Annals of the Entomological Society of America, XX(X), 2019, 1–8
doi: 10.1093/aesa/saz058
Research

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Intergenic Spacer Single Nucleotide Polymorphisms for Genotyping *Amylostereum areolatum* (Russulales: Amylostereacea) Symbionts of Native and Non-native *Sirex* Species

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Subject Editor: Jeffrey Morisette

Received 5 April 2019; Editorial decision 16 October 2019

Abstract

In North America *Amylostereum areolatum* (Chaillet ex Fr.) Boidin is a fungal symbiont associated with both the non-native *Sirex noctilio* Fabricius (Hymenoptera: Siricidae) and less commonly the native *Sirex nigricornis* Fabricius (Hymenoptera: Siricidae) woodwasps. The relationship between *S. noctilio* and *A. areolatum* constitutes a serious threat to pine plantation in the southern hemisphere. Studies have shown evidence of exchange of symbionts between non-native and native *Sirex* species. Our objectives were 1) to identify and assemble a panel of rDNA intergenic spacer–single nucleotide polymorphisms (IGS-SNPs) for genotyping strains of *A. areolatum* symbionts associated with *Sirex* species in North America, and 2) to develop genetic markers for monitoring the spread of specific *A. areolatum* haplotypes associated with *S. noctilio* across regions. The IGS-SNPs panel analyzed included haplotypes B1, B2, D1, D2 (from known IGS type B and D), E, and F. Genetic markers and haplotype-specific primers were designed to detect the IGS haplotypes D and E of *A. areolatum*. We found that haplotype D was absent in *A. areolatum* from *S. nigricornis* in Louisiana, while haplotype E was detected in all *A. areolatum* from *S. nigricornis* in Canada and Louisiana. Both haplotype D and E were co-detected in approximately 5% of samples from Canada. The IGS-SNP markers detected specific haplotypes accurately. Observing haplotype D in any *A. areolatum* from the native *S. nigricornis* likely indicates the presence of the potentially harmful *S. noctilo-A. areolatum* complex. The work highlights how IGS-SNPs can help in early detection without direct occurrence/observations of the non-native species of concern.

Key words: *Amylostereum areolatum*, haplotype, marker, *Sirex* woodwasps, *Pinus*

In North America, the siricid woodwasp *Sirex nigricornis* Fabricius (Hymenoptera: Siricidae) is a common native species occurring throughout the eastern United States and Canada. Since it is not a tree-killing pest, it received little attention until the closely related non-native *Sirex noctilio* Fabricius (Hymenoptera: Siricidae) was reported in North America for the first time in 2005 (Hoebeke et al. 2005, de Groot et al. 2006). In its native range of Eurasia and North Africa *S. noctilio* is not a significant mortality agent of pines (Slippers et al. 2015), but in some parts of its invaded range in the southern hemisphere it is a significant pest of plantation pines, including pine species native to North America.

Sirex noctilio continues to be a threat to pine plantations in invaded regions of the southern hemisphere including Africa, South America, and Oceania (Slippers et al. 2012). More recently, it has been introduced in North America (Hoebeke et al. 2005; de Groot et al. 2006) and Asia (Li et al. 2015). Following the first detection of *S. noctilio* in the United States in 2005, there were concerns that significant tree mortality and outbreaks in parts of New York and Canada could occur, but these fears have not been realized (Dodds et al. 2010, Haavik et al. 2018). Nevertheless, in the United States, *S. noctilio* has expanded its range into several states including Michigan, New Jersey, New York, Ohio, Pennsylvania, and Vermont (CERIS, 2018).

Sirex species have symbiotic relationships with fungi (e.g., *S. noctilio–Amylostereum areolatum* (Chaillet ex Fr.) Boidin), which enhance the food value of wood for developing larvae. Larvae die in the absence of the fungus; in turn, adult woodwasps are the only mechanism for introducing the fungus into new hosts, when they oviposit (Morgan 1968). The *S. noctilio–A. areolatum* relationship constitutes a serious threat to pine plantation in the southern hemisphere (Slippers et al. 2015), however, the native North American *S. nigricornis* is not known to attack healthy pines (Williams 2007). The primary fungal symbiont associated with the native *S. nigricornis* is *Amylostereum chailletii* (Gaut 1970, Bedding and Akhurst 1978, Gilbertson 1984, Smith and Schiff 2002), and this relationship is not known to have negative effects on healthy pines.

Although historically *Sirex* species were thought to have high fidelity in their relationships with fungal symbionts, Castrillo et al. (2015) recently demonstrated a lack of fidelity in the relationships between *Sirex* and *Amylostereum* species. This study investigated the genetic variation between *A. areolatum* symbionts found in a population of *S. nigricornis* in Louisiana where *S. noctilio* is currently presumed absent and *A. areolatum* symbionts found in both *S. nigricornis and S. noctilio* in Ontario, Canada; where both *Sirex* species are known to co-exist within the same range. The specific objectives of this study were 1) to identify and assemble a panel of rDNA intergenic spacer–single nucleotide polymorphisms (IGS-SNPs) from published sequences in the GenBank database for genotyping strains of *A. areolatum* symbionts associated with *Sirex* species in North America, and 2) to identify, develop, and validate genetic markers based on an *A. areolatum* IGS haplotype that might be observed in native *S. nigri*cornis but is uniquely associated with the presence of non-native *S. noctilio*. Thus, showing how IGS analysis can provide a tool for early (indirect) detection of the potentially harmful *S. noctilo–A. areolatum* complex through analysis of *A. areolatum* from native *S. nigircornis*.

