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Ultrastructure and phylogeny of *Ceratomyxa diplodae* (Myxosporea: Ceratomyxidae), from gall bladder of European seabass *Dicentrarchus labrax*

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ABSTRACT: The myxosporean parasite *Ceratomyxa diplodae* Lubat et al. 1989 sensu Sitjà-Bobadilla & Álvarez-Pellitero, 1993, originally described from the annular seabream *Diplodus annularis* in the Adriatic Sea, has subsequently been reported from several other sparid hosts, and also a moronid fish, the European seabass *Dicentrarchus labrax* from the Mediterranean Sea. Here, molecular identity and additional morphological data are given for this parasite infecting the gall bladder of *D. labrax* in a southern Portuguese fish farm. In the bile, disporic plasmodia were spherical to subspherical with a smooth surface membrane. Most myxospores were crescent-shaped, 5.1 ± 0.5 (4.8–6.7) µm long (mean ± SD) and 21.9 ± 1.0 (20.4–23.9) µm thick; a few were more arcuate, 5.7 ± 0.4 (5.3–6.3) µm long and 17.3 ± 1.0 (16.3–19.1) µm thick. The wall consisted of 2 symmetrical valves united along a slightly curved suture line, with moderately tapering to rounded ends. Two spherical polar capsules, measuring 2.9 ± 0.3 (2.5–3.4) µm in diameter, contained a polar filament forming 8 to 9 coils organized in 2 rows. Host species, tissue tropism, and myxospore morphology supported species identification. Phylogenetic analyses of the small subunit ribosomal RNA sequence positioned the parasite among most sparid-infecting *Ceratomyxa* spp., suggesting the existence of a common ancestor for these species. The acquisition of molecular data from infections of *C. diplodae* in its original host and in other sparids is essential in order to ascertain if the morphological and biological variations found among reports of this parasite are intra- or inter-specific.

KEY WORDS: Myxozoa · *Ceratomyxa diplodae* · Morphology · SSU rRNA gene · Fish farm · Portugal

INTRODUCTION

Several members of the class Myxosporea Bütschli, 1881 are recognized as pathogenic agents of fish, known to cause losses in aquaculture (Diamant 1992, Sitjà-Bobadilla & Álvarez-Pellitero 1993a, Breton & Marques 1995, Kent et al. 2001, Fioravanti et al. 2004, Yokoyama et al. 2012). The European sea bass *Dicentrarchus labrax* Linnaeus, 1758 (Teleostei: Moronidae), is an important commercial fish cultured in the European North Atlantic and Mediterranean area (FAO 2014). As such, the study and

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rapid diagnostic assessment of the parasites infecting this fish species, including myxosporeans, assumes an important role for the maintenance of its production. In the above-mentioned geographic areas, 6 myxosporean parasites are known to infect *D. labrax*: *Sphaerospora testicularis* Sitjà-Bobadilla & Álvarez-Pellitero, 1990, which intensively infects the testes and causes castration (Álvarez-Pellitero & Sitjà-Bobadilla 1993a, Rigos et al. 1999); *S. dicentrarchi* Sitjà-Bobadilla & Álvarez-Pellitero, 1992, which parasitizes the connective tissue, reducing the growth rate and increasing the possibility of secondary infections (Sitjà-Bobadilla & Álvarez-Pellitero 1992, Rigos et al. 1999, Fioravanti et al. 2004); *Enteromyxum leei* Diamant, Lom & Dyková, 1994, which causes enteritis associated with mass mortalities in several fish species, including *D. labrax* (Fioravanti et al. 2006); *Ortholinea labracis* Rangel et al. 2016, which was recently described from the urinary bladder and posterior kidney (Rangel et al. 2016); and *Ceratomyxa diplodae* Lubat et al., 1989 and *C. labracis* Sitjà-Bobadilla & Álvarez-Pellitero, 1993, which infect the gall bladder (Álvarez-Pellitero & Sitjà-Bobadilla 1993b, Sitjà-Bobadilla & Álvarez-Pellitero 1993b). In the Indo-Pacific region, *Kudoa iwatai* Egusa & Shiomitsu, 1983 has also been reported infecting the muscle of *D. labrax* and is correlated with cases of food poisoning (Diamant et al. 2005, Matsukane et al. 2011, Suzuki et al. 2012). *Ceratomyxa* spp. are considered relatively harmless, either because these parasites induce minimum tissue damage or, more likely, because no studies have investigated their pathogenicity. Consequently, little information is available on *C. labracis* and *C. diplodae*, besides their original descriptions (Lubat et al. 1989, Sitjà-Bobadilla & Álvarez-Pellitero 1993b).

Ceratomyxa Thélohan, 1892 is one of the largest genera of Myxosporea, comprising about 300 species, most of which are coelozoic in the gall bladder of marine teleosts. Traditionally, the differentiation between *Ceratomyxa* spp. and Myxosporea in general was achieved through comparative morphological criteria, such as myxospore dimensions, shape and size of the lateral processes, and number of coils of the polar filament. Species reports were solely based on light microscopy (LM) observations and schematic line drawings, leading to unreliable descriptions and hampering the recognition of some morphologically cryptic species (Lom & Dyková 1992, 2006, Eiras 2006, Heiniger & Adlard 2013). In the case of *Ceratomyxa*, its elevated number of species and dubious separation from the genus *Leptotheca* Thelohán, 1895 have further hampered taxonomic comparisons.

