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FISH PARASITOLOGY - ORIGINAL PAPER

On the occurrence and molecular identification of *Contracaecum* larvae (Nematoda: Anisakidae) in *Mugil cephalus* from Turkish waters

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Abstract

Anisakis and *Contracaecum* species are fish borne zoonotic nematodes. In our previous studies, other larval anisakid and raphidascarid nematodes, *Anisakis* and *Hysterothylacium* species, were genetically identified in marine fish from Turkish waters. However, there is no information on molecular identification of larval *Contracaecum* species in marine fish from Turkey. Therefore, the aim of this study was only to investigate the presence and molecular identification of *Contracaecum* species in commonly commercialized marine fish from Turkish waters. A total of 475 marine fish, which belong to 21 different species, were sampled from the Aegean (FAO 37.3.1), Mediterranean (FAO 37.3.2), and Black Sea (FAO 37.4.2). The prevalence of *Contracaecum* L3 larvae in the Aegean Sea was identified as 10% in *Mugil cephalus*. All *Contracaecum* L3 larvae were molecularly characterized with RFLP targeting the ITS region and *rrnS* gene. Moreover, all larvae were analyzed by sequencing of ITS region, *rrnS* and *cox2* gene. All *Contracaecum* larvae were identified as *C. overstreeti* based on the *cox2* sequence analysis. This is the first report of *C. overstreeti* larvae in *M. cephalus* as paratenic and intermediate hosts. Furthermore, the analysis reveals novel information on ITS region. Additionally, the *rrnS* gene of *C. overstreeti* was also achieved and deposited in Genbank for the first time. The PCR-RFLP patterns of the ITS region and *rrnS* gene from *C. overstreeti* were presented in the present study. Consequently, the presence of *C. overstreeti* larvae in *M. cephalus* from the Aegean Sea may also potentially capable of inducing allergic sensitization in humans.

Keywords *Contracaecum overstreeti* · ITS region · *cox2* · *rrnS* · *Mugil cephalus* · Turkish waters

Introduction

Contracaecum species that belong to the family Anisakidae are important fish-borne larval nematodes with zoonotic significance. Adult *Contracaecum* species infect marine mammals and piscivorous birds as their definitive hosts; whereas, free or encapsulated third-stage larvae infect invertebrates and fish as their paratenic and intermediate hosts (Mattiucci and Nascetti 2008). The third-stage larvae of *Contracaecum* species may infect humans when invertebrates or fish are ingested raw or upon consumption of inadequately processed seafood (Schaum and Müller 1967; Im et al. 1995; Ishikura et al. 1996; Nagasawa 2012; Shamsi and Butcher 2011). Therefore, identifying and determining the presence of *Contracaecum* species larvae in different fish species used for human consumption is crucial in terms of seafood safety and public health. Moreover, the European Food Safety Authority (EFSA) suggests that collection of systematic data on the complete life cycle, geographical and seasonal distribution, prevalence, intensity, and anatomical location of parasites in wild caught fishery products is of public health importance and should be continued (EFSA 2010). Although there are various studies on the presence of *Contracaecum* spp. larvae in marine fish from Mediterranean Sea and across other seas (Merella and Garippa 2001; Szostakowska and Fagerholm 2007; Gutiérrez-Galindo et al. 2010; Shamsi et al. 2011a, b, 2018a, b; Garbin et al. 2013; Jabbar et al. 2013; Salati et al. 2013; Mattiucci et al. 2015; Pulleiro-Potel et al. 2015), only one morphological report concerning *Contracaecum* spp.

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larvae in red mullet from Turkish Black Sea coast was studied until now (Ozturk and Yesil 2018). Several studies have explored other larval anisakid and raphidascarid nematodes, where *Anisakis* and *Hysterothylacium* species were genetically identified from different marine fish species from Turkish waters (Pekmezci et al. 2013, 2014a, b; Keskin et al. 2015; Simsek et al. 2018). However, research to date has not yet investigated the occurrence and molecular identification of *Contracaecum* species larvae in Turkish waters, requiring further examination.

The current work aims to present only the occurrence and molecular identification of *Contracaecum* species in the Turkish waters in commonly commercialized marine fish that belong to 21 species using both PCR-RFLP analysis and sequencing of the ITS, *cox2*, and *rrnS* genes to provide epidemiological information for a risk assessment for *Contracaecum* species in consumed marine fish.

Materials and methods

Sampling data

A total of 475 marine fish that belong to 23 different species of teleosts were captured from the Aegean (FAO 37.3.1), Mediterranean (FAO 37.3.2), and Black Sea (FAO 37.4.2) between September 2016 and May 2017 (Supplementary material, Table S1). Fish species were targeted by their edibility and/or their geographic distribution within Turkish coastal waters. In addition, geographical coordinates of the sampling sites were obtained from fish markets.

