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MSP-PCR and RAPD molecular biomarkers to characterize *Amanita ponderosa* mushrooms

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Abstract - *Amanita ponderosa* is a specie of wild edible mushrooms growing spontaneously in some Mediterranean microclimates, namely in Alentejo and Andaluzia, in the Iberian Peninsula. The nutritional values of these fungi make them highly exportable. Due to the wide diversity of mushrooms in nature, it is essential to differentiate and to identify the various edible species. RAPD markers have been used as a valuable tool to distinguish the different genotypes, although this method has not yet been used to *Amanita ponderosa*. Two methods were used to establish different genetic fingerprinting patterns of edible mushrooms. Samples of *Amanita ponderosa* were collected in six different regions of the southwest of the Iberian Peninsula and compared by RAPD-PCR and MSP-PCR. Additionally, to compare molecular profiles with others genera of edible mushrooms, three species of Basidiomycetes (*Pleurotus ostreatus*, *Lactarius deliciosus* and *Coriolus versicolor*) and an Ascomycete were used. Results showed that some molecular markers discriminate among an Ascomycete from Basidiomycetes (*Amanita ponderosa*, *Pleurotus ostreatus*, *Lactarius deliciosus* and *Coriolus versicolor*) and discriminate among the different genera within basidiomycetes, as it is expected. Moreover, OPF-6, OPG-2, OPG3 and M13 primes allowed to unravel a level of genetic polymorphism within *Amanita ponderosa* mushrooms collected from different geographic origin.

Key words: *Amanita ponderosa*; edible mushrooms; M13-PCR; RAPD.

INTRODUCTION

Mushrooms have been exhaustively studied. Some of these fungi are known to have both high nutritional and medicinal values, which has led to an increase in their consumption. Some others play a significant role in forest ecosystems, as it is the case of most *Amanita* species, which are obligatorily ectomycorrhizal.

The south of Portugal, due to its climate and flora diversity, is one of the European regions with a high predominance of wild edible mushrooms *Amanita ponderosa*. The gastronomic value of this species has long been appreciated especially by rural populations, but its demand has also been substantially rising in Mediterranean gourmet markets, which has contributed to the increase of its economic value.

Amanita ponderosa is characterized by a large and robust basidiomata, with a cap 8-17 cm in diameter, a hemispheric morphology when young and plane-convex in maturity, and a slight depression in the centre (Fig. 1). Although there is much research on mushrooms, there is lack of information about *A. ponderosa*. The small number of reports found in literature focus on mineral content (Moreno-Rojas *et al.*, 2004) and molecular phylogenetic analyses. Moreno *et al.* (2008) reported a study focussing on the distinction between *Amanita curtipes* and *A. ponderosa* species by sequencing the D1-D2 domains of the 28S rRNA gene and ITS1-5.8S-ITS2 region. Genetic profiles and polymorphic sequences are important tools for a rapid and

FIG. 1 - Some macroscopic aspects of *Amanita ponderosa* mushrooms. A: Young mushrooms. B: Mushrooms in maturity.

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effective characterization of these species, namely in certification processes. On the other hand, molecular approaches to establish relationships between different regions and their characteristic strains can constitute valuable tools. Literature reports some approaches such as RAPD and ITS analyses to characterize and identify edible mushrooms (Moncalvo *et al.*, 2000, Firenzuoli *et al.*, 2007, Pacioni *et al.*, 2007, Ro *et al.*, 2007). MSP-PCR fingerprinting is also used in the study of genetic variability in yeasts and filamentous fungi species (Godoy *et al.*, 2004, Alves *et al.*, 2007, Lopes *et al.*, 2007).

This study aims to characterise *A. ponderosa* mushrooms with molecular biomarkers by RAPD and MSP-PCR, in order to establish different genetic profiles of these wild edible mushrooms, collected in different sites. It focuses on the molecular identification of the Portuguese species, a kind of identification never done until now. It is important to emphasize that our study aims to a rapid method to enable the screening of *A. ponderosa* strains from different locations, especially the ones observed in the Mediterranean region. The screening of pathogenic species of the genus *Amanita* as well as the detection and quantification of toxins are out of the scope of this work, since these characteristics have already been studied and referred to in literature. Moreover, there are no studies investigating the role of bioactive compounds produced by *A. ponderosa*. A profound knowledge about this specie can improve the characterization of new bioactive compounds, for example protein-polysaccharides complex with therapeutical applications, namely with anti-tumoral activities, recognized in other edible mushrooms.

