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Topotype-based redescription of the leech *Torix tukubana* (Hirudinida: Glossiphoniiformes: Glossiphoniidae)

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Abstract.—A poorly-known proboscidate leech species, *Torix tukubana* (Oka, 1935), in which the mid-body somites are biannulate dorsally and triannulate ventrally, is redescribed based on new specimens collected from its type locality, Mt. Tsukubasan in Honshu, Japan. The redescription provides the internal digestive and genital organs of *T. tukubana* for the first time. Our observation reveals that this species possesses equal-sized 1st–6th pairs of crop ceca that are nondiverticulated and tubular ovisacs running alongside the ventral nerve cord. Additionally, phylogenetic analyses using nuclear 18S rRNA, mitochondrial cytochrome *c* oxidase subunit I, 12S rRNA, tRNA^{Leu}, and NADH dehydrogenase subunit 1 markers reveal that *T. tukubana* is closely related to the Palearctic *Hemiclepsis* Vejdovský, 1884, in which the mid-body somites are triannulate.

Keywords: ectoparasite, "Rhynchobdellida," Toricinae, internal anatomy, molecular phylogeny, COI

The leech family Glossiphoniidae is a diversified group of proboscidate leeches, with over 200 described species from worldwide (Sket & Trontelj 2008). While the mid-body somite of most glossiphoniid species consists of three annuli (triannulate), it is further subdivided in species of *Haementeria* De Filippi, 1849 exceptionally, and some species possess reduced two-annuli (biannulate) mid-body somites. Although several genera have been established for these biannulate species, recent molecular phylogenetic studies have revealed that the biannulation of mid-body somites evolved in parallel within Glossiphoniidae (Light & Siddall 1999, Siddall et al. 2005, Oceguera-Figueroa 2012). One of the biannulate genera, i.e., *Oligobdella* Mo3ore, 1928, has already been synonymized with the triannulate *Placobdella* Blanchard, 1893 (Siddall et al. 2005), and *O. brasiliensis* Cordero, 1937 has been transferred to *Haementeria* (Oceguera-Figueroa 2012).

The biannulate glossiphoniid genus *Oligoclepsis* Oka, 1935 was erected along with a description of its type species *O. tukubana* Oka, 1935 based on three specimens collected from Mt. Tsukubasan ("mont Tukuba" in the description) on the island of Honshu in Japan (Oka 1935). The genus was characterized by mid-body somites that are biannulate dorsally and triannulate ventrally, and the possession of numerous papillae on the dorsal surface. *Oligoclepsis* was later placed into the genus *Torix* Blanchard, 1893, along with other species, in which mid-body

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somites are at least biannulate dorsally, inhabiting the Eastern Palearctic as well as Northeastern Oriental regions (Sawyer 1986). However, little is known regarding internal morphology of the type species of *Torix*, *T. mirus* Blanchard, 1893 described from Cao Bang, Vietnam (Blanchard 1893, 1898), and three species of the genus (including *T. tukubana*) described from Japan (Oka 1925a, b, 1935).

Compared to the Nearctic biannulate leeches, few molecular phylogenetic analyses of the Palearctic species has been performed. *Glossiphonia baicalensis* (Stschegolew, 1922) (originally *Torix baicalensis*), which is a biannulate species endemic to Lake Baikal (Stschegolew 1922, Lukin & Epshtein 1960a, Kaygorodova 2012), is exceptional in that its phylogenetic position was estimated to be within the triannulate genus *Glossiphonia* Johnson, 1816 (Light & Siddall 1999, Siddall et al. 2005). Consequently, a precise understanding of systematic accounts of the Palearctic biannulate taxa has been hampered by a lack of both of morphological and molecular backbones.

As the first step in re-evaluating the systematic status of *Torix* leeches, especially those inhabiting Japan and adjacent areas, contemporary specimens matching the description of *T. tukubana* (Oka 1935) were collected from the type locality. In this paper, their external and internal morphological characters are fully provided, revisiting the taxonomic status of this species. In addition, the phylogenetic position of *T. tukubana* within Glossiphoniidae is estimated based on nuclear and mitochondrial genetic markers.

Materials and Methods

Sampling and morphological examination.—Leech specimens were collected from the type locality of *T. tukubana*, Mt. Tsukubasan in Ibaraki Prefecture, Japan. Leeches were found in mountain streams. Altitude and coordinates for the localities were obtained using a Garmin eTrex GPS unit.

The collected leeches were individually kept at 16°C in the laboratory to digest their blood meal. Subsequently, the specimens were relaxed by the gradual addition of 99% ethanol to freshwater. A quarter of the caudal sucker was taken from every specimen for DNA extraction and the remaining bodies were fixed in 10% formalin and preserved in 70% ethanol. The following four body measurements were taken: body length from the anterior margin of the oral sucker to the posterior margin of the caudal sucker (BL), maximum body width (BW), caudal sucker length from the anterior margin to the posterior margin of the sucker (CL), and caudal sucker width from the right to the left margins of the sucker (CW). Examination, dissection and drawing of the specimens were accomplished under stereoscopic microscopes with a drawing tube (Leica M125C and Olympus SZX7). Images of the specimens were captured with the aid of a Leica MC170 HD digital camera mounted on the Leica M125C, and assembled using Leica Application Suite v. 4.12 software. Specimens used in this study have been deposited in the Zoological Collection of Kyoto University (KUZ).

