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Highly polymorphic DNA markers to specify strains of the ectomycorrhizal basidiomycete *Tricholoma matsutake* based on σ_{marY1}, the long terminal repeat of *gypsy*-type retroelement *marY1*

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Abstract The ectomycorrhizal basidiomycete *Tricholoma matsutake* produces commercially valuable fruit bodies—matsutake—in *Pinus* sp. forest. Here we report that PCR with outward facing primers designed based on sequences comprising σ_{marY1}, the long terminal repeat of the *gypsy*-type retroelement *marY1*, specifies strains of *T. matsutake*. PCR with a primer based on the 22-bp sequence conserved at the 5′-end of σ_{marY1} conferred 73 reliable bands overall whose profiles depend upon strains of *T. matsutake* and *T. magnivelare*, the latter known as 'American matsutake'. This PCR system gave no detectable band in any other species of *Tricholoma* tested, including *T. bakamatsutake* and *T. fulvocastaneum*, symbionts closely related to *T. matsutake*, as well as a host plant, *Pinus densiflora*. Similarly, PCR with a set of primers based on 26-bp and 28-bp sequences at bp 48–73 and bp 281–308 of σ_{marY1}, internal regions that are mutated in a variant of *marY1*, conferred 90 reliable bands only in strains of *T. matsutake*. Theoretically, PCR with the 22-bp primer would allow generation of 2^{73}, or 9.4×10^{21}, types of polymorphism, and PCR with a combination of 26- and 28-bp primers, 2^{90}, or 1.2×10^{27} types. The probability of falsely specifying two different isolates as the same strain is $<1/10^{21}$. While polymorphisms conferred by the primer based on the 5′ end of σ_{marY1} rather exhibit genetic conservation of a group of *T. matsutake*, those resulting from primers based on the internal sequences more clearly demonstrate intra-specific diversification. Both systems revealed that *T. matsutake* is divergent within the species. Ectomycorrhizas formed between *P. densiflora* and *T. matsutake* were identified by the PCR systems developed in the present study. This method, using σ_{marY1} as a genetic marker, is useful in analyzing the diversity of *T. matsutake*, monitoring the behavior of individual mycorrhizas, and specifying the ecological background of fruit bodies traded in markets.

Keywords Basidiomycete · Ectomycorrhiza · Long terminal repeat · Retrotransposon · *Tricholoma* spp.

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Introduction

Tricholoma matsutake is an ectomycorrhizal basidiomycete that produces economically important edible mushrooms—matsutake—in association with *Pinus* sp. plants in the Northern Hemisphere (Hosford et al. 1997; Ogawa 1975). The annual yield of matsutake in Japan has dramatically decreased since the 1940s. The yield of 52 t in the year 2002 was only 0.4% of the yield recorded in 1941 (Ministry of Agriculture, Forestry and Fishery of Japan). The resources of *T. matsutake* are endangered if the decreased yield is due to shrinkage of the mycorrhizal fungus population, an issue that requires scientific countermeasures.

Recently, methods allowing in vitro mycorrhization between *T. matsutake* and *Pinus densiflora* were established (Guerin-Laguette et al. 2000; Yamada et al. 1999, 2003). However, there are no reports concerning culture practices that allow the fungus to produce fruit bodies. One reason for this is the lack of an effective method to specify strains of *T. matsutake*. Such a method, if developed using genetic markers taking into account ecological and evolutionary significance, will allow us to monitor the diversification of the fungus in nature, to identify strains of *T. matsutake* suited to artificial culture practice, and to trace the consequence of such practice. Selection of suitable strains from a large number of genetic resources may lead to the successful cultivation of ectomycorrhizal fungi to yield fruit bodies (Ohta 1994, 1998). While this approach is rather orthodox in the breeding of crops and of cultivated mushrooms, it has rather seldom been applied to exploration of

ectomycorrhizal resources. Conventional methods to identify strains of cultivable homobasidiomycetes, such as analysis of sexual and asexual compatibility, and morphotypes of fruit bodies, cannot be applied to ectomycorrhizal symbionts (Babasaki et al. 2003; Esser and Blaich 1994). In addition, methods based on genetic markers will be useful for identifying the origin of individual matsutake, information strongly demanded by consumers and dealers to ensure food safety, product freshness, and sensible price setting. Domestic matsutake are generally traded at much higher prices than imported products in Japan.

