Running head: Ultrastructure and phylogeny of *Ceratomyxa auratae* n. sp.

**Ultrastructure and phylogeny of *Ceratomyxa auratae* n. sp. (Myxosporea:
Ceratomyxidae), a parasite infecting the gilthead seabream *Sparus aurata* (Teleostei:
Sparidae)**

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ABSTRACT

A new myxosporean parasite is described from the gall bladder of the gilthead seabream *Sparus aurata* in a Southern Portuguese fish farm, with basis on light and transmission electron microscopy, as well as in molecular procedures. In the bile, young and mature mono- to disporic plasmodia were elliptical and presented smooth surface membrane. Crescent-shaped myxospores measured 6.7 ± 0.7 (5.3–7.6) μm in length and 27.0 ± 3.0 (19.7–31.2) μm in thickness. The myxospores wall was constituted by two symmetrical valves united along a slightly curved suture line, each presenting a lateral projection with a rounded end. Two equal-sized subspherical polar capsules, measuring 3.6 ± 0.2 (2.9–3.8) μm in length and 3.5 ± 0.3 (2.9–3.8) μm in width, were located at the same level, each displaying a polar filament coiled in 5 turns. Molecular analysis of the SSU rRNA gene confirmed the parasite as a new member of the genus *Ceratomyxa*, making this the fourth report of *Ceratomyxa* from the gall bladder of *Sparus aurata* in the Iberian Peninsula. This reinforces the assumption that species richness of ceratomyxids in South European sparids is high, but the phylogenetic analysis performed disagrees with the existence of a common ancestor for *Ceratomyxa* species infecting sparid hosts, as well as their clustering according to geographical location. The main *Ceratomyxa* clade is not monophyletic due to the inclusion of *Palliatius indecorus* and *Pseudoalatospora kovalevae*; a situation that will probably be resolved by the taxonomic revision of these genera.

Keywords:

Myxozoa; Morphology; SSU rRNA gene; Sparids; Fish farm; Portugal

1. Introduction

Several members of the family Sparidae Rafinesque, 1810 are important commercially for both wild fisheries and aquaculture in South European countries, with the gilthead seabream, *Sparus aurata* Linnaeus, 1758, being a significant example. Despite the economic importance of this fish species for Portuguese fish farms, there are only two recent studies that provide information regarding the myxosporean parasites infecting the gilthead seabream in Portuguese aquaculture facilities. Rocha et al. [1] reported *Zschokkella auratis* infecting the gall bladder of this host, and Rangel et al. [2] reported *Ortholinea auratae* infecting the urinary bladder and posterior kidney. Elsewhere another eight species of Myxosporea have been reported to infect this host, some of which are associated with increased morbidity and mortality rates. *Kudoa iwatai* [3], which is a systemic species; *Enteromyxum leei* (formerly *Myxidium leei*) [4] in the intestinal tract; *Henneguya* sp. [5] in the gills and heart; *Sphaerospora sparidarum* (formerly *Leptotheca sparidarum*) [6] and *S. sparis* (formerly *Polysporoplasma sparis*) [7–9] in the kidney; *Ceratomyxa sparusaurati* [10], *Ceratomyxa* sp. 1 ex *S. aurata* and *Ceratomyxa* sp. 2 ex *S. aurata* [11], all in the gall bladder.

The genus *Ceratomyxa* Thélohan, 1892 constitutes one of the largest myxosporean genera, comprising about 300 species, the great majority of which are coelozoic in the gall bladder of marine teleosts, exceptionally appearing in elasmobranchs. Five species have been described from the freshwater environment, but only *Ceratomyxa hongtzensis* Hsieh and Chen, 1984 infects a strictly freshwater host, the bagrid catfish *Pelteobagrus eupogon* [12, 13]. *Ceratomyxa* have elongated, crescent-shaped or arcuate myxospores with valves that are frequently conical, exceeding in length the axial diameter of the spore. Myxospores contain two subspherical polar capsules and a binucleate sporoplasm or, more rarely, two uninucleate sporoplasms [13, 14]. The majority of *Ceratomyxa* spp. appear to be relatively harmless,

probably due to their coelozoic development. Few reports document the occurrence of pathological damage to host tissues, especially in less studied non-commercial fish species [15]. An example of a pathogenic *Ceratomyxa* spp. is *C. sparusaurati*, which despite being coelozoic in the gall bladder of *S. aurata*, causes serious histopathological damages that are associated with increased mortalities [16].

