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Fidelity Among *Sirex* Woodwasps and Their Fungal Symbionts

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Abstract We report that associations between mutualistic fungi and their economically and ecologically important woodwasp hosts are not always specific as was previously assumed. Woodwasps in the genus *Sirex* engage in obligate nutritional ectosymbioses with two species of *Amylostereum*, a homobasid\iomycete genus of white rot fungi. In the present study, the *Amylostereum* species and genotypes associated with three species of *Sirex* native to eastern North America and one relatively recent invasive *Sirex* from Europe were investigated by comparing intergenic spacer regions (IGS). *Sirex* spp. were sampled over 6 years from 23 sites in six US states, ranging from Maine in the northeast to Louisiana in the southeast, to obtain samples of *Amylostereum* from mycangia of adult females. Two of the native *Sirex* species (*Sirex nigricornis* and *Sirex nitidus*) were associated with either *Amylostereum chailletii* or *Amylostereum areolatum*, refuting the hypothesis of strict species-specific relationships. However, the invasive *Sirex noctilio* and the native *Sirex cyaneus* were each collected with only *A. areolatum* or *A. chailletii*, respectively, although *S. noctilio* was associated with two different IGS genotypes of *A. areolatum* and *S. cyaneus* occurs sympatrically with the other native *Sirex*. In *Pinus*, the preferred host tree of *S. nigricornis* and *S. noctilio*, these species co-occurred in 25.9 % of trees sampled, and horizontal transmission of fungal strains from *S. noctilio* to *S. nigricornis* was documented, although only in one tree. The extent that further spread and establishment of *S. noctilio* will alter the composition of symbionts carried by native *Sirex* is unknown but will depend in part on the degree of flexibility in these host–symbiont associations.

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Introduction

Insects using wood partner with symbionts in order to gain nutrients from a recalcitrant food source. These associations with symbionts have been thought to range from species-specific obligate associations [40] to partnerships with a variable community of microbes [25]. For wood-inhabiting arthropods, specific associations with symbiotic microbes have often been assumed. However, more recent studies have shown that many of these associations are instead flexible to some extent [1, 14, 15] and that symbiont shifts have occurred due to horizontal transmission. Exposure to new symbionts, such as when exotic wood-boring insects are introduced, may increase the likelihood of symbiont shifts.

Woodwasps in the genus *Sirex* (Hymenoptera: Siricidae) engage in obligate nutritional ectosymbioses with white rot fungi in the genus *Amylostereum* (Russulales: Amylostereaceae) [40]. *Amylostereum* benefit from dispersal by the woodwasps; these fungi rarely make basidiocarps, reducing the likelihood that they are dispersed by the wind [35, 45]. Developing *Sirex* larvae bore within the wood of conifers and gain nutritive benefits either from directly eating the fungus [20] or from fungal enzymes that aid in the digestion of xylem by larval *Sirex* [18]. This mutualistic relationship is obligate for the hosts as presence of the fungus is required throughout the development of siricid larvae [28]. Vertical transmission of symbionts from mother to offspring

has been assumed as the only form of transmission [43] as female woodwasps have a pair of specialized intersegmental organs (mycangia) at the base of the ovipositor in which they transport asexual arthrospores of *Amylostereum* to insert within trees when eggs are laid or during exploratory drilling [7]. With only vertical transmission, specificity of relations between *Sirex* and *Amylostereum* has been assumed [11, 12, 40].

Sirex woodwasps are inhabitants of naturally occurring or urban coniferous forests as well as plantations throughout the Northern Hemisphere [32], typically specializing on a single genus of host tree, although alternate host tree genera may be used [36]. *Sirex* species rarely cause widespread tree mortality in their native ranges, and most species only attack the transient resource of weakened or recently dead trees [9, 36]. However, in the Southern Hemisphere, the introduced European native *Sirex noctilio*, in association with *Amylostereum areolatum*, has caused serious damage to agroforestry of introduced pines (*Pinus* spp.), killing large numbers of overcrowded trees as well as healthy trees when *S. noctilio* is present at high densities [2]. During oviposition, a phytotoxic secretion is injected into the tree by *S. noctilio* females, and the secretion acts along with the fungus to impair water relations and translocation within the tree, which usually eventually results in tree death [28]. *S. noctilio* is thought to be more aggressive than other *Sirex* species, which is supported by comparisons of European *Sirex* species that show *S. noctilio* producing the greatest amount of phytotoxic secretion [38] and having the highest oviposition densities among the species examined [37].

