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ORIGINAL PAPER

# The Morphological and Molecular Description of *Acanthogyrus* (*Acanthosentis*) *fusiformis* n. sp. (Acanthocephala: Quadrigyridae) from the Catfish *Arius* sp. (Ariidae) in the Pacific Ocean off Vietnam, with Notes on Zoogeography

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**Abstract**

**Background** Most (82%) of the 46 recognized species of *Acanthogyrus* (*Acanthosentis*) Verma and Datta, 1929 are known from Asian freshwater fishes. Only three species of *Acanthosentis* are known from marine or brackish water fishes from India and Pakistan. We have discovered another marine species of *Acanthosentis* in the Pacific Ocean, off Vietnam.

**Purpose** The purpose is to describe the new species morphologically and molecularly and provide new information of its evolutionally relationships with other species of the subgenus.

**Methods** Standard methods of collection and examination of marine hosts, processing and illustrating of specimens, and taxonomic identification of parasites using the extensive collection of the lead author were used. Specimens were further studied using energy-dispersive X-ray analysis and ion sectioning of hooks, SEM analysis, and molecular sequencing. Type specimens were deposited at the Harold W. Manter Lab. collection, Lincoln, Nebraska.

**Results** *Acanthogyrus* (*Acanthosentis*) *fusiformis* n. sp. is described from the catfish, *Arius* sp. (Ariidae: Siluriformes) off the Pacific Coast of Vietnam at Bac Lieu in the Gulf of Thailand. The three other marine Indian species include *A*. (*A*.) *arii* Bilqees, 1971 which is also described from a similar catfish, *Arius serratus* Day off the Karachi coast in the Arabian Sea, Indian Ocean. Our new species from Vietnam is distinguished from the other 46 species by a combination of characters including a small fusiform trunk, complete circles of small hollow spines covering the entire trunk, prominent double apical organs often extending posteriorly past posterior hooks, middle and posterior hooks of equal size slightly smaller than anterior hooks, large neck continuous with the outline of the proboscis without distinct separation, big drop-shaped cephalic ganglion, extension of the proboscis receptacle anteriorly past the base of the proboscis up to the insertion point of the posterior hooks, presence of two para-receptacle structures (PRSs), free unattached thick lemnisci, short female reproductive system with filamentous attachment of the distal end of the uterine bell to the ventral body wall, and small narrowly ellipsoid eggs with thickened polar ends. Partial sequences of the 18S and internal transcribed spacers (ITS1-5.8S-ITS2) of ribosomal RNA were generated and used for phylogenetic analyses to confirm the taxonomic identity of *Acanthogyrus* (*Acanthosentis*) *fusiformis* n. sp.

**Conclusions** We describe unique morphological features of *A. fusiformis* never before known in the subgenus *Acanthosentis*. The uniqueness of *A. fusiformis* is further demonstrated by its EDXA fingerprint characterized by high levels of calcium and phosphorous in hooks. The zoogeography of species of *Acanthosentis* is elucidated in the Indian subcontinent, the Caribbean, China, and Africa. Molecular data have been available only in few species of *Acanthogyrus* (*Acanthosentis*) to date on GenBank database. For 18S, only two sequences from unknown *Acanthosentis* sp. from India are available, while for the ITS1-5.8S-ITS2 region, only sequences of *A. cheni* from China and of two unidentified species from Malaysia are available. Additional studies of species of *Acanthosentis* based on morphological and molecular genetic data will be needed to reconstruct the evolutionary history and phylogenetic affinities of this group of acanthocephalans.

**Keywords** *Acanthogyrus* (*Acanthosentis*) *fusiformis* n. sp. · Acanthocephala · Description · Molecular profile · *Arius* sp. · Vietnam

Extended author information available on the last page of the article

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## Introduction

Most of the recent taxonomic work on the Acanthocephala from Vietnam was reported by the Amin-Heckmann-Ha team since 2000. We have described over 50 acanthocephalan species and higher taxa from freshwater and marine fish, amphibians, reptiles, birds, and mammals in Vietnam. A complete listing of this taxonomic literature can be found in Amin et al. [6]. Eighteen species of acanthocephalans in five families were more recently collected from fishes in the Pacific and amphibians in central Vietnam in 2016 and 2017. In the present report, we describe males and females of one new species of *Acanthosentis* Verma and Datta 1929 [43] (Quadrigyridae) from a marine fish in the Pacific coast of Vietnam. Species of *Acanthosentis* are, however, common parasites of freshwater fishes. We also discuss its zoogeography, metal composition of hooks, and report its molecular profile.

## Materials and Methods

### Collections

Thirty-two specimens of a new species of Acanthocephala were collected from 4 of 10 examined individuals of the catfish, *Arius* sp. (Ariidae: Siluriformes) off the south Pacific Coast of Vietnam at Bac Lieu in the Gulf of Thailand (9°15′N 105°45′E) on July 16, 2017.

Freshly collected specimens were extended in water until proboscides everted and then fixed in 70% ethanol for transport to our Arizona, USA laboratory for processing and further studies.

## Methods

Worms were punctured with a fine needle and subsequently stained in Mayer's acid carmine, destained in 4% hydrochloric acid in 70% ethanol, dehydrated in ascending concentrations of ethanol (24 h each), and cleared in 100% xylene and then in 50% Canada balsam and 50% xylene (24 h each). Whole worms were then mounted in Canada balsam. Measurements are in micrometers, unless otherwise noted; the range is followed by the mean values between parentheses. Width measurements represent maximum width. Trunk length does not include proboscis, neck, or bursa. Line drawing was created by using a Ken-A-Vision microprojector (Ward's Biological Supply Co., Rochester, N.Y.) which uses cool quartz iodine 150-W illumination. Color-coded objectives, ×10, ×20, and ×43 lenses, are used. Images of stained whole-mounted specimens were projected vertically on 300 series Bristol draft paper (Strathmore, Westfield, Massachusetts) and then traced and inked with India ink. Projected images were identical to the actual specimens being projected. The completed line drawings were subsequently scanned at 600 pixels, transferred on a USB and subsequently downloaded on a computer.

Type specimens were deposited in the University of Nebraska's State Museum's Harold W. Manter Laboratory (HWML) collection in Lincoln, Nebraska, USA.

### Scanning Electron Microscopy (SEM)

Four to six specimens that had been fixed and stored in 70% ethanol were processed for SEM following standard methods [29]. These included critical point drying (CPD) in sample baskets and mounting on SEM sample mounts (stubs) using conductive double-sided carbon tape. Samples were coated with gold and palladium for 3 min using a Polaron #3500 sputter coater [Quorum (Q150 TES) www.quorumtech.com] establishing an approximate thickness of 20 nm. Samples were placed and observed in a FEI Helios Dual Beam Nanolab 600 (FEI, Hillsboro, Oregon) scanning electron microscope with digital images obtained in the Nanolab software system (FEI, Hillsboro, Oregon) and then transferred to a USB for future reference. Samples were received under low-vacuum conditions using 10 kV, spot size 2, 0.7 Torr using a GSE detector.

### X-Ray Microanalysis (XEDs), Energy-Dispersive Analysis for X-Ray (EDAX)

Standard methods were used for preparation similar to the SEM procedure. Specimens were examined and positioned with the above SEM instrument which was equipped with a Phoenix energy-dispersive X-ray analyzer (FEI, Hillsboro, Oregon). X-ray spot analysis and live scan analysis were performed at 16 kV with a spot size of 5, and results were recorded on charts and stored with digital imaging software attached to a computer. The texture and elemental analytical microscopy (TEAM) software system (FEI, Hillsboro, Oregon) was used. Data were stored in a USB for future analysis. The data included weight percent and atom percent of the detected elements following correction factors.

### Ion Sectioning of Hooks

A dual-beam SEM with a gallium (Ga) ion source (GIS) is used for the LIMS (liquid ion metal source) part of the process. The hooks of the acanthocephalans were centered on the SEM stage and cross-sectioned using a probe current between 0.2 and 2.1 nA according to the rate at which the area is cut. The time of cutting is based on the nature and sensitivity of the tissue. Following the initial cut, the sample

also goes through a milling process to obtain a smooth surface. The cut was then analyzed with X-ray at the tip, middle, and base of hooks for chemical ions with an electron beam (Tungsten) to obtain an X-ray spectrum. Results were stored with the attached imaging software and then transferred to a USB for future use. The intensity of the GIS was variable according to the nature of the material being cut.

### Molecular Methods

DNA was extracted using the DNeasy™ Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. The 18S and ITS1 + 5.8S + ITS2 region of the rDNA was amplified using primers: Worm A (5′-GCGAATGGCTCATTAAATCAG-3′); 1270R (5′-CCGTCAATTCCTTTAAGT-3′) and 930F (5′-GCATGGAATAATGGAATAGG-3′); Worm B (5′-CTTGTTACGACTTTTACTTCC-3′) (Littlewood and Olson [30]) for 18S gene, while BD1 (5′-GTCGTAACAAGGTTTCCGTA-3′); BD2 (5′-TATGCTTAAATTCAGCGGGT-3′) (Galazzo et al. [21]) used for ITS1+5.8S+ITS2 region. A polymerase chain reaction (PCR) was carried out in a total volume of 25 µl consisting of 10 × PCR reaction volume, 3 µl of deoxyribonucleoside triphosphates, 0.8 µl of each forward and reverse primer, 1 µl of Taq polymerase (1 U, Biotools), and 3 µl genomic DNA. The cycling conditions were as follows: one cycle of initial denaturation at 94 °C for 3 min; 40 cycles at 94 °C for 45 s, 55 °C for 45 s for all primer pairs, and 72 °C for 1 min; plus a final extension at 72 °C for 10 min. Samples without DNA were also included in each amplification run to exclude contamination. Amplified PCR products were analyzed by electrophoresis in a 1% agarose gel stained with ethidium bromide and examined under ultraviolet light. The amplified products were then purified with the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) and sequenced with Big Dye Terminator vr. 3.1 cycle sequencing kit in ABI 3130 Genetic Analyzer, Applied Biosystems by using above-mentioned primers. The obtained sequence partial 18S and ITS1+5.8S+ITS2 was submitted to GenBank under accession numbers: MK834517–MK834520.

