

Freshwater Colonization by a Scaleless Scale Worm: *Pisione mizuchi* sp. nov. (Annelida: Sigalionidae), From Sado Island, Japan

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We describe a new species of pisionid annelid, *Pisione mizuchi* sp. nov., found from rivers on Sado Island, Japan. This species represents the second known member of the genus *Pisione* Grube, 1857 to inhabit freshwater environments. The new species can be distinguished from its congeners by the absence of elongated ventral cirri on segment 2 and dorsal cirri on segment 3, the absence of bilobed prechaetal lobes, the presence of four chaetae per parapodium, and a single pair of male copulatory organs associated with strongly modified parapodia. A phylogenetic tree of Pisioninae based on four molecular markers (cytochrome c oxidase subunit I, 16S rRNA, 18S rRNA, and 28S rRNA) is also provided.

Key words: taxonomy, phylogeny, annelid, copulatory organ, Sea of Japan, river

INTRODUCTION

Earthworms and leeches belonging to the phylum Annelida are well known to inhabit freshwater environments. Both of these groups have evolved from a common ancestor that colonized freshwater from the marine environment, with multiple subsequent transitions back to marine habitats and, in some cases, recolonizations to freshwater environments (Rousset et al., 2008; Bolotov et al., 2020; Erséus et al., 2020). In contrast, other groups of annelids inhabiting freshwater account for less than 2% of all known species (Glasby and Timm, 2008). To date, besides earthworms and leeches, more than 24 annelid families have been recorded to inhabit freshwater habitats, primarily from families such as Nereididae, Spionidae, Fabriciidae, Sabellidae, and Histriobdellidae (Glasby and Timm, 2008). In Japan, species of Nereididae and Sabellidae have primarily been reported (e.g., Glasby, 1999; Torii and Fukuhara, 2017; Torii and Satake, 2020). These annelids (excluding earthworms and leeches) are thought to have colonized freshwater environments multiple times independently (Glasby and Timm, 2008).

To survive in freshwater environments, annelids must develop effective osmoregulatory systems. In particular, reproductive adaptations to unidirectional flow are required in riverine habitats (Kuo, 2017; Glasby et al., 2021). For example, *Hediste* spp. (Nereididae) exhibit migratory life cycles involving a planktonic larval stage in marine environments followed by recolonization to rivers (Glasby et al., 2021), whereas *Manayunkia* spp. (Fabriciidae) undergo direct development that allows them to remain in freshwater throughout their life cycle (Glasby and Timm, 2008).

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Within the suborder Aphroditiformia, only a single freshwater species has been reported: *Pisione garciavaldecasasi* San Martín, López, and Camacho, 1998, which was described from rivers on Coiba Island, Panama (San Martín et al., 1998). The genus *Pisione* Grube, 1857 includes a group of sigalionid scale worms that inhabit interstitial spaces within coarse sandy substrates. Members of this genus are characterized by a slender, scaleless body, absence of prostomial antenna, presence of buccal acicula, and well-developed parapodia. Copulatory organs used for internal fertilization are also present, but only in adults (Yamanishi, 1998; Rouse et al., 2022). *Pisione* currently comprises 47 species worldwide (Read and Fauchald, 2025), with Japan recognized as a region of high diversity, where 14 species (including subspecies) have been recorded (Yamanishi, 1976, 1992, 1998; Uchida, 1988). Most *Pisione* species occur in coastal intertidal to shallow subtidal zones (Yamanishi, 1998); however, *Pisione longipalpa* Uschakov, 1956 has been found in deep-sea habitats, and *P. garciavaldecasasi* in freshwater rivers (Uschakov, 1956; San Martín et al., 1998).

In this study, we collected specimens of *Pisione* from freshwater rivers on Sado Island, Japan. We describe a new species, *Pisione mizuchi* sp. nov., representing the second known freshwater species within the genus. We also present a molecular phylogenetic analysis based on four molecular

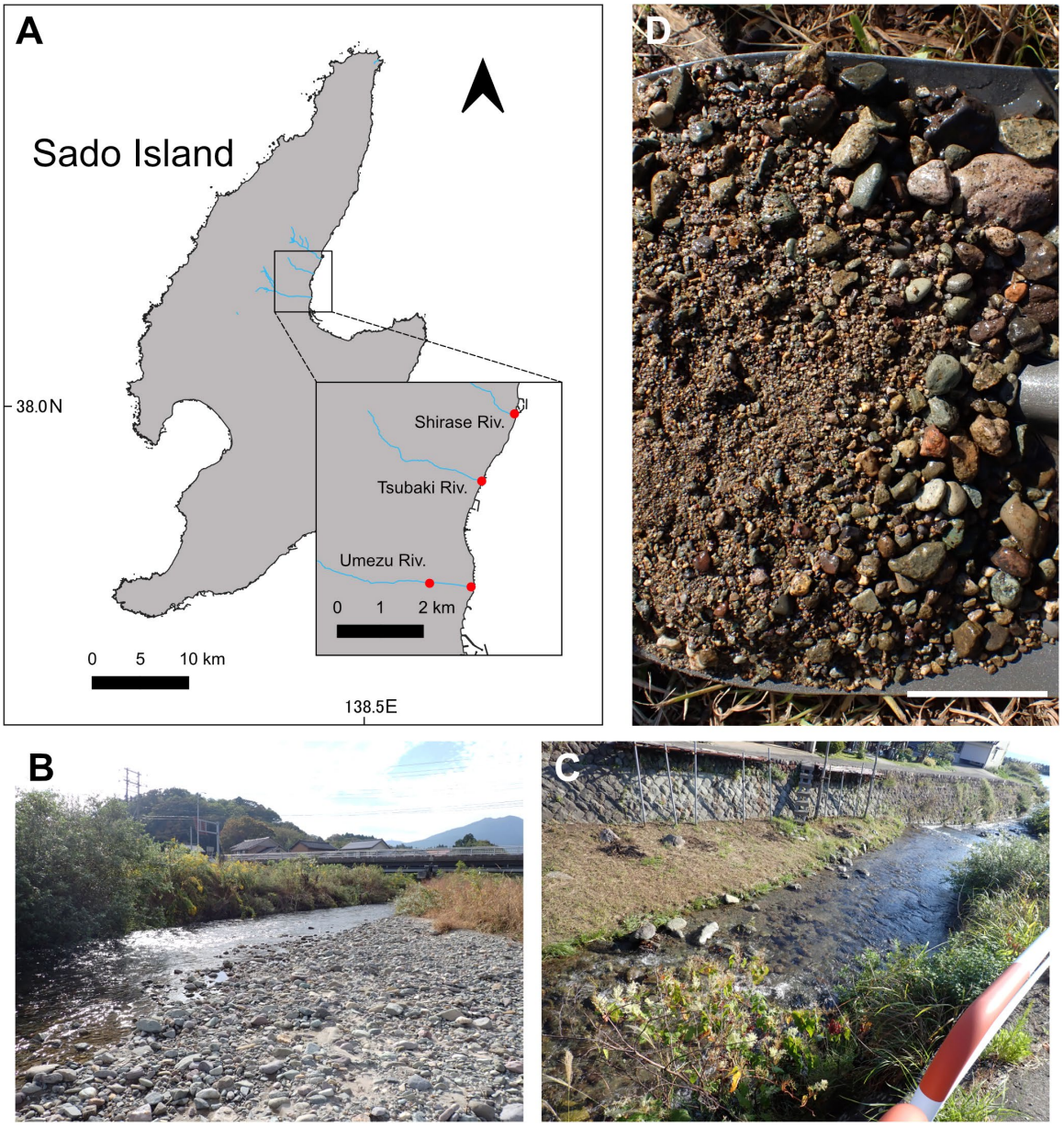

Fig. 1. Sampling sites of *Pisione mizuchi* sp. nov. **(A)** Map showing the sampling sites of *Pisione mizuchi* sp. nov., on Sado Island, Japan. Circles indicate collection sites along the Shirase, Tsubaki, and Umezu Rivers; closely situated sites are grouped together. For Umezu River, both the most upstream and the most downstream sampling localities are indicated. **(B)** Landscape at the downstream sampling locality of Umezu River. **(C)** Landscape at Tsubaki River. **(D)** River sediment at Tsubaki River. Scale bar: 50 mm.

markers—cytochrome *c* oxidase subunit I (COI), 16S rRNA (16S), 18S rRNA (18S), and 28S rRNA (28S)—incorporating data from three marine *Pisione* species newly collected in Japan.

