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Advances in Molecular Diversity of Arbuscular Mycorrhizal Fungi (Phylum Glomeromycota) in Forest Ecosystems

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1. Introduction

Glomeromycota is a fungal phylum scientifically recognized in 2001 as monophyletic group which probably diverged from the same common ancestor as the Ascomycota and Basidiomycota (Schüssler et al., 2001). Glomeromycota comprises arbuscular mycorrhizal fungi (AMF), important simbiont of land plants and the endocytobiotic fungus, *Geosiphon pyriformis*. Despite of fungi worldwide distribution, relatively few species have been described. The AM fungi are an interesting case: like other fungi they are distributed organisms with apparently widespread mycelial networks, but no sexual stage has been identified in any species in the phylum, they cannot easily be observed or located in situ as they have no conspicuous above-ground fruiting body, and they cannot yet be grown in axenic culture in the absence of plant roots. From 1,5 million microbial fungal species in soils worldwide estimated (Torsvik et al., 1990), about 214 currently described species are glomeromycetes within a universe of 97,000 species of fungal kingdom described so far.

Mycorrhizas are ubiquitous in terrestrial ecosystems. Around 80% of plant species that have been studied form the symbiosis (Wang & Qiu, 2006). There are several different types of mycorrhiza, in which different plant and fungal taxa are involved, but the arbuscular mycorrhizas (AMs) form a monophyletic group of obligate plant symbiotic fungi belonging to the Phylum Glomeromycota and around two-thirds of plant species, is both the most widespread and ancestral: all modern plants have ancestors that formed AM symbioses (Helgason & Fitter, 2009).

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Furthermore, the scientific community remains discussing several morphologic characters and its limitations to the species identification. Studies based on rDNA sequence have often confirmed the morphologically defined species and the molecular data have erected new genera and families, revealing a considerable unknown AM diversity. To identify fungi from various substrates and in different life stages to species level, the most frequently sequenced region is the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA, however other regions are informative as 18S rRNA, small subunit ribosomal of rRNA (SSU), large subunit ribosomal of rRNA (LSU). Thus, molecular methods are potential tools to cover the diversity of this group.

It is well known that mycorrhizal associations take up mineral nutrients from the soil and exchange nutritional elements with plants for photosynthetically fixed carbon (Smith & Read, 2008). The main nutrient is phosphorous (P) and AMF structures assimilates P from lower concentrations in the soil at which normal plant roots fail (Jefferies et al., 2003). Moreover, these fungi can uptake K, Zn, Fe, Cu, Mg and Ca. The mechanism of absorption is based on major surface area of roots of plants and exploring soil by extraradical hyphae beyond the root hair. The element is then biochemically transformed at external hyphae and passed to the arbuscules for being ultimately transferred to the host plant (Azcon-Aguilar & Barea, 1996).

The plant hosts of AMF are mostly angiosperms, some gymnosperms, pteridophytes, lycopods and mosses (Smith & Read, 1997) which comprises over 80% of all terrestrial plant species. The AM symbiosis is associated with a range of additional benefits for the plant including the acquisition of other mineral nutrients, such as nitrogen and resistance to a variety of stresses (Kaapor et al., 2008) or pathogens (Rabie, 1998), soil aggregation, and carbon sequestration (van der Heijden et al., 2008). AMF can mitigate the effects of extreme variations in temperature, pH and water stress (Michelsen & Rosendahl, 1990; Siqueira, 1994; Augé 2001). For water stress, recent studies have also addressed how symbiosis and water stresses interact to modify the function and expression of plant aquaporins.

Aquaporins are integral membrane proteins that function as gradient-driven water and / or solute channels present in plants. The effect of mycorrhiza interaction in aquaporin expression was described for several plant species, including the tree poplar (Marjanovic et al., 2005), though the microsymbiont was an ectomycorrhizal fungus. An aquaporin protein was recently cloned from an AMF, *Glomus intraradices*, by Aroca et al. (2009), but the interaction between the plant and fungi aquaporins remains unclear, as suggested by the authors, that the fungus aquaporin expression is due to plant. These results came from studies with culture plant species, opening a new view to investigate the expression profile of aquaporins from tree species and its rules at different ecosystems. An aquaporin (water channel) gene from an AM fungus (*Glomus intraradices*), which was named GintAQP1, was reported from experiments in different colonized host roots growing under several environmental conditions. It seems that gene expression is regulated in a compensatory way regarding host root aquaporin expression (Aroca et al., 2009). At the same time, from in vitro experiments, it was shown that a signaling communication between NaCl-treated mycelium and untreated mycelium took place in order to regulate gene expression of both GintAQP1 and host root aquaporins. The authors suggested that specific communication could be involved in the transport of water from osmotically favorable growing mycelium or host roots to salt-stressed tissues.

Despite their importance to plant productivity and sustainability of agricultural systems (Barea, 1991; Smith & Read, 1997), AMF are widely distributed through the most diverse forest ecosystems, as rain forest in southern Queensland-Australia (Gehring & Connell, 2006), Clintonia borealis roots from a boreal mixed forests in northwestern Québec (DeBellis & Widden 2006), hot-dry valley of Jinsha River, southwest China (Dandan and Zhiwei, 2007), forest with *Araucaria angustifolia* in Brazil (Moreira et al., 2007; Moreira et al. 2009; Patreze et al., 2009), in the Atlantic Forest in Southeastern Brazil (Zangaro et al., 2007, 2009), *Hepatica nobilis* Mill. site type spruce forest at central Estonia (Uibopuu et al., 2009), semi-evergreen tropical forest at southeast Mexico (Ramos-Zapata et al., 2011a), *Podocarpus cunninghamii* forests from New Zealand (Williams et al., 2011), young and old secondary forest in Western Brazilian Amazon (Stürmer & Siqueira, 2011), coastal dunes of Sisal, Mexico (Ramos-Zapata et al., 2011b) and floodplain islands as recently related at northeastern Italy (Harner et al., 2011). The observations of AMF occurrence in aquatic environment corroborate the hypothesis of coevolution with the first established plants (Berbee & Taylor, 1993).

Arbuscular mycorrhizas are abundant in herbaceous species as well as in tropical and temperate forest tree species (Harley & Harley, 1987; Newman et al., 1994). The temperate and boreal forest ecosystems are the best studied; however, AM are poorly investigated in these ecosystems since the most of tree species form ectomycorrhizas, a fungal polyphyletic group. In the Pinaceae family, for example, very common in temperate forests, a few species are able to form both ecto- and arbuscular mycorrhizas, e.g., *Pinus muricata*, *Pinus banksiana*, *Pinus strobus*, *Pinus contorta* and *Picea glauca x Picea engelmannii* (O'Dell et al., 1993; Horton et al., 1998; Wagg et al., 2008). Another temperate tree species, Douglas fir, showed over 200 morphologically distinct ectomycorrhizas in southern Oregon (Luoma et al., 1997). The authors reported that after disturbance such as fire burning or logging, roots of Douglas fir seedlings can also be colonized by arbuscular mycorrhizas.

