

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

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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 rugoensis* (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 (Nijima 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 Kłapeć, 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 Rhabditida 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.

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 $\times 40$ – 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 μ l 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 μ l PCR reaction.

The ribosomal LSU D2-D3 expansion segment was amplified with primers D2A 5'-ACAAGTACCGTGAGGGAAAGTTG-3' and D3B 5'TCGGAAGGAA 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 μ l reaction: 17.55 μ l H_2O , 2.5 μ l 10X PCR buffer, 0.5 μ l dNTP mix (10mM each dNTP), 0.75 μ l MgCl_2 , 50mM, 0.75 μ l 18S-G18S4 primer, 10 μ M, 0.75 μ l 18S-18P primer, 10 μ M, 0.2 μ l Taq (Invitrogen platinum, 1 unit), 23 μ l of the above mix + 2 μ l template DNA; cycling conditions were 94°C – 2min, 94°C – 30sec, 50°C – 30sec, 68°C – 2min, repeat 40 times: steps 2 through 4, 68°C – 10min, 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 likelihood 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 μ m, body width = 35 μ m, $V = 56.6\%$, $a = 21.7$, $c = 10.4$, $c' = 5$, stoma length = 20.6 μ m. 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.

Species	Isolate	Locality	Accession
<i>Cephaloboides nidrosiensis</i>	DF5075	The UK	EU195992
<i>Metarhabditis blumi</i>	DF5010	Spain	EU195965
<i>Metarhabditis rainai</i>	DF5091	Fiji	EU195966
<i>Oscheius carolinensis</i>		USA	FJ547239
<i>Oscheius chongmingensis</i>		China	EF503691
<i>Oscheius chongmingensis</i>	Tumian154	China	EU273599
<i>Oscheius insectivorus</i>	SB169	Germany	EU195968
<i>Oscheius myriophilus</i>	DF5020	The USA	AY602176
<i>Oscheius necromenus</i>	SB218	Australia	This paper
<i>Oscheius rugaoensis</i>	YNb59	China	AY177182
<i>Oscheius rugaoensis</i>		Japan	This paper
<i>Rhabditella axei</i>	DF5006	France	AY602177

Table 2. Nematode 18S rDNA sequences for selected taxa in Figure 4.

Species	Isolate	Locality	Accession
<i>Caenorhabditis elegans</i>	N2	The UK	AY268117
<i>Heterorhabditis bacteriophora</i>	1206	–	FJ040430
<i>Fictor stercorarius</i>	RS9003	Germany	KJ877235
<i>Fictor</i> sp. 2	RS9002	The USA	KJ877234
<i>Koerneria luziae</i>	Luc1	Japan	AB597232
<i>LeptoJacobus dorci</i>	EJR2014	Japan	KF924399
<i>Mononchoides</i> cf. <i>americanus</i>	100D10	Japan	This paper
<i>Mononchoides composticola</i>	wb31	Belgium	GU943512
<i>Mononchoides striatus</i>	MonEStr	–	AY593924
<i>Mononchoides</i> sp.	FDL-2015 M63_39	Italy	LN827618
<i>Mononchoides</i> sp 1	VS-2014 RS5441	France	KJ877210
<i>Mononchoides</i> sp 2	VS-2014 RS9007	Mexico	KJ877209
<i>Mononchoides</i> sp 3	VS-2014 RS9008	New Caledonia	KJ877211
<i>Neodiplogaster crenatae</i>	NK126	Japan	AB326310
<i>Paroigolaimella micrura</i>	VS-TU-2014-2	Germany	KJ877207
<i>Sachsia zurstrasseni</i>	VS-TU-2014-3	Germany	KJ877208
<i>Sudhausia aristotokia</i>	RS9011	Ghana	KJ877231
<i>Sudhausia crassa</i>	RS9012	South Africa	KJ877232
<i>Tylopharynx foetidus</i>	wb3	Belgium	EU306343

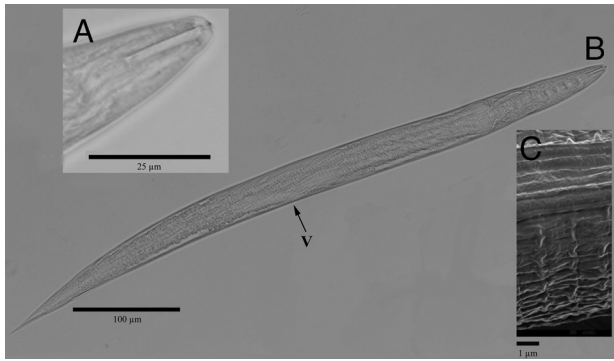


Figure 1: *Oscheius rugaoensis* Female juvenile (A) Stoma, (B) Body, ventral view, with vulva primordium (V), C. SEM lateral field cuticle.

