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Phylogeny and comprehensive revision of mugiliform-infecting myxobolids (Myxozoa, Myxobolidae), with the morphological and molecular redescription of the cryptic species *Myxobolus exiguus*

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Abstract

Mullets inhabit a wide range of habitats from tropical to temperate regions and play a critical role in their ecosystems. This commercially important fish group constitutes a significant source of food in several geographic regions, and the production of some species for consumption is an increasing trend. About 64 myxosporean species have been reported in mullets, some of which are cryptic, as is the case of *Myxobolus exiguus*, and *M. muelleri*. This paper provides, for the first time, a detailed and critical revision of the data available for myxobolids reported in mullets, determining the species that have *bona fide* mugiliform fish hosts, in accordance with the original species descriptions, the available molecular data and the currently accepted taxonomic and phylogenetic criteria. Phylogenetic analyses using Bayesian inference and maximum-likelihood methodologies suggest that the evolutionary history of myxobolids with *bona fide* mugiliform fish hosts reflects that of its vertebrate hosts, while reinforcing known evolutionary factors and old systematic issues of the clade of myxobolids. A comprehensive morphological, ultrastructural and molecular redescription is also provided for the cryptic species *M. exiguus*, from infections in the visceral peritoneum of the thinlip-grey mullet *Chelon ramada* in the River Minho, Portugal.

Introduction

The order Mugiliformes comprises of a single family, Mugilidae Cuvier, 1829, which contains about 70 species, distributed worldwide, commonly known as mullets. The great majority of mullets are highly euryahaline, inhabiting tropical and temperate habitats that include rivers, estuaries, coastal areas and seas (Hotos and Vlahos, 1998; Cardona, 2001; Durand *et al.*, 2012). Due to their omnivorous nature and benthic feeding strategy, mullets are able to feed on a great variety of materials including epiphytic algae, insects, annelids, crustaceans, mollusks and even detritus. As a result of their ecological plasticity, this family plays an important role in the ecosystem, namely by contributing to the flow of energy and matter from the lower to the upper levels (Cardona, 2001; Laffaille *et al.*, 2002; Almeida, 2003; Zetina-Rejón *et al.*, 2003). At the commercial level, the importance of mugilids depends on the geographic region and whether they are cultured for gathering roe or for food consumption. Nonetheless, the world production of mullets is an increasing trend, both in fishery and aquaculture industries (Crosetti and Cataudella, 1995; Saleh, 2006).

Several studies have been conducted on the protozoan and metazoan microorganisms parasitizing mullets worldwide (e.g. Merella and Garippa, 2001; Bahri *et al.*, 2003; Fioravanti *et al.*, 2006; Yurakhno and Ovcharenko, 2014; Özer and Kirca, 2015; Sarabeev, 2015). According to Yurakhno and Ovcharenko (2014), this fish group accounts for the description of about 64 myxosporean species from the genera of the families: Sphaeromyxidae Lom and Noble, 1984; Sphaerosporidae Davis, 1917; Myxidiidae Thélohan, 1892; Myxobilatidae Shulman, 1953 [the genus *Ortholinea* has been recently transferred to this family, and the family Ortholineidae dismantled (Karlsbakk *et al.*, 2017)]; Sinuolineidae Shulman, 1959; Alatosporidae Schulman, Kovaleva and Dubina, 1979; Chloromyxidae Thélohan, 1892; Kudoidae Meglitsch, 1960 and Myxobolidae Thélohan, 1892. The latter family is the largest within the subclass Myxozoa Grassé, 1970, namely due to the species-richness of the genera *Myxobolus* Bütschli, 1882 and *Henneguya* Thélohan, 1892. Worldwide, the genus *Myxobolus* comprises over 850 species, the majority of which are histozoic in freshwater

fish, less frequently infecting hosts from estuarine and marine environments. Few species present coelozoic development and even fewer have been reported to occur in amphibian hosts. On its turn, the genus *Henneguya* comprises about 200 species that mostly infect freshwater fish, with the exception of ca. 35 species that are known to occur in marine hosts (e.g. Lom and Dyková, 1992, 2006; Eiras, 2002; Eiras *et al.*, 2005, 2014; Eiras and Adriano, 2012; Khlifa *et al.*, 2012; Li *et al.*, 2012; Azevedo *et al.*, 2014; Rocha *et al.*, 2014*a*; Özer *et al.*, 2016*a*).

Traditionally, the taxonomy of myxosporeans was mainly based on spore morphology, and its association with a particular host and organ of infection. However, molecular analyses have been shown that the comparison of spore morphological traits is insufficient for classifying myxosporeans, both at the genus and species level (e.g. Fiala, 2006; Bartošová *et al.*, 2009; Fiala and Bartošová, 2010; Liu *et al.*, 2010). In the case of myxobolids, the artificiality of the morphological criterion hampers identification at the species level, since many species share similar spore shape and size and others present significant intraspecific variations (Lom, 1987; Mitchell, 1989; El-Matbouli *et al.*, 1992). Also, although most species are acknowledged to be host and tissue restricted (Molnár, 1994), others have been reported to indiscriminately infect a wide range of hosts and tissues (e.g. Forró and Ezsterbauer, 2016). Taxonomic comparisons are further challenged by the paucity of reliable data from most original descriptions, which relied solely on light microscopy and schematic line drawings (e.g. Lubat *et al.*, 1989), with few studies using transmission electron microscopy for ultrastructural characterization. Consequently, several species have been identified as potentially cryptic (Easy *et al.*, 2005; Ferguson *et al.*, 2008; Atkinson *et al.*, 2015), thus warranting authentication through the use of currently accepted taxonomic criteria, i.e. combined spore morphology, host specificity, tissue specificity and molecular data. Considering all of the above, this study provides, for the first time, a detailed and critical revision of the data available for myxobolids reported in mullets, evaluating the reliability of these reports through the comprehensive and careful analysis of original species descriptions, available molecular data and currently accepted taxonomic and phylogenetic criteria. Myxobolids, which occurrence is considered to be reliable in mullets, are herein referred to as having *bona fide* mugiliform fish hosts. A morphological, ultrastructural and molecular redescription is further performed for the cryptic species *Myxobolus exiguus* Thélohan, 1895 from infections in the visceral peritoneum of the thinlip-grey mullet *Chelon ramada* in the River Minho, Portugal.

Materials and methods

Fish and parasite sampling

Between March 2015 and January 2018, 18 specimens of the thinlip-grey mullet *C. ramada* (Risso, 1827) (Teleostei and Mugiliformes) were captured from the River Minho (41°56′N, 08°45′W), Vila Nova de Cerveira, Portugal. Specimens were transported live to the laboratory and, prior to dissection, anesthetized with ethylene glycol monophenyl ether until dead. The parasitological survey of several organs and tissues was performed at both the macro and microscopic levels. Cysts and parasitized tissues were prepared for light microscopy, transmission electron microscopy and molecular procedures.

Light microscopy and morphological examination

Parasitized tissues were examined and photographed using a Leitz-Dialux 20 microscope, equipped with a differential interference contrast (DIC) optics. Morphometry was determined from fresh material (Lom and Arthur, 1989). All measurements include the mean value ± standard deviation (S.D.), range of variation and number of spores measured (range, *n*).

Transmission electron microscopy

Fragments of parasitized tissue were fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.2) for 20–24 h, washed in the same buffer and postfixed in 2% osmium tetroxide also buffered with 0.2 M sodium cacodylate (pH 7.4) for 3–4 h. All these steps were performed at 4 °C. The samples were then dehydrated in an ascending graded series of ethanol, followed by embedding using a series of oxide propylene and Epon mixtures, ending in EPON. Semithin sections were stained with methylene blue-Azure II. Ultrathin sections were double-contrasted with uranyl acetate and lead citrate, and then examined and photographed using a JEOL 100 CXII TEM (JEOL Optical, Tokyo, Japan), operating at 60 kV.

DNA extraction, amplification and sequencing

Fragments of parasitized tissue were obtained from three fish specimens and preserved in 80% ethanol at 4 °C. Genomic DNA extraction was performed using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, USA), following the manufacturer's instructions. The DNA was stored in 50 μL of TE buffer at −20 °C until further use.

The SSU rRNA gene was amplified and sequenced using both universal and myxosporean-specific primers (Table 1). Polymerase chain reactions (PCRs) were performed in 50 μL reactions using 10 pmol of each primer, 10 nmol of dNTPs, 2 mM $MgCl_2$, 5 μL 10× *Taq* polymerase buffer, 2.5 units *Taq* DNA polymerase (Nzytech, Lisbon, Portugal) and approximately 50–100 ng of genomic DNA. The reactions were run on a Hybaid PxE Thermocycler (Thermo Electron Corporation, Milford, Massachusetts), with initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 53 °C for 45 s and 72 °C for 90 s. The final elongation step was performed at 72 °C for 7 min. Five microlitre aliquots of the PCR products were electrophoresed through a 1% agarose 1× tris-acetate-EDTA buffer (TAE) gel stained with ethidium bromide. PCR products were purified using the ExoFast method, in which an enzymatic clean-up that eliminates unincorporated primers and dNTPs is performed with Exonuclease I (*Escherichia coli*) and FastAP Thermosensitive (SAP).

The PCR products from different regions of the SSU rRNA gene were sequenced directly. The sequencing reactions were performed using BigDye Terminator v1.1 from the Applied Biosystems Kit (Applied Biosystems, Carlsbad, California), and were run on an ABI3700 DNA analyser (Perkin-Elmer, Applied Biosystems, Stabvida, Oeiras, Portugal).

