A new species of *Orobdella* (Hirudinida: Arhynchobdellida: Orobdellidae) from Primorye Territory, Russian Far East

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Disclosure statement

No potential conflict of interest was reported by the authors

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Geolocation information

KUZ Z2068, Z2070 (point): 43.129645°N, 132.798943°E

KUZ Z2069 (point): 43.129113°N, 132.798925°E

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ABSTRACT

A quadrannulate leech species, *Orobdella ghilarovi* sp. nov., from Primorye Territory in the southern Russian Far East is described. Phylogenetic analyses using nuclear 18S rRNA, 28S rRNA, histone H3, mitochondrial cytochrome *c* oxidase subunit I, tRNA^{Cys}, tRNA^{Met}, 12S rRNA, tRNA^{Val}, 16S rRNA, tRNA^{Leu} and NADH dehydrogenase subunit 1 markers showed that *O. ghilarovi* formed a fully supported clade with a monophyletic lineage comprising two species: *O. kawakatsuorum* and *O. koikei*, inhabiting Hokkaido, Japan. Quadrannulate *Orobdella* leeches distributed in the southern Primorye Territory have been identified as *O. whitmani*, which was originally described in Japan; these past records should be amended based on the taxonomic conclusions of this study.

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KEYWORDS

Erpobdelliformes; terrestrial; macrophagous; molecular phylogeny; misidentification

Introduction

The genus *Orobdella* Oka, 1895 is an erpobdelliform leech taxon of the terrestrial macrophagous species inhabiting Far East Asia (Sawyer 1986; Oceguera-Figueroa et al. 2011; Nakano et al. 2012). *Orobdella* leeches are characterised by their mid-body somite annulation that can be grouped into three types, i.e. 4- (quadr-), 6- (sex-), and 8- (oct-) annulate, and their possession of the gastroporal duct in their digestive tract that receives a spermatophore during copulation (Nakano 2017a, 2017b). They can be also grouped into three morpho-types according to the body length of mature individuals, i.e. small, middle, and large-types (Nakano 2017a).

This genus now consists of 20 species: most of them, i.e. 17 species, are distributed in the Japanese Archipelago (Nakano 2017a, 2017b, 2018); one species was recorded in the Korean Peninsula and adjacent islets including Tsushima Island, and two species were described in Taiwan (Nakano and Lai 2012, 2017). Until the 1960s, however, the genus *Orobdella* comprised only three species described in Japan: the quadrannulate *O. whitmani* Oka, 1895, the sexannulate *O. ijimai* Oka, 1895, and the octannulate *O. octonaria* Oka, 1895. In the late 1960s, quadrannulate *Orobdella* individuals were recorded around Vladivostok City in Primorye Territory, Russian Far East, and they were identified as *O. whitmani* (Ghilarov et al. 1969). Although their identification in the Russian Far East *Orobdella* was followed by subsequent works (Ghilarov and Perel 1971; Lukin 1976; Kurcheva 1977), it was also suggested that the Russian quadrannulate *Orobdella* might be an undescribed species rather than *O. whitmani* (Nakano 2012).

Quadrannulate *Orobdella* leeches were newly collected from near Vladivostok. In line with the results of the morphological examination and molecular phylogenetic analyses of the newly obtained specimens, they are described as a new species herein.

Material and methods

Sampling and morphological examination

Leeches were collected from a locality in Anisimovka, Primorye Territory, southern Russian Far East. When possible, elevation and geographical coordinates for the collection site were obtained using a Garmin eTrex® GPS unit.

Specimens were relaxed by the gradual addition of absolute ethanol (EtOH) to freshwater, and then fixed in absolute EtOH. For DNA extraction, botryoidal tissue was removed from the posterior part around the caudal sucker of every specimen, and then preserved in absolute EtOH. The reminder of the body was fixed in 10% formalin and preserved in 70% EtOH. 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; Leica Microsystems, Wetzlar, Germany). Specimens used in this study were deposited in the Zoological Collection of Kyoto University (KUZ).

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.

Molecular phylogenetic analyses

The phylogenetic position of the new *Orobdella* species within the genus was determined based on three nuclear and three mitochondrial markers: 1) 18S rRNA, 2) 28S rRNA, 3) histone H3, 4) cytochrome *c* oxidase subunit I (COI), 5) tRNA^{Cys}, tRNA^{Met}, 12S rRNA, tRNA^{Val} and 16S rRNA (tRNA^{Cys}–16S), and 6) tRNA^{Leu} and NADH dehydrogenase subunit 1 (tRNA^{Leu}–ND1). Methods for the genomic DNA extraction, PCR and cycle sequencing reactions were elucidated in Nakano and Lai (2016, 2017). In total, 14 sequences were newly obtained in this study and deposited with the International Nucleotide Sequence Database Collaboration (INSDC) through DNA Data Bank of Japan (Table 1).

According to the previous phylogenetic study (Nakano et al. 2018), eight OTUs were selected as the outgroup for the present phylogenetic analyses (Table 1). The alignments of H3 and COI were trivial, as no indels were observed. The sequences of the other markers were aligned using MAFFT v. 7.407 L-INS-i (Katoh and Standley 2013). The lengths of the

18S, 28S, H3, COI, tRNA^{Cys}–16S, and tRNA^{Leu}–ND1 sequences were 1844, 2823, 328, 1267, 1182, and 640 bp, respectively. The concatenated sequences yielded 8084 bp of aligned positions.

