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Description of a New Species of *Rhabdias* (Nematoda: Rhabditida: Rhabdiasidae) from Ishigakijima Island, Okinawa, Japan

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A new nematode species, *Rhabdias kiri* sp. nov., which is a lung parasite in a microhylid frog *Microhyla okinavensis* Stejneger, 1901 from Ishigakijima island in the Yaeyama Islands of Ryukyu Archipelago, Japan is described and illustrated. This new species differs from other congeners inhabiting East Asia and the Russian Far East in its features: *i.e.*, shorter body length, slender body shape, narrower and shallower buccal capsule, shorter head diameter and cuticular inflation less prominent in the anterior and middle parts of the body. This is the first record of a frog parasite in *Rhabdias* from the Yaeyama Islands. Partial sequences of mitochondrial cytochrome *c* oxidase subunit I and 12S rDNA genes were provided as DNA barcodes for the new species.

Key Words: *Microhyla okinavensis*, endoparasite, Ryukyu Islands, nematode.

Introduction

The nematode genus *Rhabdias* Stiles and Hassall, 1905 is an endoparasitic group that dwells in the lungs of amphibians and reptiles. The genus consists of *ca.* 85 nominal species that are distributed worldwide (Kuzmin 2013), and eight of these species have been recorded in the Japanese and Ryukyu Archipelagos, Japan (Wilkie 1930; Yamaguti 1935, 1941, 1954; Hasegawa 1984, 1989, 1990; Hasegawa and Iwatsuki 1993; Goldberg *et al.* 1997; Goldberg and Bursey 2002; Kuzmin 2003, 2013; Hasegawa and Asakawa 2004; Sata *et al.* 2020).

A variety of frogs and toads with highly specific genetic diversity can be found in the Ryukyu Archipelago, where most species are endemic to the area (Ota 1998; Nishikawa 2017); thus, these putative host species can also be expected to harbor diverse and unique parasites. In contrast to the great diversity of frogs and toads, little is known about the species diversity of *Rhabdias* parasites in the frogs and toads distributed in this archipelago, especially in the Yaeyama Islands, which is located at the southern part of the Ryukyu Archipelago. Accordingly, taxonomic studies of *Rhabdias* in the southern Ryukyu Archipelago are essential for improving our understanding of the true species diversity of *Rhabdias* in the Ryukyu Archipelago.

In the present study, *Rhabdias* specimens were obtained from *Microhyla okinavensis* Stejneger, 1901 endemic to the Ryukyu Archipelago collected from Ishigakijima island in the Yaeyama Islands and inspected. The nematodes are described as a new species. Additionally, mitochondrial DNA sequences from the new species are provided for DNA barcoding and future phylogenetic analyses.

Materials and Methods

Sampling and morphological examination. The *Rhabdias* specimens examined in this study were obtained from the narrow-mouthed frogs *M. okinavensis*, which were captured by the second author from Tozato (24°28′25″N, 124°14′45″E), and Ishigaki (24°22′24″N, 124°08′59″E) on Ishigakijima island in the Yaeyama Islands, Okinawa Prefecture, Japan. All captured frogs were euthanized by dipping in 10% ethyl alcohol. The body cavity of each anuran was dissected by a longitudinal incision and the lungs were removed. The excised organs were dissected longitudinally and the lumens were investigated by the first author. The obtained nematode individuals were fixed with a hot 5% solution of glycerin in 70% ethyl alcohol, and then preserved in 70% ethyl alcohol. To increase transparency of the nematode specimens, they were placed in 5% solution of glycerin in 30% ethyl alcohol and then incubated 2 days at 60°C to gradually evaporate the ethyl alcohol. The cleared specimens were observed with a light microscope (Nikon Eclipse Ni-U; Nikon, Tokyo, Japan).

The measurements are given for the holotype, followed by the mean±standard deviation of the mean and range of

paratypes in parentheses, except for the location of phasmids and eggs. The distances of phasmids from the tail ends are given as mean values, followed by ranges, and the measurements of eggs are given as mean±standard deviation of the mean, followed by range. All measurements are given in micrometers (μm) unless otherwise stated.

