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Discrimination of *Tricholoma* species by species-specific ITS primers

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Abstract The ITS region of ectomycorrhizal fungi was analyzed, and species-specific PCR primers were designed for 8 ectomycorrhizal *Tricholoma* species. Although a high degree of intraspecific homology was observed, interspecific variation was sufficient to design species-specific primers based on sequence of the ITS region. PCR amplification with the specific primers generated fragments of the expected sizes from DNA extracted from the strains of each species but gave no amplified products from the strains of the other 16 species in eight genera. These results suggest that sequence of the ITS region is appropriate to be used for species-level identification of ectomycorrhizal fungi.

Key words Ectomycorrhizal fungi · Internal transcribed spacer (ITS) region · Species-specific primer · *Tricholoma* species

Ectomycorrhizal fungi play important roles in forest ecosystems, i.e., enhancing the uptake of minerals by their hosts, influencing carbon and nitrogen cycles in forest ecosystem, and providing their hosts with enhanced resistance to attack by fungal pathogens (Smith and Read 1997). Community structure of ectomycorrhizal fungi has been studied based on surveys of the sporocarps. However, their appearance has been reported to be dependent on weather conditions and not necessarily to correspond to below-ground distribution of ectomycorrhizae (Gardes and Bruns 1996).

One of the problems to study the below-ground community structure of ectomycorrhizal fungi is the difficulty in identifying collected ectomycorrhizae to species based on their morphology. Although morphological descriptions of ectomycorrhizae now enable us to identify more than 200 species (Agerer 1987–2006, 1996–2006), high levels of skills and experiences are still required. Furthermore, not all the ectomycorrhizae can be identified based on their morphotypes (Yamada and Katsuya 1996; Matsuda and Hijii 1999).

Since the end of the last century, polymerase chain reaction (PCR)-based molecular methods have been developed, and PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA) has been shown to be effective for species-level identification of ectomycorrhizal fungi (Erland et al. 1994; Kårén et al. 1997). However, PCR-RFLP analysis may be inapplicable when the sample contains more than two fungal species, even though the ITS1F and ITS 4B primers, which have been reported to have enhanced specificity for basidiomycetes (Gardes and Bruns 1993), are used.

A species-specific ITS primer is one of the possible alternatives to specifically detect the presence of the species concerned, as reported for some fungal species (Goodwin et al. 1995; Atkins et al. 2005; Glen et al. 2007) as well as ectomycorrhizal fungi (Amicucci et al. 1998; Kikuchi et al. 2000; Landeweert et al. 2003). Many of the species belonging to the genus *Tricholoma* have been reported to be ectomycorrhizal (Agerer 1987–2002, 1996–2006), including *Tricholoma matsutake* (S. Ito & Imai) Singer, one of the most valuable edible fungi in Japan. In the present article, species-specific ITS primers were designed for eight ectomycorrhizal species belonging to the genus *Tricholoma* and their specificities were confirmed.

Fungal strains and sporocarps used in this study are shown in Table 1. All procedures to design species-specific primers were as described by Kikuchi et al. (2000). Briefly, sequencing was performed directly on purified PCR products of the ITS (ITS1/ITS4) region using an automated fluorescent DNA sequencer SQ-5500L (Hitachi, Tokyo, Japan) according to the manufacturer's instructions. When necessary, PCR products were ligated into the plasmid

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Table 1. Fungal strains used in this study

