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Characterisation of the dimorphic *Deladenus beddingi* n. sp. and its associated woodwasp and fungus

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Summary – A new dimorphic species of *Deladenus* isolated from *Sirex californicus* from Washington, USA, is described as *D. beddingi* n. sp. Evolutionary relationships of the new species with other *Deladenus* species were assessed using multilocus sequencing. Phylogenetic relationships derived from analyses of mt*CO1* and ITS showed *D. beddingi* n. sp. to be genetically distinct from other North American *Deladenus* parasitising *Sirex*. Molecular analyses indicated that *D. beddingi* n. sp. is a member of the *D. siricidicola* species complex, which also includes undescribed native *Deladenus* from *Sirex cyaneus* and *S. nitidus*, and *D. siricidicola* from *S. noctilio*. Mycophagous adults were characterised by the position of the excretory pore, which was located 32 (22-52) and 48 (38-69) μm anterior to the hemizonid in mycophagous females and males, respectively. Typologically, the new species is most similar to *D. siricidicola*, *D. proximus* and *D. nitobei*, but can be distinguished from these species by several morphometric traits, including the value of ratios a, b, c of the mycophagous females and males, ratio b of the infective females, and the morphology of the tail of the mycophagous females, which is narrow and gradually tapering. This novel nematode species feeds on the fungus *Amylostereum chailletii* during its mycophagous phase. Experimental results showed very little reproduction by *D. beddingi* n. sp. when feeding on *A. areolatum* compared to robust reproduction when feeding on *A. chailletii*.

Keywords – *Amylostereum areolatum*, *Amylostereum chailletii*, molecular, morphology, morphometrics, mycophagy, Neotylenchidae, new species, *Sirex californicus*, species description, taxonomy.

There are 26 nominal species in the neotylenchid genus *Deladenus* Thorne, 1941, including the superspecies *D. siricidicola* Bedding, 1968 and *D. wilsoni* Bedding, 1968 that contain four and two species, respectively (Chitambar, 1991; Kanzaki *et al*., 2016). Ten species of *Deladenus* are known from North America (Supplementary Table S1), with nine considered native and one thought to have been introduced from Eurasia (Yu *et al*., 2009). All *Deladenus* species feed on fungi, but some have dimorphic life cycles with morphologically different forms living as insect parasites or as mycophages (Chitambar, 1991). Dimorphic *Deladenus* are known to parasitise Siricidae, Ichneumonidae (parasitoids of Siricidae) and Buprestidae (Hartshorn *et al*., 2017). In a review of the genus, Chitambar (1991) hypothesised that perhaps all species in this genus have both forms that feed on fungus and forms that infect and parasitise hosts, but many taxa have only been found and described in their mycophagous states. Among the native *Deladenus* species already known from North America, four are only known from mycophagous forms and five are dimorphic, including *D. canii* Bedding, 1974, *D. nevexii* Bedding, 1974, *D. proximus* Bedding, 1974, *D. siricidicola* and *D. wilsoni* (Bedding, 1974) (Supplementary Table S1).

Deladenus species known to be parasitic on siricids (woodwasps) feed specifically on white rot fungi in the genus *Amylostereum* when mycophagous. The same fungi are symbionts of *Sirex*, being transported by adult females and acting as obligate external rumens, aiding larval nutrition (Thompson *et al*., 2014). The micro-

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environment created by the presence of *Sirex* larvae within trees induces the formation of the infective form of *Deladenus* that enters siricid larvae (Bedding, 1993). Juvenile nematodes migrate to the ovaries, and, depending on various factors (*i.e*., host-parasite physiology, timing, and virulence of the nematode strain), they either enter the eggs (sterilising the female) or remain around the eggs in the ovarian tissues (Bedding & Iede, 2005). For example, a *D. siricidicola* strain that completely sterilises female *Sirex noctilio* F. in Australia by parasitising all eggs is commercialised for biological control (Hajek & Morris, 2014), while a strain of *D. siricidicola* that parasitises no eggs of *S. noctilio* is present in north-eastern North America and is thought to have been introduced along with the invasive *S. noctilio* (Yu *et al*., 2009; A.E. Hajek, data unpubl.). The native North American *D. proximus* usually parasitises only some of the eggs of *S. nigricornis* F. (Fisher *et al*., 2018). Parasitised female woodwasps emerge from trees, mate and then disperse to new locations where they inject a combination of viable woodwasp eggs, dead woodwasp eggs containing nematodes, nematodes in accessory gland fluid, venom, and fungus into trees (Bedding, 2009).

Sirex noctilio, a native of Eurasia, has been introduced to many locations in the southern hemisphere where the nematode *D. siricidicola* has been used extensively as a biological control agent (Slippers *et al*., 2012). *Sirex noctilio* was first found to be established and attacking pines (*Pinus* spp.) in north-eastern North America in 2004 (Hoebeke *et al*., 2005). This was the first time that this invasive species had been found in pre-existing native *Sirex* communities. Over time, cases of fungal symbiont swapping between the invasive *S. noctilio* and native *Sirex* species in the north-eastern USA have been reported (Nielsen *et al*., 2009; Hajek *et al*., 2013; Wooding *et al*., 2013; Castrillo *et al*., 2015), as well as cases of horizontal transfer of *Deladenus* from native to invasive *Sirex* and *vice versa* (Morris *et al*., 2013; Haavik *et al*., 2015). By contrast, virtually nothing is known about the native *Sirex-Deladenus-Amylostereum* communities in the western USA and it is crucial to close this knowledge gap for two main reasons. First, several locations on the western coast of the USA have been rated as 'high risk' for eventual introduction and establishment of *S. noctilio* (Borchert *et al*., 2007). Second, a potential *S. noctilio* invasion in the west could cause population shifts in the *Sirex-Amylostereum-Deladenus* community, which could ultimately negatively impact biodiversity. It is therefore important to document the existing community associated with *Sirex* species in the western USA before *S. noctilio* becomes established there.

In 2009, *S. californicus* (Ashmead) woodwasps that emerged from a pine tree in Seattle, Washington, USA were found to be parasitised by nematodes. The nematodes were isolated and cultured on the *Amylostereum* sp. collected from the mycangia of these woodwasps. In this paper, we describe this novel *Deladenus* species using morphological and molecular approaches. Furthermore, we investigate the phylogenetic relationship of this new species to other *Deladenus* species. In addition, we identify the fungus associated with this nematode and assess the effect of two different *Amylostereum* species on nematode reproduction.

Materials and methods

Isolation and culturing of the nematodes

Sirex californicus woodwasps were initially reared from a fallen hybrid pine, *Pinus monticola* Douglas *ex* D. Don × *strobus* L. tree in Washington Park Arboretum, Seattle, King County, Washington, USA, in section #6 (N47 38.293 W122 17.860; elevation 18.9 m a.s.l.). The tree fell on 6 February 2008 and *S. californicus* emerged from 23 July to 27 August 2009. A subsample of emerging wasps was dissected to recover parasitic nematodes. Nematodes were isolated from the reproductive tracts of two *S. californicus* adult females. Nematodes were subsequently cultured by inoculating plates containing half strength potato dextrose agar at 2.5% agar (1/2PDAh, with the designation 'h' indicating that the agar is harder; Morris *et al*., 2012) with arthrospores from the female wasps' mycangia and nematodes from within the host female's body.

Culturing the mycophagous form

Nematodes were maintained in the mycophagous form by propagating them on cultures of the fungal symbiont associated with *S. californicus* (Morris *et al*., 2012). Cultures were grown in 90 mm Petri dishes at 23°C and in darkness. Nematodes predominantly fed around the growing edge of the fungal culture. Cultures were transferred to fresh media every 14 days.

Inducing the infective form

Methods described by Bedding & Iede (2005) were followed to induce the mycophagous nematodes to develop

Table 1. PCR amplification and sequencing primers used for nematode identification.