The leech collection at the National Museum of Nature and Science, Tsukuba, Japan (NSMT) including most of Oka's leech collection (Nakano 2010) was surveyed to confirm whether the type series of *T. tukubana* remains. Although a portion of Oka's collection is kept at The University Museum, The University of Tokyo (UMUT), its type series was not discovered in the UMUT zoological collection (Nakano & Itoh 2011).

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.

Molecular phylogenetic analysis.—The phylogenetic position of *Torix tukubana* within Glossiphoniidae was determined based on one nuclear and three mitochondrial gene markers: (1) 18S rRNA (18S), (2) cytochrome *c* oxidase subunit I (COI), (3) 12S rRNA, tRNA^{Val}, and 16S rRNA (12S–16S), and (4) tRNA^{Leu} and NADH dehydrogenase subunit 1 (tRNA^{Leu}–ND1). Total DNA of each specimen was extracted from the caudal sucker using phenol/chloroform extraction with "DNA sui-sui" buffer (Rizo Inc.). The primer sets, methods and cycle conditions used in the polymerase chain reactions (PCR) and cycle sequencing reactions basically followed Nakano and Lai (2016) except for the usage of a primer, LCO-inerpo2 (Nakano 2016), instead of LCO-in (Nakano 2012) for COI, and PCR reaction kit, EmeraldAmp PCR Master Mix (Takara Bio). In total, four molecular markers of four specimens of *T. tukubana* were newly obtained in this study and deposited with the International Nucleotide Sequence Database Collaboration (INSDC) through the DNA Data Bank of Japan (Table 1).

According to the previous phylogenetic studies (Siddall et al. 2005, de Carle et al. 2017), 25 glossiphoniid operational taxonomic units (OTUs) were included as ingroup taxa (Table 1). Additionally, three proboscidate oceanobdelliforms and one acanthobdellidan species were selected as the outgroup taxa. The protein and RNA genes were aligned using TranslatorX with the default setting (Abascal et al. 2010) and MAFFT v. 7.427 with the L-INS-i option (Katoh and Standley 2013), respectively. The length of the 18S, COI, 12S, and tRNA^{Leu}–ND1 sequences were 1879, 662, 370, and 704 bp, respectively. The concatenated sequences yielded 3615 bp of aligned positions.

Phylogenetic trees were inferred with maximum likelihood (ML) and Bayesian inference (BI) methods. The best-fit partition scheme and substitution models were identified with AICc using IQ-TREE v. 1.6.12 (Nguyen et al. 2015) as follows: for 18S, SYM + I + G; GTR + G for COI first position; GTR + G for COI second position; GTR + I + G for COI third position; GTR + G for 12S and tRNA^{Leu}; GTR + I + G for ND1 first position; GTR + G for ND1 second position; and HKY + G for ND1 third position. The ML phylogeny was inferred using IQ-TREE, then a nonparametric bootstrapping (BS) was conducted with 1000 pseudoreplicates. BI tree and Bayesian posterior probabilities (PPs) were obtained using MrBayes v. 3.2.6 (Ronquist et al. 2012). Two independent runs of four Markov chains were conducted for 20 million generations, and a tree was sampled every 100 generations. The parameter estimates and convergence were checked using Tracer v. 1.7.1 (Rambaut et al. 2018), and the first 20,001 trees were discarded based on the result.

Pairwise comparisons of the uncorrected *p*-distance for COI sequences (651 bp) obtained from the present *T. tukubana* specimens and another population from Hiroshima in eastern Honshu (Kambayashi et al. 2019) (Table 1), as well as those from the species closely related to *T. tukubana* revealed by the present phylogenetic analyses were calculated using MEGA X (Kumar et al. 2018).

Taxonomy

Genus *Torix* Blanchard, 1893

Torix tukubana (Oka, 1935)

(Figs. 1, 2)

Oligoclepsis tukubana Oka, 1935: 66–68, one text figure; Autrum 1936: 36, fig. 24; Lukin & Epshtein 1960b: 479; Soós 1969: 427; Lukin 1976: 290–291, fig. 138.

Torix tukubana; Sawyer 1986: 655; Yoshida 2009: 47, figs. 1, 2; Kambayashi et al. 2019: 664–665, fig. 1; Sasaki 2019: 8, figs. 3, 5.

Amended diagnosis.—Body greenish. Caudal sucker ventral, oval. Somites XIII–XXIV dorsally biannulate, ventrally

Table 1.—Samples with voucher numbers, collection country, and International Nucleotide Sequences Database Collaboration (INSDC) accession numbers used for molecular analyses. Sequences marked with an asterisk (*) were obtained for the first time in the present study; *T. tukubana* samples marked with two asterisks (**) were only used for genetic distance analyses. Acronym: KUZ, the Zoological Collection of Kyoto University.

