

# First molecular data on the validity of *Myxobolus ichkeulensis* (Cnidaria: Myxosporea) from *Mugil cephalus* (Mugilidae) in Turkish waters

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**Abstract:** To date, there is no report on molecular characterisation of *Myxobolus ichkeulensis* in *Mugil cephalus* (Mugilidae) in Turkish marine waters. The aim of the current research was to provide the first molecular data on *M. ichkeulensis* from *M. cephalus* in Turkish Black Sea coasts. A total of 40 *M. cephalus* specimens were examined for the presence of *M. ichkeulensis* in the Turkish Black Sea coasts during January 2017 and April 2017. In the present study, *M. ichkeulensis* was identified based upon the myxospore morphology, host and tissue specificity, and SSU rRNA sequence. Phylogenetic analysis using neighbor-joining also revealed that our isolate (#GZP-2018-Samsun1) was clustered with other *Myxobolus* species that infect *M. cephalus*. The current study includes the morphological and histopathological descriptions and first molecular data on *M. ichkeulensis* in *M. cephalus* in Turkish marine waters. *M. ichkeulensis* was also reported from the *M. cephalus* in the coast of the Black Sea in Turkey for the first time. Moreover, our current genetic data was recorded as the new valid SSU rRNA sequence (MH374629) for *M. ichkeulensis* in the GenBank database.

**Keywords:** Histopathology, morphology, *Myxobolus ichkeulensis*, SSU rRNA, Turkey

## Türkiye sularında *Mugil cephalus*'tan (Mugilidae) *Myxobolus ichkeulensis*'in (Cnidaria: Myxosporea) geçerliliğine ilişkin ilk moleküler veriler

**Özet:** Bugüne kadar Türkiye karasularındaki *Mugil cephalus* (Mugilidae)'da *Myxobolus ichkeulensis* türünün moleküler karakterizasyonu ile ilgili kayıt yoktur. Bu araştırmanın amacı Türkiye'nin Karadeniz kıyılarında *M. ichkeulensis* türünün *M. cephalus*'lardaki varlığını hakkında ilk moleküler verileri sağlamaktır. Ocak 2017 ile Nisan 2017 tarihleri arasında Türkiye'nin Karadeniz kıyılarında *M. ichkeulensis* türünün varlığı için toplam 40 adet *M. cephalus* incelendi. Bu çalışmada *M. ichkeulensis* türü spor morfolojisi, konak ve doku spesifitesi ile SSU rRNA sekansına dayanarak teşhis edildi. İzolatımız (#GZP-2018-Samsun1) neighbor-joining metodu kullanılan filogenetik analizde *M. cephalus*'ları enfekte eden diğer *Myxobolus* türleri ile kümelendi. Mevcut araştırma, Türkiye sularında *M. cephalus*'ta *M. ichkeulensis* türünün geçerliliği üzerine morfolojik ve histopatolojik tanımlamalar ile ve ilk moleküler verileri içermektedir. Türkiye'nin Karadeniz kıyılarında ilk kez *M. cephalus*'ta *M. ichkeulensis* türü de bildirildi. Ayrıca mevcut genetik verilerimiz GenBank veri tabanındaki *M. ichkeulensis* türü için yeni geçerli SSU rRNA dizisi (MH374629) olarak kaydedildi.

**Anahtar sözcükler:** Histopatoloji, morfoloji, *Myxobolus ichkeulensis*, SSU rRNA, Türkiye

## Introduction

Thousands of myxosporean parasites are known to cause diseases in various marine and freshwater fish (18, 19). The genus *Myxobolus* Bütschli, 1882 is the largest group of Myxobolidae contains over 850 species described (7, 8). Based on spore morphology, host/ tissue tropism with the molecular marker are especially provided

for identification of a new or existing myxozoa species and re-description of incompletely described species (11, 20). *Myxobolus ichkeulensis* was firstly described from the grey mullet *Mugil cephalus* in Ichkeul lagoon in Tunisia by Bahri and Marques (4). Later, there have been few reports on *M. ichkeulensis* in *M. cephalus* from Lake Ichkeul (Tunisia), Ebro River Delta (Spain), Baje de

Gorée (Senegal), Black and Azov Seas (Crimea, Ukraine), Camlık Lagoon (north-eastern Mediterranean, Turkey) and Japan Sea (3, 9, 23, 24, 28, 31). Whereas, this *Myxobolus* species has been reported in different marine sources of the world, there were only two SSU rRNA sequences of *M. ichkeulensis* molecularly characterized and submitted to the GenBank with the accession numbers: AF378337 and AY129315 (3, 14).

