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Genetic Diversity Analysis of *Pleurotus* spp. in Himachal Pradesh Using RAPD Fingerprints

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A B S T R A C T

Random amplified polymorphism DNA (RAPD) analysis was done to assess the diversity among 21 species/strains of *Pleurotus*. The morphologically similar species/ strains too gave a new account of the evolutionary process and taxonomy of mushrooms. A total of 150 10 mer primers were screened, out of which 10 primers viz. OPD-03, OPD-05, OPD-08, OPA-13, OPA-16, OPQ-15, OPQ-16 and OPQ-18, S-1461 and S-1462 produced consistent polymorphic banding pattern. The RAPD dendogram obtained by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) programme revealed a high genetic diversity among the isolates of *Pleurotus*. Twenty one isolates were divided into three clusters using 16 per cent similarity as a cut-off point. The cluster I accommodated 17 isolates of different species, whereas cluster III contained one strain of *P. fossulatus* I (P8) and two strains of *Pleurotus* sp. III (P15) and *Pleurotus* sp. IV (P18). RAPD bands were scored as present (1) or absent (0) for all the *Pleurotus* isolates. Each band was assumed to represent a unique genetic locus. The pattern and extent of RAPD variation were analysed with respect to primer, polymorphic locus and isolate. Total number of amplified fragment and polymorphic fragment produced by 10 decamer primer was 141 and 109, respectively with a polymorphism percentage of 77.30.

Introduction

Representatives of genus *Pleurotus* form a heterogeneous group of edible species of high commercial importance (Zervakis *et al.*, 2004). However, there are many problems in taxonomy of *Pleurotus* spp. which are still unresolved. The concept of naming species on the basis of morphological characteristics has been dominant in the fungal taxonomy. However, morphological features of higher fungi are inconsistent as they are strongly influenced by cultivation substrate and environmental conditions (Bresinsky *et al.*, 1976). Consequently, different taxonomists have given different concepts and conclusions for the same taxon on the basis of morphological features.

According to Zervakis and Balis (1996), taxonomic confusion in *Pleurotus* spp. has mainly been due to initial misidentification, absence of type specimens and instability of morphological characters due to environmental changes. In the recent years, biochemical, molecular techniques and mating

compatibility tests have been used to solve the taxonomic problems within genus *Pleurotus.*

Molecular markers being enormous in number have a property of not being affected by the environment; make them a useful tool for understanding phylogenetic relationships as well as taxonomic identification. Molecular phylogenetic studies in mushrooms have been largely based on Restriction fragment length polymorphism (RFLP) and Random amplified polymorphic DNA (RAPD). DNA fingerprinting offers reproducible and reliable genetic differentiation of isolates into species and their strains (Braithwaite, 1989; Monastyrskii *et al.*, 1990). Khush *et al.*, (1992) studied DNA amplification polymorphism in *Agaricus bisporus* and identified seven distinct genotypes among eight heterotrophic strains using RAPD markers. But, very less amount of such work has been done in *Pleurotus* in North western Himalayan region s of India. Thus the present study was proposed with the objective of *Pleurotus* species/ strain identification using RAPD markers.

Materials and Methods

i) Collection, isolation and maintenance of pure culture

Various species/strains of *Pleurotus* were collected/procured from different sources. Majority of the species/strains were collected from the natural habitat during surveys conducted in different localities of Himachal Pradesh during monsoon months. Some of the species were procured from NRCM Solan.

Isolations from the fresh specimen, collected from the wild were made following the standard tissue culture technique (Gamborg, 2002). The stock cultures were maintained in the refrigerator at 4^{o}C. Sub-culturing of the stock cultures was done after a period of 7-10 days on fresh Yeastal Potato Detrose Agar slants (Table 1).

