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A new species of *Pseudoacanthocephalus* (Acanthocephala: Pseudoacanthocephalidae) from Sado Island, Japan, with a report of its accidental occurrence in a marine elasmobranch

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Abstract: Three species of amphibian-parasitic acanthocephalans belonging to the genus *Pseudoacanthocephalus* Petrochenko, 1958 have been recorded in Japan. However, only a limited number of localities have been surveyed, and the distribution of these congeners across other regions of Japan remains unclear. In this study, acanthocephalans of the genus *Pseudoacanthocephalus* were recovered from both definitive (*Rana ornativentris* Werner – type host, and *Zhangixalus arboreus* [Okada et Kawano]) and accidental hosts (*Scyliorhinus torazame* [Tanaka]) from Sado Island, Japan. Morphological analyses indicate that those specimens represent a new species, *Pseudoacanthocephalus sadoensis* sp. n., which may be endemic to the island. Additionally, our report of the incidental occurrence of this species in a marine elasmobranch represents the first documented case of a *Pseudoacanthocephalus* species found in a marine host.

Keywords: stomach contents, molecular phylogeny, endemic species, Sea of Japan, amphibian parasites

The palaeacanthocephalan genus *Pseudoacanthocephalus* Petrochenko, 1958 currently comprises 26 valid species (Gibson and Wayland 2025), which parasitise the alimentary canals of amphibians and reptiles (Amin et al. 2008, Arredondo and Gil de Pertierra 2009, Bush et al. 2009, Tkach et al. 2013, Smales et al. 2020, Zhao et al. 2024). In Japan, three congeners have been recorded: *Pseudoacanthocephalus bufonis* (Shipley, 1903), *P. lucidus* (Van Cleave, 1925), and *P. toshimai* Nakao, 2016. *Pseudoacanthocephalus bufonis* has been reported from the Nansei Islands (Nagasawa and Yoshida 2017), while *P. lucidus* has been documented in the former Shimousa Province (now northern Chiba and western Ibaraki Prefectures) (Van Cleave 1925) and across central and western Honshu (Nakao 2016). *Pseudoacanthocephalus toshimai* has been found in Hokkaido (Nakao 2016, Nakao and Sasaki 2021) and northern Honshu (Nakao and Ishigoka 2021). Of these, *P. lucidus* and *P. toshimai* were originally described in Japan.

Additionally, *Acanthocephalus artatus* Van Cleave, 1925 was initially established based on specimens from the former Shinano Province (now Nagano Prefecture) (Van Cleave 1925). However, it was later synonymised with *P. lucidus* by Yamaguti (1939). Although Van Cleave (1925) suggested that *A. artatus* and *P. lucidus* could be distinguished by subtle morphological differences, such as egg size, this distinction was ultimately rejected in subsequent studies (Harada 1935, Yamaguti 1939, Golvan 1994, Amin 2013, Nakao 2016). While biogeographical studies on these species have been conducted (Nakao 2016, Nakao and Ishigoka 2021, Nakao and Sasaki 2021) and occurrences documented (Nagasawa and Yoshida 2017, Nagasawa and Nakamura 2018, Nakao and Sasaki 2021), research has been geographically limited. Most

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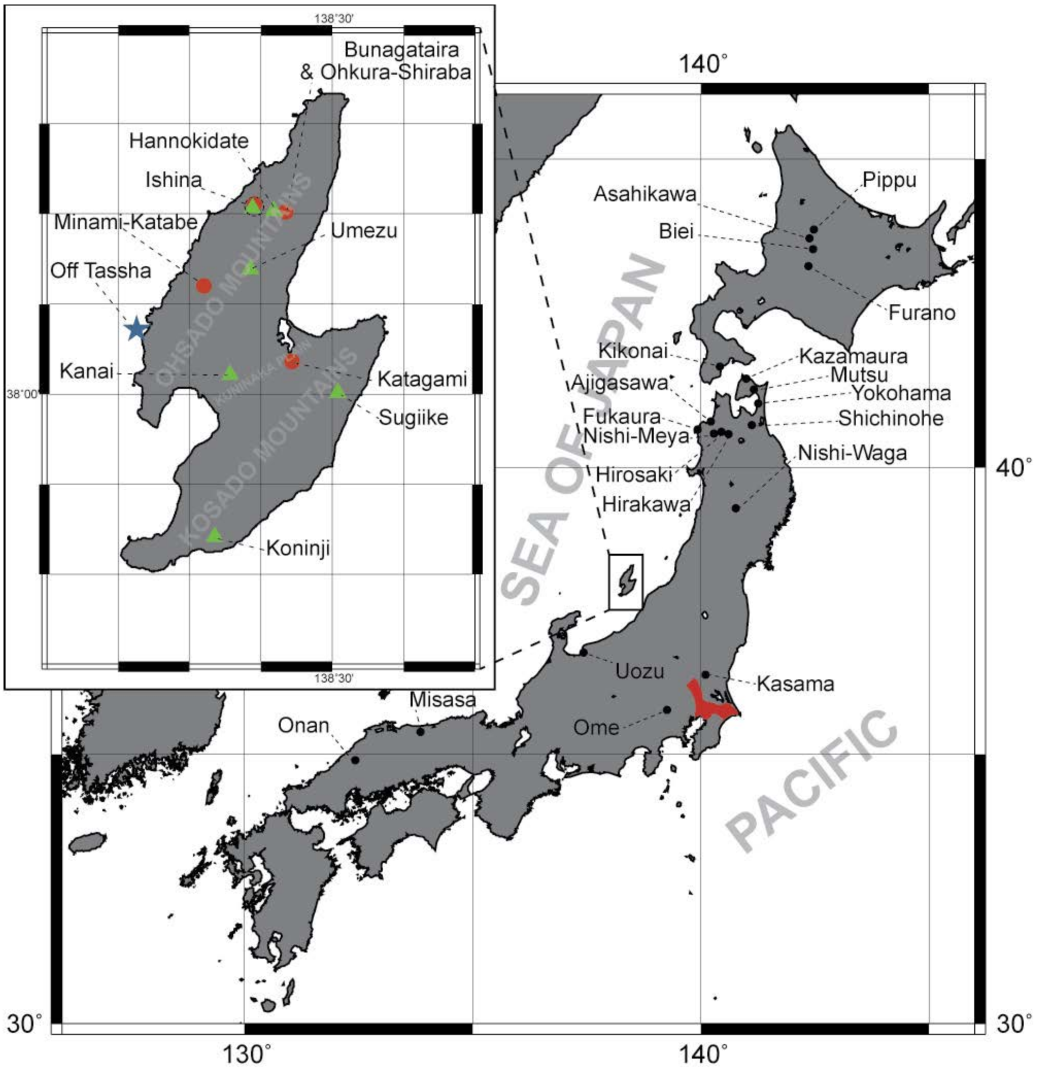

