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Isolation of *Tricholoma matsutake* and *T. bakamatsutake* cultures from field-collected ectomycorrhizas

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***Tricholoma matsutake* was isolated into pure cultures from field samples of ectomycorrhizas on *Pinus densiflora*. The mycorrhizal tips were collected at different times of the year from a colony of *T. matsutake* in a *P. densiflora* stand. The mycorrhizal tips were continuously washed with sterilized distilled water and diluted Tween 80 solution, surface-sterilized with calcium hypochlorite solution, and inoculated on several kinds of nutrient agar media. Most of the mycorrhizal tips collected in winter and spring produced colonies that were morphologically similar to cultures of *T. matsutake* isolated from basidiocarps. The identity of isolates obtained from mycorrhizas was further confirmed to be *T. matsutake* based on fungal morphology and RFLP patterns of PCR amplified rDNA. The feasibility of *T. bakamatsutake* isolation into pure culture from ectomycorrhizas on *Quercus serrata* was also confirmed. These results indicated that mycelium of matsutake mushrooms can be isolated into pure culture from ectomycorrhizas at different times of the year. Mycorrhizas of both *T. matsutake* and *T. bakamatsutake* were not observed to have any specific association with soil fungi such as *Mortierella* spp.**

Key Words——basidiomycetes; DNA identification; Matsutake mushroom; *Mortierella*; soil microorganisms.

Isolation from mycorrhizas is an ideal means to obtain the fungal symbiont of ectomycorrhiza. The isolation method has been used for several decades, and many fungal symbionts have been established as pure cultures from mycorrhizas on a variety of host plants (Harley, 1959; Björkman, 1960; Zak and Bryan, 1963; Zak and Marks, 1964; Lamb and Richards, 1970; Chu-Chou, 1979; Warcup and McGee, 1983; Danielson, 1984; Warcup, 1985; Erland and Söderstrom, 1990; Cairney et al., 1994; Yamada and Katsuya, 1995; Zelmer and Currah, 1995). However, until recently this method has not been popular due to the difficulty of isolating and identifying fungal symbionts (Zak, 1973; Molina and Palmer, 1982; Heinonen-Tanski and Holopainen, 1991; Brundrett et al., 1996).

Recently, polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) analysis of the internal transcribed spacer (ITS) region of nuclear rDNA has proven to be a reliable method for fungal identification at species level (Bruns et al., 1991; Bridge et al., 1998). This approach is also applicable to ectomycorrhizal fungi and has been used in both field and laboratory studies (Henrion et al., 1992; Gardes and Bruns, 1993; Kårén et al., 1997).

In the case of *T. matsutake* (S. Ito et Imai) Sing., one of the most famous edible ectomycorrhizal mushrooms in the world, no successful method has been established to isolate the fungus into pure culture from mycorrhizas (Hamada, 1964; Tominaga, 1963, 1971). Tominaga (1963, 1971) has reported the isolation from fine roots of *Pinus densiflora* Sieb. et Zucc. and *Juniperus rigida* Sieb. et Zucc. He obtained a fungus having characteristics such as slow-growth and sterile, clamp-less dikaryotic hyphae, which are also characteristics of *T. matsutake* isolates obtained from basidiocarps (Hiromoto, 1960; Hamada, 1964; Tominaga, 1963; Ohta, 1990; Yamada and Terasaki, 1998). However, as the isolation rates from the fine roots were low (Tominaga, 1963, 1971), an improved method is needed to promote the research on matsutake mushrooms throughout the growing season of the fungus (Hosford et al., 1997; Wang et al., 1997; Yamada et al., 1999a, 1999b).

We focused on *T. matsutake* and *T. bakamatsutake* Hongo ectomycorrhizas in the present study. The latter species is taxonomically and phylogenetically allied to *T. matsutake*, but has exclusive associations with Fagaceous trees (Ogawa and Ohara, 1978; Shimazono et al., 1979; Terashima, 1993; Murata et al., 1999; Murata and Yamada, 1999). Our primary objective was to establish a reliable method of isolation from mycorrhizas. Additionally, we sought to evaluate whether *T. matsutake* mycelium has an intimate association with *Mortierella* spp. (Tominaga, 1963; Hamada, 1964; Ogawa,

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1969), or a specific antibiotic activity against soil bacteria (Ohara and Hamada, 1967), as has been suggested.

