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Title: Ascaridoid nematode larvae in wild gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax) caught in the Tyrrhenian Sea (Western Mediterranean Sea): a contribute towards the parasitological risk assessment on two commercially important fish species

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Abstract: Gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax) are among the most popular fish on the Italian and European market. The present study aimed to assess the occurrence and localization of anisakids in wild specimens of these two species caught in a previously not investigated area of the Tyrrhenian Sea (FAO subarea 37.1.3). Forty gilthead seabream and forty-seven European seabass were analysed through visual inspection and chloro-peptic digestion. All the parasites collected were submitted to morphological and molecular analysis, targeting the cox2 and ITS genes. No Anisakis spp. were found. As regards other ascaridoid nematodes, seven larvae of Contracaecum rudolphii A (2 in the belly flaps and the rest in the viscera) (P: 2.5 95% CI: 0-7.3%; MA: 0.017; MI: 7) and one visceral larva of Hysterothylacium sp. (P: 2.5 95% CI: CI: 0-7.3%; MA: 0.025; MI: 1) were found in two different specimens of S. aurata. Seventeen larvae of C. rudolphii A were found in the viscera of six D. labrax (P: 12.7% 95% CI: 3.3-22.3%; MA: 0.15; MI: 1.17). The absence of Anisakis sp. in S. aurata agrees with literature data, while variable prevalence values had been previously reported for D. labrax, generally higher in Atlantic grounds. The occurrence of C. rudolphii A and Hysterothylacium spp., nematodes with a negligible zoonotic impact, contributes to the characterization of ascaridoid epidemiology in these commercially relevant species. In addition, the presence of C. rudolphii A in S. aurata is reported for the first time. The present results contribute towards the parasitological risk assessment of this Italian maritime area, which is also highly dedicated to the aquaculture of S. aurata and D. labrax.

Ascaridoid nematode larvae in wild gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) caught in the Tyrrhenian Sea (Western Mediterranean Sea): a contribute towards the parasitological risk assessment on two commercially important fish species

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Abstract

Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) are among the most popular fish on the Italian and European market. The present study aimed to assess the occurrence and localization of anisakids in wild specimens of these two species caught in a previously not investigated area of the Tyrrhenian Sea (FAO subarea 37.1.3). Forty gilthead seabream and forty-seven European seabass were analysed through visual inspection and chloro-peptic digestion. All the parasites collected were submitted to morphological and molecular analysis, targeting the *cox2* and ITS genes. No *Anisakis* spp. were found. As regards other ascaridoid nematodes, seven larvae of *Contracaecum rudolphii* A (2 in the belly flaps and the rest in the viscera) (P: 2.5 95% CI: 0-7.3%; MA: 0.017; MI: 7) and one visceral larva of *Hysterothylacium* sp. (P: 2.5 95% CI: CI: 0-7.3%; MA: 0.025; MI: 1) were found in two different specimens of *S. aurata*. Seventeen larvae of *C. rudolphii* A were found in the viscera of six *D. labrax* (P: 12.7% 95% CI: 3.3-22.3%; MA: 0.15; MI: 1.17). The absence of *Anisakis* sp. in *S. aurata* agrees with literature data, while variable prevalence values had been previously reported for *D. labrax*, generally higher in Atlantic grounds. The occurrence of *C. rudolphii* A and *Hysterothylacium* spp., nematodes with a negligible zoonotic impact, contributes to the characterization of ascaridoid epidemiology in these commercially relevant species. In addition, the presence of *C. rudolphii* A in *S. aurata* is reported for the first time. The present results contribute towards the parasitological risk assessment of this Italian maritime area, which is also highly dedicated to the aquaculture of *S. aurata* and *D. labrax*.

Keywords: *Anisakis* spp.; *Contracaecum rudolphii* A.; *Hysterothylacium* spp.; hazard characterization; Italy

1. Introduction

Anisakid nematodes are one of the main risks to humans associated with the consumption of seafood (EFSA, 2010). Parasites belonging to this group use marine mammals, fish-eating birds, reptiles and fish as definitive host and crustaceans as first intermediate hosts. Fish and cephalopods act as intermediate or transport hosts, carrying the zoonotic infective stage for human (third stage larva, L3) in the viscera or in the muscle. The accidental ingestion of live L3 when eating raw, or undercooked infected seafood can cause anisakidosis, a human disease clinically characterized by several gastrointestinal symptoms, and sometimes also allergic reactions (Levsen et al., 2018; Mattiucci et al., 2018). Beside the two most relevant zoonotic genera of the family Anisakidae, *Anisakis* and *Pseudoterranova*, with particular reference to the species *A. simplex*, *A. pegreffii* and *P. decipiens*, another potentially zoonotic ascaridoid larvae commonly reported in fish is *Contracaecum osculatum* (Buchmann and Merhdana, 2016; Shamsi, 2019). Finally, another parasitic genus frequently found in seafood is *Hysterothylacium* spp. (Raphidascarididae family), generally believed to be non-zoonotic (Levsen et al., 2018).

In addition to the zoonotic risk, the presence of visible nematode larvae (even if dead) reduces the quality of the product (Council Regulation EC No 2406/1996) making it unfit for human consumption (Regulation EC No 178/2002) due to consumers' repulsion (Bao et al., 2018). Therefore, all Food Business Operators (FBOs) must conduct a non-destructive visual inspection of the fishery products (fp) before placing them on the market (Commission Regulation EC No 2074/2005), to search for visible parasites in the edible portion and avoid commercialization of "*obviously contaminated products*". However, inspection by FBOs does not guarantee that the product is completely safe. Therefore, to prevent the possible ingestion of vital larvae, the Commission Regulation (EU) No 1276/2011 states that fp 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 (-20°C for 24 h or -35°C for 15 h). In addition, in Italy, fishmongers must inform

consumers on correct domestic freezing by displaying an informative note (Decree of July 17, 2013 of the Italian Ministry of Health).

