A New Quadrannulate Species of Orobdella (Hirudinida: Arhynchobdellida: Orobdellidae) from Kii Peninsula, Japan

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A quadrannulate leech species, Orobdella angustata sp. nov., from Kii Peninsula, Honshu, Japan is described. This new species is characterized by its peculiar male atrial cornua. Phylogenetic analyses using nuclear 18S 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. angustata is genetically close to the small quadrannulate species, O. brachyepididymis Nakano, 2016, O. kanaekoikeae Nakano, 2017 and O. naraharaetmagarum Nakano 2016, distributed in Chugoku District of Honshu and Shikoku, Japan.

Key Words: Erpobdelliformes, terrestrial, macrophagous, atrial cornu, molecular phylogeny.

Introduction

The terrestrial macrophagous leech genus Orobdella Oka, 1895 consists of 19 species inhabiting the Japanese Archipelago, Taiwan, and the Korean Peninsula (Nakano 2017a, b; Nakano and Lai 2017), and one unidentified species was recorded from the Russian Far East (Gilyarov et al. 1969; Nakano 2012). They can be grouped into three morpho-types each based on the mid-body somite annulation (quad-, sex-, and octannulate), and the body length of mature individuals (small, middle, and large-types), respectively (Nakano 2017a). Orobdella leeches can be also characterized by the possession of the gastroporal duct in their digestive tracts, which receives a spermatophore during copulation (Nakano 2017b). While the Orobdella species, in which the gastroporal duct is developed, possesses developed ovate or ellipsoidal atrial cornua, the species with a vestigial gastroporal duct bears undeveloped atrial cornua, or lacks a cornu-like structure in the male median reproductive system (Nakano and Lai 2017). Unidentified quadrannulate Orobdella leeches were collected from the Kii Peninsula, Honshu, Japan, and are described as a new species in this study. The phylogenetic position of the new species within Orobdella was estimated using nuclear and mitochondrial genetic markers.

Materials and Methods

Sampling and morphological examination. Leeches were collected from a montane forest in Kii Peninsula, Honshu, Japan. 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. 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 caudal sucker, and caudal sucker width (CW) from the right to the left margin of the caudal sucker. Examination, dissection, and drawing of the specimens were conducted using a stereoscopic microscope with a drawing tube (Leica M125). Specimens used in this study have been 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 newly identified Orobdella species within the genus was estimated based on two nuclear and three mitochondrial markers, i.e., 1) 18S rRNA, 2) histone H3, 3) cytochrome c oxidase subunit I (COI), 4) tRNA\(^{Cys}\), tRNA\(^{Met}\), 12S rRNA, tRNA\(^{Val}\), 16S rRNA (tRNA\(^{Cys}\)–16S), and 5) 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 (2017). In total, five sequences from the unidentified...
Orodella specimen were newly obtained in this study, and deposited with the International Nucleotide Sequence Database Collaboration (INSDC) through DNA Data Bank of Japan (Table 1).

The four eropodelliform outgroup taxa were identical to those used in the phylogenetic analyses by Nakano and Lai (2017) (Table 1). The alignments of H3 and COI were trivial, as no indels were observed. The sequences of 18S, tRNA\(^{\text{COI}}\), 16S, and tRNA\(^{\text{ND1}}\) were aligned using MAFFT v. 7.310L-INS-i (Katoh and Standley 2013). The lengths of the 18S, H3, COI, tRNA\(^{\text{COI}}\), 16S, and tRNA\(^{\text{ND1}}\) sequences were 1844, 328, 1267, 1135, and 637 bp, respectively. The concatenated sequences yielded 5211 bp of aligned positions.

Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI). The ML phylogeny was constructed using RAxML v. 8.2.8 (Stamatakis 2014) with the substitution model set as GTR\(^G\); for tRNACys, tRNAMet, 12S, tRNAVal, tRNALeu, and ND1, HKY\(^G\); for 3rd positions of COI and ND1, HKY\(^G\); and for 16S, GTR\(^G\)+I; and for H3 2nd position, JC\(^G\); for H3 3rd position, HKY\(^G\); and for tRNA\(^{\text{COI}}\), tRNA\(^{\text{Alu}}\), 12S, tRNA\(^{\text{Val}}\), tRNA\(^{\text{Leu}}\), and ND1 1st position, GTR\(^G\)+I; and for 16S, GTR\(^G\)+2. 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. 1.6.0 (Rambaut and Drummond 2013), and the first 50001 trees were discarded based on the results.

