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Genetic Diversity within the Genus *Pleurotus* Determined by RAPD Analysis

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Random amplified polymorphism DNA (RAPD) analysis was done to assess the diversity among ten species of *Pleurotus*. Understanding the pattern of fundamental not only addressing questions concerning evolutionary process and the development of conservation strategies, but also a prerequisite of the efficient use of genetic resources in breeding programme. The RAPD dendogram obtained by using a UPGMA programme grouped in to the investigated strains in to 5 clusters. RAPD bands were scored as present (1) or absent (0) for all the *Pleurotus* isolate. Each band was assumed to represent a unique genetic locus. The pattern and extent of RAPD variation were analyzed with respect to primer, polymorphic locus and isolate. Total number of amplified fragment and polymorphic fragment produced by 40 decamer primer were 229 and 226, respectively. Polymorphism percentage was 98.69. Ten primer SBSA11, SBSA13, SBSA15, SBSA16, SBSA18, SBSA19, SBSA20, SBSB14, SBSB15 and SBSB17 were not amplified the DNA from any of the isolate.

Introduction

Apart from the culinary, nutritional and health benefits of edible mushrooms, its large scale cultivation is now playing an instrumental role in solving one of the main problems facing mankind in the 21th century; the need to feed an ever-increasing population (Gokulpalan and Nair, 1990). Mushrooms have been recognized by Food and Agriculture Organization as food contributing to ameliorate the protein malnutrition in human body in the industrial waste materials; are rich in the protein, minerals and vitamins; and contain an abundance of the essential amino-acid lysine (Thayumanavan and Manikam, 1980). Therefore, mushrooms can be a good supplement to cereals. Development countries like ours need nutrients substitutes into the staple diet of man will essentially increase proteins for human consumption.

The genus *Pleurotus* is a heterogeneous group of several species are having nutritional and medicinal importance (Gunde-Cimerman, 1999 and Guzman, 2000). Some *Pleurotus* spp. Have the ability to absorb microelements from the different cultivation media, and thus

they may present an excellent dietary source (Stajic *et al*., 2002). The interest in the genetic structure of natural population has increased in the last few years owning to the necessity to broaden the knowledge of genetic variation in cultivated species. New approach to the study of genetic variation from wild species to cultivated varieties, mediated by information on molecular markers is promising avenues to exploit wild genetic resource in breeding programme. In fact, despite the economic importance of species in general or fungi in particular, little was known until recently about their natural population and the available genetic variability (Zervakis *et al.*,2004).An attempt was made in the present study to find out the genetic variability present in *Pleurotus* species by RAPD method.

Materials and Methods

DNA isolation

Pleurotus species under study were grown at room temperature on PDA for 15 to 18 days. Mycelium was collected by scrapping. The harvested mycelium (100 mg) was placed in pre-cooled mortar and pestle and liquid nitrogen was added. The mycelium was powdered then this was transferred into an Eppendrop (1.5 ml) tube containing 1 ml CTAB buffer and placed in heating block at 65^0C for 15 minutes. During incubation period, mixed by hand 2 to 3 times and centrifuged it at 13000 rpm for 5 minute. Supernatant was taken out into a fresh Eppendrop tube and 1 ml GN binding buffer was added, mixed by inversion. Then it was transferred to a mini prep spin column with a 2 ml collection tube. Let it kept for 3 minutes. Centrifuged at 13000, rpm for 30 seconds and the flow through was discarded. The rest of the solution was added in to the column and centrifuged for 30 seconds at 13000 rpm. Washing buffer (600 µl) was added and centrifuged for 30 seconds a same rpm, this step was repeated for one more time to remove impurities as much as possible then centrifuged at 13000 rpm for an additional 1 minute to remove the residual washing buffer. Spin column was placed into a 1.5 ml micro tube. T_{10} E_1 buffer (100µl) was added into the center part of the Si Max membrane in the spin column and incubated at room temperature for 3 to 5 minutes and centrifuged at 13000 rpm for 1 minute to elute DNA. The quality of the purified was determined on 1 per cent agarose gel stained with Goldview^{Tm} or ethidium bromide. The purified DNA was stored at 4^0C for further use.

RAPD primer

Random primers were procured from SBS Genetech company Ltd. the list of the primers name and sequences is listed in Table 1.

PCR-Mix

The reaction mixture of 25 µl, each containing primer – 2 µl (50 pm ml^{-1}), dNTP mix -2 µl, $MgCl_2$-1µl, Taq DNA polymerase -1 µl (5 U ml^{-1} Genetech.), 10 X PCR buffer -2.5 µl (100 mM Tris HCL (pH 8.3), and 13.5 ml of H_2O. To this 4 µl genomic DNA was added.

