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ON THREE SPECIES OF *NEOECHINORHYNCHUS* (ACANTHOCEPHALA: NEOECHINORHYNCHIDAE) FROM THE PACIFIC OCEAN OFF VIETNAM WITH THE MOLECULAR DESCRIPTION OF *NEOECHINORHYNCHUS* (*N.*) *DIMORPHOSPINUS* AMIN AND SEY, 1996

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KEY WORDS

Acanthocephala
Neoechinorhynchus longnucleatus
Neoechinorhynchus manubrianus
Neoechinorhynchus dimorphospinus
Molecular Description
New Features
Gallium Cuts
Host and Distribution Records

ABSTRACT

Specimens of 3 species of *Neoechinorhynchus* Stiles and Hassall, 1905, were collected from a number of species of marine fish along the Pacific coast of Vietnam. New information is added to the descriptions of *Neoechinorhynchus* (*Neoechinorhynchus*) *longnucleatus* Amin, Ha, and Ha, 2011, and its wider host and geographical distribution are reported. Similarly, more descriptive information and host and geographical records are added to our knowledge of *Neoechinorhynchus* (*Hebosoma*) *manubrianus* Amin, Ha, and Ha, 2011, and *Neoechinorhynchus* (*Neoechinorhynchus*) *dimorphospinus* Amin and Sey, 1996. The latter species was previously known only from the Persian Gulf. The presence of the para-receptacle structure was documented in all 3 species of *Neoechinorhynchus* reported. The molecular characterization of *N. dimorphospinus* was carried out using a partial *18S rDNA* sequence. The phylogenetic analysis showed that most species of *Neoechinorhynchus* are very closely related, while *N. dimorphospinus* was distinct from others in the tree. Metal analysis of hooks of *N. dimorphospinus* using Energy Dispersive X-ray Analysis also distinguished its characteristic finger print of high phosphorus and calcium and low sulfur levels.

Most of the recent taxonomic work on the Acanthocephala from Vietnam has been reported by the Amin-Heckmann-Ha team since 2000. A number of acanthocephalan species from freshwater fish, amphibians, reptiles, birds, and mammals were previously described in Vietnam by Amin and Ha (2008) and Amin et al. (2000, 2004, 2008a, 2008b, 2008c). Additionally, 11 other species of acanthocephalans were collected from marine fish off the eastern seaboard of Vietnam in Halong Bay in 2008 and 2009. Of these, 6 new species of *Neoechinorhynchus* Stiles & Hassall 1905, 1 new species of *Heterosentis* Van Cleave, 1931, and 2 new species of *Rhadinorhynchus* Lühe 1911 were described (Amin et al., 2011a, 2011b, 2011c). Four other species of echinorhynchid acanthocephalans from marine fishes in Halong Bay were described by Amin and Ha (2011), and 5 other new species from fishes and amphibians of 8 collected host species were described by Amin et al. (2014). More recently, Amin et al. (2018b, 2018c), Amin et al. (2019a), and Ha et al. (2018) described new echinorhynchid, Neoechinorhynchid, cavisomid, arhythmacanthid, rhadinorhynchid, and diplosentid acanthocephalans from various parts of Vietnam, mostly in marine fishes off the Pacific coast. Three other species of *Rhadinorhynchus* and 1 species of *Gorgorhynchus* were otherwise previously reported from marine fishes in Vietnam by Arthur and Te (2006).

Fifteen other species of acanthocephalans in 5 families were more recently collected from fishes in the Pacific and amphibians in central Vietnam in 2016 and 2017. In the present report, we supplement the descriptions of 3 species of *Neoechinorhynchus* Stiles and Hassall, 1905, using scanning electron microscopy (SEM) and photomicroscopy and report on their expanded host and geographical distribution. We also examined the metal composition and phylogenetic relationships of *Neoechinorhynchus dimorphospinus* Amin and Sey, 1996, vs. other species of *Neoechinorhynchus* in relationship to other acanthocephalan families using a partial *18S rDNA* sequence.

Table I. Present host and geographical distribution of *Neoechinorhynchus longnucleatus* in the Pacific Ocean off Vietnam.

Hosts	Amin et al. (2011a)	Amin et al. (2018c)	This paper
Strongylura strongylura			
Specimens	5♂♂, 7♀♀ in 2/2 fish	—	4♂♂ in 2/5 fish
Date	May 2009		April 2017
Location	Halong Bay Haiphong 20°54′N, 107°12′E		Cat Ba, Tonkin 20°54′N, 107°12′E
HWML Coll. no.*	49216, 49217		139455, 139456
S. strongylura	—	—	
Specimens			2♂♂,4♀♀ in 2/6 fish
Date			March 2017
Location			Tien Yen, Tonkin 21°20′N, 107°24′E
Leiognathus equulus	—		—
Specimens		1♀ in 1/1 fish	
Date		October 2016	
Location		Nha Trang 12°15′N, 109°11′E	
Tylosurus acus metanotus	—	—	
Specimens			1♂,3♀♀ in 3/10 fish
Date			April 2016
Location			Haiphong 20°51′54.5″N, 106°41′01.8″E
HWML Coll. no.			139457
Liza melinoptera	—	—	
Specimens			1♂,1♀ in 1/7 fishes
Date			March 2017
Location			Tien Yen, Tonkin 21°20′N, 107°24′E
HWML Coll. no.			139458
Total	5♂♂, 7♀♀	1♀	8♂♂,8♀♀

* A representative sample of each collection was deposited at the HWML collection. Other specimens are in the O.M.A. personal collection.

MATERIALS AND METHODS

Collections

Three species of neoechinorhynchid acanthocephalans were collected from marine fishes along the coast of Vietnam. Information on the collections of *Neoechinorhynchus longnucleatus* Amin, Ha, and Ha, 2011, is presented in Table I. Specimens of *Neoechinorhynchus manubrianus* Amin, Ha, and Ha, 2011, were found in *Johnius carouna* (Cuvier) (Sciaenidae) in the south at Nha Trang (12°15′N,109°11′E) on October 14, 2017, and in *Johnius* sp. in the north at Tien Yen (Gulf of Tonkin) (21°20′N,107°24′E) on March 19, 2017. Specimens of *N. dimorphospinus* were found in a mugilid fish, *Liza subviridis* (Mugilidae) (Valenciennes) in the south at Kien Giang (Gulf of Thailand) (10°0′N, 105°10′E) on July 6, 2017. Specimens of all 3 species were processed for microscopical studies, and specimens of *N. dimorphospinus* were further studied for SEM and hook metal analyses and molecular studies.

Methods

Freshly collected acanthocephalans were extended in water until proboscides were everted and fixed in 70% ethanol for transport to our Institute of Parasitic Diseases in Scottsdale, Arizona, for processing and further studies. 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 reaching 100% (24 hr each), and cleared in 100% xylene and then transferred to a solution of 50% Canada balsam and 50% xylene (24 hr 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.

A subset of the voucher specimens were deposited in the University of Nebraska's State Museum's Harold W. Manter Laboratory (HWML) collection in Lincoln, Nebraska. The remaining specimens are in the OMA personal collection.