Up to now, only four *Myxobolus* species have been morphologically identified and reported in *M. cephalus* from Turkish marine waters: *M. episquamalis*, *M. exiguus*, *M. ichkeulensis* and *M. muelleri* (2, 6, 23, 30). Whereas those species were morphologically described, there is no detailed molecular and histopathological data of *M. ichkeulensis* in *M. cephalus* in Turkish marine waters. The aim of the current research was to provide first molecular data on the validity of *M. ichkeulensis* from *M. cephalus* in Turkish marine waters.

## Material and Methods

**Sampling, morphological and histopathological examination:** For this study, ethics committee approval was not needed because no handling of live fish specimens were involved. A total of forty freshly caught specimens of *M. cephalus* were periodically purchased from commercial fishermen at Kızılırmak Delta, Samsun coast located by the Black Sea, Turkey (41°44'04.2"N 35°57'23.0"E) in the period between January 2017 and April 2017. After purchase and transportation to the laboratory, *M. cephalus* were examined for *M. ichkeulensis* infections under a dissecting microscope (18). After whitish plasmodia were detected in the gills, plasmodia were isolated with a needle and opened on a slide. Infected gills containing mature plasmodia were fixed in 10% formaldehyde and embedded in paraffin. Paraffin blocks were cut into 5 µ slices using microtome and stained with Hematoxylin-Eosin (H&E). The position of the plasmodia in the gills was classified to Molnár (21). Some fresh spores were prepared in glycerine-jelly into the slide for morphological examination. Subsamples of fresh spores were preserved in absolute ethanol for molecular identifications. Myxospores were morphologically examined as previously reported Lom and Arthur (17) by measuring 20 freshly isolated mature spores in reference slide preparations. The myxospores were photographed and measured by Nomarski DIC optics connected to a digital camera. All measurements are presented in micrometres (µm) with mean and range in parentheses.

**DNA extractions and PCR analysis:** *Myxobolus* spores were centrifuged at low speed, suspended in digestion solution and incubated at 56°C overnight. DNA was then extracted using a commercial DNA extraction kit

(GeneJET Genomic DNA Purification Kit, ThermoFisher Scientific) according to the procedure recommended by the manufacturer. Nested PCR assay was used for identification of *Myxobolus* species. The small subunit ribosomal RNA (SSU rRNA) gene (~1900 bp) was amplified by first PCR using ERIB1 and ERIB10 primer pairs (5). Fifty µl PCR reactions were contained 20-50 ng DNA, 2X Hot start PCR master mix, 0.4 µM of each primer. Amplification of first PCR conditions were: 30 sec at 95°C, 50 sec at 43°C, and 120 sec at 72°C for 30 cycles, and a 10 min extension at 72 °C. Then, second PCR were carried out in a final volume of 50 µl, which contained 1 µl of the first PCR amplicon, 2X hot start PCR master mix, 0.4 µM of each primer. The ~900 bp amplification products using the MyxospecF-MyxospecR primer sets (12) were run with 30 sec at 95°C, 50 sec at 40 °C, 90 sec at 72°C for 35 cycles, and products were subjected to a final extension at 72 °C for 10 min. After amplification, PCR amplicons were electrophoresed on 1% agarose gel in a TBE buffer. Second PCR amplicons were sequenced both directions with MyxospecF-MyxospecR primer pairs by Sanger method (Macrogen, Netherlands).

