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Putative source of the invasive *Sirex noctilio* fungal symbiont, *Amylostereum areolatum*, in the eastern United States and its association with native siricid woodwasps

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ABSTRACT

Two genotypes of the fungal symbiont *Amylostereum areolatum* are associated with the invasive woodwasp *Sirex noctilio* first found in North America in 2004. *S. noctilio* is native to Europe but has been introduced to Australasia, South America and Africa where it has caused enormous losses in pine plantations. Based on nucleotide sequence data from the intergenic spacer region (IGS) of the nuclear ribosomal DNA, the *A. areolatum* genotypes found in North America are most similar to genotypes found in Europe, and not to genotypes from the southern hemisphere. Although two IGS strains of *A. areolatum* were found in North America it cannot be stated whether *A. areolatum* was introduced to North America from Europe once or twice based on our study. Genetic groupings formed by sequencing data were in most cases supported by vegetative compatibility groups (VCGs). Other siricid woodwasp species in the genus *Sirex* are native to North America. The North American native *Sirex edwardsii* emerging from the same tree as *S. noctilio* carried the same strain of *A. areolatum* as *S. noctilio*. The North American native *Sirex* sp. '*nitidus*' collected outside the geographical range of *S. noctilio* carried a unique strain within *A. areolatum*. Our findings of *A. areolatum* in the native North American species, *S.* sp. '*nitidus*', contrast with the previous view that *A. areolatum* was not present in North America before the accidental invasion of *S. noctilio*.

Introduction

Due to international travel and trade, incursive forest pests are continuously invading ecosystems worldwide. These incursions cause damage of both economic importance and ecological concern. In North America hundreds of species of insects and plant pathogens have accidentally been introduced and biological invasions are one of the most significant environmental and socioeconomic threats to natural forest ecosystems (Liebhold *et al.* 1995; Loo 2008). Thus, several invading forest pests have had substantial long-term effects on tree mortality and reproduction (e.g., Liebhold *et al.* 1995; Orwig *et al.* 2002; Mattson *et al.* 2007; Loo 2008). Among the best known examples from North America are the chestnut blight fungus, *Cryphonectria parasitica*, and the gypsy moth, *Lymantria dispar*, since both pest species have had severe impacts on the environment after their introduction into North America (Griffin 1986; Anagnostakis 1987; Liebhold *et al.* 1995; Loo 2008).

One of the most recent inadvertent biological invasions to North America is the homobasidiomycete *Amylostereum areolatum* which arrived with its symbiotic woodwasp, *Sirex noctilio*.

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This is a pine-killing pest complex that was anticipated with dread for several years before its discovery in New York State in 2004 (Ciesla 2003; Hoebeke *et al.* 2005; Carnegie *et al.* 2006). *S. noctilio* has now also been recorded from northern Pennsylvania, a significant area in southern Ontario, and single localities in Michigan and Vermont (de Groot *et al.* 2006; USDA APHIS, 2008). As of 1995 (Farr *et al.* 1995) *A. areolatum* had not been recorded from North America but this fungal species has now been recorded in association with *S. noctilio* infestations in Canada (Bergeron *et al.* 2008) and the United States (Wilson *et al.* 2009). *A. areolatum* and *S. noctilio* are native to Europe, northern Asia and northern Africa, where they are generally considered to be secondary pests on pine (Haugen 2007). However, the *A. areolatum*/*S. noctilio* complex has been accidentally introduced into various regions of the southern hemisphere during the twentieth century, where it has devastated plantings of introduced North American pines, causing up to 70 % tree mortality (Hurley *et al.* 2007). Pines are not native to the southern hemisphere but have been planted there extensively for solid timber and pulp. The *A. areolatum*/*S. noctilio* complex was first reported from New Zealand around 1900, Tasmania in the 1950s, Australia in the 1960s, South America in the 1980s and South Africa in the 1990s (Hurley *et al.* 2007). In North America this insect-tree pathogen complex would threaten any native pine tree species. If no action is taken to control the woodwasp and its fungus, almost US$ 2 billion in losses will have occurred by the time this pest complex has spread throughout the southern pine growing region (an estimated 55 y) (USDA Forest Service, 2006). The most likely sources for introduction of the *S. noctilio*-fungus complex were solid wood packing materials or other wooden articles shipped from either its native or introduced ranges (Hoebeke *et al.* 2005).

The white rot fungus *A. areolatum* relies on *S. noctilio* for dispersal and inoculation into trees; in turn, the woodwasp relies on the fungus for wood decomposition and food. The fungus is predominantly spread by the woodwasp in the form of asexually produced oidia (=arthrospores) in a very strict symbiosis. Spread of *A. areolatum via* basidiospores released from sporocarps is only known from the palearctic whereas sporocarps have never been documented from the southern hemisphere (Thomsen 1996; Slippers *et al.* 2003) and are also not found in northernmost Europe (Solheim 2006). *S. noctilio* females emerge from trees carrying *A. areolatum* in mycangia and subsequently deposit this fungus and toxic mucous into wood when laying eggs (Bedding 2009). The fungus and mucous act together to kill the tree and create a suitable environment for larval development. All larval instars feed on the fungal-infected wood as they tunnel through the wood (Madden 1981; Kukor & Martin 1983). The larval stages generally take 10–11 mo but may require 2 y in colder areas. Adult woodwasps are strong fliers, naturally dispersing an estimated 25–50 km/y in Australia (Haugen *et al.* 1990; Sirex Science Panel 2007). Over most of North America *S. noctilio* is expected to have one generation per year (Haugen 2007).

In North America, in addition to the newly introduced *S. noctilio*, there are several native species from the genus *Sirex* (Smith & Schiff 2002; Goulet H, personal communication). At present the precise number of native species is uncertain because the taxonomy of the North American *Sirex* is presently being revised (Goulet H, personal communication). All native North American *Sirex* use conifers as hosts, but they vary in preference for pine, spruce, fir, larch, and other conifers (Krombein *et al.* 1979; Goulet H, personal communication). The native North American species have been studied less than *S. noctilio*, but the biology of the native *Sirex* resembles the biology of *S. noctilio* in most respects. However, the fungal symbionts of these native species have been studied little although it has been reported that all the native North American *Sirex* species use *Amylostereum chailletii*, a different species of fungus than the one used by *S. noctilio* (Gilbertson 1984; Smith & Schiff 2002). Although it is possible to distinguish *Amylostereum* species morphologically, many misidentifications were made in the past, especially between *A. chailletii* and *A. areolatum* (Thomsen 1996; Slippers *et al.* 2003). Recent studies, including sequences from various rDNA regions, have provided new insights regarding the identity and phylogeny of the genus *Amylostereum* (Slippers *et al.* 2000; Tabata *et al.* 2000; Maijala *et al.* 2003), as well as geographical variation and patterns and spread of *A. areolatum* in the southern hemisphere (Slippers *et al.* 2002).

The ecology and dynamics of newly introduced invasive species can best be understood by (1) identifying the source population from which they originated, and (2) elucidating associations with closely related native species. The current study addresses these two baseline questions by sequencing the internally transcribed spacer region (ITS) and the nuclear intergenic spacer region (IGS) to assay the genetic profile of *Amylostereum* isolates collected from *S. noctilio* and various *Sirex* species native to the United States. In tandem, vegetative compatibility groupings (VCGs) were determined for selected isolates to complement ITS and IGS results.

Materials and methods

Isolation of symbiont

Amylostereum samples were obtained from *Sirex* females either reared from infested wood or trapped in the field. In late June 2007, infested trees at two sites in New York State were felled and pieces of the tree trunk were marked with the section number of the tree and placed in barrels (69 cm high × 48 cm diam.) closed with window screening on top. Barrels were checked daily throughout the summer and emerging *Sirex* were collected. Females were retained at 4 °C for up to 48 h before dissection. Females were dissected following the methods of Thomsen (1996) using a 1.0 mm curette (Fine Scientific Tools, Bath, UK). For each female dissected, one mycangium was used to initiate a fungal culture and one mycangium was used for DNA extraction. All fungal *in vitro* cultures were maintained on 1.5 % Potato Dextrose Agar (PDA) at 22 ± 2 °C in the dark. Plates used for the initial transfer of the fungus contained 0.03-g/L streptomycin sulfate to decrease the risk of contaminants, whereas growth of the subsequent cultures was on PDA without antibiotics.

