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Genomic Differentiation Among Oyster Mushroom Cultivars Released in Korea by URP-PCR Fingerprinting

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URP primers of 20 mer derived from repetitive sequence of rice were used to assess genetic variation of oyster mushroom consisting of 10 cultivars of *Pleurotus ostreatus*, two cultivars of *P. florida* and two cultivars of *P. sajor-caju* which were registered in Korea. URP2F and URP38F primers produced cultivar-specific PCR polymorphic bands in the *Pleurotus* species. UPGMA cluster analysis using the URP-PCR data showed that 14 *Pleurotus* cultivars are genetically clustered into large three groups. The URP-PCR data provided important information for more efficient breeding strategies of *Pleurotus* cultivars.

KEYWORDS: ***Pleurotus* species, URP-PCR fingerprinting, Cultivar differentiation**

Oyster mushroom (*Pleurotus* species) is the most popular edible mushroom occupying about 70% of the total mushroom production in Korea. Taxonomically, the mushroom has been classified into genus *Pleurotus* of the family *Pleurotaceae* in the order Agaricales and over 30 *Pleurotus* spp. are distributed worldwide. In Korea, 14 cultivars including 10 cultivars of *P. ostreatus*, two cultivars of *P. florida*, and two cultivars of *P. sajor-caju* have been bred by protoplast fusion and cross-breeding between species or cultivars and selection of superior strains in isolates introduced from different nations. The development of methods to obtain an accurate assessment of genetic variations among cultivated *Pleurotus* species is important. Information provided by these assessments can serve as basic materials for breeding program to improve yield and quality of mushroom. In the context of cultivar registration, patent, and breeder's right for protection against a certain cultivar, a quick and reliable method for cultivar identification is particularly appealing. However, since the fruit body of *Pleurotus* species is easily affected by environmental conditions such as light, moisture, temperature, and nutrient sources, the identification based on morphological characters have been problematic. To overcome such problems, biochemical methods, like fatty acid and protein assays including isozymes (Burgess *et al.*, 1995; Lee *et al.*, 1998; Sung *et al.*, 1995) were applied to identify strains within *Pleurotus* spp., but their applications have disadvantages due to occurrence of variants depending on developmental stages.

Molecular techniques, DNA hybridization and polymerase chain reaction (PCR) have been extensively employed for confirming genotypes of microorganisms within or between species. Genomic analysis using the techniques can be evaluated independently from developmental stages and from environmental effects (Caeanto-Anolles and Gresshoff, 1997). A particular advantage of PCR is that it requires little biological materials and offers a rapid method in application to extensive sample sizes that need to be screened. Molecular analysis using IGS and ITS regions of ribosomal DNA (rDNA) has been established to assess the taxonomic and evolutionary relatedness of fungal species including mushrooms (White *et al.*, 1990; Gonzales and Labarere, 2000; Vilgalys and Sun, 1994). Although the methods have effectively been used to address them at species or genus levels, they are limited in genotype studies at strain or cultivar levels because of their low polymorphism. As alternative methods, the PCR techniques of random amplified polymorphic DNAs (RAPD) (Williams *et al.*, 1990) and arbitrary primed PCR (AP-PCR) (Welsh *et al.*, 1990; Williams *et al.*, 1990) using arbitrary primers have been used widely in studies of genetic diversity between and within fungal species (Peever *et al.*, 1999; Vakalounakis and Fragkiadakis, 1999; Yoder and Christianson, 1998). However, their sensitivity to PCR conditions tends to cause low reproducibility.

In a previous report, RAPDs, AP-PCR and PCR-RFLP of rDNA regions were applied for identifying genotypes of *Pleurotus* cultivars bred in Korea (Kim *et al.*, 1999). However, they are practically difficult to be applied for cultivar identification because of the complex analytical system with different DNA markers that require labor and time-consuming steps. Therefore, simple, rapid and reliable new methods were needed for cultivar identification of *Pleurotus* spp.

