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DETECTION OF GENETIC VARIABILITY IN *Pleurotus eryngii* USING TUBULIN-BASED POLYMORPHISM MOLECULAR MARKERS

Gabriela POPA[1,2], Catalina VOAIDES[1,2], Matilda CIUCA[3], Calina Petruta CORNEA[1]

[1]University of Agronomic Sciences and Veterinary Medicine, Faculty of Biotechnology, 59 Marasti Blvd., District 1, 011464, Bucharest, Romania, Phone: +40 (21) 318 22 66, Fax: +40 (21) 318 28 88, e-mail: popagabiro@yahoo.com
[2]Center of Applied Biochemistry and Biotechnology, BIOTEHNOL, 59 Marasti Blvd., 011464, Bucharest, Romania, phone/fax: +40 (21) 318 04 68
[3]National Agriculture Research and Development Institute Fundulea, 1 NicolaeTitulescu Street, 915200 Fundulea, Romania, Phone: +40 (21) 3150805, Fax: +40 (21) 3110722, e-mail: office@incda-fundulea.ro

Corresponding author e-mail: popagabiro@yahoo.com

Abstract

Pleurotus eryngii is an edible mushroom with important biological functions, such as anticancer, antiviral, immune potentiating, and hypolipidemic activities. Due to its importance from economically, medicinal and ecologically point of view, a special attention was given to this species worldwide. Analyses via molecular markers revealed that Pleurotuseryngii is a species complex comprises at least six varieties (natural isolates and commercial strains). Improvement of culture technologies as well as the increase of biological compounds content involves a better knowledge of the genetic properties of each isolate, candidate for specific applications. For this reason, in this study several strains of Pleurotus eryngii collected from different geographical locations were analyzed in correlation with a wild Pleurotus ostreatus isolate, in order to determine the genetic variability and relationship between species. The genetic diversity of biological material was analyzed using a TBP (tubulin-based polymorphism) molecular marker that relies on the presence of intron-specific DNA polymorphisms of the plant β-tubulin gene family. For the fungal DNA extraction mycelium obtained on plates with PDA was used. Our results revealed that TBP amplification profile of intron 1 showed eight polymorphic bands while TBP amplification profile of intron 2 indicated nine polymorphic bands.The multiple length polymorphism of β-tubulin intron 2 generated amplification profiles which resulted highly specific for each of the analyzed species. However, these results suggested that TBP molecular markers could be used for highlighting the genetic diversity both intra- and inter-specific and the genetic diversity was closely related to the geographical distribution of this fungus.

Key words :*genetic variability, Pleurotus eryngii,* polymorphism, *β*-Tubulin, *TBP molecular markers.*

INTRODUCTION

Pleurotus eryngii, known as king oyster or king trumpet, is an edible mushroom native to regions of Europe, the Middle East, and North Africa but also commercially grown in Japan and the United States. It is the largest species of the oyster mushroom, it has a thick white stem and a small tan cap and is known for its robust, somewhat earthy flavour and more meaty texture. It is a mushroom with generous amounts of nutrients including carbohydrates, proteins, vitamins, minerals and fibres. Various studies revealed that *P. Eryngii* species complex has the most abundant population diversity in the genus *Pleurotus* (De Gioia et al., 2005).Assessment of genetic and phenotypic diversity is necessary to distinguish genotypes of *Pleurotus eryngii* with traits of interest and to identify strains with high yield potential (De Gioia et al., 2005; Zhao et al., 2013).Molecular markers are the tools most widely used to assess genetic diversity (Koebner et al., 2001; Karp et al., 1998). Some of these molecular markers correspond to unknown DNA almost associate within regions of unknown function sequences. Moreover, most of the identified changes do not occur in functionally relevant regions of DNA and thus rarely lead to identification of variations occurring within genes (Bardini et al., 2004). Inter-simple sequence repeats (ISSR) markers have been widely applied for analyses of genetic variance and population structure in many types of organisms (Kauserud and Schumacher, 2003; Wang et al., 2012).

Previous studies have shown that ISSR analyses only showed a relatively low genetic diversity among peanut cultivars despite abundant morphological, physiological, and agronomic variance (Raina et al., 2001). Another molecular marker system termed start codon targeted (SCoT) polymorphism, which is a simple and novel DNA marker system, could detect more polymorphisms compared with several other molecular marker systems (Collard and Mackill, 2009; Xiong et al., 2011). Thon and Royse (1999) developed a set of primers for β-tubulin genes of basidiomycetes and showed that these genes have potential for phylogenetic studies in the Basidiomycotina. Begerow et al. (2004) analyzed 36 fungal β-tubulin sequences to study the evolution of this gene and the phylogeny of basidiomycetes. The multifunctional and essential role of the tubulin proteins is reflected in the conservation of regions within their primary amino acid sequence. The TBP technique is depending on which intron or combination of introns is used as a marker relies on an exon-primed intron-crossing PCR reaction. Therefore, a combinatorial TBP (cTBP) that uses both intron 1 and intron 2 of the β-tubulin genes as the source for genomic polymorphism is expected to produce a greater number of molecular markers associated with each original gene locus. In our studies we tested cTBP method, in order to investigate the genetic variability in several commercial strains of *Pleurotus eryngii* mushrooms collected from different geographical locations and their genetic relationship with an indigenous *Pleurotus ostreatus* isolate. According to our knowledge no studies have been so far performed on TBP method applied to *Pleurotus eryngii* mushrooms.