In summer 2007, intercept panel traps with lures of α and β pinene (Aptiv, Portland, Oregon) were placed at numerous locations in the northeastern United States. Traps were checked as frequently as possible because obtaining living

specimens optimized successful isolation of *Amylostereum* cultures. All trap-collected specimens were stored in the freezer until dissection. No ethylene glycol was present in the collecting cups in the traps because it eliminated fungal recovery from specimens within 24 h (Hajek AE & Long SJ, unpublished data). *Sirex* that were collected from traps were treated as above to obtain fungal samples.

Fungal isolates

Twenty-three isolates of *Amylostereum* originating from *S. noctilio*, two isolates from *Sirex edwardsii*, nine isolates from *Sirex nigricornis* and two isolates from *Sirex* sp. '*nitidus*', all collected in the northeastern U.S., were included in the present study (Table 1). We will use *S.* sp. '*nitidus*' to refer to the North American species preferring spruce (*Picea* spp.) that is to be named *S. nitidus* (Harris) in a New World review of this species (Goulet H, personal communication). In addition, one isolate of *A. areolatum* that accompanied the parasitic nematode *Deladenus siricidicola*, purchased from Ecogrow (Bondi Beach, New South Wales, Australia) was included. This nematode was originally obtained from *Sirex juvencus* from Sopron, Hungary in 1966–1967 (strain 198) and has been mass produced and released for *S. noctilio* control in numerous southern hemisphere countries (Bedding 2009). It is thought that the original fungal strain accompanying *D. siricidicola* from Hungary in 1966–1967 is still associated with this commercially produced nematode (RA Bedding & RJ Akhurst, personal communication).

DNA extraction

Total genomic fungal DNA from mycangia was prepared using the UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories Inc, Carlsbad, CA) following the protocol given by the manufacturer using the alternative lysis method to avoid DNA shearing. DNA was eluted in 10 mM Tris–buffer, pH 9.0, and stored at −20 °C until use.

All *in vitro* fungal isolates were cultured on PDA plates and grown in constant darkness for 2–4 weeks, after which mycelia was carefully scraped from the plates with a sterile scalpel. Total genomic fungal DNA was isolated either using the UltraClean™ Soil DNA Isolation Kit, as described above, or alternatively by using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA), following the protocol given for DNA extraction from cultured animals cells. DNA was eluted in Buffer AE (10 mM Tris–Cl, 0.5 mM EDTA; pH 9.0).

DNA concentrations were quantified by UV spectrophotometry using a Thermo Scientific NanoDrop™ 1000. When possible, DNA originating from *in vitro* cultures was used instead of DNA from mycangia for further studies.

PCR and sequencing

The internally transcribed spacer regions (ITS) were amplified and sequenced for 11 of the *Amylostereum* isolates listed in Table 1 and the nuclear intergenic spacer region (IGS) were amplified and sequenced for all thirty-seven isolates listed in Table 1. The primers used for the ITS region were the Basidiomycete-specific primer ITS1-F (5′CTTGGTCATTTAGAGGAA GTAA′3) (Gardes & Bruns 1993) and the fungal-specific primer ITS4 (5′TCCTCCGCTTCTTGATAGC′3 (White *et al.* 1990). For the nuc-IGS-rDNA region the two following Basidiomycete-specific primers were used: P-1 (5′TTGCAGACGACTTGAATGG′3) (Hsiau 1996) and 5S-2B (5′CACCGCATCCCGTCTGATCTGCG′3) (Slippers *et al.* 2002). Procedures described by Tabata *et al.* (2000) and Slippers *et al.* (2002) were used for amplification. Amplification was performed in 0.2 ml Thermowell tubes (Corning Inc., Corning, NY) in a total volume of 50 μl, with PCR mixtures containing 1 × PCR buffer with 1.5 mM $MgCl_2$ (Qiagen); 1 × Q-solution, 200 μM each of dATP, dCTP, dGTP, and dTTP (Qiagen); 0.5 μM of each primer; 2–10 ng fungal DNA; and 2.5 U Taq PCR enzyme (Qiagen). Amplification was performed in a thermal cycler (PTC-100 Peltier, MJ Research, Boston, MA) with the following parameters for the ITS region: Denaturation at 94 °C for 85 s, 13 cycles of 35 s DNA denaturation at 94 °C, 55 s annealing at 55 °C and 45 s extension at 72 °C, 13 cycles of 35 s DNA denaturation at 94 °C, 55 s annealing at 55 °C and 2 min extension at 72 °C followed by 13 cycles of 35 s DNA denaturation at 94 °C, 55 s annealing at 55 °C and 3 min extension at 72 °C, followed by a final extension at 72 °C for 10 min. Parameters for amplification of the IGS region are as follows: Initial DNA denaturation at 94 °C for 3 min, 12 cycles of 15 s DNA denaturation at 94 °C, 30 s annealing at 58 °C and 45 s, 1 min extension at 72 °C, followed by 22 cycles with settings as the first 12 IGS cycles but with the extension time increasing by 15 s per cycle.

PCR products were subjected to electrophoresis on a 1.5 % (w/v) ethidium bromide–stained gel and visualized under UV illumination. Products were purified using the QIAquick PCR purification kit from Qiagen and sequenced by the Cornell University Life Sciences Core Laboratories Center, Ithaca, NY using BIG Dye Terminator chemistry and AmpliTaq-FS DNA polymerase (Applied Biosystems). Both template and coding strands were sequenced.

For IGS results, in cases where multiple bands occurred, the PCR product was cleaned up using the QIAquick PCR purification kit from Qiagen followed by cloning using the Qiagen PCR Cloning^{plus} kit. For each PCR product, 15 bacterial colonies were chosen for seeding a PCR amplification using the same parameters as described above. PCR products were subjected to electrophoresis on a 1.5 % (w/v) ethidium bromide–stained gel and visualized under UV illumination. For each PCR product size, two replicate products were purified and sequenced as described above.

Sequences were aligned using ClustalW (Thompson *et al.* 1994) with default parameters, and manually adjusted in BioEdit Sequence Alignment Editor (Hall 1999). BLAST searches were performed in GenBank to identify related sequences. For ITS sequences two Canadian, one Japanese, one Swedish and one French *A. areolatum* isolates, and four isolates of *A. chailletii* from Canada, United Kingdom, Sweden and Germany, one *Amylostereum laevigatum* isolate from France, one *Amylostereum ferreum* isolate from Brazil as well as one *Amylostereum* sp. isolate from North America were identified as alignable sequences and included in the study for further analysis (Table 2). For the IGS region, sequences published in Slippers *et al.* (2002) were kindly provided by B. Slippers and included in further analysis (Table 3).

Table 1 – Collection data for *Amylostereum* strains used in our study. All specimens initiated from *Sirex* females, either collected in the field, trapped or reared from wood. For ID number marked by *, DNA originated from mycangia, whereas DNA from the remaining samples originated from *in vitro* cultures initiated from mycangia.

