Comparative studies on the Indian cultivated *Pleurotus* species by RAPD fingerprinting

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Abstract: The oyster mushrooms (*Pleurotus*) are the second most important cultivated mushroom in the world. The genus consists of a number of species. The genetic divergent of eight Indian species of *Pleurotus* viz. *P. florida* – P1, *P. membranaeceus* – OE128, *P. sajor-caju* – PL1140, *P. djamor* – X375, *P. cystidiosus* – P19, *P. flabelletus* – PL50, *P. sapidus* – PL40 and *P. ostreatus* – PO1803 was determined based on random amplified polymorphic DNA (RAPD) pattern. Result showed that all the species tested could be differentiated by RAPD data and even one individual primer (OPD-07) could also discriminate all tested species. Genetic similarity analysis and grouping derived from RAPD markers reveals a high level of genetic diversity. Therefore the RAPD technique can provide a powerful tool to discriminate the species and the molecular information are useful for the breeding system. [Nature and Science 2010;8(7):90-94]. (ISSN: 1545-0740).

Key words: Oyster mushroom, *Pleurotus* species, RAPD

1. Introduction

Members of the mushroom genus *Pleurotus* (Jacq. Fr.) P. Kumm. (Basidiomycotina, Pleurotaceae) form a heterogeneous group of edible species of high commercial importance. They are characterized by the production of fruit bodies with an eccentric stalk and a wide cap shaped like an oyster shell, with the widest portion of the cap being away from the stalk. They grow over a wide range of temperatures and are able to colonize a wide spectrum of unfermented, natural, lignino-cellulosic wastes. Because of their fast mycelial growth rate, they colonize the substrates rapidly; the yield of fruit bodies is also high. The bifactorial inheritance, observed in many of the species, suggests the likelihood of a high degree of genetic variability, and, hence, considerable breeding potential. Mating compatibility studies have demonstrated the existence of discrete intersterility groups (biological species) in *Pleurotus*, many of which are broadly distributed over one or more continents. Mushrooms recognized as natural and healthy foods originating from an environmentally friendly organic farming system (Moore and Chiu, 2001). To make mushroom cultivation sustainable and highly productive, novel improved strains with improved characteristics are greatly needed. However, mushroom strains are very difficult to discriminate, due to lack of clearly distinguishable characters. This makes strain protection problematic, and impedes strain improvement. Molecular markers of rDNA sequencing, RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), microsatellite and mitochondrial genotypes have all been used to discriminate mushroom species and/or strains of *Agaricus* (Castle et al., 1987; Sonnenberg et al., 1991; Khush et al., 1992; Barroso et al., 2000; Calvo-Bado et al., 2000; Moore et al., 2001; Ramirez et al., 2001), *Auricularia* (Yan et al., 1999), *Ganoderma* (Hseu et al., 1996), *Lentinula* (Chiu et al., 1996), *Stropharia rugoso-annulata* (Yan et al., 2003), and *Volvariella* (Chiu et al., 1995). These technologies provide ways to obtain reliable data for mushroom strain identification and protection. RAPD analysis was first developed to detect polymorphism between organisms, despite the absence of sequence information, to produce genetic markers, and to construct genetic maps (Williams et al., 1990). In this work, RAPD was tested in the eight Indian cultivated species of *Pleurotus* for differentiation of individual species.

2. Materials and methods:

2.1 Species studied:

The material used for RAPD analysis consisted of eight *Pleurotus* dikaryotic cultures P. *florida* – P1, *P. membraneceus* – OE128, *P. sajor-caju* – PL1140, *P. djamor* – X375, *P. cystidiosus* – P19, *P. flabelletus* – PL50, *P. sapidus* – PL40 and *P. ostreatus* – 1803 were purchased from National Research Centre for Mushroom, ICAR, Chambaghat, Solan, India. The cultures were maintained in potato dextrose agar (PDA) media for further use.

