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New perspectives on *Nephridiacanthus major* (Acanthocephala: Oligacanthorhynchidae) collected from hedgehogs in Iran

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Research Paper

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We describe morphological features not previously reported for this old acanthocephalan *Nephridiacanthus major* (Bremser, 1811 in Westrumb, 1821) Golvan, 1962 first described over 200 years ago. Our specimens were collected from long-eared hedgehog *Hemiechinus auritus* (Gmelin, 1770) (Erinaceidae) in Iran. We compare the morphometrics of our material with others previously reported from the same host in Iran, Russia, central Asia and Europe. Our specimens had markedly smaller proboscides, proboscis hooks and lemnisci than those reported from Russia and central Asia, but comparable measurements of other structures with specimens previously described from other collections. We document our new observations with scanning electron microscopy features not previously demonstrable by other observers and provide a chemical analysis of proboscis hooks using energy-dispersive X-ray analysis for the first time. The molecular profile of this acanthocephalan, based on 18S rDNA and *cox1* genes, was generated for the first time. The phylogenetic analysis showed that *N. major* is placed in a clade of the family Oligacanthorhynchidae, well separated from the families Moniliformidae and Gigantorhynchidae.

Introduction

Heckmann *et al.* (2013a) described specimens of *Nephridiacanthus major* (Bremser, 1811 in Westrumb, 1821) Golvan, 1962 from the central Asian and Middle Eastern long-eared hedgehog, *Hemiechinus auritus* (Gmelin, 1770) and the Eastern European hedgehog, *Erinaceus concolor* Martin (Erinaceidae, 1838) from Iran and provided histopathological observations. Heckmann *et al.* (2013a) summarized the records of this acanthocephalan species from hedgehogs in Germany, Morocco, central Asia, Egypt, Bulgaria, Tadzhikistan, Lebanon, Sicily, Italy, Nigeria, Turkey and Mongolia. See Petrochenko (1958) for references to descriptions by Bremser (1811), Westrumb (1821), Porta (1908), Kostylev (1916, 1918), Meyer (1931, 1933) and Golvan (1956) from European and Asian hedgehogs. Despite the long history of research and publications about this acanthocephalan species since its original description over 200 years ago, we could still find previously undescribed features and report chemical and molecular data for the first time. This study provides, for the first time, the molecular profile of *N. major* based on both small subunit ribosomal DNA (18S rDNA) and cytochrome c oxidase 1 (*cox1*) genes. Furthermore, its phylogenetic relationships with other members of the family Oligacanthorhynchidae and closest-related families are analysed and discussed.

Materials and methods

Collections

About 30 specimens of *N. major* were collected from three individuals of the hedgehog *H. auratus* collected in the Ferdowsi University campus in Mashhad, Razavi Khorasan province, northeastern Iran, between 3 and 24 October 2018. Eleven mature specimens were processed for microscopical studies and four for scanning electron microscopy (SEM) studies, metal analysis and gallium (Ga) sections. Ten juvenile specimens were processed for microscopical observations but were not measured. The remaining specimens were used for 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 Arizona, USA, 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 h each) and cleared in 100% xylene then in 50% Canada balsam and 50% xylene (24 h each). Whole worms were then mounted in Canada balsam. Measurements are given in micrometres, 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.

Voucher specimens (whole mounts) were deposited in the University of Nebraska's State Museum's Harold W. Manter Laboratory, collection no. HWML 139975, in Lincoln, Nebraska, USA.

SEM

Specimens 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 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 (Q150 TES, Quorum, Marly, Switzerland), establishing an approximate thickness of 20 nm. Samples were placed and observed in an FEI Helios Dual Beam Nanolab 600 (FEI, Hillsboro, Oregon, USA) scanning electron microscope with digital images obtained in the Nanolab software system (FEI, Hillsboro, Oregon, USA) 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 Gaseous Secondary Electron detector.

Energy-dispersive X-ray analysis (EDXA)

Standard methods were used for preparation similar to the SEM procedure. Specimens were examined and positioned with the above SEM equipment, which was equipped with a Phoenix energy-dispersive X-ray analyser (FEI, Hillsboro, Oregon, USA). 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, Hillsboro, Oregon, USA) 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 Ga ion source (GIS) is used for the liquid ion metal source part of the process. The hooks of the acanthocephalans were centred 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. The cut was then analysed 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.

DNA extraction and polymerase chain reaction (PCR) amplification

Total genomic DNA was extracted from adult worms of *N. major* using Qiagen DNeasy blood and tissue kit (Qiagen Inc., Valencia, California, USA) according to the manufacturer's instructions. Partial nuclear 18S rDNA and partial fragments of mitochondrial *cox1* gene were amplified by PCR reactions in 30 µl volumes containing 2 × red PCR premix (Ampliqon, Odense, Denmark), 20 pmol of each primer and 2 µl of isolated DNA. The partial 18S rRNA gene was amplified using the forward primer (5′-AGATTAAGCCATG CATGCGTAAG-3′) and reverse primer (5′-ACCCACCGAATC AAGAAAGAG-3′). For the *cox1* gene, the primers LCO1490 (forward, 5′-GGTCAACAAATCATAAAGATATTGG-3′) and HC O2198 (reverse, 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (1) were used. PCR conditions for 18S rDNA gene amplification comprised an initial denaturing step of 95°C for 5 min and 35 cycles followed by a denaturing step at 95°C for 30 s, an annealing step of 61°C for 30 s, 60 s of extension at 72°C and 72°C for 7 min as a final extension.

Also, the PCR cycling parameters for the *cox1* gene comprised an initial denaturation at 95°C for 6 min and 35 cycles followed by a denaturing step at 95°C for 30 s, an annealing step of 55°C for 30 s, 60 s of extension at 72°C and then a final extension stage at 72°C for 6 min. The amplicons were run on a 1.5% agarose gel and visualized with UV transluminator (Vilber Lourmat, Collégien, France). Next, the amplification products were sequenced on an ABI 3730 automatic sequencer (Applied Biosystems, Foster City, California, USA) in both directions using the same PCR primers.

