Full paper

New findings on the fungal species *Tricholoma matsutake* from Ukraine, and revision of its taxonomy and biogeography based on multilocus phylogenetic analyses

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ABSTRACT

Matsutake mushrooms are among the best-known edible wild mushroom taxa worldwide. The representative *Tricholoma matsutake* is from East Asia and the northern and central regions of Europe. Here, we report the existence of *T. matsutake* under fir trees in Eastern Europe (i.e., Ukraine), as confirmed by phylogenetic analysis of nine loci on the nuclear and mitochondrial genomes. All specimens from Japan, Bhutan, China, North Korea, South Korea, Sweden, Finland, and Ukraine formed a *T. matsutake* clade according to the phylogeny of the internal transcribed spacer region. The European population of *T. matsutake* was clustered based on the *β2* tubulin gene, with a moderate bootstrap value. In contrast, based on analyses of three loci, i.e., *rpb*2, *tef*1, and the *β2* tubulin gene, *T. matsutake* specimens sampled from Bhutan and China belonged to a clade independent of the other specimens of this species, implying a genetically isolated population. As biologically available type specimens of *T. matsutake* have not been designated since its description as a new species from Japan in 1925, we established an epitype of this fungus, sampled in a *Pinus densiflora* forest in Nagano, Japan.

Keywords: bioresource conservation, edible mycorrhizal mushroom, lectotype, population analysis

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1. Introduction

Matsutake mushrooms are gourmet foods in Japan and other Asian countries. Mushroom traders have been searching for new sources of matsutake mushrooms worldwide since the 1960s and have introduced new matsutake populations to the Japanese market (Hongo, 1971; Ogawa, 1978; Tsing, 2015). These matsutake mushrooms are identified as *Tricholoma matsutake* (S. Ito & S. Imai) Singer from eastern Asia and northern Europe, *T. anatolicum* H.H. Doğan & Intini from the Mediterranean region, *T. magnivelare* (Peck) Redhead and *T. murrillianum* Singer from North America, *T. mesoamericanum* Justo & Cifuentes from Mesoamerica, and *T. bakamatsutake* Hongo and *T. fulvocastaneum* Hongo from eastern and southeastern Asia (Hosford, Pilz, Molina, & Amaranthus, 1997; Ota et al., 2012; Endo et al., 2015; Trudell, Xu, Saar, Justo, & Cifuentes, 2017; Vaario, Yang, & Yamada, 2017; Yamanaka, Yamada, & Furukawa, 2020). All of these species are of high value in the mushroom economy, and Japanese imports of matsutake mushrooms amount to approximately 50 million dollars annually (Trade Statistics of Japan Ministry of Finance; https://www.customs.go.jp/toukei/info/tsdl.htm). In 2000, Swedish matsutake that had been identified as *Tricholoma nauseosum* (A. Blytt) Kytöv. (Kytövuori, 1988) was shown phylogenetically to be conspecific with Japanese *T. matsutake* (Bergius and Danell, 2000). This incurred a taxonomic issue, in that *T. matsutake* can be synonymized to *T. nauseosum*, based on the priority of the latter by the International Code of Nomenclature for algae, fungi, and plants. However, Ryman, Bergius, and Danell (2000) suggested retaining the name *T. matsutake* because of the established common name "matsutake mushroom" globally, and the historical and cultural importance of the mushroom in biology and the food industry. Recently, the scientific names of American matsutake mushrooms were revised, i.e., the eastern and pale tan color *T. magnivelare*, western and whitish *T. murrillianum*, and tan color Mesoamerican *T. mesoamericanum*, most of which had been identified as *T. magnivelare*, *T. ponderosum* (*Armillaria ponderosa*), *T. murrillianum* (*A. arenicola*) or merely "matsutake" (Smith, 1979; Redhead, 1984; Arora, 1986; Singer, 1986; Hosford et al., 1997; Trudell et al., 2017).

In this paper, we report a new *T. matsutake* from Eastern Europe (Ukraine). At present, the known geographic distribution of *T. matsutake* is restricted to Asia and northern and central Europe (Matsushita et al., 2005; Endo et al., 2015; Vaario et al., 2017). To fill the knowledge gap in *T. matsutake* ecology between Asia and Europe, exploration in the central region of the Eurasian continent has been desired. As we obtained a probable *T. matsutake* basidioma in 2020 from Ukraine (Fig. 1), we first clarified its species identity based on the internal transcribed spacer (ITS) sequence of nuclear ribosomal DNA (nuc rDNA). Then, we clarified how these two isolated populations of *T. matsutake* in Eurasia fit within the metapopulation based on phylogenetic analyses of multiple loci.

We designate a type of *T. matsutake* sampled in Japan. The new species description as *Armillaria matsutake* (Ito and Imai, 1925) did not accompany a detailed morphological description but cited a picture of *Matsutake*, which was identified as *Cortinellus edodes* P. Henn. by Seiichi Kawamura (1913), sampled in Honshu Island, the main island of the Japanese Archipelago. Kawamura (1913) also did not designate any biological materials for the fungal description. Therefore, the drawing of *C. edodes* by Kawamura (1913) has been designated as the lectotype of *T. matsutake* (Ryman et al., 2000). *Agaricus edodes* Berk. (Berkeley, 1877), the original scientific name of *C. edodes* adopted by Kawamura (1913) to the lectotype of *Matsutake*, is now regarded as the basionym of *Lentinula edodes* (Berk.) Pegler. This taxonomic complication is clearly summarized

in Ito and Imai (1925), but it is not necessarily sufficient in terms of the cause, so we again added a detailed account of this taxonomic history. Schröter (1886) obtained a dried specimen of Japanese *Shiitake* cultivated on log through Shinkichi Nagai and observed it, apart from the *Shiitake* specimen (No. 258) of Berkeley (1877), and described it as *Collybia shiitake* Sieboldt. Concurrently, Schröter (1886) observed a specimen of *Matsutake* (canned in salt water) obtained from Japan through S. Nagai and adopted the scientific name *Agaricus* (*Arimillaria*) *edodes* given to *Shiitake* by Berkeley (1877) for it. Schröter (1886), based on his own observation of the canned thinly-sliced basidiomata (the color and texture of the stipe surface was distinctly different above and below the annulus) with the aid of a drawing of *Matsutake* presented on a Japanese nature book focused on plant phenology (Baishiken, 1842), recognized a particularly strong relationship of *Matsutake* with the genus *Armillaria*, which might have led to the adoption of the Latin name. Hennings (1899) largely followed the identification of Schröter (1886) in these two species but took a slightly different position on *Shiitake*, changing the Latin name to *Cortinellus shiitake* (Schröter) P. Henn. On the other hand, for *Matsutake*, he followed Schröter's (1886) description and concluded that Berkeley's (1877) specimen (No. 258) is *Matsutake*. Hennings (1899) thought that Berkeley (1877) mistakenly labelled as *Shiitake* to the *Matsutake* specimen. Based on his own observation of a Japanese specimen of *Matsutake* (probably sampled in Tochigi Prefecture as described below) obtained through Mitsutaro Shirai, Hennings (1899) concluded that *Matsutake* is a species of the genus *Armillaria*, which is closely related to *A. robusta* (Alb. & Schwein.) Gillet. Hennings (1899) might have regarded *Matsutake* as *Armillaria edodes* but did not use the Latin name to *Matsutake* in the description. Hennings (1900) identified *Matsutake* as *Cortinellus edodes* (Berk.) P. Henn., which was largely dependent on the drawing of *Matsutake* sampled in Japan (probably in Kyoto) and drawn by M. Shirai (Shirai and Henning, 1899), and another drawing of *Matsutake* (originally sampled in Nagano Prefecture) by M. Shirai (Shirai and Henning, 1931) duplicated from Ichioka (1799). In Shirai and Henning (1931), which was basically compiled in 1899–1900 but released in 1931 by M. Shrai, Hennings annotated "*Cortinellus matsutake*" to the drawing of *Matsutake*. Unfortunately, Shirai and Henning (1899) lacks the drawing of *Matsutake* on the extant material stored in National Diet Library, Japan. Hennings (1901), based on his own observation of a *Matsutake* specimen immersed in alcohol from Japan sampled in Tochigi Prefecture by M. Shirai, described the species as *Armillaria edodes* Berk. Saccardo (1887) adopted *Armillaria edodes* (Berk.) Sacc. to *Shiitake*, based on the description by Berkeley (1877). We inferred that Kawamura (1913) did not adopt *A. edodes* to *Matsutake* but did provisionally *C. edodes*. Kawamura (1913) adopted *C. shiitake* (Schröter) P. Henn. to *Shiitake*.

Given the phylogenetic and other biological analyses of the *T. matsutake* population, an ideal type sampled in Japan should be designated. Currently, the Japanese *T. matsutake* population is endangered in its natural habitat, especially in western and lowland areas including Kyoto, a productive area (Kawamura, 1913) in the past, and surrounding provinces on Honshu Island due to depletion of the main host trees (i.e., *Pinus densiflora* Siebold et Zucc.) (Vaario et al., 2017; Brandrud, 2020; Yamanaka et al., 2020). Therefore, we set an epitype of *T. matsutake* sampled in a large *P. densiflora* forest in Nagano Prefecture, at a central and relatively high elevation on Honshu Island, which is currently the most productive area for this fungus in Japan.

Finally, we discuss the biogeography and taxonomy of *T. matsutake*, because its wide distribution in Eurasia implies diverse genetic variations among isolated geographic regions, underscoring

Fig. 1 – Basidiomata of *Tricholoma matsutake* in a fir–beech mixed forest in Ukraine (photographed by NB). Mature and open-veiled basidiomata (upper) and young basidiomata (lower).

the need for taxonomic reconsideration of this species.

2. Materials and Methods

2.1. Specimens examined

We used specimens of several matsutake mushrooms collected in Japan and other regions in the Northern Hemisphere (Table 1). All samples from abroad were brought to Japan legally and used for the following analyses as the collaborative research between Japan and each of these countries. Fresh materials were lyophilized and oven-dried at 60 °C overnight to inactivate DNases and other oxidative enzymes, and stored in the laboratory. Several specimens were obtained from mushroom traders or mushroom markets. Where necessary, specimens were deposited in the National Museum of Nature and Science (TNS), Japan. In addition, we used several cultured strains of *T. matsutake* for the phylogenetic characteristics and ectomycorrhizal properties of which have been reported.

