Short communication

The Digenean *Clinostomum complanatum* Found from Great Cormorant *Phalacrocorax carbo* in Japan

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ABSTRACT—Great cormorants *Phalacrocorax carbo* were found dead in a goldfish farm in Aichi Prefecture. They were infected with trematodes in the oral cavity. Based on morphological observation and genetic analysis, the trematodes were identified as *Clinostomum complanatum*. This is the first case reporting *C. complanatum* infection of great cormorants in Japan. Heavy metacercarial infection of goldfish was found in the same fish farm. The metacercariae were shown to be the same species as the adult worms from the bird by molecular analysis. These results suggest that great cormorant is involved in the life cycle of this trematode in farmed goldfish.

Key words: *Clinostomum complanatum*, *Phalacrocorax carbo*, Digenea, goldfish, life cycle

Trematodes of the genus *Clinostomum* are widely distributed parasites in the world (Locke *et al.*, 2015). They are endoparasites with a complex life cycle involving two intermediate hosts and one definitive host. The adult trematodes typically parasitize the oral cavity, pharynx or esophagus of piscivorous birds. The first intermediate hosts are snails, and the second intermediate hosts are freshwater fishes or amphibia. The metacercariae, commonly called yellow grub, form cysts in the body cavity, visceral organs, and muscles of fish (Wang *et al.*, 2017). In rare cases, this parasite occurs pharyngolaryngitis in the pharynx and larynx of humans after eating raw freshwater fish. Nineteen human cases have been reported by 2014 in Japan (Hara *et al.*, 2014).

Within genus *Clinostomum*, only *Clinostomum complanatum* has been reported from the bird and fish

hosts in Japan (Yamaguti, 1933; Shogaki, 1935; Kagei *et al.*, 1988; Aohagi *et al.*, 1992a, 1992b, 1993a, 1993b, 1995; Aohagi and Shibahara, 1994; Sato *et al.*, 2005; Uchida *et al.*, 2005; Nagasawa *et al.*, 2014). However, it is not clear if there is only one species in Japan, because detailed morphological observation or genetic analysis have not been conducted for about 30 years since the finding of adult worms by Kagei *et al.* (1988).

In the winter of 2018, we had received a request on a countermeasure on heavy *Clinostomum* metacercarial infection of goldfish *Carassius auratus* from a goldfish farm in Aichi Prefecture. In this farm, the prevalence of infection was different from pond to pond, with the highest infection of goldfish reaching 90%. We had also found adult worms of *Clinostomum* from cormorants which died in the farm and conducted species identification of the adult worms and metacercariae by morphological observation and molecular analysis.

Materials and Methods

In February 2018, three dead birds were found in a fish farm in Aichi Prefecture. They were identified as great cormorants *Phalacrocorax carbo* (Japanese name: Kawa-u) based on the appearance features (Ministry of the Environment, Government of Japan, 2013). Five adult trematodes were collected from the oral cavity of the cormorants (Fig. 1). The adults were identified as a member of the genus *Clinostomum* based on their morphological features, host, and habitat (Kanev *et al.*, 2002). Many metacercariae were also obtained from goldfish *Carassius auratus* from several ponds in the same fish farm in March 2018. The metacercariae were removed from fresh skin tissue of the fish by precision tweezers. The worms were preserved in 70% ethanol. Two adult worms (one mature and one immature) from the cormorants were gradually flattened between two microscope slides in ethanol, stained with alum carmine and mounted on slides in balsam for morphological observation under a microscope. One mature worm was used for morphological description. One immature worm from the cormorants and one metacercaria from the fish were used for DNA analyses. DNA extraction and PCR protocol followed Nakao *et al.* (2017). Mitochondrial cytochrome c oxidase subunit 1 (*cox1*) was selected as target gene. The primer set was as follows: JB3 and CO1-R trema for *cox1* (Miura *et al.*, 2005). Sequencing was outsourced to Eurofins Genomics Inc. The sequencing alignments were analyzed with software BioEdit Sequence Alignment Editor (Hall, 1999). The sequence datasets for comparison were retrieved from DDBJ/EMBL/GenBank databases.

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Fig. 1. An adult trematode obtained from a great cormorant *Phalacrocorax carbo* (stained specimen). Scale unit: 1 mm.

Results

The morphology of one mature adult worm was as follows. All measurements are in micrometers unless otherwise stated (L: length, W: width). Body elliptical and elongate, slightly narrowed at level of acetabulum, widest at level of ovary and testes, L 4.92 mm × W 1.78 mm. Oral sucker L 340 × W 361. Pharynx present. Intestinal ceca bifurcated and extending to the posterior end of body. Acetabulum positioned in anterior third of body, L 817 × W 892. Two testes lobed and tandem, the anterior one in middle third and the posterior one in posterior one-third of body, almost triangular, anterior one L 500 × W 693, posterior one L 363 × W 775. Ovary oval, intertesticular, on right side, L 249 × W 254. Genital pore opens at the right side of anterior testis. Vitelline glands follicular, begin posterior to acetabulum and extend to intestinal extremity. Eggs elliptical, L 135–140 × W 71–75. After a comparison with known

Clinostomum species (Kagei *et al.*, 1988; Matthews and Cribb, 1998; Gustinelli *et al.*, 2010; Caffara *et al.*, 2011; Rosser *et al.*, 2017; Sereno-Urbe *et al.*, 2018), it is concluded that the present trematodes are morphologically very similar to *C. complanatum*.