Fig. 1. Map of sampling sites on Sado Island (inset) and those from previous studies in Japan. Red circles: sampling sites of *Rana ornativentris* Werner in this study. Green triangles: sampling sites of *Zhangixalus arboreus* (Okada et Kawano) in this study. Blue star: sampling site of *Scyliorhinus torazame* (Tanaka) in this study. Black points: sampling sites of previous studies. The red-painted region shows the area of Shimousa Province, the type locality of *Pseudoacanthocephalus lucidus* Van Cleave, 1925.

studies focus on specific regions, such as Hokkaido, northern Honshu, the Nansei Islands, and a few scattered locations in Honshu.

The present study describes a new species of *Pseudoacanthocephalus* from Sado Island (Niigata, Japan), which has not previously been surveyed for terrestrial acanthocephalan fauna. Additionally, we report an accidental occurrence of this species in a marine elasmobranch collected off Sado Island. This represents the first recorded occurrence of a *Pseudoacanthocephalus* species in a marine fish.

MATERIALS AND METHODS

Overview of specimens

In total, 60 acanthocephalan specimens were collected from 37 individual frogs representing two species and from a single shark. Of the 60 acanthocephalans, 26 (15 from frogs, 11 from the shark) were flattened between glass slides and coverslips in either 70% or 99% ethanol, while 34 (all from frogs) were fixed in 99% ethanol without flattening.

Of the 26 flattened specimens, 13 (12 from frogs and 1 from the shark) were selected for morphological description based on their condition. One of the 10 remaining flattened specimens from the shark was used for DNA extraction, whereas the other 12 specimens (3 from frogs and 10 from the shark) were deemed unsuitable for taxonomic purposes due to the proboscis not protruding during fixation. Additionally, one of the 12 flattened specimens from frogs selected for morphological description was partially used for DNA extraction.

Of the 34 non-flattened specimens preserved in 99% ethanol from frogs, 11 were subjected to molecular analysis, one was examined using a scanning electron microscope (SEM), and the remaining 22 specimens were retained in YK's private collection.

Survey of frogs

Six specimens of the montane brown frog *Rana ornativentris* Werner (Ranidae) and 31 specimens of the forest green tree frog *Zhangixalus arboreus* (Okada et Kawano) (Rhacophoridae) were collected from various sites on Sado Island during the early summer between 2021 and 2024 (Fig. 1, Table 1). The captured

Table 1. A list of the host individuals examined in this study and obtained acanthocephalans.

Host species	Date	Number of host individuals	Number of obtained acanthocephalans	ICHUM catalogue number: sex	Locality
Scyliorhinus torazame (Elasmobranchii: Scyliorhinidae)	16 March 2022	1	11	8971-1–4: female 8972: female 8973: female 8974: female 8975-1–4: female	Off Tassha
Rana ornativentris (Amphibia: Ranidae); type host	28 June 2024	1	2	8965: male	Bunagataira
	14 June 2024	1	11	8967-1: male 8967-2: female	Minami-Katabe
	18 June 2024	1	2	8966: female	Katagami
	30 July 2024	1	11	8964-1–2: female 8968-1: male 8968-2: female	Ohkura-Shiraba
	25 August 2024	1	9	8962-1: male (holotype) 8962-2: male 8962-3: female	Ishina
	28 August 2024	1	12	8963-1: male 8963-2: female	Ishina
Zhangixalus arboreus (Amphibia: Rhacophoridae)	22 June 2021	3	1	8970: male	Sugiike
	20 June 2021	2	0	–	Kanai
	20 June 2021	5	0	–	Umezu
	23 June 2021	9	1	8969: female	Hannokidate
	17 June 2021	6	0	–	Ishina
	19 June 2021	6	0	–	Koninji

frogs were euthanised by deep freezing, identified based on Matsui (2018), and dissected at the Marine Biological Station, Sado Island Centre for Ecological Sustainability, Niigata University (Sado, Japan), and the Graduate School of Science, Hokkaido University (Sapporo, Japan). Among the 49 acanthocephalans obtained (47 from *R. ornativentris*, 2 from *Z. arboreus*), 34 specimens from *R. ornativentris* were preserved in 99% ethanol for DNA extraction and SEM study. One specimen from *Z. arboreus* was flattened in 70% ethanol, and a part of its body was cut and stored in 99% ethanol for DNA extraction. The remaining 14 specimens (13 from *R. ornativentris* and 1 from *Z. arboreus*) were flattened in 70% ethanol.

Survey of the cloudy catshark and its stomach contents

One individual of the cloudy catshark *Scyliorhinus torazame* (Tanaka) (Scyliorhinidae) was caught in a fixed net off Tassha, Sado Island (Niigata, Japan) in the Sea of Japan on 16 March 2022 (Fig. 1, Table 1). The species identification was based on Yoshino et al. (2013). Upon examination of the stomach contents, 11 mature, non-encysted acanthocephalans were collected. Each of these specimens was flattened in 99% ethanol using a glass slide and a cover slip with slight pressure for morphological observation. One of the specimens was used for DNA extraction.

In addition to the acanthocephalan specimens, several vertebrae were retrieved from the stomach contents. These vertebrae were stored in 99% ethanol for subsequent DNA analysis.