Understanding the fungal associations of North American *Sirex* has taken on pressing significance with the discovery in 2005 of the introduction and establishment of *S. noctilio* in northeastern North America [8, 16]. *A. areolatum* was subsequently reported in association with *S. noctilio* in Ontario [4] and New York state [47]. *Amylostereum chailletii* had previously been the only fungus believed to be associated with all North American *Sirex* woodwasps [11, 12, 40], and the presence of *A. areolatum* associated with any woodwasps in North America had not been reported before *S. noctilio* arrived [12]. In 2009, a study principally investigating the genotypes of *A. areolatum* carried by *S. noctilio* in North America reported a novel genotype of *A. areolatum* carried by two native North American *Sirex nitidus* females. This demonstrated for the first time that North American *Sirex* could be associated with *A. areolatum* instead of *A. chailletii* [24]. We therefore conducted a study specifically focusing on the associations between *Sirex* species and *Amylostereum* species and strains and included more samples from native *Sirex* populations to better understand the fidelity of *Sirex–Amylostereum* associations. We present results describing the symbiont specificity of the three native and one invasive *Sirex* species in eastern North America.

Methods

Collection of *Amylostereum* from *Sirex*

Samples of the three species of *Sirex* native to the USA east of the Rocky Mountains were collected from 2007 to 2012 from a total of 23 sites in Georgia, Louisiana, Maine, New York, West Virginia, and Pennsylvania. In tandem, *S. noctilio* co-occurring in some of the sampling sites in New York were collected. *S. noctilio* has not yet expanded its distribution to Georgia, Louisiana, Maine, or West Virginia and was therefore not sampled from these states [23]. Collection data for the 194 *Sirex* females that were sources for the *Amylostereum* species and genotypes used in the present study are presented in Table 1.

Living *Sirex* were collected in three different ways: (1) with aerial nets near stacks of freshly cut pine trees (6.2 % of *Sirex* specimens), (2) from panel traps with 70 % α-pinene and 30 % β-pinene lures (Aptiv, Portland, OR, USA) and ethanol lures placed at *Sirex*-infested sites (19.1 %), or (3) when emerging from *Sirex*-infested red or scots pine (*Pinus resinosa* and *Pinus sylvestris*, respectively), balsam fir (*Abies balsamea*), or Norway spruce (*Picea abies*) (74.7 %); different collection methods were not used equally for different *Sirex* species. For the latter method, trees were felled in the spring or early summer, and portions of trees infested by *Sirex* were placed in screened barrels (69–79 cm height × 24–48 cm diameter) under ambient conditions. Barrels were checked regularly for emergence from early July–mid-December. Native woodwasp species collected included *Sirex cyaneus*, which prefers fir (*Abies* spp.), *S. nitidus,* which prefers spruce (*Picea* spp.), and *S. nigricornis* [= *Sirex edwardsii*; 13], which prefers pine (*Pinus* spp.). These species can be sympatric in northeastern North America, although distributions are determined in part by distributions of host trees [32]. *S. noctilio* prefers pine and sometimes co-infests trees with *S. nigricornis*, although emergence of the relatively short-lived adult females of these two species is often separated by at least a month in northeastern North America (KJ Dodds, personal communication). Most specimens came from rearings, and all *Sirex* reared from wood emerged from the genus of tree preferred by that *Sirex* species. *Sirex* species were identified using the key in Schiff et al. [31], with adjustments and confirmations by Dr. H. Goulet, Canadian National Collection of Insects, and Dr. E.R. Hoebeke, Cornell University.

Fungal symbiont samples could be collected only from adult female *Sirex*. Living female woodwasps were killed by exposure to ethyl acetate, cadavers were swabbed with 70 % ethanol and dissected, and mycangia were removed with a microcurette [41]. To establish fungal cultures, the contents of one mycangium were transferred to a Petri dish containing potato dextrose agar (PDA) amended with

Table 1 Locations and years for collection of *Sirex*-infested wood from which *Sirex* were reared, collection of *Sirex* in traps, or collection of *Sirex* with nets

Sirex species	State	County/parish	Year	Number of specimens	*S. noctilio* in the area[a]
Sirex cyaneus	New York	Essex	2008	2	No
Sirex cyaneus	New York	Essex	2009	9	No
Sirex cyaneus	West Virginia	Tucker	2009	1	No
Sirex nigricornis	Georgia	Burke	2008	3	No
Sirex nigricornis	Louisiana	Grant	2008	18	No
Sirex nigricornis	Louisiana	Grant	2009	16	No
Sirex nigricornis	Louisiana	Grant	2010	53	No
Sirex nigricornis	Louisiana	Rapides	2010	6	No
Sirex nigricornis	Maine	Penobscot	2011	2	No
Sirex nigricornis	Maine	Penobscot	2012	2	No
Sirex nigricornis	New York	Onondaga	2007	1	Yes[b]
Sirex nigricornis	New York	Oswego	2007	4	Yes[b]
Sirex nigricornis	New York	Oswego	2009	1	Yes[b]
Sirex nigricornis	New York	Seneca	2007	1	Yes[b]
Sirex nigricornis	New York	Tompkins	2011	3	Yes[b]
Sirex nigricornis	New York	Warren	2008	1	Yes[b]
Sirex nigricornis	New York	Warren	2010	22	Yes[b]
Sirex nigricornis	Pennsylvania	Greene	2008	6	No
Sirex nigricornis	Pennsylvania	Tioga	2011	1	Yes[b]
Sirex nitidus	Maine	Penobscot	2011	2	No
Sirex nitidus	Maine	Waldo	2008	1	No
Sirex nitidus	Maine	Waldo	2012	3	No
Sirex nitidus	New York	Essex	2008	8	No
Sirex nitidus	New York	St. Lawrence	2008	1	Yes[b]
Sirex noctilio	New York	Oswego	2007	2	Yes
Sirex noctilio	New York	Warren	2010	25	Yes