ID number	Host species	State/country	County	Site	Tree/section	Coll.date	Sequenced	GenBank accession nos. (IGS)	GenBank accession nos. (ITS)
Amylostereum areolatum									
AH1-01	*S. noctilio*	NY/USA	Oswego	New Haven	AH1/QAA	16-Jul-07	ITS, IGS	GQ422452	GQ422464
AH1-06	*S. noctilio*	NY/USA	Oswego	New Haven	AH1/QAA	16-Jul-07	ITS, IGS		
AH1-15	*S. noctilio*	NY/USA	Oswego	New Haven	AH1/GJ	8-Aug-07	IGS		
AH1-17	*S. noctilio*	NY/USA	Oswego	New Haven	AH1/DF	27-Aug-07	IGS		
AH1-21	*S. noctilio*	NY/USA	Oswego	New Haven	AH1/GJ	5-Sep-07	IGS		
AH2-10	*S. noctilio*	NY/USA	Oswego	New Haven	AH2/DH	20-Aug-07	IGS		
AH2-14	*S. noctilio*	NY/USA	Oswego	New Haven	AH2/DH	21-Aug-07	IGS		
AH3-08	*S. noctilio*	NY/USA	Oswego	New Haven	AH3/LT	12-Jul-07	IGS		
AH3-29	*S. noctilio*	NY/USA	Oswego	New Haven	AH3/FK	19-Jul-07	IGS		
AH4-09	*S. noctilio*	NY/USA	Oswego	New Haven	AH4/DG	17-Jul-07	IGS		
AH4-23	*S. noctilio*	NY/USA	Oswego	New Haven	AH4/A	24-Aug-07	IGS		
P6-05	*S. noctilio*	NY/USA	Onondaga	Pompey	P6/AD	18-Jul-07	IGS		
P6-06	*S. noctilio*	NY/USA	Onondaga	Pompey	P6/IM	9-Jul-07	ITS, IGS		
P6-07	*S. noctilio*	NY/USA	Onondaga	Pompey	P6/IM	9-Jul-07	ITS, IGS		
GR-94-1	*S. noctilio*	NY/USA	Fulton	Granby	–	19-Feb-08[c]	IGS	GQ422458/GQ422459	
GR-94-2*	*S. noctilio*	NY/USA	Fulton	Granby	–	19-Feb-08[c]	IGS		
McK-12/20	*S. noctilio*	PA/USA	McKean	Kanesholm	–	20-Dec-07	IGS		
McK-12/27-1	*S. noctilio*	PA/USA	McKean	Kanesholm	–	27-Dec-07	IGS		
McK-12/27-2*	*S. noctilio*	PA/USA	McKean	Kanesholm	–	27-Dec-07	ITS, IGS		
McK-12/21*	*S. noctilio*	PA/USA	McKean	Kanesholm	–	21-Dec-07	IGS		
McK-12/26*	*S. noctilio*	PA/USA	McKean	Kanesholm	–	26-Dec-07	IGS		
McK-1/2-08*	*S. noctilio*	PA/USA	McKean	Kanesholm	–	2-Jan-08	IGS		
OtisAa	*S. noctilio*	NY/USA	Oswego	Oswego	–	Jan 2006	ITS, IGS	GQ422453	GQ422465
Ecogrow[a]	*S. juvencus*	Hungary	–	Sopron	–	1966-1967	ITS, IGS	GQ422455/GQ422456/GQ422457	GQ422466
S. cy ME-9/10	*S.* sp. '*nitidus*'[b]	ME/USA	Waldo	Winterport	TRAP	10-Sep-07	ITS, IGS	GQ422460/GQ422461	GQ422468
S. cy ME-9/04*	*S.* sp. '*nitidus*'	ME/USA	Waldo	Winterport	TRAP	4-Sep-07	IGS		
S. ed DF 9/18	*S. edwardsii*	NY/USA	Oswego	New Haven	AH 1/DF	18-Sep-07	ITS, IGS	GQ422454	GQ422467
S. ed GJ 9/18*	*S. edwardsii*	NY/USA	Oswego	New Haven	AH 1/GJ	18-Sep-07	IGS		
Amylostereum chailletii									
S. ni GJ-9/18*	*S. nigricornis*	NY/USA	Oswego	New Haven	AH 1/GJ	18-Sep-07	IGS		
S. ni DF-9/21-2	*S. nigricornis*	NY/USA	Oswego	New Haven	AH 1/DF	21-Sep-07	ITS, IGS	GQ422462	GQ422469
S. ni GJ-9/20-2	*S. nigricornis*	NY/USA	Oswego	New Haven	AH 1/GJ	20-Sep-07	ITS, IGS		
S. ni GJ-9/27-3	*S. nigricornis*	NY/USA	Oswego	New Haven	AH 1/GJ	27-Sep-07	IGS		
S. ni DF-9/25*	*S. nigricornis*	NY/USA	Oswego	New Haven	AH 1/DF	25-Sep-07	IGS		
S. ni LaF 9/27	*S. nigricornis*	NY/USA	Onondaga	Lafayette	–	27-Sep-07	IGS		
S. ni P5 9/18-1	*S. nigricornis*	NY/USA	Onondaga	Pompey	P5/CE	18-Sep-07	IGS		
S. ni INT9/11*	*S. nigricornis*	NY/USA	Seneca	Interlaken	TRAP	11-Sep-07	IGS		
DWAch2	*S. nigricornis*	NY/USA	Onondaga	Spafford	LIVE	~2 Oct-07	ITS, IGS	GQ422463	GQ422470

a Obtained from Ecogrow. Records regarding the source of this isolate were not kept but as far as is known the strain of *A. areolatum* now being used to mass produce *Deladenus siricidicola* is the same strain that was initially isolated in 1966–1967 for growing this nematode; this strain has been used for mass production of these nematodes ever since.

b This mention of an undescribed species does not constitute valid publication for purposes of zoological nomenclature.

c Reared indoors from wood cut during fall. Females emerged 27-Dec-07 and kept at 1 °C until fungal isolation.

Table 2 – ITS sequences obtained from GenBank and included in the present study.

Origin	Host insect or tree	No. of isolates	Source	GenBank accession no.
Amylostereum areolatum				
Japan	*Sirex nitobei*	1	Tabata *et al.* 2000	AF218389
Ontario, Canada	*Pinus sylvestris*	2	Bergeron *et al.* 2008	EU249343/249344
Sweden	*Picea abies*	1	Vasiliauskas *et al.* 2005	AY781245
France	Unknown	1	Lickey EB, Hughes KW & Petersen RH, unpublished data	AF454428
Amylostereum chailletii				
United Kingdom	*Urocerus gigas*	1	Tabata *et al.* 2000	AF218391
Germany	*Urocerus gigas*	1	Tabata *et al.* 2000	AF218392
Canada	Fruiting body hemlock	1	Tabata *et al.* 2000	AF218393
Sweden	*Picea abies*	1	Vasiliauskas *et al.* 2004	AY805604
Amylostereum laevigatum				
France	*Juniperus nana*	1	Tabata *et al.* 2000	AF218396
Japan	*Urocerus japonicus*	1	Tabata *et al.* 2000	AF218395
Amylostereum ferreum				
Brazil	*Podocarpus lambertii*	1	Tabata *et al.* 2000	AF218390
Amylostereum sp.				
US	*Sirex areolatus*	1	Tabata *et al.* 2000	AF218394

IGS sequences contained several insertion/deletion mutations (indels). These indels were coded by simple indel coding using the program GapCoder (Young & Healy 2003).

Cluster analysis

Unrooted parsimony analysis was implemented using the computer software PHYLIP 3.68 (Felsenstein 2008). The method of Fitch (1971) was used to minimize the number of character changes on phylogenetic trees. Support for internal branches was assessed by 1000 bootstrap replications.

VCG analysis

Selected isolates of *A. areolatum* and *A. chailletii* maintained on PDA were tested in pairs and assigned to different VCG groupings principally using the procedure described by Thomsen & Koch (1999). Inoculations were made with approximately 0.7 mm × 0.7 mm square plugs cut from the edges of actively growing cultures. Inoculation plugs were placed approximately 2 cm apart in the center of a 6 cm Petri dish containing PDA. All plates were incubated at 22 ± 2 °C in constant darkness for 2–3 weeks, although, in some cases more time was needed since some isolates grew only slowly toward the opposing isolate. 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. In cases where ambiguous reactions were observed two more replicates were carried out.

