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Helobdella viridoclypus (Hirudinea: Rhynchobdellida: Glossiphoniidae), a new free-living leech species from Chiang Mai, Thailand

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Abstract. A new species of glossiphoniid leech, *Helobdella viridoclypus*, is described from the northern region of Thailand, representing the first record of the genus *Helobdella* from the country and Southeast Asia. Its characteristics are similar to those of the type species, *H. stagnalis*, but can be distinguished by its transparent rice-shaped and triannulated body, cephalisation, a single pair of dark eyes, a smooth dorsal surface without papillae, rich dark-green pigmentation on the dorsum, six pairs of crop caeca with less developed first pair and unbranched second to sixth pairs (posterior caeca), male and female gonopore separated by a single annulus, and a triangular nuchal scute pointed backwards with yellowish-green colouring around the mid-dorsal region. The phylogenetic relationships of *H. viridoclypus*, new species, are clarified, showing that it is monophyletic within the *stagnalis* species-complex clade which includes *H. adiastola*, *H. octatestisaca*, *H. modesta*, and the type species *H. stagnalis*. The new species is a free-living leech that specifically preys on invertebrates and not on humans.

Key words. Annelida, Glossiphoniidae, Haementeriinae, jawless leech, freshwater, Huai Tueng Thao Reservoir

INTRODUCTION

Helobdella Blanchard, 1896 (Hirudinea: Glossiphoniidae) is a genus of leeches with a proboscis, characterised by a small, dorsoventrally flattened body, a pair of well-separated cephalic eye spots, and most notably, a nuchal scute on the neck region—a feature unique to this genus (Sawyer, 1986; Moser et al., 2009). This benthic predator is commonly found in organically enriched waters where it feeds on small aquatic invertebrates such as crustaceans, water snails, and oligochaetes (Sawyer, 1986; Kutschera, 2011). *Helobdella* is a diverse genus, comprising species that are widely distributed throughout the Americas, with 49 nominal species inhabiting South America and eight species identified in the United States (Siddall & Borda, 2003; Siddall & Budinoff, 2005; Christoffersen, 2009; Salas-Montiel et al., 2014; Beresic-Perrins et al., 2017). The genus has also been recorded from South Africa, Australia, New Zealand, and Taiwan, all of which also host *H. europaea* Kutschera, 1987, along with two additional species in Taiwan, *H. melananus* Lai & Chang, 2009 and *H. octatestisaca* Lai & Chang, 2009 (Kutschera, 1987; Lai et al., 2009; Oceguera-Figueroa et al., 2010). Although numerous leeches resembling *Helobdella* have been reported in various regions of Thailand through news outlets, especially aquatic areas with high tourist traffic, none have been identified and formally described. While they do not feed on blood, they are known to attach to tourists' feet and are frequently misidentified as buffalo leech larvae, establishing the need to identify and describe the species to prevent further confusion.

The use of morphological characters and pigmentation patterns alone is insufficient to compare and accurately identify these *Helobdella* leeches owing to colour variations and inconsistencies in traits (Siddall & Borda, 2003; Siddall & Budinoff, 2005; Bely & Weisblat, 2006; Lai et al., 2009). As such, a combination of morphological and molecular analysis which is the current standard for leech taxonomy (Siddall & Budinoff, 2005; Bely & Weisblat, 2006; Siddall et al., 2007; Lai et al., 2009; Oceguera-Figueroa et al., 2010), was used to identify and confirm the specimens collected from Chiang Mai Province, Thailand, in this study.

MATERIAL AND METHODS

Collection and preparation. Leeches were collected from two distinct locations in Chiang Mai Province, Thailand: (1) Huai Tueng Thao Reservoir, Don Kaeo Subdistrict, Mae Rim District (18°52′08.0″N, 98°56′29.1″E) on 12 April 2021 and 28 June 2023; and (2) Angkaew Reservoir, Su Thep Subdistrict, Mueang District (18°48′28.7″N, 98°57′01.3″E) on 28 June 2023. Specimens were collected by standing in the water body at a depth of 50–100 cm and waiting until

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the leeches crawled onto the researchers' feet. Using forceps, leeches were transferred into containers with water from the collection site before transporting them to the laboratory at the Department of Zoology, Faculty of Science, Kasetsart University for further study. Leech specimens were divided into three groups and processed differently depending on their purpose: (1) preserved in absolute ethanol for molecular analysis; (2) preserved in 70% ethanol for morphological examination; (3) preserved in 2.5% glutaraldehyde for scanning electron microscopy (SEM). Specimens used in this study are deposited at the Zoological Reference Collection (ZRC) of the Lee Kong Chian Natural History Museum, National University of Singapore, and the Zoological Museum of Kasetsart University (ZMKU) in Bangkok Province, Thailand.

Morphological study. The morphological characteristics used for taxonomic identification in this study follow the principles outlined primarily by Sawyer (1986), as well as other publications (Kutschera, 1987; Lai et al., 2009; Oceguera-Figueroa et al., 2010; Saglam et al., 2018) describing *Helobdella* species. Two levels of morphological analysis were conducted:

(1) Macro morphology: Leech specimens were examined using a DVM6 digital stereo microscope (Leica Microsystems (SEA) Pte. Ltd.) to characterise eye number and placement, annulation, features of the digestive system (including the number and structure of gastric caeca), and characteristics of the reproductive system. Some specimens were stained with Semichon's acetocarmine dye for 8–10 minutes, followed by sequential destaining with 70% ethanol, 1% acid alcohol, and 5% acid alcohol. Specimens were then rinsed in alkaline alcohol prior to examination of the digestive and reproductive systems under a light microscope (Clopton, 2012).

(2) Fine morphology: Specimens preserved in absolute ethanol were dried using critical point drying (CPD), followed by sputter coating with gold. Detailed examination of the fine external morphological features, including dorsal and ventral surfaces, annulation, oral and caudal suckers, genital pores, and—if present—eggs or juveniles beneath their abdominal surfaces, were carried out through scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS), using Quanta 450 scanning electron microscope at the Scientific Equipment Center of the Faculty of Science, Kasetsart University (Fischer et al., 2012). All visual images were processed using Photoshop CS6 (v.13.0 x32, Adobe Systems Inc., San Jose, CA, USA).

Genetic analysis. Molecular analyses were conducted on newly collected material following the protocol outlined by Chiangkul et al. (2018). The entire individual was used for DNA extraction since the caudal sucker of these leeches is insufficient in size to yield an adequate amount of genetic material. The TIANamp Genomic DNA Kit (catalogue number DP304-02; TIANGEN Biotech (Beijing) Co., Ltd., Beijing) was used for DNA isolation, following the manufacturer's instructions for the purification of total DNA from tissues using the spin-column method. Tissue samples underwent proteinase K treatment and were lysed for 2 hours at 58°C. DNA was subsequently eluted from the spin column using 200 µl of buffer.

PCR reactions were prepared using EP0402 Taq DNA Polymerase. Fragments of two mitochondrial genes, namely the cytochrome c oxidase subunit I (COI) and nicotinamide adenine dinucleotide dehydrogenase subunit I (ND1) genes, were amplified with two primers in accordance with the protocol outlined by Light & Siddall (1999). The COI universal primers employed were LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). The ND1 primers utilised were LND300 (5′-TGGCAGAGTAGTGCATTAGG-3′) and HND1932 (5′-CCTCAGCAAAATCAAATGG-3′) (Light & Siddall, 1999). Each PCR had a final volume of 50 µl, with 5 µl of leech genomic DNA added per reaction. DNA amplification was conducted accordingly: 94°C for 5 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s; and 72°C for 7 min. The purification and sequencing of PCR products were performed by Macrogen, Inc. (Korea).

The DNA sequences were generated for COI (n = 56) and ND1 (n = 29) using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/) and have been deposited in GenBank under Accession No. PP945802–PP945803 for COI and PQ064471–PQ064472 for ND1 (http://www.ncbi.nlm.nih.gov/genbank/) (Tamura et al., 2021) (Appendix 1). Comparative sequences were downloaded from GenBank and included in the analyses, comprising relevant materials (Appendix 1). All sequences were aligned using Clustal Omega and were subsequently checked manually using BioEdit v.7.2.5 (Hall, 1999). Uncorrected p-distances were calculated using MEGA v.11.0.13 (Tamura et al., 2021). Maximum likelihood analyses of COI and ND1 sequence datasets were performed with 2,000 tree search replicates, 25 initial gamma rate categories, and a final optimisation involving four gamma shape categories. Bootstrap support values were obtained from 2,000 pseudoreplicates using the rapid bootstrap algorithm, with ≥70% considered to indicate strong nodal support (Felsenstein, 2004).

