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Short Communication

Identification of the DNAs of the ectomycorrhizal fungus *Tricholoma bakamatsutake* using specific oligonucleotide probes and PCR primers

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Partial nucleotides of the 18S rDNAs of *Tricholoma bakamatsutake* were sequenced and compared with those of six ectomycorrhizal fungi and a tree. Two probes, Probes 1 and 2, and a pair of primers were designed based on the variable positions in this region. The DNAs of *T. bakamatsutake* were isolated from the colonized mycelia in the soil, field-collected fruit-bodies and artificially cultured mycelia. Hybridization with Probe 1 and PCR-amplification with the primers differentiated these DNAs of this fungus from those of eight ectomycorrhizal fungi and two tree species.

Key Words——ectomycorrhizal fungi; hybridization; PCR; ribosomal DNA; *Tricholoma bakamatsutake*.

Distribution of the mycelia of ectomycorrhizal fungi in the soil has traditionally been estimated from surveys of fruit-body occurrences above the ground (Fogel, 1981). For some ectomycorrhizal fungi, such as *Paxillus involutus* (Batsch: Fr.) Fr., fruit-body formation has been assumed to reflect the mycelial biomass and activities (Laiho, 1970). This, however, leaves unanswered the question to what extent, in general, the features of the fruit-body occurrence are proportional to the biomass of the mycelia in the soil. Although most ectomycorrhizal fungi form conspicuous fruit-bodies, like those of *Cortinarius* and *Boletus* species, fruit-bodies such as those of *Rhizopogon*, *Elaphomyces* and some Corticiaceae species are inconspicuous or rare (Dahlberg, 1991).

Recently developed DNA techniques using specific probes and polymerase chain reaction (PCR) primers allow individual fungal species to be distinguished from each other and from other organisms during taxonomic and genetic studies (Tommerup and Malajcsuk, 1993). In the field of plant pathology, mycoplasma-like organisms have been detected in infected plants and vector insects using specific PCR primers (Namba et al., 1993). These approaches using DNA techniques are also applicable to the identification and detection of mycelia of target ectomycorrhizal fungi of interest in the soil. The objective here is to identify the mycelia of *Tricholoma bakamatsutake* Hongo in the soil in these two ways, by hybridization assay with specific probes and by PCR-amplification with specific primers. Mycelial colonies of this fungus are obvious in the soil but they do not always produce fruit-bodies above them (Terashima, 1993).

Blocks of about 10 × 10 cm of colonizing mycelia of *T. bakamatsutake* (mycelial blocks), namely, the complexes of mycelia, mycorrhiza and soil, were taken from the ground after removing the organic horizon in a study field in Tateyama, Chiba. Mycelia of *T. bakamatsutake* isolate No. 16 were cultured in GPY liquid medium (Terashima, 1994). Fruit-bodies of *T. bakamatsutake* and eight other ectomycorrhizal fungi, *T. flavovirens* (Pers.: Fr.) Lund., *Amanita pantherina* (Dc.: Fr.) Krombh, *A. citrina* (Schaeff.) Pers. var. *citrina*, *Russula japonica* Hongo, *R. compacta* Frost & Peck apud Peck, *Lactarius indigo* (Schw.) Fr., *Leccinum extremiorientale* (L. Vass.) Sing., and *Cantharellus cibarius* Fr., were collected in the same field. Young leaves of the host tree species, *Castanopsis cuspidata* var. *sieboldii* Nakai and *Quercus serrata* Thunb. were also collected. All the samples were lyophilized and stored at −20°C before use.

Samples of 0.1 g of tissue were ground in a mortar with a pestle into fine powder. DNA was isolated by the modified cetyltrimethylammonium bromide method of Murrey and Thompson (1980) using NaCl-TE (1 M NaCl; 10 mM Tris-HCl, pH 8.0; and 1 mM EDTA) precipitation instead of CsCl-ethidium bromide centrifugation as described by Watanabe (1989). The precipitated DNA was resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). If necessary, the suspension was purified by passage through a column (QIAGEN-tip 5, Qiagen Inc.) according to the manufacturer's instructions, and the concentration of DNA was assayed from the optical density at 260 nm. The DNAs were stored at −20°C.

