  L    V  L  N    S  K  A    S  K  E  I    E  W  H

 961    tgcaaacatt  atgctggacg  tggattaatg  aaaaaaatta  attccggtga  agaattggca
 301     C  K  H    Y  A  G  R    G  L  M    K  K  I    N  S  G  E    E  L  A

1021    aaagaaattg  gcgtctccgt  agcacaatta  aaagcaactt  ttgatgaata  caatgatatc
 321     K  E  I    G  V  S  V    A  Q  L    K  A  T    F  D  E  Y    N  D  I

1081    gcttctggta  aaaagaaaga  tccatatggg  aagaaattct  ttcaaaacgc  gccaatgtca
 341     A  S  G    K  K  K  D    P  Y  G    K  K  F    F  Q  N  A    P  M  S

1141    attaatgaca  atttccacgt  ttctttaatg  tcaccagttt  tacattacac  catgggaggc
 361     I  N  D    N  F  H  V    S  L  M    S  P  V    L  H  Y  T    M  G  G

1201    gttgaagtta  cccctgattc  tgaagtaaaa  gatgtacaag  gaaaaactat  tccagggctt
 381     V  E  V    T  P  D  S    E  V  K    D  V  Q    G  K  T  I    P  G  L

1261    tatgcttctg  gtgaaattgc  tggaggagtt  cacggagcaa  atcgtcttgg  tggatcatct
 401     Y  A  S    G  E  I  A    G  G  V    H  G  A    N  R  L  G    G  S  S

1321    ttattaggat  gtgtcgtatt  tggtcgtgtt  gctggagata  gtgcatcacg  ccatttatta
 421     L  L  G    C  V  V  F    G  R  V    A  G  D    S  A  S  R    H  L  L

1381    caaaatttgt  ccaatgcaac  tgctactcgt  cgacttggac  aaattgctgg  tcaattggct
 441     Q  N  L    S  N  A  T    A  T  R    R  L  G    Q  I  A  G    Q  L  A

1441    ccttatcaag  ccactgttaa  cgtggatcct  acaaatcaaa  aagtacattt  ggaaatttat
 461     P  Y  Q    A  T  V  N    V  D  P    T  N  Q    K  V  H  L    E  I  Y

1501    tggggacaac  agggtggtat  ttctgtcaca  aaaTAA
 481     W  G  Q    Q  G  G  I    S  V  T    K  *
```

Figure 23 Full length genomic DNA and deduced amino acid sequence of *GiFRD*.

The cDNA was synthesised using RNA isolated from spores and extraradical hyphae and digested by restriction enzyme (*Eco*RI, *Not*I, *Hin*dIII, *Bam*HI). Then, obtained fragments were allowed to self-ligate and circulate. On obtained circular DNA, PCR was performed using 5' upstream primers (FRD1, FRD2) and 3' downstream primers (FRD3, FRD4). Obtained sequences were analyzed for ORF and *GiFRD* sequence was determined (Tab. 3). Additionally, the start codon was confirmed by BLAST comparison with fumarate reductase sequences from other organisms. *GiFRD* is 1536 bp long and codes for a protein with 512 amino acid residues with theoretical molecular weight of 55.1 kDa (JN835295) (Fig. 23).

```
S. cerevisiae_Osm1        MIRSVRRVFIYVSIFVLIIVLKRTLSGTDQTSMKQPVVVIGSGLAGLTTSNRLISKY-RI 59
S. cerevisiae_Frds1       ----------------------MSLS---------PVVVIGTGLAGLAAANELVNKY-NI 28
A. adeninivorans          --------------------MPHTEN----------VIVVGAGLAGLSAAHQILQKSPKI 30
G. irregulare_AMykor      ------------------MAS--------------QIIVVGGGLSGLSAAHTVLEHG--A 26
                                                 ::*:* **:**:::: ::.:
S. cerevisiae_Osm1        PVVLLDKAASIGGNSIKASSGINGAHTDTQQNLKVMDTPELFLKDTLHSAKGRGVPSLMD 119
S. cerevisiae_Frds1       PVTILEKASSIGGNSIKASSGINGACTETQRHFHIEDSPRLFEDDTIKSAKGKGVQELMA 88
A. adeninivorans          KVFVLEKMKSTGGNSIKASSGINGAGTPQQERANIHDSPSLFYEDTRKSAKQLGDESLAQ 90
G. irregulare_AMykor      NVLVIDKNSFFGGNSTKATSGINGALTKTQIALGIKDSAALFQEDTTRSARDLARPDLIK 86
                           * ::*    **** **:****** *  *    : *:. ** .** :**:  .  .*
S. cerevisiae_Osm1        KLTKESKSAIRWLQTEFDLKLDLLAQLGGHSVPRTHRSSGKLPPGFEIVQALSKKLKDIS 179
S. cerevisiae_Frds1       KLANDSPLAIEWLKNEFDLKLDLLAQLGGHSVARTHRSSGKLPPGFEIVSALSNNLKKLA 148
A. adeninivorans          TLSERSANAVKWLEQSFGIDLSVVSMLGGHSVARTHRGGGKIPPGFAIMKALGDKLGTYA 150
G. irregulare_AMykor      VLAGNSASAVEWLQDKFNLDLSLVSRLGGHSQPRTHRGK-EMFPGMTITYALMERLEDIA 145
                           *:  *  *:.**: .*.:.*.::: ***** .****.  :: **: *  ** ..*   :
S. cerevisiae_Osm1        SKDSNLVQIMLNSEVVDIELDNQGHVTGVVYMDENGNRKIMKSHHVVFCSGGFG---YSK 236
S. cerevisiae_Frds1       ETKPELVKINLDSKVVDIHEKD-GSISAVVYEDKNGEKHMVSANDVVFCSGGFG---FSK 204
A. adeninivorans          NCQLITEAKVLNLITKEHAGPIGKKVIGVNYLKDNKEESLFG--NVILATGGFS---ASD 205
G. irregulare_AMykor      ENQPNRARIIKKARVTNLIKEG-EEVIGVEYEKDGQTLKEYG--PVILATGGYAADFTEN 202
                          . .         .  . :        : .* * ...          *::.:**:.    ..
