Leptojacobus dorci n. gen., n. sp. (Nematoda: Diplogastridae), an Associate of *Dorcus* Stag Beetles (Coleoptera: Lucanidae)

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Abstract: A new species of diplogastrid nematode, *Leptojacobus dorci* n. gen., n. sp., was isolated from adults of the stag beetle *Dorcus ritsemae* (Coleoptera: Lucanidae) that were purchased from a pet shop in Japan. *Leptojacobus* n. gen. is circumscribed by a very thin, delicate body and by a small stoma with minute armature. A combination of other stomatal characters, namely the division of the cheilostom into adradial plates, the symmetry of the subventral stegostomatal sectors, and the presence of a thin, conical dorsal tooth, further distinguishes *Leptojacobus* n. gen. from other genera of Diplogastridae. Phylogenetic analysis of nearly full-length SSU rRNA sequences support the new species, together with an isolate identified previously as *Koerneria luziae*, to be excluded from a clade including all other molecularly characterized diplogastrids with teeth and stomatal dimorphism. Therefore, the new species will be of importance for reconstruction of ancestral character histories in Diplogastridae, a family circumscribed by a suite of feeding-related novelties.

Key words: dimorphism, evolution, morphology, new genus, new species, novelty, stoma, taxonomy, phylogeny.

The nematodes of Diplogastridae Micoletzky, 1922 are widely found in phoretic, necromenic, and parasitic associations with insects (Sudhaus and Fürst von Lieven, 2003). Targeting potential host insects for sampling is therefore likely to be a source of undocumented diversity in the family. Attention to stag beetles (Coleoptera: Lucanidae) in particular has recently revealed several new species of nematode associates, including two species of *Pristionchus* Kreis, 1932 (Kanzaki et al., 2011, 2012a, 2013) and a previously undescribed genus, *Parapristionchus* Kanzaki, Ragsdale, Herrmann, Mayer, Tanaka, and Sommer, 2012b. By continuing such collecting efforts, we have discovered a new diplogastrid species and report it in this article. We currently recognize 33 valid genera of Diplogastridae (Sudhaus and Fürst von Lieven, 2003; Kanzaki et al., 2009, 2012b; Fürst von Lieven et al., 2011; Susoy and Herrmann, 2012; Herrmann et al., 2013), and we assign the new species, based on morphological and molecular evidence, to an additional, new genus in the family.

Besides improving our understanding of the phylogenetic diversity and life histories in Diplogastridae, records of distinct new morphotypes, including new taxonomic "genera," facilitate inferences of character evolution. Diplogastrid nematodes are characterized by a suite of feeding-related evolutionary novelties, including teeth, stomatal dimorphism, and predatory behavior. Because the family is represented by an advanced model system for genetics and developmental biology, *Pristionchus pacificus* Sommer, Carta, Kim, and Sternberg, 1996 (Sommer and McGaughran, 2013), detailed mechanisms for these traits are now being revealed, including the rewiring of conserved neurons to enable predation (Bumbarger et al., 2013) and the regulation of the dimorphism by hormones (Bento et al., 2010), novel pheromones (Bose et al., 2012), and a novel developmental switch (Ragsdale et al., 2013). However, determining the order of evolutionary events still requires rigorous inference of ancestral character states. Therefore, the analysis of new taxa, particularly those representing ancient divergences relative to previously sampled diplogastrid species, will allow more accurate polarization of character transformations. Here, we show by a phylogenetic analysis of nearly full-length SSU rRNA gene sequences that the new species we describe is excluded from a clade of almost all other sequenced diplogastrids with teeth and a dimorphism. To complement this finding, we present a morphological description of the new species, the analysis of which will be essential for reconstructing the histories of novelties in the family.

Materials and Methods

Nematode isolation and cultivation: The new species was isolated from *Dorcus ritsemae* Oberthür and Houlbert, 1914 (Coleoptera: Lucanidae) by first placing adult beetles on fermented wood flakes, a common substrate for rearing stag beetles. Before introducing beetles, food flakes were sterilized by drying at 80°C for 2 d. After 2 wk of keeping beetles on this medium, beetles were removed from the substrate, after which wood flakes were kept at 25°C for 6 mon. About 5 g of subsamples were taken occasionally to be soaked in water or placed on a Baermann funnel, both methods of which yielded individuals of the new species. Alternatively, wood flakes from the beetle substrate were also placed directly on plates with of 2% water agar or NGM agar without peptone or cholesterol, with chunks of NGM agar (with peptone and cholesterol) placed on top. Approximately 3 ml of tap water were added to each culture plate, and plates were sealed with Parafilm to prevent desiccation. Microbiota originating from

the nematode body surface, gut, or both propagated voluntarily on this medium and were used as the sole food source for the nematodes.

Morphological observation and preparation of type material: Material for morphological observation was provided by 2- to 3-wk-old cultures of the new species. Observations by light microscopy (LM) and differential interference contrast (DIC) microscopy were conducted using live nematodes, which were transferred by mouth pipette from culture plates to slides. For line drawings, specimens were mounted into water on slides with silicone grease and then relaxed by applying gentle heat (Kanzaki, 2013). For morphometrics, specimens were mounted on slides with pads of 5% noble agar and 0.15% sodium azide and were additionally relaxed by heat when necessary. To prepare type material, nematodes were isolated from type strain cultures, rinsed in distilled water to remove bacteria, heat-killed at 95°C, fixed in 5% formalin, and processed through a glycerol and ethanol series using Seinhorst's method (Hooper, 1986). Nomarski micrographs were taken using a Zeiss Axio Imager Z.1 microscope and a Spot RT-SE camera supported by the program MetaMorph v.7.1.3 (Molecular Devices, Sunnyvale, CA).