On the other hand, the tropical forests are an outstanding biodiversity hotspot for vascular plants and consequently it is expected a high fungal diversity, including the AM which are symbiotic organisms of vascular plants. These fungi are important as they play a key role for nutrient cycling and nutrient retention in the humus layers. Then, tropical forests rich in plant species from diverse families are considered to be dominated by AM-forming trees; however, relatively few studies have focused at the molecular AM diversity in natural ecosystems and this knowledge is a major bottleneck in mycorrhizal ecology.

In tropical areas, most AMF propagules have shown seasonal fluctuations in abundance either as spores (Guadarrama & Alvarez-Sánchez, 1999, Silva-Júnior & Cardoso, 2006) or in colonized roots (Ramos-Zapata et al., 2011b). The approach which has been conducted is the evaluation of soil quality by direct counts of spores extracted from soil (Carvalho et al., 2003; Moreira et al., 2009), through assessment of percentage of colonized roots (Moreira et al., 2007, Patreze et al., 2009), and estimation of length and biomass of hyphae in the soil using hyphal ^{32}P-labelling (Pearson & Jakobsen, 1993). According to Rosendahl (2008), quantitative studies of arbuscular mycorrhizal fungal communities based on the presence of spore numbers are complicated as some species produce few spores on the mycelium, whereas species such as *G. intraradices*, *G. versiforme* or *G. fasciculatum* produce hundreds of spores on the same hypha. Another technique, the Most Probable Number (MPN) method is a microbiological approach that allows the detection of AMF species which do not produce

spores (Troeh & Loynachan, 2003) by soil dilution. However, there are several experimental variables which may influence the final estimations. Several experiments have been carried out at greenhouse for evaluation of dependency and responsiveness to arbuscular mycorrhizal fungi in tree species, as cedar, *Cedrela fissilis* Vell. (Rocha et al., 2006), that occurs in different biomes from Brazil. Siqueira et al. (2010) published recently a book summarizing 30 years of research with mycorrhizal fungi in Brazil. The mycotrophic tree species was discussed in respect to potential use for restoration of degraded land (Soares & Carneiro, 2010) in tropical and arid ecosystems. The use of mycotrophic species in agroforestry systems was also discussed and several examples were discussed, as the studies for peach palm (*Bactris gasipaes* Kunth) and cupuaçu (*Theobroma grandiflorum* (Willd ex Spring) K. Schum) at the central part of the Amazon region in Brazil (Silva-Júnior & Cardoso, 2006).

We propose in this chapter to discuss the advances in molecular diversity of Glomeromycota in forest ecosystems focusing on some aspects related to DNA target regions for sequencing tools, on different approaches on molecular diversity applied to fungal researches and the presence of Glomeromycota in natural and impacted forests. Finally, we will address the challenges to the development of new areas, as genomic and metagenomic applied to mycorrhizal studies.

2. New approaches from molecular techniques

Studies in planta have shown the inability to obtain axenic cultures and the difficulties associated with identifying AMF, made more difficult to establish in the past, advanced studies on their ecology, genetics, and evolution. In the past decade, considerable effort has been expended to understand the keystone ecological position of AM symbioses, most studies have been limited in scope to recording organism occurrences and identities, as determined from morphological characters and ribosomal sequence markers for characterization of AMF, leading to important advances in our understanding of the phylogeny (Schüssler et al., 2001; Schwarzott et al., 2001), ecology (Helgason et al., 1998; Helgason et al., 2002; Husband et al., 2002a, b; Kowalchuk et al., 2002), genetics (Gianinazzi-Pearson et al., 2001; Harrison, 1999), and evolution (Gandolfi et al., 2003; Sanders, 2002) of this group of obligatory symbiotic fungi. rRNA genes have become the most widely use targets for detection of AMF in environmental samples (Clapp et al., 2002). Several PCR-based strategies targeting rRNA genes have more recently been developed to detect AMF in DNA extracted from roots, soil, or spores (van Tuinen et al., 1998; Helgason et al., 1998; Kjoller & Rosendahl, 2000; Kowalchuk et al., 2002; de Souza et al., 2004). Gamper et al. (2010) proposed a shift toward plant and fungal protein-encoding genes as more immediate indicators of mycorrhizal contributions to ecological processes. A number of candidate target genes, involved in the uptake of phosphorus and nitrogen, carbon cycling, and overall metabolic activity were proposed, and advantages and disadvantages of future protein-encoding gene marker and current (phylo-) taxonomic approaches are offered as new strategy for studying the impact of AM fungi on plant growth and ecosystem functioning.

Molecular approaches to community ecology may minimize data variation in the morphological characters that hamper traditional taxonomy and have revealed a considerable unknown AM diversity from colonized roots (Rosendahl, 2008) and soils under

different land uses. In forest ecosystems, different groups of fungi, bacteria, algae, and microfauna communities living within the first soil layers can be altered by several factors. The relationship between diversity of fungal communities and resource available and the relation of fungal communities to the greater plant diversity remains under discussion. Using the Ribosomal Intergenic Spacer Analysis (RISA), Waldrop et al. (2006) found no significant effect of plant diversity on the number of fungal ITS bands. However, many other factors unrelated to plants, but inherent to soil (climate, parent material, slope), may influence fungal diversity and they are not easily controlled (Waldrop et al., 2006). The opposite also is possible, i.e. the existence of effects of AM on plants composition and development of plant communities, despite your action to the nutritional status of individual plants (Grime et al., 1987; van der Heijden et al., 1998).

The first steps to better understand the plant host-arbuscular mycorrhizal fungus (AMF) interaction in forest ecosystems could be the deeper studies on plant growth response to different natural soil inocula and upgrading of knowledge about the AM species composition from each community. Williams et al. (2011) observed that pre-inoculation of tree seedlings of *Podocarpus cunninghamii* propagated in glasshouse from cuttings with forest AMF-inoculums collected from a remnant *P. cunninghamii* forest could improve restoration success in comparison to the ex-agricultural AMF community used as inoculums. This last community was less mutualistic than the forest AMF community. These results have potential implications for forest restoration, predicting for example the effect of future forest management on understory forest vegetation. The molecular tools might complement such data helping the identification of mycorrhizal species in different forest communities. An accurate assessment of species richness and community composition is crucial to understanding the role of AMF in ecosystem functioning.

2.1 DNA target regions for sequencing tools

Molecular techniques were developed primarily for the identification of ectomycorrhizas (Gardes et al., 1991; Gardes & Bruns, 1993), and later for the analysis of arbuscular mycorrhizal fungi (AMF) (Lanfranco et al., 1999; Schüssler et al., 2001). Ectomycorrhizal fungi are a large diverse group of an estimated 5000–6000 different species belonging to the Basidiomycota and Ascomycota (Molina et al., 1992), which are very common in forest ecosystems, mainly in temperate and boreal forest ecosystems, where most tree species form ectomycorrhizas (Ducic et al., 2009).

All ribosomal genes (rRNA) are conserved in eukaryotes genomes and they are present in tandem repetition. The regions 18S, 5.8S and 28S are the most conserved, which allows the primer design to amplify the variable and informative sequences (internal transcribed spacer -ITS and intergenic spacer rRNA –IGS).

