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A new species of *Orobdella* (Hirudinida, Arhynchobdellida, Orobdellidae) from the Tsukuba Mountains in Japan

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Abstract

A small-type quadrannulate leech species, *Orobdella montipumila* sp. nov., from the Tsukuba Mountains in central Honshu island, Japan, 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 revealed that *O. montipumila* formed a monophyletic lineage with seven congeners that include the small-type quadrannulate *O. masaakikuroiwai* Nakano, 2014, which is endemic to montane regions of central Honshu. However, the present phylogenies showed that *O. montipumila* and *O. masaakikuroiwai* are not sister species, which confirms that reduction of body length has arisen independently in *Orobdella* leeches indigenous to central Honshu.

Keywords body length | Erpobdelliformes | macrophagous | molecular phylogeny | quadrannulate

1. Introduction

The genus *Orobdella* is a leech taxon that comprises 21 terrestrial macrophagous species indigenous to Far East Asia (Sawyer 1986, Nakano 2017a, Nakano & Prozorova 2019). *Orobdella* leeches are known as earthworm eaters (Nakano 2017a), and at the same time, constitute the diets of centipedes, snakes, and moles (Shibata 1968, Yokohata 1998, Fukuyama & Nakano 2018). *Orobdella* leeches are characterized by possession of the gastroporal duct in their digestive tract, which receives a spermatophore during copulation (Nakano 2017b). The 21 species of *Orobdella* can be also grouped into three morphotypes by their mid-body somite annulation—4- (quadr-), 6- (sex-) and 8- (oct-) annulate—, and by the body lengths of mature individuals—small (~5 cm), middle (~10 cm) and large (~20 cm) types—, respectively (Nakano 2017a).

All five small-type species of *Orobdella* are categorized as quadrannulate species, and are endemic to the Japanese Archipelago (Nakano 2017b): *O. koikei* Nakano, 2012 from Hokkaido island, *O. masaakikuroiwai* Nakano, 2014 from central Honshu island, *O. naraharaetmagarum* Nakano, 2016a from western Honshu, and *O. brachyepididymis* Nakano, 2016b and *O. kanaekoikeae* Nakano, 2017b from Shikoku island. Unidentified small-type quadrannulate *Orobdella* leeches were collected from the Tsukuba Mountains in eastern-central Honshu, and they are described as a new species herein. The phylogenetic position of the new species within *Orobdella* was estimated using nuclear and mitochondrial genetic markers.

2. Materials and methods

Sampling and morphological examination. Leeches were collected from montane forests in Tsukuba Mountains, Honshu, Japan. Where possible, geographical coordinates of the collection sites were obtained using a Garmin eTrex® GPS unit (Garmin Ltd., Olathe, KS, USA).

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Specimens were relaxed by the gradual addition of absolute ethanol (EtOH) to fresh water, 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 remainder of the body was fixed in 10% formalin, and preserved in 70% EtOH. Four measurements were taken: body length from the anterior margin of the oral sucker to the posterior margin of the caudal sucker, maximum body width, caudal sucker length from the anterior to the posterior margin of the sucker, and caudal sucker width from the right to the left margin of the sucker. Examination, dissection, and drawing of the specimens were conducted using a Leica M125C stereoscopic microscope with a drawing tube (Leica Microsystems, Wetzlar, Germany). Specimens examined in this study were deposited in the Zoological Collection of Kyoto University (KUZ).

Terminology and abbreviations. 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. The abbreviations for morphological characters used in the text and figures are as follows: **ac** – atrial cornu, **af** – annular furrow, **an** – anus, **at** – atrium, **BL** – body length, **BW** – body width, **CL** – caudal sucker length, **cl** – clitellum, **cod** – common oviduct, **cp** – crop, **CW** – caudal sucker width, **ed** – ejaculatory duct, **ep** – epididymis, **fg** – female gonopore, **gd** – gastroporal duct, **gp** – gastropore, **mg** – male gonopore, **np** – nephridiopore, **od** – oviduct, **ov** – ovisac, **ph** – pharynx, **ts** – testisac.

Molecular phylogenetic analyses. The phylogenetic position of the new *Orobdella* species within the genus was estimated 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, polymerase chain reactions and cycle sequencing reactions were elucidated in Nakano & Lai (2016, 2017). In total, 15 sequences were newly obtained and deposited with the International Nucleotide Sequence Database Collaboration (INSDC) through the DNA Data Bank of Japan (Table S1 supplement on www.soil-organisms.org).

