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Research Article

Isolation of a Variant Strain of *Pleurotus eryngii* and the Development of Specific DNA Markers to Identify the Variant Strain

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Abstract A degenerated strain of *Pleurotus eryngii* KNR2312 was isolated from a commercial farm. Random amplified polymorphic DNA analysis performed on the genomic DNA of the normal and degenerated strains of this species revealed differences in the DNA banding pattern. A unique DNA fragment (1.7 kbp), which appeared only in the degenerated strain, was isolated and sequenced. Comparing this sequence with the KNR2312 genomic sequence showed that the sequence of the degenerated strain comprised three DNA regions that originated from nine distinct scaffolds of the genomic sequence, suggesting that chromosome-level changes had occurred in the degenerated strain. Using the unique sequence, three sets of PCR primers were designed that targeted the full length, the 5' half, and the 3' half of the DNA. The primer sets P2-1 and P2-2 yielded 1.76 and 0.97 kbp PCR products, respectively, only in the case of the degenerated strain, whereas P2-3 generated a 0.8 kbp product in both the normal and the degenerated strains because its target region was intact in the normal strain as well. In the case of the P2-1 and P2-2 sets, the priming regions of the forward and reverse primers were located at distinct genomic scaffolds in the normal strain. These two primer sets specifically detected the degenerate strain of KNR2312 isolated from various mushrooms including 10 different strains of *P. eryngii*, four strains of *P. ostreatus*, and 11 other wild mushrooms.

Keywords Chromosome instability, Degeneration, Random amplified polymorphic DNA, Sectoring

Mushrooms are a group of fungi that produce characteristic spore-forming fruiting bodies as a means of sexual reproduction. The fruiting bodies are often consumed as food and used for medicinal purposes. Several edible mushrooms are readily available at grocery stores and food markets because they are cultivated commercially. The king oyster mushroom, *Pleurotus eryngii*, is one of the most widely cultivated mushrooms. In the commercial cultivation of *P. eryngii*, the mushroom is grown in wide-mouthed polypropylene bottles in semi-automated confined facilities and for the growth, complex substrates are used, which consist of sawdust, rice bran, powdered corncob, beet pulp, wheat bran, cottonseed hull, and shell powder [1]. Such nutritionally rich complex media are used as a result of long-term modifications of sawdust-based simple media, and these rich media are currently used in all *P. eryngii* farms to ensure rapid mycelial propagation and high-quality mushroom production.

Using the rich medium in a closed space, however, often imposes great risks in commercial cultivation because it exposes the whole plant to the attack of pathogenic microorganisms [2]. Moreover, the use of the medium frequently causes strain instability and generates abnormal morphological characteristics including the formation of thick mycelial skin on top of the substrate and weakened propagation of the mycelia into the solid substrate. The emergence of abnormal strains has caused massive losses in *P. eryngii* production. Similar phenomena have also been reported in the cultivation of the button mushroom *Agaricus bisporus*, in which “fluffy” or “stromatal” sectors are formed on the agar medium or in grain cultures [3]. “Sectoring” often results in abnormal growth and poor yields. Molecular genetic studies on the mycelial sector

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revealed that this was an inheritable trait that eventually led to the irreversible degeneration of the cultivated strain [4]. Comparative studies performed on the sectoring strains by using contour-clamped homogeneous electrophoresis and restriction fragment length polymorphism analyses revealed several abnormalities in chromosomes, including somatic recombination, chromosomal length polymorphism, and possible chromosomal translocations [5]. Therefore, the strain degeneration in the button mushroom could be a direct outcome of chromosomal instabilities. The genome instability is not fully accounted for, but appears to occur frequently in fungi. Growing successive generations of *Aspergillus flavus* in the absence of competing microorganisms was able to irreversibly turn down the expression of genes related to aflatoxin production [6]. Similarly, prolonged ethanol-limited chemostat cultivation caused the degeneration of *Penicillium chrysogenum* used in the production of penicillin [7].