Parasitological examination

A routine parasitological examination for anisakid larvae was visually performed under a stereomicroscope. Individual fish were dissected carefully and examined in the digestive track, visceral cavity, liver, kidney, gonads, and muscles. Eviscerated fish muscles were sliced in 3–5–mm-thick fillets and observed under white light to check for anisakid larvae using a dissecting microscope. Larvae were mechanically removed, counted, and placed in 70% ethanol. A total of 186 anisakid larvae were isolated from all marine fish. Anterior and posterior parts of the all anisakid larvae were individually cleared in lactophenol for morphological examination according to the morphological criteria proposed by Berland (1961), Cannon (1977), Deardorff and Overstreet (1980), Peter and Maillard (1988), and Shamsi et al. (2011b). Afterwards a total of five third stage larvae belonging to *Contracaecum* genus were detected by morphological examination of anisakid larvae. Then, five *Contracaecum* spp. larvae were subjected to PCR-RFLP and DNA sequencing to identify the species. The middle parts of *Contracaecum* larvae were used for DNA extraction. Measurements (the range, followed by the mean in parentheses) were given. Prevalence (P), mean intensity (mI), and mean abundance (mA) were calculated by Rózsa et al. (2000) and by using Quantitative Parasitology 3.0 program (Reiczigel and Rózsa 2005).

PCR amplifications and PCR-RFLP analysis

Five *Contracaecum* larvae were subjected to the molecular analysis. Total DNA was extracted from the middle part of larvae using the DNA extraction kit (Thermo Scientific). The nuclear ribosomal ITS regions (ITS-1, 5.8S subunit, ITS-2), the mitochondrial cytochrome C oxidase subunit II (*cox2*), and the small subunit of the mitochondrial ribosomal RNA (*rrnS*) gene were targeted to amplifications. ITS regions were amplified using the primers NC5 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) (Zhu et al. 1998). PCR conditions followed the protocol described by Pekmezci et al. (2014a). The *cox2* gene was amplified using 210 (5′-CAC CAA CTC TTA AAA TTA TC-3′) and 211 (5′-TTT TCT AGT TAT ATA GAT TGR TTT YAT-3′) (Nadler and Hudspeth 2000). PCR conditions and procedures followed those reported in Mattiucci et al. (2010). Then, the *rrnS* gene was amplified using MH3 (5′-TTG TTC CAG AAT AAT CGG CTA GAC TT-3′) and MH4.5 (5′-TCT ACT TTA CTA CAA CTT ACT CC-3′) (D'Amelio et al. 2007). PCR conditions were also used according to D'Amelio et al. (2007). PCR products were visualized on 1.5% agarose gel by UV transillumination. The amplified ITS and *rrnS* products were analyzed by RFLP technique, using the restriction enzymes Tsp509I (Thermo Scientific) (for the ITS), RsaI (Thermo Scientific), and DdeI (Thermo Scientific) (for *rrnS*) (D'Amelio et al. 2007; Farjallah et al. 2008). Then, digested products were electrophoresed in 2% agarose gel and photographed by UV transillumination.

DNA sequencing and phylogenetic analysis

All *Contracaecum* larvae were sequenced using ABI PRISM 310 genetic analyzer (Applied Biosystems) for ITS, *cox2*, and *rrnS* genes. The quality of the sequences were checked using Geneious R11 (Biomatters Ltd.) and Vector NTI Advance 11.5 (Invitrogen). Later, sequences were verified by forward and reverse comparisons, assembled, and edited with Contig Express in Vector NTI Advance 11.5 (Invitrogen) and Geneious R11 (Biomatters Ltd.). The obtained consensus sequences were compared with previously published data for identification by using the Basic Local Alignment Search Tool (BLAST) via GenBank database (Altschul et al. 1990). The mtDNA *cox2* sequences were aligned with other known *Contracaecum* species from waters birds in previous studies (Mattiucci et al. 2008a, 2010; Garbin et al. 2011) using ClustalW in MEGA 7.0 multiple sequence alignments (Thompson et al. 1994) and adjusted

manually. Concatenated sequences of mtDNA *cox2*, *rrnS*, and rDNA ITS (ITS-1 and ITS-2) were obtained by Geneious R11 (Biomatters Ltd.). A partition homogeneity test (Farris et al. 1994) carried out using PAUP 4.0b10 version (Swofford 2002) with 1000 replicates confirmed the homogeneity of the *cox2*, *rrnS*, ITS-1, and ITS-2 fragments ($P = 0.264$); thus, these sequences were treated as a single concatenated dataset. These sequence datasets were chosen according to the availability in GenBank database (Supplementary material, Table S2). Phylogenetic relationships were inferred using maximum likelihood (ML) with selection of the best model for nucleotide substitution by the Find Best DNA Model test implemented in MEGA 7.0 (Kumar et al. 2016). The general-time reversible model (GTR + G + I) was selected using Akaike information criterion (AIC). The phylogenetic tree on the mtDNA *cox2* and concatenated sequences of mtDNA *cox2*, *rrnS*, and rDNA ITS (ITS-1 and ITS-2) datasets obtained from *Contracaecum* specimens were examined with the ML analysis based on GTR + G + I and GTR + G values, by using MEGA 7, respectively (Kumar et al. 2016). Bootstrap confidence values were calculated with for 100 repetitions for ML (Felsenstein 1985). Bootstrap values ≥ 70 were considered well supported (Hillis and Bull 1993). The nucleotide sequences were deposited in GenBank database under the accession numbers: MG515224 for ITS regions, MG515234 for *rrnS*, and MG495095 for *cox2* gene. Reference specimens and isolated DNA samples are stored at the "Department of Aquatic Animal Diseases, Veterinary Medicine Faculty, Ondokuz Mayis University," Samsun, Turkey.

