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Detection of the ectomycorrhizal fungus *Tricholoma matsutake* and some related species with specific ITS primers

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Oligonucleotide primers (Tm1 and Tm4) were designed to amplify a 447–448 base pair fragment, comprising sections of the rDNA internal transcribed spacers (ITS) and the entire 5.8S rDNA, of *Tricholoma matsutake*. PCR products of predicted size were produced for six of eight isolates of *T. matsutake* from across its natural range in Asia, and for isolates of some closely related fungi including *T. bakamatsutake*, *T. magnivelare*, and *T. caligatum*. The closely related *T. robustum* could be discriminated from *T. matsutake* by PCR fragment size. No PCR products were produced where the primers were tested against 16 species of ectomycorrhizal fungi associated with *Pinus* spp. in the Southern Hemisphere. The specific primers were also used successfully to produce PCR products from matsutake infected roots collected in natural forests in China and Japan, and from pure culture synthesised *Pinus radiata-T. matsutake* material. These primers will be useful in research directed at establishing matsutake in the Southern Hemisphere, and also have the potential to be applied to the study of matsutake within its natural range.

Key Words——ectomycorrhizas; internal transcribed spacers; polymerase chain reaction; *Tricholoma matsutake*.

Matsutake (*Tricholoma matsutake* (Ito et Imai) Sing.), and some closely related *Tricholoma* spp., are highly valued edible ectomycorrhizal mushrooms. Apart from one record for bakamatsutake (*T. bakamatsutake* Hongo) from Papua New Guinea (Otani, 1976), matsutake and related fungi appear to be confined to the Northern Hemisphere (Wang et al., 1997). In Asia, *T. matsutake* is found in Japan, Korea, the Russian Far East, Taiwan and China, usually in association with members of the Pinaceae and less frequently with Fagaceae (Wang et al., 1997). Principal hosts include *Pinus densiflora* Sieb. et Zucc., *P. thunbergii* Parl., *P. taiwanensis* Hayata, *P. yunnanensis* Franchet and *P. densata* Masters (Zang, 1990; Wang et al., 1997), and less important hosts within the family Pinaceae include *P. pumila* (Pallas) Regel, *Picea glehnii* (Fr. Schm.) Mast., *Picea jezoensis* (Sieb. et Zucc.) Carr., *Tsuga canadensis* (L.) Carr., and *T. diversifolia* Mast. (Ogawa, 1978). The harvest of matsutake from pine forests in Japan, the principal market, has declined for several reasons since the 1940's (Iwase, 1997; Wang et al., 1997), therefore matsutake and several closely related species, including *T. magnivelare* (Peck) Redhead and *T. caligatum* (Viv.) Rick., are now exported to Japan from China, Korea, North America and North Africa. Because of its high commercial value there is now some interest in establishing matsutake in pine plantations in the Southern Hemisphere where it could be grown out of season for export to Japan, although there appear to be substantial technical difficulties to be overcome (Wang et al., 1997). In any proposed program to establish matsutake there is the need for sensitive and specific techniques to detect and assess the survival of the fungus under laboratory, glasshouse and field conditions. RFLP analysis of PCR amplified Internal Transcribed Spacers of ribosomal DNA (ITS-RFLP) has been used recently to identify specific fungi from mycorrhizal roots in some ecological studies (Gardes and Bruns, 1996a; Erland, 1995; Horton and Bruns, 1998; Horton et al., 1998). ITS-RFLP also appear to readily discriminate between tested isolates of *T. matsutake* and the entire range of known potential competitor ectomycorrhizal fungi present in south-western Australia (Dunstan, unpublished data). Other researchers have developed PCR primers based on the ITS region of ribosomal DNA for the specific detection of some *Tuber* spp. (Amicucci et al., 1998; Paolocci et al., 1997; Rubini et al., 1998). Terashima and Nakai (1996) have also developed specific 18S rDNA primers for the identification of *T. bakamatsutake*, however they were not tested against related *Tricholoma* species. Nakai also attempted to discriminate *T. matsutake* from other related species with primers based on the ITS region (personal communication).

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Specific primers may eliminate the need for the additional expense of RFLP analysis where a single species of fungus is targeted. For the reasons outlined by Bruns et al. (1991) we also chose the ITS regions of the ribosomal DNA as target sites for the development of matsutake specific PCR primers.

