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Characterization of Ectomycorrhizal species through molecular biology tools and morphotyping

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ABSTRACT: Mycorrhizae are mutualistic associations between fungi and plant roots. These symbiotic associations are abundant and occur in 75 to 80 % of plants. Ectomycorrhizal fungi are very important in ecosystems, because their mutualistic association with plants of different species helps nutrients and water absorption, as well as protection of the host plant against pathogens and abiotic stresses. Most ectomycorrhizal fungi belong to the Basidiomycota class, such as the following genera: *Amanita*, *Hebeloma*, *Hysterangium*, *Laccaria*, *Lactarius*, *Rhizopogon*, *Russula*, *Scleroderma*, *Suillus*, *Tricholoma*, among others. Morphological studies on ectomycorrhizae report important results in understanding the species biodiversity. However, the use of molecular biology nowadays is indispensable. Among the various molecular tools available, there is consensus about the use of tools based on sequencing of the Internal Transcribed Spacer (ITS) of fungi rDNA, aiding in species characterization and construction of phylogenetic studies. The ITS region is of easy amplification, it has multicopy nature and enables differentiation between species. The objective of this study was to show that the use of molecular biology tools associated with morphotyping to characterize species of ectomycorrhizae is more effective than when they are used on their own.

Keywords: PCR, ectomycorrhizal fungi, molecular identification, morphotype

Introduction

Mycorrhizae are evolved mutualistic associations between soil fungi and plant roots. Phyla of the kingdom fungi (Basidiomycota, Ascomycota and Zygomycota) and vascularized plants participate in these associations. The term symbiosis is often used to describe these interdependent mutual relationships where the host plant receives mineral nutrients, while the fungus obtains carbon-derived compounds photosynthetically.

In these associations, mycorrhizal fungi increase the capture of various soil nutrients and their translocation to the host plant. Diffuse distribution of mycelium in the soil around the roots reaches longer distances than those achieved by non-colonized roots and it provides greater absorption capacity of nutrients, particularly phosphorus (P), nitrogen (N) and potassium (K). In addition, changes in root architecture with higher branching intensity increase contact surface with the soil. Colonized roots have increased longevity, greater resistance to pathogens, toxic elements in the soil and extreme conditions of temperature, acidity and moisture. Among the different types of mycorrhizae, endomycorrhizae and ectomycorrhizae are considered the most important from an ecological and economic point of view.

Ectomycorrhizal (ECM) fungi are ubiquitous in forest ecosystems, especially in temperate regions, forming symbiotic associations with roots of woody plants. Significant advances have been made in the ecology of ECM fungal communities over the past decade. This is largely due to the development and implementation of molecular biological techniques that have been used along with traditional morphological techniques. Certainly, this "molecular revolution" has been at least partly due to the usefulness and informative nature of the internal transcribed spacer (ITS), regions of the fungal rRNA that are the focus of most ecological studies on ECM fungi, also because costs associated with the generation of molecular data having decreased considerably over the last few years. Traditional molecular approaches such as ITS-Restriction Fragment Length Polymorphism (RFLP) or ITS-Polymerase Chain Reaction (PCR) and sequencing are now more conventional in ecological studies of ECM fungi. However, their use still generates new data, promoting a better understanding of the enormous diversity and ecology of ectomycorrhizal communities. Despite all the efficiency that the molecular approach through ITS-PCR, ITS-RFLP and sequencing have provided for ECM ecology, these approaches are still considered limited due to the number of samples that can be processed in real time.

The *in situ* nature of ECM fungi below ground hinders sampling and typically, a large number of samples need to be taken for an adequate description of the community. Consequently, time is often a limiting factor as it is not uncommon that studies of ECM fungal com-

munity involve the extraction and analysis of thousands of roots.