Results

Morphological and parasitic infection levels

Third stage larvae of *Contracaecum* were morphologically identified according to the identification keys of Berland (1961), Cannon (1977), Deardorff and Overstreet (1980), Peter and Maillard (1988), and Shamsi et al. (2011b). In the present study, all *Contracaecum* larvae were whitish nematodes with finely transversely striated cuticle. The larvae ($n = 5$) were measured about 20.1–22.6 (21.4) mm in long and 1.12–1.34 (1.2) mm in width. Anterior end was with three lips. Boring tooth was present at anterior end. Excretory pore was at the base of lips. Esophagus was muscular, ending in round ventriculus, and with short posterior appendix. Esophagus was measured 2.23–2.91 (2.65) mm in length, representing 11.09–12.87 (12.3) % of body length. Ventriculus was 156–168 (161) μm long and 171–176 (174) μm wide. Ventricular appendix was narrow and 781–822 (794) μm long, representing 28.2–35.0 (29.9) % of esophagus length. Large and broad intestinal cecum was present. Intestinal cecum was 2.18–2.22 (2.19) mm long, representing 76.2–97.7 (82.6) % of esophagus length. Intestinal cecum was much longer than ventricular appendix. Ratio of ventricular appendix to intestinal cecum 1:2.70–1:2.79 (1:2.75). Tail was 220–238 (226) μm long, conical, with a sharply pointed end and a spine. Spine was measured 27–45 (34) μm long. Therefore, our larvae classified as *Contracaecum* larval type I in the present study were morphologically similar to those described by Cannon (1977) in Queensland waters and Shamsi et al. (2011b) in Victoria and Western Australia from the *M. cephalus*.

In the present study, a total of 186 anisakid larvae were collected from all marine fish. Moreover, 102 *Anisakis* type I larvae, 79 *Hysterothylacium* larvae, and 5 *Contracaecum* larvae were morphologically identified. The overall prevalence (%), mean intensity (mI), and abundance (mA) of *Contracaecum* larvae in marine fish from Turkish waters were 0.21% (1/475), 5, and 0.01, respectively. *Contracaecum* larvae were not found in the Black and Mediterranean Seas, whereas prevalence (%), mI, and mA of *Contracaecum* larvae from in the Aegean Sea were identified as 1.29% (1/77), 5, and 0.06, respectively. An overall of 5 *Contracaecum* larvae were collected in the liver, kidney, and visceral cavity, although no larvae were found from fillets in *M. cephalus* from the Aegean Sea. Two of these larvae were found in the liver parenchyma, two larvae were slightly embedded in the kidney, and one larva was found in the visceral cavity. The prevalence of *Contracaecum* larvae was 10% in *M. cephalus* from the Aegean Sea.

PCR-RFLP results

PCR-RFLP analysis of representative ITS amplicons using Tsp509I produced five different fragments of 330, 180, 140, 110, and 70 bp for all *Contracaecum* larvae from Turkish waters (Fig. 1). Moreover, PCR-RFLP analyses of *rrnS* amplicons were digested with RsaI, which displayed two fragments of 320 and 120 bp (Fig. 2). On the other hand, DdeI revealed fragments of 300 and 250 bp for the same all larvae (Fig. 3).

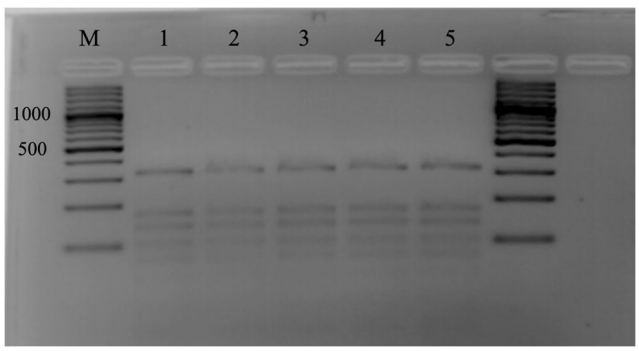

Fig. 1 RFLP patterns obtained by digestion of the ITS region with the restriction enzyme Tsp509I shown by the *Contracaecum* larvae. Lanes: 1–5 *Contracaecum* larvae, M: 100-bp DNA ladder

