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Morphological, histological, and molecular evidence of *Myxobolus spinacurvatura* (Cnidaria: Myxosporea) from *Mugil cephalus* in the Turkish Black Sea coast

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Abstract: In the present study, a total of 30 *M. cephalus* captured from the Samsun coast of the Black Sea, Turkey were examined for myxozoa infections. Different sizes of whitish and rounded cyst-like plasmodia were macroscopically detected in the liver and intestine wall of *M. cephalus*. Morphological and histological examinations were performed. The SSU rDNA was amplified using nested PCR and myxosporean-specific primers. The present *Myxobolus* species was identified as *M. spinacurvatura* based on the spore morphology and host/organ specificity. In the present study, morphological, histological, and molecular data on *M. spinacurvatura* were presented. Furthermore, BLASTn research showed that SSU rDNA sequences of *M. spinacurvatura* (accession number MH374629) were identical to that of the reference sequence of *M. spinacurvatura* (accession from AF378341) recorded previously. The current study includes the first record and molecular evidence of *M. spinacurvatura* infecting *M. cephalus* from the Turkish waters. To date, there is no report on comprehensive morphological, histopathological, and molecular data of *M. spinacurvatura* in *M. cephalus* as a typical host. Moreover, the new valid SSU rDNA sequence of *M. spinacurvatura* (accession number MH374629) was the second record in the GenBank. The present SSU rDNA sequence can also be used to construct a phylogenetic tree with other mugiliform-infecting *Myxobolus* species worldwide.

Key words: *Myxobolus spinacurvatura*, morphology, histology, SSU rDNA, *Mugil cephalus*, Turkish waters

1. Introduction

The flathead grey mullet *Mugil cephalus* (Mugilidae) is a euryhaline teleost species distributing in tropical, subtropical, and temperate areas between latitudes 42 °N and 42 °S. This species is catadromous, frequently found coastally in estuaries and freshwater environments [1]. Myxozoas are common parasites of fish and some invertebrates and are also the causative agents of several economically important diseases in aquaculture systems and wild stocks [2]. The genus *Myxobolus* Bütschli, 1882 (Cnidaria, Myxobolidae) is the largest group within the myxozoas and more than 900 species have been described [3,4]. Based on spore morphology, host/organ specificity and tissue tropism with the molecular marker are mainly provided for identification of a new or existing myxozoa and redescription of insufficiently described species [5,6].

Myxobolus spinacurvatura was first described in the mesentery, brain, spleen, and pancreas of grey mullet *Mugil cephalus* from the Ago Bay in the city of Shima, Mie Prefecture, Japan by Maeno et al. [7]. Subsequently, *M. spinacurvatura* was reported from the same fish host collected from Lake Ichkeul and Ghar El Melh (Tunisia), New South Wales coast (Australia), Delta Ebro (Spain), and the Black and Azov Seas (Crimea, Ukraine) [8–12]. Although *M. spinacurvatura* has been reported many times from different parts of the world, there was only 1 datum available on the 18S rDNA sequences of *M. spinacurvatura* in the GenBank database (accession numbers AF378341) [13].

A small number of the species of the genus *Myxobolus* have been recorded from the Turkish mullets. To date, only 4 species have been reported from *M. cephalus* in Turkish waters. *Myxobolus episquamalis*, *M. exiguus*, *M. ichkeulensis*, and *M. muelleri* were morphologically identified in *M. cephalus* [14–18]. Although these species were reported from *M. cephalus* in the different habitats, there is no comprehensive morphological, histopathological, and molecular evidence of *M. spinacurvatura* in *M. cephalus* as a typical host. Therefore, the current study aimed to provide the morphology, host

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and tissue specificity, and SSU rDNA sequence for the validity of *M. spinacurvatura* Maeno et al. 1990 in *Mugil cephalus* from the coast of the Black Sea in Turkey.

2. Materials and methods

2.1. Host sampling

Freshly caught specimens of the grey mullet, *M. cephalus* (n = 30) were periodically purchased from commercial fishermen at Kızılırmak Delta, Samsun coast located by the Black Sea, Turkey (FAO 37. 4. 2) (41°44′04.2″N 35°57′23.0″E) in the period between October 2017 and December 2017.