Gilthead seabass and European seabream are among the most marketed species in the European Union. In Italy both species, mainly marketed whole and without evisceration (EUMOFA, 2017; EUMOFA, 2019), are highly appreciated by consumers. Italy is in fact the largest EU market with 31100 t of gilthead seabream and 31122 t of European seabass sold in 2015 and 2016, respectively (EUMOFA, 2017; EUMOFA, 2019). The relevant commercial value of these two species, higher when wild and fresh, and their increasing use as a raw product in the preparation of tartare, sushi and sashimi (Armani et al., 2017; Bernardi, 2009; Legnani, 2010) underlines the need to continuously update the potential risk related to the presence of anisakid larvae in these hosts.

Previous studies on the presence of anisakids analysed gilthead seabream from the Eastern Mediterranean Sea and European seabass from the Atlantic area, while fewer fish have been investigated in the Tyrrhenian Sea (Western Mediterranean Sea) (Table 1) that represents an area highly dedicated to the aquaculture of these two species (https://www.magazinequalita.it/qualita-polo-acquacoltura-orbetello/; https://iltirreno.gelocal.it/livorno/cronaca/2017/09/24/news/allevate-ma-in-mare-le-orate-del-golfo-prendono-il-largo-1.15896632). Therefore, in addition to characterize the parasitological hazard associated with the consumption of wild gilthead seabream and European seabass coming from the Tyrrhenian Sea, data from this area can contribute towards the parasitological risk assessment in the existing offshore farms. This study aimed therefore at investigating the occurrence and localization of ascaridoid nematode larvae in wild specimens of Gilthead seabass and European seabream caught in an area of the Tyrrhenian Sea never investigated before.

2. Materials and methods

2.1 Fish sampling

A total of 40 gilthead seabream and 47 European seabass, caught by commercial anglers in the Tyrrhenian Sea, more precisely in the area between the Giglio Island and the Elba Island (FAO

105 Area 37, Division 37.1.3, Sardinia) were collected from November 2018 to November 2019. The
106 specimens were immediately frozen and then transferred to the FishLab, Department of Veterinary
107 Sciences, University of Pisa, where they were stored at -20 °C.

108 ***2.2 Parasitological analysis***

109 Each sample was registered with an internal unique code and photographed, then measured and
110 weighed registering the Total Length (TL) and the Total Weight (TW), and finally gutted. The
111 abdominal cavity and viscera were submitted to visual inspection (Commission Regulation EC No
112 2074/2005) in order to detect visible parasite larvae, i.e. with a length greater than 10 mm or 3 mm
113 when encapsulated (Codex Alimentarius 1971). Each visible parasite detected was collected and
114 microscopically identified (Nikon Eclipse E 200) to the genus level, following Sakanari and
115 McKerrow (1989), Berland (1989) and Moravec (1994). The larvae were counted and stored in 70%
116 alcohol at 4°C until subsequent molecular analysis. The stomach was then opened, and the content
117 was analysed by visual inspection. Digestion was performed on viscera and muscle using
118 Trichineasy® (CSTC srl Brescia), a recently validated tool for the parasitological analysis of fp
119 (Cammilleri et al., 2016), following the protocol described in Guardone et al., (2017). Nematode
120 larvae collected after the digestion were also counted and subsequently identified and stored as
121 described above.

122 ***2.3 Molecular identification***.

123 Total DNA was extracted from all the collected larvae as described in Guardone et al. (2016).
124 Its concentration and purity were assessed by using a NanoDrop ND-1000 spectrophotometer
125 (NanoDrop Technologies, Wilmington, DE, USA). The primers 211F and 210R (Mattiucci et al.,
126 2014) were used to amplify a fragment (~ 600-bp) of the mitochondrial cytochrome *c* oxidase
127 subunit II (*cox2*) gene while the primers NC2 and NC5 (Zhu et al., 1998) were used to amplify a
128 fragment (~900-bp) of the ITS-1 region, the 5.8S gene and the ITS-2 region plus approximately
129 70 nucleotides of the 28S gene (ITS). PCR amplifications were set up in a 20-µl reaction volume
130 as described in Guardone et al., (2018). The following cycling program were used: denaturation

at 94 °C for 3 min; 40 cycles at 94 °C for 20 s, 45 °C for 20 s, 72 °C for 25 s; and final extension at 72 °C for 10 min (*cox2*); denaturation at 94 °C for 3 min; 40 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 75 s; and final extension at 72 °C for 10 min (ITS). PCR products were analysed by electrophoresis in 2% agarose gel and those presenting the expected length were sent for standard forward and reverse Sanger sequencing to an external company. The obtained sequences were analyzed, edited and assembled with the Geneious R7 software (Kearse et al., 2012) and compared using the Basic Local Alignment Search Tool (BLAST) with sequences deposited in GenBank. In addition, a Neighbour-Joining (NJ) phylogram (Saitou & Nei, 1987) was produced using MEGA version 7.0 for both genes (in the case of ITS combining the ITS-1 and ITS-2 partial regions). The NJ trees were obtained using the sequences of different *Contracaecum* species and sibling species of *C. rudolphii* (s. l) (A, B, C, D, E), already selected for the phylogenetic analysis in the work of Mattiucci et al., (2020), together with a selection (8 for the *cox2* and 8 for the ITS) of those produced in this study (as they were all identical). In addition, 6 concatenated ITS sequences and 6 *cox2* gene sequences identified as *C. rudolphii* A *and* B in the aforesaid study (Mattiucci et al., 2020) were also included in the two NJ trees. Distances were computed using the Kimura 2-parameter model (Kimura, 1980) with 1000 bootstrap re-samplings. MEGA version 7.0 was also used to conduct pairwise distance analysis.