The adoption of other molecular techniques, such as Ribosomal Intergenic Spacer Analysis (RISA) and Automated Ribosomal Intergenic Spacer Analysis (ARISA), in studies of fungal communities may minimize some of the associated difficulties, because they are generally faster and allow working with a large number of samples, including root mass and soil samples. These approaches have been widely used in microbial ecology and are beginning to be used for ecology of soil fungi, including studies of ECM fungi. The primary importance of using these techniques is the possibility of detecting fungal mycelium in the soil, which allows testing hypotheses about communities through ECM fungal mycelia. The ability to use these techniques generates new opportunities and challenges for research on soil ECM fungi.

This literature review aims to present the importance of using morphological and molecular techniques, as well as the combination of both for identifying ectomycorrhizal fungi with greater reliability, accuracy and credibility.

Mycorrhizae

Organisms belonging to the fungal kingdom absorb nutrients from many sources, including the decomposition of organic substrates (saprophytes), predation, parasitism and involvement in mutual associations (Christensen, 1989; Brundrett et al., 1996). Many soil fungi are saprophytic and have enzymatic capacity to decompose organic substrates in different degrees of complexity, while other species require very low levels of organic or inorganic substrates. Mycorrhizal fungi are important components of soil microbiota in many ecosystems (Wainwright, 1988; Moreira and Siqueira, 2002) and generally have limited saprophytic abilities, but their endophytic properties are well developed. These properties help determine the effectiveness of mycorrhizal associations, including the number of hyphae produced in the soil in relation to mycorrhizal colonization, growth rate of hyphae, colony formation in roots and physiological characteristics that regulate the absorption or translocation of nutrients by hyphae, and their exchanges with the host plant (Smith and Gianinazzi-Pearson, 1988; Brundrett, 1996; Smith and Read, 1997).

The terms symbiosis and mutualism have been used interchangeably to describe mycorrhizal associations. Fungal symbiosis is defined as any association where the fungi have contact with the host plant from which they obtain a variety of metabolites and nutrients. The term mutualism implies association with mutual benefits involving two or more different organisms (Boucher et al., 1990; Brundrett, 2004).

The term mycorrhiza (i.e., root fungus) was proposed by Frank in 1885 when the author noted that such associations did not constitute instances of parasitism between fungi and plants, but with benefits for both. Mycorrhizae are currently classified primarily because they differ from other plant-fungus associations, as they are intimate associations with a specialized interface where a bidirectional exchange of materials between living cells occurs. Most mycorrhizae occur in roots, which evolved into the house fungi (Brundrett, 2002; Moreira and Siqueira, 2002), but they can occur in underground stems of certain plants and in bryophytes.

A broader definition covering all the diversity of mycorrhizae, excluding all other plant-fungus associations, is presented by Brundrett (2004), who describes mycorrhizae as essential symbiotic associations for one or both partners, a fungus (specialized in living in the soil and plants) and a root (or any organ in contact with the substrate) of a living plant, which is primarily responsible for transferring nutrients. Mycorrhizae occur in a specialized plant organ where intimate contact results in the synchronized plant-fungus development.

Mycorrhizal associations are regulated by the characteristics of the host plant and the mycorrhizal fungus, as well as soil conditions and environmental factors. At least seven different types of mycorrhizal associations were recognized involving different groups of fungi, host plants and distinct morphological patterns (Harley and Smith, 1983; Moreira and Siqueira, 2002). The most common associations are: (a) arbuscular mycorrhizae (endomycorrhizae) in which zygomycetes produce arbuscules, hyphae and vesicles inside root cortex cells; (b) ectomycorrhizae, where basidiomycetes and other fungi form a mantle around the roots and a Hartig net between root cells; (c) orchid mycorrhizae, where fungi produce hyphae inside the root (or plant stems) of orchids; (d) ericoid mycorrhizae, which are characterized by the intracellular presence of twisted structures located in epidermal cells. Arbutoid and monotropoid associations are present in the roots of the *Ericaceae*, *Empetraceae* and *Epacridaceae* families (Smith and Read, 1997) and (e) ectendomycorrhizae, which are similar to ectomycorrhizal associations, but have specific anatomical features (Brundrett et al., 1996; Siqueira et al., 2010).

