Molecular approach to characterize ectomycorrhizae fungi from Mediterranean pine stands in Portugal

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Abstract

Stone pine (*Pinus pinea* L.), like other conifers, forms ectomycorrhizas (ECM), which have beneficial impact on plant growth in natural environments and forest ecosystems. An *in vitro* co-culture of stone pine microshoots with pure mycelia of isolated ECM sporocarps was used to overcome the root growth cessation not only *in vitro* but also to improve root development during acclimation phase. *Pisolithus arhizus* (Scop.) Rauschert and *Lactarius deliciosus* (L. ex Fr.) S.F. Gray fungi, were collected, pure cultured and used in *in vitro* co-culture with stone pine microshoots. Samples of *P. arhizus* and *L. deliciosus* for the *in vitro* co-cultures were collected from the pine stands southwest Portugal. The *in-situ* characterization was based on their morphotypes. To confirm the identity of the collected material, ITS amplification was applied using the pure cultures derived from the sporocarps. Additionally, a molecular profile using PCR based genomic fingerprinting comparison was executed with other genera of Basidiomycetes and Ascomycetes. Our results showed the effectiveness of the techniques used to amplify DNA polymorphic sequences, which enhances the characterization

of the genetic profile of ECM fungi and also provides an option to verify the fungus identity at any stage of plant mycorrhization.

Key words - *Pisolithus arhizus*, *Lactarius deliciosus, Pinus pinea,* M13-PCR, ITS

INTRODUCTION

Ectomycorrhizal fungi (ECM) are major components of the soil fungal communities in most forests around the world and, are ecologically and economically important (Mello *et al*., 2006). Plants in Betulaceae, Pinaceae and Fagaceae families are obligate ECM (Smith and Read, 1997). ECM fungi include species from multiple families in the Basidiomycetes, Ascomycetes and some from the Zygomycetes (Bruns *et al*., 2002). Globally, as many as 10,000 fungus species and 8000 plant species maybe involved in ECM associations (Taylor and Alexander, 2005).

Development and growth of pine (*Pinus* spp.) roots are regulated in nature by ECM (Smith and Read, 1997). Inoculation with specific fungi can enhance pine root formation and/or subsequent root branching of cuttings (Normand *et al*., 1996; Karabaghli *et al*., 1998; Niemi *et al*., 2000). Some research results demonstrated the potential for use of ECM fungi in the vegetative propagation of conifers (Gay, 1990; Niemi *et al*., 2005) and during *in vitro* rooting of pine shoots (Zavattieri *et al*., 2009; Ragonezi *et al*., 20010a). Inoculations enhanced plant performance and contributed to alleviation of stress related with acclimation in a nursery and the subsequent growth in the field.

Stone pine (*Pinus pinea* L.) is one of the most important pines economically (due to the valued edible nut production) in the Mediterranean basin and it forms ectomycorrhizas. Rincón *et al*., (1999) reported that at least eight genera of ECM were associated with *P. pinea* seedlings in the nursery (*Amanita*, *Hebeloma*, *Laccaria*, *Lactarius*, *Pisolithus*, *Rhizopogon*, *Scleroderma*, and *Suillus*). Two species of fungi are commonly used for inoculation in controlled mycorrhization programs associated with *P. pinea*: *Pisolithus arhizus* (Scop.) Rauschert, (Marx *et al*., 1982; Burgess *et al*., 1995) a cosmopolitan fungus which grows in warm temperate regions of the world and is easy to propagate *in vitro* (Marx *et al*., 1982; Cline *et al*., 1987) and *Lactarius deliciosus* (L. ex Fr.) S.F. Gray, typically a Basidiomycetes which produces high commercially valuable edible fruiting bodies (Singer, 1986; Hutchison, 1999; FAO, 2004; Hortal *et al*., 2006).