In addition to the newly obtained sequences, 174 sequences of 21 *Orobdella* species and eight erpobdelliform taxonomic units, which were selected as the outgroup, were obtained from the INSDC in accordance with previous studies (Nakano 2018, Nakano & Prozorova 2019), and were included in the present dataset (Table S1). The alignments of H3 and COI were trivial, as no indels were observed. The sequences of the other markers were aligned using MAFFT v7.471 L-INS-i (Katoh & Standley 2013). The lengths of the 18S, 28S, H3, COI, $tRNA^{Cys}$–16S, and $tRNA^{Leu}$–ND1 were 1850, 2823, 328, 1267, 1195, 640 bp, respectively. The concatenated sequences thus yielded 8103 bp of aligned positions.

Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian inference (BI). The best-fit partition scheme and models were identified based on the Bayesian information criterion using PartitionFinder v2.1.1 (Lanfear et al. 2017) with the 'greedy' algorithm (Lanfear et al. 2012). The selected partition scheme and models were as follows: for 18S and H3 1st position, TRNEF+I+G (GTR+I+G for BI); for 28S, GTR+I+G; for H3 2nd position, JC+I; for H3 3rd position, K81UF+G (GTR+G for BI); for COI 1st position, TRN+G (GTR+G for BI); for 2nd positions of COI and ND1, TVM+I+G (GTR+I+G for BI); for 3rd positions of COI and ND1 and 16S, TRN+I+G (GTR+I+G for BI); for ND1 1st position, TRN+I+G (GTR+I+G for BI); and for $tRNA^{Cys}$, $tRNA^{Met}$, 12S and $tRNA^{Val}$, GTR+I+G. The ML tree was calculated using IQ-Tree v2.0.5 (Minh et al. 2020) with non-parametric bootstrapping (BS) conducted with 1000 replicates. BI tree and Bayesian posterior probabilities (PP) were estimated using MrBayes v3.2.7a (Ronquist et al. 2012). Two independent runs for four Markov chains were conducted for 40 million generations, and the tree was sampled every 100 generations. The parameter estimates and convergence were checked using Tracer v1.7.1 (Rambaut et al. 2018), and then first 100 001 trees were discarded based on the results.

ZooBank registration. This work was registred with ZooBank under urn:lsid:zoobank.org:pub:8B39C5A8-9380-472F-89A4-7F95BB534336.

3. Results

***Orobdella montipumila* sp. nov.**
(Figures 1–3)

Diagnosis. Body length of mature individuals reaching ca. 5 cm. Somite IV uniannulate, somites VIII–XXV quadrannulate. Clitellum in somite XI b5 to somite XIII a2. Male gonopore slightly anterior to middle of, or in middle of somite XI b5, female gonopore slightly anterior to middle of, or in middle of somite XIII a1, behind gastropore, gonopores separated by 1/2 + 4 + 1/2 annuli. Pharynx reaching to somite XIV a1–a2. Gastropore conspicuous, slightly anterior to middle of, or in middle of somite XIII a1. Gastroporal duct bulbous. Paired epididymides in somites XVII–XX, occupying 7–11 annuli. Atrial cornua developed, ovate.

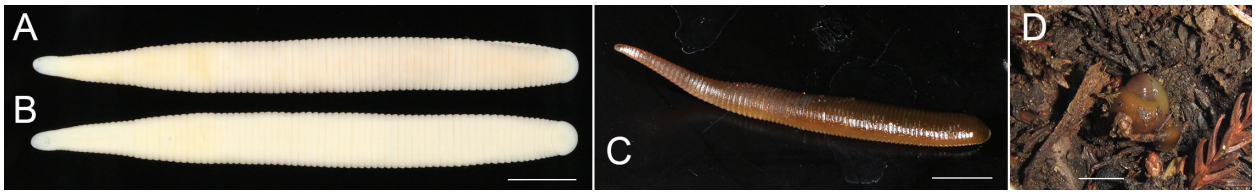

Figure 1. *Orobdella montipumila* sp. nov., holotype, KUZ Z3913. (**A**) Dorsal view. (**B**) Ventral view. (**C**) Dorsal view of live animal. (**D**) Live animal found curled up under a stone at the type locality. Scale bars: 5 mm (same bar for A and B).

