Mycorrhiza (2003) 13:27–31
DOI 10.1007/s00572-002-0193-6

ORIGINAL PAPER

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Development of microsatellite markers from an ectomycorrhizal fungus, *Tricholoma matsutake*, by an ISSR-suppression-PCR method

Received: 20 February 2002 / Accepted: 3 July 2002 / Published online: 14 August 2002

Abstract An inter-simple sequence repeat (ISSR)-suppression-PCR technique established to develop microsatellite markers of plant species was applied to an ectomycorrhizal fungus, *Tricholoma matsutake*. Six polymorphic SSR markers were developed. All six polymorphic SSR markers were single-locused and co-dominant. Alleles produced by these six single-locused markers ranged from two to nine per locus and the expected heterozygosities were calculated as values from 0.098 to 0.803. The results indicated that the ISSR-suppression-PCR technique was effective and applicable to the development of microsatellite markers from ectomycorrhizal fungi. Furthermore, the six microsatellite loci did not amplify DNA from any other ectomycorrhizal species investigated, except for *Tricholoma nauseosum* (Swedish matsutake) and *Tricholoma fulvocastaneum*, suggesting that population genetics and reproduction of *T. matsutake* could be investigated by the SSR markers developed in the present study.

Keywords Mycorrhizal fungus · Population genetics · SSR polymorphism · *Tricholoma matsutake*

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Introduction

Population genetic analysis of ectomycorrhizal fungi in the forest has revealed important aspects of their reproduction. Such analysis has usually been performed based on genetic differences among sporocarps. So far, several kinds of techniques have been employed for detecting genetic differences among sporocarps. Several early investigations analyzed genet dynamics in forests by somatic incompatibility tests (Fries 1987; Dahlberg and Stenlid 1994). However, since this method requires the isolation and culture of fungal strains, it seems to be applicable only to a few species. Molecular markers such as inter-simple sequence repeat (ISSR) markers have also been increasingly employed as more effective tools for understanding genet structures of ectomycorrhizal fungi (Gherbi et al. 1999; Zhou et al. 1999, 2000; Anderson et al. 2001; Sawyer et al. 2001). Such markers provide valuable information about the size and distribution of genets among sporocarps. However, the genetic relationship between genets and overall gene flow that reflects spore dispersal cannot be revealed using such markers.

In addition, it has been demonstrated repeatedly that the amount and distribution of ectomycorrhizal sporocarps does not necessarily reflect that of their subterranean parts, indicating that the analysis of subterranean parts of ectomycorrhizal fungi is necessary for understanding the true genet dynamics of a given ectomycorrhizal fungus (Zhou et al. 2001b). Samples from subterranean parts usually contain DNA from several fungal species and host tree roots. Therefore, use of ISSR markers is not applicable because they may simultaneously amplify too many fragments from mixed DNA templates to identify the genotype of the given fungal species.

Recently, microsatellite markers were used as more powerful molecular markers in the population genetic analysis of an ectomycorrhizal fungus. Zhou et al. (2001a) demonstrated that sporocarp analysis using such markers provides some new information on the genetic relationship between genets and gene flow in *Suillus grevillei* populations. Microsatellite markers were also

successfully applied to identify genets among mycelia in subterranean mycorrhizae (Zhou et al. 2001b). However, despite the usefulness of microsatellite markers for the investigation of population genetics, reports on the development, characterization and use of microsatellite markers in ectomycorrhizal species are still scarce (Kretzer et al. 2000; Zhou et al. 2001a). According to experiences in our laboratory, microsatellite regions seem to occur less frequently in the genome of ectomycorrhizal fungi compared with those of plants and animals, and their isolation by conventional methods seems to be much more difficult.

Last year, we established an ISSR-suppression-PCR method for developing microsatellite markers (Lian et al. 2001). This method enabled us to develop microsatellite markers from several plant species much more easily and rapidly. If this method were also effective in microsatellite marker development from ectomycorrhizal fungi, it would enable great progress in population genetics studies.

