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Putative origin of clonal lineages of *Amylostereum areolatum*, the fungal symbiont associated with *Sirex noctilio*, retrieved from *Pinus sylvestris*, in eastern Canada☆

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ABSTRACT

The Eurasian *Sirex noctilio*–*Amylostereum areolatum* complex was discovered and has become established close to the North American Great Lakes in the 2000s. This invasive forest insect pest represents a very high risk to native and exotic pines in North America. We investigated the geographical origin of clonal lineages of the fungal symbiont *A. areolatum* in the recently pest-colonized eastern Canadian region by analyzing mitochondrial and nuclear sequence variations and comparing the genetic diversity of a worldwide collection of fungal symbionts among six countries where the *Sirex* complex is native and four countries from which the insect–fungal complex has been introduced. In total, 102 isolates were analyzed. While 12 multilocus genotypes (MLGs) are observed in the areas where *S. noctilio* is native, only two MLGs are retrieved from areas where *S. noctilio* is not native, indicating the wide spread of clonal lineages in the introduced fungal symbiont of *S. noctilio*. MLG2 comprises 26 % of the Canadian isolates and is also observed in Chile and South Africa, where the insect–fungal complex has also been introduced. MLG3 comprises 74 % of the Canadian isolates and is also observed in the USA, but nowhere else in our worldwide collection. Thus, at least one of the Canadian clonal lineages shares a common origin with *A. areolatum* isolates from the Southern Hemisphere. The source of the second clonal lineage is still unknown, but phylogenetic analyses show that MLG3 is isolated. More extended sampling is necessary to determine the origin of this fungal clonal lineage and investigate its probable symbiotic association with native North American *Sirex*.
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☆ This work is dedicated to our friend and colleague Peter de Groot, who passed away during the completion of this manuscript.

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Introduction

The woodwasp, *Sirex noctilio* Fabricius, attacks pines (*Pinus*) in its native range of Eurasia and North Africa, where it appears to exist in equilibrium with its host trees and natural parasites. In these regions, it is considered a secondary pest of low economic importance and attacks sick, weakened or dying pines (Spradbery & Kirk 1978). *Sirex noctilio* has been accidentally introduced in several countries of the Southern Hemisphere: it was first recorded in New Zealand in 1900 followed by Tasmania (Australia) (1952), mainland Australia (1961), Uruguay (1980), Argentina (1985), Brazil (1988), South Africa (1994), and Chile (2001) (Rawlings 1948; Espinoza *et al.* 1986; Iede *et al.* 1988; Madden 1988; Tribe 1995). These inadvertent introductions of *S. noctilio* have been responsible for extensive mortality in North American pine species that are widely used in austral plantations to supply wood and wood fiber for forest product industries (reviewed by Ciesla 2003). *Sirex noctilio* has recently been discovered in the USA and Canada (Hoebeke *et al.* 2005; de Groot *et al.* 2006). Although early studies of the impact of *S. noctilio* in North America indicate that extensive tree mortality in New York and Ontario has not yet occurred (Dodds *et al.* 2010), it is rated as a very high-risk invasive forest insect pest for North America because of the wide distribution of native and exotic pine stands (Haugen 2006).

There is a very interesting obligate mutualistic relationship between *S. noctilio* and the white rot homobasidiomycete *Amylostereum areolatum* (Fr.) Boidin (Taylor 1981). During oviposition, oidia of *A. areolatum* carried in the female wasp mycangia, located near the ovipositor, are inoculated in the new wood together with the eggs and phytotoxic mucus. The combination of the fungus and the mucus weakens or kills the tree. The subsequent wood decay activity carried out by the fungus provides the *Sirex* larvae with an environment suitable for their development (Talbot 1977; Madden & Coutts 1979; Slippers *et al.* 2003). Dispersal of *A. areolatum* is carried out by emerging adult females that collect oidia produced in the insect galleries in their mycangia and move to lay their eggs into new hosts, sometimes kilometers away. It is believed that propagation of the fungus is largely clonal because oidia are asexual spores. Sporocarps have never been reported in the Southern Hemisphere. In the Northern Hemisphere, they are present but rare (Thomsen 1998; Slippers *et al.* 2003). The prevalence of asexual reproduction in *A. areolatum* and its close association with the woodwasp may explain the overall low genetic diversity observed within the species (Thomsen 1998; Vasiliauskas *et al.* 1998; Thomsen & Koch 1999; Vasiliauskas & Stenlid 1999; Slippers *et al.* 2001).

