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SHORT COMMUNICATION

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Genetic variation in *Armillaria mellea* subsp. *nipponica* estimated using IGS-RFLP and AFLP analyses

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Abstract In this study, genetic variation of *Armillaria mellea* subsp. *nipponica* was estimated using intergenic spacer-restriction fragment length polymorphism (IGS-RFLP) and amplified fragment length polymorphism (AFLP) analyses. Four IGS-RFLP phenotypes were produced, of which two have never been reported. AFLP analysis suggested that the 11 isolates used could be divided into five subgroups, and the isolates within the same subgroup were distributed throughout a relatively large area in Japan. A parental isolate and its offspring (single-spore isolates) showed an almost identical AFLP profile to each other. These results suggest that the large distribution of the isolates within the same subgroup were established via the basidiospore from a common parental strain.

Key words AFLP · *Armillaria mellea* subsp. *nipponica* · Homothallic sexuality · IGS-RFLP

In Japan, nine biological species have been reported in *Armillaria mellea* (Vahl) P. Kumm. sensu lato, viz. *A. cepistipes* Velen., *A. gallica* Marxm. & Romagn., *A. jezoensis* Cha & Igarashi, *A. mellea* sensu stricto, *A. nabsnona* Volk & Burds., *A. ostoyae* (Romagn.) Herink, *A. singula* Cha & Igarashi, *A. sinapina* Bérubé & Dessur., and Nagasawa E (a taxonomically unknown species) (Cha et al. 1992, 1994; Cha and Igarashi 1995; Ota et al. 1998a). Of these, the Japanese *A. mellea* s. str., which is partially compatible with the North American and European ones, is distinct in its homothallic sexuality from the North American and European populations with a heterothallic tetrapolar mating system. Cha and Igarashi (1995) recognized Japanese *A. mellea* s. str. as a taxon at the subspecies rank and gave it the name *A. mellea* subsp. *nipponica*. Over the past decade, sequence analyses of intergenic spacer (IGS) of the ribosomal DNA have revealed the phylogenetic relationship among *A. mellea* s. str., including *A. mellea* subsp. *nipponica* (Terashima et al. 1998a; Coetzee et al. 2000), and restriction fragment length polymorphism (RFLP) analyses of IGS have been applied to identify *A. mellea* subsp. *nipponica* (Harrington and Wingfield 1995; Terashima et al. 1998b; Fukuda et al. 2003).

It has been expected that populations within *A. mellea* subsp. *nipponica* have a homogeneous genetic background because this fungus has homothallic sexuality (Cha and Igarashi 1995; Ota et al. 1998b). In this regard, random amplified polymorphic DNA (RAPD) analysis and somatic compatibility tests have been carried out to estimate genetic variation in *A. mellea* subsp. *nipponica* (Ota et al. 2000). The somatic incompatibility test, however, cannot discern closely related isolates such as sib-relatives, and RAPD analysis has not proved to be a reproductive DNA marker. Therefore, to examine precise relationships between homogeneous isolates in this fungus, reproductive DNA markers are necessary.

Amplified fragment length polymorphism (AFLP) analysis was developed by Vos et al. (1995), and they demonstrated that this technique could reproducibly detect a large number of polymorphic loci. This analysis has also been used for estimation of intraspecies genetic diversity of the basidiomycete: the discrimination of sibrelatives and identification of genets in *A. ostoyae* (Terashima et al. 2001), evaluation of genetic diversity within commercial strains, and strain-typing in *Lentinula edodes* (Berk.) Pegler (Terashima et al. 2002; Matsumoto et al. 2003; Terashima

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Table 1. Origin of *Armillaria mellea* subsp. *nipponica* isolates

Isolates	Location	Host
HUA93110	Tomakomai, Hokkaido	*Acer mono* Maxim.
HUA94135	Chitose, Hokkaido	*Fraxinus lanuginosa* Koidz.
HUA96180	Kamikawa, Hokkaido	Unknown
HUA97	Hakodate, Hokkaido	Unknown
F307[a]	Hiroshima, Honshu	Unidentified broadleaf
F310[a]	Oita, Kyushu	*Quercus acutissima* Carruth.
F320[a]	Honshu	*Castanopsis cuspidata* (Thunb. ex. Murray) Schottky
F321[a]	Honshu	*Quercus acutissima*
TMIC31494	Shimane, Honshu	Unidentified broadleaf
TMIC31519	Aomori, Honshu	*Quercus serrata* Thunb. ex. Murray
TMIC32416	Tottori, Honshu	Unidentified broadleaf

[a]These isolates were contributed by J.J. Guillaumin

and Matsumoto 2004). These studies demonstrated that AFLP analysis provided reproductive DNA markers and was a reliable technique for identification of genotypes of fungal isolates.

