Identification protocol for six *Armillaria* species from northeastern North America

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Abstract: DNA sequences (~3 kb long) extending from the intergenic spacer 1 (IGS1) region to the 18S gene were obtained for isolates of *Armillaria ostoyae*, *Armillaria calvescens*, *Armillaria gallica*, and *Armillaria sinapina*. Additional investigation of 16 *A. ostoyae*, 11 *Armillaria gemina*, 21 *A. calvescens*, 18 *A. gallica*, and 15 *A. sinapina* isolates produced 117 sequences spanning the 3′ end of the IGS1 through the 5S gene and into the 5′ end of the IGS2 region. Additional sequences spanning the 3′ IGS2 to 5′ 18S gene region were obtained for two *A. ostoyae*, three *A. gemina*, two *A. calvescens*, two *A. gallica*, and three *A. sinapina* isolates. This is the first report of complete IGS2 sequences from *Armillaria* spp. A species identification protocol involving species-specific primers and restriction fragment length polymorphism analysis was devised based on species-specific polymorphisms. The protocol successfully identified all 16 *A. ostoyae*, 11 *A. gemina*, three of three *Armillaria mellea*, 18 *A. gallica*, 14 of 15 *A. sinapina* (11/12 diploid and 3/3 haploid), and 14 of 21 *A. calvescens* (13/15 diploid and 1/6 haploid) isolates included in this study. To the best of our knowledge, this success rate has not been matched by other methods.

Résumé : Les auteurs ont obtenu des séquences d'ADN (~3 kb de longueur) qui s'étendent de la région 1 de l'espaceur intergénique (IGS) jusqu'au gène 18S pour des isolats d'*Armillaria ostoyae*, *Armillaria calvescens*, *Armillaria gallica* et *Armillaria sinapina*. L'étude additionnelle de 16 isolats d'*A. ostoyae*, 11 d'*A. gemina*, 21 d'*A. calvescens*, 18 d'*A. gallica* et 15 d'*A. sinapina* a généré 117 séquences couvrant l'intervalle entre le bout 3′ de l'IGS1 et le bout 5′ de la région IGS2 en passant par le gène 5S. Des séquences additionnelles couvrant l'intervalle entre le bout 3′ de l'IGS2 et la région 5′ du gène 18S ont été obtenues pour deux isolats d'*A. ostoyae*, trois d'*A. gemina*, deux d'*A. calvescens*, deux d'*A. gallica* et trois d'*A. sinapina*. C'est la première fois qu'on obtient des séquences complètes d'IGS2 pour des espèces d'*Armillaria*. À partir de polymorphismes spécifiques à chaque espèce, les auteurs ont déterminé un protocole d'identification des espèces impliquant des amorces spécifiques à chaque espèce et l'analyse de polymorphismes de longueur des fragments de restriction. Le protocole a permis d'identifier avec succès les 16 isolats d'*A. ostoyae*, les 11 isolats d'*A. gemina*, trois des trois isolats d'*Armillaria mellea*, les 18 isolats d'*A. gallica*, 14 des 15 isolats d'*A. sinapina* (11 des 12 diploïdes et trois des trois haploïdes) et 14 des 21 isolats d'*A calvescens* (13 des 15 diploïdes et un des six haploïdes). Au meilleur de la connaissance des auteurs, ce taux de succès n'aurait pas été égalé par d'autres méthodes.

[Traduit par la Rédaction]

Introduction

Species of the genus *Armillaria* (Fr.:Fr.) Staude comprise an ecologically diverse and geographically widespread group that act as parasites and saprophytes on a wide range of woody and herbaceous plants (Kile et al. 1991; Thormann et al. 2001). Since Korhonen (1978), in Europe, and Anderson and Ullrich (1979), in North America, discovered that the causal fungus *Armillaria mellea* (Vahl:Fr.) Kummer *sensu lato* was actually a complex of biological species, numerous studies have investigated the geographic distribution, host range, and site relationships of the various species (Dumas 1988; Blodgett and Worrall 1992*a*, 1992*b*; Coetzee et al. 2000; McLaughlin 2001) as well as the phylogeny of the genus (Anderson and Stasovski 1992; Piercey-Normore et al. 1998; Terashima et al. 1998; Keča et al. 2006; Kim et al. 2006).

Underlying ecological and pathogenicity studies of *Armillaria* is the need for simple, economical, reliable, and rapid species identification of isolates obtained from host material or rhizomorphs. A variety of identification techniques have been developed and used since the 1970s. Nonmolecular species identification techniques include those based on basidiocarp morphology (Bérubé and Dessureault 1989; Fox et al. 1994), in vitro isolate pairing (Korhonen 1978; Guillaumin et al. 1991; Rizzo and Harrington 1992), and various protein-based analyses (Pérez-Sierra et al. 2000). These species identification techniques are being supplanted by the emergence and rapid development of molecular methods.

Phylogenetic analysis and comparison of DNA sequences of rDNA regions (internal transcribed spacer (ITS) and intergenic spacer 1 (IGS1)) have been conducted on *Armillaria* spp. for the purposes of species identification or clarification of the taxonomic relationships among newly identified species within a specific region or country (Bhutan: Coetzee et al. 2005) as well as for broader taxo-

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nomic studies (Northern Hemisphere: Anderson and Stasovski 1992; Europe: Chillali et al. 1998*a*, 1998*b*). Restriction fragment length polymorophism (RFLP) analysis of the IGS1 region has also become a widely used species identification technique and has been used in several national and regional surveys (British Columbia, Canada: White et al. 1998; Europe: Chillali et al. 1998*a*); North America: Kim et al. 2000; Serbia and Montenegro: Keča et al. 2006) as well as for identification of species associated with root disease on specific hosts (*Pinus* spp. in South Africa: Coetzee et al. 2000). The ITS region has also been used in RFLP analysis (Chillali et al. 1998*a*; Lochman et al. 2004). Phylogenetic and RFLP analyses of the ITS and IGS1 regions of *Armillaria* are useful tools, especially in initially characterizing species groups in a particular region or country, but they fall short as a practical means of confidently distinguishing *Armillaria* spp. because of the high sequence similarity between species (Harrington and Wingfield 1995; Banik et al.1996; White et al. 1998).

Although design of species-specific PCR primers has also been used in the detection and identification of a variety of fungal pathogens (Hamelin et al. 2000; Germain et al. 2002), wood decay fungi (Schmidt and Moreth 2000), and mycorrhizal symbionts (Kreuzinger et al. 1996), there are few reports of efforts to design species-specific primers for the detection and identification of *Armillaria* spp. (Sicoli et al. 2003). Recently, Kim et al. (2006) used amplified fragment length polymorphism (AFLP) markers and reported that these markers offer the potential for distinguishing currently recognized North American biological species of *Armillaria*.

Armillaria spp. identification studies have so far directed little attention to the 5S gene and adjacent IGS2 region. Duchesne and Anderson (1990) confirmed the location and direction of transcription of the 5S gene in *Armillaria*. White et al. (1998) distinguished British Columbia isolates of *Armillaria gallica* from *Armillaria sinapina* by *Alu*I RFLP digest of IGS2 PCR products but did not take this work further, nor did they publish any DNA sequence information for the IGS2 region. The objectives of this study were as follows: (*i*) obtain complete DNA sequences for the 5S gene and the IGS2 region of the four most commonly isolated *Armillaria* spp. in Ontario (i.e., *Armillaria ostoyae*, *A. gallica*, *Armillaria calvescens*, and *A. sinapina*) and (*ii*) identify species-specific polymorphisms in this region to develop an identification protocol for diploid isolates of *A. ostoyae*, *Armillaria gemina*, *A. gallica*, *A. calvescens*, *A. sinapina*, and *A. mellea* from field samples from northeastern North America.