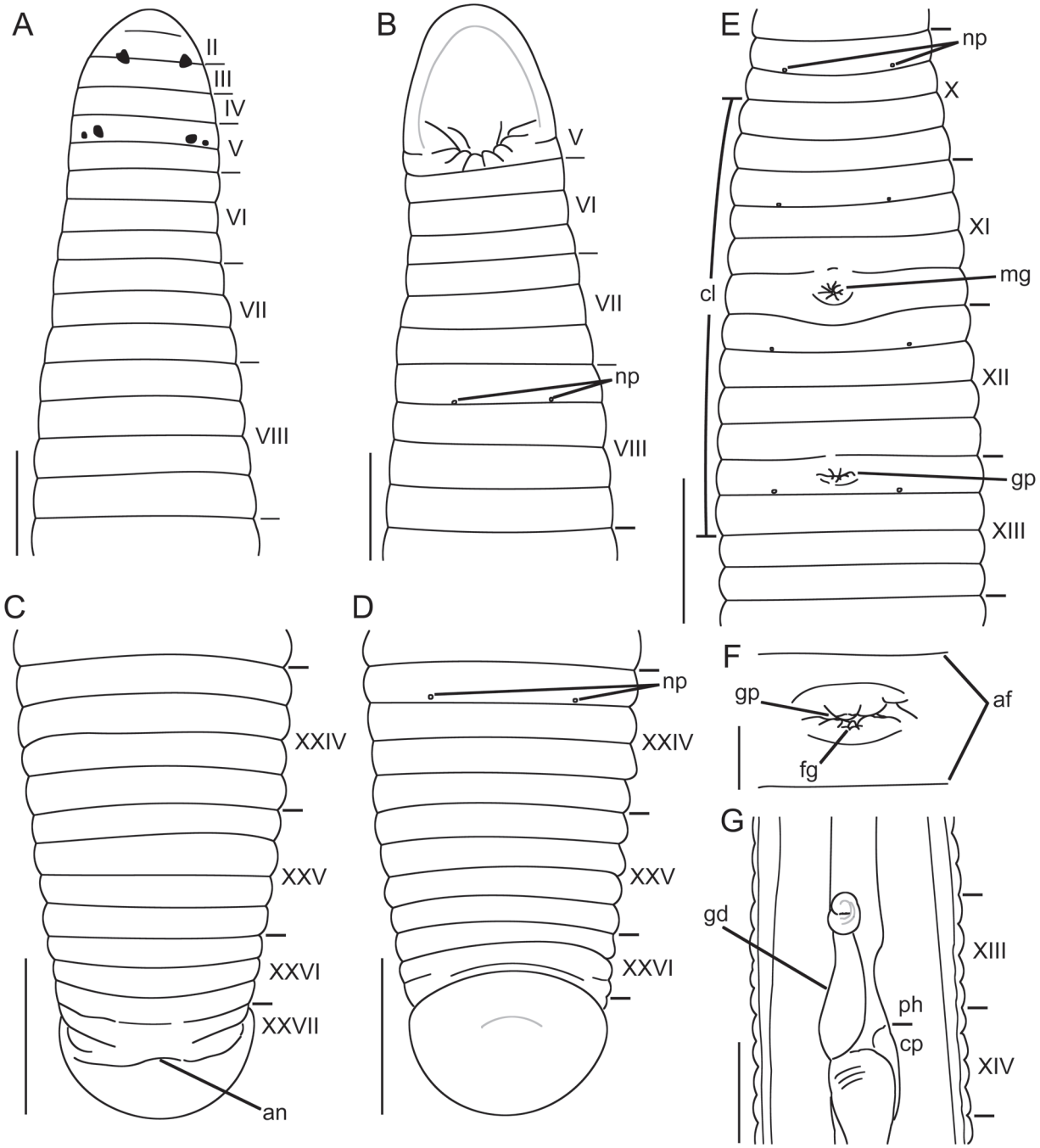

Figure 2. *Orobdella montipumila* sp. nov., holotype, KUZ Z3913. (**A**) Dorsal view of somites I–VIII. (**B**) Ventral view of somites I–VIII. (**C**) Dorsal view of somites XXIV–XXVII and caudal sucker. (**D**) Ventral view of somites XXIV–XXVI and caudal sucker. (**E**) Ventral view of somites X–XIII. (**F**) Ventral view of gastropore and female gonopore. (**G**) Ventral view of gastroporal duct. Scale bars: A, B, G = 1 mm; C–E = 2 mm; F = 0.25 mm.