2.2. DNA analysis

DNA was extracted from dried basidioma specimens as described by Gardes and Bruns (1993) with minor modifications. For PCR, we focused on several loci: the ITS and intergenic spacer 1 (IGS1) regions of nuc rDNA, the translation elongation factor 1-alpha (*tef-1*), β_2 tubulin, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), the DNA-directed RNA polymerase II subunit (*rpb*2), the small subunit (SSU) of the mitochondrial rRNA gene tandem repeat (mt rDNA), the mitochondrial ATP synthase membrane subunit 6 (*atp6*), and a macroevolutionary genomic marker specific to the phylum Basidiomycota (*megB1*) (Table 2). Although *megB1* is located in the nuc rDNA IGS1 region in diverse Basidiomycota taxa, it is not associated with nuc rDNA in section Caligata of *Tricholoma*, including *T. matsutake* (Babasaki, Neda, & Murata, 2007). The primers used are listed in Table 2. PCR was conducted using the GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The 25 μL reaction mixture consisted of 2.5 μL 10× DreamTaq buffer, 2.5 μL dNTP mixture (0.2 mM), 2.5 μL each primer (0.5 μM), 0.125 μL DreamTaq DNA Polymerase (0.625 U; Thermo Fisher Scientific, Waltham, MA, USA), and 0.5 μL the extracted DNA as the template. The PCR parameters were initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 46–55 °C for 30 s, extension at 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min. The PCR

Table 1. Samples of *Tricholoma matsutake* and related species examined in the present study

Name	Basidioma specimen	Date	Country	Location	Canopy vegetation[h]
T. matsutake	AY-2200915-001	Sep 15, 2020	Ukraine	Kosiv, Ivano-Frankivsk	Aal, Fsy
T. matsutake	Ishida F-0247	Sep 5, 2007	Sweden	Åheden, Vasterbotten	Psy
T. matsutake	Ishida F-0315	Aug 19, 2007	Sweden	Hissjön, Umeå	Psy
T. matsutake	AT-0925*	2000	Sweden	n.d.	n.d.
T. matsutake	AY-2040800-001	Aug, 2004	Finland	Haukipudas, North Ostrobothnia	Psy
T. matsutake	AY-2070925-001 (=Tn-FIN1[a])	Sep 25, 2007	Finland	Kontiolahti, North Karelia (same sampling site of isolation origin EF[b, *])	Psy
T. matsutake	AY-2040400-001	April, 2004	Bhutan	Thimphu	n.d.
T. matsutake	AY-1981000-002 (= BH1[c])	Oct, 1998	Bhutan	n.d.	n.d.
T. matsutake	Narimatsu S-1	July 27, 2017	Bhutan	Geneka	Pwa, Qsm
T. matsutake	Narimatsu S-2	July 27, 2017	Bhutan	Geneka	Qsm
T. matsutake	Narimatsu S-3-1	July 27, 2017	Bhutan	Geneka	Pwa, Qsm
T. matsutake	Narimatsu S-3-2	July 27, 2017	Bhutan	Geneka	Pwa, Qsm
T. matsutake	AY-1980831-001	Aug 31, 1998	China	n.d.	n.d.
T. matsutake	AY-1981000-001 (= CH1[c])	Oct, 1998	China	n.d.	n.d.
T. matsutake	Chi4* (= CH-HE[c])	2007	China	Heilongjiang	n.d.
T. matsutake	Chi6* (=CH-YU1[c])	2007	China	Yunnan	n.d.
T. matsutake	AY-1981000-003 (= NK1[c])	Oct, 1998	North Korea	n.d.	n.d.
T. matsutake	AT-0924* (= KFRI 432)	Sep 21, 1995	South Korea	Hongcheon, Kangwon-do	Pde
T. matsutake	AY-2150919-001	Sep 19, 2015	Japan	Nishi-okoppe, Hokkaido	Asa
T. matsutake	AY-2101004-001	Oct 4, 2010	Japan	Nikko, Tochigi	n.d.
T. matsutake	AY-1981023-003	Oct 23, 1998	Japan	Takaizuri, Hitachi-ohmiya, Ibaraki	Pde
T. matsutake	AY-2051104-001	Nov 4, 2005	Japan	Takaizuri, Hitachi-ohmiya, Ibaraki	Pde
T. matsutake	AY-2051104-002	Nov 4, 2005	Japan	Takaizuri, Hitachi-ohmiya, Ibaraki	Pde
T. matsutake	AY-2051104-003	Nov 4, 2005	Japan	Takaizuri, Hitachi-ohmiya, Ibaraki	Pde
T. matsutake	AY-2071023-002	Oct 23, 2007	Japan	Takaizuri, Hitachi-ohmiya, Ibaraki	Pde
T. matsutake	Y1[a, c, d, *] (=NBRC 33136)	Oct, 1993	Japan	Takaizuri, Hitachi-ohmiya, Ibaraki	Pde
T. matsutake	TUA-115	Oct 10, 2019	Japan	Mt. Norikuradake, Matsumoto, Nagano	Tdi
T. matsutake	AY-2131013-001 (= S-2131013-001[e])	Oct 13, 2013	Japan	Mt. Norikuradake, Matsumoto, Nagano	Ave
T. matsutake	TUA-84	Oct 10, 2018	Japan	Motoyama, Shiojiri, Nagano	Tsi
T. matsutake	AT-0740*, **	June 24, 2001	Japan	Nyu-yama, Ina, Nagano	Pde
T. matsutake	AT-0748*, **	July 2, 2003	Japan	Mt. Moriyasan, Ina, Nagano	Pde
T. matsutake	AY-2041007-002 (=TNS-F 82226, epitype)	Oct 7, 2004	Japan	Kuwahara, Nakagawa, Nagano	Pde
T. matsutake	AY-2101020-001	Oct 20, 2010	Japan	Kuwahara, Nakagawa, Nagano	Pde
T. matsutake	AY-2181005-001 (=TNS-F 82227)	Oct 5, 2018	Japan	Kuwahara, Nakagawa, Nagano	Pde
T. matsutake	AY-2021027-001	Oct 27, 2002	Japan	Matsukawa, Nagano	Pde
T. matsutake	AY-2041007-001 (=TNS-F 82228)	Oct 7, 2004	Japan	Shobuzawa, Ooshika, Nagano	Tsi
T. matsutake	AY-2101021-002	Oct 21, 2010	Japan	Shobuzawa, Ooshika, Nagano	Tsi
T. matsutake	AY-2071101-001 (isolation origin of Tm#84[f, *])	Nov 1, 2007	Japan	Takagi, Nagano	Pde
T. matsutake	AT-0742*, **, g	Oct 12, 2001	Japan	Kamihisakata, Iida, Nagano	Tsi
T. matsutake	AY-2191104-001	Nov 4, 2019	Japan	Mt. Misumiyama, Tottori, Tottori	Pde
T. magnivelare	AY-2080307-001	Mar 7, 2008	Canada	Quebec	n.d.
T. mesoamericanum	AY-1981000-004 (= MX1[a])	Oct, 1998	Mexico	n.d.	n.d.
T. murrillianum	AT-0913* (=Tp-C3[a])	1994	Canada	n.d.	n.d.
T. murrillianum	AY-2080100-001	Jan, 2008	Canada	n.d.	n.d.
T. murrillianum	AY-2071018-001	Oct 18, 2007	Canada	n.d.	n.d.
T. anatolicum	AY-1981000-005 (= MC1[a])	Oct, 1998	Morocco	n.d.	n.d.
T. anatolicum	AY-1981000-006 (= MC1[a])	Oct, 1998	Morocco	n.d.	n.d.
T. anatolicum	AY-2061109-001 (=S-3-2-1[a])	Nov 9, 2006	Turkey	Babadağ, Denizli	Cli
T. anatolicum	AY-2061108-004 (=S-2-2-1[a])	Nov 8, 2006	Turkey	Mt. Çal, Fethiye	Cli
T. anatolicum	AY-2061109-002 (=S-2-3[a])	Nov 9, 2006	Turkey	Yayla Koru, Fethiye	Cli
T. fulvocastaneum	AY-2091112-001	Nov 12, 2009	Japan	Amami-ohshima Island, Kagoshima	Qmi, Plu
T. fulvocastaneum	AY-2110606	Jun 6, 2011	Laos	n.d.	n.d.
T. bakamatsutake	AY-2080800-001	Aug, 2008	China	Jilin Province	n.d.
T. bakamatsutake	AY-2050912-001	Sep 12, 2005	Japan	Nishinouchi, Hitachi-ohmiya, Ibaraki	Qsr
T. bakamatsutake	AT-0760*	Oct, 2003	Japan	Kuwahara, Nakagawa, Nagaono	Qac
T. bakamatsutake	AY-2041007-003	Oct 7, 2004	Japan	Kuwahara, Nakagawa, Nagaono	Qsr
T. bakamatsutake	AY-2071001-002	Oct 1, 2007	Japan	Kuwahara, Nakagawa, Nagaono	Qsr

[a]–[g] Please refer to Ota et al. (2012), Vaario et al. (2010), Murata et al. (2008), Yamada et al. (1999b), Endo et al. (2015), Yamada et al. (2019), and Saito et al. (2018), respectively.

[h] Aal: *Abies alba*, Asa: *A. sachalinensis*, Ave: *A. veitchii*, Cli: *Cedrus libani*, Fsy: *Fagus sylvatica*, Pde: *Pinus deinsflora*, Plu: *P. luchuensis*, Psy: *P. sylvestris*, Pwa: *P. wallichiana*, Qac: *Quercus acutissima*, Qmi: *Q. miyagii*, Qsm: *Q. semecarpifolia*, Qsr: *Q. serrata*, Tdi: *Tsuga diversifolia*, Tsi: *T. sieboldii*

* Cultured strain. ** These cultures were isolated from monotropoid mycorrhizal root tips of *Monotropa hypopitys* that grew on the colony of *T. matsutake*.

amplicons were electrophoresed (Mupid®-exU; TaKaRa Bio, Kusatsu, Japan) on 1.5% agarose gels for fragments ≥ 1 kb (01163-76; Nacalai Tesque, Kyoto, Japan) for 30 min, stained with 0.001% ethidium bromide solution, and visualized using an ultraviolet illuminator (NM-15; UVP, Upland, CA, USA). The PCR amplicons were purified using a QIAquick PCR Purification Kit (QIAGEN, Venlo, the Netherlands) and subjected to cycle sequencing. The ITS and IGS1 regions of nuc rDNA, *gapdh*, and the mt rDNA SSU were cloned manually before cycle sequencing using a Mighty TA-Cloning Kit (TaKaRa Bio) and then inserted into competent *Escherichia coli* JM109 cells (TaKaRa Bio).

For cycle sequencing, the 10 μL reaction mixture consisted of 2 μL distilled water (DW), 2 μL 10× buffer, 1 μL 5 mM primer, 1 μL Ready Reaction Mix (BigDye Terminator v. 3.1 Cycle Sequencing Kit; Thermo Fisher Scientific), and 4 μL purified DNA solution. The reaction parameters for cycle sequencing were initial denaturation at 96 °C for 1 min, followed by 25 cycles of denaturation at 96 °C for 10 s, annealing at 46–55 °C (Table 2) for 5 s, and extension at 60 °C for 4 min. The amplicons were sequenced using an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). The obtained sequence data (chromatograms) were edited by Chromas 2.6.6 (http://technelysium.com.au/wp/chromas/) and checked the consensus between complementary sequences of each region in each sample by GeneStudio (http://genestudio.com/). The consensus sequences were deposited in DDBJ (Supplementary Table S1).

Table 2. PCR primers used in the present study.

