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| Author(s)   | Nakano, Takafumi; Jeratthitikul, Ekgachai; Nguyen, Thien Tao; Panha, Somsak  |
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## A new species of *Tritetrabdella* (Hirudinida: Hirudiniformes: Haemadipsidae) from northern Indochina

Takafumi Nakano<sup>1,2\*</sup>, Ekgachai Jeratthitikul<sup>3</sup>, Tao Thien Nguyen<sup>4</sup> & Somsak Panha<sup>5</sup>

**Abstract.** A new species of the terrestrial haemadipsid genus *Tritetrabdella*, *Tritetrabdella longiducta*, from northern Indochina is described. The new species is distinguished from all congeners by the combination of triannulate somite VII, uniannulate somite XXV, three lobes of respiratory auricles in somites XXV–XXVII, 57 friction rays on caudal sucker, male gonopore (in somite XI b5/b6) and female gonopore (in somite XII b5) positions and slightly folded vaginal sac. Phylogenetic analyses using nuclear 18S rRNA and 28S rRNA, in addition to mitochondrial cytochrome *c* oxidase subunit I markers, confirmed that the new species forms a unique lineage among the known congeners. A key to all the species of *Tritetrabdella* is provided.

**Key words.** Hirudinida, Haemadipsidae, *Tritetrabdella*, phylogenetic analyses, Thailand, Vietnam

### INTRODUCTION

The haemadipsid genus *Tritetrabdella* Moore, 1938, is a taxon of terrestrial blood-sucking leeches, for which the primary hosts are amphibians (Lai & Chen, 2010). Although members of *Tritetrabdella* are trignathous (three-jawed) leeches like the other haemadipsid genera, this genus is distinguished from other haemadipsid taxa, which have mid-body somites that are quinquannulate (5 annuli), by its quadrannulate (4 annuli) mid-body somites. Molecular phylogenetic analyses of Haemadipsidae revealed that *Tritetrabdella* is closest to the trignathous, quinquannulate *Haemadipsa cavatuses* Yang, Mo & Wang, 2009, which was found in a cave in Yunnan Province, China, among the haemadipsid species that have been sequenced (Borda & Siddall, 2011).

*Tritetrabdella* contains three species: *T. scandens* Moore, 1938 (type species), *T. kinabaluensis* Kappes, 2013, and *T. taiwana* (Oka, 1910). *Tritetrabdella scandens* was first described from Penang Hill, Malaysia, and has also been reported from southern Thailand (Borda & Siddall, 2011). *Tritetrabdella kinabaluensis* was described from Borneo,

Malaysia, and has been split into two subspecies (Kappes, 2013): *T. k. kinabaluensis* from high mountains (elevation > 1,500 m) and *T. k. inobongensis* Kappes, 2013, which inhabits lowlands (elevation < 500 m). The last species, *T. taiwana*, has been recorded in Taiwan, Hong Kong, and Guangxi Zhuang Autonomous Region, China (Keegan et al., 1968; Lai & Chen, 2010; Borda & Siddall, 2011; Lai et al., 2011; Yuen & Nakano, 2012). Molecular phylogenetic studies have revealed cryptic diversity in *T. taiwana* (Kappes, 2013; Nakano & Sung, 2014). However, no nominal species or subspecies have been established from the *T. taiwana* complex.

Distribution records for *Tritetrabdella* in Indochina are very limited. Although Ngamprasertwong et al. (2005, 2007) reported *T. scandens* and *T. taiwana* from Thailand, these records lack detailed information on collecting localities, morphological characteristics, and molecular data. Recently, the second author collected a *Tritetrabdella* specimen from northeastern Thailand, while the third author collected another *Tritetrabdella* leech from northwestern Vietnam. This is the first record of this genus from Vietnam. These specimens are described here as a new species based on morphological and molecular data. The phylogenetic position of this new species was estimated using nuclear 18S and 28S rRNA, in addition to mitochondrial cytochrome *c* oxidase subunit I sequence data.

### MATERIAL AND METHODS

**Sampling and morphological examination.** Leeches were collected from Doi PhuKa, Nan Province, Thailand, and Ta Co, Son La Province, Vietnam (Fig. 1). When possible, elevation and geographical coordinates for localities were obtained using a Garmin eTrex® GPS unit.

<sup>1</sup>Department of Science Education, Graduate School of Education, Hiroshima University, Higashihiroshima 739-8524, Japan; Email: tnakano@hiroshima-u.ac.jp (\* corresponding author)

<sup>2</sup>Department of Zoology, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan.

<sup>3</sup>Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

<sup>4</sup>Vietnam National Museum of Nature, Vietnam Academy of Science and Technology, Hanoi, Vietnam.

<sup>5</sup>Animal Systematics Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.

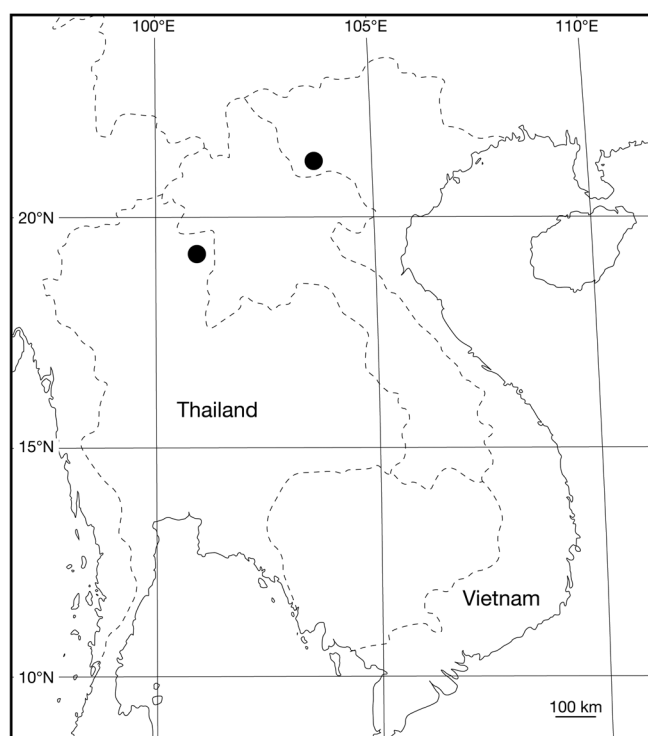


Fig. 1. Map showing the collection localities of the specimens examined in this study.

The specimen collected from Doi Phuka was relaxed by the gradual addition of absolute ethanol to freshwater. The specimen, from Son La, was directly preserved in absolute ethanol. For DNA extraction, botryoidal tissue was removed from the posterior part of the body around the caudal sucker of each specimen, and then preserved in absolute ethanol. The remainder of the body was fixed in 10% formalin and preserved in 70% ethanol. Four measurements were taken: body length (BL) from the anterior margin of the oral sucker to the posterior margin of the caudal sucker, maximum body width (BW), caudal sucker length (CL) from the anterior to the posterior margin of the sucker and caudal sucker width (CW) from the right to the left margin of the sucker. Examination, dissection, and drawing of the specimens were conducted using a stereoscopic microscope with a drawing tube (Leica M125). The specimens from Thailand and Vietnam have been deposited in the Zoological Museum of the Chulalongkorn University (CUMZ) and the biological collection of the Vietnam National Museum of Nature (VNMN), respectively. The numbering convention is based on Moore (1927): body somites are denoted by Roman numerals and the annuli in each somite are given alphanumeric designations.

