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Nuclear Distribution in Hyphal Cells of Isolates from Fruit-bodies and Single Basidiospores in Ectomycorrhizal Fungus *Tricholoma robustum* and Allied Species: Visualization of Nuclei and Septa by a Double Staining Method*

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Abstract Morphological features of hyphae were compared among *Tricholoma robustum* and its allied species: *T. matsutake, T. ponderosum, T. caligatum, T. bakamatsutake, T. fulvocastaneum,* and *T. zelleri.* The nuclear distribution in hyphal cells of isolates from fruit-body tissues and single basidiospores of these species was examined by double staining of nuclei and septa. None of these species showed clamp connection in the hyphae of isolates from fruit-body tissues, but their nuclei in hyphal cells were visualized by double staining. All of them were dikaryons. However, the hyphae of isolates from single basidiospores were monokaryons. These results suggest that the mating system for all of the species examined is heterothallic.

INTRODUCTION

In the life cycle of basidiomycetes, there are two types of hyphae with respect to the nuclear profile. One is the monokaryon, which is characterized by haploid uninucleate cells that are usually generated from single-spore cultures. Mating occurs between two monokaryons that have different incompatibility factors, and this generates a dikaryon, which is the other type of hyphae characterized by haploid binucleate cells and clamp connections at each septum. Clamp connection is often used as a criterion to distinguish between monokaryon and dikaryon. However, dikaryon of some groups of basidiomycetes have no clamp connections. In these cases, monokaryon and dikaryon must be distinguished by the nuclear profile within the hyphal cell.

Tricholoma robustum is an ectomycorrhizal fungus, associated with *Pinus* species. This fungus forms ectomycorrhiza with roots of *Pinus densi*flora (Masui, 1927; Ogawa, 1981), and is an allied species of *Tricholoma matsutake.* Pure culture of mycelia of *T. matsutake* has been established by Hamada (1964), but isolates from single basidiospores have not been established because of the very low germination frequency of the basidiospores on conventional media. Recently, butyric acid has been reported to stimulate the germination of basidiospores of *T. matsutake* (Ohta, 1986), as well as several species of genus Tricholoma including *T. robustum* (Ohta, 1988). This has facilitated the isolation of cultures from single basidiospores germinated on conventional media.

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Seven allied species of *T. Matsutake* are distributed in the world: *T. robustum*, *T. matsutake*, *T. ponderosum*, *T. caligatum*, *T. bakamatsutake*, *T. fulvocastaneum*, and *T. zelleri* (Ogawa, 1978). They are all ectomycorrhizal fungi, and their fruit-bodies are morphologically similar. There have been many reports on the ecological properties of these species (Ogawa, 1965, 1977, 1979, 1981; Ogawa and Ohara, 1978; Ohara and Ogawa, 1982), but no information is available on the properties of the hyphae of their isolates, except for a report comparing their colonial characteristics (Shimazono, 1981). Hyphae of the isolates from the fruit-body tissues of the allied species have no clamp connections (Ogawa, 1978), but the nuclear distribution in hyphal cells is unknown except for *T. matsutake* (Tominaga, 1963). Some species of basidiomycetes such as *Agaricus bisporus* (Wang and Wu, 1976) and *Volvariella volvacea* (Chang and Ling, 1978) have hyphae with no clamp connections, and are multikaryotic. Therefore, the characterization of the nuclei of hyphal cells of *T. matsutake* and its allied species is important for establishing the isolates from single basidiospores. The present study examined the nuclear distribution in hyphal cells of isolates from single basidiospores and fruit-body tissues of *T. robustum* and its allied species by double staining of their nuclei and septa.

MATERIALS AND METHODS

Organisms

Table 1 shows the isolates from a number of species of Tricholoma used in this study. Fruit-bodies were collected in Japan in 1987–1989, or purchased at markets in Kyoto in 1987–1989. Some isolates were provided by Dr. Hosford of Central Washington University, U.S.A.

Media and culture

The composition of the basal medium per liter of distilled water was: 20 g glucose, 2 g

Table 1. Isolates from tissues and spores of *T. robustum* and allied species.

