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Pathology, distribution, morphological and genetic identity of *Deladenus proximus* (Tylenchida: Neotylenchidae) a parasitic nematode of the woodwasp, *Sirex nigricornis* in the eastern United States

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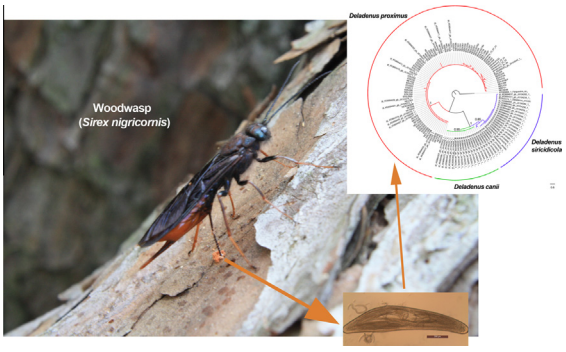
HIGHLIGHTS

- Fresh woodwasps were examined for internal nematodes to detect infected organs.
- Rearing and sequencing of specimens collected from type locality enabled unequivocal identification of *D. proximus*.
- *Sirex nigricornis* is commonly infected with *Deladenus proximus* across its range.
- *Deladenus proximus* sterilizes *S. nigricornis* females.
- Prevalence varied between years and locations likely due to environmental factors.

GRAPHICAL ABSTRACT



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ABSTRACT

The woodwasp, *Sirex nigricornis* (Hymenoptera: Siricidae), is solitary and utilizes a symbiotic fungus to extract nourishment from pine trees to feed its larvae. The woodwasp has a brief adult life, but the larvae develop for 1–3 years in the tree xylem. Infections with the nematode *Deladenus proximus* have been documented in the native woodwasp, *S. nigricornis* in the eastern United States and Canada. These nematodes appear to sterilize female woodwasps; however, the extent of the pathology and other aspects of the biology of *D. proximus* remain unknown. In this study we examined the effects of *D. proximus* on *S. nigricornis* using fresh – not preserved – specimens. Between 2009 and 2012, a total of 1639 woodwasps were examined for internal nematodes from emerging sites in Illinois, Louisiana and South Carolina. From this total, only 112 individuals were infected with the nematode *D. proximus*, with varying prevalence across localities and years. Nematodes were found inside every egg of infected females, as well as the hemocoel and the mycangia. Morphometric analyses of mycetophagous reared adult nematodes suggest that a single species is present in localities from Arkansas, Illinois, Louisiana, New York and South Carolina. The screening of the mitochondrial cytochrome oxidase subunit 1 (*cox1*) of these organisms is consistent with this pattern in that all of these individuals belong to a single clade. *Deladenus proximus* appears to be an efficient sterilizer, yet its prevalence is relatively low. Experimental infections of the invasive *Sirex noctilio* are recommended to test the viability of using this nematode as a biological control agent.

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1. Introduction

The family Siricidae (Hymenoptera: Symphyta) includes two subfamilies and eleven genera, five of which occur in North America (Schiff et al., 2012). There are around 100 named species of Siricidae worldwide with 23 in North America (Schiff et al., 2012). Siricid woodwasps possess an ovipositor that is adapted for drilling into wood and depositing eggs. The subfamily Siricinae is associated with conifers with a preference for the genus *Pinus* (Schiff et al., 2012; Talbot, 1977), although some species are associated with fir and spruce.

Sirex noctilio Fabricius is the only known woodwasp capable of killing healthy trees (Spradbery, 1977). The larvae of *S. noctilio* extract fluids from the xylem decomposed by the saprophytic action of *Amylostereum areolatum* (Chaillet) near the edge of growth of the fungus (Thompson et al., 2014). In its native range of North Africa and Eurasia, *S. noctilio* causes little damage to pines and is of minimal concern (Hall, 1968; Spradbery and Kirk, 1978). However, in locations where *S. noctilio* has invaded non-native pine plantations there is no community of predators, parasitoids and parasites to control the population (but see Yousuf et al., 2014b). As a consequence, populations of invasive *S. noctilio* have caused catastrophic damage to pine plantations in the southern hemisphere (Carnegie et al., 2005, 2006; Coutts, 1965). The first indication of an established population of *S. noctilio* in the United States was recorded near Syracuse, NY in 2004 (Hoebeke et al., 2005). As of 2010 *S. noctilio* was found across most of New York State, Ohio, Pennsylvania, Michigan, Vermont and southeastern Ontario, Canada without producing outbreaks in North American pines (Dodds et al., 2010). There are, however, fears that natural events could cause a spike in population that could lead to deaths of large numbers of trees (Yemshanov et al., 2009). Assuming that *S. noctilio* induces similar rates of pine mortality and degradation of wood prices as those seen in the southern hemisphere, the U.S. Forest Service estimates damage from *S. noctilio* in the U.S. could reach $2 billion within 55 years (Haugen, 2006), although Rabaglia and Lewis (2006) predict that damages could range between $3 and $17 billion in the U.S. over the same period.