2.4 Statistical analysis

For both the investigated species the prevalence (P), mean abundance (MA) and mean intensity (MI), as defined in Bush et al. (1997), were calculated (separately for viscera and muscle). The Pearson's correlation coefficient was used to assess the relationship between the weight and the length of the fish specimens and the parasite number.

3. Results and discussion

3.1. Occurrence, localization and identification of ascaridoid nematodes in wild gilthead seabream and European seabass

156 In this study around 40 specimens of each fish species (40 gilthead seabream and 47 European
157 seabass) were collected, in accordance with Bernardi et al., (2011) who considered this sample size
158 the best compromise between a suitable estimate of parasitological indexes and the sample costs.
159 The specimens' weight was selected based on the commercial size of aquaculture products most
160 requested from the national market (authors' note). The mean TW was 270.3 ± 63.36 g for gilthead
161 seabream and 478.4 ±188.3 g for European seabass, while the medium TL was of 22.2 ± 1.4 cm for
162 the former and 30.6 ± 4.5 cm for the latter species.
163 In this study, both the visual inspection under natural light, included in EU food hygiene and
164 safety regulations (Commission Regulation (EC) No 2074/2005), and the chloro-peptic digestion
165 method were used, as in other studies (APROMAR, 2010; Bernardi et al., 2011; Cammilleri et al.,
166 2018; Goffredo et al., 2019; Peñalver et al., 2010; Salati et al., 2013), in order to recover virtually
167 all the larvae (EFSA, 2010). All the specimens analysed in this study were negative after visual
168 inspection as parasites were found after the chloro-peptic digestion. Detailed results regarding the
169 nematode larvae found are detailed in Table 2 and discussed below in the light of available
170 literature data, although, in some cases, the utilization of different methods hampers the direct
171 comparison (Table 1).
172 All gilthead seabream and European seabass were negative for *Anisakis* spp. both in the viscera
173 and the muscle. Even though both species were found to be sensitive to experimental infection with
174 *Anisakis* spp. (Macrì et al., 2012; Marino et al., 2012) negative results for the *S. aurata* agree with
175 the available studies (Table 1). In fact, no *Anisakis* spp. were found in this species in Sardinia (same
176 FAO subarea, 37.1.3) (Culurgioni et al., 2011a; Salati et al., 2013; Culurgioni et al., 2014) and also
177 in other subareas of the Mediterranean Sea (Kalay et al. 2009; Keskin et al., 2015) different from
178 that analysed in this study. On the contrary, variable prevalence values have been reported for *D.*
179 *labrax*. No *Anisakis* spp. were detected in 56 specimens from the Southern coast of Sardinia
180 (Culurgioni et al., 2014), in agreement with our results, while a higher visceral prevalence (50%) of
181 *A. pegreffii* was found in another study conducted in the same Sardinian area (Culurgioni et al.,

182 2011b) which however analysed only 6 specimens. As regards other subareas of the Mediterranean
183 Sea, a visceral prevalence of 13% for *A. simplex s.l.* (morphologically identified) in the Northern
184 coast of Egypt was recorded (Zaid et al., 2018). On the contrary, much higher prevalence values
185 have been found in European seabass from the Northeast Atlantic Ocean (Bernardi, 2009; Bernardi
186 et al., 2011). In addition, Sterud et al. (2002) found a visceral prevalence of 15.4% of *Anisakis* sp. in
187 the Oslo fjord.
188 As regard other ascaridoid nematodes, one gilthead seabream was infected with seven larvae
189 morphologically identified as *Contracaecum* sp. and another one was infected with 1 larva
190 morphologically identified as *Hysterothylacium* sp., viscerally located. Totally, six European
191 seabass were positive, as seventeen larvae, morphologically identified as *Contracaecum* spp., were
192 found in the viscera (all details are in Table 2). No correlation was observed between the number of
193 larvae and the weight of the samples**.**
194 As for the molecular identification of the larvae, for the ITS region, the ITS-1 and ITS-2 partial
195 regions were used separately and concatenated. This approach for the ITS region was used
196 following Szostakowska & Fagerholm (2007) and also considering that sequences for the complete
197 ITS region were not available for all *Contracaecum* species. Concatenated ITS1 and ITS2 were
198 used also in the recent work of Mattiucci et al., (2020), where a multilocus approach was applied
199 analysing also the *cox2*. As regards the ITS NJ dendrogram we only report the one obtained using
200 the concatenated ITS-1 and ITS-2 regions. All the larvae morphologically identified as
201 *Contracaecum* sp. were molecularly identified as *C. rudolphii* A based on the results of the BLAST
202 analysis and of the NJ dendrogram, using both the *cox2* (Fig. 1) and the ITS region (Fig. 2). In
203 particular, all the sequences produced in this study clustered together with the sequences previously
204 identified as larvae of *C. rudolphii* A, well supported by a high bootstrap score, in both NJ trees.
205 The sequences obtained in this study were deposited in GenBank (waiting for Accession nr. XX).
206 *C. rudolphii s. l.* consists of several sibling species, named from A to F (Mattiucci et al., 2020). The
207 two sibling species *C. rudolphii* A and *C. rudolphii* B have been described in Europe (Cianchi et al.

208 1992; D'Amelio et al. 1990, 1992; Mattiucci et al., 2020). In particular, *C. rudolphii* A seems to
209 predominantly infect cormorants dwelling in brackish waters of coastal lagoons in Europe (Li et al.,
210 2005) whereas *C. rudolphii* B is harboured by cormorant colonies living in the freshwater lagoons
211 of Europe (Mattiucci et al. 2002 and personal observations; Szostakowska & Fagerholm 2007).

