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SHORT NOTE

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Intra- and inter-specific variations in the copy number of two types of retrotransposons from the ectomycorrhizal basidiomycete *Tricholoma matsutake*

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Abstract To explore intra- and inter-specific variations of the ectomycorrhizal basidiomycete *Tricholoma matsutake* that produces the fruit body "matsutake", we carried out real-time PCR analysis based on two types of retrotransposons, one designated *marY1*, which resembles a retrovirus carrying the long terminal repeat (LTR) and the other *marY2N*, which resembles mRNA carrying the polyadenylated tail. Calculation based on the average genome size of homobasidiomycetes (34 Mbp) shows that ca. 5.5% of the total genome of *T. matsutake* isolated from Asia is made up of these retrotransposons, whereas they occupy ca. 1.4% in the isolates from Morocco, ca. 0.8% in isolates from Mexico, and ca. 0.5% in *Tricholoma magnivelare*, the species which produces "American matsutake". Other *Tricholoma* spp. that produce fruit bodies similar to those of *T. matsutake*, such as *T. bakamatsutake*, *T. fulvocastaneum*, and *T. robustum*, carry them in the region less than 0.05% of their total genome. Copy number of LTR of *marY1* is consistently and markedly higher than that of the coding regions of *marY1* and *marY2N*. Data suggest that retrotransposons are deeply involved in evolution of the ectomycorrhizal symbiont.

Keywords Basidiomycetes · Ectomycorrhizas · Genome evolution · Retrotransposons · *Tricholoma matsutake*

Introduction

Tricholoma matsutake is an ectomycorrhizal basidiomycete that produces the economically important edible mushrooms "matsutake" in association with *Pinus* sp. in the northern hemisphere (Hosford et al. 1997). Recently, matsutake was also recognized in *Quercus* sp. forests in the Yunnan province of China (Nakayama and Nakanishi 2004). Some other *Tricholoma* spp. produce fruit bodies similar to those of *T. matsutake* during ectomycorrhizal symbiosis (Hosford et al. 1997). For example, *Tricholoma magnivelare* produces large white fruit bodies known as American matsutake in association with a wide variety of conifers exclusively in the Pacific northwest region of America. *Tricholoma bakamatsutake* and *Tricholoma fulvocastaneum* produce rather yellowish fruit bodies known as baka (foolish)-matsutake and nise (false)-matsutake, respectively, during symbiosis with broad-leaved trees. *Tricholoma robustum*, a symbiont of conifers, is also a member of these fungi, producing matsutake-like mushrooms. Thus far, little information is available regarding intra- and inter-specific variations of *T. matsutake* and related fungi at the level of molecular genetics and genomics.

Retrotransposons are retrovirus-like DNA parasites associated with eukaryotic genomes. In response to environmental stresses, retrotransposons may replicate through an RNA-intermediate and allow their copies to integrate into other genomic loci with activities of reverse transcriptase, RNase H, and integrase, enzymes encoded in the *pol*yprotein (*pol*) gene (Bushman 2002). Such an incidence confers the occurrence of complexity of eukaryotic genomes and influences intra- and inter-specific diversification of organisms (Bushman 2002). We previously reported for the first time in basidiomycetes two types of retrotransposons designated *marY1* and *marY2N* (Murata and Yamada 2000; Murata et al. 2001b). *marY1* is a 6.0-kb *gypsy*-type retrotransposon carrying a 426-bp long terminal repeat (LTR) designated σ_{marY1}, which is closely related to mammalian retroviruses (Murata and Yamada 2000). *marY2N* is a member of long intersperse nuclear elements (LINEs), with the size variable among copies depending mostly upon the length of 5′- and 3′-terminal regions; its structure resembles mRNA in that it carries the polyadenylation signal and the polyadenylated tail at 3′ end (Murata et al. 2001b).

