***Posthodiplostomum* sp. Metacercariae in the Trunk Muscle of Northern Snakeheads (*Channa argus*) from the Fushinogawa River, Yamaguchi, Japan**

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ABSTRACT. The northern snakehead *Channa argus*, native to China, Russia and Korea, is currently found widespread throughout Japan following its original introduction during the 1920s. A parasitological study of 10 snakeheads fished from the Fushinogawa River running through Yamaguchi City, Japan, detected 2–101 (average, 23.7) metacercariae per 100 g of trunk muscle from each fish. The trematode was identified as metacercariae of *Posthodiplostomum* sp. (Strigeidida: Diplostomidae) morphologically and characterized genetically based on the ribosomal RNA gene (rDNA). Phylogenetic trees were constructed on the basis of either the 18S, ITS or 28S region of rDNA to assess the relationship among members of the family Diplostomidae. The addition of genetic data from more diplostomid taxa, particularly *Posthodiplostomum cuticola* recorded from a variety of freshwater fish in Eurasia, would facilitate the precise identification of the present species.

KEY WORDS: *Channa argus*, Japan, *Posthodiplostomum*, rDNA.

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Posthodiplostomum cuticola (Nordmann, 1832) Dubois, 1936 is the type species of this trematode genus and a parasite of Ardeidae, such as gray heron (*Ardea cinerea*), purple heron (*A. purpurea*), squacco heron (*Ardeola ralloides*), little egret (*Egretta garzetta*) and black-crowned night heron (*Nycticorax nycticorax*) in Eurasia [19, 24]. Snails belonging to the genus *Lymnaea* are its first intermediate host, and various freshwater fish are its second intermediate host [19, 24]. In 1971, Yamaguti [24] reported that the species was found in many freshwater fish in Japan; however, no more detailed mention was made. Ishii [10] and Nagasawa *et al.* [14] recorded the presence of *Posthodiplostomum* sp. metacercariae in the crucian carp and common carp in Japan without any further parasitological data. Toyooka and Okada [21] recorded two forms of *Posthodiplostomum* metacercariae from the visceral organs of Japanese medaka (*Oryzias latipes*) and isolated *P. podicipitis* adults following oral inoculation of domestic pigeons with one of the metacercaria forms. *P. podicipitis* (syn. *Ornithodiplostomum podicipitis*) was originally described from a little grebe (*Tachybaptus ruficollis poggei*; syn. *Podiceps ruficollis japonicus*) in Kyoto Prefecture, Japan, by Yamaguti [23]. Except for these earlier records [21, 23], there are no further

records of adult trematodes of the genus *Posthodiplostomum* in Japanese birds [22].

In this study, we report a high prevalence (100%) of *Posthodiplostomum* sp. metacercariae in northern snakeheads (*Channa argus*) fished from the Fushinogawa River running through Yamaguchi City, Japan. This snakehead is native to China, Russia and Korea, and is currently one of the widespread alien freshwater fish in Japan following its original introduction during the 1920s [13]. It has been reported to be a main source of *Gnathostoma spinigerum* larvae to human patients with cutaneous larva migrans (creeping eruption) [12, 13].

Between May and October 2011, 10 northern snakeheads were collected from the Fushinogawa River running through Yamaguchi City, Japan (34°21'61"N, 131°55'72"E). Each fish was placed in an icebox and transported to the laboratory. After measuring the body length and weight, a parasitological examination of fins, gills, heart, brain, stomach and intestine was conducted. Trunk muscles from the anterior part close to the head, middle part and caudal part were removed, and an equal amount from each part was pooled. The collected muscles, 80–120 g from each fish, were minced and digested artificially for approximately three hours in physiological saline containing 0.6% (w/v) pepsin and 0.8% (v/v) HCl at 37°C. By simple sedimentation, the precipitate of muscle digestion was repeatedly washed in physiological saline. Metacercariae were collected from the final sediment. For morphological examination, a portion of the metacercariae was excysted using needles, relaxed in water for one hour, pressed between glass slides and

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fixed in 10% neutral-buffered formalin. The fixed specimens were washed in distilled water, immersed in 70% alcohol and stained with acetocarmine. Whole mounted specimens were prepared after dehydration in a series of alcohol and clearance by xylene according to a standard technique. The remaining parasites were used for DNA extraction or stored in fixative (either 70% alcohol or 10% neutral-buffered formalin).

