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Development and Characterization of New Microsatellite Markers for the Oyster Mushroom (*Pleurotus ostreatus*)

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We developed and characterized 36 polymorphic microsatellite markers for the oyster mushroom (*Pleurotus ostreatus*). In total, 169 alleles were identified with an average of 4.7 alleles per locus. Values for observed (H_O) and expected (H_E) heterozygosities ranged from 0.027 to 0.946 and from 0.027 to 0.810, respectively. Nineteen loci deviated from Hardy–Weinberg equilibrium. Significant ($P<0.05$) excess heterozygosity was observed at nine loci. Linkage disequilibrium (LD) was significant ($P<0.05$) between pairs of locus alleles. Cluster analysis revealed that five species of genus *Pleurotus* made a distinct group, and the individual cultivars were grouped into major five groups from G-1 to G-5. The diverse cultivars of *P. ostreatus* were discriminated and the other four species revealed a different section in the UPGMA tree. These microsatellite markers proved to be very useful tools for genetic studies, including assessment of the diversity and population structure of *P. ostreatus*.

Keywords: Oyster mushroom, genetic diversity, heterozygosity, microsatellite markers, *Pleurotus ostreatus*

The genus *Pleurotus* is a cosmopolitan group, including several cultivated species such as *P. ostreatus*, *P. cornucopiae*, *P. sajor-caju*, *P. eryngiim*, *P. cystidiosus*, and *P. pulmonarius* [5]. The oyster mushroom (*Pleurotus ostreatus*) is commercially important in the world mushroom market, particularly in East Asia. It is the most popular edible mushroom in Korea, consisting of approximately 32% of the entire mushroom production in Korea [12]. Besides its importance for food production, *P. ostreatus* is important in applications such as paper pulp bleaching, cosmetics, and other potential industrial uses [1, 3, 14, 15]. *Pleurotus ostreatus* also plays a role in increasing macrophage and lymphocyte activities [7], reducing cholesterol levels [2], enhancing the anti-complementary properties of polysaccharides [8], and increasing antihepatoma and antisarcoma activities [16]. These applications have stimulated research on specific aspects of the molecular biology of the organism.

Cultivars of the oyster mushroom *P. ostreatus* are readily affected by environmental conditions, making them difficult to differentiate. Disputes between farmers and spawn suppliers related to cultivated strains are becoming more frequent. As *Pleurotus* is the most commonly cultivated edible mushroom, and its consumption is continuously increasing, many attempts have been made to standardize the distribution of various *Pleurotus* cultivars for mushroom farming [5, 10, 11]. Identical strains with different commercial names or different strains with the same name often occur in the cultivation and spawn market. Incorrectly designated strains can result in huge economic losses for farmers. Therefore, precise identification and classification of commercial lines of edible *Pleurotus* spp. strains are of major importance. The aim of this work was to develop a rapid and accurate strain discrimination system for commercial *Pleurotus* strains using simple sequence repeat (SSR) markers.

A microsatellite-enriched library was constructed using a modification of the biotin-streptavidin capture method of Dixit *et al.* [4]. Briefly, total genomic DNA of oyster mushroom was digested with seven restriction enzymes (EcoRV, DraI, SmaI, PvuI, AluI, HaeIII, and RsaI) in separate reactions. The pooled digest was size-fractionated on a 1.5% agarose gel. Fragments ranging from 300 to 1,500 bp were eluted from the gel followed by purification using a gel extraction kit (QIAGEN). DNA fragments (1 μg) were ligated with 1 μg of double-stranded adaptor molecules (AP11-5'-CTCTTGCTTAGATCTGGACTA-3' and AP12-5'-

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TAGTCCAGATCTAAGCAAGAGCACA-3'). The adaptor-ligated DNA was hybridized with a mixture of biotin-labeled SSR probes [$(GA)_{20}$, $(AGC)_{15}$, $(GGC)_{15}$, $(AAG)_{15}$, $(AAC)_{15}$, $(AGG)_{15}$]. The hybridized DNA fragments were captured with streptavidin-coated magnetic beads (Promega). After stringent washing, the captured DNA fragments were eluted in 50 ml of distilled water. Final eluates were amplified with AP11 primers and cloned into a pGEM-T easy vector (Promega). In total, 638 recombinant clones were randomly picked from primary transformation plates containing ampicillin (100 μg/ml), X-gal (2 mg), and IPTG (8 μM). Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (QIAGEN) and sequenced using an ABI 3100 DNA sequencer with a BigDye terminator kit (Applied Biosystems).

