1 **DNA profiling reveals *Neobenedenia girellae* as the primary culprit in**

2 **global fisheries and aquaculture**

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ABSTRACT

19 Accurate identification of parasite species and strains is crucial to mitigate the risk of epidemics

20 and emerging disease. Species of *Neobenedenia* are harmful monogenean ectoparasites that

21 infect economically important bony fishes in aquaculture worldwide, however, the species

22 boundaries between two of the most notorious taxa, *N. melleni* and *N. girellae*, has been a topic

23 of contention for decades. Historically, identifications of *Neobenedenia* isolates have

24 overwhelmingly been attributed to *N. melleni*, and it has been proposed that *N. girellae* is

25 synonymous with *N. melleni*. We collected 33 *Neobenedenia* isolates from 22 host species

26   spanning nine countries and amplified three genes including two nuclear (*Histone 3* and *28S*

27   *rDNA*) and one mitochondrial (*cytochrome b*). Four major clades were identified using

28   Maximum Likelihood and Bayesian inference analyses; clades A-D corresponding to *N. girellae*,

29   *N. melleni*, *N. longiprostata* and *N. pacifica* respectively. All unidentified isolates and the

30   majority of *Neobenedenia* sequences from GenBank fell into clade A. The results of this study

31   indicate that *N. girellae* is a separate species to *N. melleni*, and that a large proportion of previous

32   samples identified as *N. melleni* may be erroneous and a revision of identifications is needed.

33   The large diversity of host species that *N. girellae* is able to infect as determined in this study

34   and the geographic range in which it is present (23.8426° S and 24.1426° N) makes it a globally

35   cosmopolitan species and a threat to aquaculture industries around the world.

36

37   Key words: phylogeny, host specificity, Monogenea, Capsalidae, skin fluke, *Neobenedenia*

38   *melleni*, cryptic

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40   **1. Introduction**

41

42   The Monogenea, one of three predominantly parasitic classes in the phylum

43   Platyhelminthes, predominantly comprises ectoparasitic flukes that infect the gills and skin

44   surfaces of marine and freshwater fishes (Whittington, 2004). Monogeneans in the family

45   Capsalidae (Monopisthocotylea) are recognised as virulent pathogens of finfish in sea-cage and

46   semi-open pond aquaculture and are able to multiply rapidly in high-density aquaculture

47   environments as they have a direct, single host life cycle (Jahn and Kuhn, 1932; Ogawa, 1996).

48   Some species are responsible for considerable epidemics (Bauer and Hoffman, 1976; Deveney et

49   al., 2001; Paperna and Overstreet, 1981; Whittington et al., 2004). According to current

50   taxonomic classification, the Capsalidae comprises nine subfamilies, 57 genera, and over 300

51   species, most of which are ectoparasitic on marine fishes (Gibson et al., 2010). One of the most

52   notorious of capsalid genera is *Neobenedenia*, Yamaguti 1963, which have a large host range and

53   have been implicated in causing severe pathology and devastating economic losses in

54   aquaculture worldwide.

55   *Neobenedenia* spp. harm fish by mechanical attachment and subsequent grazing on the

56   epithelial surface of their host, which can cause epidermal degradation, dermal erosion,

57   inflammation and allow the ingress of secondary pathogens (Kaneko et al., 1988; Trujillo-

58   González et al., 2015). *Neobenedenia* currently comprises six species and has gained global

59   attention primarily due to the notoriety of the type species, *Neobenedenia melleni*, MacCallum

60   1927, which was originally described from tropical fishes held in the New York Aquarium

61   (MacCallum, 1927; Whittington and Horton, 1996). While the origin of the infection is not clear,

62   it is thought that the parasite may have been introduced from the Caribbean through the

63   ornamental fish trade (Dyer et al., 1992; Whittington and Horton, 1996). *Neobenedenia melleni*

64   is infamous as a widespread pathogen in aquaria and aquaculture and the list of recorded host

65   taxa spans over 100 species from 30 families and five orders of teleosts (Bullard et al., 2000;

66   Harris et al., 2004; Whittington and Horton, 1996; Whittington, 2004; Whittington et al., 2004).

67   The broad host specificity reported for *N. melleni* is atypical for monogeneans, where it is

68   considered that approximately 80% of all monogenean species only infect one host species and

69   usually only in a single ocean (Byrnes and Rohde, 1992; Rohde, 1979; Whittington, 1998;

70   Whittington et al., 2000).

71   Given their specialised lifestyle, it is not surprising that the morphology of species of

72   *Neobenedenia* is highly conserved and accurate identification has proven challenging (Ogawa et

73   al., 1995; Whittington, 2004; Whittington et al., 2004). Of particular controversy and confusion

74   has been the delineation of *N. melleni* and *Neobenedenia girellae*, Hargis, 1955. *Neobenedenia*

75   *girellae* was originally described by Hargis (1955) infecting opaleye, *Girella nigricans,* Ayres

76   1860, from California. Twenty-six additional host species have been subsequently reported from

77   a range of localities (Nigrelli, 1947; Bravo-Hollis, 1958; Bravo-Hollis and Deloya, 1973; Gaida

78   and Frost, 1991; Love et al., 1984; Moser and Haldorson, 1982; Ogawa et al., 1995). Twenty

79   years ago, Whittington and Horton (1996) synonymised *N. girellae* with *N. melleni* based on

80   morphology and host specificity. This decision was not unanimously accepted and many authors

81   continue to use '*N. girellae*' in the scientific literature (e.g. Koesharyani et al., 1999; Ogawa et

82   al., 2006; Ogawa and Yokoyama, 1998; Wang et al., 2004; Yoshinaga et al., 2000; Zhang et al.,

83   2014).

84   Studies employing molecular techniques in systematics have revealed a large number of

85   morphologically similar parasite species that were previously recognized as a single taxon but

86   are actually genetically distinct (Blasco-Costa et al., 2010; Donald et al., 2004; Freeman &

87   Ogawa, 2010; Leung et al., 2009; Miura et al. 2005; Saijuntha et al., 2007; Sepúlveda and

88   González, 2014; Wu et al., 2005; Xiao et al. 2005). For example, Sepúlveda and González

89   (2014) found that a related capsalid species, *Benedenia seriolae*, Yamaguti 1934, is in fact a

90   complex of cryptic species and not a single taxon. The first molecular-based evidence suggesting

91   that a species complex may be present among *Neobenedenia* spp. was presented by Whittington

92   et al. (2004) where *28S rDNA* sequences were compared between two *Neobenedenia* isolates

93   identified as *N. melleni*. This study indicated that distinct taxa may be present within a complex

94   of morphologically similar individuals. Since this research, very few molecular studies have

95   addressed *Neobenedenia* taxa in order to resolve the confusion. A brief study comparing *N.*

96   *melleni* and *N. girellae* by Wang et al. (2004) used short sequences of *28S rDNA* and indicated

97   that the synonymy of these species proposed by Whittington and Horton (1996) was supported.

98   However, a more comprehensive study by Perkins et al. (2009), which addressed the phylogeny

99   of many capsalid species using multiple genes (*28S rDNA, Histone 3,* and *Elongation Factor*

100   *1α*), seems to support the opposite view; that *N. melleni* and *N. girellae* are two separate species.

101   *Neobenedenia* was first documented in Australian waters in 2000. An outbreak of *N.*

102   *melleni* was reported in farmed barramundi, *Lates calcarifer,* Bloch 1790, and resulted in the

103   death of over 200,000 fish (Deveney et al., 2001). However, it was only in 2011 that research on

104   *Neobenedenia* in Australia began to develop following the collection of *Neobenedenia* sp.

105   individuals from a fish farm in north Queensland and the subsequent establishment and

106   maintenance of a continuous *in vivo* culture in the Marine Parasitology Laboratory at James

107   Cook University, Australia (Hutson et al., 2012). Morphological identification of the species of

108   *Neobenedenia* in culture has been problematic and all previous research from this laboratory has

109   referred to the parasite as *Neobenedenia* sp. (e.g. Dinh Hoai and Hutson, 2014; Hutson et al.,

110   2012; Trujillo-González et al., 2015a,b). There is a need to accurately identify the species of

111   *Neobenedenia* currently being used as a parasite model in this laboratory so that research can be

112   ascribed to the correct taxon allowing for more meaningful application.

113   The aim of this study was to use molecular characterisation methods to generate a robust

114   diagnostic and phylogenetic framework for the identification of *Neobenedenia* spp. to underpin

115   our understanding of the ecology of the taxa implicated in causing damage to wild and

116   aquaculture fish stocks worldwide.

