Mycoscience 42: 123–127, 2001

Short Communication

Detection of genetic variation among single-spore isolates and identification of genets of *Armillaria ostoyae* by AFLP analysis with Texas Red™-labeled selective primer

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Accepted for publication 21 November 2000

AFLP (amplified fragment length polymorphism) analysis was applied to *Armillaria ostoyae* isolates (single-spore isolates and field isolates from the same forest). For detection of AFLP, we have developed a modified method using DNA sequencer with Texas Red-labeled selective primer. In analysis of single-spore isolates, this technique provided large numbers of highly polymorphic DNA markers, which can be used to identify genets. The results suggested that outbreeding might be common in *A. ostoyae*.

Key Words—AFLP; Armillaria root and butt rot; genets; population structure.

Armillaria ostoyae (Romagn.) Herink causes Armillaria root and butt rot of woody plants. In studies of the ecology and infection biology of this fungus, identification of genets in the field has been carried out using the somatic incompatibility test (Legrand et al., 1996; Rizzo and Harrington, 1993; Rizzo et al., 1995; Smith et al., 1994; Worrall, 1994). However, in earlier studies, somatic incompatibility testing was not able to distinguish about half of the inbred isolates (Kile, 1983; Korhonen, 1978). Recently, molecular markers such as isozyme profiles, RFLP (restriction fragment length polymorphism) and RAPD (randomly amplified polymorphic DNA) analysis have been used to identify genets of *Armillaria* sp. (Guillaumin et al., 1996; Legrand et al., 1996; Rizzo and Harrington, 1993; Rizzo et al., 1995; Smith et al., 1994). Guillaumin et al. (1996) compared four methods (somatic incompatibility tests, mating-type allele analysis, isozyme profiles and RAPD analysis) to identify genets and found that RAPD analysis detected the highest number of polymorphic loci and could most readily distinguish closely related isolates. However, they also suggested that RAPD analysis was not proved to be a reproductive DNA marker.

Vos et al. (1995) developed a DNA fingerprinting technique called AFLP (amplified fragment length polymorphism) analysis based on selective PCR (polymerase chain reaction) amplification of genomic DNA restriction fragments, and demonstrated that AFLP analysis could reproducibly detect a large number of polymorphic loci. AFLP analysis has been used to study genetics and population structure of many plants and some fungi (Beismann et al., 1997; Cervera et al., 1996; Julián et al., 1999; Maughan et al., 1996; Majer et al., 1996; Travis et al., 1996).

A number of different methods of AFLP analysis have been reported, and the procedure involving detection of DNA fragments with fluorescent primers is more convenient than that with radio isotope. However, the use of fluorescent primers has some significant shortcomings: 1) it is impossible to isolate the labeled DNA fragments from acrylamide gels, and 2) expensive computer analysis is necessary to detect the labeled DNA fragments. On the other hand, Texas Red-labeled DNA markers can be detected using DNA sequencers with appropriate detection ranges (Hitachi SQ5500, Vistra DNA sequencer 725 etc.). Furthermore, the labeled DNA fragments can be isolated from polyacrylamide gels using a fluorescence image scanner (Muramatsu, 1997).

We have developed a modified AFLP analysis procedure in which selective primers are labeled with Texas Red fluorescent dye. In the present study, we examined genetic variation among single-spore isolates of *A. ostoyae* and demonstrated that AFLP analysis with Texas Red-labeled primers was sufficiently powerful to identify genets. Furthermore, we discuss the breeding systems of *A. ostoyae* based on the AFLP profile similarity among

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isolates examined in this study.

Isolates Eight single-spore isolates and their parental isolate, HUA96175, were used in this study. The parental isolate originated from Teshio, Hokkaido, Japan, and was identified as *A. ostoyae* using a mating test with tester isolates. The single-spore isolates were prepared from a basidiome obtained from a pure culture of the HUA96175 isolate using Togashi's method (Togashi, 1996). In addition, six isolates (M333, M343, M350, M376, M387, and M388) originally collected from a 50 m × 50 m plot in natural forest dominated by *Quercus crispula* Blume were also analyzed. These isolates are maintained in the culture collection of the Laboratory of Forest Resource Biology, Graduate School of Agriculture, Hokkaido University. *Quercus crispula* stakes were placed five meters apart, and fungal isolates were obtained from rhizomorphs which developed on the stakes after 1 yr. These isolates were identified as *A. ostoyae* using mating tests as above. The plot is located in forest stand No. 410, Uryu Experimental Forest of Hokkaido University, Horokanai, Hokkaido, Japan.

