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Morphology, molecular characterization and phylogeny of *Bolbosoma nipponicum* Yamaguti, 1939 (Acanthocephala: Polymorphidae), a potential zoonotic parasite of human acanthocephaliasis

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ABSTRACT

Human acanthocephaliasis is a rare parasitic zoonosis mainly caused by acanthocephalans belonging to the genera *Acanthocephalus*, *Bolbosoma*, *Corynosoma*, *Macracanthorhynchus*, and *Moniliformis*. In the present paper, the juveniles of *Bolbosoma nipponicum* Yamaguti, 1939 collected from the northern fur seal *Callorhinus ursinus* (Linnaeus) (Mammalia: Carnivora) in Alaska, USA were precisely identified based on morphological characters and genetic data. Their detailed morphology was studied using light and, for the first time, scanning electron microscopy. The molecular characterization of the nuclear genes [small ribosomal subunit (18S) and large ribosomal subunit (28S)] and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) sequence data of *B. nipponicum* are provided for the first time. Moreover, in order to clarify the phylogenetic relationships of the genus *Bolbosoma* and the other genera in the family Polymorphidae, phylogenetic analyses were performed integrating different nuclear (18S + ITS+28S) and mitochondrial (*cox*1) sequence data using maximum likelihood (ML) and Bayesian inference (BI). The phylogenetic results showed that *Bolbosoma* has a sister relationship with *Corynosoma*, and also revealed that *Southwellina* is sister to *Ibirhynchus* + *Hexaglandula*. Our molecular phylogeny also indicated a possible host-switch pattern during the evolution of the polymorphid acanthocephalans. The ancestors of polymorphid acanthocephalans seem to have originally parasitized fish-eating waterfowl in continental habitats, then extended to fish-eating marine birds in brackish water and marine habitats, and finally, opportunistically infected the marine mammals.

1. Introduction

The current species identification of acanthocephalans is still mainly based on morphological methods. Recent studies have indicated that it is useful and practical to utilize some nuclear and mitochondrial DNA sequences [i.e., large ribosomal DNA (28S), internal transcribed spacer (ITS) and cytochrome oxidase subunit 1 (*cox*1)] as genetic markers for the accurate identification of acanthocephalans, especially for the delimitation of intraspecific phenotypic variation, identification of cystacanths/juveniles and discovery of cryptic species (Alcántar-Escalera et al., 2013; Kang and Li, 2018; Li et al., 2019; Lisitsyna et al., 2019; Steinauer et al., 2007; Wayland et al., 2015; Zittel et al., 2018). However, most of the currently recognized acanthocephalan species were defined only under the traditional morphospecies concept.

The genus *Bolbosoma* Porta, 1908 (Palaeacanthocephala: Polymorphida) is a small group of acanthocephalans, with adults mainly

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parasitic in marine mammals, *i.e.*, seals, whales and dolphins (Amin and Margolis, 1998; Baylis, 1929; Delyamure, 1955; Santoro et al., 2021; Skrjabin, 1972; Wang, 1980; Yamaguti, 1939). Amin (2013) listed 12 nominal species, namely *B. australis* Skrjabin, 1972, *B. balaenae* (Gmelin, 1790), *B. brevicolle* (Malm, 1867), *B. caenoforme* (Heitz, 1920), *B. capitatum* (von Linstow, 1880), *B. hamiltoni* Baylis, 1929, *B. heteracanthe* (Heitz, 1920), *B. nipponicum* Yamaguti, 1939, *B. scomberomori* Wang, 1980, *B. tuberculata* Skriabin, 1970, *B. turbinella* (Diesing, 1851) and *B. vasculosum* (Rudolphi, 1819). Some species of *Bolbosoma* are recognized as parasites that are frequently associated with human acanthocephaliasis (Beaver et al., 1983; Hino et al., 2002; Ishikura et al., 1996; Isoda et al., 2006; Kaito et al., 2019; Mori et al., 1998; Tada et al., 1983). To date, at least nine cases of human acanthocephaliasis caused by *Bolbosoma* spp. have been reported in Japan (Arizono et al., 2012; Kaito et al., 2019; Santoro et al., 2021). Humans become infected by the accidental ingestion of raw or undercooked marine fishes/squids contaminated by cystacanths of *Bolbosoma* (Kaito et al., 2019; Tada et al., 1983).

The adults of *B. nipponicum* were originally described from the common minke whale *Balaenoptera acutorostrata* Lacépède (Mammalia: Cetacea) in the North Pacific Ocean (Yamaguti, 1939). Later, this species was reported in various marine mammals, including the northern fur seal *Callorhinus ursinus* (Linnaeus) (Mammalia: Carnivora) (Delyamure 1955; Kuzmina et al., 2012, 2021). However, the detailed morphology of the juveniles of *B. nipponicum* has never been described. Furthermore, the current genetic data base for this species is still scanty, with only the partial ITS rDNA available in the GenBank now (Arizono et al., 2012).

In the present study, the juveniles of *B. nipponicum* were described using light and, for the first time, scanning electron microscopy, based on specimens collected from the northern fur seal *C. ursinus* in St. Paul Island, Alaska, USA. The molecular characterization of the small ribosomal DNA (18S), the large ribosomal DNA (28S) and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) sequence data of *B. nipponicum* are provided for the first time. Moreover, in order to clarify the phylogenetic relationships of the genus *Bolbosoma* and the other genera in the family Polymorphidae Meyer, 1931, phylogenetic analyses were performed integrating different nuclear (18S + ITS + 28S) and mitochondrial (*cox*1) genetic markers using maximum likelihood (ML) and Bayesian inference (BI).