Materials and Methods

Study site *Tricholoma matsutake* mycorrhizas were collected in a *Pinus densiflora*-dominated forest in Yamagata-machi, Ibaraki, Japan. An ecological description of the collecting site, e.g., climate, vegetation and soil texture, was reported by Yamada et al. (1999a). *Tricholoma bakamatsutake* was collected ca. 10 km south of the *T. matsutake* site. The host plant of *T. bakamatsutake* was *Quercus serrata* Thunb., which dominated the site. The other environmental conditions of the collecting site were mostly the same as those of the *T. matsutake* site.

Collecting of mycorrhizas Ectomycorrhizas colonized by *T. matsutake* were collected four times: 9 December 1997, 26 February 1998, 26 May 1998, and 27 July 1998. A soil sample was collected from inside the same mycelial colony of *T. matsutake* in each occasion. The mycelial colony was first estimated in October 1997 due to the linear fruiting of several basidiocarps of the fungus. A soil sample of ca. 100 ml was dug from inside the whitish mycelial colony of *T. matsutake* where root systems of *P. densiflora* were developed. The collected material was immediately carried on ice to the laboratory and refrigerated until preparation the following day. A portion of the collected mycorrhizas was observed microscopically according to Yamada et al. (1999a). Samples were prepared on glass slides and the ectomycorrhizal anatomy such as fungal sheath and Hartig net formation was confirmed. Mycorrhizas of *T. bakamatsutake* were collected in 30 September 1998, when two basidiocarps were found fruiting on the yellowish mycelial mat of the fungus. A mycorrhizal root system was developed just below the basidiocarp of the fungus.

Fungal isolation into pure culture Fungal isolation into pure culture from mycorrhizas followed Yamada and Katsuya (1995) with slight modification. Root systems were removed from soil and rinsed in two changes of tap water. Mycorrhizas having thin fungal sheaths but little deposition of brownish color in the root cortex were selected under a dissecting microscope and transferred to a petri dish filled with distilled water. Ten to twenty mycorrhizal tips were further selected and vortexed for one min in 1.0 ml of autoclaved 0.01% polyoxyethylene sorbitan monooleate (Tween 80) solution in an autoclaved Eppendorf microtube (1.5 ml). This washing procedure was repeated three times with fresh Tween 80 solution. Washed mycorrhizal tips were observed under a dissecting microscope to confirm the removal of soil particles, then gently washed for 30 min in a glass beaker (200 ml) with 100 ml of autoclaved 0.005% Tween 80 solution, which was set on a magnetic stirrer on a clean bench. After washing, half of the mycorrhizal tips were rinsed three times with sterile distilled water. The other mycorrhizas were surface-sterilized with 1% calcium hypochlorite solution for one min, then rinsed three times with sterile distilled water. Each rinsed mycorrhizal tip was transversally sectioned into 7–14 pieces, which were inoculated onto five kinds of agar plate media (9 cm in diam): Melin-Norklans (MN) (Norklans, 1949), modified Norklans's C (MNC) (Yamada and Katsuya, 1995), modified Melin-Norklans (MMN) (Marx, 1969), Hagem (Modess, 1941), and Ohta (Ohta, 1990). Sucrose in MN and MMN was replaced with the same weight of glucose. In the case of MNC medium, the effect of antibiotics on fungal isolation rate was examined. Streptomycin (100 μg/ml) and Tetracycline (50 μg/ml) were added to melted MNC medium after autoclaving, because these concentrations of the antibiotics efficiently suppressed bacterial growth but does not significantly affect the growth of *T. matsutake* mycelium (Yamada, unpublished data). Three plates were made in each treatment of mycorrhizas/isolation medium as replicates. Inoculated plates were incubated at ca. 22–23°C, and fungal growth was checked everyday for the first week and at intervals of several days for 2 months thereafter. All isolated fungi were separated, recorded, and observed microscopically.

