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Analyses of the genetic diversity of matsutake isolates collected from different ecological environments in Asia

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Abstract Matsutake mushrooms are able to grow in two kinds of ecological environments: coniferous forests and broadleaf forests. Japanese matsutake is a form of the matsutake mushroom that mainly appears in coniferous forests in the mid-latitude regions of Asia. In contrast, matsutake from Yunnan and Sichuan Provinces of China grows in broadleaf forests in low-latitude regions. The taxonomic relationship and the genetic distance between these two types of matsutake remain unclear. Here, we compared the random amplification of polymorphic DNA (RAPD) data and the sequences of the internal transcribed spacer (ITS) region and of variable domains V4, V6, and V9 of mitochondrial small subunit ribosomal DNA (mt SSU rDNA) to determine the genetic differences of *Tricholoma matsutake* strains collected from different ecological and geographic environments in Asia. We found few differences in the sequences of the variable domains, implying that all matsutake strains are the same biological species regardless of their host specificity and geographical differences. This conclusion is also supported by the sequences of the ITS region. On the other hand, RAPD analysis revealed that the matsutake mushrooms collected from different ecological environments have genetic differences and represent distinct varieties.

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Introduction

Matsutake mushrooms include some related forms of *Tricholoma* spp. All have the characteristic shape, taste, and smell of matsutake. The main species in Asia is *Tricholoma matsutake*. Other matsutake mushrooms include *Tricholoma magnivelare* (North America), *Tricholoma caligatum* (Europe), *Tricholoma nauseosum* (Sweden), *Tricholoma bakamatsutake* (Asia), *Tricholoma robustum* (Asia), and *Tricholoma zangii* (China).

Although matsutake mushrooms have a high commercial value and a very important role in nontimber forest products, the systematic relationships among these species remain unclear. With the accumulation of ecological knowledge and the development of phylogenetic analysis based on DNA techniques, the traditional taxonomy of *T. matsutake* and their related species has come into question. According to DNA sequence analyses, Bergius and Danell[1] suggested that Swedish (*T. nauseosum*) and Japanese matsutake (*T. matsutake*) should be considered the same species.[1,16] Also, Chapela and Garbelotto[2] revealed that the matsutake mushrooms in Asia, Europe, and eastern North America show little genetic difference, and they suggested that the traditional continental/provenance-based taxonomy of *T. matsutake*, *T. magnivelare*, *T. caligatum*, and *T. nauseosum* is incorrect. The current question is whether *T. matsutake* isolates derived from broadleaf forests are conspecific with those from coniferous forests.

Tricholoma matsutake is found in mid-latitude (Japan, Korean Peninsula, and Jilin Province of China) and low-latitude (Sichuan and Yunnan Provinces of China) regions of Asia. Many investigations have shown that, in mid-latitude regions, only coniferous trees act as hosts of *T. matsutake*; however, at low latitudes, both coniferous and broadleaf trees can be hosts for *T. matsutake*. Liao et al.[3] reported that Sichuan matsutake, which is found only in

Xiaojin County of Sichuan Province, China, mainly grows on the evergreen brush of *Quercus aquifolioides* and *Quercus pannosa*. Furthermore, studies by Xian[4] and Tang and Xian[5] showed that there are three types of vegetation that can act as hosts for Sichuan matsutake: a pure oak forest, a mixed forest of oak and pine, and a mixed forest of *Castanopsis orthacante* and *Lithocarpus rasiolozus*. Shu[6] reported that *T. matsutake* growing in Yunnan Province of southern China appears in pure forests of *Quercus* spp., pure forests of pine, and mixed forests of *Pinus* spp. and *Quercus* spp. Clearly, the incompatibility relationships and the genetic distances between Yunnan, Sichuan, and Japanese matsutakes remain to be determined.

