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MOLECULAR CHARACTERISATION OF SOME LIGNICOLOUS SPECIES FROM FUNGAL CULTURE COLLECTION

Nevena STEVIĆ[1], Eleonora ČAPELJA[1], Vladislava GALOVIĆ [2], Milana NOVAKOVIĆ[1], Maja KARAMAN[1]

[1]University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Novi Sad, Serbia
[2] Institute of Lowland Forestry and Enviroment, Novi Sad, Serbia

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Culture collections of microorganisms, including fungi, are strain deposits recognised as Biological Resource Centers (BRCs) with a great importance in science, industry and education. Their objective is to preserve the purity, viability and genomic integrity of every single strain as a member of such collection. Since improvement of molecular methods nowadays brought many novel approaches in manipulation with strains of microorganisms, they can also be useful for characterization of existing stored strains.

ITS1 region in nuclear DNA is preferred barcoding marker for taxon identification, which can be explained by its great inter-species variability. This paper presents results from analysing ITS1 region sequences (17) obtained from fungal DNA of culture collection of autochthonous, lignicolous genera Piptoporus, Pleurotus, Ganoderma and Schizophyllum cultured on malt agar plates for 14 days at 25°C. BLAST (Basic Local Alignment Search Tool) was used for comparison with online databases, while alignment of sequences was made with MEGA 5.10 software. Morphological determination of species or genus was confirmed for 13 cultures, while the others were disproved. The resulting alignment indicated small intra-species variability of ITS1 region and pointed to it as an ideal marker for verification of fungal culture collections' authenticity.

Key words: BLAST, culture collection, fungi, ITS1 region, molecular identification.

Corresponding author: Stević R. Nevena, University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia, e-mail: nevena.stevic@dbe.uns.ac.rs

INTRODUCTION

Microbial culture collections play a crucial role in providing the authenticated biological material, upon which high quality scientific research is based on (SMITH, 2003). They serve as deposits for key strains in many human activities like industry, science and education by providing them quality care and maintenance. Hence, the aim of the culture collection is to maintain the purity, viability and genomic integrity of every single strain (SMITH & RYAN, 2005), being sources of key organisms for research, available for confirmation of results and further studies (ÇAKTÜ & TÜRKOĞLU, 2011). Since the culture collection represent, not only repository of organisms, but also significant genetic database, many of them are becoming Biological Resource Centers (BRCs), as defined by the OECD Biological Resource Center Initiative, operating according to international quality criteria (SMITH, 2003). This initiative aims to create a network of collection and establish a global, virtual BRC (OECD, 2001).

Demands upon culture collections change as new technologies are developed. Techniques for the characterization of cultures are becoming numerous and various, therefore, it is essential that the methods used in the characterization, creating and maintaining cultures are optimized in order for organisms in cultures to express and maintain their full potential (ILIĆ *et al*., 2008).

Molecular identification through DNA barcoding of fungi has, during the last 20 years, become an integrated and essential part of fungal ecology research and has provided new insights into the diversity of this group of organisms (BELLEMAIN *et al*., 2010; KARAMAN *et al*., 2012). The region of *coxI* is not suitable for use as a DNA barcode in most land plants and fungi, because the mitochondrial genes in these groups evolve too slowly to allow accurate discrimination between species (CHASE & FAY, 2009). The primary amplification targets are ribosomal genes and spacers (HORTON & BRUNS, 2001), while ITS region is an area of particular importance for molecular identification of fungi. It possesses areas of high conservation and areas of high variability and presents an ideal starter for the development of specific PCR primers for identification of fungal species (ATKINS & CLARK, 2004). Therefore, the ITS region of nuclear DNA is preferred DNA barcoding marker both for the identification of taxa and official primary barcoding marker for fungi (BELLEMAIN *et al*., 2010). More than 100 000 sequences fungal ITS sequences have been deposited in the International Nucleotide Sequence Database (INSDC, http://www.insdc.org/) and/or in other databases (NILSSON *et al*., 2009).