212 Few epidemiological data are available for these ascaridoid in European seabass. The presence
213 of *C. rudolphii* was reported in Sardinia with higher visceral prevalence values (84%) (Culurgioni
214 et al., 2014) and very recently the presence of *C. rudolphii* A was demonstrated in specimens from
215 brackish water environment in the northern part of Latium (Mattiucci et al., 2020). Infection with
216 *Contracaecum* sp. in the gilthead seabream appears to be less common, but the prevalence values
217 observed before were higher than the value found herein (20% by Salati et al., 2013 and 35-80% by
218 Culurgioni et al., 2011). In this study the presence of *C. rudolphii* A in *S. aurata* is reported for the
219 first time.

220 The larva morphologically identified as *Hysterothylacium* sp. was not molecularly characterized
221 at species level because the *cox2* sequences only retrieved a highest percentage of identity of
222 99.78% with a sequence of *Hysterothylacium* sp. deposited in GenBank (KX094931) and of 98.19-
223 97.29% with sequences of *H. reliquens* (KX825838-45; MF120255). Unfortunately, no ITS
224 sequences could be obtained for this larva. The presence of *Hysterothylacium* sp. in the viscera of
225 gilthead seabream has already been reported in different sub-areas of the Mediterranean Sea. As
226 regards the same FAO subarea investigated in the present study, a slightly higher prevalence value
227 (5.9%) was found by Culurgioni et al. (2011a), while higher prevalence values (from 6.5 to 45.6 %)
228 have been reported from the Eastern Mediterranean (Dural et al., 2011; Kalay et al., 2009; Keser et
229 al., 2007; Keskin et al., 2015). Only Keskin et al. (2015) molecularly identified the larvae as *H.*
230 *aduncum.*

231 ***3.2. Ascaridoid nematodes in wild gilthead seabream and European seabass: public health***
232 ***implications***

233 Several studies on larval anisakid nematodes in many wild fish species of commercial interest
234 are available (Cavallero et al., 2012; Cipriani et al., 2018; De Liberato et al., 2013; Gazzonis et al.,
235 2017; Guardone et al., 2019; Levsen et al., 2018; Piras et al., 2014; Serracca et al., 2013). In
236 addition to producing relevant epidemiological data on the distribution of the parasites in their
237 hosts, these data have an important relapse on public health and facilitate the assessment of the risk
238 for humans to contract this zoonosis, especially in the case of fish species that can be consumed also
239 raw or slightly cooked, such as the investigated species, which are now used for the production of
240 dishes such as sushi, sashimi, *pokè, carpacci, tartare*.
241 The genus *Anisakis* comprises nine species, however only two of them (*A. simplex* s.s. and *A.*
242 *pegreffii*) have been confirmed as zoonotic (Levsen et al., 2018; Mattiucci & D' Amelio, 2014). In
243 this study, as already mentioned, no *Anisakis* larvae were found, suggesting a low or negligible risk
244 of contracting anisakiasis related to the consumption of wild gilthead seabream and European
245 seabass caught in the investigated area. In particular, data produced in this study and feedbacks
246 from local fishermen who, during the sampling, told us they never saw an infected seabream
247 confirm that the gilthead seabream is little susceptible to infection by *Anisakis* spp. larvae
248 (Mattiucci com. pers. in Kapota, 2012; Culurgioni et al., 2011a;2014; Dural et al., 2011; Kalay et
249 al., 2009; Keser et al., 2007; Keskin et al., 2015; Salati et al., 2013) (see section 3.1). The absence
250 of *Anisakis* spp. could be due to the feeding behaviour of this species, preferring gastropods and
251 bivalves (in particular mussels and oyster) to teleosts (Pita, 2002 and references herein;
252 https://www.fishbase.in/summary/Sparus-aurata.html). In agreement, also in the present study the
253 content of the stomach frequently showed the presence of mussel (*Mytilus* spp) and crabs. On the
254 contrary, the European seabass shows a trophic predatory attitude on invertebrates (at the juvenile
255 stage) and other fish (at the adult stage) (Kapota, 2012). An increase of the fish preys' size has been
256 observed as the predators' dimensions grows (Rogdakis et al., 2010). Therefore, a progressive
257 increase of nematode larvae can occur not only due to the accumulation of nematodes in the host
258 during its life, but also following diet's changes (Mattiucci et al., 2018; Smith and Wootten, 1978).

259 This trend is clearly shown in the study of Bernardi (2009) where increasing prevalence values have
260 been reported from fish categories of different weight caught in the Atlantic Ocean. However, most
261 of the specimens collected from the Mediterranean Sea (Table 1) were less than 1000 gr and
262 presented P values ranging from 0 (Brahim Tazi et al., 2016) to 13% (Zaid, 2018). These weight
263 differences could be due to different growing patterns reported for the Atlantic and Mediterranean
264 population of the European seabass: while in the Mediterranean sea the sexual maturity occurs
265 generally between 2 and 4 years of age in the Atlantic ocean sexual maturity happens later
266 (https://www.fishbase.in/summary/Dicentrarchus-labrax.html). This trend also reflects the seabass'
267 minimum landing size: 36 cm for the southern Atlantic stock, 42 cm for the Northern stock, and 25
268 cm in the Mediterranean (EUMOFA, 2019). All these factors seem to contribute to lower infection
269 levels of *Anisakis* sp. in Mediterranean seabass.

