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Use of flow cytometry, fluorescence microscopy, and PCR-based techniques to assess intraspecific and interspecific matings of *Armillaria* species

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For assessments of intraspecific mating using flow cytometry and fluorescence microscopy, two compatible basidiospore-derived isolates were selected from each of four parental basidiomata of North American Biological Species (NABS) X. The nuclear status in NABS X varied with basidiospore-derived isolates. Nuclei within basidiospore-derived isolates existed as haploids, diploids (doubled haploids), or a mixture of haploids and diploids (doubled haploids). Depending on the nuclear status of the basidiospore-derived lines of NABS X, intraspecifically mated cultures can exist as diploids or tetraploids, and possibly triploids or aneuploids under *in vitro* conditions. Based on previous *in vitro* mating studies, seven basidiospore isolates were specifically selected to assess rare, interspecific mating among *Armillaria cepistipes*, *A. sinapina*, NABS X, and NABS XI. Cultures from basidiospore-derived isolates were paired to produce four interspecifically paired cultures, and matings were assessed using flow cytometry and restriction fragment length polymorphism (RFLP) analyses. Based on flow cytometric analysis, the *A. cepistipes* isolate exhibited compatibility with a NABS X isolate, and the *A. sinapina* isolate exhibited compatibility with a NABS X isolate, and the *A. sinapina* isolates were individually compatible with isolates of NABS X and NABS XI. Mean fluorescence intensities of *A. cepistipes* × NABS X, *A. sinapina* × NABS X, and *A. sinapina* × NABS XI mated cultures revealed a triploid or tetraploid nuclear status compared to the haploid or diploid (doubled haploid) nuclear status of initial basidiospore-derived isolates. Polymerase chain reaction (PCR) and RFLP of the intergenic spacer (IGS) region generated banding patterns for basidiospore-derived isolates and mated cultures. Four species-specific RFLP banding patterns were observed in basidiospore-derived isolates of *A. cepistipes*, *A. sinapina*, NABS X, and NABS XI. PCR-RFLP analysis showed combined banding patterns from mated cultures. Flow cytometry and PCR-RFLP analysis are effective tools to assess matings of *Armillaria* species.

INTRODUCTION

The vegetative phase of most basidiomycetes exists as a dikaryon, where two haploid nuclei are paired in each cell, and the dikaryon state is maintained in new growth by clamp connections. However, clamp connections and dikaryotic cells occur only briefly in the life-cycle of many *Armillaria* species (Korhonen & Hintikka 1974, Larsen *et al.* 1992, Tommerup & Broadbent 1975) and the persistent stage is usually diploid (Franklin *et al.* 1983, Korhonen 1980, Ullrich & Anderson 1978). Microspectrophotometry was previously used to examine stages in the nuclear cycle of *A. mellea*, basidiospores were found to be haploid, monosporous isolates were generally diploid, and trama tissues were perhaps diploid (Peabody *et al.* 1978). In subsequent studies on *A. mellea*, Franklin, Filion &

Anderson (1983) demonstrated that basidiospore-derived mycelia were haploid and mated cultures were diploid.

Studies on sexual compatibility among *Armillaria* species frequently indicate low-level compatibility among isolates ascribed to separate species. Examples of low-level compatibility included paired *A. sinapina*-*A. cepistipes* (5.5 % compatible), *A. sinapina*-NABS X (3.5 % compatible), *A. sinapina*-NABS XI (5 % compatible), *A. cepistipes*-NABS X (6.5 % compatible), NABS X-NABS XI (4 % compatible) (Banik & Burdsall, 1998). Bérubé *et al.* (1996) examined the compatibility between *A. sinapina* and *A. cepistipes* and reported these species were 2.1 % to 6.7 % compatible. Anderson, Korhonen & Ullrich (1980) and McDonald *et al.* (1998) also reported that *A. cepistipes* has shown compatibility with NABS X isolates. Although such low-level compatibility does not necessarily indicate conspecificity, it does indicate a potential mechanism for gene flow among *Armillaria* species or populations.

Confirming compatibility among *Armillaria* isolates is a

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complex process. Macroscopic assessments of compatibility by characteristic culture reactions are occasionally unreliable (Banik & Burdsall 1998, McDonald, unpubl.). Compatibility assessments can be improved by examining morphological traits (e.g. clamp connections or swollen cells) of paired mycelia at the microscopic level (Larsen *et al.* 1992); however, false negative and false positive reactions still occur (McDonald & Larsen, unpubl.). If unique genetic markers are apparent in individual isolates, molecular genetic techniques can be used to demonstrate the presence of combined markers in mated cultures of compatible isolates.

Anderson & Stasovski (1992) used the 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 sequences, Harrington & Wingfield (1995) developed a simplified PCR-based method in which the IGS region is amplified, then cut with restriction endonucleases to produce restriction fragment length polymorphisms (RFLPs) that are species-specific. Mating among *Armillaria* species is evident if the mated culture contains IGS RFLPs from both basidiospore-derived lines of the mated culture. However, PCR-RFLP techniques cannot distinguish between nuclear combination and intermingled mycelia of separate genetic backgrounds. Protoplast and hyphal tip cultures can help establish uniformity of mated cultures; however, these techniques are laborious and may not reflect the overall condition of the mated culture. Because vegetative or mated mycelia of *Armillaria* species typically exist in the diploid state (Franklin *et al.* 1983, Korhonen 1980, Ullrich & Anderson 1978), determination of diploidization or nuclear combination can help verify mating among intraspecific and interspecific crosses. Flow cytometry offers the potential to assess the nuclear status resulting from intraspecific and interspecific matings of *Armillaria*.