Sirex specimens listed were all females used to obtain *Amylostereum* from mycangia

[a] Over the study period, the distribution of *S. noctilio* changed, increasing from four to seven states [19]. This column indicates whether *S. noctilio* occurred in that county at the time the sample was collected

[b] Detected by federal or state surveys [23] or from Hajek laboratory collections

antibiotics (300 mg/L SO_4–streptomycin). Cultures were grown in constant dark at 22±1 °C. The second mycangia were placed in individual tubes containing buffer (UltraClean Soil DNA Isolation Kit; MO BIO Laboratories Inc., Carlsbad, CA, USA) and stored at −20 °C until further processing.

DNA Extraction, Amplification, and Genotyping

Fungal DNA was extracted either from cultures or from mycangia using either an UltraClean Soil DNA Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) or a GeneCleanIII Kit (MP Biomedical, Salon, OH, USA) following the manufacturer's instructions. The intergenic spacer region (IGS) between the nuclear ribosomal large subunit and 5S DNA genes was PCR-amplified with the primers P1 and 5S-2B following the protocol from Nielsen et al. [24]. Products were visualized with ethidium bromide on 1–1.5 % agarose gels [24, 35]. *Amylostereum* samples were identified to species level based on the lengths of IGS amplicons. All isolates identified as *A. areolatum* were verified by direct sequencing with Applied Biosystems 3730xl DNA Analyzer (Foster City, CA, USA) at the Core Laboratories Center (CLC), Cornell University, and most isolates identified as *A. chailletii* were also verified by sequencing.

The nuc–IGS–rDNA region may be present in *A. areolatum* as multiple different copies. Heterogeneity can arise either from different nuclei having different IGS sequences within a genotype or from different ribosomal repeats within individual nuclei. Heterologous *A. areolatum* isolates were evidenced by noisy sequencing trace files. These isolates were investigated further either by cloning,

as described by Nielsen et al. [24], or with fragment analysis, which exploits size differences between copies based on indels. To generate IGS amplicons within the size range suitable for fragment analysis with an Applied Biosystems 3730xl DNA Analyzer, an internal primer that was able to amplify fragments under 500 base pairs in length was designed. By generating an alignment using the sequences found in Nielsen et al. [24], we identified a region showing 100 % site conservation. The primer IGS-intF (5′-GTTTCTTAGGGCTGTTCCAGACTTGTG-3′) was designed from this region using the program Primer3 [27]. This includes a seven-base-pair "pigtail" (GTTTCTT) added to the 5′ end to limit the addition of terminal non-template nucleotides [6]. The primer 5S-2B was labeled with a FAM fluorescent marker. The accuracy of this procedure was validated by sizing amplicons generated with the primers IGS-intF and 5S-2B from North American isolates whose IGS genotypes were determined previously by cloning. PCR reactions for fragment analysis were run under the following conditions: one cycle at 94 °C for 4 min, 35 cycles of 94 °C for 50 s, 55 °C for 45 s, 72 °C for 45 s, a final extension at 72 °C for 10 min, and then holding at 4 °C until gel visualization. Samples were mixed with formamide and LIZ500 size standard and then electrophoresed with the Applied Biosystems 3730xl DNA Analyzer at CLC, and fragment sizes were determined with PeakScanner v1.0 (Applied Biosystems Inc.) (Fig. 1).

Vegetative Compatibility Group Analysis

Vegetative compatibility group (VCG) analysis was conducted to investigate compatibility among *A. areolatum* IGS-BE cultures from Louisiana, Maine, and New York (Table 2). Thus, some of the isolates originated>2,000 km from each other. As a control, each of these isolates was also tested against *A. areolatum* IGS-BD from *S. noctilio* in New York. Isolates of *A. areolatum* maintained on PDA were tested in pairs and assigned to different VCG groupings using the procedure of Thomsen and Koch [42] and Nielsen et al. [24]. Inoculations were made with approximately 0.7×0.7-cm square plugs cut from the edges of actively growing cultures and placed approximately 2 cm apart in the center of a 6-cm-diameter Petri dish containing PDA. All plates were incubated at 23±2 °C in constant darkness for 2–4 weeks. Isolates were regarded as incompatible when a brown demarcation zone without fungal growth occurred between the isolates and compatible when hyphae intermingled freely between isolates.