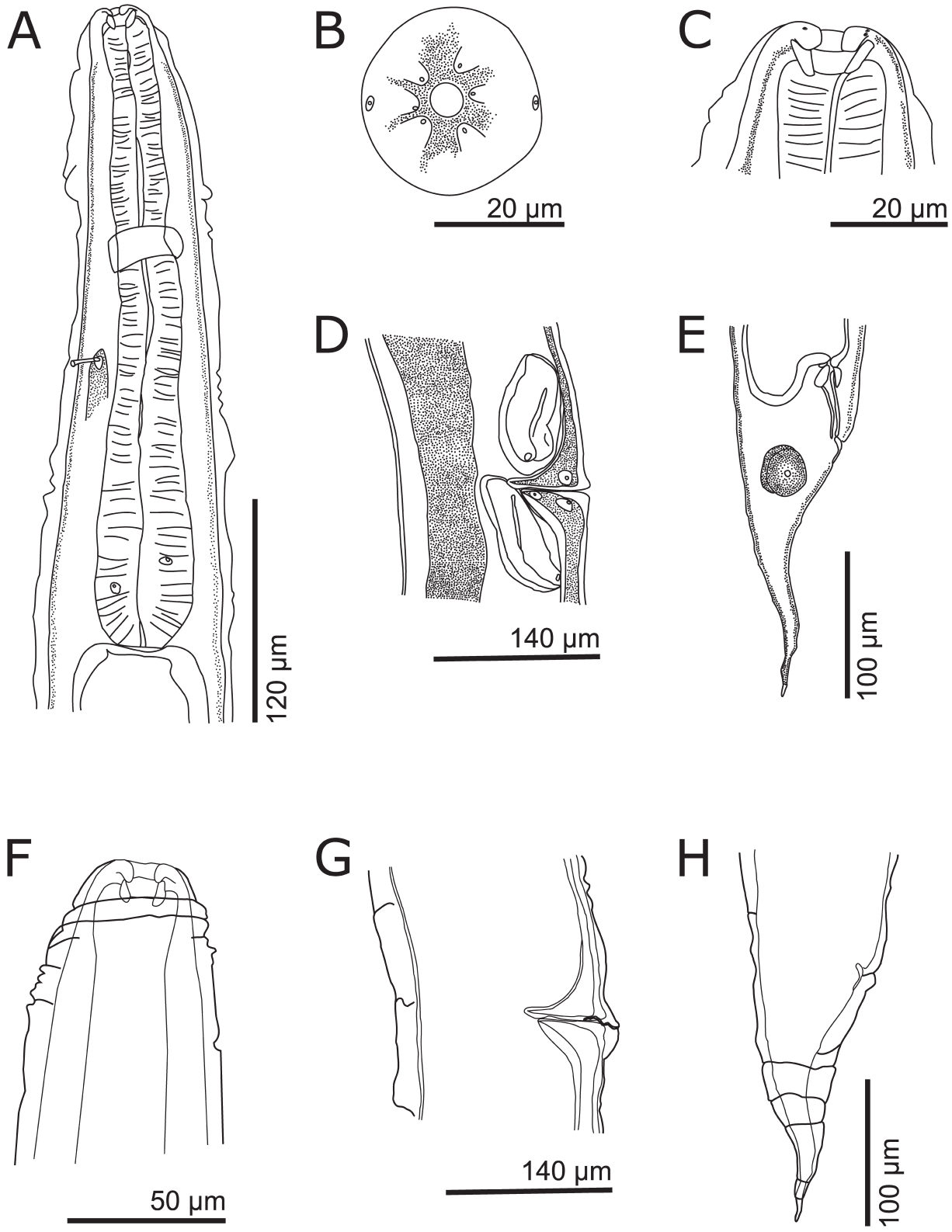

Fig. 1. *Rhabdias kiri* sp. nov., holotype (KUZ Z2959: A, C–E, G, H), paratypes (KUZ Z2968: B; KUZ Z2961: F). A, anterior region, lateral view; B, cephalic region, apical view; C, cephalic region, lateral view; D, vulvar region, lateral view; E, caudal region, lateral view; F, cuticular inflation in anterior region, lateral view; G, cuticular inflation in middle region, lateral view; H, cuticular inflation in caudal region, lateral view.

The nematode and frog specimens examined in this study have been deposited in the Zoological Collection of Kyoto University (KUZ) and at the Graduate School of Human and Environmental Studies, Kyoto University (KUHE), respectively.