Fungal isolation rates were statistically compared among mycorrhizal treatments by one-way ANOVA using StatView J ver. 4.5 (Abacus Concept Inc.). Furthermore, 10 to 20 mycorrhizal tips were washed with distilled water and stored in a deep freezer (−80°C) to serve as proof specimens for PCR-RFLP analysis.

Morphological observation of the isolated fungi Isolated fungi were observed microscopically to determine if they were the target fungi. Sporulating hyphomycetous or darkly pigmented fungi, which were obviously contaminants, were excluded from precise morphological observation at species level. The other sterile fungi were compared microscopically, because most ectomycorrhizal fungi, including *T. matsutake*, produce sterile colonies on agar media (Hutchison, 1989, 1991).

DNA analysis of isolated fungi To confirm that the isolated fungi were *T. matsutake*, the ITS region of the rDNA was analyzed by the PCR-RFLP method following Gardes and Bruns (1993). The following samples were analyzed: (1) *T. matsutake* isolates obtained under conditions of different mycorrhizal treatment/medium sets in each season, (2) a *Mortierella nana* Linneman isolate obtained in December, (3) an unidentified basidiomycete culture obtained in April, (4) basidiocarps of *T. matsutake* and *T. bakamatsutake* that were collected in the study plots in 1997 and 1998 and which had been stored in a deep freezer (−80°C), and (5) mycorrhizal tips stored in the same way.

DNA extraction: Fungal DNA was extracted from a 1×1 cm piece of fungal mycelium grown on MNC agar medium. For basidiocarps or mycorrhizas, a $5 \times 5 \times 5$ mm cut of the tissue or ca. 10 tips were used. Extracted DNA was dissolved in $0.1 \times$ TE buffer (1 mM Tris-HCl, 0.1 M EDTA, pH 8.0). An aliquot of the extracted DNA was diluted 100-fold with sterile double-distilled water,

and 1 μl of the diluted DNA solution was used as template DNA in the reaction mixture (50 μl) for PCR.

PCR amplification: We employed the primer pair ITS-1F/ITS-4B, since these primers specifically function with basidiomycetes (Gardes and Bruns, 1993). The samples were run in a Perkin Elmer DNA Thermocycler (model 9700) according to the following cycling parameters: initial denaturation for 3 min at 95°C, followed by 30 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 55°C and extension for 1.5 min at 72°C. The cycling was ended by an extension phase for 10 min at 72°C. Negative controls (no DNA template) were run for each experiment to check for DNA contamination of the reagents. The amplified PCR products were separated by electrophoresis for 1 h at 100 V (Mupid-2, Advance Inc.) on 10×6×0.5 cm gels of 3% NuSieve 3:1 agarose (FMC BioProducts Inc.) in 0.5× Tris-borate buffer (44.5 mM Tris, 44.5 mM borate, 1 mM EDTA, pH 8.0) and stained with 1 μg/ml ethidium bromide for 20 min. When only one DNA band was present in a PCR product, the product was used for RFLP analysis after estimation of its length.

RFLP analysis: Two μl of PCR product solution was digested separately with *Alu* I, *Hinf* I, *Rsa* I *Hae* III, and *Taq* I according to the manufacturer's recommendations (TaKaRa Biochemicals Inc.). The fragments were separated by electrophoresis for 45 min-4 h on 3–4% agarose gel. A 100-bp ladder marker (100–1000 bp; Superladder Low, GenSura Laboratories Inc.) was used to calculate the sizes of fragments larger than 500 bp, and a 20 bp ladder marker (20–1000 bp; Superladder Low, GenSura Laboratories Inc.) was used for fragments smaller than 500 bp. Each PCR product was digested at least twice with each enzyme to confirm the stability of the digestion pattern and precision of fragment sizes. The gel was stained with ethidium bromide, photographed (black & white film 667, Polaroid Inc.) under ultraviolet light, and scanned using a flat-bed scanner at a resolution of 300 ppi and converted to the black/white contrast. The scanned picture was expanded using Adobe Photoshop (Adobe Systems Inc.) to aid in calculating the fragment size. The fragment size was calculated following the weight curve that was drawn based on the pattern of co-electrophoresed markers. Five bp was the maximum resolution of the calculated fragment sizes by this method.

