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Molecular evaluation of some *Amanita ponderosa* and the fungal strains living in association with these mushrooms in the southwestern Iberian Peninsula

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Abstract *Amanita ponderosa* are wild edible mushrooms that grow only in some microclimates, particularly those in the southwestern part of the Iberian Peninsula. Due to the vast diversity of mushrooms in nature, as well as nutrient variability, which is highly dependent on soil type and environmental conditions, it is essential to be able to characterize fungal microbiota that lives in association with mushrooms and to differentiate *A. ponderosa* strains of different regions for certification purposes. In this study, we characterized the genetic profile of *A. ponderosa* mushrooms and the fungal strains that live in association with them in their natural habitat and compared the fingerprinting profiles obtained by M13-PCR amplification of the genomic DNA. We found that the predominant fungal isolates living in association with *A. ponderosa* were *Aspergillus* spp., *Penicillium* spp. and *Mucor* spp. M13-PCR molecular analysis showed that different fungal isolates had different genetic profiles. This approach allowed us to differentiate the different fungi strains isolated from fruiting bodies of *A. ponderosa* both rapidly and in a reproducible manner and to group them according to genus. Our fingerprinting analyses also distinguished different *A. ponderosa* mushrooms collected from different regions. Consequently, we conclude that this method is a very discriminatory approach for differentiating both *A. ponderosa* from different sites and the fungal microbiota that lives in association with these mushrooms.

Keywords *Amanita ponderosa* · M13-PCR · Molecular profile · Wild edible mushrooms · Fungal microbiota

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Introduction

The commercial harvesting of edible mushroom is an economically important business in many rural areas of some countries. Edible mushrooms are widely consumed due to their organoleptic properties, such as their texture, attractive taste and flavor (Agrahar-Murugkar and Subbulakshmi 2005; De Pinho et al. 2008; Zawirska-wojtasiak et al. 2009; Guillamón et al. 2010), their chemical composition, with a high content of protein, dietary fibre and vitamins E, B, C and D and low levels of sugar and fat, and their high mineral content, namely calcium, phosphorus, iron, potassium, selenium and cadmium (Vetter 2005; Ouzouni et al. 2007; Guillamón et al. 2010; Beluhan and Ranogajec 2011; Reis et al. 2012). In addition to their nutritional value, some edible mushrooms are known for its medicinal properties (Barros et al. 2007; Barros et al. 2008; Vaz et al. 2011; Liu et al. 2012; Pereira et al. 2012). Therefore, the consumption of edible wild-growing mushrooms has been very popular. However, as some species are highly toxic and their consumption dangerous to human health, it is very important to be able characterize wild mushrooms for certification purposes.

The genus *Amanita* Pers. is one of the best known and most characteristic genera within Agaricales Clem. and comprises edible and poisonous mushrooms that are distributed throughout the worldwide. They occupy primarily a mycorrhizal habit and play a significant role in forest ecosystems (González et al. 2002; Moreno et al. 2008). This genus has

been quite extensively studied at both the morphological and molecular levels, but some taxonomic bottlenecks remain unsolved. Some *Amanita* Pers. species are well known for their toxic or hallucinogenic properties [primarily *Amanita phalloides and A. virosa* (Section *Phalloideae*), *A. muscaria* and *A. pantherina* (Section *Amanita*)] while others are edible and highly valued in gastronomic circles [e.g. *A. ponderosa* and *A. curtipes* (Section *Amidella*) and *A. rubescens* (Section *Validae*); González et al. 2002; Moncalvo et al. 2002; Moreno et al. 2008]. *Amanita* genus includes many species, some still poorly studied, particularly *A. ponderosa* (González et al. 2002; Moreno et al. 2008; Caldeira et al. 2009)

The phylogenetic tree based on 28S rRNA gene sequences allow *Amanita* species to be clustered according to different sections (Moncalvo et al. 2002; Moreno et al. 2008). The members of section *Amidella* are macroscopically characterized by a robust fruiting body, white flesh and a very characteristic white volva with a free and membranous margin. The fleeting annulus is often friable and the cap margin is appendiculate (Moreno-Rojas et al. 2004; Moreno et al. 2008; Caldeira et al. 2009). The main microscopic features of members of section *Amidella* are the amyloid and ellipsoidal to subcylindrical spores (Pinho-Almeida 1994).

Amanita ponderosa was first described by Malençon and Heim (1942). This species is barely known in most of Europe, being abundant only in the southwestern Iberian Peninsula due to its Mediterranean characteristics—more specifically in Alentejo (Portugal) and Andalusia (Spain)— although it is found in Morocco and very rare in Italy. It appears only in the spring, even appearing in January in warmer years (Moreno et al. 2008).

