Detection and Identification of *Amylostereum areolatum* (Russulales: Amylostereaceae) in the Mycangia of *Sirex nigricornis* (Hymenoptera: Siricidae) in Central Louisiana

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ABSTRACT The woodwasp *Sirex noctilio* F. (Hymenoptera: Siricidae) has become established in North America. A primary tactic for the management of *S. noctilio* in the southern hemisphere has been the development of a biological control agent, *Deladenus siricidicola* Bedding. This nematode has a bicyclic life cycle including a mycetophagous free-living and parasitic cycle. During oviposition, female *Sirex* woodwasps inject a symbiotic fungus. Because *D. siricidicola* only develops well on *Amylostereum areolatum* (Chaillet ex Fries) Boidin (Russulales: Amylostereaceae) and North American woodwasps were thought to all have *Amylostereum chailletii* (Persoon) Boidin as their fungal symbiont, the risk of unintended impacts from *D. siricidicola* in North America was considered low. Specific polymerase chain reaction primers were designed to amplify the intergenic spacer region of *Amylostereum* symbionts in a population of the native woodwasp *Sirex nigricornis* F. located in central Louisiana (i.e., well outside the known distribution of *S. noctilio*); identity of the symbiont was confirmed by phylogenetic analyses. Overall, 95 out of 100 fungal isolates obtained from the mycangia of *S. nigricornis* were identified as *Amylostereum* species. Contrary to expectations, 60% were identified as *A. chailletii* ($N = 60$), while 35% were identified as *A. areolatum* ($N = 35$). The remaining 5% of these isolates ($N = 5$) were identified as *Bipolaris papendorfii* (Aa) Alcorn, *Alternaria alternata* (Fr.) Keissl, *Penicillium marneffei* Segretain, *Scytalidium cuboideum* (Sacc. & Ellis) Sigler & Kang, and *Hyphopichia heimii* (Pignal) Kurtzman based on sequencing of the internal transcribed spacer (ITS) region. The five non-*Amylostereum* isolates were likely contaminants during mycangia-spore extraction process. This study confirms the presence of *A. areolatum* in a population of the native woodwasp *S. nigricornis* well outside the known distribution of *S. noctilio*.

KEY WORDS *Amylostereum*, invasive species, *Sirex*, symbiont

The invasive woodwasp *Sirex noctilio* F. (Hymenoptera: Siricidae) was recently discovered in North America (Hoebeke et al. 2005, de Groot et al. 2006). This woodwasp has a symbiotic relationship with the fungus *Amylostereum areolatum* (Chaillet ex Fries) Boidin (Russulales: Amylostereaceae). During oviposition, *S. noctilio* injects both the fungus and phytotoxic mucus into host pines. The mucus and fungus play an important role in larval nutrition and in tree mortality. Spradberry and Kirk (1978) report that the principal hosts of *S. noctilio* are pines with ≈99% of 8,625 wasps collected emerging from *Pinus* spp. while the remainder were collected from species of *Picea* (0.8%) and *Abies* (0.05%). Climate models predict a potential distribution of *S. noctilio* throughout large portions of North America (Carnegie et al. 2006) and suggest that its distribution in North America will primarily be determined by the distribution of host pines.

Although it is a minor and infrequent pest in its native range of Eurasia and North Africa, in some cases introduction and establishment of *S. noctilio* to the southern hemisphere has resulted in significant mortality (up to 80%) of the North American pines *Pinus radiata* D. Don and *Pinus taeda* L. (Haugen et al. 1990, Iede et al. 1998, Tribe and Cillié 2004, Ryan and Hurley 2012). Economic analyses predict potential losses over a 30-yr period of US$48–606 million in Georgia, US$7–76 million in Minnesota, and US$7–77 million in California (U.S. Department of Agriculture [USDA] 2000). Yemshanov et al. (2009) estimate the potential impact of *S. noctilio* in Canada over the next 20 yr to be as high as US$254 million/yr in losses. There is some debate over the impact establishment of *S. noctilio* will have in North America. Mortality of pines in the south-

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ern hemisphere has primarily been associated with plantations of North American *P. radiata* and *P. taeda.* Dodds et al. (2007) point out that many North American pine forest ecosystems are more similar to Eurasian pine forests than to southern hemisphere pine plantations and that consequently a priori it is difficult to predict what impact establishment of *S. noctilio* will have in North America. Extensive pine plantations do occur in the southeastern United States and models predict that by 2040 pine plantation acreages will increase in the southern United States by as much as 67% (U.S. Department of Agriculture–United States Forest Service [USDA–USFS] 2003). Although there is uncertainty about what the impact of *S. noctilio* establishment in North America will be, because of the high value of pine resources in North America and the potential adverse impacts of *S. noctilio*, it has been rated a high-risk species for North American forests (Haugen 1999, Borchert et al. 2007).

Management programs for *S. noctilio* in the southern hemisphere have relied on coupling biological control with population monitoring and silviculture (Hurley et al. 2007). Biological control programs for *S. noctilio* are based primarily on the nematode *Deladenus siricidicola* Bedding. This nematode has a bicyclic life cycle including both a mycetophagous or free-living stage and a parasitic stage (Bedding 1967). During the free-living stage, the nematode is highly specific to *A. areolatum,* and in the parasitic stage, female nematodes enter and develop in the haemocoel of the woodwasp larvae. Nematode larvae produced by parasitic females are released inside the haemocoel of the woodwasp larvae and migrate to and infest the testes and developing eggs. As a result, although oviposition behavior is not affected, infested female *S. noctilio* are sterilized (Bedding 1967, 1972).