Contigs were assembled using MEGA7 [28] and ambiguous bases clarified using corresponding ABI chromatograms. Sequences obtained for 18S and ITS1+5.8S+ITS2 regions were aligned with sequences from other related acanthocephalan species downloaded from GenBank after BLAST search on NCBI (Tables 2, 3). Sequences for each molecular marker 18S and ITS1+5.8S+ITS2 were aligned separately using the software ClustalW with default parameters implemented in MEGA7 [28]. Phylogenetic analyses were conducted using MEGA7 with 1000 bootstrap replicates for prior testing of reliability and Bayesian inference (BI) analyses in Topali 2.5 [33]. The best-fitting nucleotide substitution model GTR+G+I for 18S and ITS1+5.8S+ITS2 were estimated using the Akaike information criterion (AIC) implemented in MEGA version 7 [28]. The phylogenetic analyses were run under Bayesian inference (BI) criteria, employing the nucleotide substitution model identified for the AIC. BI trees were generated using Topali 2.5 by independent runs of four simultaneous MCMCMC chains with every 100th tree saved. The first 25% of the sampled trees were discarded as 'burn in.' The genetic divergence among species was estimated using uncorrected 'p' distances in MEGA7. In addition, sequences of other genera, such as *Moniliformis* sp. n. (MH401043) (which is actually *Moniliformis cryptosaudi* Amin, Heckmann, Sharifdini, Albayati, 2019b) and *Mediorhynchus* sp. (AF416413), were used as out-group.

## Results

Of the 32 specimens of *A. fusiformis* n. sp. collected from four infected of ten examined individuals of *Arius* sp., twenty specimens (six males and 14 females) were processed for microscopical examination and the remaining specimens were used for SEM and molecular analysis. Catfish of the genus *Arius* appear to form a natural grouping of 26 species found in brackish and freshwaters of Eastern Africa and Southeast Asia [31].

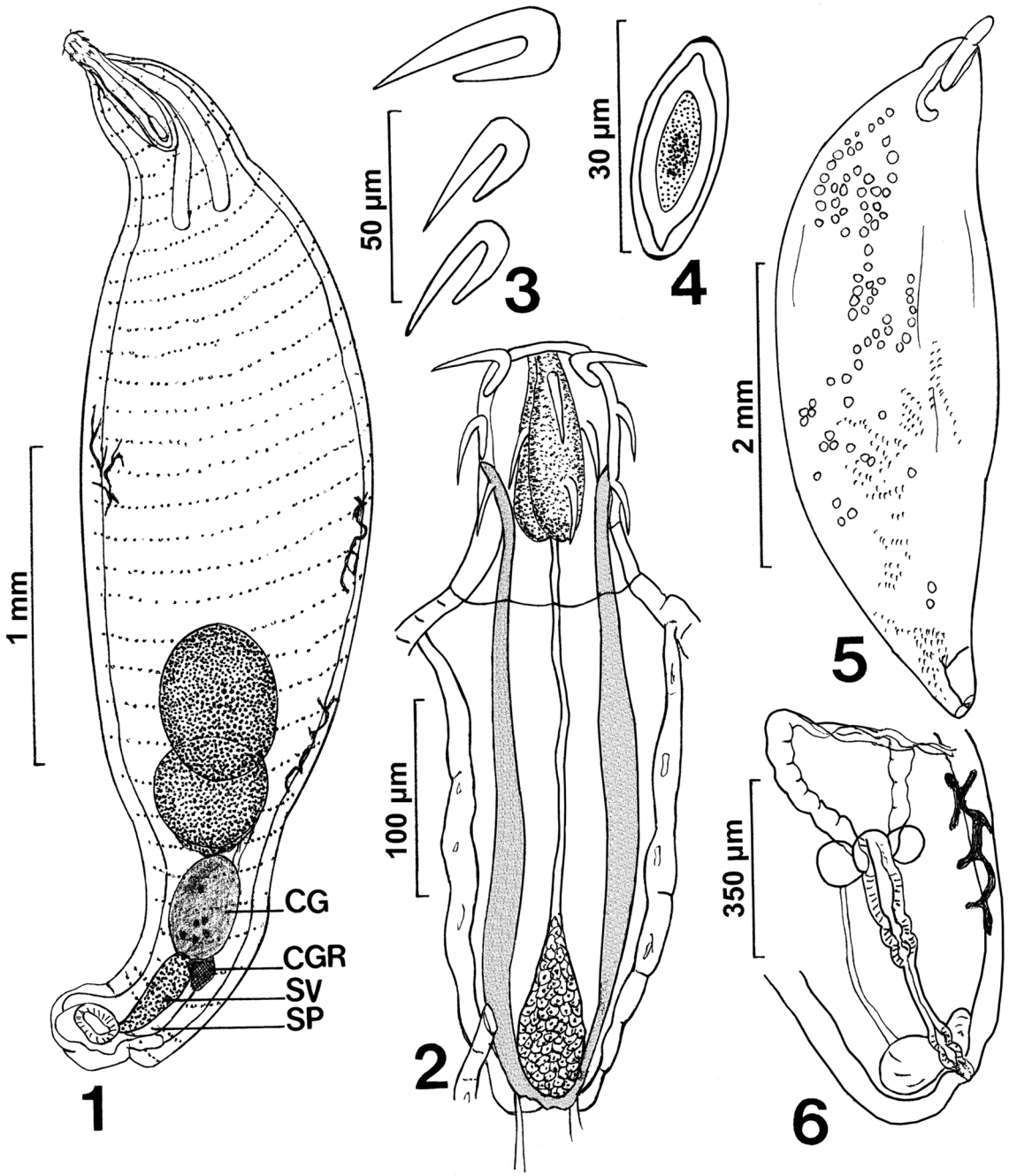
### *Acanthogyrus* (*Acanthosentis*) *fusiformis* n. sp

Specimens of this new species of the subgenus *Acanthosentis* are described from the catfish *Arius* sp. in the Pacific Ocean in the south of Vietnam at the Gulf of Thailand. It is the 46th species of *Acanthosentis* known to date and the 6th described from marine or brackish waters; three species are known from India and Pakistan and two from Japan. Most other species of *Acanthosentis* infect freshwater fishes. The recognition of *A. fusiformis* n. sp. as a new species is based on the key to 44 species of the subgenus in Amin [3], the original descriptions of all species, relevant literature, and specimens of the many species in the senior author's collections. The following is a morphological description of the new species based on microscopical examination of 20 of the 32 specimens collected from 4 of 10 individuals of *Arius* sp.

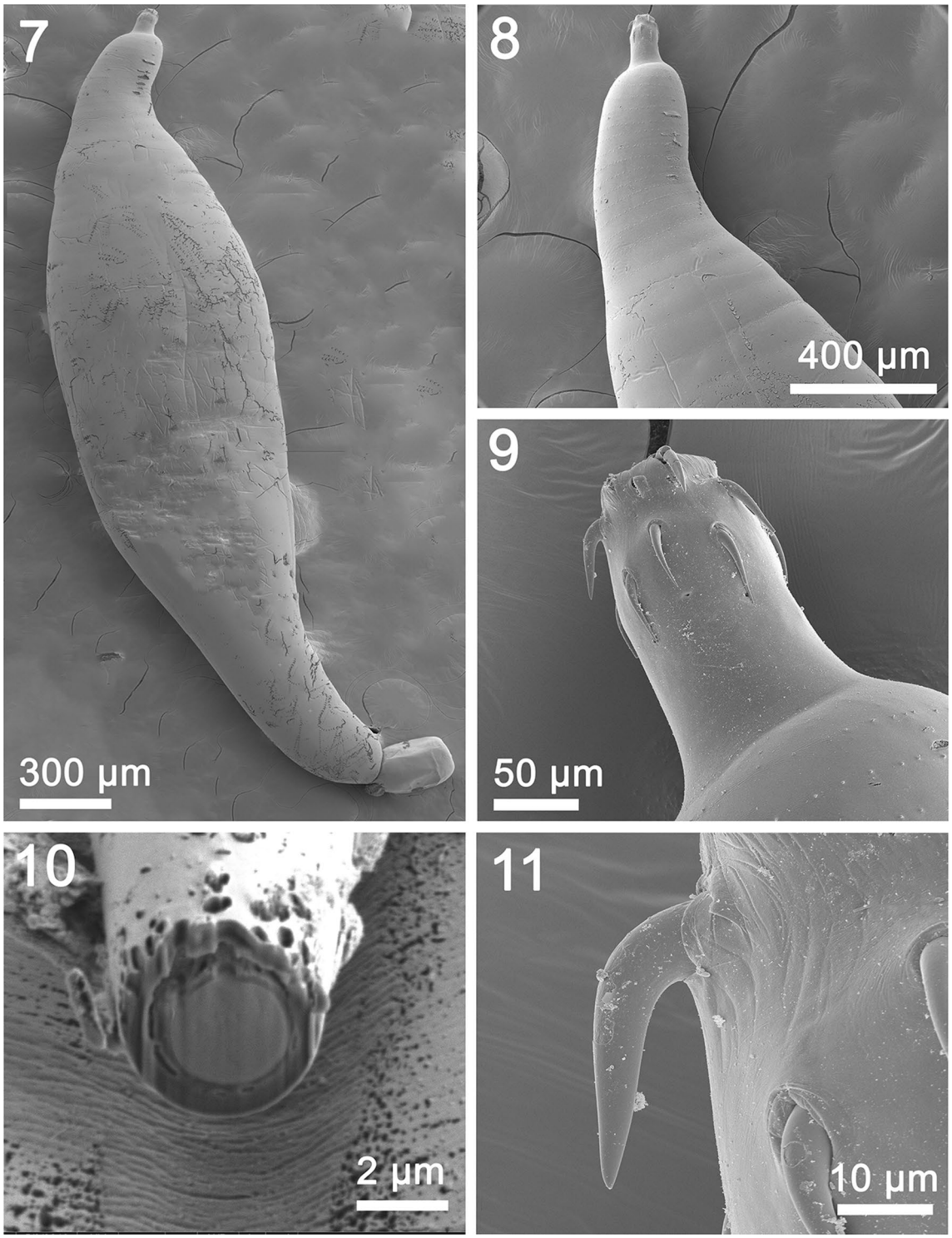
### Morphological Description (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32)

