Mycoscience (2009) 50:313–316
DOI 10.1007/s10267-009-0481-5

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Fruit body formation of *Tylopilus castaneiceps* in pure culture

Received: June 28, 2008 / Accepted: January 28, 2009

Abstract Fruit bodies of *Tylopilus castaneiceps* were formed on Ohta medium in pure culture. The mycorrhizal status of *T. castaneiceps* was confirmed by DNA analysis of the internal transcribed spacer region of mycorrhizae collected beneath its fruit bodies. However, fruiting ability was lost within 1 year of isolation, as has been reported for most of the other ectomycorrhizal species that produce fruit bodies in pure culture without host plants.

Key words DNA analysis · Ectomycorrhizal fungi · Fruit body formation · Pure culture · *Tylopilus castaneiceps*

Among the more than 2000 species of mushrooms reported to be edible (Chandra 1989), Wang et al. (2002) listed about 200 species of edible ectomycorrhizal fungi. However, the edibility of many more ectomycorrhizal species is yet to be determined (Wang et al. 2002). Although an increasing number of mushrooms are cultivated commercially (Sánchez 2004a), all but a few species [e.g., *Lyophyllum shimeji* (Kwam.) Hongo and truffles, mainly *Tuber melanosporum* Vittad.] cultivated commercially are saprotrophic fungi, which use dead organic substrates such as wood and leaf litter for growth.

Although the induction of fruit body formation in vitro has been attempted for many ectomycorrhizal fungi, most do not form fruit bodies in pure culture or even when cultivated in the presence of their host plants. Of those shown to fruit without host plants, most species belong to the Boletaceae (Modess 1941; Pontidou 1961, 1962, 1964, 1973; McLaughlin 1964, 1974; Karpiński 1967; Giltrap 1981; Yamanaka et al. 2000; Ohta and Fujiwara 2003), although the mycorrhizal status of some fungi observed in earlier work has not been confirmed. Other non-bolete species, such as *Coltricia perennis* (L.: Fr.) Murrill (Danielson 1984), *L. shimeji* (Ohta 1994), and *Hebeloma* species (Debaud and Gay 1987; Ohta 1998; Deng and Suzuki 2008), are known to form fruit bodies in pure culture.

Here we report fruit body formation of *Tylopilus castaneiceps* Hongo in pure culture. This fungus was the most dominant fruiter in a mixed stand of *Pinus densiflora* Sieb. & Zucc. and *Quercus serrata* Thunb. on the northern slope of Mt. Tsukuba, Ibaraki Prefecture, Japan (Kikuchi et al., unpublished data). The mycorrhizal status of *T. castaneiceps* was investigated by DNA analysis of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA).

Fruit bodies of *T. castaneiceps* were collected in June 1999 and 2000 in a mixed *P. densiflora*–*Q. serrata* stand in Makabe, Ibaraki Prefecture, Japan. Isolations were attempted for all collected fruit bodies on 1/5 strength Hamada medium [0.4% glucose, 0.04% yeast extract, and 1.5% agar (w/v)]. Strains were maintained on the same medium as used for isolation at 4°C in the dark. All the sporocarps collected were freeze-dried and stored at the Laboratory of Evaluation of Natural Environment, Graduate School of Frontier Sciences, the University of Tokyo, Kashiwa, Chiba, Japan.

Margins of mycelia on stock culture of the strains Tyc1 and Tyc2 were cut to about 1 cm × 1 cm in size and placed on 250 ml Ohta medium (Ohta 1990) in 500-ml Erlenmeyer flasks capped with a Silicosen culture plug (C-40; Shin-Etsu Polymar, Tokyo, Japan). The concentration of agar in the medium ranged from 0% to 1.5% (w/v). The flasks were incubated in a growth chamber MIR-253 (Sanyo, Osaka, Japan) at 23°C in the dark.

In June 2001, soil samples were collected just beneath the fruit bodies of *T. castaneiceps* in the field. Mycorrhizae collected from the soil samples were typed based on their macroscopic morphology under a stereomicroscope. The

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mycorrhizal samples were stored at –20°C until DNA extraction.

