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Full Length Research Paper

Morphological and molecular characterization of Oyster mushroom (*Pleurotus* spp.)

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Mushrooms have medicinal as well as nutritive value and extensively used as human food from the time immortal. In order to determine the genetic diversity among *Pleurotus* species of mushroom using morphological and random amplified polymorphic DNA (RAPD) markers, about seven different species were collected. Five species, naming *Pleurotus platypus* (P-6), *Pleurotus flabelatus* (P-7), *Pleurotus florida* (P-17), *Pleurotus ostreatus* (P-19) and *Pleurotus sajor-caju* (P-56) were from Canada and two *Pleurotus warm-stram* (P-9*)* and *Pleurotus eryngii* (P-16) from Philpines. Seven different morphological traits that is, mycelial growth (mm), cap diameter (cm), total yield (kg), moisture contents (%), ash contents (%), nitrogen contents (%) and protein content (%) were recorded. The dendrogram based on morphological data divided seven species in cluster 'A' and 'B' having four and three species, respectively. The dendrogram based on RAPD analysis generated 3 clusters 'A', 'B' and 'C'. Out of 14 random primers, the maximum polymorphism was observed by primers OPL_3 (72.70 %) and OPL_{11} (70%). The two species P-56 and P-17 were observed to be most similar having value 86% and constituting a cluster 'A'. The present work revealed that, morphological and RAPD markers showed different pattern of genetic diversity among different *Pleurotus* species.

Key words: Oyster mushrooms, genetic diversity, random amplified polymorphic DNA (RAPD) markers, dendrogram.

INTRODUCTION

Mushrooms are cultivated in controlled biological environment and it has been extensively used as food since ancient time, due to its nutritive and medicinal values (Manzi et al., 2001). With the passage of time, there occurred an increase in awareness about mushrooms nutritive and medicinal value (Cheung,1999). Although, mushrooms contain low amount of calories and fat, they are rich in proteins and fibers (Manzi et al., 2001).

Due to good nutritive value of mushrooms, a remarkable progress occurred in the breeding of its new strains which results in difficulty of their identification (Staniaszek et al., 2002). The determination of genotypic identity is also important to make suitable biological

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Abbreviations: RAPD, Random amplified polymorphic DNA; **PCR,** polymerase chain reaction; **PDA,** potato dextrose agar.

analysis about population, structure and evolutionary ways within and among species (Mahmood et al., 2009). There is atmost need of advance technique used for species identification beyond morphological and physiological criteria, because these characteristics are highly influenced by cultivating conditions (Staniaszek et al., 2002; Iqbal et al., 2010).

The expression of particular gene is a cumulative outcome of environment and genetic makeup of a specie/strain (Kumar, 1999; Astarini et al., 2004). Biochemical markers can be a source to reflect the genetic variability because they are direct product of genes. DNA finger printing is also one of the efficient tools of plant biotechnology used for the assessment of genetic diversity (Mehmood et al., 2008). For long, different DNA markers along with morphological traits have been used for the determination of genetic variations at molecular level (Sajida et al., 2009).

The genetic diversity of mushrooms has been worked out using molecular markers especially random amplified polymorphic DNA (RAPD) (Staniaszek et al., 2002: Yan et al., 2004; Stajic et al., 2005; Ravash et al., 2009). RAPD is used to assess genetic similarity and phylogenetic analysis due to the simplicity in its methodology (Gepts, 1993). Moharram et al. (2008) worked on characterization of Oyster mushroom on the basis of food supplements. Singh et al. (2006) characterized eighteen specialty mushroom accessions using DNA fingerprinting and ribosomal rRNA gene sequencing and reported the presence of genetic diversity. Hyeon et al. (2007) studied the diversity of *Pleurotus eryngii* using RAPD and its sequence analysis and observed that, grouping based on physiological parameters is closely related to RAPD based grouping. Stajic et al. (2005) used randomly amplified polymorphic DNA technique to asses the genetic diversity among 37 *pleurotus* species of mushrooms. In another study, RAPD- polymerase chain reaction (PCR) amplification was used to evaluate the genetic diversity among 45 pleorous strains and found that, this technique was better than morphological analysis.