Amanita ponderosa is characterized by a large and robust basidiomata (Fig. 1). The cap of the fruiting body varies in diameter from 8 to 17 cm, with a hemispheric morphology when young and plane–convex morphology in maturity and showing a slight depression in its centre (Moreno-Rojas et al. 2004). The edge of the cap is curved inwards with (occasionally) the remainder of a partial veil. The pileipellis or cuticle is organized in cutis, which consist of cylindrical hyphae that are 3–5 μm wide, hyaline, without clamps and whitish-cream in early stages or while underground, turning pale pink to brownish when old or upon contact with air. When young, they appear to be covered by a thick membrane, namely the universal veil, and the margin is short striate and appendiculate.The hymenium of the mushrooms is constituted of broad laminas, which are only slightly serrated, either free or subadnated, with few lamellae, and whitish to pinkish or brownish when old or dried. The stipe is cylindrical (4.5–10× 2–3 cm), partly smooth to slightly fibrillose and similarly colored or showing pinkish-brown hues; it has an unclear annulus, broken up by the growth of the carpophore, the remainder like threads surrounding the stipe. The flesh is very compact, firm and white, turning to pinkish when in contact

Fig. 1 Basidiomata of *Amanita ponderosa*

with the air, with a pungent flavor and a smell typical of damp earth. The base of the stem is constituted by a membranous volva, which is persistent, obconic, whitish and thick, with the same colour as the rest of the fruiting body (Moreno et al. 2008).

Basidia are tetrasporic, with basidiospores [n = (10)/11–13×6–7 μm; length/width ratio 1.83–1.85], cylindrical to cylindric–ellipsoid, amyloid and smooth. The spores are clumped or scattered and white when fresh, cream when dry. *A. ponderosa* has a semi-hypogeous habit since it grows totally underground in the first stages of development, emerging partially or completely after expansion of the pileus (cap).

This species grows on acid soils in Mediterranean forests of holm oaks and cork trees, such as *Quercus ilex* and *Q. suber*, and shrubs, such as *Cistus ladanifer*, *C. laurifolius* and *Lavandula stoechas* (Moreno-Rojas et al. 2004; Moreno et al. 2008).

Amanita ponderosa is subjected to strong commercial exploitation in some areas, with a high exportation potential in Portugal. It is so eagerly sought after by collectors that it is very often collected in the primordial stage of development as a truly hypogeous fungus before emerging to the surface (Daza et al. 2003).

The overall aim of our study was to obtain *A. ponderosa* pure cultures, characterize the genetic profile of *A. ponderosa* mushrooms collected from different areas of Alentejo (Portugal) and assess fungal strains that live in association with these mushrooms in their natural habitat. These data could then be used to construct a database of fungal biodiversity for fungal isolates living in association with *A. ponderosa.*

Several molecular genetic tools have been introduced for the classification and typing of fungi, including the

sequencing of rRNA genes and/or their flanking internal transcribed spacer (ITS) regions (Flórez et al. 2007), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism analysis (Kure et al. 2002). Although rDNA sequence analysis is currently the tool of choice in most molecular systematic studies, amplified ribosomal DNA restriction analysis of ITS (ITS-ARDRA) has been applied to the study of phenotypic relationships in several fungal genera (González et al. 2002; Alves et al. 2007). González et al. (2002) used the ARDRA technique to the study of phenotypic relationships among several Spanish *Amanita* species, and although it proved to be a powerful tool for identifying fungal species, it failed to discriminate variants within the strain. PCR-based DNA fingerprinting techniques such as RAPD and AFLP represent a very informative and cost-effective approach for assessing genetic diversity in a wide range of organisms and do not require any prior knowledge of the species' genome (Badfar-Chaleshtori et al. 2012). A recent genetic analysis of fungi has shown that fingerprinting techniques are more discriminatory than rRNA gene sequence-based methods for distinguishing strains within species (Ro et al. 2007).

RAPD is one of the most frequently applied molecular techniques and it supports the detection of polymorphism at many loci in the entire genome (Przyborowski and Sulima 2010). The main advantages of RAPD include the use of small quantities of DNA and simplicity. Several studies have demonstrated that RAPD analysis as an easy, fast and reliable tool for the characterization of edible mushrooms (Badfar-Chaleshtori et al. 2012; Ro et al. 2007).

Mini-satellite-specific primer fingerprinting is based on PCR amplification with a single primer as the core sequence of the wild-type phage M13 (Kure et al. 2002). DNA of the phage M13 has been used both as a primer and a hybridizing probe to distinguish between strains of the same fungal species (Kure et al. 2002; Alves et al. 2007; Caldeira et al. 2009).