Retroelements are retrovirus-like DNA parasites incorporated by eukaryotes as part of the genome in the course of their host evolution (Bushman 2002). In response to environmental stresses, copies of retroelements are amplified and integrated into other genetic loci of their hosts through the activity of the *pol* gene products reverse transcriptase, RNase H and integrase (Bushman 2002). Once inserted into the host genome, retroelements are stable, and are inherited through the host progenies, unlike DNA transposons that rely on 'cut-and-paste' for transposition (Bushman 2002). In *Magnaporthe grisea*, the rice blast pathogen that belongs to the filamentous ascomycetes, genetic analysis with retroelements and related repeated sequences revealed great diversification of the fungus in relation to host-parasite relationships (Dobinson et al. 1993; Farman et al. 1996; Hamer et al. 1989; Kachroo et al. 1994; Levy et al. 1991). Methods using such genetic markers have been applied to the identification of strains and pathotypes of the pathogenic fungus in epidemiological analysis (Hamer 1991; Valent and Chumley 1991). Recently, polymerase chain reaction (PCR)-polymorphism systems targeting flanking sequences of retrotransposons residing abundantly in the genome were demonstrated as a powerful molecular tool to specify strains of barley, *Hordeum vulgare* (Kalendar et al. 1999). In this system, PCR with outward-facing primers annealing to the long terminal repeat (LTR) located at both the 5′ and 3′ ends of retrotransposons, generated inter-retrotransposon-amplified polymorphism, or IRAP, and effectively specified strains of barley (Kalendar et al. 1999).

From *T. matsutake*, we previously cloned the *gypsy*-type LTR retroelement *marY1* (Murata and Yamada 2000). A PCR system targeting *pol* of *marY1* successfully identified mycorrhizae formed between *T. matsutake* and *P. densiflora* by generating DNA segments whose electrophoretic profiles are uniquely conserved in *T. matsutake* (Murata and Yamada 1999). Subsequently, the LTR of *marY1*, designated σ_{marY1} after Ty3-*gypsy* of the budding yeast *Saccharomyces cerevisiae*, which is 426-bp in length and carries the putative promoters of *marY1*, was found to express the reporter gene β-galactosidase in *S. cerevisiae* (Boeke 1989; Murata and Miyazaki 2001). In addition, σ_{marY1} allows multicopy integration of a vector DNA in the genome of the homobasidiomycete *Lentinula edodes* after transformation (Murata and Miyazaki 2004). Therefore, we hypothesize that σ_{marY1} may be deeply involved in genome evolution of *T. matsutake* through insertion and recombination, as is the case with LTRs of mammalian retroelements (Nekrutenko and Wen-Hsiung 2001; van de Lagemaat 2003). The objective of the present study was to explore the usefulness of sequences associated with σ_{marY1} as genetic markers for IRAP analysis to identify strains of *T. matsutake*, which may greatly contribute to our understanding of the behavior of the fungus and of genetic diversity of mycorrhizae in nature.

Materials and methods

Fungal samples

The 53 fungal isolates used in this study are listed in Table 1. *T. matsutake* Y1 and Y4, *T. ustale* 610, 611 and 612, *T. flavovirens* 613 and 614, *T. portentosum* 615, and *T. saponaceum* 616 were isolated from the same *P. densiflora* woodland, and *T. bakamatsutake* B1 was from a *Quercus serrata* forest adjacent to the *P. densiflora* woodland in Ibaraki Prefecture, Japan. Fungal mycelia were cultured in MMN liquid medium modified by the addition of V8 juice to a final concentration of 1.5% instead of NaCl (Campbell Soup Co., Camden, N.J.; Murata et al. 1999).

Characterization of *marY1-v*, a *marY1* variant used for primer design

The construction of a genomic library of *T. matsutake* Y1 in λEMBL3, screening of the library with a probe for *pol* (encoding reverse transcriptase) and subcloning of the library using the vector pBluescript SK^+ have been described elsewhere (Murata and Yamada 2000; Murata et al. 2001). The nucleotide sequence of *marY1-v* was determined using an ABI Prism 377 autosequencer with Big Dye terminator FS core kit (Applied Biosystems, Foster City, Calif.). Data were analyzed using Genetix Mac ver 9.0 (Software Development, Tokyo, Japan), and homology search analysis was conducted with the BLAST program provided by GenomeNet (Bioinformatics Center, Institute for Chemical Research, Kyoto University, Japan). The nucleotide sequence of *marY1* carrying σ_{marY1} and *marY1-v* carrying $\sigma_{marY1\text{-}v}$ have been deposited in the DDBJ database under the accession numbers AB028236 and AB027513, respectively.