270 Human infection with *Contracaecum* larvae has been less frequently reported than infection with
271 *Anisakis* larvae (Shamsi & Butcher, 2011). Few reports have been described, from the Baltic region
272 (Schaum and Müller, 1967), France (Dei-Cas et al., 1986), the Republic of Korea (Im et al., 1995),
273 Australia (Shamsi and Butcher, 2011) and Japan (Nagasawa, 2012). Different zoonotic potentials
274 have been hypothesized depending on the species: those occurring in marine mammals such as *C.*
275 *osculatum* are believed to be zoonotic, while those occurring in birds, as *C. rudolphii*, are not
276 considered so (Shamsi, 2019), although to-date, there has been no specific molecular identification
277 of *Contracaecum* larvae isolated from humans (Shamsi, 2019). Thus, in the case of the fish
278 analysed in this study, the risk of developing anisakidosis due to unaware ingestion of larvae of *C.*
279 *rudolphii* A can be considered very low, not only because this species seems not to be zoonotic, but
280 also because only two specimens were found in the belly flaps of a single gilthead seabream, while
281 in the six positive European seabass it was located in the viscera. Unfortunately, a comparison with
282 data found in the literature on the localization of *Contracaecum* in gilthead seabream and European
283 seabass is difficult, as the studies that found this species analysed only the viscera or give an overall
284 prevalence (Table 1). However, third stage larvae of *Contracaecum* spp. accumulate mostly in the

285 mesentery and the intestinal serosa of fish and also reside in the intestinal wall and liver. According
286 to previous studies conducted on other fish species Szostakowska & Fagerholm (2007), the
287 localization at the intestinal level seems to be the preferred one for *C. rudolphii*.

288 Finally, also the presence of *Hysterothylacium* spp. seems to represent a negligible public health
289 risk following to the consumption of wild specimens. In fact, although according to some authors
290 (Shamsi et al., 2013) its zoonotic potential is a controversial issue as two human cases (González-
291 Amores et al., 2015; Yagi et al., 1996) and possible allergenicity (Valero et al., 2003) are reported ,
292 this species is generally not believed to be zoonotic as it is not able to develop at 37°C (Cipriani et
293 al., 2019; Levsen et al. 2018). Different studies show in fact that larvae L3-L4 do not migrate into
294 the fish muscle but tend to remain in the gastrointestinal tract (Brahim Tazi et al., 2016; Culurgioni
295 e al., 2011a; Kalay et al., 2009; Keser et al., 2007; Levsen and Karl, 2014; Sterud, 2002).

296 ***3.3. Parasitological risk management in aquaculture offshore plans and market opportunities***

297 Wild gilthead seabream and European seabass are of great interest for the Italian and European
298 consumers. However, a large part of products available on the Italian market derives from farmed
299 fish, produced at national and European level, and, to a lesser extent, also in Third countries. In
300 Italy, the largest amount of national farming of both species is absorbed by domestic demand
301 (EUMOFA, 2019) and the strategy adopted by the Italian companies to face foreign competition is
302 the production of high-quality products. The presence of parasites (live and dead) could affect their
303 overall quality reducing their commercial value and marketability as well as consumers' confidence
304 (Bao et al., 2018; Guardone et al., 2019). Although mandatory preventive measures kill parasites
305 and reduce risk of human infections, preventing the infection of fish in the farmed environment is
306 the approach pursued within European aquaculture plans (Parafishcontrol, 2017). A possible
307 exemption from the preventing freezing for farmed *Atlantic salmon* (*Salmo salar*) already exists
308 (Commission Regulation (EU) No 1276/2011). This approach was based on the parasitological risk
309 assessment produced by EFSA (EFSA, 2010) that also allows the same exemption for *"farmed*
310 *fishery products other than Atlantic salmon may be considered to present a negligible risk for*

311 *parasites that may be a risk to the health of the consumer. Consequently, such farmed fishery*
312 *products may also be exempted from the freezing requirements while the high level of health*
313 *protection is still ensured*". Currently, a derogation from farmed Atlantic salmon, halibut and
314 rainbow trout bred in cages at sea exists in the United Kingdom (www.food.gov.uk/business-
315 guidance/freezing-fish-and-fishery-products).

316 Although so far no exemption has been granted to the investigated species, the topic is cutting
317 edge and has recently been addressed in a Europe-wide project called ParaFishControl
318 (https://www.parafishcontrol.eu/) aimed at investigating the occurrence of zoonotic nematodes in
319 European farmed fish. Preliminary results from over 7000 farmed specimens of different species,
320 including gilthead seabream and European seabass, showed absence of *Anisakis* spp. and only
321 found one larva of *H. fabri* in one European seabass (Gustinelli et al., 2017).

322 ***4. Conclusion***

323 Overall, data arising from this study highlight a low potential impact on public health due to the
324 consumption of gilthead seabream and European seabass caught in the analysed area of the
325 Tyrrhenian Sea (Central Mediterranean Sea). In fact, *Anisakis* spp. larvae were not found and the
326 other retrieved larvae belonged to species (*C. rudolphii* A) or genera (*Hystherothylacium*) of
327 negligible zoonotic concern. In addition, considering that most of the larvae were found in the
328 viscera and the only muscle localisation of nematode larvae was the belly flaps, the parasite
329 contamination could be easily reduced by a prompt evisceration belly-flap trimming.

330 Knowing the occurrence and localization of zoonotic parasites in wild specimens of these highly
331 marketed species could allow to clarify the interaction of biological factors such as host feeding
332 habits, occurrence of definitive and intermediate hosts, and host susceptibility that can participate to
333 determine the parasitic burden in farmed fish. Therefore, data arising from this study could be used
334 also for feeding the parasitological risk assessment in aquaculture offshore plan located in the
335 studied area. Considering that no derogation from the preventive freezing treatment still exists for
336 farmed European seabream and gilthead seabass, the possibility to obtain such an exemption by the

implementation of a correct management system, validated by the Official Authority, in offshore plants would represents a new market opportunity for aquaculture plans located in the investigated sea area.

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Figure captions

Fig. 1

NJ phylogenetic tree of the *cox2* gene sequences of specimens of *C. rudolphii* A obtained in the present study (highlighted with a dot), with other *Contracaecum* species selected in the work of Mattiucci et al, (2020) and sequences of *C. rudolphii* A (MK496476; MK496477 MK496478) and *C. rudolphii* B (MK496482; MK496483 MK496484) produced in the aforesaid work. The analysis was performed by using MEGA6 software (Tamura et al. 2013). Bootstrap values (> 70) are reported at the nodes. *Pseudoterranova ceticola* was used as outgroup.