Use of *D. siricidicola* in the southern hemisphere has been facilitated by the fact that pines are not native to areas in which *S. noctilio* has been introduced and consequently there are no native pine borers present. Unlike the southern hemisphere countries colonized by *S. noctilio*, pines are native to the United States and Canada with a diverse community of borers and mycoflora including several native species of Siricidae. All of the native North American *Sirex* spp. and some of the native *Urocerus* species attack pines (Schiff et al. 2012). In addition to *S. noctilio,* the nematode *D. siricidicola* has been reported to attack the woodwasp *Xeris spectrum* L. and the beetle *Serropalpus barbatus* (Schaller) (Bedding and Akhurst 1978). The woodwasp *X. spectrum* does not have a fungal associate but is usually associated with other woodwasps that have *A. areolatum* or *Amylostereum chailletii* (Pers.) Boidin as a symbiont (Bedding and Akhurst 1978, Fukuda and Hijii 1997). Although juvenile *D. siricidicola* were found in the hemocoel of *S. barbatus*, they were not found in the reproductive organs (Bedding 1972, Bedding and Akhurst 1978).

The primary factor that determines the exposure of woodwasps to the nematode *D. siricidicola* is their fungal symbiont. Until recently, European and North American *Sirex* species were thought to have different fungal symbionts (*A. areolatum* and *A. chailletii*, respectively) (Gilbertson 1984, Smith and Schiff 2002). Because *D. siricidicola* does not feed or reproduce well on *A. chailletii* (Williams et al. 2012), the potential for unintended impacts of *D. siricidicola* on North American woodwasps has been considered low. Further exposure of native North American woodwasps would only occur when they coinfested trees with *S. noctilio.* Uncertainty regarding the fidelity of *Sirex*–fungal associations exists in the literature. While some consider the fidelity of associations to be low (i.e., although a primary fungal associate exists, *Sirex* spp. can have more than one associate), others argue that associations are highly specific (Talbot 1977). Nielsen et al. (2009) observed that while most native *Sirex* spp. that emerged from *S. noctilio* infested trees had the fungal symbiont *A. chailletii*, a few emerged with the same strain of *A. areolatum* as coemerging *S. noctilio.* It is possible that the presence of *A. areolatum* in these native woodwasps is a result of horizontal transfer of the fungal symbiont. In the same study, a unique strain of *A. areolatum* was isolated from *Sirex nitidus* (Harris) that had emerged from a spruce tree collected outside the known range of *S. noctilio.*

Potential unintended impacts of the nematode *D. siricidicola* on native nontarget woodwasps is a concern and challenge for the development of a biological control program for *S. noctilio* in North America (Williams et al. 2012). The identity and specificity of the fungal associations of native North American woodwasps is of interest because of their impact on the exposure of woodwasps to the biological control agent *D. siricidicola.* If the fungal associations of native North American woodwasps include *A. areolatum* in the absence of *S. noctilio*, the release of *D. siricidicola* may have unintended negative impacts on native North American woodwasps. The objective of this study was to examine the *Sirex–Amylostereum* associations in a population of the native woodwasp *Sirex nigricornis* F. located well outside the North American distribution of *S. noctilio* in central Louisiana.

Materials and Methods

Sirex Samples. One hundred adult females of *S. nigricornis*, half of which comprised the dark color morph formerly known as *Sirex edwardsii* Brullé (Schiff et al. 2012), were collected preflight as they emerged from infested loblolly pine (*P. taeda*) bolts between late October and mid-November of 2011 in Grant Parish, central Louisiana (N 31.595199°, W −92.416603°). Emergence was checked daily, and *S. nigricornis* were individually collected and stored in small glass vials containing moist tissue paper. The woodwasp samples were stored in a refrigerator until processed, usually within 3 d of collection.

Symbiont Isolation. The *Sirex* specimens collected were killed by applying a drop of 70% ethyl alcohol directly on the head of each wasp while still in the collection vial. Wasp death occurred within 2 min, and dead wasps were immediately removed from the vial and placed on clean filter paper in an upright position (with the dorsal surface of the abdomen facing up),

and pinned onto a Styrofoam surface with sterilized needles. The methodology of Thomsen and Harding (2011) was modified slightly to extract fungal symbionts from the mycangia of female wasps. The mycangia, located posterior to the end of the ovipositor, were carefully exposed with sterile insect pins by lifting the last ventrite dorsally. The exposed mycangia were gently ruptured to release their contents. A small sample of the oozing spore mass from each mycangium was collected from each insect specimen ($N = 96$) using a sterile insect needle, and then placed at four different points on the surface of a potato dextrose agar culture plate (39.0 g PDA EMD Chemical Inc., Gibbstown, NJ; dissolved in liter of distilled water). Fungal symbiont isolates from *Sirex* specimens were initially plated on full strength PDA amended with 100 ppm streptomycin sulfate, and later transferred onto a new PDA plate to obtain a pure subculture. Culture plates were maintained at ambient temperature (≈25°C) in darkness. Pure subcultures were examined after 4–6 wk for identification and comparison for morphological differences. Voucher specimens of *S. nigricornis* have been deposited in the Louisiana State Arthropod Museum (Louisiana State University, Baton Rouge).

DNA Extraction Methods. The Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) was used for fungal DNA extraction according to the manufacturer's protocol. Extracted DNA was stored at −20°C until use. Because the detection turnaround time, cost of DNA extraction per sample, and quality of DNA for PCR amplification are critical factors for routine screening of fungal symbionts, we tested the efficacy of direct polymerase chain reaction (PCR) amplification from spores. We evaluated additional samples of *Amylostereum* isolates ($N = 4$) from the mycangia of four *S. nigriconis* females in a separate experiment, by extracting and suspending spores in 20 μl water in 1.5-ml microcentrifuge tubes stored at room temperature. For each isolate, 1 μl of undiluted spore suspension was used as DNA template.