It has often been said that most plants in ecosystems have mycorrhizal associations, but no attempt has been made to catalog the evidence that supports this statement since Kelley (1950). One of the main factors of this lack of data is due to the great difficulty in the identification process of these fungi (Brundrett, 2009).

Ectomycorrhizae

Ectomycorrhizae are mutualistic associations between fungi and gymnosperms or angiosperms belonging to certain families. These associations consist of a soil-mycelium system linking mycorrhizal roots and reproductive or storage structures. Ectomycorrhizal roots are characterized by the presence of a mantle and Hartig net. The Hartig net consists of entangled hyphae between the root epidermis and cell cortex. It is estimated that over 5,000 fungi species are capable of forming ectomycorrhizal symbiosis. These symbionts are found in

four divisions: Basidiomycota, Ascomycota, Zygomycota and Deuteromycota, but most species belong to Basidiomycota (Brundrett, 1996; Peterson et al., 2004).

The ectomycorrhizal fungi may have epigeous habit, when the structure called basidioma remains above the soil surface or hypogeous habit when the basidioma is developed and remains in the subsurface layer of the soil. This is an intrinsic characteristic of each fungal species (Anderson, 2006) (Figures 1A, B and C). Many of the ectomycorrhizal fungi can be identified in the field through observation of macroscopic morphological characteristics associated to the roots or through isolation in culture medium in the laboratory by other identification methods. In Brazil, Basidiomycota, which often colonize species of *Pinus* and *Eucalyptus*, belong to the genera: *Pisolithus*, *Scleroderma*, *Rhizopogon*, *Amanita*, *Lactarius*, *Russula*, *Thelephora* and *Ramaria* (Moreira and Siqueira, 2002; Siqueira et al., 2010).

Ectomycorrhizal associations are formed predominantly in root tips (fine roots) of the host plant, unevenly distributed throughout the soil profile and more abundant in superficial layers containing organic matter than in layers of mineral soil (Meyer, 1973; Harvey et al., 1976; Andreazza et al., 2008). There is little information about mycorrhizal biomass along the soil profile. However, Marks et al. (1968) Hunt and Fogel (1983) and Silva et al. (2009) suggest that ECM fungi can contribute significantly to the biomass of forest ecosystems. Hyphae of mycorrhizal fungi, which are widely distributed in the soil, provide a great contribution to the absorption of nutrients in many forest ecosystems.

According to Brundrett et al. (1996), ectomycorrhizal shapes in which the roots of the host plant and the fungi grow simultaneously are found in favorable environmental conditions. The sequence of events that results in the formation of ectomycorrhizal association can be summarized in the following steps:

1. Contact with hyphae that recognize and adhere to the epidermal cells of the root close to locations where there is growth activity in young lateral roots.

2. Mycelium proliferation on the root surface and differentiation to form the mantle.

3. Hyphae penetrate in the epidermal cells (mostly angiosperms) or in the cortex (in gymnosperms) to form the Hartig net. The host response to these changes may include the production of polyphenols in the cells and deposition of secondary metabolites in the cell walls.

4. The areas of mycorrhizal activity occur at a certain distance (mm) from the root tip, but senescence of Hartig net hyphae occurs in older areas of the root, those farthest from the tip. Consequently, Hartig net activity depends on the root age and growth.

5. The mantle in older roots typically persists long after associations have occurred, but it becomes inactive over time. Older ectomycorrhizal roots probably function as propagules and storage structures.

Figure 1 – Ectomycorrhizal association with fungi of epigeous and hypogeous habit. A; B) hypogeous basidioma, *Hysterangium* sp.; C) epigeous basidioma, *Scleroderma albidum*, *Ramaria* sp.; Photograph: Gilberto Coelho, 2013.

