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Deladenus (Tylenchida: Neotylenchidae) reproduction on species and strains of the white rot fungus *Amylostereum*

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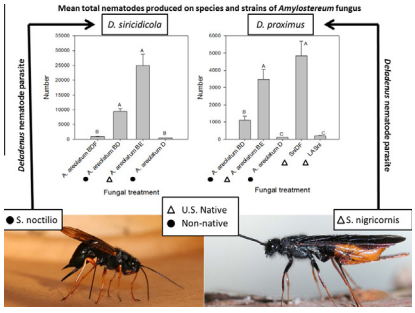
HIGHLIGHTS

- Native nematode *Deladenus proximus* can grow on both *A. chailletii* and *A. areolatum*.
- *Deladenus siricidicola* grows best on US native *Amylostereum areolatum* strain.
- *D. siricidicola* Kamona grows poorly on the fungus it will encounter in the US.
- Multiple species and strains of *Amylostereum* grew over and killed *Deladenus* eggs.

GRAPHICAL ABSTRACT



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ABSTRACT

Deladenus siricidicola nematodes are used for the biological control of invasive *Sirex noctilio* woodwasps in the Southern Hemisphere. Since the discovery of established *S. noctilio* in North America and Canada in 2005, a similar biological control program is under consideration in the United States. In this study, a culture of *Deladenus* nematodes was established from a native *Sirex nigricornis* woodwasp collected in New York State. These nematodes were identified as *Deladenus proximus* using molecular and morphometric techniques. *D. siricidicola* Kamona are mass produced for biological control when feeding on the white rot fungus *Amylostereum areolatum* in the Southern Hemisphere, so we compared the relative development and reproduction of mycophagous forms of both nematode species when feeding on native and invasive strains of *Amylostereum* fungus. *D. siricidicola* Kamona were able to reproduce on all strains of *A. areolatum* tested, but reproduced poorly on the *A. areolatum* strain they would be most likely to encounter in northeastern North America, should the nematode be released. Multiple strains of both species of *Amylostereum* were observed to grow over nematode eggs, preventing them from hatching. *D. proximus* were able to reproduce well on both *Amylostereum chailletii* and *A. areolatum*, despite prior evidence suggesting only *A. chailletii* is a suitable food source. This is the first report of the ability of *D. proximus* to survive and reproduce on *A. areolatum*, the fungal symbiont usually carried by *S. noctilio*, suggesting this native nematode should be evaluated for its ability to parasitize and sterilize *S. noctilio*.

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1. Introduction

Deladenus siricidicola Bedding (Tylenchida: Neotylenchidae) was the first nematode to be used commercially for pest control, and its use to control the invasive pine-killing woodwasp, *Sirex noctilio* F. (Hymenoptera: Siricidae), is one of the most successful classical biocontrol projects of its kind (Bedding and Iede, 2005). *D. siricidicola*, as well as some other nematodes of the genus *Deladenus*, live a remarkable dual lifestyle which alternates between fungus-feeding and insect-parasitic (Bedding, 2009). The fungal-feeding form (=mycophagous) lives inside trees while feeding on white rot fungi of the genus *Amylostereum* (Fries) Boidin (Basidiomycota: Russulales). The parasitic form of *D. siricidicola* infects woodwasp larvae of the genus *Sirex*. Parasitized adult female *Sirex* emerging from a tree act to disperse the nematodes when they oviposit in a new tree. *Sirex* woodwasp larvae are able to develop in trees due to *Amylostereum* fungus, a common resource of both the nematode and the woodwasp. Hyphal fragments, or oidia, of *Amylostereum* spp. are carried in specialized internal organs called mycangia within adult female *Sirex*, and oidia are injected into the tree at oviposition. Thus, *Sirex* woodwasps disperse both the nematodes and the food source for mycophagous *Deladenus*. Because parasitism often results in sterilization of the female woodwasp, *D. siricidicola* has been used extensively for biological control of *S. noctilio* in the Southern Hemisphere (Hurley et al., 2007).

S. noctilio is native to Eurasia and northern Africa, where it is not considered a pest. However, where it has been introduced in the Southern Hemisphere, it has caused extensive damage to plantations of introduced pines (Hurley et al., 2007). *S. noctilio* successfully infest pine trees (*Pinus* spp.) by injecting oidia of *Amylostereum areolatum* along with phytotoxic venom during oviposition. The fungus and venom inhibit the tree's ability to defend against the fungal invasion. *S. noctilio* larvae survive and grow only in the presence of *A. areolatum* (Madden and Coutts, 1979). Since being identified in the northeastern United States in 2005, *S. noctilio* has spread throughout New York and into Pennsylvania by 2006, to Vermont and Michigan by 2007, to Ohio by 2009, to Connecticut by 2010, and to New Jersey by 2012 (NAPIS, 2013).

Deladenus nematodes can produce many generations in the mycophagous phase, and this is exploited in the Southern Hemisphere in order to mass-produce *D. siricidicola* Kamona for biological control. This nematode owes its success as a biological control agent of *S. noctilio* in the Southern Hemisphere, in part, to the introduced nature of pine plantations there. In the absence of native pines, non-target effects are drastically reduced because there are no native *Sirex* species to parasitize and no native *Deladenus* species with which to compete, although several hymenopteran parasitoids have been introduced in addition to nematodes. However, pines are native to North America and North American forests are home to multiple species of *Sirex* woodwasps (Schiff et al., 2012). Some native *Sirex* species carry *Amylostereum chailletii* F. (Boidin) as their fungal symbiont, although *Sirex nigricornis* F. and *Sirex nitidus* (Harris) are now known to carry *A. areolatum* naturally at least sometimes (Hajek et al., 2013). Additionally, Wooding et al. (2013) concluded that extensive, reciprocal exchange of fungal species and strains is occurring, enabled by shifting geographic distributions of *Sirex* woodwasps. The strain of *A. areolatum* found in native *Sirex* spp., found both within and outside the current range of invasive *S. noctilio*, is different from those found in *S. noctilio* based on intergenic spacer regions (IGS), and is assumed to be native to North America (Nielsen et al., 2009; Hajek et al., 2013; Wooding et al., 2013).

