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SHORT COMMUNICATION

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Development of SSR markers from an ectomycorrhizal fungus, *Suillus bovinus*

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Abstract Nine simple sequence repeat (SSR) loci were isolated from an ectomycorrhizal fungus *Suillus bovinus* by dual-suppression PCR. Three of the SSR loci isolated were polymorphic. The number of alleles per locus was between two and seven, and expected heterozygosities ranged from 0.087 to 0.740. One of these was confirmed to be species specific and codominant, suggesting applicability for the analysis of belowground population structure and gene flow of *S. bovinus*.

Key words Codominant marker · Ectomycorrhizal fungi · Population genetics · Simple sequence repeat · *Suillus bovinus*

Ectomycorrhizal fungi form ectomycorrhizas with the roots of their host plant and thus play various important roles in forest ecosystems (Smith and Read 1997). The fungi supply mineral nutrition that is absorbed by extramatrical mycelia to their hosts and receive photosynthates in return, which are utilized as a carbon source. Although it is a challenge to elucidate how these fungi distribute and reproduce in forests, such population genetic studies have not been conducted well, primarily because of difficulties in identification of fungal individuals.

In general, fungal populations consist of many genetically distinct individuals, so-called genets or clones. Among the ectomycorrhizal species, *Suillus* species have been intensively studied in population genetics (Cairney and Chambers 1999). To distinguish each clone, several methods have been applied: the somatic incompatibility (SI) test for *Suillus luteus* (L.: Fr.) S.F. Gray, *Suillus bovinus* (L.: Fr.) Kuntze, and *Suillus variegatus* (Sw.) Kuntze (Fries 1987; Dahlberg 1997; Dahlberg and Stenlid 1990, 1994), polymerase chain reaction (PCR) single-strand conformation polymorphism (SSCP) analysis of *Suillus pungens* Thiers & A.H. Sm. (Bonello et al. 1998), intersimple sequence repeat (ISSR) polymorphism analysis for *Suillus grevillei* (Klotz.) Sing. and *Suillus pictus* (Peck) A.H. Sm. & Thiers (Zhou et al. 1999, 2000; Hirose et al. 2004), and microsatellite or simple sequence repeat (SSR) polymorphism analysis for *S. grevillei* (Zhou et al. 2001a,b).

SSRs are defined as short tandem repeat sequences of a short motif of one to six base pairs in length and are widely dispersed in eukaryotic genomes. These sequences are highly polymorphic because of variability in the number of repeats, and they are inherited in a simple Mendelian fashion, which leads to their wide use as codominant DNA markers for genetic polymorphism analysis, such as in population genetics and breeding (Ashley and Dow 1994; Jarne and Lagoda 1996).

We have studied the population dynamics of *S. bovinus* after removal of litter and humus layers in a *Pinus densiflora* Sieb. & Zucc. stand using SI tests and ISSR polymorphism analysis for 4 years (Kikuchi et al., unpublished data). However, both methods have provided little information other than genetic identity; gene flow, i.e., the genetic relationship among the genets identified, remains unclear. For further investigation of gene flow and belowground genetic structure of *S. bovinus*, the development of SSR markers from *S. bovinus* is expected to provide a powerful and informative tool.

The development of SSR markers requires the design of primers from the conserved flanking regions of the repeats. However, because these regions do not occur as frequently in the genome, the isolation of SSR loci has usually been a laborious and expensive process (Ashley and Dow 1994; Zane et al. 2002). Recently, SSR markers for the ectomycorrhizal fungi *Tricholoma matsutake* (S. Ito & Imai) Sing., *Pisolithus* sp., and *Laccaria amethystina* (Bull.) Murr. have been developed using dual-suppression PCR (Kanchanaprayudh et al. 2002; Lian et al. 2003; Wadud et al. 2006), which

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has been reported to be more efficient and less laborious for isolating SSR loci (Lian et al. 2001; Lian and Hogetsu 2004). In this study, we isolated SSR markers from *S. bovinus* using dual-suppression PCR and investigated its applicability to population studies.

Fungal samples used are listed in Tables 1 and 2. Genomic DNA was extracted from freeze-dried liquid-cultured mycelia or fruit bodies using the cetyltrimethyammonium bromide (CTAB) method described by Kikuchi et al. (2000). For SSR loci isolation, genomic DNA was extracted from freeze-dried mycelium of strain Sb1–679, which was isolated from a fruit body collected in Kake, Hiroshima Prefecture, Japan, in 2000.

