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Development of Specific Primer for *Tricholoma matsutake*

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In this study, in an effort to develop a method for the molecular detection of *Tricholoma matsutake* in Korea from other closely related Tricholomataceae, a species-specific PCR primer pair, TmF and TmR, was designed using nuclear ribosomal intertranscribed spacer (ITS) sequences. The DTmF and DTmR sequences were 5'-CCTGACGCCAATCTTTTCA-3' and 5'-GGAGAGCAGACTTGTGAGCA-3', respectively. The PCR primers reliably amplified only the ITS sequences of *T. matsutake*, and not those of other species used in this study.

KEYWORDS : ITS, *Tricholoma matsutake*, Tricholomataceae

Tricholoma matsutake, an agriculturally important mushroom due to its strong volatile flavor and taste, is an ectomycorrhizal fungus belonging to the Tricholomataceae, Agaricales, Hymenomycetes, and Basidiomycota. Molecular techniques including DNA hybridization and polymerase chain reaction (PCR) have been extensively utilized to confirm the genotypes of mushrooms. Ribosomal repeat units, composed of 18S-ITS1-5.8S-ITS2-28S (Srivastava and Schlessinger, 1991), have already proven very informative in differentiating species and genera (Fig. 1). The internal transcribed spacer (ITS) is of particular usefulness, because it is accessible with universal primers and its DNA sequence is variable at both inter- and intra-specific levels. Many molecular studies have been conducted on *T. matsutake* (Chapela and Garbelotto, 2004; Dunstan *et al.*, 2000; Kikuch *et al.*, 2000; Lee *et al.*, 1999, 2004; Lee and Hong, 1998; Lian *et al.*, 2003). However, only a minimal amount of information is currently available for the differentiation of *T. matsutake* from the closely related Tricholomataceae. The principal objective of this study was to develop a method for the molecular detection of *Tricholoma matsutake* in Korea using the specific primers, DTmF and DTmR.

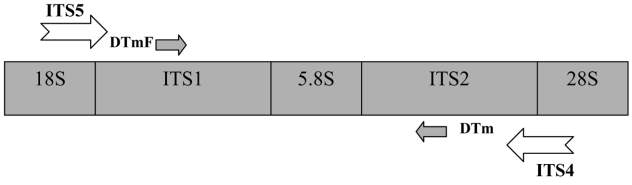

Fig. 1. Schemtic illustration of nuclear ITS region and species-specific primer sites for *T. matsutake*.

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The mycelia of strains (Table 1) were grown at 24°C for 30 days in a shaking incubator with DTM broth (Min and Han, 2000). DNA was extracted from the cultured mycelia via a modified version of the method of Raeder and Broda (1985). The mycelia were collected by filtration and then ground to fine powder in liquid N_2-after which 50 mg of powder was placed in a micro-centrifugal tube and mixed with 700 μl of modified Carlson lysis buffer (2% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 1% PEG 8000, 1% β-mercaptoethanol). DNA was precipitated with an equal volume of cold isopropanol and centrifuged for 10 min at 12,000 rpm. The supernatant was discarded and the pellet was washed with 1 *ml* of 70% ethanol and then re-dissolved in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The remaining RNA was degraded via RNase treatment (20 $\mu g/ml$).

For the amplification of the ITS sequence, ITS5 and ITS4 primers were utilized and PCR was conducted using a Mastercycler (Eppendorf, Germany) in a 50 μl reaction mixture containing 50 ng of genomic DNA, 0.2 μM of each primer, 5 μl of 10 × buffer, 200 μM of each dNTP and 1.25 unit of *Taq* DNA polymerase (Takara, Japan). PCR was conducted as follows: initial denaturation at 95°C for 2 min, followed by 24 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min, and a final 5-minute extension at 72°C. The PCR products were electrophoresed for 40 min in 0.8% agarose gel at 50 V with TAE buffer, visualized via ethidium bromide staining, and photographed under a UV transilluminator. The DNA sequence analyses and primer preparation procedures were conducted using a CoreBio system (Korea). The consensus sequence using BLAST 2 SEQUENCES (NCBI) and CLUSTAL W (v1.81; Thompson *et al.*, 1994) was used to design the specific primers

Table 1. The strains of mushroom used in this study

Species	Strain[a]	Source		GenBank Acc. No.
		Locality	Country	
Tricholoma matsutake	KCTC6468	Daejeon	Korea	
Tricholoma matsutake	KCTC26249	Gyeongju	Korea	AY736068
Tricholoma matsutake	DGUM26002	Yeongdeok	Korea	
Tricholoma matsutake	DGUM26003	Youngcheon	Korea	
Ramaria botrytis	DGUM29001	Gyeongju	Korea	
Clavicorona pyxidata	DGUM29005	Gyeongju	Korea	
Lentinula edodes	IMSNU31010	Seoul	Korea	
Lentinula lepideus	DGUM25050	Yangsan	Korea	
Lentinula nuda	DGUM26501	Gyeongju	Korea	

[a]KCTC; Korean Collection for Type Cultures, Daejeon, DGUM; Dongguk University Microbiology Lab., IMSNU; Institute of Microbiology, Seoul National University.

