1 **Remarkable morphological variation in the proboscis of *Neorhadinorhynchus nudus***

2 **(Harada, 1938) (Acanthocephala: Echinorhynchida)**

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10 **Running title:** Morphological variability of *Neorhadinorhynchus nudus*

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13

14 **Abstract** The acanthocephalans are characterized by a retractible proboscis, armed with rows
15 of recurved hooks, which serves as the primary organ for attachment of the adult worm to the
16 intestinal wall of the vertebrate definitive host. Whilst there is considerable variation in the size,
17 shape, and armature of the proboscis across the phylum, intraspecific variation is generally
18 regarded to be minimal. Consequently, subtle differences in proboscis morphology are often
19 used to delimit congeneric species. In this study, striking variability in proboscis morphology
20 was observed among individuals of *Neorhadinorhynchus nudus* (Harada, 1938) collected from
21 the frigate tuna *Auxis thazard* Lacépède (Perciformes: Scombridae) in the South China Sea.
22 Based on the length of the proboscis, and number of hooks per longitudinal row, these
23 specimens of *N. nudus* were readily grouped into three distinct morphotypes, which might be
24 considered separate taxa under the morphospecies concept. However, analysis of nuclear and
25 mitochondrial DNA sequences revealed a level of nucleotide divergence typical of an
26 intraspecific comparison. Moreover, the three morphotypes do not represent three separate
27 genetic lineages. The surprising, and previously undocumented level of intraspecific variation
28 in proboscis morphology found in the present study, underscores the need to use molecular
29 markers for delimiting acanthocephalan species.

30

31 **Keywords:** Neoechinorhynchus; proboscis armature; morphological variability; DNA
32 taxonomy; phylogeny; frigate tuna

33 **Key findings**

34 • *N. nudus* displays far greater intraspecific variation in proboscis characters than has
35 previously been reported for an acanthocephalan species.
36 • Morphotypes may erroneously be recognised as distinct taxa in the absence of genetic
37 data.
38 • Failure to use molecular markers to distinguish between intraspecific and interspecific
39 variation will confound our understanding of species diversity in the Acanthocephala.

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41

42 **Introduction**

43 The proboscis provides some of the most important morphological characters used in
44 acanthocephalan taxonomy (Van Cleave, 1953). Many acanthocephalan species are
45 discriminated from congeners on the basis of the size and form of the proboscis, and especially
46 the number, arrangement, size and shape of the proboscis hooks (Petrochenko, 1956; Yamaguti,
47 1963; Golvan, 1969). Furthermore, patterns of serial variation in hook morphometrics have
48 been used to detect cryptic species (Huffman and Bullock, 1975; Wayland, 2010).

49 During a helminthological survey of Chinese marine fishes, many specimens of
50 *Neorhadinorhynchus* Yamaguti, 1939 were collected from the frigate tuna *Auxis thazard*
51 Lacépède (Perciformes: Scombridae) in the South China Sea. Examination of this material
52 using light and scanning electron microscopy, revealed three distinct morphotypes,
53 characterized by the length of the proboscis, and the number of hooks per longitudinal row. One
54 of these morphotypes conformed to the diagnosis of *Neorhadinorhynchus nudus* (Harada,
55 1938), a common parasite of scombrid and carangid fishes in the Pacific Ocean and Red Sea