Thus, in our study we specifically sought to characterize the genetic profile of *A. ponderosa* mushrooms and fungal strains that live in association in their natural habitat using M13-PCR in order to develop metagenomic approaches that could be applied to a further certification process. This is a new approach for *A. ponderosa* characterization and fungal microbiota associated with these mushrooms.

Methodology

Samples

Fruiting bodies of *A. ponderosa* mushrooms were collected in the spring (February to April) of 2010 from different locations in the southwest of the Iberian Peninsula [Azaruja, Guadalupe, Évora (Alentejo) Portugal)]. The mushrooms collected were growing in acid soils in forests of *Quercus suber*, *Q. ilex* ssp. *ballota*, *Cistus ladanifer* and *Cistus laurifolius* at the abovementioned sites. All mushrooms were at the same growth stage to avoid the effect of size. The material was weighed and placed in sterile bags for molecular characterization in the laboratory. For the molecular study, *A. ponderosa* strains were compared with isolated fungal strains that live in association with this mushroom species in their natural habitat. *A. ponderosa* strains were also compared with four species of cultured mushrooms, namely, the Basidiomycetes *Coriolus versicolor*, *Lactarius deliciosus*, *Pleurotus ostreatus* and *Lentinula edodes*, all of which grow in the same forest environment. These four types of wild edible mushrooms were sampled and analysed per location and stored in the Laboratory of Biotechnology, Department of Chemistry of the University of Évora.

Isolation, identification and culturing of pure cultures

A primary culture was made by removing small fragments of the inside of the mushroom hat from the young fruiting body. This part of the mushroom was chosen because it contains the reproductive structures that release spores, The tissue fragments were place in a petri dishes (diameter 5.5 cm) containing Modified Morgan's medium [in g/l: glucose, 20; malt extract, 3; yeast extract, 1; $CaCl_2$, 0.05; NaCl, 0.025; $(NH4)_2HPO_4$, 0.25 g; $FeCl_3$, 0.012; thiamine, 0.003; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.150; agar, 20, pH 6.0]. All cultures were sealed with parafilm and incubated in the dark at 24 °C for 40 days prior to the first passage. Colonies that appeared during the culture period were of different morphology. Not only pure colonies of the species grew during the study but also predominant microbial flora associated with the species. Colonies were transferred to a sterilized petri dishes containing potato dextrose agar (PDA) medium (39 g/l) and the isolates were incubated at 26 °C, until the development of these fungal colonies (Cheng et al. 2008).

The colonies were successively numbered. Those with the same macro- and micromorphological characteristics were given the same number. Several transfers were made to ensure the purity of the colonies, and mycelia were kept isolated in cultures on PDA (preserved at 4 °C). Each colony was subcultured on PDA and malt-extract agar (MEA) media for identification by conventional mycological methods.

The identification of fungal strains living in association with the mushrooms was performed by classical methods, based on macro- and micro-morphological features, such as colony diameter, texture, colour, dimensions and morphology of hyphae, and reproductive structures (Domsch et al. 1980). Microscopic characteristics of the cultures were observed by light microscopy (model DM 2500P; Leica Microsystems, Wetzlar, Germany) coupled with a resolution camera (model DFC290HD; Leica Microsystems). The microscopic

preparations were stained using lactophenol blue dye to observe the isolates' mycelia. Cultures without sporulation following incubation under black light were designated as sterile mycelia.

DNA quantification

DNA samples were quantified by UV–VIS spectrophotometry using a NanoDrop 2000c spectrometer (NanoDrop Technologies, Wilmington, DE). DNA concentration and purity were determined by measuring optical density (OD) at 260 nm and by calculating the OD ratio 260 nm/280 nm, respectively.

ITS region amplification and sequencing

The region containing partial portions of the small subunit (18S), both ITS and the 5.8S of the rDNA repeat unit was amplified using the oligonucleotides primers ITS5 (5′-GGAA GTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCC GCTTATTGATATGC-3′) (Gardes and Bruns 1993). PCR reactions were carried out on a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA), and the cycling programme consisted of an initial denaturing cycle at 95 °C for 3 min, followed by 30 cycles of 92 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension for 10 min at 72 °C. The PCR products were analysed by agarose gel (1 %) electrophoresis, purified with the NucleoSpin Extract II kit (Macherey–Nagel, Dueren, Germany) and sequenced by capillary electrophoresis using the ABI PRISM 3730 XL Sequencer (Applied Biosystems, Foster City, CA) with the BDT v1.1 kit (Applied Biosystems). This approach was performed for *A. ponderosa* mushrooms, and ITS sequences were aligned with those of related fugal species retrieved from GenBank [National Center for Biotechnology Information (NCBI) databases].

