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Liolope copulans (Trematoda: Digenea: Liolopidae) parasitic in *Andrias japonicus* (Amphibia: Caudata: Cryptobranchidae) in Japan: Life cycle and systematic position inferred from morphological and molecular evidence

Takashi Baba [a], Masatomi Hosoi [b], Misako Urabe [a,*], Takeshi Shimazu [c], Takeyoshi Tochimoto [d], Hideo Hasegawa [e]

[a] Department of Ecosystem Studies, School of Environmental Science, The University of Shiga Prefecture, 2500 Hassaka, Hikone, Shiga 522–8533, Japan
[b] Department of Marine Biosciences, Faculty of Marine Biosciences, Fukui Prefectural University, Gakuen-cho 1-1, Obama, Fukui 917-0003, Japan
[c] 10486-2 Hotaka-Ariake, Azumino, Nagano 399-8301, Japan
[d] The Institute of Hanzaki, 292 Kurokawa, Ikuno, Asago, Hyogo 679-3341, Japan
[e] Department of Biology, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama, Yufu, Oita 879-5593, Japan

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ABSTRACT

The life cycle of *Liolope copulans* Cohn, 1902 (Trematoda: Digenea: Liolopidae), an intestinal parasite of the Japanese giant salamander *Andrias japonicus* (Temminck) (Amphibia: Caudata: Cryptobranchidae), was studied in the field and laboratory in Japan. This is the first description of mother sporocyst, daughter sporocyst and cercariae of a liolopid species. Non-oculate longifurcate pharyngeate cercariae were formed in lanceolate-cylindrical daughter sporocysts in *Semisulcospira libertina* (Gould) (Gastropoda: Sorbeoconcha: Pleuroceridae). They successfully developed to encapsulated metacercariae in cyprinid fishes, *Nipponocypris sieboldii* (Temminck and Schlegel) and *Rhynchocypris lagowskii* (Dybowski), by experimental infection. Cercariae had a V-shaped excretory vesicle with two looped arms, as in metacercariae and adults. Developmental stages from mother sporocyst to adult are described and illustrated. DNA sequencing was conducted for 28S and 18S rDNA of mother and daughter sporocysts, cercariae, and an adult. The result of molecular phylogenetic analysis suggests that *L. copulans* may be one of the basal taxa of the order Diplostomida Olson, Cribb, Tkach, Bray, and Littlewood, 2003, but its systematic position is still unclear because of the topological inconsistence between the 28S and 18S trees. Therefore, we tentatively place the family Liolopidae in the superfamily Diplostomoidea, mainly based on the morphology of sporocysts and cercariae.

1. Introduction

The Japanese giant salamander *Andrias japonicus* (Temminck) (syn. *Cryptobranchus japonicus* Temminck, *Megalobatrachus japonicus* (Temminck))(Amphibia: Caudata: Cryptobranchidae) inhabits upper- and middle-reaches of relatively large rivers in western Japan [1], and feeds on fish, shrimp, crayfish, crab, and other aquatic animals [2].

Liolope copulans Cohn, 1902 is a digenetic trematode parasitic in *A. japonicus* [3]. This digenean was described as a new genus and species on the basis of immature specimens found by G. W. Müller in the stomach and intestine of an individual of *A. japonicus*, which had been imported from Japan to Europe and lived for 4 weeks [3]. Later, it was redescribed from Müller's specimens [4]. In Japan, *L. copulans* was described in detail based on mature adults found in the small intestine of *A. japonicus* in Okayama Prefecture, Honshu [5]. Later, adults were reported from *A. japonicus* caught in Hiroshima Prefecture, Honshu; and metacercariae were described from a freshwater fish, *Rhynchocypris lagowskii* (Dybowski) (syn. *Moroco steindachneri* (Sauvage))(Japanese name Abura-haya) (Cyprinidae), collected in the streams in Hiroshima Prefecture, where *A. japonicus* occurred [6]. An immature specimen from *A. japonicus* captured on Mt. Kasaoka in Okayama Prefecture was described; and the generic diagnosis of *Liolope* was emended [7]. More recently, adults were recorded from two individuals of *A. japonicus* from Hyogo and Osaka prefectures, both Honshu; and one individual at Suma Aqualife Park, Kobe, Hyogo Prefecture (home river unspecified) [8]. The karyotype was also described [8]. In China, *L. copulans* was described, without mentioning the site of infection and the state of sexual maturity, from the Chinese giant salamander *Andrias davidianus* (Blanchard) (syn. *Megalobatrachus davidianus* (Blanchard)) purchased at Guiyang Market in Kweichow [Guizhou] Province in 1938–1943 [9].