Measurement of DNA content (Ormerod 1990) is one of the most widespread applications of flow cytometry. Such measurements can assist studies of ploidy levels through the fungal life cycle. Several researchers found that the vegetative stage of *Armillaria* species exists in the diploid state (Franklin *et al.* 1983, Korhonen 1980, Ullrich & Anderson 1978). In our previous work, flow cytometry was used to quantify nuclear DNA contents of 70 isolates of North American *Armillaria* species that originated from vegetative tissues (i.e. mycelial fan, rhizomorph, stipe, or context tissue) and matings of basidiospore-derived cultures. Based on these measurements, diploid nuclear DNA contents of *Armillaria* species varied with species: *A. ostoyae* (0.117 pg per 2C), *A. sinapina* (0.152 pg per 2C), NABS X (0.155 pg per 2C), NABS XI (0.152 pg per 2C), and *A. gallica* (0.173 pg per 2C) (Kim *et al.* 2000). If flow cytometry shows uniform diploidization (or nuclear combination) in a mated culture of two basidiospore-derived mycelia, such evidence would support that a mating has occurred and that the mated mycelia are of uniform nuclear status. Flow cytometry can be used to assess nuclear status and ploidy level of basidiospore-derived isolates and their mated cultures, and fluorescence microscopy can be used to determine whether mycelial cells are monokaryotic, dikaryotic, or polykaryotic.

This study used flow cytometry and fluorescence microscopy to assess the nuclear status of intraspecific mating with NABS X and interspecific matings among selected *Armillaria* species. PCR-RFLPs also were used to verify nuclear combinations in rare interspecific matings of *Armillaria* species. The objectives of this study were to: (1) assess the nuclear status and ploidy level of selected basidiospore-derived isolates, intraspecifically mated cultures, and interspecifically mated cultures using flow cytometry and fluorescence microscopy; (2) verify whether differences exist in the nuclear DNA contents of basidiospore-derived isolates and their mated cultures using flow cytometry; and (3) determine the status of nuclear combination from interspecific mating using IGS RFLP analysis.

MATERIALS AND METHODS

Fungal isolates for intraspecific and interspecific mating studies

In previous studies with NABS X, individual basidiospore-derived cultures were established in addition to cultures of parental basidioma tissue (McDonald, unpubl.). All basidiospore-derived cultures were thoroughly characterized for mating alleles using the occurrence of clamp connections and culture reactions on oak wood extract-orange juice medium (Darmono & Burdsall 1992, McDonald, unpubl.). For intraspecific mating studies, two compatible basidiospore-derived cultures were selected from each of four parental basidiomata (Table 1). Matings were accomplished by closely aligning subcultures of compatible basidiospore-derived isolates (Anderson & Ullrich 1979, 1982, Darmono & Burdsall 1992, Korhonen 1978, 1980, Larsen *et al.* 1992, Ullrich & Anderson 1978), and allowing 6 weeks for completion of mating

Table 1. *Armillaria* NABS X isolates used for the intraspecific mating study^a.

Isolate no.	Source of isolate
C7-3 (A ₁ B ₁) ^b	basidiospore
C7-11 (A ₂ B ₂)	basidiospore
C7-3 × C7-11 (A ₁ B ₁ × A ₂ B ₂)	mated culture
C7-CAP	basidioma ^c
D83-5 (A ₁ B ₁)	basidiospore
D83-6 (A ₂ B ₂)	basidiospore
D83-5 × D83-6 (A ₁ B ₁ × A ₂ B ₂)	mated culture
D83-CAP	basidioma
837-13 (A ₁ B ₂)	basidiospore
837-25 (A ₂ B ₁)	basidiospore
837-13 × 837-25 (A ₁ B ₂ × A ₂ B ₁)	mated culture
837-CAP	basidioma
C14-8 (A ₁ B ₂)	basidiospore
C14-12 (A ₂ B ₁)	basidiospore
C14-8 × C14-12 (A ₁ B ₂ × A ₂ B ₁)	mated culture
C14-CAP	basidioma

^a NABS = North American Biological Species. All NABS X isolates represent separate genets (vegetative clones) originating from ID, USA.

^b Mating alleles were identified previously, and AB allele designations pertain only to each individual cap (McDonald, unpubl.).

^c Two compatible basidiospore-derived isolates were selected from each of four basidiomata.

Table 2. *Armillaria* isolates for the interspecific mating study.

<i>Armillaria</i> species	Isolate	Source of isolates	Origin	Identifier
<i>A. cepistipes</i>	304-1	basidiospore	Finland	K. Korhonen
<i>A. sinapina</i>	HB9	basidiospore	WA, USA	H. Burdsall
<i>A. sinapina</i>	ON18	basidiospore	WA, USA	H. Burdsall
NABS ^a X	C32-4	basidiospore	ID, USA	G. McDonald
NABS X	D83-6	basidiospore	ID, USA	G. McDonald
NABS X	D84-11	basidiospore	ID, USA	G. McDonald
NABS XI	W112-4	basidiospore	WA, USA	H. Burdsall
X- <i>A. cep</i>	C32-4 × 304-1	mated culture		
XI- <i>A. sin</i>	W112-4 × ON18	mated culture		
X- <i>A. sin</i>	D83-6 × HB9	mated culture		
X- <i>A. sin</i>	D84-11 × HB9	mated culture		

^a NABS = North American Biological Species.

Table 3. Nuclear DNA content of NABS X determined by flow cytometry^a

<i>Armillaria</i> NABS X isolates	Nuclear DNA content ^b (pg per nucleus)
C7-3	0.086 ± 0.004 ^c 0.159 ± 0.008
C7-11	0.086 ± 0.002 0.156 ± 0.003
C7-3 × C7-11	0.156 ± 0.013
C7-CAP	0.158 ± 0.006
D83-5	0.078 ± 0.007 0.141 ± 0.011
D83-6	0.083 ± 0.007
D83-5 × D83-6	0.155 ± 0.009
D83-CAP	0.149 ± 0.014
837-13	0.155 ± 0.003
837-25	0.155 ± 0.003
837-13 × 837-25	0.270 ± 0.022
837-CAP	0.157 ± 0.005
C14-8	0.152 ± 0.006
C14-12	0.156 ± 0.006
C14-8 × C14-12	0.263 ± 0.031
C14-CAP	0.159 ± 0.005

^a NABS = North American Biological Species.

^b The standard used for comparison was Chicken Red Blood Cells (2.33 pg/2C), for which the DNA content is known.

