FUNGAL BIOLOGY XXX (2015) 1–10

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

Multilocus genotyping of *Amylostereum* spp. associated with *Sirex noctilio* and other woodwasps from Europe reveal clonal lineage introduced to the US

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ARTICLE INFO

Article history:
Received 12 November 2014
Received in revised form
12 February 2015
Accepted 4 March 2015
Corresponding Editor:
Joseph W. Spatafora

Keywords:
Fungal symbiont
Genetic diversity
Invasive pest
Siricid woodwasps
Somatic incompatibility

ABSTRACT

Sirex noctilio is a woodwasp of Eurasian origin that was inadvertently introduced to the southern hemisphere in the 1900s and to North America over a decade ago. Its larvae bore in *Pinus* spp. and can cause significant mortality in pine plantations. *S noctilio* is associated with a symbiotic white rot fungus, *Amylostereum areolatum*, which females inject into trees when they oviposit and which is required for survival of developing larvae. We compared the genetic diversity of *A. areolatum* isolated from *S. noctilio* and other woodwasps collected from Europe and from northeastern North America to determine the origin of introduction(s) into the United States. Multilocus genotyping of nuclear ribosomal regions and protein coding genes revealed two widespread multilocus genotypes (MLGs) among the European samples, one of which is present in the US. The other two MLGs associated with *S. noctilio* in the US represented unique haplotypes. These latter two haplotypes were likely from unrepresented source populations, and together with the introduced widespread haplotype reveal multiple *A. areolatum* MLGs introduced by *S. noctilio* and indicate

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Abbreviations; IGS, intergenic spacer region; ITS, internal transcribed spacer region; mtssu, mitochondrial small subunit; RPB2, DNA directed ribosomal polymerase II subunit; tef1, translation elongation factor alpha 1

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http://dx.doi.org/10.1016/j.funbio.2015.03.004

possible multiple *S. noctilio* introductions to North America from Europe. Our results also showed a lack of fidelity between woodwasp hosts and *Amylostereum* species.

Introduction

Amylostereum areolatum (Basidiomycetes: Russulales) is a saprotrophic decay fungus that can be found in stumps or logs of various conifers and is often effectively injected by woodwasps of the genus *Sirex* spp. (Hymenoptera: Siricidae) into living trees. The fungus is carried in a pair of specialized intersegmental mycangia located at the base of the female woodwasp's ovipositor and opening into the oviducts. Fragments of the fungus, or arthrospores, are deposited into host trees with the woodwasp eggs during oviposition. Larval nutrients are derived from wood decomposed by the fungus (Thompson *et al.* 2014). Surveys on the genetic diversity of this fungus collected from woodwasps, basidiocarps or wounded trees from Denmark, Lithuania, and Sweden and tested using somatic or vegetative compatibility grouping (VCG) assays have revealed widespread and conserved clonal populations (Thomsen & Koch 1999; Vasiliauskas & Stenlid 1999). These results along with the general low occurrence of *A. areolatum* fruiting bodies on logs versus its prevalence in wounded trees in some locations within central and northern Europe indicate that this species uses primarily asexual reproduction due to its close association with woodwasp hosts (Thomsen & Koch 1999; Vasiliauskas & Stenlid 1999).

In Europe, *A. areolatum* is associated with the woodwasps *Sirex juvencus* and *Sirex noctilio* (Gaut 1970). Two other species of *Amylostereum* are also associated with siricid woodwasps: *Amylostereum chailletii* is carried by *Sirex cyaneus*, *Sirex areolatus*, and *Urocerus* spp. in Europe (Gaut 1970; Tabata *et al.* 2000), while *Amylostereum laevigatum* is associated with *Urocerus antennatus* and *Urocerus japonicus* in Japan (Tabata & Abe 1997, 1999).

Amylostereum areolatum benefits from its association with the woodwasp *S. noctilio* in multiple ways: the insect transports the fungus to suitable hosts and also injects the host with a venom gland secretion that facilitates lethal infection by the fungus (Bordeaux *et al.* 2014). The venom, noctilisin, induces a variety of physiological changes in the host resulting to weakened defense, enabling the fungus to grow and colonize the surrounding wood (Bordeaux *et al.* 2014). This woodwasp attacks primarily *Pinus* (99 %) but has been found to also infest *Abies* and *Picea* (Spradbery & Kirk 1978).

In the last century, *S. noctilio* was introduced, likely via the movement of infested logs or packing materials, to the southern hemisphere, being found in New Zealand in the 1900s and Australia in 1952, South America in 1980, and South Africa in 1994 (Gilbert & Miller 1952; Morgan & Stewart 1966; Madden 1988; Tribe 1995; Iede *et al.* 2010). This woodwasp is of little economic importance across its native distribution in Eurasia and North Africa as it attacks stressed, dying, or recently dead hosts (Spradbery & Kirk 1978). *S. noctilio*, however, has become a major pest in areas of introduction. Woodwasp outbreaks in exotic *Pinus radiata* plantations in the southern hemisphere in the 1940s and 1950s resulted in up to 80 % tree mortality, making this woodwasp a serious threat to the softwood industry (Madden 1988). *S. noctilio* was first detected in North America in New York in 2004 (Hoebeke *et al.* 2005), and since then has been collected in six other states and also in the provinces of Ontario and Quebec in Canada (NAPIS, 2014), causing serious concerns for the possible impact of this invasive pest on forest ecosystems in North America. In addition, the surveillance for *S. noctilio* in the US forests has decreased in recent years; thus, its present geographic distribution likely is underestimated.

