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rDNA BASED ANALYSIS OF AUTOCHTONOUS FUNGAL SPECIES FROM SERBIA

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Determination of fungal species by traditional morphological approach can often be problematic. In the phylum Basidiomycota, sporocarps of different species can share very similar morphoanatomical characteristics. Using molecular markers and phylogenetic species concept this problem can be reduced. In this study identification of six autochtonous fungal species, collected from several locations in Serbia (Tara, Kopaonik, Stara planina) was done by comparison between morphological and molecular data of fungal species, as well as information obtained from phylogenetic tree. ITS sequences amplified from 11 specimens of two genera of ph. Basidiomycota: *Marasmius* and *Ganoderma*, were compared with ITS sequences from database using basic local alignment search tool (BLAST). Phylogenetic tree was constructed using Neighbor joining method based on differences between analyzed ITS sequences. Our results showed that within genera *Marasmius* and *Ganoderma* morphological and molecular determinations are usually in accordance, but for proper species delimitation both approaches should be used.

Key words: Basidiomycota, BLAST, *Ganoderma* internally transcribed spacer, species delimitation, *Marasmius*

INTRODUCTION

Determination of fungal species was mainly based on macroscopic and microscopic morphological features of fungal reproductive structures until recently (KARAMAN *et al*., 2012). This approach can be often ambiguous because sporocarps of different species might share very similar morphological characteristics. Species delimitation based on molecular markers can provide new solution to this problem (JARGEAT *et al.,* 2010). Furthermore, it was suggested that phylogenetic species concept can be more applicable for determination of fungal species than

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morphological and biological species concepts which were traditionally used (TAYLOR *et al.,* 2000).

In fungal genomes, among eukaryotic organisms, genes for rRNA are organized in tandem units. One unit consists of three rRNA genes (18S; 5,8S; 28S) which are separated by two ITS (internally transcribed spacer) sequences (SCHOCH *et al.,* 2012). These sequences show high interspecific and low intraspecific variability in the phylum Basidiomycota, which makes them a good tool for phylogenetic species determination and they were used for that purpose in many investigations (e.g. GOMES *et al*., 2002, GULDBERG FRŘSLEV *et al*., 2007, NILSSON *et al.*, 2008, OLARIAGA *et al*., 2009).

In this work, two species of the genus *Ganoderma*: *Ganoderma adspersum* (Schulzer) Donk and *Ganoderma applanatum* (Pers.: Wall.) and three species of the genus *Marasmius*: *Marasmius androsaceus* (L. : Fr.) Fr*., Marasmius rotula* (Scop. : Fr.) Fr. and *Marasmius scorodonius* (Fr. : Fr.) Fr. were analyzed.

Genus *Ganoderma* includes widespread species of lignicolous, white-rot causing fungi. Although many of *Ganoderma* species represent parasites of economically important plants (FLOOD *et al*., 2000), their fruiting bodies have medicinal properties (LU *et al*., 2004, PATERSON, 2006, KARAMAN *et al*., 2009a, KARAMAN *et al.*, 2009b, KARAMAN *et al.*, 2010, KARAMAN *et al.*, 2013, KOZARSKI *et al*., 2011) and were used for centuries in traditional medicine of Asian cultures. Due to the high variability of the macroscopic and microscopic characteristics of basidiocarps of these fungi and similar habitat (NOVAKOVIĆ *et al*., 2012), the taxonomy of this genus is still unclear and a large number of synonyms is present (SEO and KIRK, 2000, GOTLIEB *et al.*, 2000). Moreover, analysis of SSU rDNA suggests that genus *Ganoderma* represents polyphyletic and relatively young group of fungi (HONG and JUNG, 2004).

The genus *Marasmius* is comprised of around 600 widely spread saprotrophic and litter-decomposing species (WANNATHES *et al.*, 2009). They form relatively small and marcescent (i.e. reviving in situ) basidiocarps with convex to conical and striate pilei, and typically tough, filiform stipes (WILSON and DESJARDIN, 2005). Species of this genus could be confused with collybioid and mycenoid mushrooms (http://www.mushroomexpert.com/marasmius.html).