Distance estimation and phylogenetic analysis

The partial sequences obtained for the case isolate were aligned in MEGA 6.06, allowing the construction of the parasite's assembled SSU rRNA sequence, with a total of 2013 bp. In order to calculate distance estimation, a dataset was created solely for the SSU rRNA sequence of the case isolate and all other SSU rRNA sequences available for *Myxobolus* spp. that have been reported from mullets. This includes *M. bramae* Reuss, 1906, *M. branchialis* (Markevitsch, 1932) Landsberg and Lom, 1991 and *M. rotundus* Nemeczek, 1911, despite these species having not been sequenced from hosts of the order Mugiliformes. Of the several SSU rRNA sequences available in the GenBank for *M. rotundus*, only one (FJ851447) was chosen to represent this species in the dataset,

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Table 1. PCR primers used for the amplification and sequencing of the SSU rRNA gene.

Name	Sequence (5′–3′)	Position	Pared with	Source
18e	CTG GTT GAT CCT GCC AGT	1	ACT3r, MYX4R	Hillis and Dixon, 1991
ACT3f	CAT GGA ACG AAC AAT	900	18r	Hallett and Diamant, 2001
MYX4F	GTT CGT GGA GTG ATC TGT CAG	1300	18r	Rocha *et al.*, 2015
ACT3r	ATT GTT CGT TCC ATG	900	18e	Rocha *et al.*, 2014*a*
MYX4R	CTG ACA GAT CAC TCC ACG AAC	1300	18e	Hallett and Diamant, 2001
18r	CTA CGG AAA CCT TGT TAC G	1832	ACT3f, MYX4F	Whipps *et al.*, 2003

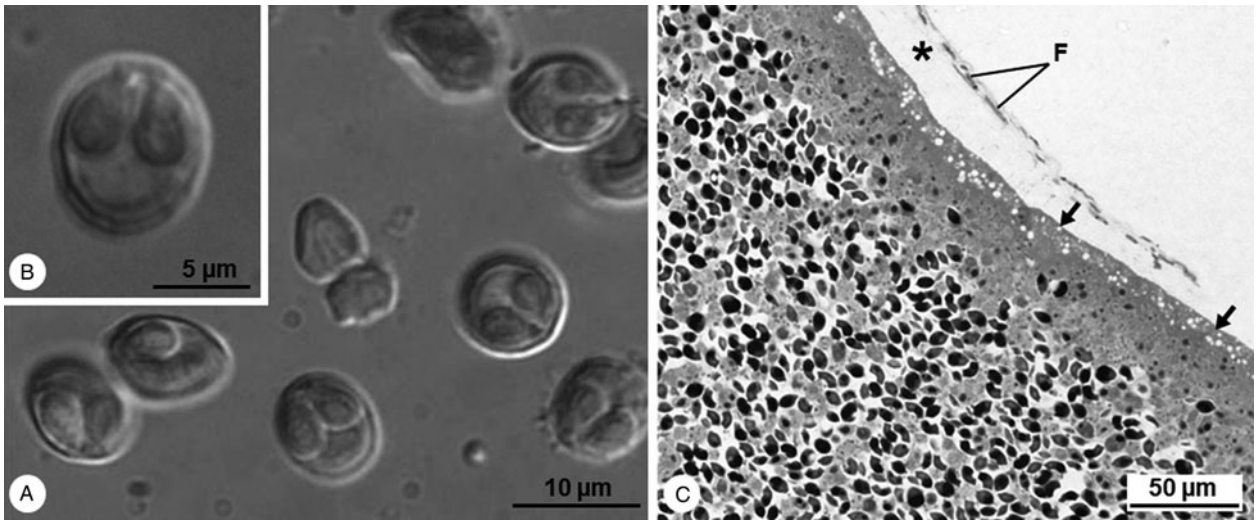

Fig. 1. Light micrographs of *Myxobolus exiguus* infecting the peritoneum of *Chelon ramada* in the River Minho. (A) DIC micrograph showing some free fresh mature myxospores, subspherical in valvular view and ellipsoidal in sutural view, and containing two polar capsules. (B) Free fresh mature myxospore displaying several markings near the suture line. (C) Semithin section of the periphery of a cyst evidencing the vacuolated ectoplasm (arrow) adhering to loose connective tissue (*), where some fibroblasts (F) are observed.

considering that all sequences were identical among each other, with the exception of one (AY165179) that is misidentified (Zhang *et al.*, 2010) and, therefore, was not included. Similarly, two SSU rRNA sequences of *M. bramae* (AF085177 and AF507968) are available in the GenBank but, despite having both been obtained from infections occurring in the cypriniform host *Abramis brama* Linnaeus, 1758, only one is considered valid (AF507968), thus having been included in the dataset. Sequences were aligned using software MAFFT version 7 available online, and distance estimation was performed in MEGA 6.06, with the *p*-distance model and all ambiguous positions were removed for each sequence pair.

For phylogenetic analyses, the dataset was widened to encompass other representatives of the clade of myxobolids. The final dataset comprised of a total of 93 SSU rRNA sequences, and included *Chloromyxum riorajum* (FJ2624481), *Myxidium lieberkuehni* (X76638) and *Sphaerospora oncorhynchi* (AF201373) as the outgroup. Sequences were aligned using software MAFFT version 7 available online, and posteriorly manually edited in MEGA 6.06. Phylogenetic trees were calculated from the sequence alignments using maximum-likelihood (ML) and Bayesian inference (BI). Models of nucleotide substitution were evaluated using MEGA 6.06. The general time reversible substitution model with estimates of invariant sites and gamma distributed among site rate variation (GTR + I + Γ) was chosen as the best suited model for the dataset, and was used in both ML and BI analyses. ML analyses were also conducted in MEGA 6.06 (Tamura *et al.*, 2013), with bootstrap confidence values calculated from 1000 replicates. BI analyses were performed using MrBayes v3.2.6 (Ronquist and Huelsenbeck, 2003), with posterior probability distributions generated using the Markov Chain Monte Carlo method, with four chains running, simultaneously, for 500 000 generations, and every 100th tree sampled.

Results

Revised description and taxonomic summary of M. exiguus

Diagnosis

Cysts whitish and spherical, about 1 mm in diameter, located adjacent to the peritoneum lining the viscera. Mature myxospores subspherical in valvular view and ellipsoidal in sutural view, measuring 9.3 ± 0.6 (8.4–10.7) μm in length and 8.2 ± 0.5 (7.6–8.9) μm in width (n = 25). Valves are smooth presenting several markings near the suture line. Two pyriform equal-sized polar capsules located side by side at the myxospores' anterior pole, 4.8 ± 0.2 (4.4–5.3) μm long and 2.8 ± 0.3 (2.2–3.1) μm wide (n = 25), each containing an isofilar polar filament forming five coils (Fig. 1A and B).

Ultrastructural description

Cysts' wall with cytoplasmic expansions forming ladder-like junctions that strongly adhere to mesothelial cells of the peritoneum. Detachment of cytoplasmic portions of mesothelial cells by insertion of the cysts' wall expansions into connective tissue. Several fibroblasts widely separated by bundles of collagen fibres in

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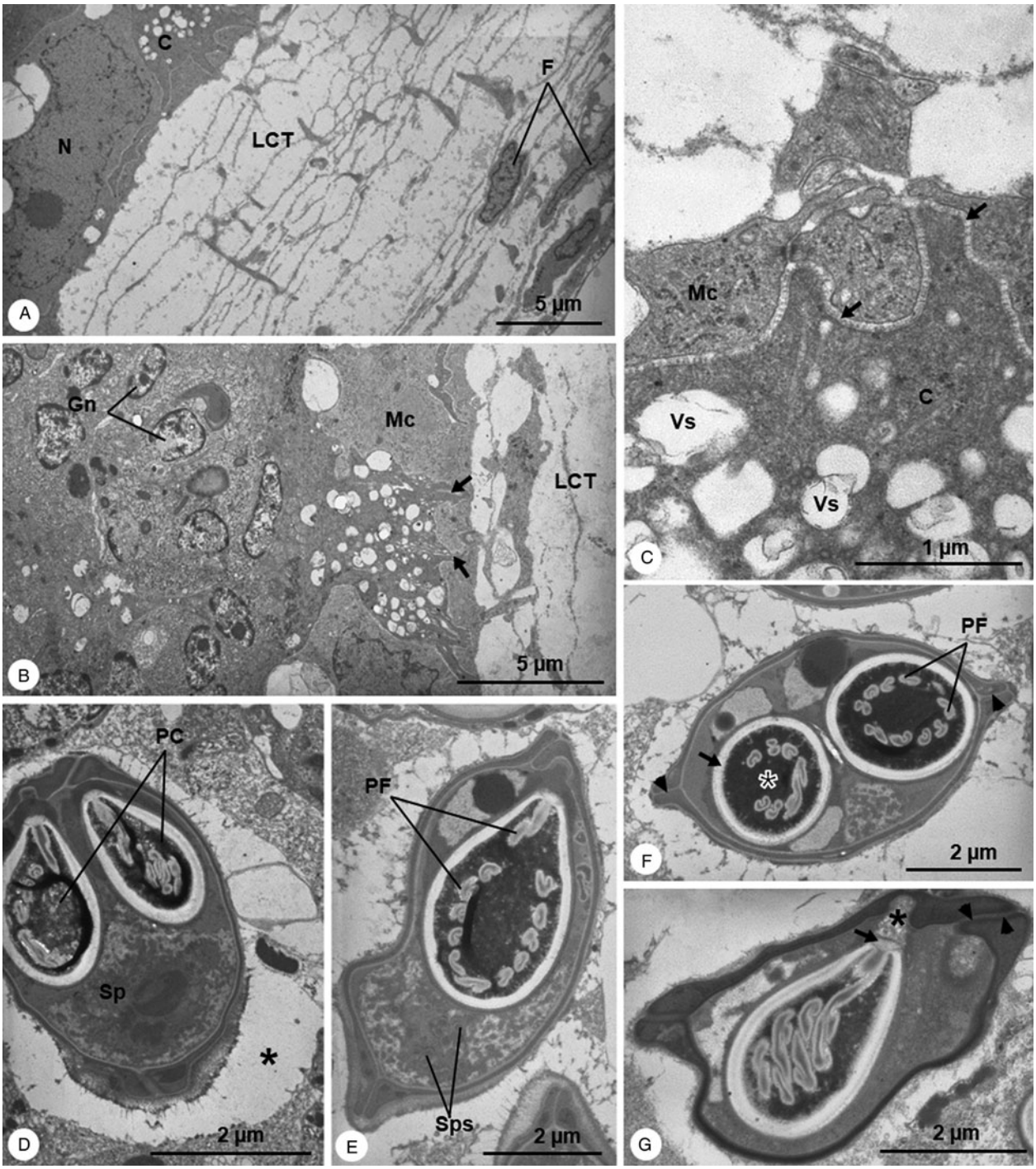

