

Association of *Pinus banksiana* Lamb. and *Populus tremuloides* Michx. seedling fine roots with *Sistotrema brinkmannii* (Bres.) J. Erikss. (Basidiomycotina)

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Received: 4 January 2012 / Accepted: 13 March 2012 / Published online: 5 April 2012
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Abstract *Sistotrema brinkmannii* (Bres.) J. Erikss. (Basidiomycotina, Hydnaceae), commonly regarded as a wood decay fungus, was consistently isolated from bareroot nursery *Pinus banksiana* Lamb. seedlings. *S. brinkmannii* was found in ectomycorrhizae formed by *Thelephora terrestris* Ehrh., *Laccaria laccata* (Scop.) Cooke, and *Suillus luteus* (L.) Roussel. In pure culture combinations with sterile *P. banksiana* and *Populus tremuloides* Michx. seedlings, *S. brinkmannii* colonized root cortical cells while not killing seedlings. Colonization by *S. brinkmannii* appeared to be intracellular but typical endo- or ectomycorrhizae were not formed. The fungus did not decay roots, although it was shown to produce cellulase in enzyme tests. Results suggest a unique association between *S. brinkmannii* and seedling roots that is neither mycorrhizal nor detrimental; its exact function remains to be elucidated.

Keywords *Sistotrema brinkmannii* · *Pinus banksiana* · *Populus tremuloides*

Introduction

Sistotrema brinkmannii (Bres.) J. Erikss. is associated with soil, moss, and wood of angiosperms and gymnosperms in natural environments and forest products and is considered a saprotroph producing brown rot decay (Wang and Zabel 1990; Ginns and Lefebvre 1993). *S. brinkmannii* is unique in culture by possessing hyphae composed of bulbous, oleiferous cells resembling chlamydo-spores, which account for its common name “the chain chlamydo-spore fungus” (Wang and Zabel 1990). Hallenberg (1984) demonstrated that *S. brinkmannii* occurs within a complex taxon, based on compatibility tests and basidiome morphology, of species still requiring taxonomic resolution. This is reflected by ongoing changes in genera and epithets for this species, including *Odontia brinkmannii* Bres., *Odontia brassicicola* Bres., *Grandinia brinkmannii* (Bres.) Bourd & Galzin, *Grandinia brassicicola* (Bres.) Bourd & Galzin, *Grandinia sordidissima* Rick, *Trechispora brinkmannii* (Bres.) D.P. Rogers & Jackson, *Trechispora brassicicola* (Bres.) Melo & Telleria, *Corticium varians* Kneip, and *Corticium masculi* Sprau (see Parmasto et al. 2009). In addition, this species is geographically widespread (Ginns and Lefebvre 1993; Breitenback and Kranzlin 1986) and has been isolated from a variety of substrates including utility poles (Wang and Zabel 1990), diseased *Pinus sylvestris* roots (Menkis et al. 2006), and wood of *Pinus contorta* killed by mountain pine beetles (Son et al. 2011).

Sistotrema Fr. is placed in the Hydnaceae of the Cantharellales (Kirk et al. 2008). This highly polyphyletic genus is comprised of about 50 morphologically and ecologically diverse fungi species (Larsson et al. 2004; Binder et al. 2005; Moncalvo et al. 2006; Parmasto et

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al. 2009). DNA-based phylogenetic studies have determined that it belongs to the cantharelloid clade that contains genera that are lignicolous (Binder et al. 2005), lichenicolous (Lawrey et al. 2007), or ectomycorrhizal (Nilsson et al. 2006; Di Marino et al. 2008). Well-known ectomycorrhizal species of angiosperm and gymnosperm forest trees found in this clade include *Hydnum repandum* L., *Cantharellus cibarius* Fr., and *Craterellus tubaeformis* (Bull) Quel. (Di Marino et al. 2008).

