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Phylogenetic analysis of *Deladenus* nematodes parasitizing northeastern North American *Sirex* species

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ABSTRACT

The parasitic nematode *Deladenus siricidicola* is a biological control agent of the invasive woodwasp, *Sirex noctilio*. Since the discovery of *S. noctilio* in pine forests of northeastern North America in 2005, a biological control program involving the Kamona strain of *D. siricidicola* has been under consideration. However, North American pine forests have indigenous *Sirex* spp. and likely harbor a unique assemblage of associated nematodes. We assessed phylogenetic relationships among native *Deladenus* spp. in the northeastern United States and the Kamona strain of *D. siricidicola.* We sequenced three genes (mtCO1, LSU, and ITS) from nematodes extracted from parasitized *Sirex* spp. collected inside and outside of the range of *S. noctilio*. Our analyses suggest cospeciation between four North American *Sirex* spp. and their associated nematode parasites. Within two *S. noctilio* individuals we found nematodes that we hypothesize are normally associated with *Sirex nigricornis*. One individual of the native *S. nigricornis* contained *Deladenus* normally associated with *S. noctilio*. We discuss nematode-host fidelity in this system and the potential for non-target impacts of a biological control program using *D. siricidicola* against *S. noctilio*.

1. Introduction

Deladenus species are parasitic nematodes that attack siricid woodwasps and also some siricid parasitoids and associates (Bedding and Akhurst, 1978) that develop in conifers (Williams et al., 2012). Some species in the genus *Deladenus* (=*Beddingia*) have a dicyclic life history, consisting of a free-living mycophagous life cycle and an insect-parasitic life cycle (Chitambar, 1991). These different strategies allow *Deladenus* to increase within trees as the specific wood rot fungi they eat grows, then switch to the parasitic form when siricid hosts are present, facilitating their dispersal to new trees.

In the eastern United States, there are three native species of *Sirex*: *S. nigricornis*, *S. cyaneus*, and *S. nitidus* (Schiff et al., 2012). Additionally one introduced species, *Sirex noctilio*, was first collected in New York state in 2004 (Hoebeke et al., 2005). *Sirex* species infest trees by depositing eggs along with a symbiotic white rot fungus into the tree during oviposition. The symbiotic fungi, in the genus *Amylostereum*, grow throughout the wood and provide nutrition to the *Sirex* larvae. North American *Sirex* are not considered serious pests as they only infest dead or dying trees (Furniss and Carolin, 1977; Madden, 1988); however, the invasive *S. noctilio* is capable of killing healthy trees (Spradbery, 1973).

Most of what is known about *Deladenus* comes from in-depth studies of its use as a biological control agent against *S. noctilio* (Bedding and Iede, 2005). One part of the *Deladenus siricidicola* life cycle is spent living within the tracheids of pine trees. There, nematodes eat the growing hyphal tips of the white rot fungus *Amylostereum areolatum* (Basidiomycota: Russulales), the fungal symbiont of *S. noctilio*. Adult mycophagous nematodes mate via amphimixis, lay eggs, and develop from larvae into adults. This free-living cycle can continue indefinitely. In the presence of *S. noctilio* larvae, however, chemical cues stimulate nematode larvae to develop into preinfective adults. Preinfective adults also mate via amphimixis and the mated females use a long tubular stylet to pierce the *Sirex* larval cuticle and enter the host. Once inside, the nematode becomes parasitic after it sheds its outer cuticle and develops microvilli on the outside of its body, facilitating absorption of nutrients from the host. This leads to rapid growth of the nematode. When the host pupates, the nematode lays eggs that will develop into mycophagous adults (Bedding, 2009). The eggs hatch and juvenile nematodes migrate to the host's reproductive organs. Depending on the species and strain of *Deladenus*, juveniles can be found either within host egg shells, or around viable host eggs. In parasitized male *S. noctilio*, juvenile nematodes migrate to the testes, but a male *S. noctilio* host is a dead end for the nematode. When a parasitized female emerges from the tree as an adult, it mates and then oviposits on a new tree, injecting nematodes either with or around the eggs (Bedding, 2009).

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D. siricidicola juveniles migrate into *S. noctilio* eggs, effectively sterilizing the host. This sterilizing effect the nematode has on the host has been exploited in order to reduce populations of *S. noctilio* in the Southern Hemisphere in numerous biological control programs (Hurley et al., 2007). Upon the arrival of *S. noctilio* to North America, controlled release studies were conducted on the use of the *D. siricidicola* to control *S. noctilio* in the United States (Williams et al., 2012). The use of *D. siricidicola* in the United States may be more complex than in the Southern Hemisphere, however, in that unlike the Southern Hemisphere, where pines are introduced and there are no native *Sirex*, North America has native pines, indigenous *Sirex*, and associated nematodes.