Fig. 2. Transmission electron micrographs of *Myxobolus exiguus* infecting the peritoneum of *Chelon ramada* in the River Minho. (A) Periphery of a cyst (C) adhering to a mesothelial cell near its nucleus (N), and reaching the loose connective tissue (LCT), where fibroblasts (F) are observed widely separated by bundles of collagen fibres. (B) Periphery of a cyst displaying numerous generative nuclei (Gn) and forming cytoplasmic expansions (arrows) that strongly adhere to the mesothelial cells (Mc) and reach the LCT. (C) Detailed aspect of the cytoplasmic expansions forming ladder-like junctions (arrows) that connect the cyst (C) to the mesothelial cells (Mc). Notice the numerous vacuoles (Vs) occupying the cyst's ectoplasm. (D) Longitudinal section of a myxospore in valvular view, located within a vacuole-like structure (*), and displaying its two polar capsules (PC) and binucleate sporoplasm (Sp). **E.** Longitudinal section of a myxospore in sutural view, depicting the number of polar filament (PF) coils, as well as some sporoplasmosomes (Sps) randomly distributed in the sporoplasm. (F) Transverse section of a myxospore showing its two valves united along a straight suture line (arrowheads), and its two PCs presenting a double-layered wall (arrow) that surrounds an electron-dense matrix (*) and a coiled polar filament (PF). (G) Longitudinal oblique section of a polar capsule displaying its cap-like structure (arrow) in continuity with the valve's extrusion pore (*), located near the suture line (arrowheads).

loose connective tissue (Figs 1C and 2A–C). Cysts' ectoplasm highly vacuolated and devoid of cytoplasmic organelles (Figs 1C and 2C); endoplasm rich in mitochondria, vegetative nuclei and containing all sporogonic stages. Sporogony asynchronous and centripetal: generative cells and young sporoblasts located at the cysts' periphery; immature and mature myxospores in the centre, each within a vacuole-like structure (Figs 1C and 2B, D). Myxospores wall thin and smooth, comprised of two symmetrical valves adhering together along a straight suture line. Polar capsules with a double-layered wall formed by an outer thin electron-dense layer and an inner thick electron-lucent layer. Polar filament coils in an electron-dense homogenous matrix (Fig. 2D–F). Cap-like structure at the apex of polar capsule, directed at the corresponding extrusion pore. Extrusion pores near the

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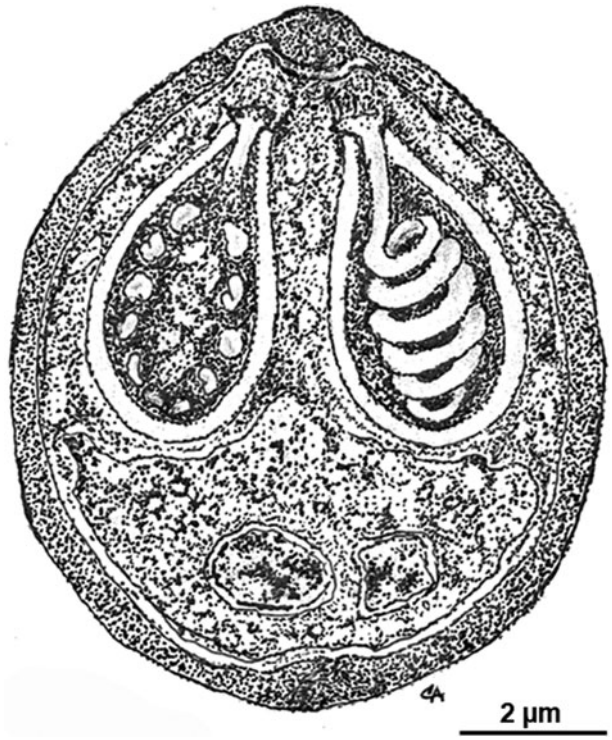

Fig. 3. Schematic drawing depicting the ultrastructural organization of a myxospore of *Myxobolus exiguus* in sutural view.

suture line, corresponding to the portions of the valves with diminished thickness (Fig. 2G). Sporoplasm at the myxospores' posterior pole, with two nuclei and several sporoplasmosomes randomly distributed in a heterogeneous matrix (Fig. 2D and E).

Morphology of the myxospores is represented in a schematic drawing (Fig. 3) depicting the ultrastructural features here described.

Type host: the thinlip-grey mullet *C. ramada* (Risso, 1827) (Teleostei, Mugiliformes).

Site of infection: the visceral peritoneum.

Prevalence: three infected in 18 specimens analysed (16.7%).

Type locality: France (Vivier-sur-Mer, Marseille, Banyuls).

Other localities: Tunisia (Ichkeul Lake); Portugal (River Minho).

Pathogenicity: long-term pathological assessments were not performed, but collected and analysed fish did not present evident external symptoms of infection or disease.

Vouchers: one glass slide containing semi-thin sections of the hapantotype was deposited in the Type Slide Collection of the Laboratory of Animal Pathology at the Interdisciplinary Centre of Marine and Environmental Research, Porto, Portugal, reference CIIMAR 2018.19.

Sequences: one assembled SSU rRNA gene sequence with a total of 2013 bp and GenBank accession no. MH236070.

Molecular comparison of the case isolate to other Myxobolus *spp. reported from mullets*

Pairwise comparisons between the SSU rRNA sequence here obtained and all others available in the GenBank for *Myxobolus* spp. reported from mugiliform fish hosts (Table 2) revealed the case isolate presenting 100% of similarity to two sequences: one identified as *M. exiguus* (AY129317), and the other identified as *M. muelleri* Bütschli, 1882 (AY129314), with these also sharing 100% of similarity between each other. The other SSU rRNA sequences of *M. exiguus* (AY129316) and *M. muelleri* (AY129313) obtained from infections in mugiliform fish hosts followed, with 99.4% of similarity to the case isolate, and 100% of similarity between each other. The two remaining available sequences of *M. muelleri* (AY325284 and DQ439806), which correspond to infections of this species in the cypriniform fish host *Squalius cephalus* (Linnaeus, 1758), shared 97.0% of similarity between each other, but differed significantly from its conspecific sequences available from mugiliform fish hosts, as well as from those of *M. exiguus* and the sequence in study, with percentages of identity that varied between 72.2 and 73.4%. All other species resulted in percentages of similarity lower than 90%.

Phylogenetic positioning of M. exiguus *and other myxobolids reported from mugiliform fish hosts*

BI and ML analyses resulted in similar topologies, with some entropy in the middle of the tree, namely due to the unstable positioning of the subclade comprising SSU rRNA sequences from myxobolids that infect marine perciform fish hosts. The phylogenetic analyses here performed revealed the case isolate clustering to form a well-supported clade together with most of the SSU rRNA sequences available for myxobolid species described from mugiliform fish hosts: *M. exiguus* (AY129316 and AY129317); *M. muelleri* (AY129313 and AY129314); *M. parvus* Schulman, 1962 (KX242161); *M. episquamalis* Egusa, Maeno and Sorimachi, 1990 (KC733437); *M. bizerti* Bahri and Marques, 1996 (AY129318); *M. ichkeulensis* Bahri and Marques, 1996 (AY129315); *Myxobolus* sp. Kim, Kim and Oh, 2013 (KC733438); *M. spinacurvatura* Maeno, Sorimachi, Ogawa and Egusa, 1990 (AF378341); and also with members of the sphaeractinomyxon, endocapsa and triactinomyxon collective groups. Exceptions to the mugiliform-infecting clade are the other two SSU rRNA sequences available for *M. muelleri* (DQ439806 and AY352284), as well as those of *M. bramae* (AF507968), *M. branchialis* (JQ388887) and *M. rotundus* (FJ851447), which cluster among cypriniform-infecting myxobolids (Fig. 4).