In a study of jack pine (*Pinus banksiana* Lamb.) seedling stunting in a nursery in northern Michigan, USA (Potvin 2008), *S. brinkmannii* was isolated from 6 % of ectomycorrhizal root tips formed by *Thelephora terrestris* Ehrh., *Laccaria laccata* (Scop.) Cooke, and *Suillus luteus* (L.) Roussel. The isolate of *S. brinkmannii* produced cellulase by a simple enzyme test, while the isolates of the known ectomycorrhizal fungi lacked this ability. In corresponding pure culture synthesis experiments with jack pine and *S. brinkmannii*, no ectomycorrhizae were formed, but seedling growth was stimulated and the fungus was again isolated from root tips. Although Potvin (2008) reisolated *S. brinkmannii* from the synthesis seedlings and root tips were examined macroscopically for mycorrhizae and none were found. To better understand the association between *S. brinkmannii* and jack pine seedling roots, a second mycorrhiza synthesis experiment was conducted to more closely examine the resulting root/fungus association through staining and microscopy. In addition, aspen (*Populus tremuloides* Michx.) seedlings were included to examine the association of this fungus with a hardwood host.

Methods

The isolate of *S. brinkmannii* (LP T-1) was obtained in July 2006 from mycorrhizal root tips on first year (1+0) bareroot jack pine seedlings at the USDA Forest Service, J.W. Toumey Nursery in Watersmeet, MI, USA (Potvin 2008). Briefly, 240 root tips from 40 seedlings were surface sterilized using a 1:10 ($v v^{-1}$) Clorox® solution (5.25 % sodium hypochlorite) water solution, rinsed three times with sterile water (modified methods of Zak and Bryan 1963), and plated on a 2 % malt agar medium with additions of 100 ppm streptomycin and 100 ppm tetracycline and 10 ppm of the fungicide benomyl (Benlate®). Cultures identified as putative ectomycorrhizal fungi were transferred to modified Melin-Norkrans (MMN) agar (Marx 1969), incubated at 22°C for 3 weeks, and sorted into types using macro- and micromorphological characteristics (Hutchison 1991; Wang and Zabel 1990). Of the 240 root tips, 15 yielded the LP T-1 culture type, later identified as *S. brinkmannii* using a combination of culture and molecular methods. DNA was extracted

from hyphae scraped from the surface of one of the actively growing LP T-1 cultures using the CTAB mini-prep method.

Fungal-specific primers ITS1F and ITS4 were used for the PCR amplification of the internal-transcribed spacer (ITS) region (White et al. 1990; Gardes and Bruns 1993). Samples were prepared for sequencing using the QIAquick PCR Purification Kit with the primers ITS1F and ITS4 and sent to the University of Nevada—Reno Nevada Genomics Center. The sequence was edited using Bioedit 7.1.3 (Tom Hall, Ibis Biosciences, Carlsbad, CA) and submitted to GenBank (Benson et al. 2011). The sequence was matched with highly similar ITS sequences in the GenBank database using the BLAST search (Altschul et al. 1997). From the BLAST results, 25 sequences were selected for further phylogenetic analysis based on percentage of query coverage, percentage of ITS similarity, and detailed source information (Appendix). The sequences were aligned in Unipro UGENE (<http://ugene.unipro.ru>) using Kalign (Lassman and Sonnhammer 2005). The alignment was checked manually for errors and trimmed. A Bayesian phylogenetic analysis was carried out on the aligned sequences using MrBayes 3.2 (Ronquist and Huelsenbeck 2003). Default settings were applied: two Markov chain Monte Carlo runs for 1,000,000 generations, the first 25,000 trees discarded as the burn-in phase, and trees sampled every 1,000 generations. The phylogram was viewed using Dendroscope (Huson et al. 2007).

Cellulase production by the isolate of *S. brinkmannii* was examined following the methods of Smith (1977). Two tubes each of cellulose azure media were inoculated with *S. brinkmannii*, *Gloeophyllum trabeum* Pers.: Fr (ATCC11539) (a brown rot fungus), and *L. laccata* (DR137) (an ectomycorrhizal fungus) and evaluated after 10 days and 1-month incubation (26°C).

