Vegetative Growth and Phylogenetic Relationship of Commercially Cultivated Strains of *Pleurotus eryngii* based on ITS sequence and RAPD

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Pleurotus eryngii, known as king oyster mushroom has been widely used for nutritional and medicinal purposes. This study was initiated to screen the suitable conditions for mycelial growth and to determine the phylogenetic relationship of the selected strains. Optimal mycelial growth was observed at 30°C and minimum mycelial growth observed at 10°C. This mushroom tolerates a broad pH range for mycelial growth, with most favorable growth observed at pH 6. Results also indicated that glucose peptone, yeast malt extract and mushroom complete media were favorable growth media, while Hennerberg and Hoppkins media were unfavorable. Dextrin was the best and xylose the least effective carbon sources. Results revealed that inorganic nitrogen sources were less effective than organic sources for the mycelial growth of *P. eryngii*. Investigation of genetic diversity is necessary to identify the strains. The ITS region of rDNA were amplified using PCR. The size of the ITS1 and ITS2 regions of rDNA from the different strains varied from 214 to 222 bp and 145 to 236 bp, respectively. The sequence of ITS2 was more variable than that of ITS1, and the 5.8S sequences were identical. A phylogenetic tree based on the ITS region sequences indicated that selected strains could be classified into six clusters. Fourteen IUM and ATCC-90212 strains were also analyzed by RAPD with 20 arbitrary primers. Fourteen of these primers were efficiently amplified the genomic DNA. The number of amplified bands varied with the primers and strains, with polymorphic fragments in the range from 0.2 to 2.3 kb.

KEYWORD : ITS, Phylogenetic relation, *Pleurotus eryngii*, RAPD, Vegetative growth

Pleurotus eryngii is also known as the king oyster mushroom. It is tetrapolar heterothallic and forms an edible fruiting body on lignocellulosic substrates. The fungus is a weak parasite on the roots and stems of umbellifers (family Apiaceae, genera *Eryngium, Ferula, Ferulago, Cachrys, Laserpitium, Diplotaenia* and *Elaeoselinum*). It grows wild in the forests of hilly areas and is cultivated in temperate and subtropical regions of the world (Ro *et al.*, 2007). The distribution of *P. eryngii* extends from France and Spain to western China, including the Mediterranean regions (Lewinsohn *et al.*, 2001). This mushroom has been known to produce various biologically active molecules and novel enzymes (Choi *et al.*, 2005). Laccase is a ligninolytic enzyme, typically produced as multiple isoenzymes, which have been isolated and characterized in different strains of *P. eryngii* (Soden and Dobson, 2001; Mayer and Staples, 2002).

Watling (1992) reported that commercial strains of mushrooms sometimes decline in production performance or yield, as a result of several consecutive subcultures and/or a long period of storage in culture medium. Bio-logical efficiency can sometimes be raised by optimization of cultural conditions, such as combining different substrates and/or adding nutritional supplements. Mycelium propagation is an essential precursor step to cultivate fruiting bodies of mushrooms, and can be enhanced by different environmental and nutritional factors.

To identify *P. eryngii* strains with high yield potential and other traits of interest, assessment of genetic and phenotypic diversity is necessary. Various molecular genetics tools have been established for the verification of fungi, such as RFLP, RAPD, and SSU rDNA and ITS sequence analyses. Phylogenetic analysis of *P. eryngii* based on DNA sequencing, ITS1-5.8S-ITS2 of rDNA sequence analysis, is frequently used for fungal verification (Tuckwell *et al.*, 2005). RAPD was particularly successfully when applied to the verification of *P. eryngii* strains from various hosts with a wide range of geographical origins (Zervakis *et al.*, 2001). The aim of this study was to investigate molecular genomic polymorphisms among the selected strains of *P. eryngii* using both ITS sequence and RAPD analysis. Experiments also explored the physical and chemical conditions for optimal mycelial growth.

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Materials and Methods

Mushroom strains. Fourteen cultivated strains of *P. eryngii* were collected from the different ecological regions of China (IUM-3487, IUM-3501, IUM-3865 and IUM-3920), Japan (IUM-2253 and IUM-2263), Korea (IUM-1550, IUM-2106, IUM-3140 and IUM-3939), and Taiwan (IUM-2126, IUM-2137, IUM-2143 and IUM-2161). Pure cultures were deposited in the Culture Collection of Mushrooms (CCM) and acquired Incheon University Mushroom (IUM) accession numbers. Ten strains of *P. eryngii* AY-581425, AY-589046, AY-589047, AY-589048, AY-589049, DQ-342326, EF-514246, EU-395845, EU-520192 and ATCC-90212, were used as control strains for the phylogenetic comparison with our selected IUM strains. Sequencing data of the control strains were collected from the National Center for Biotechnology Information (NCBI) gene bank data base and ATCC-90212 strains was acquired from USA.