**PCR and DNA sequencing.** The extraction of genomic DNA from botryoidal tissues preserved in absolute ethanol followed Nakano (2012a). Primer sets for the PCR and cycle sequencing (CS) reactions used in this study were as follows: for partial 18S rRNA, A and L (PCR and CS), C and Y (PCR and CS), as well as O and B (PCR and CS) (Apakupakul et al., 1999); for partial 28S rRNA, LR0R (PCR and CS) (Cubeta et al., 1991), LR3 (CS) (Vilgalys & Hester, 1990) and 28nn (PCR & CS) (Passamanek et al., 2004), 28F1-2

(Passamanek et al., 2004) and 28ff (Hillis & Dixon, 1991) (PCR and CS), 28F2-2 and 28R2 (PCR and CS) (Passamanek et al., 2004), as well as 28F2\_3 (Jördens et al., 2004) and 28R3 (Passamanek et al., 2004) (PCR and CS); for partial cytochrome *c* oxidase subunit I (COI), LCO 1490 (PCR and CS) and HCO2198 (CS) (Folmer et al., 1994), and LCO-inTri (CS) (Nakano & Sung, 2014) and HCO-out (PCR and CS) (Nakano, 2012a). The PCR reaction and DNA sequencing were performed using the modified methods mentioned in Nakano (2012b). The PCR reactions were performed using a GeneAmp PCR System 2700 and a GeneAmp PCR System 9700 (Applied Biosystems) as well as a T100 Thermal Cycler (Bio-Rad). The PCR reaction mixtures were heated to 95°C for 5 min, followed by 35 cycles at 94°C (10 s each), 60°C, 60°C and 42°C, respectively, for each part of 18S, 50°C for 28S, or 48°C for COI (20 s), and 72°C (42 s for 18S, 48S for 28S, or 1 min 12 s for COI), and a final extension at 72°C for 6 min. The sequencing mixtures were heated to 96°C for 2 min, followed by 40 cycles at 96°C (10 s), 50°C (5 s), and 60°C (48 s each). The obtained sequences were edited using DNA BASER (Heracle Biosoft S.R.L.). The DNA sequences newly obtained in this study were deposited with the International Nucleotide Sequence Database Collaboration (INSDC) through the DNA Data Bank of Japan (DDBJ) (Table 1).

#### Molecular phylogenetic and genetic distance analyses.

Thirty-four published sequences were obtained from the INSDC for use in molecular phylogenetic analyses (Table 1). Three *Tritetrabdella* species were included in the analyses along with the following four haemadipsid species as outgroup taxa according to results of molecular phylogenetic analyses by Borda & Siddall (2011) and Tessler et al. (in press) (see also Figs. 6, 7 later): *Chtonobdella whitmani* (Lambert, 1899), *C. australis* (Richardson, 1969), *H. cavatuses*, and *H. zeylanica* (Moquin-Tandon, 1827).

The phylogenetic position of the newly identified *Tritetrabdella* species within the genus was estimated based on the gene fragments of 18S, 28S and COI sequences. The alignment of COI was trivial, as no indels were observed. 18S, and 28S were aligned using MAFFT v. 7.245 L-INS-i (Kato & Standley, 2013). The lengths of the 18S, 28S, and COI sequences were 1,832, 2,080, and 1,267 bp, respectively. The concatenated sequences yielded 5,179 bp of aligned positions.

Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI). ML phylogenies were constructed using RAxML v. 8.1.5 (Stamatakis, 2014) with the substitution model set as GTRCAT, immediately after nonparametric bootstrapping (Felsenstein, 1985) conducted with 1,000 replicates. The best-fit partitioning scheme for the ML analysis was identified with the Akaike information criterion (Akaike, 1974) using PartitionFinder v. 1.1.1 (Lanfear et al., 2012) with the “all” algorithm: 18S/28S/1st position of COI/2nd position of COI/3rd position of COI. BI and Bayesian posterior probabilities (PPs) were estimated using MrBayes v. 3.2.5 (Ronquist et al., 2012). The best-fit partition scheme and models for each partition were selected based on the Bayesian information

Table 1. Samples with voucher or isolate numbers, collection country and INSDC accession numbers used for molecular analyses. Sequences marked with an asterisk (\*) were obtained for the first time in the present study. Acronyms: CUMZ, Zoological Museum of the Chulalongkorn University; KUZ, Zoological Collection of Kyoto University; SP, Sabah Park; VNMN, Vietnam National Museum of Nature.

| Taxon                         | Voucher or Isolate Number | Country (Island)  | INSDC Accession Numbers |           |           |
|-------------------------------|---------------------------|-------------------|-------------------------|-----------|-----------|
|                               |                           |                   | 18S                     | 28S       | COI       |
| <i>Tritetrabdella</i>         |                           |                   |                         |           |           |
| <i>Tritetrabdella</i> sp.     | CUMZ 5100                 | Thailand          | LC099532*               | LC099533* | LC099534* |
| <i>Tritetrabdella</i> sp.     | VNMN 04733                | Vietnam           | LC099535*               | LC099536* | LC099537* |
| <i>T. scandens</i>            | TI49                      | Thailand          | HQ203117                | HQ203155  | HQ203194  |
| <i>T. k. kinabaluensis</i>    | SP13306                   | Malaysia (Borneo) |                         |           | KF839949  |
| <i>T. k. kinabaluensis</i>    | SP13398                   | Malaysia (Borneo) |                         |           | KF839948  |
| <i>T. k. inobongensis</i>     | SP13380                   | Malaysia (Borneo) |                         |           | KF839944  |
| <i>T. k. inobongensis</i>     | SP13381                   | Malaysia (Borneo) |                         |           | KF839945  |
| <i>T. k. inobongensis</i>     | SP13382                   | Malaysia (Borneo) |                         |           | KF839946  |
| <i>T. k. inobongensis</i>     | SP13383                   | Malaysia (Borneo) |                         |           | KF839947  |
| <i>T. taiwana</i>             | L141A                     | Taiwan            |                         |           | HQ322463  |
| <i>T. taiwana</i>             | L142A                     | Taiwan            |                         |           | HQ322464  |
| <i>T. taiwana</i>             | L143A                     | Taiwan            |                         |           | HQ322465  |
| <i>T. taiwana</i>             | L144A                     | Taiwan            |                         |           | HQ322466  |
| <i>T. taiwana</i>             | L146A                     | Taiwan            |                         |           | HQ322467  |
| <i>T. taiwana</i>             | L147A                     | Taiwan            |                         |           | HQ322468  |
| <i>T. taiwana</i>             | L150A                     | Taiwan            |                         |           | HQ322469  |
| <i>T. taiwana</i>             | TICH                      | China             | HQ203118                | HQ203156  | HQ203195  |
| <i>T. taiwana</i>             | KUZ Z196                  | China (Hong Kong) |                         |           | AB685259  |
| <i>T. taiwana</i>             | KUZ Z611                  | China (Hong Kong) |                         |           | AB823740  |
| <i>T. taiwana</i>             | KUZ Z612                  | China (Hong Kong) |                         |           | AB823741  |
| Outgroup                      |                           |                   |                         |           |           |
| <i>Chtonobdella australis</i> | AU76                      | Australia         | HQ203086                | HQ203123  | HQ203162  |
| <i>Chtonobdella whitmani</i>  | AU78C                     | Australia         | HQ203087                | HQ203124  | HQ203163  |
| <i>Haemadipsa cavatuses</i>   | HABL                      | Laos              | HQ203092                | HQ203129  | HQ203168  |
| <i>Haemadipsa zeylanica</i>   | HZSL                      | Sri Lanka         | HQ203106                | HQ203144  | HQ203183  |