Sterile seedlings of jack pine and aspen were grown with the isolate of *S. brinkmannii* LP T-1 in 1:1 ($v v^{-1}$) sphagnum peat/vermiculite in 0.5 L jars following methods of Richter and Bruhn (1989). Upon planting three sterile germinated seeds per jar (ten jars with jack pine, four jars with aspen), a colonized agar plug (approx. 2 mm in diameter from 2 % malt, 1.5 % agar (2MA)) from an actively growing culture was placed alongside each seed. Lids of jars were replaced loosely and wrapped with Parafilm®; the jars were tipped at a 45° angle and placed in a Conviron PGR15 growth chamber at 18 h 20°C, 6 h 15°C ($875 \mu\text{mol m}^{-2}\text{s}^{-1}$). For comparison, six jars were prepared with jack pine and a known ectomycorrhizal fungus, *L. laccata* (Scop.: Fr.) Berk. & Br (DR-137).

After 5 months, the jars were removed from the growth chamber and seedlings were evaluated for vigor. A small amount of substrate from each jar was plated on 2MA to detect viability of the fungus in the peat/vermiculite. Six (jack pine) and three (aspen) jars were randomly selected for detailed examination of roots. Seedlings were gently

removed, root systems were washed in tap water, and total length was estimated; three root tips from each seedling were removed with fine sterile forceps, rinsed for 10–15 s with 10 % chlorine bleach, and plated on 2MA. Roots were first examined with a low-power ($\times 10$) microscope for gross evidence of ectomycorrhizae. Then, sections of fine root from each seedling bearing short laterals and root tips were cleared and stained with trypan blue using methods of Koske and Gemma (1989) and examined at $\times 400$ for evidence of intracellular infection.

Results

Culture morphology and characteristics

In pure culture, chain chlamydospore hyphae with dense, irregular hyphal growth patterns, clamped hyphal connections, bleaching of agar, and moderate to fast growth were characterized with the isolate *S. brinkmannii* LP T-1 on MMN. *S. brinkmannii* and the brown rot fungus *G. trabeum* produced a strong blue reaction in cellulose azure tubes, indicating the production of cellulase. Conversely, the

ectomycorrhizal fungus, *L. laccata*, produced no reaction on cellulose azure.

Molecular data

BLAST results of this study's *S. brinkmannii* sequence (accession # GQ478194) yielded known *S. brinkmannii* specimens and unknown uncultured and environmental samples with diverse ecological roles. The ITS alignment consisted of 26 species with 598 bp. The 50 % majority-rule consensus phylogram from the Bayesian analysis is shown in Fig. 1. The results placed the *S. brinkmannii* isolate from this study in a highly supported clade with a known *S. brinkmannii* sequence from an American Type Culture Collection specimen (DQ899094), as well as showing it closely related to another clade containing almost exclusively *S. brinkmannii*.

Jack pine \times *S. brinkmannii*

After 5 months, inoculated seedlings appeared green, healthy, and 5 to 10 cm tall. Substrate from each jar plated