Current biological control strategies in the southern hemisphere utilize the nematode *Deladenus siricidicola*, which parasitizes *S. noctilio* as well as feeds on its fungal symbiont (Bedding 2009). The nematode life cycle has alternating parasitic and mycetophagous phases, and the latter phase has been exploited for mass rearing the nematode for biological control programs (Bedding 1972). The efficacy of this biological control agent, however, relies in part on its suitability with the *Sirex–Amylostereum* complex, since the virulence of nematode-wasp associations may be specific to certain geographical populations (Bedding 1972). The potential use of *D. siricidicola* in the US is complicated further by the presence of North American *Sirex* species that also carry *Amylostereum* spp. and, thus, could serve as alternate hosts for the introduced nematode. Thirteen *Sirex* species are endemic to the New World and the genus is widespread in North America (Schiff *et al.* 2012). In the northeastern US, three *Sirex* species -*S. cyaneus*, *S. nigricornis*, and *S. nitidus* – have overlapping geographic distributions and host ranges with the introduced *S. noctilio*. Although major host tree preferences vary among the North American woodwasps, all three attack *Pinus* (Schiff *et al.* 2012), and this allows the possibility of host trees with mixed *Sirex* infestations. *S. cyaneus* has been reported to carry only *A. chailletii*, but *S. nigricornis* and *S. nitidus* carry *A. areolatum* or *A. chailletii* (Hajek *et al.* 2013; Olatinwo *et al.* 2014).

As part of our effort to study the biology of the introduced *S. noctilio* and its associated *A. areolatum* genotypes in the US, we determined the genetic diversity of *Amylostereum* spp. associated with *S. noctilio* and other woodwasps from Europe and compared observed genotypes with those from *S. noctilio* collected in the northeastern US.

Materials and methods

Isolate collection and maintenance

Amylostereum samples were obtained from the mycangia of adult female *Sirex* and *Urocerus* spp. either trapped in the field

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during flight season or reared from infested logs (Hajek *et al.* 2013). These logs were placed in screened barrels (77.5 cm high × 51.4 cm diameter) for *Sirex* emergence and handled as described in Morris *et al.* (2014) and Hajek *et al.* (2013). Collected siricid specimens were identified morphologically to species using the key in Schiff *et al.* (2012). Female adults were dissected as described by Thomsen & Harding (2011) and the content of one mycangium was plated on potato dextrose agar (PDA) amended with antibiotics (300 mg L^{-1} streptomycin sulfate). Cultures were incubated at 22 ± 1 °C for at least 2 weeks under continuous darkness. The other mycangium was stored in cetyl trimethylammonium bromide (CTAB) solution (2 % CTAB, 100 mM Tris–HCl pH 8.0, 20 mM EDTA pH 8.0, and 1.4 M sodium chloride) until processing. When culturing the fungus was not possible due to sampling location, the mycangia or the whole wasp was stored in CTAB solution for later processing. Woodwasps were collected from Denmark, Hungary, Italy, Spain, and the US between 2007 and 2012. In addition to these collections, strains obtained by I. Thomsen in 1994 from earlier studies (Thomsen & Koch 1999) and reference strains of *A. areolatum*, *Amylostereum chailletii*, and *Amylostereum laevigatum*, courtesy of T. Harrington (Dept. of Plant Pathology, Iowa State University, IA), were also included in the survey. A total of 45 *A. areolatum*, seven *A. chailletii*, and two *A. laevigatum* isolates from nine siricid species from eight countries were used in this study (Table S1). Although the main focus of our study is associating symbiotic fungi with woodwasp species, two strains from host trees from Canada and Germany were also included for comparison. We will refer to previously uncharacterized fungal samples from woodwasps or host trees as isolates and samples that have been characterized here or in other studies as strains. Fungal strains are maintained for long-term storage at the Department of Entomology at Cornell University in Ithaca, NY.

DNA extraction, PCR amplification and sequencing

Fungal genomic DNA was extracted from either 2-week old cultures grown on PDA overlaid with cellophane and under conditions described earlier or from the wasp mycangia using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Mycelia (≤100 mg wet weight) from pure cultures were transferred to a 2-ml bead beating tube (BioSpec, Bartlesville, OK) using a sterile scalpel and homogenized at 5000 rpm for 60 s using 0.5 g of 0.5 mm zirconia-silica beads (BioSpec) and 400 μL of the kit-provided lysis buffer amended with 0.4 μL of RNase A (100 mg mL^{-1}). Mycangia from woodwasps were triturated using a sterile pellet pestle prior to homogenization under the conditions described. Extracted DNA was eluted in AE buffer, quantified using a spectrophotometer and stored at −20 °C until use.

The intergenic spacer region (IGS), internal transcribed spacer region (ITS), mitochondrial small subunit (mtssu), DNA-directed ribosomal polymerase II (RPB2), translation elongation factor alpha 1 (tef1), and laccase loci were amplified and sequenced following a study by Bergeron *et al.* (2011) showing the utility of these regions and genes for genotyping *A. areolatum* associated with *Sirex* woodwasps. Further, these sequences would allow comparison of our samples with those collected by Bergeron *et al.* (2011) from pine trees in Canada attacked by *Sirex noctilio*. PCR assays were conducted using published primers (Table S2) and the following conditions: initial denaturation at 95 °C for 4 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 58, 55, or 52 °C (IGS, ITS/tef1/mtssu, or laccase, respectively) for 1 min, and extension at 72 °C for 1 min; final extension at 72 °C for 10 min; and a holding temperature of 4 °C prior to gel electrophoresis. For the RPB2 gene, PCR conditions were as follows: initial denaturation at 95 °C for 4 min; 35 cycles of 95 °C for 1 min, 50 °C for 2 min with a ramp rate of 1 °C per sec and 72 °C for 2 min; a final extension at 72 °C for 10 min; and a holding temperature of 4 °C. PCR products were visualized in 1 % agarose stained with ethidium bromide, followed by purification using the QIAquick PCR purification kit (Qiagen Inc.) and quantified using a spectrophotometer. Purified PCR products were submitted to Cornell University Biotechnology Resource Center for bidirectional sequencing using an automated 3730xl DNA analyzer and BigDye Terminator chemistry (Applied Biosystems).