Morphological observations

The methods of morphological observations largely followed those described by Kita and Kajihara (2021). Twenty-five flattened specimens (13 from *R. ornativentris*, 2 from *Z. arboreus* and 10 from *S. torazame*) were stained with Heidenhain's iron haematoxylin, dehydrated through a graded ethanol series, cleared with xylene, and mounted on glass slides using Canada balsam diluted with xylene. Unless otherwise noted, measurements are given in micrometres. Voucher specimens were deposited in the Invertebrate Collection of Hokkaido University Museum (ICHUM), Sapporo, Japan, under the catalogue numbers 8962–8975.

For observation by SEM, one specimen in 99% ethanol was air-dried and mounted on an aluminium stub with double-sided tape. The stub with the specimen was coated with gold in E-1045 ion sputter coater (Hitachi, Japan) and observed with SEM S-3000N (Hitachi, Japan) at 15 kV acceleration voltage.

Molecular phylogenetic study

In total, 13 specimens of acanthocephalans (11 from *R. ornativentris*, 1 from *Z. arboreus* and 1 from *S. torazame*) were analysed. The methods for DNA extraction, amplification of cytochrome *c* oxidase I (COI) and 18S rRNA (18S) genes, and direct sequencing followed Kita et al. (2023). The obtained sequences have been deposited in the International Nucleotide Sequence Database Collaboration (INSDC) via the DNA Data Bank of Japan (DDBJ) under accession numbers LC853414–LC853427.

For molecular phylogenetic analysis, we used COI sequences, while the 18S sequence was not used due to the limited availability of other 18S sequences in public databases. Additional COI sequences were retrieved from GenBank (Table 2). The primary analytical methods, including sequence alignment, trimming of ambiguous sites and phylogenetic tree reconstruction, followed Kita et al. (2024). The final COI dataset had a length of 480 bp. The edited sequence dataset was concatenated using MEGA ver. 7 (Kumar et al. 2016), resulting in a final dataset comprising 79 terminal taxa.

Phylogenetic trees were reconstructed using both the maximum-likelihood (ML) method and Bayesian inference (BI). For ML analysis, IQ-TREE ver. 2.3.6 (Minh et al. 2020) was used under a partition model (Chernomor et al. 2016). For BI analysis, MrBayes ver. 3.2.7 (Ronquist et al. 2012) was employed. The optimal substitution models and partition schemes were determined following Kita and Kajihara (2021). The selected models were GTR + I + G for the first and the second codon positions and GTR + G for the third codon position. Branch support values in the ML tree were assessed using 1,000 pseudoreplicates with ultrafast bootstrap approximation (Hoang et al. 2018).

In BI analysis, the Markov chain Monte Carlo process was initiated with a random tree and ran for 1,000,000 generations

Table 2. A list of using DNA partial sequences of COI for the reconstruction of the molecular phylogenetic tree, with the accession number of DDBJ/EMBL/GenBank for reconstruction of molecular phylogenetic trees.

Taxon	COI	References
Paracanthocephalidae		
Acanthocephalus clavula	AM039866	Benesh et al. (2006)
Acanthocephalus lucii	MT682949	Benesh et al. (2006)
Pseudoacanthocephalidae		
Pseudoacanthocephalus bufonis	MZ958236; PP477056	Zhao et al. (2013); Zhao et al. (2024)
Pseudoacanthocephalus lucidus	LC100057– LC100069; LC790532	Nakao (2016); Ansai et al. (2025)
Pseudoacanthocephalus nguyenthileae	PP476963– PP476964	Zhao et al. (2024)
Pseudoacanthocephalus previatesticulus	PP481149	Zhao et al. (2024)
Pseudoacanthocephalus sichuanensis	PP469226– PP469227	Zhao et al. (2024)
Pseudoacanthocephalus toshimai	LC100044– LC100056; LC515228– LC515234; LC601914– LC601937	Nakao (2016); Nakao and Sasaki (2021); Nakao and Ishigoka (2021)
***Pseudoacanthocephalus sadoensis* sp. n.**	LC853414– LC853426	This study
Pseudoacanthocephalus sp.	OP680759	Liu et al. (unpublished)

across two parallel runs, each consisting of four chains. The first 25% of the samples were discarded as burn-in. Convergence was evaluated based on the average standard deviation of split frequencies (0.016717) and the potential scale reduction factors for all parameters (1.000). Additionally, genetic distances (*p*-distance and Kimura's 2-parameter [K2P]) for COI (446 bp) were calculated using MEGA ver. 7 (Kumar et al. 2016).

DNA barcoding of an unknown organism using DNA extracted from the vertebrae

The largest vertebrae fragment extracted from the stomach of the cloudy catshark consisted of a portion of the skull and vertebral column. DNA was extracted from the spinal cord remaining within the vertebral column using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). To amplify partial sequences of the 12S rDNA (12S), 16S rDNA (16S) and COI, three sets of primers were used: MiFish-U-F (5′-GTCGGTAAAACTCGTGCCAGC-3′) in conjunction with MiFish-E-R (5′-CATAGTGGGGTATCTAATCCTAGTTTG-3′) (Miya et al. 2015); 16S rDNA forward (5′- CGCCTGTTTAACAAAAACAT -3′) paired with 16S rDNA reverse (5′-CCGGTCTGAACTCAGATCATGT-3′) (Bouchon et al. 1994); and FishF1 (5′-TCAACCAACCACAAAGACATTGGCAC-3′) with FishR1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) (Ward et al. 2005). PCR amplification was performed in a 15.0 µl volume containing 7.5 µl of KOD One PCR Master Mix (Toyobo, Osaka, Japan), 0.45 µl of each 10 µM primer, 1.0 µl of extracted DNA, and 5.6 µl of distilled water.

