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Phylogenetic Status and Morphological Characters of *Rhabditolaimus anoplophorae* (Rhabditida: Diplogastridae)

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Abstract: Rhabditolaimus anoplophorae Kanzaki and Futai was re-isolated from its type host (carrier), the cerambycid beetle *Anoplophora malasiaca*, collected in an experimental field of the Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, Japan. The nematode was cultured on nematode growth medium plates seeded with *Escherichia coli* OP50, and its morphological characters and molecular profile were examined to modernize the description. Scanning electron microscopic and light microscopy revealed the presence of four stomatal flaps, a very long gymnostom, a single ventral papilla in males, and a horizontal slit-like vulval opening in females. The positions of the deirids, hemizonids, phasmids, and rectal glands are additionally described, and the absence of a male bursa was confirmed. Phylogenetically, the genus forms a well-supported clade in the family Diplogastridae. *Rhabditolaimus anoplophorae* is a member of the monophyletic *Rhabditolaimus* clade and is closely related to *R. leuckarti* and several undescribed species.

Key words: molecular profiles, morphology, re-isolation, *Rhabditolaimus anoplophorae*, taxonomy.

The nematode *Rhabditolaimus anoplophorae* (Kanzaki and Futai, 2004) Susoy and Herrmann 2012 was originally described from a long-horn beetle, *Anoplophora malasiaca* (Thomson). The species, collected in Kyoto, was originally named "*Cylindrocorpus*" *anoplophorae*. Although the original culture was lost after description, the same species was re-isolated from *A. malasiaca* collected in Tsukuba, Japan, by one of the present authors (NK). In the present study, molecular sequences of near-full-length of small subunit (SSU) and D2/D3 expansion segments of large subunit (D2/D3 LSU) of ribosomal RNA, which are often used for modern molecular phylogenetic analysis and a species-specific molecular profile, were determined, and some additional morphological characters were described. Further, several typological characters were compared among several *Rhabditolaimus* species and discussed in relation to their phylogenetic relationships to modernize the current morphological and phylogenetic information of this long neglected genus.

Materials and Methods

Nematode isolation: An adult male *A. malasiaca* was collected from an experimental field of the Forestry and Forest Products Research Institute (FFPRI), Tsukuba, Ibaraki, Japan, in June 2012. The beetle was dissected, and dispersal third-stage (dauer) juveniles of nematodes were collected from the genitalia of the insect. The dauer juveniles were casually observed to determine feeding habit (i.e., bacteriophagous or mycophagous), determined to be a diplogastrid, and then transferred to nematode growth medium (NGM) agar plates seeded with *Escherichia coli* OP50. The nematodes successfully propagated and were maintained as a laboratory culture through occasional subculture.

Identification and observation: The cultured nematodes were observed under a light microscope and were identified as *R. anoplophorae* based on general morphology, isolation from a phoretic host (*A. malasiaca*), and (limited) unpublished molecular data, which was not sufficiently complete in the original description to permit a phylogenetic analysis. Detailed morphological observations were conducted on live material employing the methods described by Kanzaki (2013). Adult nematodes from a 10-d-old culture were killed by heat (60°C, 1 min), fixed in triethanolamine formalin (TAF), processed in a glycerol-ethanol series of baths using the methods of Minagawa and Mizukubo (1994), and stored as voucher specimens in the Forest Pathology Laboratory Collection of the FFPRI.

For scanning electron microscopy (SEM), adult males and females of *R. anoplophorae* were collected using the Baermann funnel technique, fixed in 3% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 wk, and postfixed in 2% (w/v) OsO_4 for 2 h. Specimens were dehydrated in a series of ethanol baths, critical point-dried using CO_2, mounted on stubs, sputter-coated with gold, and observed using a JXA-840A (Jeol, Tokyo, Japan) scanning electron microscope operating at 5 to 8 kV.

Undescribed species used in phylogenetic analysis: Because the number of operational taxonomic units (OTUs) deposited in GenBank was not sufficient to allow for comparison of molecular phylogeny with morphological characters, two undescribed *Rhabditolaimus* species isolated by the present authors were included in the analysis.