MATERIALS AND METHODS

Samples. Fruiting bodies of the *Amanita ponderosa* mushrooms were collected between February and April 2007 in six different regions of the southwest of the Iberian Peninsula, namely Évora, Beja, Mina de São Domingos, Santo Aleixo da Restauração and Vila Nova de São Bento (Alentejo, Portugal) and Cabeza Rubia (Andaluzia, Spain). The mushrooms under analysis were collected in acid soils, in forests of *Quercus ilex* ssp. *ballota*, *Cistus ladanifer* and *Cistus laurifolius* of the abovementioned sites, at the same growth stage to avoid the effect of size. For the molecular study, *A. ponderosa* strains were also compared with one wild edible truffle (*Terfezia arenaria*) that grows in the same forest environment and with three species of cultured mushrooms (*Coriolus versicolor*, *Lactarius deliciosus* and *Pleurotus ostreatus*). At least 3 individuals of wild edible mushrooms were sampled and analysed per location and stored in the Laboratory of Biotechnology, at the Department of Chemistry of the University of Évora.

Molecular analysis. The extraction of the genomic DNA from the small fragments of fruiting bodies was done by means of the modified microsphere method (Sambrook *et al*., 1989; Martins, 2004). The obtained DNA was then quantified and diluted to a final concentration of 0.1 ng/L for PCR assays. RAPD-PCR was carried out with the microsatellite primers OPF-1 (5′-ACGGATCCTG-3′); OPF-6 (5′-GGGAATTCGG-3′); OPF-9 (5′-CCAAGCTTCC-3′); OPF-13 (5′-GGCTGCAGAA-3′); OPG-2 (5′-GGCACTGAGG-3′); OPG-3 (5′-GAGCCCTCCA-3′); OPH-2 (5′-TCGGACGTGA-3′); OPH-4 (5′-GGAAGTCGCC-3′). The M13 primer (5′- GAGGGTGGCGGTTCT-3′) was used to MSP-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 36 °C (RAPD) or 1 min at 50 °C (MSP-PCR) 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. PCR samples were separated in a 1.5% agarose gel and visualized by means of a UV transilluminator (Bio-Rad) and the Quantity One 1-D Analysis software (Biorad). To evaluate the reproducibility of RAPD and MSP-PCR assays, each sample has been analysed in at least three independent PCR reactions. A negative control (without DNA template) has been included in every run.

Statistical analysis. The phylogenetic tree was generated by the UPGMA method, through the use of the Dice coefficient of similarity, based on the fingerprinting obtained from *A. ponderosa* and other species of Basidiomycetes and Ascomycetes.

RESULTS AND DISCUSSION

The fingerprinting obtained by RAPD-PCR through the use of OPF-1, OPF-6, OPF-9, OPF-13, OPG-2, OPG-3, OPH-2, OPH-4 and by MSP-PCR using M13 primer are presented in Fig. 2. Table 1 summarizes the band profile obtained in this study. The different samples have generated distinct patterns. RAPD assays have produced polymorphism for all different species tested, showing 2-14 bands ranging from 0.1 to 2.4 kb. The amplification using the M13 primer has generated a profile with 9-20 bands ranging from 0.2 to 1.3 kb.

Figure 3 shows a dendogram analysis based on the RAPD-PCR through the use of OPF6, OPG2 and OPG3 and M13-PCR fingerprinting patterns for different strains of *A. ponderosa* and for *L. deliciosus*, *P. ostreatus* and *T. arenaria*. RAPD primers OPF-6, OPG-2 and OPG-3 allowed the distinction of Basidiomycetes from Ascomycetes. These three primers have showed clusters for all Basidiomycetes, with a similarity of 30, 25 and 20%, respectively. The fingerprinting obtained with the M13 primer allowed us to distinguish *T. arenaria*. from the studied Basiodiomycetes with 44% of similarity. This result is coherent since Truffle is an ectomycorrhizal ascomycete which produces subterranean ascomata, usually known as truffles, while all the other samples are basidiomycetes. OPF-6, OPG-2 and OPG-3 RAPD markers and M13-PCR highlighted a differentiation at the species and strain level. *Amanita ponderosa* strains formed clusters with 65% of similarity for OPF-6 and OPG-2 primers, and 80% of similarity with the OPG-3 primer. In the M13-PCR fingerprinting, *A. ponderosa* strains were grouped in a cluster with a similarity of 67% (Fig. 3).