Furthermore, since cultures grow in a form of white mycelia in collections, without developing fruiting bodies or ultrastructural characteristics necessary for their identification, they precisely demand an analysis of the genetic material before the use of these cultures for further scientific research. Considering also the high morphological similarity of the studied species with other members within their genera, it is decided to confirm traditional determination based on morpho-anatomical characteristics using molecular approach (GALOVIC *et al*., 2008, GALOVIC *et al*., 2009, GALOVIC *et al*. 2010). The aim of this study was the molecularly based verification of morphological identification of lignicolous fungi obtained from the Culture Collection of Department of Biology and Ecology, Faculty of Sciences in Novi Sad.

MATERIALS AND METHODS

Fungal cultivation and DNA isolation: 17 cultures of fungi used in this study (Table 1) were obtained from the Laboratory of Microbiology at Faculty of Sciences in Novi Sad. All species belong to a group of lignicolous macrofungi, which are members of the phylum Basidiomycota.

Material from cultures, stored at +4 ° C in distilled water, was subcultured from stock cultures on malt agar Petri plates at for 10-14 days as well as in malt broth in 1.5 ml microtubes (Eppendorf), incubating on room temperature with shaking for 3 days. Genomic DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen, CA, www.qiagen.com) in accordance with the manufacturer's instructions. DNA concentration and purity was determined spectrophotometrically by measuring the absorbance of DNA solution at 260 and 280 nm (DU-65 Spectrophotometer, Beckman Instruments Inc. CA, www.beckmancoulter.com). Afterword, DNA is quantified to get the concentration of 10ng/µl.

Table 1. Comparison between obtained sequences and sequences from the database

	Morphological determination of culture	Culture symbol	BLAST results	Length in bp	Query coverage[1]	Max identity[2]
1	*Piptoporus betulinus*	PB-21	*Piptoporus betulinus*	652	98 %	99 %
2	*Piptoporus betulinus*	PB-53	*Piptoporus betulinus*	339	83 %	85 %
3	*Piptoporus betulinus*	PB-57	No similarity	206	/	/
4	*Pleurotus ostreatus*	PO-1	*Pleurotus ostreatus*	586	97 %	95 %
5	*Pleurotus ostreatus*	PO-3	*Pleurotus ostreatus*	749	85 %	98 %
6	*Pleurotus dryinus*	PD-13	*Panus lecomtei* *	659	95 %	99 %
7	*Schizophyllum commune*	SC-1	*Schizophyllum commune*	704	85 %	99%
8	*Schizophyllum commune*	SC-20	*Schizophyllum commune*	414	79 %	95 %
9	*Schizophyllum commune*	SC-34	*Schizophyllum commune*	712	83 %	99 %
10	*Schizophyllum commune*	SC-44	*Schizophyllum commune*	622	97 %	99 %
11	*Schizophyllum commune*	SC-46	*Schizophyllum commune*	623	97 %	99 %
12	*Ganoderma sp.*	G-14	*Ganoderma resinaceum**	647	96 %	98 %
13	*Ganoderma sp.*	G-30	*Ganoderma adspersum**	716	86 %	97 %
14	*Ganoderma sp.*	G-33	*Ganoderma resinaceum*	647	96 %	99 %
15	*Ganoderma sp.*	G-36	*Ganoderma resinaceum*	1093	56%	99 %
16	*Ganoderma sp.*	G-47	*Trametes versicolor*	623	95 %	99 %
17	*Ganoderma sp.*	G-49	*Trametes ochracea*	617	97 %	99 %

[1] percent of the query sequence that overlaps the subject sequence

[2] percent similarity between the query and subject sequences over the length of the coverage area

DNA amplification and preparation of PCR products for sequencing: ITS1 region of DNA was amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3 ') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (WHITE *et al*., 1990). PCR master mix was prepared in 200 µl PCR microtubes in total volume of 25 µl, reaction mixture including: 10ng/µl gDNK, 0.2 µM each primer, 2.5 mM MgCl2, 0.2 mM dNTPs, 1xPCR buffer, Taq polymerase (5U/µl)

