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Characterization of North American *Armillaria* species by DNA content and RFLP analysis[1]

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Abstract: Twenty-six isolates representing nine North American *Armillaria* species were investigated with flow cytometry and RFLP (restriction fragment length polymorphism) analyses to determine their genome size and RFLP profile. Three putatively diploid isolates of *A. ostoyae*, *A. gemina*, *A. calvescens*, *A. sinapina*, *A. mellea*, *A. gallica*, *A. nabsnona*, and North American Biological Species (NABS) X were analyzed, and two putative diploid isolates of NABS XI also were analyzed. Genome sizes of *Armillaria* species were 0.11--0.17 pg per nucleus (55--84 _ 10^6 bp/C), depending on species. Among the nine North American *Armillaria* species tested, *A. ostoyae*, *A. gemina*, and *A. mellea* possessed relatively small genome sizes (0.11--0.12 pg per nucleus), whereas *A. gallica* possessed a relatively large genome size (0.17 pg per nucleus). *A. nabsnona* has a slightly larger genome size (0.13 pg per nucleus) than *A. ostoyae, A. gemina*, and

A. mellea. Other species (*A. calvescens*, *A. sinapina*, NABS X, and NABS XI) possessed moderate genome sizes (*ca* 0.15 pg per nucleus). Polymerase chain reaction (PCR) and RFLP of the intergenic spacer region-1 (IGS-1) generated banding patterns for nine *Armillaria* species. In addition to previously reported banding patterns, new banding patterns are presented for *A. gemina*, *A. calvescens*, *A. mellea*, and *A. gallica*.

Key Words: flow cytometry, genome size, intergenic spacer, North American Biological Species

INTRODUCTION

The genus *Armillaria* consists of about nine species in North America. Species were originally defined using biological and morphological characteristics (Anderson and Ullrich 1979, Korhonen 1978); however, molecular genetic studies of these species allow new evaluations of taxonomic relationships. Information on genome size and DNA sequence data for *Armillaria* species will facilitate investigations of their phylogenetic relationships, ecological roles in forest ecosystems, and gene flow within and among different species. Since 1985, several studies report qualitative comparisons of DNA content in nuclei of *Armillaria* species; however, this study represents the first quantitative determinations of genome sizes in *Armillaria* species.

In previous studies, microspectrophotometric techniques were used to detect differences in nuclear DNA content between two geographically distinct isolates of *Armillaria* species (Motta 1985). Microspectrophotometric analysis also revealed relative differences in nuclear DNA content in basidioma tissue of *A. mellea* s. s. and *A. bulbosa* (=*A. gallica*) (Motta et al 1986). Similar techniques were used to determine that monokaryotic cells of mature *A. bulbosa* basidiocarps contained haploid nuclei (Peabody and Peabody 1985). Subsequently, nuclear

volume and DNA content were estimated for three life-cycle stages of *A. bulbosa* (Peabody and Peabody 1986). Fluorescence microscopy also allowed relative comparisons of nuclear DNA content at various developmental stages of Japanese *A. mellea* s. s. (Ota et al 1998). Although results from microspectrophotometry methods can be informative, the methods are slow, difficult to use, and measurement is practically limited to about 100 nuclei per fungal sample (Eilam et al 1994).

Laser flow cytometry is a relatively new technique that allows quantitative measurement of DNA content. The laser flow cytometer measures the fluorescence intensity of a DNA-binding stain such as propidium iodide to determine DNA content of cell nuclei (Arumuganathan and Earle 1991, Hultquist et al 1996). Thousands of propidium iodide-stained nuclei can be measured in a few seconds, and data can be automatically analyzed using software that determines the mean position of nuclear peaks from the sample. Although the availability of laser flow cytometry has led to its widespread use in basic biology and medicine, relatively few reports utilizing flow cytometry in mycological studies have been published (Allman 1992, Bianciotto and Bonfante 1992, Bianciotto et al 1995, Eilam et al 1994). Laser flow cytometry was conducted in conjunction with protoplast isolation to demonstrate comparatively a haploid nuclear condition in secondary mycelium of *A. mellea* s. s. (Darmono and Burdsall 1993). However, quantification of DNA content in *Armillaria* species has not been reported previously. Previous difficulties in DNA quantification were associated with deficiencies in methodologies including measuring equipment and general techniques to separate and uniformly stain mycelial nuclei for precise quantitative analysis.