Despite spore morphology constituting a useful tool for species comparison, phylogenetic studies show that it is not the most reliable criterion for recognizing myxosporean taxonomy, and further reveal that the type of aquatic environment, tissue tropism and host relatedness are more phylogenetically informative criteria [1, 17–23]. For the molecular analysis of *Ceratomyxa*, GenBank provides the SSU rRNA sequences of a significant number of species that, however, are all coelozoic in teleosts exclusively inhabiting brackish/marine environments. The only exception is the SSU rRNA sequence of *Ceratomyxa diamanti*, which was obtained from the gall bladder of a catadromous teleost, *Polydactylus macrochir*, during its freshwater rearing in the Brisbane River, Queensland, Australia [15]. No molecular data are available for *Ceratomyxa* spp. infecting freshwater hosts; at least not since the genus *Ceratonova* was erected to encompass *Ceratonova shasta* syn. *Ceratomyxa shasta* (Noble 1950) as type species, and *Ceratonova gasterostea* Atkinson, Foott and Bartholomew, 2014 as its only congener [23]. *Ceratonova shasta* constituted 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 [15, 24, 25].

The present study seeks to describe the fourth ceratomyxid species found infecting the gall bladder of *S. aurata*, providing a reliable comparative basis for future parasitological surveys concerning this parasite genus and fish host.

2. Materials and methods

2.1. Fish and parasite sampling

Between June 2012 and June 2014, two hundred and sixty specimens of the gilthead seabream, *Sparus aurata* Linnaeus, 1758 (Teleostei: Sparidae) were obtained from a fish farm in the Alvor estuary, near the Atlantic coast (37°08'N/08°37'W), Portimão, Algarve, Portugal. The fish was collected from monthly stocks for commercialization, so it was slaughtered in accordance with the animal ethical laws stipulated for the aquaculture industry in Portugal and then shipped to the laboratory within thermic containers filled with chipped ice. Upon arrival, the dissection of fish was performed for the parasitological survey of several organs and tissues, with preliminary microscopic observations revealing myxosporean plasmodia and myxospores parasitizing the gall bladder of some specimens. The bile of the parasitized fish was collected and prepared for light microscopy, transmission electron microscopy (TEM) and molecular procedures.

2.2. Light microscopy and morphological analysis of myxospores

The parasitic stages in the bile were examined and photographed using a Zeiss Axiophot microscope (Grupo Taper, Sintra, Portugal), 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, in accordance to Lom and Arthur [26]. All measurements include the mean value $\pm$ standard deviations (S.D.), range of variation and number of spores measured (range, n).

2.3. Transmission electron microscopy

Free myxospores and plasmodia were isolated from the bile and fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.4) 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.

2.4. DNA extraction, amplification and sequencing

Free myxospores and plasmodia were obtained from the bile of four infected specimens and then fixed and preserved in absolute 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 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 MgCl₂, 5 µl 10× *Taq* polymerase buffer, 1.5 units *Taq* DNA polymerase (Nzytech, Lisbon, Portugal), and 3 µl (approximately 100–150 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-µl 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 cleanup that eliminates unincorporated primers and dNTPs is performed with Exonuclease I (E. 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 a BigDye Terminator v1.1 from the Applied Biosystems Kit (Applied Biosystems, Carlsbad, California), and were run on an ABI3700 DNA analyzer (Perkin-Elmer, Applied Biosystems, Stabvida, Oeiras, Portugal).

2.5. Distance and phylogenetic analysis

The forward and reverse segments sequenced were manually aligned with ClustalW [29] in MEGA 5 software [30], and ambiguous bases were clarified using corresponding ABI chromatograms. To determine the phylogenetic position of the new sequence of *Ceratomyxa* amongst its closest relatives sequenced to date, 61 myxosporean SSU rRNA sequences were obtained from GenBank and analysed. Thirty of these sequences belong to species of the genus *Ceratomyxa* and were chosen according to highest similarity score; as do *Palliatius indecorus* (DQ377712) and *Pseudoalataspora kovalevae* (JX467675). *Tetracapsuloides bryosalmonae* (U70623) and *Buddenbrockia plumatellae* (AY074915) were selected as outgroup. The remaining SSU rRNA sequences used in the analysis were selected according to aquatic environment and organ of infection. Phylogenetic and molecular evolutionary analyses were conducted using MEGA 5.05. Alignments were performed using ClustalW, with an opening gap penalty of 10 and a gap extension of four for both paired and multiple alignments [30].