^c Each number represents the mean ± standard deviation of the mean of five replications. Each replication of *Armillaria* isolate DNA content was based on 10,000 scanned nuclei.

processes before subsequent subculture. Based on a previous interspecific pairing study (McDonald, unpubl.), seven isolates of *A. cepistipes*, *A. sinapina*, NABS X, and NABS XI were specifically selected for strong potential of interspecific matings based on multiple criteria including culture morphology and the occurrence of clamp connections (Table 2). These isolates were derived from basidiospores, and were previously identified by various investigators using pairing tests (Table 2). Interspecific matings were conducted following previously described methods (Anderson & Ullrich 1979, 1982, Darmono & Burdsall 1992, Korhonen 1978, 1980, Larsen *et al.* 1992, Ullrich & Anderson 1978). For use in flow cytometry, fluorescence microscopy, and RFLP analysis, all 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.

Cultures and long-term archives of the *Armillaria* species used in this study are maintained in the USDA Forest Service, RMRS, Forestry Sciences Laboratory (Moscow, ID). Cultures are publicly accessible and will be shipped world-wide, provided that the requestor has appropriate regulatory permits. Representative cultures are also being sent to the American Type Culture Collection (ATCC). Original basidiomes were not preserved.

Flow cytometry and fluorescence microscopic analysis

Flow cytometric techniques were used to assess the nuclear status of basidiospore-derived, basidioma-derived, and mated cultures. After incubating for 6 weeks at 22 °, each mated culture was subcultured by excising a portion of dark- or brown-colored, crustose mycelia with a sterile scalpel blade, then placing it on fresh 3 % malt agar medium. Following a 5-week period, mycelia (ca. 30 mg) of each isolate were dissected away from the medium, and 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 nuclear suspension, 2.5 µl RNAse (500 µg ml⁻¹) were added, followed by gentle mixing with 20 µl (5 mg ml⁻¹) of propidium iodide. All nuclear isolation procedures were conducted on ice, and resulting suspensions were incubated in the dark for 20 min at room temperature. Fluorescence of the propidium iodide stained nuclei was analyzed at the University of Nebraska-Center for Biotechnology Flow Cytometry Core Research Facilities using a FACScan Flow Cytometer (Becton Dickinson, San Jose, CA). Data were registered on logarithmic scales. Debris was excluded from the analysis by raising the FL3 threshold, and the appropriate threshold value was determined experimentally. For each sample and the internal standard, the mean positions of nuclear peaks were determined by analysis using CellQuest software (Becton Dickinson, San Jose, CA). Each measurement of fungal DNA content was

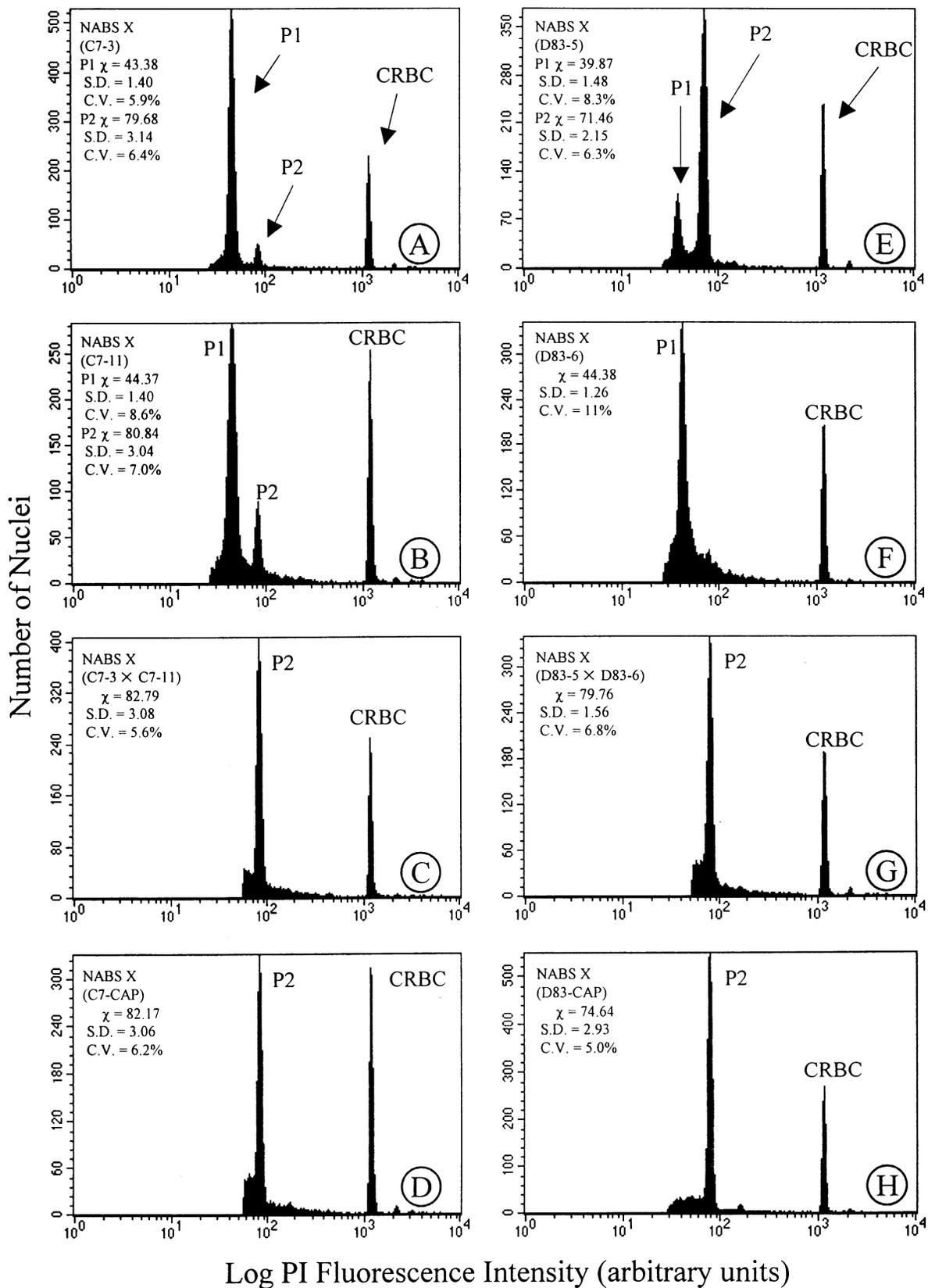


Fig. 1A-H. For legend see facing page.

based on 10,000 scanned nuclei. The 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). 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) \times DNA content of the standard (2.33 pg). Flow cytometric analyses were conducted four or five times for each basidiospore-derived or basidioma-derived isolate and five or eight times for each mated culture.