Phylogenetic analysis

The IGS, ITS, mtssu, RPB2, tef1 and laccase sequence data were assembled and edited separately using Clustal W (Thompson *et al.* 1994), and confirmed as belonging to the genus *Amylostereum* by BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as well as comparison (nucleotide identity) to authenticated sequences (Bergeron *et al.* 2011). New DNA sequences generated for this study and representative of each multilocus genotype (MLG) were deposited in GenBank (Table S3). For phylogenetic analyses, combined ITS, mtssu, RPB2, and tef1 sequence data for 12 and 5 representative isolates of *Amylostereum areolatum* and *Amylostereum chailletii*, respectively, as well as the sister taxon *Amylostereum laevigatum* was constructed in Mesquite v3.00 (Maddison & Maddison 2014) and aligned in Clustal X v2.1 (Larkin *et al.* 2007). An incongruence length difference test (ILD; Farris *et al.* 1995) as implemented by the partition homogeneity test in PAUP* 4.0b10 (Swofford 2003) was conducted to examine the possibility of a combined analysis, consisting of 10 000 ILD replicates with ten random taxon additions per ILD replicate. The final alignment was deposited in the public database TreeBASE (http://purl.org/phylo/treebase/phylows), study no. 16496.

Three separate phylogenetic approaches were utilized: maximum-likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) of phylogeny. ML and MP analyses were performed using PAUP*, whereas BI was executed via the Markov Chain Monte Carlo (MCMC) method in MrBayes v3.2.0 (Ronquist *et al.* 2012). For the ML analysis, the best model of sequence evolution for the concatenated alignment was the Tamura-Nei model with equal base frequencies plus gamma distribution (TrNef+G; Tamura & Nei 1993) and was selected by the Akaike Information Criterion (AIC; Akaike 1974) implemented in jModelTest 2.1.4 (Guindon & Gascuel 2003; Darriba *et al.* 2012). The heuristic search was performed with 1000 random-addition-sequence (RAS) replicates, tree bisection-reconnection (TBR) branch swapping, and MULTREES in effect. All nucleotides were included in the phylogenetic analysis and gaps were treated as missing characters. Branch support was

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evaluated using 1000 bootstrap replicates and 100 RAS per pseudo-replicate. Similarly, the MP analysis was also performed with the heuristic search option with 1000 RAS replicates and TBR as the branch-swapping algorithm. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replicates. Nodes receiving ML and MP bootstrap values of ≥70 % were considered significantly supported.

Unlike ML analysis performed in PAUP*, BI in combination with MrBayes makes it possible to partition combined datasets and, concomitantly, apply an independent model of evolution to each partition as unlinked genes often have different evolutionary constraints. Thus the combined dataset was divided into four unlinked partitions (ITS, tef1, RBB2, and mtssu) and the best-fit evolutionary model per partition (gene) – executable in MrBayes – was determined using jModelTest as previously described. A heterogeneous likelihood model was then applied to the partitioned dataset; one model with priors for each gene. The ITS and tef1 gene were fitted to a symmetrical (SYM; Zharkikh 1994) and general time reversible (GTR; Rodriquez *et al.* 1990) model, respectively; each with equal base frequencies. Likewise, a Kimura 2-parameter model with gamma distributed rate variation (K2P+G; Kimura 1981) was used for RPB2, whereas the likelihood model Felsenstein 1981 plus invariant sites (F81+I; Felsenstein 1981) was fitted to the mtssu gene. One cold and three heated Markov chain(s) were run, and samples were taken every 100 generations over 5.0×10^6 generations for a total of 50 000 sampled generations. The potential scale reduction factor (PSRF) for each of the model parameters was >1.0 when the program was terminated. Stationarity (chain convergence) was accessed by examining the average standard deviations of split frequencies and likelihood values. Burn-in value (10 %) was determined using Tracer v1.5 (Rambaut & Drummond 2009); removing 5000 burn-in (sampled) generations. The remaining trees were utilized to calculate a 50 % majority-rule consensus tree and calculate posterior probabilities (PP).

To further compare the inter- and intraspecific genetic variability among *Amylostereum* spp., mean pairwise genetic distances were calculated by gene (ITS, tef1, RPB2, and mtssu) among the representative isolates of *A. areolatum* (N = 12) and *A. chailletii* (N = 5) using the custom distance matrix option in PAUP*, the best model of sequence evolution per gene, and the statistical software JMP® Pro 10.0.2 (SAS Institute Inc., Cary, NC). Mean nucleotide difference (N) and nucleotide similarity (%) within and between species were assessed also in Geneious® v.6.1.7 (Biomatters Ltd., Auckland, New Zealand).

The laccase sequence data, which were obtained only from *A. areolatum* samples and were omitted from the phylogenetic analysis, were utilized in further determination of MLGs among isolates of this species and in the selection of isolates for compatibility analysis. The IGS sequence data were incomplete and were also omitted from phylogenetic analysis.