56(Harada, 1938; Yamaguti, 1939, 1963; Amin and Nahhas, 1994; Hassanine, 2006), and was
57 used in a redescription of the taxon (Li *et al.* 2018). The other two morphotypes were suspected
58 to represent a different, as yet undescribed species. In the present study we tested the hypothesis
59 that these three morphotypes correspond to three distinct lineages,, by analysing nuclear and
60 mitochondrial DNA sequences from a few individuals of each morphotype. The *cox*1 gene and
61 ITS region were selected with the expectation that they would reveal population structure and
62 discriminate congeneric species (Král'ová-Hromadová *et al*. 2003; Steinauer *et al*. 2007). The
63 more slowly evolving 18S rRNA was chosen to provide resolution at higher taxonomic ranks
64 (García-Varela and Nadler, 2005).
65
66
67 **Material and methods**
68 *Morphological observation*
69 Specimens of *N. nudus* collected from the intestine of *A. thazard* in the South China Sea (off
70 Shanwei, Guangdong Province, China), were kept in tap water for several hours until the
71 proboscis everted, and then fixed and stored in 80% ethanol until studied. Acanthocephalans
72 were identified to the specific level based on the following morphological characters: the
73 morphology and size of the trunk, proboscis, proboscis receptacle and uterine bell, the number
74 of longitudinal rows of proboscis hooks and the hooks per longitudinal row, the number and
75 size of the cement glands and testes, and the length of the neck and uterus. For light
76 microscopy, the worms were cleared in lactophenol and examined as wet mounts. Drawings of
77 the proboscis were made with the aid of a Nikon microscope drawing attachment. Voucher

78 specimens (accession numbers: HBNU–F-A-2017005L, HBNU–F-A-2018002L, HBNU–F-A-
79 2018003L) are deposited in College of Life Sciences, Hebei Normal University, Hebei
80 Province, P. R. China.
81
82 *Molecular procedures*
83 A total of 12 specimens representing the three morphotypes were selected for molecular
84 analysis (see Table 1 for details). Genomic DNA from each individual was extracted using a
85 Column Genomic DNA Isolation Kit (Shanghai Sangon, China) according to the
86 manufacturer's instructions. DNA was resuspended in elution buffer and kept at -20°C until use.
87 Part of the gene encoding the small subunit 18S rRNA was amplified by polymerase chain
88 reaction (PCR) using the forward primer (5'-AGATTAAGCCATGCATGCGT -3') and the
89 reverse primer (5'-GCAGGTTCACCTACGGAAA-3') (Garey *et al*. 1996). A segment of the
90 cytochrome c oxidase subunit 1 (*cox*1) gene was amplified by PCR using the forward primer
91 (5'- GGTCAACAAATCATAAAGATATTGG-3') and the reverse primer (5'-
92 TAAACTTCAGGGTGACCAAAAAATCA-3') (Goméz *et al*. 2002). A region of the internal
93 transcribed spacer (ITS) was amplified by PCR using the forward primer (5'-
94 GTCGTAACAAGGTTTCCGTA-3') and the reverse primer (5'-
95 TATGCTTAAATTCAGCGGGT-3') (Král'ová-Hromadová *et al*. 2003). The cycling conditions
96 were as described previously (Li *et al*. 2017). PCR products were checked on GoldView-stained
97 1.5% agarose gels and purified with a Column PCR Product Purification Kit (Shanghai Sangon,
98 China). Sequencing was carried out using a DyeDeoxyTerminator Cycle Sequencing Kit (v.2,
99 Applied Biosystems, California, USA) and an automated sequencer (ABI-PRISM 377).

100 Sequencing of each sample was carried out for both strands.

101 Sequences were aligned using ClustalW2 (Larkin *et al*. 2007) with some manual

102 adjustment. The DNA sequences obtained herein were compared (using the algorithm

103 BLASTn) with those available in the National Center for Biotechnology Information (NCBI)

104 database (http: //www.ncbi.nlm. nih.gov). The functional consequences of nucleotide

105 polymorphisms in the *cox*1 sequences were assessed by translating them into amino acid

106 sequences using the EMBOSS program Transeq (Rice *et al*. 2000; Li *et al*. 2015) with the

107 invertebrate mitochondrial codon table.

