NOTE

Armillaria sinapina in herbaceous plant material from a peatland in Alberta, Canada

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Abstract: *Armillaria sinapina* Bérubé & Dessureault mycelium was isolated once from living *Carex aquatilis* Wahlenb. rhizomes, twice from decomposing *C. aquatilis* leaves, and three times from decomposing *Salix planifolia* Pursh leaves in a southern boreal sedge-dominated fen in Alberta, Canada. Restriction fragment length polymorphism (RFLP) analyses of the IGS-1 region with the *Alu*I restriction enzyme were used to identify the isolates, because conspicuous, epigeous basidiomes could not be found in or near the fen. During these analyses, two previously unpublished fragment patterns for *A. sinapina* were found, consisting of 399–240–135 base pairs (bp) and 399–240–183–135 bp. Interspecific somatic incompatibility and interfertility tests confirmed the identifications obtained from the RFLP analyses. This is the first report of an annulate species of *Armillaria* being actively involved in the colonization and decomposition of herbaceous plant material in an ecosystem having only an organic soil horizon. Surveys for species of *Armillaria* should be expanded to include peatlands and herbaceous plant materials, because they may serve as vast potential biomass and genetic diversity reservoirs for this genus.

Key words: peatland, *Carex* rhizomes, *Salix* leaves, RFLP, interspecific somatic incompatibility, interfertility.

Résumé : Les auteurs ont isolé du mycélium d'*Armillaria sinapina* Bérubé & Dessurault, une fois à partir de rhizomes vivants du *Carex aquatilis* Wahlenb., deux fois à partir de feuilles du *C. aquatilis* en décomposition, et trois fois à partir de feuilles du *Salix planifolia* Pursh en décomposition, dans une tourbière basse boréale à carex, du sud de l'Alberta, au Canada. Pour identifier les isolats, les auteurs ont utilisé les analyses RFLP de la région IGS-1 avec l'enzyme de restriction *Alu*I, puisqu'il était impossible de trouver visuellement des basidiomes épigées, dans ou près de la tourbière. Au cours de ces analyses les auteurs ont trouvé deux patrons de fragments jamais publiés pour l'*A. sinapina*, comportant 399–240–135 bp et 399–240–183–135 bp. Les essais d'incompatibilité et d'interfertilité confirment les identifications obtenues à partir des analyses RFLP. Il s'agit de la première mention d'une espèce d'*Armillaria* annelée activement impliquée dans la colonisation et la décomposition de matériel végétal herbacé, dans un écosystème ne possédant qu'un horizon de sol organique. La recherche des espèces d'*Armillaria* devrait être étendue aux tourbières et aux matériaux végétaux herbacés, puisqu'ils peuvent servir de vastes réservoirs potentiels de biomasse et de diversité génétique pour ce genre.

Mots clés : tourbière, rhizomes de *Carex*, feuilles de *Salix*, RFLP, incompatibilité somatique inter-spécifique, interfertilité.

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Introduction

Species of *Armillaria* (Fr.:Fr.) Staude (Agaricales, Tricholomataceae) are some of the most important tree and shrub pathogens, causing root and butt rot in many forest, orchard, and ornamental plant species worldwide (Raabe 1962; Shaw and Kile 1991). This taxon consists of a complex of reproductively isolated groups, or biological species, in North America (NABS) (Anderson and Ullrich 1979; Anderson et al. 1980), Europe (Korhonen 1978), and Australia (Kile and Watling 1983). Currently, nine biological species are recognized in North America. Of these, only three have been found in the Canadian prairie provinces (Mallett 1990) of which *Armillaria ostoyae* (Romagn.) Herink (NABS I), and *Armillaria sinapina* Bérubé & Dessureault (NABS V) are most frequently encountered (Mallett 1992).

