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Phylogenetic Relationship in Different Commercial Strains of *Pleurotus nebrodensis* Based on ITS Sequence and RAPD

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The molecular phylogeny in nine different commercial cultivated strains of *Pleurotus nebrodensis* was studied based on their internal transcribed spacer (ITS) region and RAPD. In the sequence of ITS region of selected strains, it was revealed that the total length ranged from 592 to 614 bp. The size of ITS1 and ITS2 regions varied among the strains from 219 to 228 bp and 211 to 229 bp, respectively. The sequence of ITS2 was more variable than ITS1 and the region of 5.8S sequences were identical. Phylogenetic tree of the ITS region sequences indicated that selected strains were classified into five clusters. The reciprocal homologies of the ITS region sequences ranged from 99 to 100%. The strains were also analyzed by RAPD with 20 arbitrary primers. Twelve primers were efficient to applying amplification of the genomic DNA. The sizes of the polymorphic fragments obtained were in the range of 200 to 2000 bp. RAPD and ITS analysis techniques were able to detect genetic variation among the tested strains. Experimental results suggested that IUM-1381, IUM-3914, IUM-1495 and AY-581431 strains were genetically very similar. Therefore, all IUM and NCBI gene bank strains of *P. nebrodensis* were genetically same with some variations.

KEYWORDS : ITS, *Pleurotus nebrodensis*, phylogenetic relation, RAPD

Pleurotus nebrodensis is known as the Ballin oyster and white sanctity mushroom (Shen *et al.*, 2005). It is cultivated mainly on cotton seed hulls, sawdust or maize cobs (Tan *et al.*, 2005). According to Tan *et al.* (2005), optimum spawn run temperature range from 25 to 28°C for 22 days. After wards, the temperature should be maintained below 25°C to avoid an excessive mycelial growth. To induce pining, the temperature should be dropped to 10~15°C for 10~15 days (Chang and Miles, 1988). Basidiocarp development requires temperature of 12~15°C. The basidiospores are widely cylindrical, 15~18 × 6~8 μm. Host plants and spore size differ between Italian and Chinese strains. The host plants of Chinese *P. nebrodensis* are *Ferula sinkiangensis* and *F. ferulaeoides* and the Italian host of *P. nebrodensis* is *Cachrys ferulacea* (Venturella, 2000). *P. nebrodensis* is abundant in nutrition including sub-oleic acid, non-saturate fatty acids and many microelements such as calcium, zinc and manganese. It is a good source of dietary fiber and other valuable nutrients. They also contain a number of biologically active compounds with therapeutic activities such as modulation of the immune system, inhibition of tumor growth and inflammation, hypoglycemic and antithrombotic activities, decreasing blood lipid concentrations, prevention of high blood pressure and atherosclerosis (Choi *et al.*, 2005; Wang and Ng, 2004).

Pleurotus nebrodensis has complicated morphological variations of basidiospores, resulting in taxonomic confusion and difficulties in delimiting species boundaries (Venturella, 2000). Recent molecular phylogenetic studies have demonstrated that the internal transcribed spacer (ITS) region of genomic DNA is very useful for assessing phylogenetic relationships at lower taxonomic levels. ITS sequence comparisons are becoming increasingly popular tools for phylogenetic analysis and for the differentiation of populations. The internal transcribed spacer of rDNA is considered as a variable region among the species and even among the strains (Paul, 2002).

Among the molecular approaches, the random application of polymorphic DNA (RAPD) is a convenient method for detecting genetic diversity (Park *et al.*, 2004; Tuchwell *et al.*, 2005). Recent genetic analysis on the fungal species has shown that RAPD was superior to rDNA sequence based methods when distinguishing strains within species. RAPD was particularly successful when applied for verifying mushroom strains from various hosts with a wide range of geographical origins (Lopandic *et al.*, 2005). The present work was carried out to investigate the genetic relationship in different cultivated stains of *P. nebrodensis* using both ITS and RAPD analysis.