108 *Phylogenetic analyses* A haplotype network was constructed from the *cox*1 DNA

109 sequence data using the method of statistical parsimony (Templeton, 1992) and an infinite sites

110 model, as implemented in the R package pegas (Paradis, 2010). Phylogenetic trees for the *cox*1

111 haplotypes were inferred using both maximum parsimony (MP) and maximum likelihood (ML),

112 as implemented in MEGA7 (Kumar *et al*. 2016). *Filisoma bucerium* Van Cleave, 1940

113 (DQ089722) was selected as the outgroup, because it is the only other species of the family

114 Cavisomidae for which a DNA barcode is available. The *N. nudus* and *F. bucerium cox*1

115 sequences were aligned using the MUSCLE algorithm (Edgar, 2004) implemented in MEGA7,

116 with the default alignment parameters, and then refined manually. The optimal nucleotide

117 substitution model for the *cox*1 data-set was determined to be the Hasegawa-Kishino-Yano with

118 invariant sites (Hasegawa *et al*. 1985). Bootstrap resampling (n=1,000) was used to quantify

119 clade support.

120

121

122 **Results**

123 *Morphological analysis*

124 A total of 38 individuals of *N. nudus* were collected from three specimens of *A. thazard.* Based

125 on the number of hooks per longitudinal row and the length of proboscis (Figs. 1, 4, Table 1),

126 these acanthocephalans were readily classified into three morphotypes, designated I, II and III.

127 All three morphotypes were present in each of the host fish examined. Morphotype I had the

128 longest proboscis (1.24–1.78 mm) and the most hooks per longitudinal row (24–27).

129 Morphotype III had the shortest proboscis (0.59–0.81 mm) and the smallest number of hooks

130 per longitudinal row (10–13). Morphotype II had a proboscis that was intermediate in form

131 between those of the other two morphotypes, with a length of 0.99–1.53 mm and 16–20 hooks

132 per longitudinal row. Morphometric and meristic data for the three morphotypes are provided in

133 Table 2. Anatomical differences between the three morphotypes appear to be restricted to the

134 number of hooks per longitudinal row and the length of the proboscis. Pairwise scatter plots and

135 principal component analysis (PCA) failed to find additional combinations of morphometric

136 and/or meristic variables which might discriminate the three morphotypes.

137

138 *Molecular characterization*

139 *Partial 18S rDNA region*

140 Partial 18S rDNA sequences, 1201 bp in length, were obtained from ten specimens of *N. nudus*

141 (four from morphotype I, four from morphotype II and two from morphotype III). All ten

142 sequences are identical. They have been deposited in GenBank

143 (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MG838936–MG838945.

144 There are only two other cavisomid species *Filisoma bucerium* Van Cleave, 1940 and *F.*
145 *rizalinum* Tubangui & Masilungan, 1946 with 18S rDNA data registered in GenBank
146 (https://www.ncbi.nlm.nih.gov/genbank/). Nucleotide divergence between *N. nudus* and *F*
147 *bucerium* (AF064814) was 14.5%, whereas divergence between *N. nudus* and *F. rizalinum*
148 (JX014229) was 21.7%.
149
150 *Partial ITS region*
151 Partial ITS sequences, 559 bp in length, were obtained from 12 specimens of *N. nudus* (four
152 from morphotype I, five from morphotype II and three from morphotype III). All sequences are
153 identical. They have been deposited in Genbank under accession numbers MG757440–
154 MG757443 and MG838923–MG838930. GenBank contains ITS sequence data for only one
155 other cavisomid species, *F. bucerium* (AF286305). The ITS sequences of *F. bucerium* and *N.*
156 *nudus* display over 40% nucleotide divergence.
157
158 *Partial cox1 region*
159 Partial *cox*1 sequences, 669 bp in length, were obtained from nine specimens of *N. nudus* (four
160 from morphotype I, three from morphotype II and two from morphotype III) and represented
161 eight unique haplotypes. The two specimens sharing the same haploptype belonged to
162 morphotype II. Nucleotide polymorphisms were found at 33 (4.9%) of the sites; 30 (4.5%) at a
163 third codon position and 3 (0.4%) at a first codon position (sequences were read in frame 2).
164 Translation of the partial *cox*1 nucleotide sequences demonstrated that they all encode the same
165 sequence of amino acids. Nucleotide divergence between pairs of haplotypes ranged from