The phylogenetic analysis was performed using 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, Kimura 2-parameter as substitution model with gamma distribution (shape parameter = 1.4) was performed. For MP, the close neighbour interchange (CNI) heuristic option with a search factor of 1 and random initial trees addition of 500 replicates was performed. All positions with less than 95% site coverage were eliminated from all trees, resulting in a total of 872 positions in the final dataset. The bootstrap consensus tree was inferred from 500 replicates for ML, NJ and MP.

Distance estimation was performed for a second alignment of the SSU rRNA sequences clustering together with the parasite here described and the other three *Ceratomyxa* spp. that occur in the gall bladder of *S. aurata*, resulting in a total of 867 positions in the final dataset. This analysis was also carried out in MEGA 5.05, using the Kimura-2 parameters model distance matrix for transitions and transversions, with all gaps and missing data eliminated.

3. Results

3.1. Characterization of *Ceratomyxa auratae* n. sp.

3.1.1. Taxonomic placement

Phylum Myxozoa Grassé, 1970

Class Myxosporea Bütschli, 1881

Order Bivalvulida Shulman, 1959

Family Ceratomyxidae Doflein, 1899

Genus *Ceratomyxa* Thélohan, 1892

3.1.2. Morphological description and taxonomic summary

Light microscopy: subspherical to elliptical plasmodia, in different stages of maturation, as well as mature myxospores were observed floating free in the bile (Figs. 1A, B). Myxospores elongated and crescent-shaped with rounded ends, 6.7 ± 0.7 (5.3–7.6) μm long ($n = 16$) and 27.0 ± 3.0 (19.7–31.2) μm thick ($n = 18$). Two equal-sized subspherical polar capsules, 3.6 ± 0.2 (2.9–3.8) μm long ($n = 19$) and 3.5 ± 0.3 (2.9–3.8) μm wide ($n = 19$), located at the same level at the myxospores' anterior pole (Figs. 1A, B).

Ultrastructure: young plasmodia subspherical and mature plasmodia elliptical, all presenting a smooth surface membrane and the cytoplasm riddled with mitochondria and vesicles containing granular material of different electronic density, as well as some lipidic droplets and large vacuoles. Sporogenic development mono- to disporic (Figs. 1C, D). The earliest stages of sporogenesis observed were young sporoblasts comprised by two large and single nucleated valvogenic cells surrounding the developing sporoplasmogenic cell and capsulogenic cells (Figs. 1E-G, 2A, B). At the beginning of its differentiation, each capsulogenic cell formed a capsular primordium composed by a cylindrical formation of microtubules surrounded by rough endoplasmic reticulum and numerous mitochondria, and containing a single nucleus (Figs. 1E, F). In more advanced stages, the capsular primordium assumed a globular shape and extended into an external tubule (Fig. 1G). The inversion and coiling of this tubule along the inner wall of the globular shape, originates the polar filament that characterizes the polar capsules (Figs. 2C, D). Myxospores constituted by two symmetrical valves, thick and smooth, united along a slightly curved suture line (Fig. 2E) and each presenting a lateral projection with a rounded end (Figs. 1A, B, 2B). Within, two polar capsules displaying a double-layered wall, comprised by a thinner outer electron-dense layer and a thicker inner electron-lucent layer, and containing a dense and homogeneous matrix where the polar filament coils in 5 turns (Figs. 2A, C, D). The sporoplasm, located at the posterior pole, presented two nuclei and many electron-dense sporoplasmosomes. A

semischematic drawing of a myxospore in sutural view is presented in Fig. 3, also incorporating ultrastructural features.

Type material: [Upon acceptance] One glass slide with semithin sections displaying plasmodia containing several myxospores of the hapantotype 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 2015.7. Identical sequences of the SSU rRNA gene from four samples collected from four infected fish specimens; a total of 1,846 bp obtained and deposited in GenBank (Accession number KP765721).

Type locality: the Alvor estuary, near the Atlantic coast (37°08'N/08°37'W), Portimão, Algarve, Portugal.

Type host: the gilthead seabream *Sparus aurata* Linnaeus, 1758 (Teleostei: Sparidae).

Site of infection: within the gall bladder.

Prevalence: ten infected in a total of 260 examined (3.8%).

Pathogenicity: collected and analyzed fish did not present external symptoms of infection or disease, neither was mortality recorded from the stock used.

Etymology: the specific epithet “*auratae*” derives from the specific epithet of the host species.