Primer	Sequence (5′ → 3′)	Amplified gene	Reference
CO1F	5′-CCTACTATGATTGGTGGTTTTGGTAATTGAATAC-3′	mtCO1	Morris *et al*. (2013)
CO1R	5′-CAGGCAGTAAAATAAGCACGAGAATCTAAATCTAT-3′	mtCO1	Morris *et al*. (2013)
TW81	5′-GTTTCCGTAGGTGAACCTGC-3′	ITS	Subbotin *et al*. (2001)
AB28	5′-ATATGCTTAAGTTCAGCGGGT-3′	ITS	Subbotin *et al*. (2001)

into the infective form for morphological characterisation. Fungal cultures were inoculated onto 2% lactic acid PDA medium and placed in a desiccator which was flushed with 10% CO_2. The desiccator was then sealed and stored at 23°C for 7 days, at which point each plate was removed from the desiccator and inoculated with approximately 1000 nematode eggs. Plates were then placed back into the desiccator, which was flushed again with 10% CO_2, sealed, and returned to 23°C for approximately 2 weeks, at which point nematodes were killed and fixed.

Morphological and morphometric observations

Mycophagous males and females and infective females were examined after heat-killing and relaxation in M9 buffer in a water bath heated to 60°C. Heat-killed specimens were fixed in formaldehyde-acetic acid solution (FA 4:10) (Franklin & Goodey, 1949) at 50-60°C, slowly dehydrated, and processed to anhydrous glycerin (Seinhorst, 1959). Specimens were mounted on glass slides with Pliobond® industrial contact cement to both seal and provide cover glass support to avoid their flattening (Lee *et al*., 2009). Quantitative measurements of each nematode were made using an Olympus BX51 microscope equipped with differential interference contrast optics and Olympus Microsuite software (Soft Imaging System). Illustrations were prepared from digitised camera lucida images. Morphological characters measured considered both fixed and live specimens.

Nematode DNA extraction, amplification, sequencing, and analysis

Nematodes were obtained from cultures by flooding the dishes with 95% ethanol. Approximately 10 μl of the suspension containing several nematodes was placed in a 1.5 ml microcentrifuge tube containing 180 μl ATL buffer. DNA was extracted using a QIAamp DNA Micro Kit (Qiagen) after holding the microcentrifuge tube in a water bath at 56°C overnight for lysis. DNA was eluted in double distilled H_2O and stored at –20°C until use as a PCR template.

Primers used for PCR amplification and sequencing are listed in Table 1. Reaction conditions for amplification of mt*CO1* and ITS genes are described in Ye *et al*. (2007) and Subbotin *et al*. (2001, 2006), respectively.

A QIAQuick PCR Purification Kit (Qiagen) was used to purify PCR products according to the manufacturer's instructions and purified DNA was eluted in EB buffer. PCR products were sequenced in both directions at the Cornell University Biotechnology Resource Center (BRC). Raw sequence data were assembled and edited with BioEdit (version 7.2.5) (Hall, 1999). The sequence data were combined with a selection of representative sequence data included in Morris *et al*. (2013) and a Genbank sequence for *Howardula aoronymphium* Welch, 1959, in the same suborder as *Deladenus*, was included as an outgroup (Ye *et al*., 2007) (Supplementary Table S2). Sequence alignment was done for both genes using MAFFT (Katoh *et al*., 2002; Katoh & Toh, 2008) and inspected by direct examination in Mesquite (v. 2.74). jModelTest (v. 0.1.1) (Guindon & Gascuel, 2003; Posada, 2008) was used to select the most appropriate model of nucleotide substitution for each gene under the AIC criterion. Indels were treated as missing data. Maximum likelihood (ML) analyses were performed in RAxML with 100 bootstrap replicates for individual gene datasets. Resulting tree configurations did not reveal any conflict among mt*CO1* and ITS genes, and a combined dataset was created from both genes. A GTRgamma model, the default, was used for all ML analyses in RAxML, which was applied individually to four partitions in the concatenated mt*CO1*-ITS dataset: one partition for each codon position in mt*CO1*, and a separate partition for ITS. This dataset was analysed using the GTRgamma model for each of the three mt*CO1* partitions and GTRinvgamma for the ITS partition. Posterior probability of the branches was calculated on this partitioned alignment using MrBayes on the Cipres Science Gateway (Miller *et al*., 2010) under the following conditions: two independent runs, each with 1×10^6 generations, using four Markov chains (one cold, three

heated) for the COX1-ITS dataset. Each chain was sampled every 50 generations. Following analysis, the results were analysed using Tracer (v. 1.6) to determine that an effective sample size had been used and stationarity had been reached (Rambaut *et al*., 2014). The consensus tree was obtained using the 50% majority rule and default parameters. Results of the analysis were interpreted with MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), using a 25% burn in. Maximum parsimony (MP) analysis was conducted in TNT (v. 1.1) with TBR with 20 replications to find the most parsimonious tree. Symmetric resampling was used to calculate node support (Goloboff *et al*., 2003). The tree resulting from the inference of its posterior probability was visualised using FigTree (v. 1.31).

Isolation and identification of the fungal symbiont

One adult female *S. californicus* emerging in 2009 was dissected and one mycangium was used to initiate a fungal culture on PDA following the method described by Thomsen (1996). Fungal genomic DNA was extracted from a 2-week-old culture and from the other mycangium using the Qiagen DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. Eluted DNA was quantified using a spectrophotometer (NanoDrop 3000, ThermoScientific) and stored at −20°C until further use. The fungus, isolate number SAC203 for the mycangial sample and isolate SAC119 for the culture, has been deposited in the Center for Forest Mycology at the USDA Forest Service in Madison, WI, USA.

To identify the fungal symbiont, the ITS and small subunit of the mitochondrial (mtssu) ribosomal DNA regions and the protein coding gene RNA polymerase II (RPB2) were amplified and sequenced using the following primers: ITS1F/ITS4B (Gardes & Bruns, 1993); MS1/MS2 (White *et al*., 1990); and RPB2-6F/fRPB2-7cR (Matheny *et al*., 2007), respectively. PCR conditions were as reported for each gene. The PCR products were purified using QiaQuick PCR Purification Kit (Qiagen) following the manufacturer's protocol. Sequencing was performed using the Big Dye Terminator chemistry and AmpliTaq-FS DNA polymerase (Applied Biosystems) at the Cornell University BRC. Obtained sequences were compared with available sequence data from reference *Amylostereum* spp. strains, including six *Amylostereum chailletii* (Pers.) Boidin strains (Castrillo *et al*., 2015), and were also subjected to BLAST (National Center for Biotechnical Information, Bethesda, MD, USA) to determine fungus identity. Sequences for ITS (KT337458), mtssu (KT337459), and RPB2 (KT337460) regions were submitted to GenBank.

Nematode propagation assays

Nematode propagation assays used 1/2 PDAh medium for growing *A. chailletii* and *Amylostereum areolatum* (Chaillet *ex* Fr.) Boidin and were conducted at 23°C in darkness. The *A. chailletii* isolate used for these studies was the same strain isolated with these nematodes. The *A. areolatum* IGS BE strain was isolated from mycangia of *S. nigricornis* emerging from *P. sylvestris* L. from New Haven, NY. Fungal cultures were established in 90 mm diam. Petri dishes by transferring a 3 mm diam. plug from the growing edge of a culture. New cultures were maintained for 5 days, at which point each plate was inoculated with approximately 500 nematode eggs. To inoculate plates with eggs, nematode colonies producing many eggs were flooded with sterile distilled water, and this suspension containing eggs, nematodes and fungal fragments was filtered once through a Swinnex filter holder (Millipore) equipped with a 60 μm filter which allowed eggs to pass through into the filtrate. Some small juvenile nematodes also passed through the filter. The filtrate was vortexed to ensure an even suspension, after which ten 20 μl samples were taken. Eggs in each sample droplet were counted with a dissecting microscope at 20× to determine volumetrically the total number of eggs in the stock suspension. The suspension was then diluted to a mean of 167 eggs per 20 μl droplet. One 60 μl droplet of the suspension, containing approximately 500 eggs, was then added to each fungal plate. The drop was gently streaked around the periphery of the fungal colony. After inoculation, plates were kept at 23°C in darkness for 21 days.

After 21 days, the plates were flooded with water and ten 20 μl samples of the washings were examined under a dissecting microscope to volumetrically determine the total number of eggs and nematodes per dish. The amount of water used for flooding differed by plate due to the varying fungus and nematode densities, but it was always measured for use in calculation of the total nematodes produced per plate. A total of four replicate plates were set up for each of the two fungal species and this experiment was conducted three times.