Scanning electron microscopy: Nematodes were prepared for scanning electron microscopy (SEM) by fixation in 2.5% glutaraldehyde in PBS buffer and then postfixed with 1% osmium tetroxide. After several rinses with water, samples were dehydrated through a graded ethanol series, followed by critical point drying from liquid carbon dioxide. Specimens were mounted on polylysine-coated cover slips, sputter-coated with 20-nm gold/palladium, and then imaged with a Hitachi S-800 field emission scanning electron microscope operating at 20 kV.

Molecular characterization and phylogenetic analysis: We amplified a nearly full-length sequence of the SSU rRNA gene of the new species, using the forward primer SSU18A (AAA GAT TAA GCC ATG CAT G) (Eyualem and Blaxter, 2003) and reverse primer VL26346 (GGT TCA AGC CAC TGC GAT) (Susoy et al., 2013). The gene was sequenced using the above amplification primers as well as forward primer VL26345 (GCG AAA GCA TTT GCC AAG A) (Susoy et al., 2013) and reverse primer SSU26R (CAT TCT TGG CAA ATG CTT TCG) (Eyualem and Blaxter, 2003). PCR conditions were as described by Mayer et al. (2007). The sequence was assembled using the program SeqMan Pro 7.2.2 (DNASTAR) and has been deposited in the GenBank database under the accession number KF924399.

Phylogenetic analysis was performed on an alignment of sequences of the SSU rRNA gene. Homologous sequences for all other taxa included in the analysis were retrieved from GenBank. Nearly full-length SSU rRNA sequences were aligned using MAFFT version v. 7.0 (Katoh et al., 2002), followed by manual improvement of the alignment in Geneious version 6.1, including deletion of ambiguously aligned positions. The phylogeny was inferred under maximum likelihood (ML) and Bayesian criteria, as implemented in RAxML v.7.2.8 (Stamatakis, 2006) and MrBayes 3.2 (Ronquist et al., 2012), respectively. For the ML analysis, which invoked a general time reversible model with a gamma-shaped distribution of rates across sites, 50 independent runs with different starting trees were performed. Bootstrap support (BS) was evaluated by 1,000 pseudoreplicates. Bayesian analyses were initiated with random starting trees and were run with four chains for 4 million generations. Markov chains were sampled at intervals of 100 generations. The analysis was performed four times. After confirming convergence of runs, the first 25% generations were discarded as burn-in and the remaining topologies used to generate a 50% majority-rule consensus tree with clade credibility values given as posterior probabilities (PP). Bayesian analysis invoked a mixed model of substitution with a gamma-shaped distribution across sites. Model parameters were unlinked across character partitions in all analyses.

RESULTS

*Leptojacobus** n. gen.

Description: Diplogastridae. The body is cylindrical, very thin, and delicate. The cuticle is finely annulated, although annulations are less distinct when cuticle detaches from body, as is common in the type species. The proclivity for the cuticle to detach from the body and become "baggy" is tentatively considered a diagnostic character for the genus. The cuticle also has fine longitudinal striations, lending a cross-hatched appearance to the body surface where the cuticle is tightly bound to the body. Six lips are present but are not clearly separated from each other or from the rest of the body. Six setiform labial sensilla are present in both sexes. Four additional cephalic papillae are present in males, as typical for diplogastrid nematodes. The stoma is small and stomatal armature minute. Collectively, the cheilostom and gymnostom are at least as wide as long, such that the stoma is not tube-shaped (i.e., "rhabditiform"). The cheilostom is apically divided into six adradial plates, which extend into minute flaps. Divisions of the cheilostom within the stoma cannot be distinguished with certainty by DIC microscopy. The cheilostom overlaps the gymnostom to give the appearance of a ring around the stoma. The stegostom is dorsoventrally anisomorphic, as typical for diplogastrid nematodes. A moveable dorsal tooth, which is thin and conical, is present. The left and right subventral sectors of the stegostom are symmetrical and form thinly cuticularized ridges. The postdental telostegostom is cup-shaped. Stegostomatal