The SSR motifs targeted were CA and CGA, because $(CA)_n$ type SSR loci have been reported for many biological species (Hamada et al. 1982), and ISSR polymorphism analysis using a $(CGA)_5$ primer gave the most reproducible polymorphism for *S. bovinus* (Kikuchi et al., unpublished data). SSR loci were isolated according to the procedures described by Lian et al. (2001) and Lian and Hogetsu (2004), with slight modifications: (i) to prevent the shorter strand of the adapter from misannealing with the primer during the first PCR of the second step, we used a primer called AP1–2 (5′-ATCGTAATACGACTCACTATAGGGCAC-3′) instead of the AP1 primer because the sequence of the four 3′-terminal bases of the AP1 primer was complementary to those of the shorter strand of the adapter, and nonspecific amplifications frequently occurred in preliminary experiments; (ii) DNA was digested with *Alu*I blunt-ends restriction enzyme; (iii) PCR products were ligated into pCR2.1 (Invitrogen, Carlsbad, CA, USA) or pT7Blue (Novagen, Madison, WI, USA) vectors and the plasmids were transferred to *Escherichia coli* strain INVαF'. At the first step, 15 positive clones were chosen for each motif and sequenced for the second step.

PCR amplification of the isolated SSR loci was performed with a DNA Thermocycler 9600 or 9700 (Applied Biosystems, Foster City, CA, USA) in a 10-μl reaction mixture containing 10 ng genomic DNA, 0.2 μM each primer, 1× buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin), 200 μM each dNTP, and 0.5 U Ampli Taq Gold DNA polymerase (Applied Biosystems). The PCR parameters were as follows: 9 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at the annealing temperature of each primer pair listed in Table 3, and 30 s at 72°C.

Reaction products were electrophoresed on 6% Long Ranger sequencing gels (FMC BioProducts, Rockland, ME, USA) using an SQ-5500L sequencer (Hitachi, Tokyo, Japan) or 12%–15% polyacrylamide gels. Samples electrophoresed using the sequencer were amplified in a reaction

Table 1. Ectomycorrhizal species used in the study

Species	Sample[a]
Boletaceae	
Suillus spectabilis (Peck) Sing.	m
S. laricinus (Berk.) Kuntze	m
S. grevillei(Klotz.) Sing.	m
S. luteus (L.: Fr.) S.F. Gray	m
Gyroporus castaneus (Bull.: Fr.) Quél.	f
Boletinus cavipes (Opat.) Kalchbr.	m
Xerocomus nigromaculatus Hongo	f
Xerocomus sp.	f
Boletus griseus var. *fuscus* Hongo	f
B. ornatipes Peck	m
B. speciosus Frost	f
B. pseudocalopus Hongo	f
Tylopilus neofelleus Hongo	f
T. castaneiceps Hongo	m
Leccinum extremiorientale (L. Vass.) Sing.	m
L. holopus (Rostk.) Watling	m
Boletellus chrysenteroides (Snell) Snell	m
B. russelli (Frost) Gilb.	m
Tricholomataceae	
Lyophyllum shimeji (Kawam.) Hongo	m
Laccaria amethystea (Bull.) Murr.	m
L. laccata (Scop.: Fr.) Berk. & Br.	m
Tricholoma auratum (Fr.) Gill.	m
T. robustum (Alb. & Schw.: Fr.) Ricken	m
T. fulvocastaneum Hongo	m
T. matsutake(S. Ito & Imai) Sing.	m
T. bakamatsutake Hongo	m
T. ustale (Fr.: Fr.) Kummer	m
Amanitaceae	
Amanita hemibapha (Berk. & Br.) Sacc.	m
A. pseudoporphyria Hongo	m
A. citrina var. citrina (Schaeff.) Pers.	m
A. castanopsidis Hongo	f
Amanita sp.	f
Russulaceae	
Russula sp.	f
Lactarius chrysorrheus Fr.	f
Gasteromycetidae	
Rhizopogon rubescens Tul.	m
Pisolithus tinctorius (Pers.) Coker & Couch	m
Calvatia craniiformis (Schw.) Fr.	m
Ascomycotina	
Cenococcum geophilum Fr.	m

[a] DNA was extracted from hyphae sampled directly from the fruit bodies (f) or cultured mycelia (m)