Ectomycorrhizal associations are abundant in soils rich in organic matter. In Brazil, ectomycorrhizae are found predominantly in forest plantations of pine, eucalyptus and occasionally in species of *Rosaceae*, *Caesalpiniaceae* and in "Campinarama" type of vegetation (flooded shrublands) in the Amazon region. In the Cerrado vegetation, there is *Bauhinia forficata* and *Campomanesia xanthocarpa*, which can form ectomycorrhizae (Moreira and Siqueira, 2002). Among the genera or species of fungi already identified and associated to vegetation are: *Thelephora terrestris*, *Rhizopogon* spp., *Scleroderma* spp., *Suillus granulatus* and *Pisolithus tinctorius* (Krugner and Tomazello Filho, 1981). Other species such as *Amanita muscaria*, *Inocybe curvipes*, *Lactarius deliciosus*, *Russula consobrina*, *Suillus cothurnatus*, *Suillus granulatus*, *Laccaria fraterna*, *Ramaria toxica*, *Tricholoma* sp. were also recorded in association to tree species by Carvalho and Amazonas (2002).

The ECM fungi are very important in ecosystems because their mutualistic association with plants of different species helps the absorption of nutrients and water, as well as protection of the host plant against pathogens, abiotic stresses and environments contaminated with heavy metals (Smith and Read, 1997; Peterson et al., 2004; Silva et al., 2013). The role of ectomycorrhizae in forest ecosystems is even more pronounced, strongly acting in biogeochemical cycles, in the dynamics of plant communities and in the maintenance of soil structure (Rillig and Mummey, 2006; Morris et al., 2009). Morphological techniques and/or molecular techniques can be used for the characterization and identification of ectomycorrhizal fungi.

Morphological techniques

ECM fungal species develop a sexual reproductive structure called basidioma and have epigeous habits, developing above the soil surface. Therefore, they may be collected, identified and used in investigations of their community structure. Some ECM fungi, however, produce hypogeous basidioma, unnoticeable or difficult to detect and therefore often ignored in field studies. The diversity of ECM fungi requires analysis of both habit structures, whether epigeous or hypogeous (Brundrett et al., 1996).

The morphological and anatomical characteristics of the tip of roots formed by different ECM fungi are often unique and allow classification in morphological types that can be used as a guide to identify fungal species (Agerer, 1991; Mello et al., 2006).

Although morphotyping has been successfully used in many ecological studies, its approach has several disadvantages. It is an extremely lengthy procedure and requires a significant skill, which requires training and practice. In addition, several morphotypes are often not identified in ecological studies because of the lack of distinctive features or time required to perform morphotyping more precisely. Morphotyping also provides morphological and anatomical characteristics of mycorrhizae, producing useful information on functional roles of fungi in the community regarding nutrients and water absorption (Anderson, 2006).

Taxonomy provides a framework on the biological basis for works by providing names of organisms and data on their biology, as well as its analogy with related organisms. The name of a fungus is of paramount importance, as it provides access to all information on the species and their possible relationships. For example, which host plants a particular fungus is known to form mycorrhizal association with is significant information for ecological studies, especially in habitats where many species of ectomycorrhizal plants live closely. If the fungus is not identified, determining it as an ectomycorrhizal fungus may require time-consuming procedures such as isolation and testing of pure cultures (Brundrett et al., 1996; Peterson et al., 2004; Sulzbacher et al., 2013).

According to Brundrett et al. (1996), the identification of fungi depends on precise recognition and detailed description of the macroscopic and microscopic features. The identification may be performed as described below, depending on the precision intended.

1. First, one must determine the large group of fungi to which the specimen belongs, for example, if it is an Ascomycota or Basidiomycota, as well as the family to which it belongs. This level of identification may often be insufficient to determine whether or not it is an ectomycorrhizal specimen.