For construction of *T. bakamatsutake*-specific probes and primers, we examined nucleotide sequence of the partial small subunit ribosomal RNA-encoding gene (18S rDNA), because it was more easily accessible for

GGTGGTGCAT GGCCGTTCTT AGTTGGTGGA GTGATTTGTC TGGTTAATTC
CGATAACGAA CGAGACCTTA ACCTGCTAAA TAGCCAGGCC GGCTTTTGCT
GGTCGCAGGC TTCTTAGAGG GACTGTCAGC GTCTAGCTGA CGGAAGTTTG
AGGCAATAAC AGGTCTGTGA TGCCCTTAGA TGTTCTGGGC CGCACGCGCG
CTACACTGAC AGAGCCAGCG AGTTTTTCAC CTTGGCCGGA AGGTCTGGGT
AATCTTGTGA AACTCTGTCG TGCTGGGGAT AGAGCATTGC AATTATTGCT
CTTCAACGAG GAATACCTAG TAAGCGCGAG TCATCAGCTT GCGTTGATTA
CGTCCCTGCC CTTTGTACAC ACCGCCCGTC GCTACTACCG ATTGAATGGC
TTAGTGAGGT CTCCGGATTG GCTTTGGGGA GCCGGCAACG GCACCTTATT
GCTGAGAAGC TGATCAAACT TGGTCATTTA GAGGAAGTAA AAGTCGTAAC
AAGGTTTCCG TAGGTGAACC TGCGG

Fig. 1. The 525 nucleotide 18S rDNA sequence of *T. bakamatsutake*. Underlines indicate the forward (UF5) and reverse (UR6) primers. The sequence is from the 5′ terminus.

study than other parts of DNAs (Hibbett, 1992). Part of the 18S rDNA gene was amplified by PCR with the primers UF5: 5′-CGCCAGGGTTTTCCCAGTCACGAC-GGTGGTGCATGGCCG-3′, and UR6: 5′-AGCGGATAAC-AATTTCACACAGGA-CCGCAGGTTCACCTAC-3′. These primers were designed based on the conserved regions of the eukaryotic 18S rDNA and tagged with M13/pUC forward or M13/pUC reverse primer sequence (Nakai and Hishinuma, 1992). A portion of the extracted DNA (about 2.5 ng) from each sample was used as PCR template. Reaction mixture contained 0.2 mM of each dNTP (Ultrapure dNTP Set, Pharmacia Biotech), 2.5 mM $MgCl_2$, 0.5 μM of each primer, 1.25 U of Taq DNA polymerase (Promega Corp.) with buffer (50 mM KCl; 10 mM Tris-HCl, pH 9.0; and 0.1% (v/v) Triton X-100) supplied by the manufacturer in a total volume of 50 μl. Reactions were carried out in a thermal cycler (GTU-16, Taitec Corp.) programmed as follows: 95°C for 2 min; 30 cycles of 95°C for 40 sec, 55°C for 2 min and 72°C for 2 min; 72°C for 10 min.

The PCR-amplified fragments of 18S rDNA were subjected to low-melting agarose gel electrophoresis; a 5-μl portion of the PCR reaction mixture with 0.05% (w/v) of bromophenol blue was run on a 1.0% (w/v) agarose gel containing 0.05% (w/v) ethidium bromide. The band was excised from the gel, recovered from the gel matrix through QIAGEN-tip columns, precipitated with isopropanol and resuspended in TE buffer, and its UV absorption was then measured.

The sequence of the PCR fragment was determined by direct sequencing of each side of a double-stranded DNA fragment using a commercial sequencing kit (fmol™ DNA sequencing system, Promega Corp.) and commercially available biotinylated M13/pUC forward sequencing primer, # 1224-BT (5′-biotinyl-CGCCAGGG-TTTTCCCAGTCACGAC-3′), or reverse primer, # 1233-BT (5′-biotinyl-AGCGGATAACAATTTCACACAGGA-3′) (New England BioLabs Inc.). After polyaclylamide gel electrophoresis, the sequence ladders were detected by the chemiluminescent detection method using a Phototope™ 6K detection kit (New England BioLabs Inc.) according to the manufacturer's protocol.

Figure 1 shows the 525 nucleotides of the 18S rDNAs of *T. bakamatsutake* including the forward and reverse primers. The sequences of this region from the fruit-bodies, mycelial blocks and cultured mycelia of this fungus matched exactly. This demonstrated that the 18S rDNAs extracted from the mycelial blocks could be ascribed to this fungus.