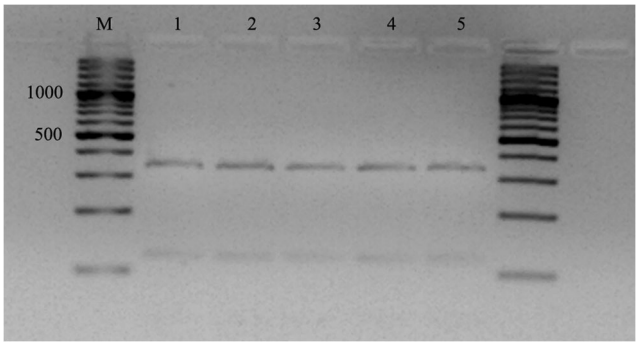

Fig. 2 RFLP patterns obtained by digestion of the *rrnS* gene with the restriction enzyme RsaI shown by the *Contracaecum* larvae. Lanes: 1–5 *Contracaecum* larvae, M: 100-bp DNA ladder

DNA sequencing and phylogenetic analysis

In the present study, no intraspecific nucleotide variability within all *Contracaecum* larvae (isolates GZP-1 to GZP-5) in *M. cephalus* from the Aegean Sea were observed in the ITS region, *cox2*, and *rrnS* genes. Therefore, the sequences of ITS, *cox2*, and *rrnS* of one *Contracaecum* larva (GZP-1) from the Aegean Sea, Turkey, was submitted to GenBank with the accession numbers MG515224, MG495095, and MG515234, respectively. The specimens of all *Contracaecum* larvae (isolate GZP-1 (MG495095) to GZP-5) in *M. cephalus* matched 98.6–99.8% of the previously reported reference gene sequences for the mtDNA *cox2* in *C. overstreeti* (Mattiucci et al. 2010) from Dalmatian pelican *Pelecanus crispus* in Greek waters, which was examined previously and deposited in GenBank (EU852343-EU852348) (Mattiucci et al. 2010). The *Contracaecum* larvae (MG495095) from *M. cephalus* in the Aegean Sea, Turkey, and *C. overstreeti* (EU852345-EU852348) from *P. crispus* in the Greek waters were clustered in the same clade, which were very well supported in the ML tree (Fig. 4) inferred from the mtDNA *cox2* sequence analysis. Moreover, the concatenated phylogenetic tree of the mtDNA *cox2*, *rrns*, and rDNA ITS (ITS-1 and ITS-2) sequences using ML analyses indicated that *C. overstreeti* clades were distinct

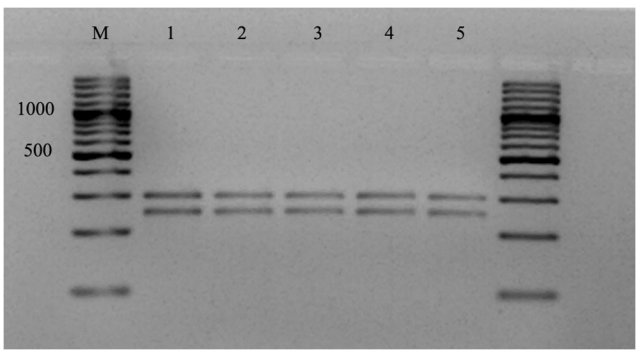

Fig. 3 RFLP patterns obtained by digestion of the *rrnS* gene with the restriction enzyme DdeI shown by the *Contracaecum* larvae. Lanes: 1–5 *Contracaecum* larvae, M: 100-bp DNA ladder

species by high bootstrap values (Fig. 5). Also, the mtDNA *cox2* sequence (isolate GZP-1, MG495095) was aligned with the same gene for *C. overstreeti* (EU852343-EU852348), which was previously deposited in GenBank (Fig. 6). In addition, the nucleotide percent identities among *rrnS* sequences of *Contracaecum* larvae from Aegean Sea, Turkey (MG515234), showed 98.1% identity with *C. multipapillatum* from Florida, USA, (EF030717) from GenBank (D'Amelio et al. 2007).

Discussion

The mtDNA *cox2* sequence analysis confirmed that third-stage larvae parasitizing the flathead gray mullet *M. cephalus* (Mugilidae) belong to the species *C. overstreeti*, a parasite at the adult stage of the Dalmatian pelican *P. crispus* from Greek waters (Mattiucci et al. 2010). Mitochondrial gene region has also been used in many studies (Mattiucci et al. 2003, 2008a, b, 2010; Garbin et al. 2011, 2013) due to its ability to species-based identification of *Contracaecum* specimens in a wide taxonomic range. Mitochondrial markers may provide reliable evidence for species identification of *Contracaecum* larvae occurring in fish (Mattiucci et al. 2010). Therefore, the occurrence of *C. overstreeti* larvae from Turkish waters was also proved by molecular evidence inferred from a mitochondrial marker used in the present study.