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* Derived from the Greek λεπτός ("slim") + Ιάκωβος ("Jim") and refers to the unusually thin bodies of these diplogastrid nematodes.
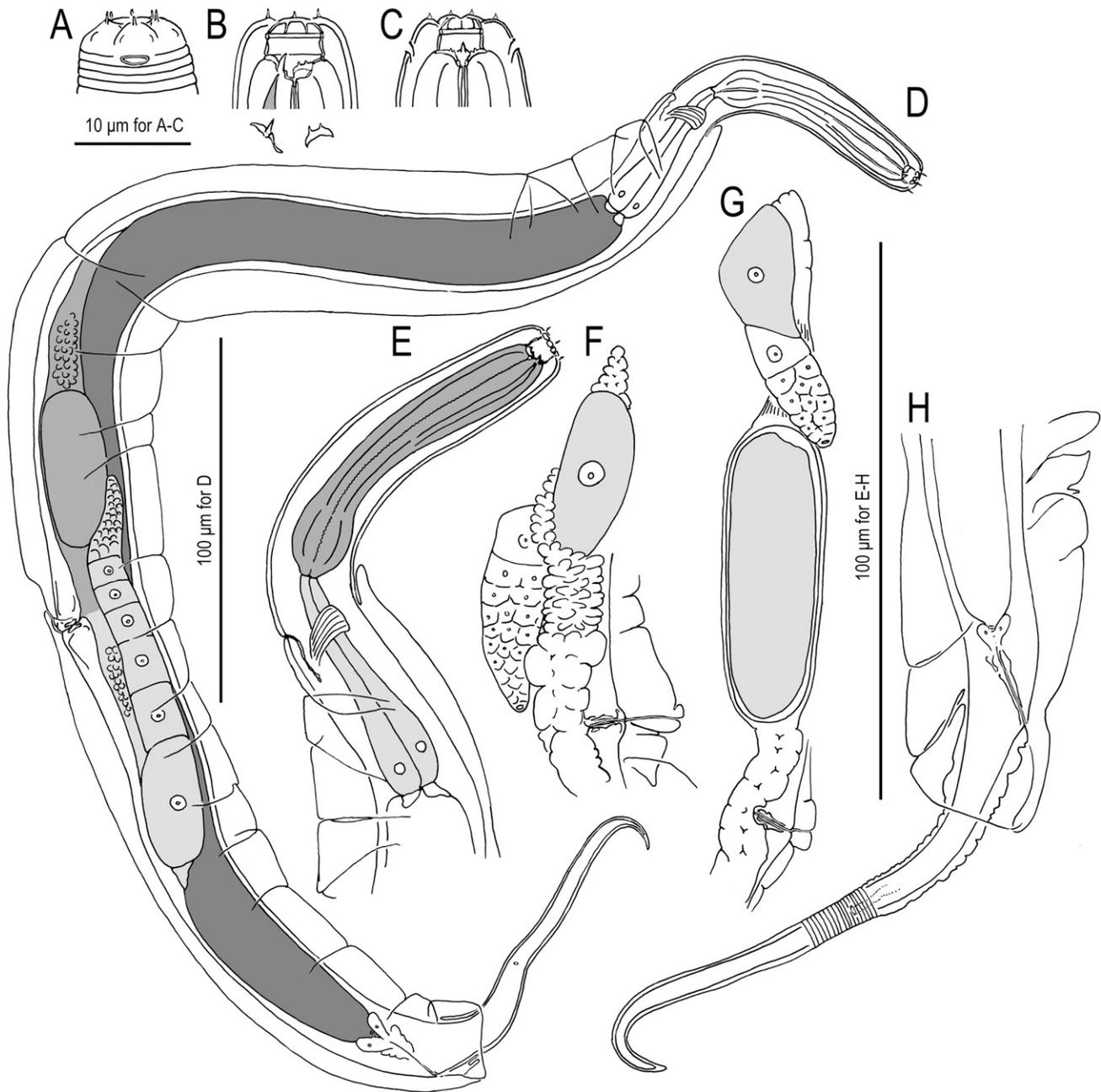
FIG. 1.   *Leptojacobus dorci* n. gen., n. sp. A. Surface of anterior region of adult male. B. Left lateral view of stomatal region. C. Ventral view of stomatal region. D. Adult female whole body. E. Anterior region of adult female in left lateral view. F,G. Anterior gonad of female in right lateral view. H. Female tail in right lateral view.

apodemes are absent. Dimorphism of the typically small stoma was not observed. The dorsal gland is large and distinct, and the dorsal musculature of the procorpus is more developed than the subventral musculature, both as typical for diplogastrids. The metacorpus is muscular, forming a well-developed median bulb. The isthmus is narrow and not muscular, and the basal bulb is glandular. In both sexes, the gonads occupy most of or the entire small body diameter. Female gonads are didelphic, amphidelphic, and reflexed. The testis is not reflexed. Males have nine pairs of genital papillae as well as one small, unpaired, ventral genital papilla on the anterior

cloacal lip. A bursa or bursal flap is absent. The life cycle consists of one molt inside of the egg and three molts after hatching, as typical for diplogastrid nematodes.

Type and only species: Leptojacobus dorci n. gen., n. sp.

Relationships: Leptojacobus n. gen. is distinguished from all other diplogastrids (Fürst von Lieven and Sudhaus, 2000; Sudhaus and Fürst von Lieven, 2003) by its very thin, delicate body, which is atypical of diplogastrids and other rhabditids, and by its minute stomatal structures. Although these characters clearly distinguish the new genus, *Leptojacobus* n. gen. is further diagnosed by a combination of several stomatal characters in particular,

namely the apical division of the cheilostom into adradial plates and flaps, the symmetry of left and right subventral sectors, the presence of a thin, conical dorsal tooth. The presence of adradial plates (i.e., per- and interradial cheilostomatal divisions) distinguishes the new genus from all other Diplogastridae except for *Acrostichus* Rahm, 1928, *Diplogasteriana* Meyl, 1960, and some species of *Paroigolaimella* Paramonov, 1952, but it is distinguished from the former two by the lack of subventral teeth or projections and is distinguished from all of them by its thin, conical dorsal tooth and the small size of its stoma. Although symmetry of the subventral sectors is also present in several diplogastrid genera without a tube-shaped stoma, namely the above three genera and *Sachsia* Meyl, 1960, *Leptojacobus* n. gen. is distinguished from all four genera as described above. The new genus is distinguished from all other taxa with symmetrical subventral sectors, namely *Demaniella* Steiner, 1914, *Diplogasteroides* de Man, 1912, *Diplogastrellus* Paramonov, 1952, *Goffartia* Hirschmann, 1952, *Mehdinema* Farooqui, 1967, *Pseudodiplogasteroides* Körner, 1954, *Rhabditidoides* Rahm, 1928, *Rhabditolaimus* Fuchs, 1914, and *Sudhausia* Herrmann, Ragsdale, Kanzaki, and Sommer, 2013, by the absence of a tube-shaped gymnostom. All of the generic characters above distinguish the new genus from *Koerneria* Meyl, 1960, including a Japanese strain tentatively identified as *Koerneria luziae* (Körner, 1954) Meyl, 1960 (Kanzaki et al., 2011), which is a possible sister taxon to *Leptojacobus* n. gen. (see below). Bearing a superficial resemblance to the new genus is *Deleyia* Holovachov and Boström, 2006, which like the type host of the type species of *Leptojacobus* n. gen. lives in rotting wood and has a small and slender body, baggy cuticle, a pharynx without a grinder, and a small stoma. However, *Leptojacobus* n. gen. is clearly distinguished from *Deleyia* by a stoma with teeth and other armature vs. a reduced stoma, one vs. two circles of labial papilla, and a well-developed muscular corpus.