In this study, we applied the ISSR-suppression-PCR method to develop microsatellite markers for an ectomycorrhizal fungus, *Tricholoma matsutake*, which forms economically important and edible mushrooms in Japan.

Materials and methods

Fungal samples

T. matsutake sporocarps were collected from different places in Japan. *Tricholoma bakamatsutake* (No. 30663), *Tricholoma fulvocastaneum* (No. 6940 and 6949), *Tricholoma ponderosum* (syn. *T. magnivelare*) (No. 6939) and *Tricholoma robustum* (No. 6936 and 8332) mycelia were obtained from IFO, Osaka, Japan. *Tricholoma caligatum* and *Tricholoma nauseosum* (Swedish matsutake) were collected in Italy and Sweden. All sporocarps of other species were collected in Japan.

DNA isolation

DNA was extracted from air-dried or freeze-dried sporocarps of *T. matsutake* by a modified CTAB method as described previously (Zhou et al. 1999; Lian et al. 2001) and stored at –30°C.

Microsatellite retrieval

Microsatellites were isolated by the ISSR-suppression-PCR technique outlined in Lian et al. (2001). As the first step in isolating microsatellite loci, ISSR fragments flanked by two microsatellite sequences at both ends were separately amplified from the *T. matsutake* genome by one or a pair of SSR primers, namely $(AC)_{10}$, $(GA)_{10}$, $(GTG)_6$, $(GCT)_5$, a combination of $(AC)_{10}$ and $(GTG)_6$, or a combination of $(GA)_{10}$ and $(GTG)_6$. The amplified fragments were directly ligated into pT7 Blue vectors (Novagen, Wis.) according to the manufacturer's instructions and were subcloned into *Escherichia coli*, strain XL1-Blue MRF′. Plasmid DNA was extracted from positive clones and sequenced using a Thermo Sequenase Pre-mixed Cycle Sequencing Kit (Hitachi, Tokyo) with the T7 or M13 forward primer labeled with Texas Red (Hitachi, Tokyo) in a SQ-5500 sequencer (Hitachi, Tokyo). In this step, the sequence of the region between two microsatellite loci, i.e. one of two regions flanking each microsatellite locus was determined. To walk the other unknown region, a primer (IP1) designed from the sequenced region and, for nested PCR, another primer (IP2) based on the sequence between IP1 and the microsatellite were prepared. As the second step, DNA was separately digested with a restriction enzyme: *Eco*RV, *Ssp*I, *Alu*I, *Afa*I, *Acc*II or *Hae*III (Takara Shuzo, Tokyo). The fragments were then ligated with a suppression PCR adaptor consisting of a 48-mer single-stranded oligonucleotide (5′-GTAATACGACTCACTATAGGGC-ACGCGTGGTCGACGGCCCGGGCTGGT-3′) and a complementary 8-mer single-stranded oligonucleotide with the 3′-end capped by an amino group (5′-ACCAGCCC-NH_2-3′) using a DNA Ligation Kit (Takara Shuzo, Tokyo), resulting in six restriction enzyme libraries. As adaptor-primers for nested PCR, AP1 (5′-CCAT-CGTAATACGACTCACTATAGGGC-3′) and AP2 (5′-CTATAG-GGCACGCGTGGT-3′) were also prepared. The primary PCR reaction was conducted with each constructed DNA library using primers IP1 and AP1. The secondary PCR reaction was conducted using primers IP2 and AP2 and a 100-fold dilution of the primary PCR products. Single-banded fragments were derived from some libraries and one of the single-banded fragments was subcloned and sequenced as described above. A second primer (IP3) from the newly defined flanking sequence was designed for amplification of the region containing a microsatellite. The primer pair, IP1 or IP2 and IP3 (Table 1), was examined as a microsatellite marker.