Characterization of the incompatibility relationships should clarify the taxonomy of *T. matsutake* isolates from different ecological and geographic environments. The mating test is often used to study the incompatibility of basidiomycetes, but it is very difficult to apply to matsutake mushrooms because of technical obstacles, such as difficulties in spore germination and the lack of an indicator of a clamp connection for dikaryotic mycelia. For these reasons, DNA sequencing should be useful for elucidating the taxonomic relationships between *T. matsutake* growing in different ecological environments.

Different DNA techniques have been successfully used to determine the genetic differences within and among basidiomycetes. Gonzalez and Labarere[7] found that the variable domains V4, V6, and V9 of the mitochondrial small subunit ribosomal DNA (mt SSU rDNA) were conserved within species, but show a high degree of interspecies variation within the genus *Agrocybe*. They suggested that these variable domains be used as species-specific markers for the basidiomycetes. Wu et al.[8] used nuclear ITS sequences to determine the phylogenetic relationship of *Suillus* species and further analyze their biogeographic distribution. Also, Zervakis et al.[9] used the random amplification of polymorphic DNA (RAPD) technique to successfully demonstrate that the *Pleurotus eryngii* species complex contains five clusters, in agreement with their host specificity. Zervakis et al.[10] further showed that the different geographic populations of the *Pleurotus cystidiosus* group have significant genetic differences in their internal transcribed spacer (ITS) regions. Finally, Bao et al.[11] analyzed the V4 domain sequence of 19 *Pleurotus* strains and obtained three phylogenetic lineages, each corresponding to a separate biological species.

In this study, we used the RAPD data and the sequence data for the V4, V6, and V9 domains and the ITS region to analyze the genetic diversity of *T. matsutake* strains derived from different ecological and geographic environments in Asia.

Materials and methods

Strains and culture conditions

Seventeen strains of matsutake mushrooms were examined in this study (Table 1). The host trees of seven of them (Tm-JK, Tm-2, IFO30605, samples of Tm-K, Tm-NK, CB-054, and CB-05) were conifers. Tm-JK, Tm-2, Tm-K, CB-054, and CB-05 were collected from Japan, Korea, and Jilin Province of China. IFO30605 was bought as a culture from the Institute for Fermentation, Osaka, Japan. The fruiting body of Tm-NK was imported from North Korea. Ten *Tricholoma matsutake* strains (X1, X3, N1, N3, N4, W1, S1, S2, SC-05, and XZ-05), the hosts of which are broadleaf trees, were collected from Sichuan Province, Yunnan Province, and the Tibet Autonomous Region of southern China. *Tricholoma bakamatsutake* strain No. 51 was obtained from the Chiba Prefecture Forestry Research Center, Japan, and *Tricholoma magnivelare* strain Tm-C from Canada was bought at a market in Japan.

Table 1. *Tricholoma* strains used in this study

Stock no.	Species	Strain	Geographic origin	Material type
1	*T. matsutake*	Tm-JK	Japan, Kyoto Prefecture	Dried basidiocarp
2	*T. matsutake*	TM-2	Japan, Hiroshima Prefecture	Culture
3	*T. matsutake*	IFO30605	Japan, Institute for Fermentation	Culture
4	*T. matsutake*	Tm-K	Korea	Dried basidiocarp
5	*T. matsutake*	Tm-NK	North Korea	Dried basidiocarp
6	*T. matsutake*	CB-054	China, Jilin Province	Dried basidiocarp
7	*T. matsutake*	CB-05	China, Jilin Province	Dried basidiocarp
8	*T. matsutake*	X1	China, Sichuan Province	Dried basidiocarp
9	*T. matsutake*	X3	China, Sichuan Province	Dried basidiocarp
10	*T. matsutake*	SC-05	China, Sichuan Province	Dried basidiocarp
11	*T. matsutake*	N1	China, Yunnan Province	Dried basidiocarp
12	*T. matsutake*	N3	China, Yunnan Province	Dried basidiocarp
13	*T. matsutake*	N4	China, Yunnan Province	Dried basidiocarp
14	*T. matsutake*	W1	China, Yunnan Province	Dried basidiocarp
15	*T. matsutake*	S1	China, Yunnan Province	Dried basidiocarp
16	*T. matsutake*	S2	China, Yunnan Province	Dried basidiocarp
17	*T. matsutake*	XZ-05	China, Tibet Autonomous Region	Dried basidiocarp
18	*T. magnivelare*	Tm-C	Canada	Dried basidiocarp
19	*T. bakamatsutake*	No. 51	Japan, Chiba Prefecture Forestry Research Center	Culture

