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RESEARCH ARTICLE

Supplementary studies on *Myxobolus talievi* Dogiel, 1957 (Cnidaria, Myxozoa) from the skeletal muscle of the cottoid fish *Cyphocottus eurystomus* in Lake Baikal: Morphological, histological and molecular characterisations

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ABSTRACT

Myxobolus talievi Dogiel, 1957 was originally described from the eyes, skeletal muscles and body cavity of endemic cottoid fish from Lake Baikal. In the present study, we supply new information on the myxospore morphology and histopathology of *M. talievi*; furthermore, we complete the original species description by Dogiel and Bogolepova (1957) with 18S ribosomal DNA (rDNA) sequence data. Histopathological analysis showed that the plasmodium was encapsulated by a thin layer of connective tissue and located in the intermuscular connective tissue among muscle cells. No inflammation was observed. Phylogenetic analysis revealed that *M. talievi* clustered with *Myxobolus* sp. 2 (NCBI Acc. No. U13830), an unidentified *Myxobolus* species from cottoid fish studied by Smothers et al. (1994), and located in the sister clade of *Myxobolus* spp. developing spores in the nerves of salmonids.

KEYWORDS

Myxozoa, *Myxobolus*, cottoid fish, morphology, molecular data, muscle

INTRODUCTION

Myxosporeans (Cnidaria) are metazoan parasites that have complex life cycles in vertebrate (mainly fish, less often in amphibians, reptiles, birds, and mammals) and invertebrate (aquatic annelid worms and bryozoans) hosts (Lom and Dyková, 2006; Hallett et al., 2015). Fish myxosporeans in the territory of the former USSR were studied more than half a century ago (Schulman, 1966). Now, there is a need to revise descriptions and complete species characterisations using modern molecular biological techniques.

Although *Myxobolus* is a species-rich genus (Eiras et al., 2005, 2014, 2020), apart from *Myxobolus talievi* only five *Myxobolus* species were reported in cottoid fish worldwide: *Myxobolus spatulatus* Dogiel and Bogolepova (1957) from *Paracottus kneri*; *M. koryakovii* Pronina and Pronin (2002) from *Comephorus baikalensis* and *C. dybowskii*; *Myxobolus cotti* El-Matbouli and Hoffman, 1987, *Myxobolus jiroveci* Lom et al. (1989) from *Cottus gobio*, *Myxobolus cognati* (Cone et al., 1996) from *Cottus cognatus* (Lom et al., 1989; El-Matbouli et al., 1990; Cone et al., 1996; Pronina and Pronin, 2002). *Myxobolus* sp. was detected in the stomach, the muscle tissue near the pectoral fin, and the gill arches of mottled sculpin *Cottus bairdii* (Heckmann et al., 1987). Furthermore, in the same cottoid fish species, Smothers et al. (1994) found two unidentified species, *Myxobolus* sp. 1 and *Myxobolus* sp. 2, whose 18S rDNA sequences are available in Genbank. Unfortunately, all these species descriptions from cottoid fish are incomplete, as they lack either molecular or morphological data.

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The myxosporean fauna of the Baikalian sculpins consists of nine species: three common, gallbladder-infecting *Myxidium* species, three *Myxobolus* spp., two *Henneguya* spp. from different inner organs, and *Myxobilatus paragasterostei* Zaika, 1963 from the renal tubules. The prevalence of infection of cottoid fish by other myxozoan species is usually very low (<10%). *Myxobolus spatulatus* and *M. paragasterostei* were not found during our 20-year investigation of the parasite fauna of cottoid fish in Lake Baikal.

The host of *M. talievi, Cyphocottus eurystomus* is a member of the genus *Cottus*, and inhabits the Middle and South Baikal at depths of 50–600 m. This species is one of 33 cottoid species endemic to Lake Baikal except for *P. kneri* and *Cottus kessleri,* which are distributed in the neighbouring water reservoirs (Taliev, 1955; Sideleva, 2000). In the course of evolution, the Baikalian sculpins have shifted from an ancestral shallow-water, benthic lifestyle, to deep-water life in environments even below 1,500 m, and also colonised the pelagic habitat (Goto et al., 2015). The taxonomic status of sculpins is still being revised, and new species have been described (Sideleva, 2009; Sideleva and Goto, 2012).