Targeted locus	Primer name	Sequence (5'→3')	Tm value (°C)
nuc rDNA ITS (N[*])	ITS1F (F)[1]	CTTGGTCATTTAGAGGAAGTAA	55
	ITS4B (R)[1]	CAGGAGACTTGTACACGGTCCAG	65
	ITS4 (R)[2]	TCCTCCGCTTATTGATATGC	60
nuc rDNA IGS1 (N)	CNL12 (F)[3]	CTGAACGCCTCTAAGTCAG	56
	5S-Anderson (R)[4]	CAGAGTCCTATGGCCGTGGAT	66
RPB2 (N)	rpb2_tu_f1 (F)[5]	CTGTCGGYTCYTATTCTGC	53
	rpb2_tu_r1 (R)[5]	GCTRGGATGAATCTCACAATG	52
GAPDH (N)	gpd_tm_f1 (F)[6]	CTTGCCGACGGCCTTTG	57
	gpd_tm_r1 (R)[6]	CCCTTCATCGATCTCGAATACATGG	57
TEF1 (N)	tef1_tm_f1 (F)[6]	GTCAAACVCGAGAGCAYG	54
	tef1_tm_r1 (R)[6]	CACAAGCTTGACRATRCAAGC	55
	tef1_tm_2f1 (F)[6]	CTCGAGSGATTTACCTGTCC	55
	tef1_tm_2r1 (R)[6]	GACCGATTCAACGAAATCGTG	54
β₂ tubulin gene (N)	b-tub1_tm_f1 (F)[6]	GCGTGAAATCGTCCACCTTC	56
	b-tub1_tm_r1 (R)[6]	CAGGGAAYCGCAAGCAAG	56
	b-tub1_tm_2f1 (F)[6]	CCAGTTGGTGGACAGAAAG	53
	b-tub1_tm_2r1 (R)[6]	CTGGATCAGGTGCGAAGTT	55
mt rDNA SSU (M[*])	MS1 (F)[2]	CAGCAGTCAAGAATATTAGTCAATG	52
	MS2 (R)[2]	GCGGATTATCGAATTAAATAAC	48
ATP6 (M)	ATP-3f (F)[7]	TCTCCTTTAGAACAATTTGA	46
	ATP-6r (R)[8]	AACTAATARAGGAACTAAAGCTA	48
megB1 (N)	TS-1_fws2(F)[9]	CCCGTCTTAATCGACCATG	52
	TS-2_rvc1(R)[9]	TCAGACATCCAAAGGAAGGT	53

[*] N: nucleus; M: mitochondrion

[1] Gardes & Bruns (1993); [2] White et al. (1990); [3] Anderson & Stasovski (1992); [4] Henrion et al. (1992); [5] Aoki et al. (2021);
[6] This study; [7] Kretzer & Bruns (1999); [8] Binder & Hibbett (2003; http://www2.clarku.edu/faculty/dhibbett/Protocols_
Folder/Primers/Primers.pdf); [9] Babasaki et al. (2007).

Several sequences were downloaded from GenBank and UNITE (Supplementary Table S2). For phylogenetic analysis of each DNA region, sequences were aligned using ClustalW (Larkin et al., 2007) and MEGA7 (Kumar, Stecher, & Tamura, 2016). Alignment gaps were treated as missing data, and ambiguous positions were excluded from the analysis. Datasets for the ITS (ITS1–5.8S–ITS2; 651 bp) and IGS1 (420 bp) regions of nuc rDNA, rpb2 (461 bp), gapdh (629 bp), atp6 (347 bp), tef-1 (695 bp), SSUs of mt rDNA cluster (581 bp), β₂ tubulin (710 bp), and megB1 (445 bp) were prepared. Maximum likelihood (ML) and Bayesian inference analyses were conducted to clarify the phylogenetic relationships among species and among specimens within T. matsutake. ML trees were constructed using RAxML version 8.2.4 with 1000 bootstrap replicates, which followed a general time-reversible model with a gamma distribution and invariant sites (Stamatakis, 2014). For Bayesian inference analysis, the SYM model of nucleotide substitution with a discrete gamma distribution was performed using MrBayes 3.2.1 (Ronquist et al., 2012). Two runs with four chains of Markov Chain Monte Carlo iterations were performed for 1,000,000 generations when the average standard deviation of split frequencies was below 0.01 (the first 25% of generations were treated as burn-in). Trees were kept for every 100 generations, and the remaining 75% of trees were used to calculate the 50% majority-rule consensus topology and to determine the posterior probabilities for individual branches.

2.3. Microscopy observations of selected specimens

In this study, we observed basidiospores and basidia and compared their sizes among the specimens distinguished by the phylogenetic analyses. Several gills were cut from each dried specimen, rehydrated in a few milliliters of 5% KOH solution for 30 min, transferred to several milliliters of DW, and incubated for several minutes. Fully rehydrated gills were sectioned with a razor, mounted on glass slides using lactic acid, and observed under a differential interference contrast (DIC) microscope with a 100× oil-immersion objective lens (BX51N-33; Olympus, Tokyo, Japan or AXIO Scope 2; Carl Zeiss, Göttingen, Germany). When using Melzer's reagent (Clémençon, 2012), the dried material was rehydrated in 10 mL 70% ethanol solution for a few minutes, transferred to 20 mL DW for 1 h, and mounted in Melzer's reagent. The lengths and widths of cells were measured at a resolution of 0.1 µm on the recorded micrographs using ImageJ software (https://imagej.nih.gov/ij/). The Q-value (length/width) of each spore was calculated. At least 100 spores and basidia in each specimen were measured. As some samples had fewer spores and basidia, smaller numbers thereof were measured.

Measured numerical data were subjected to further statistical analyses. For most samples, the shortest 5% of all measured spore-length data ($n \geq 100$) were excluded from the evaluation because such spores were most likely immature. In the case of samples with an insufficient number of measured spores ($n < 100$), 25% of the shortest samples were excluded from the evaluation. Because these specimens had much possibility to be measured the size of immature basidiospores. To compare numerical data, one-way analysis of variance was conducted using R (https://www.r-project.org/), and the significance of differences was determined by Tukey's post hoc test ($p < 0.05$). To indicate the range of cell size in the morphological description, we ordered the mean value of each specimen as minimum–average–maximum.

For the morphological definition of T. matsutake, we followed Kawamura (1930, 1955), Imazeki and Hongo (1957), and Christensen and Heilmann-Clausen (2013). In addition, as Kytövuori (1988) described sclerobasidia and sclerospores in T. nauseosum, we observed their presence/absence and measured their wall thickness. For the description of T. matsutake based on the epitype spec-

imen, which was newly designated in this study, we conducted additional DIC microscopy observations and observed the sizes of the sterigma, pileipellis, and stipitipellis (Clémençon, 2012; Christensen and Heilmann-Clausen, 2013).

3. Results

3.1. Phylogenetic relationships among matsutake mushrooms

The nuc rDNA ITS phylogenetic tree included the Ukraine specimen in the *T. matsutake* clade that comprised all Japanese, Bhutanese, Chinese, North Korean, South Korean, Swedish, and Finnish specimens examined (Fig. 2). One Chinese *T. bakamatsutake* specimen, AY-2080800-001, sampled in Jilin Province and labeled as *T. matsutake* was included in the *T. bakamatsutake* clade. Other related species (*T. magnivelare, T. anatolicum, T. mesoamericanum, T. murrillianum,* and *T. fulvocastaneum*) formed an independent clade. The phylogenetic tree of nuc rDNA IGS1 (Fig. 3) showed a different topology from that of ITS. The *T. magnivelare* specimen AY-2080307-001 from Canada was included in the *T. matsutake* clade, demonstrating a *T. matsutake–T. magnivelare* continuum. In addition, *T. anatolicum* and *T. murrillianum* were regarded as parallel subclades to *T. matsutake*. The *rpb2* phylogenetic tree (Fig. 4) showed unique topology; i.e., Bhutanese and Chinese *T. matsutake* specimens formed a subclade (B/C clade) independent from that of the other *T. matsutake* specimens (the A/E clade accompanying *T. mesoamericanum* and *T. magnivelare*). In addition, *T. matsutake* specimens in the A/E clade were found on branches that consisted of three Japanese *T. matsutake* specimens sampled in Nagano Prefecture. The *tef-1* phylogenetic tree (Fig. 5) showed a similar topology to the *rpb2* phylogenetic tree, i.e., separation of Bhutanese and Chinese *T. matsutake* specimens (B/C clade) from the other *T. matsutake* specimens (A/E clade). The β_2 tubulin gene phylogenetic tree (Fig. 6) showed similar topology to that of ITS. In addition, European and Bhutanese–Chinese specimens consisted of subclades within the *T. matsutake* clade, with moderate support values.

The *gapdh* phylogenetic tree (Supplementary Fig. S1), as well as the nuc rDNA ITS, *tef-1* and β_2 tubulin phylogenetic trees (Figs. 2, 5, 6), showed independency of *T. matsutake* from the other related species. The *atp6* phylogenetic tree (Supplementary Fig. S2) showed independency of *T. matsutake* from the other related species, except for a Japanese specimen (AY-2101004-001). The *megB1* phylogenetic tree (Supplementary Fig. S3) showed two isolated clades of *T. matsutake*, with the *T. murrillianum* clade positioned between them. The mt rDNA SSU phylogenetic tree (Supplementary Fig. S4) showed three isolated clades of *T. matsutake*, two of which included *T. anatolicum*. Notably, the Ukrainian specimen of *T. matsutake* was isolated from the other *T. matsutake* specimens.

3.2. Morphological characteristics of basidiospores and basidia in matsutakes

Based on the phylogenetic data (Figs. 2–6; Supplementary Figs. S1–S4), we compared the morphological characteristics of basidiospores and basidia among matsutake species, as well as among *T. matsutake* populations, i.e., the Bhutan–China group (B/C group; corresponding to specimens in the B/C clade of *rpb2* and *tef-1* phylogenies), and the other specimens sampled from far east Asia (the Japanese Archipelago and the Korean Peninsula) and Europe (A/E group; corresponding to specimens in the A/E clade of *rpb2* and *tef-1* phylogenies). Of the 31 specimens selected for microscopic observation (Supplementary Table S3), several had a limited num-

ber of measurements of basidium size. The sizes of about 3600 spores and 2100 basidia were measured.

The mean spore size of 10 Japanese *T. matsutake* specimens was 6.54–7.87 × 5.08–5.94 μm, the mean Q-value was 1.19–1.37, and the mean basidium size was 32.89–42.22 × 6.94–8.02 μm (Supplementary Table S3). Specimens in the A/E group other than the Japanese specimens (Ukraine, Sweden, North Korea, and Finland) had a mean spore size of 6.69–7.37 × 5.31–5.64 μm, a mean Q-value of 1.23–1.32, and a mean basidium size of 36.49–41.11 × 7.34–7.42 μm. Specimens in the B/C group (Bhutan and China) had a mean spore size of 6.84–7.43 × 5.61–6.05 μm, a mean Q-value of 1.22–1.24, and a mean basidium size of 37.59–41.00 × 6.94–7.50 μm. Mean spore lengths and mean Q-values did not differ significantly between the A/E and B/C groups according to *t*-tests ($p = 0.210$ and 0.379, respectively). However, mean spore width was significantly larger in the B/C group than in the A/E group ($p = 0.024$), although the limitation that only three specimens were evaluated in the B/C group should be noted. Mean basidium lengths and widths did not differ significantly between the A/E and B/C groups ($p = 1.0$ and 0.428, respectively).

The mean basidiospore sizes and shapes of specimens of *T. anatolicum*, *T. magnivelare*, *T. mesoamericanum*, *T. murrillianum*, and *T. fulvocastaneum* were similar to those of *T. matsutake* (Fig. 7; Supplementary Table S3). However, the spore Q-value of *T. anatolicum* sampled in Morocco was large (> 1.4). The spore size (length and width) of *T. bakamatsutake* was significantly smaller than that of *T. matsutake* ($p < 0.01$). The basidium lengths and shapes of *T. anatolicum*, *T. mesoamericanum* and *T. murrillianum* were within the range of those of *T. matsutake*. However, *T. murrillianum* had a larger length-to-width ratio and *T. bakamatsutake* had a smaller length-to-width ratio (due to a shorter length) compared with *T. matsutake*. The Chinese *T. bakamatsutake* specimen AY-2080800-001 (sliced segments and dried basidioma samples) labeled as *T. matsutake* but identified as *T. bakamatsutake* by the phylogenetic analyses and morphological observations had a few larger basidiospores on some sliced segments, implying spore contamination of *T. matsutake*. However, additional ITS analyses in each of the subsamples ($n = 17$) showed a single *T. bakamatsutake* signal (data not shown).

