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Short Communication

Identification of ectomycorrhizae formed between *Tricholoma matsutake* and *Pinus densiflora* by polymerase chain reaction (PCR) targeting retroelement coding regions

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We previously reported that conservation and diversification of repetitive sequences carrying motifs of retroposons have occurred in *Tricholoma matsutake* and related ectomycorrhizal basidiomycetes through their evolution. Here we report that the polymerase chain reaction using primers designed to amplify retroelement coding regions specified ectomycorrhizae formed between *T. matsutake* and *Pinus densiflora*.

Key Words——ectomycorrhizae; *Pinus densiflora*; retroelement; *Tricholoma matsutake*.

Matsutake mushrooms, the fruiting bodies of the ectomycorrhizal basidomycete *Tricholoma matsutake* (S. Ito et Imai) Sing., are an economically important product of *Pinus* forest (Hosford et al., 1997). Like many other ectomycorrhizal basidomycetes, *T. matsutake* produces fruiting bodies only when the organism forms a mycorrhizal association with conifers, mostly *Pinus densiflora* Sieb. et Zucc. in Japan (Smith and Read, 1997). Because ectomycorrhizal resources are endangered in nature, and because of the difficulty of sustainable culture in the field as well as in spawns, measures to conserve them are urgently needed (Hosford et al., 1997; Torigoe, 1998). To achieve this objective, it is important to develop an efficient method to identify mycorrhizae and monitor their ecology, because the visual examination of the direct connection between *shiro* and the fruiting bodies currently used requires skill and does not index them efficiently taking genetic diversity into account (Agerer, 1991; Hosford et al., 1997; Ogawa, 1985).

For the past decade, various attempts have been made to analyze the diversity of ectomycorrhizae at the molecular level in other fungal genera, and in some cases it became possible to monitor the ecology of mycorrhizae, for example, by analysis of genes encoding rRNA (Amicucci et al., 1998; Bruns et al., 1998), chitin synthase (Mehmann et al., 1994), or glycerol-3-phosphate dehydrogenase (Kreuzinger et al., 1996). In species of *Tricholoma*, Nakai et al. developed a polymerase chain reaction (PCR) system that allows *T. matsutake* to be distinguished from other related fungi by specifying the variable positions in the 18S rRNA gene (Nakai and Hishinuma, 1992; Nakai and Ohno, 1994). Subsequently, this approach was applied to the identification of ectomycorrhizae formed between *T. bakamatsutake* Hongo and Fagaceae trees (Terashima and Nakai, 1996). While these methods are highly reliable at species-specific level, they generally require nucleotide sequencing analysis of each targeted gene of each specimen, which is not feasible for those who do not have access to a sequence analysis system. In addition, these genes are too highly conserved to allow differentiation of strains within the species.

We previously reported for the first time in ectomycorrhizal basidiomycetes that two types of repetitive sequences carrying motifs of retrotransposons are linked to the genome of *T. matsutake* (Fig. 1A; Murata et al., 1999): one with a high copy and the other with a low copy in the genome of strains of *T. matsutake*. These sequences have been cloned in a vector generating the plasmids pHHM145 and pHHM146, respectively (Fig. 1A; Murata et al., 1999). The cloned genetic elements are markedly conserved in *T. matsutake*, segmentally conserved in *T.* (Peck) Redhead (=American matsutake), and extensively diversified in 11 other *Tricholoma* species and three other related genera (Murata et al., 1999). Here we report that PCR with primers designed to amplify the repetitive elements successfully identified ectomycorrhizae formed between *T. matsutake* and *P. densiflora*.

Axenic seedlings of *P. densiflora* strain Ypd1 were co-cultivated with mycelia of *T. matsutake* strain Y1 in a

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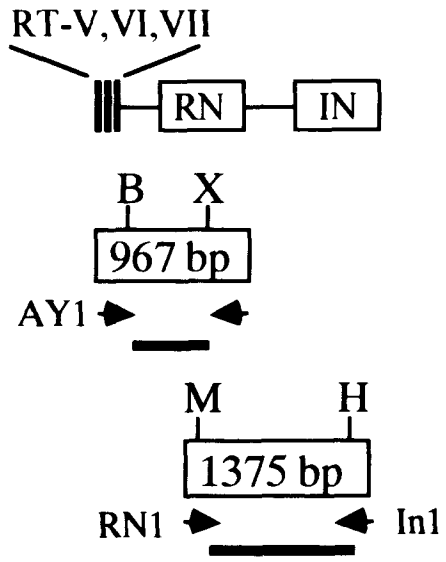

Description of primers and PCR products

AY = 5'-GCCTATTTGGACGACATCCTC-3'
Anealing temperature = 50-55 °C
Original clone = the plasmid pHHM145
(Murata et al., 1999)