The obtained sequence results were manually edited and trimmed using Chromas version 2.01 (Technelysium Pty Ltd., Brisbane, Queensland, Australia) and BioEdit software version 7.0.9, Nucl. Acids. Symp. Ser. 41: 95–98. The basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov/) was used to compare the sequences with available data in GenBank. The sequences of 18S rDNA and *cox1* genes were submitted to GenBank database (accession numbers MN612079 for 18S rDNA and MN612080 for *cox1*).

Phylogenetic analysis

The maximum-likelihood method and Tamura three-parameter model were used for phylogenetic tree reconstruction, and genetic distances were calculated with the maximum composite likelihood model using Molecular and Evolution Genetic Analysis software version 6 (MEGA 6, Institute of Molecular Evolutionary Genetics, University Park, Pennsylvania, USA). A bootstrap value with 1000 replications was also implemented to evaluate the reliability of the tree topologies. The sequences used for the phylogenetic analysis are listed in table 1.

Results

We provide a morphometric description of the present population of *N. major* from *H. auritus* in Mashhad, Iran, compared with the other population from *H. auritus* and *E. concolor*, also from Iran at Shahrood (Heckmann *et al.*, 2013a) and from two other selected European populations – one from the west European hedgehog *Erinaceus europeanus* Linn, 1758 in Novocherkassk and Buzuluk, Russia, and from *H.*

Table 1. Acanthocephalan species represented in the phylogenetic analysis with their family, host species, GenBank accession numbers, locations and references.

Species	Host	GenBank acc. no. 18S rDNA	GenBank acc. no. *cox1*	Location	Reference
Oligacanthorhynchidae					
Macracanthorhynchus ingens (von Linstow, 1879)	*Procyon lotor*	_____	KT881244	USA	Richardson *et al*. (unpublished)
Macracanthorhynchus ingens	Na	AF001844	_____	Na	Near *et al*. (1998)
Macracanthorhynchus ingens	Na		AF416997	Na	Garcia-Varela *et al*. (unpublished)
Macracanthorhynchus hirudinaceus (Pallas, 1781)	*Sus scrofa leucomystax*	LC350002	LC350021	Japan	Kamimura *et al*. (2018)
Macracanthorhynchus hirudinaceus	Na	_____	FR856886	Na	Weber *et al*. (2013)
Oligacanthorhynchus tortuosa (Leidy, 1850) Schmidt, 1972	Na	AF064817		Na	García-Varela *et al*. (2000)
Oligacanthorhynchus tortuosa	*Didelphis marsupialis*	_____	KM659345	USA	Richardson *et al*. (unpublished)
Oligacanthorhynchus tortuosa	*Virginia opossum*	_____	KT881245	USA	Richardson *et al*. (unpublished)
Oncicola luehei (Travassos, 1917) Schmidt, 1972	Na	_____	JN710452	Na	Gazi *et al*. (2012)
Oncicola sp.	Na	_____	AF417000	Na	Garcia-Varela *et al*. (unpublished)
Oncicola sp.	Na	AF064818		Na	García-Varela *et al*. (2000)
Prosthenorchis elegans (Diesing, 1851)	*Saguinus leucopus*	_____	KT818501, KT818504	Colombia	Falla *et al*. (2015)
Gigantorhynchidae				Na	
Mediorhynchus grandis Van Cleve, 1916	Na	AF001843	_____	Na	Near *et al*. (1998)
Mediorhynchus africanus Amin, Evans, Heckmann & El-Naggar, 2013	*Numida meleagris*	_____	KC261351	South Africa	Amin *et al*. (2013)
Mediorhynchus gallinarum (Bhalerao, 1937)	*Gallus gallus*	_____	KC261352	Indonesia	Amin *et al*. (2013)
Mediorhynchus sp.	Na	AF064816	_____	Na	García-Varela *et al*. (2000)
Moniliformidae				Na	
Moniliformis moniliformis (Bremser, 1811)	*Rattus rattus*	HQ536017	_____	Spain	Foronda Rodriguez *et al*. (unpublished)
Moniliformis moniliformis	Laboratory rat	Z19562		England	Telford & Holland (1993)
Moniliformis moniliformis	Na	_____	AF416998	Na	Garcia-Varela *et al*. (unpublished)
Moniliformis kalahariensis Meyer, 1931	*Atelerix frontalis*	MH401042	MH401040	South Africa	Amin *et al*. (2018)
Moniliformis saudi sp. n Amin, 2016	*Paraechinus aethiopicus*	KU206782	KU206783	Saudi Arabia	Amin *et al*. (2016)
Echinorhynchidae (out-group)				Na	
Echinorhynchus truttae (Schrank, 1788)	*Thymallus thymallus*	AY830156	_____	Na	García-Varela & Nadler (2005)
Echinorhynchus truttae	*Thymallus thymallus*	_____	DQ089710	Na	García-Varela & Nadler (2006)

Na, not available.

Table 2. Morphometric comparisons among populations of *Nephridiacanthus major* from Iran and others from Europe and Russia.