The sequences of *T. bakamatsutake* and six other field-collected ectomycorrhizal fungi, *T. flavovirens*, *A. pantherina*, *A. citrina* var. *citrina*, *R. japonica*, *R. compacta*, *L. indigo* and a tree, *C. cuspidata* var. *sieboldii*, were compared in order to detect the highly variable positions in the region between UF5 and UR6 of the 18S rDNAs of this fungus for oligonucleotide probes and PCR primers. The alignments showed high homology levels (96.4% on average) between *T. bakamatsutake* and the other samples. But the 81-110, 221-240 and 401-470 positions from the 5′ terminus of the forward primer UF5 in the sequence of *T. bakamatsutake* were found to consist of comparatively highly variable nucleotides (Fig. 2). Two probes, Probes 1 and 2, and a pair of primers, Primers 1 and 2, were designed (Table 1) based on these variable

Table 1. Designed probes and PCR primers for identification of *T. bakamatsutake*.

	Sequence (5′-3′)	Position[a]	Length (mer)
Probe 1	Biotin-TGCGACCAGCAAAAGCC	91-107	17
Probe 2	Biotin-GATCAGCTTCTCAGCAATAA	446-465	20
Primer 1	GGCTTTTGCTGGTCGCA	91-107	17
Primer 2	GATCAGCTTCTCAGCAATAAGG	444-465	22

a) Positions were from the 5′ terminus of the forward primer, UF5.

	81	91	101	
T. bakamatsutake	TAGCCAGGCC	GGCTTTTGCT	GGTCGCAGGC	
T. flavovirens	TAGCCAGGC**T**	GGCTTTTGCT	**A**GT**T**GC**C**GGC	
A. pantherina	TAGCC**C**GA**C**T	GGC**A**T**C**TGCT	GG**C**TGCTGGC	
A. citrina var. *citrina*	TAGCC**T**GGC**T**	GGCTTT**C**GCT	G**ACGTAT**GGC	
R. japonica	TAGCC**T**GGCC	GGCTTTTGCT	GG**C**CA**C**C**G**GC	
R. compacta	TAGCCAGGCC	GGCTTT**C**GCT	GG**C**TGCTGGC	
L. indigo	TAGCC**T**GGCC	GGC**A**TT**C**GCT	GG**C**CA**C**TGGC	
C. cuspidata var. *siebodii*	TAGCTATGC**G**	**AGGTGACC**CT	**CCGG**GC**C**A**G**C	
	401	411	421	431
T. bakamatsutake	TTAGTGAGGT	CTCCGGATTG	GCTTTGGGGA	GCCGGCAACG
T. flavovirens	TTAGTGAGGT	CTCCGGATTG	GCTTT**A**GGGA	GCCGG**A**AACG
A. pantherina	TTAGTGAGGT	CTC**T**GGATTG	GCTT**C**GAGGA	GCCGG**A**AACG
A. citrina var. *citrina*	TTAGTGAGGT	CTCCGGATTG	GCTTTGGGGA	GCCGGCAACG
R. japonica	TTAGTGAG**AC**	CTCCGGAT**C**G	GCTTTG**A**GGA	**A**CGG**A**AACG
R. compacta	TTAGTGAGGT	CTCCGGAT**C**G	GCTT**C**GGGGA	GCCG**CA**AACG
L. indigo	TTAGTGAG**AC**	CTCCGGATTG	GCTTTG**A**GGA	GTCGG**A**AACG
C. cuspidata var. *sieboldii*	**CCGTGA**AG**TG**	**TTCGGATCGC**	GC**GAC**GTGG**G**	**CGGTTCGCT**G
	441	451	461	
T. bakamatsutake	GCACCTTATT	GCTGAGAAGC	TGATCAAACT	
T. flavovirens	GCACCTTATT	GCTGAGAAGC	TGATCAAACT	
A. pantherina	GCACCTT**G**TT	GCTGAGAAGC	TGATCAAACT	
A. citrina var. *citrina*	GCACC**CG**TT	GCTGAGAAGC	TGATCAAACT	
R. japonica	GC**G**CCT**CG**T**C**	GCTGA**C**AAGC	TG**G**TCAAACT	
R. compacta	GCACC**CCG**TG	GCTGAGAAGC	TGATCAAACT	
L. indigo	**A**CACC**CCC**TT	GCTGAGAAG**T**	TG**G**TCAAACT	
C. cuspidata var. *sieboldii*	**CCGGCGACG**T	**CGC**GAGAAG**T**	**CCACTG**AACC	

Fig. 2. Partial 18S rDNA sequence of *T. bakamatsutake* aligned with those of field-collected ectomycorrhizal fungi and a tree species.
Numbers are from the 5′ terminus of the forward primer in Fig. 1. Straight and wavy lines indicate the variable positions used for Probe 1 and Primer 1, and Probe 2 and Primer 2, respectively. Bold letters indicate the nucleotides of each sample that differ from those of *T. bakamatsutake*.

positions and on the basic requirements for PCR primers (Sommer and Tautz, 1989).