Fragment analysis of heterologous IGS types

Fragment analysis was utilized to examine the IGS of the rDNA of *A. areolatum*, which has heterogenic sequences. At least five sequences have been observed and have been shown to occur in different patterns: five combinations of two haplotypes each (AB, AC, BC, BD, or BE) or two patterns containing only one haplotype (C or D) (Nielsen *et al.* 2009; Slippers *et al.* 2002). Strains with heterogeneous IGS 'types' were evident by the amplification of two bands that require either separation on agarose gel prior to PCR product purification and sequencing or cloning of PCR products. Another method to determine IGS haplotypes is by fragment analysis, which utilizes the size differences among these heterogenic sequences (Hajek *et al.* 2013). The IGS was PCR-amplified using the primers IGS-intF (5′-GTTTCTTAGGGCTGTTCCAGACTTGTG) and FAM-labelled 5S-2B (Table S2) and the following thermocycler program: initial denaturation at 94 °C for 4 min; 35 cycles of 94 °C for 50 s, 55 °C for 45 s and 72 °C for 45 s; a final extension at 72 °C for 10 min; and holding at 4 °C until electrophoresis of amplicons (Hajek *et al.* 2013). PCR products were mixed with formamide and GeneScan 500 LIZ dye size standard (Applied Biosystems, Grand Island, NY) prior to electrophoresis with the Applied Biosystem 3730xl DNA Analyzer. Fragment sizes were determined using Peak Scanner v1.0 (Applied Biosystems). All strains, with either single or combination IGS haplotypes, were analysed by fragment analysis.

Vegetative compatibility testing

Vegetative compatibility among representatives of different *A. areolatum* MLGs was investigated to determine association between VCG and MLG. Representatives of each MLG were grown on PDA plates (100 × 15 mm) for 2 weeks at 22 ± 1 °C under continuous darkness and a 0.5 mm plug from the growing edge of each culture was cut using clipped sterile P1000 pipet tips (Rainin, Oakland, CA) and used for inter isolate pairings. Two plugs were placed approximately 2 cm apart in the centre of PDA plates (60 × 15 mm) and incubated at 22 ± 1 °C in the dark for 2–3 weeks or longer as needed for slower growing strains. A control plate with two plugs of the same strain was set up for each strain tested. Strains were scored as compatible if mycelia continued to grow freely and intermingle along the line of contact, and were assigned the same VCG number. In contrast, the presence of a clear zone between inoculated cultures, often accompanied by dark brown colouration, between the two growing strains indicated incompatibility. A total of 16 strains were tested, with two plates per assay and assays were replicated twice.

Results

Phylogenetic analysis

The concatenated alignment utilized for ML, MP, and BI analyses consisted of 2817 characters (ITS: 691 characters; tef1: 928 characters; RPB2: 685 characters; mtssu: 513 characters) of which 2540 were constant, 43 variable and parsimony-uninformative, and 234 parsimony-informative. Results of the ILD test (P = 0.0140) for the combined dataset indicated weak incongruence caused by the absence of genetic differences within the tef1 gene between isolates of *Amylostereum chailletii* and its sister taxon *Amylostereum laevigatum* (Table 1). Topological uncertainty imposed by tef1 was

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Table 1 – Pairwise comparisons of genetic identity, nucleotide similarity and nucleotide difference among the *Amylostereum* spp. and four loci sequenced.[a]

Species/Loci	*Amylostereum areolatum*				*Amylostereum chailletii*			
	ITS	tef 1	RPB2	mtssu	ITS	tef 1	RPB2	mtssu
A. areolatum	0.0017 (0.0003)	0.0013 (0.0006)	0.0030 (0.0007)	0.0026 (0.0005)				
	99.9 (0.0)	99.8 (0.1)	99.7 (0.1)	99.7 (0.0)				
	1.2 (0.2)	1.7 (0.6)	2 (0.5)	1.3 (0.2)				
	66	66	66	66				
A. chailletii	0.026 (0.0004)	0.1288 (0.0002)	0.0987 (0.0008)	0.037 (0.0004)	0.0029 (0.0012)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	97.4 (0.1)	86.6 (0.0)	90.7 (0.0)	95.4 (0.0)	99.7 (0.1)	100 (0.0)	100 (0.0)	0.0 (0.0
	17.7 (0.5)	124 (0.2)	63.3 (0.3)	22.8 (0.2)	2.3 (0.9)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	60	60	60	60	10	10	10	10
A. laevigatum	0.0338 (0.0006)	0.1288 (0.0005)	0.0910 (0.0008)	0.0321 (0.0007)	0.0215 (0.0025)	0.0 (0.0)	0.0451(0.0)	0.0102 (0.0)
	96.6 (0.2)	86.6 (0.1)	91.5 (0.1)	93.9 (0.1)	97.8 (0.3)	100 (0.0)	95.6 (0.0)	95.3) (0.0)
	23.3 (1.2)	124 (0.5)	58.7 (0.5)	31.5 (0.3)	15.5 (2.1)	0.0 (0.0)	30 (0.0)	24 (0.0)
	12	12	12	12	5	5	5	5

a Comparison by gene region: Genetic distance (95 % CI); % nucleotide similarity (95 % CI); no. of nucleotide difference (95 % CI); no. of pairwise comparisons.

confirmed by pruning these sequence data from the four-gene concatenated alignment and performing a second ILD test ($P = 0.0980$). A total evidence approach (Kluge 1998) for the ML and MP analyses was undertaken, utilizing the combined four-gene dataset as tef1 provided the greatest pairwise genetic differences (TrN+I: mean = 0.1288 substitution/site [s s^{-1}]) among *Amylostereum areolatum* and *A. chailletii* (Table 1) – the two species of primary interest in the present study.