Myxobolus talievi was noted by Dogiel et al. (1949), Dogiel and Bogolepova (1957) and Zaika (1965) in freshwater sculpins of the families Cottinae (*Leocottus kessleri*), Comephorinae (*P. kneri, Batrachocottus baikalensis, Batrachocottus nikolskii*), and Abyssocottinae (*Abyssocottus pallidus, A. bergianus, Asprocottus megalops, Procottus jeittelesi, Cottinella boulengeri*). Since the first description of *M. talievi*, the taxonomy of the cottoid hosts has been revised several times (Taliev, 1955; Sideleva, 1982, 2003). Taliev (1955) described the subspecies *A. megalops eurystomus*. Then, *A. megalops* and *A. megalops eurystomus* were transferred to the genus *Cyphocottus* Sideleva, 2003, and renamed as *Cyphocottus megalops* and *C. eurystomus*, respectively. *Myxobolus talievi* myxospores were first observed in some sculpins studied by Smirnova during a business trip from the Zoological Institute of the USSR Academy of Sciences in 1944 (Dogiel et al., 1949). Subsequently, Dogiel and Bogolepova (1957) described *M. talievi* as a new species based on the material collected in 1944. *C. eurystomus* can be considered as type host, as the fish species was named *A. megalops* at that time, and only one myxosporean species (*M. talievi*) has been reported from *C. eurystomus* so far.

Except Baikalian sculpins, *M. talievi* was also detected in *Gymnocypris przewalski przewalski* and *Sinogastromyzon* sp. (Chen and Ma, 1998) and *Carasobarbus luteus* by Sheyaa (2019) (reviewed by Mhaisen and Al-Jawda, 2020). However, molecular characterisation would be required to clarify the identity of these *M. talievi* isolates from various fish species. In the present study, new material is used to provide further details on the spore morphology, the site preference, and the 18S rDNA sequence data of *M. talievi*.

MATERIALS AND METHODS

Fish collection

In total, 5 specimens of *C. eurystomus* were collected in Lake Baikal (53°47′N; 108°338′E) in the winter of 2020. The fish were caught with gill nets and were transported to the Laboratory of Parasitology of the Institute of General and Experimental Biology SB RAS, Ulan-Ude for parasitological analysis. A thorough inspection for the presence of myxosporean infections, including skins, fins, gills, brains, musculature, and viscera was conducted macroscopically, whereas the kidney, liver and muscles were examined in squash preparations at × 10–25 magnification with an MBS-15 dissecting microscope (LOMO, Saint-Petersburg, Russia).

Morphometric and histologic study

Plasmodia were mechanically isolated from the muscle. Myxospores from the isolated plasmodium were first studied in a wet mount, and then some myxospores were fixed in glycerol gelatin, according to Donec and Schulman (1973). Myxospores were identified and measured from one plasmodium. Measurements were taken from 50 myxospores, as recommended by Lom and Arthur (1989); using Nikon Elements BR software. All measurements are given in micrometres (μm) as mean values with standard deviation (SD), with minimum–maximum range.

To study histology, two pieces of infected musculature with plasmodium were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, stained by Nissl, Perls Prussian blue, and Ziehl–Neelsen, and then examined and photographed. Histological analysis and the examination of myxospore morphology was performed with a light microscope (Axio Imager M.2, Carl Zeiss, Germany).

Scanning electron microscopy

Myxospores from a plasmodium were transferred to a poly-L-lysine-coated coverslip, left to adhere to the slide for 15 min, fixed in 2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate (pH 7.4) at 4 °C for 24 h, and finally dehydrated in a series of ascending concentrations of ethanol. Finally, coverslips were critical point dried and broken on a stub before being coated with gold. The fixed 50 myxospores were observed and photographed by a scanning electron microscope (LEO 1430VP, Carl Zeiss, Germany).

Molecular characterisation

To determine the small subunit ribosomal DNA (18S rDNA) sequence of the myxozoan sample, DNA was extracted with DNA-Extran-2 (Syntol, Moscow, Russia) from the myxospores from one plasmodium (cyst) preserved in ethanol, according to the manufacturer's protocol for animal tissue.

The 18S rDNA fragment was amplified using a nested PCR assay with the 18S rDNA universal eukaryotic primers, 18e and 18g in the first round (Hillis and Dixon, 1991). The PCR cycle consisted of an initial denaturation step of 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min.

This was followed by a second-round PCR with the myxozoan-specific primer pair, Myx1F and Myx4R (Hallett and Diamant, 2001). Amplification conditions in the second

round were 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min.

PCR products were visualised on 0.9% agarose gel in TAE buffer. PCR products were purified using a PCR Product Purification Kit (Biosilica, Novosibirsk, Russia) and directly sequenced with the primers Myx1F and Myx4R used in the second round PCR. Sequencing was made using a BigDye Terminator Cycle Sequencing Kit v3.1 (Thermo-Fisher Scientific, Waltham, USA), and run on an ABI3700 DNA analyzer (Thermo-Fisher Scientific, Waltham, USA). In total, ten forward and reverse sequence segments were aligned in BioEdit (Hall, 1999) and ambiguous bases were clarified using corresponding ABI chromatograms. The consensus sequence was deposited in GenBank under the accession number MZ474686.