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Materials and Methods

Mushroom strains and DNA extraction. IUM-1381, IUM-1495, IUM-2210, IUM-2235, IUM-3061, IUM-3424, IUM-3514, IUM-3914 and IUM-3918 strains of *Pleurotus nebrodensis* were used in this study. These strains were obtained from the Culture Collection of Mushrooms (CCM) in the Department of Biology, University of Incheon and were collected in various locations of China in different times. Five strains of *P. nebrodensis* such as AY-581429, AY-581430, AY-581431, AY-581432 and AY-581433 were used as control strains for the comparative study of our selected strains. Sequencing data of control strains were collected from the NCBI gene bank data base.

Genomic DNA was extracted according to the procedure of Lee and Taylor (1990) with some modifications as follows. Fresh mycelia were collected from the 10 days old culture on PDA medium and were frozen with liquid nitrogen. Frozen mycelia were grounded with sterilized mortar-pestle and kept in 1.5 *ml* micro tube. As extraction buffer, equal amount 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 min. After incubation, same amount of PCI (25 *ml* phenol: 24 *ml* chloroform: 1 *ml* isoamyl-alcohol) was added, vortexed and centrifuged at 4°C, 10 min, 12000 rpm. After wards, only supernatant of upper part was taken in 1.5 *ml* micro tube, added 1000 μl of 99.9% alcohol and centrifuged at 4°C, 5 min, 12000 rpm. In this case, supernatant was removed, added 500 μl of 70% alcohol with precipitated DNA, vortexed and centrifuged at 4°C, 5 min, 12000 rpm. Again supernatant was removed and waited until residual alcohol evaporated. Finally 500 μl of sterilized distilled water was added. 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. nebrodensis* was amplified by polymerase chain reaction (PCR) using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Amplification reactions were performed in a total volume of 20 μl containing 10 × PCR buffer 2 μl, dNTP 1.6 μl, 0.5 μl of each primer, 0.2 μl of Taq polymerase, 1 μl of genomic DNA and 14.2 μl of sterilized distilled water. PCR reaction was performed using 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 extension for 10 minutes at 72°C. Amplification products were electrophoresed by a 1.5% agarose gel with a 1 kb DNA ladder as a marker. ITS sequences were aligned for phylogenetic analysis using the program Cluster W (Thompson *et al.*, 1994). Phylogenetic tree was constructed by Neighbor-joining method using 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 sorts of arbitrary 10-base oligonucleotide primers (Operon Technologies Inc.) were used to produced amplified fragments. The primer sequences are listed in Table 1. 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 extension for 7 minutes at 72°C. RAPD products were electrophoresed on 1.4% agarose gel in 1 × TAE 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% $\mu g/ml$). 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). The similarity coefficients were calculated between strains across band for all primers.

Table 1. 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

Results and Discussion

ITS sequence analysis. To study the genetic variation of selected strains of *P. nebrodensis*, the ITS region was amplified using ITS1 and ITS4 primers and sequenced. The PCR products of the ITS region in nine different strains were confirmed to be in the range of 575 to 625 bp (Fig. 1). Results indicated that a length polymorphism at the sequence level ranged from 592 to 614 bp. The size of the ITS1 and ITS2 regions varied among the strains from 219 to 228 bp and 211 to 229 bp, respectively (Table 2). Total C+G and A+ T contents of ITS region varied from 262 to 270 bp and 330 to 368 bp. The DNA sequence for multiple alignments including all of the ITS1, 5.8S and ITS2 regions are presented in Fig. 2. Sequence analysis showed that the 5.8S rDNA sequence was identical (158 bp) for all of the tested strains of *P. nebrodensis*. Kawai *et al.* (2008) reported that the ITS region consisting of ITS1, 5.8S and ITS2 range from 633 to 635 bp in the Bai-ling-Gu and A-Wei-Mo strains of *P. nebrodensis*. The size variation was caused by different nucleotide sequences, revealing that these strains were clearly distinguished from each other based on substitution, insertion or deletion polymorphism of the base position except IUM-