Recently, molecular genetic methods were developed to augment identification of

Armillaria species (Anderson and Stasovski 1992, Anderson et al 1987, 1989, Harrington and Wingfield 1995, Jahnke et al 1987, Miller et al 1994, Smith and Anderson 1989, Smith et al 1990, Schulze et al 1995, 1997). Anderson and Stasovski (1992) used polymerase chain reaction (PCR) to amplify the Intergenic Spacer (IGS) of the ribosomal RNA operon, and sequenced this region for several *Armillaria* species to determine phylogenetic relationships. Based on the IGS-1 sequences, Harrington and Wingfield (1995) developed a simplified PCR-based method in which the IGS-1 region is amplified, then cut with restriction endonucleases to produce RFLPs that are species specific. Their PCR-based method is especially practical because mycelial scrapings frequently provide sufficient template DNA for species identification. Subsequently, this method has been widely applied to augment identification of *Armillaria* species (Banik and Burdsall 1998, Banik et al 1996, Baumgartner et al 1997, Baur et al 1997, Chillali et al 1997, Coetzee et al 1997, Frontz et al 1998, Sierra and Whitehead 1997, Sung et al 1997, Volk et al 1996, White et al 1998).

The objectives of this study were to: (i) quantify nuclear DNA content of nine North American *Armillaria* species, (ii) verify whether differences exist in the genome sizes among nine *Armillaria* species investigated, and (iii) determine the IGS-1 diagnostic banding patterns using the PCR-RFLP method for species identification of nine *Armillaria* species. These objectives provide a characterized set of diploid tester strains for use in biological and taxonomic studies of *Armillaria* species.

MATERIALS AND METHODS

Tested species included *A. ostoyae* (Romagn.) Henr., *A. gemina* Bérubé & Dessureault, *A. calvescens* Bérubé & Dessureault, *A. sinapina* Bérubé & Dessureault, *A. mellea* (Vahl: Fr.)

Kummer, *A. gallica* Marxmüller & Romagnesi, *A. nabsnona* Volk & Burdsall, NABS X, and NABS XI. Three isolates of each of the nine described species of *Armillaria* in North America were included in the study, with the exception of NABS XI for which only two isolates were included (TABLE I). Isolates originated from basidioma (stipe or context) tissue or mass-spore cultures, and were previously identified by various investigators using haploid x haploid mating or haploid x diploid pairing tests (TABLE I). To ensure that each isolate represented a distinct genet (vegetative clone), isolates were selected from different geographic regions or were tested by somatic pairing (Anderson and Kohn 1995) (TABLE I). For use in flow cytometry and RFLP analyses, isolates were maintained in Petri dishes on 3% malt agar medium (3% malt extract, 1% peptone, 3% glucose, 1.5% agar) and incubated at 22 C in the dark.

Armillaria isolates were grown for 6 wk on 3% malt agar medium. Mycelia (*ca* 30 mg) of each isolate were dissected away from the medium, fixed in 4% (w/v) formaldehyde in Tris buffer (10 mM Tris, 10 mM Na-EDTA, 100 mM NaCl, 0.1% Triton-100, pH 7.4) (Bianciotto et al 1995) for 15 min, and then washed twice in Tris buffer. Intact nuclei were isolated in Tris buffer by repeatedly chopping the mycelia with a sharp scalpel blade. Suspended nuclei were filtered through nylon filters with a 30-µm pore diameter. To a 0.7 mL suspension of nuclei, 2.5 µL RNAse (500 µg/mL) was added, followed by gentle mixing with 20 µL propidium iodide solution (5 mg/mL). All nuclear isolation procedures were conducted on ice. Nuclear suspensions were incubated in the dark for 20 min at room temperature. Fluorescence of the nuclear stain propidium iodide was measured at the University of Nebraska-Center for Biotechnology Flow Cytometry Core Research Facilities using a FACScan Flow Cytometer (Becton Dickinson, San Jose, California). Debris was excluded from the analyses by raising the