criterion (Schwarz, 1978) using PartitionFinder with the “all” algorithm: for 18S and 28S, GTR+I; for the 1st position of COI, GTR+G; for the 2nd position of COI, F81+I; and for the 3rd position of COI, HKY+I+G. Two independent runs of four Markov chains were conducted for 10 million generations, and the tree was sampled every 100 generations. The parameter estimates and convergence were checked using Tracer v. 1.6.0 (Rambaut & Drummond, 2009) and the first 25,001 trees were discarded based on these results. Nodes with bootstrap support (BS) values higher than 70% were considered sufficiently resolved (Hillis & Bull, 1993). Nodes with PPs higher than 95% were considered statistically significant (Leaché & Reeder, 2002).

Pairwise comparisons of uncorrected *p*-distances for 20 COI sequences (655–1267 bp) obtained from the present *Tritetrabdella* specimens and those of its known species obtained from the INSDC (Table 1) were calculated using MEGA6.06 (Tamura et al., 2013). All missing positions were eliminated for each sequence pair.

For verifying whether the COI differences calculated above are synonymous or non-synonymous substitutions, amino acid substitutions per site were also calculated using MEGA6 based on the COI corresponding protein sequences

of 20 *Tritetrabdella* individuals and four outgroup taxa. The COI sequences were translated to the corresponding peptide sequences using transeq command implemented in EMBOSS v. 6.6.0 (Rice et al., 2000) with the invertebrate mitochondrial codon table, then aligned using MAFFT v. 7.266 FFT-INS-2. The lengths of the COI peptide sequences were 218–422 amino acid positions. Amino acid divergences were calculated with Poisson correction model. All missing positions were eliminated for each sequence pair. Based on these divergences, neighbour-joining (NJ) tree was constructed with nonparametric bootstrapping based on 1,000 replicates.

## TAXONOMY

### Family Haemadipsidae Blanchard, 1892

#### Genus *Tritetrabdella* Moore, 1938

#### *Tritetrabdella longiducta*, new species (Figs. 2–5)

**Material examined.** Holotype: hermaphrodite (CUMZ 5100), dissected, Doi PhuKa, Tambon Phu Kha, Nan Province, Thailand (19.201306°, 101.082250°; elevation 1286 m), coll.



Fig. 2. *Tritetrabdella longiducta*, new species, holotype (CUMZ 5100). A, dorsal view; B, ventral view. Scale bar = 3 mm.

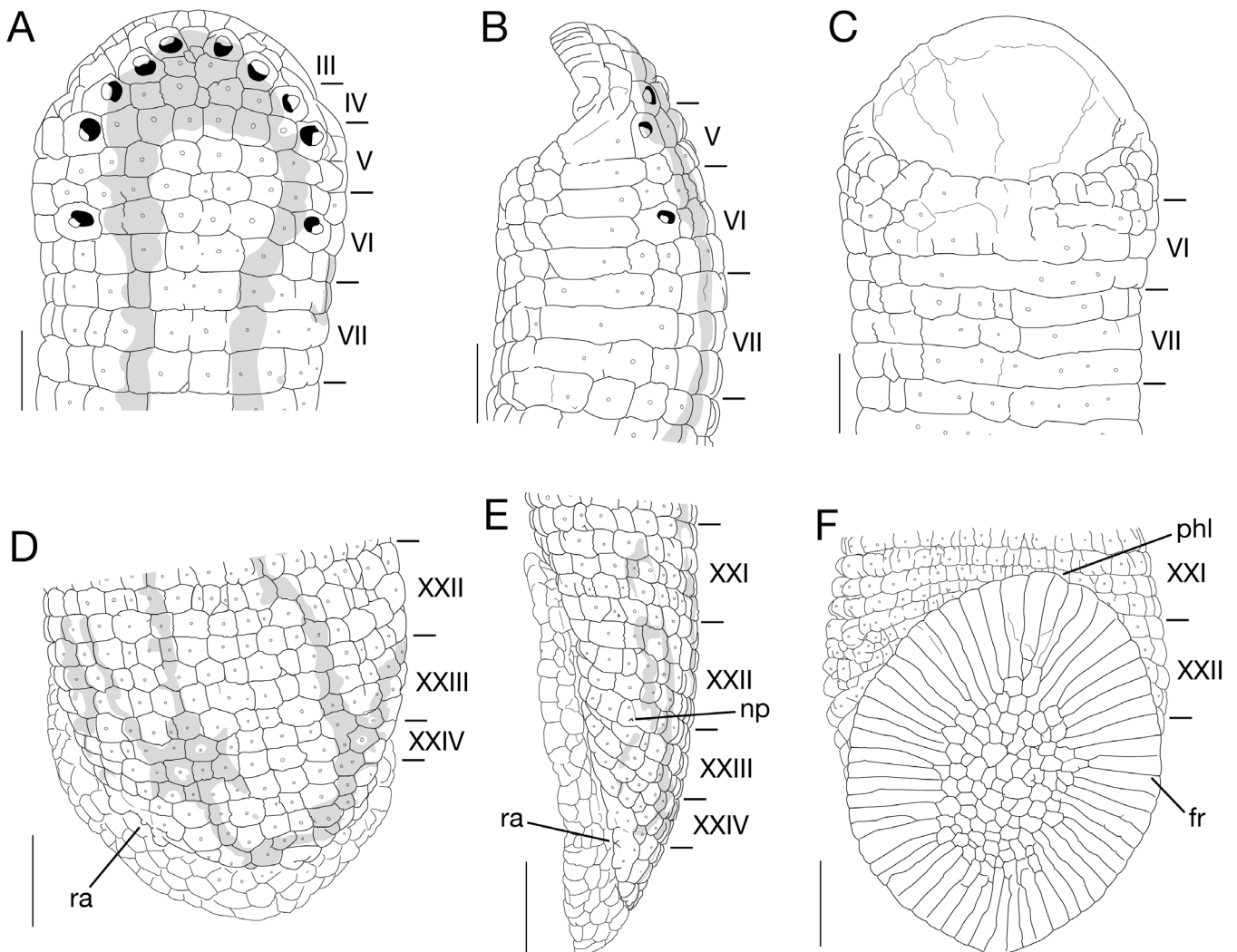


Fig. 3. *Tritetrabdella longiducta*, new species, holotype (CUMZ 5100). A, dorsal view of somites I–VII; B, lateral view of somites I–VII; C, ventral view of somites I–VII; D, dorsal view of somites XXII–XXVII and caudal sucker; E, lateral view of somites XXI–XXVII and caudal sucker; F, ventral view of somites XXI, XXII and caudal sucker. Abbreviations: fr, friction ray; np, nephridiopore; phl, prehensile lobe; ra, respiratory auricle. Scale bars = 1 mm.