The introduction of *S. noctilio* also resulted in the introduction of its parasitic nematode *Deladenus siricidicola* Bedding 1968 (Yu et al., 2009). This species of nematode has been used as a biological control agent against *S. noctilio* in Australia (Bedding, 1967), and several other countries in the southern hemisphere (Slippers et al., 2012). However, *in vitro* rearing of some strains of *S. noctilio* renders them unable to form the infective form that is able to penetrate woodwasp larvae (i.e., entomopathogenic females) and therefore, sterilize woodwasps (Bedding and Iede, 2005). A strain originally from Sopron, Hungary, (and later replaced by the Kamona strain from Tasmania) has been used extensively as a biological control agent of *S. noctilio* (Bedding and Akhurst, 1974, 1978). The strain of *D. siricidicola* found in North America, does not sterilize the woodwasp and may not have any impact on the spread of *S. noctilio* (Yu et al., 2009); however, infection has a negative effect on the size of females (Kroll et al., 2013). The presence of this non-sterilizing form of the Kamona strain of *D. siricidicola* in North America, raises concerns relative to the efficacy of a widespread biological control program and highlights the need to find a different agent.

The life cycle of the native woodwasp, *Sirex nigricornis* Fabricius, is assumed to be similar to the invasive woodwasp, *S. noctilio* (Bedding and Akhurst, 1978; Nielsen et al., 2009; Stillwell, 1966). Yet, unlike *S. noctilio*, females of *S. nigricornis* do not attack and kill healthy trees (Schiff et al., 2012). Females of *S. nigricornis* inoculate and distribute the wood-rotting fungi *Amylostereum chailletii* (Persoon) and *A. areolatum* to pines (*Pinus* spp.) that are dead or nearly dead (Furniss and Carolin, 1977; Hajek et al., 2013; Nielsen et al., 2009). Larvae of *S. nigricornis* create galleries in the wood that assist in the dispersal of the fungi, colonization by other insects and accelerate decomposition.

The parasitic nematode *Deladenus proximus* Bedding 1974 was originally described from infections detected in *S. nigricornis* collected in Sumter National Forest, South Carolina (Bedding, 1974). This nematode has a facultative dicyclic life history that is nearly identical to that of the biocontrol agent, *D. siricidicola* and other North American species in the genus including *Deladenus canii* Bedding (1974) and *Deladenus nevexii* Bedding (1974) (Bedding, 1974). *Deladenus proximus* sterilizes female woodwasps and can be cultured on agar plates with the symbiotic fungus of *S. nigricornis*, *A. areolatum* (Bedding, 1974; Morris and Hajek, 2014). Although the prevalence and effects of an unidentified species of *Deladenus* on *S. nigricornis* was characterized across Arkansas (Keeler, 2012), the extent of sterilization induced by *D. proximus* across the range of *S. nigricornis* and its geographic range remain unknown. Prior to this study, few have investigated the natural history of *D. proximus*, therefore little is known on its prevalence, pathology and distribution. With the invasion of the European woodwasp it is critically important to explore these aspects of the biology of *D. proximus* and potential impacts on the native woodwasp-nematode relationship as well as the potential to control the spread of *S. noctilio* with this native nematode. According to Morris et al. (2013), *D. proximus* is able to infect *S. noctilio* and the nematode has the ability to feed, grow and reproduce on cultures of the fungus *A. areolatum* (Morris et al., 2014). These findings suggest that the native nematode *D. proximus* may be a possible candidate as a biocontrol agent of *S. noctilio*.

The focus of this study is the native woodwasp *S. nigricornis* [junior synonym *Sirex edwardsii* (Schiff et al., 2012, 2006; Wilson and Schiff, 2006)] and the nematode *D. proximus*. The objectives of this work are as follows: (a) to identify and document nematodes associated with *S. nigricornis* across the eastern United States; (b) to characterize the prevalence of *D. proximus* in *S. nigricornis* and their ability to penetrate eggs; (c) to determine the effect of infection on the size of the host, and (d) to determine genetic diversity of these population(s) of nematodes and identify reliable characters that can be used to identify both adult and larval nematodes.

2. Methods

2.1. Woodwasp collection, nematode screening and effects of infection on host

We collected live adult female woodwasps from locations in Illinois (37.760223N, 89.157282W) during October 2011 and 2012, and South Carolina (34.467682N, 81.669316W) on 24–27 October and 9–14 November 2012 by felling eastern (*Pinus strobus*), shortleaf (*Pinus echinata*) and loblolly (*Pinus taeda*) pines. These pines were cut into 1-meter billets, which were then piled in a space of 3 m^2. A multiple funnel trap (Lindgren, 1983) with a cup filled with propylene glycol was positioned above the log pile. Live woodwasps were collected by hand as they attempted to oviposit on the logs; the multiple funnel traps yielded few woodwasps trapped in propylene glycol; both of these trapping methods only yield females. In Louisiana (31.743935N, 92.620239W) trees were felled and woodwasps were allowed to oviposit in them. The tree was then cut into billets and these were placed in 208.3 l barrels with screens covering them; woodwasps were collected from the barrels the following fall (October 2009, 2010, and 2011), thus yielding males and females.