#### General

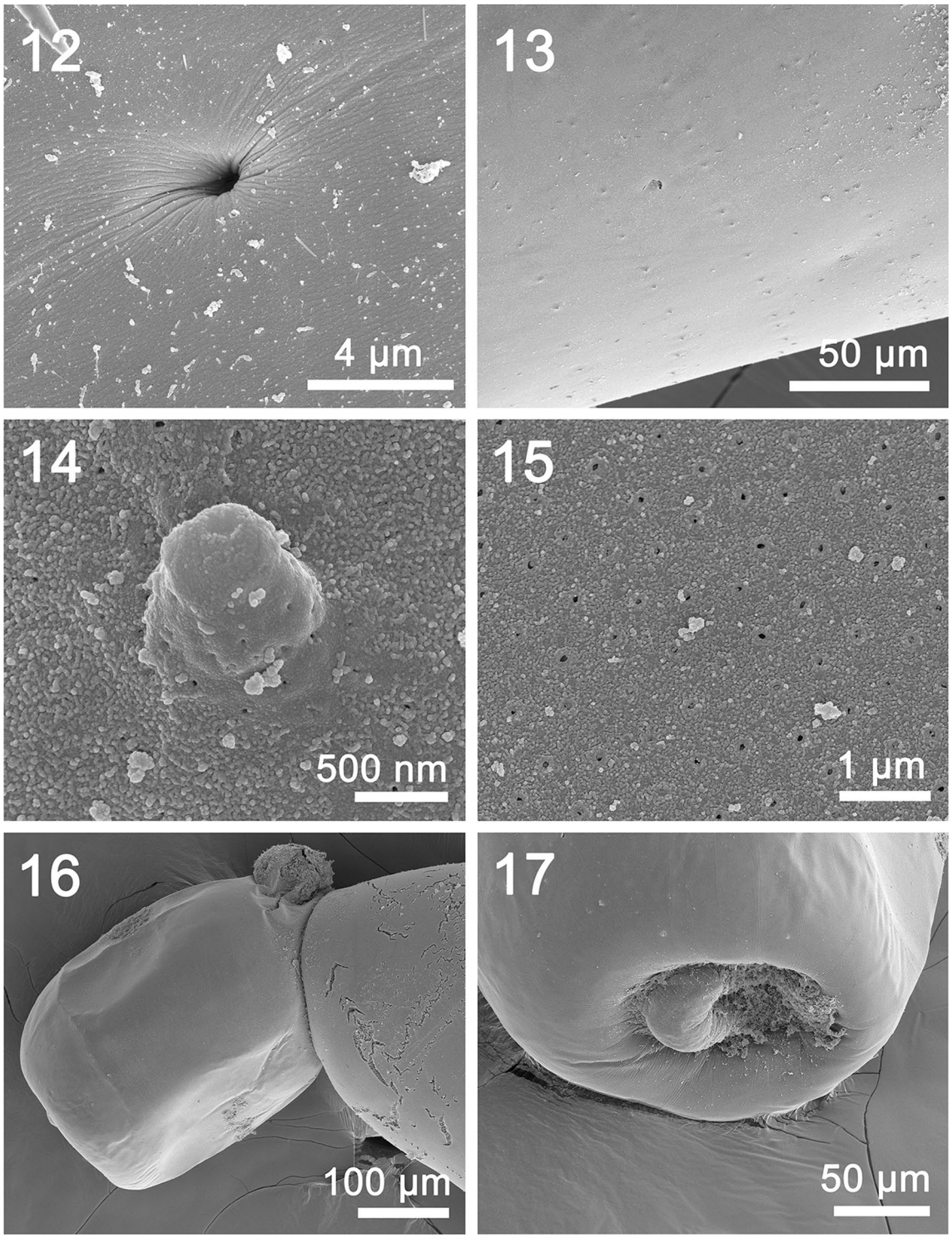
With characters of the genus *Acanthogyrus* Thapar, 1927 and the subgenus *Acanthosentis* Verma and Datta, 1929



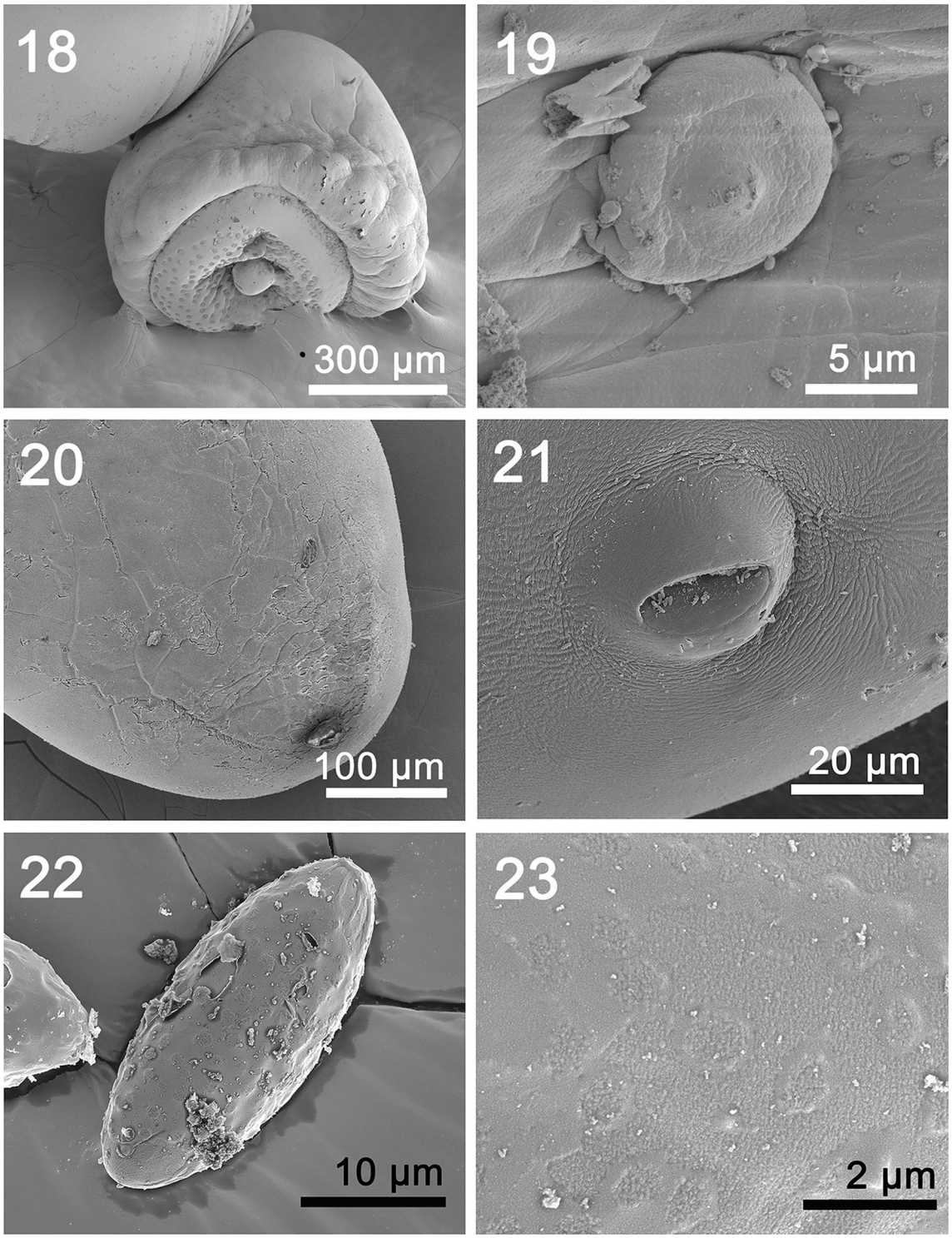

**Figs. 1–6** Line drawings of male and female specimens of *Acanthosentis fusiformis* n. sp. collected from *Arius* sp. in the Pacific coast of Vietnam. **1** A male paratype. Note the regularly spaced spines and the posteriorly widening circles of trunk spines, the stringy-branched giant hypodermal nuclei, and the posterior position of the reproductive system. *CG* cement gland, *CGR* cement gland reservoir, *SP* Saefftigen's pouch, *SV* sperm vesicle. **2** Anterior part of a male specimen. Note the double apical organ, long widening neck, shaded proboscis receptacle, drop-shaped cerebral ganglion connected to the apical organ with apical sensory cord, and two nucleated para-receptacle structures (PRS). **3** One longitudinal row of hooks and hook roots. **4** Egg. Note the thickening of the polar ends of the outer shell. **5** A paratype female with the same fusiform trunk shape as males. **6** Female reproductive system. Note the attachment of the uterine bell to the ventral body wall, few uterine bell glands, stringy-branched giant nucleus, and the large para-vaginal pouch attached to uterine bell gland with straight dorsal duct