During a worldwide survey conducted in the 1970s to find natural enemies of *S. noctilio*, seven species of *Deladenus* parasitizing *Sirex* species and their associates were described from North America (Bedding and Akhurst, 1978; Bedding, 1974). However, the distribution of these nematodes was not well defined as samples were only collected from a few areas (see Table 1). Further complicating matters, a non-sterilizing strain of *D. siricidicola* has been found parasitizing *S. noctilio* in Ontario, Canada and in New York state (Shields, 2009; Williams et al., 2009), which presumably arrived with this invasive (Yu et al., 2009). This strain is referred to as the "North American strain" by Williams et al. (2012) and the" non-sterilizing form" by Yu et al. (2009). The relationships among species within *Deladenus* are poorly understood and no phylogenetic analyses focused solely on the group have been conducted. The seven North American insect-parasitic species of *Deladenus* were described based on the morphology of mycophagous adults (Bedding, (1968, 1974)). However, in a review of this entire genus, Chitambar (1991) stated that morphology cannot be used to distinguish species of *Deladenus* that parasitize *Sirex* except for defining two major groups, or superspecies. The two proposed superspecies are *Deladenus wilsoni* and *D. siricidicola*.

In this paper, we use molecular methods to characterize the diversity and relationships of *Deladenus* species associated with both native and introduced *Sirex* woodwasps in the eastern United States. Understanding the diversity of these nematodes and their host associations may inform decisions about the impacts of nematodes introduced for *S. noctilio* biocontrol in this region.

2. Materials and methods

2.1. Specimen acquisition and DNA extraction

Sirex woodwasps were obtained from multiple sites in New York, Pennsylvania, and Louisiana from 2008–2011 through a combination of intercept-panel traps (www.alphascents.com), insect collecting nets, and rearing from infested wood. Intercept-panel traps were placed 1–2 m high in pine trees and checked one to four times monthly. After collection, *Sirex* woodwasps were kept at 4 °C in 29.6 mL plastic cups with lids until dissection. The majority of *Sirex* specimens were obtained by felling red pine (*Pinus resinosa*) and Scots pine (*Pinus sylvestris*) trees exhibiting symptoms of *Sirex* infestation described by Haugen and Hoebeke (2005) and cutting the felled trees into bolts approximately one meter in length. The bolts were placed in barrels with a screened lid and kept in a lab at room temperature or at ambient conditions in a barn. During *Sirex* emergence periods, barrels were checked several times each week. All *Sirex* were kept alive at 4 °C until dissection could confirm the presence of nematodes. We obtained a live culture of *D. siricidicola* Kamona strain (specimen "noc172"), a biological control agent of *S. noctilio* that is mass produced by the company Ecogrow Environment (Queanbeyan, N.S.W., Australia). Another live culture, of the non-sterilizing North American strain of *D. siricidicola* (specimen "noc173"), was isolated from *S. noctilio* from Manlius, New York (D.W.W.). Specimen data for nematodes and *Sirex* hosts are given in Table 2.

Nematodes were preserved in 95% ethanol until subsequent tissue lysis and DNA extraction. DNA was extracted using a QIAamp DNA Micro Kit (Qiagen, Valencia, CA) after removing nematodes from the ethanol and lysing them by soaking in a waterbath at 56 °C overnight. For female *Sirex*, approximately 4 eggs showing symptoms of nematode infection either inside or on the outside of the eggs were used in the extraction process. Nematode DNA was obtained from male *Sirex* specimens by extraction from testes. Two samples were obtained from live cultures of nematodes, and in this case, the nematode colony was flooded with 95% ethanol and approximately 10 μL of the suspension was included in the DNA extraction for all samples. DNA was eluted in double distilled H_2O and stored at −20 °C until use as a PCR template.

2.2. DNA amplification, sequencing, and analysis

Primers used for PCR amplification and sequencing are listed in Table 3. Reaction conditions for amplification of mtCO1 and LSU were the same as those in Ye et al. (2007). For the ITS gene, the thermal cycling program was the same as that used by Subbotin et al. (2001, 2006).