Results

Fungal isolation of *T. matsutake* Most mycorrhizal tips that were collected in the winter exclusively produced a fungus having the same colony morphology as that of known *T. matsutake* isolates established from basidiocarps (Yamada and Terasaki, 1998; Yamada et al., 1999b). A few other mycelial fungi were also isolated from these mycorrhizal tips (Table 1). The most dominant fungus was confirmed to be *T. matsutake*, because it had the same micromorphological and DNA characteristics as known *T. matsutake* isolates (Table 2; Fig 1). Although mycorrhizal tips tested in other seasons also produced *T. matsutake* mycelia, the isolation rate conspicuously decreased in April and July (Table 1).

Table 1. Fungal isolation of *T. matsutake* on MNC medium.

Date of isolation	Treatment[a]	Number of mycorrhizas		Mean isolation rate (%) of the following fungi and bacteria per mycorrhiza[c]				
		tested	produced *T. matsutake* mycelium	*T. matsutake*	*Mortierella nana*	unknown basidio-mycete	other mycelial fungi	bacteria
Dec. 97	C (control)	2	2	71.4	7.1	0.0	0.0	7.1
	C+A	2	2	42.9	0.0	0.0	0.0	0.0
	C+ss	2	2	78.6	0.0	0.0	0.0	0.0
	C+ss&A	2	2	64.3	0.0	0.0	0.0	0.0
Feb. 98	C	3	3	70.0 (80.0)[b]	0.0	0.0	0.0	10.0
	C+A	3	3	70.0	0.0	0.0	0.0	0.0
	C+ss	3	2	40.2 (43.9)[b]	0.0	0.0	0.0	3.7
	C+ss&A	3	3	81.5	0.0	0.0	0.0	0.0
Apr. 98	C	6	3 (5)[b]	9.7 (27.2)[b]	0.0	1.7	0.0	41.4
	C+A	3	2	35.0	0.0	0.0	0.0	0.0
Jul. 98	C	4	2	15.7	0.0	0.0	33.6 (38.9)[b]	11.4
	C+A	4	2	11.0	0.0	0.0	53.8	0.0
	C+ss	4	3	34.6	0.0	3.1	38.9 (43.1)[b]	4.2
	C+ss&A	4	2	23.1	2.5	0.0	49.3	0.0

[a] C: Washed mycorrhizas were inoculated on MNC medium. C+A: Washed mycorrhizas were inoculated on MNC medium containing antibiotics. C+ss: Surface-sterilised mycorrhizas were inoculated on MNC medium. C+ss&A: Surface-sterilised mycorrhizas were inoculated on MNC medium containing antibiotics.

[b] Numbers in parentheses were obtained when the bacterium-contaminated mycorrhizal fragments secondarily produced a fungus after re-inoculation on MNC medium containing antibiotics.

[c] Isolation rate per mycorrhiza: number of mycorrhizal fragments that produced a fungus or a bacterium ×100/number of tested mycorrhizal fragments.

Table 2. Comparison of morphological characteristics of a known *T. matsutake* isolate and mycorrhiza-derived isolates grown on MNC medium.