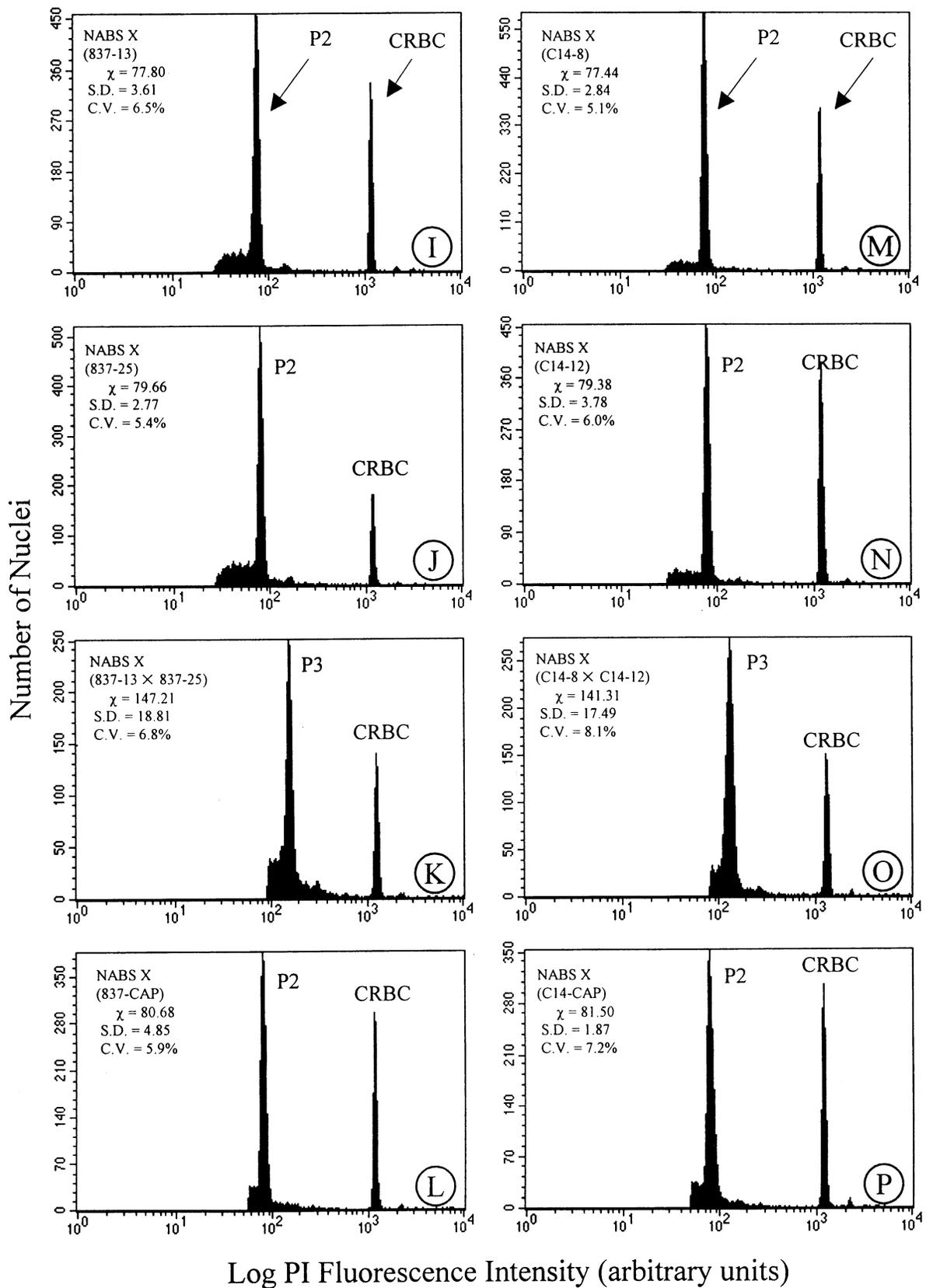


Fig. 1. Histograms showing numbers of given fluorescence intensities obtained by flow cytometry for propidium iodide-stained nuclei of *Armillaria* North American Biological Species X and chicken red blood cells (CRBC; 2.33 pg/nucleus). Isolates of *Armillaria* are described in Table 1. The mean of log fluorescence intensities (χ), standard deviation (s.d.), and coefficient of variation (c.v.) are listed for the peak from each isolates.

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 °, 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⁻¹ 4',6-diamidino-2 phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA). Specimens were observed using an oil immersion lens and UV excitation light on a fluorescence microscope (Olympus BX60).

Polymerase chain reaction, restriction endonuclease digestions, and electrophoresis

The IGS-1 region between the 3' end of the 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 weeks old). The following primers were used: LR12R, 5'-CTGAACGCCTCTAAGTCAGAA-3' (Veldman *et al.* 1981) and O-1, 5'-AGTCCTATGGCCGTGGAT-3' (Duchesne & Anderson 1990). 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, NJ), PCR reaction buffer (supplied with *Taq* enzyme), 4 mM MgCl₂, 200 µM dNTPs, and 0.5 µM of each primer (LR12R and O-1). After an initial denaturation step at 95 ° for 90 s, PCR was conducted using 35 cycles of 90 ° for 30 s (denaturation), 60 ° for 40 s (annealing), and 72 ° for 2 min (extension) using a Perkin-Elmer (480) thermocycler (Harrington & Wingfield 1995). A final extension step at 72 ° for 10 min was conducted to ensure complete amplification into a double-stranded product.

Restriction digestions were conducted with aliquots (i.e., 10–15 µl) from PCR amplification mixtures. Because the enzyme *Alu* I (New England BioLabs, Beverly, MA) typically produces the most polymorphisms useful for species identification, it was the primary restriction endonuclease used on all PCR products (Harrington & Wingfield 1995). Digestions were conducted using 5–10 units of restriction endonucleases for 8–12 h at 37 °.

Intact and restriction-digested PCR products were electrophoresed in 2.5 % MetaPhor® agarose (FMC BioProducts, Rockland, ME) 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⁻¹) and bands were visualized using UV light. Gel images were analyzed using SigmaGel™ gel analysis software (SPSS Science, Chicago, IL).

