

Risk of introduction of *Clinostomum complanatum* (Digenea: Clinostomidae) to Sicily through use of *Cobitis bilineata* (Canestrini, 1865) as live baits

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Abstract

Clinostomum complanatum (Rudolphi, 1814) is a digenetic trematode infecting fish-eating birds, reptiles and occasionally humans. Freshwater snails are the first intermediate hosts and many fish species and amphibians are second intermediate hosts. The aim of this study was to report *Cobitis bilineata* as a new intermediate host of *C. complanatum*. In this study, we found infections of *C. complanatum* metacercariae in 12 out of 30 (P= 40%) *C. bilineata* originating from northern Italy. Infected fish had 2 or 3 encysted metacercariae close to the gill operculum and lower jaw. Parasites were removed and identified both morphologically and molecularly using sequences of a mitochondrial gene, cytochrome c oxidase I, and the ribosomal markers. Some cysts extracted from dead fish were processed for histopathological investigations. The risk of the introduction of the parasitosis also in other new areas, such as Sicily, using live baits is discussed.

Introduction

Italian spined loach *Cobitis bilineata* (Canestrini, 1865) is a little fish belonging to the Cobitidae family reaching the maximum length of 6.5 cm and 10.0 cm in males and females, respectively. The natural environment includes northern Italy and Slovenian Adriatic basin, but it is present also in the Zrmanja river basin in Croatia and is native to southern Switzerland (Po/Ticino drainage). It has been accidentally introduced to peninsular Italy and Sardinia Island but not in Sicily, Spain and to an area north of the Alps,

and southeastern France. The habitat includes different freshwater environments such as lakes, rivers, brooks and irrigation channels with still or moderately flowing water, sand, gravel or mud bottom, often with vegetation (Kottelat and Freyhof, 2007). Today this fish is one of the most preferred live baits used for game fishing in fresh water basins. *Clinostomum complanatum* (Rudolphi, 1814) is a digenetic trematode living at the adult stage in the mouth and pharynx of fish-eating birds, mainly Ardeidae, reptiles and occa-

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sionally humans, confined so far to the Palearctic area of the world. The life cycle involves several snail species as first intermediate hosts, while freshwater fish and amphibians serve as second intermediate hosts. This warm-water parasite is typical of subtropical and tropical regions of Africa, Asia, and America. In Northern Europe, the species is sporadically found during the spring in herons coming to breed. In Italy *C. complanatum* was described for the first time in 1934 (Maccagno, 1934) in *Cobitis taenia* and *Ardea cinerea* from Vercelli province (Piedmont region). Lately in 1994, Andreucci et al. described metacercariae of *C. complanatum* from barbell (*Barbus barbus*) and chub (*Squalius cephalus*) and adults from *A. cinerea* and *A. purpurea* all from Bologna province (Emilia-Romagna region). Caffara et al. (2011) described *C. complanatum* coupling the morphological description by molecular characterization of both metacercariae and adults from barbell, chub and fish-eating birds respectively, collected in Emilia-Romagna region, followed by other reports by the same authors (Gustinelli et al., 2012; Caffara et al., 2013). Finally Caffara et al. (2014) described for the first time *C. complanatum* in European newts (*Triturus carnifex* and *Lissotriton vulgaris*) from a protected area in Tuscany.

The parasite's life cycle is confined to warm climate and involves snails like *Lymnaea stagnalis* and *Radix ovata* as the first intermediate hosts, while various freshwater fish (perch, bream) and amphibians serve as second intermediate hosts and piscivorous birds being the definitive hosts (Caffara et al., 2014).

In definitive hosts, pathological study revealed acute inflammation of the submucosa of the oral cavity and esophagus which can lead to impair-

ment of deglutition and malnutrition which in turn can weaken the immune system (Shamsi et al., 2013). Although infections in humans are extremely rare, by eating raw freshwater fish infected by metacercariae of *C. complanatum*, the larvae will excyst in the stomach, migrate through the esophagus, and attach to the throat and mature, causing parasitic pharyngitis or laryngitis. Human laryngitis caused by *C. complanatum* is a rare disease; although there are records in Japan, Korea, and other Asian countries where eating raw fish is a tradition. The dietary changes in European Countries like eating raw fish increases the risk of infestation by this parasite (Kitagawa et al., 2003; Park et al., 2009; Hara et al., 2014).

The aim of this paper is to report *C. complanatum* in *C. bilineata* as a new intermediate host.

Materials and methods

During July 2014, 30 specimens of *C. bilineata*, bought as live bait for fishing, coming from natural fresh water basins in Piemonte (Northern Italy) were brought to the Centre of Experimental fish pathology of Sicily (CISS) located at the Department of Veterinary Science for the presence, in some subjects, of whitish nodules mainly located near the operculum. Live fish were anesthetized with MS-222 using 0.2 mg/L (buffered with 0.4 mg/L of sodium bicarbonate) (Harper and Lawrence, 2011) of water and the nodules were surgically opened in order to extract the parasite. After surgery, antibiotic therapy was administered dissolved in water using enrofloxacin (0.5 mL/3 L). The parasites collected were preserved in 70% ethanol and then clarified in Amman's lactophenol for morphological identification according to Caffara et al. (2011). A little piece of the posterior end of the

same specimens were used for molecular identification amplifying and sequencing the COI mtDNA as described by Caffara et al. (2011). The nodules containing the encysted metacercariae of five fish were fixed in 10% neutral buffered formalin for histopathological studies following routine methods: tissue samples were rinsed in tap water, dehydrated by rinsing in alcoholic solutions, clarified in xylene and finally embedded in paraffin wax. Five μm thick tissue sections were stained with Haematoxylin-Eosin and Masson's modified Goldner trichrome technique and photographed under a light microscope Zeiss axiophot.

