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Neobenedenia girellae infection of aquarium-raised snubnose pompano (*Trachinotus blochii*) in Korea

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We found skin flukes in snubnose pompano (*Trachinotus blochii*) from a public aquarium and attempted clear identification of them to the species level by morphology and molecular analyses. Skin flukes were collected from snubnose pompano showing dyspnea, anorexia and mild hemorrhage on the skin. All the fish samples (n=2) were infected with the flukes on the skin, gill and eyes, covered with excessive mucus. The isolated worms were transferred for making slide specimen and PCR amplification targeting 18S rDNA, 28S rDNA, mitochondrial cytochrome c oxidase subunit 1 (mt *cox*1) and cytochrome b (*Cytb*) genes for further analyses. Morphology and measurements data of our slide specimen coincided with those of *Neobenedenia girellae*. The sequence data of 2 genes (28S rDNA and *Cytb*) and the phylogenetic trees revealed that our specimen consistently belonged to the *N. girellae* clade. For 18S rDNA and mt *cox*1 genes, there was no sequence of either of these 2 *Neobenedenia* species from the type host available in GenBank. This is the first record of *N. girellae* in snubnose pompano, but it is still unclear if the snubnose pompano is a natural host for *N. girellae* or not because *N. girellae* is known to have an unusual broad host range and the host-switching can occur particularly in captive conditions such as aquarium or aquaculture facilities.

Key words: *Neobenedenia girellae*, skin fluke, monogenean, capsalidae,

The capsalid monogeneans constitute approximately 200 species living predominantly on the skin and gills of marine fishes. They are considered as virulent pathogens in aquaculture and public aquaria, and some of them are responsible for considerable epidemics in finfish aquaculture (Whittington, 2004). For example, *Benedenia seriolae*, *Neobenedenia melleni* and *Neobenedenia girellae* have caused serious economic losses of finfish aquaculture worldwide (Bullard et al., 2000; Ogawa et al., 1995; Sepúlveda and González, 2014).

These capsalid monogeneans affect fish by mechanical attachment and subsequent grazing on the epithelium of the infected hosts, causing epidermal damage, loss of mucous cells and subsequent impairment of immune response to other pathogens (Trujillo-González et al., 2015). Genus *Neobenedenia* is of particular interest to marine finfish aquaculture industry because the type species, *Neobenedenie melleni* (Mac Callum, 1927) Yamaguti, 1963 is recognized as a notorious widespread pathogen in aquaria and aquaculture, with the broad host range (e.g., more than 100 species), which is unusual for monogeneans (see Brazenor et al., 2018a). Another *Neobenedenia* species, *Neobenedenia girellae* (Hargis, 1955) Yamaguti, 1963 has been also recorded from approximately 30 host species (Brazenor et al., 2018a).

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Given the unusual broad host specificity of the 2 *Neobenedenia* species mentioned above, identification of *Neobenedenia* at the species level, particularly for *N. melleni* and *N. girellae* has been considered problematic; There are no clear characters which distinguish these 2 species and no type host species exist for *N. melleni*. Whittington and Horton (1996) synonymized *N. girellae* with *N. melleni*, while Ogawa et al. (1995; 2006) recognized *N. girellae* as a distinct species from *N. melleni*. Molecular studies also revealed that *N. melleni* and *N. girellae* are 2 separate species (Brazenor et al., 2018a; Perkins et al., 2009)

In this study, we isolated capsalid monogeneans from the skin of snubnose pompano (*Trachinotus blochii*) reared in a public aquarium. We conducted morphological observation of the stained specimen and molecular analysis of several DNA markers for identification.

Materials and Methods

Fish examination and parasite slide specimen preparation

Snubnose pompano was transported to the laboratory for postmortem examination on May 27, 2019. They were imported from China and maintained in a public aquarium located in Gangneung, Korea. The 2 individual fish were moribund during sampling but dead when arrived at the laboratory. The fish were measured and examined for any abnormal clinical signs and external parasites. Then, they were dissected and checked for routine health examination.