Temperature and pH. Six different temperatures, 10, 15, 20, 25, 30 and 35°C were used to find the optimum temperature for the mycelial growth of *P. eryngii*. A 5 mm diameter agar plug removed from a 10 day old culture and placed in the centre of a PDA plate. The medium was adjusted to pH 6 and incubated for 10 days at 10, 15, 20, 25, 30 and 35°C. To determine the optimum pH, the medium was adjusted to pH 4, 5, 6, 7, 8 and 9 by adding 1 N NaOH or HCl before autoclaving. Samples were incubated for 10 days at 25°C. The measurement of mycelial growth was performed according to the methods described by Shim *et al.* (1997).

Culture media. Ten different culture media (Czapek dox, glucose peptone, glucose tryptone, Hamada, Hennerberg, Hoppkins, Lilly, mushroom complete, potato dextrose agar and yeast malt extract) were used to investigate the mycelial growth of *P. eryngii* (Table 1). The media were adjusted to pH 6 before being autoclaved.

Carbon and nitrogen sources. These experiments were performed on basal medium (0.05 g $MgSO_4$, 0.46 g KH_2PO_4, 1.0 g K_2HPO_4, 120 μg thiamine-HCl, 20 g agar and 1 litter of distilled water) supplemented with one of 10 carbon sources (dextrin, fructose, galactose, glucose, lactose, maltose, mannose, sorbitol, sucrose and xylose) and ten nitrogen sources (alanine, ammonium acetate, ammonium phosphate, arginine, calcium nitrate, glycine, histidine, methionine, potassium nitrate and urea). To evaluate the most favorable carbon and nitrogen sources for mycelial growth, each carbon source with 5 g of peptone was added to the basal medium separately at the concentration of 0.1 M and mixed thoroughly. Each nitrogen source, with 20 g of glucose was supplemented to the basal medium at the concentration of 0.02 M (Sung *et al.*, 1993). In both cases, the basal medium was adjusted to pH 6 before autoclaving.

Table 1. Composition of culture media used in this study

Composition	Media (g/l)									
	CZA	GLP	GLT	HAM	HEN	HOP	LIL	MUC	PDA	YEM
Agar	20	20	20	20	20	20	20	20	20	20
Asparagine							2			
Dextrose				10					20	10
Ebiose				5						
Hyponex				3						
Glucose		10	5		50	10				
Malt extract		15						20		3
Maltose						10				
Peptone		10						2		5
Potato									200	
Sucrose	30									
Triptone			10							
Yeast extract		10	3	3				2		3
$NaNO_3$	3				2					
K_2HPO_4	1							1		
$MgSO_4$	0.5				0.5	0.5	0.5	0.5		
KCl	0.5									
$FeSO_4$	0.01									
$CaCl_2$					0.1					
KH_2PO_4					1	0.1	1	0.5		
KNO_3					2	2				

CZA: Czapek dox, GLP: Glucose peptone, GLT: Glucose tryptone, HAM: Hamada, HEN: Hennerberg, HOP: Hoppkins, LIL: Lilly, MUC: Mushroom complete, PDA: Potato dextrose agar, YEM; Yeast malt extract.

DNA extraction. Genomic DNA was extracted according to the procedure of Lee and Taylor (1990) with some modifications as follows. Fresh mycelia were collected from 10 day old culture on PDA medium and were frozen with liquid nitrogen. Frozen mycelia were ground with sterilized mortar-pestle and kept in 1.5 *ml* micro tubes. 500 *μl* extraction buffer [equal volumes of 50 mM Tris-HCl (pH 7.5), 50 mM EDTA (pH 8) and 1% sarkosyl] was added to the micro tube and incubated at 65°C for 30 minutes. After incubation, same volume of PCI (25 *ml* phenol: 24 *ml* chloroform: 1 *ml* isoamyl-alcohol) was added, vortexed and centrifuged at 4°C, 10 minutes, 12000 rpm. Afterwards, the upper phase was transferred to a 1.5 *ml* micro tube, 1000 *μl* of 99.9% alcohol was added and centrifuged at 4°C, 5 minutes, 12000 rpm. Subsequently, supernatant was removed, 500 *μl* of 70% alcohol was added to the precipitated DNA, vortexed and centrifuged at 4°C, 5 minutes, 12000 rpm. Again supernatant was removed and the residual alcohol evaporated. The DNA pellet was resuspended in 500 *μl* of sterilized distilled water. DNA concentration was measured using spectrophotometer (Cubero *et al.*, 1999).