Rhabditolaimus sp. 1 was isolated from *Euwallacea fornicatus* Eichhoff collected from a dead log of an unidentified broad-leaved tree in Okinawa, Japan, in early spring 2013. *Rhabditolaimus* sp. 2 was isolated from *Episcapha gohrami* Lewes collected in Sapporo, Hokkaido, Japan, in 2012 together with *Pristionchus bucculentus* Kanzaki, Ragsdale, Herrmann, Röseler, and Sommer,

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2013. Collection details have been reported by Kanzaki et al. (2013a). Both undescribed species have a long tube-like stoma, a short pro/metacorpus, didelphic female gonads, and a leptoderan male bursa (Kanzaki, unpublished data). Although the female gonad and male tail of *Rhabditolaimus* sp. RS5514 have not been observed by the present authors, the species has a long and tube-like stoma and a short pro/metacorpus (see the photographs of Susoy and Herrmann, 2012), and *Rhabditolaimus* sp. RGD808, which was isolated from an unidentified cerambycid from La Selva, Costa Rica, in 2008, is morphologically and biologically very close to *R. walkeri* (Hunt, 1980) Susoy and Herrmann, 2012 and may in fact be conspecific with that species. Thus, the species has a long tube-like stoma, a short pro/metacorpus, didelphic female gonads, and a leptoderan male bursa, and is associated with cerambycid beetles in Central America and the Antilles Islands (Kanzaki and Giblin-Davis, unpublished data; see Hunt, 1980).

Molecular sequencing and phylogenetic analysis: For molecular analysis, nematodes were individually transferred to 30-µl amounts of nematode digestion buffer (Kikuchi et al., 2009; Tanaka et al., 2012) and digested at 60°C for 20 min. The obtained crude DNA solutions were used as PCR templates. Partial DNA sequences of ribosomal DNA (the *ca.* 1.6-kb near-full-length SSU RNA and the 0.7-kb D2/D3 LSU RNA) were obtained using the methods of Kanzaki and Futai (2002) and Ye et al. (2007).

The molecular phylogenetic status of *R. anoplophorae* was determined by reference to the SSU sequence, using Maximum Likelihood (ML), Maximum Parsimony (MP), and Bayesian analysis. The species (OTUs) compared herein with *R. anoplophorae* were identified on the basis of a BLAST homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and OTUs used in previous studies of diplogastrid and rhabditid phylogeny (Kanzaki et al., 2011; Susoy and Herrmann, 2012) were also included. The details of molecular phylogenetic analysis have been described previously (Kanzaki et al., 2011).

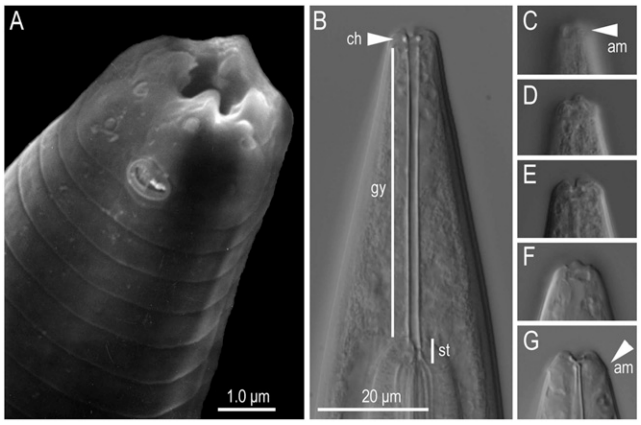

Fig. 1. Lip and stomatal morphology of *Rhabditolaimus anoplophorae*. A. A scanning electron micrograph of the anterior region. B. A right lateral view of the mouthparts of an adult male (ch: cheilostom; gy: gymnostom; st: stegostom). C–E. Left lateral views of an adult male in different focal planes (am: amphid). F,G. Ventral views of an adult female.

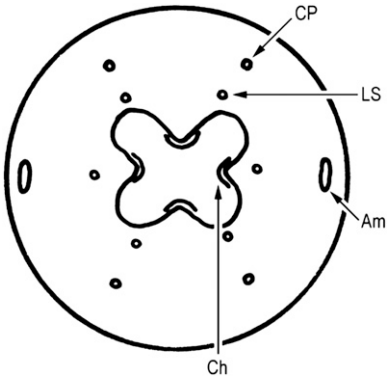

Fig. 2. Schematic drawing of *en face* view of male of *Rhabditolaimus anoplophorae* (Am: amphid; Ch: extended cheilostomatal element; CP: cephalic papilla; LS: labial sensillum).