Collected monogeneans were washed with sterile PBS, flattened between a slide glass and coverslip, and fixed in AFA (Alcohol-Formalin-Acetic acid; 70% ethanol 20 parts, formalin 1 part, acetic acid 1 part). They were stained with alum carmine, dehydrated, cleared in xylene and mounted in Canada balsam for morphological observation under a light microscope.

Molecular analysis

The collected parasites were washed with sterilized PBS several times, fixed with 100% ethanol and placed individually into 1.5 ml Eppendorf tube until DNA extraction. DNA was extracted using QIAamp DNA kit (QIAGEN, France) according to the manufacturer's protocols. Polymerase chain reaction (PCR) amplifications of partial 18S rDNA, 28S rDNA, mitochondrial (mt) cytochrome c oxidase subunit 1 (*cox*1) and cytochrome b (*Cytb*) gene sequence were conducted using 2 μl extracted DNA as template, 1 μl for each primer in total volume of 20 μl *Accu Power*® PCR Premix (Bioneer, Korea), containing 1U Taq DNA polymerase, 250 μM dNTPs (dATP, dCTP, dGTP, dTTP), 10mM Tris-HCl (pH 9.0), 30 mM KCl and 1.5 mM $MgCl_2$. Amplification was conducted using MyCycler™ (BioRad, USA), with the cycling conditions in Table 1. PCR products were purified by *AccuPrep*® Gel Purification Kit (Bioneer, Korea)

Table 1. Primers and PCR conditions for *Neobenedenia* species gene amplification in this study

Target genes	Primers	Sequence(5′-3′)	PCR Condition	Reference
18S rDNA	18F2 IR5	GGAGGGCAAGTCTGGTGCCAG TACGGAAACCTTGTTACGAC	95℃(30sec)-50℃(30sec)- 72℃(1min), 35 cycles	Badets et al., 2011
28S rDNA	C1 D2	ACCCGCTGAATTTAAGCAT TGGTCCGTGTTTCAAGAC	95℃(30sec)-55.5℃(30sec)- 72℃(1min), 35 cycles	Hassouna et al., 1984
cox1	JB3 JB4.5	TTTTTTGGGCATCCTGAGGTTTAT TAAAGAAAGAACATAATGAAAATG	95℃(30sec)-48℃(30sec)- 72℃(1min), 35 cycles	Bowles et al. 1995 Littlewood et al. 1997
cytb	M1676 M1677	TGAGTTATTATTGATGTAGAGG AAAATATCAKTCAGGCTTWA	95℃(30sec)-45℃(30sec)- 72℃(1min), 35 cycles	Brazenor et al., 2018

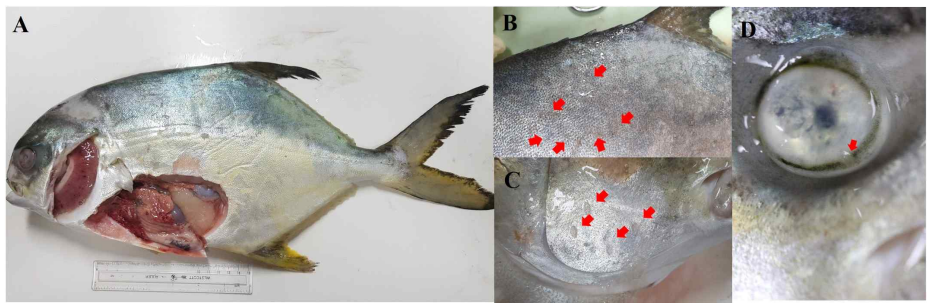

Fig. 1. Snubnose pompano (*Trachinotus blochii*) heavily infected with *Neobenedenia girellae* (arrows). A. The whole body. Note the excessive mucus around the head; B. Excessive mucus on the dorsal part the body; C. operculum; D. opaque eyes due to excessive mucus and *N. girellae* infection.

according to the manufacturer's instruction. 10 ng/μl of purified PCR products were directly sequenced by ABI Prism 3730 XL DNA Analyzer (PE Applied Biosystems, USA).