PCR and DNA sequencing. Genomic DNA of nematode specimens was extracted from whole body of each individual using the DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany). The partial sequence of cytochrome *c* oxidase subunits I (COI) and 12S ribosomal DNA (12S) were amplified by polymerase chain reaction (PCR) using a TaKaRa Ex Taq kit (Takara Bio, Kusatsu, Japan) and a T100 Thermal Cycler instrument (Bio-Rad Laboratories, Hercules, CA, USA). The primer set used for PCR of COI was LCO1490 and HCO2198 (Folmer *et al.* 1994), and that of 12S was 12SF and 12SR (Casiraghi *et al.* 2004). PCR conditions were as follows: 95°C for 1 min, followed by 35 cycles of 30 s at 95°C, 40 s at 42°C, and 1 min at 72°C and then a final extension at 72°C for 5 min for COI; 94°C for 3 min, followed by 35 cycles of 45 s at 94°C, 45 s at 48°C, and 1 min at 72°C and then a final extension at 72°C for 5 min for 12S. The PCR products were purified with ExoSAP-IT reagent (Thermo Fisher Scientific, Waltham, MA, USA), then sequenced directly using the primer sets that used for the PCR reaction (DNA sequencing service was provided by Eurofins Genomics K. K., Tokyo, Japan). In total, four DNA sequences from the present *Rhabdias* specimens were determined, and deposited with the International Nucleotide Sequence Database Collaboration (INSDC) through the DNA Data Bank of Japan.

Genus ***Rhabdias*** Stiles and Hassall, 1905

Rhabdias kiri sp. nov.

(Fig. 1)

Diagnosis. The East-Asian *Rhabdias*, with short and slender body. Buccal capsule small, 8.5 (8.6±1.2, 7.3–11.9) wide and 6.9 (6.6±1.1, 5.3–8.8) deep. Cuticular inflation less prominent in anterior and middle part of body.

Material examined. *Holotype*: KUZ Z2959, whole specimen, adult female, obtained from the lung of *M. okinavensis* (KUHE 61751), collected from Tozato (24°28′25″N, 124°14′45″E), Ishigakijima island, Okinawa Prefecture, Japan on 7 August 2019. *Paratypes*: KUZ Z2960–Z2967, eight whole specimens obtained from the lung of the holotype's host and other *M. okinavensis* specimens (KUHE 61750, 61751), data as for holotype; KUZ Z2968, one section of anterior end of a female and remaining body; KUZ Z2968 was obtained from the lung of the holotype's host specimen.

Additional materials. KUZ Z2969, three whole-body specimens, and two non-deposited specimens for the present molecular studies, obtained from the same specimen of the holotype's host (KUHE 61751); one non-deposited specimen for the present morphological and molecular studies obtained from KUHE 61750; and one non-deposited specimen for the present molecular studies, obtained from a *M. okinavensis* specimen (KUHE 61752), collected from Ishigaki (24°22′24″N, 124°08′59″E), Ishigakijima island, on 9 August 2019.

Type locality. Japan, Okinawa: Ishigakijima island, Tozato (24°28′25″N, 124°14′45″E).

Type host. *Microhyla okinavensis* Stejneger, 1901 (Amphibia: Microhylidae); site of infection: lung. A total of 17 individuals were obtained from three specimens of *M. okinavensis*; mean intensity, followed by ranges: 5.7, 1–14.

Description. *General*. Body short and slender with tapered extremities, body becoming wider at region of vulva or slightly behind vulva. Cephalic end with six lips, each lip with one minute papilla. Amphids situated on posterior surface of lateral lips. Oral opening almost round. Cuticular inflation slightly inflated in anterior and middle part of body, slightly more prominent in caudal region. Anterior end of cuticular inflation possessing two prominent annulations.

Body length 4.54 mm (5.18±0.99 mm, 4.14–7.07 mm), and maximum width 142 (146±14.6, 124–165). Body length/maximum body width=32.0 (29.2–43.7). Body width at junction of esophagus and intestine 97 (96±11.4, 79–121), at vagina 142 (145±15.8, 117–165), and at anus 68 (76±8.8, 65–90). Head diameter, 26 (25±1.5, 24–29) (n=9). Vestibulum funnel-shaped, 6.3 (7.1±1.2, 5.5–9.5) long. Buccal capsule small, possessing thick wall, cup-like in lateral view, nearly half or almost completely surrounded by anterior end of esophagus, 6.9 (6.6±1.1, 5.3–8.8) deep, 8.5 (8.6±1.2, 7.3–11.9) wide. Esophagus possessing a slight dilation at anterior region of nerve ring. Esophagus 352 (348±37.5, 302–430) long [7.8% (5.6–8.9) of body length] with width of 21 (24±3.0, 20–30) at anterior end, 28 (31±2.8, 28–36) at dilated muscular part, 29 (30±3.4, 23–34) at nerve ring, 54 (57±2.6, 52–60) at bulbar part. Nerve ring and excretory pore 118 (123±10.8, 111–150) [2.6% (1.6–3.1%) of body length] and 191 (193±12.6, 167–207) [4.2% (2.7–4.9%) of body length], respectively, from cephalic end, and 112 (114±10.6, 100–137) [31.8% (26.3–36.7%) of esophagus length] and 187 (186±12.3, 160–199) [53.1% (45.8–63.6%) of esophagus length], respectively, from anterior end of esophagus. Intestine thick walled. Anterior end of intestine wider than bulbar part of esophagus. Rectum, short, thin walled. Vulva 2.80 mm (2.89±0.47 mm, 2.10–3.62 mm) from cephalic end [61.7% (49.9–61.7%) of body length], having slightly salient lips. Genital system amphidelphic, anterior limb turned posteriorly at 0.74 mm (1.15±0.26 mm, 0.74–1.60 mm) from cephalic end, and posterior limb turned anteriorly at 0.54 mm (0.77±0.29 mm, 0.52–1.40 mm) from tail end. Uteri long, tubular, filled with eggs, less than 100 (n=9). Tail long conical, sharply pointed, abruptly tapering from anus posteriorly, gradually tapering from mid-region to tip, 197 (208±32.4, 157–265) long [4.3% (3.1–4.9%) of body length]. Lateral pores observed in most specimens. Phasmids lateroventral, 145 (85–228) from tail end (n=6). Eggs elliptical, 80±5.68 (68–91) by 39±4.47 (30–50) (n=52), thin shelled, most eggs containing first stage larva.