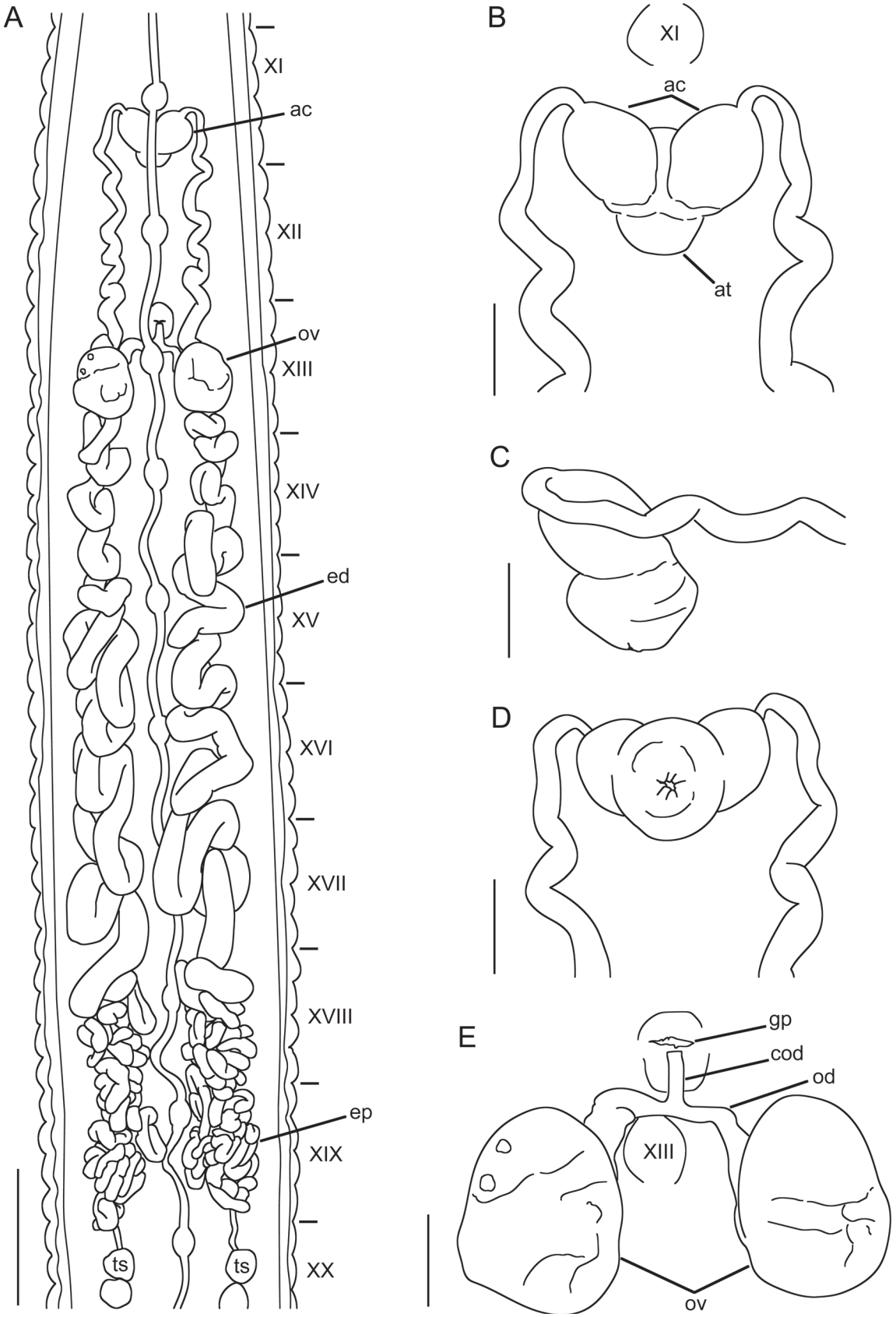

Figure 3. *Orobdella montipumila* sp. nov., holotype, KUZ Z3913. (**A**) Dorsal view of reproductive system, including ventral nervous system. (**B**) Dorsal view of male atrium including position of ganglion XI. (**C**) Left lateral view of male atrium. (**D**) Ventral view of male atrium. (**E**) Dorsal view of female reproductive system, including position of ganglion XIII. Scale bars: A = 2 mm; B–E = 0.5 mm.