The fungal internal transcribed spacer (ITS) region of genomic DNA was characterized from single AMF spores by restriction fragment length polymorphism analysis (PCR-RFLP) (Sanders et al., 1995). Then, the ITS region was used to detect AMF in different roots systems (van Tuinen et al., 1998; Colozzi-Filho & Cardoso, 2000; Redecker, 2000) and in the field (Renker et al., 2003, Mergulhão et al., 2008). Using specific PCR primers to identify AMF within colonized roots of *Plantago media* and *Sorghum bicolor*, Redecker (2000) defined five groups of AMF. Later on, the same groups were detected by Shepherd et al. (2007) in roots

of twelve tree legumes and non-legume trees, but these primers did not discriminate the AMF species. A set of primers amplifying a SSU-ITS-LSU fragment was developed (Kruger et al., 2009) allowing phylogenetic analyses with species level resolution. Such primers are useful to monitor entire AMF field communities, but they present a drawback related to their size of 1500 bp. Candidate regions to be DNA barcoding of arbuscular mycorrhizal fungi were analysed (Stockinger et al., 2010), but there was intraspecific variation heterogeneous and high in some groups.

Glomeromycota has a distribution of ITS fragment lengths concentrated between 550 and 650 bp, found in 96.4% of in silico analyzed sequences by Patreze et al. (2009). These authors rescued eight Glomeromycota genera and 31 species from 422 ITS sequences. The sub-regions ITS1 and ITS2 show high evolution rate and they are typically specific-species (Bruns & Shefferson, 2004). Furthermore, the great number of ITS copies per cell (more than 250) characterize this region as good target to sequencing where the DNA initial quantity is low, as environmental samples (Nilsson et al., 2009a). Due some taxonomic discrepancies between ITS1 and ITS2 analyzed separately and also in relation to the full ITS region (Nilsson et al., 2009b), it is suggested starting the study using the ITS2 region because it is as variable and long as ITS1, but ITS2 (White et al., 1990) has a major number of access at INSD (International Nucleotide Sequence Databases; Benson et al., 2008) to perform comparisons. Although the ITS sequencing allows species identification, the number of samples required for environmental studies can be unviable. The region ITS1-5.8-ITS2 was used to assess the genetic diversity of geographical isolates of *Glomus mosseae* (Avio et al., 2009).

The target regions Large Subunit (LSU) rDNA and the Small Subunit (SSU) rDNA have been useful at AMF's detection, whereas most studies are based on this last one. The resolution of these genes is different, and a direct comparison of phylogenetically defined taxa is not possible. Van Tuinen et al. (1998) detected four AMF species using one region from LSU rDNA in stained mycorrhizal root fragments by nested PCR. Species and in some cases isolates, also were separated based on polymorphism found in the gene coding for the large ribosomal subunit (LSU) by Single Stranded Conformation Polymorphism (SSCP) method (Kjøller and Søren Rosendahl, 2000). In this later method, nucleotide differences between homologous sequence strands are detected by electrophoresis of single-stranded DNA under non-denaturing conditions (Orita et al., 1989). The question was if it was possible to apply to field roots with unknown arbuscular mycorrhizal symbionts. Some years after, Rosendahl & Stukenbrock (2004) used with success the LSU rDNA sequences to analyze the community structure in coastal grassland in Denmark. The LSU provides a better resolution, but several primers are necessary for amplifying all genera of Glomeromycota. Lee et al. (2008) developed new primers using the small subunit rRNA gene (SSU) as target, providing another alternative to detect AMF directly from field roots. The sequencing of DNA target regions can reveal high variability of taxon richness and composition between particular ecosystems. Öpik et al. (2006) surveyed 26 publications that report on the occurrence of natural root-colonizing AM fungi identified using rDNA region (ITS, SSU and/or LSU), of which nine reports were in forest ecosystems. The number of AM fungal taxa per host plant species in tropical forests was 18.2 and temperate forests were 5.6. The Table 1 summarizes the results of surveys about AMF detection using rDNA regions in forest ecosystems after 2006. Data from two reports were obtained using second-generation sequencing technologies where taxa at very low abundances may be recorded.

Forest ecosystem	Plant species	N° of root samples	N° of clones screened	N° of AMF sequenced	OTU	Diversity index	Marker region	Primers used	Reference
seminatural woodland, North Yorkshire	*Hyacinthoides non-scripta*	33	141	62	**	**	SSU	NS31/AM1	Helgason et al. 1999
	Cajanus cajan	5			48	2.67			
	Heteropogon contortus	5			24	1.88			
boreonemoral forest, Central Estonia	10 species	458	*	158 358	47	9.96 to 38.32	SSU	NS31/AM1	Opik et al. 2009
gypsum area, Southern Spain	*G. struthium* L.	24	3072	1443	19	1.13	SSU	NS31 and AM1+AM2+AM3	Alguacil et. al 2009
	Teucrium libanitis Schreber	24						Nested PCR (NS31 / NS41 and ARCH1311/NS8)	
	Ononis tridentata L.	24							
	Helianthemum squamatum (L.) Dum.Cours	24							
hot and arid valley, Southwest China (undisturbed land)	*Bothriochloa pertusa*	5	1168	241	25	2.38	LSU	Nested PCR (LR1/FLR2 and 28G1/28G2)	Li et al. 2010
northern hardwood forests, Michigan, USA	maple (*Acer* spp.) roots	144	2160	38	27	1.94	18S	AM1/NS31	Van Diepen et al. 2011
mosaic of grassland, wood and heath, UK	soils (area of 7 m²)	66 soil cores	*	108 245	70	2.45	SSU	Nested PCR (NS31/AM1 and WANDA/AM1)	Dumbrel et al. 2011

Table 1. Overview of arbuscular mycorrhizal (AM) fungal community surveys from forest ecosystems. Data from sampling and sample screening were included. The asterisk means that the manuscript applied the pyrosequencing approach.

The fungal communities analyses by sequencing is based on PCR amplification using specific primers for the taxa in study, followed of cloning of fragments which represent the

species richness. This kind of approach generates a library of clones, which many times are high (above one hundred). In order to minimize costs, the clones obtained can be select by restriction fragment length polymorphism (RFLP), grouped according a restriction standard revealed using restriction enzymes. Helgason et al. (1998) made use of this technique for the first time to evaluate the AMF diversity changes comparing agriculture soils and forest adjacent. They suggested that the low taxonomic diversity of arbuscular mycorrhizal fungi in arable fields indicates that their functional contribution may be less there than in woodland.

To date, almost all information on sequence differences in this interesting fungal group comes from ribosomal genes. Other coding regions of the genome were investigated as the variability of β-tubulin and H^+-ATPase genes in the AMF *Glomus intraradices* (Corradi et al., 2004). For this purpose, the authors used degenerate primers in order to sequence the most gene variants possible including any that might have originated from other fungal and eukaryotic groups. Following this idea, it is important to check the sequences available on databases of additional fungal groups to improve the consistence of phylogenetic analysis for arbuscular mycorrhizal fungi, mainly when the objective is evaluate the variability in other than ribosomal genes.