Contracaecum multipapillatum (von Drasche, 1882) Lucker, 1941 (sensu lato) consists of a complex of sibling species, which are the two species *C. multipapillatum* sp. A and sp. B, described as *C. gibsoni* and *C. overstreeti*, which are found in fish-eating birds that belong to the family Pelicanidae from Greek waters (Mattiucci et al. 2010). To date, the molecular identification of larval stages of *Contracaecum* spp. and *C. rudolphii* from different marine fish species from the Mediterranean Sea have been reported (Jabbar et al. 2013; Dezfuli et al. 2016); however, much less is known about the molecular identification of *C. overstreeti* larvae in marine fish from the Mediterranean Sea. Therefore, this is the first report of *C. overstreeti* larvae in *M. cephalus* (Mugilidae) as paratenic and intermediate hosts from the Mediterranean Sea. Moreover, *C. multipapillatum* s.l. larvae were reported in Mugilidae from Mexico, other regions of America, and Australia (Deardorff and Overstreet 1980; Iglesias et al. 1998, 2011; Valles-Ríos et al. 2000; Valles-Vega 2011; Valles-Vega et al. 2017; Shamsi et al. 2011b, 2018a; Jabbar et al. 2013).

Contracaecum sp. was morphologically reported as an adult only in Dalmatian pelican *P. crispus* in Turkey (Giriskin et al. 2012). However, to date, there is no molecular identification of *Contracaecum* species as an adult stage from fish-eating birds of the Pelicanidae and Ardeidae families from Turkey. For this reason, accurate *Contracaecum* species

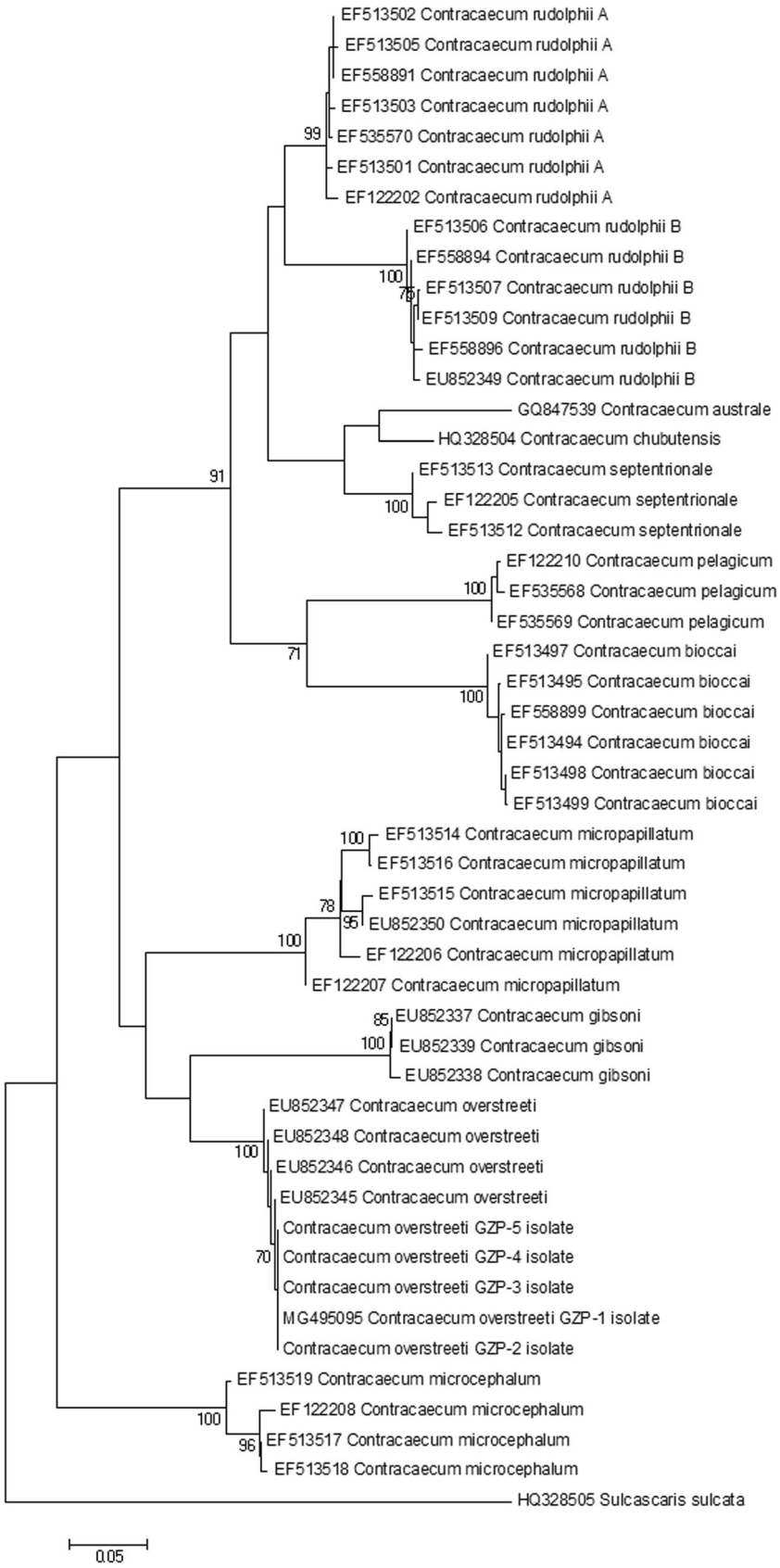

Fig. 4 Phylogenetic relationships between *C. overstreeti* (GZP isolate-Turkey) from the present study and other *Contracaecum* species as inferred by maximum likelihood obtained from mtDNA *cox2* gene. The scale bar indicates the distance in substitutions per nucleotide. Bootstrap values were calculated over 100 replicates and percentages ≥ 70% are shown at the internal nodes. *Sulcascaris sulcata* was used as outgroup

