First Record of *Poecilobdella nanjingensis* (Hirudinida: Arhynchobdellida: Hirudinidae) from Taiwan and its Molecular Phylogenetic Position within the Family

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(Received 22 April 2016; Accepted 3 October 2016)

The terrestrial hirudinid leech *Poecilobdella nanjingensis* Yang, 1996 is reported for the first time from Taiwan, having previously been found only in continental China. A morphological description of the new material from Taiwan is provided here, confirming that, contrary to the diagnosis of *Poecilobdella* Blanchard, 1893, *P. nanjingensis* lacks a vaginal caecum in its female reproductive system. In addition, molecular phylogenetic analyses reveal that *P. nanjingensis* is part of a clade consisting of the hirudinid genera defined by the lack of a vaginal caecum. These results highlight the need for further taxonomic studies on the generic position of this species.

**Key Words:** *Poecilobdella nanjingensis*, cattle leech, new record, phylogenetic analysis, Taiwan.

**Introduction**

The hirudinid cattle leech genus *Poecilobdella* Blanchard, 1893 and its ally *Hirudinaria* Whitman, 1886 contain large, blood-sucking leeches that are distributed mainly in Southeast and East Asia (Sawyer 1986). Both genera are distinguished by the presence of a caecum in the female vagina. *Poecilobdella* species possess a developed vaginal stalk in the female reproductive system, while members of *Hirudinaria* lack a vaginal stalk (Sawyer 1986).

The type species of *Poecilobdella*, *P. granulosa* (Savigny, 1822), the type locality of which is Pondicherry ([sic]; Pondicherry = currently Puducherry, India), was revisited by Moore (1927a) based on specimens collected from various places in India. Although three species, *P. guangdongensis* Tan, 2006, *P. hubeiensis* Yang, 1980, and *P. nanjingensis* Yang, 1996, are known in East Asia, all three have been found only in continental China. Of the *Poecilobdella* species inhabiting East Asia, only *P. granulosa* is distributed in Taiwan as well (Lai and Chen 2010).

Although the Taiwanese leech fauna has been well documented (Lai and Chen 2010), we collected several specimens of a mountain-dwelling hirudinid species newly found in Taiwan, which we have identified as *P. nanjingensis*. In addition to their morphological description, the phylogenetic position of these specimens within the family Hirudinidae was estimated using nuclear 18S and 28S rRNA as well as mitochondrial cytochrome c oxidase subunit I and 12S rRNA sequence data.

**Materials and methods**

Leeches were collected from two localities in Taiwan. When possible, their elevation and geographical coordinates were obtained using a Garmin eTrex® GPS unit. Almost all of the specimens were relaxed by the gradual addition of absolute ethanol (EtOH) to freshwater. For DNA extraction, botryoidal tissue was removed from the posterior part of the body around the caudal sucker of every specimen, and then preserved in absolute EtOH. The remainder of the body was fixed in 10% formalin and preserved in 70% EtOH. Four measurements were taken: body length from the anterior margin of the oral sucker to the posterior margin of the caudal sucker (BL), maximum body width (BW), caudal sucker length from the anterior margin to the posterior margin of the sucker (CL), and caudal sucker width from the right to the left margin of the sucker (CW). Examination, dissection, and drawing of the specimens were conducted using a stereoscopic microscope with a drawing tube (Leica M125): examination of their jaw morphology was done using a light microscope (Olympus BX60). Specimens used in this study have been deposited in the Zoological Collection of Kyoto University (KUZ). The numbering convention of leech morphology is based on Moore (1927b): body somites are denoted by Roman numerals, and the annuli in each somite are given alphanumeric designations.
The extraction of genomic DNA from botryoidal tissues preserved in absolute EtOH followed Nakano (2012a). Primer sets for the PCR and cycle sequencing (CS) reactions used in this study were as follows: for 18S rRNA, A and L (PCR and CS), C and Y (PCR and CS), and O and B (PCR and CS) (Apakupakul et al. 1999); four primer sets were used for ca. 2,500 bp sequence of 28S rRNA, 1) LR0R (PCR and CS) (Cuberta et al. 1991), LR3 (CS) (Vilgalys and Hester 1990) and 28nn (PCR and CS) (Passamanek et al. 2004), 2) 28F1-2 (Passamanek et al. 2004) and 28ff (Hillis and Dixon 1991) (PCR and CS), 3) 28F2-2 and 28R2 (PCR and CS) (Passamanek et al. 2004), and 4) 28F2_3 (Jördens et al. 2004) and 28R3 (Passamanek et al. 2004) (PCR and CS); for cytochrome c oxidase subunit I (COI), LCO1490 and HCO2198 (PCR and CS) (Folmer et al. 1994); four primer sets used for 12S ribosomal RNA and tRNAVal (12S), 12SAI and 12SB-out (PCR and CS) (Borda and Siddall 2004), and 12SA-BI (PCR and CS) (Nakano 2012a). The PCR reaction and DNA sequencing were performed using the ABI PRISM® 377 DNA Sequencer equipped with the GeneAmp PCR System 2700 and a GeneAmp PCR System 9700 (Applied Biosystems, Waltham, USA) as well as the DNA Data Bank of Japan (DDBJ) (Table 1).