Phylogenetic trees were inferred using maximum likelihood (ML) and Bayesian inference (BI). The ML phylogeny was inferred using RAxML v. 8.2.8 (Stamatakis 2014) with the substitution model set as GTRCAT, immediately after nonparametric bootstrapping (BS) was conducted with 1000 replicates. The best-fit partition scheme was identified with the Bayesian information criterion using PartitionFinder v. 2.1.1. (Lanfear et al. 2017) with the “greedy” algorithm (Lanfear et al. 2012): 18S, plus H3 1st and 2nd positions/28S/H3 3rd position/COI 1st position/each COI and ND1 2nd position/each COI and ND1 3rd position/tRNA^{Cys}, tRNA^{Met}, 12S and tRNA^{Val}, tRNA^{Leu} and ND1 1st position/16S. BI and Bayesian posterior probabilities (PPs) were estimated using MrBayes v. 3.2.6 (Ronquist et al. 2012). The best-fit partition scheme and models for each partition were selected with the Bayesian information criterion using PartitionFinder with the “greedy” algorithm: for 18S and H3 1st position, K80+I+G; GTR+I+G for 28S; JC+I for H3 2nd position; HKY+G for H3 3rd position; GTR+G for COI 1st position; GTR+I+G for 2nd positions of COI and ND1; HKY+I+G for 3rd positions of COI and ND1; GTR+I+G for tRNA^{Cys}–tRNA^{Val} and ND1 1st position; and GTR+G for 16S. Two independent runs of four Markov chains were conducted for 20 million generations, and the 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 50001 trees were discarded based on the results.

Pairwise comparisons of uncorrected *p*-distances for respective COI (1267 bp), tRNA$^{\mathrm{Cys}}$–16S (1032 bp of aligned positions), and tRNA$^{\mathrm{Leu}}$–ND1 (630 bp) sequences obtained from the newly identified *Orobdella* were calculated using MEGA7.0.16 (Kumar et al. 2016). Prior to the calculation, tRNA$^{\mathrm{Cys}}$–16S sequences were aligned using MAFFT FFT-NS-2.

Taxonomy

Genus ***Orobdella*** Oka, 1895

Orobdella ghilarovi sp. nov.

(Figures 1–3)

Orobdella whitmani: Ghilarov et al., 1969: 235–236, fig. 1; Ghilarov and Perel, 1971: table 1; Lukin, 1976: 464–466, figs. 288, 289.

Type material

Holotype. KUZ Z2068 (Figure 1), dissected, collected from under a stone along a forest road, Anisimovka (43.129645°N, 132.798943°E; elev. 435 m), Shkotovsky District, Primorye

Territory, Russia, by Takafumi Nakano, on 21 August 2018.

Paratypes. In total 2 individuals collected from around the type locality on 21 August 2018: KUZ Z2069 (43.129113°N, 132.798925°E; elev. 435 m) by Takafumi Nakano, and KUZ Z2070 (43.129645°N, 132.798943°E; elev. 435 m) by Larisa Prozorova.

Diagnosis

Body length of mature individual exceeding 60 mm (67.2 mm in maximum length). Somite IV uniannulate, somites VIII–XXV quadrannulate. Clitellum in somite XI b5 to somite XIII a2. Male gonopore in middle of somite XI b6, female gonopore in middle of somite XIII a1, behind gastropore, gonopores separated by 1/2 + 4 + 1/2 annuli. Pharynx reaching to somite XIII/XIV. Gastropore conspicuous, in middle of somite XIII a1. Gastroporal duct bulbous. Paired epididymides in somites XVI–XIX, occupying 10 annuli. Atrial cornua developed, ovate.

Description

Body firm and muscular, elongate, with constant width in caudal direction, dorsoventrally compressed, BL 67.2 mm, BW 5.2 mm (Figure 1(a), 1(b)). Caudal sucker ventral, elliptic, CL 2.8 mm, CW 3.2 mm (Figures 1(b), 2(d)).

Somite I completely merged with prostomium (Figure 2(a)). Somites II (= peristomium), III and IV uniannulate (Figure 2(a)). Somite V biannulate, (a1 + a2) = a3; a3 forming posterior margin of oral sucker (Figure 2(a), 2(b)). Somites VI and VII triannulate, a1 = a2 = a3 (Figure 2(a), 2(b)). Somites VIII–XXV quadrannulate, a1 = a2 = b5 = b6 (Figure 2(a–e)). Somite XXVI triannulate, a1 > a2 < a3 (b5 = b6 dorsally); a3 being ventrally last complete annulus (Figure 2(c), 2(d)). Somite XXVII uniannulate with slight dorsolateral furrow on respective sides (Figure 2(c)). Anus behind somite XXVII; post-anal annulus absent (Figure 2(c)).

Somite X b5 and somite XIII a2, respectively, being first and last annuli of clitellum (Figure 2(e)).

Male gonopore in middle of somite XI b6 (Figure 2(e)). Female gonopore in middle of somite XIII a1, inconspicuous, located posterior to gastropore (Figure 2(e), 2(f)). Gonopores separated by 1/2 + 4 + 1/2 annuli (Figure 2(e)).

Anterior ganglionic mass in somite VI a2, a3 and somite VII a1. Ganglion VII in a2. Ganglion VIII in a2 and b5. Ganglia IX–XII, of each somite, in a2 (Figure 3(c)). Ganglion XIII in a2 and b5 (Figure 3(c)). Ganglia XIV–XXIV, of each somite, in a2 (Figure 3(c)). Ganglion XXV in a1. Ganglion XXVI in somite XXV b6. Posterior ganglionic mass in somite XXVI a1 and a2.