Additionally, native *Deladenus* nematode species parasitize North American *Sirex* (Morris et al., 2013). In particular, the pine-dwelling native *S. nigricornis* is parasitized by *Deladenus proximus* (Bedding, 1974; Yu et al., 2011). There is potential for interaction between fungal and nematode communities associated with *S. noctilio* and *S. nigricornis*, due to their mutual affinity for pine trees. These *Sirex* species have been found infesting the same trees, and *S. nigricornis* has been found to carry a strain of *A. areolatum* known to have originated from *S. noctilio* (Nielsen et al., 2009; Hajek et al., 2013). In addition to fungal exchange, two instances of nematode exchange have been recorded. Two *S. nigricornis* specimens collected in New York State were parasitized by a strain of non-sterilizing *D. siricidicola* that was introduced to North America with *S. noctilio* and one *S. noctilio* in New York State was found carrying *D. proximus*, which is normally associated with *S. nigricornis* (Morris et al., 2013).

D. proximus was originally described from parasitized *S. nigricornis* in South Carolina, USA in 1974 (Bedding and Akhurst, 1978). Recently, Yu et al. (2011) found nematodes parasitizing *S. nigricornis* in Ontario, Canada, and identified them as *D. proximus* via morphological and molecular methods. The nematodes are difficult to identify in that they must first be reared to adulthood in culture before morphology can be used; additionally, there has been a scarcity of molecular work on known *Deladenus* species with which to compare sequences from unidentified *Deladenus*. *D. proximus* has not previously been recorded from the state of New York, so to confirm the identification of nematodes parasitizing *S. nigricornis* included in this study, nematodes were reared to adults and identified using both morphological and molecular methods.

Whereas *D. siricidicola* has been studied extensively due to its status as a biological control agent, relatively little is known about the biology of *D. proximus*. When Bedding and Akhurst (1978) described *D. proximus* in *S. nigricornis* in the Southeastern US, they found that the mycophagous form of the nematode was only able to eat *A. chailletii,* and not *A. areolatum*. Thus, it was eliminated as a potential biological control agent of *S. noctilio* (Bedding and Iede, 2005). Similarly Yu et al. (2011) found that *D. proximus* parasitizing *S. nigricornis* in Ontario, Canada, only matured to the adult stage when feeding on *A. chailletii* isolates. With the lone exception of *Deladenus wilsoni* Bedding, most insect-parasitic *Deladenus* reproduce when feeding on either *A. areolatum* or *A. chailletii* but not both (Bedding and Akhurst, 1978). However, Morris et al. (2013) found multiple *S. nigricornis* parasitized by *D. proximus* while simultaneously carrying *A. areolatum* in their mycangia, indicating the possibility that the *D. proximus* parasitizing those *S. nigricornis* developed in association with *A. areolatum.*

To explore possible new associations between the fungus and nematode communities associated with *S. noctilio* and *S. nigricornis* in North America, we were interested in the ability of *D. siricidicola* and *D. proximus* to feed on species and strains of *Amylostereum* currently present in North America. We hypothesized that *D. proximus* would be able to reproduce on the native North American strain of *A. areolatum*, in addition to *A. chailletii*. Several strains of *A. areolatum*, including the strain used for mass production of *D. siricidicola* in Australia, were established and subsequently inoculated with *D. siricidicola* or *D. proximus* eggs in order to investigate the relative increase of the nematode species on the different strains of fungus.

2. Materials and methods

2.1. D. siricidicola culture

The strain of *D. siricidicola* used for studies, now commonly called Kamona, was obtained in 2006 from Ecogrow Environment© where it is mass-produced for biological control. This strain

originated in Sopron, Hungary around 1967. However, due to the loss of nematode infectivity following years of laboratory culture was discovered in the 1980s, the strain of nematode was reisolated from an early field site where it had been released in Kamona, Tasmania (Bedding, 2009).

D. siricidicola Kamona were kept under USDA, APHIS permit in a quarantine facility (the Sarkaria Arthropod Research Laboratory, Ithaca, New York) for the duration of the experiment. These nematodes were initially grown on 100 mm diameter Petri dishes containing cultures of the strain of *A. areolatum* used in the commercial production of *D. siricidicola*, IGS strain BDF, using half-strength potato dextrose agar (Difco, Sparks, MD) with a total of 25 g/L agar to make this medium harder (½PDAh) (R.A. Bedding, pers. comm.). Petri dishes with fungus and nematodes were maintained inside brown paper bags in a 23 °C incubator with no lights.

2.2. Isolation of D. proximus

S. nigricornis specimens were obtained by felling red pine (*Pinus resinosa*) and Scots pine (*Pinus sylvestris*) trees exhibiting symptoms of *S. noctilio* infestation described by Hoebeke et al. (2005). Felled trees were cut into bolts approximately one meter in length and ends were waxed to prevent the wood from drying quickly. Bolts were put into fiber barrels (77.5 cm tall × 51.4 cm diam.) with window screening covering the top and kept at ambient conditions in a barn. During *Sirex* emergence periods, barrels were checked several times each week. *S. nigricornis* males were kept alive at 4 °C in 29.6 mL clear plastic cups with lids until dissection could confirm the presence of juvenile nematodes in the testes. A culture of *D. proximus* was established by inoculating the juvenile nematodes from a parasitized *S. nigricornis* male specimen that emerged from red pine (*P. resinosa*) from Warren County, New York on September 7, 2012, onto 100 mm diameter Petri dishes containing cultures of *A. areolatum* BE strain, using ½PDAh medium. Nematodes were maintained as a mycophagous culture inside brown paper bags in a 23 °C incubator with no light.

2.3. Fungal isolates

The geographic and host origins of the seven species and strains of *Amylostereum* included in this study are listed in Table 1. Two isolates of *A. chailletii* were included in the study. Four of the *A. areolatum* isolates were collected in North America and chosen to represent the diversity of genotypes present in North America based on intergenic spacer regions (IGS) (Nielsen et al., 2009; Hajek et al., 2013). Three of the isolates used in this study are thought to be native to North America (WB9/19, ScyME, and LLASni11/20-10). WB9/19 and ScyME are IGS-BE type, which occurs outside of the range of *S. noctilio* in the US, and LLASni11/20-10 also was collected where no *S. noctilio* occur. Two isolates are thought to have been introduced along with *S. noctilio* (Gr94-1, and SedDF).

Table 2
Morphometric features used to differentiate species in this study.