*Leptojacobus dorci** n. sp.
(Figs. 1–4)

Measurements: See Table 1.

Adults. Outer layers of cuticle often detach from the rest of the body wall. When the cuticle separates from the rest of the body, it consistently detaches between the excretory pore and cloaca/anus. Annulation, but not the longitudinal striation, is most apparent in the neck and tail of the nematodes, i.e., where the cuticle does not detach. Conversely, striation is the predominantly visible pattern in areas of detached cuticle. Where the cuticle has detached, horizontal striations can also be observed in the layer of cuticle still attached to the body wall. The head does not narrow relative to

* The species epithet is the Latin genitive of *Dorcus*, the type host genus for the new species.
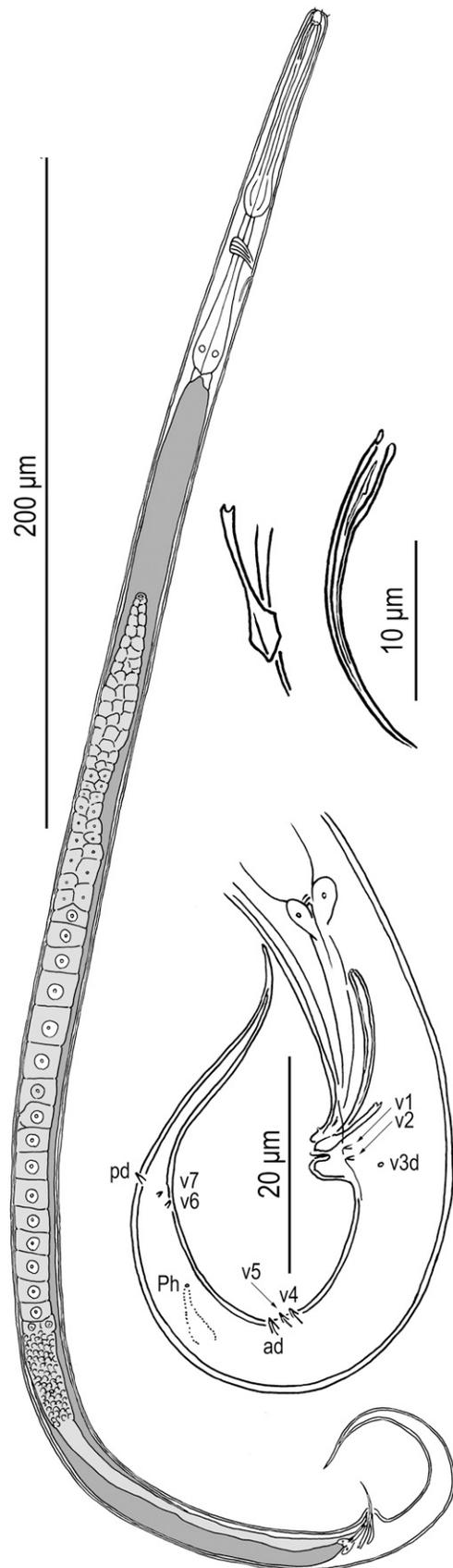
FIG. 2.   *Leptojacobus dorci* n. gen., n. sp. A. Whole body of adult male. B: Spicule and gubernaculum in right lateral view. C. Male tail in left lateral view.
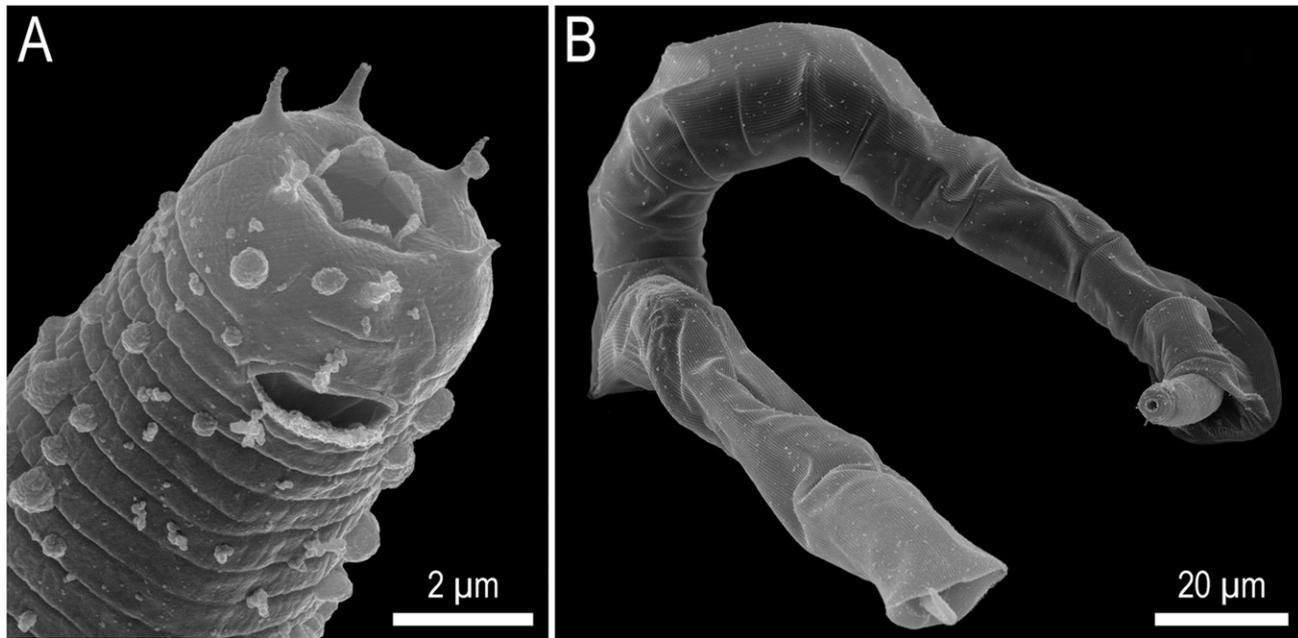
Fig. 3.   Scanning electron micrographs of *Leptojacobus dorci* n. gen., n. sp. A. Female, oblique lateral and en face view. Lips are only slightly separated, and cheilostomatal flaps are adradial. Globules or contaminants on body surface, observed on several specimens, are of unknown origin. B. Whole body of female, showing detached, "baggy" body-wall cuticle.