2. Materials and methods

2.1. Morphological study

Acanthocephalans were collected from the intestine of northern fur seal *C. ursinus* in St. Paul Island, Alaska, USA in 2014 (see Kuzmina et al., 2021 for details); fresh helminths were washed in tap water, then fixed and preserved in 70% ethanol. For light microscopical studies, three juvenile acanthocephalans were placed in temporary mounts and cleared in glycerin. Photomicrographs were recorded using a Nikon® digital camera coupled to a Nikon® optical microscopy (Nikon ECLIPSE Ni–U, Nikon Corporation, Tokyo, Japan). For scanning electron microscopy (SEM), the anterior part of one juvenile specimen was post-fixed in 1% OsO4, dehydrated via an ethanol series and acetone, and then critical point dried. The specimen was coated with gold at about 20 nm and examined using a Hitachi S-4800 scanning electron microscope at an accelerating voltage of 20 kV. Measurements (the range, followed by the mean in parentheses) are given in micrometres unless otherwise stated.

2.2. Molecular procedures

The mid-body of one male and one female juvenile specimens were used for molecular analysis (the anterior and posterior ends of body deposited for morphological study). Genomic DNA from each sample was extracted using a Column Genomic DNA Isolation Kit (Shanghai Sangon, China) according to the manufacturer's instructions. The partial 18S region was amplified in one fragment by polymerase chain reaction (PCR) using forward primer (5′-AGATTAAGCCATGCATGCGTAAG-3′) and the reverse primer (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Garey et al., 1996). The partial 28S region was amplified in 4 overlapping fragments (amplicons 1–4) by PCR using the following primers (García-Varela and Nadler, 2005), including amplicon 1: forward 5′-CAAGTACCGTGAGGGAAAGTTGC-3′ and reverse 5′-CAGCTATCCTG AGGGAAAC-3′; amplicon 2: forward 5′-ACCCGAAAGATGGTGAACTA TG-3′ and reverse 5′-CTTCTCCAAC(T/G)TCAGTCTTCAA-3′; amplicon 3: forward 5′-CTAAGGAGTGTGTAACAACTCACC-3′ and reverse 5′-AATGACGAGGCATTTGGCTACCTT-3′; amplicon 4: forward 5′-GAT CCGTAACTTCGGGAAAAGGAT-3′ and reverse 5′-CTTCGCAATGATAG GAAGAGCC-3′. The partial ITS region was amplified by PCR using the forward primer ITS-F (5′-GTC GTA ACA AGG TTT CCG TA-3′) and the reverse primer ITS-R (5′-TAT GCT TAA ATT CAG CGG GT-3′) (Král'ová-Hromadová et al., 2003). The partial *cox*1 region was amplified by PCR using the forward primer COI–F (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and the reverse primer COI-R (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Gómez et al., 2002). Cycling conditions were as previously described by (Li et al., 2019). PCR products were checked on GoldView-stained 1.5% agarose gels and purified with Column PCR Product Purification Kit (Shanghai Sangon, China). Sequencing of each sample was carried out for both strands. Sequences were aligned using ClustalW2. The DNA sequences obtained herein were compared (using the algorithm BLASTn) with those available in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The 18S, ITS, 28S and *cox*1 sequences of *B. nipponicum* were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) (accession numbers: 18S: ON358429, ON361558; ITS: ON361563, ON361566; 28S: ON359851, ON359856; cox1: ON359908, ON359909).

2.3. Phylogenetic analyses

Phylogenetic trees were constructed based on the 18S + ITS+28S + cox1 sequence data using maximum likelihood (ML) inference with IQTREE (Nguyen et al., 2015) and Bayesian inference (BI) with Mrbayes 3.2.7 (Ronquist et al., 2012), respectively. *Centrorhynchus clitorideus* (Palaeacanthocephala: Polymorphida: Centrorhynchidae) was chosen as the out-group according to the previous studies (García-Varela et al., 2013). The in-group includes 25 polymorphid species representing 10 genera with sequences available on GenBank. The detailed information on acanthocephalan species included in the present phylogenetic analyses was provided in Table 1. The nucleotide sequences of each gene were aligned in batches using MAFFT v7.313 under iterative refinement method of E–INS–I (Katoh and Standley, 2013), poorly aligned regions were excluded using BMGE v1.12 (h = 0.4) (Criscuolo and Gribaldo, 2010). Further, partially ambiguous bases were manually inspected and removed. PhyloSuite v1.2.2 (Zhang et al., 2020) was then used to concatenate these alignments into a single alignment and generate phylip and nexus format files for the phylogenetic analyses.

The maximum likelihood inference was conducted in IQTREE v2.1.2 (Minh et al., 2020). Substitution models were compared and selected according to the Bayesian Information Criterion by using ModelFinder (Kalyaanamoorthy et al., 2017). The TVM + F + R3 model was identified as the optimal nucleotide substitution model for 18S + 28S + ITS + cox1 sequence data. Reliabilities for maximum likelihood inference were tested using 1000 bootstrap replications and Bayesian Information Criterion analysis was run for 5×10^6 MCMC generations and sampling a tree with every 1000 generations. The first 25% trees were treated as "burn-in". The phylogenetic trees were visualized in iTOL v6.1.1 (Letunic and Bork, 2021). In the ML tree, the bootstrap values ≥ 85 were considered to constitute strong nodal support, whereas BS values ≥ 50 and <85 were considered to constitute moderate nodal support. In the BI tree, the Bayesian posterior probabilities values ≥ 0.90 were considered to constitute strong nodal support, whereas Bayesian posterior

Table 1
Detailed information of representatives of the family Polymorphidae used for phylogenetic analyses.