AFLP procedure *Armillaria ostoyae* isolates were cultured in liquid medium (2% soluble starch, 2% sucrose, 0.15% polypeptone, 0.3% malt extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2$, 10 ppm $FeCl_2$, 10 ppm $MnSO_4 \cdot 4$-$5H_2O$, 4 ppm $ZnCl_2$, 10 mM phosphate buffer) with shaking (105 strokes/min) at 25°C in the dark for 4 wk. Mycelia were harvested onto cheesecloth, rinsed with distilled water, ground in liquid nitrogen, rinsed three times with methanol containing 0.1% mercaptoethanol, and dried for 1 h at room temperature.

Genomic DNA was then extracted according to the method of Möller et al. (1992) and treated with RNase A for 30 min at 55°C.

AFLP analysis was carried out using a modification of the procedure described by Vos et al. (1995) and the instruction manual of the AFLP Core Reagent Kit (Life Technologies, Rockville, USA).

Digestion of genomic DNA and ligation with adaptors were carried out using the AFLP Core Reagent Kit. Genomic DNA (200 ng) was digested with restriction enzymes (*Eco* RI and *Mse* I) using half of the reagent volumes recommended by the manufacturer.

For preselective amplification, 20 μl PCRs were performed in a PE-9600 or PE-2400 thermocycler (Perkin-Elmer, Foster, USA) with the primers E+0 (5′-GAC TGC GTA CCA ATT C-3′) and M+0 (5′-GAT GAG TCC TGA GTA A-3′) (Vos et al.,1995). Reaction conditions and the thermocycler programming for preselective amplification were as described by Vos et al. (1995). Preselective amplification reaction mixtures were diluted 50-fold with TE (10 mM Tris-HCl, 0.1 mM EDTA pH 8.0) before being further amplified with selective primers.

Selective amplification was performed in a PE-9600 thermocycler using 1 pmol of the E+AT primer (5′-GAC TGC GTA CCA ATT CAT-3′) labeled with Texas Red and 15 ng either of the primers M+2 (M+CA (5′-GAT GAG TCC TGA GTA ACA-3′) or M+CC (5′-GAT GAG TCC TGA GTA ACC-3′)) in a total reaction volume of 10 μl. The size of nucleotides extentions of these selective primer was according to Majer et al. (1996). The E+AT primer was labeled with Texas Red using the 5′ oligonucleotide Texas Red labeling kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions, except that 2 nmol of E+AT primer was used instead of the recommended 1 nmol, and purified using Sep-Pak C18 cartridges (Waters, Milford, USA). Thermocycling conditions and concentrations of all reagents except primers were as described by Vos et al. (1995).

Electrophoresis and detection of amplified fragments were carried out using a SQ5500 DNA sequencer (Hitachi, Tokyo, Japan). Electrophoresis was carried out on 6% Long Ranger™ (FMC bioproducts, Rockland, USA) gels for 6 h, and gel images were saved at intervals of 60 min.

AFLP in single-spore isolates and inbred isolates The presence (1) or absence (0) of specific DNA fragments was scored and recorded in binary notation as indicated. The pairwise similarities between samples were calculated using Nei's similarity index (Nei and Li, 1979). We calculated the binary notation and the similarity index of inbred isolates as well as those of single spore isolates. The data of inbred isolates were based on the composite AFLP profiles of two single-spore isolates which were sexually compatible with different mating alleles.

Part of a gel image from analysis of single-spore isolates is shown in Fig. 1A. In this examination, data analysis was performed using DNA fragments of 100–350 bp, because fragments of less than 100 bp showed low reproductibility, and fragments longer than 350 bp were not clearly resolved.