Results and Discussion

Additional morphological characters and correction of original description: The general morphology of the nematode was as described previously (Kanzaki and Futai, 2004). However, additional morphological characters were noted in the present study and are described below.

The most significant addition to the original description concerns lip and stomatal morphology. The lip has six outer labial sensilla and two lateral ellipsoid amphids but is without typical tri- or hexaradiate symmetry because of asymmetric fusion in the dorsal and subventral lip sectors that manifests as one dorsal, two lateral, and one ventral triangular projections (Figs. 1; 2). The anterior end of the cheilostomatal wall extends to form short flaps located at the position of the lip projections. In other words, the cheilostomatal flaps are

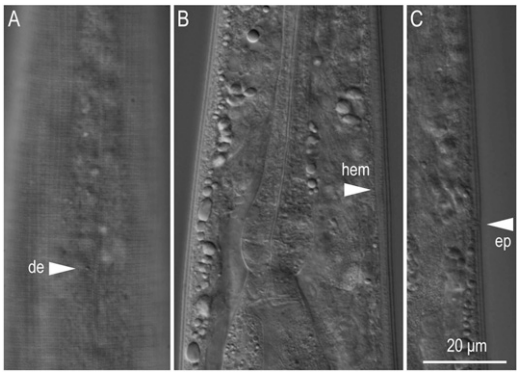

Fig. 3. The basal bulb region of *Rhabditolaimus anoplophorae*. A–C. Right lateral views in different focal planes showing the deirid ("de" in A), the hemizonid ("hem" in B), and the excretory pore ("ep" in C).

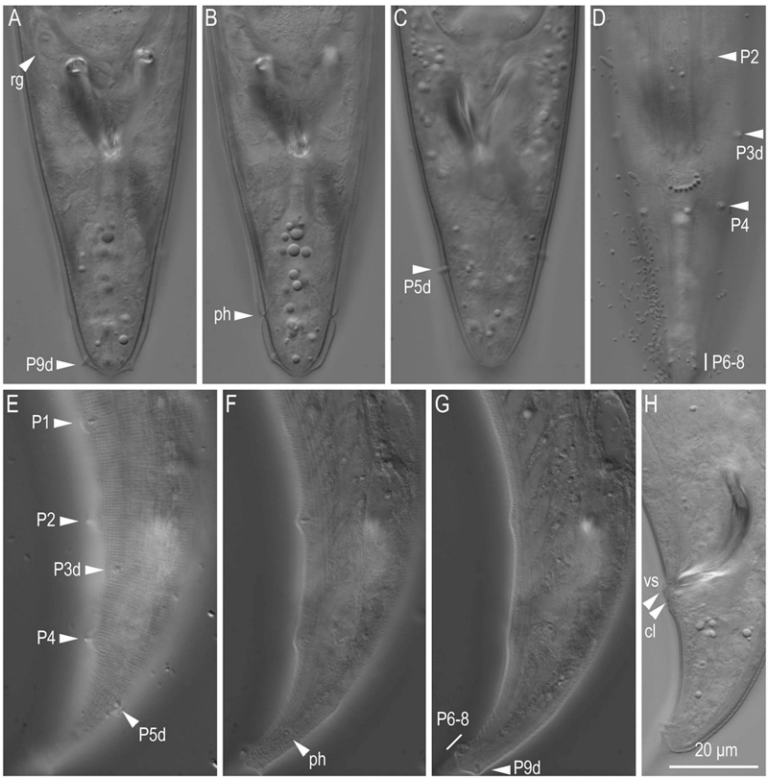

FIG. 4. The male tail region of *Rhabditolaimus anoplophorae*. A–D. Ventral views in different focal planes. E–H. Left lateral views in different focal planes (rg: rectal gland; ph: phasmid; P + number: genital papillae; vs: ventral single papilla; cl: cloacal opening).