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). The longer sequence of *Oscheius rugaoensis* from Japan (vs that of China) had 5/288 nucleotide differences (98.3% similarity), compared to the closest species, *Oscheius necromenus* SB218. In contrast, two conspecific populations of *Oscheius chongmingensis* (Zhang et al., 2008; Ye

et al., 2010) had only 4 out of 488 nucleotide differences between them (0.8%). The three millipede-associated nematode species resided in a clade with 100% support in a Bayesian likelihood tree (Fig. 3).

Oscheius necromenus Sudhaus and Schulte, 1989

Description

Measurements

N = 10 heat-killed hermaphrodite specimens, body length = $1,661 \pm 220$ (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* OP50 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

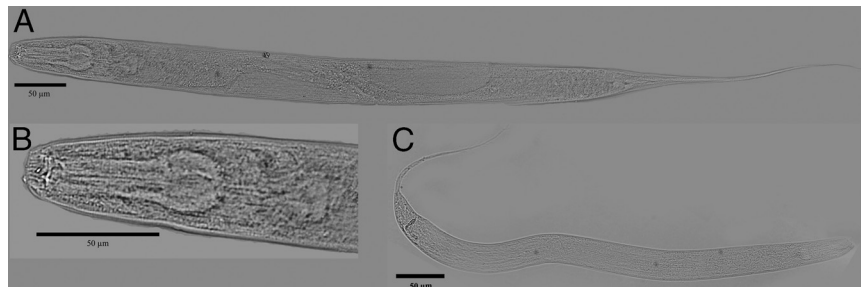


Figure 2: *Mononchoides* sp. cf. *americanus* (A) Female body, ventral view, (B) Female pharynx, (C) Male body, lateral view.