Molecular analyses

Genomic DNA was extracted from the small fragments of the fruiting bodies using the modified microsfer method (Sambrook et al. 1989; Martins 2004; Caldeira et al. 2009). DNA (25 ng) amplification by PCR was carried out in a final volume of 25 μl sterilized ultrapure water containing 2 μM M13 primer (5′-GAGGGTGGCGGTTCT-3′) (Invitrogen, Carlsbad, CA), Taq polymerase buffer (1×), 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotideo and 1 U Taq DNA polymerase (Fermentas, Thermo Fisher, Vilnius, Lithuania), using the Mastercycler (Eppendorf, Hamburg, Germany). The PCR cycling 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 72 °C for 1 min, with a final extension at 72 °C for 5 min followed by cooling and holding at 4 °C.

The PCR products were separated electrophoretically through a 1.5 % (w/v) agarose gel in 0.5× TBE buffer (tris borate–EDTA), at 90 V for 2 h. The gel was stained with ethidium bromide (3 μg/ml), and the DNA was visualized on a UV transilluminator (Bio-Rad) using Quantity One 1-D Analysis software (Bio-Rad). A negative control (without DNA template) was included in every run.

Data analysis

The nucleotide sequences of the ITS region were aligned with those of related fungal species retrieved from the GenBank (NCBI) databases for the homology analysis using the BLASTN 2.2.25+ programme.

For the M13-PCR analysis, the phylogenetic tree was generated by the unweighted pair group method with arithmetic average through the use of the Dice coefficient of similarity using Quantity One 1-D Analysis software (Bio-Rad), based on the fingerprinting profile *A. ponderosa* and other species of class Basidiomycetes, Ascomycetes and Zygomycetes.

Results and discussion

Characterization of *A. ponderosa* cultures and fungal strains which live in symbiosis

Representative samples of *A. ponderosa* sporocarps (Fig. 1) were sequenced and submitted to GenBank under accession number HQ529373. During the isolation of *A. ponderosa,* different fungal strains that live in association with these mushrooms were isolated. These fungi exhibited higher growth kinetics than the mushrooms, thereby interfering with the isolation process of *A. ponderosa*. In terms of the production of pure cultures of *A. ponderosa* and the development of methagenomic methodologies, it is crucial to characterize the associated microbiota. With the aim to characterize these fungi, we cultured the samples on solid PDA and MEA media, ultimately obtaining ten different fungal strains (Fig. 2). The identification of these strains was based on the macroscopic features of colonies growing on agar plates, such as colony diameter, texture, colour and dimensions, and micromorphological characteristics, such as morphology of hyphae and reproductive structures (for sporulating isolates), following standard methods.

Figure 2 shows the macroscopic and microscopic features of the isolated fungal cultures. The main fungal strains isolated in association with *A. ponderosa* mushrooms belong to the genera *Aspergillus*, *Cladobotryum*, *Mucor* and *Penicillium*. Isolates numbers 2 and 3 were identified as belonging to the genus *Penicillium*. Their reproductive structures, the conidiophores, with chains of single-celled conidia called a phialide can be observed in this figure. Isolate number 4 was identified