PCR Amplification of IGS Region. Amplification of extracted DNA from pure cultures ($N = 96$) and direct spores ($N = 4$) from mycangia was performed in 10-μl PCR reactions using an *Eppendorf Mastercycler Pro* PCR machine (Eppendorf AG, Hamburg Germany). Extracted DNA template from each of the 96 isolates was diluted 1:100 in sterile deionized distilled water before use in PCR amplification reactions, while spores suspended in 20 μl water were used without further dilution in direct PCR amplification for the remaining four isolates. A reaction mixture contained 5 μl *Taq*PCR Master Mix (Qiagen), 2 μl of a 5 μM solution of each forward and reverse primer, and 1 μl of diluted DNA template. The basidiomycete specific primers P1 (5′TTGCAGACGACTTGAATGG'3; Hsiau 1996) and 5S-2B (5′CACCGCATCCCGTC TGATGTGCG'3; Slippers et al. 2002) were used to amplify the first nuclear ribosomal intergenic spacer region or the ribosomal DNA repeat (IGS) that lies between the 3′ end of the large subunit rDNA gene and the 5′ end of the 5S gene. The two primers (P1 and 5S-2B) were used for amplification of the IGS region in this study based on results of successful amplifications of *Amylostereum* isolates described by Nielsen et al. (2009).

Species-Specific IGS Primers. Because results from the basidiomycete specific primers were inconclusive in differentiating between *Amylostereum* species, six sets of newly designed species-specific primers amplifying only a small portion of the IGS1 were evaluated, with the goal of ultimately using them in assay for quick identification of species, without the need for sequencing. The primers were designed according to the protocol in Drenth et al. (2006), based on information from published studies (Vasiliauskas et al. 1999; Slippers et al. 2000, 2002, 2003; Tabata et al. 2000; Nielsen et al. 2009; Van der Nest et al. 2009; Bergeron et al. 2011), and sequences obtained using the basidiomycete-specific primers (P1 and the 5S-2B). The two optimal sets of specific primers for identifying *Amylostereum* species in native *Sirex* species were AA1 F (5′TTCAACCTCGGTTGGACTTC'3) and AA1R (5′CAAGCACCCCCTACATTTTG'3) for *A. areolatum*, and AC2 F (5′TGAGGTTAAGCCCTTGT TCG'3) and AC2R (5′CCCCCTTTCATTTTTCC AAT'3) for *A. chailletii*.

A. areolatum and *A. chailletii* species have similar biologies, including symbiotic relationships with *Sirex* species, so the specific primers (AA1 F and AA1R, and AC2 F and AC2R) were evaluated for their ability to discriminate between them. Primers AA1 F and AA1R and AC2 F and AC2R were tested against *A. areolatum* and *A. chailletii* using a subsample of isolates (*s20*, *s22*, *s23*, *s24*, *s25*, *s26*, *s27*, *s28*, *s29*, *s30*, *s31*, and *s32*) initially identified as *Amylostereum* by the IGS region-specific primers (P1/5S-2B). Primers AA1 F and AA1R and AC2 F and AC2R were designed to amplify 193 bp of *A. areolatum* DNA and 204 bp of *A. chailletii* DNA, respectively. The PCR amplification conditions for the two primer sets were designed to be identical for ease of use in simultaneously processing a large number of samples. The PCR conditions consisted of initial denaturation at 95°C for 3 min, followed by 35 cycles of 35 s denaturation at 95°C, 55 s annealing at 60°C, and 1 min extension at 72°C, and a final extension at 72°C for 10 min. Non-*Amylostereum* fungal isolates were identified by PCR amplifications and sequencing of the internal transcribed spacer (ITS) region using same PCR conditions as *Amylostereum* species with ITS1 F (5′CTTGGTCATTTAGAGGAAGTAA'3) and ITS4R (5′TCCTCCGCTTATTGATATGC'3) as forward and reverse PCR primers, respectively.

Electrophoresis was used to examine amplified products by loading 4 μl PCR products mixed with 1 μl Qiagen GelPilot DNA loading Dye 5× on 1% agarose gels. After 30 min of electrophoresis, the agarose was stained with ethidium bromide, and the resulting bands were visualized under ultraviolet illumination. In cases where multiple bands were observed, gel extraction of each band was performed, and the two products were purified separately using the QIAquick PCR purification kit (Qia-

Table 1. General information about representative isolates and GenBank sequences included in the multiple alignment and phylogenetic tree analysis

Name/GenBank accession no.	Host species	Country	State	County/parish	Date collected
s20 (KC858267)[a]	*S. nigricornis*	USA	LA	Grant	24 Oct. 11
s22 (KC858268)[a]	*S. nigricornis*	USA	LA	Grant	24 Oct. 11
s23 (KC858269)[a]	*S. nigricornis*	USA	LA	Grant	24 Oct. 11
s24 (KC858270)[a]	*S. nigricornis*	USA	LA	Grant	24 Oct. 11
s25 (KC858271)[a]	*S. nigricornis*	USA	LA	Grant	24 Oct. 11
s26 (KC858272)[a]	*S. nigricornis*	USA	LA	Grant	24 Oct. 11
s112 (KC858273)[a]	*S. nigricornis*	USA	LA	Grant	1 Nov. 11
s113 (KC858274)[a]	*S. nigricornis*	USA	LA	Grant	1 Nov. 11
s117 (KC858275)[a]	*S. nigricornis*	USA	LA	Grant	8 Nov. 11
s120 (KC858276)[a]	*S. nigricornis*	USA	LA	Grant	8 Nov. 11
GQ422452[b]	*S. noctilio*	USA	NY	Oswego	16 July 07
GQ422453[b]	*S. noctilio*	USA	NY	Oswego	Jan.-06
GQ422454[b]	*S. edwardsii*	USA	NY	Oswego	18 Sept. 07
GQ422455[b]	*S. juvencus* (Linnaeus)	Hungary	—	—	1966–1967
GQ422456[b]	*S. juvencus* (Linnaeus)	Hungary	—	—	1966–1968
GQ422457[b]	*S. juvencus* (Linnaeus)	Hungary	—	—	1966–1969
GQ422458[b]	*S. noctilio*	USA	NY	Fulton	19 Feb. 08
GQ422459[b]	*S. noctilio*	USA	NY	Fulton	19 Feb. 08
GQ422460[b]	*S. sp. 'nitidus'*	USA	ME	Waldo	10 Sept. 07
GQ422461[b]	*S. sp. 'nitidus'*	USA	ME	Waldo	10 Sept. 07
GQ422462[b]	*S. nigricornis*	USA	NY	Oswego	21 Sept. 07
GQ422463[b]	*S. nigricornis*	USA	NY	Onondaga	02 Oct. 07