Species	Strain	Sample[a]	Geographical origin	DDBJ accession no.	Culture accession no.
Tricholoma japonicum Kawamura	Tj 1	m	Kounan, Shiga	AF204809	
	Tj 3	m	Shigaraki, Shiga	AF204810	IFO32820
T. auratum (Paulet) Gillet	Tk 3	m	Kounan, Shiga	AB289659	ATCC76003
	Tk 6	m	Kounan, Shiga	AB289660, AB289661[b]	
	ISK1	m	Kaga, Ishikawa	AB289662	IFO32788
	Tk 1	m	Mikuni, Fukui	AB289663	IFO32821
T. matsutake (S. Ito & Imai) Singer	Tm 1	m	Ina, Nagano	AF204868	
	Tm A-5	m	Kake, Hiroshima	AF202772	
T. bakamatsutake Hongo	Tb 1	m	Miwa, Kyoto	AF204807	
	WK1B	m	Ryujin, Wakayama	c	
	Tb30663	m	unknown	c	IFO30663
T. robustum (Alb. & Schwein.) Ricken	Tr 1	f	Ina, Nagano	AB289664	
	Tr 3	m	Kounan, Shiga	AB289665	ATCC76000
	Tr 6936	m	unknown	AB289666	IFO6936
T. fulvocastaneum Hongo	WK1N	m	Nakahechi, Wakayama	AF204808	
	TN 6940	m	unknown	AB289667	IFO6940
	TN 6941	m	unknown	AB289668	IFO6941
T. ustale (Fr.) Kummer	Tu 1	m	Hino, Shiga	AF204812	IFO32825
	HyTu1	m	Kouduki, Hyogo	c	
T. caligatum (Viv.) Ricken	Tc 1	m	Algeria	AF204813	
	Tc 2	m	Morocco	c	
T. magnivelare (Peck) Redhead	Tp 1	m	Canada	AF204811	
	Tp 4	m	Canada	c	
Lyophyllum shimeji (Kawam.) Hongo	Ls 1	m	Kake, Hiroshima		
	Ls 13	m	Kutsuki, Shiga		IFO32810
Laccaria laccata (Scop.) Fr.	Ll 1	m	Hurano, Hokkaido		
Amanita muscaria (L.) Lam.	Amus	m	Kaida, Nagano		
A. citrina var. *citrina* (Schaeff.) Pers.	Ac	f	Kake, Hiroshima		
Rozites caperata (Pers.) Karst.	Rc1	m	Ayabe, Kyoto		
Suillus luteus (L.) Roussel	Sl 9	m	Hino, Shiga		IFO32818
	Sl 10	m	Kounan, Shiga		
S. bovinus (Pers.) Roussel	Sb 15	m	Kounan, Shiga		
	Sb 2-77	m	Kake, Hiroshima		
	Sb 744	m	Katano, Osaka		
Russula emetica (Schaeff.) Pers.	Re	f	Kake, Hiroshima		
Pisolithus tinctorius (Pers.) Coker & Couch	Pt 3	m	Ogasawara, Tokyo		
	Pt 4	m	Ogasawara, Tokyo		
Rhizopogon rubescens Tul.	Rr 1	m	Amino, Kyoto		
	Rr 2	m	Miyazu, Kyoto		

DDBJ: DNA Data Bank of Japan; IFO: Institute for Fermentation, Osaka; ATCC: The American Type Culture Collection
[a] DNA was extracted from the fruit bodies (f) or cultured mycelia (m)
[b] The strain contained the two different sequences of the ITS region
[c] The sequence of the ITS region was completely identical to that of the strains of the same species in the table

vector pCR2.1 (Invitrogen, Carlsbad, CA, USA) and then sequenced. DNA sequences obtained were deposited in the DDBJ database; the accession numbers are shown in Table 1. The sequence data were aligned using the ClustalX program (Thompson et al. 1997), and specific primers for each *Tricholoma* species were designed based on the alignments (Table 2, Fig. 1). Levels of sequence similarity (S) were calculated between all the strains of *Tricholoma* species used in the study, according to $S\,(\%) = N_C/N_T \times 100$, where N_C and N_T are the number of nucleotides in common and the total length of the alignment compared, respectively. Inter- and intraspecific sequence similarities are presented in Table 3 as the average of those between the strains of the species compared.

PCR amplification with putatively species specific primers (see Table 2) was performed in a 20-µl reaction mix containing 5 ng genomic DNA, 0.2 µM each primer, 2 µl 10× buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 0.01% gelatin], 200 µM each dNTP, and 0.5 units Ampli Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The reaction was performed in a Perkin Elmer DNA Thermocycler 9600 (Applied Biosystems), and the parameters of PCR amplification were as follows: an initial denaturation at 94°C for 5 min was followed by 35 cycles of denaturation at 94°C for 30 s, and annealing and extension at suitable temperature for each specific primer pair (see Table 2) for 1.5 min. Presence or absence of amplified fragment was examined by electrophoresis on 1.5% agarose gel in a 0.5× Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) buffer and visualized by ethidium bromide staining and UV irradiation.