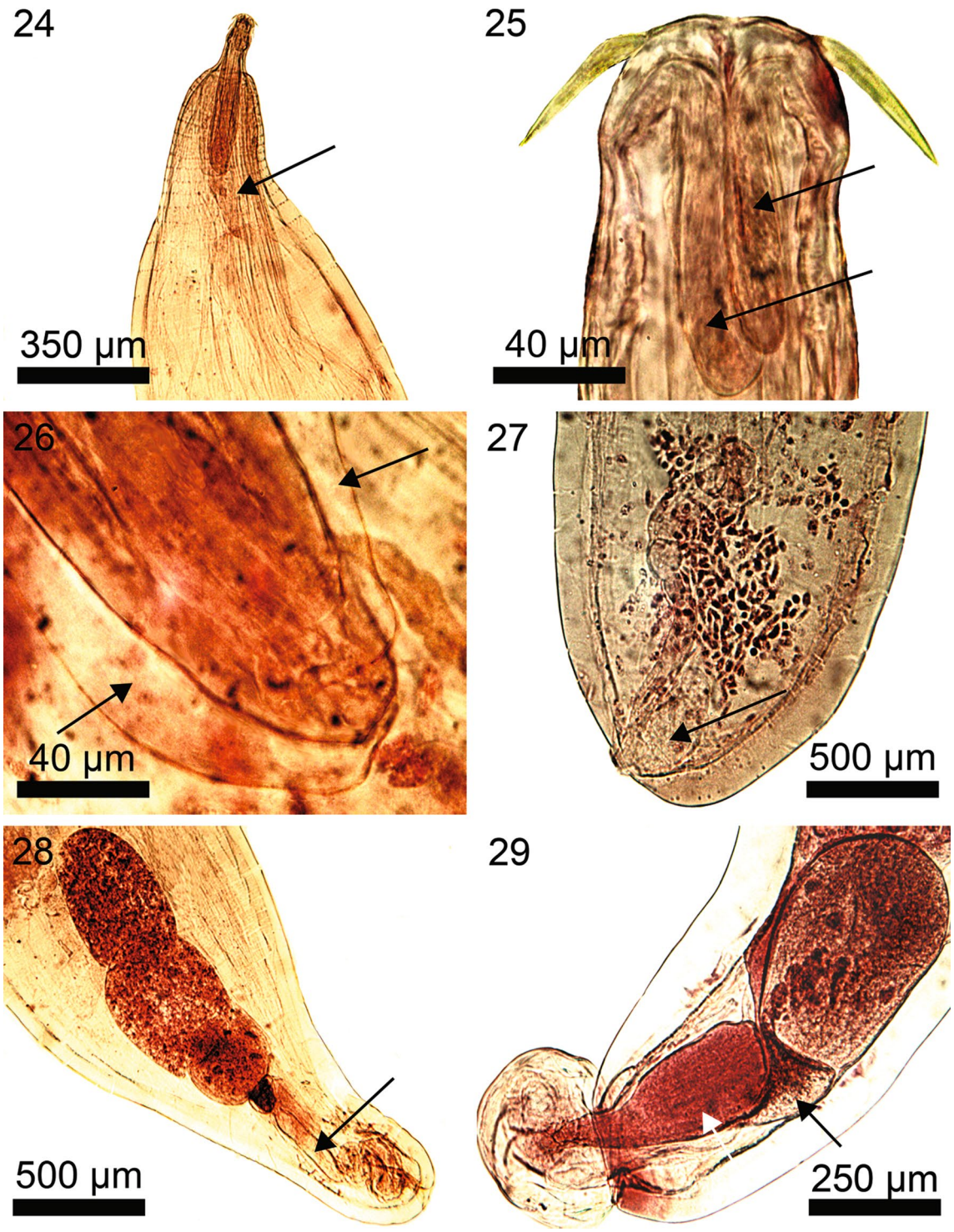

**Figs. 7–11** SEM of male and female specimens of *Acanthosentis fusiformis* n. sp. collected from *Arius* sp. in the Pacific coast of Vietnam. **7** A male specimen. Females have the same fusiform shape except they are wider. **8** The sharply tapering anterior end of another specimen. **9** The proboscis of the specimen in Fig. 8 showing its truncated anterior end swelling posteriorly and prominent neck with two sensory pores on 1 side. **10** A gallium cut hook showing is solid core and dense cortical layer. **11** A high magnification of a lateral hook from the proboscis in Fig. 9




**Figs. 12–17** SEM of male and female specimens of *Acanthosentis fusiformis* n. sp. collected from *Arius* sp. in the Pacific coast of Vietnam. **12** A high magnification of a sensory pore from the neck of a specimen. **13** A view of circles of trunk spines. **14** A high magnification of a truncated trunk spine. Note the extension of the body wall micropores on the cuticle of the spine. **15** Micropores from the mid-trunk of a specimen. **16** A lateral view of a bursa. **17** A view of a retracted bursa showing its terminal position




**Figs. 18–23** SEM of male and female specimens of *Acanthosentis fusiformis* n. sp. collected from *Arius* sp. in the Pacific coast of Vietnam. **18** A semi-lateral view of a bursa showing the prevalence of rings of sensory structures and the thickness of the bursa rim. **19** A higher magnification of 1 sensory cup from the bursa of a specimen. **20** The posterior end of a female specimen showing the sub-ventral position of the gonopore. **21** A high magnification of another female gonopore showing its prominent orifice. **22** An egg. **23** A high magnification of the cortical surface of an egg showing its shallow tuberculate surface




**Figs. 24–29** Microscope images of male and female specimens of *Acanthosentis fusiformis* n. sp. collected from *Arius* sp. in the Pacific coast of Vietnam. **24** Anterior end of a male specimen showing the pattern of distribution of trunk spines. Note the size of the lemniscus (arrow) compared to that of the receptacle. **25** The proboscis extending into the neck of a male specimen showing the shape and orientation of the anterior hooks and the double apical organs (arrows). **26** The posterior portion of the proboscis receptacle and the insertion of the dorsal and ventral para-receptacle structures through the receptacle wall (arrows). **27** The posterior part of a female reproductive system showing the prominent para-vaginal pouch (arrow). **28** A male reproductive system. Note the darkly stained cement reservoir directly posterior to the cement gland and Saefftigen's pouch (arrow). **29** The posterior part of a male reproductive system showing the cement reservoir (black arrow) and the seminal vesicle (white arrow)

as outlined in Amin [3]. Shared structures markedly larger in females than in males. Trunk small, fusiform (Figs. 1, 5, 7), with many electron dense micropores extending on trunk spines (Figs. 14, 15). Body wall markedly thicker ventrally, with 1 or 2 dorsal or ventral, barely visible, stringy-branched giant hypodermal nuclei (Fig. 1). Trunk spines small, 3–8 long, hollow (Fig. 14), smallest to barely detectable most posteriorly, covering the whole body in complete circles (Fig. 13), closely set anteriorly but wider apart more posteriorly (Figs. 1, 8, 24), in 25–41 (31) complete circles in males and 41–64 (48) circles in females. Trunk spines about equal distance apart in each circle, fewest anteriorly, increasing in number in following circles corresponding to widening of mid-trunk then decreasing in number as trunk tapers posteriorly. Number of spines in half circles for first 20 circles from anterior: 15–22 (19), 15–27 (22), 16–29 (22), 18–27 (24), 20–31 (26), 21–33 (27), 24–40 (30), 23–45 (30), 22–40 (29), 26–42 (31), 21–39 (31), 28–39 (32), 29–40 (32), 28–39 (33), 27–39 (33), 30–40 (34), 34–40 (37), 33–50 (42), 32–51 (41), 33–51 (42). Proboscis almost rectangular, longer than wide, truncated and abruptly narrowing anteriorly, with double prominent apical organs usually reaching level of posterior hooks (Figs. 2, 25), sensory pits at posterior tip of middle and posterior hooks (Figs. 9, 12), and three circles of hooks. Middle and posterior hooks directed posteriorly, about equal and only slightly shorter than laterally directed anterior hooks (Figs. 2, 9, 11, 25). Hooks with solid central core and somewhat moderately think cortical layer (Fig. 10). Roots of all hooks simple, stout, about half as long as blades (Fig. 3). Neck large, continuous with outline of the proboscis without distinct separation, and gradually widening posteriorly (Figs. 2, 8, 9). Proboscis receptacle about 4 times as long as proboscis, with single strong wall extending anteriorly past posterior end of proboscis to level of base of posterior hooks, and lined anteriorly and at middle with thin multilayered nucleated cellular sheath. Cerebral ganglion at posterior end of receptacle, large, drop-shaped, markedly longer than apical organ and slightly longer than proboscis, connected to apical organ via apical sensory cord (Fig. 2). Two para-receptacle structures (PRS), dorsal and ventral, in all specimens (Figs. 2, 26). Lemnisci digitiform, thick, equal, about twice as long as receptacle with one large, oval, barely discernible giant nucleus each.