In the past decade, the implementation of molecular analyses has allowed the resolution of old taxonomic inaccuracies, through the establishment of informative biological correlates to myxosporean phylogeny. These include the type of aquatic environment, tissue tropism, and host relatedness, but not spore morphology due to its plasticity and the limited number of defining characters that can be observed using LM (Kent et al. 2001, Eszterbauer 2004, Holzer et al. 2004, Fiala 2006, Heiniger et al. 2008, Bartošová et al. 2009, Fiala & Bartošová 2010, Carriero et al. 2013, Rocha et al. 2013). Gunter & Adlard (2010) proposed the abolition of the genus *Leptotheca*, with the reassignment of its species to *Ceratomyxa*, *Sphaerospora* Thélohan, 1982, *Ellipsomyxa* Køie, 2003, and *Myxobolus* Bütschli, 1882, mainly on the basis of tissue tropism. More recently, the genus *Ceratonova* Atkinson et al., 2014 was erected to encompass *Ceratonova shasta* syn. *Ceratomyxa shasta* (Noble 1950), a long-standing taxonomic outlier to all other *Ceratomyxa* spp. due to its histozoic development in the intestinal epithelium, its freshwater life cycle, and its phylogenetic distance (Gunter et al. 2009, Atkinson & Bartholomew 2010, Atkinson et al. 2014).

Nonetheless, myxospore morphology cannot be disregarded, particularly in the case of *Ceratomyxa* spp., for which the molecular comparison is hindered by the general lack of molecular data available for more diversified members of the genus (Rocha et al. 2015).

Considering the importance of having reliable morphological and molecular data for the proper identification and rapid diagnosis of myxosporean species, this study provides the morphological, ultrastructural, and molecular redescription of *C. diplodae* based on material collected from the gall bladder of *D. labrax* sampled from a southern Portuguese fish farm.

MATERIALS AND METHODS

Fish and parasite sampling

Specimens (n = 141) of European seabass *Dicentrarchus labrax* were obtained between June 2012 and June 2013 from a fish farm in the Alvor estuary, near the Atlantic coast (37° 08′ N, 08° 37′ W), Portimão, Algarve, Portugal. Monthly fish collections were performed from batches intended for commer-

cialization. Collected specimens were slaughtered in accordance with the animal ethical laws stipulated for the aquaculture industry in Portugal and then shipped to the laboratory in insulated containers filled with chipped ice. Upon arrival, fish specimens were dissected and a parasitological examination of several organs and tissues was performed. LM revealed infection of the gall bladder with the myxosporean parasite *Ceratomyxa diplodae*. In some specimens, co-infection with *C. labracis* was observed. As such, infected bile containing solely, or mainly, young developmental stages was not used for further microscopic and molecular procedures. Parasitic material was chosen based on the morphological distinction of mature myxospores, which are not cryptic, since *C. labracis* possesses very thin and long lateral processes, overall displaying very different morphological aspects from *C. diplodae*.

LM and morphological analysis of myxospores

The parasitic stages in the bile were examined and photographed using a Zeiss Axiophot microscope (Grupo Taper), equipped with a Zeiss Axiocam digital camera Icc3. Axiovision 4.6 software (Grupo Taper) was used for image analysis. Morphometry was determined from fresh material (Lom & Arthur 1989). All measurements include the mean ± SD, range of variation, and number of spores measured (range, n).

Transmission electron microscopy (TEM)

Samples of bile infected with *C. diplodae* were fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.4) for 20 to 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 to 4 h. All of 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), operating at 60 kV.

DNA extraction, amplification, and sequencing

Samples of bile infected with *C. diplodae* were obtained from 3 fish specimens and preserved in absolute ethanol at 4°C. Genomic DNA extraction was performed using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich), following the manufacturer's instructions. The DNA was stored in 50 µl of TE buffer at −20°C until further use.

The small subunit ribosomal RNA (SSU rRNA) gene was amplified and sequenced using both universal primers and myxosporean-specific primers (Table 1). PCRs were performed in 50 µl reactions using 10 pmol of each primer, 10 nmol of each dNTP, 2.5 mM $MgCl_2$, 5 µl of 10× *Taq* polymerase buffer, 1.5 units of *Taq* DNA polymerase (Nzytech), and 3 µl (approximately 100–150 ng) of genomic DNA. The reactions were run on a Hybaid PxE Thermocycler (Thermo Electron), 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. Aliquots (5 µl) 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 cleanup that eliminates unincorporated primers and dNTPs is performed with Exonuclease I (*E. coli*) and FastAP Thermosensitive (SAP).

Table 1. Polymerase chain reaction primers used for the amplification and sequencing of the small subunit ribosomal RNA gene of *Ceratomyxa diplodae*

Name	Sequence (5′–3′)	Position	Paired with	Source
18e	CTG GTT GAT CCT GCC AGT	1	CeratR1, MYX4R	Hillis & Dixon (1991)
MyxospecF	TTC TGC CCT ATC AAC TTG TTG	312	MYX4R, 18r	Fiala (2006)
MYX4F	GTT CGT GGA GTG ATC TGT CAG	1300	18r	Rocha et al. (2015)
CeratR1	CCA ATG TCT GGA TTG GGT A	420	18e	Rocha et al. (2015)
MYX4R	CTG ACA GAT CAC TCC ACG AAC	1300	18e, MyxospecF	Hallett & Diamant (2001)
18r	CTA CGG AAA CCT TGT TAC G	1832	MyxospecF, MYX4F	Whipps et al. (2003)

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

Distance and phylogenetic analysis

The forward and reverse segments sequenced were manually aligned with ClustalW in MEGA 6.06 software (Thompson et al. 1994, Tamura et al. 2013), and ambiguous bases were clarified using corresponding ABI chromatograms. To determine the phylogenetic position of the parasite amongst its closest relatives sequenced to date, 55 myxosporean SSU rRNA sequences were chosen from GenBank according to the highest similarity score: 52 belonging to the genus *Ceratomyxa*, *Palliatus indecorus* Shulman et al. 1979 (accession number DQ377712), *Pseudoalataspora kovalevae* Kalavati et al., 2013 (JX467675), and *Myxodavisia bulani* Fiala et al., 2015 (KM273030). *Zschokkella lophii* Freeman et al., 2008 (DQ301509), *Kudoa iwatai* Egusa & Shiomitsu, 1983 (AY514038), and *Myxidium incurvatum* Thélohan, 1892 (DQ377708) were selected as outgroups. Phylogenetic and molecular evolutionary analyses were conducted using MEGA 6.06. Alignments were performed using ClustalW, with an opening gap penalty of 10 and a gap extension of 4 for both paired and multiple alignments (Tamura et al. 2013); ambiguous regions were eliminated manually.