The first introductions of the *S. noctilio*–*A. areolatum* complex in the Southern Hemisphere in New Zealand probably originated from Europe before the 1900s (Rawlings 1948). Subsequent dispersal within the Southern Hemisphere probably took place across countries and continents within this hemisphere rather than through additional introductions from the Northern Hemisphere. Vegetative incompatibility testing and genetic analyses of the intergenic spacer (IGS) region of the nuclear ribosomal RNA (nuclear IGS rRNA) conducted on *A. areolatum* concurred with this hypothesis (Slippers *et al.* 2001, 2002). The first recorded introductions of *A. areolatum* in North America have been documented by Bergeron *et al.* (2008) in Canada and Wilson *et al.* (2009) in USA. Based on nucleotide sequence data from the nuclear IGS rRNA region, two North American clonal lineages have been retrieved. They are most similar to European ones, and not to non-native clonal lineages from the Southern Hemisphere (Nielsen *et al.* 2009).

Tracing the sources of the *S. noctilio*–*A. areolatum* complex could help determine the routes of its spread and potentially prevent future introductions. In addition, understanding the genetic structure of the fungal population is important to determine suitability of inoculation of the biological control agent of *S. noctilio*, *Deladenus siricidicola* (=*Beddingia*) Bedding. In its free-living form, this entomopathogenic nematode feeds on *A. areolatum* (Bedding 2006). This nematode was recently reported in Canada (Yu *et al.* 2009). The aim of the present study is to use mitochondrial and nuclear sequence variations to investigate the geographical origin of *A. areolatum* clonal lineages in areas recently colonized by the *S. noctilio*–*A. areolatum* complex in eastern Canada.

Material and methods

Collection

Following the discovery of *Sirex noctilio* in Ontario in 2005 (de Groot *et al.* 2006), a wide ranging survey for *S. noctilio* was conducted in 2006 in Ontario, Québec, Nova Scotia, and New Brunswick (CFIA 2006). A total of 335 sites were surveyed of which 39 were positive for *S. noctilio* in Ontario. No positive sites were reported in other provinces (CFIA 2007). Twelve of these positive sites in Ontario were sampled to collect *Amylostereum areolatum* in September and October 2006 (Fig 1) from Scots pines (*Pinus sylvestris*) showing evidence of *S. noctilio* attacks (suppressed trees with brown foliage showing resin beads, resin drips, fungal staining; see Dodds *et al.* 2010).

Log sections from trees exhibiting current attack by *S. noctilio* were collected and split lengthwise to reveal decay columns of *A. areolatum*. Wood chips from these columns were removed under sterile conditions and placed on 2 % malt extract agar (MEA) and incubated at 20 °C for approximately 1 m after which characteristic colonies were sub-cultured to fresh MEA. Eighty-two fungal isolates were identified as *A. areolatum* (Bergeron *et al.* 2008). A worldwide collection of *A. areolatum* was also obtained to include 16 native and three other non-native isolates, as well as the Ecogrow nematode culture on which the entomopathogenic nematode *Deladenus siricidicola* is mass-reared in Australia. The related species *Amylostereum chailletii* (Pers.) Boidin (n = 8), *Amylostereum laevigatum* (Fr.) Boidin (n = 2), and *Amylostereum ferreum* (Berk. & M.A. Curtis) Boidin & Lanq. (n = 2) were included in the collection (Table S1).

DNA isolation and amplification

DNA was extracted from growing cultures of the fungus by scraping the mycelium and soaking it in 200 μL of 0.5 M Tris–HCl pH 9.0, 1 % Triton solution overnight with rotation, at room temperature. The suspension was boiled for 3 min,