SSR identification within cloned sequences and primer design were carried out using the SSR MANAGER program [6]. Among 638 sequenced clones, 144 (22.6%) were redundant. Of the remaining 494 unique clones, 201 (40.7%) were suitable for the design of SSR primer pairs for microsatellite sequences, while other clones' SSR motif leaned to the end side of the sequence which was difficult to design primer sets. In total, 201 primer pairs were designed and evaluated for polymorphism against a panel of 10 oyster mushroom samples using a procedure described earlier [4]. Thirty-six primer pairs produced reproducible polymorphic bands and were further characterized using a diverse set of 20 accessions. The M13-tail PCR method of Schuelke was used to measure the size of PCR products [13]. The method involves three primers; the forward SSR-specific primer with the M13 (5'-TGTAAAACGACGGCC AGT-3') tail at the 5' end, the reverse SSR-specific primer, and a phosphoramidite fluorescent dye-labeled (FAM, HEX, or NED) M13 (5'-TGTAAAACGACGGCCAGT-3') universal primer. The amount of forward primer was adjusted to less than half of the reverse primer. Microsatellite alleles were resolved on an ABI PRISM 3100 DNA sequencer (Applied Biosystems) using GENESCAN 3.7 software and sized using GeneScan 500 ROX (6-carbon-X-rhodamine) molecular size standards (35 to 500 bp) with GENOTYPER 3.7 software (Applied Biosystems) (Fig. 1).

A total of 37 cultivars belonging to genus *Pleurotus*, collected from the Agricultural Sciences Institute, were chosen for variablilty test and cluster analysis (Table 1). The variability at each locus was measured in terms of the number of alleles, observed heterozygosity (H_O), and expected heterozygosity (H_E), using the genetic analysis package POPGENE Version 1.31 [17]. The same program was used to test Hardy–Weinberg equilibrium (HWE) and pair–wise linkage disequilibrium (LD). In total, 169 alleles were detected with an average of 4.7 alleles per locus. Values for H_O and H_E ranged from 0.027 to 0.946 (mean=0.398) and from 0.027 to 0.810 (mean=0.549), respectively (Table 2). Nineteen loci deviated from HWE ($P<0.001$). The analysis also revealed significant ($P<0.05$)