Phylogenetic analyses

The 18S rDNA sequences of myxosporeans were identified using the Basic Local Alignment Search Tool (BLAST). The created alignment contained 44 ingroup taxa of the genera *Myxobolus* and *Henneguya* and the outgroup species *Ceratonova shasta* (GenBank: AF001579). Multiple alignments were performed with Clustal X (Thompson et al., 1997) and manually edited applying BioEdit (Hall, 1999). The alignment of the 18S rDNA sequence dataset included 633 characters after the removal of ambiguously aligned regions using trimAL (Capella-Gutiérrez et al., 2009). Maximum likelihood (ML) analysis was performed with MEGA 6 (MEGA) software (Tamura et al., 2013) with the Kimura 2 parameter K2+G+I model, and ML bootstrap support was calculated in 1,000 replicates. Bayesian inference (BI) analysis was carried out using MrBayes (Ronquist et al., 2012) under the best-fit model GTR+G+I, which was selected by jModeltest 3.0 software (Posada, 2008) using the Akaike Information Criterion (AIC). Posterior probabilities were calculated over 1,000,000 generations via two independent runs of four simultaneous Markov chain Monte Carlo chains with every 100th tree saved. Trees were visualised in FigTree v.1.3.1 (Rambaut and Drummond, 2010) (https://tree.bio.ed.ac.uk/software/figtree/) and then edited in Adobe Illustrator (Adobe Systems, USA).

RESULTS

Myxobolus talievi Dogiel 1957 (Figs 1–3)

Phylum Cnidaria Hatschek, 1888
Unranked Sub-phylum Myxozoa Grassé (1970)
Class Myxosporea Bütschli, 1881
Order Bivalvulida Shulman, 1959
Family Myxobolidae Thélohan, 1892
Genus *Myxobolus* Bütschli, 1882

Type host: C. eurystomus (Taliev, 1955) (Cottidae: Scorpaeniformes, Abyssocottidae Jordan, 1923)

Locality: Lake Baikal near underwater ridge Akademichesky, Russia (53°47′N; 108°338′E)

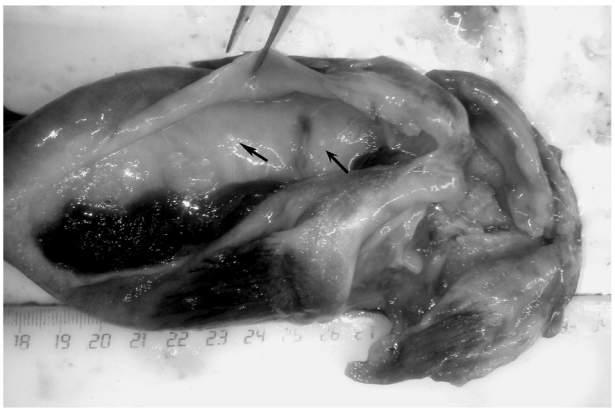

Fig. 1. Photography of *Cyphocottus eurystomus* musculature infected by *Myxobolus talievi*, showing the milky white round pseudocyst (arrows)

Site of infection: The plasmodia were located in the intermuscular connective tissue of skeletal muscle.

Prevalence: 1/5 (20%). Five plasmodia were found in the affected fish specimen.

Myxospores: Myxospores were ellipsoidal in frontal view, lemon-shaped in lateral view (Figs 2 and 3). Myxospore measurements are given in Table 1, in comparison to those of other *Myxobolus* species from cottoid fishes. Polar capsules were pyriform, equal in size, and slightly converging anteriorly. On stained myxospores, an iodinophilous vacuole was visible, located on the side sporoplasm (Fig. 2A). The polar tubules formed four coils (Fig. 2B). The apical end of the polar capsules were located slightly distant from each other (Figs 2D and 3A). A sutural protrusion formed a circular rim around the myxospore. A prominent groove was visible on the valve edge surrounding the suture. Myxospores showed folds either on the prominent groove (Fig. 2E and F). In some myxospores, the sutural rim was wide, while in others it was narrow. A thin mucous layer was observed around the spores (Fig. 2A). The surface of the spore was covered by an irregular network of intertwined strands, probably constituents of the mucous envelope (Fig. 2E and F). One oval, laterally compressed extrusive hole of polar tubules (about 0.50 µm in size), was observed at the anterior end of the myxospore (Fig. 2E). The holes were visible in 54% of the spores and had a similar shape.