In an investigation of the microfungal communities of living and decomposing peatland vegetation, six fungal isolates were obtained that produced rhizomorphs in culture and resembled *Armillaria*. Surveys for conspicuous, epigeous basidiomes of *Armillaria* were unsuccessful in the fen and the surrounding uplands (within a 100-m radius from the location where the isolates were obtained) in the fall of 1998 and 1999. Therefore, the focus of this note is to outline the iden-

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tification process of the six isolates, report on two new IGS-1 fragment patterns obtained from these isolates, and address the ecology and distribution of species of *Armillaria* in peatlands.

Methods

Study area and site description

The riverine sedge fen (54°28′N, 113°18′W) lies within the subhumid low boreal ecoclimatic region of Canada (Ecoregions Working Group 1989). The climate of the area is characterized by mild summers and cold, snowy winters with a long-term mean annual temperature of 1.7°C and a total mean annual precipitation of approximately 500 mm (Environment Canada 1982).

The fen is dominated by *Carex aquatilis* Wahlenb., *Carex lasiocarpa* Ehrh., *Salix planifolia* Pursh, and *Equisetum fluviatile* L. The bryophyte stratum is sparse and discontinuous and consists primarily of *Brachythecium mildeanum* (Schimp.) Schimp. ex Milde and *Tomenthypnum nitens* (Hedw.) Loeske. The surface water of the fen has a pH of 6.7, the mean growing season (May–October) water level is 1 cm above the peat surface, and the depth of the acrotelm (oxygenated peat horizon) is 6 cm. The fen has 1 m of peat, consisting primarily of sedge remains. A more detailed treatise of this fen is in Thormann et al. (1999).

Isolates

The microfungus communities of the dominant vegetation of a southern boreal peatland in Alberta, Canada, were investigated in a related study. Briefly, the top 10 cm of ten *C. aquatilis* leaves, ten 10-cm segments of living *C. aquatilis* rhizomes, ten entire *S. planifolia* leaves, and ten 10-cm terminal segments of *S. planifolia* roots were collected in early May, July, and September 1997 in the riverine sedge fen. A 2-year decomposition study using nylon mesh bags (3 × 6 cm, 1-mm gauge) containing senesced plant materials of these plant species was initiated in early September 1997 (Thormann et al. 2001). Between five and eight individual fresh segments of each plant substrate were placed separately into each of 18 decomposition bags. These were placed horizontally approximately 2–5 cm below the peat surface (*C. aquatilis* rhizomes and *S. planifolia* roots) or on top of the peat surface (*S. planifolia* and *C. aquatilis* leaves) in the fen to mimic natural conditions of decomposition for these plant tissues. The bags containing the *Carex* materials were deployed in the sedge-dominated central area of the fen, while the bags containing the *Salix* materials were deployed in the shrub-dominated fringe area of the fen. Sets of triplicate decomposition bags of each litter were retrieved after 20 and 50 days in 1997, after 8 and 12 months in May and September 1998, respectively, and after 20 and 24 months in May and September 1999, respectively.

All plant materials were cleaned by removing roots and other plant tissues using fine forceps and a dissecting microscope. Each of 10 randomly selected plant segments of each plant tissue was cut with a flame-sterilized scalpel into approximately 10 smaller segments. These were then surface sterilized for 5 min in 10% hydrogen peroxide and washed with sterilized distilled water (dH_2O) prior to placing them onto primary isolation plates containing potato dextrose agar (PDA; 39.0 g Difco potato dextrose agar, 1.0 L dH_2O) amended with benomyl (selective against most ascomycetes and deuteromycetes, 0.0002% w/v) and oxytetracycline (to suppress bacterial growth, 0.01% w/v). Six rhizomorph-forming isolates resembling *Armillaria* were obtained from different plant segments from different primary isolation plates, suggesting that these isolates were different genets of *Armillaria*. These isolates subsequently were transferred onto 1.5% malt extract agar (MEA; 15.0 g Difco malt extract agar, 20.0 g Difco agar, 1.0 L dH_2O) and maintained on that medium on a slant culture at 4°C. Attempts to induce basidiome formation in vitro using autoclaved oranges and an alternating regime of light – room temperature and darkness–10°C (Guillaumin et al. 1989) were unsuccessful. Representative *Armillaria* cultures from each of the three different plant materials (living *C. aquatilis* rhizomes and decomposing *C. aquatilis* leaves and *S. planifolia* leaves) have been deposited in the University of Alberta Microfungus Collection and Herbarium and at the Northern Forestry Centre (NoF), Edmonton.