Fig. 2

NJ phylogenetic tree of the concatenated ITS1+ITS2 rDNA gene sequences of specimens of *C. rudolphii* A obtained in the present study (highlighted with a dot), with other *Contracaecum* species selected in the work of Mattiucci et al, (2020) and sequences of *C. rudolphii* A (MK496488+MT096410; MK496489+ MT096411; MK496490+MT096410) and *C. rudolphii* B (MK496494+MT096416; MK496495+ MT096417; MK496496+ MT096418) produced in the aforesaid work. The analysis was performed by using MEGA6 software (Tamura et al. 2013). Bootstrap values (> 70) are reported at the nodes. *Ascaris suum* was used as outgroup.

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Highlights

- Occurrence of anisakid nematodes were assess in wild gilthead seabream and European seabass
- Fish were sampled in an area of the Tyrrhenian Sea dedicated to fish farming
- No *Anisakis* spp. were found, suggesting a low risk of anisakiasis
- Other ascaridoid larvae with a lower or negligible zoonotic impact were found
- Data on wild specimens may be useful to feed the parasitological risk for aquaculture

Table

Table 1 Epidemiological studies on wild gilthead sea bream (*Sparus aurata*) and European European seabass (*Dicentrarchus labrax*) available in the literature (2000-2020). V: viscera; M: muscle; P%: prevalence

References	Geographical area	N° examined specimens and species	Weight	Examined tissue	Analythical method	Parasite identification	Anisakid species (n of larvae, when available)	V P%	M P%
Sterud *et al*., 2002	Oslo fjord (FAO area 27)	13 European seabass	495- 2431 g	V	Visual inspection Stereomicroscope Phase contrast light microscope	Morphology	*Hysterothylacium aduncum* *Anisakis* spp.	46.1 15.4	-
Keser *et al*., 2007	Dardanelles (FAO area 37.4.1)	12 gilthead seabream	-	V	Visual inspection Stereomicroscopy	-	*Hysterhothylacium aduncum* (1)	8.3	-
Bernardi *et al*., 2009	North East Atlantic (FAO area 27)	561 European seabass	<2 kg: 334 2 - 3 kg: 180 >3 kg: 47	V	Visual inspection	-	Anisakidae	< 2 kg: 65.3 2 - 3 kg: 85.00 > 3 kg: 89.4	-
Kalay et al. 2009	Southern coast of Turkey (FAO area 37.3.2)	208 gilthead seabream	85.50 ± 19.05 g (65.68-130.52 g)	V and M	Not mentioned	Morphology	*Hysterhothylacium sp.* (adults)	6.25	-
Bernardi *et al*., 2011	North East Atlantic (FAO area 27)	40 European seabass	1450 ± 800 g	V and M	Visual inspection Magnifier desk lamp dissection against fluorescent lamp Digestion	PCR (*cox2*)	*A. simplex* (3696)	95 (95% CI 82.9-99.1); 96.4 (95% CI 67.9-137.6)	42.5 (95% CI 27.7-58.8); 1.94 (CI 1.41-2.59)
Culurgioni *et al*., 2011a	Southern coast of Sardinia (3 lagoons) (FAO area 37.1.3)	75 gilthead seabream	81.5-283 g	V	Standard parasitological techinque	Morphology	*Hysterothylacium* spp. *Contracaecum rudolphii*	5.9 35.3-80	-
Culurgioni *et al*., 2011b	Southern coast of Sardinia	6 European seabass	-	V and M (*belly*	Visual inspection, dissection under	Morphology PCR (*cox2*)	*A. pegreffii*[b] (41)	50	-

	(3 lagoons) (FAO area 37.1.3)			*flaps*)	stereomicroscope Candling				
Dural et al., 2011	Iskenderun Bay on March 2008.	46 gilthead seabream	Positive: 139.34 ± 25.52 g Negative: 145.07 ± 32.83 g		Visual inspection	-	*Hysterothylacium aduncum*	45.6	
Kapota 2012	Greek ionic coast (Missolongi)	30 gilthead seabream 30 European seabass	200-250 g 240-370 g	V and M	Visual inspection Candling	-	-	0	
Salati et al., 2013	Sardinia (FAO area 37.1.3)	10 gilthead seabream	-	V and M	Visual inspection Digestion	Morphology PCR (12S)	*Contracaecum* sp.	20	
Culurgioni *et al*., 2014	Southern coast of Sardinia (FAO area 37.1.3)	56 European seabass	-	V	Visual inspection	Morphology PCR (*cox2*)	*Contracaecum rudolphii*	83.9; 95.1±28.8	-
Emre et al., 2014	Antalya, Turkey (FAO area 37.3.2)	87 European seabass	-	V	Visual inspection Dissection	Morphology	*Hysterothylacium* sp. (1)	1.1	-
Keskin et al., 2015	Mersin Bay (FAO area 37.3.2)	385 gilthead seabream	Negative: 78.83 ± 19.02 g; Positive: 85.64 ± 22.2 g	V	Standard parasitological procedures	Morphology PCR (ITS region, *cox1*)	*Hysterothylacium aduncum*	14.55	-