Species	Host	Locality	GenBank ID				References
			18S	ITS	28S	cox1	
Outgroup							
Centrorhynchus clitorideus	*Athene noctua*	Pakistan	MN661371	–	MN661375	MT113355	Zhao et al. (2020); Muhammad et al. (2020)
Ingroup							
Arhythmorhynchus frassoni	*Uca spinicarpa*	Mexico	JX442164	–	JX442176	EU189484	García-Varela et al. (2013); Guillén-Hernández et al. (2008)
Andracantha gravida	*Phalacrocorax auritus*	Mexico	EU267802	–	EU267814	EU267822	García-Varela et al. (2009)
Bolbosoma nipponicum	***Callorhinus ursinus***	**Alaska, USA**	*	*	*	*	**Present study**
Bolbosoma sp.	*Callorhinus ursinus, Homo sapiens*	USA, Japan	JX442167	LC375174	JX442179	JX442190	García-Varela et al. (2013); Kaito et al. (2019)
Bolbosoma turbinella	*Eschrichtius robustus, Paralichthys isosceles*	USA, Brazil	JX442166	KU314819	JX442178	JX442189	García-Varela et al. (2013); Fonseca et al. (2019)
Corynosoma australe	*Phocarctos hookeri, Otaria byronia*	New Zealand	JX442168	AF286307	JX442180	JX442191	García-Varela et al. (2005, 2013)
Corynosoma enhydri	*Enhydra lutris*	USA	AF001837	AF286311	AY829107	DQ089719	Near et al. (1998); García-Varela and Nadler (2005, 2006)
Corynosoma magdaleni	*Pusa hispida botnica, Pusa hispida, Pusa hispida saimensis*	Finland	EU267803	AY532065	EU267815	EF467872	García-Varela et al. (2005, 2009); García-Varela & Perez-Ponce de Leon (2008)
Corynosoma obtuscens	*Callorhinus ursinus*	USA	JX442169	–	JX442181	JX442192	García-Varela et al. (2013)
Corynosoma semerme	*Halichoerus grypus, Phoca largha*	Germany, Japan	–	MF001279	LC461963	MF001277	Waindok et al. (2018); Sasaki et al. (2019)
Corynosoma strumosum	*Phoca vitulina, Neophocaena phocaenoides*	USA, Japan	EU267804	AF286313	EU267816	LC465402	García-Varela et al. (2005, 2009); Sasaki et al. (2019)
Corynosoma validum	*Callorhinus ursinus, Odobenus rosmarus*	USA	JX442170	AF286314	JX442182	JX442193	García-Varela et al. (2005, 2013)
Hexaglandula corynosoma	*Nyctanassa violacea*	Mexico	EU267808	–	EU267817	EF467869	García-Varela et al. (2009); García-Varela & Perez-Ponce de Leon (2008)
Ibirhynchus dimorpha	*Eudocimus albus*	Mexico	GQ981436	–	GQ981437	GQ981438	García-Varela et al. (2011)
Polymorphus minutus	*Gammarus pulex*	France	EU267806	–	EU267819	EF467865	García-Varela et al. (2009); Garcia-Varela & Perez-Ponce de Leon (2008)
Polymorphus obtusus	*Ahythya affinis*	Mexico	JX442172	–	JX442184	JX442195	García-Varela et al. (2013)
Polymorphus trochus	*Fulica america*	Mexico	JX442173	–	JX442185	JX442196	García-Varela et al. (2013)
Pseudocorynosoma anatarium	*Bucephala albeola*	Mexico	EU267801	–	EU267813	EU267821	García-Varela et al. (2009)
Pseudocorynosoma constrictum	*Spatula clypeata*	Mexico	EU267800	–	EU267812	EU267820	García-Varela et al. (2009)
Pseudocorynosoma sp.	*Oxyura jamaicensis*	Mexico	JX442175	–	JX442187	JX442198	García-Varela et al. (2013)
Profilicollis altmani	*Enhydra lutris*	USA	AF001838	AY532066	AY829108	DQ089720	Near et al. (1998); García-Varela et al. (2005); García-Varela and Nadler (2005, 2006)
Profilicollis botulus	*Somateria mollissima*	Denmark	EU267805	–	EU267818	EF467862	García-Varela et al. (2009); García-Varela & Perez-Ponce de Leon (2008)
Profilicollis chasmagnathi	*Emerita analoga*	Chile	JX442174	–	JX442186	JX442197	García-Varela et al. (2013)
Southwellina hispida	*Tigrisoma mexicanum, Ardea cinerea*	Mexico	EU267807	–	EU267811	NC026516	García-Varela et al. (2009); Gazi et al. (2015)

probabilities values ≥ 0.70 and < 0.90 were considered to constitute moderate nodal support. The bootstrap values ≥ 50 and Bayesian posterior probabilities values ≥ 0.70 were shown in the phylogenetic trees.

3. Results

3.1. Morphology of juveniles of *Bolbosoma nipponicum* Yamaguti, 1939 (Figs. 1–3)

General. Trunk medium, more or less cylindrical, elongate, with bulbous enlargement in anterior part (Fig. 1A, D, 2A, 3A). Trunk spines scaly, extending from base of neck to just posterior of maximum enlargement (Fig. 1A, D, E, 2A, 3A, B). Proboscis cylindrical, with 20–22 longitudinal rows of 6–7 hooks per row (Fig. 1A–D, 2A, B, 3A, C, D). Apical proboscis hooks slender, middle hooks robust, basal hooks smallest, all hooks with simple posteriorly directed roots (Fig. 1B and C, 2B, 3A, C, D). Proboscis receptacle double-walled, cerebral ganglion at base of proboscis receptacle (Fig. 1A, D, 2A). Neck trapezoid (Fig. 1A, D, 2A, 3A). Lemnisci subequal, stubby, distinctly shorter than proboscis receptacle (Fig. 1A, D, 2A).

Male [Based on 2 juvenile male specimens] (Table 2). Trunk 7.48–8.35 (7.91) mm long, maximum width 1.08–1.23 (1.15) mm. Proboscis 525–584 (554) long, 376–416 (396) wide. Size of proboscis hooks from anterior: 88–93 × 18–19 (90 × 18); 93–98 × 18–23 (95 × 20); 93–95 × 18–23 (94 × 20); 93–95 × 23–28 (94 × 25); 75–80 × 20–23 (78 × 21); 53–55 × 13–15 (54 × 14). Neck 158–188 (173) long, 485–525 (505) wide. Proboscis receptacle 1.22–1.33 (1.28) mm long, 416–475 (446) wide. Longer lemniscus 446–851 (648) long, 158–238 (198) wide. Shorter lemniscus 396–822 (609) long, 119–178 (149) wide. Testes two, very small, oval; nearly equal in size, 141–169 (154) long, 80–127 (105) wide (Fig. 1D, F, 2C). Cement-glands two pairs, slender, tubular, 1.68–2.18 (1.93) mm long, 30–40 (35) wide (Fig. 1D, F, 2C). Copulatory bursa not everted, 802–822 (812) long, 525–545 (535) wide. Gonopore terminal.