The weights of the 10 northern snakeheads ranged from 0.20 to 2.60 kg (average, 1.43 kg). Their body lengths ranged from 34 to 68 cm (average, 52.5 cm). From the 10 fish, 2 to 101 encysted metacercariae (average, 23.7) were recovered per 100 g of trunk muscle. A part of these cysts was darkened due to pericystic melanophores (Fig. 1). The dimensions of the cyst ($n=11$) were 0.79–1.40 (1.04 ± 0.18) mm by 0.54–0.94 (0.69 ± 0.14) mm. Excysted metacercariae consisted of a forebody and hindbody, with the latter attached to the dorsal side of the former (Fig. 2). The holdfast organ with a median slit was located in the posterior one third of the forebody. The oral sucker and the acetabulum were relatively small, and their sizes were comparable (Table 1). Calcareous corpuscles were distributed along the whole body, but larger particles were observed only in the anterior two thirds of the forebody. Genital organs such as testis and ovary remained as primordial. These morphological features were identical to the *P. cuticola* metacercariae reported in a variety of freshwater fish across Eurasia [6, 19, 24], but morphometrically these two species appeared distinct; the present species being markedly smaller than *P. cuticola* (Table 1). Metacercariae of *P. podicipitis* and of another unidentified diplostomid species, belonging to either *Posthodiplostomum* or *Ornithodiplostomum*, found in the viscera of *Oryzias latipes* in Japan were reported to have well-developed testes and ovaries upon isolation from the fish host [21]. Thus, at present, it would appear most likely that the metacercariae collected in the present work from northern snakeheads are *P. cuticola* rather than other known diplostomids [19, 24]. Voucher specimens are deposited in the National Museum of Nature and Science, Tokyo, Japan (accession nos. NSMT-PI5922–PI5929).

Parasite DNA was extracted from three metacercariae from three different snakeheads using Illustra™ Tissue and Cell Genomic Prep Mini Spin Kit (GE Healthcare, Buckinghamshire, U.K.) according to the manufacturer's instructions. PCR amplification of overlapping ribosomal RNA gene (rDNA) segments was performed using five primer combinations [18]: NSF4/18 and 18S-1192R/20; NSF573/19 and NSR1787/18; NSF1419/20 and Dig.5.8SR; NC13 (ITS2)/F and Dig.LSU-270R; and Dig.LSU-F1/C1 and 28S-1270R/22. PCR products for sequencing were purified using a High Pure PCR Cleanup Micro Kit (Roche Diagnostics GmbH, Mannheim, Germany). Following direct sequencing of PCR amplicons, the sequences were assembled manually with the aid of the Clustal W multiple alignment program [20]. For some rDNA segments, the amplicon was cloned into a plasmid vector, pTA2 (Target Clone™, TOYOBO, Osaka, Japan), and transformed into *Escherichia coli* JM109 (TOYOBO) according to the manufacturer's instructions.

Fig. 1. *In situ* view of an encysted metacercaria of the *Posthodiplostomum* sp. in the trunk muscle of a snakehead. This cyst with a clear border to muscle is surrounded by several melanophores.

Fig. 2. Artificially excysted metacercariae of the *Posthodiplostomum* sp. A fresh specimen with numerous larger calcareous corpuscles in the anterior two thirds of the forebody (A) and a mounted stained specimen (B). AC, acetabulum; BC, bursa copulatrix; CC, calcareous corpuscles; GP, genital primordia; HG, holdfast gland; HiB, hindbody; HO, holdfast organ; and OS, oral sucker.

Following propagation, plasmid DNA was extracted using a NucleoSpin® Plasmid kit (MACHEREY-NAGEL GmbH, Düren, Germany), and inserts from multiple independent clones were sequenced using universal M13 forward and reverse primers. At least three clones were sequenced for each segment. The rDNA nucleotide sequence of the present *Posthodiplostomum* sp. is deposited in the DDBJ/EMBL/GenBank databases under accession no. AB693170.

