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Genetic Diversity Characterization of *Pleurotus* strains by Random Amplified Polymorphic DNA Fingerprinting

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Wild edible mushroom are the special product of the forest. The diversity of mushroom and their natural beauty inhabit major threat in the biological world and India has been a frame for these fungi. The previous attempts for genetic improvement in mushrooms had little success due to low genetic diversity amongst commercially cultivated mushrooms strains and non- inclusion of wild collections in the breeding programs. This limited availability of genetic variation significantly slowed down the progress of genetic improvement in this commercially most important mushroom. The present investigation of genetic diversity of *Pleurotus* species were ranged from 30 to 70%. The Cluster analysis were using Unweighted Pair-Group Method with Arithmetic Mean (UPGMA), clearly separate dendrogram was obtained from the molecular marker systems for all the eight *Pleurotus* species. Clustering on the basis of Random Amplified Polymorphic DNA (RAPD) marker using Jaccard's coefficient generated two (I and II) broad groups of six and two *Pleurotus* species. Clusters also confirmed 70% of genetic diversity among the *Pleurotus* species.

Introduction

The use of wild mushrooms for food in all probability began with the prehistoric man. During the long period human as a hunter gathered the fungi of the forest that has served as an important source of nourishment. There are many edible mushrooms *i.e.* Volvarias, Polypores and tubers fungi that used ethno-botanical food by the tribal of forest regions. These are obviously non toxic as these have been in intimate human consumption by native and tribal, since antiquity (Pandey and Srivastava, 1994). However, the food value and acceptance of these edible fungi by the scientific and civilized world have not been recognized. These fungi are more important for a tropical/ subtropical country like India, which has a climate most congenial for the natural growth of such fungi (Purkayastha and Chandra, 1985). Describing the number of fungi on earth has been a position of conversation and several studies have focused on enumerating the world's fungal diversity (Crous *et al.,* 2006). Only a part of total fungal wealth has been subjected to scientific

study and mycologists continue to disentangle the unknown and hidden wealth one third of fungal diversity of the globe exists in India and of this only 50 % are characterized until now (Manoharachary *et al.,* 2005). Mushrooms have been extensively studied in the Western countries, while tropical countries like India especially in Vindhya forest of Northern India. However no determined efforts have been made for a detailed study of them. Till today no research have been done on natural mushroom flora, further attempt on isolation, characterization and maintenance of cell mycelial culture both *in vitro* and *ex vitro* yet to carry out. Mean while in Vindhya region several mushrooms have been reported and described on morphological basis by the various workers (Rahi, 2001).

The main objectives was to typical diversity of fleshy fungi, characterize, collect preserve and evaluate edibility of these different species in dry deciduous forest of Vindhya region of Northern India. RAPD fingerprinting has been used for genetic diversity analysis, varietal identification and strain protection in various field crops. RAPD markers have also been successfully used to detect genetic variation in a wide variety of fungal species including mushrooms.

Materials and Methods

Random amplified polymorphic DNA (RAPD) molecular marker

Some of the collected different strains of edible mushroom were characterized by RAPD analysis by the modified method (Yadav *et al.,* 2003). Random amplified polymorphic DNA (RAPD) markers were used for evaluation of genetic diversity in selected group of *Pleurotus*s trains. Six RAPD primers used and all the strains were distinguished using aggregated RAPD data. This study indicates that RAPD analysis is a sensitive and powerful tool for assessment of genetic variation at DNA level among *Pleurotus* strains.

DNA isolation and purification

Genomic DNA from fruit body was isolated by CTAB modified method (Dellaporta *et al.,* 1983).

DNA extraction solutions

Isolation buffer 20ml

2% w/v CTAB (Cetyl Tri-methyl Ammonium Bromide)	0.30ml
1.4 M NaCl	1.638g
20 mM EDTA (pH 8.0)	0.148g
100 mMTrisHCl (pH 8.0)	0.315g

Note

0.2%mercaptoethanol was added immediately before use.