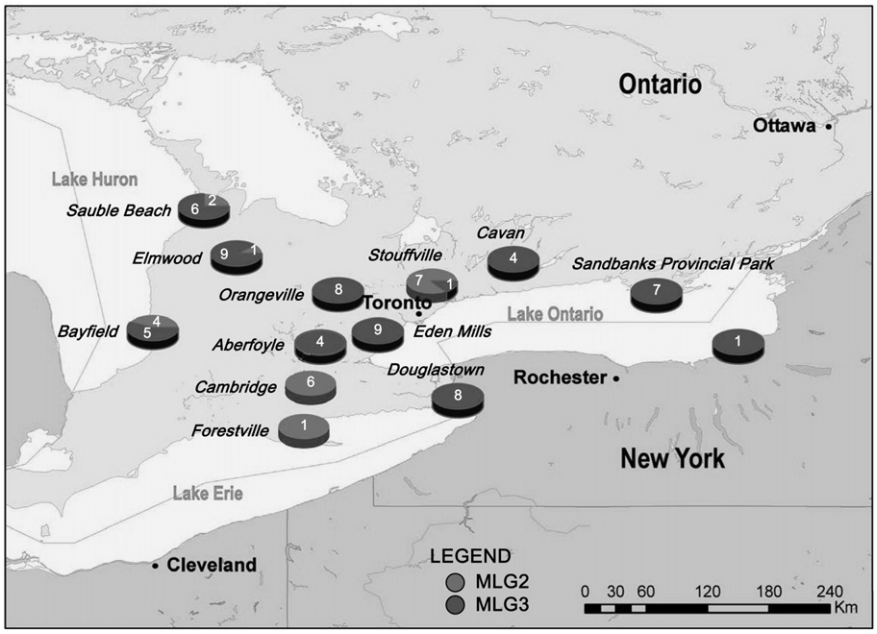

Fig 1 – Southern Ontario locations where *Amylostereum areolatum* was collected from logs of Scots pines (*Pinus sylvestris*) exhibiting current attack by *Sirex noctilio*, in September and October 2006. For each sampling location, the occurrence of MLGs is associated with the pie charts. The location where *A. areolatum* was collected in USA for this study was also mapped.

centrifuged at full speed for a few seconds, cooled down at room temperature, and applied on a QIAquick column (Qiagen Sciences, Germantown, MD, USA). The QIAquick PCR purification kit protocol provided by the manufacturer was followed and the DNA was eluted with 30 μL of buffer EB. One microliter of purified DNA solution was used as template for the PCR amplification by Platinum Taq DNA polymerase (Invitrogen, Life Technologies, Carlsbad, CA, USA), in a total reaction volume of 25 μL. Primer pair MS1/MS2 was used to obtain a fragment of the small mitochondrial ribosomal RNA subunit gene (mtSSU rRNA) (White *et al.* 1990). The basidiomycete-specific ITS1F/ITS4b or fungus-specific ITS5/ITS4 primer pairs were used to amplify the nuclear 5.8S rRNA gene and the flanking internal transcribed spacers (ITS) (White *et al.* 1990; Gardes & Bruns 1993). The basidiomycete-specific P-1/5S-2B primer pair was used to amplify the IGS region included between the nuclear large subunit and 5S rRNA genes (Hsiau 1996; Slippers *et al.* 2002). The region between conserved domains 6 and 7 of the RNA polymerase II second largest subunit gene (RPB2) was amplified with degenerate basidiomycete-specific primers bRPB2-6F/bRPB2-7.1R (Matheny 2005). The N-terminal copper-binding region of the putative laccase gene (LAC-like) was amplified with degenerate primers laccasef/laccaserc (D'Souza *et al.* 1996). Degenerate primers tef1f-(TCMAHGARATYA-TYAAGGAGAC)/tef1rc-(DGGGTCGTTYTTSGAGTCA) were designed from fungal sequence alignment and used for amplification of a fragment of the translation elongation factor 1-alpha gene (TEF1). Primer design was performed using Primer Premier software V5.00 (PREMIER Biosoft International, Palo Alto, CA, USA). Thermocycling conditions included an initial denaturation step of 5 min at 94 °C followed by 35 cycles of denaturation at 92 °C for 30 s, primer annealing at 55 °C (except LAC-like and nuclear IGS rRNA region at 52 °C and 58 °C, respectively) for 45 s, and elongation at 72 °C for 1 min. A final elongation step at 72 °C for 10 min concluded the program.

Multilocus genotyping

Multilocus genotypes (MLGs) of the Canadian *Amylostereum areolatum* isolates were obtained by both single-strand conformation polymorphism (SSCP) analysis, as previously described by Hamelin *et al.* (2005), and sequencing. Thus, a screening for polymorphism was first carried out using the SSCP method for all loci, except the nuclear ITS rRNA region, which had already been sequenced (Bergeron *et al.* 2008), and the nuclear IGS rRNA region, for which direct sequencing was restricted to 16 isolates representative of each ribotype (ITS) encountered within the 12 sampling sites in southern Ontario as well as to the American, Chilean, South African, and three European isolates. Specific running conditions, including temperature, electrical power and migration time, were empirically determined for each of the following four loci: mtSSU rRNA, RPB2, and TEF1: 4 °C, 2 W, 18 h; LAC-like: 4 °C, 2 W, 5 h. Alleles were scored visually according to migration profiles. Then, at least eight representative samples of each migration profile were selected for sequencing.