Phylogenetic analyses included maximum likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP) methodologies. For ML, the general time reversible substitution model with 4 gamma-distributed rate variation among sites was performed. For NJ, a Kimura 2-parameter substitution model with a gamma distribution (shape parameter = 1.4) was performed. For MP, the close neighbor interchange heuristic option was selected, with a search factor of 1 and addition of random initial trees, and 500 replicates were performed. All positions with less than 100% site coverage were eliminated from all trees, resulting in a total of 1082 positions in the final dataset. The bootstrap consensus tree was inferred from 500 replicates for ML and MP and 1000 replicates for NJ. Distance estimation was also carried out in MEGA 6.06, using the p-distance model with all ambiguous positions removed for each sequence pair.

RESULTS

Our parasitological survey revealed the presence of several myxosporean parasites that are known to infect *Dicentrarchus labrax* in other geographical areas. *Sphaerospora dicentrarchi* was observed infecting the connective tissue of several organs; *S. testicularis* was observed in the testes of fully matured males; and, less frequently, both *Ceratomyxa diplodae* and *C. labracis* were observed in the gall bladder, being co-infective in some of the analyzed specimens. Also resulting from this parasitological survey was the description of *Ortholinea labracis* from the urinary bladder and posterior kidney. Infections by *Enteromyxum leei* and *Kudoa iwatai* were not observed.

Ceratomyxa diplodae Lubat et al., 1989 sensu Sitjà-Bobadilla & Álvarez-Pellitero, 1993

Diagnosis: Plasmodia, in different stages of maturation, as well as mature myxospores, were observed floating free in the bile (Fig. 1A–D). Myxospores are crescent-shaped, 5.1 ± 0.5 (4.8–6.7) µm long and 21.9 ± 1.0 (20.4–23.9) µm thick (n = 15) (Fig. 1B); occasionally more arcuate, 5.7 ± 0.4 (5.3–6.3) µm long and 17.3 ± 1.0 (16.3–19.1) µm thick (n = 6) (Fig. 1C); total length 5.7 ± 0.5 (4.8–6.7) µm and total thickness 20.0 ± 2.5 (16.3–24.0) µm (n = 21). Abnormal myxospores with 3 valves and 3 polar capsules were occasionally observed (Fig. 1D). Two equal-sized spherical polar capsules measure 2.9 ± 0.3 (2.5–3.4) µm in diameter (n = 30) (Fig. 1B,C).

Description: Plasmodia are spherical to subspherical, with smooth cellular membrane and present disporic development. Cytoplasmic contents include mitochondria, vesicles containing granular material of different electronic density, and lipidic globules. The earliest stages of sporogenesis observed were developing sporoblasts (Fig. 1E,F). The 2 valves are roughly equal, smooth, and united along a slightly curved suture line, with moderately tapering to rounded ends. The 2 polar capsules are located at the same level at the myxospores' anterior pole and each contain a polar filament forming 8 to 9 coils organized in 2 rows. The polar capsule wall is double-layered, comprising a thinner outer electron-dense layer and a thicker inner electron-lucent layer, and containing a dense and heterogeneous matrix (Fig. 1G–J). Sporoplasm is binucleate and contain electron-dense sporoplasmosomes (Fig. 1K). A semi-schematic drawing of a myxospore in sutural view is presented in Fig. 2.