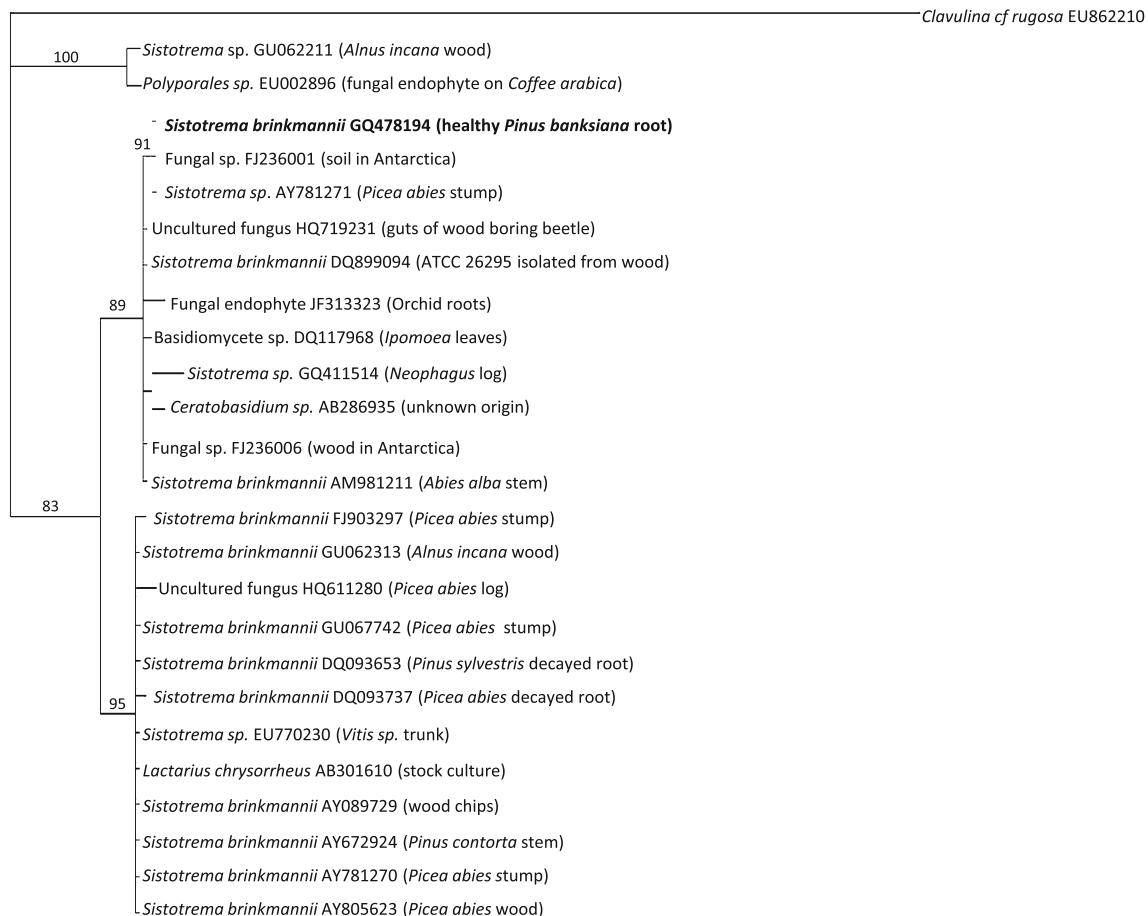


Fig. 1 Consensus ITS phylogram from Bayesian analysis with Genbank ID, accession number, and substrate (if known) where the sequence was isolated from. Support values are Bayesian posterior probabilities (BPP). The sequence in **bold** is the isolate of interest for this study

on 2MA yielded pure cultures of *S. brinkmannii*. Total length of root systems per seedling was typically 10–20 cm (up to 30 cm); main roots bore numerous branches that in turn bore single (unbranched) root tips, mostly 1–3-mm long \times 0.5–1-mm diameter; occasional root hairs were present along the main roots and on root tips (Fig. 2). Upon gross microscopic examination, no mycorrhizae or mantle was observed, although occasional clamped hyphae were observed along roots. All root tips plated on 2MA yielded pure cultures of *S. brinkmannii*.

Following clearing and staining at $\times 400$, bulbous intracellular hyphae of *S. brinkmannii* were observed in root cortical surface cells and within cells several layers inward (Fig. 3). Colonized cortical cells were rectangular, typically $80\text{--}150 \times 20\text{--}30 \mu\text{m}$; stained intracellular hyphal elements were bulbous, globular to oblong, typically $8\text{--}15 \times 8\text{--}12 \mu\text{m}$; in many cases, cortical cells were entirely filled with bulbous hyphae that were often separated by a short clamp connection (Fig. 4). Fungi were not observed in all sections of roots examined. Of the 18 jack pine root sections (8–10 cm) examined (one section per seedling), nine sections clearly showed blue areas due to presence of the fungus; colonized areas typically measured 1–3-mm long \times 0.25–1.0-mm wide.