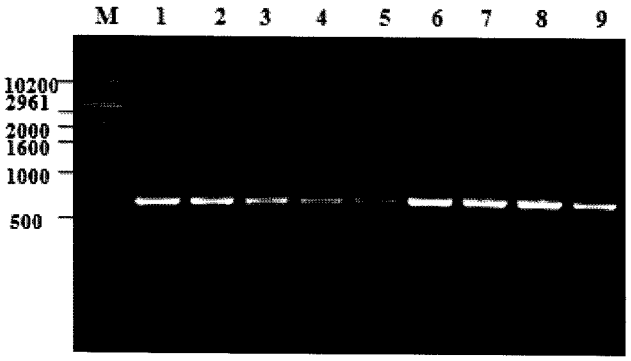

Fig. 1. PCR products of the ITS region in nine different strains of *Pleurotus nebrodensis*. M, molecular size marker (1 kb DNA ladder); lane 1, IUM-3514; 2, IUM-3918; 3, IUM-2235; 4, IUM-3061; 5, IUM-3424; 6, IUM-1381; 7, IUM-1495; 8, IUM-3914 and 9, IUM-2210.

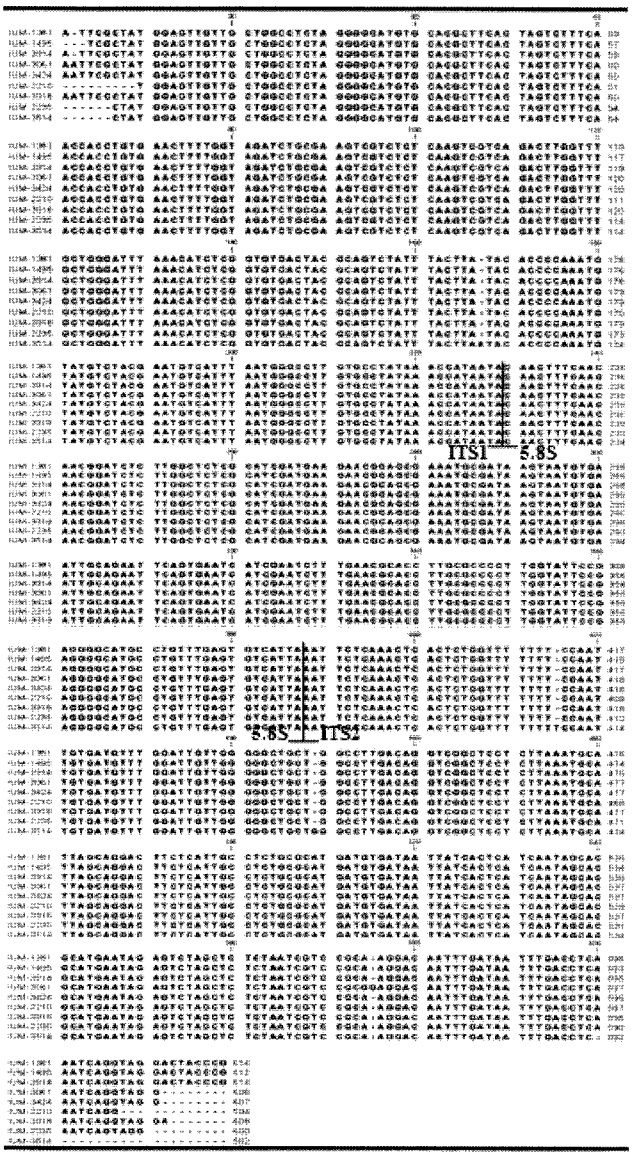

Fig. 2. Multiple sequence alignments of the ITS1 region in different strains of *Pleurotus nebrodensis*.