Successive transfer in a rich complex medium is a protocol widely used for maintaining the production strains of *P. eryngii*. Unlike in the case of *A. bisporus*, however, no scientific research has been conducted on the strain abnormality of *P. eryngii*, because the commercial cultivation of this mushroom has been established only recently and the strain development has been driven by industrial sector. Nonetheless, the imposed risk is present in almost every *P. eryngii* farm and, currently, detecting and removing the degenerated strain at the stage of spawn development is the most effective method available for reducing the economic damage caused. Accordingly, in this study, we developed a PCR-based method to specifically detect the degenerated strain of *P. eryngii* KNR2312, which has been the most cultivated strain in Korea. Comparing the sequences of the PCR amplicons, which were acquired through random priming, revealed that the strain degeneracy originated from chromosomal rearrangement. The use of primer sets targeting the rearranged chromosomal region enabled the specific detection of the degenerated strain.

MATERIALS AND METHODS

Strains and culture conditions. A cultivated strain of *P. eryngii* (KNR2312) was obtained from its developer, Gyeongnam Agricultural Research and Extension Services (GNARES). The degenerated strains of KNR2312, designated KNR2312-M1 and KNR2312-M2, were obtained from Greenpeace Mushroom Co., Korea. Strains of *P. ostreatus* and *P. sajor-caju* were collected from mushroom farms located in the southern part of Korea. Wild mushrooms with listed IUM numbers were obtained from Culture Collection of Wild Mushroom (CCWM), Incheon University, Korea (Table 1). The strains were grown on potato-dextrose agar (PDA) plates at 25°C. Bottle cultures were grown at 25°C for 35 days, with the substrate containing pine sawdust (23%), corncob (29%), rice bran (18%), beet pulp (4%), wheat bran (14%), cottonseed hull (4%), shell powder (4%), and soybean powder (4%). The water content of the solid substrate was adjusted to 75%.

Extraction of genomic DNA and random amplified polymorphic DNA analysis. Genomic DNA was extracted from the mycelia grown on PDA plates as previously described [8]. In brief, frozen mushroom mycelia harvested from PDA were ground using a mortar and pestle. The ground powder (0.4 g) was suspended in 0.5 mL of buffer containing 50 mM Tris-HCl (pH 8.0), 170 mM EDTA (pH 8.0), and proteinase K (10 μg/mL). The suspension was incubated for 10 min at 65°C and then centrifuged at 13,000 rpm for 3 min. The supernatant was collected and nucleic acids were precipitated using 40% isopropanol. To examine the chromosomal DNA anomaly, random amplified polymorphic DNA (RAPD) analysis was conducted using the random primers OPS1 (5'-CTACTGCGCT-3'), OPS10 (5'-ACCGTTCCAG-3'), and OPL13 (5'-ACCGCCTGCT-3'). PCR was performed using a PCR premix (Dyemix; Promega Co., Madison, WI, USA) in a Px2 Thermal Cycler (Thermo Electron Co., Waltham, MA, USA) by using these conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 40°C for 30 sec,

Table 1. Mushroom strains used in this study

Species	No.	Strain name	Species	No.	Strain name
Pleurotus eryngii	1	KNR2312	*P. ostreatus*	15	Sinnong
	2	KNR2312-M1		16	Chunchu
	3	KNR2312-M2		17	Chunchu2
	4	KNR2510	*P. sajor-caju*	18	Yeureum
	5	KNR2512	*Lentinula edodes*	19	IUM1508
	6	KNR2514	*Microporus vernicipes*	20	IUM3147
	7	KNR2517	*Flammulina velutipes*	21	IUM1324
	8	KNR2519	*Trametes suaveolens*	22	IUM1732
	9	KNR2520	*Coriolus versicolor*	23	IUM0072
	10	KNR2523	*Stereum ostrea*	24	IUM1296
	11	KNR2524	*Fomes fomentarius*	25	IUM0204
	12	KNR2525	*Pycnoporus cinnabarinus*	26	IUM4209
	13	KNR2577	*Naematoloma fasciculare*	27	IUM4075
P. ostreatus	14	Suhan	*Ganoderma lucidum*	28	IUM1727