117

2. Materials and Methods

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2.1. Sample collection

121

Neobenedenia specimens were collected between 2000 and 2015 from wild and captive fish in nine countries. The majority of Australian isolates were collected by the authors while many of the international samples were donated by research colleagues (Table 1). Samples were collected by removing live parasites from the skin of their hosts using a scalpel blade or after bathing the host in freshwater, which kills the parasites. Parasites were fixed and stored in 70% analytical grade ethanol.

A slice of tissue (sliver) was removed from the right-hand side margin of individual parasites, opposite the reproductive organs, taking care to avoid the gut. The remainder of the specimen was washed three times using distilled H_2O and stained with Haematoxylin. Parasites were then dehydrated through an alcohol series, cleared in methyl salicylate or cedarwood oil, and mounted on microscope slides in Canada balsam for further study (Hutson et al., 2012). At least one mounted specimen collected per host by A.K. Brazenor and K.S. Hutson was accessioned into the Queensland Museum helminthology collection. Specimens from the late I.D. Whittington's collection were accessioned to the Australian Helminthological Collection at the South Australian Museum (Table 1).

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2.2. DNA preparation, PCR amplification, and amplicon sequencing

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140   DNA was extracted from parasite slivers using either the PUREGENE DNA purification

141   system (Gentra Systems) protocol for DNA purification from solid tissue or a QIAgen DNeasy

142   Kit (QIAGEN Inc., Valencia, California, USA) according to the manufacturers' protocols. PCR

143   amplifications of partial *H3*, *28S rDNA*, and *Cytb* sequence were carried out in 25μl reactions

144   using the primers listed in Table 2 and either Amplitaq Gold DNA polymerase, or Phusion high-

145   fidelity polymerase with the following reaction and cycling conditions: Amplitaq Gold – we

146   followed Perkins et al. (2009) except that we standardised the annealing temperature at 55°C and

147   used a maximum of 34 PCR cycles: Phusion - we used a final concentration of 5μL of

148   5xPhusion® HF buffer, 0.5 μL of 10mM dNTPs, 1.25 μL of each primer (10mM), 0.25 μL of

149   Phusion® Tag DNA polymerase, and 4 μL of DNA template with an initial denaturation step of

150   98 °C for 30 s, followed by 35 cycles of PCR; denaturation at 98 °C for 10 s, annealing 53-62 °C

151   for 20 s, extension at 72 °C for 30 s, with an additional final extension at 72 °C for 7.5 min. The

152   double-stranded amplification products were visualised on 1.5% agarose gels and purified using

153   a Multiscreen – PCR Plate (Millipore Corporation). Purified products were sent to the Australian

154   Genome Research Facility for cycle-sequencing in both directions using the BigDye Terminator

155   v3.1 cycle-sequencing kit (Applied Biosystems) on an AB3730xl capillary sequencer.

156

157   *2.3. Phylogenetic analyses*

158   Sequence chromatograms were edited with SeqEd 1.0.3 (Applied Biosystems) and

159   aligned using Se-Al2.0a11 (http://tree.bio.ed.ac.uk/software/seal/) using inferred amino acid

160   translations (*H3* and *Cytb*) and the predicted *28S rDNA* secondary structure model for

161   *Gyrodactylus salaris*, Malmberg 1957, (Matejusová and Cunningham, 2004). Subsequently, the

162   *28S rDNA* alignment was trimmed to 411bp to remove sequences that could not be aligned

163 unambiguously and sequences from all three genes concatenated to give a final alignment length

164 of 1409bp. We were unable to amplify all target fragments for some samples and these are coded

165 in the final alignment as "missing data". All gene sequences have been deposited in GenBank

166 (codes to be added following acceptance of manuscript). PartitionFinder v1.1.1 (Lanfear et al.,

167 2012) was used to partition the data and select the most optimal model of nucleotide substitution

168 for each partition based on the Akaike Information Criterion.

169 Bayesian phylogenetic analyses were run using the MPI version of MrBayes 3.2.6

170 (Ronquist et al., 2012) on a 12-core virtual machine on the NeCTAR research cloud under an

171 Ubuntu 16.04 LTS image using Open MPI version 2.0.1 (https://www.open-mpi.org/). Analyses

172 employed two runs, each with four chains, with ten million steps, sampling every 1000 steps.

173 The first 20% of sampled topologies was discarded as burn-in based on stability of log likelihood

174 values and that sampled topologies were essentially identical across runs, with standard deviation

175 of split frequencies ~0.01 or less. Samples for numerical parameters were also essentially

176 identical, with variance between versus within runs approaching unity (Ronquist et al., 2012).

177 The majority-rule consensus tree was constructed from the combined post-burn-in samples.

178 Maximum likelihood (ML) analyses was conducted using the RAxML BlackBox server

179 (http://embnet.vital-it.ch/raxml-bb/) implementing the methods of Stamatakis et al. (2008). Data

180 were partitioned as recommended by PartitionFinder and run using the Gamma model of rate

181 heterogeneity.

182 All *Neobenedenia Cytb* and *28S rRNA* sequences on GenBank were downloaded and

183 aligned to our data. We also included the *Cytb* sequence of the only full mitochondrial genome

184 of this genus (JQ038228). A neighbor-joining analysis of the aligned data for each locus was

185   conducted in MEGA 6.06 (Tamura et al., 2013), using uncorrected *P* distance, in an attempt to

186   reconcile the GenBank sequences with our phylogenetic framework.

187

188   **3. Results**

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190   *3.1. DNA sequence characteristics*

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192   Amplified *Cytb* and *H3* sequences did not contain any premature stop codons or

193   frameshift mutations, contributing 704 and 292 characters respectively to the overall

194   concatenated alignment. Even though we were able to amplify over 800bp of sequence for *28S*

195   *rDNA*, the sequence spans a highly variable region which we were unable to unambiguously

196   align, even when using the secondary structure of the *28S rDNA* sequence for *G. salaris* as a

197   guide. Thus, we were forced to trim our *28S rDNA* sequences to 411 characters. Some *28S rDNA*

198   and *H3* sequence chromatograms contained overlapping peaks indicative of heterozygous alleles

199   and these sites were scored with IUPAC ambiguity codes for dimorphic sites. All individuals

200   included in the analyses are represented by sequences of at least two of the genes (*Cytb* and *H3*

201   or *28S rDNA*).

202

203   *3.2. Phylogenetic analyses*

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205   Analysis of the concatenated dataset in PartitionFinder v1.1.1 suggested that three

206   partitions (*Cytb* codon position 3; *Cytb* codon positions 1 and 2; *H3* codon positions 1, 2 and 3

207   and *28S rDNA*) were optimal for the data. PartitionFinder selected the General Time Reversible

208   (GTR) model with a gamma distribution for rates across sites (GTR + G), GTR + G

209   incorporating a proportion of invariable sites (GTR + I + G) and the Symmetrical model with a

210   gamma distribution for rates across sites (SYM + G) for the partitions respectively.

211   Bayesian (Fig. 1) and Maximum Likelihood (ML) (Fig. 2) analyses yielded essentially

212   identical topologies, with *Neobenedenia* samples forming a monophyletic clade to the exclusion

213   of the outgroups and separated into four major clades (A-D). The *Neobenedenia* isolate

214   recovered from *Lutjanus argentiventris*, Peters 1869, was the exception and was recovered as

215   part of clade A in the Bayesian analysis (Fig. 1) but as a sister to clade A in the ML analysis

216   (Fig. 2; labelled A* for clarity) albeit with low bootstrap support (BS 24%).

217   Samples that comprise clades C and D have been morphologically identified by us (IDW)

218   as *N. longiprostata*, Bravo-Hollis 1971 and *N. pacifica*, Bravo-Hollis 1971, respectively, while

219   samples from both A and B are morphologically '*N. melleni*'. Clade B contains a single

220   specimen collected from *Sphoeroides annulatus*, while clade A contains 28 of the 33 in-group

221   samples (excluding the sample from *L. argentiventris*) included in the study. The isolate

222   currently in culture at the Marine Parasitology Laboratory at James Cook University, Townsville,

223   Australia collected from *Lates calcarifer* (Bowen, Queensland) also fell into clade A

224   The neighbor-joining tree constructed from all available *Cytb* sequences is presented in

225   Figure 3. Given the proportions of informative sites (Table 3 – Supplementary Material), it is not

226   surprising that the topology is reflective of the Bayesian and ML analyses. Furthermore, the

227   sample collected from *L. argentiventris* is recovered as the sister of the clade A in congruence

228   with the ML analysis (Fig. 2; clade A*). All of the *Cytb* sequences downloaded from Genbank

229   fall into clade A, except for accession HMM222533 collected from *S. annulatus* (Perkins et al.