Primer screening and PCR amplification

Forty-one sporocarps of *T. matsutake* collected from various locations in Japan were used for primer screening. Microsatellite amplification was performed by a PCR thermal cycler (TP3000; Takara Shuzo, Tokyo) in a reaction mixture (10 µl) containing 20 ng template DNA, 0.4 mM of each dNTP, 0.2 µM of each designed primer pair (ten in total, see Table 1) of which one primer was labeled with Texas Red (Hitachi, Tokyo), 1× GC buffer I (Takara Shuzo, buffer composition unknown) with 2.5 mM Mg^{2+}, and 0.5 U LA *Taq* DNA polymerase (Takara Shuzo). The PCR conditions were as follows: 1 min at 94°C for 1 cycle, followed by 29 cycles of 30 s at 94°C, 30 s at the annealing temperature of each primer pair (shown in Table 1) and 30 sec at 72°C, followed by 1 cycle of 30 s at 94°C, 30 s at the annealing temperature of each primer pair and 5 min at 72°C. The reaction products were electrophoresed on a 6% Long Ranger sequencing gel (FMC BioProducts, Me.) using an SQ-5500 sequencer (Hitachi). Electrophoretic patterns were analyzed with FRAGLYS version 2 software (Hitachi).

Microsatellite amplification from DNA of different ectomycorrhizal species

PCR fragments were amplified using markers developed from DNA of other ectomycorrhizal species. The amplified products were visualized by 1.5% agarose gel electrophoresis followed by ethidium bromide staining.

Results

SSR development

PCR fragments were amplified from the *T. matsutake* genome using different SSR primers. Their electrophoretic patterns in agarose gels are shown in Fig. 1. No PCR product was detected using the $(GA)_{10}$ primer. The $(AC)_{10}$ primer produced only a single band pattern. Primer $(GTG)_6$ and a mixture of primers $(GTG)_6$ and $(GA)_{10}$ produced several bands. In the case of $(GCT)_5$ and mixture of $(AC)_{10}$ and $(GTG)_6$, faint bands against a smeared background were observed. The products amplified by $(AC)_{10}$, $(GTG)_6$, $(GCT)_5$, and $(AC)_{10}$ and $(GTG)_6$ were

Table 1 Characteristics of microsatellite markers isolated from *Tricholoma matsutake*. T_a Annealing temperature, H_o observed heterozygosity, H_e expected heterozygosity

Locus	Repeat	Primer sequence(5′–3′)	T_a (°C)	Size (bp)	Allele no.	H_o	H_e
Trma01	$ACGAAC(ACGACC)_2$ $(ACGAAC)_4ACG...(AC)_3$	GTCTACCCCATGTGCTCATCC CTTCCCCCACACCTATCTACG	60	228	3	0.267	0.316
Trma02	$(AAC)_2(AC)_5$	GTGGATAAGAACGAGGTCACC TGTGGACCTGGTACGTACTAG	60	106	2	0.100	0.098
Trma06	$(TC)_3(TTC)_2CT(TC)_3$ $(CT)_4...(GTG)_2$	CACCCTATTCTGATGGGTGTG CTTGAGCCTTGTGGTTCAAGG	60	186	1	–	–
Trma07	$(CAC)_6$	GTGTGACTGCTCGTGGGCTGGATAATG CACAATCCACCCTACAAGCAG	60	125	5	0.367	0.421
Trma08	$(CAC)_3$	CATCACACCTGCCAGCTCATC CCCAAATGACATCCATCATCG	60	200	4	0.233	0.221
Trma10	$(AC)_3TC(CAC)_4$	CTTGTGAGCCACCTGCCATTC TTGAGGCGGTGATATGGGGTG	60	116	1	–	–
Trma12	$(GCA)_5$	CTGCGGCATCCTTTCTAGAGC ACATGCTGACTCAGGCTTGCG	60	200	1	–	–
Trma13	$(CAG)_5$	ATGACAACGACAACTCCCAGG GGACTGGCAACTTTGCAGCAG	60	210	1	–	–
Trma14	$GCTGGT(GCT)_6$	GCTTTGTCTCAGCCTTCAAAG AAGACAACCACAAATCCTCCC	60	140	5	0.600	0.575
Trma16	$C_{10}AGTCAT_8...(GA)_3(GT)_2$	CTGTGACTCAGTGTTCTTGTG ATGTGATAGTGTCAGAGGCAC	60	136	9	0.500	0.803