The three newly obtained rDNA sequences of *Posthodiplostomum* sp. from three different fish were absolutely identical, consisting of 18S (1,958 bp), ITS1 (569 bp), 5.8S (157 bp), ITS2 (288 bp) and 28S (1,370 bp). This newly obtained sequence and retrieved sequences of related diplostomid and

Table 1. Morphometric comparison of metacercariae of major *Posthodiplostomum* spp. from freshwater fish*

Parasite species	<i>Posthodiplostomum</i> sp.		<i>P. cuticola</i>	<i>P. podicipitis</i>	<i>Posthodiplostomum</i> sp.		<i>P. minimum</i>
Host	<i>Channa argus</i>		<i>Phoxinus laevis</i>	<i>Oryzias latipes</i>	<i>Oryzias latipes</i>		<i>Eupomotis gibbosus</i> ; <i>Helioperca incisor</i>
Location	Trunk muscle		Skin, fins, muscle	Liver, kidney, gonads			Liver, mesentery, heart
Reference	The present study		Dönges (1964) [6]	Toyooka & Okada (1954) [21]	Toyooka & Okada (1954) [21]		Hughes (1928) [9]
Locality	Yamaguchi Pref., Japan		Germany	Tokushima Pref., Japan	Tokushima Pref., Japan		U.S.A.
State of specimen	Fresh n=10	Mounted n=6	Mounted n=5-9	Fresh n=?	Fresh n=?	Mounted n=5-10	
Cyst size (mm)	0.79-1.40 (1.04 ± 0.18) by 0.54-0.94 (0.69 ± 0.14)		0.80-0.96 (0.89 ± 0.05) by 0.69-0.88 (0.81 ± 0.05)	0.54-0.99 by 0.27-0.68	No cyst (Free in the body cavity)	1.05-1.45 (-) by 0.65-0.75 (-)	
Body length (mm)	0.53-0.90 (0.69 ± 0.13)		0.68-1.08 (0.81 ± 0.15)	0.63-0.81	0.81-1.12	0.56-1.06 (0.73)	
Body width (mm)	0.28-0.54 (0.39 ± 0.08)		0.31-0.42 (0.37 ± 0.03)	0.25-0.37	0.22-0.38	0.30-0.42 (-)	
Forebody length (mm)	0.43-0.75 (0.58 ± 0.11)		0.53-0.81 (0.64 ± 0.11)	—	—	0.30-0.41 (0.35)	
Forebody width (mm)	0.28-0.54 (0.39 ± 0.08)		0.31-0.42 (0.37 ± 0.03)	—	—	0.28-0.44 (0.35)	
Hindbody length (mm)	0.13-0.23 (0.18 ± 0.04)		0.12-0.27 (0.17 ± 0.05)	—	—	0.22-0.33 (0.28)	
Hindbody width (mm)	0.13-0.26 (0.20 ± 0.04)		0.19-0.24 (0.21 ± 0.02)	—	—	0.25-0.38 (0.31)	
Oral sucker (μm)	45-58 (49 ± 4) by 28-53 (39 ± 8)		32-42 (38 ± 4) by 30-46 (38 ± 8)	35-46 by 30-46	35-60 by 35-46	18-28 (20)	
Pharynx (μm)	38-48 (43 ± 3) by 18-33 (24 ± 4)		28-32 (30 ± 2) by 20-30 (24 ± 4)	24-30 by 22-26	24-25 by 28-30	28-39 (33) by 18-28 (21)	
Acetabulum (μm)	33-58 (49 ± 8) by 30-50 (41 ± 7)		38-49 (43 ± 5) by 36-40 (39 ± 2)	45-50	40-48	42-53 (46) by 60-70 (64)	
Holdfast organ (μm)	108-155 (128 ± 16) by 100-145 (129 ± 13)		70-144 (114 ± 25) by 86-120 (106 ± 12)	120-160	120-136 by 140-170	67-88 (74) by 81-105 (95)	
Bursa copulatrix (μm)	— by 54-74 (63 ± 8)		46-80 (63 ± 13)	—	—	53-88 (68)	

* The unit for measurement is shown for each object under microscopic observation.

Fig. 3. A ML phylogenetic tree of members of the families Diplostomidae and Strigeidae based on an alignment of 18S rDNA sequences. Branch support scores calculated by aLRT (see text) and expressed after multiplication by 100 are given at nodes.

Fig. 4. An unrooted ML phylogenetic tree of members of the family Diplostomidae based on an alignment of ITS1–5.8S rDNA–ITS2 sequences. Branch support scores calculated by aLRT (see text) and expressed after multiplication by 100 are given at nodes.

strigeid species from DDBJ/EMBL/GenBank were aligned using the Clustal W multiple alignment program, with subsequent manual adjustment. Regions judged to be poorly aligned and characters with a gap in any sequences were excluded from subsequent analyses. Maximum likelihood (ML) analysis was performed with the program PhyML [5, 7] provided on the ‘phylogeny.fr’ website (<http://www.phylogeny.fr/>). The probability of inferred branch was assessed by the approximate likelihood ratio test (aLRT), an alternative to the non-parametric bootstrap estimation of branch support [2].

