The Journal of Eukaryotic Microbiology

Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

ORIGINAL ARTICLE

Molecular and Morphometric Characteristics of *Ceratomyxa hamour* n. sp. (Myxosporea: Bivalvulida) Infecting the Gallbladder of the Orange-spotted Grouper *Epinephelus coioides* from the Arabian Gulf, Saudi Arabia

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Keywords
Bile; Myxozoa; new species; parasite; phylogeny.

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Received: 14 March 2014; revised 10 May 2014; accepted May 13, 2014.

doi:10.1111/jeu.12148

ABSTRACT

Ceratomyxa hamour n. sp. was found to infect the gallbladder of the orange-spotted grouper, *Epinephelus coioides* located off the Saudi Arabian coast of the Arabian Gulf. The infection was reported as a free-floating spore in the bile, and pseudoplasmodia were not observed. Mature spores were crescent-shaped and measured on average 7 μm in length and 16 μm in thickness. The polar capsule, meanwhile, had length to width measurements of 4 μm and 3 μm on average. A periodical survey was conducted throughout a sampling period between December 2012 and December 2013, with the results showing that the parasite was present throughout the year with a mean prevalence of 32.6%. The objective of this study was to characterize this new species based on its morphological and molecular differences from previously described species. Molecular analysis based on the partial sequence of the SSU rDNA gene, showed the highest similarity (97.8%) to *Ceratomyxa buri*, reported in the cultured yellow tail *Seriola quinqueradiata* in Japan. Indeed, *C. buri* and the new species described here formed an individual cluster with a high degree of bootstrap support. This is the first reported species of genus *Ceratomyxa* from the Arabian Gulf fishes off Saudi Arabia.

THE orange-spotted grouper, *Epinephelus coioides*, belongs to the family Serranidae (Randall 1997) and occurs in the western Indian Ocean from the southern Red Sea to Natal and east to the western Pacific, where it is distributed from the Ryukyu Islands to New South Wales. It ranges east into Oceania as far as Palau in the Northern Hemisphere and Fiji in the Southern (Grandcourt et al. 2005; Russell and Houston 1989). *Epinephelus coioides*, common name hamour, is the commonest fish species in the Arabian Gulf and is one of the most important commercially exploited species in Saudi Arabia (Grandcourt et al. 2005; Randall 1995).

Myxosporean parasites have a worldwide distribution, mostly in fish hosts, although rarely in platyhelminths, reptiles, and amphibians. Recently, a myxozoan-like parasite was found in the brain of a mole *Talpa europrea* (Friedrich et al. 2000; Kent et al. 2001; Lom and Dykova 2006). *Ceratomyxa* is the second largest genus of myxozoan parasites, with more than 280 species recounted globally (Gunter et al. 2010). Members of the genus *Ceratomyxa* are mostly coelozoic, parasitizing the gallbladder of marine fishes. *Ceratomyxa* spp. are distinguished by spores of elongated shape with shell valves exceeding in extent the axial diameter of the spore (Lom and Dykova 2006). There are two sub-spherical polar capsules near the sutural line at the anterior pole of the spore (Sobecka et al. 2013). Binucleate sporoplasm does not fill the spore cavity completely, and, in some species, two uninucleate sporoplasms have been reported (Lom and Dykova 2006).

Until recently, detection and identification of *Ceratomyxa* species are performed with microscopy-based methods by examination of morphometrical features of the mature spores. This could mask multiple distinct cryptic species because of the plasticity on the spore morphometry (Heiniger et al. 2008; Smothers et al. 1994).

Actually new species descriptions should include both molecular and morphological data, and eventually ecobiological parameters of parasite (Fiala 2006; Smothers et al. 1994). Molecular analysis, mainly based on the SSU rDNA sequence and sometimes completed with the LSU rDNA sequence, offer the possibility of more accurate and efficient methods for *Ceratomyxa*-specific characterization and may help on the elucidation of misclassified species (Gunter and Adlard 2010; Smothers et al. 1994).