width of neck or the rest of the body, and the apex of the lip region is almost perpendicular to the body wall at the region of the anterior neck. Body annulation extends anteriad to the amphid apertures and posteriad almost to the tail tip. Amphidial apertures are lateral, are positioned at the level of the gymnostom, and are large relative to the size of the lip region, being almost as wide as individual lips. In addition to stomatal characters as described for the genus, the stegostom anterior to the tooth forms a sclerotized ridge, and the two subventral ridges of the stegostom each bear two minute denticles. The pharyngeal corpus (procorpus and median bulb) is about 1.2 times as long as the postcorpus (isthmus and basal bulb). The pharyngo-intestinal junction is well developed. The hemizonid was not observed. Deirids were not easily observed, presumably because of the common detachment of the body-wall cuticle, but in at least one individual they were localized to the region of the basal bulb.

Male. The excretory pore is usually posterior to the nerve ring. The testis is to the right of the intestine, stretches about half of the body length, and occupies almost the entire body diameter and so does not run either ventrally or dorsally. Spermatogonia are arranged in two to three rows for the distal third of gonad and in a single row for the middle third. The vas deferens is long, comprising the proximal third of the gonad, and is wide only distally, where it contains mature, amoeboid spermatids. Three cloacal glands, two subventral and one dorsal, are present but not obvious. Spicules are paired and separate, and they are simple and slender. In lateral view, spicules are smoothly ventrally arcuate, giving spicules a curve of about 100°. The manubrium is

long, being almost a fourth of the spicule length, and is not set off from the rest of the spicule, and the spicule shaft and blade (i.e., calomus and lamina complex) gradually thins posteriorly, being only slightly expanded at the anterior fourth of its length. The gubernaculum is also slender, about or slightly less than half of the length of spicules, possessing small ventral and dorsal peaks at its posterior end, thinning midway along its length, forming a ventral tube enclosing spicules at posterior fourth of its length, and expanding into a small ventral keel at its anterior tip. When the body cuticle is detached, the cuticle extending posteriad overlaps the cloaca. The phasmid (Ph) is difficult to observe by DIC microscopy and is located at about half of the tail length. Nine pairs of genital papillae and a pair of phasmids are arranged as <(v1, C, v2, v3d), (v4, v5, ad), Ph, (v6, v7), pd> in the nomenclature of Sudhaus and Fürst von Lieven (2000), or <(P1, C, P2, P3d), (P4, P5, P6d), Ph, (P7, P8), P9d)> in the homology-agnostic nomenclature of Kanzaki et al. (2012a). Papilla v1 is thus very close to v2, which is at about the same level as v3d; putative v4 is unusually posterior, being closely anterior to ad; putative v6 is unusually far anterior, even anterior to ad; Ph is midway between ad and v6; pd is clearly posterior to v6 and v7. The tail is conical, strongly ventrally curved when killed by heat, about five cloacal body widths in length, and it has a thinly pointed, hyaline tip.

Female. The excretory pore is usually at the level of or anterior to the nerve ring. The ovary, oviduct, and uterus are distinct in each genital system. Gonads are reflexed. The anterior gonadal branch in young individuals extends along the right of the intestine, with the reflexion to the right of the proximal part; however,
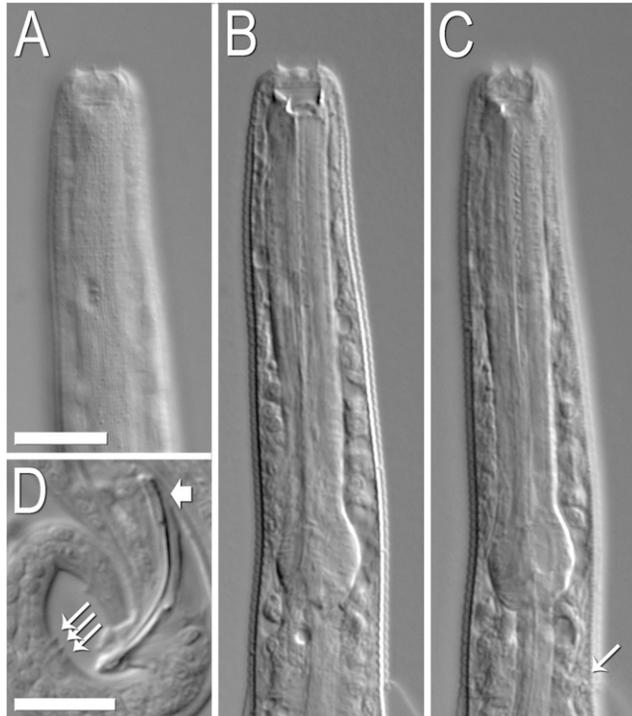
Fig. 4.   DIC micrographs of *Leptojacobus dorci* n. gen., n. sp. Scale bars are 10 μm. A–C, which are at the same scale, show the neck of a single adult female in right lateral view and at several focal planes. A. Body surface, including amphid aperture. Plane is right lateral. B. Stomatal characters shown are the small, conical dorsal tooth, a subventral ridge with two minute denticles, and the ring of overlap of cheilostom and gymnostom. The cuticle is detached posteriorly, as shown at the bottom of image. Plane is sagittal at stoma. C. Cuticle detaches immediately posterior to the excretory pore (arrow). D. Male genitalia in left lateral view. The cluster of three genital papillae (putative v4, v5, and pd; long arrows) is unusual among diplogastrids. Shown are the long, slender manubrium (short arrow) of spicule and the distal (posterior) keel of the distinctively thin gubernaculum. Plane is sagittal at excretory pore.

