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Title: Eustrongylides excisus (Nematoda: Dioctophymatidae) in big-scale sand smelt (Atherina boyeri) from the lake Massaciuccoli (Northwest Tuscany, Italy): epidemiological findings, potential public health impact and implications for seafood quality

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Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

Eustrongylides excisus (Nematoda: Dioctophymatidae) in big-scale sand smelt (*Atherina boyeri*) from the lake Massaciuccoli (Northwest Tuscany, Italy): epidemiological findings, ~~and~~ potential public health impact and ~~-~~implications for seafood quality

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Abstract

Eustrongylides spp. are cosmopolitan parasitic nematodes with several freshwater fish species as intermediate or paratenic hosts, rarely reported as zoonotic agents. This work aims to report, for the first time, the occurrence of this parasite in specimens of big scale sand smelt (*Atherina boyeri*, Risso 1810) caught in the lake Massaciuccoli (Northwest Tuscany, Italy). ~~In addition, the occurrence of the highly invasive cestode *Schyzocotyle* (syn. *Bothriocephalus*) *acheilognathi*, is reported.~~ Totally 3317 specimens of *A. boyeri* were collected in three different samplings (August-October 2019). Fishes were visually examined and then submitted to artificial digestion. Visible parasites were collected and counted after both procedures, then they were identified to genus level by microscopic examination and to species level by molecular analysis of the ITS gene region ~~for nematodes and of the 28S for cestodes~~. Overall, 75 nematodes identified as larval stages of *Eustrongylides excisus* (Jägerskiöld, 1909) were found (P: 2.3% 95% CI 1.8-2.8; MA: 0.02; MI: 1), in the viscera and in the muscle. ~~For *S. acheilognathi* only the MA (0.036) could be calculated due to partial abdomen rupture of fish.~~ *A. boyeri* is a highly appreciated fish species in several Italian areas as well as in various Mediterranean countries. Beside an epidemiological interest due to its role in the parasite cycle, the report of *E. excisus* in the study area arises possible quality and public health issues. In fact, although the involvement of *E. excisus* in human cases has not yet been proven, further molecular data are needed to assess the zoonotic potential of the single species of the genus. Therefore, also considering the method of preparation of *A. boyeri* and that lately the increasing consumption of raw fish has also targeted freshwater fish species, it is essential to communicate the presence of this hazard and the need of applying appropriate sanitary measures to all FBOs along the supply chain and consumers.

Keywords

Waterborne zoonoses; freshwater ecosystems; ~~fish;~~ visible parasites; nematode; seafood inspection~~; Cestode; freshwater ecosystems~~

1.Introduction

The genus *Eustrongylides* includes parasitic nematodes of the family Dioctophymatidae that have been reported worldwide (Guagliardo et al., 2019; Mazzone et al., 2019; Metin et al., 2014; Xiong et al, 2013). These parasites have an indirect life cycle, with adults typically inhabiting the mucosa of the esophagus, proventriculus or intestine of freshwater piscivorous birds (definitive hosts). The first-stage larva develops in the egg passed in the faeces by infected birds and is ingested by aquatic oligochaetes, acting as first intermediate hosts. Here the parasite develops into the second and third larval stages, which are infective to the second intermediate hosts, planktivorous and benthivorous fishes in which the third-stage larvae moult into the fourth stage and remain~~ in the fish~~ until ingestion by the definite hosts (Measures, 1988a; Spalding and Forrester, 2008; Spalding et al., 1993).~~ Coyner et al. (2003), however, were able to~~ Successful direct fish infection ~~fish directly~~ with larvated eggs of *Eustrongylides* ~~*E.*~~ *ignotus* (Jägerskiöld, 1909), without development in oligochaetes, has been also reported (Coyner et al. (2003). Predatory fish species consuming infected fish may serve as paratenic hosts (Goncharov et al., 2018; Measures, 1988a), a role that can also be taken by amphibian and reptile species (Bjelić-Čabrilo et al., 2013; EFSA, 2007).

Eustrongylides spp. have attracted considerable attention due to their wide geographical distribution and great potential for transmission and pathogenicity (Xiong et al., 2009). Death of nestlings, especially for herons, but also for other wading bird species, has~~ve in fact~~ been reported (Spalding and Forrester, 1993; Spalding and Forrester 2008). Furthermore, ~~the~~ fish infection~~ of fishes~~ may result in pathological alterations (Guagliardo et al., 2019), and even in behavioural changes favouring predation ~~have been described~~ (Coyner et al., 2001).

Even though ~~T~~the genus *Eustrongylides* is ~~also~~ listed ~~together~~ with other parasites characterized by a potential public health impact, including other nematodes ~~(~~, ~~among other nematodes (~~*~~Anisakis~~*

spp., *Pseudoterranova* spp., and *Gnathostoma* spp.), as well as cestodes (*Diphyllobothrium* spp.), and trematodes (*Clonorchis sinensis*, *Opisthorchis* spp., *Heterophyes* spp., *Metagonimus* spp., *Nanophyetus salmincola*, and *Paragonimus* spp.) as a seafood parasite of concern (Food and Drug Administration, 2012; 2019), because it can is only be responsible for rare zoonotic infections (Eiras et al., 2018). To date, at least five cases have been described in the USA and two in South Sudan. In the USA, the infection was generally associated to the consumption of live minnows by anglers, but with the exception ofin one case which it was attributed to domestically prepared sushi. All subjects showed sSevere abdominal pain developed within 24 hours after the ingestion of contaminated fish, following penetration of the worm into the gut wall. Four of the five cases reported from the USA required surgery, during which worms were removed from the abdomen (Table 1 and cited references).