Co-occurrence of S. *noctilio* and *S. nigricornis* in Pines

To explore the frequency with which *S. noctilio* and *S. nigricornis* emerged from the same pine trees, pines from which all *Sirex* that emerged within barrels (both males and females) had been counted were included. *Sirex* rearings from 58 pine trees from New York and Pennsylvania were included in this analysis (Table 3). In 2007 and 2010, all *Sirex* were reared from adjacent sections of trees in the same barrel, and for nine of these trees both *S. nigricornis* and *S. noctilio* emerged, allowing quantification of co-occurrence within individual sections of trees.

Results

Amylostereum Genotyping

The nuc–IGS–rDNA regions were successfully amplified for all isolates included in this study, both for in vitro isolates as well as when fungal DNA was extracted directly from *Sirex* mycangia. Samples originating from the same *Sirex* individual always resulted in identical sequence results independently of whether DNA was isolated from cultures or mycangia. Fragment sizes were homogeneous and species specific for *A. chailletii* (552 bp). In contrast, IGS was in some cases present as multiple divergent copies in *A. areolatum*, possibly arising from balanced heterokaryosis in the fungal thallus. IGS copies differed by SNPs and indels. Fragment analysis successfully distinguished between amplicons belonging to the different IGS groups described in Slippers et al. [35] and Nielsen et al. [24] (Fig. 1) and can be more time efficient than cloning PCR products. The IGS A-type was estimated at 476 bp, the B-type at 492 bp, the C-type at 424 bp, the D-type at 472 bp, the E-type at 452 bp, and the F-type at 443 bp. In the present study, only the B, D, and E types were documented in *A. areolatum* from North America, with sequences being identical to the sequences reported previously by Slippers et al. [35] and Nielsen et al. [24].

Sirex–Amylostereum Associations

Only one *Amylostereum* species or genotype was present in the individual mycangium of each *Sirex* female that was sampled. Overall, three *A. areolatum* genotypes, hereafter referred to as IGS-BE, IGS-D, and IGS-BD, and one *A. chailletii* genotype were documented based on IGS amplicons/sequences. Females of both the *Pinus*-preferring *S. nigricornis* and the *Picea*-preferring *S. nitidus* carried either *A. areolatum* or *A. chailletii* (Table 4). For females of *S. nigricornis*, *A. chailletii* was more commonly carried than *A. areolatum* (Fisher's exact test; $P<0.0001$). However, the percentage of *S. nigricornis* carrying *A. areolatum* was greater among samples from Pennsylvania and sites further north (47.6 %) compared with samples from the southern sites in Georgia and Louisiana (13.5 %) (Fisher's exact test; $P<0.0001$) (Fig. 2). For *S. nitidus*, there was no significant difference in the number of females