Discussion

Overview of mugiliform-infecting myxobolids

In this paper, we summarize the data available for myxobolids that have mugiliforms as *bona fide* fish hosts (Tables 3 and 4), with measurements from the original descriptions (whenever given), and updated scientific names for host species (according to FishBase). The vast majority of mugiliform-infecting *Myxobolus* species are registered from the genus *Mugil* Linnaeus, 1758, with the flathead grey mullet *M. cephalus* Linnaeus, 1758 accounting for an astonishing number of 19 species infecting several of its organs in coastal waters of the Mediterranean Sea, Atlantic Ocean, Indian Ocean and North Pacific Ocean; while white mullet *M. curema* Valenciennes, 1836 accounts for two species from the Atlantic coast off Senegal, and lebranche mullet *M. liza* Valenciennes, 1836 for one species in Brazilian waters. The genus *Chelon* Artedi, 1793 accounts for 12 species: four from several organs of golden grey mullet *C. auratus* (Risso, 1810) (syn. *Liza aurata*) in the Mediterranean Sea and North Atlantic Ocean; three from thicklip grey mullet *C. labrosus* in European and North African waters; two from several organs of the leaping mullet *C. saliens* (Risso, 1810) [syn. *L. saliens* (Risso, 1810)] in Eurasian coastal waters; one from goldspot mullet *C. parsia* (Hamilton, 1822) in India; one from tade grey mullet *C. planiceps* (Valenciennes, 1836), also in India; and one from *C. ramada* (Risso, 1827) [syn. *L. ramada* (Risso, 1827)] in Europe and North Africa. Seven species have been reported from hosts of the genus *Planiliza* Whitley,

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Table 2. Comparison between the SSU rRNA sequences of the case isolate and all other *Myxobolus* spp. infecting mugilids: percentage of identity (top diagonal) and nucleotide difference (bottom diagonal)

ID	*Myxobolus* spp.	GenBank	pb	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)
(1)	*M. exiguus* (this study)	MH236070	2013	–	100	100	99.4	99.4	89.6	88.4	85.3	85.2	84.8	84.1	84.0	74.5	74.4	73.4	72.6	72.0
(2)	*M. exiguus*	AY129317	1591	0	–	100	100	100	88.8	87.0	84.1	85.2	84.8	83.8	84.0	72.5	74.3	73.3	72.5	72.0
(3)	*M. muelleri*	AY129314	1591	0	0	–	100	100	88.8	87.1	84.2	85.2	84.8	83.9	84.1	72.5	74.3	73.4	72.6	72.0
(4)	*M. exiguus*	AY129316	828	5	0	0	–	100	85.5	87.1	82.7	77.2	79.9	78.4	79.6	75.3	71.8	72.5	72.2	71.3
(5)	*M. muelleri*	AY129313	828	5	0	0	0	–	85.5	87.1	82.7	77.2	79.9	78.4	79.6	75.3	71.8	72.5	72.2	71.3
(6)	*M. parvus*	KX242161	1727	179	175	175	94	94	–	86.9	84.1	85.2	84.1	83.0	83.5	74.0	73.6	73.5	73.0	73.1
(7)	*M. episquamalis*	KC733437	1881	218	205	204	95	95	224	–	88.1	88.7	87.7	84.2	86.3	75.7	75.1	73.8	74.2	73.7
(8)	*Myxobolus* sp.	KC733438	1938	283	250	249	134	134	271	222	–	85.1	93.7	82.5	94.7	75.7	73.8	73.2	74.0	73.2
(9)	*M. bizerti*	AY129318	1594	233	233	233	108	108	229	179	235	–	85.2	82.8	84.8	73.0	74.0	72.0	71.8	71.6
(10)	*M. ichkeulensis*	AY129315	1594	240	240	239	96	96	245	194	99	234	–	82.7	93.8	74.4	73.6	73.7	73.9	73.5
(11)	*M. supamattayai*	HQ166720	1666	259	255	254	109	109	268	257	283	272	272	–	82.1	73.5	72.9	72.8	72.3	71.8
(12)	*M. spinacurvatura*	AF378341	1537	244	244	243	97	97	251	210	81	233	95	273	–	73.8	73.1	73.2	73.2	73.3
(13)	*M. rotundus*	FJ851447	1985	496	420	420	192	192	431	440	454	410	388	419	385	–	79.1	88.3	88.7	87.7
(14)	*M. branchialis*	JQ388887	1333	334	334	334	135	135	345	325	341	338	344	358	350	271	–	78.4	78.7	78.2
(15)	*M. muelleri*	AY325284	1616	420	414	413	138	138	409	414	422	434	407	430	402	184	287	–	97.0	96.0
(16)	*M. muelleri*	DQ439806	1589	426	425	424	134	134	413	402	403	436	403	434	401	176	282	48	–	97.5
(17)	*M. bramae*	AF507968	1580	433	433	433	135	135	409	407	413	440	409	439	399	190	288	63	40	–

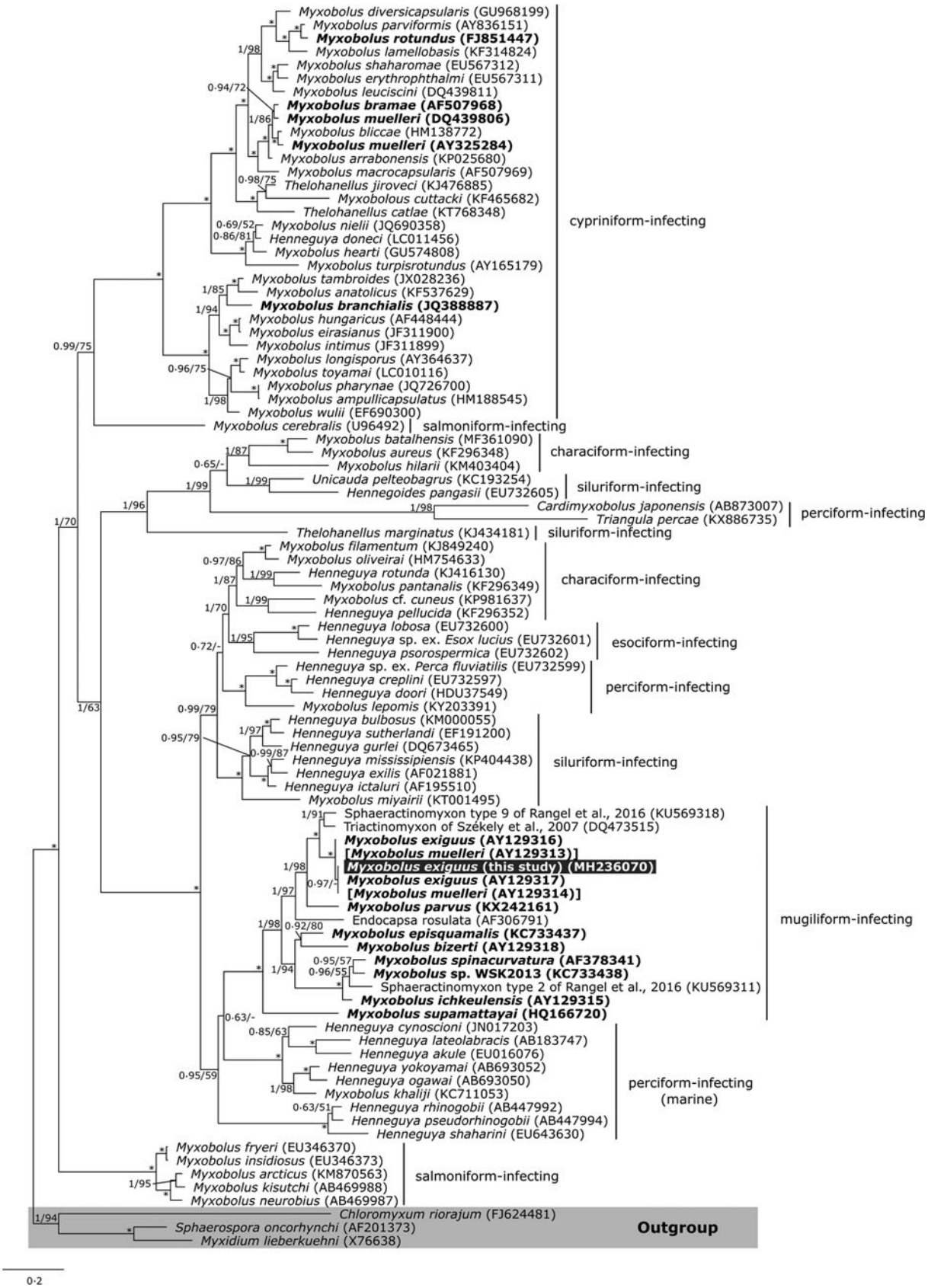

Fig. 4. Tree topology resulting from the Bayesian analysis of 93 SSU rRNA sequences representative of the clade of myxobolids. Numbers at the nodes are Bayesian posterior probabilities/ML bootstrap values; asterisks represent full support in both methodologies; dashes represent a different branching for the ML tree or a bootstrap support value under 50. Bold taxa correspond to species that have been reported from mugiliform fish hosts, with the invalid sequences of *M. muelleri* contained within square brackets. The SSU rRNA sequence obtained in this study for *M. exiguus* is marked with a dark grey box. Final host groups are indicated by vertical lines.