Eyes in 3 pairs, 1st pair dorsally on somite II/III, 2nd and 3rd pairs dorsolaterally on posterior margin of somite V (a1 + a2) (Figure 2(a)). Papillae numerous, minute, hardly visible, one row on every annulus.

Nephridiopores in 17 pairs, each situated ventrally at posterior margin of a1 of each somite in somites VIII–XXIV (Figure 2(b), 2(d), 2(e)).

Pharynx agnathous, euthylaematous, reaching to somite XIII/XIV (Figure 3(a)). Crop tubular, acaecate, reaching to somite XIX b5/b6 (Figure 3(b)). Intestine tubular, reaching to somite XXIII b5, with 1 pair of pouch-shaped intestinal caeca in somite XIX a2–b6 (Figure 3(b)); each caecum opening in behind junction between crop and intestine in somite XIX b6, then ascending to somite XIX a2. Rectum tubular, thin-walled, straight. Gastropore conspicuous, ventral, on middle of somite XIII a1 (Figure 2(e), 2(f)). Gastroporal duct bulbous, winding at junction with gastropore, reaching to somite XIII b6 (Figure 3(a)).

Testisacs multiple; on right side, in somite XIX b5 to smite XXV a2, in total ~28 testisacs, 1 in XIX, 5 in XX, 4 in XXI, 6 in XXII, 5 in XXIII, 5 in XXIV, 2 in XXV; on left side, in somite XIX b6 to somite XXV b5, in total ~30 testisacs, 1 in XIX, 5 in XX, 5 in XXI, 6 in XXII, 5 in XXIII, 5 in XXIV, 3 in XXV. Paired epididymides in somite XVI b6 to somite XIX a1, occupying 10 annuli (Figure 3(c)). Paired ejaculatory duct in somite XI b5 to somite XVI b6 (Figure 3(c)); coiled in position posterior to ovisacs; each duct crossing ventrally beneath each ovisac, then nearly straight in position anterior to ovisacs; each widening from respective junction with epididymis, narrowing at junction with atrial cornua, then turning proximally toward atrial cornua without pre-atrial loop (Figure 3(c–f)). Pair of muscular atrial cornua developed, ovate, in somite XI b5 and b6 (Figure 3(c–f)). Atrium short, muscular, globular in somite XI b5 and b6 (Figure 3(d–f)). Penis sheath and penis absent.

Paired ovisacs globular, in somite XIII a1–b5 (Figure 3(c), 3(g)). Oviduct thin-walled, left oviduct crossing ventrally beneath nerve cord (Figure 3(c), 3(g)); both oviduct converging into common oviduct in somite XIII a1/a2. Common oviduct thin-walled, short, directly descending to female gonopore (Figure 3(g)).

Variation

Measurements (*n* = 2, paratypes only): BL 35.0–36.1 mm, BW 3.1 mm, CL 1.5–1.6 mm, CW 1.9–2.1 mm. Somite XXVI biannulate, a1 < (a2 + a3), (a2 + a3) with slight dorsolateral furrow on respective sides, or triannulate a1 > a2 = a3. Small unpaired eye dorsally on right posterior margin of somite IV (KUZ Z2069).

Colouration

In life, dorsal surface yellow (Figure 1(c)), or yellowish brown; ventral surface whitish yellow, or greyish white; clitellum, when obvious, paler than other body parts (Figure 1(c)). Colour faded in preservative; dark mid-dorsal line present from somite VIII a1 to somite XXVII in holotype (Figure 1(a)).

Distribution

According to the previous records of quadrannulate *Orobdella* leeches from around Vladivostok (Ghilarov et al. 1969; Ghilarov and Perel 1971), *O. ghilarovi* inhabits the Ussuri Nature Reserve in addition to the type locality.

Natural history

This species was found curled up under rocks in moist habitats along a forest road. Since a mature individual (= holotype) bearing a slight clitellum and developed testisacs as well as ovisacs was collected on 21 August 2018, the reproductive season of this species may be around August.

Etymology

The specific name is a noun in the genitive case formed directly from the name of the late Dr Merkuriy Sergeevich Ghilarov, who provided the first record of *Orobdella* leeches from Primorye Territory (Ghilarov et al. 1969).

Molecular analyses results

The BI tree (mean ln $L = -45227.02$; Figure 4) for determining the phylogenetic position of

O. ghilarovi had an almost identical topology to that of the ML tree (ln $L = -46910.15$; not shown). *Orobdella ghilarovi* was a sister taxon of a well-supported clade (BS = 100%, PP = 1.0) including the two species, *O. kawakatsuorum* Richardson, 1975 and *O. koikei* Nakano, 2012, inhabiting Hokkaido, northern Japan, and adjacent islets (Nakano 2012; Nakano and Gongalsky 2014). The monophyly of the continental *O. ghilarovi* + Hokkaido clade was fully supported (BS = 100%, PP = 1.0). This clade was a sister lineage to a monophyletic group (BS = 100%, PP = 1.0) consisting of the other 18 species known from the Korean Peninsula, Taiwan, and the other Japanese islands in the south of Hokkaido. This major monophyletic group was divided into two sub-lineages: a lineage corresponded to *O. tsushimensis* Nakano, 2011 that is distributed in the Korean Peninsula and adjacent islands; and another clade (BS = 77%, PP = 0.97) contained the remaining 17 species.