Table 2. The sequences of primers used in this study and other reports for *T. matsutake*

Primer	Orientation	Sequence (5' to 3')	Report
ITS5	Forward	GGAAGTAAAAGTCGTAACAAGG	In this study
ITS4	Reverse	TCCTCCGCTTATTGATATGC	
DTmF	Forward	CCTGACGCCAATCTTTTCA	
DTmR	Reverse	GGAGAGCAGACTTGTGAGCA	
Tm1	Forward	AAATATGTCTCGAGGAAGCTC	Dunstan *et al.*, 2000
Tm4	Reverse	CAATGGCGTAGATAATTATCACACC	
TmF	Forward	CATTTTATTATACACTCGGT	Kikuchi *et al.*, 2000
TmR	Reverse	GACGATTAGAAGCCGACCTA	

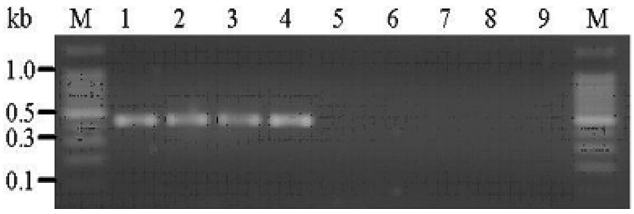

Fig. 2. Amplification of the ITS region of *T. matsutake* with the primer of DTmF and DTmR. No PCR sequence was shown in other mushrooms. M; size marker, Lane 1; *T. matsutake* KCTC6468, Lane 2; *T. matsutake* KCTC26249, Lane 3; *T. matsutake* DGUM26002, Lane 4; *T. matsutake* DGUM26003, Lane 5; *R. botrytis* DGUM 29001, Lane 6; *C. pyridata* DGUM29005, Lane 7; *L. edodes* IMSNU31010, Lane 8; *L. lepideus* DGUM25050, Lane 9; *L. nuda* DGUM26501.

1 AGCTTGGTTAGGTTGTCGCTGGCTCTCCGGGGCATGTGCACG**CCTGACGCCAATCTTTTC**
61 ACCACCTGTGCACATTTTGTAGGCTTGGATAAATATGTCTCGAGGAAGCTCGGTTTGAGG
121 ACTGCCGTGCTGCAAAAGCCAGGCTTTCCTTGTATTTTTCCAGCCTATGCATTTTATTAT
181 ACACTCGGTATGTCATGGAATGTTATTTGGTTGGCTTAATTGCCAGTAAACCTTATACAA
241 CTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAG
301 TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTG
361 GTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTTTTCAGCTTTTTG
421 TTGAATAGGCTTGGATTTTGGGAGTTGTTGCAGGC**TGCTCACAAGTCTGCTCTCC**TTAAA
481 TGTATTAGCGGGGCCCTTGTTGTCTAGCATTTGGTGTGATAATTATCTACGCCATTGTGA
541 ACAATGTAATAGGTCGGCTTCTAATCGTCTTAGCATTTGGTGTGATAATTATCTACGCCA
601 TTGTGAACAATGTAATAGGTCGGCTTCTAATCGTCTCGTAAAGAGACAATCTCTGACATT
661 TGA

Fig. 3. Sequence of the ITS region of *T. matsutake* KCTC26249 binding with the specific primer of DTmF (upper; bold type) and DTmR (lower; bold type underlined), respectively.

of *T. matsutake*, and deposited into the GenBank database (accession no.; AY736068, Table 1).

Based on the sequence of the *T. matsutake* ITS region, a specific primer set (DTmF-DTmR) was designed using a multiple sequence alignment program (http://prodes.toulouse.inra.fr/multalin/multalin.html, Table 2). When the DTmF-DTmR primer was used, a ca. 400 bp PCR product of ITS region was detected only in *T. matsutake*, and not in the other species utilized herein (Fig. 2). The primer binding regions of DTmF and DTmR were confirmed using the Primer Show program on the web site (www.bioinformatics.org/sms/primer_show.html) (Fig. 3). These results demonstrate that the DTmF-DTmR primer pair is species-specific for *T. matsutake* in Korea, thus suggesting that the primer may prove useful in differentiating *T. matsutake* from other mushrooms related to the Tricholomataceae.

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