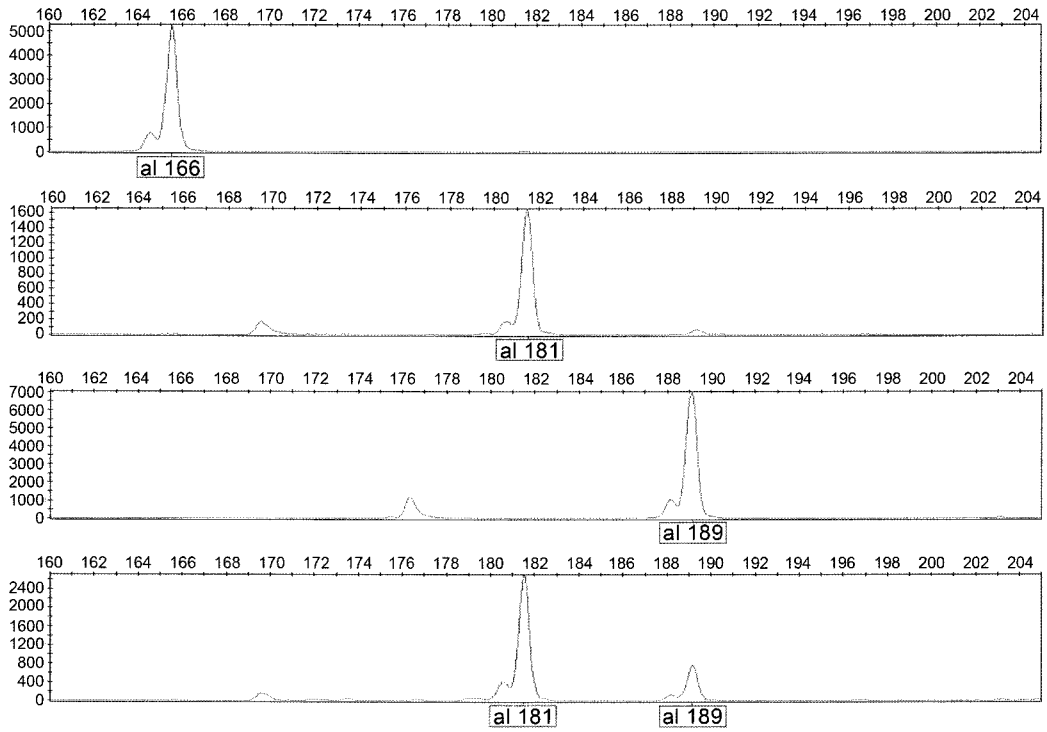

Fig. 1. An example of the M13-tail PCR method.
The PCR products were resolved on an ABI PRISM 3130 DNA sequencer.

Table 1. Thirty-seven strains of genus *Pleurotus* used in the study.

No.	ASI No.	Species	Commercial Name	Source	Year
1	2830	*P. ostreatus*	Baekdu1	Korea	2005
2	2001	*P. ostreatus*	Nonggi2-1	Korea (MKACC52243)	1971
3	2072	*P. ostreatus*	Nonggi202	Korea (MKACC51410)	1980
4	2180	*P. ostreatus*	Wonhyeong	Korea (MKACC51493)	1990
5	2183	*P. ostreatus*	Wonhyeong2	Korea (MKACC51496)	1990
6	2240	*P. ostreatus*	Wonhyeong3	Korea (MKACC52342)	1994
7	2706	*P. ostreatus*	Heukpyeong	Korea (MKACC52328)	2001
8	2506	*P. ostreatus*	Gyunhyeop1	Korea (KACC360)	2000
9	2488	*P. ostreatus*	Myeongweol	Korea (MKACC51732)	1999
10	2549	*P. ostreatus*	Sinnong94	Korea (MKACC52282)	2000
11	2595	*P. ostreatus*	Suhan2	Korea (MKACC51818)	2001
12	2593	*P. ostreatus*	JanganPK	Korea (MKACC52326)	2001
13	2707	*P. ostreatus*	Kimjae9	Korea (MKACC52311)	2001
14	2708	*P. ostreatus*	Kimjae10	Korea (MKACC52312)	2001
15	2710	*P. ostreatus*	Heungrim1	Korea (MKACC52314)	2001
16	2722	*P. ostreatus*	Jangan6	Korea (MKACC52324)	2002
17	2724	*P. ostreatus*	Nongong99	Korea	2003
18	2727	*P. ostreatus*	Sinnong12	Korea	2003
19	2789	*P. ostreatus*	Samguhwanghak	Korea	2004
20	2790	*P. ostreatus*	SamguPJ	Korea	2004
21	2793	*P. ostreatus*	Hanra2	Korea	2004
22	2858	*P. cornucopiae* var. *citrinopileatus*	Geumbit	Korea	2007
23	2851	*P. ostreatus*	Nongmin59	Korea	2006
24	2228	*P. ostreatus*	Chunchu1	China (MKACC51529)	1994
25	2344	*P. ostreatus*	Chunchu2	Netherland (MKACC51632)	1995
26	2333	*P. sajor-caju*	Yeoreum2	Korea (MKACC51621)	1995
27	2394	*P. eryngii*	Keunneutari3	Japan (MKACC52327)	1997
28	2720	*P. nebrodensis*	Baeksongi	Korea (MKACC52323)	2002
29	2825	*P. ostreatus*	Sinnong14	Korea	2005
30	2018	*P. ostreatus*	Nonggi201	Korea (MKACC51362)	1978
31	2535	*P. ostreatus*	Byeongneutari1	Korea (MKACC51778)	2000
32	2477	*P. ostreatus*	Heukjinju	Korea (MKACC51724)	1999
33	2594	*P. ostreatus*	Ilseong2	Korea (MKACC51817)	2001
34	2721	*P. ostreatus*	Jangan5	Korea	2002
35	2728	*P. ostreatus*	Sinnong13	Korea	2003
36	2791	*P. ostreatus*	Samgu01	Korea	2004
37	2070	*P. sajor-caju*	Yeoreum	India (MKACC52247)	1982

ASI, Agricultural Sciences Institute, Suwon, Korea; KACC, Korea Agricultural Culture Collection; MKACC, Mushroom Korea Agricultural Culture Collection; Year, Collected year.