3.1.3. Molecular analysis

Four partial sequences of the SSU rRNA gene were obtained from parasitic material collected from four infected host individuals. These sequences were identical and a 1,846 bp consensus DNA sequence was deposited in GenBank (accession number KP765721). To determine the phylogenetic position of the new sequence here obtained and its relation to closely related taxa, a total of 63 SSU rRNA sequences were retrieved from GenBank, 30 of which refer to *Ceratomyxa* spp., and included those with higher BLAST scores and

maximum identity. ML, NJ, and MP phylogenetic trees constructed for the selected SSU rRNA sequences revealed the parasite clustering with all other *Ceratomyxa* spp. analyzed. The formation of this large *Ceratomyxa* clade, marine and coelozoic, appears supported by bootstrap values of 96% for ML, 97% for NJ and 86% for MP. Also clustering within this clade are *Pseudoalatospora kovalevae* and *Palliatius indecorus*. Other marine myxosporeans that are coelozoic in the gall bladder (*Sphaeromyxa kenti*, *S. zaharoni*, *S. hellandi*, *Zschokkella* sp. PS030203 and *Z. auratis*) do not cluster within the previous clade, but rather with freshwater myxosporeans that are also coelozoic in the gall bladder (*Sphaerospora* sp. 2 EE-2004, *Zschokkella* sp. DDI-2008 and *Z. parasiluri*). Also considered in the phylogenetic analysis, the freshwater and histozoic parasite *Ceratonova shasta* and its congener, *Cn. gasterostea*, appear related to a clade containing marine myxosporeans that are histozoic (*Enteromyxum scopthalmi*, *E. leei*, *Kudoa ogawai*, *K. iwatai*, *K. hypoepicardialis* and *K. leptacanthae*) or that infect the excretory system (*Zschokkella* sp. 4 IF-2006, *Z. lophii* and *Sinuolinea phyllopteryxa*) rather than to the clade containing freshwater species that are histozoic (*Myxobolus pyramidis*, *M. pellicides*, *M. bibullatus*, *Henneguya rhinogobii* and *H. pseudorhinogobii*) or that infect the excretory system of their fish hosts (*Myxobilatus gasterostei*, *Ortholinea auratae*, *Zschokkella* sp. AH2003, *Myxidium giardi* and *Sphaerospora oncorhynchi*).

Pairwise comparisons among the SSU rRNA sequences that cluster together with the parasite here described or share the same organ in the gilthead seabream resulted in percentages of similarity lower than 90%. The only exception is *Ceratomyxa gurnardi*, with a percentage of identity of 92% (Table 2).

4. Discussion

1 The characteristics of the myxosporean species here described are consistent with
2 those defined for the genus *Ceratomyxa* according to traditional criteria, namely in terms of
3 myxospore morphology [13, 24]. Nevertheless, phylogenetic studies show that this criterion
4 is not the most reliable for myxosporean differentiation, which should include molecular
5 analyses, typically of the SSU rRNA gene [1, 17–23]. In fact, the use of molecular tools have
6 recently led to the erection of a new ceratomyxid taxon, the genus *Ceratonova*, in order to
7 more appropriately incorporate the species that being morphologically very similar to
8 *Ceratomyxa*, are phylogenetically distant [24]. Molecular comparison between *Ceratomyxa*
9 spp. is restricted by the general lack of molecular data available for more diversified species
10 of this genus, specifically in terms of host taxonomy, aquatic environment and geographic
11 location. As such, the morphological criterion regains its importance as a taxonomic tool for
12 species differentiation. Acknowledging the widely accepted phylogenetic criteria of tissue
13 tropism, aquatic environment and host affinity for Myxosporea [1, 17– 23], the
14 morphological comparison performed in this study first took into consideration all the
15 *Ceratomyxa* spp. that are coelozoic in the gall bladder of sparid hosts (and more specifically
16 those previously described from the gall bladder of *S. aurata*) (Table 3). No significant
17 morphometric and morphologic resemblance to *Ceratomyxa auratae* n. sp. was found.
18 Further morphological comparison to other *Ceratomyxa* infecting the gall bladder of
19 brackish/marine teleosts, especially those without molecular data, also revealed no gross
20 similarity to *Ceratomyxa auratae* n. sp., despite the fact that many reports are outdated and
21 lack reliable data for comparative purposes (for instance, *Ceratomyxa herouardi* and
22 *Ceratomyxa coris* are without measurements) [12].