The life cycle of *L. copulans* reflects the habitat and food habit of *A. japonicus*. Metacercariae were found "encysted" [sic; should be encapsulated] in cyprinid fishes of many species [6,10]. A freshwater

* Corresponding author. Tel.: +81 749 28 8308; fax: +81 749 28 8463.
E-mail address: urabe@ses.usp.ac.jp (M. Urabe).

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snail, *Semisulcospira libertina* (Gould) (Gastropoda: Sorbeoconcha: Pleuroceridae), was experimentally determined as a first intermediate host [10]. However, the cercaria was not obtained at that time.

As regards the systematic position, the family Liolopidae Odhner, 1912 with *Liolope* as the type genus [11] has previously been placed, together with the family Clinostomidae Lühe, 1901, in the superfamily Clinostomoidea Lühe, 1901 [12]. Recently, the classification of the Digenea has been reconstructed based on the molecular phylogenetic analysis of nuclear ribosomal DNA [13], and the Clinostomidae was included in the superfamily Schistosomatoidea Stiles and Hassall, 1898 [13]. However, the larval morphology and DNA sequence data of no liolopid species have been analyzed, and the taxonomic position of the Liolopidae has remained uncertain [13].

Recently, we found mother and daughter sporocysts and cercariae of *L. copulans* in *S. libertina*. Their morphology is described herein along with the descriptions of the metacercaria and adult. Moreover, we conducted DNA sequencing of 28S and 18S rDNA of *L. copulans*. Based on the morphological and molecular evidence, the systematic position of the Liolopidae is discussed.

2. Materials and methods

2.1. Daughter sporocysts and cercariae in naturally infected snails

Host snails (*S. libertina*) were collected in the following rivers and ditch, where *A. japonicus* was abundant: (1) the Kamo River at Kamigamo and Kumogahata, Kyoto City, Kyoto Prefecture, in July 2007; (2) the Tenno River at Tenno, Nose Town, Osaka Prefecture, in July 2007; (3) the Taki River at Nagasaka and Shorenji, Nabari City, Mie Prefecture, on 21 August 2007; (4) the Minoo River at Minoo-koen, Minoo City, Osaka Prefecture, in July 2007, 19 October 2008, 11 May 2009, and 3 March 2010; and (5) the ditch with outflow from a fish pond at Jugo, Hidaka, Toyooka City, Hyogo Prefecture, on 21 November 2007. In the last pond, about 400 individuals of *A. japonicus* from the Izushi River in Hyogo Prefecture had been reared during the river improvement work after a flood in 2004.

The snails were crushed for cercariae with the excretory system characteristic of *L. copulans* [5,6]. Such cercariae were found to develop in daughter sporocysts. Some snails were kept alive to collect spontaneously emerged cercariae.

Daughter sporocysts and cercariae were observed on both living specimens and stained whole-mounts in balsam under a light microscope. Daughter sporocysts and cercariae were either flattened and fixed in AFA or fixed in hot 5% nonbuffered formalin, stained either with Heidenhain's iron hematoxylin or with Grenacher's alum carmine, and mounted in Canada balsam. Spontaneously emerging cercariae were fixed in hot 5% nonbuffered formalin and measured.

The preparation of those samples for molecular purposes will be described in Section 2.5.

2.2. Experimental infection of snails with miracidia

Some adult *S. libertina* were collected in the Ichi River, the Nagano River (a tributary of the Ichi River), and a ditch at Kurokawa, Ikuno, all in Asago City, Hyogo Prefecture, on 30 November 2007. Three hundred seventeen laboratory-raised young snails were obtained from these adults.