Characters	Present paper	Heckmann *et al.* (2013a)	Petrochenko (1958)	Meyer (1933)
Hosts	*Hemiechinus auritus*	*Hemiechinus auritus* *Erinaceus concolor*	*Hemiechinus auritus* *Erinaceus europeanus*	*Erinaceus europeanus*
Locality	Mashhad, Iran	Shahrood, Iran	Central Asia and Russia	Central Europe
Sample size	Five males, six females	Seven males, six females	2–10 specimens per host	–
Males				
Trunk L × W (mm)	35.38–78.75 (59.70) × 2.50–3.50 (2.96)	18.87–85.75 (40.77) × 1.00–3.17 (1.94)	***c.* 20.00 × 1.50**	40.00–75.00 × 1.20
Proboscis L × W	**364–395 (372) × 333–406 (382)**[a]	478–572 (523) × 395–468 (430)	**630–670 × 470**	500 × 375 (Fig. 211)
Hook L from ant.	**94–107 (100), 73–94 (86), 52–83 (67), 42–73 (61)**	107–120 (113), 80–107 (96), 65–80 (73), 55–70 (60)	**116–157, 110–125, 94, 94, 94, 63–78, 63–78**, 75 in six spiral rows	In six spiral rows of eight hooks
Neck L × W	400–525 (470) × 325–425 (385)	260–416 (347) × 364–416 (388)	310 ×–	Long
Prob recept. L × W (mm)	1.27–1.50 (1.43) × 0.42–0.57 (0.50)	0.94–1.61 (1.27) × 0.36–0.57 (0.50)	1.4–1.6 × 0.30	1.50
Long lemniscus L × W (mm)	6.75–8.00 (7.37) × 0.30–0.47 (0.37)	3.90–15.00 (6.97) × 0.20–0.50 (0.33)	**13.5 ×**–	8.00–10.00 ×–
Short lemniscus L × W (mm)	5.12–7.00 (5.90) × 0.20–0.40 (0.32)	3.42–14.50 (6.38) × 0.17–0.55 (0.33)	**12.5 ×**–	8.00–10.00 ×–
Ant. testis L × W (mm)	3.02–5.37 (4.23) × 0.77–2.07 (1.25)	1.37–6.50 (3.44) × 0.27–1.50 (0.79)	–	3.00 ×–
Post. testis L × W (mm)	3.02–4.87 (4.11) × 0.82–1.40 (1.17)	1.32–6.50 (3.36) × 0.35–1.20 (0.71)	–	3.00 ×
Cement glands L × W (mm)	1.12–1.70 (1.30) × 0.70–1.25 (0.94)	0.57–2.25 (1.22) × 0.37–1.25 (0.69)	In four pairs	In a mass of 3.50 ×–
Large cem. Gl. L × W (mm)	1.62–2.00 (1.78) × 0.88–1.17 (1.02)	0.57–2.25 (1.22) × 0.37–1.25 (0.69)	–	–
Saefftigen's pouch L × W	3.00–4.25 (3.87) × 0.62–1.00 (0.85)	1.14–4.00 (2.53) × 0.26–0.82 (0.55)	–	–
Females				
Trunk L × W (mm)	18.00–135.00 (55.70) × 1.10–5.00 (2.57)	6.25–126.25 (85.00) × 0.67–3.77 (2.06)	**200.00–215.00 × 3.10–3.20**	38.00–125.00 (80.00) × 2.50
Proboscis L × W	**364–468 (419) × 354–499 (421)**	416–562 (473) × 332–520 (447)	**630–670 × 470**	500 × 375 (Fig. 211)
Hook L from ant.	**114–125 (113), 94–114 (102), 72–87 (78), 52–62 (57)**	104–135 (121), 104–112 (106), 62–94 (83), 47–83 (65)	**116–157, 110–125, 94, 94, 94, 63–78, 63–78, 75** in six spiral rows	In six spiral rows of eight hooks
Neck L × W	325–572 (404) × 275–437 (369)	291–592 (485) × 364–530 (433)	310 ×–	Long
Prob. recept. L × W (mm)	1.25–1.82 (1.42) × 0.38–0.60 (0.48)	1.12–1.77 (1.43) × 0.35–0.80 (0.44)	1.40–1.60 × 0.30	150 × –
Long lemniscus L × W (mm)	6.50–6.75 (6.62) × 0.32–0.35 (0.33)	5.30–8.75 (7.33) × 0.16–0.38 (0.28)	**13.5 ×**–	8.00–10.00 × –
Short lemniscus L × W (mm)	5.62–6.12 (5.87) × 0.16–0.27 (0.21)	4.65–8.37 (6.90) × 0.15–0.34 (0.26)	**12.5 ×**–	8.00–10.00 × –
Reprod. system L.	1.25–3.87 (2.85); 2.8%–6.9% of trunk	–	–	–
Egg L × W	83–114 (99) × 50–73 (60)	94–114 (107) × 52–73 (61)	110–116 × 61–65	80–100 × 50

[a]Range (mean). Bolded numbers exhibit extreme sizes.
Notes: L, length; W, width.

auritus in central Asia (Petrochenko, 1958); and another from *E. europeanus* in central Europe (Meyer, 1933) (table 2). Moreover, we describe additional features of *N. major* by SEM (figs 1 and 2), characterize the metal composition of hooks using EDXA (fig. 3 and table 3), use Ga cuts of hooks to further explain their composition and structure (figs 1e, f and 2d) and produce a molecular profile for this unusual acanthocephalan species.