Thus, public health issues may arise, especially considering that *Eustrongylides* larvae are found not only in the viscera but also in the flesh of a wide variety of fish (Food and Drug Administration, 2012). Even though the *Eustrongylides* larvae are clearly visible, also due to their pink-red colour and dimension, and thus more easily removed/discarded by Food Business Operators (FBOs), tThe correct application of the preventive procedures required by the European and Italian legislation for managing the parasitological risk in seafood (D'Amico et al., 2014) is extremely important also for freshwater fish species. In fact, these fish could have an important role in the transmission of zoonosis, not only in countries where they are largely consumed (Bjelić Čabrilo et al., 2013) but also in lake areas in countries where their consumption is tied to local tradition (Pozio et al., 2013; Scaramozzino et al., 2018). In particular, a correct freezing treatment should be applied to products intended for raw consumption, also considering the implication that changes in human food habits can have in the transmission of fish borne zoonosis (D'Amico et al., 2014; Pozio et al., 2013; Scaramozzino et al., 2018; Scholz et al., 2009). For example, over 200 confirmed cases of opistorchiasis occurred from 2003 to 2011, due to the consumption of raw fillets of tench (*Tinca tinca*) fished from two lakes, Bolsena and Bracciano, located in central Italy (Pozio et al., 2013).

~~Similarly, an increase in reports of diphyllobothriosis has been described due to the consumption of raw or undercooked perch (*Perca fluviatilis*) (Scholz et al., 2009).~~

To the best of our knowledge, no human infection by *Eustrongylides* spp. has been described in Italy so far. However, its occurrence was reported in edible and commercially exploited fish species and also in the great cormorant, a definitive host, from lake Trasimeno (Agnetti et al., 2016; Branciari et al., 2016; Dezfuli et al., 2015; Mazzone et al., 2019) and recently in fish in Piedmont (Menconi et al., 2020). Studying its presence in other Italian lakes ~~may~~ contributes to understand its epidemiology, as well as to evaluate the need for appropriate monitoring and sanitary measures (Agnetti et al., 2016), also considering the increased consumers and media attention towards parasites in seafood. In fact, beside public health concerns, the presence of visible parasites, such as *Eustrongylides* spp., makes fish products unfit for human consumption (Reg. EC No 178/2002), and has an obvious impact on their marketability (Branciari et al. 2016; Mazzone et al., 2019).

The big-scale sand smelt *Atherina~~.~~ boyeri* (Risso, 1810) is an autochthonous euryhaline species (ARPAT, 2008) of the lake Massaciuccoli (Northwest Tuscany). Other than representing a forage species for many fish and bird species inhabiting the lake (https://lago-massaciuccoli.webnode.it/la-fauna/) this small fish is among the most commercially appreciated edible species in this area (Alessio et al., 1997; ARPAT, 2008), and it is also consumed in other Italian regions and European countries (Çolak, 2013; ISTAT, 2001; Lorenzoni et al, 2015; Maci and Basset, 2010). The aim of this work is to report the occurrence of *Eustrongylides* spp. in specimens of *A. boyeri* caught in the Massaciuccoli lake, to update the nematode geographical distribution and to discuss the potential related potential public health ~~and commercial~~ and implications for seafood quality. ~~In addition, the occurrence of the highly invasive cestode *Schyzocotyle* (syn. *Bothriocephalus*) *acheilognathi* is reported.~~

2. Materials and methods

2.1 Sampling

A total of 3317 specimens of *A. boyeri* were collected from the central area of the Massaciuccoli lake (Lucca, Northwest Tuscany) in three different samplings (August 2019: 1150 specimens, September 2019: 1197 specimens, October 2019: 970 specimens). The specimens were immediately frozen and then transferred to the Laboratory of fish pathology, Experimental Zooprophylactic Institute of Lazio and Toscana (local unit of Pisa), where they were stored at -20 °C until the parasitological analysis~~, which were performed at the FishLab, University of Pisa~~.

2.2 Parasitological analysis

Each batch of samples was registered with an internal unique code and weighed. The fishes were then counted and visually inspected (Commission Regulation EC No 2074/2005) to search for visible parasites (parasites longer than 10 mm or 3 mm when encapsulated, Codex Alimentarius Commision, 1971): the ventral surface of each fish was opened longitudinally ~~starting from the abdomen~~ to examine the visceral cavity, in good lighting conditions. Considering the small size of the examined fish species, this procedure allowed to fully open each specimen and thus to examine the translucent muscle tissue. Visible nematodes were collected, rinsed in saline, counted and microscopically identified (Nikon Eclipse E 200) to the genus level, following Mazzone et al., (2019), Measures (1988a) and Panesar and Beaver (1979). In addition, t~~T~~he samples were ~~then~~ digested using Trichineasy® (CTSV srl Brescia), ~~following~~ according to the operating manual (http://www.ctsv.biz/image-ctsv/PDF/TrichinEasy-anisakis.pdf). A maximum of 200 g of tissue was digested per time. The digested material was then filtered and examined to verify the presence of further visible nematodes. ~~. Visible nematodes parasites detected during visual inspection (nematodes and cestodes) and after the digestion (only nematodes) were collected and microscopically identified (Nikon Eclipse E 200) to the genus level, following Mazzone et al., (2019), Measures (1988a) and Panesar and Beaver (1979) for nematodes, Choudhury et al. (2006), Poll and Chub (1985) and Scholz (1997) for cestodes. The~~ All the collected parasites were ~~counted and~~ stored in 70% alcohol at 4°C for subsequent molecular analysis.