All other samples, including those belonging to *Amylostereum chailletii*, *Amylostereum laevigatum*, and *Amylostereum ferreum* species, were directly sequenced (Genome sequencing and genotyping platform of the CHUL Medical Research Centre, Québec, QC, Canada). In case of heterozygosity (more than one heterozygous site) or length heterogeneity, cloning was performed. PCR products were then purified with the QIAquick PCR purification kit (Qiagen Sciences) and ligated into the pDrive cloning vector supplied in the Qiagen PCR cloning^{plus} kit (Qiagen Sciences), in accordance with the protocols

provided by the manufacturers. Sequences were edited in Sequencher software V4.8 (Gene Codes Corporation, Ann Arbor, MI, USA) and deposited in GenBank (EU249343, EU249344; HM461062–HM461110).

Data analyses

Since asexual reproduction is predominant in *Amylostereum areolatum*, genetic analyses were conducted with a clone-corrected data set, i.e. within each sampling site, only one representative of each MLG was included in the data set (Boeger *et al.* 1993). Genotypic diversity within native and non-native populations was estimated from the sum of squares of genotype frequencies. If p_{lu} was the frequency of the uth genotype for the lth population, genotypic diversity for this population was $G_l = 1/\Sigma p_{lu}^2$ (Stoddart & Taylor 1988).

Evolutionary history of the *A. areolatum* clonal lineages was reconstructed by incorporating allelic variation within mtSSU rRNA, RPB2, LAC-like, and TEF1 loci. First, an allelic distance matrix was calculated for each gene using PAUP* V4.10b (Swofford 2002), according to the most appropriate model of nucleotide substitution selected with MODELTEST V3.7 (Posada & Crandall 1998). Then, these allelic distance matrices were converted into distance matrices of organisms (or clonal lineages) using POFAD V1.03 (Joly & Bruneau 2006). The matrix of organisms obtained from one locus could either be used alone or combined with matrices obtained from other loci. When combined, all matrices were standardized to have the same weight. Phylogenetic networks of clonal lineages were reconstructed using the NeighborNet algorithm (Bryant & Moulton 2004) implemented in SplitsTree V4 (Huson & Bryant 2006). Missing data was present only at the TEF1 locus. However, using the program POFAD made it possible to estimate missing distance values between two organisms by calculating the mean distance values observed between these two same organisms in the data sets with complete information.

The Bayesian inference and Markov chain Monte Carlo sampling (B/MCMC) methods made possible the phylogenetic estimation of the *Amylostereum* genus, based on the combined mtSSU rRNA, RPB2, TEF1, and nuclear ITS rRNA region data sets. First, characters were partitioned into the following categories: 1st, 2nd, and 3rd positions of exons and each intron for coding genes and ITS1, 5.8S rRNA and ITS2. Best-fit models of nucleotide substitution were selected for each sub-partition (mixed-model approach) using the hierarchical likelihood-ratio tests implemented in MODELTEST. After models were selected, one cold and three incrementally heated chains were run in MrBayes V3.1.2 (Ronquist & Huelsenbeck 2003) for one million generations, with a random starting tree. Trees were sampled every 100th generations, for a total of 10 000 trees, and trees sampled during the burn-in phase (set at 2500) were discarded. Two simultaneous runs, starting from different randomly chosen trees, were made to ensure that the individual runs had converged on the same target distribution. The 50 % majority rule consensus tree was edited in FigTree V1.3.1 (available at http://tree.bio.ed.ac.uk/software). Clonal lineages with missing data for the TEF1 locus were excluded from the analysis. There was a total of 2413 sites in the final data set. Heterozygous positions were coded using degenerate bases, according to IUPAC-IUB nomenclature. The LAC-like locus was excluded from the analysis since there was no sequence for *Amylostereum chailletii*, *Amylostereum laevigatum*, and *Amylostereum ferreum*.