FL3 threshold, and the appropriate threshold value was determined experimentally. Each measurement of fungal DNA content was based on 10 000 scanned nuclei. The internal standard used for comparison was chicken red blood cells (CRBC), for which the DNA content is known (2.33 pg/2C) (Galbraith et al 1983). All flow cytometry data were evaluated using a linear and logarithmic scales to assure normal distribution. For each sample and the internal standard, the mean positions of nuclear peaks were determined by analysis using CellQuest software (Becton Dickinson, San Jose, California). The formula used for converting fluorescence intensity values to DNA content was: Nuclear DNA content = (mean fluorescence intensity of sample peak) / (mean fluorescence intensity of the standard peak) x DNA content of the standard (2.33 pg). All flow cytometry analyses were conducted four times per isolate. The analysis of variance (ANOVA) for species means was performed using the General Linear Model (GLM) procedure (SAS 1996). A Tukey's Honestly Significant Differences test ($\alpha = 0.05$) was used to determine differences among *Armillaria* species.

Fluorescent stained nuclei were observed with fluorescence microscopy to determine the number of nuclei per cell. Actively growing mycelia were dissected away from the 3% malt agar medium with a scalpel, then fixed in FAA (4% formaldehyde, 2.5% acetic acid, and 47.5% ethanol in 0.1 M phosphate buffer, pH 7.0) for 6 h at 4 C, then rinsed four times in 0.1 M phosphate buffer. Fixed nuclei were stained and mounted in Vectashield® mounting medium with 1.5 µg/mL 4',6-diamidino-2 phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, California). Specimens were observed using an oil immersion lens and UV excitation light on a fluorescence microscope (Olympus BX60).

The IGS-1 region between the 3' end of the 25S (large subunit) ribosomal RNA (rRNA)

gene and the 5' end of the 5S rRNA gene was amplified using PCR. Template DNA was derived from scrapings of actively growing mycelial cultures (3--4 wk old). Primers LR12R (Veldman et al 1981) and O-1 (Duchesne and Anderson 1990) were used to amplify the IGS-1 region. Each 100-µL reaction mixture contained template DNA from a mycelial scraping (or no DNA template for negative control), 2.5 units *Taq* polymerase (Perkin-Elmer, Branchberg, New Jersey), PCR reaction buffer (supplied with *Taq* enzyme), 4 mM $MgCl_2$, 200 µM dNTPs, and 0.5 µM of each primer (LR12R and O-1). After an initial denaturation step at 95 C for 90 s, PCR was conducted using 35 cycles of 60 C for 40 s (annealing), 72 C for 2 min (extension), and 90 C for 30 s (denaturation) using a Perkin-Elmer (480) thermocycler (Harrington and Wingfield 1995). A final extension step at 72 C for 10 min was conducted to ensure complete amplification into a double-stranded product.

Restriction digestions were conducted with aliquots (e.g., 10–15 µL) from PCR amplification mixtures. Because the enzyme *Alu* I typically produces the most polymorphisms useful for species identification, it was the primary restriction endonuclease used on all PCR products (Harrington and Wingfield 1995). When *Alu* I (New England BioLabs, Inc., Beverly, Massachusetts) restriction cuts were insufficient to distinguish species, other restriction endonucleases (e.g., *Bsm* I, *Dde* I, *Eco*R I, *Hae* III, *Hin*c II, *Nde* I) (New England BioLabs, Inc., Beverly, Massachusetts) were used. Digestions were conducted using 5--10 units of restriction endonucleases for 8--12 h at 37 C.

Intact and restriction-digested PCR products were electrophoresed in 2.5% MetaPhor® agarose (FMC BioProducts, Rockland, Maine) gels using 0.5X TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) buffer. Electrophoresis was conducted at a constant 100 V for 4 h. Gels

were stained with ethidium bromide (0.5 μg/mL) and bands were visualized using UV light. Gel images were analyzed with SigmaGel™ gel analysis software (SPSS Science, Chicago, Illinois).

