Two nematodes (Nematoda: Diplogastridae, Rhabditidae) from the invasive millipede Chamberlinius hualienensis Wang, 1956 (Diplopoda, Paradoxosomatidae) on Hachijojima Island in Japan

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This paper was edited by Johnathan Dalzell. Received for publication September 29, 2015.

Abstract

Millipedes may cause unexpected damage when they are introduced to new locations, becoming invaders that leave behind their old parasites and predators. Therefore, it was interesting to find numerous rhabditid nematodes within the gut of the invasive phytophagous millipede Chamberlinius hualienensis Wang, 1956 (Diplopoda, Paradoxosomatidae) from Hachijojima (Japan) in November, 2014. This millipede originated in Taiwan but was discovered in Japan in 1986. The nematodes were identified as juvenile Oscheius rugaoensis (Zhang et al., 2012) Darsouei et al., 2014 (Rhabditidae), and juvenile and adult Mononchoides sp. (Diplogastridae) based on images, morphometrics, and sequences of 18S and 28S rDNA. A novel short 28S sequence of a separate population of Oscheius necromenus SB218 from Australian millipedes was also included in a phylogenetic comparison of what can now be characterized as a species complex of millipede-associated Oscheius. The only other nematode associates of millipedes belong to Rhigonematomorpha and Oxyuridomorpha, two strictly parasitic superorders of nematodes. These nematode identifications represent new geographic and host associations.

Key words

Invertebrate phoresy, Nematode ecology, Ribosomal DNA, Phylogeny, Systematics, Taxonomy.

The invasive phytophagous millipede Chamberlinius hualienensis Wang, 1956 was originally described from Taiwan, where it is extremely common (Chen et al., 2011). It was discovered in Okinawa, Japan in 1986 (Higa and Kishimoto, 1986) and outlying islands of the Ryukyu Archipelago (Nakamura and Korsós, 2010). Fourteen years ago it was detected on Hachijo Island, Japan (Fujiyama et al., 2012). Remarkably these millipedes are not known to swarm in Taiwan (Chen et al., 2011), but in Japan mass occurrences of the species have even led to disruptions of railway traffic (Niijima and Arimura, 2002). While most millipedes in the world are harmless to humans, they can vector plant and animal diseases caused by bacteria such as Citrobacter, Enterobacter, Salmonella, and Raoultella species (Kania and Klapec, 2012). Millipedes may cause unexpected damage when they are introduced to new locations, becoming invaders that leave behind their old parasites and predators. A taxonomic inventory of their invertebrate associates may shed light on their biology and ecology. Therefore, it was interesting that nematodes of the Rhabditidae were recently discovered within the gut of the invasive species C. hualienensis (Meyer-Rochow, 2015). Their morphological and molecular characterization, phylogenetic relationships and ecological associations are detailed in this report.
Two nematodes (Nematoda: Diplogastridae, Rhabditidae) from the invasive millipede *Chamberlinius hualienensis* Wang,

Materials and methods

Nematodes were discovered to be associated with the phytophagous millipede *C. hualienensis*, specimens of which were collected in November on Hachijojima (33°05’N; 139°47’E) from mass aggregations on the concrete walls of a freeway (Meyer-Rochow, 2015). Nematodes were rinsed under tap water strictly from the internal cavity (hemocoel and intestine were not specified) of millipedes after dissection and placed in 65% ethanol before sending to Beltsville in December, 2014 and January 2015 to process for slides and PCR. Culture was not possible due to inadequate laboratory facilities in Japan.