230   2009), which falls into clade B.

231   Similarly, the neighbor-joining analysis (not shown) from all available *28S rRNA*

232   sequences also recovered all previously identified clades. The specimen collected from *L.*

233   *argentiventris* was once again recovered as a separate lineage but clusters with another specimen

234   collected from *Sebastes rubrivinctus*, Jordan and Gilbert 1880, that was part of clade A in all

235   other analyses. Several sequences fell into clade B but all these were sampled from *S. annulatus*.

236

237   **4. Discussion**

238   The correct identification of species underpins all biological study and for pathogens is

239   critical in understanding infection dynamics, predicting outbreaks and determining effective

240   control strategies. However, in the genus *Neobenedenia*, morphological plasticity, attributed to

241   host induced morphological variation (Whittington and Horton, 1996), failure to accession

242   specimens (including wet and mounted material) to curated collections (e.g. Cowell et al., 1993;

243   Ellis and Watanabe, 1993; Koesharyani et al., 1999; Jahn and Kuhn, 1932; Müeller et al., 1992;

244   Nigrelli and Breder, 1934; Nigrelli, 1935; Nigrelli, 1937; Nigrelli, 1947; Robinson et al., 1992;

245   Zhang et al. 2014) and the lack of a robust genetic framework to aid in identification, has

246   hampered these efforts. This study presents the most comprehensive phylogenetic investigation

247   of *Neobenedenia* spp. to date, incorporating 33 isolates spanning 22 host fish species and nine

248   countries and used both mitochondrial and nuclear gene markers.

249   Our results clearly show that there is a single species of *Neobenedenia* (Fig 1-3; clade A)

250   that is both widespread geographically, able to infest a large number of different host fishes, and

251   can be found on wild, farmed, and captive fish. Most importantly, clade A does not contain

252   *Neobenedenia* specimens from *Sphoeroides annulatus*, which is likely to be the type host of *N.*

253   *melleni* according to the description by McCallum (1927) and analysis by Whittington and

254 Horton (1996). Our sample from *S. annulatus* (Fig 1-3; clade B), which was collected from wild

255 fish in southern Mexico, is well differentiated from clade A and is the sister species to *N.*

256 *longiprostata* in our analyses. Furthermore, except for HM222533, which was sampled from *S.*

257 *annulatus* (Perkins et al., 2009) and clusters with our sample in clade B, all other *CytB* sequences

258 obtained from Genbank which are named both *N. melleni* and *N. girellae* all fall in clade A,

259 including the *CytB* sequence from the full mitochondrial genome (JQ038228), which is

260 supposedly *N. melleni* (Zhang et al., 2014). Similarly, all Genbank *28S rRNA* sequences fall into

261 clade A unless they were sampled from *S. annulatus*, where they clustered with clade B. Thus,

262 the results of the present study support the findings by Whittington et al. (2004) and Perkins et

263 al. (2009) that *N. melleni* and *N. girellae* are cryptic species in that they are morphologically very

264 similar but genetically distinct. In an effort to stabilise the naming of *Neobenedenia* species, it

265 seems prudent to ascribe *N. melleni* to individuals in clade B and retain *N. girellae* for those

266 individuals in clade A.

267 It is likely that clade A* (Fig. 2, Fig. 3) represents another species of *Neobenedenia,*

268 however at this stage, it is unclear whether this sample is a previously recorded *Neobenedenia*

269 species or if it is novel to science. The single specimen represented in this clade is the only one

270 collected from a lutjanid host (*Lutjanus argentiventris* collected from La Paz, Mexico) and the

271 assessment of this family of hosts for potential *Neobenedenia* species is an important avenue of

272 research to pursue. The Bayesian placement of this specimen into clade A appears incorrect

273 given the long branch length and ML and neighbor-joining analyses clearly differentiate it from

274 other samples in this study. Further work is underway to assess that status of this individual.

275 The findings of this study suggest that, historically, both prior and post the proposed

276 synonymy by Whittington and Horton (1996), many of the parasites identified as *N. melleni* may

277 in fact be *N. girellae* given how few isolates were identified as *N. melleni* in this study (e.g.

278 Bullard et al., 2000; Deveney et al., 2001; Kerber et al., 2011; Landos, 2012; Wang et al., 2004;

279 Zhang et al., 2014). Accurate identification of *Neobenedenia* species may be hampered by

280 intraspecific morphological plasticity thereby resulting in false identifications. Despite the

281 recognised difficulty in differentiating *N. melleni* and *N. girellae*, the majority of studies

282 focussing on *Neobenedenia* spp. have not stated that they have accessioned samples to museum

283 or private collections. This makes clarification of previous identifications almost impossible and

284 accurate identification of historical host species and geographic locations for each of these two

285 taxa unachievable. However, given the number of isolates that were included in this study and

286 the varied host species and locations they were collected from, it is likely that the number of

287 hosts and locations that *N. melleni* has been credited infecting and inhabiting has been

288 overestimated. To facilitate morphological and genetic comparison of individuals in the future, it

289 is recommended to accession reference material (both mounted and fixed specimens).

290   Using our genetic framework, it will be possible to reassess the morphological

291 differences between *N. girellae* and *N. melleni a posteriori*. The *CytB* neighbor-joining analysis

292 shows significant substructuring in *N. girellae*, which may account for the variable morphology

293 noted by Whittington and Horton (1996). Morphological variation can be due to epigenetic

294 factors which influences the phenotype expressed depending on the particular environment being

295 experienced (Agrawal 2001; Mati et al., 2014; Olstad et al., 2009; Via et al., 1995). The

296 flexibility displayed by these parasites makes the identification of species solely through

297 morphological means considerably challenging (Barcak et al., 2014; Bickford et al., 2006). As

298 closely related cryptic parasite species may exhibit variable traits to one another such as host

299 susceptibility (Reversat et al., 1989), pathogenicity (Haque et al., 2003; Homan and Mank, 2001;

300   Skovgaard, et al., 2002), and epidemiology (Murrell and Pozio, 2000), accurate identification is

301   crucial to understand the risk posed by the presence of a particular species in a system.

302   *Neobenedenia girellae* infects a large number of fish species of economic importance

303   including commercial fisheries, aquaculture, and the ornamental trade (Ogawa et al., 1995;

304   2006). *Neobenedenia girellae* isolates were collected from several fish species that support major

305   aquaculture production or commercial fisheries including *Lates calcarifer* (barramundi or Asian

306   sea bass), *Epinephelus coioides* (gold-spot grouper), *Coryphaena hippurus* (mahi mahi or

307   dolphinfish), *Plectropomus leopardus* (coral trout), *Rachycentron canadum* (cobia), *Seriola*

308   *lalandi* (yellowtail kingfish)*, and *Seriola rivoliana* (almaco jack), Valenciennes 1873 (Fig. 1).

309   Similarly, attractive tropical species are also at risk. Parasites infecting eight species of popular

310   ornamental fish species from seven families were among the isolates included in this study. All

311   represent new host records for *N. girellae* and show the diversity of host species this parasite can

312   infect (Table 1; see *3.1.*). Ornamental fish support a huge, multi-national industry that involves

313   hundreds of fish species (and their associated pathogen communities) being transported all

314   around the globe (Bruckner, 2004). This provides an excellent opportunity for the dispersal of *N.*

315   *girellae*, further encouraged by its lack of host specificity. Surveillance of this pathway for *N.*

316   *girellae* is advised given its broad host specificity.

317   Isolates collected from two outbreaks on *L. calcarifer* which occurred in Queensland

318   Australia, the first on a fish farm in Hinchinbrook in 2000 and the second in Gladstone in 2014

319   were included in this study (Table 1) and were determined to be a part of the *N. girellae* clade

320   (Figure 1-2). The parasites of concern were initially identified as *N. melleni* by Deveney et al.

321   (2001) and Landos (2012), however, our study suggests that these were erroneous identifications

322   and the species associated with these mortality events was in fact *N. girellae*. Similarly, the

323   previously unidentified species of *Neobenedenia* which is currently being cultured at the Marine

324   Parasitology Laboratory at James Cook University, Townsville has also been identified as *N.*

325   *girellae* using this analysis. As such, all previous research on this species from the Marine

326   Parasitology Laboratory is now ascribed to *Neobenedenia girellae* (i.e. Brazenor and Hutson

327   2015; Dinh Hoai and Hutson 2014; Hutson et al. 2012; Militz et al. 2013a; 2013b; Militz and

328   Hutson 2015; Trujillo-Gonzalez et al. 2015a,b).

329   The large diversity of host species that *N. girellae* is able to infect (23 host species

330   determined from the present study) and the geographic range in which it is present (found

331   between latitudes 23.8489° S and 24.1422° N (wild isolates) and 23.8426° S and 24.1426° N (all

332   isolates) from samples included in this study) makes it a globally cosmopolitan species and a

333   threat to aquaculture industries around the world.