RESULTS

Genome sizes of nine *Armillaria* species as determined by laser flow cytometry are presented in TABLE II. Genome sizes varied from 0.109 pg to 0.237 pg per nucleus and showed small variances with a CV (coefficient of variation) ranging from 6.4% to 10.8% (8.5% of mean CV) (TABLE II, FIG. 1). *Armillaria ostoyae, A. gemina*, and *A. mellea* have comparatively small genome sizes (0.109--0.125 pg per nucleus) (TABLE II); however, a significant difference was noted between *A. ostoyae* and *A. mellea* (TABLE III). No significant differences in genome sizes were observed among *A. calvescens*, *A. sinapina* (except isolate ST13), NABS X, and NABS XI (0.148--0.159 pg per nucleus) (TABLES II , III). Compared to the other species, *A. gallica* shows a relatively large genome size (0.165--0.169 pg per nucleus) (TABLE II). *Armillaria nabsnona* has a slightly larger genome size (0.131--0.134 pg per nucleus) than *A. ostoyae, A. gemina*, and *A. mellea* (TABLE II), and a significant difference was noted between *A. nabsnona* and *A. ostoyae*, *A. gemina*, or *A. mellea* (TABLE III). Among 26 isolates, *A. sinapina* isolate ST13 showed the largest genome size (0.237 pg per nucleus), a size indicative of a triploid nuclear condition (TABLE II, FIG. 1E). A histogram of fluorescent events versus amount of fluorescence intensity revealed well-defined peaks with a normal distribution for all nine *Armillaria* species (FIG. 1). Fluorescence microscopic observations revealed a monokaryotic condition in all investigated isolates of *Armillaria* species.

Amplified DNA products corresponding to the intergenic spacer (IGS-1) region were *ca*

875 bp for *A. mellea*, and *ca* 920 bp for *A. ostoyae*, *A. gemina*, *A. calvescens*, *A. sinapina*, *A. gallica, A. nabsnona*, NABS X, and NABS XI. The restriction enzymes *Alu* I, *Bsm* I, *Dde* I, *Eco*R I, *Hae* III, *Hin*c II, and *Nde* I were used to examine restriction sites within the amplified IGS-1. One to three *Alu* I digestion patterns were found in each of the 9 taxa tested (TABLE II, FIG. 2). Sizes of *Alu* I digestion fragments for the nine *Armillaria* species are given in TABLE II, and fragment patterns of *Alu* I digestion are illustrated in FIG. 2. Some isolates (e.g., *A. sinapina* ST13) displayed additional bands with a pattern that represented composites of other isolates of the same species (FIG. 2). In these isolates, the total size of the fragments was greater than the size of the uncut IGS-1 region. Digestion with *Alu* I produced characteristic diagnostic banding patterns for *A. ostoyae* (ST1, ST2, P1404) (FIG. 2 lanes 1--3), *A. gemina* (ST9, ST11) (FIG. 2 lanes 5, 6), *A. sinapina* (ST12, ST13, M50) (FIG. 2 lanes 10--12), *A. mellea* (ST20, ST21) (FIG. 2 lanes 14, 15), *A. gallica* (ST23) (FIG. 2 lane 18), *A. nabsnona* (C21, M90, ST16) (FIG. 2 lanes 19--21), NABS X (837, D82, POR100) (FIG. 2 lanes 22--24), and NABS XI (M110, S20) (FIG. 2 lanes 25, 26) (TABLE II) (Banik et al 1996, Banik and Burdsall 1998, Harrington and Wingfield 1995, Volk et al 1996, White et al 1998). Restriction digestions with *Alu* I could not differentiate between isolates of *A. ostoyae* (ST1, ST2, P1404) (FIG. 2 lanes 1--3) and *A. gemina* (ST9, ST11) (FIG. 2 lanes 5, 6) (TABLE II). However, restriction digestion with *Nde* I produced a unique banding pattern for *A. ostoyae*. Amplified products of all *A. ostoyae* isolates were digested with *Nde* I (552, 372 bp); however, the product of only one *A. gemina* isolate (ST9) was digested with *Nde* I (913, 552, 461, 372 bp). Using *Alu* I, *Bsm* I, *Dde* I, *Hae* III, *Hin*c II, and *Nde* I, isolates of *A. calvescens* (ST3, ST17, ST18) (FIG. 2 lanes 7--9) and *A. sinapina* (ST12) (FIG. 2 lane 10) showed the same RFLP banding pattern (TABLE II), and were indistinguishable. Compared to

earlier studies (Banik and Burdsall 1998, Banik et al 1996, Harrington and Wingfield 1995, Volk et al 1996, White et al 1998), previously unreported RFLP patterns of *A. gemina* (ST8, FIG. 2 lane 4), *A. calvescens* (ST3, ST17, ST18, FIG. 2 lanes 7--9), *A. mellea* (ST5, FIG. 2 lane 13), and *A. gallica* (M70, ST22, FIG. 2 lanes 16, 17) were also observed.