**Orodella angustata** sp. nov.

[New Japanese name: kubire-kugabiru]

(Figs 1–3)

**Diagnosis.** Body length of pre-mature individuals exceeding 5 cm; possibly middle-type. Somite IV unannulate, somites VIII–XXV quadrannulate. Male gonopore in middle of somite XI b6, female gonopore slightly anterior to

Table 1. Samples used for the phylogenetic analyses. The information on the vouchers is accompanied by the INSDC accession numbers. Sequences marked with an asterisk (*) were obtained for the first time in the present study. Acronyms: KUZ, the Zoological Collection of Kyoto University; and UNIMAS, the Universiti Malaysia Sarawak.

| Species Voucher | INSDC accession # |
|-----------------|-------------------|
| **Species**     | **Voucher**       | **18S** | **H3** | **tRNA\(^{\text{COI}}\)–16S** | **tRNA\(^{\text{Leu}}\)–ND1** |
| Orodella        | KUZ Z1433 Holotype | LC323140* | LC323138* | LC323139* | LC323141* | LC323137* |
| O. angustata sp. nov. | KUZ Z1673 Holotype | LC106319 | LC106321 | LC106320 | LC106318 | LC106322 |
| O. brachyepidymis Nakano, 2016 | KUZ Z120 Holotype | AB663665 | AB698876 | AB679680 | AB679681 | AB828558 |
| O. dolichopharynx Nakano, 2011 | KUZ Z29 Holotype | AB663655 | AB698873 | AB679664 | AB679665 | AB828553 |
| O. esakuta Nakano, 2010 | KUZ Z110 Holotype | AB663659 | AB698877 | AB679672 | AB679673 | AB828559 |
| O. ittani Oka, 1895 | KUZ Z110 Topotype | LC184551 | LC184553 | LC184552 | LC184550 | LC184554 |
| O. kawakatsuworum Richardson, 1975 | KUZ Z167 Holotype | AB663661 | AB698878 | AB679704 | AB679705 | AB828561 |
| O. ketagalan Nakano and Lai, 2012 | KUZ Z208 Holotype | AB704785 | AB704786 | AB704787 | AB828582 | AB828563 |
| O. kokie Nakano, 2012 | KUZ Z156 Holotype | AB698883 | AB698882 | AB679688 | AB679689 | AB828560 |
| O. massaikirinai Nakano, 2014 | KUZ Z694 Holotype | AB938003 | AB938013 | AB938006 | AB937997 | AB938016 |
| O. mesai Nakano and Lai, 2017 | KUZ Z1917 Holotype | LC314423 | LC314425 | LC314424 | LC314422 | LC314426 |
| O. mononoke Nakano, 2012 | KUZ Z224 Holotype | AB698868 | AB698869 | AB698866 | AB698867 | AB828564 |
| O. nakahama Nakano, 2016 | KUZ Z1672 Holotype | LC106330 | LC106332 | LC106331 | LC106329 | LC106333 |
| O. narashinaemacharum Nakano, 2016 | KUZ Z1652 Holotype | LC087143 | LC087145 | LC087144 | LC087142 | LC087146 |
| O. octonaria Oka, 1895 | KUZ Z181 Holotype | AB698870 | AB698871 | AB679708 | AB679709 | AB828562 |
| O. okamoto Nakano, 2016 | KUZ Z1671 Holotype | LC106341 | LC106343 | LC106342 | LC106340 | LC106344 |
| O. shimadae Nakano, 2012 | KUZ Z128 Holotype | AB663663 | AB698875 | AB679676 | AB679677 | AB828557 |
| O. shishiminesis Nakano, 2011 | KUZ Z134 Holotype | AB663653 | AB698872 | AB679662 | AB679663 | AB828554 |
| O. whitmani Oka, 1895 | KUZ Z45 Topotype | AB663657 | AB698874 | AB679668 | AB679669 | AB828556 |
| O. yamanaca Nakano, 2016 | KUZ Z1678 Holotype | LC106349 | LC106351 | LC106350 | LC106348 | LC106352 |