334

335   **Acknowledgments**

349   **References**

350

351   Agrawal, A.A., 2001. Phenotypic Plasticity in the Interactions and Evolution of Species. Science

352   294, 321–326.

353   Barcak, D., Oros, M., Hanzelova, V., Scholz, T., 2014. Phenotypic plasticity in *Caryophyllaeus*

354   *brachycollis* Janiszewska, 1953 (Cestoda: Caryophyllidea): does fish host play a role?

355   Syst. Parasitol. 88, 153–166.

356   Bauer, O.N., Hoffman, G.L., 1976. Helminth range extension by translocation of fish. In: Page,

357   L.A. (Ed.), Wildlife Diseases, Plenum, New York, pp. 163–172.

358   Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K.K., Das,

359   I., 2006. Cryptic species as a window on diversity and conservation. Trends Ecol. Evol.

360   22, 148–155.

361   Blasco-Costa, I., Balbuena, J.A., Raga, J.A., Kostadinova, A., Olson, P.D., 2010. Molecules and

362   morphology reveal cryptic variation among digeneans infecting sympatric mullets in the

363   Mediterranean. Parasitology 137, 287–302.

364   Bravo-Hollis, M., 1958. Trematodes de peces marinos de aguas mexicanas. XIV. Cuarto

365   monogéneos de la familia Capsalidae Baird, 1853, de las costas del Pacifíco, incluyendo

366   una especie nueva. An. Inst. Biol. 28, 195–216.

367   Bravo-Hollis, M., Deloya, J.C., 1973. Catálogo de la colección helmintológica del Instituto de

368   Biologia. Instituto de Biología Universidad Nacional Autonóma de México 2, 137.

369   Brazenor, A.K., Hutson, K.S., 2015. Effects of temperature and salinity on the life cycle of

370   *Neobenedenia* sp. (Monogenea: Capsalidae) infecting farmed barramundi (*Lates*

371   *calcarifer*). Parasitol. Res.114, 1875–1886.

372   Bullard, S.A., Benz, G.W., Overstreet, R.M., Williams, E.H., Hemdal, J., 2000. Six new host

373   records and an updated list of wild hosts for *Neobenedenia melleni* (MacCallum)

374   (Monogenea: Capsalidae). Comp. Parasitol. 67, 190–196.

375   Byrnes, T., Rohde, K., 1992. Geographical distribution and host specificity of ectoparasites of

376   Australian bream, *Acanthopagrus* spp. (Sparidae). Folia Parasitol. 39, 249–264.

377   Chisholm, L.A., Morgan, J.A.T., Adlard, R.D., Whittington, I.D., 2001. Phylogenetic analysis of

378   the Monocotylidae (Monogenea) inferred from the 28s rDNA sequences. Int. J. Parasitol.

379   31, 1253-1263.

380   Colgan, D.J., McLauchlan, A., Wilson, G.D.F., Livingston, S.P., Edgecombe, G.D., Macaranas,

381   J., Cassis, G., Gray, M.R., 1998. Histone H3 and U2 snRNA DNA sequences and

382   arthropod molecular evolution. Aust. J. Zool. 46, 419-437.

383   Cowell, L.E., Watanabe, W.O., Head, W.D., Grover, J.J., Shenker, J.M., 1993. Use of tropical

384   cleaner fish to control the ectoparasite *Neobenedenia melleni* (Monogenea: Capsalidae)

385   on seawater-cultured Florida red tilapia. Aquaculture 113, 189–200.

386   Deveney, M., Chisholm, L., Whittington, I., 2001. First published record of the pathogenic

387   monogenean parasite *Neobenedenia melleni* (Capsalidae) from Australia. Dis. Aquat.

388   Organ. 46, 79–82.

389   Dinh Hoai, T., Hutson, K.S., 2014. Reproductive strategies of the insidious fish ectoparasite,

390   *Neobenedenia* sp. (Capsalidae: Monogenea). Plos One 9, 1–7.

391   Donald, K.M., Kennedy, M., Poulin, R., Spencer, H.G., 2004. Host specificity and molecular

392   phylogeny of larval Digenea isolated from New Zealand and Australian topshells

393   (Gastropoda: Trochidae). Int. J. Parasitol. 34, 557–568.

394   Ellis, E.P., Watanabe, W.O., 1993. The effects of hyposalinity on eggs, juveniles and adults of

395   the marine monogenean, *Neobenedenia melleni* Treatment of ecto-parasitosis in

396   seawater-cultured tilapia. Aquaculture 117, 15–27.

397   Freeman M. A., Ogawa K. 2010. Variation in the small subunit ribosomal DNA confirms that

398   *Udonella* (Monogenea: Udonellidae) is a species-rich group. Int. J. Parasitol. 40(2), 255–

399   264.

400   Gaida, I.H., Frost, P., 1991. Intensity of *Neobenedenia girellae* (Monogenea: Capsalidae) on the

401   halfmoon, *Medialuna californiensis* (Perciformes: Kyphosidae), examined using a new

402   method for detection. Proceedings of the Helminthological Society of Washington 59,

403   129–130.

404   Gibson, D., Harris, P., Bray, R. (2010). Capsalidae Baird, 1853. Accessed through: World

405   Register of Marine Species at

406   http://www.marinespecies.org/aphia.php/aphia.php?p=taxdetails&id=119225 on 2016-

407   11-19.

408   Hargis, W. J., 1955. A New Species of Benedenia (Trematoda: Monogenea) from *Girella*

409   nigricans, the Opaleye. J. Parasitol. 41(1), 48–50.

410   Harris P. D., Shinn A. P., Cable J., Bakke T. A., 2004. Nominal species of the genus

411   *Gyrodactylus* von Nordmann 1832 (Monogenea: Gyrodactylidae), with a list of principal

412   host species. Syst. Parasitol. 59, 1–27.

413   Hutson, K., Mata, L., Paul, N., de Nys, R., 2012. Seaweed extracts as a natural control against

414   the monogenean ectoparasite, *Neobenedenia* sp., infecting farmed barramundi (*Lates*

415   *calcarifer*). Int. J. Parasitol. 42, 1135–1141.

416   Jahn, T.L., Kuhn, L.R., 1932. The life history of *Epibdella melleni*, MacCallum, 1927, a

417   monogenetic trematode parasitic in marine fishes. Biol. Bull. 62, 89–111.

418   Koesharyani, I., Zafran., Yuasa, K., Hatai, K., 1999. Two Species of Capsalid Monogeneans

419   Infecting Cultured Humpback Grouper *Cromileptes altivelis* in Indonesia. Fish Pathol.

420   34, 165–166.

421   Landos, M., 2012. Investigation of the Causes of Aquatic Animal Health Problems in the

422   Gladstone Harbour and Nearshore Waters. Report commissioned by the Gladstone

423   Fishing Research Fund, pp. 191.

424   Lanfear, R., Calcott, B., Ho, S., Guindon S., 2012. PartitionFinder: combined selection of

425   partitioning schemes and substitution models for phylogenetic analyses. Mol. Biol. Evol.

426   29 (6), 1695–1701.

427   Leung, T.L.F., Keeney, D.B., Poulin, R., 2009. Cryptic species complexes in manipulative

428   echinostomatid trematodes: when two become six. Parasitology 136, 241–252.

429   Littlewood, D.T.J., Rohde, K., Clough, K.A., 1997. Parasite speciation within or between host

430   species? Phylogenetic evidence from site-specific polystome monogeneans. Int. J.

431   Parasitol. 27, 1289–1297.

432   Love, M.S., Shriner, K., Morris, P., 1984. Parasites of olive rockfish, *Sebastes serranoides*

433   (Scorpaenidae) off central California. Fish. Bull. 82, 530–537.

434   Matejusová, I., Cunningham, C.O., 2004. The First Complete Monogenean Ribosomal RNA

435   Gene Operon: Sequence and Secondary Structure of the *Gyrodactylus salaris* Malmberg,

436   1957, Large Subunit Ribosomal RNA Gene. J. Parasitol. 90(1), 146–151.