Results

Deladenus beddingi[*] n. sp.
= *Deladenus* sp. Morris & Hajek, 2014
(Figs 1-3)

Measurements

See Table 2.

Description

Mycophagous female

Body straight when heat-killed, cuticle with fine annulations, annules 1 μm apart. Lateral field narrow. Lip region low, not offset from body. Stylet 10.5 $\pm$ 1.3 (8.5-12.5) μm long, with small basal knobs; shaft slightly longer than cone. Pharynx with cylindrical corpus, slightly swollen in middle and tapering gradually to isthmus; metacorpus absent. Pharyngeal gland region non-muscular. Pharyngo-intestinal valve not visible. Dorsal pharyngeal gland opening located immediately posterior to stylet base. Pharyngeal subventral gland overlapping subdorsal gland, its opening in mid-region of corpus. Excretory pore located 89 $\pm$ 7.5 (79-101) μm from anterior end. Hemizonid located 120 $\pm$ 16.0 (84-138) μm from anterior end. Pharyngo-intestinal junction located immediately posterior to nerve ring, with an oval non-muscular valve. Intestinal lumen wide. Rectum conspicuous. Ovary prodelphic, sometimes overlapping dorsal gland, post-uterine sac absent. Spermatheca usually ovoid. Uterus ending in a deep vagina. Vulva wide with protruding lips. Post-vulval region straight and tapering. Tail gradually narrowing to a pointed terminus. Phasmids not observed.

Mycophagous male

Body thinner than mycophagous female. Straight or slightly curved when heat-killed. Stylet structure similar to that of mycophagous female. Pharynx corpus cylindrical, slightly swollen in mid-region. Pharyngeal glands overlapping anterior end of intestine. Single testis, with short *vas deferens*. Spicules tylenchoid, 21.5 $\pm$ 2.5 (18-25) μm long. Spicule manubrium well developed, rounded, shaft well defined, lamina curved with one or two internal ribs, terminal end pointed. Gubernaculum thin, boat-shaped. Bursa peloderan, well developed. Tail conoid with a pointed terminus.

Infective female

Body slender, straight when heat-killed, with faint cuticle annulations. Lips fused. Stylet robust, 21 $\pm$ 1.4 (19-23) μm long. Shaft, cone and basal knobs fused, three guiding rings present. Pharynx cylindrical, pharyngo-intestinal junction near nerve ring. Pharyngeal dorsal gland opening located near stylet base. Ovary small and immature, narrow oviduct, spermatheca elongated, uterus short and reduced. Vulva small, in form of a narrow slit, non-protruding. Short post-uterine sac present. Rectum visible, located 31 $\pm$ 1.5 (28-33) μm from tail end. Tail narrow with pointed terminus.

Type habitat and locality

Specimens were isolated from a female *S. californicus* emerging from a *P. monticola* $\times$ *strobus* hybrid in section No. 6 of the Seattle Arboretum in Seattle, WA, USA, in 2009. GPS coordinates: 47°38.293′N, 122°17.860′W. Altitude: 18.9 m a.s.l.

Type material

Holotype mycophagous female, five paratype mycophagous females, five paratype mycophagous males and five paratype infective females deposited in the University of California Davis Nematode Collection (UCDNC), Davis, CA, USA. Five paratype mycophagous females, five paratype mycophagous males and five paratype infective females deposited in the USDA Nematode Collection, MD, USA.

Diagnosis and relationships

Mycophagous adults are characterised by the position of the excretory pore, which is 32 (22-52) and 48 (38-69) μm anterior to the hemizonid in mycophagous females and males, respectively.

Deladenus beddingi n. sp. morphologically resembles *D. siricidicola*, *D. proximus* and *Deladenus nitobei* Kanzaki, Tanaka, Fitza, Kosaka, Slippers, Kimura, Tsuchiya & Tabata, 2016 (Kanzaki *et al*., 2016), but can be distinguished from these species by several morphometric traits, including the value of ratios a, b, c of the mycophagous females and males, ratio b of the infective females, and the morphology of the tail of the mycophagous females, which is narrow and gradually tapering.

[*] Named after Robin A. Bedding, a leading expert in studies of dimorphic species in the genus *Deladenus* and in the implementation of *D. siricidicola* for biological control of *S. noctilio*.

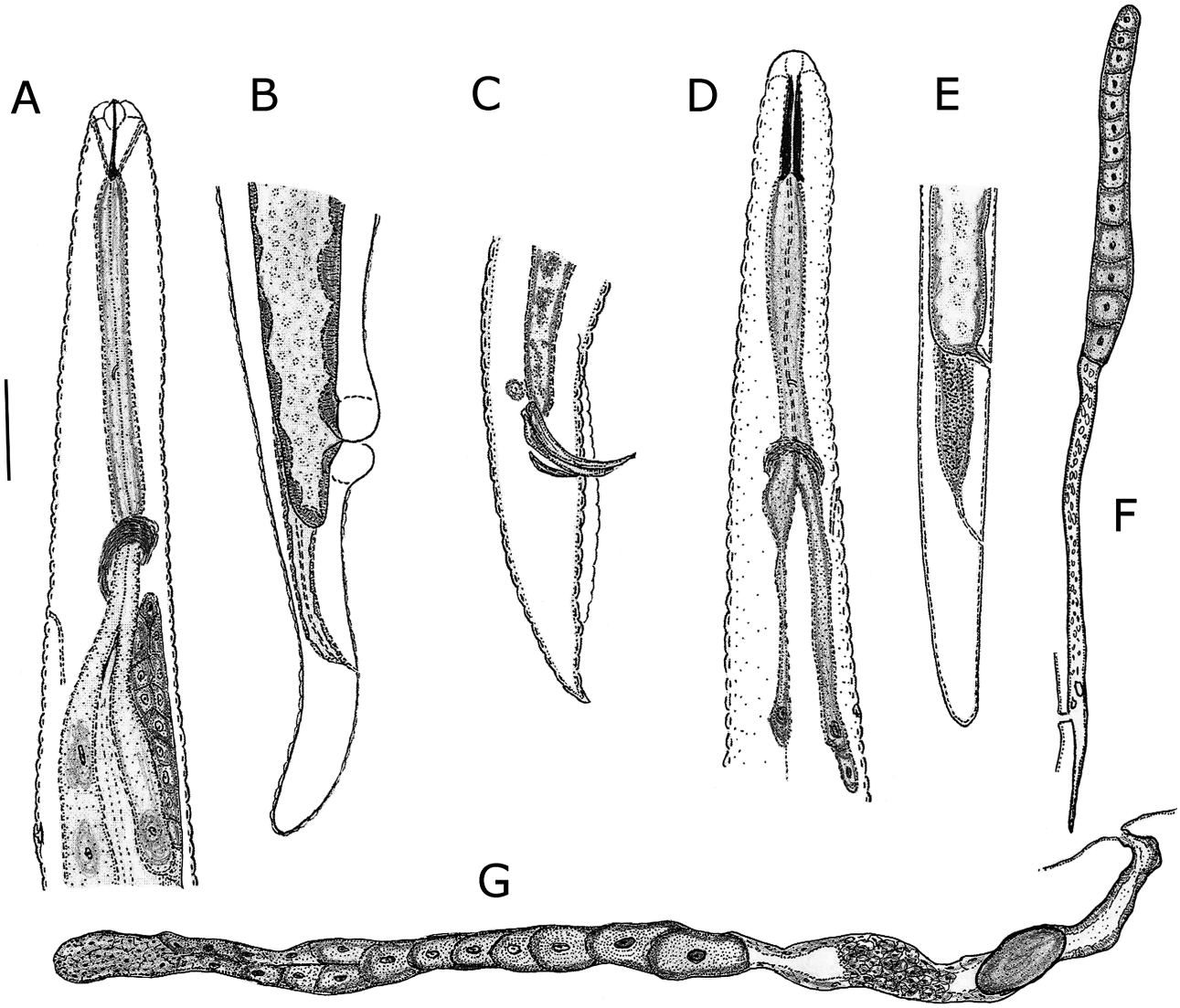

Fig. 1. Line drawings of *Deladenus beddingi* n. sp. A, B: Mycophagous female, anterior end, lateral view (A) and posterior end, lateral view (B); C: Mycophagous male, posterior end, lateral view: D, E: Infective female, anterior end, lateral view (D), posterior end, lateral view (E); F: Infective female, reproductive system; G: Mycophagous female, reproductive system with well-developed oocytes. (Scale bars are based on that in A: A = 13.5 μm; B, C = 15 μm; D = 20 μm; E = 17.5 μm; F = 28 μm; G = 60 μm.)