Molecular characterization

Extraction of genomic DNA

Total genomic DNA of each isolate was extracted following the standard procedure (Sharma *et al.*, 2005). The amount of DNA was quantified by recording the absorbance at 260 nm wavelength using UV/VIS Spectrophotometer (BioRad Smart Spec 3000). DNA was stored at -20^{o}C for further use.

Assessment of genetic diversity by RAPD analysis

Random amplified polymorphic DNA (RAPD) based fingerprinting was used to study variation in *Pleurotus* species/strains.

Primer screening

One hundred and fifty 10-mer primers (Operon Technologies Inc. Almedea, USA and Life Technologies, India Pvt. Ltd.) were screened twice with two randomly selected isolates of *Pleurotus* to select primers showing maximum polymorphism with consistent banding pattern. Ten most polymorphic and reproducible primers *viz.* OPD-03, OPD-05, OPD-08, OPA-13, OPA-16, OPQ-15, OPQ-16, OPQ-18, S-1461 and S-1462 were finally used in RAPD analysis.

PCR amplification

The PCR amplification was carried out in 0.2 ml PCR tubes with 25 µl reaction volume containing 2.5 µl of 10 x buffer (20 mM Tris HCl, pH 8.0, 50 mM KCl); 1.5 µl of $MgCl_2$ (25 mM $MgCl_2$), 2.0 µl of dNTP's (2.5 mM each) (Eppendorf, India Pvt. Ltd.), 1.0 µl primer, 0.2 µl of Taq polymerase (Bangalore

Genei, India, 5U/µl), 2 µl of DNA template and 15.8 µl of sterilized distilled water to make total reaction volume of 25 µl. Reaction mixture was vortexed and centrifuged in a microfuge (Bangalore Genei, India) for the proper mixing of the contents. Amplifications were performed using thermal cycler (Gene Amp PCR System 9700, Applied Biosystems, USA) programmed with initial denaturation at 94^{o}C for 5 minutes, followed by 40 cycles at 94^{o}C for 1 minute, 37^{o}C for 1 minute, 72^{o}C for 2 minutes and a final extension at 72^{o}C for 5 minutes.

Gel electrophoresis

The amplified PCR products were resolved by electrophoresis using 1.2 per cent agarose gel in 0.5X Tris borate EDTA buffer (0.5 M Tris, 0.05 M boric acid and 1 mM EDTA, pH 8.0). The gels were stained with 0.5 µg/ml of Ethidium bromide. 100 bp DNA ladder (Biobasic, Lifetech, India Pvt. Ltd.) and lambda DNA / *EcoR* / Hind III double digest (MBI Fermentas) were used as markers. The gels were run at 80 V for two hours (Bangalore Genei System) and were viewed under the gel documentation system (AlphaImager 2200, Alpha Infotech Corporation, and USA) and scored.

DNA bands that could be scored univocally for presence (1) and absence (0) were included in analysis. Binary matrices were analysed by NTSYS pc V 2.0 (Rholf, 1998) and Jaccard's coefficient was used to construct dendrogram using SHAN clustered programme, selecting the unweighted pair group arithematic mean (UPGMA). The dendrogram with best fit for to similarity matrix based on cophentic value (COPH) and matrix comparison (MXCOMP) was chosen.

Results and Discussion

DNA based markers have increased the potential to study the genetic diversity of various fungal isolates of same or different species, as these markers are not affected by the environment. In the present study, RAPD markers were used to determine the genetic diversity among *Pleurotus* isolates. RAPD analysis revealed the existence of high genetic diversity among 21 *Pleurotus* isolates. Cluster analysis of RAPD data divided test isolates into three major and five sub-clusters.

RAPD analysis of *Pleurotus* species/strains

Random amplified polymorphic DNA (RAPD) based fingerprinting was used to study variation in *Pleurotus* species/strains. Initially 150 primers were used for the amplification of two randomly selected isolates of *Pleurotus* with a view to screen primers exhibiting maximum polymorphism.