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Table 3. Summary of data available for *Myxobolus* spp. with *bona fide* mugiliform fish hosts

Myxobolus spp.	Hosts	Location	Site of infection	SL	SW	ST	PCL	PCW	PFc	Source
M. achmerovi Schulman, 1966	*M. cephalus*, *Planiliza haematocheila*	Japan Sea off Russia	Fins, gills, mesentery	12.0–14.0	9.0–10.0	–	4.0–5.3	2.3–3.5	–	Eiras *et al.*, 2005; Yurakhno and Ovcharenko, 2014
M. adeli (Isjumova, 1964) Yurakhno and Ovcharenko, 2014	*Chelon auratus*	Mediterranean Sea off Spain, Azov and Black Seas	Digestive tract, swim bladder, gills, muscle	6.2 ± 0.3 (5.6–6.8)	7.2 ± 0.3 (6.6–7.8)	4.6 ± 0.4 (3.5–5.3)	3.1 ± 0.3 (2.4–3.8)	1.8 ± 0.2 (1.3–2.3)	4	Yurakhno and Ovcharenko, 2014
M. anili Sarkar, 1989	*R. corsula*, possibly also in *Planiliza macrolepis*	India Ocean: Bay of Bengal	Duodenal mesentery	10.7 (9.8–11.1)	8.6 (7.9–9.8)	–	4.8 (4.7–5.0)	3.0 (2.4–3.1)	5–6	Sarkar, 1989
M. bankimi Sarkar, 1999	*S. cascasia*	India	Gall bladder	10.6 ± 0.4 (10.0–11.0)	8.7 ± 0.3 (8.0–9.0)	–	3.9 ± 0.5 (3.4–4.5)	2.7 ± 0.3 (2.5–3.0)	4–6	Sarkar, 1999
M. bizerti Bahri and Marques, 1996 (syn. *M. hannensis* Fall *et al.*, 1997)	*M. cephalus*	Mediterranean off Tunisia, Atlantic Ocean off Senegal	Gills	14.2 (14.0–14.5)	14.2 (14.0–14.5)	–	6.5 (6.0–7.0)	5.8 (5.5–6.0)	6–7	Bahri and Marques, 1996
M. cephalis (Iversen *et al.*, 1971) Landsberg and Lom, 1991	*M. cephalus*	Atlantic Ocean off Florida	Brain meninges, gills, oral cavity, jaw bone, crop tissue	14.1 (14.0–15.0)	11.0 (10.0–11.0)	9.0 (8.0–10.0)	4.7 (4.0–5.0)	3.2 (3.0–4.0)	4–5	Iversen *et al.*, 1971
M. cheni Schulman, 1962	*M. cephalus*, *L. haematocheila*	China	Trunk muscle	8.0–8.5	6.0–6.5	–	4.5–5.0	2.0	–	Eiras *et al.*, 2005
M. chiungchowensis Chen in Chen and Ma, 1998	*M. cephalus*	Off China	Intestine	10.8 (10.2–11.8)	10.5 (9.6–11.0)	6.2 (6.0–6.6)	6.0 (5.6–6.2)	3.6 (3.4–3.8)	6–8	Eiras *et al.*, 2005
M. dasguptai Haldar *et al.*, 1996	*C. planiceps*	India	Gills, muscle	14.1 (11.4–19.5)	6.4 (4.9–8.1)	–	9.1 (7.3–11.4)	2.6 (1.6–4.0)	–	Eiras *et al.*, 2005
M. egyptica Abdel-Baki, 2011	*O. labiosus*	Red Sea off Egypt	Gills	10.0 ± 0.6 (9.5–10.5)	8.5 ± 0.4 (8.0–9.0)	8.7 ± 0.5 (8.4–9.2)	5.2 ± 0.5 (5–6)	2.3 ± 0.4 (2.0–3.0)	3–4	Abdel-Baki, 2011
M. episquamalis Egusa *et al.*, 1990	*M. cephalus*	Off Japan	Scales	8.6 (7.5–9.5)	6.8 (6.0–7.5)	5.1 (4.5–5.5)	4.4 (3.8–5.0)	2.2 (2.0–3.0)	–	Egusa *et al.*, 1990
M. exiguus Thélohan, 1895 (syn. *Myxosporidium mugilis* Perugia, 1891)	*C. ramada*, possibly also in *C. auratus*, *C. saliens*, *C. labrosus*, and *M. cephalus*	France (Vivier-sur-Mer, Marseille, Banyuls), Tunisia (Ichkeul Lake), Portugal (River Minho)	Visceral peritoneum	9.3 ± 0.6 (8.4–10.7)	8.2 ± 0.5 (7.6–8.9)	–	4.8 ± 0.2 (4.4–5.3)	2.8 ± 0.3 (2.2–3.1)	5	Thélohan, 1895; Kudo, 1919; Schulman, 1966; Siau, 1978; Lubat *et al.*, 1989; Bahri *et al.*, 2003 (measurements from this study)

M. goensis Eiras and D' Souza, 2004	*M. cephalus*	Off India	Gills	9.7 (9.5–10.5)	6.6 (6.0–7.5)	5.2 (5.0–6.0)	L 5.3 (4.5–6.0) S 2.4 (2.0–3.0)	L 2.4 (2.0–3.0) S 1.8 (1.5–2.0)	5	Eiras and D'Souza, 2004
M. hani Faye *et al.*, 1999	*M. curema*	Off Senegal	Branchial spines	8.0 ± 0.5 (7.0–9.0)	7.3 ± 0.3 (7.0–8.0)	–	–	–	–	Faye *et al.*, 1999
M. ichkeulensis Bahri and Marques, 1996 (syn. *M. goreensis* Fall *et al.*, 1997)	*M. cephalus*	Mediterranean Sea off Tunisia, Turkey and Spain, Atlantic Ocean off Senegal	Gills	13.5 (13.0–14.0)	12.5 (12.0–13.0)	–	5.5 (5.0–6.0)	4.1 (4.0–4.3)	7–8	Bahri and Marques, 1996; Bahri *et al.*, 2003; Maíllo-Bellón *et al.*, 2011; Özak *et al.*, 2012
M. lizae (Narasimhamurti and Kalavati, 1979) Landsberg and Lom, 1991	*P. macrolepis*	India	Outer wall of gut	9.0–9.5	4.6–5.2	–	3.2	2.0	5–7	Narasimhamurti and Kalavati, 1979
M. macrolepi Dorothy and Kalavati, 1992	*P. macrolepis*	India	Intestine	6.3 (5.2–6.9)	5.3 (4.3–6.9)	–	2.8 (1.7–3.4)	2.0 (1.7–2.5)	6–7	Eiras *et al.*, 2014
M. mugauratus (Pogoreltseva, 1964) Landsberg and Lom, 1991 [syn. *M. mugilis* (Pogoreltseva, 1964) (praeocc. Perugia, 1981)]	*C. auratus*	Black Sea off Ukraine	Abdominal serosa	6.5	5.0	–	4.0	3.0	–	Eiras *et al.*, 2014
M. mugcephalus (Narasimhamurti *et al.*, 1980) Landsberg and Lom, 1991	*M. cephalus*	Off India	Gills	4.8–5.2	4.8–5.2	–	1.6–2.0	1.0–1.2	–	Narasimhamurti *et al.*, 1980
M. mugchelo (Parenzan, 1966) Landsberg and Lom, 1991	*C. labrosus*	Off Italy	Gills or mesentery?	5.9 (5.8–6.0)	4.6 (4.0–5.0)	–	–	–	–	Eiras *et al.*, 2005
M. mugilii Haldar *et al.*, 1996	*M. cephalus*	Off India	Gills	11.7 (8.1–16.3)	5.5 (4.0–7.3)	–	6.1 (2.4–8.1)	2.7 (1.6–4.0)	–	Haldar *et al.*, 1996
M. narasii (Narasimhamurti, 1970) Landsberg and Lom, 1991	*E. vaigiensis*	Off India	Gut epithelium	12.5–13.5	8.6–9.5	–	2.9–3.6	1.6–1.8	–	Eiras *et al.*, 2005
M. nile Negm-Eldin *et al.*, 1999	*M. cephalus*	Off Egypt	Gills	7.4	7.3	–	L 3.6 S 2.4	L 2.1 S 1.2	L 6–8 S 5–6	Eiras *et al.*, 2005
M. parenzani Parenzan (1966) Landsberg and Lom, 1991	*C. labrosus*	Off Italy	Gills	5.4 (5.0–6.0)	5.4 (5.0–6.0)	–	–	–	–	Eiras *et al.*, 2005
M. parsi Das, 1996	*C. parsia*	India	Gills	9.1 (9.0–9.5)	8.1 (8.0–8.5)	–	4.4 (4.0–4.5)	2.8 (2.5–3.0)	5	Eiras *et al.*, 2014
M. parvus Schulman, 1962	*M. cephalus*, *C. auratus*, *C.*		Gills, kidney, liver,	6.5–7.0	5.5–6.0	4.0–4.2	3.8–4.2	2.0	–	Iskov, 1989; Eiras and D'Souza, 2004;

(*Continued*)

Table 3. (*Continued.*)