23 Overall, the results presented here are consistent with previous studies [15, 17, 20],
24 through supporting the existence of a close phylogenetic relationship among all *Ceratomyxa*
25 species. Now that the position of *Ceratonova shasta* has been resolved by the erection of its

own genus within the family Ceratomyxidae [24], the remaining exceptions to monophyly in the *Ceratomyxa* clade are *Palliatius indecorus* and *Pseudoalatospora kovalevae*. The placement of *Palliatius indecorus* within the large *Ceratomyxa* clade was noticed by Fiala [20] and, since then, supported by other phylogenetic studies [11, 15]. Lom and Dyková [13] describe *Palliatius* species as coelozoic in the gall bladder of marine fish, characterized by myxospores that are subspherical with an anteriorly prominent sutural ridge and enveloped in a membranaceous veil that in non-mature spores is twisted in two cords around the myxospore. The genus *Palliatius* Kovaleva and Dubina, 1979 currently belongs to the family Sphaerosporidae Davis, 1917 but several studies have suggested that a taxonomic revision of this group may be required. Shulman et al. [39] proposed the erection of a new taxon, the family Palliatidae, for this genus. Conversely, Gunter et al. [15] believed that this genus would be more appropriately assigned to the family Ceratomyxidae, based both on the molecular analysis of the SSU rRNA gene, and on tissue tropism (members of Sphaerosporidae mainly infect the urinary system of their hosts, while all *Palliatius* infect the gall bladder). Considering the phylogenetic placement of *P. indecorus* in our study and, without disregarding tissue tropism and aspects of the generic morphology, it seems correct that this genus should be attributed to the family Ceratomyxidae. Nevertheless, further SSU rRNA sequences from *Palliatius*, particularly from the type species *Palliatius mirabilis* Shulman, Kovaleva and Dubina, 1979 are necessary before the taxonomic position of this genus is clarified. The SSU rRNA sequence of *Pseudoalatospora kovalevae* constitutes the only molecular data available for the genus *Pseudoalatospora* Kovaleva and Gaevskaya, 1983 [39], which Lom and Dyková [14] describe as comprising myxosporean parasites that are coelozoic in the gall bladder of marine fish, with myxospores that are extremely elongated in the plane perpendicular to the central straight sutural line and display membranaceous projections adhering along their posterior half. These projections are

1 doubled to form parachute-like pockets. The genus *Pseudoalatospora* currently belongs to the
2 family Alatosporidae Shulman, Kovaleva and Dubina, 1979 but shares similar morphological
3 characters with *Ceratomyxa*, differing only in the presence/absence of delicate alate
4 processes. According to Kalavati et al. [40], these alate processes are fragile and difficult to
5 observe, and raised the possibility that some *Pseudoalatospora* may be erroneously assigned
6 to the genus *Ceratomyxa*. Nevertheless, taking into account the close phylogenetic
7 relationship of *P. kovalevae* to *Ceratomyxa* spp. revealed in this study, as well as the shared
8 tissue tropism and faint morphological differences between these genera, its species would
9 probably be more adequately assigned within the same genus, namely within the family
10 Ceratomyxidae, which represents a stronger taxon than the family Alatosporidae. The latter
11 contains only another genus, *Alatospora* Shulman, Kovaleva and Dubina, 1979, which also
12 contains species that are coelozoic in the gall bladder of marine fish and display myxospore
13 morphology highly similar to *Pseudoalatospora* and, therefore, also to *Ceratomyxa*, but lacks
14 molecular data to corroborate phylogenetic similarity to ceratomyxids. Thus, further
15 sequencing of both *Pseudoalatospora* and *Alatospora* will probably lead to the demise of the
16 family Alatosporidae, with subsequent assignment of its species to the genus *Ceratomyxa*.

17 *Ceratonova shasta* clusters with its only congener, *Cn. gasterostea*, and together form
18 a subclade positioned within a clade containing marine myxosporeans that are histozoic or
19 infect the excretory system, and that is sister to the large *Ceratomyxa* clade. This position was
20 constant in all tree topologies and supports the idea that *Ceratonova* spp. represent an
21 independent lineage of ceratomyxids, as suggested by Fiala [20] for *Cn. shasta*. It is also
22 noticed that *Ceratonova* spp. constitute exceptions to the Myxosporea main phylogenetic
23 division into a marine clade and a freshwater clade.

