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EVALUATION OF THE GENETIC DIVERSITY OF *Pleurotus ostreatus* STRAINS USING COMBINATORIAL TUBULIN BASED POLYMORPHISM (cTBP) AND ISSR MARKERS

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Abstract

Pleurotus ostreatus is one of the most widely cultivated and popular edible fungi from the genus Pleurotus. In the last years, genetic variation of wild strains and cultivated varieties was investigated by use molecular markers and revealed a reduction of the diversity in commercial cultures. In this paper are presented the results obtained by application of combinatorial tubulin based polymorphism (cTBP) and inter simple sequence repeat (ISSR) markers, in order to investigate the genetic variability in several commercial strains of P. ostreatus collected from different geographical locations and their genetic relationships with wild isolates. Five polymorphic PCR products were obtained with primers designated for intron 1 of beta-tubulin gene, and 12 polymorphic amplicons were detected when primers for intron 2 of the same gene were used. Molecular analysis performed with ISSR primers displayed a polymorphism at intra specific level. The data achieved with the cTBP and ISSR markers revealed a reduced genetic differentiation among the commercial lines dispersed in Europe. However, comparing with commercial lines originated from USA, differences at molecular level were observed, suggesting a possible influence of environmental conditions. Regarding the wild strain of P. ostreatus, the molecular data indicated similarity with all European commercial lines. The results demonstrated that the applied molecular methods could be considered efficient tools to evaluate the genetic diversity in Pleurotus ostreatus.

Key words: *genetic diversity, ISSR markers, Pleurotus ostreatus* polymorphism, *β*-Tubulin, c*TBP molecular markers.*

INTRODUCTION

The genus *Pleurotus* (Jacq. Fr.) Kumm. (*Pleurotaceae*, *Basidiomycetes*) comprises a wide group of edible mushrooms with high nutritional value, therapeutic properties, and various environmental and biotechnological applications (Cohen et al., 2002).

Pleurotus is one of the most diverse groups among cultivated fungi with many taxonomic problems (Vilgalys et al., 1996).

In addition, the cultivated lines of *Pleurotus* may undergo a drastic loss of diversity resulting from human selection (Hamrick and Godt, 1989; Iracabal et al., 1995).

To clarify the taxonomic status of species in the genus *Pleurotus*, researchers started to classify these fungi also by genetic criteria (Bao et al., 2004).

In the last years, the interest in the genetic structure of natural populations has increased the necessity to expand the knowledge of genetic variation in cultivated species.

Currently, new approaches for assessing genetic variability of the wild and cultivated of *Pleurotus* strains represent an important goal of molecular genetics research.

Molecular approaches included techniques based on random amplified polymorphic DNA (RAPD) markers (Wang et al., 2003), amplified fragment length polymorphism (AFLP) markers (Pawlik et al., 2012), inter simple sequence repeat (ISSR) markers (Zhao et al, 2013) and they are currently used to analyse genetic variation in basidiomycetes.

ISSR markers were frequently used for the analysis of genetic variability and population structure in many types of organisms

(Kauserud and Schumacher, 2003; Wang et al., 2012). In 1999, Thon and Royse developed a set of primers for the β-tubulin genes which are coding for basidiomycete β-tubulin proteins and showed that these genes may have a great potential for phylogenetic studies of mushroom species. Tubulin-Based-Polymorphism (TBP) was introduced as a novel method for assaying genetic diversity of some plant species and varieties (Bardini et al, 2004), and is based on the analysis of amplification products resulted with primers directed to the first intron from the coding region of the β-tubulin gene family. The disadvantage of this method is the low number of molecular markers due to limited variation in the nucleotides sequence of this intron of β-tubulin gene family. To solve this inconvenient Breviario et al (2007) proposed a new set of primers targeted to the second intron of the β-tubulin genes. The combined application of the primers for these introns caused increased number of the molecular markers and allowed a more reliable evaluation of relationships between species/varieties (Breviario et al., 2007). No information about this approach was encountered for *Pleurotus* genetic analysis. In this respect, the aim of present study was to evaluate both cTBP and ISSR markers for the investigation of the genetic diversity in several commercial strains of *Pleurotus* collected from different geographical locations and their genetic relationship with natural isolates.

MATERIALS AND METHODS

Mushrooms samples. Seven *P. ostreatus* commercial lines collected from different geographic regions (Romania, Belgium, Germany and USA) and one *P. ostreatus* natural isolated were used for genetic diversity studies (Table 1). Mostly of the fungal material was kindly provided by Ph.D. Eng. Ioana Tudor and Eng. Paul Covic.