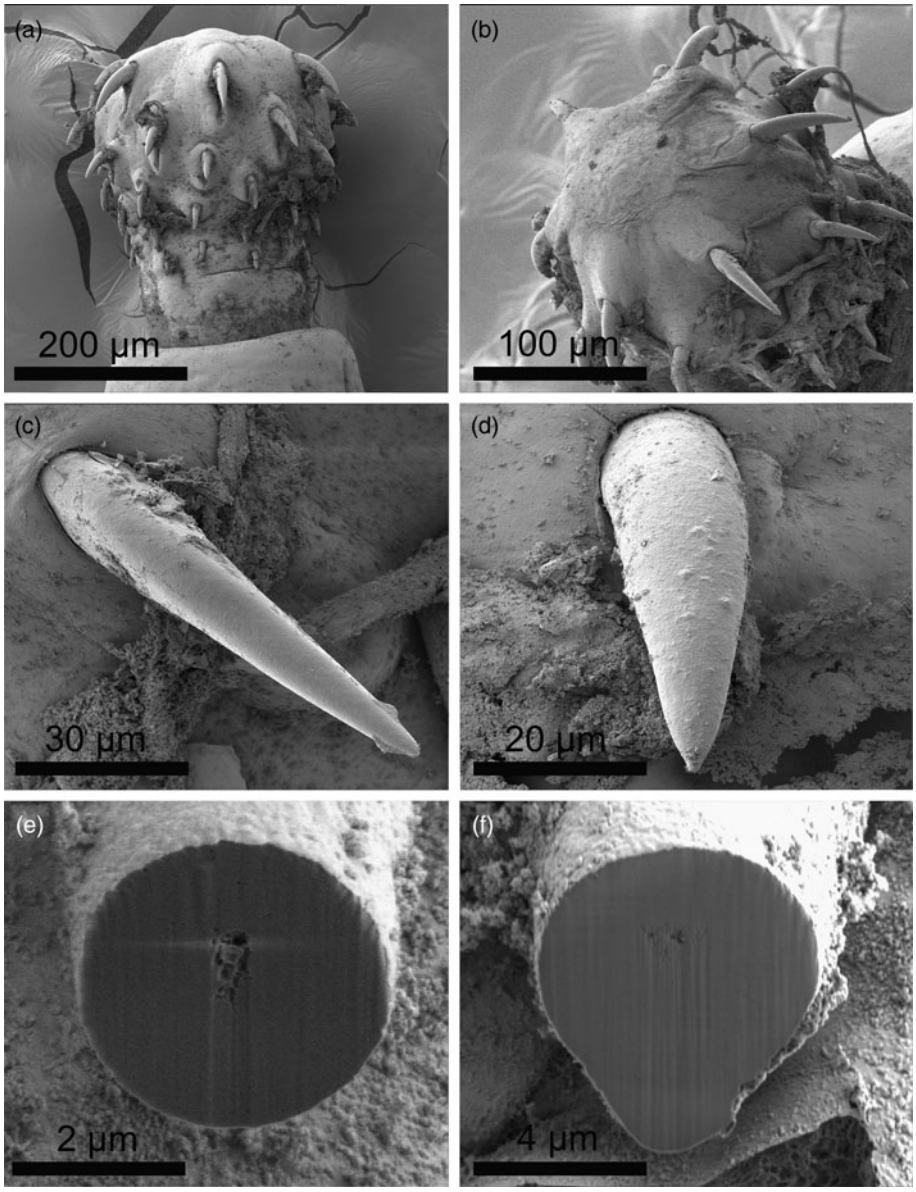


Fig. 1. SEM of specimens of *Nephridiacanthus major* from *Hemiechinus auratus* in Mashhad, Iran. (a) Lateral view of a male proboscis, showing its shape, hook arrangement and prominent neck. (b) Dorso-lateral view of another proboscis, showing anterior hooks insertions and large, rounded, slightly elevated apical organ. (c) Dorsal view of an anterior hook. (d) Face view of a shorter and plumper posterior hook. (e) Perfectly spherical gallium-cut section of a hook near its terminal end. (f) A gallium-cut section of another hook near its basal end with prominent ventral expansion. Note the solid core and thin cortical layer in both sections.

New anatomical observations

The specimens studied from *H. auratus* in Mashhad, Iran, had similar qualitative features as the other population that we described from the same host species and from *E. concolor* in Shahrood, Iran, by Heckmann *et al.* (2013a), and the measurements were comparable except that the sizes of the proboscis and hooks were larger in both sexes of the Shahrood specimens as well as the two European populations. Comparative measurements are provided in table 2, and new and more demonstrable morphological features are noted in figs 1 and 2. The profile of the proboscis, its shape and the comparative size and organization of its hooks, and prominent neck are shown in fig. 1a. The apical topography of the proboscis, demonstrating the slight elevation of the apical organ and the arrangement of the anterior hooks, is shown in fig. 1b. The variable shapes and sizes of anterior and posterior hooks are demonstrated in figs 1c and 1d, respectively. Hooks have a solid core and a very thin cortical layer, and are rounded at the middle (fig. 1e) but extended ventrally near their thicker base (fig. 1f). Figure 2a demonstrates the anatomy of the anterior hook of a male specimen and the massive anterior portion of its root with prominent anterior angulate manubrium. Figure 2b shows the comparable structures of the second hook of the same specimen. The sub-ventral position of the female gonopore is shown in fig. 2c, and a lateral Ga cut of a middle hook demonstrates the solid core and the thin cortical layer previously demonstrated in the cross sections (figs 1e, f).

Micropores

The variations in size and distribution of micropores in the middle and posterior sections of the trunk are demonstrated in figs 2e and 2f, respectively.

EDXA

Figure 3 and table 3 give the chemical composition of a proboscis hook at the middle centre, with calcium and phosphorous present in high concentrations, but with very low levels of sulphur and sodium.