Type material. Holotype. KUZ Z3913 (Fig. 1), dissected, collected from under a stone along a mountain trail, Onokawa, Mt. Tsukubasan in Tsukuba Mountains, Sakuragawa, Ibaraki Prefecture (Honshu island), Japan (36.234870°N, 140.099470°E; elev. 520 m), by T. Nakano on 22 May 2019. Paratypes. In total 4 specimens collected in Tsukuba Mountains, Ibaraki Prefecture: KUZ Z1349, from under a stone along a mountain stream, Mt. Kabasan, Ishioka (~36.2°N, ~140.18°E), by K. Eto on 27 Mar 2010; KUZ Z1367, litter, Mt. Wagakunisan, Kasama (~36.32°N, ~140.20°E), by R. Ueshima on 27 Nov 2011; KUZ Z1557, litter, along a forest road, Uwaso, Ishioka (36.254361°N, 140.132222°E; elev. 403 m), by T. Nakano on 16 Jun 2014; and KUZ Z1560, from under soil along a mountain trail, Menokawa, Mt. Tsukubasan, Sakuragawa (36.2337°N, 140.1039°E; elev. 510 m) by Y. Yamane on 16 Jun 2014; KUZ Z1349, Z1367, Z1557, dissected.

Description. Body firm and muscular, elongate, with constant width in caudal direction, dorsoventrally compressed, BL 39.7 mm, BW3.8 mm (Fig. 1A, B). Caudal sucker ventral, elliptic, CL 1.9 mm, CW 2.5 mm (Figs 1B, 2D).

Somite I completely merged with prostomium. Somite II (= peristomium), III, IV uniannulate (Fig. 2A). Somite V biannulate, (a1 + a2) = a3; a3 forming posterior margin of oral sucker (Fig. 2A, B). Somites VI and VII triannulate, a1 = a2 = a3 (Fig. 2A, B). Somites VIII–XXV quadrannulate, a1 = a2 = b5 = b6 (Fig. 2A–E). Somite XXVI dorsally triannulate, a1 = a2 = a3, ventrally biannulate a1 < (a2 + a3); (a2 + a3) being ventrally last complete annulus (Fig. 2C, D). Somite XXVII biannulate (Fig. 2C). Anus behind somite XXVII; post-anal annulus absent (Fig. 2C).

Somite X b5 and somite XIII a2, respectively, first and last annuli of clitellum (Fig. 2E).

Male gonopore slightly anterior to middle of somite XI b6 (Fig. 2E). Female gonopore in middle of somite XIII a1, inconspicuous, located posterior to gastropore (Fig. 2E, F). Gonopores separated by 1/2 + 4 + 1/2 annuli (Fig. 2E).

Anterior ganglionic mass in somite VI a2 and a3, Ganglia VII–IX, of each somite, in a2. Ganglia X and XI, of each somite, in a2 and b5 (Fig. 3A). Ganglia XII–XVIII, of each somite, in a2 (Fig. 3A). Ganglion XIX in a1 and a2 (Fig. 3A). Ganglion XX in a1 (Fig. 3A). Ganglia XXI and XXII, of each somite, in a1 and a2. Ganglion XXIII in a1. Ganglion XXIV in a1 and a2. Ganglion XXV in somite XXIV b6 and somite XXV a1. Ganglion XXVI in somite XXV b5 and b6. Posterior ganglionic mass in somite XXVI a1 and (a2 + a3).

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

Nephridiopores in 17 pairs, each situated ventrally at posterior margin of a1 of each somite in somites VIII–XXIV (Fig. 2B, D, E).

Pharynx agnathous, euthylaematous, reaching to somite XIV a1 (Fig. 2G). Crop tubular, acecate, reaching to somite XIX b5/b6. Intestine tubular, reaching to XXIV b5/b6, with 1 pouch-shaped intestinal ceca on left side in XIX a2 and b5, opening in behind junction between crop and intestine in somite XIX b5/b6, then ascending to somite XIX a2. Rectum tubular, thin-walled, straight. Gastropore conspicuous, ventral, in middle of somite XIII a2 (Fig. 2E, F). Gastroporal duct bulbous, slightly winding at junction with gastropore, reaching to somite XIV a2/b5 (Fig. 2G).