Table 2. Nucleotide distribution, ITS-1, 5.8S and ITS-2 sequence in nine different strains of *Pleurotus nebrodensis*

Strain	Nucleotide distribution (bp)						Sequence information (bp)			
	A	C	G	T	C+G	A+T	ITS-1	5.8S	ITS-2	Total Length
IUM-3514	143	133	129	187	262	330	223	158	211	592
IUM-3918	150	136	132	190	268	340	228	158	222	608
IUM-2235	147	134	131	188	265	335	222	158	220	600
IUM-3061	148	135	135	190	270	368	228	158	222	608
IUM-3424	149	135	133	190	268	339	228	158	221	607
IUM-1381	150	139	134	191	273	341	227	158	229	614
IUM-1495	149	139	134	190	273	339	225	158	229	612
IUM-3914	150	139	134	191	273	341	227	158	229	614
IUM-2210	145	133	130	186	263	331	219	158	217	594

A, Adenine; C, Cytosine; G, Guanine and T, Thymine

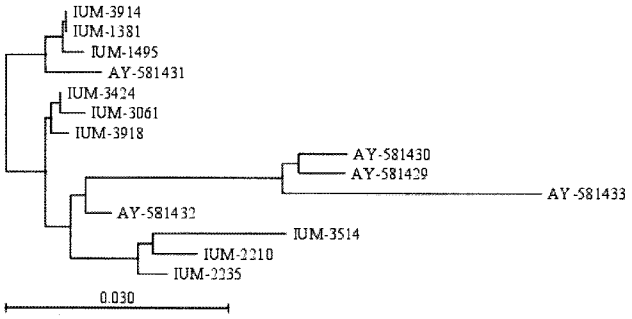

Fig. 3. Phylogenetic tree of fourteen strains of *Pleurotus nebrodensis* based on the nucleotide sequence of the ITS region using neighbor joining method with 1000 bootstrapping.

1381 and IUM-3914.

The phylogenetic tree based on the nucleotide sequence of ITS region in fourteen different strains of *P. nebrodensis* was obtained by the neighbor joining methods (Fig. 3). Reciprocal homologies of the ITS region sequences ranged from 99 to 100%. White *et al.* (1990) reported that ITS sequences are generally constant, or show little variation within species, but vary between species in a genus. The ITS region is relatively short and can be easily amplified by PCR using universal single primer pairs. Genetic distance exhibited high level of similarity with identical ITS sequences. The maximum difference was observed between IUM-3424 and AY-581433 strains, while maximum similarity (99.53%) was recorded in between AY-581431 and IUM-1381, IUM-1495 and IUM-3914 strains. Results on the phylogenetic tree in fourteen strains of *P. nebrodensis* indicated that nine IUM strains were very similar to five NCBI gene bank strains. Base sequences of the ITS region of rDNA were variable among the tested strains and can be used to estimate genetic distances and provide information on phylogenetic study. Our results are comparable to the study made by Bruns *et al.* (1991).

RAPD analysis. Twenty primers were used to amplify the segments of DNA in nine different strains of *P. nebrodensis*. Among the 20 primers, 12 primers, OPA-01, OPA-02, OPA-3, OPA-05, OPA-07, OPA-09, OPA-10, OPA-11, OPA-12, OPA-13, OPA-18 and OPA-20 were found to be efficient for amplifying the genomic DNA (Table 3). These 12 primers showed significant band profiles on the tested strains and high possibilities to screening of each strain (Fig. 4, 5 and 6). The size of these polymorphic fragments was in the range of 0.2 to 2.0 kb. Polymorphism of DNA bands showed the same characters in the replication tests. Therefore, if a certain strain is tested for DNA polymorphisms using the same primers, it could be identified whether the strain is the similar or not by consulting Table 3. The dendrogram was made by average linkage cluster analysis with the statistics on the presence or absence of bands by strains in Table 3.

Table 3. DNA bands in different strains of *Pleurotus nebrodensis* by RAPD assay on 10 base OPA primers