Phylogenetic analyses

Sequence chromatograms were aligned using Clustal W method (Thompson et al., 1994), with DNA sequences. The obtained sequences of each 4 genes were trimmed to remove sequences that could not be aligned unambiguously. All *Neobenedenia* and other monogenean gene sequences were retrieved from GenBank and aligned to our data. A neighbor-joining analysis of the aligned data for each gene was conducted with MEGA 7 (Kumar et al., 2016).

Results and Discussion

Two snubnose pompano (average total length : 51.6 cm, average body weight : 1,985.7g) showed dyspnea and anorexia for a week while reared in the public aquarium. The fish had mild hemorrhage on the body trunk with excessive mucus on head, operculum and eyes (Fig. 1A, B, C, D). A large number of monogeneans were found on the skin, operculum and eyes of all 2 individual fish (Fig. 1B, C, D).

The morphological characteristics of collected parasites matched those of the genus *Neobenedenia* redescribed by Whittington and Horton (1996). In brief, the parasite had an elongated oval body, an aseptate haptor with 3 pairs of median sclerites and 7 pairs of marginal hooks, anterior attachment organ consisted of disc-shaped pads, lacked vagina and the accessory gland reservoir was in the penis sac (Fig. 2).

The measurement data of the parasites (n=10) were as follow: total body length including haptor 4.2-5.8 mm and maximum width 2.2-3.0 mm; anterior attachment organs 0.29-0.56 mm long by 0.23-0.52 mm wide; haptor 0.75-1.43 mm long by 0.87-1.30 mm wide; anterior hamuli 0.25-0.41 mm long; posterior

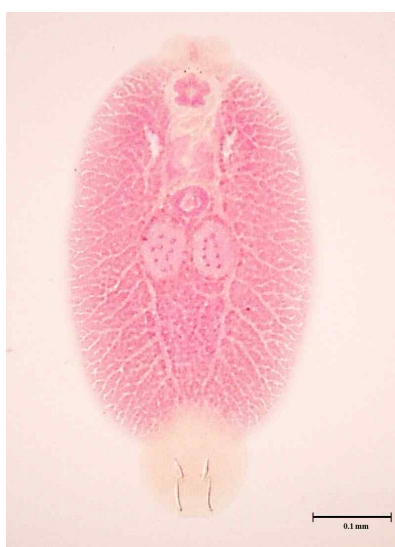

Fig. 2. *Neobenedenia girellae* (Hargis, 1955) Yamaguti, 1963 from snubnose pompano, ventral view.

hamuli 0.10-0.17 mm long; accessory sclerites 0.17-0.38 mm long; a pair of testes 0.55-0.77 mm long by 0.42-0.50 mm wide; ovary 0.31-0.45 mm long by 0.31-0.50 mm wide. All of these measurements data coincided with those of Ogawa et al. (1995), although some measurements range in our data were slightly wider. Therefore, the parasites in this study was identified as *N. girellae*. The slide specimen was deposited in the Department of Marine Bioscience, Gangneung-Wonju National University (specimen number GWFPC-190527).

We obtained 4 target gene sequences (18S rDNA, 28S rDNA, mt *cox*1, *Cytb*), however, we could use 28SrDNA and *Cytb* genes for further analyses because only these 2 genes of both *Neobenedenia* species (*N. girellae* and *N. melleni*) were available in GenBank. For the 28S rDNA gene (partial sequence length 638 bp), we compared the sequence of our specimen with those of *Neobenedenia* species retrieved from GenBank. The phylogenetic tree showed that *Neobenedenia* species formed a monophyletic clade and separated into 4 major clades. Most of the *Neobenedenia* sequences in the phylogenetic tree were obtained and identified as *N. girellae* by Brazenor et al. (2018a), and one *N. melleni* sequence (Gen Bank accession number: MH843696.1) were obtained by Perkins et al. (2009). Our sequence fell into the *N. girellae* clade (Fig. 3). For *Cytb* gene (partial sequence length 704 bp), the phylogenetic tree also showed the same trend and our specimen was clustered with *N. girellae* (Fig. 4). All the obtained gene sequences in this study were registered in GenBank (GenBank accession number: MT542140, 542866, 549677, 559979).