Testisacs multiple in somite XX a2 to XXV a2 (Fig. 3A); on each side, in total ~21 testisacs, 3 in XX, 4 in each somite of somites XXI–XXIV, 2 in XXV. Paired epididymides; right epididymis in somite XVIII a1/a2 to somite XIX/XX, occupying 7 annuli; left epididymis in somite XVIII a1 to somite XIX/XX, occupying 8 annuli (Fig. 3A). Paired ejaculatory ducts; right duct in somite XI b5 to somite XVIII a1/a2; left duct in somite XI b5 to somite XVIII a1; coiled in position posterior to ovisacs; each duct crossing ventrally beneath each ovisac, then loosely coiled in position anterior to ovisacs; each widening from respective junction with epididymis, narrowing at junction with atrial cornua, then loosely turning proximally toward atrial cornua without pre-atrial loop (Fig. 3A, B). Pair of muscular atrial cornua developed, ovate, in somite XI b5 and b6 (Fig. 3A–D). Atrium short, muscular, globular in somite XI b5 and b6 (Fig. 3B–D).

Paired ovisacs globular, in somite XIII a2 and b5 (Fig. 3A, E). Oviducts thin-walled, left oviduct crossing ventrally beneath nerve cord (Fig. 3A, E); both oviducts converging into common oviduct in somite XIII a1/a2. Common oviduct thin-walled, short, directly descending to female gonopore (Fig. 3E).

Variation. Measurements (mean ± 1SD, followed by ranges in parentheses; n = 5, including holotype): BL 37.8 ± 9.6 mm (21.3–50.3 mm), BW 3.9 ± 1.1 mm (2.3–5.1 mm), CL 1.9 ± 0.4 mm (1.2–2.4 mm), CW 2.4 ± 0.6 mm (1.4–2.8 mm). Somite XXVI triannulate a1 = a2 < a3; a3 being ventrally last complete annulus. Male gonopore slightly anterior to middle of somite XI b6, or in middle of somite XI b6. Female gonopore slightly anterior to middle of somite XIII a1, or in middle of somite XIII a1. Pharynx reaching to XIV a2. Crop reaching to somite XIX b5/b6–XIX/XX. Intestine reaching to somite XXIII/XXIV–XXIV a1. Gastroporal duct reaching to somite XIV a2–somite XIV b5/b6. Testisacs; right side ~26 sacs in somite XX b5

to somite XXV a1–b5; left side ~26 sacs in somite XX a2 to somite XXV a1–b6. Paired epididymides; right epididymis in somite XVII a2/b5–b6 to somite XIX b5–somite XX a2, occupying 9–11 annuli; left epididymis in somite XVII a2/b5–b5/b6 to somite XIX b6–somite XX a1/a2, occupying 10 annuli. Paired ejaculatory ducts; right side in somite XI b5 to somite XVII a2/b5–b6; left side in somite XI b5 to somite XVII a2/b5–b5/b6; loosely coiled, or nearly straight in position anterior to ovisacs. Paired ovisacs in somite XIII a2 and b5, or in somite XIII a2–b6. Right or left oviduct crossing ventrally beneath nerve cord.

Coloration. In life, dorsal surface yellowish brown (Fig. 1C); ventral surface whitish yellow; clitellum, when obvious, paler than other body parts (Fig. 1C). Color faded in preservative.

Distribution. This species was collected only from Tsukuba Mountains.

Natural history. This species was found curled up under rocks or fallen leaves in moist habitats in the Tsukuba Mountains (Fig. 1D). Because mature individuals bearing an obvious clitellum were collected on 22 May 2019 and 16 June 2014, it is estimated that the reproductive season of this species begins before early June.

Etymology. The specific name is a compound adjective in nominative singular derived from the Latin words, *mons* (mountain) and *pumilus* (dwarf), referring to the fact that this small-type new species inhabits Tsukuba Mountains.

