

1 **RH: KAMBAYASHI ET AL.—*ORIENTOBDELLOIDES TRIDENS* FROM**

2 **INDONESIA**

3 **First Record of *Orientobdelloides tridens* (Hirudinea: Glossiphoniidae) from**

4 **Indonesia, with Remarks on Its Taxonomic Characters**

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13 **ABSTRACT:** The freshwater leech genus *Orientobdelloides* Bolotov, Eliseeva &

14 Kondakov, 2022 is reported for the first time in Indonesia. Morphological and

15 mitochondrial DNA sequence data identified the specimens as *Orientobdelloides tridens*

16 (Chiangkul, Trivalairat, Kunya & Purivirojkul, 2021), a species previously known only

17 from Thailand. The external and internal morphological features of the Indonesian *O.*

tridens are provided in detail here, and the species diagnosis is amended based on our observations.

KEY WORDS: *Orientobdelloides tridens*, Glossiphoniidae, Hirudinea, leech, *Siebenrockiella crassicollis*, Black marsh turtle, Banten, Indonesia, molecular phylogeny, mitochondrial DNA

Freshwater leech species from Africa, Asia, and Oceania, classified into the genus *Placobdelloides* Sawyer, 1986, are ectoparasitic to vertebrates (Sawyer, 1986). However, recent molecular phylogenetic studies revealed *Placobdelloides* to be polyphyletic (Siddall et al., 2005; Chiangkul et al., 2018; Bolotov et al., 2022). Consequently, *Orientobdelloides* Bolotov, Eliseeva & Kondakov, 2022 was established for turtle-specific species from the Indochinese region, with *Orientobdelloides siamensis* (Oka, 1917) (syn. *Hemiclepsis siamensis*), designated as the type species. *Orientobdelloides* species are mainly characterized by a single pair of eyespots, esophageal glands, and male and female gonopores separated by two annuli (Bolotov et al., 2022). Currently, the genus comprises three turtle-associated species: *O. siamensis*, *Orientobdelloides sirikanchanae* (Trivalairat, Chiangkul & Purivirojkul, 2019), and *Orientobdelloides tridens* (Chiangkul, Trivalairat, Kunya & Purivirojkul, 2021), all described from Thailand. *Orientobdelloides siamensis* has

a wider distribution, with records from China and Malaysian Borneo (Yang, 1996; Asad et al., 2021), whereas the remaining two species have not been recorded since their original descriptions.

MATERIALS AND METHODS

A Black marsh turtle, *Siebenrockiella crassicollis* (Gray, 1831), was collected in October 2018 from Mancak, Serang, Banten Province, Java Island, Indonesia (6°07'16"S, 106°00'10"E), and was found to be parasitized by *Orientobdelloides* leeches. The leeches were removed from the turtle body, fixed in 99% ethanol, and a quarter of the caudal sucker was taken from representative specimens for DNA extraction. The remaining bodies were then fixed in 10% formalin and preserved in 70% ethanol. The following four body measurements were recorded: 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 margins of the sucker (CW). Specimens were examined, dissected, and drawn under stereomicroscopes (Leica M125C and M165C; Leica Microsystems GmbH, Wetzlar, Germany) equipped with drawing tubes. Specimens used in this study have been deposited in the Museum Zoologicum Bogoriense (MZB). The somite

numbering convention is based on Moore (1927): body somites are denoted by Roman numerals and the annuli in each somite are given alphanumeric designations.

Total DNA of a specimen (MZB.Ann 147) was extracted from the caudal sucker using phenol/chloroform extraction (Sambrook and Russell, 2001). The partial sequence of mitochondrial cytochrome *c* oxidase subunit I (COI) and the region from tRNA^{Leu} to NADH dehydrogenase subunit 1 (ND1) were PCR-amplified using the primers LCO1490–HCO2198 (Folmer et al., 1994) and LND300–HND1932 (Light and Siddall, 1999), respectively. The PCR and cycle sequencing reactions were performed according to Nakano (2016) except for the use of the PCR reaction kit, EmeraldAmp PCR Master Mix (Takara Bio). The newly determined sequences were deposited in the International Nucleotide Sequence Databases (INSD) through the DNA Data Bank of Japan.