For the identification of *T. bakamatsutake* by using probes, the designed Probes 1 and 2 were subjected to hybridization assay. The PCR products amplified with the primers UF5 and UR6 from *T. bakamatsutake* and the eight other fungi and two tree samples were electrophoresed on an agarose gel and immobilized on a membrane (Immobilon™-S, Millipore Corp.) using the Southern blotting method under the manufacturer's recommended conditions. The calculated melting temperatures (Ologo™ primer analysis software for Macintosh, National Bioscience Inc.) were 66°C for Probe 1 and 64°C for Probe 2 in hybridization solution (125 mM NaCl; 173 mM sodium dodecylsulfate; 12.5 mM polyethylene glycol 7,500; and 25 mM phosphate buffer, pH 7.2). The membrane was washed four times with washing solution (12.5 mM NaCl; 17.3 mM sodium dodecylsulfate; and 2.5 mM phosphate buffer, pH 7.2) at room temperature. Hybridized probes were detected on X-ray film using the Phototope™ 6K detection kit.

Figure 3 shows the bands between UF5 and UR6 photographed and exposed on a X-ray film. Assayed with Probe 1 at 68°C, the DNA fragments from the fruit-bodies, cultured mycelia and field-collected mycelial blocks of *T. bakamatsutake* gave the clearest exposures and were most readily differentiated from those of the other samples. Probe 2 reacted with the DNA fragments of two other fungal species, *T. flavovirens* and *A. pantherina*, as clearly as with those of *T. bakamatsutake*. None of the nucleotides of *T. flavovirens* in the Probe 2 positions (446–465) differed from those of *T. bakamatsutake*, and only one nucleotide of *A. pantherina* did. Probe 2 could not differentiate this variation of the nucleotides even if it was hybridized under more stringent conditions such as at 90°C (data not shown). Probe 1 was then better for the identification of *T. bakamatsutake* among the samples used in this study.

To identify *T. bakamatsutake* by using primers, Primers 1 and 2 were used for PCR-amplification of the template DNAs from the fruit-bodies, mycelial blocks and cultured mycelia of this fungus and those of the other samples. PCR was conducted under the previously mentioned conditions except for the various annealing temperatures of 55–66°C.