In nature, and also in controlled inoculations, genetically distinct mycelia of the same ECM species were found on the root system of a single tree (Guidot *et al*., 1999). This was also demonstrated by others studies with *Pinus banksiana* (De La Bastide *et al*., 1995) and *Pinus pinaster* (Gryta *et al*., 1997). Even in cases where the *in vitro* inoculation was controlled, genetic diversity has been found in *ex vitro* phases caused by the lack of effective sterilization of the mixed substrates, contamination from the environment in the growth chamber and in some cases from the irrigation source. On the other hand, ECM fungi are relatively selective of host plant species (Allen *et al*., 1995) and host responses could be partially attributable to variation between different fungus taxa and strains. For all these reasons, accurate characterization and identification of the ECM fungi are fundamental requirements for *in vivo* or *in vitro* mycorrhization programs.

The traditional method of fungal identification by colour, shape and other macroscopic features and microscopic characteristics (Agerer, 1987-2002) could be applied only to a limited number of fungal species (Iotti and Zambonelli, 2006).Neverthless, today a wide range of molecular techniques can be used to distinguish DNA sequence for the identification of ECM fungi (Gardes *et al*.; 1991a, 1991b; Henrion *et al*., 1992; Hortal *et al*., 2006) and also to verify the genetic variation within a specific group (Alves *et al.,* 2007; Caldeira *et al*., 2009).

Amplification of the internal transcribed spacer (ITS) regions in the ribosomal genes (rDNA) usually reveals interspecific variations (Bruns *et al*., 1991, Gomes *et al.,* 2000, Horton 2002). This region has four primary advantages over other regions: 1 - it is multicopy, so the amount of sample material needed for successful amplification is low; 2 - it has well-conserved fungal specific priming sites directly adjacent to multiple highly variable regions; 3 - there are many sequences already available for comparison, which facilitates the identification of unknown samples; and 4 - it correlates well with morphologically defined species in many groups (Smith *et al*., 2007).

Genetic profiles and polymorphic sequences on the other hand, are important tools for rapid and effective characterization of ECM species (Caldeira *et al*., 2009). The polymerase chain reaction (PCR) based genomic fingerprinting is a good alternative to methods that rely on specifically targeted primers. This technique, which analyzes the whole genome, has been

shown to be relatively robust and discriminatory (Alves *et al*., 2007). PCR fingerprinting is also used in the study of genetic variability in yeast and filamentous fungi (Godoy *et al.,* 2004; Alves *et al.,* 2007; Lopes *et al.,* 2007).

The goals of the present study were, first to identify ECM fungi associated with stone pine stands through PCR amplification of the ITS region of the ribosomal genes and to use them in *in vitro* mycorrhization experiments. Second goal was to test the applicability of the M13-PCR fingerprinting methodology for monitoring different species of Basidiomycetes and Ascomycetes which can be found in association between *P. pinea* and ECM fungi.

MATERIAL AND METHODS

Collection of mushrooms from stone pine (*Pinus pinea* L.) stand

Fruiting bodies of *Pisolithus arhizus* (Scop.) Rauschert and *Lactarius deliciosus* (L. ex Fr.) S.F. Gray were collected from a pure stand of stone pine (N 38° 25'; W 7° 56') in January of 2010. Morphological identification was done *in situ* at the collection time. Specimens were stored at 4ºC prior to sterilization and isolation procedures. Voucher specimens of *Pisolithus arhizus* and *Lactarius deliciosus* were deposited at Évora University Herbarium with the numbers UEVH-FUNGI 2001610 and UEVH-FUNGI 2001712, respectively.

Mycelia isolation and fungal cultures

For the asepsis, the fruiting bodies were cut into large pieces, placed in running water for 10 min and then in 70% ethanol for 2 min. Then, pieces were rinsed with sterile distilled water in a laminar flow unit, placed in 20% (v/v) commercial bleach (≤ 5% active chlorine) for 10 min and rinsed four times with sterile water. The larger pieces were then cut in smaller pieces (50 mm^3) for growth and subsequently DNA extraction or were stored at -20ºC. Isolates were cultured in Hagen medium (Modess, 1941). The formulation of modified Hagen per liter was: KH_2PO_4 0.5 g, NH_4CL 0.5 g, $MgSO_4$ $7H_2O$ 0.5 g, $FeCL_3$ (1%) 0.5 ml, glucose 5 g, malt extract 5 g, thiamine HCL 50 µg and agar 15 gand the pH was adjusted to 4.5-5.0. With the purpose to avoid the contamination by bacteria, 100 mg ml^{-1} of Rifampicin (Sigma-Aldrich®) was added to the media after cooling. Pieces of sporocarps were kept in petri dishes Hagen medium, grown