<table>
<tr><th rowspan="2">Characteristics</th><th rowspan="2">Known T. matsutake isolate Y1[b]</th><th colspan="3">Fungi isolated from mycorrhizas</th></tr>
<tr><th>T. matsutake</th><th>unknown basidiomycete</th><th>Mortierella nana</th></tr>
<tr><td>Diam of hyphae (μm)</td><td colspan="2">1.5–4.0</td><td>2.0–5.5</td><td>1.0–5.0</td></tr>
<tr><td>Regular septa</td><td colspan="2">present</td><td>present</td><td>indefinite</td></tr>
<tr><td>Clamp connection</td><td colspan="2">absent</td><td>present</td><td>absent</td></tr>
<tr><td>rhizomorph</td><td colspan="2">present, mycelium differentiation poor</td><td>absent</td><td>absent</td></tr>
<tr><td>Chlamydospore:</td><td colspan="2"></td><td></td><td></td></tr>
<tr><td>frequency</td><td colspan="2">low (vesicle ?)</td><td>high (conidia ?)</td><td>high</td></tr>
<tr><td>size (μm)</td><td colspan="2">4.5–22 × 5.5–24</td><td>3.5–7.0 × 5.5–8.5</td><td>4.5–20</td></tr>
<tr><td>formation site</td><td colspan="2">apex or intercalary of hyphae</td><td>lateral of hyphae</td><td>apex of hyphae</td></tr>
<tr><td>Autofluorescence to UV:</td><td colspan="2"></td><td></td><td></td></tr>
<tr><td>hyphae</td><td colspan="2">weak, whitish blue</td><td>weak, bluish green</td><td>weak, whitish blue</td></tr>
<tr><td>spore</td><td colspan="2">weak, whitish blue</td><td>moderate, bluish green</td><td>trace, brown</td></tr>
<tr><td>Nuclear phase[a]</td><td colspan="2">mainly dikaryotic</td><td>dikaryotic hyphae present</td><td>coenocytic</td></tr>
</table>

[a] Fluorescent die staining of nucleic acids was used (Yamada and Terasaki, 1998).
[b] The isolate was established from a *T. matsutake* basidiocarp (Yamada et al., 1999b).

Contamination by mycelial fungi, e.g., *Trichoderma*, *Penicillium*, and *Paecilomyces* spp., drastically increased in July. *Mortierella nana*, one of the *Mortierella* species that has been suggested to be closely associated with *T. matsutake* mycelium in soil (Kawai, 1966), was isolated at a very low rate, as was another sterile basidiomycetous fungus that had clamp connections (Tables 1, 2). Bacterial contamination was confirmed on the MNC medium without antibiotics, and peaked in April. Most bacterial colonies were yellow or yellow-orange in color.

In many cases, *T. matsutake* mycelium started to grow from mycorrhizal fragments several days after inoculation on MNC medium and formed a colony on the surface of the medium 2–3 wk after inoculation. In contrast, contaminating fungi grew more rapidly than *T. matsutake*. There was no significant difference in the mean isolation rate of *T. matsutake* among mycorrhizal treatments within any given season ($p<0.05$). However, surface-sterilization of mycorrhizas slightly decreased the bacterial contamination. Furthermore, some bacteria-contaminated mycorrhizal fragments produced *T. matsutake* mycelia after they were transferred onto MNC medium containing antibiotics (Table 1). There were significant differences ($p<0.01$) in the mean isolation rates of *T. matsutake* between winter and the other seasons (Table 3).

Mycelium of *T. matsutake* was also established from mycorrhizas on MN, MMN, and Hagem media with isolation rates as high as that on MNC medium (Table 4). There was no significant difference in the mean isolation rate among medium conditions ($p<0.05$). However, no mycelium was isolated from mycorrhizas inoculated on Ohta medium. Mycorrhizal fragments darkened strongly on Ohta and slightly on Hagem media. Colony morphology of *T. matsutake* mycelia that were first isolated on media other than MNC was subsequently confirmed on MNC medium. Mycelial growth was relatively slow on MN medium.

Fungal isolation of *T. bakamatsutake* Mycelium of *T. bakamatsutake* was also isolated from mycorrhizas on three kinds of media (Table 5). Mycelium of. *T. bakamatsutake* started to grow from mycorrhizal fragments 1–3 wk after inoculation on the media and subsequently formed a colony on the surface of the medium 2–3 wk after inoculation. Fungal identification was also conducted as in *T. matsutake*, i.e., comparison of colony morphology, microscopic characters of the mycelium, and RFLP pattern of ITS region of rDNA (Fig. 1).

