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Marine microturbellarians from Japan, with descriptions of two new species of *Reinhardorhynchus* (Platyhelminthes, Rhabdocoela, Koinocystididae)

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Abstract

Marine microturbellarians are an assemblage of meiofaunal flatworms abundant in sediments and on seaweeds around the world. The diversity and distribution of these animals in Japan are poorly understood. Here, we provide an overview of all recorded species in Japan and characterize two new species of the rhabdocoel genus *Reinhardorhynchus* based on morphological features and a molecular phylogeny inferred from 18S and 28S rDNA sequences. *Reinhardorhynchus ryukyuensis* **sp. nov.** can be distinguished from other species in the genus by the lack of an armed cirrus and by the presence of two larger opposing hooks and five smaller interconnected hooks in its male copulatory organ. *Reinhardorhynchus sagamianus* **sp. nov.** differs from its congeners because its male copulatory organ combines a bipartite cirrus armed with a belt of overlapping scale-like spines, an unarmed accessory cirrus, and two large distal accessory hooks. Our molecular phylogenetic analyses show that *R. ryukyuensis* **sp. nov.** and *R. sagamianus* **sp. nov.** form a clade with all the other species of *Reinhardorhynchus* for which DNA sequence data are available. Within this clade, *R. sagamianus* **sp. nov.** is in a clade that also includes *R. riegeri* and *R. anamariae*. The discovery of these new species highlights the importance of uncovering and documenting the hidden biodiversity along Japan's coastal margin.

Key Words

Distribution, flatworms, Japanese invertebrates, Kalyptorhynchia, marine meiofauna

Introduction

Microturbellarians are microscopic and mostly free-living flatworms that are common in marine meiofaunal communities around the globe (Schockaert et al. 2008; Armonies 2017; Fegley et al. 2020). They inhabit various types of interstitial substrates (e.g., algae and sediments) in intertidal and subtidal habitats and have also been recorded at depths of up to ~600 m (Artois et al. 2000; Aramayo 2018; Armonies 2023). Our understanding of the diversity and distribution of marine microturbellarians is mostly limited to regions where dedicated research has been conducted on these animals, including the coastal areas in Europe (e.g., Casu et al. 2014; Schockaert 2014; Gobert et al. 2020; Armonies 2023), Brazil (e.g., Marcus 1950, 1951, 1952; Braccini et al. 2016), Cuba (e.g., Diez et al. 2018, 2023a, 2023b), or Canada (e.g., Van Steenkiste and Leander 2018a, 2018b; Stephenson et al. 2019). However, even in some of these well-studied areas, the diversity of marine microturbellarians can be significantly higher than initial studies have shown. For example, ecological estimations suggest the presence of ~200 species in Cuba (Diez et al. 2023b) and ~400 species on the island of Sylt (northern Germany, in the North Sea) (Armonies 2023).

For Japan, only scattered records of marine and brackish water microturbellarians are known from the literature. The first marine microturbellarians described from Japan were prolecithophorans (Tozawa 1918). Since the 1950's, new representatives of Macrostomorpha, Rhabdocoela, Proseriata, and Prolecithophora have been reported intermittently (Westblad 1955; Karling 1966; Tajika 1977, 1978, 1979, 1980, 1981a, 1981b, 1982a, 1982b, 1982c, 1983a, 1983b, 1983c, 1984; Ax 2008; Omi 2018, 2020; Takeda and Kajihara 2018). However, these studies are confined to a limited number of localities in Japan. With numerous islands and inlets bordering the Sea of Okhotsk, the Sea of Japan, the East China Sea, the Philippines Sea, and the Northwest Pacific Ocean, the coastal margin of Japan represents an important but poorly explored part of the wider Pacific Ocean. It is expected that the diversity of marine microturbellarians in Japan is far from being adequately described.

Here, we characterize two new species of Koinocystididae (Rhabdocoela) with morphological and molecular data. Their phylogenetic positions are determined based on analyses using 18S and 28S rDNA sequences. Additionally, we provide a concise overview of the marine and brackish microturbellarian diversity of Japan and highlight the importance of such research.

Materials and methods

Specimen collection and fixation

The specimens of *Reinhardorhynchus ryukyuensis* sp. nov. were collected by Niels Van Steenkiste and Kevin Wakeman at Onna, Okinawa, Japan (26°28'52.7"N, 127°50'18.8"E) in February 2019 from a coarse mixture of sand, coral fragments, and shell hash in seagrass meadows in a shallow intertidal bay. The specimens of *Reinhardorhynchus sagamianus* sp. nov. were collected by Aoi Tsuyuki and Yuki Oya at Sangashita beach, Hayama, Kanagawa (35°15'58.3"N, 139°34'19.64"E) in April and August 2023, from clean, coarse sandy sediments in the upper intertidal zone (Fig 1). The upper centimeters

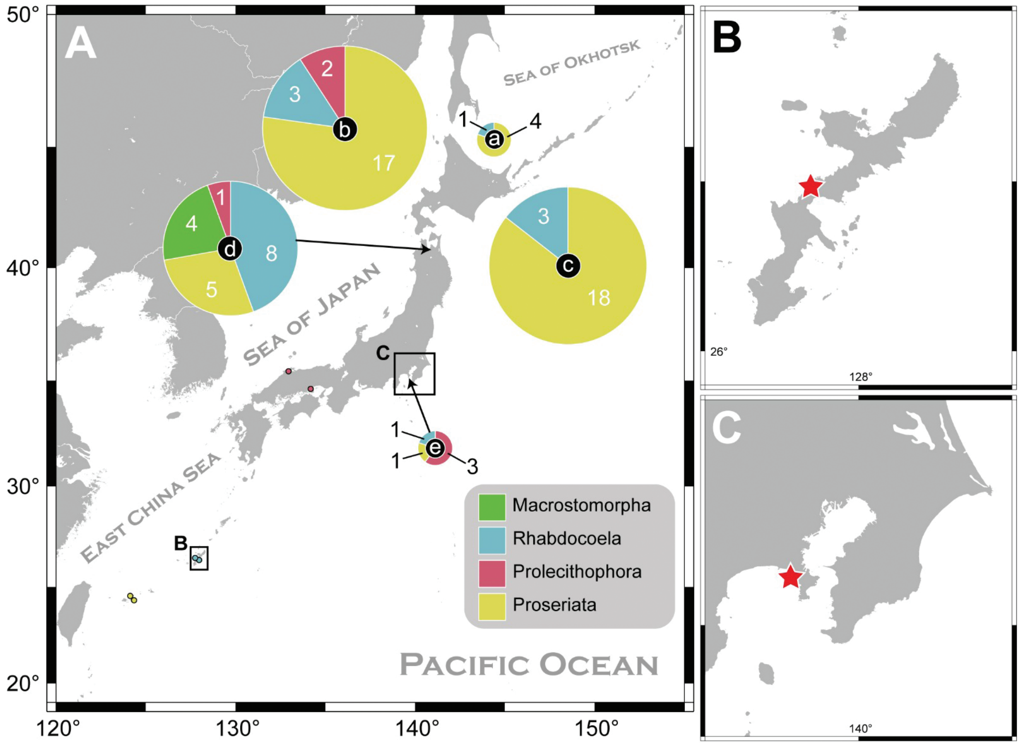

Figure 1. Records of marine microturbellarians in Japan. **A.** Map shows the documented occurrences of marine microturbellarians reported from Japan. The size and numbers within the pie chart represent the number of recorded species from a) the Okhotsk Sea coast of Hokkaido, b) the Sea of Japan coast of Hokkaido, c) the Pacific coast of Hokkaido, d) Mutsu Bay and the brackish water areas of Aomori, and e) the Pacific coast of Kanagawa. The small circles without numbers indicate single species from the Inland Sea, East China, and the Pacific coasts of Okinawa and Ishigaki Islands, respectively. **B.** Magnification of the area, including Okinawa, with a star designating the type locality of *Reinhardorhynchus ryukyuensis* n. sp. **C.** Magnification of the area, including Kanagawa, with a star designating the type locality of *R. sagamianus* sp. nov.

of sediment were collected using a shovel. Specimens of *R. ryukyuensis* sp. nov. were separated from sediments using the $MgCl_2$ decantation method (Schockaert 1996). Individual worms were isolated under a stereoscope and whole-mounted alive in seawater to be studied and photographed at the Okinawa Institute of Science and Technology (OIST) under a compound microscope (Zeiss Axioscope) equipped with DIC. Two specimens were whole-mounted in lactophenol to study the sclerotized parts of the male copulatory organ. Two additional specimens were frozen in 3 µl of filtered seawater for DNA extraction. The specimens of *R. sagamianus* sp. nov. were procured by meticulously rinsing the collected sandy substrate with seawater, employing a dip net with an approximately 1-mm mesh size. Subsequently, the specimens were pipetted into petri dishes to facilitate isolation, whole-mounted in seawater to observe morphological characters on two life specimens, mounted in Entellan New (Merck) to study the sclerotized structures, or fixed in 99.5% ethanol for DNA extraction.

Morphological observations

Measurements and descriptions were made based on squashed preparations. Measurements of the sclerotized structures such as hooks and spines, as well as for soft body tissues, are expressed in micrometers (µm) and were taken using ImageJ software (Schneider et al. 2012). The sclerotized structures in live and fixed specimens were photographed with a Nikon D5600 digital camera affixed to an Olympus BX51 light microscope and with an Olympus DP20 digital camera affixed to an Olympus BX21 compound microscope. Figures were created with Adobe Illustrator CC 23.0.3 (Adobe Systems Inc., USA). All the whole mounted specimens fixed in lactophenol and Entellan New were deposited at the National Museum of Nature and Science, Tokyo (NSMT). A comprehensive bibliographic compilation of historic records and distribution data of free-living microturbellarians from marine and brackish water environments in Japan was also conducted.