Woodwasps were dissected in Petri dishes with sterile water under a calibrated dissecting microscope. The ovipositor was

separated from the body, keeping both ovipositor and mycangia intact. The remainder of the abdomen was opened to reveal the ovaries and other internal organs. The ovaries were then separated and examined for signs of nematodes. Intensity of infection of larval and adult *D. proximus* is an estimate of the number of nematodes present in the hemocoel, mycangia, gonads and eggs (Bush et al., 1997). Nematodes present in the hemocoel were processed with a sample divider based on the concept of a Folsom Plankton separator; this was made with a 30 ml collection vial with a divider half the diameter of the tube glued at its equator (Fig. 1). Nematodes were separated from the body of the woodwasp and were suspended in 5 ml sterile water. The water was gently mixed to ensure even distribution of suspended nematodes and the tube was rolled on a flat surface to divide the sample evenly. This was repeated 5 times to dilute the sample in half. Nematodes present in final dilution were counted and multiplied by 32, this number is the result of halving the sample 5 times ($2^5 = 32$). The accuracy of this method was compared against the actual tallied number of organisms, both numbers being within 5% of the total count. All woodwasp eggs from infected female wasps were examined for the presence on nematodes. From each individual infected, five woodwasp eggs were isolated and dissected to count the number of individual nematodes inside each; these numbers were averaged and this value was multiplied by the total number of eggs present.

Using the length of the abdomen of the woodwasp as a standard measurement, a two-way analysis of variance (ANOVA) was performed to compare size of infected and uninfected (healthy) woodwasps, as well as size depending on locations, using PROC GLM in SAS 9.2 (SAS Institute Inc., 2013). Males and females were treated as separate groups due to the known size differences between sexes (Schiff et al., 2006). Nematode prevalence – number of woodwasps infected divided by number of woodwasps examined (Bush et al., 1997) – was compared from year to year and across localities using a χ^2 test of independence in SAS 9.2.

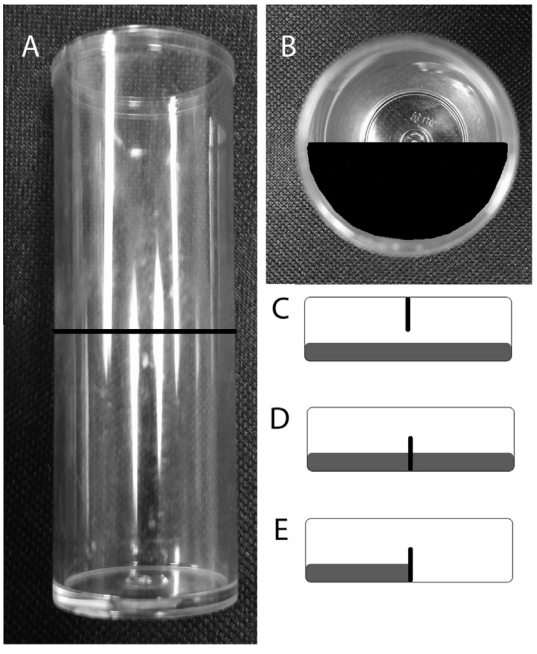

Fig. 1. Diagram showing the concept of the sample divider used to calculate the number of nematodes free in the hemocoel. (A) Insect vial with a line representing the location of the division. (B) View from the mouth of the vial with a representation of the sample divider. (C) Represents the liquid sample (gray area) before mixing and dividing. (D) Sample once rotated and divided. (E) Retained liquid sample. White scale bar is 1 cm.

2.2. Nematode identification

2.2.1. Morphological identification

To observe diagnostic features in adults, nematodes were reared on cultured fungus; thus spores of *A. chailletii* were inoculated in growth media and subsequently larval nematodes were added to the culture following the process described below. The fungal symbiont was isolated from the woodwasp and cultured on a modified pine potato dextrose agar (pPDA) plate. This pPDA included extracts from 500 g of fresh pine-wood (in 2.5 cm cubes); which were boiled in 1200 ml of distilled water for 1 h. This process extracted oils and resins from the pine-wood which can help prevent growth of non-target fungi and bacteria, as well as potentially enable growth of *A. chailletii* in culture. The solution containing these extracts was then filtered to remove particulate matter and used as the base for the agar. The recipe for the pPDA is 39 g of potato dextrose agar (Oxoid Ltd., Basingstoke, Hampshire, England) to 1 l of solution.

Under a laminar flow hood and using sterilized instruments the intact mycangia were removed from the woodwasps, dipped in 95% ETOH and rinsed with sterile water. The mycangia were then streaked on a modified pPDA plate and incubated at 21 °C. After 4 days, live nematodes were isolated from woodwasps and rinsed several times with sterile water and any non-nematode material removed. Nematodes were transferred to the fungal culture using a 10 µl pipette. Culturing continued at 21 °C and nematodes were monitored daily for growth. Larval nematodes removed from a single infected female woodwasp from Arkansas (34.399618, −94.270444) were also processed in this manner.

Mature nematodes were removed from cultures, fixed with TAF – triethanolamine, formaldehyde and water – (Courtney et al., 1955), and mounted on slides. Several key morphometric features proposed by de Man (1880) were taken and compared to previous studies of *D. proximus* (Bedding, 1974; Yu et al., 2011; Morris et al., 2014). These characters were compared using a multivariate analysis of variance (MANOVA) in SAS 9.2 (SAS Institute Inc., Cary, NC). The nematodes were deposited in the Harold W. Manter Laboratory Parasitology collection at the University of Nebraska-Lincoln (HWML49939–49944).