2.3 Molecular identification.

155 *2.3.1 Total DNA extraction and evaluation.* Total DNA extraction and quantity and quality
156 evaluation were conducted on a subsample ~~(~~of 30 nematodes ~~and 20 cestodes) of the collected~~
157 ~~parasites~~, following the procedure in Guardone et al. (2016).
158 *2.3.2 ITS region amplification.* For nematodes, a fragment of about 900-bp of the ITS-1 region,
159 the 5.8S gene and the ITS-2 region plus approximately 70 nucleotides of the 28S gene (ITS), was
160 amplified using the primers NC2 and NC5 (Zhu et al., 1998). PCR amplifications were set up in a
161 20 µl reaction volume containing 4 µl of a 5× buffer (biotechrabbit GmbH, Berlin, Germany), 200
162 µM of each dNTP (dNTPmix, EurocloneS.p.A-Life Sciences Division, Pavia, Italy), 250 nM
163 primers, 2.5 U Taq DNA Polymerase (biotechrabbit GmbH, Berlin, Germany) and 1–2 µl of DNA
164 (50-100 ng/ µl) and DNase free water (Water Mol. Bio. Grade, DNase-RNase and Protease free,
165 5Prime GmbH, Hamburg, Germany) with the following cycling program: 95 °C for 3 min; 40
166 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 75 s; and final extension at 72 °C for 10 min. PCR
167 products were analyzed by electrophoresis in 2% agarose gel.
168 ~~*2.3.3 D2 region of the large subunit (LSU) ribosomal DNA.* For cestodes, a 780 bp fragment of~~
169 ~~the variable D2 region of the LSU rDNA gene was amplified with the primer pair TrypFOR1 (5'-~~
170 ~~AGTCGGGTTGTTTGAGAATG 3') and TrypREV (5' CGTGTTTCAAGACGGGTC 3'),~~
171 ~~designed and routinely used in FishLab for cestode species identification. PCR amplifications were~~
172 ~~set up in a 20 µl reaction volume, as described above, with the following cycling program: 95 °C for~~
173 ~~3 min; 35 cycles at 95 °C for 25 s, 50 °C for 25 s, 72 °C for 35 s; 72 °C for 5 min. PCR products~~
174 ~~were analyzed by electrophoresis in 2% agarose gel.~~
175 *2.3.4 Sequencing and post sequencing analysis.* Amplicons presenting the expected length were
176 forward and reverse Sanger sequenced ~~at the Experimental Zooprophylactic Institute of Lazio and~~
177 ~~Toscana (local unit of Pisa)~~. The obtained sequences were analyzed, edited and assembled with
178 Geneious R7 software (Kearse et al., 2012) and compared with sequences deposited in GenBank
179 using the Basic Local Alignment Search Tool (National Center for Biotechnology Information
180 database). ~~In addition, t~~The Maximum Likelihood method and Kimura 2-parameter model (Kimura,

181 1980) with 1000 bootstrap re-samplings were used to produce a tree in MEGA-X (Kumar et al.,
182 2018) using five sequences randomly selected among those produced in this study (as they were all
183 identical, see Section 3) and the sequences deposited as *E. excisus* (Jägerskiöld, 1909) and
184 *Eustrongylides* sp. already selected in the ML analysis by Mazzone et al., (2019). The sequences
185 produced in this study and used for the phylogenetic analysis were deposited in GenBank as
186 *Eustrongylides* aff. *excisus* (MT415236-MT415240).

187 ***2.4 Statistical analysis***

188 The prevalence (P) (and 95% confidence intervals - CI), mean abundance (MA) and mean
189 intensity (MI) were calculated according to Bush et al. (1997).

190 **3. Results**

191 Overall, 75 nematodes morphologically identified as larval stages of *Eustrongylides* sp. were
192 found (P: 2.3% 95 CI 1.8-2.8; MA: 0.02; MI: 1) with visual inspection. No additional nematode was
193 found after the digestion. Details of the results for the different batches are reported in Table 2. The
194 larvae were variously located in the host body, most of them were in the viscera (Fig. 1a) while
195 others had invaded the muscle. Some of these, given the transparent aspect of the fishes and its
196 small dimension, were even visible from an external examination (Fig. 1b). Assignment to this
197 genus was done considering that collected worms were pink-red, 4.5 cm long on average (range 3-
198 5.5 cm) and 0.5-1 mm wide (Fig. 1c-d). The cephalic extremity presented a small oral cavity
199 surrounded by 12 cephalic papillae of similar size arranged in two concentric rings (Fig. 2a). The
200 development of the genital primordia at the posterior extremity allowed identification of the L4
201 larvae as male and female (Fig. 2b-c) (Mazzone et al., 2019; Measures, 1988a). While
202 morphological species specific identification of larval stages is not possible (Mazzone et al. 2019),
203 molecular identification was achieved, as the BLAST analysis of all the 30 obtained sequences
204 retrieved 100% of identity with sequences of *E. excisus* from the Trasimeno lake produced in the
205 recent study of Mazzone et al., (2019). In addition, 100% of identity was also obtained with
206 sequences of *Eustrongylides* spp. from Iran (1 sequence - KU963206, unpublished reference) and

China (Xiong et al., 2013). Very high identity values (99.75-99.87%) were also retrieved with 1 sequence of *Eustrongylides* spp. from Turkey (MK007967, unpublished reference) and with other sequences from China (Xiong et al., 2013). The ML tree produced confirmed the BLAST analysis, as the five sequences produced in this study clustered with the sequences of *E. excisus* (Mazzone et al., 2019), the sequences of clade 3 of Xiong et al. (2013) and the sequences of *Eustrongylides* sp. from Iran and Turkey (Fig. 3).