Morphometric characters	Symbol[a]
Body length	L
Body length/maximum body width	A
Body length/esophageal length	B
Body length/tail length	C
(Distance from cloacal aperture to anterior end of testes/body length) * 100	T
% Distance of vulva from anterior	V
(Distance from cloacal aperture to anterior end of ovaries/body length) * 100	G_1
Excretory pore to anterior end	
Excretory pore anterior to hemizonid	
Tail length	

[a] Characters are referred to by these symbols in Table 3.

2.4. S. nigricornis nematode identification

2.4.1. Molecular characterization

Nematode colonies obtained by rearing nematodes from *S. nigricornis* were identified using molecular characterization via amplification and sequencing of the mtCO1 gene. The primers used, PCR conditions, and sequencing methods were described in Morris et al. (2013). The sequence was then compared to *D. proximus* mtCO1 sequences in GenBank (JX104268).

2.4.2. D. proximus morphometrics

Adult mycophagous *D. proximus* were preserved according to Bedding (pers. comm.). Nematodes were washed from a Petri plate and the suspension, containing approximately 500 nematodes in 1 mL tap water, was placed into a 15 mL centrifuge tube. The tube was put into a 60 °C water bath for 1 min to kill the nematodes. The tube was next filled with 10 mL TAF fixative (2% triethanolamine and 8% formalin). Nematodes were allowed to settle, at which point excess TAF was removed. Adult nematodes were placed on slides, with five per slide. Two pieces of vellum paper were placed under either side of each 22 × 22 mm cover slip to prevent the weight of the cover slip from crushing the nematodes. Slides were then placed on a slide warmer (Chicago Surgical Electrical Co., Melrose Park, IL). The TAF was replaced slowly with a 50:50 ethyl alcohol (ETOH):glycerol solution by adding 10 µl of the solution to the edge of the cover slip and allowing it to diffuse. This was done to prevent the high density glycerol from crushing or distorting the nematodes. Slides were then left on the slide warmer at 50 °C for seven days, adding ETOH:glycerol daily as the TAF and ETOH evaporated. Twenty each of adult male and female *D. proximus* were measured and morphometrics were recorded using characters described by De Man (1880) (Table 2). Nematodes were examined and under a Leica DM2500 microscope with DIC capabilities and an attached DFC295 camera (Leica Microsystems, Wetzlar, Germany). Measurements were taken in Leica Application

Table 1
Strains and isolates of *Amylostereum* included in the reproduction assays.

SAC #	Isolate ID	Species	Isolation from	Original host	Date isolated	IGS strain
132	WB9/19[ac]	*A. areolatum*	Warrensburg, NY	S. nigricornis	19 Sep 2009	BE
081	ScyME[b,c]	*A. areolatum*	Waldo Co., ME	*S. nitidus*	13 Sep 2007	BE
101	Gr94-1[a,b]	*A. areolatum*	Fulton County, NY	*S. noctilio*	19 Feb 2008	BD
085	SedDF[a,b]	*A. areolatum*	Oswego County, NY	*S. nigricornis*	19 Sep 2007	D
001	Aussie[b]	*A. areolatum*	Sopron, Hungary	*S. juvencus*	1967–1968	BDF
091	SniDF[a,c]	*A. chailletii*	New Haven, NY	*S. nigricornis*	21 Sep 2007	G
152	LLASni11/20-10[c]	*A. chailletii*	Kisatchie NF, LA	*S. nigricornis*	20 Nov 2008	G

[a] Isolate used for *D. proximus* reproduction assay.
[b] Isolate used for *D. proximus* reproduction assay.
[c] Isolate thought to be native to US.

Suite 3.7 (LASv3.7) and compared to measurements of *D. proximus* provided by Yu et al. (2011) and Bedding (1974).

2.5. Nematode propagation assay

The goal of these studies was to compare *D. siricidicola* or *D. proximus* increase when feeding on native *A. areolatum* (IGS BE), introduced *A. areolatum* (IGS BD, D), or the commercial strain (IGS BDF). All nematode growth assays used ½PDAh medium for growing *Amylostereum* isolates and were conducted at 23 °C in darkness. The fungal isolates were grown in 100 mm diameter Petri dishes by transferring a 3 mm diameter plug from the growing edge of a culture. The fungal cultures were allowed to propagate for 5 d, at which point each dish was stored at 4 °C until it was inoculated with nematode eggs. To inoculate plates with nematode eggs, *D. siricidicola* Kamona and *D. proximus* colonies were flooded with sterile distilled water containing 6.25 mg/L gentocin. The liquid containing nematodes and eggs was filtered three times through a Swinnex filter holder (Millipore) equipped with a 60 μm filter, which allowed eggs to pass through into the filtrate. In some instances, it was unavoidable that a few small juveniles passed through with the eggs, but this happened equally for all treatments. Ten 20 μL samples were taken from this stock suspension and all eggs were counted under a dissecting microscope at 20× magnification to determine the total number of eggs in the stock solution volumetrically. The suspension was then diluted so that a mean of 167 eggs were present per 20 μL drop. Three drops were added to each fungal dish, equidistant from one another and half-way between the fungal plug and the edge of the Petri dish. Therefore, each dish received approximately 500 eggs. Inoculated Petri dishes were placed in paper bags and allowed to propagate for up to 34 d.

Starting at 7 d following nematode inoculation, plates were destructively sampled every 3 d for the remainder of the experiment. For each collection date, two to four plates per treatment were flooded with water. The amount of water used for flooding differed by plate due to the varying fungal and nematode densities, but it was always measured for calculation of the total nematodes produced per plate. Ten 20 μL samples of each of the washings were examined under a dissecting microscope at 20× magnification to volumetrically determine the total number of eggs and nematodes per dish. For the *D. siricidicola* study, the number of available nematode eggs for inoculating plates was a limiting factor, so fewer replicate plates were set up (from two to four), and the experiment was conducted on five separate dates for four isolates of *A. areolatum*. *A. chailletii* was not tested due to the inability of *D. siricidicola* to grow on *A. chailletii* (Bedding and Akhurst, 1978). For the *D. proximus* experiment, there were a total of 30 dishes of nematodes per each of the five fungal treatments (3 *A. areolatum*, 2 *A. chailletii*) for a total of 150 plates per experiment, and the experiment was conducted on three separate dates. *D. proximus* was not tested on *A. areolatum* BDF due to the authors' expectation that *A. areolatum* BDF will not be released in the US.