2.2. Morphological and histological examination

All fish were transferred in ice to the laboratory. *Mugil cephalus* were examined for myxozoa infections [19]. For studying the occurrence of plasmodia; skin, fins, and internal organs were examined externally and under a dissecting microscope. After mature plasmodia were detected in the organs, plasmodia were isolated with a needle and opened on a slide. Infected liver containing mature plasmodia were fixed in 10% formaldehyde and embedded in paraffin. Paraffin blocks were cut into 5-µm slices using a microtome and stained with Hematoxylin-Eosin (H&E). Some fresh spores were prepared in glycerine jelly onto the slide for morphological examination. Subsamples of fresh spores were preserved in absolute ethanol for molecular identifications. Morphological and morphometric characters of 20 myxospores were characterized as previously reported by Lom and Arthur [20] in reference to slide preparations. The myxospores were photographed and measured with a light microscope equipped with differential interference contrast (Nomarski DIC) optics connected to a digital camera. All measurements are presented in micrometers (µm). Morphological measurements were presented as the mean followed by the minimum and maximum values in parenthesis.

2.3. DNA extractions and PCR analysis

Myxobolus spores were centrifuged at low speed, suspended in digestion solution, and incubated at 56 °C overnight. DNA was extracted using a commercial DNA extraction kit (Thermo Scientific), according to the manufacturer's instructions. The SSU rRNA gene was amplified with the primers ERIB1 and ERIB10 [21]. A total of 50 µL PCR reactions including 20–50 ng DNA, 2X Hot start PCR master mix (Thermo Scientific), 0.4 µM of each primer. Amplification of first PCR conditions was: 30 s at 95 °C, 50 s at 43 °C, and 120 s at 72 °C for 35 cycles, and a 10 min extension at 72 °C. Then, the second PCR was carried out in a final volume of 50 µL and included 1 µL of amplified DNA, 2X Hot start PCR master mix (Thermo Scientific), 0.4 µM of each primer. Amplification using the MyxospecF-MyxospecR primer set [22] was run for 30 s at 95 °C, 50 s at 40 °C, and 90 s at 72 °C for 35 cycles, and products were subjected to a final extension at 72 °C for 10 min and held at 4 °C. PCR amplicons were loaded into the 1.5% agarose gel (Prona) and electrophoresis was applied in a TBE buffer. The second PCR amplicon was sequenced by the Sanger method (Macrogen, Amsterdam, Netherlands) using MyxospecF-MyxospecR primer pairs.

2.4. Phylogenetic analyses

The obtained sequences were checked by Vector NTI Advance 11.5 (Invitrogen) and Geneious R11 (Biomatters Ltd) using Phred values. Then, sequences were assembled and edited using Contig Express in Vector NTI Advance 11.5. The consensus sequences were compared with previously published data for identification by using BLAST via GenBank [23]. The 18S rDNA sequences were aligned with other known *Myxobolus* species from *M. cephalus* in previous studies [10,13,18,24,25] using ClustalW in MEGA 7.0 multiple sequence alignments [26] and adjusted manually. The phylogenetic tree was constructed using neighbor-joining (NJ) analysis in MEGA 7.0 [27]. The Kimura 2-parameter (K2P) model was used in the analysis. The species *Ceratomyxa shasta* was chosen as the out group. The mugilid infecting myxobolid sequence sets were built with 1000 bootstrap replications for the NJ reconstruction [28]. Bootstrap values ≥ 70 were considered well supported [29].

3. Results

We concluded that our isolate (GZP-2018-Samsun2) is *M. spinacurvatura* Maeno, Sorimachi, Ogawa & Egusa, 1990 based on the spore morphology, biological traits (host/organ specificity and tissue tropism), and molecular data.