		INSDC accession number			
Species (Voucher number)	Country	18S	COI	12S–16S (or 12S)	tRNA^{Leu}–ND1
Glossiphoniidae					
Torix tukubana (Oka, 1935) (KUZ Z2970)**	Japan	LC538283*	LC538263*	LC538279*	LC538267*
Torix tukubana (KUZ Z2971)**	Japan	LC538284*	LC538264*	LC538280*	LC538268*
Torix tukubana (KUZ Z2972)**	Japan	LC538285*	LC538265*	LC538281*	LC538269*
Torix tukubana (KUZ Z2973)	Japan	LC538286*	LC538266*	LC538282*	LC538270*
Alboglossiphonia heteroclita (Linnaeus, 1761)	USA	AF115983	AF116016	AF099955	AY047339
Batracobdelloides koreanus Bolotov, Klass, Bespalaya, Konopleva, Kondakov & Vikhrev, 2019	South Korea	MN312194	MN295424		
Batracobdelloides tricarinata (Blanchard, 1897)	South Africa	AY962430	AY962457		AY962445
Glossiphonia baicalensis (Stschegolew, 1922)	Russia	AY962425	AY047329		AY047355
Glossiphonia complanata (Linnaeus, 1758)	UK	AF115982	AY047321	AY425414	AY047344
Haementeria brasiliensis (Cordero, 1937)	Brazil		JN850911	JN850874	JN850936
Haementeria ghilianii De Filippi, 1849	Brazil	AF115985	AF329035	AY425417	AF329058
Haementeria gracilis (Weyenbergh, 1883)	Uruguay	AF115984	AF329034	AY425418	AF329057
Helobdella elongata (Castle, 1900)	USA	AY962419	AF329045	JN850882	AF329068
Helobdella paranensis (Oka, 1930)	Uruguay	AF115987	AF329037	AY425412	AF329060
Helobdella triserialis (E. Blanchard, 1849)	Bolivia	AY962435	AF329054	JN850883	AF329077
Hemiclepsis kasmiana Oka, 1910	South Korea	MN312193	MN295423		
Hemiclepsis khankiana Bolotov, Klass, Bespalaya, Konopleva, Kondakov & Vikhrev, 2019	Russia	MN312192	MN295420		
Hemiclepsis marginata (O. F. Müller, 1774)	France	AF115981	AF003259	AY425425	AY047336
Hemiclepsis myanmariana Bolotov, Klass, Bespalaya, Konopleva, Kondakov & Vikhrev, 2019	Myanmar	MN312180	MN295394		
Hemiclepsis schrencki Bolotov, Klass, Bespalaya, Konopleva, Kondakov & Vikhrev, 2019	Russia	MN312189	MN295415		
Hemiclepsis tumniniana Bolotov, Klass, Bespalaya, Konopleva, Kondakov & Vikhrev, 2019	Russia	MN312166	MN295371		
Marsupiobdella africana Goddard & Malan, 1912	South Africa	AF115979	AF116015	AY425433	AY047347
Placobdella biannulata (Moore, 1900)	USA	AF115989	AF116021	AY425435	AY047343
Placobdella costata (F. J. Müller, 1846)	Portugal	AY962436	MF067143	MF067198	MF067204
Placobdella papillifera (Verrill, 1872)	USA	AY962421	MF067125	MF067181	MF067217
Placobdella phalera (Graf, 1899)	USA	AY962413	MF067117	MF067173	MF067225
Placobdella picta (Verrill, 1872)	Canada	AF115988	MF067113	MF067171	MF067227
Theromyzon tessulatum (O. F. Müller, 1774)	France	AF115980	AY047318	AF099957	AY047338
Torix tukubana (KUZ Z2016)**	Japan		LC413905		
Torix tukubana (KUZ Z2017)**	Japan		LC413906		
Torix tukubana (KUZ Z2019)**	Japan		LC413907		
Torix tukubana (KUZ Z2020)**	Japan		LC413908		
Torix tukubana (KUZ Z2025)**	Japan		LC413909		
Torix tukubana (KUZ Z2026)**	Japan		LC413910		
Torix tukubana (KUZ Z2028)**	Japan		LC413911		
Torix tukubana (KUZ Z2056)**	Japan		LC413912		
Outgroup					
Acanthobdella peledina Grube, 1851	Sweden	AY040701		AF099953	AF115978
Calliobdella vivida (Verrill, 1872)	USA	AF115992	AF003260	AY425409	AY047333
Ozobranchus margoi (Apathy, 1890)	USA	AF115991	AF003268		AY047331
Piscicola geometra (Linnaeus, 1761)	France	AF115995	AF003280	AY425437	AY047334