Results and discussion

Clonal lineage characterization of Amylostereum areolatum *in eastern Canada*

A total of 15 MLGs are observed in our worldwide collection of 102 *A. areolatum* isolates (Table 1): MLG1 associated with the Ecogrow nematode culture, MLG2 and MLG3 found among the 85 isolates from countries where the *Sirex noctilio*–*A. areolatum* complex is non-native, and MLG4–MLG15 observed among the 16 native European isolates. As expected, a much higher diversity is observed in samples from Europe (Austria, Czech Republic, Denmark, France, Germany, and Switzerland), the putative center of origin of the insect–fungal complex. Genotypic diversity, estimated from the genotype frequencies, is higher in the native population ($G = 10.9$, $n = 14$) than in the non-native one ($G = 1.95$, $n = 19$). Thus, the low diversity observed in the non-native population of *A. areolatum* is consistent with a population bottleneck following a recent introduction and clonal spread by *S. noctilio*.

Table 1 – MLGs and IGS profiles retrieved from areas where the *Sirex noctilio*–*Amylostereum areolatum* complex is native (European countries) and non-native (pest-colonized countries).

MLGs		MS–RPB2–LAC–TEF1–ITS[a]	IGS profiles[b]
MLG1	Ecogrow nematode culture	a–ab–aa–aa–1&3	–
Pest-colonized countries[c]			
MLG2	Canada (6); Chile (1); South Africa (1)	a–ab–aa–aa–1	BD
MLG3	Canada (10); USA (1)	b–ab–ab–bc–1&2	D
European countries[c]			
MLG4	Austria (1)	d–ab–cc–*n.a.*–1	–
MLG5	Austria (1)	e–ef–cc–bb–1	–
MLG6	Austria (1)	e–ee–cc–dd–1	–
MLG7	Austria (1)	e–aa–cc–*n.a.*–1	–
MLG8	Austria (1); Denmark (1)	e–bb–aa–ab–1&7	–
MLG9	Czech Republic (1)	e–eh–cc–ee–1	–
MLG10	Czech Republic (1)	d–bb–ac–bb–1	–
MLG11	Czech Republic (1)	e–ag–ac–df–1	–
MLG12	Denmark (1)	a–ad–ac–*n.a.*–1&8	–
MLG13	France (1)	e–bb–cc–aa–1	–
MLG14	Germany (1)[d]; Switzerland (1)[d]	a–bc–cc–aa–1	D
MLG15	Germany (1)[d]	c–ab–aa–aa–1	BD

a MS: mtSSU rRNA; LAC: laccase-like.
b Our IGS profile designation is based on the fragment sizes shown in Table 3 by Nielsen *et al.* (2009), not on the sequence-level variation.
c Number of sampling sites per country is in parentheses.
d Selected as representative native samples for the sequence-level variation analysis of the nuclear IGS rRNA region.

Among the 82 Canadian *A. areolatum* isolates genotyped, MLG2 and MLG3 are encountered, suggesting the occurrence of two clonal lineages of *A. areolatum* in eastern Canada (Fig 1). There is no perfect match between these MLGs and those observed in Europe. However, 21 Canadian isolates (26 %) belong to MLG2, which is also found in Chile and South Africa. The remaining 61 Canadian isolates (74 %) belong to MLG3, which is found in New York State but nowhere else in the world (Fig 2). This suggests that at least one of the Canadian clonal lineages shares a common origin with *A. areolatum* isolates from the Southern Hemisphere. This lineage could either 1) have been introduced from a Southern Hemisphere source, or 2) have come directly from Eurasia or North Africa to North America, and it is the same lineage that was previously introduced into the Southern Hemisphere but has still not been isolated in its native range.

All alleles observed in MLG2 are shared with the European population. The allelic combination for the RPB2, LAC-like, and TEF1 loci in MLG2 is identical to those in MLG1 (Ecogrow nematode culture, presumably originating from Hungary) and MLG15 (German isolate) (Table 1). On the other hand, the most frequently isolated clonal lineage in eastern Canada MLG3 possesses some unique alleles at individual loci. For example, the ribotype 2, allele B of the mtSSU rRNA and LAC-like loci, and allele C of the TEF1 locus are not observed elsewhere in our world collection (Fig 2 and Table 1). The ribotype 2 differs by only a single indel from ribotype 1, the most common ribotype worldwide.

Based on nucleotide sequence data from the nuclear IGS rRNA region, Nielsen *et al.* (2009) also observed the presence of two lineages of *A. areolatum* in the USA, whose genotypes are most similar to genotypes found in Europe, and not to genotypes retrieved from the Southern Hemisphere. To compare our results, we conducted an analysis using the nuclear IGS rRNA region on a subset of our samples. We also identified two IGS profiles in eastern Canada. MLG2 is linked with the 'IGS-BD' profile, while MLG3 is linked with the 'IGS-D' profile described in Table 3 by Nielsen *et al.* (2009).