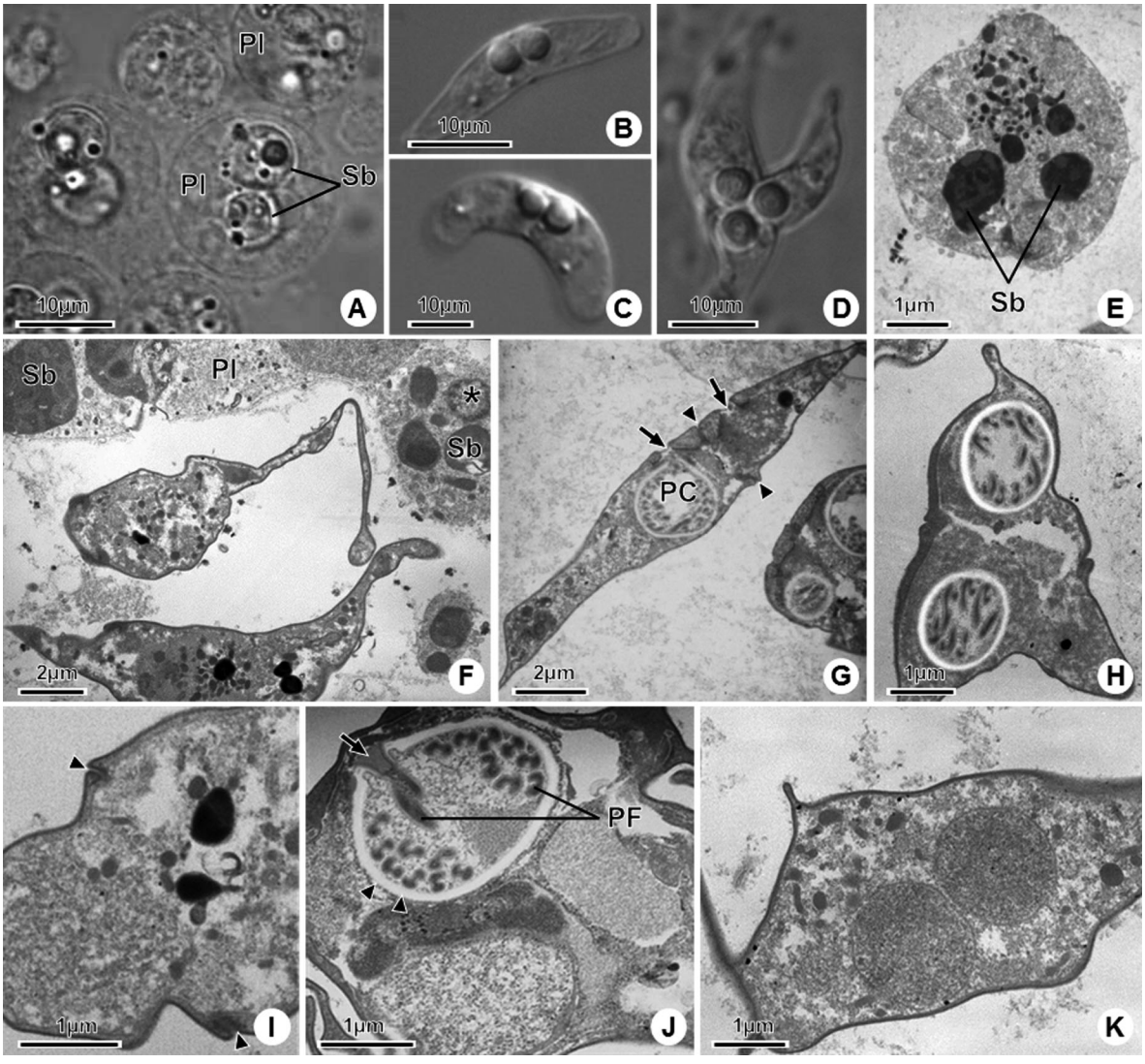

Fig. 1. Light and transmission electron micrographs of *Ceratomyxa diplodae* infecting the gall bladder of *Dicentrarchus labrax*. (A) Aggregate of spherical to subspherical plasmodia (Pl) presenting smooth cellular membrane and containing developing sporoblasts (Sb), observed under differential interference contrast (DIC) optics. (B) Mature crescent-shaped myxospore, as observed under DIC optics. (C) Occasional more arcuate myxospore, as observed under DIC optics. (D) Rare aberrant myxospore displaying 3 valves and 3 polar capsules, as observed under DIC optics. (E) Ultrathin section showing a subspherical plasmodium containing 2 developing sporoblasts (Sb). (F) Ultrathin section depicting 2 mature myxospores surrounded by an aggregate of plasmodia (Pl) containing developing sporoblasts (Sb). Notice the vegetative nucleus (*) in 1 of the plasmodia. (G) Oblique longitudinal section of a mature myxospore displaying 1 of its 2 polar capsules (PC), and allowing recognition of the extrusion pores (arrows) through which the polar filaments exit, located near the suture line (arrowheads). (H) Oblique section of a rare aberrant myxospore showing 2 of its 3 polar capsules. (I) Ultrastructural detail of the slightly curved suture line (arrowheads) uniting the 2 valves. (J) Longitudinal section of a polar capsule showing the polar filament (PF) coiled along its double-layered wall (arrowheads) and capped at the apex by a stopper (arrow). (K) Ultrastructure of the binucleate sporoplasm

Prevalence. One year sampling: 10 infected in a total of 141 examined (7.1%).

Pathogenicity. The collected and analyzed fish did not present external symptoms of infection or disease, and no mortality was recorded from the stock used.

Vouchers. One glass slide with semithin sections displaying plasmodia containing several myxospores has been 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 2016.11).

Sequences. Sequence of the SSU rRNA with a total of 1819 bp obtained and deposited in GenBank (accession number KX099691).

Molecular analysis: ML, NJ, and MP phylogenetic trees revealed the parasite clustering within the

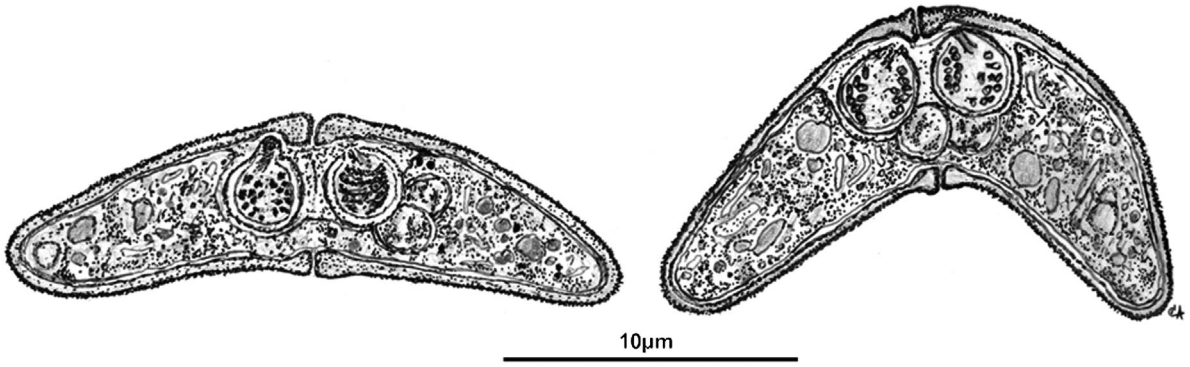

Fig. 2. Schematic of *Ceratomyxa diplodae* depicting the internal and external organization of the myxospores: common crescent-shaped type (left) and rare more arcuate shaped type (right)