2.6. Statistical analysis

To compare the increase in nematode numbers when feeding on different fungal isolates, the total numbers of either *D. siricidicola* or *D. proximus* (including eggs, juveniles and adults) produced over the course of the experiments were transformed to log $(x + 1)$. Fungal isolate and days post nematode inoculation were the main effects using a multifactorial ANOVA with experimental replicate coded as a random effect, and means were separated with least squared means Tukey's HSD (JMP, 1989–2007). To determine differences in production of either *D. siricidicola* or *D. proximus* among fungal isolates for specific times, separate analyses were conducted, in which day was the main effect in one-way ANOVAs. The total number of nematodes produced per fungal isolate was compared and means were separated with least squared means Tukey's HSD (JMP, 1989–2007). To determine differences in total egg production of either *D. siricidicola* or *D. proximus* among isolates, the mean numbers of eggs for each treatment were calculated and then transformed to log $(x + 1)$. Fungal isolate was the main effect in a one-way ANOVA with experimental replicate coded as a random effect, and means were separated with least squared means Tukey's HSD (JMP, 1989–2007).

3. Results

3.1. S. nigricornis nematode identification

The twenty reared nematodes of each sex that were evaluated matched the original description of *D. proximus* (Bedding, 1974) as well as recent data from *D. proximus* parasitizing *S. nigricornis* in Canada (Yu et al., 2011). The sample size ($n = 20$ of each sex) was greater than the original description ($n = 12$ of each sex) and greater than the Yu et al. (2011) study ($n = 11$ females, $n = 7$ males). The results from the morphometric analysis of the nematodes included in the study fit within the previously defined parameters for *D. proximus* (Table 3).

The mtCO1 sequence for the *S. nigricornis* nematode used in the experiment was identical to previously identified *D. proximus* included in Morris et al. (2013). The sequence was submitted to Genbank (KJ573794).

3.2. Nematode growth assay

3.2.1. D. siricidicola Kamona feeding on strains of A. areolatum

D. siricidicola Kamona consistently attained a population density above the initial inoculation number per plate of 500 nematodes on all fungal isolates except *A. areolatum* IGS D (Fig. 1). There was an interaction between isolate and day ($F_{3,404} = 31.04$; $p < 0.0001$). Separate analyses on isolate and day showed that *A. areolatum* BE and *A. areolatum* BD produced significantly more nematodes than *A. areolatum* BDF and *A. areolatum* D over time ($t_{1,103} = 3.79$; $p < 0.0001$) (Fig. 2). *A. areolatum* BE and *A. areolatum* BD produced the most nematodes over all days combined, with averages of $2.5 \times 10^4 \pm 3.78 \times 10^3$ (mean + SE) and $9.40 \times 10^3 \pm 9.63 \times 10^2$, respectively ($F_{3,404} = 40.1$; $p < 0.0001$). *A. areolatum* strains BDF and D produced the fewest nematodes, with $9.10 \times 10^2 \pm 1.53 \times 10^2$, and $4.17 \times 10^2 \pm 34$, respectively.

Nematodes on *A. areolatum* strains BE and BD exceeded 500 by days 10 or 13, and nematodes on *A. areolatum* strain D never exceeded 500 (Fig. 1). *A. areolatum* BE and *A. areolatum* BD also produced the most eggs over all days combined, with averages of 7.9×10^3 and 1.9×10^3, respectively ($F_{3,41} = 26.0$; $p < 0.0001$) (Fig. 3).

3.2.2. Growth of D. proximus on strains of A. areolatum and isolates of A. chailletii

D. proximus consistently maintained a population well above the initial inoculation number of 500 nematodes when feeding on *A. areolatum* BE and the New York isolate of *A. chailletii* (Fig. 1). There was an interaction between isolate and day ($F_{4,438} = 7.14$; $p < 0.001$). Separate analyses on isolate and day showed that *A. chailletii* New York, *A. areolatum* BE, and *A. areolatum* BD produced significantly more nematodes than *A. chailletii* Louisiana and *A. areolatum* D over time ($t_{1,88} = 2.79$; $p = 0.007$) (Fig. 4). *A. chailletii* New York and *A. areolatum* BE produced the most nematodes over all days combined, with averages of $4.84 \times 10^3 \pm 8.60 \times 10^2$ and $3.48 \times 10^3 \pm 5.73 \times 10^2$, respectively ($F_{4,443} = 89.86$; $p < 0.001$)

Table 3
Morphometric measurements of mycophagous adults of *D. proximus*. Range is given in parentheses in the row under the mean ± SE.

Characters[a]	Yu et al. (2011) Measurements		Bedding (1974) Measurements		Deladenus from *S. nigricornis* Measurements	
	Female	Male	Female	Male	Female	Male
# Specimens	11	7	12	12	20	20
Body length (mm) (L)	2.38 ± 0.21 (1.93–2.91)	1.39 ± 0.18 (1.02–1.59)	2.03 (1.76–2.2)	1.52 (1.34–1.59)	2.04 (1.46–2.49)	1.43 (1.19–1.68)
Body width (μm)	49.12 ± 11.23 (32.55–71.19)	16.81 ± 1.73 (13.83–19.02)	– –	– –	45.48 (33.55–56.49)	19.99 (17.80–23.77)
Stylet length (μm)	10.06 ± 0.67 (8.07–10.13)	10.06 ± 0.61 (8.82–10.05)	11.4 (11–12)	11 (11)	11.18 (9.98–12.7)	10.85 (9.46–11.77)
Excretory pore from the anterior end (μm)	145.11 ± 25.73 (134.23–150.89)	95.33 ± 12.76 (89.99–111.96)	127 (127–159)	126 (111–142)	149.08 (142.98–155.17)	– –
Excretory pore anterior to the hemizonid (μm)	7.23 ± 4.41 (2.21–11.14)	7.33 ± 1.19 (4.57–8.80)	8.52 (1–11)	2.1 (0–7)	8.01 (6.46–9.55)	– –
Tail length (μm)	33.93 ± 3.96 (30.24–44.68)	40.02 ± 5.03 (27.94–45.15)	39 (31–47)	48 (44–52)	35.86 (30.41–43.38)	41.33 (34.66–46.10)
A	50.19 ± 6.47 (35.50–50.33)	81.47 ± 7.82 (65.00–91.87)	47.6 (40–53.7)	56 (48–62)	45.20 (38.00–58.41)	71.66 (55.58–83.46)
B	16.78 ± 3.33 (13.13–19.90)	18.56 ± 4.66 (12.56–22.91)	19.1 (16.3–21.3)	15.5 (13.6–17.5)	– –	– –
C	65.19 ± 6.39 (55.15–75.53)	34.30 ± 3.19 (28.84–38.00)	53 (44–60)	32 (27–35)	55.89 (47.49–64.53)	34.61 (28.3–40.74)
V	96.14 ± 0.86 (95.69–97.11)	– –	90.3 (87.3–93.9)	– –	95.58 (92.32–96.49)	– –
G_1	83.23 ± 6.91 (75.55–89.12)	– –	88.9 (84.8–94.8)		– –	– –
T	– –	78.59 ± 6.45 (71.00–86.45)	– –	90.3 (87.3–93.9)	– –	86.67 (81.98–90.45)

[a] Symbols are defined in Table 2.

