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NOTE

Advances toward DNA-based identification and phylogeny of North American *Armillaria* species using elongation factor-1 alpha gene

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Abstract The translation elongation factor-1 alpha (EF-1α) gene was used to examine the phylogenetic relationships among 30 previously characterized isolates representing ten North American *Armillaria* species: *A. solidipes* (=*A. ostoyae*), *A. gemina*, *A. calvescens*, *A. sinapina*, *A. mellea*, *A. gallica*, *A. nabsnona*, North American biological species X, *A. cepistipes*, and *A. tabescens*. The phylogenetic relationships revealed clear separation of all ten North American *Armillaria* species, with the exception of one *A. gallica* isolate that perhaps represents an unnamed cryptic species. These results indicate that the EF-1α gene could potentially serve as a diagnostic tool for distinguishing among currently recognized North American biological species of *Armillaria*.

Keywords Armillaria root disease · DNA sequencing · Phylogenetics · Physalacriaceae · Species identification

The genus *Armillaria* (Fr.) Staude is widely distributed across North America, where it displays diverse ecological behaviors ranging from beneficial saprobe to virulent root/butt-disease pathogen. In North America, *Armillaria* currently comprise one undescribed, unnamed biological species (NABS X; Anderson and Ullrich 1979) and nine formally described, named species (Volk and Burdsall 1995), as follows: *A. solidipes* Peck (=*A. ostoyae* (Romagnesi) Herink; Burdsall and Volk 2008), *A. gemina* Bérubé & Dessureault, *A. calvescens* Bérubé & Dessureault, *A. sinapina* Bérubé & Dessureault, *A. mellea* (Vahl) Kummer, *A. gallica* Marxmüller & Romagnesi, *A. nabsnona* T.J. Volk & Burdsall, *A. cepistipes* Velenovský, and *A. tabescens* (Scopoli) Emel. Previous studies have attempted to distinguish these species using DNA-based methods, such as rDNA restriction fragment length polymorphisms (RFLPs) (Harrington and Wingfield 1995; Volk et al. 1996; White et al. 1998; Kim et al. 2000; McLaughlin and Hsiang 2010), anonymous DNA sequences (Piercey-Normore et al. 1998), rDNA sequences [internal transcribed spacer (ITS), intergenic spacer (IGS), small subunit (SSU), large subunit (LSU); Anderson and Stasovski 1992; Kim et al. 2006], amplified fragment length polymorphisms (AFLPs) (Kim et al. 2006), and nuclear DNA content (Kim et al. 2000) with varying degrees of success. Because *Armillaria* species can display great differences in pathogenicity, it is critical to accurately identify *Armillaria* spp. that occur on a site to assess disease risks. Unfortunately, many *Armillaria* spp. are difficult to identify based on morphology alone, and isolates are typically found in the vegetative state. Thus, DNA-based diagnostic tools have been sought to aid in species identification for assessments of disease risks and other purposes. Recent studies showed that sequences of the translation elongation factor-1 alpha (EF-1α) were useful to separate *Armillaria* spp. (Maphosa et al. 2006; Hasegawa et al. 2010); however, the EF-1α gene has not yet been used to distinguish all currently recognized North American *Armillaria* species. The objectives of this study were to (1) use the EF-1α gene to examine phylogenetic relationships among 30 well-characterized isolates representing the ten North American *Armillaria* species; and (2) assess whether EF-1α sequence differences have potential use as a diagnostic tool to

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distinguish among these North American *Armillaria* species, particularly those that are morphologically similar or closely related.