Species	Collected from	Isolated from	Origin
T. robustum	Kyoto	Tissue	
	Nara	Tissue	
	Kyoto	Spore	
T. matsutake	Kyoto	Tissue	
	Kyoto	Spore	
T. caligatum	Market[a)]	Tissue	
	Market[a)]	Spore	
T. ponderosum	Market[b)]	Tissue	
	Market[b)]	Spore	
	U.S.A.	Tissue	Dr. Hosford
T. bakamatsutake	Wakayama	Tissue	
	Chiba	Tissue	
T. fulvocastaneum	Kyoto	Tissue	
T. zelleri	U.S.A.	Tissue	Dr. Hosford

[a)] imported from Morocco, [b)] imported from Canada.

yeast extract (Difco Laboratories, U.S.A.), 0.2 g polypeptone (Daigo Nutritive Chemicals, Osaka), 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.5 g KH_2PO_4. To obtain isolates from single basidiospores, diluted medium was used (per liter of distilled water): 2 g glucose, 0.2 g yeast extract, 0.2 g polypeptone, 0.05 g $MgSO_4 \cdot 7H_2O$, and 0.05 g KH_2PO_4. Agar was added at 2% (w/v) when necessary. The media were sterilized by autoclaving at 120°C for 15 min.

To obtain isolates from fruit-body tissues, internal tissues or gills of the fruit-bodies were aseptically inoculated onto agar basal medium in test tubes, and cultured at 20°C. To obtain isolates from single basidiospores, basidiospores were collected as spore prints, and suspended in sterilized distilled water at 10^5–10^6 spores/ml. The suspension (1 ml) was inoculated onto agar medium in a Petri dish (9 cm in diameter). The medium was supplemented with 0.001% butyric acid as a germination stimulator. Germinated basidiospores were isolated under a stereomicroscope and maintained on agar basal medium in test tubes.

Cover glass culture for double staining

A small piece of the cultured mycelia was placed on a sterilized cover glass (0.17- to 0.25-mm thick) and mounted with a drop of 30 μl of liquid basal medium. The cover glasses were placed in a sterilized Petri dish, which then was sealed with Parafilm (American Can Co., U.S.A.). The dishes were incubated at 20°C for about 2–3 weeks.

Double staining of nuclei and septa

After incubation, the samples were fixed with a mixture of ethanol and acetic acid (3:1). Nuclei were stained with 500 ng/ml 4',6-diamidino-2-phenylindole (DAPI), 100 μg/ml ethidium bromide, or 100 μg/ml propidium iodide for 10 min, and septa were stained with 600 μg/ml Calcofluor white M2R (Polysciences, Inc., U.S.A.). After nuclear staining, RNA was digested by 1 mg/ml RNase A type XII-A (Sigma, U.S.A.) for 20–60 min at 37°C, when necessary.

Fluorescence microscopy

The stained samples were observed under an epifluorescence microscope (Nikon VFD-R) equipped with an epifluorescence unit, which provides four types of excitation settings, U, V, B, and G. The nuclei stained with DAPI were observed with the U setting (excitation filter UV330–380, absorbing filter 420K, and dichroic mirror DM400), and those stained with ethidium bromide or propidium iodide were observed with the G setting (excitation filter IF535–550, absorbing filter 580W, and dichroic mirror DM575). The septa stained with Calcofluor white M2R were observed with the U setting.

RESULTS

Properties of hyphae

In order to compare the properties of hyphae of isolates from different species, the isolates listed in Table 1 were cultured on agar basal medium. They all grew slowly, *T. bakamatsutake* being the slowest. Aerial hyphae and brown pigments were formed in several species (Table 2). Mycelial cords were found in isolates from fruit-body tissues of *T. robustum* (Fig. 1), but not in those from single basidiospores. Chlamydospore-like

Table 2. Properties of hyphae of isolates from *T. robustum* and allied species.

Species	Isolated from	Aerial hyphae	Brown pigments	Notes
T. robustum	Tissue	+	+	Mycelial cord
	Spore	+	+	
T. matsutake	Tissue	−	−	
	Spore	−	−	
T. ponderosum	Tissue	−	−	
	Spore	−	−	
T. caligatum	Tissue	−	−	
	Spore	−	−	
T. bakamatsutake	Tissue	+	+	Chlamydospore
T. fulvocastaneum	Tissue	+	−	
T. zelleri	Tissue	+	+	

All isolates were cultured on agar basal medium.