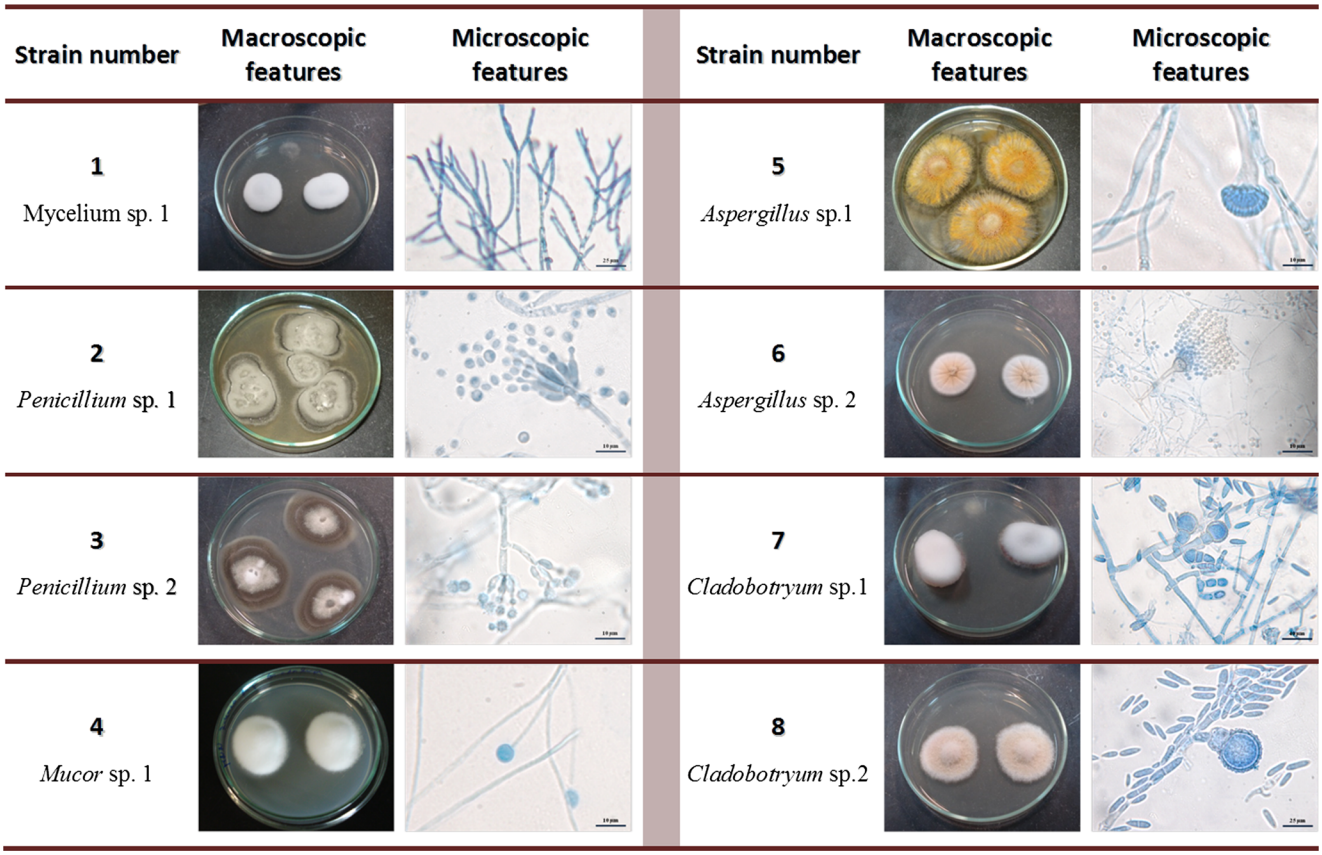

Fig. 2 Macroscopic and microscopic features of ten isolated strains of *A. ponderosa* microflora

as a zygomycota, belonging to the genus *Mucor*. This genus is characterized by coenocytic and multinucleated hyphae, a branched sporangiophore and the production of sporangiospores in closed global sporangia with a round columela (Hermet et al. 2012). Isolates 5 and 6 were identified as belonging to the genus *Aspergillus*. These strains present conidiophores which terminate in a vesicle covered with bases of phialides (strain 9) or metulae (strain 8). Isolates 7 and 8 were identified as belonging to the phylum Ascomycota. These fungi have septated mycelia and asexual spores designated as conidia, which are elongated and median septated and produced from the top of the hyphae, as can be seen in the microscopic features of strain 7 (Fig. 2). Most of these species produce thick-walled, subglobose cells referred to as chlamydospores, both in nature as well as in culture (Põldmaa 2011). On natural substrata, the chlamydospores occur as single cells (Poldmaa 2003; Põldmaa 2011), while in cultures they grow out from a similar or simple intercalary cell on submerged or aerial hyphae and can be followed by the formation of more complex aggregations.

Members of genera *Mucor*, *Penicillium* and *Aspergillus* are ubiquitous in nature, found in environmental samples such as soil, air and dust and are mainly saprophytic; however, some of *Penicillium* and *Aspergillus* strains may cause diseases (Hermet et al. 2012). Members of *Cladobotryum* genus are fungal parasites, and their chlamydospores serve as survival structures to overcome periods between the availability of host fruiting bodies as well as unfavourable environmental conditions, such as drought (Põldmaa 2011). Although seemingly more important as parasites of soft, ephemeral fruiting bodies of mushrooms, they are found also in cultures of species isolated from the more persistent basidiomata of wood-rotting fungi (Douhan and Rizzo 2003; Poldmaa 2003; Põldmaa 2011). Some species are obligate parasites and only found growing on their host where they cause systemic infections and result in the mummification of host fruiting bodies (Põldmaa 2011). Others are considered to be facultative saprotrophic because they are sometimes found on decaying wood and litter.