MATERIALS AND METHODS

Specimen collection and preservation

Specimens of *Pisione* were collected from three rivers (Shirase, Tsubaki, and Umezu Rivers) on Sado Island, Niigata Prefecture, along the Sea of Japan coast (Fig. 1). In 2019, YM and KK-Y together with students from the Program of Field Research in the Environmental Sciences at Niigata University conducted sampling by sifting river sediment. In 2024, SS performed additional sampling by scooping river sediment with a shovel into buckets and washing the sediment with water to extract specimens. Live specimens were photographed using a Nikon D7500 digital camera, and an Olympus DP23 digital camera mounted on an Olympus SZX16 stereo microscope. After anaesthetization by gradual addition of ethanol, specimens were fixed and preserved in 70% ethanol.

Morphological examination

External morphology was examined using a Nikon SMZ18 stereo zoom microscope and a Nikon ECLIPSE Ni light microscope. For more detailed observations, we performed scanning electron microscopy (SEM): one paratype was dehydrated through a graded ethanol series, dried in a Vacuum Device freeze dryer (VFD-21S) using t-butyl alcohol, coated with osmium using a Nippon Laser & Electronics plasma coater (OPC80), and observed using a JEOL Scanning Electron Microscope (SEM, JSM-7610F). Additionally, a non-type specimen was dehydrated through a graded ethanol series, dried using a HITACHI critical-point dryer (HCP-2) with liquid CO_2, gold-coated with a HITACHI ion sputter (E-1045), and examined with a JEOL SEM (JSM-5900LV). For observations of the internal morphology, specimens preserved in 70% ethanol were processed for histological analysis. Specimens were dehydrated through an ethanol series, cleared in xylene, and embedded in paraffin. Paraffin-embedded specimens were sectioned at a thickness of 7 μm, stained with hematoxylin and eosin, and examined using a Nikon ECLIPSE E200 light microscope. All specimens are deposited at the National Museum of Nature and Science, Tsukuba (NSMT-Pol H-1032; P-1033–1035; 113625–113631), and the Invertebrate Collection of Hokkaido University Museum (ICHUM 9093). The morphological terminology in the taxonomic descriptions follows that used in the report by Murugesan et al. (2023). The number of segments excludes the prostomium and pygidium.

Table 1. List of Pisioninae species and outgroup species included in the phylogenetic analysis, together with accession numbers in GenBank. Classification of copulatory groups is based on Yamanishi (1998).

Family	Species	18S	28S	COI	16S	Copulatory group	References
Sigalionidae	*Pisione bulbifera*	KY657613	KY657626	KY657660	KY657643	*remota*	Gonzalez et al. (2017)
	***Pisione* cf. *crassa* Sado**	**PV490728**	**PV490721**	**PV483718**	**PV490724**	***crassa***	This study
	Pisione guanche	KY657614	KY657627	KY657661	KY657644	*africana*	Gonzalez et al. (2017)
	Pisione hartmannschroederae	KY657617	KY657630	KY657663	KY657648	*crassa*	Gonzalez et al. (2017)
	***Pisione mizuchi* sp. nov. NSMT-Pol H-1032**	**PX529171**	–	**PX431615**	**PX529018**	***gopalai***	This study
	***Pisione mizuchi* sp. nov. NSMT-Pol P-1033**	**PV490727**	**PV490720**	**PV483717**	–	***gopalai***	This study
	Pisione papuensis	KY657618	KY657632	KY657664	KY657650	*papuensis*	Gonzalez et al. (2017)
	Pisione puzae	KY657619	KY657633	KY657665	KY657651	*remota*	Gonzalez et al. (2017)
	Pisione remota	KY657620	KY657636	KY657668	KY657654	*remota*	Gonzalez et al. (2017)
	Pisione wolfi	KY657622	KY657639	KY657670	KY657657	*crassa*	Gonzalez et al. (2017)
	Pisione cf. *vestigalis*	KY657623	KY657640	KY657671	KY657658	*crassa*	Gonzalez et al. (2017)
	Pisione sp. A	KY657621	KY657637	KY657669	KY657655	–	Gonzalez et al. (2017)
	***Pisione* sp. Sado**	**PV490729**	**PV490722**	–	**PV490725**	–	This study
	***Pisione* sp. Shizuoka**	**PV490730**	**PV490723**	**PV483719**	**PV490726**	–	This study
	Pisionidens ixazaluohae	KX282503	KX282504	KX282505	KX282502	–	Petersen et al. (2016)
	Pisionidens sp. outgroup	JN852842	JN852876	JN852943	JN852915	–	Norlinder et al. (2012)
Sigalionidae	*Euthalenessa* cf. *digitata*	KY657612	KY657625	–	KY657642	–	Gonzalez et al. (2017)
	Neoleanira tetragona	AY839570	JN852872	AY839582	JN852911	–	Wiklund et al. (2005), Norlinder et al. (2012)
	Pholoides asperus	JN852841	JN852875	JN852942	JN852914	–	Norlinder et al. (2012)
	Sigalion spinosus	AY894304	DQ790062	AY894319	–	–	Struck et al. (2005, 2007)
Acoetidae	*Panthalis oerstedi*	AY839572	JN852845	AY839584	JN852881	–	Wiklund et al. (2005), Norlinder et al. (2012)
Iphionidae	*Iphione* sp.	KY753852	KY753852	KY753835	KY753835	–	Zhang et al. (2018)
	Thermiphione fijiensis	MG994960	MH000402	MG981044	MG994953	–	McCowin and Rouse (2018)
	Thermiphione rapanui	MG994955	MH000397	MG981038	MG994948	–	McCowin and Rouse (2018)
Polynoidae	*Halosydna brevisetosa*	JN852827	JN852857	AY894313	JN852895	–	Struck et al. (2005), Norlinder et al. (2012)
	Harmothoe imbricata	AY340434	AY340400	AY839580	AY340463	–	Rousset et al. (2007)
	Lepidasthenia elegans	JN852832	JN852863	JN852933	JN852901	–	Norlinder et al. (2012)

DNA extraction, PCR amplification, and phylogenetic analysis

DNA extraction and sequencing were performed in accordance with the methods described by Shimooka et al. (2025). Genomic DNA was extracted from the middle body region of the holotype and paratype specimens. The primers used for PCR amplification were as follows: polyLCO (5′-GAYTATWTTCAA CAAATCATAAAGATATTGG-3′) and polyHCO (5′-TAMACTTCW GGGTGACCAAARAATCA-3′) for part of the mitochondrial COI gene (Carr et al., 2011); 16SarL (5′-CGCCGTTTATCAAAAA CAT-3′) and 16SbrH (5′-CCGGTCTGAACTCAGATCACGT-3′) for part of the mitochondrial 16S gene (Palumbi et al., 1991); 1F (5′-TACCTGGTTGATCCTGCCAGTAG-3′) and 9R (5′-GATCCTTC CGCAGGTTCACCTAC-3′) (Giribet et al., 1996) for part of the nuclear 18S gene, with internal primers 18Sbi (5′-GAGTCTCGTTC GTTATCGGA-3′) and a2.0 (5′-ATGGTTGCAAAGCTGAAAC-3′) (Whiting et al., 1997); and LSU5 (5′-TAGGTCGACCCGCTGAAYT TAAGCA-3′) and rd5b (5′-CCACAGCGCCAGTTCTGCTTAC-3′) (Littlewood, 1994; Schwendinger and Giribet, 2005) for part of the nuclear 28S gene. The newly obtained sequences – Holotype: COI (665 bp), 16S (493 bp), 18S (822 bp); Paratype: COI (647 bp), 18S