2.2 Molecular methods for fungal diversity and applications

Many methods allow the elucidation of microbial structure has been intensively applied to bacterial and fungal communities, as PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Terminal-Restriction Fragment Length Polymorphism (T-RFLP), or Oligonucleotide Fingerprinting of rRNA Genes (OFRG). Here, we select studies which were developed from these methods in Glomeromycetes communities which were applied or have a potential for assessment in natural ecosystems, as forests. All possibilities are described in Figure 1, in an adaptation from Theron & Cloete (2000). The actual function of AMF symbiosis in nature should be considered at the community level of both the AMF and host plants, but we are focusing at fungal partner. All primers sequences and regions of ribosomal RNA genes used in such molecular approaches are shown at Figure 2.

2.2.1 PCR-RESTRICTION Fragment Length Polymorphism (PCR-RFLP) and Single Stranded Conformation Polymorphism (SSCP)

The technique PCR-RFLP was employed with success (Sanders et al., 1995) to distinguish AMF species from DNA isolated of spores; however, when applied to field samples, this technique can generate polymorphism in not target organisms. Avio et al. (2009) were able to discriminate *Glomus mosseae* isolates from G. coronatum, *G. intraradices* and G. viscosum by using of a single enzyme (HinfI) with ITS-RFLP profiles. For field samples, this technique remains insufficiently tested, Mergulhão et al. (2008) detected AMF species in an impacted semiarid soil using the ITS1-5.8S-ITS2 region and Börstler et al. (2010) analyzed for the first time the intraspecific genetic structure of an AMF directly from colonized roots in the field comparing between agricultural and semi-natural sites. To our knowledge, there are not studies using solely PCR-RFLP to characterize AMF communities in forest ecosystems. The work from van Diepen et al. (2011) used the PCR-RFLP to select clones representatives of each type to be re-amplified and sequenced.

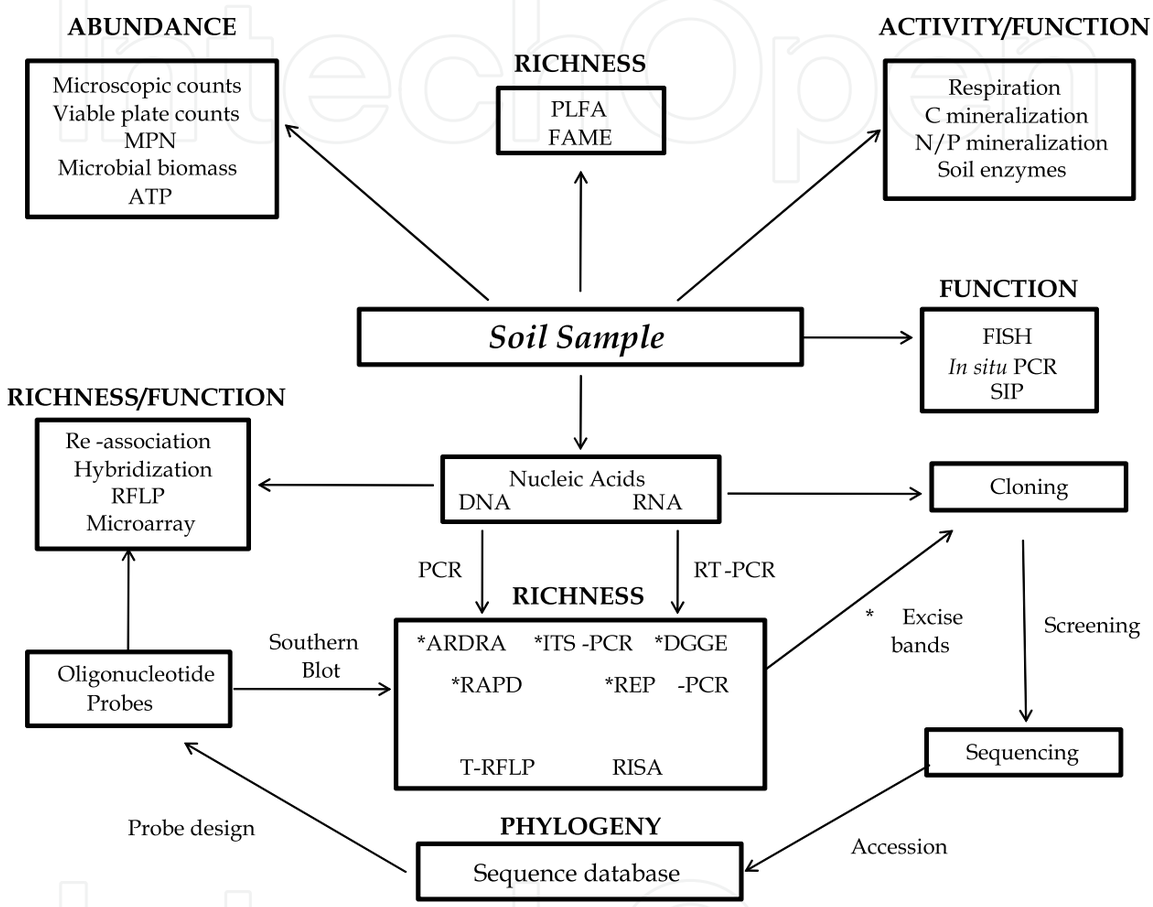
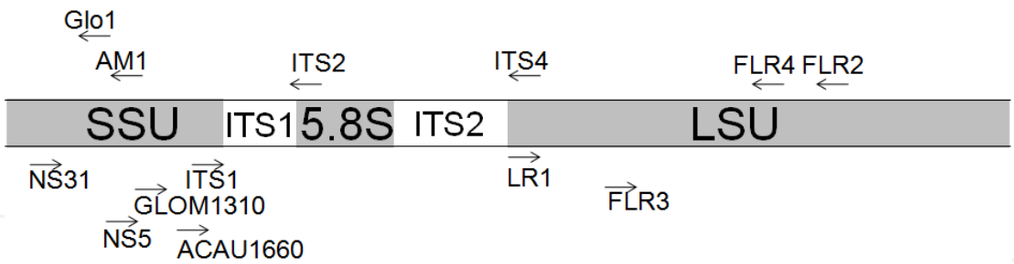


Fig. 1. Commonly used molecular approaches in microbial ecology. PLFA - Phospholipid Fatty Acids; FAME – Fatty Acid Methyl Ester; FISH – Fluorescence in Situ Hybridization; SIP - Stable Isotope Probing; RFLP - Restriction Fragment Length Polymorphism; PCR - Polymerase Chain Reaction; RT-PCR – Real Time Polymerase Chain Reaction; ARDRA – Amplified Ribosomal DNA Restriction Analysis; ITS-PCR – Internal Transcribed Spacer Polymerase Chain Reaction; DGGE – Denaturing Gradient Gel Electrophoresis; RAPD – Random Amplified Polymorphic DNA; T-RFLP – Terminal Restriction Fragment Length Polymorphism; REP-PCR – Repetitive Element Palindromic Polymerase Chain Reaction; SIP – Stable Isotope Probing.