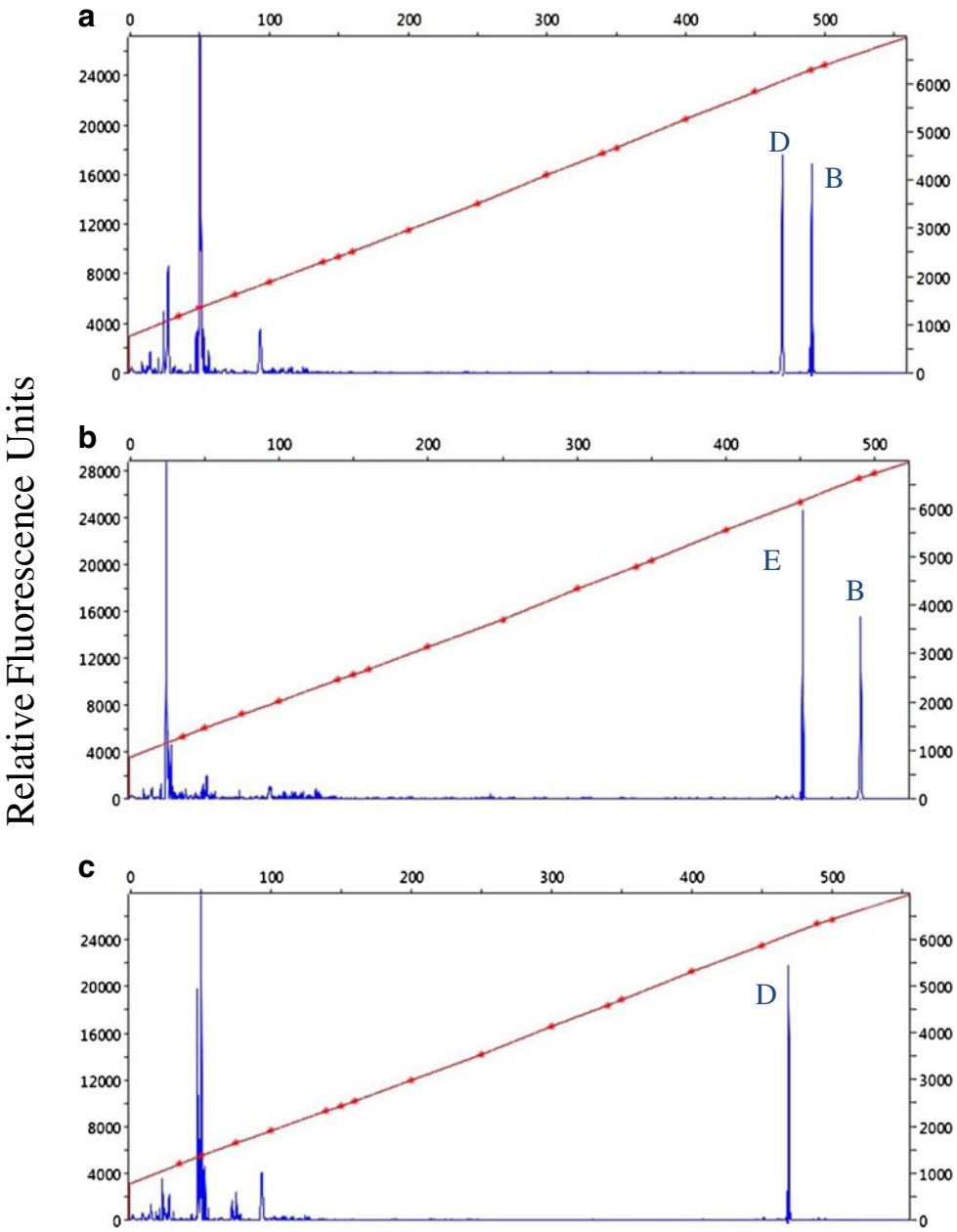

Fig. 1 Fluorescence profile for three IGS genotypes of *A. areolatum*. The peaks on the *far right* are IGS fragments. **a** Peaks for B and D, **b** peaks for B and E, **c** peak for D alone

carrying *A. areolatum* versus *A. chailletii* (Fisher's exact test; $P=0.2429$). Although the number of samples of *S. nitidus* females was low ($n=15$), individuals carrying either *A. areolatum* or *A. chailletii* were found both in New York and Maine states. All individuals of *S. cyaneus* carried *A. chailletii*, and *S. noctilio* always carried *A. areolatum*.

For *Sirex* that were carrying *A. areolatum* (i.e., all *S. noctilio* and some *S. nigricornis* and *S. nitidus*), either one or two IGS

Table 2 *A. areolatum* isolates used for vegetative compatibility tests

Sirex host	Isolate (SAC #)[a]	IGS	Collection location	County/parish	State	Collection method	Date collected
Sirex nigricornis	132	BE	Warrensburg	Warren	New York	Trap	19 Sept. 2008
Sirex nigricornis	146	BE	Kisatchie Natl. For.	Grant	Louisiana	Insect net	20 Nov. 2008
Sirex nitidus	81	BE	Winterport	Waldo	Maine	Trap	10 Sept. 2007
Sirex noctilio	101	BD	Granby	Fulton	New York	Reared from *Pinus sylvestris*	27 Dec. 2007

[a] *Sirex*/*Amylostereum* culture collection (SAC) maintained by the Hajek laboratory at Cornell University

Table 3 Pines from which all *S. noctilio* and/or *S. nigricornis* that emerged were counted to evaluate the extent that these *Sirex* species develop within the same trees and the same sections of the same trees

Year when trees were harvested	State	Location	County	Tree species	Number of trees
2007	NY	New Haven	Oswego	*Pinus sylvestris*	4
2007	NY	Pompey	Onondaga	*Pinus sylvestris*	2
2008	NY	Fingerlakes Natl. Forest	Schuyler	*Pinus resinosa*	4
2010	NY	Cameron State Forest	Steuben	*Pinus resinosa*	5
2010	NY	Heiberg Forest	Cortland	*Pinus resinosa*	4
2010	NY	Pack Forest	Warren	*Pinus resinosa*	8
2010	NY	Chaffee Road	Tompkins	*Pinus resinosa*	2
2011	NY	Pompey	Onondaga	*Pinus sylvestris*	3
2011	NY	Heiberg Forest	Cortland	*Pinus resinosa*	2
2011	NY	Arnot Forest	Tompkins	*Pinus sylvestris*	1
2011	NY	Waterburg Road	Tompkins	*Pinus sylvestris*	3
2011	PA	Government Road	Tioga	*Pinus resinosa*	10
2011	PA	Mountain Ridge Road	Tioga	*Pinus resinosa*	5
2011[a]	NY	Unknown location	Onondaga or Oswego	*Pinus sylvestris*	1
2011[a]	NY	Unknown location	Onondaga or Oswego	*Pinus sylvestris* or *Pinus resinosa*	3
2011	NY	Triangle	Broome	*Pinus resinosa*	1

[a] Infested wood was obtained from USDA APHIS. Detailed records of collections were not available

amplicons were identified from each female. Whether within the distribution of *S. noctilio* or to the northeast and south, all *S. nitidus* and the vast majority of *S. nigricornis* with *A. areolatum* carried the IGS-BE genotype of *A. areolatum* (Fig. 2). This IGS type has never been documented outside the USA. The *A. areolatum* IGS-BE genotype was never found in *S. noctilio* females. *S. noctilio* females carried *A. areolatum* of the IGS-D or IGS-BD genotypes, with D being more abundant (Fisher's exact test; $P<0.0001$) as it was carried by 92.6 % of females.