E. Jeratthitikul, 13 October 2013. Paratype: hermaphrodite (VNMN 04733), dissected, Ta Co, Sop Cop District, Son La Province, Vietnam (20.96°, 103.6°; elevation 720–920 m), coll. T. T. Nguyen, 15 September 2014.

**Diagnosis.** Caudal sucker circular, with 57 friction rays. Somite VII triannulate, somites VIII–XXII quadrannulate, somite XXV uniannulate. Somites XXV–XXVII forming respiratory auricles. Male gonopore in somite XI b5/b6, female gonopore slightly anterior to middle of somite XII b5, gonopores separated by  $3 + 1/2$  annuli. Jaws trignathous, each jaw without salivary papillae. Paired epididymides in somite XIII b6 to somite XIV a2, occupying more than one full somite; from each epididymal mass, thick sperm ducts reaching to each ejaculatory bulb. Ejaculatory bulbs ellipsoidal, in somite XI b6 to somite XIII a1. Common oviduct length nearly equal with length of vaginal duct. Vaginal sac slightly folded, reaching to somite XIV a1/a2–b5.

**Description of holotype.** Body firm, muscular, with constant width posteriorly, BL 19.7 mm, BW 3.7 mm (Fig. 2). Caudal sucker ventral, elliptical, CL 4.3 mm, CW 2.57 mm; prehensile lobe undeveloped, not sharply hooked; with 57 friction rays (Figs. 2B, 3F).

Somite I completely merged with prostomium (Fig. 3A). Somite II uniannulate, comprising two paramedian ocular plates with one interocular plate and margins (Fig. 3A). Somite III uniannulate, comprising two ocular plates and two interocular plates with median small plates and margins (Fig. 3A). Somite IV uniannulate, comprising two ocular plates and four interocular plates with median small plate and margins (Fig. 3A). Somite V biannulate,  $(a1 + a2) > a3$ ,  $(a1 + a2)$  comprising two ocular plates and six interocular plates (Fig. 3A, B). Somite VI dorsally triannulate/ventrally biannulate,  $a1 = a2 = a3/(a1 + a2) > a3$ . Somite V to somite VI ( $a1 + a2$ ) unite altogether, forming posterior margin of oral sucker (Fig. 3A–C). Somite VII triannulate,  $a1 = a2 = a3$  (Fig. 3A–C). Somites VIII–XXII quadrannulate,  $a1 = a2 = b5 = b6$  (Figs. 3D–F, 4A). Somite XXIII triannulate,  $a1 = a2 = a3$  (Fig. 3D, E). Somite XXIV biannulate,  $(a1 + a2) = a3$  (Fig. 3D, E). Somites XXV–XXVII uniannulate; ambilateral margins of each of somites XXV–XXVII forming three lobes of one pair of trilobate respiratory auricles, median lobe of somite XXVI smaller than other two lobes (Fig. 3D, E). Anus at posterior margin of somite XXVII.

Male gonopore in somite XI b5/b6 (Fig. 4A). Female gonopore slightly anterior to middle of somite XII b5 (Fig. 4A). Gonopores separated by  $3 + 1/2$  annuli.

Anterior ganglionic mass in somite VII a1 and somite VIII a1. Ganglion VII in somite VIII a1 and a2. Ganglion VIII in b5 and b6. Ganglion IX in b5. Ganglia X and XI of each somite, in a2 and b5 (Fig. 4B). Ganglion XII in b5. Ganglia XIII–XV of each somite, in a2 and b5 (Fig. 4B). Ganglia XVI–XVIII of each somite, in a2 (Fig. 4B). Ganglia XIX and XX of each somite, in a2 and b5 (Fig. 4B). Ganglion XXI in somite XX b6 and somite XXI a1 (Fig. 4B). Ganglion XXII in somite XXI a2 and b5 (Fig. 4B). Ganglion XXIII

in somite XXII a2 and b5 (Fig. 4B). Ganglion XXIV in somite XXIII a1 (Fig. 4B). Ganglion XXV in somite XXIII a2 (Fig. 4B). Ganglion XXVI in somite XXIII a2 and a3 (Fig. 4B). Posterior ganglionic mass in somite XXIII a3 to somite XXV (Fig. 4B).

Eyes 5 pairs, in parabolic arc; first pair on somite II, second pair on somite III, third pair on somite IV, fourth pair on somite V ( $a1 + a2$ ), and fifth pair on somite VI a2 (Fig. 3A, B). Sensillae developed, one row on every annulus. Furrow pit undetectable.

Nephridiopores in 13 pairs, one each situated laterally at posterior margin of a1 of each somite in somites XI–XXIII (Fig. 3E). Nephridiopores anterior to somite XI undetectable. Nephridiopores of somite XXIV under respiratory auricles.

Each jaw without salivary papillae; monostichodont, number of teeth of each jaw uncounted. Pharynx reaching to somite VIII/somite IX. Crop reaching to somite XIX b6–somite XX a2, bearing 10 pairs of crop caeca: first pair in somite X b6 and somite XI a1; second pair in somite XI b5 and b6; third pair in somite XII a2–b6; fourth pair in somite XIII a2–b6; fifth pair in somite XIV a1–b5; sixth pair in somite XV a2–b6; seventh pair in somite XVI a1 and a2; eighth pair in somite XVII a1–b5; ninth pair in somite XVIII a2 and b5; and 10th pair being post-crop caeca, right post-crop caecum in somite XIX a1 to somite XXIV ( $a1 + a2$ ), left post-crop caecum in somite XIX a1 to somite XXII b6. Intestine reaching to somite XXIII a1–somite XXIV ( $a1 + a2$ ). Rectum simple, tubular.

Testisacs nine pairs with an additional sac on left side: first pair, right testisac in somite XIII b6 and somite XIV a1, left testisac in somite XIV a1; second pair, right sac in somite XIV b6 and somite XV a1, left sac in somite XV a1 and a2; third pair in somite XV b5 to somite XVI a1; fourth pair in somite XVI b5 and b6; fifth pair, right sac in somite XVII b5 to somite XVIII a1, left sac in somite XVII a2–b6; sixth pair, right sac in somite XVIII b5 to somite XIX a1, left sac in somite XVIII b5 and b6; seventh pair in somite XIX a2–b6; eighth pair, right sac in somite XX b5 to somite XXI a1, left sac in somite XX a2–b6; ninth pair, right sac in somite XXI a1–b5, left sac in somite XX b6 to somite XXI a2; and an additional testisac on left side in somite XXI b6 to somite XXII a2 (Fig. 4B). Paired epididymides developed; right epididymis in somite XIII b6 to somite XV a1; and left epididymis in somite XIV a1 to somite XV a2; from each epididymal mass, thick sperm ducts reaching to each ejaculatory bulb (Fig. 4B–D). Ejaculatory bulbs developed, ellipsoidal; right bulb in somite XI b6 to somite XII b5; and left bulb in somite XI b6 to somite XII a2 (Fig. 4B–D). Ejaculatory ducts narrow, running inward toward male atrium in somite XI b5; left ejaculatory duct crossing ventrally beneath nerve cord (Fig. 4C, D). Male atrium globular in somite XI b5 and b6 (Fig. 4B–D).