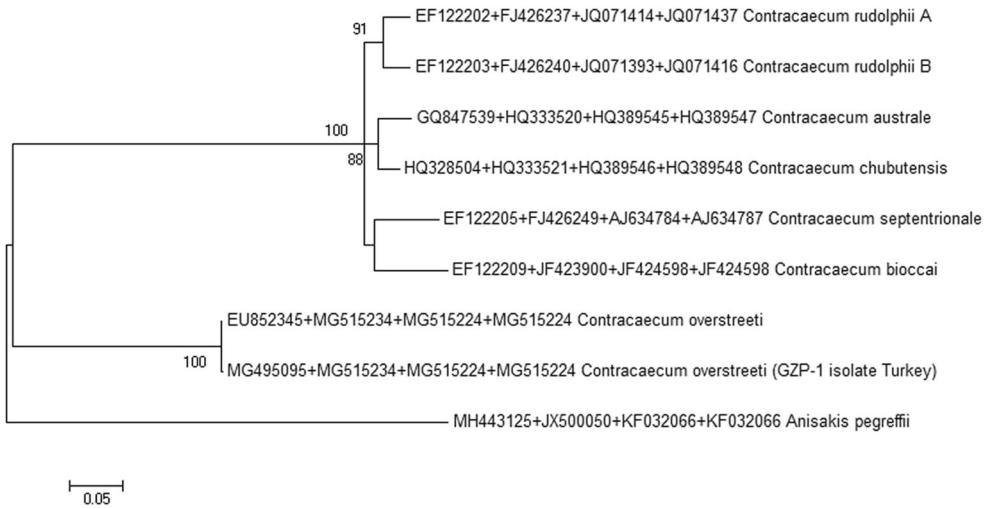

Fig. 5 Phylogenetic relationships between *C. overstreeti* (GZP isolate-Turkey) from the present study and other *Contracaecum* species as inferred by maximum likelihood obtained from the concatenated sequences of mtDNA *cox2*, *rrnS*, and rDNA ITS (ITS-1 and ITS-2). The scale bar indicates the distance in substitutions per nucleotide. Bootstrap values were calculated over 100 replicates, and percentages ≥ 70% are shown at the internal nodes. *Anisakis pegreffii* was used as outgroup

as an adult stage are exactly unknown from Turkish waters. Furthermore, molecular identification of *Contracaecum* larvae in fish species have also not been studied, and there is still no correct identification of the *Contracaecum* species in marine and freshwater fish species from Turkish waters. Until now, morphological identification has also been used to identify larvae of *Contracaecum* spp. in Turkey. Recently, *Contracaecum* sp. larvae in red mullet *Mullus barbatus ponticus* from the Turkish Black Sea coast was identified by only using light microscopy in Turkey (Ozturk and Yesil 2018). Therefore, we also provide the first molecular evidence of *Contracaecum* infection in fish from Turkish waters in the present study.

Flathead gray mullet *M. cephalus* (Mugilidae) is a frequently consumed marine fish in the Turkish and European markets. This mugilid species are commonly found in the Eastern Atlantic, Mediterranean, and Black Sea (Froese and Pauly 2017). Previously, *Contracaecum* larvae found in the liver, kidney, and muscle of *M. cephalus* originating in the Gulf of Mexico, California, and Australia were identified as *C. multipapillatum* (Deardorff and Overstreet 1980; Iglesias et al. 1998; Olivero-Verbel et al. 2005; Shamsi et al. 2011b). However, to date, no data has been reported about the occurrence of *C. overstreeti* larvae in this food fish. In the present study, *C. overstreeti* was reported for the first time infecting the liver, kidney, and visceral cavity of *M. cephalus* as paratenic and intermediate hosts. The occurrence of *C. overstreeti* in *M. cephalus* may present a health risk for consumers. *Contracaecum multipapillatum* larvae were previously found in the muscle of *M. cephalus* (Iglesias et al. 1998), while *Contracaecum* larvae in the muscle of *M. cephalus* were not detected in the present study. Moreover, definitive host of *C. gibsoni* and *C. overstreeti* are the Dalmatian pelican *P. crispus* from Greek waters as a type-locality. *Pelecanus crispus* feeds specifically on eels, mullets, and gobies in brackish waters (Mattiucci et al. 2010). Similarly, *C. overstreeti* larvae were detected here from the mullet and *C. overstreeti* larvae in marine fish hosts caught in the Aegean Sea coasts of Turkey, which are closed to type-locality of *C. overstreeti* as an adult stage from type-host. This is the first data to be collected on the larval stage of *C. overstreeti* in fish hosts that are caught close to the type-locality.