Phenol : Chloroform: iso-amyl alcohol (PCI)-25:24:1

Chloroform : iso-amyl alcohol (CI)-24:1

100% iso-propanol : 0.30ml

Washing solution : 70% ethanol, 10mM ammonium acetate

TE buffer

10mM TrisHCl	0.029g
1mM EDTA (pH 8.0)	0.037g

3 M ammonium acetate 2.312g

DNA isolation protocol

100mg fruit body of mushroom were quick freeze in liquid nitrogen (-196^{0} C) and grind to a fine powder in pre-chilled autoclaved

mortar and pestle. Powder was transferred to a sterile 2 ml centrifuge tube. Thawing was carefully avoided and 1000 µl of extraction buffer was added and mixed thoroughly. The centrifuge tubes were incubated for 30 minutes at 60^0C in water bath with intermittent swirling at every 10 minutes. One volume of phenol: chloroform: iso-amyl alcohol (25:24:1) was added into the tubes, capped and extracted for 10 minutes on a rotary shaker. Mixing was done gently but thoroughly to ensure emulsification. The tubes were centrifuged at 10 K rpm for 10 minutes. Depending upon the purity of DNA preparation; the upper aqueous phase was extracted 2-3 times with fresh chloroform: iso-amyl alcohol (24:1). The final aqueous phase was transferred to other centrifuge tubes. To these, 0.6 volume of ice cold iso-propanol was added and mixed gently by inverting the tube. At this stage, DNA-CTAB complex was found to precipitate as a whitish matrix. The pellet was gently agitated for few minutes and collected by centrifugation at 4°C). Residual CTAB buffer was removed in this step. The tubes were inversed and drained on a paper towel for 1hr with care so that the pellet does not slip down. Pellet was neither containing ethanol nor was too dry. An appropriate (50µl) volume of TE buffer was used to dissolve the pellet.

Purification of DNA

To remove the RNA, RNase A @ 10µg/ml was added into the DNA solution and incubated at 37°C for 30 minutes. Equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) was added into it and centrifuged at 10000 rpm for10 minutes. Aqueous phase was taken and equal volume of chloroform: iso-amyl alcohol (24:1) was added and centrifuged at 10000 rpm for 10 min. To the aqueous phase, $1/20^{th}$ volume of Na acetate (3M, pH-5.2) and 2.5 volume of ethanol was added and incubated at -20°C for 1h (or-70°C for 30 min). Then the solution was centrifuged at10000 rpm for 10 min, pellets were washed with 70% ethanol (10000 rpm, 5 min), air dried and dissolved in distilled water. Yield of DNA was estimated using DNA markers by electrophoresis.

PCR amplification

PCR amplification was performed by Williams *et al.,* (1990). PCR amplification was carried out in 0.2 ml thin-wall PCR tubes using an Eppendorf (model AG 22331 Hamburg) thermal cycler. A total of 6 RAPD primers were screened in our present study (6 primers from kit S) was amplified. 6 primers (viz. S43, S1017, S159, S485, S75 and S24) could produce unambiguous polymorphic bands and was selected for the present study. The preparation of Polymerase chain reaction (PCR) mixture of 25 µl was in table 1. PCR cycle conditions were as follows: initial denaturing step at 94°C for 3 min followed by 44 cycles of 94°for 1 min, 37°C for 1 min and 72°C for 2 min. In the last cycle, primer extension at 72°C for 7 min was provided.

Gel electrophoresis and documentation

PCR products were electrophoretically separated on a 1.2% Agarose gel containing Ethidium Bromide using 1X TAE buffer (pH 8.0). Agrose gel 0.480g was dissolved in 40ml 0f 1X TAE buffer and boiled. After that add 2 µl of EDTA dye mixed and poured in plate of gel. After that solidify the gel were kept in gel electrophoresis instruments; load the samples and Ladder and switch on the electric charge for running gel electrophoresis.

The amplified products were visualized and photographed under UV light source. As molecular marker, 100 bp DNA Ladder Plus (ready-to-use) (Fermentas Inc.) was used. The ranges of fragments were measured in this

particular molecular weight marker varied from 3000 bp to 100bp.