The PCR cycling protocol included an initial denaturation at 98°C for 10 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 48°C for 5 s, extension at 68°C for 10 s, and a final extension at 68°C for 60 s. The resulting PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, Cleveland, USA) and sequenced commercially (Azenta Life Sciences, Tokyo, Japan) with the same primers used for PCR. The obtained sequences and electropherograms were manually reviewed and edited utilising MEGA ver. 11 (Tamura et al. 2021). The sequences were then submitted to the DDBJ and compared with existing sequences in the INSDC via a BLASTn search (www.ncbi.nlm.nih.gov/) conducted on 2 December 2024. The specimen was deposited in the ICHUM under the catalogue number 8976. The accession numbers of the three sequences registered in the INSDC through the DDBJ are LC853428–LC853430.

RESULTS

Morphological observation

The field survey revealed that acanthocephalans were present in all examined individuals of *Rana ornativentris* (n = 6: intensity 1–10), whereas they were found in only two individuals of *Zhangixalus arboreus* (n = 31: intensity 0–1). The acanthocephalan specimens obtained from frogs exhibited the following morphological characteristics: (i) a cylindrical to conical proboscis; (ii) an aspinose trunk; (iii) six filiform to fusiform cement glands. Based on these morphological traits and DNA barcoding of the vertebrae samples from the shark, we identified the acanthocephalans as a congener of *Pseudoacanthocephalus*.

Additionally, specimens from the two species of frogs were genetically and morphologically identical to those obtained from the shark. However, comparison with other members of *Pseudoacanthocephalus* revealed that the morphological characteristics of our specimens did not match those of any known species in the genus. Therefore, we conclude that these specimens represent an undescribed species, which we describe herein as a new species. A detailed description and taxonomic information are provided below.

Order Echinorhynchida Southwell et Macfie, 1925

Family Pseudoacanthocephalidae Petrochenko, 1958

Genus *Pseudoacanthocephalus* Petrochenko, 1958

Remarks. The two names Pseudoacanthocephalidae and *Pseudoacanthocephalus* were introduced without diagnoses (Petrochenko 1956: 160) and were *nomina nuda* at that time. These names became available when Petrochenko (1958) provided a detailed diagnosis of the family and the genus. Therefore, the date of publication of these names is 1958 instead of 1956.

Pseudoacanthocephalus sadoensis sp. n.

ZooBank number for species:
urn:lsid:zoobank.org:act:5068EFBC-DD37-405E-A337-17A4D1CA57F9

Holotype: ICHUM 8962-1 (male).
Paratype: ICHUM 8962 (2 males and 1 female), 8963 (1 male and 1 female), 8964-1 (1 female), 8965 (1 male), 8967 (1 male and 1 female), 8968 (1 male and 1 female), 8970 (1 male), and 8974 (1 female).
Type locality: Ishina (38.2111N, 138.3911E) (Sado, Niigata, Japan).

