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Larval stages of *Neoplagioporus elongatus* (Goto and Ozaki, 1930) (Opecoelidae: Plagioporinae), with notes on potential second intermediate hosts

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ABSTRACT

The morphology of sporocysts and cercariae of *Neoplagioporus elongatus* (Goto and Ozaki, 1930) is described for the first time. A cotylomicrocercous cercaria obtained from the sorbeoconch snail *Semisulcospira nakasekoae* was confirmed to be the cercaria of *N. elongatus*, based on the degree of sequence identity of the COI gene to that of adult worms. Freshwater annelids (oligochaetes and leeches) and some aquatic insects (odonates) were demonstrated experimentally to be potential second intermediate hosts.

1. Introduction

Neoplagioporus elongatus (Goto and Ozaki, 1930) (Opecoelidae: Plagioporinae) was first described by Goto and Ozaki (1930) as *Labouria elongate* [1]. Shimazu [2] established a new genus *Neoplagioporus* with the type species *N. zacconis* (Yamaguti, 1934), and moved *Labouria elongata* into the new genus as *Neoplagioporus elongatus*. At present, four *Neoplagioporus* species (*N. zacconis*, *N. elongatus*, *N. ayu* (Takahashi, 1928) and *N. kajika* Urabe et Higa, 2006) have been reported from freshwater fishes in Japan. A recent molecular study suggests that *N. elongatus* is related more closely to the genus *Urorchis* Ozaki, 1927 than to the other congeneric species *N. zacconis* (type species of *Neoplagioporus*) and *N. ayu* [3]. Although its generic status is in dispute, the same study confirmed that *Neoplagioporus* and *Urorchis* from Japan belong to the subfamily Plagioporinae with freshwater opecoelid species from North America [3].

The life cycle of *Neoplagioporus* species has not been described. In general, digeneans of the subfamily Plagioporinae produce cotylomicrocercous cercariae, which develop in daughter sporocysts in snails and metamorphose into metacercariae in arthropods [4–7]. In rare cases, metacercariae develop in mollusks [8,9], fish [10], annelids [11], or develop into adults directly in sporocysts [12]. Shimazu [13] described the cercariae and the first intermediate host of *Urorchis goro* Ozaki, 1927, which is related to *N. elongatus* [3]. The cercaria is cotylomicrocercous with a candle-like stylet and five pairs of penetration glands that develops in the freshwater semisulcospirid snail *Semisulcospira libertina* (Gould, 1859) [13]. Urabe [14] obtained a type of cotylomicrocercous cercariae from some species of *Semisulcospira* in the Lake Biwa water system. The cercariae had a candle-like stylet and six pairs of penetration glands and their experimental hosts were chironomid larvae. However, Urabe did not demonstrate to which species the cercariae belong [14].

We obtained cotylomicrocercous cercariae from *Semisulcospira nakasekoae* Kuroda, 1929 in the Uji River, Kyoto, which is one of the sampling sites of "opecoelid cercariae" in Urabe [14] and where *N. elongatus* are abundant in cyprinid fish such as *Hemibarbus labeo* (Pallas, 1776) [15] (*Hemibarbus barbus* in [15] is now identified as *H. labeo*; see [16]). We demonstrated that the cercariae are the larval stage of *N. elongatus* using molecular techniques. In this paper, we describe the morphology of daughter sporocysts and cercariae of *N. elongatus*; several potential second intermediate hosts are reported, based on the results of experimental infections.

2. Materials and methods

Individuals of the freshwater snail *Semisulcospira nakasekoae* were collected to obtain cotylomicrocercous cercariae at Ingen Bridge, Uji River, Makishima, Uji City, Kyoto Prefecture (135°47′23″E, 34°54′59″N), on August 1 and October 25, 2014, November 23, 2015, and April 9, 2016. At each sampling, 100–200 snails were collected. The snails were transported to the laboratory and kept in 300-mL plastic cups individually to obtain free-shed cercariae. The live cercariae were observed under a microscope equipped with Nomarski interference contrast. Vital staining with Nile blue sulfate was conducted to facilitate observation of the penetration glands. Some cercariae were flattened

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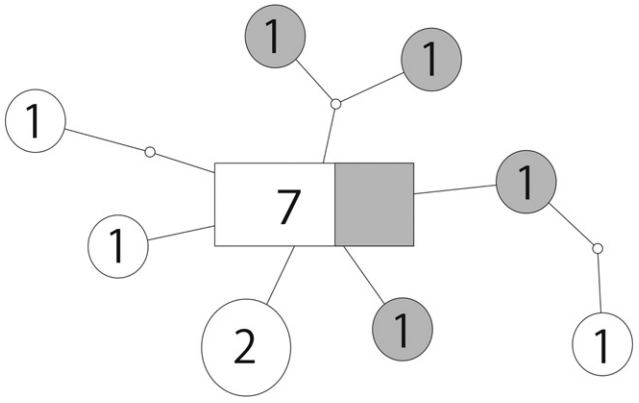

Fig. 1. Haplotype network of *Neoplagioporus elongatus* obtained from Uji. Gray, haplotypes of adult *N. elongatus*; white, haplotypes of sporocysts. Numbers in circles/square indicate numbers of samples.

slightly, fixed with alcohol-formalin-acetic acid (AFA), stained with Heidenhain's iron hematoxylin, and mounted in Canada balsam for morphological observation and measurements except body proper and tail. The others were fixed in 10% hot formalin without flattening for measurement of body proper and tail.