2. At a second identification level, fresh samples of the basidioma collected in the field are required for the classification of the fungi. Features like color, color of the spores and texture add an important taxonomic value to some fungi groups. Dichotomous keys are essential for the identification at the level of genus and species. Identification at the genus level is typically sufficient to determine whether or not mycorrhizal association exists.

3. At the third investigation level, more precision is needed to accurately identify species of ectomycorrhizal fungi, because it typically involves a detailed analysis of macroscopic and microscopic features, as well as a review of studies specialized in each genus of fungi. It may also be necessary to consult a taxonomist, preferably specialized in a specific fungi group. Descriptive data and samples that are submitted to a herbarium should allow its identification, but it is a slow process.

Macromorphological features

In order to identify ectomycorrhizal fungi in the field during the collection, observation and recording of morphological details of the specimens are important. Taxonomists often require rich details to conduct rigorous taxonomic identification. Some equipment for the collection of fungi is essential, such as hand lenses, digital camera, ruler, data sheet, paper bags and GPS. After collection, macroscopic features should be analyzed with both the naked eye and under a stereoscopic microscope. Each group of fungi has characteristics that distinguish them. For mushroom basidioma, one should consider the characteristics of the pileus, lamellae and stipe, noting details of shape, size, color, texture, diameter, volva, presence and absence of ring, among other special features. The morphology of fungi is extremely diverse, and in some cases, some features may require special attention to detail (Brundrett et al., 1996; Moreira and Siqueira, 2002; Castellano et al., 2004; Lupatini et al., 2008).

Micromorphological features

Microscopic characteristics can be observed with fresh or dry herbarium (oven at 50 °C for 48 h) material. Only general characteristics can be observed with low power microscope, and an oil immersion lens is essential to observe minute details such as the ornamentation of spores and other important taxonomic characteristics (Brundrett et al., 1996; Peterson et al., 2004). According to Brundrett et al. (1996), there is an order of importance for the most important parts of the mushroom basidioma that need to be examined under a microscope: 1) The basidiospores are most often the most important structures. 2) The hymenium and the peridium are also important. 3) For more detailed examinations, other structures, such as the trama of the hymenium, caulocystidium, pileus and stipe, are also analyzed.

For a rapid assessment, only one basidioma can be examined. For a more detailed identification, it is typically necessary to assess the developmental stage of the group species in order to observe structures in immature and mature stages, as well as recording characteristics

such as size of the spores, types of spores, chemical reactions carried out using specific dyes (such as Phloxine (3 %), Melzer's reagent, Cotton Blue) more accurately. In mushrooms and wood-degrading fungi, the Melzer's reagent is widely used in which two amyloid reactions are observed (blue color) and dextrinoids (brown color) (Brundrett et al., 1996; Peterson et al., 2004; Sulzbacher et al., 2010). Table 1 presents some fungi and their morphological descriptions according to the authors who described them.

Molecular techniques

Most molecular techniques used in the identification of mycorrhizal fungi are based on the analysis of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) (Siqueira et al., 2010) through the polymerase chain reaction (PCR), which allows amplification and characterization of DNA from a small sample of the genetic material of the fungus. The advantage of these processes is that they are quick and highly sensitive, and they are not subject to phenotypic variation, environmental action, developmental stage and other factors that may affect the organism morphology (Anderson, 2006).

As with all PCR-based methods, the specificity of the analysis is determined by the choice of the oligonucleotide primer used in the initial PCR. The oligonucleotide primers amplify regions of the ribosomal DNA (rDNA), especially the ITS regions, for ECM fungi (Anderson et al., 2003). There are no specific oligonucleotide primers for ECM fungi, as the ectomycorrhizae are formed by a diverse range of fungi belonging to different phyla, families and genera of which other non-mycorrhizal species are also part (Siqueira et al., 2010). Thus, studies on molecular ecology of ECM fungi involve the extraction of nucleic acids from individual ectomycorrhizal tips. "Universal" oligonucleotide primers, which have been used for fungi, may be used for ECM fungi, and nucleic acids should be extracted from root tips colonized by a single fungus. In studies on ECM fungi, oligonucleotide primers of the ITS region are most used due to greater amount of information available in the database of this region for these organisms and, most importantly, because they provide genomic print for each ECM fungus. Oligonucleotide primers for 18S rDNA have also been used in molecular studies on soil fungi in general (Anderson and Cairney, 2004). However, they are more limited to ECM fungi due to their low taxonomic resolution of 18S rRNA genetic sequences and the limited amount of information currently available in the public database for many ECM groups (Anderson, 2006; Siqueira et al., 2010).