RESULTS

Intraspecific matings of *Armillaria* NABS X isolates

Nuclear DNA contents were quantified as shown in Table 3. The coefficients of variation for measurements of fluorescence intensity of all NABS X isolates ranged from 5 % to 11 % with

a mean of 6.7 % (Fig. 1). Nuclear DNA contents varied from 0.078 pg (D83-5) to 0.159 pg (C7-3) per nucleus for basidiospore-derived isolates and from 0.155 pg (D83-5 × D83-6) to 0.270 pg (837-13 × 837-25) per nucleus for mated cultures (Table 3). Nuclear DNA contents of basidioma (CAP)-derived isolates were 0.149 pg (D83-CAP) to 0.159 pg (C14-CAP) per nucleus (Table 3). Basidiospore-derived isolates C7-3, C7-11, and D83-5 showed two mean positions of nuclear peaks; the first peak (P1) corresponded to the haploid genome size (0.078 pg–0.086 pg per nucleus) and the other peak (P2) corresponded to a diploid (doubled haploid) nuclear DNA content (0.141 pg–0.159 pg per nucleus) (Fig. 1A–B, E). Other basidiospore-derived isolates, 837-13, 837-25, C14-8, and C14-12, showed only one mean position of the nuclear peak; this peak (P2) corresponded to the diploid (doubled haploid) nuclear condition (DNA contents: 0.152 pg–0.156 pg per nucleus) (Fig. 1I–J, M, N). One basidiospore-derived isolate, D83-6, exhibited a single mean position of the nuclear peak (P1), which corresponded to a haploid genome size (0.083 pg per nucleus) (Fig. 1F).

In mated cultures, a single mean position of the nuclear peak was observed (Fig. 1C, G, K, O). Nuclear peaks of C7-3 × C7-11 and D83-5 × D83-6 corresponded to the diploid nuclear DNA contents: 0.156 pg per nucleus for C7-3 × C7-11 and 0.155 pg per nucleus for D83-5 × D83-6 (Fig. 1C, G). Nuclear peaks of 837-13 × 837-25 and C14-8 × C14-12 indicated their nuclear DNA contents was tetraploid: 0.270 pg per nucleus for 837-13 × 837-25 and 0.263 pg per nucleus for C14-8 × C14-12 (Fig. 1K, O). Nuclei of all basidioma-derived isolates (C7, D83, 837, and C14) were diploid (0.149 pg per nucleus) (Fig. 1D, H, L, P). After DAPI staining, fluorescence microscopic observations revealed that all basidiospore-derived and mated cultures were monokaryotic.

Interspecific matings among *A. cepistipes*, *A. sinapina*, NABS X, and NABS XI

Nuclear DNA contents of *Armillaria* isolates as determined by laser flow cytometry are shown in Table 4. The coefficients of variation for measurements of fluorescence intensity of all isolates ranged from 6 % to 10 % with mean of 7.9 % (Fig. 2). Nuclear DNA contents varied from 0.083 pg (*A. sinapina* ON18) to 0.159 pg (NABS XI W112-4) per nucleus for basidiospore-derived isolates and from 0.232 pg (NABS XI W112-4 × *A. sinapina* ON18) to 0.260 pg (NABS X D84-11 × *A. sinapina* HB9) per nucleus for mated cultures (Table 4). Basidiospore-derived isolates C32-4, ON18, and W112-4 showed two mean positions of nuclear peaks; the first peak (P1) corresponded to the haploid genome size (0.083 pg–0.087 pg per nucleus) and the other peak (P2) corresponded to a diploid (doubled haploid) nuclear DNA content (0.149 pg–0.159 pg per nucleus) (Fig. 2A, D–E). Isolates 304-1, HB9, and D84-11 showed only one mean position of the nuclear peak; this peak (P2) corresponded to the diploid (doubled haploid) nuclear condition (nuclear DNA contents: 0.142 pg–0.151 pg per nucleus) (Fig. 2B, H, J). In addition, isolate D83-6 exhibited only one mean position of the nuclear peak (P1), which corresponded to a haploid genome size (0.089 pg per nucleus) (Fig. 2G).

Table 4. Nuclear DNA content of *Armillaria* species determined by flow cytometry and *Alu* I digestion fragment sizes (bp) of the intergenic spacer (IGS-1) region from seven single-spore derived *Armillaria* isolates and four interspecifically mated cultures.

<i>Armillaria</i> species	Isolates no.	DNA content ^a (pg per nucleus)	<i>Alu</i> I fragment sizes ^b (bp) of IGS-1 region
<i>A. cepistipes</i>	304-1	0.147 ± 0.004 ^c	309, 199, 137, 92
<i>A. sinapina</i>	HB9	0.142 ± 0.002	401, 241, 186
<i>A. sinapina</i>	ON18.	0.083 ± 0.001	401, 241, 186
		0.149 ± 0.002	
NABS ^d X	C32-4	0.084 ± 0.001	401, 186, 144
		0.155 ± 0.001	
NABS X	D83-6	0.089 ± 0.002	401, 186, 144
NABS X	D84-11	0.151 ± 0.003	401, 186, 144
NABS XI	W112-4	0.087 ± 0.001	401, 197, 186
		0.159 ± 0.004	
NABS X- <i>A. cepistipes</i>	C32-4 × 304-1	0.251 ± 0.006	403, 307, 199, 185, 144, 137, 92
NABS XI- <i>A. sinapina</i>	W112-4 × ON18	0.232 ± 0.011	400, 241, 197, 185
NABS X- <i>A. sinapina</i>	D83-6 × HB9	0.239 ± 0.005	400, 240, 186, 144
NABS X- <i>A. sinapina</i>	D84-11 × HB9	0.260 ± 0.021	401, 240, 186, 144

^a The standard used for comparison was Chicken Red Blood Cells (2.33 pg/2C), for which the DNA content is known.

^b Fragment sizes were determined from DNA size standards (20 bp or 50 bp) using SigmaGel™ gel analysis software. Values represent the mean of three replications from three different gels. Standard deviation of fragment size estimation was *ca.* ± 3 bp.