and 72°C for 2 min, and finally 72°C for 10 min. The PCR products were resolved in 1.5% agarose gels, and unique DNA bands were extracted from the gels and purified using a PCR purification kit (Solgent Co., Daejeon, Korea). The purified DNA was ligated into the pGEM Teasy vector (Promega) and the DNA sequence of the insert was determined. The determined DNA sequences were deposited in GenBank under the accession numbers KC461497 (DNA No. 6 in Fig. 4A) and KC461498 (DNA No. 1 in Fig. 4B).

Detection of the degenerated strain by using PCR. Primer sets targeting the degenerated strains were designed based on the two DNA sequences obtained from RAPD analysis. The degenerated strains were detected by performing PCR with the primer sets and using the following conditions: pre-denaturation at 95°C for 5 min; 25 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 2 min; and a final extension at 72°C for 10 min. The PCR products were resolved in 1.0% agarose gels by using 50 V and the TAE buffer.

RESULTS AND DISCUSSION

Characteristics of the variant strain. The complete propagation of mycelia inside culture bottles is a prerequisite for successfully cultivating *P. eryngii*. However, mushroom growers often find substrate bottles containing substantially diminished mycelial densities among the bottles that contain normal, fully propagated mycelia. The low-density mycelia in such bottles showed several abnormal morphological characteristics, which include the formation of a thick mycelial skin on top of the substrate and a weakened propagation of the mycelia inside the solid substrate (Fig. 1). Moreover, the mycelia failed to form primordia under fruiting conditions and thus failed to produce fruiting bodies. The growing mycelia exhibited a floppy morphology and featured an increased number of aerial hyphae, which eventually blocked the aeration holes in the solid substrate (Fig. 1). This blocking of the aeration holes resulted in the inhibition of mycelial propagation in the bottom half of the substrate bottle (Fig. 1D). The mycelia also secreted yellowish-brown mucus on top of the culture media (Fig. 1C). This type of mucus has been reported in various mushroom cultures infected with bacteria or fungi [9], but the exact nature of the mucus remains unclear in this case because neither bacteria nor fungi were detected in the culture media.

Two variants of *P. eryngii* KNR2312 were isolated from the abnormal bottle cultures. The two isolates, designated KNR2312-M1 and KNR2312-M2, which were collected from distinct bottles but were derived from the same spawn, showed several abnormal morphological characteristics including a thick mycelial skin formation on top of the substrate and weakened propagation of mycelia inside the solid substrate (Fig. 1). The anomaly was not reversed even after serial transfer on PDA, which suggested that the anomaly was inherited. The growth of the M1 and M2 strains on PDA was investigated in terms of morphology and growth rate. Both variants exhibited more aerial

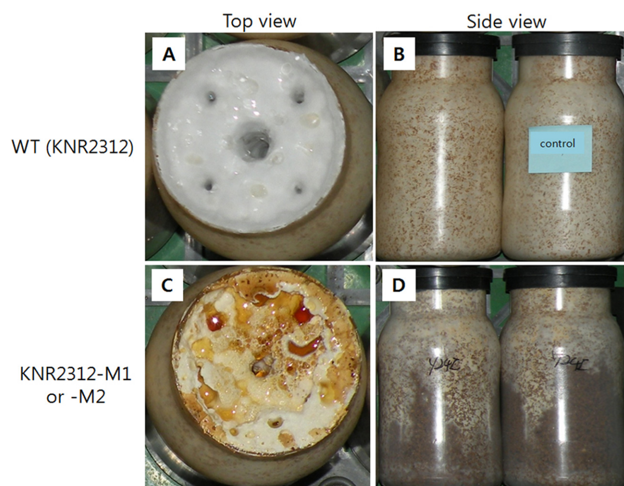
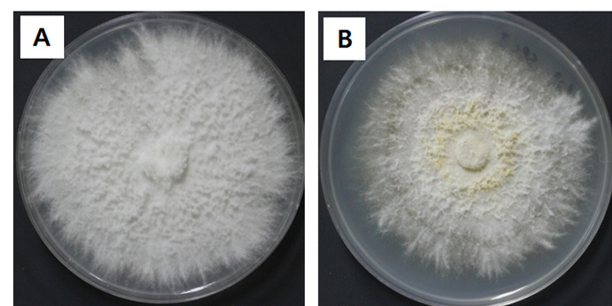


