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 autochthonous 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...
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 FRÆSLEV 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 (NOVAKOVIĆĆ 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 (WANNA THES 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 (RATANACH OMSRIT 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

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

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

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'-TCCGTAAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATGATGC-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, Ge ml 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.

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

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

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 FRÆSLEV 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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http://www.svims.ca/council/Marasm.htm
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