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Note

Agar plate assays using dye-linked substrates differentiate members of *Tricholoma* sect. *Caligata*, ectomycorrhizal symbionts represented by *Tricholoma matsutake*

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ABSTRACT

Tricholoma matsutake is an ectomycorrhizal fungus that produces the prized mushrooms "matsutake" in association with the Pinaceae. Other species of *Tricholoma* sect. *Caligata* are also ectomycorrhizal symbionts that produce a variety of "*quasi*-matsutake" mushrooms. Here we developed agar plate assays using the dye-linked substrates azurine-crosslinked (AZCL) hydroxyethyl cellulose and AZCL-amylose to differentiate *T. matsutake* strains and related species based on their polysaccharide-degrading activities. This method may be useful for screening strains that adapt well to spawn cultivation for mushroom fruiting.

Tricholoma matsutake is an ectomycorrhizal (EM) agaricomycete that produces the prized, but uncultivable, mushrooms "matsutake" (Hosford et al. 1997). Other closely related taxa that belong to *Tricholoma* sect. *Caligata* are also EM symbionts that produce a variety of "*quasi*-matsutake" mushrooms. The most closely related taxa include *T. anatolicum* from the Mediterranean region, *T. magnivelare* from North America, and unidentified *Tricholoma* species from Mexico, all of which are symbionts of the Pinaceae (Hosford et al. 1997; Intini et al. 2003; Ota et al. 2012; Murata et al. 2013a). Taxa that diversified phylogenetically before these conifer symbionts, but produce *quasi*-matsutake, are *T. bakamatsutake* and *T. fulvocastaneum*, both of which associate with the Fagaceae (Hosford et al. 1997; Murata et al. 2013b). *Tricholoma caligatum*, which associates with the Pinaceae in Italy and Spain, is classified phylogenetically as an intermediate species between these pinaceous and fagaceous symbionts (Murata et al. 2013a).

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Tricholoma matsutake has been reported to produce hydrolases, such as β-glucosidase, endoglucanase, xylanase, β-xylosidase, and α-glucuronidase, which could be involved in cell wall polysaccharide decomposition (Terashita et al. 1995; Vaario et al. 2002, 2003, 2012; Kusuda et al. 2006, 2008). Based on its whole-genome sequence (JGI Genome Portal; http://genome.jgi-psf.org/Trima3/Trima3.home.html), *T. matsutake* should have seven putative β-glucosidases (EC 3.2.1.21) and one endoglucanase (EC 3.2.1.4), but no cellobiohydrolases (EC 3.2.1.91). In addition, putative genes that could encode amylose-catabolizing enzymes have also been recognized in the whole genome of *T. matsutake*. Therefore, *T. matsutake* is predicted to produce some cellulolytic and amylose-degrading enzymes.

In the present study, we developed agar plate assays using two dye-linked substrates, azurine-crosslinked hydroxyethyl cellulose (AZCL-HE-cellulose) and AZCL-amylose, to detect endoglucanase and amylase activities, respectively. This system allowed us to differentiate strains among given populations, and classify species belonging to *Tricholoma* sect. *Caligata*.

See Table 1 for descriptions of the fungal strains used in this study. Some *T. matsutake* strains were newly isolated, while others were present in public databanks. The taxa of newly isolated *T. matsutake* were ascertained using DNA markers that are present solely in this species (Yamaguchi et al. 2016).

Potato dextrose agar (BD Difco, Franklin Lakes, NJ, USA) medium containing 0.1% AZCL-HE-cellulose or AZCL-amylose (Megazyme, Bray, Ireland) was sterilized, and then 8 mL of each medium was poured into a 5-cm Petri dish. Mycelial plugs (6 mm diam) were prepared with a sterile cork borer for each potato dextrose agar plate, and they were cultivated for 4–6 wk at 23 °C in the dark to inoculate the plugs onto the

Table 1 – Fungal strains.

Sample no.	Species	Strain no.[a]	Voucher specimen no.[b]	Sampling site
1	*Tricholoma matsutake*	NBRC 6932		*Pinus densiflora* forest, Nagano, Japan
2		NBRC 6933		*P. densiflora* forest, Nagano, Japan
3		NBRC 33136 (ATCC MYA-915)		*P. densiflora* forest, Ibaraki, Japan
4		NBRC 33137		*P. densiflora* forest, Fukushima, Japan
5		NBRC 108255		*P. densiflora* forest, Iwate, Japan
6		NBRC 108256		*P. densiflora* forest, Iwate, Japan
7		NBRC 108264		*P. densiflora* forest, Hiroshima, Japan
8		NBRC 108684		*P. densiflora* forest, Shiga, Japan
9		NBRC 108688		*P. densiflora* forest, Shiga, Japan
10		NBRC 108713		*P. densiflora* forest, Ibaraki, Japan
11		TO-1	TFM S-08006	*P. densiflora* forest, Nagano, Japan
12		Hokkaido Tm13		*P. densiflora* forest, Hokkaido, Japan
13		Iwate kuzumaki 1		*P. densiflora* forest, Iwate, Japan
14		Iwate MYI1305		*P. densiflora* forest, Iwate, Japan
15		Iwate site 2-1		*P. densiflora* forest, Iwate, Japan
16		Shiga site 2-A		*P. densiflora* forest, Shiga, Japan
17		Kyoto site 41		*P. densiflora* forest, Kyoto, Japan
18		Matsuyama kihoku 2		*P. densiflora* forest, Ehime, Japan
19	*T. bakamatsutake*	NBRC 107029		*Pasania edulis/Castanopsis sieboldii* forest, Chiba, Japan
20		NBRC 108265		*P. edulis/C. sieboldii* forest, Chiba, Japan
21		NBRC 108266		*P. edulis/C. sieboldii* forest, Chiba, Japan
22		NBRC 108267		Chiba, Japan
23		WK-Tb1		*P. edulis/C. sieboldii* forest, Wakayama, Japan
24	*T. fulvocastaneum*	NBRC 6947		–[c]
25		NBRC 108268		*Quercus phillyraeoides* forest, Wakayama, Japan
26		NBRC 108269	TFM M-R20	*Q. phillyraeoides* forest, Wakayama, Japan
27		NBRC 108270	TFM M-R27	*Q. phillyraeoides* forest, Wakayama, Japan
28		NBRC 108271	TFM M-L914	*C. sieboldii* forest, Kagoshima, Japan
29	*T. caligatum*	NBRC 109035	TFM M-R106	*Pinus. pinea* forest, Carabria, Italy
30		NBRC 109036	TFM M-R107	*P. pinea* forest, Carabria, Italy
31	*Tricholoma* sp.	ATCC MYA-921(Originally described as *T. matsutake*)		Commodity, Mexico
32	*T. anatolicum*	ATCC MYA-929 (Originally described as *T. matsutake*)		Commodity, Morocco
33	*T. magnivelare*	ATCC MYA-930		Commodity, Canada

[a] NBRC, NITE Biological Resource Center, National Institute of Technology and Evaluation, Kisarazu, Chiba, Japan; ATCC, American Type Culture Collection, Manassas, VA, USA.
[b] TFM, Mycological herbarium of Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, Japan.
[c] No information is available at NBRC.