Fig. 1. Mycelial development in the degenerated *Pleurotus eryngii* strain in bottle cultures. A, B, Normal strain KNR2312; C, D, Degenerated strain. The mycelia were propagated for 35 days at 25°C in the dark.



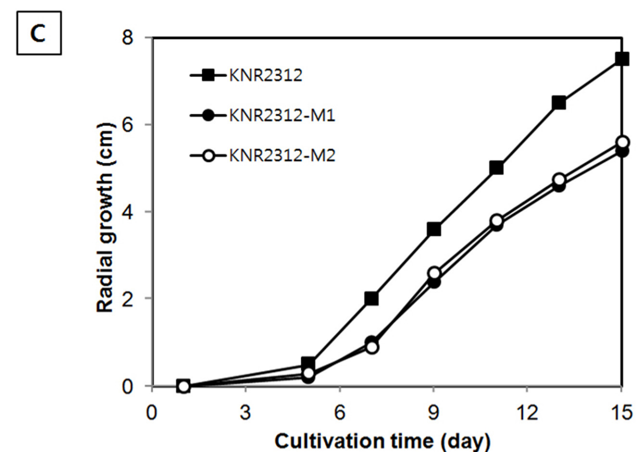


Fig. 2. Growth of the degenerated *Pleurotus eryngii* strain on potato dextrose agar (PDA). Mycelial morphology of KNR2312 (A) and the degenerated strain (B) grown on PDA plates. C, Growth of the normal (KNR2312) and the degenerated strains (KNR2312-M1 and -M2) measured based on the diameter of the mycelia grown at 25°C.

hyphae and a lower mycelial density than the wild-type strain (Fig. 2B) and a markedly retarded growth rate (Fig. 2C).

RAPD and DNA sequence analysis of the variant strain-specific marker. RAPD is a useful technique available for verifying fungal groups at the species level [10] and for developing strain-specific markers [11]. To use RAPD in developing variant-specific markers, we performed PCR by using three 10-mer random primers and the normal and variant chromosomes. As shown in Fig. 3, RAPD yielded five unique DNA bands, with sizes ranging from 0.5 to 2.2 kbp (DNA bands Nos. 1~5). Three of these bands were unique to the variant strains (Nos. 1~3), whereas two were specific to the normal strain (Nos. 4 and 5). The overlap of the DNA banding patterns of the two variant strains (M1 and M2) indicated that they both represented the same strain, which was also suggested by the similarities in their morphology and growth characteristics (Fig. 2).