Remarks

In the original description by Dogiel and Bogolepova (1957), the myxospores were almost round, with a slightly narrowed anterior end. Pyriform polar capsules converged towards the anterior pole and usually occupied less than half of the myxospore cavity (Table 1). The measurements of *M. talievi* myxospores have a wider range in size in the original description, and some parameters were absent. We compared the myxospores of *M. talievi* with the ones of five *Myxobolus* species from cottoid fish (Table 1). The myxospores of these species have shapes different from that of *M. talievi*, besides, they have larger polar capsules, and a

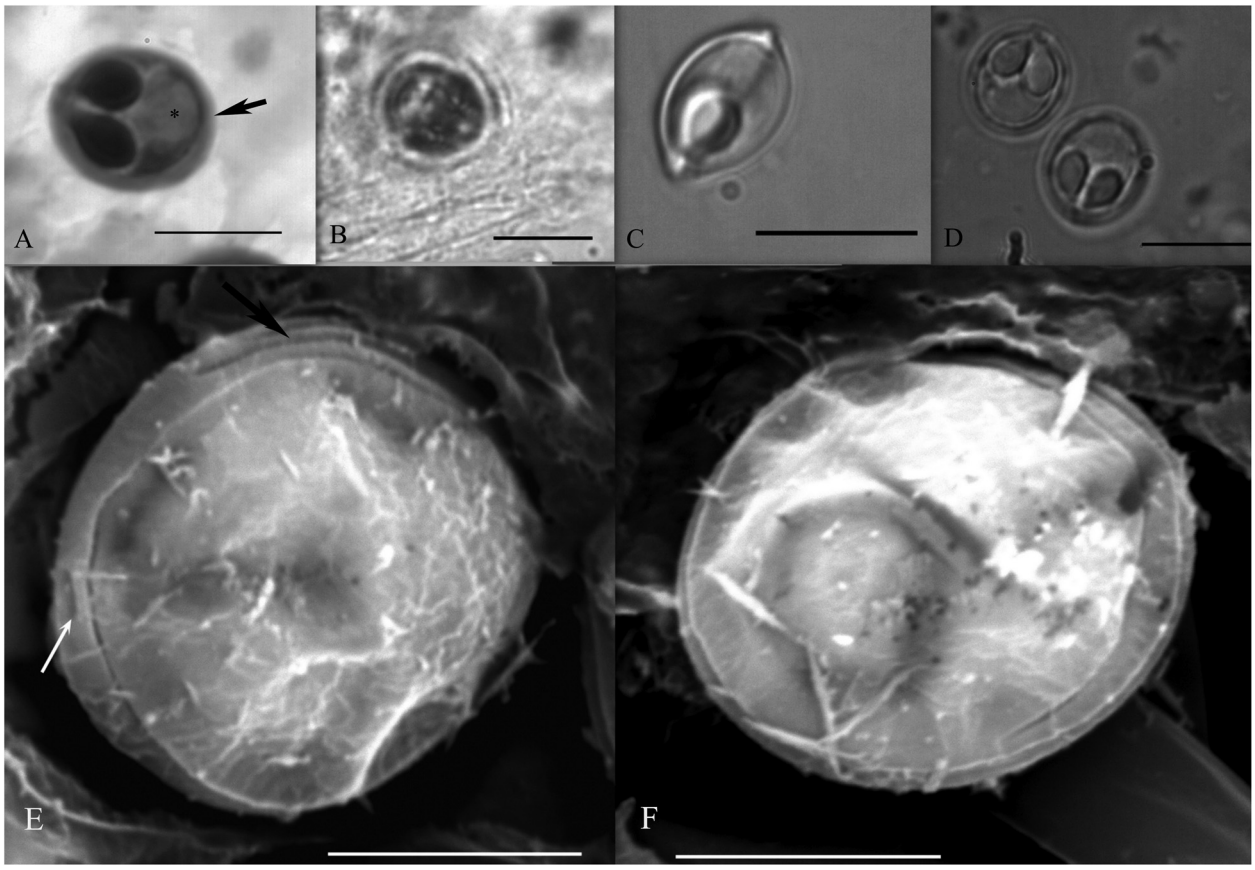

Fig. 2. Myxospores of *Myxobolus talievi* (A, B) – Photomicrographs of myxospores in frontal view using Ziehl–Neelsen stain. A – An iodinophilous vacuole (asterisk) and mucous envelope (arrow) are clearly visible in the sporoplasm; B – Polar tubules are visible in polar capsules. (C, D) Fresh myxospores in lateral view and frontal view; (E, F) Scanning electron micrograph of myxospores. E – The extrusive hole of polar tubule (white arrow) and the folds in the sutural rim (black arrow); F – The folds on the valves near the sutural rim (arrow). Scale bars = 10 µm