Microscopy

Nematodes were imaged at ×40–60 on an Olympus BX51 microscope with a DP71 camera (Olympus America Inc., Center Valley, PA) equipped with polarization optics. Measurements in micrometers were made with an ocular micrometer on a Zeiss Ultraphot II compound microscope with Nomarski optics on alcohol-distorted specimens before formalin fixation, and images were also directly measured with CellSens ver 1.6 imaging software integrated with the camera (Olympus America LLC, Center Valley, PA). Fixed specimens of Rhabditidae juveniles were processed for permanent slides according to the formalin-glycerine method (Golden, 1990). Imaged specimens of Diplogastridae were subsequently processed for PCR so no vouchers are available. For scanning electron microscopy (SEM) *Oscheius* nematodes were fixed according to a method recently described by Takaku et al. (2013). Specimens were observed in a JEM7100F JEOL high-pressure environmental scanning electron microscope at 1 kV.

DNA analysis

Specimens of each nematode were mechanically disrupted in 20 µl of extraction buffer (Baldwin et al., 1997) then stored in PCR tubes at –80°C until needed. Extracts were prepared from thawed pools by incubating the tubes at 60°C for 60 min, followed by 95°C for 15 min. to deactivate proteinase K. Two microliters of the extract was used for each 25 µl PCR reaction.

The ribosomal LSU D2-D3 expansion segment was amplified with primers D2A 5’-ACAAGTACCG TGAGGGAAAGTTG-3’ and D3B 5’TGGAGAAGGAA CCAGCTACTA-3’ (Nunn et al., 1996) using previously published amplification procedures (Baldwin et al., 1997 for *O. necromenus*; Ye et al., 2007 for others).

The 18S sequences reaction components included, per 25 µl reaction: 17.55 µl H₂O, 2.5 µl 10X PCR buffer, 0.5 µl dNTP mix (10 mM each dNTP), 0.75 µl MgCl₂, 50 mM, 0.75 µl 18S-G18S4 primer, 10 µM, 0.75 µl 18S-18P primer, 10 µM, 0.2 µl Taq (Invitrogen platinum, 1 unit), 23 µl of the above mix + 2 µl template DNA; cycling conditions were 94°C – 2 min, 94°C – 30 sec, 50°C – 30 sec, 68°C – 2 min, repeat 40 times: steps 2 through 4, 68°C – 10 min, 4°C – Hold, with primers of Thomas et al. (1997) used for PCR and sequencing.

PCR products were visualized and purified within the Lonza FlashGel™ DNA system (VWR International, Radnor, PA), and sequence was generated with an ABI BigDye Terminator v3.1 kit with sample sequence data analyzed on an ABI 3130XL Automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The 28S sequence was determined on both strands using d2A and D3B primers.

The 28S rDNA sequences related to *Oscheius* (Table 1) and 18S rDNA sequences of *Mononchoides* Rahm, 1928 and relatives (Table 2) were aligned with MAFFT ver 7.017 (Katoh, et al., 2005). Bayesian likehood trees were made with the MrBayes (Huelsenbeck and Ronquist, 2001) plugin within Geneious 7.1.7 (Biomatters, Auckland, New Zealand) using ModelTest ver. 3.7 (Posada and Crandall, 1998) AIC parameters generated within PAUP* (Sinauer Associates, Sunderland, MA).

Results

Systematics

*Oscheius rugaoensis* Zhang et al., 2012.

(Fig. 1)

Description

Measurement

*Oscheius rugaoensis* juvenile dauer stage N = 1: body length = 754 µm, body width = 35 µm, V = 56.6%, a = 21.7, c = 10.4, c’ = 5, stoma length = 20.6 µm. The stoma opening (Fig. 1A) was occluded. The pharynx especially (Fig. 1B) was shrunken and not well defined due to initial preservation in alcohol, so the ‘b’ ratio was not reliable. The lateral field was composed of five ridges (Fig. 1C).

Differential diagnosis

This juvenile of *Oscheius rugaoensis* has no clear counterpart for comparison in the descriptive
Table 1. Nematode 28S rDNA sequences for selected taxa in Figure 3.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
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</tr>
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<td>China</td>
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<td>Rhabditella axei</td>
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Table 2. Nematode 18S rDNA sequences for selected taxa in Figure 4.