In recent studies, possibility of using these fungi in industry was investigated, especially for production of enzymes like peroxidase (SCHEIBNER *et al.*, 2008) and thermostable xylanase (RATANACHOMSRI *et al.*, 2006).

Genus *Marasmius* was considered to be homogenous group of fungi, but analysis of ITS, 5,8S and nLSU (nuclear ribosomal large subunit) DNA sequences showed that this genus have polyphyletic origin (WILSON and DESJARDIN, 2005). Members of this group can be classified in a number of distinct clades (/omphalotaceae, /physalacriaceae, /marasmiaceae) (ANTONIN *et al.*, 2010).

Considering complexity of determination in specific groups of fungal genera, the aim of this study was an identification of autochtonous species by comparison of morphological and molecular data, as well as obtaining information from phylogenetic tree.

MATERIALS AND METHODS

The analyzed basidiocarps (11) were collected from different locations on the territory of the Republic of Serbia (Table 1), which represents permanent sites designed for longterm monitoring of macrofungi in forest ecosystems (NOVAKOVIĆ *et al*., 2013a; NOVAKOVIĆ *et al*., 2013b). Fungi were identified on the basis of macroscopic and microscopic morphological

characteristics of fruiting bodies using specific identification keys (BON, 1988, UZELAC, 2009, http://www.mushroomexpert.com/,http://www.rogersmushrooms.com/,http://www.svims.ca/council/_Marasm.htm).

Pieces of the basidocarps which were used for DNA extraction were preserved in 96% ethanol on +4˚C after obtaining from the inner part of fresh fruiting bodies using the method of sterile clippings.

Table 1. Analyzed species and locations information

No.	Taxon	Locality	Locality designation
1	*Marasmius androsaceus* (L.) Fr.	Vazganica, mt. Vidlič	3ASTR2
2	*Marasmius androsaceus* (L.) Fr.	Crveni potok, mt. Tara	12TARA5
3	*Marasmius rotula* (Scop.) Fr.	Crveni potok, mt. Tara	7TARA5
4	*Marasmius scorodonius* (Fr.) Fr.	Crveni potok, mt. Tara	4TARA5
5	*Marasmius scorodonius* (Fr.) Fr	Vazganica, mt. Vidlič	6STR2
6	*Marasmius sp./ Micromphale perforans* (Hoffm.) Gray	Suva reka, mt. Kopaonik	KOPSP
7	*Ganoderma applanatum* (Pers.) Pat.	Mitrovac, mt. Tara	G1TARA4
8	*Ganoderma applanatum* (Pers.) Pat	Mitrovac, mt. Tara	G2TARA4
9	*Ganoderma applanatum* (Pers.) Pat	Mitrovac, mt. Tara	G3TARA4
10	*Ganoderma applanatum* (Pers.) Pat	Crveni potok, mt. Tara	GTARA5
11	*Ganoderma adspersum* (Schulzer)Donk	Crveni potok, mt. Tara	GTARA5

DNA extraction

Approximately 100 mg of fungal material was used for the extraction of DNA, and it was performed according to CTAB protocol (CULLINGS, 1992). DNA concentration was determined using spectrophotometric analysis. DNA was quantified to 20 ng/µl.

PCR amplification of ITS sequences

The ITS rDNA regions were PCR-amplified using the primer set ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (WHITE *et al*., 1990). Reactions were performed in Thermocycler (Eppendorf, NY, http://www.eppendorf.com). Protocols for preparation of PCR master mix and PCR amplification conditions were done according to GALOVIĆ *et al*., 2010.

PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide (EtBr). Marker used for evaluation of length of PCR products was GeneRuler™ 50 bp DNA Ladder (Fermentas, http://www.fermentas.com). Separation of the PCR products was performed for 45 minutes in 0,5xTBE buffer at constant voltage of 80V. Visualization was done using DIAS system (SERVA Electrophoresis GmbH, D, www.serva.de).