Results

ITS sequences

The nuc-ITS-rDNA region targeted with primers ITS4 and ITS1-F was successfully amplified for all *Amylostereum* isolates (Table 1). The ITS region was highly conserved in all *Amylostereum* species based on a fragment of about 650 bp. For *A. chailletii* the PCR products were successfully sequenced in both directions and all isolates gave PCR products of 643 bp. For *A. areolatum* 287 bp were successfully sequenced with primer ITS1-F and 356 bp were successfully sequenced using the primer ITS4 and, after these points, double peaks occurred suggesting ITS heterogeneity. No overlap was found between the two sequenced strands, giving a total of 643 successfully sequenced base pair. Among the sequences found in GenBank two Canadian isolates included the entire sequence we were targeting in the present study; one of the isolates (EU249343) had a target length of 643 bp and the other had a target length of 644 bp (EU249344). These two Canadian isolates had identical sequences differing only in the presence of one additional nucleotide (A) in EU249344, exactly at the point where we noticed double peaks in our sequences. All other isolates of *Amylostereum* found in GenBank lacked nucleotide A at this same location. In the complete data set, including sequences from GenBank, there were 502 alignable characters, excluding the ambiguous character found after 287 bp/356 bp. The ITS region was highly conserved in all *Amylostereum* species, with 92 % (460 base pairs) conserved among all taxa. Within *A. areolatum* only eight characters differed among the isolates included in the present study and for *A. chailletii* seven characters differed among the included isolates. Seven most parsimonious trees of 59 steps were found (Fig 1). Further neighbor-joining analysis of the same data resulted in a tree with the same topology as for the most parsimonious trees (neighbor-joining tree not shown). Strong bootstrap support was found for the inferred clade that included all *A. chailletii* isolates as well as the inferred clade that included all *A. areolatum* isolates (Fig 1). For North American *A. areolatum*, there were no differences between sequences originating from the invasive species *S. noctilio* (six isolates from three sites in New York and one site in Pennsylvania) and the native species *S. edwardsii* (one isolate from New York). However, the isolate originating from the native species *S.* sp. '*nitidus*' collected

Table 3 – IGS grouping. A: 622 bp, B: 638, C: 570, D: 618, E: 598, F: 589, G: 552, H: 538, I: 558, J: 569.

Origin	Host or source	No. of isolates	IGS group	Source
Amylostereum areolatum				
Tasmania	*Sirex noctilio*	1	AB	Slippers *et al.* 2002
New Zealand	Wood with *Sirex noctilio*	1	AB	Slippers *et al.* 2002
Brazil	*Sirex noctilio*	2	AB	Slippers *et al.* 2002
South Africa	*Sirex noctilio*	2	AB	Slippers *et al.* 2002
Denmark	Fruiting body *Picea abies*	1	AB	Slippers *et al.* 2002
Lithuania	Wood of wounded *Picea abies*	1	AC	Slippers *et al.* 2002
Lithuania	Wood of wounded *Picea abies*	1	BC	Slippers *et al.* 2002
Sweden	Wood of wounded *Picea abies*	1	AC	Slippers *et al.* 2002
Denmark	Fruiting body *Picea abies*	1	BC	Slippers *et al.* 2002
Germany	Wood of *Picea abies*	1	D	Slippers *et al.* 2002
France	Unknown	1	C	Slippers *et al.* 2002
Australia (Ecogrow)[a]	*Sirex juvencus*	1	BDF	This study
US New Haven NY	*Sirex noctilio*	10	D	This study
US Pompey NY	*Sirex noctilio*	2	D	This study
US Granby NY	*Sirex noctilio*	2	BD	This study
US Lafayette NY	*Sirex noctilio*	1	D	This study
US Kanesholm PA	*Sirex noctilio*	5	BD	This study
US Kanesholm PA	*Sirex noctilio*	1	D	This study
US Winterport ME	*Sirex* sp. '*nitidus*'	2	BE	This study
US New Haven NY	*Sirex edwardsii*	2	D	This study
Amylostereum chailletii				
Scotland	Mycangium of *Urocerus gigas*	1	G	Slippers *et al.* 2002
Canada	Fruiting body *Abies balsamea*	1	G	Slippers *et al.* 2002
Canada	Fruiting body *Tsuga* sp.	1	G	Slippers *et al.* 2002
US New Haven NY	*Sirex nigricornis*	4	G	This study
US Lafayette NY	*Sirex nigricornis*	1	G	This study
US Pompey NY	*Sirex nigricornis*	1	G	This study
US Interlaken NY	*Sirex nigricornis*	1	G	This study
Amylostereum laevigatum				
France	*Juniperus nana*	1	H	Slippers *et al.* 2002
Amylostereum ferreum				
Brazil	*Podocarpus lambertii*	1	I	Slippers *et al.* 2002
Amylostereum sp.				
US California	*Sirex areolatus*	1	J	Slippers *et al.* 2002

a Isolate used for mass production of the *S. noctilio*-parasitic nematode *D. siricidicola*; this is not the isolate of *A. areolatum* recorded from the field in South Africa, New Zealand, Tasmania, or Brazil (=AB in the field) (Slippers *et al.* 2002), although fungal strains used for nematode mass propagation (either BC or BDF) have been released there along with nematodes.

outside the range of *S. noctilio* could be differentiated from all other North American *A. areolatum* isolates based on two characters. North American *A. chailletii* fell into two clades. One clade included two isolates; one from New Haven, NY (Sni DF-9/21-2) and one from Spafford, NY (DWAch2), and the other clade included one isolate from New Haven only (SniGJ-9/20-2).

IGS sequences

The nuc-IGS-rDNA regions were successfully amplified with the primer pair P-1/5S-2B for all isolates included in this study, both for *in vitro* isolates as well as for rDNA isolated directly from *Sirex* mycangia. The sequences amounted to a total of 695 characters after alignment. Fragment sizes were homogeneous and species specific for *A. chailletii* (552 bp), *A. laevigatum* (538 bp), *A. ferreum* (558 bp) and *Amylostereum* sp. (569 bp). In contrast, isolates of *A. areolatum* contained from one to three different sequences in each isolate. The PCR fragment sizes for the *A. areolatum* isolates were reproducible in each isolate in which they occurred. Altogether five sequences ranging from 622 to 570 bp were documented in *A. areolatum* and they will hereafter be referred to as strains A–F (Fig 2, Table 3), with sequences A–D being identical to the sequences reported by Slippers *et al.* (2002). Sequences B–F were basically identical except for several major indels, whereas sequence A differed from the other isolates at several positions (Fig 3). Based on PCR fragment sizes *A. areolatum* could be divided into seven major groups each consisting of either a single PCR product or double or triple PCR products (AB, AC, BC, D, BDF, BD and BE) (Fig 2, Table 3). Two sequences of *A. areolatum* were isolated from North American *S. noctilio*: B and D (Fig 2, Table 3). The B sequence was only found in association with D although the D sequence was frequently found alone. Both BD and D sequences were isolated from the same site (Kanesholm, Pennsylvania) in one instance. The B sequence is also present in isolates from Europe (Denmark) and the southern hemisphere (Tasmania, New Zealand, Brazil, South Africa), whereas the D sequence is only known from Europe (Germany). *S. noctilio* isolates originating from the southern