Aspen \times *S. brinkmannii*

The inoculated aspen seedlings were 4 to 8 cm tall, had numerous small (1–2 cm diameter) green leaves, and appeared healthy. Substrate from each jar plated on 2MA yielded pure cultures of *S. brinkmannii*. Total length of root systems per seedling was typically 10–15 cm; roots were highly branched with numerous laterals that in turn bore single (unbranched) root tips, mostly 2–10 mm-long \times 0.1–0.5-mm diameter. Root hairs were abundant along all roots and root tips. Upon gross microscopic examination, no



Fig. 2 Total root system of jack pine seedling grown for 5 months in axenic culture with *S. brinkmannii* (bar=5 cm)

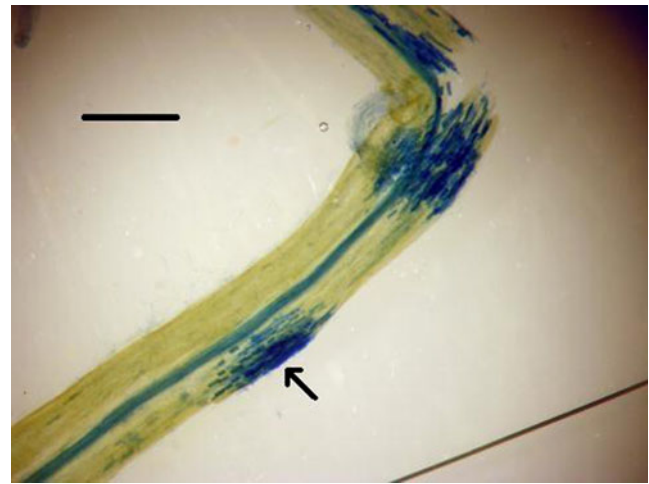


Fig. 3 Jack pine root section showing patchy colonization of cortical cells by *S. brinkmannii* ($\times 10$); blue areas indicate areas of fungus-colonized cortical cells which were typically 1–3-mm long \times 0.25–1.0-mm wide and several cortical cell layers inward (bar=1 mm)

mycorrhizae or mantle was observed, although occasional clamped hyphae were observed along roots. All root tips plated on 2MA yielded pure cultures of *S. brinkmannii*.

Following clearing and staining at $\times 400$, bulbous intracellular hyphae of *S. brinkmannii* were observed in root cortical surface cells and within cells several layers inward (Fig. 5). Colonized cortical cells were rectangular, typically $50\text{--}120 \times 15\text{--}25 \mu\text{m}$. Stained intracellular hyphal elements were similar in size and shape to those seen in jack pine cortical cells (Fig. 6). Fungi were not observed in all sections of roots examined; of the nine aspen root sections (6–8 cm) that were examined (one section per seedling), only two sections clearly showed several blue areas due to the

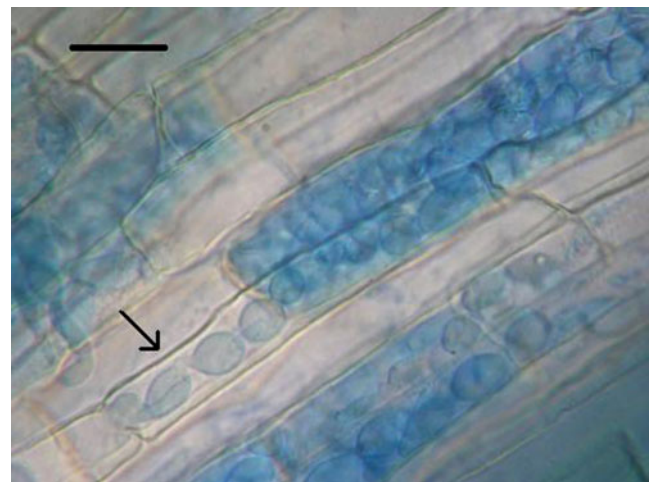


Fig. 4 High-power ($\times 400$) micrograph of jack pine root cortical cells showing intracellular infection by *S. brinkmannii*; blue areas indicate areas of fungus-colonized cortical cells; note chains of globular fungus cells typically $8\text{--}15 \times 8\text{--}12 \mu\text{m}$ separated by a short clamp connection showing the characteristic “chain chlamydo spores” of *S. brinkmannii* (bar=100 μm)