3.3. Sclerobasidia and sclerospores in matsutakes

Sclerobasidia and sclerospores, which are reportedly common in *T. nauseosum* (Kytövuori, 1988), are characteristic of matsutakes. Although distinct sclerobasidia and sclerospores were present in some specimens, their wall thickness varied. Therefore, we categorized wall thickness into three types: that of type A was 0.2–0.25 μm in spores and 0.25–0.3 μm in basidia, that of type B was 0.25–0.4 μm in spores and 0.3–0.7 μm in basidia, and that of type C was 0.4–0.6 μm in spores and 0.7–1.2 μm in basidia (Fig. 9). Types B and C fully correspond to sclerobasidia and sclerospores, respectively (Kytövuori, 1988). The type A sclerospores and sclerobasidia were sometimes difficult to distinguish from general basidiospores due to the slight differences in their wall thicknesses determined via DIC microscopy. Of the 10 Japanese *T. matsutake* specimens examined, 4 did not show a sclerobasidium or sclerospore, 6 showed type A sclerospores accounting for less than 30% of all spores, and 3 showed both type A sclerospores and sclerobasidia occupying less than 30% of all spores and basidia, respectively (Table 3). A limited number of type B basidia and a single type C basidium were observed on the AY-2051104-002 specimen. Specimens in the A/E group of *T. matsutake* other than the Japanese ones had type B or C sclerospores and sclerobasidia occupying approximate-
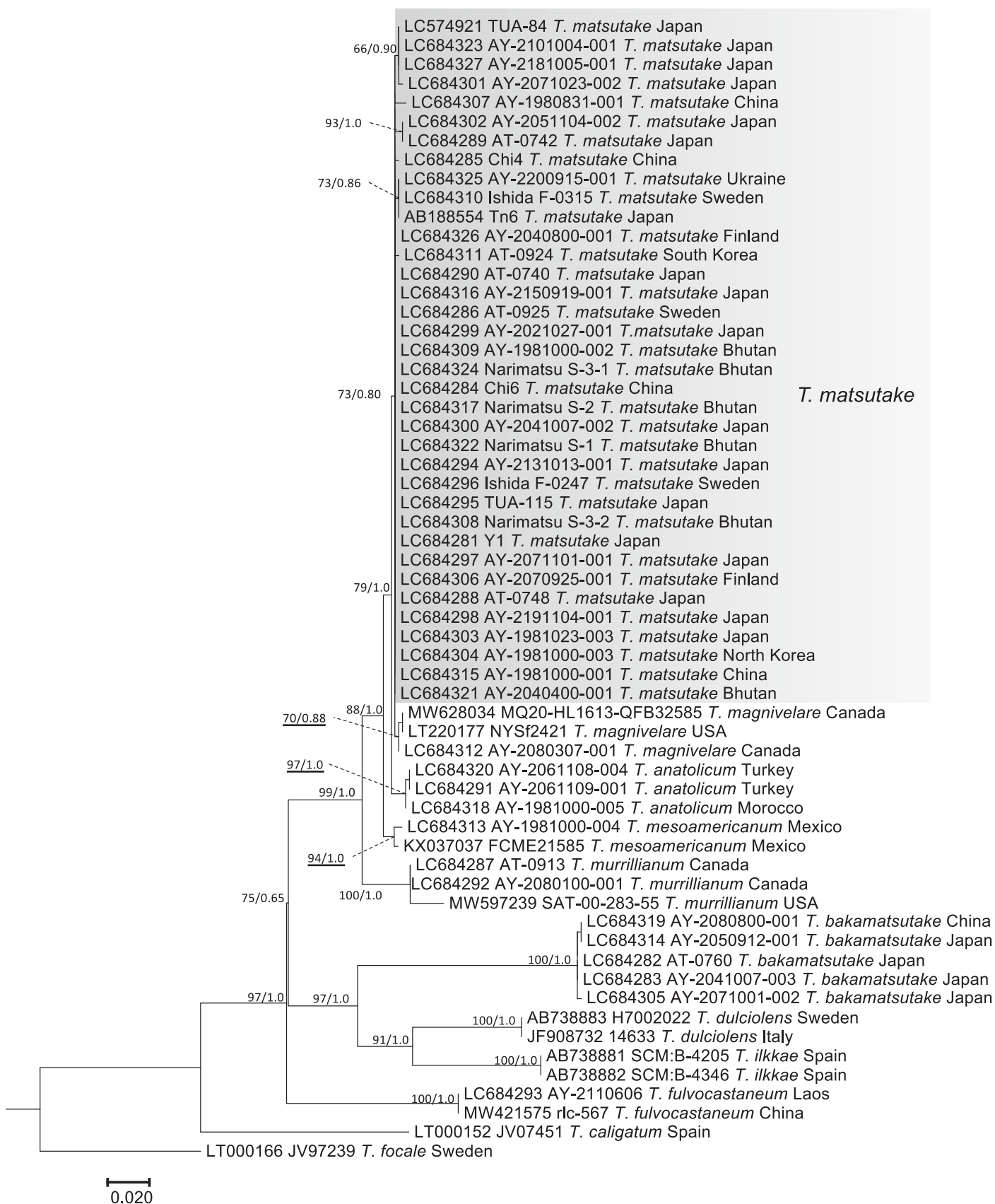
Fig. 2 – ML phylogenetic tree of the nuc rDNA ITS region of *Tricholoma matsutake* and closely related species in the section Caligata. Bootstrap (BS) values > 60% from ML trees (left) and Bayesian posterior probabilities (PP) > 0.60 (right) are shown near the nodes.
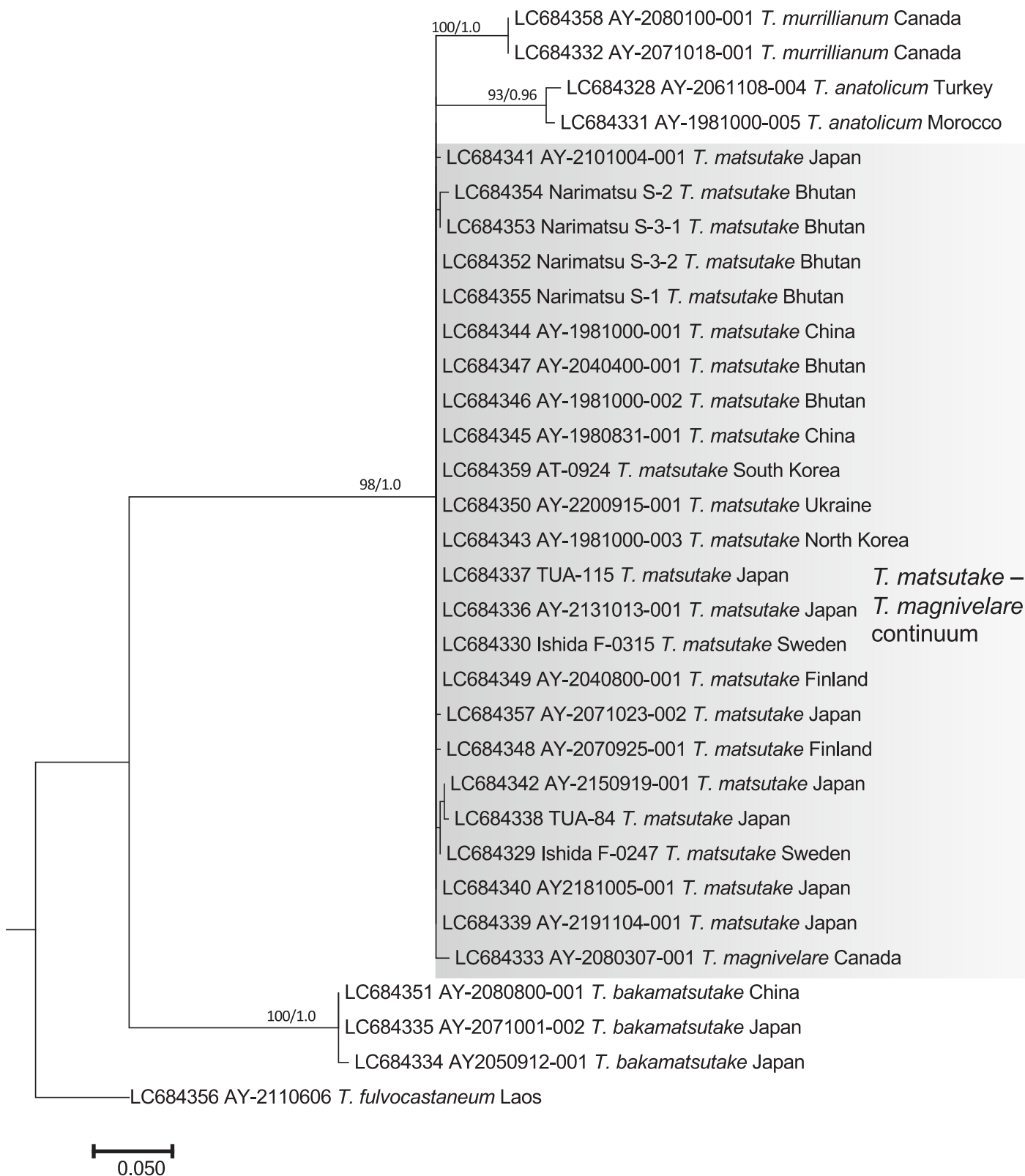
Fig. 3 – ML phylogenetic tree of the nuc rDNA IGS1 region of *Tricholoma matsutake* and closely related species in the section Caligata. Bootstrap (BS) values > 60% from ML trees (left) and Bayesian posterior probabilities (PP) > 0.60 (right) are shown near the nodes.

ly 5–30% of all spores and basidia, respectively. A Swedish specimen had type B sclerobasidia accounting for less than 1% of all basidia. The Finnish specimen AY-2070925-001 frequently had type C sclerospores and sclerobasidia, some with a wall thickness > 1 μm.