VCG Compatibility

The three *A. areolatum* IGS-BE genotypes isolated from native *Sirex* from different geographic locations were all incompatible with the *A. areolatum* IGS-BD genotype. However, the three *A. areolatum* IGS-BE from Louisiana, New York, and Maine were all vegetatively compatible with each other.

Co-occurrence of *S. noctilio* and *S. nigricornis* in Pines

Among the *Sirex* emerging from 58 pine trees, *S. noctilio and S. nigricornis* co-occurred in 25.9 % of the trees. For the nine trees where co-occurrence within sections of trees could be determined, 98.0 % of *S. nigricornis* emerged from sections of trees where *S. noctilio* emerged ($n=100$ total specimens) and 50.1 % of *S. noctilio* emerged from sections of trees where *S. nigricornis* emerged ($n=791$ total).

For four of the pines within which both *S. noctilio* and *S. nigricornis* females had developed, *Amylostereum* carried by adult females was determined. For three of these trees, *S. nigricornis* carried either IGS-BE *A. areolatum* or *A. chailletii*. However, among ten *S. nigricornis* females emerging from one *P. sylvestris* tree in New York, nine females carried *A. chailletii* and one female carried IGS-D. *S. noctilio* from the same sections of co-infested tree also carried *A. areolatum* IGS-D.

Table 4 Species and IGS strains of *Amylostereum* associated with *Sirex* species in eastern North America, 2007–2012

Sirex host	Samples, *n*	*A. chailletii*	*A. areolatum*		
			IGS-BE	IGS-D	IGS-BD
Sirex nigricornis[a]	138	105	32	1	0
Sirex cyaneus	12	12	0	0	0
Sirex nitidus	15	9	6	0	0
Sirex noctilio	27	0	0	25	2

[a]IGS genotypes could not be determined for two additional fungal samples carried by *S. nigricornis* that were known to be *A. areolatum*