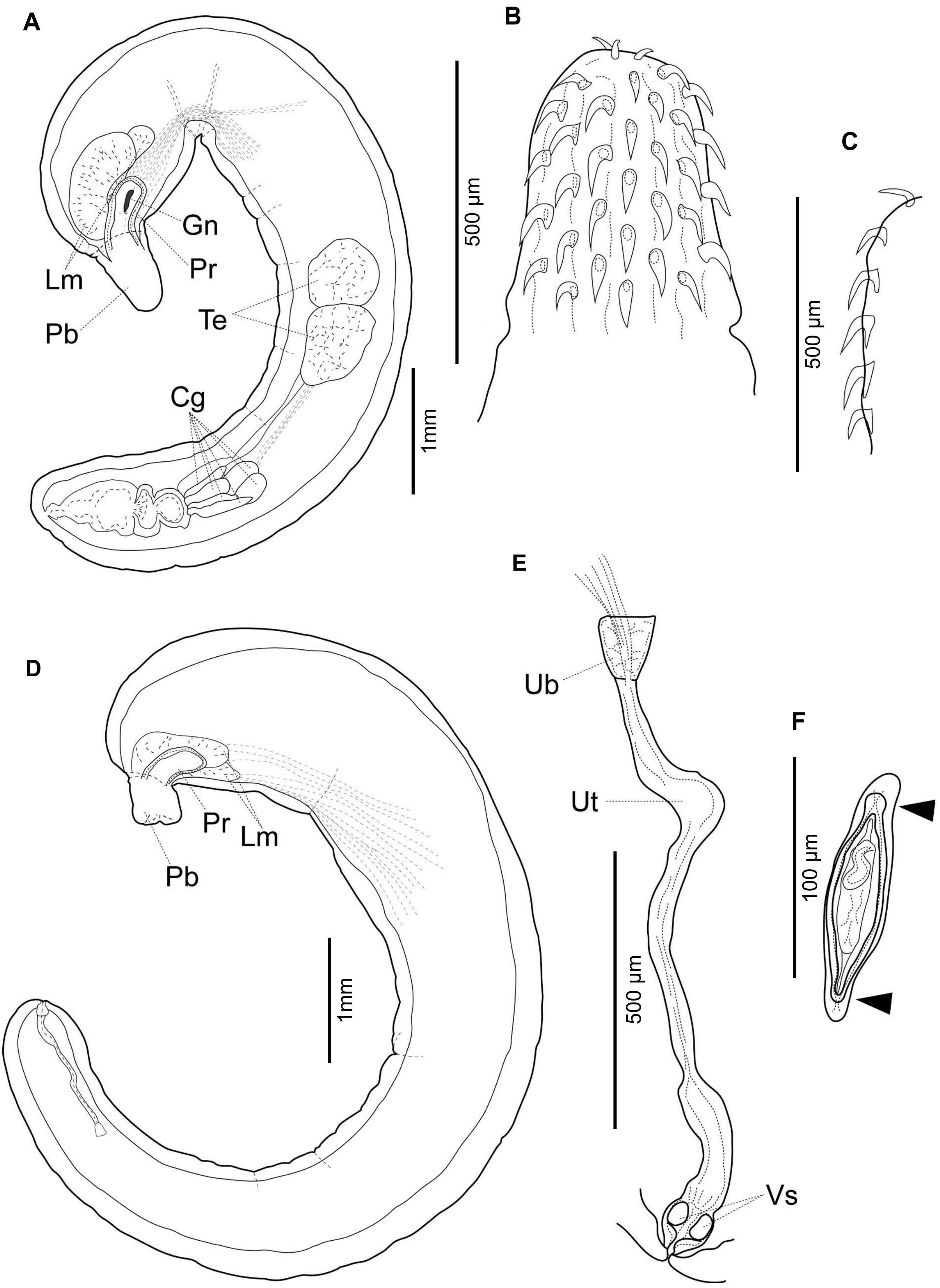

Fig. 2. Line drawings of *Pseudoacanthocephalus sadoensis* sp. n. from *Rana ornativentris* Werner, Japan. Arrowheads indicate polar prolongation of egg. **A** – whole male body (ICHUM 8962); **B** – proboscis (ICHUM 8962); **C** – a hook row (ICHUM 8962); **D** – whole body of female (ICHUM 8964); **E** – genital organs of female (ICHUM 8964); **F** – egg (ICHUM 8964). *Abbreviations*: Cg – cement glands; Gn – cephalic ganglion; Lm – lemnisci; Pb – proboscis; Pr – proboscis receptacle; Te – testes; Ub – uterine bell; Ut – uterus; Vs – vaginal sphincter.

Other localities: Bunagataira (Sado, Japan); Hannokidate (Sado, Japan); Katagami (Sado, Japan); Minami-Katabe (Sado, Japan); off Tassha (Sado, Japan: accidental); Ohkura-Shiraba (Sado, Japan); Sugiike (Sado, Japan).

Type host: *Rana ornativentris* Werner (Ranidae).

Other hosts: *Zhangixalus arboreus* (Okada et Kawano) (Rhacophoridae); *Scyliorhinus torazame* (Tanaka) (Scyliorhinidae: accidental).

Description: General (n = 14, including SEM specimen). Trunk cylindrical, aspinose, slightly swollen in anterior one-fourth to middle. Proboscis cylindrical to conical, armed with 18–19 longitudinal rows (n = 8) of 6–7 hooks (n = 3) (Figs. 2B,C, 3B). Size of hooks gradually increasing toward middle of row: female hooks relatively larger than male. Proboscis receptacle short, double-walled. Cephalic ganglion positioned middle of proboscis receptacle. Lemnisci longer than receptacle, broad.

Male (n = 7). Trunk cylindrical, 4.6–7.4 mm (average 6.4 mm) × 1.1–1.3 mm (1.3 mm) (Fig. 2A). Proboscis cylindrical to conical, 500–550 (530) (n = 2) × 280–400 (350) (n = 5) (Fig. 2A–C). Size of hooks gradually increasing toward middle of row: hook blade 30–40 (40) in anterior first and second hooks, 50–60 (54) in third hook, 50–70 (63) in fourth hook; 60–70 (67) in fifth hook; and 40–70 (55) in sixth hook; hook root 10–20 (16) in first and second hooks; 20–30 (27) in third hook; 20–40 (35) in fourth hook; 30–40 (40) in fifth hook; and 20–30 (31) in sixth hook. Proboscis receptacle short, double-walled, 400–770 (559) × 230–370 (291) (Fig. 2A). Lemnisci longer than receptacle, broad, 600–1,130 (843) in length (Fig. 2A). Testes globular to oval, tandem (Fig. 2A). Anterior testis 380–630 (483) × 280–540 (430). Posterior testis 360–650 (510) × 240–470 (388). Cement glands six, clustered, filiform to fusiform (Figs. 2A, 3D). Saefftigen's pouch 400–680 (558) in length. Copulatory bursa opening subterminally, 440–540 (497) (n = 2) in width (Fig. 2B).

Female (n = 6). Trunk cylindrical, 5.1–9.7 mm (7.9 mm) × 1.1–1.3 mm (1.3 mm) (Fig. 2D). Proboscis cylindrical, 260–430 (336) (n = 5) in width (Fig. 3D) (not fully extended). Size of hooks gradually increasing toward middle of row: hook blade 50–70 (60) in third hook, 60–80 (74) in fourth hook; 70–80 (77) in fifth hook; and 50–70 (61) in sixth hook; hook root 20–30 (25) in third hook; 30–40 (36) in fourth hook; 30–40 (40) in fifth hook; and 20–40 (32) in sixth hook. Proboscis receptacle short, double-walled, 290–1,000 (561) × 190–340 (248) (Fig. 2D). Lemnisci longer than receptacle, broad, 560–820 (735) (n = 3) in length (Fig. 2D). Uterus 940–1,100 (1,014) in length (Fig. 2E). Vaginal sphincter single. Eggs elliptical with polar prolongation, 80–100 (96) (n = 10) in length (Figs. 2F, 3C).

Etymology. The new specific name is an adjective, after the type locality, Sado Island.

Remarks. Based on the key to species provided by Zhao et al. (2024) and the original descriptions of all congeners, the new species is distinguished within the genus by (i) the

Table 3. Morphological comparison among *Pseudoacanthocephalus* species. The characteristics similar to *P. sadoensis* sp. n. are in bold type.