Molecular phylogenetic position. The BI (mean ln $L = -46390.05$; Fig. 4) and ML (ln $L = -46373.90$; not shown) trees had almost identical topologies, and very closely matched those of previous analyses (Nakano 2018, Nakano & Prozorova 2019). *Orobdella montipumila* was clustered into a well-supported clade (BS = 80%, PP = 0.99) that comprised seven other *Orobdella* species. This lineage was split into three clades. The first clade (BS = 100%, PP = 1.0) comprised the middle-type sexannulate *O. ijimai* Oka, 1895 and the large-type octannulate *O. octonaria* Oka, 1895, both of which are distributed in Honshu (Nakano 2017a). The second clade (BS = 90%, PP = 1.0) included the middle-type quadrannulate *O. whitmani* Oka, 1895, the small-type quadrannulate *O. masaakikuroiwai*, the middle-type sexannulate *O. okanoi* Nakano, 2016b and *O. yamaneae* Nakano, 2016b, and the large-type octannulate *O. nakahamai* Nakano, 2016b; while the former two species are indigenous to Honshu (Nakano 2017a), the latter three species have been described from Shikoku and an adjacent islet (Nakano 2016b). The last lineage comprised only *O. montipumila*. Precise relationships among the three clades could not be elucidated.

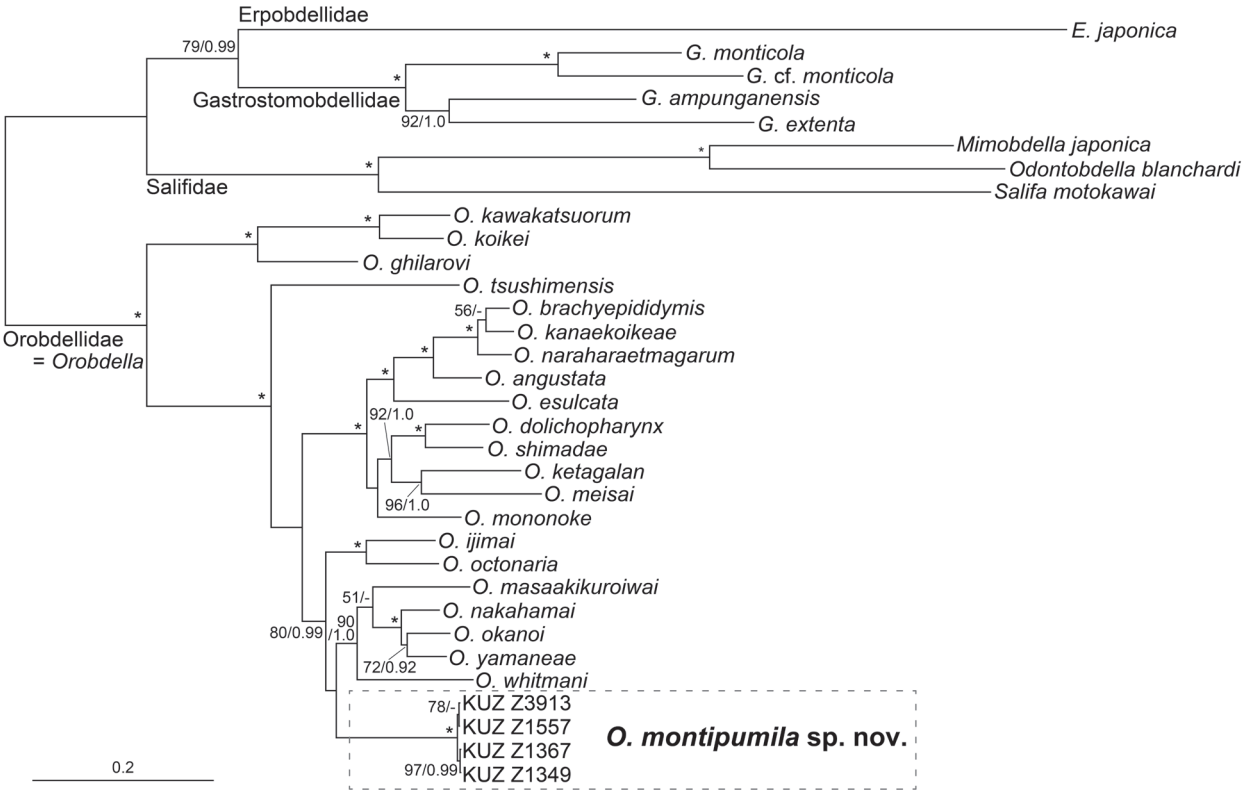

Figure 4. Bayesian inference tree for 8103 bp of nuclear 18S rRNA, 28S rRNA and H3, and mitochondrial COI, $tRNA^{Cys}$, $tRNA^{Met}$, 12S rRNA, $tRNA^{Val}$, 16S rRNA, $tRNA^{Leu}$ and ND1 markers. Numbers on nodes indicate bootstrap (BS) values for maximum likelihood ≥ 50% and Bayesian posterior probabilities (PP) ≥ 0.90. An asterisk denotes the node with BS = 100% and PP ≥ 1.0.

