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Research Article

Studies on the detection of genetic variation of commercially cultivated Mushroom species using RAPD markers

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ABSTRACT

Edible mushrooms are nutritionally endowed fungi (mostly Basidiomycetes) that grow naturally. Besides containing high protein, vitamins, fibers and low calories, it also has many medicinal properties. In this study, seven samples of mushrooms which are commercially cultivated were screened for phytochemicals such as Cardiac glycosides, Anthraquinones, Terpenoids, Proteins, Flavonoids, Saponins, Tannins, Lignins and Phenol. All the samples showed a positive results for Terpenoids and Proteins and showed a negative results for Anthraquinones, Flavonoids, Tannins, Lignins and Phenol. Most of the samples were found to be positive for Cardiac glycosides and Saponins. In this study, genomic DNA was extracted from seven samples of mushroom and molecular characterization was taken up using ten decamer oligo-nucleotides as single primers in Polymerase Chain Reaction (PCR), DNA fingerprinting, dendrogram analysis and genetic similarity matrix were estimated, revealing variations between selected seven samples of mushrooms. From the analyzed samples, White beach, Shimeji and Chinese shimeji were found to be most closely related. The described approach holds great promise for further analyses and gives support to discrimination as well as for conservation of genetic resources.

Keywords: Genetic discrimination; Mushroom; Phytochemicals; RAPD

INTRODUCTION

Mushrooms are recognized as natural and healthy foods (Iwalokun B. A. et al., 2007) and credited to be the third largest macro-fungus cultivated for food and industrial purposes worldwide. Over 5,000 species of mushrooms has been found in The United States out of which 4-6% are safe to eat and approximately 2% are poisonous, about twelve species are known to be lethal if ingested. Outbreaks of severe mushroom poisoning have been documented in Europe, Russia, the Middle East, and the Far East. Ninety percent of deaths from mushroom ingestions are due to amatoxin, which possesses a fatality rate of up to 25% when ingested (Poucheret P. et al., 2010). A mushroom can actually consume field waste and other solid wastes and participates in controlling solid waste, building organic soil and returning minerals to the soil. They convert cotton waste, coffee waste, straw, maize stalks, many other field wastes, sugar cane bagasse, agro-industrial waste and even animal manure into wholesome, vitamin and protein rich food (Kurtzman RH Jr., 1975; Kurtzman RH Jr., 1997). Use of medicinal mushrooms in Western countries has increased during the last decade but in Asian countries, it has long tradition (Sharma N., 2003). Mushrooms and their metabolites are used as adaptogens, immune stimulants and antitumor agent and for curing many of the human diseases (Jose N. et al., 2000). It has reported that mushrooms are a source of physiologically beneficial and non-toxic medicines, low in calories and high in minerals, essential amino acids, high proteins, vitamins and fibers (Mattila P. et al., 2002; Lindequist U. et al., 2005; Diéz V.A. et al., 2001; Agahar-Murugkar D. et al., 2005; Barros L. et al., 2007c) as well as nutritionally functional food (Wasser S.P. et al., 1999). A food or part of a food that provides medical or health benefits like the prevention and treatment of disease can be considered as a nutraceuticals (Andlauer W. et al., 2002; Kruger C.L. et al., 2003). Mushrooms are rich sources of nutraceuticals (Çagˇlarirmak N., 2007; Elmastas M. et al., 2007; Ribeiro B. et al., 2007) and their bioactive properties have been proven by the scientific community (Lindequist U. et al., 2005). Reports show that mushrooms are effective as antitumor, antibacterial, antiviral and haematological agents and in immune modulating treatments (Yang J.H. et al., 2002) also possess antioxidant capacity (Ribeiro B. et al., 2007; Ribeiro B. et al., 2006) and can be used as food supplement as well as in the pharmaceutical industry.

