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FULL PAPER

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Genetic relationship of *Tricholoma matsutake* and *T. nauseosum* from the Northern Hemisphere based on analyses of ribosomal DNA spacer regions

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Abstract The genetic relationship among *Tricholoma matsutake* and *T. nauseosum* strains collected from various parts of the Northern Hemisphere was investigated using sequence analysis of the rDNA ITS region and PCR-RFLP analysis of the rDNA IGS-1 region. ITS sequence similarity between *T. matsutake* and *T. nauseosum* ranged between 98.1% and 100%. The strains of *T. matsutake* from coniferous forests and those from broad-leaved forests showed more than 99.8% similarity in their ITS sequences. Three distinct RFLP types were detected when IGS-1 regions were digested with *Cfr*13I. RFLP patterns showed no variability among the strains of *T. nauseosum* and those of *T. matsutake* from broad-leaved forests. This pattern corresponded to the dominant RFLP type in the Japanese population of *T. matsutake*. Thus, strains belonging to this RFLP type are widely distributed throughout East Asia and Europe and associated with many tree species of Pinaceae and Fagaceae. The result suggests that *T. matsutake* in coniferous and broad-leaved forests and *T. nauseosum* should be treated as the same species genetically.

Key words Ectomycorrhizal fungi · Intergenic spacer (IGS) · Internal transcribed spacer (ITS) · *Tricholoma matsutake* · *Tricholoma nauseosum*

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Introduction

Tricholoma matsutake (S. Ito & Imai) Sing. is one of the most economically important ectomycorrhizal mushrooms in the world. In the early 1940s, about 12000 tons of *T. matsutake* were harvested each year in Japan, but since then production has dramatically decreased to less than 100 tons per year. In recent years, more than 2000 tons of *T. matsutake* or closely related species are imported annually, mainly from Bhutan, Canada, China, Korea, Mexico, Morocco, Turkey, and the United States (Japan Tariff Association 1994–2003; Suzuki 2005).

Among closely related species, *T. bakamatsutake* Hongo, *T. caligatum* (Viv.) Ricken, and *T. magnivelare* (Peck) Redhead are clearly distinguished from *T. matsutake* by morphological features (Hosford et al. 1997; Wang et al. 1997). Ito and Imai (1925) reported that *T. matsutake* (syn. *Armillaria matsutake*) and *T. caligatum* (syn. *A. caligata*) are quite different in the form of stipe base, i.e., the stipe of *T. matsutake* is enlarged and that of *T. caligatum* is tapered downward. However, the characteristics of stipe base could not be differentiated between *T. matsutake* and *T. nauseosum* because the stipe of *T. nauseosum* varies from tapered downward to enlarged (Kytövuori 1988). According to morphological studies with 139 specimens from Europe, North Africa, and Japan, Kytövuori (1988) proposed that *T. matsutake* was identical to *T. nauseosum*. Because the sequences of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) region were 98%–99% matched between *T. nauseosum* and *T. matsutake*, they were considered to be the same species (Bergius and Danell 2000). However, only four strains of *T. nauseosum* from Sweden and one strain of *T. matsutake* from Japan were examined for sequencing. It seems to be too small a number to examine among the many strains throughout Japan.

Table 1. Nucleotide sequence length of the internal transcribed spacer (ITS) region and restriction fragment sizes of the amplified IGS-1 region by *Cfr*13I of *Tricholoma matsutake* and *T. nauseosum* strains analyzed