Isolate 1 in Fig. 2 has branched septate hyphae in the end region, forming a "fork-like" structure. Based on the mycelium aspect and the observed macroscopic characteristics, we suspected this strain to be a basidiomycete, probably *A. ponderosa* (Crous et al. 2009).

Genetic profile of *A. ponderosa* mushrooms and fungal strains that live in association in their natural habitat

The molecular fingerprinting profile of three *A. ponderosa* strains was compared with four edible mushrooms and with

the ten isolated fungal strains that live in association with *A. ponderosa* mushrooms in their natural habitat. The RAPD-PCR analysis using the M13 primer generated different patterns for the *A. ponderosa* strains, edible mushrooms species and the isolated strains, respectively, as shown in Fig. 3.

The M13-PCR assays produced polymorphism for all of the different species tested, resulting in 2–14 bands that ranged in size from 0.1 to 2.4 kb. The M13-PCR yielded 8–20 band profiles for the *A. ponderosa* strains assayed, ranging in size from approximately 266 to 1,482 bp. (Fig. 4). PCR with the M13 primer enabled fungal strains of class Basidiomycetes to be distinguished from those of class Ascomycetes and class Zigomycetes strains. This approach showed a cluster with a similarity of 19 %.

Dendogram analysis showed that the four known Basidiomycetes strains formed a cluster with 29 % of similarity. *Pleurotus ostreatus* and *Coriolus versicolor,* a white rot fungi, are the most similar, presenting a similarity of 61 %, followed by the strain *Lentinula edodes* that shows a similarity of 44 % with the former two species and finally by *Lactarius deliciosus* which is the most distant.

The isolated strains identified as *Penicillium* sp. 1 and *Penicillium* sp. 2 formed a cluster with 68 % similarity, *Aspergillus* sp. 1 and *Aspergillus* sp. 2 formed a cluster with 53 % similarity and grouped between the former with 27 % of similarity. All of these strains belong to the phylum Ascomycota. The strain mycelium sp. 2 had a degree of similarity of 10 % with these four strains, which may actually indicate that it belongs to the same phylum. The isolated strains identified as *Cladobotryum* sp. 1 and *Cladobotryum* sp. 2 were grouped in a cluster with 81 % similarity. The isolated strain mycelium sp. 3 presented a proximity of 56 % with these strains, and based on the above analysis it also can be included as an Ascomycota. All of these strains are grouped in a cluster with 4 % similarity (Fig. 4), which reveals some degree consistency in the sense that both genera belong to the same phylum (Ascomycota). It should also be noted that the cluster which includes all of the Ascomycetes shows a similarity of 4 % and that these have a proximity of just 2 % with a cluster that includes all of the Basidiomycetes.

Mucor sp. 1 is the most distant strain from the remaining species (0 % of similarity with the Basidiomycetes and Ascomycetes), which is explained by the fact that it is the only Zigomycete identified between samples.

The dendogram analysis showed that *A. ponderosa* mushrooms formed a cluster with 71 % similarity (Fig. 3). Mycelium sp. 1 formed a cluster with *A. ponderosa* mushrooms and was attributed to an isolate of these fruiting bodies, denoted *A. ponderosa* sp.1.

The MSP-PCR fingerprinting method described herein allowed us to distinguish all tested strains with different fingerprinting profiles and then to group all samples in clusters according to their genera. This technique led to clear differentiation of all the isolates at the species level. Consequently, we believe that M13-PCR analysis is a rapid method to characterize the genetic profile of *A. ponderosa* strains with high reproducibility and similarity for the same species. This approach, although producing fingerprints that are very similar within strains of the same species (Alves et al. 2007; Caldeira et al. 2009; Salvador et al. 2012), is also able to