437   Mati, V.L.T., Freitas, R.M., Bicalho, R.S., Melo, A.L., 2014. Phenotypic plasticity of male

438   *Schistosoma mansoni* from the peritoneal cavity and hepatic portal system of laboratory

439   mice and hamsters. J. Helminthol. 29, 294–301.

440   Militz, T.A., Southgate, P.C., Carton, A.G., Hutson, K.S., 2013. Efficacy of garlic (*Allium*

441   *sativum*) extract applied as a therapeutic immersion treatment for *Neobenedenia* sp.

442   management in aquaculture. J. Fish Dis. 1–11.

443   Militz, T.A., Southgate, P.C., Carton, A.G., Hutson, K.S., 2013. Dietary supplementation of

444   garlic (*Allium sativum*) to prevent monogenean infection in aquaculture. Aquaculture

445   408-409, 95-99.

446   Militz, T.A., Hutson, K.S., 2015. Beyond Symbiosis: Cleaner Shrimp Clean Up in Culture. Plos

447   One 10(2): e0117723.

448   Miura, O., Kuris, A.M., Torchin, M.E., Hechinger, R.F., Dunham, E.J., Chiba, S., 2005.

449   Molecular-genetic analyses reveal cryptic species of trematodes in the intertidal

450   gastropod, *Batillaria cumingi* (Crosse). Int. J. Parasitol. 35, 793–801.

451   Moser, M., Haldorson, L., 1982. Parasites of two species of surfperch (Embiotocidae) from

452   seven Pacific coast locales. J. Parasitol. 68, 733–735.

453   Müeller, K.W., Watanabe, W.O., Head, W.D., 1992. Effect of Salinity on Hatching in

454   *Neobenedenia melleni*, a Monogenean Ectoparasite of Seawater-Cultured Tilapia. Journal

455   of the World Aquac. Soc. 23, 199–204.

456   Murrell, K.D., Pozio, E., 2000. Trichinellosis: the zoonosis that won't go quietly. Int. J. Parasitol.

457   30, 1339–1349.

458   Nigrelli, R.F., Breder, C.M., 1934. The susceptibility and immunity of certain marine fishes to

459   *Epibdella melleni,* a monogenetic trematode. J. Parasitol. 20, 259–269.

460   Nigrelli, R.F., 1935. Studies on the acquired immunity of the pompano, *Trachinotus carolinus*, to

461   *Epibdella melleni*. J. Parasitol. 21, 438–439.

462   Nigrelli, R.F., 1937. Further studies on the susceptibility and acquired immunity of marine fishes

463   to *Epibdella melleni*, a monogenetic trematode. Zoologica 22, 185–192.

464   Nigrelli, R.F., 1947. Susceptibility and immunity of marine fishes to *Benedenia* (= *Epibdella*)

465   *melleni* (MacCallum), a monogenetic trematode. III. Natural hosts in the West Indies. J.

466   Parasitol. 33, 25.

467   Ogawa, K., Bondad-Reantaso, M.G., Fukudome, M., Wakabayashi, H., 1995. *Neobenedenia*

468   *girellae* (Hargis, 1955) Yamaguti, 1963 (Monogenea: Capsalidae) from Cultured Marine

469   Fishes of Japan. J. Parasitol. 81, 223–227.

470   Ogawa, K., 1996. Marine parasitology with special reference to Japanese fisheries and

471   mariculture. Vet. Parasitol. 64, 95–105.

472   Ogawa, K., Yokoyama, H., 1998. Parasitic diseases of cultured marine fish in Japan. Fish Pathol.

473   33, 303-309.

474   Ogawa, K., 2006. Impacts of diclidophorid monogenean infections on fisheries in Japan. Int. J.

475   Parasitol. 32, 373–380.

476   Olstad, K., Bachmann, L., Bakke, T. A., 2009. Phenotypic plasticity of taxonomic and diagnostic

477   structures in gyrodactylosis-causing flatworms (Monogenea, Platyhelminthes).

478   Parasitology 136, 1305–1315.

479   Paperna, I., Overstreet, R., 1981. Parasites and diseases of mullets (Muglidae). In: Oren, O.H.E.

480   (Ed.), Aquaculture of Grey Mullets, Cambridge University Press, Cambridge, pp. 411–

481   493.

482   Perkins, E.M., Donnellan, S.C., Bertozzi, T., Chisholm, L.A., Whittington, I.D., 2009. Looks can

483   deceive: Molecular phylogeny of a family of flatworm ectoparasites (Monogenea:

484   Capsalidae) does not reflect current morphological classification. Mol. Phylogenet. Evol.

485   52, 705–714.

486   Robinson, R.D., Khalil, L.F., Hall, R.N., Steele, R.D., 1992. Infection of red hybrid tilapia with a

487   monogenean in coastal waters off southern Jamaica. Proc. Gulf Caribb. Fish. Inst. 42,

488   441–447.

489   Rohde, K., 1979. A critical evaluation of intrinsic and extrinsic factors responsible for niche

490   restriction in parasites. Am. Nat. 114, 648–671.

491   Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D., Darling, A., Höhna, S., Larget, B., Liu,

492   L., Suchard, M.A. and Huelsenbeck, J.P., 2012. MrBayes 3.2: Efficient Bayesian

493   phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–

494   542.

495   Saijuntha, W., Sithithaworn, P., Wongkham, S., Laha, T., Pipitgool, V., Tesana, S., Chilton,

496   N.B., Petney, T.N., Andrews, R.H., 2007. Evidence of a species complex within the food-

497   borne trematode *Opisthorchis viverrini* and possible co-evolution with their first

498   intermediate hosts. Int. J. Parasitol. 37, 695–703.

499 Skovgaard, K., Bødker, L., Rosendahl, S., 2002. Population structure and pathogenicity of

500 members of the *Fusarium oxysporum* complex isolated from soil and root necrosis of pea

501 (*Pisum sativum* L). FEMS Microbiol. Ecol. 42, 367–374.

502 Stamatakis, A., Hoover, P., Rougemont, J. 2008. A Rapid Bootstrap Algorithm for the RAxML

503 Web-Servers, Syst. Biol. 75(5): 758–771.

504 Sepúlveda, F.A., González, M.T., 2014. Molecular and morphological analyses reveal that the

505 pathogen *Benedenia seriolae* (Monogenea: Capsalidae) is a complex species:

506 Implications for yellowtail *Seriola* spp. aquaculture. Aquaculture 418-419, 94–100.

507 Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar S., 2013. MEGA6: Molecular

508 Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

509 Trujillo-González, A., Johnson, L.K., Constantinoiu, C.C., Hutson, K.S., 2015a. Histopathology

510 associated with haptor attachment of the ectoparasitic monogenean *Neobenedenia* sp.

511 (Capsalidae) to barramundi, *Lates calcarifer* (Bloch). J. Fish Dis. 38(12), 1063–1067.

512 Trujillo-González, A., Constantinoiu, C.C., Rowe, R., Hutson, K.S., 2015b. Tracing transparent

513 monogenean parasites on fish from infection to maturity. Int. J. Parasitol. 4, 316–322.

514 Via, S., Gomulkiewica, R., Jong, G., Scheiner, S.M., Schlichting, C.D., Van Tienderen, P.H.,

515 1995. Adaptive phenotypic plasticity: consensus and controversy. Trends Ecol. Evolut.

516 10, 212–217.

517 Wang, J., Zhang, W., Su, Y., Ding, S., 2004. Genetic relationship between *Neobenedenia*

518 *girellae* and *N. melleni* inferred from 28S rDNA sequences. Acta Oceanol. Sin. 23, 709–

519 716.

520 Whittington, I., 1998. Diversity "down under": monogeneans in the Antipodes (Australia) with a

521 prediction of monogenean biodiversity worldwide. Int. J. Parasitol. 28, 1481–1493.

522 Whittington, I.D., 1996. Benedeniine capsalid monogeneans from Australian fishes: pathogenic

523   species, site-specificity and camouflage. J. Helminthol. 70, 177–184.

524 Whittington, I.D., Cribb, B.W., Hamwood, T.E., Halliday, J.A., 2000. Host-specificity of

525   monogenean (platyhelminth) parasites: a role for anterior adhesive areas? Int. J. Parasitol.

526   30, 305–320.

527 Whittington, I.D., 2004. The Capsalidae (Monogenea: Monopisthocotylea): a review of diversity,

528   classification and phylogeny with a note about species complexes. Folia Parasitol. 51,

529   109–122.