DNA extraction, polymerase chain reaction, and sequencing

Total genomic DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. For phylogenetic inference, fragments of the 18S rDNA and 28S rDNA were PCR amplified using the primers and thermocycling conditions in Table 1. For the 18S and 28S rDNA of *R. sagamianus* sp. nov., 10-µl reaction volumes were used, each of which contained 1 µl of total DNA template, 1 µl of 10 × ExTaq buffer (Takara Bio), 2 mM of each dNTP, 1 µM of each primer, and 0.25 U of Takara Ex Taq DNA polymerase (5 U/µl; Takara Bio) in deionized water. For the 18S and 28S rDNA of *R. ryukyuensis* sp. nov., Illustra™ PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare) were suspended in a 25-µl volume of water, primers (0.2 µM), and DNA template (1.5 µl). Amplicons were visualized on 1.5% agarose gels stained with GelRed™ (Biotium) (*R. ryukyuensis* sp. nov.) or 1.0% agarose gels stained with FluoroDye DNA Fluorescent Loading Dye (SMOBIO) (*R. sagamianus* sp. nov.) and purified enzymatically using Illustra™ ExoProStar S (GE Healthcare) (*R. ryukyuensis* sp. nov.) or Exonuclease I and SAP (Takara Bio) (*R. sagamianus* sp. nov.). Amplicons of *R. ryukyuensis* sp. nov. were subsequently sequenced by Genewiz (Azenta Life Sciences) through standard Sanger DNA sequencing, while amplicons of *R. sagamianus* sp. nov. were sequenced with a BigDye Terminator Kit ver. 3.1 and a 3730 Genetic Analyzer (Life Technologies), using the amplification and internal sequencing primers shown in Table 1. Trace files were assembled into full sequences in either Geneious v11.0.15 (Kearse et al. 2012) or MEGA ver. 7.0 (Kumar et al. 2016) and subjected to a BLAST search on the NCBI website (http://blast.ncbi.nlm.nih.gov) to verify the specimens' taxonomic identity. Sequences were deposited in DDBJ/EMBL/GenBank, with accession numbers provided in Table 2.

Molecular phylogenetic analyses

For phylogenetic analyses, a concatenated dataset (3,264 bp) comprising partial 18S rDNA (1,642 bp) and 28S rDNA (1,622 bp) was prepared using DNA sequences of 24 koinocystidids in addition to the sequences of two individuals of *Reinhardorhynchus ryukyuensis* sp. nov. and one individual of *R. sagamianus* sp. nov. (Table 2). *Cystiplex axi* Karling, 1964, and *Cystiplex* sp. were included as outgroup taxa. Sequences were aligned using MAFFT ver. 7.472 (Katoh et al. 2019) with the L-INS-I strategy selected under the "Auto" option. Ambiguous sites were trimmed with Clipkit ver. 1.0 using the "kpic" option (Steenwyk et al. 2020). The optimal substitution models selected with PartitionFinder ver. 2.1.1. (Lanfear et al. 2016) were GTR+I+G for both the 18S and 28S rDNA partitions. A maximum likelihood (ML) analysis was performed using IQTree ver. 1.6 (Nguyen et al. 2015) under a partition model (Chernomor et al. 2016). Bayesian inference (BI) of the phylogeny was performed using MrBayes ver. 3.2.3 (Ronquist and Huelsenbeck 2003; Altekar et al. 2004) with two independent runs of Metropolis-coupled Markov chain Monte Carlo (MCMC), each consisting of four chains of 1,000,000 generations. All parameters (*statefreq*, *revmat*, *shape*, and *pinvar*) were unlinked between each position; trees were sampled every 100 generations. The first 25% of the trees were discarded as burn-in before a 50% majority-rule consensus tree was constructed based on the remaining 7,500 trees. Convergence was confirmed with the average standard deviation of split frequencies (0.008833), potential scale reduction factors for all parameters (0.999–1.006), and effective sample sizes for all parameters (≥322). Nodal support within the ML tree was assessed by analyses

Table 1. Primers and thermocycling conditions used in this study.

Primers	Primer name	Sequence (5‘–3‘)	Application	Reference
R. ryukyuensis sp. nov.				
18S rDNA	TimA	AMCTGGTTGATCCTGCCAG	Amplification and sequencing	Norén and Jondelius (1999)
18S rDNA	TimB	TGATCCATCTGCAGGTTCACCT	Amplification and sequencing	Norén and Jondelius (1999)
18S rDNA	600F	GGTGCCAGCAGCCGCGGT	Sequencing	Norén and Jondelius (1999)
18S rDNA	600R	ACCGCGGCTGCTGGCACC	Sequencing	Norén and Jondelius (1999)
18S rDNA	1100F	CAGAGGTTCGAAGACGATC	Sequencing	Norén and Jondelius (1999)
18S rDNA	1100R	GATCGTCTTCGAACCTCTG	Sequencing	Norén and Jondelius (1999)
18S rDNA	18S7F	GCAATAACAGGTCTGTGATGC	Sequencing	Norén and Jondelius (1999)
18S rDNA	18S7FK	GCATCACAGACCTGTTATTGC	Sequencing	Norén and Jondelius (1999)
28S rDNA	LSU5	TAGGTCGACCCGCTGAAYTTA	Amplification and sequencing	Littlewood et al. (2000)
28S rDNA	LSUD6-3B	GCTGTTCACATGGAACCCTTCTC	Amplification and sequencing	Van Steenkiste et al. (2013)
28S rDNA	L300F	CAAGTACCGTGAGGGAAAGTTG	Sequencing	Littlewood et al. (2000)
28S rDNA	L300R	CAACTTTCCCTCACGGTACTTG	Sequencing	Littlewood et al. (2000)
28S rDNA	L1200F	CCCGAAAGATGGTGAACTATG	Sequencing	Littlewood et al. (2000)
28S rDNA	L1200R	GCATAGTTCACCATCTTTCGG	Sequencing	Littlewood et al. (2000)
R. sagamianus sp. nov.				
18S rDNA	hrms18S_F	ATCCTGCCAGTAGTCATATGC	Amplification and sequencing	Oya and Kajihara (2020)
18S rDNA	hrms18S_Fi1	GCCGCGGTAATTCCAG	Sequencing	Oya and Kajihara (2020)
18S rDNA	hrms18S_R	CTACGGAAACCTTGTTACGAC	Sequencing	Oya and Kajihara (2020)
18S rDNA	hrms18S_Ri1	CTTTAATATACGCTATTGGAGCTGG	Sequencing	Oya and Kajihara (2020)
18S rDNA	hrms18S_Ri2	CTATTTAGTGGCTAGAGTCTCGTTCG	Amplification and sequencing	Oya and Kajihara (2020)
28S rDNA	LSU5	TAGGTCGACCCGCTGAAYTTA	Amplification and sequencing	Littlewood et al. (2000)
28S rDNA	Rd4.8a	ACCTATTCTCAAACTTTAAATGG	Sequencing	Whiting (2002)
28S rDNA	rD5b	CCACAGCGCCAGTTCTGCTTAC	Sequencing	Whiting (2002)
28S rDNA	LSUD6-3B	GCTGTTCACATGGAACCCTTCTC	Amplification and sequencing	Van Steenkiste et al. (2013)
Thermocycling conditions				
R. ryukyuensis				
18S rDNA	95 °C for 3m, touch down in 9 cycles (94 °C for 30 s, 60 °C down to 56 °C for 30 s, 72 °C for 1 m 30 s), 31 cycles (94 °C for 30s, 55 °C for 30 s, 72 °C for 1 m 30 s), 72 °C for 5m			
28S rDNA	95 °C for 3m, touch down in 9 cycles (94 °C for 30 s, 60 °C down to 56 °C for 30 s, 72 °C for 1 m 30 s), 31 cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 m 30 s), 72 °C for 5m			
R. sagamianus				
18S rDNA	94 °C for 1 m, 35 cycles (94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 m), 72 °C for 7 m			
28S rDNA	94 °C for 1m, 35 cycles (94 °C for 30 s, 50 °C for 30 s, 72 °C for 1.5 m), 72 °C for 7 m			

of 1,000 pseudoreplicates of ultrafast bootstrap (UFBoot) (Minh et al. 2013) and SH-aLRT branch tests (Guindon et al. 2010). ML UFBoot values ≥95%, SH-aLRT values ≥85%, and posterior probability (PP) values ≥0.90 were considered to indicate clade support.

Abbreviations used in Figures

a: apicomplexan; br: brain; bs: bursal stalk; bu: bursa; cg: common gonopore; cds: spines of the distal part of the spiny belt; ciu: unarmed accessory cirrus; cia: armed cirrus; cm: circular muscles; cms: spines of the middle part of the spiny belt; cps: spines of the proximal part of the spiny belt; ed: ejaculatory duct; fa: female atrium; fd: female duct; fg: female glands; h: hook; i: intestine; ilm: inner layer of longitudinal muscles; lh: larger hooks; ma: male genital atrium; oe: oesophagus; olm: outer layer of longitudinal muscles; om: oblique muscles; ov: ovary; pg: prostate glands; ph: pharynx; pp: penis papilla; pr: proboscis; s: spine; sh: smaller hook; scl: sclerotized layer; sv: seminal vesicle; t: testis; u: uterus; vi: vitellaria.

Table 2. List of species and respective GenBank accession numbers used for the molecular phylogenetic analyses in this study.