Materials and Methods

IGS-SNPs Panel

Thirty-three *A. areolatum* sequences (IGS region) from Nielsen et al. (2009), Wooding et al. (2013), Olatinwo et al. (2013), and an ovipositional discharge study (Olatinwo et al. unpublished) were used to identify IGS-SNPs sites. Multiple alignments of the above sequences obtained from the GenBank database was done to assemble a panel of IGS-SNPs for developing haplotype-specific markers. Sequence alignments, editing, and analyses of *A. areolatum* extracted from *S. nigricornis* and *S. noctilio* were conducted in MEGA7 (Kumar et al. 2016). SNPs markers and the corresponding primers were designed using the Ecogrow clone L (GenBank Assession GQ422457.1) isolated from *Sirex juvencus* (L.) as the reference sequence. The panel analysis focused on nucleotide position in the range from 206 to 276, row 1 (Fig. 1) from multiple aligned sequences. Information on sources of all sequences and the associated IGS haplotype categories: 1) BD, 2) BE, 3) D, and 4) E (Nielsen et al. 2009, Bergeron et al. 2011, Wooding et al. 2013) included in this study are provided in Table 1. A subcategory of haplotypes (B1 and B2, and D1 and D2 identified for the first time from known IGS type B and D respectively), were included in the IGS-SNPs panel analysis. The relatedness of haplotypes sequence (B1, B2, D1, D2, E, and F) to *A. areolatum* strains and their specificity to native or non-native *Sirex* species (Table 1) were analyzed by hierarchical clustering analysis (Ward method) in the SAS-JMP 13.1 version software (SAS Institute Inc.) by scoring each haplotype as present (1) or absent (0).

The constellation plot calculated from the hierarchical clustering analysis (Fig. 2) illustrates the dissimilarity between the native and non-native *Sirex* species clusters (*Y*-axis), and differences across locations (*X*-axis) based on the IGS haplotypes in the *A. areolatum* symbiont. The plot arranges the *A. areolatum* isolates as endpoints and each cluster join as a new point, while the lines represent membership in a cluster. The length of a line between cluster joins, approximates the distance between the clusters that were joined, where *X* and *Y*-axis enable comparison of the relative distance between clusters. Longer lines represent greater distances between clusters. Observed differences or similarities among clusters facilitated marker identification and the design of corresponding specific primers to detect haplotypes associated with *A. areolatum* from non-native *S. noctilio* and haplotypes associated with *A. areolatum* from native *S. nigricornis*.

Sirex Samples

Sirex nigricornis and *S. noctilio* adult females investigated in this study were obtained from two sources: 1) infested pines collected in Sandbanks Provincial Park, Ontario, Canada. Infested pines were identified by the presence of characteristic resin beading on the bole and felled in the summer of 2012. Infested logs were transported to and stored at the Ontario Ministry of Natural Resources and Forests Tree Seed Plant facility in cardboard rearing tubes in a covered shed with open sides (Haavik et al. 2013). In 2013, tubes were checked several times per week and freshly emerged wasps were collected, identified, sexed, counted and stored individually until use; and 2) live *S. nigricornis* collected between late October and mid-November 2011 in Grant Parish, central Louisiana USA (N 31.595199°, W –92.416603°) as they emerged from bolts of felled infested loblolly pine (*P. taeda*) (Johnson et al. 2013, Olatinwo et al. 2013). Fungal symbionts were isolated from the mycangium of each specimen as described in Olatinwo et al. (2013). Voucher *S. nigricornis* adult female specimens from Louisiana were deposited in the Louisiana State Arthropod Museum (Louisiana State University, Baton Rouge).

DNA Extraction

Two sets of *Amylostereum* DNA samples (i.e., Ontario and Louisiana) were evaluated. The first set (n = 69) was extracted directly from spores in the mycangia of dead *S. nigricornis* (n = 39) and *S. noctilio* (n = 30) specimens collected in Ontario Canada, using a MoBio Powerplant PRO DNA kit (catalog number 13400) following the manufacturer's instructions. In the second set (n = 78), DNA was extracted from pure culture of individual isolates obtained from the mycangia of *S. nigricornis* specimens collected in Louisiana. Individual isolates were plated and maintained on potato dextrose agar (39.0 g PDA EMD Chemical Inc., dissolved in a liter of distilled water and amended with 100 ppm streptomycin sulfate) at ambient temperature (ca. 25°C). Genomic DNA was extracted from the mycelia of each isolate using the Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. DNA was stored at –20°C and used in PCR amplification to validate markers and corresponding designed primers.