Methods

Armillaria isolates

Eighty-one isolates of *A. ostoyae*, *A. gemina*, *A. calvescens*, *A. sinapina*, and *A. gallica*, which represent five of the six *Armillaria* spp. found in Ontario (Dumas 1988; McLaughlin 2001), were selected for intensive study, and three isolates of a sixth species, *A. mellea*, were included in a later stage of the study (Table 1). Most of the isolates were collected during earlier studies (McLaughlin 2008) from a variety of coniferous and deciduous hosts from various parts of Ontario, but some originated from other parts of northeastern North America.

DNA extraction

Isolates were incubated on cellophane sheets (Flexel; Atlanta, Georgia) on 3% malt agar for 2–4 weeks at room temperature. Isolates that produced very slow-growing, crusty cultures were transferred to cellophane sheets on 3% malt agar amended with 20 μg/mL ethanol to stimulate rhizomorph production (Weinhold 1963). Mycelia and rhizomorphs were scraped from the cellophane and used fresh or were stored at –20 °C in 1.5 mL microcentrifuge tubes until needed. DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Mississauga, Ontario) following the manufacturer's instructions with the following modification of the cell lysis step: 100 mg of fresh or frozen harvested mycelium and rhizomorphs was combined with 100 mg of sterile, sharp sand and 400 μL of DNeasy AP1 buffer and ground with plastic pestles in a 1.5 mL microcentrifuge tube for 1–2 min. Extracted DNA was stored at –20 °C.

Primer design and PCR amplification

PCR amplification and sequencing of the IGS2 was achieved in a stepwise fashion starting with a primer pair, 5S02R and TW1L (Howlett et al. 1992), based in the 5S and 18S genes, respectively. Internal primers within the IGS2 region were designed stepwise from sequences produced in the study. Primers 5S-1f, 5S-3f, and 5S-1r, used to amplify and sequence the 5S gene region including portions of the adjacent IGS1 and IGS2 regions, were designed from downstream portions of IGS1 sequences obtained from GenBank and from the IGS2 upstream end sequences obtained from this study. Two new primers were designed to facilitate more thorough study of the 3′ end of the IGS2 region and for species identification: Ao-f700, a forward primer based in the IGS2 region, and 18S-rev, comprising the last 21 bp of the reverse primer TW1L. The complete list of primers used in the study is presented in Table 2 and their approximate locations are shown in Fig. 1.

The PCR contained 1× PCR buffer (supplied with the polymerase), 200 μmol/L dNTPs, 2.5 mmol/L $MgSO_4$, 0.17–0.5 μmol/L primers, 0.5–1 unit of *Tsg* polymerase (Bio Basic, Markham, Ontario), and 1 μL (~5–10 ng) of genomic DNA template with water to a final volume of 15 μL. Primer and polymerase concentrations were adjusted to optimize the PCR results. Most amplifications of the IGS2 region were performed in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer,Woodbridge, Ontario), while a TC-312.2 (Techne, Burlington, New Jersey) was used in the work on the 3′ IGS1 to 5′ IGS2 and 3′ IGS2 to 18S regions. The PCR conditions are presented in Table 2. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) before being sent for sequencing by the University of Guelph Lab Services Division on an ABI 3700 DNA sequencer.

DNA sequence alignment and editing

Sequences were aligned first with the software CLUSTAL X (Chenna et al. 2003) using default parameters and the results were manually edited. For some samples (which typically produced multiple band sizes in gel electro-

Table 1. *Armillaria* isolates used in the species identification study.

Isolate	Host	Origin[a]	Ploidy[b]	IGS2 haplotype[c]	Collector/identifier
***Armillaria ostoyae* (NABS I)**					
168	*Betula papyrifera*	Simcoe Co., S ON	D	Ao-4[d]	J.A. McLaughlin
255	*Fraxinus americana*	Hastings Co. S ON	D	Ao-5	J.A. McLaughlin
P162b	*Pinus resinosa*	Simcoe Co., S ON	D	Ao-5	J.A. McLaughlin
Ya	*Pinus resinosa*	York Reg., S ON	D	Ao-2 and Ao-4	J.A. McLaughlin
286	*Pinus resinosa*	Durham Reg., S ON	D	Ao-2	J.A. McLaughlin
A1	*Pinus resinosa*	Simcoe Co., S ON	D	Ao-2	J.A. McLaughlin
W5	*Pinus resinosa*	Simcoe Co., S ON	D	Ao-1	J.A. McLaughlin
P165a	*Pinus resinosa*	Simcoe Co., S ON	D	Ao-1	J.A. McLaughlin
DnH1	*Pinus resinosa*	Dufferin Co., S ON	D	Ao-1	J.A. McLaughlin
B249c	*Pinus resinosa*	Simcoe Co., S ON	D	Ao-3	J.A. McLaughlin
SS59	*Picea mariana*	Sioux Lookout, NW ON	D	Ao-1	J.A. McLaughlin
DMR20	Unknown	NH	D	Ao-5	USDA FS, Madison
I JB 14a	*Betula papyrifera*	Chicoutimi, QC	H	Ao-1	J.A. Bérubé
I JB 29e	*Betula alleghaniensis*	Quebec City, QC	H	Ao-1 and Ao-2	J.A. Bérubé
I JB 77a	*Picea glauca*	St. Benjamin, QC	H	Ao-1	J.A. Bérubé
I JB 87c	Unknown	Quebec City, QC	H	Ao-1	J.A. Bérubé
***Armillaria gemina* (NABS II)**					
5	*Prunus serotina*	Muskoka Dist., C ON	D	Age-1	J.A. McLaughlin
18	*Acer saccharum*	Muskoka Dist., C ON	D	Age-2	J.A. McLaughlin
21	*Acer saccharum*	Muskoka Dist., C ON	D	Age-1	J.A. McLaughlin
95	*Betula alleghaniensis*	Muskoka Dist., C ON	D	Age-1	J.A. McLaughlin
118	*Acer saccharum*	Renfrew Co., C ON	D	Age-2 and Age-3	J.A. McLaughlin
124	*Quercus rubrum*	Renfrew Co., C ON	D	Age-2	J.A. McLaughlin
JJL153	Unknown	NY	D	Age-1	J. Worrall
JJW64	Unknown	NY	D	Age-1 and Age-4[e]	J. Worrall
MIELKE	Unknown	WV	D	Age-1	USDA FS, Madison
II JB 38a	*Acer saccharum*	Duchesnay, QC	H	Age-1	J.A. Bérubé
II JB 85d	*Acer saccharum*	Bromont, QC	H	Age-1	J.A. Bérubé
***Armillaria calvescens* (NABS III)**					
7	*Betula alleghaniensis*	Muskoka Dist., C ON	D	Ax-C[f] and Ax-A	J.A. McLaughlin
10a	*Acer saccharum*	IL	H	Ax-A	M.T. Banik
13	*Acer saccharum*	Muskoka Dist., C ON	D	Ac-5 and Ac-6	J.A. McLaughlin
15	*Acer saccharum*	Muskoka Dist., C ON	D	Ax-C and Ax-A	J.A. McLaughlin
20	*Acer saccharum*	Muskoka Dist., C ON	D	Ax-C and Ax-A	J.A. McLaughlin
21-5	Unknown	Unknown	H	Ax-C	J.B. Anderson
27	*Fagus grandifolia*	Parry Sound Dist., C ON	D	Ax-C and Ax-A	J.A. McLaughlin
28	Unknown	Parry Sound Dist., C ON	D	Ax-C	J.A. McLaughlin
44	*Acer saccharum*	Parry Sound Dist., C ON	D	Ax-A	J.A. McLaughlin
155	*Fraxinus americana*	Simcoe CO., S ON	D	Ax-C	J.A. McLaughlin
207	*Tilia americana*	Simcoe CO., S ON	D	Ac-3 and Ax-C	J.A. McLaughlin
223	*Acer saccharum*	Leeds & Grenville Co., S ON	D	Ax-C and Ac-4	J.A. McLaughlin
295	*Fagus grandifolia*	Peel Reg., S ON	D	Ax-A and Ac-3	J.A. McLaughlin
402	*Liriodendron tulipifera*	Elgin Co., S ON	D	Ax-C and Ac-7	J.A. McLaughlin
PR-3	Unknown	MI	D	Ax-A	USDA FS, Madison
FFC-7	Unknown	MI	D	Ax-A and Ac-7	USDA FS, Madison
423	*Pinus strobus*	Elgin Co., S ON	D	Ax-C	J.A. McLaughlin
IIIJB53c	Unknown	Loretteville, QC	H	Ax-A	J.A. Bérubé
IIIJB55c	*Acer saccharum*	St. Ange, QC	H	Ax-A	J.A. Bérubé
IIIJB56a	*Acer saccharum*	St. Ange, QC	H	Ax-A	J.A. Bérubé
IIIJBMD 52a	Unknown	Loretteville, QC	H	Ax-A	J.A. Bérubé
***Armillaria sinapina* (NABS V)**					
9	*Pinus strobus*	Muskoka Dist., C ON	D	As-1	J.A. McLaughlin
14	*Thuja occidentalis*	MI	H	As-1	M.T. Banik
23	*Tsuga canadensis*	Muskoka Dist., C ON	D	As-1	J.A. McLaughlin
47	*Populus tremuloides*	Sudbury Dist., C ON	D	As-1 and As-2	J.A. McLaughlin