**Outgroup**

| Species Voucher | INSDC accession # |
|-----------------|-------------------|
| Erpobdella japonica Pawlowski, 1962 | KUZ Z178 | AB663648 | AB698879 | AB679654 | AB679655 | AB828542 |
| Gastrostomobdella monticola Moore, 1929 | UNIMAS/A3/BH01/10 | AB663649 | AB698880 | AB679656 | AB679657 | AB828543 |
| Mimobdella japonica Blanchard, 1897 | KUZ Z179 | AB663650 | AB698881 | AB679658 | AB679659 | AB828544 |
| Odontobdella blanchardi (Oka, 1910) | KUZ Z180 | AB663651 | AB938012 | AB938004 | AB937995 | AB938014 |
middle of somite XIII a1, behind gastropore, gonopores separated by 1/2 + 4 + 1/3 annuli. Pharynx reaching to somite XIV a1/a2–a2. Gastropore conspicuous, slightly anterior to middle of somite XIII a1. Gastroporal duct bulbous. Paired epididymides in somites XVIII–XX, occupying 6–7 annuli. Paired ejaculatory ducts thick. Atrial cornua developed, hyperboloidal, i.e., each middle part constricted, then expanding at respective junction with ejaculatory duct.

Material examined. Holotype: KUZ Z1439, dissected, collected from Mt. Gomadanzan, Ryujinmura, Tanabe, Wakayama Prefecture (Kii Peninsila, Honshu island), Japan

![Fig. 1. Preserved specimen of Orobdella angustata sp. nov., holotype, KUZ Z1439. A, dorsal view; B, ventral view. Scale bar: 5 mm.](image)

![Fig. 2. Orobdella angustata sp. nov., holotype, KUZ Z1439. 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 XI–XIII; F, ventral view of gastropore and female gonopore; G, ventral view of gastroporal duct. Scale bars: 1 mm (A, B, G); 2 mm (C–E); 0.25 mm (F). Abbreviations: af, annular furrow; an, anus; cp, crop; fg, female gonopore; gd, gastroporal duct; gp, gastropore; mg, male gonopore; np, nephridiopore; ph, pharynx.](image)
**Description.** Body firm and muscular, elongate, with constant width in caudal direction, dorsoventrally compressed, BL 54.3 mm, BW 5.5 mm (Fig. 1A, B). Caudal suck-
er ventral, elliptic, CL 2.6 mm, CW 3.2 mm (Figs 1B, 2D).

Somite I completely merged with prostomium (Fig. 2A). Somites II (=peristomium), III and IV uniannulate (Fig. 2A); somite II not separated from somite I. 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 quadran- nulate, a1+a2=b5=b6 (Fig. 2A–E). Somite XXVI dorsally quadran- nulate, a1+a2>b5+b6, ventrally triannulate, a1>a2<a3; a3 (=dorsally b5 and b6) being ventrally last complete annulus (Fig. 2C, D). Somite XXVII biannulate; a3 being dorsally last annulus with slight furrow (Fig. 2C). Anus behind somite XXVII; post-anal annulus absent (Fig. 2C).

Clitellum undeveloped.

Male gonopore in middle of somite XI b6 (Fig. 2E). Female gonopore slightly anterior to middle of somite XIII a1, inconspicuous, located posterior to gastropore (Fig. 2E, F). Gonopores separated by 1/2+4+1/3 annuli (Fig. 2E).