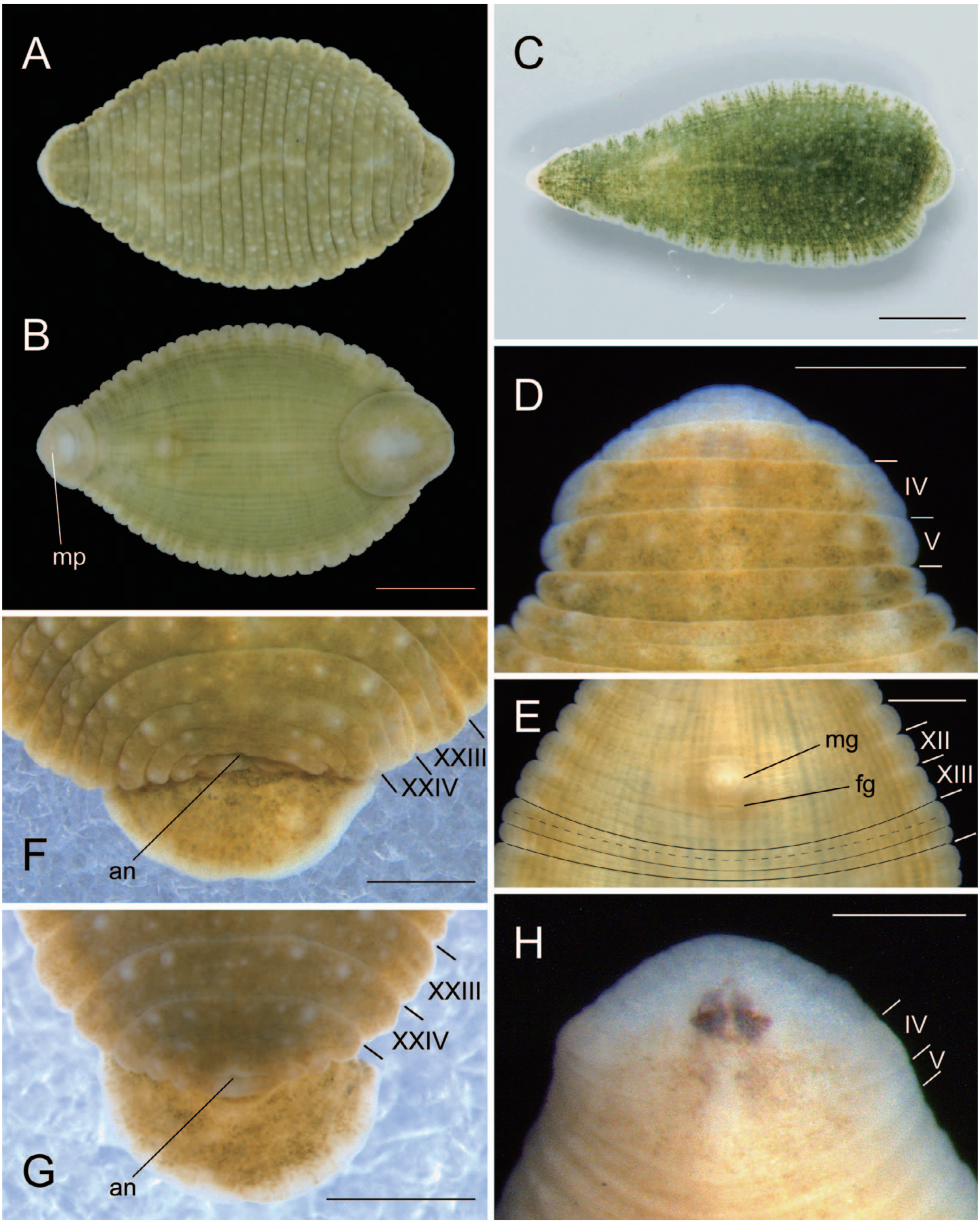

Fig. 1. *Torix tukubana* (Oka, 1935), collected from Mt. Tsukubasan, Japan; KUZ Z2973 (A–F), KUZ Z2972 (G), KUZ Z2970 (H). A, Dorsal view; B, ventral view; C, dorsal view of live animal; D, dorsal view of anterior end; E, ventral view of mid-body somites; F, G, dorsal view of posterior end; H, dorsal view of anterior end. Scale bars: 5 mm (A–C), 1 mm (D–G), 0.25 mm (H). Abbreviations: an, anus; fg, female gonopore; mg, male gonopore; mp, mouth pore.