Keeping in view the usefulness of morphological and molecular markers, the present study was planned to investigate the genetic diversity among different strains of cultivated mushroom (*Pleurotus spp.*).

MATERIALS AND METHODS

Out of seven species, culture of 5 species; *Pleurotus platypus* (P-6), *Pleurotus flabellatus* (P-7), *Pleurotus florida* (P-17), *Pleurotus Ostreatus* (P-19) and *Pleurotus sajor-caju* (P-56) were taken from Vineland Agriculture Station, Ontario Canada and culture of remaining two species *Pleurotus warm-stram* (P-9) and *P. eryngii* (P-16) were taken from Dr. Qumio, Plant Pathology Department University of Philpines.

Mushroom culture

All the species were cultured on potato dextrose agar (PDA) media having composition, potato starch 20 g, dextrose 20 g, agar 20 g and distill water to make final volume one litre. Streptomycin was added at 1 g/l as antibiotic. The media was wet sterilized at 121°C for 30 min at 15 psi, then, it was cooled to 40°C (Khan et al., 2006) for culturing of mushrooms.

Mushroom cultivation

Cotton waste material was used as substrate for growth of mushrooms. The substrate was prepared by soaking in H2O for 72 hrs. and then, it was allowed to extrude extra moisture by spreading on the inclined plane. This substrate was filled in ploythene bags @ 1 kg/bag and dry sterilized. The experiment was laid out in completely randomized design with three replications and ten bags in each replication for each treatment (Khan et al., 2006). Data for different morphological traits that is, mycelial growth (mm) (after seven days on PDA), cap diameter (cm), total yield (kg) after four flushes and biochemical traits that is. moisture contents (%), ash (%), nitrogen contents (%), protein (%) of fruiting body were collected from mushrooms grown on cotton waste.

Morphological data analysis

Analysis of the variance was performed for all morphological and biochemical traits in order to test the significance of variance among all species (Steel et al., 1997) and a dendrogram was constructed using Minitab statistical software.

Molecular analysis

DNA extraction

In the fungi, the release of DNA is often poor due to cell walls or capsules that are not readily susceptible. Filamentous fungi have strong cell walls which are often difficult to rupture in traditional DNA extraction procedures (Van Burik et al., 1997). The protocol suggested by Sambrook et al. (2001) was followed for DNA isolation from fungal species.

DNA of seven species was extracted from mycelial threads after multiplication on potato dextrose medium at 25°C for three days. Mycelial mat was centrifuged for 5 min at 10000 rpm followed by a washing with 500 µl TE buffer. The resulting palette was grinded in extraction buffer (200 mm Tris-HCl, pH-8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) with a conical grinder followed by addition of 150 µl of 3M sodium acetate (pH-5.2). The lysates were incubated at -20°C for 10 min followed by centrifugation. DNA was precipitated from the supernatant by adding equal volumes of isopropanol and resultant pallet was washed with 70% ethanol. The DNA palette was air dried and dissolved in 20 µL TE buffer. DNA quantification was performed and a dilution of 15 ng/µl was used in downstream application.

RAPD analysis

Individual PCR reactions were performed for each isolate in 0.2 ml tubes using 14 random decamer primers (Table 1) synthesized from Operon Technologies, USA. Amplification were performed in 25 µl reactions containing PCR buffer, 5 mM each of the deoxyribonucleotide triphosphate, 10 pmol each of the appropriate primers, DNA having concentration of 15 ng/µl and 1unit of Taq polymerase. The amplification protocol include initial denaturation at 94°C for 5 min followed by 40 cycles of amplification, 95°C for 1 min (denaturing), 36°C for 1 min (annealing), 72°C for 2 min (extension) and final extension step at 72°C for 10 min.

Table 1. Operon random primers.