To our knowledge, no *Ceratomyxa* spp. have so far been described in fishes from the Arabian Gulf. Herein, we describe a new species of *Ceratomyxa* from the gallbladder of an economically significant fish, the orange-spotted grouper, *E. coioides*. The identification was based on spore morphometry and molecular phylogeny.

MATERIALS AND METHODS

Host and parasite

Between December 2012 and December 2013, a total 85 orange-spotted grouper *E. coioides* were collected from the boat landing site at Dammam (26°33′20″N, 50°0′25″E) on the eastern coast of Saudi Arabia. The fish were examined for myxosporean infection. Gallbladders were extracted with caution and bile was collected by puncturing the gall bladder with a delicately pointed glass pipette for light microscopic examination. Some positive gallbladders with microscopic examination were preserved in 85% ethanol for molecular analysis. Fresh spores were photographed and measured using an Olympus microscope equipped with a digital camera. Spore description and measurements followed the guidelines of Lom and Arthur (1989). Measurements were based on 50 fresh spores using ocular micrometer and data were presented as mean ± SD (range).

SSU rDNA analysis

DNA extraction was carried out from 50 μl of the infected bile preserved in ethanol using the QIAGEN DNeasy kit (QIAGEN Inc., Valencia, CA). SSU rDNA was amplified using the PCR technique. The partial 18S sequence was amplified using the forward primer Myxospec F (5′-TTC TGCCGT ATC AAC TWG TTG) (Fiala 2006) and the reverse primer18R (5′-CTACGG AAA CCT TGT TAC G) (Whipps et al. 2003). Amplifications were performed in a final volume of 30 μl of PCR mixture containing 1X Taq DNA polymerase buffer (MBI, Fermentas), 0.2 mmol of mixed dNTP, 1.5 mmol of $MgCl_2$, 0.2 pmol of each primer, 1 U of Taq DNA polymerase and 50–100 ng of DNA, and ultra-pure water. The amplification was done in a thermocycler apparatus (Techne TC-Plus Satellites, Staffordshire, UK). The PCR cycling program used consisted of an initial step of denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s, and a final step of extension at 72 °C for 5 min. Subsequently, PCR products were separated in 1% agarose gel electrophoresis in a Tris-borate-EDTA buffer (0.045 M Tris-borate, 0.001 M EDTA pH 8.0), stained with ethidium bromide, and visualized on an UV transilluminator using a gel documentation system (BioRad Gel225 Doc™XR+, Ontario, Canada). PCR products obtained from three different specimens were sequenced by Macrogen Inc. (Seoul, South Korea), using the two primers mentioned above and two additional internal primers, MyxF1338 and MyxR1437 (Mansour et al. 2013).

Phylogenetic analysis

Overlapping sequences were clipped and edited by visual inspection and assembled. The consensus sequence of 1,603 bp was used to extract the most similar sequence from GenBank using a BLAST (Basic Local Alignment Search Tool) search at NCBI (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/). Thirty-three sequences of *Ceratomyxa* species and one *Tetracapsuloides bryosalmonae* isolate were clipped (accession numbers and coordinates listed in Table 1) and aligned with ClustalX 2.1.0.12, applying the default parameters (Larkin et al. 2007). A phylogenetic tree, based on the obtained alignment was generated using maximum likelihood and neighbor joining methods, using version 5 of the MEGA software (Tamura et al. 2011). The parameters for the maximum likelihood analyses were General Time Reversible model, 2,000 bootstrap replications, Gamma distributed (G), number of discrete Gamma rates of 5, and complete deletion. The genetic distance matrix was estimated using the Kimura two-parameter model distance for transition and transversion, for a total of 1,092 positions in the final dataset, and the rate variation among sites was modeled with a gamma distribution (shape parameter = 4).