530 Whittington, I.D., Deveney, M.R., Morgan, J.A.T., Chisholm, L.A., Adlard, R.D., 2004. A

531   preliminary phylogenetic analysis of the Capsalidae (Platyhelminthes: Monogenea:

532   Monopisthocotylea) inferred from large subunit rDNA sequences. Parasitology 128, 511–

533   519.

534 Wu, X.Y., Chilton, N.B., Zhu, X.Q., Xie, M.Q., Li, A.X., 2005. Molecular and morphological

535   evidence indicates that *Pseudorhabdosynochus lantauensis* (Monogenea: Diplectanidae)

536   represents two species. Parasitology 130, 669–677.

537 Xiao, N., Qui, J., Nakao, M., Li, T., Yang, W., Chen, X., Schantz, P.M., Craig, P.S., Ito, A.,

538   2005. *Echinococcus shiquicus* n. sp., a taeniid cestode from Tibetan fox and plateau pika

539   in China. Int. J. Parasitol. 35, 693–701.

540 Yoshinaga, T., Segawa, I., Kamaishi, T., Sorimachi, M., 2000. Effects of temperature, salinity

541   and chlorine treatment on egg hatching of the monogenean *Neoheterobothrium hirame*

542   infecting Japanese flounder. Fish Pathol. 35, 85–88.

543 Zhang, J., Wu, X., Li, Y., Zhao, M., Xie, M., Li, A., 2014. The complete mitochondrial genome

544   of *Neobenedenia melleni* (Platyhelminthes: Monogenea): mitochondrial gene content,

545 arrangement and composition compared with two *Benedenia* species. Mol. Biol. Rep. 41,

546 6583–6589.

Table 1: Host fish species from which *Neobenedenia* were sampled and used for sequencing from Australia and overseas.

Host species	Figure codes	Origin	Location	Genbank accession codes *		
				H3	28s	Cytb
Lates calcarifer	Latcal1	Wild	Gladstone, QLD, Australia	XXXX	XXXX	XXXX
Lates calcarifer	Latcal2	Captive-Farm	Bowen, QLD, Australia	XXXX	XXXX	XXXX
Lates calcarifer (2011)	Latcal3	Captive-Farm	Hinchinbrook, QLD, Australia	XXXX	XXXX	XXXX
Lates calcarifer	Latcal4	Captive-Research	MARF, Townsville, QLD, Australia	XXXX	XXXX	XXXX
Lates calcarifer	Latcal5	Captive-Farm	Singapore	XXXX	XXXX	XXXX
Lates calcarifer (2000)	Latcal6	Captive-Farm	Hinchinbrook, QLD, Australia	XXXX	XXXX	XXXX
Seriola lalandi	Serlal	Captive-Farm	WA, Australia	XXXX	XXXX	XXXX
Trachinotus kennedeyi	Traken	Wild	La Paz, Mexico	XXXX	XXXX	XXXX
Oligoplites altus	Olialt	Wild	La Paz, Mexico	XXXX	XXXX	XXXX
Gnathanodon speciosus	Gnaspec1	Wild	La Paz, Mexico	XXXX	XXXX	XXXX
Gnathanodon speciosus	Gnaspec2	Captive-Aquarium	Durban, South Africa	XXXX	XXXX	XXXX
Seriola rivoliana	Serriv	Captive-Farm	Hawaii, USA	XXXX	XXXX	XXXX
Rachycentron canadum	Raccan1	Captive-Research	MARF, Townsville, QLD, Australia	XXXX	XXXX	XXXX
Rachycentron canadum	Raccan2	Captive	Panama	XXXX	XXXX	XXXX
Rachycentron canadum	Raccan3	Captive	Italy	XXXX	XXXX	XXXX
Mycteroperca rosacea	Mycros	Wild	La Paz, Mexico	XXXX	XXXX	XXXX
Epinephelus coioides	Epicoi	Captive-Research	Cairns, QLD, Australia	XXXX	XXXX	XXXX
Arothron caercaeruleopunctatus	Arocae	Captive-Ornamental	Townsville, QLD, Australia	XXXX	XXXX	XXXX
Canthigaster bennetti	Canben	Captive-Ornamental	Townsville, QLD, Australia	XXXX	XXXX	XXXX
Sphoeroides annulatus	Sphann	Wild	La Paz, Mexico	XXXX	XXXX	XXXX
Coryphaena hippurus	Corhip1	Captive	WA, Australia	XXXX	XXXX	XXXX
Coryphaena hippurus	Corhip2	Captive-Aquarium	Durban	XXXX	XXXX	XXXX
Mugil curema	Mugcur1	Wild	La Paz, Mexico	XXXX	XXXX	XXXX
Mugil curema	Mugcur	Wild	La Paz, Mexico	XXXX	XXXX	XXXX
Nemateleotris decora	Nemdec	Captive-Ornamental	Townsville, QLD, Australia	XXXX	XXXX	XXXX
Pseudocheilinus hexataenia	Psehex1	Captive-Ornamental	Townsville, Q QLD, Australia (June 2012)	XXXX	XXXX	XXXX
Pseudocheilinus hexataenia	Psehex2	Captive-Ornamental	Townsville, QLD, Australia (December 2012)	XXXX	XXXX	XXXX
Pseudochromis fridmani	Psefri	Captive-Ornamental	Townsville, QLD, Australia	XXXX	XXXX	XXXX
Verasper variegatus	Vervar	Captive-Farm	Japan	XXXX	XXXX	XXXX
Sebastes rubrivinctus	Sebrub	Captive-Farm	USA	XXXX	XXXX	XXXX
Neocirrhites armatus	Neoarm	Captive-Ornamental	Townsville, QLD, Australia	XXXX	XXXX	XXXX
Echeneis naucrates	Echnau	Captive-Ornamental	Woods Hole, MS, USA	XXXX	XXXX	XXXX
Sparus aurata	Spaaur	-	Eilat, Israel	XXXX	XXXX	XXXX
Lutjanus argentiventris	Lutarg	Wild	La Paz, Mexico	XXXX	XXXX	XXXX

MARF = Marine Aquaculture Research Facility, James Cook University, Townsville; USA = United States of America, WA = Western Australia; QLD = Queensland, XXXX = Codes forthcoming upon acceptance of manuscript.* If no code present for a particular gene, it was unable to be sequenced for analysis.

Table 2: Primers used for PCR amplification of *Neobenedenia* spp. isolates.

Gene	Primer ID	Sequence ('5-'3)	Forward/Reverse	Source
H3	G926	GACCGCYCGYAAAAGYAC	F	[a]
	G927	AGCRTGRATDGCRCACAA	R	[a]
	H3aF	ATGGCTCGTACCAAGCAGACVGC	F	[b]
	H3R2	ATRTCCTTGGGCATGATTGTTAC	R	[b]
28S rDNA	C1	ACCCGCTGAATTTAAGCAT	F	[c]
	D1	TGGTCCGTGTTTCAAGAC	R	[c]
	EC-D2	CCTTGGTCCGTGTTTCAAGACGGG	R	[d]
Cytb	M1676	TGAGTTATTATTGATGTAGAGG	F	[e]
	M1677	AAAATATCAKTCAGGCTTWA	R	[e]