DISCUSSION

This study provides genome size and IGS-1 RFLP data that further characterize a set of tester strains for use in biological and taxonomic studies of *Armillaria* species. Flow cytometry provided an effective means to measure genome sizes of *Amillaria* spp. The IGS-1 RFLP data is essential preliminary information for species identification and provides a basis for future molecular genetic studies.

Following their quantification of nuclear DNA in fungi, Duran and Gray (1989) suggested that genome size could assist their taxonomic classification of 70 smut fungi and two *Neurospora* species. Studying arbuscular mycorrhizal fungi, Bianciotto and Bonfante (1992) showed a 65% difference between nuclear DNA content of *Gigaspora margarita* Becker & Hall (0.74--0.77 pg per nucleus) and *Glomus versiforme* (Karsten) Berch (0.25--0.27 pg per nucleus). Although more studies are needed to determine genome sizes of fungal taxa, available data suggest that genome size is useful for assessing a taxonomic relationships.

The DNA-propidium iodide fluorescence measurements revealed marked differences among the genome sizes of nine *Armillaria* species (FIG. 1). Because this is the first report of quantitative measurements of genome size in *Armillaria* species, other independent measurements are needed for comparison. In our preliminary studies, flow cytometry was used on basidiospore derived cultures to determine haploid genome sizes of *A. sinapina* (*ca* 0.08 pg

per nucleus), NABS X (*ca* 0.08 pg per nucleus), and NABS XI (*ca* 0.08 pg per nucleus). Matings of basidiospore-derived cultures of NABS X isolates produced diploid mycelia with genome sizes of *ca* 0.15--0.16 pg per nucleus which corresponded to the genome size of cultures derived from the parental basidiomata (Kim et al unpublished).

Genome sizes of *A. ostoyae*, *A. gemina*, *A. calvescens*, *A. sinapina*, *A. mellea*, *A. nabsnona*, NABS X, and NABS XI were significantly different from *A. gallica* (TABLE III). *Armillaria gallica* had a genome size approximately 40% larger than *A. ostoyae*, *A. gemina*, or *A. mellea.* In addition, the *A. gallica* genome size was about 25% larger than *A. nabsnona,* and about 10% larger than *A. calvescens, A. sinapina* (except ST13), NABS X, and NABS XI. Motta et al (1986) reported a 32% difference between nuclear DNA content of *A. mellea* s. s. and *A. bulbosa* (= *A. gallica*) using microspectrophotometric analysis (n = 50). Thus, genome size represents a useful diagnosic characteristic to distinguish select species of *Armillaria*.

Flow cytometric quantification of DNA is also a useful tool for basic studies in fungal cell biology. Using flow cytometric analysis of nuclei isolated from spores and mycorrhizal roots of leek, Bianciotto et al (1995) demonstrated that colonization of a host by an arbuscular mycorrhizal fungus, *Glomus versiforme*, is linked to activation of its cell cycle. In this same manner, flow cytometry could also assist the study of ploidy levels through the life cycle of *Armillaria* species. Vegetative mycelia (e.g., mycelial fan or rhizomorphs) and mated cultures of *Armillaria* species typically exist in the diploid state (Franklin et al 1983, Korhonen 1980, Ullrich and Anderson 1978), in contrast to the dikaryotic state of most basidiomycetes. In our preliminary flow cytometry work with 45 vegetative (rhizomorph or mycelial fan) isolates, we also found that genome sizes were consistent with a primarily diploid condition, as exemplified

by *A. ostoyae* (*ca* 0.12 pg per nucleus), *A. sinapina* (*ca* 0.15 pg per nucleus), NABS X (*ca* 0.16 pg per nucleus), and *A. gallica* (*ca* 0.18 pg per nucleus). With these 45 vegetative isolates, only one isolate of *A. ostoyae* showed a genome size indicative of a triploid condition (*ca* 0.175 pg per nucleus) (Kim et al unpublished). Our present results showed the nine *Armillaria* species possess nuclei with a homogeneous unimodal distribution, pointing to a single ploidy level in all isolates (FIG. 1). Therefore, diploid genome sizes of nine North American *Armillaria* species are from 0.113 pg (*A. ostoyae*) to 0.168 pg (*A. gallica*) per 2C, depending on species (TABLE III).

