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Short Communication

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Molecular Identification of *Armillaria gallica* from the Niobrara Valley Preserve in Nebraska

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Abstract

Armillaria isolates were collected from a unique forest ecosystem in the Niobrara Valley Preserve in Nebraska, USA, which comprises a glacial and early postglacial refugium in the central plains of North America. The isolates were collected from diverse forest trees representing a unique mixture of forest types. Combined methods of rDNA sequencing and flow cytometric measurements of nuclear DNA content determined that all *Armillaria* isolates collected from the site were *A. gallica*.

Introduction

Armillaria species are diverse in pathogenicity, host specificity, and environmental requirements. Some *Armillaria* spp. are aggressive pathogens, while other *Armillaria* spp. are predominately saprophytic, and may help sustain forest productivity via nutrient cycling. Thus, *Armillaria* species identification is a critical component for monitoring forest health and understanding forest ecosystem functions. Current classification of *Armillaria* spp. is based in part on morphology and *in vitro* compatibility of isolates. In recent decades, molecular genetic methods were developed to augment identification of *Armillaria* species (Kim et al. 2006), and the nuclear DNA content of North American *Armillaria* spp. has been determined using laser flow cytometry (Kim et al. 2000).

The Niobrara Valley Preserve is a glacial and early postglacial refugium in the central plains of North America where six major ecosystems converge. The middle Niobrara Valley contains a unique mixture of three forest types (northern boreal, western coniferous, and eastern deciduous) (Kaul et al. 1988). This forest refugium offers a unique opportunity to study biological diversity of this ecosystem that has harboured enriched flora and fauna since early postglacial to glacial times (ca. 9000–12 000 years B.P.). However, little is known about the fungal community associated with tree species in this ecologically unique area, and *Armillaria* species have not been previously reported from this ecosystem. Because *Armillaria* fungi can be major drivers of forest ecosystem processes, our objectives were to (i) determine if *Armillaria* spp. are present on diverse tree species within the Niobrara Valley Preserve forest refugium and (ii) identify any *Armillaria* spp. that were collected in this forest refugium.

Materials and Methods

On the Niobrara Valley Preserve in Nebraska, USA (Latitude 42°55′16″N, Longitude 100°26′21″ W, elevation 791 m), 10 trees were randomly selected within the three forest types present (Table 1). On each tree, major lateral roots were excavated and inspected for external rhizomorphs or internal mycelial fans of *Armillaria* spp. Samples of *Armillaria* spp. were collected along with host tree data, which included species and general health status. *Armillaria* spp. collections were established in culture following the protocol of Hanna et al. (2007).

The protocol of Kim et al. (2006) was used for PCR amplification of the intergenic spacer 1 (IGS-1) region of rDNA. The PCR products of IGS-1 region were sequenced with an ABI 3700 DNA sequencer at the Davis Sequencing Facility (Davis, CA, USA), and the IGS-1 sequences of the *Armillaria* isolates have been deposited in GenBank (Table 1).

Methods of Kim et al. (2000) were used to determine nuclear DNA content. Nuclei were isolated from mycelia and stained with propidium iodide (PI). Fluorescence of the PI-stained nuclei was analysed at the University of Nebraska-Flow Cytometry Core Research Facilities using a FACScan Flow Cytometer

Table 1
Species identification of *Armillaria* isolates collected from the Niobrara Valley Preserve in Nebraska, USA

Isolate[a] no.	Substrate	Species identification by DNA sequence data of IGS-1[b]	Genome size[c] (pg per nucleus)	GenBank accession no.
NE01	*Ulmus americana*	*A. gallica*, *A. calvescens*, or *A. sinapina*	0.184 ± 0.001	GU734129
NE02	*Betula papyrifera*	*A. gallica*, *A. calvescens*, or *A. sinapina*	0.180 ± 0.001	GU734130
NE03	*Betula papyrifera*	*A. gallica*, *A. calvescens*, or *A. sinapina*	0.180 ± 0.001	GU734131
NE04	*Betula papyrifera*	*A. gallica*, *A. calvescens*, or *A. sinapina*	0.177 ± 0.002	GU734130
NE05	*Tilia americana*	*A. gallica*, *A. calvescens*, or *A. sinapina*	0.169 ± 0.002	GU734131
NE06	*Quercus macrocarpa*	*A. gallica*, *A. calvescens*, or *A. sinapina*	0.177 ± 0.002	GU734130
NE07	*Pinus ponderosa*	*A. gallica*, *A. calvescens*, or *A. sinapina*	0.175 ± 0.001	GU734132
NE08	*Betula papyrifera*	*A. gallica*, *A. calvescens*, or *A. sinapina*	0.170 ± 0.001	GU734130
NE09	*Fraxinus pennsylvanica*	*A. gallica*, *A. calvescens*, or *A. sinapina*	0.179 ± 0.001	GU734133
NE10	*Populus deltoides*	*A. gallica*, *A. calvescens*, or *A. sinapina*	0.179 ± 0.001 0.177 ± 0.001[d]	GU734133

[a]Ten isolates (NE01 – NE10) of unknown *Armillaria* were collected from the Niobrara Valley Preserve.
[b]IGS-1, Intergenic spacer-1 of rDNA. *Armillaria* isolates were compared with known *Armillaria* spp. using the GenBank BLAST.
[c]The standard used for comparison was chicken red blood cell (2.33 pg/2C). The formula used for converting fluorescence intensity values to DNA content is provided by Kim et al. (2000). Each number represents the mean ± standard deviation of the mean of four replications. Each replication of *Armillaria* isolate DNA content was based on 10 000 scanned nuclei.
[d]Mean nuclear DNA content of 10 isolates of *A. gallica*.