Discussion

Mycelium of *T. matsutake* and *T. bakamatsutake* was successfully isolated from field-collected mycorrhizas at higher isolation rates than have generally been suggested for ectomycorrhizas, e.g., less than 20% by Molina and

Table 3. Seasonal change in fungal isolation rate of *T. matsutake* on MNC medium.

Date of isolation	Number of mycorrhizas tested	Mean isolation rate (%) with standard error in parentheses[a]
Dec. 97	8	64.3 (8.9)
Feb. 98	12	65.4 (9.6)
Apr. 98	9	20.7 (8.9)
Jul. 98	16	20.4 (7.4)

[a] Mean isolation rate was calculated as in Table1. Dec. and Feb. isolation rates are significantly greater than Apr. and Jul. isolation rates ($p<0.01$).

Table 4. Fungal isolation of *T. matsutake* on several media.

Date of isolation	Medium and treatment[a]	Number of mycorrhizas		Mean isolation rate (%) of the following fungi and bacteria permycorrhiza[c]			
		tested	produced *T. matsutake* mycelium	*T. matsutake*	unknown basidio-mycete	other mycelial fungi	bacteria
Dec. 97	Ohta	2	0	0.0	0.0	0.0	0.0
	Ohta+ss	1	0	0.0	0.0	0.0	0.0
Feb. 98	MN	3	3	52.4	0.0	0.0	0.0
	MN+ss	3	2	24.1	0.0	0.0	0.0
	MMN	3	3	70.0 (80.0)[b]	4.8	0.0	10.0
	MMN+ss	3	2	57.7	0.0	0.0	0.0
	Hagem	3	2 (3)[b]	17.3 (21.4)[b]	0.0	0.0	4.2
	Hagem+ss	3	3	71.4	0.0	0.0	0.0

[a] +ss: Surface-sterilised mycorrhizas were inoculated on the medium.
[b] Numbers in parentheses are the same as in Table 1.
[c] Mean isolation rate was calculated as in Table1.

Palmer (1982). Tominaga (1963) isolated *T. matsutake* mycelium from vascular tissue of fine root (2–8 mm in diam) of *P. densiflora* at isolation rates of 2.7–11.1%, but failed from mycorrhizas. Mycelium was also isolated from fine roots of *Juniperus rigida* at isolation rates of 14.3% (Tominaga, 1971). These results suggest that, in addition to ectomycorrhizal colonization of lateral roots of *P. densiflora* (Yamada et al., 1999a, 1999b), *T. matsutake* has the ability to colonize root vascular tissue of both Pinaceae and Cupressaceae. The latter family has been reported to have both ecto- and arbuscular mycorrhizal associations (Harley and Harley, 1987; Molina et al., 1992, Smith and Read, 1997). However, as the *T. matsutake* isolates from root vascular tissues of *P. densiflora* and *J. rigida* (Tominaga, 1963; 1971) lack enough characteristics for species identification using both DNA analysis and mycorrhizal synthesis (Danell, 1999), further research is desired.

PCR-RFLP analysis of the ITS region of rDNA was able to separate matsutake colonies from other soil fungi. The ITS primer pair of IF and 4B (Gardes and Bruns, 1993) specifically produced a PCR product in *T. matsutake*, *T. bakamatsutake*, and an unidentified basidiomycete, but not in *M. nana*, and the PCR products were easily separated between the two *Tricholoma* species. It has been reported that the base of the stipe of *T. matsutake* is often contaminated by *Mortierella*, e.g., *M. pusilla* Oudemans, *M. vinacea* Dixon-Stewart, *M. nana* and other unidentified species (Fujioka and Uehara, 1957; Tominaga, 1963; Hamada, 1964; Kawai, 1966). These fungal species have been suggested to have close relationships with *T. matsutake* in nature because they also have been dominantly isolated from inside the mycelial colonies and around mycorrhizal root systems of *T. matsutake* in *P. densiflora* stands (Tominaga, 1963; Hamada, 1964; Ogawa, 1969; Terashita et al., 1993). However, in our present study, intimate association between *T. matsutake* and *M. nana* was poorly observed, at least on the living mycorrhizas (Table 2). Furthermore, *Mortierella* has been suggested to consist of two subgenera that are phylogenetically distant (Gams, 1977; Gams et al., 1993). Therefore, the *T. matsutake-Mortierella* association must be reevaluated based on the valid taxonomic criteria of the genus *Mortierella*. On the other hand, especially in summer, diverse Hyphomycetous fungi and some bacteria were isolated.