The respective COI (LC431609–LC431610), and tRNA$^{\mathrm{Leu}}$–ND1 (LC431613–LC431615) sequences, which were obtained from the holotype and two paratypes of *O. ghilarovi*, did not exhibit any variations among them. Although base-compositions of the tRNA$^{\mathrm{Cys}}$–tRNA$^{\mathrm{Val}}$ regions in the three tRNA$^{\mathrm{Cys}}$–16S sequences (LC431616–LC431618) dovetailed completely with each other, the remaining 16S sequences were slightly variable, and thus the pairwise tRNA$^{\mathrm{Cys}}$–16S uncorrected *p*-distance within three individuals of *O. ghilarovi* was 0.20%.

Remarks

Orobdella ghilarovi clearly belongs to *Orobdella* as it possesses the generic diagnostic features defined by Nakano (2016a). Its molecular phylogenetic position also supports the present genus-level affinity of this new species. Additionally, the mitochondrial DNA sequences obtained from the present three specimens fully supported their taxonomic identity belonging to the same species. Although only one mature individual of the new species (= holotype) could be obtained, the dissected holotype provided sufficient morphological features of the new species along with the two undissected paratypes.

According to previous studies (Nakano 2010, 2011, 2012; Nakano and Lai 2012; Nakano 2014; Nakano and Seo 2014; Nakano 2016a, 2016b, 2017b; Nakano and Lai 2017; Nakano 2018), therefore, the new species can be distinguished from the 12 quadrannulate congeners, i.e. *O. angustata* Nakano, 2018, *O. brachyepididymis* Nakano, 2016a, *O. esulcata* Nakano, 2010, *O. kanaekoikeae* Nakano, 2017b, *O. kawakatsuorum*, *O. koikei*, *O. masaakikuroiwai* Nakano, 2014, *O. meisai* Nakano and Lai, 2017, *O. naraharaetmagarum* Nakano, 2016b, *O. tsushimensis* and *O. whitmani*, by the following combination of characteristics (Table 2): middle-type body length, uniannulate somite IV, quadrannulate somite XXV, 1/2 + 4 + 1/2 annuli between gonopores, pharynx reaching to posterior XIII, bulbous gastroporal duct, epididymides in somites XVI–XIX that occupy 10 annuli, and developed ovate atrial cornua. *Orobdella ghilarovi* is clearly distinguishable from the six sexannulate and two octannulate species by its mid-body somites that are quadrannulate.

Since internal characteristics of the Russian quadrannulate *Orobdella* leeches, which were reported as *O. whitmani*, were not provided by Ghilarov et al. (1969), their precise taxonomic account remains unclarified. However, it was stated that their male and female gonopores opened in the middle of the respective annuli, and the individuals possessed 4 complete annuli between the gonopores (Ghilarov et al. 1969), i.e. they bore 1/2 + 4 + 1/2 annuli between the gonopores. This characteristic is coincident with the feature of *O. ghilarovi*, and the Ghilarov's specimens were collected from a forest near Kangauz Settlement (Ghilarov et al. 1969), which is the old Chinese name of the type locality of the new species, Anisimovka Settlement renamed in 1972. Accordingly, the *Orobdella* leeches reported by Ghilarov et al. (1969) and Ghilarov and Perel (1971) are judged herein as *O. ghilarovi*. Additionally, *Orobdella* individuals were also collected from two other locations of Primorye Territory, which are more distant from Vladivostok, i.e. in the Khasan Settlement, the vicinity near Russian/North Korean state boundary, and nearby the Chuguevka Settlement (Kurcheva 1977). Their taxonomic account should be revised by future taxonomic studies.

The obtained molecular phylogenies revealed that *O. ghilarovi* is genetically close to *O. kawakatsuorum* and *O. koikei* inhabiting Hokkaido and adjacent islets; the new species did not form a monophyletic group with *O. tsushimensis*, which is distributed in the continental Korean Peninsula and adjacent islets (Nakano 2011; Nakano and Seo 2012, 2014). This result highlighted the close relationships among the three species, i.e. *O. ghilarovi*, *O.*

kawakatsuorum and *O. koikei*, with insight into the biogeographical history of the genus *Orobdella*. Future faunal and systematic studies of *Orobdella* leeches in the Russian Far East including Sakhalin Island will lead us to a better understanding of the evolutionary and biogeographical histories of this terrestrial macrophagous leech group. Moreover, a recent molecular phylogenetic study shed light onto a distinctive phylogenetic status of the American terrestrial macrophagous species *Americobdella valdiviana* (Philippi, 1872), and split Arhynchobdellida Blanchard, 1894 into the three suborders (Tessler et al. 2018), i.e. Americobdelliformes Siddall, de Carle and Tessler in Tessler et al., 2018, Erpobdelliformes Sawyer, 1986 and Hirudiniformes Caballero, 1953. Since the genus *Orobdella* forms a basal phylogroup within Erpobdelliformes (Nakano et al. 2012, 2018), future evolutionary studies that focus on phylogenetic positions of this genus and the American terrestrial macrophagous taxa will elucidate a key evolutionary event of the arhynchobdellidan leeches.