~~In addition, during the visual inspection, cestodes 6-10 cm long, with a flattened fleshy unarmed scolex almost spherical, with two bothria and an antero laterally directed narrow slit like opening were found (Fig. 4). Due to the fact that several of them were found externally of the hosts following partial abdomen rupture occurred during defrosting, only the MA and not P and MI rates could be calculated. The overall MA was 0.036 (0.032 in August, 0.037 in September 2019 and 0.040 in October). The cestodes were identified as *Schyzocotyle* (syn. *Bothriocephalus*) sp. based on their morphological characteristics. Specific identification was later achieved by molecular analysis, as all the 28S sequences produced in this study (MT416078-MT416082) retrieved percentage of identity above 99.5% with sequences of *S. acheilognathi* deposited in GenBank. Details of the molecular results are reported in Table 1SM.~~

4. Discussion

4.1 Eustrongylides excisus in A. boyeri from the lake Massaciuccoli (Northwest Tuscany): epidemiological findings and public health impact and implications for seafood quality

The genus *Eustrongylides* was created by Jägerskiöld in 1909. Karmanova (1968) recognized 14 species, later revised by Measures (1988b) who established only three species (*E. tubifex*, *E. ignotus* and *E. excisus*) as valid, which were distinguished according to differences in the labial papillae of adults and in the caudal extremity of adult males (Measures 1988b), while, as mentioned, larvae do not show specific morphological features (Mazzone et al., 2019; Xiong et al., 2013). However, only *E. ignotus* and *E. excisus* are listed as valid in WoRMS (WoRMS, 2020). The larvae collected in this study were molecularly identified as *E. excisus* thanks to the recent work of Mazzone et al.,

(2019) in which, for the first time, adult and larval stages of this species were morphologically and molecularly characterized. ~~In particular, o~~Our results support the hypothesis suggested by Mazzone et al., (2019) that some of the sequences from China deposited as *Eustrongylides* sp. by Xiong et al. (2013) were in fact *E. excisus*. Interestingly, these molecular outcomes seem to concord with the geographical distribution across Europe and Asia reported by Measures (1988b) for this species ~~*E. excisus*~~. However, further molecular studies are needed to elucidate the geographical distribution, as well as the epidemiology and the taxonomical classification (Abe, 2011).

4.1.1 Epidemiological findings. A. boyeri plays a role as second intermediate hosts in *Eustrongylides* sp. cycle ~~as it represents a second intermediate hosts~~ (Branciari et al., 2016). It is a generalist and opportunistic carnivore, preying copepods in deep waters and benthic fauna in shallow waters (Vizzini e Mazzola, 2005). The species is indigenous of the lake Massaciuccoli, that is part of the Massaciuccoli basin, extending over 9500 hectares flanked by the Tyrrhenian Sea and the Apuan Alps, between the cities of Pisa, Lucca and Viareggio. The lake falls within the territory of the Migliarino, San Rossore, Massaciuccoli Regional Natural Park (Spandre and Meriggi, 1997). Here this species is known to feed also on zooplankton and zoobenthos, including oligochaetes (Alessio et al., 1997), which act as first intermediate hosts. ~~It has been highlighted that p~~Proliferation of oligochaetes, due to anthropic alteration of the environment, may have an effect on the presence of *Eustrongylides* sp. in fish, even provoking epizootic phenomena (Coyner et al., 2002; Spalding et al., 1993).

The prevalence found in this study for *A. boyeri* (2.3%, 95% CI 1.8-2.8%) is higher than the rate reported for the Trasimeno lake (0.13%) ~~by Branciari et al., (2016)~~, where the nematode was found in only one of the 764 analysed samples (Branciari et al., 2016). On the contrary, in another study o~~i~~n the Trasimeno, *A. boyeri* was found to be the most parasitized among the several fish species analysed (Agnetti et al., 2019). As regards other countries, this fish species was only investigated in Turkey, where a prevalence of 6.6% in Iznik lake was found (Çolak, 2013). As for other epidemiological parameters, the mean intensity (MI) of 1 found in our study can probably be

~~probably~~ related to the small size of the big scale sand-smelt, as ~~(~~the mean total length is about 8-10 cm ~~, as also observed by~~ (Çolak, 2013; Lorenzoni et al., ~~(~~2015~~) and Çolak, (2013~~). It agrees with the MI values reported for *A. boyeri* (Çolak, 2013) and for other species of similar ~~size~~ (Brugni and Viozzi 1999; Centers for Disease Control, 1982; İnnal et al., 2019). The parasite localization both in the viscera and in the muscle also agrees with previous studies on *A. boyeri* (Branciari et al., 2016; Çolak, 2013). In addition, as already described by Agnetti et al. (2019), a few (n=2) worms were found partially out of the fish indicating *post-mortem* migration.

The big scale sand smelt has a central role in structuring food webs, being able to fill empty niches in the ecosystem and thus ~~representing~~ acting as a link between different trophic levels (Partal et al., 2019). In particular, *A. boyeri* is a forage species, representing a natural prey of piscivorous fish species including perch, pike, largemouth bass, sun bass and royal perch, as well as numerous ichthyophagous birds (Mantilacci et al., 1990; Moretti et al., 1959). Fish-eating birds, especially migratory species, may play a relevant role in spreading pathogens, including parasites like *Eustrongylides* (El-Dakhly et al., 2012; Švažas et al., 2011). Thus, considering that many fish and birds species acting as paratenic and definitive host for *Eustrongylides* spp. are present also in the Massaciuccoli Lake (https://lago-massaciuccoli.webnode.it/la-fauna/), further investigations on the parasite occurrence in the other fish species inhabiting this freshwater environment ~~would be~~are needed. In this respect, it is worth noting that parasitological surveys conducted in 2010-2012 on different fish species (Macchioni et al., 2015a; Macchioni et al., 2015b) did not find the parasite. On the contrary, the presence of *Eustrongylides* sp. in other predatory fish in Italy was first described in the muscle of 31 European perch (*P. fluviatilis*) out of 510 examined from the Trasimeno lake (Dezfuli et al., 2015). This species was found positive also in the study of Branciari et al. (2016) (P 6.84%) and Agnetti et al. (2016) (P 16.9%). In addition, its presence in the same fish species was also informally reported from lakes Ceresio (https://www.tio.ch/ticino/attualita/1397786/trovato-un-parassita-nel-pesce-persico-del-ceresio) and from lake Montorfano (https://www.parcovallelambro.it/news/presenza-parassiti-nel-pesce-del-lago-montorfano) in

Lombardy, North Italy. Recently, Menconi et al. (2020) described the presence of *Eustrongylides* sp. in *Lepomis gibbosus* (P 18.3%), *Micropterus salmoides* (P 16.7%), and in *P. fluviatilis* (P 10%), while no parasites were detected in the other five fish species analysed (*Ameiurus melas*, *Ictalurus punctatus*, *Squalius cephalus*, *Carassius carassius*, and *Scardinius erythrophthalmus*) in a subalpine lake in Piedmont. Interestingly, several reports of *Eustrongylides* spp. in predatory fish species such as perch and pike perch are available from East Europe, Middle East and the Russian area, where these species are commonly consumed. High prevalence values (58-100%) have been found in European perch, pike perch and pike in Ukraine (Goncharov et al., 2018), as well as in perch and in pike perch in in Turkey (Metin, 2014; Soylu, 2013). A 14% prevalence rate was also found in the muscle of pike-perch in Serbia (Bjelić-Čabrilo et al., 2013).