2.2 Isolation of genomic DNA:

Petri dishes containing PDA were inoculated with each species and incubated at 22ºC for 7 days. Next, 4 discs (7 mm diameter) were cut with a sterile cork borer, from the border of the mycelial colony and placed (using a sterile scalpel) into 250 ml flask

containing 100 ml of PDA broth and incubated at 22ºC for 12 days. When species colonized the surface of the flask, the mycelia were harvested from the liquid by being drained through a funnel containing Whatman No. 1 filterpaper. The mycelia were frozen in liquid nitrogen and ground to power in a mortar (Lewinsohn et al., 2001). DNA isolation used SDS as lysis buffer (3% SDS, 1% 2marcaptoethanol, 50 mM EDTA, 50 mM Tris/HCl pH 7.2) and phenol/chloroform/isoamyl alcohol (25:24:1) as extractant. The DNA was precipitated with 0.1 volume of 3 M sodium acetate and 0.6 volume of isopropanol, washed with 70% ethanol and resuspended in sterile double distilled water (Zervakis et al., 2004). The purity and quality of genomic DNA was determined spectrophotometrically and confirmed by use of 1.2% agarose against a known concentration of unrestricted lambda DNA. Standard 30 µg/ml DNA working solution were formulated for each sample in sterile double distilled water. DNA stock solution was kept in freezer for further test (Lewinsohn et al., 2001).

2.3 RAPD-PCR analysis:

Amplification reactions were performed in a final volume of 25 µl containing 10 ng of genomic DNA. The reaction solution consisted of 200 µM each of dATP, dCTP, dGTP and dTTP (Sigma) 50 µmol oligonucleotide primer (Sigma) and 2 units *Taq* polymerase (Fermentas) in 10 mM Tris pH 8.3, 2 mM $MgCl_2$, 0.001% gelatin, 0.05% Tween 20, 50 mM KCl. Amplification was performed in Applied Biosystems 2720 thermal cycler: one cycle at 94ºC for 5 min, 39.5ºC for 1 min, 72ºC for 1 min, and 44 cycles at 94ºC for 1 min, 39.5ºC for 1min and 72ºC for 1 min. The final synthesis was performed at 72ºC for 5min (Zervakis et al., 2001). Total ten primers were used for the amplification. Positive results obtained against three primers were chosen for the final amplification. The amplified fragments were resolved on a 1.2% agarose gel, run under standardized conditions, and stained by ethidium bromide. A 100 bp ladder DNA marker (Fermentas) was used as a size standard. The size of the obtained DNA was calculated against the ladder.

2.4 Data scoring and analysis:

The gels were scored for presence or absence of reproducible bands. Following Lynch and Milligan (1994), each band was regarded as a locus with two alternative alleles: present (1) or absent (0). The identification of 76 scorable bands led to the construction of 8 isolates x 76 loci data matrix, which was analyzed for diversity between populations (Lewinsohn et al., 2001). A binary matrix combined all the data records for all strains used in this study from all three primers. The matrix was then used as input for the prerelease version 0.9.1.50 of *FreeTree* software package with the similarity coefficient set to Dice. Clustering was performed by bootstrap neighbor joining (NJ) method and the tree was constructed by using the software TreeView (Win 16) 1.4.

3. Results and discussion:

The RAPD-PCR reaction was setup with eight Indian cultivated species of *Pleurotus* using the different ten-mar primers (Table 1). The amplifications were carried out twice to check for reproducibility. Occasionally, the intensity of some bands were reduced or increased slightly, but the total number of bands obtained with a primer remains the same. A negative control without the *Pleurotus* genomic DNA template was kept for amplification along with the *Pleurotus* genomic DNA with its primer. This was to confirm the quality of the primer and to avoid the scoring of bands which may arise due to primer for possible contaminants. Among the 10 primers scanned, 3 were chosen for amplifying genomic DNA of all the eight *Pleurotus* species. Primer OPA-4 amplified the lowest number of scorable bands and primer OPD-07 amplified the highest number of scorable bands. The third primer OPD-11, which also gave almost similar scorable bands like OPD-7. The primer OPA-4 produced bands ranging between 1.2 to 0.18 kb in size except *P. membranaeceus* showed no visible band with this RAPD primer. OPD-7 showed 1-5 bands with size ranging from 0.9 kb to 0.12 kb. *P. sapidus, P. flabelletus, P. sajor-caju* and *P. florida* showed 4 bands but they differed from each other by at least one band position. In case of OPD-11, 3-4 bands appeared in each of the seven *Pleurotus* species (ranging 1.1 kb to 0.14 kb), but only one band (0.9 kb) appeared in case of *P. ostreatus*. In total three primers yielded 76 scorable bands ranging from 1.2 kb to 0.14 kb for all eight *Pleurotus* species. All the three primers showed the specific RAPD profile. Primer OPD-7 and OPD-11 gave the most number of RAPD fingerprints and by using these two primers all the eight *Pleurotus* species can be differentiated individually.