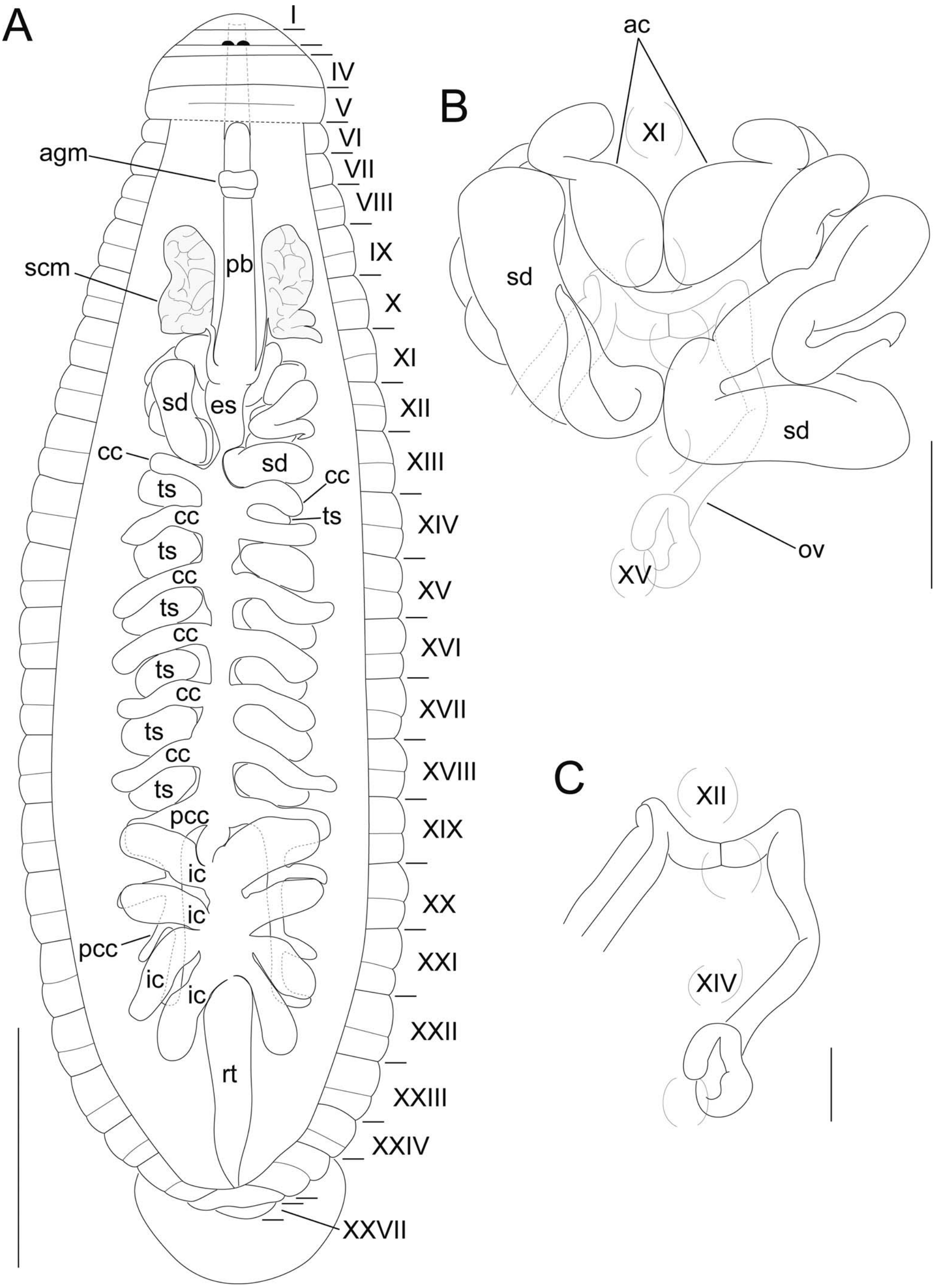

Fig. 2. *Torix tukubana* (Oka, 1935), KUZ Z2972, collected from Mt. Tsukubasan, Japan. A, Dorsal view of digestive tract and male genital organs; B, dorsal view of male atrium including ovisacs and positions of ganglia XI–XV; C, dorsal view of female reproductive system including positions of ganglia XII–XV; a portion of left ovisac being lost during dissection. Scale bars: 2 mm (A), 0.5 mm (B), 0.25 mm (C). Abbreviations: ac, atrial cornu; agm, anterior ganglionic mass; cc, crop cecum; es, esophagus; ic, intestinal cecum; ov, ovisac; pb, proboscis; pcc, post-crop cecum; rt, rectum; sd, sperm duct; scm, salivary cells mass; ts, testisac.

triannulate. Anus between somites XXVI/XXVII. Male gonopore between somites XI/XII, female gonopore between somite XII (a1 + a2)/a3, gonopores separated by 1 annulus XII (a1 + a2). Eyes in 2 pairs, in "placobdellid" arrangement; 1st pair inconspicuous on somite II, often coalescing with large conspicuous 2nd pair on somite III. Dorsal papillae on IV–XXV, ca. 13–20 on every mid-body annulus of somites XIV–XXI. Mouth pore on anterior margin of oral sucker. Salivary cells in 1 pair of compact mass. Esophagus simple, esophageal grand absent. Bacteriosomes absent. Crop giving rise to 7 pairs of crop ceca, 1st–6th pairs simple, nondiverticulated, 7th pair (post-crop ceca) diverticulated into 4 sections. Intersomital testisacs in 6 pairs. Paired sperm ducts thick, strongly coiled. Atrial cornua directed anterolaterally, developed ovate. Paired ovisacs tubular, reaching to somite XII (a1 + a2).

Material examined.—A total of four specimens newly collected at the type locality, Mt. Tsukubasan, Ibaraki Prefecture, Honshu island, Japan, by CK and TN, on 23 May 2019. Three free-living specimens attached to the underside of stones in mountain streams: KUZ Z2971–Z2972 (36°23′40″N, 140°10′77″E; elev. 521 m), and KUZ Z2973 (36°23′09″N, 140°10′45″E; elev. 660 m). One juvenile specimen, KUZ Z2970, attached to the surface of the Tago's brown frog, *Rana tagoi* Okada, 1928, collected in a mountain stream (36°23′45″N, 140°10′68″E; elev. 527 m). Internal morphology was provided based on the two specimens (KUZ Z2972 and Z2973).

Name-bearing types.—The three specimens in the original description of *T. tukubana* (Oka 1935) are automatically fixed as the syntypes according to the Article 73.2 of the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature 1999). However, no type series of *T. tukubana* was found at NSMT. Syntypes are believed to have been lost or destroyed in the past according to the present survey and Nakano & Itoh (2010), nonetheless, a neotype is not designated for *T. tukubana* in the present study. There is no doubt over the identity of the species as *Torix tukubana*, given the fact that no other biannulate glossiphoniid species were not found at the type locality of this species.

Redescription.—Body ovate (Fig. 1A–C). Caudal sucker ventral, oval (Fig. 1B). Measurements (mean, followed by ranges in parentheses; n = 3, KUZ Z2971–Z2973): BL 9.73 mm (7.36–11.1), BW 4.11 mm (2.47–6.48), CL 2.16 mm (1.41–2.93), CW 1.93 mm (1.38–2.57).