In addition to the newly obtained sequences, nine sequences of three *Orientobdelloides* species were included as ingroup, and two sequences of other glossiphoniid species were used as the outgroup in accordance with the previous phylogenetic study (Bolotov et al., 2022) (Table 1). The sequences retrieved from INSD were aligned using MAFFT v. 7.427 with the L-INS-i option (Kato and Standley, 2013) for each gene. The lengths of the COI and ND1 sequences were 658 and 594 bp,

respectively.

Phylogenetic trees were reconstructed with maximum likelihood (ML) and Bayesian inference (BI) methods. The best-fit partition scheme and substitution models for each alignment were identified with AICc (Sugiura, 1978) using IQ-TREE v. 1.6.12 (Nguyen et al., 2015): for COI, GTR + F + G; for ND1, HKY + F + I. The ML phylogeny was inferred using IQ-TREE and nonparametric bootstrapping (BS) was subsequently conducted with 1000 replicates. BI tree and Bayesian posterior probabilities (PP) were obtained using MrBayes v. 3.2.6 (Ronquist et al., 2012). Two independent runs of four Markov chains were conducted for two million generations and a tree was sampled every 1000 generations. Parameter estimates and convergence were checked using Tracer v. 1.7.1 (Rambaut et al., 2018) and the first 10% of trees were discarded based on the result. Pairwise comparisons of the uncorrected *p*-distance for COI between the specimen obtained in this study and other *Orientobdelloides* species were calculated using MEGA X (Kumar et al., 2018).

RESULTS

The trident-shaped crop caeca, elongated fourth intestinal caeca, and mitochondrial sequence data confirmed the identity of the *Orientobdelloides* specimens as *O. tridens*. ML

(ln L = -4724.04; Fig. 1) and BI (ln L = -4744.25; not shown) phylogenetic analyses yielded identical topologies. The Indonesian specimen was clustered into a well supported clade (BS = 100, PP = 1.0) that comprised two other *Orientobdelloides* species. Within this clade, it was sister to *O. tridens* from Thailand (BS = 75, PP = 0.98), with an uncorrected p -distance of 5.3% for the mitochondrial COI marker.

***Orientobdelloides tridens* (Chiangkul, Trivalairat, Kunya & Purivirojkul, 2021)**

(Figs. 2–6)

(Syn. *Placobdelloides tridens* Chiangkul et al., 2021; *Orientobdelloides tridens* Bolotov et al., 2022)

Diagnosis (amended from Chiangkul et al., 2021)

Caudal sucker ventral, oval. Anus between somites XXVI/XXVII. Male gonopore between somites XI/XII, female gonopore between somite XII a2/a3, gonopores separated by two annuli. Eyespots in 1 pair, close to each other. Dorsal papillae on every mid-body annulus. Mouth pore on anterior margin of oral sucker. Salivary glands in 1 pair, compact. Oesophagus long, surrounded by gland cells (oesophageal organ) close to its proximal end. Crop giving rise to 7 pairs of crop caeca, 1st–6th pairs diverticulated into two or three sections, 7th pair (post-crop caeca) into multiple sections. Intestine giving rise to 4 pairs of

103 intestinal caeca, 4th pair elongated. Intersomital testisacs in 6 pairs. Paired sperm ducts
104 thick, highly coiled. Atrial cornua developed ovate. Paired ovisacs tubular, reaching to
105 somite XV a2.