excess heterozygosity for nine loci. LD was significant ($P<0.05$) between pairs of locus alleles. Cluster analysis was performed in PowerMarker version 3.23 software with the SharedAllele method to calculate genetic distance and was checked for discriminative power of the microsatellite markers [9]. The individual cultivars were grouped to mainly five groups from G-1 to G-5. All four cultivars in G-1 were *P. ostreatus*, and G-2 included one distinct cultivar of *P. sajor-caju*. G-3 included 12 cultivars; one *P. eryngii* and 11 *P. ostreatus*. G-4 included 14 cultivars; one *P. cornucopiae* and 13 *P. ostreatus*. G-5 included seven cultivars; one *P. nebrodensis*, one *P. sajor-caju*, and five *P. ostreatus* (Fig. 2). The diverse cultivars of *P. ostreatus* were discriminated, and the other four species revealed a different section in the UPGMA tree. The microsatellite markers reported here provide very useful tools for several genetic studies, including assessment of the diversity and population structure of *P. ostreatus*.

Table 2. Characteristics of 36 microsatellite loci developed from an enriched library of oyster mushroom (*Pleurotus ostreatus*).

Locus	GenBank Accession No.	Primer sequence		Repeat motif	T_A (°C)	N_A	Size range (bp)	H_O	H_E	F_{IS}
		Forward	Reverse							
GB-PO-001	EU502619	CGCAAGCTACAAACGGAC	AGCAGCAAGCACAAGAGC	$(GCA)_3(ATTGGC)(GCA)_1$	52	2	307–313	0.182	0.298	0.402
GB-PO-006	EU502620	TGTGGCAAACCCAAGTTC	CCCAAAGGATGAGGAAGG	$(GGC)_4(GAC)(GGC)_1$	52	3	203–227	0.324	0.517	0.387
GB-PO-011[a]	EU502622	TCCCATACCCTGACATCG	ATCATCAAGCGCCACAAC	$(CTG)_4(TA)(CTG)_1$	52	7	166–262	0.265	0.783	0.670
GB-PO-025	EU502624	TGATCATGGCGAGTAGGG	GGAACTGTCAGCAGACGC	$(GGA)_{10}$	52	3	211–217	0.314	0.269	−0.154
GB-PO-026	EU502625	AATCGCATGGGCTCTG	CTGTCCCTCCGTGTACCA	$(TTG)_3(TTC)(TTG)_3$	52	2	293–296	0.027	0.027	0.000
GB-PO-028[a]	EU502626	CTGGAGAATCGTAGCCCC	ACAAGCGCTCGGAATACA	$(GTC)_{13}(CAT)_5$	52	8	285–327	0.622	0.805	0.240
GB-PO-039	EU502627	TGTGGATGTGATGTGATGTG	ACGTCCAGCGTCGAGTTA	$(GGT)_2(GGC)(GGT)_5$	52	5	191–236	0.568	0.594	0.058
GB-PO-050[a]	EU502628	CATCCGATACAGACCCGA	AGGCATCCCACAACACTG	$(GTT)_5$	52	8	145–175	0.351	0.810	0.576
GB-PO-051[a]	EU502629	CATAGGGACGACAGCGAG	ACTGAGCCTTCAGCACCA	$(GCT)_6$	52	4	269–278	0.568	0.653	0.144
GB-PO-061[a]	EU502631	TAACTTGGGCGCTTGAAA	TGGAACGCGTAGACTTGG	$(GCA)_2(CAGTAC)(GCA)_3$	52	3	252–270	0.206	0.355	0.432
GB-PO-064[a]	EU502632	GTTCTGAGGGTTGAGGGG	CCAACCACACTCTTCCCA	$(GTT)_5(ATT)(GTT)_3$	52	5	209–230	0.086	0.236	0.645
GB-PO-076	EU502633	TCGATTGTCAGATTGTTGGA	CGGAGAAGCAGTTGGTTG	$(GGC)_6$	52	5	233–251	0.405	0.663	0.400
GB-PO-079[a]	EU502634	ACCCAGACGATTTGGGAG	AGGCTGGCGTGGAATACT	$(GGA)_4(AGA)(GGA)_2$	52	9	221–329	0.541	0.739	0.281
GB-PO-080	EU502635	CACCCATGTGCCTCAGTC	TGTCTATGGGTTACGGCG	$(GGC)_6$	52	2	230–233	0.324	0.394	0.191
GB-PO-086[a]	EU502636	CATCTTCGATGAACCGGA	CGAAGATGAGCCAGCAAC	$(GA)_3(GGAGAAGC)(GA)_3$, $(CGA)_3$	52	4	376–446	0.081	0.242	0.673
GB-PO-094[a]	EU502637	CGCGAGACAATTAAACGC	ACAGTTCCTGGAGCCCAT	$(CCT)_2(TCT)(CCT)_2$	52	4	303–324	0.243	0.349	0.316
GB-PO-097[a]	EU502640	CATGGAGAGAGGGCGG	CGTTTCATCGTTCGCTGT	$(GGT)_5$	52	5	241–253	0.486	0.679	0.296
GB-PO-102[a]	EU502638	TGTCTATGGGTTACGGCG	TGCAAAGCAAATCGGAAC	$(GCC)_4(GC)(GCC)_1$	52	2	266–269	0.222	0.494	0.560
GB-PO-113[a]	EU502639	GTTCATCTGAACGCCGTC	CCTATGACGAGGGGAAGG	$(CGC)_5$	52	2	270–375	0.108	0.339	0.688
GB-PO-115	EU502641	TGGTAGCAGGTTGTTGGG	CCGCTAAGCCACTGTTTG	$(TGC)_5(GCTGGC)(TGC)_4$	52	6	213–234	0.811	0.665	−0.205
GB-PO-117	EU502642	TCAAACTCACGTGGTACGC	TCACATATCCGCCGGTAG	$(TGC)_7$	52	4	218–227	0.622	0.623	0.017
GB-PO-124	EU502643	TGCGTTTGCTCGGTTAAT	CGCTACTACGTCGATCCG	$(CG)_5$	52	8	264–300	0.919	0.732	−0.243