**Phylogenetic analyses:** The obtained sequences were controlled by Vector NTI Advance 11.5 (Invitrogen, USA) using phred values. Then, sequences were de novo assembled and edited with using Contig Express (NTI Advance 11.5, Invitrogen, USA) and the created sequences were compared with GenBank accessions using the BLAST research (1). The SSU rRNA sequences were aligned with other known *Myxobolus* species from *M. cephalus* in previous studies (3, 14, 15, 28). Sequence alignments were performed with ClustalW in MEGA 7.0 (29) and adjusted manually. Phylogenetic tree was constructed using neighbor-joining (NJ) analysis in MEGA 7.0 (16). The Kimura two-parameter (K2P) model was used in the analysis. The species *Ceratomyxa shasta* was chosen as the out-group. The mugilid infecting *Myxobolus* sequence sets were built with 1000 bootstrap replications for the NJ reconstruction (10). Bootstrap values ≥ 70 were considered well supported (13).

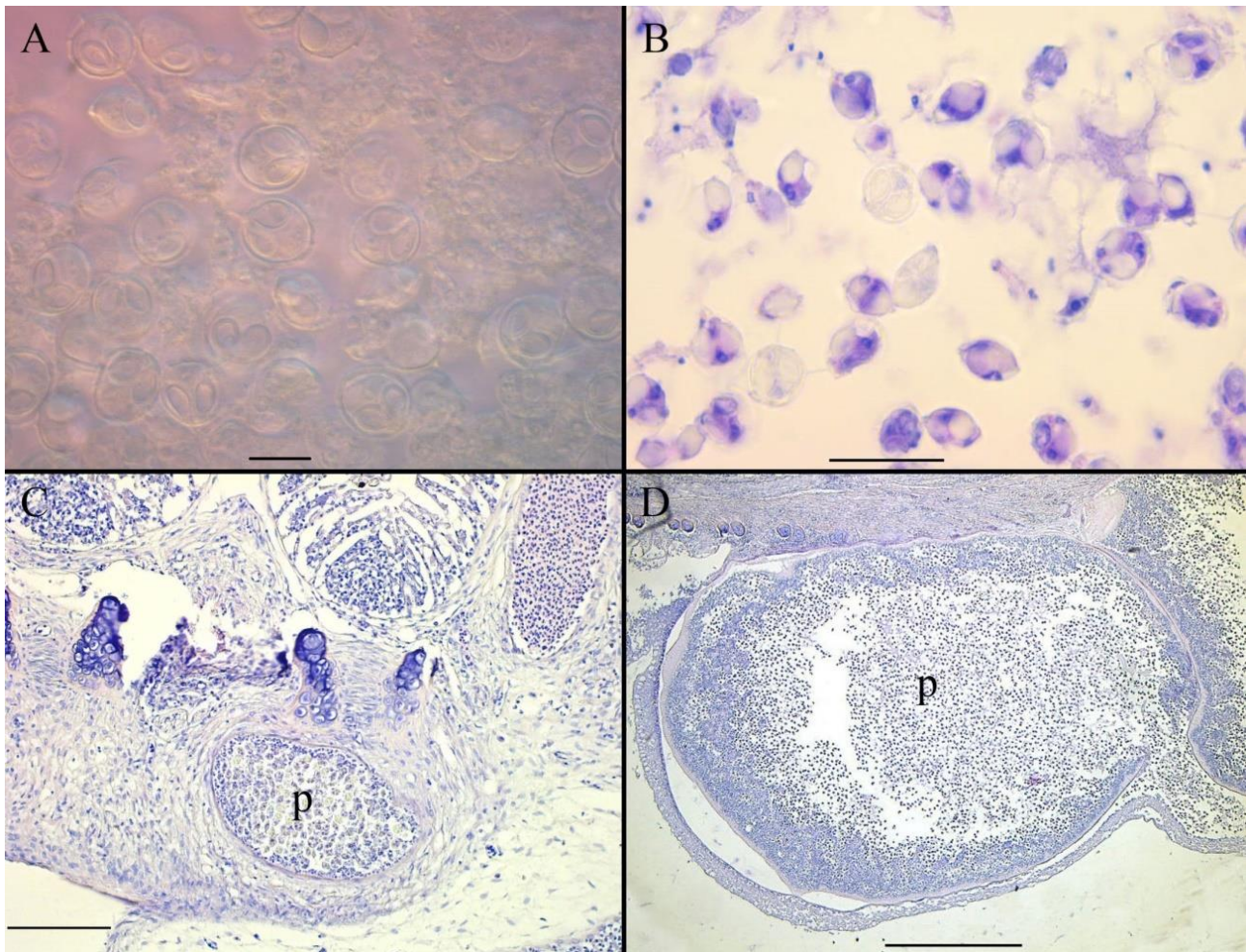
## Results

Whitish cyst-like plasmodia were detected macroscopically along the conjunction line of the gill filaments and arches of *M. cephalus*. We concluded that our isolate (#GZP-2018-Samsun1) is *M. ichkeulensis* based on the spore morphology, biological traits (host/organ specificity and tissue tropism) and molecular data.

### Taxonomy of *Myxobolus ichkeulensis* (4)

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

**Locality:** Kızılırmak Delta, Samsun, Turkey (41°44'04.2"N 35°57'23.0"E)



**Figure 1.** Microscopic photographs of *M. ichkeulensis* infecting the gill arch of *M. cephalus*.

**A.** Fresh myxospores of *M. ichkeulensis* in glycerine-jelly, scale: 10  $\mu$ m. **B.** Myxospore of *M. ichkeulensis* inside plasmodium (p), histopathological section, H&E staining, scale: 10  $\mu$ m. **C.** *M. ichkeulensis* plasmodium (p) located in the connective tissue elements of the gill arch, histopathological section, H&E staining, scale: 100  $\mu$ m. **D.