S. cerevisiae_Osm1        EMLKEYSPNLIHLPTTNGKQTTGDGQKILSKLGAELIDMDQVQVHPTGFIDPNDRENNWK 296
S. cerevisiae_Frds1       EMLKEYAPELVNLPTTNGQQTTGDGQRLLQKLGADLIDMDQIQVHPTGFIDPNDRSSSWK 264
A. adeninivorans          QLVSKYRPDLVGLPTTNSEGTLGEGLEMCQAVNAELIDADQVQVHPTGFVDPKDPASQSK 265
G. irregulare_AMykor      SLLKKYRPDIYDLPTTNGDHCTGDGHKMVLAIGGKAIDLEKVQVHPTGLVDPKDPDSKIK 262
                          .::.:* *::  *****..   *:* .:   :... ** :::******::**:*  .. *
S. cerevisiae_Osm1        FLAAEALRGLGGILLHPTTGRRFTNELSTRDTVTMEIQSKCPKNDNR--ALLVMSDKVYE 354
S. cerevisiae_Frds1       FLAAESLRGLGGILLNPITGRRFVNELTTRDVVTAAIQKVCPQEDNR--ALLVMGEKMYT 322
A. adeninivorans          FLAGEALRREGGILL--IGGKRFTDELQTRDFVTAKVLESCKRANVSPGSVIALNPAGYD 323
G. irregulare_AMykor      FLAAEALRGVGGLLIN-AEGKRFCDELGHRDYVTGEIWKT--KGPVR----LVLNSKASK 315
                          ***.*:**  **:*:    *:** :**  ** **  : .   :        :.:.
S. cerevisiae_Osm1        NYTNNINFYMSKNLIKKVSIN-DLIRQYDLQTTASELVTELKSYSDVNTK-DTFDRPLII 412
S. cerevisiae_Frds1       DLKNNLDFYMFKKLVQKLTLS-QVVSEYNLPITVAQLCEELQTYSSFTTKADPLGRTVIL 381
A. adeninivorans          KIKHHVDFYVFKGLMKKGTLT-EMCTELNWDVDLVRAEFESYNKVVEGQEADSKGRNILV 382
G. irregulare_AMykor      EIEWHCKHYAGRGLMKKINSGEELAKEIGVSVAQLKATFDEYNDIASGKKKDPYGKKFFQ 375
                          .   : ..*  : *::* .   ::  : .      .   :  .      : *. .: .:
S. cerevisiae_Osm1        NAFDKDISTESTVYVGEVTPVVHFTMGGVKINEKSQVIKKNSESVLSNGIFAAGEVSGGV 472
S. cerevisiae_Frds1       NEFGSDVTPETVVFIGEVTPVVHFTMGGARINVKAQVIGKNDERLLK-GLYAAGEVSGGV 440
A. adeninivorans          LAS-SQPANDGELFWGLTTPVLHFTMGGVHINTSAQVLGAGGNAIEG--LYAAGEVSGGV 439
G. irregulare_AMykor      NAP---MSINDNFHVSLMSPVLHYTMGGVEVTPDSEVKDVQGKTIPG--LYASGEIAGGV 430
                                : :  .. .  :**:*:****..:. .::*    .: :    ::*:**::***
S. cerevisiae_Osm1        HGANRLGGSSLLECVVFGKTAADNIAKLY------------------------------ 501
S. cerevisiae_Frds1       HGANRLGGSSLLECVVFGRTAAESIANDRK------------------------------ 470
A. adeninivorans          HGANRLGGSSLLECVVFGRIAADSSTGAGSSL---------------------------- 471
G. irregulare_AMykor      HGANRLGGSSLLGCVVFGRVAGDSASRHLLQNLSNATATRRLGQIAGQLAPYQATVNVDP 490
                          ************ *****: *.:. :
S. cerevisiae_Osm1        ----------------------
S. cerevisiae_Frds1       ----------------------
A. adeninivorans          ----------------------
G. irregulare_AMykor      TNQKVHLEIYWGQQGGISVTK 511
```

Figure 24 Amino acid sequence alignment of Gifrdp with fumarate reductase protein sequences from other organisms. The alignment was performed using ClustalW software (www.ebi.ac.uk/ClustalW). Identical residues in all sequences are marked with an asterix. The GeneBank accession numbers of FR proteins are as followed: Gifrdp (JN835295), *S. cerevisiae* Osm1p (NP_012585.1), *S. cerevisiae* Frds1p (P32641.1), *A. adeninivorans* Afrd1p (JN835294).

The genomic sequence was obtained by PCR amplification on *G. irregulare* AMykor isolate genomic DNA using primers based on the first and last 20 nucleotides of the *GiFRD* coding sequence (Tab. 4, 5). The genomic *GiFRD* sequence was identical to cDNA sequence, indicating that the open reading frame of *GiFRD* consists of only one exon and no introns.

The predicted protein sequence of Gifrdp has a high degree of similarity to the fumarate reductase of the yeast *A. adeninivorans* Afrd1p (45%) and both proteins from *S. cerevisiae* (Osm1 - 38% and Frds1 - 38%). Alignment of the fumarate reductase protein sequences from those organisms showed a low degree of conservation over the entire sequence of the proteins (Fig. 24). Further examinations (InterProScan, BLAST) showed that the Gifrdp sequence contains the consensus motif which is proposed to be the FAD / NAD(P) binding domain at amino acids position 1 - 461 (1 - 1383 bp), typical for all the known fumarate reductases of yeast and fungi. Also a domain of the redox protein flavocytochrome c was present at amino acid position 4 - 453 (12 - 1359 bp) (InterProScan, EMBL-EBI).

5.2 *Fumarate reductase* for AMF identification

Genomic DNA was isolated from two isolates of *G. irregulare* and one isolate of *Glomus mosseae*. A fumarate reductase sequence fragment (approx. 740 bp) was PCR amplified from isolated genomic DNA using the primers GiFRD_EcoRI (fwd) and GiFRD_SalI (rev) (Tab. 3). The PCR products were cloned into pCR4 vector in *E. coli* TOP10 cells and sequenced with primers M13forw and M13rev (Tab. 3). After sequencing, the forward and reverse sequences were manually edited and combined in single consensus sequences using CHROMASPRO 1.41. Alignment of the sequences obtained both *G. irregulare* isolates and *G. mosseae* isolate was done using Geneious 5.5 programme (Fig. 25) (Drummond *et al*., 2011).

Between both isolates of *G. irregulare* a difference of 6 - 7 bp in this sequence region was demonstrated. Two *Glomus* species, *G. irregulare* and *G. mosseae*, showed difference of 3 - 4 bp in fumarate reductase sequence. This variation influenced amino acids sequence, where 2 - 4 amino acids differed between analyzed organisms (Fig. 25).

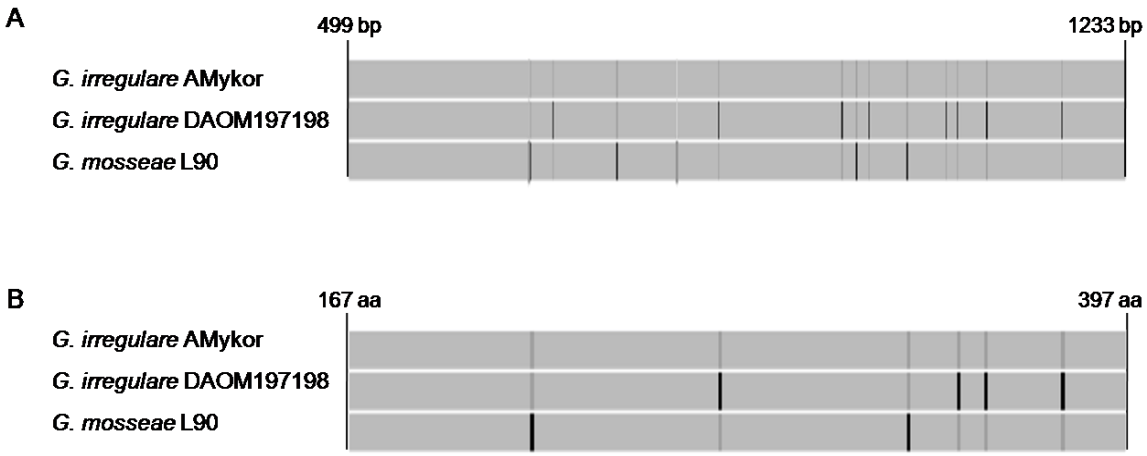

Figure 25 Nucleotides (A) and amino acids (B) sequence comparison between two *G. irregulare* isolates and one *G. mosseae* isolate. Light grey indicates consensus sequences, dark lines indicate sequence variations.

5.3 Transcript analysis in AMF developmental stages

GiFRD RNA accumulation was studied by RT-PCR. PCR primers used in this study are listed in Table. 3 and were designed to amplify gene fragments of the following sizes: 735 bp for *GiFRD*, 575 bp for *β - tubulin*, and 326 bp for *TEF*.

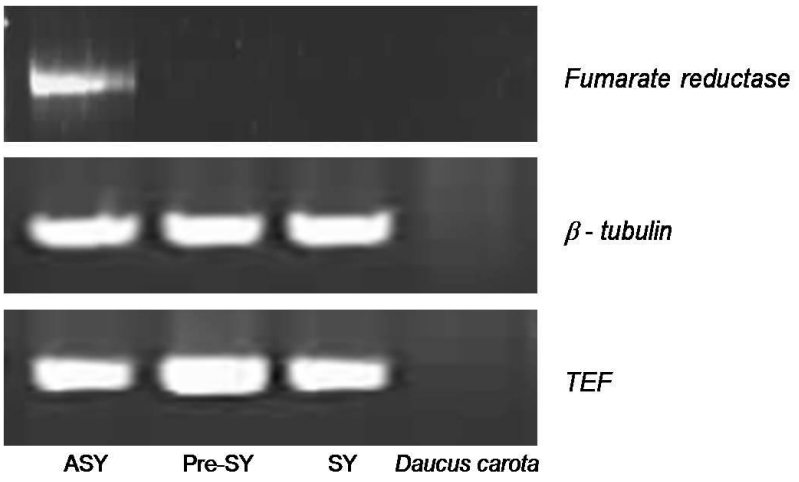

Figure 26 *GiFRD* expression shown by RT-PCR in three developmental stages of *G. irregulare* AMykor (ASY-spores from *in vitro* culture cultivated for 14 days with absence of roots, Pre-SY - spores cultivated for 14 days in the presence of roots exudates, SY1 secondary spores with extra- and intraradical mycelium and roots from an *in vitro* culture). The expression of two housekeeping genes, encoding β-tubulin and TEF, was used as a control to monitor cDNA synthesis.

The transcript analysis was established in three AMF developmental stages: asymbiotic stage (ASY - spores from *in vitro* culture cultivated for 14 days in the absence of roots), presymbiotic stage (Pre-SY - spores from *in vitro* culture cultivated for 14 days in the presence of root exudates in medium but without physical contact with roots) and symbiotic stage (SY - secondary spores with extra- and intraradical mycelium and roots from an *in vitro* culture).