The polymorphism in the DNA banding pattern suggested that the variant featured major changes at the chromosome level. To confirm this, DNA band No. 1 (1.7 kbp) from the M1 strain was cloned into a TA cloning vector and its sequence was determined. Moreover, DNA band No. 6 (2.0 kbp), which was present in all three strains, was analyzed as a control. The acquired sequences were compared with the genomic sequence of the KNR2312 strain, which was recently determined by GNARES (but is not yet publicly available). The sequence of DNA No. 6 was found within

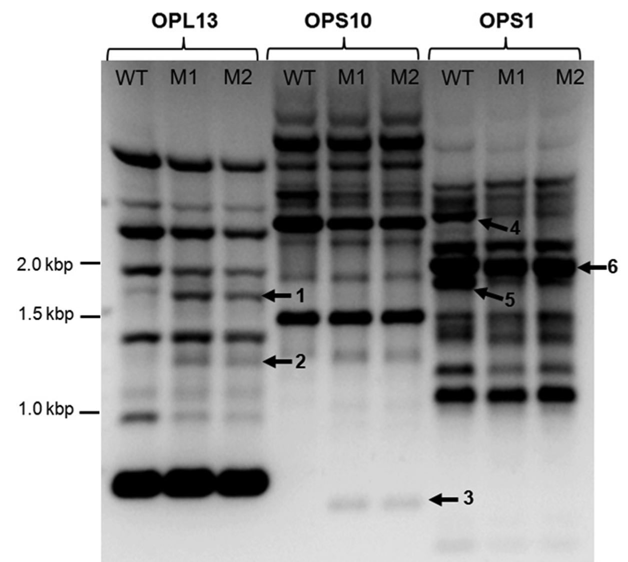


Fig. 3. Random amplified polymorphic DNA analysis performed on the normal (WT) and the degenerated strains (M1 and M2) by using the random primers OPL13, OPS10, and OPS1. Arrows with numbers indicate the unique DNA bands for the degenerated strain (Nos. 1~3) and the normal strain (Nos. 4 and 5). The sequence of DNA band No. 1 was analyzed together with that of DNA band No. 6 as a control.

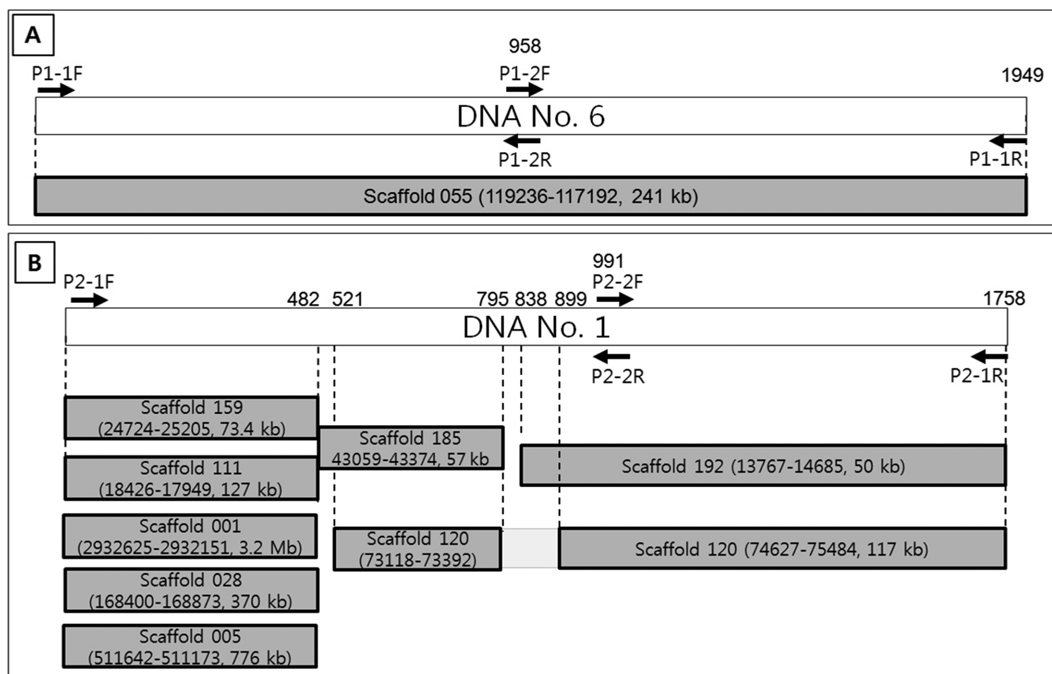


Fig. 4. Sequence analyses of DNA bands obtained from random amplified polymorphic DNA. BLAST analysis was conducted on the sequences of DNA band No. 6 (A) and DNA band No. 1 (B) against the genomic sequence database of KNR2312 (not yet publically available). The grey bars are the sequence regions that reside within the genomic scaffolds of KNR2312. The matched regions in the scaffolds are written within the parentheses together with the size of the scaffolds. Priming sites are indicated by arrows.