TAXONOMY AND SYSTEMATICS

Order Rhynchobdellida Blanchard, 1894

Suborder Glossiphoniiformes Tessler & de Carle, 2018 in Tessler et al., 2018

Family Glossiphoniidae Vaillant, 1890

Subfamily Haementeriinae Autrum, 1939

Genus *Helobdella* Blanchard, 1896

Type species. *Helobdella stagnalis* (Linnaeus, 1758), designated by Blanchard (1896), redescribed by Iwama et al. (2019).

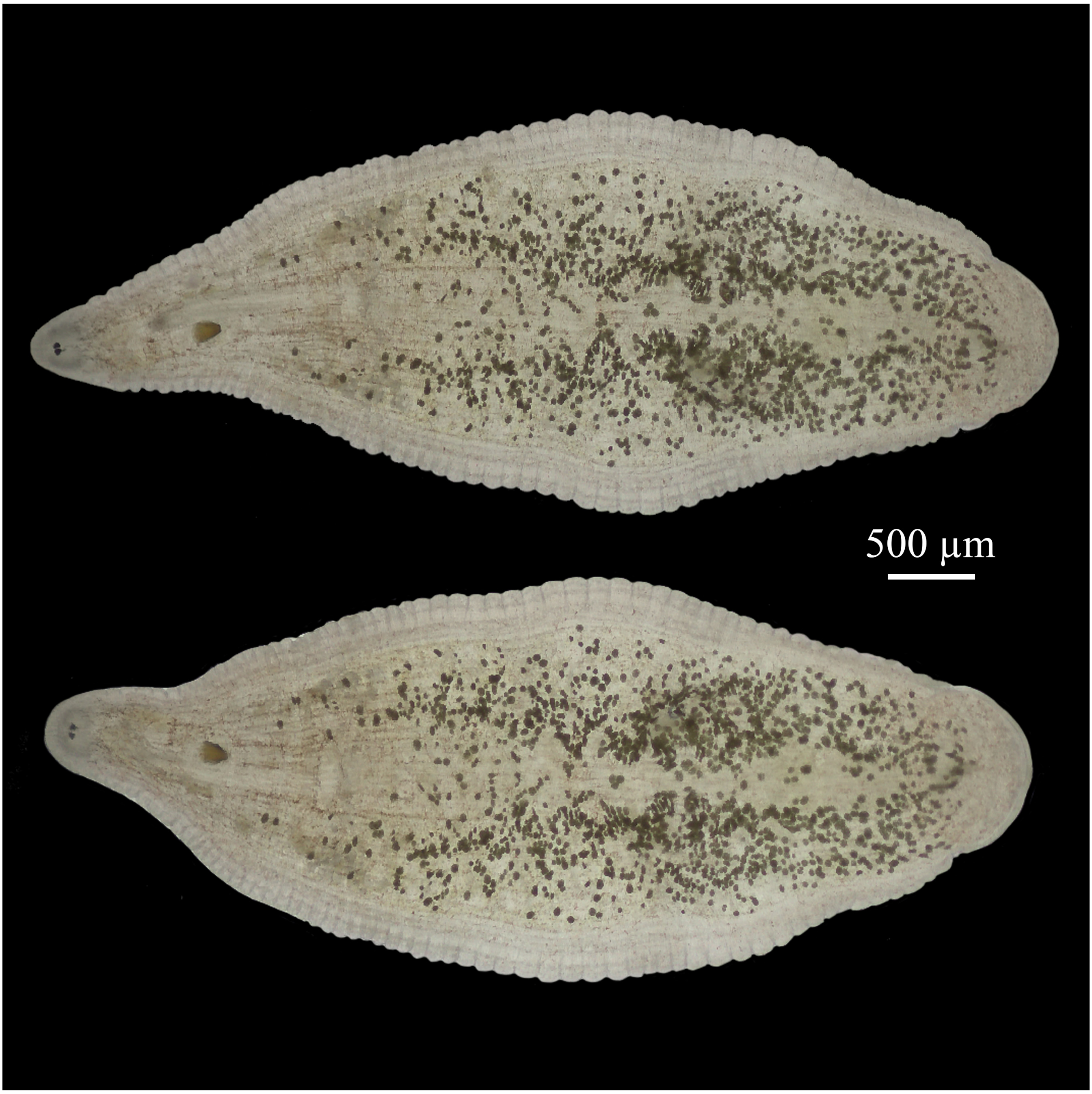

Fig. 1. *Helobdella viridoclypus*, new species, live holotype (ZRC.ANN.1623). A, dorsal view; B, ventral view.

Diagnosis. Small, two eyes, transparent papillae on segment, nuchal gland or scute on dorsum on VIIIa1/a2, gonopores separated by one annulus, compact salivary glands, crop with less developed first pair and unbranched second to sixth pairs of caeca, liquidosomatophagous (Blanchard, 1896; Sawyer, 1986).

Distribution. North America, South America (Siddall & Borda, 2003; Siddall et al., 2005; Christoffersen, 2009; Beresic-Perrins et al., 2017), South Africa, Europe (neotype locality: Sweden), Australia, New Zealand, Russian Far East (Bolotov et al., 2022), Japan (Oka, 1910), continental China (Yang, 1996), Taiwan (Blanchard, 1896; Lai et al., 2009; Oceguera-Figueroa et al., 2010; Salas-Montiel et al., 2014) and Thailand (this study).

Helobdella viridoclypus, new species

(Figs. 1–8, 11)

Material examined. Holotype (Figs. 1, 2): ZRC.ANN.1623 (in ethanol), Huai Tueng Thao Dam, Don Kaeo Subdistrict, Mae Rim District, Chiang Mai Province, Thailand (18°52′08.0″N, 98°56′29.1″E), coll. P. Trivalairat & K. Trivalairat, 12 April 2021 (Figs. 1, 2). Paratypes: ZRC. ANN.2307–2310 (4 specimens, stained whole on slides), same locality as holotype, coll. P. Trivalairat & K. Trivalairat, 28 June 2023; ZMKU-ANN-0038–0039 (2 specimens, in ethanol), same locality as holotype, coll. P. Trivalairat & K. Trivalairat, 28 June 2023; ZMKU-ANN-0040–0041 (2 specimens, in ethanol), Angkaew Reservoir, Su Thep Subdistrict, Mueang District, Chiang Mai Province, Thailand

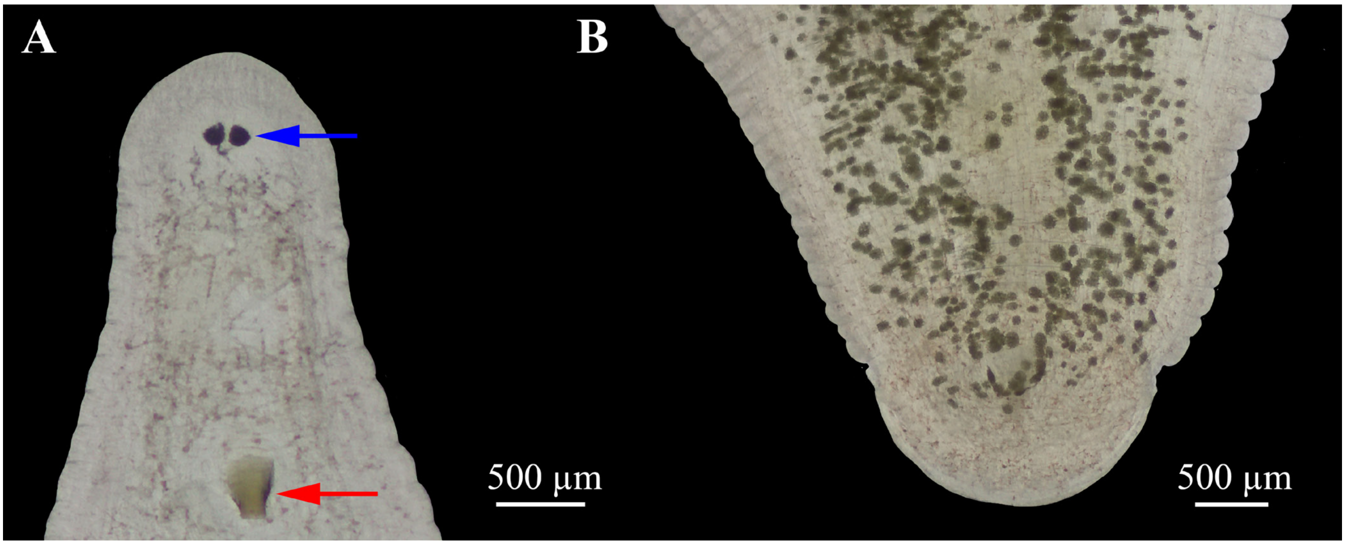

Fig. 2. *Helobdella viridoclypus*, new species, live holotype (ZRC.ANN.1623). A, anterior dorsal section with a pair of eyes (blue arrow) and nuchal scute (red arrow); B, posterior dorsal section.