106 **Description**

107 Body ovate or lanceolate (Fig. 2–3). Caudal sucker ventral, oval. Measurements
108 (mean $\pm$ 1SD, followed by ranges in parenthesis; n = 4): BL 12.0 ± 1.80 mm (9.73–13.9
109 mm), BW 3.78 ± 1.34 mm (2.65–5.63 mm), CL 2.03 ± 0.57 mm (1.65–2.85 mm), CW 1.94
110 ± 0.40 mm (1.67–2.53 mm). Somite I completely merged with prostomium. Somites II (=
111 peristomium), III–IV uniannulate; Somites V uniannulate with slight dorsal furrow. Somites
112 VI–VII biannulate, $(a1 + a2) > a3$. Somites VIII–XXIV triannulate, $a1 = a2 = a3$. Somites
113 XXV–XXVII uniannulate. Anus in XXVI/XXVII. Clitellum unobservable. Male gonopore
114 in XI/XII. Female gonopore in XII a2/a3. Gonopores separated by two annuli. Eyes in one
115 pair, close to each other, on Somites II. Dorsal papillae on III–XXVII, ≤ 24 on every mid-
116 body annulus and caudal sucker of mature individuals. Dorsal papillae unobservable in
117 juveniles. Nephridiopores undetectable. Mouth pore on anterior margin of oral sucker.
118 Proboscis cylindrical, slender, in membranous sheath reaching to XI/XII (Fig. 4). One pair
119 of retractor muscles slender, attached to dorsal side of basal part of proboscis. Salivary

120 glands in one pair, compact between X a1–XI a2, bundle of ductules inserting into base of
121 proboscis in XI (Fig. 5). Oesophagus long, slender, surrounded by gland cells (oesophageal
122 organ) just short of its junction with crop, folds with retraction of proboscis. Oesophageal
123 organ pigmented (Fig. 5), in XII a2–XIII a1. Crop in XIII–XIX, giving rise to seven pairs
124 of crop caeca (Fig. 4); 1st–6th pairs of crop caeca diverticulated into two or three sections
125 when engorged: 1st pair in XII a3–a3; 2nd pair in XIV a1–a3; 3rd pair in XV a1–a3; 4th
126 pair in XVI a1–a3; 5th pair in XVII a1–a3; 6th pair in XVIII a1–a3; and 7th pair (post-crop
127 caeca) diverticulated into multiple sections, in XIX a1–XXIII a1. Intestinal caeca in four
128 pairs (Fig. 4), overlying post-crop caeca on dorsal side, in XX–XXIII: 1st pair in XX a1–
129 a3, directed antero-laterally; 2nd pair in XX a3–XXI a2, directed antero-laterally; 3rd pair
130 in XXII a1–a2, initially directed antero-laterally, bends backwards to be directed postero-
131 laterally; 4th pair in XXII a3–XXIII a3, directed postero-laterally, elongated more than
132 other three pairs. Rectum simple tubular (Fig. 4). Testisacs in six pairs, intersomital (Fig.
133 4): 1st pair in XIII a3–XIV a1; 2nd pair in XIV a3–XV a1; 3rd pair in XV a3–XVI a1; 4th
134 pair in XVI a3–XVII a1; 5th pair in XVII a3–XVIII a1; 6th pair in XVIII a3–XIX a1.
135 Paired sperm ducts thick, coiled, usually running posteriorly alongside ventral nerve cord,
136 subsequently turned anteriorly toward male gonopore (Fig. 6). One pair of atrial cornua

muscular, developed ovate in XI a3–XII a1 (Fig. 6). One pair of ovisacs tubular, thin walled, running alongside ventral nerve cord, XII a3 to XIII a1–XV a2 (Fig. 6).

Taxonomic summary

Type host: Malayan giant turtle, *Orlitia borneensis* Gray, 1873 (Testudines, Geoemydidae) (Chiangkul et al., 2021).

Type locality/collection date: Nakhon Ratchasima Zoo, Chai Mongkhon Subdistrict, Mueang District, Ratchasima Province, Thailand, 14°51'01.1"N; 102°04'28.8"E, 16 January 2019 (Chiangkul et al., 2021).

Current record: Black marsh turtle, *Siebenrockiella crassicollis* (Testudines, Geoemydidae), Mancak, Serang, Banten Province, Java Island, Indonesia, 6°07'16"S; 106°00'10"E.