IGS Primers and PCR Amplification

The nuclear ribosomal intergenic spacer (IGS) region is located between the 3′ end of the large subunit rDNA gene and the 5′ end of the 5S gene. The PCR amplification for individual haplotypes

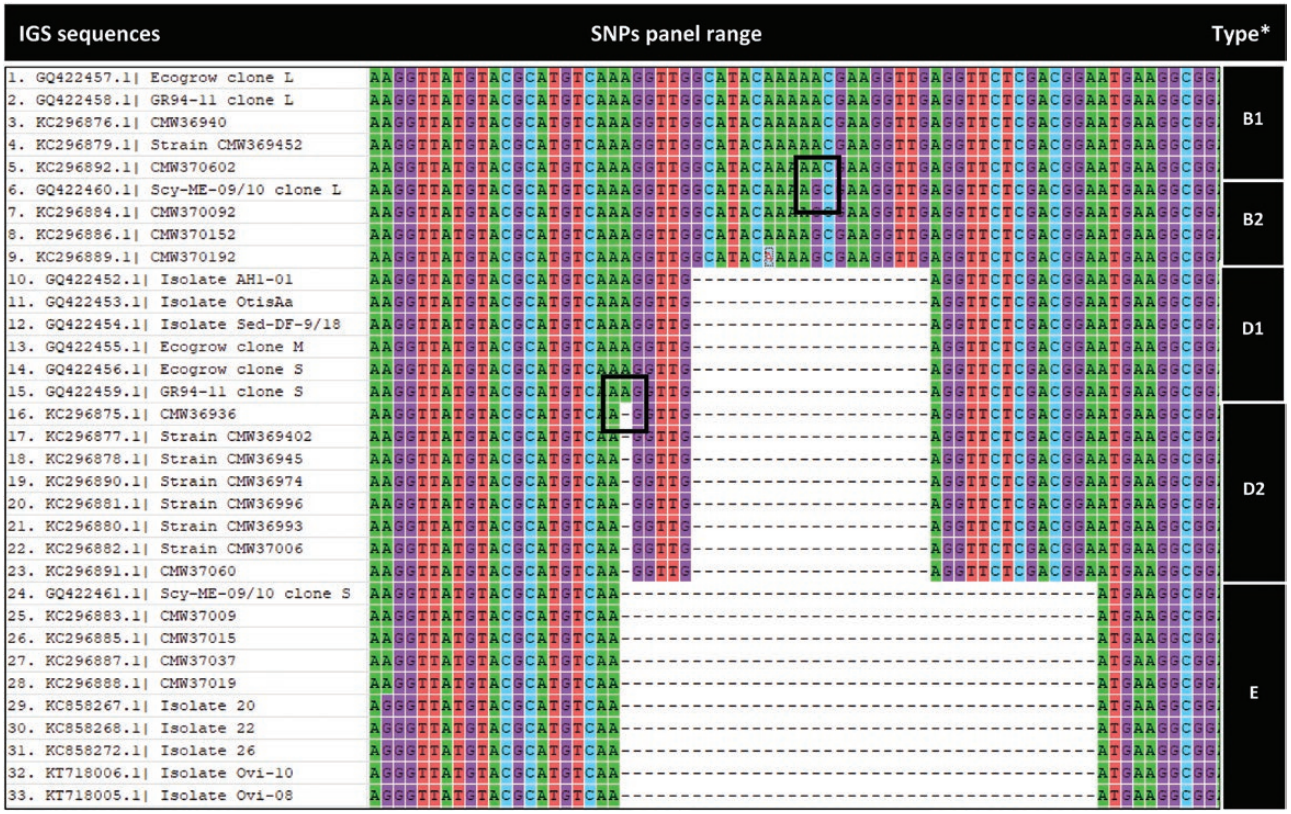

Fig. 1. Multiple alignment of the IGS haplotype sequences (Type B, D, and E) included in this study found in the *Amylostereum areolatum* isolates from *Sirex nigricornis* and *Sirex noctilio* specimens conducted in MEGA 7 software. The IGS sequences were obtained from the GenBank database and other sources including 1) Nielsen et al. 2009, 2) Wooding et al. 2013, 3) Olatinwo et al. 2013, and 4) Olatinwo et al. (unpublished). The alignment was used to identify SNPs panel range and nucleotide positions for markers and primer development. Ecogrow clone L (GenBank Assession GQ422457.1 position 206 to 276) was used as the reference sequence from multiple aligned sequences (AAGGTTATGTACGCATGTCAA**A**GGTTGGCATACAAAA**A**CGAAGGTTGAGGTTCTCGACGGAATGAAGG CGG). The IGS-SNPs panel included haplotypes B1, B2, D1, D2 (from known IGS type B and D), and haplotype E denoted by * and the label boxes on the right. The B1 and B2 were identified based on SNP at position 243, while D1 and D2 were identified based on SNP at position 227 in haplotype B and D, respectively.

followed a two-step approach involving a separate set of IGS primers to achieve different objectives: 1) to distinguish between *A. areolatum* and *A. chailletii* using species-specific primers, and 2) to detect *A. areolatum* IGS haplotypes (i.e., D and E) using separate haplotype-specific primers. In the first step, two sets of species-specific IGS primers previously developed (Olatinwo et al. 2013), AA1F and AA1R were used to detect *A. areolatum*, while AC2F and AC2R were used to detect *A. chailletii* in separate PCR reactions to prove the species-specificity of the *A. areolatum* primers (Table 2). In the second step, another two sets of specific primers were designed to amplify the IGS haplotypes D and E individually. The forward primer (in each set) exploited the SNPs positions within the identified IGS-SNPs panel range to discriminate between haplotypes. Haplotype primers IGSD-F and IGSD-R were designed to detect the presence of IGS haplotype (D), while IGSE-F and IGSE-R were designed to detect the presence of IGS haplotype (E) in *A. areolatum* DNA samples evaluated (Table 2).