2.2.2. Screening for cryptic species of nematodes

In addition to our field-collected woodwasps, six larval nematodes recovered from one infected female *S. nigricornis* collected in Arkansas were included in this analysis. Individual larval nematodes collected from woodwasps were dried for 30 min at 30 °C. DNA was extracted using Proteinase-K and Chelex solution (Bio-Rad., Hercules, California) heating the sample at 56 °C for 2 h. After the initial heating the sample was heated at 95 °C for an additional 8 min. The sample was then centrifuged to bring the Chelex beads to the bottom and 70 µl of the supernatant was removed. A Taq PCR master mix kit (Qiagen Inc., Valencia, CA) was used to carry out polymerase chain reactions. Each reaction contained the following; 10.2 µl molecular grade water, 2.0 µl CoralLoad buffer (containing: Tris–Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, gel loading reagent, orange dye, red dye; pH 8.7), 4 mM each dNTP's, 0.5 µl forward primer, 0.5 µl reverse primer, 4.0 µl Q solution and 2.5 u Taq polymerase. A Bio-Rad C1000 Thermal cycler was used to perform the PCR. The primers NCO1f1 (5′-CCTACTATGATTGGTGGTTTT GGTAATTG-3′) and NCO1r.2 (5′-GTAGCAGCAGTAAAATAAGCAC-3′) were used to amplify a region of about 600 bp of the Cytochrome 1 (*cox1*) gene using the following profile: 3 min at 94 °C, [0:30, at 94 °C, 0:30 at 48 °C, 1:00 at 72 °C] × 35, 10:00 at 72 °C.

Successfully amplified products were cleaned with Exonuclease I/Shrimp alkaline phosphate (ExoSAP-IT, Affymatrix, Inc., Cleveland, OH), following manufacturer recommendations. The products were then used in a sequencing reaction. Each reaction

contained the following: Terminator BigDye kit version 3.1 (Applied Biosystems) 0.75 μl, 5× BigDye Buffer 3.0 μl, molecular grade water 9.25 μl, DNA template 1.0 μl, and DNA primer (only one direction each reaction, at concentration of 3.2 pM) 1.0 μl for a final volume of 15.0 μl. The thermal cylcer profile was 1 min at 96 °C, [0:15 at 96 °C, 0:10 at 50 °C, 4:00 at 60 °C] × 40. Products were purified with the aid of Sephadex columns (GE Healthcare, Buckinghampshire, UK), treated with 10 μl highly deionized formamide (Hi-Di, The Gel Company, San Francisco, CA), and direct sequenced in a 3130XL Genetic Analyzer (Applied Biosystems, Grand Island, NY).

The sequence data obtained was initially annotated and assembled into contigs using Sequencher (Gene Codes Corp., Ann Arbor, MI). Sequences were also downloaded from a previous study by Morris et al. (2013). A matrix was assembled with two congeneric species (*D. siricidicola* and *D. canii*) and a species from a different genus (*Fergusobia* sp.) acting as a sister group. Sequences were aligned using ClustalW (Larkin et al., 2007). Following alignment, the best-fitting substitution model was determined using JModelTest 2 (Darriba et al., 2012). The model of evolution indicated as the best fit using the Akaike information criterion is the general time reversible plus gamma (GTR + G). Posterior probabilities of branches were reconstructed using MrBayes 3.2 (Ronquist et al., 2012). The Bayesian analyses were run under the following conditions; 4 chains, 3 runs, and 10,000,000 generations. Each chain was sampled every 1000 generations. Tracer (Drummond and Rambaut, 2007) was used to determine if the appropriate burn-in was used.

2.3. Screening for genetic variation across color morphs in S. nigricornis

The woodwasp *S. nigricornis* features wide color and size variation; in the past up to five different nominal species, including *S. edwardsii*, were proposed as junior synonyms of *S. nigricornis* (Schiff et al., 2012). To identify the possible role of including different species of woodwasps in the analysis, DNA was amplified for woodwasps as well. A small sample of muscle was taken from the leg of the woodwasp and DNA was extracted using the protocol described above. The mitochondrial gene *cox1*, was amplified using primers LCO (5′ GGTCAACAAATCATAAAGATATTGG 3′) and HCO (5′ TAAACTTCAGGGTGACCAAAAAATCA 3′) and reactions were completed using a thermal profile consisting of 1 min at 95 °C, [1:00 at 95 °C, 1:00 at 50 °C, 1:30 at 72 °C] × 35, 7:00 at 72 °C.

3. Results

3.1. Prevalence and effects on host

Nematodes were located in the hemocoel, eggs, ovaries and testes, and in the mycangia in all infected woodwasps examined. The prevalence ranged from 1.1% to 33.3% based on location and year (Table 1). There was a significant difference among years and sites ($\chi^2_{6,1} = 77.82$, $p < 0.001$). Nematodes were found in 100% of eggs in infected female woodwasps (Fig. 2). In Illinois the number of nematodes in each egg ranged from 3 to 119. The average number of nematodes per egg was 30.37 and the average ranged from 18.2 to 93.8 among woodwasps. Mean intensity for Louisiana was 2298.25 (range = 1311–3930) in 2011; mean intensity in Illinois was 3100.27 (range = 1063–9808), and mean intensity in South Carolina was 13,006, (range = 520–42,096). A one-way ANOVA indicated that the difference in intensities was not significant ($F_{2,22} = 2.639$, $p = 0.096$).