Table 1 (*concluded*).

Isolate	Host	Origin[a]	Ploidy[b]	IGS2 haplotype[c]	Collector/identifier
50	*Picea glauca*	Sudbury Dist., C ON	D	As-1 and As-4	J.A. McLaughlin
69	*Betula alleghaniensis*	Sudbury Dist., C ON	D	As-5	J.A. McLaughlin
93	*Fagus grandifolia*	Muskoka Dist., C ON	D	As-1 and As-3	J.A. McLaughlin
96	*Fagus grandifolia*	Muskoka Dist., C ON	D	As-1 and As-3	J.A. McLaughlin
431	*Tsuga canadensis*	Mark S. Burnham Park, S ON	D	As-1	J.A. McLaughlin
K144	*Picea mariana*	Kapuskasing, NE ON	D	As-1	J.A. McLaughlin
K176	*Populus tremuloides*	Kapuskasing, NE ON	D	As-1	J.A. McLaughlin
CF-2	Unknown	MI	D	As-1 and As-4	USDA FS, Madison
TS190	*Populus tremuloides*	Timmins, NE ON	D	As-1	J.A. McLaughlin
V JB 05	*Acer saccharum*	Bromont, QC	H	As-1	J.A. Bérubé
V JB 19a	*Pinus strobus*	Chicoutimi, QC	H	As-1	J.A. Bérubé
***Armillaria mellea* (NABS VI)**					
232	*Fagus grandifolia*	Leeds & Grenville Co., S ON	D		J.A. McLaughlin
253	*Betula papyrifera*	Hastings Co. S ON	D		J.A. McLaughlin
323	*Fagus grandifolia*	Niagara Reg., S ON	D		J.A. McLaughlin
***Armillaria gallica* (NABS VII)**					
5	Unknown	Unknown	H	Ax-A	J.B. Anderson
7	Unknown	Unknown	H	Ax-A	J.B. Anderson
104	*Fraxinus americana*	Hastings Co. S ON	D	Ax-A	J.A. McLaughlin
101	*Acer saccharum*	Hastings Co., S ON	D	Ax-A	J.A. McLaughlin
111	*Acer saccharum*	Hastings Co. S ON	D	Ax-A and Ax-C	J.A. McLaughlin
173	*Fagus grandifolia*	Dufferin Co., S ON	D	Ax-A	J.A. McLaughlin
188	*Pinus resinosa*	Simcoe Co., S ON	D	Ax-A	J.A. McLaughlin
243	*Fagus grandifolia*	Leeds & Grenville Co., S ON	D	Ax-A and Ax-B	J.A. McLaughlin
251	*Ostrya virginiana*	Hastings Co. S ON	D	Ax-A	J.A. McLaughlin
260	*Prunus serotina*	Hastings Co. S ON	D	Ax-A	J.A. McLaughlin
266	*Pinus strobus*	Hastings Co. S ON	D	Ax-A	J.A. McLaughlin
273	*Prunus serotina*	York Reg., S ON	D	Ax-A and Ax-C	J.A. McLaughlin
283	*Betula papyrifera*	Durham Reg., S ON	D	Ax-A	J.A. McLaughlin
347	*Quercus rubra*	Elgin Co., S ON	D	Ax-A	J.A. McLaughlin
410	*Sassafras albidum*	Elgin Co., S ON	D	Ax-A	J.A. McLaughlin
Gc	*Pinus resinosa*	Ganaraska Forest, S ON	D	Ax-A	J.A. McLaughlin
MA-1	Unknown	WI	D	Ax-A and Ax-C	USDA FS, Madison
EL-1	Unknown	MI	D	Ax-A	USDA FS, Madison

[a]C ON, central Ontario; S ON, southern Ontario; NW ON, northwestern Ontario; NE ON, northeastern Ontario; QC, Quebec; NH, New Hampshire; IL, Illinois; MI, Michigan; NY, New York; WI, Wisconsin; WV, West Virginia.

[b]D, diploid culture; H, haploid culture from single-spore isolate.

[c]One or more haplotypes comprise each diploid isolate.

[d]For example, "Ao-4" is a unique haplotype (sequences with single nucleotide polymorphisms and indels >1 nucleotide in length) identified in isolates of this species.

[e]Identical to Ao-4.

[f]For example, "Ax-C" is a haplotype common to several isolates from more than one species.

phoresis), polymorphisms in the form of insertions or deletions (indels) between haplotypes produced long stretches of double peaks in sections of the chromatograms and muddled sequence results. These sequences, representing heterogeneous rDNA repeats, were separated into homogeneous sequences by closely examining chromatographs and selecting a previously obtained complete and unambiguous sequencing result for comparison. The matching and nonmatching nucleotides and multiple peaks were recorded. Subsequent alignment of separated sequences using CLUSTAL X or ALIGN (Feng and Doolittle 1987) revealed polymorphisms. The different sequence types within an isolate were labelled with letter designations appended to their genet label (e.g., Ac13-a, Ac13-b).

Species identification protocol

A step-by-step species identification protocol was devised based on the number and size of the PCR amplicons produced by the primer pair Ao-f700/18S-rev and digests of the 5S-1f/5S-1r amplicons with *Mph*1103I (Fermentas Life Sciences, Burlington, Ontario), *Tai*I (Fermentas Life Sciences), and *Bst*EII (Promega/Fisher Scientific, Nepean, Ontario). Primers Ao-f700 and18S-rev were designed from *A. ostoyae* IGS2 and 18S sequence data obtained from PCR products of the primer pair 5S02R/TW1L and were used to separate the isolates into species groups that could then be further separated by RFLP analysis of the 5S-1f/5S-1r amplicons. The specificity of the identification protocol to *Armillaria* spp. was tested by probing DNA extracted from

Table 2. Primers and thermal cycler conditions used in amplifying and sequencing the 5S gene and IGS2 rDNA regions of *Armillaria* spp. found in Ontario.