at 25ºC in the dark and sub-cultured at weekly intervals. Isolates have been growing in Hagen slants for 14 days at 25ºC and stored at 4ºC. Fungal isolates of *Pisolithus arhizus* and *Lactarius deliciosus* were deposited in the Culture Collection of the Biotechnology Laboratory of University of Évora and preserved at −80°C in cryovials containing 10% glycerol.

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DNA extraction

The extraction of the genomic DNA from the smaller fragments of sporocarps and from the mycelia (after 14 days of culture) was performed using the modified microsphere method (Martins, 2004; Guimarães *et al.,* 2011). The quality and quantity of the obtained DNA was evaluated by agarose gel.

ITS region amplification and sequencing

The region containing partial portions of the small subunit (18S), both internal transcribed spacers (ITS) and the 5.8S of the rDNA repeat unit was amplified using the oligonucleotides primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns, 1993). PCR reactions were carried out on a MyCycler Thermal Cycler (BIO-RAD) and consisted of initial denaturing at 95ºC for 3 min followed by 30 cycles at 92ºC each 30 s, 55ºC for 30 s, and 72ºC for 1 min. The reaction was completed by a 10-min extension at 72ºC. PCR products were analyzed by agarose gel (1%) electrophoresis, purified with the NucleoSpin Extract II Kit (Macherey-Nagel) and sequenced by capillary electrophoresis using the ABI PRISM 3730 xl sequencer (Applied Biosystems) with the Kit BDT v1.1 (Applied Biosystems).

M13-PCR amplification

The M13 primer (5'- GAGGGTGGCGGTTCT-3') was used for the PCR. The PCR conditions consisted of an initial denaturing step of 5 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C. The reaction was completed with a final extension at 72 °C for 5 min and then cooled at 4 °C. A sample of each PCR reaction product was electrophoresed in a 1.5% agarose gel and visualized, by staining with ethidium bromide, in

a UV transilluminator (BIO-RAD). To evaluate the reproducibility of the assay, each sample has been analyzed in at least three independent PCR reactions. A negative control (without DNA template) has been included in every run. Subsequently, DNA sequence analysis was employed for confirmation of the fingerprint technique characterization.

Data analysis

The nucleotide sequences of the ITS region were aligned with those of related fungal species retrieved from the GenBank (National Center for Biotechnology Information - NCBI) databases for the homology analysis using the BLASTN 2.2.25+ program. The phylogenetic relationships between different species were inferred after multiple alignments using CLUSTAL W (Thompson *et al*., 1994). The distances of the DNA arrays were calculated with the option of Jukes-Cantor and from these matrixes, using the Neighbor-Joining method, the phylogenetic tree was constructed, using the program Mega 5 (Tamura, 2011).

For the M13-PCR analysis, the phylogenetic tree was generated by the Unweighted Pair Group Method with arithmetic Average (UPGMA), through the use of the Dice coefficient of similarity using Quantity One 1-D Analysis software (BIO-RAD).

RESULTS AND DISCUSSION

Collection of fruiting bodies from stone pine stand

Representative voucher specimens of *Pisolithus arhizus* and *Lactarius deliciosus* fruiting bodies are shown in Fig. 1a and Fig. 2a. Based on preliminary tests, we have selected Hagen medium as the most suitable for isolation and growth of the mycelia from sporocarps. The cultured mycelia were characterized by yellowish-ochraceous with paler margin in the case of *P. arhizus* (Fig. 1b) and pinkish with paler margin for *L. deliciosus* (Fig. 2b). The microscopic features showed the secondary mycelia at the septa of a Basidiomycota hypha (Fig. 1c e 2c).