4.1.2 Public health impact and implications for seafood quality. In the past few decades, there has been growing attention towards *Eustrongylides* spp. nematodes as zoonotic agents (Mazzone et al., 2019). In fact, even though responsible for rare infections (Table 1), this genus is worldwide distributed, and larval stages have been found in the flesh and viscera of a wide variety of fish (Food and Drug Administration, 2012). To better characterize the potential pathogenic impact *Eustrongylides* spp., it is worth mentioning the ability of the nematode from the Dioctophymatidae family to infect laboratory animals, such as rabbits, causing serious pathological alterations of the gastrointestinal tract (Barros et al., 2004; Shirazian et al., 1984).

Other than in the big scale sand smelt and in the previously mentioned predatory species (see section 4.1.1) *Eustrongylides* spp. have been reported in species of great commercial appeal, such as the Persian sturgeon (*Acipenser persicus*) in the Caspian Sea (Noei et a., 2015), brown trout (*Salmo trutta*) in Norway (Haugen et al., 2008), tilapia (*O. niloticus*) (Bekele & Hussien, 2015), kutum (*Rutilius frisii*) (Mohammad et al., 2011) and various species of African catfish (*Clarias* spp.) in the African continent (Ibiwoye et al., 2004). Even though freshwater fish, together with crustaceans and cephalopods, represent only 5% of the EU production (EUMOFA, 2019), they may have a local commercial appeal (Pozio et al., 2013; Scaramozzino et al., 2018; Scholz et al., 2009). Together

311 with eel, carp, pike and prawns, *A. boyeri*, commonly known as "*latterino* or *aquadella*" in Italy
312 (Ministerial Decree MIPAAF n. 19105 of September the 22nd, 2017) and as "*crognolo*" in Tuscany
313 (https://agroalimroma.it/latterino-o-lattarino/), is among the most appreciated species in the
314 Massaciuccoli area (ARPAT, 2008)., ~~where, however, c~~Currently it cannot be exploited ~~due~~ as a
315 ~~fishing~~ ban for fishing in the lake exists (Ordinanza N. 32 del 20.07.2007 and N. 27 del
316 22.07.2011), issued ~~following~~ due to the presence of microcystins ~~contamination~~ (Bruno et al.,
317 2009). However, illegal fishing activities are known, and~~. In addition,~~ the Massaciuccoli basin in
318 well connected to neighbouring water/coastal areas were *A. boyeri* is fished (authors' note).
319 Furthermore, *A. boyeri* is one of the main commercial species in Mediterranean coastal lagoons
320 (Cataudella et al., 2014) and of high appeal in Italian areas facing waterbodies that host this species
321 (Lorenzoni and Ghetti, 2012). In the Trasimeno lake it represents more than 50% of the total catch
322 and it is one of the main source of fishing revenues (Lorenzoni et al., 2015). This species also
323 account for a large majority of fresh water species total catches in Emilia Romagna and Veneto
324 (https://www.venetoagricoltura.org/upload/File/osservatorio_economico/PESCA%20IN%20NUME
325 RI/Pescainnumeri_06.pdf) and it is frequently caught also in the area around the lakes Bolsena
326 (Latium) and Lesina (Apulia) (Scaramozzino et al., 2018;
327 http://www.regione.puglia.it/documents/10192/37569966/Delibera+-+2152+2018+-
328 +documento+2.pdf). In addition, it is commercially exploited in several Mediterranean countries,
329 such as Croatia, Greece, Spain and Turkey (Atalay et al., 2017; Lorenzoni et al., 2015; Maci and
330 Besset, 2010 and references therein).
331 *Eustrongylides* spp. larvae (L4) are clearly visible due to their dimension (3-5 cm) and their
332 pink-red colour. In addition, they are normally very active after the death of the fish. Their presence
333 can have implications for seafood quality determininge a negative impact on the marketability of
334 fish as already happened in the past, when outbreaks in Romania in 1927 decreased the value of
335 commercial freshwater fish from lakes, (Spalding and Forrester, 2008). Fish processing, such as
336 evisceration, trimming, and preparation, in particular the type of cooking, can greatly influence the

risk of transmission to humans if preventive measures for ensuring the safety of freshwater fish products are not properly applied. *A. boyeri*, due to its small dimension, is usually consumed whole without evisceration. This habit can greatly increase the risk of transmitting the infection considering that, other than fried (~~http://www.carlozucchetti.it/latterini-del-lago-di-bolsena-fritti-e-passati-pe-locchio/; https://www.agraria.org/pesci/cucina_latterino.htm~~), this species is also frequently consumed marinated (~~https://tanteideeincucina.altervista.org/latterini-marinati-in-aceto/?doing_wp_cron=1585500089.6598529815673828125000; https://www.campagnamica.it/2020/02/07/l-lattarino-piccolo-pesce-grandi-qualita/~~). While in the first case the temperature reached during frying should allow the parasite to be inactivated (Food and Drug Administration, 2012; 2019), the marinating process probably does not ensure to obtain safe products as for other nematodes. ~~For example, it is well known that marinated anchovies are the main responsible of human anisakiasis (Guardone et al., 2018). This kind of preparation could be particular hazardous, also in the light of the high prevalence found in this and other studies for *A. boyeri*.~~