The respective COI (LC616660–LC616663), $tRNA^{Cys}$–l6S (LC616664–LC616667) and $tRNA^{Leu}$–ND1 (LC616668–LC616671) sequences, which were obtained from the holotype and three paratypes of *O. montipumila*, were almost concordant with each other. The present phylogenies certified that the holotype and three paratypes of *O. montipumila* belong to a single species (BS = 100 %, PP = 1.0).

4. Remarks

Orobdella montipumila clearly belongs to *Orobdella* because this new species possesses the generic diagnostic features described by Nakano (2016b). The obtained phylogenies also corroborate that the new species is an unquestionable member of the genus *Orobdella*.

Orobdella montipumila clearly differs from the six sexannulate species and two octannulate species by its mid-body somites that are quadrannulate. According to previous studies (Nakano 2010, 2011, 2012, 2014, 2016a, 2016b, 2017b, Nakano & Lai 2012, 2017, Nakano & Seo 2014, Nakano & Prozorova 2019), *O. montipumila* is distinguishable from the 13 quadrannulate congeners, *O. angustata* Nakano, 2018, *O. brachyepididymis*, *O. esulcata* Nakano, 2010, *O. ghilarovi* Nakano & Prozorova, 2019, *O. kanaekoikeae*, *O. kawakatsuorum* Richardson, 1975, *O. ketagalan* Nakano & Lai, 2012, *O. koikei*, *O. masaakikuroiwai*, *O. meisai* Nakano & Lai, 2017, *O. naraharaetmagarum*, *O. tsushimensis* Nakano, 2011, *O. whitmani*, by the following combination of characteristics (Table 1): small-type body length, uniannulate somite IV, quadrannulate somite XXV, 1/2 + 4 + 1/2 annuli between gonopores, pharynx reaching to anterior of somite XIV, bulbous gastroporal duct, epididymides in somites XVII–XX that occupy 7–11 annuli, and developed ovate atrial cornua. *Orobdella montipumila* shares almost all of these diagnostic features with the small-type *O. masaakikuroiwai*, which inhabits central Honshu;

Table 1. Comparisons of morphological characters between *Orobdella montipumila* sp. nov. and 13 quadrannulate *Orobdella* species.

Species	Body length	Somite IV	Somite XXV	Annuli between gonopores	Pharynx length	Gastroporal duct	Epididymides	Atrial cornua
O. montipumila sp. nov.	small	1	4	1/2 + 4 + 1/2	to anterior XIV	bulbous	XVII to XX	developed, ovate
O. angustata	middle?	1	4	1/2 + 4 + 1/3	to anterior XIV	bulbous	XVIII to XX	developed, hyperboloidal
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. ghilarovi	middle	1	4	1/2 + 4 + 1/2	to posterior XIII	bulbous	XVI to XIX	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

nevertheless, the new species differs from the latter in a characteristic of the epididymides (located in somites XVII–XX in *O. montipumila*, vs. being located in somites XVI–XVIII in *O. masaakikuroiwai*; Nakano 2014).

The present phylogenies showed the monophyly of *O. montipumila* and the species that are indigenous to Honshu and Shikoku. Previous studies have already highlighted that the three small-type *Orobdella* inhabiting western Honshu and Shikoku—*O. brachyepididymis*, *O. kanaekoikeae* and *O. naraharaetmagarum*—form a well-supported clade among *Orobdella* leeches (Nakano 2017b); their close affinity was also confirmed in the present analyses. In contrast, the present results did not support a sister relationship of the small-type *O. montipumila* and the small-type *O. masaakikuroiwai*, and moreover showed deep divergence between these two species, although both species are distributed in montane regions of central Honshu. The obtained phylogenies thus suggest that size reduction arose at least twice within the lineage that contains *O. montipumila*, *O. masaakikuroiwai* and the other six congeners.

5. Acknowledgments

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