^c Each number represents the mean ± standard deviation of the mean of four (single spore-derived isolates) and eight (mated pair) replications. Each replication of *Armillaria* isolate DNA content was based on 10,000 scanned nuclei.

^d NABS = North American Biological Species.

In mated cultures, a single mean position of the nuclear peak was found (Fig. 2C, F, I, K). Nuclear peaks of NABS X × *A. sinapina* (D83-6 × HB9) and NABS XI × *A. sinapina* (W112-4 × ON18) corresponded to the triploid nuclear DNA contents: 0.239 pg per nucleus for D83-6 × HB9 and 0.232 pg per nucleus for W112-4 × ON18 (Fig. 2F, I). In addition, nuclear peaks of NABS X × *A. cepistipes* (C32-4 × 304-1) and NABS X × *A. sinapina* (D84-11 × HB9) were indicative of a tetraploid (or possibly triploid or aneuploid) nuclear DNA contents: 0.251 pg per nucleus for C32-4 × 304-1 and 0.260 pg per nucleus for D84-11 × HB9 (Fig. 2C, K). Observations of DAPI stained mycelia under the fluorescence microscope indicated a monokaryotic condition was present in cells from basidiospore-derived and interspecifically mated cultures.

The amplified DNA products corresponding to the intergenic spacer (IGS-1) region were *ca.* 920 bp for all isolates. Sizes of *Alu* I digestion fragments from seven basidiospore-derived isolates and four interspecifically mated cultures are presented in Table 4, and fragment patterns of *Alu* I digestion are shown in Fig. 3. Four distinct RFLP banding patterns were observed in basidiospore-derived isolates of *A. cepistipes*, *A. sinapina*, NABS X, and NABS XI (Fig. 3 lanes 1, 3, 6, 8). PCR-RFLP analyses showed mated cultures contained combined banding patterns that represented a combination of both basidiospore-derived lines (Fig. 3 lanes 2, 4, 5, 7).

DISCUSSION

Armillaria cultures are slow growing, and the only nuclei that are dividing are those at the hyphal tips. Thus, the majority of nuclei analyzed by flow cytometry were not undergoing replication, and the few nuclei that were dividing were doing so at a relatively slow rate. In our flow cytometry studies on *Armillaria* spp., the peaks were consistent and uniform. As

expected, relatively few nuclei possessed DNA undergoing replication, and flow cytometric analyses showed that the proportion of nuclei in G2 was negligible. Thus, peaks representing fused nuclei are quite distinct from those observed in S or post-S phase nuclei.

It appears that basidiospore-derived isolates can exist in cultures as haploid, diploid (doubled haploid), or a mixture of haploid and diploid (doubled haploid). Basidiospore-derived cultures that showed a diploid nuclear DNA content are considered to be doubled haploids because they behaved as typical haploids in all previous mating reactions designed to identify mating alleles (McDonald, unpubl.). In addition, diploid nuclear DNA contents of *A. sinapina*, NABS X, and NABS XI were measured as 0.152 pg to 0.155 pg based on our previous studies (Kim *et al.* 2000). From these measurements, we anticipate that haploid genome sizes of *A. sinapina*, NABS X, and NABS XI range from 0.076 pg to 0.078 pg. Because basidiospores were unavailable for our flow cytometry studies, we cannot verify when basidiospore-derived cultures attained a diploid (doubled haploid) status. Presently, it is unknown whether cultures existing as doubled haploids and mixed nuclear status were naturally derived or represent a response to extended growth in culture. Although haploid isolates have been cultured for 7–12 years except isolate 304-1 (32 years), these isolates continue to behave as typical haploids in mating reactions (McDonald, unpubl.). Cultures of mixed nuclear status appeared to possess only one nucleus per cell. This condition suggests that spontaneous chromosomal doubling may occur during haploid culture. In previous studies on relative DNA content of *A. mellea*, Franklin *et al.* (1983) reported that basidiospores and monosporous isolates were haploid and that strains derived from matings of isolates of single spores were diploid; however, the results of Peabody, Motta & Therrien (1978) differed from Franklin *et al.* (1983). Using Feulgen staining and