The genome size (0.237 pg per nucleus) of *A. sinapina* isolate ST13, which originated from a multisporous isolation, is indicative of a triploid nuclear condition. Genome sizes of the other isolates of *A. sinapina* (ST12, M50) were 0.149 pg and 0.151 pg per 2C, respectively (TABLE II). The fluorescence intensity of ST13 showed a homogeneous unimodal distribution (FIG. 1E), and microscopic observation of DAPI-stained nuclei revealed a monokaryotic condition. In previous studies on genetic exchange between diploid and haploid mycelia of *A. gallica*, Carvalho et al (1995) presented evidence that triploidy occasionally occurs. The biological significance and origin of triploidy, exemplified by *A. sinapina* isolate ST13, is unknown at this point. The natural prevalence of triploidy or other polyploidy is also unknown for other *Armillaria* species. Ploidy level could potentially influence mating, compatibility, adaptation, reproduction, pathogenicity, or other interactions with the biotic and abiotic environments. Continued studies with flow cytometry can help determine the role of polyploidy in the ecology of *Armillaria* species.

Diploidization of mated *Armillaria* cultures among interspecific species can also be assessed using flow cytometry. Verification of diploidization (or nuclear combination) can help

verify mating among intraspecific and interspecific crosses. Studies on compatibility among *Armillaria* species frequently indicate a low-frequency compatibility among isolates ascribed to separate species. Examples of low-frequency compatibility include isolates of *A. sinapina* paired with isolates of *A. cepistipes*, NABS X, or NABS XI (Anderson et al 1980, Banik and Burdsall 1998, Bérubé et al 1996). Isolates of *A. cepistipes* have shown a low-frequency compatibility with isolates of NABS X (Anderson et al 1980, McDonald et al 1998), and NABS X isolates have shown low-frequency compatibility with NABS XI isolates (Banik and Burdsall 1998). Although low-frequency compatibility does not necessarily indicate conspecificity, it does represent a possible mechanism for gene flow among species. Thus, our results show that flow cytometric techniques can help assess questions of nuclear status during the cell cycle of *Armillaria* species and address gene flow issues among *Armillaria* species.

For most isolates, RFLP patterns from *Alu* I cut IGS-1 were similar to those reported previously. However, isolates used in this study also produced IGS-1 RFLPs that were previously unreported. The IGS-1 RFLPs of *A. gemina* (ST8), *A. calvescens* (ST3, ST17, and ST18), *A. mellea* (ST5), and *A. gallica* (M70 and ST22) were not observed in previous reports of these species.

Amplified IGS-1 of *A. ostoyae* and *A. gemina* (except ST8) showed identical RFLP banding patterns following an *Alu* I cut (FIG. 2). Previous studies have shown that these species possess similar morphological (Bérubé and Dessureault 1988) and molecular characteristics (Anderson and Stasovski 1992, Piercey-Normore 1998). In addition, our study shows that genome sizes of these two species are not significantly different (TABLE III). Previous studies imply that speciation has occurred quite recently in the *A. ostoyae-A. gemina* group; however, it

is not completely clear which species is ancestral (Anderson and Stasovski 1992, Piercey-Normore 1998). *Armillaria gemina* ST8 had an additional fragment of 168 bp, possibly resulting from a 28 bp deletion in the 196 bp fragment (FIG. 2 lane 4). White et al (1998) reported a similar deletion pattern from *A. ostoyae*. With isolates in our study, *A. ostoyae* and *A. gemina* were distinguishable with an *Nde* I cut IGS-1.