***Myxobolus spinacurvatura* Maeno, Sorimachi, Ogawa & Egusa, 1990 (Figures 1 and 2)**

Host: Grey mullet, *Mugil cephalus* Linnaeus, 1758 (Mugilidae)

Location: Kızılırmak Delta, Samsun, Turkey (41°44′04.2″N 35°57′23.0″E)

Site of infection: Different sizes of whitish and rounded cyst-like plasmodia were macroscopically detected in the liver and intestine wall of *M. cephalus* (Figures 1A and 1B). The intensity of infection was variable ranging from 20 to 40 plasmodia/fish. None of the dissected fish presented external symptoms of infection or disease.

Type material: Reference glycerine jelly and histopathological preparations were deposited in the Department of Aquatic Animal Diseases, Veterinary Faculty, Ondokuz Mayıs University, Samsun, Turkey, coll. No. OMU DAA, 2017. 11–15. The SSU rRNA gene sequence of *M. spinacurvatura* was submitted to the GenBank database (accession number: MH377061).

Prevalence of infections: 16.6% (5 out of 30), 10–21 cm sized fish.

Histological findings: The histological examination revealed the development of plasmodia (p) surrounded by connective tissues encapsulated in liver tissue in various sizes. Moreover, degenerative changes in hepatocytes and severe hyperemia were especially apparent in regions around the plasmodium (Figures 1C and 1D).

Myxospores (Figures 2A–2C): Mature myxospores were round. The spores were 11.47 (10.36–12.19) µm