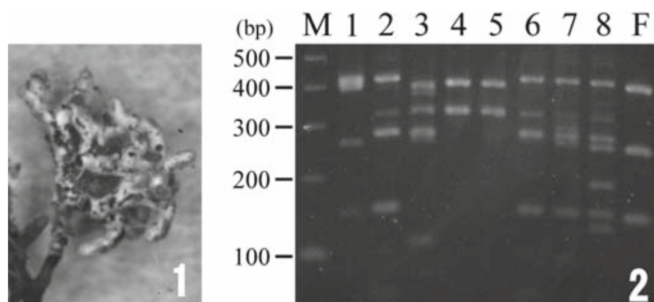
Total DNA extraction from each type of mycorrhiza and polymerase chain reaction (PCR) amplification of the ITS region of nuclear rDNA were conducted following the procedures described by Kikuchi et al. (2000). The primers used for PCR amplification were ITS1 and ITS4 (White et al. 1990). After digestion of the PCR products with *Hin*fI for 12 h at 37°C, electrophoresis was conducted on 2.0% agarose gel, and restriction fragment length polymorphism (RFLP) banding patterns were visualized by ethidium bromide staining and UV irradiation.

Sequencing was performed directly on purified PCR products of the ITS region with an automated fluorescent DNA sequencer (SQ-5500L; Hitachi, Tokyo, Japan) according to the manufacturer's instructions using the ITS1, ITS3, and ITS4 primers (White et al. 1990).

Among the morphotypes classified, one (Fig. 1) had an RFLP banding pattern identical to that of *T. castaneiceps* (lane 1 in Fig. 2). The ITS region sequences were also identical, indicating that *T. castaneiceps* is a mycorrhizal fungus. The sequence data from the fruit body of *T. castaneiceps* (specimen no. Tsukuba 227, deposited in the Herbarium of the University of Tokyo as TOFO-F99) was submitted to DDBJ (accession no. AB289669).

Formation of primordia was observed 25–30 days after inoculation on the medium (Fig. 3). The primordia developed into immature fruit bodies with pilei and stipes in 1 week (Fig. 4). Lowering of temperature was not necessary for primordia formation.

When the concentration of agar was 1.5% (w/v), fruit bodies grew to no more than 1.5 cm, and formation of pores was not observed (Fig. 5). When the agar concentration was 0.1% (w/v), fruit bodies grew to 3–4 cm and pores were formed, but the fruit bodies collapsed into the media, primarily because of the structural weakness of the agar (Fig. 6). When the concentration of agar was 0.5% or 0.25% (w/v), immature fruit bodies grew to 4–5 cm in height after 1 week (Figs. 7, 8). Although formation of pores (Fig. 9) and differentiation of hymenia (Fig. 10) were observed on young



Figs. 1, 2. DNA analysis of the ectomycorrhizae collected beneath sporocarps of *Tylopilus castaneiceps*. **1** Mycorrhiza with restriction fragment length polymorphism (RFLP) banding patterns identical to those of *T. castaneiceps.* **2** RFLP pattern of the internal transcribed spacer (ITS) region digested by *Hin*fI. *Lane 1* showed an RFLP pattern identical to that of *T. castaneiceps* (*lane F*). *Lanes 1–8*, mycorrhizae collected beneath a *T. castaneiceps* fruit body. *F*, fruit body of *T. castaneiceps*; *M*, molecular weight marker (100-bp ladder)