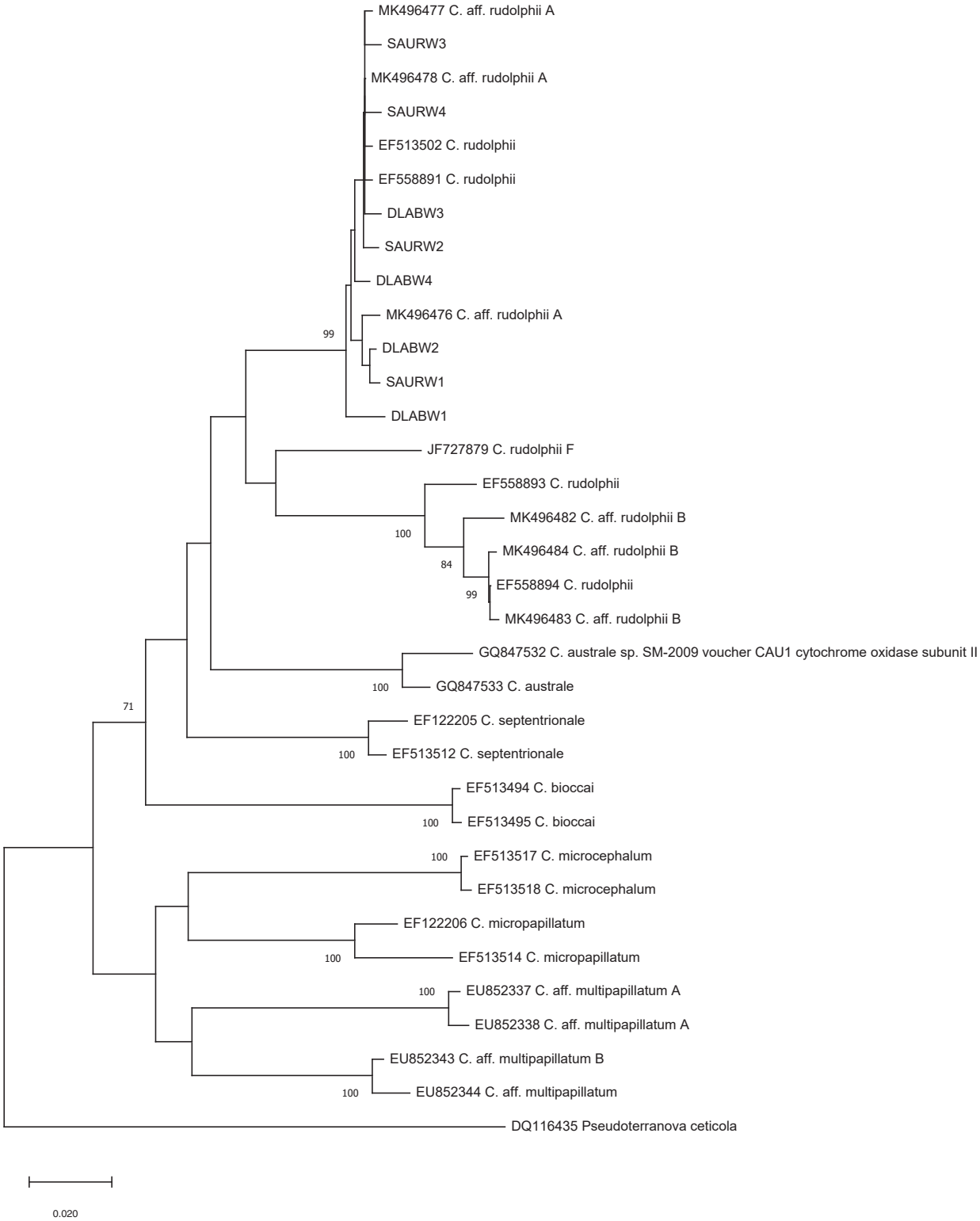
Brahim Tazi et al., 2016	Fish markets Oran, Algeria (FAO area 37.1.1)	10 European seabass	-	V	Visual inspection low and high magnification	Morphology	*Hysterothylacium aduncum*	60	-
Zaid et al., 2018	Northern coast of Egypt (FAO area 37.3.2) (from fish market)	100 European seabass	40-250 g	V	Visual inspection, dissection under stereomicroscope	Morphology SEM	*Anisakis simplex*	13	-
							Hysterothylacium aduncum	19	-
Mattiucci et al., 2020	Tarquinia coast and Mouth of Fiora river (FAO area 37.1.3)	37 European seabass	-	V and M	Visual inspection, dissection under stereomicroscope, UV press method	Morphology, multilocus allozyme electrophoresis (MAE), PCR (ITS region, *cox2*)	*Contracaecum rudolphii A*	46.2	