Polymerase chain reaction

Genomic DNA to be used as a template was isolated from frozen mycelia of fungal strains using a lysis buffer containing hexadecyltrimethylammonium bromide and phenol-chloroform as described elsewhere (Dobinson et al. 1993). PCR was conducted in 50 μl reaction mixtures containing 250 μM dNTP, 0.5 μM primers, 30 ng template DNA, 0.5 U *Taq* polymerase (Gene Taq NT, Wako Pure Chemicals, Osaka, Japan) and a universal buffer provided with the enzyme. Cycle reactions were performed as follows; 1× (94°C/2 min), 25× (94°C/30 s, annealing temperature/30 s, and 72°C/5 min), and 1× 72°C/10 min in a GeneAmp 9700 device (Applied Biosystems). The best annealing tem-

Table 1 Fungal strains used in this study

Species	Strains	Sampling site	Year
Tricholoma matsutake	IW-92602	*Pinus densiflora* forest, Iwate Prefecture, Japan	2002
	TM-15	*P. densiflora* forest, Iwate Prefecture, Japan	1992
	Y1	*P. densiflora* forest, Ibaraki Prefecture, Japan	1993
	Y4	*P. densiflora* forest, Ibaraki Prefecture, Japan	1996
	Tm-8	*P. densiflora* forest, Kyoto Prefecture, Japan	1992
	MR32	*P. densiflora* forest, Hyogo Prefecture, Japan	1987
	Tm029	*P. densiflora* forest, Shiga Prefecture, Japan	1983
	Tm040	*P. densiflora* forest, Shiga Prefecture, Japan	1991
	OK-T4	*P. densiflora* forest, Okayama Prefecture, Japan	1992
	OK-T5	*P. densiflora* forest, Okayama Prefecture, Japan	1992
	Tm-H001	*P. densiflora* forest, Hiroshima Prefecture, Japan	1982
	Tm-H102	*P. densiflora* forest, Hiroshima Prefecture, Japan	1992
	Tm-Y59AFB	*P. densiflora* forest, Yamaguchi Prefecture, Japan	1984
	Tm-A59C	*P. densiflora* forest, Yamaguchi Prefecture, Japan	1984
	Tm-T4	*P. densiflora* forest, Tokushima Prefecture, Japan	1993
	K1[a]	Republic of Korea	1997
	K3[a]	Republic of Korea	2003
	K4[a]	Republic of Korea	2003
	Tm-K2[a]	Republic of Korea	1992
	Tm-31[a]	*P. densiflora* forest, Kyongsang North, Republic of Korea	1998
	NK1[a]	Democratic People's Republic of Korea	1998
	CHI1[a]	People's Republic of China	1998
	Tm-9[a]	People's Republic of China	1992
	CH381[a]	People's Republic of China	2003
	CH382[a]	People's Republic of China	2003
	CH383[a]	People's Republic of China	2003
	CH384[a]	People's Republic of China	2003
	CH385[a]	People's Republic of China	2003
	CH387[a]	People's Republic of China	2003
	BH1[a]	Kingdom of Bhutan	1998
	MC1[a]	Kingdom of Morocco	1998
	TM-5[a]	Kingdom of Morocco	1992
	MX1[a]	Mexico	1998
	TM-4[a]	Mexico	1992
Tricholoma magnivelare	Tp-C3[a]	Canada	1994
	TM-10[a]	Canada	1992
Tricholoma bakamatsutake	B1	*Quercus serrata* forest, Ibaraki Prefecture, Japan	1993
	CB-Tb1	*Pasania edulis*/*Castanopsis cuspidata* var. *Sieboldii* forest, Chiba Prefecture, Japan	1989
	CB-Tb2	*P. edulis*/*C. cuspidata* var. *Sieboldii* forest, Chiba Prefecture, Japan	1990
	CB-Tb3	*P. edulis*/*C. cuspidata* var. *Sieboldii* forest, Chiba Prefecture, Japan	1989
	CB-Tb4	*P. edulis*/*C. cuspidata* var. *Sieboldii* forest, Chiba Prefecture, Japan	1987
Tricholoma fulvocastaneum	WK-N1	*Quercus phillyraeoides*forest, Wakayama Prefecture, Japan	1988
Tricholoma robustum	KB1	*P. densiflora* forest, Nagano Prefecture, Japan	2000
Tricholoma ustale	610	*P. densiflora* forest, Ibaraki Prefecture, Japan	1997
	611	*P. densiflora* forest, Ibaraki Prefecture, Japan	1997
	612	*P. densiflora* forest, Ibaraki Prefecture, Japan	1997
Tricholoma flavovirens	613	*P. densiflora* forest, Ibaraki Prefecture, Japan	1997
	614	*Pinus densiflora* forest, Ibaraki Prefecture, Japan	1997
Tricholoma portentosum	615	*P. densiflora* forest, Ibaraki Prefecture, Japan	1997
Tricholoma saponaceum	616	*P. densiflora* forest, Ibaraki Prefecture, Japan	1997
Tricholoma japonicum	FK-J1	*P. densiflora* forest, Fukui Prefecture, Japan	1988
	MR28	*P. densiflora* forest, Hyogo Prefecture, Japan	1992
Tricholoma sejunctum	NA12	*P. densiflora/Q. serrata* forest, Nagano Prefecture, Japan	1993