Female [Based on 1 juvenile female specimen] (Table 2). Trunk 9.93 mm long, maximum width 1.13 mm. Proboscis 594 long, 446 wide. Size of proboscis hooks from anterior to posterior: 79 × 18; 103 × 21; 101 × 25; 91 × 24; 82 × 21; 59 × 15. Neck 347 long, 545 wide. Proboscis receptacle 1.35 mm long, 465 wide. Longer lemnisci 1.16 mm long, 228 wide. Shorter lemnisci 1.12 mm long, 188 wide. Uterine bell 842 long,

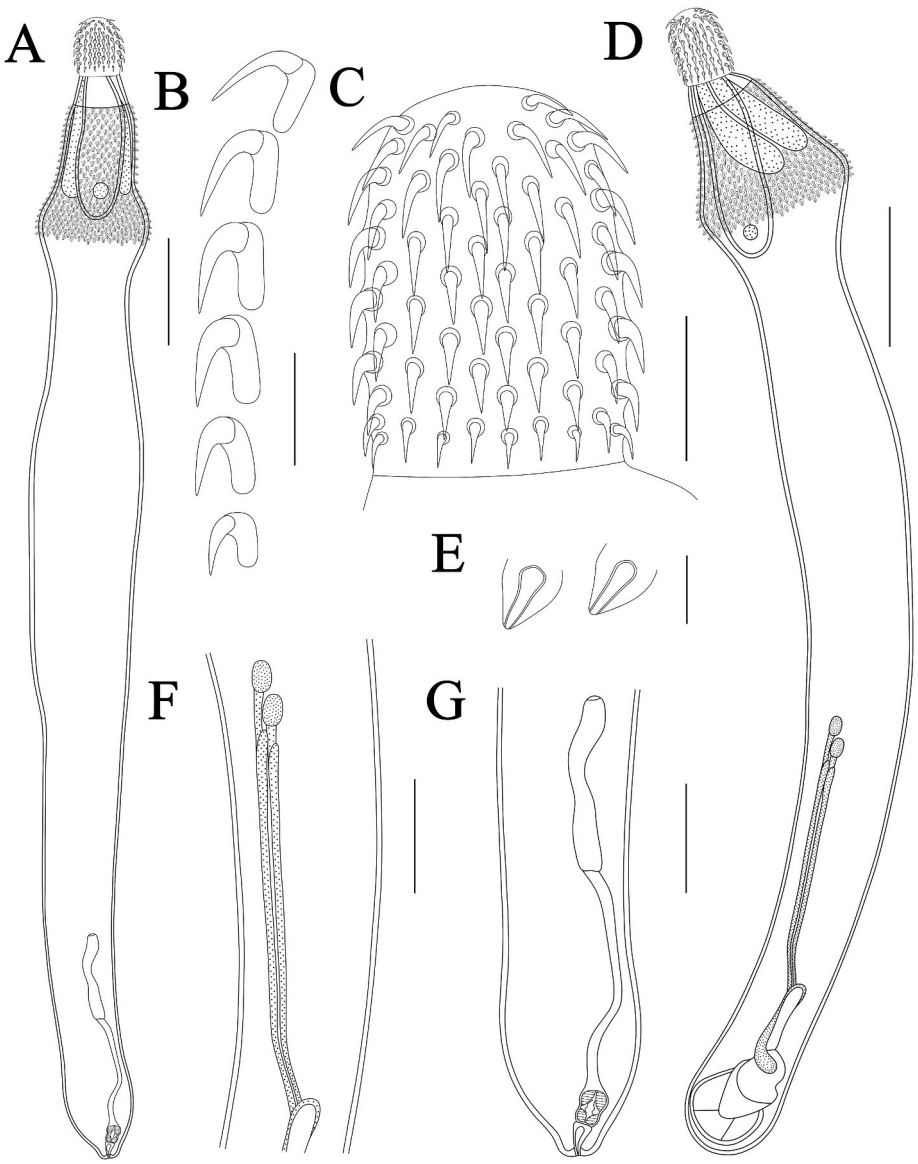

Fig. 1. *Bolbosoma nipponicum* collected from *Callorhinus ursinus* (Linnaeus) (Carnivora: Otariidae) in St. Paul Island, Alaska. A: female; B: hooks; C: proboscis; D: male; E: trunk spines; F: testes and cement-glands; G: poster part of female. Scale bars: A, D = 1000 μm; B = 100 μm; C = 200 μm; E = 50 μm; F, G = 500 μm.

139 wide. Uterus 1.07 mm long, 30 wide, vagina 327 long, 129 wide (Fig. 1A, G, 2D). Eggs not observed. Gonopore terminal (Fig. 1A, G, 2D).

Hosts of present material: Callorhinus ursinus (Linnaeus) (Carnivora: Otariidae).

Locality: St. Paul Island, Alaska, USA (57° 09′ N, 170° 13′ W).

Site infection: Intestine.

Voucher specimens: 2 juvenile males, 1 juvenile female (HBNU–A-2022M002L), deposited in the College of Life Sciences, Hebei Normal University, Hebei Province, P. R. China.

3.2. Molecular characterization

3.2.1. Partial 18S region

Two 18S sequences of *B. nipponicum* obtained herein are both 1182 bp in length, with no nucleotide divergence detected. In the genus *Bolbosoma*, the 18S sequence data are available in GenBank for *B. turbinella* (JX442166), *B. balaenae* (MZ047218–MZ047227, JQ040304–JQ040306, MT233305), *B. vasculosum* (JX014225), *B. caenoforme* (KF156879) and *Bolbosoma* sp. MGV-2012 (JX442167). Pairwise comparison of 18S sequences of *B. nipponicum* with these five species of *Bolbosoma* produced 0–0.42% of nucleotide divergence.