A set of new techniques has been developed for the identification of microorganisms in environmental samples and, although their use at a fungal ecology level is still at an early stage, these techniques have revolutionized studies and have the potential to promote understanding of ECM fungal communities in the field of bacterial ecology (Anderson and Cairney, 2004; Yang, 2011; Kõljalg and Grebenc, 2013).

An important advantage of these techniques is that is enables analysis of communities with a high DNA yield extracted from soil samples or symbiotic root tissue, thus allowing good replication. Perhaps the main advantage of these techniques is the direct detection of ECM mycelium in the soil, allowing to investigate the distribution, dynamics and abundance of ECM fungal communities in the field (Cairney, 2005; Lupatini et al., 2013).

The most promising molecular techniques for the study of ECM ecology are Polymerase Chain Reaction (PCR), Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE), Restriction fragment length polymorphism (RFLP) and Terminal Restriction fragmented length polymorphism (TRFLP), Ribosomal intergenic spacer analysis (RISA) and automated RISA (ARISA), as well as cloning (Siqueira et al., 2010). Recently, new generation sequencing, coupled with bioinformatics tools, has identified arbuscular fungal microbes in environmental samples, such as the soil (Xiang et al., 2016) and roots (Hart et al., 2014), without the use of clones. This same methodological approach can also be used for ectomy-

Table 1 – Examples of morphological characterization of ectomycorrhizal fungi.

Ectomycorrhizal fungi	Characteristic(s)	Author
Amanita	Ring and volva in stipe. Smooth hyaline spores with thin walls and shape ranging from subglobose to ellipsoid and non-amyloid.	Largent, 1986 Brundett et al., 1996
Laccaria	Hyaline echinulate (with thorns) spores with thin walls and shape ranging from globose to ellipsoid. They do not react with the Melzer's reagent: non-amyloid.	Brundrett et al., 1996
Russula	The most striking macroscopic feature is the "limestone" texture of its basidioma, which is very brittle. The most striking microscopic feature is the presence of spherocysts.	Largent, 1986 Brundrett et al., 1996
Lactarius	The distinctive feature of the *Lactarius* genus is latex exudation of the gills or pulp when they are damaged.	Largent, 1986 Largent and Baroni, 1988
Boletinus	Poroid or tubular hymenium. Voluminous spores with color ranging from brown to olive, and smooth basidiospores with fusoid elongate. The basidia are small compared to the basidiospores.	Brundrett et al., 1996
Scleroderma	Gasteroid basidia. Gleba (interior) with greyish tones, olive, almost black, pulverulent. Peridium is hard, inelastic and brittle. Colored basidiospores with brown tones, shape varies from globose to subglobose and may have echinulate, subreticulate and reticulate ornamentation.	Guzmán, 1970. Brundrett et al., 1996

corrhizal fungi (Gao et al., 2015). These tools, besides allowing the identification of ectomycorrhizae, can contribute to a better understanding of the mutualistic interaction between plant and fungus (Larsen et al., 2011; Sebastiana et al., 2014). The use of a technique varies depending on the purpose of the study.