Statistical analysis

One-way ANOVA was carried out, and the statistical comparisons among the seasons were performed with Holm–Sidak method using a statistical package program (Sigma Plot version 11.0, Systat Software, San Jose, CA). All p values are two-tailed, and $p < 0.001$ was considered as significant for all statistical analysis in this study.

RESULTS

The infection was observed in the form of free-floating spores in the bile. Plasmodia were not observed.

Table 1. The relationship between the seasons and the prevalence of *Ceratomyxa hamour* n. sp. infecting the gallbladder of *Epinephelus coioides* from the Arabian Gulf

Seasons	No. examined fish	No. infected fish	Percent of infection
Spring	36	12	33.3
Summer	36	11	30.5
Autumn	36	7	19.4
Winter	36	17	47.2
Total	144	47	32.6

Spore description

Spores were typical of the genus *Ceratomyxa*. Mature spores were crescent-shaped with two smooth identical valves in the frontal view (Fig. 1, 2). Spores measured 7 ± 0.3 (6–8) μm in length and 16 ± 0.4 (15–18) μm in thickness. A straight sutural line was clearly visible between the valves (Fig. 1A, 2). The polar capsules were equal in size, pyriform in shape, and measured 4 ± 0.2 (3–5) μm in length and 3 ± 0.3 (2–4) μm in width. The polar filament generally formed three turns somewhat oblique to the longitudinal axis of the capsules. The sporoplasm filled the entire spore cavity (Fig. 2).

Prevalence and seasonal variation

The infection was encountered in all seasons with overall prevalence 32.6% (47/144). The highest prevalence was reported in winter 47.2% (17/36) followed by spring 33.3% (12/36), summer 30.5% (11/36), and autumn 19.4% (7/36). There was a significant difference observed between winter and summer (p 0.001), winter and autumn (p 0.001), spring and summer ($p = 0.023$), spring and autumn ($p = 0.004$), and winter and spring ($p = 0.010$), but not between autumn and summer ($p = 0.272$) (Table 1; Fig. 3).

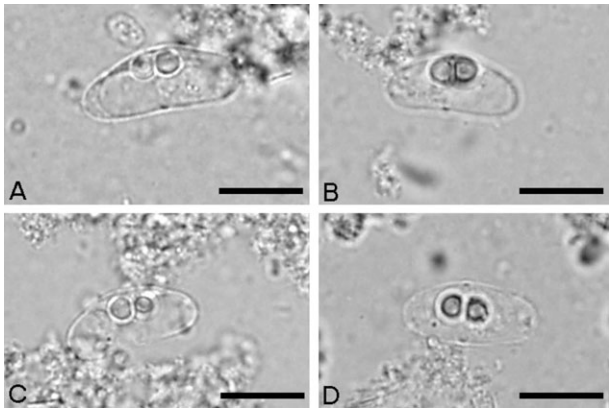

Figure 1 **A–D.** Fresh spores of *Ceratomyxa hamour* n. sp. from gallbladder of the *Epinephelus coioides*. Scale bar = 10 μm.

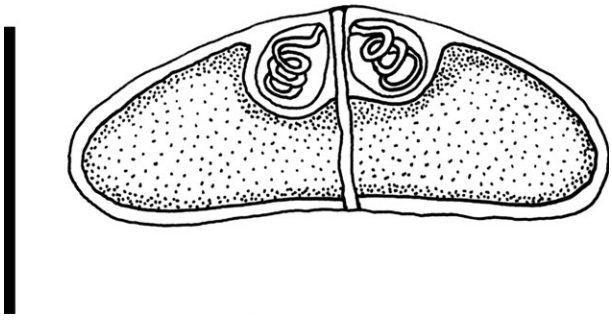

Figure 2 Schematic drawing of a mature spore of *Ceratomyxa hamour* n. sp. Scale bar = 10 μm.