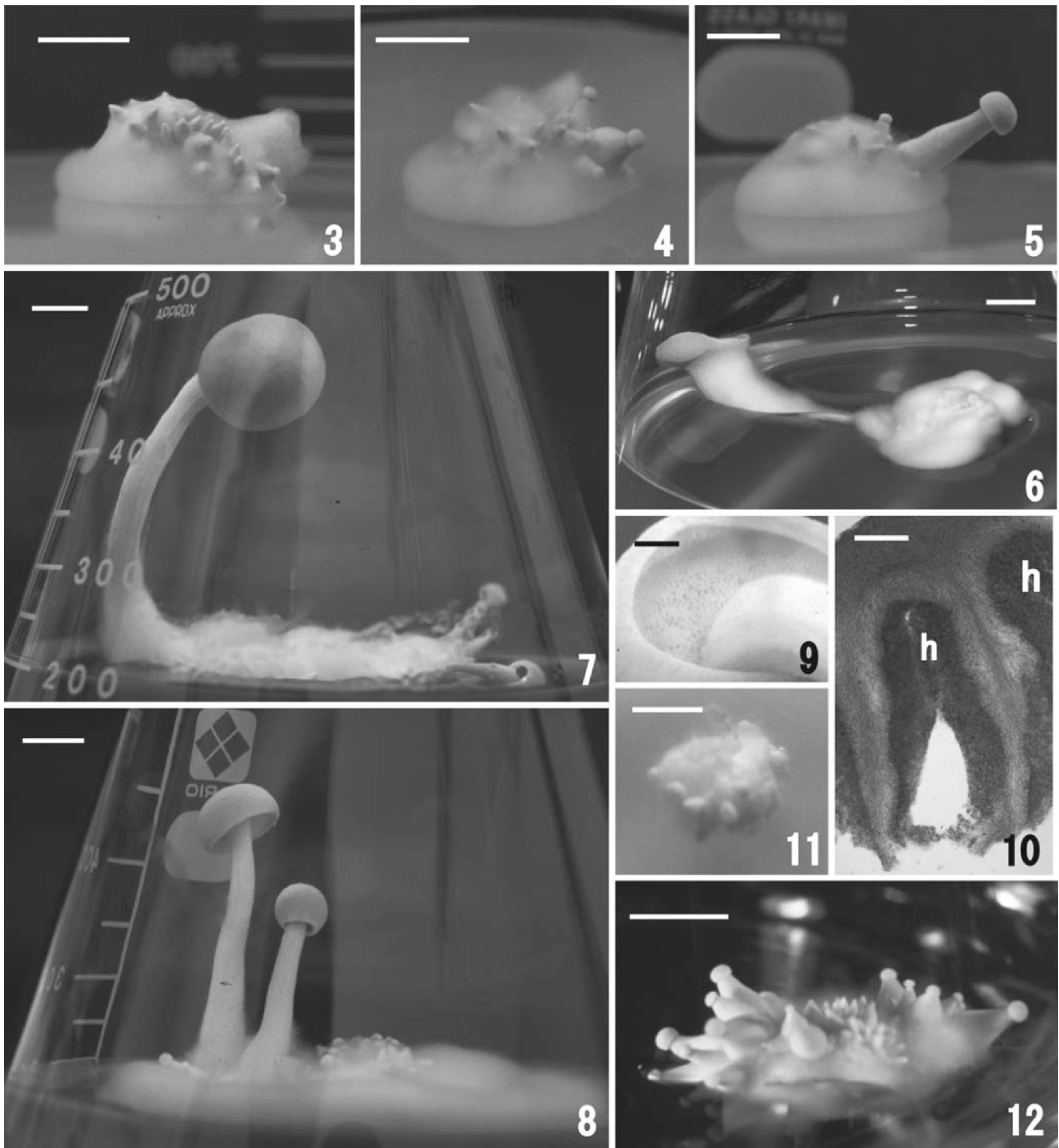
fruit bodies formed on media solidified with 0.25% agar, basidiospore formation was not confirmed. In all cases, only one or two primordia grew to young fruit bodies, although numerous primordia were formed.

Agar concentration affects diffusion of solutes (Stoltz 1971; Patil and Adhyapak 1982; Arnikar and Tattitali 1986) and the water potential of the medium (Stoltz 1971; Debergh et al. 1981; Ghashghaie et al. 1991). Therefore, our results suggest that the amount of nutrients and/or water supplied after primordia formation is an important factor determining the final size of fruit bodies grown from primordia.