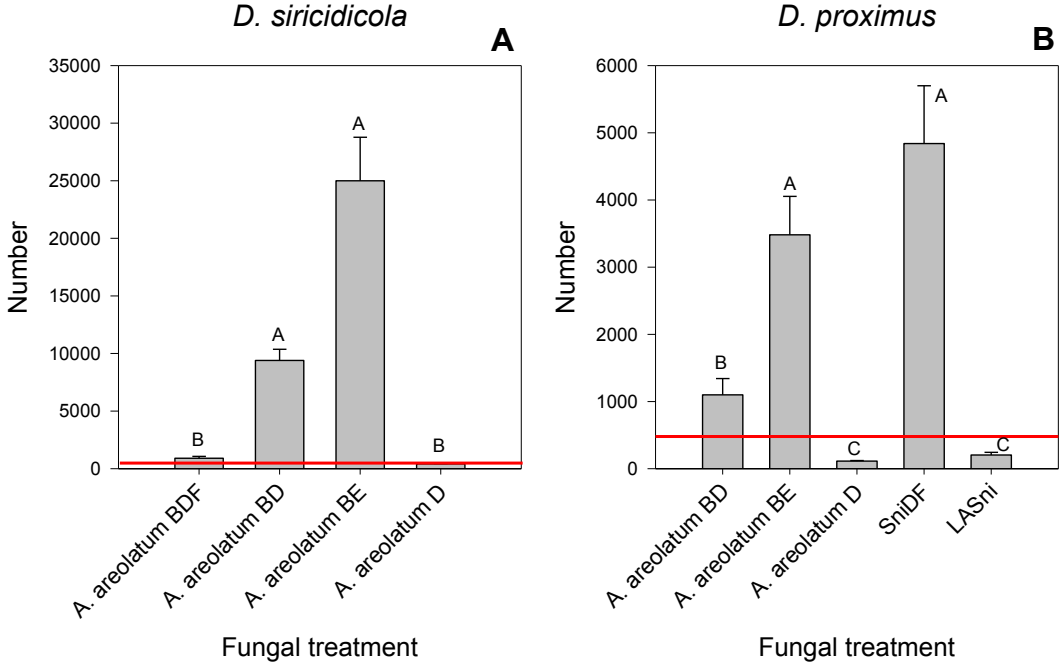

Fig. 1. Total combined numbers (±SE) *D. siricidicola* (A) and *D. proximus* (B) nematodes and eggs produced on fungal isolates. Capital letters denote significant differences between total nematodes and eggs produced on a given fungal isolate. Horizontal line indicates initial number of eggs inoculated.

(Fig. 1). Limited reproduction occurred by *D. proximus* on *A. areolatum* D and the Louisiana isolate of *A. chailletii*, with average numbers of nematodes of $1.13 \times 10^2 \pm 9$ and $2.04 \times 10^2 \pm 40$, respectively.

Nematodes were able to lay eggs on all isolates except *A. areolatum* strain D, on which nematodes never reproduced. The time at which eggs laid exceeded 500 was 10–13 d for the New York isolate of *A. chailletii*, and ranged from 10 to 16 d for plates of *A. areolatum* strain BE (Fig. 4). In only one experimental replicate of three did *D. proximus* growing on *A. areolatum* strain BD exceed 500, and this occurred between 16–19 d. *A. chailletii* New York and *A. areolatum* BE also produced the most eggs over all days combined, with averages of 7.9×10^3 and 1.9×10^3, respectively ($F_{3,38} = 50.1$; $p < 0.0001$) (Fig. 3).