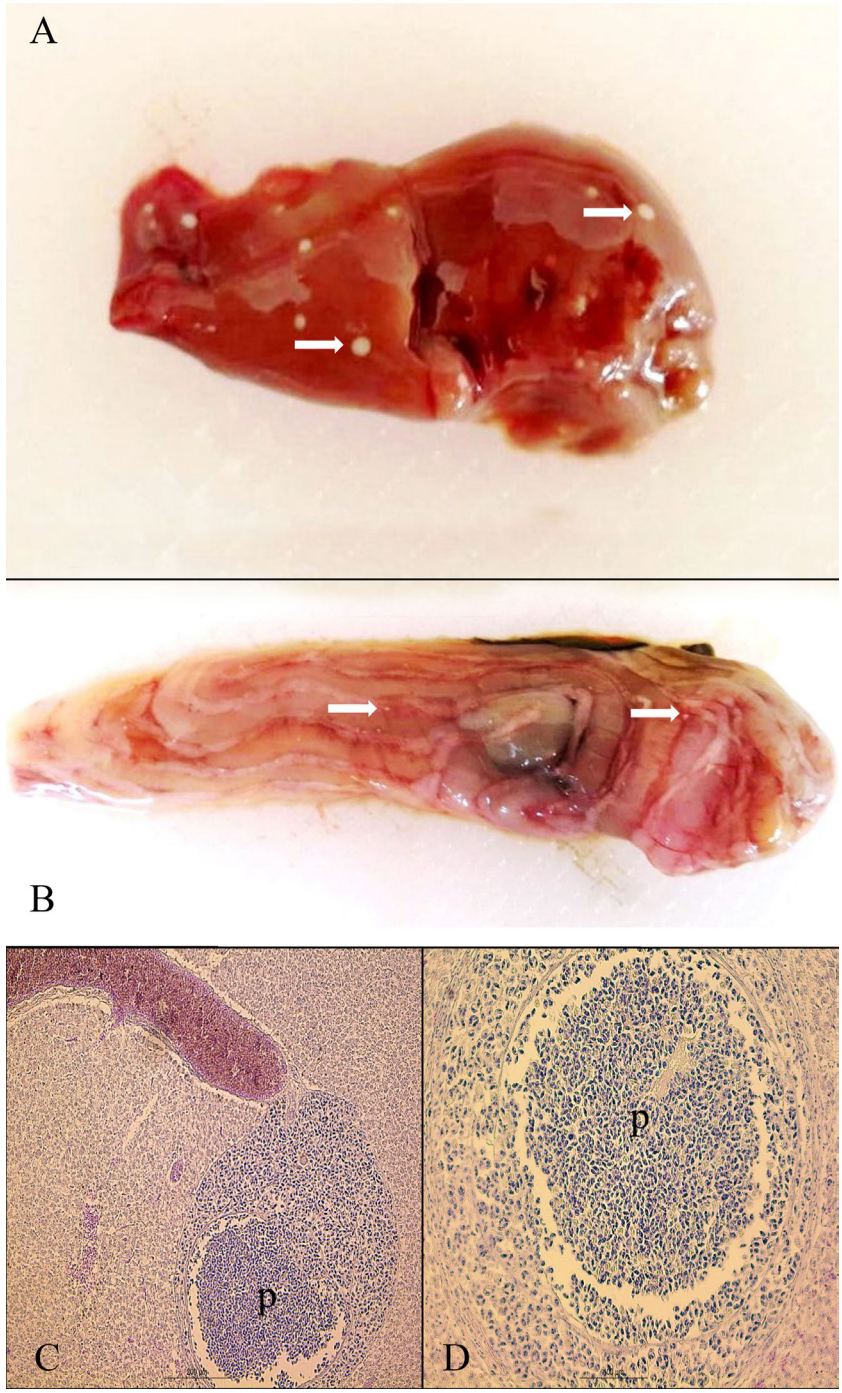

Figure 1. Photographs of *Myxobolus spinacurvatura* plasmodia. Whitish and rounded plasmodia (white arrows) in the liver (A) and the intestine wall (B). Histological section of liver from *Mugil cephalus* and Hematoxylin-Eosin (H&E) sections showing plasmodium (p) of *Myxobolus spinacurvatura* (C: H&E, X100, D: H&E, X200).

long (n = 20), 9.8 (9.31–10.49) µm wide (n = 20), and 7.03 (6.64–7.12) µm thick (n = 10). The 2 polar capsules were pear-shaped, not equal in size, 4.74 (4.02–5.85) µm long (n = 20) and 3.23 (2.96–3.49) µm wide (n = 20). Polar filaments were coiled 4–5 turns in the polar capsule (Figure 2C). Sutural edge markings were distinctly seen in fresh spores.

Molecular data: No intraspecific nucleotide variability within both the liver and intestine isolate of *M. spinacurvatura* from the Black Sea was observed in the 18S rDNA sequences. The BLAST search indicated that the 18S rDNA sequence of our isolate GZP-2018-Samsun2 (MH377061) from *M. cephalus* showed 99.21% similarity to *M. spinacurvatura* (AF378341). For this reason, our *Myxobolus* isolate (GZP-2018-Samsun2) molecularly identified to belong to *M. spinacurvatura* Maeno et al. 1990.

4. Discussion

The flathead grey mullet *M. cephalus* Linnaeus, 1758 is a cosmopolitan coastal fish species distributed in the coastal waters of Europe, Asia, Africa, Australia, America, and Oceania [1]. Several myxosporean parasites have been reported as serious pathogens of mugilid fish species [12,30]. Moreover, a great number of myxosporean species were recorded in *M. cephalus* among other mugiliform fish species. To date, 36 species of myxosporea have been reported, and among them are *M. muelleri*, *M. ichkeulensis*, *M. spinacurvatura*, *M. exiguus*, *M. parvus*, and *M. episquamalis* only 6 cosmopolite species in *M. cephalus* [12]. However, *M. spinacurvatura* was found in the liver of *Trachurus trachurus* from the Aegean Sea [31].

The morphological, biological (host/organ specificity), and molecular data obtained in the current study confirm the identification of the species as *M. spinacurvatura*, initially described by Maeno et al. [7]. This *Myxobolus* species was firstly described in the mesentery, brain, liver, spleen, and pancreas of *M. cephalus* based on traditional criteria, including tissue tropism and detailed light and electron microscopic examination of spore morphology [7] and afterward Kent et al. [13] provided a supplemental data on *M. spinacurvatura* from the type host with molecular data of the SSU rDNA sequence (AY129315). In the present study, the 18S rDNA sequences of our isolate (GZP-2018-Samsun2) showed 99.21% identity with *M. spinacurvatura* (AF378341). Therefore, we molecularly identified our *Myxobolus* species as *M. spinacurvatura*. To date, 1 SSU rDNA sequences of *M. spinacurvatura* were molecularly characterized and submitted in the GenBank database under accession numbers AF378341 [13]. Furthermore, the new valid SSU rDNA sequence of *M. spinacurvatura* (accession number MH374629) obtained from *M. cephalus* was the second record in the GenBank database.