MATERIALS AND METHODS

Mushrooms culture. Pure cultures of three strains of *P. eryngii: P. eryngii*2600 (Belgium), *P.eryngii* (Romanian producers), *P. eryngii* (German producers) and an indigenous *Pleurotus ostreatus* strain, were obtained by isolating tissue cultures from the fruiting bodies (fig. 1). A small piece of tissue was removed aseptically and transferred into a culture tube containing potato dextrose agar (PDA), and incubated in the dark at 25°C for 7-9 days. These samples were stored at 4°C until further used.

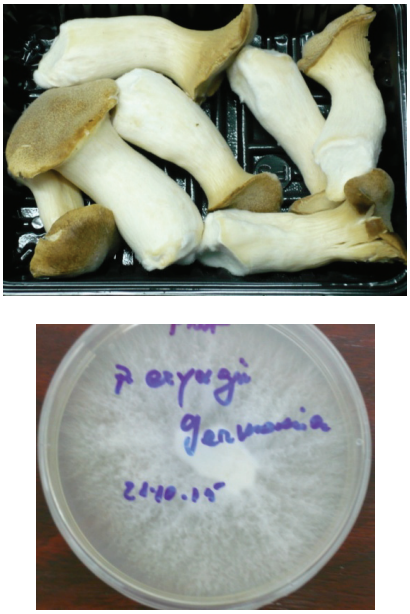

Figure1. Fruiting body and mycelium of *P.eryngii*

DNA isolation

Mycelia for DNA extraction were cultured on PDA Petri dishes with cellophane at 25°C for 5 days. 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 through electrophoresis on 0.8 % agarose gel. The DNA solution was stored at -20°C.

TBP PCR Amplification

Intron1 and intron2of the *β*-tubulin gene family were PCR amplified using each 50 ng of template genomic DNA and the following forward and reverse oligonucleotide primers combination:
TBPfex1(5'AACTGGGCBAARGGNCAYTAYAC3');TBPrex1(5'ACCATRCAYTCRTCDGCRTTYTC-3') pair of primers for intron1, TBPfin2(5'GARAAYGCHGAYGARTGYATG3');TBPrin2(5'CRAAVCCBACCATGAARAARTG-3') pair of primers for intron2

(Brevario et al., 2007). PCRs (20 µL) were performed in 1× PCR buffer (10 mMTris.HCl (pH 8.8), 50 mMKCl, 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 (Promega). Following the initial denaturation step at 94 °C for 3 min, the PCR consisted of 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& al., 2004). Highlighting products was performed on agarose gel (1.5 %), staining with ethidium bromide and visualization under UV light using UVP BioDocIt system.

Data analysis. Bands generated by TBP-PCR amplification were scored as either present (1) or absent (0) across all accessions to build separate binary data matrices. The dendrogram was constructed with UPGMA cluster analysis using TREECON for windows software (version 1,3b).

RESULTS AND DISCUSSIONS

Molecular analysis results using the markers for intron 1 and intron 2 of β- tubulin gene, previously mentioned, showed polymorphism for both markers. Following TBP - PCR amplification, generated profile of intron1 exhibited 8 polymorphic bands. There were two polymorphic areas: one range between 320-500pb and other between 900 -1500pb (fig. 2)

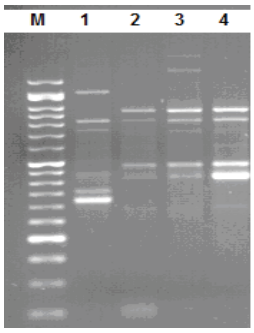

Figure 2. TBP - PCR amplification profile of intron1
M = Molecular marker size (GeneDirex 50 bp);
1= *Pleurotus ostreatus*(wild strain); 2= *P. eryngii* 2600 (Belgium); 3= *P. eryngii* (Romania);4= *P. eryngii* (Germany).

This polymorphism clearly separated *Pleurotus ostreatus* from *P. eryngii* strains. On the other hand, small polymorphic differences were observed between *P.eryngii* strains investigated. These results obtained with TBPfex1/TBPrex1 pair primers for intron1 suggests a polymorphism both at intra- and inter-species level. More information was obtained following TBP - PCR amplification of intron2 with TBPfin2/TBPrin2 pair of primers. Generated profile of intron2 of β- tubulin gene showed nine polymorphic bands (fig.3).