For use in molecular phylogenetic analyses, 72 published sequences were obtained from INSDC (Table 1). Along with two xerobdellid species as outgroup taxa, 18 hirudinid species were included in the analyses (Table 1).

The phylogenetic position of the Taiwanese P. nanjingensis within Hirudinidae was estimated based on the sequences obtained for the 18S, 28S, COI, and 12S genes. The alignment of COI was trivial, as no indels were observed. The 18S, 28S, and 12S sequences were aligned using MAFFT v. 7.266 L-INS-i (Katoh and Standley 2013). The lengths of the 18S, 28S, COI, and 12S sequences were 1858, 2067, 1267, and 715 bp, respectively. Prior to constructing a phylogenetic tree based on the concatenated sequences, maximum likelihood (ML) trees were constructed based on each of the 18S, 28S, COI, and 12S markers using RAxML v. 8.1.5 (Stamatakis 2014) with the substitution model set as GTRCAT, immediately after nonparametric bootstrapping (Felsenstein 1985) conducted with 1000 replicates. Based on the four phylogenies we ob-

Table 1. Samples with voucher numbers and INSDC accession numbers of sequences for four genes used for molecular analyses. Sequences obtained in this study were deposited with the International Nucleotide Sequence Database Collaboration (INSDC) through the DNA Data Bank of Japan (DDBJ) (Table 1).

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<th>Taxon</th>
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Acronyms: AMNH, American Museum of Natural History; KUZ, Zoological Collection of Kyoto University.
Poecilobdella nanjingensis from Taiwan

2014: 128, fig. 4-5.

**Material examined.** Two specimens collected from Neidong Forest Trail, Wulai District, New Taipei City, Taiwan, by Takafumi Nakano on 3 February 2013: KUZ Z1742 (elev. 565 m, 24.830°N, 121.5320°E) and KUZ Z1743 (elev. 569 m, 24.8297°N, 121.5324°E), dissected. Two specimens collected from Rueiyan River Major Wildlife Habitat, Renai Township, Nantou County, Taiwan: KUZ Z1744 (elev. 2238 m, 24.1151°N, 121.1969°E) collected by Yu-Chang Yang on 19 January 2009, and KUZ Z1745 (elev. 2200 m, 24.1056°N, 121.1836°E), dissected, collected by Huei-Ping Shen on 2 July 2009.

**Description.** Body firm, muscular, with constant width posteriorly, BL 12.9–49.4 mm, BW 2.8–8.7 mm (Fig. 1). Caudal sucker ventral, elliptical, CL 2.1–5.5 mm, CW 2.0–5.9 mm; ventral surface with several radial rays (Figs 1B, 2D).

X b5 and XIII a2, respectively, being first and last annuli of clitellum.

Male gonopore in XI b5/b6 (Fig. 2E). Female gonopore in XII b5/b6 (Fig. 2E). Gonopores separated by 5 annuli.

Eyes in 5 pairs, in parabolic arc; 1st pair on II, 2nd pair on
III, 3rd pair on IV (a1+a2), 4th pair on V (a1+a2), and 5th pair on VI a2. Sensillae developed, 1 row on every annulus. Somitic papillae undeveloped.

Nephridiopores in 17 pairs, one each situated ventrally at posterior margin of a1 of VIII, and at posterior margin of b2 of each somite in IX–XXIV (Fig. 2B, D, E).

One median longitudinal furrow on ventral surface of oral sucker (Fig. 2B). Three jaws in oral cavity, 1 dorsal and 2 ventrolateral; each jaw with uncountable barely detectable salivary papillae; monostichodont, median jaw bearing 85 diminutive teeth (KUZ Z1743 only), right jaw bearing 82 (KUZ Z1745) or 88 (KUZ Z1743) teeth, and left jaw bearing 80 (KUZ Z1745) or 81 (KUZ Z1743) teeth. Pharynx reaching to IX a2/b5. Crop reaching to XIX a2/b5, giving rise to 10 pairs of crop caeca: 1st pair in XI b1 and b2; 2nd pair in XI a2–b6; 3rd pair in XII a2–b6; 4th pair in XIII b2–b5 or a2–b6; 5th pair in XIV a2–b6 or XIV b2–XV b1; 6th pair in XV a2–b6 or XV b2–XVI b1; 7th pair in XVI a2–b6 or XVI b2–XVII b1; 8th pair in XVII b2–b6 or XVII b2–XVIII b2; 9th pair in XVIII a2–b6 or XVIII b2–XIX b1; and 10th pair (post-crop caeca) in XIX b2–XXIV a3. Intestine reaching to XXIII b1. Rectum simple tubular.