Strain[a]	Geographical origin		Sample[b]	Dominant host tree[c]	ITS[d]			IGS-1	
	Locality	Country			Accession no.	Length (bp)	Reference[e]	RFLP type[f]	Fragment size (bp)
T. matsutake									
TmK1	Yangyang	Korea	f	*Pinus densiflora*	AB 188533	634	–	C	348, 215, 133, 63, 45
TmC1	Jiaohe, Jilin	China	f	*Pinus densiflora*	AB 188534	634	–	B	348, 63, 45
TmC2	Fuyu, Jilin	China	f	*Pinus densiflora*	AB 188535	634	–	A	215, 133, 63, 45
TmC3	Antu, Jilin	China	f	*Pinus densiflora*	AB 188536	634	–	C	348, 215, 133, 63, 45
TmC4	Wangqing, Jilin	China	f	*Pinus densiflora*	–	–	–	A	215, 133, 63, 45
TmY1	Lufeng, Yunnan	China	m	*Castanopsis orthacantha*	AB 188537	634	–	A	215, 133, 63, 45
TmY2	Lufeng, Yunnan	China	m	*Castanopsis orthacantha*	AB 188538	634	–	A	215, 133, 63, 45
TmY3	Lijiang, Yunnan	China	m	*Quercus* spp.	AB 188539	634	–	A	215, 133, 63, 45
TmY4	Lijiang, Yunnan	China	m	*Quercus* spp.	AB 188540	634	–	A	215, 133, 63, 45
TmY5	Lijiang, Yunnan	China	m	*Quercus* spp.	AB 188541	634	–	A	215, 133, 63, 45
TmY6	Lijiang, Yunnan	China	m	*Pinus yunnanensis*	AB 188542	634	–	A	215, 133, 63, 45
TmY7	Lijiang, Yunnan	China	m	*Pinus yunnanensis*	AB 188543	634	–	A	215, 133, 63, 45
TmY8	Lijiang, Yunnan	China	m	*Pinus yunnanensis*	AB 188544	634	–	A	215, 133, 63, 45
TmY9	Lijiang, Yunnan	China	m	*Pinus yunnanensis*	AB 188545	634	–	A	215, 133, 63, 45
TmY10	Zhongdian, Yunnan	China	m	*Quercus pannosa*	AB 188546	634	–	A	215, 133, 63, 45
TmY11	Zhongdian, Yunnan	China	m	*Quercus pannosa*	AB 188547	634	–	A	215, 133, 63, 45
TmY12	Zhongdian, Yunnan	China	m	*Quercus pannosa*	AB 188548	634	–	A	215, 133, 63, 45
Tm1	Ina, Nagano	Japan	–	*Pinus densiflora*	AF 204868	634	K	–	–
Tm4	Iwaizumi, Iwate	Japan	–	*Pinus densiflora*	–	634	K	–	–
Tm33	Minakuchi, Shiga	Japan	–	*Pinus densiflora*	AF 204806	634	K	–	–
Tm0945	Miyazu, Kyoto	Japan	–	*Pinus densiflora*	–	634	K	–	–
TmA-5	Kake, Hiroshima	Japan	–	*Pinus densiflora*	AF 202772	634	K	–	–
T. nauseosum									
Tn1	Valtellina	Italy	f	*Picea abies*	AB 188549	634	–	A	215, 133, 63, 45
Tn2	Lario	Italy	f	*Picea abies*	AB 188550	634	–	A	215, 133, 63, 45
Tn3	Varese	Italy	f	*Picea abies*	AB 188551	634	–	A	215, 133, 63, 45
Tn4	Grognardo	Italy	f	*Castanea setiva*	AB 188552	634	–	A	215, 133, 63, 45
Tn5	Val Gerola	Italy	f	*Picea abies*	AB 188553	634	–	A	215, 133, 63, 45
Tn6	Ticino	Switzerland	f	*Picea abies*	AB 188554	634	–	A	215, 133, 63, 45
Tn7	unknown	Sweden	f	*Pinus sylvestris*	AB 188555	634	–	A	215, 133, 63, 45
Tn8	unknown	Sweden	f	*Pinus sylvestris*	AB 188556	634	–	A	215, 133, 63, 45
Tn9	unknown	Sweden	f	*Pinus sylvestris*	AB 188557	634	–	A	215, 133, 63, 45
F-013392	Uppsala	Sweden	–	*Pinus sylvestris*	AF 145447	637	B	–	–
F-013393	Arjeplog	Sweden	–	*Pinus sylvestris*	AF 145448	636	B	–	–
F-013394	Vagnhärad	Sweden	–	*Pinus sylvestris*	AF 145446	635	B	–	–
F-013395	Kiruna	Sweden	–	*Pinus sylvestris*	AF 145449	639	B	–	–

[a] Species names were according to collectors
[b] DNA was extracted from hyphae sampled directly from the fruiting body (f) or from pure cultured mycelium (m)
[c] Dominant tree(s) at the collection site
[d] TmC4 was not used for ITS sequence analysis
[e] B, published by Bergius and Danell (2000); K, published by Kikuchi et al. (2000); –, determined in this study
[f] Published by Guerin-Laguette et al. (2002)