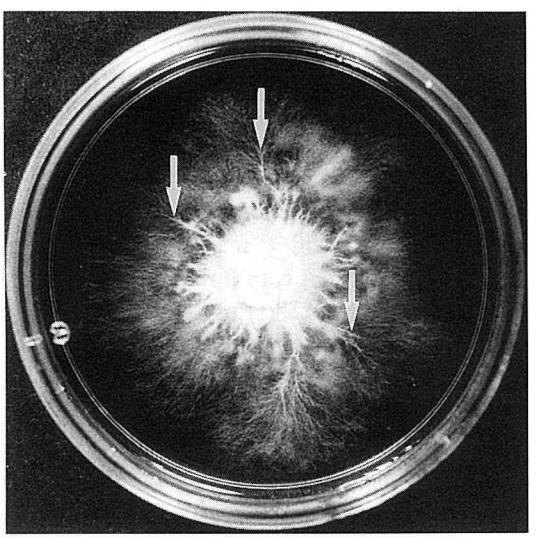

Fig. 1. Colony of isolates from fruit-body tissues of *T. robustum* showing mycelial cords (arrows).

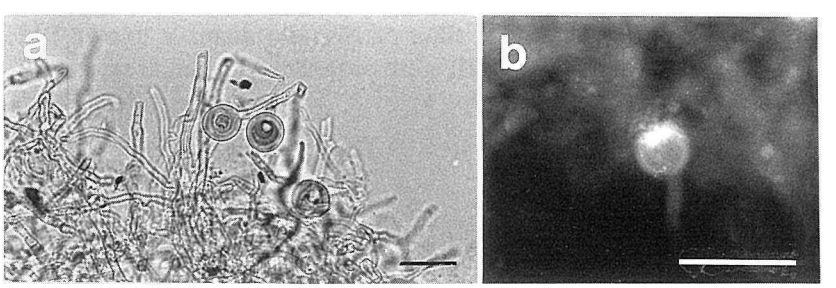

Fig. 2. Photomicrographs of hyphae from fruit-body tissues of *T. bakamatsutake* showing chlamydospores (a), and the nuclei within a chlamydospore stained with DAPI (b). Scale bars=20 μm.

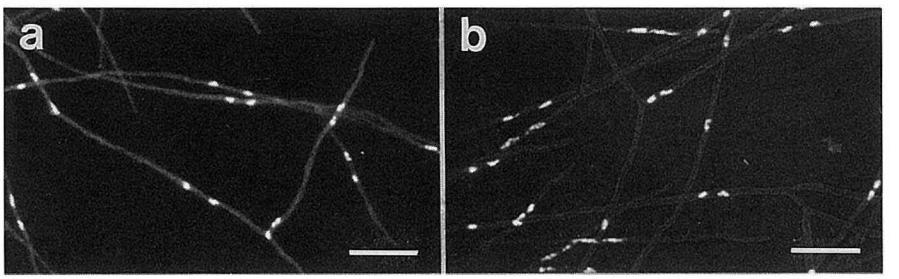

Fig. 3. Hyphae of *T. robustum* stained with ethidium bromide without (a) or with (b) RNase digestion. Scale bars = 20 μm.