(Fermentas, http://www.fermentas.com) and PCR water was added to reach the final volume. DNA fragments were amplified in a Thermal Cycler (Eppendorf, NY, http://www.eppendorf.com). The protocol for PCR amplification was performed according to GALOVIĆ *et al*., 2010a

PCR products were quantified by electrophoresis on an agarose gel (2% agarose, 0.5 X TBE buffer) stained with EtBr dye. DNA ladder used was 50 bp GeneRuler ™ (Fermentas). Electrophoretic separation of PCR products was carried out in 0.5 X TBE buffer for 60 minutes at a constant current intensity of 50V. Visualisation was performed under UV light exposure on DIAS photodocumentation system (SERVA Electrophoresis GmbH, D, www.serva.de). Electrophoretically separated DNA fragments extracted by QIAquick Gel Extraction Kit, in accordance with the manufacturer's instructions (Qiagen). The concentration of purified DNA was determined spectrophotometrically and samples were quantified to 5ng/µl. Samples were sent for sequencing (Macrogen, NL, http://www.macrogen.com/) by automatic DNA sequencer (ABI 3730XL) using capillary electrophoresis.

Sequence analysis: The analysis of the ITS sequence included their alignment using software MEGA 5.10 (TAMURA *et al*., 2011) and their comparison with sequences from the NCBI database using the BLAST program (ALTCHUL *et al.* 1990), Available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

The success of DNA amplification, sequencing and sequence alignment: DNA amplification of all tested cultures was successful and the length of obtained fragments was between 600 and 700bp (Figure 1). Sequencing of ITS1 region was successful for a total of 16 of the 17 fungi in culture, whereas ITS1 region of No. 3 fungal culture with the length of 206bp is not sequenced (Table 1). The results of BLAST searches, with significant parameters, as well as the length of all sequences in base pairs are presented in Table 1. Sequence's lengths are corresponding to the literature data for the ITS regions of examined species (BUZINA *et al.*, 2001; YANG, HUANG & YAO, 2007).

Alignment of obtained ITS sequences of *Sch. commune*, *P. ostreatus*, *P. betulinus* and species of the genus *Ganoderma* was performed using the software MEGA 5.10 (TAMURA *et al*, 2011) (not shown). The homology of sequences within the same species or genus was calculated using the same software. The percentage of conserved nucleotides within the four samples of *Sch. commune* was 95.3%, while between two sequences of *P. ostreatus* it was 89.5%. 79.6% conserved nucleotides was observed between two sequences of *P. betulinus,* while for the four samples in the genus *Ganoderma* it was 84.1%. Alignment of sequences in the software MEGA 5.10 shows low intraspecific variability of ITS region, which is evident even in this small group of samples. The high degree of sequence conservation obtained for *Sch. commune* conservation is consistent with different study which examined the variability of strains of this species with fungi from culture, herbarium, different substrates and clinical samples (BUZINA *et al*., 2001).

Comparison between obtained sequences and sequences from the database: sequencing of ITS region confirmed the morphological determination of 9 cultures (*P. betulinus*: PB-21 and PB-53, *P. ostreatus*: PO-1 and PO-3, *Sch. commune*: SC -1, SC-20, SC-34, SC-44, SC-46). Among these sequences, the percentage of identity with sequences from the database was high, ranging from 95 to 99% (except for PB-53, where this parameter was 85%). Thus, considering the length of the sequence and the sequence coverage when compared with a database, reliable

evidence of taxonomic and morphological confirmation of morphological determination was provided.