Species	18S rDNA	28S rDNA
Itaipusa divae	MW081596	MW054455
Itaipusa biglandula	MW081601	MW054460
Itaipusa karlingi	MW081598	MW054457
Itaipusa novacaledonica	KJ887481	KJ887528
Itaipusa sp. 1	KJ887451	KJ887557
Koinogladius sinensis YTP1	MF443159	MF443174
Koinogladius sinensis YTP2	MF443160	MF443175
Koinogladius sinensis YTP3	MF443161	MF443176
Mesorhynchus terminostylis	AY775741	KJ887500
Reinhardorhynchus anamariae	MW081597	MW054456
Reinhardorhynchus hexacornutus	MW054464	MW054451
Reinhardorhynchus riegeri	MW081595	MW054454
Reinhardorhynchus riegeri (CU1272)	OR490859	OR490875
Reinhardorhynchus ryukyuensis sp. nov.	LC807766	LC807768
Reinhardorhynchus ryukyuensis sp. nov.	–	LC807769
Reinhardorhynchus sagamianus sp. nov.	LC807767	LC807770
Reinhardorhynchus tahitiensis A	MW054463	MW054452
Reinhardorhynchus tahitiensis B	MW054462	MW054453
Rhinolasius dillonicus	MW081602	MW054461
Sekerana stolci	–	KJ887537
Utelga heinckei	MW081600	MW054459
Utelga heinckei (QU4)	OR490861	OR490876
Utelga heinckei (QU43)	OR490862	–
Utelga heinckei (QU44)	OR490863	OR490877
Utelga pseudoheinckei	MW081599	MW054458
Koinocystididae sp. 1	KR339027	–
Outgroup		
Cystiplex axi	KJ887437	KJ887549
Cystiplex sp.	KJ887469	KJ887495

Results

Taxonomic Account

Rhabdocoela Ehrenberg, 1831
Kalyptorhynchia von Graff, 1905
Eukalyptorhynchia Meixner, 1928
Koinocystididae Meixner, 1924
Reinhardorhynchus Diez, Monnens, & Artois, 2021

Reinhardorhynchus ryukyuensis **Van Steenkiste, Wakeman, & Leander, sp. nov.**

https://zoobank.org/57D2EE7F-0934-4BB1-AF9A-84E1D0D804FB

Fig. 2

Material examined. ***Holotype:*** **Japan •1**; Okinawa Prefecture, Onna; 26°28'52.7"N, 127°50'18.8"E; Feb. 2019; coarse mixture of sand, coral fragments, and shell hash from an intertidal seagrass bed; Niels Van Steenkiste and Kevin Wakeman leg.; one individual worm in a single slide [Holotype: NSMT-Pl 6458];

Paratype: **Japan •1**; locality same as for holotype; Feb. 2019; Niels Van Steenkiste and Kevin Wakeman leg.; one individual worm in a single slide; [Paratype: NSMT-Pl 6459].

Other material. Japan •1; locality same as for holotype; Feb. 2019; Niels Van Steenkiste and Kevin Wakeman leg.; two genomic DNA extracts from two individuals stored at -20 °C; GenBank: LC807766 (18S rDNA; 1,760 bp), LC807768, LC807769 (28S rDNA; 1,675 bp).

Type locality. Japan, Okinawa Prefecture, Onna (26°28'52.7"N, 127°50'18.8"E).

Diagnosis. Species of *Reinhardorhynchus* with conjuncta-duplex type male copulatory organ composed of a proximal globular part, a weakly sclerotized cylindrical middle part, and a distal penis papilla. Sclerotized structures of the copulatory organ consist of two large, separate hooks at the transition between the middle part and penis papilla and a distal girdle of two semi-elliptical plates bearing five smaller hooks. One larger hook with collared striated base, 29–31 µm long, pointing proximally; the other larger hook straight, with striated base, 29–34 µm long, pointing distally. The smaller distal hooks are 9–17 µm long. Female system with bipartite female duct, muscular bursal stalk, large bursa, and pouch of female glands.

Description. ***General morphology.*** Animals are 840–1060 µm long ($\bar{x}$ = 935 µm; n = 4), transparent, and have two eyes (Fig. 2A, B). General organization and internal morphology are consistent with other species of *Reinhardorhynchus*, as described by Diez et al. (2021). The large,

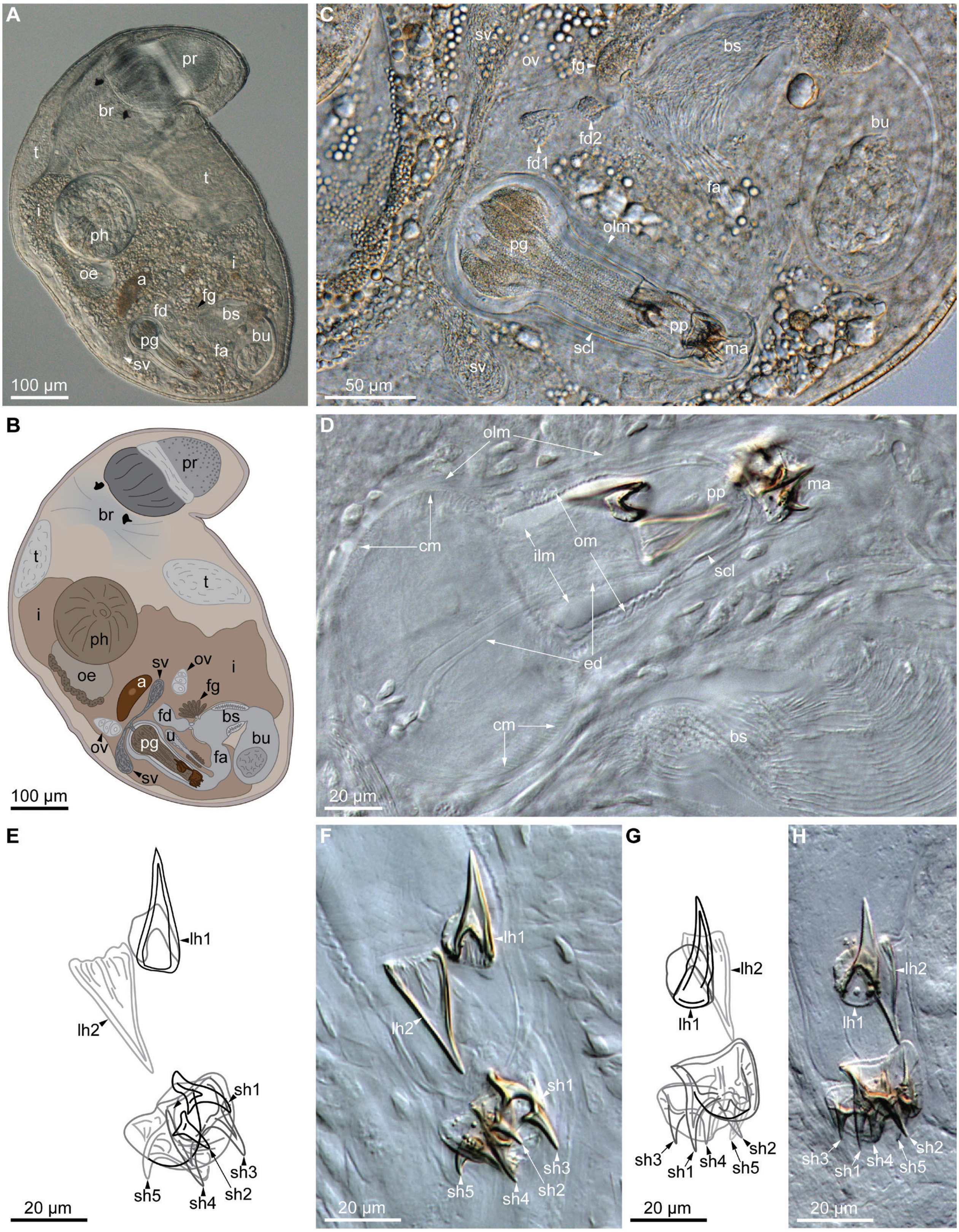

Figure 2. *Reinhardorhynchus ryukyuensis* sp. nov. **A, B.** Micrograph and drawing of a live animal. **C.** Detail of the atrial organs in a live specimen. **D.** Male copulatory organ in a whole-mounted specimen fixed in lactophenol. **E, F.** Drawing and micrograph of the sclerotized parts of the male copulatory organ in the holo/paratype. **G, H.** Drawing and micrograph of the sclerotized parts of the male copulatory organ in the holo/paratype.

typical koinocystidid proboscis (pr, Fig. 2A, B) is about 1/4 of the body length (215–233 µm; x̄ = 224 µm; n = 3), with a well-developed juncture sphincter. Ciliated cellular epidermis with needle-like rhabdites is present all over the body.

The globular pharynx (ph, Fig. 2A, B) is at about 50% of the body length, followed by an intestine (i, Fig. 2A, B) that runs all the way to the posterior end. The oesophagus (oe, Fig. 2A, B) is visible as a clear, transparent zone bordered by oesophageal glands right behind the pharynx.

Male reproductive system. Paired testes are located in front of the pharynx (t, Fig. 2A, B). The conjuncta-duplex type male copulatory organ (165–195 µm; x̄ = 180 µm; n = 4) is inverted-pear shaped and encompasses the prostate vesicle (pg, Fig. 2A–C). This prostate vesicle is composed of gland necks of prostate glands originating extracapsularly, extending into the middle part of the copulatory organ, and opening distally into the ejaculatory duct. The bulbous proximal part of the prostate vesicle is surrounded by circular muscles (cm, Fig. 2D), while the cylindrical middle part is surrounded by two layers of oblique muscles (om, Fig. 2D) and an inner layer of longitudinal muscles (ilm, Fig. 2D). The walls of the middle and distal parts of the copulatory organ are weakly sclerotized (scl, Fig. 2C, D) and distally form a penis papilla (pp, Fig. 2C, D), which enters into the male genital atrium (ma, Fig. 2C, D). The entire copulatory organ is encased in an outer layer of longitudinal muscles (olm, Fig. 2C, D). Paired seminal vesicles (sv, Fig. 2A–C) merge right before entering the copulatory organ proximally and form the ejaculatory duct (ed, Fig. 2D). The necks of the prostate glands and the ejaculatory duct run throughout the entire length of the copulatory organ and into the penis papilla.