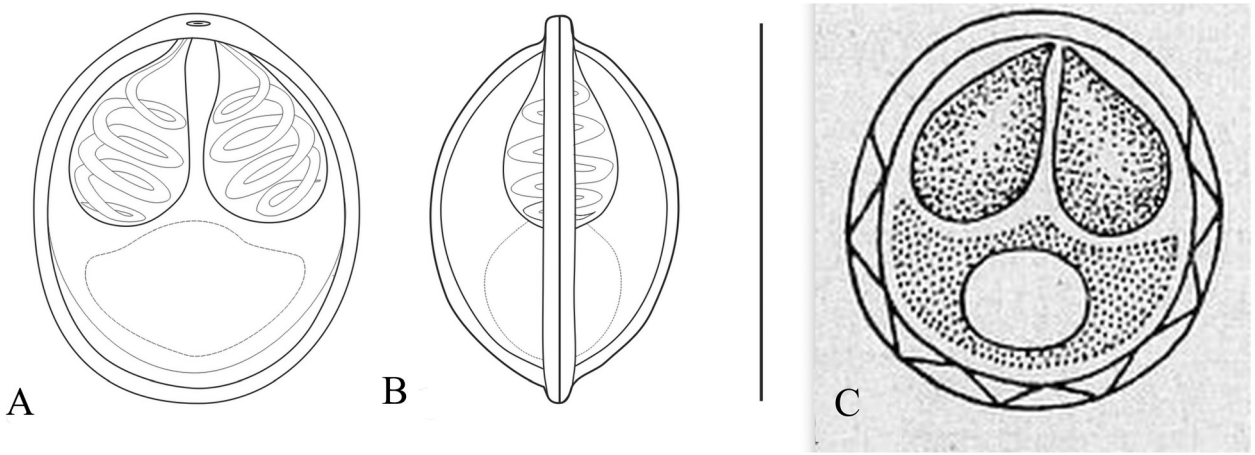

Fig. 3. Line drawings of *Myxobolus talievi* examined in the present study (A: frontal view, B: lateral view). Scale bar: 10 µm. (C) Myxospore illustration from the original species description by Dogiel and Bogolepova (1957). No scale bar on the original drawing

greater number of coils of polar tubules. In the myxospores of *M. spatulatus*, the posterior end was extended into a spatulate process. *Myxobolus cotti* and *M. jirovecii* myxospores are pear-shaped.

Histology

Plasmodia were oval in longitudinal section and rounded in transverse section, from 3 to 7 mm in size, and localised intermuscularly in the connective tissue of skeletal muscle

Table 1. Comparison of phenotypic features of *Myxobolus talievi* myxospores with those of *Myxobolus* spp. infecting cottoid fishes. Data are mean values ±SD and the range is in parentheses; measurements are given in µm. NCF – number of polar tubule coils, n/d = data not available, Ref. = reference

Species	Host	Myxospore			Polar tubule		NCF	Organ/tissue tropism	Type locality	Ref.
		Length	Width	Thickness	Length	Width				
M. talievi	*Cyphocottus eurystomus*	10.9 ± 0.07 (10.2–11.9)	9.4 ± 0.07 (8.8–10.3)	7.3 ± 0.06 (6.9–7.6)	4.8 ± 0.05 (4.9–5.5)	2.9 ± 0.02 (2.6–3.3)	4	skeletal muscle/ connective tissue	Lake Baikal	Present study
M. talievi	*Abyssocotus pallidus, A. bergianus, Batrachocottus nikolskii, B. baicalensis, B. uschkani, Procottus jeittelesi, Paracottus kessleri, P. kneri, Asprocottus megalops, Cottinella boulengeri*	9.3–12	9–11	n/d	3.9–5	n/d	n/d	Eye cavity, muscle, body cavity/unidentified tissue	Lake Baikal	Dogiel and Bogolepova (1957)
M. spatulatus	*Paracottus kneri*	9–12	6–7	n/d	5–6	n/d	n/d	Eyes and gills/ unidentified tissue	Lake Baikal	Dogiel and Bogolepova (1957)
M. koryakovii	*Comephorus baikalensis, C.dybowskii*	15.8–18.7 (17.6 ± 0.3)	8.7–10.3 (9.1 ± 0.2)	0.8–1.1	4.7–6.3	3.2–3.9	n/d	kidney/unidentified tissue	Lake Baikal	Pronina and Pronin (2002)
M. cognati	*Cottus cognatus*	13.3 (12–14)	10 (9.5–10.5)	8.5 (8.0–9.0)	6.6 (5.5–7.5)	3.0	8–11	operculum/connective tissue	USA	Cone et al. (1996)
M. cotti	*Cottus gobio*	12.7–17.7	8.9–10.1	n/d	5.1–7.6	n/d	n/d	brain, spinal cord/ unidentified tissue	Europe	El-Matbouli et al. (1990)
M. jiroveci	*Cottus gobio*	13.8 (12.5–14.1)	10.2 (9.4–11.0)	n/d	7.3 (6.3–7.8)	3.8 (3.1–4.3)	7–8	brain/nerve fibres	Europe	Lom et al. (1989)