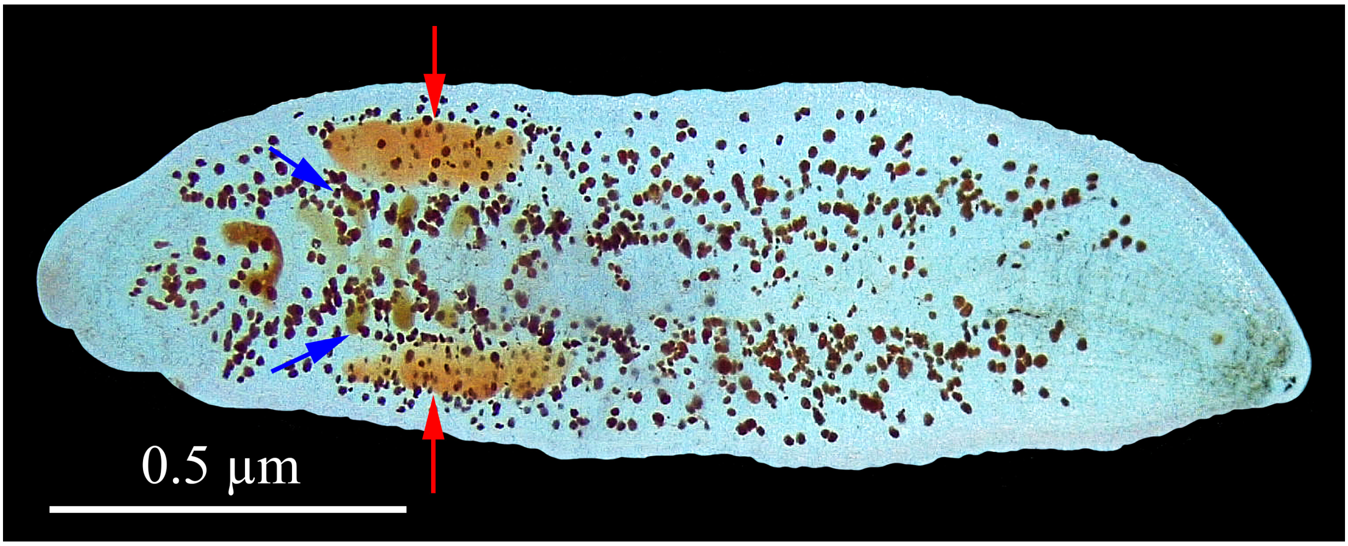

Fig. 3. *Helobdella viridoclypus*, new species, live paratype (ZMKU-ANN-0040), with blood remaining in the post-caeca (red arrows) and intestines (blue arrows) after a meal.

(18°48′28.7″N, 98°57′01.3″E), coll. P. Trivalairat & K. Trivalairat, 28 June 2023 (Fig. 3). Specimens are deposited at the Zoological Reference Collection (ZRC) of the Lee Kong Chian Natural History Museum, National University of Singapore, and the Zoological Museum of Kasetsart University (ZMKU) in Thailand.

Diagnosis. Rice-shaped, transparent body; cephalisation; a pair of large, distinctly separated, dark eyes on somite III; anterior sucker twice as large as prostomium without pits inside; central mouth in anterior sucker; triangular nuchal scute pointed backward with yellowish-green colouring around mid-dorsal region (VIIa1–a3, annuli 11–13); smooth surface without dorsal papillae; rich dark-green pigmentation on dorsum, particularly around crop caeca; lesser or almost absent pigmentation along crop; one male gonopore on XIIa1/XIIa2 (annuli 26/27); one female gonopore on XIIa2/a3 (annuli 27/28); six pairs of crop caeca with unbranched posterior caeca; maximum body width wider than anterior and posterior suckers by four and two times, respectively.

Description. Specimen length 5.92 mm (mean 4.13 ± 1.66 mm, range 2.31–5.96 mm, n = 30), width 2.36 mm (mean 1.69 ± 6.97 mm, range 0.54–2.37 mm, n = 30) (Table 1). Dorsum colour ranges from translucent to translucent with dark green. Dorsal papillae absent. One pair of large, distinct, dark eyes present on somite III; eye size 46.88 µm (mean 40.94 ± 4.42 µm, 33.13–46.88 µm, n = 30) and distance between eyes 8.13 µm (mean 14.53 ± 4.73 µm, range 8.23–21.25 µm, n = 30). Nuchal scute triangular, pointed backwards around mid-dorsal region, yellowish green in colour (VIIa1–a3, annuli 11–13). Nuchal scute made up of carbon (59.38% weight), oxygen (37.75% weight), and calcium (2.87% weight), dorsal surface area surrounding the nuchal scute made up of carbon (70.11% weight) and oxygen (29.89% weight) (Figs. 4, 5). One annulus separates male and female gonopores, with one male gonopore on XIIa1/a2 (annuli 26/27) and one female gonopore on XIIa2/XIIa3 (annuli 27/28). Anterior sucker diameter 0.50 mm (mean 0.41 ± 0.09 mm, range 0.24–0.50 mm, n = 30). Mouth located subterminally in oral sucker. Posterior sucker diameter 0.87 mm (mean 0.79 ± 0.16 mm,

Table 1. Morphological comparison of *Helobdella* species in the *H. stagnalis* complex clade.

Characteristics	***H. viridoclypus*, new species**	***H. stagnalis* (sensu Iwama et al., 2019)**	***H. stagnalis* (sensu Linnaeus, 1758)**	***H. adiastola***	***H. octatestisaca***	***H. modesta***
Body length (mm)	2–6	4.8–5.0	8–15	8–13	9–14	8–12
Body colour	transparent to pale greenish yellow with dark-green pigment	body yellowish, with scattered black chromatophores, no metameric pigmentation on dorsum or venter	transparent to light grey or yellowish grey with dark-brownish pigment	–	transparent to light grey with transversely light brown dots and irregular dark green or olive pigments	transparent to light grey with dark-brownish pigment
Annulation	68	–	67	–	68	67
Eyes	1 pair on III	1 pair on III	1 pair on II/III or III	1 pair	1 pair on III	1 pair on II/III or III
Distance between eyes (µm)	8–21	–	–	–	–	–
Dorsal surface	smooth without papillae	papillae and sensillae absent	smooth without papillae	–	smooth without papillae	smooth without papillae
Nuchal scute	triangular, pointed backwards, yellowish-green colour	ovoid, brown colour	circular top and square bottom, reddish-brown colour	oval with truncated posterior end, brown colour	round or backwards-pointed triangle with black, brown, or sometimes transparent grey colour	square with brown colour
Proboscis length (mm)	0.92–1.96	–	–	–	–	–
Salivary glands	compact	diffuse	diffuse	diffuse	diffuse	diffuse
Crop caeca	6 pairs, first pair less developed, second to sixth pairs unbranched	5–6 pairs digitiform, last pair forming post caeca	5 pairs	5 pairs	5 pairs, unlobed and unbranched	5 pairs, unbranched
Post-caeca	one pair, elongated lobed	one pair, well developed	one pair, short	one pair	one pair, elongated	one pair, elongated
Male gonopore	XIIa1/a2 (26/27)	XII a1/a2	XIIa1/a2	XIIa1/a2	XIIa1/a2 (26/27)	XIa2/a3 (24/25)
Testisacs	6 pairs	6 pairs	6 pairs	–	4 pairs	6 pairs
Female gonopore	XIIa2/a3 (27/28)	XII a2/a3	XIIa2/a3	XIIa2/a3	XIIa2/a3 (27/28)	XIa3/XIIa1 (25/26)
Brooding period	3–4 weeks	–	–	–	–	6–7 weeks

Characteristics	***H. viridoclypus*, new species**	***H. stagnalis* (sensu Iwama et al., 2019)**	***H. stagnalis* (sensu Linnaeus, 1758)**	***H. adiastola***	***H. octatestisaca***	***H. modesta***
Clutch size	12–24	–	–	–	17–74	12–35
Egg diameter (mm)	0.34–0.44	–	–	–	–	–
Egg colour	transparent to yellowish-pink eggs	–	–	–	–	pink
Distribution	Asia (Thailand)	Europe (Sweden, Norway, Iceland, England, France, Italy, Slovenia, Turkey), West Asia (Russia, Iran)	Europe (Germany, UK), North America (Mexico)	North America (Mexico)	Africa, Asia (Taiwan), North America (California, Mexico)	North America (Arizona, New Jersey, Ohio, Washington)
References	This study	Iwama et al., 2019	Sawyer, 1986; Beresic-Perrins et al., 2017; Saglam et al., 2018	Sawyer, 1986	Lai et al., 2009; Beresic-Perrins et al., 2017	Sawyer, 1986; Kutschera, 1988; Beresic-Perrins et al., 2017; Saglam et al., 2018