Paired ovisacs globular, in somite XII b5 to somite XIII a1 (Fig. 4B, E, F). Oviducts short; left oviduct crossing ventrally beneath nerve cord; both oviducts converging into

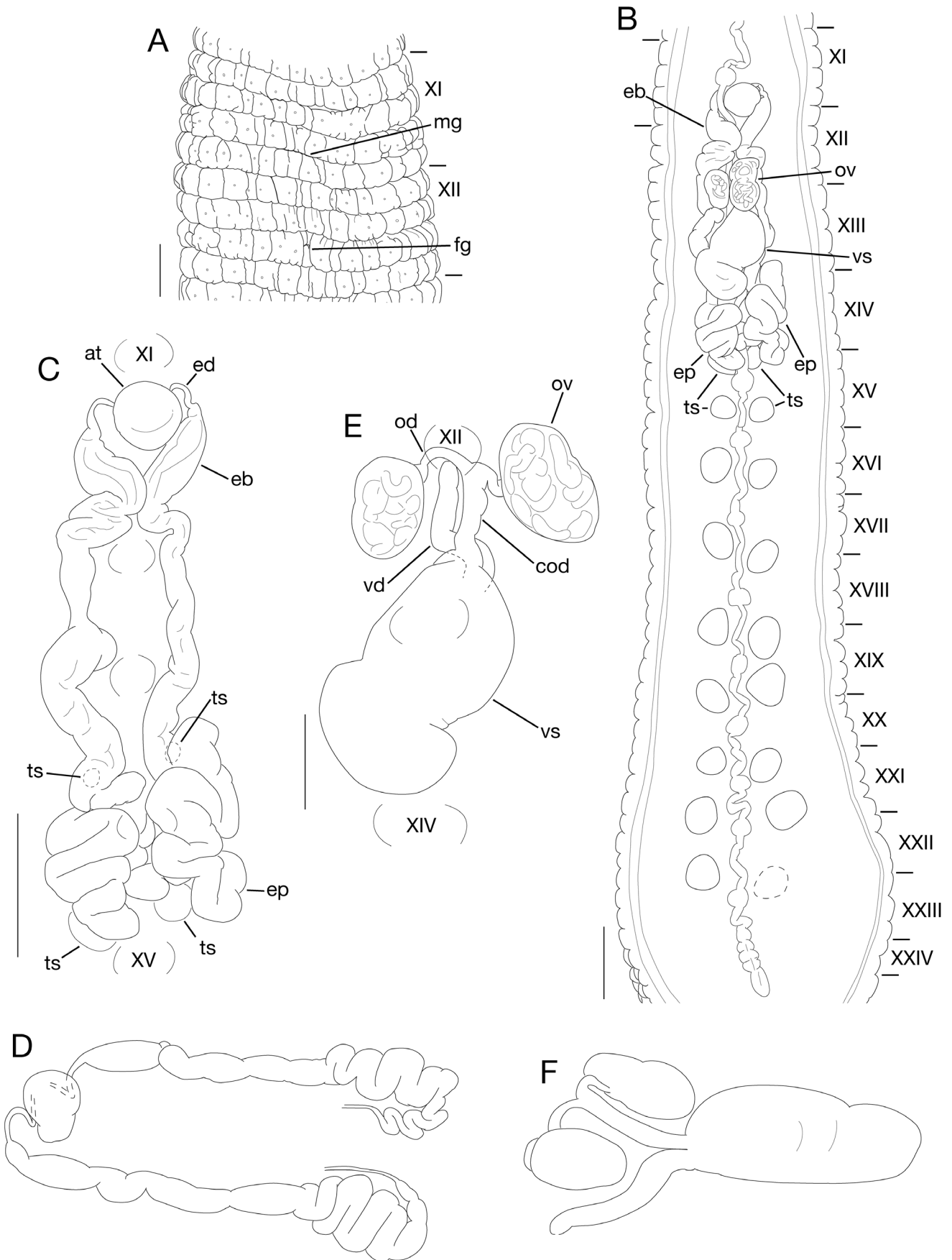


Fig. 4. *Tritetrabdella longiducta*, new species, holotype (CUMZ 5100). A, ventral view of somites XI and XII; B, dorsal view of reproductive system including ventral nervous system; C, dorsal view of male median reproductive system including positions of ganglia XI–XV; D, left lateral view of schematic drawing of male median reproductive system; E, dorsal view of female reproductive system including positions of ganglia XII–XIV; F, left lateral view of schematic drawing of female reproductive system. Abbreviations: at, atrium; cod, common oviduct; eb, ejaculatory bulb; ed, ejaculatory duct; ep, epididymis; fg, female gonopore; mg, male gonopore; od, oviduct; ov, ovisac; ts, testisac; vd, vaginal duct; vs, vaginal sac. Scale bars = 0.5 mm [A, E]; 1 mm [B, C].

common oviduct in somite XII b6 (Fig. 4E, F). Common oviduct descending to female vaginal sac in somite XII b6 to somite XIII a1/a2 (Fig. 4E, F). Vaginal sac slightly folded, reaching to somite XIV a1/a2 (Fig. 4B, E, F). Vaginal duct from somite XIII a2 to somite XII b5, directly descending to female gonopore (Fig. 4E, F).

**Variation.** BL 8.5 mm, BW 2.7 mm, CL 2.8 mm, CW 2.6 mm. Somite III uniannulate, comprising two ocular plates and three interocular plates with median small plates and margins. Somite IV uninnulate, comprising two ocular plates and five interocular plates with margins. Somite V biannulate,  $(a1 + a2) = a3$ ,  $(a1 + a2)$  comprising two ocular plates and eight interocular plates. Nephridiopores in somites IX and X detected. Male gonopore in somite XI b5/b6. Female gonopore in anterior margin of somite XII b5. Gonopores separated by 3 annuli at least. Paired epididymides; right epididymis in somite XIV b5 to somite XVI a1; and left epididymis in somite XIV b6 to somite XVI a2. Ejaculatory bulbs; right bulb in somite XII a2–b5; and left bulb in somite XII a2 to somite XIII a1. Paired ovisacs; right ovisac in somite XII b5 to somite XIII a1; left ovisac somite XII b5 and b6. Oviducts; right oviduct crossing ventrally beneath nerve cord. Common oviduct descending to female vaginal sac in somite XII b6 to somite XIII b6. Vaginal sac slightly folded, reaching to somite XIV b5. Vaginal duct from somite XIII b6 to somite XII b5.

**Colouration.** In life, dorsal surface yellowish clay with three brown longitudinal stripes with blackish borders; median stripe wider than lateral stripes, with irregular asymmetrical loops expanding toward lateral stripes (Fig. 5); ventral surface paler than dorsal surface. Color faded in preservative, but longitudinal stripes with blackish borders on dorsal surface remain.

**Distribution.** Known only from the type locality and Ta Co, Son La Province in northern Vietnam (Fig. 1). The elevations of the localities were more than 700 m above sea level. The individual from Doi Phuka was found on soil around a decayed banana tree containing very high moisture.