DNA isolation and polymerase chain reaction (PCR) amplification

The total DNA of test strains were extracted using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) from frozen dried fruiting bodies or from cultivated mycelia grown in MMN medium [5 g glucose, 2 g malt extract, 1 g yeast extract, 0.5 g KH_2PO_4, 0.25 g $(NH_4)_2HPO_4$, 0.15 g $MgSO_4 \cdot 7H_2O$, 5 ml $CaCl_2$ (1% solution), 2.5 ml NaCl (1% solution), 1.2 ml $FeCl_3$ (1% solution), 1 l distilled water, pH 5.5].

The V4, V6, and V9 domains of the mt SSU rDNA were amplified using the following primer pairs: for V4, MS1 (5′-CAGCAGTCAAGAATATTAGTCAATG-3′) and MS2 (5′-GCGGATTATCGAAATAAATAAC-3′); for V6, V6U (5′-TTAGTCGGTCTCGGAGCA-3′) and V6R (5′-TGACGCAGCCATGCAAC-3′); and for V9, V9U (5′-CCGTGATGAACTAACCGT-3′) and V9R (5′-TTCCAGTAAAGCTACCT-3′). The ITS region of nuclear rDNA was amplified using primers ITS1 (5′-TCC GTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCC GCTTATTGATATGC-3′). The PCR was carried out in a 100-μl volume containing 2.5 units of Takara Ex Taq (Takara, Shiga, Japan), 0.2 mM dNTP mixture, 1× PCR buffer (0.1 M Tris-HCl, pH 8.3, 0.5 M KCl, and 0.015 M $MgCl_2$), 0.8 pmol primers, and 100 ng of template total DNA. Amplifications were carried out as follows: initial denaturation for 1 min at 94°C; amplification for 30 cycles of 30 s at 94°C, 30 s at 60°C (V4 domain and ITS region), 54°C (V6 domain), or 50°C (V9 domain), and 30 s at 72°C; and a final annealing for 10 min at 72°C.

RAPD analyses were carried out using the following eight primers: L02, 5′-TGGGCGTCAA-3′; L03, 5′-CCAG CAGCTT-3′; L05, 5′-ACGCAGGCAC-3′; L09, 5′-TGC GAGAGTC-3′; L13, 5′-ACCGCCTGCT-3′; L17, 5′-AGC CTGAGCC-3′; L19, 5′-GAGTGGTGAC-3′; and L20, 5′-TGGTGGACCA-3′. PCRs for RAPD were performed in a final volume of 25 μl containing 1.5 units of *Taq* DNA polymerase (Bioneer, Daejeon, Korea), 0.2 mM dNTPs mixture, 1× PCR buffer (0.1 M Tris-HCl, 0.4 M KCl, 0.015 M $MgCl_2$, pH 9.0), 0.8 pmol primers, and 150 ng of template total DNA. Amplifications were carried out as follows: initial denaturation for 1 min at 94°C; amplification for 45 cycles of 1 min at 94°C, 2 min at 35°C, and 3 min at 72°C; and final annealing for 10 min at 72°C. Amplified DNA fragments were separated by 2% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Purified PCR products and cycle sequencing

PCR products were purified by ultrafiltration through a Microcon YM-100 filter (Millipore, Bedford, MA, USA). A 1-μl sample of purified PCR products was separated by electrophoresis on a 2% agarose gel along with OneSTEP Ladder 100 standards (Nippon Gene, Tokyo, Japan) to estimate the concentrations of the PCR product. Direct sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Four primer pairs (MS1/MS4, V6U/V6R, V9U/V9R, and ITS1/ITS4) were used to sequence both DNA strands of the V4, V6, and V9 domains and the ITS region, respectively. The extension products were purified with Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ, USA) and analyzed with an ABI Prism 3100 Genetic Analyzer.