PCR region	Primer name[a]	Primer sequence (5′–3′)	Thermal cycler conditions[b]	Product size
IGS2 step 1	5S02R[c]	GCGGATCCCGTCCGATCTGCGAAGT	(94 °C 30 s, 60 °C 30 s, 68 °C 120 s) × 30[d]	~2.9–3.2 kb
	TW1L[c]	GCGGATCCACAAGCATATGACTACTGGC		
IGS2 step 2	IGS2-F3	TTACTTAGCTTCGTTAACGGAACG	(94 °C 30 s, 56 °C 60 s, 72 °C 120 s) × 35	~2–2.3 kb
	IGS2-2r	TTGGAGTACTAGACCGACATCA		
IGS2 step 3	IGS2-3f	GTCTCTCTTTCCTACCCTTAC	(94 °C 30 s, 56 °C 45 s, 72 °C 90 s) × 32	~1.3–1.5 kb
	IGS2-3r	GACCCGTTCGACGTTCAAGAC		
IGS2 step 4	IGS2-4fb	GCTYYATAGGATCAATATTCACCAC GTTTTAACTTCAAATTTGAGCG	(94 °C 30 s, 62 °C 45 s, 72 °C 90 s) × 35	~480–670 bp
	IGS2-4r			
IGS1(3′)–5S–IGS2(5′)	5S-1f	GTTTGCAACAAWTTTGGTGG	(94 °C 30 s, 61 °C 40 s, 72 °C 50 s) × 35	507–547 bp
	5S-3f	GAGTTTTCATTGACTTGGC	(94 °C 30 s, 56 °C 40 s, 72 °C 50 s) × 35	475–517 bp
	5S-1r	CTGWCATTTTAACTTMGTCTCAG		
IGS2(3′)–18S(5′)	Ao-f700	TGAGTACATGCTGTTAATGTCG	(94 °C 30 s, 58 °C 45 s, 72 °C 90 s) × 35	731, 622, and ~185 bp
	18S-rev	GACAAGCATATGACTACTGGC		

[a]Unless otherwise indicated, all primers were designed by J.A. McLaughlin for this study.
[b]Initial denaturation in all cases was at 94° for 3 min and the final extension was at 72° for 10 min.
[c]Howlett et al. (1992).
[d]Cycle modified from White et al. (1998).

other basidiomycete wood decay fungi associated with tree roots and the rhizosphere with the primers Ao-f700 and 18S-rev.

Sequences of the 3′ IGS1 to 5′ IGS2 amplicons obtained with the primer pair 5S-1f/5S-1r were examined for the presence of restriction sites that would allow species identification of the isolates. Similarly, sequences of Ao-f700/18S-rev amplicons were examined for species-specific restriction sites. Endonuclease enzyme digestions were performed with *Mph*1103I, *Tai*I, and *Bst*EII according to the manufacturers' recommendations.

Two instruments were used for incubating samples, a Temp-Block Module Heater water bath (Lab Line Instruments, Melrose, Illinois) and a Techne TC-312.2 thermal cycler. Electrophoretic separation of the digestion products was performed on 1.0% or 2.5% agarose (Promega) TBE gels, stained with ethidium bromide, and photographed under ultraviolet illumination. The 100 – 10 000 bp O'GeneRuler™ ladder (Fermentas Life Sciences) was used as a size marker.

Results

5S gene and IGS2 sequences

Full sequences of the 5S gene and partial sequences for the 3′ end of the IGS1 and 5′ end of the IGS2 regions were obtained for 16 *A. ostoyae* (four haploid, 12 diploid), 11 *A. gemina* (two haploid, nine diploid), 21 *A. calvescens* (six haploid, 15 diploid), 18 *A. gallica* (two haploid, 16 diploid), and 15 *A. sinapina* (three haploid, 12 diploid) isolates. The 118 bp 5S gene was highly conserved over the five species sequenced, with only one exception, a G for A transition single nucleotide polymorphism (SNP) at nucleotide position 60 in one haploid isolate, *A. calvescens* III-JB-MD-52a, from Quebec (GenBank accession Nos. FJ494982 to FJ495028, FJ495031 to FJ495067, and GQ924087 to GQ924103).

Full sequences of the IGS2 region, ranging from 2775 to 3063 nucleotides in length, were obtained for two *A. ostoyae*, two *A. calvescens*, two *A. gallica*, and three *A. sinapina* diploid isolates (Table S1[2]). Sequence heterogeneity between strands of five of the nine diploid isolates produced 14 sequences for analysis (GenBank accession Nos. FJ494968 to FJ494981). The *A. ostoyae* sequences differed noticeably from those of the other species, with portions of sequence as well as large indels not shared by the other species. For example, *A. ostoyae* isolates had a nine nucleotide deletion spaced at 2629–2637 bp and three insertions, 64, 11, and 95 nucelotides in length, spaced at 2291–2354, 2427–2437, and 2467–2561 bp, respectively, all relative to the overall alignment counting from the 5′ end of the IGS2. The full-length IGS2 sequence was not obtained for *A. gemina*, but the amplicon produced by the primer pair Ao-f700/18S-rev targeting the IGS2 did share the above-mentioned 95 bp insert observed in the *A. ostoyae* sequences.

Focussed study of the 3′ end of the IGS2 region of 21 isolates conducted with the primer pair Ao-f700/18S-rev for the purpose of designing a species identification protocol added sequences from two *A. ostoyae*, three *A. sinapina*, two *A. gallica*, two *A. calvescens*, and three *A. gemina* isolates (GenBank accession numbers FJ495068 to FJ495079) in ad-

Fig. 1. Map of locations of primers used in sequencing the 3′ IGS1–5S–5′ IGS2 18S region of *Armillaria* spp.

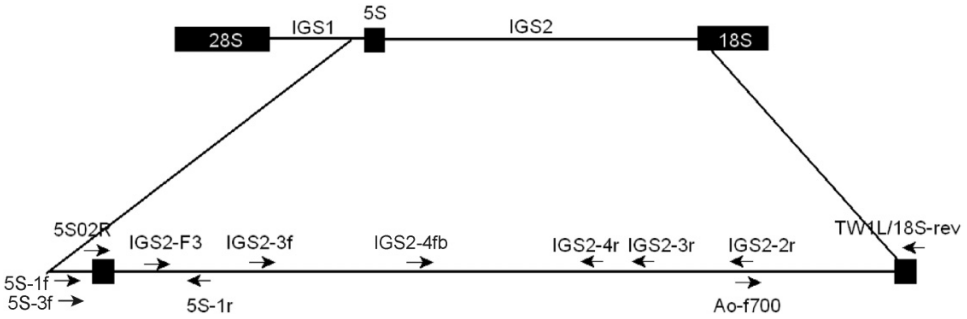
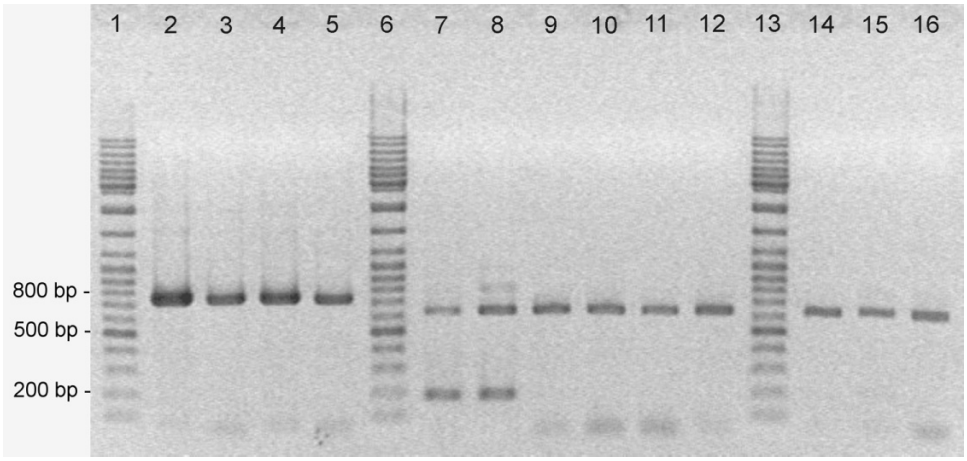


Table 3. Number of isolates studied and number of haplotypes identified in the 265–344 bp 5′ portion of the IGS2 region of five *Armillaria* spp.