T. matsutake grows widely in Japanese red pine (*Pinus densiflora* Sieb. & Zucc.) forests throughout Japan, Korea, and northeastern China (Hosford et al. 1997) and forms ectomycorrhizae on *P. densiflora* (Gill et al. 1999, 2000; Yamada et al. 1999). *T. matsutake* is also associated with coniferous trees such as *P. thunbergii* Parl., *P. pumila* Regel, *Picea glehnii* Mast., *Tsuga sieboldii* Carr., and *T. diversifolia* Mast. in Japan (Imazeki and Hongo 1987). *T. nauseosum* is primarily associated with *Pinus sylvestris* L. (Bergius and Danell 2000). However, *T. matsutake* in southwestern China grows in broad-leaved forests mainly consisting of *Castanopsis* spp. and *Quercus* spp. (Wang et al. 1997; Matsushita et al. 2004). There is still the question of whether *T. matsutake* in coniferous forests, *T. matsutake* in broad-leaved forests, and *T. nauseosum* are conspecific.

rDNA regions are generally informative for species and genera differentiation of fungi (Hibbett 1992). The ITS regions often vary at the intergeneric and interspecific level (Gardes and Bruns 1993; Karen et al. 1997), whereas the intergenic spacer (IGS) regions vary significantly up to the intraspecific level (Bruns et al. 1991). Polymorphisms of the ITS and the first IGS (IGS-1) regions of *T. matsutake* in Japan have been investigated (Guerin-Laguette et al. 2002; Kikuchi et al. 2000), and distinct ribotypes were identified within the species.

The objective of this study was to investigate the genetic relationship among *T. matsutake* and *T. nauseosum* strains collected from various parts of the Northern Hemisphere using sequence analysis of the ITS region and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the IGS-1 region.

Materials and methods

Fungal material and DNA extraction

Seventeen strains of *T. matsutake* and 9 strains of *T. nauseosum* were used in this study (Table 1). DNA was extracted using a DNeasy Plant kit (Qiagen, Hilden, Germany) either from fragments (~50 mm^3) of fresh mycelia grown on Ohta's agar medium (Ohta 1990), or from fragments (~100 mm^3) of air-dried or freeze-dried fruiting body (Guerin-Laguette et al. 2002). DNA extracts were diluted 5- or 25 fold in sterile distilled water to be used as a template for PCR.

Sequencing and RFLP analysis

The primer pair ITS5/ITS4 was used to amplify the ITS region (White et al. 1990), and the primer pair 5SA/CNL12 was used to amplify the IGS-1 region (Henrion et al. 1992). The concentration of components in PCR mixtures was 50 μM of each deoxynucleoside triphosphate (dNTP) (Applied Biosystems), 0.2 μM of each primer (Kurabo, Osaka, Japan), 1 unit of *Taq* polymerase, and 1× PCR buffer supplied by the manufacturer (Perkin Elmer,

Table 2. Sequence similarities of rDNA ITS region between the *T. matsutake* and *T. nauseosum*

Strain	Sequence similarity									
	T. matsutake					*T. nauseosum*				
	TmK1	TmC1, TmC3	TmC2	Tm1, Tm4, Tm33, Tm0945, TmA-5	TmY1, Y2, Y3, Y4, Y5, Y6, Y7, Y8, Y9 Y10, Y11, Y12	Tn1, Tn2, Tn3, Tn4Tn5, Tn6, Tn7	Tn8, Tn9	F-013392	F-013393	F-013394	F-013395
TmK1	–	639/640	638/640	639/640	639/640	638/640	639/640	634/640	635/640	637/640	628/640
TmC1, TmC3	99.8%	–	638/640	639/640	639/640	638/640	639/640	634/640	635/640	637/640	628/640
TmC2	99.7%	99.7%	–	639/640	639/640	638/640	639/640	634/640	635/640	637/640	629/640
Tm1, Tm4, Tm33, Tm0945, TmA-5	99.8%	99.8%	99.8%	–	640/640	639/640	640/640	635/640	636/640	638/640	629/640
TmY1, Y2, Y3, Y4, Y5, Y6, Y7, Y8, Y9, Y10, Y11, Y12	99.8%	99.8%	99.8%	100.0%	–	639/640	640/640	635/640	636/640	638/640	629/640
Tn1, Tn2, Tn3, Tn4 Tn5, Tn6, Tn7	99.7%	99.7%	99.7%	99.8%	99.8%	–	639/640	636/640	636/640	637/640	630/640
Tn8, Tn9	99.8%	99.8%	99.8%	100.0%	100.0%	99.8%	–	635/640	636/640	638/640	629/640
F-013392	99.1%	99.1%	99.1%	99.2%	99.2%	99.4%	99.2%	–	637/640	636/640	631/640
F-013393	99.2%	99.2%	99.2%	99.4%	99.4%	99.4%	99.4%	99.5%	–	637/640	630/640
F-013394	99.5%	99.5%	99.5%	99.7%	99.7%	99.5%	99.7%	99.4%	99.5%	–	630/640
F-013395	98.1%	98.1%	98.3%	98.3%	98.3%	98.4%	98.3%	98.6%	98.4%	98.4%	–