Sources: [a] Current study.
[b] Nielsen et al. 2009.

gen). Products were sequenced by GENEWIZ Inc. (http://www.genewiz.com).

Phylogenetic Analyses. Phylogenetic analyses was performed on 10 sequences from purified PCR products that include six isolates from PDA culture (*s20*, *s22*, *s23*, *s24*, *s25*, and *s26*) and four isolates from direct spores (*s112*, *s113*, *s117*, and *s120*), which were amplified with basidiomycetes-specific IGS primer pair (P1 and 5S-2B). Overall, a subsample of 10 *Amylostereum* isolates that captured the patterns of bands observed in gel electrophoresis, were sequenced to confirm identities of the corresponding isolates. Sequences from the 10 isolates ranged between 432 and 507 bp. BLAST searches of sequences were performed at GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify individual isolates. Sequences were compared among isolates and with 12 other sequences previously submitted to the GenBank by Nielsen et al. (2009) with phylogenetic analyses.

PCR products from the same six isolates from PDA cultures (*s20*, *s22*, *s23*, *s24*, *s25*, and *s26*) were amplified using the two species-specific IGS primers (AA1 F and AA1R, and AC2 F and AC2R) and sequenced. Primers AA1 F and AA1R amplified a 193 bp sequence of *A. areolatum*, while primers AC2 F and AC2R amplified a 204 bp sequences of *A. chailletii*. BLAST searches of sequences from the two sets of primer pairs (AA1 F and AA1R and AC2 F and AC2R) were performed to confirm isolate identity, and compared with sequences from P1 and 5S-2B. Only sequences from basidiomycetes-specific IGS primers were included in multiple alignments and phylogenetic analyses.

Sequence alignments were constructed in MAFFT (Katoh and Toh 2010), using the online (MAFFT version 6; http://mafft.cbrc.jp/alignment/server/) multiple alignment program for amino acid or nucleotide sequences. Ten subsample sequences from this study and 12 sequences from Nielsen et al. (2009) available from Genbank were included for analyses. Aligned sequences were edited using in *Jalview* (Waterhouse et al. 2009), an online program for multiple sequence alignment editing, visualization, and analysis provided on the same Web site, and edited sequences were saved as a FASTA format file. Maximum likelihood (ML) analyses were conducted with RAxML-HPC2 on XSEDE v7.2.8 (Stamatakis 2006) via the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway portal using default parameters (Miller et al. 2010). The GenBank accession numbers and published sources for the sequences analyzed are provided in Table 1.

Culture Morphology. Differences in morphological characteristic of *Amylostereum* isolates on PDA plates were examined between 4 and 6 wk after spores were extracted from mycangia of native females *S. nigricornis* specimens.

Results

Identification of Isolates. Gel electrophoresis of DNA extracted from fungal isolates and amplified using the IGS primer pair (P1 and 5S-2B) produced different sized bands. Overall, 95 out of 100 fungal isolates obtained from mycangia of 100 native wood-

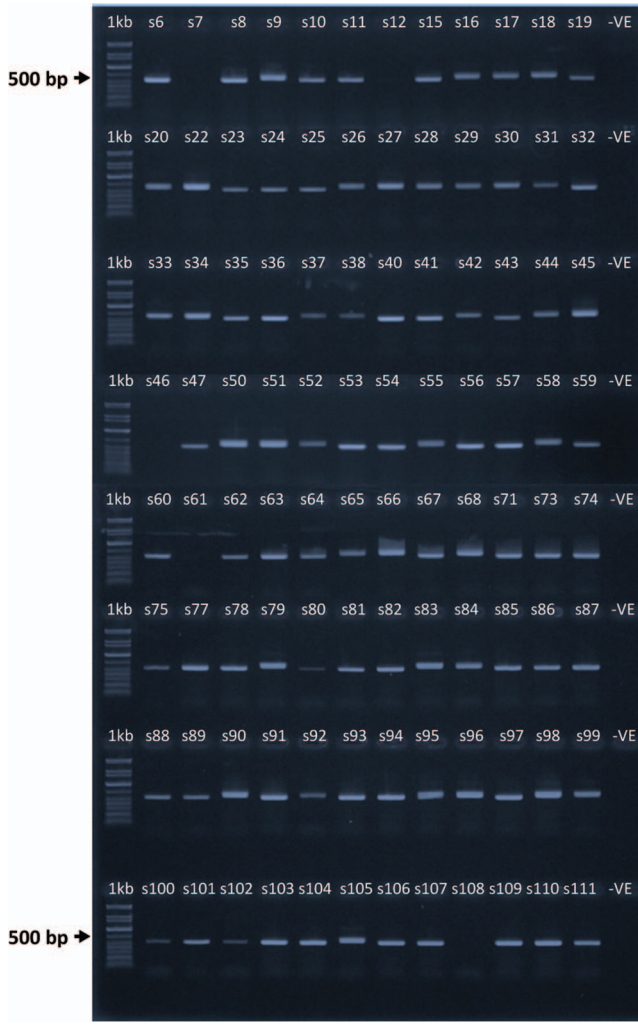

Fig. 1. Amplification of *Amylostereum* isolates from native *S. nigricornis* using the IGS region basidiomycetes-specific primers P1 (Hsiau 1996) and 5S-2B (Slippers et al. 2002). (Figure in color online only.)