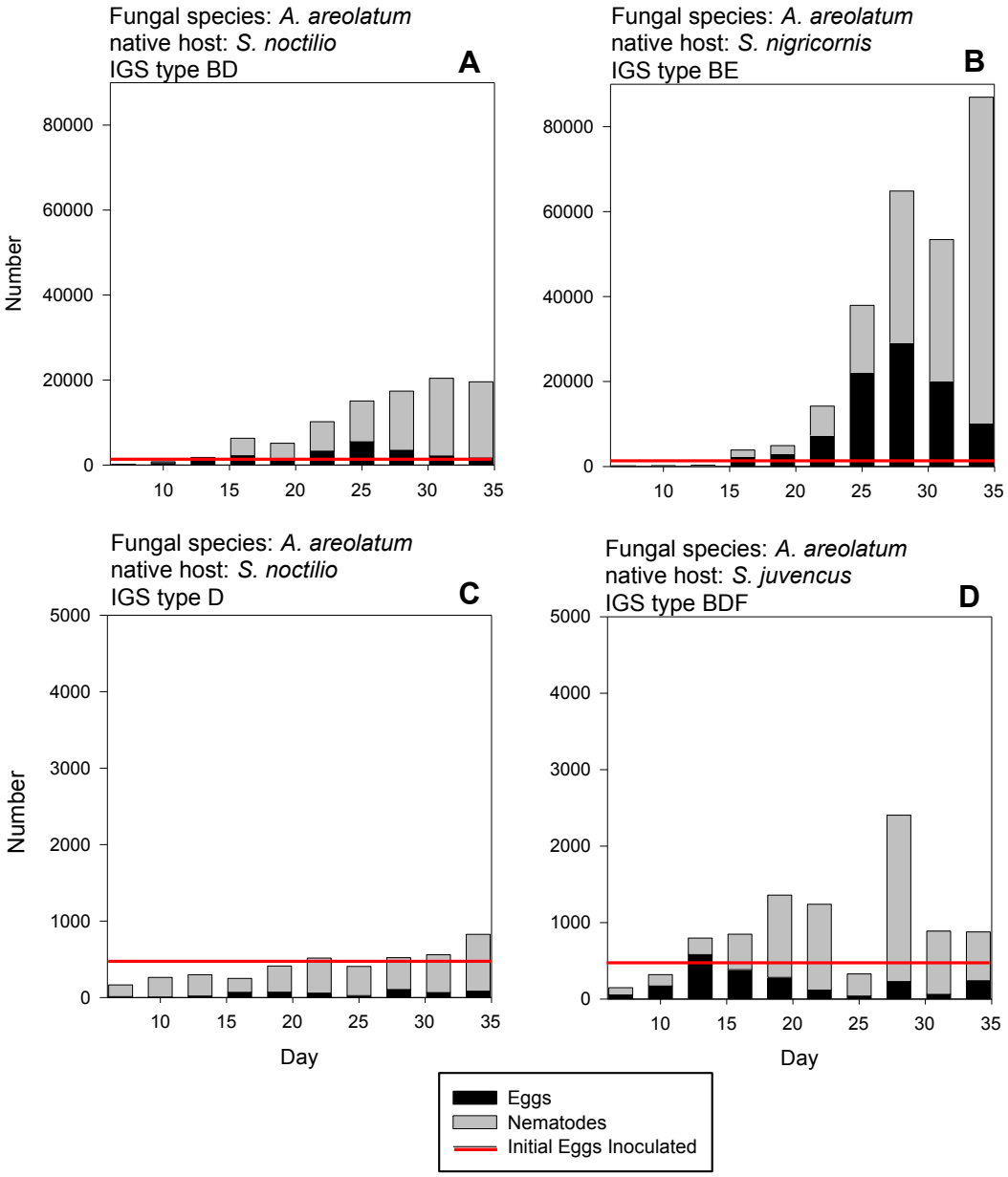

Fig. 2. Mean numbers of *D. siricidicola* nematodes and eggs produced on four strains of *A. areolatum* over 34 d. Upper case letters denote significant differences in the total of nematodes and eggs produced on different fungal treatments over all days combined. Horizontal line indicates initial number of eggs inoculated.

4. Discussion

Deladenus nematodes are introduced into a tree as the mycophagous form, whether via parasitized *S. noctilio* or manually as part of a biological control program. This makes the type of fungus available in the tree extremely important to the growth, survival, and reproduction of the nematodes, which ultimately will affect the ability of the nematodes to form infectives and parasitize new *S. noctilio* larvae. Previous studies found that *D. proximus* exclusively feeds on *A. chailletii*; Bedding and Akhurst (1978) and more recently Yu et al. (2011) found that *D. proximus* juveniles could only be reared to adults on strains of *A. chailletii*. Based on this finding, *D. proximus* was not evaluated as a possible biological control agent of *S. noctilio* (Bedding and Iede, 2005). In this study, however, *D. proximus* developed into adults and reproduced on two of the three *A. areolatum* isolates tested, in addition to *A. chailletii*. Attention has been called towards the possible need to find additional strains of *D. siricidicola* for biological control of *S. noctilio*, because it is hypothesized that *S. noctilio* had multiple founding populations, making the introduction of a single strain of nematode for biological control insufficient to control the genetically heterogeneous mixture of *S. noctilio* (Mlonyeni et al., 2011). Based on this need for nematode diversity, *D. proximus* should be evaluated for its ability to parasitize and sterilize *S. noctilio* in North America.

D. proximus has been found in New York State parasitizing *S. nigricornis* that carried *A. areolatum* BE strain in their mycangia, leading to the speculation that perhaps this fungal strain would be a suitable host food for the nematode (Morris et al., 2013). In this study, the two fungal isolates which produced the most *D. proximus* were *A. chailletii* (New York) and *A. areolatum* BE strain. These two isolates are notable in that both are thought to be native to North America and were therefore fungal symbionts of *S. nigricornis* prior to the *S. noctilio* invasion (Nielsen et al., 2009; Hajek et al., 2013; Wooding et al., 2013). A prior history between *D. proximus* and *A. areolatum* BE strain may be the reason that the

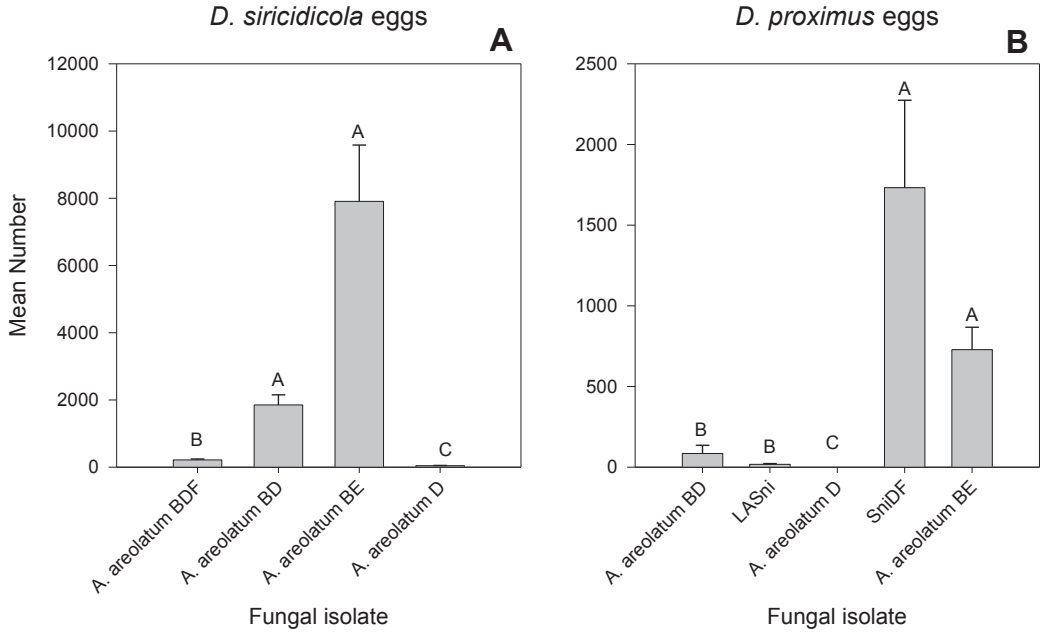

Fig. 3. Mean numbers (±SE) of *D. siricidicola* and *D. proximus* eggs produced on fungal isolates. Capital letters denote significant differences between eggs produced among fungal isolates.