S/N	Primer code	Primer sequence	No. of bands	No. of polymorphic bands	Polymorphism (%)
1	OPQ_1	GGGACGTATGG	9	5	55.60
2	OPQ_6	GAGCGCCTTG	6	0	00.00
3	OPI_2	GGAGGAGAGG	7	4	57.10
4	OPL_3	CCAGCAGCTT	11	8	72.70
5	OPL_{11}	ACGATGAGCC	10	7	70.00
6	OPL_{15}	AAGAGAGGGG	8	3	37.50
7	OPL_{13}	ACCGCCTGCT	16	7	43.75
8	OPL_2	TGGGCGTCAA	8	3	37.50
9	OPI_5	TGTTCCACGG	10	5	50.00
10	OPQ_{17}	CCGTACGTAG	7	0	00.00
11	OPP_{11}	AACGCGTCG	11	3	27.27
12	OPP_{17}	TGACCGCCT	5	0	00.00
13	OPN_{10}	ACAACTGGGG	6	2	33.33
14	OPL_8	AGCAGGTGGA	9	4	44.44

Gel electrophoresis and RAPD data scoring

The amplicons after PCR were analyzed by electrophoresis on 1.2% (W/V) agarose gels by running in 0.5X TBE buffer. After staining with ethidium bromide the gels were visualized under a UV transilluminator and photographed using Bio-Rad gel documentation system. The amplification product generated by each RAPD primer were scored as ‘‘1’’ or ‘‘0’’ for presence or absence of specific allele, respectively. To estimate the similarity and genetics distance among different *Pleurotus* species, cluster analysis based on Nei’s unweighted pair-group with arithmetic average (UPGMA) was performed using the Popgen-32 software (Yeh et al., 2002) and a dendrogram was constructed.

RESULTS

The analysis of variance (Table 2) indicated that, all the traits studied except protein contents were statistically significant ($P \leq 0.05$). The dendrogram (Figure 1) grouped the seven mushroom species in two main clusters. The cluster ‘A’ contains four species while cluster ‘B’ contain three species. In cluster ‘A’, species P-6 (*P. platypus*) and P-19 (*P. ostreatus*) were more similar and fall in same subgroup and the comparison of means for total yield, ash contents and protein contents showed that these species were statistically alike. On the other hand, in cluster ‘B’ the species P-9 (*P. warm-starm*) and P-16 (*P. eryngii*) were in same subgroup and comparison of means for total yield, nitrogen contents and protein contents revealed that these species were also statistically similar.

The genomic DNA of seven *Pleurotus* species were analyzed using 14 ten mer random primers. All the primers except OPQ_6, OPQ_{17} and OPP_{17} were polymerphic. The number of bands and banding pattern were variable depending upon the primer and type of species tested and it ranged from 5 to 16 in counting. The maximum polymorphism was produced by the primer OPL_3 and OPL_4 having polymorphism percentage 72.70 and 70%, respectively.

The dendrogram based on similarity matrix differentiated the species into three distinct clusters A, B and C. Cluster A constituted species P-56 (*P. sajor-caju*), P-17 (*P. florida*) and P-6 (*P. platypus*), cluster B contained species P-19 (*P. oysterus*) and P-7 (*P. flabellatus*) and cluster C comprised of species P-9 (*P. warm-starm*) and P16 (*P. eryngii*) (Figure 2). The genetic similarities between species ranged from 75.5 and 86% (Table 3). The similarity among three species of cluster A was from 84 to 86%. The cluster ‘B’ and ‘C’ comprised of two species in each group, which showed 80 and 81% similarity, respectively. The similarity matrix also depicted that most closely related isolates (P-56 and P-17) were 86% similar.

DISCUSSION

The mean square and means for the consideration traits in cluster analysis revealed that, there were significant differences among all the species. The existence of species P-6 (*P. platypus*) and P19 (*P. ostreatus*) in same sub cluster of main cluster ‘A’ and species P-9 (*P. warm-starm*) and P-16 (*P. eryngii*) in same subgroup of cluster ‘B’ revealed that, the morphological behaviour of these species was similar or they may have same ancestors, but sometimes, morphological based grouping did not match molecular/genomic relationship among the species (Stajic et al., 2005). In this regard, the morphological traits do not provide a meaningful frame work for evolutionary classifications. Based on the relatively simple fruiting structures and considerable developmental plasticity of fungi, it is accepted that accumulating differences in developing sub-populations are not always expressed in terms of morphological divergence. Closely

Table 2. Means, mean square and LSD value of morphological traits.