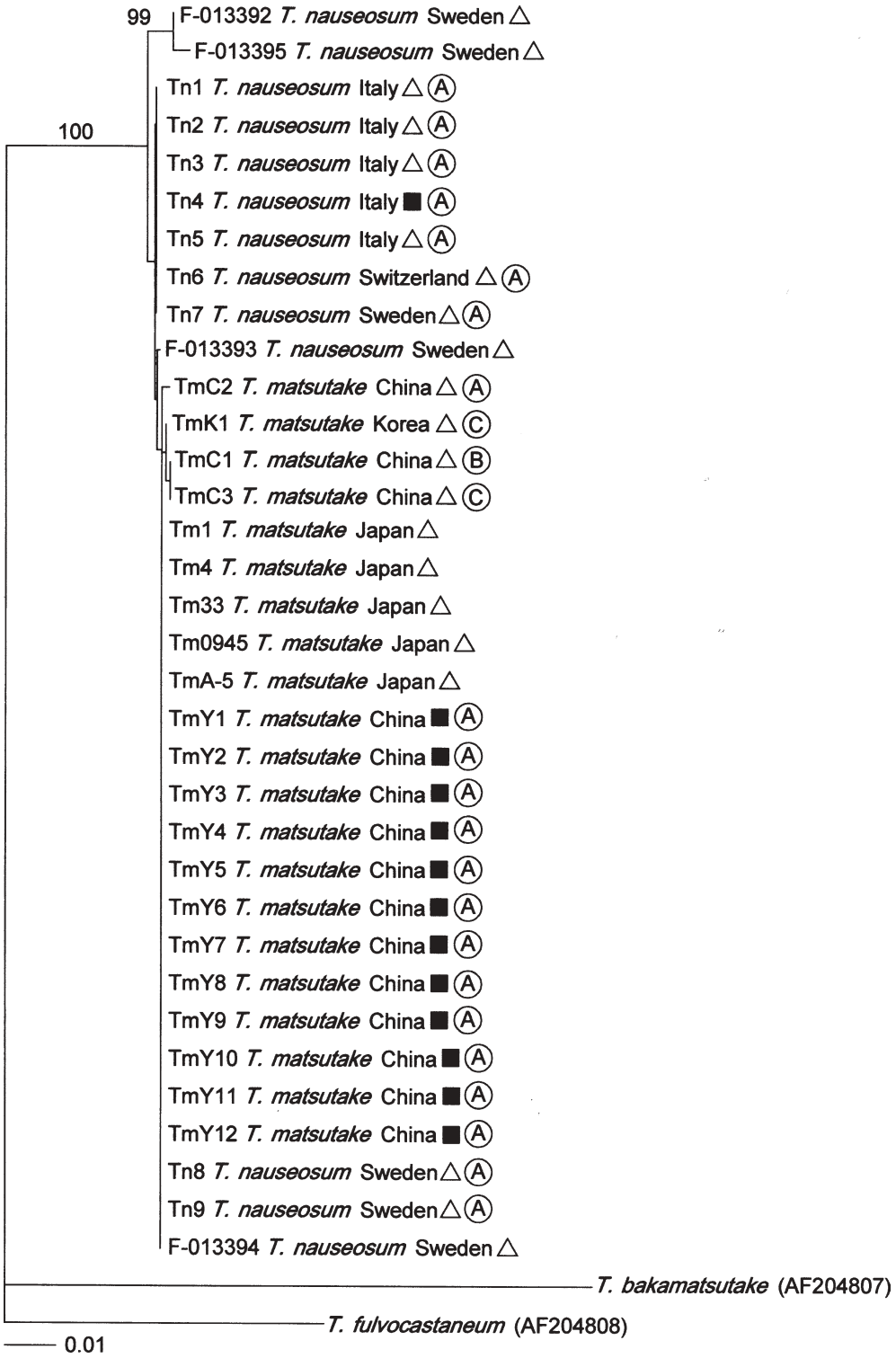

Fig. 1. Neighbor-joining (NJ) phylogenetic dendrogram of *Tricholoma matsutake* and *T. nauseosum* based on internal transcribed spacer (ITS) sequences. *T. bakamatsutake* and *T. fulvocastaneum* were used as an outgroup. *Numerical values on branches* are the bootstrap values as percentage bootstrap replication from a 1000-replicate analysis. *Bar* 0.01 genetic distance between strains; *triangles*, strains from coniferous forests; *squares*, strains from broad-leaved forests; *encircled letters*, intergenic spacer (IGS)-1 restriction fragment length polymorphism (RFLP) type

Norwalk, USA). Reactions were run in a Perkin Elmer GenAmp 9700 thermocycler under the conditions described by Kraigher et al. (1995). A negative control (no DNA template) was used in each experiment to test for the presence of DNA contamination in reaction mixtures.