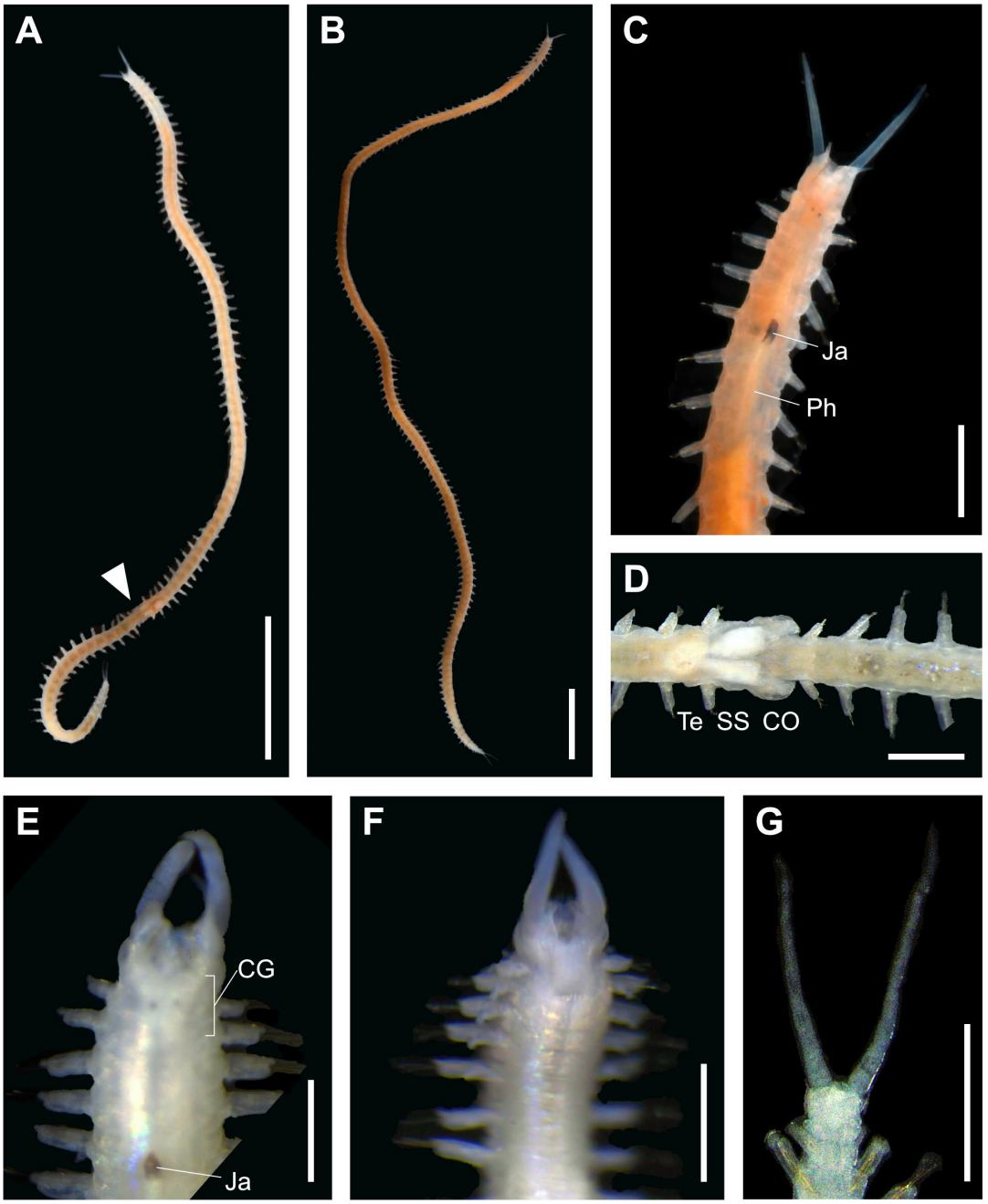

Fig. 2. *Pisione mizuchi* sp. nov. **(A, D–G)** holotype (NSMT-Pol H-1032), male; **(B, C)** paratype (NSMT-Pol P-1033), sex unknown. **(A–D, G)** living specimen, **(E, F)** preserved specimen. **(A)** Whole body, dorsal view; a white triangle indicates the position of the male copulatory organs. **(B)** Whole body, dorsal view. **(C)** Anterior end, dorsal view. **(D)** Segments near male copulatory organs, dorsal view. **(E)** Anterior end, dorsal view. **(F)** Anterior end, ventral view. **(G)** Pygidium, dorsal view. Abbreviations: Ja, jaws; Ph, pharynx; Te, testis; SS, sperm sac; CO, copulatory organ; CG, cerebral ganglia. Scale bars: **(A, B)** 5 mm; **(C)** 1 mm; **(D–G)** 500 μm.

(1213 bp), and 28S (999 bp) – have been deposited in GenBank (Table 1). Additional operational taxonomic units (OTUs) of marine *Pisione* species were also included in the analysis: *Pisione* cf. *crassa* Yamanishi, 1976 from Koda (38°13′52.3″N 138°23′08.0″E), Sado Island, subtidal zone, 0–0.5 m depth; *Pisione* sp. Sado from Tassha Beach (38°04′40.5″N 138°14′52.5″E), Sado Island, subtidal zone, 0–0.5 m depth; and *Pisione* sp. Shizuoka from Yawatano (34°52′08.3″N 139°06′14.5″E), Shizuoka Prefecture, Japan, Pacific Ocean, subtidal zone, 30 m depth. A total of 103 sequences from 26 species across the COI (25 sequences), 16S (25 sequences), 18S (27 sequences), and 28S (26 sequences) loci were analyzed. All sequences were aligned using MAFFT v. 7.511 (Katoh and Standley, 2013). Alignment-ambiguous positions were removed using trimAL v. 1.4 with the gappyout method (Capella-Gutierrez et al., 2009). The trimmed sequences of the four genes, COI (680 bp), 16S (305 bp), 18S (1688 bp), and 28S (979 bp), were concatenated using PartitionFinder 2.1.1, which recommended a GTR + G evolutionary model for each of the genes (Lanfear et al., 2016). A phylogenetic tree was constructed using the maximum likelihood (ML) method in RAxML v. 8 (Stamatakis, 2014). The robustness of the ML tree was evaluated by 1000 bootstrap (BS) pseudo-replicates. Bayesian inference (BI) analysis was conducted using MrBayes 3.2.7 (Ronquist et al., 2012), with Markov chains of 1 million generations. The model choice for each partition was also based on PartitionFinder 2.1.1. Run convergence was analyzed using Tracer v1.7.2 (Rambaut et al., 2018); the first 20,000 generations were discarded as burn-in. The untrimmed ML and BI trees are presented in Supplementary Figure S1. All OTUs belong to subfamily Pisioninae. *Euthalenessa* cf. *digitata* (McIntosh, 1885), *Neoleanira tetragona* (Örsted, 1845), *Pholoides asperus* (Johnson, 1897), and *Sigalion spinosus* (Hartman, 1939) from the family Sigalionidae were included as outgroups, following the procedure reported by Gonzalez et al. (2018). In addition, based on the results of Gonzalez et al. (2018), who recovered Acoetidae, Iphionidae, and Polynoidae as sister groups to Sigalionidae, one to three species from each of these families were also included as outgroups.

RESULTS

Systematics

Family **Sigalionidae** Kinberg, 1856
[Japanese name: Norari-urokomushi-ka]
Subfamily **Pisioninae** Ehlers, 1901
[New Japanese name: Sunagokai-aka]
Genus ***Pisione*** Grube, 1857
[Japanese name: Sunagokai-zoku]
Pisione mizuchi Shimooka and Jimi, sp. nov.
[New Japanese name: Kawa-sunagokai]
(Figs. 2–7)

Material examined. Holotype (NSMT-Pol H-1032): male, complete, Umezu River (38°06′09.6″N 138°26′13.5″E), coarse sand, 0.1–0.3 m depth, 18 October 2024, collected by SS. Paratypes: (NSMT-Pol P-1033); three male and one unknown sex specimens, complete, one of male specimen on stubs, Umezu River (38°06′09.6″N 138°26′13.5″E), coarse sand, 0.1–0.3 m depth, 18 October 2024, collected by SS. (NSMT-Pol P-1034); one male specimen, complete, Tsubaki River (38°07′30.1″N 138°26′23.6″E), coarse sand, 0.1–0.3 m depth, 18 October 2024, collected by SS. (NSMT-Pol P-1035); one unknown sex specimen, complete, Shirase River (38°08′21.5″N 138°26′56.4″E), coarse sand, 0.1–0.3 m depth, 26 August 2019, collected by YM and KK-Y.

Non-type material: (NSMT-Pol 113625); five male and two unknown sex specimens, complete, Umezu River (38°06′09.6″N 138°26′13.5″E), coarse sand, 0.1–0.3 m depth, 18 October 2024, collected by SS. (NSMT-Pol 113626); one male and one unknown sex specimens, complete, Tsubaki River (38°07′30.1″N 138°26′23.6″E), coarse sand, 0.1–0.3 m depth, 18 October 2024, collected by SS. (NSMT-Pol 113627); one unknown sex specimen, complete, Umezu River (38°06′09.6″N 138°26′13.5″E), coarse sand, 0.1–0.3 m depth, 26 August 2019, collected by YM and KK-Y. (NSMT-Pol 113628); two unknown sex specimens, complete, Umezu River (38°06′10.8″N 138°26′06.0″E), coarse sand, 0.1–0.3 m depth, 26 September 2019, collected by YM. (NSMT-Pol 113629); one unknown sex specimen, incomplete, Umezu River (38°06′13.1″N 138°25′38.4″E), coarse sand, 0.1–0.3 m depth, 26 August 2019, collected by YM and KK-Y. (NSMT-Pol 113630); one unknown sex specimen, complete, Tsubaki River (38°07′30.1″N 138°26′23.6″E), coarse sand, 0.1–0.3 m depth, 26 September 2019, collected by YM. (NSMT-Pol 113631); two unknown sex specimens, complete, Shirase River (38°08′21.5″N 138°26′56.4″E), coarse sand, 0.1–0.3 m depth, 26 August 2019, collected by YM and KK-Y. (ICHUM 9093); one male specimen on stubs, Umezu River (38°06′09.6″N 138°26′13.5″E), coarse sand, 0.1–0.3 m depth, 26 August 2019, collected by YM and KK-Y.