when a large oocyte is in the uterus, the proximal part of the gonad is sometimes forced to the left of the intestine, in addition to pushing the intestine to the dorsal wall. The posterior branch is consistently to the left of the intestine, the reflexion being to the left of the proximal part. The reflexed part of each gonadal branch comprises the ovary, in which oocytes are arranged in two to three rows for the distal half or less of the reflexion, and has a single row of oocytes in the remainder of the ovary. The flexure of each gonad is usually twisted 180° around the axis of the gonad, except for when a fully developed oocyte in the oviduct or uterus stretches the ovary longitudinally, thereby untwisting the flexure (Fig. 2F,G). The oviduct, which comprises most of the length of the proximal part of the gonad, has a bunched appearance, similar to a crustaformeria, but can be stretched smooth by the internal passage of an oocyte. Sperm are stored throughout the oviduct, and a distinct spermatheca is absent. The uterus is short and consists of flattened, diamond-shaped cells. Up to a single egg is observed in the oviduct or uterus at one time.

A distinct receptaculum seminis is absent. The vagina is perpendicular to the body surface and is thinly but distinctly sclerotized. In lateral view, the vulva is slightly protuberant where the cuticle adheres to the body wall; the vulva extends as a canal through the detached body cuticle, when it is detached, and apart from this canal the cuticle surrounding the vulva is completely detached from the rest of the body wall. The rectum is about one anal body width long, and the intestinal-rectal junction is surrounded by well-developed sphincter muscle. Three rectal glands, two subventral and one dorsal, are present. When the body cuticle is detached, the cuticle extending posteriad overlaps the cloaca. The phasmid is difficult to observe by DIC microscopy and is located at about midtail length. The tail is conical, about eight anal body widths in length, and has a conical tip.

Type host (carrier) and locality: The culture from which the type specimens were obtained was originally isolated by N. Kanzaki from adults of the stag beetle *Dorcus ritsemae* as described above. The beetles were purchased from a pet shop in Aichi Prefecture, Japan, and were marketed as wild-collected individuals, purportedly from their native range in Indonesia, presumably Java. Nematodes were not isolated directly from the beetles, although failure to isolate them from the bodies of beetles may have been because of procedural artifact. Specifically, dissecting beetles on agar plates is a method typically successful for isolating larger species in other diplogastrid genera, as well as other rhabditids, from their beetle carriers (Herrmann et al., 2006). As a consequence, these larger nematodes develop and proliferate quickly and could have easily obscured the small, more slowly developing individuals of *L. dorci* n. gen., n. sp. Therefore, although *D. ritsemae* is considered to be the type carrier of the new species, the nature of the nematode's association with the host is still unknown.

Type material, type strain, and nomenclatural registration: A holotype male (accession 31368), three paratype males, and seven paratype females (31369-31372) have been deposited in the University of California Riverside Nematode Collection (UCRNC), CA. Two paratype males and four paratype females have been deposited in the Swedish Natural History Museum, Stockholm, Sweden. The type strain is available in living culture from the Department of Evolutionary Biology, Max Planck Institute (MPI) for Developmental Biology, Tübingen, Germany and can be provided to other researchers upon request. Attempts to achieve frozen stocks of this species have been unsuccessful. The new genus has been registered in the ZooBank database (zoobank.org) under the identifier 0978210B-FD0C-4553-A425-0B684CA44D8C, and the new species binomial under the identifier CEFC571F-72EC-484B-92D2-59C483EF6B7C.

Molecular characterization and phylogenetic analysis: The alignment comprised 1,605 sites, 872 of which were variable and 699 parsimony informative. Only the ML tree is shown (Fig. 5). Monophyly of the family

TABLE 1.   Morphometrics of male holotype (in glycerin) and male and female specimens (temporary water mounts) of *Leptojacobus dorci* n. gen., n. sp. All measurements are in μm and in the form: mean ± sd (range).