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<td>RS9011</td>
<td>Ghana</td>
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<td>Sudhausia crassa</td>
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<td>Tylopharynx foetidus</td>
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</table>
Two nematodes (Nematoda: Diplogastridae, Rhabditidae) from the invasive millipede *Chamberlinius hualienensis* Wang,

morphological literature, being intermediate in length between the limits for dauer juveniles (666 µm) and adults (921 µm) (Zhang et al., 2012), yet having rudimentary gonad arms, similar stoma length (Darsouei et al., 2014) and five ridges (Zhang et al., 2012) as in adult females.

**Molecular sequences and phylogeny**

The small distance tree based on a 175 base pair (bp) MAFFT alignment (Fig. 3, Table 1) demonstrated that *O. rugaoensis* from Japan had a nearly identical sequence to that of a population from China (1/144 nucleotide difference or 0.7%), which had been described as *Heterorhabditoides rugaoensis* by Zhang et al. (2012), and formally synonymized four years later (Darsouei et al., 2014) and five ridges (Zhang et al., 2012) as in adult females.

*Oscheius necromenus* Sudhaus and Schulte, 1989

**Description**

**Measurements**

N = 10 heat-killed hermaphrodite specimens, body length = 1,661 ± 220 (1,340–2,120) µm, body width = 130 ± 24 (100–180) µm, a = 13.0 ± 1.8 (8.7–15.1), b = 7.5 ± 0.7 (6.1–8.8), c = 14.3 ± 1.6 (12–17.4), c’ = 2.6 ± 0.2 (2.3–2.9), V = 50 ± 2 (47–54)% , stoma length = 23 ± 1 (21–25) µm, stoma width = 5.7 ± 0.5 (5–6) µm, stoma length/stoma width = 4.5 ± 0.5 (3.3–4.9).

The original description (Sudhaus and Schulte, 1989) is supplemented by these measurements of adult females made from specimens cultured on *E. coli* in 1996. Some measurements of this cultured population interface with measures of the original population, extending the range compared to *O. necromenus* types for body length (vs 830–1,500 µm), body width (vs 54–90 µm), and stoma length (vs 18–20 µm). These measurements overlap with *O. necromenus* stoma width, ‘c’ and ‘c’’ ratios better than with *O. myriophilus* (vs 4–6 µm, 9–13, 3–5, respectively).

**Locality and host**

*Oscheius necromenus* Sudhaus and Schulte (1989) population SB169 from the diplopod *Oncocladosoma castaneum*, Adelaide Hills, South Australia (Sudhaus and Schulte, 1989), kept in culture since 1988 by the Sudhaus laboratory in Germany and later the Fitch laboratory at New York University (NYU), from where we obtained it in 1996. Although a LSU 28S rDNA D3 sequence was incorporated into a larger *Oscheius* tree
(Carta et al., 2001), this culture is no longer available, and molecular marker sequences other than the one generated in this work are not available in GenBank. *Mononchoïdes* sp. (Fig. 2)

**Description**

**Measurements**

Female body N = 1 (Fig. 2A) length = 890 µm, body width = 44.2 µm, stoma length = 14.9 µm, pharynx (Fig. 2B) length = 139 µm, anterior pharynx/posterior pharynx ratio = 1.7, vulva-anus distance = 278.8 µm, tail length = 254.0 µm, V = 40%, a = 20.1, b = 6.4, c = 3.5, c′ = 11, Paired gonads = 24.4% body length, anterior gonad = 116, posterior gonad = 101.

Male body n = 1 (Fig. 2C) length = 642 µm, body width = 29.2 µm, stoma length = 13.6 µm, tail length = 192 µm, spicule length = 26.2 µm, a = 22, b = 6.6, c = 3.4, c′ = 7.5.

Stoma of male and female with eight prominent cheilostom striations visible laterally defining seven plates, anterior esophagus about 1.7 × the posterior esophagus. Vulva-anus distance = 1.2 × tail length, long female tail ending in a thread. Pharynx, intestine, gonad, and genital papillae of male poorly defined.