Species identification

Molecular studies

The method based on polymerase chain reaction (PCR) that was used to identify the *Armillaria* isolates by restriction fragment length polymorphism (RFLP) in the rDNA IGS-1 region was modified from the protocol established by Harrington and Wingfield (1995). Isolates were grown for 2–3 weeks on 2% malt yeast extract agar (MYEA; 20.0 g Difco malt extract agar, 2.0 g yeast extract, and 15.0 g Difco agar, 1.0 L d H_2O) at room temperature. Approximately 25 mm^3 of fungal mycelium was taken from the plate, added to a 1.5-mL microcentrifuge tube containing 500 μL of TE buffer pH 8 (10mM of Tris-HCl pH 8, 1mM EDTA–NaOH (pH 8)), ground using a disposable pellet pestle, and centrifuged at 14 000 × *g* for 10 min. A 1:50 dilution of the supernatant was made with sterile dH_2O to create the template DNA. The PCR reaction mixture contained 2.5 units of *Taq* polymerase (Biological Sciences Department, University of Alberta, Edmonton Alta.), 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 0.25 μM of each primer, and 5 μL template DNA in a final volume of 100 μL. The primers used were LR12R (5′-TGA-ACGCCTCTAAGTCAGAA-3′) and O-1 (5′-AGTCCTATGGCC-GTGGAT-3′) (Canadian Life Technologies Inc., Burlington, Ont.). Two or three drops of mineral oil was used to overlay the reaction mixture in the microcentrifuge tube.

After preheating the thermocycler (Model 60, COY Laboratory Products Inc., Ann Arbor, Mich.) to 72°C, tubes containing the reaction mixture were introduced. Thermocycler conditions were as follows: 50-s ramp to 95°C, 95 s at 95°C (initial denaturation) followed by 30 cycles of 80-s ramp to 55°C, 20 s at 55°C (annealing), 30-s ramp to 72°C, 60 s at 72°C (elongation), 40-s ramp to 95°C, and 30 s at 95°C (denaturation). This was followed by a final elongation cycle at 72°C for 10 min after which the temperature was held at 4°C until use. Following amplification, 15 μL of unpurified PCR product was digested with four units *Alu*I (Canadian Life Technologies Inc., Burlington, Ont.) for 16 h at 37°C. Ten microlitres of digested sample was mixed with 2 μL loading buffer (0.25% bromophenol blue, 0.25% xylene cyanole FF, 30% glycerol (v/v) in dH_2O) and loaded into the gel. Gel electrophoresis to separate digestion products was performed on a 2% agarose gel (AG; 20.0 g agarose gel, 1.0 L dH_2O) in Tris – boric acid – EDTA buffer (pH 8) (TBE) at 5 $V \cdot cm^{-1}$ for 1–2 h.

Digested samples of known *Armillaria* species with predetermined fragment lengths (supported by sequences published by Anderson and Stasovski (1992)) and a 50 base pair (bp) DNA ladder standard (Canadian Life Technologies Inc., Burlington, Ont.) were run alongside the "unknown" samples on AG. Gels were stained with ethidium bromide (0.5 $\mu g \cdot mL^{-1}$) for 30–45 min, visualized using ultraviolet light, and photographed. The lengths of the fragments were determined by comparing their migration distances to those of the known *Armillaria* species and the DNA standard. Only fragments larger than 100 bp were scored.