The shell of infected snails was crushed using a pair of pliers to obtain sporocysts from the rectum. Some sporocysts were stored at −30 °C for DNA analysis. The others were prepared as whole mounts using the same protocols as for cercariae, or fixed in 10% hot formalin for measurements.

Adult *N. elongatus* individuals were obtained from the intestine of *Hemibarbus labeo* caught at the same locality as snails on July 14, 2014. The removed worms were washed in TAE buffer and stored at 30 °C for DNA analysis.

Genomic DNA was extracted using a Wizard® SV Genomic DNA Purification System (Promega, Madison, WI, USA). A partial region of the COI was amplified by PCR using the primer set JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and COI R-Trema (5′-CAACAAATCATGATGCAAAAGG-3′) [17] as a forward and a reverse primer, respectively. PCR amplification was performed in a 20-μL reaction mixture containing 0.5 U ExTaq (TaKaRa Bio Inc., Otsu, Shiga, Japan), 10 × ExTaq Buffer (TaKaRa), dNTPs (0.2 mM each), 0.5 μL template, and 0.5 μM forward and reverse primers, using a Mycycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The thermocycling profile was as follows: 40 cycles of 10 s at 94 °C, 30 s at 50 °C, and 60 s at 72 °C. Gel electrophoresis was conducted on the PCR products and bands correspond to a length of approximately 800 bp were excised. They were purified using a Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced directly (DNA sequencing services provided by FASMAC Co., Ltd., Atsugi, Kanagawa, Japan). Sequence divergences (uncorrected p distance) were calculated between all pairs of obtained haplotypes using Mega (ver. 7.0.16) [18], and a haplotype network was drawn using TCS ver. 1.21 [19]. The analyzed sequences have been deposited in DDBJ (http://ddbj.sakura.ne.jp/) (accession no. LC196185-LC159193).

Some of the newly collected specimens were deposited in the National Museum of Science (NMST Pl-6265–6269), and the others are in M. Urabe's personal collection.

2.1. Experimental infection

Cercariae of *N. elongatus* were kept with invertebrates to detect potential second intermediate hosts. Snails infected with *N. elongatus* were collected at the same locality on August 1 and October 25, 2014, and April 9, 2016. Host candidates were collected from canals, ditches and rice fields in Hikone or Moriyama City, where the definitive hosts of *N. elongatus* were absent, or cultured organisms were purchased. One to fifteen host candidates were placed in 300-mL plastic cups containing dechlorinated tap water and living cercariae (8–28 individuals/trial), and stored for 1–2 days in the laboratory at room temperature. All host snails which provided cercariae were dissected after the experiment and sporocysts in them were molecularly identified. Host candidates were dissected under a stereoscopic microscope to detect metacercariae. When encysted metacercariae were found, they were observed under a microscope and their stylets were examined. Only metacercariae immediately after metamorphosis have stylets in the oral sucker, and the stylets fall out during the process of maturation; thus, metacercariae with stylets were regarded as having been infected

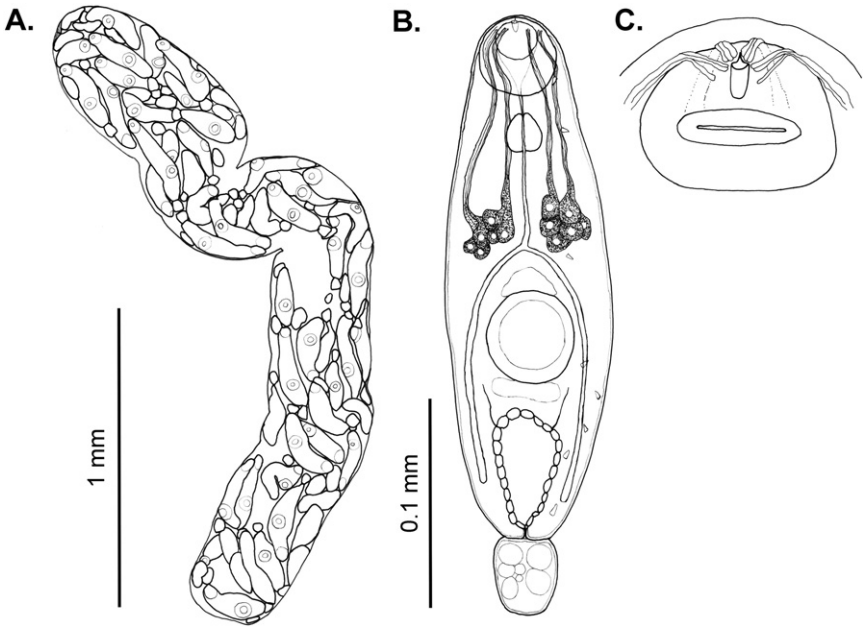

Fig. 2. A, Daughter sporocyst of *Neoplagioporus elongatus* obtained from the rectum of *Semisulcospira nakasekoae*; B, free-shed cercaria of *Neoplagioporus elongatus*; C, anterior terminal of cercariae, showing the arrangement of penetration ducts.