[a]Loci deviated from the Hardy–Weinberg equilibrium (HWE); T_A, annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, Wright's fixation index.

Table 2. Continued

Locus	GenBank Accession No.	Primer sequence		Repeat motif	T_A (°C)	N_A	Size range (bp)	H_O	H_E	F_{IS}
		Forward	Reverse							
GB-PO-128	EU502644	TGATTGGTTTGAATGGGC	GCACGATGAGGATGCAGT	$(GTT)_{10}$	52	11	214–247	0.568	0.801	0.304
GB-PO-131	EU502645	CTCCCTCCTCCGTGTACC	CGTAACGTTCGCTTCCTG	$(CCT)_2(CCCC)(CCT)_2$	52	3	200–212	0.730	0.528	−0.371
GB-PO-134	EU502647	GAGTGTGAAGAATCGGCG	GTGCACTCTGCCTATCGC	$(GA)_2(GT)(GA)_3$	52	3	115–285	0.757	0.561	−0.337
GB-PO-135	EU502646	AGGAGGGGGTGCTTGATA	TCCTCCGCCTTCTCTACC	$(GGA)_2(GGGA)(GGA)_2$	52	3	217–271	0.162	0.151	−0.061
GB-PO-138[a]	EU502648	TATGGAACGGTGCGAAGT	GCCGTCAAAAGGGAACTC	$(CCG)_4$, $(TTC)_3$	52	4	191–209	0.946	0.723	−0.296
GB-PO-149[a]	EU502649	AGTGCATATGCCCGACAC	CGTCGTAGATGCAGGCTC	$(TCC)_8$	52	7	193–277	0.297	0.746	0.610
GB-PO-152	EU502650	ACTGAGCCTTCAGCACCA	CATAGGGACGACAGCGAG	$(AGC)_5$	52	4	272–395	0.500	0.615	0.201
GB-PO-154[a]	EU502651	GTCGTAGCCAGCCATGAG	AGGGTATCTCGGGTGCAT	$(CGA)_7$	52	5	254–269	0.649	0.698	0.084
GB-PO-157[a]	EU502652	ATGGACGTGGTGTTCTGC	AAACCAAGCCTACCCAGC	$(GCT)_4$	52	6	281–305	0.514	0.656	0.231
GB-PO-171[a]	EU502654	TCTCGGGCATCATTCTTG	ACGTCAGGGTGTCAAACG	$(TTG)_3$, $(TA)_4$, $(TA)_4$	52	4	295–310	0.194	0.674	0.718
GB-PO-172	EU502655	GCAGAAGTTGCCCAAAGA	ATGTCCAGCGGAAGACCT	$(TGC)_2(TAC)(TGC)_5$	52	3	169–229	0.306	0.422	0.290
GB-PO-173[a]	EU502656	ATGAAGTGTGAGCCGTGG	TGTCCATTCATGCGTTCA	$(GT)_8$	52	5	294–309	0.114	0.748	0.851
GB-PO-181[a]	EU502657	TTATTGTGAAGCCCCCG	GACATCGGCAGAAGGTCA	$(CAG)_1(CAA)(CAG)_5$	52	4	240–249	0.054	0.595	0.911
GB-PO-190	EU502658	TTTCCATTTCCGTTGGTG	CAGGGGGTGATTATGCAA	$(TGC)_3(TGT)(TGC)_2$	52	6	186–222	0.278	0.579	0.531
Mean						4.7		0.398	0.549	
SD						2.16		0.25	0.21	