Ten 10-mer primers viz. OPD-03, OPD-05, OPD-08, OPA-13, OPA-16, OPQ-15, OPQ-16 and OPQ-18 (Operon Technologies Inc. Almedea, USA), S-1461 and S-1462 (Life Technologies, India Pvt. Ltd.) produced consistent polymorphic banding pattern with 11-17 bands of 0.3-3.0 kb (Table 2). Finally these 10 primers were used for RAPD analysis of 21 *Pleurotus* species/strains.

The number of scorable and polymorphic bands obtained with each primer ranged from 11-17 and 9-14, respectively (Table 2). The maximum polymorphism was found with primer OPD-05 (87.5%) followed by S-1462 (85.71%), S-1461 (81.81%) and OPQ-16 (80.00%). Among 141 scorable bands, 109 were polymorphic with 77.30 per cent polymorphism.

Cluster analysis of scorable RAPD bands generated a dendrogram revealing high genetic diversity in *Pleurotus* species/strains. Twenty one isolates were divided into three clusters using 16 per cent similarity as a cut-off point.

Table.1 Source of collection of various *Pleurotus* species / strains

SOURCE	NAME	SPECIES /STRAINS
Collection from wild	P11	*Pleurotus sp.*II
	P5	*Pleurotus cystidiosus* I
	P21	*Pleurotus ostreatus* IV
	P3	*Pleurotus flabellatus* II
	P4	*Pleurotus cornucopiae*
	P12	*Pleurotus cystidiosus* II
	P6	*Pleurotus pulmonarius*
	P8	*Pleurotus fossulatus* I
	P10	*Pleurotus fossulatus* II
	P18	*Pleurotus sp.*IV
	P19	*Pleurotus sp.*V
	P20	*Pleurotus ostreatus* III
	P7	*Pleurotus* sp.I
	P15	*Pleurotus sp.*III
	P17	*Pleurotus eryngii* II
NRCM, Solan		
	P1	*Pleurotus sapidus*
	P2	*Pleurotus flabellatus* I
	P9	*Pleurotus florida*
	P13	*Pleurotus ostreatus* I
	P14	*Pleurotus eryngii* I
	P16	*Pleurotus ostreatus* II

Table.2 Number of scorable and polymorphic RAPD bands obtained by PCR amplification of DNA of *Pleurotus* species/strains with primers showing polymorphism

Primers	Scorable bands	Polymorphic bands	Per cent polymorphism
OPD-03	17	12	70.5
OPD-05	16	14	87.5
OPD-08	14	10	71.4
OPA-13	13	10	76.92
OPA-16	14	10	71.42
OPQ-15	12	9	75.00
OPQ-16	15	12	80.00
OPQ-18	15	11	73.33
S-1461	11	9	81.81
S-1462	14	12	77.30
Total	**141**	**109**	**77.30**