Figure 4 is a photograph of the 375 nucleotides am-

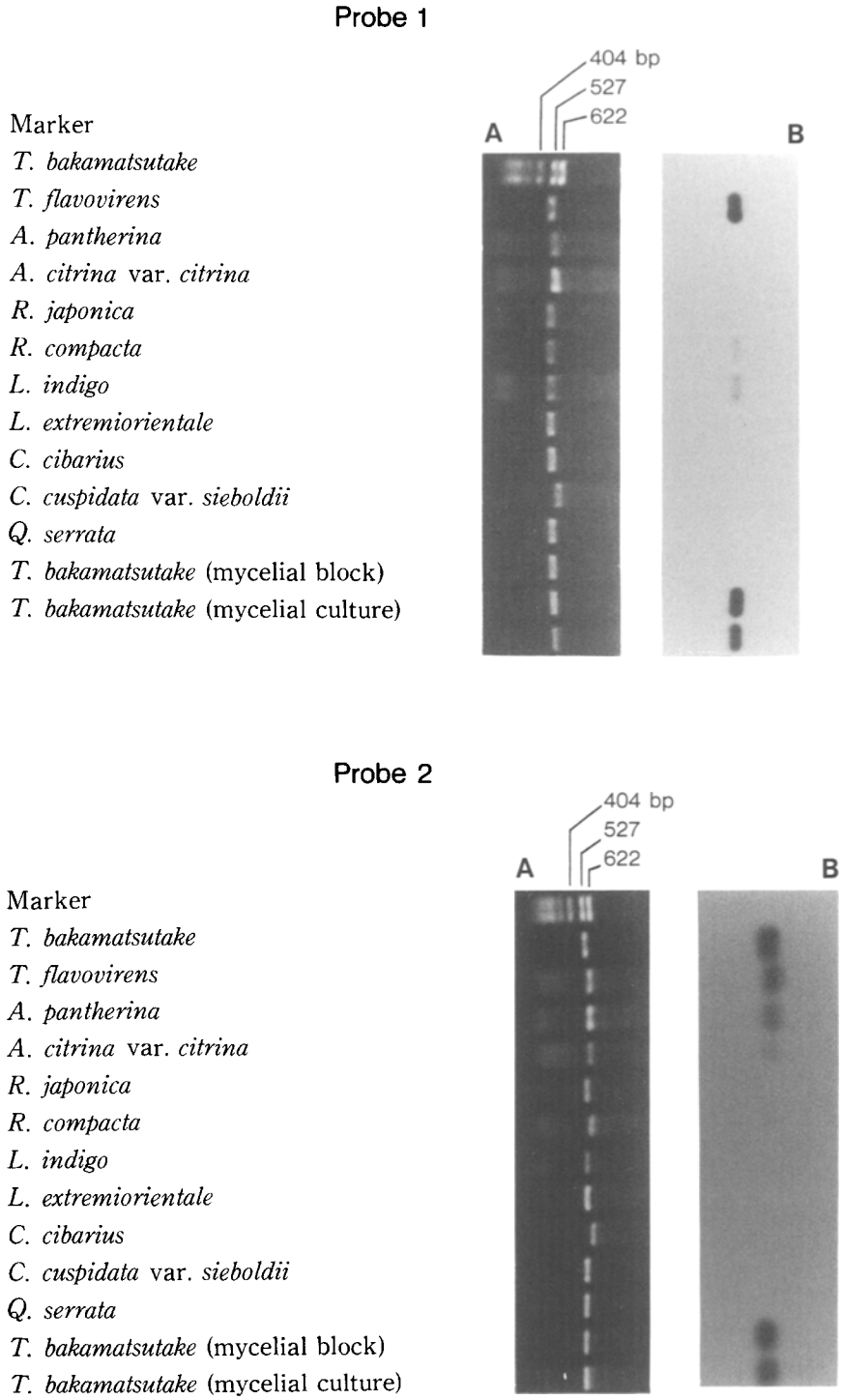

Fig. 3. PCR-amplified 18S rDNA fragments using the primaers UF5 and UR6, resolved on agarose gel; (A) photograph visualized with ethidium bromide under UV-irradiation, and (B) photogram after Southern hybridization assay with the designed Probes 1 and 2 and chemiluminescent detection.
Except where indicated, sample fungi were from fruit-bodies.

plified using the designed primers at the annealing temperature of 65.5°C. The primers effectively distinguished DNA from *T. bakamatsutake* from the DNAs of the eight ectomycorrhizal fungi and two tree species. Few attempts have been reported in the field of DNA studies of ectomycorrhizal fungi to use specific PCR primers as probes (Nakai and Hishinuma, 1992).

In this study, although the universal primers, UF5 and UR6, amplified the DNAs of both fungi and plants, the DNAs of the mycelial blocks of *T. bakamatsutake*

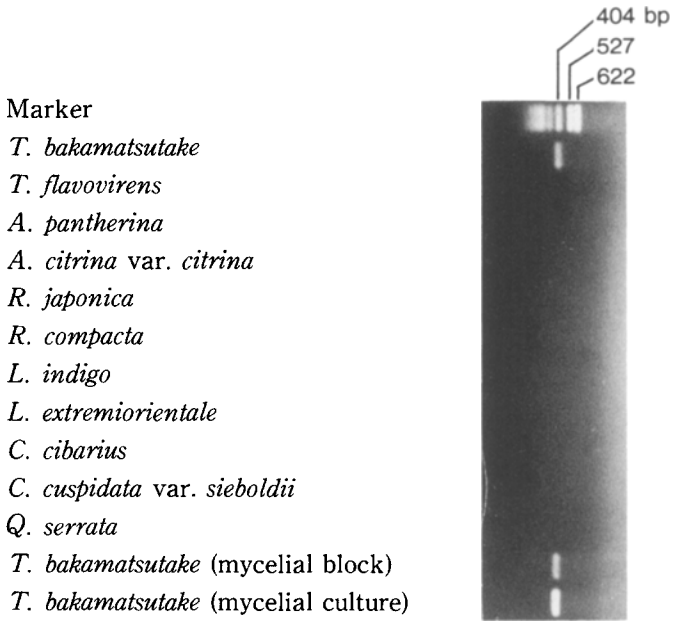

Fig. 4. Photograph of PCR-amplified 18S rDNA fragments using the designed PCR primers resolved on agarose gel and visualized with ethidium bromide under UV-irradiation.
Except where indicated, sample fungi were from fruit-bodies.

were identified by the hybridization assay using Probe 1 and the PCR-amplification using the specific PCR primers. This might lead to further detection studies of this fungus from mycelia in the soil.

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