In the present study, we investigated the copy number of *marY1* and *marY2N* in the genome of *T. matsutake* and

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closely related fungi by a real-time PCR system. Such a system should allow PCR products to be quantified during the exponential multiplication phase of the reaction through the measurement of fluorescent light emitted by SYBR Green dye incorporated into double-stranded DNA (Higuchi et al. 1992). Thus, real-time PCR provides us with information on the number of specific genes and expressed genes, as well as on the size of the population of microbial cells in nature (Gibson et al. 1996; Heid et al. 1996). Through this novel approach, we were able to promote our understanding of the biology and, potentially, the ecology of commercially important ectomycorrhizal resources.

Materials and methods

Fungal strains and culture condition

Fungal strains used in this study are shown in Table 1. Fungal mycelia were cultured in a modified Melin-Norkrans liquid medium containing V8 juice (Campbell Soup Co., Camden, NJ, USA) instead of NaCl at the final conc. of 1.5%.

Plasmids for calibration of real-time PCR

The plasmid pHHM147 is the vector pSK+ carrying the 5.5-kb *Kpn*I-*Bgl*II genomic DNA of *T. matsutake* Y1 that is predicted to encode part of the *pol*yprotein designated pol_{marY1} (Murata and Yamada 2000; see also Fig. 1 for the structure of *marY1*). The plasmid pHHM149 is the vector pCR2.1 carrying the 0.65-kb 5′-σ_{marY1} region, i.e., the LTR of *marY1* (Murata and Miyazaki 2001). The plasmid pHHM166 is pSK+ carrying the 4.9-kb *Eco*RI genomic DNA of *T. matsutake* Y1, on which the *pol*yprotein gene designated $pol\text{-}1_{marY2N}$ resides (Murata et al. 2001b; see also Fig. 1 for the structure of *marY2N*).

Primers used for real-time PCR

Primers used in real-time PCR analysis are depicted in Fig. 1. The primers 5B and 3L were designed based on the sequence located onward at bp 309 and the complementary sequence backward at bp 426 of *marY1*, respectively (Fig. 1). These primers allowed the amplification of a 118-bp σ_{marY1} DNA segment in a variety of higher fungi (Fig. 1; Murata et al. 2001a). For the analysis of a coding region of *marY1*, the primers Y1-p and Y1-r were designed based on the sequence at bp 3,121 and the complementary one at bp 3,234 of *marY1*, respectively, which allowed the amplification of a 113-bp pol_{marY1} DNA segment (Fig. 1). We previously noted in *marY2N* two ORF-encoding *pol*yproteins (*pol*), one apparently encoding a protein not holding any catalytic domains ($pol\text{-}1_{marY2N}$) and the other encoding reverse transcriptase and RNase H ($pol\text{-}2_{marY2N}$) (Fig. 1; Murata et al. 2001b). Of these two *pol* genes, $pol\text{-}1_{marY2N}$, located between *gag* and $pol\text{-}2_{marY2N}$, was consistently noted in cloned copies of *marY2N*, a situation different from $pol\text{-}2_{marY2N}$, which is not always associated depending upon copies (Fig. 1; Murata et al. 2001b). Therefore, the primers Y2N-p and Y2N-r for assaying a coding region of *marY2N* were designed based on the sequence at bp 217 and the complementary one at bp 321 of $pol\text{-}1_{marY2N}$ (Fig. 1).

Table 1 Fungal strains used in this study

Species	Strains	Sampling site	Year
Tricholoma matsutake	Y1	*Pinus densiflora* forest, Ibaraki Prefecture, Japan	1993
	Y4	*P. densiflora* forest, Ibaraki Prefecture, Japan	1996
	K1	Republic of Korea[a]	1997
	K3	Republic of Korea[a]	2003
	K4	Republic of Korea[a]	2003
	Tm-9	People's Republic[a]	1992
	CHI1	People's Republic of China[a]	1998
	MC1	Kingdom of Morocco[a]	1998
	TM-5	Kingdom of Morocco[a]	1992
	MX1	Mexico[a]	1998
	TM-4	Mexico[a]	1992
Tricholoma magnivelare	Tp-C3	Canada[a]	1994
	TM-10	Canada[a]	1992
Tricholoma bakamatsutake	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
Tricholoma fulvocastaneum	WK-N1	*Quercus phillyraeoides* forest, Wakayama Prefecture, Japan	1988
Tricholoma robustum	KB1	*P. densiflora* forest, Nagano Prefecture, Japan	2000