Etymology. The specific name *kiri* is from a Japanese word kiri (=drill), which refers to the long and pointed tail of the new species, and thus treated as indeclinable.

DNA sequences. In total, 4 sequences were determined: one specimen obtained from the same host frog (KUHE 61751) of the holotype, 2 sequences—COI (LC547915; 655 bp), and 12S (LC547917; 475 bp); one specimen obtained from a *M. okinavensis* specimen (KUHE 61752) from Ishigaki, Ishigakijima island, 2 sequences—COI (LC547916; 655 bp), and 12S (LC547918; 475 bp). Intraspecific variations were not observed in the 12S sequences; single nucleotide variation was detected in the COI sequences.

Remarks. *Rhabdias kiri* sp. nov. can be discriminated from the other 11 congeners recorded from East Asia and the Russian Far East as follows. The new species, of which body length ranges from 4.14 to 7.07 mm, can be distinguished from the following five *Rhabdias* species: *R. bicornis* Lu, 1934 (13.056–15.428 mm; Lu 1934), *R. incerta* Wilkie, 1930 (14.3 mm; Wilkie 1930), *R. kafunata* Sata, Takeuchi, and Nakano, 2020 (10.99–16.77 mm; Sata *et al.* 2020), *R. rhacophori* Yamaguti, 1941 (10.55 mm; Yamaguti 1941) and *R. tokyoensis* Wilkie, 1930 (12 mm; Wilkie 1930). *Rhabdias kiri* sp. nov. can be differentiated from *R. bermani* Rausch, Rausch, and Atrashkevich, 1984 and *R. polypedatis* Yamaguti, 1941 by body shape because the latter two species possess much larger body widths relative to their body lengths than that of the new species [*R. bermani*: 6.06–10.68 mm long by 294–458 wide (Rausch *et al.* 1984); and *R. polypedatis*: 4.2–5.2 mm long by 200–300 wide (Yamaguti 1941)]. The new species is also distinguishable from *R. montana* Yamaguti, 1954 and *R. nipponica* Yamaguti, 1935 by the smaller width and depth of the buccal capsule, respectively [*R. montana*: 15–16 wide (Yamaguti 1954); and *R. nipponica*: 9–12 deep (Yamaguti 1935)]. Additionally, *R. kiri* sp. nov. differs from *R. nipponica* because the new species possesses a much smaller head diameter (see Yamaguti 1954), and differs from *R. globocephala* Kung and Wu, 1945 and *R. japalurae* Kuzmin, 2003 in the absence of a cuticular inflation at the anterior and middle parts of the body (Kung and Wu 1945; Kuzmin 2003, 2005).

The discovery of *Rhabdias kiri* sp. nov. from Ishigakijima island is the first record of the frog-parasitizing *Rhabdias* species from the Yaeyama Islands. A recent phylogeographic study of *M. okinavensis*, the only known host of *Rhabdias kiri* sp. nov., suggested that the Yaeyama Islands' population of this frog is phylogenetically closer to a population of the continental species, *M. mixture* Liu and Hu, 1966, than to the population of *M. okinavensis* from the central Ryukyus (Tominaga *et al.* 2019). Thus, comprehensive taxonomic and phylogenetic studies of *Rhabdias* nematodes parasitizing *Microhyla* frogs in the East Asia would provide a new insight into the evolutionary history of parasites in the amphibians and reptiles of this area.

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