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Identification of *Armillaria* species from Hokkaido by analysis of the intergenic spacer (IGS) region of ribosomal DNA using PCR-RFLP

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The intergenic spacer (IGS) region, which is located between the 3′ end of 26S ribosomal DNA (rDNA) and the 5′ end of 5S rDNA, of six *Armillaria* species from Hokkaido was investigated using polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP). Restriction with only *Alu* I could distinguish *A. mellea* subsp. *nipponica* from the other species. With *Alu* I and *Dde* I, *A. ostoyae* and *A. gallica* could be distinguished from the other species. Digestion with *Alu* I resulted in two patterns (types A and B) of *A. singula* and three patterns (types A, B, and C) of *A. jezoensis*. One pattern (type B) of the former species and two patterns (types B and C) of the latter species were each different from those of the other species. *Armillaria sinapina* gave only one *Alu* I digestion pattern, which was identical to that of *A. jezoensis* (type A) and *A. singula* (type A). However, by digestion with *Dde* I, *A. singula* (type A) could be distinguished from *A. jezoensis* (type A) and *A. sinapina*.

Key Words——*Armillaria*; IGS (intergenic spacer) region; PCR-RFLP; ribosomal DNA.

Armillaria mellea (Vahl: Fr.) Kummer sensu lato is distributed world-wide and is well known as a cause of *Armillaria* root and butt rot in forests. It has an extremely wide host range and shows a great variability in culture and basidiome morphology. Studies of its sexual mating system showed that this fungus consists of several biological species which function as reproductively isolated groups. Biological species from Europe and North America have been identified (Anderson, 1986; Anderson and Ullrich, 1979; Anderson et al., 1980; Bérubé and Dessureault, 1987, 1989; Hansen, 1985; Volk et al., 1996). In Japan, at least eight biological species have been reported (Cha and Igarashi, 1995a; Cha et al.,1992, 1994; Nagasawa et al., 1991).

Haploid-haploid and diploid-haploid pairing tests are usually used as criteria for the identification of biological species. In these pairings, fresh haploid culture is always necessary, but basidiomes of most biological species cannot easily be produced in vitro. Furthermore, it takes a long period to obtain results.

Isozyme profiles and analyses of nuclear and mitochondrial DNA have been applied to identify different species of *Armillaria*. (Anderson et al., 1987, 1989; Cha and Igarashi, 1995b; Jahnke and Bahnweg, 1987; Matsushita et al., 1996; Miller et al., 1994; Schulze et al., 1995). Anderson and Stasovski (1992) indicated that the nucleotide sequence of the intergenic spacer (IGS) region, which is located between the 3′ end of the 26S ribosomal DNA (rDNA) and the 5′ end of 5S rDNA of *Armillaria*, was useful for phylogenetic study. Recently, polymerase chain reaction – restriction fragment length polymorphisms (PCR-RFLP) of the IGS region has been applied to the identification of the species of *Armillaria* (Banik et al., 1996; Harrington and Wingfield, 1995; Volk et al., 1996). Harrington and Wingfield (1995) indicated that PCR-RFLP analysis of the IGS region allowed discrimination of all *Armillaria* species from Europe and North America, except *A. gallica* Marxmüller & Romagnesi and *A. calvescens* Bérubé & Dessurealt, which were considered to be closely related (Anderson and Stasovski, 1992). Banik et al. (1996) described that this technique was particularly useful for identification of isolates from a limited geographical area, once a baseline using known isolates has been established.

Thus, identification of *Armillaria* species using PCR-RFLP analysis of the IGS region was useful for almost all *Armillaria* species from North America and Europe. In Hokkaido, Japan, the profiles of biological species have been established (Cha and Igarashi, 1995a; Cha et al., 1992, 1994). Therefore, it is necessary to estimate the suitability of PCR-RFLP analysis of the IGS region for *Armillaria* species from Hokkaido. Furthermore, we think that a comprehensive data base is important for rapid identification of species using PCR-RFLP analysis. In this paper, the IGS region of *Armillaria* species from Hokkaido were investigated using PCR-RFLP analysis.

Materials and Methods

Isolates and culture Six biological species originating

from Hokkaido, Japan, which were identified by mating test (Cha and Igarashi, 1995a; Cha et al., 1992, 1994; Mohammed et al., 1994), were used, together with the JD10 isolate of *A. mellea* subsp. *nipponica* Cha & Igarashi, which originated from Fukuoka, Kyushu, Japan. Diploid isolates are listed in Table 1. For DNA extraction, the isolates were cultured in a liquid synthetic medium (Mwangi et al., 1989) at 25°C in the dark for 2–3 wk. The mycelia were harvested and frozen in liquid nitrogen, then freeze-dried.