large *Ceratomyxa* clade, in a subclade with *Ceratomyxa* spp. from sparids and siganids. This subclade was supported by strong bootstrap values in all phylogenetic analyses. *C. labracis* (AF411472), which also infects the gall bladder of *D. labrax*, never grouped with *C. diplodae* in this subclade, and displayed an unresolved positioning in the overall tree topology, despite consistently clustering together with *C. verudaensis* Fiala et al., 2015 (KM273027) from the gall bladder of *Scorpaena porcus* (Linnaeus, 1758). Also clustering within the large *Ceratomyxa* clade are *Pseudoalatospora kovalevae* (JX467675), *Palliatus indecorus* (DQ377712), and *Myxodavisia bulani* (KM273030) (Fig. 3).

Pairwise comparisons among the SSU rRNA sequences closely related to *C. diplodae* revealed the greatest similarity to *Ceratomyxa* sp. (JF820293), *C. puntazzi* (JF820290), *Ceratomyxa* sp. (JF820291), *C. sparusaurati* (AF411471), and *Ceratomyxa* sp. (JF820292), with percentages of identity higher than 95%. *C. labracis* (AF411472) showed only 89.9% identity to *C. diplodae* (Table 2).

DISCUSSION

At present, it is widely accepted that the resolution of taxonomic and phylogenetic issues within the class Myxosporea can only result from the combined consideration of both morphological and molecular characters. For many years, species reports were solely based on LM observations, sometimes leading to poor descriptions that now warrant validation through the use of more accurate analyses. Consequently, in the past few years, several species have been redescribed and, in some cases, taxonomically revised according to the molecular input (Gunter & Adlard 2010, Liu et al. 2013, Atkinson et al. 2014, Rocha et al. 2014).

In the present study, the combined use of LM, TEM, and molecular procedures allowed the redescription of *Ceratomyxa diplodae* collected from the gall bladder of *Dicentrarchus labrax* in a southern Portuguese fish farm. This species was originally described from the gall bladder of the annular seabream *Diplodus annularis* (Teleostei, Sparidae) in

Table 2. Comparison between the small subunit ribosomal RNA sequences closely related to *Ceratomyxa diplodae* (shown in **bold** type): percentage of identity (upper right) and nucleotide difference (lower left)

Species	GenBank acc. no.	Sequence (bp)	ID	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Ceratomyxa diplodae	**KX099691**	1819	(1)	–	97.1	97.1	96.6	96.6	96.4	91.7	91.3	91.3	89.9
Ceratomyxa sp.	JF820293	1743	(2)	50	–	98.5	98.5	98.6	97.1	91.5	91.5	91.2	89.9
Ceratomyxa puntazzi	JF820290	1708	(3)	50	26	–	98.4	97.7	97.0	91.8	91.9	91.6	89.7
Ceratomyxa sp.	JF820291	1766	(4)	60	26	27	–	98.2	96.9	91.7	91.6	91.3	89.5
Ceratomyxa sparusaurati	AF411471	1741	(5)	59	23	37	31	–	98.2	91.5	91.2	91.0	90.1
Ceratomyxa sp.	JF820292	1769	(6)	64	50	52	54	31	–	90.7	90.8	90.6	88.9
Ceratomyxa sp.	DQ333431	1732	(7)	143	141	133	138	146	155	–	94.6	93.6	90.3
Ceratomyxa barnesi	FJ204245	1445	(8)	125	121	116	121	121	132	74	–	96.7	90.7
Ceratomyxa sp.	DQ333429	1705	(9)	147	145	133	143	151	154	108	45	–	90.6
Ceratomyxa labracis	AF411472	1763	(10)	173	169	167	174	170	186	166	128	158	–

Adriatic waters (Lubat et al. 1989). The description lacked photographs of the parasite and did not present a comparative discussion to the morphological traits of other *Ceratomyxa*, thus making future identifications of the parasite difficult. Lubat et al. (1989) apparently did not deposit any museum specimens (type material). Later, Sitjà-Bobadilla & Álvarez-Pellitero (1993b) identified *C. diplodae* from the gall bladder of *D. labrax*, based on myxospore morphometry, proximity of the sampling areas, and relatedness of the host families. Their redescription improved the morphological characterization and provided some LM micrographs of the myxospores, as well as a referenced holotype. *C. diplodae* was also reported occurring from the gall bladder of the cultured sparids common dentex *Dentex dentex* Linnaeus, 1758 and sharpsnout seabream *Diplodus puntazzo* (Rigos et al. 1997, Katharios et al. 2007). Identification was based on myxospore morphometry, geographic location, and host relatedness. The lack of molecular data supporting species identification, combined with the morphological and biological disparities among these reports, namely in terms of host species and tissue tropism, question their reliability.