** A large plasmodium (p) of *M. ichkeulensis* in the gill arch, histopathological section, H&E staining, scale: 500  $\mu$ m.

**Site of infection:** Plasmodia were observed macroscopically as whitish cysts along the conjunction line of the gill filaments and arches.

**Type material:** Reference glycerine-jelly and histopathological sections were deposited in the laboratory. The SSU rRNA sequence of *M. ichkeulensis* was recorded in GenBank as MH374629.

**Prevalence of infections:** 12.5 % (5 out of 40), 12 to 18-cm-sized fish

**Myxospores:** The myxospores were round or spherical. The spores were 13.3 (12.06 to 13.72)  $\mu$ m long (n = 20), 11.42 (10.5 to 12.4)  $\mu$ m wide (n = 20), and 8.24 (7.75 to 8.53)  $\mu$ m thick (n = 10). The two polar capsules were oval, equal in size, 5.97 (5.7 to 6.65)  $\mu$ m long (n = 20) and 3.81 (3.4 to 4.18)  $\mu$ m wide (n = 20), and their posterior end reached half the length of the spore. Eight to ten sutural edge markings were easily observed (Figure 1A-B).

**Histopathological findings:** 250 to 2000  $\mu$ m in diameter plasmodia were found. The histopathological analysis revealed the development of the cyst-like plasmodia as gill arch type. Moreover, plasmodia were located in the connective tissue elements of the gill arch (Figure 1C-D).

**Molecular data:** No intraspecific nucleotide variability within three isolate of *M. ichkeulensis* from the Black Sea were observed in the SSU rRNA sequences. A BLAST search indicated that SSU rRNA sequence of our isolate GZP-2018-Samsun1 (MH374629) from *M. cephalus* showed 99.05% similarity to *Myxobolus* sp. voucher Spain6-tp (MF118764), which is also a tail-infecting species and identified as *M. ichkeulensis*, and shared 98.82% similarity with that of *M. ichkeulensis* (AF378337; AY129315). For this reason, our *Myxobolus* isolate (GZP-2018-Samsun1) thus identified molecularly to belong to *M. ichkeulensis*.