PCR-RFLP analysis The template DNA for PCR amplification was prepared using the modified method of Volk et al. (1996) described in our previous report (Terashima et al., 1998). PCR-RFLP analysis was carried out using the technique of Harrington and Wingfield (1995). The primers LR12R and O-1 (Anderson and Stasovski, 1992) were used, which were synthesized using a DNA synthesizer (Applied Biosystems, Perkin-Elmer, Model 380B, Tokyo, Japan). When PCR amplification of the IGS region gave poor yields, 0.16 units of cloned *Pfu* DNA polymerase (Stratagene, Heidelberg, Germany) was added to the PCR solution and the PCR cycle was modified to 36 cycles. Two restriction enzymes were used to digest amplified DNA: *Alu* I (Takara, Shiga, Japan), and *Dde* I (Toyobo, Tokyo, Japan). Electrophoresis of the restriction fragments was carried out on 3% NuSieve GTG agarose (FMC Bioproducts, Rockland, Maine, USA). Due to gel to gel variation in fragment size within the same species, the average size of fragments considered identical was used. Because of slight differences in the average value between species, fragments differing in size by less than 16 bp were considered identical across the different species. Fragments smaller than 100 bp were not counted because they could not be distinguished from primer dimers.

Results and Discussion

The fragments amplified by PCR were of about 890 bp in all isolates except two isolates of *A. mellea* subsp. *nipponica*, in which they were 830 bp in size. The fragment sizes of the IGS region produced by digestion with *Alu* I and *Dde* I are listed in Table 2. PCR-RFLP analysis with *Alu* I resulted in eight fragments, named *h*, *i*, *j*, *k*, *l*, *m*, *n*, and *o*, with sizes respectively of 410–423 bp, 371 bp, 308–317 bp, 249–266 bp, 207–210 bp, 184–190 bp, 162 bp, and 135–137 bp (Table 2). Single digestion patterns were seen in *A. mellea* subsp. *nipponica* with fragments *i* and *n*; *A. gallica* with fragments *j*, *l*, and *o*; *A. ostoyae* (Romagnesi) Herink with fragments *j*, *l*, and *o*; and *A. sinapina* Bérubé & Dessureault with fragments *h*, *k*, and *m*. Two digestion patterns (types A and B) were

Table 1. Origin of the isolates of *Armillaria* from Hokkaido.

Species	Isolate	Locality and regions	Host
A. ostoyae	HUA9112	Moshiri, Horokanai, Sorachi	*Betula ermanii*
	HUA93123	Akan, Kusiro	*Abies sachalinensis*
	236	Akan, Kusiro	*Betula platyphylla* var. *japonica*
A. gallica	HUA9104	Otoinepppu, Kamikawa	*Ulmus japonica*
	HUA9105	Otoinepppu, Kamikawa	*Quercus mongolia* var. *grosseserrata*
	HUA9125	Tomakomai, Iburi	*Prunus ssiori*
	HUA9264	Moshiri, Horokanai, Sorachi	Unidentified
	HUA93105	Yoshino, Fukushima, Oshima	*Cryptomeria japonica*
A. jezoensis	HUA9116	Moshiri, Horokanai, Sorachi	*Ulmus japonica*
	HUA93114	Nopporo, Ebetsu, Ishikari	Unidentified
	HUA93119	Moshri, Horokanai, Sorachi	*Tilia japonica*
	HUA9293	Kaminokuni, Hiyama	*Galeola septentrionalis*
	HUA9294	Kaminokuni, Hiyama	*Galeola septentrionalis*
	HUA9298	Kaminokuni, Hiyama	*Galeola septentrionalis*
A. mellea subsp. *nipponica*	HUA93110	Tomakomai, Iburi	*Acer mono*
	JD10	Fukuoka Pref. (Kyushu)[a]	*Camaecyparis obstusa*
A. sinapina	HUA9124	Tomakomai, Iburi	*Ulmus japonica*
	HUA9261	Moshiri, Horokanai, Sorachi	*Salix sachalinensis*
	HUA9262	Moshiri, Horokanai, Sorachi	*Salix sachalinensis*
	JH1	Shintoku, Tokachi	*Tilia japonica*
A. singula	HUA9109	Tomakomai, Iburi	*Abies sachalinensis*
	HUA9270	Sapporo, Ishikari	*Alnus japonica*
	HUA9276	Shari, Abasiri	Unidentified
	HUA9278	Shari, Abasiri	*Quercus mongolica* var. *grosseserrata*
	HUA9280	Shari, Abasiri	Unidentified

a) The only isolate from Kyushu, southwestern Japan.

seen in *A. singula* Cha & Igarashi which had respectively fragments *h*, *k*, and *m* and *h*, *l*, and *m*. Three digestion patterns (types A, B, and C) were seen in *A. jezoensis* Cha & Igarashi, respectively with fragments *h*, *k*, and *m*; *j*, *k*, and *m*; and *h*, *j*, *k*, and *m*. The *Alu* I digestion patterns observed in *A. mellea* subsp. *nipponica*, *A. singula* (type B), and *A. jezoensis* (types B and C) were each unique (Fig. 1).