(Becton Dickinson, San Jose, CA, USA). Each measurement of *Armillaria* DNA content was based on 10 000 scanned nuclei. All flow cytometric analyses were conducted four times per isolate.

Results and Discussion

Rhizomorphs of *Armillaria* spp. were found on each of the 10 trees inspected. No signs of pathogenicity (e.g., mycelial fans) were found, and all trees appeared healthy with no symptoms of disease. A total of 10 isolates of *Armillaria* were collected, representing each of the diverse host trees inspected, and included seven different host species (Table 1).

Based on a GenBank BLAST search, IGS-1 sequences of all *Armillaria* isolates had maximum sequence identity (99%) with *A. gallica* (AY509173.1 for NE01-NE06 and NE08-NE10; AY509172.1 for NE07), *A. sinapina* (AY509168.1 for NE01-NE10), and *A. calvescens* (AY509165.1 and AY509166.1 for NE01-NE06 and NE08-NE10; AY509163.1 and AY509164.1 for NE07) (Table 1). As noted in a previous study, rDNA sequence data alone are not always sufficient for definitive identifications among these closely related *Armillaria* species (Kim et al. 2006); however, no other DNA sequence-based methods are presently available to identify all North American *Armillaria* spp.

Nuclear DNA contents of the 10 *Armillaria* isolates (0.17–0.18 pg per nucleus) are shown Table 1. A histogram of fluorescent events versus amount of fluorescence intensity revealed well-defined peaks of *Armillaria* isolate nuclei (Fig. 1). A previous study reported that *A. gallica* possesses the largest nuclear DNA content (0.17 pg per nucleus) among nine North American *Armillaria* spp. (0.11–0.17 pg per nucleus), which can be considered a diagnostic characteristic of the species (Kim et al. 2000). Thus, all isolates from this study can be identified as *A. gallica*, because their nuclear DNA content is very similar to that previously reported for *A. gallica*.

The identification of *Armillaria* spp. is an essential step in monitoring and predicting *Armillaria* root disease in forest ecosystems. In this survey, combined methods of DNA sequencing of IGS-1, which showed a strong relationship to *A. gallica* and closely related species, and measurements of nuclear DNA content

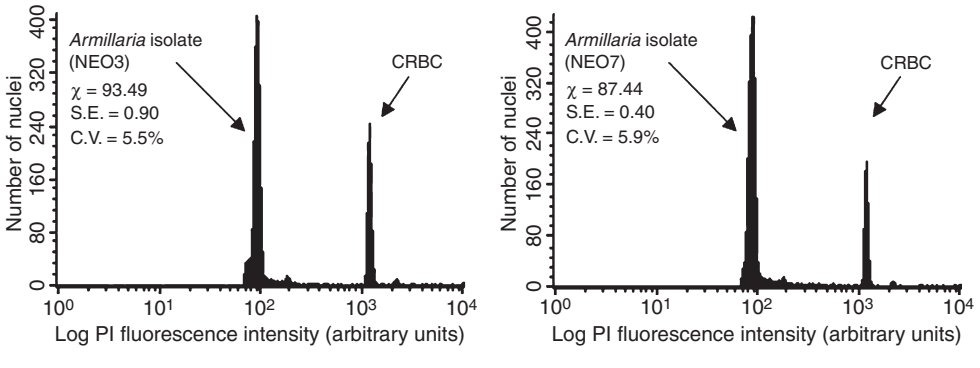

Fig. 1 Representative histograms showing numbers of given fluorescence intensities obtained by flow cytometry for propidium iodide (PI)-stained nuclei of *Armillaria* isolates (NE03 and NE07) and chicken red blood cell (CRBC; 2.33 pg/nucleus). Isolates of *Armillaria* are described in Table 1. The mean of fluorescence intensities (χ), standard deviation (SE), and coefficient of variation are (C.V.) listed for the peak

determined that all *Armillaria* isolates from this study are *A. gallica*. To our knowledge, this is the first confirmed report of *A. gallica* (or any other *Armillaria* spp.) in the state of Nebraska, USA. *Armillaria gallica* has been widely categorized as a beneficial saprophyte, an opportunistic pathogen, or an aggressive pathogen (Brazee and Wick 2009). Smith et al. (1992) reported that a single genet of *A. gallica* (= *A. bulbosa*) remained genetically stable for more than 1500 years. Because the Niobrara Valley Preserve is a protected area with no known tree planting, it seems reasonable to speculate that *A. gallica* may have co-existed in the forest ecosystem of this forest refugium for several millennia. All of the *A. gallica* isolates in our study were collected from trees that appeared healthy, indicating that *A. gallica* was not behaving as a pathogen at the time of the survey. However, it should be noted that *A. gallica* has recently been observed as an aggressive pathogen in some areas of the eastern USA (Brazee and Wick 2009), and it has the potential to contribute to forest decline, especially under increasing stressors such as climate change. Armillaria root disease is typically more severe in highly susceptible tree species and in trees that are maladapted to climate-induced stress (Kliejunas et al. 2009). Thus, it appears likely that climate change will further exacerbate damage from Armillaria root disease; however, accurate identification of *Armillaria* spp. is needed to monitor and predict climate-change impacts of Armillaria root disease (Klopfenstein et al. 2009). More intensive studies and surveys are needed to understand factors associated with the distribution and ecological behaviour of *A. gallica* in these forest ecosystems.

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