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Note

Monokaryotic hyphae germinated from a single spore of the ectomycorrhizal basidiomycete *Tricholoma matsutake*

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ABSTRACT

We document here that monosporous isolates of the ectomycorrhizal basidiomycete *Tricholoma matsutake* were initially uni- or bi-nucleate. During pure culture, however, many uninucleate isolates became multinucleate. While the parent strain had two patterns of single nucleotide polymorphisms (SNPs) within its single-copy DNA, 19 of 20 monosporous isolates exhibited one of the two parent SNPs, and an isolate that was binucleate upon germination had both, indicating the former were monokaryotic and the latter dikaryotic. Of those, two isolates carrying SNPs different from one another have been predominantly uninucleate for 9 years. These isolates may be useful in genetics/breeding of "matsutake" mushrooms.

In homobasidiomycetes, monokaryotic basidiospores are generally produced after meiosis in the dikaryotic mycelia that result from mating between two monokaryotic mycelia or between monokaryotic and dikaryotic mycelia. This typical sexual reproduction process has been reported in model organisms, e.g., *Coprinopsis cinerea* and *Schizophyllum commune*, and other saprophytic cultivated mushrooms, e.g., *Pleurotus ostreatus* and *Pholiota nameko* (Buller 1931; Raper 1966; Babasaki et al. 2003; Fraser et al. 2007; Lin and Heitman 2007; Raudaskoski and Kothe 2010). Based on this well-

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documented mating system, better cultivars of saprophytic cultivated mushrooms have been bred to produce desirable commercial qualities.

Tricholoma matsutake is an ectomycorrhizal basidiomycete that associates as a symbiont with Pinaceae plants and produces the prized but yet uncultivable "matsutake" mushrooms in natural habitats (Ogawa 1975; Tominaga 1978; Yamada et al. 2010, 2014). Although genomic information is currently available (JGI; http://genome.jgi-psf.org/Trima3/Trima3.home.html), the genetics of *T. matsutake* have not been well elucidated because of difficulty in mating due to the absence of "clamp connections" in the secondary mycelia (see below). In addition, the nuclear phase of the secondary mycelium has not been clarified as a result of unexplained technical difficulty in visualizing nuclei along with septa, unlike in many saprophytic mushrooms, although the species is said to be dikaryotic on the basis of the nuclear phase of the spores (Tominaga 1978). Tominaga (1978) reported that *T. matsutake* produces both binucleate and uninucleate spores. Hyphal regeneration from *T. matsutake* spores, however, had not been achieved until a unique spore germination method using organic acids as inducers was established (Ohta 1986a,b, 2006). In fact, no monokaryotic cultures of *T. matsutake*, which could be useful in genetics and breeding of matsutake, have been available.

The aims of the present study were to (i) isolate monosporous hyphae of *T. matsutake* and (ii) characterize the nuclear phases of these isolates by both microscopic examination and DNA-based analysis. The ultimate goal was to obtain monokaryons of the symbiotic mushroom.

Tricholoma matsutake SF-Tm172 is an isolate from a fruit body harvested from "shiro no. 6" (= a rhizospheric colony of *T. matsutake*) growing at the Kohnan study site, Shiga Prefecture, Japan, on 18 October, 2004 (Murata et al. 2005). Twenty monosporous isolates were obtained from this isolate using a protocol described by Ohta (1986a,b, 2006; Table 1). The spore isolates have been deposited in the Forestry and Forest Products Research Institute (FFPRI) gene bank, Tsukuba, Japan (Table 1).

Spores were germinated on F5+Bu agar containing crude hot water extracts of *P. densiflora* leaves (50 g/L), butyric acid (50 μL/L), and agar (8 g/L), then transferred to a standard culture medium using a micromanipulator. Unless stated otherwise, the nuclear phases of monosporous isolates were examined using DAPI staining (1 ppm) and a fluorescence microscope; the fungal hyphae were cultured between an agar medium and a slide glass so that hyphae grew horizontally. Nuclei stained with DAPI lit up in ca. 20–40 cells of the mycelia, but not all of them, when observed at 40× magnification. At least four independent mycelial areas per specimen were examined. Note that *T. matsutake* barely exhibits septa

Table 1 – *Tricholoma matsutake* SF-Tm172 and its siblings obtained by single spore isolation.

Strain	FFPRI[a] accession numbers	Source[b]	Number of nuclei in predominant cells:[c] days after spore dispersal				SNP types[d]
			154	189	479	3100	
SF-Tm172	435312	P	2	2	2	N[e]	α, β
04ss50	435313	S	2	2	2	N	α, β
04ss26	435314	S	1	1	1	1	α
04ss27	435315	S	1	1	1	1	β
04ss17	435316	S	1	2	2	N	β
04ss24	435317	S	1	1	1/2	2	β
04ss40	435318	S	1	2	2	2	β
04ss45	435319	S	1	2	2	N	β
04ss46	435320	S	2	N	2	N	β
04ss48	435321	S	1	2	2	N	β
04ss58	435322	S	1	2	2	N	β
04ss65	435323	S	1	2	2	N	β
04ss71	435324	S	1	1	1	2	β
04ss142	435325	S	1	1	1	2	β
04ss147	435326	S	1	1	1	2	β
04ss150	435327	S	1	1	1	2	β
04ss43	435328	S	2	N	2	2	α
04ss54	435329	S	2	N	2	N	α
04ss63	435330	S	1	2	2	N	α
04ss122	435331	S	1	1	1/2	2	α
04ss141	435332	S	2	N	2	N	α

[a] FFPRI, Forestry and Forest Products Research Institute; the spore isolates are available at FFPRI gene bank.
[b] P, The parent isolated from a fruit body grown in the shiro no. 6 of the Konan study site, Shiga, on October 19, 2004. S, Spore isolates derived from SF-Tm172 (see text for a protocol used for spore germination on an agar plate).
[c] Nuclear phase was determined by microscopic analysis with DAPI fluorescent staining.
[d] SNP, Single nucleotide polymorphisms within the 431-bp single copy DNA segment, in which a set of closely linked two SNP markers are localized at bp 238 and 278, as determined by MEGA5-based multiple alignment analysis of 12–16 PCR cloned DNA segments of each specimen (Fig. 3). α, C/C at bp 238/278. β, A/T at bp 238/278.
[e] N, Not determined.