URPs (universal rice primers), which can be used in PCR fingerprinting of various organisms including plants, animals and microorganisms, were developed from repetitive sequence of rice genome (Kang *et al.*, 1998; Kang *et al.*, 2000). The URP-PCR technique is a useful tool for the characterization and grouping of fungal species at

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Table 1. *Pleurotus* strains used in this study

Cultivar	Strain No. (ASI)	Scientific name	Source and characteristic
Wonhyeongneutari 1	2180	*P. ostreatus*	Korea, Somatic hybrid of protoplast
Wonhyeongneutari 2	2183	*P. ostreatus*	Korea, Somatic hybrid of protoplast
Wonhyeongneutari 3	2240	*P. ostreatus*	Korea, Somatic hybrid of protoplast
Chunchuneutari 1	2228	*P. ostreatus*	China, Mid-temp.
Chunchuneutari 2	2344	*P. ostreatus*	Netherlands, Mid-temp.
Nonggi 2-1	2001	*P. ostreatus*	Korea, Low-temp.
Nonggi 201	2018	*P. ostreatus*	Korea, Mid-temp.
Nonggi 202	2072	*P. ostreatus*	Korea Mid-temp., Interspecific hybrid (ASI 2018 × ASI 2016)
Aeneutari 1	2042	*P. ostreatus*	Japan
Heuckpyong	2223	*P. ostreatus*	China
Sachulneutari 1	2016	*P. florida*	German, High-temp.
Sachulneutari 2	2181	*P. florida*	Thailand, High-temp.
Yeoreumneutari 1	2070	*P. sajor-caju*	India, High-temp.
Yeoreumneutari 2	2333	*P. sajor-caju*	Korea, High-temp. hybrid (ASI 2070 × ASI 2139)

interspecific and intraspecific level (Kang *et al.*, 2000). Our objective was to develop a useful molecular tool that can reliably differentiate each genome of *Pleurotus* cultivars released to mushroom-growers in Korea. The present study describes identification of each cultivar by PCR technique using URP primers.

Materials and Methods

***Pleurotus* cultivars.** The isolates in *Pleurotus* spp. used in this study are listed in Table 1. Ten isolates of *P. ostreatus* consist of three cultivars of wonhyeongneutaris, two cultivars of chunchuneutaris, a cultivar of aeneutari , a cultivar of heuckpyong and three cultivars of nonggi produced by protoplast fusion (Yoo, 1994; Yoo *et al.*, 1993) and cross-breeding among species or isolates (Go *et al.*, 1981) and selection of superior cultivars from the strains. The remaining isolates include two cultivars of sachulneutaris in *P. florida* and two cultivars of yeoreumneutaris in *P. sajor-caju* introduced from German, Thailand and India.

DNA extraction. *Pleurotus* species was grown in shaking culture of potato dextrose broth (PDB) for two weeks at 25°C. The mycelia were then harvested and freeze-dried. For the DNA extraction, the mycelium was placed in microcentrifuge tube (1.5 m*l*) and ground with a toothpick and 400 μl of extraction buffer (200 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM EDTA) with proteinase K (50 μg) was added in the tube. The tube was incubated at 37°C for 1 hour and 2% CTAB (cetyltrimethylammonium bromide) solution of the same volume was added in the tube and then incubated at 65°C for 15 min. The mixture was gently extracted with chloroform:isoamylalcohol (24 : 1) containing 5% phenol and centrifuged at 12,000 rpm at room temperature for 5 min and then the supernatant was transferred to a new tube. Isopropanol with 0.6 volume was added in the solution and centrifuged for pelleting DNA for 5 min at 12,000 rpm. The pellet was washed with 70% ethanol and then dissolved in 100 μl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). RNA in the sample was removed by adding RNase.

Primers and PCR amplification. PCR reactions were performed in a 50 μl PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 2.5 mM of dNTP, each 200 ng for each of primers URP2F (5'-GTGTGCGATCAGTTGCTGGG-3') and URP38F (5'-AAGAGGCATTCTACCACCAC-3'), and 2.5 unit *Taq* polymerase (Promega, Madison, WI, USA). The total amount of genomic DNA from various organisms added to the PCR mixture was approximately 50 ng. PCR amplification was carried out in a PTC-200TM Gradient cycler (MJ Research, Inc., Waltham, MS, USA) using the following profile: one cycle of 4 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C; one cycle of a final extension for 7 min at 72°C. Fifteen μl URP-PCR products were electrophoresed in 1.8% agarose gel at 6 voltage/cm with TAE buffer for 4 hours. DNA fragments in the gel were visualized by staining with ethidium bromide and photographed under UV transilluminator.

Data analysis. Individual URP-PCR products of each *Pleurotus* cultivar were scored for their presence (value = 1) or absence (value = 0). The phylogenic analysis was done following the method by Nei. (1987). The similarity coefficient (F) was calculated as the fraction of shared fragments between pairs of cultivars. For cultivars, x and y, $F = 2Nxy/(Nx + Ny)$, where Nxy is the number of DNA fragments shared by cultivars X and Y, while Nx and Ny are the number of fragments scored from cultivars X and Y, respectively. On the basis of the similarity coefficient, a

dendrogram was constructed with the statistical program NTSYSpc (version 2.0, Exter Software, Setauket, NY) using the unweighted pair-group method with arithmetic mean (UPGMA).