New improved samples with improved characteristics of mushroom are needed to make mushroom cultivation sustainable and highly productive. But due to lack

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of clearly distinguishable characters, mushroom samples are very difficult to discriminate and hence create problems in sample protection and improvement (Pei-Sheng Yan et al., 2004). Molecular markers of rDNA sequencing, RAPD (random amplified polymorphic DNA), microsatellite, RFLP (restriction fragment length polymorphism) and mitochondrial genotypes have all been used to discriminate mushroom species and/or samples of Auricularia (Pei-Sheng Yan et al., 1999), Agaricus (Barroso G. et al., 2000; Calvo-Bado L. et al., 2000; Moore A.J. et al., 2001; Ramirez L. et al., 2001), Stropharia rugoso-annulata (Pei-Sheng Yan et al., 2003), Ganoderma (Hseu R.S. et al., 1996), Volvariella (Chiu S.W. et al., 1995) and Lentinula (Chiu S.W. et al., 1996). All these technologies have proven to gain reliable data for sample identification and protection of mushroom. Using molecular markers especially RAPD, the genetic diversity of mushrooms has been worked out (Staniaszek M. et al., 2002; Stajic M. et al., 2005; Ravash R. et al., 2009) and this technique is used to assess the genetic diversity among 37 pleurotus species of mushrooms and found that, this technique was better than morphological analysis (Stajic M. et al., 2005).

In this study, the evaluation of nutraceutical composition included the determination of Cardiac glycosides, Anthraquinones, Terpenoids, Proteins, Saponins, Tannins, Lignins and Phenol in seven mushroom species and discrimination has been done by RAPD technique to achieve the genetic diversity among them.

MATERIALS AND METHODS

Sample Collection

Total seven samples of mushroom viz. King Oyster, Portobello, White beach, Shimeji, Chinese shimeji, Button mushroom and Normal Oyster were collected from different malls and local market of Bangalore. The mushroom samples were air dried under shade and powdered, which was used for extraction procedure and further analysis.

Preliminary Phytochemical Analysis

Phytochemical screening of mushroom extracts was done by the standard procedure by Harbone (Harbone J.B. et al., 1998). All the prepared plant extracts were subjected to preliminary phytochemical screening for the presence of Cardiac glycosides, Anthraquinones, Terpenoids, Proteins, Flavonoids, Saponins, Tannins, Lignins and Phenol.

DNA Extraction

Approximately, 50 mg of fresh mushroom samples were cut into small pieces and grounded with homogenization buffer (50 mM Tris, 10 mM EDTA and 50 mM glucose), extraction buffer (100 mM Tris, 10 mM EDTA and 250 mM NaCl, pH= 8.0 and 1% Sodium Dodecyl Sulfate) with proteinase-K was added and incubated in dry bath at 60^0 C for 1 h. After centrifugation at 3,000 rpm for 5 min., supernatant was treated with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and incubated at 37^0 C for 30 min. Centrifugation was carried out at 8,000 rpm for 6 min., phenol: chloroform: isoamyl alcohol (25:24:1) step was repeated and DNA was precipitated using 0.8 volumes of chilled isopropanol. The DNA samples were tested qualitatively on 0.8% agarose gel and quantified by using a Nanodrop spectrophotometer (Thermo Scientific).

PCR Reaction

A set of 10 decamer oligo-nucleotides was used in this study as single primers in the Polymerase Chain Reaction [Table- 1]. The polymerase chain reaction was carried out in final volume of 25 µl containing 100 ng DNA, 2 U of Taq DNA polymerase (Chromous Biotech, Bangalore), 2.5 mM $MgCl_2$ (Chromous Biotech, Bangalore), 2.5 mM each dNTPs (Chromous Biotech, Bangalore) and 100 p mol of primers (Eurofins Genomics, Bangalore). The DNA amplification was performed in the Corbett RG 6000 thermo cycler using the following conditions: complete denaturation (94°C for 5 min), 10 cycles of amplification (94°C for 45 sec, 32°C for 1 min and 72°C for 1.5 min) followed by 30 cycles of amplification (94°C for 45 sec, 34°C for 1 min and 72°C for 1 min) and the final elongation step (72°C for 5 min).