In this study, we estimated genetic variation in *A. mellea* subsp. *nipponica* using IGS-RFLP and AFLP analyses and discuss the life cycle, dispersion, and molecularly based identification of this fungus.

The isolates used in this study are listed in Table 1. These isolates are maintained in the culture collection of the Laboratory of Forest Resource Biology, Graduate School of Agriculture, Hokkaido University. All isolates except HUA96180 and HUA97 were identified as *A. mellea* subsp. *nipponica* using mating tests with tester isolates (Mohammed et al. 1994; Cha and Igarashi 1995). HUA96180 and HUA97 isolates were identified based on morphological features of basidiomes and single spore-derived mycelia. Four single-spore isolates (HUA93110-2, -3, -4, and -5) from HUA93110 were also used for AFLP analysis. These isolates were cultured under conditions described in Terashima et al. (2001). Genomic DNA was then extracted according to the method of Möller et al. (1992) by addition of RNaseA treatment for 30 min at 55°C.

IGS-RFLP analysis was carried out using the technique of Harrington and Wingfield (1995) with small modification (Terashima et al. 1998b). The IGS region of the ribosomal RNA gene was amplified with the LR12R and O-1 primers (Anderson and Stasovski 1992), yielding an amplified fragment of approximately 830 bp in all isolates examined in this study. The fragment was digested separately with *Alu*I (Takara, Shiga, Japan) and *Dde*I (Toyobo, Tokyo, Japan).

AFLP analysis was carried out using the procedure described by Vos et al. (1995) and the instruction manual of the AFLP Core Reagent Kit (Invitrogen, Carlsbad, CA, USA) with small modification (Terashima et al. 2001). For preselective amplification, the E + 0 (5′-GAC TGC GTA CCA ATT C-3′) and M + 0 (5′-GAT GAG TCC TGA GTA A-3′) primer pairs were used. Selective amplification was performed using the E + AT (5′-GAC TGC GTA CCA ATT CAT-3′) and M + CA (5′-GAT GAG TCC TGA GTA ACA-3′) primers. Similarity indexes (Nei and Li 1979) between AFLP profiles were calculated based on DNA fragments that were clearly identified from the isolates used.

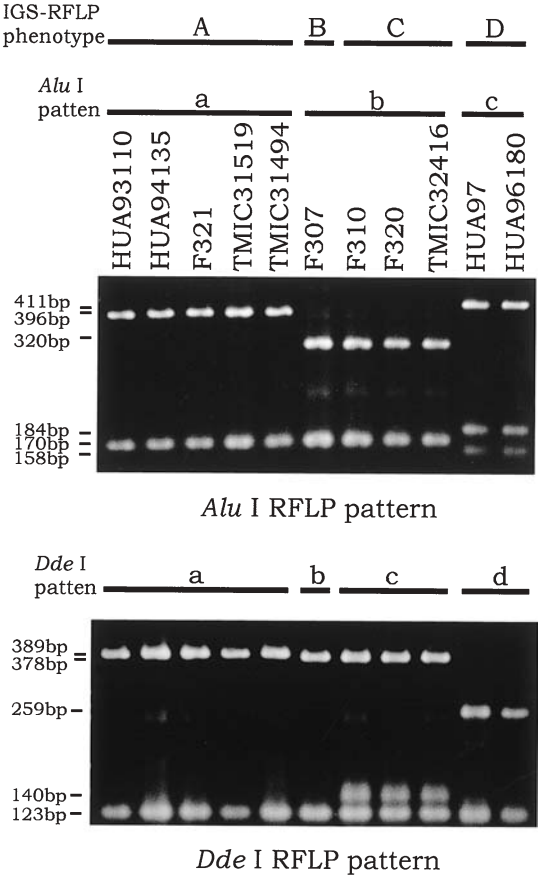

Fig. 1. Agarose gel electrophoresis images of intergenic spacer-restriction fragment length polymorphism (IGS-RFLP) analysis of 11 *Armillaria* subsp. *nipponica* isolates. *Upper* and *lower panels* show the image of IGS-RFLP analysis with *Alu*I and *Dde*I as restriction endonucleases, respectively

Cluster analysis based on the similarity indexes was carried out using the unweighted-pair group method arithmetic clustering (UPGMA) with the program Neighbor in PHYLIP version 3.572 (Felsenstein 1995).