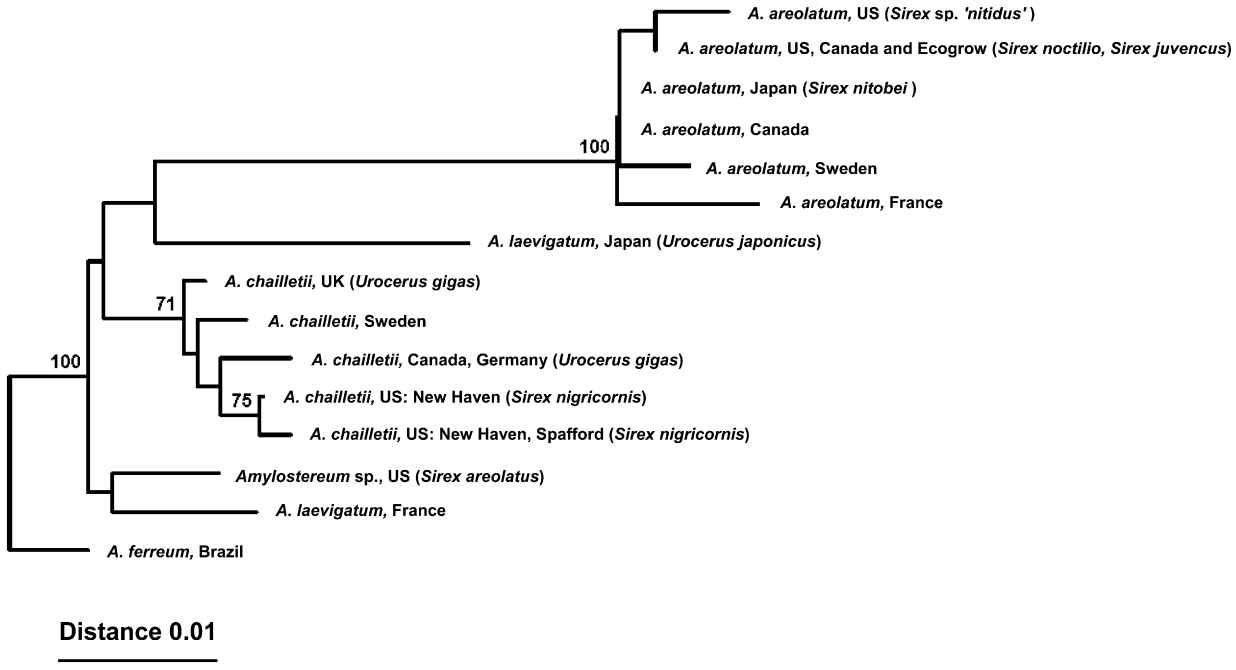

Fig 1 – One of the seven most parsimonious ITS trees of 59 steps based on 502 alignable characters of the ITS-1, 5.8S, and ITS-2 regions of the nuclear rDNA of *Amylostereum*. Siricids from which fungal strains were isolated are included in the figure, whereas trees from which fungal strains were isolated are excluded in the figure but are listed in Table 2. Bootstrap values above 70 % (1000 replications) are indicated above branching. The tree was generated in the Phylogeny Inference Package PHYLIP v. 3.68 with the "More thorough search" option in order to save multiple tied trees, without collapsing internal branches that have no evidence of change on them.

hemisphere contained in all instances the A sequence, a sequence also occurring in Europe but not found from North American samples.

Two isolates (one *in vivo* and one *in vitro*) originating from the native species *S. edwardsii* emerged from tree AH1 at the New Haven site in NY, USA were also included in this study. Both isolates were homogeneous and contained the D sequence only, which was also the only sequence documented from *S. noctilio* at the New Haven site (Fig 2, Table 3). Two isolates originating from the North American native species *S.* sp. '*nitidus*' collected outside the range of *S. noctilio* were documented to yield two IGS–PCR fragments, namely the B sequence as well as E sequence; the E sequences were unique to the *A. areolatum* isolate originating from *S.* sp. '*nitidus*', whereas the B sequence is also known from the host insects *S. noctilio* and *Sirex juvencus* (Fig 2, Table 3).

VCG compatibility

As expected, *A. areolatum* strains were incompatible with *A. chailletii* strains (Table 4). *A. areolatum* D strains were all compatible with each other as were BD strains. The *A. areolatum* strain (BE) from the native siricid *S.* sp. '*nitidus*' was not compatible with either of the *S. noctilio* strains from New York and Pennsylvania. Vegetative compatibility of the BDF strain used to mass produce *D. siricidicola* (Ecogrow) in Australia was less clear. The BDF strain was compatible with both BD strains tested as well as the BE strain from the native *S.* sp. '*nitidus*'. However, the VCG test between Ecogrow and D strains was in one case compatible (AH1-15), and in three cases ambiguous or weakly incompatible (Table 4).

Discussion

Sirex noctilio was introduced to the southern hemisphere from Europe and was first detected in the North Island of New Zealand around 1900 (Chou 1991). One genotype of its symbiotic fungus, *A. areolatum*, was introduced with it (nuc-IGS-rDNA strain AB; Slippers *et al.* 2001, 2002). This same genotype has now been found throughout the southern hemisphere, as *S. noctilio* and *A. areolatum* were spread to the South Island of New Zealand, Australia, South America and South Africa. *Sirex noctilio* was first found in North America in New York State in samples collected in 2004 (Hoebeke *et al.* 2005). In contrast to the introduction to the southern hemisphere, we found two genotypes of *A. areolatum* associated with *S. noctilio* in New York State and Pennsylvania, one homogeneous strain (D) and one heterogeneous strain (BD). The homogeneous *A. areolatum* sequence is equivalent to an isolate obtained from spruce (*Picea abies*) in Lautenthal, Germany in 1967 (CBS 305.82). The heterogeneous *A. areolatum* IGS sequence from *S. noctilio* containing two IGS sequences had not previously been reported but includes the B sequence previously known from Europe and the southern hemisphere (Slippers *et al.* 2002) along with the D sequence. Thus the *A. areolatum* nuc-IGS-rDNA genotypes found in North America are most similar to genotypes found in Europe, which is in accordance with findings by B. Slippers (personal communication).

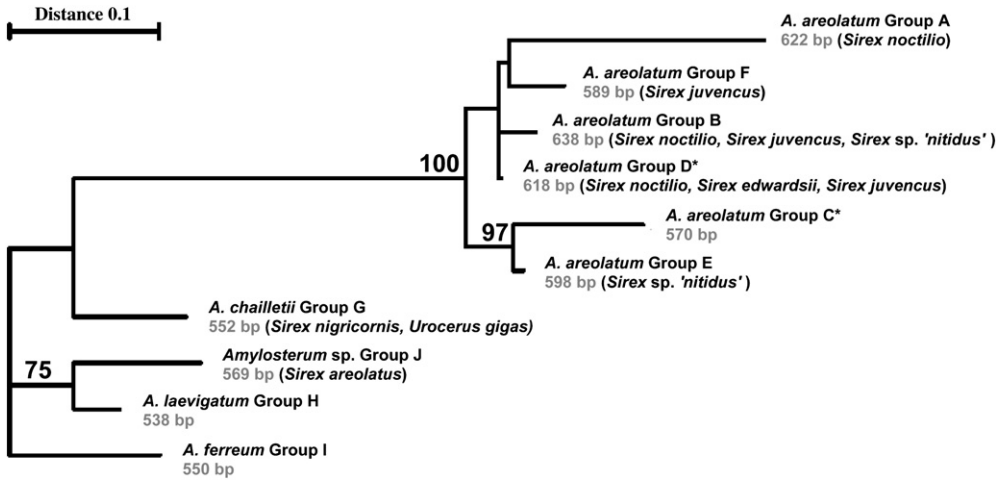

Fig 2 – One of the three most parsimonious IGS tree of 709 steps based on 774 alignable characters of the IGS region of the nuclear rDNA of *Amylostereum*. Siricids from which fungal strains were isolated are included in the figure, whereas trees from which fungal strains were isolated are excluded in the figure but are listed in Table 3. Bootstrap values above 70 % (1000 replications) are indicated above branching. The tree was generated in the Phylogeny Inference Package PHYLIP v. 3.68 with the "More thorough search" option in order to save multiple tied trees, without collapsing internal branches that have no evidence of change on them. * In Slippers *et al.* (2002) the CBS culture numbers were accidentally switched for the French and German isolates included in their study. According to B Slippers (personal communication) sequence C originated from France (CBS334.66) and sequence D from Germany (CBS305.82).