PCR amplification with primer pairs putatively specific for each targeted species generated the fragments of the expected sizes (see Table 2) from DNA extracted from strains of each species but gave no amplified products from those of the other species (Fig. 2). Therefore, the primers designed in the present study were suggested to be specific for each *Tricholoma* species.

Table 2. Primers and annealing temperatures for specific PCR amplification of *Tricholoma* species

Species	Primer	Sequence (5′ to 3′)	Ta (°C)	Expected size (bp)
Tricholoma japonicum	Tj	GCACTTTTTGTAGACCTAA	58	577
	ITS4[a]	TCCTCCGCTTATTGATATGC		
T. auratum	Tk	AGACTTTCTTGGTTTGGCGC	62	547, 548, 550
	ITS4[a]	TCCTCCGCTTATTGATATGC		
T. robustum	Tr	CTTTTGTAGACCTGGATATC	60	518, 520
	Tr-R	GAAATGTCCGAAGACGATT		
T. fulvocastaneum	TN	TAATATTTCTAGGTTTATGT	52	494, 495
	ITS4[a]	TCCTCCGCTTATTGATATGC		
T. bakamatsutake	Tb	TGTTTGTAGACTTGGGTAAGG	60	563
	ITS4[a]	TCCTCCGCTTATTGATATGC		
T. ustale	ITS1[a]	TCCGTAGGTGAACCTGCGG	60	636
	Tu	GTCAGAAAAGGTCCCATAGG		
T. magnivelare	Tp	TGTTTTATTATATACTCAGC	59	403
	Tc-R	GACGATTAGAAGCCGAACTC		
T. caligatum	Tm[b]	CATTTTATTATACACTCGGT	59	404
	Tc-R	GACGATTAGAAGCCGAACTC		

Ta: annealing temperature
Sequence of the primers were reported by White et al. (1990) (a) or Kikuchi et al. (2000) (b)

Table 3. Average sequence similarity (%) of the ITS region among the *Tricholoma* species used in the study

Species	% Sequence Similarity								
	1.	2.	3.	4.	5.	6.	7.	8.	9.
1. *Tricholoma japonicum* (2)	100.0								
2. *T. auratum* (4)	79.6	99.5							
3. *T. robustum* (3)	82.1	78.0	99.5						
4. *T. matsutake* (2)	80.4	77.6	83.5	100.0					
5. *T. bakamatsutake* (3)	78.6	76.1	78.0	82.3	100.0				
6. *T. fulvocastaneum* (3)	76.2	71.1	75.4	81.2	78.8	99.0			
7. *T. ustale* (2)	83.4	79.3	94.7	85.2	78.8	76.0	100.0		
8. *T. caligatum* (2)	80.3	77.5	83.6	98.1	83.6	80.3	84.7	100.0	
9. *T. magnivelare* (2)	80.3	77.3	83.3	96.3	82.3	80.4	85.1	96.7	100.0

The values are the averages of sequence similarities between the strains of the species compared used in the study
The number in parenthesis after each species is that of the strains of each species used in the study

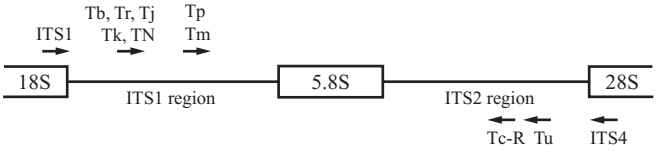

Fig. 1. Schematic diagram showing the positions of the polymerase chain reaction (PCR) primer used in the study. The *arrows* represent the 3′-end and approximate positions of each primer. Sequences of the primers are provided in Table 2