Results

Twelve out of thirty (Prevalence = 40%, Mean abundance = 0.8, Mean Intensity = 2) fish showed whitish nodules under the skin (Figure 1). Fish

were 5 to 8 cm in length (mean = 7.2 cm). The preferred site of infection of the parasite was the gill operculum and the lower portion of jaw. Positive specimens had 1 to 3 nodules (mean = 2). Larvae were excised and observed at stereoscope (Figure 2).

The larvae ($n = 10$) had an oral collar. Ventral sucker larger than oral one. Glandular structure was present in forebody, anterior to ventral sucker. Intestinal ceca lateral to ventral sucker and genital complex, with prominent lateral pouches. Testes were smaller than in adults, with more digitations evident. Cirrus sac was well developed; Ovary irregular in shape and, smaller than the cirrus sac. Vitellaria were not evident. Excretory ducts connecting at the posterior extremity in the V-shaped vesicle were strictly juxtaposed to the ceca ends.



Figure 1. *Cobitis bilineata*: macroscopic features of the yellow grub disease.



Figure 2. *Clinostomum complanatum* metacercaria at microscopical examination. Scale bar 1mm.

The morphometrical study (Table 1) enabled identification of the parasites collected to the species *C. complanatum*. BLAST analysis of the sequences obtained (GenBank accession number KU236382) gave 100% identity with *C. complanatum*.

Metacercariae were usually encysted in derma as well as in lower muscular tissue. Each parasite was surrounded by a unique capsule produced by the fish host. In some histological sections some microanatomical characteristics of the parasites were observed, such as oral and ventral suckers, the typical spines on the external surface of the body. The most evident tissue changes were represented by a slight atrophy of the muscle and/or connective tissue surrounding the nodules due to a space occupying mass. Only few lymphocytes were detected at the site of attachment of the parasite, close to the oral sucker (results not shown).

Discussion

The occasional finding of metacercariae of *C. complanatum* in *C. bilineata* represents the first record on this species. In this paper our findings extended the host range of *C. complanatum* in Italy and consequently in Europe. The common second intermediate hosts in our country, restricted to the Emilia-Romagna region, are mainly barbel (*Barbus barbus*) and chub (*Squalius cephalus*) (Caffara et al., 2011, 2013; Gustinelli et al., 2012) and recently European newts (*Lissotriton vulgaris* and *Triturus carnifex*) from Tuscany (Caffara et al., 2014). *C. bilineata* is not a fish of commercial importance for human consumption, but it's role as intermediate host of *C. complanatum* could contribute to the distribution of this parasite even in areas outside of the common distribution range recorded so far.

Table 1. Measurement of ten metacercariae after surgical removal from *Cobitis bilineata*. Length and width of oral sucker, length and width of ventral sucker and distance between suckers

ID Metacercaria	Body length/ width (mm)	Oral sucker length/width (μm)	Ventral sucker length/width (μm)	Distance between suckers (μm)
1	4.9/2.6	262/1083	707/803	887
2	5.6/3.1	289/1155	781/833	923
3	6.3/3.5	301/1266	801/845	998
4	6.8/3.9	305/1352	828/897	1028
5	5.8/3.3	293/1163	778/866	922
6	6.1/3.3	299/1232	796/838	982
7	5.5/2.9	283/1132	764/822	918
8	4.7/2.5	260/1036	742/801	872
9	7.1/4.2	312/1324	851/932	1,042
10	6.9/4.1	309/1287	835/912	1,033
Mean	5.97/3.3	291.3/1099	788.3/854.9	960.5

All the metacercariae isolated from *C. bilineata* were mainly encysted in muscular tissue, but also in the areas under the mouth and behind the operculum. The histopathological observations confirmed the low tissue damages due to the presence of *C. complanatum* in second intermediate hosts. Tissue changes included atrophy of surrounding tissues due to the protracted pressure depending by the presence of the cyst. Only rarely cellular infiltration was demonstrated in or close to the sites of attachment of the parasites as a result of immune reaction (results not shown). It could be interesting to study the early phase of the encapsulation of the cercariae in the host tissue to better clarify the impact on the immune system and its involvement.

Fish eating birds have not been checked to date in Sicily for parasites. Thus, maybe this study can form a platform for studying the parasites

of birds to see if *Clinostomum* has already established the life cycle or not in southern Italian regions. Considering that if the *Cobitis* fish are released into the natural habitat, and if fish eating birds are present, its life cycle could be established and have harmful implications for other endemic fish.

Our report of *C. complanatum* metacercariae in *C. bilineata* represents an important finding considering that the diffusion of the fish species, used as live bait particularly for many wild carnivorous freshwater fish, such as perch, bass, pike, etc., could increase the distribution of the parasitosis in freshwater basins throughout Europe in general, but also in Sicily where the parasite has never been described to date.

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