The expression of *GiFRD* was detected only in the asymbiotic stage, ASY (Fig. 26). Expression of the *fumarate reductase* gene was not observed in stages with roots or root exudates present. The expression of two housekeeping genes encoding β-tubulin (Olsson *et al*., 2005) and TEF (Gianinazzi-Pearson *et al*., 2009), was used as a control for cDNA synthesis. The expression level was similar in all stages and no expression was observed in a control sample with *D. carota* cDNA only.

5.4 Role of Gifrdp during oxygen deficiency in yeast cells

To assess whether *GiFRD* encodes a functional enzyme and to verify the physiological role of the *G. irregulare* fumarate reductase, the gene was functionally expressed in *S. cerevisiae.* A fumarate reductase mutant was generated by deletion of both *S. cerevisiae* fumarate reductase genes (*OSM1* and *FRDS1*).

To construct the *S. cerevisiae Δosm1 Δfrds1* mutant, strains Y06849 and Y10288 (Tab. 1) were allowed to mate and sporulate (Spencer *et al*., 1989). The haploid sporulation product of *S. cerevisiae* S0010 *Δosm1 Δfrds1* (BY4741 MATα *his3Δ1; leu2Δ0; met15Δ0; lys2Δ0; ura3Δ0; osm1::kanMX4; frds1::kanMX4*) was identified by PCR reaction on genomic DNA, using primers amplifying DNA sequence upstream and downstream of the *OSM1* gene (OSM1_delet_fwd and OSM1_delet_rev) or *FRDS1* gene (FRDS1_delet_fwd and FRDS1_delet_rev) (Tab. 3). The *S. cerevisiae* S0010 double mutant was transformed with the basic vector pYES2 (S0010/pYES2) and the *GiFRD* expression vector pYES2-GiFRD (S0010/pYES2-GiFRD) (Tab. 2). Strain Y15271 (Tab. 1), which contains both *S. cerevisiae* fumarate reductase genes, was transformed with an empty pYES2 vector, and used as a positive control for all experiments.

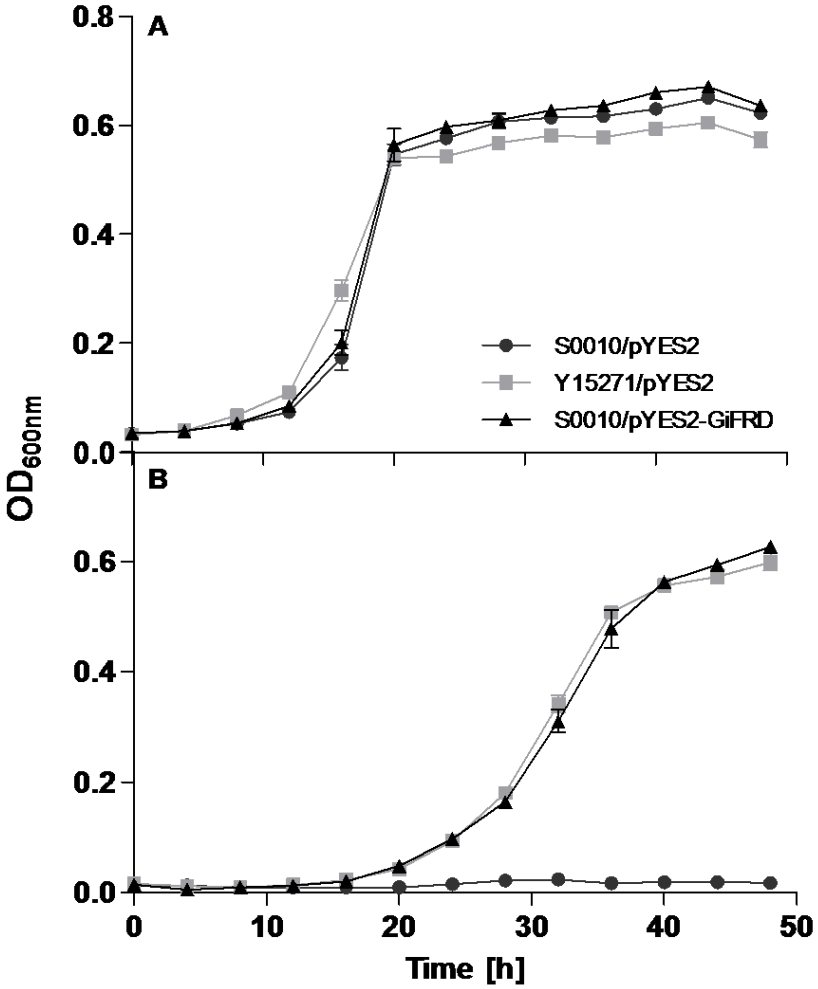

Figure 27 Contribution of *G. irregulare* fumarate reductase to yeast aerobic and anaerobic growth. The *S. cerevisiae* Y15271/pYES2 control strain (■), the S0010/pYES2-GiFRD transformant (▲) and the S0010/pYES2 mutant (●) were grown in 2% galactose SD medium under aerobic (A) and anaerobic (B) conditions. Standard deviations were calculated from three independent experiments (GraphPad Prism 5.04).

Yeast cells were pre-cultured in 1% glucose SD medium for 48 h at 30˚C, with or without shaking, to generate aerobic and anaerobic conditions, respectively. Main cultures, in 2% galactose SD medium, were inoculated with cells from pre-cultures, incubated in corresponding conditions and grown for analysis. Aerobic cultures were grown in Erlenmeyer flasks at 30˚C with shaking (180 x rpm). The anaerobic cultures were prepared as follows. Anaerobic conditions were created by bubbling nitrogen through the medium, for 30 min at 70˚C. The 12 ml plastic tubes were completely filled with the deoxygenated medium containing 2 mg l^{-1} resazurin to monitor anaerobiosis. Resazurin in presence of oxygen is oxidized to the pink coloured resorufin.

The tubes were closed with plastic stoppers and covered with Parafilm®. Inoculation and collection of the samples were done through a needle installed in the stopper for the complete incubation period. The needle also allowed CO_2 to escape and prevented the entry of air to the tube. Cells were grown at 30˚C without shaking.

Wild type-like growth was observed when all transformants were grown under aerobic conditions (Fig. 27A). However, the S0010 mutant (*Δosm1 Δfrds1*) was unable to grow under anaerobic conditions in 2% galactose SD medium, which is in agreement with previous reports (Camarasa *et al*., 2007; Enomoto *et al*., 2002). The transformant S0010/pYES2-GiFRD was able to grow under anaerobic conditions in the same to the control strain Y15271/pYES2 (Fig. 27B). Thus, it was demonstrated that functional substitution of *S. cerevisiae* enzymes by Gifrdp in yeast cells does occur.

5.5 Gifrdp enzymatic activity

To confirm functionality of the enzyme encoded by *GiFRD* in *S. cerevisiae* cells, the *G. irregulare* gene was cloned into the yeast expression vector pYES2 under the control of the *GAL1* promoter and tested for its ability to complement the deletion of the *OSM1* and *FRDS1* genes in the *S. cerevisiae* mutant S0010 under anaerobic conditions.

Permeabilized cells prepared by the procedure described by Miozzari *et al*. (1978) were used for the detection of intracellular fumarate reductase activities in *S. cerevisiae* S288C and S0010/pYES2, Y15271/pYES2 and S0010/pYES2-GiFRD transformants. Cells were grown at 30°C in 2% galactose SD medium under anaerobic conditions, harvested and then permeabilized. The concentration of permeabilized cells was determined from the OD_{600nm} value. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

The activity of fumarate reductase was assayed according to Camarasa *et al*. (2007). The assay mixture contained 0.1 M potassium phosphate buffer, pH 7.5, 0.4 mM FMN and 10 mM fumarate. FMN was completely reduced by the addition of sodium hydrosulfite (70 mM) before addition of the cell extracts. The enzymatic activity was measured spectrophotometrically at 445 nm after 20 min incubation at 30˚C, as the rate of oxidation of $FMNH_2$.

Measurement of the enzymatic activity in the *GiFRD* transformants showed reduced fumarate reductase activity compared to control strains, which confirms the functional substitution of *S. cerevisiae* genes with *G. irregulare* gene (Tab. 12). The pH optimum of the enzyme reaction was 7.2 - 8.0 and the Michaelis constant (K_M), measured with fumarate as substrate, was 1.1 mM.

The fumarate reductase activity was also analyzed in fungal material, in three AMF developmental stages. Permeabilized cells were incubated for 20 min and the enzyme activity was measured. Only in the asymbiotic stage of the AMF development fumarate reductase activity was detected in range of 24 nmol of oxidized $FMNH_2$ per mg of wet weight (Tab. 12).