In1 = 5'-GCAAGAGGTGAACTTCGAG-3'
RN1 = 5'-GGACAGTCCTTCCAGTGGC-3'
Anealing temperature = 48 °C
Original clone = the plasmid pHHM146
(Murata et al., 1999)

[A]

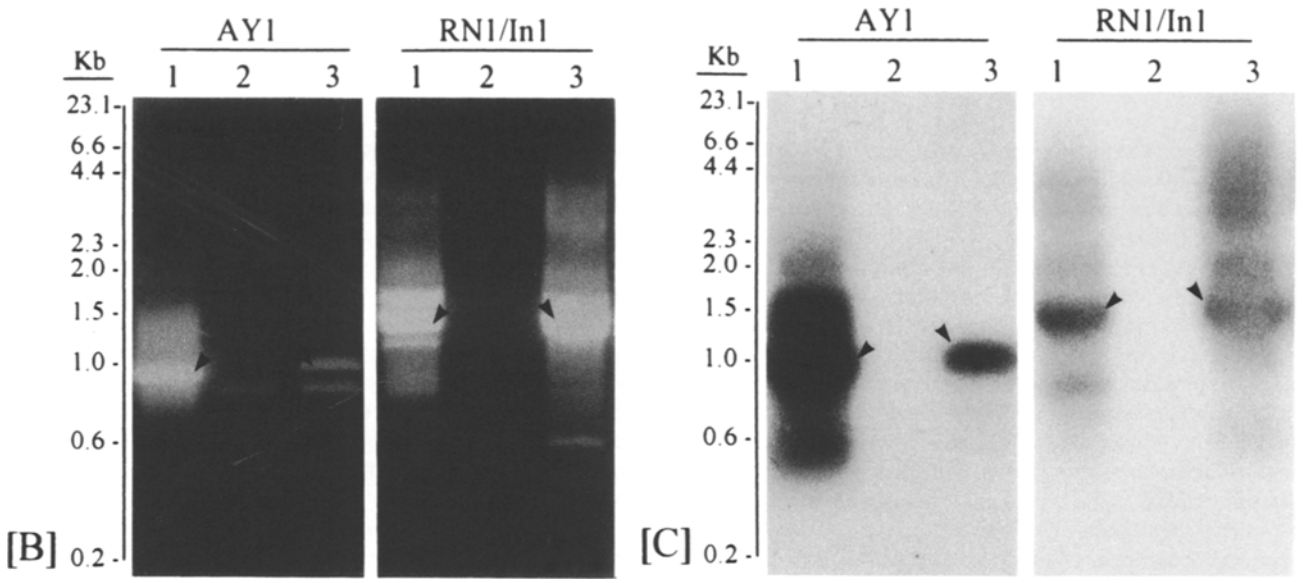

Fig. 1. Polymerase chain reaction (PCR) designed to probe ectomycorrhizae formed between *T. matsutake* and *P. densiflora*. [A] Schematic representation of the PCR products previously cloned in the plasmids pHHM145 and pHHM146, and description of primers designed to amplify the fragments. The cloned 967-bp and 1375-bp fragments are aligned relative to reverse transcriptase domains V, VI and VII (RT-V, VI, VII), RNase H (RN), integrase (IN), and intervening sequences (thin line) of a retrotransposon. Arrows indicate location and direction of primers AY1, RN1, and In1. Solid lines depict the fragments used for Southern hybridization analysis shown in [C] as a probe. Abbreviations: B, *Bam*HI; H, *Hin*dIII; M, *Mun*I; X, *Xba*I. [B] PCR profiles of samples obtained from ectomycorrhizae formed between *T. matsutake* strain Y1 and *P. densiflora* strain Ypd1. Representative data of four independent analyses using four independent samples prepared by two axenic culture methods are given (Ohta, 1994; Yamada et al., 1999). Primers used for the analysis are indicated on top of each panel. Lane 1, mycelia of *T. matsutake* strain Y1; lane 2, lateral roots of *P. densiflora* strain Ypd1; lane 3, mycorrhizae formed between *T. matsutake* strain Y1 and *P. densiflora* strain Ypd1. Bands corresponding to fragments originally cloned in the plasmids pHHM145 and pHHM146 are highlighted by arrowheads (see [A]; Murata et al., 1999). Molecular markers (kb) are indicated in the left column. [C] Southern hybridization analysis conducted on the PCR profiles shown in [B] by using the *Bam*HI-*Xba*I fragment of pHHM145 and the *Mun*I-*Hin*dIII fragment of pHHM146 as probes. Panels, lanes, and marks correspond to those shown in [B].

spawn containing a mixture of peatmoss, vermiculite and components of the MNC medium, or the medium used for cultivation of *Lyophyllum shimeji* (Kawam.) Hongo. at 20°C for 6 months (Ohta, 1994; Yamada et al., 1999). After the cultivation, the lateral roots of *P. densiflora* surrounded by mycelia, i.e., the fungal sheaths, were microscopically examined for the intercellular penetration of the hyphae to confirm the formation of the Hartig net. Prior to the examination for the Hartig net, the fungal sheaths were carefully removed with a fine forceps. The samples were then freeze-dried and used for the extraction of DNA (Dobinson et al., 1993). As a control, genomic DNA of individual organisms cultured separately in the same way was used as a template for the analysis.