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

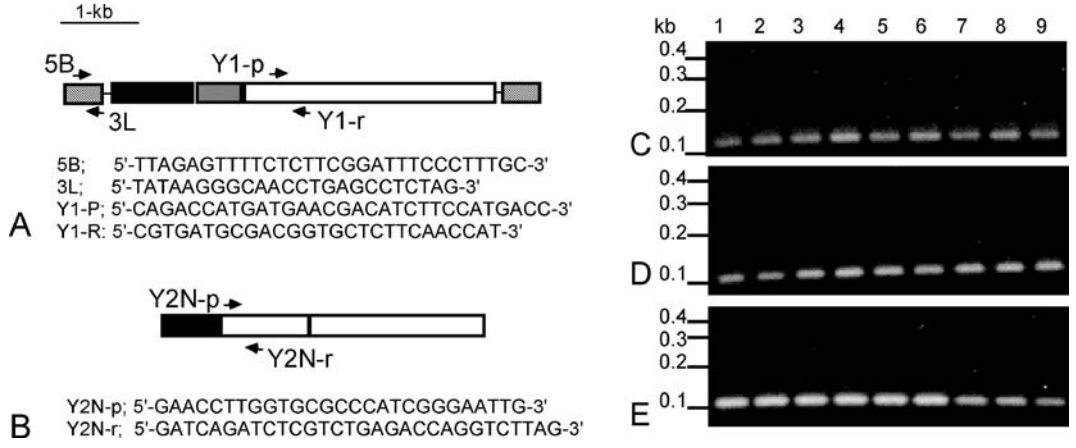

Fig. 1 Primer design to determine copy number of the retrotransposons *marY1* and *marY2N* in the genome of *Tricholoma* by the real-time PCR analysis. **A** Schematic representation of *marY1* and description of primers 5B, 3L, Y1-p, and Y1-r. Long terminal repeat designated σ_{marY1} and the pol_{marY1} coding region analyzed in this study are shown by the *hatched bar* and the *open bar*, respectively. Likewise, other coding regions, such as gag_{marY1} and prt_{marY1}, are given by the *solid bar* and the *shaded bar*, respectively. Locations of primers are indicated by the *arrows*. **B** Schematic representation of *marY2N* and description of primers Y2N-p and Y2N-r. gag_{marY2N} and two *pol* coding regions, i.e., $pol\text{-}1_{marY2N}$ and $pol\text{-}2_{marY2N}$, are given by the *solid bar* and the *open bar*, respectively. Locations of primers are indicated by the *arrows*. **C**, **D** and **E** Profiles of fragments amplified by real-time PCR with the primer set 5B/3L, Y1-p/Y1-r, and Y2N-p/Y2N-r, respectively. 1, *T. matsutake* Y1; 2, *T. matsutake* K1; 3, *T. matsutake* TM-9; 4, *T. matsutake* TM-5; 5, *T. matsutake* TM-4; 6, *T. magnivelare* TM-10; 7, *T. bakamatsutake* Cb-Tb2; 8, *T. fulvocastaneum* WK-N1; and 9, *T. robustum* KB

Real-time PCR analysis

Genomic DNA to be used as a template was isolated from frozen mycelia of fungal strains using a lysis buffer containing hexadecyltrimethylammonium bromide (Murata and Yamada 2000). Real-time PCR was carried out using primers described above in the LightCycler system (Roche Diagnostics Japan, Tokyo). The enzymatic reaction was conducted in 10 μl of a standard reaction mixture containing SYBR Green with 1.0 ng of an appropriate template DNA and 300 nM primers. Cycle reactions were performed in the sequence of 1×[95°C/10 min], followed by 40× [95°C/10 sec, 61°C/5 sec, and 72°C/3 sec]. Each set of PCR runs, which was carried out in duplicate, was calibrated with reference to a standard curve and a water control. The standard curve was constructed by real-time PCR with a plasmid containing a target DNA, in which a 1.0-ng/μl plasmid sample was serially diluted up to 10^{-9}, and 1 μl of each dilution was added to the 10-μl reaction mixture. The standard curve of the σ_{marY1}, based on the 5′-LTR of *marY1* cloned in the plasmid pHHM149, thus produced the slope (S) of log concentration (ng/ml) vs a cycle number of −3.077 with error (E) of 0.146 and regression coefficient (R) of −1.0. Similarly, the standard curves of coding regions of *marY1* and *marY2N*, i.e., pol_{marY1} and $pol\text{-}1_{marY2N}$, were drawn based on the plasmid pHHM147 and pHHM166, respectively. The former showed S of −3.214 with E of 0.169 and R of −1.0, and the latter showed S of −3.266 with E of 0.132 and R of −1.0. PCR products were recognized as showing a single melting curve that fits the melting curve of a control plasmid used for constructing a standard curve in the LightCycler system, confirming the authenticity of quantified PCR products. Furthermore, the amplified fragments were recognized as a single band of a predicted size in TBE–1.5% agarose gel electrophoresis (Fig. 1).