the 241-kbp-long genomic scaffold 055 of KNR2312 and it exhibited 99% sequence identity (Fig. 4A; the sequence alignment data are provided in Electronic Supplementary Fig. 1). However, the sequence of DNA No. 1, whose exact size was 1,758 bp, did not belong to any single genomic scaffold. The DNA was fragmented and scattered over at least nine genomic scaffolds (Fig. 4B; the sequence alignment data are provided in Electronic Supplementary Fig. 2). The 5 482-bp fragment was found in the scaffolds 159, 111, 001, 028, and 005. The middle fragments covering 483~795 bp or 521~795 bp of DNA No. 1 were present in the scaffolds 185 or 120, respectively. The 3 fragments covering 838~1,758 bp or 899~1,758 bp were in the scaffolds 192 or 120, respectively. This, coupled with the RAPD data, demonstrated that DNA No. 1 of the variant strain was a product of the rearrangement of the wild-type chromosomes. Notably, chromosome-level alterations in fungi appear to be common: The "sectoring" strain of button mushroom showed a variety of chromosomal anomalies including chromosomal length polymorphism, chromosomal loss, and somatic recombination [5]. Moreover, a loss of conditionally dispensable chromosomes was reported in the virulent strains of the insect pathogenic fungus *Metarhizium anisopliae* [12], and human pathogenic fungi such as *Candida albicans* and *Cryptococcus neoformans* showed chromosomal rearrangements that enabled diversification in response to environmental changes [13, 14].

Development of variant-specific primers. Primers specific for the variant strain were designed using the sequences of DNA No. 1 (unique for M1 or M2) and DNA No. 6 (common to all strains), which targeted the full length, the 5' half, and the 3' half of the DNA fragments (Fig. 4). The sequences of the primers and the target sizes are summarized in Table 2. The specificity of the primers for the variant strains was examined by performing PCR at an elevated annealing temperature (60°C). As shown in Fig. 5A, the primer sets P1-1, P1-2, and P1-3, which target DNA No. 6, yielded PCR products of the expected sizes from all three strains. By contrast, the primer sets P2-1 and P2-2, targeting DNA No. 1, yielded the corresponding 1.76 and 0.97 kbp PCR products, respectively, only in the case of the variant strains (M1 and M2). This was the expected result because the DNA regions targeted by P2-1 and P2-2 were present only in the variant strains (Fig. 4B). The priming sites for the forward and reverse primers were located at distinct genomic scaffolds in the wild-type strain

Table 2. PCR primer sets for the detection of degenerated strain

Primer set	Primer name	Sequence (5' to 3')	Expected size (bp)
P1-1	P1-1F	CTACTGCGCTCTCATT	1,949
	P1-1R	TCTACTGCGCTCATGG	
P1-2	P1-1F	CTACTGCGCTCTCATT	954
	P1-2R	CGCCGTCCCATTCTAC	
P1-3	P1-2F	GTAGAATGGGACGGCG	1,007
	P1-1R	TCTACTGCGCTCATGG	
P2-1	P2-1F	ACCGCCTGCTACCAGC	1,758
	P2-1R	TACCGCCTGCTAAGCA	
P2-2	P2-1F	ACCGCCTGCTACCAGC	966
	P2-2R	CCAAGGATGATGCTCC	
P2-3	P2-2F	GGAGCATCATCCTTGG	808
	P2-1R	TACCGCCTGCTAAGCA	