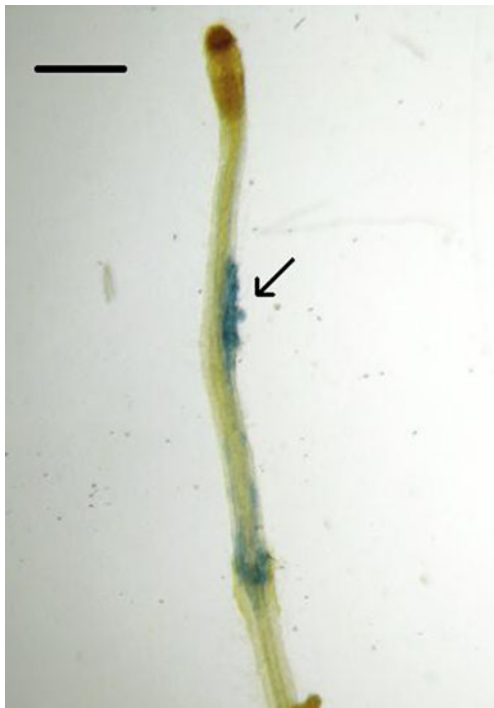


Fig. 5 Aspen root section showing patchy colonization of cortical cells by *S. brinkmannii* ($\times 10$). Roots were cleared and stained with trypan blue; blue areas indicate areas of fungus-colonized cortical cells which were typically 0.5–2-mm long \times 0.1–0.25-mm wide and several cortical cell layers inward ($bar=1$ mm)

presence of the fungus. Colonization typically measured 0.5–2-mm long \times 0.1–0.25-mm wide.

Jack pine \times *L. laccata*

Height and health of jack pine seedlings grown with *L. laccata* were similar to their cohorts grown with *S. brinkmannii*. Total

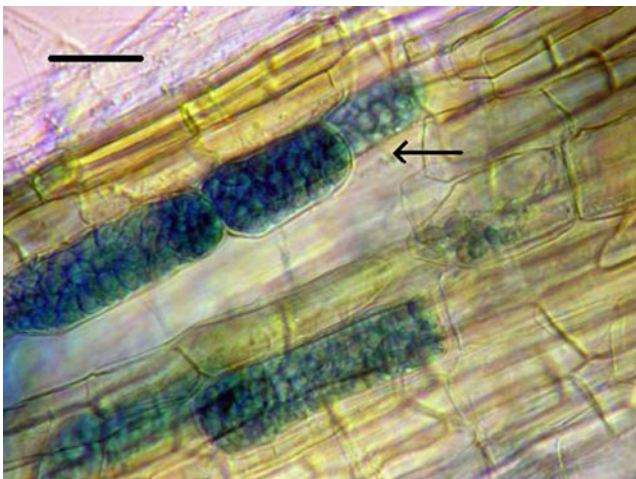


Fig. 6 High-power ($\times 400$) micrograph of aspen root cortical cells showing intracellular infection by *S. brinkmannii*; blue areas indicate areas of fungus-colonized cortical cells; note chains of globular fungus cells typically 8–15 \times 8–12 μ m showing the characteristic “chain chlamydospores” of *S. brinkmannii* ($bar=100$ μ m)

root length per seedling was shorter, typically 8–15 cm. Main roots bore numerous branches that in turn bore single (unbranched) and double (bifurcated) root tips, appearing as mycorrhizae, mostly 1–3-mm long \times 0.5–1-mm diameter; root hairs were not observed. Following clearing and staining at $\times 400$, interdigitating hyphae of a mycorrhizal mantle and intercellular hyphae of a Hartig net were observed, and occasional clamped hyphae were observed along roots. All root tips plated on 2MA yielded pure cultures of *L. laccata*.