PCR products were purified for sequencing using a QIAQuick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and eluted in double distilled H_2O. PCR products were sequenced in both directions by the Core Laboratory Center (CLC) at Cornell University. Raw sequence data were assembled and edited with CodonCode Aligner (version 3.7.1). The sequence data for the outgroup included in the study was obtained from Genbank. The outgroup was the nematode *Howardula aoronymphium* (Tylenchida: Allantonematidae), which is in the same suborder as *Deladenus* (Hexatylina) and is parasitic on mycophagous *Drosophila* (Ye et al., 2007). Sequence alignment for each gene was performed in MAFFT (Katoh et al., 2002; Katoh and Toh, 2008) and improved by direct examination in Mesquite (version 2.74). Gaps were treated as missing data. jModelTest (version 0.1.1) (Posada, 2008; Guindon and Gascuel, 2003) was used to select the most appropriate model of nucleotide substitution for each gene under the AIC criterion. Tree configurations resulting from maximum likelihood (ML) analyses performed in RAxML with 100 bootstrap replicates for individual gene datasets did not reveal any conflict among mtCO1 and ITS genes, so a combined dataset was created for mtCO1 and ITS. Significant tree differences were observed in the LSU dataset (ML bp > 70), and this gene was analyzed

Table 1
Species of *Deladenus* found in eastern United States and Canada.

Nematode	Insect host	Tree host	Fungal food source	Collection location	Citation
D. canii	*S. cyaneus*	*Abies balsamea*	*Amylostereum chailletii*	New Brunswick, Canada	Bedding (1974)
D. proximus	*S. nigricornis*	*Pinus* spp.	*Amylostereum chailletii*	South Carolina, United States	Bedding (1974)
D. wilsoni	*S. cyaneus, Rhyssa* spp.	(any tree with rhyssines or *S. cyaneus*)	*Amylostereum chailletii, Amylostereum areolatum*	United States, Canada, wherever rhyssines parasitizing *Sirex* occur	Bedding (1968)
D. siricidicola (North American strain)	*S. noctilio*	*Pinus* spp.	*Amylostereum areolatum*	New York, United States, and Canada	Yu et al. (2009)

Table 2
Sirex nematodes and outgroup samples used for DNA sequencing.