Having the excretory pore more than 20 μm anterior to the hemizonid was recognised by Chitambar (1991) as a key trait of many dimorphic *Deladenus* spp. However, this feature separates the new species from *D. proximus*, which has the hemizonid located less than 15 μm from the excretory pore for both mycophagous females and males (Tables 3-5). By contrast, this feature overlaps between *D. beddingi* n. sp. and *D. siricidicola*, *D. canii* and *D. valveus* Yu, Popovic & Gu, 2014. The distance between the excretory pore and hemizonid in mycophagous females of *D. nitobei* is marginally shorter than in *D. beddingi* n. sp. Similarly, the distance between the excretory pore and hemizonid in mycophagous males of *D. nitobei* is shorter than *D. beddingi* n. sp. Additionally, mycophagous females of *D. nitobei*, *D. siricidicola*, and *D. canii* all have a broad tail, whereas *D. beddingi* n. sp. has a more narrow, gradually tapering tail.

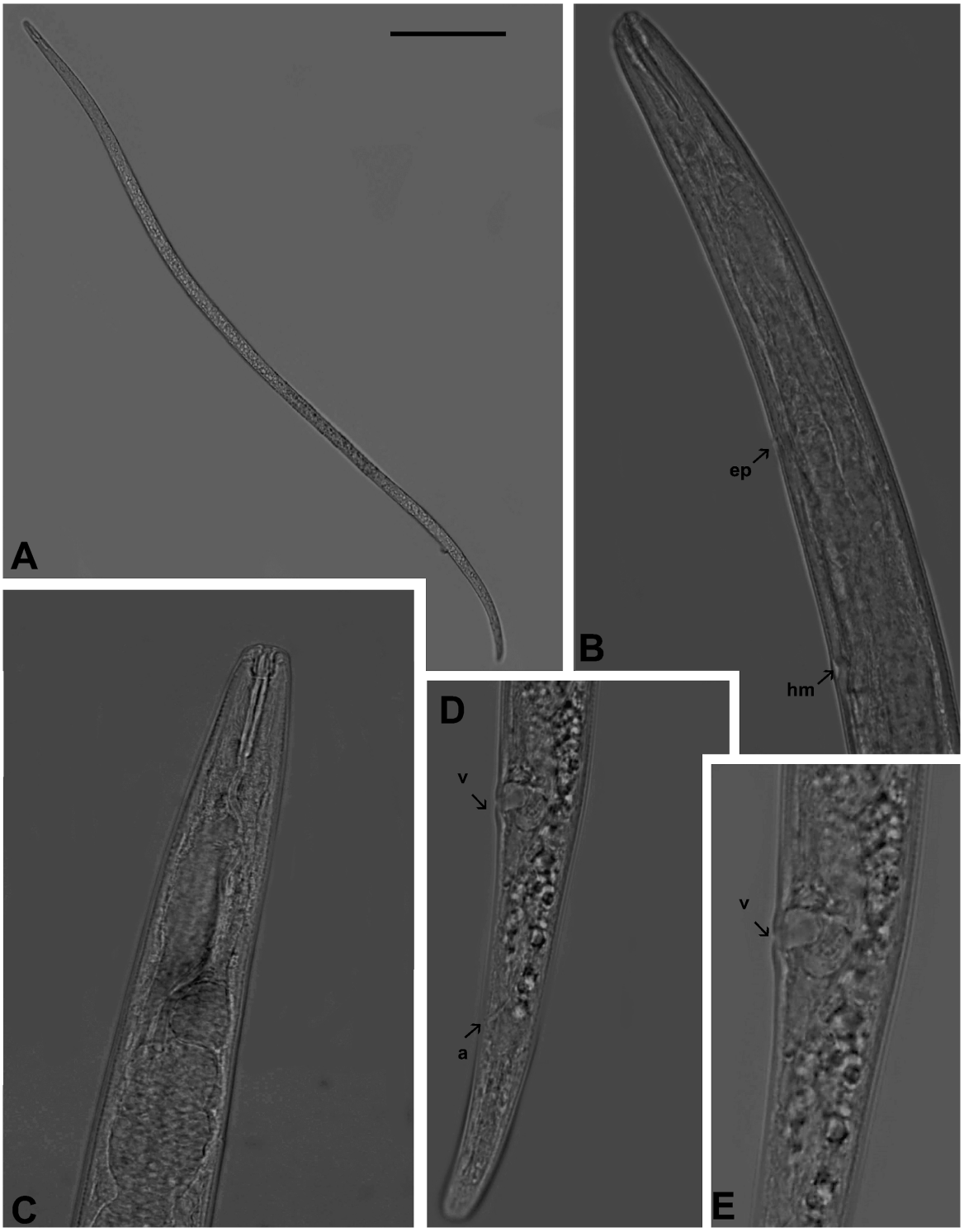

Fig. 2. Light microscopy images of infective female of *Deladenus beddingi* n. sp. A: Entire body, lateral view; B, C: Anterior end, lateral view of two individuals, excretory pore (ep) and hemizonid (hm) are shown in B; D: Posterior end showing vulva (v) and anus (a); E: Vulval region (v). (Scale bars: A = 132 μm; B = 22 μm; C = 17.5 μm; D, E = 13.5 μm.)