Thermal cycler condition

Amplification was performed in a master cycler with lid heating option at 105^0C with initial denaturation of genomic DNA at 95^0C for 2 min. followed by 35 cycles of template denaturation at 94^0C for 1 min. primer annealing at 36^0C for 45 sec, extension at 72^0C for 2 min and a final extension at 72^0C for 10 min.

Gel electrophoresis

A 200 ml (1XTAE) agarose gel was prepared. For making Gel, 180 ml distilled water + 20 ml TAE buffer (10 X) + agarose 2 g was

added and boiled to dissolved, and then kept it for cooling. The comb in gel casting tray was fixed and agarose solution was poured slowly. It was kept for 30 minutes to solidify the gel. The comb was pulled out. 1kb DNA ladder (2 µl + 2µl TAE) + 2µl 6X loading dye was loaded in first well, in subsequent wells the PCR amplified product (5µl) was loaded. The gel was run in 1X TAE buffer at 60V for 2 hrs. The gel was stained with ethidium bromide for 20 minute.

Visualization of gel

The gel was visualized with gel documentation system (UVI Tek, UK) and the photograph was taken.

Statistical data analysis

RAPD bands were scored as present (1) or absent (o) for all the *Pleurotus* isolates. Each band was assumed to represent a unique genetic locus. Its presence was interpreted as either a heterozygote or dominant homozygote and absence of the band as a recessive homozygote. The pattern and extent of RAPD variation were analyzed with respect to primer, polymorphic locus and isolate. Data entry was done into binary data matrix and statistical analysis was carried out using NTSYS-PC, 2.01 version (Rohlf, 1997). Pair wise comparison of samples was used to estimate Jaccards similarity coefficient. Genetic distances (GD) between pair of lines were estimated at 1-GS. Jaccard's similarity coefficient was used to generate dendogram using unweighted pair group method with arithmetic mean UPGMA (Sneath and Sokal, 1973).

Results and Discussion

All *Pleurotus* species included in this study showed unique banding pattern as observed from amplification band comparisons obtained used by 40 decamer primers. Total number of amplified fragments and polymorphic fragments produced by 40 decamer primers were 229 and 226, respectively.

Table.1 The list of the primers name and sequences used in this study

1.	SBSA-01	CAG GCC CTT C	SBSB-01	GTT TCG CTC C
2.	SBSA-02	TGC CGA GCT G	SBSB-02	TGA TCC CTG G
3.	SBSA-03	AGT CAG CCA C	SBSB-03	CAT CCC CCT G
4.	SBSA-04	AAT CGG GCT G	SBSB-04	GGA CTG GAG T
5.	SBSA-05	AGG GGT CTT G	SBSB-05	TGC GCC CTT C
6.	SBSA-06	GGT CCC TGA C	SBSB-06	TGC TCT GCC C
7.	SBSA-07	GAA ACG GGT G	SBSB-07	GGT GAC GCA G
8.	SBSA-08	GTG ACG TAG G	SBSB-08	TGC CAC ACG G
9.	SBSA-09	GGG TAA CGC C	SBSB-09	TGG GGG ACT C
10.	SBSA-10	GTG ATC GCA G	SBSB-10	CTG CTG GGA C
11.	SBSA-11	CAA TCG CCG T	SBSB-11	GTA GAC CCG T
12.	SBSA-12	TCG GCG ATA G	SBSB-12	CCT TGA CGC A
13.	SBSA-13	CAG CAC CCA C	SBSB-13	TTC CCC CGC T
14.	SBSA-14	TCT GTG CTG G	SBSB-14	TCC GCT CTG G
15.	SBSA-15	TTC CGA ACC C	SBSB-15	GGA GGG TGT T
16.	SBSA-16	AGC CAG CGA A	SBSB-16	TTT GCC CGG A
17.	SBSA-17	GAC CGC TTG T	SBSB-17	AGG GAA CGA G
18.	SBSA-18	AGG TGA CCG TA	SBSB-18	CCA CAG CAG T
19.	SBSA-19	CAA ACG TCG G	SBSB-19	ACC CCC GAA G
20.	SBSA-20	GTT GCG TCG G	SBSB-20	GGA CCC TTA C