[a] Perkins et al. (2009); [b] Colgan et al. (1998); [c] Chisholm et al. (2001); [d] Littlewood et al. (1997); [e] present study.
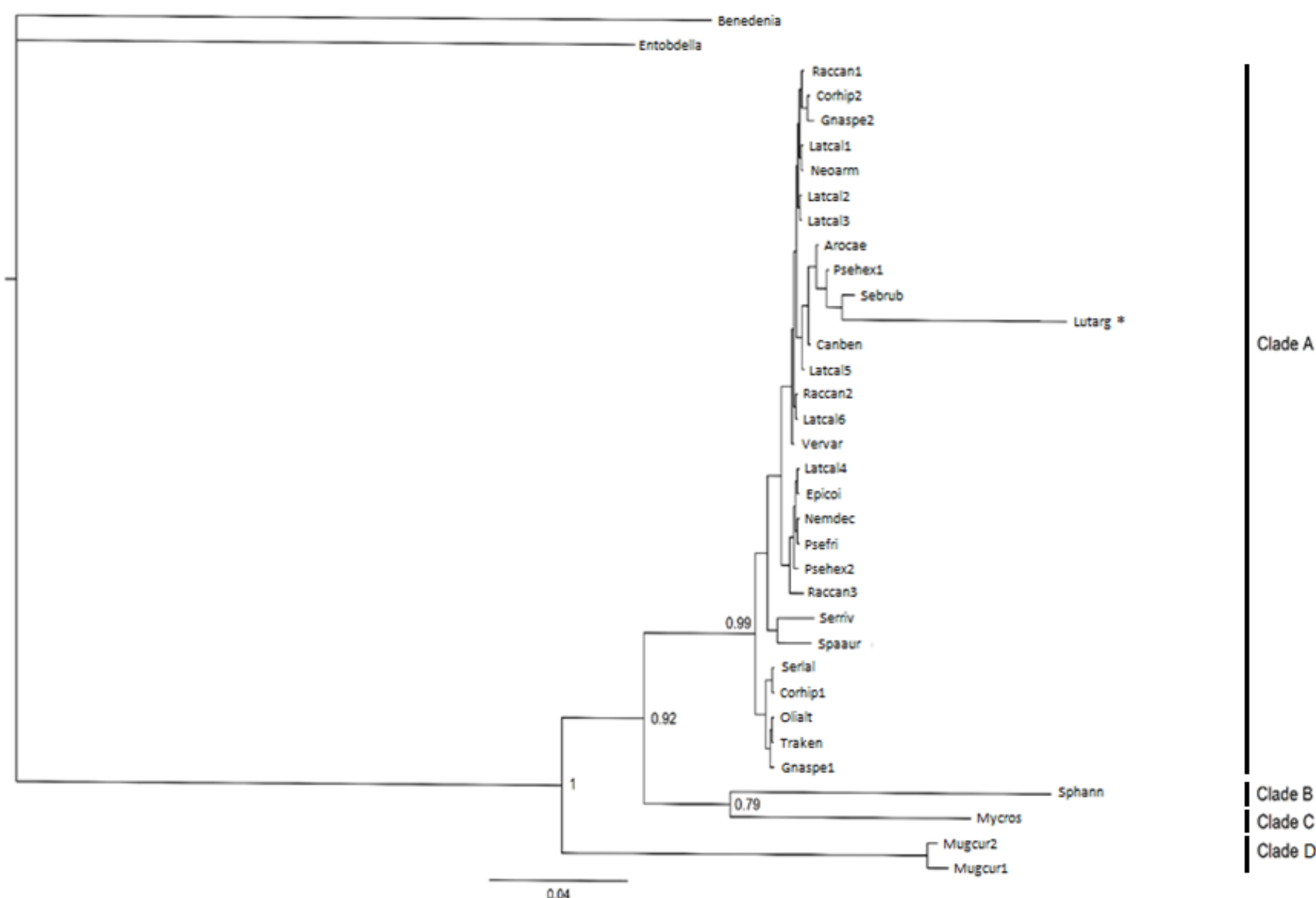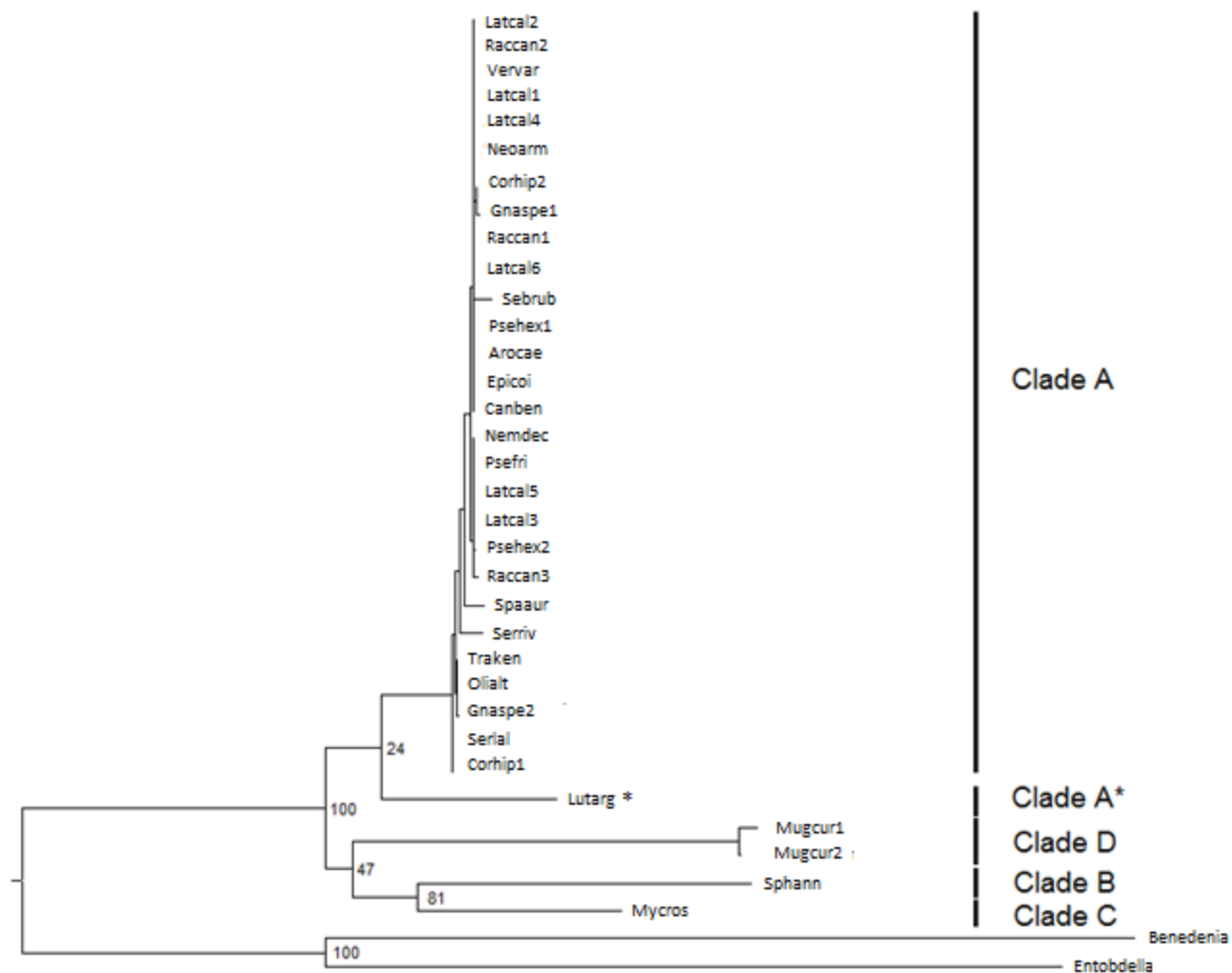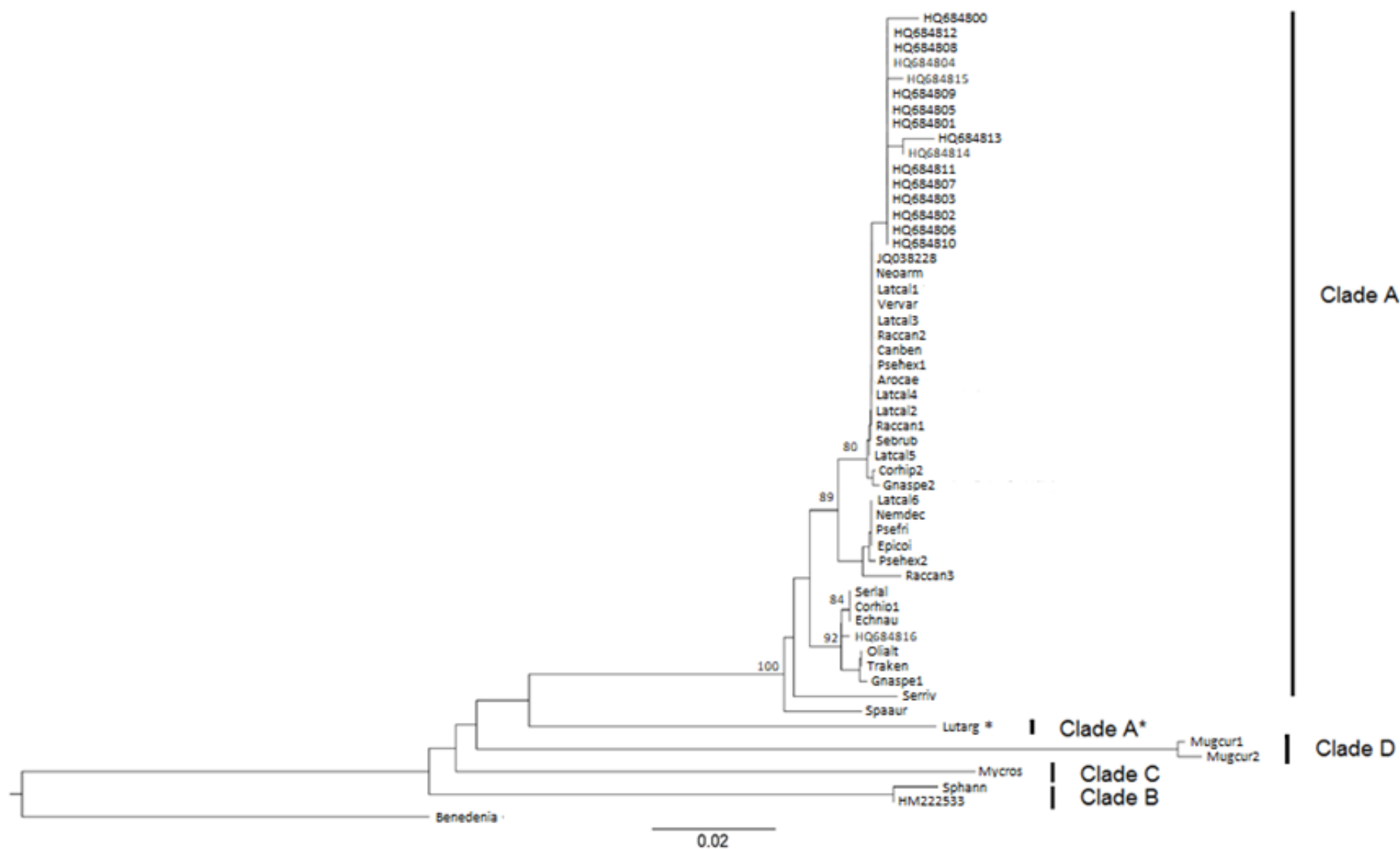
Fig. 1. Relationships of species of *Neobenedenia* isolates collected from wild and captive host fish based on Bayesian inference and maximum likelihood analyses of the *H3*, *28S rDNA*, and *cytochrome b* concatenated dataset. Posterior probability values are next to major nodes. "Benedenia" = *Benedenia seriolae* (HM222526.1 (*Seriola hippos* – South Australia, Australia)*,* AY033941.1 (*Seriola quinqueradiata* – Japan), and FJ972088.1 (*Seriola hippos* – South Australia, Australia)) and "Entobdella" = *Entobdella soleae* (FJ972108.1 (*Solea solea* – United Kingdom), AY486152.1 (*Solea solea* – United Kingdom), HQ684799.1 (unknown host – China)) sequences obtained from Genbank were included as outgroups.