Somite I completely merged with prostomium (Figs. 1D, 2A). Somites II (= peristomium), III and IV uniannulate (Figs. 1D, 2A); somite IV forming posterior margin of oral sucker. Somites V–VII uniannulate, often with slight dorsal furrow respectively. Somites VIII–XII both dorsally and ventrally biannulate, (a1 + a2) > a3. Somites XIII–XXIV dorsally biannulate, (a1 + a2) > a3, ventrally triannulate, a1 = a2 < a3, ventral annular furrow between a1 and a2 slightly shallow (Fig. 1E–G); somite XXIV a3 being ventrally last complete annulus. Somite XXV biannulate, (a1 + a2) > a3 (Fig. 1F, G). Somites XXVI and XXVII both dorsally and ventrally uniannulate (Fig. 1F, G). Anus between somites XXVI/XXVII; somite XXVII being post-anal annulus (Fig. 1F, G).

Clitellum unobservable.

Male gonopore between XI/XII (Fig. 1E). Female gonopore between XII (a1 + a2)/a3 (Fig. 1E). Gonopores separated by 1 annulus.

Eyes in 2 pairs, almost completely coalesced, right and left eyes well separated from each other, on posterior margin of II, or on II–III (Fig. 1D). Dorsal papillae on IV–XXV, ca. 13–20 on every mid-body annulus of XIV–XXI, forming 1 inconspicuous transverse row on posterior margin of (a1 + a2), and on middle of a3 of each mid-body somite, respectively;

slightly larger on both distal margins on (a1 + a2); 2 faint longitudinal rows detectable (Fig. 1A, D, F, G). Ventral sensillae in 1 pair located posterior margin of (a1 + a2) or a2 of each of VIII–XXIII.

Nephridiopores undetectable.

Mouth pore on anterior margin of oral sucker (Fig. 1B). Proboscis in membranous sheath reaching to XI/XII, without forming loop (Fig. 2A). Salivary cells arranged in 1 pair of compact mass between somites VIII a3–XI (a1 + a2) (Fig. 2A); ductules not forming bundle, inserting independently into base of proboscis in XI (Fig. 2A). Esophagus simple, not recurved (Fig. 2A); esophageal grand absent. Bacteriosomes absent. Crop reaching to XIX/XX, giving rise to 7 pairs of crop ceca (Fig. 2A); 1st–6th pairs crop ceca simple, nondiverticulated, almost equal in size: 1st pair in XIII (a1 + a2)–XIV (a1 + a2); 2nd pair in XIV (a1 + a2)–a3; 3rd pair in XV (a1 + a2)–a3; 4th pair in XVI (a1 + a2)–a3; 5th pair in XVII (a1 + a2)–a3; 6th pair in XVIII (a1 + a2)–a3; and 7th pair (post-crop ceca) diverticulated into 4 sections, in XIX (a1 + a2)–XXII (a1 + a2). Intestinal ceca in 4 pairs (Fig. 2A): 1st pair in XIX (a1 + a2)–XX (a1 + a2); 2nd pair in XX (a1 + a2)–a3; 3rd pair in XXI (a1 + a2)–XXII (a1 + a2); 4th pair in XXI a3–XXII a3. Rectum simple tubular (Fig. 2A).

Testisacs in 6 pairs, intersomital (Fig. 2A): 1st pair in XIII a3–XIV (a1 + a2); 2nd pair in XIV a3–XV (a1 + a2); 3rd pair in XV a3–XVI (a1 + a2); 4th pair in XVI a3–XVII (a1 + a2); 5th pair in XVII a3–XVIII (a1 + a2); 6th pair in XVIII a3–XIX (a1 + a2). Paired sperm ducts thick, strongly coiled, in XI–XIII (Fig. 2A, B). Pair of muscular atrial cornua directed anterolaterally, developed ovate, in XI a3–XII (a1 + a2) (Fig. 2A, B).

One pair of ovisacs tubular, thin-walled, slightly folded running alongside ventral nerve cord, in XII (a1 + a2) to XIV a3–XV a3 (Fig. 2B, C); both ovisacs reaching to XII (a1 + a2), then turned posteromedially toward female gonopore.

Juvenile morphology.—Measurements (KUZ Z2970): BL 2.64 mm, BW 1.19 mm, CL 0.94 mm, CW 0.96 mm. Somites I–XXIV uniannulate, with slight dorsal furrow on each of VII–XXIV, and with slight ventral furrow of each of XV–XXIV. Eyes in 2 pairs: 1st pair on posterior margin of II; 2nd pair on anterior margin of III (Fig. 1H). Male and female gonopore undetectable. Dorsal papillae and ventral sensillae undeveloped.

Coloration.—In life, dorsal surface uniform green, white and green mottled on distal margin (Fig. 1C); ventral surface transparent. Color faded in preservative; uniform pale green or yellowish grey (Fig. 1A, B).

Host preference.—Two out of three individuals of the Tago's brown frog, *Rana tagoi*, were infested by *T. tukubana*. One host frog and the attaching leech were collected. Although six individuals of the Tsukuba clawed salamander, *Onychodactylus tsukubaensis* Yoshikawa & Matsui, 2013, were also observed, none were parasitized by leeches.