A two-way ANOVA showed no significant difference in size between infected and uninfected female woodwasps ($F_{1,188} = 0.62$, $p = 0.4302$). There was no significant interaction between site and location ($F_{5,188} = 0.51$, $p = 0.599$). There was a significant difference in size when location was used alone, indicating that woodwasps in Louisiana and South Carolina were larger than those in Illinois ($F_{1,188} = 6.44$, $p = 0.002$). Male woodwasps were evaluated from Louisiana only because our trapping method in Illinois and South Carolina yielded females. A one-way ANOVA detected that infected male woodwasps in Louisiana were significantly smaller than uninfected individuals ($F_{1,113} = 8.65$, $p = 0.0040$).

Table 1
Prevalence (% wasps infected) and mean intensity (mean number of individuals per infected wasp) of infections caused by *D. proximus* in three localities in the eastern United States *N* = number of wasps collected.

Location/ infection	2009	2010	2011	2012
Louisiana				
Prevalence (*n*)	7.53% (*N* = 431)	1.11% (*N* = 721)	4.58% (*N* = 240)	–
Intensity (±SD)	–	–	2298.25 (±1191.75)	
Illinois				
Prevalence (*n*)	–	-	23.47% (*N* = 196)	33.3% (*N* = 30)
Intensity (±SD)	–	–	3100.27 (±2974.43)	–
South Carolina				
Prevalence (*n*)	–	–	–	31.25% (*N* = 21)
Intensity (±SD)	–	–	–	13,006.20 (±17821.02)

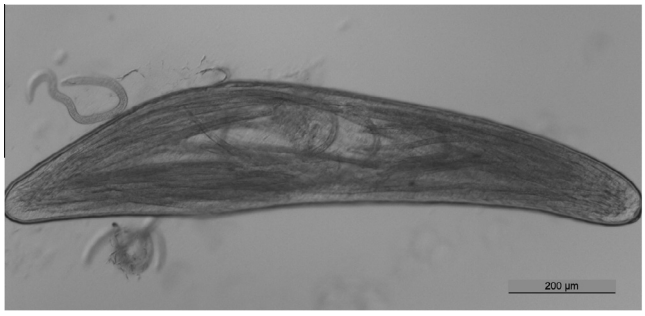

Fig. 2. Egg of *Sirex nigricornis* extracted from the ovarioles. Larvae of *Deladenus proximus* are visible inside the egg. An individual larva is visible on the upper left.

3.2. Nematode identification

3.2.1. Morphological homogeneity of adult nematodes

Morphometric features were measured in 20 adult male and female nematodes from Arkansas, Illinois, Louisiana and South Carolina (Table 2 and Fig. 3). The nematodes showed variability in size and morphometric features. Our morphometric data were compared to data from 3 previous accounts of *D. proximus* (Bedding, 1974; Yu et al., 2011; Morris et al., 2014). A comparison across the trapping locations shows there was no significant difference among sampled populations (females Wilks' $\lambda = 0.0001$, $F_{33,3.6502} = 2.11$, $p = 0.2618$, males Wilks' $\lambda = 0.549$, $F_{36,181.62} = 0.88$, $p = 0.6717$).

3.2.2. Haplotype heterogeneity in D. proximus and relationships with other nematodes

A total of 73 nematodes were successfully sequenced for *cox1* (Genbank accession numbers KJ193975–KJ194038). This included 37 from Illinois, 26 from Louisiana, 6 from Arkansas, and 4 from South Carolina. In addition to our sequences we downloaded 12

Table 2
Morphometric features of adult mycetophagous individuals of *Deladenus proximus* reared from infected wasps collected across four localities in the eastern United States. Values presented represent mean ± standard error and (range).