Fresh mycelia of each culture were used to inoculate stone pine microshoots at the rooting phase (Fig. 3). The mycorrhization of the plants were confirmed in the acclimatization phase (Fig. 4a and b) revealing the typical ECM structure. The ECM fungi presence was

monitored and confirmed during the mycorrhization process, by applying two molecular complementary approaches: ITS sequencing and M13-PCR amplification.

Species identification of *P. arhizus* and *L. deliciosus*

In the past, the most common approach to evaluate fungal biodiversity in various plants/ systems has been the sample collection, fungal isolation and identification based on classical methods (Genilloud *et al.,* 1994; De Jager *et al.,* 2001; Moreira *et al.,* 2001; Schmit and Lodge, 2005), but nowadays several molecular techniques can be used namely for ECM fungi identification (Rosling, 2003; Leake *et al.,* 2004; Hortal *et al.,* 2006; Caldeira *et al.,* 2009).

Amplification of the ITS region is a common approach in molecular identification strategies (Hortal *et al.,* 2006; Alves *et al.,* 2007). PCR products of ITS4/ITS5 primers, corresponding to the ITS1, 5.8S and ITS2 regions of the rDNA were approximately 644 bp and 400 bp obtained from dikariontic isolates from *Pisolithus sp*. P1001 and *Lactarius deliciosus* UEZB1 respectively. Sequence alignments of *P. arhizus* showed identities that ranged from 99-100% among isolates belonging to *P. arhizus.* Sequences were aligned at the NCBI for isolates corresponded to other *Pisolithus spp.* In the case of *L. deliciosus* the homology was over 99%. The most similar sequences of *P. arhizus* and *L. deliciosus* are shown in Table 1. The phylogenetic tree (Fig. 5) was obtained from the alignment of these sequences. We identified two different clusters, *Pisolithus* sp. P1001 and *L. deliciosus* isolate UEZB1 (Fig. 5). Multiple alignment of *Pisolithus sp.* cluster corresponded to a partial sequence of 18S RNA gene and ITS1, 5.8S ribosomal RNA gene and ITS2, and partial sequence of 28S RNA ribosomal region. *L. deliciosus* UEZB1 corresponded to partial sequence of ITS1, 5.8S ribosomal RNA gene, ITS2 and partial sequence of 28S ribosomal RNA. Both sequences were published in GenBank with accession number HQ896485 and JQ066791, respectively.

Intraspecies identification by M13-PCR

The amplification using the M13 primer has generated a profile with 7-14 DNA fragments ranging from 100 to 2700 bp in the Basidiomycetes sporocarps species (*P. arhizus*, *L. deliciosus* and *R. roseolus*), *Pisolithus sp.* isolated P1001 and select Ascomycetes (*P.*

brevicompactum, *A. niger*, *Cladosporium sp*.1, *and F. oxysporum*). These Ascomycetes species could live in association with ECM fungi and were commonly found in the isolation process. Reproducibility of the M13-PCR fingerprinting techniques was checked by comparing the banding profiles resulting from independent extractions and amplifications of the same fungi strain. The different samples have generated distinct patterns in the electrophoresis analysis (Fig.6). The M13-PCR band profile generated in each fingerprint varied according to the species included in this study: 10 fragments ranging from 200-900 bp for *P. arhizus,* 7 fragments for *L. deliciosus* (from 200-550 bp) and 9 fragments for *R. roseolus* (from 200-750 bp). The Ascomycetes, *P. brevicompactum* presented 9 fragments (from 200-1100 bp), *A. niger* 10 fragments (from 200-1850 bp), *Cladosporium* sp.1 presented 13 fragments (from 200-1850 bp) and *F. oxysporum* 14 fragments (from 200- 2700 bp). The Ascomycetes group presented a higher DNA fragments (ranging from 950-2700 bp) than the Basidiomycetes, with fragments from 150-900 bp. Figure 7 shows a phylogenetic tree based on M13-PCR fingerprinting. In the analysis of the phylogenetic tree, *P. arhizus* formed a cluster of 41% similarity with *R. roseolus*, which forms a cluster with *L. deliciosus* with a homology of 34%. This approach also allowed the distinguishing between Basidiomycetes and Ascomycetes group, which formed a cluster with 15% of similarity for *P. brevicompactum*, *8% for A. niger, 5%* for *F. oxysporum and 2% for Cladosporium* sp.1.