Myxobolus spp.	Hosts	Location	Site of infection	SL	SW	ST	PCL	PCW	PFc	Source
	saliens, *P. haematocheila*	China, Ukraine, Black Sea, Indian Ocean	mesentery, gall bladder, intestine, lower jaw							Eiras *et al.*, 2005; Özer *et al.*, 2016*b*
M. platanus Eiras *et al.*, 2007	*M. liza*	Brazil	Spleen	10.7 (10.0–11.0)	10.8 (10.0–11.0)	5.0	7.7 (7.0–8.0)	3.8 (3.5–4.0)	5–6	Eiras *et al.*, 2007
M. raibauti Fall *et al.*, 1997	*M. cephalus*	Off Senegal	Liver	15.3 (14.0–16.0)	12.1 (12.0–13.0)	–	5.9 (5.0–6.5)	3.6 (3.0–4.0)	–	Fall *et al.*, 1997
M. rohdei Lom and Dyková, 1994	*M. cephalus*	Off Australia	Kidney	11.0 (9.8–11.8)	8.9 (8.4–9.1)	6.5–7.0	4.3 (3.7–5.0)	2.8 (2.5–3.1)	3–4	Lom and Dyková, 1994
M. sphaeralis Dorothy and Kalavati, 1992	*P. macrolepis*	India	Gills	14.1 (13.0–15.0)	7.8 (6.5–8.0)	–	7.5 (7.0–8.0)	2.3 (2.0–3.0)	4–5	Dorothy and Kalavati, 1992
M. spinacurvatura Maeno *et al.*, 1990	*M. cephalus*	Off Japan, off Tunisia	Brain, mesentery, liver, spleen and pancreas	10.5–12.5	9.0–11.0	6.0–7.5	3.5–5.0	2.5–3.5	–	Maeno *et al.*, 1990; Bahri *et al.*, 2003
M. supamattayai U-taynapun *et al.*, 2011	*M. seheli*	Off Thailand	Skin	6.6 (6.2–7.0)	6.5 (6.2–6.7)	–	3.5 (3.4–3.6)	2.0 (1.9–2.2)	4–5	U-taynapun *et al.*, 2011
Myxobolus sp. Faye *et al.*, 1997	*M. curema*	Atlantic Ocean off Senegal	Heart	–	–	–	–	–	–	Fall *et al.*, 1997
Myxobolus sp. Kim *et al.*, 2013	*M. cephalus*	Korea	Intestine	10.4 (9.0–11.9)	8.4 (7.3–10.1)	–	3.7 (2.5–4.5)	2.2 (1.8–2.9)	–	Kim *et al.*, 2013
Myxobolus sp. I Yemmen *et al.*, 2012	*M. cephalus*	Mediterranean Sea off Tunisia	Liver	12.0–14.0	10.0–12.0	–	5.0–7.0	3.0–3.5	5–6	Yemmen *et al.*, 2012
Myxobolus sp. II Yemmen *et al.*, 2012	*M. cephalus*	Mediterranean Sea off Tunisia	Heart	10.0–12.0	10.0–11.0	–	4.0–5.5	2.0–2.5	4–5	Yemmen *et al.*, 2012

SL, myxospore length; SW, myxospore width; ST, myxospore thickness; PCL, polar capsule length; PCW, polar capsule width; PFc, number of polar filament coils; S, smaller; L, larger. Measurements are means ± S.D. (range) (when available), given in μm.

Table 4. Summary of data available for *Henneguya* spp. with *bona fide* mugiliform fish hosts

Henneguya spp.	Hosts	Location	Site of infection	SBL	SBW	SBT	STL	LCA	PCL	PCW	PFc	Source
Henneguya ouakamensis Kpatcha *et al.*, 1997	*M. cephalus*	Atlantic Ocean off Senegal	Gills and heart	10.9 (9.0–13.0)	6.9 (5.0–9.0)		20.8 (16.0–24.0)	9.9 (6.0–14.0)	3.7 (3.0–4.0)	2.4 (2.0–3.0)	–	Kpatcha *et al.*, 1997
Henneguya sp. Faye *et al.*, 1997	*M. cephalus*	Atlantic Ocean off Senegal	Heart	–	–	–	–	–	–	–	–	Faye *et al.*, 1997

SBL, myxospore body length; SBW, myxospore body width; SBT, myxospore body thickness; STL, total length of myxospore; LCA, length of caudal appendages; PCL, polar capsule length; PCW, polar capsule width; PFc, number of polar filament coils; S, smaller; L, larger. Measurements are means ± s.d. (range) (when available), given in μm.

1945: four from the gills and gut of large-scale mullet *P. macrolepis* (Smith, 1846) [syn. *C. macrolepis* (Smith, 1846)] in India; and three from several organs of so-iuy mullet *P. haematocheila* (Temminck and Schlegel, 1845) [syn. *L. haematocheila* (Temminck and Schlegel, 1845)] in Eurasian coastal waters. Other hosts accounting for a single species are: the corsula *Rhinomugil corsula* (Hamilton, 1822), the yellowtail mullet *Sicamugil cascasia* (Hamilton, 1822) and the squaretail mullet *Ellochelon vaigiensis* (Quoy and Gaimard, 1825), all from in India; the hornlip mullet *Oedalechilus labiosus* (Valenciennes, 1836) in the Red Sea off Egypt; and the bluespot mullet *Moolgarda seheli* (Forsskål, 1775) off Thailand. On its turn, only two species of *Henneguya* have been reported from the gills and brain of *M. cephalus* off Senegal (Table 4). Some of these parasite species have been reported, and even originally described, from more than one mugiliform fish host; it is the case of *M. achmerovi* Schulman, 1966, *M. anili* Sarkar, 1989, *M. cheni* Schulman, 1962, *M. exiguus* and *M. parvus*. Also, some have been indiscriminately reported from several organs and tissues; it is the case of *M. achmerovi*, *M. adeli*, *M. cephalis*, *M. dasguptai*, *M. parvus* and *M. spinacurvatura* (Table 3). The great biodiversity of *Myxobolus* spp. parasitizing mugiliform fish hosts reflects not only the species-richness of this myxosporean genus, which is the most common in freshwater environments, but probably correlates with the migratory patterns and feeding strategies of mullets. The catadromous nature of mullets allows these fish species to move into freshwater and brackish environments, thus increasing risk of exposure to typically freshwater parasites, such as *Myxobolus* spp. Also, being benthic feeders, mullets have increased proximity to infected annelids and, therefore, are more prone to contact with waterborne actinosporean stages. The high number of *Myxobolus* spp. parasitizing *M. cephalus* in particular, might suggest that this species possesses higher susceptibility than other mullets to myxosporean infection, but most likely simply reflects the higher number of parasitological surveys that have been conducted in this fish species, as a result of its economic importance in fisheries and aquaculture.

Most mugiliform-infecting species are without molecular data, so that their reports (original and subsequent) have been solely based on morphological traits, which molecular-based systematics reveal are artificial for the reliable description of myxobolids (e.g. Fiala, 2006; Bartošová *et al.*, 2009; Fiala and Bartošová, 2010; Liu *et al.*, 2010). Thus, the legitimacy of these species, and their occurrence in the several reported sites of infection and hosts, must be evaluated through the use of molecular tools; a task that it might prove more difficult than expected, not only due to the frequent occurrence of co-infections (e.g. Molnár *et al.*, 2006), but also due to the vague boundary between intraspecific and interspecific variability of myxobolids. For instance, our molecular analysis shows that the SSU rRNA sequence provided by Molnár *et al.* (2006) for *M. muelleri* displays only 97% of similarity to its conspecific sequence by Eszterbauer (2004), while it also shares a similar percentage of identity (97.0–97.5%) to the SSU rRNA sequences of *M. arrabonensis* (KP025680), *M. bliccae* (HM138772) and *M. bramae* (AF507968). Similarly, high values of intraspecific variability have been reported for different isolates of *M. koi* (3.0%), *M. flavus* (1.9%), *H. corruscans* (2.3%) and *H. maculosus* (1.9%) (Camus and Griffin, 2010; Carriero *et al.*, 2013). On the other hand, very low interspecific variability has been reported between *M. pseudodispar*, *M. musculi* and *M. cyprini* (0.3–0.6%); *M. pendula* and *M. pellicides* (0.4%); *M. fryeri* and *M. insidiosus* (0.5%); *M. intramusculi* and *M. procerus* (2.1%); *M. paksensis* and *M. cycloides* (2.4%); and *M. szentendrensis* and *M. intimus* (2.8%) (Kent *et al.*, 2001; Molnár *et al.*, 2002; Easy *et al.*, 2005; Ferguson *et al.*, 2008; Cech *et al.*, 2015). Considering all of the above, it is clear that the reliable

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classification of myxobolids can only result from the comprehensive evaluation of biological, morphological and molecular features. Another problem that researchers face when studying myxobolids, as well as myxosporeans in general, is the amount of unpublished, incomplete, erroneous and confusing data in the GenBank. Thus, it is important to recognize and avoid the use of poor records. In fact, Molnár (2011) identified and deemed invalid several SSU rRNA sequences of *Myxobolus* spp. In this study, it is further acknowledged that some SSU rRNA sequences of *M. exiguus* are erroneously attributed to *M. muelleri* (AY129313 and AY129314), as are the cases of the SSU rRNA sequences of *M. turpisrotundus*, *M. toyamai* and *M. cutanei*, erroneously designated as *M. rotundus* (AY165179), *T. toyamai* (HQ338729) and *U. caudatus* (JQ388890), respectively.

Assessment of the legitimacy of mugiliform fish as hosts for cryptic species, with the redescription of M. exiguus

The great majority of myxobolids are host specific (Molnár, 1994), with few having been recognized to infect a wide range of hosts belonging to the same taxonomic family or order. For instance, *M. pseudodispar* has been shown to infect a wide range of cypriniforms (Molnár *et al.*, 2002; Forró and Ezsterbauer, 2016) and, in the same manner, *M. cerebralis* is known to parasitize several species of salmonids (El-Matbouli *et al.*, 1999; Hoffman, 1999; Hedrick *et al.*, 2001; Ferguson *et al.*, 2008). Similarly, most myxobolids have well-defined sites of infection (Molnár, 1994; 2002; Molnár *et al.*, 2006), but several species have been indiscriminately reported from multiple organs, either due to misidentifications, or to the parasite's specificity to a given tissue. For instance, the plasmodia of *M. diaphanus* develop in the connective tissue of several different organs of the banded killifish *Fundulus diaphanus* (Lesueur, 1817) (Cone and Easy, 2005). As such, indicating specific tissue tropism, rather than just the organ of infection, is necessary for the correct characterization of myxobolids, and myxosporeans in general. Furthermore, recent phylogenetic studies have consistently shown vertebrate host group as the strongest evolutionary signal for myxobolids (e.g. Ferguson *et al.*, 2008; Carriero *et al.*, 2013; Rocha *et al.*, 2014*a*), followed by the aquatic environment of the host species and tissue tropism (Kent *et al.*, 2001; Eszterbauer, 2004; Holzer *et al.*, 2004; Fiala, 2006).