Determination of the copy number of the target DNA

The copy number of the target DNA in a single copy of the *T. matsutake* genome was established by the following equation, which used the average genome size of basidiomycetes published elsewhere (34 Mbp; Royer et al. 1991; Walz 2004); such a value was not available in *T. matsutake* due to the difficulty of generating protoplast cells.

$$\mathrm{Cn} = [V/(\mathrm{Fs} \times \mathrm{Mw}) \times N]/[\mathrm{Ga}/(\mathrm{Gs} \times \mathrm{Mw}) \times N] = (V \times \mathrm{Fs})/(\mathrm{Ga} \times \mathrm{Gs}),$$

where Cn is the copy number per genome; V, value of row data; Fs, size of a fragment used to construct a standard curve (bp); Mw, molecular weight of one bp nucleotide (Dalton); N, Avogadro constant; Ga, amount of chromosome used in the reaction (10 ng); and Gs, size of the genome (3.4×10^7 bp).

Alternatively, it might be possible to determine the copy number of target DNA referring to a single copy gene. However, such a gene is not identified in *T. matsutake*, as in the case with many other homobasidiomycetes, and it is possible that the presence of even a few pseudogenes homologous to the single copy gene used for the reference dramatically changes the result. Therefore, it should be much more reliable to use the average genome size worked out from data available in basidiomycetes such as *Agaricus bisporus* (34 Mbp), *Pleurotus ostreatus* (35 Mbp), *Phanerochaete chrysosporium* (32 Mbp), and *Schizophyllum commune* (35–36 Mbp) (Royer et al. 1991; Walz

2004). The average genome size thus obtained also corresponds to the genome size of the other group of higher fungi, ascomycetes such as *Aspergillus nidulans* (31 Mbp), *Curvularia lunata* (29.7 Mbp), *Penicillium chrysogenum* (32.8–34.1 Mbp), and *Trichoderma longibranchiatum* (33 Mbp) (Royer et al. 1991; Walz 2004). Although we may have to recalculate the data once the precise genome size of *T. matsutake* and related fungi becomes available, the change of the results should be marginal. Furthermore, the recalculation should affect all the data in parallel, rendering the comparative studies unimpaired.

Results

Both *T. matsutake* Y1 and Y4 were found to contain ca. 1,500 copies of σ_{marY1}, the LTR of *marY1*, in their genomes (Fig. 2). Such a high copy number was noted in *T. matsutake* K1 and K3 from Korea and CHI1 and TM-9 from China (Fig. 2). *T. matsutake* TM-5 and MC1, and TM-4 and MX1 that represent isolates from Morocco and Mexico, respectively, as well as *Tricholoma magnivelare* TM-10 and Tp-C3, contained ca. 300 copies of σ_{marY1}. All other fungi related to *T. matsutake* contained a far smaller number (less than 10 copies) of σ_{marY1}. As compared with σ_{marY1}, the copy number of the coding region of *marY1* was markedly low (Fig. 2). For example, Asian isolates of *T. matsutake* contained ca. 200 copies of pol_{marY1} in the genome, whereas the isolates from Morocco and Mexico had ca. 60 and 20 copies, respectively (Fig. 2). *T. magnivelare* TM-10 and Tp-C3 contained less than 10 copies of pol_{marY1} (Fig. 2) Other fungi related to *T. matsutake* were found to contain a single copy of pol_{marY1}. The copy number of a coding region of *marY2N* followed a similar trend to that of *marY1*, although the values of *marY2N* were consistently lower than those of *marY1* (Fig. 2). Based on the copy number and molecular size, we estimated that *marY1* and *marY2N* occupied ca. 5.5% of the genome of Asian isolates of *T. matsutake*, ca. 0.5–0.8% of the Morocco and Mexican isolates and *T. magnivelare*, and less than 0.01% in other closely related *Tricholoma* spp.