Sequencing of PCR products

PCR products were extracted from agarose gel using QIAquick Gel Extraction Kit (Qiagen, http://www.qiagen.com/default.aspx). Extracted DNA was quantified to 5 ng/µl and sequenced (Macrogen, NL, http://www.macrogen.com/) by automatic DNA sequencer (ABI 3730XL) using capillary electrophoresis method.

Analysis of the sequence data

Obtained ITS sequences were aligned using MEGA 5.10 (TAMURA *et al.*, 2011) software. The sequences were identified by comparison against nucleotide collection (nr/nt) database using basic local alignment search tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The program was optimized for highly similar sequences (megablast). Word size was set to 20, and low complexity regions were not filtered. All other parameters were set to default.

Phylogenetic tree was constructed in the MEGA 5.10 software by applying Neighbor-Joining method (SAITOU and NEI, 1987). The evolutionary distances were computed using the number of differences method (NEI and KUMAR, 2000) and are in units of the number of base differences per sequence. The tree was drawn to scale, with branch lengths in the same units. For the construction of the phylogenetic tree, only the sequences which were identified using BLAST were used. The downloaded ITS sequence of *Stereum hirsutum* (GenBank number: JX082331.1) was used as an outgroup.

RESULTS AND DISCUSSION

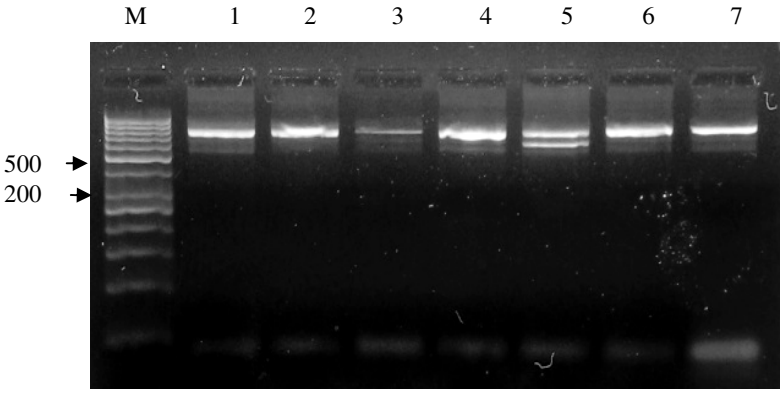
Amplification of ITS rDNA region was successful for all 12 samples, and obtained sequences were approximately 750 bp long (Figure 1), which is in consent with data of other authors (e.g. GOMES *et al.*, 2002, GEML *et al*., 2004). In a sample of *M. androsaceus* one extra sequence of approximately 650 bp was amplified (Figure 1). This sequence was also extracted from gel and sequenced (specimen no. 3, Table 2)

Comparison of analyzed sequences with sequences from database showed various results (Table 2). For six, among 11 analyzed specimens morphological identification was confirmed (samples no. 2, 4, 6, 9, 10, 11, Table 2).