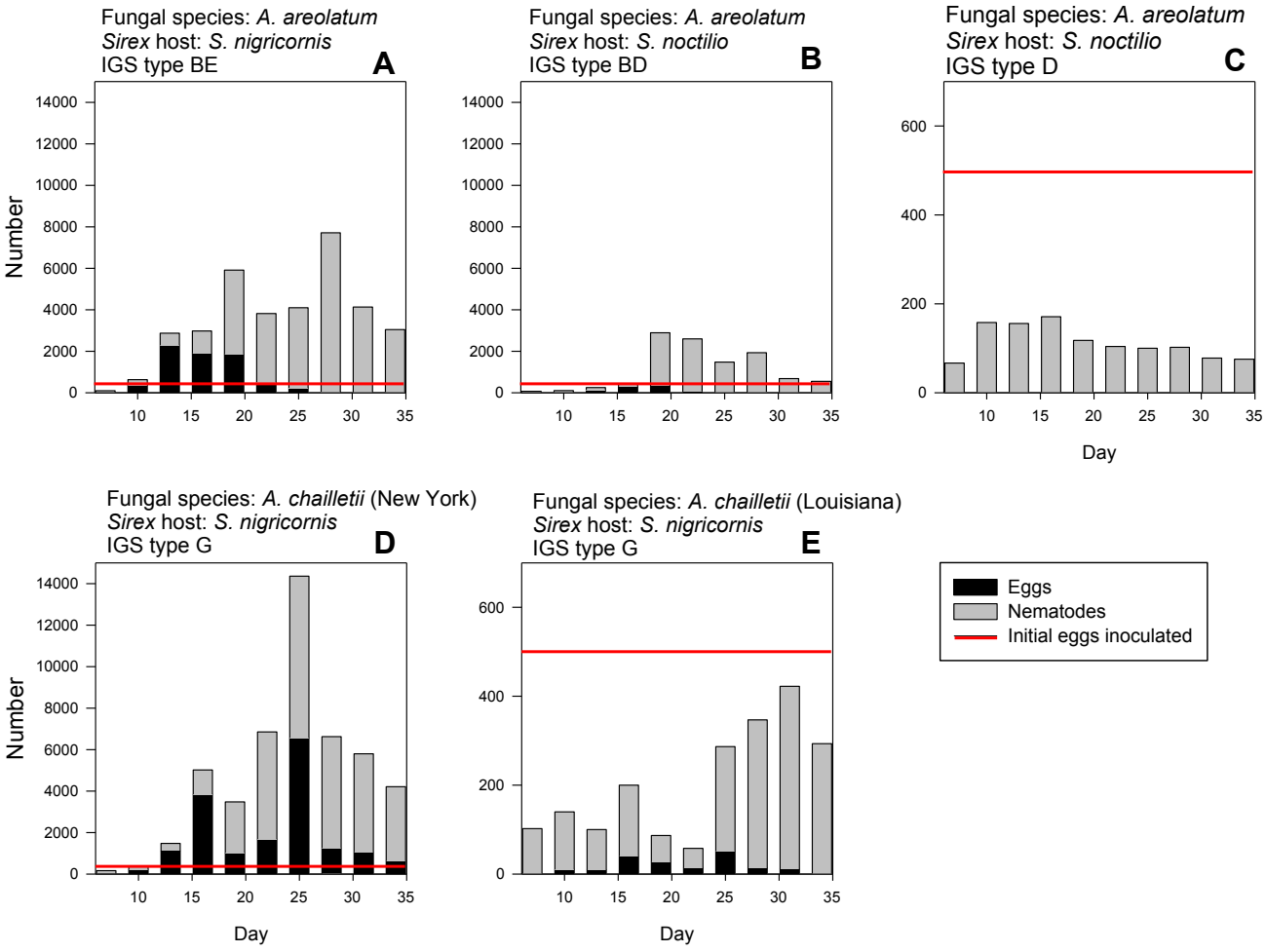

Fig. 4. Mean numbers (±SE) of *D. proximus* nematodes and eggs produced every 3 d on three strains of *A. areolatum* and two isolates of *A. chailletii* over 34 d. Horizontal line indicates initial number of eggs inoculated.

nematode can thrive on *A. areolatum* BE strain but not on *A. areolatum* BD and D strains, which are both thought to have been introduced to North America with the *S. noctilio* invasion.

Chitambar (1991) reported that insect-parasitic *Deladenus* nematodes only could be separated via morphometric measurements into one of two supergroups – the *D. wilsoni* supergroup, including *D. wilsoni* and *D. proximus*, and the *D. siricidicola* supergroup, containing all of the other insect-parasitic *Deladenus*. Since then, Yu et al. (2011) separated *D. wilsoni* and *D. proximus* based on distances between the anterior of the nematode, the excretory pore, and the hemizonid. However, given that *D. wilsoni* can grow on both species of *Amylostereum* (Bedding and Akhurst, 1978), it is reasonable that *D. proximus*, which is hypothesized to be closely related to *D. wilsoni*, also is able to grow on both fungal species. Notably, *D. wilsoni* historically was considered for the control of *S. noctilio*, but was eliminated due to its propensity to parasitize hymenopteran parasitoids of *S. noctilio* (Bedding and Iede, 2005).

Interestingly, the strain of *A. areolatum* on which *D. proximus* reproduced well, *A. areolatum* BE strain, is also the strain on which *D. siricidicola* Kamona produced numerically the greatest number of offspring, despite the apparent lack of evolutionary history of *D. siricidicola* with this fungal strain, which has not yet been found in Europe (Nielsen et al., 2009; Hajek et al., 2013; Wooding et al., 2013). Likewise, both species of nematode reproduced poorly on *A. areolatum* strain D, which originated from invading *S. noctilio*. *D. siricidicola* Kamona reproduced very little on this strain, despite developing into adults, while *D. proximus* never matured into adults, and thus did not reproduce at all when feeding on it.

This association between *D. siricidicola* Kamona and *A. areolatum* D strain could have unintended consequences in a biological control program. This fungal symbiont is widespread within *S. noctilio* populations in North America. Hajek et al. (2013) found that 25 out of 27 *S. noctilio* tested carried this strain as their fungal symbiont, based on IGS sequence data. *A. areolatum* symbionts of *S. noctilio* also have been commonly found with this IGS type in North America in other studies (Bergeron et al., 2011; Wooding et al., 2013). Given the poor reproduction of *D. siricidicola* Kamona strain on this particular fungal strain, adequate biological control may be difficult to achieve, if target *S. noctilio* are surrounded by a protective layer of non-host fungus, upon which the nematode will not develop properly.