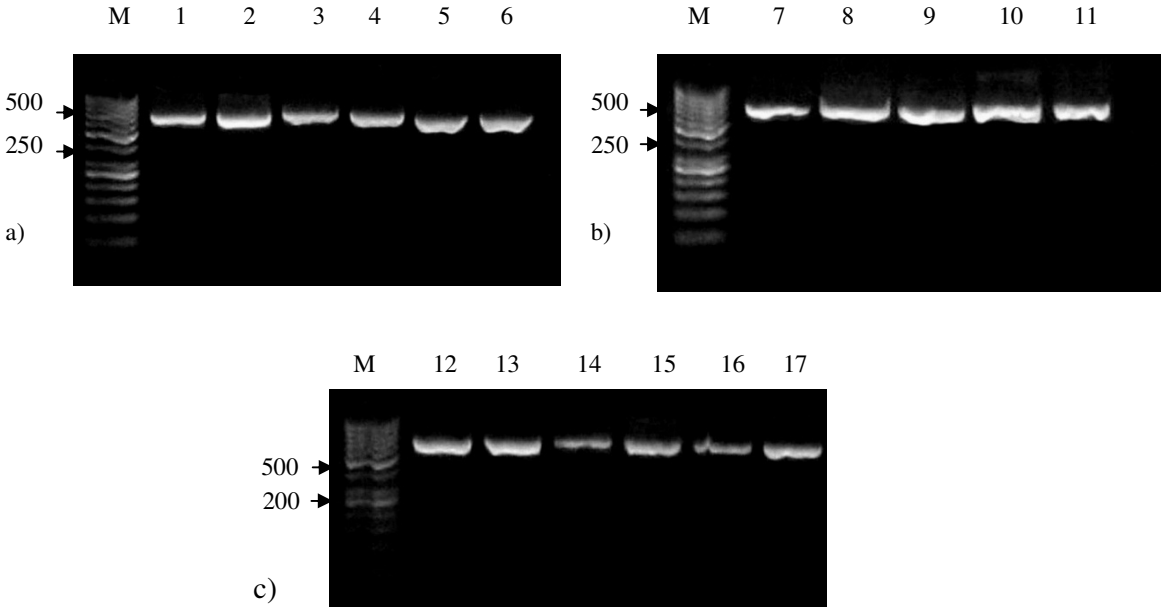

Figure 1. Electrophoresis of PCR amplification products on 2% agarose gel. M: molecular weight marker 50-bp DNA ladder;
a) lane 1: PB-21; lane 2: PB-53; lane 3: PB-57; lane 4: PO-1; lane 5: PO-3; lane 6 PD-13;
b) lane 7: SC-1; lane 8: SC-20; lane 9: SC-34; lane 10: SC-44; lane 11: SC-46;
c) lane 12: G-14; lane 13: G-30; lane 14: G-33; lane 15: G-36; lane 16: G-47; lane 17: G-49.

Cultures G-14, G-33 i G-36 were identified as *G. resinaceum* and G-30 was identified as G. *adspersum.* Considering the fact that lignicolous species share the same habitat and substrate as well as possess the high morphological similarity, the possible reason for incorrect or incomplete identification of species within the genus prior to the formation of culture is probably morphological determination of fungi itself.

Analysis of the ITS region did not confirm the morphoanatomical determination for cultures PD-13, G-47 and G-49 (Table 1). Identification of *P. dryinus* as *Panus lecomtei* can also be explained by the morphological similarities. Although taxonomically distant, distinguishing etween *P. dryinus* and *Panus* species (e.g., *P. strigosus* and *P. levis*) is difficult (WILSON, 2006, Retrieved October 11, 2012.), especially when young fruting bodies are collected.

Cultures G-47 and G-49 (*Ganoderma*) were identified as two separate species of the genus *Trametes* (*T. versicolor* and *T. ochracea*). As *Ganoderma* and *Trametes* species use the same substrate, contamination of the fruiting bodies with fungi of other species is possible.