Primer	Sequence (5'-3')	Reference
ITS1	TCCGTAGGTGAACCTGCGG	White et al. 1990
ITS2	GCTGCGTTCTTCATCGATGC	White et al. 1990
ITS4	TCCTCCGCTTATTGATATGC	White et al. 1990
NS5	AACTTAAAGGAATTGACGGAAG	White et al. 1990
NS31	TTGGAGGGCAAGTCTGGTGCC	Simon et al. 1992
LR1	GCA TAT CAA TAA GCG GAG GA	van Tuinen et al. 1998
AM1	GTTTCCCGTAAGGCGCCGAA	Helgason et al. 1998
FLR2	GTC GTT TAA AGC CAT TAC GTC	Trouvelot, et al. 1999
ACAU1660	TGAGACTCTCTCGGATCGG	Redecker et al. 2000
GLOM1310	AGCTAGGYCTAACATTGTTA	Redecker et al. 2000
FLR3	TTGAAAGGGAAACGATTGAAGT	Gollotte et al. 2004
FLR4	TACGTCAACATCCTTAACGAA	Gollotte et al. 2004
Glo1	GCCTGCTTTAAACACTCTA	Cornejo et al. 2004

Fig. 2. Diagram of the ribosomal DNA cluster of fungi containing the location and sequences of the primers cited in this chapter and used in molecular methods to fungal community studies. The ribosomal RNA (rRNA) gene is a tandem array of at least 50–100 copies in the haploid genome of all fungi. SSU=small ribosomal subunit; ITS1=intergenic transcribed spacer region 1; ITS2=intergenic transcribed spacer region 2; LSU=large ribosomal subunit. The arrows refer to the approximate annealing sites of primers, but the diagram is not to scale.

In order to detect AMF species, nested-PCR, based on sequence differences in the gene coding for the large ribosomal subunit can be coupled to a method known as SSCP (Single Stranded Conformation Polymorphism) and the differences among species are visualized in polyacrylamide gels under non-denaturing conditions. This method allowed Kjøller & Rosendahl (2000) detect four species of Glomus in root tissues of four culture plant species. Jansa et al. (2002) could detect different ITS sequences types within of the single-spore isolates of *Glomus intraradices* using the SSCP technique. A recent study assessed the AMF community at field, in arid gypsophilous plant communities in south-eastern Spain (Alguacil et al., 2009) by sequencing (Table 1), using SSCP to select clones. In such work, representatives of each SSCP pattern were chosen for sequencing while the remaining clones

(almost two thousand) were classified by SSCP typing. These sequences showed high degree of similarity to sequences from taxa belonging to the phylum Glomeromycota. In a savanna area, Alguacil et al. (2010) related the AMF diversity in roots of *Centrosema macrocarpum* to soil parameters and sources of phosphorus. The authors amplified by PCR the AM fungal small-subunit (SSU) rRNA genes and selected clones by SSCP to sequencing and phylogenetic analyses. They identified nine fungal types: six belonged to the genus Glomus and three to Acaulospora. The single-stranded conformation polymorphism (SSCP) approach is a very sensitive and reproducible technique that has potential to be applied successfully in studies in order to analyze the sequence diversity of AM fungi within roots from forest ecosystems, not reported so far.

2.2.2 Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

Another technique, derived from a combination of PCR, RFLP and electrophoresis of nucleic acids (Liu et al., 1997) nominated as Terminal-Restriction Fragment Length Polymorphism (T-RFLP) use oligonucleotides fluorescent-labeled that enables generation of fingerprinting data of microbial communities efficiently (Marsh, 1999). Figure 3 exemplifies the steps needed for the T-RFLP analysis. As PCR-RFLP, the selection of restriction enzymes is a fundamental step. It is important to use 2-4 enzymes in each study for obtaining of different amplicons (Tiedje et al., 1999). These authors recommend the enzymes HhaI, RsaI, and MspI, which have given the greatest resolution based on restriction analysis of the database as well as natural communities for soil microbial, but others may be of use under special circumstances, as specific AMF communities. The initial works using T-RFLP for fungal community analysis focused on temperate forests (Klamer et al., 2002) or ectomycorrizas (Zhou and Hogetsu, 2002; Dickie et al., 2002). Combining LSU rDNA sequencing and T-RFLP analysis, Mummey et al. (2009) investigated if the pre-inoculation may play a role in arbuscular mycorrhizal fungi (AMF) community assembly within the roots. Another application of T-RFLP for arbuscular mycorrhizal can be developed to measure the effect of soil inoculums representing different AM fungal communities on the growth of three plant species. Uibopuu et al. (2009) used Glomeromycota specific primers NS31 and AM1 labeled with fluorescent dyes to perform the method known as T-RFLP, comparing the inoculums from a young forest stand, an old forest stand and an arable field at growing of the three plant species and showed that the old and young forest resulted in similar root AMF communities whilst plants grown with AM fungi from arable field hosted a different AMF community from those grown with old forest inocula. However the AMF richness in plant roots was not related to the origin of AMF inoculums. Previous works using the T-RFLP technique were performed in AMF community colonizing roots from herbaceous (Wu et al. 2007), grass (Mummey et al., 2005, Mummey and Rillig, 2008; Hausmann and Hawkes, 2009), addressing studies of impact of various agricultural practices on AMF biodiversity (Lekberg et al., 2007; Verbruggen et al., 2010). The average number of AMF taxa reached in this work was 8.8 OTU and the authors stressed the importance of organic management in agro-ecosystems maintenance of mycorrizal fungi. Van de Voorde et al. (2010) compared the AMF communities from roots of one species of interest in both situations: bioassay plants and plants collected from the field. Although the species had not been a forest, wood or tree plant, this kind of study can be applicable to address similar questions for them.

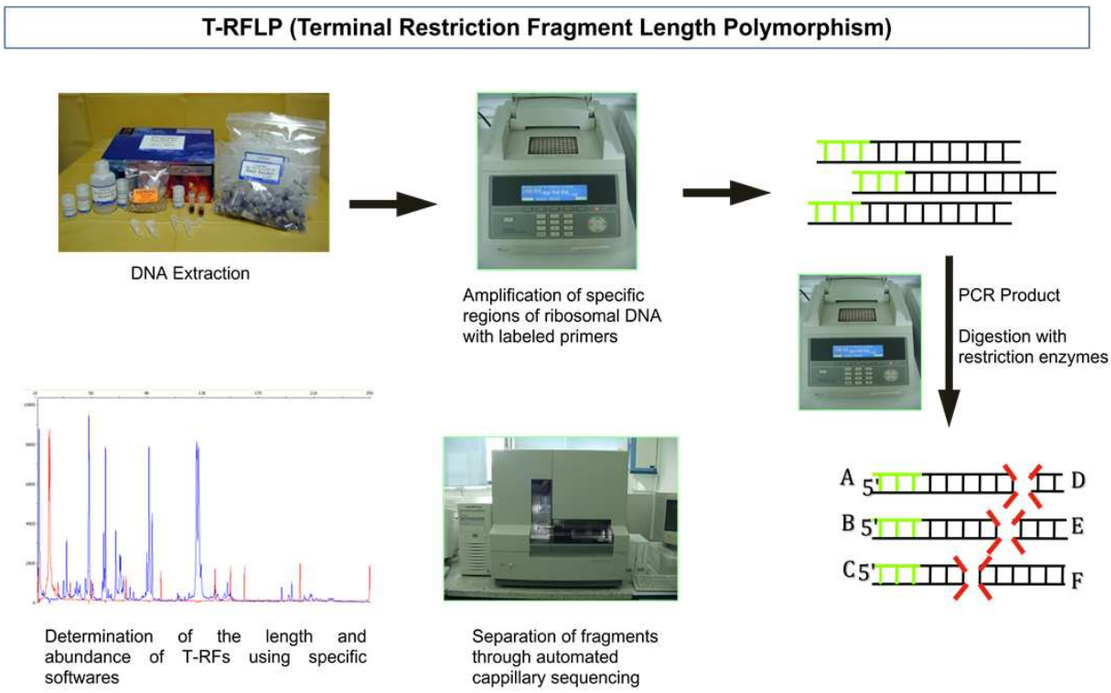


Fig. 3. A schematic design for the steps of a T-RFLP analysis on the soil microbial community structures or for determination of the presence of a target species using specific primer.