To avoid releasing another non-native *A. areolatum* strain in North America during biological control introductions, Morris et al. (2012) suggested that *A. areolatum* BE strain be considered for mass production of *D. siricidicola* Kamona, should this nematode be released to control *S. noctilio*. At that time, however, it was not known that *D. proximus* and *D. siricidicola* could cross-infect their respective hosts (Morris et al., 2013), nor was it known that *D. proximus* could thrive when feeding on certain strains of *A. areolatum*.

A. areolatum BE strain, which has not been found in *S. noctilio*, is vegetatively incompatible with *A. areolatum* BD strain, which is carried by *S. noctilio* (Hajek et al., 2013). This suggests the possibility that using *A. areolatum* BE strain to mass produce *D. siricidicola* Kamona for biocontrol could lead to a vegetative incompatibility barrier in a tree between the injected nematodes and target *S. noctilio* larvae. Would *D. siricidicola* Kamona cross the barrier to parasitize the larvae? Or, consider another scenario involving injecting *D. siricidicola* Kamona and *A. areolatum* BE strain into a pine tree in which *D. proximus* are present. If the *D. proximus* benefitted from a population boost due to the presence of more of their fungal food source, they might then outcompete *D. siricidicola* Kamona and parasitize the invasive *S. noctilio*. Parasitism by a *Deladenus* nematode does not necessarily lead to sterilization of a given *Sirex* host. Indeed, *S. noctilio* invading North America brought with them a different strain of *D. siricidicola*, which is genetically distinguishable from the Kamona strain (Leal et al., 2012; Kroll et al., 2013). This nematode has been referred to as the "non-sterilizing strain" because it invades the *S. noctilio* reproductive system; however, juvenile nematodes remain external to the eggs, leading to viable *S. noctilio* offspring (Yu et al., 2009; Williams et al., 2012). Even in the absence of sterilization, however, this strain of *D. siricidicola* can have physiological impacts on the host, including reduced body size and lowered fecundity (Kroll et al., 2013). It is presently unknown whether *D. proximus* parasitizing *S. noctilio* would be sterilizing or non-sterilizing.

Fungal host can influence reproduction of mycophagous nematodes (Townshend, 1964). In the present study, *D. siricidicola* and *D. proximus* did not reproduce equally on different fungal treatments. This may be attributed to multiple factors ranging from poor nutrition (Pillai and Taylor, 1967) to nematicidal toxins (Jansson and Lopez-Llorca, 2001) of a given fungal strain.

In another scenario, eggs and nematodes instead could be overgrown and parasitized by fungus. An advantage to the destructive sampling design of this study was the ability to observe the numbers of eggs produced in a given treatment, and when those eggs appeared. A prior study showed that *D. siricidicola* Kamona reproduced poorly on *A. areolatum* IGS D, and this study investigated this further (Morris et al., 2012). We found that low yields of nematodes in different fungal treatments could not necessarily be attributed to the same cause. For example, although numbers of *D. proximus* on *A. chailletii* (Louisiana) and *A. areolatum* D strain were statistically the same, there were observable differences. On *A. chailletii* (Louisiana), nematode eggs present at inoculation were frequently overgrown by fungus and subsequently never hatched, but the few surviving juveniles were sometimes able to develop into adults and reproduce (EEM unpublished data). On the contrary, most *D. proximus* eggs on *A. areolatum* D hatched but the juveniles did not develop to the adult stage and therefore did not reproduce.

Prior literature suggests a "balance" must be achieved when growing *D. siricidicola* on *A. areolatum*, stating that fungus can overgrow a nematode culture (Bedding, 1972). The phenomenon was not discussed in detail, however. Other lignicolous fungi are fed upon by mycophagous nematodes (Townshend, 1964) and fungi have evolved different mechanisms of dealing with this grazing pressure. Barron (1977) suggested that wood-rotting fungi in particular could benefit from an antagonistic relationship with nematodes. First, nitrogen is generally limited in wood, so preying on nematodes could provide a nutritional boost for wood-rotting fungi. Second, when nematodes are fungivorous, there would be an added benefit for these fungi if there are fewer nematodes to graze upon fungal hyphae. In this study, when nematodes were able to lay large numbers of eggs, isolates of both species of fungus were often observed growing over eggs en masse (EEM, unpublished data); after nematode eggs were overgrown by *Amylostereum*, they became shrunken and never hatched, suggesting that the fungus is killing the nematode eggs. Whether this naturally occurs within trees is not known, nor is it known how the fungal colony is ultimately affected by using the nematode eggs for nutrition or by reducing the overall number of nematodes. Tanney and Hutchison (2011) described a novel nematode antifeedant mechanism employed by the basidiomycete *Sphaerobolus* spp., in which the stylets of *Aphelenchoides* nematodes pierced special fungal structures called gloeocystidia, resulting in the anterior portion of the nematodes becoming swollen and covered with an encapsulating matrix. This matrix prevented further nematode feeding, leading to death of the immobilized nematodes, possibly due to starvation. *Amylostereum* fungi have a different type of cystidia called "encrusted cystidia," which are thought to have a function in secretion (Gull and Newsam, 1975). Tanney and Hutchison (2011) discuss the role that cystidia play in fungal defense, and it

is possible that *Amylostereum* cystidia play a similar role against fungal grazers.

Nutrition also could play a role in the ability of *Deladenus* to successfully reproduce when feeding on *Amylostereum* species and strains. Pillai and Taylor (1967) tested the influence of fungal species on the population increase of multiple species of fungivorous nematodes and attributed lower populations of nematodes to an absence in some fungi of an essential nutrient required for nematode reproduction. The authors also successfully linked nematode morphometric measurements to fungal food source. Nutritional differences between species and strains of *Amylostereum* have not been described or quantified, though they certainly could exist.

In conclusion, this study provides evidence that species and strains of the symbiotic *Amylostereum* fungus carried by *Sirex* woodwasps impact reproduction of *Deladenus* nematodes. *D. proximus* can thrive on native North American *A. areolatum* BE strain, despite prior evidence suggesting it only eats *A. chailletii*. *D. siricidicola* Kamona, the biological control agent of *S. noctilio* in the Southern Hemisphere, is able to thrive on multiple strains of *A. areolatum*, including a strain isolated from *S. nigricornis* which historically had no association with *D. siricidicola* Kamona.

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