[a]A fruit body was obtained in a 'matsutake'market in Japan

perature, desirably near or above T_m, which should confer reproducible results by specifying sequences perfectly matching with those of primers, was determined in a gradient thermal cycler using ramp conditions equivalent to that of the GeneAmp 9700 (Takara Shuzo, model TP600, Otsu, Japan). For example, primer pS1 [GCACCCCCC TAGTCCCCTTACA, T_m (°C)=64.2] was annealed to template DNA at 68°C, and the set of primers pS48 (GAG GTGGGGAAAAATATGGGACGAAC, T_m =62.1)/pL281 (CTTCACATATACTGGGCATCAGCAAGGG, T_m=63.4) at 62°C. PCR products were electrophoresed in TBE-1.8% agarose (Nusieve GTG agarose, FMC Bio Products, Rock-

land, Me.) gels containing 0.1 μg/ml ethidium bromide in a 27 cm ×42 cm apparatus (Nippon Eido, Tokyo, Japan), which was enough to run 37 samples simultaneously, at 195 V for 4 h. Data were reproducible based on three independent PCRs per primer set. Therefore, representative data are presented here.

Phylogenetic analysis

PCR products manifesting reproducible solid bands between 0.1 and 1.0 kb in triplicate agarose gel electrophoresis were visually scored line by line using two letter codes, marking P for a positive band and N for a negative position. Neighbor-joining analysis was conducted with bootstrap analysis based on 1,000 replications using the CLUSTAL X program (Thompson et al. 1997). Phylogenetic trees were drawn and visualized using the program TreeView PPC.

Mycorrhiza synthesis in vitro

Axenic seedlings of *P. densiflora* were co-cultivated with mycelia of *T. matsutake* Y1 in a spawn containing a mixture of peatmoss, vermiculite and components of the MNC medium, or the medium used for cultivation of *Lyophyllum shimeji*, at 20°C for 6 months (Murata and Yamada 1999). After cultivation, the lateral roots of *P. densiflora* surrounded by mycelia were examined microscopically for intercellular penetration of the hyphae to confirm the formation of the Hartig net.

Results

Primer design based on conserved and mutated portions of σ_{marY1}

From a genomic library of *T. matsutake* Y1, we obtained a variant of *marY1* designated *marY1-v* (Fig. 1). $\sigma_{marY1\text{-}v}$, the homologue of σ_{marY1} in *marY1-v*, which contains various mutations, notably in the internal portion, is associated with a tandem *pol* gene, apparently degenerated by deletions and insertions, presenting evidence that the genome of *T. matsutake* has evolved accompanying recombination among copies of *marY1* (Fig. 1). Based on the data, two sets of primers, which point towards regions outwith σ_{marY1}, were designed to amplify DNA segments associated with σ_{marY1} by PCR (Fig. 1, see Materials and methods for sequences). Primer pS1 was designed based on the complementary strand of the 5′-terminal sequences of σ_{marY1}, which are apparently conserved (Fig. 1). Another set of primers, pS48/pL281, was designed based on internal sequences at bp 48–73 and bp 281–308 of σ_{marY1}; the sequence of pS48 and sequences associated with that of pL281 are mutated in $\sigma_{marY1\text{-}v}$ (Fig. 1). Therefore, we expected that polymorphisms observed with pS1 and those with pS48/pL281 would not be identical, and may provide us with different information.

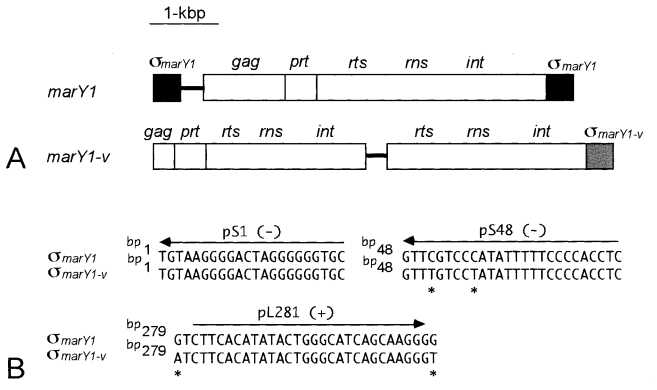

Fig. 1 **A** Schematic representation of *marY1* and its variant, *marY1-v*. A description of *marY1* has been published in detail elsewhere (Murata and Yamada 2000). *Closed boxes* LTR, *open boxes* coding regions, *solid lines* non-coding regions, *gag* group specific antigen domain, *prt* protease domain, *rts* reverse transcriptase domain, *rns* RNase H domain, *int* integrase domain. **B** Alignment of sequences used to design outward-facing primers for PCR. *Arrows* and (+/−) indicate the direction and strand of sequences of primers *pS1*, *pS48* and *pL281*. *Asterisks* Non-identical nucleotides σ_{marY1} and $\sigma_{marY1\text{-}v}$. Numbers represent positions (bp) of sequences relative to the 5′ of σ_{marY1} and $\sigma_{marY1\text{-}v}$