Taxon	Number of hook rows	Number of hooks per row	Proboscis shape	Number of cement glands	Eggs polar prolongation	References
P. betsileo	16	**6–7**	**cylindrical**	**6**	absent	Golvan (1969)
P. bigueti	14	4	**conical, cylindrical**	**6**	absent	Golvan (1969)
P. bufonicola	16	**6–7**	**cylindrical**	**6**	absent	Golvan (1969)
P. bufonis	**16–20**	**3–5**	**cylindrical**	**5–6**	absent	Golvan (1969); Zhao et al. (2013)
P. caucasicus	22 (male); 22–24 (female)	**6–7**	**cylindrical**	**6**	**present**	Golvan (1969)
P. caspanensis	**18–19**	**6–7**	**cylindrical**	**5–6**	absent	Fernández and Ibarra Vidal (1992)
P. coniformis	13	7	**cylindrical**	8	–*	Amin et al. (2014)
P. elongatus	16	13–15	elongate	–**	–**	Van Cleave (1937)
P. goodmani	**14–18**	**4–6**	**cylindrical**	**6**	absent	Smales et al. (2020)
P. lucidus	12–16	**4–5**	**cylindrical**	**6**	**present**	Nakao (2016)
P. lutzi	**14–18**	**5–7**	**cylindrical**	**4–6**	absent	Arredondo and Gil de Pertierra (2009)
P. nguyenthileae	**15–19**	**5–6**	**cylindrical, rounded anteriorly**	8	absent	Amin et al. (2008)
P. nickoli	**14–18**	**4–5 (male); 4–6 (female)**	**cylindrical**	**5–7**	absent	Tkach et al. (2013)
P. perthensis	12–14	**4–5**	subcylindrical to ovoid	**6**	absent	Edmonds (1971)
P. previatesticulus	**16–18**	**4–5**	**cylindrical**	**6**	absent	Zhao et al. (2024)
P. rauschi	20–22	10–11	oval	8	not mentioned	Gupta and Fatma (1986)
P. reesei	12–15	**4–5**	**cylindrical**	**6**	absent	Bush et al. (2009)
P. rhampholeontos	14	3–4 (male); 3 (female)	**conical**	not mentioned	absent	Smales (2005)
***P. sadoensis* sp. n.**	**18–19**	**5–6**	**conical, cylindrical**	**6**	**present**	This study
P. shillongensis	14	**5**	not mentioned	**6**	absent	Bhattacharya (2007)
P. sichuanensis	**18–20**	**4–6**	**cylindrical**	**6**	absent	Zhao et al. (2024)
P. smalesae	11–13 (male); 12–13 (female)	**3–5 (male); 5–6 (female)**	**conical, cylindrical**	4	absent	Tkach et al. (2013)
P. toshimai	14–16	**4–5**	**cylindrical**	**6**	**present**	Nakao (2016)
P. xenopeltidis	8–12	**–*** **	not mentioned	not mentioned	not mentioned	Golvan (1969)

* Data of female unavailable ** Data based on immature worm ***Could not be observed due to the condition of the type materials

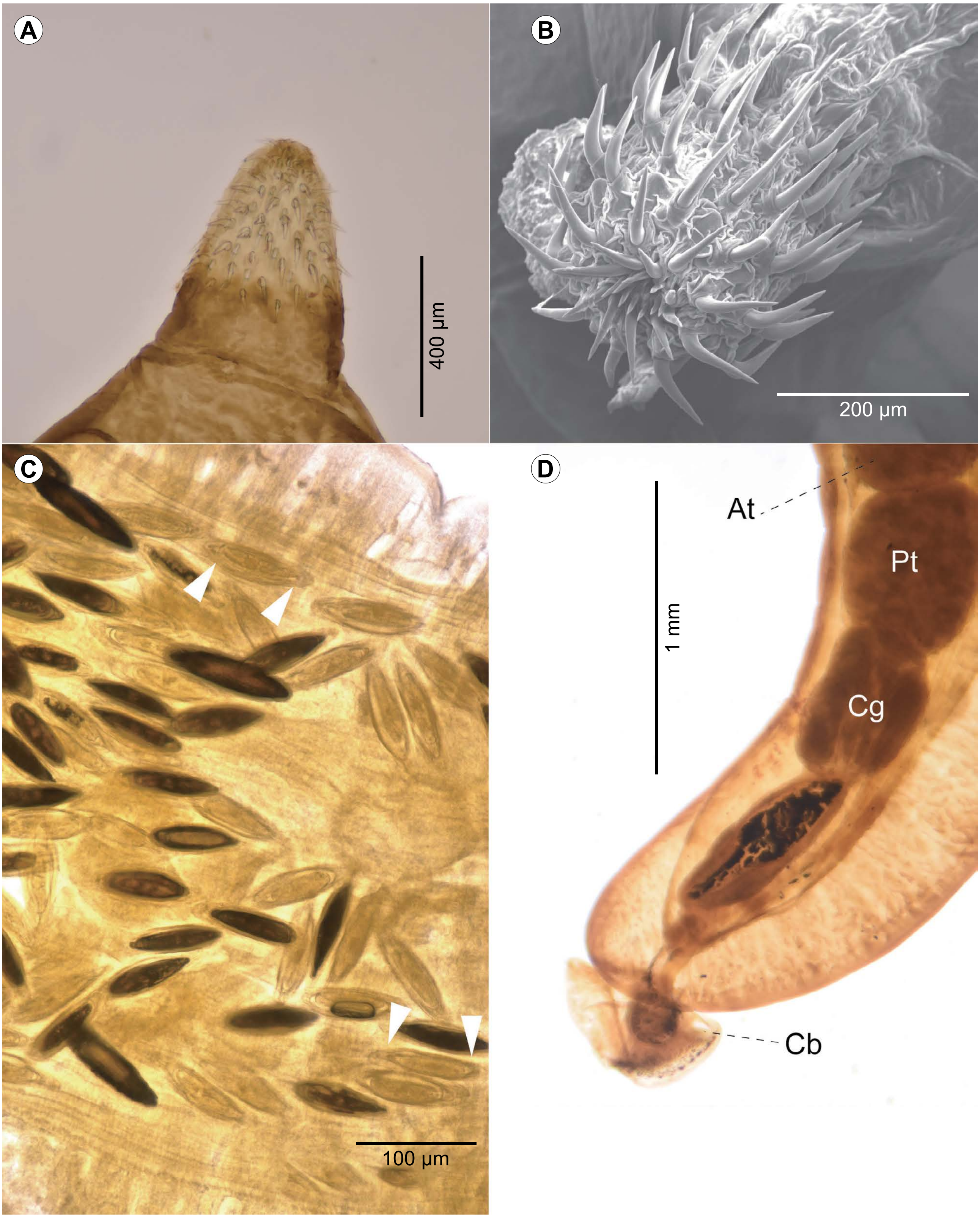

Fig. 3. SEM image and microphotographs of *Pseudoacanthocephalus sadosensis* sp. n. from *Rana ornativentris* Werner, Japan, or *Scyliorhinus torazame* (Tanaka), Japan. Arrowheads indicate polar prolongation of egg. **A** – proboscis of female specimen (ICHUM 8974) from *S. torazame;* **B** – SEM image of proboscis viewed from the anterior end; **C** – eggs of female specimen (ICHUM 8971) from *S. torazame*; **D** – posterior end of male (ICHUM 8962). *Abbreviations*: At – anterior testis; Cb – copulatory bursa; Cg – cement glands; Pt – posterior testis.