166 0.30% to 2.54% (see Table 3 for details). The *cox*1 sequences of *N. nudus* are deposited in
167 GenBank under accession numbers MG757444–MG757447 and MG838931–MG838935.
168 There is only one other cavisomid species *F. bucerium* with *cox*1 data (DQ089722) registered in
169 GenBank, and pairwise comparison between *N. nudus* and *F. bucerium* showed over 30.0%
170 nucleotide differences in the *cox*1 region.
171
172 *Phylogenetic analyses*
173 The *cox*1 haplotype network (Fig. 3) shows no evidence that the three morphotypes represent
174 distinct genetic lineages. For example, haplotypes IV and VII, exhibiting the most divergent
175 phenotypes (morphotypes I and III respectively) differ by only two nucleotide substitutions.
176 Moreover, one of the two joint longest links in the minimum spanning tree (13 nucleotide
177 substitutions) connects haplotypes II and IV, both of which exhibit morphotype I. Phylogenetic
178 trees constructed from the *cox*1 data-set using ML and MP had almost an identical topology, but
179 there were small differences in clade support (Fig. 4). None of the three morphotypes formed a
180 monophyletic group in these trees. Moreover, there was strong bootstrap support (97% in the
181 ML tree) for the clade comprising samples Nn3, Np3, Nn4 and Ns2, which includes
182 representatives of all three morphotypes.
183
184
185 **Discussion**
186 In recent years, acanthocephalan taxonomists have started to augment their morphological
187 descriptions of new species with DNA sequence data (*e.g.* Tkach *et al*. 2013; Amin *et al*. 2013;
188 Braicovich *et al*. 2014; Li *et al*. 2017). Nevertheless, the vast majority of the *c.* 1,300 currently

189 recognised species (Amin, 2013) were defined under the traditional morphospecies concept

190 (Cain, 1953; Ruse, 1969). Within *Neorhadinorhynchus*, none of the eight currently recognised

191 species were characterised using molecular markers when they were originally described

192 (Harada, 1938; Fukui and Morisita, 1937; Golvan, 1969; Mordvinova, 1988; Amin and Nahhas,

193 1994; Amin and Ha, 2011; Smales *et al*. 2015). However, Li *et al.* (2018) used both

194 morphological and molecular characters in their redescription of *N. nudus*.

195 The acanthocephalans collected in the present study exhibit far greater variation in

196 proboscis characters than has previously been reported among conspecifics. The three

197 morphotypes are clearly delimited by disjunct ranges for the number of hooks per longitudinal

198 row, and so under the morphospecies concept they would be considered distinct taxa. However,

199 analysis of the *cox*1 sequence data provides convincing evidence that these morphotypes do not

200 represent monophyletic groups. Moreover, the level of genetic variation within this collection

201 of worms is a strong indication that they are systematically homogeneous.

202 Across the animal kingdom, uncorrected nucleotide divergence in the *cox*1 gene between

203 populations, sibling species, and morphologically distinct congeneric species is on average

204 0.89%, 3.78% and 11.06% respectively (Kartavtsev, 2011). More specifically, within the

205 acanthocephalan order Echinorhynchida, Steinauer et al. (2007) found that for pairwise

206 comparisons of six closely related species of *Leptorhynchoides* Kostylev, 1924, the proportion

207 of nucleotide substitutions in the *cox*1 gene ranged from 6.3% to 11.6%. Divergence within

208 these six taxa ranged from 0.4 to 2.8%. Therefore, the 0.3% to 2.54% divergence observed in

209 the present study is strongly indicative of an intraspecific comparison. Identical ITS sequences

210 for all sequenced individuals of *N. nudus* are further evidence for the systematic homogeneity