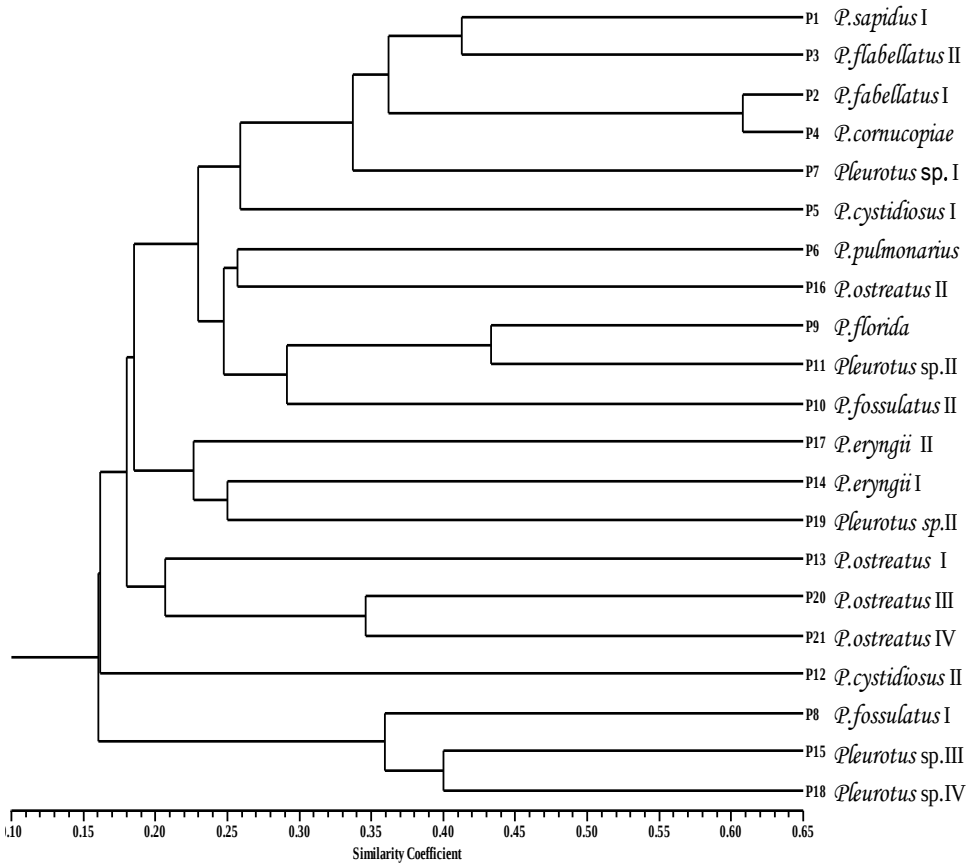

FIG. 4.1 : Dendrogram of 21 isolates of *Pleurotus* spp. generated by UPGMA (Unweighted pair group method arithmetic mean) analysis with 10 RAPD primers.

The cluster I accommodated 17 isolates of different species, whereas cluster III contained one strain of *P. fossulatus* I (P8) and two strains of *Pleurotus* sp. III (P15) and *Pleurotus* sp. IV (P18). Besides this cluster II possessed only one strain of *P. cystidiosus* (P12) as shown in figure 1. The cluster I was sub-divided into two sub-clusters I_a and I_b at 18 per cent similarity. The sub-cluster I_a included 14 isolates and sub-cluster I_b contained three strains of *P. ostreatus* (P13, P20 and P21) (Fig. 1). However, RAPD was unable to differentiate the various *Pleurotus* species into distinct clusters. There was no congruence between RAPD and morphological groupings of the test isolates. Similar diversity in different Agaricales including *Pleurotus* has also been reported by many workers (Khush *et al.,* 1992; Liu *et al.,* 1995; Singh *et al.,* 2000; Lewinsohn *et al.,* 2001). Singh *et al.,* (2000) observed wide variation among different genera of Agaricales and also within *Pleurotus* species and strains using RAPD analysis. Lewinsohn *et al.,* (2001) observed 68 and 32 per cent genetic diversity in twelve populations (144 isolates) of *Pleurotus eryngii* using RAPD markers. However, Stajic *et al.,* (2005) categorized 37 strains of ten *Pleurotus* species using RAPD into six clusters and concluded that morphology does not necessarily coincide genetics. In our study, 10 mer primers OPD-

03 and OPA-13 amplified a distinct 500 bp fragment in all the isolates of *Pleurotus cystidiosus* and *P. eryngii*. Whereas 2350 bp fragment was noticed only in *P. ostreatus* II isolate with primer OPA-16, thus differentiating them from other species/strains. Similar distinction of *Pleurotus* species using RAPD fingerprint has also been reported by Marongiu *et al.*, (2001) who found a 1200 bp fragment only in *P. eryngii* samples collected from host plant *Ferula communis* but not in those collected from *Eryngium* species. However, Lee *et al.*, (2000) observed a 600 bp fragment in all *P. ostreatus* isolates using RAPD markets.

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