However, AY-2200915-001 from Ukraine had only type A sclerospores and sclerobasidia. In the B/C group specimens of *T. matsutake*, AY-2040400-001 from Bhutan and AY-1980831-001 from China had both type A sclerospores and sclerobasidia occupying
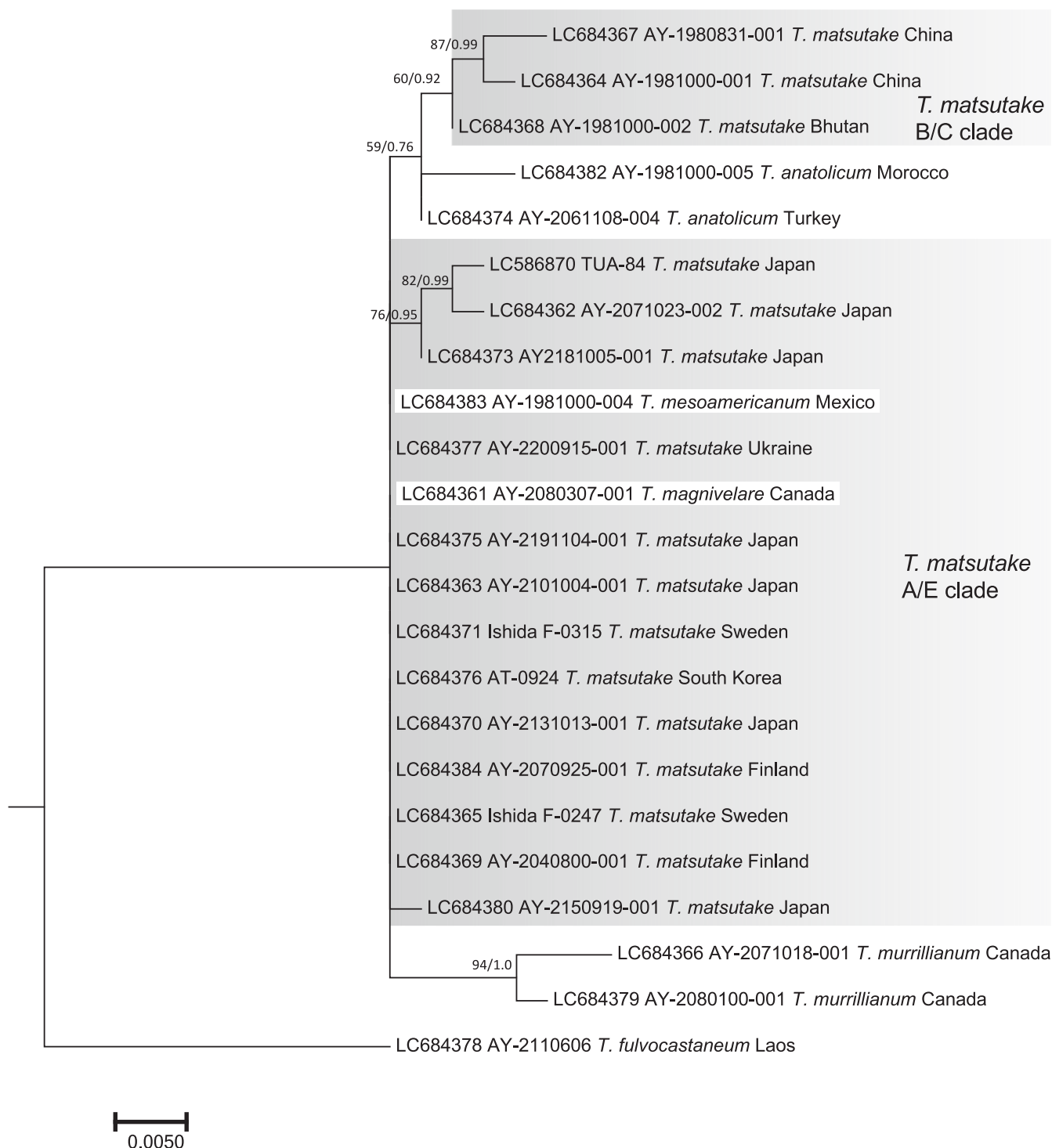
Fig. 4 – ML phylogenetic tree of the *rpb2* gene of *Tricholoma matsutake* and closely related species in the section Caligata. Bootstrap (BS) values > 60% from ML trees (left) and Bayesian posterior probabilities (PP) > 0.60 (right) are shown near the nodes.

approximately 5–10% of all spores and basidia, respectively. However, AY-1981000-001 from China had both type A and type B sclerospores and sclerobasidia accounting for approximately 10–20% of all spores and basidia, respectively. Both sclerospores and sclerobasidia had inamyloid (indextrinoid) walls under Melzer's reagent staining, in contrast to the results of Kytövuori (1988) (data not shown). Specimens of *T. anatolicum* and *T. fulvocastaneum* had type C sclerospores and sclerobasidia, and *T. bakamatsutake* had type B sclerospores and type C sclerobasidia.

3.4. Morphological definition of T. matsutake

Based on the descriptions of *T. matsutake* by Kawamura (1913, 1930, 1955), Ito and Imai (1925), Imazeki and Hongo (1957), Kytövuori (1988), and Christensen and Heilmann-Clausen (2013), we redescribed the morphological characteristics of this fungus. We cited the macroscopic characteristics from Kawamura (1955) and Imazeki and Hongo (1957), as well as our own observations, and revised the microscopic characteristics based on Japanese
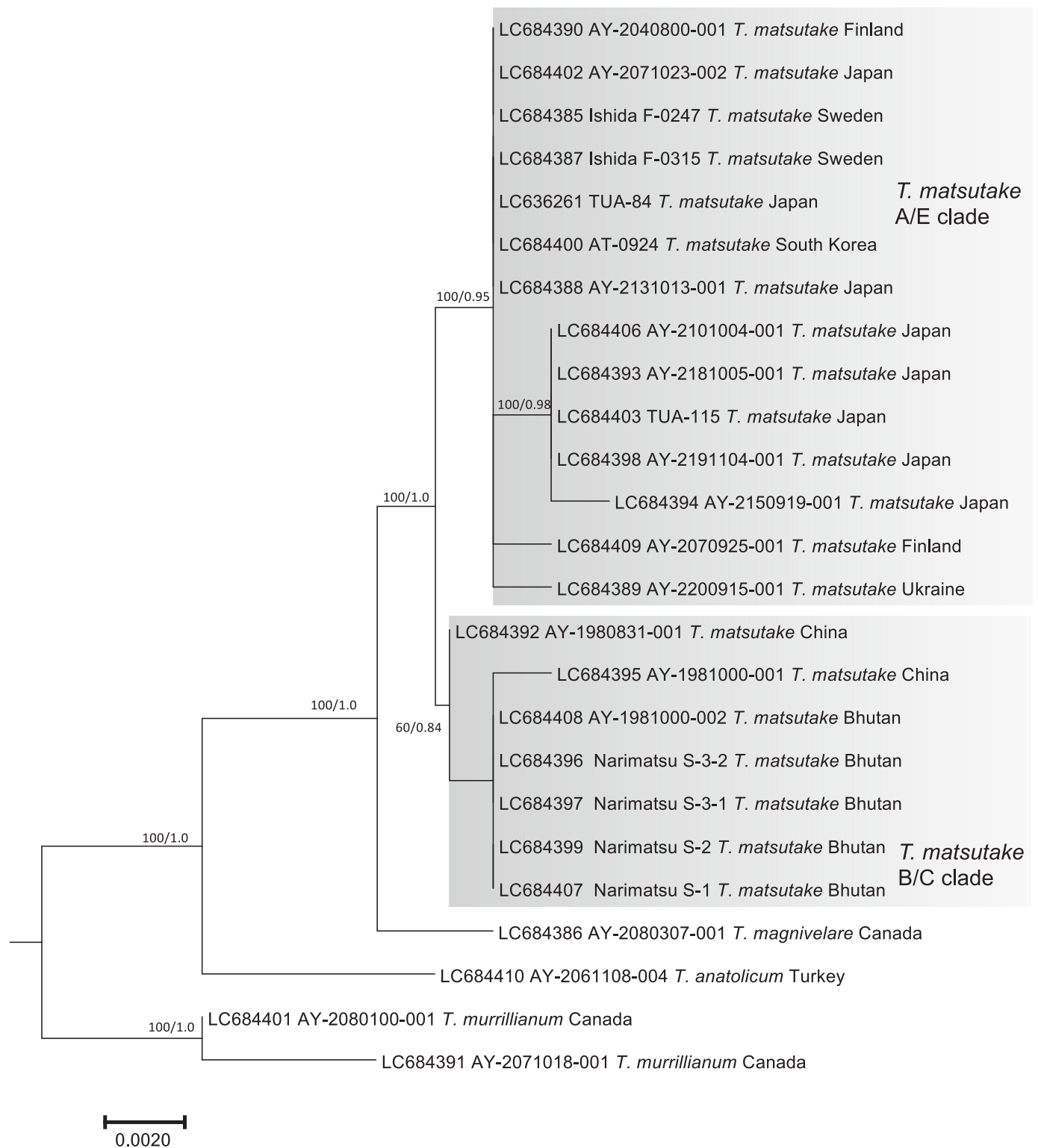
Fig. 5 – ML phylogenetic tree of the *tef-1* gene of *Tricholoma matsutake* and closely related species in the section Caligata. Bootstrap (BS) values > 60% from ML trees (left) and Bayesian posterior probabilities (PP) > 0.60 (right) are shown near the nodes.

specimens, including the designated epitype, as follows.

4. Taxonomy

Tricholoma matsutake (S. Ito & S. Imai) Singer, Annls mycol. 41(1/3): 77, 1943. Fig. 8.

≡ *Armillaria matsutake* S. Ito & S. Imai, Bot Mag 39: 327, 1925.

= *Armillaria nauseosa* A. Blytt, Norges Hymenomyceter: 22, 1905.

= *Tricholoma nauseosum* (A. Blytt) Kytöv., Karstenia 28: 69, 1988.

Misapplied synonyms: *Agaricus edodes* Berk., J. Schröter, Gartenflora 35: 135, 1886; *Cortinellus edodes* (Berk.) P. Henn., P. Hen-
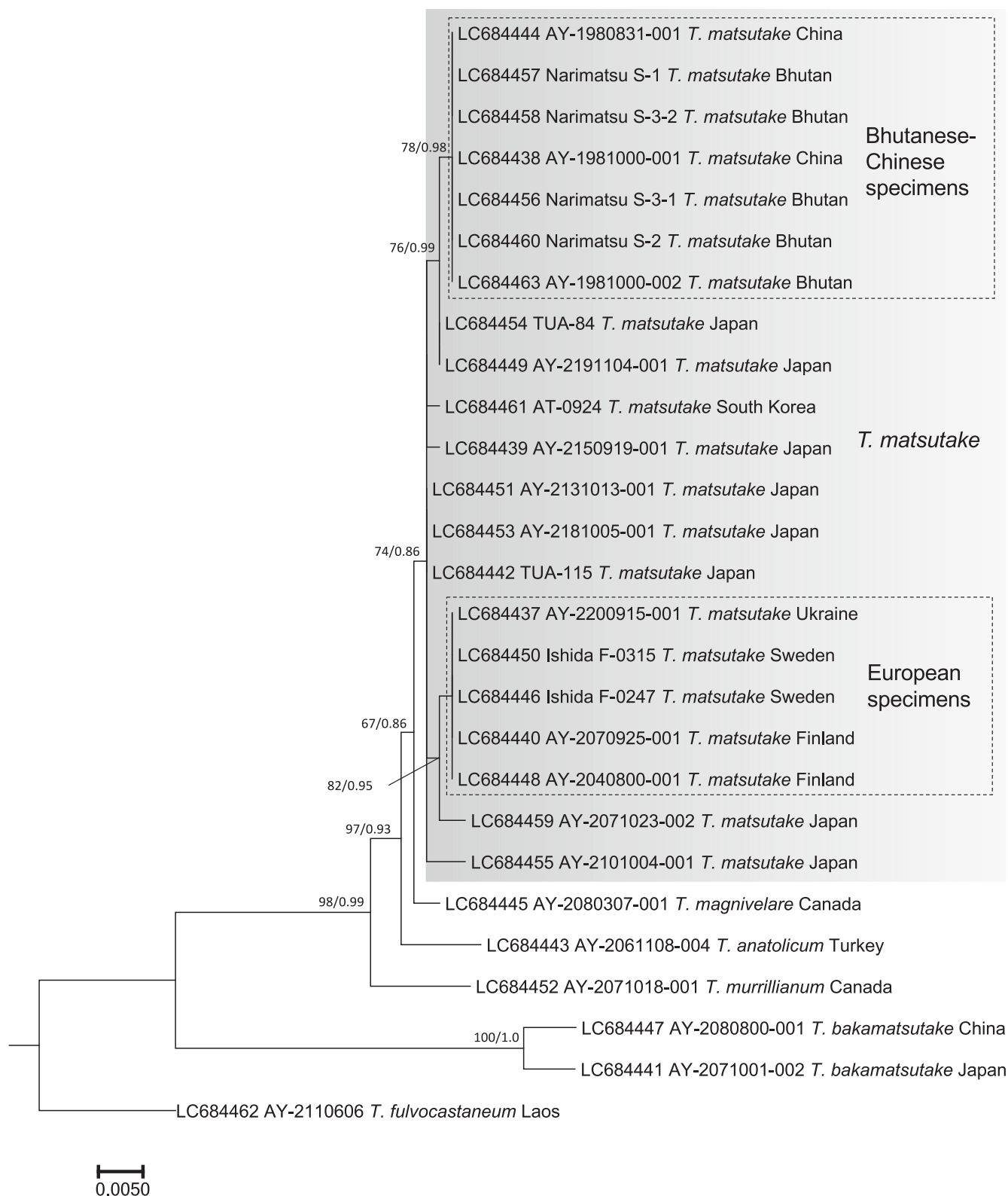
Fig. 6 – ML phylogenetic tree of the β$_2$ tubulin gene of *Tricholoma matsutake* and closely related species in the section Caligata. Bootstrap (BS) values > 60% from ML trees (left) and Bayesian posterior probabilities (PP) > 0.60 (right) are shown near the nodes.

nings, Hedwigia 39: 156, 1900; *Armillaria edodes* Berk.*,* P. Hennings, Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie 28: 270, 1901; *Armillaria caligata* Viv., P. Hariot & N. Patouillard, Bull Mus Hist Nat 8: 132, 1902.