RAPD data analysis

DNA bands were scored '1' for its presence and '0' for its absence for each primer-genotype combination. Only unambiguous bands were scored for identifying presence and absence of bands. A similarity matrix was constructed, using Jaccard's coefficient, and the resulting similarity data were used to construct a dendrogram, using UPGMA and the NTSYS-pc software version 2.1 developed by Rohlf (1997).

Results and Discussion

Genetic diversity among the collected edible mushrooms (*Pleurotus sp.*) analysis by Random Amplified Polymorphic DNA

Genetic diversity of collected edible mushroom of eight species of *Pleurotus* is revealed by Random Amplified Polymorphic DNA (RAPD). DNA bands were scored '1' for its presence and '0' for its absence for each primer-genotype combination (Fig. 1). Only unambiguous bands were scored for identifying presence and absence of bands. A similarity matrix was constructed, using Jaccard's coefficient, and the resulting similarity data were used to construct a dendrogram, using UPGMA and the NTSYS-pc software version 2.1 developed by Rohlf (1997).

A total 8 *Pleurotus* sp. were tested for their genetic variability by RAPD analysis, using 6 random primers. These primers viz. S43, S1017, S159, S485, S75 and S24 produced easily scorable and consistent banding patterns, which were used for RAPD analysis of test. The amplified products were ranged from 200 bp to 1250 bp. Analysis of the genetic coefficient, derived from the scores of RAPD profile, showed that minimum and maximum % diversities among the *Pleurotus sp.* were ranged from 30 to 70%, respectively (Fig. 2). Cluster analysis, using Unweighted Pair-Group Method with Arithmetic Mean (UPGMA), clearly separate dendrogram was obtained from the molecular marker systems for all the 8 *Pleurotus sp.* Clustering on the basis of RAPD marker using Jaccard's coefficient generated two (I and II) broad groups of six and two *Pleurotus sp.* Clusters also confirmed 70% of genetic diversity among the *Pleurotus sp.* (Fig. 2). Cluster I consisted 6 (PL-1, PL-2, PL-3, Psc-1, Psc-2 and PF-1) and cluster II consisted 2 (PO-1 and PE-1) *Pleurotus* species. Cluster I were further separated in to two sub cluster (A and B) with 67.5% diversity.

Table.1 Detail of polymerase chain reaction (PCR) mixture of 25 µl

S. No.	Component	Quantity
1	Sterile Milli Q water	19.05 µl
2	10 x Taq buffer	2.5 µl
3	d NTPs	0.25 µl
4	Random primer	1.0 µl
5	Taq DNA polymerase	0.2 µl
6	Template DNA	2.0 µl

Fig.1 Genetic diversity of eight *Pleurotus* sp. Analysis by Random Amplified Polymorphic DNA by Gel Electrophoresis