Data on the occurrence and distribution of *Eustrongylides* sp. in fish species of commercial interest that could have implications for public health and seafood quality are of great interest especially considering the increasing tendency of raw or undercooked fish consumption. In fact, due to the growing interest of Western consumers for both exotic tastes and "lightly preserved" seafood products (Bestor, 2000) also freshwater fish have been recently used for these kind of recipes ~~included~~. For example, the consumption of raw fillets ~~of common whitefish (*Coregonus lavaretus*), but also~~ of tench (*Tinca tinca*) at home, in small restaurants along the shores of lakes, and at gastronomic events, which has lately become popular~~. As mentioned, this new habit~~ has ~~caused~~ an outbreak of opistorchiasis with over 200 confirmed cases between 2003 and 2011 (Pozio et al., 2013; Scaramozzino et al., 2018).

Food Business Operators (FBOs) should conduct a visual inspection of fishery products to avoid ~~hazard~~ risk to human health (Branciari et al., 2016; Menconi et al, 2020). However, this practice

does not guarantee that the product is completely safe and the EU legislation (Commission Regulation (EU) No 1276/2011) states that fish intended to be eaten raw or almost raw, or products submitted to processing unable to devitalize the larvae, have to undergone a preventive freezing treatment. In addition, ~~i~~In Italy, fishmongers must inform consumers on correct domestic freezing by displaying a note at the retail level (Decree of July 17, 2013 of the Italian Ministry of Health).

All this considered, educating FBOs and the general public about the risks of eating raw or undercooked fish, and the need to intensify surveillance of fish is advised, also in areas surrounding lakes (Scaramozzino et al., 2018). In particular, anglers, restaurateurs and final consumers should be correctly informed also around the lake Massaciuccoli, considering that this hazard had not been highlighted before (Macchioni et al., 2015a; Macchioni et al., 2015b). This is important also considering that the Regulation EC No 852/2004 does not apply to the direct supply of small quantities of primary products from the producer to the final consumer or to local retailers directly supplying the final consumer. Consumers education should also aim to explain that parasite may naturally occur in wild seafood products in order to avoid excessive and unnecessary alarmism.

~~***4.2 Schyzocotyle acheilognathi in A. boyeri from the lake Massaciuccoli***~~

~~*Schyzocotyle* (syn. *Bothriocephalus*) *acheilognathi*, commonly called the Asian fish tapeworm, is a bothriate cestode mainly parasitizing cyprinid fish (Salgado-Maldonado and Pineda-López, 2003; Kuchta et al., 2018). Its life cycle is indirect: the adult worms live in the intestine of fish that act as final hosts and acquire the infection by eating a copepod (intermediate host) parasitized by ingestion of the coracidium larvae hatched from the cestode eggs expelled through the faeces of the parasitized fish (Salgado-Maldonado and Pineda-López, 2003). *S. acheilognathi* represents a significant, commercially important fish pathogen with a global impact (Brabec et al., 2016) as it can cause serious damage in fry and small fish and massive kills in fish farms. However, it can also have pathogenic effects in wild fish species. Pathology is usually associated with high parasite intensities (Salgado-Maldonado and Pineda-López, 2003) or with newly acquired host species (Kuchta et al., 2018).~~

Like its original host, the carp *Cyprinus carpio*, this parasite is believed to be native to the Amur River Basin in China. Until the end of the 1950s it occurred only in East Asia, but, following the export of common carp (*C. carpio*) and grass carp (*Ctenopharyngodon idella*) it has been distributed on every continent, except Antarctica. The rapid expansion of *S. acheilognathi* in many countries was mainly caused by poor preventive measures and veterinary control during fish transfer (Dove and Fletcher 2000; Salgado Maldonado and Pineda López, 2003). In addition, this parasite performance as an invader is favoured by its ability to colonize depauperate helminthic communities with many vacant niches (Salgado Maldonado and Pineda López, 2003).

This highly invasive parasite is not only unique in its extraordinary wide geographical distribution (Yera et al., 2013), but also in the wide host spectrum that make this species probably the most successful invasive metazoan parasite worldwide, as it has been reported from over 300 freshwater fish species, belonging to 38 families and 14 orders (Kuchta et al., 2018). Its presence in *A. boyeri* is not exceptional, as it has been reported in this species in Turkey (Çolak et al., 2013; Öztürk, T., & Özer) and reports from the Atherinidae family account for 4% of the global cases (Kuctha et al., 2018).

Most of the global records are from North America, followed by Asia and Europe (Kuchta et al., 2018). In Italy the first report of this cestode occurred in 1980's in a carp (*C. carpio*) fish farm near Lake Trasimeno (Minervini et al., 1985). Despite an apparently successful treatment, the cestode was later found in the same farm also in tench (*Tinca tinca*) (Scholz and Di Cave, 1992). Despite the importance of this parasite, reports in Italy are scarce and, other than in the Trasimeno lake (Giovinazzo et al., 2006), its current distribution seems unclear. The presence of *Bothriocephalus* sp. in the lake Massaciuccoli had already been reported by Macchioni et al. (2015b), in *C. carpio* and *Carassius auratus*.