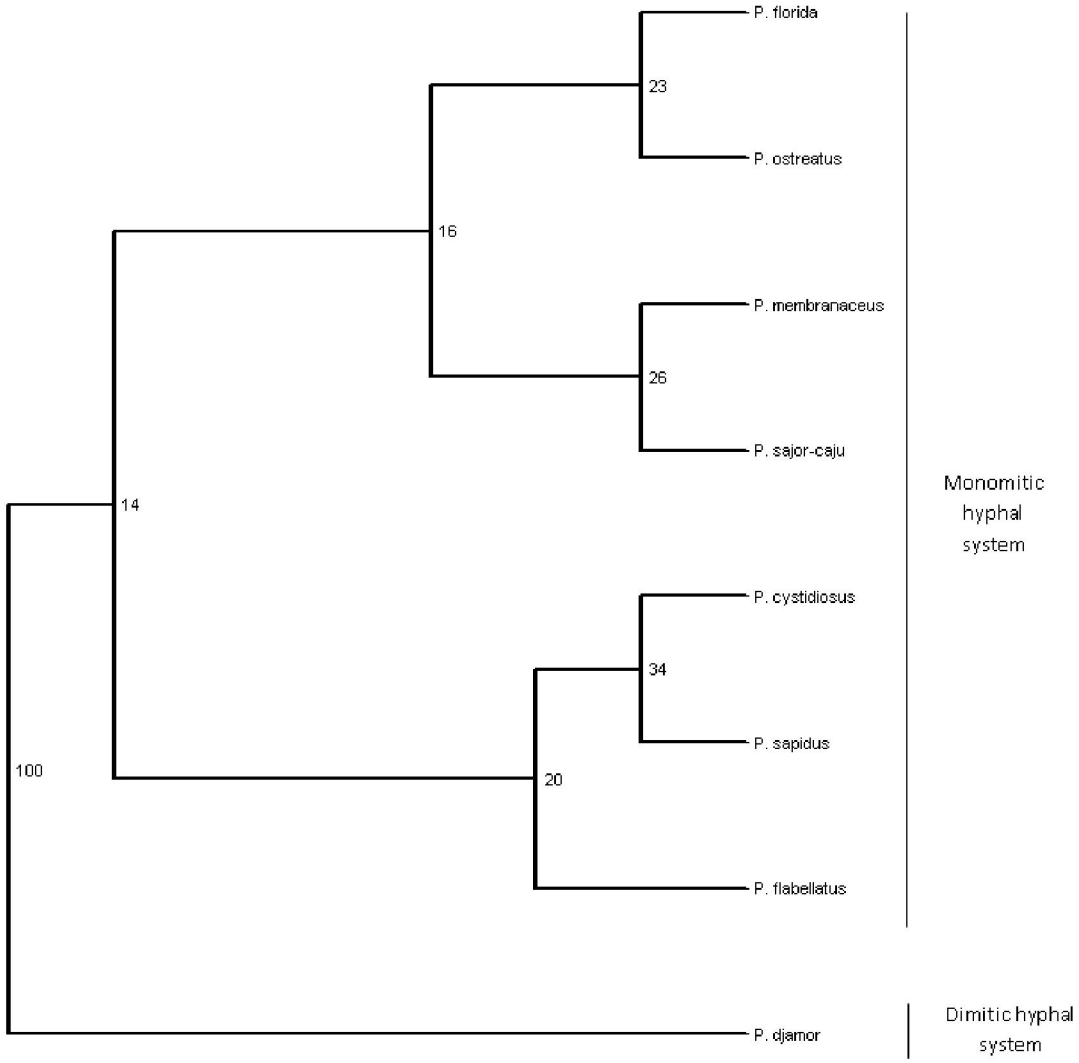

Figure 1. Consensus tree obtained by the bootstrap neighbour-joining method from the RAPD analysis.