Testisacs in 9 pairs (Fig. 3A): 1st pair in XIII b5–XIV b1; 2nd pair in XIV b5–XV b1; 3rd pair in XV b5–XVI b1, or right sac in XV b5 and b6 (KUZ Z1743); 4th pair in XVI b5–XVII b1; 5th pair in XVII a2–b6 or XVII b5–XVIII b1; 6th pair in XVIII b5–XIX b1, or left sac in XVIII a2–b6 (KUZ Z1743); 7th pair in XIX b5–XX b1, or left sac in XIX a2–b2 (KUZ Z1743); 8th pair in XX b5–XXI b1 (KUZ Z1745), or right sac in XX b5 and b6, left sac in XX a2–b6 (KUZ Z1743); 9th pair in XXI a2–b6. Paired epididymides developed, globular (Fig. 3A–C); right epididymis in XII b1 and b2 (KUZ Z1745) or XII b1–b5 (KUZ Z1743) and left epididymis in XII b1–a2 (KUZ Z1745) or XI b6–XII a2 (KUZ Z1743); from each epididymal mass, thick sperm duct reaching to right or left ejaculatory bulb, respectively. Ejaculatory bulbs developed, ovoidal (Fig. 3A–C); right bulb in XI b5 and b6 (KUZ Z1743) or XI b6–XII b2 (KUZ Z1745) and left bulb in XI b5 and b6 (KUZ Z1743) or XI b5–XII b1 (KUZ Z1745). Ejaculatory ducts narrow, running inward toward male atrium in somite XI b5–b6 (Fig. 3B, C); right duct crossing ventrally beneath nerve cord. Male atrium covered with prostate gland and continuous with penis sheath (Fig. 3A–C). Penis sheath hook-like, reaching to XII b2–XIII b1, then turning anteriorly to male gonopore (Fig. 3B, C).

Paired ovisacs globular, in XII b6 and XIII b1 (Fig. 3A, D, E). Oviducts short (Fig. 3D, E); right oviduct crossing ventrally beneath nerve cord; both oviducts converging into common oviduct in XII b6–XII/XIII. Common oviduct thick, short, descending to female vagina in XII b6–XIII b1 (Fig. 3D, E). Female vagina covered with prostate gland,
Fig. 3. Poecilobdella nanjingensis Yang, 1996, from Neidong, Wulai, KUZ Z1743. A, dorsal view of reproductive system including ventral nervous system; B, dorsal view of male median reproductive system with outline of prostate gland including positions of ganglia XI and XII; C, left lateral view of schematic drawing of male median reproductive system; D, dorsal view of female reproductive system with outline of prostate gland including position of ganglion XIII; E, lateral view of schematic drawing of female reproductive system. Scale bars: 2 mm (A); 0.5 mm (B, D). Abbreviations: at, atrium; eb, ejaculatory bulb; ep, epididymis; od, oviduct; ov, ovisac; pes, penis sheath; pg, prostate gland; ts, testisac; vd, vaginal duct; vg, vagina.
continuous to vaginal duct (Fig. 3A, D, E); vaginal duct hook-like, slightly folded, reaching to XIII a2–XIII/XIV, then turning anteriorly to female gonopore (Fig. 3D, E); vaginal caecum absent.

**Colouration.** In life, dorsal surface greenish yellow with 7 discontinuous black longitudinal stripes, each stripe present in b2–b5 and absent in b1 and b6 of each mid-body somite (Figs 1A, 4); median stripe darker than lateral stripes, with weaker striping in cephalic region (somites I–X) and darker striping from somites XI to XXV; 4 lateral inner stripes, lighter than 2 lateral outer stripes; irregular black markings present in b1 and b6 of each mid-body so-

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**Fig. 4.** *Poecilobdella nanjingensis* Yang, 1996, from Neidong, Wulai, KUZ Z1743. A, dorsal view of live animal; B, live animal found under a stone at the locality. Scale bar: 5 mm (B).

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**Fig. 5.** Map showing the localities where *Poecilobdella nanjingensis* Yang, 1996 has been recorded. Closed circles indicate localities reported herein, open circles those from previous studies: *Yang* (1996) and *Yang* (2014).