Genus *Neobenedenia* can be diagnosed as lacking haptoral septa and a vagina, but having accessory sclerites, haptoral hamuli, paired anterior circular discs and 2 juxtaposed testes, which is a unique combination to this genus (Perkins et al., 2009). However, the interspecific morphological variations are subtle in this genus and accurate identification are known to be challenging (Ogawa et al., 1995, Whittington et al., 2004). In particular, delineation of *N. melleni* and *N. girellae* has been very controversial; both of these 2 species have the broad host specificity, which is atypical for monogeneans and the subsequent morphological variation is probably associated with the broad host range and diverse environmental conditions (Brazenor et al., 2018a, b; Whittington and Horton, 1996). Due to these characteristics, Whittington (1996) synonymized *N. girellae* with *N. melleni*, however, it was not unanimously accepted and many authors still have used *N. girellae* in their papers (see Brazenor et al., 2018a).

Recently, Molecular markers have become powerful tools for taxonomy, systematics and phylogeny of morphologically very similar monogeneans but are actually genetically distinguishable. For example, *Benedenia seriolae* was found in fact as a complex of cryptic species, not a single taxon (Sepúlveda and Gonzalez, 2014). Moreover, 2 morphologically very similar *Neobenedenia* species (*N. girellae* and *N. melleni*) are reported to be different species, by phylogenetic analyses of 2 nuclear and one mitochondrial genes (Brazenor et al., 2018a; Perkins et al., 2009). We included only one *N. melleni* sequence (GenBank accession number: MH853747.1) from *Sphoeroides annulatus*, which is thought to be the type host of *N. melleni,* registered by Brazenor et al. (2018a) for the phylogenetic analysis. Several other *N. melleni* sequences are available in GenBank but all of them are probably misidentified because they were located in the *N. girellae* clade (Brazenor et al., 2018a).

Snubnose pompano (*Trachinotus blochii*) distributes extensively in the Indo-Pacific region (Red Sea and East Africa to the Marshall Islands and Samoa, north to southern Japan, south to Australia) and the aquaculture has been successfully established in several Asia-Pacific countries such as China, Indonesia and India (Abdul Nazar et al., 2012). There has been no report describing snubnose pompano is a natural host for either of these 2 *Neobenedenia* species, but

several other congeneric species are known as natural hosts of *Neobenedenia* species (e.g., *T. kennedeyi* is recorded as a host for *N. gerellae*, and *T. carolinus, T. falcatus, T. glaucus, T. goodei* are recorded as hosts for *N. melleni*) (Brazenor et al., 2018a; Whittington and Horton, 1996). Therefore, *T. blochii* is added to the host list for *N. girellae* in this study.

But it is not clear if the snubnose pompano in this study was naturally infected with *N. girellae* when introduced to the aquarium or newly infected with *N. girellae* after introduction, from other host fish species inhabiting in the same aquarium. It should be noted that host-switching of *Neobenedenia* can often occur under the aquarium or aquaculture conditions.