Molecular analyses.—The ML (ln L = –23026.468; Fig. 3) and BI (mean ln L = –22972.413; not shown) trees had the same topology. In the trees, the monophyly of the family Glossiphoniidae was fully supported (BS = 100%, PP = 1.0). The 10 glossiphoniid genera analyzed here consisted of three monophyletic lineages: *Placobdella* (BS = 93%, PP = 0.91), *Helobdella* Blanchard, 1896 + *Haementeria* (BS = 90%, PP = 1.0), and remaining seven genera (BS = 66%, PP = 0.93) divided into two subclades. The first subclade includes *Glossiphonia* and *Alboglossiphonia* Lukin, 1976 (BS = 100%, PP = 1.0), and the second subclade consist of the genus *Hemiclepsis* Vejdovský, 1884, *Marsupiobdella* Goddard & Malan, 1912, *Batracobdelloides* Oosthuizen, 1984, *Theromyzon* Philippi, 1867, and *T. tukubana*, but the monophyly of this subclade was not fully supported (BS = 60%, PP = 0.84). *Torix*

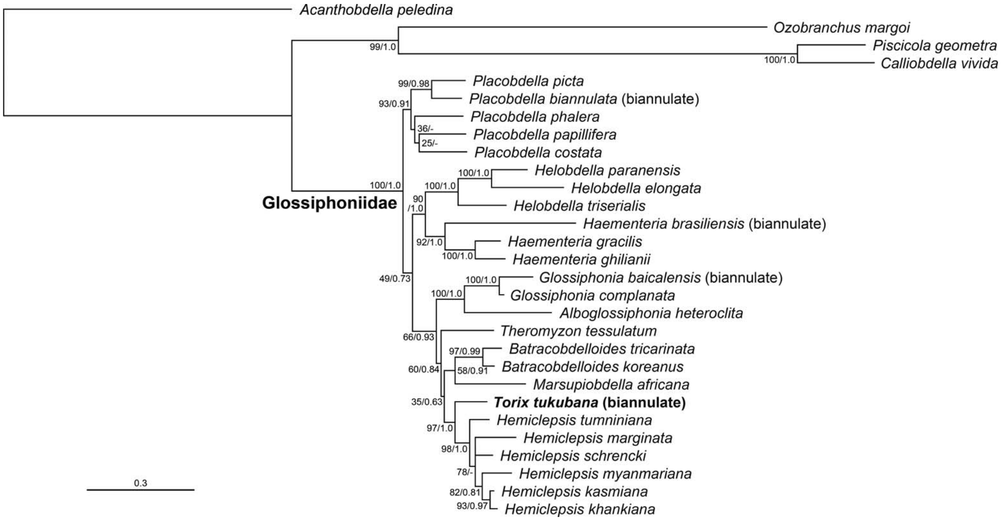

Fig. 3. Maximum likelihood tree for 3615 bp of nuclear 18S rRNA and mitochondrial COI, 12S rRNA, tRNA^{Leu} and ND1 markers. Numbers on nodes represent bootstrap values for maximum likelihood and Bayesian posterior probabilities.

tukubana formed a highly supported clade with *Hemiclepsis* (BS = 97%, PP = 1.0).

The pairwise COI uncorrected *p*-distances between the present *T. tukubana* specimens and the population from Hiroshima was 3.2–4.0% (mean = 3.6%), and those between the topotypic *T. tukubana* and *Hemiclepsis* species was 14–16% (mean = 15%) (Table 2).

The four COI sequences obtained from three matured and one juvenile leeches are almost concordant with each other. Although four polymorphic sites were found from 1267 sequenced sites (0.32%), this value fell into the intraspecific divergence among the other glossiphoniid leeches (de Carle et al. 2017), indicating that all four specimens used here are unquestionably conspecific.

Remarks.—The present mature individuals with testisacs that were fully developed were unquestionably identified as *T. tukubana* because of the fact that they possessed mid-body somites that were biannulate dorsally and triannulate ventrally, and ca. 13–20 developed papillae on the dorsal surface of somites XIV–XXI (16 or 17 papillae on each annulus of somites XVI–XVIII were depicted in the original description) (Oka 1935). However, the morphological characteristics of the immature individual, in which the gonopores are undeveloped, highlighted the fact that these diagnostic features are ontogenetic traits in this species as already stated in

Table 2.—Uncorrected *p*-distances calculated for the available COI sequences of the topotypic *Torix tukubana*, the Hiroshima population identified as *T. tukubana* by Kambayashi et al. (2019), and *Hemiclepsis* species included in the phylogenetic analyses.

Species	INSDC accession numbers	1	2	3
1. *Torix tukubana* (topotypic population)	LC538263–LC538266	–		
2. *Torix tukubana* (Hiroshima population)	LC413905–LC413912	3.2–4.0%	–	
3. *Hemiclepsis* species	AF003259, MN295371, MN295394, MN295415, MN295420, MN295423	14–16%	13–15%	–

Table 3.—Comparisons of morphological characters between *Torix tukubana* (Oka, 1935) and four congeneric species.

Character	*Torix tukubana* (Oka, 1935)	*Torix cotylifer* Blanchard, 1898	*Torix mirus* Blanchard, 1893	*Torix orientalis* (Oka, 1925)	*Torix tagoi* (Oka, 1925)
Male gonopore	XI/XII	XI/XII	XI (a1 + a2)/a3	XI/XII	XI/XII
1st–6th pairs of crop ceca	equal-sized, nondiverticulated	1st pair extending to lateral margin of body, nondiverticulated	?	equal-sized, diverticulated	equal-sized, nondiverticulated
Ovisacs	tubular	globular	?	globular	globular

another population from Hiroshima in eastern Honshu identified as *T. tukubana* in a previous study (Kambayashi et al. 2019). The immature specimen bore mid-body somites that were also biannulate ventrally and a smooth dorsal surface without papillae. Additionally, the number of eyes can be considered ontogenetic because mature individuals have one pair of eyes and an immature individual has two pairs of eyes.