The male copulatory organ is provided with sclerotized structures consisting of two larger separate hooks and a girdle of five smaller interconnected hooks (lh1–lh2, sh1–sh5, Fig. 2E–H). The two larger hooks are positioned at the transition of the cylindrical middle part to the penis papilla and point in opposite directions. The proximally pointing larger hook (lh1, Fig. 2E–H) measures 29–31 µm (n = 2) and is provided with a collared striated base. The distally pointing larger hook (lh2, Fig. 2E–H) is straight, measures 29–34 µm (n = 2), and has a striated base that seems to be continuous with the weakly sclerotized layer. The five distal, smaller hooks are connected by a complex sclerotized girdle surrounding the distal tip of the penis papilla. The smaller hooks sh1 (15–17 µm; n = 2) and sh2 (10–13 µm; n = 2), and sh3 (10–13 µm; n = 2) and sh4 (9–12 µm; n = 2) are each connected through a semi-elliptical base, respectively. The base of the smaller hook sh5 (10–12 µm; n = 2) seems to connect both semi-elliptical bases into an open girdle.

Female reproductive system. The female structures are located caudal to the pharynx and include paired ovaries (ov, Fig. 2B, C), a female duct (fd, Fig. 2B) consisting of two parts (fd1 and fd2, Fig. 2C), and a very muscular bursal stalk (bs, Fig. 2B–D) guarding the entrance to a large bursa (bu, Fig. 2B, C). At its proximal end, the female atrium (fa, Fig. 2B, C) receives the female duct, the bursal stalk, and a pouch of female glands (fg, Fig. 2B, C). The female duct is separated from the female atrium by a small sphincter. A uterus (u, Fig. 2B) is present between the male and female atria. Vitellaria and the common gonopore could not be observed in the live animals.

Etymology. Species epithet based on its occurrence on the Ryukyu Islands.

Distribution. Okinawa Islands, Japan.

Reinhardorhynchus sagamianus Tsuyuki, Reyes, Oya, & Van Steenkiste, sp. nov.

https://zoobank.org/B2534AFD-3461-4329-A62F-3C6AC8467FB2

Fig. 3

Material examined. ***Holotype:*** **Japan •1**; Kanagawa Prefecture, Hayama, Sangashita beach; (35°15'58.3"N, 139°34'19.6"E); 21 April 2023; sandy substrates; Aoi Tsuyuki and Yuki Oya leg.; one individual worm in a single slide; [Holotype: NSMT-Pl 6460].

Paratype: **Japan •1**; locality same as for holotype; 30 Aug 2023; Yuki Oya leg.; genomic DNA extract from one individual stored at -20 °C; GenBank: LC807767 (18S rDNA; 1,654 bp), LC807770 (28S rDNA; 1,667 bp); [Paratype: NSMT-DNA 56985].

Type locality. Japan, Kanagawa Prefecture, Hayama, Sangashita Beach (35°15'58.3"N, 139°34'19.6"E).

Diagnosis. Species of *Reinhardorhynchus* with a copulatory organ encompassing an armed cirrus, an unarmed accessory cirrus, and two distal hooks. Bipartite armed cirrus consisting of two sacs lined with a continuous ±295.2-µm-long sclerotized belt of overlapping scale-like spines. Larger sac with more spaced-out, triangular, ±20.1 µm-long spines on the proximal end of the belt. Spines gradually decrease in size distally as the belt runs towards and folds into the smaller sac, increasing in size (±6.1 to ±22.2 µm long) towards the proximal tip of the smaller sac, and decreasing in size again from the proximal to the distal tip of the smaller sac. Unarmed accessory cirrus as an elongated sac. The larger distal hook is slightly curved, ±111.5 µm long and ±43.5 µm wide at its base; its base is provided with a slightly curved, ±42.3 µm-long projection with a blunt distal tip forming a ~90° angle with the axis of the hook. The smaller hook is funnel-shaped, ±58.9 µm long and ±46.6 µm wide at its base.

Description. ***General morphology.*** Live mature specimens are 1500–1800 µm long (n = 2), with two eyes (Fig. 3A, B). The proboscis is 297–304 µm (n = 2) long in swimming animals and is characteristic for koinocystidids (Brunet 1972; Karling 1980; Diez et al. 2021). The pharynx (ph, Fig. 3A, B) is positioned near the body's midpoint and has an approximate diameter of 231–258 µm (n = 2) in the live specimens. The oesophagus is visible as a clear zone surrounded by oesophageal glands behind the pharynx. It empties into the intestine, which is situated in the posterior portion of the body. The male and female reproductive systems are mainly located in the third posterior region of the body.

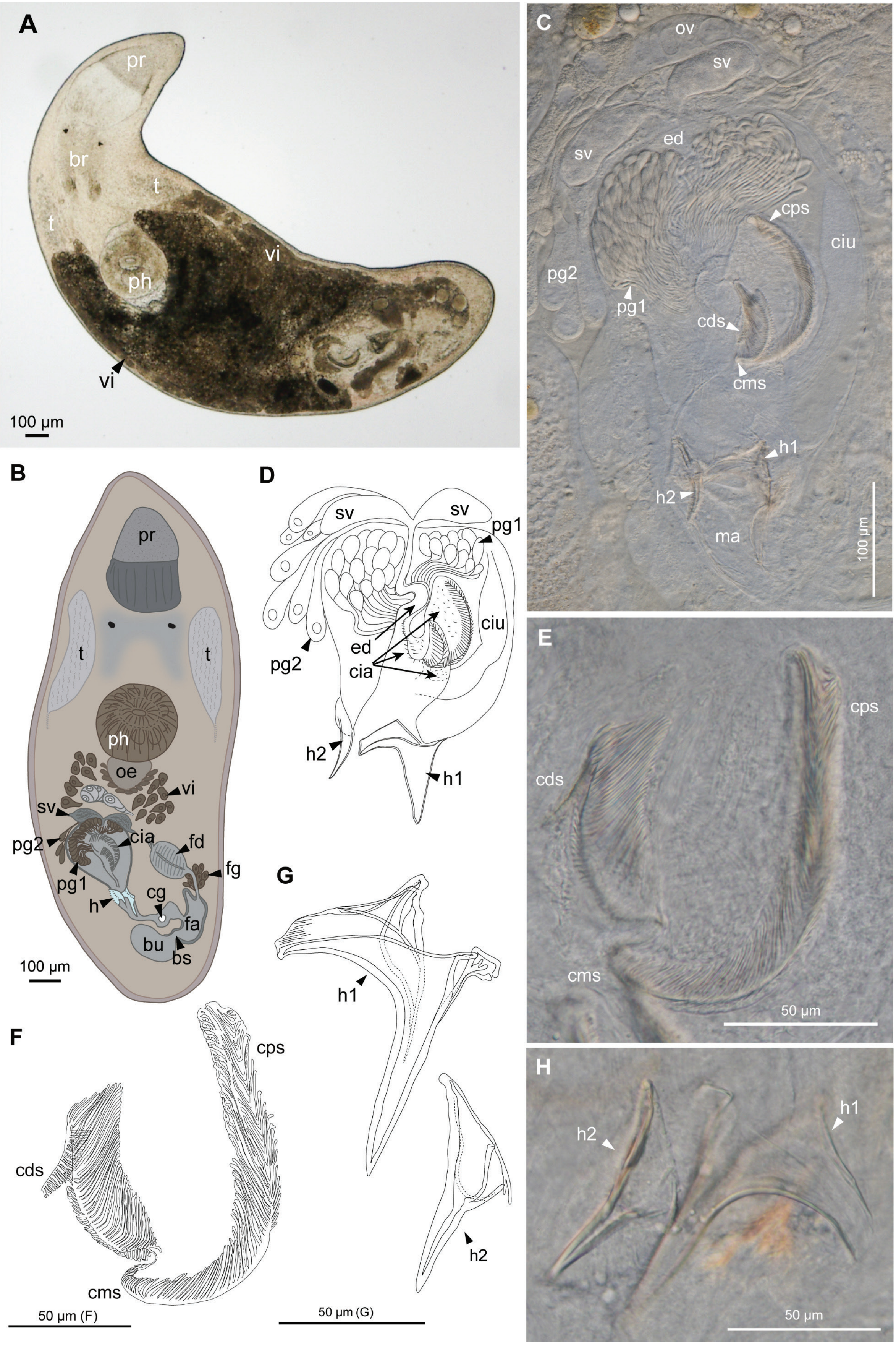

Figure 3. *Reinhardorhynchus sagamianus* n. sp., NSMT-DNA 56985 (paratype) (**A**) and NSMT-Pl 6460 (holotype) (**B**–**H**). **A, B.** Micrograph and drawing of live animals. **C.** Detail of the male copulatory organ in a live animal. **D, E.** Drawing and micrograph of the belt of overlapping spines in the armed cirrus of the male copulatory organ in a whole mounted specimen fixed in Entellan New. **F–H.** Drawings and micrograph of the distal hooks associated with the male copulatory organ in a whole mounted specimen fixed in Entellan New.