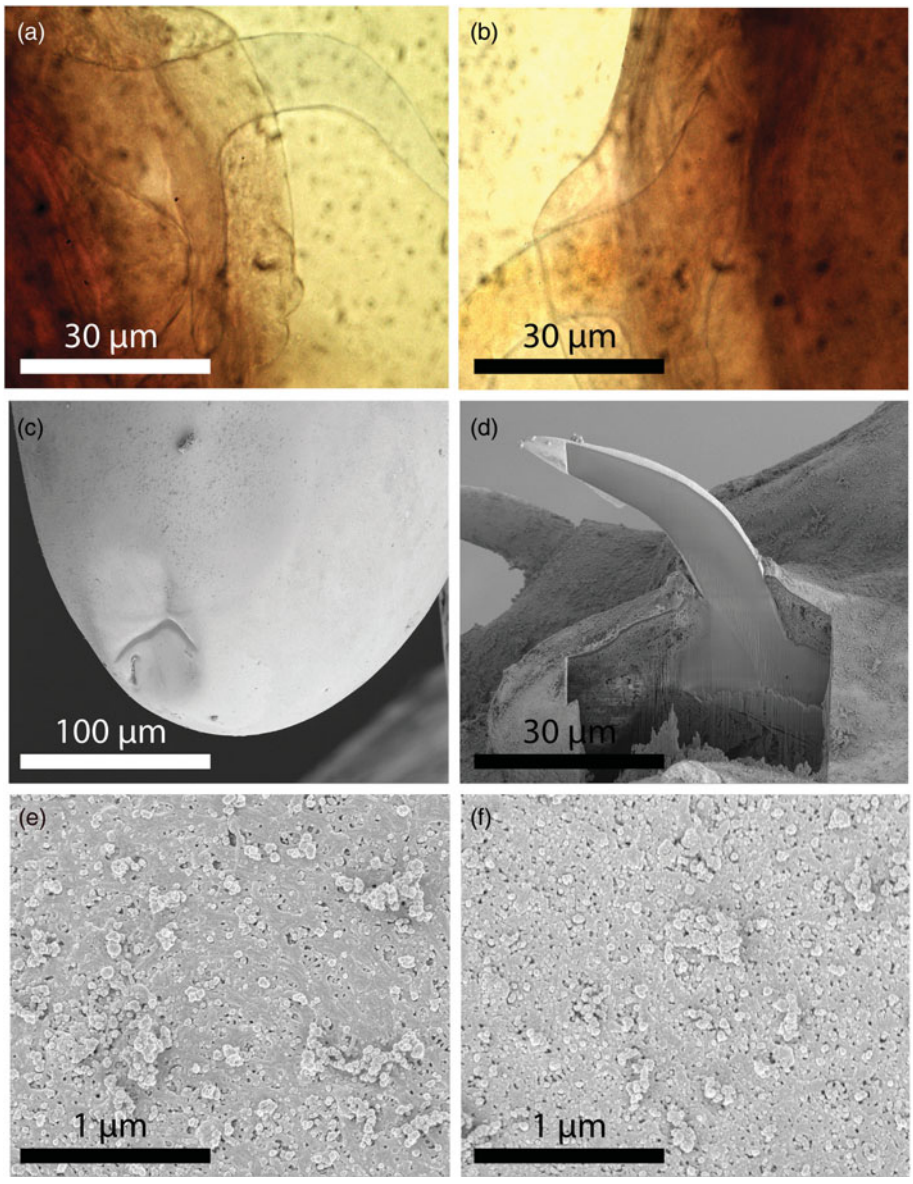


Fig. 2. SEM and microscope images of specimens of *Nephridiacanthus major* from *Hemiechinus auratus* in Mashhad, Iran. (a) A profile of an anterior hook of a male specimen, showing the shape of the large root and angulate anterior manubrium. (b) A similar view of the second hook of the same specimen. (c) The posterior end of a female specimen, showing the subventral position of the unassuming gonopore. (d) A lateral gallium-cut section of a hook, showing the same core–cortical relationship demonstrated in cross sections (fig. 1e, f). (e) Micropores of different diameters and distributions from the middle and posterior section of the trunk. (f) Micropores of different diameters and distributions from the middle and posterior section of the trunk.

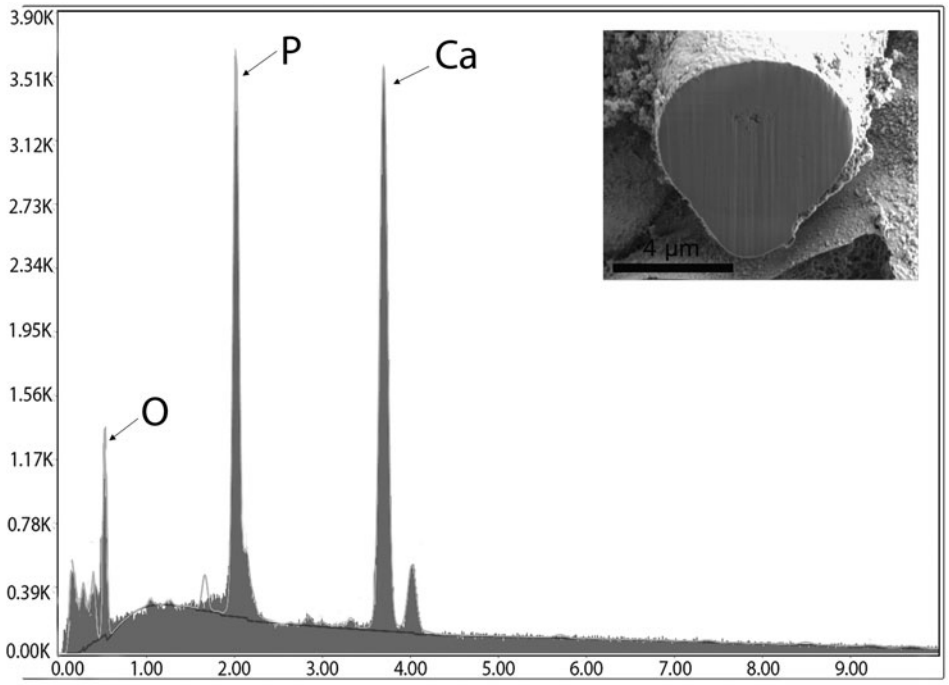
Molecular analysis

The specimens of *N. major* successfully presented amplification of about 1300 bp for the partial 18S rDNA gene and about 700 bp for the partial *cox1* gene. The 18S rDNA dataset (1240 nt) included ten sequences for the species of three families including Oligacanthorhynchidae, Moniliformidae and Gigantorhynchidae within the Archiacanthocephala class and the novel sequence of *N. major*. The *cox1* dataset (597 nt) included 16 sequences from three families of class Archiacanthocephala (Oligacanthorhynchidae, Moniliformidae and Gigantorhynchidae) and the sequence of *N. major*.