Several factors affected the isolation rate of matsutake. The most important was the season when isolation was conducted, although most mycorrhizal tips tested throughout the study period showed the same morphological and anatomical characteristics (Yamada et al., 1999a). This might be attributed to the higher activity of microorganisms around mycorrhizas in the warmer seasons, which caused much contamination in the fungal

Table 5. Fungal isolation of *T. bakamatsutake* on several media.

Date of isolation	Medium and treatment[a]	Number of mycorrhizas		Mean isolation rate (%) of the following fungi and bacteria per mycorrhiza[b]		
		tested	produced *T. bakamatsutake* mycelium	*T. bakamatsutake*	other mycelial fungi	bacteria
Sep. 98	MNC	3	3	23.8	0.0	4.8
	MNC+ss	3	1	14.3	0.0	0.0
	MN+ss	3	1	4.8	0.0	0.0
	Hagem+ss	3	2	14.3	0.0	0.0

[a] +ss: Surface-sterilised mycorrhizas were inoculated on the medium.
[b] Mean isolation rate was calculated as in Table1.

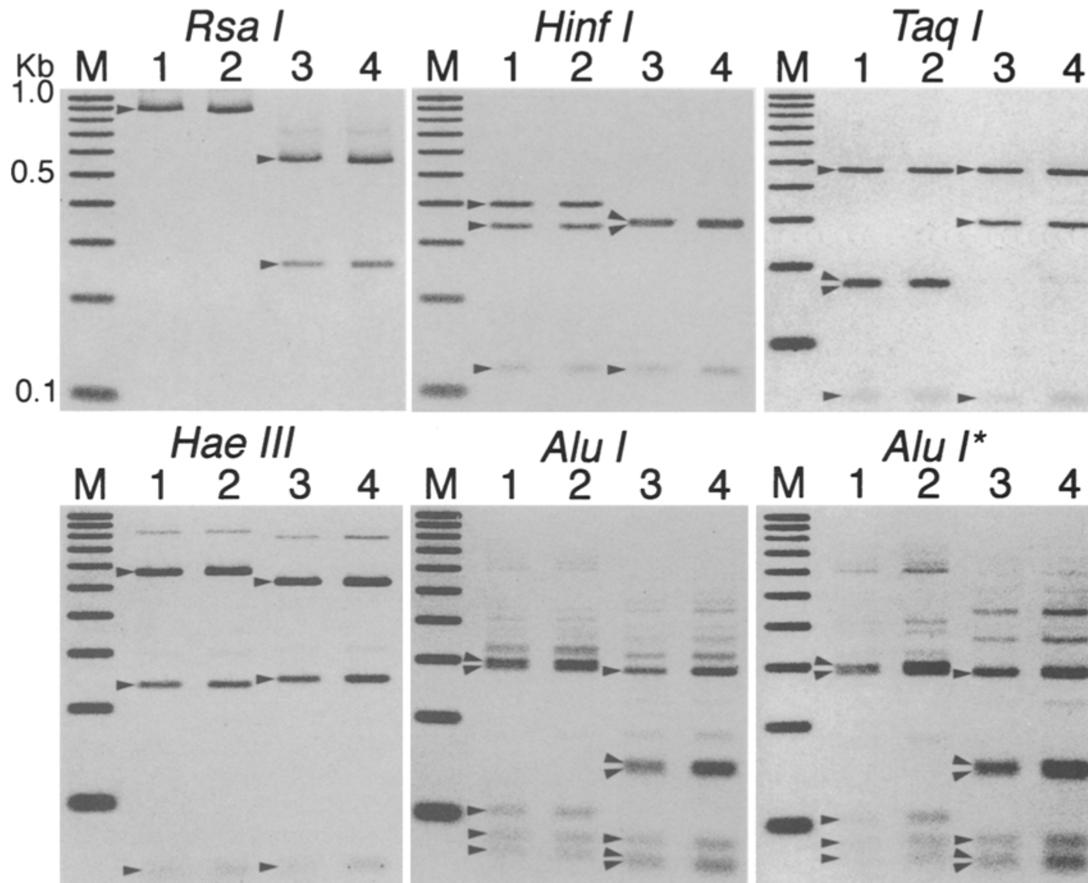

Fig. 1. RFLP patterns of ITS regions of rDNA in *T. matsutake* and *T. bakamatsutake*. Lanes: M, molecular markers, 0.1–1.0 Kbp; 1, *T. matsutake* basidiocarp; 2, *T. matsutake* isolate from a mycorrhiza; 3, *T. bakamatsutake* basidiocarp; and 4, *T. bakamatsutake* isolate from a mycorrhiza. Arrows indicate concrete bands. Lanes 1 and 2, and 3 and 4 show the same RFLP patterns in any enzyme treatment, respectively. In the case of *Alu* I digestions, RFLP patterns were different to some extent depending on the enzyme used (*: *Alu* I of Nippon gene Inc.). The figure shows the RFLP pattern of only one isolate each of both *T. matsutake* and *T. bakamatsutake*, because all tested isolates of each species showed the same pattern. The RFLP patterns of both species matched the patterns predicted from the sequence data described in the GenBank database and those obtained from analysis of established *T. matsutake* isolates Y1, Y4 and F (Yamada et al., 1999b).