Etymology. The specific name is derived from the Japanese term "*mizuchi*", a legendary dragon-like creature inhabiting rivers. It refers to the riverine habitat and the elongated body of the species.

Description. Body long, nearly circular in cross section. Reddish to pale orange in living specimens (Fig. 2A–C), yellowish to whitish in ethanol preserved specimens. Dorsal and ventral surfaces smooth, with iridescent cuticle. Segmentation well-marked laterally, but not dorsally and ventrally. Ventral longitudinal groove shallow. Pores on tip of postchaetal lobes (Fig. 5B), not visible on dorsal and ventral surface (Fig. 5A).

Prostomium rectangular, completely fused to peristomium and segment 1, both lateral sides parallel (Figs. 2C, E, F; 3A, 4A). Cerebral ganglia extending from segments 2–3 (Fig. 2E). Two pairs of eyes between segment 2 and segment 3, each pair so tightly arranged that it appears as a single eyespot (Fig. 2C). Eyes indistinct in preserved specimens (Fig. 2E).

Segment 1 anterior to prostomium, supported by strong buccal aciculae. Tentacular cirri emerging frontally. Palps inserted ventrally to tentacular lobes, slender, finely annulated, reaching to segment 4 (Figs. 2A–C, 3A, 4A–C, 5A). Dorsal tentacular cirri inserted above palps, half as wide as palps width, elongated. Dorsal cirri longer than ventral, about half length of palps length, basally annulated. Ventral cirri emerging at base of dorsal cirri, small, globular, smooth (Figs. 2A–C, 3A, 4A–C, 5A). Buccal acicula light yellow, extending from front of first parapodia to internal tentacular lobe border, emerging off body wall. Tips expanded to distal plates with irregular denticles (Figs. 3A, B; 4A–C). Pharynx extending from segments 6–9 (Fig. 2C). Distal part of pharynx surrounded by about 14 equal-sized papillae (paratype: NSMT-Pol P-1033). Two pairs of jaws visible in segment 6, with sharp tips (Fig. 2C, E).

Dorsal cirri at base of parapodia, similar in size throughout. Small, articulated, bottle-shaped, with globular base twice length of upper globular papillose article (Figs. 2E, 3A,

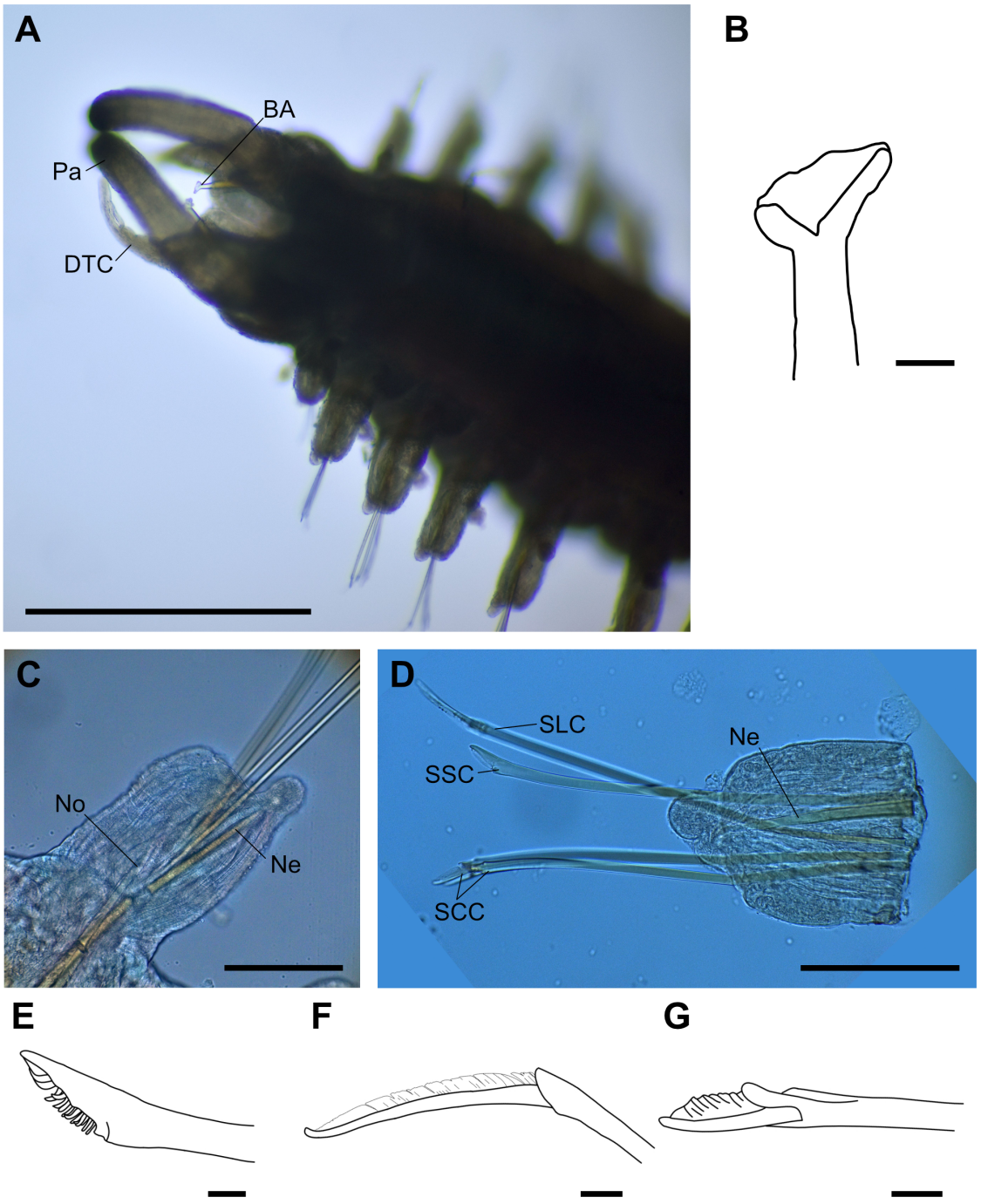

Fig. 3. Micrographs **(A, C, D)** and line drawings **(B, E–G)** of *Pisione mizuchi* sp. nov. holotype (NSMT-Pol H-1032). **(A)** Anterior end, dorsal view. **(B)** Left buccal acicula, dorsal view. **(C)** Left parapodium of segment 11, dorsal view. **(D)** Left parapodium of segment 9, rear view. **(E)** Supra-acicular simple chaeta of middle segment. **(F)** Subacicular long-bladed compound chaeta of middle segment. **(G)** Subacicular compound chaetae of middle segment. Abbreviations: Pa, palp; DTC, dorsal tentacular cirrus; BA, buccal acicula; No, notoacicula; Ne, neuroacicula; SSC, supra-acicular simple chaeta; SLC, subacicular long-bladed compound chaeta; SCC, subacicular compound chaetae. Scale bars: **(A)** 500 μm; **(B, E–G)** 10 μm; **(C, D)** 100 μm.

4A, B, D; 5A, B). Ventral cirri on segment 2 emerging at base of parapodia. From segment 3, ventral cirri emerging in the middle of parapodial lobe. Similar in size throughout, similar-shaped to dorsal cirri (Figs. 2F, 4B, D; 5A, B).

Parapodia long, reaching maximum length at segment 5; segment 3 half length of segment 5; length about half body width posteriorly (Figs. 2C, E, F; 3A, 4A, B, D). Notopodia reduced, only notoacicula present. Neuropodia cylindrical, truncated. Prechaetal lobe distally rounded, longer than postchaetal. Postchaetal lobe distally rounded (Figs. 3D, 4B, D; 5B). Notoacicula slightly curved dorsally, with colorless pointed tips. Neuroacicula twice length of notoacicula, with tips curved ventrally. Neither acicula protruding from parapodia (Fig. 3C, D).