3.2.2. Partial 28S region

Two 28S sequences of *B. nipponicum* obtained herein are both 2755 bp in length, with 0.04% nucleotide divergence detected. In the genus *Bolbosoma*, the 28S sequence data are available in GenBank for *B. turbinella* (JX442178), *B. balaenae* (MZ047231–MZ047239) and *Bolbosoma* sp. MGV-2012 (JX442179). Pairwise comparison of 28S sequences of *B. nipponicum* and these three species of *Bolbosoma* produced 0–1.12% (*B. turbinella*) of nucleotide divergence.

3.2.3. Partial ITS region

Two ITS sequences of *B. nipponicum* obtained herein are both 754 bp in length, with 0.13% nucleotide divergence detected. In the genus *Bolbosoma*, the ITS sequence data are available in GenBank for *B. nipponicum* (AB706183), *B.* cf. *capitatum* (AB706182), *B. turbinella* (KU314817–KU314819) and *Bolbosoma* sp. KMC (LC375174) (Table 1). Pairwise comparison of ITS sequences of *B. nipponicum* obtained herein with *B. nipponicum* (AB706183) available in GenBank displayed 0–0.13% of nucleotide divergence, and with the other three species of *Bolbosoma* produced 9.42–11.6% of nucleotide divergence.

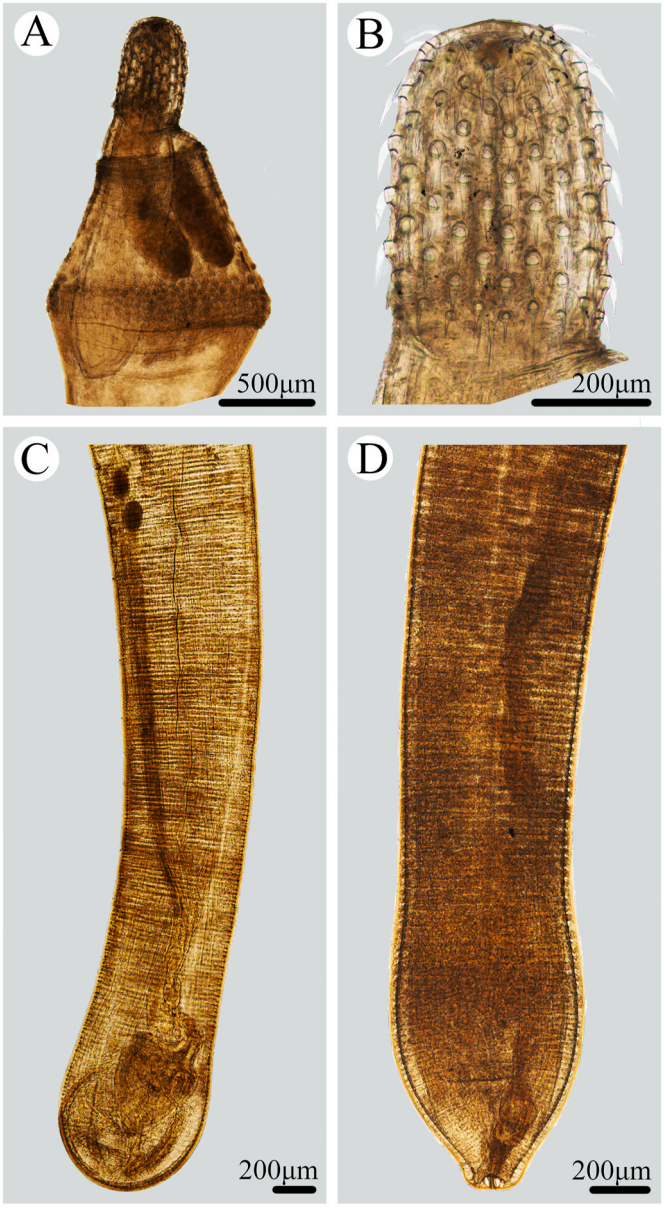

Fig. 2. Photomicrographs of *Bolbosoma nipponicum* collected from *Callorhinus ursinus* (Linnaeus) (Carnivora: Otariidae) in St. Paul Island, Alaska. A: anterior part of male; B: proboscis; C: posterior part of male; D: posterior part of female.

3.2.4. Partial *cox*1 region

Two *cox*1 sequences of *B. nipponicum* obtained herein are both 655 bp in length, with 0.31% nucleotide divergence detected. In the genus *Bolbosoma*, the *cox*1 sequence data are available in GenBank for *B. turbinella* (JX442189, KU314821, KU314823), *B. balaenae* (MZ047272–MZ047281), *B. caenoforme* (KF156891) *Bolbosoma* sp. MJA-2016 (KX098556) and *Bolbosoma* sp. KMC (LC377776) (Table 1). Pairwise comparison of *cox*1 sequences of *B. nipponicum* with *B. caenoforme* (KF156891) showed 0.15% of nucleotide divergence, and with the other four species of *Bolbosoma* produced 13.9–31.7% of nucleotide divergence.

3.3. Phylogenetic analyses (Figs. 4 and 5)

The phylogenetic results based on the 18S + ITS+28S + *cox*1 sequence data using ML and BI methods, respectively, were very similar (Figs. 4 and 5). In the ML tree (Fig. 4), *Southwellina hispida* clustered together with the representatives of *Ibirhynchus* + *Hexaglandula* with strong support, which formed a sister relationship with all the representatives of *Pseudocorynosoma* + *Arhythmorhynchus* + *Profilicollis* + *Polymorphus* with moderate support. Species of *Bolbosoma* clustered together with the representatives of *Corynosoma* with strong support, which constituted a sister group with *Andracantha gravida* (Fig. 4). In the BI tree (Fig. 5), *Southwellina hispida* was at the base of the tree, forming a sister clade to the remaining Polymorphidae with low support value. The representatives of Ibirhynchus + Hexaglandula formed a sister relationship with strong support. The representatives of *Pseudocorynosoma* + *Arhythmorhynchus* + *Profilicollis* + *Polymorphus* constituted a separated branch with strong support. Species of *Bolbosoma* also showed sister relationship with the representatives of *Corynosoma*, both clustered together with *A. gravida* with strong support.