The stock cultures of fungal strains were maintained on PDA **(**potato dextrose agar**)** slants at 4°C.

DNA isolation. Mycelia developed on PDA medium in Petri dishes with cellophane were used for DNA extraction. The total DNA was extracted using a Plant Genomic DNA Miniprep Kit (Sigma) according to the protocol specified by the manufacturer. The purity and quality of the genomic DNA were determined spectrophotometrically and by 0.8 % agarose gel electrophoresis. The DNA solution in TE buffer was stored at -20°C.

Table 1. Fungal strains used in experiments

No.	***P. ostreatus* strains**	Source
1	*P. ostreatus* – wild isolate	Chitila forest
2	*P. ostreatus var.* Florida2125	Belgium
3	*P. ostreatus var.* Florida	Romania
4	*P. ostreatus* 1220	USA
5	*P. ostreatus* 50	Romania
6	*P. ostreatus* HK35	Belgium
7	*P. ostreatus* 375	Belgium
8	*P. ostreatus* 2191	Belgium

cTBP - PCR Amplification. Two pairs of primers for the introns of beta-tubulin gene proposed by Brevario et al. (2007) were used for PCR amplification (Table 2).

Table 2. The sequences of primers used for cTBP-PCR amplification

TBP markers	Primers	Nucleotide sequence (5'-3')
Intron 1	TBP fex1	AACTGGGCBAARGGNCAYTAYAC
	TBP rex1	ACCATRCAYTCRTCDGCRTTYTC
Intron 2	TBP fin2	GARAAYGCHGAYGARTGYATG
	TBP rin2	CRAAVCCBACCATGAARAARTG

PCRs were performed in a 20 µl total volume which consisted of 1× PCR buffer (10 mM Tris.HCl (pH 8.8), 50 mM KCl, and 0.1% v/v Triton X-100), 0.2 mM each dNTP, 2.5 mM MgCl2, 50 ng of template DNA, 1 µM each primer, and 1 U *Taq* polymerase 360 (*AmpliTaq360DNA Polymerase-Promega*). All amplification reactions were performed in a thermal cycler (Eppendorf, Germany) with the following program: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 90 s. The reactions were held at 15 °C after a final extension at 72 °C for 8 min (Bardini et al., 2004). Highlighting products was performed on agarose gel (1.5 %), staining with ethidium bromide and visualization under UV light using UVP BioDocIt system.

PCR Amplification with ISSR primers. For PCR amplifications were used six ISSR primers (Table 3).

Table 3. ISSR primers used for PCR amplification

ISSR Primers	Nucleotide sequence (5'-3')
HB15	(GTG)3 GC
17898A	(CA)7-AC
17898B	(CA)7-GT
UBC810	(GA)8 - T
UBC866	(CTC)6
UBC876	GAT AGA TAG ACA GAC A

The reactions for the amplification of the DNA were carried out in a thermocycler (Eppendorf) under the following conditions: 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 44 °C for 30 s, and 72 °C for 90 s. The DNA fragments were separated by 1.0 % agarose gel electrophoresis, in 1× TBE buffer. The gels were stained with ethidium bromide and examined using UVP BioDocIt system.

Data analysis. Amplicons generated by PCR amplification were scored as either present (1) or absent (0) across all accessions to build separate binary data matrices. The dendrograms were constructed with UPGMA cluster analysis using TREECON for windows software (version 1,3b). UPGMA cluster analysis was based on Nei and Li's (1979) genetic distance.

RESULTS AND DISCUSSIONS

cTBP analysis. The electrophoretic patterns of the DNA fragments obtained for both the first and the second intron of beta-tubulin gene from eight lines of *P. ostreatus* were analysed. Clear five polymorphic bands with 340 bp, 430 bp, 500 bp, 780 bp and 970 bp were detected for the first intron (Figure1).

Differences regarding the presence/absence of some amplicons were observed among the mushrooms varieties, both wild type (natural isolate) and commercial lines.

Among the European varieties the differences regarding the first intron were reduced. However, the electrophoretic pattern of the amplicons obtained from USA variety presented some differences: few supplementary amplicons were scored.

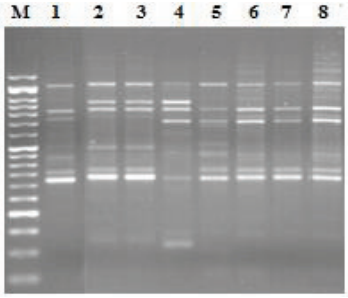

Figure 1. Electrophoretic pattern of amplicons generated with primers for the first intron of β -tubulin gene

Legend:

M = Molecular marker standard (GeneDirex 50 bp); 1= *P. ostreatus* (Romania - Chitila forest); 2 = *P. ostreatus* var.Florida 2125 (Belgium); 3 = *P. ostreatus* var. Florida (Romania); 4 = *P. ostreatus* 1220 (USA); 5 = *P. ostreatus* 50 (Romania); 6 = *P. ostreatus* HK 35 (Belgium); 7 = *P. ostreatus* 375 (Belgium); 8 = *P. ostreatus* 2191 (Belgium).