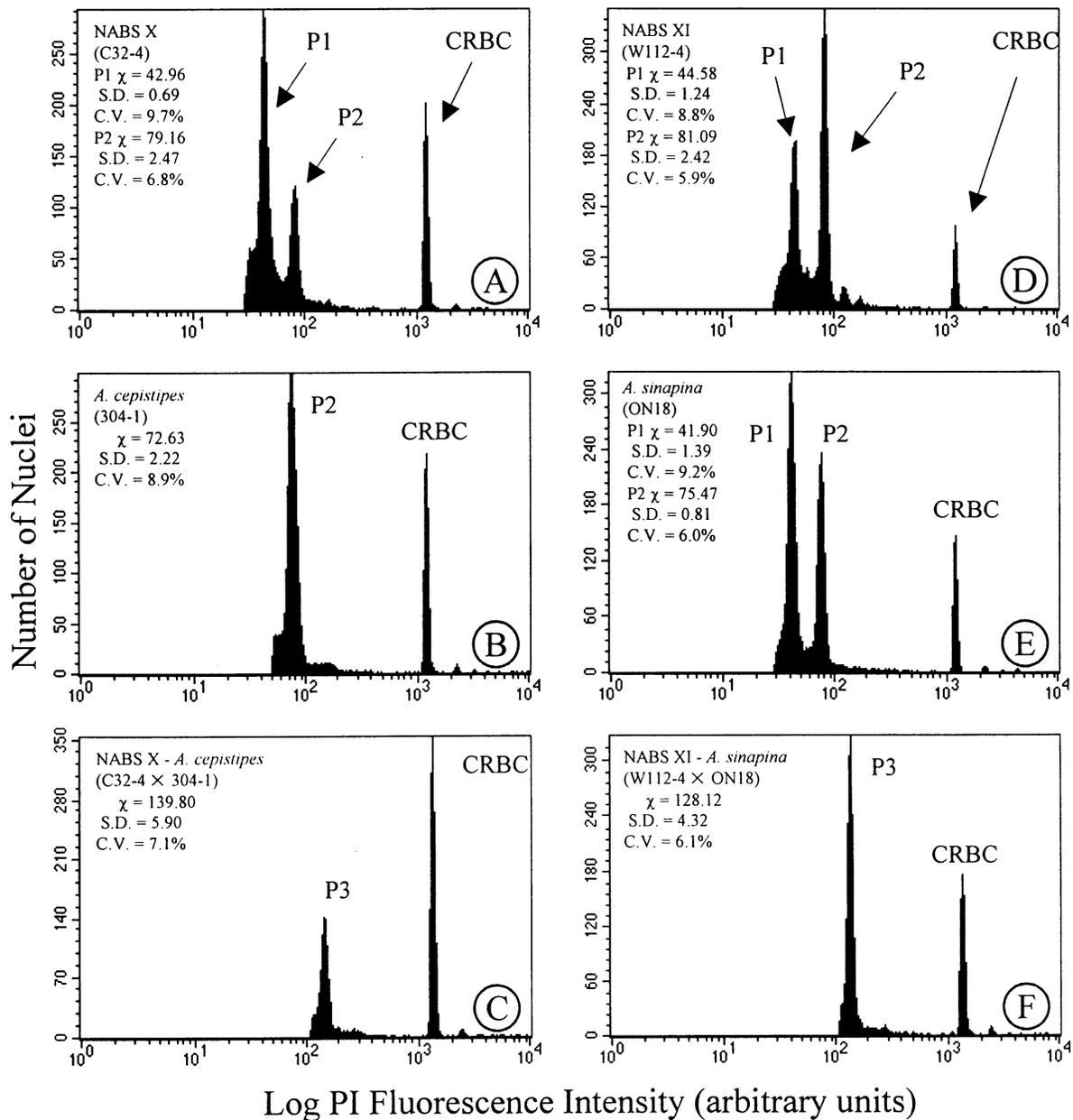


Fig. 2 A–F. For legend see facing page.

microspectrophotometry, Peabody *et al.* (1978) compared nuclear DNA content of several cell types in *A. mellea*. They concluded that basidiospores were haploid, but many monosporous isolates were diploid. Franklin *et al.* (1983) argued that diploid monosporous isolates would be difficult to reconcile based on observed mating behavior and bifactorial heterothallism. It appears that diploid monosporous isolates of Peabody *et al.* (1978) occurred by spontaneous chromosomal doubling. Such evidence further supports that the diploid nuclei in our basidiospore-derived cultures represent a doubled haploid state generated by spontaneous chromosomal doubling in culture.

Determination of diploidization or nuclear combination can help verify intraspecific and interspecific matings. Depending on the status of the basidiospore-derived lines, intra- and interspecific mating cultures can potentially exist as diploids, triploids, tetraploids, or aneuploids. Our results show that

cultures from intraspecific and interspecific matings possessed nuclei with a homogeneous unimodal distribution, demonstrating a single ploidy level resulting from nuclear fusion or combination (Fig. 1C, G, K, O, Fig. 2C, F, I, K). The ecological role and biological significance of ploidy level in the natural life cycle and *in vitro* culture of *Armillaria* species is not yet determined. Continued studies are needed to determine the ploidy of naturally produced basidiospores, basidiomata, rhizomorphs, and mycelial fans. Further investigations are also required to determine the origin of triploidy or tetraploidy, exemplified by both intra- and interspecifically mated cultures. The mechanisms regulating nuclear combination also warrant further study. The natural occurrence of aneuploidy, triploidy, tetraploidy, or other ploidy level is not well understood for *Armillaria* species. In our previous work, diploidy was a common nuclear condition among culture isolates derived from vegetative mycelia (e.g. rhizomorphs or