211 of this collection of acanthocephalans. In an interspecific or even an interpopulation

212 comparison, nucleotide variation in the ITS region would be expected (Král'ová-Hromadová *et*

213 *al*. 2003). Nucleotide polymorphisms in the slowly evolving 18S rRNA gene were neither

214 anticipated nor found, but this DNA sequence will be a valuable reference for future studies

215 examining the phylogenetic relationships of higher taxa within the Acanthocephala (García-

216 Varela and Nadler, 2005).

217 Molecular genetic studies on other morphologically variable acanthocephalan taxa have

218 shown that the morphospecies concept often conflates cryptic species (*e.g.* Buron *et al*. 1986;

219 Väinölä *et al*. 1994; Steinauer *et al*. 2007; Martínez-Aquino *et al*. 2009). This study highlights

220 the opposite, less commonly reported problem, where the use of the morphospecies concept

221 would result in the splitting of a biological species into genetically indistinct taxa. The present

222 study is not the first record of clearly defined morphotypes within an acanthocephalan species.

223 Li *et al*. (2017) observed two phenotypes in *Pomphorhynchus zhoushanensis* Li, Chen, Amin &

224 Yang, 2017. In one the neck bulb was symmetrical and in the other asymmetrical. Analysis of

225 molecular markers confirmed that the two forms were conspecific (Li *et al*. 2017).

226 The most recent key to the species of *Neorhadinorhynchus* Yamaguti, 1939 relied almost

227 exclusively on proboscis characters (Amin and Nahhas, 1994), which the current study has

228 shown may lack the stability required for delimiting taxa in this genus. When the key was

229 devised, *N. nudus* was thought to exhibit 24–25 hooks per longitudinal row (Amin and Nahhas,

230 1994). The number of hooks per row observed in the present study (10–27) encompasses almost

231 the full range of variation found across the entire genus. *N. basrahiensis* Smales, Al-Hasson, Al-

232 Hasson & Al-Azizz, 2015 has the fewest hooks per longitudinal row, with a reported range of

233 9–10 (Smales *et al*. 2015). *N. atypicalis* Amin & Ha, 2011 also displays 27 hooks per
234 longitudinal row (Amin and Ha, 2011), which is the maximum number reported for a species of
235 this genus. Tandem molecular phylogenetic and morphological analyses will be required to
236 determine the true species diversity in *Neorhadinorhynchus* and to identify the best anatomical
237 characters for differential diagnosis of these taxa.
238 The cause of the broad anatomical variation in the proboscis of *N. nudus* can only be
239 speculated. It may be the result of phenotypic plasticity, *i.e.* "the property of individual
240 genotypes to produce different phenotypes when exposed to different environmental
241 conditions", such as temperature or nutrient availability (Pigliucci *et al*. 2006). Many of the
242 studies that have attempted to identify sources of phenotypic variation in acanthocephalans (*e.g.*
243 Grabda-Kazubska and Ejsymont, 1969; Amin, 1975; Buckner and Nickol, 1975; Amin and
244 Redlin, 1980; Amin, 1984; Shostak *et al*. 1986; Brown, 1987) were conducted at a time when
245 molecular systematics was in its infancy, and so did not make use of genetic data to differentiate
246 intraspecific from interspecific variation. Recently, Sobecka *et al*. (2012) investigated
247 intraspecific morphological variation in a species of the *Echinorhynchus gadi* Zoega in Müller,
248 1776 group, after first using molecular markers to confirm that their collection of
249 acanthocephalans was systematically homogenous. Morphological variation was subtle,
250 compared to that found in the present study, and showed an association with geographical
251 locality, definitive host subspecies, and size of the acanthocephalan infrapopulation.
252 Morphogenesis of the acanthocephalan proboscis, including hooks, occurs in the
253 acanthella larva, within the intermediate host (Schmidt, 1985). Therefore to identify factors that
254 might influence this process, both the immediate environment of the acanthella and the habitat