Results and Discussion

URP-PCR fingerprinting of *Pleurotus* cultivars. As in RAPD (Williams *et al.*, 1990) and AP-PCR (Welsh and McClelland, 1990; Welsh *et al.*, 1990) reactions, each single URP was used for each PCR reaction, but a high stringent temperature was employed in the annealing step to give high PCR reproducibility. Twelve URP primers were screened for amplifying genomic DNA of *Pleurotus* spp. Of them, primers URP2F and URP38F produced cultivar-specific polymorphic bands (one to 18) varying in size from 100 bp to 4,000 bp and allowed to discriminate each 14 cultivar in three *Pleurotus* spp. (Fig. 1). URP-PCR profiles could distinguish *P. sajor-cajur* cultivars (yeoreumneutaris 1 and 2) from 12 cultivars of other two species. Moreover, the profile was different between both isolates by presence or absence of two bands of 2,000 and 1,500 bp that were amplified by URP2F, but URP38F-PCR pattern between both isolates was identical. On the other hand, *P. florida* and *P. ostreatus* isolates including 12 cultivars shared URP-PCR bands generated by each of URP2F and URP38F and each URP-PCR profile of the cultivars was characterized by its band pattern. URP-PCR patterns between wonhyeongneutaris 1 and 2 appeared to be identical, suggesting that wonhyeongneutaris 1 and 2 originated from the same genomic background. Nevertheless, primers URP2F and URP38 differentiated wonhyeongneutari 3 from them by the presence of a band around 1,000 bp and two polymorphic bands of 650 bp in size. Wonhyongneutari cultivars have been developed through selection procedures of somatic hybrids obtained by protoplast fusion among sachulneutari 1, nonggi 2-1 and 201 (You, 1994) and thus the genomic backgrounds of wonhyeongneutari cultivars may share those of the parental cultivars. As shown in Fig. 1 (lanes 3, 4 and 5), URP-PCR bands of wonhyeongneutari cultivars share those that are observed on the parental cultivars.