Amplification of the ITS region and analysis of sequences. The ITS region of the rDNA of selected strains of *P. eryngii* was amplified by polymerase chain reaction (PCR) using universal primers ITS1 (5'-TCCGTAG-GTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTAT-TGATATGC-3'). Amplification reactions were performed in a total volume of 20 *μl* containing 2 *μl* 10 × PCR buffer, 1.6 *μl* dNTP, 0.5 *μl* of each primer, 0.2 *μl* Taq polymerase, 1 *μl* genomic DNA and 14.2 *μl* of sterilized distilled water. PCR was performed using a thermal cycler (Veriti thermal cycler, Applied Biosystems, USA) with an initial denaturation stage of 5 minutes at 95°C, followed by 35 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 52°C, extension for 1 minute at 72°C and a final 10 minutes extension at 72°C. Amplification products were analyzed by gel electrophoreses on a 1.5% agarose gel with a 1 kb DNA ladder as a marker. ITS sequences were aligned for phylogenetic analysis using the program Clustal W (Thompson *et al.*, 1994). The phylogenetic tree was constructed by neighbor-joining method using the CLC free Workbench program. Bootstrap analysis was repeated 1000 times to examine the reliability of the interior branches and the validity of the trees obtained (Felsenstein, 1985; Saitou and Nei, 1987).

RAPD analysis. Genomic DNA was amplified by the RAPD technique (Williams *et al.*, 1990) in which 20 arbitrary 10-base oligonucleotide primers (Operon Technologies Inc., Alameda, CA, USA) were used to produced amplified fragments. The primer sequences are listed in

Table 2. List of RAPD primers used in this study

| Primers	Sequence (5' to 3')
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-12	TGCGCGATAG
OPA-13	CAGCACCCAC
OPA-14	TCTGTGCTGG
OPA-15	TTCCGAACCC
OPA-16	AGCCAGCGAA
OPA-17	GACCGCTTGT
OPA-18	AGGTGACCGT
OPA-19	CAAACGTCGG
OPA-20	GTTGCGATCC

Table 2. RAPD-PCR reaction was performed using a thermal cycler with an initial denaturation stage of 5 minutes at 94°C, followed by 35 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 36°C, extension for 2 minutes at 72°C and a final 7 minutes extension at 72°C. RAPD products were run on a 1.4% agarose gel in 1 × Tris-acetate-EDTA buffer for 1.15 hour at 100 V, with a 1 kb DNA ladder as a size marker, and then stained while agitated in an EtBr solution (0.5% *μg/ml*). The stained gels were visualized and photographed using a UV transilluminator. RAPD bands were recorded as present (1) or absent (0) to generate the data matrix. The similarity coefficients (S) were calculated between isolates across bands for all primers using the formula S = 2Nxy/(Nx + Ny), where Nx and Ny are the number of bands shared by the two strains (Nei and Li, 1979).