These results demonstrated that M13-PCR discriminated among between species and taxonomic groups. Based on the specific PCR fingerprints and the high interspecies variation of these banding patterns, a clear distinction among all species was feasible. M13-PCR highlighted differentiation at the species and strain level (Caldeira *et al.,* 2009). In this study, the M13-PCR approach was a rapid method to amplify DNA polymorphic sequences, with a high level of similarity for the same species, which enhances the characterization of the genetic profile of sporocarps such as *L. deliciosus*, *P. arhizus* and *R*. *roseolus*.

The advantages of this DNA amplification method are the technique simplicity, universal availability of PCR primers, reproducibility and amenability to the computer database analysis. Using only a single primer M13-PCR, it was possible to achieve high levels of resolution. This makes the procedure much faster and easier, and greatly reduces the cost (Alves *et al.,* 2009).

Hence, PCR fingerprinting offers a simple and reliable alternative method to resolve taxonomic problems and to "label" strains of filamentous fungi (Meyer *et al*., 1991).

CONCLUSIONS

Results of this study demonstrate that the combined use of sequence analysis of the ITS regions of the rDNA and the PCR fingerprinting technique can be successfully applied as an excellent tool to examine the species collected in the field associated with *Pinus pinea* and also as a methodology to monitor the fungi species involved in all the steps in a mycorrhization program. The applied molecular techniques accurately characterized field collected sporocarps and confirmed the presence of the fungus in inoculated plants.

Owing to its low cost and rapidity, the M13-PCR has a wide application in applied mycology as was demonstrated in this study. Also, we confirmed that the M13-PCR technique has a high level of reproducibility because the fungal samples amplified in independent PCRs displayed similar banding pattern profiles.

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Table 1. ITS rDNA homology from fungal strains used in the phylogenetic tree construction. The nucleotide sequences of the ITS region were aligned with those of related fungal species retrieved from the NCBI databases (www.ncbi.nlm.nih.gov).

Strains	Identification nº (NCBI)	Homology
Pisolithus sp. KH-NC09	gb|GQ429212.1	99%
Pisolithus microcarpus	emb|AM084706	100%
Pisolithus tinctorius R15	gb|AF374695	99%
Pisolithus microcarpus VIC30598	gb|HQ693097	100%
Lactarius deliciosus H:6002989	gb|GU373514.1	100%
Lactarius sp. isolate cm130.ps	gb|EU668299.1	100%
Lactarius deliciosus isolate CSUFTXY7	gb|HQ635086.1	100%
Lactarius deliciosus LDTA30	gb|FJ858745.1	100%

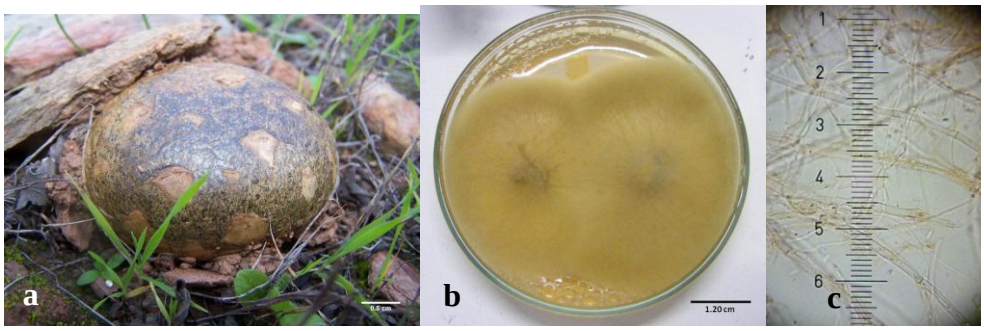

Figure 1. *Pisolithus arhizus* sporocarp collected in a stone pine stand selected for *Pinus pinea*-ECM associations study (a). The mycelia cultured in Hagen medium (b). Secondary mycelia. Each interval 2.5 µm (c).