On 1/5 strength Hamada medium, primordia were formed (Fig. 11) but did not show further growth, i.e., pilei and stipe formation. However, when some primordia were transferred to liquid Ohta medium, they developed into immature fruit bodies 3–6 mm in height with pilei and stipes after about 1 week (Fig. 12). Similarly, in some saprobic fungi, fruit body formation is affected by the media used (Davis and Jong 1976; Lahaie 1982; Sánchez 2004b). Some ectomycorrhizal fungi, such as *L. shimeji* (Ohta 1994), *Hebeloma* species (Ohta 1998; Deng and Suzuki 2008), and several Boletaceae species (McLaughlin 1964, 1974; Ohta and Fujiwara 2003), form mature fruit bodies that produce basidiospores without host plants. Among these species, *L. shimeji* (Ohta 1994), two *Hebeloma* species (Ohta 1998), and *Boletus* sp. (Ohta and Fujiwara 2003) grow well on barley-sawdust medium. In *L. shimeji* and the two *Hebeloma* species, good ability to use starch as a carbon source has been reported (Ohta 1997), which contributes to their ability to form mature fruit bodies on barley-sawdust medium. However, supplements of micronutrients and/or vitamins to the basic medium were required for increased fruit body formation in *Boletus* sp. (Ohta and Fujiwara 2003). Therefore, formation of mature fruit bodies of *T. castaneiceps* may be accomplished through a more detailed investigation of the nutrients and/or growth conditions such as humidity, light, and gas composition (e.g., CO_2 concentration), which affect fruit body formation in *Chalciporus rubinellus* (McLaughlin 1970).

All the 32 strains collected at the research site formed primordia on 1/5 strength Hamada medium, although only 2 strains were used for fruit body formation. By inter-simple sequence repeat (ISSR) polymorphism analysis, the population of *T. castaneiceps* at the research site was shown to consist of several genetically different clones (Kikuchi et al., unpublished data). These results imply that all genetically different strains of *T. castaneiceps* potentially form fruit bodies on Ohta medium.

Fruiting ability, however, was lost when mycelia isolated in the previous year were used as inocula in 2000 and 2001. Similarly, some of the boletes reported to form primordia and immature fruit bodies in pure culture lost that ability 3 or 4 months after strain isolation (Giltrap 1981), whereas the fruiting ability of a strain of *B. reticulatus* declined but was retained 1.5 years after isolation (Yamanaka et al. 2000). In contrast, strains of *L. shimeji* formed mature fruit bodies in pure culture even 7 years after isolation (Ohta 1994). The loss of the ability to form sporocarps in *T. castaneiceps* on chemically defined Ohta medium may have



Figs. 3–12. Fruit body formation of *T. castaneiceps* in pure culture. **3** Primordia formed on Ohta medium 25 days after inoculation (agar concentration, 1.5%). **4** Young fruit bodies with pilei and stipes formed 30 days after inoculation (agar concentration, 1.5%). **5** Young fruit bodies formed 40 days after inoculation (agar concentration, 1.5%). **6** Young fruit bodies formed on Ohta medium (agar concentration, 0.1%). **7** Young fruit bodies formed on Ohta medium (agar concentration, 0.25%). **8** Young fruit bodies formed on Ohta medium (agar concentration, 0.5%). **9** Pores formed on the fruit bodies in **7**. **10** Differentiation of hymenia (*h*) on the fruit bodies in **7**. **11** Primordia formed on 1/5 strength Hamada medium. **12** Young fruit bodies with pilei and stipes formed by transferring the primordia formed on 1/5 strength Hamada medium to liquid Ohta medium (this photograph was taken 1 week after transplanting). *Bars* **3–8, 12** 1 cm; **9, 11** 5 mm; **10** 200 μm

considerable potential for studying the genetics and physiology of fruit body formation of ectomycorrhizal fungi through elucidation of the cause of the loss of fruiting ability using molecular techniques.

Acknowledgments This research was funded in part by a Japan Society for the Promotion of Science (JSPS) Research Fellowship for Young Scientists (No. 12–09714).

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