integrated with the lip projections and share the unusual lip symmetry with four rhabdions (versus the usual six), which makes the stomatal opening appear as an X or a four-leaved clover (Fig. 2). This morphology is basically identical to that of *R. ulmi* (Goodey, 1930) Susoy and Herrmann, 2012 (coded as "*Goodeyus* sp."), examined by Fürst von Lieven and Sudhaus (2000). This lip and cheilostomatal morphology was also observed in *Rhabditolaimus* sp. RGD808, sp. 1 (*Euwallacea fornicatus* isolate) and sp. 2 (*Episcapha gorhami* isolate). In terms of the phylogenetic relationship between *R. ulmi*, *R. anoplophorae*, and the other three *Rhabditolaimus* spp. (see below; these species belong to different clades within the genus), such morphology is a shared (symapomorphic) character. Males of *R. anoplophorae* have an additional four cephalic sensilla located slightly posterior to the ventral and dorsal labial sensilla as is typical for the family Diplogastridae. The stomatal morphology may be described using the terminology of Sudhaus and Fürst von Lieven (2003) as follows: the cheilostom short, ring-like, visible as refractal dots in a lateral view; the gymnostom very long, flexible, forming a narrow tube, the dorsal side a little shorter than the ventral side; and the stegostom short, with an unclear pro-meso stegostom, a cuticularized (sclerotized) metastegostom, and a narrow nonsclerotized telostegostom. The long pharyngeal sleeve described by Kanzaki and Futai (2004) is thus considered to be formed by arcade syncytial cells. This type of long pharyngeal sleeve has been described in some other species, e.g., *R. inevectus* (Poinar, Jackson, Bell & Wahid) Susoy & Herrmann (Poinar et al., 2003). We posit that

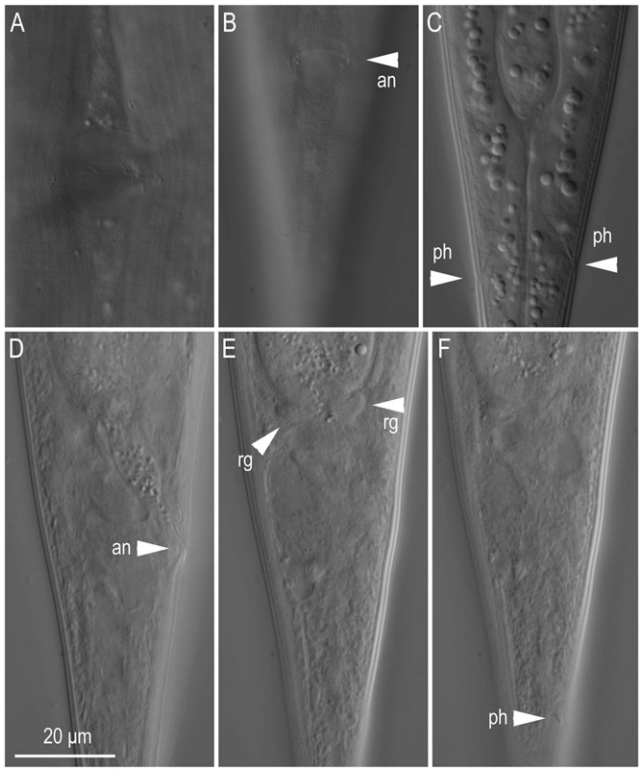

FIG. 5. Female tail region of *Rhabditolaimus anoplophorae*. A–C. Ventral views in different focal planes. D–F. Right sublateral views in different focal planes (an: anus; ph: phasmid; rg: rectal gland).

these long sleeves were also formed by the arcade syncytial cells and therefore represent a synapomorphy.

A hemizonid and a deirid were evident in the anterior regions of both males and females (Fig. 3). The hemizonid is located at the level of the anterior end of the basal bulb (thus at the junction between the isthmus and the basal bulb), and the deirid is laterally located at the level of the posterior end of the basal bulb or cardia.