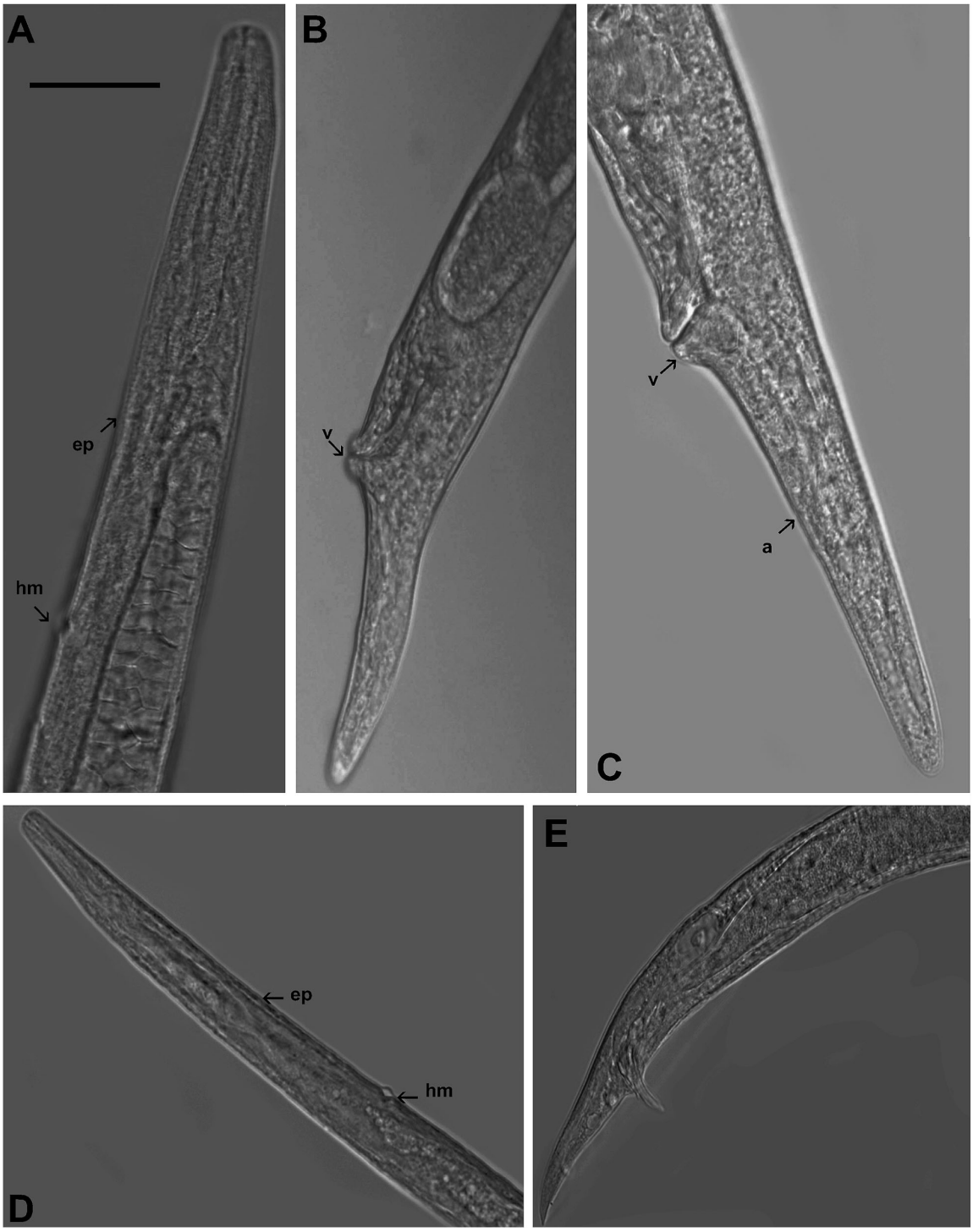

Fig. 3. Light microscopy images of mycophagous adults of *Deladenus beddingi* n. sp. A-C: female. A: Anterior end, lateral view showing position of excretory pore (ep) and hemizonid (hm); B, C: Posterior end of two individuals, lateral view showing protruding vulva (v) and anus (a); D, E: male. D: Anterior end, lateral view. Excretory pore (ep) and hemizonid (hm) are shown in D; E: Tail showing spicules and bursa, lateral view. (Scale bars: A = 17.5 μm; B = 8.5 μm; C = 6 μm; D = 16 μm; E = 12 μm.)

Table 2. Morphometrics of *Deladenus beddingi* n. sp. All measurements are in μm and in the form: mean ± s.d. (range).

Character	Mycophagous stage			Infective stage
	Female		Male	Female
	Holotype	Paratypes	Paratypes	Paratypes
n	–	20	10	10
L	1850	1549 ± 157 (1061-1935)	1376 ± 137 (1171-1560)	1106 ± 130 (830-1452)
a	49.3	48.0 ± 6.2 (37-61)	55.0 ± 8.3 (41-67)	54.0 ± 5.4 (43-60)
b	19.5	15.1 ± 2.6 (12.1-18.4)	15.4 ± 1.8 (11.2-17.4)	10.0 ± 0.9 (9.5-12.0)
c	50.9	50.0 ± 6.3 (36-61)	35.0 ± 3.1 (29.5-39.3)	40.0 ± 4 (36-50)
V	94.5	94.0 ± 5.3 (81-98)	–	94.0 ± 0.6 (93-96)
G_1 or T	55	54 ± 2.9 (51-57)	75 ± 2.5 (70-80)	53 ± 1.9 (48-57)
Max. body diam.	38	33 ± 6.5 (21-44)	26 ± 5 (20-34)	20 ± 3.8 (17-27)
Stylet length	10.1	10.5 ± 1.3 (8.5-12.5)	9.7 ± 1.2 (8.3-11.6)	21.0 ± 1.4 (19.0-23.0)
Pharynx base from anterior	95	107 ± 18 (78-126)	90 ± 6.8 (83-105)	118 ± 2.8 (113-121)
Nerve ring from anterior	89	80 ± 11.6 (71-100)	75 ± 12 (67-99)	72 ± 3.4 (67-77)
Excretory pore to anterior	100	89 ± 7.5 (79.5-101)	77 ± 16 (57-103)	89 ± 3.4 (75-110)
Excretory pore to hemizonid	35	32 ± 12.8 (22-52)	48 ± 7.8 (38-69)	71 ± 1.5 (68-73)
Hemizonid from anterior	125	120 ± 16 (84-138)	108 ± 17 (84-134)	111 ± 1.4 (108-113)
Tail length	36	31 ± 3.0 (25-34)	39 ± 3.0 (36-43)	31 ± 1.5 (28-33)
Cloacal/anal body diam.	14	12.6 ± 1.2 (11.0-15.0)	13.0 ± 1.4 (10.0-15.0)	13.0 ± 1.3 (10.5-14.5)
Spicule length	–	–	21.5 ± 2.5 (18-25)	–
Gubernaculum length	–	–	12.0 ± 1.3 (9-14)	–

The vulva in the mycophagous females of the novel species is more anteriorly located than that of *D. siricidicola* and *D. proximus* (Table 3). The value of the anterior gonad (G_1) for *D. beddingi* n. sp. mycophagous females is smaller (54 (51-57) μm) than that reported for the mycophagous stage of *D. siricidicola*, *D. proximus* and other related species (see Table 3). The stylet of the mycophagous females of the new species is slightly shorter than that of *D. proximus* (10.5 (8.5-12.5) *vs* 11.4 (11-12) μm, respectively), and slightly longer than that of *D. nitobei* (10.5 (8.5-12.5) *vs* 9.4 (9.0-9.7) μm, respectively) (see Table 3).

The percentage length of the G_1 in the infective females is larger than that observed in *D. siricidicola* and other related taxa (See Table 4). The excretory pore of the infective females of *D. beddingi* n. sp. is more anteriorly located than that of *D. proximus* and more posteriorly located than the excretory pore in *D. nitobei* (see Table 4).

Table 3. Comparison of key diagnostic morphometric traits of mycophagous females *Deladenus beddingi* n. sp. and related species. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*D. beddingi* n. sp.	*D. siricidicola*[1]	*D. proximus*[2]	*D. canii*[2]	*D. valveus*[3]	*D. nitobei*[4]	*D. ipini*[5]
L	1549 ± 157 (1061-1935)	1910 ± 276 (1500-2710)	2030 ± 139 (1760-2200)	1890 ± 179 (1610-2390)	647 ± 101 (509-970)	1842 ± 90 (1708-2000)	973 ± 238 (896-1080)
a	48 ± 6.2 (37-61)	50.9 ± 6.9 (33-69.1)	47.6 ± 4.1 (40-53.7)	53.5 ± 4.6 (47.9-62)	18.5 ± 3.2 (17.2-29.9)	60.4 ± 3.7 (54.6-65.2)	37 ± 6.2 (35-40)
b	15.1 ± 2.6 (12.1-18.4)	19 ± 0.4 (15.2-26.6)	19.1 ± 0.4 (16.3-21.3)	19 ± 1.9 (14.8-22.5)	8.3 ± 0.9 (7.1-12.4)	26.0 ± 1.6 (23.7-28.5)	12.1 ± 6.7 (10.4-15.2)
c	50 ± 6.3 (36-61)	44.6 ± 5.2 (32.6-58.9)	53 ± 7.6 (44-60)	47.9 ± 3.6 (42-54)	16.4 ± 5.5 (13.6-20.8)	50.8 ± 4.3 (42.7-56.9)	35.2 ± 12.3 (29.6-38.8)
V	94 ± 5.3 (81.0-98.0)	94.8 ± 0.6 (93.2-96.2)	95.6 ± 0.3 (95.1-95.9)	95.1 ± 0.4 (94.5-95.8)	90.1 ± 1.5 (88.3-92.1)	95.4 ± 0.3 (94.9-95.9)	93 ± 0.9 (92-93)
G_1	54 ± 2.9 (51-57)	83.3 ± 7.5 (60.7-92.2)	88.9 ± 4.1 (84.8-94.8)	86.4 ± 3.2 (80.1-89.8)	69.6 ± 3.6 (60.1-74.7)	–	–
Stylet length	10.5 ± 1.3 (8.5-12.5)	10.0	11.4 ± 0.5 (11-12)	9.5 ± 0.6 (8.5-10)	11 ± 1.7 (9-13)	9.4 ± 0.3 (9-9.7)	11.8 ± 0.5 (11.6-12)
Excretory pore to anterior	89 ± 7.5 (79.5-101)	104 (80-140)	140 ± 10.7 (127-159)	103 ± 8.0 (81-116)	61 ± 9.3 (59-74)	77 ± 5.4 (65-85)	–
Excretory pore to hemizonid	32 ± 12.8 (22-52)	40.7 ± 8.0 (24-58)	8.5 ± 2.9 (1.0-11.0)	52 ± 9.6 (29-63)	46 ± 12.6 (37-54)	25 ± 3.8 (18-30)	–
Hemizonid from anterior	120 ± 16.0 (84-138)	146 (112-191)	145 ± 10.4 (132-162)	145 ± 8.7 (108-176)	–	102 ± 4.2 (95-107)	–
Tail length	31 ± 3.0 (25-34)	–	39 ± 4.8 (31-47)	40 ± 2.7 (33-64)	41 ± 2.6 (38-47)	36 ± 1.8 (34-40)	28 ± 9.7 (24-32)

[1]After Bedding (1968).
[2]After Bedding (1974).
[3]After Yu *et al*. (2014).
[4]After Kanzaki *et al*. (2016).
[5]After Chitambar (1991).