Primers	DNA band (kb)	IUM-Strains								
		1	2	3	4	5	6	7	8	9
OPA-01	1.7	+	–	–	–	+	+	+	–	–
	1.4	+	–	–	–	–	+	+	+	+
	1.2	+	+	+	+	+	+	+	+	+
OPA-02	2.0	+	+	–	–	+	+	+	+	–
	1.5	+	–	–	–	+	–	–	–	–
	0.9	–	+	+	+	+	+	+	+	+
	0.6	+	+	–	v	+	–	–	–	–
OPA-03	1.0	+	+	–	–	–	+	+	+	–
	0.8	+	+	+	+	–	+	+	+	+
	0.7	–	+	+	+	–	+	+	+	–
	0.4	+	+	+	+	+	+	+	+	+
OPA-05	1.5	+	–	–	–	–	+	+	–	–
	0.7	+	–	–	–	+	–	–	–	–
	0.2	–	+	+	+	+	+	+	+	+
OPA-07	1.5	+	+	+	+	+	+	+	+	+
	1.3	+	+	+	+	–	+	+	+	–
	1.0	–	+	+	+	+	+	+	+	–
	0.6	–	+	+	+	+	+	+	+	+
OPA-09	2.1	+	–	–	–	–	–	–	–	–
	1.3	+	+	–	–	+	+	+	+	–
	1.0	+	+	+	+	–	+	+	+	+
	0.9	+	+	+	+	–	+	+	+	+
OPA-10	1.2	–	–	–	–	–	+	+	+	–
	1.0	–	+	+	+	–	+	+	+	+
	0.7	+	+	+	+	–	+	+	+	+
	0.5	–	–	–	–	+	–	–	–	–
OPA-11	1.7	–	–	–	–	+	–	–	–	–
	1.2	+	–	–	–	–	–	–	–	–
	0.6	–	+	+	+	+	+	+	+	+
	0.3	–	+	+	+	+	+	+	+	+
OPA-12	1.5	+	–	–	–	–	–	–	–	–
	0.7	+	–	–	–	–	–	–	–	–
	0.2	–	+	+	+	+	+	+	+	+
OPA-13	1.7	+	–	–	–	–	–	–	–	–
	1.1	+	+	+	+	+	+	+	+	+
	0.9	+	+	+	+	+	+	+	+	+
	0.5	–	+	+	+	–	+	+	+	+
OPA-18	1.1	–	–	–	+	+	+	+	+	–
	0.8	–	–	–	–	+	–	–	–	–
	0.6	–	+	+	+	–	+	+	+	+
	0.2	+	+	+	+	–	–	–	–	–
OPA-20	1.5	+	–	–	–	–	+	+	+	–
	0.8	+	+	+	+	+	+	+	+	+
	0.4	–	+	+	+	+	+	+	+	+

1, IUM-3514; 2, IUM-3918; 3, IUM-2235; 4, IUM-3061; 5, IUM-3424; 6, IUM-1381; 7, IUM-1495; 8, IUM-3914; 9, IUM-2210, - indicate absence of DNA band, + indicate presence of DNA band.

The dendrogram based on RAPD markers in nine different strains of *P. nebrodensis* is shown in Fig. 7. RAPD

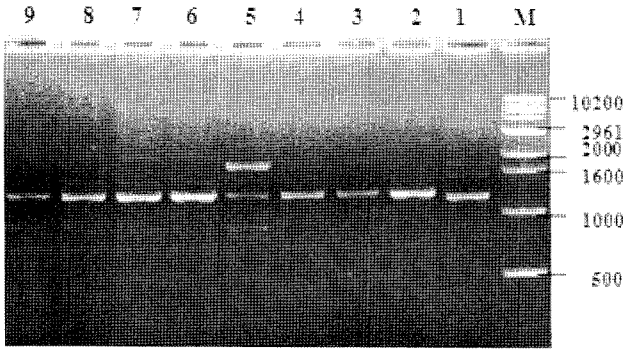

Fig. 4. RAPD profiles in different strains of *Pleurotus nebrodensis* with primer OPA-1. M, molecular size marker (1 kb DNA ladder); lane 1, IUM-3514; 2, IUM-3918; 3, IUM-2235; 4, IUM-3061; 5, IUM-3424; 6, IUM-1381; 7, IUM-1495; 8, IUM-3914 and 9, IUM-2210.