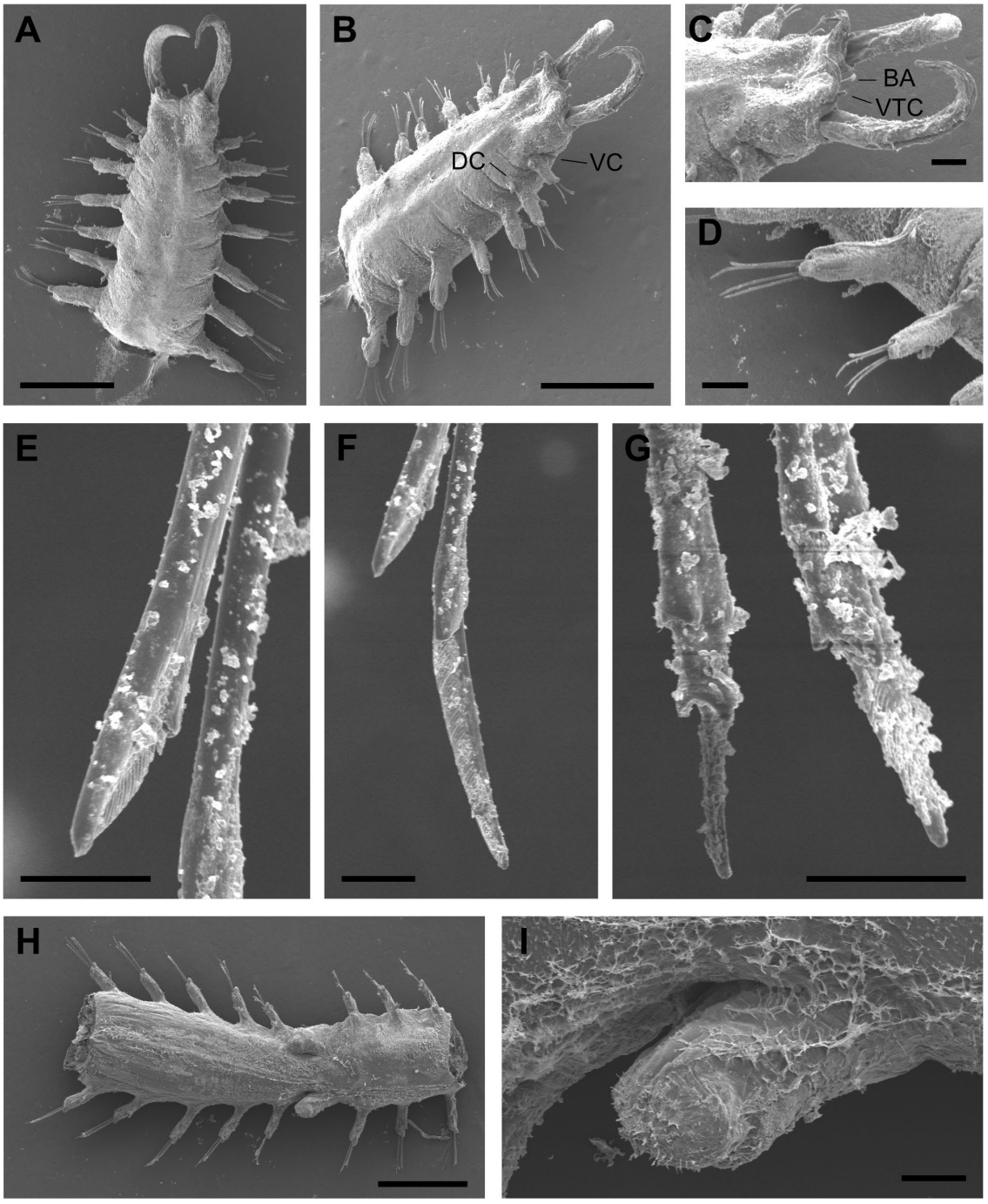

Fig. 4. Scanning electron microscope (SEM) micrographs of *Pisione mizuchi* sp. nov. paratype (NSMT-Pol P-1033), male. **(A–C)** Anterior end, dorsal view. **(D)** Right parapodium of segments 5 and 6, frontal view. **(E)** Supra-acicular simple chaeta of segment 6. **(F)** Subacicular long-bladed compound chaeta of segment 6. **(G)** Subacicular compound chaetae of segment 6. **(H)** Segments near male copulatory organs, ventral view. **(I)** Left male copulatory organ of segment 58, ventral view. Abbreviations: DC, dorsal cirrus; VC, ventral cirrus; VTC, ventral tentacular cirrus; BA, buccal acicula. Scale bars: **(A, B, H)** 500 μm; **(C, D)** 100 μm; **(E–G)** 10 μm; **(I)** 50 μm.

Four neurochaetae per parapodium, of three kinds (Figs. 3D, 4D). One uppermost supra-acicular simple chaeta, thick, stout, obliquely truncated, with dense, fine spines on subdistal margin (Figs. 2E, 4E). One subacicular long-bladed compound chaeta, heterogomph, with long blade distally unidentate. Blade half width of shaft, twice length of other lower compound chaetae, with dense, fine spines (Figs. 2F, 4F). Two lowermost subacicular compound chaetae, heterogomph, with short blades distally unidentate. Blades with dense, fine spines (Figs. 2G, 4G).

Pygidium subtriangular, two slender pygidial cirri present, anus terminal (Fig. 2G).

Copulatory segment twice length of normal segments, with one pair of male copulatory organs on segment 60

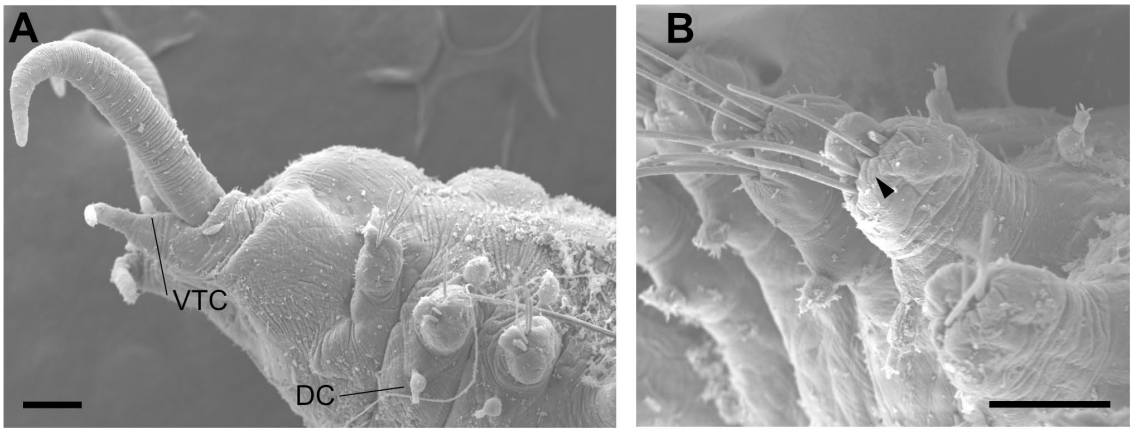

Fig. 5. Scanning electron microscope (SEM) micrographs of *Pisione mizuchi* sp. nov. non-type specimen (ICHUM 9093), male. **(A)** Anterior end, lateral view. **(B)** Parapodia of middle segments, rear view. A black triangle indicates a pore. Abbreviations: DC, dorsal cirrus; VTC, ventral tentacular cirrus. Scale bars: 100 μm.

(Figs. 2D, 4H, I; 6A–F). Parapodia with copulatory organs strongly modified. Chaetae absent, two aciculae present; notoacicula straight, with colorless tips; neuroacicula twice as long as notoacicula, slightly curved dorsally, with pointed tips (Fig. 6A, B). Dorsal cirri unmodified, ventral cirri absent (*gopalai*-group organs) (Fig. 6A, B). Copulatory organs rounded, arising as thick cylindrical processes from lateral side of segment, extending obliquely downward and posteriorly. Surface with cilia and pores (Fig. 6C–F). Distal part branches into three; innermost papilla curved 270-degree; outer papilla curved 135-degree; outermost pilose papilla extending outwards (Fig. 6A–F). Frontal side with wide, short fan-like appendage, with less than 30 spinous papillae arranged in band at base (Fig. 6A–C, F). Large clusters of spinous papillae absent on surface. Nephridial duct with distinct wall, located inside copulatory organs, continuing anteriorly (Fig. 6A, B). Nephridial duct opening embedded in parapodium, penis not distinguishable (Fig. 6A–F). In living specimen, sperm sacs visible dorsally as a pair of bright white oval spheres (Fig. 2D), extending from segment 59 to segment 60; testis visible dorsally as buried pale orange sphere, located between segment 58 and segment 59 (Fig. 2D).

Internal structures. Internal morphology based on sectioned specimens of paratype (NSMT-Pol P-1033) (Fig. 7): a pair of nerve cords at ventral side; a pair of large, circular-shaped sperm sacs, in center of body cavity, narrower at posterior, connected with sperm duct of copulatory organs (Fig. 7A, C, D). Stained spermatozoa inside sperm sacs appear as oval spheres measuring 10–17 μm (Fig. 7B). Oocytes not seen from August to October.