PCR amplification was performed in a 10 µl reaction mixture using an *Eppendorf Mastercycler Pro* PCR machine. A reaction mixture contained 5µl *TopTaq* PCR Master Mix (Qiagen), 1.5 µl of a 5µM solution of each forward and reverse primer, 1 µl of 10× CoralLoad, and 1 µl of the diluted (1:10) DNA template, and 1 µl of sterile water was used as the negative check. For the species-specific IGS amplification (step 1), 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.

For the IGS haplotype amplification (step 2), the PCR conditions consisted of an initial denaturation at 94°C for 2 min, followed by 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 59°C and 1 min extension at 72°C, and a final extension at 72°C for 15 min.

Detection of haplotype D (IGSD-F /IGSD-R) and haplotype E (IGSE-F/IGSE-R) were validated with DNA from previously identified *A. areolatum* isolates (from *S. nigricornis*), and *A. areolatum* isolates (from *S. noctilio*) from Ontario, Canada (n = 69), where both *Sirex* species co-occur. Subsequently, DNA samples from *Amylostereum* species (n = 78) extracted from native *S. nigricornis* adult females collected in central Louisiana (Olatinwo et al. 2013) were tested and evaluated for the presence of haplotype D. In Louisiana the native *S. nigricornis* has no history of co-occurrence with *S. noctilio* and consequently these specimens were checked to determine whether haplotype D was absent or present in populations of *A. areolatum* from *S. nigricornis* in Louisiana.

Gel electrophoresis was conducted to examine the amplified PCR products by loading 5 µl of each product into separate wells on 1% agarose gels. After 15 min of electrophoresis, the agarose was stained with ethidium bromide for 20 min, and positive bands of PCR products on the gel were visualized under UV illumination.

Table 1. The intergenic spacer sequences included in the identification of SNP markers for detection of specific haplotypes in *Amylostereum areolatum* isolated from *Sirex nigricornis* and *Sirex noctilio*

Sirex host species	Location/ Country	Isolate code[a]	IGS group	Accession No.	IGS Type I	Accession No.	IGS Type II
S. noctilio	New York, United States	Isolate AH-01a	D	_	_	GQ422452.1	D1
S. noctilio	New York, United States	OtisAaa	D	_	_	GQ422453.1	D1
S. edwardsii	New York, United States	Sed-DF-9/18a	D	_	_	GQ422454.1	D1
S. juvencus	Hungary	Ecogrowa	BDF[a]	GQ422456.1	D1	GQ422457.1	B1
S. noctilio	New York, United States	GR94-11a	BD	GQ422458.1	B1	GQ422459.1	D1
S. sp. 'nitidus' (Harris)	Maine, United States	Sym-ME-09/10a	BE	GQ422461.1	E	GQ422460.1	B2
S. noctilio	Ontario, Canada	CMW36936b	D	_	_	KC296875.1	D2
S. noctilio	Ontario, Canada	CMW36940b	BD	KC296876.1	B1	KC296877.1	D2
S. noctilio	Ontario, Canada	CMW36945b	D	KC296878.1	D2	KC296879.1	B1
S. noctilio	Ontario, Canada	CMW36974b	D	_	_	KC296890.1	D2
S. noctilio	Ontario, Canada	CMW36993b	D	_	_	KC296880.1	D2
S. noctilio	Ontario, Canada	CMW36996b	D	_	_	KC296881.1	D2
S. noctilio	Ontario, Canada	CMW37006b	D	_	_	KC296882.1	D2
S. nigricornis	Ontario, Canada	CMW37009b	BE	KC296883.1	E	KC296884.1	B2
S. nigricornis	Ontario, Canada	CMW37015b	BE	KC296885.1	E	KC296886.1	B2
S. nigricornis	Ontario, Canada	CMW37037b	E	KC296887.1	E	_	_
S. nigricornis	Ontario, Canada	CMW37019b	BE	KC296888.1	E	KC296889.1	B2
S. noctilio	Ontario, Canada	CMW37060b	BD	KC296891.1	D2	KC296892.1	B1
S. nigricornis	Louisiana, United States	ISOLATE20c	_	KC858267.1	E	_	_
S. nigricornis	Louisiana, United States	ISOLATE22c	_	KC858268.1	E	_	_
S. nigricornis	Louisiana, United States	ISOLATE26c	_	KC858272.1	E	_	_
S. nigricornis	Louisiana, United States	OVIPOS08d	_	KT718005	E	_	_
S. nigricornis	Louisiana, United States	OVIPOS10d	_	KT718006	E	_	_

[a]Source (a) Nielsen et al. 2009, (b) Wooding et al. 2013, (c) Olatinwo et al. 2013, and (d) Olatinwo et al. (unpublished).

[a]Ecogrow IGS group BDF has S (GQ422456.1), L (GQ422457.1), & M (GQ422455.1), haplotypes respectively. Haplotypes D, E, and F were included in the hierarchical clustering analysis in SAS-JMP 13.1 version (SAS Institute Inc.).