wasp *S. nigricornis* in this study were identified as *Amylostereum* species. Contrary to expectations, 60% of these fungal isolates were identified as *A. chailletii* ($N = 60$), while 35% were identified as *A. areolatum* ($N = 35$). The remaining 5% of these fungal isolates ($N = 5$) were identified as *Bipolaris papendorfii* (Aa) Alcorn (*s7*), *Alternaria alternata* (Fr.) Keissl (*s12*), *Penicillium marneffei* Segretain (*s46*), *Scytalidium cuboideum* (Sacc. & Ellis) Sigler & Kang (*s61*), and *Hyphopichia heimii* (Pignal) Kurtzman (*s108*) based on sequencing of the ITS region (Fig. 1).

Amplifications with New Specific IGS Primers. A subsample of 12 isolates (*s20*, *s22*, *s23*, *s24*, *s25*, *s26*, *s27*, *s28*, *s29*, *s30*, *s31*, and *s32*) initially identified as *Amylostereum* with primers P1 and 5S-2B captured the variation in band patterns observed from gel electrophoresis (Fig. 2a). The specific primers AA1 F and AA1R identified eight of the isolates (*s20*, *s22*, *s26*, *s27*, *s28*, *s29*, *s30*, and *s31*) as *A. areolatum*, while primers AC2 F and AC2R identified the remaining four isolates (*s23*, *s24*, *s25*, and *s32*) as *A. chailletii*. Isolates *s20*, *s22*, *s26*, *s27*, *s28*, *s2*, *s30*, and *s31* produced bands that were later resolved to be double bands after gel electrophoresis was run for a longer duration. Amplification from isolates *s23*, *s24*, *s25*, and *s32* each produced a single and slightly smaller band PCR product as indicated by the corresponding mark on the 1-kb ladder and the number of base pairs from the sequence analyses (Fig. 2a). The two primer sets used identical PCR conditions, which enabled a single run of PCR in the thermal cycler. We used this approach to reduce processing time, which is critically important in routine screening of large samples. Direct amplification of *Amylostereum* spores (*s112*, *s113*, *s117*, and *s120*) from mycangia of four *Sirex* specimens, with the basidiomycetes-specific IGS primer pair (P1 and 5S-2B) gave good sequences similar to Qiagen DNeasy extraction kit. The four isolates were identified as *A. chailletii*.

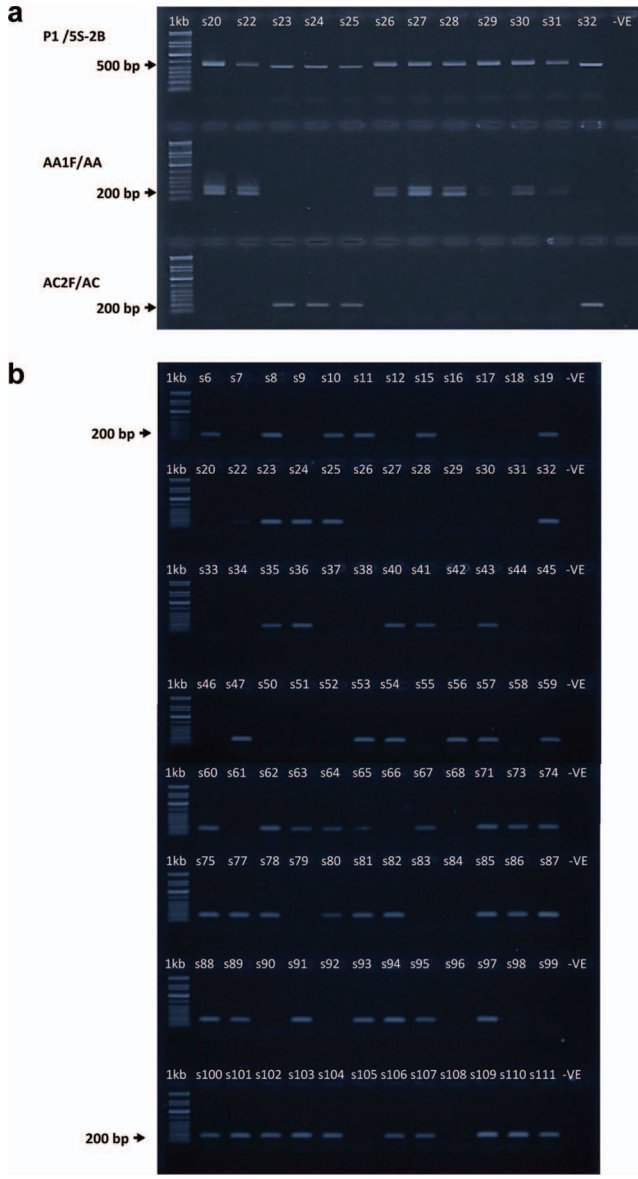

Fig. 2. (a) Amplification of 12 Amylostereum isolates from *S. nigricornis* using the IGS region basidiomycetes-specific primers P1 and 5S-2B, specific primers AA1 F and AA1R for *A. areolatum*, and AC2 F and AC2R for *A. chailletii*. (b) Distinguishing *A. chailletii* from the 96 fungal isolates extracted from the mycangia of native *S. nigricornis* using the IGS region-specific primers AC2 F and AC2R. (Figure in color online only.)