For 18S, the newly obtained sequence of *Posthodiplostomum* sp. and 19 taxa of the superfamily Diplostomoidea [15, 16], along with Troglotrema sp. HS-2009 (accession no. AB521803) and *Euryhelminx costaricensis* (Heterophyidae: accession no. AB521797) were analyzed first. When the latter two were used as an out-group for the

construction of a phylogenetic tree, *Holostephanus dubinini* (Cyathocotylidae: accession no. AY245707) branched off first, followed by *Hysteromorpha triloba* (Diplostomidae: accession no. AY245763), and 18 taxa of either Diplostomidae or Strigeidae formed a major clade of Strigeidae. Then, *H. triloba* was used as an out-group for the construction of a phylogenetic tree, using 1,731 characters, of which 239 were variable (Fig. 3). The present *Posthodiplostomum* sp. formed a clade with *P. minimum* (accession no. AY245767) of unknown host origin and strigeid cercariae from a physid snail (accession no. EU371593) [1] from North America. Furthermore, different genera of the family Strigeidae were segregated in two clades of the family Diplostomidae, forming a polyphyletic group. The 18S rDNA sequences of the present *Posthodiplostomum* sp. and *P. minimum* showed a 99.2% identity (1,936/1,951 bp) with no gaps. Metacercariae of the latter species are encysted in the viscera of a wide

range of freshwater fish from North America [8, 9, 17] and are morphologically different from the present species as well as *P. cuticola* (Table 1); e.g., *P. minimum* has distinct oral sucker and acetabulum dimensions, whereas they are comparable in the present specimens and *P. cuticola*.

Two additional phylogenetic trees based on the ITS region including 5.8S rDNA (16 taxa with 746 characters, of which 230 were variable) or 28S rDNA (11 taxa with 1,024 characters, of which 390 were variable) were constructed. Again, the same pattern of clusterings of Diplostomidae genera as in the ML phylogenetic tree based on the 18S rDNA was observed in both ML phylogenetic trees based on the ITS region (Fig. 4) and 28S (data not shown). The precise relationships of members of Diplostomidae might be assessed by the addition of more sequences of congeners of such genera in the future.

Metacercariae of *P. cuticola* have been recorded from the skin, fins, gills or muscles of fish classified in the families Cyprinidae (*Scardinius*, *Abramis*, *Blicca*, *Cyprinus*, *Carassius*, *Leuciscus*, *Phoxinus*, *Alburnus*, *Barbus*, *Capoetobrama*, *Chalcalburnus*, *Squalius*, *Gobio*, *Chondrostoma*, *Pelecus* and *Aspius*), Cobitidae (*Cobitis*), Balitoridae (*Nemacheilus*), Siluridae (*Silurus*), Percidae (*Perca*) and Acipenseridae (*Acipenser*) on the Eurasian continent [19, 24]. To the best of our knowledge, *Channa argus* (Channidae) has not been identified as a host of *P. cuticola*. During the development of *P. cuticola* metacercariae in fish hosts, melanophores surround the parasite cysts in the tissue, leading to clearly recognizable black spots [6]. Due to this characteristic, the disease is known as 'black spot' and major infections are associated with spinal deformities, poor growth and muscle damage. The snakeheads examined in this study exhibited only a few melanophores surrounding the cysts of *Posthodiplostomum* sp. metacercariae. *P. minimum* is a congeneric species in North America [9] causing white spots primarily in the kidneys, liver and heart and occasionally in the spleen, connective tissue of the gut and ovaries [3, 4, 11]. Currently, it is not clear whether the present *Posthodiplostomum* sp. metacercariae do not elicit accumulation of melanophores around the cysts in any freshwater fish or just in snakeheads. This requires examination of freshwater fish other than snakeheads from the Fushinogawa River where this trematode species is endemic.

The present study provides for the first time morphological data and the rDNA sequence of a *Posthodiplostomum* sp. distributed in Japan. It is currently uncertain whether this species is identical to the previously observed *Posthodiplostomum* sp. in crucian carp and common carp in Japan [10, 14]. In addition, the relationship with *P. cuticola*, the major congener on the Eurasian continent, should be clarified.

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