Phylogenetic analyses

RAPD fragments were scored as presence (+) or absence (−) and used to calculate the genetic distance with the RESTDIST program in the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html).[12] The sequence data were edited with Genetyx-SV/RC Ver. 6.1 (Software Development, Tokyo, Japan). Multiple alignments of all the sequences were performed using CLUSTAL W (http://www.ddbj.nig.ac.jp/search/clustalw-j.html), followed by manual adjustments. The location of the V4 variable domain in the 5′-portion of the mt SSU rDNA from each strain was determined by comparing the aligned sequences with the corresponding sequences in the GenBank database. The genetic distance based on sequence was calculated using the DNADIST programs in the PHYLIP package.

The phylogenetic relationships were analyzed with UPGMA (unweighted pair group method with arithmetic mean) methods[13] using the NEIGHBOR programs in the PHYLIP package.[12] The TreeView program[14] was used to view the phylogenetic tree.

Results

Comparison of the sequence of the V4, V6, and V9 domains in the mt SSU rDNA among *Tricholoma* strains

Comparison of the sequence of the V4, V6, and V9 domains in the mt SSU rDNA among the *Tricholoma* strains tested revealed that the size of the V4 domain is variable. The lengths of the V4 domain range from 277 nucleotides for *Tricholoma magnivelare* strain Tm-C to 309 nucleotides for *Tricholoma bakamatsutake* strain No. 51. The lengths of the V4 domains for all tested strains of *Tricholoma matsutake* except for XZ-05 (282 nucleotides) were the same (283 nucleotides). In contrast, the V6 and V9 domains have the same lengths for all of the *Tricholoma* strains tested (140 and 256 nucleotides, respectively).

Alignment of the V4 sequences shows that 14 strains of *T. matsutake* have identical sequences. Strains IFO30605 and SC-05 have a single nucleotide change, and XZ-05 has three nucleotide changes. For the V6 and V9 domains, 13 of the *Tricholoma* strains have the same sequences (data for strains CB-054, CB-05, SC-05, and XZ-05 are absent from this study). These data show that 17 of the *T. matsutake* strains from broadleaf and coniferous forests are the same biological species.

Comparison of the sequences of *T. matsutake* shows that *T. magnivelare* strain Tm-C has 2 different nucleotides and 6 deletions in the V4 domain, 1 different nucleotide in the V6 domain, and no differences in the V9 domain. *Tricholoma bakamatsutake* strain No. 51 has 12 different nucleotides and 24 insertions in the V4 domain. Therefore, we suggest that *T. magnivelare*, *T. bakamatsutake*, and *T. matsutake* are distinct biological species.

Phylogenetic analyses based on the sequences of the V4 domain

We performed phylogenetic analyses using sequence data of the V4 domain from the tested strains of *Tricholoma*. Identical sequences were merged into one input sequence when running the programs in the PHYLIP package. When the phylogenetic tree based on the V4 domain sequences was constructed by the UPGMA method, a strain of *Tricholoma subaureum* (Genbank database accession number AF357082) was used as an outgroup (Fig. 1). This rooted tree shows two phylogenetic lineages arising from the root *T. subaureum:* one leads to a terminal node corresponding to *T. bakamatsutake*, and the other subdivides further into three branches leading to two terminal nodes corresponding to *T. magnivelare* and *T. matsutake* strain XZ-05 as well as a closed third-level subcluster. The third-level subcluster contains three terminal nodes: 1 strain of *T. matsutake* (IFO30605), 1 strain of *T. matsutake* (SC-05), and the other 14 strains of *T. matsutake*. The slight phylogenetic divergences among the 17 strains of *T. matsutake* are due only to several nucleotide differences, which could be sequencing artifacts. Therefore, it appears that all strains under the name *T. matsutake* are the same species regardless of whether they grow in coniferous or broadleaf forests.