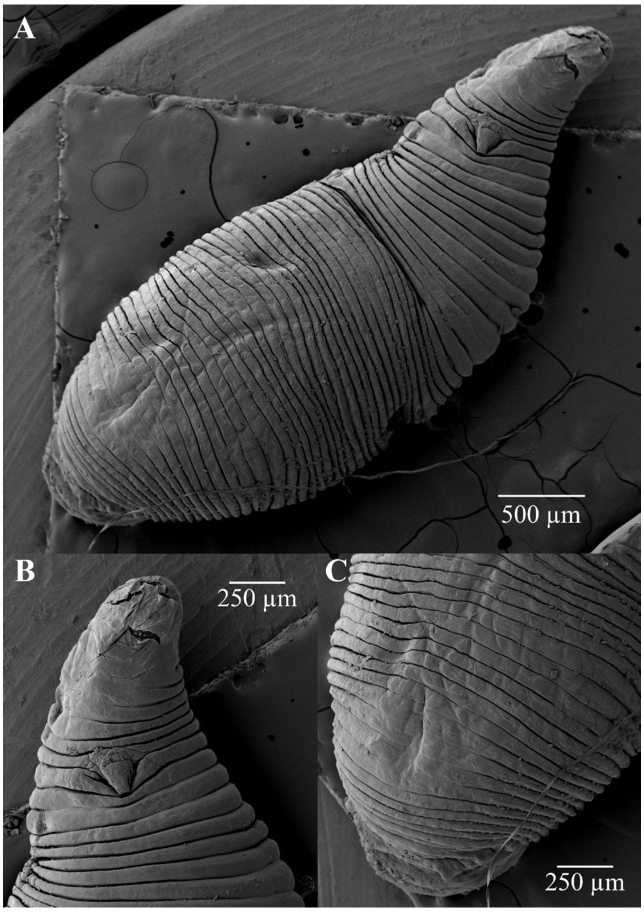

Fig. 4. Scanning electron micrograph of the dorsal surface of *Helobdella viridoclypus*, new species. A, whole body; B, anterior section, with nuchal scute; C, posterior section.

range 0.53–0.96 mm, n = 30) (Fig. 6). Soft, thin-walled, transparent cocoons (diameter 0.55 ± 0.11 mm, 0.39–0.71 mm, n = 5) (in total, diameter 0.61 ± 0.12 mm, 0.39–0.72 mm, n = 12, 5–6 cocoons per clutch), each containing 1–2 eggs (diameter 0.37 ± 0.03 mm, 0.34–0.42 mm, n = 12) (in total, diameter 0.39 ± 0.04 mm, 0.34–0.44 mm, n = 21) laid on posterior ventral side of parent (Fig. 7). Anus in furrow of XXVI/XXVII (annuli 66/67).

Body transparent and pale greenish yellow in colour when alive. Dorsum has tiny dark-green dots distributed posteriorly around crop caeca, particularly fourth to sixth crop caeca (Figs. 1, 2). Dark green or olive chromatophores arranged irregularly under body surface. Venter lacks chromatophores. Dorsum of posterior sucker has irregularly distributed brown spots. Nuchal scute yellowish green. Venter of posterior sucker lacks dots or spots. Body pale yellow in preserved specimens, except for nuchal scute which remains yellowish green.

68 annuli present in holotype specimen: I, II, and III uniannulate; IV–V biannulate; VI–XXIII triannulate; XXIV–XXV biannulate; and XXVI–XXVIII uniannulate.

Digestive system (Fig. 8). Proboscis pore opens at centre of anterior sucker. Robust proboscis uniformly cylindrical and in membranous sheath on IVa1–XIIa2 (annuli 4–27).

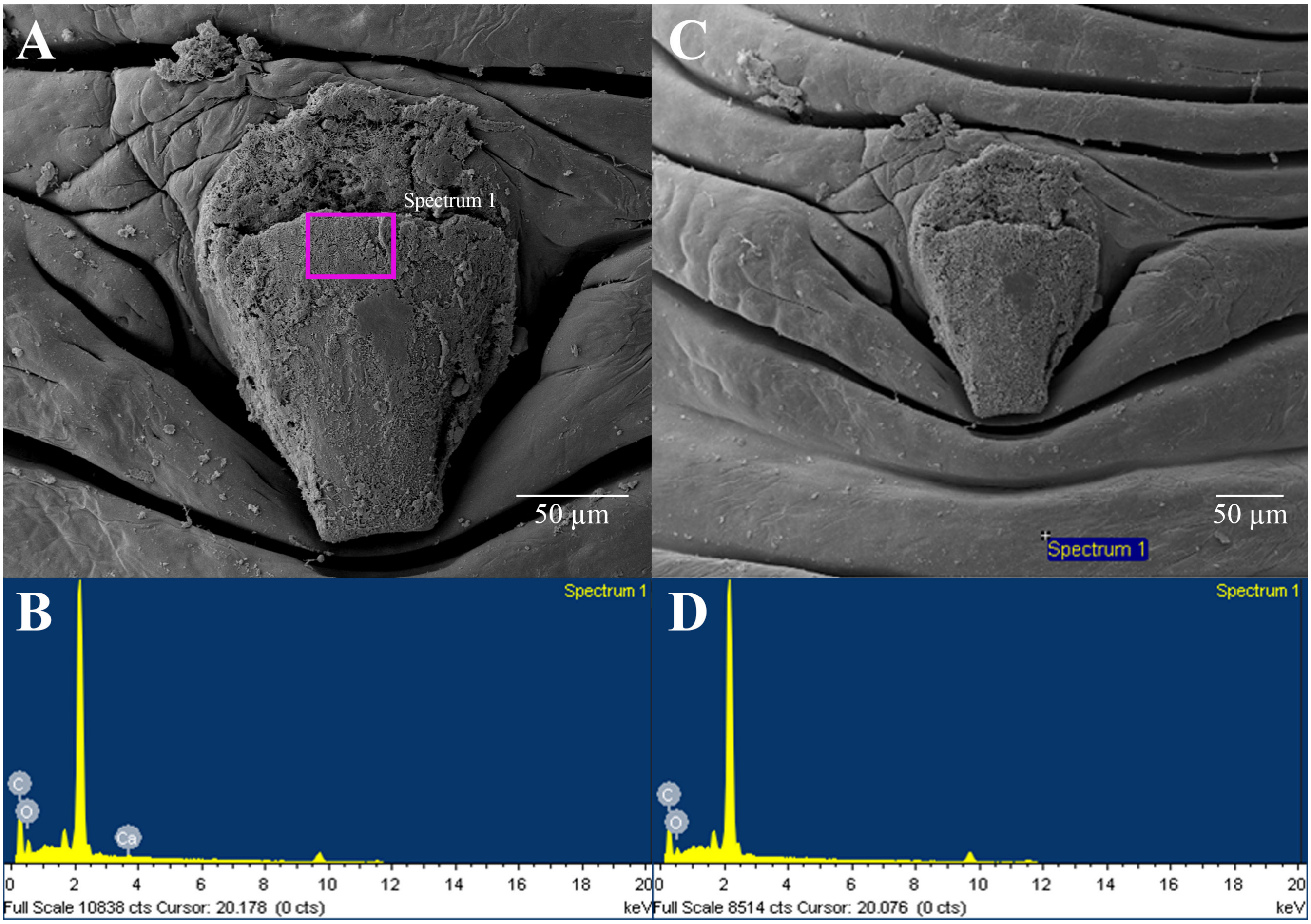

Fig. 5. *Helobdella viridoclypus*, new species. Scanning electron micrograph of A, nuchal scute surface and C, dorsal surface surrounding the nuchal scute. Energy dispersive x-ray spectroscopy (EDS) analysis of B, nuchal scute surface and D, dorsal area surrounding the nuchal scute. Elements detected in high components were on the nuchal scute surface, and only carbon and oxygen on the dorsal surface surrounding the nuchal scute.

Proboscis length 1.49 ± 0.43 mm (0.92–1.96 mm, n = 30). Salivary glands compactly scattered on either side of proboscis, and salivary ductule bundles attached at each side of base of proboscis on XIIa1–a3 (annuli 26–28). Short, simple oesophagus present on XIIa3–XIIIa2 (annuli 28–30). Six pairs of short, simple, unbranched crop caeca on XIIIa2–XXIIIa2 (annuli 31–60), with last lobed pair on XIXa2–XXIIIa2 (annuli 48–60). Four pairs of short, thick, diverticulated intestines on XXa3–XXIIIa3 (annuli 52–62). Simple, robust rectum recurves on XXVa2–XXVII (annuli 65–67) and opens dorsally at anus in furrow between somites XXVI–XXVII (annuli 66–67).