Inter-generic differences based on a partial 18S rDNA sequence of *N. major* with *Macracanthorhynchus hirudinaceus*, *Macracanthorhynchus ingens*, *Oligacanthorhynchus tortuosa* and *Oncicola* sp. as other members of family Oligacanthorhynchidae was 0.2%, 0.3%, 0.7% and 0.5%, respectively. The sequence divergence based on the partial sequence of *cox1* of *N. major* with *M. ingens*, *M. hirudinaceus*, *Oncicola sp.*, *Oncicola luehei*, *Prosthenorchis elegans* and *O. tortuosa* from Oligacanthorhynchidae was 21.8–22.3%, 33.5–35.9%, 36.6%, 36.3%, 36.0–36.1% and 44.2–46.2%, respectively.

The phylogenetic tree of the 18S rDNA illustrated that the *N. major* sequence grouped with *M. hirudinaceus* (LC350002), *M. ingens* (AF001844) and an unidentified species of *Oncicola* (AF064818) in the family Oligacanthorhynchidae with strong support. The sequence of *O. tortuosa* (AF064817) (Oligacanthorhynchidae) is located at the basal position to the members of the clade of Oligacanthorhynchidae. Indeed, as a major sister group, families Moniliformidae and Gigantorhynchidae clustered close to the family Oligacanthorhynchidae in the tree (fig. 4).

The phylogenetic reconstruction based on the partial sequence of *cox1* showed that our sequence of *N. major* clustered with *M. ingens* (AF416997 and KT881244) and *M. hirudinaceus* isolates (FR856886 and LC350021) with high statistical support (fig. 5). This clade appeared to be a sister group consisting of sequences of an unidentified species of *Oncicola* (AF417000), *O. luehei* (JN710452) and *P. elegans* (KT818501 and KT818504). These two clades belong to a larger clade of the



Element	Weight %	Atomic %	Error %	Net Int.	K Ratio
C	0.56	3.50	99.99	14.64	0.0067
N	9.80	18.84	13.57	37.14	0.0186
O	15.92	26.80	10.65	159.34	0.0394
Na	0.26	0.30	64.23	6.85	0.0014
Ga	0.06	0.02	73.60	0.87	0.0004
Mg	0.18	0.20	62.3	7.24	0.0012
P	19.65	7.19	2.65	702.92	0.1642
Au	3.18	17.09	11.82	52.22	0.0268
S	0.04	0.04	99.99	1.39	0.0003
Cl	0.04	0.03	99.99	1.15	0.0003
Pd	0.90	0.23	39.60	12.72	0.0064
K	0.61	0.42	30.2	14.53	0.0051
Ca	47.78	32.10	2.49	927.22	0.4070

Fig. 3. Energy-dispersive X-ray spectrum of a gallium-cut mid hook of a *Nephridiacanthus major* specimen, showing high levels of calcium (Ca), phosphorous (P) and oxygen (O). The X-ray data are the elemental analysis of the hook centre (see bold figures in table 3). Inset: SEM of a gallium-cut hook base.

family Oligacanthorhynchidae. In addition, *Moniliformis moniliformis* (AF416998), *Moniliformis kalahariensis* (MH401040) and *Moniliformis saudi* sp. n. (KU206783) of the Moniliformidae family, grouped as a sister clade of the family Oligacanthorhynchidae. Additionally, *O. tortuosa* isolates (KT881245 and KM659345) (Oligacanthorhynchidae) located as the sister group of the major clade. Moreover, *Mediorhynchus africanus* (KC261351) and *Mediorhynchus gallinarum* (KC261352) (Gigantorhynchidae) are placed at the basal position to the members of the tree.

Discussion

Specimens of *N. major* are reported from only three species of hedgehogs in the Middle East, Europe and Asia, but they exhibit considerable morphological variation in size of certain taxonomically important structures that appear to be related to the geographical distribution of hosts. For instance, the trunk length of specimens from Russia and central Asia (Petrochenko, 1958) show extreme small and large values of 20.00 mm and 200.00 mm in males and females, respectively, compared to males and females from the same host species in the two collections from Iran, 18.87–85.75 mm in males and 6.25-135.00 mm in females (table 2). The lemnisci were also considerably longer in Petrochecko's (1958) specimens than in the two Iranian populations. Similar relationships have also been observed in other acanthocephalans. For example, populations of *Mediorhynchus papillosus* Van Cleave, 1916 have a wide distribution in North and South America, Eastern Europe, Asia and many former Soviet republics, east to China and Taiwan. Morphometric comparisons of specimens of *M. papillosus* from Maryland, Colorado, Taiwan, Trans-Baikal, Lower Yenesei River, Volga,

Table 3. Chemical composition of a gallium-cut hook of a specimen of *Nephridiacanthus major*.

	Hook tip[a]		Mid area hook[a]		Hook base[b]	
Element[c]	Edge	Centre	Edge	Centre	Entry	Bottom
Sodium (Na)	0.24	0.08	0.42	**0.26**[d]	0.81	0.45
Magnesium (Mg)	0.26	0.17	0.22	**0.18**	0.54	0.22
Phosphorous (P)	18.54	19.98	17.91	**19.65**	15.70	16.09
Sulphur (S)	0.00	0.00	0.00	**0.04**	0.00	0.00
Potassium (K)	0.14	0.44	0.14	**0.61**	0.30	0.32
Calcium (Ca)	38.17	48.89	37.10	**47.78**	34.77	34.24

[a]Cross-section cut.
[b]Longitudinal cut.
[c]Common protoplasmic elements (C, N, O) and processing elements (Ga, Au, Pd) omitted from table. Listed in WT%.
[d]Bold weight % figures are used to generate the spectrum (Fig. 21).