Genotype	Mycelial growth (mm)	Cap diameter (cm)	Total yield (kg)	Moisture contents (%)	Ash content (%)	Nitrogen content (%)	Protein content (%)
P-6	34.00 bc	34.00 b	294.7 bc	73.00 e	0.960 b	6.700a	41.870a
P-7	29.00d	29.00 c	253.3 c	81.50 bc	0.980 b	6.400 b	39.680a
P-9	25.00 e	25.00 d	351.3a	79.00 cd	1.097a	6.200 b	38.810a
P-16	31.00cd	29.33 c	351.0a	82.50 bc	0.970 b	6.167 b	38.430a
P-17	36.00 b	36.00 b	361.7a	77.00 d	1.000 b	6.200 b	38.860a
P-19	40.00a	40.50a	291.7 bc	84.50 b	0.950 b	6.400 b	40.000a
P-56	42.00a	42.00a	316.7ab	88.70a	0.990 b	6.400 b	40.130a
LSD	*3.959*	*3.376*	*54.25*	*3.690*	*0.713*	*0.259*	*3.880*
Mean squares	112.61**	118.68**	4762**	78.97**	0.00722**	0.1043**	4.21^{NS}

* Significant ($p \geq 0.05$); ** highly significant ($p \geq 0.01$); ^{NS} non-significant.

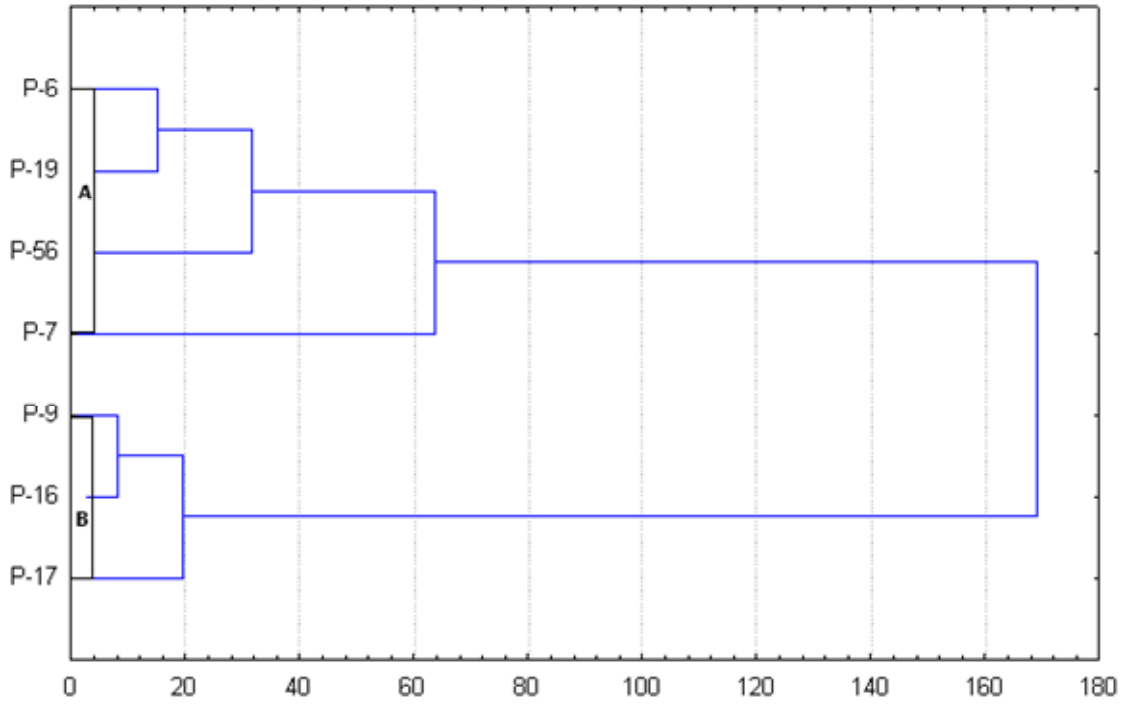

Figure 1. Dendrogram based on morphological traits.