Collectively, the ML, MP, and BI analyses effectively delineated *A. areolatum* and *A. chailletii* as isolates identified morphologically to species *a priori* as *A. areolatum* and *A. chailletii* and grouped these separately into two monophyletic clades (Fig. 1). Moreover, a monophyletic subclade with strong support probabilities – ML and MP bootstraps as well as BI PPs – was also revealed within each clade (species). In the *A. areolatum* clade, the subclade of MLGs Aa3, Aa4, and Aa5 from Hungary, Japan, and Denmark, respectively, was well supported; and in *A. chailletii*, a subclade of MLGs Ac2 from Denmark and Ac4 from Canada and Germany. The pairwise genetic distances, and hence phylogenetic resolution, between *A. areolatum* and *A. chailletii* varied by gene (Table 1); comparisons of tef1 provided the greatest genetic distance followed by RPB2 (K2P+G: mean = 0.0987 s s^{-1}), mtssu (TPM3uf+I; mean = 0.0370 s s^{-1}), and the ITS gene region (TrNef: mean = 0.0262 s s^{-1}). Yet, as previously indicated, homogeneous tef1 sequence data was found when comparing *A. chailletii* and *A. laevigatum*. Interspecific genetic

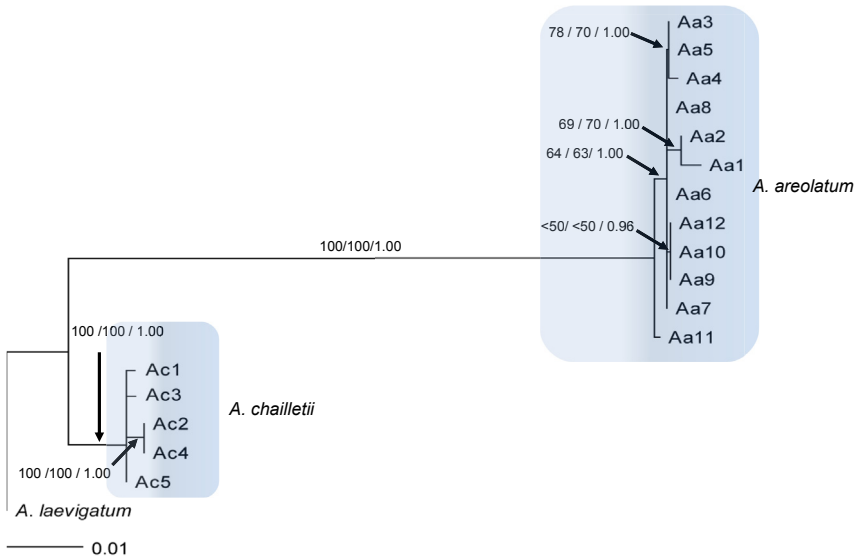

Fig. 1 – Phylogenetic relationships of *Amylostereum* spp. associated with *Sirex noctilio* and other woodwasps. Majority-rule consensus tree based on the combined analysis of the ITS, tef1, RPB2, and mtssu genes inferred from maximum likelihood (ML), maximum parsimony (MP), and Bayesian (BI) analyses. Numbers above branches, from left-to-right, indicate ML bootstrap values, followed by MP bootstrap values and BI posterior probabilities. Nodes receiving less than 70 % bootstrap and <0.95 posterior probability support were collapsed; however, those with conflicting support were retained.

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differences between the latter two species, however, was greatest for the RPB2 gene (mean = 0.0215 s s^{-1}) followed by the ITS (mean = 0.0215 s s^{-1}) and mtssu (mean = 0.0102 s s^{-1}) genes.

Addition of the laccase data, with three haplotypes, to the *A. areolatum* ITS, mtssu, RPB2 and tef1 sequences resolved the samples into 12 MLGs (Table 2). Eight of these MLGs had only one sample, with the rest consisting of 2–16 samples. The most widespread MLG, Aa1, was observed from *Sirex juvencus, Sirex noctilio, Urocerus albicornis*, and *Urocerus gigas* collected from Denmark, Hungary, or Spain. The other widespread MLG, Aa6, was detected from *S. juvencus* and *S. noctilio* collected from five countries, including the US. A BLAST search comparing DNA sequences of this MLG with samples from pine trees attacked by *S. noctilio* in Canada revealed that the Aa6 genotype was closely similar to the second more common MLG that Bergeron *et al.* (2011) found in Canada. Sequence comparisons were limited to the ITS, mtssu, RPB2 and laccase loci, due to less than 40 % coverage between the tef1 data sets.

Among the other *A. areolatum* strains from the US, two from *S. noctilio* and one from the North American *Sirex nigricornis* shared identical sequences for all loci except for the laccase locus. One of these strains, OtisAa, had sequences closely matching the more common *A. areolatum* MLG observed by Bergeron *et al.* (2011) in Canada. The *A. areolatum* strain carried by another North American woodwasp, *S. nitidus*, showed the least similarity with all the other *A. areolatum* strains studied.

The two strains of *A. areolatum* from *S. noctilio* collected in Italy revealed identical mtssu and tef1 loci but different ITS sequences, and alignments showed these gene haplotypes to be present in the other European samples (Table 2). MLG designation of the Italian samples, in comparison with the rest of the *A. areolatum* strains, was not possible due to inadequate amounts of DNA isolated from the mycangial samples for sequence analysis.

IGS sequences and fragment analysis data

IGS sequences were obtained from only seven strains of *Amylostereum areolatum* each carrying a single haplotype. Three US strains, AH1-17, OtisAa, and SedDF, were identified as having IGS type D. The IGS of two strains from Japan and two from Italy differed by one base pair from that of the US strains and was designated type D2 (Table 2). Fragment analysis of these strains also revealed only one peak of approximately 472 bp. The rest of the *A. areolatum* strains had heterogenic sequences and their combination haplotypes were determined using fragment analysis. Two heterogeneous types, BC (peaks of 492 bp and 424 bp) and BD (two peaks of 492 bp and 472 bp), were observed. Comparison of MLG data with IGS types indicated one type associated with each MLG (Table 2), with no specific type associated with a given cluster of strains. For example, IGS type BC was found associated with MLG Aa1, which clustered with MLG Aa2 associated with IGS type BD (Table 2, Fig. 1). Three IGS types were found among *Sirex* spp. from Europe, D/D2, BC and BD, whereas only two, D and BD, were found associated with *Sirex noctilio* in the US. Two North American woodwasps, *Sirex nigricornis* and *Sirex nitidus*, were found to carry IGS D and BE (peaks of approximately 492 bp and 452 bp), respectively.