24 Considering the phylogenetic setting within the large clade of *Ceratomyxa*, it is noted
25 that *Ceratomyxa auratae* n. sp. does not cluster within the subclade containing other

Ceratomyxa spp. sequenced from the gall bladder of sparid hosts (*C. sparusaurati*, *C. puntazzi*, *Ceratomyxa* sp. 1 *Sparus aurata*, *Ceratomyxa* sp. 2 *Sparus aurata*, *Ceratomyxa* sp. ex *Diplodus annularis*) [11, 41], but rather within a sister subclade containing *Ceratomyxa* spp. that infect fish hosts of the families Triglidae (*C. gurnardi*), Apogonidae (*C. rueppellii*, *C. ostorhinchii* and *C. cyanosomae*), Serranidae (*C. gleesoni*), Lethrinidae (*C. hallettae*) and Labridae (*C. thalassomae*) of the order Perciformes [15, 42–45], as well as of the family Mugilidae (*C. robertsthompsoni*) of the order Mugiliformes [15]. *Ceratomyxa arabica*, which was also reported from the gall bladder of a sparid fish host, *Acanthopagrus bifasciatus*, in the Arabian Gulf in Saudi Arabia [31], also clusters in a separate subclade. This placement disagrees with previous studies that suggest a common ancestor for *Ceratomyxa* species infecting sparid hosts, as well as their clustering according to geographical location [11], since *Ceratomyxa auratae* n. sp. clusters closely with species described from Australian waters [15, 42–44] and *C. gurnardi* from the Atlantic Ocean off Scotland [45]. The inclusion of *C. gurnardi* in this clade is, however, dubious since its sequence is rather small (949 bp) in comparison to all the other ceratomyxid SSU rRNA sequences used in the phylogenetic analysis, which account for at least 1300 bp. Overall, this type of clustering suggests that radiation with host taxa and geographical location does not drive the overall phylogeny of *Ceratomyxa*.

With the exception of *C. mylionis*, *C. arabica* and *C. acanthopagri*, all other *Ceratomyxa* spp. infecting the gall bladder of sparid hosts have been described from the North Atlantic and Mediterranean waters bathing South European countries, as seen in table 3. Thus, *Ceratomyxa auratae* n. sp. is the ninth species of its genus described from sparid hosts in South European waters, and the fourth from the gall bladder of *Sparus aurata* in the Iberian Peninsula. The description of this new ceratomyxid reinforces the assumption that species richness of *Ceratomyxa* in South European sparids is high and that a single sparid

1 host species can be infected by more than one species of *Ceratomyxa* [11, 43], with or
2 without the occurrence of co-infection. On the other hand, molecular systematics have shown
3 that most marine *Ceratomyxa* are restricted to a single host [15, 44, 46], so reports based on
4 morphology alone of several species of this genus from multiple hosts suggest that a
5 significant number of known species are yet to be described. For instance, *Ceratomyxa*
6 *diplodae* was originally described from *Diplodus annularis* [35] and later from *Dicentrarchus*
7 *labrax* [34] and *D. puntazzo* [33], but no molecular data were provided in any of these
8 reports. Therefore, it is probable that the morphological variations between *C. diplodae*
9 descriptions are not host dependent but inter-specific and represent further species richness in
10 South European sparids.
11

12 The ultrastructural study of the sporogenic development of *Ceratomyxa auratae* n. sp.
13 revealed asynchronous development, with both young and mature plasmodia present
14 simultaneously in infected gall bladders. The ultrastructural features of the plasmodial
15 development described here are congruent with those generally recognized in coelozoic
16 myxosporeans, in that the size and shape of the plasmodia vary according to the stage of the
17 sporogenic development [2, 6, 34, 47]. However, the differentiation of peripheral projections
18 does not occur, and the morphometric and morphological alterations incurred by the
19 plasmodia appear associated with the growth of the myxospores, rather than with nutritional
20 purposes. Also, although no long term study was performed on the fish specimens parasitized
21 by *Ceratomyxa auratae* n. sp., no gross ultrastructural damages to the gall bladder epithelium
22 were observed, and clinical signs of infection or disease were absent, indicating that this
23 species does not affect gravely the gilthead seabream.
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25 **Acknowledgments**

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Figure legends

Fig. 1. Light and transmission electron micrographs of *Ceratomyxa auratae* n. sp. infecting the gall bladder of *Sparus aurata*. **A.** Young and mature plasmodia (Pl) and myxospores (S) observed in DIC optics. Scale bar: 15 µm. **B.** Mature plasmodia (Pl), each containing a crescent-shaped myxospore, as observed in DIC optics. Scale bar: 10 µm. **C.** Ultrathin section of young and subspherical plasmodia containing developing myxospores (S) and displaying the cytoplasm riddled with vesicles (V) and lipidic droplets (Li). Scale bar: 5 µm. **D.** Ultrathin section of a mature elliptical plasmodium (Pl) containing a myxospore (S). Scale bar: 5 µm. **E.** Ultrathin section of a plasmodium (Pl) showing two capsulogenic cells (CC) in different developmental stages: one displaying the capsular primordium constituted by a nucleus (N) and rough endoplasmic reticulum (RER), and the other, more developed, showing the polar filament (PF) coiled within the globular structure of the maturing polar capsule. Scale bar: 2 µm. **F.** Ultrastructural detail of the capsular primordium evidencing some of its constituents: the nucleus (N), the rough endoplasmic reticulum (RER) and mitochondria (Mt). Scale bar: 2 µm. **G.** Globular capsular primordium (Cp) extending into an

external tubule (arrow), which will latter invert and coil along the inner wall of the polar capsule, originating the polar filament. Scale bar: 2 μ m.