Materials and Methods

Fungal isolates Fungal material used for design and testing of primers (Table 1) included isolates of *T. matsutake* from some locations within its range in Asia (Wang et al., 1997), some closely related *Tricholoma* spp., and a selection of ectomycorrhizal fungi found in the Southern Hemisphere associated with *Pinus* spp., (Dunstan et al., 1998; U. Kõljalg and W. Dunstan, unpublished data), where most have been introduced.

Field and pure culture synthesised material Soil including mycorrhizal roots and mycelium was collected from beneath *T. matsutake* fruitbodies associated with *Pinus armandii* Franchet and *Castanopsis* sp. (Zi Xi Mountain, Yunnan Province, PRC) and *P. densiflora* (Mukaibayashi Forest, Iwaizumi, Japan). Soil was stored at ca. 4°C for up to 12 mo before DNA was extracted from roots putatively mycorrhizal with *T. matsutake*. DNA was extracted from mycelium infected roots of 18 mo old *P. radiata* D. Don seedlings which had been inoculated with *T. matsutake* and grown under aseptic conditions (T. Shiraishi, unpublished).

Plant material Seeds of *Pinus densiflora* and *P. radiata* were sterilised after the method of Marx and Bryan (1970), and were germinated on water agar with 10 μM B as H_3BO_3 and 1 mM Ca as $Ca(NO_3)_2 \cdot 4H_2O$. DNA was extracted from entire uncontaminated 2 wk old seedlings.

DNA extraction Mycelial cultures (100–200 mg), or

Table 1. Isolates and origins of Matsutake and related species, and some other ectomycorrhizal fungi associated with pines, used in development and testing of PCR primers.

Code[1]	Species	Origin
E869	*Tricholoma matsutake* (Ito et Imai) Singer	Kangwon Province, Korea
E876	*T. matsutake*	Iwate Prefecture, Japan
E878	*T. matsutake*	Iwate Prefecture, Japan
E916	*T. matsutake*	Hiroshima Prefecture, Japan
E5049	*T. matsutake*	Hiroshima Prefecture, Japan
FY105	*T. matsutake*	Iwate Prefecture, Japan
GM924	*T. matsutake*	Yunnan Province, China
SR3	*T. matsutake*	Yunnan Province, China
ZX02	*T. bakamatsutake* Hongo	Yunnan Province, China
E5051	*T. bakamatsutake*	Hiroshima Prefecture, Japan
E874	*T. magnivelare* (Peck) Redhead	North America[2]
323	*T. robustum* (Alb. et Schw.: Fr.) Rick.	Nagano Prefecture, Japan
305	*T. caligatum* (Viv.) Rick.	Morocco
E412	*Amanita muscaria* (L.: Fr.) Hook.	Australia (eastern)
E902	*Boletus edulis* Bull.: Fr.	France
H200	*Cenococcum geophilum* (Sowerby) Ferd. & Winge	USA
DE051	*Chalciporus piperatus* (Bull.: Fr.) Bat.	Australia (eastern)
DE011	*Hebeloma crustuliniforme* (Bull.: Fr.) Quel.	Australia (western)
DE017	*Laccaria laccata* (Scop.: Fr.) Berk. & Br.: Pat.	Australia (western)
E3044	*Lactarius deliciosus* (L.: Fr.) S. F. Gray	Australia (eastern)
DE014	*Paxillus involutus* (Batsch: Fr.) Fr.	Australia (western)
DH005	*Rhizopogon luteolus* Fr. apud Fr. & Nordh.	Australia (western)
DH004	*R. roseolus* (Corda) Th. Fr.	Australia (western)
DE005	*Suillus collinitus* Fr.	Australia (western)
DE007	*S. luteus* (L.: Fr.) S. F. Gray	Australia (western)
DE044	*Thelephora terrestris* Ehrh.: Fr.	Australia (western)
M010	*Tomentella* sp.	Australia (western)
920	*Tricholoma saponaceum* (Fr.: Fr.) Kummer	Japan
DE085	*T. terreum* (Schaeff.: Fr.) Kummer	Australia (eastern)