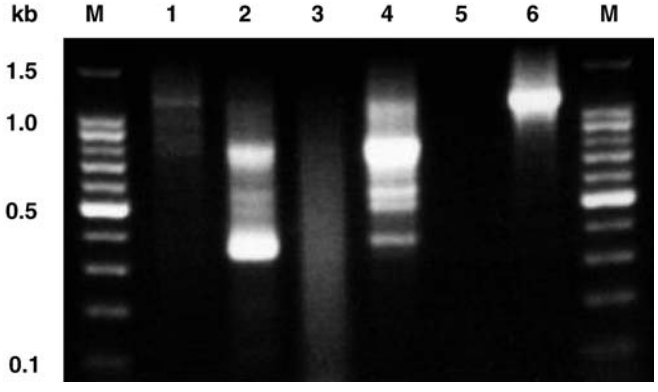

Fig. 1 PCR products amplified by a single, or pairs of, simple sequence repeat (SSR) primers from the genomic DNA of *Tricholoma matsutake*. Lanes: *1–6* Products amplified by primers $(GCT)_5$, $(GA)_{10}$ and $(GTG)_6$, $(AC)_{10}$ and $(GTG)_6$, $(GTG)_6$, $(GA)_{10}$, and $(AC)_{10}$, respectively; *M* DNA size markers (100 bp ladder; Takara Shuzo, Tokyo)

used for the subsequent subcloning and sequencing. Fourteen microsatellite sequences were chosen to design the nested PCR primers (IP1 and IP2) for the determination of the unknown flanking region; four microsatellite sequences from the fragments amplified by $(AC)_{10}$, four from $(GTG)_6$, three from $(AC)_{10}$ and $(GTG)_6$, and three from $(GCT)_5$. In the second step, nested PCRs for 12 microsatellite sequences produced single bands from some restricted DNA libraries. All of these microsatellite sequences were successfully cloned and sequenced from one of the single-banded PCR products. All of them contained microsatellites. Of these 12 microsatellite loci, a primer (IP3) from the newly defined flanking sequence was designed for ten microsatellite loci. Each of the ten microsatellite loci was amplified using a pair of primers, IP1 or IP2 and IP3 (Table 1). The remaining two loci, Trma04 [motif: $(GT)_2GG(GT)_2G(GT)_3$] and Trma05 [motif: $(CCA)_2$] were not used for further analysis since the sequence repeats were too short.

SSR characterization

Of the ten primer pairs designed, four were monomorphic for all samples and the other six were polymorphic. These six polymorphic SSR markers amplified one or two bands, being single-locused and co-dominant. Alleles produced by the markers ranged from 2 to 9 per locus and the expected heterozygosities were calculated as values from 0.098 to 0.803 (Table 1). Only one allele had a markedly larger frequency than other alleles in five SSR markers (Fig. 2). The other marker was the most polymorphic locus (Trma16), showing a more uniform allele frequency distribution. One (allele 106) of the two alleles in Trma02 represented more than 95% of the total frequency. Some alleles appeared only at single locations; allele Trma01–203 appeared only at Seto in Aichi Province, Trma07–124 at Minamiaiki in Nagano, Trma07–134 at Ina in Nagano, Trma08–181 at Hiyoshi in Nagano, Trma08–188 at Yahiko in Niigata, Trma14–127 at Kanayama in Gifu, Trma16–142 at Maruko in Nagano, and Trma16–143 at Tatsuno in Nagano. The 41 sporocarp samples were divided into 34