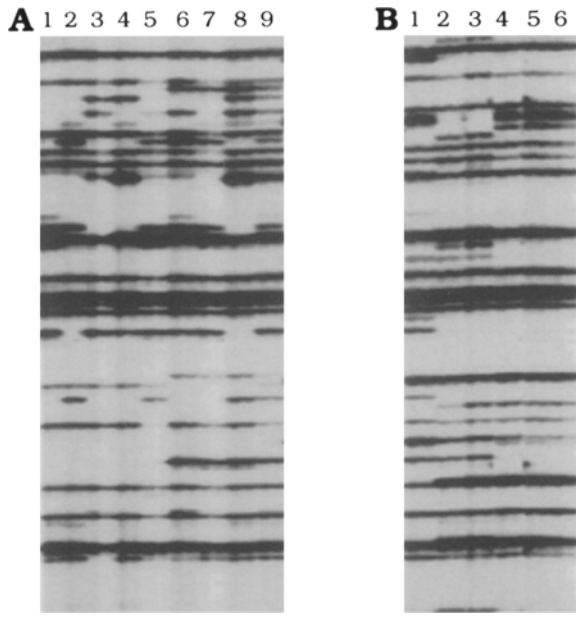

Fig. 1. Gel images of AFLP analysis with Texas Red-labeled selective primer. The digitized image shows fragments between 100 bp and 200 bp in length. These DNA fragments were produced with the E+AT/M+CA primer combination. A. Lanes 1–8, single-spore isolates; lane 9, parental isolate HUA96175. B. Lanes 1–6, isolates M333, M343, M376, M350, M387, M388.

Table 1. Number of DNA fragments, and similarity indices among the single-spore isolates and among the inbred isolates.

	Single-spore isolates	Inbred isolatesc
E+AT/M+CA[a]		
Total number of DNA fragments	45	45
Total number of polymorphic DNA fragments	22	13
Number of DNA fragments in each isolate	32–41 (35.4)[b]	35–44 (40.6)
Similarity index	0.771–0.911 (0.848)[b]	0.846–0.977 (0.921)
E+AT/M+CC		
Total number of DNA fragments	49	49
Total number of polymorphic DNA fragments	32	23
Number of DNA fragments in each isolate	29–37 (34.5)	38–44 (42.1)
Similarity index	0.667–0.873 (0.762)	0.775–0.976 (0.889)
Combined data with two primer combinations		
Total number of DNA fragments	94	94
Total number of polymorphic DNA fragments	54	36
Number of DNA fragments in each isolate	63–75 (69.9)	77–86 (82.8)
Similarity index	0.755–0.867 (0.806)	0.840–0.959 (0.901)

a) Selective primer combination used in AFLP analysis.
b) Number in parenthesis indicates the average value.
c) These data were calculated based on composite AFLP profiles of two single-spore isolates, which were sexually compatible with different mating type alleles.

The results of AFLP analysis with eight single-spore isolates and eight inbred isolates are summarized in Table 1. The use of the two primer combinations produced a total of 94 DNA fragments in single-spore isolates, in which 45 and 49 DNA fragments were detected with E+AT/M+CA and E+AT/M+CC primer combinations, respectively. Each single-spore isolate showed 32-41 (average 35.4) and 29–37 (average 34.5) DNA fragments with primer combinations E+AT/M+CA and E+AT/M+CC, respectively. All of the DNA fragments detected in single-spore isolates also appeared in the parental isolate. Polymorphism in the single-spore isolates was revealed in 22 and 32 DNA fragments with the E+AT/M+CA and E+AT/M+CC primer combinations, respectively. The eight single-spore isolates produced eight sexually compatible combinations (inbred isolate), and the data were inferred from composite AFLP profiles of eight compatible single-spore combinations (Table 1). Primer combinations E+AT/M+CA and E+AT/M+CC produced a total of 45 and 49 DNA fragments and a total of 13 and 23 polymorphic fragments, respectively. Overall similarity index ranged 0.840–0.959 with an average of 0.901.

Zolciak et al. (1997) examined single-spore isolates of *A. ostoyae* using RAPD analysis, and detected 5–11 DNA fragments per primer, of which 2–5 DNA fragments revealed polymorphism. In this study, AFLP analysis displayed 4–16 times as many polymorphic DNA fragments per reaction as RAPD analysis, and DNA fragments of 100–350 bp were reproductively detected.

In this study, we used preselective primers with no restriction site-specific nucleotide extensions and selective primers with two nucleotide extensions. The useful size of nucleotide extensions depends on the genome size of the organism examined. In earlier studies of *Thanatephorus cucumeris* (Frank) Donk [anamorph: *Rhizoctonia solani* Kühn] (Julián et al., 1999), *Cladosporium fulvum* Cooke and *Pyrenopeziza brassicae* B. Sutton and Rawl. (Majer et al., 1996), primers with the same extension size, two base pairs, as those used in the present study were shown to generate useful AFLP profiles. We therefore recommend the use of primers with these extension sizes for attempts to apply AFLP analysis to other fungi.