Notes: [1] Isolate numbers prefixed with E or H refer to cultures held at the CSIRO Herbarium, CSIRO Forestry and Forest Products, Perth, Western Australia. Other cultures are maintained at Biol. Environ. Inst., KEEC Co. Ltd., Uji, Japan, or at Murdoch University, Western Australia.
[2] Obtained in Japan from imported material; precise origin not known.

dried herbarium material (70–110 mg), were frozen with liquid nitrogen and slurried in a mortar with 200 µl of extraction buffer of Raeder and Broda (1985). Alternatively, samples of roots from pure culture syntheses (15 mg f.w.) and root samples from field sites (7–14 mg f.w.) were frozen in liquid nitrogen and ground in extraction buffer in 1.5 ml Eppendorf tubes using an electric pellet mixer. Slurried samples were incubated in a waterbath at 65°C for 1–2 h, centrifuged for 5 min (14 000 rpm), and supernatants were transferred to a fresh tube. DNA extracts were further purified, by a silica binding method, using a Bresa-Clean™ DNA purification kit according to the manufacturer's instructions.

DNA bound to silica was eluted in TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8), (30 µl for root samples, 50 µl for other extracts), and quantified by fluorometry. Extracts were incubated with RNAse A (100 pg μl^{-1}, final concentration) at 37°C for 10 min., diluted to 1 ng μl^{-1} in H_2O and stored at −20°C.

DNA sequencing For fungal isolates where the ITS were sequenced, the ITS regions were amplified in 20 µl reactions using 1ng of template genomic DNA with 25 pmol of each of the primers ITS1 and ITS4 (primer sequences previously described by White et al., 1990). Reaction mixtures contained 0.2 mM dNTPs, 2 mM $MgCl_2$, 67 mM Tris-HCl (pH 8.8 at 25°C), 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/ml Gelatin, and 0.7 U Tth Plus* Polymerase (Biotech International Ltd.). Thermal cycler sequence comprised an initial denaturation at 94°C for 10 min followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 1 min, extension at 72°C for 2 min, and a final extension cycle at 72°C for 10 min.

PCR amplified fragments of the ITS were subjected to agarose gel electrophoresis (1% agarose), excised from the gel after staining with 0.5 µg/ml ethidium bromide, and extracted and purified using a QIAGEN® QIAquick™ Gel Extraction Kit according to the manufacturer's instructions. Purified products were eluted from spin columns in 10 mM Tris-HCl, pH 8.5 and quantified by fluorometry. Double stranded ITS fragments were sequenced from both ends using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions, with 100 ng of template ITS fragments per reaction, and 2 pmole of one of the primers ITS1, ITS2, ITS3 or ITS4 (primers described by White et al., 1990) per reaction, in 10 µl reactions. Sequences of selected isolates of *T. matsutake* were edited and aligned using SEQUED 1.04 and CLUSTAL W (1.4), (PE Applied Biosystems, Foster City, California). Potential forward and reverse primers were selected manually and using Primer Express™ primer design program, (PE Applied Biosystems, Foster City, California), from aligned ITS1 and ITS2 sequences of *T. matsutake* isolates E916, E5049, E876 and E878. Primers were selected to include segments of ITS1, ITS2 and the entire 5.8S rDNA regions. Primers were synthesised by GibcoBRL Custom Primers.

Amplification with specific primers Specific PCR reactions were performed under the following conditions in 20 µl reactions: 10 pmol of each primer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 67 mM Tris-HCl (pH 8.8 at 25°C), 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/ml Gelatin, and 0.7 U Tth Plus* Polymerase per reaction. Genomic DNA templates were 1 ng per reaction for fungal, plant and synthesised material, or 10^0–10^{-2} dilutions for extracts from field samples (where DNA concentrations could not be determined by fluorometry). Thermal cycle parameters, modified after Gardes and Bruns (1993), comprised the following: initial denaturation step at 95°C for 85 s; followed by 13 cycles of denaturation at 95°C for 35 s, annealing at 58°C for 55 s, and extension at 72°C for 45 s. For cycles 14–26 and 27–35 extension times were increased to 120 and 180 s respectively, followed by a final extension at 72°C for 10 min and a final holding temperature of 15°C.