Character	Male		Female
	Holotype	Temporary water mounts	Temporary water mounts
n	–	12	10
L	475	546 ± 62 (455-664)	658 ± 68 (534-737)
L'	417	480 ± 56 (401-590)	558 ± 66 (445-634)
a	43	43 ± 5.9 (35-57)	29 ± 4.1 (22-36)
b	5.5	5.3 ± 0.4 (4.8-6.2)	6.0 ± 0.7 (4.9-6.7)
c	8.2	8.4 ± 0.6 (7.1-9.0)	6.6 ± 0.6 (5.6-7.5)
c'	6.9	5.3 ± 1.1 (4.2-7.7)	7.8 ± 1.1 (6.5-9.4)
V or T	47	54 ± 3.7 (46-58)	50 ± 4.4 (42-55)
Maximum body diam. (not detached cuticle)	11	13 ± 2.0 (8.0-15)	23 ± 1.8 (20-26)
Stoma diam.	2.3	2.9 ± 0.5 (2.0-3.8)	4.6 ± 0.6 (3.5-5.3)
Stoma length	3.1	4.1 ± 0.3 (3.6-4.6)	4.7 ± 0.5 (4.1-5.4)
Anterior end to amphidial aperture	2.0	2.4 ± 0.2 (2.0-2.8)	2.2 ± 0.5 (1.3-3.0)
Corpus length	42	52 ± 4.7 (39-55)	60 ± 2.8 (53-63)
Pharynx length	79	99 ± 7.4 (79-107)	106 ± 3.7 (97-112)
Corpus as % pharynx length	53	52 ± 1.5 (49-55)	56 ± 1.4 (54-58)
Neck length	86	102 ± 7.5 (82-110)	109 ± 3.9 (62-72)
Median bulb diam.	6.5	6.7 ± 0.3 (6.0-7.2)	10 ± 0.9 (8.0-11)
Basal bulb diam.	5.3	6.1 ± 1.0 (4.7-8.3)	10 ± 0.7 (9.1-12)
Excretory pore from anterior end	60	70 ± 8.2 (51-82)	70 ± 3.5 (66-78)
Nerve ring from anterior end	57	66 ± 7.5 (47-75)	72 ± 6.4 (63-81)
Vulva from anterior end	–	–	329 ± 58 (223-381)
Vulva to anus distance	–	–	230 ± 14 (210-256)
Length of anterior gonad branch	–	–	95 ± 16 (72-116)
Length of anterior flexure	–	–	80 ± 17 (61-116)
Length of posterior gonad branch	–	–	100 ± 12 (85-127)
Length of posterior flexure	–		85 ± 14 (67-108)
Testis length	223	294 ± 47 (213-379)	–
Cloacal or anal body diam.	8.4	13 ± 2.8 (7.0-16)	13 ± 2.0 (10-16)
Tail length	58	65 ± 7.7 (54-74)	99 ± 5.6 (89-105)
Spicule length (curve)	23	25 ± 1.6 (23-27)	–
Spicule length (chord)	20	22 ± 1.7 (18-25)	–
Gubernaculum length	10	11 ± 1.2 (9.0-13)	–
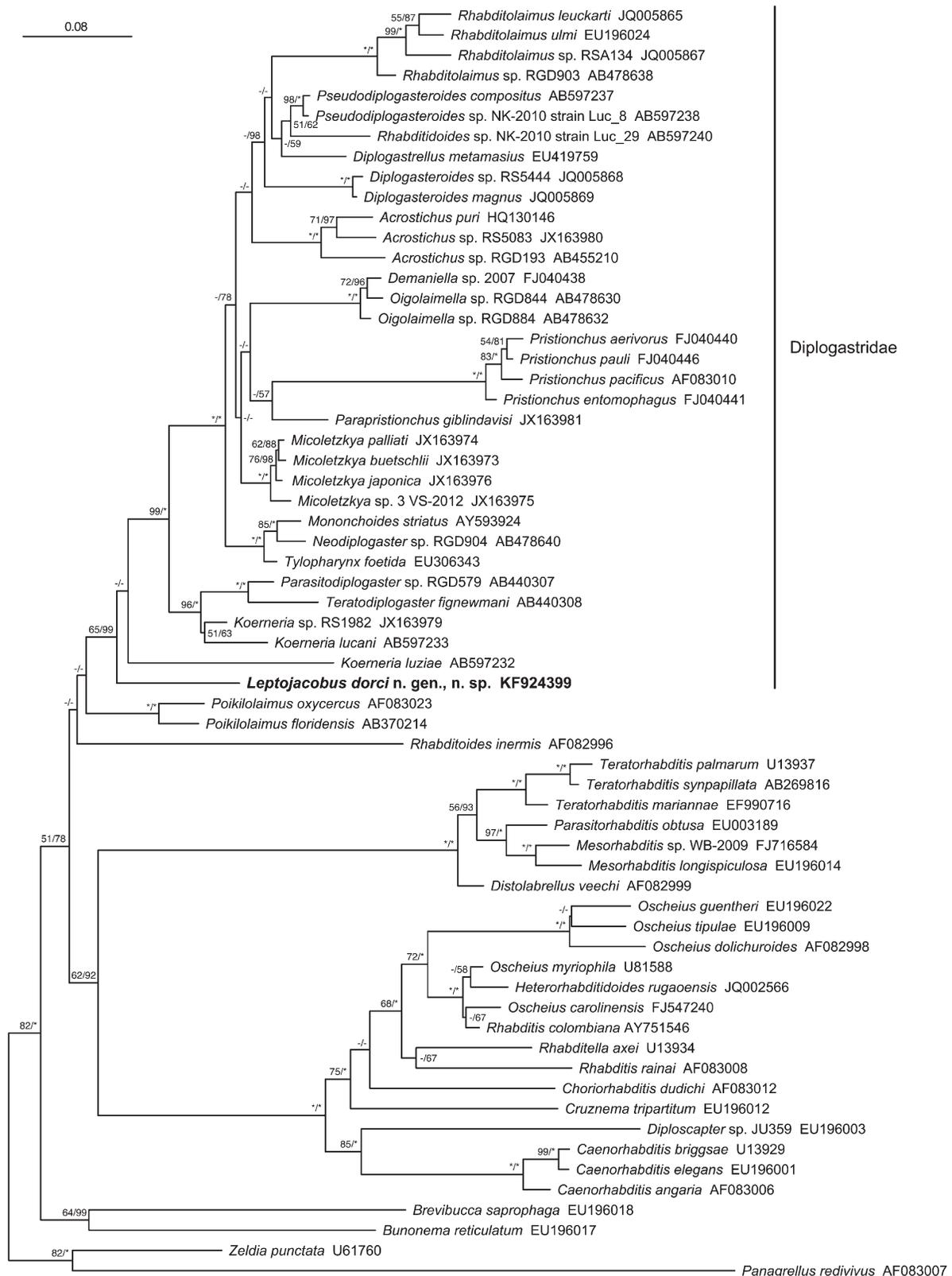
FIG. 5. Phylogenetic relationships of Diplogastridae and outgroups, as inferred by maximum likelihood (ML) of nearly full-length SSU rRNA sequences. The tree with the highest log likelihood is shown. For nodes that were present in the topology inferred by Bayesian analysis, posterior probabilities (PP) are also given. Support values are shown next to the nodes: left value is the proportion of trees in which the associated taxa clustered together in 1,000 bootstrap pseudoreplicates in the ML analysis; right value, PP of that node according to Bayesian inference. Support values above 50% are shown. Asterisks indicate 100% support; dashes indicate < 50% BS (left value) or the absence of the node in the Bayesian tree (right value). Tree is drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank accession numbers are given to right of taxon names.