Comparison of the sequences of the nuclear rDNA ITS regions among *Tricholoma* strains

We used the nuclear rDNA ITS region to further analyze the variation amongst the matsutake strains. We found that the ITS region contains 601 nucleotides in the majority of tested samples, which included six strains from Yunnan Province (X1, X1, N1, N3, N4, and W1), two strains from Sichuan Provinces (S1 and S2), strain TM-2 from Japan, strain Tm-K from Korea, and strain Tm-NK from North Korea. In two strains (IFO30605 and TM-JK from Japan), the ITS region was 602 nucleotides long, and for *T. magnivelare* strain Tm-C, it was 605 nucleotides long.

Alignment of the ITS sequences show that almost all *T. matsutake* strains in this study have identical sequences except for a one-nucleotide insert in the ITS1 region in strains IFO30605 and TM-JK. Thus, the matsutake strains isolated from different ecological environments could not be resolved using the ITS region sequences.

The ITS sequence of *T. magnivelare* strain Tm-C has approximately 30 nucleotide differences and insertions/

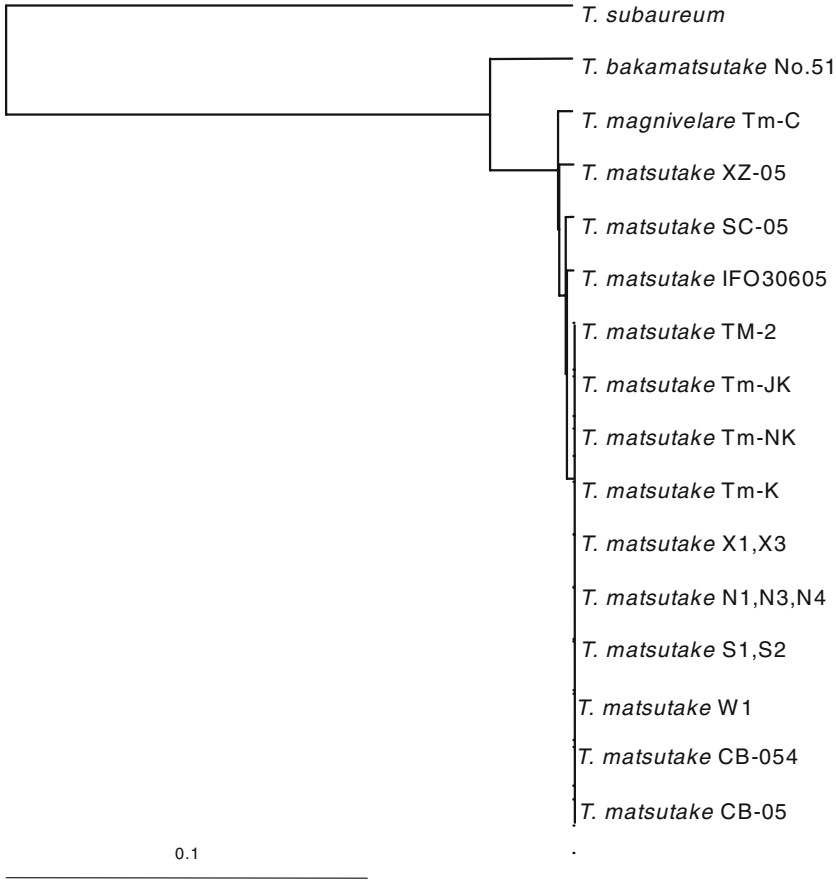

Fig. 1. Phylogenetic tree constructed by the UPGMA (unweighted pair group method with arithmetic mean) method based on the sequences of the V4 domain

deletions compared with the sequences of other *T. matsutake* strains. This indicates that *T. magnivelare* can be distinguished from *T. matsutake* based on the ITS sequences.