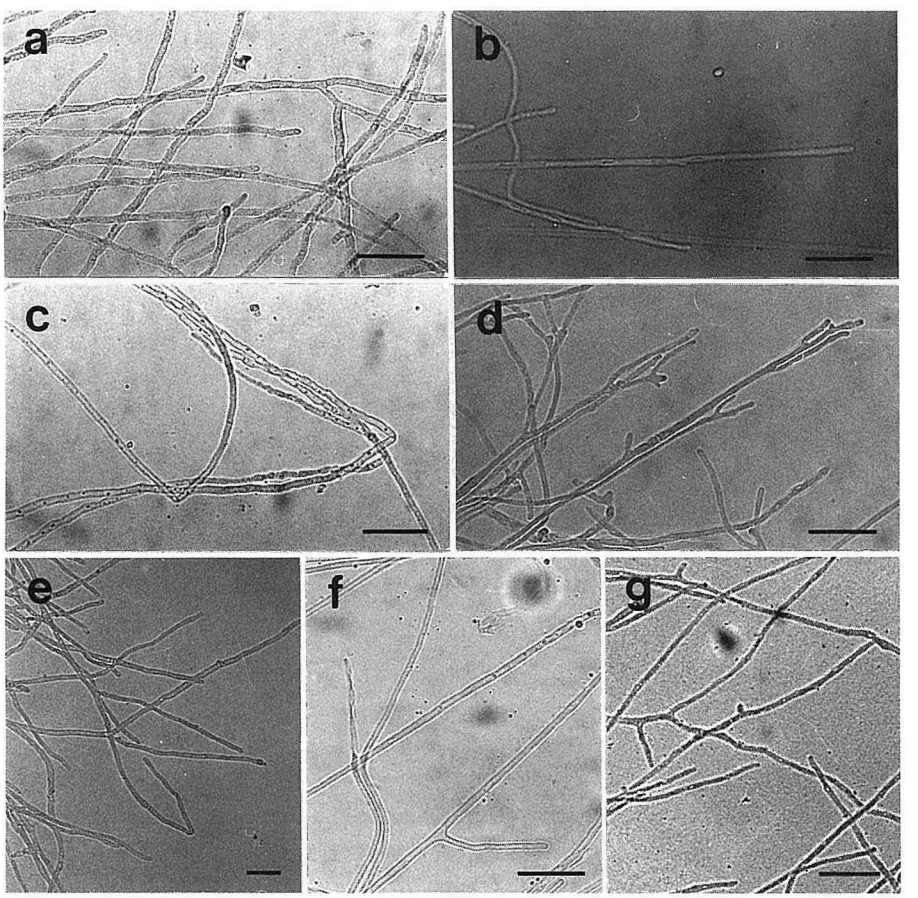

Fig. 4. Hyphae of isolates from fruit-body tissues of *T. robustum* and its allied species. a, *T. robustum*; b, *T. matsutake*; c, *T. caligatum*; d, *T. ponderosum*; e, *T. bakamatsutake*; f, *T. fulvocastaneum*; and g, *T. zelleri*. None have a clamp connection. Scale bars = 20 μm.