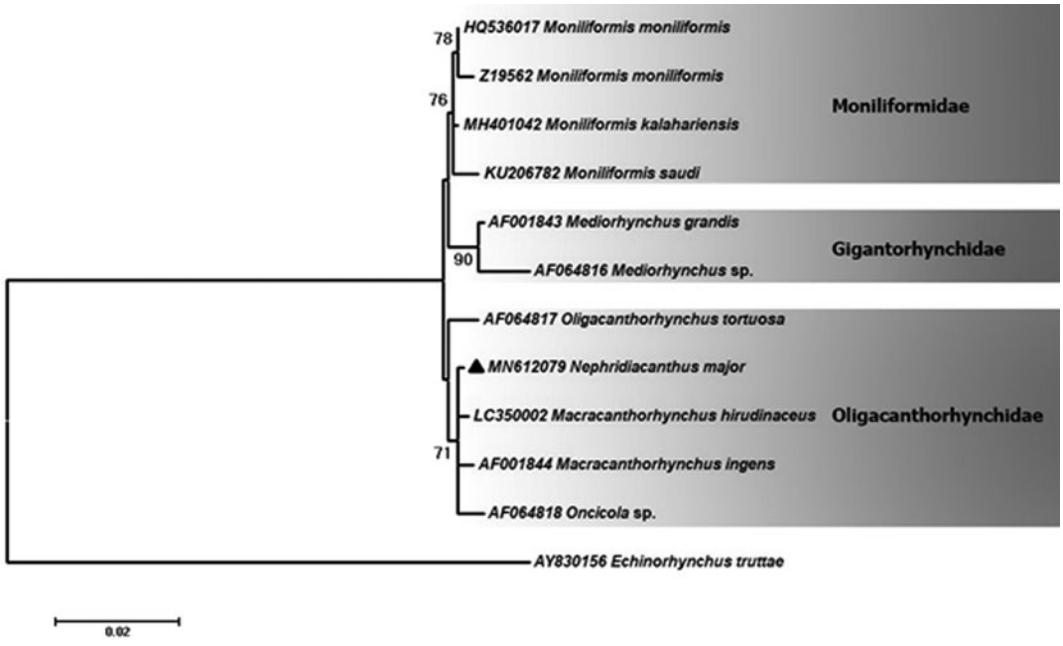


Fig. 4. Phylogenetic analysis of isolate of *Nephridiacanthus major* obtained in this study (▲) and closest-related members of class Archiacanthocephala retrieved from GenBank based on partial 18S rDNA gene. The tree was constructed using the maximum likelihood test and the Tamura three-parameter model in MEGA software version 6. *Echinorhynchus truttae* sequence was used as the out-group. Bootstrap values lower than 70 are omitted. The scale bar indicates number of substitutions per site.

Ukraine and Bulgaria exhibited distinctive morphological variability in certain key characters, 'with specimens from Colorado and Taiwan being at the opposite ends of the variability spectrum' (Amin & Dailey, 1998). Populations of *N. major* may have a disjunct distribution similar to that demonstrated in *M. papillosus* that was attributed to 'geographical restriction of intermediate (and adult hosts) in the apparent absence or scarcity of paratenic/reservoir hosts. Disjunct populations would promote distinct geographical diversity with consistent morphological difference from each other' (Amin & Dailey, 1998, p. 199). New species would have been created from disjunct populations if it was not for their presence along the geographical west–east gradient. This proposition was not considered viable in the case of *N. major*.

The additional morphological features depicted in figs 1 and 2 throw additional light on the morphology of *N. major* that have not been presented before.

Micropores

The electron-dense micropores present throughout the epidermal surface of the trunk of *N. major* have not been reported previously. The micropores of *N. major*, 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 (Heckmann *et al.*, 2013b) and in a few more since, and demonstrated the tunnelling from the cuticular surface into the internal crypts, using transmission electron micrographs (TEM). Amin *et al.* (2009) gave a summary of the structural-functional relationship of the micropores in various acanthocephalan species, including *Rhadinorhynchus ornatus* Van Cleave, 1918, *Polymorphus minutus* (Goeze, 1782) Lühe, 1911, *M. moniliformis* (Bremser, 1811) Travassos (1915), *M. hirudinaceus* (Pallas, 1781)