Host	Host source	Coll. Date	Coll. Location	Sirex sex	Nematode ID#	GenBank accession no.		
						MTCO1	LSU	ITS
S. nigricornis	Trap	9-Sep-09	Oswego, NY	f	nig4	JX104234	JX104233	
S. nigricornis	Trap	5-Oct-10	Warrensburg, NY	f	nig159	JX104269		
S. nigricornis	Trap	5-Oct-10	Warrensburg, NY	f	nig161	JX104270	JX104269	JX212772
S. nigricornis	Trap	5-Oct-10	Warrensburg, NY	f	nig162	JX104271		JX212773
S. nigricornis	*Pinus taeda*	1-Nov-10	Grants Parrish, LA	f	nig163	JX104272	JX104271	JX212774
S. nigricornis	*Pinus taeda*	1-Nov-10	Grants Parrish, LA	f	nig164	JX104273		JX212775
S. nigricornis	*Pinus taeda*	1-Nov-10	Grants Parrish, LA	f	nig165	JX104274	JX104273	
S. nigricornis	Trap	15-Oct-08	Mt. Morris, PA	f	nig12	JX104240	JX104239	JX212752
S. nigricornis	Trap	19-Oct-08	Garards Fort, PA	f	nig14	JX104242	JX104241	JX212754
S. nigricornis	Caught in field	18-Sep-10	Warrensburg, NY	f	nig157	JX104268	JX104267	JX212771
S. nigricornis	*Pinus resinosa*	2008	Fabius, NY	m	nig175	JX104278	JX104277	JX212779
S. nigricornis	*Pinus resinosa*	2008	Fabius, NY	m	nig174	JX104277		JX212778
S. nigricornis	Trap	14-Oct-08	Mount Morris, PA	f	nig13	JX104241		JX212753
S. nigricornis	*Pinus resinosa*	2008	Fabius, NY	m	nig176	JX104279		JX212780
S. nitidus	*Picea abies*	2-Sep-09	Newcomb, NY	m	nit30	JX104245		JX212756
S. nitidus	*Picea abies*	28-Aug-08	Newcomb, NY	f	nit17	JX104243		JX212755
S. cyaneus	*Abies balsamea*	31-Aug-09	Newcomb, NY	f	cya2	JX104232		JX212748
S. cyaneus	*Abies balsamea*	31-Aug-09	Newcomb, NY	f	cya3	JX104233		
S. cyaneus	*Abies balsamea*	31-Aug-09	Newcomb, NY	m	cya5	JX104235	JX104234	
S. cyaneus	*Abies balsamea*	31-Aug-09	Newcomb, NY	m	cya6	JX104236	JX104235	
S. cyaneus	*Abies balsamea*	29-Aug-09	Newcomb, NY	m	cya7	JX104237	JX104236	JX212749
S. cyaneus	*Abies balsamea*	29-Aug-09	Newcomb, NY	m	cya8	JX104238	JX104237	JX212750
S. cyaneus	*Abies balsamea*	25-Aug-09	Newcomb, NY	m	cya9	JX104239	JX104238	JX212751
S. cyaneus	*Abies balsamea*	29-Aug-09	Newcomb, NY	f	cya24	JX104244	JX104243	
S. cyaneus	*Abies balsamea*	31-Aug-09	Newcomb, NY	m	cya32	JX104246	JX104245	
S. cyaneus	*Abies balsamea*	14-Aug-09	Newcomb, NY	m	cya34	JX104247		JX212757
S. cyaneus	*Abies balsamea*	21-Aug-09	Newcomb, NY	m	cya36	JX104248	JX104247	JX212758
S. cyaneus	*Abies balsamea*	21-Aug-09	Newcomb, NY	m	cya37	JX104249		JX212759
S. cyaneus	*Abies balsamea*	26-Aug-09	Newcomb, NY	m	cya38	JX104250	JX104249	
S. cyaneus	*Abies balsamea*	17-Aug-09	Newcomb, NY	m	cya41	JX104251		
S. cyaneus	*Abies balsamea*	21-Aug-09	Newcomb, NY	m	cya43	JX104252	JX104251	
S. cyaneus	*Abies balsamea*	25-Aug-09	Newcomb, NY	m	cya50	JX104253		
S. cyaneus	*Abies balsamea*	26-Aug-09	Newcomb, NY	f	cya52	JX104254	JX104253	JX212760
S. noctilio	*Pinus sylvestris*	22-Jun-09	Oswego, NY	m	noc76	JX104255		JX212761
S. noctilio	*Pinus sylvestris*	25-Jun-09	Oswego, NY	m	noc78	JX104256	JX104255	
S. noctilio	*Pinus sylvestris*	30-Jun-09	Oswego, NY	m	noc79	JX104257		JX212762
S. noctilio	*Pinus sylvestris*	22-Jun-09	Oswego, NY	m	noc80	JX104258	JX104257	JX212763
S. noctilio	*Pinus resinosa*	10-Jul-09	Tioga, PA	m	noc101[b]	JX104259		JX212764
S. noctilio	*Pinus sylvestris*	2-Jul-09	Onondaga, NY	f	noc115	JX104260	JX104259	
S. noctilio	*Pinus sylvestris*	1-Jul-09	Onondaga, NY	m	noc119	JX104261		JX212765
S. noctilio	*Pinus sylvestris*	6-Jul-09	Onondaga, NY	m	noc120	JX104262	JX104261	JX212766
S. noctilio	*Pinus sylvestris*	25-Jun-09	Onondaga, NY	m	noc121	JX104263		JX212767
S. noctilio	*Pinus sylvestris*	19-May-09	Onondaga, NY	m	noc124	JX104264	JX104263	JX212768
S. noctilio	*Pinus sylvestris*	26-May-09	Oswego, NY	m	noc148	JX104265		JX212769
S. noctilio	*Pinus sylvestris*	26-May-09	Oswego, NY	m	noc149	JX104284	JX104283	JX212770
S. noctilio	Trap	3-Sep-10	Warrensburg, NY	f	noc155	JX104266	JX104265	
S. noctilio	*Pinus resinosa*	2011	Huron, NY	m	noc180	JX104281		JX212782
S. noctilio	Net	27-Aug-10	Warrensburg, NY	f	noc156	JX104267		
S. noctilio	*Pinus resinosa*	2011	Huron, NY	m	noc179	JX104280	JX104279	JX212781
S. noctilio	*Pinus resinosa*	11-Jul-11	Triangle, NY	m	noc192	JX104282	JX104281	
S. noctilio	*Pinus resinosa*	2011	Huron, NY	m	noc193	JX104283		
S. noctilio	NA		NA		noc172[c]	JX104275		JX212776
S. noctilio	*Pinus sylvestris*		Manlius, NY	f	noc173[d]	JX104276	JX104275	JX212777
S. noctilio			Australia		AY633450[e]	AY633450		
S. noctilio			Ontario, CA		EU545474[f]	EU545474		
S. noctilio			Ontario, CA		FJ004889[f]			FJ004889
S. nigricornis			Ontario, CA		JF304744[g]			JF304744
Drosophila neotestacea			Rochester, NY		how367[h]	AY589466	AY589395	

[a] *Sirex*-nematode collection numbers, Cornell University, Ithaca, NY.
[b] *Sirex* emerged in quarantine.
[c] Live culture mass produced by the company Ecogrow Environment (Queanbeyan, N.S.W., Australia); originally isolated from *S. juvencus* in Sopron, Hungary. It was then reisolated from *S. noctilio* in Kamona, Tasmania in 1991 (=Kamona strain).
[d] Live culture obtained from D.W. Williams at the USDA, APHIS Otis Laboratory in Buzzards Bay, MA.
[e] *D. siricidicola*, sequence data from Ye et al. (2007).
[f] *D. siricidicola*, sequence data from Yu et al. (2009).
[g] *D. proximus*, sequence data from Yu et al. (2011).
[h] Outgroup. *Howardula aoronymphium*, sequence data from Ye et al. (2007).