RAPD analyses have been a valuable tool to distinguish different genotypes in edible mushrooms such as *Ganoderma lucidum* (Hseu *et al.*, 1996), *Lentinula edodes* (Zhang and Molina, 1995), *Agaricus bisporus* (Khush *et al.*, 1992) and *Pleurotus ostreatus* (Lee *et al.*, 2000) as well as to evaluate genetic similarities. Nevertheless, it is a new approach to the study of *A. ponderosa* strains. The second fingerprinting approach, employing the minisatelite M13, makes use of the occurrence of a great number of copies of interspersed repeat motifs in most genomes. This method has been successfully used to amplify hypervariable repeated DNA sequences with a high level of reproducibility (Martin *et al.*, 1998, Lopes *et al.*, 2007).

In this study, the degree of discrimination of RAPD-PCR fingerprinting depended largely on the primers used. MSP-PCR has shown to be 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 edible mushrooms such as *T. arenaria*, *L. deliciosus*, *P. ostreatus*

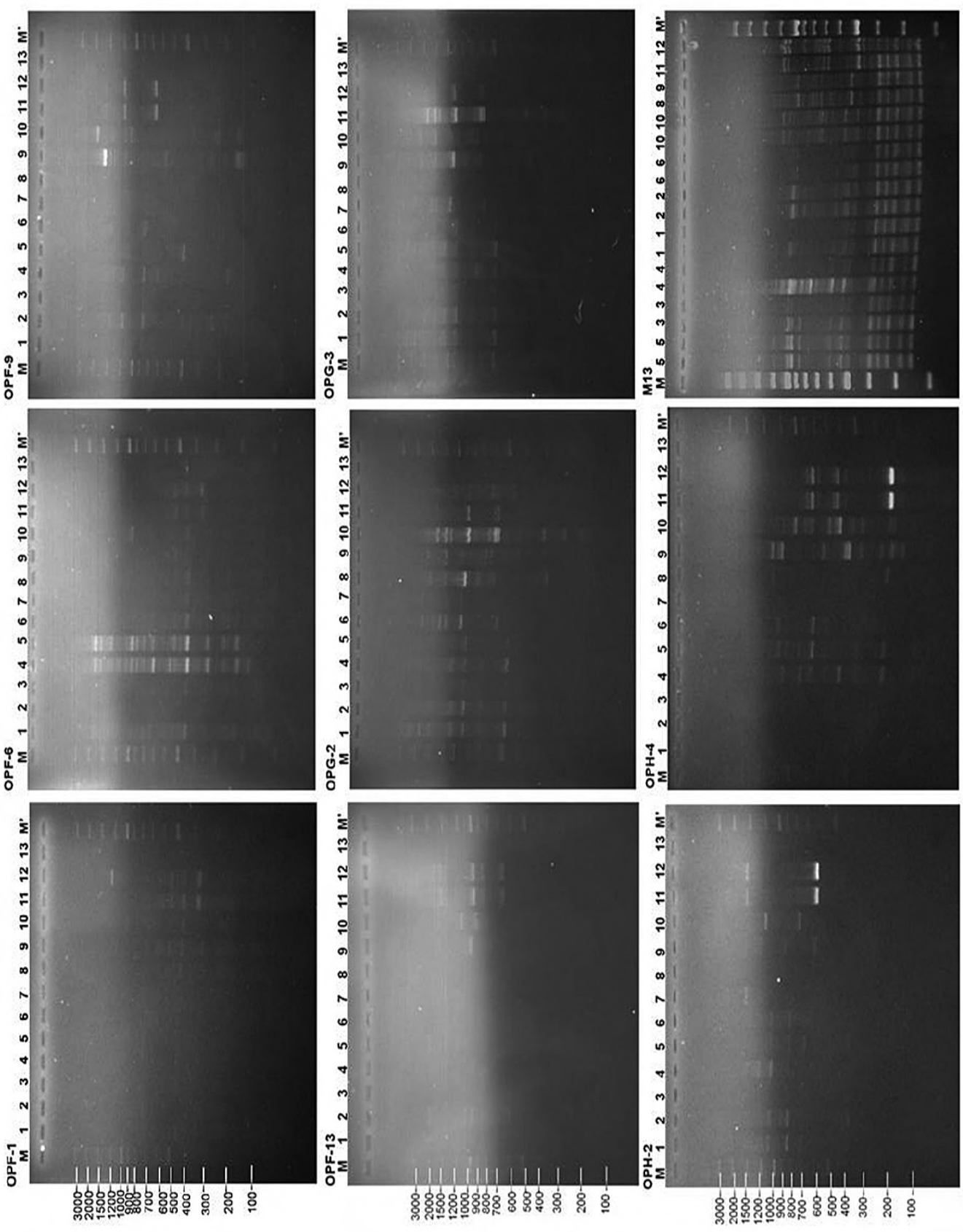

FIG. 2 - Fingerprinting patterns obtained from *Amanita ponderosa* mushrooms collected from different sites*, Coriolus versicolor, Pleurotus ostreatus, Lactarius deliciosus*, and *Terfezia arenaria*. Lanes M and M': DNA molecular ladder 100 bp plus (Fermentas), line 1: *A. ponderosa* (Évora), line 2: *A. ponderosa* (Cabeza Rubia), line 3: *A. ponderosa* (Vila Nova de São Bento), line 4: *A. ponderosa* (Mina de São Domingos), line 5: *A. ponderosa* (Beja), line 6: *A. ponderosa* (Santo Aleixo da Restauração), line 7: *Coriolus versicolor,* lines 8 and 9: *P. ostreatus,* line 10: *L. deliciosus,* lines 11 and 12: *T. arenaria,* line 13: Control.