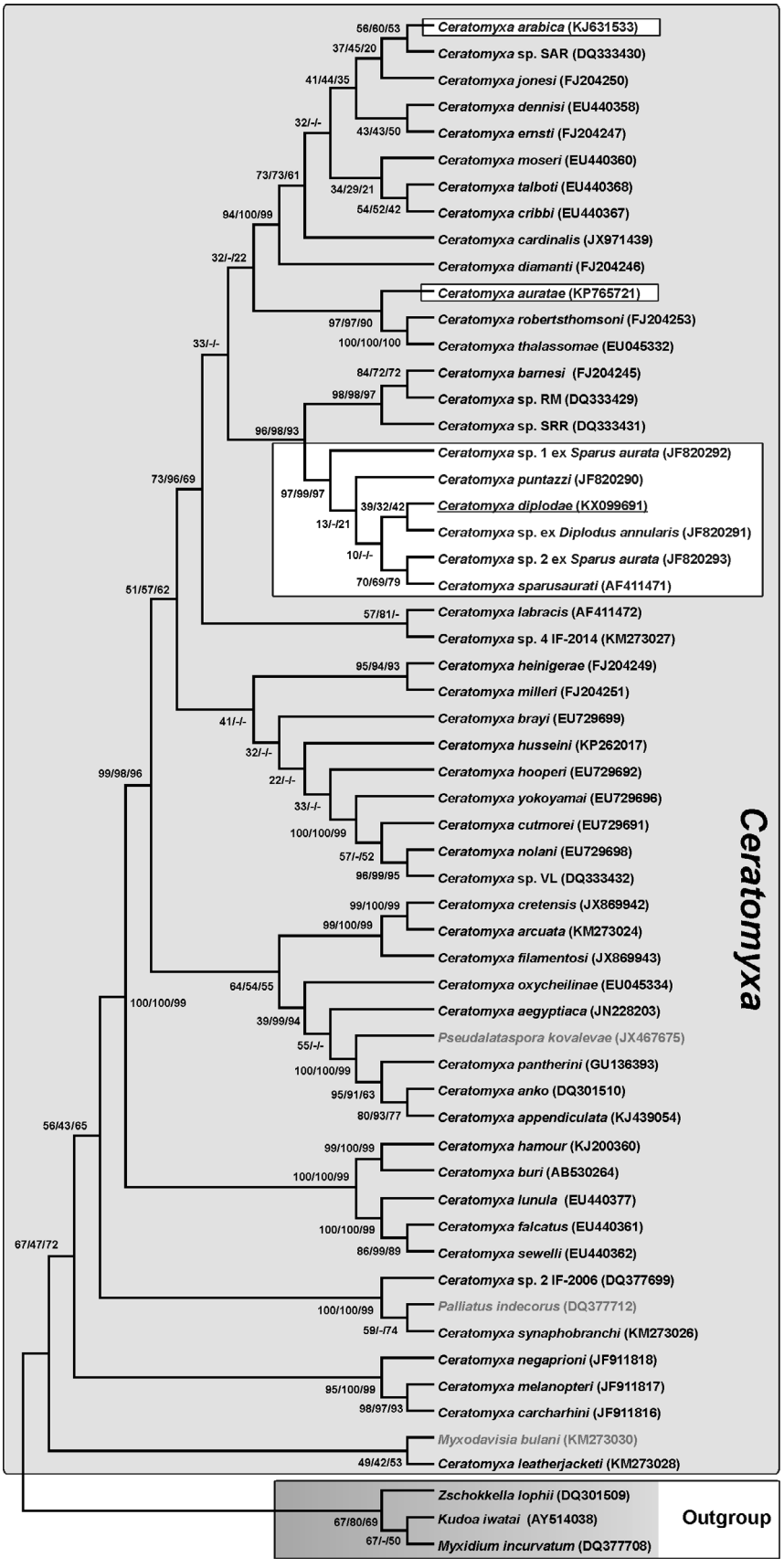

Fig. 3. Maximum likelihood tree of the small subunit ribosomal RNA (SSU rRNA) sequence of *Ceratomyxa diplodae* (underlined; white boxes: species described from sparid hosts) and other selected myxozoan species (grey type: other myxospore genera clustering with the *Ceratomyxa* clade). Numbers on the branches are bootstrap confidence levels corresponding to maximum likelihood/neighbor-joining/maximum parsimony trees. The final dataset included a total of 1082 positions. GenBank accession numbers are provided in parentheses

Although broad host specificity is a known trait of several myxosporean species (Hoffman et al. 1965, Diamant et al. 2006, Jirků et al. 2006), molecular systematics suggest that the genus *Ceratomyxa* is mostly host specific (Gunter & Adlard 2008, 2009, Gunter et al. 2009, Alama-Bermejo et al. 2011, Heiniger & Adlard 2013), so that the molecular redescription of several species reported from multiple hosts on a morphological basis will probably expand the genus further. Taking this into consideration, it is possible to assume that the morphological and biological variations between reports of *C. diplodae* may be inter-specific, rather than intra-specific (Rocha et al. 2015). Alama-Bermejo et al. (2011) attempted to sequence *C. diplodae* from its original host, *D. annularis*, but found yet another morphologically distinct *Ceratomyxa* species; this is in agreement with several studies that have reported the occurrence of infection by multiple ceratomyxids in some fish hosts (Gunter & Adlard 2009, Heiniger & Adlard 2013, Rocha et al. 2015). In addition to the above mentioned records of *C. diplodae* from the gall bladder of sparids, 10 other species and 3 records of *Ceratomyxa* exist from fish hosts of the family Sparidae, mostly in the Mediterranean and North Atlantic coasts off southern European countries (Table 3).