separately. For all ML analyses in RAxML, the GTRgamma model was used, as the model suggested by jModeltest was unavailable. This model was applied individually to four partitions in the concatenated mtCO1-ITS dataset: one partition for each codon position in mtCO1, and a separate partition for ITS. Bayesian analyses were conducted in MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), under the following conditions: 4 chains, 2 runs, and 1,000,000 generations for the COX1-ITS

Table 3
PCR amplification and sequencing primers used in the study.

Primer	Sequence	Amplified gene	Reference
CO1F	5′-CCTACTATGATTGGTGGTTTTGGTAATTGAATAC-3′	mtCO1	Designed for this study by Steven M. Bogdanowicz
CO1R	5′-CAGGCAGTAAAATAAGCACGAGAATCTAAATCTAT-3′	mtCO1	Designed for this study by Steven M. Bogdanowicz
D2A	5′-ACAAGTACCGTGAGGGAAAGTTG-3′	LSU	Subbotin et al. (2006)
D3B	5′-TCGGAAGGAACCAGCTACTA-3′	LSU	Subbotin et al. (2006)
TW81	5-GTTTCCGTAGGTGAACCTGC-3	ITS	Subbotin et al. (2001)
AB28	5-ATATGCTTAAGTTCAGCGGGT-3	ITS	Subbotin et al. (2001)

dataset. Each chain was sampled every 50 generations. The concatenated dataset was partitioned as in ML analyses, using the GTRgamma model for each of the three mtCO1 partitions and GTRinvgamma for the ITS partition. The LSU dataset was analyzed with 3,000,000 generations, under the GTRinvgamma substitution model. Maximum parsimony (MP) analyses were conducted in TNT (version 1.1), using TBR with 20 replications to find the best tree. Support for nodes was calculated via symmetric resampling (Goloboff et al., 2003). Trees were edited in FigTree (version 1.3.1) (Drummond and Rambaut, 2007).

2.3. Distinguishing strains of D. siricidicola in silico

Two specimens known to be *D. siricidicola*, "noc172", which is *D. siricidicola* Kamona strain, and "noc173," which is the North American strain of *D. siricidicola* were included to develop rapid diagnostic methods to distinguish Kamona from the North American strain. To distinguish strains of *D. siricidicola*, DNA sequences from the mtCO1 gene of nematodes were subjected to in silico enzyme digestion with CodonCode Aligner (version 3.7.1) to search for diagnostic restriction site patterns.

2.4. Identification of symbiotic fungal associates of Sirex

Because only female *Sirex* have mycangia containing *Amylostereum* fungus, there is no information on fungal identity for any of the male specimens included in the study. For eleven of the nematode specimens included in the study, isolation and characterization of the fungal associate of the host *Sirex* was conducted based on the intergenic spacer (IGS) region. The methods used to remove fungal symbionts, extract DNA, conduct PCR and sequencing, and analyze the results were the same as those used in Nielsen et al. (2009) for *Amylostereum chailletii* isolates, and fragment analysis was conducted to determine strain of *A. areolatum* (Hajek et al., 2013).

3. Results

3.1. Phylogenetic relationships among nematodes

Phylogenetic relationships resulting from the concatenated mtCO1-ITS dataset were largely congruent across all tree reconstruction algorithms. Since phylogenetic trees resulting from the LSU dataset were in conflict with the other two genes, this gene was excluded from further consideration. In general, there were four monophyletic clades of nematodes, mostly corresponding to *Sirex* host species (Fig. 1). Nematodes from *S. nigricornis*, S. *noctilio*, *S. cyaneus*, and *S. nitidus* each formed their own monophyletic clades.

There was strong support for host specificity in *Deladenus* species, except in three cases. Two samples from the *S. nigricornis* clade were found parasitizing *S. noctilio*. In another case, a sample from the *S. noctilio* clade was found parasitizing *S. nigricornis*. These exceptions are indicated by a solid black background in Fig. 1.

3.2. Distinguishing strains of D. siricidicola in silico

The combined presence of one AciI restriction site and three RsaI sites indicated the North American strain of *D. siricidicola*, whereas the absence of an AciI restriction site and the presence of at least one RsaI restriction site indicated *D. siricidicola* Kamona strain.