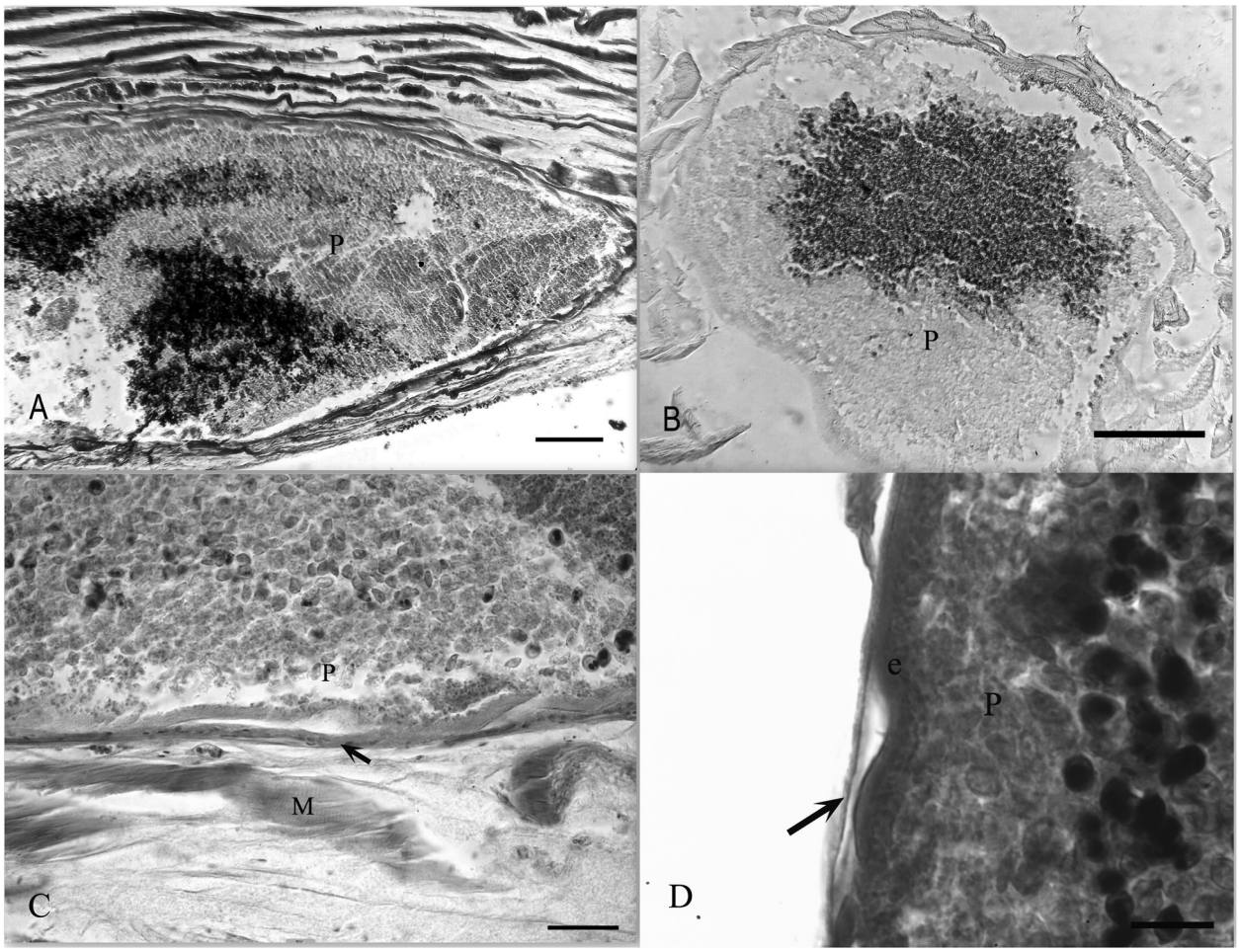

Fig. 4. Histological sections of *Cyphocottus eurystomus* skeletal muscle infected with *Myxobolus talievi* (A, B). An infected muscle showing a plasmodium with myxospores (P). (A – longitudinal section, Ziehl–Neelsen staining; B – cross section, Perls Prussian Blue staining). Scale bars = 500 µm. (C, D) An infected muscle (M) showing host encapsulation by a thin layer of connective tissue (arrows). Nissl staining. C – The capsule consisting of fibroblasts (arrow) associated with plasmodia (P). Scale bar = 50 µm. D – The plasmodia (P) contained ectoplasm (e) and developmental stages at the periphery and mature myxospores close to the centre. A thin layer on the capsule consists of fibres (arrow). Scale bar = 20 µm