	Isolates		
Species	Haploid	Diploid	Haplotypes[a]
Armillaria ostoyae (NABS I)	4	12	5
Armillaria gemina (NABS II)	2	9	4
Armillaria calvescens (NABS III)	6	15	7
Armillaria sinapina (NABS V)	3	12	5
Armillaria gallica (NABS VII)	2	16	3

[a]Haplotype refers to sequences with the same indels and were of the same length but could have also contained a few polymorphic sites.

Fig. 2. PCR products of primer pair Ao-f700/18S-rev. Lanes 1, 6, and 13, O'GeneRuler DNA ladder (Fermentas); lanes 2–4, *Armillaria ostoyae* (731 bp); lane 5, *A. gemina* (731 bp); lanes 7 and 8, *A. mellea* (~622 and ~185 bp); lanes 9 and 10, *A. calvescens* (622 bp); lanes 11 and 12, *A. gallica* (622 bp); lanes 14–16, *A. sinapina* (622 bp). Appearance of the ~185 bp band was not consistent over all thermal cyclers included in testing the identification protocol.



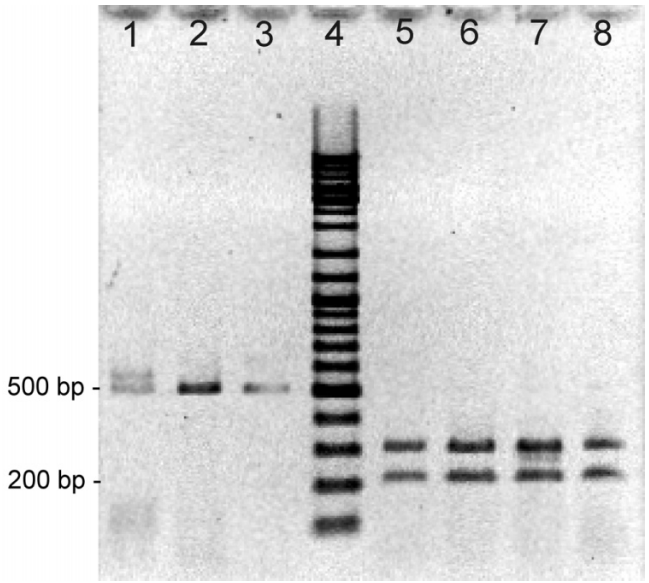
dition to the nine isolates that were sequenced over the full IGS2 region (Table S2[2]).

Sequence heterogeneity

Intraspecific and interspecific sequence heterogeneity was observed in the IGS2. Most notable was the 5′ end of the IGS2, where aside from SNPs that occurred in many sequences, several indels were observed. Indels ranged in size from 1 to 63 nucleotides in length within the first 300 nucleotides of the IGS2. Some indels in this region were species specific, such as an AGT insertion located in 11 of 12 diploid and three of three haploid isolates of *A. sinapina* at nucleotide ~249, while others varied intraspecifically and even among IGS2 repeats within a single haploid isolate (*A. ostoyae* isolate I JB 29e) (Tables S3 and S4[2]). Another area of large intraspecific and interspecific indels was located at the approximate midpoint of the IGS2. These included a 41 bp insertion in two of three *A. ostoyae* sequences as well

[2] Supplementary data for this article are available on the journal Web site (http://cjfr.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 5360. For more information on obtaining material, refer to http://cisti-icist.nrc-cnrc.gc.ca/eng/ibp/cisti/collection/unpublished-data.html.

Fig. 3. *Tai*I digests of 5S-1f/5S-1r PCR products of isolates of *Armillaria ostoyae* and *A. gemina*. Lane 4, O'GeneRuler DNA ladder (Fermentas); lanes 1–3, *A. ostoyae* isolates; lanes 5–8, *A. gemina* isolates. Multiple bands in lane 1 result from differences in template size due to haplotype heterogeneity.



as a 185 bp deletion in one of four *A. calvescens* sequences, a 92 bp deletion in two of five *A. sinapina* sequences, and a different 92 bp deletion in three of three *A. ostoyae* sequences. The primary sources of heterogeneity in the downstream third of the IGS2 region were the indels described above as distinguishing *A. ostoyae* from *A. gallica*, *A. calvescens*, and *A. sinapina*. Only *A. sinapina* isolate As69 produced a homogeneous sequence over the entire amplified region.

Twenty-four intraspecific IGS2 haplotypes were identified through comparison of 117 sequences (ranging in size from 265 to 344 nucleotides) obtained from the IGS2 portion of the 477–556 bp 5S-1f/5S-1r amplicons of 81 isolates (Table 3; Tables S5 and S6[2]). Each haplotype typically included several SNPs. When the15 haplotypes identified in *A. calvescens*, *A. sinapina*, and *A. gallica* were compared across species, some haplotypes were shared between species giving a total of 11 haplotypes. Haplotype Ax-A (comprising haplotypes Ag-1, Ac-2, and As-4) was represented in each of these three species by 38 sequences (21 *A. gallica*, 15 *A. calvescens*, and two *A. sinapina*). Haplotype Ax-B (comprising haplotypes Ag-3, Ac-3, and As-5) was represented in *A. gallica* (one sequence), *A. calvescens* (two sequences), and *A. sinapina* (two sequences). Haplotype Ax-C (comprising haplotypes Ac-1 and Ag-2) was represented in *A. calvescens* (15 sequences) and *A. gallica* (three sequences). With reference to Ax-A, which served as the benchmark sequence for the *A. gallica* – *A. calvescens* – *A. sinapina* species group, Ax-B had a 14 bp deletion between positions 75 and 88 and Ax-C had a 45 bp deletion between positions 27 and 71 from the 5′ end of the IGS2. *Armillaria ostoyae* had five haplotypes and *A. gemina* had four. Unlike the *A. gallica* – *A. calvescens* – *A. sinapina* species group, there was only one case, *A. gemina* isolate AgeJJW64, where interspecies sharing of haplotypes (Age-4 and Ao-4) was observed. Haplotype Ao-4 shared sequence characteristics of *A. ostoyae* and *A. gemina* and was observed in two *A. ostoyae* isolates. Another haplotype with a blend of *A. ostoyae* and *A. gemina* characteristics, Ao-5, was observed in three *A. ostoyae* isolates.

Sequences of some *A. sinapina* isolates exhibited characteristics of interspecies heterogeneity. Diploid isolates As50 (GenBank accession Nos. FJ495036 and FJ495037), As93 (GenBank accession Nos. FJ495038 and FJ495039), CF-2 (GenBank accession Nos. GQ924100 and GQ924101), and As96 (GenBank accession Nos. FJ495040 and FJ495041) each comprised two haplotypes, one halotype the distinct *A. sinapina* type containing the AGT insert at position ~249 and a repeat of five Ts just upstream from the 5S-1r primer annealing site and the other haplotype either Ax-A (isolates As50 and AsCF-2) or Ax-B (isolates As93 and As96), both lacking the AGT insert and containing the eight Ts repeat characteristic of most of the *A. gallica* and *A. calvescens* isolates.

Thirteen of 15 diploid *A. calvescens* isolates had a C/T SNP at position five at the 5′ end of the IGS2. Five of six haploid sequences had a T and one had a C at this site. All other isolates of the other four species did not have this SNP, with the exception of one isolate (Ag101) that had been identified as *A. gallica* in haploid–diploid pairing tests in an earlier study (McLaughlin 2001).