Morphomeptric feature	Illinois		Louisiana		South Carolina		Arkansas	
	Female (n = 20)	Male (n = 20)	Female (n = 20)	Male (n = 20)	Female (n = 20)	Male (n = 20)	Female (n = 20)	Male (n = 20)
Body length (μm) (L)	2045.81 ± 62.82 (1517.25–2491.94)	1420.98 ± 31.71 (1109.89–1696.18)	2064.90 ± 70.79 (1397.54–2614.74)	1413.03 ± 33.02 (1140.56–1660.20)	2032.73 ± 66.31 (1543.19–2494.81)	1445.97 ± 29.88 (1189.33–1632.16)	1990.05 ± 59.11 (1595.49–2463.25)	1411.73 ± 33.36 (1147.92–1701.82)
Body width (μm)	45.20 ± 1.42 (31.69–53.842)	20.49 ± 0.32 (18.06–23.82)	45.15 ± 1.48 (31.83–58.65)	20.39 ± 0.37 (17.45–23.09)	46.01 ± 1.55 (34.59–57.46)	20.93 ± 0.35 (18.04–23.69)	44.78 1.47 (33.56–56.92)	20.31 ± 0.35 (17.16–23.61)
Stylet length (μm)	11.41 ± 0.12 (10.20–12.21)	10.91 ± 0.13 (9.65–11.86)	11.49 ± 0.17 (9.98–12.47)	10.97 ± 0.07 (10.43–11.48)	10.78 ± 0.13 (9.67–11.73)	11.02 ± 0.10 (10.02–11.84)	11.19 ± 0.21 (9.78–12.67)	10.92 ± 0.13 (9.89–11.91)
Excretory pore from anterior end (μm)	150.45 1.04 (143.59–157.81)	–	152.07 ± 0.96 (142.65–157.14)	–	153.52 ± 0.65 (148.70–156.66)	–	152.98 0.65 (149.04–156.84)	123.82 ± 1.96 (109.48–139.95)
Excretory pore anterior to the hemizonid (μm)	8.10 ± 0.242 (6.47–9.79)	–	7.44 ± 0.27 (6.12–9.86)	–	7.42 ± 0.67 (1.41–9.49)	–	8.36 ± 0.24 (6.48–10.03)	–
Tail length (μm)	40.15 ± 0.86 (33.18–44.69)	40.93 ± 0.74 (33.25–47.70)	39.57 0.96 (32.65–44.34)	41.58 ± 0.65 (36.44–46.32)	38.07 ± 1.02 (29.90–43.45)	42.08 ± 0.71 (35.94–45.99)	37.59 0.90 (31.78–42.98)	41.37 ± 0.55 (36.92–46.51)
a	45.40 ± 0.77 (39.13–54.59)	69.34 ± 1.15 (57.33–77.75)	46.00 ± 0.87 (39.90–54.49)	69.52 ± 1.64 (55.68–84.03)	44.35 ± 0.80 (38.76–50.37)	69.24 ± 1.36 (59.91–82.80)	44.66 ± 0.81 (39.77–53.75)	69.62 ± 1.39 (56.99–79.52)
c	51.44 ± 1.17 (44.07–57.03)	34.78 ± 0.66 (29.48–41.19)	55.11 ± 0.66 (52.56–61.88)	34.03 ± 0.70 (26.88–38.79)	53.24 ± 0.57 (49.57–56.91)	34.48 ± 0.78 (29.15–40.34)	56.38 ± 1.42 (50.33–64.95)	34.18 ± 0.80 (27.62–40.71)
V	95.10 ± 0.28 (91.80–96.97)	–	94.50 ± 0.31 (90.79–96.12)	–	94.46 ± 0.18 (92.83–95.95)	–	94.99 ± 0.19 (93.84–96.71)	–
T	–	86.31 ± 0.62 (81.09–90.95)	–	85.90 ± 0.76 (80.60–92.00)	–	86.45 ± 0.64 (79.77–90.21)	–	85.64 ± 0.84 (77.39–91.90)

Symbols, a, body length/maximum body width; c, body length/tail length; T, (distance from cloacal aperture to anterior end of testes/body length) * 100, V, hemizonid to anterior end.

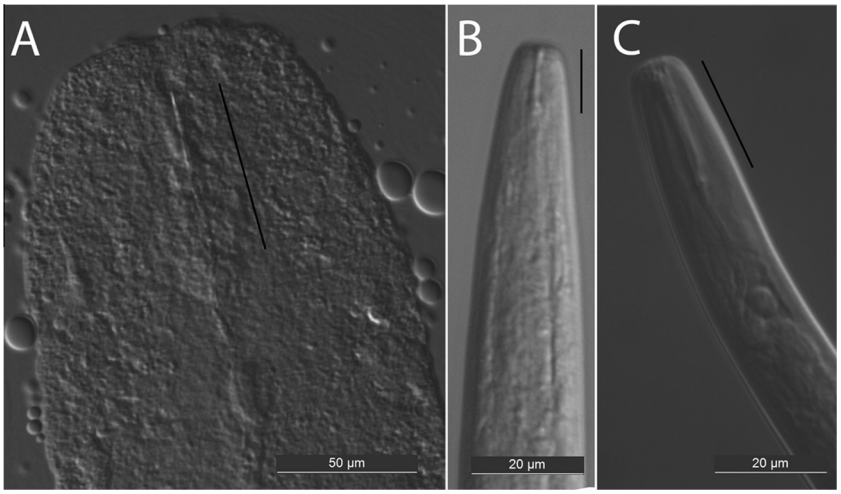

Fig. 3. Comparison of anterior ends of female *D. proximus* at different stages. (A) Entomopathogenic stage; (B) mycetophagous stage, and (C) pre-adult infective. The black line indicates the length of the stylet. Note that scale bar for both 1B and 1C is 20 µm.

sequences of *D. proximus* from Genbank deposited by Morris et al. (2013). These sequences included 7 nematodes collected from New York, 2 from Pennsylvania, and 3 from Louisiana. The sequences we obtained and those from GenBank did not match perfectly and had to be trimmed to match. The length of sequence that did line up was 520 base pairs. Phylogenetic analysis (Fig. 4) indicates that all individuals identified as *D. proximus* form a monophyletic clade with strong support, this includes specimens collected for the completion of this project and sequences deposited in GenBank by Morris et al. (2013). The divergence from *D. siricidicola* and *D. canii* had a posterior probability of 1.

3.3. Genetic homogeneity across color morphs in S. nigricornis

Amplification of *cox1* in the woodwasps was also successful (Genbank Accession numbers KF908922–KF908993). After sequencing and aligning the 666 bp of the *coxI* amplicon, we found no genetic variation among the woodwasps we collected. Further, we compared our sequences to those from previous studies (Schiff et al., 2012) and found no differences (100% match).