Measurements and Variations. The holotype measured 16.1 mm in length and 0.5 mm in maximum width (excluding parapodia), with a total of 87 segments. Male copulatory organs were observed on segment 60. A total of 22 complete specimens were examined (NSMT-Pol H-1032, NSMT-Pol P-1033–1035, and NSMT-Pol N-113625–113628, 113630, 113631) (Table 2). Specimen lengths ranged from 10.2 to 46.8 mm, with maximum widths (excluding parapodia) ranging from 0.5 to 0.9 mm, and segment counts ranged from 62 to 142. Male copulatory organs were consistently present as a single pair, with their position varying between segments 52 and 68. Specimens lacking externally visible copulatory organs were presumed to be female; however, dissection did not reveal female reproductive structures in any of these individuals.

Phylogenetic analysis. The ML and BI analyses produced phylogenetic trees with largely similar topologies, except for the placement of *Pisione guanche* San Martín, López, and Núñez, 1999, which was strongly supported in the ML tree (bootstrap support [*BS*] = 93) but only weakly supported in the BI tree (posterior probability [*PP*] = 0.52) (Fig. 8). *Pisione mizuchi* sp. nov. was strongly supported as a member of the subfamily Pisioninae (*BS* = 100, PP = 1.0) and was strongly placed within *Pisione* (*BS* = 93, *PP* = 1.0) (Fig. 8). Within *Pisione*, *P. mizuchi* sp. nov. was identified as sister to *Pisione* cf. *vestigalis* Yamanishi, 1998, with moderate to strong support (*BS* = 62, *PP* = 0.99). Japanese *Pisione*, *P. mizuchi* sp. nov., *Pisione* cf. *crassa*, *Pisione* sp. Sado, and *Pisione* sp. Shizuoka were placed in separate clades, indicating that Japanese *Pisione* do not form a monophyletic assemblage. Additional analyses based on alignment without trimming yielded largely congruent results (see Supplementary Figure S1).

Distribution and habitat. *Pisione mizuchi* sp. nov. inhabits shallow freshwater environments in the downstream sections of rivers (Shirase, Tsubaki, and Umezu rivers) on the central eastern coast of Sado Island, Japan. Specimens were primarily found in coarse sand deposits among stones within the stream (Fig. 1). Salinity at the sampling sites in Tsubaki and Umezu rivers was measured using an Atago seawater refractometer (MASTER-S/Millα) as 0‰. A sampling site in the Tsubaki River was located above two small elevation steps and is considered to be separated from the sea most of the time. A sampling site in Umezu River was approximately 700 m upstream from the river's mouth. At both sites, freshwater-inhabiting insect larvae such as Plecoptera and Ephemeroptera were collected. These observations suggest that the habitat is consistently maintained as a freshwater environment.

Remarks. Based on De Wilde and Govaere (1995), Yamanishi (1998), Salcedo et al. (2015), and Murugesan et al. (2023), we reviewed the synoptic comparisons of *Pisione* species and added information on new species. *Pisione*

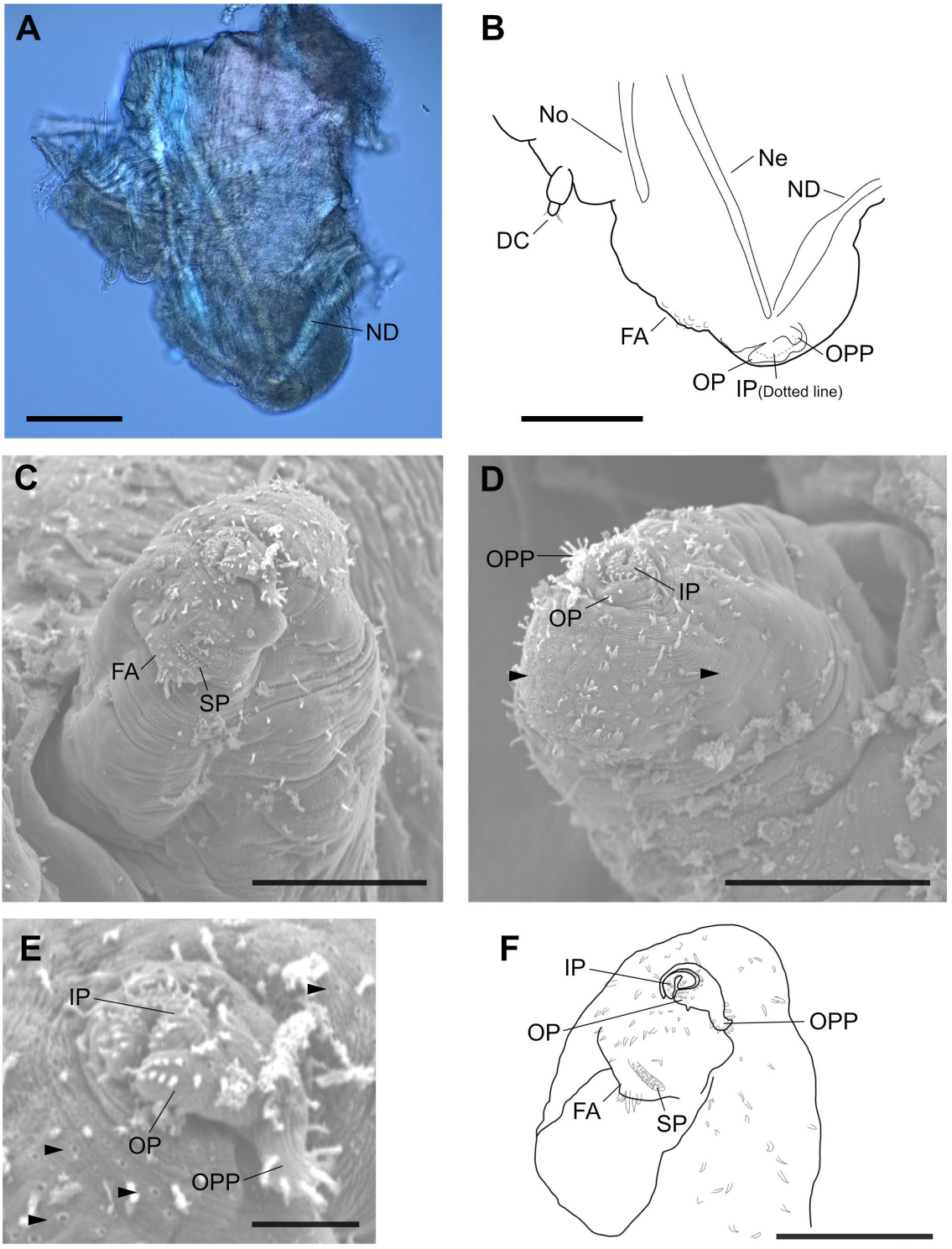

Fig. 6. Male copulatory organ of *Pisione mizuchi* sp. nov. **(A, B)** Left male copulatory organ of segment 60 of holotype (NSMT-Pol H-1032), lateral view. **(C–E)** Scanning electron microscope (SEM) micrographs and line drawing **(F)** of right male copulatory organ of non-type specimen (ICHUM 9093). Black triangles indicate some of the pores. **(C, F)** Frontal view. **(D)** Rear view. **(E)** Enlarged view of the tip, frontal view. Abbreviations: ND, nephridial duct; No, notoacicula; Ne, neuroacicula; DC, dorsal cirrus; FA, fan-like appendage; SP, spinous papillae; IP, innermost papilla; OP, outer papilla; OPP, outermost pilose papilla. Scale bars: **(A–D, F)** 100 μm; **(E)** 20 μm.

mizuchi sp. nov. is characterized by the following combination of morphological traits: ventral cirri on segment 2 are not elongated; dorsal cirri on segment 3 are also not elongated; prechaetal lobes are undivided; protruding notoacicula are absent; infra-acicular simple chaetae are absent; long-bladed compound chaetae are present; male copulatory organs are consistently present as a single pair, occurring between segments 52 and 68 and classified as the *gopalai-*