Among the seven *Amylostereum chailletii* strains, two IGS sequence variations were observed (Table S3). A BLAST search identified one sequence as the IGS type G from *Urocerus gigas* collected from Denmark and Germany, *S. nigricornis* from the

Table 2 – Multilocus genotypes based on ITS, mtssu, RPB2, tef 1 and laccase sequence data and IGS type of *Amylostereum areolatum* from *Sirex* spp. and *Urocerus* spp. from various countries.

MLG	ITS-mtssu-RPB2-tef1-laccase types[a]	Woodwasp/tree host	Collection site	No. samples	IGS type[b]
Aa1	A-A-A-A-A	*S. juvencus, S. noctilio U. albicornis, U. gigas*	Denmark, Hungary, and Spain	16	BC
Aa2	B-B-A-B-B	*S. juvencus*	Hungary	1	BD
Aa3	B-B-B-B-A	*S. noctilio*	Hungary	1	BD
Aa4	D-B-B-B-A	*S. nitobei*	Japan	2	D2[c]
Aa5	B-B-B-B-B	*S. juvencus*	Denmark	3	BD
Aa6	C-B-C-B-A	*S. juvencus, S. noctilio*	Australia, Denmark, Hungary, Spain, and US	14	BD
Aa7	C-B-A-B-B	tree source (*Picea abies*)	Germany	1	D
Aa8	C-F-C-B-A	*S. juvencus*	Germany	1	BD
Aa9	C-E-C-B-A	*S. noctilio*	US	1	D
Aa10	C-E-C-B-C	*S. noctilio*	US	1	D
Aa12	C-E-C-B-B	*S. nigricornis*	US	1	D
Aa11	E-D-C-C-B	*S. nitidus*	US	1	BE
	A-B-x-B-x[d]	*S. juvencus*	Italy	1	D2
	C-B-x-B-x[d]	*S. juvencus*	Italy	1	D2

a Each letter represents a unique sequence for each locus, and sequence data for representative strains with different types were deposited to GenBank (Supplementary Table 3).

b IGS type was determined by sequencing of strains with one haplotype and by fragment analysis of strains with either one or a combination of two haplotypes.

c Fragment analysis revealed a peak of the expected IGS D type, but sequencing revealed a variation of one base.

d *Amylostereum areolatum* samples from the mycangia of *S. noctilio* collected from Italy provided insufficient amounts of DNA for sequencing all five loci. X indicates missing data.

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US, and a tree source in Canada. The other IGS sequence observed, designated G2, varied from the G type by two bases, and was found also from *U. gigas* from Denmark. The two *Amylostereum laevigatum* strains had heterologous IGS and were not analysed further.

Vegetative compatibility groups

Vegetative compatibility assays pairing fungal representatives of the same *Amylostereum areolatum* MLG had compatible reactions (Fig. 2a, Table S4). Pairings of strains from Denmark and Spain for the MLG Aa1 from *Sirex juvencus, Urocerus. albicornis,* and *Urocerus gigas* resulted in compatible reactions. Compatible results were also observed between strains from the US and Spain for Aa6. Pairings between strains from different MLGs showed incompatible reactions, except in a few cases (Fig. 2, Table S4), e.g., strains AH1-17, OtisAa, SedDF, representing MLGs 9, 10 and 12 (all IGS type D), respectively, had hyphae intermingling freely along the line of contact.

Discussion

Multilocus sequencing of *Amylostereum areolatum* isolates collected from woodwasps from Europe revealed two widespread MLGs associated with *Sirex* spp. One of these, Aa1, appears absent from the US. This MLG is also associated with the IGS BC type, which has not been detected in the US. MLG Aa1 includes strains from Denmark that have been identified previously by Thomsen & Koch (1999) as representatives of VCG DK A, also found in Lithuania and Sweden (VCG A1) by Vasiliauskas & Stenlid (1999). Thomsen & Koch (1999) observed this VCG among samples collected between 1983 and 1994 from basidiocarps on *Picea abies* and the woodwasp *Sirex juvencus*, and made up 61 % (19 of 31) of their European samples. Likewise, Vasiliauskas & Stenlid (1999) found this VCG as one of two more widespread clones among their samples from wounded stems of *P. abies*, present in one plot of three sampled in Sweden and one plot of seven sampled in Lithuania. Vegetative compatibility requires identical *het* or *vcg* genes, thus