Polymerase chain reaction (PCR)

PCR is a technique involving enzymatic *in vitro* synthesis of millions of copies of a specific segment of DNA in the presence of the DNA polymerase enzyme. The reaction of PCR relies on annealing and enzymatic extension of a pair of oligonucleotides (small single-stranded DNA molecules) used as primers, delimiting the sequence of double-stranded DNA, which is the target of amplification (Anderson et al., 2006). A PCR cycle consists of three steps: denaturation, annealing and extension. This cycle is repeated dozen of times to ensure that, after only 20 cycles, the initial amount of the target sequence is produced over one million times. Therefore, this amplification range allows starting with minimal amounts of DNA (in picograms or nanograms), and finishing the reaction with large quantities of a specific DNA sequence of interest. The easiness, speed, versatility and sensitivity of PCR make it particularly important for molecular genetic studies involving large numbers of individuals of any living organism (Siqueira et al., 2010).

Studies using PCR and sequencing have shown that ECM communities can be vertically stratified, with some species present even in the deepest mineral layer of the soil (Rosling et al., 2003), and PCR has been used to describe spatial and temporal variations in ECM communities (Izzo et al., 2005).

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE)

DGGE and TGGE are techniques that separate DNA fragments of the same size, but with different base composition, based on the denaturation behavior of DNA. DNA sequences with different base compositions denature at different positions in polyacrylamide gels containing a linear (DGGE) or temperature (TGGE) denaturing gradient on the basis of guanine (G) and cytosine (C) contents (Anderson, 2006) .

DGGE/TGGE have been widely used in studies on microbial communities, because they are fast and visual methods to investigate communities of soil fungi, especially when the objective is to investigate changes or alterations in community composition (Anderson et al., 2003). DGGE has been used to evaluate the diversity of fungi in forest soils with ECM fungi mycelia (Pennanen et al., 2001; Anderson et al., 2003; Smit et al., 2003; Landeweert et al., 2005).

One of the main advantages of the gel-based techniques is the ability to remove target bands and sequence them to produce taxonomic information on members of the selected community through research in databases and phylogenetic analysis. This is an advantage over the TRFLP technique, which does not have a database for the study site. It is also effective when ECM root samples are analyzed without prior morphotyping or knowledge of the species composition (Anderson, 2006).

Restriction fragment length polymorphism (RFLP)

The RFLP technique analyzes polymorphism in the length of fragments obtained by cutting double-stranded DNA. This polymorphism is evidenced by DNA fragmentation using restriction enzyme and observed by hybridization of these fragments with homologous DNA sequences labeled with radioactivity or compounds, which trigger a luminescence reaction (Siqueira et al., 2010). Polymorphism observed in RFLP occurs because DNA of genetically distinct individuals differs in the nucleotide sequence along the strand. The presence or absence of specific sequences of 4 to 8 base pairs, recognized and cleaved by restriction enzymes, may vary between different individuals thus generating polymorphism. When subjected to cleavage with a restriction enzyme, DNA from genetically distinct individuals is cut at restriction sites, generating fragments of different sizes. The genetic basis of the polymorphism observed results from mutations at restriction or insertion sites, deletions and rearrangements between these sites (Anderson, 2006; Siqueira et al., 2010).

Terminal Restriction fragment length polymorphism (TRFLP)

TRFLP is perhaps the most promising technique to characterize ECM communities, and to date, it has provided the most significant insights in ECM ecology. The use of this technology allows comparison of different environmental samples and it is the combination of three other techniques (PCR, RFLP and nucleic acid electrophoresis) (Siqueira et al., 2010). It is a quantitative molecular method for rapid analysis of complex communities. This technique is not restricted to the study of the 18S rRNA gene and it can be used for any gene to uncover differences in mycorrhizal fungal communities (Jesus and Moreira, 2008).

In the TRFLP technique, primers used for PCR are labeled with fluorochrome. Therefore, only the terminal restriction fragments are detected, reducing the complexity of the analysis. The richness of fungal species is estimated by determining the number of terminal fragments observed from PCR amplification. This technique has been widely used for the analysis of microbial communities including studies with ECM fungi. Midgley et al. (2007) used the TRFLP to evaluate the diversity of ECM fungal communities in Australian soils and greater richness of local species with natural vegetation.