In addition to the observation of fragment size differences among the *A. areolatum* IGS rRNA sequences, we also paid attention to single nucleotide polymorphisms (SNPs). Among the MLG2 and MLG15 lineages, both linked with the 'IGS-BD' profile, there is no sequence variation at the nuclear IGS rRNA region (six Canadian, one Chilean, one South African and one German isolates analyzed). This complete homology between nuclear IGS rRNA region sequences of Canadian, Chilean, and South African clonal lineages, which is corroborated by our multilocus genotyping approach, contrasts with the results reported by Nielsen *et al.* (2009) that showed different IGS grouping between their American isolates (BD or D) and the isolates from the Southern Hemisphere (AB) genotyped by Slippers *et al.* (2002). This highlights the importance of comparing isolates with reference strains.

Moreover, among IGS rRNA sequences of MLG3 and MLG14 lineages, both linked with the 'IGS-D' profile, there are SNPs at positions 119, 408, and 437 that segregate MLG3 from MLG14 (10 Canadian, one American, one German and one Swiss isolates analyzed) (Fig S1 and Table 1). Based on this observation, it seems likely that the American lineage identified by Nielsen *et al.* (2009) as 'IGS-D' belongs to the North American MLG3 lineage that we characterized. This is further supported by their observation, within the ITS rRNA region of their North American 'IGS-D' strain, of double peaks downstream of positions 287 and 356 with the primers ITS1F and ITS4, respectively, which is characteristic of the occurrence of ribotypes 1 and 2 associated with the MLG3 lineage.

Phylogenetic relationships of Amylostereum areolatum

Incorporation of allelic variation for multiple genes for reconstructing the evolutionary history of individuals is particularly interesting since it gives twice the amount of information per locus for dikaryotic individuals. In addition, it is more

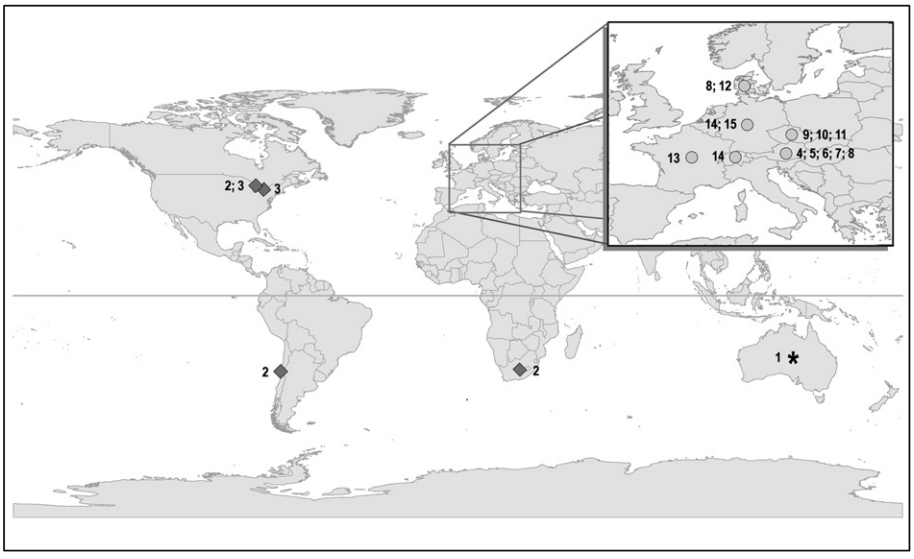

Fig 2 – Multilocus genotyping of *Amylostereum areolatum* sampled from areas where the *Sirex noctilio*–*Amylostereum areolatum* complex is native (green circle) and non-native (red lozenge). Asterisk refers to the Ecogrow nematode culture on which the entomopathogenic nematode *Deladenus siricidicola* is mass-reared in Australia. Numbers refer to MLGs (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

powerful than an approach based on a single gene which, if incongruent with the evolutionary history of the species, could lead to erroneous interpretations. The phylogenetic network approach adopted for the current study makes it possible to incorporate such information. Phylogenetic relationships among the *A. areolatum* clonal lineages are estimated from the allelic variation found in the mtSSU rRNA, RPB2, LAC-like, and TEF1 loci. The combination of these four loci for reconstructing the phylogenetic network (Fig 3) gives higher resolution than when loci are analyzed individually (data not shown). As expected, the combined network shows that MLG1, MLG2, and MLG15 cluster together. However, the clonal lineage MLG3, which has been found only in North America, is phylogenetically isolated (associated with the highest bootstrap support value).