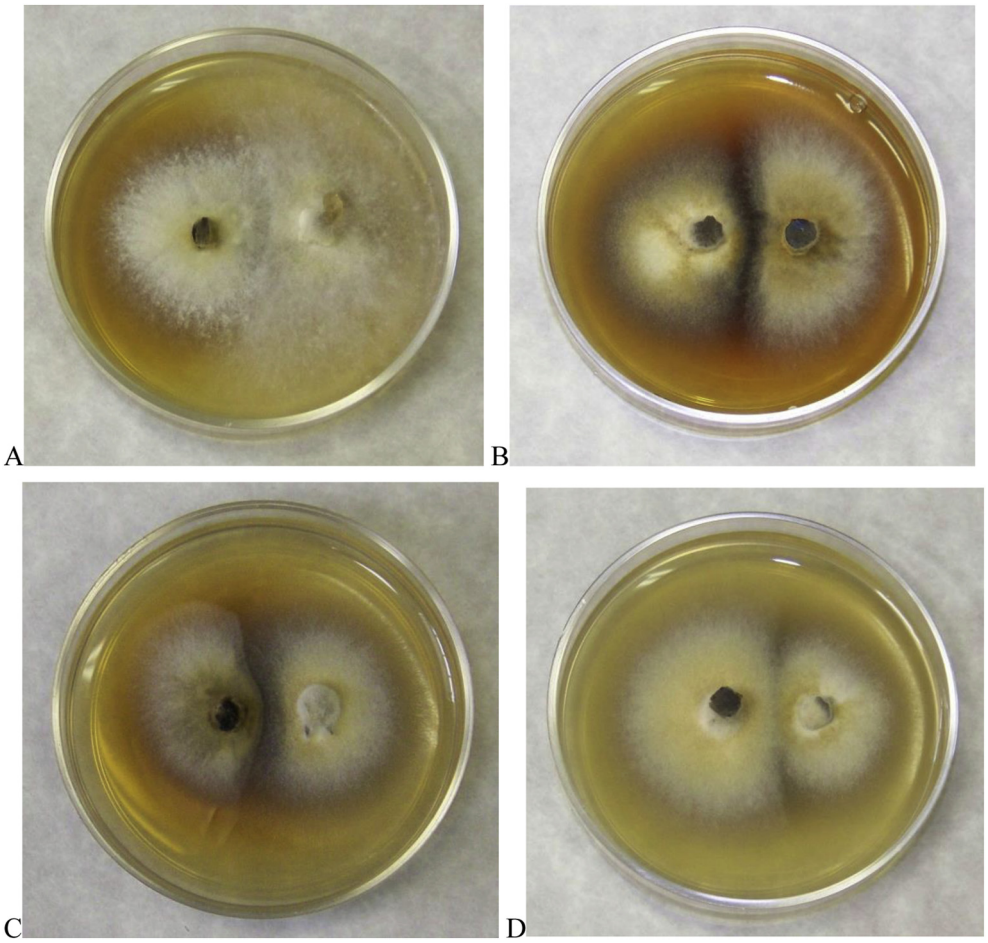

Fig. 2 – Vegetative compatibility assays between *Amylostereum areolatum* strains representing the same (A) or different (B, C, and D) multilocus genotypes (MLGs) on potato dextrose agar plates incubated for 2–3 weeks. (A) Strains of the same MLG Aa6, GR94 and SP12-E2, showing compatible reactions evidenced by the free intermingling of growing hyphae along the line of contact. Incompatible reactions evidenced by a clear zone between the growing strains, often accompanied by a dark brown pigmentation, were observed between strains GR94 and AH1-17 (B) and between strains B1352 and OtisAa (C). The former pair has different IGS types, while the latter pair shares the same IGS type D. Compatible reaction was observed between strains OtisAa and SedDF, which have different MLGs but the same IGS type D (D).

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compatibility is likely limited to genetically very similar isolates (Glass et al. 2000). Our results also demonstrated that representative strains of MLG Aa1 to be vegetatively compatible. Thus, the collection of this MLG/VCG from another site in Denmark after more than two decades and from different sites in Hungary and in Spain collected in 2011 and 2012, respectively, documents the occurrence of at least one widespread *A. areolatum* lineage potentially preserved over time and dispersed by its *Sirex* woodwasp host(s) from one forest stand to another. The observation of this lineage also provides additional support to the importance of *Sirex* woodwasp-fungal symbiont associations in the predominantly asexual distribution *A. areolatum* (Thomsen & Koch 1999; Vasiliauskas & Stenlid 1999).

The other widespread MLG, MLG Aa6, has been introduced to the US, and is associated with the IGS type BD. VCG testing of representative strains GR94 (US) and SP12-E2 (Spain) also showed compatible reaction. Compatibility testing, however, was limited to only two strains. Among the US samples included in this study, only one, GR94, was found to belong to MLG Aa6. Analysis of other US strains of the IGS type BD (Hajek et al. 2013) may reveal more representatives of this MLG and allow more compatibility testing between US and European samples and also between samples from the US. Surveys of *A. areolatum* associated with *Sirex noctilio* in the US have revealed only two IGS types, D and BD, with the former being more prevalent (Nielsen et al. 2009; Hajek et al. 2013). Nielsen et al. (2009) found the D type present in 67 % (14 of 21) *S. noctilio* samples in NY and PA. Likewise, Bergeron et al. (2011) also found both IGS types associated with *A. areolatum* from pine trees infested with *S. noctilio* in Canada, with the D type also more prevalent (74 % or 61 of 82 samples). Bergeron et al. (2011) also found the IGS type D associated with the Canadian MLG3 and the IGS type BD associated with MLG2.

Of the other MLGs reported herein and associated with *S. noctilio* in the US, Aa9 (AH1-17) and Aa10 (OtisAa), the latter had sequences closely similar to MLG3 reported by Bergeron et al. (2011) and is also associated with the IGS type D. Bergeron et al. (2011) found no close European or southern hemisphere connection to this MLG3 and suggested an unrepresented parent population. Likewise, we found no European sample of the same MLG as Aa10. MLGs Aa9 and Aa10, however, are closely related to Aa12 (SedDF) (Fig. 1), and all three have the IGS type D (Table 2). Determining the source of this lineage, however, will require additional fungal samples not only from Europe and S. America but also from N. America. The close similarity of Aa9 and Aa10 from *S. noctilio* to Aa12 from *S. nigricornis* and the prevalence of this MLG and its associated IGS type in N. America highlights the need to examine further *A. areolatum* genotypes associated with native siricids.