*Males* (based on six whole-mounted adult specimens with sperm and four specimens studied by SEM). Trunk 2.25–3.62 (2.85) mm long by (0.69–1.00) mm wide. Proboscis 87–115 (105) long by 75–95 (86) wide. Apical organ 70–100 (82) long by 25–42 (31) wide. Proboscis hooks from anterior 45–55 (49), 35–40 (37), 35–40 (39) long. Neck 75–100 (87) long by 100–125 (112) posteriorly. Proboscis receptacle 330–388 (352) long by 75–104 (88) wide. Cerebral ganglion 100–130 (115) long by 32–50 (42) wide posteriorly. Lemnisci 425–625 (560) long by 50–83 (68) wide. Reproductive system at posterior quarter of trunk. Testes and other reproductive structures contiguous and often overlapping (Fig. 1). Anterior testis 333–676 (501) long by 239–572 (367) wide, larger than posterior testis 250–572 (423) long by 250–426 (376) wide. Cement gland spheroid, 208–385 (291) long by 208–312 (243) wide with at least six small giant nuclei. Cement reservoir ovoid-elongate to triangulate, 94–166 (123) long by 110–125 (115) wide. Saefftigen's pouch 262–364 (312) long by 62–83 (70) wide overlapping sperm vesicle 281–354 (326) long by 135–187 (152) wide anteriorly (Fig. 1). Bursa globular, muscular, with thick rim and many sensory cups clustered around center and in few peripheral circles (Figs. 16–19, 28, 29), 198–228 (213) long by 218–291 (254) wide.

*Females* (based on 14 whole-mounted sexually mature specimens with eggs and ovarian balls and four specimens studied by SEM). Trunk 2.87–6.00 (4.43) mm long by 0.82–1.75 (1.22) mm wide. Proboscis 105–132 (116) long by 80–125 (93) wide. Apical organ 77–112 (92) log by 22–45 (32) wide. Proboscis hooks from anterior 40–65 (52), 36–50 (41), 36–50 (42) long. Neck 50–100 (78) long by (75–156) 111 wide posteriorly. Proboscis receptacle 339–492 (409) long by 87–114 (99) wide. Cerebral ganglion 117–150 (134) long by 47–57 (51) wide posteriorly. Lemnisci 728–884 (803) long by 45–115 (80) wide. Reproductive system 625–1060 long with subterminal gonopore, often winding short uterus multi-bulbar distally, few large uterine bell glands, prominent funnel-shaped uterine bell with filamentous attachment of its distal end to ventral body wall. Large para-vaginal pouch attached to uterine bell glands with straight dorsal duct (Fig. 6, Fig. 27). Eggs narrowly ellipsoid with thickening of opposite outer shell poles, some prolongation of nuclear membrane and disk-like cortical ornamentation (Figs. 4, 22, 23), 20–37 (30) long by 7–15 (11) wide.

**Table 1** Chemical composition of gallium (Ga) cut hooks for *Acanthosentis fusiformis*

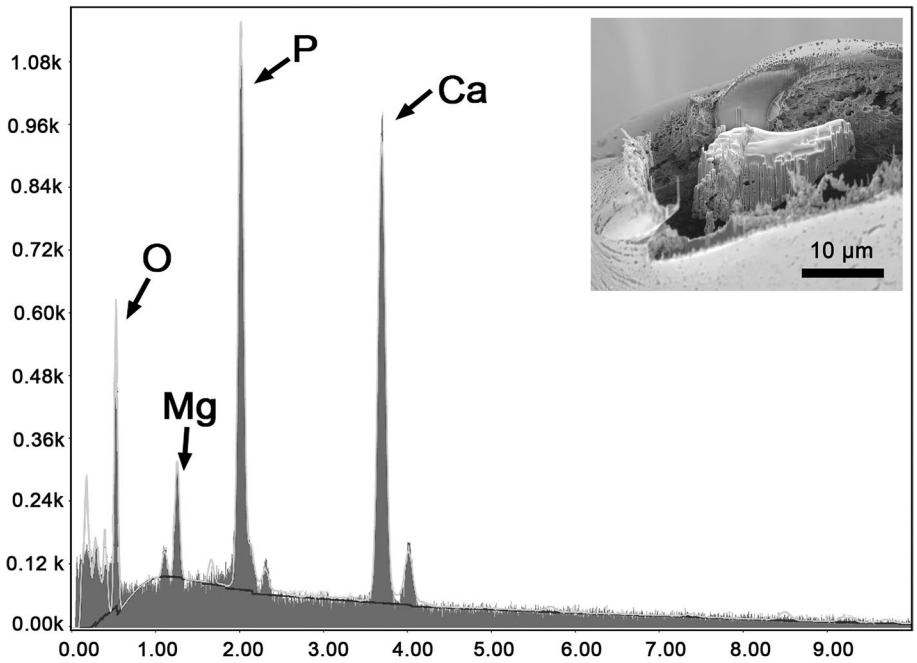
| Element[c] | Hook tip[a] | Mid hook[a] | Mid hook[a] | Hook base[b] | |
|---|---|---|---|---|---|
| | | Center | Edge | Arch | Center |
| Magnesium (Mg) | 0.20 | 2.98 | 2.18 | 1.89 | **2.93**[d] |
| Phosphorus (P) | 4.04 | 15.69 | 10.43 | 14.55 | **20.62** |
| Sulfur (S) | 13.49 | 4.05 | 9.42 | 0.22 | **0.81** |
| Calcium (Ca) | 5.05 | 27.98 | 15.97 | 37.56 | **40.39** |

[a]Cross-sectional cuts

[b]Longitudinal cut

[c]All elements are listed in wt%. Common protoplasm elements (C, N, O) and processing elements (Au, Pd, Ga) omitted from the table

[d]Bolded figures are those used in the EDXA spectrum (Fig. 30)




| Element | Weight % | Atomic % | Error % | Net Int. | K Ratio |
|---|---|---|---|---|---|
| C | 2.19 | 4.55 | 19.45 | 18.05 | 0.0086 |
| N | 10.37 | 18.48 | 14.75 | 41.58 | 0.0219 |
| O | 19.75 | 30.82 | 10.11 | 206.22 | 0.0533 |
| Ga | 1.25 | 0.45 | 20.92 | 18.47 | 0.0089 |
| Mg | 2.93 | 3.01 | 7.77 | 111.41 | 0.02 |
| P | 20.62 | 16.62 | 3.13 | 688.71 | 0.1678 |
| Au | 1.33 | 0.17 | 31.67 | 21.04 | 0.0113 |
| S | 0.81 | 0.63 | 30.81 | 25.02 | 0.0064 |
| Cl | 0.07 | 0.05 | 99.99 | 1.89 | 0.0005 |
| Pd | 0.29 | 0.07 | 59.83 | 3.8 | 0.002 |
| Ca | 40.39 | 25.16 | 3.21 | 738.98 | 0.3382 |

**Fig. 30** X-ray elemental scan (XEDS) of the center of a hook showing high levels of calcium and phosphorus and low level of magnesium. Insert: SEM of a gallium cut hook showing its solid core

## Energy-Dispersive X-Ray Analysis (EDXA)

The results of the x-ray microanalysis are presented in Table 1 and the spectrum (Fig. 30) based metal analysis of a longitudinal cut of the center of hook base. The data show high levels of calcium and phosphorous. Common protoplasm elements (C, N, O) and processing elements (Au, Pd, Ga) are omitted from the table.