Currently, based on spore morphology, host/organ specificity and tissue tropism with the molecular

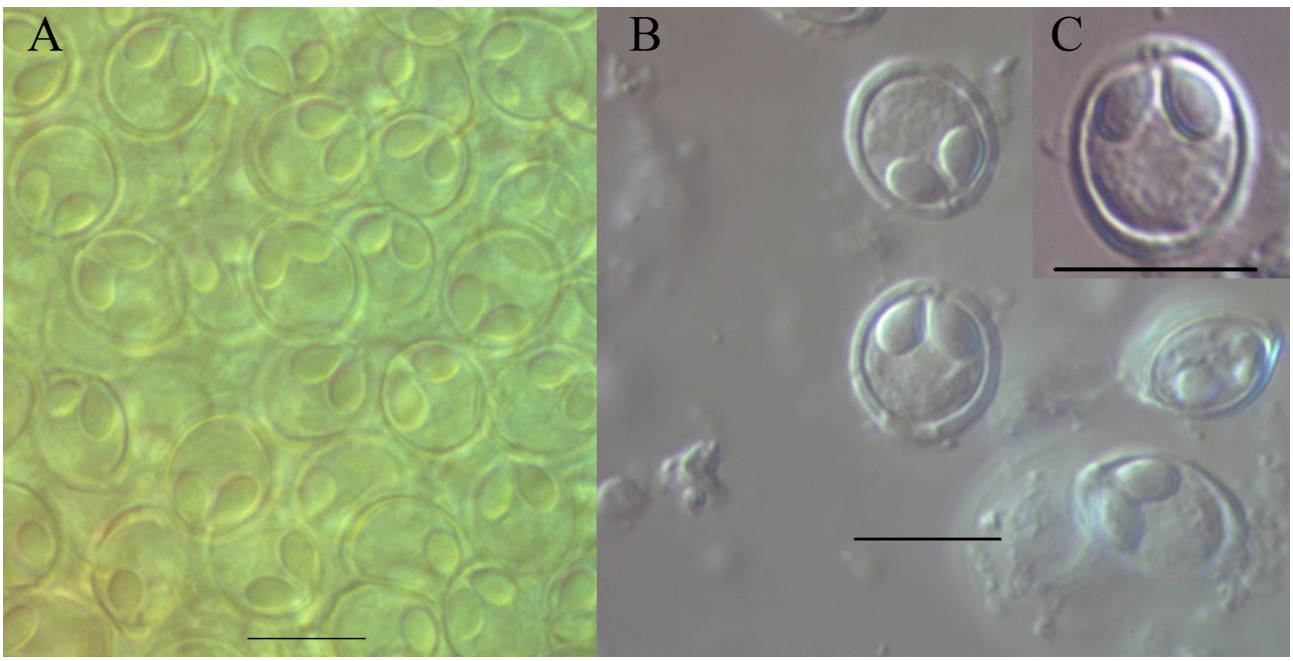

Figure 2. Nomarski differential interference contrast images of *Myxobolus spinacurvatura* from the liver of the *Mugil cephalus* (A-C). Scale Bar = 10 µm.