Armillaria gallica and *A. ostoyae* showed identical *Alu* I digestion patterns consisting of fragments *j*, *l*, and *o* (Fig. 1). However, on PCR-RFLP analysis with *Dde* I, these two species gave different digestion patterns (Fig. 2). The *Alu* I digestion patterns of *A. sinapina*, *A. singula* (type A) and *A. jezoensis* (type A) were also identical, consisting of fragments *h*, *k*, and *m* (Fig. 1). On PCR-RFLP analysis with *Dde* I, however, *A. singula* (type A) was distinguished from *A. sinapina* and *A. jezoensis* (type A) (Fig. 2).

The *Alu* I digestion pattern of *A. ostoyae* isolates from Hokkaido was identical to those of *A. ostoyae* isolates from North America and Europe (Banik et al., 1996; Harrington and Wingfield, 1995). The *Alu* I digestion patterns of *A. singula* (type B) from Hokkaido and North American Biological Species XI (NABS XI) from North

Table 2. *Alu* I and *Dde* I restriction fragment sizes in the isolates of *Armillaria* from Hokkaido.

Species	Isolate	Fragment sizes (bp) (*Alu* I digest)	Fragment sizes (bp) (*Dde* I digest)
A. mellea subsp. *nipponica*	HUA93110, JD10[a)]	371 (*i*)[b)], 162 (*n*)	
A. gallica	HUA9104, HUA9105, HUA9125, HUA9264, HUA93105	317 (*j*), 209 (*l*), 135 (*o*)	237, 211, 148
A. ostoyae	HUA9112, HUA913123, 236	312 (*j*), 210 (*l*), 137 (*o*)	214, 179, 120
A. sinapina	HUA9124, HUA9261, HUA9262, JH1	423 (*h*), 258 (*k*), 190 (*m*)	235, 218, 148, 111
A. singula (type A)	HUA9278, HUA9280	417 (*h*), 266 (*k*), 186 (*m*)	234, 150, 113
A. singula (type B)	HUA9109, HUA9270, HUA9276	410 (*h*), 207 (*l*), 184 (*m*)	
A. jazoensis (type A)	HUA93114, HUA9293, HUA9294, HUA9298	417 (*h*), 252 (*k*), 187 (*m*)	235, 222, 147, 112
A. jazoensis (type B)	HUA93119	312 (*j*), 250 (*k*), 185 (*m*)	
A. jazoensis (type C)	HUA9116	413 (*h*), 308 (*j*), 249 (*k*), 185 (*m*)	

a) This isolate originated from Kyushu, southwestern Japan.
b) Fragments with the same letter in parenthesis were considered to be identical.