With the exception of "noc172" and "noc101" all *D. siricidicola* in the *S. noctilio* clade were the non-sterilizing North American strain of *D. siricidicola*. This included the nematode specimen "nig4," which was dissected from a *S. nigricornis*, indicating that in this instance the *S. nigricornis* was parasitized with the North American strain of *D. siricidicola*.

3.3. Identification of symbiotic fungal associates of Sirex

The *Amylostereum* fungus was identified to species from twelve of the fourteen *S. nigricornis* specimens from which nematodes were included in the study (Table 4). Four of the *S. nigricornis* carried *A. areolatum* in their mycangia, and five of the *S. nigricornis* carried *A. chailletii* in their mycangia. As the majority of *S. noctilio* included in the study were male, no successful fungal identification was possible. Of the two *S. nitidus* specimens included, only one was a female, and it was found to carry *A. chailletii* in its mycangia. Of the two nematode-parasitized *S. cyaneus* females from which fungus was identified, both carried *A. chailletii*.

4. Discussion

4.1. Nematode diversity and identification

For determining closely related isolates of *Deladenus* in the present study, the two-gene concatenated dataset for mtCO1 and ITS provided the finest resolution. Phylogenetic analyses showed that, for the most part, each *Sirex* host species included in the study has a corresponding nematode. With one exception, *S. nigricornis* included in the present study all contained the same nematode genotype, which matched the *Deladenus proximus* identified by Yu et al. (2011). Based on this information, we feel that the nematodes in our study collected from *S. nigricornis* are likely conspecific with those Bedding and Akhurst (1978) called *D. proximus* and collected in *S. nigricornis*.

D. wilsoni is another possible name to apply to nematodes obtained from *S. nigricornis*. Morphologically, the two species would be difficult if not impossible to distinguish. However, Bedding and Akhurst (1978) reported that although *D. wilsoni* can parasitize some *Sirex* spp., it had not been found in *S. nigricornis.* Additionally *D. wilsoni* mostly parasitizes rhyssine wasps, which are parasitoids of *Sirex* species, and it rarely produces the parasitic stage when in the presence of *Sirex* larvae in nature (Bedding and Akhurst, 1978). Future studies comparing gene sequences from nematodes found parasitizing rhyssines to nematodes parasitizing *S. nigricornis* could help elucidate the identities of these nematodes.

Nematodes infecting *S. cyaneus* likely represent *Deladenus canii,* described from *S. cyaneus* in fir (*Abies*) in New Brunswick, Canada (Bedding, 1974). *D. canii* also was said to be found in the southwestern United States (Bedding and Akhurst, 1978). However, it is likely that the woodwasp referred to as *S. cyaneus* in the southwest was actually a different species. In fact, southwestern *Sirex* woodwasps were collected from a different genus of conifer (*Picea*)