Interspecific somatic incompatibility

The L-DOPA (L-3,4-dihydroxyphenalanine) technique of Hopkin et al. (1989), based on interspecific somatic incompatibility ("black line"), was used for species identification. Three of the unknown isolates (1, 4, and 6) representing the three banding patterns ob-

tained by RFLP were identified by this means. A 5 mg·L^{-1} solution of L-DOPA (Sigma Chemicals, St. Louis, Mo.) was used to intensify the black line produced between incompatible colonies. *Armillaria ostoyae* (NoF-1076), *Armillaria gallica* Marxmüller & Romagn. (NoF-735), *A. sinapina* (NoF-758), and *Armillaria calvescens* Bérubé & Dessureault (NoF-1468), isolated from basidiomata and whose identity had been determined by haploid–haploid crosses, were paired with the unknown isolates. Pairings were replicated twice.

Diploid–haploid pairing method

The diploid–haploid pairing method of Korhonen (1978) was used to confirm identification of isolates 1, 4, and 6. Two haploid "testers" of *A. ostoyae* (C-970, C-940), *A. gallica* (C-971, C-1191), *A. sinapina* (C-964, C-983), and *A. calvescens* (C-945, C946) were paired with the three "unknown" isolates on MEA and allowed to grow for 1 month. The resulting colonies were observed for evidence of conversion, i.e., the formation of a continuous "crustose" colony type indicating that the unknown was the same species as the tester.

Results and discussion

Identification of the isolates

We obtained over 3000 fungal isolates during the investigation of the fungal communities of living and decomposing peatland vegetation from September 1997 to September 1999. Among these, *Armillaria* was represented six times, once from living *C. aquatilis* rhizomes, twice from decomposing *C. aquatilis* leaves, and three times from decomposing *S. planifolia* leaves (Table 1). These isolates were identified as *A. sinapina* using RFLP and confirmed by interspecific somatic incompatibility and interfertility techniques.

Using the *Alu*I restriction enzyme, three distinct fragment patterns were obtained from the six isolates. These consisted of 399–240–183 bp (pattern b), 399–240–183–135 bp (pattern g), and 399–240–135 bp (pattern i) (Table 1). White et al. (1998) and Banik et al. (1996) previously reported pattern b for *A. sinapina*; however, the other two patterns have never been reported in the literature. White et al. (1998) found *A. sinapina* to be the most variable *Armillaria* species in its fragment patterns, obtaining five different fragment patterns from 17 isolates from British Columbia, Canada. Pattern g was a composite of the other two patterns and possibly represents a heterozygote resulting from mating of haploids with patterns b and i as described by Volk et al. (1996) for *Armillaria nabsnona* Volk & Burdsall. Based on Anderson and Stasovski's (1992) sequence data for *A. sinapina* and the very closely related species *A. gallica* (Miller et al. 1994), *Alu*I restriction maps of the IGS-1 region are proposed consistent with the fragment patterns found in this study (Fig. 1).

Interspecific somatic incompatibility and interfertility tests confirmed the results of the molecular studies, showing that our six isolates were *A. sinapina*. At least one of the isolates originating from decomposing *S. planifolia* leaves (isolate 4) appeared to be haploid based on the results of its pairing with a known *A. sinapina* haploid. Both isolates in this pairing changed from individual, definitely fluffy colonies to a single suppressed crustose colony type. The other two paired isolates (1 and 6) appeared to be diploid, suggesting that they have the potential to produce basidiomes and that nearby living *Salix* and *Betula* shrubs and *Picea* trees may have been colonized by *A. sinapina*. However, basidiomes were not found, and we did not investigate the presence of *Armillaria* rhizomorphs in their roots or stems, because we did not anticipate to isolate species of *Armillaria* from the vegetation in this fen.