In the Mediterranean Sea, *Contracaecum* larvae were observed most prevalent in *Diplodus* spp. (16%), *Sparus aurata* (15.8%), *Mullus* spp. (14.6%), and *Liza ramada* (13.6%) (Salati et al. 2013). Gutiérrez-Galindo et al. (2010) found *Contracaecum* larvae in *Scomber scombrus* and *Trachurus trachurus* with prevalence values of 2% and 0.7%, respectively, while for *Liza aurata* prevalence value was 9.0% (Merella and Garippa 2001). In our study, *Contracaecum* larvae were only found in *M. cephalus* and showed 10% prevalence as shown in Table S1. Although *Contracaecum* larvae were localized in the muscles of *Trachurus* sp., *Sparus aurata*, and *L. ramada* (Salati et al. 2013), no *Contracaecum* larvae were found in the muscle of *M. cephalus* in the present study. In addition, we did not find *Contracaecum* larvae in the farmed *Dicentrarchus labrax* and *S. aurata* reared in floating cages, in correspondence to the study of Salati et al. (2013). However, high prevalence of *Contracaecum* larvae was recorded in *S. aurata*, *D. labrax*, and *Diplodus* sp. reared in land-based semi-intensive tanks with water lagoon (Salati et al. 2013).

The present study shows that prevalence and abundance of *Contracaecum* larvae in the examined marine fish is relatively low. It is important to note that only routine visual examination was used to isolate *Contracaecum* larvae in this study. The prevalence and number of larvae may have been higher than *Contracaecum* larvae found in marine fish in the present study if an incubation method had been used (Shamsi and Suthar 2016; Shamsi et al. 2018a, b).