Phylogenetic analyses based on the sequences of the ITS region

Phylogenetic analyses were performed using sequences for the ITS region of the tested strains of *Tricholoma*. Identical sequences were merged into a single input sequence when running the programs in the PHYLIP package.

When the phylogenetic tree based on sequences of the ITS region was constructed by the UPGMA method, a *T. bakamatsutake* strain (Genbank accession number AB036898) was used as an outgroup (Fig. 2). One of two lineages arising from the root leads to *T. magnivelare* Tm-C and the other to a subcluster. This subcluster comprises 13 of the *T. matsutake* strains tested in this study.

RAPD pattern analysis

Thirteen strains of *T. matsutake* and one strain of *T. magnivelare* were examined by RAPD analyses. A total of 84 bands were obtained using eight primers. Among the isolates from the broadleaf forests, only strains S1 and S2 and strains N3 and N4 shared the same pattern. The patterns between Yunnan matsutake strains N1 and W1 were different with 94.0% identity. On the other hand, the Sichuan matsutake X1 and X3 strains also have different RAPD patterns. Comparison of RAPD patterns of Yunnan and Sichuan matsutakes shows that these matsutake strains, which are derived from broadleaf forests, are distinct.

All tested matsutakes growing in the coniferous forest were collected from Japan, Korea, and North Korea. The RAPD patterns from the three Japanese matsutakes are different from each other and from the isolates from Korea and North Korea. In contrast, the RAPD patterns from matsutake strains are 52.4% to 61.9% identical with that from *T. magnivelare* strain Tm-C. This result suggests that *T. matsutake* is a different species from *T. magnivelare*.

Phylogenetic analyses based on RAPD data

A phylogenetic tree was constructed by the UPGMA method using the 84 RAPD bands from the 14 tested strains (Fig. 3). According to the topological shape of this tree, 4