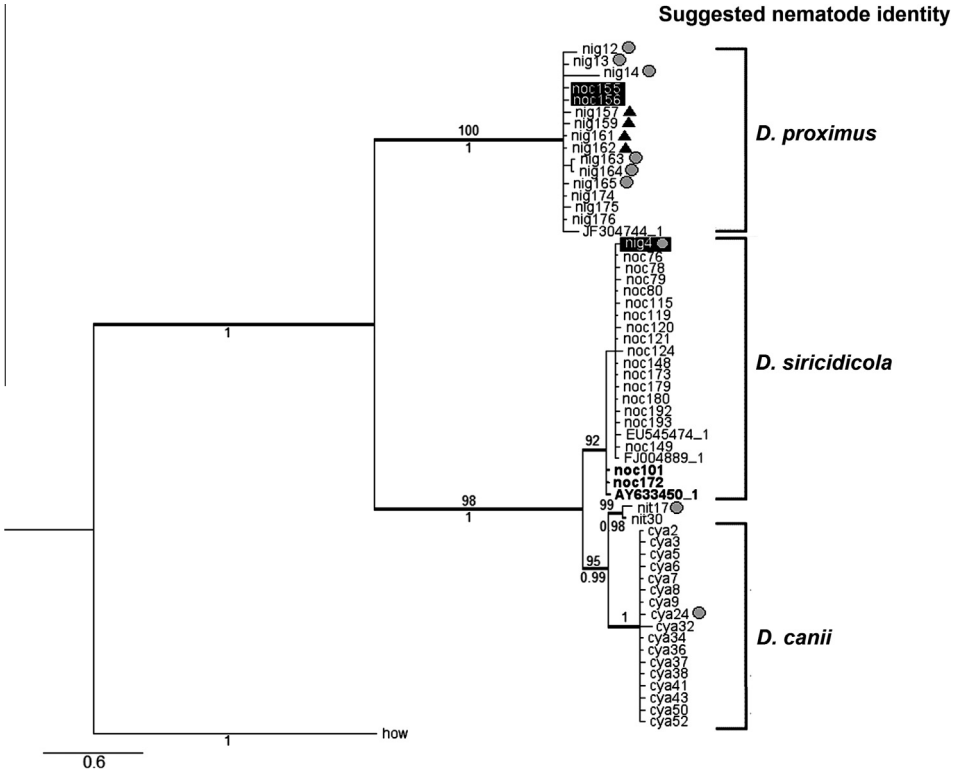

Fig. 1. Bayesian tree for *Deladenus* inferred from combined mtCO1 and ITS sequences. Values above branch points represent bootstrap support for clades well-supported by ML analysis (>70). Values below branch points represent the Bayesian posterior probability for well-supported clades (>0.95). Where MP analysis symmetric resampling values indicated well-supported clades (>75), branch lines are in bold. Branches without bold lines or numbers indicate the relationship was not supported above the aforementioned thresholds. *D. siricidicola* Kamona strain indicated in bold (noc101 and noc172). Suggested nematode species are indicated to the right of the clade. Instances of nematodes parasitizing unexpected *Sirex* hosts are highlighted in black. Known fungal associations of *Sirex* host are indicated with a circle (*A. chailletii*) or a triangle (*A. areolatum*). The outgroup is *Howardula aoronymphium*, a nematode parasitic on mycophagous *Drosophila*.

Table 4
Species of symbiotic *Amylostereum* fungus associated with *Sirex* specimens included in the study.

Species of host *Sirex*	*Deladenus* specimen number	Species of *Amylostereum*	Strain of *A. areolatum*	Genbank accession no.
S. nigricornis	nig159	*A. areolatum*	BE	
S. nigricornis	nig161	*A. areolatum*	BE	
S. nigricornis	nig162	*A. areolatum*	BE	
S. nigricornis	nig157	*A. areolatum*	BE	
S. nigricornis	nig163	*A. chailletii*		
S. nigricornis	nig165	*A. chailletii*		
S. nigricornis	nig12	*A. chailletii*		KC411828
S. nigricornis	nig14	*A. chailletii*		KC411829
S. nigricornis	nig13	*A. chailletii*		KC411827
S. nitidus	nit17	*A. chailletii*		KC411826
S. cyaneus	cya24	*A. chailletii*		KC411830
S. cyaneus	cya52	*A. chailletii*		

and were likely *S. nitidus* (H. Goulet, pers. comm.); therefore, it is possible that the nematode found in the southwestern *Sirex* was not the same nematode as was described from *S. cyaneus* in the eastern United States and Canada.

The sample "noc172" is the biological control agent *D. siricidicola* Kamona strain. Based on the presence of restriction sites (with the exception of "noc101"), all nematodes found parasitizing *S. noctilio* were the non-sterilizing North American strain of *D. siricidicola*. In some cases this was corroborated with data on whether the nematodes were present inside the eggs of the host or not (D.W.W., unpublished data). In many cases, however, this was difficult to diagnose, as many specimens originated from male *Sirex*, so there are no data regarding eggs. Moreover, the nematodes and their *Sirex* hosts had been stored in ethanol, which made it difficult to determine whether nematodes were inside of *Sirex* eggs or merely in the sheath of the egg as reported for the non-sterilizing strain of *D. siricidicola* by Yu et al. (2009). Yu et al. (2009) reported that the non-sterilizing strain of *D. siricidicola* is present in New York and Ontario. In the present study, the North American strain was found in New York as well as Pennsylvania. The sample "noc101," which is the *D. siricidicola* Kamona strain, originated from a controlled release study (D.W.W., unpublished data). This distinction between the non-sterilizing North American strain of *D. siricidicola* and *D. siricidicola* Kamona was also found by Leal et al. (2012), who reported that mtCO1 sequences for the two isolates could be used to differentiate the strains. Additionally, they were able to develop a PCR-RFLP tool to differentiate the strains.

Linking the nematodes collected in the present study to those previously described from *Sirex* hosts (Bedding, 1974; Bedding and Akhurst, 1978) is challenging for several reasons. First, none of the nematodes collected by Bedding and Akhurst (1978) were collected where our samples originated, so it is difficult to use geo-

graphic range as a guide. Second, the taxonomy of *Sirex* hosts from which the nematodes were collected has been in flux over the past several decades (Goulet, 2012), making it difficult to link the *Sirex* hosts mentioned in Bedding and Akhurst (1978) to the *Sirex* hosts included in the present study. Moreover, it is difficult to obtain adults of these nematodes, which express the morphological characters key to species identification, as parasitized *Sirex* hosts contain only juvenile nematodes. Even if adults were available, using morphological characters to differentiate species of *Deladenus* may be problematic, however, as Chitambar (1991) stated that, among the species of *Deladenus* that have been identified as having an insect parasitic stage, morphological characters alone cannot be used to distinguish species. In fact, due to intergrading morphological characters, the nematodes could only be placed into one of two superspecies: the *D. wilsoni* superspecies, containing *D. wilsoni* and *D. proximus*; and the *D. siricidicola* superspecies, containing *D. siricidicola*, *D. canii*, *Deladenus rudyii*, and *Deladenus nevexii* (Chitambar, 1991; Siddiqi, 2000).