Discussion

Unlike the pol_{marY1} coding region of *marY1*, the LTR of *marY1* designated as σ_{marY1} is widely distributed in *T. matsutake* worldwide and in *T. magnivelare* with a markedly high copy number. This observation suggests that the majority of σ_{marY1} is a solo element without coding regions of *marY1* in the genome, and that σ_{marY1} itself exerted great influence on the evolution of *T. matsutake* in the genus *Tricholoma*. Coincidentally, we detected some features characteristic of short interspersed nuclear elements (SINEs), which are 75- to 500-bp DNA elements that replicate through an RNA intermediate, in the complementary strand of σ_{marY1} (DDBJ accession no. AB028236).

In humans, 45% of the genome draft sequence is derived from transposable elements, mostly retrotransposons (The

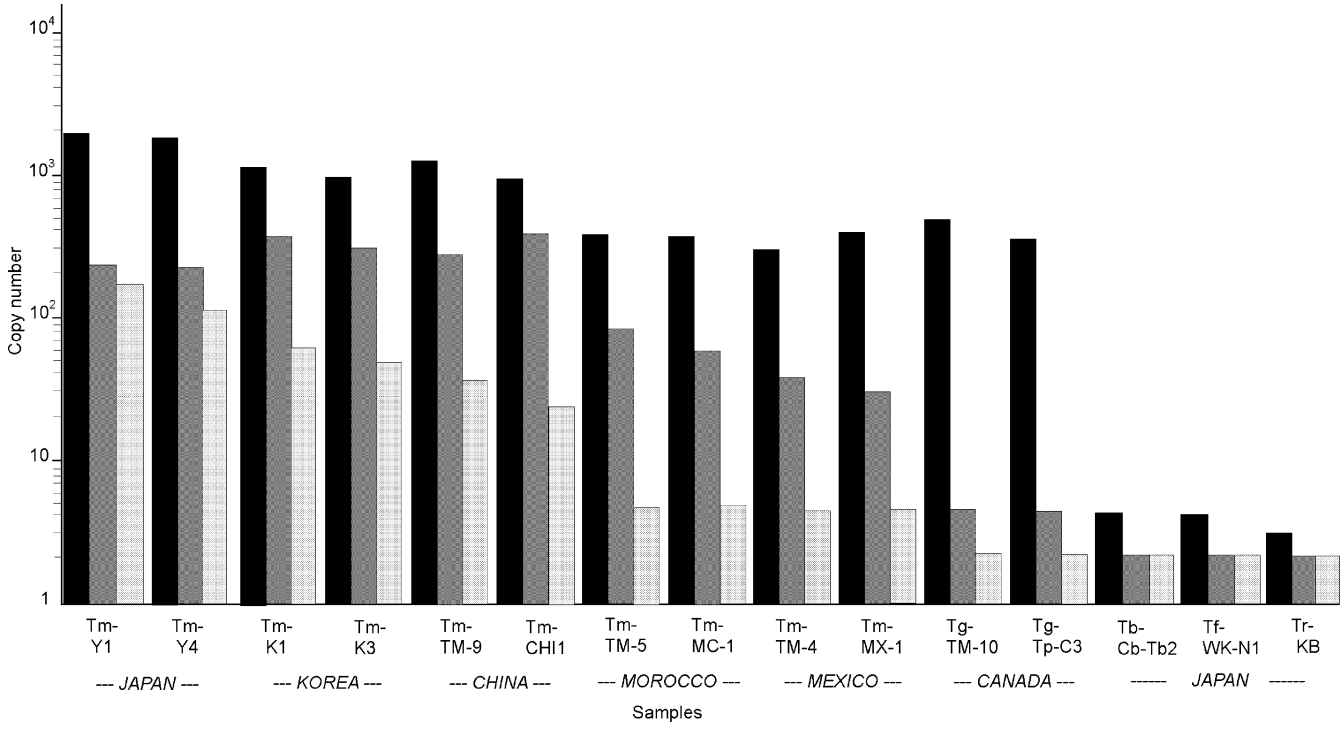

Fig. 2 Copy number of σ_{marY1} and pol_{marY1} derived from the retrotransposon *marY1*, and $pol\text{-}1_{marY2N}$ from *marY2N* in the genome of *T. matsutake* and closely related fungi. *Solid bar* represents the copy number of σ_{marY1}; *shaded bar*, copy number of pol_{marY1}; *dotted bar*, copy number of $pol\text{-}1_{marY2N}$. Following are the abbreviations used: Tm, *T. matsutake*; Tg, *T. magnivelare*; Tb, *T. bakamatsutake*; Tf, *T. fulvocastaneum*; and Tr, *T. robustum*. Fungal strains and their geographical origins are given

Human Genome Sequencing Consortium 2001; Nekrutenko and Li 2001). Of those, LINEs and SINEs are predominant in the human genome (Deininger 1989; Hutchison et al. 1989). In contrast to LINEs of humans, the copy number of a LINE designated *marY2N* is much less than that of the LTR retrotransposon *marY1* in *T. matsutake* and related fungi. This situation is apparently consistent with the fact that only a few LINEs have been observed in higher fungi, including filamentous ascomycetes, whereas LTR retrotransposons, which resemble retroviruses, have been ubiquitously identified (Pöggeler and Kempken 2004). We previously documented that reverse transcriptase genes of *marY2N*-like elements cloned from some ectomycorrhizal *Tricholoma* samples showed a vertical phylogenetic relatedness, consistent with their host–parasite relations and fungal taxonomy, whereas reverse transcriptase genes of *marY1*-like elements did not show such a relatedness, their mutual relations being horizontal (Murata et al. 2002). Therefore, the genome of *T. matsutake* could have dramatically evolved during a rather later evolutionary stage by the heavy involvement of LTR retrotransposons.