Because of the presence of unique alleles in the MLG3 lineage (Table 1) which cause its phylogenetic isolation, we wanted to determine its position within a larger phylogenetic framework. A Bayesian phylogenetic tree was reconstructed from the combination of mtSSU rRNA, RPB2, TEF1, and ITS rRNA loci and included all known species within the genus *Amylostereum* (Fig 4). The tree clearly shows that all *A. areolatum* clonal lineages from our study, including MLG3, cluster together in a well-supported clade. Its topology is concordant with that of trees produced using the nuclear ITS rRNA region, mtSSU rRNA, manganese-dependent peroxidase A exons or nuclear IGS rRNA region data (Vasiliauskas *et al.* 1999; Slippers *et al.* 2000, 2002). According to Slippers *et al.* (2002), the divergence of *A. areolatum* can be attributed to the obligate relationship of *A. areolatum* with its insect vectors and to the predominance of asexual reproduction compared with other *Amylostereum* species.

Amylostereum areolatum *and its insect vectors*

Compared with other *Sirex noctilio*-colonized countries in the Southern Hemisphere, the occurrence of native *Sirex* species is a situation that is unique to North America. They all use *Pinus* spp. as hosts but, unlike introduced *S. noctilio*, none of them attack healthy trees (Williams 2007). One interesting and important observation reported by Nielsen *et al.* (2009) is that *S. noctilio* and a North American native species, *Sirex edwardsii*, which emerged from the same tree both carrying the 'IGS-D strain' of *A. areolatum*. Although it has been reported that *A. areolatum* could be carried by more than one woodwasp species, *S. edwardsii* is known to carry *Amylostereum chailletii*, the fungal symbiont associated with native North American *Sirex* (Gaut 1970; Bedding & Akhurst 1978; Gilbertson 1984; Smith & Schiff 2002). Nielsen *et al.* (2009) also found another native