Table 12 Fumarate reductase activities in parental and mutant *S. cerevisiae* strains (Tab. 1) and in three AMF developmental stages.

S. cerevisiae	Fumarate reductase activity ($OD_{445\,/\,600nm}$)
S288c	0.34
Y15271/pYES2	0.35
S0010/pYES2	0.0
S0010/pYES2-GiFRD	0.13
G. irregulare stage	Fumarate reductase activity (nmol / mg of wet weight)
ASY	24.0
Pre-SY	0.0
SY	0.0

Data is given as the mean of three experiments, and the standard error of the mean is less than 20%.

5.6 Localization of Gifrdp in *S. cerevisiae* cells

The subcellular localization of fumarate reductases in yeast has been reported to be in the cytoplasm and mitochondria (Besteiro *et al*., 2002; Enomoto *et al*., 1996; Muratsubaki & Enomoto, 1998). Sequence analysis of Gifrdp detected no transmembrane domains in the protein sequence (TMHMM Server v.2.0 provided by the Centre for Biological Sequence Analysis at the Technical University of Denmark). SOSUI sequence analysis (Hirokawa *et al*., 1998)

predicted that Gifrdp is a soluble protein. Prediction of subcellular locations, using fuzzy *k*-NN method, indicated a 47.8% possibility that Gifrdp is a cytoplasmic protein (Huang Y & Li Y, 2004). To confirm that prediction, the *GiFRD* genomic sequence was fused with *GFP* gene at its C terminus.

The *GiFRD* sequence was PCR amplified from genomic DNA and subcloned first to pCR4 vector and then to pYES2 expression vector (Fig. 10). Next, amplified *GFP* sequence was cloned into linearized vector pYES2-GiFRD (Fig. 11). The control vector pYES2-GFP was constructed in analogous way (Fig. 12).

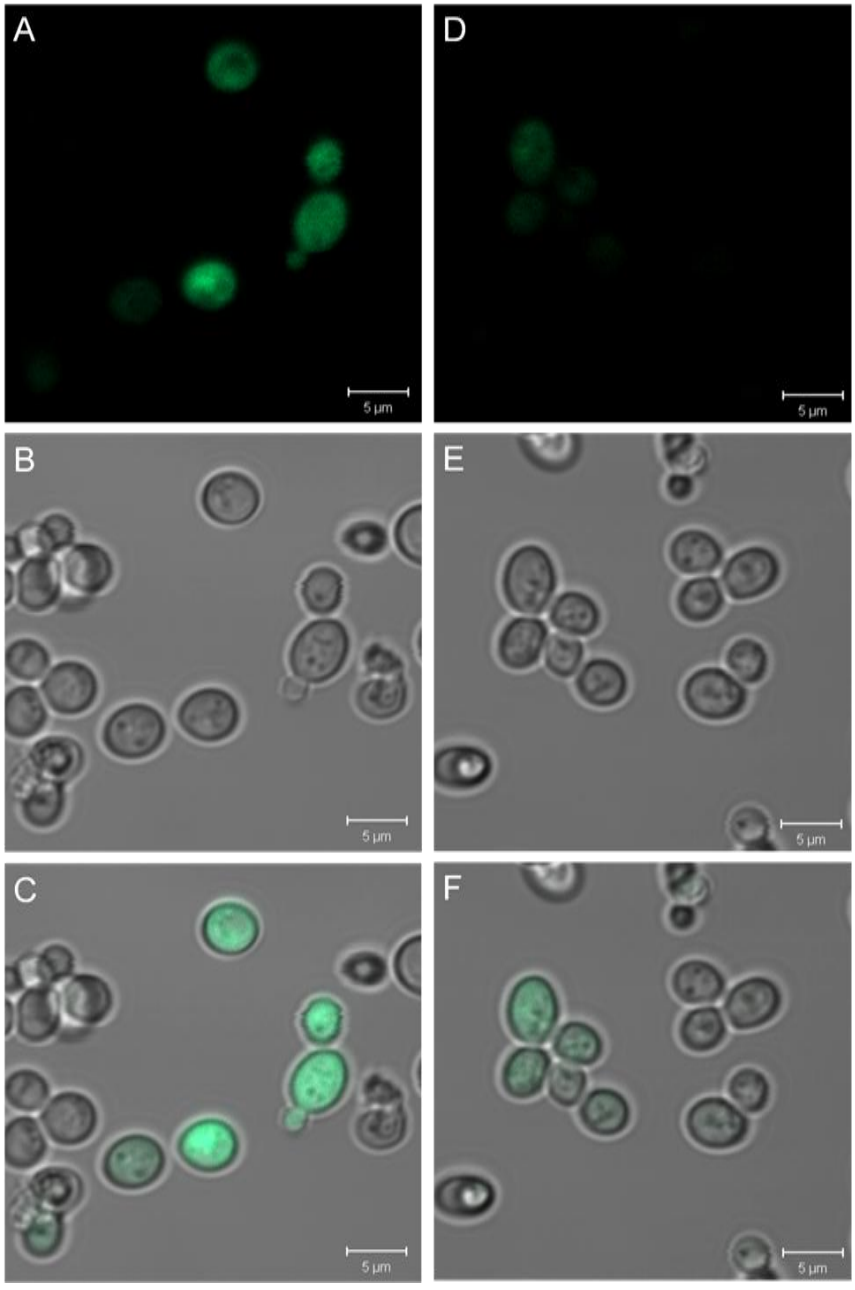

Figure 28 Subcellular localization of Gifrdp. The *S. cerevisiae* double mutant *Δosm1 Δfrds1* transformed with pYES2-GiFRD::GFP (Clone B, Fig. 26) (A-C) or with pYES2-GFP (D-F) were visualized by epifluorescence microscopy (A, D), bright field (B, E) and merged image (C, F).

GFP-tagged Gifrd protein, as well as GFP only, were expressed in the *S. cerevisiae* S0010 mutant (*Δosm1 Δfrds1*). *S. cerevisiae* S0010 mutants transformed with pYES2-GiFRD::GFP or with pYES2-GFP vector were pre-cultured in 1% glucose SD medium for 24 h which were used to inoculate 5 ml of 2% galactose SD medium to a density of 0.1 OD_{600nm}. The cells were incubated for 24 h at 30°C. Then cells were examined with Zeiss Axioskop fluorescence microscope equipment with Zeiss AxioCam HRc camera system.

The yeast cells expressing Gifrd::GFP and GFP protein alone showed cytoplasmic fluorescence. Since previous observation of *S. cerevisiae* S0010 cells showed presence of small vacuoles compare to other strains, such as wild type S288C, obtained results indicated that Gifrdp was targeted to the cytoplasm in *S. cerevisiae* cells (Fig. 28).

To confirm presence of GFP in the transformants, protein gel blot analysis was performed on a total protein extract of transformed yeast cells using a specific antibody raised against GFP. Predicted sizes of proteins were ~82 kDa for Gifrd::GFP and ~28 kDa for GFP. Bands of expected size were observed in all GFP transformants (Fig. 29).

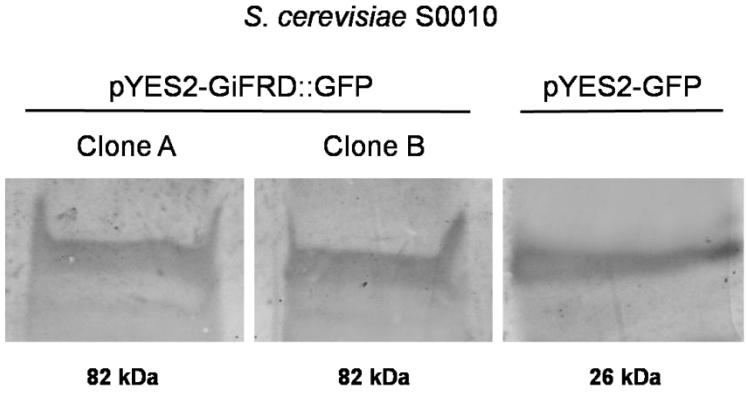

Figure 29 Immunodetection of GFP and fused Gifrd::GFP in *S. cerevisiae* S0010 (*Δosm1 Δfrds1)* mutant transformed with pYES2-GiFRD::GFP (Fig. 28 A-C) or with pYES2-GFP (Fig. 28 D-F). Predicted sizes of target proteins are ~82 kDa for Gifrd::GFP and ~28 kDa for GFP. Total protein of yeast cells was separated by 10% SDS-PAGE gel electrophoresis (Tab. 6), blotted to PVDF membrane and incubated to rabbit anti-GFP.