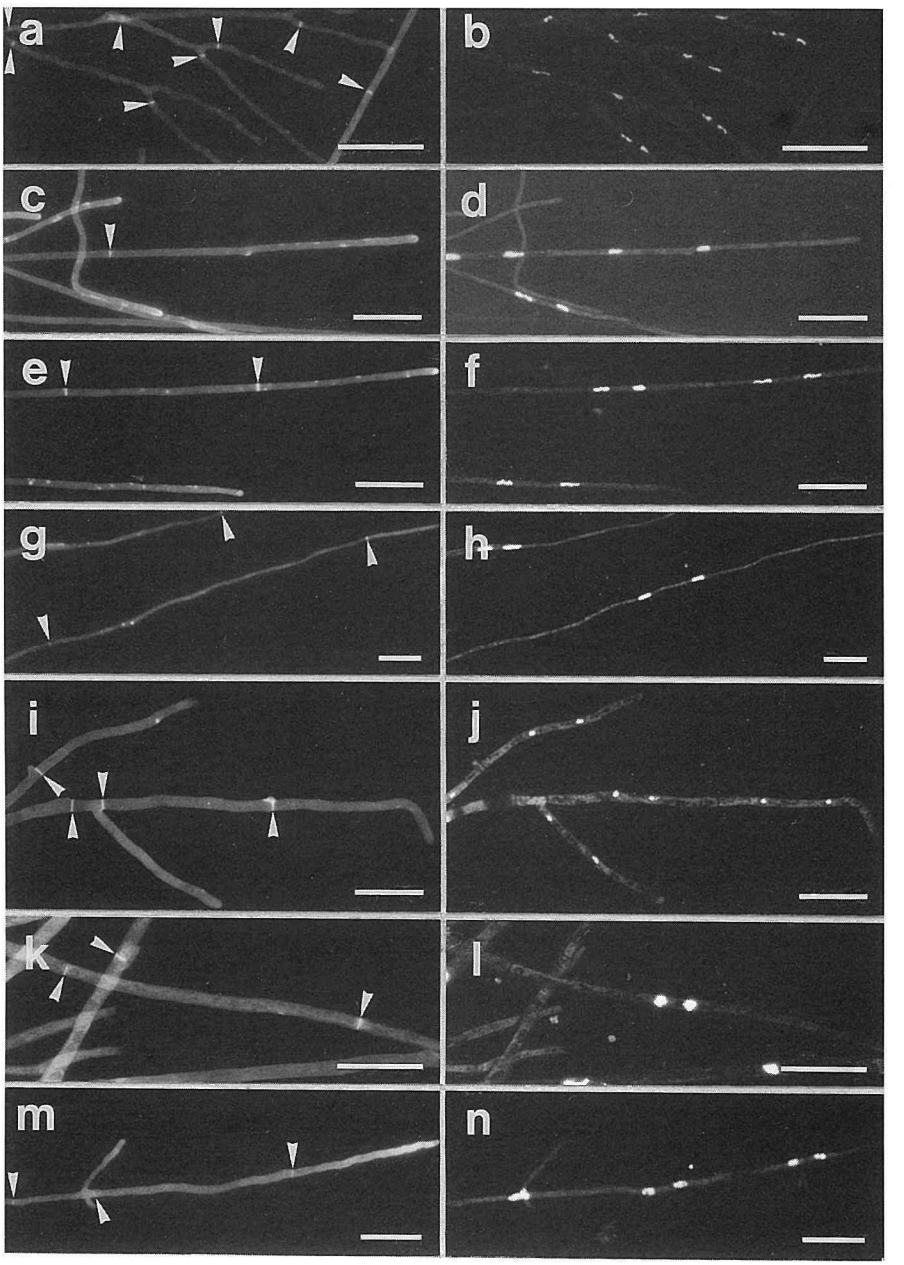

Fig. 5. Hyphae of isolates from fruit-body tissues of *T. robustum* and its allied species stained with ethidium bromide and Calcofluor white. a and b, *T. robustum*; c and d, *T. matsutake*; e and f, *T. caligatum*; g and h, *T. ponderosum*; i and j, *T. bakamatsutake*; k and l, *T. fulvocastaneum*; and m and n, *T. zelleri*. Photographs in the left column were taken under U excitation and show septa (arrowheads), and those in the right column were taken under G excitation and show nuclei. Scale bars =20 μm.

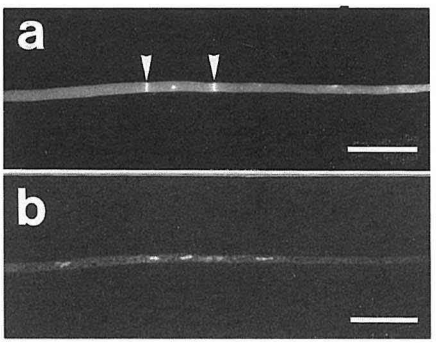

Fig. 6. Hypha having compact cell in subapical region of *T. fulvocastaneum* showing septa (a) and nuclei (b). Scale bars = 20 μm. Arrowheads in **a** indicate septa.