S. acheilognathi normally does not infect humans. However, in a male patient from French Guiana, regularly eating raw freshwater and brackish fish, and presenting abdominal pain during a holiday in France, Diphyllobothrium like eggs were isolated from the stool. The eggs were then

~~specifically attributed to *S. acheilognathi* using molecular analysis (Yera et al., 2013). The need to apply molecular methods is essential also for the identification of adult worms, especially in hosts other than cypriniforms and cyprinidontiforms (Kuchta et al., 2018).~~

~~Finally, also *S. acheilognathi*, being a visible parasitic cestode 6-10 cm long (Fig. 4), can concur to make commercial species disgusting for consumers, especially if they become evident during evisceration due to partial rupture of the intestine.~~

5. Conclusions

This report of *E. excisus* in *A. boyeri* contributes to describe the distribution of this parasite in Italian lakes, reporting it for the first time in the Massaciuccoli lake (NW Tuscany). Beside an epidemiological interest due to its role in the parasite cycle, the presence of this species suggests possible public health issues and implication for seafood quality, as *A. boyeri* is a locally highly appreciated species in Italy as well as in various Mediterranean countries such as Croatia, Greece, Spain and Turkey. Although *E. excisus* has not yet been proven as a causal agent of human cases, the zoonotic potential cannot be ruled out until further molecular data clarifying the species involved in human cases and the geographical distribution of the different species are available. Therefore, also considering the method of preparation and processing of *A. boyeri* and the growing tendency of consumption of raw or undercooked fish, it is essential to communicate the need of applying appropriate sanitary measures to all FBOs along the freshwater supply chain and educate consumers on the natural occurrence of ~~this~~ parasites in ~~certain kind of~~ wild fish, also to avoid excessive and unnecessary alarmism ~~along the freshwater supply chain~~. Finally, given the lack of available data, it would be of interest to investigate other Italian lakes.

Figures caption

Fig. 1

Macroscopic aspect of the collected specimens of *Eustrongylides excisus* (Jägerskiöld, 1909) from big scale sand smelt (*Atherina boyeri*, Risso, 1810), showing visceral (a) and muscle (b) localization, as

well as an overview of~~. The~~ collected worms~~ were pink-red, 4.5 cm long on average (range 3-5.5 cm) and 0.5-1 mm wide~~ (c-d).

Fig. 2

Microscopic aspect of the collected specimens of *Eustrongylides excisus* (Jägerskiöld, 1909) from big scale sand smelt (*Atherina boyeri*, Risso, 1810), showing the cephalic extremity presenting a small oral cavity surrounded by 12 cephalic papillae of similar size arranged in two concentric rings (a) and the posterior extremity with male (b) and female (c) genital primordia. Scale bar 100µ.

Fig. 3

Dendrogram produced combining ITS1 and ITS2 sequences, using Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980) with 1000 bootstrap re-samplings using the five sequences deposited in this study the sequences deposited as *Eustrongylides excisus* (Jägerskiöld, 1909) by Mazzone et al., (2019) and the sequences deposited as *Eustrongylides* sp. already selected in the ML analysis by Mazzone et al., (2019).

~~**Fig. 4**~~

~~Macroscopic and microscopic aspect of the collected specimens of *Schyzocotyle* (syn. *Bothriocephalus*) *acheilognathi* in *Atherina boyeri*. Cestodes were 6-10 cm long (a-b), with a flattened fleshy unarmed scolex almost spherical, with two bothria and an antero-laterally directed narrow slit-like opening were found (c-d).~~

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***Highlights (for review)**

- *Eustrongylides excisus* was found in *Atherina boyeri* from Tuscany for the first time
- The presence of the highly invasive cestode Schyzocotyle acheilognathi was also confirmed
- Molecular characterization of the ITS region allowed specific identification (78)
- Public health and quality issues may arise

***Conflict of Interest Form**

Declarations of interest: none

***Credit Author Statement**

Guardone L. : Data curation, Formal analysis, Writing - original draft

Ricci E. : Formal analysis, Data curation, Writing - review & editing

Susini F. : Conceptualization, Writing - review & editing

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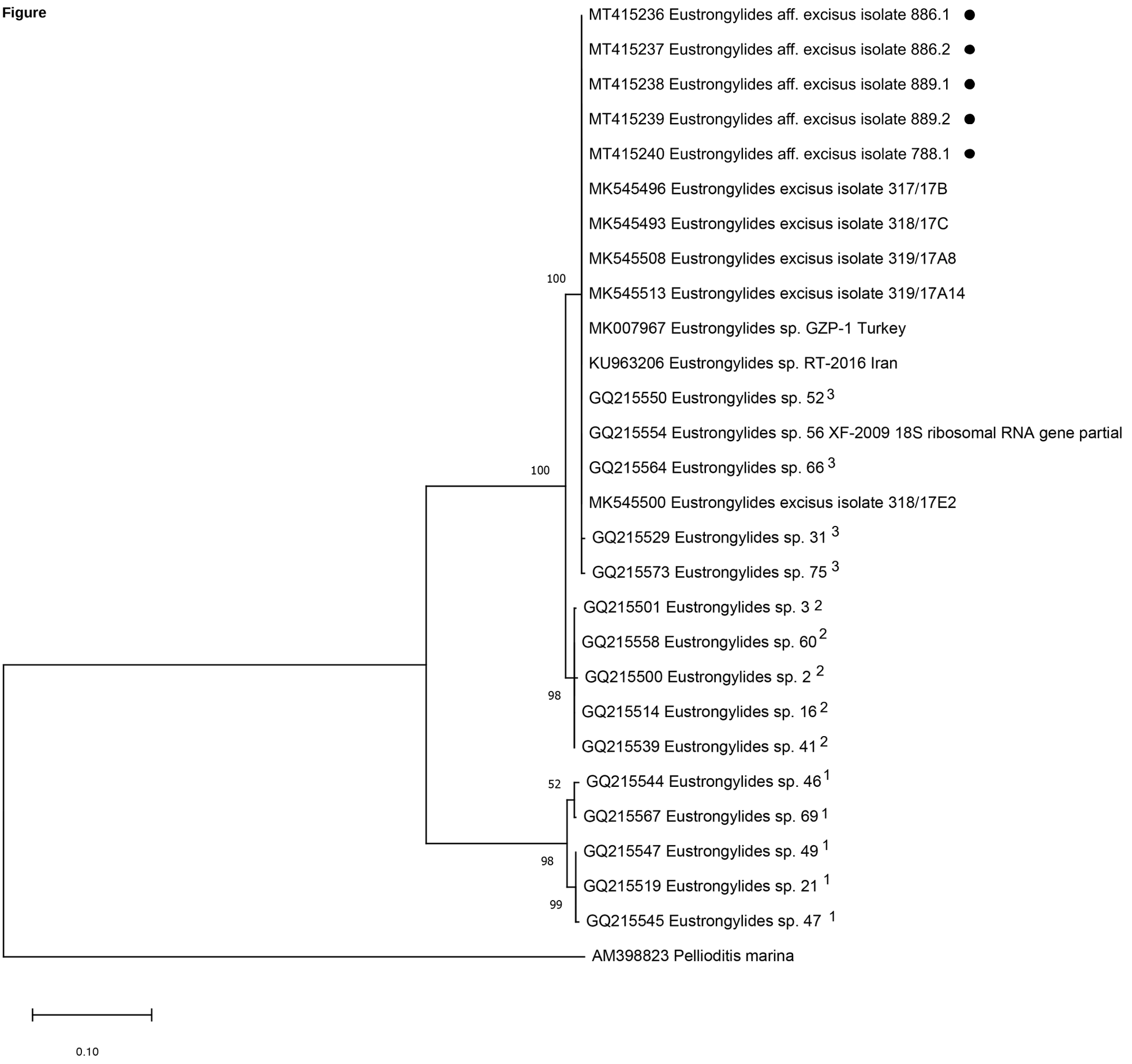
Figure