4. Discussion

4.1. Prevalence and effects on host

Larvae of *D. proximus* were found in all eggs of infected female woodwasps indicating that this nematode sterilized female hosts, a phenomenon reported in the original description of the species (Bedding, 1974). The pattern is consistent across 3 different locations that represent different climatic zones in southeastern United States. In contrast to our results, an examination of *S. nigricornis* across Arkansas using different methods revealed that only a fraction of infected females harbored *Deladenus* in every egg (Keeler, 2012). As such, it is unknown whether *S. nigricornis* experiences incomplete sterilization in Arkansas due to (1) different behavior of *D. proximus* in that region, (2) infection with a different *Deladenus* species entirely, or (3) co-occurrence of multiple species of *Deladenus* with differing sterilization capacity. Incomplete sterilization has been documented in *S. noctilio* infected with *D. siricidicola* (Yousuf et al., 2014a), and in both *Sirex cyaneus* Fabricius and *Urocercus californicus* (Ashmead) infected with the generalist *D. nevexii* (Bedding, 1974). However our results, in combination with the known ability of *S. nigricornis*, *S. noctilio* and *D. proximus* to feed on *A. chailletii* and *A. areolatum* (Hajek et al., 2013; Hurley et al., 2012; Leal et al., 2012; Morris et al., 2012), suggest that the native nematode, *D. proximus* collected from Louisiana, Illinois and South Carolina, should be explored as a possible agent to control *S. noctilio*.

Host size reduction due to nematode infection is a significant factor in decreasing dispersal range for *S. noctilio* because smaller woodwasps have a shorter maximum flight distance (Bedding, 1979; Villacide and Corley, 2008). Bedding (1979) suggested that size reduction in *S. noctilio* may be the result of competition with the nematodes for the fungal symbiont food source. We found no statistical difference in the size of infected and uninfected female *S. nigricornis*, yet males were significantly smaller when infected with *D. proximus*. However, male woodwasps do not disperse far, since they may simply fly to the top of the trees from which they emerged and wait for a mate (Slippers et al., 2012). Dispersal is critical for a successful biocontrol campaign and our findings suggest that nematode infection would have little effect on dispersal of female hosts, which would assist on the spreading of the nematode. Our results contrast with experimental and field tests of the effect of the infection of *D. siricidicola* on *S. noctilio* (Villacide and Corley, 2008; Yousuf et al., 2014a) and *Deladenus* sp. on *S. nigricornis* (Keeler, 2012). Since there are observed differences in males herein tested, the lack of significant differences between infected and uninfected females should be considered with caution. It may be convenient to select a set or even a single body trait to allow cross comparison in the body size of these wasps. These body parts may include not flexible parts of the thorax and legs.

The efficacy of *D. siricidicola* as a biocontrol agent of *S. noctilio* appears to be directly proportional to the density of the woodwasps (Bedding, 1993; Slippers et al., 2012). In addition, this efficacy may be affected by environmental factors that regulate the survival and growth of *D. siricidicola* (Slippers et al., 2012), and even the presence of other exotic species (Yousuf et al., 2014b). This variability may also explain the inability of *D. siricidicola* to infect the eggs of invasive woodwasps in North America. At a first glance, the varying prevalence of *D. proximus* does not make this species stand out as a promising biological control agent. However, it must be noted that larvae are very efficient at invading all eggs in the infected woodwasp and that the nematode is able to grow on cultures of both woodwasp fungal symbionts *A. chailletii* and *A. areolatum* (Morris et al., 2014). In addition, the nematode has been found in nature infecting the invasive woodwasp