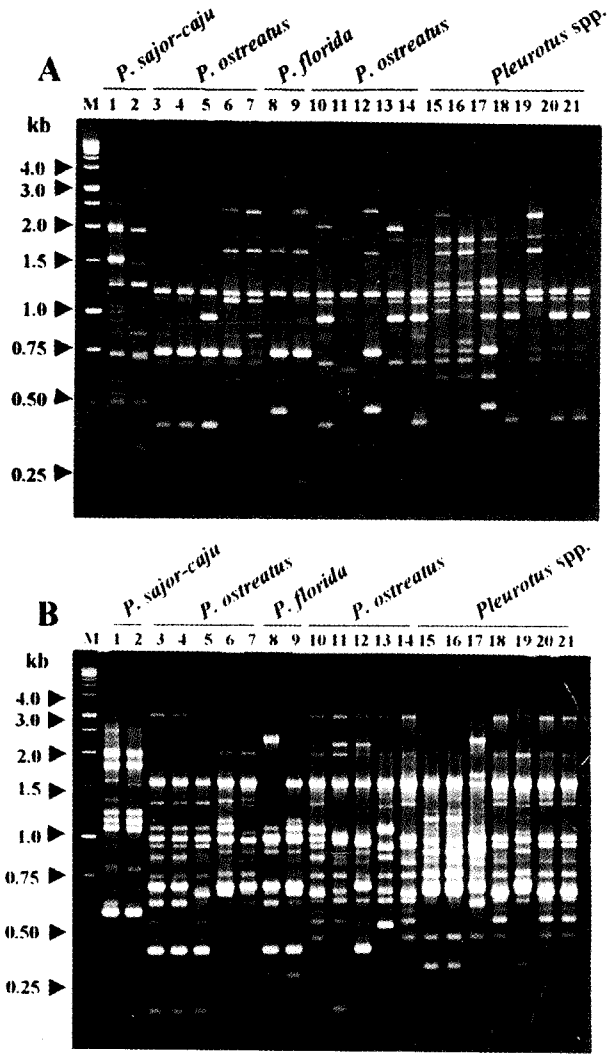

Fig. 1. PCR profiles of *Pleurotus* spp. generated by primers URP2F (A) and URP38F (B). PCR products were resolved by agarose gel (1.5%) electrophoresis and visualized by staining with ethidium bromide. M: 1 kb ladder (Promega), Lanes 1-14 (registered cultivars): Yeoreumneutari 1 (ASI 2070), Yeoreumneutari 2 (ASI 2333), Wonhyeongneutari 1 (ASI 2180); Wonhyeongneutari 2 (ASI 2183); Wonhyeongneutari 3 (ASI 2240); Chunchuneutari 1 (ASI 2228); Chunchuneutari 2 (ASI 2344), Sachulneutari 1(ASI 2016), Sachulneutari 2 (ASI 2181), Nonggi 2-1 (ASI 2001), Nonggi 201 (ASI 2018), Nonggi 202 (ASI 2072), Aeneutari (ASI 2042), Heukpyong (ASI 2223). Lanes 15-21: unregistered *Pleurotus* isolates.

Intra- and interspecific protoplast fusion of *Pleurotus* species has been used as a tool for developing new cultivars and the identification of hybrids have been assisted by isozymes and DNA markers (Yoo *et al.*, 1993; Yoo, 1994). As such, URP-PCR polymorphic bands could be used as potential genetic markers in future studies for identifying somatic hybrids. In case of chunchuneutari, different URP-PCR patterns were also detected between two cultivars and the major difference was observed on 750 bp-band and double band around 1,100 bp of URP2F-PCR and URP38F-PCR products. In addition, double URP2F-PCR bands of 2,500 and 450 bp and URP38F-PCR band of 2,500 bp discriminated between sachulneutaris 1 and 2. Furthermore, each cultivar of oyster mushroom (*Pleurotus*) including nonggi 2-1, 201, 202, aeneutari 1and Heuckpyong was characteristically profiled by URP2F and URP38F primers. The results demonstrated that PCR fingerprinting technique using the primers is a powerful tool to address cultivar identification of oyster mushroom.

In previous studies, some approaches, based on isozyme

assay (Lee *et al.*, 1998), rDNA and mitochondria DNA (Toyomasu *et al.*, 1992; Kim *et al.*, 1998; Gonzalez and Labarere, 2000) were used in an attempt to study phylogenic relationships among different *Pleurotus* species. Although the methods were capable of identifiying species or partial strains, they did not discriminate respective cultivars within *Pleurotus* spp. Alternatively, RAPD and AP-PCR methods were used for identifying respective 13 *Pleurotus* cultivars in Korea. However, they failed to characterize each PCR profile depending upon the cultivars, although some partial cultivars could successfully be distinguished from them. The methods specifically required a complicated marker system using a number of primer. In contrast, URP-PCR method used in this study could address each cultivar using PCR fingerprints produced by only two URP primers (URP2F and URP38F), reflecting that URP-PCR can alternatively be used as a practical, rapid and simple system to identify *Pleurotus* cultivars.

The RAPD method uses short arbitrary primers of 10 nucleotides and low stringency conditions of annealing temperatures ranging from 35 to 37°C throughout the thermo-cycling reaction (Williams *et al.*, 1990). AP-PCR is a versatile method that generates fingerprints of genomes using arbitrarily selected long primers over 20 mer, and low annealing temperatures (36~48°C) during the initial two cycles which results to induction of many DNA segments with a variety of mismatches (Welsh and McClelland, 1990). Since the conditions in both methods have low stringency in the annealing stages, both can amplify non-specific bands resulting in low reproducibility, a problem that has been the subject of considerable discussion (Caetano-Anolles and Gresshoff, 1997). The main difference between URP-PCR and above-mentioned methods which are both PCR techniques, is the use of URP primers that are relatively long (20 bp), designed for fingerprinting any organism at a relatively high annealing temperature. Generally, long primer and high annealing temperatures improve the specificity between primers and template DNA (Caetano-Anolles *et al.*, 1992; Wu *et al.*, 1991). Thus, hot start amplification of URP-PCR protocol may be expected to increase the PCR reproducibility. In repeating the experiment, we confirmed that URP-PCR patterns of *Pleurotus* cultivars are reliable and reproducible.

In Korea, 14 *Pleurotus* cultivars used in this study were formally registered and have been distributed as mushroom spawns to a farm household for producing fruitbodies. However, *Pleurotus* isolates of uncertain sources have been cultivated in mushroom farms and morphologically, such isolates are difficult to distinguish from the registered cultivars (Kim *et al.*, 1998).