Figure

Figure


MT415236 Eustrongylides aff. excisus isolate 886.1
MT415237 Eustrongylides aff. excisus isolate 886.2
MT415238 Eustrongylides aff. excisus isolate 889.1
MT415239 Eustrongylides aff. excisus isolate 889.2
MT415240 Eustrongylides aff. excisus isolate 788.1
MK545496 Eustrongylides excisus isolate 317/17B
MK545493 Eustrongylides excisus isolate 318/17C
MK545508 Eustrongylides excisus isolate 319/17A8
100
MK545513 Eustrongylides excisus isolate 319/17A14
MK007967 Eustrongylides sp. GZP-1 Turkey
KU963206 Eustrongylides sp. RT-2016 Iran
GQ215550 Eustrongylides sp. 52 3
GQ215554 Eustrongylides sp. 56 XF-2009 18S ribosomal RNA gene partial
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GQ215564 Eustrongylides sp. 66 3
MK545500 Eustrongylides excisus isolate 318/17E2
GQ215529 Eustrongylides sp. 31 3
GQ215573 Eustrongylides sp. 75 3
GQ215501 Eustrongylides sp. 3 2
GQ215558 Eustrongylides sp. 60 2
GQ215500 Eustrongylides sp. 2 2
98
GQ215514 Eustrongylides sp. 16 2
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52
GQ215544 Eustrongylides sp. 46 1
GQ215567 Eustrongylides sp. 69 1
GQ215547 Eustrongylides sp. 49 1
98
GQ215519 Eustrongylides sp. 21 1
99
GQ215545 Eustrongylides sp. 47 1
AM398823 Pellioditis marina
0.10

Table

Table 1. Studies reporting human cases of *Eustrongylides* sp. available in the literature

Reference	Geographical area	N cases	Infection source	Symptoms and treatment	Parasite identification and nr (if available)
Centers for Disease Control, (1982)	Baltimore, Maryland (USA)	3 fishermen (23, 25 years old and n.d.)	Swallowing live minnows	severe abdominal pain within 24 hours from ingestion; surgical removal of two worms from the abdominal cavity of each of the two patients	80-120 mm long and 1-2 mm in diameter; morphologically identified as 4th-stage larval nematodes of *Eustrongylides* sp.
Eberhard et al., 1989	New Jersey (USA)	1 fisherman (17 years old)	Swallowing live minnows while fishing	Severe abdominal pain, vomiting; surgical treatment for the removal of 2 live worms in the peritoneal cavity	The worms measured 55.2 and 59 mm long and were 0.80-0.85 mm wide. Morphologically identified as *Eustrongylides* spp., although the larger size of inner papillae compared to the outer papillae suggests it may be *E. ignotus*
Wittner et al., 1989	New York	1 young man (24 years old)	Sushi/sashimi prepared at home with fish (unknown species) bought at a local fish market	Abdominal pain (mimicking appendicitis) 1 day after raw fish consumption; removal of 1 nematode from the abdominal cavity	Pinkish-red, sinuous worm 4.2 cm long and 1 mm wide. Cleared in lactophenol for identification, two circles of 6 cephalic papillae present. The size and morphology indicated that it was probably an early 4th stage larva of the genus *Eustrongylides*, species undetermined
Eberhard and Ruiz Tiben, 2014	South Sudan	2 women (23 and 24 years old)	unknown	2 large living worms were collected as they emerged from the skin of the lower limb of two persons	The worms were robust, red-brown in color, and measured approximately 7 and 8.5 cm long, respectively and were approximately 0.8 mm in maximum diameter. Mophological and morphometric analysis allowed identification as *Eustrongylides* sp.

Table

Table 2. Results of the parasitological analysis conducted on specimens of *Atherina boyeri* (Risso, 1810) collected from the Massaciuccoli lake (NW Tuscany, Italy). N: number; P: prevalence; A: abundance: I: intensity

Sampling	**N collected specimens**	**Average weight/specimen (g)**	***Eustrongylides excisus***				~~***Schyzocotyle acheilognathi***~~
			N positive	**P% (95% CI)**	**MA**	**MI**	~~**A**~~
August 2019	1150	0.72	22	1.9 (1.1-2.7)	0.02	1	~~0.032~~
September 2019	1197	0.68	26	2.2 (1.3-3.0)	0.02	1	~~0.037~~
October 2019	970	0.68	27	2.8 (1.7-3.8)	0.03	1	~~0.040~~
Total	**3317**	**0.69**	**75**	**2.3 (1.8-2.8)**	**0.02**	**1**	~~**0.036**~~