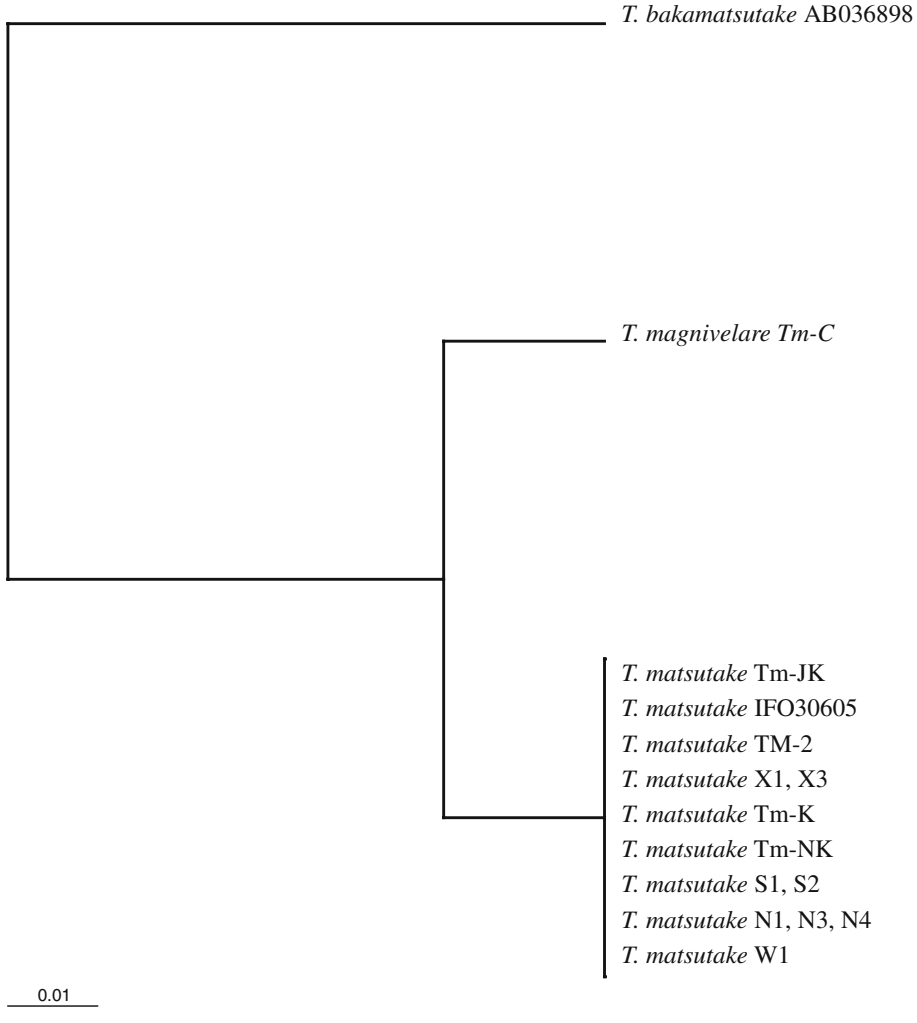

Fig. 2. Phylogenetic tree constructed by the UPGMA method based on the sequences of the internal transcribed spacer regions

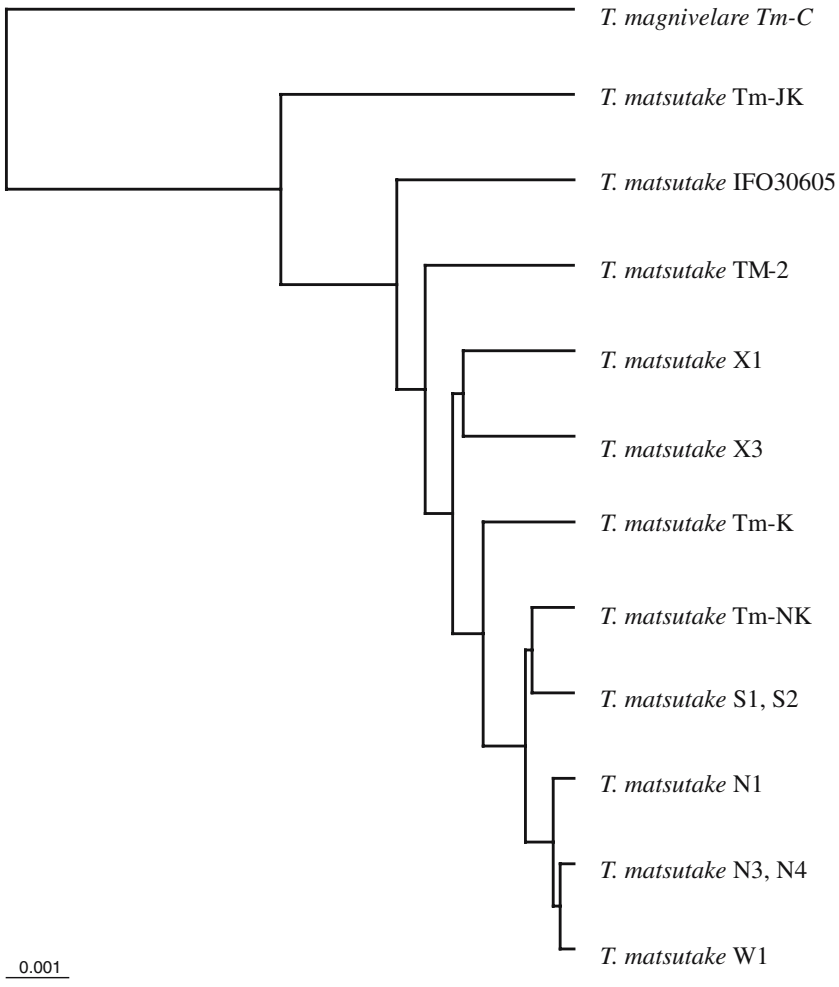

Fig. 3. Phylogenetic tree constructed by the UPGMA method based on random amplification of polymorphic DNA data

Yunnan matsutake strains (N1, N3, N4, and W1) correspond to three terminal nodes and are closely gathered in a cluster located at the bottom of the tree. Another 2 Yunnan matsutake strains (S1 and S2) and 2 Sichuan matsutake strains (X1 and X3) comprise independent clusters. The Korean (Tm-K) and North Korean strains (Tm-NK) distribute in distinct clusters. Three Japanese matsutake strains (TM-JK, IFO30605, and TM-2) lead independently to three terminal nodes located at the top of the tree. A strain of *T. magnivelare* (Tm-C) has a long genetic distance from all of the matsutake strains and forms the root of the tree.