SEM (Scanning Electron Microscopy)

Specimens of *N. manubrianus* and *N. dimorphospinus* that had been fixed and stored in 70% ethanol were processed for SEM following standard methods (Lee, 1992). These included critical point drying in sample baskets and mounting on aluminum stubs using conductive double-sided carbon tape. Samples were coated with gold and palladium for 3 min using a Polaron no. 3500 sputter coater (Quorum [Q150 TES] (Quorum Technologies Ltd., East Sussex, U.K.) establishing an approximate thickness of 20 nm. Samples were placed and observed in an FEI Helios Dual Beam Nanolab 600 (FEI, Hillsboro, Oregon) scanning electron microscope with digital images obtained in the Nanolab software system (FEI) and then transferred to a USB drive for future reference. Samples were received under low vacuum conditions using 10 KV, spot size 2, 0.7 Torr using a GSE detector.

Table II. Metal composition of gallium cut hooks of *Neoechinorhynchus dimorphospinus*.

	Tip cut†		Mid cut‡	Hook base‡	
Element*	Edge	Center	Edge	Middle	Entry
Magnesium (Mg)	1.43	1.44	1.46	1.58	1.56
Phosphorus (P)	12.16	17.34	9.56	17.78	15.23
Sulfur (S)	4.41	0.88	2.50	0.86	1.07
Calcium (Ca)	23.61	37.53	16.60	37.67	31.01
Sodium (Na)	0.00	0.00	0.00	1.20	1.89

* Common protoplasmic elements (C, N, O) and processing elements (Ga, Pd, Au) omitted. Wt. % listed.
† Cross-sectional cuts.
‡ Longitudinal cuts.

Energy Dispersive X-Ray Analysis (EDXA)

Standard methods were used for preparation similar to the SEM procedure. Specimens of *N. dimorphospinus* were examined and positioned with the SEM instrument described above, which was equipped with a Phoenix Energy-dispersive X-ray Analyzer (FEI). 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 TEAM (Texture and Elemental Analytical Microscopy) software system (FEI) was used. Data were stored on a USB drive for future analysis. The data included weight percentage and atom percentage 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 Liquid Ion Metal Source (LIMS) 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 nA 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 using an XL30 ESEM FEG (FEI). The XL30 ESEM scope can do transmission EM work or transmission electron microscopy (TEM) as well as scanning SEM while the sample is still on the stage. The scope is equipped with software using TEAM for EDXA analysis. The cut was then analyzed with an 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 drive for future use. The intensity of the GIS was variable according to the nature of the material being cut.

Molecular methods

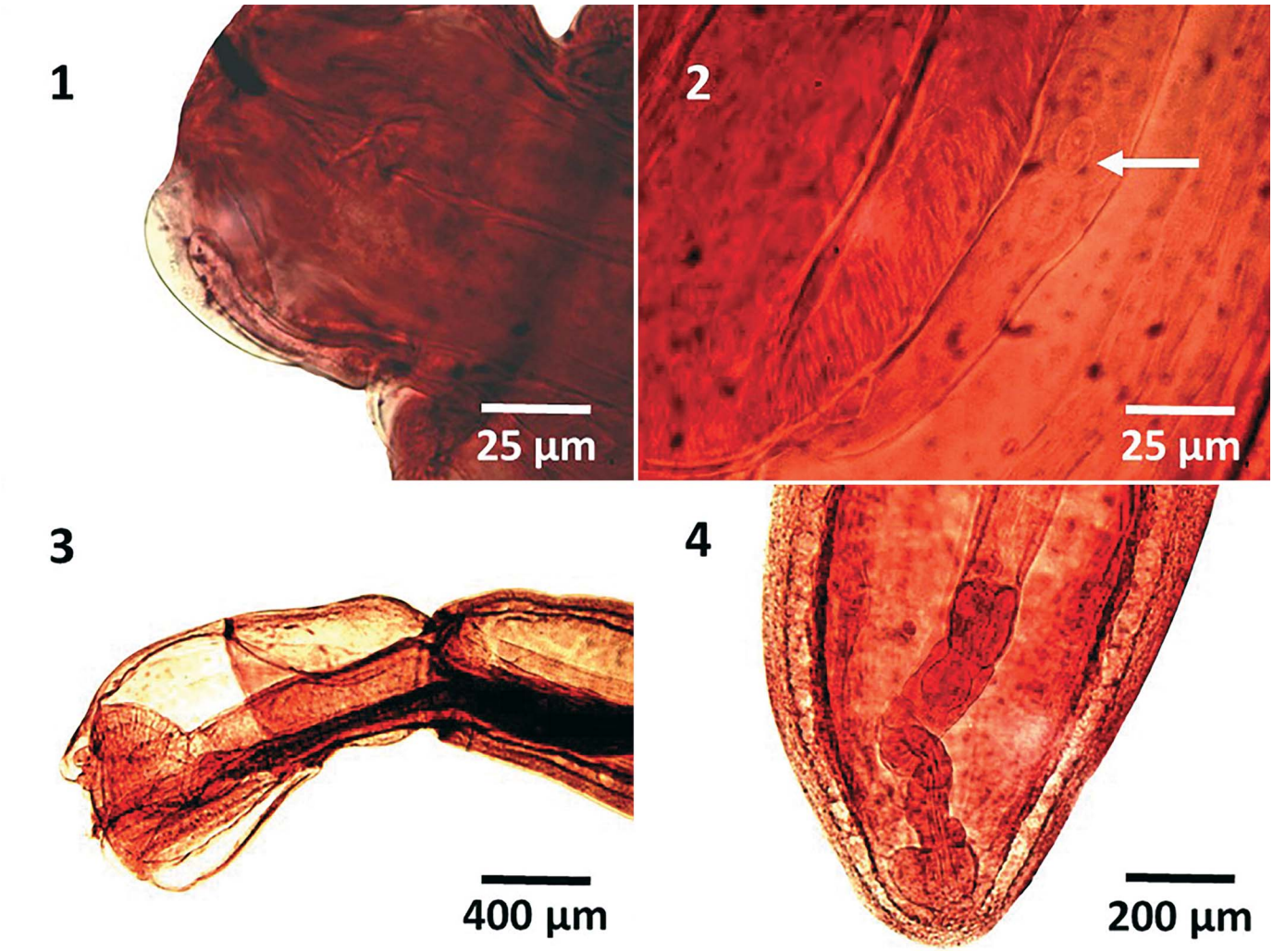
Adult worms of *N. dimorphospinus* were washed 3 times in sterile distilled water in order to remove the ethanol. Total genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and stored at –20 C until PCR amplification. PCR reactions were performed in 30 µl volumes containing 2 × red PCR premix (Ampliqon, Odense, Denmark), 20 pmol of each primer, and 3 µl of extracted DNA. Forward primer (5′-AGATTAAGCCATGCATGCGTAAG-3′) and reverse primer (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) were used to amplify a 1,685 base pair (bp) fragment of the *18S rRNA* gene (Near et al., 1998). The cycling condition included initial denaturation step at 95 C for 6 min, 35 cycles of 95 C for 30 sec, 57 C for 30 sec, and 72 C for 90 min, followed by a final extension at 72 C for 6 min. Finally, PCR product was electrophoresed on 1.5% of agarose gel and visualized with an ultraviolet transluminator. Next, the PCR product was submitted to Bioneer Company (Daejeon, Korea) and sequenced in both directions using the same PCR primers. Sequence results were manually edited and analyzed with BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and Chromas software (https://technelysium.com.au/wp/chromas/). The sequence was compared with GenBank submitted sequences using the BLAST system (http://www.ncbi.nlm.nih.gov/), and multiple sequence alignment was carried out using the Clustal W method for Bioedit software. Phylogenetic analysis was performed using the maximum-likelihood method and Tamura 3-parameter model with Molecular and Evolution Genetic Analysis software version 6 (MEGA 6). A bootstrap with 1,000 replications was also implemented to determine the reliability of the tree topologies obtained. If the nodal support was lower than 70%, there was no hard conflict and explained as being due to chance alone. The partial *18S rDNA* sequence of *N. dimorphospinus* was deposited in GenBank (accession number MK510080). The sequences used for the phylogenetic analysis are listed in Table II.