4.2. Host specificity and potential for non-target effects

The invasion of *S. noctilio* has led to new possible *Sirex-Deladenus* associations. Because *S. noctilio* and *S. nigricornis* can co-infest pine trees, there is potential for nematodes to switch hosts. Three samples from the present study appear to be cases of host switching. Among nematodes collected from *S. noctilio*, two specimens out of 19 were found to be the nematode more often associated with *S. nigricornis.* These samples, "noc155" and "noc156", were collected from Warrensburg, New York, where a number of *Deladenus* nematodes carried by *S. nigricornis* specimens included in the study were collected. Likewise, among the nematodes collected from *S. nigricornis* specimens, one out of 14 was found to be the non-sterilizing strain of *D. siricidicola*. This sample, "nig4", was collected in Oswego, New York, where a number of *S. noctilio* containing the non-sterilizing strain of *D. siricidicola* were collected. This indicates a potential for non-target effects, should the *D. siricidicola* Kamona strain be released in North America. It is not known, however, whether parasitization of *S. nigricornis* by the *D. siricidicola* Kamona strain would lead to sterilization in *S. nigricornis*, nor is it known how frequently such a host switch might occur.

4.3. Host associations with Amylostereum species

With the exception of *D. wilsoni*, native North American *Deladenus* species have been thought to exclusively consume *A. chailletii* (Bedding and Akhurst, 1978); however, four of the *S. nigricornis* specimens included in the study were found to carry *A. areolatum* in their mycangia (Table 4). Bedding and Akhurst (1978) stated that *Deladenus* nematodes (with the exception of *D. wilsoni*) are highly fungus-specific. Additionally, Morris et al. (2012) found differences in the reproductive output of the *D. siricidicola* Kamona strain when feeding on different strains of *A. areolatum*. This suggests the possibility that nematodes associated with *S. nigricornis* are able to eat either *A. areolatum* or *A. chailletii*. If this nematode is indeed *D. proximus*, then this observation may not be too surprising, as *D. proximus* and *D. wilsoni* together comprise the *D. wilsoni* superspecies proposed by Chitambar (1991). Perhaps both nematodes in this superspecies are able to eat either species of *Amylostereum*. However, it could be possible that nematodes found in these *S. nigricornis* specimens had access to *A. chailletii* also in the tree at the same time.

4.4. Usefulness of mtCO1, ITS, and LSU for distinguishing Deladenus nematodes

Yu et al. (2009) were able to distinguish the non-sterilizing strain of *D. siricidicola* from the *D. siricidicola* Kamona strain based on mtCO1 sequence data, and in the present study, mtCO1 also was useful for distinguishing among *Deladenus* nematodes. ITS has been useful for identifying species of entomopathogenic nematodes, as well as assessing their evolutionary history (Stock, 2009). In the present study, MP analysis failed to resolve monophyletic clades based on ITS data, although the ML and Bayesian analyses both recovered well supported clades that reflect the two proposed superspecies. Ribosomal large subunit sequences (LSU) have been used to resolve taxonomic and phylogenetic issues at the genus and species level for nematodes, especially among *Steinernema* spp. (Stock et al., 2001). In the present study, no method of analysis was able to resolve clades based on LSU data (see Supplementary materials), and resolution became worse with the inclusion of outgroup taxa in the genus *Howardula*. The two *Deladenus* superspecies proposed by Chitambar (1991) were supported in all three analyses of mtCO1; however, this locus offered poor resolution among nematodes collected from *S. noctilio*, *S. cyaneus*, and *S. nitidus*.

The native *Deladenus* fauna is poorly known, and this study has helped clarify *Sirex-Deladenus* associations in eastern North America. Understanding the native fauna and *Sirex*-nematode specificity is important for determining non-target effects, should the *D. siricidicola* Kamona strain be introduced for biological control of *S. noctilio*. In particular, the apparent cross-infectivity of nematodes typical of *S. nigricornis* and *S. noctilio* indicates a possibility for native *S. nigricornis* to become parasitized by the primary biological control agent of *S. noctilio*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2013.03.003.

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