The 5′-region of *marY1* containing σ_{marY1} expresses in a heterologous *Saccharomyces cerevisiae* system, indicating that the promoters of *marY1* are recognizable by *trans*-acting factors shared in common among higher fungi (Murata and Miyazaki 2001). Although we cannot yet confirm this, as evidenced by the full-length transcript unit, σ_{marY1} may also be a component of various genes and *cis*-regulatory regions. In humans and mice, a large number of transposable elements, mostly retrotransposons, are identified in protein coding regions as well (Jordan et al. 2003; Nekrutenko and Li 2001; van de Lagemaat et al. 2003). Such a phenomenon is observable especially in genomes that have evolved lately (Jordan et al. 2003; Nekrutenko and Li 2001; van de Lagemaat et al. 2003).

In the phytopathogen *Magnaporthe grisea*, retrotransposons are tightly linked to genes that determine host plant-specificity (Farman et al. 2002; Shull and Hamer 1996; Talbot et al. 1993). Such a trend is also observed in another eukaryotic phytopathogen *Pyrenophora tritici-repentis* (Lichter et al. 2002; Martinez et al. 2004). In addition, phenotypic variants were generated by de novo integration of transposable elements in *M. grisea* (Kang et al. 2001; Nishimura et al. 2000; Shull and Hamer 1996). In *T. matsutake*, insertion sites of σ_{marY1} are highly polymorphic within the species (Murata et al. 2005). In fact, the present study shows that inter- and intra-specific variations could have occurred even regarding the copy number of retrotransposons in *T. matsutake*. We are currently trying to identify the yet unearthed transposons and characterize the genomics associated with transposable elements. Further transposon-based genome analysis in the future may greatly contribute to the understanding of biology and biodiversity of commercially valuable *T. matsutake*.

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