4. Discussion

Arizono et al. (2012) reported the ITS sequence data of *B. nipponicum* based on the adult specimen collected from the type host (*Balaenoptera acutorostrata*) in the type locality (North Pacific Ocean), which enabled us to more accurately identify our present material. Pairwise comparison of ITS sequences of our material and *B. nipponicum* (AB706183) sequenced by Arizono et al. (2012), showed 0–0.13% of nucleotide divergence, which is accordant with the level of intraspecific genetic variation between different individuals detected herein. Consequently, we considered our material to be conspecific with *B. nipponicum*.

Although Kuzmina et al. (2012, 2021) found *B. nipponicum* in the northern fur seal *C. ursinus*, they did not provide a detailed morphological description of their specimens. Because the present specimens are all juvenile male (testis very small) and female (no eggs found in the uterus), we speculated that the northern fur seal *C. ursinus* possibly acts as an unsuitable final host for *B. nipponicum*, or the juvenile worms have only recently infected the northern fur seal and have not had time to mature. The similar situation also occurred in *B. vasculosum*. Costa et al. (2000) reported the juvenile worms of *B. vasculosum* from the stranded common dolphin *Delphinus delphis* in the Atlantic Ocean. In spite of our present specimens being immature, their morphology and morphometrics are more or less identical to the original description of mature adults regarding several features, including the morphology of the neck and trunk, the arrangement of trunk spines, the number and arrangement of the proboscis hooks and the size of proboscis receptacle. However, comparison with Yamaguti's (1939) material, our specimens have distinctly smaller trunk and testis, and shorter trunk spines region and neck (see Table 2 for details). Moreover, the proboscis receptacle is much longer than the lemnisci in the present juvenile specimens (*vs* the proboscis receptacle distinctly shorter than the lemnisci in the mature specimens), and the maximum enlargement of trunk is distinctly narrower than the mature material of Yamaguti (1939). The shape of proboscis of the juvenile is also slightly different from that of mature specimens. Fukui and Morisita (1939) redescribed *B. turbinella* based on specimens collected from *Balaenoptera borealis* Lesson (Cetacea: Balaenopteridae). However, Yamaguti (1939) considered that Fukui and Morisita's (1939) material identified as *B. turbinella* may be the species *B. nipponicum*. We also compared the morphology and measurements of these two species (see Table 2 for details) and did not find remarkable differences between Fukui and Morisita's (1939) material and Yamaguti's (1939) specimens, except proboscis of Fukui and Morisita's (1939) material with 7–8 hooks per row (*vs* proboscis usually with 6 hooks per row in Yamaguiti's specimens).

The molecular characterization of the 18S, 28S and *cox*1 genes of *B. nipponicum* are provided for the first time. Our molecular analysis revealed that the level of intraspecific genetic variation in the 18S and 28S sequence data is distinctly lower than that of the interspecific genetic variation in the ITS and *cox*1 regions. It seems more useful and practical to utilize the ITS and/or *cox*1 regions than the 18S and 28S sequences as genetic markers for identifying and distinguishing acanthocephalans, especially for the diagnosis of the closely related species.

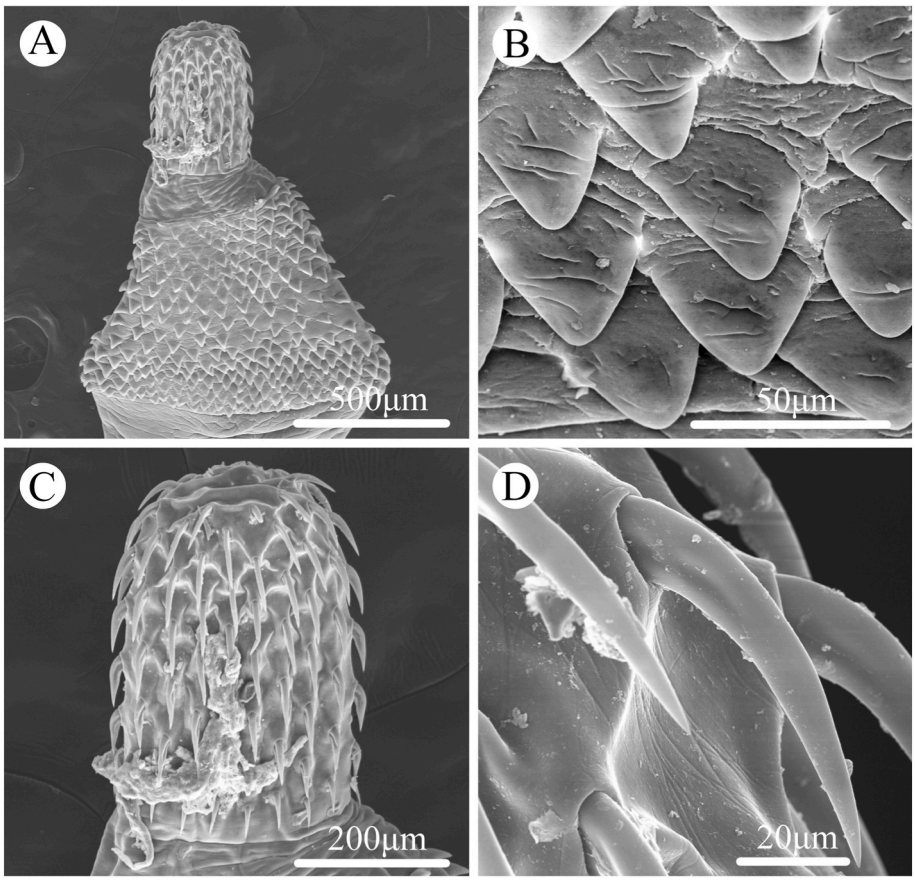

Fig. 3. Scanning electron micrographs of *Bolbosoma nipponicum* collected from *Callorhinus ursinus* (Linnaeus) (Carnivora: Otariidae) in St. Paul Island, Alaska. A: anterior part of male; B: trunk spines; C: proboscis; D: hooks.