Discussion

The pure cultural characteristics of the isolate in this study, in conjunction with the molecular data and subsequent Bayesian analysis of phylogeny, confirm the identification of the fungus isolated from jack pine roots as *S. brinkmannii*. The highly supported clade where this species was placed contains isolates with geographical and ecological diversity (Fig. 1 and Appendix). A number of the closely related sequences were isolated from living and dead wood and two of the *S. brinkmannii* sequences were obtained from decayed conifer seedling roots (Menkis et al. 2006). Conversely, another sequence classified as a fungal endophyte (JF313323) was isolated from the healthy roots of the orchid *Cypripedium irapeanum* (Valdes et al. 2011), and another, classified as epibiotic fungus (DQ117968), was isolated from the healthy leaves of *Ipomoea asarifolia* (Steiner et al. 2006). These results point to the apparent generalist nature of *S. brinkmannii*. The *S. brinkmannii* specimen obtained in the present study was isolated from *Pinus* seedling roots, which showed no visible signs of decay, and in pure culture synthesis it did not appear to affect seedling growth.

Hardwood sawdust and small wood chips are used as a soil amendment at the nursery where the jack pine seedlings were harvested; *S. brinkmannii* was possibly introduced to seedling roots through this material. A wood block decay test using the *S. brinkmannii* isolate (LP T-1) from this study found no mass loss after 12 weeks (Potvin 2008). Similarly, Son et al. (2011) found that *S. brinkmannii* produced little to no mass loss in a wood block decay test with *P. contorta*, even though it was isolated from mountain pine beetle killed trees. Although *S. brinkmannii* has been shown to be capable of decaying of wood (Wang and Zabel 1990; Ginns and Lefebvre 1993, Vasiliauskas 1998), recently Vasiliauskas et al. (2007) demonstrated in pure culture experiments that certain wood decay fungi (*Phlebiopsis gigantea*, *Phlebia centrifuga*, and *Hypholoma fasciculare*) can colonize fine roots of tree seedlings with no visible deleterious effects on seedling health and found intracellular colonization, but no mantle formation, with *P. gigantea* in *Picea abies* roots.

Nilsson et al. (2006) established the ectomycorrhizal association of two *Sistotrema* spp. (*Sistotrema albuluteum*

(Bourdôt & Galzin) Bondartsev & Singer and *Sistotrema muscicola* (Pers.) S. Lundell) in mixed forests in northern Europe by correlating molecular data between fruiting bodies and root-tip mantle mycelia. DNA sequencing of ectomycorrhizal fungi growing on mature *Pseudotsuga menziesii* (Mirbel) Franco (Douglas-fir) roots also yielded a *Sistotrema* species, whose sequence most closely matched *S. muscicola* (Dunham et al. 2007). Di Marino et al. (2008) morphologically characterized the ectomycorrhizae of *Sistotrema* sp. with *Castanea sativa* L.; the species was most similar to the ITS sequence of *S. muscicola*. Anatomically, the mycorrhizae formed by *S. muscicola* were similar to that formed by *H. repandum* (Di Marino et al. 2008). Most recently, Münzenberger et al. (2012) conducted pure culture synthesis with *P. sylvestris* and a *Sistotrema* sp. that was suspected to form ectomycorrhiza and confirmed mantle formation. This was the first study to show an ectomycorrhizal association produced by *Sistotrema* in pure culture.

This pure culture combination of *S. brinkmannii* with sterile jack pine and aspen seedlings demonstrates an asso-

ciation with roots that is neither detrimental to the seedling nor typically mycorrhizal. While it is likely that the fungus is absorbing plant cell nutrients, the intracellular bulbous hyphae are not suspected to be involved in nutrient exchange, such as that in endomycorrhizae, because plasma membrane penetration was not observed and the cell walls of seedlings appear to be intact in areas colonized by *S. brinkmannii*. Thus, the ecological role of the association of *S. brinkmannii* with tree roots remains unclear.

Acknowledgments We thank Dr. Erik Lilleskov who provided substantial assistance with phylogenetic analysis; Dr. Harold H. Burdsall Jr. who provided valuable comments on the early taxonomy of *S. brinkmannii*; Drs. Linda van Diepen and Carrie Andrew for assistance with staining and molecular methodology; and Karena Schmidt for assistance with figures. We also thank the anonymous reviewers for their role in shaping our manuscript. This work was supported in part by the USDA Forest Service J.W. Toumey Nursery, the National Center for Reforestation, Nurseries, and Genetics Resources, the Rocky Mountain Research Station, and Michigan Technological University.