We therefore, further tested the practical applicability of URP-PCR method for differentiating the isolates from the 14 *Pleurotus* cultivars registered in Korea. Seven unregistrated *Pleurotus* isolates that have been circulated as spawns in mushroom farms were used as representative samples. Their genomic DNA was amplified by primers URP2F and URP38F and the profiles were compared to those of 14 *Pleurotus* cultivars. Interestingly, URP-PCR profile of isolates on lanes 18, 20 and 21 showed an identical pattern to that of Hueckpyong (Fig. 1A, B), suggesting the isolates are likely originated from cultivar Hueckpyong. On the contrary, those of isolates on lanes 19, 16, 15 and 17 were different to those of 14 cultivars used in this study, showing that they are characteristic genotypes that are different to the cultivars analyzed. From the result, it was considered that URP-PCR profiles could be effectively used as a DNA standard index for confirming genotypes of *Pleurotus* isolates.

Genetic relationship among *Pleurotus* cultivars. The genetic similarity index calculated from the PCR fingerprinting bands amplified by URP2F and URP38F was used to estimate the genetic relatedness among 14 *Pleurotus* cultivars. Based on the URP-PCR fingerprint data, the genetic distance was used to construct a dendrogram for the 14 *Pleurotus* cultivars analyzed. Fig. 2 shows the genetic relationships of the varieties on the basis of URP-PCR data. It is interesting to note that most of the cultivars were clustered into three large groups corresponding to their species. The genetic relationship of the cultivars based on URP-PCR fingerprinting data is relatively low with an average value of 55% as compared to the results of previous studies based on isozymes and rDNA (Kim *et al.*, 1998; Lee *et al.*, 1998). It was assumed that such phenomenon is due to the high polymorphism among cultivars as revealed by URP-PCR analysis. Previously, PCR-RFLP analysis using IGR and ITS regions in rDNA showed a high genetic similarity ranging from 93% to 82% among *P. ostreatus*, *P. florida* and *P. sajor-caju* (Kim *et al.*, 1998). Cultivars of *P. sajor-caju* evaluated showed an 87% similarity and were noted to be distantly related to other species with a 38% similarity. The cultivars within *P. ostreatus* were clustered into three small groups

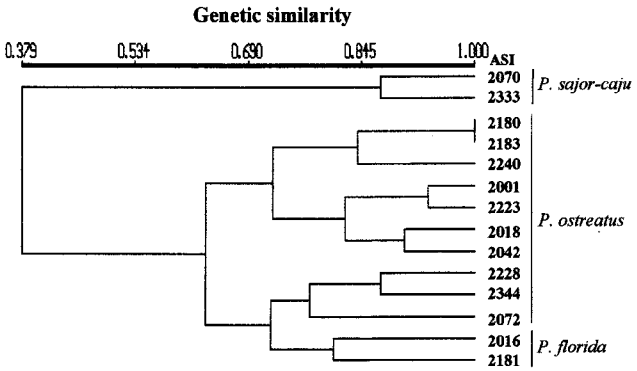

Fig. 2. Dendrogram of genetic relationships among 14 *Pleurotus* cultivars.

and the genetic similarity values which ranged from 100% to 57% were observed on cultivars of *P. ostreatus*. Unexpectedly, *P. ostreatus* including cultivars Nonggi 202, chunchuneutaris 1 and 2 showed a closer genetic relationship as shown by their 70% genetic similarity to cultivars of *P. florida* than those of *P. ostreatu*. In fact, *P. florida* have been taxonomically confused as *P. ostreatus* due to morphological similarity. Thus, Eger *et al.* (1997) insisted that *P. florida and P. ostreat* can be reasonably classified as same species. Cultivar Nonggi 202 was developed by hyphal mating between *P. ostreatus* (Nonggi 201) and *P. florida* (Sacheolneutari 1). It was reported that Nonggi 202 is more similar to Sacheolneutari 1 than to Nonggi 201 in the aspects of the physiological and cultivate characters (Go *et al.*, 1981). It is interesting to note that Nonggi 202 shared URP-PCR bands with sacheolneutari and Nonggi 201 (Fig. 1). A high genetic similarity between Nonggi 202 and Sacheolneutari was also noted (Fig. 2). Chunchuneutari cultivars were taxonomically classified into *P. ostreatus*. However, the results of this study showed that they are more closely related to cultivars of *P. florida* than those of *P. ostreatus*. We could not offer explanation on these results in the absence of information on the genetic background of Chunchuneutari cultivars.

In conclusion, genetic analysis among cultivars using the URP-PCR data can serve as a useful tool in breeding programs because it permits the organization of germplasm and provides important information for more efficient breeding strategies.

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