Ecological implications

This is the first report of an annulate *Armillaria* species in a peatland. *Armillaria ectypa* (Fr.) Lamoure, an exannulate species of *Armillaria*, represents an exception to the generally accepted rule that species of *Armillaria* are absent from purely organic soils, as it is found exclusively in European bogs (Zolciak et al. 1997). Morrison (1982) showed that an increasing organic matter content in soil resulted in increased growth and branching of *Armillaria mellea* (Vahl:Fr.) Kumm. rhizomorphs, which he attributed, in part, to increased access to nutrients by the fungus. However, the organic matter content of those soils did not exceed 8%, unlike the soil in the riverine sedge fen, which consists of nearly 100% organic matter. Hintikka (1974) reported that, in Finland, *A. mellea* rhizomorphs are absent from organic soil horizons, except in areas with shallow peat deposits and flowing ground water. He did not provide an explanation for the absence of this plant pathogen in organic soils; however, low nutrient concentrations common to most peatlands may restrict the growth and dispersal of *Armillaria* species. Generally, peatlands are characterized by low concentrations of available nutrients such as nitrogen, phosphorus, and mineral ions (Szumigalski and Bayley 1997; Thormann and Bayley 1997), all of which have been shown to affect the growth and dispersal of *Armillaria* species (Mallett and Maynard 1998).

The growth of species of *Armillaria* is negatively affected by low soil oxygen concentrations (Smith and Griffin 1971; Shaw and Kile 1991). Most of the isolates obtained in this study originated from decomposing plant leaf litters (five of six) that were placed on the surface of the peat. Therefore, *A. sinapina* was not limited by low oxygen concentrations (depth of acrotelm was 6 cm), despite being submerged for part of the year (e.g., in the spring following snow and ice melt) (mean growing season water level is 1 cm above the peat surface). One isolate originated from living *C. aquatilis* rhizomes. These rhizomes are located predominantly within the top 10 cm of the peat profile and have aerenchyma tissues that facilitate the diffusion of oxygen from above- to below-ground plant tissues (roots and rhizomes) (Fagersted 1992). Wargo and Shaw (1985) and Whiting and Rizzo (1999) determined that moist soil conditions favour the formation of rhizomorphs; however, Smith and Griffin (1971) determined that rhizomorph growth was inhibited in water-saturated soils. Therefore, it is not surprising that we did not find rhizomorphs associated with living and decomposing plant material in the fen, where the water level is above the peat surface for most of the year.

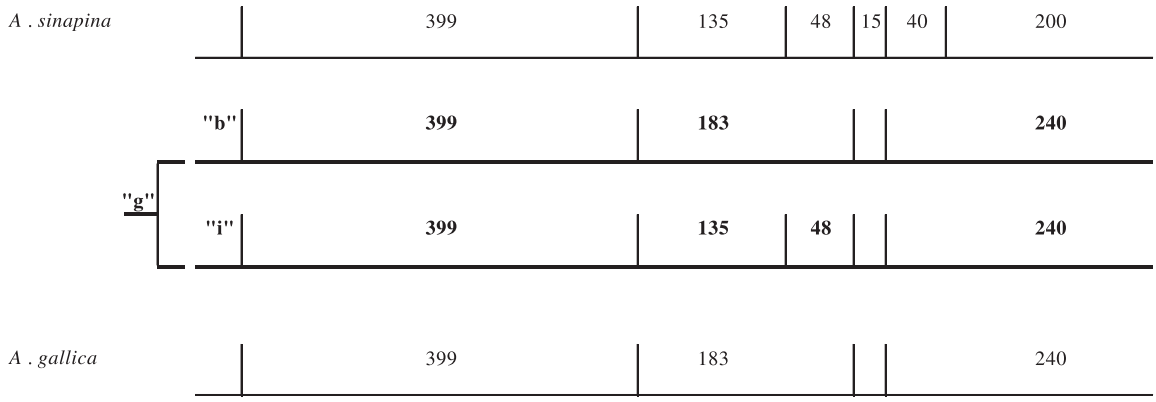
Klein-Gebbinck and Blenis (1991) found *A. ostoyae* rhizomorphs attached to and infecting herbaceous plant species (*Epilobium angustifolium* L. and *Arctostaphylus uva-ursi* (L.) Spreng.) in situ. Klein-Gebbinck et al. (1993) later determined that *A. mellea* and *A. ostoyae* were able to colonize roots of *E. angustifolium* in vitro. They hypothesized that

Table 1. Isolation substrates, fragment length patterns and identification letters, and accession numbers for the six isolates of *Armillaria sinapina*.