**Natural history.** The hosts are not known because the specimens examined were collected free-living, but are presumed to include amphibians (Lai & Chen, 2010). The Vietnamese individual was collected when it was climbing the third author; therefore, mammals may also be hosts of *T. longiducta*.

**Etymology.** The specific name is a compound adjective derived from the Latin words, *longus* (long), and *ductus* (duct), referring to the fact that the common oviduct of this species nearly equals the length of its vaginal duct, a diagnostic character of the species.

**Phylogenetic relationships and genetic distances.** Topologies of the BI (Fig. 6) and ML ( $\ln L = -13574.98$ ; not shown) trees for estimating the phylogenetic position of *T. longiducta* differed: *T. taiwana* did not form a clade in the BI tree but did in the ML tree (BS = 77%); *T. kinabaluensis*



Fig. 5. *Tritetradella longiducta*, new species, holotype (CUMZ 5100). Dorsal view of a live animal.

was a sister lineage of the clade consisting of *T. scandens* and *T. longiducta* in the BI tree, but was a sister lineage of the clade comprising of the other three species in the ML tree (BS = 66%). According to the ML and BI trees, *Tritetradella* comprised five lineages (hereafter referred to as lineages A–E). Lineage A (BS = 98%, PP = 0.99) included only Taiwanese *T. taiwana*. Lineage B (BS = 94%, PP = 0.99) contained *T. taiwana* collected from Taiwan and mainland China, including Hong Kong. Within lineage B, the monophyly of the Taiwanese (L00141A, L00146A, L00147A, and L00150A) and mainland Chinese (TICH, KUZ Z196, Z611, and Z612) specimens was recovered (Taiwanese, BS = 99%, PP = 0.99; Chinese, BS = 73, PP = 0.98). Lineage C (BS = 98, PP = 1.0) contained only *T. kinabaluensis*. The monophyly of the subspecies *T. k kinabaluensis* (SP13306 and SP13398) was not supported by the either analysis. Lineage D consisted only of *T. scandens*. In lineage E, the monophyly of our specimens was well-supported (BS = 100, PP = 1.0). Lineages D and E formed a clade, but this relationship was not supported by the BI analysis (BS = 82%, PP = 0.54).

The COI uncorrected *p*-distance between the *T. longiducta* specimens was 2.8% (Table 2). The COI uncorrected *p*-distances between lineage E (= *T. longiducta*) and each of the remaining lineages were as follows: lineage A (= Taiwanese *T. taiwana*), 15.7–16.3%; lineage B (Taiwanese and mainland Chinese *T. taiwana*), 11.6–13.7%; lineage C (= *T. kinabaluensis*), 14.1–15.2%; and lineage D (= *T. scandens*), 12.8%.

The COI amino acid divergence between the *T. longiducta* specimens was 0.24% (Table 3). Therefore, only one nonsynonymous substitution was detected among the 36 variable sites of their two COI sequences. The COI amino acid divergences within each of the remaining lineages were as follows: lineage A, 0.46%; lineage B, 0.46%; lineage C, no variable site was detected. The NJ tree based on the COI corresponding peptide sequences clearly recovered the monophyly of lineage E (BS = 93%) (Fig. 7).

**Remarks.** The new species unambiguously belongs to *Tritetradella*, as it has the following generic diagnostic characteristics: mid-body somite quadrannulate; and jaws trignathous, each jaw without salivary papillae. The two specimens from Thailand and Vietnam both possess the following morphological characteristics: triannulate somite



Table 2. Uncorrected *p*-distances for the available cytochrome *c* oxidase subunit I sequences of *Tritetrabdella* leeches. Acronym: CUMZ, Zoological Museum of the Chulalongkorn University; KUZ, Zoological Collection of Kyoto University; SP, Sabah Park; VNMIN, Vietnam National Museum of Nature.

| Species                         | Voucher or Isolate Number | Length (bp) | (1)   | (2)   | (3)   | (4)   | (5)   | (6)   | (7)   | (8)   | (9)   | (10)  | (11)  | (12)  | (13)  | (14) |
|---------------------------------|---------------------------|-------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------|
| (1) <i>T. longiducta</i>        | CUMZ 5100                 | 1267        |       |       |       |       |       |       |       |       |       |       |       |       |       |      |
| (2) <i>T. longiducta</i>        | VNMIN 04733               | 1267        | 0.028 |       |       |       |       |       |       |       |       |       |       |       |       |      |
| (3) <i>T. scandens</i>          | TI49                      | 1215        | 0.128 | 0.128 |       |       |       |       |       |       |       |       |       |       |       |      |
| (4) <i>T. taiwana</i>           | TICH                      | 655         | 0.137 | 0.133 | 0.128 |       |       |       |       |       |       |       |       |       |       |      |
| (5) <i>T. taiwana</i>           | KUZ Z196, Z611, Z612      | 1267        | 0.118 | 0.116 | 0.126 | 0.018 |       |       |       |       |       |       |       |       |       |      |
| (6) <i>T. taiwana</i>           | L00141A, 00147A, 00150A   | 658         | 0.125 | 0.126 | 0.123 | 0.049 | 0.049 |       |       |       |       |       |       |       |       |      |
| (7) <i>T. taiwana</i>           | L00146A                   | 658         | 0.128 | 0.126 | 0.123 | 0.052 | 0.052 | 0.003 |       |       |       |       |       |       |       |      |
| (8) <i>T. taiwana</i>           | L00142A                   | 658         | 0.163 | 0.157 | 0.141 | 0.119 | 0.114 | 0.119 | 0.119 |       |       |       |       |       |       |      |
| (9) <i>T. taiwana</i>           | L00143A                   | 658         | 0.160 | 0.157 | 0.141 | 0.116 | 0.120 | 0.116 | 0.119 | 0.058 |       |       |       |       |       |      |
| (10) <i>T. taiwana</i>          | L00144A                   | 658         | 0.157 | 0.153 | 0.133 | 0.110 | 0.109 | 0.109 | 0.112 | 0.049 | 0.049 |       |       |       |       |      |
| (11) <i>T. k. kinabaluensis</i> | SP13306                   | 658         | 0.141 | 0.144 | 0.139 | 0.128 | 0.128 | 0.117 | 0.120 | 0.144 | 0.132 | 0.129 |       |       |       |      |
| (12) <i>T. k. kinabaluensis</i> | SP13398                   | 658         | 0.143 | 0.146 | 0.147 | 0.131 | 0.131 | 0.117 | 0.120 | 0.149 | 0.134 | 0.134 | 0.009 |       |       |      |
| (13) <i>T. k. inobongensis</i>  | SP13380                   | 658         | 0.149 | 0.152 | 0.150 | 0.134 | 0.132 | 0.131 | 0.134 | 0.144 | 0.141 | 0.144 | 0.046 | 0.049 |       |      |
| (14) <i>T. k. inobongensis</i>  | SP13381–13383             | 658         | 0.147 | 0.150 | 0.150 | 0.133 | 0.131 | 0.131 | 0.134 | 0.147 | 0.143 | 0.146 | 0.043 | 0.046 | 0.005 |      |

VII; uniannulate somite XXV; somites XXV–XXVII forming respiratory auricles; male gonopore in somite XI b5/b6; female gonopore slightly anterior to middle of somite XII b5; paired epididymides in posterior of somite XIII to anterior of somite XIV, occupying more than one full somite; ejaculatory bulbs ellipsoidal, in posterior of somite XI to anterior of somite XIII; common oviduct length nearly equal with length of vaginal duct; and vaginal sac slightly folded, reaching to anterior to middle of somite XIV. The obtained molecular phylogenies showed that the two specimens examined in this study formed a well-supported clade. In addition, the calculated COI uncorrected *p*-distance between the two individuals was 2.8%. The calculated COI corresponding peptide sequence divergence between them was 0.24% (only one mutation was detected among 422 amino acid positions). This value was smaller than the values calculated within two lineages of *T. taiwana* (Table 3). Therefore, both specimens can be considered to belong to the same species, *T. longiducta*, based on their morphological characteristics along with molecular analyses results.