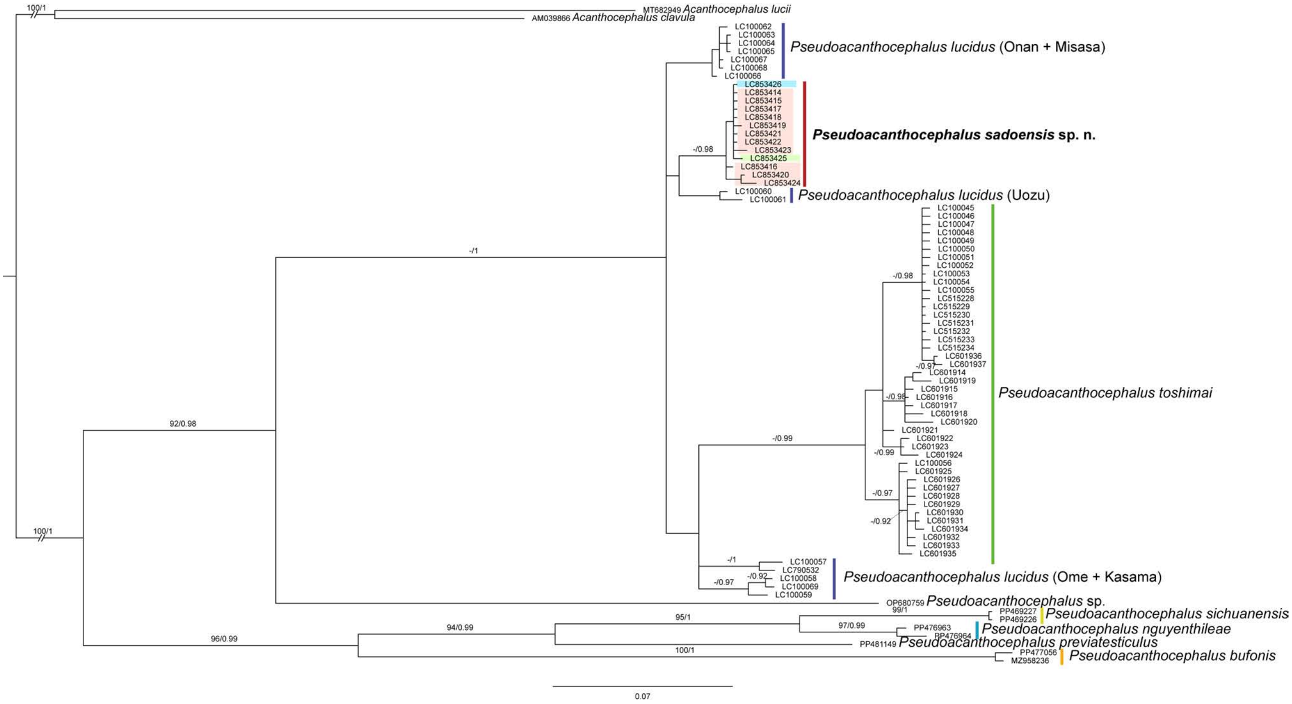

Fig. 4. Bayesian inference phylogenetic tree of species of *Pseudocacanthocephalus* Petrochenko, 1958 based on COI. Branching support values are indicated as numbers near nodes (ML bootstrap value/BI posterior probability); BI posterior probabilities less than 0.90 were not shown; nodes with hyphens in place of ML bootstrap value denote that the topology differed between the ML and BI trees.

arrangement of proboscis hooks, (ii) the number of cement glands, and (iii) the presence of eggs with polar prolongation (Table 3). We can distinguish *Pseudoacanthocephalus sadoensis* sp. n. from other congeners by the presence of polar prolongation in the eggs, except *P. caucasicus* (Petrochenko, 1953), *P. lucidus* and *P. toshimai*. These three species differ from *P. sadoensis* sp. n. in the proboscis hook arrangement: *P. sadoensis* sp. n. possesses 18–19 hook rows, compared to 12–16 rows in *P. lucidus* and *P. toshimai*, and 22–24 in *P. caucasicus*. In addition, we found that the proboscis hooks in females were relatively larger than those in males. Such sexual dimorphism has not been observed in previous studies of *P. lucidus* or *P. toshimai* (Van Cleave 1925, Nakao 2016), the closest relatives of *P. sadoensis* sp. n.

DNA barcoding of the unknown organism using DNA extracted from the vertebrae

For DNA barcoding, sequences of 12S (171 bp; LC853429), 16S (537 bp; LC853430) and COI (620 bp; LC853428) were obtained from the unidentified vertebrae found in the shark's stomach. A BLASTn analysis of the 12S fragment returned an identical match to *R. ornativentris*, with 100% query cover (AB058857 – Sumida et al. 2003). Similarly, the closest matches for the newly generated 16S and COI sequences were also *R. ornativentris*, with 100% query cover. The 16S sequence showed 99.07% similarity to KX269187 (Yuan et al. 2016), AB058875 (Sumida et al. 2003) and LC014157 (Igawa et al. 2015), while the COI sequence exhibited 97.42% similarity to LC682587 (unpublished data). Based on these results, the vertebrae found in the shark's stomach was identified as *R. ornativentris*.