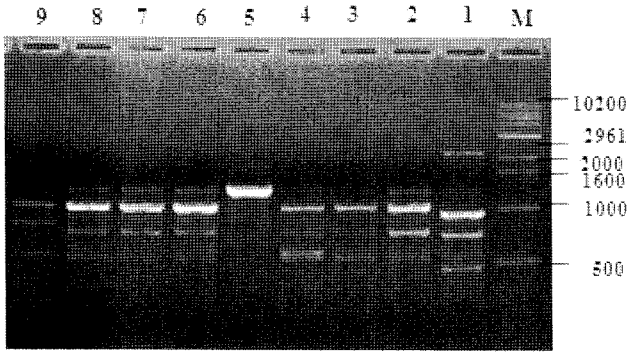

Fig. 5. RAPD profiles in different strains of *Pleurotus nebrodensis* with primer OPA-9. M, molecular size marker (1 kb DNA ladder); lane 1, IUM-3514; 2, IUM-3918; 3, IUM-2235; 4, IUM-3061; 5, IUM-3424; 6, IUM-1381; 7, IUM-1495; 8, IUM-3914 and 9, IUM-2210.

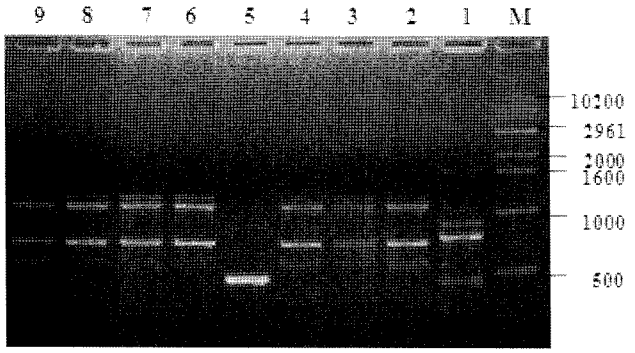

Fig. 6. RAPD profiles in different strains of *Pleurotus nebrodensis* with primer OPA-10. M, molecular size marker (1 kb DNA ladder); lane 1, IUM-3514; 2, IUM-3918; 3, IUM-2235; 4, IUM-3061; 5, IUM-3424; 6, IUM-1381; 7, IUM-1495; 8, IUM-3914 and 9, IUM-2210.

data indicated that strains 6 (IUM-1381), 7 (IUM-1495) and 8 (IUM-3914) were very similar with few exceptions compared to others strains. In most of cases, strain 5

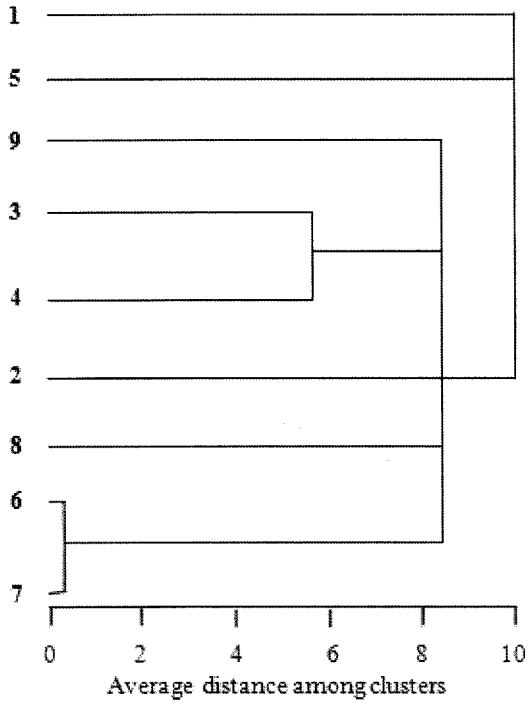

Fig. 7. Dendrogram constructed based on RAPD markers of *Pleurotus nebrodensis* strains determined by average linkage cluster. 1, IUM-3514; 2, IUM-3918; 3, IUM-2235; 4, IUM-3061; 5, IUM-3424; 6, IUM-1381; 7, IUM-1495; 8, IUM-3914 and 9, IUM-2210.

(IUM-3424) had different bands compared to all the remaining strains. The results of RAPD analysis were almost similar to the results obtained by the analysis of ITS region sequences. Similar results have been reported by Ro *et al.*, 2007 and Lee *et al.*, 1997 in the phylogenetic classification of some strains of *Pleurotus eryngii* and *Lentinus edodes* mushrooms, respectively.

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