Sequencing was performed directly on purified PCR products using an automated fluorescent DNA sequencer SQ-5500L (Hitachi, Tokyo, Japan) according to the sequencer manufacturer's instructions. Both strands of the ITS region were sequenced using Texas Red labeled primers ITS3, ITS4, and ITS5. DNA sequences obtained in this study have been deposited in the GenBank database, and the accession numbers are shown in Table 1. The sequence data were aligned using Clustal X (Thompson et al.

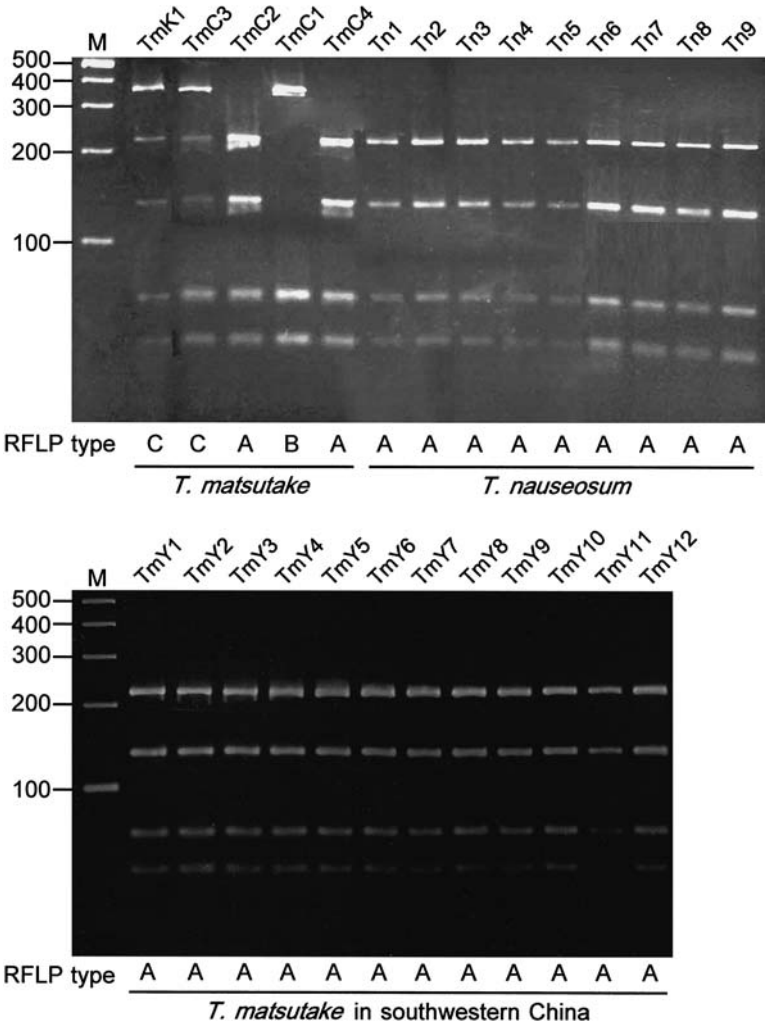

Fig. 2. Restriction fragment patterns of the polymerase chain reaction (PCR)-amplified IGS-1 region of *Tricholoma matsutake* and *T. nauseosum* with the restriction endonuclease *Cfr*13I. *Lane M*, fragment size marker