5.7 Influence of osmotic stress on Gifrdp expression

There are contradictory reports about a possible regulation of fumarate reductase expression by osmotic stress. It was reported that deletion of *OSM1* increases the sensitivity of *S. cerevisiae* cells to hypertonic medium (Singh & Shermann, 1978). In contrast to those results, Camarasa *et al*. (2007) showed that expression of the *OSM1* gene is not controlled by osmotic stress or by oxygen availability. In an attempt to clarify, an experiment was performed to determine whether fumarate reductase presence or absence increases osmosensitivity of *S. cerevisiae* cells. Additionally, AMF are often used for increasing the plant growth on dry and salty soils. The experiments were performed to verify if *GiFRD* gene product might be involved in the AMF adaptation mechanism under salt stress.

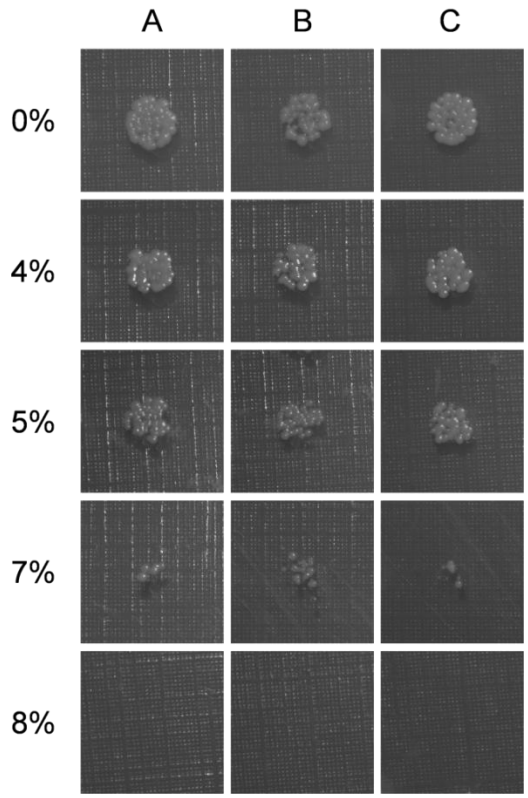

Figure 30 Influence of Gifrdp on the osmotolerance in *S. cerevisiae.* Aliquots (ca. 10^3 cells) of *S. cerevisiae* S0010/pYES2 (A), Y15271/pYES2 (B) and S0010/pYES2-GiFRD (C) grown on 2% galactose SD agar plates supplemented with the indicated NaCl concentrations and cultured for 4 days at 30°C. Pictures were taken on a grey checkered background at the end of the growth period.

The growth of the *S. cerevisiae* S0010 mutant harbouring an empty pYES2 vector (negative control), or a vector containing *GiFRD* and of the *S. cerevisiae* Y15271 mutant harbouring an empty pYES2 vector (positive control) was monitored in presence of different concentrations of sodium chloride (0 - 8%) on selective 2% galactose SD medium plates. After 4 days of incubation at 30°C, all strains were able to grow up to 7% NaCl with the growth rate correlating with the NaCl concentration, higher NaCl concentration higher inhibition of growth. Increasing the NaCl concentration up to 8% NaCl however caused a cessation of growth (Fig. 30). Also, no difference in growth was observed in S0010/pYES2 (negative control) or Y15271/pYES2 (positive control) and the *GiFRD* transformant. Thus, the transformant expressing Gifrdp did not have greater sensitivity to a hypertonic medium than negative control without any fumarate reductase gene.

6. DISCUSSION

6.1 Genetics of *Glomus irregulare* AMykor

AMF have a great influence, directly and indirectly, on life on land, thus, their obvious importance makes it necessary to understand the fundamental biology, genetics, and genomics of Glomeromycotan fungi. Correct identification of AMF isolates is becoming more crucial for basic and applied studies. Accurate recognition of reference fungal isolates, routinely used in different laboratories and deposited in public collections, is also highly desirable.

The first aim of this work was to determine species affiliation of an industrial isolate of AM fungus (AMykor, GmbH, Bitterfeld-Wolfen, Germany) and show the variation in AMF genome sequences within a single spore culture (one isolate) and within and among nuclei.

The Glomeromycotan species were for a long time only defined by morphological characterization. Recent species discrimination of the *Glomus* group A (GlGrA) is based on morphological criteria resulting in some isolates being misplaced. For this reason species verification, based on molecular analysis, of known *Glomus* isolates is important.

This analysis confirmed that the model AM fungus DAOM197198 isolate can't be phylogenetically assigned as *G. intraradices* because it clearly falls into a well-defined clade that included isolates classified as a *G. irregulare* (BEG195 / Att1485-12, DAOM197198 / Att690-23, Błaszkowski and DAOM197198 / Att119-53) (Błaszkowski *et al*., 2008; Stockinger *et al*., 2009). The *Glomus* AMykor isolate, which was the main object in this study, was also a *G. irregulare* species. All AMykor sequences were clustered together with the previously analysed isolate BEG195, which was characterized as *G. irregulare* (Stockinger *et al*., 2009). Even though *G. irregulare* seemed to be morphologically different from the DAOM197198 and the AMykor isolates (Błaszkowski *et al*., 2008), according to molecular data obtained in this study, they represent a separate phylogenetic entity. Although G. *intraradices* and G. *irregulare* appeared as clearly separated groups, they are however, closely related species (Fig. 14). This can be explained by the morphological plasticity making it difficult to microscopically distinguish species within GlGrAb of AMF. In conclusion, it was determined that

two *Glomus* isolates analysed in this study (AMykor and DAOM197198) belong to the *Glomus irregulare* species.

All phylogenetic analyses resulted in a well-supported tree topology. Comparison of the isolates using different length of partial rDNA sequence (~ 1000 bp and ~1700 bp) showed similar resolution (Fig. 14, 15). It can be concluded that partial SSU - ITS1 - 5.8S - ITS2 - partial LSU rDNA sequences of approx. 1000 bp length applied in this study, can be successfully used for species determination in closely related isolates of AMykor and DAOM197198 with statistically good support.

An additional interesting outcome of these analyses was the comparison of partial rDNA sequences isolates from an individual spore. Sanders *et al*. (1995) demonstrated that the evaluation of intraspecific variation within Glomeromycota is complicated by the presence of several different ribosomal variants within the same isolate. Present analysis showed that high polymorphism within the rDNA encoding the SSU, 5.8S, LSU subunits and internal transcribed spacers (ITS) occurs, even in individual spores of both *G. irregulare* isolates (AMykor and DAOM197198) (Fig. 14).

Two different groups of sequences were detected within the 5.8S rDNA region from a single spore isolates of *G. irregulare* DAOM19871 and AMykor (Fig. 16, 17). The main difference between these two sequences-families was deletion of the six-nucleotide fragment GGNAAC. This removal happened as a single deletion event and thereby resulted in relatively high divergence within a very short time. Jansa *et al*. (2002) and Redecker *et al*. (1999) reported similar deletion of a 6 bp fragment from the same rDNA region in another *Glomus* isolate. It looks as though these two variants of the sequence developed independently since clear separation and accumulation of extra mutations occurred within each sequence.

The nuclei are thought to be genetically independent and, as Kuhn *et al*. (2001) suggested, randomly segregated into the spores of offspring. However, it is not clear whether intra-individual genetic diversity of nuclei in the AMF plays role in their physiology and ecological tolerance. The high genetic diversity in single spore could be an evidence for as adaptive mechanism that allowed it to establish a symbiotic relationship with different plants in a whole range of ecosystems (Lanfranco *et al*., 1999).

Using these approaches, variation at the level of single gene sequences, nuclei, and single spores was analyzed and provided valuable insight into the organization and evolution of the AMF genome and its consequences for studies on AMF ecology, molecular biology and AMF commercial inoculum production. Further studies are needed to fully understand the role of AMF intrasporal diversity in their genetics.

To complement information concerning basic genetics of *G. irregulare* genome size and ploidy level was examined. Knowledge of the genome size of *G. irregulare* will be useful in a genome sequencing project and the success of this project will lead to more accure and faster recognition of the genes involved in plant-fungi symbiosis which will result in an improvement of the application of this relationship in agriculture and industry.

To investigate if both isolates differ in their number of rDNA sites, fluorescence *in situ* hybridization (FISH) analysis was performed. While FISH has been repeatedly successfully applied, for example in plants for estimation of the ploidy level and localization of genes on chromosomes (Dhar *et al*., 2002; Dolezel *et al*., 1998; Jiang & Gill, 1994), there are so far only two reports on the use of this method in AMF (Trouvelot *et al*., 1999; Kuhn *et al*., 2001). Here, FISH was successfully used to localize rDNA sequences in flow-sorted interphase nuclei of AM fungus *G. irregulare* (Fig. 19).