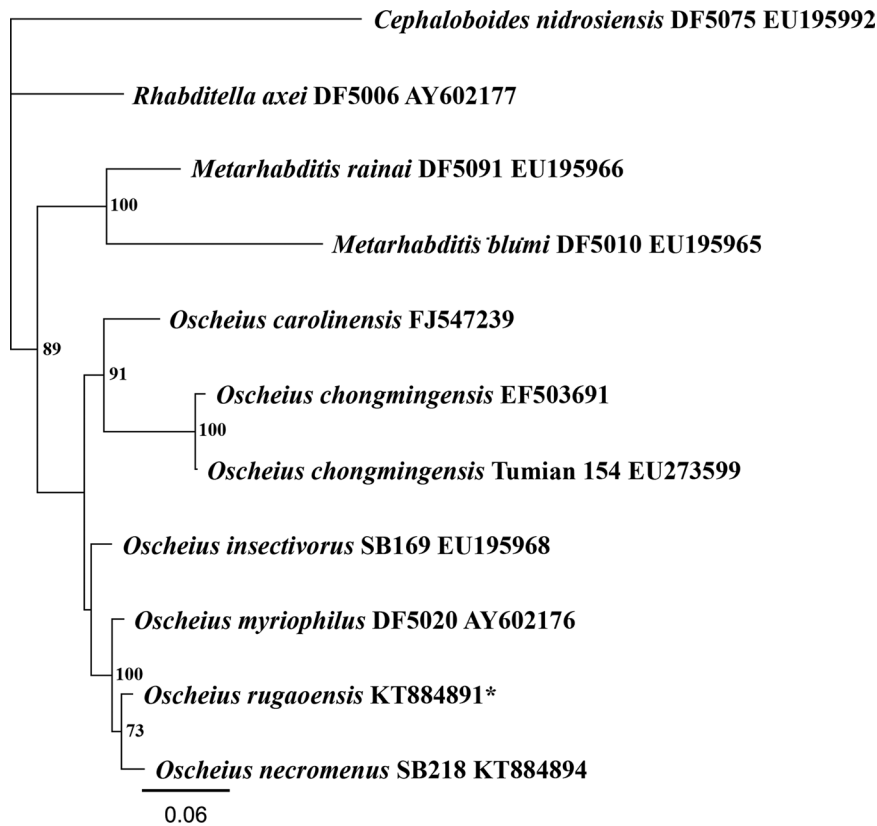


Figure 3: *Oscheius rugaoensis* 28S rDNA MrBayes phylogenetic tree of MAFFT aligned 175 base pair (bp) sequences of *Oscheius* with *Metarhabditis*, *Rhabditella*, *Cephaloboides* outgroups as implemented in Geneious ver. 7.1.7. ModelTest AIC GTR+G+I model with gamma rates, alpha shape parameter = 0.77. Chain Length 1,100,000, Burnin 110,000, Average LnL for two runs = -11529.5. Bayesian likelihood support values indicated above branches.

(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.

Mononchooides 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 *Mononchooides 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, *Mononchooides*

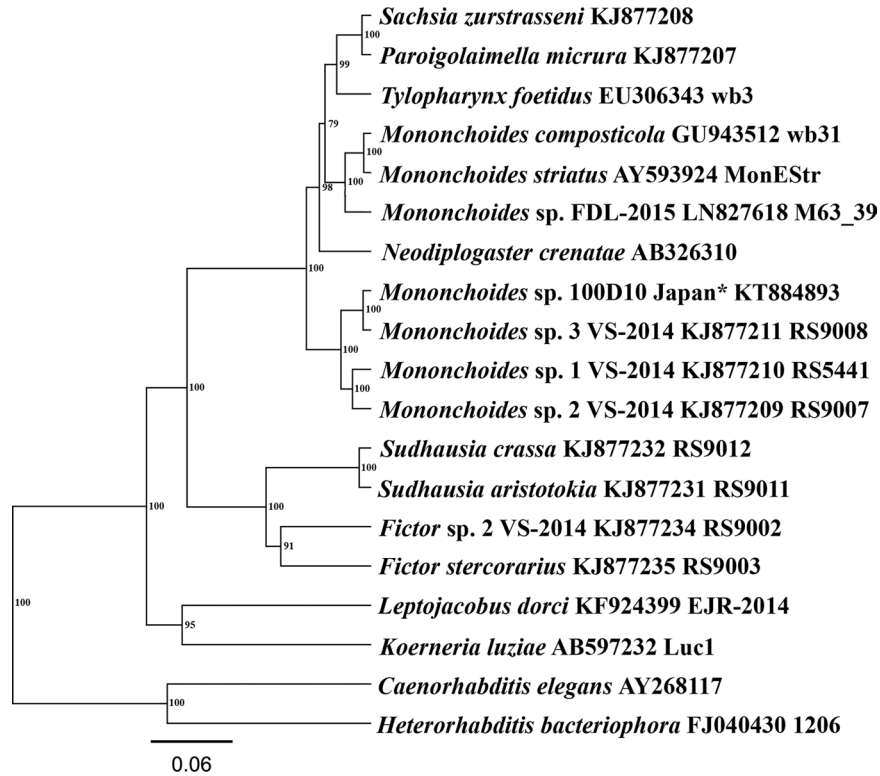


Figure 4: *Mononchoides* and diplogastrid relatives MrBayes phylogenetic tree of 18S rDNA based on a 3,410bp MAFFT alignment as implemented in Geneious ver. 7.1.7. ModelTest AIC GTR+G+I model with gamma rates, alpha shape parameter = 0.60. Chain Length 1,100,000, Burnin 110,000, Average LnL for two runs = -9630.4. Support values indicated above branches for Bayesian likelihood.

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 *Heterorhabditidis 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*, *Koerneria* 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 *Mononchoides 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 *Mononchoides* out of a possible 43 nominal morphospecies (Sudhaus and Fürst von Lieven, 2003). This *Mononchoides* sp. represents an exceptional ecological association with millipedes. Other species in the genus *Mononchoides* 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 *Mononchoides* due to the small numbers of adults that were recovered.

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