Genetic distances

The genetic distances (COI; length 446 bp) between our specimens and *P. lucidus* ranged from 0.031 to 0.058 (*p*-distance) and from 0.032 to 0.061 (K2P) (Supplementary material, Table S1). In contrast, the genetic distances between our specimens and *P. toshimai* were higher, ranging from 0.081 to 0.108 (*p*-distance) and from 0.084 to 0.118 (K2P). Additionally, the genetic distances between specimens obtained from the shark and those from the two species of frogs ranged from 0 to 0.018 (*p*-distance and K2P), indicating close genetic similarity.

Molecular phylogenetic analysis

In our molecular phylogenetic analysis based on COI, the sequences of *P. sadoensis* sp. n. formed a monophyletic group with high support (Fig. 4). In contrast, the sequences of *P. lucidus* were recovered as polyphyletic, although their support values were low.

DISCUSSION

To determine whether *Pseudoacanthocephalus sadoensis* sp. n. is endemic to Sado Island, further surveys are required in the surrounding areas of Honshu. Among documented records of *Pseudoacanthocephalus* species, including *P. lucidus*, the closest relative of *P. sadoensis* sp. n., the nearest known locality to Sado Island is Uozu (middle Honshu), approximately 160 km away (Fig. 1) (Nakao 2016). Currently, no records of this genus exist from regions closer to Sado, such as Niigata City. *Pseudoacanthocephalus sadoensis* sp. n. primarily parasitises *Rana ornativentris*, a frog species also found on the Japanese mainland (Matsui 2018). However, we did not examine other frog species inhabiting Sado Island, such as *Glandirana susurra* (Sekiya, Miura et Ogata), an anuran species endemic to the island (Sekiya et al. 2012). In total, seven species of frogs are believed to inhabit the island (Sado Geopark Promotion Council 2022).

To accurately determine the distribution of *P. sadoensis* sp. n., it is essential to identify which frog species on Sado Island serve as hosts and to investigate *Pseudoacanthocephalus* species in unexplored regions of Honshu. Furthermore, phylogenetic analyses conducted in this study indicate that *P. sadoensis* sp. n. is most closely related to the *P. lucidus* population from Uozu (Fig. 4). The genetic distances (COI) among *P. sadoensis* sp. n. and *P. lucidus* from Uozu were 0.031–0.040 (*p*-distances) and 0.032–0.042 (K2P).

Although previous studies have not clearly defined threshold values for COI-based species delimitation within this genus, data from other acanthocephalan genera – e.g., approximately 2% in *Rhadinorhynchus* Lühe, 1911 (Kita et al. 2024) and 5% in *Echinorhynchus* Zoega in Müller, 1776 (Wayland et al. 2015) – suggest that these values fall within the range typically considered interspecific divergence. However, the genetic diversity within *P. lucidus* is also high: COI genetic distances range from 0.000 to 0.065 (Supplementary Table S1), and this species is divided into three distinct clades in the phylogenetic analysis (Fig. 4).

Considering the type locality of *P. lucidus* is Shimousa Province, the Ome + Kasama clade may represent topotypic populations. In contrast, the Uozu clade and the Onan + Misasa clade may indicate the presence of cryptic species. Unfortunately, Nakao (2016), who determined these sequences, examined only the morphology of the Ome population, and no voucher specimens were deposited except for those from Ome.

Therefore, it remains unclear whether specimens from other localities truly share the same morphological characteristics as *P. lucidus*. Future research should incorporate morphological and genetic data from *Pseudoacanthocephalus* populations on and off Sado Island to elucidate the species' distribution and boundary. A combined morphological and molecular approach would be instrumental in achieving this goal.

The difference in the prevalence of *P. sadoensis* sp. n. between *R. ornativentris* and *Zhangixalus arboreus* may reflect the difference in predation pressure between the two species on intermediate host organisms. In *P. lucidus* and *P. toshimai*, intermediate hosts have been identified as terrestrial isopods of the genus *Ligidium* Brandt (Nakao 2016, Ansai et al. 2025).

There are no available records of terrestrial isopods on Sado Island, particularly in the inland woodlands inhabited by *R. ornativentris* and *Z. arboreus*. However, similar terrestrial isopods or ground insects likely serve as intermediate hosts for *P. sadoensis* sp. n., which can be inferred to some extent from the feeding habits of *R. ornativentris* and *Z. arboreus*. *Rana ornativentris* primarily feeds on the forest floor, mainly on ground insects and isopods (Matsui 2018), while *Z. arboreus* feeds mainly on flying insects in the trees (Toda and Kusano 2013).

Furthermore, the female acanthocephalans discovered in the shark were already gravid rather than encysted (cystacanths), supporting the hypothesis that the shark ingested a dead frog in the water. Additionally, *Scyliorhinus torazame* is a generalist feeder (Park et al. 2019), making it reasonable to assume that the shark consumed a dead frog, either floating or resting on the bottom, without distinguishing it from other prey. Ultimately, *P. sadoensis* sp. n. cannot be considered a true parasite of *S. torazame* because it did not reach the intestine. However, unexpected host infections can occur through accidental events such as those described above (Nagasawa 2014, Nakao and Sasaki 2021). If *P. sadoensis* sp. n. were able to establish a viable life cycle in sharks, it could serve as a driver of parasite speciation.

A major limitation in the study of *Pseudoacanthocephalus* is the scarcity of sequence data other than COI. Although various molecular phylogenetic studies have been conducted in recent years (e.g., Zhao et al. 2024), nuclear DNA sequence data remain inconsistent across studies, and no comprehensive dataset is currently available for genus-wide analysis. In this study, only 18S was determined from one individual of the acanthocephalan. Therefore, additional sequence data, such as from the ITS regions, will be necessary for more robust phylogenetic assessments in the future.

Author contributions. Y. Kita supervised this study. H. Abe, S. Kaburagi and T. Fujita collected the frog specimens. K. Toyota collected the specimen of a shark. Y. Kita dissected the frog specimens. Y. Kondo dissected the shark specimen. Y. Kita and M. Nitta prepared acanthocephalan specimens. M. Nitta analysed the stomach contents of the shark. Y. Kita conducted the molecular phylogenetic study on the acanthocephalans. Y. Kita wrote the draft of this manuscript. All authors checked and edited this manuscript.

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