Although phylogenetic analysis separated the AMykor and DAOM197198 isolates into different subclades, both mainly showed one or two hybridization signals per nucleus. In a small proportion of nuclei, three or four signals were detectable (Tab. 9). Even though these numbers deviate slightly from the findings of Trouvelot *et al*. (1999) who reported, for *G. irregulare* DAOM197198 isolate, mostly three or five rDNA loci in 25 evaluated nuclei, the order of magnitude is the same. Deviations in the observed numbers can be explained by the possibility that several rDNA loci fuse inside the interphase nuclei into one or two clusters, because of their close association with the nucleolus. However, a splitting of the hybridization signal due to different condensations of the rDNA tracts might also occur, especially when un-sorted nuclei are used.

Assuming *G. irregulare* is haploid (Hijri & Sanders, 2004) this would indicate that the *G. irregulare* genome harbours at least two major rDNA sites

and possibly two minor sites. However, genome polyploidization has been detected in many asexual taxa, and it is an effective way to undermine negative effects of accumulated mutations (Pawlowska & Taylor, 2004). Genome polyploidization, in absence of sexual reproduction in Glomeromycota, might be important for their long-term evolutionary persistence. Presented not conclusive results from FISH analysis favours polyploidy status for *G. irregulare*. Therefore, further analysis on *G. irregulare* ploidy level has to be preformed.

After determination of the species to which both analyzed isolates belong, the genome size of each was also analyzed using flow cytometry. Flow cytometry was previously successfully applied for evaluating the amount of nuclear DNA per nucleus of different Glomeromycotan species (Hosny *et al*., 1998). From such measurements, it is possible to estimate the genome size if the ploidy of the organism is also known.

In present study, the nuclei isolation method was improved, which allowed a rapid analysis of a large number of nuclei and reliable estimation of DNA content (Fig. 20). The average DNA content per nucleus was calculated to be 153.0 ± 3.6 Mb for the *G. irregulare* AMykor isolate and 154.8 ± 6.2 Mb for the *G. irregulare* DAOM197198 isolate based on the converting formula of Dolezel *et al.* (2003). Thus, the nuclei DNA content of both isolates can be estimated to be approx. 150 Mb which is similar to the genome size of *A. thaliana* (157 Mb/1C).

These values are in contrast to data published by Hijri and Sanders (2004) who reported a genome size of 15.74 ± 1.69 Mb for the *G. irregulare* DAOM197198 isolate. The differing values might be explained at least partially by methodological variations. In comparison to Hijri and Sanders (2004), *A. thaliana* was used as an internal reference standard instead of *S. cerevisiae* as an external reference. Although the isolation of nuclei was, due to technical reasons, performed separately for fungal and plant material, fixation, staining and measurement were done under identical conditions. Feulgen densitometry measurements and additional flow cytometry analysis using *R. sativus* as an internal reference standard, resulted in very similar values as the flow cytometry analysis (Fig. 20; Tab. 11). Furthermore, the initial results of genome sequencing of *G. irregulare* DAOM197198 confirmed that the genome is much larger than value suggested by Hijri and Sanders (2004) (Francis Martin, pers. comm.) and are therewith in favour of presented here estimation.

To summarize, the phylogenetic analysis of two *Glomus* isolates, AMykor and DAOM197198 classified them to *G. irregulare* species. The high genetic diversity of rDNA sequences within single spore isolate and individual spore was confirmed and two main variants of 5.8S rDNA with sub-types were determined. The main difference of both variants was deletion of the six-nucleotide fragment GGNAAC. Ploidy level analysis showed that genome of *G. irregulare* harbours at least two major rDNA sites and possibly two minor sites. Nuclei DNA content was similar in both analyzed *G. irregulare* isolates and estimated to be approx. 150 Mb.

Although there are still a number of questions concerning the genetics of AMF to be investigated, the data presented here will be useful in future investigations focusing on understanding the genetics of arbuscular mycorrhizal fungi.

6.2 *Glomus irregulare* fumarate reductase (Gifrdp)

Recently, criticisms have been made of the use of rDNA sequences for fungal barcoding. It is therefore necessary to develop another system for the identification to species level of Glomeromycotan fungi. Kuhn *et al*., (2001) showed that even in single copy gene, such as *BiP* high genetic diversity occurs, so the use of single copy gene for species-level resolution is possible. The focus of this study was a gene encoding fumarate reductase enzyme as a potential candidate for species determination.

Comparison of partial sequences from two *G. irregulare* isolates (AMykor and DAOM197198) and one *G. mosseae* isolate, showed a difference of 3 - 7 bp in an approx. 740 bp length sequence of the *GiFRD* gene (Fig. 25). Observed variations do not allowed resolving Glomeromycotan species. However, further analysis of enzyme encoded by *GiFRD* showed a possible role for fumarate reductase in AMF energy production under oxygen deficiency conditions.

The cellular electron transport chain in the mitochondrion generates a proton motive force which is used to produce chemical energy. This electrochemical proton gradient across a membrane can be used for the synthesis of ATP in aerobically functioning organisms. The respiratory chain oxidizes reduced cofactors, such as NADH, $FMNH_2$ or $FADH_2$, which are produced by catabolic processes like Krebs cycle or β-oxidation. This process results in the reduction of oxygen as it acts as the terminal electron acceptor.

In oxygen deficiency conditions the cell adapts so that it can maintain its redox balance. In eukaryotes two mechanisms can be described that maintain redox balance during hypoxia or anoxia, namely fermentation and dismutation (Van Hellemond & Tielens, 1994).

Fermentation of carbohydrates can be found in many organisms. Production of ethanol from glucose by alcohol dehydrogenase (alcohol fermentation) or glucose degradation to lactate by lactate dehydrogenase is necessary for growth under oxygen deficiency conditions. Unfortunately, fermentation produces a smaller amount of energy compared with the respiratory chain. Aerobic dissimilation of glucose results in 30 ATP molecules per one glucose molecule, while alcoholic fermentation can form only two ATP molecules per glucose molecule. These results from the fact that a proton gradient is not

generated and substrates are not completely oxidized (Snoek & de Steensma, 2007).

In addition to simple fermentation processes, many facultative anaerobic eukaryotes possess glycolytic processes linked to an electron transport chain, such as dismutation. Malate dismutation degrades carbohydrates by the glycolytic pathway to malate. Further reduction of malate to succinate occurs in two reactions that are a reversal of part of the Krebs cycle. One of these processes is mediated by fumarate reductase and it's the reverse reaction of succinate oxidation. This reaction is essential for oxidation of cofactors used to maintain redox balance during dismutation (Camarasa *et al*., 2007; Van Hellemond & Tielens, 1994). Through fermentation via malate dismutation, fumarate is used as an electron sink and linked to electron transport in an anaerobically functioning electron transport chain (Tielens & Van Hellemond, 1998).

The reduction of fumarate is a common process in facultative anaerobic bacteria and eukaryotes, like green algae, parasites and fungi, where at least part of the life cycle occurs in reduced level or complete absence of oxygen. Other ways of maintaining the redox balance during oxygen deficiency is via the action of Gpd3, a glycerol-3-phospate dehydrogenase and Adh2, which is a mitochondrial alcohol dehydrogenase, both of which have been reported in *S. cerevisiae* (Snoek & de Steensma, 2007). These enzymes, together with fumarate reductase, may cooperatively function in maintaining the redox state during hypoxia or anoxia.

The AMF, like other organisms, have to maintain a redox balance under oxygen deficiency conditions that occur in soil. Average oxygen concentration in a clay soil remains below 10% while in the atmosphere this level is 20.9% (Smith & Dowdell, 2006). Additionally, AMF living in wetland habitats are exposed periodically to anaerobic soils. However, the current knowledge is insufficient to answer the question of how they are able to survive in such conditions. Miller and Bever (1999) suggested mechanism by which the fungus could be concentrated near the plant root, obtaining oxygen directly from the root or as oxygen diffuses from the root into the rhizosphere. Brown and Bledsoe (1996) observed AMF in the plant aerenchyma, tissue with large intercellular spaces, the formation of which is associated with plant adaptation to hypoxia.

This suggests that AMF are adapted to life in oxygen-deficient soil during symbiotic stages of their life cycle. However, it is not known how hypoxic conditions are managed during the asymbiotic stage without presence of plant roots. Additionally, none of the genes, that has been characterized in other organisms and demonstrated to be involved in redox balance preservation during absence of oxygen, have yet been identified in AMF.