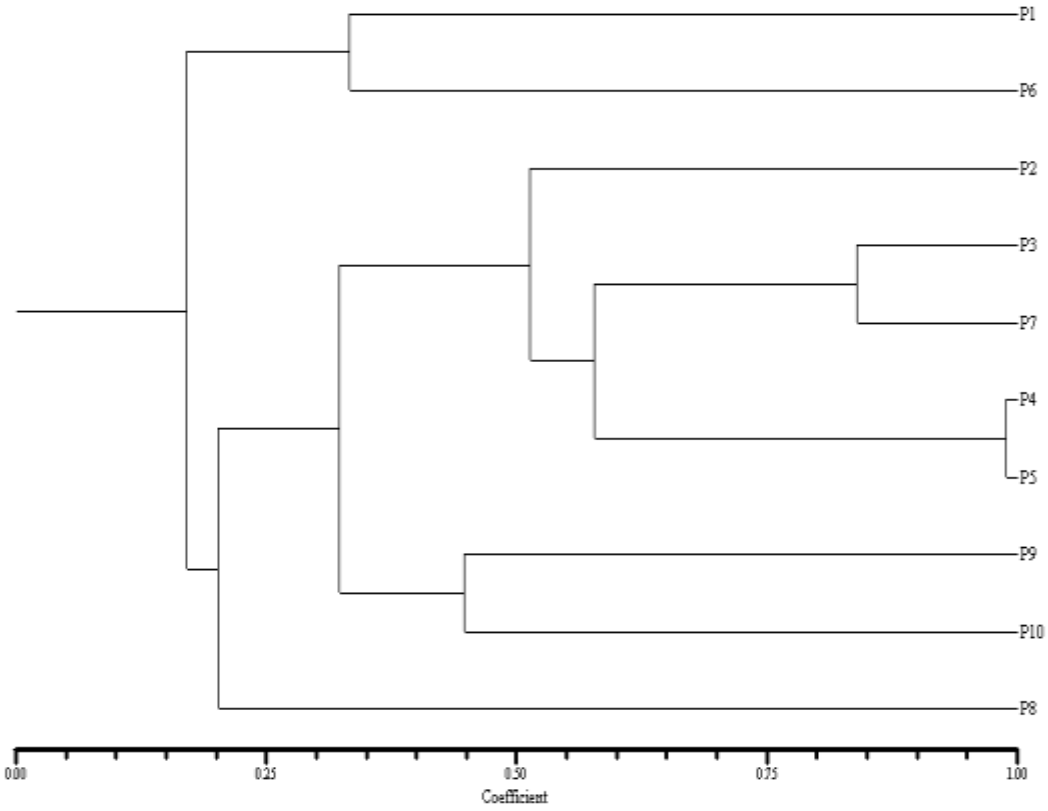
Table.2 List of decamer primer and the polymorphism observed in the study

Sl. No.	Primer	Primer Sequence	Total no. of bands	No.of Polymorphic bands	Polymorphism Per cent	Isolate Distinguished
1.	SBSA-01	CAG GCC CTT C	7	7	100	P1(325,500 bp)
2.	SBSA-02	TGC CGA GCT G	8	8	100	P5(225, 675 bp) and P8(300 bp)
3.	SBSA-03	AGT CAG CCA C	9	9	100	P6(350 bp)
4.	SBSA-04	AAT CGG GCT G	8	6	75	P8(400 bp)
5.	SBSA-05	AGG GGT CTT G	9	9	100	P6(575 bp)
6.	SBSA-06	GGT CCC TGA C	8	8	100	P6(740 bp) and P8(500, 625 bp)
7.	SBSA-07	GAA ACG GGT G	10	10	100	P2(450 bp) and P8(750 bp)
8.	SBSA-08	GTG ACG TAG G	8	8	100	P6(500,800 bp);P9(650 bp) and P10(375 bp)
9.	SBSA-09	GGG TAA CGC C	5	5	100	P1(450,775 bp);P6(730,925 bp) and P8(425 bp)
10.	SBSA-10	GTG ATC GCA G	8	8	100	P8(475 bp)
11.	SBSA-11	CAA TCG CCG T	-	-	-	-
12.	SBSA-12	TCG GCG ATA G	10	10	100	P1(690bp);P7(375,650bp)and P9(250,675 bp)
13.	SBSA-13	CAG CAC CCA C	-	-	-	-
14.	SBSA-14	TCT GTG CTG G	7	6	85.7	P10(525,600 bp)
15.	SBSA-15	TTC CGA ACC C	-	-	-	-
16.	SBSA-16	AGC CAG CGA A	-	-	-	-
17.	SBSA-17	GAC CGC TTG T	8	8	100	P4(300,525,600bp);P8(850bp)and P9(650,1000 bp)
18.	SBSA-18	AGG TGA CCG T	-	-	-	-
19.	SBSA-19	CAA ACG TCG G	-	-	-	-
20.	SBSA-20	GTT GCG TCG G	-	-	-	-
21.	SBSB-01	GTT TCG CTC C	6	6	100	P6(750 bp) ; P9(250 bp)
22.	SBSB-02	TGA TCC CTG G	8	8	100	P1(490 bp);P6(1000 bp)
23.	SBSB-03	CAT CCC CCT G	5	5	100	-
24.	SBSB-04	GGA CTG GAG T	8	8	100	P10(625 bp)
25.	SBSB-05	TGC GCC CTT C	7	7	100	P8(675 bp)
26.	SBSB-06	TGC TCT GCC C	9	9	100	P6(375 bp);P8(650 bp)
27.	SBSB-07	GGT GAC GCA G	6	6	100	-
28.	SBSB-08	TGC CAC ACG G	9	8	88.8	P6(750 bp); P8(625 bp)
29.	SBSB-09	TGG GGG ACT C	7	7	100	P2(300bp) and P9(750 bp)
30.	SBSB-10	CTG CTG GGA C	7	7	100	P19275 bp); P8(250bp) and P9,P10(625 bp)
31.	SBSB-11	GTA GAC CCG T	8	8	100	P9(375 bp)
32.	SBSB-12	CCT TGA CGC A	8	8	100	P3(750 bp); P8(300,990 bp); P9(550 bp)
33.	SBSB-13	TTC CCC CGC T	7	7	100	P9(900 bp) and P10(450 bp)
34.	SBSB-14	TCC GCT CTG G	-	-	-	-
35.	SBSB-15	GGA GGG TGT T	-	-	-	-
36.	SBSB-16	TTT GCC CGG A	4	4	100	P1(675 bp)
37.	SBSB-17	AGG GAA CGA G	-	-	-	-
38.	SBSB-18	CCA CAG CAG T	8	8	100	P9(350, 500 bp)
39.	SBSB-19	ACC CCC GAA G	9	9	100	P8(350 bp) and P9(400 bp)
40.	SBSB-20	GGA CCC TTA C	10	9	90	P10(375 bp)
	Total		**229**	**226**	**98.69**	