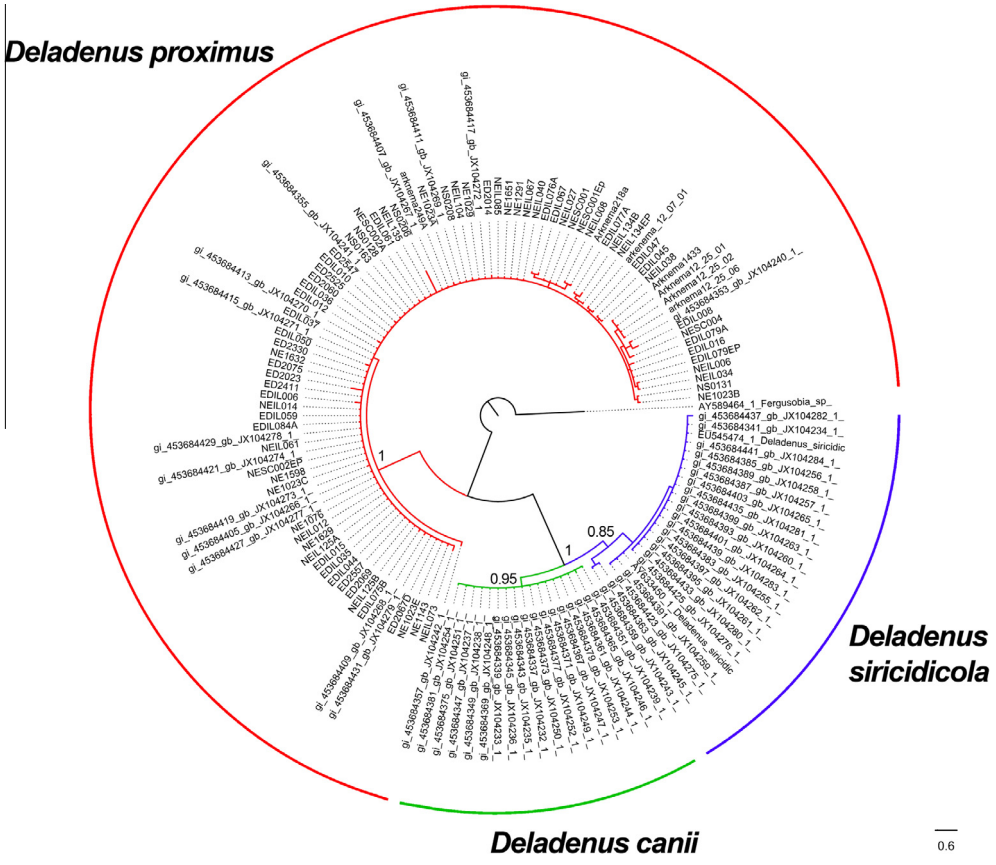

Fig. 4. Phylogenetic relationships for *Deladenus proximus* across the eastern United States, featuring posterior probabilities for three species present in North America: *D. proximus* in red, *D. siricidicola* in blue and *D. canii* in green. Bayesian posterior probabilities were inferred using MrBayes 3.5 under a GTR + G as model of evolution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Morris et al., 2013). These characteristics would suggest that the nematode could be used in the control of the invasive *S. noctilio*. Experimental infections of the invasive *S. noctilio* with the native *D. proximus* will help testing the hypothesis of the viability of *D. proximus* as a biocontrol agent of *S. noctilio*. Perhaps, both species of nematodes could be used in the control of *S. noctilio* in pine plantations.

4.2. Nematode identification

4.2.1. Morphological homogeneity and haplotype heterogeneity in D. proximus

Based on our analysis of the morphometric features and the genetic similarities, we found that a single species of nematode infects *S. nigricornis* across the range of locations sampled. This species matched the species description of *D. proximus* and subsequent characterizations for this species in Canada and New York (Bedding, 1974; Yu et al., 2011; Morris et al., 2014). Our trapping locations covered a large portion of the range of *S. nigricornis* and included Sumter National Forest, South Carolina, which is the type locality for *D. proximus*. While it is likely that *D. proximus* is present in all locations where *S. nigricornis* is present, data presented elsewhere raises questions of whether co-occurring species of *Deladenus* overlap in some areas (Keeler, 2012). Genetic analyses to identify individual nematodes from Keeler (2012) to the species level would be highly informative to understand variation in sterilization capacity and nematode community structure.

We expected to find variability in the morphological features of the examined nematodes, as it had been seen in previous studies of the species (Bedding, 1974; Morris et al., 2014; Yu et al., 2011). Although the locations sampled represent a significant portion of the geographic range of the native woodwasp, we were not able to detect a significant difference in the specimens across locations Previous studies only examined a few individual nematodes from each location (Bedding, 1974) or single locations (Morris et al., 2014; Yu et al., 2011). Our study includes several localities and measurements for a large number of nematodes from each locality. The nematodes were grown under identical conditions; therefore, if different species were present at least one phenotypic difference would likely be detectable.

4.3. Genetic homogeneity across color morphs in S. nigricornis

The *cox1* gene tree suggests that all individuals infecting *S. nigricornis* in the eastern U.S. belong to the same species. It indicates that all of our samples were closely related and therefore are considered the same species. This species was identified as *D. proximus* and included specimens collected from the type locality (Sumter National Forest, South Carolina). In addition, it reinforces the notion that *D. proximus* is more distantly related to *D. siricidicola* and *D. canii* than these two are to each other (Morris et al., 2013), suggesting that the diversity of *Deladenus* in North America includes several species infecting woodwasps associated with pines, fir, and spruce. We also conclude that specimens recovered from the hemocoel of *S. nigricornis* and sequenced by Morris et al. (2013) are part of the same species as the nematodes we observed sterilizing *S. nigricornis*. These data are important because they show that the association *D. proximus–S. nigricornis* can be consistently found from Louisiana to southeastern Canada, yet

cross infections (i.e., *D. proximus–S. noctilio*, and *D. siricidicola–S. nigricornis*) are observed in nature.

5. Conclusion

Rearing and sequencing nematodes infecting *S. nigricornis* across three localities in the United States, including the type locality, enabled us to identify them as *D. proximus*. We have established that the nematode *D. proximus* is able to induce sterility in all female woodwasps it infects, by invading the eggs and destroying the embryo. Although the nematode does not occur in high prevalence, it is easily reared in the laboratory. While *D. proximus* has been observed infecting *S. noctilio* in the wild, further research needs to be conducted to determine if the nematode is capable of infecting *S. noctilio* routinely and inflicting the same pathology as we observed in *S. nigricornis*. Additionally, a study of the genetics of the nematodes at closer distances and with larger sample sizes at each location may yield data on the dispersion of the nematodes via their woodwasp hosts in their native range. The utility of native nematodes as biocontrol agents may have been overlooked due to the assumption that these species are highly host specific and fungus specific.

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