(Fig. 4). A thin connective tissue capsule consisting of fibres was formed around the plasmodia, and fibroblasts were visible in some places (Fig. 4B). Denser and more brightly coloured plasmodia ectoplasm (about 10 µm in width) was found adjacent to the capsule (Fig. 4C). The development of myxospores within the plasmodia was asynchronous; mature myxospores occupied the interior part of the plasmodia, whereas developing myxospores were located at the periphery.

Molecular characterisations and phylogenetic analysis

A consensus 18S rDNA sequence obtained for *M. talievi* was 1,330 bp in length (GenBank accession no. MZ474686). A BLAST search indicated that the obtained partial 18S rDNA sequence was the most similar to that of *Myxobolus* sp. 2 (U13830, 96%), and a relative close relationship was detected to the 18S rDNA of *M. fryeri* (EU346371, 94%), *M. articus* (EU346378, 93%), *M. insidiosus* (EU346377, 91%), and *M. neurobius* (AB469986, 90%).

The topologies of phylogenetic trees inferred from BI and ML were the same, and thus, the supports of both analyses were incorporated into one tree based on the ML tree (Fig. 5). *Myxobolus talievi* grouped with *Myxobolus* sp. 2, the parasite of another cottoid fish, the mottled sculpin *Cottus bairdii*, with very high nodal support. These species clustered as a sister clade of *Myxobolus* spp. infecting the nervous system of salmonid fish.

DISCUSSION

Nowadays, the combination of spore morphology, ultrastructure, host and tissue specificity completed with molecular biological characterisation is required for the adequate identification of a new myxosporean species, and for the redescription of incompletely described myxozoan species.

In the original description, the plasmodia of *M. talievi* were round, milky white cysts with a diameter of 0.5–5 mm,