A few studies on specific primers for some *Tricholoma* species based on the 18S rDNA region sequence have been already reported. Alignments of the 18S rDNA showed high homology (96.4% on average) between *T. bakamatsutake* Hongo and other species both in *Tricholoma* and other genera (Terashima and Nakai 1996). Furthermore, the sequence of the 18S rDNA showed no differences among *T. matsutake*, *T. magnivelare* (Peck) Redhead, and *T. caligatum* (Viv.) Ricken (Nakai and Ohno 1994). By contrast, the sequence of the ITS region showed high interspecific variation: the average sequence similarities between *T. bakamatsutake* and other *Tricholoma* species used in the present study were not more than 83.6% (see Table 3), and sequence similarities between *T. matsutake* and *T. magniverale*, *T. matsutake* and *T. caligatum*, and *T. magniverale* and *T. caligatum* were 96.3%, 98.1%, and 96.7%, respectively (Table 3). On the other hand, intraspecific homology of the ITS region was sufficient to design species-specific primers based on the sequence (see Fig. 2): the strains of the same species had a completely identical sequence, except for those of *T. auratum* (Paulet) Gillet, *T. robustum* (Alb. & Schwein.) Ricken, and *T. fulvocastaneum* Hongo, which also showed high intraspecific homology, 99.5%, 99.5%, and 99.0% on average, respectively (Table 3). This result indicates that the ITS region sequence is more informative than the 18S rDNA sequence for species-level identification of ectomycorrhizal fungi.

Sequence characterized amplified regions-PCR (SCAR-PCR) has been reported to be an effective strategy for designing specific primers at various levels of taxonomy (Paran and Michelmore 1993). For ectomycorrhizal fungi, a species-specific primer pair for *Tuber borchii* Vittad. was designed by this method (Bertini et al. 1998). SCAR-PCR consists of two steps: randomly amplified polymorphic