IGS-RFLP analysis with *Alu*I and *Dde*I revealed that *A. mellea* subsp. *nipponica* isolates used in this study had four IGS-RFLP phenotypes (A, B, C, D) (Fig. 1). Phenotype A

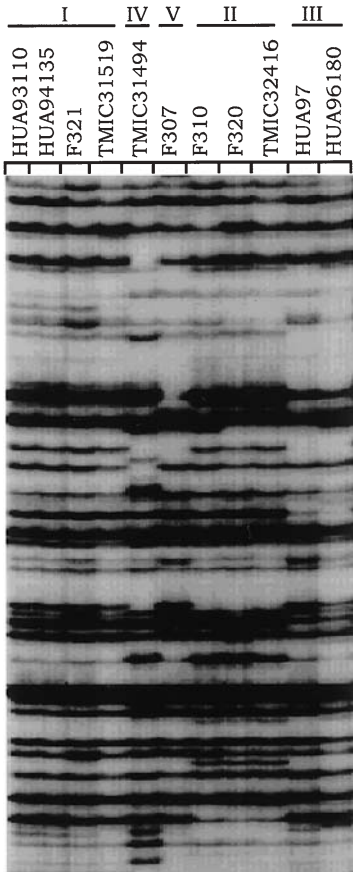

Fig. 2. Electrophoresis image of amplified fragment length polymorphism (AFLP) analysis of 11 *Armillaria mellea* subsp. *nipponica* isolates. The image shows DNA fragments between 100 and 200 bp in length from *bottom* to *top*. These DNA fragments were produced with an E + AT/M + CA primer combination. *Roman numbers above the image* indicate isolates with an almost identical AFLP profile. The numbers correspond to the subgroup numbers in Fig. 3

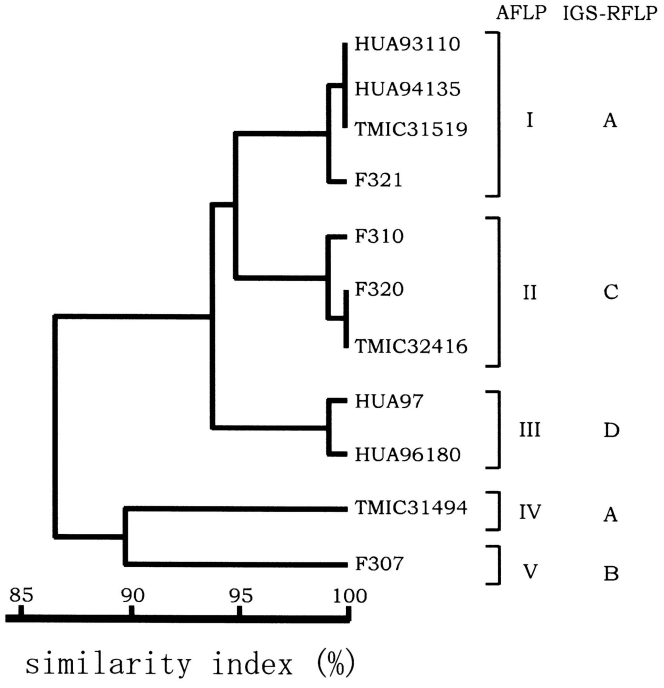

Fig. 3. Unweighted pair-group matched with arithmetic averages (UPGMA) dendrogram based on AFLP profile similarities among *Armillaria mellea* subsp. *nipponica* isolates. Similarity indexes between AFLP profiles were calculated according to Nei and Li (1979)

(*Alu*I-a and *Dde*I-a pattern) was found in HUA93110, HUA94135, F321, TMIC31519, and TMIC31494. Phenotype B (*Alu*I-b and *Dde*I-b pattern) was obtained in F307. Phenotype C (*Alu*I-b and *Dde*I-c pattern) was presented in F310, F320, and TMIC32416, and phenotype D (*Alu*I-c and *Dde*I-d pattern) was found in HUA97 and HUA96180. Additional IGS-RFLP analysis with *Msp*I, *Hae*III, *Hha*I, *Hin*fI, *Rsa*I, *Eco*RV, and *Mbo*I could not divide the isolates with the identical phenotype into subgroups (data not shown). The IGS-RFLP phenotype *Alu*I-a pattern was reported by Terashima et al. (1998b), and the *Alu*I-b pattern was similar to *A. mellea* B type reported by Harrington and Wingfield (1995), but the *Dde*I pattern was not examined for their isolates.