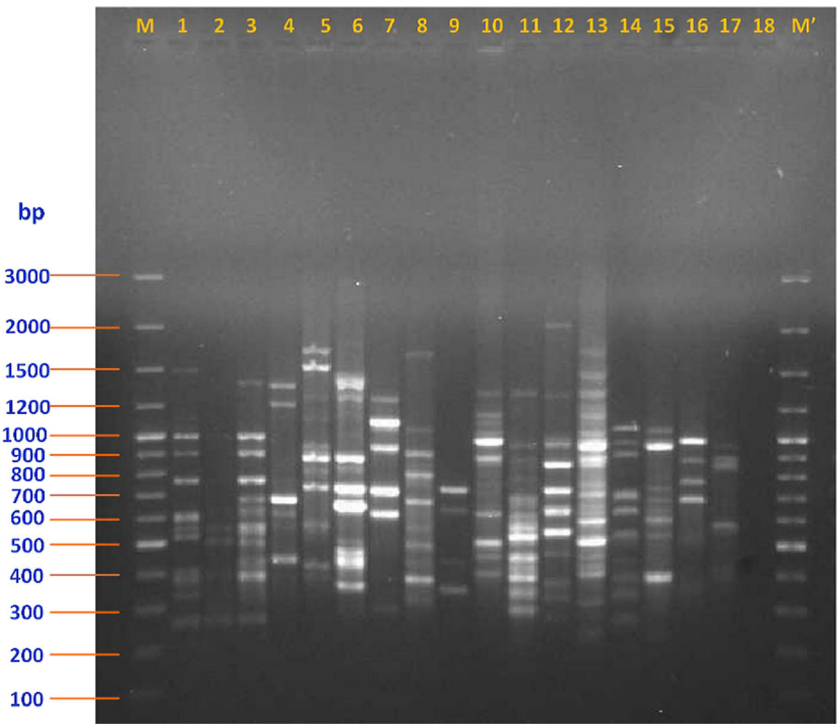

Fig. 3 M13-PCR fingerprinting patterns obtained from *A. ponderosa* mushrooms collected from different sites, four strains of identified basidiomycetes and ten isolated fungal strains that live in association with this species of mushroom in their natural habitat. *Lanes*: *M*, *M'* DNA molecular ladder 100 bp plus (Fermentas), *1 A. ponderosa* (Azaruja), *2 A. ponderosa* (Guadalupe), *3 A. ponderosa* (Évora), *4* mycelium sp. 1, *5* mycelium sp. 2, *6 Penicillium* sp.1, *7 Mucor* sp. 1, *8* mycelium sp. 3, *9 Penicillium* sp.2, *10 Cladobotryum* sp. 1, *11 Aspergillus* sp.1, *12 Aspergillus* sp. 2, *13 Cladobotryum* sp. 2, *14 Lentinula edodes*, *15 Pleurotus ostreatus*, *16 Coriolus versicolor*, *17 Lactarius deliciosus*, *18* control