Results of the VCG assays also showed compatibility among MLGs Aa9, Aa10, and Aa12, which was surprising, but not impossible given that these genotypes shared identical sequences in four loci and differed only in their laccase sequence. In ascomycetes, genetic diversity is generally greater between than within compatible groups, but considerable variation may still be present within some groups (Worrall 1997). In basidiomycetes, better correspondence between molecular data and somatic compatibility has been observed (Malik & Vilgalys 1999). Our results indicate that while VCG testing could provide a measure of genetic similarity in *A. areolatum*, multilocus sequence data provide better resolution of genotype diversity.

Vegetative compatibility among US strains of *A. areolatum* with the same IGS type, determined by sequencing or fragment analysis, has also been documented by Nielsen et al. (2009) and Hajek et al. (2013). In these cases, the IGS types reflected closely related genotypes isolated from *S. noctilio* collected in the northeastern US. The utility of IGS typing, however, is limited given that it represents only one locus and cannot resolve clonal lineages (Table 2). In addition, our IGS fragment data indicated that the full characterization of IGS types would require gene sequencing given that nucleotide variations could be present among fragments of the same size. Nevertheless, fragment analysis could provide a convenient method for initial IGS grouping of isolates prior to subsampling for large population studies.

The detection of at least three distinct *S. noctilio*-associated *A. areolatum* MLGs in North America (Table 2 this study; Bergeron et al. 2011) revealed multiple *A. areolatum* haplotypes introduced by *S. noctilio*, indicating possible multiple *S. noctilio* introductions to North America from Europe. Whether these introductions occurred by a direct route from Europe or indirectly by way of South America (Boissin et al. 2012), or a combination of both would be difficult to determine without additional samples and heterogeneous genetic markers for assessing the population genetics of *A. amylostereum*. Given the difficulty of collecting *Sirex* spp. in Europe, where they are not considered major pests, we had a relatively small sample size to fully assess the population genetics of the *A. areolatum* associated with *S. noctilio* in Europe. And it is unfortunate that we have only two samples from Germany since Ciesla (2003) reported that this country was the source of the most woodwasp interceptions (37.9 %) at US ports from 1993 to 2001. Abundance of basidiocarps in this part of Central Europe (Vasiliauskas & Stenlid 1999) would suggest a more genetically diverse population of outcrossing *A. areolatum* that could be associated with siricid woodwasps. And although the four loci used in this study were useful for phylogenetic analyses, further analyses of additional European samples using microsatellite markers or other single nucleotide polymorphism DNA techniques could help sufficiently characterize other populations associated with *Sirex* spp. For example, the strain GR94, collected from NY in 2007, which represented the introduced MLG Aa6, could have come directly from Europe or by way of the southern hemisphere and South America. This MLG was observed in samples not only from Europe, but also from Australia (Table 2), and the Canadian complement, MLG2, also included Chile and South Africa as collection sites (Bergeron et al. 2011). The difficulty in delimiting the route and corresponding number of introductions was previously noted by Boissin et al. (2012). Boissin and colleagues analysed microsatellite and sequence data from over 300 *S. noctilio* samples across five continents, yielding multiple scenarios regarding the history of introduction of *S. noctilio* to N. America. Their results indicate the presence in N. America of a mixture of specimens from Europe and South America, resulting possibly from independent introduction(s) from Europe and subsequent spread from invaded South America and Oceania.

Isolation of the same fungus of the same MLG from different *Sirex* spp. provided further evidence of the lack of fidelity

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between *Sirex* spp. and their symbiont. Although it was generally believed that the transmission of *Amylostereum* is uniparental and vertical between generation, which would result in the tight co-evolution of woodwasp host and fungal symbiont, recent studies have shown that one *Amylostereum* species can be carried by different woodwasp species (van der Nest *et al.* 2012; Hajek *et al.* 2013; Wooding *et al.* 2013). Our finding of *A. areolatum* from *Urocerus* spp., previously only reported as being associated with *Amylostereum chailletii*, extends the fungal symbiont sharing between two siricid genera.

The lack of siricid host-fungal symbiont fidelity underscores the need to study North American woodwasps and their associated *Amylostereum* spp. genotypes as *S. noctilio* continues to expand its geographic distribution. Our results also highlight the importance of obtaining fungal samples directly from woodwasps compared to collecting *Amylostereum* from infested trees, especially where native siricids are present for basic and applied ecological studies. For example, evaluations of control strategies against *S. noctilio* utilizing the nematode *Deladenus siricidicola* would require specific information on woodwasp–symbiont associations to accurately assess potential impact on North American woodwasps. Morris *et al.* (2013) observed that a strain of the nematode *D. siricidicola* introduced with *S. noctilio* also parasitizes North American *S. nigricornis* carrying *A. areolatum*. In addition, comparative studies on the reproduction of *D. siricidicola* strain Kamona, the strain used in the southern hemisphere for biological control and currently commercially available, revealed its ability to utilize different strains of *A. areolatum*. Nematode reproduction, however, varied, with higher numbers produced on strains GR94 (IGS BD) and ScyME (IGS BE) compared with strain SedDF (IGS D) (Morris *et al.* 2014). These results indicate lack of specificity, but variation in fitness among nematodes feeding on *Amylostereum*, and suggest the potential for non-target effects on North American *Sirex* species if *D. siricidicola* Kamona is used in North America.

Acknowledgements

We would like to thank Thomas Harrington and Doug McNew (Dept. of Plant Pathology, Iowa State University) for the cultures of *Amylostereum* spp.; and Stefanie Kroll, Stefan Long and Erin Morris (Dept. of Entomology, Cornell University) for technical assistance; and David Williams and Robert Rabaglia for valuable input. Funding was provided by USDA-APHIS, USDA AFRI NIFA (grant # 2009-02182), and USDA Forest Service (grant # 12-CA-11420004-043).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2015.03.004.

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