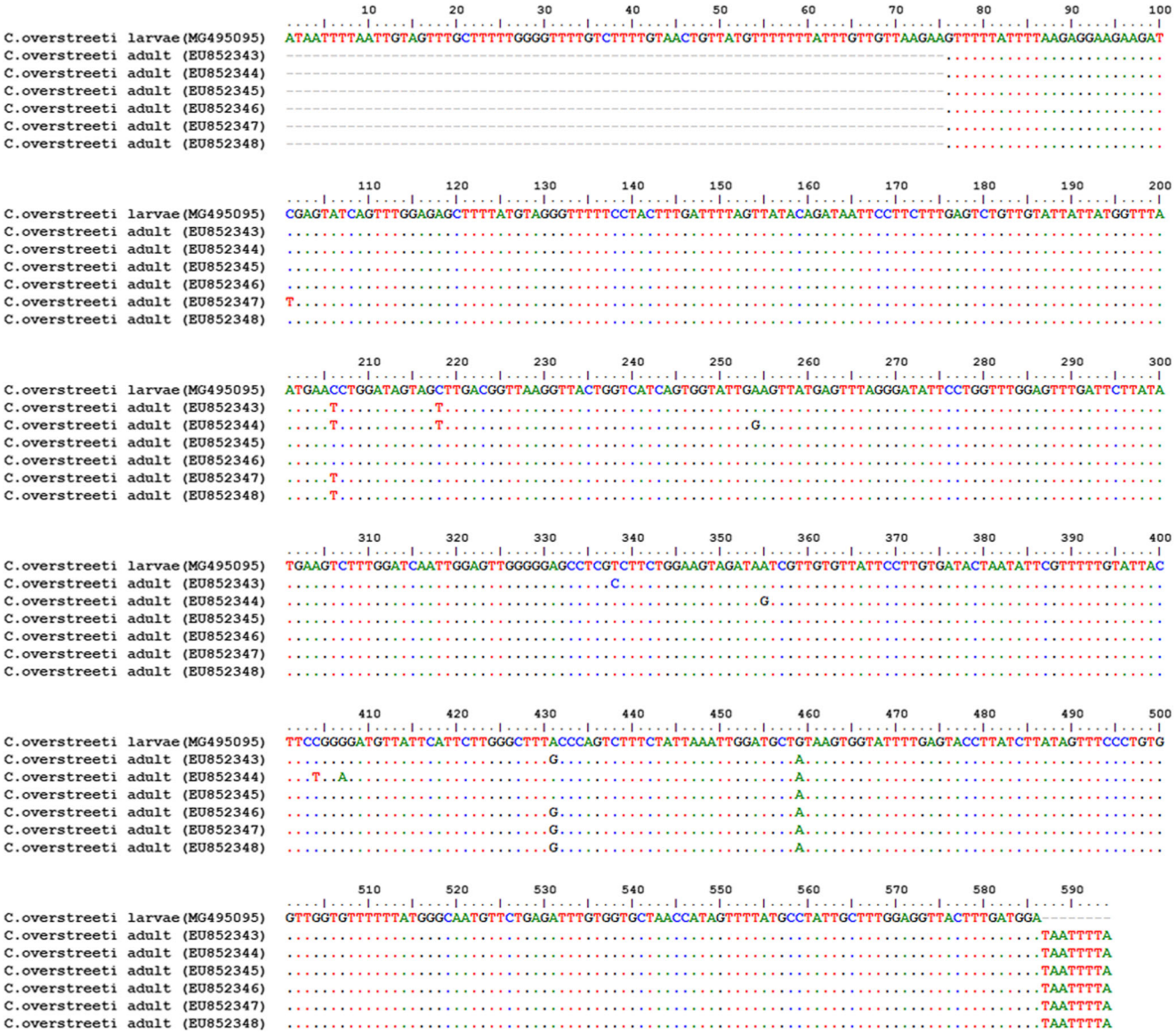

Fig. 6 Alignment of the mtDNA *cox2* (586 bp) sequence of the *C. overstreeti* larvae isolated from *Mugil cephalus* with respect to the adult *C. overstreeti* from *Pelecanus crispus* have previously been sequenced and deposited in GenBank under the accession numbers as published in Mattiucci et al. (2010). The alignment was performed using BioEdit. Dots indicate identity with the first sequence and dashes are inferred insertion–deletion events

The sequencing of multiple loci (i.e., mtDNA *cox2*, *rrnS* genes, and rDNA ITS regions) have been widely recommended for an accurate differentiation and identification of *Contracaecum* species (D'Amelio et al. 2007; Farjallah et al. 2008; Garbin et al. 2011; Shamsi et al. 2008, 2011a, b, 2018a, b; Mattiucci et al. 2003, 2008a, 2010, 2015). However, only the ITS and *rrnS* PCR-RFLP patterns of *C. rudolphii* B and C have been previously reported from fish-eating birds (D'Amelio et al. 2007; Farjallah et al. 2008). Up to date, there has been no report on the PCR-RFLP patterns of *C. overstreeti*. Moreover, our PCR-RFLP patterns show that the restriction enzymes Tsp509I and RsaI were found to be differ from *C. rudolphii* B and *C. rudolphi* C by the fragment lengths and patterns (D'Amelio et al. 2007; Farjallah et al. 2008). More specifically, PCR-RFLP analysis of the ITS amplicons using the Tsp509I produced fragments of 330, 180, 140, 110, and 70 bp for *C. overstreeti* compared with fragments of 480, 220, 170, and 80 bp for *C. rudolphii* C and fragments of 480, 360, and 80 bp for *C. rudolphii* B (Farjallah et al. 2008). PCR-RFLP analysis of *rrnS* amplicons from *C. overstreeti* was digested with RsaI displayed 320 and 120 bp whereas, displayed patterns of 271, 81, 64, and 37 bp for *C. rudolphii* C, a pattern similar to that of *C. rudolphii* B produced 270, 82, 64, and 49 bp (D'Amelio et al. 2007). Thus, these different sizes of PCR-RFLP patterns may only have a diagnostic value among *C. rudolphi* B, C, and *C. overstreeti.*

The only sequence registered in GenBank is the mtDNA *cox2* sequence of *C. overstreeti* (=*Contracaecum multipapillatum* sp. B) (Mattiucci et al. 2010). Within the present study, sequences of the mtDNA *cox2* gene (isolate GZP-1 (MG495095) to GZP-5) were clustered in the same well-supported clade, where same species have previously been sequenced and deposited in GenBank database (Fig. 4). According to the result of the mtDNA *cox2* analysis, all larvae were classified as *C. overstreeti* (Fig. 4). In addition, novel information on ITS region and *rrnS* gene of C. *overstreeti* was also achieved and deposited in Genbank for the first time.

Conclusion

The Aegean Sea coast of Turkey is a popular tourist destination for national and international visitors, where marine fish is among commonly consumed food. *Contracaecum multipapillatum* sensu lato is a species complex comprising *C. gibsoni* (=*C. multipapillatum* sp. A), and *C. overstreeti* (=*C. multipapillatum* sp. B) are found in fish-eating birds from Greek waters (Mattiucci et al. 2010). Moreover, *C. multipapillatum* may cause a potential public health problem (Vidal-Martínez et al. 1994). Recently, *C. multipapillatum* larval antigens were shown to being potentially capable of inducing allergic sensitization, which was proven in mice (Fontenelle et al. 2018). Therefore, *C. overstreeti* larval antigens may also potentially capable of inducing allergic sensitization in humans. These results are also consistent with our previous studies about fish consumers (Pekmezci 2014; Pekmezci et al. 2014a). Therefore, precautions in the Hazard Analysis Critical Control Point (HACCP) program should be revised to decrease the risk of anisakid-related allergies. As hazards from fish-borne parasitic zoonoses are tremendously important for public health, it is crucial to take appropriate precautions, such as including RASFF (Rapid Alert System for Food and Feed) in the legal system for fish that are captured or imported in Turkey.

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