Results and Discussion

Effect of temperature and pH. A range of 10~35°C was considered to determine the most suitable temperature for mycelia growth. The highest average mycelial growth (84.71 mm) was recorded at 30°C and the lowest (12.58 mm) was observed at 10°C (Fig. 1). Almost similar mycelial growth was observed at the temperature of 20 and 35°C. In case of maximum mycelial growth, there are no significant difference between the temperature of 25 and 30°C. Therefore, experimental results indicate that the optimum temperature range is 25~30°C for the mycelial growth of *P. eryngii*. The findings of this study are comparable to the previous study of Alam *et al.* (2008), which reported that 30°C is the optimum temperature for the
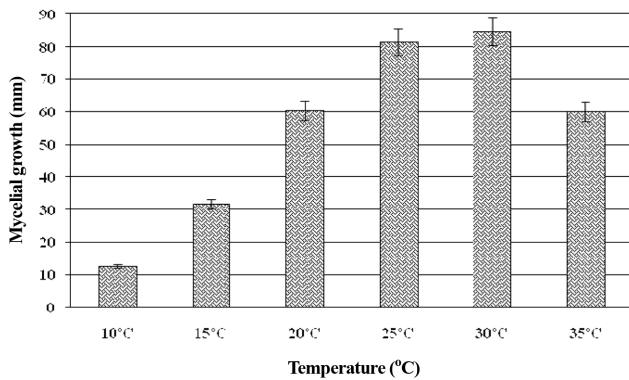
Fig. 1. Effect of temperature on the mycelial growth of *P. eryngii* on PDA after 10 days of incubation. Vertical bars show standard errors.
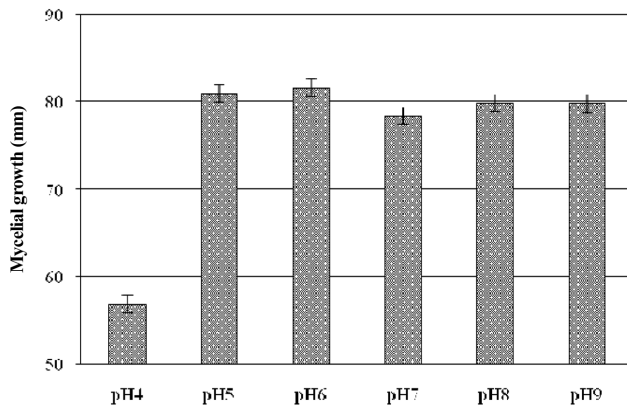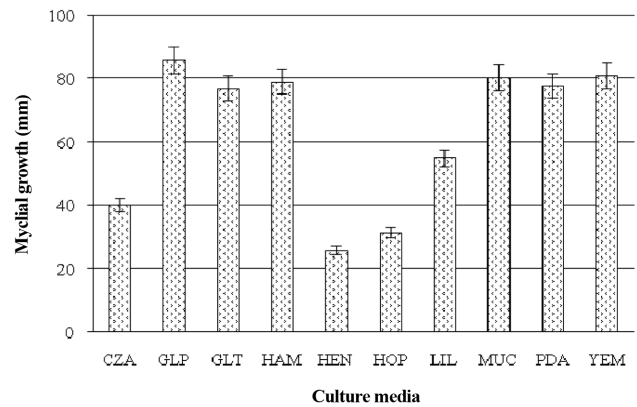

Fig. 2. Effect of pH on the mycelial growth of *P. eryngii* on PDA after 10 days of incubation at 25°C. Vertical bars show standard errors.
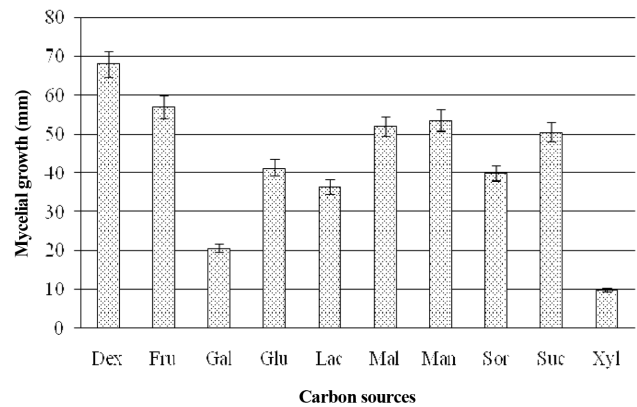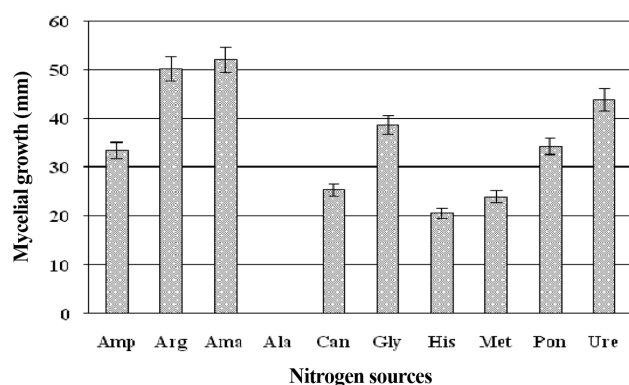

Fig. 3. Effect of different media on the mycelial growth of *P. eryngii* after 10 days of incubation at 25°C. Vertical bars show standard errors. CZA: Czapek dox, GLP: Glucose peptone, GLT: Glucose tryptone, HAM: Hamada, HEN: Hennerberg, HOP: Hoppkins, LIL: Lilly, MUC: Mushroom complete, PDA: Potato dextrose agar, YEM: Yeast malt extract.