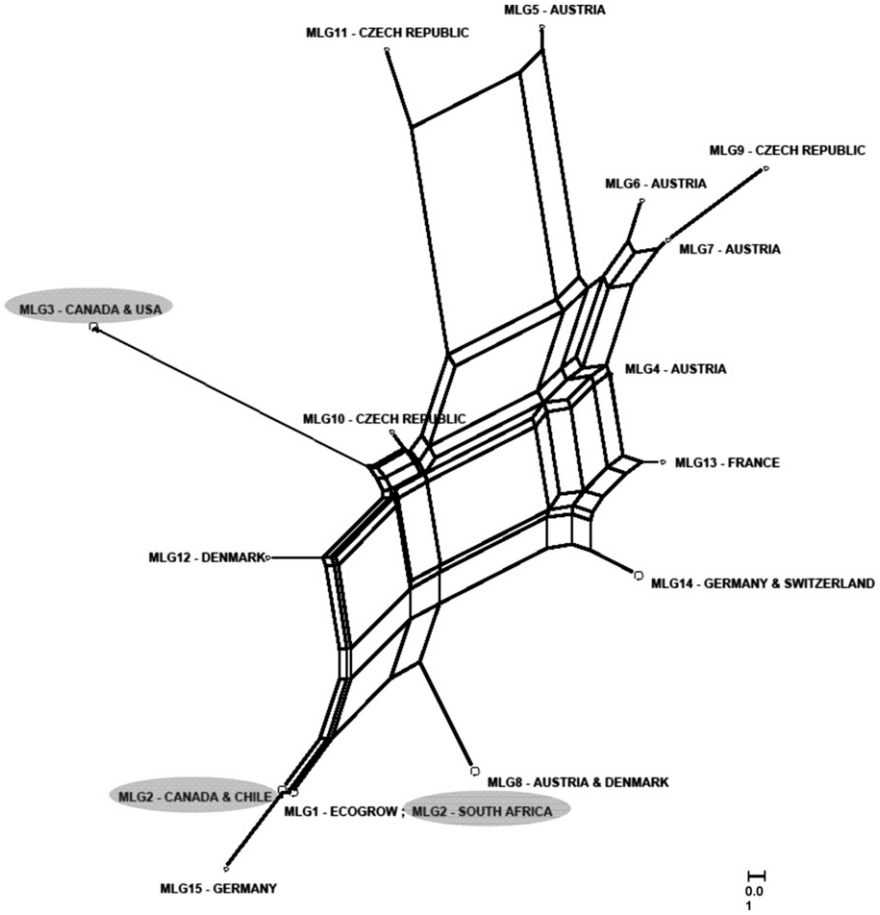

Fig 3 – Phylogenetic network (NeighborNet) representing the relationships between the *Amylostereum areolatum* clonal lineages obtained from the combined analysis of the mtSSU rRNA, RPB2, LAC-like, and TEF1 loci. MLGs retrieved from areas where the *Sirex noctilio*–*Amylostereum areolatum* complex is non-native are within gray ellipses. The scale only gives a relative indicator of distance because the matrices were standardized.

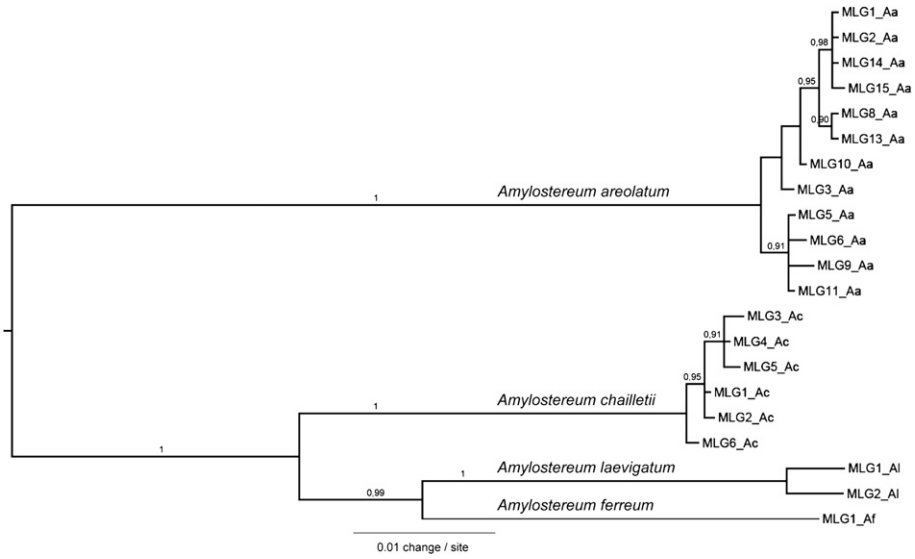

Fig 4 – Phylogram of relationships among *Amylostereum areolatum* clonal lineages and related species within *Amylostereum* genus inferred from combined Bayesian analysis of the mtSSU rRNA, RPB2, TEF1, and nuclear ITS rRNA region data sets, under mixed models. Bayesian posterior probabilities ≥0.90 are shown above the branches. Branch lengths are optimized using estimated mean parameter values and drawn proportional to character changes (base substitutions) as indicated by the scale bar. Clonal lineages with missing data for the TEF1 locus were excluded from the analysis.

species, *Sirex* sp. '*nitidus*', carrying a unique strain of *A. areolatum*, the 'IGS-BE' strain, but it was located far from the known area colonized by *S. noctilio*. Although the new species *Sirex* sp. '*nitidus*' has not been formally described (based on taxonomic studies by H. Goulet and cited as a personal communication by Nielsen *et al.* 2009), it is currently recognized as *Sirex juvencus*, but differs from the European *S. juvencus* (Smith & Schiff 2002). As stated by Nielsen *et al.* (2009), their findings do reopen the debate regarding the identity and specificity of *Sirex*/*Amylostereum* associations. Further investigation is needed to verify this phenomenon and to determine if other native species of *Sirex* also carry *A. areolatum* because the findings by Nielsen *et al.* (2009) were based on only a few samples.

Thus, a plausible explanation for the large divergence between the North American clonal lineage MLG3 and other MLGs found in our study and its high occurrence in eastern Canada could be the evolutionary relationship between native North American *Sirex* and *A. areolatum*. Although the first reports of *A. areolatum* in North America resulted from the discovery of *S. noctilio* (Bergeron *et al.* 2008; Wilson *et al.* 2009), it is conceivable that the fungus was present before, but it was associated with native *Sirex* spp., thereby generating the divergent fungal lineage MLG3. However, it is also possible that European (e.g. southern Europe), North African or Asian populations of *A. areolatum*, which have not been investigated, comprise the origin of the MLG3 lineage. Further sampling is needed to explore these avenues.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.funbio.2011.05.009.

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