Male reproductive system. Paired testes anterior to the pharynx are on each side of the body (t, Fig. 3A, B). A pair of sac-like seminal vesicles (sv, Fig. 3B–D) fuse distally before entering the copulatory organ and forming an ejaculatory duct (ed; Fig. 3C, D). The piriform copulatory organ is 323–376 µm (n = 2) long and encompasses a proximal prostate vesicle, a bipartite armed cirrus, and an unarmed accessory cirrus. Distally, it bears two large hooks of varying shapes (h1, h2, Fig. 3D). The prostate vesicle consists of one type of intracapsular prostate gland (pg1; Fig. 3B–D) opening into the transition zone between the ejaculatory duct and armed cirrus through filiform ducts (Fig. 3B–D; Suppl. material 1). A second type of extracapsular prostate gland (pg2; Fig. 3B–D) enters the copulatory organ proximally. The armed cirrus bears small spines on its entire surface (cia, Fig. 3B–D; Suppl. materials 1, 2) and has two sacs of differing sizes, both of which are equipped with an interconnecting belt of overlapping, scale-like spines (Fig. 3B–F; Suppl. material 1). The spines of the proximal part of the spiny belt start in the larger sac. Here, the scale-like spines are triangular, more spaced out, and 20.1 µm long (cps, Fig. 3B–F). Gradually, these spines become less triangular, more overlapping, and decrease in size (8.8 µm long) until they reach the transition to the smaller sac, where they fold backwards and continue into the smaller sac (cms, Fig. 3C, E, F; Suppl. materials 1–3). From this fold, the spines gradually increase in size (from 6.1 to 22.2 µm long) towards the proximal tip of the smaller sac, where they fold again to continue along the distal side of this sac while gradually decreasing in size again (cds, Fig. 3C, E, F). The total length of this spiny belt is 295.2 µm. The unarmed accessory cirrus (ciu, Fig. 3C, D; Suppl. material 1) runs alongside the armed cirrus from the proximal end of the copulatory organ to the distal end of the armed cirrus. It appears as an elongated sac that narrows proximally. It is possible that this narrow proximal part of the unarmed accessory cirrus connects to the bundles of extracapsular prostate glands (pg2, Fig. 3C) visible around the copulatory organ and seminal vesicles, but this connection could not be established with certainty. The larger distal hook is 111.5 µm long and 43.5 µm wide at its base (h1, Fig. 3G, H; Suppl. material 1). One side of the base features a sturdy, slightly curved projection with a blunt distal tip. The projection itself is 42.3 µm long and forms an ~90° angle with the axis of the hook. The smaller distal hook is 58.9 µm long and has a 46.6 µm-wide base (h2, Fig. 3G, H).

Female reproductive system. The vitellaria (vi; Fig. 3A) extends from the rear end of the pharynx to the posterior body end. Paired ovaries with oocytes arranged in a single line are situated anterior to the copulatory organ (ov, Fig. 3B, C). The bursa (bu, Fig. 3B) opens into the female atrium through a muscular bursal stalk (bs, Fig. 3B). The female atrium also receives the female duct (fd, Fig. 3B), which consists of a narrow distal duct in which bundles of female glands (fg, Fig. 3B) discharge, and a wide, muscular proximal part that receives the oviducts.

Etymology. The species epithet *sagamianus* refers to the type locality, which is located in Sagami Bay.

Distribution. Kanawaga, Sangashita Beach, Japan.

Molecular phylogeny

The resulting ML and BI trees were congruent with each other in terms of topology, so only the ML tree is shown in Fig. 4. All of the examined koinocystidid species form a clade with full support. Within this clade, four major clades can be recognized: (1) a clade composed of *Utelga heinckei* (Attems, 1897), *Utelga pseudoheinckei* Karling, 1980, and *Parautelga* sp. (with full support); (2) a clade with *Koinogladius sinensis* (Wang & Lin, 2017) and *Rhinolasius dillonicus* Karling, 1980 (‘*sinensis*’ clade in Diez et al. (2021)) (PP = 1.00; SH-aLRT = 99.5%; UFBoot = 99%), (3) a clade composed of four representatives of *Itaipusa* (‘*divae*’ clade in Diez et al. (2021)) (with full support), and (4) a clade with several representatives of *Reinhardorhynchus*, *Itaipusa* sp. 1, and Koinocystididae sp. 1 (‘*riegeri*’ clade in Diez et al. (2021)) (PP = 0.82; SH-aLRT = 99.1%; UFBoot = 82%). Our two new species, *R. ryukyuensis* sp. nov. and *R. sagamianus* sp. nov., from Japan, are nested in the ‘*riegeri*’ clade. *Reinhardorhynchus ryukyuensis* sp. nov. is the sister taxon to a clade including *R. hexacornutus* Jouk, Diez, Reygel & Artois, 2021, *R. tahitiensis* Jouk, Diez, Yurduseven, Reygel & Artois, 2021, and an unidentified species of Koinocystididae, albeit with relatively low support (PP = 0.75; SH-aLRT = 99.3%; UFBoot = 54%). *Reinhardorhynchus sagamianus* sp. nov. is sister to a clade consisting of *R. riegeri* (Karling, 1978), *R. anamariae* Diez, Reygel & Artois, 2021, and *Itaipusa* sp. 1 with full support (Fig. 4).

Faunistic account

A total of 58 taxa of marine and brackish water microturbellarians that have been identified to species level have been recorded from the coastal areas of Japan, including the two new species of *Reinhardorhynchus* described in this study; four taxa were only identified up to genus level (Table 3). All these taxa belong to the Macrostomorpha, Rhabdocoela, Prolecithophora, and Proseriata. Nineteen species were found in brackish water habitats, all of which belong to genera that are either considered typical to these environments or euryhaline marine taxa. Most of the records are from Hokkaido (33 taxa) and the northern part of Honshu (18 taxa) (Table 3; Fig. 1). A few records are from locations in southern Honshu (Kanagawa, Okayama, and Shimane) and the Ryukyu Islands (Okinawa).

Discussion

Morphology

Koinocystididae is one of the most species-rich groups of kalyptorhynch rhabdocoels. Its representatives are found globally in marine sediments and on seaweeds; however, some species also occur in freshwater habitats. Most koinocystidids have a large proboscis with a sphincter at the

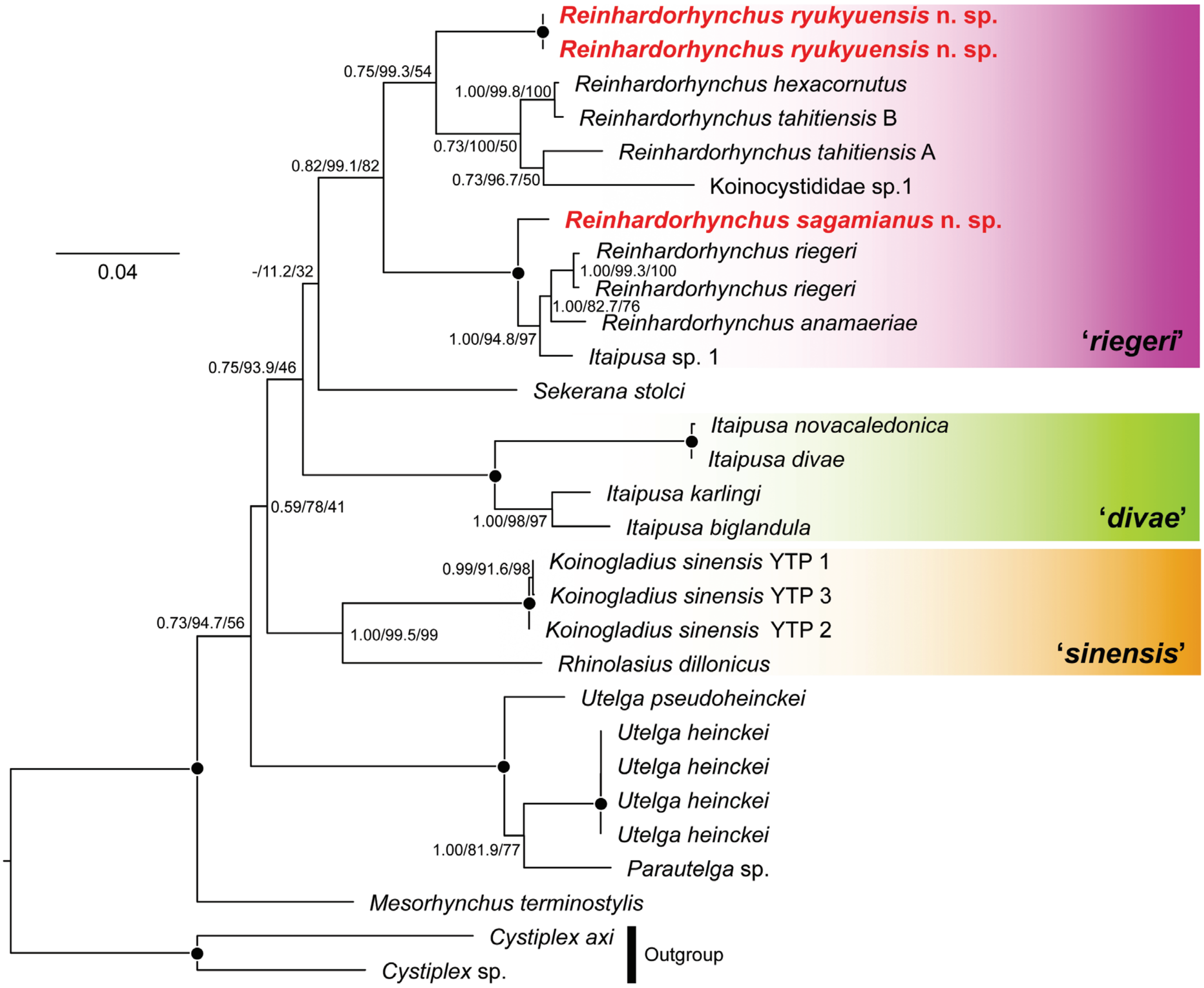

Figure 4. ML phylogenetic genetic tree based on a concatenated dataset of partial 18S and 28S rDNA sequences. Branch support values are indicated next to the nodes as posterior possibilities/SH-aLRT values/UFBoot values. Black dots indicate the maximum values for all support measures.

base of the cone and complex atrial organs with sclerotized structures and a copulatory bursa (Brunet 1972; Karling 1980; Diez et al. 2021). The two new koinocystidid species from Japan exhibit the characteristics described for the recently proposed genus *Reinhardorhynchus*. At present, this genus accommodates sixteen species, half of which were newly described by Diez et al. (2021). Representatives of *Reinhardorhynchus* all have large accessory hooks or spikes associated with the male copulatory organ, in contrast to species of *Itaipusa*, which lack such accessory structures (Karling 1978, 1980; Diez et al. 2021).