Phylogenetic Analyses. Purified PCR products from six isolates from culture (*s20*, *s22*, *s23*, *s24*, *s25*, and *s26*) and four isolates from direct spores (*s112*, *s113*, *s117*, and *s120*) were sequenced with sizes ranging between 431–507 bp. Sequences were obtained from the IGS locus using the basidiomycete-specific primers P1 and 5S-2B (Hsiau 1996, Slippers et al. 2002, Nielsen et al. 2009). When blasted against the sequence database at GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), three isolates (*s20*, *s22*, and *s26*) shared 99% sequence identity to an accession of *A. areolatum* (GQ422461), whereas three other isolates (*s23*, *s24*, and *s25*) shared 99% identity to accessions of *A. chailletii* (GQ422462 and GQ422463; reported by Nielsen et al. 2009). Phylogenetic analyses of the IGS locus confirmed that both species were present within the sampled population (Fig. 3).

The primers AA1 F and AA1R amplified sequences of 193 bp that were identified as *A. areolatum* in BLASTN analyses; product amplified from isolates *s20*, *s22*, and *s26* with these primers shared 99% sequence identity with *A. areolatum* Scy-ME-09/10 (GenBank GQ422461) from Waldo Co. Maine (Nielsen et al. 2009). The primers AC2 F and AC2R amplified sequences of 204 bp that were identified as *A. chailletii*

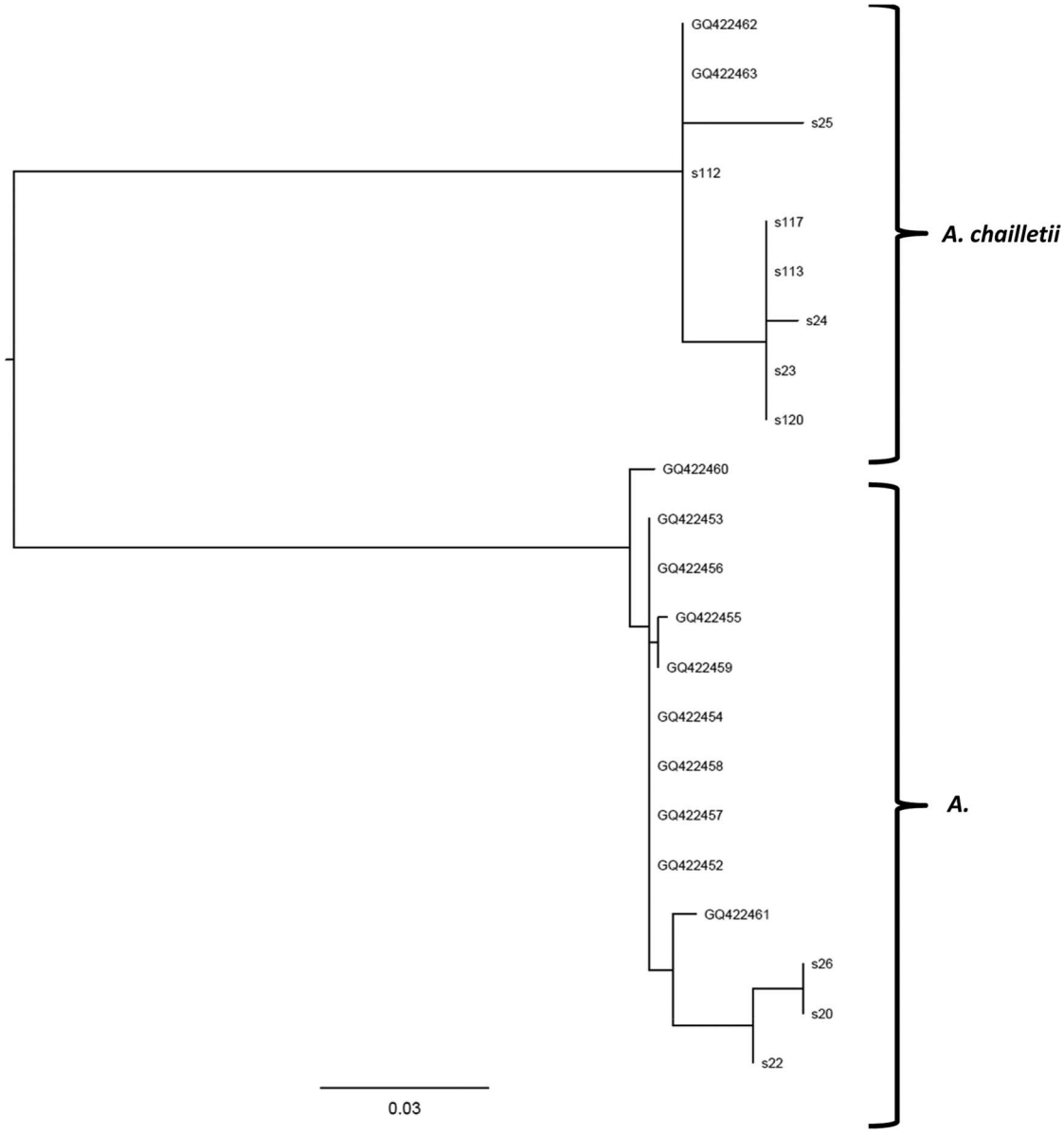

Fig. 3. Phylogenetic tree based on 486 alignable characters of the IGS region of the nuclear rDNA of *Amylostereum* species, using sequences of 10 isolates from this study and 12 sequences from Nielsen et al. (2009) in the GenBank. Sequence alignments were constructed in MAFFT (Katoh and Toh 2010) and ML analyses were conducted in with RAxML-HPC2 on XSEDE v7.2.8 (Stamatakis 2006) via the CIPRES Science Gateway portal using default parameters (Miller et al. 2010).

in BLASTN analyses; product amplified from isolates *s23*, *s24*, and *s25* with these primers shared 100% sequence identity with *A. chailletii* Sni-DF-09/21–2 and DWAch2 28S (GenBank GQ422462 and GQ422463, respectively) from Oswego and Onondaga Co. New York (Nielsen et al. 2009). Comparison of the aligned sequences based on basidiomycete-specific IGS primers, P1 and 5S-2B, highlights the differences in the amplified IGS region (161–218) and (356–396) of *A. areolatum* and *A. chailletii* (Fig. 4).