Overall, about 38 species and four records of *Myxobolus* have been performed from mugiliform fish hosts worldwide (Table 3; Naidenova *et al.*, 1975; Donets, 1979; Ibragimov, 1987; Yurakhno and Maltsev, 2002; Yurakhno, 2004; Eiras *et al.*, 2007, 2014), with *C. ramada* being host to *M. exiguus* and *M. muelleri* (Siau, 1978; Lubat *et al.*, 1989; Thélohan, 1895; Bahri *et al.*, 2003). Both these species are cryptic, having been described from a wide range of tissues and hosts. The original description of *M. exiguus* simultaneously reported the parasite from the stomach epithelium, pyloric caeca, kidney and spleen of the mugilids *C. labrosus* and *C. ramada* in France, and from the gills of the cypriniform fish host *A. brama* (Linnaeus, 1758), with basis on a single schematic line drawing and some spores' measurements (Thélohan, 1895). Since then, this parasite was reported from several other cypriniform and mugiliform fish hosts inhabiting freshwater across Europe and the Mediterranean Sea, including: *Alburnus alburnus* (Linnaeus, 1758), *Leuciscus aspius* (Linnaeus, 1758), *Blicca bjoerkna* (Linnaeus, 1758), *Chondrostoma nasus* (Linnaeus, 1758), *L. idus* (Linnaeus, 1758), *Pelecus cultratus* (Linnaeus, 1758), *Rutilus rutilus* (Linnaeus, 1758), *Scardinius erythrophthalmus* (Linnaeus, 1758), *C. auratus*, *C. saliens* and *Mugil cephalus* (Kudo, 1919; Siau, 1978; Lubat *et al.*, 1989; Bahri *et al.*, 2003). Bahri *et al.* (2003) sequenced the SSU rRNA gene of this parasite (AY129316, AY129317) using samples obtained from the intestine of *C. ramada* from the Ichkeul Lake, Tunisia. However, these authors also provided two SSU rRNA sequences for *M. muelleri* (AY129313 and AY129314) obtained from the mesenteric vessels of *C. ramada*, which our analysis shows, are equal to the ones provided for *M. exiguus*. According to the phylogenetic analysis performed by Bahri *et al.* (2003), the sequences obtained for *M. muelleri* and *M. exiguus* differed solely by three nucleotide substitutions, with the myxospores exhibiting subtle morphological differences and undergoing sporogony in different organs (*M. muelleri* in mesenteric vessels and *M. exiguus* in the intestine). However, acknowledging that *C. ramada* should not be considered as a *bona fide* host for *M. muelleri*, it seems probable that Bahri *et al.* (2003) sequenced the SSU rRNA gene of the same parasite, *M. exiguus*, from the visceral peritoneum, which lines the intestine and double-folds to form the mesentery attaching to the gastrointestinal organs. Molecular comparison of the SSU rRNA sequence here obtained to those obtained by Bahri *et al.* (2003) identified the parasite in study as *M. exiguus*. This identification is corroborated by the specificity of the site of infection and host species, but also by the morphological characters of the myxospores, which dimensions are congruent with those provided in the original description of *M. exiguus* by Thélohan (1895), as well as those provided by Bahri *et al.* (2003) (albeit being slightly bigger, and presenting fewer polar filament coils (five) than those (six to seven) described for the myxospores collected from the mesenteric vessels and misidentified as *M. muelleri*). Bahri *et al.* (2003) further described the valves as smooth, with 10–12 markings appearing near the suture line, as it was also observed in this study. Considering that *M. exiguus* was originally described on the basis of a single schematic line drawing, and that its morphometrics were obtained from myxospores that most probably belong to different *Myxobolus* spp., as shown by the several tissues of infection and hosts accounted for in the original description, this paper aims to present a comprehensive morphological and molecular redescription of this myxobolid species. *Chelon ramada* is here suggested as type host for *M. exiguus*, not only because it is among the host species included in the original description, but also because the parasite gained its molecular identity from infections in the visceral peritoneum of this mullet species. Thus, other fish species, namely cypriniforms, should be disregarded as *bona fide* hosts for *M. exiguus*. Similarly, the visceral peritoneum is suggested as type tissue, so that other tissues and organs of infection, such as the gills, should also be disregarded as sites of infection for *M. exiguus*.

On its turn, *M. muelleri*, type species of the genus, was originally described from the gills of several cypriniforms, without indication of a type host, and since then reported from several different tissues and organs in a great number of fish hosts from Eurasia and North America, including: the kidney and ovaries of *Phoxinus phoxinus* (Linnaeus, 1758); the eyes of *Symphodus melops* (Linnaeus, 1758) and *A. alburnus*; the gills of *Zingel asper* (Linnaeus, 1758), *Barbus barbus* (Linnaeus, 1758), *R. rutilus* and *Lota lota* (Linnaeus, 1758); the pseudobranches of *Cottus gobio* (Linnaeus, 1758); and the gills, fins, eyes, mesentery, intestine, gall bladder, urinary bladder, liver, kidney, spleen, gonads, heart and muscle of *M. cephalus*, *C. auratus*, *C. saliens* and *C. ramada* (Kudo, 1919; Shulman, 1984; Lom and Dyková, 1992; Bahri *et al.*, 2003; Molnár *et al.*, 2006; Umur *et al.*, 2010; Yurakhno and Ovcharenko, 2014). Considering the currently accepted evolutionary signals of Myxosporea, namely the importance of host affinity for myxobolids (Carriero *et al.*, 2013), it is clear that *M. muelleri* constitutes a species-complex, comprising several species that are phenotypically similar and, therefore, have been misidentified. This is further supported by the astonishing variation in the shape and size of the myxospores of *M. muelleri* between reports. In their taxonomic revision of the genus *Myxobolus*, Landsberg

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and Lom (1991) suggested settling *S. cephalus* as the type host for *M. muelleri*. Eszterbauer (2004) provided a SSU rRNA sequence (AY325284) for the parasite sampled from the gills of the chub *S. cephalus* from the River Danube, Hungary; but it was Molnár *et al.* (2006) who characterized *M. muelleri* by providing a comprehensive morphological and molecular redescription of the species from samples obtained from the gills, as well as from the swimbladder of *S. cephalus* in Hungary (DQ439806). Bahri *et al.* (2003) had supposedly sequenced *M. muelleri* from the mesenteric vessels of *C. ramada* from the Ichkeul Lake, Tunisia. Nonetheless, our molecular analyses show that the sequences obtained from the mugilid fish host (AY129313 and AY129314) display lower percentage of identity (72.2–73.4%) than those obtained by Eszterbauer (2004) and Molnár *et al.* (2006), revealing that the parasite infecting *C. ramada* is not *M. muelleri*, but *M. exiguus*, as previously stated. Thus, our study agrees with Molnár (2011) in that the SSU rRNA sequences obtained by Bahri *et al.* (2003) for *M. muelleri* should be deemed invalid. We further suggest disregarding *C. ramada* and other mugilids has *bona fide* hosts for *M. muelleri*, as well as gadids, percids and scorpaenids.

Analysing the legitimacy of mugiliform fish as hosts for other *Myxobolus* spp. reported from mullets, some species require obvious attention, as their original descriptions were performed from cypriniforms. *Myxobolus acutus* (Fujita, 1912) Landsberg and Lom, 1991, originally *S. acuta*, was first described from the gills of *Carassius auratus gibelio* in Japan, and later reported from the scales of *M. cephalus* and *P. haematocheila* from several Russian Rivers, and from the Sea of Japan (Landsberg and Lom, 1991; Eiras *et al.*, 2005; Yurakhno and Ovcharenko, 2014). *Myxobolus bramae* was originally described from the gills of *A. brama* in Russia, and later reported from a wide range of organs and tissues of *M. cephalus* in the Black Sea, including the gills, skin, fins, heart, muscle, mouth, oesophagus, intestine, swim bladder, liver, gall bladder, spleen and kidney (Iskov, 1989; Eiras *et al.*, 2005; Yurakhno and Ovcharenko, 2014). Both Andree *et al.* (1999) and Eszterbauer (2004) deposited an SSU rRNA sequence for this parasite obtained from the gills of its type host in Hungary (AF085177 and AF507968, respectively), which turned out shared very low percentage of similarity between each other. Considering that the common bream is the host for several other gill-infecting *Myxobolus* spp. in Hungary (Molnár and Székely, 1999), Eszterbauer (2004) and Ferguson *et al.* (2008) suggested that the samples used by Andree *et al.* (1999) were probably contaminated by myxospores of another species and the corresponding SSU rRNA sequence was ultimately deemed invalid (Molnár, 2011). *Myxobolus rotundus*, which was also originally described from the gills of *A. brama* in Germany, as well as from the gudgeon *Gobio gobio* (Linnaeus, 1758), was later reported from the gills, heart and other internal organs of *C. auratus* in the Black Sea (Donets, 1979; Iskov, 1989; Eiras *et al.*, 2005). This parasite ultimately had its SSU rRNA gene characterized from infections in its type tissue and host (Székely *et al.*, 2009). *Myxobolus branchialis*, originally *Myxosoma branchialis*, was first described from the gills of *B. barbus* in Ukraine, but then reported from the gills, kidney and spleen of *M. cephalus*, *C. auratus* and *C. saliens* in the Caspian Sea and Black Sea (Schulman, 1966; Ibragimov, 1987; Iskov, 1989; Eiras *et al.*, 2005). Molnár *et al.* (2012) gave molecular identity to the parasite upon its redescription from the gills of common barbel and Iberian barbel *Luciobarbus bocagei* (Steindachner, 1864) in Hungary and Portugal. Finally, *M. circulus* (Achmerov, 1960) Landsberg and Lom, 1991, originally *M. circulus*, was first described from the gills of *Cyprinus carpio* Linnaeus, 1758 in Russia, being later reported from the gills, fins, muscle and kidney of *M. cephalus* in the Black Sea (Naidenova *et al.*, 1975; Iskov, 1989; Yurakhno, 2004; Eiras *et al.*, 2005). Given the molecular trends accepted for myxobolids (Andree *et al.*, 1999; Kent *et al.*, 2001; Eszterbauer, 2004; Fiala, 2006; Carriero *et al.*, 2013), it is highly unlikely for a cypriniform-infecting species to parasitize members of the order Mugiliformes. Thus, we suggest disregarding mullets as legitimate hosts for all these species, which should be considered restricted to their original hosts, and others proven through means of molecular tools (as is the case of *M. branchialis*) (Table 5). For the same reason, we suggest disregarding the cyprinid *C. carpio haematopterus* as a *bona fide* host for *M. achmerovi* Schulman, 1966, which original description was performed from the gills, fins and mesentery of *M. cephalus* and *P. haematocheila* (Eiras *et al.*, 2005; Yurakhno and Ovcharenko, 2014).