PCR was conducted in 20-μl reaction mixtures containing 250 μM dNTP, 0.5 μM primers, 40 ng of template DNA, 0.7 U of Taq polymerase (Gene Taq NT, Wako Pure Chemicals, Osaka, Japan), and the universal buffer provided with the enzyme. Cycle reactions were performed as follows: 1× [94℃/2 min], 30× [94℃/1 min, annealing temperature/1 min and 72℃/5 min] and 1× [72℃/10 min]. All the PCR reactions were performed in a 200-μl microtube using TaKaRa thermal cycler TP3000 (Takara Shuzo Co, Otsu, Japan). Description of primers and annealing temperature used in this study are given in Fig. 1A.

The PCR reaction with a primer AY1, which amplifies the conserved 967-bp fragment carrying reverse transcriptase domains V, VI and VII, and RNase H of retroelements in the samples of *T. matsutake*, generated products of the same size in the samples prepared from ectomycorrhizae, whereas no product corresponding to the fragment was noted in those of lateral roots of *P. densiflora* (Fig. 1A and B). Similarly, the reaction with a pair of primers, RN1/ In1, that generated the 1375-bp fragment encoding RNase H and integrase of retroelements in the samples of *T. matsutake* also yielded the distinctive products in samples of ectomycorrhizae (Fig. 1A and B). Southern hybridization analysis was conducted to confirm that the products corresponding to the 967-bp and the 1375-bp fragments obtained from ectomycorrhizae contained homologues of the repetitive sequence originally cloned in the plasmids pHHM145 and pHHM146, respectively (Fig. 1A; Murata et al., 1999). The internal *Bam*HI-*Xba*I of pHHM145 and the *Mun*I-*Hin*dIII of pHHM146 were labeled with [^{32}P]dCTP and used as probes (Fig. 1A). Hybridization signals were detected in samples obtained from *T. matsutake* mycelia and its mycorrhizae formed in the lateral roots of *P. densiflora*, unlike samples from the lateral roots alone (Fig. 1C).

We previously reported that PCR using primers AY1 and RN1/In1 to amplify the 967-bp and the 1375-bp DNA, respectively, which are predicted to encode a motif of retroposons, clearly distinguished 11 strains of *T. matsutake* isolated from various locations worldwide from 21 strains of 10 other species of *Tricholoma* and related ectomycorrhizal fungi including *T. magnivelare*, *Tricholoma ustale* (Fr.: Fr.) Kummer, *T. flavovirens* (Pers.: Fr.) Lund, *T. portentosum* (Fr.) Quél., *T. saponaceum* (Fr.) Kummer, *T. japonicum* Kawamura, *T. sejunctum* (Sow.: Fr.) Quél., *T. bakamatsutake*, *T. fulvocastaneum* Hongo, *T. giganteum* Massee, *Suillus bovinus* (L.: Fr.) O. Kuntze, *Rhizopogon rubescens* (Tul.) Tul., and *L. shimeji* (Murata et al., 1999). In addition, conservation and diversification of the repetitive sequences in *T. matsutake* and other fungi markedly correlate to our current interpretation of polyphyletics of fungi, and of evolution of *Pinus* sp. plants and ectomycorrhizae (Bruns et al., 1991; Hibbett et al., 1997; Murata et al., 1999; Richardson, 1998; Sugiyama, 1998). In these respects, the method described in this report is a simple and powerful way to identify ectomycorrhizae formed between *T. matsutake* and *P. densiflora*. Recently, Ito et al. (1998) have reported the application of random amplified polymorphic DNA analysis for the identification of *T. matsutake* cultured *in vitro*, to distinguish it from three other *Tricholoma* species. Our method could be used to monitor mycorrhizae by specifying the putative retroelements which could be acquired by the origin of *T. matsutake* and ectomycorrhizal and plant parasitic clades, and diversified through their evolution in terms of host specificity and/or geological niches (Murata et al., 1999). Further development of the methodology based on the present study may contribute significantly to the conservation of the ectomycorrhizal species, the selection of specific strains of *T. matsutake* and *P. densiflora* for a reinoculation program, and commercial production of fruiting bodies of matsutake.

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