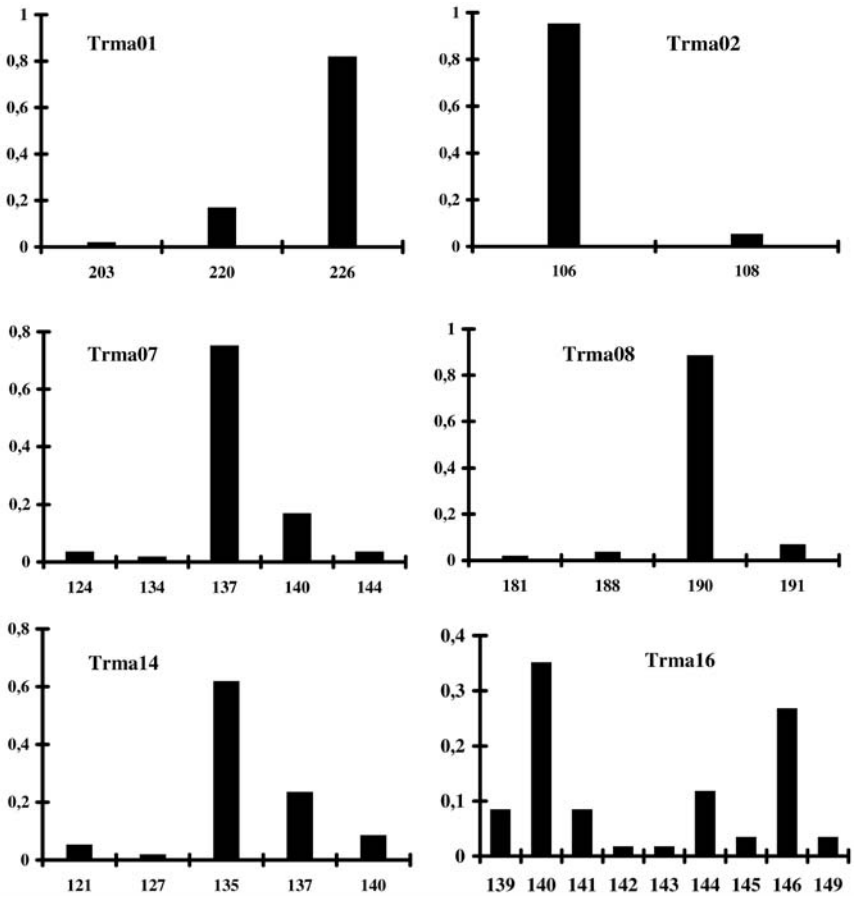

Fig. 2 Allele frequency distribution for six microsatellite loci. The x- and y-axes indicate allele size in base pairs and allele frequency, respectively

Table 2 Ectomycorrhizal species used in this study

Species	Origin
Amanita flavipes	Japan
Boletinus cavipes	Japan
Boletus fraternus	Japan
Boletus pulverulentus	Japan
Chroogomphus rutilus	Japan
Hebeloma crustuliniforme	Japan
Hebeloma mesophaeum	Japan
Inocybe fastigiata	Japan
Inocybe lacera	Japan
Lactarius hatsudake	Japan
Laccaria laccata	Japan
Ramaria botrytis	Japan
Russula cyanoxantha	Japan
Russula mariae	Japan
Russula nigricans	Japan
Suillus grevillei	Japan
Suillus laricinus	Japan
Suillus luteus	Japan
Suillus spectabilis	Japan
Tricholoma bakamatsutake	Japan (IFO)
Tricholoma caligatum	Italy
Tricholoma fulvocastaneum	Japan (IFO)
Tricholoma imbricatum	Japan
Tricholoma matsutake	Japan
Tricholoma nauseosum	Sweden
Tricholoma nauseosum	Italy
Tricholoma ponderosum	Japan (IFO)
Tricholoma robustum	Japan (IFO)

genotypes by the combination of six polymorphic SSR markers. Most of genotypes were represented by only one individual. Four individuals from Ina in Nagano, four from Izumozaki in Niigata, and two from Yahiko in Niigata, were identified as the same genotype.