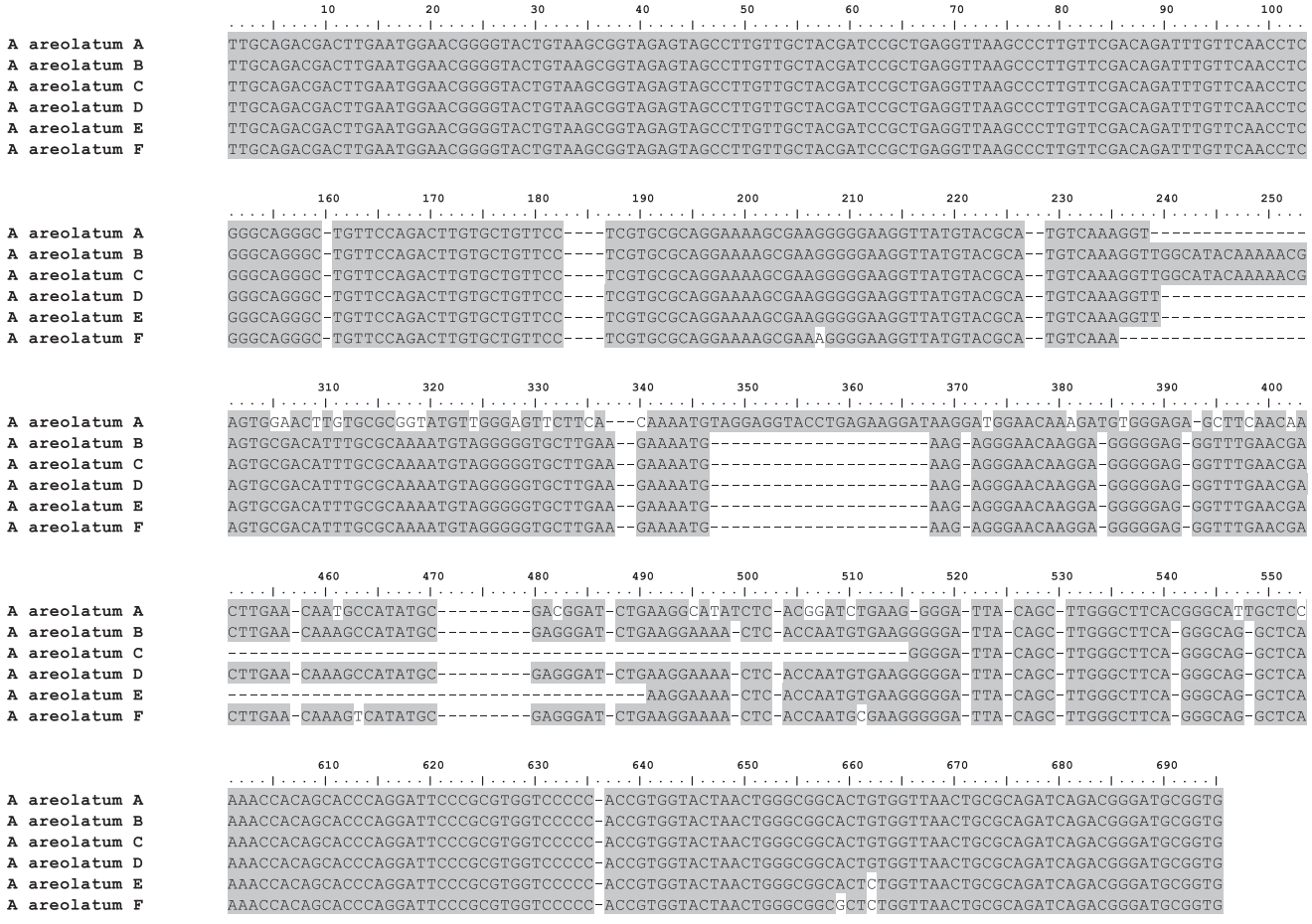

Fig 3 – Nucleotide sequences of IGS regions from groups A–F identified during this study.

Table 4 – Isolates used for vegetative compatibility tests. + = compatible, − = incompatible, ± = weakly incompatible in at least one of three replicate pairings.

Isolate no. (IGS group)	AH1-15 (D)	P6-05 (D)	OtisAa (D)	Mck-12/27-1 (BD)	gr-94-1 (BD)	Ecogrow (BDF)	SedDF09/18 (D)	ScyME09/10 (BE)	SniGJ09/27-3 (G)	SniDF09/21-2 (G)	DWAch2 (G)
Amylostereum areolatum											
AH1-15 (D)	+	+	+	−	−	+	+	−	−	−	−
P6-05 (D)	+	+	+	−	−	±	+	−	−	−	−
OtisAa (D)	+	+	+	−	−	±	+	−	−	−	−
McK-12/27-1 (BD)	−	−	−	+	+	+	−	−	−	−	−
GR-94-1 (BD)	−	−	−	+	+	+	−	−	−	−	−
Ecogrow (BDF)	+	+	±	+	+	+	−	−	−	−	−
SedDF09/18 (D)	+	+	+	−	−	±	+	−	−	−	−
ScyME09/10 (BE)	−	−	−	−	−	+	−	+	−	−	−
Amylostereum chailletii											
SniGJ09/27-3 (G)	−	−	−	−	−	−	−	−	+	+	+
SniDF09/21-2 (G)	−	−	−	−	−	−	−	−	+	+	+
DWAch2 (G)	−	−	−	−	−	−	−	−	+	+	+

The D strain that we found in all *A. areolatum* from *S. noctilio* in North America has only been identified from Germany previously (Slippers *et al.* 2002, see above). By coincidence, the single country that was the source of the most woodwasp interceptions at U.S. ports of entry between 1993–2001 was Germany (37.9 % of interceptions (Ciesla 2003).

By combining results from the present study with results reported by Slippers *et al.* (2002) five sequences of *A. areolatum* have been detected and these were found in two homogeneous states (C, D) and in six heterogeneous states (AB, AC, BC, BD, BE and BDF). The AB genotype that is so widely distributed in the southern hemisphere was never found among our samples from New York State and Pennsylvania. The homogeneous C sequence, known only from Europe, was also not found among our samples. *Sirex noctilio* in North America always contained the D genotype but some isolates also contained the B genotype. In the past, it was reported that species of *Sirex* native to North America only carried *A. chailletii* (Gilbertson 1984; Smith & Schiff 2002) and *A. areolatum* was not recorded from North America (Farr *et al.* 1995). However, specimens of *S.* sp. '*nitidus*' included in this study, carried the BE strain of *A. areolatum*. This siricid was previously referred to as *S. juvencus* (Smith & Schiff 2002) and was erroneously thought to have been introduced from Europe, although current taxonomic studies recognize that this species differs from the European *S. juvencus*. The *S.* sp. '*nitidus*' from which BE was obtained were collected in Maine in 2007, far from the known distribution of the invasive *S. noctilio*. To our knowledge, the fungal symbiont associated with *S.* sp. '*nitidus*' in Maine has not been examined previously and our results suggest that *A. areolatum* could indeed be native to North America. Slippers *et al.* (2002) reported that the presence of the B sequence in *A. areolatum* from *S. juvencus* as well as *S. noctilio* suggests that gene flow occurred between these isolates at some time in the past. Our findings of an *A. areolatum* B sequence in the native North American *S.* sp. '*nitidus*' would also suggest that gene flow occurred between European and North American isolates in the past.