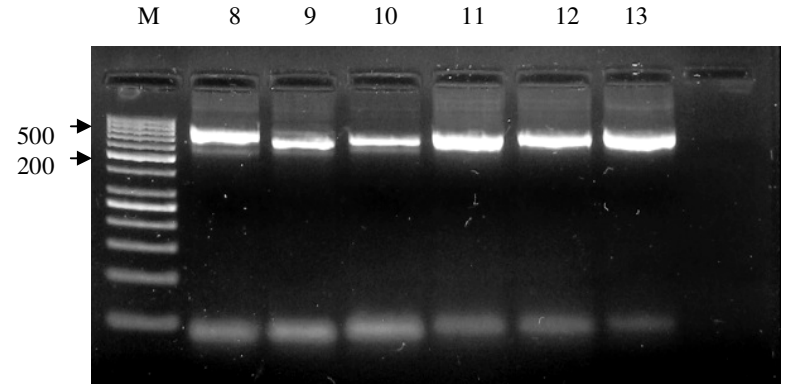
Samples no. 8 and 12 (Table 2) were identified, but results were not in accordance with data obtained after morphological identification. Sample no. 8 was identified as *G. applanatum* by morphological identification and sample no. 12 was identified as *G. adspersum*. After comparison of these sequences with sequences from the database, they were identified as *Trametes hirsuta* and *Fomes fomentarius,* respectively. Because *G. applanatum, G. adspersum, T. hirsuta,* and *F. fomentarius* share the same habitat (UZELAC, 2009) it is possible that cross contamination may occurred. To avoid environmental contamination, DNA should be extracted from single spore cultures (CHOI *et al*., 1999). Moreover, all these species are members of the same order Polyporales, so it is possible that their ITS sequences are very similar. However, according to SCHOCH and coworkers (2012), ITS sequences have the highest resolving power for discriminating closely related species, so this assumption might be discarded.

For sequences 1, 3 and 5 (Table 2) there were no significant similarity found in the database. This usually happens when query sequences are short and/or are of low complexity (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=FAQ#nohits), just like it was the case with sequences 1 and 5. Sequence number 3 (Table 2) was

not short, but since it represents one extra sequence which was amplified from specimen number 2 (Table 2) it is not an ITS sequence, so it remained unidentified.



a)



b)

Figure 1. Electrophoresis of PCR amplification products on 2% agarose gel. M: molecular weight marker 50-bp DNA ladder;

a) 1 and 2: *M. rotula*; 3 - 5: *M. androsaceus;*; 6 and 7: *M. scorodonius*;

b) 8: *Marasmius sp./ Micromphale perforans*; 9 - 12:*G. applanatum*; 13: *G. adspersum*;

Table 2. Results obtained after comparison of analyzed sequences with sequences from database

Morphological identification	Locality	Identification based on ITS sequences	Sequence length [pb]	Total score	E-value
Marasmius androsaceus	3ASTR2	No similarity found	242		
Marasmius androsaceus	12TARA5	*M. androsaceus*	276	286	2e-75
Marasmius androsaceus 2	12TARA5	No similarity found	673		
Marasmius rotula	7TARA5	*M. rotula*	831	324	3e-86
Marasmius scorodonius	4TARA5	No similarity found	273		
Marasmius scorodonius	6STR2	*M. scorodonius*	269	88.8	8e-16
Marasmius sp./ Micromphale perforans	KOPSP	*Micromphale sp.*	671	711	0.0
Ganoderma applanatum	G1TARA4	*Trametes hirsuta*	338	76.1	7e-12
Ganoderma applanatum	G2TARA4	*G applanatum*	424	603	1e-170
Ganoderma applanatum	G3TARA4	*G. applanatum*	541	784	0.0
Ganoderma applanatum	GTARA5	*G.applanatum*	421	545	6e-153
Ganoderma adspersum	GTARA5	*Fomes fomentarius*	640	916	0.0

For specimen number 7 (Table 2), clear morphological identification was not possible. After comparing it's ITS sequence with database, it was determined that this specimen belong to genus *Micromphale sp.*

As can be seen from the Figure 2, phylogenetic tree confirmed the results obtained after the comparison of the sequences with the database. ITS sequences of specimens identified as *G. applanatum* (samples 9, 10, 11, Table 2) showed almost no differences amongst them, which means they are undoubtedly members of the same species. This is in accordance with other investigations (GOMES *et al*., 2002, GULDBERG FRŘSLEV *et al*., 2007, NILSSON *et al*., 2008, OLARIAGA *et al*., 2009) which confirmed that intraspecific variability of ITS sequences is low.

Branch lengths of specimens identified as *T. hirsuta* and *F. fomentarius* are longer than those of *G. applanatum* specimens, which means that their ITS sequences are more variable, and clearly represent two distinct species. They also have shown a degree of similarity with sequences of *G. applanatum* and are placed in the same clade Polyporales.