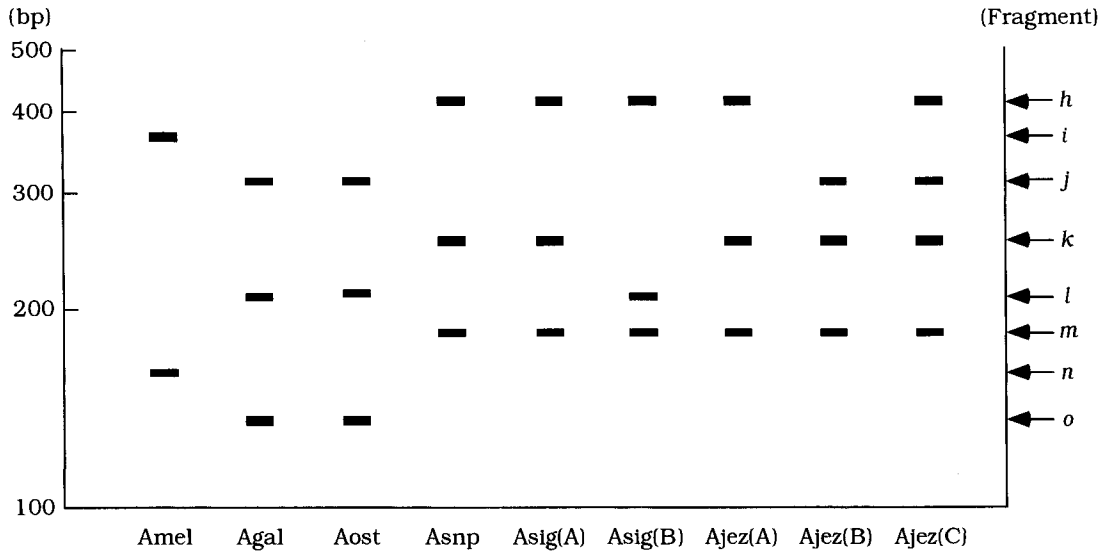

Fig. 1. Scheme of the agarose gel on electrophoresis in PCR-RFLP analysis with *Alu* I as the restriction enzyme.
Abbreviations under the fragment patterns indicate the following: Amel, *A. mellea* subsp. *nipponica*; Agal, *A. gallica*; Aost, *A. ostoyae*; Asnp, *A. sinapina*; Ajez, *A. jezoensis*; Asig, *A. singula*. The letters in parenthesis is indicate the IGS type in the species. The arrows on the right of the figure indicate the following eight fragments: *h* (410–423 bp), *i* (371 bp), *j* (308–317 bp), *k* (249–266 bp), *l* (207–210 bp), *m* (184–190 bp), *n* (162 bp), *o* (135–137 bp).

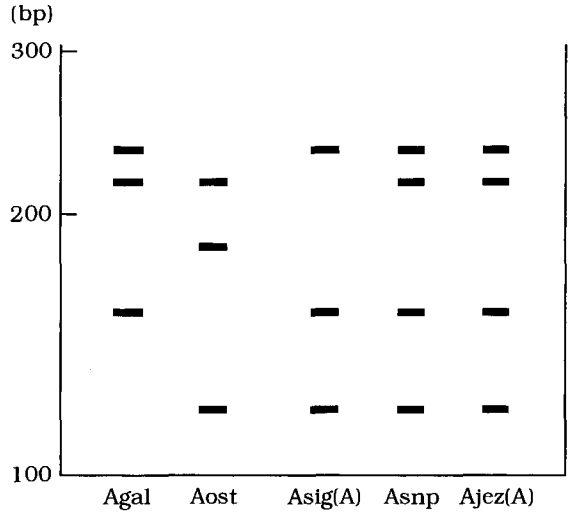

Fig. 2. Scheme of the agarose gel on electrophoresis in PCR-RFLP analysis with *Dde* I as the restriction enzyme. Abbreviations under the fragment patterns are as described in the legend to Fig. 1. Fragments differing by less than 4 bp are illustrated as identical fragments.

America (Banik et al., 1996) were also identical. *Armillaria gallica* is distributed in North America, Europe, and Japan (Cha et al.,1992; Korhonen, 1995), and *A. sinapina* is found in North America and Japan (Bérubé and Dessureault, 1989; Cha et al., 1994). However, the *Alu* I digestion patterns of the two species from Hokkaido were not similar to those of the same species from North America and Europe. Furthermore, *A. jezoensis* and *A. mellea* subsp. *nipponica* were reported only in Japan (Cha and Igarashi, 1995b; Cha et al., 1994), and their *Alu* I digestion patterns were different from those of foreign *Armillaria* species.

PCR-RFLP analysis of the IGS region with *Alu* I and *Dde* I is useful for identification of all *Armillaria* species from Hokkaido except *A. sinapina* and *A. jezoensis* (type A). From the results, by digestion with *Alu* I, *Armillaria* species from Hokkaido showed six IGS types of *A. mellea* subsp. *nipponica*, *A. ostoyae* and *A. gallica*, *A. singula* (type B), *A. jezoensis* (type B), *A. jezoensis* (type C), and *A. sinapina*, *A. singula* (type A) and *A. jezoensis* (type A). However, by digestion with *Dde* I, *A. ostoyae*, *A. gallica*, *A. singula* (type A), and *A. sinapina* and *A. jezoensis* (type A) were classified.

We attempted to distinguish *A. sinapina* and *A. jezoensis* (type A) by digestion with *Alu* I, *Dde* I, *Acc* I, *Bam* HI, *Dra* I, *Hind* III, *Eco* RI, *Sal* I, *Pst* I, *Sac* I, *Xba* I, *Sma* I, *Kpn* I, *Eco* RV, *Bgl* II, *Xho* I, *Sac* II, and *Hinf* I, a total of 18 restriction enzymes, but all of them yielded identical digestion patterns for *A. sinapina* and *A. jezoensis* (type A). (data not shown). We previously reported that *A. singula* and *A. jezoensis* are closely related, by analysis of the sequence of the IGS region (Terashima et al., 1998). Furthermore, it is very interesting for phylogenetic relationships that some isolates of *A. jezoensis* and *A. sinapina* in this study showed same IGS type.

To understand the evolution in *Armillaria* better and to identify *Armillaria* species by PCR-RFLP analysis, it is necessary to investigate the IGS region of more isolates of *A. jezoensis* and to obtain the nucleotide sequence of the IGS region for *A. jezoensis* (type A) and *A. sinapina*, which should provide more information than the digestion pattern by PCR-RFLP analysis. Furthermore, we should investigate other regions of the *Armillaria* genome.

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