To avoid ambiguity derived from misidentified isolates, this study focused on the only available set of North American *Armillaria* isolates that has been extensively characterized by multiple methods (Kim et al. 2006). Three isolates each of ten North American *Armillaria* species were included in this study (Table 1). More detailed information on the *Armillaria* isolates used in this study is available in Kim et al. (2006). We used the protocol of Kim et al. (2006) for fungal culture and DNA extraction. To amplify the EF-1α gene, each 50-μl polymerase chain reaction (PCR) mixture contained 5 μl (~100 ng) template DNA, 2.5 U *Taq* polymerase (Applied Biosystems, Foster City, CA, USA), PCR reaction buffer, 4 mM $MgCl_2$, 200 μM dNTP, and 0.5 μM of each primer. Two sets of primers were used for PCR and sequencing: EF595F/EF1160R (Kauserud and Schumacher 2001) and 983F/2218R (Rehner and Buckley 2005). The PCR conditions were as follows: 94°C for 2 min; 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min and 30 s; then 72°C for 7 min. The PCR products of EF-1α gene were sequenced with an ABI 3700 DNA Sequencer at the University of Wisconsin, Biotechnology Center (Madison, WI, USA). Sequences were edited and aligned manually with BioEdit software (Hall 1999). For isolate ST11, the method of Hanna et al. (2007) was used to decipher and edit a "frame-shift" because of length variation between two gene copies, from which two separate sequences (ST11A and ST11B) were generated. These edited EF-1α gene sequences of the *Armillaria* isolates have been deposited in GenBank (see Table 1). Sequence alignments can be found in TreeBASE (S11603).

The fungal cultures used in this study were deposited at the USDA Forest Service, Rocky Mountain Research Station, Forestry Sciences Laboratory, Moscow, Idaho, USA, and living subcultures are available upon request.

Table 1 *Armillaria* isolates used in this study

Species	Isolate	Origin	GenBank accession no.
A. solidipes (=*A. ostoyae*)	ST1	New Hampshire, USA	JF313141
	ST2	Washington, USA	JF313139
	P1404	Idaho, USA	JF313140
A. gemina	ST8	New York, USA	JF313136
	ST9	New York, USA	JF313135
	ST11	West Virginia, USA	JF313133[a], JF313134[a]
A. calvescens	ST3	Quebec, Canada	JF313138
	ST17	Michigan, USA	JF313130
	ST18	Michigan, USA	JF313129
A. sinapina	M50	British Columbia, Canada	JF313114
	ST12	Washington, USA	JF313132
	ST13	Michigan, USA	JF313131
A. mellea	ST5	Virginia, USA	JF313137
	ST20	Wisconsin, USA	JF313128
	ST21	New Hampshire, USA	JF313127
A. gallica	M70	British Columbia, Canada	JF313123
	ST22	Michigan, USA	JF313126
	ST23	Wisconsin, USA	JF313125
A. nabsnona	C21	Idaho, USA	JF313119
	M90	British Columbia, Canada	JF313122
	ST16	Alaska, USA	JF313124
NABS X	837	Idaho, USA	JF313120
	D82	Idaho, USA	JF313118
	POR100	Idaho, USA	JF313117
A. cepistipes	M110	British Columbia, Canada	JF313121
	S20	British Columbia, Canada	JF313116
	W113	Washington, USA	JF313115
A. tabescens	ATMUS2	South Carolina, USA	JF313113
	OOI99	Georgia, USA	JF313112
	OOI210	Georgia, USA	JF313111

More detailed information on the *Armillaria* isolates used in this study is available in Kim et al. 2006

NABS, North American biological species

[a] JF313133 (ST11A), JF313134 (ST11B); for isolate ST11, the method of Hanna et al. (2007) was used to decipher and edit a "frame-shift" caused by length variation between two gene copies, from which two separate sequences (ST11A and ST11B) were generated

A Bayesian phylogenetic analysis was inferred using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). To incorporate insertion and deletion events as phylogenetic characters, the sequence of each isolate was partitioned into two sections. The first section contained 1,065 DNA characters that were assigned to a STM + G (symmetrical model plus gamma) nucleotide substitution model based on Akaike information criterion (AIC) data obtained using jModeltest 0.1.1 (Akaike 1974; Guindon and Gascuel 2003; Posada 2008). The second section consisted of eight binary characters representing gaps within the EF-1α alignment coded using a simple gap-coding method (Simmons and Ochoterena 2000). This section was assigned a simple F81-like substitution model in MrBayes (Felsenstein 1981).