Table

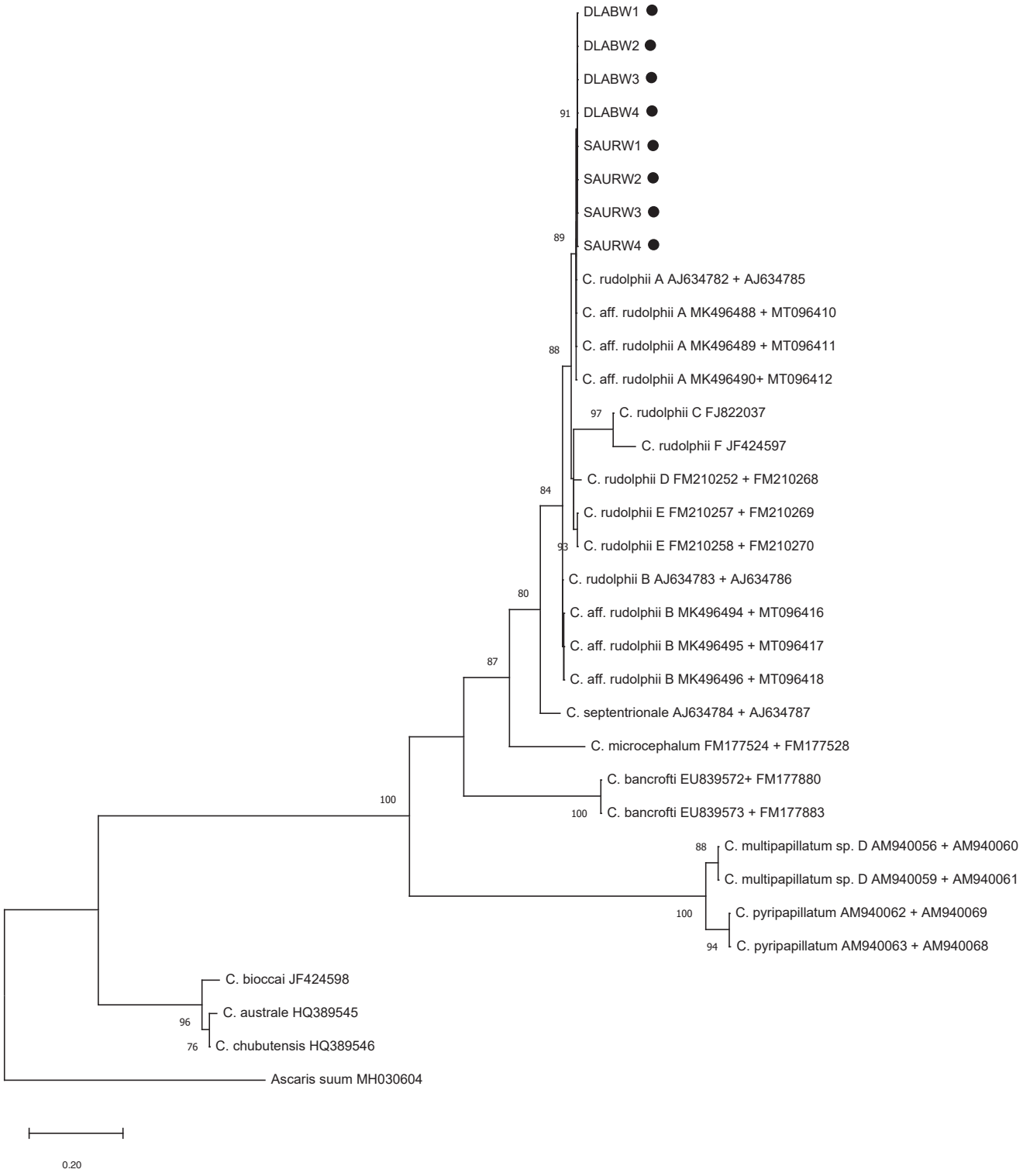
Table 2 Results of the parasitological examination (viscera and muscle) of the Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). P%: prevalence expressed in percentage; CI: confidence interval MA: mean abundance; MI: mean intensity

	Gilthead seabream (n=40)			**European seabass (n=47)**		
	N positive, P%, MA, MI	N larvae viscera	N larvae muscle	N positive, P%, MA, MI	N larvae viscera	N larvae muscle
Anisakis sp.	0	0	0	0	0	0
Contracaecum rudolphii A	1, 2.5% (95% CI 0-7.3), 0.17, 7	5	2	6, 12.7% (95% CI 3.3-22.3), 0.36, 1.17	17	0
Hysterothylacium sp.	1, 2.5% (95% CI 0-7.3), 0.02, 1	1	0	0	0	0

Figure


MK496477 C. aff. rudolphii A
SAURW3
MK496478 C. aff. rudolphii A
SAURW4
EF513502 C. rudolphii
EF558891 C. rudolphii
DLABW3
SAURW2
DLABW4
MK496476 C. aff. rudolphii A
99
DLABW2
SAURW1
DLABW1
JF727879 C. rudolphii F
EF558893 C. rudolphii
MK496482 C. aff. rudolphii B
100
MK496484 C. aff. rudolphii B
84
EF558894 C. rudolphii
99
MK496483 C. aff. rudolphii B
GQ847532 C. australe sp. SM-2009 voucher CAU1 cytochrome oxidase subunit II
GQ847533 C. australe
100
71
EF122205 C. septentrionale
100
EF513512 C. septentrionale
EF513494 C. bioccai
100
EF513495 C. bioccai
100
EF513517 C. microcephalum
EF513518 C. microcephalum
EF122206 C. micropapillatum
100
EF513514 C. micropapillatum
100
EU852337 C. aff. multipapillatum A
EU852338 C. aff. multipapillatum A
EU852343 C. aff. multipapillatum B
100
EU852344 C. aff. multipapillatum
DQ116435 Pseudoterranova ceticola
0.020

Figure


DLABW1
DLABW2
DLABW3
DLABW4
SAURW1
SAURW2
SAURW3
SAURW4
C. rudolphii A AJ634782 + AJ634785
C. aff. rudolphii A MK496488 + MT096410
C. aff. rudolphii A MK496489 + MT096411
C. aff. rudolphii A MK496490+ MT096412
C. rudolphii C FJ822037
C. rudolphii F JF424597
C. rudolphii D FM210252 + FM210268
C. rudolphii E FM210257 + FM210269
C. rudolphii E FM210258 + FM210270
C. rudolphii B AJ634783 + AJ634786
C. aff. rudolphii B MK496494 + MT096416
C. aff. rudolphii B MK496495 + MT096417
C. aff. rudolphii B MK496496 + MT096418
C. septentrionale AJ634784 + AJ634787
C. microcephalum FM177524 + FM177528
C. bancrofti EU839572+ FM177880
C. bancrofti EU839573 + FM177883
C. multipapillatum sp. D AM940056 + AM940060
C. multipapillatum sp. D AM940059 + AM940061
C. pyripapillatum AM940062 + AM940069
C. pyripapillatum AM940063 + AM940068
C. bioccai JF424598
C. australe HQ389545
C. chubutensis HQ389546
Ascaris suum MH030604
91
89
88
97
84
93
80
87
100
100
88
100
94
96
76
0.20