The confirmation of the molecular variability among *P. ostreatus* varieties at beta-tubulin gene was performed by analysing the electrophoretic patterns of the amplicons generated with primers for the second intron of this gene. Clear 12 polymorphic bands were evaluated for this intron: 370 bp, 550 bp, 600 bp, 740 bp, 875 bp, 900 bp, 950 bp, 1000 bp, 1100 bp, 1200 bp and two bands > 1500bp (Figure 2).

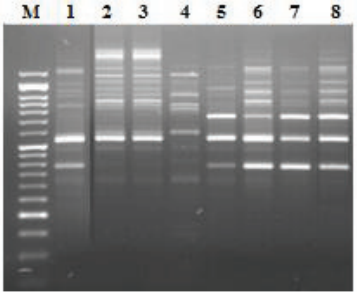

Figure 2. Electrophoretic pattern of amplicons generated with primers for the second intron of β -tubulin gene (Please repeat the legend)

The results obtained with the primers for the second intron were similar with those generated with the primers for the first intron: all the commercial European varieties were similar and comparable with the Romanian natural isolate. Variations were observed among *P. ostreatus* var. Florida, *P. ostreatus* from USA and European commercial/wild type lines.

These data suggest a possible influence of environmental conditions on the molecular variability in *P. ostreatus*. The reduced genetic variability among the commercial lines could be explained by the successive breeding cycles applied for mushrooms in order to increase the productivity or for other characteristics. In contrast, the molecular polymorphism observed at beta-tubulin gene level of the natural isolates of *P. ostreatus* suggests new sources for valuable genes, useful in commercial approaches.

Cluster analysis. The genomic relationships between the studied *Pleurotus* strains based on the data obtained from cTBP - PCR amplification with intron 1 and 2 primers are presented in the dendrogram constructed with an UPGMA cluster analysis (Nei and Li's, 1979)(fig. 3). Two main clusters comprising eight *Pleurotus* strains were obtained. In the first cluster are included five strains of *P. ostreatus*: three strains originated from Belgium (*P. ostreatus* HK 35, *P. ostreatus* 375, and *P. ostreatus* 2191) that were similar at beta-tubulin amplicons profiles level, and two other varieties, *P. ostreatus* wild strain and *P. ostreatus* 50 commercial strain. The second cluster included *P. ostreatus* 1220 strain (USA) and two cultivated strains of *P. ostreatus* var. Florida, one from Belgium and another from Romania. The higher genetic similarity between the commercial strains *P. ostreatus* var. Florida 2125 from Belgium and *P. ostreatus* var. Florida from Romania suggests the possibility that these varieties are identical and have the same origin (Figure 3).

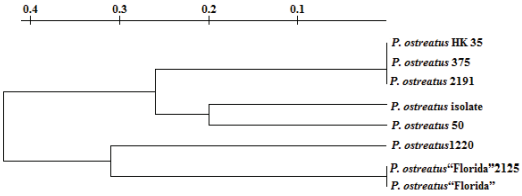

Figure 3. UPGMA dendrogram of genetic relationships among *Pleurotus* strains based on intron1 and intron2 polymorphisms

Analysis of genetic distance between the eight *P. ostreatus* varieties showed a lower variation between the European varieties: 0.44 (*P. ostreatus* natural isolate vs. *P. ostreatus* var. Florida), 0.30 (*P. ostreatus* natural isolate vs. *P. ostreatus* 50) and 0.27 (Romanian natural isolated vs. Belgian *P. ostreatus*). However differences at molecular level were found between Romanian *P. ostreatus* wild strain and USA originated *P. ostreatus* 1220 (0.58).

The combinatorial version of original TBP method has increased the possibility of detection the variability by addition of the second intron present within the vast majority of beta-tubulin genes (Breviario et al, 2007). Preliminary experiments performed on different plant species such as bean (*Phaseolus vulgaris*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabaccum*) and rice (*Oryza sativa*) confirmed that the second intron of the β-tubulin genes also could be a reliable source for DNA polymorphism, and could be used for studies in more plant or even mushroom species. The multifunctional and essential role of the β-tubulin proteins is the conservation of the regions with the main sequence of amino acids from a polypeptide chain. This data are relevant in the studies on the evolution of the genes for β -tubulin and for investigation of the relationships between basidiomycete species (Begerow et al., 2004).