Anterior ganglionic mass in somite VI a2 and a3. Ganglion VII in a1 and a2. Ganglia VIII–XIII, of each somite, in a2 (Fig. 3A). Ganglia IX–XV, of each somite, in a1 and a2 (Fig. 3A). Gangion X–XV, of each somite, in a2 (Fig. 3A). Ganglia X–XVII, of each somite, in a1 and a2 (Fig. 3A). Ganglia X–XVIII, of each somite, in a1 and a2 (Fig. 3A). Ganglia XIX–XX b6 (Fig. 3A); on right side, in total ~22 testisacs, 1 in XX, 4 in XXI, 4 in XXII, 6 in XXIII, 4 in XXIV, 3 in XXV; on left side, in total ~22 testisacs, 2 in XX, 4 in XXI, 4 in XXII, 5 in XXIII, 4 in XXIV, 3 in XXV. Paired epididymides in somite XVII–XIX to somite XX b5, occupying 7 annuli (Fig. 3A). Paired ejaculatory ducts thick, in somite XI b5 to somite XVII–XIX (Fig. 3A); loosely coiled in position posterior to ovisacs; each duct crossing ventrally beneath each ovisac, then nearly straight in position anterior to ovisacs; each noticeably winding from respective junction with epididymis,

terior to middle of somite XIII a1 (Fig. 2E, F). Gastroporal duct bulbous, winding at junction with gastropore, reaching to somite XIV a2 (Fig. 2G). Intestine tubular, accenate, reaching to somite XXIV a1/a2. Rectum tubular, thin-walled, straight.

Testisacs multiple, in somite XX b5 to somite XXVIII b6 (Fig. 3A); on right side, in total ~22 testisacs, 1 in XX, 4 in XXI, 4 in XXII, 6 in XXIII, 4 in XXIV, 3 in XXV; on left side, in total ~22 testisacs, 2 in XX, 4 in XXI, 4 in XXII, 5 in XXIII, 4 in XXIV, 3 in XXV. Paired epididymides in somite XVII–XIX to somite XX b5, occupying 7 annuli (Fig. 3A). Paired ejaculatory ducts thick, in somite XI b5 to somite XVII–XIX (Fig. 3A); loosely coiled in position posterior to ovisacs; each duct crossing ventrally beneath each ovisac, then nearly straight in position anterior to ovisacs; each noticeably winding from respective junction with epididymis,

| Table 2 | Comparisons of morphological characters between Orobdella angustata sp. nov. and 11 quadranulate congeneric species. |
|---------|---------------------------------------------------------------------------------------------------------------|
| Species | Body length | Somite IV | Somite XVX | Annuli between gonopores | Pharynx length | Gastroporal duct | Epididymides | Atrial cornua |
|---------|-------------|-----------|------------|--------------------------|----------------|----------------|--------------|--------------|
| 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+1/1/2 | to anterior XIV | tubular | XX to XIX | small, ovate |
| O. esulcata | middle | 1 | 4 | 2/3+4+1/3 | to anterior to posterior XIV | bulbous | XVI to XX | developed, ovate |
| O. kawakatsuorum | middle | 1 | 4 | 1/2+4+1/2 | to posterior XIII to XIV | bulbous | XIV to XVIII | developed, ovate |
| O. ketagalan | middle | 1 | 4 | 2/3+4+1/2 | to anterior to posterior XIV | simple tubular | XVI to XVII | undeveloped |
| O. koikei | small | 1 | 3 | 1/2+4+1/2 | to posterior XIII to XIV | simple tubular | absent | undeveloped |
| O. masaakikuroiwai | small | 1 | 4 | 1/2+4+1/2 | to anterior to middle XIV | bulbous | XVI to XVIII | developed, ovate |
| O. meissi | middle | 1 | 4 | 5+1/4 | to posterior XV | rudimentary tubular | absent | absent |
| O. naraharaisetmagarum | small | 1 | 4 | 1/2+4+1/2 | to posterior XIV | bulbous | XVII to XIX | developed, ovate |
| O. tsuashimensis | middle | 1 | 4 | 1/2+5 | to posterior XIII to posterior XIV | bulbous | XVI to XVIII | developed, ovate |
narrowing at junction with atrial cornua, then turning proximally toward atrial cornua without pre-atrial loop (Fig. 3A, B). Pair of muscular atrial cornua developed, hyperboloidal, in somite XI b5 and b6 (Fig. 3A–D); each middle part constricted, then expanding at respective junction with ejaculatory duct. Atrium short, muscular, globular, in somite XI b5 and b6 (Fig. 3B–D). Penis sheath and penis absent.