Electrophoresis Products from specific PCR amplification reactions were analysed using 2% agarose (Progen Industries Ltd., DNA grade) run at 70 V (4.7 V/cm^{-1}) for 90–120 min., and visualised after staining in 0.5 µg ml^{-1} ethidium bromide.

Results and Discussion

Sequences for the forward primer Tm1 and reverse primer Tm4, corresponding to positions 98–119 in ITS1 and 95–120 in ITS2 respectively of *T. matsutake* E916, are detailed in Table 2.

The primer pair Tm1–Tm4 was used successfully to obtain PCR products of predicted size for most isolates of *T. matsutake*, but also for *T. bakamatsutake* (one of two isolates), *T. magnivelare* and *T. caligatum* (Fig. 1; Table 2). No PCR product was obtained for the single *T. matsutake* isolate from Korea (E869, Fig. 1). Subsequent DNA sequencing revealed a 10 bp deletion in *T. matsutake* E869 at the forward primer site, although there was otherwise 98–100% homology between the ITS and 5.8S rDNA sequences between this isolate and the other fungi used in the design of primers. The isolate of *T. robustum* and one isolate of *T. matsutake* (GM924) produced products ca. 10 bp shorter than other isolates (Fig. 1), which suggests that the identity of *T. matsu-*

Table 2. Primers selected from ITS 1 and ITS 2 sequences.

Primers	PCR product size (bp)
Tm1 5′-AAA TAT GTC TCG AGG AAG CTC-3′ (forward)	
Tm4 5′-CAA TGG CGT AGA TAA TTA TCA CAC C-3′ (reverse)	447–448[1] (447±18)[2]

Notes: Product size estimated from [1] DNA sequences; [2] electrophoresis of *T. matsutake*/Tm1–Tm4 PCR fragments on 2% agarose.

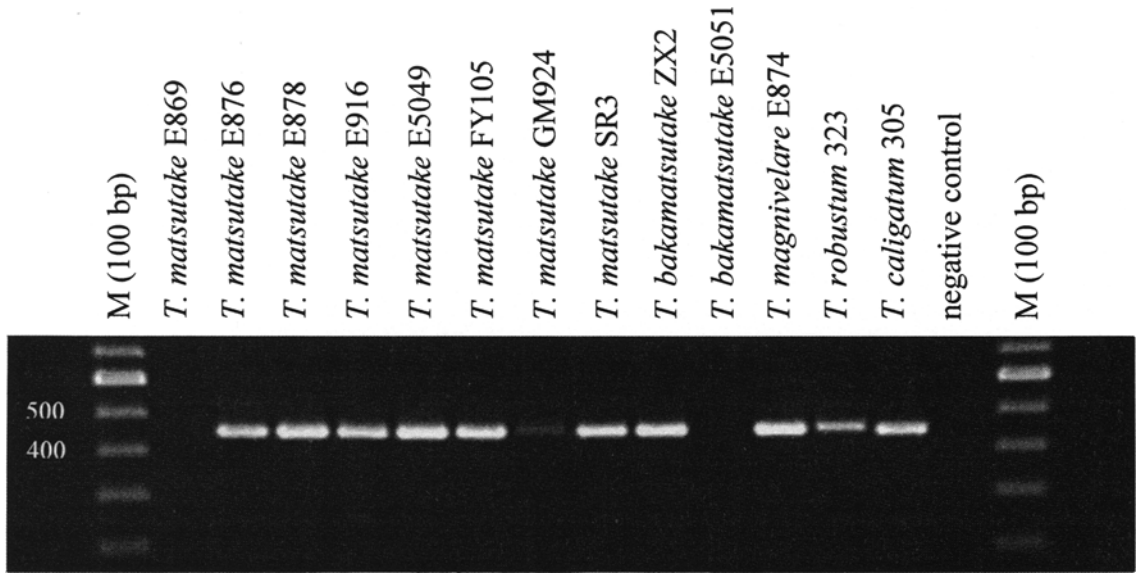

Fig. 1. Amplification products obtained with the primers Tm1 and Tm4 and DNA of isolates of *Tricholoma matsutake* and some related fungi.