Development of an *Armillaria* species identification protocol

Species-specific primers targeting the 3′ end of the IGS2 and RFLP analysis digest of the 5S-1f/5S-1r PCR product were combined in the development of a species identification protocol for the six *Armillaria* spp. found in Ontario. The primer pair Ao-f700/18S-rev proved highly effective for initial screening of isolates collected from field samples into three groups: (*i*) *A. ostoyae* and *A. gemina*, (*ii*) *A. calvescens*, *A. gallica*, and *A. sinapina*, and (*iii*) *A. mellea* (Fig. 2). These primers, designed from *A. ostoyae* sequence data obtained in this study, produced PCR products of the expected size (731 bp) from *A. ostoyae* and *A. gemina* isolates. These two species were separated through RFLP digests with *Tai*I of the 489–511 bp (minimum 477 bp, maximum 541 bp) PCR products produced by the primer pair 5S-1f/5S-1r. This product brackets the 5S gene by 94 bases upstream in the IGS1 and 265–329 bases into the 5′ end of the IGS2. The *Tai*I (ACGT/) digest of *A. gemina* removed a 303 bp fragment from the 5′ end of the amplicon. *Armillaria ostoyae* isolates lack this restriction site due to a C for G substitution in the third base of the recognition sequence (Fig. 3).

Ao-f700/18S-rev also amplified a 622 bp product from *A. calvescens*, *A. gallica*, and *A. sinapina*, apparently due to mismatched forward primer annealing downstream from the principal binding site. The mismatched annealing occurred because 12 of the first13 nucleotides (from the 3′ end) of primer Ao-f700 and the *A. calvescens*, *A. gallica*, and *A. sinapina* isolate sequences matched, with the first polymorphism at position 7. With *A. mellea*, this primer pair could produce two bands, one ~622 bp and another smaller band of equal strength ~185 bp in size. This smaller product, however, was not consistently found with all thermal cyclers used in further testing of the protocol. In the absence of the

Fig. 4. *Tai*I digests of Ao-f700/18S-rev PCR products of *Armillaria mellea*, *A. sinapina*, *A. calvescens*, and *A. gallica*. Lanes 5 and 12, O'GeneRuler DNA ladder (Fermentas); lanes 1–4, pairings of undigested and digested *A. mellea* with diagnostic ~320 bp fragment; lanes 6 and 7, pairing of undigested and digested *A. sinapina*; lanes 8 and 9, pairing of undigested and digested *A. calvescens*; lanes 10 and 11, pairing of undigested and digested *A. gallica*.

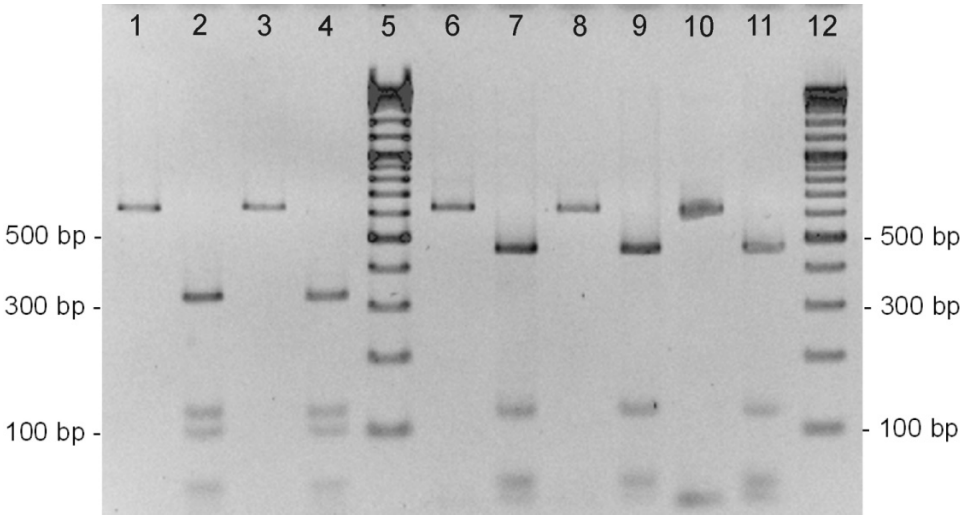
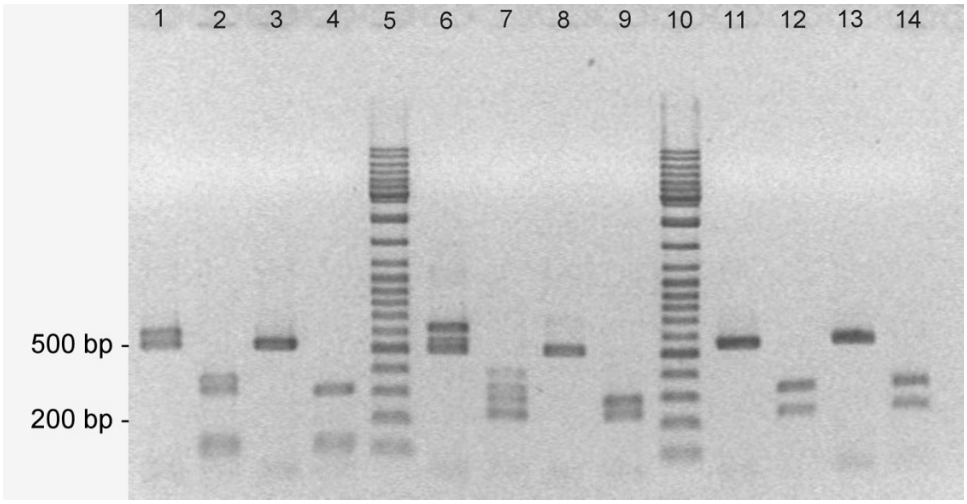


Fig. 5. *Bst*EII digest of 5S-1f/5S-1r PCR products of *Armillaria sinapina*, *A. calvescens*, and *A. gallica*. Lanes 5 and 10, O'GeneRuler DNA ladder (Fermentas); lanes 1–4, pairings of undigested and digested *A. sinapina*; lanes 6–9, pairings of undigested and digested *A. calvescens*; lanes 11–14, pairings of undigested and digested *A. gallica*. Multiple bands in undigested lanes result from differences in template size due to haplotype heterogeneity.

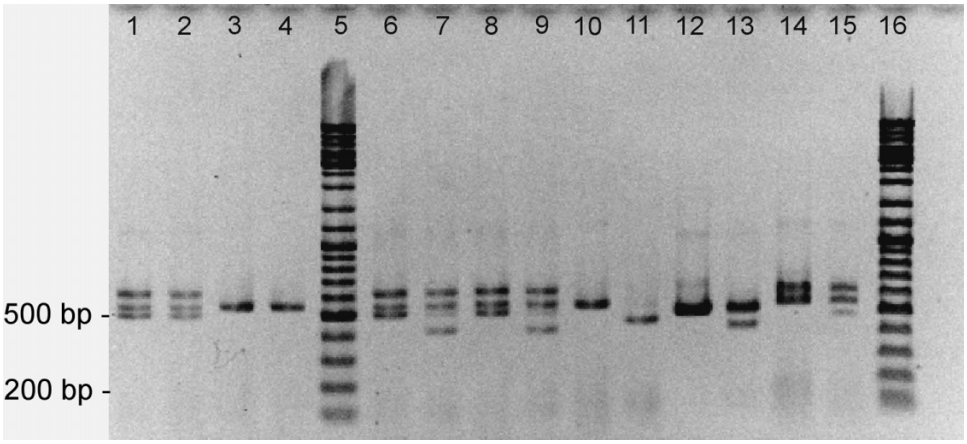


~185 bp band, *A. mellea* was identified following the next stage of the identification protocol, where PCR of *A. mellea* DNA with the primer pair 5S-1f/5S-1r did not produce the strong ~500–550 bp amplicon typical of the other five species. Positive proof of the identity of these *A. mellea* isolates was then obtained by digesting the ~622 bp PCR product produced by the primer pair Ao-f700/18S-rev with the restriction enzyme *Tai*I. This digest produced a diagnostic fragment ~320 bp in size along with fragments of 122, 98, 49, and 30 bp in length. Digest of the similar 622 bp PCR products from *A. calvescens*, *A. gallica*, and *A. sinapina* produced fragments 455, 118, and 49 bp in size (Fig. 4).