RESULTS

The nomenclature of the species of *Neoechinorhynchus* follows that of Amin (2002) splitting the genus into 2 subgenera, *Neoechinorhynchus* Stiles and Hassall, 1905, and *Hebesoma* Van Cleave, 1928, based primarily on egg anatomy. The following is a treatment of species of *Neoechinorhynchu*s that have been previously described from marine fish in Vietnamese and Persian Gulf waters. These collections brought to light new features that have not been demonstrable in the past. The partial *18S rDNA* sequence of *N. dimorphospinus*, among other new features, is revealed for the first time.

Neoechinorhynchus (*Neoechinorhynchus*) *longnucleatus* Amin, Ha, and Ha, 2011

This acanthocephalan was first described from *Strongylura strongylura* (van Hasselt) (Belonidae) in Halong Bay by Amin et al. (2011b) and subsequently reported from a new host, *Leiognathus equulus* (Forsskål) (Leiognathidae) in Nha Trang by Amin et al. (2018a). Two additional host species, *Tylosurus acus metanotus* (Bleeker) (Belonidae) and *Liza melinoptera* (Valenciennes) (Mugilidae) in Tien Yen and Haiphong, respectively, were also found (Table I). *Neoechinorhynchus longnucleatus* appears to be widely distributed in 4 species of fish in families along the Vietnamese coastline from Tien Yen in the north to Nha Trang in the south. The shape and measurements of the new specimens were comparable to those in the original description. Additional documentation of above noted descriptions of the anterior proboscis hook and root (Fig. 1), the para-receptacle structure (PRS) on both sides of the proboscis receptacle (Fig. 2) not previously observed, the bursa (Fig. 3), and the female



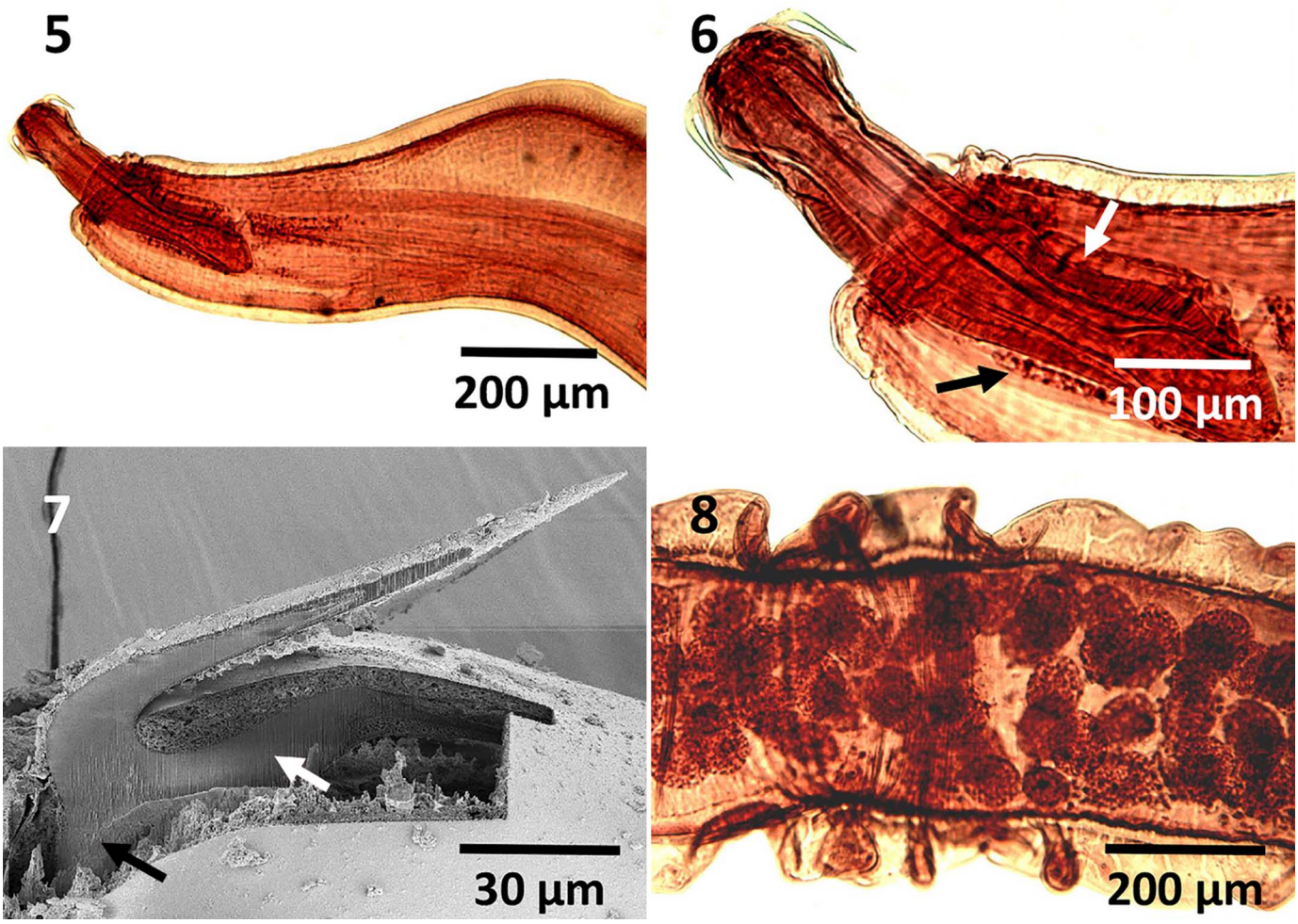
Figures 1–4. Microscopic images of specimens of *Neoechinorhynchus* (*N.*) *longnucleatus* from *Tylosurus acus melanotus* in Vietnam. (**1**) The proboscis of a female specimen showing a perfect anterior hook and root. (**2**) The posterior part of the proboscis receptacle showing the posterior end of the para-receptacle structure (arrow) and its insertion into the receptacle wall. (**3**) A lateral view of the bursa in a male specimen showing the extension of the posterior ducts of the reproductive system into it. (**4**) The posterior part of the female reproductive system showing the shape of the vagina and the sphincter bulbs. Color version available online.

reproductive system (Fig. 4) were made possible with microscopic images for the first time.

Specimens: HWML collections nos. HWML139455, HWML139456 (from *S. strongylura*), HWML139457 (from *L. melanoptera*), and HWML139458 (from *Tylosurus acus metanotus*).