isolation procedure. It has been reported that no bacteria were isolated from soil samples where *T. matsutake* mycorrhizas were dominant (Ohara and Hamada, 1967). This phenomenon has been explained by the growth inhibition effect of *T. matsutake* mycelium on the soil bacterial community, which resulted in the disappearance of most bacteria within *T. matsutake* mycelia (Ohara and Hamada, 1967). However, our isolation results showed the presence of certain bacteria on the mycorrhizas throughout the study period, especially in warmer seasons. In terms of the media used, most worked well for recovery of matsutake mycelia, except for Ohta medium, on which inoculated mycorrhizal fragments became dark brown. Such a change in root coloration, probably an oxidative reaction of the plant tissue, might have a lethal effect on the mycorrhizal mycelium, limiting its ability to resume growth on the agar medium. The use of antibiotics was quite efficient because bacteria-contaminated mycorrhizal fragments could secondarily produce matsutake mycelium, although the treatment did not significantly increase the mean isolation rate per mycorrhiza. On the other hand, surface-sterilization of mycorrhizas was less effective in raising the fungal isolation rate. Although stronger surface-sterilization by calcium hypochlorite solution (more than 0.7% for 10 min) is known to decrease the isolation rate in *Rhizopogon* from mycorrhizas (Chu-Chou, 1979), our sterilization method (1.0% for 1 min) did not show such an effect on the isolation rate of matsutake mycelium.

The present isolation method allows the establishment of matsutake isolates even in seasons that lack fruiting of the basidiocarp. It is difficult to isolate *T. matsutake* even from basidiocarps except from the hymenial tissue (Hiromoto, 1960; Hamada, 1964). Furthermore, hymenial tissue isolation is often followed by basidiospore germination (unpublished data), which has a potential to produce genetic heterogeneity within an established colony. Therefore, the present isolation method from mycorrhizas is ideal to establish genetically homogeneous isolates of matsutake mushrooms. The

findings of the present study should promote research on matsutake mushrooms, including ecophysiological studies such as those carried out in cultivation studies of truffles (Pacioni and Comandini, 1999).

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