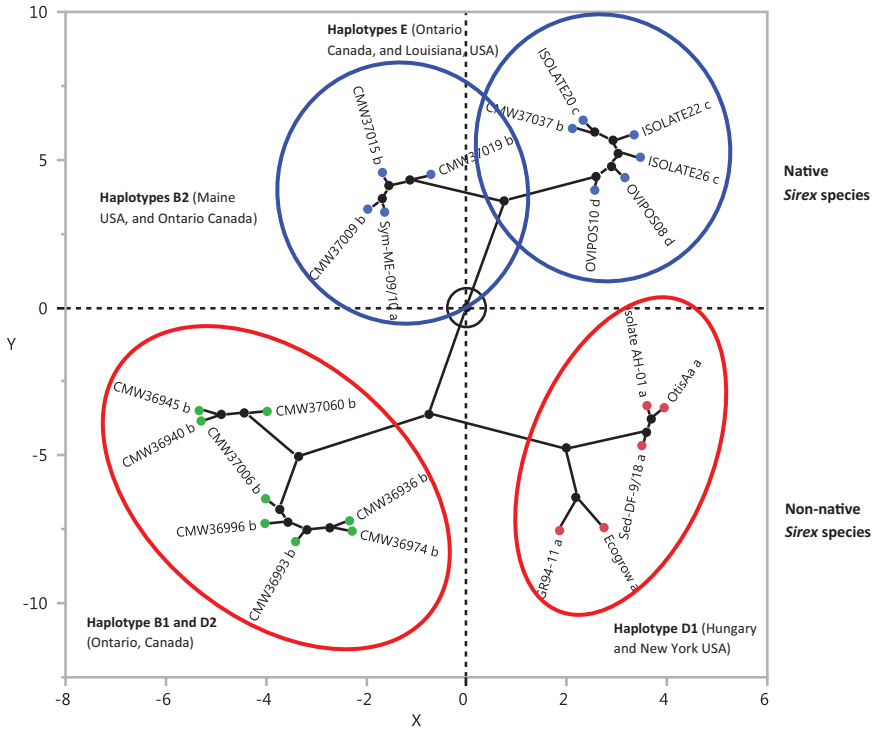

Fig. 2. Hierarchical clustering analysis and constellation plot based on the 33 sequences from the GenBank (Table 1) and relatedness of haplotypes in the *Amylostereum areolatum* isolates from native and non-native *Sirex* specimens from multiple locations. The intergenic spacer sequences included in the analysis are indicated with the GenBank accession number followed by the source of sequence 1) Nielsen et al. 2009, 2) Wooding et al. 2013, 3) Olatinwo et al. 2013, and 4) Olatinwo et al. (unpublished). The constellation plot illustrates the dissimilarity between the native and non-native *Sirex* species clusters (y-axis), and differences across locations (x-axis) based on the IGS haplotypes in the *A. areolatum* symbiont. The plot arranges the *A. areolatum* isolates as endpoints and each cluster join as a new point, while the lines represent membership in a cluster. The length of a line between cluster joins, approximates the distance between the clusters that were joined, where *X* and *Y*-axis enable comparison of the relative distance between clusters. Longer lines represent greater distances between clusters. The axis scaling, orientation of points, and angles of the lines on the constellation plot are arbitrary with no assigned unit in the SAS-JMP 13.1.

Table 2. Specific primers for detection of IGS haplotypes in the DNA of *Amylostereum* species isolated from *Sirex nigricornis* and *Sirex noctilio*

Target symbiont	*Sirex* species[a]	Oligo- name	Primer direction	Primer sequence (5′–3′)
A. areolatum	*S. nigriconis*/	AA1F	Forward	TTCAACCTCGGTTGGACTTC
	S. noctilio	AA1R	Reverse	CAAGCACCCCCTACATTTTG
A. chailletii	*S. nigriconis*	AC2F	Forward	TGAGGTTAAGCCCTTGTTCG
		AC1R	Reverse	CCCCCTTTCATTTTTCCAAT
A. areolatum	*S. noctilio*	IGSD-F	Forward	TACGCATGTCAAGGTTGAGG
(Haplotype D)		IGSD-R	Reverse	CCTGGGTGCTGTGGTTTTAT
A. areolatum	*S. nigriconis*	IGSE-F	Forward	GTTATGTACGCATGTCAAATGAA
(Haplotype E)		IGSE-R	Reverse	GGGAATCCTGGGTGCTGT
A. areolatum	*S. nigriconis*/	P1	Forward	TTGCAGACGACTTGAATGG
A. chailletii	*S. noctilio*	5S-2B	Reverse	CACCGCATCCCGTCTGATGTGCG

[a]*Sirex* species expected to carry target symbiont in their mycangia.