Mycophagous males of *D. beddingi* n. sp. also differ from *D. nitobei* in the values of the ratios a (55 (41-61) *vs* 64.3 (55.8-74.1)) and b (15.4 (11.2-17.4) *vs* 21.5 (19.5-24.3)) (Kanzaki *et al*., 2016) (see Table 5). The length of the spicules of the mycophagous males is also shorter than that observed in *D. proximus* (21.5 (18-25) *vs* 25 (24-38) μm) (Table 5; Bedding, 1974) and *D. nitobei* (40 (38-44) μm) (Kanzaki *et al*., 2016). The hemizonid and excretory pore of the males of the new species are located more posteriorly than in *D. nitobei* (108 (84-134) *vs* 94 (85-99) μm, and 77 (57-103) *vs* 69 (65-76) μm, respectively) (Kanzaki *et al*., 2016).

Deladenus ipini Massey, 1974 also occurs in North America and, like the novel species, is found in pine trees, although these two species have allopatric distributions as far as is known. From a morphological standpoint, mycophagous females of *D. ipini* are shorter and wider than *D. beddingi* n. sp. (Table 3), and have shorter stylets than *D. beddingi* n. sp. Furthermore, while free-living mycophagous females of *D. ipini* have a post-uterine sac, this is absent in *D. beddingi* n. sp.

Phylogenetic analyses

Phylogenetic relationships derived from the concatenated dataset for mt*CO1* and ITS showed *D. beddingi* n. sp. to be genetically distinct from other North American *Deladenus* parasitising *Sirex* (Fig. 4). It grouped within the *D. siricidicola* clade (the *D. siricidicola* superspecies), as well as two unidentified *Deladenus* nematodes found parasitising *S. cyaneus* F. (cya2) and *S. nitidus* (T.W. Harris) (nit30) from the eastern USA, as reported by Morris *et al*. (2013). This placement was strongly supported by all three phylogenetic analyses, which had the same tree topology. Within that clade, *D. beddingi* was more closely related to the two undescribed *Deladenus* spp. than to *D. siricidicola*, although this was only strongly supported by

Table 4. Comparison of key diagnostic morphometric traits of infective females of *Deladenus beddingi* n. sp. and related species. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*D. beddingi* n. sp.	*D. siricidicola*[1]	*D. proximus*[2]	*D. canii*[2]	*D. valveus*[3]	*D. nitobei*[4]
L	1106 ± 130 (830-1452)	1220 ± 191 (800-1600)	1280 ± 69 (1170-1370)	1190 ± 139 (910-1370)	683 ± 36 (551-727)	1433 ± 115 (1250-1583)
a	54 ± 5.4 (43-60)	61.2 ± 10.0 (44.0-109.1)	58 ± 3.2 (53-62)	60.8 ± 6.6 (54.2-69.9)	27.8 ± 3.1 (24.7-31.5)	52.2 ± 4.6 (42.6-56.9)
b	10 ± 0.9 (9.5-12)	10.9 ± 0.9 (9.3-13.7)	10.2 ± 0.7 (9.5-11.6)	10.3 ± 1.0 (8.4-11.3)	6.2 ± 0.8 (5.5-7.3)	15.0 ± 1.2 (13.5-17.5)
c	40 ± 4.0 (36-50)	35.1 ± 3.7 (27.0-43.8)	39 ± 5.0 (34-42)	36.6 ± 4.0 (29.7-50.6)	12.4 ± 2.1 (11.3-14.8)	40.5 ± 3.3 (34.8-45.0)
V	94.0 ± 0.6 (93.0-96.0)	94.1 ± 4.2 (92.8-95.0)	94.6 ± 0.3 (94.2-95.3)	93.9 ± 1.2 (92.4-94.7)	75.7 ± 1.4 (74.3-77.2)	94.4 ± 0.3 (93.8-94.8)
G_1	53 ± 1.9 (48-57)	36.7 ± 5.5 (27.6-52.0)	28.3 ± 4.4 (20.9-34.3)	43.4 ± 6.4 (39.5-54.6)	24.5 ± 2.5 (22.5-26.9)	–
Stylet length	21 ± 1.4 (19-23)	21 ± 1.6 (19-25)	26.4 ± 1.8 (22-31)	21 ± 1.6 (19-24)	22 ± 1.7 (20-24)	26 ± 0.1 (24-27)
Excretory pore to anterior	89 ± 3.4 (75-110)	97 (78-116)	127 ± 8.1 (111-142)	83 ± 10.2 (73-111)	71 ± 6.6 (57-78)	76 ± 8.1 (65-89)
Excretory pore to hemizonid	71 ± 1.5 (68-73)	33 ± 0.85 (22-45)	3.2 ± 2.9 (0-8)	41 ± 7.4 (24-54)	44 ± 5.5 (39-49)	27 ± 4.7 (22-36)
Hemizonid from anterior	111 ± 1.4 (108-113)	131 (103-154)	130 ± 6.0 (118-142)	125 ± 10.8 (110-143)	–	102 ± 8.0 (90-114)
Tail length	31 ± 1.5 (28-33)	32 ± 0.67 (17-42)	32 ± 2.8 (30-35)	33 ± 1.8 (31-36)	48 ± 6.5 (41-55)	36 ± 4.6 (30-46)

[1]After Bedding (1968).
[2]After Bedding (1974).
[3]After Yu *et al*. (2014).
[4]After Kanzaki *et al*. (2016).

Bayesian analysis. For mt*CO1*, genetic similarity between *D. beddingi* n. sp. and cya2 and nit30 was 98.2% (11 base pairs difference) and 98.5% (9 base pairs difference), respectively. For ITS, genetic similarity between *D. beddingi* and cya2 (3 base pairs difference) and nit 30 (1 base pair difference) was 99.7% and 99.9%, respectively.

Fungal symbiont

The fungal symbiont associated with *S. californicus* was identified as *A. chailletii*. Comparison of SAC 203 ITS, mtSSU, and RPB2 sequences with those from reference strains of *Amylostereum* spp. showed 99-100% genetic similarity with reference *A. chailletii* strains. The mtSSU sequence was identical to those of six reference *A. chailletii* strains and several other strains using a BLAST search, whereas the ITS and RPB2 loci sequences differed at most by only a few bases.

Testing whether *D. beddingi* n. sp. was able to utilise a North American strain of the other major symbiont associated with *Sirex* in North America showed that significantly more eggs and nematodes were produced when *D. beddingi* n. sp. was cultured on *A. chailletii* than on *A. areolatum* BE strain ($F_{1,20} = 377.5$; $p < 0.0001$), with means of $4.5 \times 10^4 \pm 3.5 \times 10^3$ (mean ± SE) and $2.7 \times 10^1 \pm 1.1 \times 10^1$ produced, respectively. Nematodes growing on *A. areolatum* IGS BE strain never produced more than 66 eggs per plate and nearly half of all replicate plates produced zero eggs (five of 12 plates). Nematodes growing on *A. chailletii*, however, never produced fewer than 6.4×10^3 eggs per plate. Nematodes growing on both fungal treatments were observed to reach the adult stage.