In our study, the nuc-ITS-rDNA region was informative for distinguishing between *Amylostereum* species, whereas no or only little variation was found among isolates within an *Amylostereum* species. The ITS region demonstrated homology among *A. areolatum* isolates originating from *S. noctilio* but isolates from *S. noctilio* differed with isolates from *S.* sp. '*nitidus*'. However, for all *A. areolatum* isolates, from one to a few ambiguous characters were excluded from our alignments. The ambiguous characters probably originated from different ribosomal repeats, differences among nuclei in mycelia or possibly variability in the mycelium because isolates were not single spored. In a recent paper, Wilson *et al.* (2009) reported an ITS sequence for *A. areolatum* from New York (based on nine isolates) that differed by <98.8 % from two ITS sequences obtained from Canadian *A. areolatum* isolates from Ontario (Bergeron *et al.* 2008). Wilson *et al.* (2009) hypothesized that the lack of complete homology between Canadian and New York *A. areolatum* isolates indicated multiple *S. noctilio* introductions to North America. In the present study however, only IGS-D strains were included for ITS sequencing and no IGS-BD strains were included; it is therefore unknown at present

whether isolates belonging to different IGS groups display different ITS sequences. Although we found two IGS strains of *A. areolatum* in New York State and Pennsylvania, based on current results, it cannot be stated whether *A. areolatum* was introduced to North America from Europe once or twice. The occurrence of two different strains could be explained by two introductions but there are other plausible explanations. Based on past interceptions (Hoebeke *et al.* 2005), it is generally assumed that *S. noctilio* was introduced from Europe in infested wood, with adults emerging and dispersing after their arrival. During the present study, we found both *A. areolatum* and *A. chailletii* in mycangia from *Sirex* females of different species emerging from the same section of a tree. Thomsen & Koch (1999) reported finding different strains of *A. chailletii* from the same section of a tree. During the present study, we also found both strains of *A. areolatum* (BD and D) in *S. noctilio* from the same collection site (i.e., Kanesholm, Pennsylvania). Our results suggest that it is possible that *S. noctilio* in North America could have arrived in one infested log, containing both strains of *A. areolatum*.

A. areolatum rarely makes sporocarps and basidiospores in northern Europe and is not known to make sporocarps in the southern hemisphere (Thomsen 1996; Slippers *et al.* 2003). Dispersal of this fungus by basidiospores is not needed for spread because the woodwasps disperse asexual spores (oidia) of this fungus. Studies in northern Europe have demonstrated that populations of *A. areolatum* are highly clonal, with low genetic variability among clones and it is therefore believed that *A. areolatum* primarily disperses *via* its winged symbionts in these areas (Vasiliauskas *et al.* 1998). In agreement with trends from European vegetative compatibility studies (Thomsen & Koch 1999; Vasiliauskas & Stenlid 1999), we found different VCG groups of *A. areolatum* carried by *S. noctilio*. VCG groups were in most cases supported by IGS groupings. However, we also found that in some cases pairings between D and BDF strains gave ambiguous results. This might, however, not be surprising since Vasiliauskas *et al.* (1998) found that vegetative compatibility tests differentiated several genets with identical minisatellite DNA markers. Vegetative compatibility in *A. areolatum* has recently been documented to be controlled by at least two loci, and these loci appear to be multiallelic (van der Nest *et al.* 2008). Therefore it is expected that *A. areolatum* would have many different VCGs and that related heterokaryons differing at a single locus could display weak incompatibility (van der Nest *et al.* 2008). van der Nest *et al.* (2008) concluded that vegetative compatibility tests often will not differentiate clearly between genetically different, but closely related, *A. areolatum* strains; this may explain our inconsistent or weak incompatibility between the BDF strain from Ecogrow and North American D strains. We therefore conclude that to elucidate clonality and genetic variation in *A. areolatum*, studies including both molecular characterization and vegetative compatibility are recommended. Due to only little variations in the ITS and IGS regions, future research including other molecular methods with higher resolution such as AFLP or Simple Sequence Repeats (SSRs) might provide additional useful information.

There has been controversy in the literature regarding the specificity of associations between *Sirex* species and fungal species; Francke-Grosmann (1939; as reported in Talbot 1977) stated that different *Sirex* species are not always associated with the same fungal species, although for individual *Sirex* species there is a dominant fungal species, while Talbot (1977) stated that woodwasp/fungus specificity was very strict. In this study, we found that *S. noctilio* and the native *S. edwardsii* emerging from the same wood carried the same *A. areolatum* strain. In contrast, previous authors reported that *S. edwardsii* carries *A. chailletii* (Bedding & Akhurst 1978; Gilbertson 1984). Surprisingly, the native *S. nigricornis* also emerging from that same tree section carried *A. chailletii*, supporting the woodwasp/fungus association reported by others (Bedding & Akhurst 1978; Gilbertson 1984). Our findings reopen the debate regarding the specificity of the *Sirex*/*Amylostereum* association. These results will have broader impact because the fungal association of *Sirex* also influences the potential for biological control with parasitic nematodes.

The IGS results from *A. areolatum* fit expectations for basidiomycetes, in which a mycelium composed of two different nuclei is the most common form of vegetative mycelia (Johannesson & Stenlid 2004); five *A. areolatum* strains included two different IGS sequences while two strains were homogeneous and one strain included three IGS sequences. Nuclear transfer and reassortment during anastomosis have been suggested as a source of such variation in the white rot fungus *Heterobasidion annosum* in which four nuclear types were identified in the same mycelium (Johannesson & Stenlid 2004). However, it is also possible that there are different ribosomal repeats within a single nucleus since there are numerous repeats of ribosomal genes. The strain of *A. areolatum* from Australia included three different IGS sequences, including the F, which was not found in other isolates during our study. The Australian *A. areolatum* strain has been used for mass production of *Deladenus* (=*Beddingia*) *siricidicola* since 1966–1967; the long history of laboratory propagation for this strain makes it quite different from the other strains used in this study. The *A. areolatum* strain used for propagation of *D. siricidicola* in Australia was also isolated in 1995 and included in a study by Slippers *et al.* (2002); these authors reported that the IGS for this strain was BC, which differs from our results of BDF when we isolated the fungus that accompanied the nematode purchased from Ecogrow in Australia in late 2006. Differences among these strains used for mass-producing *D. siricidicola* must be investigated further.

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REFERENCES

Anagnostakis SL, 1987. Chestnut blight: the classical problem of an introduced pathogen. *Mycologia* **79**: 23–37.

Bedding RA, 2009. Controlling the pine-killing woodwasp, *Sirex noctilio*, with nematodes. In: Hajek AE, Glare TR, O'Callaghan M (eds), *Use of Microbes for Control of Invasive Arthropods*. Springer, Dordrecht, Netherlands, pp. 213–235.

Bedding RA, Akhurst RJ, 1978. Geographical distribution and host preferences of *Deladenus* species (Nematoda: Neotylenchidae) parasitic in siricid woodwasps and associated hymenopterous parasitoids. *Nematologica* **24**: 286–294.

Bergeron MJ, Hamelin RC, Leal I, Davis C, de Groot P, 2008. First report of *Amylostereum areolatum*, the fungal symbiont of *Sirex noctilio*, on *Pinus* spp. in Canada. *Plant Disease* **92**: 1138.

Carnegie AJ, Matsuki M, Hurley BP, Ahumada R, Haugen DA, Klasmer P, Jianghua S, Iede ET, 2006. Predicting the potential distribution of *Sirex noctilio* (Hymenoptera: Siricidae), a significant exotic pest of *Pinus* plantations. *Annals of Forest Science* **63**: 119–128.

Chou CKS, 1991. Perspectives of disease threat in large-scale *Pinus radiata* monoculture – the New Zealand experience. *European Journal of Forest Pathology* **21**: 71–81.

Ciesla WM, 2003. European woodwasp: a potential threat to North America's conifer forests. *Journal of Forestry* **101**: 18–23.

de Groot P, Nystrom K, Scarr T, 2006. Discovery of *Sirex noctilio* (Hymenoptera: Siricidae) in Ontario, Canada. *The Great Lakes Entomologist* **39**: 49–53.

Farr DF, Bills GF, Chamuris GP, Rossman AY, 1995. *Fungi on Plants and Plant Products in the United States*, second edn. APS Press, St. Paul, Minnesota, USA.

Felsenstein J, 2008. *PHYLIP, Version 3.68*.http://evolution.genetics.washington.edu/phylip.html (accessed 11.01.08).