Site of infection: Skin of toes, hands, and neck.

Intensity: 511 leeches in one host turtle.

Specimens deposited: Four leech specimens, MZB.Ann 144–147; remaining 507 specimens, MZB.Ann 143. One host turtle specimen, MZB.Test 741. Partial sequences of mitochondrial COI (LC812271) and ND1 (LC812272) for MZB.Ann 147, in INSD.

Specimens examined: Four specimens, MZB.Ann 144–147, dissected.

DISCUSSION

Orientobdelloides tridens is characterized by its trident-shaped crop caeca and elongated fourth intestinal caeca (Chiangkul et al., 2021). These unique features are absent in other congeneric species but are present in our Indonesian specimens. The type specimen of *O. tridens* shows more slender and deeply branched crop caeca compared to the Indonesian specimens (Chiangkul et al., 2021), which may be due to differences in the degree of engorgement, as known in other glossiphoniid leeches (Kambayashi and Nakano, 2023).

While the male gonopore of the Thai *O. tridens* was previously described as opening in XII a2/a3 (Chiangkul et al., 2021), our re-examination of annulation patterns in the original figures suggests a location in the furrow between the two somites, possibly in XI/XII.

Given that male and female gonopores were shown to be separated by two annuli, the female gonopore of the Thai specimens likely resides in XII a2/a3, which aligns with our observations on the Indonesian specimens. The molecular analyses further supported a close relationship between the Indonesian specimens and *O. tridens* from Thailand. The genetic distance between them (5.3%) was comparable to the intraspecific variation observed in *O. sirikanchanae* (5.2%). It also fell within the range of the intraspecific variation in *O. siamensis* (1.4%–14.5%) and below the reported interspecific variation in

other glossiphoniid species (6%–7%) (Kaygorodova et al., 2020). Therefore, we herein identified our specimens as *O. tridens*.

Chiangkul et al. (2021) described *O. tridens* with sperm ducts that initially bulge, extend posteriorly, and then recurve anteriorly in front of the post-crop caeca to connect to the testisacs. In contrast, the present specimens exhibit highly coiled paired sperm ducts situated beside the ovisacs and running along the ventral nerve cord. The original description of *O. tridens* by Chiangkul et al. (2021) included a rugby ball-shaped “spermatheca” with two ducts entering bifurcated ovisacs, a structure not observed in our specimens. Based on the positions of the reproductive organs in the Thai *O. tridens*, this “spermatheca” likely represents the highly coiled sperm ducts and ovisacs we observed in the Indonesian specimens. Consequently, the original diagnosis of *O. tridens* is likely to include such misinterpretations, and herein amended to account for these differences.

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FIGURE LEGENDS

Figure 1. Maximum-likelihood phylogenetic tree inferred from 1252 bp of concatenated mitochondrial COI and ND1 gene sequences. Numbers on nodes represent bootstrap values for maximum likelihood and Bayesian posterior probabilities.

Figures 2–3. *Orientobdelloides tridens* (Chiangkul, Trivalairat, Kunya & Purivirojkul, 2021), from Indonesia, MZB. Ann 147. **2.** Dorsal view. **3.** Ventral view. A portion of the caudal sucker was removed from the specimen for DNA extraction.

Figures 4–6. *Orientobdelloides tridens* (Chiangkul, Trivalairat, Kunya & Purivirojkul, 2021), from Indonesia, MZB. Ann 147. **4.** Dorsal view of the digestive tract and male genital organs. **5.** Dorsal view of the anterior part of the digestive tract. **6.** Dorsal view of the reproductive system and positions of ganglia XI and XII. Abbreviations: ac, atrial cornu; cc, crop cecum; fg, female gonopore; ic, intestinal cecum; mg, male gonopore; oes, oesophagus; ov, ovisac; pb, proboscis; pcc, post-crop cecum; rm, retractor muscle; rt, rectum; sd, sperm duct; sg, salivary gland; ts, testisac.