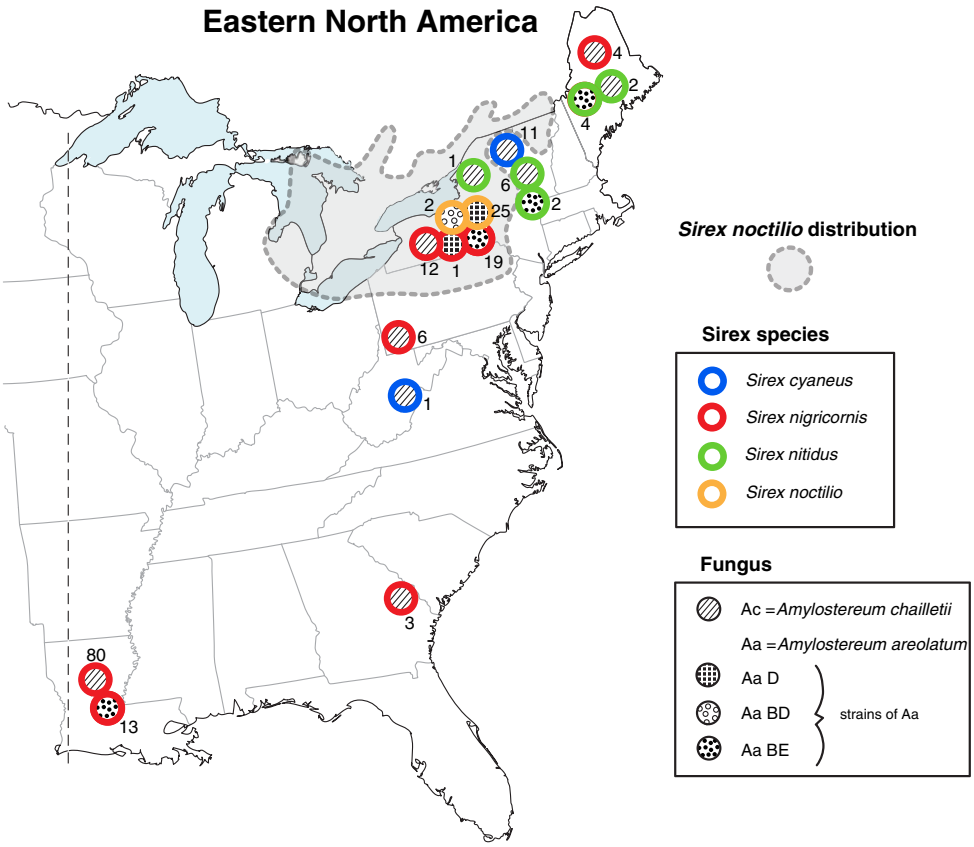

Fig. 2 Distribution of associations of *Sirex* species with *Amylostereum* species and strains in eastern North America. *Circles* designating the same *Sirex* species that are in contact indicate fungal samples from the same collection region. Distribution of *S. noctilio* from [23] and the Hajek laboratory (illustration by Frances Fawcett)

Discussion

Results from this study demonstrated that one *Sirex* species can be associated with more than one species or genotype of fungal symbiont. It was previously assumed that each *Sirex* species was associated with only one species of fungal symbiont, and in North American *Sirex* spp., this fungal species was always *A. chailletii* [11, 12, 40]. However, two of the three native *Sirex* species were found to be associated with either *A. areolatum* or *A. chailletii* (Fig. 3). In addition, associations of North American native *Sirex* with *A. areolatum* extended throughout the geographical range of sampled specimens, with many collections far from known *S. noctilio* infestations. In all cases, the two native *Sirex* species associated with *A. areolatum* carried what we believe is a North American indigenous genotype of *A. areolatum*, the IGS-BE genotype, always when outside the range of *S. noctilio* and even when within the *S. noctilio* range. Our results confirm the data of Nielsen et al. [24] which suggested that North American *S. nitidus* is sometimes associated with the IGS-BE genotype of *A. areolatum*. These findings also affirm Francke–Grosmann's predictions [10] that *Sirex* species could be associated with more than one species of fungal symbiont, although each *Sirex* species was more commonly associated with one particular fungal species. This prediction had been rejected by other authors stating that only strict species-specific associations occurred between *Sirex* and their fungal symbionts [e.g., 11, 12, 40].

Our data thus demonstrate that the associations between *S. nigricornis* or *S. nitidus* and *Amylostereum* are not strictly species or genotype specific. However, our data suggest that *S. cyaneus* could be specific to *A. chailletii*, although sample sizes for this species were low. We also found that *S. noctilio* only carried *A. areolatum*, as is known from North America [5, 24], the Southern Hemisphere [35], and Europe [2, 45]. In North America, *S. noctilio* carries two different genotypes of *A. areolatum* [5, 24]. Interestingly, in Europe, *Sirex torvus* (previously incorrectly known as *S. cyaneus* [13]) has been reported as being associated with either *A. areolatum* or *A. chailletii*, although association with *A. areolatum* was described as "occasional" [3]. While Gaut

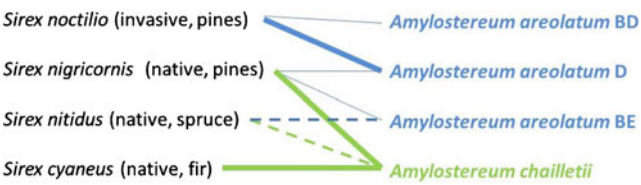

Fig. 3 Associations of *Amylostereum* species and strains with native and invasive *Sirex* species in eastern North America. *Thicker lines* denote more abundant associations, *dashed lines* denote common associations, and *thinner lines* denote less frequent associations

[11] stated that *S. torvus* was only associated with *A. areolatum*, his samples came from a small geographic distribution. Thus, it seems possible that the degree of fungal specificity of *Sirex* species could differ by species, with some *Sirex* being highly specific and others less so, although more samples from more species must be examined. At present, it is still unknown whether the fitness of *Sirex* that develop feeding on less prevalent *Amylostereum* genotypes is equal to fitness when larvae feed on more prevalent symbiont genotypes. In contrast with *Sirex*, in the woodwasp family Xiphydriidae, five species of *Xiphydria* are associated with one to four species of symbiotic fungi. In this system, each *Xiphydria* species is associated with numerous different species and genera of hardwood trees, which influences the species of symbiotic fungi with which they are associated [26].