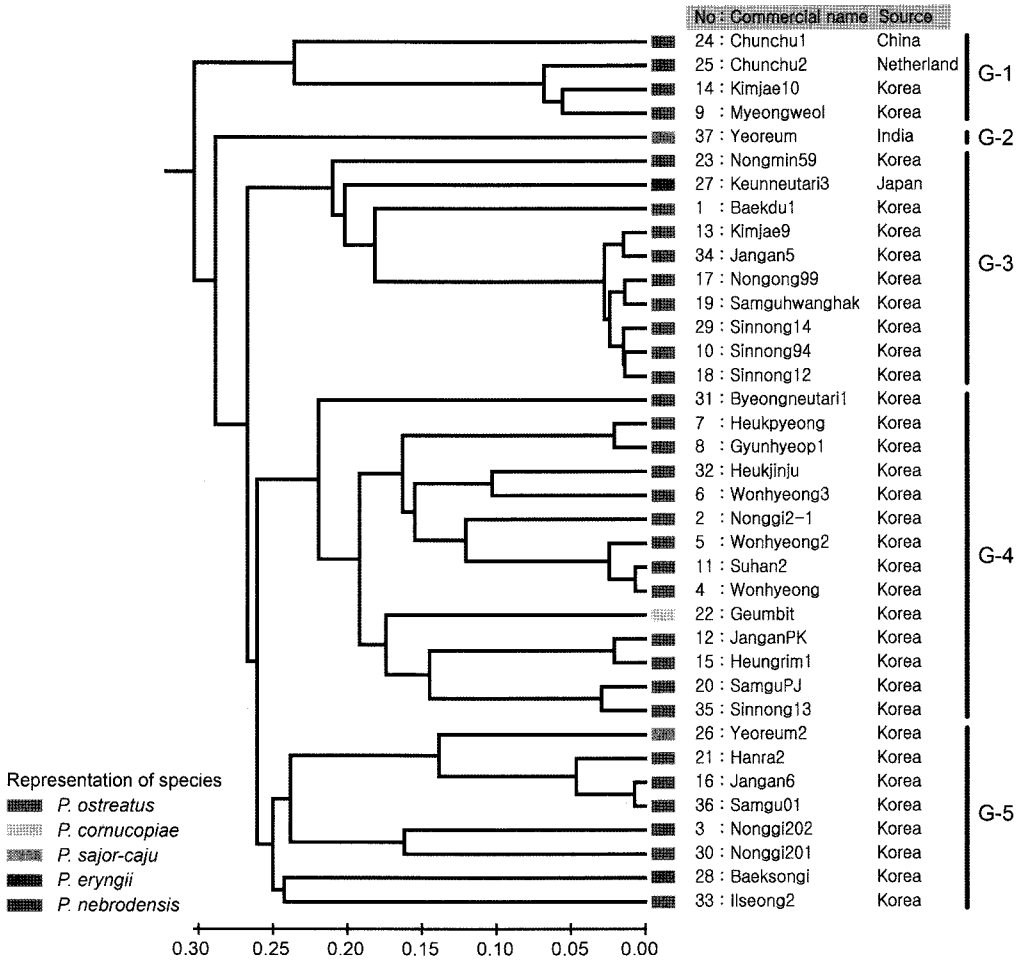

Fig. 2. UPGMA dendrogram showing phylogenetic relationships among different oyster mushroom (*Pleurotus ostreatus*) including other *Pleurotus* species.

Acknowledgments

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