Species specificity of the designed PCR

Primer pS1 amplified fragments in samples of *T. matsutake* and *Tricholoma magnivelare* (known as 'American matsutake'; Fig. 2), while the primer pair pS48/pL281 did so only in the sample of *T. matsutake* (Fig. 3). Under the conditions of the assay, neither PCR system produced a detectable fragment in any of the other species of *Tricholoma* listed in Table 1, including *T. robustum* (pseudo-matsutake), *T. bakamatsutake* (foolish-matsutake), *T. fulvocastaneum* (false-matsutake), *T. flavovirens*, *T. portentosum*, *T. ustale*, *T. saponaceum*, *T. japonicum*, and *Tricholoma sejunctum*. Ectomycorrhizas formed in vitro between *P. densiflora* and *T. matsutake* were also specified by PCR with pS1 and pS48/pL281 (Fig. 4). In addition, both systems produced polymorphic fragments in a sample of an ectomycorrhiza formed between *P. densiflora* and *T. matsutake* collected in Iwate Prefecture, Japan, but did not produce any detectable fragments in a sample of the uninfected host plant obtained in the same area (Fig. 4).

Strain specificity of the designed PCR

PCR with pS1 conferred 73 reliable bands in all in strains of *T. matsutake* and *T. magnivelare*, and PCR with pS48/pL281 produced 90 reliable bands exclusively in strains of *T. matsutake* in agarose gel electrophoresis (Figs. 2, 3). PCR product polymorphisms varied depending on the fungal strain. Theoretically, PCR with pS1 would allow generation of 2^{73} or 9.4×10^{21} types of polymorphism, and that with pS48/pL281 would produce 2^{90}, or 1.2×10^{27} types. These calculations were based on those used in randomly amplified polymorphic DNA (RAPD) analysis, in which the total number of types of polymorphism equals two types per

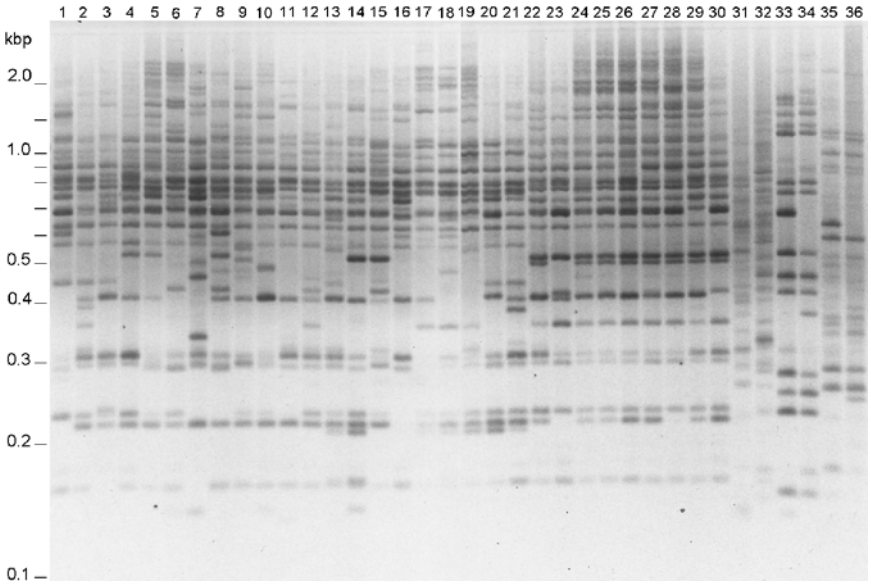

Fig. 2 Polymorphisms of strains of *Trichloma matsutake* and *Trichloma magnivelare* generated by PCR with primer pS1. A negative image is presented according to Kalendar et al. (1999). Marker sizes (kb) are on the left. Lanes: *1–34 T. matsutake*;*1* TM-15, *2* IW-92602, *3* Y1, *4* Y1, *5* Tm029, *6* Tm040, *7* Tm-8, *8* MR32, *9* OK-T4, *10* OK-T5, *11* Tm-H001, *12* Tm-H102, *13* Tm-Y59AFB, *14* Tm-A59C, *15* Tm-T4, *16* K1, *17* Tm-K2, *18* K3, *19* K4, *20* Tm-31, *21* NK1, *22* CHI1, *23* Tm-9, *24* CH381, *25* CH382, *26* CH383, *27* CH384, *28* CH385, *29* CH387, *30* BH1, *31* MC1, *32* TM-5, *33* MX1, *34* TM-4; *35–36 T. magnivelare*;*35* Tp-C3, *36* TM-10

single band position, i.e., positive or negative, to the power of the number of total band positions (Williams et al. 1990). Therefore, the probability of falsely specifying two different isolates as the same strain is $<1/10^{21}$.