With *A. tabescens* used as an outgroup, Bayesian analysis of the partial EF-1α gene revealed two additional major clades: (1) a clade containing *A. solidipes* (=*A. ostoyae*), *A. gemina*, *A. sinapina*, and *A. mellea*, and (2) a clade containing *A. cepistipes*, *A. nabsnona*, NABS X, *A. calvescens*, and *A. gallica* (Fig. 1). Partial sequence data for the EF-1α gene delineated all but one of the North American species of *Armillaria* into separate clades or subclades (Fig. 1). Isolates of *A. gallica* did not cluster within a single subclade (Fig. 1). The first major clade consisted of *A. solidipes* (=*A. ostoyae*) and *A. mellea*, which are aggressive pathogens, *A. gemina*, which is similar to *A. solidipes* (=*A. ostoyae*) (Kim et al. 2006), and *A. sinapina*, which has occasionally been associated with disease centers (Dettman and van der Kamp 2001). This

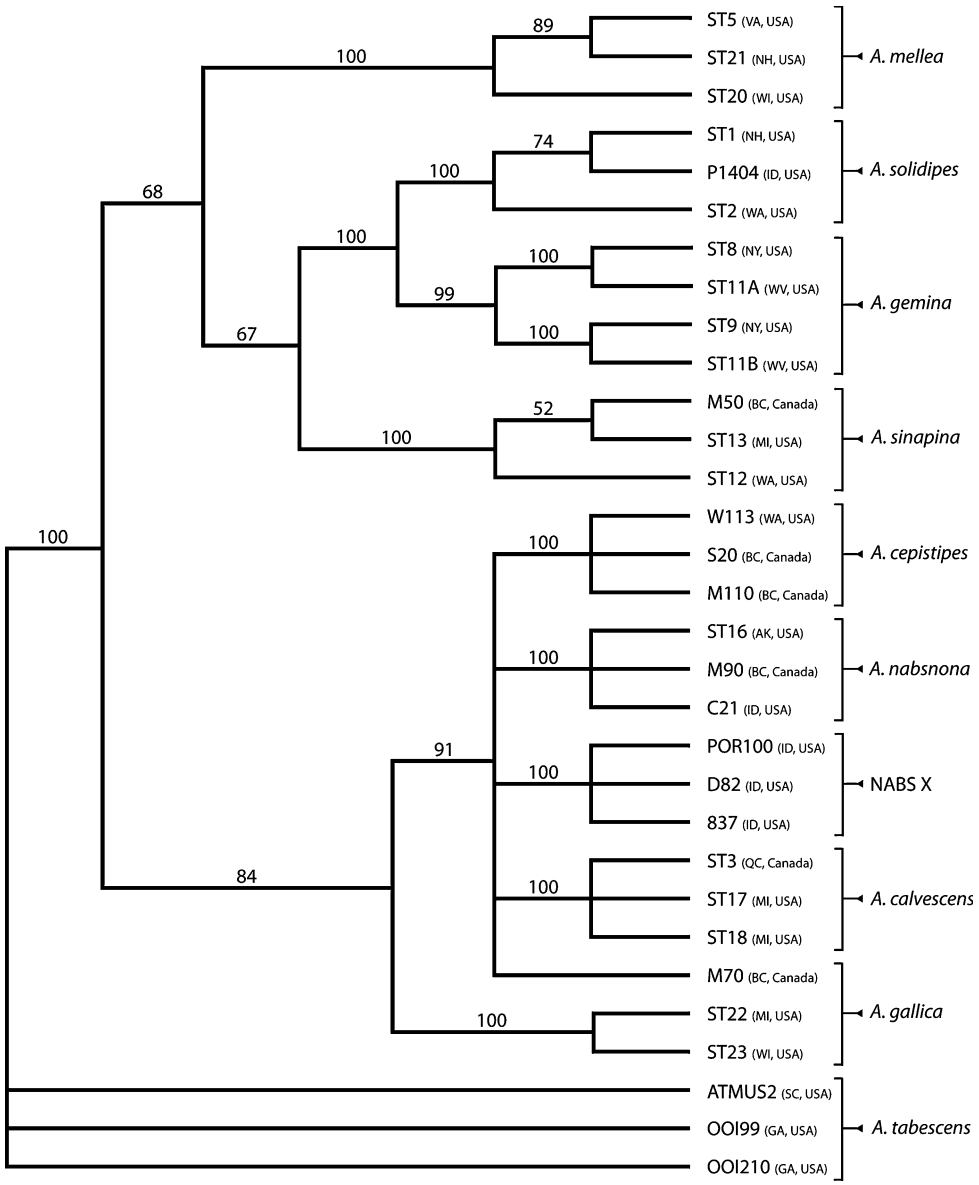

Fig. 1 A 50% majority-rule consensus tree of ten North American *Armillaria* species based on 24,001 trees from Bayesian inference analysis of the translation elongation factor 1-alpha gene. *Numbers between clades* indicate posterior probability. Sequence alignments can be found in TreeBASE (S11603)

result differs from previous phylogenetic studies using rDNA sequences (Anderson and Stasovski 1992; Kim et al. 2006) and anonymous nucleotide sequences (Piercey-Normore et al. 1998) in which *A. sinapina* clustered with *A. calvescens*, *A. gallica*, and *A. cepistipes*.

The second major clade included *A. cepistipes* and *A. nabsnona*, saprobes or occasionally weak pathogens found on hardwoods in western North America. The second major clade also included the unnamed NABS X, known only from British Columbia, Washington, Oregon, California, and Idaho, as well as *A. calvescens* and *A. gallica*, which are morphologically very similar species. Interestingly, *A. gallica* (ST22 and ST23) from eastern North America grouped together, although the isolate (M70) from western North America was separate (Fig. 1). Although isolate M70 has been previously identified as *A. gallica* by pairing tests (Morrison et al. 1985) and nuclear DNA content (Kim et al. 2000), AFLP data also showed this isolate as genetically distinct from the other two *A. gallica* isolates (ST22 and ST23) (Kim et al. 2006). These data, as well as other DNA sequence data (Kim et al. 2006; Hanna et al. 2007), suggest that *A. gallica* is a diverse species that comprises multiple, genetically distinct groups which may include cryptic species (Hawksworth 2010). Confirmation of cryptic species within *A. gallica* will require sequence analysis of more genetic regions within multiple isolates from diverse sources. Further examination of more isolates could also determine if genetic divergence occurs between *A. gallica* isolates from eastern and western North America, as was observed with *A. mellea* (Baumgartner et al. 2010).