The experimental infection of snails with miracidia was attempted in the swimming pool on the campus of the Institute of Hanzaki at Kurokawa. About 60 individuals of *A. japonicus* had been reared in the swimming pool ($20\times3\times1$ m) as a temporary refuge during the repairs of their home river, the Ichi River. The above-mentioned 317 parasite-free young snails were put in 1-mm mesh bags, which were then set on the bottom of the swimming pool to expose them to miracidia from 22 July 2008 to 31 October 2008, on the assumption that some of the individuals of *A. japonicus* must be infected with *L. copulans*. The water temperature ranged between 10 and 24 °C during the exposure. On 31 October 2008 (0–101 days after exposure), the snails were moved from the swimming pool to the laboratory (neither room nor water temperature controlled) and were kept in plastic cups. The snails were dissected for larvae of *L. copulans* under a stereoscopic microscope on 30th, 61st, 92nd, 121st, and 223rd days of maintenance in the laboratory.

2.3. Experimental infection of fish with cercariae

Host snails (*S. libertina*) were collected in the Taki River on 7 October 2007 and the Minoo River on 11 May 2009 (Section 2.1). The snails were kept individually in a 300-ml plastic cup to collect spontaneously emerged cercariae.

Host fish used were: one *Nipponocypris sieboldii* (Temminck and Schlegel) (Japanese name: Numamutsu) (42.4 mm in standard body length) caught at an artificial stream on the campus of the University of Shiga Prefecture; and one *R. lagowskii* (98.5 mm) caught in the Inukami River, Kaideima, both in Hikone City, Shiga Prefecture. Both the stream and the river were known to be free from *A. japonicus*.

The first experiment was made using cercariae from the Taki River and one individual of *N. sieboldii*. Many cercariae (not counted) collected from one snail were put into a glass tank ($18\times18\times18$ cm) with the fish on 21 and 30 January and 5 February 2008. The fish was dissected for metacercariae on 22 April 2008 (77–92 days after infection).

The second experiment was conducted using cercariae from the Minoo River and an individual of *R. lagowskii*. Many cercariae (not counted) collected from seven snails were put into a glass tank ($18\times30\times24$ cm) with the fish on 14 and 17 May 2009. The fish was dissected for metacercariae on 12 June 2009 (28–31 days after infection).

Some metacercariae recovered were removed from their capsules, flattened, fixed in AFA, stained with Heidenhain's iron hematoxylin, and mounted in Canada balsam.

2.4. Specimens for morphological observation and methods

Besides the newly obtained material, specimens of *L. copulans* were loaned from Meguro Parasitological Museum (MPM), Tokyo; and the National Museum of Science and Nature (NMSN), Tokyo (formerly the National Science Museum, Tokyo (NSMT)).

Drawings were made with the aid of a camera lucida. Measurements (length by width) are given in millimeters unless otherwise stated. Voucher specimens of the present material have been deposited in the NMSN.

The material used for description of the metacercaria consisted of three lots: (1) many whole-mounted and serially sectioned specimens (NSMT-Pl 4588 and 4589; encapsulated, and removed from their capsules and flattened, undescribed) from the cyprinid *Acheilognathus typus* (Bleeker) (Japanese name: Zeni-tanago) on 5 December 1997 and 27 April 1998, and specimens (NSMT-Pl 4592; removed from their capsules and flattened, and serially sectioned, undescribed) from the cyprinid *R. lagowskii* (syn. *Phoxinus lagowskii steidachneri* Sauvage) on 23 June 1997 [10]; (2) 3 whole-mounted specimens (NSMT-Pl 4593; removed from their capsules and flattened, undescribed) from the cyprinid *Tribolodon hakonensis* (Günther) (Japanese name: Ugui), and many whole-mounted and serially sectioned specimens (NSMT-Pl 4594; removed from their capsules and flattened, and serially sectioned *in situ* in the host fish, undescribed) from the cyprinid *R. lagowskii* [10]; and (3) many whole-mounted specimens obtained by the present experimental infection: from *R. lagowskii* on day 28–31 after infection and from *N. sieboldii* on day 77–92.

The host fishes of the first lot were found naturally infected during the exhibition at Inokashira Park Zoo (Inokashira Shizen Bunka-en