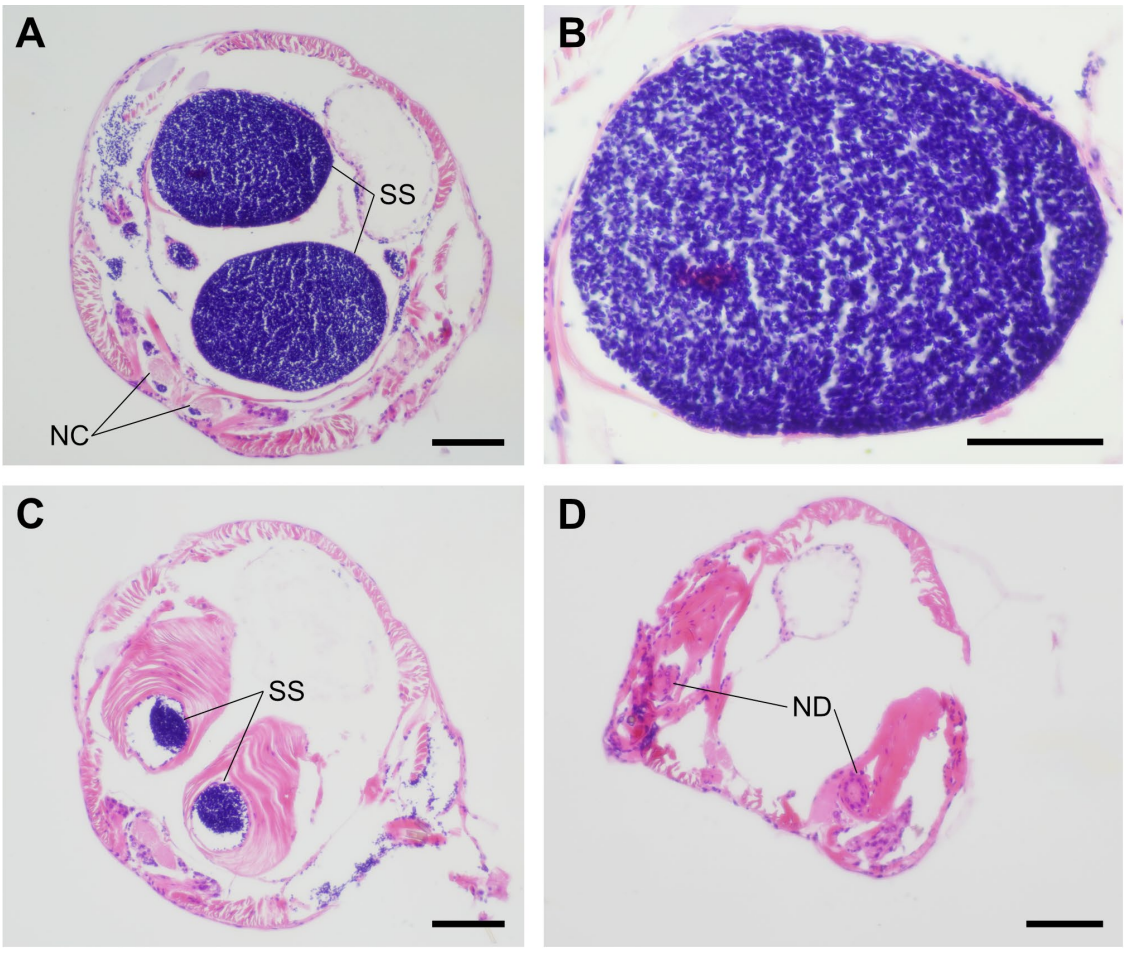

Fig. 7. Micrographs of paraffin-embedded specimens of *Pisione mizuchi* sp. nov. paratype (NSMT-Pol P-1033), male, segment 61, transverse view. **(A)** Middle region of the sperm sacs. **(B)** Enlarged view of middle region of left sperm sac. **(C)** Posterior region of sperm sacs. **(D)** Anterior region of male copulatory organs. Abbreviations: SS, sperm sac; NC, nerve cord; ND, nephridial duct. Scale bars: **(A, C, D)** 100 μm; **(B)** 50 μm.

group; the body comprises 87 segments.

The only previously described freshwater species, *P. garciavaldecasasi*, is found in a riverine environment similar to that of *P. mizuchi* sp. nov. The two species are morphologically similar, both with elongated bodies of over 30 mm in length and orangish coloration (San Martín et al., 1998). However, *P. garciavaldecasasi* differs in having elongated ventral cirri on segment 2 and elongated dorsal cirri on segment 3, although these differences may be subtle and difficult to confirm. While the parapodial shape is generally similar between the two species, they can be differentiated by the number of chaetae per middle parapodium: *P. mizuchi* sp. nov. bears four chaetae per parapodium, whereas *P. garciavaldecasasi* has five. Specifically, *P. mizuchi* sp. nov. has two lowermost compound chaetae, whereas *P. garciavaldecasasi* has three. The most apparent difference is found in mature males: *P. mizuchi* sp. nov. has a small, inconspicuous distal portion of the copulatory organs with a small fan-like appendage. The copulatory organs are restricted to one segment. In contrast, *P. garciavaldecasasi* has an enlarged, well-developed distal portion with a prominent fan-like appendage. The copulatory organs span three–five non-successive segments.

Among the 14 species of *Pisione* recorded in Japan, four possess male copulatory organs classified as the *gopalai*-group (Yamanishi, 1998). Each species can be distinguished as follows:

i) *Pisione papillata* Yamanishi, 1976, can be distinguished in having thick, digitiform ventral cirri on the copulatory segment, elongated ventral cirri on segment 2, elongated dorsal cirri on segment 3, and bilobed prechaetal lobes (Yamanishi, 1976).

ii) *Pisione gopalai vannifera* Yamanishi, 1998, can be distinguished by its male copulatory organs, which bear clusters of spinous papillae. It also has elongated dorsal cirri on segment 3, bilobed prechaetal lobes in the anterior parapodia, and five chaetae per parapodium (Yamanishi, 1998).

iii) *Pisione parva* De Wilde and Govaere, 1995, can be distinguished by bilobed prechaetal lobes in the anterior parapodia and the absence of long-bladed compound falcigers (De Wilde and Govaere, 1995).

iv) *Pisione paucisetosa* Yamanishi, 1998, bears three clusters of spinous papillae on the male copulatory organs and bilobed prechaetal lobes on the anterior parapodia (Yamanishi, 1998).

Based on the work by Murugesan et al. (2023), the spe-

Table 2. Body size and position of male copulatory organs in complete specimens of *Pisione mizuchi* sp. nov.

Collection date	Location	Voucher number	Sex	Length (mm)	Width (mm)	Number of segments	Position of segment with male copulatory organs
2019/8/26	Shirase Riv.	NSMT-Pol P-1035	?	21.0	0.8	116	
2019/8/26	Shirase Riv.	NSMT-Pol 113631	?	13.8	0.7	79	
2019/8/26	Shirase Riv.	NSMT-Pol 113631	?	17.9	0.6	113	
2019/8/26	Umezu Riv.	NSMT-Pol 113627	?	23.5	0.6	142	
2019/9/26	Tsubaki Riv.	NSMT-Pol 113630	?	10.4	0.7	68	
2019/9/26	Umezu Riv.	NSMT-Pol 113628	?	17.0	0.7	97	
2019/9/26	Umezu Riv.	NSMT-Pol 113628	?	21.1	0.9	102	
2024/10/18	Tsubaki Riv.	NSMT-Pol P-1034	♂	15.6	0.5	97	68
2024/10/18	Tsubaki Riv.	NSMT-Pol 113626	♂	16.3	0.6	77	57
2024/10/18	Tsubaki Riv.	NSMT-Pol 113626	?	10.2	0.6	62	
2024/10/18	Umezu Riv.	NSMT-Pol H-1032	♂	16.1	0.5	87	60
2024/10/18	Umezu Riv.	NSMT-Pol P-1033	♂	28.4	0.7	108	58
2024/10/18	Umezu Riv.	NSMT-Pol P-1033	♂	32.4	0.6	124	61
2024/10/18	Umezu Riv.	NSMT-Pol P-1033	♂	16.1	0.5	108	62
2024/10/18	Umezu Riv.	NSMT-Pol 113625	♂	19.4	0.7	111	65
2024/10/18	Umezu Riv.	NSMT-Pol 113625	♂	23.7	0.6	106	57
2024/10/18	Umezu Riv.	NSMT-Pol 113625	♂	36.3	0.7	120	58
2024/10/18	Umezu Riv.	NSMT-Pol 113625	♂	20.1	0.5	89	52
2024/10/18	Umezu Riv.	NSMT-Pol 113625	♂	11.7	0.5	77	52
2024/10/18	Umezu Riv.	NSMT-Pol P-1033	?	28.7	0.9	126	
2024/10/18	Umezu Riv.	NSMT-Pol 113625	?	46.8	0.8	133	
2024/10/18	Umezu Riv.	NSMT-Pol 113625	?	28.3	0.9	131	