The basal clade (outgroup) consists entirely of *A. tabescens*, the only exannulate species of *Armillaria* in North America. It is pathogenic on hardwoods, particularly on oaks and fruit trees, in eastern North America (Schnabel et al. 2005). Maximum likelihood and maximum parsimony analyses of the dataset showed similar results (data not shown).

It should be noted that the primer set 983F/2218R is more appropriate to amplify and sequence the EF-1α gene for the North American *Armillaria* species than is the primer set EF595F/EF1160R. For these *Armillaria* species, the 983F primer is located upstream (5′-end) of the EF595F primer (despite numbering). The sequences from the 983F/2218R primer set overlap the EF595F/EF1160R primer locations and reveal a one-nucleotide difference from EF595F and two-nucleotide differences from EF1160R. Thus, improved primers should be designed for broader application to diverse *Armillaria* species and isolates.

DNA-based identification of *Armillaria* is an essential step to monitor and predict Armillaria root disease in forest ecosystems. Previously, Kim et al. (2006) reported that rDNA sequence data are not sufficient to definitively confirm species identification among the closely related North American species *A. calvescens*, *A. sinapina*, *A. gallica*, and *A. cepistipes*. The results from this study indicate that the EF-1α gene could potentially serve as a diagnostic tool to distinguish among the North American species of *Armillaria*. Furthermore, the EF-1α gene sequences provide additional evidence that NABS X is genetically distinct from other North American *Armillaria* species, which indicates that NABS X should be formally described when sufficient supporting information is available. Because this study focused on previously characterized isolates to ensure the validity of results, further work associated with morphological characterization, genetic characterization, and mating/pairing tests of more isolates representing the intra- and interspecific diversity of North American *Armillaria* is required to verify the utility of this region as a diagnostic tool.

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