A single ventral papilla and a pair of phasmids were seen in the male tail (Fig. 4). The ventral papilla is very small, difficult to observe in a ventral view, and located just posterior to the cloacal opening. Each phasmid is small and pore-like, and is located between the P5d and P6 papillae. No narrow leptoderan bursa was observed in the present study, but a thickened lateral cuticle that has the appearance of a narrow bursa was observed in the tail region, from the level of the P4 papilla to the base of the tail spike. As stated previously (e.g., Kanzaki et al., 2013b), a thickened cuticle around the tail region can falsely appear as a narrow leptoderan bursa in ventral view in many diplogastrids. Previously described species with tape-like leptoderan bursa (summarized by Poinar et al., 2003) will need to be confirmed for this character.

In females, the vulval opening was observed ventrally as a horizontal slit-like pore surrounded by an oval structure, and a small pore-like phasmid is located approximately 1.5 anal body diameters posterior to the anal opening (Fig. 5).

Although the morphological characters have thus been revised, this species remains distinguishable from its close relatives based principally on the clearest definitive character, the midlength tail spike of males. Additionally, a biological characteristic, association with *A. malasiaca*, seems to be unique (Kanzaki and Futai, 2004). Thus the taxonomic validity of the species is retained.

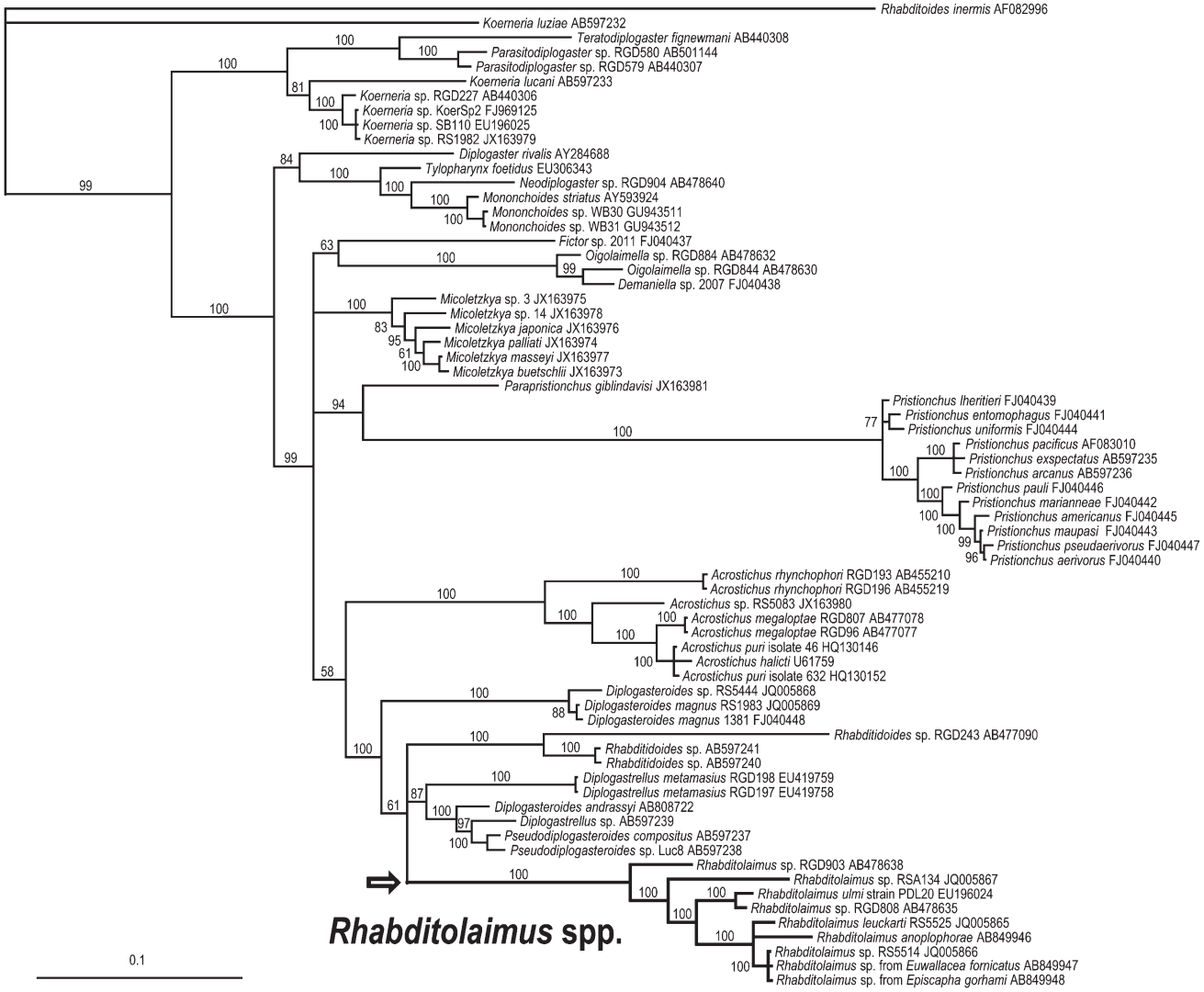

FIG. 6. Molecular phylogenetic status of *Rhabditolaimus anoplophorae* inferred from near-full-length SSU sequence. The Bayesian tree #10001 constructed using the GTR+I+G model is shown (lnL = 15640.4268; freqA = 0.2379; freqC = 0.2124; freqG = 0.2683; freqT = 0.2813; R(a) = 1.1953; R(b) = 2.9845; R(c) = 2.4567; R(d) = 0.4328; R(e) =5.2976; R(f) = 1; Pinva = 0.4185; Shape = 0.5594). Posterior probability values exceeding 50% are shown for appropriate clades.

Molecular phylogenetic status: The molecular sequences determined in the present study were deposited in GenBank under accession numbers AB849946 to AB849951. Although several minor differences are apparent, all three phylogenetic analyses (Bayesian, ML, and MP) conducted suggest that *Rhabditolaimus* spp. forms a well-supported (100% bootstrap and posterior probability values) clade located at a relatively derived position within the family, and the three phylogenetic trees constructed are almost identical topologically. Therefore, only