Fig. 4. Effect of carbon sources on the mycelial growth of *P. eryngii* on basal medium after 10 days of incubation at 25°C. Vertical bars show standard errors. Dex: Dextrin, Fru: Fructose, Gal: Galactose, Glu: Glucose, Lac: Lactose, Mal: Maltose, Man: Mannose, Sor: Sorbitol, Suc: Sucrose, Xyl: Xylose.

mycelial growth of *P. adiposa*.

Next to determine the suitable pH culture conditions for mycelial growth, pH values ranging from 4~9 were studied. The highest level of radial growth of mycelium was found at pH 6 (Fig. 2). There was no significant variation in mycelial growth within pH ranging from 5~9. This result agrees with the data from the study of Hur (2008). He observed that pH 6 was the optimum for the myclial growth of *P. linteus*. Present results indicate that *P. eryngii* grows well at acidic, neutral or alkaline conditions.

Effect of culture media. Ten different culture media were tested to determine the optimum medium for mycelial growth of the selected strains of *P. eryngii*. According to the mycelial growth results indicate that glucose peptone, yeast malt extract and mushroom complete media were the most favorable, while Hennerberg and Hoppkins media were the least favorable for the vegetative growth of *P. eryngii*. Average highest and lowest mycelial growth was recorded in glucose peptone (86.04 mm) and Hennerberg (25.98 mm) media, respectively (Fig. 3).

Hur (2008) reported that excellent mycelial growth of *P. linteus* was observed in mushroom complete medium which was also one of the most favorable medium for *P. eryngii*.

Effect of carbon and nitrogen sources. Among the ten different carbon sources tested, dextrin was found to be the best for mycelial propagation of *P. eryngii*, followed by fructose, mannose and maltose. On the other hand, xylose and galactose were the most unfavorable carbon sources (Fig. 4). This result is similar to that of Shim *et al.* (2005). Ten different nitrogen sources were assayed to determine vegetative growth conditions for *P. eryngii*. Among the nitrogen sources, ammonium acetate was

Fig. 5. Effect of nitrogen sources on the mycelial growth of *P. eryngii* on basal medium after 10 days of incubation at 25°C. Vertical bars show standard errors. Amp: Ammonium phosphate, Arg: Arginine, Ama: Ammonium acetate, Ala: Alanine, Can: Calcium nitrate, Gly: Glycine; His: Histidine, Met: Methionine, Pon: Potassium nitrate, Ure: Urea.

Fig. 6. Phylogenetic tree of twenty three strains of *P. eryngii* based on the nucleotide sequence of the ITS region using the neighbor-joining method with 1000 bootstrapping trails.

found to be the best, followed by arginine and urea. Mycelial growth was totally absent in alanine, however, lowest growth levels were recorded in histidine and methionine (Fig. 5). Shim *et al.* (2005) reported that glycine was the most favorable nitrogen source, which diverges from our findings. They also determined that histidine was the most unfavorable nitrogen sources for the mycelial growth of *M. procera*, which is similar to our result. In general, organic nitrogen sources are more effective then inorganic nitrogen sources.

ITS sequence analysis. The goal was to investigate the genetic characteristics of fourteen strains of *P. eryngii*, collected from various ecological regions of China, Japan, Korea and Taiwan at different times. The ITS region was

amplified using ITS1 and ITS4 primers and sequenced. Results revealed that lengths of the sequences among the selected strains ranged from 518 to 616 bp. The size of the ITS1 and ITS2 regions varied among the strains from 214 to 222 bp and 145 to 236 bp, respectively. Total C + G and A + T content of ITS region varied from 230 to 273 bp and 288 to 343 bp (Table 3). Sequence analysis indicated that the 5.8S rDNA sequence was identical (158 bp) for all of the tested strains of *P. eryngii*. Ro *et al.* (2007) reported that the average sequence lengths of ITS1, 5.8S and ITS2 of rDNA were 231, 160 and 378 bp, respectively. They also mentioned that 5.8S rDNA sequence was identical for the *P. eryngii* strains, which is similar to

Table 3. Nucleotide distribution, ITS1, 5.8S and ITS2 sequences in fourteen different strains of *P. eryngii*