Within ITS sequences of *Marasmius* and *Micromphale* specimens, higher variability is observed than within Polyporales clade. According to phylogenetic relationship inferred from ITS and nLSU sequences, genus *Marasmius* represents polyphyletic group (WILSON and DESJARDIN, 2005). So, it is possible that observed variability of ITS sequences of genus *Marasmius* can be explained by its polyphyletic origin.

Our results show that molecular and morphological determinations are usually in accordance, and it is the best identification method to use both approaches for proper species determination. Molecular approach can be especially useful in determination of morphologically similar specimens. It can be also equally useful among the specimens whose morphological data are missing or are incomplete.

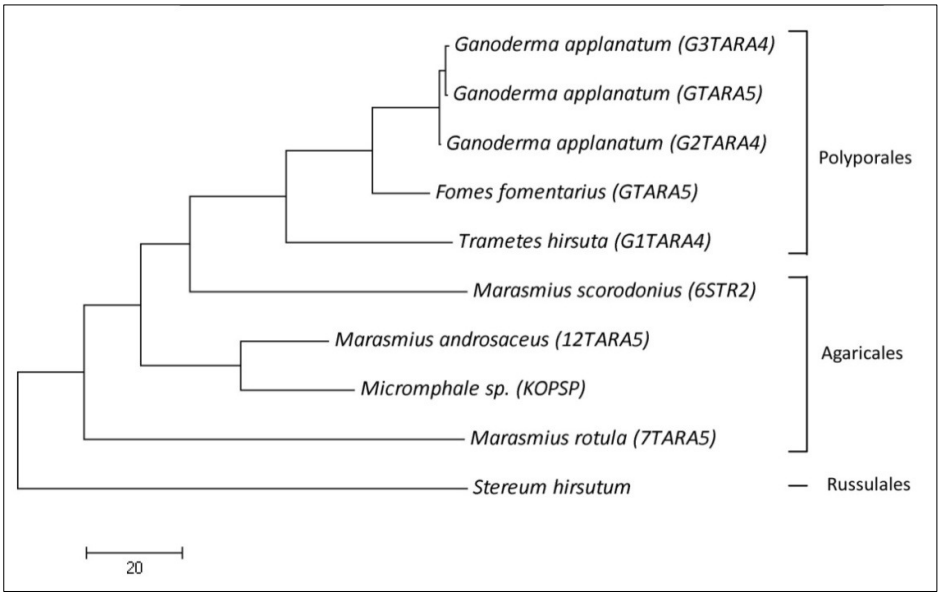


Figure 2. Phylogenetic tree constructed on the basis of differences of ITS sequences

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rDNK BAZIRANA ANALIZA AUTOHTONIH VRSTA GLJIVA SRBIJE

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Izvod

Plodna tela gljiva razdela Basidimycota često poseduju veoma slične morfoanatomske karakteristike, pa determinacija vrsta gljiva iskuljučivo na osnovu morfoloških karakteristika često može biti problematična. Primenom molekularnih markera i filogenetskog koncepta vrste ovaj problem može biti smanjen. U ovom radu, izvršena je analiza morfoloških i molekularnih podataka dobijenih za šest autohnotih vrsta gljiva koje su sakupljene sa nekoliko lokaliteta u Republici Srbiji (Tara, Kopaonik, Stara planina), sa ciljem da se izvrši pravilna identifikacija. ITS sekvence amplifikovane iz 11 uzoraka gljiva rodova *Marasmius* i *Ganoderma* poređene su sa ITS sekvencama iz baze podataka koristeći BLAST program za pretraživanje. Filogenetsko stablo je konstruisano na osnovu razlika između ispitivanih ITS sekvenci koristeći Neighbor joining metod. Naši rezultati pokazuju da se u okviru rodova *Marasmius* i *Ganoderma* determinacija na osnovu morfoloških i molekularniih podataka najšeće podudara, ali za pravilnu identifikaciju vrsta gljiva najbolje je koristit oba pristupa.

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