Table 1: primer name and sequences:

PRIMERS	SEQUENCES	POLYMORPHISM
OPA-04	5' AATCGGGCTG 3'	+
OPA-16	AGCCAGCGAA	-
OPA-20	GTTGCGATCC	**X**
OPB-7	GGTGACGCAG	**X**
OPB-12	CCTTGACGCA	**X**
OPC-4	CCGCATCTAC	**X**
OPC-15	GACGGATCAG	**X**
OPD-07	TTGGCACGGG	+
OPD-11	AGCGCCATTG	+
OPD-20	ACCCGGTCAC	-

+ With polymorphism; - one or two species showed amplification; **X** without amplification.

Coefficients of genetic similarity were calculated form paired comparison of the eight species, based on normalized identity of each locus in each of species (Nei, M., 1978). The results are given in Table 2. The mean value of similarity coefficient was 0.357 (range 0.111 to 0.727). The highest similarity coefficient was obtained 0.727 between *P. cystidiosus* – P19 and *P. sapidus* – PL40 and lowest similarity coefficient was obtained 0.111 between *P. flabelletus* – PL50 and *P. ostreatus* – 1803. The cladogram produced by 8 RAPD phenotypes was evaluated by Bootstrap method and are presented in figure 1. Phylogenetic study revealed that species of *Pleurotus* having monomitic hyphal system might be originated from the *Pleurotus* having dimitic hyphal system (Figure 1). Previous phylogenetic studies have given rise to various ambiguities in the genus *Pleurotus*. According to the molecular markers used, *P. ostreatus*, *P. colombinus* and *P. cornucopiae*

have been in turn associated in the same clade (Iraçabal et al., 1995) or clearly separated (Zervakis et al., 1994), and the position of *P. sapidus* has remained uncertain (Zervakis and Balis, 1996).

Table 2: Similarity matrix of eight species of *Pleurotus*.

	P. flo	*P. mem*	*P. saj*	*P. dja*	*P. cys*	*P. fla*	*P. sap*	*P. ost*
P. flo	1.000							
P. mem	0.375	1.000						
P. saj	0.273	0.375	1.000					
P. dja	0.300	0.286	0.300	1.000				
P. cys	0.476	0.400	0.476	0.421	1.000			
P. fla	0.500	0.444	0.250	0.364	0.696	1.000		
P. sap	0.435	0.353	0.435	0.476	0.727	0.560	1.000	
P. ost	0.250	0.200	0.125	0.143	0.133	0.111	0.118	1.000

P. flo: *P. florida* – P1; ***P. mem:*** *P. membranaeceus* – OE128; ***P. saj:*** *P. sajor-caju* – PL1140; ***P. dja:*** *P. djamor* – X375; ***P. cys:*** *P. cystidiosus* – P19; ***P. fla:*** *P. flabelletus* – PL50; ***P. sap:*** *P. sapidus* – PL40 *and* ***P. ost:*** *P. ostreatus* – PO1803.

The RAPD technique has also been successfully used to distinguish other genera of cultivated mushrooms, such as for the discrimination of different strains of *Agaricus bisporus* (Khush et al., 1992, Moore and Chiu, 2001), *Ganoderma lucidum* complex (Hseu et al., 1996), *Lentinula edodes* (Chiu et al., 1996), and for the identification and genetic evaluation of single-spore progenies of *Agaricus bisporus* (Calvo-Bado et al., 2000) and *Stropharia rugoso-annulata* (Yan et al., 2003). Therefore, the RAPD genetic variability in *Pleurotus* mushroom will also provide useful information for breeding of commercial strains.

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