## Phylogenetic Analysis

Two partial 18S rDNA (1710–1735 nt) and two ITS1-5.8S-ITS2 (750–758 nt) sequences were generated for *A. fusiformis* n. sp. No intraspecific sequence divergence was found in different isolates of *A. fusiformis* regarding 18S and ITS1-5.8S-ITS2 rDNA sequences. Tables 2 and 3 present data for the sequences used in the phylogenetic analyses, retrieved from GenBank. Both algorithms, ML and BI, produced trees with identical topology for the 18S gene and ITS1-5.8S-ITS2 region (Figs. 31, 32). The ML and Bayesian inference (BI) tree of 18S sequences of *A. fusiformis* n. sp. were well supported with posterior probability values 100/1.00 (Fig. 31). The phylogenetic analyses of 18S sequences show *Neoechinorhynchus* species as a sister group, while the data for 18S gene sequences are virtually lacking in GenBank as only two sequences are available for molecular comparison (Fig. 31). Phylogenetic analyses based on the ITS1-5.8S-ITS2 data set of *A. fusiformis* n. sp., grouped with high support (99/1.00) with a clade formed by isolates of *A. cheni* from China (82.69–85.76% similarity), and with the species of *Neoechinorhynchus* (Fig. 32). Although for ITS1-5.8S-ITS2 cluster, only 45 sequences from *A. cheni* isolates and

**Table 2** Acanthocephalan species information used for phylogenetic analysis based on the 18S gene cluster

| Species | Host | Host origin | GenBank accession no. |
|---|---|---|---|
| *Acanthosentis* Verma and Datta 1929 | | | |
| *Acanthosentis* sp.1 NKG-2016 | Na | India | KY305529 |
| *Acanthosentis* sp.2 NKG-2016 | Na | India | KY305530 |
| *Acanthosentis fusiformis* n. sp. isolate 1 | *Arius* sp. | Vietnam | MK834520* |
| *Acanthosentis fusiformis* n. sp. isolate 2 | *Arius* sp. | Vietnam | MK834518* |
| *Floridosentis* Ward 1953 | | | |
| *Floridosentis mugilis* | *Mugil cephalus* | Mexico | AF064811 |
| *Hebesoma* Van Cleave 1928 | | | |
| *Hebesoma violentum* | *Perccottus glenii* | Russia | KF156881 |
| *Neoechinorhynchus* Stiles and Hassall 1905 | | | |
| *Neoechinorhynchus dimorphospinus* | *Liza subviridis* | Thailand | MK510080 |
| *Neoechinorhynchus* sp. | *Siganus fuscescens* | China | HM545898 |
| *Neoechinorhynchus* sp. | Na | China | KM507363 |
| *Neoechinorhynchus* sp. | *Capoeta aculeata* | Iran | KU363972 |
| *Neoechinorhynchus buttnerae* | Na | Brazil | MK249749 |
| *Neoechinorhynchus crassus* | *Capoeta aculeata* | Iran | KU363974 |
| *Neoechinorhynchus pseudemydis* | Na | USA | NPU41400 |
| *Neoechinorhynchus cylindratus* | *Micropterus salmoides* | USA | MF974925 |
| *Neoechinorhynchus salmonis* | *Oncorhynchus nerka* | Russia | KF156878 |
| *Neoechinorhynchus saginata* | Na | USA | AY830150 |
| *Neoechinorhynchus tumidus* | *Coregonus nasus* | Russia | KF156876 |
| *Neoechinorhynchus simansularis* | *Salvelinus alpinus* | Russia | KF156877 |
| Out-group | | | |
| *Moniliformis* sp. n. OMA-2018 | *Hemiechinus auritus* | Iraq | MH401043 |

Sequences marked with an asterisk were obtained in this study

*Na* not available

two from Malaysia (MK184204 and MK184205) are available in database.

## Taxonomic Summary

Type host. the catfish, *Arius* sp. (Ariidae: Siluriformes).

Type locality. The south Pacific Coast of Vietnam at Bac Lieu in the Gulf of Thailand (9°15′N 105°45′E).

Site of infection. Intestine.

Specimens. HWML collections nos. 139484 (holotype and paratypes on 1 slide), 139485 (allotype female and paratypes on 1 slide)'.

Infection parameters. Four infected of ten examined (40%) fish were infected with 32 specimens; mean: 3.20.

Etymology. The species is named for the fusiform shape of the trunk.

## Remarks

*Acanthogyrus* (*A.*) *fusiformis* is the 47 species of the subgenus *Acanthosentis* known from fishes mostly but not only from the Indo-West Pacific, African, and European regions of the world. The key by Amin [3] recognizes 44 valid species. *Acanthogyrus* (*Acanthosentis*) *barmeshoori* Amin, Gholami, Alkhalghi, Heckmann, 2013 and *A.* (*A.*) *kashmirensis* Amin, Heckmann, Zargar, 2017 are the 45th and 56th species. Only three other species of *Acanthosentis* are known from marine or brackish water fishes in India and Pakistan. The Indian species include *A.* (*A.*) *arii* Bilqees, 1971 off the Karachi coast, *A.* (*A.*) *oligospinus* Anantaraman, 1980 off the Madras coast, and *A.* (*A.*) *indicus* Tripathi, 1956 in estuaries of Hooghly and Matla rivers.

*Acanthosentis fusiformis* n. sp. is readily distinguished from all other species of *Acanthosentis* by the keys in Amin [3] and Amin et al. [11] and by its following unique combination of characters: small fusiform trunk with complete circles of small hollow spines covering the whole trunk, prominent double apical organs often extending posteriorly past posterior hooks, large neck continuous with the outline of the proboscis without distinct separation, extension of the proboscis receptacle anteriorly past the base of the proboscis up to the insertion point of the posterior hooks, presence of two para-receptacle structures (PRS), short female reproductive system with filamentous attachment of the distal end of the uterine bell to the ventral body wall. It is also appropriate to further separate it from *A. arii*, the only other species

**Table 3** Species of acanthocephalan, their host, origin and GenBank accession numbers used for phylogenetic analysis based on the ITS1-5.8S-ITS2 gene cluster

| Species | Host | Host origin | GenBank accession no. |
|---|---|---|---|
| *Acanthosentis* Verma and Datta 1929 | | | |
| *Acanthosentis cheni* isolates | *Coilia nasus* | China | JX960708–JX960752 |
| *Acanthosentis fusiformis* n. sp. isolate 1 | *Arius* sp. | Vietnam | MK834517* |
| *Acanthosentis fusiformis* n. sp. isolate 2 | *Arius* sp. | Vietnam | MK834519* |
| *Acanthosentis* sp. | *Barbonymus schwanefeldii* | Malaysia | MK184204 |
| *Acanthosentis* sp. | *Barbonymus schwanefeldii* | Malaysia | MK184205 |
| *Neoechinorhynchus* Stiles and Hassall 1905 | | | |
| *Neoechinorhynchus salmonis* | *Ptychocheilus oregonensis* | USA | MK238094 |
| *Neoechinorhynchus golvani* | *Dormitator latifrons* | Mexico | FJ968116 |
| *Neoechinorhynchus golvani* | *Dormitator latifrons* | Mexico | FJ968117 |
| *Neoechinorhynchus golvani* | *Dormitator latifrons* | Mexico | FJ388972 |
| *Neoechinorhynchus brentnickoli* | *Dormitator latifrons* | Mexico | MG870674 |
| *Neoechinorhynchus brentnickoli* | *Dormitator latifrons* | Mexico | MG870683 |
| *Neoechinorhynchus brentnickoli* | *Dormitator latifrons* | Mexico | MG870681 |
| *Neoechinorhynchus brentnickoli* | *Dormitator latifrons* | Mexico | KC004181 |
| *Neoechinorhynchus brentnickoli* | *Dormitator latifrons* | Mexico | KC004183 |
| *Neoechinorhynchus mexicoensis* | *Dormitator maculatus* | Mexico | MG870825 |
| *Neoechinorhynchus mexicoensis* | *Dormitator maculatus* | Mexico | MG870822 |
| *Neoechinorhynchus* sp. | *Dormitator maculatus* | Mexico | MG870826 |
| *Neoechinorhynchus mamesi* | *Dormitator latifrons* | Mexico | MG870869 |
| *Neoechinorhynchus mamesi* | *Dormitator latifrons* | Mexico | MG870866 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870626 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870627 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870628 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870629 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870630 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870631 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870632 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870633 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870634 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870635 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870636 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870637 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870638 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870639 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870640 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870641 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870642 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870643 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870644 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870645 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870646 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870647 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870648 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870649 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870650 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870651 |
| *Neoechinorhynchus* sp. | *Dormitator latifrons* | Mexico | MG870652 |
| Out-group | | | |
| *Mediorhynchus* sp. | Na | Mexico | AF416413 |

Sequences marked with an asterisk were obtained in this study

*Na* not available

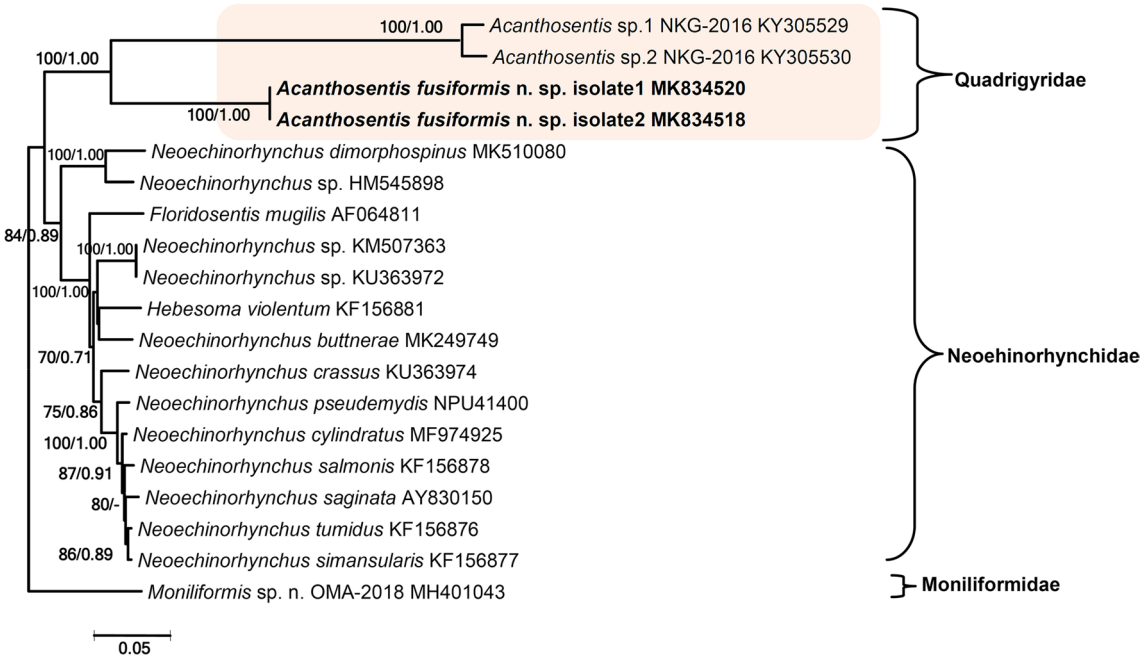



**Fig. 31** Phylogenetic tree based on analysis of ribosomal DNA sequences (18S) for *Acanthosentis fusiformis* n. sp. Bootstrap percentages refer to maximum likelihood/Bayesian inference analysis. Only bootstrap values above 70% are shown. GenBank accession numbers are indicated after species names. Name of the studied species collected for present study is shown in bold. Hyphen indicates node unsupported by BI. *Moniliformis* sp. was selected as the outgroup