Genetic variation among the natural forest isolates Part of a gel image from analysis of the natural forest isolates is shown in Fig. 1B. Using two primer combinations, 81 and 82 DNA fragments were detected in the isolates M343 and M376, respectively, and all but one of them were identical between the two isolates (similarity index greater than 0.99). Analysis of the isolates M350, M388 (80 DNA fragments each) and M387 (81 DNA fragments) showed that the AFLP profiles of these isolates were identical with the exception of one fragment which was not detected in the M387 isolate (similarity index greater than 0.99). The M333 isolate had an unique AFLP profile. The AFLP profile similarity indices between M333 and M343, between M333 and M350 and between M343 and M350 were 0.78, 0.77 and 0.81, respectively.

AFLP profile similarity indices among the isolates M350, M387 and M388, and between M343 and M376, were much higher than among the inbred isolates. Somatic incompatibility tests demonstrated compatibility between these isolates which showed similar AFLP profiles (data not shown). Therefore, we considered isolates with highly similar AFLP profiles (similarity index greater than 0.99) to be genets which originated from

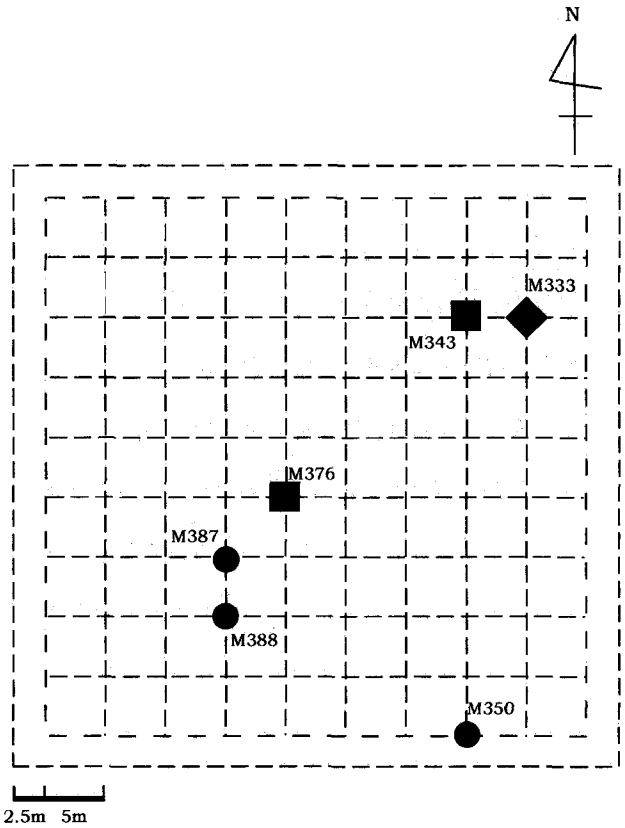

Fig. 2. Collection map of *A. ostoyae* natural forest isolates. Isolates represented by the same symbol showed almost all identical AFLP profiles and are considered as putative genets.

one mating event and spread by vegetative growth. The maximum distance between the collection points of genets was 25 m (Fig. 2). Slight differences in AFLP profiles between genets were observed in this study. Smith et al. (1994) also indicated the same phenomenon in DNA fingerprints of RFLP between isolates belonging to the identical genet. They suggested these minor differences in DNA fingerprints are due to: 1) somatic mutation during vegitative growth, or 2) partial digestion by restriction enzyme or chemical modification of the enzyme recognition site. To clarify the cause of the minor differences in DNA fingerprints, it is necessary to analyze the sequence of the loci showing these DNA fragments.

The level of AFLP profile similarity between genets was lower than that among the inbred isolates, suggesting that outbreeding might be common in *A. ostoyae*. In this study, genetic heterogeneity among single-spore isolates was also indicated, which meant that many loci in the parental isolate HUA96175 were heterozygous. These results also supported the notion that an outbreeding system was effective in *A. ostoyae*, because inbreeding would cause loss of heterozygosity in many loci. Smith et al. (1994) proposed that breeding populations of *A. ostoyae* are panmictic over a considerable area based on similarity value of DNA fingerprints among genets in a red pine plantation. The outbreeding system lies at the base of panmictic population. Therefore, we considered that their results also supported the outbreeding system in *A. ostoyae*. However, we have to accumulate more information on the breeding system in *A. ostoyae*. For example, inbreeding depression should be examined, and it might be useful to investigate gene frequency among the population.

Acknowledgements——The authors are grateful to Dr. I. Togashi of Hokkaido Forest Products Research institute, Asahikawa, Japan, for technical advice on cultivation of basidiomes, to Dr. Y. Tamai of the Graduate school of Agriculture, Hokkaido Univ., Sapporo, Japan, for valuable advice, and to Mr. H. D. Stankov of the Faculty of Agriculture, Hokkaido Univ., Sapporo, Japan, for reading the entire text in its original form.

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