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Table 1. Samples used for phylogenetic analyses; the information on the vouchers is accompanied by the INSDC accession numbers

Species	Voucher[1]	INSDC#[2]					
		18S	28S	Histone H3	COI	tRNA^{Cys}–16S	tRNA^{Leu}–ND1
Orobdella							
O. ghilarovi sp. nov.	KUZ Z2068 Holotype	LC431608*	LC431607*	LC431612*	LC431609*	LC431616*	LC431613*
O. ghilarovi sp. nov.	KUZ Z2069 Paratype				LC431610*	LC431617*	LC431614*
O. ghilarovi sp. nov.	KUZ Z2070 Paratype				LC431611*	LC431618*	LC431615*
O. angustata	KUZ Z1439 Holotype	LC323140	LC431606*	LC323138	LC323139	LC323141	LC323137
O. brachyepididymis	KUZ Z1673 Holotype	LC106319	LC274535	LC106321	LC106320	LC106318	LC106322
O. dolichopharynx	KUZ Z120 Holotype	AB663665	LC274541	AB698876	AB679680	AB679681	AB828558
O. esulcata	KUZ Z29 Holotype	AB663655	LC274538	AB698873	AB679664	AB679665	AB828555
O. ijimai	KUZ Z110 Topotype	AB663659	LC274542	AB698877	AB679672	AB679673	AB828559
O. kanaekoikeae	KUZ Z1747 Holotype	LC184551	LC274533	LC184553	LC184552	LC184550	LC184554
O. kawakatsuorum	KUZ Z167 Topotype	AB663661	LC274544	AB698878	AB679704	AB679705	AB828561
O. ketagalan	KUZ Z208 Holotype	AB704785	LC274546	AB704786	AB704787	AB828582	AB828563
O. koikei	KUZ Z156 Holotype	AB698883	LC274543	AB698882	AB679688	AB679689	AB828560
O. masaakikuroiwai	KUZ Z694 Holotype	AB938003	LC274530	AB938013	AB938006	AB937997	AB938016
O. meisai	KUZ Z1917 Holotype	LC314423	LC431605*	LC314425	LC314424	LC314422	LC314426
O. mononoke	KUZ Z224 Holotype	AB698868	LC274547	AB698869	AB698866	AB698867	AB828564
O. nakahamai	KUZ Z1672 Holotype	LC106330	LC274534	LC106332	LC106331	LC106329	LC106333
O. naraharaetmagarum	KUZ Z1652 Holotype	LC087143	LC274531	LC087145	LC087144	LC087142	LC087146
O. octonaria	KUZ Z181 Topotype	AB698870	LC274545	AB698871	AB679708	AB679709	AB828562
O. okanoi	KUZ Z1671 Holotype	LC106341	LC274532	LC106343	LC106342	LC106340	LC106344

O. shimadae	KUZ Z128 Holotype	AB663663	LC274540	AB698875	AB679676	AB679677	AB828557
O. tsushimensis	KUZ Z134 Holotype	AB663653	LC274537	AB698872	AB679662	AB679663	AB828554
O. whitmani	KUZ Z45 Topotype	AB663657	LC274539	AB698874	AB679668	AB679669	AB828556
O. yamaneae	KUZ Z1678 Holotype	LC106349	LC274536	LC106351	LC106350	LC106348	LC106352
Outgroup							
Erpobdella japonica	KUZ Z178	AB663648	LC274512	AB698879	AB679654	AB679655	AB828542
Gastrostomobdella ampunganensis	ZRC.ANN.0083 Holotype	LC274517	LC274516	LC274552	LC274551	LC274564	LC274571
Gastrostomobdella extenta	MUMNH-Hir0001 Holotype	LC274519	LC274518	LC274554	LC274553	LC274565	LC274572
Gastrostomobdella monticola	UNIMAS/A3/BH01/10	AB663649	LC274515	AB698880	AB679656	AB679657	AB828543
Gastrostomobdella cf. *monticola*	KUHE 56264	LC274514	LC274513	LC274550	LC274549	LC274563	LC274570
Mimobdella japonica	KUZ Z179	AB663650	LC274528	AB698881	AB679658	AB679659	AB828544
Odontobdella blanchardi	KUZ Z180	AB663651	LC274529	AB938012	AB938004	AB937995	AB938014
Salifa motokawai	VNMN 2015.65 Holotype	LC029434	LC274548	LC029435	LC029431	LC029432	LC029433

[1] Acronyms: KUHE, Kyoto University, Human and Environmental Studies; KUZ, Zoological Collection of Kyoto University; MUMNH, Mahidol University Museum of Natural History; UNIMAS, Universiti Malaysia Sabah; VNMN, Vietnam National Museum of Nature; ZRC, Zoological Reference Collection, Lee Kong Chian Natural History Museum.

[2] Sequences marked with an asterisk (*) were obtained for the first time in the present study.