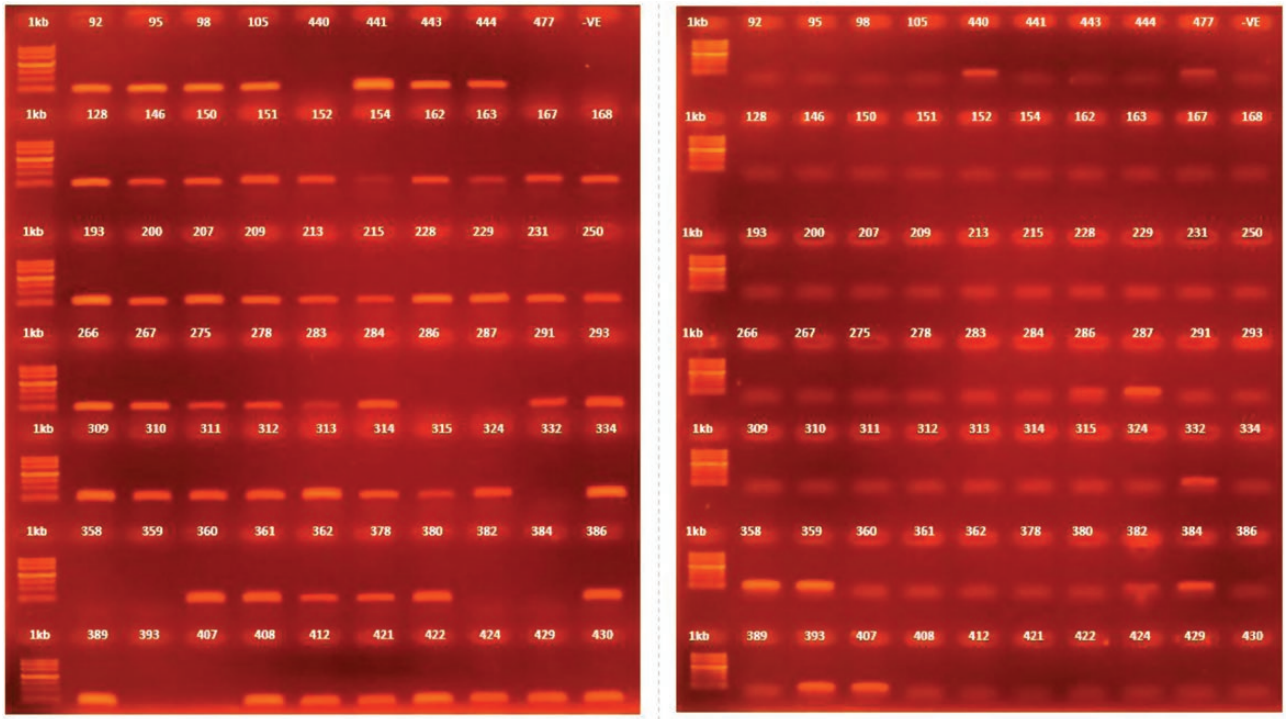

Fig. 3. The first validation step of specific primers using: (a) AA1F/AA1R for detection of only *Amylostereum areolatum* and (b) AC2F/AC2R for detection of only *Amylostereum chailettii* isolates from *Sirex nigricornis* and *Sirex noctilio* specimens (n = 69) in Ontario, Canada according to the information on Table 3.

Longer sequences of each *A. areolatum* identified in step two (from Louisiana) were obtained by PCR amplification using the IGS forward P1 (Hsiau 1996) and reverse 5S-2B (Slippers et al. 2002) primers (Table 2), according to previously used PCR protocol (Nielsen et al. 2009, Olatinwo et al. 2013). PCR products were sent to MCLAB (San Francisco, CA) for purification and sequencing using the forward (P1) and reverse (5S-2B) primers. Sequences were submitted to the GenBank database (GenBank Accessions, MG063752 to MG036781). The purpose of sequencing was to enable identification of genetic variation and differences compared to available sequences in the GenBank database.

Results

We identified SNPs in the intergenic spacer region of *A. areolatum* sequences from multiple sources, developed two sets of specific primers to detect the presence of haplotypes D and E in *A. areolatum* DNA, and validated the accuracy of the markers-primers using *A. areolatum* DNA samples from *Sirex* species from Ontario, Canada compared with *A. areolatum* DNA samples from native *S. nigricornis* in Louisiana.

Cluster analysis, of the relatedness of haplotype sequence (B1, B2, D1, D2, E, and F) for *A. areolatum* strains and their specificity to native or non-native *Sirex* species (Table 1), showed that haplotype

D1 was associated with AH-01, OtisAa, Sed-DF-9/18 and GR94-11 isolates from non-native *S. noctilio* found in New York, US, and the Ecogrow isolate from *S. juvencus* from Hungary (Fig. 2). Haplotype D2 was consistently associated with B1 found in *A. areolatum* from non-native *S. noctilio* in Ontario, Canada, while B2 was consistently associated with haplotype E found in *A. areolatum* from native *Sirex* species in Ontario, Canada.

The gel electrophoresis analyses confirmed the specificity of the *A. areolatum* specific primers AA1F/AA1R in detecting only *A. areolatum* among *Amylostereum* isolates from *S. nigricornis and S. noctilio* specimens in Canada (Fig. 3a), while the *A. chailettii* specific primers AC2F and AC2R served as a check to verify the accuracy of the *A. areolatum* specific primers (Fig. 3b). A band indicates detection, while no band indicates negative. Among the *A. areolatum* detected in Fig. 3a, those with the haplotype D were identified using primers IGSD-F and IGSD-R (Fig. 4a), and those with haplotype E were identified with primers IGSE-F and IGSE-R (Fig. 4b). Although, haplotype D was consistently found in *A. areolatum* isolates from the non-native *S. noctilio* from Ontario, Canada, it was absent in all *A. areolatum* (n = 30) from the native *S. nigricornis* in Louisiana (Tables 3 and 4). Haplotype D was also detected in 6 of the 28 *A. areolatum* evaluated from native *S. nigricornis* in Ontario Canada. Interestingly both haplotypes (D and E) were co-detected in 3 of those 6 *A. areolatum* DNA samples (Fig. 4). Haplotype E was consistently absent in *A. areolatum* isolates from the non-native *S. noctilio* (Table 3).