Table 3. Similarity matrix for Nei's and Li's coefficient of seven fungal species

Specie	P-9	P-56	P-17	P-19	P-16	P-6	P-7
P-9	1.000						
P-56	0.723	1.000					
P-17	0.784	0.862	1.000				
P-19	0.808	0.754	0.755	1.000			
P-16	0.812	0.767	0.755	0.755	1.000		
P-6	0.793	0.843	0.838	0.791	0.811	1.000	
P-7	0.783	0.805	0.804	0.807	0.760	0.785	1.000

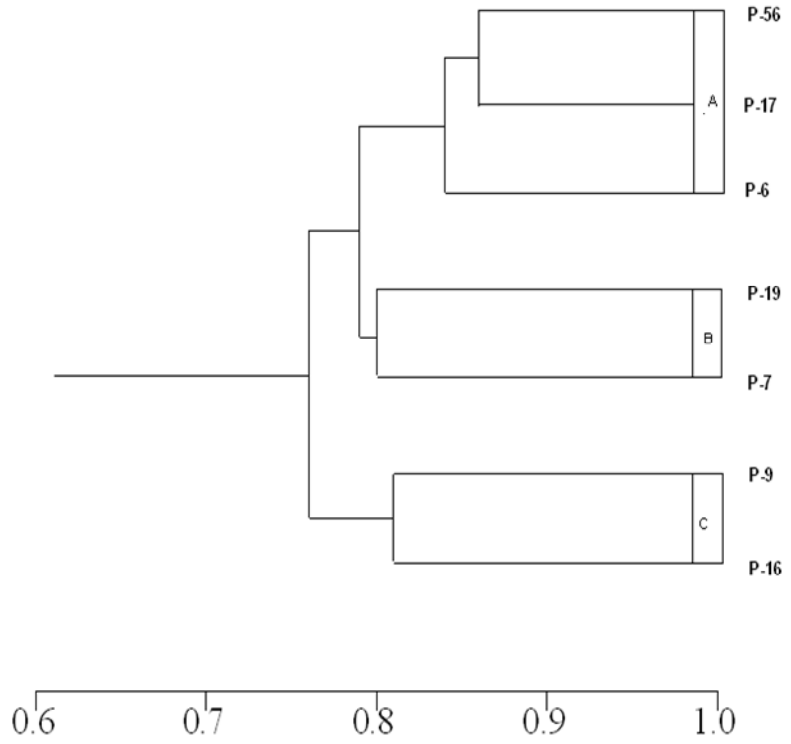

Figure 2. Dendrogram of seven fungal strains developed from RAPD data using the unweighted pair group method of arithmetic means (UPGMA). The scale is based on Nei's and Li's coefficients of similarity.

related or sibling species may therefore, lack taxonomically useful morphological differences long after the initial speciation event. Morphological features that had been used to identify *Pleurotus* spp. in the past do not clearly distinguish the different phylogenetic species of which it is comprised. The basic DNA sequence of an organism can be presumed to be insensitive to short term environmental change and thus should provide a more stable alternative for strain/ species identification. Therefore, the random and genomic wide nature of the RAPD technique is best to indicate over all genetic relatedness/dissimilarity than the morphological analysis (Ravash et al., 2009).

The different primers produced different number of bands in PCR. This variation in the number of bands may be due to the sequence of primer, availability of annealing sites in the genome and template quality (Kernodle et al., 1993). Despite this, all the species were grown on similar culture but the variations in the banding pattern were reflected. Chandra et al. (2010) used the RAPD markers to discriminate the eight *Pleurotus* species of mushrooms and also found variations in banding pattern. The polymorphism produced by 11 RAPD primers except OPQ_6, OPR_{17} and OPP_{17} may be due to the base substitution, insertion and deletion or collection of genetic material from different sources (Chopra, 2005; Jusuf, 2010).