Therefore, the sampling of mycelium for purposes of culture forming is advised to be performed by taking the inner, protected part of the fruiting body. Since these cultures were obtained on this manner it is possible that also a different source of culture contamination happened in the laboratory environment itself. The possible reasons for this could be the following: inappropriate maintenance of cultures in collections (medium change or the incorrect subcultivation of specific strain from the stock cultures), the lack of separated sterilized equipment, the existence of spores of other species existing at the same fruiting body. Furthermore, since fungi grow in culture in the form of white mycelia, determination or verification of their taxonomical position is necessary to be checked by biochemical tests, such as staining with diazonium blue B reagent (SUMMERBELL, 1985) or using molecular techniques; RFLP of ITS regions or direct sequence analysis and its comparison with online databases (HORTON & BRUNS, 2001; SEIFERT, 2009).

There was no significant overlap in the database for ITS region of culture PB-57, therefore, it remained unidentified. The main reason for this is the short length of the sequence of 206bp, which does not cover the entire ITS region of fungi, whose length is between 450 and 700bp (BELLEMAIN *et al*., 2010). When the tested sequences are too short, E-value parameter can have high value, and thus can lead to misidentification, or in cases where the E-value exceeds threshold value (which is set in the parameters of the algorithm for searching the database and is usually 10), identification does not occur (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=FAQ#nohits Retrieved October 11, 2012.).

At some of the cultures where analysis of ITS region did not confirm the morphological determination (PD-13, G-14 and G-30) the processes of subcultivation, DNA isolation, amplification and sequencing of the ITS region were repeated (marked with " * " in Table 1). Hence the comparison of the base sequences yielded the same results both times, the contamination in the laboratory manipulation may be excluded and pointed to the false morphological determination.

The results of this study suggest small interspecies variability of ITS region among fungal cultures confirming it as an ideal marker for the species identification and verification of their integrity in the cultures. Confirmation of morphological determination at the molecular level is the initial step in further research (NOVAKOVIĆ *et al*., 2012). However, in the case when ITS region analysis does not confirm the identity of the culture, that can point to the need of an improvement of the steps in its characterization or maintenance inside the culture collection protocols.

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MOLEKULARNA KARAKTERIZACIJA NEKIH LIGNIKOLNIH GLJIVA IZ KOLEKCIJE KULTURA GLJIVA

Nevena STEVIĆ[1], Eleonora ČAPELJA[1], Vladislava GALOVIĆ[2], Milana NOVAKOVIĆ[1], Maja KARAMAN[1]

[1] Univerzitet u Novom Sadu, Prirodno-matematički fakultet, Departaman za biologiju i ekologiju, Novi Sad, Srbija
[2] Institut za nizijsko šumarstvo i životnu sredinu, Novi Sad, Srbija

Izvod

Kolekcije kultura mikroorganizama, uključujući i gljive, su depozit sojeva priznatih kao Biološki resursni centri (Biological Resource Centers – BRCs) sa velikim značajem za nauku, industriju i obrazovanje. Njihov cilj je održavanje čistoće, vijabilnosti i genomskog integriteta svakog soja kao člana takve kolekcije. Kako je napredak molekularnih metoda doneo brojne načine za manipulaciju sojevima mikroorganizama, one mogu biti korisne i za karakterizaciju postojećih.

ITS1 region nuklearne DNK je prioritetni DNK barkoding marker za identifikaciju taksona zbog svoje velike interspecijske varijabilnosti. U ovom radu su prikazani rezultati analize 17 sekvenci ITS1 regiona dobijenih iz gljiva iz kultura, autohtonih, lignikolnih vrsta, pripadnika rodova *Piptoporus*, *Pleurotus*, *Ganoderma* i *Schizophyllum* kultivisanih na čvrstoj hranljivoj podlozi sladnog agara tokom 14 dana 25°C. BLAST (Basic Local Alignment Search Tool) pretraživač je upotrebljen za upoređivanje sa bazama podataka, dok je poravnavanje sekvenci urađeno pomoću softvera MEGA 5.10. Morfološka determinacija vrste ili roda je potvrđena za 13 kultura, dok je kod ostalih opovrgnuta. Rezultati poravnavanja sekvenci ukazuju na malu intraspecijsku varijabilnost ITS1 regiona. ITS1 regioni predstavljaju idealan marker za potvrdu autentičnosti kolekcije kultura gljiva.

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