Identification of the *A. gallica*, *A. calvescens*, and *A. sinapina* isolates was accomplished by first separating *A. sinapina* from the other two by *Bst*EII (G/GTTACC) digest of the 502–556 bp 5S-1f/5S-1r PCR products produced from these isolates. *Armillaria sinapina* had two restriction sites, producing three products, one of 305–311 bp (maximum 321 bp) and two smaller products of 100 and 125 bp. The 100 and 125 bp fragments were difficult to clearly distinguish on 1% agarose gels but their obvious presence and the >175 bp difference between them and the larger residual fragment (Fig. 5) made identification simple. *Armillaria calvescens* and *A. gallica* had only one restriction site and digest produced two products, a larger product in the range of 276–321 bp and a smaller product typically in the 226–228 bp range but with minimum and maximum sizes of 222 and 280 bp, respectively (Fig. 5).

The final step of separating *A. gallica* and *A. calvescens* isolates was accomplished through digest of the 5S-1f/5S-1r PCR product with *Mph*1103I (ATGCA/T), exploiting the presence of a C for T SNP two bases upstream from the re-

Fig. 6. *Mph*1103I digest of 5s-1f/5S-1r PCR products of isolates of *Armillaria gallica* and *A. calvescens*. Lanes 5 and 16, O'GeneRuler DNA ladder (Fermentas); lanes 1–4, pairings of undigested and digested *A. gallica*; lanes 6–15, pairings of undigested and digested *A. calvescens*. Lanes 10 and 11 are from a haploid *A. calvescens* isolate with the restriction site. Multiple bands in undigested lanes result from differences in template size due to haplotype heterogeneity.



striction site (ATGYA/T) on one strand of *A. calvescens* isolates, very close to the 3′ end of the IGS1. The result is removal of an 86 bp fragment from the 5′ end of one strand of the *A. calvescens* PCR product. Because the restriction site is on only one strand, some of the full-sized product remains after the digest (Fig. 6, lanes 7, 9, 13, and 15). *Armillaria gallica* lacks the C SNP and it is therefore not cut by *Mph*1103I (Fig. 6, lanes 2 and 4). Due to variability in the overall size of the PCR product, this was most easily detected by comparing the digested and undigested products (Fig. 6). The step-by-step species identification protocol is presented in Fig. 7.

PCR of genomic DNA extracted from *Coniophora puteana*, *Fomitopsis pinicola*, *Hypholoma sublateritium*, *Inonotus circinatus*, *Inonotus tomentosus*, *Peniophora gigantea*, *Phellinus pini*, *Perenniporia subacida*, *Scytinostroma galactinum*, and *Stereum sanguinolentum* demonstrated the specificity of the primer pair Ao-f700/18S-rev to *Armillaria* spp. These primers did not amplify PCR products from these other species.

Discussion

5S gene and IGS2 region sequences

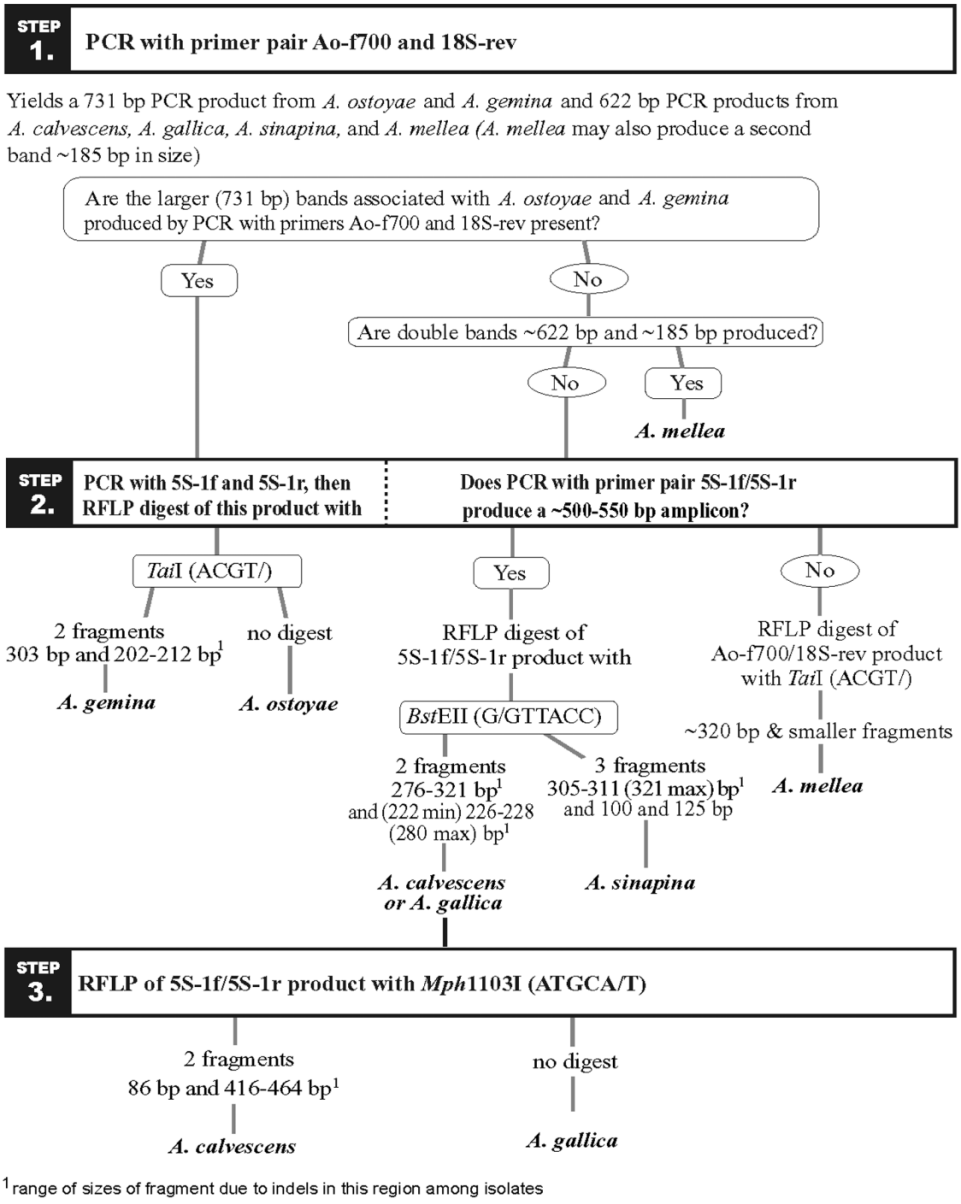
Complete sequences of the 118 bp 5S gene were obtained for five of the six *Armillaria* spp. found in Ontario, *A. ostoyae*, *A. gemina*, *A. gallica*, *A. calvescens*, and *A. sinapina*. The 5S gene was found to be highly conserved, with only one sequence exhibiting an SNP out of 81 obtained from isolates from Ontario and Quebec in Canada and Michigan, Illinois, Wisconsin, New York, and West Virginia in the United States.

The IGS2 region of the species sequenced in this study was highly polymorphic, with many SNPs and indels particularly at the 5′ end but also to a lesser degree midway and farther through the ~2.8–3.1 kb IGS2 region. Guidot et al. (1999) made similar observations in their study of the 2.6 kb IGS2 region of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*. In the current study, *A. calvescens* isolates were the most polymorphic, with 21 isolates (six haploid and 15 diploid) representing seven distinct IGS2 haplotypes.