Substrate	Fragment size (bp)	Accession No.*
Carex aquatilis rhizomes (living, Sept. 1997)	399–240–135, "i"	NoF 2380, UAMH 9792
Carex aquatilis leaves (decomposing for 20 days, Oct. 1997)	399–240–183, "b"	NoF 2378
Carex aquatilis leaves (decomposing for 20 months, May 1999)	399–240–183, "b"	NoF 2376
Salix planifolia leaves (decomposing for 20 months, May 1999)	399–240–183, "b"	NoF 2375
Salix planifolia leaves (decomposing for 20 months, May 1999)	399–240–183, "b"	NoF 2377
Salix planifolia leaves (decomposing for 20 months, May 1999)	399–240–183–135, "g"	NoF 2379

Note: All isolates originated from a riverine sedge fen in southern boreal Alberta, Canada.
*NoF, Northern Forestry Centre; UAMH, University of Alberta Microfungus Collection and Herbarium.

Fig. 1. IGS-1 restriction maps for *Armillaria sinapina* and the closely related *Armillaria gallica* using the *Alu*I restriction enzyme. Light lines are based on sequences published by Anderson and Stasovski (1992); heavy lines show the three restriction patterns seen in the six isolates from this study ("b", "i", and its composite "g"). Values above the lines are the approximate fragment lengths (base pairs).



herbaceous plant species may be involved in the epidemiology of *Armillaria* root disease by serving as an inoculum reservoir. The role of *A. sinapina* in this fen is unclear because of the absence of woody plant material; however, what is relevant is that *A. sinapina* was isolated from living and decomposing herbaceous plant litters, indicating that it can colonize these litters and that they may serve as inocolum sources or biomass reservoirs. Furthermore, species of *Armillaria* synthesize a suite of enzymes, including polyphenol oxidases and proteases, that permit them to parasitize trees and shrubs or acquire nutrients saprophytically from accumulating litter in ecosystems (Mallett and Colotelo 1984). Therefore, it is not surprising that we isolated this basidiomycete from different plant litters as well.

Terashita and Chuman (1987, 1989) isolated several species of *Armillaria* (*Armillaria borealis* Marx. & Korh., *A. gallica* (identified as *Armillaria lutea* Gillet), *Armillaria cepestipes* Velen., *A. mellea*, and *Armillaria tabescens* (Scop.:Fr.) Emel.) from roots of the orchid *Galeola septentrionalis* Rchb. f. in Japan. The basidiomycete formed pelotons in the roots of the orchid and was mutualistic. This suggests that some species of *Armillaria* can not only colonize roots of herbaceous plant species, but can form mutualistic relationships with their plant hosts as well. It is possible that a similar relationship exists between *A. sinapina* and *C. aquatilis* rhizomes in this fen.

The majority of surveys for *Armillaria* species have been concentrated in forest ecosystems because of the potentially devastating effects this fungus can have on the forest industry (Hiratsuka 1987; Mallett 1992). While the majority of the *Alu*I restriction enzyme fragment patterns of species of *Armillaria* likely have been found in forest ecosystems (Harrington and Wingfield 1995), there may well be many additional fragment patterns from *Armillaria* species in nonforest ecosystems, such as peatlands, suggesting a high molecular diversity of individual North American and European biological species. The presence of *A. sinapina* not only in herbaceous plant material but also in a peatland suggests that its distribution is more widespread than previously thought and surveys for species of *Armillaria* should include ecosystems other than forests and their woody plant substrata. Approximately 16% of Alberta's (Vitt et al. 1996) and 14% of Canada's (National Wetlands Working Group 1988) land base is covered by peatlands. Thus, these ecosystems may represent vast biomass and genetic diversity reservoirs for species of *Armillaria* and have to be considered in future surveys.

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