Paired ovisacs globular; right ovisac in somite XIII a2 and b5; left ovisac in somite XIII a2 (Fig. 3A, E). Oviducts thin-walled, left oviduct crossing ventrally beneath nerve cord (Fig. 3A, E); both oviducts converging in to 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=4, including holotype): BL 51.9±2.7 mm (48.9–54.3 mm), BW 5.1±0.28 mm (4.8–5.5 mm), CL 2.4±0.14 mm (2.4–2.6 mm), CW 2.8±0.45 mm (2.3–3.2 mm). Somite XXVI generally triannulate, a1–a2<2<3. Pharynx reaching to somite XIV a2. Crop reaching to somite XX a2/b5. Gastroporal duct reaching to somite XIV a2/b5. Intestine reaching to somite XXIV a2/b5. Testisacs in somite XIX a2 to somite XXV b5; both sides, ~29 sacs, respectively. Paired epididymides occupying 6 annuli, respectively; right side in somite XVII/XVIII to somite XIX a2/b5; left side in somite XVIII a1 to somite XIX a2/b5. Paired ejaculatory ducts; right side in somite XI b5 to somite XVII/XVIII; left side in somite XI b5 to somite XVIII a1; coiled in position posterior to ovisacs. Paired ovisacs in somite XIII a2 and b5.

**Coloration.** In life, dorsal surface bluish black; ventral surface bluish white. Color faded in preservative.

**Distribution.** This species was collected only from its type locality.

**Natural history.** Although both dissected individuals possessed developed testisacs, their clitella were undeveloped, and hardly detected. The reproductive season of the new species thus remains unclear.

**Etymology.** The specific name is a participle in nominative singular derived from the Latin word *angustatus* (narrowed) referring to the fact that the male atrial cornua of this new species are constricted in their upper-middle parts.

**Molecular analyses results.** The BI tree (mean ln L=−28673.07; Fig. 4) for estimating the phylogenetic position of the new species had an identical topology to that of the ML tree (In L=−29119.04; not shown). *Orobdella angustata* belonged to a well-supported clade (BS=99%, PP=1.0) containing the other four quadranulate species inhabiting the western part of Honshu, Shikoku and northern Kyushu. *Orobdella esulcata* Nakano, 2010 formed a sister clade to the other four species including *O. angustata* and the well-supported clade belonging to *O. brachyepidymis* Nakano, 2010 (BS=99%, PP=1.0). Nakano and Lai 2012; Nakano 2014; Nakano and Seo 2014; Nakano 2016a, b, 2017b; Nakano and Lai 2017), the new species clearly differs from the other 11 quadranulate congeners in having short epididymides in somite XVIII–XIX to somite XIX–XX that are occupying 6–7 annuli, thick ejaculatory ducts, and hyperboloidal developed atrial cornua (see Table 2). The new species differs from the six sex-annulate species and two octanulate species by its mid-body somites that are quadranulate.

Because all the present individuals of *O. angustata* bear undeveloped citella, it remains uncertain whether this new species is assigned to the small or middle types. The obtained phylogenies showed that *O. angustata* is phylogenetically close to the three “small-type” species, *O. brachyepidymis*, *O. kanaekoikeae* and *O. naraharaetmagarum*. However, the dissected specimens possessed fully developed genital organs, and thus they could be identified at least as pre-mature leeches. Because the body lengths of the dissected individuals exceed 5 cm, it is highly possible that *O. angustata* can be designated as a “middle-type” species. Future field surveys should collect individuals of *O. angustata* with obvious citella, and reveal the natural history as well as the body length group of this species.

The male atrial cornua of *Orobdella angustata* show quite unique characteristics among those of the other *Orobdella* species. However, in this new species, the gastroporal duct, which is a spermatophore receptor of *Orobdella* leeches (Nakano 2017b), bears the bulbous feature that is common in the other congeners whose gastroporal ducts are developed. The characteristics of the gastroporal duct of *O. angustata* imply that its spermatophore may not be distinctive despite the noteworthy atrial cornua of the new species.

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