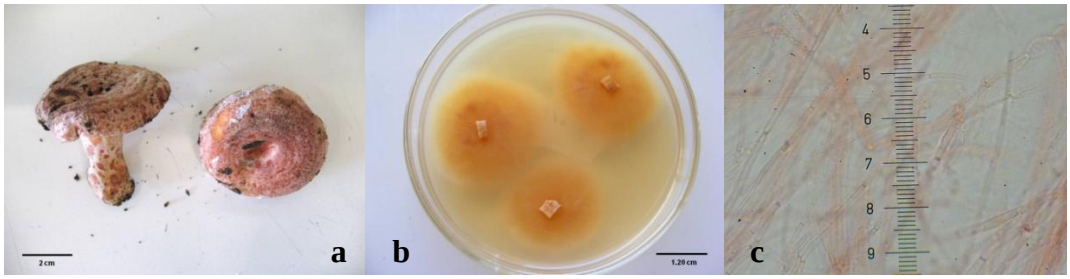

Figure 2. *Lactarius deliciosus* sporocarps collected in a pine stand selected for *Pinus pinea*-ECM associations study (a). The mycelia cultured in Hagen medium (b). Secondary mycelia. Each interval 2.5 µm (c).

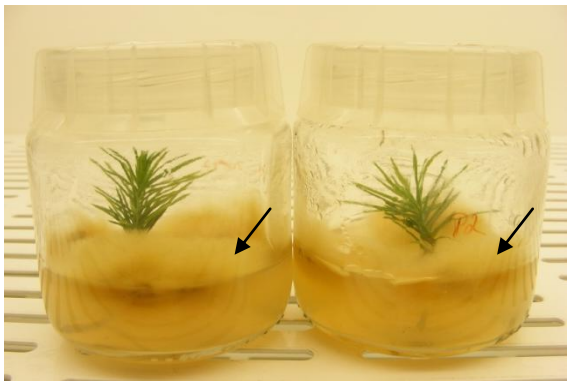

Figure 3. *In vitro* co-culture of *Pinus pinea* and *P. arhizus* mycelium (arrows)

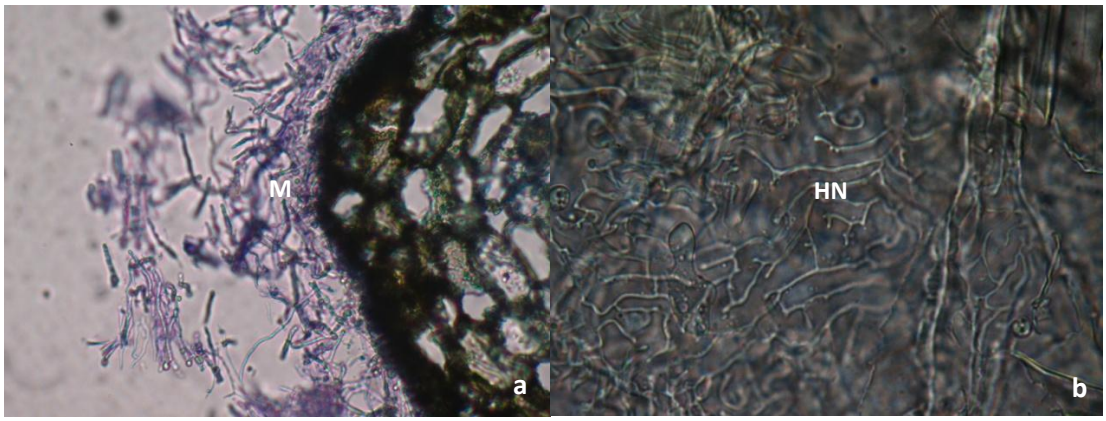

Figure 4. Cryostat transversal root section of the colonized by *P. arhizus* pine root showing the mantle hyphae (M) (100x); Scale bar 20 µm (a). Details of the transversal section showing well-differentiated Hartig-net (HN) in cortical cells (1250x); Scale bar 7.5 µm (b).