In a recent study of North American *Armillaria* spp. focussing on the ITS, IGS1, and LSU regions of rDNA, Hanna et al. (2007) reported that heterogeneous rDNA products were common in all of these regions. rDNA heterozygosity has also been reported in diploid European *A. gallica* isolates (Pérez-Sierra et al. 1999), *A. sinapina* (White et al. 1998), and *Armillaria nabsnona* (Volk et al. 1996). Kim et al. (2000), in their investigation of the nuclear DNA contents of North American *Armillaria* spp., found evidence of triploidy in one *A. ostoyae* isolate. In this study, IGS2 sequence heterogeneity was observed at all levels: genus, species, diploid individual, and even in one haploid individual (AoI JB 29e), which provides evidence of heterogeneity among rDNA repeats within single nuclei. One putatively diploid isolate (Ac223) produced three sequences of the 5S-1f/5S-1r PCR product, two of which differed only in a few SNPs whereas the third also contained a 51 nucleotide repeat section near the 3′ end of the amplicon. Whether Ac223 is polyploid or simply contains heterogeneous IGS2 repeats within one nucleus cannot be determined by the data obtained. The strength of the peaks in the extended section of the sequence (where the longer sequence extends beyond the other two, shorter sequences), however, suggests that there may be numerous copies of the third type. Rauscher et al. (2002), in an investigation of hybridization in the *Glycine tomentella* (Leguminosae) polyploid complex, found that direct sequencing of ITS PCR products from hybrids produced secondary peaks when the secondary repeat type comprised at least 10% of the PCR product and that relative overall peak size was a good indicator of the relative concentration of each sequence.

Heterogeneity within diploid isolates and even within-nucleus heterogeneity may explain the ambiguous sequence results that are commonly obtained from IGS2 PCR products from diploid isolates of *Armillaria* spp. In this study, diploid isolates containing different haplotypes in the first 90 nucleotides of the IGS2 were common, especially in *A. calvescens* where 60% (nine of 15) of the isolates each was

Fig. 7. Step-by-step identification protocol for *Armillaria* spp. based on amplification of the 3′ IGS2 (~700 bp) followed by restriction of the 3′ IGS1–5S–5′ IGS2 (~500 bp) or 3′ IGS2 with restriction enzymes (*Bst*EII, *Tai*I, and *Mph*1103I).



composed of distinctly different haplotypes followed by *A. sinapina* with 42% (five of 12) and *A. gallica* with 19% (three of 16). In the other species groups, this occurred in 22% (two of nine) of *A. gemina* and 8% (one of 12) of *A. ostoyae* isolates.

Aside from the polymorphism described above, the IGS2 was characterized by areas of single nucleotide repeats of T and C up to 12 bases in length near positions 270 and 830. Kauserud and Schumacher (2003) observed similar G and TT indels, which they termed "single motif microsatellites" in their investigation of IGS1 sequences of *Trichaptum abietinum*. In this study, variations in the number of repeats at these loci were often sources of heterogeneity between haplotypes, which appeared in chromatograms as "frame shifts" (Kim et al. 2006) due to the different lengths among the rDNA units. These loci were often identified through combined forward and reverse sequencing, each producing unambiguous peaks until they reached the repeat area, at which point double peaks or a "frame shift" would become evident in the chromatogram. Future study of the IGS2 needs to consider and include primer design strategies that minimize the impact of these heterogeneous regions.

Considering the high level of polymorphism encountered in the IGS2 region of the five *Armillaria* spp. that were sequenced, the most unusual observation was the completely homogeneous, unambiguous sequence obtained for *A. sinapina* isolate As69, the only isolate that was homogeneous over its entire 3191 bp length from the 3′ end of the IGS1 to the 5′ end of the 18S gene. This isolate showed greater sequence similarity to *A. gallica* or *A. calvescens* than to other isolates of *A. sinapina*.

Sequences common among species

Interspecific sharing of distinct haplotypes was previously

observed in a study by Kim et al. (2006), where heterogeneous IGS1 sequences of a diploid *A. gemina* isolate clustered with both *A. gemina* and *A. ostoyae* genets in phylogenetic analyses. In this study, only one case of interspecific sharing of a haplotype was observed for these species (i.e., isolate AgeJJW64), but some *A. ostoyae* isolates (i.e., Ao168, Ao162b, AoYa-b, Ao255, and AoDMR20) had 5′ IGS2 sequences that included typical *A. gemina* characteristics.

An *Armillaria* identification protocol

Although the protocol provided an efficient method of identifying isolates to species in almost all cases, there were exceptions. Fourteen of 15 *A. sinapina* isolates contained sequences necessary for successful identification; however, isolate As69 lacked the first *Bst*EII restriction site in the 5S-1f/5S-1r PCR product due to a 34 bp deletion at positions 296–329 of the 5S-1f/5S1r PCR product (positions 76–109 of the IGS2 region relative to the alignment of all 117 sequences) that included the first G in the restriction sequence. It also lacked the second site due to a T for G substitution at the first G of the restriction sequence at position 428 of the 5S-1f/5S-1r PCR product or position 226 of the IGS2 region (relative to the alignment of all 102 sequences). Due to these polymorphisms, isolate As69 would be misidentified in screening by this protocol. The first Ao-f700/18S-rev step would correctly place it in the *A. calvescens* – *A. gallica* – *A. sinapina* group but the next step, the *Bst*EII digest, would place it with *A. calvescens* and *A. gallica.* The final *Mph*1103I digest would identify it incorrectly as *A. gallica*. Two diploid isolates, As93 and AsCF-2, lacked the *Bst*EII site on one strand due to a C for G substitution at the first G of the restriction sequence. In both cases, the other strand possessed the correct sequence, so this heterogeneity would not prevent isolates As93 and AsCF-2 from being properly identified by this protocol.

Thirteen of 15 diploid *A. calvescens* isolates contained one strand with the *Mph*1103I restriction sequence at positions 82–87. The two exceptions were isolates Ac7 and Ac402, which had been identified in an earlier study (McLaughlin 2001) through haploid–diploid pairing. In that study, Ac7 had been represented (after diploid–diploid somatic incompatibility pairing) by another isolate from the same sample plot in the diploid–haploid pairing test. That surrogate isolate was identified as *A. calvescens* by two of three *A. calvescens* haploid test isolates reacting positively, while one *A. gallica* tester had an ambiguous reaction. Ac402 also reacted ambiguously with two of three *A. calvescens* and one of three *A. gallica* haploid test isolates. It is common that haploid test isolates do not all produce positive reactions when paired with diploid isolates of the same species (Guillaumin et al. 1991). Until the mechanisms of diploidization in *Armillaria* are better understood, species identification by this method will retain an unavoidable subjective component. Only one of the six haploid *A. calvescens* isolates possessed the C for T substitution in the *Mph*1103I restriction sequence necessary for identification as *A. calvescens* with this protocol. As with isolate As69, the lack of the C for T substitution in isolates Ac7 and Ac402 would result in their misidentification as *A. gallica* rather than *A. calvescens*. Since only one strand of the DNA in *A. calvescens* and *A. sinapina* was found to contain the restriction site necessary for correct species identification, haploid isolates of these species could therefore could be misidentified. DNA from diploid isolates is more reliable for species identification using this protocol than DNA from haploids.

The protocol successfully identified 16 of 16 *A. ostoyae* (diploid and haploid), 11 of 11 *A. gemina* (diploid and haploid), three of three *A. mellea* (diploid), 18 of 18 *A. gallica* (diploid and haploid), 14 of 15 *A. sinapina* (11/12 diploid and 3/3 haploid), and 14 of 21 *A. calvescens* (13/15 diploid and 1/6 haploid) northeastern North American isolates included in this study. To the best of our knowledge, this success rate over this range of species has not been matched by other methods. Future work will attempt to improve the efficiency of separation of *A. calvescens* and may involve the use of real-time PCR with highly sensitive, sequence-specific probes.

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