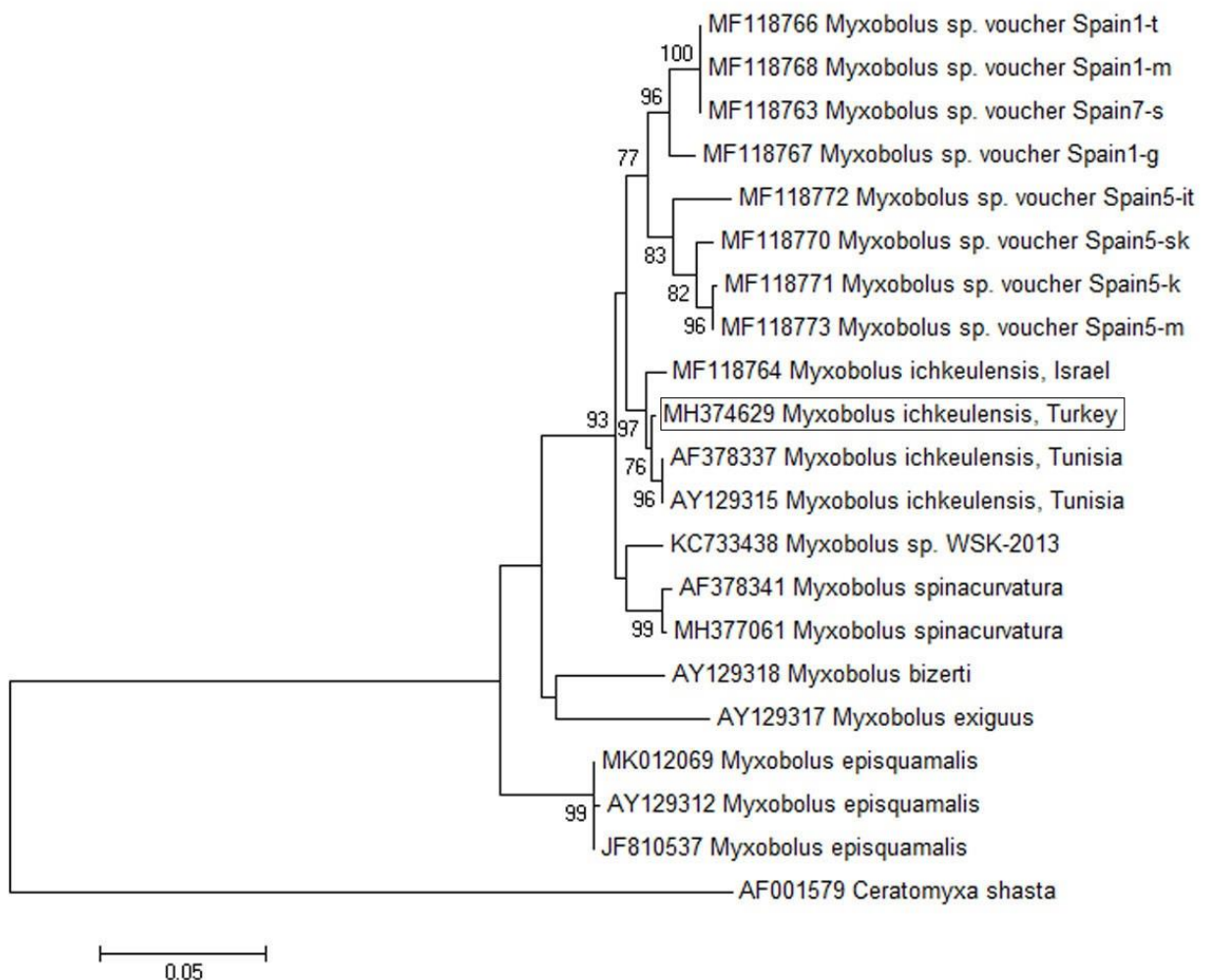


### Discussion and Conclusion

The flathead grey mullet *M. cephalus* (Mugilidae) is a cosmopolitan coastal fish species distributed worldwide. Several myxosporean parasites have been reported as serious pathogens of mugilid fish species (22, 31). Moreover, a great number of myxosporean species were recorded in *M. cephalus* among other mugiliform fish species. To date, thirty six species of myxosporeans have been reported and amongst them are *M. muelleri*, *M. ichkeulensis*, *M. spinacurvatura*, *M. exiguus*, *M. parvus* and *M. episquamalis* are only six cosmopolite species in *M. cephalus* from worldwide (31).