Discussion

Whilst several new species of dimorphic *Deladenus* nematodes have been described recently (Nasira *et al*., 2013; Yu *et al*., 2013, 2014; Kanzaki *et al*., 2016), no new species thought to be a North American native

Table 5. Comparison of key diagnostic morphometric traits of mycophagous males *Deladenus beddingi* n. sp. and related species. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*D. beddingi* n. sp.	*D. siricidicola*[1]	*D. proximus*[2]	*D. canii*[2]	*D. valveus*[3]	*D. nitobei*[4]	*D. ipini*[5]
L	1376 ± 137 (1171-1560)	1490 ± 148.4 (1150-1920)	1520 ± 69.0 (1340-1590)	1460 ± 69.0 (1300-1640)	483 ± 47.0 (457-504)	1538 ± 110 (1375-1708)	636
a	55 ± 8.3 (41-67)	53.4 ± 7.0 (43.2-77.5)	56 ± 4.1 (48-62)	66.5 ± 7.1 (59.1-74.0)	27.6 ± 2.2 (24.7-30)	64.3 ± 4.7 (55.8-74.1)	38
b	15.4 ± 1.8 (11.2-17.4)	15.7 ± 1.7 (12.1-22.4)	15.5 ± 0.7 (13.6-17.5)	15.7 ± 1.5 (13.8-19.1)	–	21.5 ± 1.6 (19.5-24.3)	8.4
c	35 ± 3.1 (29.5-39.3)	31.6 ± 2.3 (26.1-37.0)	32 ± 2.4 (27-35)	33.1 ± 3.4 (30.2-40.0)	14.4 ± 0.9 (13.4-15.8)	37.7 ± 3.4 (31.6-42.7)	25.1
T	75 ± 2.5 (70-80)	84.0 ± 4.0 (76.3-91.9)	90.3 ± 2.4 (87.3-93.9)	86.3 ± 2.8 (81.1-90.0)	55.4 ± 2.4 (49.8-58.2)	84.3 ± 3.4 (80.6-90.5)	66
Excretory pore to anterior	77 ± 16 (57-103)	91 (72-109)	126 ± 9.0 (111-142)	82 ± 7.8 (63-92)	59 ± 5.5 (53-69)	69 ± 3.4 (65-76)	–
Excretory pore to hemizonid	48 ± 7.8 (38-69)	36.5 ± 5.5 (22-46)	2.1 ± 2.2 (0-7)	44 ± 6.0 (29-53)	36 ± 3.4 (31-49)	25 ± 2.8 (19-28)	–
Hemizonid from anterior	108 ± 17.0 (84-134)	126 (108-154)	129 ± 8.9 (114-143)	126 ± 10.2 (116-140)	–	94 ± 4.3 (85-99)	–
Tail length	39 ± 3.0 (36-43)	–	48 ± 2.5 (44-52)	44 ± 1.3 (41-48)	36 ± 3.4 (30-43)	41 ± 2.3 (35-43)	–
Spicule length	21.5 ± 2.5 (18-25)	10	25 ± 1.4 (24-38)	17.7 ± 1.2* (16-20)	25 ± 2.3 (20-27)	40 ± 1.8 (38-44)	14

*According to Bedding (1974) the range is '16-10'. However, we believe this is a mistake and it should be '16-20' instead.
[1]After Bedding (1968).
[2]After Bedding (1974).
[3]After Yu *et al*. (2014).
[4]After Kanzaki *et al*. (2016).
[5]After Chitambar (1991).

has been reported or described from North America for several decades (Bedding & Akhurst, 1978; Yu *et al*., 2014). In general, species of *Deladenus* associated with the siricids found in western USA are poorly known. Only one species has been described, *D. nevexii* from *Xeris* sp. emerging from *Abies concolor* (Gordon) Lindley *ex* Hildebrand in Nevada, USA (Bedding, 1974). However, Bedding & Akhurst (1978) reported an 'undescribed genus' of parasitic nematodes associated with *S. californicus* in North America and we hypothesise that this could refer to *D. beddingi* n. sp.

Phylogenetic analysis showed the fungal symbiont of *S. californicus* included in this study to be *A. chailletii*, this finding agreeing with previous literature (Gaut, 1970). In 1978, Bedding & Akhurst suggested that *Deladenus* species are more specific to their fungal food than to the *Sirex* species that they parasitise (Bedding & Akhurst, 1978). *Deladenus beddingi* n. sp. did not have sufficient survivorship and reproduction on *A. areolatum* for it to be considered a fungal host, but colonies were easily sustained on *A. chailletii*. However, this does not necessarily mean that *D. beddingi* n. sp. cannot survive when feeding on any *A. areolatum* strains, as only one strain of *A. areolatum* was tested and different strains of *Amylostereum* can differentially impact nematode reproduction (Morris *et al*., 2012, 2014; Caetano *et al*., 2016). It is thought that mycophagous *Deladenus* feed on either *A. areolatum* or *A. chailletii*, with the exception of *D. wilsoni* and the closely related *D. proximus* (both in the *D. wilsoni* superspecies group), which feed on both species (Morris *et al*., 2014). If *D. beddingi* n. sp. only survives when feeding on *A. chailletii* in the field, this would rule it out as a potential new biological control agent of *S. noctilio*, since this invasive woodwasp is predominantly associated with *A. areolatum*. Additionally, Morris & Hajek (2014) showed that both *A. areolatum* and *A. chailletii* are able to parasitise their nematode predators by growing over and into nematode eggs and capturing adult females. It should be noted