Table 2. Strains of *Suillus bovinus* used in the study

Strain	Geographic origin
Tsukuba-1	Makabe, Ibaragi
Tsukuba-2	Makabe, Ibaragi
Tsukuba-3	Makabe, Ibaragi
Ina04-1	Ina, Nagano
Ina02-1	Ina, Nagano
Ina02-2	Ina, Nagano
Ina 1	Ina, Nagano
Ina 2	Ina, Nagano
Ina 3	Ina, Nagano
Ina 4	Ina, Nagano
Sb 3	Yasu, Shiga
Sb 6	Kamitonda, Wakayama
Sb 12	Konan, Shiga
Sb 15	Konan, Shiga
Sb 744	Katano, Osaka
KY1S	Mizuho, Kyoto
ME1S	Hakusan, Mie
ME3S	Hokusei, Mie
ME4S	Aoyama, Mie
OK1S	Chuou, Okayama
Sb 1-679	Kake, Hiroshima
Kake 2-77	Kake, Hiroshima
Kake 4-41	Kake, Hiroshima
Kake 4-88	Kake, Hiroshima

DNA was extracted from cultured mycelia

Table 3. Characteristics of simple sequence repeat (SSR) loci isolated from *Suillus bovinus*

Locus	Repeat motif	Primer sequence	Ta (°C)	No. of alleles	Ho	He[a]	Size range (bp)	DDBJ accession no.
Sb-CA1	$(CA)_{13}$	5′-TGGACTCAATCAAATTGCTG-3′ 5′-TTGAGTTCTCTAAGTCTCAG-3′	60	7	0.409	0.740	117–129	AB205330
Sb-CA3	$(CA)_{7}$	5′-GTATCTCTCATTCCCCCG-3′ 5′-TTGTTCCAGAGCACATGCGT-3′	60	2	0.000	0.087	94–98	AB205331
Sb-CA5	$(GCGT)_{3}GTGA(TG)_{6}$	5′-AGGGGCTGGCGCAGT-3′ 5′-GGAAGTCACTGCGTCA-3′	48	1	–	–	92	AB205332
Sb-CGA3	$(GTC)_{4}$	5′-GGTGCCGGTGTGGAT-3′ 5′-ACTTACGTCTGCGGCTT-3′	56	1	–	–	107	AB205333
Sb-CGA4	$(CGA)_{3}\ldots(TGC)_{2}AGA(TGC)$	5′-TATGCGCTCTCTGGGTCCAAGTTT-3′ 5′-GCTGATCCTGACTTTGGT-3′	56	1	–	–	176	AB205334
Sb-CGA5	$(CGT)_{3}A(CGT)$	5′-TGAGGTCCGCTCCACTT-3′ 5′-GCTGAGTCTGACCAGAG-3′	48	1	–	–	121	AB205335
Sb-CGA6	$(ACG)_{2}ACA(ACG)_{3}$	5′-GCAGAGAGAGGGGGCAGAACTAA-3′ 5′-TAGCGGGGTAGATGCAGA-3′	52	1	–	–	223	AB205336
Sb-CGA7	$(TCG)_{2}TCT(TCG)_{2}$	5′-TTGGACCCTGAACACTT-3′ 5′-GATGATTATGCAGCGAG-3′	56	1	–	–	133	AB205337
Sb-CGA8	$(ACG)_{4}ATG(ACG)_{2}$	5′-GTCAGATGACATGGACTCTT-3′ 5′-GAAAATTAATGTTATGTAGC-3′	60	2	0.045	0.201	127–133	AB205338

Ta, annealing temperature; Ho, observed heterozygosity; He, expected heterozygosity; DDBJ, DNA Data Bank of Japan
[a] Expected heterozygosity at each locus was estimated according to the formula $He = 1 - \Sigma p_i^2$, where p_i is the frequency of the *i*th allele