Fig. 2. Transmission electron micrographs of *Ceratomyxa auratae* n. sp. infecting the gall bladder of *Sparus aurata*. **A.** Developing myxospore showing two valvogenic cells (VC) surrounding two capsulogenic cells (CC). The latter exhibit each a remaining nucleus (N). Also notice one of the two nuclei (*) of the sporoplasmogenic cell. Scale bar: 2 μ m. **B.** Plamodium (Pl) containing two myxospores (S), one of which displaying the sporoplasmogenic cell (SPC) and its nucleus (*) appearing adjacent to one of the capsulogenic cells (CC). Notice the nucleus (arrow) of one of the two valvogenic cells. Scale bar: 2 μ m. **C.** Capsulogenic cell (CC) displaying its nucleus (N) and the almost matured polar capsule in longitudinal section. Notice the stopper (arrow) located at the apex of the latter, and the polar filament (PF) coiling within. Scale bar: 2 μ m. **D.** Longitudinal section of a polar capsule, displaying its double-layered wall (arrowheads) capped by a stopper (arrow) at the apex and containing a homogenous matrix (*) where the polar filament (PF) coils. Scale bar: 2 μ m. **E.** Ultrastructural detail of the two valves united along a slightly curved suture line (arrowheads). Scale bar: 1 μ m.

Fig. 3. Schematic drawing of a myxospore of *Ceratomyxa auratae* n. sp., depicting the internal and external organization described in the text. Scale bar: 5 μ m.

Fig. 4. Maximum likelihood tree of the SSU rRNA sequence of *Ceratomyxa auratae* n. sp. and other selected myxozoan species. The numbers on the branches are bootstrap confidence levels on 500 replicates corresponding to ML/NJ/MP trees. There were a total of 872

positions in the final dataset. GenBank accession numbers in parentheses after the species
name; scale is given under the tree.

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Figure

Table 1. Polymerase chain reaction primers used for the amplification and sequencing of the SSU rRNA gene of *Ceratomyxa auratae* n. sp.

Name	Sequence (5'-3')	Position	Pared with	Source
18e	CTG GTT GAT CCT GCC AGT	1	CeratR1, MYX4R	[27]
MyxospecF	TTC TGC CCT ATC AAC TTG TTG	312	MYX4R, 18r	[20]
MYX4F	GTT CGT GGA GTG ATC TGT CAG	1300	18r	Present study
CeratR1	CCA ATG TCT GGA TTG GGT A	420	18e	Present study
MYX4R	CTG ACA GAT CAC TCC ACG AAC	1300	18e, MyxospecF	Present study
18r	CTA CGG AAA CCT TGT TAC G	1832	MyxospecF, MYX4F	[28]

Table 2. Percentage of similarity for the small subunit rRNA sequences of *Ceratomyxa* species that cluster together with *Ceratomyxa auratae* n. sp., as well as for those that also infect the gall bladder of *Sparus aurata*.

ID	Species	GenBank	1	2	3	4	5	6	7	8	9	10	11	12
(1)	<i>Ceratomyxa auratae</i> n. sp.	(xxxxxxx)	-											
(2)	<i>Ceratomyxa gurnardi</i>	(JQ071439)	92.0	-										
(3)	<i>Ceratomyxa hallettae</i>	(FJ204248)	89.9	89.3	-									
(4)	<i>Ceratomyxa cyanosomae</i>	(JX971424)	89.4	88.8	91.5	-								
(5)	<i>Ceratomyxa</i> sp. 2 ex <i>S. aurata</i>	(JF820293)	89.3	88.0	88.4	87.0	-							
(6)	<i>Ceratomyxa gleesoni</i>	(EU729693)	89.0	88.4	90.2	88.7	85.3	-						
(7)	<i>Ceratomyxa sparusaurati</i>	(AF411471)	88.9	87.7	88.6	87.2	99.1	85.1	-					
(8)	<i>Ceratomyxa ostorhinchii</i>	(JX971425)	88.5	88.9	91.2	96.0	86.8	88.9	86.8	-				
(9)	<i>Ceratomyxa thalassomae</i>	(EU045332)	88.3	89.1	90.9	95.8	86.0	89.0	86.7	95.7	-			
(10)	<i>Ceratomyxa rueppellii</i>	(JX971423)	88.3	88.8	94.4	91.0	86.4	90.1	85.9	90.5	91.0	-		
(11)	<i>Ceratomyxa robertsthomsoni</i>	(FJ204253)	87.6	87.9	93.8	90.6	85.5	89.8	85.1	90.1	90.2	98.1	-	
(12)	<i>Ceratomyxa</i> sp. 1 ex <i>S. aurata</i>	(FJ820292)	87.2	86.0	87.2	85.1	96.9	84.1	97.7	84.7	83.5	83.9	83.0	-