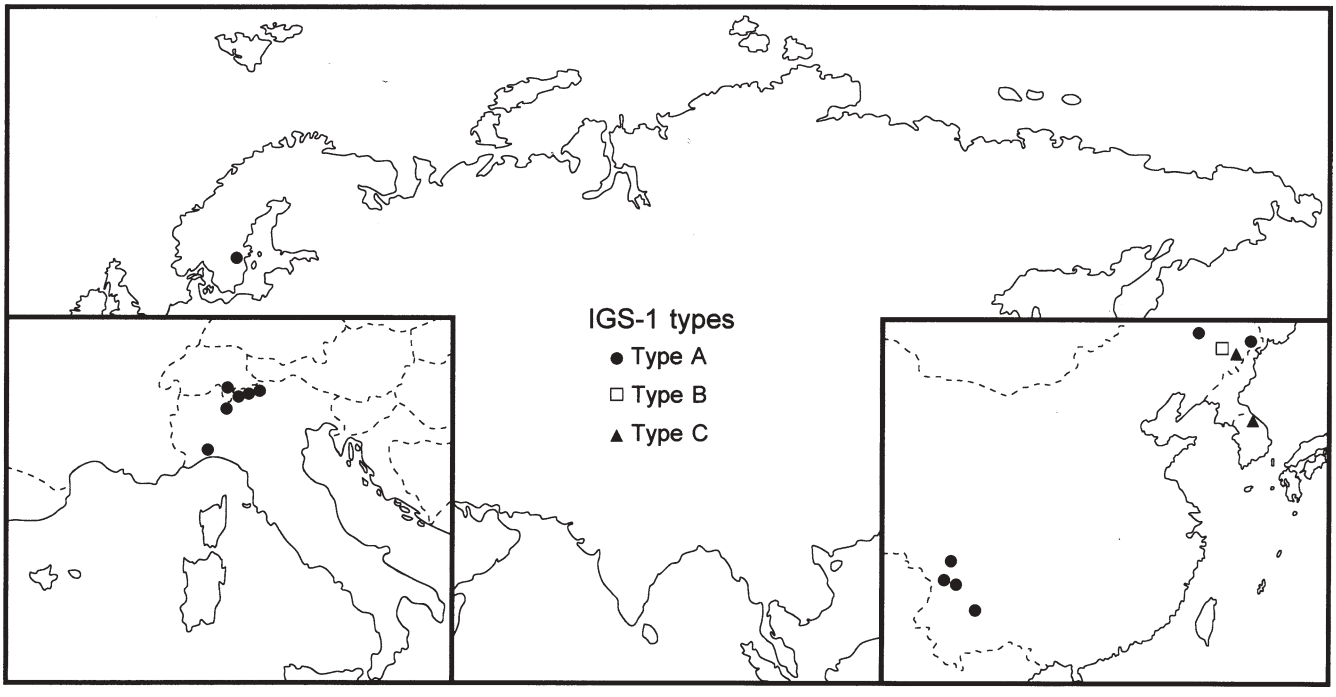

Fig. 3. Distribution of IGS-1 RFLP types of *Tricholoma matsutake* and *T. nauseosum* in the Northern Hemisphere

1997), and phylogenetic analyses were performed using neighbor joining (NJ) of PHYLIP systems (Felsenstein 1993). ITS sequences of *T. bakamatsutake* (GenBank: AF204807) and *T. fulvocastaneum* (GenBank: AF204808) (Kikuchi et al. 2000) were included as outgroup species. Bootstrap values were calculated by 1000 replications.

PCR products of the IGS-1 region were digested overnight with 2.5 units of endonuclease *Cfr* 13I (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The restriction fragments were separated by electrophoresis on 3% high resolution (Sigma-Aldrich, Missouri, USA) agarose gels. Electrophoreses were carried out in 0.5× Tris-borate + EDTA (TBE) for 2–4 h at 4 V cm^{-1}. The gels were stained with ethidium bromide and photographed with an EDAS 290 system (Kodak, New York, USA) using the program 1D Image Analysis Software (Kodak). The 100-bp DNA Ladder (Takara, Bio) was used as a size standard.