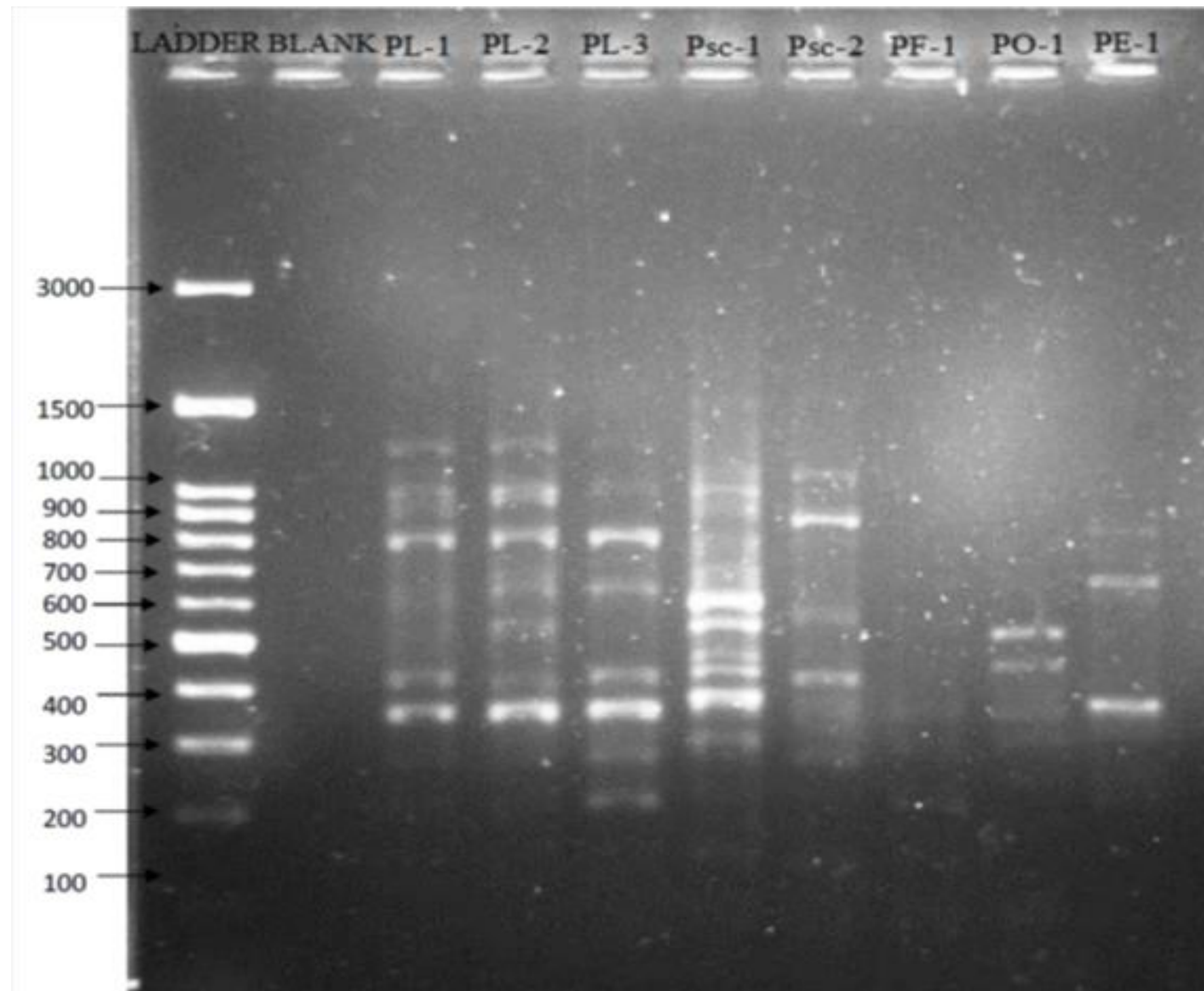
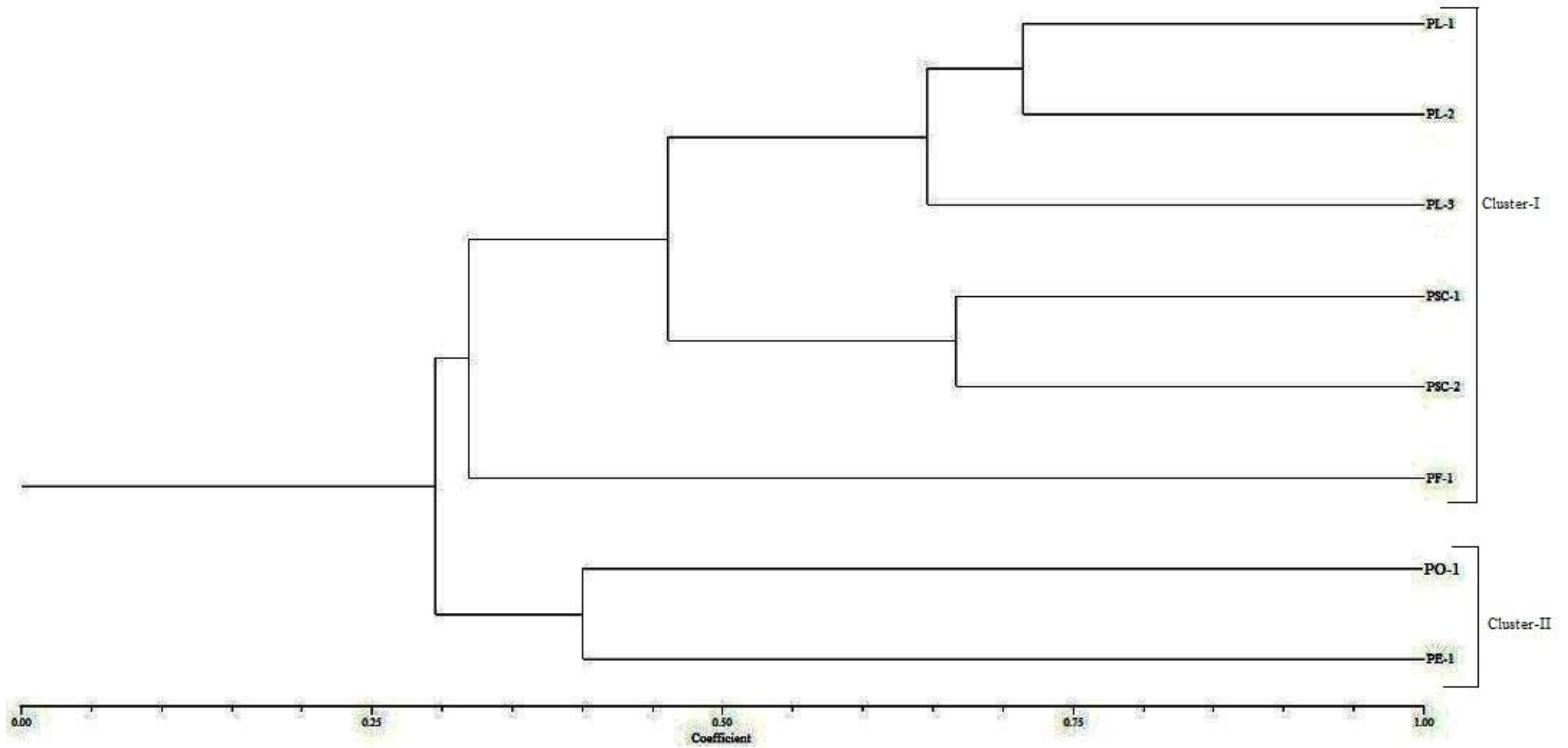