TABLE 1 – Band profile obtained with the different primers

Primers	Strains	*Amanita ponderosa* (Évora)	*Amanita ponderosa* (Cabeza Rubia)	*Amanita ponderosa* (V. N. São Bento)	*Amanita ponderosa* (Mina São Domingos)	*Amanita ponderosa* (Beja)	*Amanita ponderosa* (Sto Aleixo Restauração)	*Coriolus versicolor*	*Pleurotus ostreatus*	*Lactarius deliciosus*	*Terfezia arenaria*
OPF-1	Bands (n)	8	8	8	8	8	8	0	7	9	8
	bp	118-738	118-738	118-738	118-738	118-738	118-738	–	118-591	166-1539	166-661
OPF-6	Bands (n)	9	6	6	13	13	7	2	3	2	3
	bp	166-1763	166-532	166-532	166-1763	166-1763	166-932	259-445	509-584	376-584	368-532
OPF-9	Bands (n)	4	6	4	6	5	4	0	7	6	3
	bp	274-948	274-1236	274-948	274-1236	274-948	342-948	–	24-1945	229-1855	727-1310
OPF-13	Bands (n)	2	3	2	2	2	2	2	2	4	6
	bp	946-1900	586-1900	946-1900	946-1900	946-1900	946-1900	657-1362	744-984	803-1117	653-1552
OPG-2	Bands (n)	8	8	8	8	8	8	8	8	9	14
	bp	486-2317	486-2317	486-2317	486-2317	486-2317	486-2317	559-1706	285-1520	429-1520	150-1520
OPG-3	Bands (n)	8	8	8	8	8	8	7	7	9	8
	bp	434-2354	434-2354	434-2354	434-2354	434-2354	434-2354	606-1873	272-1563	432-1563	225-1439
OPH-2	Bands (n)	5	5	5	5	5	5	1	3	4	4
	bp	413-1339	413-1339	413-1339	413-1339	413-1339	413-1339	1621	621-1299	644-1193	619-1621
OPH-4	Bands (n)	7	5	6	11	12	10	1	13	12	11
	bp	210-1069	210-1069	210-1069	210-1069	210-1069	246-1069	1248	210-1293	322-1532	322-1185
M13	Bands (n)	9	13	11	15	13	9	–	20	18	16
	bp	237-1001	237-1189	237-1001	237-1189	237-1189	237-1001	–	237-1299	237-1140	237-1154