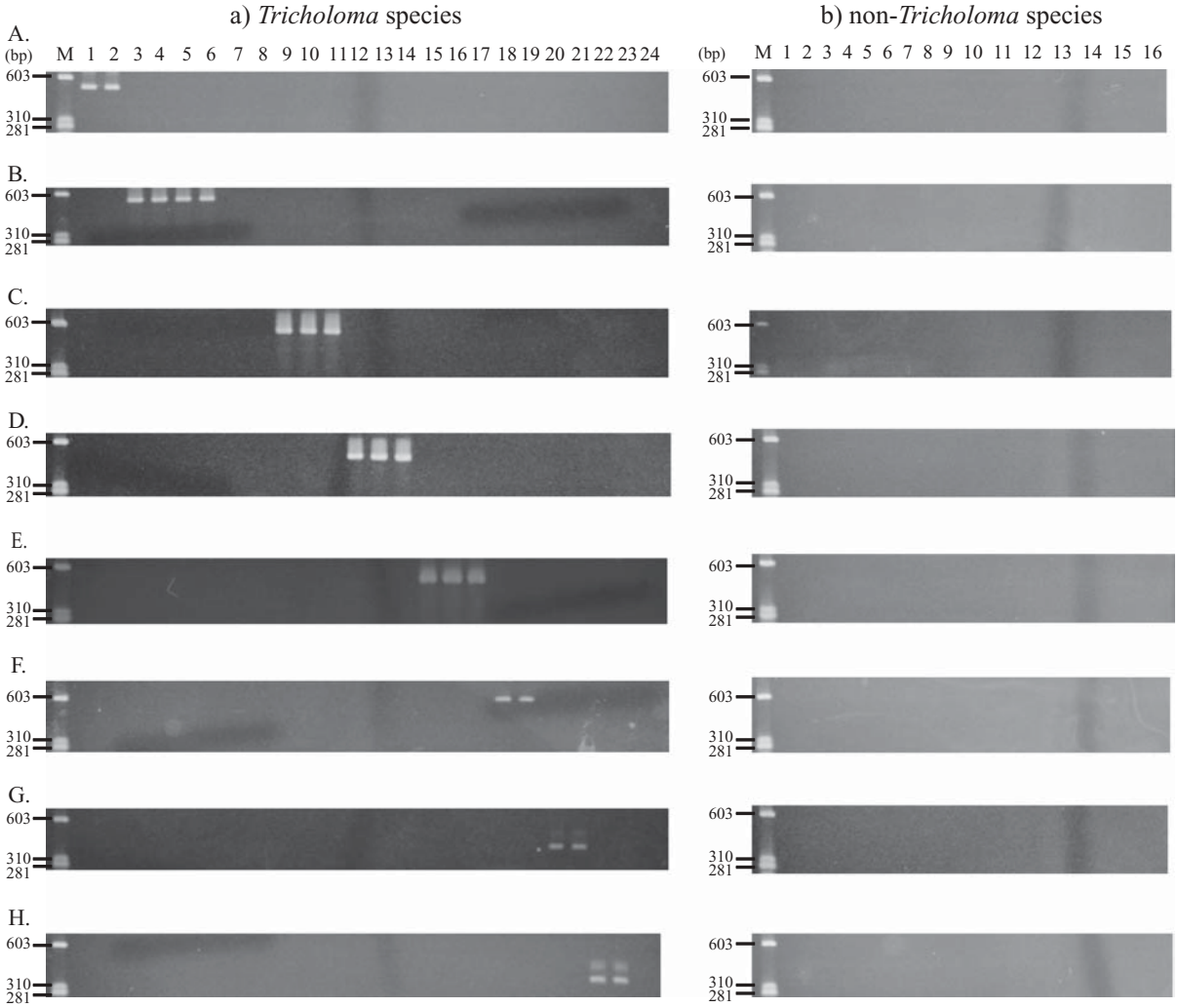

Fig. 2. Agarose gel electrophoresis of PCR amplification products using species-specific internal transcribed spacer (ITS) primers for (**A**) *Tricholoma japonicum*; (**B**) *T. auratum*; (**C**) *T. bakamatsutake*; (**D**) *T. robustum*; (**E**) *T. fulvocastaneum*; (**F**) *T. ustale*; (**G**) *T. magnivelare*; (**H**) *T. caligatum*. *a Tricholoma* species: *lane 1*, Tj1; *lane 2*, Tj3; *lane 3*, Tk3; *lane 4*, Tk6; *lane 5*, ISK1; *lane 6*, Tk1; *lane 7*, Tm1; *lane 8*, TmA-5; *lane 9*, Tb1; *lane 10*, WK1B; *lane 11*, Tb30663; *lane 12*, Tr1; *lane 13*, Tr3; *lane 14*, Tr6936; *lane 15*, WK1N; *lane 16*, TN6940; *lane 17*, TN6941; *lane 18*, Tu1; *lane 19*, HyTu1; *lane 20*, Tp1; *lane 21*, Tp4; *lane 22*, Tc1; *lane 23*, Tc2; *lane 24*, negative control; *M*, molecular marker. *b* Non-*Tricholoma* species: *lane 1*, Ls1; *lane 2*, Ls13; *lane 3*, Ll1; *lane 4*, Amus; *lane 5*, Ac; *lane 6*, Rc1; *lane 7*, Sl9; *lane 8*, Sl10; *lane 9*, Sb15; *lane 10*, Sb2-77; *lane 11*, Sb744; *lane 12*, Re; *lane 13*, Pt3; *lane 14*, Pt4; *lane 15*, Rr1; *lane 16*, Rr2; *M*, molecular marker. The abbreviation for each fungal strain is listed in Table 1. Molecular size marker used was φX174/*Hae* digest

DNA (RAPD) analysis to find a specific amplified fragment and design of specific primers based on the sequence of the fragment. Of these steps, the former RAPD analysis is easy but laborious and time consuming. Furthermore, the specific fragments need to be subcloned to vectors before sequencing as both termini of the amplified fragments have the same sequence. In contrast, PCR products of the ITS region were able to be sequenced by direct sequencing for most of the strains used in the present study (see Table 1). Therefore, the approach of designing species-specific primers based on the ITS region sequence, which was employed in the present study, was supposed to be an appropriate strategy for designing species-specific primers.

In the present study, although PCR amplification from DNA extracted from field-collected samples such as ectomycorrhizae or extramatrical mycelia was not performed, applicability of specific primers for specific detection of each species from field samples is strongly possible, as shown in other studies (Terashima and Nakai 1996; Bertini et al. 1998; Kikuchi et al. 2000; Guerin-Laguette et al. 2005). Species-specific primers have been shown to have a wide range of possible applications: combination of real-time PCR and species-specific ITS primers have provided a qualitative and quantitative assay for detection of the fungal species concerned from field-collected samples (Landeweert et al. 2003; Atkins et al. 2005), and in situ PCR using specific primers allowed the localization of low abundance nucleic acids targets directly within tissue sections of fungal samples (Bago et al. 1998; Bindslev et al. 2002). Therefore, development of specific ITS primers for the ectomycorrhizal species belonging to other than *Tricholoma* will contribute to the study of ectomycorrhizal fungi.

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