The new species of *Reinhardorhynchus* from Okinawa, *R. ryukyuensis* sp. nov., has a unique combination of features. Firstly, *R. ryukyuensis* sp. nov. has no armed cirrus in its male copulatory organ. However, the presence of a short, unarmed cirrus cannot be excluded, as the exact ending of the prostate glands and ejaculatory duct in the tip of the penis papilla could not be observed. In all other known species of *Reinhardorhynchus*, except for *R. scoticus* (Karling, 1954), the male copulatory organ possesses one (*R. hexacornutus* Jouk, Diez, Reygel & Artois, 2021, *R. renei* (Reygel, Willems & Artois, 2011)), two (*R. riegeri* (Karling, 1978), *R. unicornis* Diez, Aguirre, Reygel, & Artois, 2021, *R. variodentatus* (Karling, Mack-Fira, & Doerjes, 1972)) or three (*R. pacificus* Diez, Reygel & Artois, 2021) armed cirri provided with fields of spines, or a single cirrus armed with belts/rows of overlapping spines (*R. anamariae* Diez, Reygel & Artois, 2021, *R. beatrizae* Diez, Aguirre, Reygel & Artois, 2021, *R. bispina* (Karling, 1980), *R. curvicirrus* (Karling, 1980), *R. evelinae* (Marcus, 1954), *R. riae* Diez, Reygel & Artois, 2021, *R. ruffinjonesi* (Karling, 1978), *R. soror* Diez, Reygel & Artois, 2021, *R. tahitiensis* Jouk, Diez, Yurduseven, Reygel, & Artois, 2021) (Karling et al. 1972; Karling 1980; Reygel et al. 2011; Diez et al. 2021). *Reinhardorhynchus ryukyuensis* sp. nov. differs from *R. scoticus* because it has a girdle of five smaller interconnected hooks around the distal tip of the penis papilla rather than a circular plate with fine needle-like spines as in *R. scoticus*, and because it has two larger separate hooks instead of three to five foldable hooks at the base of the penis papilla (Karling 1954, 1963; Ax 2008). Secondly, the configuration of the larger and smaller hooks in *R. ryukyuensis* sp. nov. markedly deviates from the sclerotized hooks and spines found in other species of *Reinhardorhynchus*. The complex arrangement of the five smaller hooks on a girdle composed of two semi-elliptical plates surrounding the penis papilla is unique among representatives of the genus. Another species, *R. riae*, also has sclerotized structures associated with a pseudocuticular penis papilla, but in this species, these structures consist of two flattened hooks with a broad and rounded distal end (Diez et al. 2021). In addition, *R. riae* also has a cirrus armed with two spinous rows. The above-mentioned differences make it easy to distinguish *R. ryukyuensis* sp. nov. from its congeners and thus warrant the description of a new species.

The new species of *Reinhardorhynchus* from Kanagawa, *R. sagamianus* sp. nov., is also unique among its congeners because it is the only species with the combination of a bipartite armed cirrus with a belt of spines, an unarmed accessory cirrus, and two distal accessory hooks associated with the male copulatory organ. Only three other species of *Reinhardorhynchus*, *R. riegeri*, *R. unicornis*, and *R. variodentatus*, possess two cirri and one (*R. unicornis*) or two (*R. riegeri* and *R. variodentatus*) accessory hooks in their male copulatory organ. However, in these three species, both the cirri are provided with spines. Eight other species of *Reinhardorhynchus*, *R. anamariae*, *R. beatrizae*, *R. bispina*, *R. curvicirrus*, *R. riae*, *R. ruffinjonesi*, *R. soror*, and *R. tahitiensis*, possess a single cirrus armed with belts or rows of spines and two distal hooks (Karling 1978, 1980; Diez et al. 2021). One species, *R. evelinae*, has a single cirrus with hooked denticles and two or three rows of spines in combination with three distal spines (Marcus 1954). Apart from the lack of an unarmed accessory cirrus in all these species, other noticeable differences in the morphology of the male copulatory organ are present when compared to *R. sagamianus* sp. nov. The two distal hooks in *R. riae* and *R. tahitiensis* are symmetrical and have broad and rounded distal ends. In addition, these two species have multiple spinous belts – two in *R. riae* and four in *R. tahitiensis* (Diez et al. 2021) – and are, therefore, very distinct from *R. sagamianus* sp. nov. and the other six species mentioned above. The morphology of the spiny cirrus belt, including its overall length and shape and the shape and size of the individual spines, is unique in every species of *Reinhardorhynchus* with such a belt. In *R. bispina*, these spines are mostly uniform in size, whereas in *R. anamariae*, *R. beatrizae*, *R. curvicirrus*, *R. ruffinjonesi*, *R. sagamianus* sp. nov., and *R. soror*, there are clear sections with a gradual increase or decrease in the size of the spines (Karling 1978, 1980; Diez et al. 2021). In *R. soror* and *R. ruffinjonesi*, a row of larger hook- or claw-shaped spines is also present in the belt (Diez et al. 2021). Another unique feature of the new species from Kanagawa is the fact that the armed cirrus has two sacs, which results in the unique curved and twisted contour of the spinous belt as it spans the different compartments of the cirrus. While described as a "fold in the cirrus wall," the configuration of the armed cirrus in *R. curvicirrus* also implies the presence of a blind sac (Karling 1980). A small "spiny diverticulum" as part of the armed cirrus has also been described for *R. ruffinjonesi* (Karling 1978; Diez et al. 2021). Finally, only *R. beatrizae* and *R. soror* share the presence of a projection on the base of one of the distal hooks with *R. sagamianus* sp. nov. In *R. sagamianus* sp. nov., this projection has a blunt, angled distal tip and is oriented at a ~90° angle to the main axis of the larger distal hook. In *R. beatrizae*, this projection is more funnel-shaped, distally more pointed, and at an angle of more than 100° to the main axis of the hook (Diez et al. 2021). In *R. soror*, the tip of the largest hook is noticeably curved compared to *R. sagamianus* sp. nov., which has a straight tip. Also, there are two projections on the base of the largest hook of *R. soror*: one long straight, funnel-shaped projection with a blunt tip making an angle of 90° to the main axis of the hook, and one shorter, more or less square and folded projection (Diez et al. 2021). These characteristics are clearly different from *R. sagamianus* sp. nov.

Molecular phylogeny

The interrelationships of the Koinocystididae have been extensively discussed by Diez et al. (2021) based on the results of molecular phylogenetic analyses inferred from 18S and 28S rDNA sequences. Our reconstructed tree is congruent with these results and shows that the new species *R. ryukyuensis* sp. nov. and *R. sagamianus* sp. nov. form a clade with five other species of *Reinhardorhynchus* and the unidentified taxa *Itaipusa* sp. 1 and Koinocystididae sp. 1 with high support ('*riegeri*' clade in Fig. 4). With 12 out of 18 species of *Reinhardorhynchus* still lacking from the analyses, the molecular phylogenetic interrelationships within the *'riegeri'* clade are difficult to interpret within the context of character evolution. *Reinhardorhynchus ryukyuensis* sp. nov., *R. hexacornutus*, and *R. tahitiensis* belong to the same clade and have a clearly different morphology from other species of *Reinhardorhynchus*. For example, the lack of an armed cirrus, the presence of two large separate hooks, and a distal girdle of five smaller hooks in the male copulatory organ are unique features of *R. ryukyuensis* sp. nov. However, it does share this relatively large number of hooks with *R. hexacornutus*, which has six separate hooks associated with the male copulatory organ; moreover, these six hooks are also organized in two groups (4+2) (Diez et al. 2021). In all other species of *Reinhardorhynchus*, the number of hooks or spines not associated with a cirrus is either one, two, or three, except for *R. scoticus*, which has five separate sclerotized structures associated with its copulatory organ. The male copulatory organ of *R. tahitiensis* has an armed cirrus with four rows of spines that vary in size and two similar, blunt accessory hooks. *Reinhardorhynchus sagamianus* sp. nov. forms a clade with *R. anamariae*, *R. riegeri*, and *Itaipusa* sp. 1. The first three

Table 3. Species of free-living microturbellarians from marine (M) and brackish water (B) habitats in Japan and territories claimed by Japan, including their geographical distributions and reference literature.