Fig.2 Dendrogram of eight *Pleurotus* sp. analysis of genetic dissimilarities based on random amplified polymorphic DNA data with 6 primers



The sub cluster A was also divided into two groups Aa and Ab with 58.5% diversity. Aa group consisted two *Pleurotus sp.* (Psc-1 and Psc-2) having 35% variability while sub cluster Ab divided into two having 37.5% variability; one cluster consisted only one *Pleurotus sp.*(PL-3) and another consisted two *Pleurotus sp.* (PL-1 and PL-2) with having 30% variability. The sub cluster B was consisted one species PF-1.

The result is confirmative with finding of Yin *et al.,* (2013) who investigated that the molecular markers were used to analyze the genetic diversity of 15 Chinese *P. pulmonarius* cultivars. In total, 21 RAPD primers selected for generating data based on their clear banding profiles produced. With the use of these RAPD primers, a total of 361 RAPD fragments were detected, of which 287 (79.5 %) RAPD fragments were polymorphic. UPGMA trees of these three methods were structured similarly, grouping the 15 tested strains into four clades. Subsequently, visual DNA fingerprinting and cluster analysis were performed to evaluate the resolving power of the combined RAPD markers in the differentiation among these strains.

The confirming result of Theochari *et al.,* (2002) who studies an approach of the biodiversity was achieved by analyzing allelic polymorphism at mating type loci in a sample of *P. ostreatus* isolates, which made evident the variability likely to exist in this biological material.

In conclusion, the genetic diversity of *Pleurotus* species was ranged from 30 to 70%. Cluster analysis, using UPGMA, clearly separate dendrogram was obtained from the molecular marker systems for all the 8 *Pleurotus* species. Clustering on the basis of RAPD marker using Jaccard's coefficient generated two (I and II) broad groups of six and two *Pleurotus* species. Clusters also confirmed 70% of genetic diversity among the *Pleurotus* species.

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