Neoechinorhynchus (*Hebosoma*) *manubrianus* Amin, Ha, and Ha, 2011

Since its original description from *Johnius carouna*, *Nibea albiflora* (Richardson), and *Pennahia argentata* (Houttuyen) (Sciaenidae), by Amin et al. (2011b) in Halong Bay, an expanded description of *Neoechinorhynchus manubrianus* was also previously reported by Amin and Heckmann (2012) from *Nibea albiflora* from the same northern location in Halong Bay. In our present study, female specimens of *N. manubrianus* were collected from 2 individuals of *Johnius* sp. on 19 March 2017, off Tien Yen in the northern Gulf of Tonkin. Two female specimens were also obtained from 1 individual of *J. carouna* in a mixed infection with 7 specimens of *Neoechinorhynchus johnii* on 14 October 2017, off Nha Trang in the south. It appears that *N. manubrianus* has a presence in sciaenid fishes at least along the Pacific coast of Vietnam that extend beyond the limited type location in Halong Bay. The specimens at hand were morphologically similar to those described by Amin et al. (2011b) and Amin and Heckmann (2012) except for the additional documentation of the presence of the PRS (Figs. 5, 6). New microscopic and SEM images shed new light on the anterior trunk showing the antero-dorsal hump (Fig. 5), proboscis, receptacle, and PRS (Fig. 6), the anterior proboscis hook showing the prominent posteriorly directed root (Fig. 7, white arrow) with anterior manubrium (Fig. 7, black arrow), and the cuticular folding of the trunk (Fig. 8). The micropores on the body wall of all specimens of *N. manubrianus* (Amin and Heckmann, 2012; Figs. 9, 10) have also been demonstrated in specimens of the other 2 reported species of *Neoechinorhynchus*.



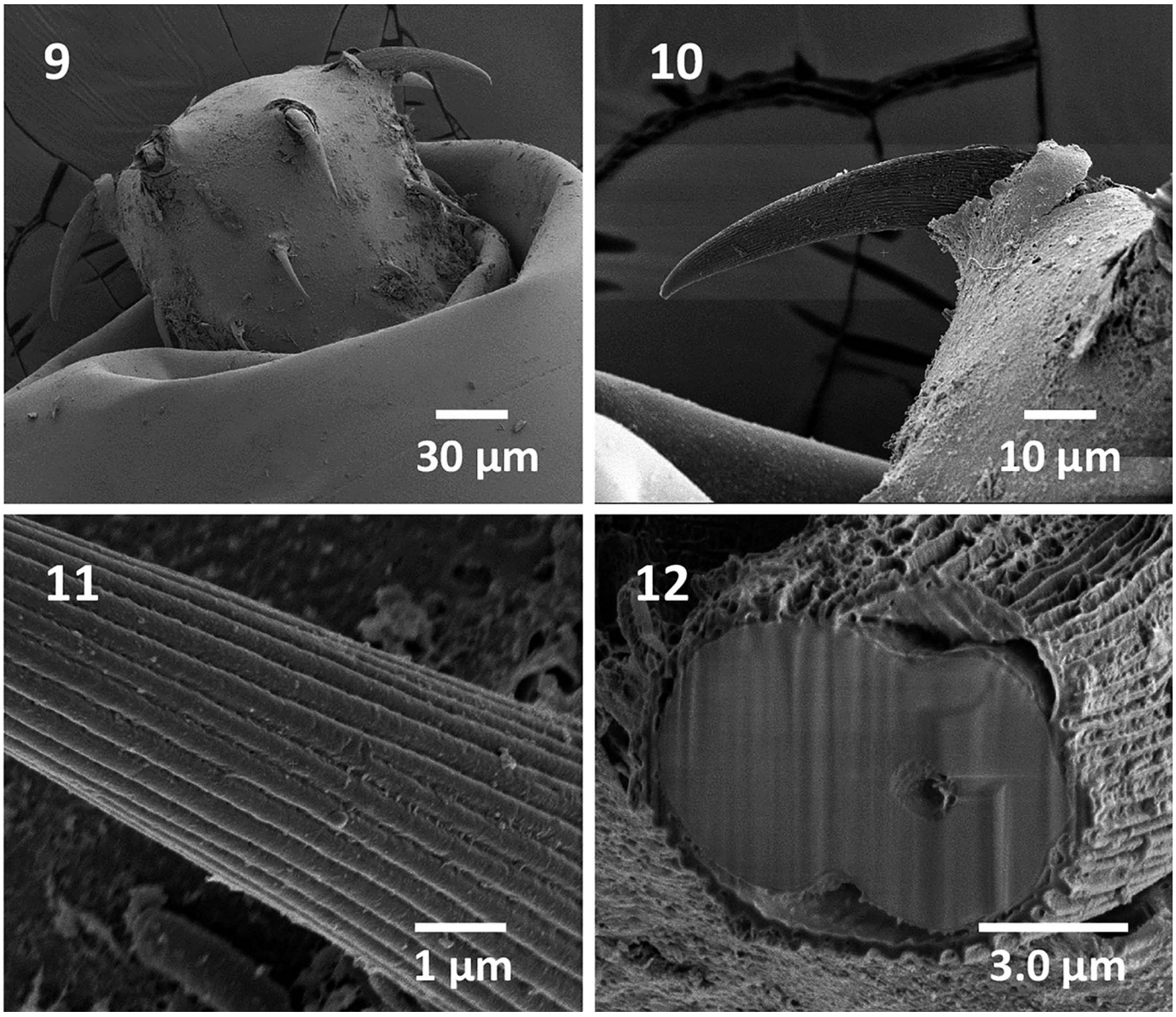
Figures 5–8. Light micrographs of specimens of *Neoechinorhynchus* (*Hebosoma*) *manubrianus* from *Johnius* sp. in Vietnam. (**5**) The anterior part of a female specimen showing the characteristic anterior dorsal trunk hump. (**6**) A higher magnification of the anterior portion of the specimen in figure 5 showing 2 para-receptacle structures (arrows). (**7**) SEM of a gallium cut anterior hook showing the prominent posteriorly directed root (white arrow) and the extended anterior manubrium (black arrow). (**8**) The undulating dorsal and ventral cuticular extensions of the body wall are shown. Color version available online.

Specimens: HWML collection nos. HWML139453, 130454 (from *Johnius* sp. and *J. carouna*).

Neoechinorhynchus (*Neoechinorhynchus*) *dimorphospinus* Amin and Sey 1996

Fourteen specimens of *N. dimorphospinus* were found in 4 of 20 examined individuals of *Liza subviridis* (Valenciennes) on 6 July 2017 on the south Vietnamese Pacific coast at Kien Giang, Gulf of Thailand. Four specimens were used for SEM and future molecular analysis, and 10 were processed for microscopical examination. Specimens of *N. dimorphospinus* was originally described from 4 other species of fish from the Persian Gulf off Kuwait by Amin and Sey (1996): *Dorosoma nasus* (Bloch) (Clupeidae) (type), *Allanetta forskali* (Ruppell) (Atherinidae), *Liza macrolepis* (Smith) (Mugilidae), and *Pseudorhombus arsius* (Hamilton-Buchanan) (Bothiridae). This acanthocephalan was previously reported also in the Persian Gulf off Kuwait from *L. macrolepis* by Amin et al. (1984) and its description expanded from specimens collected from another mullet, *Liza klunzingeri* (Day) in the Shatt Al-Arab estuary near Fao City, Basrah, Iraq. There are only 2 other reports of *N. dimorphospinus* from the Persian Gulf. Abdul-Rahman (1999) reported it from the freshwater mullet, *Liza abu* (Heckel), in the Kurmat Ali River, which drains into the Hammar Marsh of the Shatt Al-Arab drainage system. Bannai (2002) also reported it from the marine mullet *L. subviridis* in the northwest Persian Gulf. The latter fish is the same fish species from which our Vietnamese specimens were collected. The reports by Abdul-Rahman (1999) and Bannai (2002) added nothing new to our taxonomic knowledge of *N. dimorphospinus*. Altogether, this acanthocephalan is presently known from 7 marine and freshwater species of fish in 4 families spread from the Persian Gulf across the Indian Ocean to the Pacific coast of Vietnam. The potential link between these 2 distant locations is the marine, freshwater, brackish, demersal, and catadromous fish, *L. subviridis*, an Indo-West Pacific fish, extending west to the Persian Gulf and Red Sea and east and north to Samoa and Japan (Wright, 1988; Fricke et al., 2011).