Results and discussion

Sequence analysis of the ITS region

The ITS region including 5.8S rDNA was completely sequenced in both directions. These sequences and those of five strains of *T. matsutake* from Japan (Kikuchi et al. 2000) and four strains of *T. nauseosum* from Sweden (Bergius and Danell 2000), ranging from 634 to 639 bases (see Table 1), were aligned by inserting gaps. A total alignment of 640 bases was obtained and used in the comparisons among strains. Of the 640 aligned bases, 12 sites in ITS1, 1 in 5.8S rDNA, and 2 in ITS2 were variable.

The dissimilarities within the *T. matsutake* from Japan, Korea, and northeastern China were less than 0.3%, and those within the *T. nauseosum* from Italy, Switzerland, and Sweden were less than 1.7% (Table 2). *T. matsutake* and *T. nauseosum* were more than 98.1% similar in their ITS sequences. In the NJ tree constructed from the ITS and 5.8S rDNA regions, *T. matsutake* and *T. nauseosum* formed a single genetic clade (Fig. 1). These results suggest that *T. matsutake* and *T. nauseosum* are genetically conspecific, in concurrence with Bergius and Danell (2000).

The sequences of all strains from southwestern China were identical. Sequence similarity between the strains of *T. matsutake* from *P. densiflora* forests in Japan, Korea, and northeastern China and those from broad-leaved forests in southwestern China ranged between 99.8% and 100% (Table 2). The NJ tree constructed from the ITS region showed that *T. matsutake* from coniferous forests and those from broad-leaved forests formed a single genetic clade (see Fig. 1). Results from the sequence data of the ITS region suggest that strains of *T. matsutake* in broad-leaved forests are genetically the same species as those in coniferous forests. However, we should investigate the morphological characteristics of *T. matsutake* in broad-leaved forests, the ability of *T. matsutake* to form ectomycorrhizae with broad-leaved trees, and the compatibility between strains from broad-leaved forests and those from coniferous forests to answer the question of whether *T. matsutake* strains in broad-leaved forests and those in coniferous forests are conspecific.

PCR-RFLP analysis of the IGS-1 region

Amplified products of the IGS-1 region were ~460 bp for all strains of *T. matsutake* and *T. nauseosum* used in this study. The endonuclease *Cfr*13I was used to examine restriction sites within the amplified IGS-1. As enzyme *Cfr*13I produced the highest sequence polymorphisms in Japanese *T. matsutake* (Guerin-Laguette et al. 2002), this enzyme was used for the present study. Three different patterns were found among five strains of *T. matsutake* from *P. densiflora* forests in Korea and northeastern China (Fig. 2). These digestion patterns corresponded to IGS-1 types A, B, and C of Guerin-Laguette et al. (2002), which they observed in Japanese *T. matsutake* (see Table 1). Type C was regarded as a combination of types A and B by cloning and sequencing of the PCR products (Guerin-Laguette et al. 2002). *Cfr*13I digestion patterns showed no variability among strains of *T. nauseosum* and *T. matsutake* in southwestern China, and this pattern was identical with IGS-1 type A (Fig. 2).

In Japan, eight distinct IGS-1 rDNA types were identified using the restriction endonuclease *Cfr*13I (Guerin-Laguette et al. 2002). Among these types, the strains belonging to type A were by far the most frequent and found throughout Japan. In the present study, type A was detected in strains of the *T. matsutake* from China and those of the *T. nauseosum* from Italy, Switzerland, and Sweden (Fig. 3). The strains displaying type A could be collected under the five host tree species of Pinaceae in Japan (Guerin-Laguette et al. 2002) and the seven host tree species of Pinaceae and Fagaceae in China and Europe (see Table 1). These results indicate that the strains belonging to type A are widely distributed throughout East Asia and Europe and are associated with many species of Pinaceae and Fagaceae. Types B and C were only found in the southern part of Japan (Guerin-Laguette et al. 2002), Korea, and northeastern China (Fig. 3). This observation suggests that the genetic diversity of *T. matsutake* may be high in these regions.

There was little sequence variation in the ITS region between *T. nauseosum* and *T. matsutake*, and the IGS-1 RFLP type of *T. nauseosum* was identical to that of *T. matsutake*. These results show that *T. matsutake* and *T. nauseosum* should be treated as the same species genetically. Furthermore, we should investigate the compatibility of the two species to confirm whether the two species are biologically conspecific.

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