The maximum similarity (86%) was observed between P-17 (*Pleurotus florida*) and P-56 (*Pleurotus sajor-caju*) belonging to cluster 'A'. Chandra et al. (2010) found mini-

mum similarity level (27 %) between *Pleurotus florida* and *Pleurotus sajor-caju.* This contradiction in the finding may be due to different growth conditions. The minimum (72%) similarity was observed between P-56 (*Pleurotus sajor-caju*) and P-9 (*Pleurotus warm-starm*) which belonged to two different clusters 'A' and 'C', respectively. During growth studies, both of these, P-56 *(P. sajor-caju)* and P-9 *(Pleurotus warm-starm),* proved to be fast and slow growing respectively and showed both belong to two different habitat that is why these species were observed to join different clusters, A and C, respectively. These findings revealed that, the genetic make up is correlated with environmental heterogeneity (Nevo, 1998). The study depicted that cultural variations was also retained at molecular level. P-19 (*Pleurotus ostreatus*) is a low temperature adopted species while P-7 (*Pleurotus flabelatus*) is tropical in nature but despite this distinction, they formed a separate cluster 'B'. P-9 (*Pleurotus warm-starm*) and P-16 (*Pleurotus eryngii*) formed a distinct sub-group in cluster. At the molecular level, both of these specie also retained their grouping based on morphological data. The results depicted that, there is strong correlation between molecular and morphological criteria (Zervakis et al., 2004). Jusuf (2010) suggested that, mushrooms belonging to *Pleurotus* specie have common growing habbit that's why they may have common genetic background.

Formation of sub-groups by P-56 (*Pleurotus sajor-caju*) and P - 17 (*Pleurotus florida*) in cluster A, P-19 (*Pleurotus ostreatus*), P-7 (*Pleurotus flabelatus*) in cluster B and P-9 (*Pleurotus warm-starm*) and P-16 (*Pleurotus .eryngii*) in cluster 'C' is due to genetic distance of 0.86, 0.80 and 0.81, respectively. The species within a distinct subgroup might be due to their same genus *Pleurotus* and same ancestry.

Conclusions

The species of fungi belonging to ecological proximity or different geographical origins can be classified through morphological and molecular markers. The current study demonstrated that, the RAPD analysis and morphological evaluation both are useful for characterization, genetic diversity and identifying relationships among *Pleurotus* species of mushrooms. Study also revealed that, RAPD analysis can be very useful tool for mushroom grower for classification and maintenance of good quality spawns.

REFERENCES

Astarini AI, Plummer AJ, Lancaster AR, Yan G (2004). Fingerprinting of cauliflower cultivars using RAPD markers. Aust. J. Agri. Res. 55: 112-124.

Chandra S, Ghosh K, Acharya K (2010). Comparative study on the Indian cultivated Pleurotus species by RAPD fingerprinting. Nature & Sci. 8: 90-93.

Cheung ST (1999). World production of cultivated edible and medicinal mushrooms in (1997). with emphasis on lentinus edodes (berk). Sing in China. J. Med. Mushrooms. 1: 291-300.

Chopra VL (2005). Mutagenesis: Investigating the process and processing the outcome for crop improvement. Curr. Sci. 89: 353-359.

Gepts P (1993). The use of molecular and biochemical markers in crop Evolution studies. In: Hecht MK (ed.). Evol. Biol. 27: 51-94.

Hyeon-Su RO, Sung SK, Jae SR, Che-Ok J, Tae SL, Hyun-Sook L (2007). Comparative studies on the diversity of the edible mushroom *Pleurotus eryngii*: ITS sequence analysis, RAPD fingerprinting, and physiological characteristics. Mycological Res. 111: 710-715.