Discussion

Comparison of the sequences of the V4 domain in the mt SSU rDNA from 3 Japanese strains, 1 Korean strain, and 1 North Korean strain of matsutake shows that all sequences are identical except for a single nucleotide difference in Japanese strain IFO30605. Therefore, we suggest that these 5 matsutake strains, which were obtained from coniferous forests, are the same biological species. On the other hand, comparison of the V4 sequences of the 10 strains growing in broadleaf forests of Sichuan Province, Yunnan Province, and the Tibet Autonomous Region of southern China reveals that, except for one to three nucleotide differences in strains SC-05 and XZ-05, all of the remaining nucleotides are identical. Strains CB-054 and CB-05 from the coniferous forest of Jilin Province of northern China have almost identical V4 sequences as the 10 strains from southern China. We found fewer nucleotide differences in the V6 and V9 domains than in the V4 domain in the mt SSU rDNA of matsutake strains. Therefore, the V6 and V9 sequence data also support the conclusion obtained from the V4 domain sequences, that is, that all matsutake strains are the same biological species regardless of their host specificity and geographic differences. An additional published result on *Tricholoma matsutake* by Cao and Yao[15] also supports this conclusion. Cao and Yao[15] reported that different matsutake samples obtained from conifer and broadleaf hosts have similar morphological characteristics. Furthermore, in this study, we showed that the V4 domain sequences of matsutake isolates and those of closely related species *Tricholoma bakamatsutake* and *Tricholoma magnivelare* differed by 36 and 8 nucleotides, respectively, proving that they are different species.

We suggest that, like the variable domains of the mt SSU rDNA, the rDNA ITS region can be used to determine the relationships of the various biological species of matsutake

mushrooms. In this study, phylogenetic analysis of the sequences of the ITS region showed that 13 matsutake isolates from China, Japan, Korea, and North Korea are included in the same cluster. Matsushita et al.[16] also treated *T. matsutake* from coniferous and broadleaf forests as the same species on the basis of sequence analysis of the ITS region. On the other hand, *T. bakamatsutake* and *T. magnivelare* strains are located in a different cluster from *T. matsutake*, and, based on analysis of the mt SSU rDNA, are distinct species.

Because the sequences of the ITS region and the variable domains of the mt SSU DNA (V4, V6, and V9 domains) are highly conserved within the species, they do not reveal the genetic divergence between the different *T. matsutake* isolates. The RAPD data, however, were more variable, allowing analysis of the genetic differences. Specifically, RAPD analysis on the 13 Matsutake isolates from different ecological environments (southern China, Korean Peninsula, and Japan) show that they comprise 11 phylogenetic subclusters, which may correspond to 11 varieties of matsutake mushrooms. Based on the comparison of the subclusters, the matsutake isolates from the broadleaf forests of Yunnan Province are closely related, and the 2 Sichuan matsutake isolates are also closed related. Zeng et al.,[17] however, suggested that the matsutake mushrooms growing in Sichuan, Yunnan, and Jilin Provinces are the same species based on RAPD analysis of around 20 isolates from different geographic environments. In this study, in contrast, we showed that the matsutake mushrooms collected from different ecological environments have genetic differences and represent distinct varieties. On the other hand, compared to the five strains from Japan, Korea, and North Korea, the Japanese isolates were more closely related to each other, and the Korean isolate is closely related to that from North Korea. Based on these RAPD findings, it appears that the matsutake isolates are related to each other by geographic distribution rather than host specificity. This hypothesis remains to be confirmed using a mating test.

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