Description: ***Pileus*** 8–20(–30) cm diam, first convex, later plano-convex, finally plane, surface with pale yellowish brown to pale chestnut brown, appressed squamulose (Fig. 8A) but rarely recurved squamulose (Fig. 8C), lately darkish in color and sometimes radial cracks showing whitish flesh tissue, margin inrolled and

Table 3. Types of sclerospores and sclerobasidia

Species	Specimen	Phylogrop	Country	Sclerospores			Sclerobasidia		
				State	Type	Frequency (%)	State	Type	Frequency (%)
T. matsutake	AY-2200915-001	A/E	Ukraine	+/−	A	< 1	+/−	A	< 1
	Ishida F-0315	A/E	Sweden	+/−	A	< 1	+/−	A, B	< 1
	AY-2040800-001	A/E	Finland	+	A, B	< 5	+	A, B	< 5
	AY-2070925-001	A/E	Finland	++	A–C	~ 30	++	A–C	~ 30
	AY-1981000-003	A/E	North Korea	++	A, B	~ 10	++	A	~ 20
	AY-1981023-003	A/E	Japan	+	A	< 5	+	A	< 5
	AY-2051104-001	A/E	Japan	++	A	~ 30	−		
	AY-2051104-002	A/E	Japan	++	A	~ 20	++	A (B, C)	~ 10
	AY-2051104-003	A/E	Japan	+	A	< 5	−		
	AY-2071023-002	A/E	Japan	−			−		
	AY-2131013-001	A/E	Japan	+/−	A	< 1	+/−	A	< 1
	AY-2041007-002	A/E	Japan	−			−		
	AY-2041007-001	A/E	Japan	−			−		
	AY-2021027-001	A/E	Japan	−			−		
	AY-2071101-001	A/E	Japan	+	A	< 5	−		
	AY-2040400-001	B/C	Bhutan	+	A	< 5	−		
	AY-1981000-002	B/C	Bhutan	++	A	~ 10	+	A	< 5
	AY-1980831-001	B/C	China	+	A	< 5	+	A	< 5
	AY-1981000-001	B/C	China	++	A, B	~ 20	++	A, B	~ 20
T. bakamatsutake	AY-2041007-003		Japan	++	A, B	~ 20	++	A	< 5
	AY-2071001-002		Japan	++	A, B	~ 10	+	A, B	< 5
	AY-2080800-001		China	+++	A, B	~ 50	++	A–C	~ 10
T. fulvocastaneum	AY-2091112-001		Japan	+++	A–C	~ 60	+	B, C	< 5
T. magnivelare	AY-2080307-001		Canada	+/−	A	< 1	+/−	B	< 1
T. murrillianum	AY-2080100-001		Canada	+/−	A	< 1	−		
	AY-2071018-001		Canada	+/−	A	< 1	−		
T. mesoamericanum	AY-1981000-004		Mexico	+/−	A	< 1	−		
T. anatolicum	AY-2061109-001		Turkey	++	B, C	~ 20	+/−	B, C	< 1
	AY-2061108-004		Turkey	++	B, C	~ 30	+/−	B, C	< 1
	AY-2061109-002		Turkey	++	A–C	~ 10	+/−	B, C	< 1
	AY-1981000-005		Morocco	++	B, C	~ 10	++	B, C	~ 20
	AY-1981000-006		Morocco	++	B, C	~ 30	++	B, C	~ 20

+++: Commonly Present, ++: Present, +: Present with low frequency, +/−: Rarely present, −: Absent.
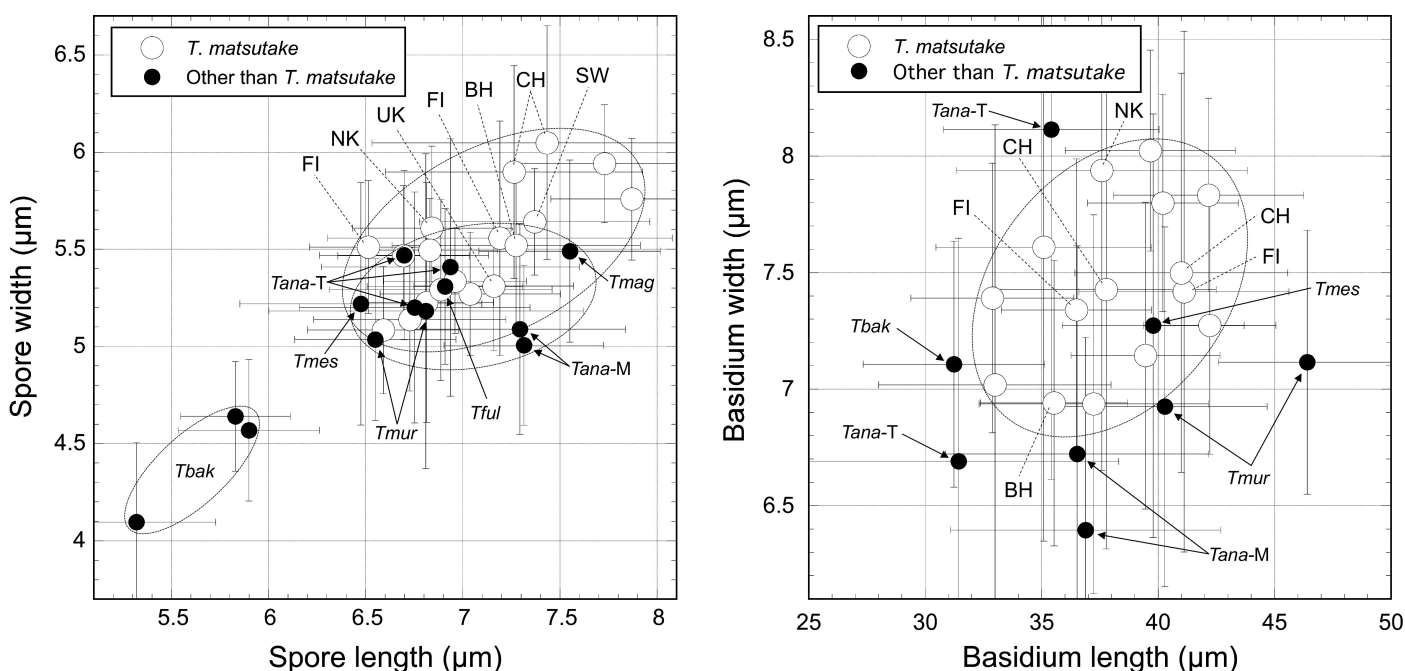

Fig. 7 – Morphological comparisons of basidiospores (left) and basidia (right) between *Tricholoma matsutake* and its closely related species. The open and closed circles show mean values in specimens from *T. matsutake* and other species, respectively. Bars in each plot show the standard deviations. Dotted circle lines show the mean values in *T. matsutake* and other species. Abbreviations: BH: Bhutan; CH: China; FI: Finland; NK: North Korea; SW: Sweden; UK: Ukraine; *Tana*-M: *T. anatolicum* Morocco; *Tana*-T: *T. anatolicum* Turkey; *Tbak*: *T. bakamatsutake*; *Tful*: *T. fulvocastaneum*; *Tmag*: *T magnivelare*; *Tmes*: *T. mesoamericanum*. The detailed data are presented in Supplementary Table S3.
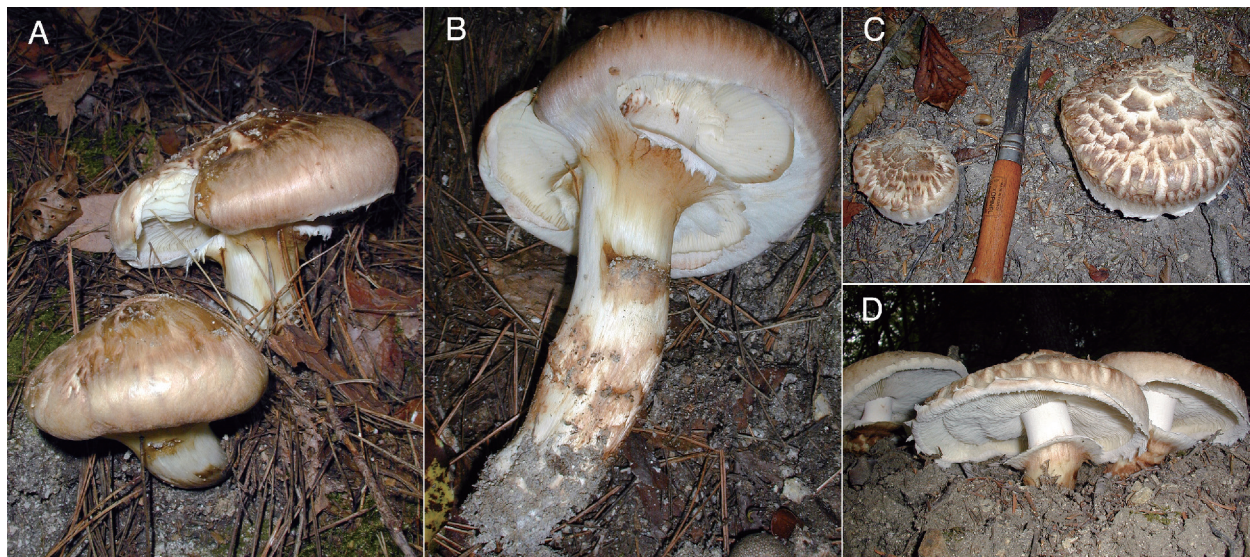
Fig. 8 – External morphology of Japanese *Tricholoma matsutake*. A, B: External morphology of basidiomata specimen AY-2101020-001 showing appressed squamulose on the pileus surface. This specimen may be the same as, or at least a sibling of, the epitype AY-2041007-002 and paratype AY-2181005-001 specimens (Table 1) because they were sampled from almost the same site in a *P. densiflora* stand. C, D: External morphology of basidiomata specimen AY-2101021-002, showing recurved squamulose on the pileus surface. This specimen may be the same as, or at least a sibling of, AY-2041007-001 (Table 1) and another specimen AY-2101021-001 (Trudell et al., 2017) because they were sampled from almost the same site in a *T. sieboldii* stand (Table 1).