Table 3. Morphological comparison between *Ceratomyxa* spp. that infect 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 given in μm .

<i>Ceratomyxa</i> spp.	Hosts	Location	SL	ST	PCL	PCW	PFc	References
<i>Ceratomyxa auratae</i> n. sp.	<i>Sparus aurata</i>	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	Present study
<i>Ceratomyxa arabica</i> Al-Qahtani, Mansour, Al-Quraishy & Abdel-Baki 2015	<i>Acanthopagrus bifasciatus</i>	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	[31]
<i>Ceratomyxa</i> sp. 1 ex <i>S. aurata</i> Alama-Bermejo, Raga & Holzer, 2011	<i>Sparus aurata</i>	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)	–	[11]
<i>Ceratomyxa</i> sp. 2 ex <i>S. aurata</i> Alama-Bermejo, Raga & Holzer, 2011	<i>Sparus aurata</i>	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)	–	[11]
<i>Ceratomyxa</i> sp. ex <i>Diplodus annularis</i> Alama-Bermejo, Raga & Holzer, 2011	<i>Diplodus annularis</i>	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)	–	[11]
<i>Ceratomyxa puntazzi</i> Alama-Bermejo, Raga & Holzer, 2011	<i>Diplodus puntazzo</i>	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	[11]
<i>Ceratomyxa acanthopagri</i> syn. <i>Leptotheca acanthopagri</i> (Zhao & Song, 2003)	<i>Acanthopagrus schlegelii</i>	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)	–	[32]
<i>Ceratomyxa sparusaurati</i> Sitjà-Bobadilla, Palenzuela & Alvarez-Pellitero, 1995	<i>Sparus aurata</i>	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	[10]
<i>Ceratomyxa diplodae</i> Lubat, Radujkovic, Marques & Bouix, 1989	<i>Diplodus puntazzo</i>	Not indicated, probably Mediterranean off Greece	6.6 ± 0.5	24.0 ± 0.8	2.7 ± 0.2	2.7 ± 0.2	–	[33]
	<i>Dicentrarchus labrax</i>	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	[34]
	<i>Diplodus annularis</i>	Mediterranean off Montenegro	6.0 (5.0–7.0)	20.0 (18.0–22.0)	2.2	2.0	–	[35]
<i>Ceratomyxa mylionis</i> syn. <i>Leptotheca mylionis</i> (Ishizaki, 1960)	<i>Acanthopagrus schlegelii</i>	Off Japan	6.2	13.3	2.3	2.3	–	[32]
<i>Ceratomyxa herouardi</i> Georgévitch, 1916	<i>Sarpa salpa</i>	Mediterranean off Monaco	–	–	–	–	–	[36]
<i>Ceratomyxa pallida</i> Thélohan, 1894	<i>Sarpa salpa</i> , <i>Boops boops</i>	Mediterranean of France, Monaco and Croatia	5.0	25.0-30.0	–	–	–	[37]
<i>Ceratomyxa arcuata</i> Thélohan, 1892	<i>Pagellus bogaraveo</i>	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	[38]

Fig. 1. Gallbladder of the gilthead seabream *Sparus aurata*. Infection by a myxosporean of the genus *Ceratomyxa* was detected, with a prevalence of infection of 3.8%.

Fig. 2. *Ceratomyxa auratae* n. sp. (A) Plasmodia and spores as observed in DIC optics. Scale bar: 20 μ m. (B) Transmission electron micrograph. Scale bar: 5 μ m.

Fig. 3. 1% agarose 1 $\times$ tris-acetate-EDTA buffer (TAE) gel stained with ethidium bromide, in which the PCR products were electrophoresed.