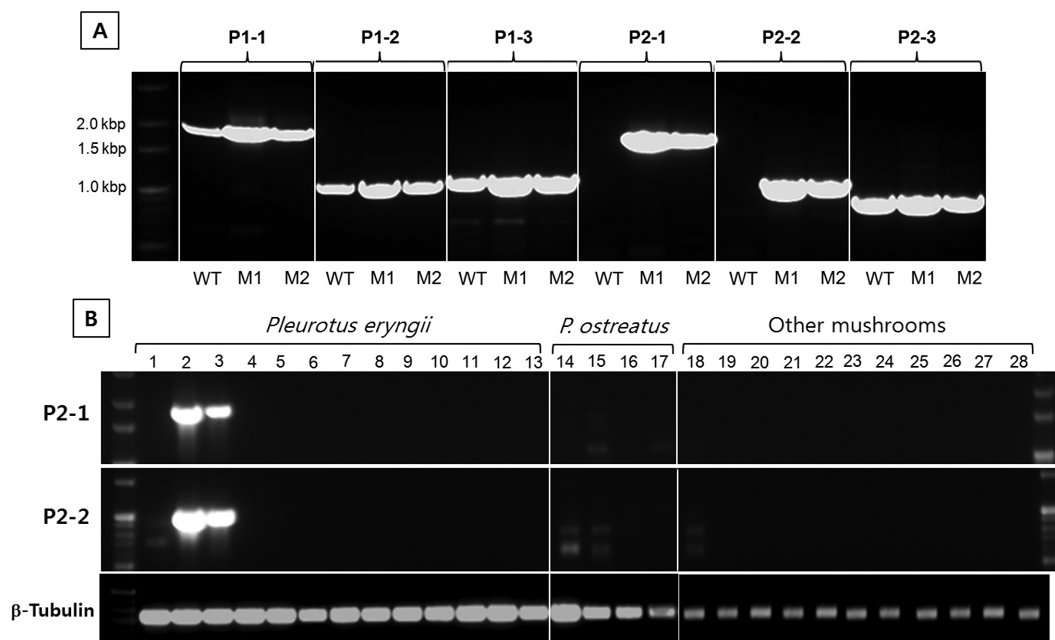


Fig. 5. PCR performed using the designed primer sets. A, Amplification of marker DNA sequences of the normal (WT) and degenerated strains (M1 and M2); B, Specificity of the primer sets P2-1 and P2-2. The mushroom strains are summarized in Table 1.

(Fig. 4B). Unlike the P2-1 and P2-2 sites, the priming site for P2-3 remained intact at two independent scaffolds of the wild-type strain, and thus produced the 0.8 kbp product in all three strains (Fig. 5A). This result further demonstrated genomic rearrangements in the variant strains. PCR analysis performed using these primer sets revealed that the primer sets P2-1 and P2-2 can be employed to verify the variant strain. For this purpose, we examined the specificity of the primer sets by using various mushroom strains including 10 different strains of *P. eryngii*, four strains of *P. ostreatus*, and 11 other wild mushrooms. Both primer sets were specific only to the variant strain of *P. eryngii* (Fig. 5B, lanes 2 and 3), indicating their applicability in the detection of the variant strain.

In conclusion, our results suggest that abnormal *P. eryngii* strains occasionally emerge because of chromosome-level rearrangements that possibly occur through somatic recombination, as in the case of the "sectoring" strain of *A. bisporus* [5]. The primer sets specific to the variant chromosome designed here can distinguish the variant from the normal strain, and thus performing PCR by using these primer sets is an easy and rapid method to detect anomalies in *P. eryngii* strains.

ELECTRONIC SUPPLEMENTARY MATERIALS

Supplementary data including two figures can be found with this article online at http://www.mycobiology.or.kr/src/sm/mb-42-46-s001.pdf.

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