Table 1: List of primers

Primer	Sequence (5' → 3')	Primer	Sequence (5' → 3')
OPD-18	GAG AGC CAA C	OPT-7	GGC AGG CTG T
OPD-19	CTG GGG ACT T	OPW-1	CTC AGT GTC C
OPD-20	ACC CGG TCA C	OPW-2	ACC CCG CCA A
OPT-5	GGG TTT GGC A	OPX-6	ACG CCA GAG G
OPT-6	CAA GGG CAG A	OPX-7	GAG CGA GGC T

Agarose gel electrophoresis of PCR products

Total volume of the amplified product (25µl) of each sample was subjected to electrophoresis on 2.0 % agarose gel containing ethidium bromide in 1x TBE buffer at 120V for 1 h. Finally, the DNA bands were observed on a Gel Doc system and the photographs were captured.

Data Analysis

The RAPD profiles were analyzed based on the presence or absence of individual RAPD bands. The genetic distance was calculated by the Jaccard co-efficient. The matrix of genetic distance was used for grouping the mushroom samples based on the dendrogram con-

Table 2: Preliminary phytochemical analysis of screened mushroom samples

S.No	Test	*White beach*	*Portobello*	*King oyster*	*Oyster*	*Button mushroom*	*Chienese Shimeji*	*Shimeji*
1	Cardiac glycosides	-	+	+	+	-	+	+
2	Anthraquinones	-	-	-	-	-	-	-
3	Terpenoids	+	+	+	+	+	+	+
4	Proteins	+	+	+	+	+	+	+
5	Flavonoids	-	-	-	-	-	-	-
6	Saponins	+	+	+	+	-	-	-
7	Tannins	-	-	-	-	-	-	-
8	Lignins	-	-	-	-	-	-	-
9	Phenol	-	-	-	-	-	-	-