The 3 male specimens of *N. dimorphospinus* fit perfectly within the range of characters and measurements in the original description except that the lemnisci appeared more unequal than



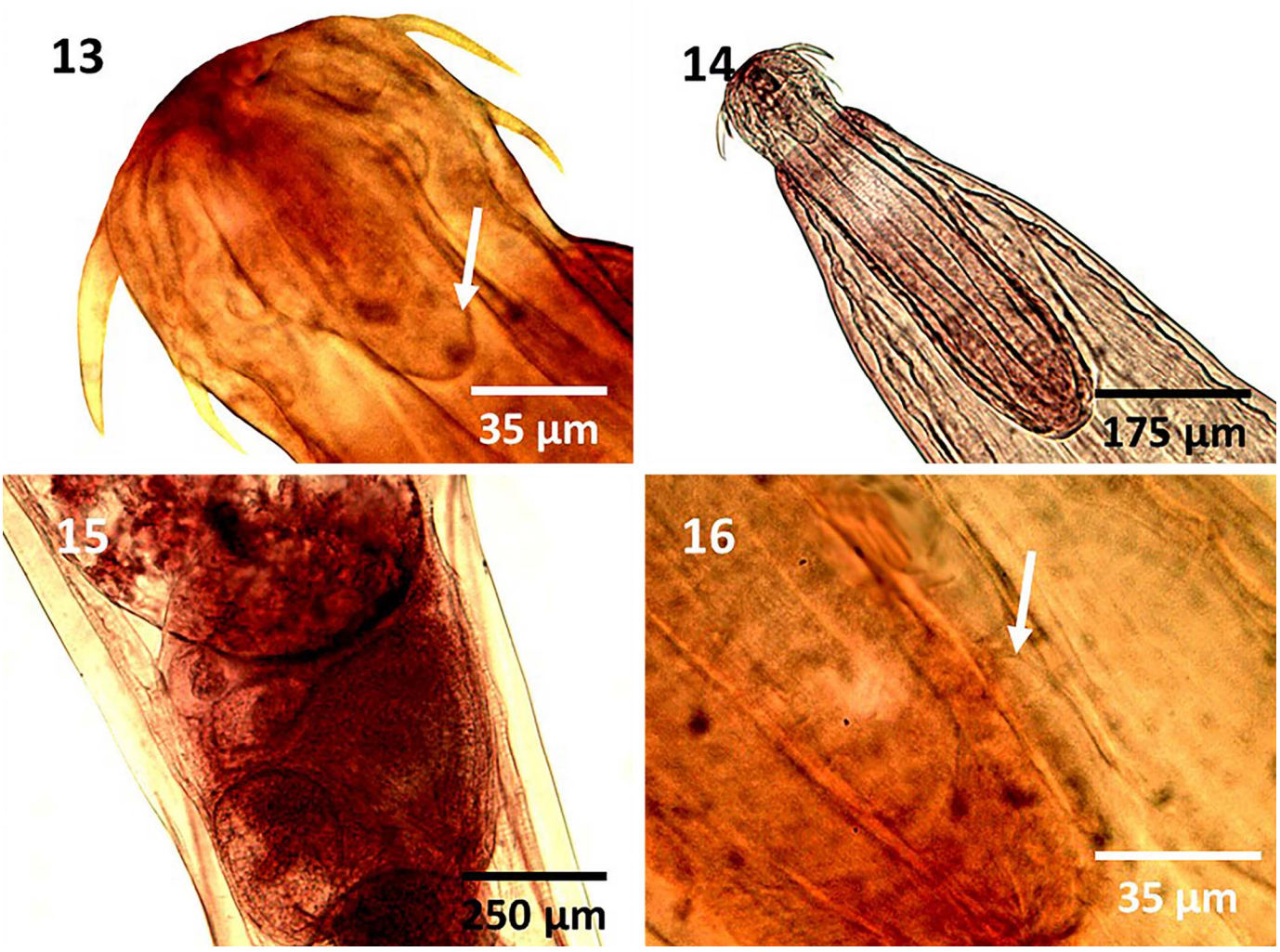
Figures 9–12. SEM of specimens of *Neoechinorhynchus* (*Neoechinorhynchus*) *dimorphospinus* from *Liza subviridis* in Vietnam. (**9**) The proboscis of a female specimen showing the extreme dimorphism in the size of anterior proboscis hooks. (**10**) A high magnification of the anterior left lateral hook of the proboscis in Figure 9. (**11**) A high magnification of a hook showing the detail of the ribbed form characteristic of this acanthocephalan species**.** (**12**) A gallium-cut cross section of a hook showing the almost detached corrugated cortical layer and the solid core resembling the bark on a tree trunk.

"near equal," a not unusual observation of this contractile structure. Characteristic and new features of the shape and size of the unequal anterior corrugated hooks (Figs. 9–12), hook roots (Fig. 13), receptacle (Fig. 14), cement gland (Fig. 15), and the PRS (Fig. 16) were well demonstrable and documented. The number of giant nuclei in the cement gland was not noted in the original description but is now determined to be 6, and the presence of the PRS is established in that species for the first time.

EDXA of proboscis hooks of *N. dimorphospinus* at hook tip (Fig. 17) and at base entry into the proboscis (Fig. 18) showed high levels of calcium and phosphorus. The hook tip had a moderate level of sulfur and no sodium, but the hook base at entry had a negligible level of sulfur and low sodium levels (Table II). This appears to be the characteristic metal signature of hooks typical of *N. dimorphospinus*.

Molecular studies

Acanthocephalan species, host species, locality names, and GenBank accession numbers used in the phylogenetic analysis are shown in Table III. The specimen of *N. dimorphospinus* successfully presented amplification of about 1,685 bp for the *18S rDNA* gene. Inter-species variation among species of *Neoechinorhynchus* was 4.1% to 13.7%. The BLASTN results indicated that *N. dimorphospinus* has 96% identity with *Neoechinorhynchus* sp. (HM545898) considering a 100% query cover, and max score 2,723; *N. buttnerae* Golvan, 1956 (MK249749)



Figures 13–16. Light micrographs of specimens of *Neoechinorhynchus* (*Neoechinorhynchus*) *dimorphospinus* from *Liza subviridis* in Vietnam. (**13**) The proboscis of a specimen showing the nucleated apical organ; arrow points to its posterior margin. (**14**) The anterior end of another specimen showing the relationship between the proboscis, neck, and receptacle. (**15**) The nucleated cement gland overlapping the posterior end of the posterior testis. (**16**) The posterior extension of the para-receptacle structure (arrow) into the posterior end of the receptacle. Color version available online.