Taxonomic idenity			Locality in Japan	Prefecture in Japan	Reference	Distribution outside of Japan
Identitified species						
Macrostomorpha	**Macrostomidae**	*Macrostomum flexum* Ax, 2008 (B)	Jusan Lake	Aomori	Ax (2008)	–
		Macrostomum guttulatum Ax, 2008 (B)	Jusan Lake, Noheji River, Obuchi Pond	Aomori	Ax (2008)	–
		Macrostomum semicirculatum Ax, 2008 (B)	Takase River, Obuchi Pond, Takahoko Pond	Aomori	Ax (2008)	–
		Macrostomum uncinatum Ax, 2008 (B)	Takase River	Aomori	Ax (2008)	–
Rhabdocoela	**Koinocystididae**	*Reinhardorhynchus ryukyuensis* sp. nov. (M)	Onna (26°28'52.7"N, 127°50'18.8"E)	Okinawa	This study	–
		Reinhardorhynchus sagamianus sp. nov. (M)	Hayama (35°1'58"N, 139°3'19.4"E)	Kanagawa	This study	–
		Utelga monodon Ax, 2008 (B)	Obuchi pond, Takase River	Aomori	Ax (2008)	–
	Polycystididae	*Palladia nigrescens* (Evdonin, 1971) Evdonin, 1977 (B)	Kominato River, Obuchi Pond, Takase River	Aomori	Ax (2008)	Posyet, Russia (Evdonin 1971, 1977)
		Phonorhynchoides japonicus Ax, 2008 (B)	Takase River	Aomori	Ax (2008)	–
	Cheliplanidae	*Cheliplana setosa* Evdonin, 1971 (B)	Takase River	Aomori	Ax (2008); Gobert et al. (2021)†	Posyet, Russia (Evdonin 1977), British Columbia, Canada (Gobert et al. 2021); California, USA (Karling 1983); Asturias, Spain (Noreña et al. 2007)
		Cheliplana terminalis Brunet, 1968 (M)	Igei (26°27'17.9"N, 127°52'27.5"E)	Okinawa	Van Steenkiste et al. (2023)	Port Lincoln, Australia (Gobert et al. 2021); Southern France (Brunet 1968; Gobert et al. 2021); Blanes, Spain (Gobert et al. 2021); Djezira, Somalia (Schockaert 1982); Mombasa, Kenya (Jouk and De Vocht 1989); Santiago de Cuba, Cuba (Diez et al. 2019)
		Freddius tricaudatus Takeda & Kajihara, 2018 (M)	Akkeshi (43°01'16"N, 144°50'13"E)	Hokkaido	Takeda and Kajihara (2018)	–
	Schizorhynchidae	*Proschizorhynchella caudociliata* Takeda & Kajihara, 2018 (M)	Mukawa (42°33'25"N, 141°55'42"E)	Hokkaido	Takeda and Kajihara (2018)	–
		Proschizorhynchella magnoliae Takeda & Kajihara, 2018 (M)	Akkeshi (43°01'16"N, 144°50'13"E)	Hokkaido	Takeda and Kajihara (2018)	–
		Proschizorhynchella shibazakii Takeda & Kajihara, 2018 (M)	Oshoro (43°12'33"N, 140°51'31"E)	Hokkaido	Takeda and Kajihara (2018)	–
		Proschizorhynchella shuttlecock Takeda & Kajihara, 2018 (M)	Obira (44°03'03"N, 141°39'46"E), Soya (45°29'16"N, 141°58'05"E)	Hokkaido	Takeda and Kajihara (2018)	–
		Proschizorhynchella pacificus (Evdonin, 1969) (M)	Kunashiri	Hokkaido‡	Evdonin (1969)	–
Rhabdocoela	**Provorticidae**	*Pogaina japonica* Ax, 2008 (B)	Takase River, Obuchi Pond, Noheji River, Takahoko Pond	Aomori	Ax (2008)	–
		Pogaina scypha Ax, 2008 (B)	Obuchi Pond	Aomori	Ax (2008)	–
	Trigonostomidae	*Trigonostomum vanmecheleni* Artois et al., 2013 (M)	Otaru (43°13'31.2"N, 141°01'04.0"E)	Hokkaido	Van Steenkiste et al. (2023)	Venice, Italy (Artois et al. 2013); Guangdong, China (Hu et al. 2019); Santiago de Cuba (Diez et al. 2023a)
		Ptychopera japonica Ax, 2008 (B)	Takase River, Obuchi Pond	Aomori	Ax (2008)	British Columbia, Canada (Van Steenkiste and Leander 2018a)
	Promesostomidae	*Promesostoma teshirogii* Ax, 1992 (B)	Takase River, Jusan Lake, Takahoko Pond	Aomori	Ax (1992)	–
Prolecithophora	**Plagiostomidae**	*Vorticeros lobatum* Tozawa, 1918 (M)	Misaki	Kanagawa	Tozawa (1918)	–

Taxonomic idenitity			Locality in Japan	Prefecture in Japan	Reference	Distribution outside of Japan
Prolecithophora	**Plagiostomidae**	*Vorticeros ijimai* Tozawa, 1918 (M)	1) Misaki; 2) Ushimado	1)Kanagawa; 2) Okayama	Tozawa (1918)	–
		Plagiostomum lobatum kurilense Kulinitch, 1979 (M)	Rishiri Island	Hokkaido	Omi (2018)	–
	Pseudostomidae	?*Allostoma durum* (Fuhrmann 1896) (M)	Misaki	Kanagawa	Westblad (1955); Omi (2020)	Concarneau, France (Fuhrmann 1896); Isle of Man, UK (Graff 1913; Westblad 1955); Plymouth, UK (Graff 1913); Trieste, Italy (Graff 1913); Sevastopol, Ukraine (Graff 1913); Falkland Islands, UK (Westblad 1952); Gullmarn, Sweden (Westblad 1955); Vestland, Norway (Karling 1940; Westblad 1955); Istria, Croatia (Westblad 1955)
		Cylindrostoma monotrochum (Graff, 1882) Westblad, 1955 (M)	Wakkanai, Rishiri Island (from Kelps)	Hokkaido	Omi (2018)	Kınalıada, Turkey (Ax 1959); Adriatic Sea, Italy (Graff 1882, 1913; Ritter-Zahony 1908); Sevastopol, Ukraine (Graff 1913); Devon, UK (Westblad 1955); Ilha de São Sebastião, Brazil (Marcus 1951); Hawaii, USA (Karling et al. 1972); California, USA (Karling 1962); Bermuda (Karling 1978).
		Enterostomula densissimabursa Omi, 2020 (B)	Shinji Lake, Nakaumi	Shimane	Omi (2020)	–
	Multipeniatidae	*Multipeniata kho* Nasonov, 1927 (B)	Jusan Lake, Kominato River, Takahoko Pond, Takase River	Aomori	Ax (2008)	Posyet, Russia (Nasonov 1927)
		Japanoplana insolita Ax, 1994 (B)	Kominato River, Takase River	Aomori	Ax (1994; 2008)	–
Proseriata	**Monocelididae**	*Minona pelvivaginalis* Tajika, 1982 (M)	Rumoi, Raigishi, Setana	Hokkaido	Tajika (1982b)	–
		Tajikina juliae (Tajika, 1982) (M)	Onbetsu, Akkeshi	Hokkaido	Tajika (1982b)	–
		Monocelis tenella japonica Tajika, 1982 (M)	Oshoro	Hokkaido	Tajika (1982b)	–
		Monocelis colpotriplicis Tajika, 1982 (M)	Oshoro, Abuta, Shakubetsu, Akkeshi, Habomai, Abashiri, Saruru	Hokkaido	Tajika (1982b)	–
		Minona dolichovesicula Tajika, 1982 (M)	Muroran, Habomai, Nemuro	Hokkaido	Ax (2008)	–
		Duplominona filiformis Ax, 2008 (B)	Takase River	Aomori	Ax (2008)	–
		Duplominona japonica Ax, 2008 (B)	Jusan Lake	Aomori	Ax (2008)	–
		Archilina japonica Ax, 2008 (B)	Noheji River, Takase River	Aomori	Ax (2008)	–
		Minona minuta Ax, 2008 (B)	Obuchi pond	Aomori	Ax (2008)	–
	Nematoplanidae	*Ezoplana oxygona* Tajika, 1982 (M)	1) Cape Erimo, Harutachi; 2) Yaeyama Islands	1)Hokkaido; 2) Okinawa	Tajika (1982a)	–
		Ezoplana masacoae Tajika, 1982 (M)	1) Raigishi, Kameda Peninnsula, Hidaka, Abashiri; 2) Joga-Shima Island; 3) Hateruma Island	1)Hokkaido; 2) Kanagawa; 3) Okinawa	Tajika (1982a)	–
	Coelogynoporidae	*Ezona habomaiensis* Tajika, 1980 (M)	Habomai	Hokkaido	Tajika (1980)	–
		Coelogynopora coniuncta Tajika, 1978 (M)	Oshoro, Hakodate, Akkeshi	Hokkaido	Tajika (1978)	–
		Ezona pinnigera Tajika, 1980 (M)	Oshoro, Cape Aikappu, Raigishi, Habomai	Hokkaido	Tajika (1980)	–
		Invenusta paracnida (Karling, 1966) (M)	Akkeshi, Ishikari, Harutachi, Erimo, Habomai, Saruru	Hokkaido	Tajika (1981b)	Alaska, USA (Ax and Armonies 1990); Washington, USA (Ax and Sopott-Ehlers 1979; Ehlers and Sopott-Ehlers 1987); California, USA (Karling 1966)
		Coelogynopora birostrata Tajika, 1978 (M)	Oshoro, Hakodate, Akkeshi, Okushiri Island	Hokkaido	Tajika (1978)	–
		Pseudovannuccia hirutai (Tajika, 1981) (M)	1) Oshoro, Tomamae, Rebun Island, Rishiri Island, Muroran, Abuta, Akkeshi; 2) Ishigaki Island	1)Hokkaido, 2) Okinawa	Tajika (1981b)	–