connected to stipe with a cottony veil at first, incurved lately. **Stipe** 10–20(–30) × 1.5–3(–5) cm length and width, equal to subclavate, sometimes slightly curved, solid, surface white to pale cream on the upper area than apical annuls, pale yellowish brown or chestnut brown appressed or incurved squamulose below the annuls, annuls single, cottony with pale yellowish brown or chestnut brown in the outer side and whitish in the inner side, permanent (Fig. 8B, D). **Gills** white to pale cream color, medium spaced to crowded, smooth edge, sinuate. **Flesh** white, dense, with specific fragrance similar to cinnamon or the lumber of hinoki cypress. **Basidiospores** (5.8–)6.5–7.1–7.9(–10.3) × (4.1–)5.1–5.5–6.1(–9.6) μm length and width, Q = (1.02)1.19–1.28–1.37(–1.83), subglobose to broadly ellipsoidal, normally thin-walled, most specimens include type A sclerospores with low or moderate frequency, some specimens include type B and C sclerospores with low frequency (Fig. 9A–E). **Basidia** (22.8–)32.9–38.1–42.2(–59.7) × (4.9–)6.9–7.5–8.0(–11.9), clavate, curved at the one-third point from the base, basal clamp connection absent, normally thin-walled, but some Japanese and a North Korean and Bhutanese specimens included type A sclerobasidia, and Swedish, Finnish, and Chinese specimens included type B and type C sclerobasidia (Fig. 9G–M; Table 3). **Sterigmata** mostly 4 in number, (1.5–)1.9–3.3–5.4(–6.3) × (0.9–)1.0–1.5–2.0(–2.1) μm length and basal width, straight or incurved (Fig. 9F). Neither cheilocystidia nor pleurocystidia are observed. **Pileipellis** cutis, superpellis hyphae 23.8–58.8–119.4 × 4.2–9.0–18.2 μm, cylindrical, straight, or slightly curved, wall and intracellular space often show light brownish pigment, no clamp connections, subpellis hyphae are narrower than superpellis hyphae, often slightly curved, almost transparent, sometimes anastomosed with parallel hyphae and showing barrel-shaped swelling on the dolipore septum (Fig. 9N–O).

Epitype examined: Japan, Nagano Prefecture: AY-2041007-002 (= TNS-F 82226, *A. Yamada*).

Other specimens examined: Japan, Nagano Prefecture: AY-2181005-001 (= TNS-F 82227, *W. Aoki*), AY-2041007-001 (= TNS-F 82228, *A. Yamada*), AY-2021027-001 (*T. Sawahata*), AY-2071101-001 (*A. Yamada*), AY-2131013-001 (*N. Endo*); Ibaraki Prefecture: AY-1981023-003 (*A. Yamada*), AY-2051104-001 (*H. Kobayashi*), AY-2051104-002 (*H. Kobayashi*), AY-2051104-003 (*H. Kobayashi*), AY-

2071023-002 (*H. Kobayashi*); North Korea: AY-1981000-003 (*H. Murata*); Finland: AY-2040800-001 (*S. Anttila*), AY-2070925-001 (*L-M. Vaario*); Sweden: Ishida F-0315 (*T.A. Ishida*); Ukraine: AY-2200915-001 (*N. Bergius*); Bhutan: AY-2040400-001(*K. Matsushima*), AY-1981000-002 (*H. Murata)*; China: AY-1980831-001 (*A. Yamada*), AY-1981000-001(*H. Murata*).

GenBank accession numbers of DNA sequences: see Supplementary Table S1.

Ecology: Japanese *T. matsutake* is distributed in temperate to alpine zones on Honshu, Hokkaido, Shikoku, Kyushu, and Sadogashima Islands under various *Pinaceae* trees, including *Pinus densiflora*, *P. thunbergii* Parl., *P. pumila* (Pall.) Regel, *Picea glehnii* (F. Schmidt) Mast., *Abies veitchii* Lindl., *A. sachalinensis* (Fr. Schmidt) Mastersand, *Tsuga diversifolia* (Maxim.) Mast., *T. sieboldii* Carrière, forming ectomycorrhizal associations with these hosts, fruiting (Jul–)Aug–Nov(–Dec), occasionally in Mar–May in warmer coastal areas (Matsutake Research Association, 1964; Ogawa, 1978; Endo et al., 2015; Gisusi et al., 2019). Fruiting events at a stand level are, however, limited to within a few weeks. It is not clear whether five-needle pines other than *P. pumila* (*P. parviflora* var. *parviflora* Siebold et Zucc., *P. parviflora* var. *pentaphylla* (Mayr) A. Henry, and *P. koraiensis* Siebold et Zucc.) are hosts in Japan, although *P. koraiensis* is reportedly a harvest host of *T. matsutake* in northeastern China (Wang, Yu, Zhang, & Li, 2017). In the Nagano area, Japan, *T. matsutake* colonies are sometimes parasitized by the achlorophyllous *Ericaceae* plant *Monotropa hypopitys* L. (Kitamura, 2004; Fujiwara, 2011; Saito et al., 2018), which is a similar plant–fungus association to that between *Allotropa virgata* Torr. & Gray and *T. murrillianum* in the west Pacific region of North America (Bidartondo and Bruns, 2002; Hosford, 1997; Trudell et al., 2017). Geographic regions other than the Japanese Archipelago include Taiwan Island, the Korean Peninsula, southwestern and northeastern China, Bhutan, the Primorskaya Oblast, Sakhalin, the Kuril Islands in East Asia, and eastern, northern, and central Europe, under various coniferous trees. However, in southwestern China and Bhutan, oak trees are also associated with *T. matsutake* defined as the B/C group (e.g., Narimatsu, Terashima, Watanabe, Matsushita, & Penjor, 2019).
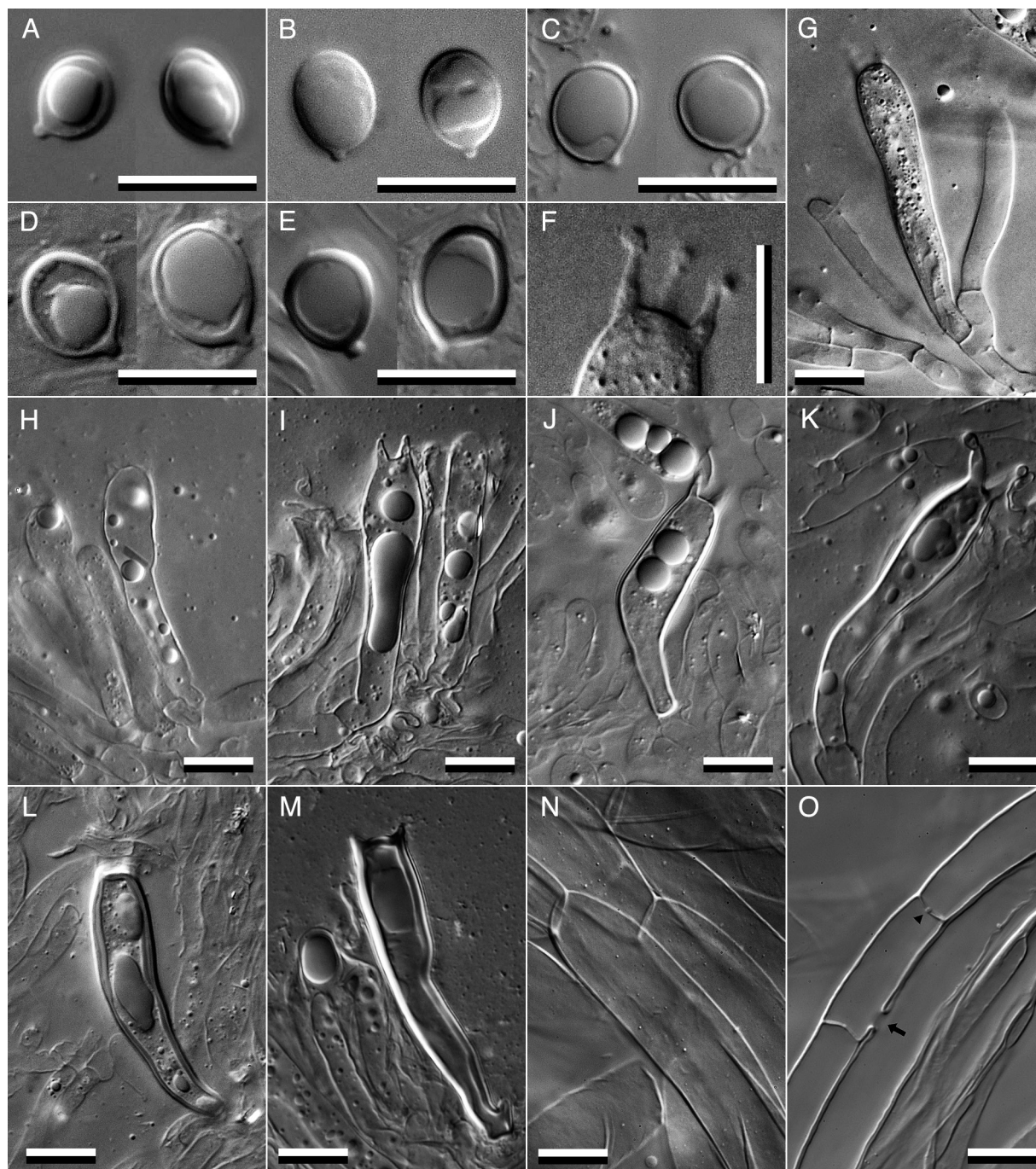
Fig. 9 – Microscopic features of *Tricholoma matsutake*. A–E: basidiospores of AY-2200915-001 (Ukraine; A), epitype AY-2041007-002 (Japan; B), its thick-walled sclerospore type A of AY-2051104-002 (Japan; C), type B of AY-1981000-001 (China; D), and type C of AY-2070925-001 (Finland; E). F: sterigmata of the epitype AY-2041007-002, in which three of four sterigmata on a basidium can be seen in the same depth of field. G–M: Basidia of the epitype AY-2041007-002 (G), its thick-walled sclerobasidium type A of AY-2051104-002 (H) and AY-2070925-001 (I), type B of AY-2051104-002 (J) and AY-2070925-001 (K), and type C of AY-2051104-002 (L) and AY-2070925-001 (M). Superpellis (N) and subpellis (O) hyphae in the pileipellis layers of the epitype AY-2041007-002. The arrow indicates anastomosis between parallel hyphae, and the arrowhead indicates a barrel-shaped swelling on the dolipore septum. *Bars*: 10 μm.

Comment: Our microscopic data on the Asian and European populations of *T. matsutake* matched the descriptions by Kawamura (1930, 1955) and Imazeki and Hongo (1957). In addition, our microscopic data largely matched the *T. nauseosum* description by Kytövuori (1988), except for the reaction of the sclerobasidium and sclerospore walls to Melzer's reagent. Although Kytövuori (1988) reported dextrinoid walls, our DIC microscopy observations revealed both inamyloid walls and intracellular contents. This difference might be attributed to the microscopy technique used, because Kytövuori (1988) conducted normal compound microscopy. We suggest that the dextrinoid walls (of probably thick-walled sclerospores) reported by Kytövuori (1988) might be an invalid interpretation. We did not find a distinct sclerospore or sclerobasidium in most Japanese *T. matsutake* specimens. Based on the phylogenetic data, the variations in sclerospores and paired sclerobasidia can be regarded as infraspecific characteristics of *T. matsutake*.