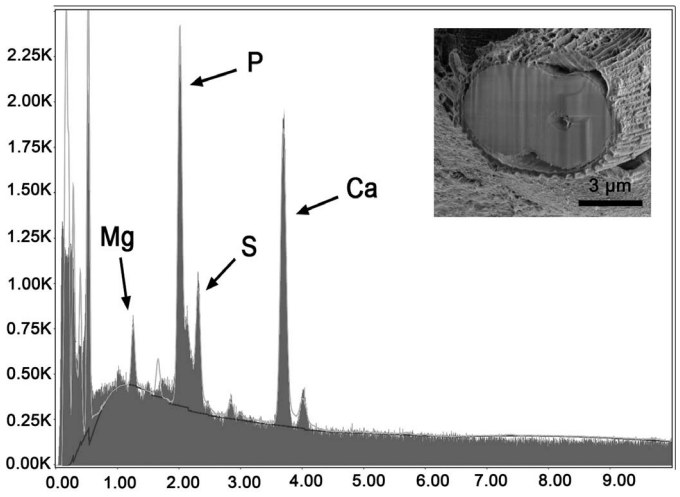
with 93% identity, 93% query cover, and max score 1,454; *Neoechinorhynchus* sp. (KU363972) with 94% identity, 93% query cover, and max score 1,426; *N. pseudemydis* Cable and Hopp, 1954 (U41400) with 92% identity, 93% query cover, and 1,395 max score; *N. cylindratus* (Van Cleave, 1913) Van Cleave, 1919 (MF974925) with 93% identity, 85% query cover, and max score 1,384; *N. saginatus* Van Cleave and Bangham, 1949 (AY830150) with 92% identity, 93% query cover, and max score 1,378; *N. crassus* Van Cleave, 1919 (KU363974) with 91% identity, 93% query cover, and max score 1,351; and *Hebesoma violentum* (Van Cleave, 1928) Salgado-Maldonado, 1978 (KF156881) with 92% identity, 51% query cover, and max score 1,212.

The phylogenetic reconstruction showed that our sequence of *N. dimorphospinus* is grouped with *Neoechinorhynchus* sp. (HM545898) with a statistical support of 100% in a clade of the family Neoehinorhynchidae. Also, the majority of species of *Neoechinorhynchus* appear very closely related, while *N. dimorphospinus* and *Neoechinorhynchus* sp. (HM545898) were separated from others in the tree. Of the Class Eoacanthocephala Van Cleave, 1936, the Quadrigyridae clade (Order Gyracanthocephala Van Cleave, 1936) represented by species of *Pallisentis* Van Cleave, 1928, appears as a sister group of the clade of the family Neoehinorhynchidae (Order Neoechinorhynchida Southwell and Macfie, 1925) with 100% of bootstrap support. The genera *Rhadinorhynchus* Lühe, 1911, *Pomphorhynchus* Monticelli, 1905, *Acanthocephaloides* Meyer, 1932, *Echinorhynchus* Zoega in Müller, 1776, *Pseudoacanthocephalus* Petrochenko, 1956, *Acanthocephalus* Koelreuther, 1771, and *Filisoma* Van Cleave, 1928, are placed in another major clade as sister groups with 100% of bootstrap support.

DISCUSSION

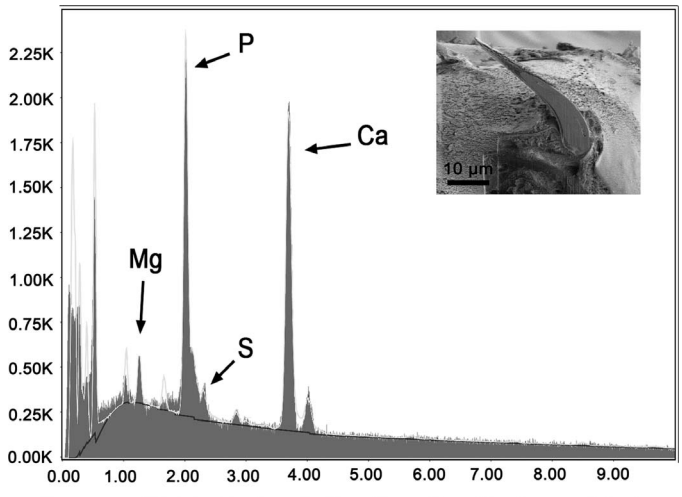
Variability

Much of the reported variability in the size of taxonomically important structures such as the trunk, proboscis hooks, proboscis, testes, etc., in the reported acanthocephalan species



Element	Weight %	Atomic %	Error %	Net Int.	K Ratio
C	11.98	20.51	8.44	263.91	0.053
N	15.03	22.06	11.44	168.63	0.0373
O	25.21	32.41	9.12	679.36	0.0739
Mg	1.43	1.21	8.07	128.35	0.0097
P	12.16	8.07	2.82	953.87	0.0979
Au	4.55	0.47	11.32	157.95	0.0358
S	4.41	2.83	5.85	324.01	0.035
Pd	1.63	0.31	31.76	49.93	0.011
Ca	23.61	12.12	3.27	999.67	0.1927

Figure 17. Energy dispersive X-ray spectrum for the tip cut edge of an anterior hook of a *Neoechinorhynchus dimorphospinus* specimen (see Table II). Insert: SEM of a cross gallium cut hook near anterior end.



Element	Weight %	Atomic %	Error %	Net Int.	K Ratio
C	9.68	17.58	8.56	219.98	0.0449
N	13.15	20.48	11.83	139.26	0.0312
O	21.82	29.76	9.26	568.45	0.0624
Na	1.89	1.79	9.5	112.99	0.0104
Mg	1.56	1.4	8.24	136.17	0.0103
P	15.23	10.73	2.79	1165.51	0.1198
Au	3.16	0.35	14.35	108.91	0.0249
S	1.07	0.73	17.09	76.52	0.0083
Pd	1.43	0.29	34.49	43.28	0.0096
Ca	31.01	16.88	2.92	1293.15	0.2494

Figure 18. Energy dispersive X-ray spectrum for the base entry of an anterior hook into the proboscis of a *Neoechinorhynchus dimorphospinus* specimen (see Table II). Insert: SEM of a longitudinal gallium cut hook showing insertion into proboscis.

has been attributed to host species. Such relationships have been previously reported in other species of acanthocephalans including *Echinorhynchus salmonis* Müller, 1784, in Lake Michigan, United States, where male and female specimens from bloater, *Coregonus hoyi* (Gill) (Salmonidae), achieved not only larger size but also different body form (broad anteriorly) compared to the slender and smaller specimens from rainbow smelt, *Osmerus mordax* (Mitchell) (Osmeridae) (Amin and Redlin, 1980). The larger and heavier worms from bloater almost invariably showed higher regression coefficient (adjusted coefficient of determination) compared to those from smelt in all characters, including size of trunk, proboscis, longest proboscis hooks, receptacle, testes, lemnisci, and eggs. The taxonomic implications of this variability were discussed (Amin and Redlin, 1980).

Earlier Amin (1975) demonstrated a similar relationship for *Acanthocephalus dirus* (Van Cleave, 1931) Van Cleave and Townsend, 1936, in Wisconsin fishes. Females of the same developmental stage recovered during the same period were found to have attained larger sizes in certain hosts than in others with the largest females being found in *Lepomis macrochirus* Rafinesque. The size of trunk in males was also found to follow the same pattern. Similarly, testes also attained a larger size in males recovered from *Catostomus commersoni* Lacépède (Catostomidae) than in males from *Semotilus atromaculatus* (Mitchill) (Cyprinidae). Amin (1975, p. 308) stated that these size variations "result from differential growth rates of these worms in the various host intestinal environments (and) are probably mediated by certain host specific factors."