The new RFLP pattern for *Alu* I cut IGS-1 of *A. calvescens* (ST3, ST17, and ST18) is identical with one isolate of *A. sinapina* (ST12) (TABLE II, FIG. 2). Several other studies have also shown this banding pattern is shared by *A. gallica* and *A. sinapina* (Banik et al 1996, Harrington and Wingfield 1995, White et al 1998). However, previously reported banding patterns of the *A. gallica* isolates were distinct from the pattern of *A. calvescens* or *A. sinapina* isolates in our study (TABLE II, FIG. 2 lanes 16--18). One banding pattern of *A. gallica* (585, 234 bp, FIG. 2 lane 18) was observed previously with *A. gallica* (582, 240 bp) and *A. calvescens* (582, 240 bp) (Harrington and Wingfield 1995). Harrington and Wingfield (1995) could not differentiate between *A. gallica* and *A. calvescens* using several restriction enzyme digestions. In our study, restriction digestions with *Bsm* I, *Dde* I, *Eco*R I, *Hae* III, *Hin*c II, and *Nde* I failed to differentiate between isolates of *A. calvescens* and *A. sinapina* (data not shown). We also attempted to differentiate these two species using *Alu* I, *Eco*R I, *Hin*c II, and *Nde* I restriction cuts of PCR-amplified ITS (internal transcribed spacer) region, but found identical banding patterns with both species (data not shown). Somatic pairing tests can distinguish among *A. calvescens*, *A. sinapina*, and *A. gallica* (McDonald unpublished). However, definitive PCR-RFLP species identification among these species may require additional restriction enzymes or assessments of variation in other DNA regions. Sequence analysis of IGS, ITS, or other

diagnostic regions may provide information useful for distinguishing these species and addressing questions concerning evolution of this group.

An overriding goal of this study was to better characterize a set of diploid tester strains for use as references in future research. The determination of genome size and RFLP characterization of IGS-1 provide a critical basis for biological characterization of *Armillaria* spp. Further studies will characterize additional features of these and other isolates of *Armillaria* species. Directly or indirectly, such efforts should contribute to a better understanding of biological, ecological, and taxonomic relationships among *Armillaria* species.

ACKNOWLEDGMENTS

The paper was supported in part by the Biotechnology Graduate Research Associateship program of the Center for Biotechnology (UNL), the Storkan-Hanes Foundation, the Department of Plant Pathology at the University of Nebraska-Lincoln, and Nebraska Statewide Arboretum Graduate Research Fellowship. The authors thank Drs. Alan Harvey, Mike Larsen, Gary Yuen, and Phil Wargo for their comments on earlier versions of this manuscript. We also thank Drs. Harold Burdsall and Duncan Morrison for *Armillaria* isolates, and Mr. James Donley and Mr. Richard Carman for technical assistance. Use of trade names in this paper does not constitute endorsement by the USDA Forest Service.

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Fig. 1. Representative histograms showing numbers of given fluorescence intensities obtained by flow cytometry for propidium iodide-stained nuclei from nine *Armillaria* species and chicken red blood cells (CRBC; 2.33 pg/nucleus). Isolates of *Armillaria* are described in TABLE I. The mean of log fluorescence intensities (χ), standard error (S.E.), and coefficient of variation (C.V.) are listed for the peak from each isolate.

Fig. 2. PCR-RFLP band patterns of *Armillaria* species intergenic spacer (IGS-1) region digested with *Alu* I. Lanes: 1 (ST1), 2 (ST2), and 3 (P1404) = *A. ostoyae*; 4 (ST8), 5 (ST9), and 6 (ST11) = *A. gemina*; 7 (ST3), 8 (ST17), and 9 (ST18) = *A. calvescens*; 10 (ST12), 11 (ST13), and 12 (M50) = *A. sinapina*; 13 (ST5), 14 (ST20), and 15 (ST21) = *A. mellea*; 16 (M70), 17 (ST22), and 18 (ST23) = *A. gallica*; 19 (C21), 20 (M90), and 21 (ST16) = *A. nabsnona*; 22 (837), 23 (D82), and 24 (POR100) = North American Biological Species (NABS) X; 25 (M110) and 26 (S20) = NABS XI. Size markers (50 bp) in base pairs are shown on far left and far right.

[1]This manuscript has been assigned Journal Series No. 12652, Agricultural Research Division, University of Nebraska.

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