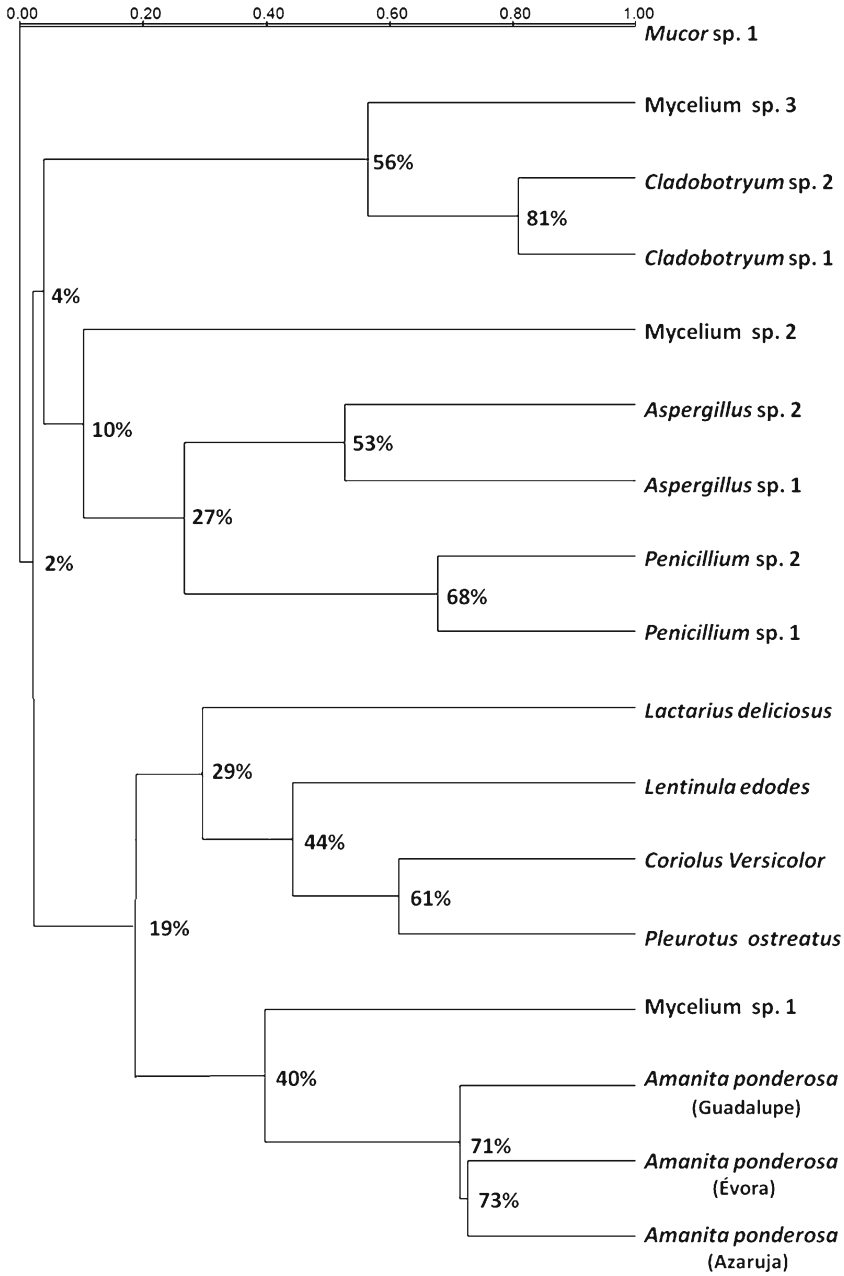

Fig. 4 Dendogram analysis based on the M13-PCR fingerprinting patterns for different strains of *A. ponderosa*, four strains of basidiomycetes (*L. deliciosus*, *P. ostreatus*, *C. versicolor*, and *L. edodes*) and ten isolated fungal strains. The distance values between branches are reported as percentage of similarity (0–100 %)

detect intraspecific variation and identify different polymorphisms between strains of the same species (Fig. 4) (Caldeira et al. 2009; Salvador et al. 2012).

DNA-based techniques provide practical markers for molecular typing of a range of species of fungi. These methods are commonly used as tools in fungal taxonomy as they enable the discrimination of fungal isolates from intrageneric to strain levels (Alves et al. 2007). A range of molecular approaches based on ITS analyses for used in characterizing and identifying edible mushrooms has been described in the literature (Moncalvo et al. 2000; Douhan and Rizzo 2003; Firenzuoli et al. 2007; Ro et al. 2007; Moreno et al. 2008), such as the study of Spanish *Amanita* species using ITS-ARDRA (González et al. 2002), and a more recent study on distinguishing *A. ponderosa* and *A. curtipes* species by restriction analysis of the ITS region (Moreno et al. 2008).

The fingerprinting methods referred to as M13-PCR (microsatellite-primed PCR) and rep-PCR (repetitive-sequence-based PCR) and RAPD have been used to discriminate between species of fungi (Alves et al. 2007). The advantages of these three methods are simplicity, universal availability of PCR primers, reproducibility and amenability to computer database analysis. It is possible to build databases of fingerprints that can be used for routine identification of

isolates by a simple comparison of fingerprints with those of reference cultures.

The major advantage of M13-PCR and rep-PCR over ARDRA is that in the former there is no need for a step involving the digestion of PCR amplicons with restriction endonuclease (Alves et al. 2007). In fact, M13-PCR is easy to implement, requires less time than other molecular approaches with restriction enzymes, only uses a single primer or set of primers and achieves higher levels of resolution. This makes the procedure much faster and easier and greatly reduces the cost. Furthermore, it involves the lowest labour imput and instrumentation costs and requires little knowledge of the molecular biology of the species being examined and no sequence information.

M13-PCR fingerprinting has been used to study genetic variability in filamentous fungi species (Godoy et al. 2004; Alves et al. 2007; Lopes et al. 2007; Guimarães et al. 2011b), showing that these methodologies have a great potential as diagnostic tools. The technique has been used also in studies on fungal biodiversity in a ecosystem (Guimarães et al. 2011a, b), differentiation of species and strains among filamentous fungi (Meyer et al. 1991), and characterization of fungal species' isolates collected from plant hosts (Ma and Michailides 2002; Guimarães et al. 2011a). It is considered the first choice DNA fingerprinting method in clinical laboratories involved in routine epidemiological studies.

Conclusions

Amanita ponderosa strains and some fungal species living in association with these mushrooms were distinguished using the M13-PCR approach. These assays produced polymorphism for all of the different species tested, showing different patterns for the three *A. ponderosa* strains when compared with other four edible mushrooms and with ten isolated fungal strains that live in association with this species of mushrooms in their natural habitat. Molecular analysis by M13-PCR revealed that the fungi isolates and *A. ponderosa* had different genetic profiles, which enabled the different strains to be differentiated quickly and reproducibly and to be subsequently grouped according to genus. M13-PCR enabled the molecular profiles from *A. ponderosa* strains to be distinguished from those of the basidiomycetes. This fingerprinting technique also distinguished different *A. ponderosa* mushrooms collected from different regions, thereby displaying a very discriminatory approach. Based on these results, we suggest that this methodology could be useful in developing a strategy for *A. ponderosa* certification process.

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