Morphological distinction between the A/E and B/C groups of *T. matsutake* is almost impossible, although we did find a significant difference in spore width. As there were only a limited number of specimens in the B/C group, additional research is needed to validate such morphological differences. The very similar *T. magnivelare* species distributed in North America can be distinguished from *T. matsutake* by the external color of basidiomata and spore size (Trudell et al., 2017). However, these characteristics may not distinguish the species in some cases due to infraspecific variations. As indicated in Figure 7, the spores of our *T. magnivelare* specimen were larger than the size (5–7.5 × 3.5–5.5 μm) reported by Trudell et al. (2017).

5. Discussion

This is the first valid report of *T. matsutake* from eastern Europe. The finding that Scandinavian *T. nauseosum* is conspecific to the Japanese *T. matsutake* (Kytövuori, 1988) and their phylogenetic congruence (Bergius and Danell, 2000; Matsushita et al., 2005) revised our understanding of this fungal biogeography. The present findings of this mushroom from Ukraine imply that *T. matsutake* is probably widely distributed throughout the Eurasian region from East Asia to Europe via the central region of Eurasia, i.e., circumboreal area in Eurasia, as has already been suggested by Endo et al. (2015) and Vaario et al. (2017). At present, there is no valid report of *T. matsutake* in these areas, such as in the Ural Mountains and Central Siberian Plateau, although its presence has been suggested based on specimen and observation records (GBIF, 2021). The hosts of *T. matsutake* in central and northern Europe include *Pinus sylvestris* L. and *Picea abies* (L.) H. Karst. (Kytövuori, 1988; Vaario et al., 2017). However, the *Abies* association was first found in Ukraine. Even in Japan, the association between *T. matsutake* and *Abies* has not been accepted until recently (Endo et al., 2015; Gisusi et al., 2019), after root associations were confirmed by mycorrhizal analyses. Therefore, surveys of *T. matsutake* habitats under fir forests may be another valuable approach to elucidate their fungal biogeography.

The *rpb2*, *tef-1*, and β₂ tubulin gene phylogenies included the Ukraine *T. matsutake* specimen in the A/E clade (Figs. 4, 5) or European population (Fig. 6), implying the significance of these loci for characterization of geographic origin. In contrast, the mt rDNA SSU phylogeny (Supplementary Fig. S4) revealed the unique and independent position of the Ukrainian specimen. At present, we cannot explain this. As the mt rDNA SSU phylogeny showed a mixed relationship among the four matsutake species *T. matsutake*, *T. magnivelare*, *T. mesoamericanum*, and *T. anatolicum*, this locus may not mirror the speciation and evolutionary processes of these taxa. In this study, the conspecificity of the Ukrainian specimen with Japanese *T. matsutake* was determined by phylogenetic analysis of eight loci (ITS, IGS1, *rpb2*, β₂ tubulin, *gapdh*, *tef-1 atp6,* and *megB1*), strongly implying the taxonomic identity. These results also suggest that describing species based on the phylogeny of a single locus can be problematic for taxonomic study.

In contrast, our phylogenetic analyses showed divergence of the *T. matsutake* population within Asia, i.e., the A/E (far east Asia and Europe) and B/C (Bhutan and China) clades, based on the *rpb2* and *tef-1* phylogenetic analyses (Figs. 4, 5). It has been suggested that Asian *T. matsutake* has two distinct genetic groups, i.e., the far eastern population, including Japan (genotype A), and that at the foot of the Tibetan Plateau (genotype B), based on genomic analysis of the LTR retroelement DNA markers (Murata et al., 2008). Therefore, our phylogenetic data support genetic isolation between these two *T. matsutake* populations in Asia. Of the samples distin-

guished as the B/C clade in this study, specimens AY-1981000-002 and AY-1981000-001 from Bhutan (=BH1) and China (=CH1), respectively, were classified as genotype B by Murata et al. (2008). This implies that another Chinese specimen, AY-1980831-001, was sampled in a western region, such as Yunnan or Szechwan Province, not an eastern region, such as Heilongjiang or Jilin Province. In addition, specimen AY-1981000-003 from North Korea (=NK1), classified as genotype A (referred to as genotype A-2) by Murata et al. (2008), was grouped in the A/E clade (Figs. 4, 5). Most of the Japanese *T. matsutake* specimens analyzed by Murata et al. (2008) were sampled from lowland *P. densiflora* forests and classified as genotype A (referred to as genotype A-1 in the eastern population and as A-2 in several western populations). Therefore, our present *T. matsutake* samples harvested in subalpine *Tsuga* and *Abies* spp. stands as well as *P. densiflora* and sympatric *T. sieboldii* stands, all of which were grouped in the A/E clade, possibly correspond to genotype A-1 proposed by Murata et al. (2008). We suggest that these two genetically isolated *T. matsutake* groups in Asia correspond to two geographically isolated populations, or two incompatible populations, i.e., different biological species. Although we did not find a clear morphological difference between the two groups except in spore width, the reported ecological characteristics, i.e., host relationships, support their divergence from a geographic viewpoint. Japanese *T. matsutake* is associated solely with *Pinaceae* plants in nature (Ogawa, 1978; Yamada, Kanekawa, & Ohmasa,1999a; Endo et al., 2015; Gisusi et al., 2019), which is similar to European populations (Christensen and Heilmann-Clausen, 2013; Vaario et al., 2017) and has been largely confirmed by *in vitro* mycorrhizal synthesis experiments (Yamada, Maeda, & Ohmasa, 1999b; Yamada, Maeda, Kobayashi, & Murata, 2005; Vaario, Pennanen, Sarjala, Savonen, & Heinonsalo, 2010; Yamada et al., 2010; Saito et al., 2018). However, *T. matsutake* in Bhutan and southwestern China, such as in Yunnan and Sichuan Provinces, have been associated with both *Pinaceae* (e.g., *Pinus wallichiana* A. B. Jacks., *P. yunnanensis* Franch., *Picea*, and *Tsuga*) and *Fagaceae* (e.g., *Quercus*, *Lithocarpus*, and *Castanopsis*) (Cao, Yao, & Pegler, 2003; Yamanaka, Aimi, Wan, Cao, & Chen, 2011; Vaario et al., 2017; Wang et al., 2017; Narimatsu et al., 2019). Therefore, from a genetic viewpoint, the two diverged *T. matsutake* populations in Asia can be characterized based on host associations. In this respect, we need to consider another *T. matsutake*-related population (*T. zangii* Z. M. Cao, Y. J. Yao & Pegler, distributed at the foot of the Tibetan Plateau (Cao et al., 2003; Cao and Yao, 2004)). The geographic distribution of this fungus overlaps that of *T. matsutake*, but the habitats differ due to the higher elevation of *T. matsutake*. However, phylogenetic analysis of *T. zangii* has not been reported, which would allow verification of its position in relation to closely related species (Bao et al., 2007; Amend, Garbelotto, Fang, & Keeley, 2010; Zeng and Chen, 2015). As we did not analyze a *T. zangii* specimen in the present study, we did not conduct taxonomic treatment in the B/C clade of *T. matsutake*.

In this study, we set the epitype of *T. matsutake* because biologically available type specimens have not been designated in Japan since Kawamura (1913) identified "*Matsutake*" as *Cortinellus edodes* P. Henn. and described it with color drawings and a brief description. A detailed description of this fungus with microscopic features was provided by Seiichi Kawamura based on basidiomata sampled under a *P. densiflora* stand in the Kitayama area of Kyoto in November 1909 (Kawamura, 1930, 1955), which might have been the motif of the color drawings of *C. edodes* by Kawamura (1913). The basidioma specimens of *Armillaria matsutake* by Kawamura (1930, 1955) are also not extant. As the color drawings by Kawamura (1930, 1955) show the basidioma pileus as a reddish

tan color, the specimen can be regarded as not fresh; Kawamura (1955) commented that the specimens consisted of "one young and two overmatured basidiomata." Fresh basidiomata of *T. matsutake* are lighter in color, as reported by Imazeki and Hongo (1957), Imazeki, Otani, and Hongo (1988), Kytövuori (1988), and Christensen and Heilmann-Clausen (2013). Currently, *T. matsutake* habitats in Japan are endangered in western and lowland areas, including Kyoto, due to the decline of *Pinus densiflora*, the main host, in lowland areas caused by nematode disease and ongoing global warming (Vaario et al., 2017; Brandrud, 2020; Yamanaka et al., 2020). Therefore, we decided to designate an epitype of *T. matsutake* as that sampled in Nagano Prefecture, where the mountain ranges still harbor large forest areas of *P. densiflora* at mid-elevations, as well as the other hosts, *Tsuga sieboldii*, *T. diversifolia*, and *Abies veitchii*, at higher elevations. The designated epitype, AY-2041007-002 (= TNS-F 82226), was sampled from a *P. densiflora* forest associated with many productive sites of this fungus (Furukawa, Masuno, & Takeuchi, 2016). The specimen is thought to be a typical genetic resource in the diverse genetic pool of this fungus in Japan. The specimen AY-2181005-001 (= TNS-F 82227) is probably a sibling of (or of the same clone as) the epitype, AY-2041007-002, because the two specimens were sampled from the same site in a *P. densiflora* stand.

The *T. bakamatsutake* specimen AY-2080800-001 from Jilin Province, China, labeled as *T. matsutake*, was identified as *T. bakamatsutake* based on phylogenetic data. In fact, similar cases were found in traceability tests of *T. matsutake* imported from abroad (Murata et al., 2008), from which large and typical basidiomata were selected for DNA analysis, but small or atypical basidiomata were excluded. In fact, some samples corresponding to the latter case were identified as *T. bakamatsutake* by ITS sequence analysis (unpublished data). *Tricholoma bakamatsutake* has been identified in northeastern China and the neighboring Primorskaya Oblast in Russia under *Quercus mongolica* (Ogawa, 1978) trees, as well as in Japan under various Fagaceae trees (Hongo, 1971; Terashima, 1996). Therefore, if basidiomata of *T. matsutake* and *T. bakamatsutake* occur sympatrically in the same forest, such as in mixed *Pinus–Quercus* stands, mushroom harvesters may collect the two together. Our field research in Nagano Prefecture showed such coexistence in *P. densiflora–Q. serrata* and *T. sieboldii–Q. serrata* mixed stands, where basidiomata of both species occurred only a few meters apart (unpublished data). Although we did not analyze *T. bakamatsutake* specimens collected in southwestern China, these regions potentially have at least four matsutake mushroom species associated with *Fagaceae*: *T. matsutake* (B/C clade), *T. zangii*, *T. bakamatsutake*, and *T. fulvocastaneum* (Liu, Yuan, Wang, Sun, & Yang, 1999; Cao et al., 2003; Cao and Yang, 2004; Sanmee, Lumyong, Dell, & Lumyong, 2007; Yamada et al., 2010; Yamanaka et al., 2011) or more diverse taxa (Wang et al., 2017). Matsutake may be difficult to identify in the market, even in North America. At present, three species (*T. murrillianum*, *T. magnivelare*, and *T. mesoamericanum*) are exported to Japan. In addition, the presence of North American *T. dulciolens*, *T. focale*, and "*T. caligatum*" has been reported (Chapela and Garbelotto, 2004; Bessette, Bessette, Roody, & Trudell, 2013; Murata et al., 2013; Benazza-Bouregba, Savoie, Fortas, & Billette, 2016; Heilmann-Clausen, Christensen, Frøslev, & Kjøller, 2017). This suggests that these species may be mixed in the exported American matsutake, as is the case of *T. bakamatsutake* presence in exported Chinese matsutake.

Disclosure

The authors declare no conflicts of interest. All experiments in this study were performed in compliance with the current laws of Japan.

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