Latcal2
Raccan2
Vervar
Latcal1
Latcal4
Neoarm
Corhip2
Gnaspe1
Raccan1
Latcal6
Sebrub
Psehex1
Arocae
Epicoi
Canben
Nemdec
Psefri
Latcal5
Latcal3
Psehex2
Raccan3
Spaaur
Serriv
Traken
Olialt
Gnaspe2
Serial
Corhip1
Lutarg *
Mugcur1
Mugcur2
Sphann
Mycros
Benedenia
Entobdella

Clade A

Clade A*
Clade D
Clade B
Clade C

100
24
47
81
100

0.2

1

2 Fig. 2. Relationships of species of *Neobenedenia* isolates collected from wild and captive host fish based on Maximum likelihood

3 analysis of the *H3*, *28S rDNA*, and *cytochrome b* concatenated dataset. Maximum likelihood bootstrap proportions are next to major

4 nodes. "Benedenia" = *Benedenia seriolae* (HM222526.1 (*Seriola hippos* – South Australia, Australia)*,* AY033941.1 (*Seriola*

5 *quinqueradiata* – Japan), and FJ972088.1 (*Seriola hippos* – South Australia, Australia)) and "Entobdella" = *Entobdella soleae*

6 (FJ972108.1 (*Solea solea* – United Kingdom), AY486152.1 (*Solea solea* – United Kingdom), HQ684799.1 (unknown host – China))

7 sequences obtained from Genbank were included as outgroups. Clades labelled as per the topology of Figure 1. * indicates the

8 *Lutjanus argentiventris* sample from Clade A in Figure 1.

9

10

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13

14   Fig. 3. Relationships of species of *Neobenedenia* isolates collected from wild and captive host fish based on a Neighbor-joining

15   analysis of partial *cytochrome b* sequences collected from Genbank and the present study. Genbank samples included in this analysis

16   are titled using their Genbank identification code and were collected from a variety of host species. "Benedenia" = *Benedenia seriolae*

17   (FJ972088.1 (*Seriola hippos* – South Australia, Australia)) sequence obtained from Genbank was included as an outgroup. Clades

18   labelled as per the topology of Figure 1. * indicates the sample collected from *Lutjanus argentiventris* identified in Clade A in Figure

19   1.

20   Supplementary Material

21   Table 1: Museum accession numbers for *Neobenedenia* used in this study. XXXX = code forthcoming upon acceptance of the manuscript.

Host species	Figure codes	Museum Accession Code
Lates calcarifer	Latcal1	XXXX
Lates calcarifer	Latcal2	XXXX
Lates calcarifer	Latcal3	XXXX
Lates calcarifer	Latcal4	XXXX
Lates calcarifer	Latcal5	XXXX
Lates calcarifer	Latcal6	XXXX
Seriola lalandi	Serlal	XXXX
Trachinotus kennedeyi	Traken	XXXX
Oligoplites altus	Olialt	XXXX
Gnathanodon speciosus	Gnaspec1	XXXX
Gnathanodon speciosus	Gnaspec2	XXXX
Seriola rivoliana	Serriv	XXXX
Rachycentron canadum	Raccan1	XXXX
Rachycentron canadum	Raccan2	XXXX
Rachycentron canadum	Raccan3	XXXX
Mycteroperca rosacea	Mycros	XXXX
Epinephelus coioides	Epicoi	XXXX
Arothron caercaeruleopunctatus	Arocae	XXXX
Canthigaster bennetti	Canben	XXXX
Sphoeroides annulatus	Sphann	XXXX
Coryphaena hippurus	Corhip1	XXXX
Coryphaena hippurus	Corhip2	XXXX
Mugil curema	Mugcur1	XXXX
Mugil curema	Mugcur	XXXX
Nemateleotris decora	Nemdec	XXXX
Pseudocheilinus hexataenia	Psehex1	XXXX
Pseudocheilinus hexataenia	Psehex2	XXXX
Pseudochromis fridmani	Psefri	XXXX
Verasper variegatus	Vervar	XXXX
Sebastes rubrivinctus	Sebrub	XXXX
Neocirrhites armatus	Neoarm	XXXX
Echeneis naucrates	Echnau	XXXX
Sparus aurata	Spaaur	XXXX
Lutjanus argentiventris	Lutarg	XXXX

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23 Table 2: Sequences of *Neobenedenia* (identified as "*Neobenedenia melleni*" by researchers) retrieved from Genbank, the lodging

24 researchers and associated codes

Genbank code	Species	Host species/species code	Collection location	Gene	Reference authors
HQ684800	*Neobenedenia melleni*	LsaHZ3	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684801	*Neobenedenia melleni*	SduXM	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684802	*Neobenedenia melleni*	CliGZ2	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684803	*Neobenedenia melleni*	EcoZJ1	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684805	*Neobenedenia melleni*	EchZH	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684806	*Neobenedenia melleni*	LarYJ	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684807	*Neobenedenia melleni*	LstYJ1	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684808	*Neobenedenia melleni*	SduZH2	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684809	*Neobenedenia melleni*	SduZH1	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684810	*Neobenedenia melleni*	LsaZJ1	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684811	*Neobenedenia melleni*	CliGZ1	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684812	*Neobenedenia melleni*	PciYJ1	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684813	*Neobenedenia melleni*	TblHD1	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684814	*Neobenedenia melleni*	PciZJ2	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684815	*Neobenedenia melleni*	EmoJAP1	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HQ684816	*Neobenedenia melleni*	EmoJAP2	China	Mitochondrial - Cytochromeb	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
JQ038228	*Neobenedenia melleni*	*Seriola dumerili*	China – Fujian Province	Mitochondrial – partial genome	Zhang,J., Wu,X.Y., Xie,M.Q.. and Li,A.X.
HM222533	*Neobenedenia* sp.	*Sphoeroides annulatus*	Mexico – La Paz	Mitochondrial - Cytochromeb	Perkins,E.M., Donnellan,S.C., Bertozzi,T. and Whittington,I.D.

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28   Table 3: Proportion of informative sites of three genes; 28S, H3, and cytochrome b for sequences included in analyses.

Gene	Length	Parsimony informative sites
28S	411	24
H3	292	26
Cytochrome b	704	166

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