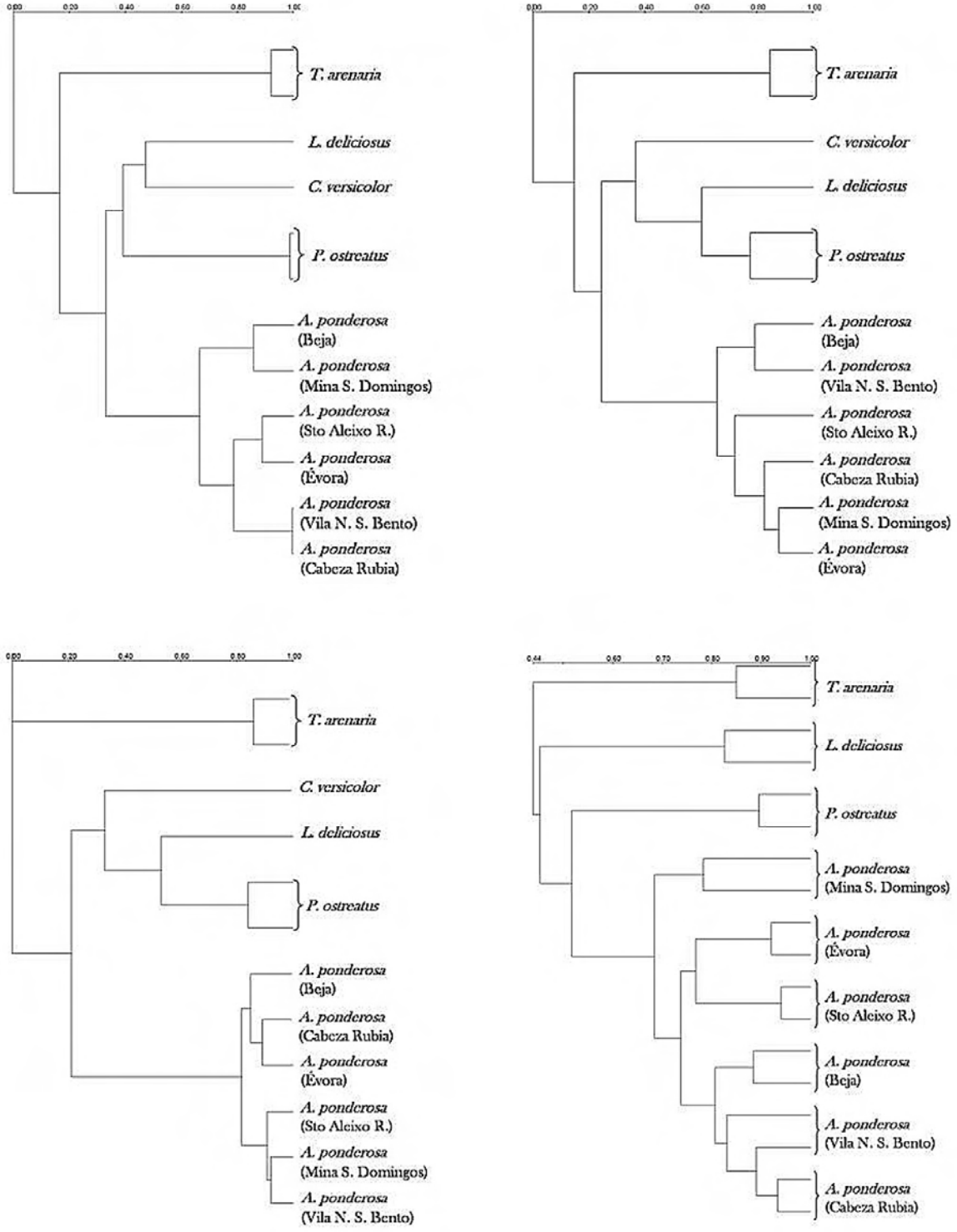

FIG. 3 - Dendogram analysis based on the RAPD-PCR through the use of OPF6, OPG2 and OPG3 and M13-PCR fingerprinting patterns for different strains of *Amanita ponderosa* and for *Lactarius deliciosus*, *Pleurotus ostreatus* and *Terfezia arenaria*. The distance values between branches are reported as percentage of similarity (0-100%).

and *A. ponderosa*. Furthermore, MSP-PCR is a valuable tool to distinguish the different genotypes of *A. ponderosa* strains showing a polymorphism probably related to geographically differentiated populations.

RAPD-PCR and MSP-PCR analyses establish different genetic profiles for *T. arenaria, P. ostreatus, L. deliciosus* and *A. ponderosa* strains. Additionally, the amplified DNA polymorphic sequences by means of OPF-6, OPG-2, OPG-3 and M13 primers allowed to find the differences between *A. ponderosa* strains collected in different sites. Both methods, RAPD-PCR and MSP-PCR, could be used for a higher discriminatory power of these types of fungi as well as to categorize species of wild edible mushrooms usually found in Mediterranean ecosystems. This kind of approach could be useful in the study of other species of wild edible mushrooms found in Alentejo (Portugal), namely in the characterization of the strains at the molecular level.

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