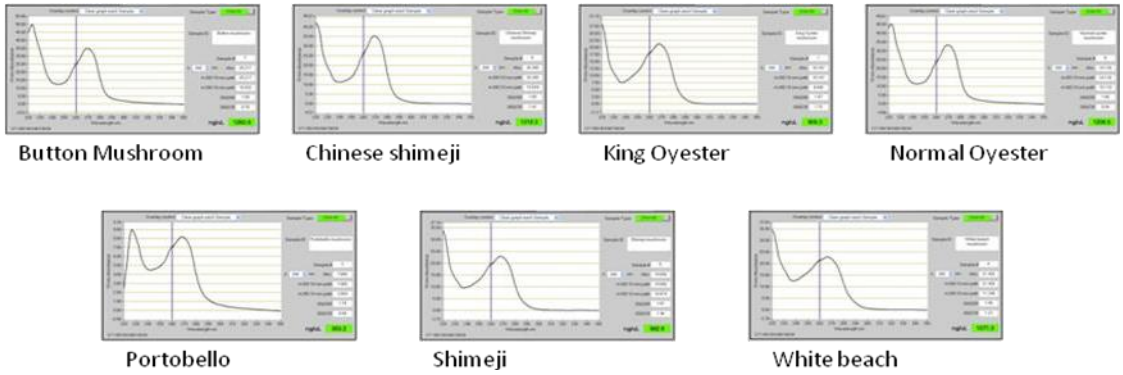

Figure 1: DNA quantification by Nano-drop spectrophotometer

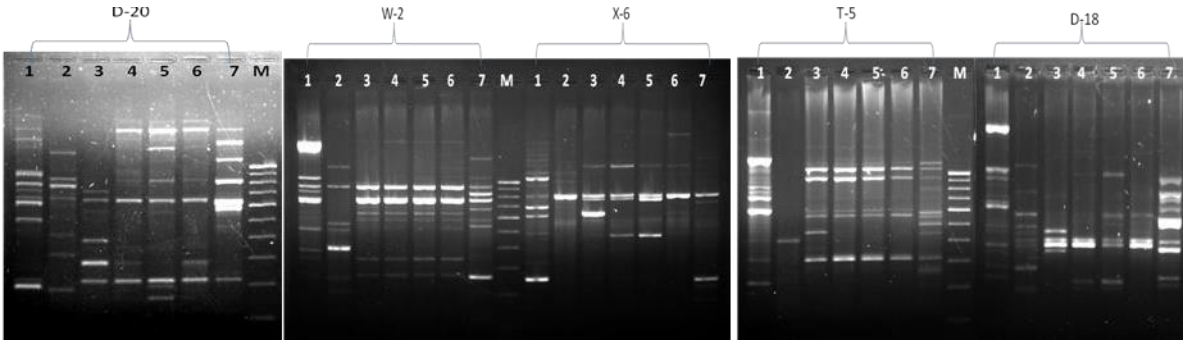

Figure 2: DNA fingerprinting of mushroom samples by primers OPD-20, OPW-2, OPX-6, OPT-5 and OPD-18

1- King Oyster, 2- Portobello, 3- White beach, 4- Shimeji, 5- Chinese shimeji, 6- Button mushroom, 7- Normal Oyster and M- DNA marker (100 – 1000bp)

structed by UPGMA (Unweighed Pair Group Method with Arithmetic averages).

RESULT AND DISCUSSION

Recently there has been a renewed interest in improving health and fitness through the use of more natural products (deSouza E.L. et al., 2005). Phytochemicals are compounds that are used as food and medicine to protect against illness and to maintain human health (Afolabi C.A. et al., 2007). Selected samples of mushrooms were screened for Cardiac glycosides, Anthraquinones, Terpenoids, Proteins, Flavonoids, Saponins, Tannins, Lignins and Phenol. Among seven samples screened, terpenoids and proteins were found to be present in all samples. Cardiac glycosides found present in all except White beach and Button mushroom. Saponins was present in White beach, Portobello, King oyster and normal oyster. The test for Anthraquinones, Flavonoids, Tannins, Lignin and Phenol were negative for all mushroom samples. The result of preliminary phytochemical analysis is shown in table 2.

Genomic DNA was extracted from all seven samples and quality was observed in Agarose gel, bands were found to be intact and without RNA contamination. In quantitative analysis, a ratio of 260:280 was found in

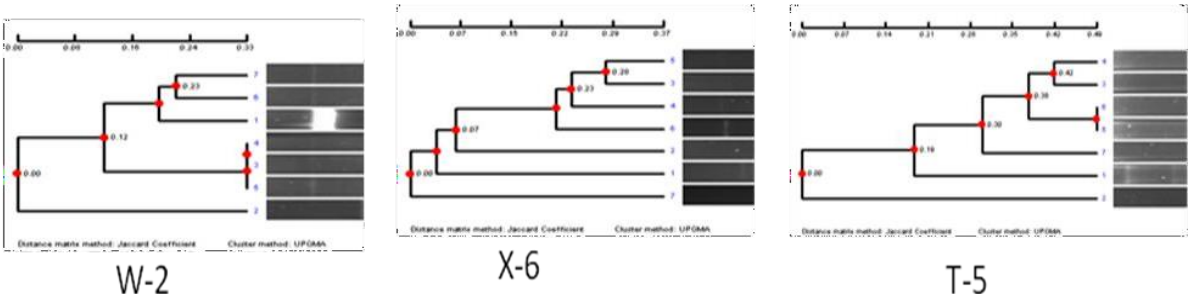

Figure 3: Dendrogram analysis of amplified DNA for primer OPW-2, OPX-6 and OPT-5 primers