Para-receptacle structure (PRS)

Inserts anteriorly in the body wall near the neck and posteriorly at the posterior end of the receptacle. The presence of the PRS in eoacanthocephalans with a weak single proboscis receptacle wall was first demonstrated in *Neoechinorhynchus* (*N.*) *qatarensis* Amin, Saoud, Alkuwari, 2002, by Amin et al. (2002). It had since been reported in other species of *Neoechinorhynchus* Stiles and Hassall, 1905, and *Acanthogyrus* (*Acanthosentis*) Verma and Datta, 1929, reviewed in part by Amin et al. (2011b) and reported here for the first time in various other species of *Neoechinorhynchus* from marine fishes off the east coast of Vietnam. In the description of the PRS, Amin et al. (2002, 2007) proposed that it may regulate the hydrostatic pressure in the receptacle to facilitate the retraction and eversion of the proboscis.

Electron dense micropores

Electron dense micropores present throughout the epidermal surface of the trunk of reported species of *Neoechinorhynchus*, like those reported in other species of Acanthocephala, are associated with internal crypts and vary in diameter and distribution in different trunk regions corresponding with differential absorption of nutrients. The present authors have documented this phenom-

Table III. Acanthocephalan species, host species, locality names, and GenBank accession numbers used in the phylogenetic analysis.

Species	Host	Locality	GenBank accession no.
Neoechinorhynchus simansularis	*Barbatula toni*	Russia	KF156877
Neoechinorhynchus saginatus	Na*	Na	AY830150
Neoechinorhynchus pseudemydis	Na	Na	U41400
Neoechinorhynchus pseudemydis	*Capoeta aculeate*	Iran	KU363973
Neoechinorhynchus crassus	*Capoeta aculeate*	Iran	KU363974
Neoechinorhynchus crassus	*Capoeta aculeate*	Iran	KU363971
Neoechinorhynchus cylindratus	*Micropterus salmoides*	USA	MF974925
Neoechinorhynchus beringianus	*Pungitius pungitius*	Russia	KF156875
Neoechinorhynchus buttnerae	Na	Na	MK249749
Neoechinorhynchus dimorphospinus	*Liza subviridis*	Thailand	MK510080
Neoechinorhynchus sp.	*Capoeta aculeate*	Iran	KU363972
Neoechinorhynchus sp.	Na	China	KM507363
Neoechinorhynchus sp.	*Siganus fuscescens*	China	HM545898
Hebesoma violentum	*Perccottus glenii*	Russia	KF156881
Rhadinorhynchus pristis	*Gempylus serpens*	Indonesia	JX014226
Rhadinorhynchus lintoni	*Selar crumenophthalmus*	USA	JX014224
Pseudoacanthocephalus toshimai	*Rana pirica*	Japan	LC129278
Pseudoacanthocephalus lucidus	*Rana ornativentris*	Japan	LC129279
Pomphorhynchus tereticollis	*Gammarus pulex*	France	AY423347
Pomphorhynchus laevis	*Rutilus rutilus*	Germany	JX014223
Filisoma rizalinum	*Scatophagus argus*	Indonesia	JX014229
Filisoma bucerium	*Kyphosus elegans*	Na	AF064814
Echinorhynchus truttae	*Thymallus thymallus*	Na	AY830156
Acanthocephalus lucii	*Perca Fluviatilis*	Na	AY830152
Acanthocephalus dirus	*Asellus aquaticus*	Na	AY830151
Pallisentis sp.	Na	Na	KY305516
Pallisentis sp.	Na	Na	KY305517
Pallisentis sp.	Na	Na	KY305518
Pallisentis sp.	Na	Na	KY305519
Pallisentis sp.	Na	Na	KY305520
Pallisentis sp.	Na	Na	KY305521
Pallisentis sp.	Na	Na	KY305522

* Na = not available.

enon in 16 species of acanthocephalans (Heckmann et al., 2013) and a few more since. The functional aspects of micropores in a few other acanthocephalan species including *Rhadinorhynchus ornatus* Van Cleave, 1918, *Polymorphus minutus* (Goeze, 1782) Lühe, 1911, *Moniliformis moniliformis* (Bremser, 1811) Travassos, 1915, *Macracanthorhynchus hirudinaceus* (Pallas, 1781) Travassos, 1917, and *Sclerocollum rubrimaris* Schmidt and Paperna, 1978, were reviewed earlier by Amin et al. (2009). We demonstrated the tunneling from the cuticular surface into the internal crypts with TEM. Wright and Lumsden (1969) and Byram and Fisher (1973, p. 557) 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 (1979, p. 64) estimated a 44-fold increase at a surface density of 15 invaginations per 1 μm^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 *Pusa caspica* (Gmelin) in the Caspian Sea were demonstrated by transmission electron micrographs in Amin et al. (2011d).

Neoechinorhynchus longnucleatus is documented from 4 host species in families along the Pacific coast of Vietnam (Table I). Apparently, this acanthocephalan is of wider host and geographical distribution than originally reported. The hosts of *N. longnucleatus* are of Indo-West-Pacific distribution, and it is likely to find this species in wider geographical localities in the future. Of equal interest is the fact that there was no significant morphological variability between our specimens and those examined or described earlier except for the discovery of para-receptacle structure.

Neoechinorhynchus manubrianus is documented from 2 sciaenid fish species in the north and the south of the Pacific coast of Vietnam extending its range of distribution beyond the original northern type location in Halong Bay. Microscopic images augmented prior morphological accounts and documented the presence of the PRS, which was not previously reported in that species.

Neoechinorhynchus dimorphospinus is probably one of the most versatile and widespread species of the genus.

Distribution

We report *N. dimorphospinus* from 7 species of marine and freshwater fishes in 4 different families spread between the Persian Gulf in the west and the West Pacific coast of Vietnam in the east. This wide host and geographical distribution "suggest that this is a highly diverse and adaptable acanthocephalan that likely infects