Part of an electrophoresis image of AFLP analysis is shown in Fig. 2. In this study, 57–64 AFLP bands in each isolate (total, 77 bands) were identified. Cluster analysis based on the AFLP profiles revealed five subgroups (I, II, III, IV, V) (Fig. 3). Subgroup I was composed of HUA93110, HUA94135, TMIC31519, and F321; subgroup II was composed of F310, F320, and TMIC32416; subgroup III consisted of HUA97 and HUA 96180; and subgroups IV and V were TMIC31494 and F307, respectively. Subgroups I, II, III, IV, and V had IGS-RFLP phenotypes A, C, D, A, and B, respectively. The average of AFLP profile similarities within the 11 isolates was 92.1%, and that within each subgroup was greater than 99.1%, suggesting that isolates constituting each of the subgroups have very similar genetic composition. AFLP profiles of a parental isolate (HUA93110) derived from a basidiome and four single-spore isolates (HUA93110-2, -3, -4, and -5) were also almost identical: of four single-spore isolates, three (HUA93110-2, -3, and -4) showed the same AFLP profile (61 bands) to that of the parental isolate, and one (HUA93110-5) had 60 AFLP bands identical to those of the remaining isolates, indicating that the similarities between the spore-derived isolates were greater than 99.2%. These results indicate that the genetic composition of the spore-derived isolates and their parental isolates is almost identical.

IGS-RFLP analysis have been applied to molecular-based identification of *Armillaria* species; it has been known that several IGS-RFLP phenotypes exist within one species of *Armillaria* (Harrington and Wingfield 1995; Terashima et al. 1998b). Comprehensive databases of IGS-RFLP phenotypes are, therefore, necessary for rapid identification of *Armillaria* species using the IGS-RFLP phenotype. Hereafter, IGS-RFLP phenotypes of more isolates in *A. mellea* subsp. *nipponica* should be examined to make this technique more reliable for rapid identification of this fungus.

IGS sequence data have been used to infer phylogenetic relationships among isolates in *A. mellea* s. str. from Europe, North America, and Asia, including Japan and Korea (Coetzee et al. 2000). Two types of IGS sequences in *A. mellea* subsp. *nipponica* isolates (HUA93110 and B731) were reported (Terashima et al. 1998a; Coetzee et al. 2000): the IGS sequence of HUA93110 was identical to Korean *A. mellea* s. str. isolates (B916, B917); the B731 isolate prob-

ably had IGS-RFLP phenotype B or C. Comparison of the other IGS sequences of *A. mellea* subsp. *nipponica* with those of *A. mellea* s. str. outside Japan would reveal phylogenetic relationships between heterothallic *A. mellea* s. str. and homothallic *A. mellea* subsp. *nipponica*, providing insights into evolution of the life cycle in *Armillaria*.

In this study, isolates with an identical IGS-RFLP phenotype showed relatively low similarity of AFLP profiles: the IGS phenotype A was found in HUA93110, HUA94135, F321, TMIC31519, and TMIC31494, whereas these isolates were divided into two subgroups by AFLP analysis (subgroup I: HUA93110, HUA94135, TMIC31519, and F321; subgroup IV: TMIC31494) (see Fig. 3). It is unknown whether IGS sequences of these isolates are identical because RFLP analysis can detect the polymorphism at only the nucleotide sequences recognized by the restriction endonuclease. Sequencing analysis of the IGS region and/or the other gene in these isolates might offer a clue to the relationships between these subgroups.

Ota et al. (1998b) have described that the life cycle of *A. mellea* subsp. *nipponica* is a kind of secondary homothalism: a meiotic division in the basidium produces four haploid nuclei, the haploid nuclei fuse in pairs to form two diploid nuclei in the basidium, and each of the diploid nuclei migrates into a basidiospore. These observations prompted us to consider that meiotic recombination would confer the distinction in genotypes between basidiospore-derived and basidiome-derived isolates if one of two genomes constituting the diploid nucleus in the basidiome cell were dissimilar to the other. In contrast to this possibility, in this study it was indicated that genetic compositions of the parental isolate and its offspring (spore-derived isolates) were very similar, suggesting that the two genomes constituting the diploid nucleus in the basidiome cell are almost identical.

In this study, it was suggested that the isolates within the same AFLP subgroup were distributed throughout a relatively large area in Japan (see Fig. 1, Table 1). It was also pointed out that spore-derived isolates show very similar AFLP profiles to that of their parental isolate. Production of asexual spores such as oidia or conidia has never been reported in *A. mellea* subsp. *nipponica*. These results, therefore, suggest that the large distribution of the isolates within the same subgroup was established via the basidiospore, but not the asexual spore, from a common parental strain.

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