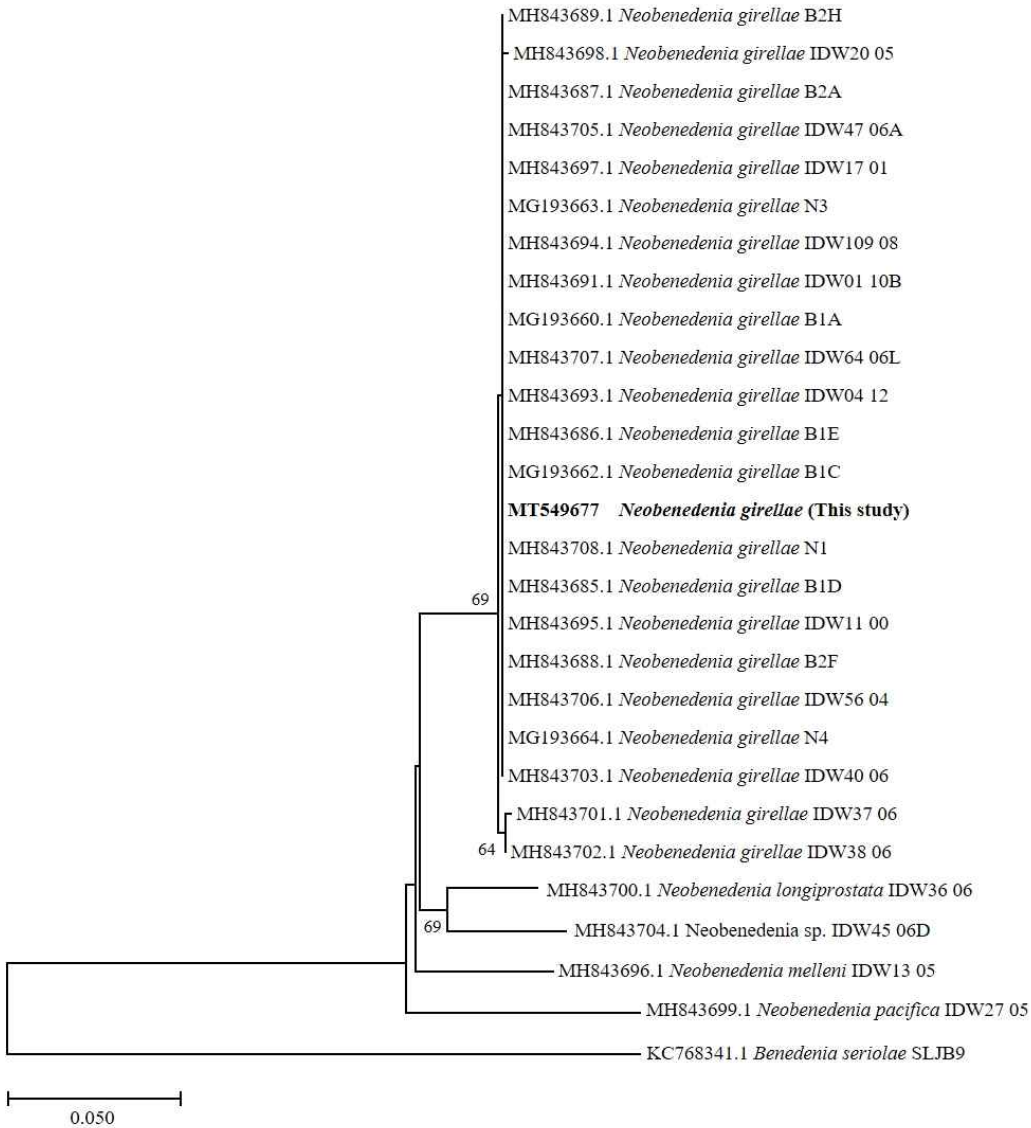

Fig. 3. Phylogenetic relationships of *Neobenedenia* species, based on a Neighbor-joining analysis of partial 28S rDNA gene sequences (638 bp) retrieved from GenBank and our specimen. GenBank sequences in this analysis were selected based on the information by Brazenor et al. (2018a).