Twenty-seven other ectomycorrhizal species (Table 2) were also tested for amplification using the ten primer pairs designed from *T. matsutake*. Although all ten loci were also amplified in *T. nauseosum* (in isolates from Sweden and Italy), only locus Trma01 was amplified in *T. fulvocastaneum*, and no fragments were amplified from templates of the other 24 species using any of the markers (data not shown).

Discussion

In previous work, we amplified ISSR fragments from DNA templates of plants such as *Salix reinii*, *Pinus densiflora* and *Robinia pseudoacacia* using the $(AC)_{10}$ primer (Lian et al. 2001) or the $(GA)_{10}$ primer (unpublished data). The fragments exhibited both clear and smeared banding patterns on agarose electrophoresis and contained a number of different ISSR loci. In the present study, ISSR fragments were amplified by primers corresponding to GA, AC, GTG, and GCT repeats from *T. matsutake* templates of the same amount and under the

same conditions as those used previously for plants. These amplifications produced either no band, only a few clear bands without smeared background, or faint bands against a thin smeared background (Fig. 1). The number of ISSR fragments of *T. matsutake* detected in agarose gels seems to be much less than that in plant species. Since the number of ISSR fragments in agarose gels may correspond to the number of SSR loci distributed in the genome, the results indicate that microsatellites of GA, AC, GTG, and GCT are much rarer in *T. matsutake* than in plants. The fact that fewer ISSR loci were detected after subcloning these amplified products in *T. matsutake* than in plants also suggests the rareness of *T. matsutake* microsatellites. Although microsatellites seem to be scarce in ectomycorrhizal genomes as mentioned above, subcloned ISSR fragments of *T. matsutake* at the first step all showed different sequences. This indicates that, independent of the SSR frequency in the genome, relatively abundant ISSR loci in the genome could be immediately obtained by this method.

High allele frequency was biased to only one allele at five of the six polymorphic SSR loci in *T. matsutake* (Fig. 2). A similar bias was also observed in the polymorphic SSR markers of ectomycorrhizal *Suillus grevillei* (Zhou et al. 2001a). Such bias is disadvantageous to detection of genotype polymorphism. However, most of the *T. matsutake* individuals investigated here were divided into different genotypes by the six polymorphic SSR markers. This suggests that the SSR markers are polymorphic enough to analyze population genetics and reproduction.

Most microsatellite markers of *S. grevillei* are also species-specific, and distribution of subterranean genets was clearly indicated by use of one of the markers (Zhou et al. 2001b). All ten primer pairs isolated from *T. matsutake* amplified DNA from *T. nauseosum*, indicating that these two species might be same. This result is in agreement with the suggestion that Japanese and Swedish matsutake (*T. matsutake* and *T. nauseosum*) should be treated as the same species (Bergius and Danell 2000). Since the *T. matsutake* markers were not amplified from DNA of most other ectomycorrhizal species, they could be also employed as species-specific markers to investigate the subterranean genets of *T. matsutake*. *T. matsutake* sometimes forms sporocarps in a fairy ring upon subterranean mycelial aggregation called “Shiro”. The mechanism of Shiro initiation and development could be investigated using these markers.

In conclusion, the present study showed that the ISSR-suppression-PCR method is applicable and effective in the development of microsatellite markers from ectomycorrhizal fungi. This method could contribute greatly to understanding of reproduction in ectomycorrhizal fungi.

Acknowledgements We are grateful to Kazuhide Nara and Zhihua Zhou (Symbiotic Function Research Unit, Asian Natural Environmental Science Center, University of Tokyo) for providing ectomycorrhizal samples in Japan. We also would like to thank Dr Marcello Intini (National Research Council, Italy) and Dr Niclas Bergius (SLU, Sweden) for providing the samples of *T. caligatum* and *T. nauseosum*. This work was supported in part by a grant from PROBRAIN and Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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