Culture Morphology. Morphological variability was observed in the appearance of *Amylostereum* isolates examined in this study with varying levels of diffused pigments (changes in agar color) on PDA plate over several weeks. We noticed variability in color and mycelia growth as seen between dense-white mycelia in isolate *s24* (*A. chailletii*) and the dark pigmentation in isolates *s26* (*A. areolatum;* Fig. 5). Previous studies (Thomsen and Harding 2011, Wermelinger and Thomsen 2012) have similarly reported differences in color and growth rate between the two *Amylostereum* species on artificial media. Thomsen (1998) noted that the basidiospores from the fruiting body are smaller in *A. areolatum* than those of *A. chailletii*, and the dif-

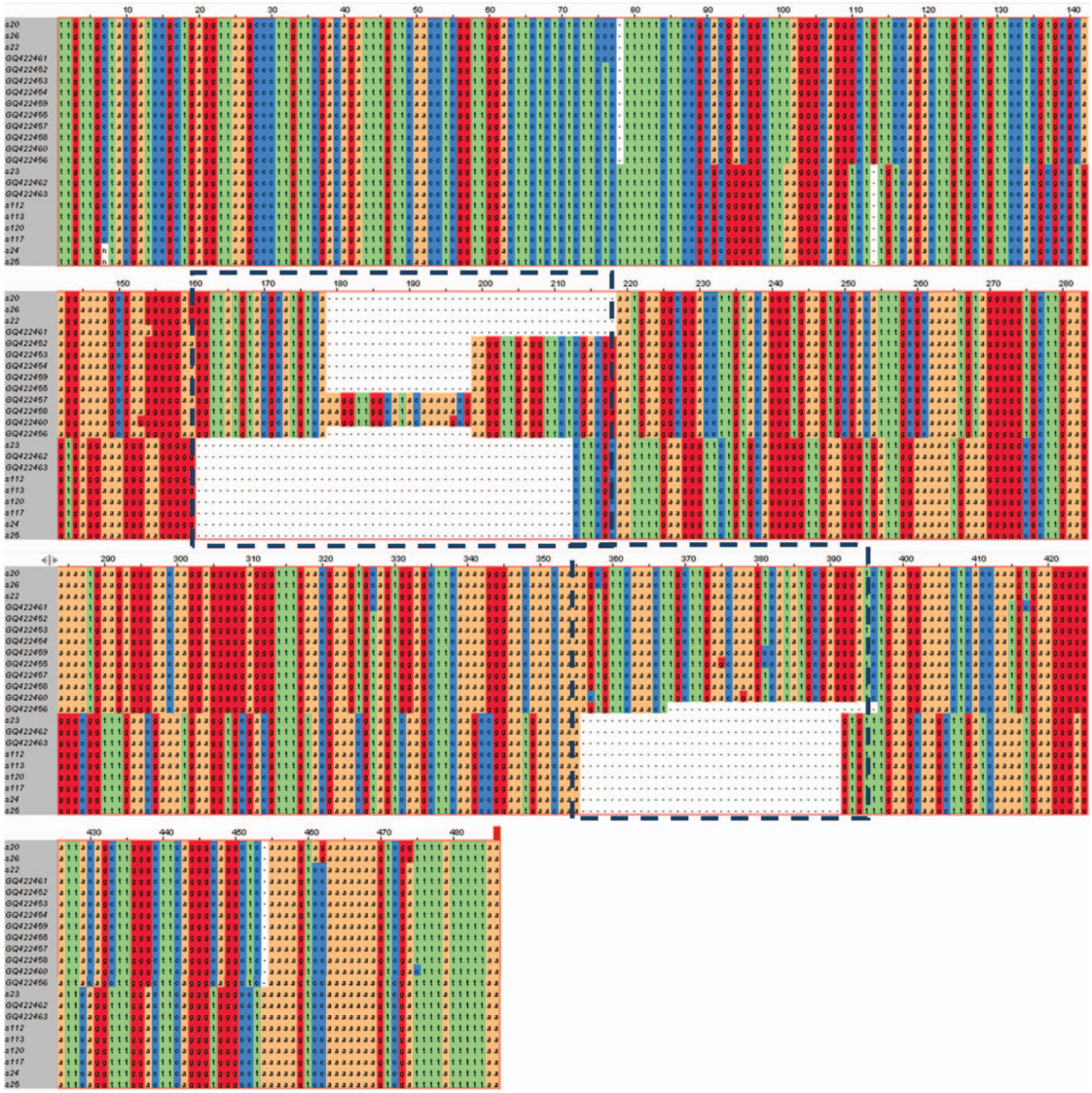

Fig. 4. Multiple alignment of nucleotide sequences obtained from 10 isolates included in this study and 12 related sequences from Nielsen et al. (2009). Comparison of the aligned sequences highlights differences in the amplified IGS region of *A. areolatum* and *A. chailletii.* (Figure in color online only.)

ferences are obvious when the basidiospores are viewed through a microscope.