Table 2. Comparisons of morphological characters between *Orobdella ghilarovi* sp. nov. and 12 quadrannulate species

Species	Body length	Somite IV	Somite XXV	Annuli between gonopores	Pharynx length	Gastroporal duct	Epididymides	Atrial cornua
O. ghilarovi sp. nov.	middle	1	4	1/2 + 4 + 1/2	to posterior XIII	bulbous	XVI to XIX	developed, ovate
O. angustata	middle?	1	4	1/2 + 4 + 1/3	to anterior XIV	bulbous	XVIII to XX	developed, hyperbologidal
O. brachyepididymis	small	1	4	1/2 + 4 [+ (< 1/2)]	to anterior XIV	tubular	XX to XXI	small, ovate
O. esulcata	middle	1	4	2/3 + 4 + 1/3	to anterior to posterior XIV	tubular, but bulbous at junction with gastropore	XVI to XX	developed, ovate
O. kanaekoikeae	small	1	4	1/2 + 4 + 1/2	to posterior XIII to anterior XIV	bulbous	XIV to XVIII	developed, ovate
O. kawakatsuorum	middle	2	4	6	to middle to posterior XIV	simple tubular	XVI to XVII	undeveloped
O. ketagalan	middle	1	4	1/2 + 4 + 1/2	to posterior XIV	simple tubular	absent	undeveloped
O. koikei	small	1	3	1/2 + 4 + 1/2	to posterior XIII to anterior XIV	bulbous	XV to XX	developed, ovate
O. masaakikuroiwai	small	1	4	1/2 + 4 + 1/2	to anterior to middle XIV	bulbous	XVI to XVIII	developed, ovate
O. meisai	middle	1	4	5 + 1/4	to posterior XV	rudimentary tubular	absent	absent
O. naraharaetmagarum	small	1	4	1/2 + 4 + 1/2	to posterior XIII	bulbous	XV to XX	developed, ovate
O. tsushimensis	middle	1	4	1/2 + 5	to posterior XIII to posterior XIV	bulbous	XVII to XIX	developed, ovate
O. whitmani	middle	1 or 2	4	1/2 + 4 + 1/2	to anterior to posterior XIV	bulbous	XVI to XVIII	developed, ovate

Figure captions

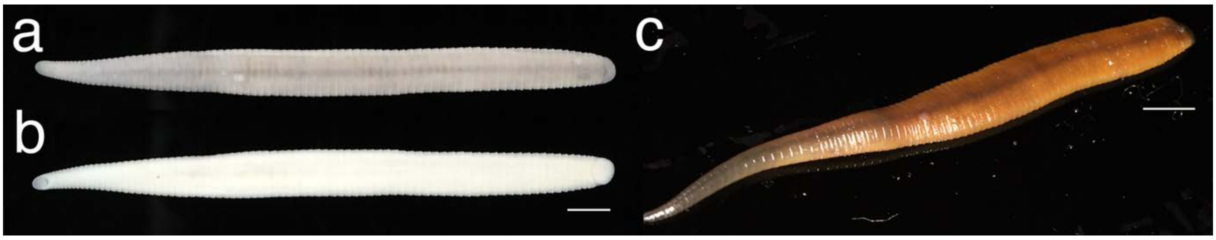
Figure 1. *Orobdella ghilarovi* sp. nov., holotype, KUZ Z2068. (a) Dorsal view. (b) Ventral View. (c) Dorsal view of live animal. Scale bars, 5 mm.

Figure 2. *Orobdella ghilarovi* sp. nov., holotype, KUZ Z2068. (a) Dorsal view of somites I–VIII. (b) Ventral view of somites I–VIII. (c) Dorsal view of somites XXIV–XXVII and caudal sucker. (d) Ventral view of somites XXIV–XXVI and caudal sucker. (e) Ventral view of somites X–XIII. (f) Ventral view of gastropore and female gonopore. Abbreviations: af, annular furrow; an, anus; cl, clitellum; fg, female gonopore; gp, gastropore; mg, male gonopore; np, nephridiopore. Scale bars, 2 mm (a–e); 0.2 mm (f).

Figure 3. *Orobdella ghilarovi* sp. nov., holotype, KUZ Z2068. (a) Ventral view of gastroporal duct. (b) Ventral view of junction between crop and intestine with intestinal caeca. (c) Dorsal view of reproductive system including ventral nervous system. (d) Dorsal view of male atrium including position of ganglion XI. (e) Left lateral view of male atrium. (f) Ventral view of male atrium. (g) Dorsal view of female reproductive system including position of ganglion XIII. Abbreviations: ac, atrial cornua; at, atrium; cod, common oviduct; cp, crop; ed, ejaculatory duct; ep, epididymis; gd, gastroporal duct; gp, gastropore; ic, intestinal caecum;

in, intestine; od, oviduct; ov, ovisac; ph, pharynx. Scale bars, 2 mm (a, b); 5 mm (c); 0.5 mm (d–g).

Figure 4. Bayesian inference tree for 8084 bp of nuclear 18S rRNA, 28S rRNA and histone H3 plus mitochondrial COI, tRNA^{Cys}, tRNA^{Met}, 12S rRNA, tRNA^{Val}, 16S rRNA, tRNA^{Leu} and ND1 markers. Inset shows real branch lengths. Numbers on nodes represent bootstrap values for maximum likelihood and Bayesian posterior probabilities.