255 of the intermediate host must be considered. For example, different species of intermediate
256 hosts might present different environments for the acanthella in terms of nutrient availability,
257 physical space and, if they occupy different habitats, temperature regime.
258 The development of *Echinorhynchus truttae* Schrank, 1788 (another member of the
259 Echinorhynchida) in its intermediate host *Gammarus pulex* (L.) is influenced by infection
260 intensity, which is presumably a proxy for the magnitude of competition for resources, such as
261 nutrients and space. Awachie (1966) found that in heavily infected intermediate hosts,
262 cystacanths of this species were smaller and more slender than those developing under less
263 crowded conditions, and in some cases were malformed or irregularly shaped. If the
264 intermediate host(s) of *N. nudus* can be identified, and maintained in the laboratory, phenotypic
265 plasticity could be investigated via experimentation.
266 In summary, the present study has shown that *N. nudus* displays far greater intraspecific
267 variation in proboscis characters than has previously been reported for an acanthocephalan
268 species. This variation is partitioned into three morphotypes, each of which might erroneously
269 be recognised as adistinct taxon in the absence of genetic data. We recommend that when
270 species of Acanthocephala are described or redescribed, appropriate molecular markers (*e.g.*
271 *cox*1 or ITS sequences) should be used to distinguish intraspecific from interspecific
272 morphological variation, and to provide a reference for diagnostic purposes.
273
274
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278

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282

283 **Conflict of interest** The authors declare that they have no conflict of interest.

284

285 **Ethics approval**

286 This study was conducted according to the Hebei Normal University Experiments in Animals

287 Policy and was approved by the Animal Ethics Committee of Hebei Normal University as

288 complying with the Animal Protection Law of the People's Republic of China.

289

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431 **Figure legends:**

432

433 **Fig. 1.** Scanning electron micrographs of the different number of hooks per longitudinal row

434 and the length of the proboscis of *Neorhadinorhynchus nudus* (Harada, 1938) from *Auxis*

435 *thazard* (Lacépède) (Perciformes: Scombridae) in the South China Sea. A, morphotype I with

436 the longest proboscis and 24–27 hooks per longitudinal row; B, morphotype III with the

437 shortest proboscis and 10–13 hooks per longitudinal row; C, morphotype II with a proboscis of

438 intermediate length and 16–20 hooks per longitudinal row. *Scale bars*: A = 100 µm; B = 40 µm;

439 C = 100 µm.

440

441 **Fig. 2.** Scatterplot of proboscis length (mm) *vs* the maximum number of hooks per longitudinal

442 row. Plotting symbols indicate the morphotype.

443

444 **Fig. 3.** Haplotype network based on the partial *cox*1 sequences. Node size is proportional to

445 haplotype frequency. Node colour indicates the morphotype. The minimum spanning tree is

446 represented by solid black links (edges). Alternate links are shown as grey dashed-lines.

447 Mutations are indicated by small segments on the links. Haplotypes correspond to the following

448 samples: I, Nn1; II, Nn2; III, Nn3; IV, Nn4; V, Np1 and Np2; VI, Np3; VII, Ns2; VIII, Ns3.

449

450 **Fig. 4.** ML tree showing the phylogenetic relationships of nine specimens of

451 *Neorhadinorhynchus nudus* (Harada, 1938) from *Auxis thazard* (Lacépède) (Perciformes:

452 Scombridae) in the South China Sea based on the mitochondrial cox1 sequences. Numbers at

453 the nodes are the clade credibility values (%) for each method of phylogeny reconstruction

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454 (ML/MP). The tree is rooted on the outgroup *Filisoma bucerium* Van Cleave, 1940

455(DQ089722). A, morphotype I with 24-27 hooks per longitudinal row; B, morphotype II with

456 16-20 hooks per longitudinal row; C, morphotype III with 10-13 hooks per longitudinal row.

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