Based on taxonomic studies (Moore, 1938; Lai et al., 2011; Kappes, 2013), *T. longiducta* could be distinguished from congeners by the following combination of characteristics (Table 4): somite VII triannulate (versus quadrannulate in *T. scandens*); somite XXV uniannulate (versus biannulate in *T. kinabaluensis* and *T. taiwana*); somites XXV–XXVII forming three lobes of respiratory auricles [versus somite XXV (a1 + a2) to somite XXVI forming respiratory auricles in *T. kinabaluensis* and *T. taiwana*]; friction rays 57 (versus 55 in *T. kinabaluensis*, 56–59 in *T. scandens* and 57–61 in *T. taiwana*); male gonopore in somite XI b5/b6 (versus in somite XI a2/b5 in *T. scandens*); female gonopore in somite XII b5 (versus in somite XII a2–a2/b5 in *T. scandens*); and vaginal sac slightly folded (versus unfolded in *T. kinabaluensis* and *T. taiwana*).

The phylogenetic trees failed to reconstruct the precise phylogenetic relationships among the *Tritetrabdella* species. However, our ML tree indicated that *T. longiducta* formed a supported clade with *T. scandens*. Both species shared the following characteristics: somites XXV–XXVII forming respiratory auricles; and vaginal duct slightly folded. However, this relationship was not recovered by our BI analysis. To elucidate the precise phylogenetic position of *T. longiducta*, further molecular sequence data and morphological examination based on a large number of *Tritetrabdella* specimens would be essential.

The discovery of *T. longiducta* from northern Indochina sheds light on the cryptic diversity of *Tritetrabdella* in this region. Ngamprasertwong et al. (2005, 2007) stated that *T. taiwana* and *T. scandens* were distributed in Thailand. Therefore, the taxonomic status and distribution records of *Tritetrabdella* leeches provided by Ngamprasertwong et al. (2005, 2007) should be revisited. The *T. longiducta* specimens were collected from localities at elevations greater than 700 m; therefore, this species might be more broadly distributed in the mountainous regions of northern Indochina, including Thailand, Laos, and Vietnam. Further faunal surveys and

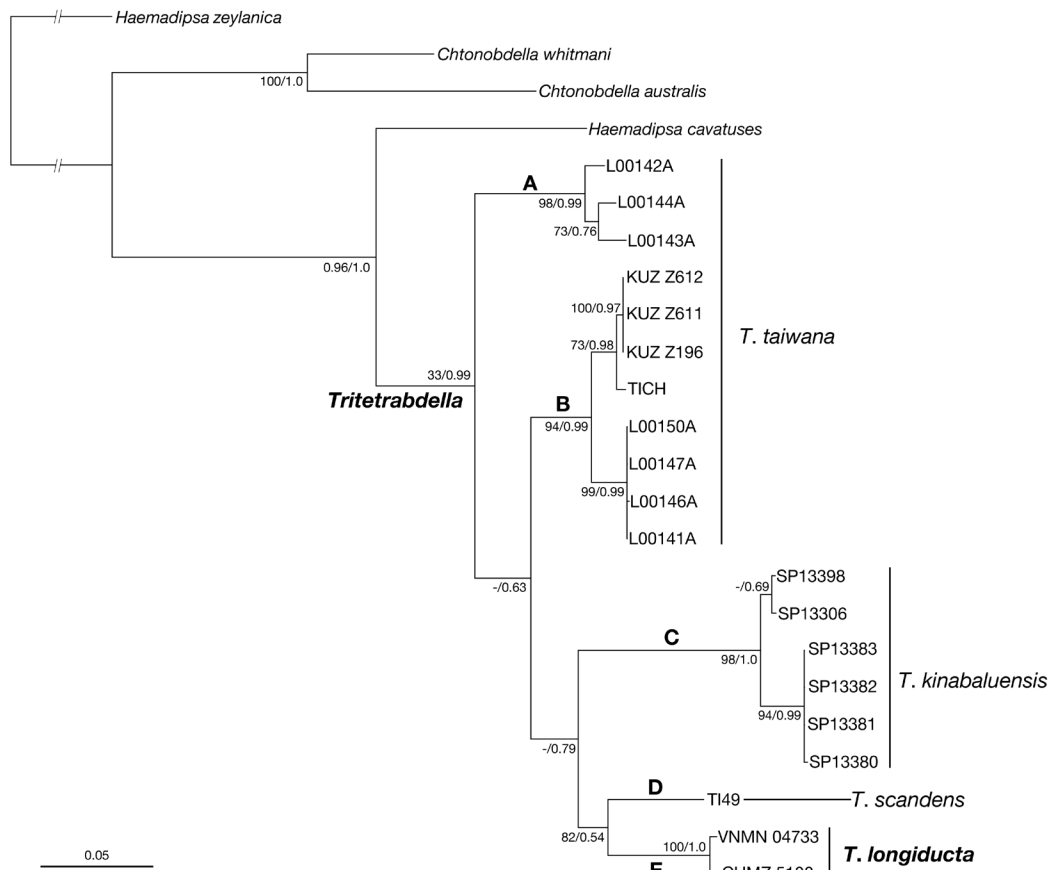


Fig. 6. Bayesian inference tree for 5,179 bp alignment positions of nuclear 18S rRNA and 28S rRNA and mitochondrial cytochrome *c* oxidase subunit I markers. Numbers on nodes indicate bootstrap values for maximum likelihood and Bayesian posterior probabilities.