The results presented in this part of the thesis describe the characterization of a new fumarate reductase gene (*GiFRD*) from the AM fungus *G. irregulare* AMykor isolate. Sequence analysis demonstrated the presence of a gene encoding fumarate reductase protein with high homology to both of the *S. cerevisiae* isoenzymes (38%) (Fig. 24). Based on amino acid identity, cytoplasmic localization and biochemical characteristics, the *G. irregulare* fumarate reductase (Gifrdp) was classified as a equivalent of *S. cerevisiae* Frds1p, the most abundant isoform (Fig. 24, 28). This indicates that Gifrdp belongs to the second class of fumarate reductases, which regenerates the reduced cofactors independently from the electron transport chain.

Using a yeast expression system, it was demonstrated that the protein encoded by *GiFRD* has fumarate reductase activity (Tab. 12).

It was shown that the *S. cerevisiae Δosm1 Δfrds1* mutant is not able to grow under anaerobic conditions, which was expected because fumarate reductase is essential for maintaining an intracellular redox balance during oxygen deficiency. The presence of the *GiFRD* gene however restored the ability of *Δosm1 Δfrds1* mutant to grow under oxygen deficiency conditions, confirming that the *G. irregulare* fumarate reductase is an active enzyme capable of functional complementation of the missing *S. cerevisiae* genes (Fig. 27). However the lack of *S. cerevisiae* fumarate reductases or presence of *G. irregulare* protein was not shown to have any influence on growth in aerobic conditions (Fig. 27) indicating that fumarate reductase enzyme is only essential under anaerobic conditions and is dispensable under aerobiosis.

Functionality of the fumarate reductase, imply that Gifrdp may play a key role in the ability of the fungus to deal with oxygen deficiency present in soil by regulation of the redox state, as previously shown for *S. cerevisiae* (Enomoto *et al*., 2002).

Additionally, it was shown that *GiFRD* transformants were not affected by the presence of salt in the medium because the *S. cerevisiae* negative (S0010/pYES2) and positive (Y15271/pYES2) controls and the *GiFRD* transformant showed no difference in their growth in hypertonic medium (Fig. 30). This indicates that the presence of fumarate reductase is not required for yeast growth under osmotic stress.

Transcription analysis in the developmental stages of the fungus and the presence of enzymatic activity in *S. cerevisiae Δosm1 Δfrds1* mutant expressing *GiFRD* gene confirms the existence in AM fungus *G. irregulare* of a functional fumarate reductase which reoxidise cellular $FMNH_2$ / $FADH_2$ generated by anaerobic metabolism. Analysis of *GiFRD* expression and fumarate reductase activity in the life cycle revealed that it was only expressed in the asymbiotic phase of *G. irregulare* life cycle, where spores exist without physical contact with roots and roots exudates (Fig. 26).

It has been suggested that root exudates influence fungal respiratory activity (Tamasloukht *et al*., 2003; Besserer *et al*., 2006) and that O_2 consumption clearly showed higher fungal respiratory activity after the addition of root exudates. The gene encoding pyruvate carboxylase showed 10 - fold RNA levels after addition of the root exudates. This could be caused by the need to more effectively incorporate additional carbon sources obtained from the plant into oxaloacetate, which is required for a range of processes important to maintain fungal growth, such as gluconeogenesis for cell wall or ribonucleotide synthesis, amino acid biosynthesis. Tamasloukht *et al*., (2003) showed that during asymbiotic growth, AMF spores demonstrate a low respiratory activity. Probably, they are using alternative electron transport pathways to minimize carbon consumption from their own resources such as glycogen and lipids.

The fact that *GiFRD* expression was present only in the asymbiotic stage demonstrates existence of at least one metabolic pathway involved in anaerobic metabolism in the only plant-independent phase of fungus life (Fig. 26). Further, the absence of *GiFRD* expression in the presymbiotic stage indicates that the switch from the asymbiotic to the symbiotic phase coincides with change from anaerobic to aerobic metabolism. This suggests that the AMF behave as facultative anaerobes in the asymbiotic stage.

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8. SUBSCRIPTION

8.1 Figures

8.2 Tables

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Educational Background

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10/2006 - 07/2007	Master's thesis: "Comparison of replisome and segregosome localization in *Streptomyces coelicolor* mycelium" in Laboratory of Molecular Biology of Microorganisms in Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław, Poland, under the supervision of Prof. Dr. Hab. Jolanta Zakrzewska - Czerwińska
10/2002 - 07/2007	Master student at Wrocław University of Technology in Wrocław, Poland. Faculty of Chemistry, Major – Biotechnology, specialization– Molecular Biotechnology and Biocatalysis.
09/1998 - 06/2002	Juliusz Slowacki Secondary School in Kielce, Poland, specialization: Biology and Chemistry.
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Publications

Sedzielewska Kinga A, Jörg Fuchs, Eva M. Temsch, Keith Baronian, Roland Watzke and Gotthard Kunze (2011) Estimation of the *Glomus intraradices* nuclear DNA content. New Phytologist 192: 794- 797.

Sedzielewska Kinga A, Katja Vetter, Rüdiger Bode, Keith Baronian, Roland Watzke and Gotthard Kunze (2012) *GiFRD* encodes a protein involved in anaerobic growth in the arbuscular mycorrhizal fungus, *Glomus intraradices*. Fungal Genetics and Biology 49: 313-321.

Sedzielewska Kinga A, Erik Böer, Carmen Bellebna, Thomas Wartmann, Rüdiger Bode, Michael Melzer, Keith Baronian and Gotthard Kunze (2012) Role of the *AFRD1* encoded fumarate reductase in hypoxia and osmotolerance in *Arxula adeninivorans* (accepted).

Sedzielewska Kinga A, Dorothee Klemann, Nicolai M. Nürk, Thomas Boller and Gotthard Kunze. Intrasporal rDNA variations in AMF *Glomus irregulare* (in preparation).

Conference attendance

2011 March 15th - 20th	The 26th Fungal Genetics Conference at Asilomar, CA, USA. Poster presentation: A molecular approach to characterize *Glomus* AMykor isolate.
2010 Nov 25th - 26th	14. Tagung der PG Mikrobielle Symbiosen, Halle (Saale), Germany.
2010 June 15th - 18th	6th Plant Science Student Conference, IPK, Gatersleben, Germany. Oral presentation: Symbiotic glomeromycota *Glomus intraradices* – diagnostic investigation.
2009 June 23th - 26th	5th Plant Science Student Conference, Leibniz Institute of Plant Biochemistry, Halle/Saale, Germany. Poster presentation: Isolation of a gene cluster and characterization of enzymes for nitrate assimilation from symbiotic glomeromycota *Glomus intraradices*.

Professional experience

02/2009-05/2012	PhD project in Yeast Genetics Group in IPK, Gatersleben, Germany.
05/2008 - 02/2009	Scientific employee on the project "Hormone biosensor system in *Arxula adeninivorans*" in Yeast Genetics Group in IPK, Gatersleben, Germany.
11/2007 - 05/2008	Traineeship under the Leonardo da Vinci Scholarship in Yeast Genetics Group in IPK, Gatersleben, Germany. Theme of practice: "Differential expression of *Glomus intraradices* genes during the asymbiotic and symbiotic phase of the fungus".
7/2007 - 10/2007	Internship in Nestle-Winiary Factory in Kalisz, Poland (production control).
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7/2004 - 8/2004	Training in Microbiology Laboratory in Sanitary Inspection in Kielce, Poland.

ERKLÄRUNG

Hiermit erkläre ich,

dass diese Arbeit bisher von mir weder an der Mathematisch - Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich,

dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Gatersleben, 31.01.2012

Kinga Anna Sedzielewska

APPENDIX

Tables of all sequence used in phylogenetic analysis with the proper database accession numbers.

Table 13 Sequences used for analysis of ~ 1000 bp rDNA region.