Iqbal A, Sadia B, Khan AI, Awan FS, Kainth RA, Sadaqat HA (2010). Biodiversity in the sorghum (Sorghum bicolor L. Moench) germplasm of Pakistan. Genet. Mol. Res.9: 756-764.

Jusuf M (2010). Amplified fragment length polymorphism diversity of cultivated white Oyster mushroom *Pleurotus ostreatus*. HAYATI J. Biosci. 17: 21-26

Kernodle SP, Cannon RE, Scandalios JG (1993). Rapid and simple phage DNA isolation. Biotechniques. 14: 360-362.

Khan SM, Nazir J, Zahoor HK, Sultan MK (2006). Yield performance of oyster mushroom. Pak. J. Phytopathology. 18: 89-93.

Kumar LS (1999). DNA markers in plant improvement. An overview. Biotechnol. Advances. 17: 143-182.

Mahmood Z, Raheel F, Dasti AA, Shazadi S, Athar M, Qayyum M (2009). Genetic diversity analysis of the species of Gossypium by using RAPD markers. Afr. J. Biotechnol. 8: 3691-3697

Manzi P, Aguzzi A, Pizzoferrato L (2001). Nutritional value of mushrooms widely consumed in Italy. Food Chem. 73: 321-325.

Mehmood S, Bashir A, Ahmad A, Akram Z (2008). Molecular characterization of regional sorghum bicolor varieties from Pakistan. Pak. J. Bot. 40: 2015-2021.

Moharram AH, Salama MF, Hissien AA (2008). Characterization of Oyster mushroom mycelia as a food supplement. Aust. J. Basic & Appl. Sci. 2: 632- 642.

Nevo E (1998) Molecular evolution and ecological stress at global, regional and local scale: the Israeli prospective. J. Exp. Zool. 282: 95-119

Ravash R., Shiran B, Alavi A, Zarvagis J (2009). Evaluation of genetic diversity in Oyster mushroom (*Pleurotus eryngii*) isolates using RAPD marker. J. Sci. & Technol. Agric. & Natur. Resour. 13: 739-741.

Sajida B, Imtiaz AK, Bughio HR, Iftikhar AO, Muhammad AA, Abdullah K (2009). Genetic differentiation of rice mutants based on morphological traits and molecular marker (RAPD). Pak. J. Bot. 4: 737-743.

Sambrook J D, Russell W, Sambrook J (2001). Molecular cloning: a laboratory manual, 3rd ed. NY: Cold Spring Harbor Laboratory Press.

Singh SK, Sharma, VP, Sharma S, Kumar RS, Tiwari M (2006). Molecular characterization of Trichoderma taxa causing green mould disease in edible mushrooms. Curr. Sci. 90: 427-431.

Stajic M, Sikorski J, Wasser SP, Nevo E (2005). Genetic similarity and taxonomic relationships within the genus *Pleurotus* (higher Basidiomycetes) determined by RAPD analysis. Mycotoxon. 93: 247-255

Staniaszek M, Marczewski W, Szudyga K, Maszkiewicz J, Czaplicki A, Qian G (2002). Genetic relationship between Polish and Chinese strains of the mushroom Agaricus bisporus (Lange) Sing., determined by the RAPD method. J. Appl. Genet. 43: 43-47.

Steel RGD, Torrie JH, Dickey DA (1997). Principles and Procedures of Statistics, A Biometrical Approach. McGraw Hill Book Co., New York, USA.

Van Burik, JH, Schreckhise RW, Myerson D, White TC, Bowden RA (1997). Comparison of six extraction techniques for isolation of DNA from filamentous fungi. Abstracts of the 97th General Meeting of the American Society for Microbiology, Washington, D.C : 262.

Yeh FC, Yang R, Boyle TJ, Ye Z. (2002) Popgene 32, Microsoft ware windows based freeware for population genetic analysis. Molecular Biology and Biotechnology Centre, Edmonton.

Zervakis GI, Moncalvo JM, Vilgalys R (2004). Molecular phylogeny, biogeography and speciation of mushroom species Pleurotus cystidiosus and allied taxa. Microbiology, 150: 715-726.