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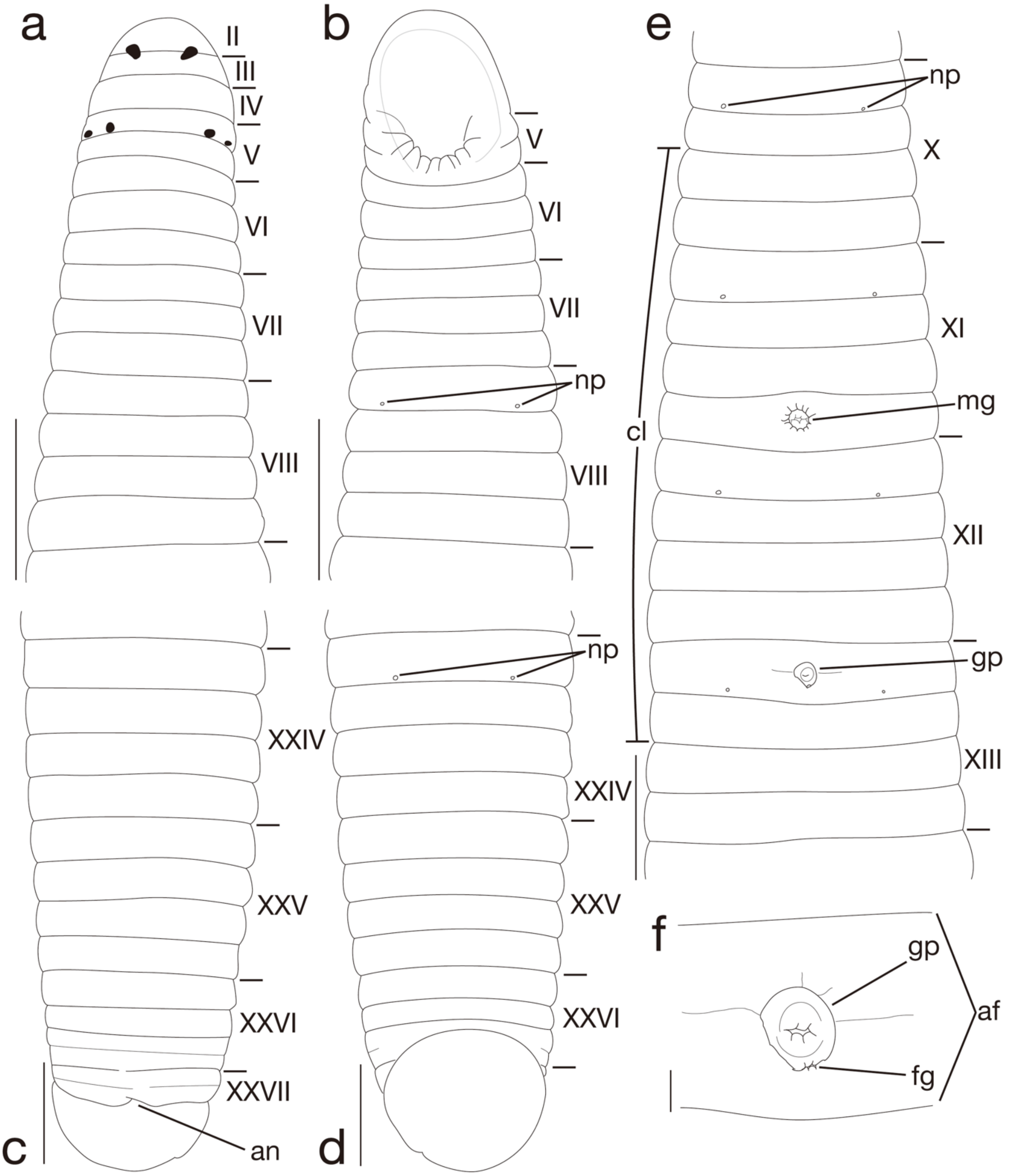

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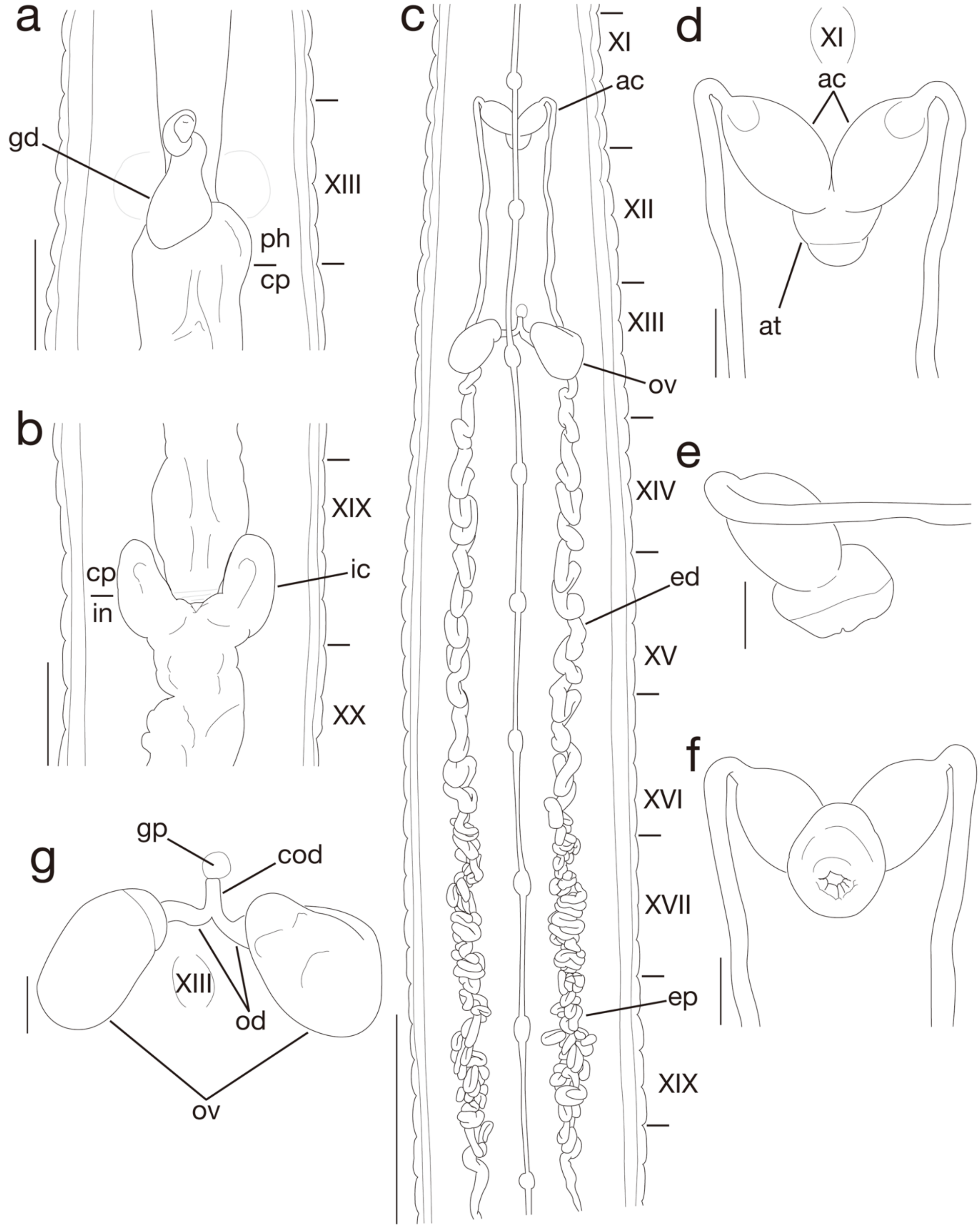