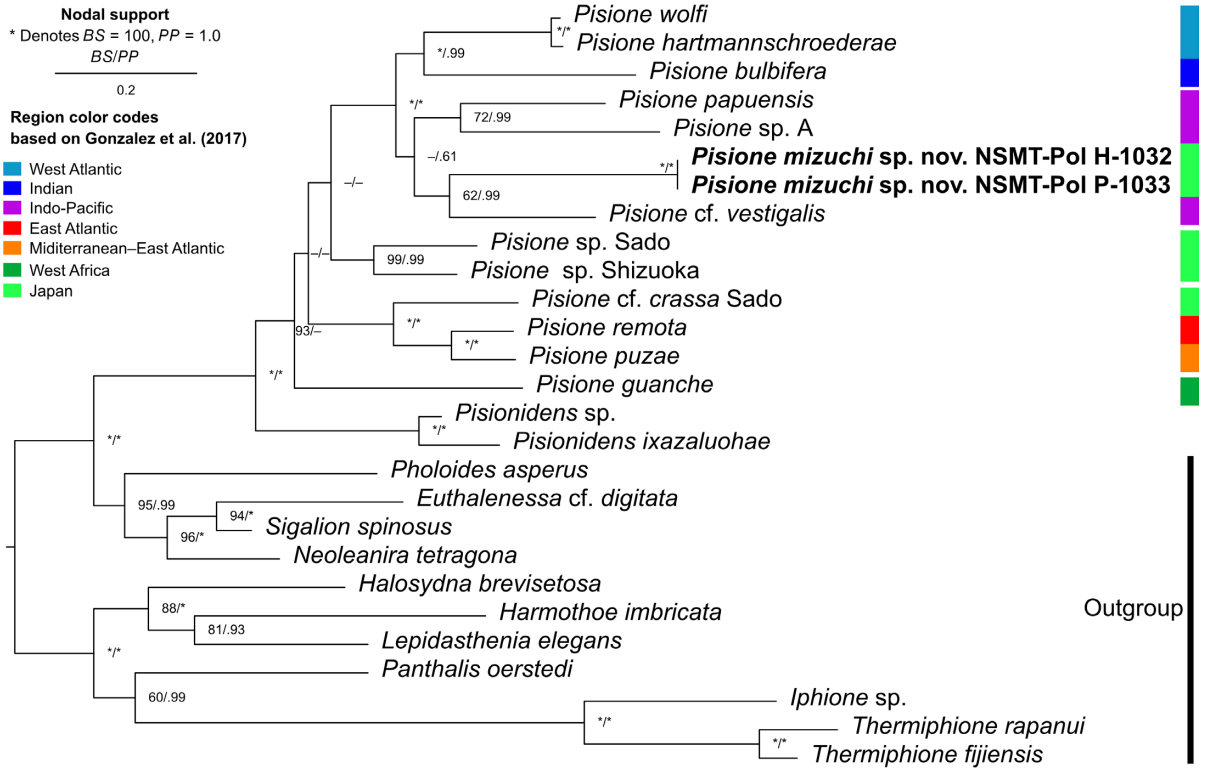

Fig. 8. Phylogenetic tree of Pisioninae based on trimmed COI, 16S, 18S, and 28S sequences. Tree topology based on the maximum-likelihood (ML) analysis in combination with the Bayesian inference (BI). Sigalionidae from other subfamilies, Acoetidae, Iphionidae, and Polynoidae, were used as an outgroup. Nodal support values (bootstrap support [*BS*] and posterior probability [*PP*]) are indicated on each branch. Only nodal support above *BS* = 50 and *PP* = 0.5 is displayed. Nodes not recovered or with low support are illustrated with a dash (–). A single asterisk (*) denotes maximum support in a specific analysis (*BS* = 100 or *PP* = 1.0). Colors indicate biogeographical regions within *Pisione* based on Gonzalez et al. (2017). Light green represents the operational taxonomic units (OTUs) collected from Japan by the present study.

cies most morphologically similar to *P. mizuchi* sp. nov., outside of Japan, are *Pisione gopalai* (Alikuhni, 1941) and *Pisione laubieri* Hartmann-Schröder, 1970:

v) *P. gopalai* differs in having clustered spinous papillae on the male copulatory organs and five chaetae per parapodium (Alikuhni, 1941).

vi) *P. laubieri* may include two species and was described based on two morphotypes that differ in their copulatory organs (Hartmann-Schröder, 1970; Yamanishi, 1998). Compared to the holotype description of *P. laubieri*, it differs in possessing five chaetae per parapodium and one to three pairs of male copulatory organs with ventrally curved tips (Hartmann-Schröder, 1970).

In addition, the known *Pisione* species with body length exceeding 30 mm, as in *P. mizuchi* sp. nov., are *Pisione oerstedii* Grube, 1857, *Pisione hermansi* Gradek, 1991, and *P. garciavaldecasasi*:

vii) *P. hermansi* is differentiated by elongated ventral cirri on segment 2, elongated dorsal cirri on segment 3, bilobed prechaetal lobes, and the absence of long-bladed compound falcigers (Gradek, 1991).

viii) *P. oerstedii* differs in having elongated ventral cirri on segment 2 and elongated dorsal cirri on segment 3 (Grube, 1857).

Notably, *P. mizuchi* sp. nov. can be morphologically distinguished from all previously described *Pisione* species by the structure of the male copulatory organs and by a unique combination of morphological features, such as ventral cirri on segment 2, dorsal cirri on segment 3, shape of the prechaetal lobes, the existence of protruding notoacicula, and the composition and number of chaetae.

DISCUSSION

Previous studies have documented the presence of a planktonic larval stage in *Pisione* (Åkesson, 1961; Stecher, 1968; Rouse, 2000). A life history involving a planktonic larval stage is also observed in the catadromous annelid *Hediste* Malmgren, 1867 (Nereididae) and in the nemertean genus *Potamostoma* Kajihara, Gibson, and Mawatari, 2003, both identified in upper brackish waters of rivers (Sato, 1999, 2017; Kan et al., 2020; Hookabe et al., 2024). However, in *Hediste*, species that lack a planktonic larval stage and complete their entire life cycle in low-salinity environments such as estuaries have also been reported to occur sympatrically with catadromous species (Sato, 1999, 2017; Kan et al., 2020). Indeed, interstitial freshwater species of Nerillidae and freshwater species of *Manayunkia* Leidy, 1859 are known to undergo direct development (Jouin and Swedmark, 1965; Glasby and Timm, 2008). It remains unclear whether *P. mizuchi* sp. nov. establishes itself in freshwater river habitats via a planktonic larval stage or whether it completes its life cycle within these habitats through direct development, as observed in interstitial freshwater annelids. To clarify the reproductive mode of *P. mizuchi* sp. nov., future studies should collect specimens throughout different seasons and include ovigerous females, which were not obtained during the present study.

Phylogenetically, *P. mizuchi* sp. nov. was found to be distinct from the two other species from Sado Island—*P.* cf. *crassa* and *Pisione* sp.—each of which belongs to separate clades within *Pisione* (Fig. 8). This suggests that these species have independent evolutionary histories. Gonzalez et al. (2017) hypothesized that *Pisione* species may form clades reflecting biogeographical regions. However, our analysis revealed that Japanese *Pisione* species are distributed across three distinct clades, one including species from the Indo-Pacific and another including species from the Mediterranean–East Atlantic. This suggests that this hypothesis may not hold universally.

Furthermore, Gonzalez et al. (2017) examined, within a phylogenetic framework, the evolutionary succession of the increasing number and complexity (*africana* → *remota* → *crassa* groups) observed in male copulatory organs, as proposed by Yamanishi (1998). Their results indicated that the evolution of male copulatory structures in *Pisione* is more complex than the previously suggested linear evolution. In this study, *P.* cf. *crassa*, which possesses male copulatory organs classified as the *crassa* group, was found to belong to a clade distinct from other *crassa* group species, namely, *Pisione* cf. *vestigalis*, *Pisione wolfi* San Martín, López, and Núñez, 1999, and *Pisione hartmannschroederae* Westheide, 1995, supporting the polyphyly within the copulatory organ group (Fig. 8; Table 1). This study also represents the first inclusion of *P. mizuchi* sp. nov., a *gopalai* group species in a phylogenetic framework; given that the copulatory organs of this group are highly modified and differ markedly from those of the other groups, further sampling of additional species will be required to test the monophyly of the *gopalai* group.

The apparent evolutionary transition of *P. mizuchi* sp. nov. from marine to freshwater environments is particularly noteworthy and raises important questions regarding the mechanisms underlying habitat shifts in *Pisione*. To better understand the evolutionary trajectory of this transition, it would be highly beneficial to obtain molecular data for *P. garciavaldecasasi*, the only other known freshwater *Pisione* species. Comparative genomic analyses between freshwater and marine *Pisione* species could reveal the genetic adaptations necessary for osmoregulation, reproduction, and survival in freshwater habitats.

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COMPETING INTERESTS

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

SS, YM, and KK-Y, collected the samples; SS and NJ carried out morphological observation; SS conducted molecular analyses, AO, NH, and NJ helped with the observation and analyses; SS wrote the text; NJ supervised the study; and all authors read and approved the final manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available online. (URL: https://doi.org/10.2108/zs250042)

Supplementary Figure S1. Phylogenetic tree of Pisioninae based on untrimmed COI, 16S, 18S, and 28S sequences. Tree topology based on the maximum-likelihood (ML) analysis in combination with the Bayesian inference (BI).

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