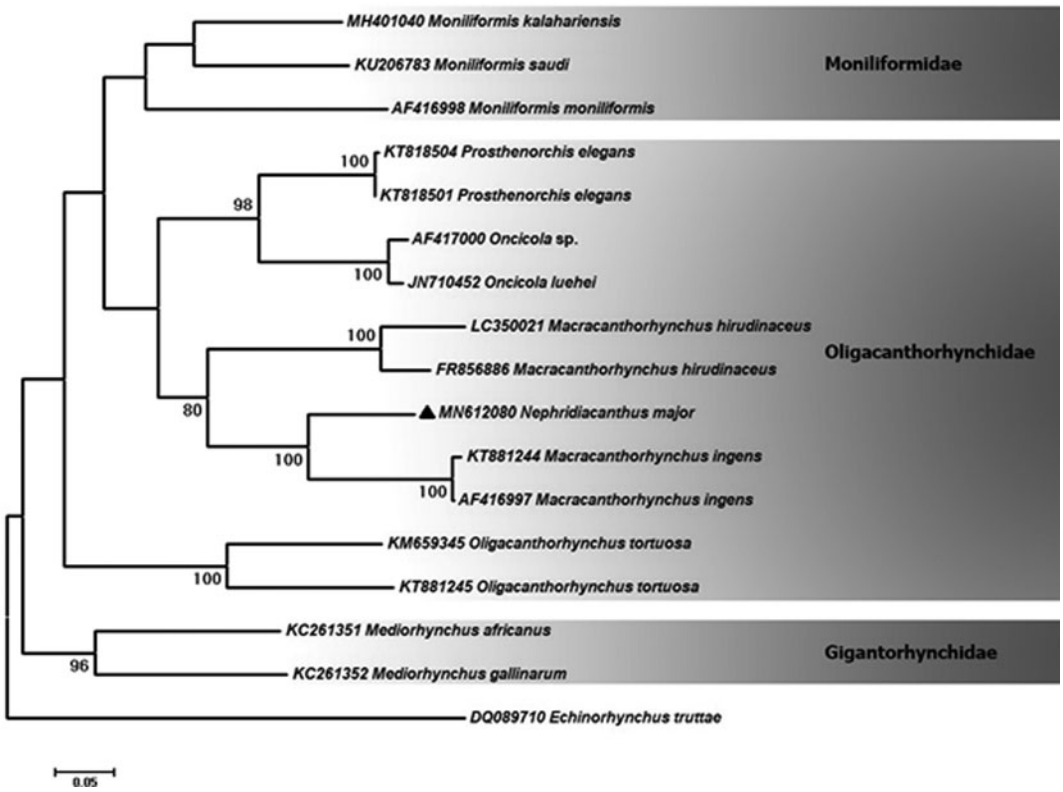


Fig. 5. Phylogenetic analysis of isolate of *Nephridiacanthus major* obtained in this study (▲) and closest-related members of class Archiacanthocephala retrieved from GenBank based on partial *cox1* gene. The tree was constructed using the maximum likelihood test and the Tamura three-parameter model in MEGA software version 6. *Echinorhynchus truttae* sequence was used as the out-group. Bootstrap values lower than 70 are omitted. The scale bar indicates the expected number of substitutions per site.

Travassos (1916, 1917) and *Sclerocollum rubrimaris* Schmidt & Paperna, 1978. Wright & Lumsden (1969) and Byram & Fisher (1973) 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) estimated a 44-fold increase at a surface density of 15 invaginations per 1 μm^2 of *M. moniliformis* 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, 1788) in the Caspian Sea were demonstrated by TEM in Amin *et al.* (2011).

EDXA

Results of the X-ray scans of the Ga cut hooks (dual-beam SEM) of *N. major* show differential composition and distribution of metals in different hook parts with the calcium and phosphorus being considerably higher at the centre of the middle of hooks where tension and strength are paramount compared to other metals (table 3). The chemical elements present in the hooks are typical for acanthocephalans (Amin & Heckmann, 2017; Amin *et al.*, 2017, 2018; Ha *et al.*, 2018). Note the thin outer layer (figs 1e, f and 2d) of the hook that relates to the sulphur content in the hook of *N. major*, which is less than what was found in other acanthocephalans (Amin & Heckmann, 2017; Amin *et al.*, 2018). The hook centre in middle cuts has a completely different calcium profile than the cortical layer (table 3). X-ray scans (EDXA) provide insight into the hardened components – for example, calcium and phosphorus – of acanthocephalan hooks. The EDXA appears to be species specific, as in fingerprints, and is shown to have significant diagnostic value in acanthocephalan systematics – for example, *Moniliformis cryptosaudi* Amin, Heckmann, Sharifdini, Albayati, 2019 was erected based primarily on its EDXA pattern (Amin *et al.*, 2019).

Molecular analysis

In recent years, DNA sequence-based methods were applied for the species identification, classification and evaluation of phylogenetic relationships among acanthocephalan species (García-Varela *et al.*, 2002). This is the first genetic study of *N. major*. It resolves its phylogenetic relationships with other members of the family Oligacanthorhynchidae and also with its closest families. Inter-generic differences are noted between *N. major* and other members of the family Oligacanthorhynchidae based on both partial 18S rDNA and *cox1* genes being 0.2–0.7% and 21.8–46.2%, respectively. These results illustrate that sequence differences between *N. major* and other members of the family based on the *cox1* gene are higher than those for 18S rDNA. Due to the high level of divergence in the *cox1* gene, it is appropriate to consider it for phylogenetic and taxonomic studies (García-Varela & de Leon, 2015). Based on the results of the *cox1* analyses, the similarity of *N. major* with *M. ingens* and *M. hirudinaceus* was 77.7–78.2% and 64.1–66.5%, respectively. Its phylogenetic tree also confirmed the higher similarity of *N. major* with *M. ingens*, with these two genera grouped together with higher bootstrap value. Meanwhile, based on 18S rDNA, *N. major* illustrated

more identity with *M. hirudinaceus* (99.8%) than with *M. ingens* (99.7%), which is in agreement with the 18S rDNA tree. Also, considering both genetic markers, *N. major* had the highest sequence differences with *O. tortuosa* among the members of the family Oligacanthorhynchidae. The phylogenetic analysis of the 18S rDNA sequence (fig. 1a) showed that our sequence of *N. major* is clustered with *M. hirudinaceus* (LC350002), *M. ingens* (AF001844), *Oncicola* sp. (AF064818) and *O. tortuosa* (AF064817), forming a clade of the family Oligacanthorhynchidae. The phylogenetic tree (fig. 4) demonstrated that the clade Oligacanthorhynchidae is well separated from those of the families Moniliformidae and Gigantorhynchidae. The phylogenetic reconstruction based on the partial sequence of *cox1* illustrated that our sequence of *N. major* is grouped with isolates of *M. ingens* (AF416997 and KT881244) and *M. hirudinaceus* (FR856886 and LC350021) (fig. 5). This group, along with a sister clade including *Oncicola* sp. (AF417000), *O. luehei* (JN710452) and *P. elegans* isolates (KT818501 and KT818504), were located in a major clade of the family Oligacanthorhynchidae. Meanwhile, *O. tortuosa* isolates (KT881245 and KM659345) were placed separately from other members of Oligacanthorhynchidae. Due to the lack of molecular data for *N. major* from other parts of the world, it would be necessary to obtain specimens from other geographical regions in order to determine the genetic diversity of the species. Also, obtaining sequence data from other members of the genus *Nephridiacanthus* would be useful for gaining a better understanding of the phylogenetic relationships in this genus.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

Author contributions. O. Amin made the taxonomic decisions and comparisons, wrote the manuscript and made the microscopic images. M. Sharifdini made the genetic analysis and wrote the molecular text. R. Heckmann created the SEM images and the EDXA analysis. M. Zarean collected and provided specimens of *N. major* from trapped hosts for the study. All authors critically reviewed the manuscript.

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