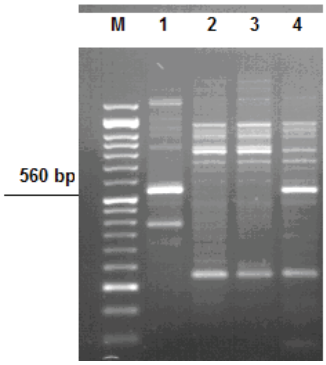

Figure 3. TBP - PCR amplification profile of intron2
M = Molecular marker size (GeneDirex 50 bp);
1= *Pleurotus ostreatus*(wild strain); 2= *P. eryngii* 2600 (Belgium); 3= *P. eryngii* (Romania); 4= *P. eryngii* (Germany)

Regarding the samples of *P. eryngii 2600* (Belgium) and *P. eryngii* originated from Romania we can observe that resulting amplification products showed high uniformity. This suggests that *P. eryngii* from Romania maybe identical with *P. eryngii 2600* from Belgium. But, considering the results obtained with TBPfex1/TBPrex1 pair primers for intron1 that show in *P. eryngii*–Romania sample two non-specific products (in the size of ~1000 - 1500bp) we can concluded there is a small genetic variability between the two *P. eryngii* strains. Contrariwise, in the case of *P. eryngii* originated from Germany we obtained a major amplification product, in the size of ~560 bp, which has similarity with an amplification product with the same size from the *P. ostreatus* isolate (fig. 3).

Clusters analysis

The genetic relationship between the studied *Pleurotus* strains is shown in dendrograms constructed with UPGMA cluster analysis based onpolymorphism for each intron, separately. Thus, UPGMA dendrogram based on polymorphism of intron1 shows two clusters: one includes *P. eryngii* 2600 (Belgium) and *P. eryngii*(Germany) strains, and one includes *P. eryngii* (Romania). The *P. eryngii* group belong to the same branch which shows us a low genetic diversity into this group (fig. 4).

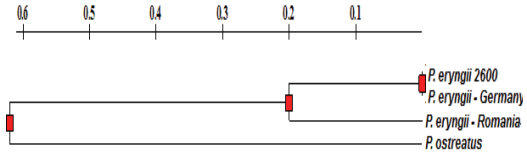

Figure 4. UPGMA dendrogram of *P. Eryngii* strains and *P. Ostreatus* isolate based on polymorphism for intron1.The scale bar means the genetic distance.

UPGMA dendrogram based on polymorphism of intron2 shows also two clusters, one includes *P. eryngii* 2600 (Belgium) and *P. eryngii* (Romania) and the other cluster include *P. eryngii*(Germany) (fig. 5).

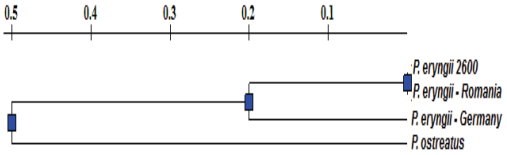

Figure 5. UPGMA dendrogram of *P. Eryngii* strains and *P. Ostreatus* isolate based on polymorphism for intron2.The scale bar means the genetic distance

When the analysis was performed with the amplicons obtained for both introns, clearer results were shown: based onthe molecular polymorphism it could be concluded that the *P.eryngii* commercial strains presented intraspecific differences at beta-tubulin gene level (fig. 6).

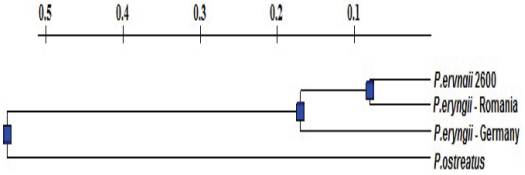

Figure 6. UPGMA dendrogram of *P.eryngii* strains based on the polymorphisms of introns 1 and 2

Moreover, significant interspecific diversity was observed that allow the idea that the primers for intron 1 and 2 of beta-tubulin gene could be used for identification of *Pleurotus* species. However, supplementary studies are necessary for the validation of this idea.Similar polymorphism levels were previously observed in other edible mushrooms, such as *Lentinulae dodes*(99.6%) (Xiao et al., 2010) and *Auricularia polytricha* (99.8%) (Du et al., 2011). 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

Based on the combined data from the cTBP patterns obtained in the PCR amplification with two pair of primers, our result showed a high similarity between *Pleurotus eryngii*2600 (Belgium) and *Pleurotus eryngii*(Romania) and a small genetic diversity between these strains and *Pleurotus eryngii* originated from Germany. This preliminary study regarding determination of genetic variability in *Pleurotus* genus using cTBP method suggests that this technique could be useful for species-specific identification. Whatever, in the future we will increase the number of the mushrooms species / strains that will be analyzed using cTBP technique. According to our knowledge this is the first study performed on *P. Eryngii* using TBP method, but future experiments with an increased number of strains are needed.

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