Ribosomal intergenic spacer analysis (RISA) and Automated RISA (ARISA)

The RISA method evaluates the existing heterogeneity in 18S-28S intergenic spacer in fungi. The PCR

Table 2 – Examples of molecular characterization of ectomycorrhizal fungi.

Ectomycorrhizal fungi	Technique(s)	Author
Chondrogaster angustisporus	PCR, agarose gel electrophoresis	Lupatini et al., 2008
Hysterangium sp.	PCR, agarose gel electrophoresis	Smith et al., 2007
Pisolithus sp.	PCR, agarose gel electrophoresis, RFLP	Anderson et al., 2001
Pisolithus indicus	PCR, agarose gel electrophoresis, RFLP	Kanchanaprayudh et al., 2003
Pisolithus tinctorius	PCR, agarose gel electrophoresis	Martin et al., 2002
Scleroderma UFSMSc1	PCR, agarose gel electrophoresis	Lupatini et al., 2008

PCR = Polymerase chain reaction; RFLP = Restriction Fragment Length Polymorphism.

product is separated using polyacrylamide gel electrophoresis and the DNA pattern is displayed. The result is a complex pattern of DNA fragments of different sizes that provides a specific profile for the community, where each fragment corresponds to at least one organism in the sample. Such fragments are typically purified in gel and used for sequencing (Siqueira et al., 2010).

The automation of the RISA method is ARISA and it was developed to improve resolution and reduce analysis time. Through ARISA, the intergenic spacer between the larger and smaller subunits of the rRNA gene is amplified. The evaluations using intergenic spacer have been effective in studies on intraspecific diversity or isolated groups with great phylogenetic affinity, as the region has greater variability both in the composition of bases and in its size, compared to the 18S and 28S gene regions of the rRNA gene (Anderson and Carney, 2004; Siqueira et al., 2010).

Cloning

Different DNA sequences amplified from DNA samples of microbial community can be separated by cloning all products from the sample through a specific vector such as a plasmid, and subsequently screening the clones using PCR, restriction digest, sequencing, or a combination of these techniques. It is a quick method to determine identity and diversity of species present in a sample. However, if the number of samples is high, it may become a costly and time-consuming activity (Anderson et al., 2006). An initial screening of clones using RFLP to group them into operational taxonomic units (OTUs) may be useful to reduce the number of samples, but it is difficult to determine how many clones require analysis in order to fully describe the diversity contained within a single sample (Siqueira et al., 2010).

Cloning has been used to show the vertical distribution of ECM mycelium in the soil profile (Landeweert et al., 2005), investigate the effects of fires in ECM and other soil fungi in Australian forests (Chen and Cairney, 2002), and show that ITS sequences with affinity for ECM can also be detected in soil collected near glaciers (Jumpponen, 2003). The description of some ectomycorrhizal fungi characterized by molecular techniques is shown in Table 2.

Final Remarks

The identification of ectomycorrhizal fungi species based on morphological criteria is performed using dichotomous keys and guides for specific fungal species (Mello et al., 2006). Although taxonomy based on morphological characteristics offers description and identification methods of species, there are still difficulties to classify fungi reliably only by their morphological characteristics.

Therefore, the use of molecular techniques is presented as an effective alternative, through DNA analysis, composition and gene sequence of individuals. These methods allow one to ensure issues not defined by morphological description, such as the differentiation of similar species of ectomycorrhizal fungi (Sanon et al., 2009). Some studies have used the combination of morphological and molecular techniques for fungi characterization (Baldoni et al., 2012; Lupatini et al., 2008).

There are a number of methods available to study soil microbial diversity, but each method has its limitations. Therefore, it is advisable to use a variety of methods, incorporating results of traditional and molecular methods to obtain reliable results.

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