Table 3: Similarity matrix calculated by Jaccard coefficient for OPW-2 and OPX-6

	1	2	3	4	5	6	7
1	100.00						
2	41.67	100.00					
3	58.33	33.33	100.00				
4	58.33	33.33	100.00	100.00			
5	58.33	33.33	100.00	100.00	100.00		
6	75.00	27.27	60.00	60.00	60.00	100.00	
7	75.00	40.00	60.00	60.00	60.00	80.00	100.00

	1	2	3	4	5	6	7
1	100.00						
2	35.71	100.00					
3	35.71	42.86	100.00				
4	28.57	50.00	80.00	100.00			
5	42.86	37.50	83.33	66.67	100.00		
6	28.57	28.57	80.00	60.00	66.67	100.00	
7	28.57	28.57	28.57	33.33	25.00	14.29	100.00

between 1.58 to 1.89 for all samples [Fig-1]. The RAPD patterns of genomic DNA of mushroom samples by Eurofins Genomics primers OPD-18, OPD-19, OPD-20, OPT-5, OPT-6, OPT-7, OPU-10, OPU-11, OPW-2 and OPX-6 were analyzed for polymorphism, total five primers gave a clear distinctive band patterns as shown [Fig-2]. The band patterns shown in [Fig-2] are developed by primer OPD-20, OPW-2, OPX-6, OPT-5 and OPD-18 primers respectively. Total 62 bands were generated by OPD-20 in which approximately 650 bp band was found common in all samples similarly out of 61 bands produced by OPW-2 primer, approximately 900 bp band was present in all samples and 750 bp and 250 bp was found common in most of the samples. 800 bp band was common in 52 bands produced by primer OPX-6. 300 bp, 550 bp, 900 bp and 1000 bp bands were common in all samples except Chinese shimeji, in DNA fingerprinting produced by primer OPT-5. Furthermore, for DNA fingerprinting produced by OPD-18 primer, 300 bp and 400 bp band was common in all the mushroom samples.

There are some different methods to form clusters like MCLUST which fits a very similar model to fine structure, K-Means which places individuals in the population closest to them and 'Unweighted Pair Group Method with Arithmetic Mean' (UPGMA) which iteratively merges the closest groups. Genetic similarity was calculated using a Similarity frequency coefficient and dendrogram was generated to access the genetic relationship among seven selected samples [Fig-3]. Dendrogram constructed by cluster analysis of RAPD markers showed that three clusters are 100 % similar for samples 3, 4 and 5 (White beach, Shimeji and Chinese shimeji) with respect to primer OPW-2. Also a result of dendrogram analysis by primer OPX-6 shows that samples 3, 4 and 5 are closely related. The aim of a genetic similarity measure is to identify pairs of individuals who are 'closely related' by assigning them higher similarity than those who are distantly related. Similarity matrices can be related to many population genetics methods in a two-stage approach to population genetics by first computing the pairwise similarities, and then perform clustering or other analyses on this summary of the dataset (Biswas S. et al., 2009; . Gao X. et al., 2007). The similarity index [Table-3] shows that sample 3, 4 and 5 are most closely related.

CONCLUSION

The analyzed mushrooms contain very useful phytochemics which could be extracted for the purpose of being used as functional ingredients. Public health authorities consider prevention and treatment with phytochemicals a powerful instrument in maintaining and promoting health, longevity and life quality. The beneficial effects of phytochemicals will undoubtedly have an impact on nutritional therapy; they also represent a growing segment of today's food industry. Besides, these mushrooms might be used directly in diet and promote health, taking advantage of the additive and effects of all the bioactive compounds present. The RAPD profiles in the present study displayed a high degree of polymorphism. This confirms the suitability of RAPD markers for discrimination of mushroom samples. It is important to mention the fact that data results from RAPD assays can be extended to further dissect traits in a more refined way to exactly knowledge on specific genes and genetic pathways using other molecular methodologies. There is also the opportunity and need to study sequences of specific polymorphic bands, to determine the genes detected by RAPD experiments. Further studies with other molecular methodologies are essential to clarify and confirm genetic relationships among mushroom samples depicted using RAPD.

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