Strain	Nucleotide distribution						Sequence information			
	A	C	G	T	C + G	A + T	ITS-1	5.8S	ITS-2	Length (bp)
IUM-1550	153	137	136	190	273	343	222	158	236	616
IUM-2106	149	133	134	185	267	334	216	158	227	601
IUM-2126	150	132	134	186	266	336	214	158	230	602
IUM-2137	153	132	134	189	266	342	219	158	231	608
IUM-2143	149	132	134	185	266	334	215	158	227	600
IUM-2161	151	132	134	186	266	337	215	158	230	603
IUM-2253	148	134	134	184	268	332	216	158	226	600
IUM-2263	149	132	133	185	265	334	215	158	226	599
IUM-3140	148	132	131	184	263	332	215	158	222	595
IUM-3487	120	114	116	168	230	288	215	158	145	518
IUM-3501	127	116	117	167	233	294	214	158	155	527
IUM-3865	148	132	131	184	263	332	215	158	222	595
IUM-3920	126	115	117	168	232	294	220	158	148	526
IUM-3939	152	138	135	188	273	340	220	158	235	613

A: Adenine, C: Cytosine, G: Guanine, T: Thymine.

our findings. The size variation was caused by different nucleotide sequence, revealing that these strains were clearly distinguished from each other based on ecological distribution, substitution, insertion or deletion polymorphisms of the base position.

Based on the aligned sequences, we constructed a molecular phylogenetic tree of selected strains. The phylogenetic tree separated out into six groups (Fig. 6). Maximum differences were observed between IUM-3487 (China) and IUM-2263 (Japan). IUM-3487, 3501 and 3920 were collected from China, which was very similar to AY-581425, NCBI gene bank strain, while IUM-3865 (China) was genetically similar (100%) to IUM-3140 (Korea)

strain. Both Korean IUM-3939 and1550 strains were very similar to EU-520192 and DQ-342326, NCBI gene bank strains. Therefore, results indicated that fourteen IUM strains were similar to NCBI gene bank strains of *P. eryngii*. White *et al.* (1990) reported that ITS sequences are genetically constant or show little variation within species, but vary between species in a genus. The sequence of the ITS region of rDNA was variable among the tested strains. Genetic variation within groups was greater than between groups. The high genetic diversity detected within groups is probably due to efficient gene flow and to high genetic compatibility within the tested strains. These results support the data of Zervakis *et al.* (2001).

Table 4. DNA bands in different strains of *P. eryngii* by RAPD assay on 10 base OPA primers

Primers	DNA band (kb)	IUM Strains														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
OPA-01	0.9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.8	+	−	+	+	+	+	+	+	−	+	+	+	−	+	−
	0.6	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+
	0.5	−	−	−	+	+	+	+	+	−	+	+	+	+	−	−
OPA-07	1.2	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+
	0.7	+	−	−	−	−	−	+	+	+	+	−	−	+	+	−
	0.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
	0.5	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+
OPA-08	1.0	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
	0.6	−	−	+	−	−	−	−	−	−	−	+	+	−	−	+
	0.5	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+
OPA-09	1.1	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+
	0.9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.6	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
	0.4	−	+	+	+	+	+	+	+	+	+	−	+	+	−	+
OPA-11	1.4	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
	0.9	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−
	0.7	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+
	0.6	+	−	−	+	+	+	+	+	+	+	+	+	−	+	+
	0.4	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+
OPA-12	1.3	+	−	−	−	−	−	+	+	+	+	−	+	−	+	+
	1.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.7	−	−	−	−	−	+	−	−	−	−	−	−	+	+	−
OPA-13	1.5	+	+	+	+	+	+	+	+	+	+	−	+	−	+	−
	1.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.2	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+
OPA-14	2.0	+	−	−	−	−	−	+	+	+	+	−	+	+	+	−
	0.9	+	+	−	−	+	−	+	+	+	+	+	+	+	+	−
	0.6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
	0.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA-15	1.5	−	−	−	−	−	−	+	+	+	+	−	−	+	+	+
	1.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.7	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+
	0.6	−	−	+	+	+	−	+	−	−	−	+	−	−	−	+