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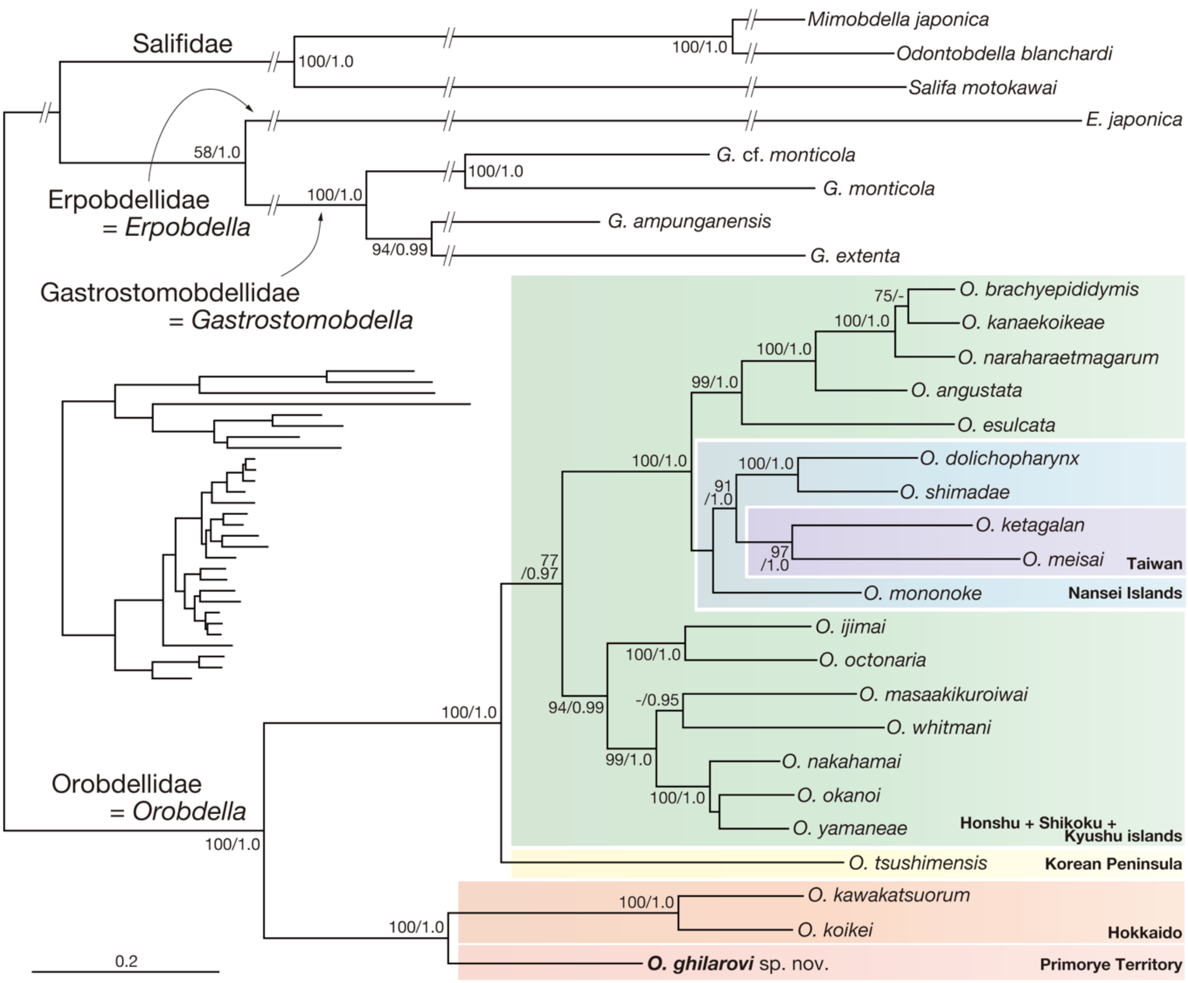

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