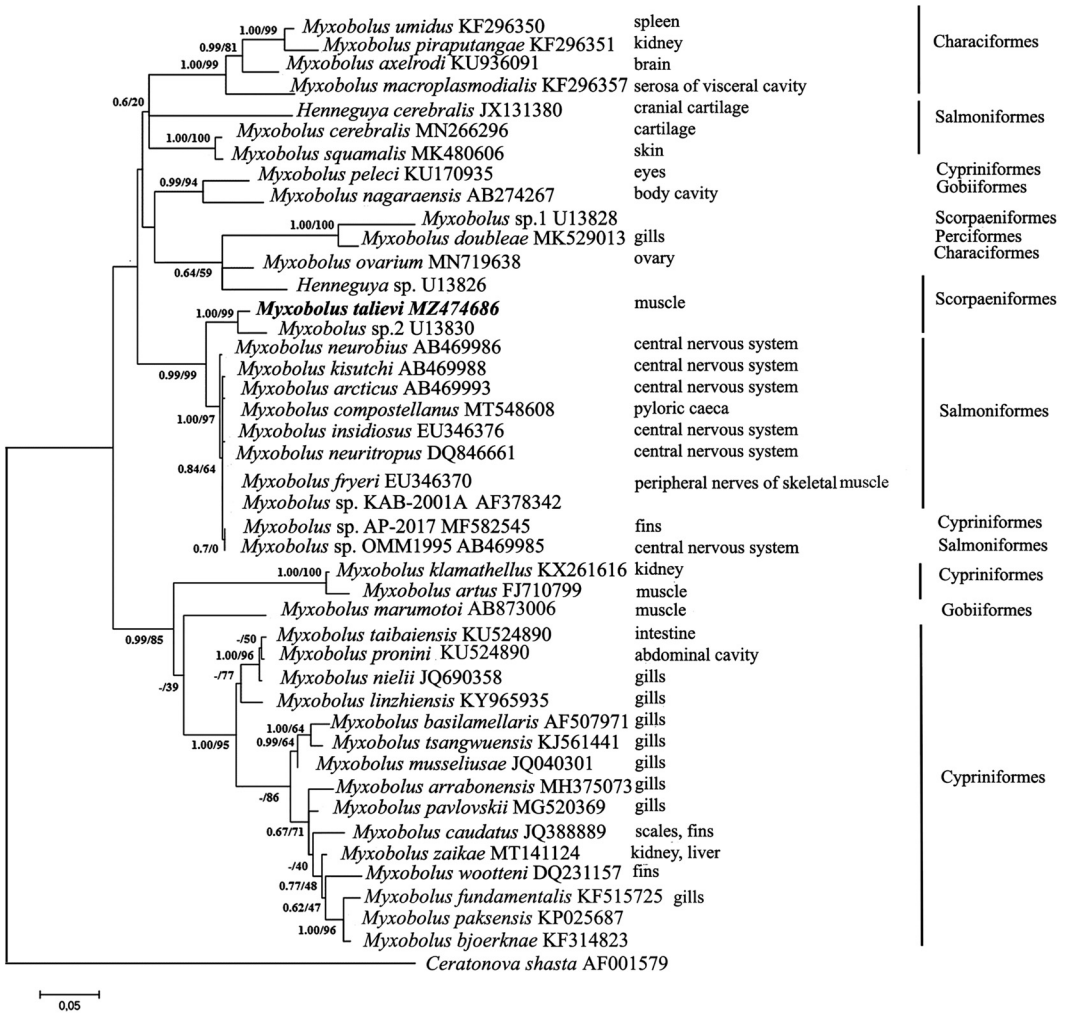

Fig. 5. Phylogenetic tree generated by maximum likelihood analysis inferred from the 633-bp-long, partial 18S rDNA sequences of *Myxobolus talievi* and related myxozoan species. Numbers near branches indicate posterior probability (BI) and bootstrap values (ML). *Ceratonova shasta* AF001579 was used as outgroup. *Myxobolus talievi* in bold

and were localised in the muscles, in the walls of the eye socket, and in the body cavity. The shape and the size of the plasmodia and the myxospores, according to the description by Dogiel and Bogolepova (1957), were similar to *M. talievi* examined by us. However, the original description of myxospores does not indicate the presence of a mucous envelope, which probably remained unnoticed while examining the spores under a light microscope. Mitchell (1989) suggested that the mucous envelope is an unreliable character, because its presence appears to be variable in myxospores of *M. muelleri* and in myxospores from a single cyst of *Myxobolus* sp. It is possible that the mucous envelope is a polymorphic character or, alternatively, that its presence may be ontogenetically determined.

The surface of *M. talievi* myxospores is similar to that of *Myxobolus cerebralis* (Hedrick et al., 1991), which also has a mucous membrane and grooves with folds parallel to the suture ridge.

While most species of the genus *Myxobolus* have strict host and tissue specificity, we assume that *M. talievi* may have a large number of hosts and it is a stenoxenic species, such as *M. cerebralis*, which can infect at least four salmonid genera (Hedrick et al., 2001). The broad organ specificity of *M. talievi* may be due to its tropism to connective tissue. As known for species with a tropism to connective tissue (e.g., *Myxobolus gayerae*, *M. pfeifferi*, *M. fundamentalis*), the organ specificity is less emphasised and plasmodia are developed in the same tissue type in different organs (Molnár et al., 2007, 2014; Molnár and Eszterbauer, 2015; Borzák et al., 2018).

The close phylogenetic relationship of *M. talievi* and *Myxobolus* sp. 2 from a freshwater sculpin may reflect the common freshwater origin of cottid hosts; however, this theory would need further investigation. On the other hand, the sequences of *Myxobolus* sp. 1 and *Henneguya* sp. from *Cottus bairdii* were located far from *M. talievi* on the phylogenetic tree. Perhaps this arrangement is due to the peculiarity of tissue tropism of myxozoans, playing an important role in phylogenetic relationships among related species of *Myxobolus* (Andree et al., 1999; Eszterbauer, 2004; Molnár and Eszterbauer, 2015). The close position of the *M. talievi* – *Myxobolus* sp. 2 group to the clade of *Myxobolus*

spp. from the nervous system of salmonids is also noteworthy. However, no similarities were found between *M. talievi* and *Myxobolus* spp. from salmonids either in tissue tropism or in the morphology of myxospores and vegetative forms. It is difficult to analyse the relationships in the phylogenetic tree due to the lack of detailed morphological data on *Myxobolus* sp. 2, as well as due to the missing molecular data for most *Myxobolus* spp. species from cottoid fish. When these data become available, it will be possible to carry out a thorough phylogenetic analysis to shed light on the genetic relationship among these species. Nevertheless, our findings complete the description of *M. talievi* from its type host, *C. eurystomus*, to aid the precise identification of this myxozoan species in the future.

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