Taxonomic idenitity			Locality in Japan	Prefecture in Japan	Reference	Distribution outside of Japan
Proseriata	**Coelogynoporidae**	*Vannuccia tripapillosa* Tajika, 1977 (M)	Oshoro, Akkeshi, Rebun Island, Cape Erimo	Hokkaido	Tajika (1977)	–
		Coelogynopora alata Tajika, 1981 (M)	Okushiri Island, Muroran, Harutachi	Hokkaido	Tajika (1981b)	–
	Otoplanidae	*Archotoplana abutaensis* Tajika, 1983 (M)	Abuta	Hokkaido	Tajika (1983b)	–
		Zygotoplana ezoensis Tajika, 1983 (M)	Ishikari	Hokkaido	Tajika (1983b)	–
		Archotoplana yamadai Tajika, 1983 (M)	Ishikari	Hokkaido	Tajika (1983b)	–
		Polyrhabdoplana perforata Tajika, 1983 (M)	Muroran	Hokkaido	Tajika (1983c)	–
		Notocaryoplana geminofollicularis Tajika, 1983 (M)	Ishikari, Raigishi, Muroran, Samani, Akkeshi, Habomai, Abashiri, Sawaki	Hokkaido	Tajika (1983a)	–
		Itaspiella macrostilifera Tajika, 1984 (M)	Muroran, Raigishi	Hokkaido	Tajika (1984)	–
	Nematoplanidae	*Nematoplana ciliovesiculae* Tajika, 1979 (M)	Raigishi	Hokkaido	Tajika (1979)	–
		Nematoplana pullolineata Tajika, 1979 (M)	Toya	Hokkaido	Tajika (1979)	–
	Archimonocelididae	*Tajikacelis itoi* (Tajika, 1981) Curini-Galletti & Schockaert, 2021 (M)	Uchikabuto, Satokabuto (Oshoro)	Hokkaido	Tajika (1981a)	–
Unidentified species						
Macrostomorpha	**Macrostomidae**	*Bradburia* sp.[§] (M)	Kataya Port, Miura Peninnsula (35°08'31.24"N, 139°40'14.50"E)	Kanagawa	Kobayashi (2009)	
		Macrostomum sp. (M)	Hanami Beach, Noto Peninnsula (37°17'19.09"N, 137°0'00.76"E)	Ishikawa	Kobayashiw (2009)	
Rhabdocoela	**Koinocystididae**	*Parautelga* sp. (M)	Igei (26°27'17.9"N, 127°52'27.5"E)	Okinawa	Van Steenkiste et al. (2023)	
	Schizorhynchidae	*Carcharodorhynchus* sp. (M)	Onna (26°29'05.1"N, 127°50'25.6"E)	Okinawa	Van Steenkiste et al. (2023)	

The numbers in each locality correspond to the numbers assigned in each prefecture.

[†] Gobert et al. (2021) considered the specimens from Japan to belong to a different species of *Cheliplana*, most likely *C. hawaiiensis* Gobert, Reygel, Van Steenkiste & Artois, 2021, although *C. evdonini* Karling, 1983 was also considered.

[‡] Kunashir Island is claimed by both Japan and Russia.

[§] The genus *Bradburia* is currently considered as incertae sedis by Brand et al. (2022).

species in this clade all have the combination of at least one armed cirrus and two large, heteromorphic accessory hooks (Diez et al. 2021; this study). In addition, *R. sagamianus* sp. nov. and *R. anamariae* both have a conspicuous belt consisting of overlapping lamellar spines of varying sizes in the armed cirrus. It is possible that other species of *Reinhardorhynchus* with such a combination of characters, including *R. beatrizae*, *R. curvicirrus*, *R. ruffinjonesi*, and *R. soror*, also gather in this clade, but further analyses with more dense taxon sampling are needed to confirm its synapomorphic traits.

Marine microturbellarians in Japan

Most of the species recorded in Japan have been accurately identified except for a prolecithophoran that most likely belongs to the species *Allostoma durum* (Fuhrmann, 1896) and four unidentified species of Macrostomorpha (*Bradburia*, *Macrostomum*) and Rhabdocoela (*Carcharodorhynchus*, *Parautelga*), respectively (Table 3). The putative representative of *Allostoma durum* lacks a specified collection locality in Japan based on our literature survey (Westblad 1955; Omi 2020). The four unidentified species await further description. Some microturbellarians found in Japan, including specimens likely belonging to Plagiostomidae (*Vorticeros*, unidentified genus) and Cylindrostomidae (unidentified genus) collected from Rishiri Island (Hokkaido), are not listed in Table 3 because the individuals were immature (Omi 2018). Resampling will be required for an accurate identification.

Macrostomorphs, rhabdocoels, prolecithophorans, and proseriates are the most commonly encountered microturbellarians in marine and brackish water environments around the world. It is, therefore, not surprising that all microturbellarians collected in Japan so far belong to these groups (Table 3). Other marine taxa of free-living microturbellarians, including catenulids and gnosonesimids, are rarely encountered, and while they seem to have widespread distributions, they have not yet been found in Japan. Most recorded species were collected in Hokkaido and northern Honshu as a result of the research activities conducted in these areas over the years (Evdonin 1969; Tajika 1978, 1981b, 1982a, 1982b, 1983b, 1983c; Ax 1994, 2008; Omi 2018; Takeda and Kajihara 2018; Van Steenkiste et al. 2023). Japan extends from 20° to 45° north latitude and consists of more than 14,000 islands and almost 30,000 km of coastline. This results in a wide variety of marine habitats and climatic conditions, from seasonal sea ice along the northern coasts of Hokkaido to tropical coral reefs around the atolls and islands of the Ryukyu and Ogasawara Islands. Undoubtedly, rich communities of microturbellarians are also present in these diverse but unexplored marine areas of Japan.

Only nine out of 58 species of marine or brackish water microturbellarians from Japan have also been collected in other parts of the world (Table 3). Our overview indicates that some of these species might be confined to the regional seas around Japan (*Palladia nigrescens* (Evdonin, 1971), *Multipeniata kho* Nasonov, 1927) or the Northern Pacific (*Ptychopera japonica* Ax, 2008, *Invenusta paracnida* (Karling, 1966)), while others have widespread distributions (*Cheliplana setosa* Evdonin, 1971, *Cheliplana terminalis* Brunet, 1968, *Trigonostomum vanmecheleni* Artois, Schockaert, Beenaerts & Reygel, 2013, *Allostoma durum* (Fuhrmann, 1896), *Cylindrostoma monotrochum* (von Graff, 1882)). The fact that 49 species have only been recorded from Japan does not necessarily indicate a high degree of endemism for microturbellarians in the marine areas of Japan, but rather exemplifies how little species discovery and exploration has been done in the surrounding coastal areas of the Russian Far East, the Korean peninsula, Eastern China, and the Philippines.

The scarcity of researchers focusing on various meiofaunal groups, such as microturbellarians, has been recognized as a significant challenge that needs urgent attention (Schockaert et al. 2008; Balsamo et al. 2020). Marine microturbellarians play crucial roles in the trophic dynamics of coastal marine environments (Urban-Malinga 2011; Leasi et al. 2016, 2018; Schratzberger and Ingels 2018; Martínez et al. 2019; Balsamo et al. 2020). Hence, understanding their diversity and interactions with other organisms, including prokaryotes, protists, and other micro-invertebrates, is essential for evaluating their impact on marine ecosystems along the Japanese coasts. Microturbellarians also establish symbiotic relationships with other micro-organisms. The rhabdocoel representatives of *Pogaina*, of which two species have been recorded from Japan (Table 3), are known to practice kleptoplasty by sequestering plastids from diatom prey cells (Van Steenkiste et al. 2019). Recent studies identified single-celled parasites in microturbellarians from Japan and other parts of the world as apicomplexans belonging to the genus *Rhytidocystis* (Holt et al. 2022; Van Steenkiste et al. 2023). An apicomplexan cell was also observed inside the intestine of *R. ryukyuensis* sp. nov., which was collected in the same location as *Carcharodorhynchus* sp. (Table 3), one of the host taxa in the study of Van Steenkiste et al. (2023). It is therefore likely that the apicomplexan in *R. ryukyuensis* sp. nov. also belongs to the genus *Rhytidocystis*. The effects of these interactions on the flatworm hosts and their symbionts or parasites are still largely unknown. Understanding these complex ecological relationships is crucial for addressing the challenges in environmental management and conservation in the region (Zeppilli et al. 2015).

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Supplementary material 1

Reinhardorhynchus sagamianus **sp. nov – detail of the male copulatory organ in a live animal**

Authors: Aoi Tsuyuki, Jhoe Reyes, Yuki Oya, Kevin C. Wakeman, Brian S. Leander, Niels W. L. Van Steenkiste

Data type: mov

Link: https://doi.org/10.3897/zse.100.120244.suppl1

Supplementary material 2

Reinhardorhynchus sagamianus **sp. nov – detail of the male copulatory organ in a live animal**

Authors: Aoi Tsuyuki, Jhoe Reyes, Yuki Oya, Kevin C. Wakeman, Brian S. Leander, Niels W. L. Van Steenkiste

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Supplementary material 3

Reinhardorhynchus sagamianus **sp. nov – detail of the male copulatory organ in a live animal**

Authors: Aoi Tsuyuki, Jhoe Reyes, Yuki Oya, Kevin C. Wakeman, Brian S. Leander, Niels W. L. Van Steenkiste

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