Once the belowground feedbacks may lead to changes in species diversity, the knowledge about AMF diversity and distribution both in soil and roots, as well as plant performance is very useful to sustainability of an ecosystem. Evaluating the AMF diversity in roots from seven different shrub species, Martínez-García (2011) suggested that the generate islands of fertility which differ in nutrient content and, therefore, support different AMF communities, increasing AMF diversity at the landscape level. These authors used the primers sequences LR1 and FLR2 for the amplification of the 5′ end of LSU rDNA sequences in general fungi (Van Tuinen et al., 1998; Trouvelot et al., 1999) and in the second amplification, they used the AMF specific primers FLR3 and FLR4 (Gollotte et al., 2004). The same set of primers were used by Koch et al. (2011) to investigate the impacts of introduced plants and exotic AM fungi on local AM fungi at Canada.

2.2.3 Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE)

Denaturing gradient gel electrophoresis (DGGE) and terminal-restriction fragment length polymorphism (T-RFLP) approaches also were applied to AMF studies. These techniques are based on extraction of community DNA followed by the PCR amplification of rRNA genes from the community DNA using universal, domain or group specific primers. The resulting products are separated based on relative helix stabilities in a denaturant (DGGE) or thermal (TGGE) gradient gel (Muyzer et al., 1993). Such techniques are very sensitive and have been used to detect single base differences. However, the gel system employed has low resolving power and there is no way of defining with accuracy the Tm of the helix.

Moreover, there is no comparative sequence database for AMF. In addition, reports have been published and stimulate to better focus on this issue. Kowalchuk et al. (2002) first coupled an AMF-specific PCR strategy targeting the 18S rRNA gene (Helgason et al., 1998) with denaturing gradient gel electrophoresis (DGGE) to detect AMF in roots of a grass species at two coastal sand dune locations in the Netherlands. In this report, the primer reverse NS31, described by Simon et al. (1992) had a GC clamp sequence, as described by Kowalchuk et al. (1997). The same gene (18S rRNA), though a different sub-region, was used by de Sousa et al. (2004) who applied the DGGE method to discriminate Gigaspora isolates and Gigasporaceae populations from environmental samples. Although the environmental samples were collected in a grassland field, a cattle farm in Brazil, that work brings new possibilities for studying the ecology of Gigaspora under field conditions, including forest ecosystems, without the need for trap cultures.

The DGGE from sequential amplification of 18S rDNA fragments by nested PCR using primer pairs AM1-NS31 and Glo1-NS31GC yielded a high-resolution band profile to soil samples from different ecosystems, including a eastern red cedar (Juniperus virginiana L.) forest ecosystems (Liang et al., 2008). The primer Glo1 was described by Cornejo et al. (2004). Although the primer pair AM1/NS31 is one of the most widely used group-specific primer pairs in studies of AMF communities, amplifying the three well-established families of the Glomeromycota (Glomaceae, Acaulosporaceae, and Gigasporaceae), this primer pair does not amplify 18S rDNA fragments from all known AMF or they can amplify some non-AMF sequences. Nevertheless, an advantage for the use of primer pair AM1/NS31 is the relatively large amounts of DNA sequence information derived from this primer pair available. A subsequent study (Zhang et al., 2010) was aimed the variable V3-V4 region of the 18S rDNA of AMF gene, by using nested PCR in three steps: (1) first round PCR, using primers GeoA2 and Geo11 (Schwarzott and Schüssler, 2001); (2) second round, using primers above-mentioned AM1/ NS31-GC; and (3) third round using NS31-GC/Glol. In that study, the AMF community from rhizosphere of two shrubs species was investigated and the species richness ranged from 17 to 25 AMF species. Internal Transcribed Spacer (ITS) specific primers for Acaulosporaceae (ACAU1660/ITS2) and Glomaceae (GLOM1310/ITS2) (Redecker et al., 2000; White et al., 1990) have been used successfully in DGGE analysis on differentiating of composition of mycorrhizal communities in maize genotypes (Oliveira et al., 2009; Pagano et al., 2011).

Two other studies aiming at the molecular community analysis of AMF had as target the fungal small subunit (SSU) rRNA gene. Their objectives were related to role of AMF in plant tolerance to heavy metals stress (Long et al., 2010) and the interplay between soil properties and crop yield (Wu et al., 2011).

Few studies have applied molecular tools as DGGE analysis in forest species. The scarcity of works of this nature in forest species reinforces our goal of encouraging research in this area. Öpik et al. (2003) had surveyed the mycorrhizal status of plants grown in soils from a boreal forest by DGGE plus restriction analysis and sequencing. The region analyzed by them was the SSU region. Recently, using specific PCR conditions for Glomaceae family (nested system with NS5/ITS2 and GLOM1310/ITS2 primers) in DGGE system, Pagano et al. (2011) showed the applicability of this technique to understand the role of AMF in woody and shrub species from Caatinga, a dry deciduous forest at Brazil. The region studied had different agroforestry systems which were implanted in a degraded area in order to be an

attractive alternative to conventional afforestation systems. An important conclusion from this work is the existence of functional diversity among AMF, supporting the theory that the AMF are considered as one of the factors that determine how plant species coexist. As observed by Pagano et al. (2011) for semi-arid soil, the analysis of AMF population of an experimental area may inform the state of land restoration depending how close they are from those of climax vegetation.

2.2.4 F-RISA and Automated-RISA (ARISA)

The Fungal Ribosomal Intergenic Spacer Analysis (F-RISA) method exploits the variability on the length of the nuclear ribosomal DNA (rDNA) region that contains the two internal transcribed spacers (ITS1 and ITS4) and the 5.8S rRNA gene (ITS1-5.8S-ITS2). Gleeson et al. (2005) characterized the fungal community structure on mineral surface using this region and Hong, Fomina and Gadd (2009) showed the applicability of this assay to examine the potential role of fungi as bioindicator of effects of organic and metal contamination in soil. It is possible to use the same approach to Glomeromycetes, but it is suggested to sequence some F-RISA fragments from AMF species known to establish standards. Thus, differential fragments when experimental communities are compared can be excised from gel, purified and sequenced in order to detect core AMF species in each environment or ecosystem.