ISSR-PCR amplification. Six ISSR primers (HB15, 17898A, 17899B, UBC 810, UBC 866, and UBC 876) were used for detection molecular polymorphism among *P. ostreatus* varieties. Molecular analysis performed with HB15 primer displayed a polymorphism at intraspecific level: distinctive PCR product of 850 bp was observed at *P. ostreatus* wild strain and *P. ostreatus* 50 commercial strain (fig.4). PCR amplification with UBC866 primer highlighted a genetic polymorphism for P. *ostreatus* 1220 (USA), *P. ostreatus* 50 (Romania), and *P. ostreatus* 2191 (Belgium) (fig.4). The amplicons obtained with 17898A, 17899B, UBC810 and UBC876 ISSR primers also displayed an intraspecific polymorphism in the group of *P. ostreatus* var. "Florida"2125 (Belgium), *P. ostreatus* var. "Florida" (Romania), *P. ostreatus* 1220 (USA) and *P. ostreatus* 50 (Romania and the wild strain of *P. ostreatus* (Figure 4).

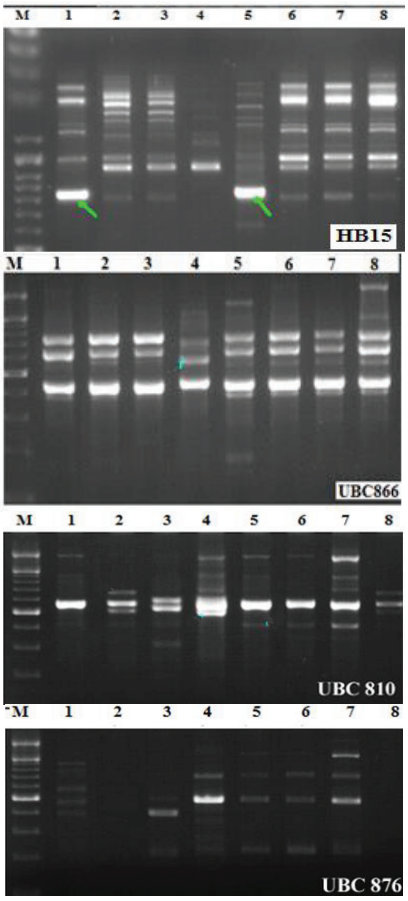

Figure 4. PCR amplification profiles of ISSR primers
Legend:
M = Molecular marker standard (GeneDirex 50 bp); 1= *P. ostreatus* (Romania - Chitila forest); 2 = *P. ostreatus* var. Florida 2125 (Belgium); 3 = *P. ostreatus* var. Florida (Romania); 4 = *P. ostreatus* 1220 (USA); 5 = *P. ostreatus* 50 (Romania); 6 = *P. ostreatus* HK 35 (Belgium); 7 = *P. ostreatus* 375 (Belgium); 8= *P. ostreatus* 2191 (Belgium).

Similar intraspecific polymorphism was observed by other authors: according to Santos Araújo et al. (2016) the difference in the profile of amplification of the primers is related to the kind of DNA sampled. This is possible because using DNA samples in bulk, alleles with low frequency in the population cannot be amplified (Yanaka *et al.* 2005).
In our studies the data obtained with the ISSR markers revealed a lower genetic differentiation among mushroom varieties dispersed in Europe and a higher genetic diversity between European varieties and American ones. Based on such observations, specialists consider that mushroom populations must be divided into groups according to their geographical origins which indicate that the genetic diversity is closely related to the geographical distribution (Zhao et al., 2013).

CONCLUSIONS

In order to investigate the genetic diversity in *P. ostreatus* commercial and natural varieties collected from different geographical locations, cTBP and ISSR markers were used. The results revealed a polymorphism at intraspecific level. Molecular analysis of *P. ostreatus* strains both with primers directed to beta-tubulin gene or ISSR primers showed molecular differences according to their geographical origins. Among the European *P. ostreatus* varieties the genetic variation is reduced. On the contrary, higher genetic diversity was found between European mushroom varieties versus *P. ostreatus* 1220 strain originated from USA. However, the wild isolate of *P. ostreatus* shows similarity with the European commercial strains.
The results obtained allowed the conclusion that the markers generated by cTBP and ISSR primers are very useful to detect the genetic polymorphism among varieties of *P. ostreatus*. According to our knowledge it is the first study concerning the use of cTBP and ISSR markers to investigate the genetic diversity in *Pleurotus ostreatus*.

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