Appendix

Table 1 ITS sequences in the GenBank database used for Bayesian phylogenetic analysis. Query coverage, No. of bp compared, and ITS similarity are in relation to the sequence of interest for this study, which is indicated in *bold*

Genbank accession #	Query coverage (%)	No. of bp compared	ITS similarity (%)	Sample type	Host	Geographic origin	Author
AB286935	90	549	99	<i>Ceratobasidium</i> sp.	NA	NA	Sharon et al. 2008
EU862210	82	359	88	<i>Clavulina cf rugosa</i>	Spruce forest	Finland	Olariaga et al. 2009
AB301610	90	553	97	<i>Lactarius chrysorrheus</i>	NA	NA	Maeta et al. 2008
EU002896	92	571	93	<i>Polyporales</i> sp.	Fungal endophyte on <i>Coffea arabica</i>	Puerto Rico	Vega et al., unpublished
AM981211	89	543	99	<i>Sistotrema brinkmannii</i>	<i>Abies alba</i> stem	Slovenia	Jurc et al., unpublished
AY089729	90	555	97	<i>Sistotrema brinkmannii</i>	Wood chips	NA	Adair et al. 2002
AY672924	90	551	97	<i>Sistotrema brinkmannii</i>	<i>Pinus contorta</i>	British Columbia, Canada	Kim et al. 2005
AY781270	86	531	97	<i>Sistotrema brinkmannii</i>	<i>Picea abies</i> stump	Sweden	Vasiliauskas et al. 2005
AY805623	86	533	96	<i>Sistotrema brinkmannii</i>	<i>Picea abies</i> wood disc	Sweden	Menkis et al. 2004
DQ093653	94	577	97	<i>Sistotrema brinkmannii</i>	<i>Picea sylvestris</i> seedling decayed root	Lithuania	Menkis et al. 2006
DQ093737	93	573	97	<i>Sistotrema brinkmannii</i>	<i>Picea abies</i> seedling decayed root	Lithuania	Menkis et al. 2006
DQ899094	95	579	99	<i>Sistotrema brinkmannii</i>	Wood	Nova Scotia, Canada	Marek, unpublished
FJ903297	99	606	97	<i>Sistotrema brinkmannii</i>	<i>Picea abies</i> stump	Latvia	Arhipova et al., unpublished
GQ478194	100	100	100	<i>Sistotrema brinkmannii</i>	<i>Pinus banksiana</i> roots	Michigan, USA	This study
GU062313	96	586	98	<i>Sistotrema brinkmannii</i>	<i>Alnus incana</i> wood	Latvia	Arhipova et al. 2011
GU067742	92	566	98	<i>Sistotrema brinkmannii</i>	<i>Picea abies</i> stump	Finland	Vasaitis et al., unpublished
AY781271	86	524	100	<i>Sistotrema</i> sp.	<i>Picea abies</i> stump	Sweden	Vasiliauskas et al. 2005
EU770230	91	559	97	<i>Sistotrema</i> sp.	<i>Vitis</i> sp. trunk	New Zealand	Graham et al. 2009
GQ411514	93	571	99	<i>Sistotrema</i> sp.	<i>Neophagus</i> log	New Zealand	Fukami et al., unpublished
GU062211	96	591	94	<i>Sistotrema</i> sp.	<i>Alnus incana</i> wood	Latvia	Arhipova et al. 2011
HQ719231	99	605	99	Uncultured/environmental	Guts of <i>Prionoplus reticularis</i>	New Zealand	Williams and Morgan, unpublished
DQ117968	93	569	99	Uncultured/environmental	<i>Ipomoea asarifolia</i> leaves	Germany	Steiner et al. 2006
FJ236001	86	528	100	Uncultured/environmental	Soil	Antarctica	Arenz and Blanchette, unpublished
FJ236006	90	551	99	Uncultured/environmental	Wood	Antarctica	Arenz and Blanchette, unpublished
JF313323	96	588	99	Uncultured/environmental	<i>Cypripedium irapeanum</i> roots	NA	Valdes et al. 2011
HQ611280	97	595	97	Uncultured/environmental	<i>Picea abies</i> log	Sweden	Lindner et al. 2011

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