Table 4. Continued

| Primers	DNA band (kb)	IUM Strains														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
OPA-16	1.8	+	+	−	+	+	+	+	+	+	+	−	−	−	−	+
	1.6	+	−	−	−	+	+	+	+	+	+	−	−	−	+	+
	1.0	+	−	−	+	+	+	+	+	+	+	−	−	+	+	+
	0.7	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+
	0.5	−	−	+	−	+	+	−	+	+	−	+	+	−	−	+
OPA-17	2.0	+	−	−	−	−	−	+	+	+	+	−	−	+	−	+
	1.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA-18	1.3	+	+	−	−	+	−	+	+	+	+	−	+	+	+	−
	1.0	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
	0.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.4	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−
OPA-19	1.5	+	−	−	−	−	−	+	+	+	+	−	−	+	−	+
	0.9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA-20	2.3	+	−	−	−	−	−	+	+	+	+	−	−	+	−	−
	1.5	+	−	−	−	+	+	+	+	+	+	−	−	+	+	+
	1.2	+	−	−	−	−	−	+	+	+	+	−	−	+	−	−
	0.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−

Lane 1, IUM-1550; 2, IUM-2106; 3, IUM-2126; 4, IUM-2137; 5, IUM-2143; 6, IUM-2161; 7, IUM-2253; 8, IUM-2263; 9, IUM-3140; 10, IUM-3487; 11, IUM-3501; 12, IUM-3865; 13, IUM-3920; 14, IUM-3939; 15, ATCC-90212; − indicates absence of DNA band; + indicates presence of DNA band.

RAPD analysis. Twenty arbitrary 10 base oligonucleotide primers were used to amplify segments of DNA from fourteen IUM and ATCC-90212 strain of *P. eryngii*. ATCC-90212 strain was used as a control for comparison with IUM strains. Among the tested primers, fourteen primers such as OPA-1, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20 were found efficiently amplify the genomic DNA (Table 4). These efficient primers showed signifi-

cant band profiles on the tested strains, which made them good candidates for screening each strain (Fig. 7, 8 and 9). RAPD-PCR generated distinct, multiple products with considerable variability among the tested strains. The number of amplified bands was variable with both the primers and the strains. The size of the polymorphic fragments obtained ranged from 0.2 to 2.3 kb. Patterns of the DNA bands were consistent between replication tests.
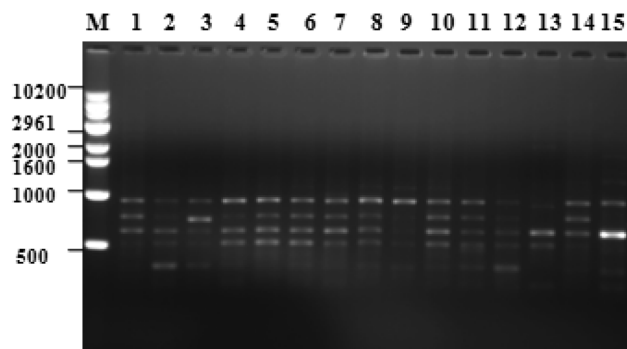