Reproductive system (Figs. 6B, 8). Male gonopore opening thick and curled, wider than female gonopore. One annulus separates male and female gonopores, with male gonopore on XIIa1/a2 (annuli 26/27) and female gonopore on XIIa2/XIIa3 (annuli 27/28). Ejaculatory bulb on XIa3–XIIa2 (annuli 25–27) a bean-shaped sac that receives opening of vas deferens. Two long, constricted vas deferens extend posteriorly from ejaculatory bulb and connect laterally to each testis. Six pairs of small, round testisacs present, with each located in front of each pair of crop caeca. Female gonopore rim more cramped than that of male. Rounded-square spermatheca is located on XIIa3–XIIIa3 (annuli 28–31), receiving opening of ovaries, and opening to bifurcated ovisacs.

Helobdella viridoclypus, new species, deposits five to six transparent cocoons per clutch, each containing one to two transparent to yellowish-pink eggs inside (Fig. 7). Brooding period lasts approximately 3–4 weeks before juveniles reach maturity and become ready to mate.

Molecular results. A Maximum Likelihood analysis was conducted based on 518 COI nucleotides from *Helobdella* species and the outgroup containing *Batracobdelloides bangkhenensis* Chiangkul et al., 2020, *Orientobdelloides siamensis* (Oka, 1917), *O. sirikanchanae* (Trivalairat et al., 2019), and *O. tridens* (Chiangkul et al., 2021) in Fig. 9. Our analysis revealed the monophyletic divergence of *H. viridoclypus*, new species, within the *octatestisaca* complex clade which also contained *H. adiastola* Ringuelet, 1972 (MK354119 from Argentina, MK354120 from Mexico), *H. octatestisaca* Lai & Chang, 2009 (MH729329 from California, HQ179855 from Mexico, FJ000342 from Taiwan [most likely introduced from South America]), and *H. stagnalis* (HQ179860 from South Africa), with a robust bootstrap support value of 89 (Fig. 9). The *octatestisaca*

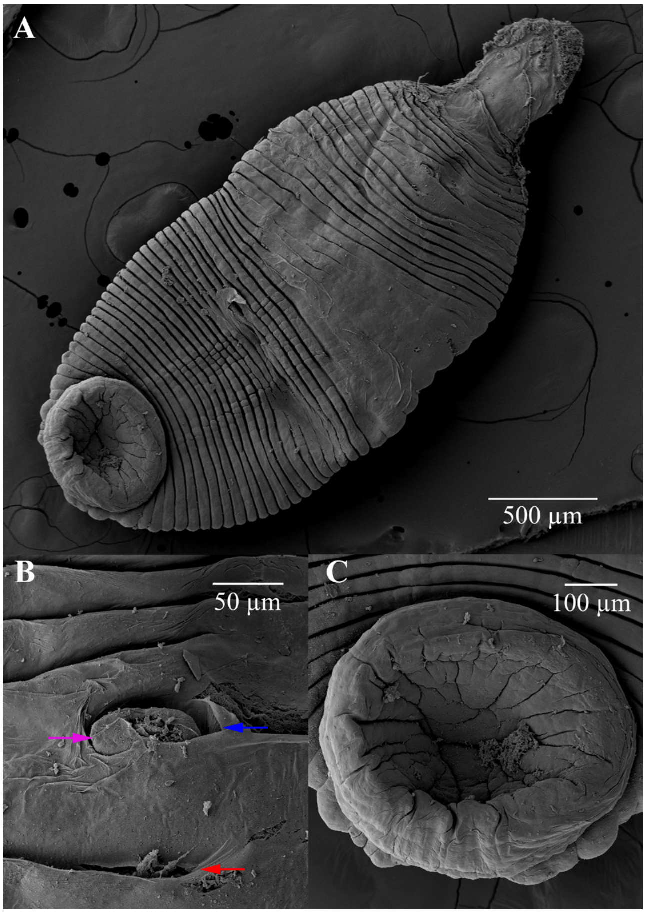

Fig. 6. Scanning electron micrograph of the ventral surface of *Helobdella viridoclypus*, new species. A, whole body; B, gonopores (blue arrow = male gonopore, red arrow = female gonopore, purple arrow = spermatophore); C, posterior sucker.

complex clade was recovered as sister to the clade containing the paraphyletic *modesta* complex clade (*H. modesta* (Verrill, 1872), AF329040 from Ohio, HQ179853 from Washington) and the other specimen of *H. stagnalis* sensu stricto (AF329041 from the UK), within the larger *stagnalis* complex clade.

The phylogenetic analysis based on the combined data set of 518 COI nucleotides and 555 ND1 nucleotides also supported the monophyly of *H. viridoclypus*, new species, and its separation from *H. modesta* of the *modesta* complex clade, with a medium bootstrap support value of 60 (Fig. 10).

Molecular comparison based on p-distances between two specimens of *H. viridoclypus*, new species, from Chiang Mai Province, Thailand revealed a difference of 0.42% using the COI gene (518 bp) (GenBank MT431621–MT431623) and 1.14% using the ND1 gene (555 bp) (GenBank MT430980–MT430982) (Table 2). The COI distance between *H. viridoclypus*, new species, and members of the *octatestisaca* complex clade (FJ000342, HQ179855, HQ179860, MH729329, MK354119, MK354120) ranged between 0.15%–2.32%. More specifically, the difference between *H. viridoclypus* and the two closely related species *H. adiastola* (Mexico and Argentina) and *H. octatestisaca* (Mexico, USA, and Taiwan) was 0.15%–0.61% and 0.46%–2.32%, respectively. These were lower than the distances of more than 16% between *H. viridoclypus*, new species, and other *Helobdella* species in this study, including both *H. modesta* (USA) and *H. stagnalis* sensu stricto (UK) from the *stagnalis* complex clade, which ranged between 17.53%–18.57% and 18.10%–18.69%, respectively (Table 2). Using the ND1 gene, the difference between *H. viridoclypus*, new species, and other *Helobdella* species ranged between 29–35% (Table 2).

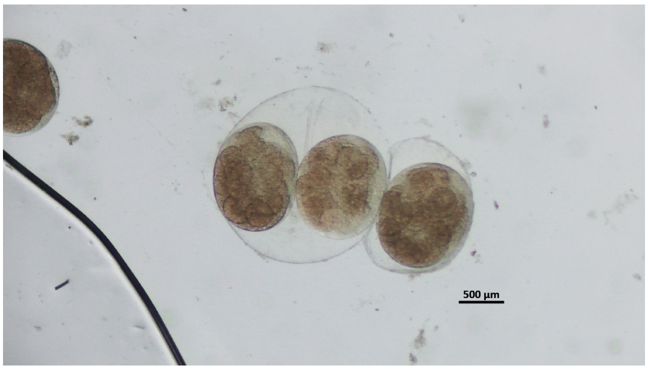

Fig. 7. Transparent cocoons of *Helobdella viridoclypus*, new species, viewed under a light microscope. Each cocoon contains one or two developing eggs.

Habitat. *Helobdella viridoclypus*, new species, is found in the sandy benthic zones of various large freshwater reservoirs in Chiang Mai Province (Fig. 11A).

Etymology. The species epithet refers to the description of the nuchal scute on the neck region, which resembles a shield and is a yellowish green colour. The following common names are suggested: "Emerald-charm leech" (English), "Pling soi mor ra kot" (ปลิงสร้อยมรกต) (Thai), and "Der Smaragdt-charm Plattegel" (German).

Remarks. *Helobdella viridoclypus*, new species, is similar to other cryptic species in the *H. stagnalis* complex in both external and internal characteristics. However, it can be distinguished by its significantly smaller body size, a backward-pointing triangular scute with a yellowish-green colour, six pairs of crop caeca with less development on the first pair, and elongated, lobed post-caeca (Table 1).

In surveys, *Helobdella viridoclypus*, new species, was commonly found in large, sandy reservoirs where human activities such as the operation of waterside restaurants and swimming occur. Although it has been observed to attach to human bodies in response to vibrations, it does not feed on humans (Fig. 11B). Instead, this species primarily feeds on small freshwater invertebrates such as *Tubifex* worms (Supplementary Material).

DISCUSSION

Helobdella viridoclypus, new species, is a free-living leech described from northern Thailand based on both morphological and genetic characteristics. It is difficult to

Table 2. Range of p-distance values for COI and ND1 genes within *Helobdella viridoclypus*, new species, and between *H. viridoclypus* and other *Helobdella* species listed in this study.