Fitch WM, 1971. Towards defining the course of evolution: minimum change for a specific tree topology. *Systematic Zoology* **20**: 406–416.

Francke-Grosmann H, 1939. Über das Zusammenleben von Holzwespen (Siricinae) mit Pilzen. *Zeitschrift für Angewandte Entomologie* **25**: 647–680.

Gardes M, Bruns TD, 1993. ITS primers and enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.

Gilbertson RL, 1984. Relationships between insects and wood-rotting Basidiomycetes. In: Wheeler Q, Blackwell M (eds), *Fungus–Insect Relationships: Perspectives in Ecology and Evolution*. Columbia University Press, New York, pp. 130–165.

Griffin GJ, 1986. Chestnut blight and its control. *Horticultural Reviews* **8**: 291–336.

Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Haugen DA, Bedding RA, Underdown MG, Neumann FG, 1990. National strategy for control of *Sirex noctilio* in Australia. *Australian Forest Grower* **13**: 8 Special liftout section No. 13.

Haugen DA, 2007. Sirex woodwasp: biology, ecology and management. *USDA Forest Service, General Technical Report* **NRS-P-10**: 48–49.

Hoebeke ER, Haugen DA, Haack R, 2005. *Sirex noctilio*: discovery of a Palearctic siricid wasp in New York. *Newsletter of the Michigan Entomological Society* **50**: 24–25.

Hsiau PT-W, 1996. *The Taxonomy and Phylogeny of the Mycangial Fungi from Dendroctonus brevicomis (Coleoptera: Scolytidae)*. Ph.D. thesis, Iowa State University, Ames, Iowa.

Hurley BP, Slippers B, Wingfield MJ, 2007. A comparison of control results for the alien invasive woodwasp, *Sirex noctilio*, in the southern hemisphere. *Agricultural and Forest Entomology* **9**: 159–171.

Johannesson H, Stenlid J, 2004. Nuclear reassortment between vegetative mycelia in natural populations of the basidiomycete *Heterobasidion annosum*. *Fungal Genetics and Biology* **41**: 563–570.

Krombein KV, Hurd PD, Smith DR, Burks BD, 1979. *Catalogue of Hymenoptera in America North of Mexico*. In: *Symphyta and Apocrita*, vol. 1. Smithsonian Institution Press, Washington, DC.

Kukor JJ, Martin MM, 1983. Acquisition of digestive enzymes by siricid woodwasps from their fungal symbiont. *Science* **220**: 1161–1163.

Liebhold AM, Macdonald WL, Bergdahl D, Mastro VC, 1995. Invasion by exotic forest pests: a threat to forest ecosystems. *Forest Science Monograph* **30**, 49 pp.

Loo JA, 2008. Ecological impacts of non-indigenous invasive fungi as forest pathogens. *Biological Invasions* **11**: 81–96.

Madden JL, 1981. Egg and larval development in the woodwasp, *Sirex noctilio* F. *Australian Journal of Zoology* **29**: 493–506.

Maijala P, Harrington TC, Raudaskoski M, 2003. A peroxidase gene family and gene trees in *Heterobasidion* and related genera. *Mycologia* **95**: 209–221.

Mattson W, Vanhanen H, Veteli T, Sivonen S, Niemela P, 2007. Few immigrant phytophagous insects on woody plants in Europe: legacy of the European crucible? *Biological Invasions* **9**: 957–974.

Orwig DA, Foster DR, Mausel DL, 2002. Landscape patterns of hemlock decline in New England due to the introduced hemlock woolly adelgid. *Journal of Biogeography* **29**: 1475–1487.

Sirex Science Panel, 2007. Sirex *Science Panel Report*. http://www.aphis.usda.gov/plant_health/plant_pest_info/sirex/downloads/sap12-14-06.pdf (accessed 15.11.08).

Slippers B, Wingfield MJ, Wingfield BD, Coutinho TA, 2000. Relationships among *Amylostereum* species associated with siricid woodwasps inferred from mitochondrial ribosomal DNA sequences. *Mycologia* **92**: 955–963.

Slippers B, Wingfield MJ, Wingfield BD, Coutinho TA, 2001. Population structure and possible origin of *Amylostereum areolatum* in South Africa. *Plant Pathology* **50**: 206–210.

Slippers B, Wingfield D, Coutinho A, Wingfield MJ, 2002. DNA sequence and RFLP data reflect geographical spread and relationships of *Amylostereum areolatum* and its insect vectors. *Molecular Ecology* **11**: 1845–1854.

Slippers B, Coutinho TA, Wingfield BD, Wingfield MJ, 2003. A review of the genus *Amylostereum* and its association with woodwasps. *South African Journal of Science* **99**: 70–74.

Smith DR, Schiff NM, 2002. A review of the siricid woodwasps and their ibaliid parasitoids (Hymenoptera: Siricidae, Ibaliidae) in the eastern United States, with emphasis on the mid-Atlantic region. *Proceedings of the Entomological Society of Washington* **104**: 174–194.

Solheim H, 2006. Treveps og assosierte sopper i Norge. *Agarica* **26**: 87–95.

Tabata M, Harrington TC, Chen W, Abe Y, 2000. Molecular phylogeny of species in the genera *Amylostereum* and *Echinodontium*. *Mycoscience* **41**: 583–593.

Talbot PHB, 1977. The *Sirex-Amylostereum-Pinus* association. *Annual Review of Phytopathology* **15**: 41–54.

Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

Thomsen IM, 1996. *Amylostereum areolatum and Amylostereum chailletii, symbiotic fungi of woodwasps (Sirex* sp. and *Urocerus* sp.). PhD thesis, Danish Forest and Landscape Research Institute, Hørsholm, Denmark.

Thomsen IM, Koch J, 1999. Somatic compatibility in *Amylostereum areolatum* and *A. chailletii* as a consequence of symbiosis with siricid woodwasps. *Mycological Research* **103**: 817–823.

USDA APHIS (United States Department of Agriculture, Animal and Plant Health Inspection Service), 2008. Plant Health: *Sirex noctilio (*Sirex woodwasp).http://www.aphis.usda.gov/plant_health/plant_pest_info/sirex/index.shtml (accessed 23.11.08).

USDA Forest Service (Forest Health Protection), 2006. *Sirex noctilio* Fabricius in New York State. http://www.fs.fed.us/foresthealth/news/news_sirexnoctilio.shtml (accessed 23.11.08).

Vasiliauskas R, Stenlid J, 1999. Vegetative compatibility groups of *Amylostereum areolatum* and *A. chailletii* from Sweden and Lithuania. *Mycological Research* **103**: 824–829.

Vasiliauskas R, Stenlid J, Thomsen IM, 1998. Clonality and genetic variation in *Amylostereum areolatum* and *A. chailletii* from Northern Europe. *New Phytologist* **139**: 751–758.

Vasiliauskas R, Lygis V, Larsson K-H, 2004. *National Center for Biotechnology Information*.http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=55783671 (accessed 6.01.09).

Vasiliauskas R, Larsson E, Larsson K-H, Stenlid J, 2005. Persistence and long-term impact of Rotstop biological control agent on mycodiversity in *Picea abies* stumps. *Biological Control* **32**: 295–304.

van der Nest MA, Slippers B, Stenlid J, Wilken PM, Vasaitis R, Wingfield MJ, Wingfield BD, 2008. Characterization of the systems governing sexual and self-recognition in the white rot homobasidiomycete *Amylostereum areolatum*. *Current Genetics* **53**: 323–336.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), *PCR Protocols: a guide to methods and application*. Academic Press, San Diego, California, pp. 315–322.

Wilson AD, Schiff NM, Haugen DA, Hoebeke ER, 2009. First report of *Amylostereum areolatum* in pines in the United States. *Plant Disease* **93**: 108.

Young ND, Healy J, 2003. GapCoder automates the use of indel characters in phylogenetic analysis. *BMC Bioinformatics* **4**: 6.