Phylogenetic analysis of various strains of *T. matsutake* showed that the pS1 system exhibited primarily genetic conservation of a group of specimens, while the pS48/pL281 system emphasized diversification within the group (Fig. 5). For example, PCR with pS1 conferred polymorphisms indicating that Asian isolates of *T. matsutake* are closely related to each other, but occupy positions distant from isolates from Morocco and Mexico (Fig. 5). Among Asian isolates, those from Japan and the Korean Peninsula were much more diversified within their local regions compared with isolates from China and Bhutan (Fig. 5). The occurrence of tremendous diversification among Japanese and Korean isolates, and that of some among Chinese and Bhutanese isolates, can be clearly demonstrated by analysis with pS48/pL281 (Fig. 5).

Discussion

This is the first report describing IRAP analysis targeting the flanking sequences of an LTR for identification of strains as well as species of an ectomycorrhizal fungus, unearthing for the first time the occurrence of considerable genetic diversification within the species of *T. matsutake* that produces economically important fruit bodies. IRAP analysis generally requires retroelements that are present

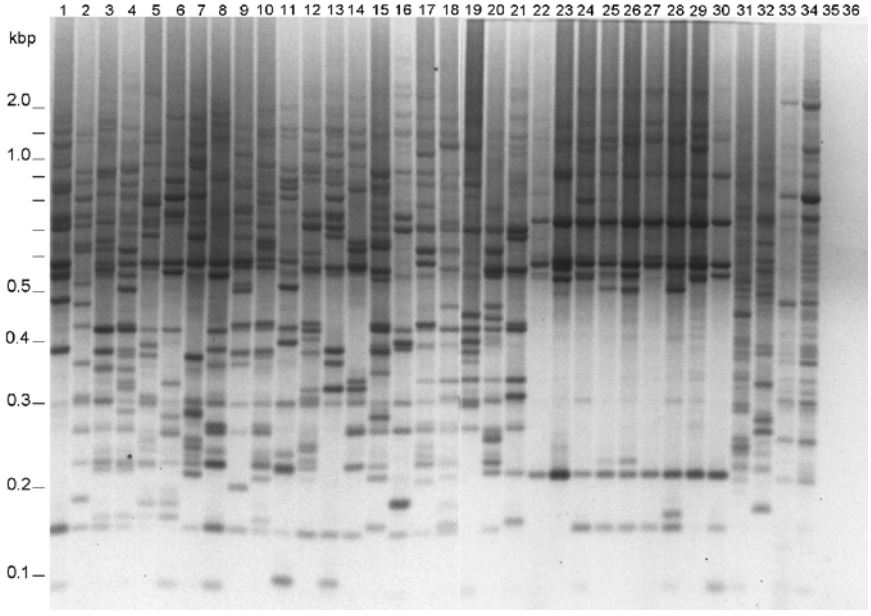

Fig. 3 Polymorphisms of strains of *T. matsutake* and *T. magnivelare* generated by PCR with primers pS48/pL281. A negative image is presented according to Kalendar et al. (1999). Marker sizes (kb) are on the left. Lanes: *1–34 T. matsutake*;*1* TM-15, *2* IW-92602, *3* Y1, *4* Y1, *5* Tm029, *6* Tm040, *7* Tm-8, *8* MR32, *9* OK-T4, *10* OK-T5, *11* Tm-H001, *12* Tm-H102, *13* Tm-Y59AFB, *14* Tm-A59C, *15* Tm-T4, *16* K1, *17* Tm-K2, *18* K3, *19* K4, *20* Tm-31, *21* NK1, *22* CHI1, *23* Tm-9, *24* CH381, *25* CH382, *26* CH383, *27* CH384, *28* CH385, *29* CH387, *30* BH1, *31* MC1, *32* TM-5, *33* MX1, *34* TM-4; *35–36 T. magnivelare*;*35* Tp-C3, *36* TM-10