of *Acanthosentis* described from another marine fish of the same genus as *A. fusiformis*, *Arius serratus* Day (Ariidae: Siluriformes) from the Karachi coast, in the Arabian Sea, Indian Ocean. Specimens of *A. arii* are larger worms with posteriorly enlarged trunk that differ from *A. fusiformis* n. sp. by having comb-like trunk spines covering the whole body, lemnisci enclosed in common sheath fused terminally, much longer female reproductive system about half as long as trunk, larger round eggs, and no neck or Saefftigen's pouch [15, 16].

## Zoogeography

Petrochenko [35] recognized four species of *Acanthosentis*, Golvan [23] eight species, Yamaguti [48] 11 species, Bullock [17] 12 species, Amin [2] 26 species, Golvan [22] 37 species, and Amin [4] 43 species. Discrepancies are related to the description of new species and a number of synonymies. Currently, 47 species of *Acanthosentis*, including *A. fusiformis* n. sp., have been described. All species were evaluated and are treated as valid, even though some are hardly recognizable, e.g., *A.* (*A.*) *arii* Bilqees, 1971, *A.* (*A.*) *holospinus* Sen, 1938 and *A.* (*A.*) *shuklai* Agrawal and Singh, 1982. Twelve species were recognized as taxonomically problematic and discussed by Amin [3].

The following notes update and amend the account in Amin [3]. Almost 50 years after Bullock [17] remarked that "*Acanthosentis* (with 12 species) appears to be primarily a genus of parasites in southern Asia and Africa," of a total of 47 species are considered valid, 39 (83%) are known from Asia, 6 (13%) from Africa, 1 (2%) from Central America, and 1 (2%) from Europe. They occur in cyprinid fish hosts, but there are notable exceptions. Of the 39 Asian species, 23 (58%) are known from the Indian subcontinent, 1 (3%) from Iran, 1 (3%) from Malaya, 1 (3%) from Thailand; 1 (3%) from Russia, 9 (23%) from China, 2 (6%) from Japan, and 1 (3%) from Vietnam. The Indian subcontinent appears to be the major center of radiation and diversification of *Acanthosentis.* Worms with all forms of proboscis armature and trunk spination and with some unusual features of the reproductive systems are found in this region.

In the Indian subcontinent, most species have been found in freshwater fishes [1, 38, 40]. However, 2 marine and 1 estuarine species have also been reported: *A.* (*A.*) *arii* Bilqees, 1971 in *Arius serratus* Day (syn. *A. thalassinus* Rüppell) (Ariidae) off the Karachi coast in the Arabian Sea, Indian Ocean [15, 16]; *A.* (*A.*) *oligospinus* Anantaraman, 1980 in *Mystus gulio* (Hamilton) (Bagridae) off the Madras Coast, Bay of Bengal, Indian Ocean [14]; and *A.* (*A.*) *indicus* Tripathi, 1956 in *Setipinna phasa* (Hamilton) (Engraulidae) and *Hilsa ilisha* (Hamilton) (= *Tenualosa ilisha* Hamilton, 1822) (Clupeidae) from the estuary of the Hooghly and Matla rivers as well as from the River Ganges [42]. All four species of fish are native to the northern Indian Ocean,

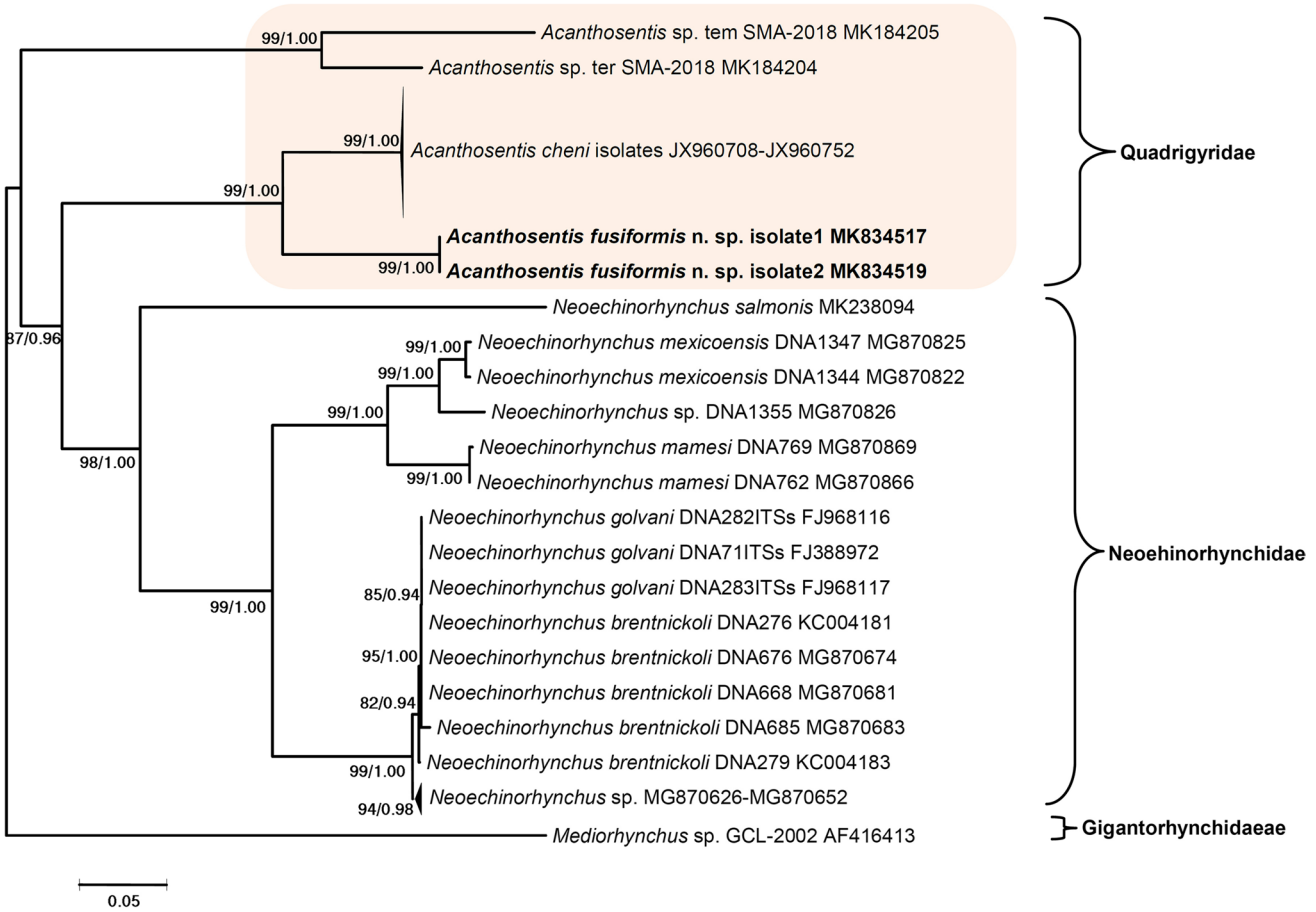



**Fig. 32** Phylogenetic analysis for *Acanthosentis fusiformis* n. sp. based on the ITS1-5.8S-ITS2 region of rDNA data set. *Mediorhynchus* sp. is used as out-group. The bootstrap values are listed in the order: ML/BI. GenBank accession numbers are provided alongside the species names. *Acanthosentis fusiformis* n. sp. collected for present study is shown in bold

but were projected to spread as far as Australia, the Gulf of Thailand, Polynesia, the Red Sea, Vietnam, and Japan [34, 36, 44, 45]. This makes *A.* (*A.*) *fusiformis* n. sp. the fourth species of marine/estuarine distribution of known Asian species of the subgenus. There are no unusual anatomical distinctions of these four marine/estuarine species that distinguish them from the freshwater representatives. There are, however, distinctive anatomical features that distinguish *A. fusiformis* from the above-mentioned marine species as well as from all other species of the subgenus. These features are summarized in the Remarks section.