Species	**COI (%)**	**ND1 (%)**
Helobdella viridoclypus, new species	0.42	1.14
H. adiastola	0.15–0.61	–
H. alti	19.83–21.12	–
H. austinensis	21.92–22.55	–
H. blinni	27.69–29.57	–
H. bolivianita	22.07–22.68	31.90–32.15
H. bowermani	16.78–16.95	–
H. californica	22.08–22.69	–
H. elongata	19.62–19.83	30.96–31.20
H. europaea	17.75–18.42	31.08–32.83
H. fusca	22.48–23.10	35.82
H. lineata	21.64–22.24	33.38–34.16
H. melananus	19.70–20.30	–
H. michaelseni	24.40–24.84	29.13–29.47
H. modesta (*modesta* complex clade)	17.53–18.57	31.68–31.97
H. nunununojensis	18.60–19.25	32.44–33.12
H. octatestisaca	0.46–2.32	–
H. papillata	21.26–22.07	32.57–33.58
H. papillornata	20.89	31.29
H. paranensis	16.44–17.01	30.90–31.14
H. pichipanan	20.34–21.03	32.32
H. robusta	21.56–22.33	–
H. simplex	17.57–17.98	–
H. socimulcensis	19.41–19.98	–
H. sorojchi	18.47–20.27	30.96–31.69
H. sp. *Xochimilco*	18.64–19.26	–
H. stagnalis sensu stricto (AF329041)	18.10–18.69	31.45–31.69
H. transversa	21.06–21.66	31.94–32.19
H. triserialis	18.75–20.08	33.58–33.83
H. virginiae	22.42–22.64	–
H. octatestisaca complex clade (including *H. adiastola*, *H. octatestisaca*)	0.15–2.32	–

distinguish this species from other scutiferous species in the *H. stagnalis* complex owing to various similarities such as annulation, eyes, dorsal surface, nuchal scute, gonopores, salivary glands, crop caeca, and intestines (Autrum, 1936). The phylogenetic tree constructed in our study using the COI gene supported the monophyly of *H. viridoclypus*, new species, as well as its divergence from other species in the *stagnalis* complex, with robust bootstrap support values (Fig. 9). These include the paraphyletic *H. modesta* and the *H. stagnalis* specimen AF329041 from England, both of which were well separated (17.53%–18.57% and 18.10%–18.69%) from *H. viridoclypus* in terms of COI distance (Table 2). While *H. viridoclypus*, new species, was shown to diverge from *H. adiastola* and *H. octatestisaca* within the smaller *octatestisaca* complex clade, the COI distances between *H. viridoclypus* and these species were

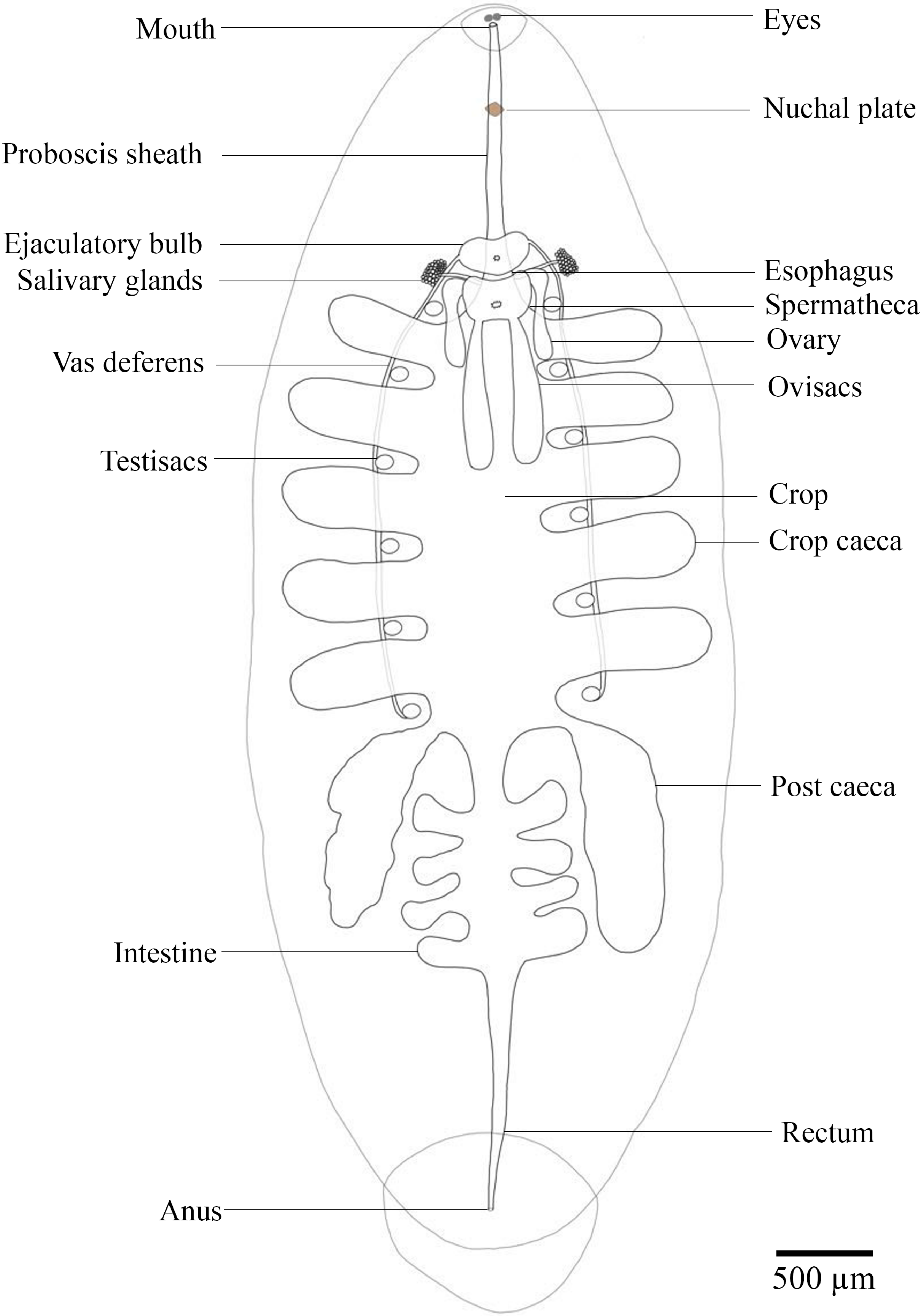

Fig. 8. Illustration of internal anatomy of *Helobdella viridoclypus*, new species, based on observations of multiple live specimens.

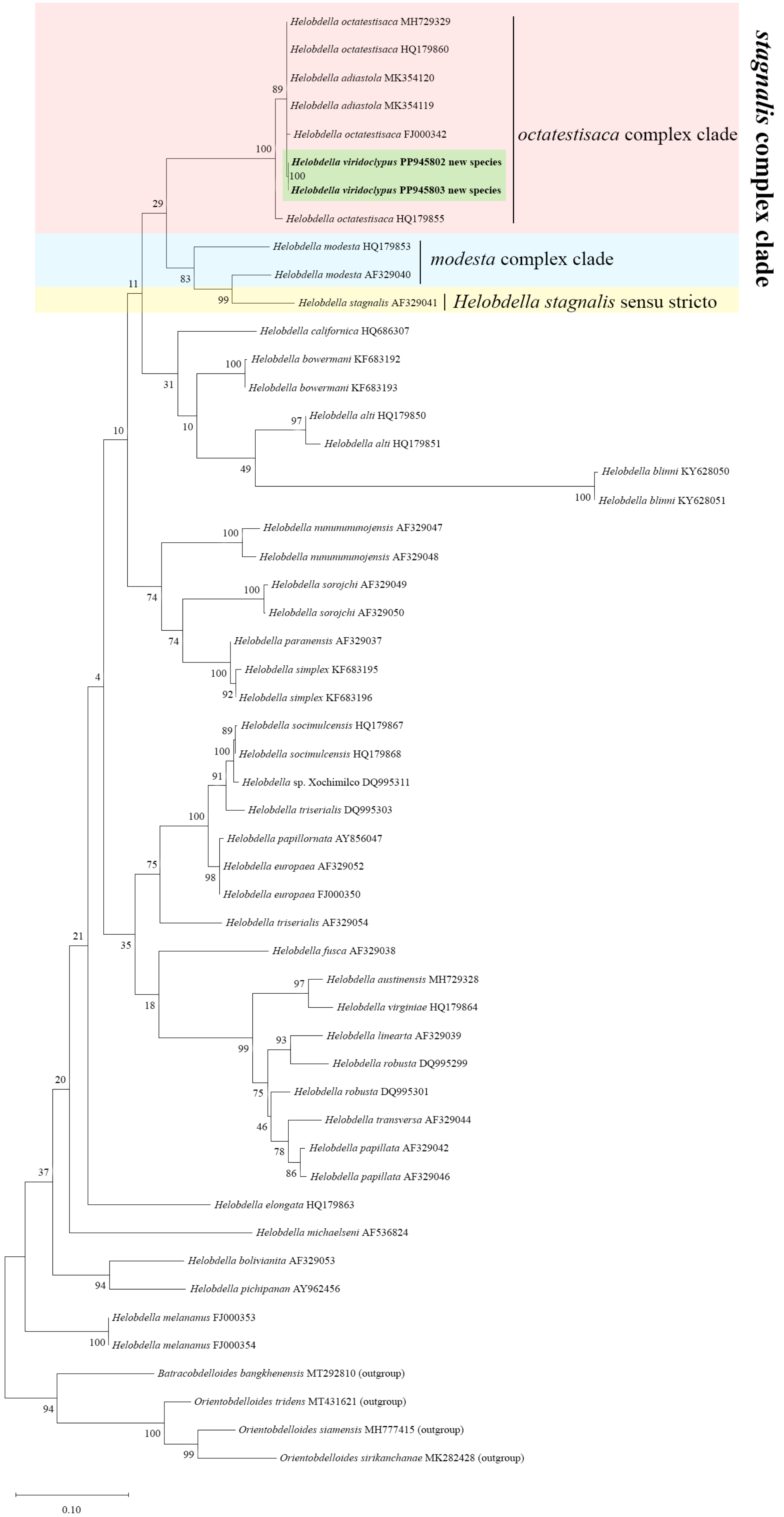

Fig. 9. Phylogenetic tree of *Helobdella* species, based on a Maximum Likelihood analysis of 518 COI nucleotides. Bootstrap values are indicated at the branch nodes. The scale bar (0.10) represents the genetic distance. Taxa in bold represent sequences generated in this study. GenBank accession numbers are indicated after the species names.