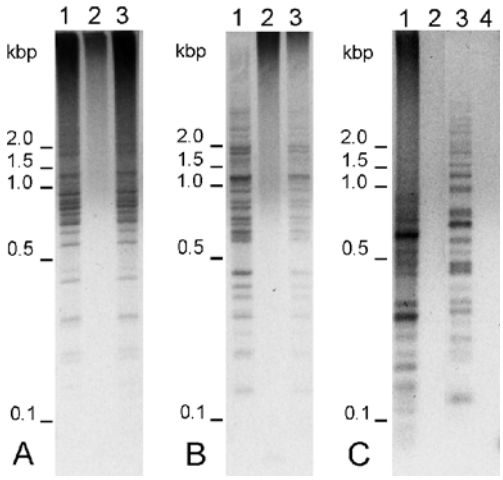

Fig. 4A–C PCR profiles of samples obtained from ectomycorrhizas formed between *T. matsutake* and *Pinus densiflora*, and those of lateral roots of *P. densiflora*. Negative images according to Kalendar et al. (1999) are presented. Marker sizes are on the left. **A**, **B** PCR profiles with primer pS1 (**A**) or primer set pS48/pL281 (**B**) of samples obtained from ectomycorrhizas formed between *T. matsutake* Y1 and *P. densiflora* in vitro. Lanes: *1* Mycelia of *T. matsutake* Y1, *2* lateral roots of *P. densiflora*, *3* mycorrhizas formed between *T. matsutake* Y1 and *P. densiflora*. **C** PCR profiles of *T. matsutake-P. densiflora* ectomycorrhiza collected in Iwate Prefecture. Lanes: *1* Ectomycorrhiza (pS1), *2* lateral root (pS1), *3* ectomycorrhiza (pS48/pS281), *4* lateral root (pS48/pS281)

abundantly in the genome, occupying locations close to each other, so that the flanking sequences can be amplified by PCR (Kalendar et al. 1999). Homology search analysis revealed that the sequences corresponding to primers pS1, pS48 and pL281 are widely distributed to eukaryotes such as primates, fish, mouse and plants. According to RAPD theory (Williams et al. 1990), a single copy 1.0-kb DNA segment bound by a specific 22-bp sequence could occur in a 35-Mbp genome with a probability of 1.1×10^{-16}, an estimation based on the calculation $(3.5\times10^{7}\times10^{3})/(4^{22}\times4^{22})$. Therefore, the sequences of σ_{marY1} used as primers are ubiquitous in eukaryotes, but rather uniquely dispersed throughout the genome of *T. matsutake*, or clustered in a species-specific fashion in the fungal genome, in association with *marY1*. This notion implies that application of IRAP-PCR targeting σ_{marY1} may be effective in detection of strains of *T. matsutake* in nature, though it may still be necessary to examine a number of plant and fungal specimens associated with the symbiosis to establish a standardized methodology.

In general, retroelements are powerful genetic markers since they are stable as a part of the genome and are inherited through progenies of eukaryotes, while they may also replicate and integrate copies at other genetic loci in response to environmental stresses (Bushman 2002). Such a unique replication process allows a vertical radiation of phylogeny among related organisms, leaving traceable footprints of special events that triggered self-replication (Shimamura et al. 1997). From this viewpoint, it is interesting to note that pS1 generated polymorphic DNA in *T. magnivelare* as well as in *T. matsutake* while pS48/pL281 did so only in *T. matsutake*. The divergence observed in the fingerprints between these symbionts could be attributed to the occurrence of mutations in the internal portion of σ_{marY1}. In retroviruses, this part of LTR, designated R, corresponds to the end of the RNA form and is the site of the template switch (or 'jump') from one end to the other during reverse transcription, consequently generating extensive direct repeats to complete the DNA form, often inserting mutations (Lewin 2004). The observation indicates that *T. magnivelare*