Fig. 7. RAPD profiles in different strains of *P. eryngii* with primer OPA-1. M, molecular size marker (1 kb DNA ladder); lane 1, IUM-1550; 2, IUM-2106; 3, IUM-2126; 4, IUM-2137; 5, IUM-2143; 6, IUM-2161; 7, IUM-2253; 8, IUM-2263; 9, IUM-3140; 10, IUM-3487; 11, IUM-3501; 12, IUM-3865; 13, IUM-3920; 14, IUM-3939; 15, ATCC-90212.
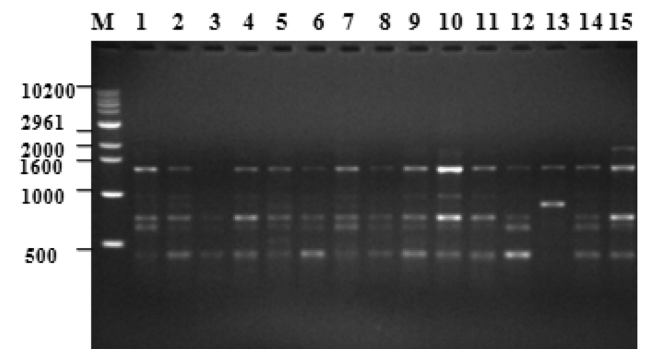
Fig. 8. RAPD profiles in different strains of *P. eryngii* with primer OPA-11. M, molecular size marker (1 kb DNA ladder); lane 1, IUM-1550; 2, IUM-2106; 3, IUM-2126; 4, IUM-2137; 5, IUM-2143; 6, IUM-2161; 7, IUM-2253; 8, IUM-2263; 9, IUM-3140; 10, IUM-3487; 11, IUM-3501; 12, IUM-3865; 13, IUM-3920; 14, IUM-3939; 15, ATCC-90212.
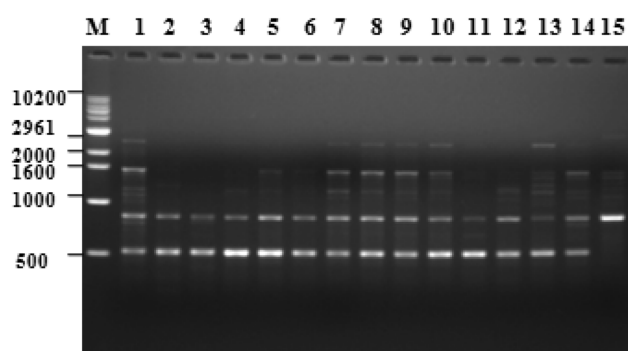
Fig. 9. RAPD profiles in different strains of *P. eryngii* with primer OPA-20. M, molecular size marker (1 kb DNA ladder); lane 1, IUM-1550; 2, IUM-2106; 3, IUM-2126; 4, IUM-2137; 5, IUM-2143; 6, IUM-2161; 7, IUM-2253; 8, IUM-2263; 9, IUM-3140; 10, IUM-3487; 11, IUM-3501; 12, IUM-3865; 13, IUM-3920; 14, IUM-3939; 15, ATCC-90212.
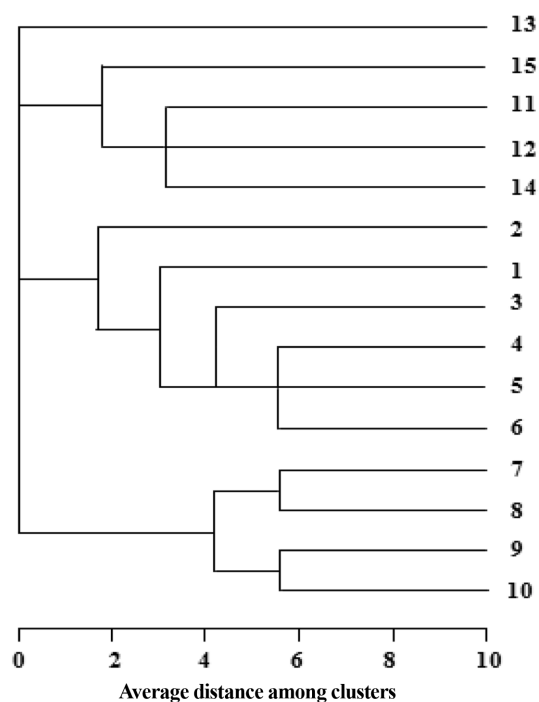
Fig. 10. Dendrogram constructed based on RAPD markers of *P. eryngii* strains determined by average linkage cluster.1, IUM-1550; 2, IUM-2106; 3, IUM-2126; 4, IUM-2137; 5, IUM-2143; 6, IUM-2161; 7, IUM-2253; 8, IUM-2263; 9, IUM-3140; 10, IUM-3487; 11, IUM-3501; 12, IUM-3865; 13, IUM-3920; 14, IUM-3939; 15, ATCC-90212.

Therefore, if a strain is tested for DNA polymorphisms using the same primers, the strain can be determined to be similar or not to the strains in this study, as listed in Table 4. To maximize the specificity of polymorphic patterns, a combined dendrogram was constructed by using RAPD-PCR amplified bands obtained from the fourteen RAPD

primers. Four putative groups among the 15 strains of *P. eryngii* were obtained by cluster analysis based on banding patterns and size of amplified products (Fig. 10). Among the fourteen IUM strains, IUM-3501, IUM-3865 and IUM-3939 were similar to the ATCC-90212 strain. IUM-2253, IUM-2263, IUM-3140 and IUM-3487 were similar and formed a second group. The IUM-3920 strain had different bands compare to all the remaining strains. Our results are comparable to the study made by Alam *et al.* (2009). Results on RAPD analysis were similar to the results obtained by analysis of ITS region sequences. RAPD primers are useful to clarify genetic relationships among strains. Development of a molecular map and strain selection for breeding would be a realistic way to improve yield and quality of this mushroom.

Acknowledgements

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