The present results, which revealed the internal digestive and genital features of *T. tukubana* for the first time, and previous taxonomic studies (Blanchard 1893, 1898, Oka 1925a, b, Moore 1930a, b, Lukin & Epshtein 1960b) show that *T. tukubana* clearly differs from other Asian congeners in having a male gonopore between XI/XII, nondiverticulated equal-sized 1st–6th crop ceca, and tubular ovisacs (see Table 3). In addition to the *Torix* species, a glossiphoniid *Parabdella quadrioculata* (Moore, 1930) was recorded as the amphibian-reptile parasitic leech from Japan (Yamauchi et al. 2013). *Torix tukubana* is clearly distinguishable from *P. quadrioculata* by its mid-body somites that are biannulate (triannulate in *P. quadrioculata*; Moore 1930b), anus opening between XXVI/XXVII (behind XXVII), and nondiverticulated crop ceca (diverticulated; Yang 1996).

It is noteworthy that the COI uncorrected *p*-distances between the topotypic *T. tukubana* and the Hiroshima population investigated by Kambayashi et al. (2019) were higher than the intraspecific divergence calculated for the glossiphoniid *Placobdella* species (de Carle et al. 2017). Therefore, the population indigenous to Hiroshima may belong to a different species from *T. tukubana*. Its taxonomic status should be clarified in future studies.

Discussion

The topology of the obtained phylogenies was almost concordant with the previous study (de Carle et al. 2017) showing that glossiphoniid leeches comprise three clades, i.e., *Placobdella*, *Helobdella* + *Haementeria*, and the other genera including *Alboglossiphonia*, *Glossiphonia*, *Hemiclepsis*, *Marsupiobdella*, *Batracobdelloides*, and *Theromyzon*. Our phylogenies revealed that *T. tukubana* forms a monophyletic lineage with *Hemiclepsis*. The present phylogenies and those in previous studies (Light & Siddall 1999, Siddall et al. 2005) also clarified that *Glossiphonia* includes the Palearctic biannulate species *G. baicalensis*. Moreover, the clade consisting of *Glossiphonia* and *Alboglossiphonia* was phylogenetically distinct from the clade comprising *Hemiclepsis* and *T. tukubana*. Therefore, the present results highlight that biannulation of the mid-body somites has evolved independently within the Palearctic glossiphoniid species, as can be seen in glossiphoniid leeches indigenous to the New World (Oceguera-Figueroa 2012).

The genus-level classification of *T. tukubana* still remains problematic. The present results led to the following alternatives for the generic status of *T. tukubana*: (1) *T. tukubana* would remain within *Torix*; (2) *T. tukubana* would be placed under *Hemiclepsis*; or (3) *Oligoclepsis* would be resurrected for *T. tukubana*. Members of *Hemiclepsis* in which mid-body somites are triannulate are endemic to the Palearctic region, and they infest freshwater fish, mollusks including bivalves, and amphibians (e.g., Nagasawa & Miyakawa 2006, Tanaka et al. 2017, Bolotov et al. 2019). *Hemiclepsis* leeches having 9–11 pairs of crop ceca (Sawyer 1986) clearly differ from *T. tukubana* and the other *Torix* species that possess seven pairs of crop ceca. Given this clear morphological difference, we do not transfer *T. tukubana* into the genus *Hemiclepsis*. It is also difficult to conclude whether *T. tukubana* should be placed within *Torix* or classified under its original genus *Oligoclepsis*, because the internal anatomy and phylogenetic position of *T. mirus*, which is the type species of *Torix*, remain veiled. Consequently, we tentatively regard *T. tukubana* as a member of *Torix* without revalidating *Oligoclepsis* for this species.

According to the external morphology of *T. mirus* and other congeners, *Torix* can be characterized by the mid-body somites that are at least biannulate dorsally. Based on the internal anatomy of *T. cotylifer* Blanchard, 1898, *T. orientalis* (Oka, 1925), *T. tagoi* (Oka, 1925) and *T. tukubana* (Oka 1925a, b, Moore 1930b, Lukin & Epshtein 1960b, present study), additionally, the seven pairs of crop ceca can be also treated as a tentative diagnostic character of this genus. Further morphological and phylogenetic studies are needed to elucidate the precise systematic status of *Torix* and its species.

Torix tukubana inhabiting Mt. Tsukubasan was observed to suck only the Tago's brown frog, *Rana tagoi*, although the individuals of the Tsukuba clawed salamander, *Onychodactylus tsukubaensis*, were found in the same location. Therefore, this ranid frog was deemed to be a preferred host species of *T. tukubana* in the type locality. On the other hand, *T. tukubana* from the other population in Hiroshima, Japan is known to infest two ranid frog species as well as a salamander *Onychodactylus japonicus* (Houttuyn, 1782) (Kambayashi et al. 2019), suggesting that the host specificity of *T. tukubana* could be diverse among populations. Our study based on topotypic specimens of *T. tukubana* sheds light on the systematic account and host preferences of this species. Further faunal surveys and taxonomic studies of *T. tukubana* as well as other *Torix* species are necessary to clarify the species diversity and evolutionary history of Sino-Japanese biannulate glossiphoniid leeches.

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