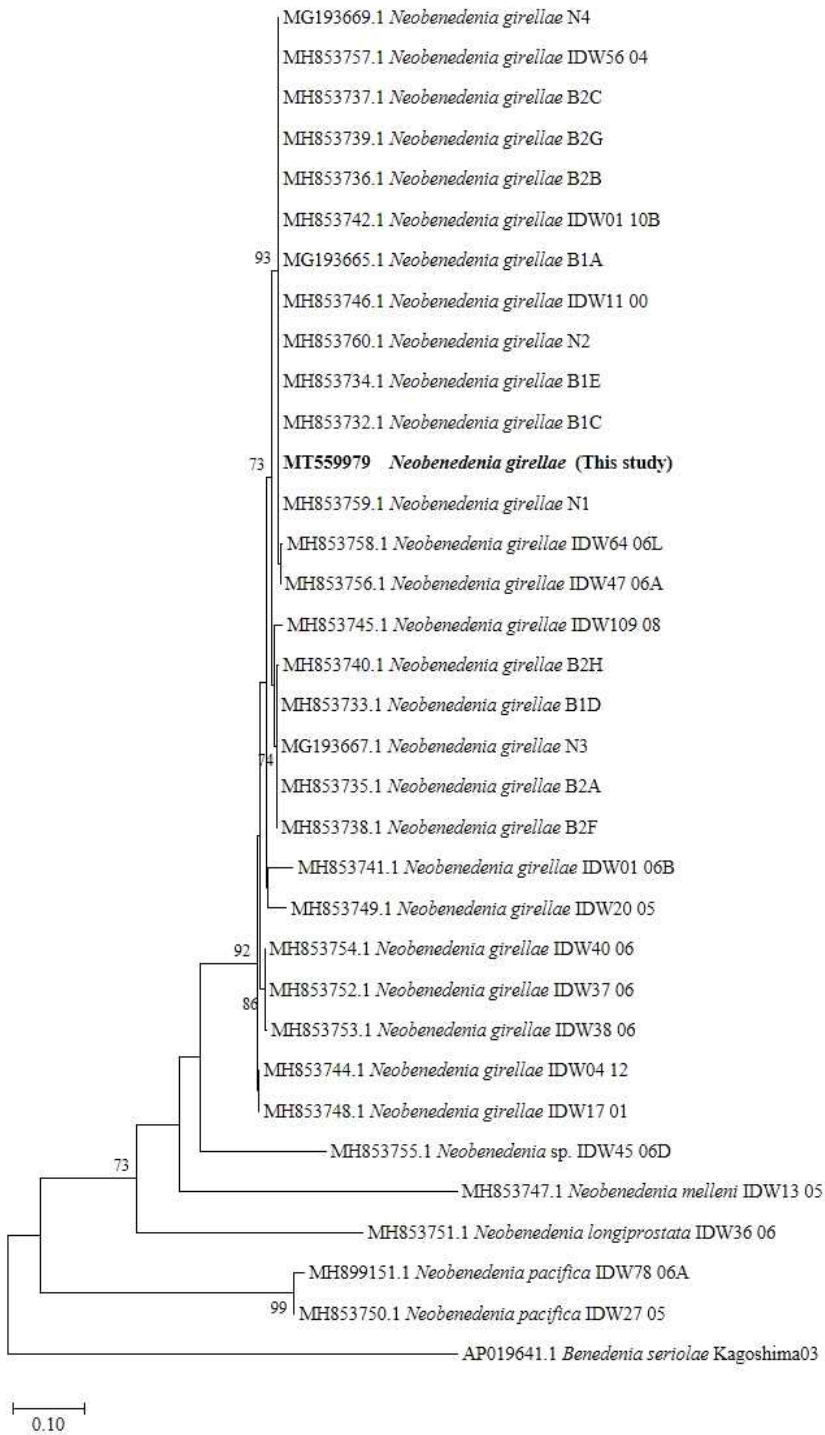

Fig. 4. Phylogenetic relationships of *Neobenedenia* species, based on a Neighbor-joining analysis of partial *Cytb* gene sequences (704 bp) retrieved from GenBank and our specimen. GenBank sequences in this analysis were selected based on the information by Brazenor et al. (2018a).

Acknowledgements

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