Table 2
Comparative morphometric data for *Bolbosoma nipponicum* (all measurements are in millimetres).

Hosts	*Callorhinus ursinus*		*Balaenoptera rostrata*		*Balaenoptera borealis*	
Localities	Alaska, USA		North Pacific Ocean		North Pacific Ocean	
Sources	Present study		Yamaguti (1939)		Fukui and Morisita (1939)	
Characteristics/sex	Immature male	Immature female	Mature male	Mature female	Mature male	Mature female
Trunk length	7.48–8.35	9.93	up to 45.0	up to 60.0	20.0–28.0	25.0–33.0
Trunk width	1.08–1.23	1.13	1.20–4.00	1.20–4.00	–	–
Proboscis length	0.52–0.58	0.59	0.40–0.65	0.40–0.65	0.86	0.86
Proboscis width	0.38–0.42	0.45	0.30–0.44	0.30–0.44	0.40	0.40
Hook rows	20–22	22	17–23	17–23	19–21	19–21
Hooks/per row	6–7	6	5–6	5–6	7–8	7–8
Lemnisci length	0.40–0.85	1.12–1.16	1.00–2.30	1.00–2.30	–	–
Lemnisci width	0.12–0.24	0.19–0.23	0.12–0.30	0.12–0.30	–	–
Proboscis receptacle length	1.22–1.33	1.35	1.10–1.75	1.10–1.75	1.65	1.65
Proboscis receptacle width	0.42–0.48	0.47	0.35–0.48	0.35–0.48	0.55	0.55
Neck length	0.16–0.19	0.35qw	0.50–0.60	0.50–0.60	–	–
Neck width	0.49–0.52	0.54	0.62–0.80	0.62–0.80	–	–
Testis length	0.14–0.17	–	1.00–2.00	–	–	–
Testis width	0.08–0.13	–	0.60–1.05	–	–	–
Cement gland length	1.68–2.18	–	2.70	–	–	–
Size of egg	–	–	–	0.12–0.19 × 0.03–0.04	–	0.15 × 0.03
Uterus length	–	1.07	–	0.23–3.85	–	–

However, we noted that a pairwise comparison of *cox*1 sequences of our specimens and *B. caenoforme* (KF156891) provided by Malyarchuk et al. (2014), showed only 0.15% of nucleotide divergence. The voucher specimen of *B. caenoforme* (KF156891) in Malyarchuk et al. (2014) was collected from the *Salvelinus malma* (Walbaum) (Salmoniformes: Salmonidae) in the Taui Gulf, which acts as a paratenic host for species of *Bolbosoma* (Santoro et al., 2021). Although Malyarchuk et al. (2014) claimed their samples to be *B. caenoforme*, this species identification is almost certainly erroneous. Due to the scarcity of the material of this species, although the numbers of our present specimens are limited, the morphological and genetic data of *B. nipponicum* presented here will be a valuable reference for future studies on the diagnosis of different developmental stages, population genetics and phylogenetics of this group.

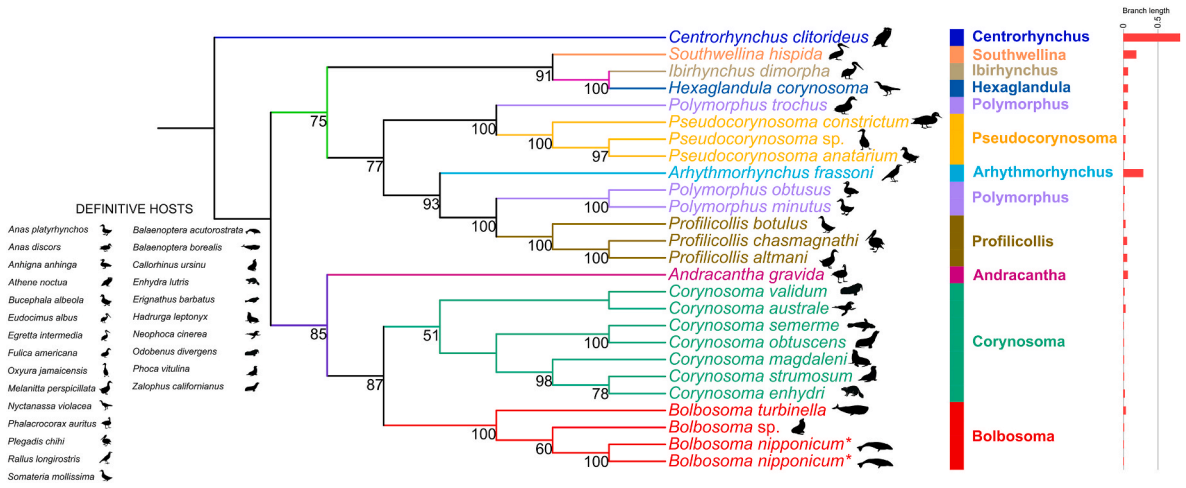

Fig. 4. Phylogenetic relationships of representatives of the family Polymorphidae using maximum likelihood method based on the 18S + ITS +28S + *cox*1 sequence data. *Centrorhynchus clitorideus* (Polymorphida: Centrorhynchidae) was chosen as outgroup. Bootstrap values > 50 are shown in the phylogenetic tree.