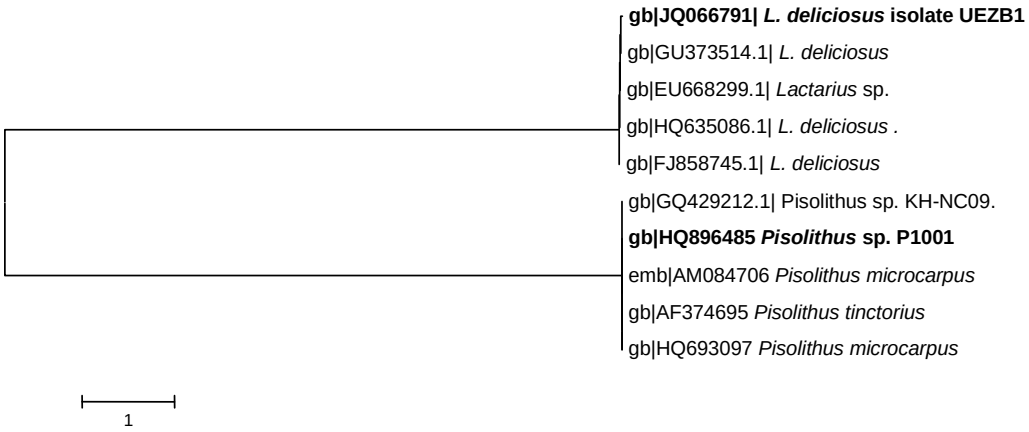

Figure 5. Phylogeny tree based on the ITS sequence

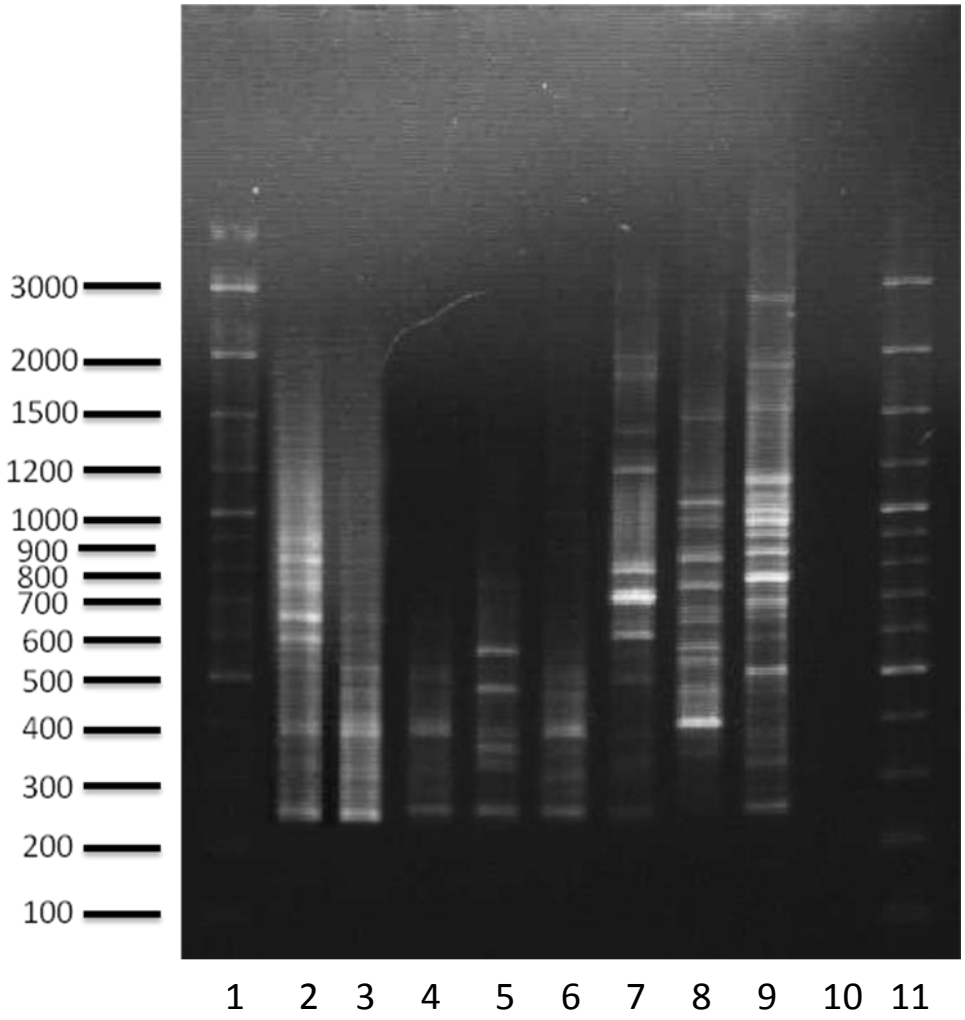

Figure 6. Fingerprinting patterns obtained from obtained by amplification of genomic DNA. Lanes: 1 and 11 DNA molecular ladder 100 bp plus (Fermentas), 2: *Pisolithus arhizus* culture, 3: *Pisolithus arhizus* sporocarps, 4: *Lactarius deliciosus* sporocarps, 5: *Rhizopogon roseolus* sporocarps, 6: *Penicillium brevicompactum* sporocarps, 7: *Aspergillus niger* sporocarps 8: *Cladosporium sp*., 9: *Fusarium oxysporum* sporocarps, 10: Control.

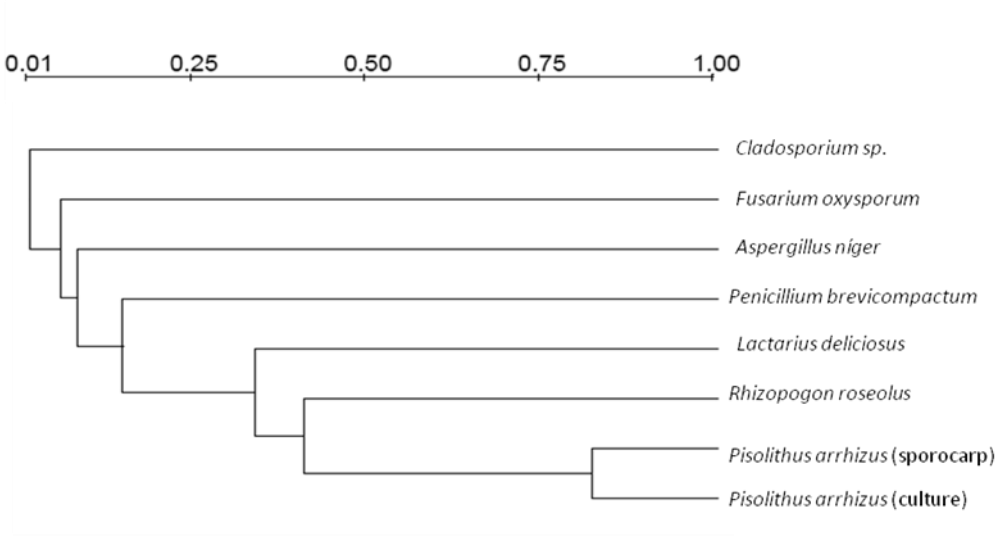

Figure 7. Phylogenetic tree analysis based on the PCR fingerprinting patterns for different species of Basidiomycetes and Ascomycetes. To evaluate the reproducibility of the assay, each sample has been analyzed in at least three independent PCR reactions. The distance values between branches are reported as percentage of similarity (0-100%).