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**Fig. 6.** Bayesian inference tree for 5519 bp alignment positions of nuclear 18S rRNA, 28S rRNA, mitochondrial cytochrome c oxidase subunit I, and 12S rRNA markers. Numbers on nodes indicate bootstrap values for maximum likelihood and Bayesian inference posterior probabilities.
mite; in cephalic region, irregular markings and lateral inner stripes forming chainlike pattern; ventral surface paler than dorsal surface. Colour faded in preservative, but longitudinal stripes and markings still present on dorsal surface (Fig. 1A).

Distribution. In addition to the Taiwanese localities, this species has been found at two locations in continental China (Fig. 5): the Nanjing University campus, Nanjing, Jiangsu (type locality) (Yang 1996) and Tianmu Mountain, Zhejiang (Yang 2014).

Natural history. The Neidong individuals were found curled up under rocks in moist mountainous habitats (Fig. 4B). Thus, they are indeed terrestrial as was mentioned by Yang (2014). A mature leech, KUZ Z1745, with an obvious clitellum was collected on 2 July; therefore, P. nanjingensis is thought to enter its reproductive season before early July in Taiwan.

Phylogenetic position. The obtained BI (Fig. 6) tree had a topology identical to that of the ML tree (in L = −17190.50; not shown). The Taiwanese P. nanjingensis belong to a well supported clade (BS = 98%, PP = 0.99) that consists of Goddardobdella Richardson, 1969, Hirudinaria, Aliolimnatis Richardson, 1972 species, and Asiaticobdella fenestra (Moore, 1939). In the ML analyses, the monophyly of the Taiwanese P. nanjingensis and Aliolimnatis + As. fenestra clade was supported (BS = 89%); however, this relationship was not recovered in the BI analysis (PP = 0.85). The two Taiwanese individuals formed a fully supported clade (BS = 100%, PP = 1.0).

Remarks. Chinese P. nanjingensis possess over 100 teeth on each jaw with salivary papillae arranged in four longitudinal stripes (Yang 1996, 2014). The present specimens possess jaws without developed salivary papillae, and they have a lower number of teeth (~80) on each jaw compared to Chinese individuals. The Taiwanese specimens were identified as P. nanjingensis based on the presence of the following characteristics: dorsal surface with seven dark, discontinuous stripes; somital annulation excluding unianulate somite XXVII (biannulate in Chinese leeches); male gonopore in XI b5/b6 and female gonopore in XII b5/b6; crop caeca in 10 pairs, 10th pair being post-crop; globose epididymides in posterior part of XI to anterior part of XII; atrium continuous with penis sheath; penis sheath hook-like, reaching to body area between posterior part of XII and anterior part of XIII, and then turning to male gonopore; vaginal duct hook-like, reaching to XIII b2, then turning to female gonopore.

Yang (1996) stated that this species has a vaginal caecum. However, according to the original description and figure of P. nanjingensis, its common oviduct clearly descends to the terminus of its female vagina. Therefore, the vagina does not consist of a vaginal ‘caecum,’ but forms a simple ‘duct’-like structure. The Taiwanese P. nanjingensis also has a vaginal duct only. This morphological characteristic sheds light on its generic position, because Poecilobdella has been differentiated by a developed vaginal caecum in the female median structure. It is thus highly possible that P. nanjingensis should be placed under another hirudinid genus. To determine the precise generic position of P. nanjingensis, further taxonomic studies on the nominal genera of Hirudinidae should be conducted.

The molecular phylogenies showed that P. nanjingensis forms a monophyletic lineage with a clade of Aliolimnatis/Asiaticobdella, which are diagnosed by an acaecate female vagina (Richardson 1969, 1972). In contrast, Goddardobdella and Hirudinaria, both of which have a vaginal caecum (Richardson 1969; Sawyer 1986), forms a distinctive lineage. However, the present phylogenetic analyses did not cover all of the known hirudinid genera. A future phylogenetic analysis based on a larger number of the known hirudinid taxa will lead to a better understanding of whether the characteristics of the vagina reflect hirudinid phylogenetic relationships.

Acknowledgments

The authors are grateful to Mr Yu-Chang Yang and Dr Huei-Ping Shen (Endemic Species Research Institute, Taiwan) for providing valuable specimens of P. nanjingensis. TN expresses his sincere thanks to Professor Tong Yang (Institute of Hydrobiology, Chinese Academy of Sciences) for providing valuable information on P. nanjingensis. Part of this study was financially supported by JSPS KAKENHI Grant Numbers JP13J00450, JP15J00720, and JP26840127 to TN.

References