To improve the resolution of this technique was developed an automated variation (ARISA) by Fisher & Triplett (1999) for characterization of bacterial communities. This PCR-based technique is based on the use of a fluorescent primer in the amplification of microbial ribosomal intergenic spacers, using DNA extracted from environmental samples as a template. ARISA was first used to fungal soil communities by using a pair of primers that targeted the 3'end of the 18S rDNA sequence and the 5'end of the 25S rDNA sequence (Ranjard et al., 2001). These authors examined the fungal database for the size of the ITS1-5.8S-ITS2 region in fungi, totalizing 104 genera and 251 species. However, the Glomeromycetes were not recognized as a phylum that date. Patreze et al. (2009) repeated the same in silico analysis including data updated to January 2008. The authors followed the classification of Hibbett et al. (2007), which consider the Glomeromycota as a phylum. Representatives of this phylum have a distribution of ITS fragment lengths concentrated between 550 and 650 bp (Patreze et al., 2009). The authors concluded that a clear distinction among the fungi kingdom is not possible considering the ITS sequence length. However, the method RISA was useful to characterize soil fungal communities from three forest ecosystems from Brazil: a native forest of *Araucaria angustifolia* and two replanted forest.

2.3 Microsatellites

Genetic diversity of arbuscular mycorrhizal fungi also can be investigated from the viewpoint of the population or individual, aside from the community level. Multilocus genotyping of AMF using microsatellites have been useful as marker suitability for population genetics. Microsatellites or Simple Sequence Repeats (SSRs) are regions with at least five identical repeats of two, three or four nucleotides, or a stretch of at least 10 identical single nucleotides. The length polymorphisms at microsatellite regions are caused by changes in the numbers of repeat lengths, which are repeated up to about 100 times (Tautz, 1989). This marker was used to explore the AMF diversity, simultaneously published by Croll et al. (2008a) and Mathimaran et al. (2008a) for the *Glomus intraradices*

species. Previous works of distinct nature had reveled genetic variation within AMF species (Vandenkoornhuyse & Leyval, 1998), which affect plant growth and nutrition (Koch et al., 2006). Previously, the possibility of using a tandem repeated DNA sequence as a diagnostic probe for detection in colonized roots was demonstrated from the arbuscular mycorrhizal fungus *Scutellospora castanea* (Zézé et al., 1996). Then Zézé et al. (1997) employed the M13 minisatellite-primed PCR technique to explore the intersporal genetic variation of *Gigaspora margarita*. In the same year, the microsatellites were used as target to detect mycorrizal fungi (ecto and endo-mycorrhiza), including AMFs, however the isolates of *Glomus mosseae* could not be separated by microsatellites analysed (Longato & Bonfante, 1997). In addition, Douhan and Rizzo (2003) had developed a technique to isolate and detect microsatellite loci in AM fungi from single spores of *Glomus etunicatum* and *Gigaspora gigantea*. The authors were not certain that the microsatellite motifs found by them were from the target organism due the possible contaminants. A fingerprinting technique widely used in studies of closely organisms (Lim et al., 2004) known as Inter-Simple-Sequence Repeat (ISSR-PCR) allowed

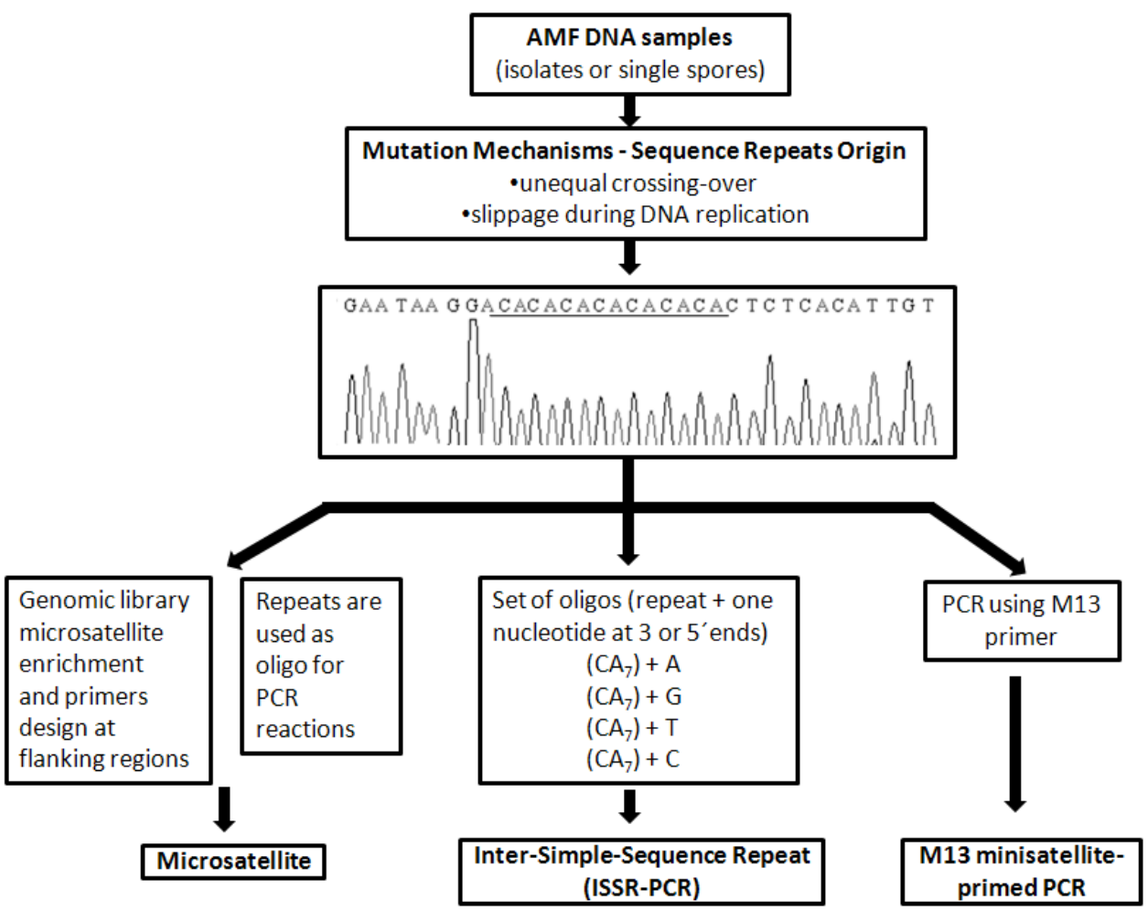


Fig. 4. Microsatellites regions origin and different approaches to explore the variation at length repeats: Microsatellite or Simple Sequence Repeats; Inter-Simple-Sequence Repeat and M13 minisatellite-primed PCR. These techniques were applied to arbuscular mycorrhizal fungi studies.

the characterization of genetic diversity of *Glomus mosseae* isolates (Avio et al., 2009). The authors amplified the minisatellite and microsatellite regions from fungal extracted DNA using the primers M13, $(GTG)_5$ and $(GACA)_4$. The same strategy above-described has potential application to several AMF species. In our opinion, this study can be a precursor of a field research related to the functional diversity of arbuscular mycorrhizal fungi, which remains poorly explored.