Table.3 Genetic similarity co-efficient based on 40 RAPD primers among 10 species of *Pleurotus*

Sl. No.	Species	*P. sajor caju*	*P. flabellatus*	*P. platypus*	*P. fossulatus*	*P. florida*	*P. citrinopileatus*	*P. sapidus*	*P. dajmor*	*P. ostreatus*	*H. ulmarious*
1	***P. sajor caju***	1.000									
2	*P. flabellatus*	0.198	1.000								
3	*P. platypus*	0.191	0.527	1.000							
4	*P. fossulatus*	0.198	0.509	0.568	1.000						
5	*P. florida*	0.200	0.513	0.574	0.988	1.000					
6	*P.citrinopileatus*	0.333	0.122	0.169	0.150	0.151	1.000				
7	*P.sapidus*	0.204	0.500	0.840	0.581	0.5871	0.174	1.000			
8	*P. dajmor*	0.177	0.221	0.203	0.183	0.184	0.133	0.189	1.000		
9	*P.ostreatus*	0.152	0.350	0.314	0.263	0.265	0.192	0.317	0.188	1.000	
10	*H. ulmarious*	0.166	0.401	0.313	0.344	0.347	0.141	0.305	0.241	0.447	1.000

Fig.1 UPGMA dendogram depicting relationship among ten Pleurotus species



Polymorphism percentage was 98.69. Ten primers namely SBSA 11, SBSA 13, SBSA 15, SBSA 16, SBSA 18, SBSA 19, SBSA 20, SBSB 14, SBSB 15, SBSB 17 were not amplified the DNA from any of the isolate.

Some of the RAPD primers were found to be specific to distinguished the *Pleurotus* species. Primer SBSA 01 would distinguished *P. sajor caju* (325 bp, 500 bp). Primer SBSA 07 & SBSB 09 would distinguished *P. flabellatus* (450bp & 300bp respectively). *P. platypus* be distinguished by primer SBSB 12

(700bp), primer SBSA 17 will be identified the strain *P. fossulatus* (300 bp, 525 bp, 600 bp), primer SBSA 02 would distinguished sp. *P. florida* (225 bp, 375 bp, 875 bp), SBSA 05, SBSA 08, SBSB 06 will distinguish *P. citrinopileatus* (575 bp, 500 bp & 800 bp, 375 bp respectively), SBSA 12 will be able to identify *P. sapidus* (375 bp, 650 bp), *P. djamor* will be distinguished by SBSB 19 & SBSB 08 with 350 bp & 625 bp respectively, *P.ostreatus* by SBSB 11 with (375 bp), *H.ulmarious* by SBSA 14, SBSB 13 with 525 bp & 600 bp respectively (Table 1). RAPD markers revealed genetic diversity among the *Pleurotus* species with genetic similarity ranging from 0.22 to 0.98 (Table 2 and 3).

In UPGMA dendogram (Fig. 1), there were 5 clusters which made several group and sub group. Cluster A with strains namely *P.sajor caju & P. citripileatus.* Cluster B distinguished a separate species *P. djamor.* Cluster D & E made from cluster C. *P. flabellatus* separate from cluster E. Cluster D sub grouped with *P. ostreatus & H. ulmarious.* Cluster F grouped into two sub group, first sub group with *P. fossulatus & P. florida*, second sub group with *P. platypus* & *P. sapidus.*

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