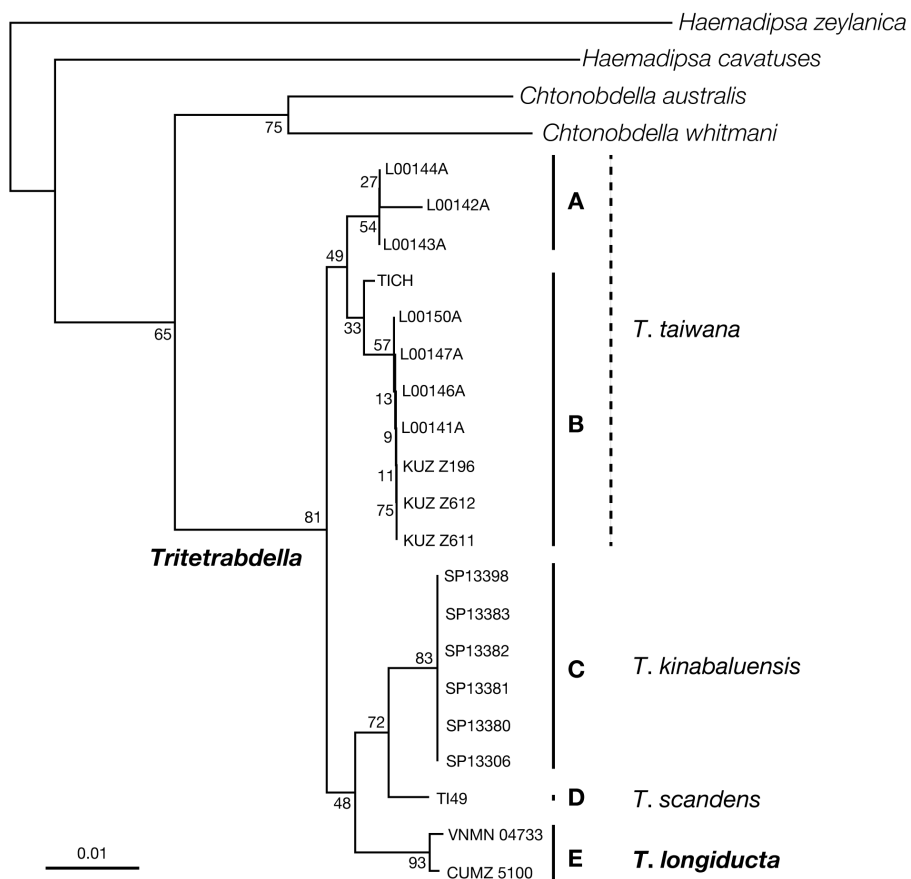


Fig. 7. Neighbour-joining tree for 422 amino acid alignment positions of mitochondrial cytochrome *c* oxidase subunit I corresponding peptide sequences. Numbers on nodes indicate bootstrap values.

Table 3. Amino acid divergences with Poisson correction model for the available cytochrome *c* oxidase subunit I corresponding peptide sequences of *Tritetrabdella* leeches. Acronym: CUMZ, Zoological Museum of the Chulalongkorn University; KUZ, Zoological Collection of Kyoto University; SP, Sabah Park; VNMN, Vietnam National Museum of Nature.

| Species                        | Voucher or Isolate Number       | Amino Acid Positions | (1)    | (2)    | (3)    | (4)    | (5)    | (6)    | (7)    | (8)    | (9)    | (10) |
|--------------------------------|---------------------------------|----------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|------|
| (1) <i>T. longiducta</i>       | CUMZ 5100                       | 422                  |        |        |        |        |        |        |        |        |        |      |
| (2) <i>T. longiducta</i>       | VNMN 04733                      | 422                  | 0.0024 |        |        |        |        |        |        |        |        |      |
| (3) <i>T. scandens</i>         | TI49                            | 405                  | 0.0174 | 0.0174 |        |        |        |        |        |        |        |      |
| (4) <i>T. taiwana</i>          | TICH                            | 218                  | 0.0232 | 0.0232 | 0.0196 |        |        |        |        |        |        |      |
| (5) <i>T. taiwana</i>          | KUZ Z196, Z611, Z612            | 422                  | 0.0191 | 0.0216 | 0.0250 | 0.0046 |        |        |        |        |        |      |
| (6) <i>T. taiwana</i>          | L00141A, 00146A, 00147A, 00150A | 219                  | 0.0184 | 0.0184 | 0.0147 | 0.0046 | 0.0000 |        |        |        |        |      |
| (7) <i>T. taiwana</i>          | L00142A                         | 219                  | 0.0231 | 0.0231 | 0.0196 | 0.0092 | 0.0138 | 0.0138 |        |        |        |      |
| (8) <i>T. taiwana</i>          | L00143A, 00144A                 | 219                  | 0.0184 | 0.0184 | 0.0147 | 0.0046 | 0.0092 | 0.0092 | 0.0046 |        |        |      |
| (9) <i>T. k. kinabaluensis</i> | SP13306, 13398                  | 219                  | 0.0184 | 0.0184 | 0.0098 | 0.0232 | 0.0184 | 0.0184 | 0.0231 | 0.0184 |        |      |
| (10) <i>T. k. inobongensis</i> | SP13380–13383                   | 219                  | 0.0184 | 0.0184 | 0.0098 | 0.0232 | 0.0184 | 0.0184 | 0.0231 | 0.0184 | 0.0000 |      |

Table 4. Morphological comparison between *Tritetrabdella longiducta*, new species and four congeneric species-group taxa.

| Taxon                      | Annulation of Somite VII | Annulation of Somite XXV | Respiratory Auricles                | Number of Friction Rays | Male Gonopore   | Female Gonopore                              | Vaginal Sac     |
|----------------------------|--------------------------|--------------------------|-------------------------------------|-------------------------|-----------------|--|-----------------|
| <i>T. longiducta</i>       | triannulate              | uniannulate              | in somites XXV–XXXVII               | 57                      | somite XI b5/b6 | slightly anterior to middle of somite XII b5 | slightly folded |
| <i>T. k. inobongensis</i>  | triannulate              | biannulate               | in somite XXV (a1 + a2)–somite XXVI | 55                      | somite XI b5/b6 | posterior margin of somite XII b5            | unfolded        |
| <i>T. k. kinabaluensis</i> | triannulate              | biannulate               | in somite XXV (a1 + a2)–somite XXVI | 55                      | somite XI b5/b6 | posterior margin of somite XII b5            | unfolded        |
| <i>T. scandens</i>         | quadrannulate            | uniannulate              | in somites XXV–XXXVII               | 56–59                   | somite XI a2/b5 | somite XII a2–a2/b5                          | slightly folded |
| <i>T. taiwana</i>          | triannulate              | biannulate               | in somite XXV (a1 + a2)–somite XXVI | 57–61                   | somite XI b5/b6 | middle of somite XII b5                      | unfolded        |

taxonomic studies are necessary to reveal the species diversity and distributions of *Tritetrabdella* species in Indochina.

**Key to species of *Tritetrabdella*.** Although Kappes (2013) provided a key to three known species of *Tritetrabdella*, it was based only on colourations, distributions and base compositions of their COI sequences. The present key addresses all the *Tritetrabdella* species including *T. longiducta*, and based on both external and internal anatomical characteristics.

1. Somite VII triannulate. Male gonopore in somite XI b5/b6. Female gonopore in somite XII b5.....2
  - Somite VII quadrannulate. Male gonopore in XI a2/b5. Female gonopore in somite XII a2–a2/b5. Vaginal sac slightly folded..... *Tritetrabdella scandens* Moore, 1938
2. Somite XXV biannulate. Respiratory auricles in somite XXV (a1 + a2) to somite XXVI. Vaginal sac unfolded.....3
  - Somite XXVI biannulate. Respiratory auricles in somites XXV–XXVII. Vaginal sac slightly folded..... *Tritetrabdella longiducta*, new species
3. Friction rays 55. Female gonopore in posterior margin of somite XII b5 ..... *Tritetrabdella kinabaluensis* Kappes, 2013
  - Friction rays 57–61. Female gonopore in middle of somite XII b5 ..... *Tritetrabdella taiwana* (Oka, 1910)

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