Taking into account the AM fungal populations, the molecular techniques have also shown that natural populations exhibit unexpectedly high genetic diversity, despite the assumption that diversity in these seemingly asexual fungi should be low. Thus, microsatellites are an interesting alternative as markers since the ribosomal gene sequences in AM fungi present high diversity, which might cause problems in their use in field studies.

3. Conclusion

Advances at understanding of genome structure of AMF were done in specifics and common species, as *G. intraradices*, which can easily be cultured in large amounts. Analyzing the regions of both LSU rDNA (*Glomus intraradices*) and POL1-like (PLS) sequences (*Glomus etunicatum*), Boon et al. (2010) confirmed high intra-isolate genetic polymorphism at the genome level. Their showed that genetic variation persists at the transcript level and suggested that in AMF, multiple nuclear genomes contribute to a single phenotype. Thus, it is supposed AMF connect plants together by a hyphal network, and that these different genomes may potentially move around in this network. In addition, genomic changes do not only appear among highly divergent lineages but can also occur among highly related species and individuals from the same population (Corradi et al., 2006).

The reassociation kinetics on *G. intraradices* experiments conducted by Hijri and Sanders (2004) revealed that 1.59% from haploid genome size is repetitive DNA, the category that includes the microsatellites regions. However the repetitive regions had low frequency, these authors had suggested that the very small genome size of *G. intraradices* makes it an excellent candidate for a genome sequencing project, beyond this species to be one of the most commonly studied AM fungi which colonizes host plants rapidly. The production of a completely annotated and assembled *G. intraradices* genome was initiated in 2004, having been shown especially arduous challenge. Martin et al. (2008) summarized the main difficulties found to complete this project and presented a nice historical perspective about the advances and approaches used to sequencing.

The recently developed massively parallel ('454') pyrosequencing enables metagenomic and metagenetic analyses in a manner that increase the capacity of traditional Sanger sequencing-based approaches by several orders of magnitude (Tedersoo et al., 2010). Pyrosequencing of fungi in diverse environments, such as soil or roots, elevates the number of recovered taxa several fold (Table 1). Similarly to study of Tedersoo et al. (2010) which compares a ectomycorrhizal fungi community of a tropical rainforest ecosystem by pyrosequencing and Sanger's sequencing, such analysis need to be performed to arbuscular mycorrhizal fungi in order to improve technical biases and to interpreted the data accordingly. Recently, the suitability of species abundance models in arbuscular mycorrhizal fungi were addressed (Unterseher et al., 2011) using output data from a boreonemoral forest in Estonia (Öpik et al., 2009), described in the Table 1. The authors proposed the use of lognormal species abundance distributions (SAD) as a working

hypothesis to elucidate MOTU richness and biodiversity of AMF communities with low to medium sampling coverage. Such analyses are recommended to new studies in AMF communities emerging from pyrosequencing.

Studies on the evolutionary ecology of the AMF are on demand in order to approach measuring selection and host specificity as variations in AMF phenotypes were observed more recently with the development of molecular techniques. New approaches based on protein-encoding genes are expected to open opportunities to advance the mechanistic understanding of ecological roles of mycorrhizas in natural and managed forest ecosystems as well. And the idea that direct selection on AM fungal traits related to their survival and performance in the environment independent of the host is being reviewed as extraradical mycelium can be shown to be responsible for a significant part of the diversity of the AM fungi. As proposed by Helgason & Fitter (2009), the fungal response to the abiotic environment is that it would be expected for there to be substantial uncharacterized diversity in the Phylum Glomeromycota, since its members are globally distributed but poorly dispersed, and soil conditions vary greatly in time and space.

There have been significant advances in the plant-microbe interaction studies. As example, laser microdissection (LMD), a method which has been used widely by human and animal biologists to study gene expression in specific cell types and to elucidate the associated molecular events, has been adapted to plant tissues (Day et al. 2005; 2006) and applied successfully to study root-mycorrhizal fungus interactions for the identification of differentially expressed transcripts from LMD-derived RNA for the development of the arbuscule–cortical cell interface (Gomez & Harrison, 2008) or identify transcripts of different phosphate transporters in the same arbusculated cell population provides (Balestrini et al., 2007). The special advantage of LMD for arbuscular mycorrhiza is the isolating of cortical cells containing the fungus from the rest of the root cells. One objective is to rescue the RNA to perform transcript profiles analysis. LMD opens a new scenario for the understanding of the molecular basis of the AM symbiosis. Although the preparation protocols needs to be optimized for each tissue type and plant species, LMD can be adapted to detect and quantify mycorrhizal fungus in forest roots.

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5. Glossary

AMF = Arbuscular Mycorrhizal fungi
ARISA = Automated Variation
DGGE = Denaturing Gradient Gel Eletrophoresis
F-RISA = Fungal Ribosomal Intergenic Spacer Analysis
ITS = Internal Transcribed Spacer
LMD = Laser Microdissection
LSU = Large Subunit ribosomal of rRNA
OFRG = Oligonucleotide Fingerprinting of rRNA Genes

PCR = Polymerase Chain Reaction
RFLP = Restriction Fragment Length Polymorphism
RISA = Ribosomal Intergenic Spacer Analysis
rRNA = Ribosomal RNA
SSCP = Single Stranded Conformation Polymorphism
SSRS – Microsatellites or Single Sequence Repeats
SSU = Small Subunit ribosomal of rRNA
TGGE = Temperature Gradient Gel Eletrophoresis
T-RFLP = Terminal Restriction Fragment Length Polymorphism

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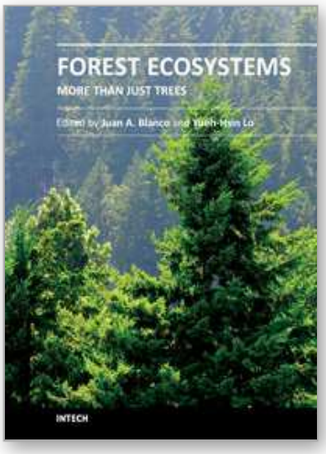

Forest Ecosystems - More than Just Trees

Edited by Dr Juan A. Blanco

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The common idea for many people is that forests are just a collection of trees. However, they are much more than that. They are a complex, functional system of interacting and often interdependent biological, physical, and chemical components, the biological part of which has evolved to perpetuate itself. This complexity produces combinations of climate, soils, trees and plant species unique to each site, resulting in hundreds of different forest types around the world. Logically, trees are an important component for the research in forest ecosystems, but the wide variety of other life forms and abiotic components in most forests means that other elements, such as wildlife or soil nutrients, should also be the focal point in ecological studies and management plans to be carried out in forest ecosystems. In this book, the readers can find the latest research related to forest ecosystems but with a different twist. The research described here is not just on trees and is focused on the other components, structures and functions that are usually overshadowed by the focus on trees, but are equally important to maintain the diversity, function and services provided by forests. The first section of this book explores the structure and biodiversity of forest ecosystems, whereas the second section reviews the research done on ecosystem structure and functioning. The third and last section explores the issues related to forest management as an ecosystem-level activity, all of them from the perspective of the ⿰other⿰ parts of a forest.

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