In the present study, the identification of *C. diplodae* was achieved through morphological comparison to the features reported by Sitjà-Bobadilla & Álvarez-Pellitero (1993b), as the latter was performed from the same host and constitutes the most reliable account of the parasite species. The myxospores studied here presented similar measurements to those reported in the description from *D. labrax*, further coinciding with the observation of different shapes: myxospores are frequently crescent-shaped, occasionally arcuate, with the rare occurrence of abnormal forms with 3 valves and 3 polar capsules. The only difference between this and the previous report is the number of polar filament coils, which are 3 to 4 according to the observations of Sitjà-Bobadilla & Álvarez-Pellitero (1993b). Here, the combined use of LM and TEM revealed the polar filament forming 8 to 9 coils organized in 2 rows. It is possible that, having relied solely on LM observations, the previous description considered only 1 of the 2 polar filament rows, thus presenting a smaller number of coils. The occurrence of aberrant spores with 3 valves and 3 polar capsules has been documented for other species of the genus, such as *C. bassoni* Abdel-Ghaffar et al., 2008, *C. protopsettae* Fujita, 1923, and *C. gurnardi* Sobecka et al., 2013 (Cho et al. 2004, Abdel-Ghaffar et al. 2008, Sobecka et al. 2013). Co-infections of *C. diplodae* and *C. labracis* were observed in infected specimens, with the parasites being differentiated on the basis of morphological and molecular information. Our comparative study further took into consideration the morphological and molecular traits of all other *Ceratomyxa* spp. known to infect sparid fish hosts (Tables 2 & 3). Comparison to *C. arcuata* Thélohan, 1892 relied on the morphological data provided in its original description from the sparid fish *Pagellus bogaraveo* (Brünnich, 1768), since its SSU rRNA sequence was obtained from non-sparid fish hosts. *C. pallida* Thélohan, 1894, *C. mylionis* (Ishizaki, 1960), and *C. acanthopagri* (Zhao & Song, 2003) are without molecular data, but differ morphologically from *C. diplodae*. *C. herouardi* Georgévitch, 1916 was described without providing measurements, and molecular data are not available, so a comparison to this species is difficult.

Although the genus *Ceratomyxa* represents one of the most cohesive myxosporean lineages, the internal phylogenetic relationships of its species remains unclear. Fiala et al., (2015) resolved 5 subclades within the main *Ceratomyxa* clade: (1) basal subclade comprising *M. bulani* and *Ceratomyxa leatherjacketi* Fiala et al., 2015; (2) subclade containing the species sequenced from elasmobranchs; (3) subclade in which *Palliatus indecorus* clusters with *Ceratomyxa* sp. 2 and *C. synaphobranchi* Fiala et al., 2015; (4) subclade comprising some sub-tropical and tropical species from the Pacific and Indian Oceans; (5) most derived taxon-rich subclade containing the majority of species with unresolved deeper nodes. The phylogenetic analysis performed here agrees with these findings and further acknowledges the phylogenetic position of *P. kovalevae* in a poorly resolved group of the most derived subclade, closely related to *C. pantherini* Gunter et al. 2010, *C. anko* Freeman et al., 2008, and *C. appendiculata* Thélohan, 1892. Molecular systematics reveals that the *Ceratomyxa* clade is not monophyletic due to the inclusion of *P. indecorus*, *P. kovalevae*, and *M. bulani*; this condition will probably be resolved by the taxonomic revision of these species, as suggested in previous studies (Fiala 2006, Gunter et al. 2009, Fiala et al. 2015, Rocha et al. 2015).

The SSU rRNA sequence obtained here for *C. diplodae* also clustered within the most derived subclade, forming a well-supported group with most of the *Ceratomyxa* spp. that have been sequenced from sparid hosts (*C. puntazzi*, *C. sparusaurati*, the 2 *Ceratomyxa* sp. from *S. aurata*, and *Ceratomyxa* sp.

Table 3. Comparison of *Ceratomyxa diplodae* to other *Ceratomyxa* spp. reported from the gall bladder of sparid hosts. SL: myxospore length; ST: myxospore thickness; PCL: polar capsule length; PCW: polar capsule width; PFc: polar filament coils. Measurements are means ± SD (range) (where available), given in µm