It has previously been assumed that the symbiotic fungi carried by *Sirex* species were always transferred from a *Sirex* female to her offspring vertically during oviposition. However, results from this study as well as previous results with *S. nigricornis* [24] suggest that at times horizontal transmission occurs. We found that *A. areolatum* IGS-D was carried by one *S. nigricornis* emerging from the same sections of a pine tree as *S. noctilio*; in a previous study, two *S. nigricornis* co-occurring in the same tree as *S. noctilio* carried *A. areolatum* IGS-D [24]. Studies with siricids in Europe have found identical genotypes of *Amylostereum* in pairs of *Sirex* and *Urocerus* species, and this has been interpreted as being suggestive of horizontal transmission [43]. In the present study, we also found that *S. noctilio* and *S. nigricornis* infested the same trees, and even the same sections of the same trees, fairly frequently, which would provide the proximity necessary for horizontal transmission. Co-occurrence of *S. noctilio* and *S. nigricornis* within pines in northeastern North America has also been documented in other recent studies [19, 29, 30]. Development of multiple *Sirex* species within the same trees creates the potential for horizontal transmission of fungal symbionts among *Sirex* species. We hypothesize that as *S. noctilio* becomes more established and spreads further, the potential for horizontal transmission of the *A. areolatum* genotypes it carries to native *Sirex* will increase. Curiously, in this study, *S. noctilio* females were never found carrying fungal species and genotypes assumed to be native to North America, even when they emerged from the same trees as native *Sirex* individuals. The phenology of this system may help to explain this. In northeastern North America, most *S. noctilio* females oviposit in July and August [49], while *S. nigricornis* oviposit in September and October (KJ Dodds, personal communication). Thus, based on phenology, the fungus carried by *S. noctilio* would often have become established within trees before *S. nigricornis* females emerged from trees and oviposited.

Our study documented two IGS genotypes, B and D, associated with *S. noctilio* in northeastern North America. In agreement, a study by Bergeron et al. [5] documented two different multi-locus genotypes that were associated with *A. areolatum* IGS-BD or IGS-D in 2006 in Ontario, Canada. We found the *A. areolatum* IGS-BE genotype associated with *S. nigricornis* and *S. nitidus* in Maine and New York and with *S. nigricornis* in Louisiana. The *A. areolatum* IGS-BE sequences for isolates from these distant locations were exactly the same and isolates were vegetatively compatible, suggesting that regardless of the distance between sites, these isolates are the same or very similar, although more detailed comparisons are necessary.

Studies from a variety of host–symbiont associations have reported diversity and flexibility in host/symbiont associations [e.g., 1, 14, 15, 17, 22, 33, 44]. The ability to utilize different symbionts could prevent hosts from becoming entirely aposymbiotic. It has been hypothesized that different redundant partners may confer the same type of benefit for a host, although not necessarily to the same degree. In particular, the bark beetle *Dendroctonus ponderosae* is associated with different fungal symbionts having different temperature tolerances under warmer versus cooler conditions, which has been hypothesized as allowing these aggressive tree-killing beetles to occupy variable habitats [34]. We hypothesize that flexibility in symbiont associations can be advantageous for *Sirex*. Adult females do not live very long but must locate weakened trees and oviposit during their short lives [28]. It could be advantageous for *Sirex* to be flexible regarding acceptability of a potential symbiont that was not carried by its mother. This change in symbiont usage could occur when females lay eggs into areas already colonized by a different *Amylostereum* genotype or when larvae tunnel into such areas. Also, *Sirex* females have been reported to sometimes eclose as adults with fungus-free mycangia [39], and in these situations, horizontal acquisition of *Amylostereum* would be required for subsequent *Sirex* larval development.

Implications for Biological Control

The fungal symbiont specificity of native *Sirex* has implications for the biological control of *S. noctilio*. At many locations where *S. noctilio* has been introduced in the Southern Hemisphere, a genotype of the parasitic nematode *Deladenus siricidicola* originating from Hungary has been effectively used as a biological control agent [2]. This nematode species has a complex life cycle with mycophagous forms that feed on *A. areolatum* and parasitic forms that parasitize *Sirex* larvae and subsequently sterilize adult females. *D. siricidicola* is very specifically associated only with *A. areolatum* [21, 48] but is less specific regarding which *Sirex* host it will parasitize as it parasitizes both *S. noctilio* and *S. juvencus* in Europe [3]. As *S. noctilio* populations increase and spread in North America, authorities are considering whether *D. siricidicola*

should be introduced for classical biological control [46]. The fungal species and genotypes used by the native North American *Sirex* could impact whether this biological control nematode will be associated with developing *Sirex* larvae. Based on the present study, it seems possible that *D. siricidicola* used against *S. noctilio* could be near either *S. nigricornis* or *S. nitidus* within trees, when these *Sirex* are associated with *A. areolatum*. However, further studies are needed to evaluate whether the genotype of *D. siricidicola* used for biological control will successfully parasitize these non-target siricids.

Conclusions

Our studies have demonstrated that associations between *Sirex* and *Amylostereum* are not always specific. Flexibility in association of *Sirex* with different *Amylostereum* species and genotypes could provide siricids using ephemeral resources with the ability to thrive under a variety of conditions, e.g., use of weakened trees for larval development, that have already been colonized by *Amylostereum*. We have found that fungal symbionts of *Sirex* species can be swapped, but the effect of this on fitness of both exotic and native *Sirex* has yet to be determined. Whether the flexibility in associations of *Sirex* with fungal strains could have an impact on the biological control nematode associated with one fungal species remains to be determined.

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