the Bayesian tree is shown (Fig. 6). The genus *Rhabditolaimus* (Fuchs, 1915) Susoy and Herrmann, 2012, has a rather complicated taxonomic history. The genus was erected by Fuchs (1915), but synonymized with *Diplogasteroides* de Man, 1912, along with several other genera, by Sudhaus and Fürst von Lieven (2003). Thereafter, Susoy and Herrmann (2012) resurrected the genus based on morphological and molecular phylogenetic analyses, and synonymized *Myctolaimus* Cobb, 1920, into the genus. The genus *Myctolaimus* also had several junior synonyms and was previously separated into several genera by stomatal/pharyngeal morphology, the number of female gonads, and the presence/absence and shape of the male bursa. These genera were *Myctolaimus*, *Cylindrocorpus* Goodey, 1939, *Goodeyus* Chitwood, 1933, *Myctolaimellus* Andrassy, 1984, and *Protocylindrocorpus* (Rühm, 1959) Paramonov, 1964.

The results of the present study support the relevance of molecular phylogeny-based classification as developed by Susoy and Herrmann (2012). Although more detailed morphological observations on many more *Rhabditolaimus* species are required, the currently described morphological characters did not easily corroborate inferred molecular phylogenetic relationships. For example, *R. leuckarti* Fuchs, 1914, which has a relatively short stoma and a long pro/metacorpus, lies within the clade containing species with a long stoma and a short pro/metacorpus (Fuchs, 1914; Kanzaki and Futai, 2004; Kanzaki, unpublished observations); *R. ulmi*, which has a single female gonad is closely related to a didelphic species, *Rhabditolaimus* sp. RGD808 (Goodey, 1930; Kanzaki and Giblin-Davis, unpublished observations); and a bursa or bursal flap (lacking in *R. anoplophorae*) is also absent in the clade containing the species with a peloderan bursa (Fuchs, 1914; Kanzaki and Futai, 2004; Kanzaki unpublished observations).

The highly divergent morphological characters of the genus *Rhabditolaimus* are interesting in terms of the morphological evolution of diplogastrid nematodes. However, current morphological data are insufficient to allow easily supported reconstruction of the phylogenetic relationships of *Rhabditolaimus* species without independent molecular phylogenetic evidence. More detailed observations using SEM and high-resolution light microscopy are required to better understand the phylogenetic relationships among and morphological evolution of *Rhabditolaimus* species.

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