***Ceratomyxa* spp.**	**Host(s)**	**Location**	**SL**	**ST**	**PCL**	**PCW**	**PFc**	**Reference**
Ceratomyxa diplodae Lubat et al., 1989	*Dicentrarchus labrax*	North Atlantic off Portugal	5.7 ± 0.5 (4.8–6.7)	20.0 ± 2.5 (16.3–24.0)	2.9 ± 0.3 (2.5–3.4)	2.9 ± 0.3 (2.5–3.4)	8–9	Present study
	Dicentrarchus labrax	Mediterranean off Spain	6.2 ± 0.4 (5.0–7.5)	20.9 ± 2.8 (17.0–27.0)	3.0 ± 0.3 (2.5–3.8)	2.8 ± 0.4 (2.0–3.8)	3–4	Sitjà-Bobadilla & Álvarez-Pellitero (1993a)
	Diplodus puntazzo	Not indicated, probably Mediterranean off Greece	6.6 ± 0.5	24.0 ± 0.8	2.7 ± 0.2	2.7 ± 0.2	–	Katharios et al. (2007)
	Diplodus annularis	Mediterranean off Montenegro	6.0 (5.0–7.0)	20.0 (18.0–22.0)	2.2	2.0	–	Lubat et al. (1989)
Ceratomyxa auratae Rocha et al., 2015	*Sparus aurata*	South Atlantic off Portugal	6.7 ± 0.7 (5.3–7.6)	27.0 ± 3.0 (19.7–31.2)	3.6 ± 0.2 (2.9–3.8)	3.5 ± 0.3 (2.9–3.8)	5	Rocha et al. (2015)
Ceratomyxa arabica Al-Qahtani et al., 2015	*Acanthopagrus bifasciatus*	Arabian Gulf	8.0 ± 0.4 (7.0–9.0)	12.0 ± 0.4 (10.0–14.0)	3.0 ± 0.3 (2.5–3.5)	2.0 ± 0.2 (1.5–2.5)	3	Al-Qahtani et al. (2015)
Ceratomyxa sp. 1 ex *S. aurata* Alama-Bermejo et al., 2011	*Sparus aurata*	Mediterranean off Spain	5 ± 0.5 (3.9–5.6)	17.2 ± 3.4 (13.1–22.5)	2.2 ± 0.4 (1.6–2.7)	2.1 ± 0.3 (1.5–2.5)	–	Alama-Bermejo et al. (2011)
Ceratomyxa sp. 2 ex *S. aurata* Alama-Bermejo et al., 2011	*Sparus aurata*	Mediterranean off Spain	9.9 ± 0.6 (8.7–11.4)	20.0 ± 2.1 (16.7–24.7)	3.8 ± 0.3 (3.2–4.5)	3.8 ± 0.4 (3.2–4.5)	–	Alama-Bermejo et al. (2011)
Ceratomyxa sp. ex *Diplodus annularis* Alama-Bermejo et al., 2011	*Diplodus annularis*	Mediterranean off Spain	9.8 ± 0.8 (7.1–13.0)	28.8 ± 3.7 (21.5–32.7)	4.1 ± 0.6 (3.2–5.2)	4.1 ± 1.1 (3.1–5.1)	–	Alama-Bermejo et al. (2011)
Ceratomyxa puntazzi Alama-Bermejo et al., 2011	*Diplodus puntazzo*	Mediterranean off Spain	9.2 ± 0.7 (8.0–10.7)	29.0 ± 291 (23.8–34.5)	4.1 ± 0.4 (3.0–4.8)	4.0 ± 0.4 (2.9–4.6)	5	Alama-Bermejo et al. (2011)
Ceratomyxa acanthopagri syn. *Leptotheca acanthopagri* (Zhao & Song, 2003)	*Acanthopagrus schlegelii*	Off China	9.4 (8.7–10.0)	17.9 (16.0–20.0)	1.9 (1.7–2.3)	1.9 (1.7–2.3)	–	Gunter & Adlard (2010)
Ceratomyxa sparusaurati Sitjà-Bobadilla et al., 1995	*Sparus aurata*	Mediterranean off Spain and South Atlantic	5.6 ± 0.7 (4.5–7.5)	15.8 ± 1.0 (14.0–17.5)	2.8 ± 0.3 (2.2–3.4)	2.8 ± 0.3 (2.2–3.4)	6	Sitjà-Bobadilla et al. (1995)
Ceratomyxa mylionis syn. *Leptotheca mylionis* (Ishizaki, 1960)	*Acanthopagrus schlegelii*	Off Japan	6.2	13.3	2.3	2.3	–	Gunter & Adlard (2010)
Ceratomyxa herouardi Georgévitch, 1916	*Sarpa salpa*	Mediterranean off Monaco	–	–	–	–	–	Georgévitch (1916)
Ceratomyxa pallida Thélohan, 1894	*Sarpa salpa*, *Boops boops*	Mediterranean off France, Monaco and Croatia	5.0	25.0–30.0	–	–	–	Thélohan (1894)
Ceratomyxa arcuata Thélohan, 1892	*Pagellus bogaraveo*	Mediterranean off France, Monaco, Italy and Northeast Atlantic	6.8 ± 0.9 (6.0–9.0)	36.2 ± 2.7 (32.5–40.0)	3.7 ± 0.7 (2.5–5.0)	3.0 ± 0.2 (2.5–4.0)	4–5	Kalavati & MacKenzie (1999)

from *Diplodus annularis*), and closely related to *C. barnesi* and 2 other *Ceratomyxa* spp. from siganid hosts. The other sparid-infecting *Ceratomyxa* spp. included in the analysis (*C. arabica*, *C. auratae*, and *C. arcuata*) clustered separately, within different groups of the subclade. The inclusion of *C. diplodae* from the moronid fish host *D. labrax* among most of the sparid-infecting *Ceratomyxa* suggests the existence of a recent common ancestor for these species, which is also supported by geographic clustering (all described from the Mediterranean area and North Atlantic off Portugal). Nonetheless, geography has been shown not to correlate well with *Ceratomyxa* evolution (Gunter et al. 2009, Heiniger & Adlard 2013, Rocha et al. 2015), and this is supported by the present phylogenetic analysis, in which species from several different locations clustered together. For instance, *C. auratae* from the North Atlantic off Portugal and *C. arabica* from Saudi Arabia clustered separately, together with species from Australian waters. At this point, molecular data from infections of *C. diplodae* in its sparid hosts would be valuable to confirm those fish species as hosts for the parasite.

Information on the pathogenic action of coleozoic myxosporeans is very sparse, and the majority of *Ceratomyxa* spp. are considered to be relatively harmless. The few exceptions usually produce lesions characterized by vacuolization, deformation, and necrosis of the gall bladder epithelial cells, as has been described for the infection of *Ceratomyxa* spp. in *D. labrax*, *C. puntazzi* in *D. puntazzo*, and *C. dehoopi* Reed et al., 2007 in *Clinus superciliosus* (Linnaeus, 1758) (Álvarez-Pellitero & Sitjà-Bobadilla 1993b, Reed et al. 2007, Alama-Bermejo et al. 2011). In the case of *C. diplodae*, Katharios et al. (2007) reported high mortalities of *D. puntazzo* due to heavy infestations of the parasite in potentially immunosuppressed specimens, and Merella et al. (2005) correlated *C. diplodae* with the enlargement of the gall bladder in fish intensively infected with the polyopisthocotylean *Atrispinum salpae* (Parona & Perugia, 1890). Nonetheless, it cannot be disregarded that the latter studies were subject to a lack of reliable characters for species diagnosis, as previously mentioned. In our study, mortality was not reported from the sampling stocks, and infected specimens of *D. labrax* did not display symptoms of disease. Gross pathology of the gall bladder was not observed, demonstrating that only low-intensity infections were recorded. Therefore, histopathological studies of highly parasitized individuals may produce different results.

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