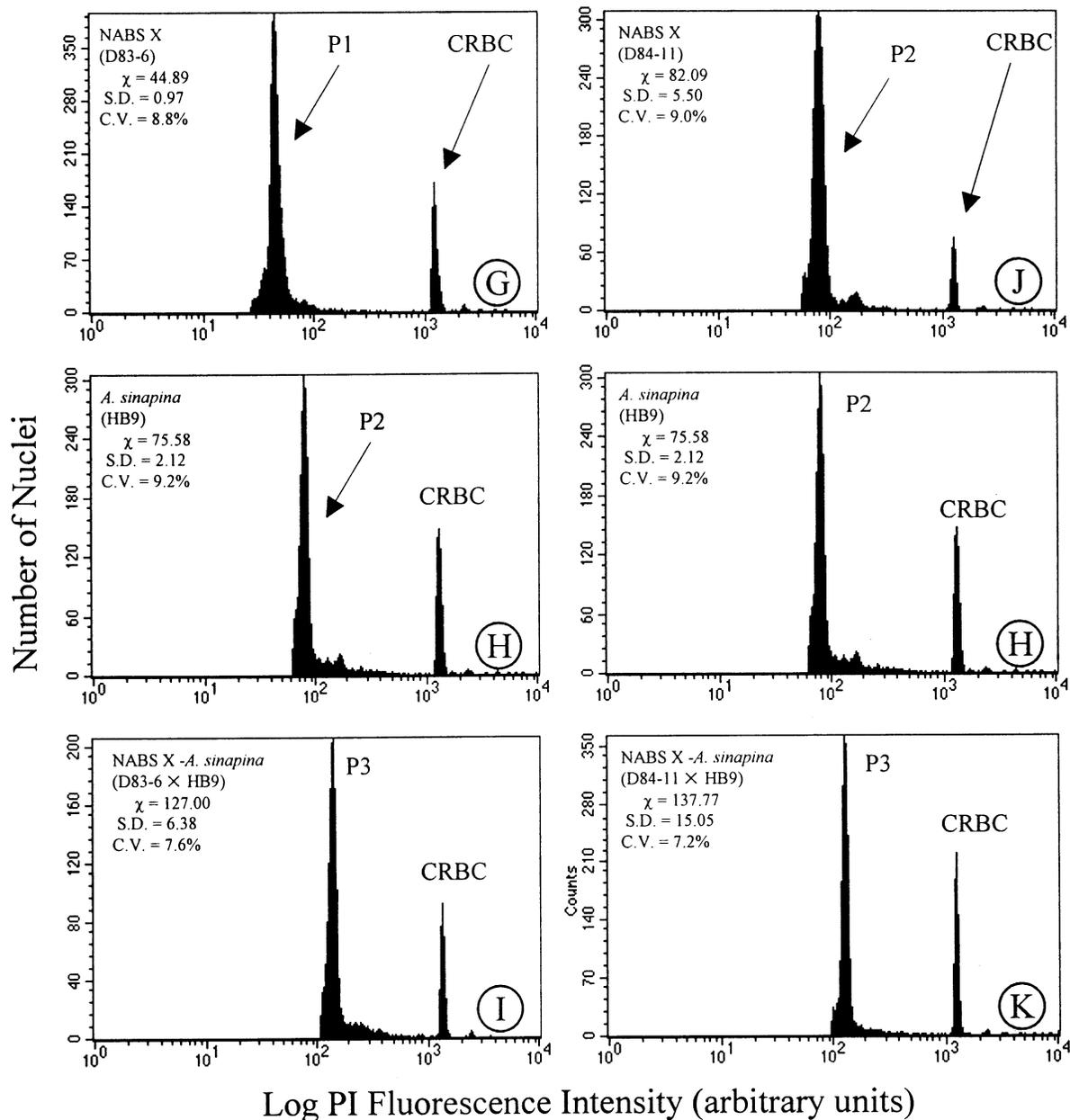


Fig. 2. Histograms showing numbers of given fluorescence intensities obtained by flow cytometry for propidium iodide-stained nuclei of *Armillaria* isolates and chicken red blood cells (CRBC; 2.33 pc/nucleus). Isolates of *Armillaria* are described in Table 2. The mean of log fluorescence intensities (χ), standard deviation (S.D.), and coefficient of variation (C.V.) are listed for the peak from each isolate.

mycelial fans) of North American *Armillaria* species (Kim *et al.*, unpubl.). After examining 35 cultures isolated from vegetative mycelial samples in northern Idaho, only one isolate, *A. ostoyae* (isolated from a mycelial fan) was found to exist in the triploid nuclear state (Kim *et al.*, unpubl.). Ploidy level could potentially influence mating, adaptation, compatibility, reproduction, pathogenicity, or other interactions with biotic and abiotic environments. Continued studies with flow cytometry, fluorescence microscopy, and genetic markers can help determine the role of ploidy level and nuclear combination in the ecology and *in vitro* studies of *Armillaria* species.

All of the RFLP banding patterns of *A. cepistipes*, *A. sinapina*, NABS X, and NABS XI observed for the IGS-*Alu* I digestions correspond to those reported earlier (Banik & Burdsall 1998,

Banik *et al.* 1996, Harrington & Wingfield 1995, White *et al.* 1998). Nuclear combination of interspecific matings was verified by identification of RFLP combined patterns that were a combination of both basidiospore-derived lines (Fig. 3, lanes 2, 4, 5, 7). Banik & Burdsall (1998) reported IGS RFLPs banding patterns are very useful genetic markers for assessing nuclear combination among *Armillaria* species. When morphological ratings (e.g. crustose) indicated successful mating among *A. sinapina*, *A. cepistipes*, NABS X, and NABS XI, these paired cultures generally showed combined IGS RFLPs banding patterns from both basidiospore-derived lines of the paired culture (Banik & Burdsall 1998). Only one RFLP banding pattern of IGS-1 or ITS (internal transcribed spacer) regions was present in NABS X (Fig. 3 lane 3), and thus this

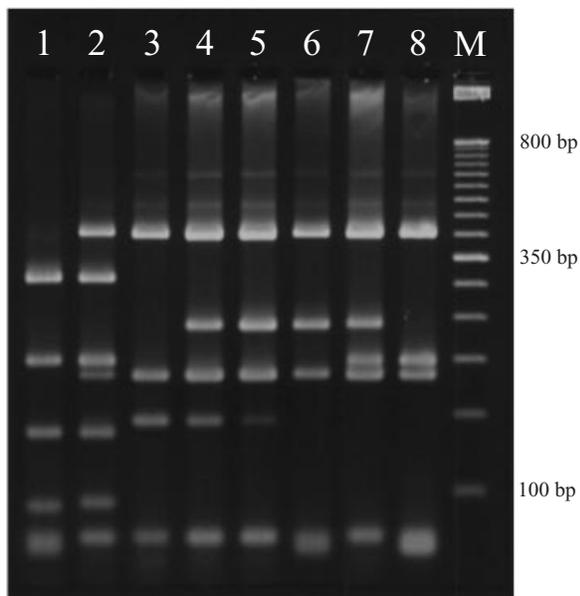


Fig. 3. PCR-RFLP band patterns of *Armillaria* species intergenic spacer (IGS-1) digested with *Alu* I. Lanes: 1 = *A. cepistipes* (304-1); 2 = North American Biological Species (NABS) X *A. cepistipes* (C32-4 x 304-1); 3 = NABS X (32-4); 4 = NABS X x *A. sinapina* (D83-6 x HB9); 5 = NABS X x *A. sinapina* (D84-11 x HB9); 6 = *A. sinapina* (HB9); 7 = NABS XI x *A. sinapina* (W112-4 x ON18); 8 = NABS XI (W112-4). Size markers (M; 50 bp) in base pairs are shown on far right.

RFLP analysis could not be used to assess nuclear combinations for the intraspecific matings.

In previous work, Anderson *et al.* (1980) also studied the monosporous isolate 304-1 (corresponds to isolate 2.3/1 in Anderson *et al.* 1980) which was used in our study of interspecific matings. In the previous work, isolate 304-1 appeared compatible in pairings with isolates of NABS X (Anderson *et al.* 1980). These studies demonstrate conclusively that select basidiospore-derived isolates of NABS X can undergo genetic recombination with select basidiospore-derived isolates of *A. cepistipes* or *A. sinapina* under laboratory conditions. Similarly, select basidiospore-derived isolates of NABS XI can genetically recombine *in vitro* with select basidiospore-derived isolates of *A. sinapina*. Macroscopic and microscopic observations of interspecific pairing indicate that interspecific mating occurs at relatively low frequency within the *A. sinapina*, NABS X, NABS XI, and *A. cepistipes* group. Continued use of flow cytometry with molecular genetic markers can address issues regarding phylogenetic and ecological significance of such matings. Further studies are also needed to more broadly characterize the genetic basis of interspecific matings and potential avenues of gene flow within the important genus *Armillaria*.

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