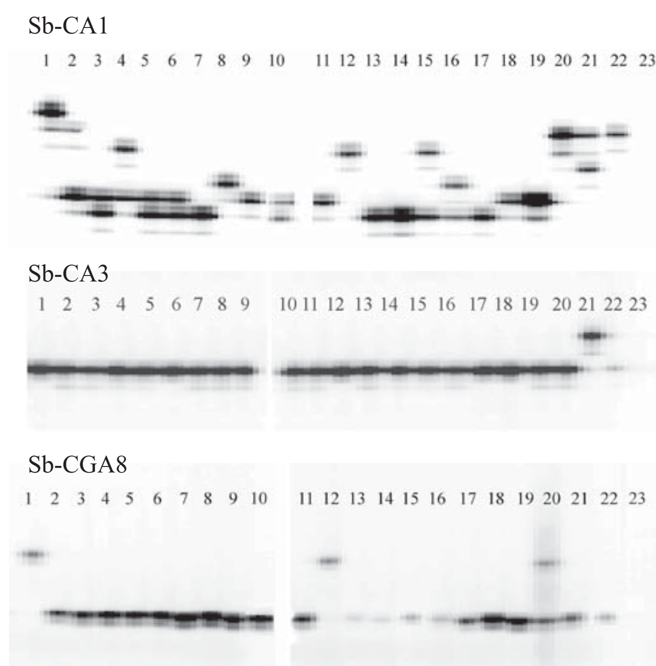

Fig. 1. Polymerase chain reaction (PCR) amplification of the three polymorphic SSR loci of *Suillus bovinus* strains. Lanes: *1*, Tsukuba-1; *2*, Tsukuba-2; *3*, Ina04-1; *4*, Ina02-1; *5*, Ina02-2; *6*, Ina1; *7*, Ina2; *8*, Ina3; *9*, Ina4; *10*, Sb3; *11*, Sb6; *12*, Sb12; *13*, Sb15; *14*, Sb744; *15*, KY1S; *16*, ME1S; *17*, ME3S; *18*, ME4S; *19*, OK1S; *20*, Kake2-77; *21*, Kake4-41; *22*, Kake4-88; *23*, nonmycorrhizal root of *Pinus densiflora*

mixture containing primers labeled with Texas Red fluorescent dye. The banding patterns were visualized by ethidium bromide staining and UV irradiation for the polyacrylamide gels; for Long Ranger gels, analysis was conducted with the sequencer software version 1.5 and FRAGLYS version 3 software (Hitachi Electronics Engineering, Tokyo, Japan).

The isolated SSR loci are listed in Table 3. From a total 24 strains of *S. bovinus* from various geographic regions listed in Table 2, nine loci were isolated of which two CA repeats and one CGA repeat loci were polymorphic (Fig. 1) and the others were monomorphic (data not shown).

PCR amplification of the Sb-CA1 locus gave no amplified products from the DNA of the species listed in Table 1 other than *S. bovinus* (data not shown), indicating its possible application as a species-specific marker. In contrast, PCR amplification of the Sb-CA3 and Sb-CGA8 loci gave amplified products for most of the species used in the study, although intensities of the fragments were usually lower than those amplified from DNA of *S. bovinus* (data not shown).

To confirm segregation of the alleles at the isolated SSR loci, single-spore isolation was conducted for some of the sporocarps collected in Kake, Hiroshima Prefecture and Mt. Tsukuba, Ibaragi Prefecture in 2001 and 2002, respectively. Spore germination was induced by combination of charcoal treatment of water agar plates and co-culture with *P. densiflora* seedlings, as described by Kikuchi et al. (2006). Among the parental strains of the monokaryons obtained, as the Tsukuba-3 strain was confirmed to be heterozygous at the locus Sb-CA1, its monosporous progeny (15 specimens) were subjected to analysis of the SSR locus. Either size of the bands (117 or 119 bp), which were amplified from the DNA of the parental strain Tsukuba-3, was detected when those extracted from its monosporous progeny were used as templates (Fig. 2). This result indicated that the Sb-CA1 locus could be used as a codominant marker. For the other two polymorphic loci, it could not be confirmed whether they were codominant, because monosporous strains were not available from fruit bodies heterozygous at either of the two loci. However, they were presumably codominant because one or two fragments were amplified by PCR reaction (see Fig. 1).

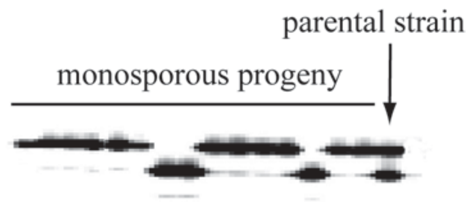

Fig. 2. PCR amplification of the locus Sb-CA1 from a dikaryotic strain and its monosporous progeny

In general, DNA extracted from field-collected mycorrhizal samples contains that of at least two biological species, i.e., the host plant and the mycorrhizal fungus, and sometimes other fungal species. Therefore, DNA fingerprinting methods, such as ISSR or randomly amplified polymorphic DNA analysis, are presumably inapplicable for belowground population genetics because it is impossible to determine if each fragment simultaneously amplified from the DNA of several biological species is actually from the mycorrhizal fungus of interest. The Sb-CA1 locus showed promise as a species-specific codominant marker for use in studies of belowground genetic structure and gene flow of *S. bovinus* populations, as reported for *S. grevillei* (Zhou et al. 2001b).

Recently, CT repeats have been isolated from *Heterobasidion annosum* (Fr.) Bref. (Johannesson and Stenlid 2004) and *Laccaria bicolor* (Maire) Orton (Jany et al. 2006), and CAC and CAT repeats from *L. amethystina* (Wadud et al. 2006). The development of SSR markers for other motifs targeted in this study will provide the necessary numbers of polymorphic SSR markers to clarify the population genetics of *S. bovinus*.

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