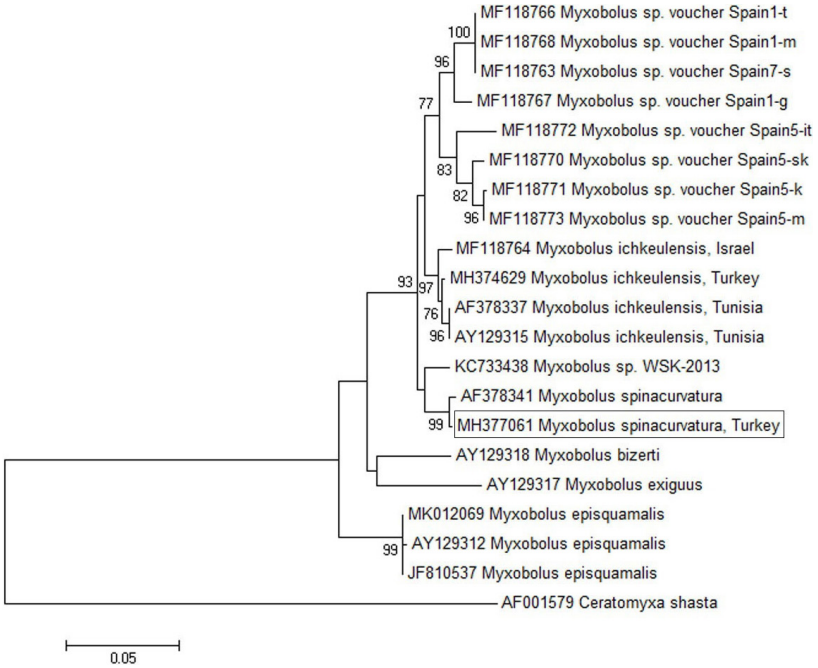

Figure 3. Phylogenetic tree generated by NJ analysis of the SSU rRNA sequences of *M. spinacurvatura* and other mugiliform-infecting *Myxobolus* species. Numbers at nodes indicate the bootstrap values. *Ceratomyxa shasta* was used as the out-group.

marker are mainly useful for new myxosporean species and redescription of insufficiently described species [10,12,18,25,32–35]. In Turkey, *M. ichkeulensis* was previously identified from the gills of *M. cephalus* based on the myxosporean morphology, host and tissue specificity, and SSU rRNA sequence [18]. This study has 3 approaches in combination (morphology, biological traits, and molecular markers) for the validity of *M. spinacurvatura* Maeno et al. 1990 in *M. cephalus* in Turkish waters for the first time. Moreover, this is also the first report of *M. spinacurvatura* Maeno et al. 1990 in *M. cephalus* from Turkish waters. Although the present and previous studies [18] on the Turkish Black Sea coast were performed on the same mugilid fish species, 2 different *Myxobolus* species were morphologically and molecularly identified in these studies. The phylogenetic tree showed that our isolate was clustered with reference and the first sequence of *M. spinacurvatura* (accession from AF378341) from *M. cephalus* (Figure 3). Additionally, comparisons of spore morphometric data among the *M. spinacurvatura* spores in *M. cephalus* were presented in Table.

The myxosporea species is strictly connected to a specific tissue of the host [6]. *Myxobolus spinacurvatura* plasmodia were described in the mesentery, brain, spleen, and pancreas of *M. cephalus* as a typical host [7]. Besides, *M. spinacurvatura* infection was also caused by the spinal curvature [36]. Within the present study, lordosis or scoliosis and abnormal findings in *M. cephalus* were not externally observed and *M. spinacurvatura* plasmodia were only found in the liver and intestine wall (Figures 1A and 1B).

Table. Comparison of spore morphometric data (dimensions in μm) of *Myxobolus spinacurvatura* infection in *Mugil cephalus*.

Spore length	11.47 (10.36–12.19)	11.5 (10.5–12.5)
Spore width	9.8 (9.31–10.49)	9.8 (9–11)
Spore thickness	7.03 (6.64–7.12)	6.7 (6–7.5)
Polar capsule length	4.74 (4.02–5.85)	4.6 (3.5–5.5)
Polar capsule width	3.23 (2.96–3.49)	2.9 (2.5–3.5)
Locality	Turkey: Black Sea coast, Kızılırmak Delta	Japan: Bay of Mie Prefecture
Reference	Present study	Maeno et al. 1990

In conclusion, the current research provides the morphology, host, and tissue specificity and SSU rDNA

sequence for the validity of *M. spinacurvatura* Maeno et al. 1990 in *M. cephalus* as typical host. This is the first record and molecular evidence of *M. spinacurvatura* in Turkish waters. Moreover, our molecular data of *M. spinacurvatura* was the second record in the GenBank. The present SSU rDNA sequence can also be used to construct a phylogenetic tree with other mugiliform-infecting *Myxobolus* species worldwide.

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