Molecular phylogenetic analysis

A sequence of 1,603 bp of the 18S rRNA gene (GenBank accession number: KJ200360) of the present *Ceratomyxa* species was used in the comparison with other available and similar sequences in GenBank. Blast research revealed that there is no identical sequence deposited in GeneBank. The highest level of identity was provided by the GenBank entry AB530264 corresponding to the 18S sequence of *Ceratomyxa buri* Yokoyama, Fukuda, 2001. Pairwise comparisons based on the Kimura two-parameter model showed that the minimum genetic distance was 0.022 corresponding to 97.8% identity with *C. buri* (Table 2). The percentages of similarities ranged between 93.5% and 82% for the other *Ceratomyxa* species included in this study (Table 2). The distance with *T. bryosalmonae* (chosen as outgroup) was 58.2%. The phylogenetic trees generated by the NJ and ML methods are similar and show the close association between the present *Ceratomyxa* species and *C. buri* in the same branch with high bootstrap support of 100 (NJ) and 99% (ML) (Fig. 4).

TAXONOMIC SUMMARY

Type host. Orange-spotted grouper *E. coioides* Hamilton, 1822 (Teleostei, Perciforme, Serranidae).
Type locality. Saudi Arabian coast of the Arabian Gulf.
Site of infection. Gallbladder.
Prevalence. 32.6% (47/144).
Type-material. Syntypes of spore of *C. hamour* n. sp. in gallbladder content of the orange-spotted grouper *E. coioides*—sample in 80% ethanol are deposited in Hungarian

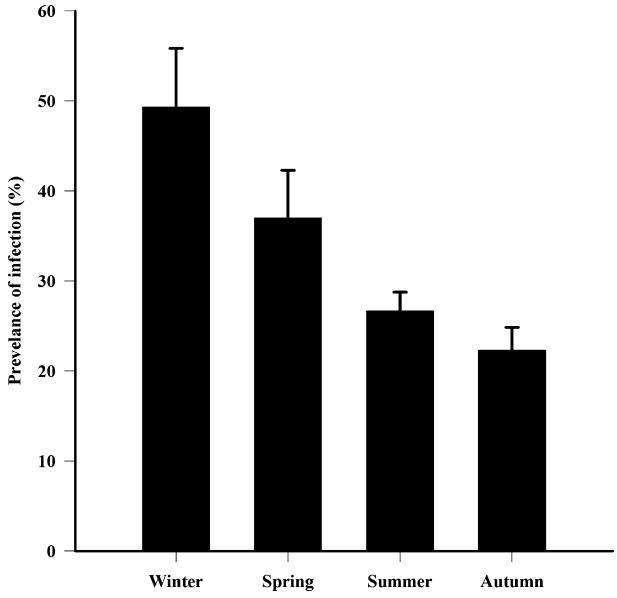

Figure 3 The relationship between the seasons and the prevalence of *Ceratomyxa hamour* n. sp. infecting *Epinephelus coioides* from the Arabian Gulf.

Table 2. *Ceratomyxa* species used for multiple alignment and phylogenetic tree construction