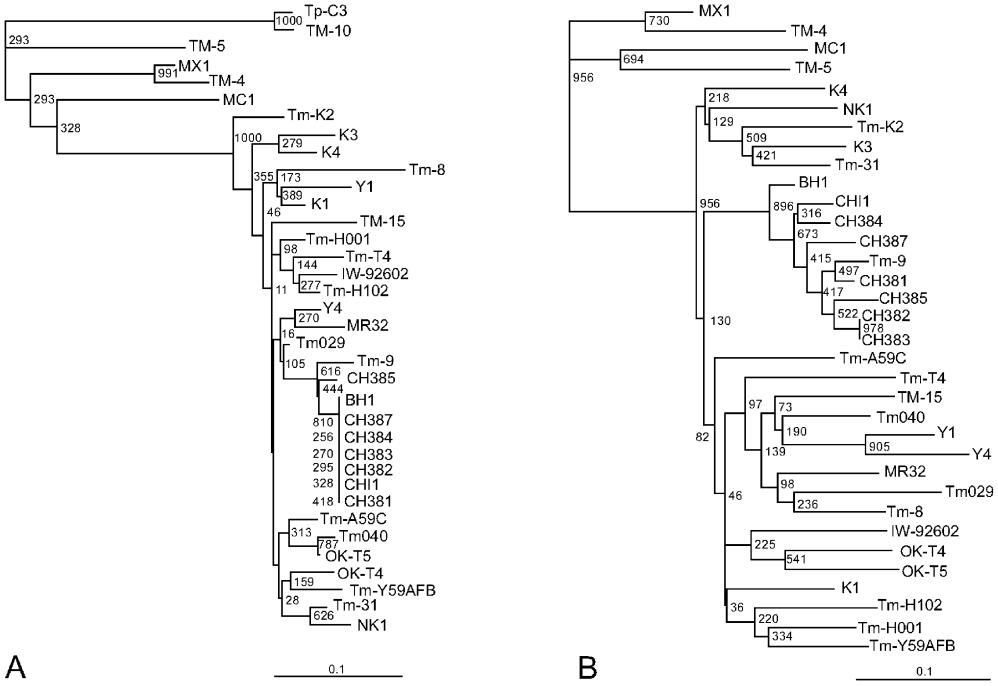

Fig. 5 Phylograms of strains of *T. matsutake* and *T. magnivelare* based on polymorphisms generated by PCR with pS1 (**A**) and pS48/pL281 (**B**). Neighbor-joining analysis was carried out with the Clustal X program (Thompson et al. 1997). Numbers of bootstrap samplings derived from 1,000 replications are given

(American matsutake), which is a much closer clade of *T. matsutake* than *T. bakamatsutake* and *T. fulvocastaneum* growing in Japan, has evolved uniquely after being isolated in the pacific northwest of America by a dramatic climate change that exerted a great impact on the pine ecosystem (Richardson 1998). This notion strongly suggests that the tremendous genome evolution involving replication and integration of *marY1* progressed during, but not prior to, the process of evolution of species that eventually brought about *T. matsutake* and *T. magnivelare*.

While polymorphisms of *T. matsutake* strains generally fell into groups reflecting their global origins, those of Japanese isolates were poorly correlated with their local sampling sites. In addition, the polymorphisms of Japanese isolates did not lead to a solid phylogenetic profile with generally low bootstrap values. Such complexity suggests that diverse genetic origins could have been involved in their intra-specific differentiation. Recently, eight distinct IGS1 rDNA types were identified in a population of *T. matsutake* basidiomata collected in Japan, in which one ribotype dominated (Guerin-Laguette et al. 2002). Our σ_{marY1}-based analysis indicated, however, that *T. matsutake* could have diversified locally involving recombination through a sexual cross, or even asexual mycelial interactions, generating mosaic or chimera, respectively, phenomena that confer further complex phylogenetic profiles. Asexual interactions among heterologous mycelia leading to phenotypic variations have been observed to occur rather frequently in homobasidiomycetes (Babasaki et al. 2003, Fukuda and Fukumasa-Nakai 1996, Peabody et al. 2000). It is likely that an apparently single uniform mycelial culture isolated from fruit bodies in vitro or a single mycorrhiza in nature may also consist of heterologous genetic resources, making typing difficult. Under such circumstances, σ_{marY1}-IRAP analysis, when applied to a number of *T. matsutake* specimens from a limited sampling area on a local scale, may help identify the composition of heterologous genetic resources in the mycorrhizas as well as in the fruit bodies of *T. matsutake*. Should such data be available, they may be much more informative than that based on the assay of specimens locally sampled on a global scale as presented in this study.

Recently, an inter-simple sequence repeat (ISSI)-PCR method using microsatellite markers was developed to detect strains of *T. matsutake* from mycorrhizas (Lian et al. 2003). However, such a method requires PCR with various sets of primers to complement insufficient polymorphic information consisting of only one or two bands, albeit the bands are specific to both species and strains (Lian et al. 2003). Unlike ISSI-PCR, a single set of PCR targeting sequences associated with σ_{marY1} will generate highly polymorphic genetic profiles, allowing *T. matsutake* to be specified in order to detect diversification within the species and to classify strains. Although the σ_{marY1}-based system is highly specific to *T. matsutake* and cannot be applied directly to the analysis of other ectomycorrhizal fungi, similar analytical methods can be conveniently applied once an LTR of a species-specific retroelement is identified. The system involves simple PCR without any further treatment such as restriction digests or nucleotide sequencing, unlike systems targeting the ITS region of rDNA (Kårén et al. 1997).

Díez et al. (2003) described a similar analysis in the ecotomycorrhizal homobasidiomycete *Laccaria bicolor* by targeting flanking sequences of the putative Ty1-*copia*-type reverse transcriptase domain (Ty1-RT), the internal coding region of the retroelement, instead of the LTR which is directly associated with the integration sites. Although the Ty1-RT system also successfully revealed species variations within *L. bicolor*, it is not clear whether it can clearly distinguish species as well as strains, the critical part in analyzing unknown samples from mycorrhizas in nature. In fact, the Ty1-RT system in *L. bicolor* was reported also to generate non-specific DNA bands from samples of the host plant *Pseudotsuga menziesii*. In this respect, the LTR-based PCR system can be a powerful tool to monitor *T. matsutake* mycorrhizas, since this system did not generate any detectable fragments in tested samples of closely related fungi and *P. densiflora*, the major host of *T. matsutake* in Japan. In fact, the usefulness of the method was demonstrated by the assay of mycorrhizas formed between *T. matsutake* and *P. densiflora* in vitro and in the field.

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