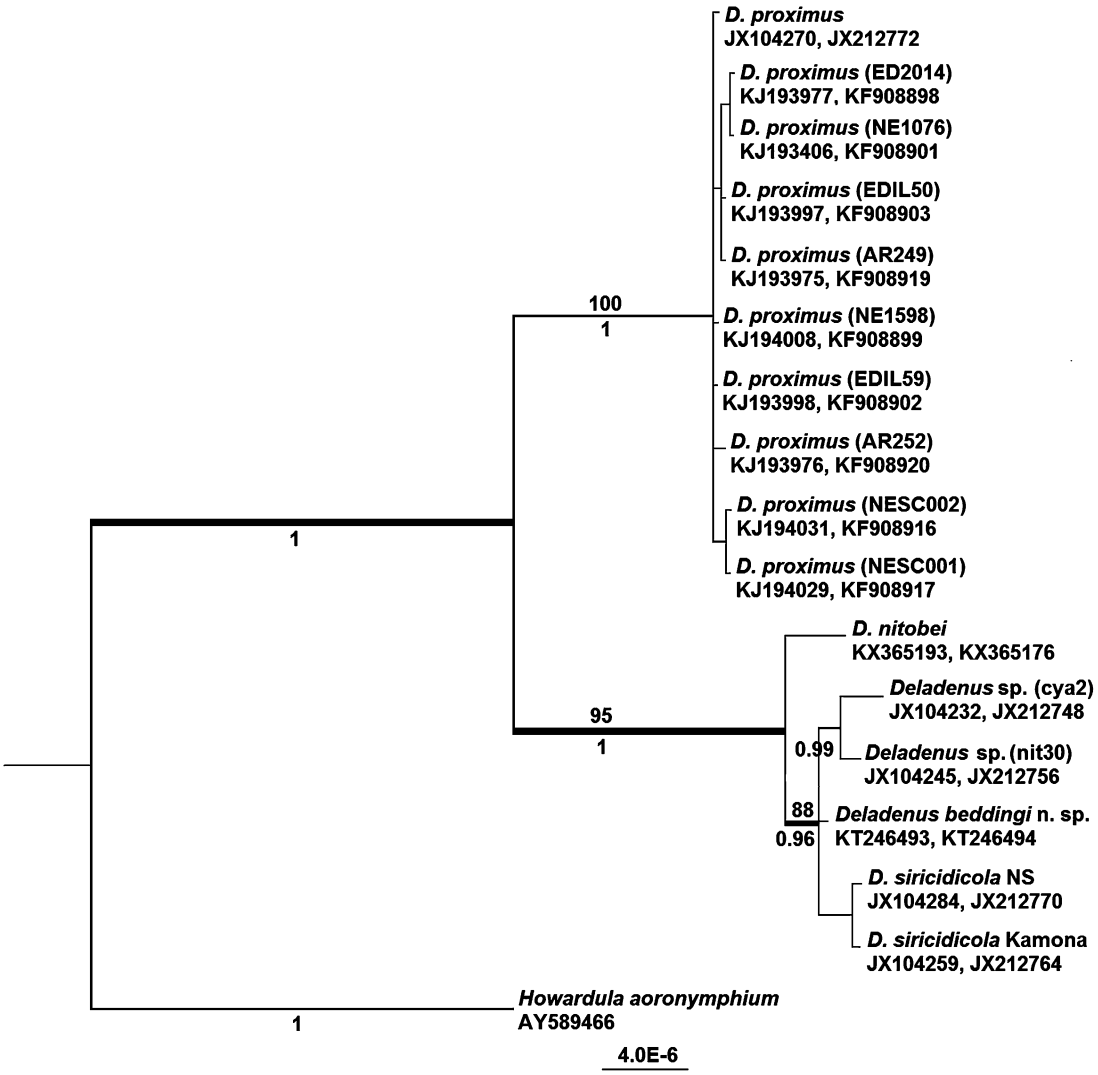

Fig. 4. Bayesian tree for *Deladenus* inferred from combined mt*CO1* and ITS sequences. Values above branch points represent bootstrap support for clades well supported by ML analysis (>70). Values below branch points represent the Bayesian posterior probabilities for well-supported clades (>0.95). Where maximum parsimony (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. Nematode species are indicated to the right of the taxa. Accession numbers are given for mt*CO1* and ITS, respectively. The outgroup is *Howardula aoronymphium*, a nematode parasitic on mycophagous *Drosophila*.

that the "undescribed *Deladenus* species" parasitised by *A. chailletii* in Morris & Hajek (2014) is the *D. beddingi* n. sp. described in the present study. Thus *D. beddingi* n. sp. both feeds on *Amylostereum* and can be killed and eaten by *A. chailletii*.

A previous study found that each *Sirex* species in the eastern USA has its own corresponding *Deladenus* parasite (Morris *et al*., 2013), and the present study also shows this trend. Phylogenetic analysis showed *D. beddingi* n. sp. to be most closely related to two isolates, *S. cyaneus* 'cya 2' and *S. nitidus* 'nit30', from the eastern USA. Unfortunately, molecular data are only available for a limited number of *Deladenus* spp: *D. siricidicola* (Yu *et al*., 2009; Mlonyeni *et al*., 2011; Leal *et al*., 2012; Morris *et al*., 2013), *D. proximus* (Yu *et al*., 2011; Morris *et al*., 2013; Zieman *et al*., 2015; Hartshorn *et*

al., 2017), and *D. prorsus* Yu, Gu & Ye, 2013 (Yu *et al*., 2013), hampering inclusion of other described species in our analysis. Molecular data have proven to be increasingly important for discriminating these nematode species. Morphological data are challenging to obtain because cultures of nematodes are required and these are difficult to establish, limiting the availability of specimens for morphological diagnosis and requiring special expertise. Thus, studies including both types of data are important to link the morphology and molecular approaches and to identify and/or characterise species of *Deladenus* properly.

We herein report and describe a new *Deladenus* species, *D. beddingi* n. sp., isolated from *S. californicus* from the state of Washington in the USA. This is the first report of a native dimorphic *Deladenus* species present in North America since 1978 and is only the second dimorphic *Deladenus* species described from the western USA after *D. nevexii*, which also parasitises siricids. Research conducted in this study expands current knowledge on the diversity and distribution of native *Deladenus* spp. in North America. Furthermore, this study adds novel data on insect-host associations, which is especially important given the recent establishment of the invasive pine-killing *S. noctilio* in North America.

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Table S1. Species of *Deladenus* known from North America.

Species	Distribution*	References
D. apopkaetus Chitambar, 1991	USA (FL)	Chitambar (1991)
D. beddingi n. sp.**	USA (WA)	Morris *et al.*, this paper
D. canii Bedding, 1974**	Canada (NB)	Bedding (1974)
D. durus (Cobb, 1922)	USA (VA, OR, NM), Canada (Arctic), Europe, Asia	Cobb (1922)
D. ipini Massey, 1974	USA (LA)	Massey (1974)
D. lonchites Massey, 1974	USA (AZ)	Massey (1974)
D. nevexii Bedding, 1974**	USA (NV)	Bedding (1974)
D. proximus Bedding, 1974**	Eastern USA and Canada	Bedding (1974); Yu *et al.* (2011); Morris *et al*. (2013); Zieman *et al.* (2015); Hartshorn *et al.* (2017)
D. posteroporus Yu, Gu, Ye, Li & He, 2017**	USA and Canada	Yu *et al.* (2017)
D. siricidicola Bedding, 1968**,***	USA, Canada, Europe, Asia, South America, Africa, Oceania	Bedding (1968); Bedding & Akhurst (1978); Yu *et al.* (2009); Kroll *et al.* (2013); Hajek & Morris (2014)
D. wilsoni Bedding, 1968**	SE and SW USA, Canada, Europe, Asia	Bedding (1968); Bedding & Akhurst (1978)

*Based on the emphasis for this study, distributional details are provided for North American areas only.
**Species with known parasitic as well as mycophagous stages.
****D. siricidicola* is invasive in North America, where it has been reported from New York and Pennsylvania, USA, and Ontario, Canada (Yu *et al*., 2009; Kroll *et al.*, 2013) and has been introduced for biological control to Oceania, South America and Africa (Hajek & Morris, 2014).

Table S2. Host, location and accession data for nematodes included in the phylogenetic analysis.

Host	Host source	*Sirex* coll. date	Coll. location	*Sirex* sex	Nematode ID	Genbank Accession No.	
						MTCO1	ITS
Sirex californicus	*Pinus monticola* × *strobus*	Aug 2009	Seattle, WA	F	*Deladenus beddingi* n. sp.	KT246493	KT246494
S. nigricornis	Trap	5 Oct 2010	Warrensburg, NY	F	*D. proximus*	JX104270	JX212772
S. nigricornis	Trap	*	*		ED2014[a]	KJ193977	KF908898
S. nigricornis	Trap	*	*		NE1598[a]	KJ194008	KF908899
S. nigricornis	Trap	*	*		NE1076[a]	KJ194006	KF908901
S. nigricornis	Trap	*	*		EDIL59[a]	KJ193998	KF908902
S. nigricornis	Trap	*	*		EDIL50[a]	KJ193997	KF908903
S. nigricornis	Trap	*	*		AR249[a]	KJ193975	KF908919
S. nigricornis	Trap	*	*		AR252[a]	KJ193976	KF908920
S. nigricornis	Trap	*	*		NESC002[a]	KJ194029	KF908916
S. nigricornis	Trap	*	*		NESC001[a]	KJ194029	KF908917
S. nitidus	*Picea abies*	2 Sep 2009	Newcomb, NY	M	nit30[b]	JX104245	JX212756
S. cyaneus	*Abies balsamea*	31 Aug 2009	Newcomb, NY	F	cya2[b]	JX104232	JX212748
S. noctilio	*Pinus resinosa*	10 Jul 2009	Tioga, PA	M	*D. siricidicola*[c]	JX104259	JX212764
S. noctilio	*Pinus sylvestris*	26 May 2009	Oswego, NY	M	*D. siricidicola*[d]	JX104284	JX212770
S. nitobei	*Pinus densiflora*	Jul 2015	Aomori, Japan	F	*D. nitobei*[e]	KX365193	KX365176
Drosophila neotestacea			Rochester, NY		*Howardula aoronymphium*[f]	AY589466	

*Dates and collection locations not provided for specific samples.
[a]Refers to Zieman *et al*. (2015).
[b]Refers to samples included in Morris *et al*. (2013).
[c]*Deladenus siricidicola* Kamona strain. This is the commercial strain available for biological control of *S. noctilio*, and this sample emerged in quarantine.
[d]*Deladenus siricidicola* non-sterilising strain (NS). This is the strain that occurs in *S. noctilio* established in the northeastern USA, probably introduced by the invading *S. noctilio*.
[e]Refers to Kanzaki *et al*. (2016).
[f]Outgroup. *Howardula aoronymphium*, sequence data from Ye *et al*. (2007).