Isolate CMW37037 from Canada clustered with the Louisiana samples, perhaps representing the strain naturally associated with *S. nigricornis* in Canada before the new strains associated with *S. noctilio* species were introduced. The isolates from Louisiana evaluated in this study were assumed to represent a portion of the *A. areolatum* strains associated with the native *Sirex* species in Louisiana.

Discussion

An earlier study of the fungal symbionts associated with native adult female *S. nigricornis* in Louisiana found that approximately 35% were carrying *A. areolatum*, instead of *A. chailletii* as expected. In contrast to Louisiana where *S. noctilio* does not occur but *S. nigricornis* does, the two species are sympatric in Ontario, Canada. Consequently, the exchange of symbionts between the two *Sirex* species is possible in Ontario but unlikely in Louisiana (Wooding et al. 2013). Results from this study suggest that symbiont exchange (i.e., haplotype D and E) has occurred (Table 3), as both haplotype D and E were detected in 3 of the 69 DNA samples from Ontario Canada (<5% of samples), while haplotype D was never detected in any of the DNA samples from Louisiana (Table 4). We found evidence of symbiont exchange from the non-native *S. noctilio* to the native *S. nigricornis* in the Canadian samples but no evidence of exchange from *S. nigricornis* to *S. noctilio*.

Wooding et al. (2013) similarly observed that the IGS type E is from eastern North America. It is very interesting that haplotype D and E were co-detected in this study. It is likely that the use of *A. areolatum* spores extracted directly from mycangia for all samples used in this study would have resulted in more co-detections.

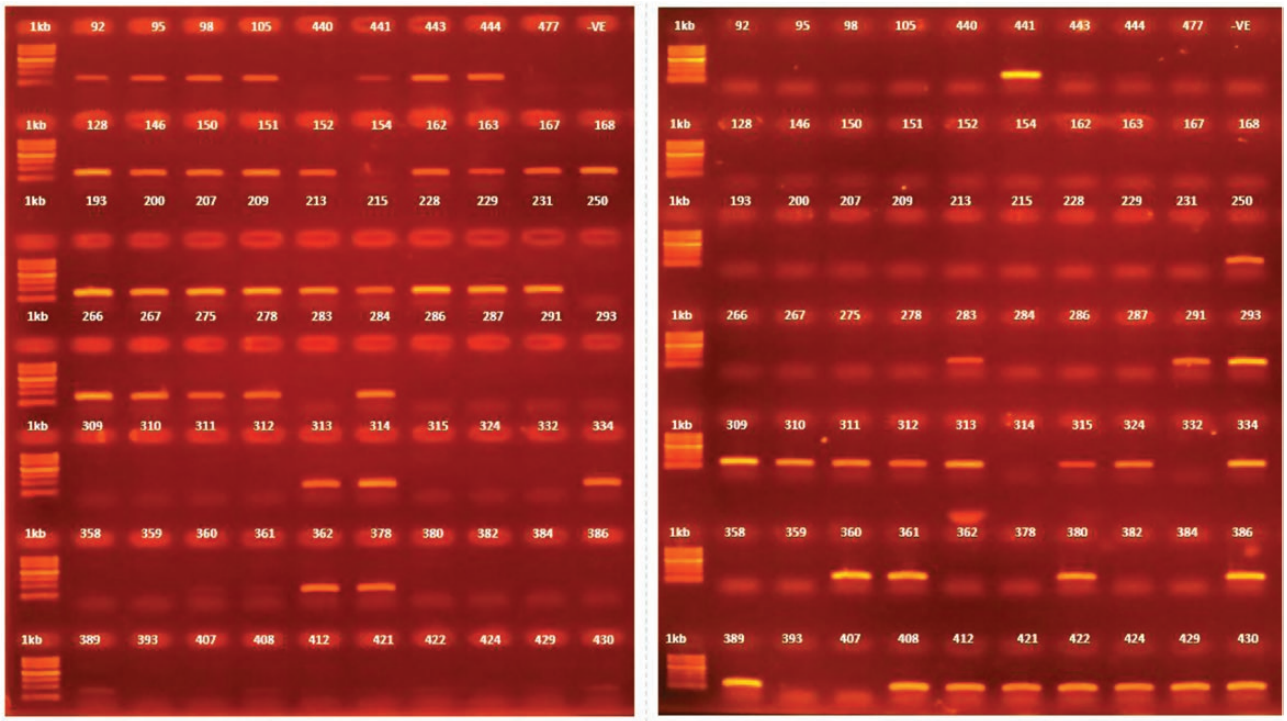

Fig. 4. The second validation step of specific primers using: (a) IGSD-F/IGSD-R for detection of only IGS haplotype D and (b) IGSE-F/IGSE-R for detection of only IGS haplotype E in the DNA of *Amylostereum areolatum* isolates from *Sirex nigricornis* and *Sirex noctilio* specimens (n = 69) in Ontario, Canada according to the information on Table 3.