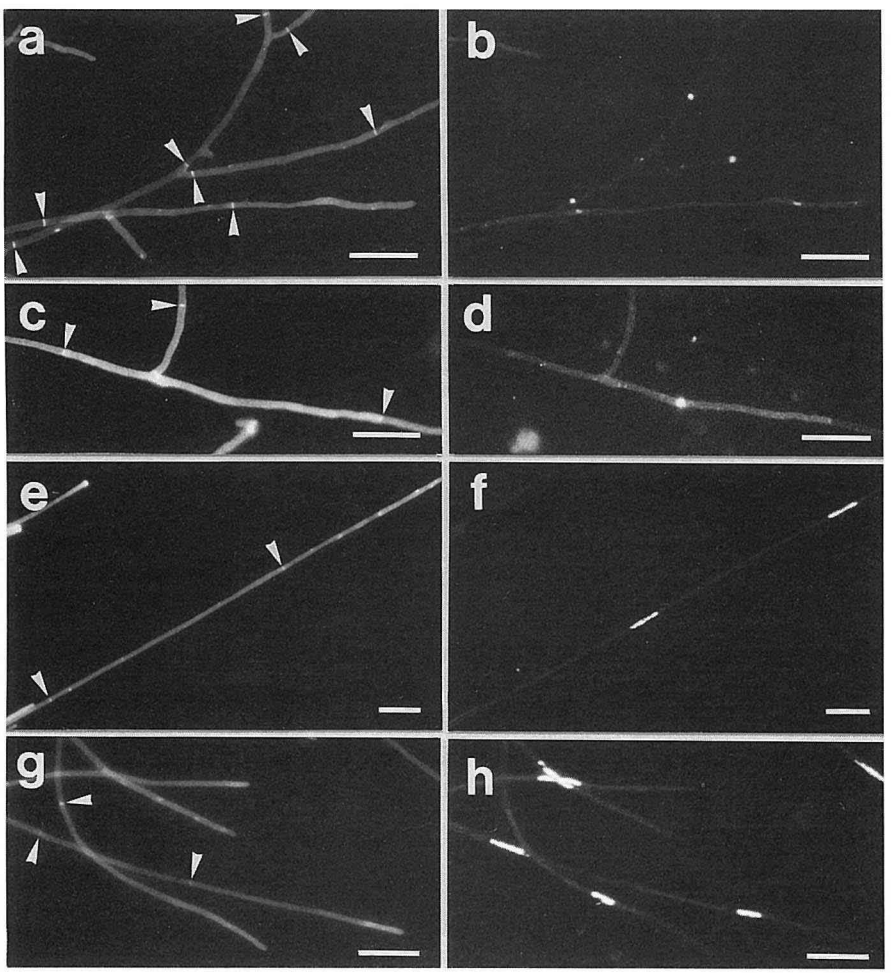

Fig. 7. Hyphae of isolates from single basidiospores of *T. robustum* and its allied species stained with ethidium bromide and Calcofluor white. a and b, *T. robustum*; c and d, *T. matsutake*; e and f, *T. caligatum*; and g and h, *T. ponderosum*. Photographs in the left column were taken under U excitation and show septa (arrowheads), and those in the right column were taken under G excitation and show nuclei. Scale bars = 20 μm.

Table 3. Nuclear distribution in hyphal cells of isolates from single spores and fruit-body tissues of *T. robustum*.

Source	Number of isolates		
	monokaryon	dikaryon	total
Basidiospore	56	4	60
Fruit-body	0	5	5

structures were found in the marginal region of colonies of *T. bakamatsutake* (Fig. 2a), and most of them contained two nuclei (Fig. 2b).

Selection of fluorescent dyes for nuclear staining

Nuclei were stained with DAPI, ethidium bromide, or propidium iodide, and then septa were stained with Calcofluor white. In the case of a combination of DAPI and Calcofluor white, nuclei could not be visualized by epifluorescence microscopy with the U excitation setting. With the G excitation setting, ethidium bromide and propidium iodide showed red fluorescence of nearly the same wavelength. Therefore, nuclei were observed with a combination of ethidium bromide and Calcofluor white or propidium iodide and Calcofluor white. In the following experiments, the former combination was used for observation of nuclei and septa.

Effect of RNase treatment on the staining with ethidium bromide

When cytoplasm of hyphal cells were stained with ethidium bromide so strongly that it interfered with the observation of nuclei (Fig. 3a), samples were treated with RNase to decrease the cytoplasm staining (Fig. 3b). However, cytoplasm stainability varied with the isolate and species.

Nuclear distribution in hyphal cells of isolates from fruit-body tissues

Hyphae of the isolates were observed under a microscope. None of the species showed a clamp connection in the hyphae of isolates from fruit-body tissues (Fig. 4). To determine the nuclear distribution in hyphal cells, hyphae were double-stained with ethidium bromide and Calcofluor white, and were observed under an epifluorescence microscope. Septa and nuclei were detected by using the U excitation setting (Fig. 5a,c,e,g,i,k, and m) and the G excitation setting (Fig. 5b,d,f,h,j,l, and n), respectively. Most hyphal cells of all species had two nuclei. In *T. fulvocastaneum*, compact cells contained two nuclei (Fig. 6) and were located in the subapical region. More than 10% of subapical cells were compact cells. In other species, however, compact cells were rarely found.