Two other species of *Acanthosentis* are also known from marine fishes in the Caribbean and the Mediterranean waters. The occurrence of *A.* (*A.*) *acanthuri* in three species of marine surgeon fishes (Acanthuridae) of the Caribbean (off Puerto Rico and Tobago, West Indies) distant from the larger endemic species clusters in Asia and Africa is noteworthy. The three host species of *A.* (*A.*) *acanthuri*, *Acanthurus bahianus* Castelnau, *A. chirurgus* (Bloch) and *A. coeruleus* Bloch and Schneider are found in the western Atlantic Ocean from Massachusetts to Brazil and in the eastern Atlantic Ocean off Angola, Ascension Island and Senegal [37]. The two species of *Acanthosentis* collected off the Pakistani and Indian coasts, *A.* (*A.*) *arii* and *A.* (*A.*) *oligospinus*, parasitize the marine fishes *Arius serratus* and *Mystus gulio*, respectively. The first fish species is found in the NW Indian Ocean, the Red Sea, the Philippines and the Gulf of Thailand [36]; the latter off countries bordering the eastern Indian Ocean from India to Indonesia and off Vietnam and Pakistan [34]. The presence of *A.* (*A.*) *adriaticus* Amin, 2005 in the Mediterranean region and *A.* (*A.*) *acanthuri* in the Caribbean may suggest a Tethysian relationship between the Caribbean, Mediterranean, and the Indian/Pakistani species. Otherwise, it must be concluded, accordingly, that *A.* (*A.*) *acanthuri* and *A.* (*A.*) *adriaticus* have diversified independently over a long period and that their possible ancestor remains unknown. *Acanthogyrus* (*A.*) *adriaticus* was described from *Liza aurata* (Risso) in the Adriatic Sea off Yugoslavia. Its distributional range is in the eastern Atlantic (from Scotland to the Cape Verde Islands,

Mediterranean and Black Sea, as well as coastal waters from southern Norway to Morocco) [41]. The possibility of a relationship with such North African species as *A.* (*A.*) *maroccanus* is considered highly unlikely in light of the considerable anatomical differences between the two species.

China represents the second major Asian center of diversification of the subgenus. Most species were reported from freshwater fishes in the southeastern province of Fukien. There are probably more species of *Acanthosentis* elsewhere in China. The three remaining Asian species of *Acanthosentis* appear to represent extensions of the range of distribution of the Chinese species. A southern extension from the Fukien Province would include *A.* (*A.*) *siamensis* Farooqi and Sirikanchana, 1987 in Thailand [19] and *A.* (*A.*) *partispinus* Furtado, 1963 in Malaya [20]. A northern extension would include *A.* (*A.*) *intermedius* Achmerov and Dombrowskaja-Achmerova, 1941 in the Amur River Basin in Russia. The known Japanese representatives include *A.* (*A.*) *alternatspinus* Amin, 2005 and *A.* (*A.*) *parareceptaclis* Amin, 2005 in Lake Biwa [3]. *Acanthogyrus* (*A.*) *alternatspinu* was probably introduced to Japan with its type-host *Rhodeus o. ocellatus* from China in the 1950s [27]. The other Japanese species, *A.* (*A.*) *parareceptaclis*, appears to be native to the Lake Biwa basin because of its unique para-receptacle structures and vaginal sleeve and on the fact that it is found exclusively in *Cobitis biwae*, which is endemic to Japan [32, 49].

The distribution of the six known species of *Acanthosentis* in Africa cannot be accounted for by the dispersal of their fish hosts, except for *A.* (*A.*) *tilapiae* which has been reported from 30 ubiquitous species of cichlid (28 of the genus *Tilapia*) and three non-cichlid species in Tanzania, Congo, Uganda, Chad, Nigeria, Egypt and Malawi [12]. The other five species have regional distributions in geographically distant and unrelated parts of the continent: *A.* (*A.*) *malawiensis* in Malawi, *A.* (*A.*) *maroccanus* in Morocco, *A.* (*A.*) *nigeriensis* in Niger, *A.* (*A.*) *papilio* in Senegal and *A.* (*A.*) *phillipi* in South Africa.

## Discussion

### The Energy-Dispersive X-Ray Analysis

Our studies of acanthocephalan worms have usually involved X-ray scans (EDXA) of gallium cut hooks and other worm structures [25, 26, 39]. Hooks are evaluated for chemical ions with sulfur (S), calcium (Ca) and phosphorus (P) being the prominent elements in most acanthocephalan attachment structures. Calcium and phosphorus were major ions in the base of hooks of *A. fusiformis* but sulfur was highest in hook tips. This appears to be the characteristic profile of that species. Like finger prints, the EDXA appears to be species specific and have significant diagnostic value in acanthocephalan systematics [6]. For example, *Moniliformis cryptosaudi* Amin, Heckmann, Sharifdini, and Albayati, 2019b was erected based primarily on its EDXA pattern [9].

### The Para-Receptacle Structure (PRS)

The PRS inserts anteriorly in the body wall near the neck and posteriorly at the posterior end of the receptacle [10]. The presence of the PRS in eoacanthocephalans with weak single proboscis receptacle wall was first demonstrated in *Neoechinorhynchus* (*N.*) *qatarensis* Amin, Saoud, Alkuwari, 2002 [13] and had since been reported in other species of *Neoechinorhynchus* Stiles and Hassall, 1905 and *Acanthogyrus* (*Acanthosentis*) Verma and Datta, 1929 reviewed in part in Amin et al. [5]. In the description of the PRS, Amin et al. [10, 13] proposed that it may regulate the hydrostatic pressure in the receptacle to facilitate the retraction and eversion of the proboscis.

### The Micropores

The electron dense micropores present throughout epidermal surface of the trunk of *A. fusiformis*, like those reported from other species of the Acanthocephala, are associated with internal crypts and vary in diameter and distribution in different trunk regions corresponding with differential absorption of nutrients. We have reported micropores in a large number of acanthocephalan species summarized in Heckmann et al. [24] and in a few more since, and demonstrated the tunneling from the cuticular surface into the internal crypts by TEM. Amin et al. [8] gave a summary of the structural–functional relationship of the micropores. The functional aspects of micropores in a few other acanthocephalan species including *Rhadinorhynchus ornatus* Van Cleave, 1918, *Polymorphus minutes* (Goeze, 1782) Lühe, 1911, *Moniliformis moniliformis* (Bremser, 1811) Travassos (1915), *Macracanthorhynchus hirudinaceus* (Pallas, 1781) Travassos (1916, 1917), and *Sclerocollumrubrimaris* Schmidt and Paperna, 1978 were reviewed by Amin et al. [8]. Wright and Lumsden [47] and Byram and Fisher [18] reported that the peripheral canals of the micropores are continuous with canalicular crypts. These crypts appear to "constitute a huge increase in external surface area implicated in nutrient up take." Whitfield [46] estimated a 44-fold increase at a surface density of 15 invaginations per 1 $\mu m^2$ of *Moniliformis moniliformis* (Bremser, 1811) Travassos, 1915 tegumental surface. The micropores and the peripheral canal connections to the canaliculi of the inner layer of the tegument of *Corynosoma strumosum* (Rudolphi, 1802) Lühe, 1904 from the Caspian seal *Pusacaspica* (Gmelin) in the Caspian Sea were demonstrated by transmission electron micrographs [7].

## Molecular Analysis

The phylogenetic trees inferred with the 18S gene generated in this study showed that the new sequenced isolates of *Acanthosentis fusiformis* n. sp. from Vietnam formed independent lineage supported by high bootstrap (ML) and posterior probability (BI) values. Based on 18S gene among the two isolates of *Acanthosentis fusiformis* n. sp., no intraspecific genetic divergence was found. For 18S, molecular data have been available only for two sequences from unknown *Acanthosentis* sp. from India to date on GenBank database (Table 2). The genetic divergence among isolates of *Acanthosentis fusiformis* n. sp. and the two unknown *Acanthosentis* species from India shows 0.5% variation. For the phylogenetic trees inferred with the ITS1-5.8S-ITS2 region, only sequences of *A. cheni* from China and of two unidentified species from Malaysia are available (Table 3). The phylogenetic trees based on ITS1-5.8S-ITS2 region showed a clear resolution of *Acanthosentis fusiformis* n. sp. isolates, generated a separate lineage within a clade also formed by isolated of sister species *A. cheni* from China. There is no intraspecific genetic divergence found among the isolates of *Acanthosentis fusiformis* n. sp in ITS1-5.8S-ITS2 region. The interspecific genetic divergence among *Acanthosentis fusiformis* n. sp. isolates and *A. cheni* are 0.14% while with two unidentified species from Malaysia ranged from 0.4 to 0.5%. Finally, the species diversity of *Acanthosentis* species can be different from what we know today, as currently most of the species are described based only on the morphology and we do not have much data for molecular comparisons. The scarcity of molecular data for the species of *Acanthogyrus* (*Acanthosentis*) creates problems for deducing correct phylogenetic relationships with other genera. Therefore, additional studies of species of *Acanthosentis* from other congeneric species based on morphological and molecular genetic data will be needed to reconstruct the evolutionary history and phylogenetic affinities of this group of acanthocephalans. Addressing the questions regarding the mono-/paraphyletic nature of the species of *Acanthosentis* also requires additional analysis. We do hope that the current study adds importance to the use of molecular data in the present and future research on the species of *Acanthosentis* and that comparison can be more readily made.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare no conflict of interest and compliance with all relevant ethical standards.

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