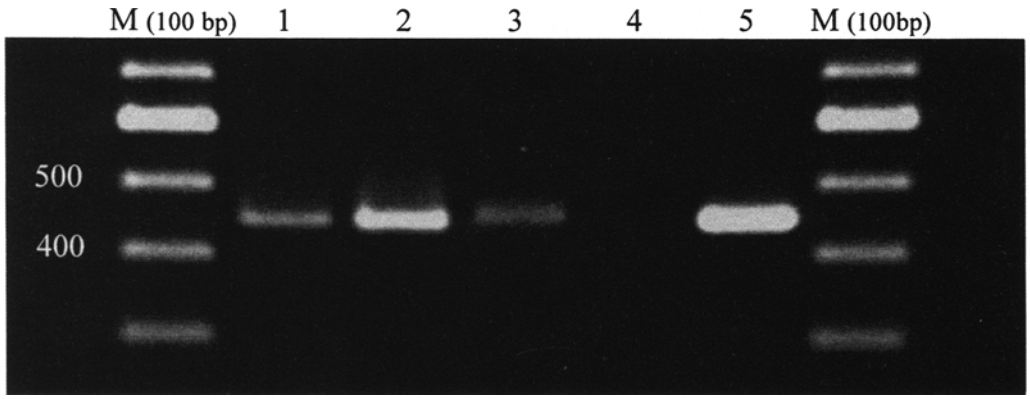

Fig. 2. Amplification products obtained with the primers Tm1 and Tm4 and DNA extracted from roots putatively mycorrhizal with *Tricholoma matsutake*, or from pure culture synthesised material. Lane1, *Pinus radiata-T. matsutake* pure culture synthesis; lane 2, *T. matsutake* mycorrhizal roots (China); lane 3, *T. matsutake* mycorrhizal roots (Japan); lane 4, negative control; lane 5, positive control (*T. matsutake* E5049, pure culture).

take GM924 should be re-examined. The anomalies with *T. matsutake* identified in this study indicate the importance of testing PCR based methods against positively identified local or regional material (sporocarps and /or cultures) before screening of mycorrhizas, which has been previously suggested by Kårén et al. (1997). *Tricholoma matsutake* E916 Tm1–Tm4 PCR products were also sequenced using primers ITS2 and ITS3. With the exception of the primer sites plus ca. 20 bp of poor quality sequence 3′ of each primer, sequence of the E916 Tm1–Tm4 fragment was found to be 100 percent homologous with a similar section of the previously sequenced ITS and 5.8S rDNA of *T. matsutake* E916. No PCR products were produced where the primer pair Tm1–Tm4 was tested against the 16 isolates of species of ectomycorrhizal fungi included in Table 1, that are found in association with introduced pines in the Southern Hemisphere. No PCR products were obtained using genomic DNA of *Pinus densiflora* or *P. radiata* as templates. The primer pair Tm1–Tm4 was used successfully to obtain PCR products, of size consistent with matsutake, from pure culture synthesised and field material (Fig. 2). Serial dilutions 10^{-1} and 10^{-2} of DNA extracts from field samples (1 μl template) consistently yielded PCR products. Successful amplification with Tm1–Tm4 using field material stored at sub-optimal conditions highlights the sensitivity and specificity of this method. Although the number of field samples used in this study is very small, the results presented here contrast with the findings of Erland (1995). Using the universal primers ITS1 and ITS4 she found that a larger number of dilutions had to be used as template to obtain a PCR product, and a reduced success rate with time of storage of samples. Annealing temperatures of 55°C and 62°C were also

tested using Tm1–Tm4, and did not appear to affect yield.

The apparent specificity of this primer pair for matsutake and some related fungi clearly makes them useful in research aimed at establishing matsutake in the Southern Hemisphere. The primers detailed in this study have not been tested against the large and diverse range of other *Tricholoma* spp. found in the Northern Hemisphere; this requires further investigation. If this set of primers does amplify rDNA from as yet untested *Tricholoma* spp. they may still have some application in ecological studies of matsutake within its natural range if it can be established that matsutake and/or closely related fungi can be discriminated using RFLP analysis. To develop primers even more specific for *T. matsutake*, the intergenic spacer (IGS), between 25S rDNA and 5S rDNA, may be an appropriate region for further investigation because of the possibly greater variation within this region of rDNA at the species level than available within the ITS (Gardes and Bruns, 1996b).

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