*M. ichkeulensis* was firstly described in *M. cephalus* by Bahri and Margues (4) based on traditional criteria, including tissue tropism and detailed light and electron microscopic examination of spore morphology and subsequently Bahri et al. (3) provided a supplemental data on *M. ichkeulensis* from the host type with molecular data of the SSU rDNA sequence (AY129315). Within the current study, the SSU rDNA sequences of our isolate

(#GZP-2018-Samsun1) showed 98.82% identity with reference sequence of *M. ichkeulensis* (AY129315). Therefore, we molecularly identified our *Myxobolus* species as *M. ichkeulensis*. Currently, based on spore morphology, host/organ specificity and tissue tropism with the molecular marker are mainly useful for new myxosporean species and re-description of insufficiently described species (3, 25-28, 31). Thus, these approaches combined (morphology, biological traits, and molecular markers) were used for the validation of *M. ichkeulensis* in *M. cephalus* from Turkish waters for the first time. Moreover, this is also first report of *M. ichkeulensis* in *M. cephalus* from the coast of the Black Sea in Turkey. Furthermore, the phylogenetic tree showed that our isolate was clustered with *M. ichkeulensis* species previously known to be sequenced from *M. cephalus* (Figure 2). A comparison of *M. ichkeulensis* Bahri and Marques (4), spore morphometric data isolated from *M. cephalus* at different geographical areas is presented in Table 1.



**Figure 2.** Phylogenetic tree generated by NJ analysis of the SSU rRNA sequences of *M. ichkeulensis* and other *Myxobolus* species infecting mugiliform.

Numbers at nodes indicate the bootstrap values. *Ceratomyxa shasta* was used as the out group.

**Table 1.** Comparison of spore morphometric data ( $\mu\text{m}$ ) of *M. ichkeulensis* infection in *M. cephalus*.

Spore length	Spore width	Spore thickness	Polar capsule length	Polar capsule width	Locality	Reference
13.5 (13-14)	12.5 (12-13)	-	5.5 (5-6)	4.2 (4-4.3)	Tunisia: Ichkeul Lagoon	Bahri and Marques (4)
13.5 $\pm$ 0.54	12.5 $\pm$ 0.54	-	5.5 $\pm$ 0.54	4.25 $\pm$ 0.27	Tunisia: Ichkeul Lake	Bahri et al. (3)
13.32 (12.49-14.15)	12.24 (11.58-12.9)	7.69 (7.24-8.14)	6.38 (6-6.76)	4.18 (3.95-4.41)	Turkey: Mediterranean coast, Camlık Lagoon	Ozak et al. (23)
13.13 (12.06-13.72)	11.42 (10.5-12.4)	8.24 (7.75-8.53)	5.97 (5.7-6.65)	3.81 (3.4-4.18)	Turkey: Black Sea coast, Kızılırmak Delta	Present study

Myxosporean plasmodia localize in or among gill lamellae, in gill filaments and inside the gill arch cartilage (20). In the present study, histopathological sections showed *M. ichkeulensis* plasmodia in the connective tissue elements of the gill arch (Figure 1C-D). Myxosporean species is strictly connected to a specific tissue of the host (20). Myxosporean plasmodia may develop within the connective tissue layer in skin doublets between the fin rays (20, 21). Supportively, *Myxobolus* plasmodia embedding in the fin tail connective tissue was found in *M. cephalus* and was 99% similar to *M. ichkeulensis* (28). Our study and Sharon et al. (28) indicated that *M. ichkeulensis* in *M. cephalus* has an affinity for connective tissues both in the gill arch and tail fin. Moreover, small and single cysts of *M. ichkeulensis* reveal a basi-filamental type of plasmodial development in the *M. cephalus* (23).

In conclusion, supplementary data of histopathology and SSU rDNA analysis of *M. ichkeulensis* infecting *M. cephalus* as host type were provided in the present study for the first time from Turkish waters. Moreover, the new valid SSU rRNA sequence (MH374629) obtained from *M. cephalus* from Turkish waters has been submitted to the GenBank. This sequence can be also used to construct a phylogenetic tree with other mugiliform-infecting *Myxobolus* species.

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### Conflict of Interest

The authors declared that there is no conflict of interest.

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