Ultrastructure of M. exiguus

Ultrastructural studies of the plasmodial and sporogonic development can provide valuable supplementary information for the distinction of individual species and, more importantly, for understanding host–parasite interactions (Hallett and Diamant, 2001; Rocha *et al.*, 2013; 2014*b*). Current (1979) further suggested that certain ultrastructural differences in the plasmodium wall may partially correlate with the degree of pathogenicity of the parasite.

In general, the wall of cyst-forming myxosporeans is structured similarly between different species and genera, with few variations that probably result from the physical and biological conditions of the tissue/organ of infection, as well as the host immune response (Lom and Dyková, 1992; Hallett and Diamant, 2001; Rocha *et al.*, 2013). Most histozoic species present a smooth plasmodial wall, with pinocytosis or phagocytosis being widely accepted as the processes supplying the nutrients necessary for the parasite's development (Current and Janovy, 1976; Mitchell, 1977; Current, 1979; Current *et al.*, 1979; Casal *et al.*, 2006; Azevedo *et al.*, 2011). The ultrastructural features of the plasmodial development of *M. exiguus* is similar to that of other histozoic myxosporeans only in that pinocytotic activity is evidenced by the large number of vacuoles occupying the ectoplasmic layer. Its plasmodial wall, however, displays an irregular outline, with peripheral projections expanding the parasite–host interface, probably for optimizing nutrient intake. This feature has been reported for few other histozoic species, e.g. *M. insignis* (Azevedo *et al.*, 2013) and *M. filamentum* (Naldoni *et al.*, 2015), since the differentiation of peripheral projections is common to the plasmodial development of coelozoic species (Sitjà-Bobadilla and Alvarez-Pellitero, 1993; 2001; Rocha *et al.*, 2011). Overall, the ultrastructural study performed revealed significant unique features of the plasmodial development of *M. exiguus*, namely in its attachment to the mesothelial cells. In turn, the sporogony of *M. exiguus* is essentially similar to that of other myxobolids with centric asynchronous development (e.g. Current, 1979; Current *et al.*, 1979; Naldoni *et al.*, 2015), in that the ectoplasm appears highly vacuolated and devoid of cytoplasmic organelles, while the endoplasm is riddled with organelles and the parasite's different developmental stages: generative cells and developing sporoblasts at the periphery, and immature myxospores at the centre.

Phylogenetic analysis

The phylogenetic analysis here performed is congruent with previously published cladograms (e.g. Kent *et al.*, 2001; Fiala, 2006; Ferguson *et al.*, 2008; Carriero *et al.*, 2013; Rocha *et al.*, 2014*a*), in that it shows the vertebrate host group as the most relevant evolutionary signal for myxobolids, with tissue tropism and aquatic environment playing less conspicuous roles. Accordingly, all SSU rRNA sequences available for myxobolids with legitimate

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Table 5. Summary of valid data for *Myxobolus* spp. erroneously reported from mugiliform fish hosts

Myxobolus spp.	Hosts	Location	Site of infection	SL	SW	ST	PCL	PCW	PFc	Source
M. acutus (Fujita, 1912) Landsberg and Lom, 1991	*C. auratus gibelio*	Russia	Gills	8.0–10.0	7.0–8.0	5.0–6.0	5.0	4.0	–	Landsberg and Lom, 1991; Eiras *et al.*, 2005
M. bramae Reuss, 1906	*A. brama*	Russia	Gills	10.0–12.0	8.0–10.0	4.5–6.5	4.0–5.5	2.3–3.5	4–5	Eiras *et al.*, 2005
M. branchialis (Markevitsch, 1932) Landsberg and Lom, 1991	*B. barbus*, *L. bocagei*	Ukraine	Gills	6.8–8.4	5.8–6.4	4.0–4.8	2.5–3.2	1.6–2.0	–	Landsberg and Lom, 1991; Eiras *et al.*, 2005; Molnár *et al.*, 2012
M. circulus (Achmerov, 1960) Landsberg and Lom, 1991	*C. carpio*	Russia	Gills	8.5–12.0	7.5–12.0	–	3.5–6.0	2.0	–	Landsberg and Lom, 1991; Eiras *et al.*, 2005
M. muelleri Bütschli, 1882*	*S. cephalus*, and possibly in *P. phoxinus*, *A. alburnus*, *B. barbus*, *R. rutilus*, and other cypriniforms	Hungary	Gills	9.8 ± 0.2 (9.5–10.0)	7.5 ± 0.2 (7.5–8.0)	5.2 ± 0.2 (5.0–5.5)	4.6 ± 0.5 (4.0–5.0)	3.6 ± 0.5 (3.0–4.0)	5–6	Kudo, 1919; Lom and Dyková, 1992; Eiras *et al.*, 2005; Molnár *et al.*, 2006
M. rotundus Nemeczek, 1911	*A. brama*, the oligochaete *Tubifex tubifex*, and possibly *G. gobio*	Germany, Hungary	Gills	10.0	9.8	3.0	3.8–5.0	–	–	Eiras *et al.*, 2005; Molnár *et al.*, 2009; Székely *et al.*, 2009

SL, myxospore length; SW, myxospore width; ST, myxospore thickness; PCL, polar capsule length; PCW, polar capsule width; PFc, number of polar filament coils; S, smaller; L, larger. Measurements are means ± s.d. (range) (when available), given in μm.
*Data from the morphological redescription and molecular identification of the parasite from its original site of infection and host species in Hungary (Molnár *et al.*, 2006).

mugiliform fish hosts are here shown clustering together to form a well-supported subclade of the clade of myxobolids. Members of the sphaeractinomyxon, endocapsa and triactinomyxon collective groups that probably play a role in the life cycles of myxobolid species from mullet hosts also cluster within this subclade. In turn, the valid SSU rRNA sequences available for *M. bramae*, *M. branchialis*, *M. muelleri* and *M. rotundus*, which were obtained from their cypriniform type hosts, all cluster within the clade comprising cypriniform-infecting myxobolids. This emphasizes the incongruence of reporting these species from mugiliform fish hosts, as well as the artificiality of using morphological characters for species identification. The fallibility of morphology as an evolutionary signal for myxobolids has been well reported in several studies (e.g. Fiala, 2006; Bartošová *et al.*, 2009; Fiala and Bartošová, 2010; Liu *et al.*, 2010). For instance, traditional taxonomy separates the genera *Myxobolus* and *Henneguya* according to the absence or presence of caudal appendages, respectively (Lom and Dyková, 2006). Nonetheless, molecular-based taxonomy has consistently shown the convergent evolution of caudal appendages (Fiala and Bartošová, 2010; Liu *et al.*, 2010), revealing that this morphological trait bares little insight into the relationships of myxobolids. In fact, abnormal spore extensions have been reported for some *Myxobolus* spp. (Mitchell, 1989; Cone and Overstreet, 1997; Bahri, 2008; Liu *et al.*, 2010, 2013, 2014, 2015; Zhang *et al.*, 2014), including *M. bizerti* from the gills of *M. cephalus*, and *M. exiguus* (misidentified as *M. muelleri*, as previously mentioned) from the mesenteric vessels of *C. ramada* (e.g. Longshaw *et al.*, 2003; Eiras *et al.*, 2005; Kaur and Singh, 2010; Camus *et al.*, 2017).

It has been suggested that the origins and radiations of myxosporean parasites probably reflect the evolution of their fish hosts (e.g. Carriero *et al.*, 2013; Kodádková *et al.*, 2015). Evolutionary phylogenies of fish reveal that the order Mugiliformes is monophyletic in relation to its sister taxa, despite the polyphyly and/or paraphyly that takes place at the genera-level, due to systematics based in poorly informative anatomical characters (Durand *et al.*, 2012). The phylogenetic analyses here performed supports the coevolutionary history of myxosporeans and their vertebrate hosts, as it shows all legitimate mugiliform-infecting myxobolids clustering together to form a monophyletic well-supported subclade within the clade of myxobolids. In the future, it would be interesting to unravel the significance that this coevolutionary history had in the adaptive strategies of myxosporeans to different micro- and macroenvironments.

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Conflicts of interest. None.

Ethical standards. The work developed in this study was performed in accordance with European ethical standards.

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