Discussion

This study confirms the presence of *A. areolatum* in the mycangia of the native woodwasp *S. nigricornis* collected in central Louisiana. Sixty percent of fungal isolates obtained from *S. nigricornis* in this study were identified as *A. chailletii;* 35% were identified as *A. areolatum*, while the remaining 5% were identified as *B. papendorfii*, *A. alternata*, *P. marneffei*, *S. cuboideum*, and *H. heimii* based on sequencing of the ITS region (Figs. 1 and 2b). The five non-*Amylostereum* isolates were likely contaminants during mycangia-spore extraction process.

At one time *A. chailletii* was thought to be the only fungal isolate of North American *Sirex* spp. (Bedding and Akhurst 1978); however, *A. areolatum* was recently isolated from the native North American woodwasps *S. nitidus* and *S. nigricornis* (Nielsen et al. 2009). Although the presence of *A. areolatum* in *S. nigricornis* may have been the result of horizontal transfer from *S. noctilio* coinfesting host material, this is unlikely to be the case for *S. nitidus* sampled in this study, which were all collected from outside the known range of *S. noctilio.* The isolation of *A. areolatum* from *S. nigricornis* collected in central Louisiana (i.e., well outside the known distribution of *S. noctilio*) is consistent with Nielsen et al. (2009) and demonstrates that fungal associations involving

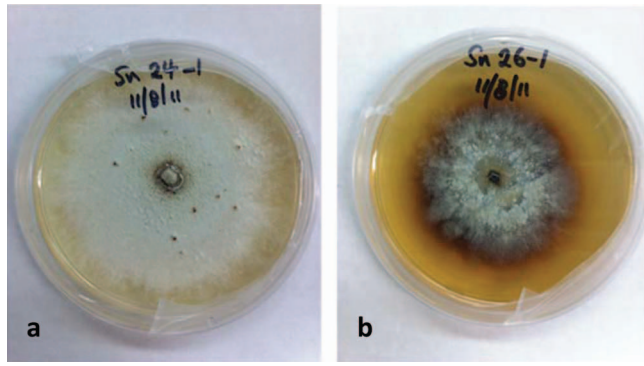

Fig. 5. Cultures of *Amylostereum* isolates; (a) *A. areolatum* and (b). *A. chailletii* on PDA plates 4–6 wk after spores were extracted from mycangia of native female *S. nigricornis* samples collected in central Louisiana. (Figure in color online only.)

native *Sirex* spp. in North America are more complex than previously believed.

The nematode *D. siricidicola* has a bicyclic life cycle, alternating between a mycetophagous and parasitic cycle (Bedding 1967, Slippers et al. 2012). Although nematodes from this genus have low specificity during the parasitic cycle, they have high specificity during the mycetophagous cycle (Bedding and Akhurst 1978, Williams et al. 2012). *D. siricidicola* has been released as a biocontrol agent in all southern hemisphere countries where *S. noctilio* has become established and a pest of pines. Although high levels of parasitism have been observed in some cases, the performance of the nematode has been low in others. For example, although parasitism levels between 70–100% have been observed with the Kamona strain, releases of the same strain in some regions of South Africa have resulted in <10% parasitism (Hurley et al. 2007, 2012). Slippers et al. (2001) reported differences in the strain of *A. areolatum* present in South Africa and the strain from Australia used to culture the nematode. Incompatibility between the nematode and fungal strain could result in low nematode performance during the mycetophagous portion of the life cycle and ultimately low levels of parasitism. Hurley et al. (2007) hypothesized that the fungal strain associated with *S. noctilio* could influence the success of *D. siricidicola* and that the low levels of parasitism observed in some regions of South Africa might be because of fungal–nematode incompatability. Although Hurley et al. (2012) did not observe support for the incompatibility hypothesis, they did observe evidence that the fitness of specific nematode strains was not independent of the *A. areolatum* strain they develop on. Similarly, Morris et al. (2012) demonstrated that the relative fitness of *D. siricidicola* varied significantly among *A. areolatum* strains and was significantly higher on the strain isolated from the native *S. nitidus* (ScyME hereafter) than any of the other strains of *A. areolatum*, including the two strains isolated from *S. noctilio.* The *A. areolatum* isolates identified in this study are similar to the ScyME strains (Fig. 3). The biocontrol agent *D. siricidicola* has not been released in North America; however, a strain of *D. siricidicola* has been found in North America (Yu et al. 2009). The presence of the nematode in North America appears similar to the situation in New Zealand where the nematode was not intentionally introduced and likely originally entered with *S. noctilio* (Zondag 1969). In North America, parasitism of *S. noctilio* by the strain of *D. siricidicola* present does not result in sterilization of female *S. noctilio* (Yu et al. 2009).

Evidence is accumulating that in North America associations between *Sirex* spp. and their fungal symbionts are complex. Boissin et al. (2012) developed a molecular data set and used it to test invasion scenarios for the worldwide spread of *S. noctilio.* Their analyses revealed a much more complex invasion scenario than previously believed with populations on most continents likely the result of introductions from several sources. North American *S. noctilio* appeared to be a mixture of introductions from South America and Europe. Similar results were reported by Bergeron et al. (2011) for the *S. noctilio* fungal symbiont *A. areolatum.* In North America associations between native *Sirex* spp. and *A. areolatum* may limit the potential for the use of *D. siricidicola* as a management tactic because 1) exposure of woodwasps to *D. siricidicola* is determined by the identity of their fungal symbiont (i.e., there may be nontarget effects of *D. siricidicola* on native woodwasps associated with *A. areolatum*); and 2) the impact of *D. siricidicola* has been observed to vary both among populations of *S. noctilio* (Hurley et al. 2012) and strains of the nematode (Williams et al. 2012). As a result a priori it is difficult to predict what impact commercial releases of *D. siricidicola* will have on native North American *Sirex* spp.

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