Isolate	rDNA sequence / clone	Accession number
Glomus AMykor isolate	AMykor_ITS_1.1	JF820390
Glomus AMykor isolate	AMykor_ITS_1.2	JF820391
Glomus AMykor isolate	AMykor_ITS_1.3	JF820392
Glomus AMykor isolate	AMykor_ITS_1.4	JF820393
Glomus AMykor isolate	AMykor_ITS_1.5	JF820394
Glomus AMykor isolate	AMykor_ITS_1.6	JF820395
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Glomus AMykor isolate	AMykor_ITS_1.10	JF820399
Glomus AMykor isolate	AMykor_ITS_2.1	JF820400
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Glomus AMykor isolate	AMykor_ITS_2.4	JF820403
Glomus AMykor isolate	AMykor_ITS_2.5	JF820404
Glomus AMykor isolate	AMykor_ITS_2.6	JF820405
Glomus AMykor isolate	AMykor_ITS_2.7	JF820406
Glomus AMykor isolate	AMykor_ITS_2.8	JF820407
Glomus AMykor isolate	AMykor_ITS_2.9	JF820408
Glomus AMykor isolate	AMykor_ITS_2.10	JF820409
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Glomus AMykor isolate	AMykor_ITS_3.4	JF820413
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Glomus AMykor isolate	AMykor_ITS_4.8	JF820427

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Glomus DAOM197198 isolate	DAOM197198_ITS_9.4	JF820553
Glomus DAOM197198 isolate	DAOM197198_ITS_9.5	JF820554
Glomus DAOM197198 isolate	DAOM197198_ITS_9.6	JF820555
Glomus DAOM197198 isolate	DAOM197198_ITS_9.7	JF820556
Glomus DAOM197198 isolate	DAOM197198_ITS_9.8	JF820557
Glomus DAOM197198 isolate	DAOM197198_ITS_9.9	JF820558
Glomus DAOM197198 isolate	DAOM197198_ITS_9.10	JF820559
Glomus DAOM197198 isolate	DAOM197198_ITS_10.1	JF820560
Glomus DAOM197198 isolate	DAOM197198_ITS_10.2	JF820561
Glomus DAOM197198 isolate	DAOM197198_ITS_10.3	JF820562

Glomus DAOM197198 isolate	DAOM197198_ITS_10.4	JF820563
Glomus DAOM197198 isolate	DAOM197198_ITS_10.5	JF820564
Glomus DAOM197198 isolate	DAOM197198_ITS_10.6	JF820565
Glomus DAOM197198 isolate	DAOM197198_ITS_10.7	JF820566
Glomus DAOM197198 isolate	DAOM197198_ITS_10.8	JF820567
Glomus DAOM197198 isolate	DAOM197198_ITS_10.9	JF820568
Glomus DAOM197198 isolate	DAOM197198_ITS_10.10	JF820569
Glomus DAOM197198 isolate	DAOM197198_ITS_11.1	JF820570
Glomus DAOM197198 isolate	DAOM197198_ITS_11.2	JF820571
Glomus DAOM197198 isolate	DAOM197198_ITS_11.3	JF820572
Glomus DAOM197198 isolate	DAOM197198_ITS_11.4	JF820573
Glomus DAOM197198 isolate	DAOM197198_ITS_11.5	JF820574
Glomus DAOM197198 isolate	DAOM197198_ITS_11.6	JF820575
Glomus DAOM197198 isolate	DAOM197198_ITS_11.7	JF820576
Glomus DAOM197198 isolate	DAOM197198_ITS_11.8	JF820577
Glomus DAOM197198 isolate	DAOM197198_ITS_11.9	JF820578
Glomus DAOM197198 isolate	DAOM197198_ITS_11.10	JF820579
Glomus DAOM197198 isolate	DAOM197198_ITS_12.1	JF820580
Glomus DAOM197198 isolate	DAOM197198_ITS_12.2	JF820581
Glomus DAOM197198 isolate	DAOM197198_ITS_12.3	JF820582
Glomus DAOM197198 isolate	DAOM197198_ITS_12.4	JF820583
Glomus DAOM197198 isolate	DAOM197198_ITS_12.5	JF820584
Glomus DAOM197198 isolate	DAOM197198_ITS_12.6	JF820585
Glomus DAOM197198 isolate	DAOM197198_ITS_12.7	JF820586
Glomus DAOM197198 isolate	DAOM197198_ITS_12.8	JF820587
Glomus DAOM197198 isolate	DAOM197198_ITS_12.9	JF820588
Glomus DAOM197198 isolate	DAOM197198_ITS_12.10	JF820589
Glomus irregulare BEG195	*G_irreg*_BEG195_FM865593	FM865593
Glomus irregulare BEG195	*G_irreg*_BEG195_FM865588	FM865588
Glomus irregulare BEG195	*G_irreg*_BEG195_FM865589	FM865589
Glomus irregulare BEG195	*G_irreg*_BEG195_FM865596	FM865596
Glomus irregulare Błaszkowski	*G_irreg*_Blaszkowski_FJ009604	FJ009604
Glomus irregulare Błaszkowski	*G_irreg*_Blaszkowski_FJ009602	FJ009602
Glomus irregulare Błaszkowski	*G_irreg*_Blaszkowski_FJ009635	FJ009635
Glomus irregulare Błaszkowski	*G_irreg*_Blaszkowski_FJ009600	FJ009600
Glomus irregulare DAOM197198	*G_irreg*_DAOM197198_FM865617	FM865617
Glomus irregulare DAOM197198	*G_irreg*_DAOM197198_FM865611	FM865611
Glomus irregulare DAOM197198	*G_irreg*_DAOM197198_FM992383	FM992383
Glomus irregulare DAOM197198	*G_irreg*_DAOM197198_FM992386	FM992386
Glomus irregulare DAOM197198	*G_irreg*_DAOM197198_FM992379	FM992379
Glomus irregulare DAOM197198	*G_irreg*_DAOM197198_FM992387	FM992387
Glomus irregulare DAOM197198	*G_irreg*_DAOM197198_FM865609	FM865609
Glomus irregulare DAOM197198	*G_irreg*_DAOM197198_FM865656	FM865656
Glomus proliferum MUCL4182	*G_prolif*_MUCL4182_FM992399	FM992399
Glomus proliferum MUCL4182	*G_prolif*_MUCL4182_FM992391	FM992391

Glomus proliferum MUCL4182	*G_prolif*_MUCL4182_FM992388	FM992388
Glomus intraradices INVAM	*G_intra*_INVAM_FL208_FM865567	FM865567
Glomus intraradices INVAM	*G_intra*_INVAM_FL208_FM865561	FM865561
Glomus intraradices INVAM	*G_intra*_INVAM_FL208_FM865571	FM865571
Glomus intraradices INVAM	*G_intra*_INVAM_FL208_FM865584	FM865584
Glomus intraradices MUCL49410	*G_intra*_MUCL49410_FM865547	FM865547
Glomus intraradices MUCL49410	*G_intra*_MUCL49410_FM865548	FM865548
Glomus intraradices MUCL49410	*G_intra*_MUCL49410_FM865545	FM865545
Glomus intraradices MUCL49410	*G_intra*_MUCL49410_FM865549	FM865549
Glomus clarum Att894_7	*G_clar*_Att894_7_FM865539	FM865539
Glomus clarum Att894_7	*G_clar*_Att894_7_FM865540	FM865540
Glomus clarum Att894_7	*G_clar*_Att894_7_FM865538	FM865538
Glomus clarum Att894_7	*G_clar*_Att894_7_FM865541	FM865541

Table 14 Sequences used for analysis of ~ 1700 bp rDNA region.

Isolate	rDNA sequence_clone	Accession number
Glomus DAOM197198 isolate	DAOM_ITS_1.2b	JN417510
Glomus DAOM197198 isolate	DAOM_ITS_1.2a	JN417511
Glomus DAOM197198 isolate	DAOM_ITS_1.3	JN417512
Glomus DAOM197198 isolate	DAOM_ITS_1.8	JN417513
Glomus DAOM197198 isolate	DAOM_ITS_1.12	JN417514
Glomus DAOM197198 isolate	DAOM_ITS_2.19	JN417515
Glomus DAOM197198 isolate	DAOM_ITS_2.14	JN417516
Glomus DAOM197198 isolate	DAOM_ITS_2.10	JN417517
Glomus DAOM197198 isolate	DAOM_ITS_2.4	JN417518
Glomus DAOM197198 isolate	DAOM_ITS_2.18	JN417519
Glomus AMykor isolate	AMykor_ITS_6.14	JN417520
Glomus AMykor isolate	AMykor_ITS_6.38	JN417521
Glomus AMykor isolate	AMykor_ITS_6.23	JN417522
Glomus AMykor isolate	AMykor_ITS_6.11	JN417523
Glomus AMykor isolate	AMykor_ITS_6.39	JN417524
Glomus AMykor isolate	AMykor_ITS_6.12	JN417525
Glomus AMykor isolate	AMykor_ITS_8.53	JN417526
Glomus AMykor isolate	AMykor_ITS_8.23	JN417527
Glomus AMykor isolate	AMykor_ITS_8.22	JN417528
Glomus AMykor isolate	AMykor_ITS_8.33	JN417529
Glomus AMykor isolate	AMykor_ITS_8.21	JN417530
Glomus AMykor isolate	AMykor_ITS_8.7	JN417531