Table 3. Detection of IGS haplotypes in DNA of *Amylostereum* species isolated from *Sirex nigricornis* and *Sirex noctilio* specimens in Ontario, Canada

		Symbiont detected		Haplotype detected		
Sirex species	Symbiont species	Ac	Aa	D	E	D/E[a]
S. nigricornis	*A. chailleti* (Ac)	11	-	-	-	-
S. nigricornis	*A. areolatum* (Aa)	0	28	6/28	25/28	3/28
S. noctilio	*A. areolatum* (Aa)	0	30	30/30	0/30	0/30
	n = 69	11	58			

[a]Both haplotype D and E were detected in DNA sample.

Table 4. Detection of IGS haplotypes in DNA of *Amylostereum* species isolated from *Sirex nigricornis* specimens in Louisiana, United States

		Symbiont detected		Haplotype detected		
Sirex species	Symbiont species	Ac	Aa	D	E	D/E[a]
S. nigricornis	*A. chailleti* (Ac)	48	-	-	-	-
S. nigricornis	*A. areolatum* (Aa)	0	30	0/30	30/30	0/30
	n = 78	48	30			

All *A. areolatum* identified from the above evaluation (*n* = 30) were amplified and sequenced using the IGS primers; P1 (5′TTGCAGACGACTTGAATGG′3 - forward primer) [15], and 5S-2B (5′CACCGCATCCCGTCTGATGTGCG ′3 - reverse primer) [16] and submitted to the GenBank database (GenBank Accessions, MG063752 to MG036781).

[a]Both haplotype D and E were detected in DNA sample.

Multi-locus genotype (MLG) studies (Nielsen et al. 2009, Bergeron et al. 2011) identified the significance of both haplotype D and BD, which were linked with MLG2/ MLG15, and MLG3/ MLG14 respectively, and both MLG2 and MLG3 were detected among *A. areolat*um isolates from *S. noctilio* specimens found outside their native range. MLG3 was found only in North America, while MLG14 and MLG15 were observed among native European isolates (Bergeron et al. 2011). Clustering analysis from the current study found haplotype B2 in association with E were consistently linked to *A. areolatum* from native *Sirex* species, while haplotype B1 in association with D2 were linked to *A. areolatum* from non-native *Sirex* species in Ontario, Canada. Nielsen et al. (2009) observed that IGS type B was only found in association with D (i.e., BD), while D was frequently found alone.

The genetic variation (i.e., SNPs) within the IGS region provides important information that facilitates detection of specific haplotypes as described in this study. The use of haplotype-specific markers may facilitate monitoring and management efforts. Although, currently there is no monitoring program for *S. noctilio* in North America, the specific markers presented could be useful for the detection of haplotype D in the native *Sirex* population as a proxy for detecting *S. noctilio* (i.e., for use characterizing the distribution of *A. areolatum* strains). In the future, it may also be possible to use these markers to improve the efficacy of biological control programs by identifying populations of specific *A. areolatum* strains that improve nematode growth. For example, *Deladenus siricidicola*, has a bicyclic life cycle which includes a mycetophagous free-living form that targets the *A. areolatum* symbiont of the adult female *Sirex* species (Bedding 1972, Bedding 2006). Morris et al. 2012 found that *D. siricidicola* grows well on the native *A. areolatum* (SymME), with variable growth observed on strains carrying haplotype D (SedDF and OtisAa) from *S. noctilio* found in North America. The preference and specificity of *D. siricidicola* (Morris et al. 2012) in targeting *A. areolatum* strains could be critical in achieving effective management of populations of *S. noctilio*. Additionally, haplotype-specific markers may facilitate the avoidance or mitigation of unintended non-target effects on native *Sirex* populations carrying other *A. areolatum* haplotypes in their mycangia. It is important to note that presently, no biological control agents have been approved for release against *S. noctilio* in North America.

To date, the impact of *S. noctilio* appears to be limited where it has been reported in the United States and Canada (e.g., Haavik et al. 2018). Although the impact of *S. noctilio* in Canada is consistent with the conclusion that it is a low priority invasive, it should be noted that *S. noctilio* was not recognized as a pest in New Zealand until several decades after its arrival (Bain et al. 2012). Because of this history of delayed impacts, monitoring programs should be developed and implemented in North America, to track the spread and impact of the *A. areolatum-S. noctilio* complex within and beyond the current reported distribution (Hajek et al. 2013, Wooding et al. 2013). This is particularly true in the southern United States where climate, ecology, tree species, soils, management, and landscape configurations of pine stands are different than in northeastern North America, with unknown impacts on *S. noctilio* population dynamics (Ayres et al. 2014).

Genetic markers and haplotype-specific primers developed and presented in this study could also complement existing monitoring tools such as semiochemical-baited flight intercept traps (Haavik et al. 2014) or predictions based on the emergence patterns of female adults (Haavik et al. 2013; Hartshorn et al. 2016). In particular, these genetic tools could facilitate tracking further spread of *A. areolatum* strains, across forests and regions, especially strains associated with the non-native *S. noctilio* (i.e., carrying the IGS haplotype D). Markers may also facilitate other future investigations of the dynamics of *Sirex-Amylostereum* interactions.

Acknowledgments

We thank Dr. James Meeker, Billy Bruce, and Chris Steiner, USDA Forest Service, Forest Health Protection, Pineville, LA for specimens provided for this study. Funding for this research was provided by the USDA, Forest Service, Southern Research Station (14-DG-11330129-041), supported by the USDA Forest Service, Forest Health Protection and the Canadian Forest Service.

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