Nuclear distribution in hyphal cells of isolates from single basidiospores

Single basidiospore cultures were obtained in *T. robustum*, *T. matsutake*, *T. ponderosum*, and *T. caligatum*. There are two kinds of isolates from single basidiospores in T. robustum. In the first main group, hyphae were monokaryotic (Fig. 7a,b). In the other group, hyphae were dikaryotic and the same as those of isolates from fruit-body tissues. About 93% of the isolates from single-spore cultures were monokaryons (Table 3).

In *T. matsutake*, *T. ponderosum*, and *T. caligatum*, all of the isolates from single basidiospores were monokaryotic (Fig. 7c–h).

DISCUSSION

There are few reports comparing the microscopic properties of hyphae among *T. matsutake* and allied species. Formation of chlamydospore-like structures in *T. bakamatsutake* has been reported in mycelia from the field (Ohara and Ogawa, 1981) and from culture (Shimazono, 1976). The present study showed that these chlamydospore-like structures contained two nuclei. Therefore, they are considered to be real chlamydospores. However, their germination was not tested. In compact cells, which were found in *T. fulvocastaneum*, two nuclei were also compact (Fig. 6b). They may be resting or dormant cells.

DAPI, ethidium bromide, and propidium iodide were used to stain nuclei for fluorescence microscopy. Among them, DAPI is excited by the U excitation setting, while others by the G setting. Calcofluor white is used to stain cell walls for fluorescence microscopy, and this dye is excited by the U setting. DAPI and Calcofluor white generate bright blue to white fluorescence of about the same wavelength when excited by the U setting. These two dyes were used to visualize nuclei and septa of *Uromyces* (Freytag et al., 1988). In *T. robustum* and allied species, however, nuclei could not be identified because of interference by the fluorescence from cell walls stained with Calcofluor white.

None of the species examined showed clamp connection in the hyphae of isolates from fruit-body tissues (Fig. 4), but these hyphae were dikaryotic (Fig. 5). In the dikaryon of *Schizophyllum commune*, conjugate nuclear division occurs during mitosis, one nucleus passes through a clamp cell, while the other directly migrates through hyphae (Niederpruem et al., 1971). This must prevent the mixing of the two nuclei during mitosis and help maintain dikaryotic state. In the species without clamp connection, nuclear behavior during mitosis and migration through hyphae has been reported with *V. volvacea* (Chang, 1970). Nuclei are not divided synchronously and the hyphae are multikaryotic in this species. However, the nuclear behavior in the dikaryotic hyphae without clamp connections has not been studied yet. The hyphae of all species examined here were dikaryotic.

Although dikaryotic as well as monokaryotic isolates were obtained from single basidiospores of *T. robustum*, the number of dikaryotic isolates was small. Therefore, the dikaryotic isolates may be exceptional or have resulted from mating among hyphae derived from two or more basidiospores picked up together in the isolation procedure. The details of nuclear behavior during formation and germination of basidiospores are under investigation.

In *T. matsutake*, *T. ponderosum*, and *T. caligatum*, the isolates from single basidiospores were also monokaryon, which suggests that the mating system of these species including *T. robustum* is heterothallic. This remains to be determined by examining whether the mating actually occurs between two monokaryotic isolates from the same species.

Among ectomycorrhizal fungi, monokaryotic hyphae have been obtained from single basidiospores in five species of *Laccaria* (Fries and Mueller, 1984) and *Hebeloma cylindrosporum* (Debaud et al., 1986). However, the hyphae of the dikaryons of these

species have clamp connections. Therefore, it is easy to distinguish monokaryons from dikaryons. This is the first report that establishes the isolates from single basidiospores in *T. matsutake* and allied species and also in a group of ectomycorrhizal fungi whose dikaryons have no clamp connection in hyphae. The double staining method presented here is also useful for the cytological studies in other species, especially for examination of the nuclear distribution in hyphal cells.

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