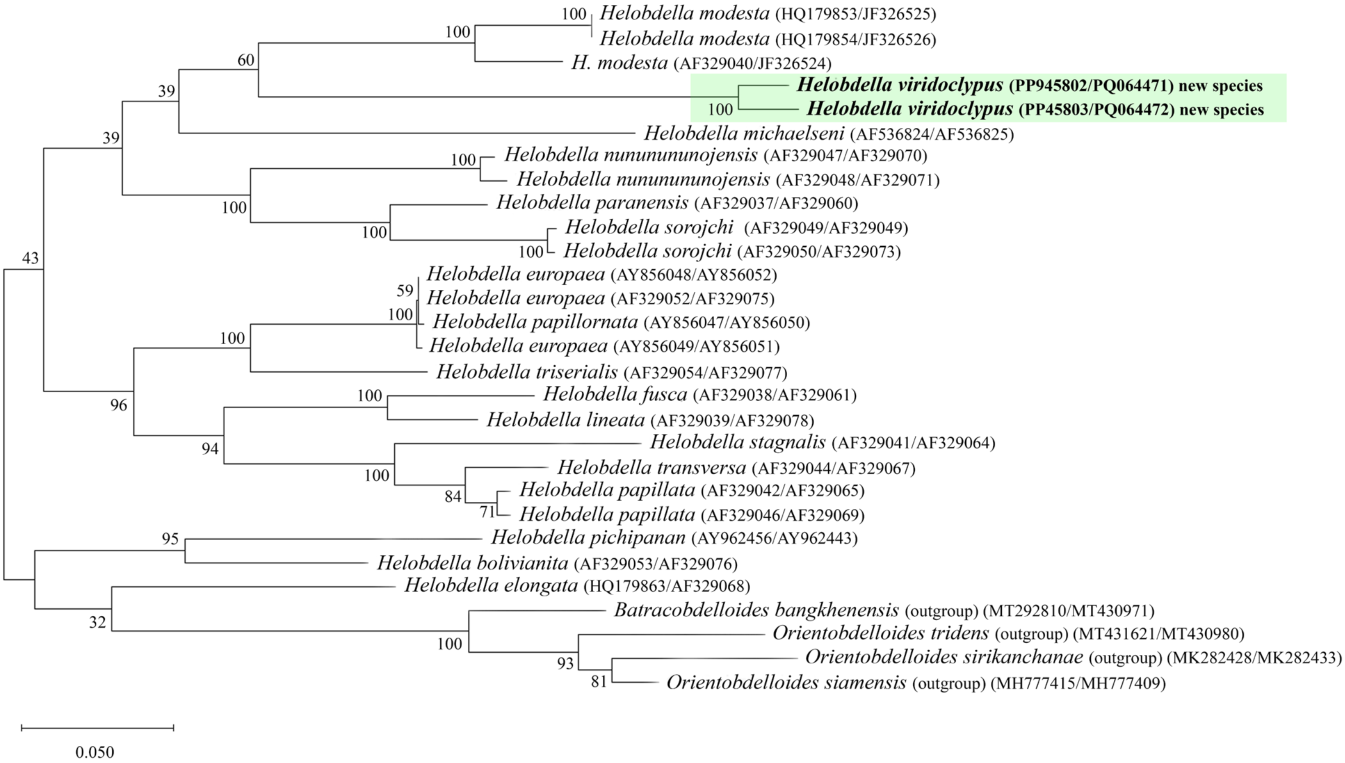

Fig. 10. Phylogenetic tree of *Helobdella* species based on a Maximum Likelihood analysis on the combined data set of 518 COI nucleotides and 555 ND1 nucleotides. Bootstrap values are indicated at the branch nodes. The scale bar (0.050) represents the genetic distance. Taxa in bold represent sequences generated in this study. GenBank accession numbers are indicated after the species names.

Fig. 11. A, type locality of *Helobdella viridoclypus*, new species, Huai Tueng Thao Reservoir, Chiang Mai Province, Thailand; B, multiple individuals of *H. viridoclypus* (red arrows) attached to feet. Photographs by Poramad Trivalairat.

much smaller (0.15%–2.32%) suggesting they are more closely related. The presence of the *H. stagnalis* specimen (HQ179860) from Mexico within the *octatestisaca* complex clade while the *H. stagnalis* specimen (AF329041) from England was in a clade well separated from the complex, supports the results of Saglam et al. (2018) and Iwama et al. (2019) who re-described and designated a neotype for the type species, clarifying that *H. stagnalis* sensu stricto is not present in North America but only in Europe and West Asia. This further clarifies the possible misidentification of the specimen HQ179860, which was assigned to *H. octatestisaca* in both studies.

Although the two western American species *H. adiastola* and *H. octatestisaca* were also shown to be closely related (COI distance of 0.15%–0.61% and 0.46%–2.32%, respectively) to *H. viridoclypus*, new species, within the *octatestisaca* complex, the bootstrap values in Fig. 9 still suggest a reliable separation between *H. viridoclypus* and the other members of the complex clade, indicating that it is genetically distinct but shares close evolutionary relationships with them.

Hellobdella viridoclypus, new species, is morphologically distinct from closely related members of the *stagnalis* complex, based on body length, salivary glands, number of crop caeca, post-caeca, and the nuchal scute, as listed in Table 1. Most species within the *stagnalis* complex are significantly larger (8–15 mm) than *H. viridoclypus*, new species (2–6 mm), possess diffuse salivary glands (vs. compact salivary glands in *H. viridoclypus*), and have five pairs of crop caeca (vs. six pairs). The only exception is the redescribed *H. stagnalis* sensu stricto, which can have either five or six pairs of crop caeca but differs by the absence of pigmentation on both dorsal and ventral surfaces (vs. dark green pigment in *H. viridoclypus*).

The original description of the type species *H. stagnalis* states that it has short post-caeca, while *H. stagnalis* sensu stricto is described to have well-developed post-caeca, as compared to *H. viridoclypus*, new species, which has elongated, lobed post-caeca. The new species also has a yellowish-green triangular scute pointed backwards, whereas the original description of *H. stagnalis* states that it has a reddish-brown scute with circular top and square bottom, the neotype of *H. stagnalis* sensu stricto (see Iwama et al., 2019) has a brownish ovoid scute, *H. adiastola* which is the species most closely related to *H. viridoclypus* in terms of COI distance has a brown, oval scute with truncated posterior end, and *H. modesta* has a square scute, brown in colour. Lastly, *H. octatestisaca*, the species with the closest geographical distribution to *H. viridoclypus*, has fewer pairs of testisacs (four pairs vs. six pairs). (Sawyer, 1986; Kutschera, 1988; Lai et al., 2009; Beresic-Perrins et al., 2017; Saglam et al., 2018).

In addition, *H. viridoclypus*, new species, has bifurcated ovisacs, a character also observed in some *Helobdella* species (Saglam et al., 2018) and in other freshwater leeches, including *Orientobdelloides* species (Chiangkul et al., 2018; Trivalairat et al., 2019; Chiangkul et al., 2021) and *Alboglossiphonia* species (Moser et al., 2022; Kambayashi & Nakano, 2025).

The above morphological analysis not only highlights the distinctive characters of *H. viridoclypus* compared to closely related species within the *H. stagnalis* complex, but also suggests that the use of only COI gene may not provide sufficient evidence for species identification or separation. Consequently, a combination of COI-ND1 genes was used in our phylogenetic analysis to further support the monophyletic divergence of *H. viridoclypus*, new species, although a combined comparison of both genes could only be conducted for some of the species in this study due to a lack of molecular data (Fig. 10, Table 2).