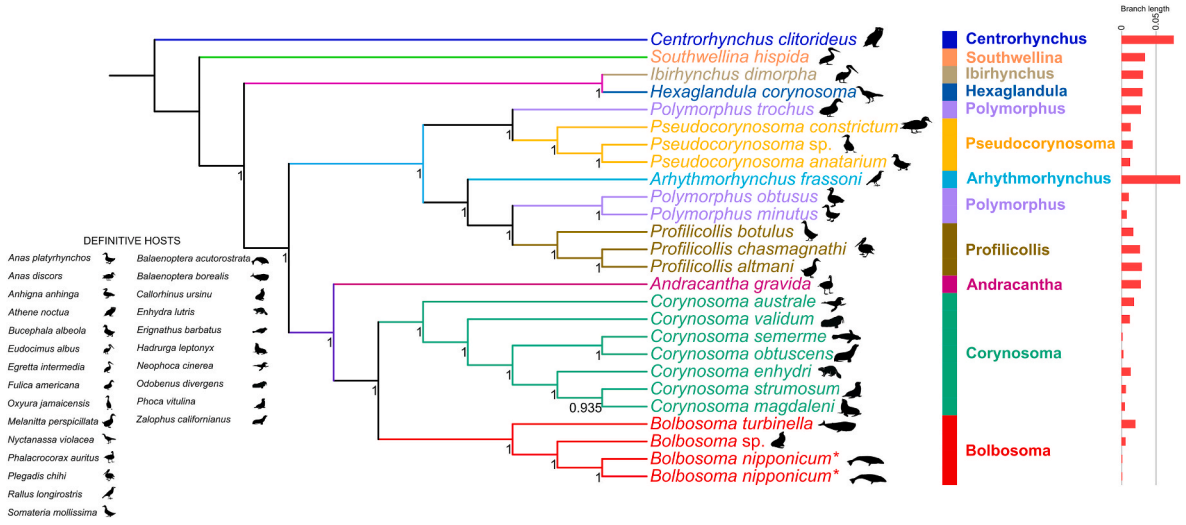

Fig. 5. Phylogenetic relationships of representatives of the family Polymorphidae using Bayesian inference based on the 18S + ITS +28S + *cox*1 sequence data. *Centrorhynchus clitorideus* (Polymorphida: Centrorhynchidae) was chosen as outgroup. Bayesian posterior probabilities values > 0.70 are shown in the phylogenetic tree.

The family Polymorphidae is a large group of acanthocephalans, currently including over 120 species mainly parasitic in marine mammals, fish-eating marine birds and waterfowls (Amin, 2013; Aznar et al., 2006; Delyamure, 1955; Dimitrova and Georgiev, 1994; García-Varela et al., 2011, 2013; Schmidt, 1975). Amin (2013) listed 12 genera in this family, namely *Andracantha*, *Ardeirhynchus*, *Arhythmorhynchus*, *Bolbosoma*, *Corynosoma*, *Diplospinifer*, *Filicollis*, *Ibirhynchus*, *Polymorphus*, *Profilicollis*, *Pseudocorynosoma* and *Southwellina*. Some previous phylogenetic studies supported Polymorphidae to be a monophyletic group (García-Varela and Pérez-Ponce de León, 2008; Martín García-Varela et al., 2011, 2013; Verweyen et al., 2011). Amin (1992, 2013) considered the genus *Hexaglandula* Petrochenko, 1950 to be a synonym of *Polymorphus*. However, the recent molecular phylogenetic studies supported that *Hexaglandula* represents an independent valid genus (García-Varela et al., 2009, 2011, 2013; García-Varela and Pérez-Ponce de León, 2008; Presswell et al., 2017).

Our phylogenetic results using ML and BI methods both showed that the genus *Bolbosoma* has a sister relationship with *Corynosoma* with strong support, which agreed well with the recent molecular studies based on 28S + *cox*1 (Presswell et al., 2017) and 18S + 28S + *cox*1 sequence data (García-Varela et al., 2013). The close relationship of *Bolbosoma* and *Corynosoma* can be easily understood, when we considered they have the same type of definitive hosts. In the family Polymorphidae, only adults of *Bolbosoma* and *Corynosoma* can parasitize marine mammals, and some species of *Bolbosoma* and *Corynosoma* [*i.e.*, *B. capitatum*, *Bolbosoma* sp., *C. strumosum* (Rudolphi, 1802), *C. validum* Van Cleave, 1953 and *C. villosum* Van Cleave, 1953] are important zoonotic pathogens for human acanthocephaliasis (Arizono et al., 2012; Fujita et al., 2016; Kaito et al., 2019). The present phylogenetic results also supported the validity of the genus *Hexaglandula*, which is sister to *Ibirhynchus* with strong support in both ML tree and BI tree. The results are accordant with the previous phylogenetic analyses (García-Varela et al., 2013; Presswell et al., 2017). However, our results revealed the genus *Southwellina* formed a sister clade to the remaining representatives of Polymorphidae with low support value in the BI tree and constituted a sister relationship with *Ibirhynchus* + *Hexaglandula* with strong support only in the ML tree, which is different from all the previous phylogenetic studies (García-Varela et al., 2009, 2011, 2013; García-Varela and Pérez-Ponce de León, 2008; Presswell et al., 2017). The present different results obtained herein may be related to the different representatives included in the phylogeny or supplementary the ITS sequence data (all the previous studies did not use the ITS

sequence data for phylogenetic analyses). However, a more rigorous molecular phylogenetic study including broader representatives of the Polymorphidae using more nuclear and mitochondrial sequence data is required to further clarify the phylogenetic relationships of *Southwellina* and the other genera of Polymorphidae in the future.

The evolutionary relationships of the polymorphid acanthocephalans revealed by the present molecular analyses enabled us to speculate a possible host-switch pattern (parasitic sequence of changes in definitive host) during the evolution of the polymorphid acanthocephalans: the ancestor of polymorphid acanthocephalans seems to have originally parasitized fish-eating waterfowl in continental habitats (*i.e.*, *Southwellina*, *Ibirhynchus*, *Hexaglandula*, *Pseudocorynosoma*, *Arhythmorhynchus*, *Polymorphus* and *Profilicollis*), then extended to fish-eating seafowls in brackish water and seawater habitats (*i.e.*, *Andracantha*), and finally, opportunistically infected marine mammals (*i.e.*, *Bolbosoma* and *Corynosoma*). However, this evolutionary issue is still open. We need some more direct evidence to support the present hypothesis.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Ethical approval

The authors declare that they have observed all applicable ethical standards.

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