Diplogastridae, including *L. dorci* n. gen., n. sp., was recovered in both ML and Bayesian analyses, albeit with variable support (65% BS, 99% PP). The new species was excluded from a highly supported clade (99% BS, 100% PP) that includes all but one other diplogastrid species analyzed in this study. The Japanese isolate of *K. luziae* was the other species excluded from this clade, consistent with a previous study that included this isolate (Kanzaki et al., 2011). The phylogenetic position of *L. dorci* n. gen., n. sp. relative to that of *K. luziae* differed between the ML and Bayesian analyses, and was poorly supported in both: in the ML tree, the new species was sister to all other analyzed Diplogastridae (< 50% BS); in the Bayesian tree, it was the sister of *K. luziae* (73% PP). Finally, the inclusion of *L. dorci* n. gen., n. sp. in our analysis yielded poorly supported and conflicting results regarding the closest outgroup to all sampled diplogastrid taxa: whereas the closest clade was *Poikilolaimus* spp. Fuchs, 1930 in the ML analysis (< 50% BS), the outgroup was predicted to be *Rhabditoides inermis* (Schneider, 1866) Dougherty, 1955 in the Bayesian analysis (58% PP). Therefore, *L. dorci* n. gen., n. sp. is clearly basal to most or all of the Diplogastridae included in this study, although the precise position of the new species and the identity of the nearest outgroup to the family remain ambiguous.

Discussion

Leptojacobus dorci n. gen., n. sp. has been shown by an analysis of nearly full-length SSU rRNA sequences to represent an ancient divergence from other taxa in the family (as defined by Sudhaus and Fürst von Lieven, 2003). Specifically, *L. dorci* n. gen., n. sp. is excluded from a well-supported monophyletic clade that includes all other diplogastrid taxa so characterized to date, with the exception of *K. luziae*. The new species therefore provides a basis for reconstructing ancient character histories in Diplogastridae, a group characterized by a number of novelties relative to outgroup rhabditids. Although the phylogenetic positions of *L. dorci* n. gen., n. sp. and *K. luziae* were not well supported in our analyses, the relatively basal divergence of *L. dorci* n. gen., n. sp. in the family directs the search for possible plesiomorphic or intermediate states for diplogastrid-specific characters. The new species clearly shows characters diagnostic of diplogastrids: the first molt inside the egg (Fürst von Lieven, 2005), as well as a glandular pharyngeal postcorpus, anisomorphy of the anterior pharynx, and a moveable dorsal tooth (Sudhaus and Fürst von Lieven, 2003). Based on only these characters, *L. dorci* n. gen., n. sp. is not an obvious morphological intermediate between diplogastrids and outgroups.

In contrast to the above traits, stomatal dimorphism was not observed in the new species. This trait, although absent in many diplogastrid genera, is unique to the family. The apparent absence of dimorphism in *L. dorci* n. gen., n. sp., however, might have been observational artifact. For example, particular environmental cues triggering the development of an alternative mouthform may not have been met. Alternatively, the small size of stoma structures in *L. dorci* n. gen., n. sp., which are at the limit of what is accessible by LM and DIC microscopy, might have confounded interpretation of the trait. On the other hand, a true absence of dimorphism in *L. dorci* n. gen., n. sp., if this species were the sister to a clade including all dimorphic diplogastrids, would suggest that this trait was gained only after the evolution of a moveable tooth. Clearly, better phylogenetic resolution, deeper sampling of taxa and characters, and perhaps more intensive morphological analysis of *L. dorci* n. gen., n. sp., will be required to address the question of which trait came first in the evolution of Diplogastridae. Knowing this will be necessary to determine the role, if any, of plasticity in the evolution of moveable teeth as well as other mouth armature, as might be predicted by theory (e.g., West-Eberhard, 2003; Moczek, 2007).

In addition to the interesting morphological questions our discovery of *L. dorci* n. gen., n. sp. poses, the new species provides further evidence for unknown diversity to be discovered in association with beetles. With increased taxon sampling, it may be possible to infer ancient ecological associations in Diplogastridae. As with *L. dorci* n. gen., n. sp., *K. luziae* has also been reported from *Dorcus* stag beetles (Körner, 1954; Kanzaki et al., 2011), while the potentially close outgroup *Rhabditoides inermis* is known from carrion beetles (Coleoptera: Silphidae) (Völk, 1950). Notwithstanding a possible sampling bias toward beetle hosts, these observations support an ancestral association with them. Corroborating this is our discovery of a morphologically close but genetically distinct isolate of *Leptojacobus* n. gen. from rearing substrate of *Dorcus rectus* (N.K., unpubl. data), although we unfortunately lost that strain. Thus the continued sampling of stag beetles, including by the isolation strategy herein, holds promise for the discovery of more undescribed diversity in anciently divergent diplogastrid lineages.

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