**Differential diagnosis**

The specimens from the millipede come closest to *Mononchoïdes americanus* Steiner (1930) (=Glauxinema americanus; Steiner, 1930; Tsalolikhin, 2009) in major morphometrics except a shorter male spicule (26 vs 30–45 µm) and smaller value for female ‘c’ (7.5 vs 8.7–12).

**Molecular sequences and phylogeny**

The DNA sequences were submitted to GenBank: for *Oscheius necromenus* SB 169 28S KT884894, *Oscheius rugaoensis* 28S KT884891, *Mononchoïdes*
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Two nematodes (Nematoda: Diplogastridae, Rhabditidae) from the invasive millipede *Chamberlinius hualienensis* Wang, sp. cf. *americanus* 28S KT884892, 18S KT884893. The closest taxon for both 18S and 28S rDNA markers was *Mononchoides* sp. 3 from New Caledonia (in Susoy et al., 2015). For the 18S marker, a 1,088bp alignment showed 99% similarity between those isolates, and a 28S 779bp alignment showed a 96.8% similarity. A MrBayes phylogenetic tree for 18S rDNA (Fig. 4, Table 2) demonstrated 100% support for two independent clades of three and four isolates of putative *Mononchoides*, separated by *Neodiplogaster*. The second clade with four isolates included this millipede isolate of *Mononchoides* cf. *americanus*.

**Discussion**

Three primary groups of nematodes have been reported as associates of millipedes: Rhigonematomorph millipede gut parasite species (Hunt, 1996; Malysheva and Spiridonov, 2013), Oxyuridomorph, Thelastomatoidea and Coronostomatoidea (Adamson and van Waerebeke, 1982; Jex et al., 2005; Phillips et al., 2016), and Rhabditida: *Oscheius myriophilus* (Poinar, 1986) and *O. necromenus* (Sudhaus and Schulte, 1989). The first two nematode groups are true parasites, but the rhabditids may have a spectrum of parasitic to phoretic associations (Sudhaus, 2008).

The *Oscheius rugaoensis* population described in this work had the same 28S rDNA sequence as a nematode population incorrectly identified as an entomopathogenic *Heterorhabditis* sp. in 2003 (AY177182), later published under the name *Heterorhabditis rugaoensis* (Zhang et al., 2012), and recently synonymized as *Oscheius* (Darsouei et al., 2014). *Oscheius necromenus* from Australia and *O. rugaoensis* are closely related (Fig. 3). Since *Oscheius myriophilus* (Poinar, 1986) and *O. necromenus* (Sudhaus and Schulte, 1989) were previously reported from millipedes, *O. rugaoensis* becomes now the third species from a millipede.

The nematode family Diplogastridae is one of the most phenotypically diverse within Rhabditida (Susoy and Herrmann, 2012). It is especially difficult to diagnose genera such as *Mononchoides*, *Koeneria* Meyl,
1960, and Fictor Paramonov, 1952 with superficially similar and highly plastic mouthparts (Huang et al., 2010). These three genera are only distantly related to one another in a recent, comprehensive phylogenetic tree of diplogastrids (Susoy et al., 2015). That tree did not include Mononchoïdes composticola and M. striatus seen here in the Figure 4 three-taxon clade. It demonstrates a lack of monophyly among the few GenBank sequences of Mononchoïdes out of a possible 43 nominal morphospecies (Sudhaus and Fürst von Lieven, 2003). This Mononchoïdes sp. represents an exceptional ecological association with millipedes. Other species in the genus Mononchoïdes were associated with rotting plant material and beetles (Sudhaus and Fürst von Lieven, 2003). The millipede association is probably secondary rather than primary (Sudhaus, 2008) for these Mononchoïdes due to the small numbers of adults that were recovered.

Acknowledgments

The authors thank Shiguang Li of the Nematology Laboratory and Krystalynne Morris from the Thomas laboratory for technical help, and Y. Takaku for providing the scanning electron micrograph from which an area of interest is shown. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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