The *cox1* sequences (879 bp) of the adult and the metacercaria were identical to each other. Moreover, the partial *cox1* sequences showed 99% (876 / 879 bp) sequence similarities with those of *C. complanatum* deposited on the database (KM923964) (Chen *et al.*, 2016). The present *cox1* sequences have been deposited on DDBJ/EMBL/GenBank databases with accession numbers MH491530.

Discussion

The taxonomy of the genus *Clinostomum* had been complicated. According to recent studies, the genus currently contains 20 described species (Briosio-Aguilar *et al.*, 2018; Rosser *et al.*, 2018; Sereno-Urbe *et al.*, 2018). Twelve out of the 20 species have been validated using a combined morphological and molecular approach. Besides, several putative species recognized solely through DNA sequences are proposed (Rosser *et al.*, 2018; Sereno-Urbe *et al.*, 2018). Among these species, the present worm is morphologically identified as *C. complanatum*. Moreover, *cox1* sequences of the present worms showed high similarities to the reference sequences of *C. complanatum* (KM923964, from *Carassius auratus* in China) (Chen *et al.*, 2016), supporting the identification based on the morphological features.

In Japan, only one species, *C. complanatum* has been reported, and grey heron *Ardea cinerea*, great egret *Ardea alba*, intermediate egret *Egretta intermedia*, little egret *Egretta garzetta* and night heron *Nycticorax nycticorax* are known as final hosts (Shogaki, 1935; Kagei *et al.*, 1988; Aohagi *et al.*, 1992b, 1993b; Sato *et al.*, 2005; Uchida *et al.*, 2005). Yamaguti (1933) obtained adult *C. complanatum* from a heron that was experimentally fed with metacercariae obtained from freshwater fish. As second intermediate hosts, common carp *Cyprinus carpio*, Japanese crucian carp *Carassius cuvieri*, Gin-buna *Carassius auratus langsdorffii*, *Carassius* spp., slender bitterling *Tanakia lanceolata*, rosy bitterling *Rhodeus ocellatus kurumeus*, Japanese dace *Tribolodon hakonensis*, stone moroko *Pseudorasbora parva*, Japanese sea bass *Lateolabrax japonicus* are known in Japan (Aohagi *et al.*, 1992a, 1993a, 1995; Aohagi and Shibahara, 1994; Nagasawa *et al.*, 2014). This is the first case reporting *C. complanatum* infection of great cormorants in Japan. Internal parasites of cormorants have rarely been investigated in Japan, though habitat surveys and extermination of cormorants have been widely implemented due to problems of economic losses to aquaculture caused by

them. *C. complanatum* infections have already been reported from cormorants worldwide (Gibson *et al.*, 2005).

Generally, the metacercariae of *C. complanatum* lay quiescently in the cyst, and no great harm was done to fish unless there were massive numbers (Lo *et al.*, 1985). However, Yasumoto *et al.* (2018) reported mass mortalities of goldfish infected with *Clinostomum* metacercariae, caused by elevating water temperature. Cysts containing *Clinostomum* metacercariae were accompanied by hemorrhage and congestion, and metacercariae were activated and excysted during the high temperature water treatment. In Korea, many encysted metacercariae of *C. complanatum* were found in farmed goldfish. Fish infected with more than 200 metacercariae became moribund with listless swimming and finally died (Kim and Nagasawa, 1996). In Taiwan, the yellow grub parasitizing cultivated ayu *Plecoglossus altivelis*, and loach *Misgurnus anguillicaudatus* was one of the most serious problems in fish farms. Lo *et al.* (1981) and Wang *et al.* (2017) reported the burden due to massive infection by *C. complanatum* metacercariae, such as retarded growth, unusual host behavior, and even death, in ayu, loach and other freshwater fish, with consequent serious economic losses to aquaculture. Lo *et al.* (1981) considered that high mortality could result from numerous lesions caused by excystation of the yellow grub with hemorrhage around cysts in the body wall following treatment of an insecticide for fish lice.

The metacercaria infecting a goldfish in the fish farm was shown to be the same species as the adult worm from the great cormorant by DNA identification based on *cox1* sequences. The results suggest that great cormorants are involved in the life cycle of this trematode in farmed goldfish. In Japan, *C. complanatum* infection in cormorants is considered not to be rare, and the cormorants can also be a source of the infection to farmed fish as well as herons. Therefore, attention and countermeasure to this parasite will be necessary.

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