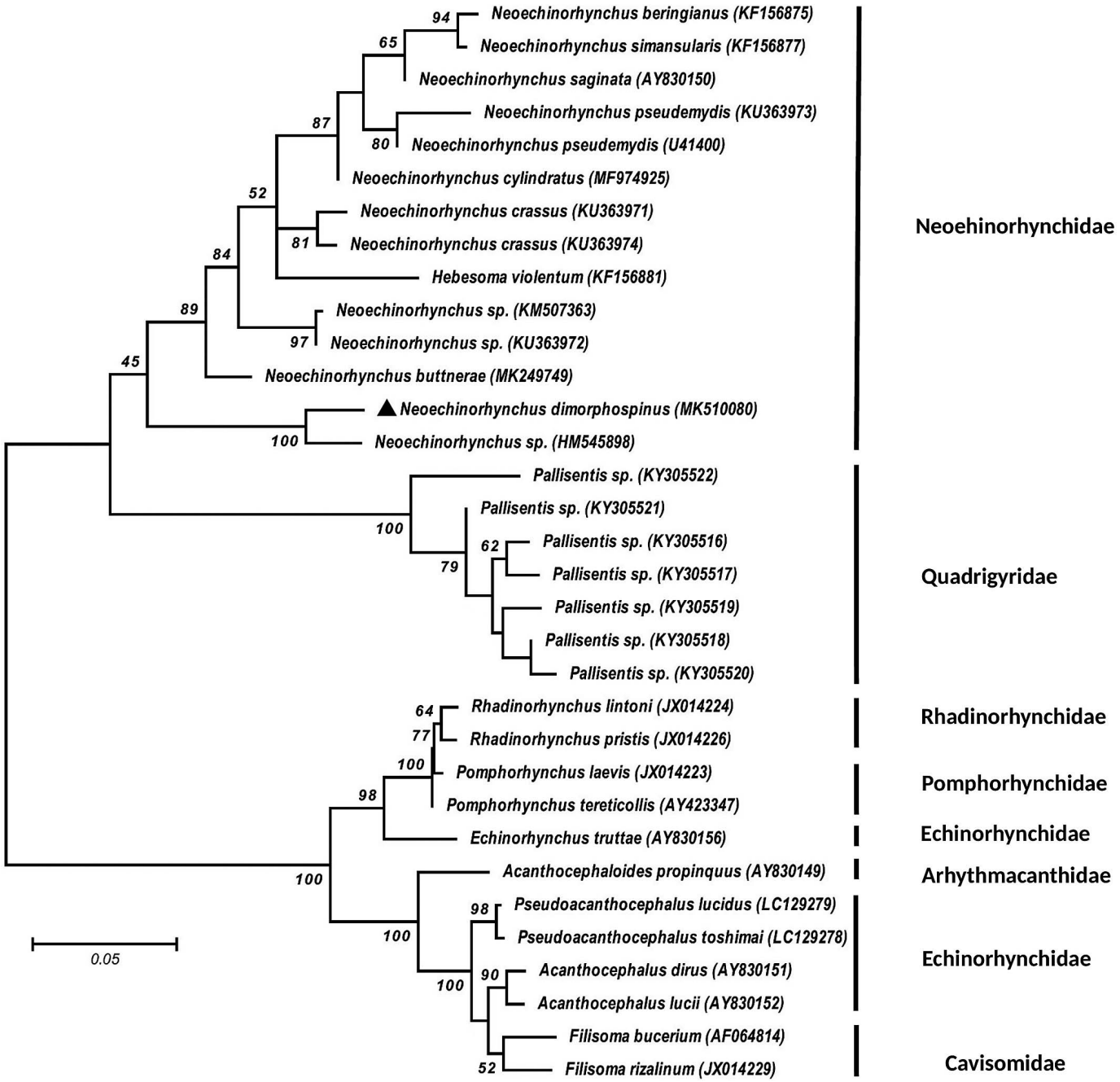


Figure 19. Phylogenetic tree of isolates of *Neoechinorhynchus dimorphospinus* obtained in this study (▲) and reference sequences retrieved from GenBank based on partial *18S rDNA* sequences and constructed using the Tamura 3-parameter model in MEGA software version 6.

a wider assortment of fish species and whose range may extend into Iraq as well as into additional marine fishes beyond the Persian Gulf. Only additional collections into those areas will reveal the extent of its distribution, which must also be regulated by the distribution of its currently unidentified crustacean intermediate host(s)" (Amin et al., 2015, p. 64). This prediction has finally come true with the finding of *N. dimorphospinus* well beyond its original distribution in the Persian Gulf to Vietnam into fish hosts like *L. subviridis*, whose Indo-West Pacific distribution from the Persian Gulf and the Red Sea extends east to the Pacific, north to Japan, and south to Australia (Thomson, 1984; Heemstra, 1995; Froese and Pauly, 2013). Other known or new host species may also be involved. The PRS presence in this acanthocephalan is established for the first time, and some of its usual features such as the serrated surface, different sizes, and roots of the anterior hooks are further elucidated and detailed with microscopic and SEM images.

EDXA

Results of the X-ray scans of the gallium cut hooks (dual-beam SEM) of *N. dimorphospinus* are given in Table II and Figures 17

and 18. The chemical elements present in the hooks are typical for acanthocephalans (Amin and Heckmann, 2017; Amin et al., 2018c; Ha et al., 2018). The hook contains the common elements for protoplasm (C, O, N, H) and those used to form the hardened hook structure (Ca, P, S) similar to the layers of a mammalian tooth. Note the thin outer layer (Fig. 12) of the hook for the worm, which relates to the sulfur (S) content (Table II) in the hook of *N. dimorphospinus*, which is less than for other acanthocephalans (Amin and Heckmann, 2017; Amin et al., 2018c), The high sulfur content shows up in the outer edge of X-ray scans of the hook (Table II, Amin et al., 2018c). The absence of sodium at the hook tip and its presence at the hook base and the opposite pattern of sulfur are characteristic of *N. dimorphospinus* hooks. X-ray scans (EDXA) provide insight into the hardened components, e.g., calcium and phosphorus, of acanthocephalan hooks. The EDXA appear to be species specific and have significant diagnostic value in acanthocephalan systematics; e.g., *Moniliformis cryptosaudi* Amin, Heckmann, Sharifdini, and Albayati, 2019, was erected based primarily on its EDXA pattern (see Amin et al., 2019b).

DNA analysis

Some investigations have showed that the *18S rDNA* gene is a useful tool to analyze the genetic variations and phylogenetic relationships among Acanthocephalans (Near et al., 1998; García-Varela and Nadler, 2005; Malyarchuk et al., 2014). In the current study, molecular results obtained from the *18S rDNA* gene explain the morphological differences between *N. dimorphospinus* and other species of *Neoechinorhynchus*. The phylogenetic analysis showed that the sequence of *N. dimorphospinus* is grouped with *Neoechinorhynchus* sp. (HM545898) forming a clade in the family Neoehinorhynchidae that is separated from other species of *Neoechinorhynchus*. Our phylogenetic tree reveals that *H. violentum* is placed among species of *Neoechinorhynchus*. The genus of *Neoechinorhynchus* was split in 2 subgenera of *Hebesoma* and *Neoechinorhynchus* (see Amin, 2002). Malyarchuk et al. (2014) reported that the *18S rDNA* gene tree shows a sister relationship among *Hebesoma* and *Neoechinorhynchus*. The mitochondrial *COI* gene tree by Malyarchuk et al. (2014) erroneously places *H. violentum* as a member of subgenus *Neoechinorhynchus*. However, these authors did not use relevant species of *Neoechinorhynchus* including *Neoechinorhynchus buttnerae* (MK249749), *Neoechinorhynchus* sp. (KU363972), *Neoechinorhynchus* sp. (KM507363), and *Neoechinorhynchus* sp. (HM545898) in their ribosomal gene tree. Therefore, using more sequences of other species in the family Neoehinorhynchidae and the application of more gene targets are necessary to better understand the relationship between species of this family, especially species in the West Pacific that are scarcely studied. The phylogenetic tree (Fig. 17) illustrated that clades in the family Neoehinorhynchidae are well separated from the Quadrigyridae, Echinorhynchidae, Arhythmacanthidae, Rhadinorhynchidae, Cavisomidae, and Pomphorhynchidae families. The very wide range of geographical distribution of *N. dimorphospinus* and its distinction from others on the tree by having the unique dimorphism in the size of the anterior proboscis hooks appears to be associated with this distinction.

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