At least 80 percent of *Helobdella* species have been reported in North and South America (Weber, 1915; Sawyer, 1986; Kutschera, 1988, 2004, 2011; Govedich & Davies, 1998; Siddall & Borda, 2003; Oceguera-Figueroa & Leon-Regagnon, 2005; Kutschera et al., 2013; Moser et al., 2013, 2016; Beresic-Perrins et al., 2017; Saglam et al., 2018; Iyer et al., 2019; Jiménez-Armenta & Oceguera-Figueroa, 2019), including all members of the *stagnalis* complex clade in our study, except for *H. stagnalis* sensu stricto (see Iwama et al., 2019) and *H. viridoclypus*, new species. Some species have also been recorded on other continents, such as *H. europaea* which has been reported in South America, Asia (Taiwan, GenBank accession number FJ000350), Australia (AF329052), New Zealand (AY856049), and South Africa (AY856048) (Kutschera, 2004; Reyes-Prieto et al., 2014); *H. octatestisaca* in South Africa (HQ179860, formerly *H. stagnalis*), North America (Mexico, HQ179855, California, MH729329), and Asia (Taiwan, FJ000342) (Oceguera-Figueroa et al., 2010; Reyes-Prieto et al., 2014; Richardson et al., 2017); *H. robusta* Shankland et al., 1992 in North America (California, DQ995301) and Asia (Japan, LC761207) (Bely & Weisblat, 2006; Gerardo, 2019; Kambayashi & Nakano, 2023); and *H. stagnalis* in Europe (UK, AF329041 and Germany) (Siddall & Borda, 2003). As the genus *Helobdella* is not known to survive marine conditions, some of these species may have been introduced to other continents through passive means such as waterbird migration or the introduction of aquatic plants and snails via the commercial trade, examples being the *H. europaea* and *H. octatestisaca* specimens from Taiwan which were found attached to the apple snail *Pomacea canaliculata* introduced from South America (Lai et al., 2009). While *H. viridoclypus*, new species, is shown to be closely related to other members of the *octatestisaca* complex clade—particularly *H. octatestisaca* and *H. adiastola*—it has not been reported in the Americas and there are distinct morphological differences between these species. The results suggest the ancestor of *H. viridoclypus* and the *octatestisaca* complex may have originated in North America, before dispersing elsewhere, and the possibility remains that *H. viridoclypus*, new species, was introduced to Thailand with host animals. More comparative material of *Helobdella* species from Thailand and elsewhere in Asia would be beneficial in further resolving the evolutionary relationships and origins of *H. viridoclypus*, new species.

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SUPPLEMENTARY MATERIAL

Supplementary Video. Feeding behaviour of *Helobdella viridoclypus*, new species, on *Tubifex* worms. Video can be accessed online at https://doi.org/10.6084/m9.figshare.30016228.v1

All video clips included in this submission were recorded and produced by the authors, who hold full rights for their use in this publication.

APPENDIX

Appendix 1. GenBank accession numbers for leech sequences used in the phylogenetic analysis of the genus *Helobdella.*

Taxon	Locality	GenBank accession numbers	
		COI	ND1
Helobdella viridoclypus, new species	Chiang Mai, Thailand	PP945802	PQ064471
Helobdella viridoclypus, new species	Chiang Mai, Thailand	PP945803	PQ064472
Helobdella adiastola Ringuelet, 1972	Argentina	MK354119	–
Helobdella adiastola Ringuelet, 1972	Nuevo León Mexico	MK354120	–
Helobdella alti Oceguera-Figueroa & Leon-Regagnon, 2005	Aljojuca, Puebla, Mexico	HQ179850	–
Helobdella alti Oceguera-Figueroa & Leon-Regagnon, 2005	Totolcingo, Tlaxcala, Mexico	HQ179851	–
Helobdella austinensis Kutschera et al., 2013	Austin, Texas, USA	MH729328	–
Helobdella blinni Beresic-Perrins et al., 2017	Montezuma Well, Arizona, USA	KY628050	–
Helobdella blinni Beresic-Perrins et al., 2017	Montezuma Well, Arizona, USA	KY628051	–
Helobdella bolivianita Siddall, 2001	Bolivia	AF329053	AF329076
Helobdella bowermani Moser et al., 2013	Oregon, USA	KF683192	–
Helobdella bowermani Moser et al., 2013	Oregon, USA	KF683193	–
Helobdella californica Kutschera, 1988	Golden Gate Park, California, USA	HQ686307	–
Helobdella elongata Castle, 1900	Jalisco, Mexico	HQ179863	AF329068
Helobdella europaea Kutschera, 1987	Taiwan	FJ000350	–
Helobdella europaea Kutschera, 1987	Aura Vale Lake, Australia	AF329052	AF329075
Helobdella europaea Kutschera, 1987	South Africa	AY856048	AY856052
Helobdella europaea Kutschera, 1987	New Zealand	AY856049	AY856051
Helobdella fusca (Castle, 1900)	Wild Goose Lake, Michigan, USA	AF329038	AF329061
Helobdella lineata (Verrill, 1874)	Michigan, USA	AF329039	AF329078
Helobdella melananus Lai & Chang, 2009	Taiwan	FJ000353	–
Helobdella melananus Lai & Chang, 2009	Taiwan	FJ000354	–
Helobdella michaelseni Blanchard, 1900	Lago Calafquen, Chile	AF536824	AF536825
Helobdella modesta (Verrill, 1872)	Columbus, Ohio, USA	AF329040	JF326524
Helobdella modesta (Verrill, 1872)	Washington, USA	HQ179853	JF326525
Helobdella modesta (Verrill, 1872)	Washington, USA	HQ179854	JF326526
Helobdella nununununojensis Siddall, 2001	Bolivia	AF329047	AF329070
Helobdella nununununojensis Siddall, 2001	Bolivia	AF329048	AF329071
Helobdella octatestisaca Lai & Chang, 2009	Taiwan	FJ000342	–
Helobdella octatestisaca Lai & Chang, 2009	Sacramento, California, USA	MH729329	–
Helobdella octatestisaca Lai & Chang, 2009	Mexico	HQ179855	–
Helobdella octatestisaca Lai & Chang, 2009 (formerly *Helobdella stagnalis* (Linnaeus, 1758)	South Africa	HQ179860	–
Helobdella papillata (Moore, 1906)	Michigan, USA	AF329042	AF329065
Helobdella papillata (Moore, 1906)	Virginia, USA	AF329046	AF329069

Taxon	Locality	GenBank accession numbers	
		COI	ND1
Helobdella papillornata Govedich & Davies, 1998	Australia	AY856047	AY856050
Helobdella paranensis (Oka, 1930)	Arroyo Espinas, Uruguay	AF329037	AF329060
Helobdella pichipanan Siddall & Borda, 2004	Lago Chico, Chile	AY962456	AY962443
Helobdella robusta Shankland, Bissen & Weisblat, 1992	Sacramento, California, USA	DQ995299	–
Helobdella robusta Shankland, Bissen & Weisblat, 1992	Sacramento, California, USA	DQ995301	–
Helobdella aff. *robusta* Shankland, Bissen & Weisblat, 1992	Kanagawa, Japan	LC761207	–
Helobdella simplex (Moore, 1911)	Argentina	KF683195	–
Helobdella simplex (Moore, 1911)	Argentina	KF683196	–
Helobdella socimulcensis Caballero, 1931	Xochimilco, Mexico	HQ179867	–
Helobdella socimulcensis Caballero, 1931	Xochimilco, Mexico	HQ179868	–
Helobdella sorojchi Siddall, 2001	Madidi, Bolivia	AF329049	AF329072
Helobdella sorojchi Siddall, 2001	Madidi, Bolivia	AF329050	AF329073
Helobdella sp. Xochimilco	Xochimilco, Mexico	DQ995311	–
Helobdella stagnalis (Linnaeus, 1758)	Cotswolds, United Kingdom	AF329041	AF329064
Helobdella transversa Sawyer, 1972	Michigan, USA	AF329044	AF329067
Helobdella triserialis (Blanchard, 1849)	Laguna Volc*á*n, Bolivia	AF329054	AF329077
Helobdella triserialis (Blanchard, 1849)	California, USA	DQ995303	–
Helobdella virginiae Oceguera-Figueroa, 2007	Catemaco, Veracruz, Mexico	HQ179864	–
Orientobdelloides siamensis (Oka, 1917)	Bangkok, Thailand	MH777415	MH777409
Orientobdelloides sirikanchanae (Trivalairat, Chiangkul & Purivirojkul, 2019)	Songkhla, Thailand	MK282428	MK282433
Orientobdelloides tridens (Chiangkul, Trivalairat, Kunya & Purivirojkul, 2021)	Nakhon Ratchasima, Thailand	MT431621	MT430980
Batracobdelloides bangkhenensis (Chiangkul, Trivalairat & Purivirojkul, 2020)	Bangkhen, Bangkok, Thailand	MT292810	MT430971