Myxozoa species	18S coordinates	Accession numbers	Percentage of identity with *C. hamour*
C. hamour	213-1564	KJ200360	ID
C. buri	395-1757	AB530264	97.8
C. sewelli	51-1352	EU440362	93.5
C. falcatus	51-1437	EU440361	92.8
C. lunula	51-1354	EU440378	92.6
C. diamanti	75-1385	FJ204246	86.8
C. ireneae	2-1309	JX971430	86.6
C. puntazzi	327-1641	JF820290	86.5
C. barnesi	68-1390	FJ204245	85.5
C. dennisi	51-1363	EU440359	85.5
C. gunterae	2-1311	JX971422	85.3
C. moseri	51-1378	EU440360	85.5
C. hooperi	43-1363	EU729692	84.4
C. labracis	420-1763	AF411472	85.8
C. anko	416-1814	DQ301510	77.7
C. cardinalis	63-1385	JX971436	85.2
C. seriolae	405-1767	AB530265	86.1
C. talboti	51-1378	EU440375	85.5
C. atkinsoni	54-134	JX971424	84.7
C. cyanosomae	2-1364	FJ204244	83.4
C. nolani	43-1321	EU729698	82.9
C. pantherini	58-1426	GU136393	77.7
C. whippsi	43-1448	EU729694	83.9
C. brayi	43-1394	EU729697	85.2
C. sp. 2 ex *Sparus aurata*	376-1690	JF820293	85.9
C. ostorhinchi	2-1380	JX971425	83.9
C. robertsthomsoni	76-1439	FJ204253	83.2
C. auerbachi	407-1679	EU616734	86.0
C. thalassomae	65-1430	EU045332	83.5
C. bartholomewae	51-1373	GU136391	85.4
C. rueppellii	2-1376	JX971423	83.7
C. cribbi	51-1384	EU440367	84.6
C. gleesoni	44-1472	EU729693	82.3
Tetracapsuloides bryosalmonae[a]	409-1686	U70623	58.2

Accession number in SSU rDNA database entry and coordinates for used sequences are given. Percentage of similarity is based on the Kimura two-parameter model obtained after pairwise analysis between *C. hamour* n. sp. and the selected myxozoa species.
[a]*Tetracapsuloides bryosalmonae* was used as outgroup.

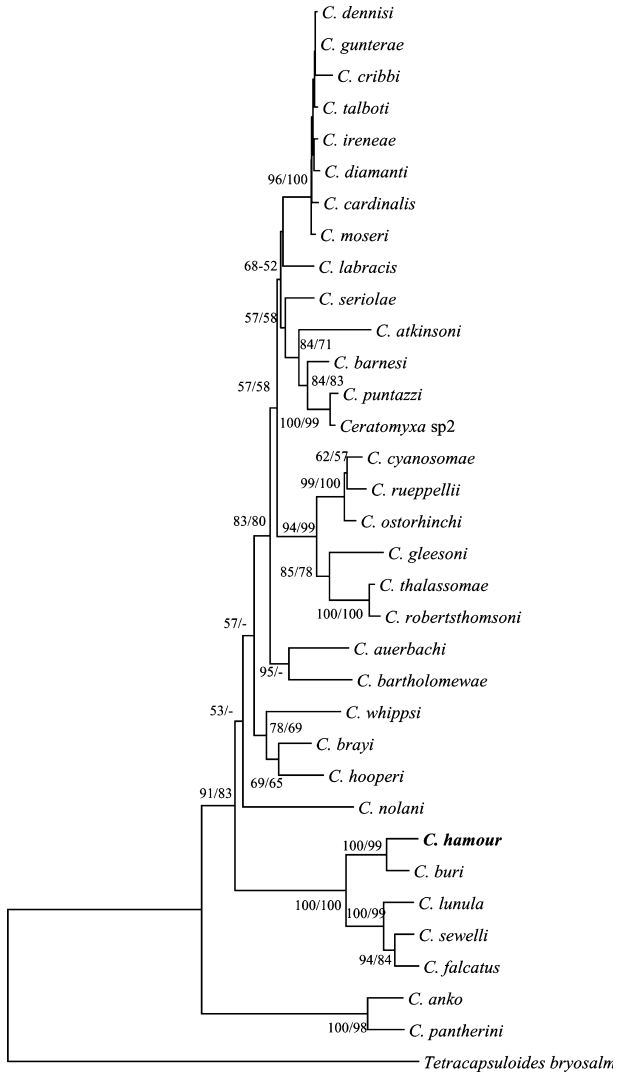

Figure 4 Phylogenetic tree, based on the obtained alignment was generated using maximum likelihood and neighbor joining methods. Numbers in branches represent bootstrap percentages of neighbor joining/maximum likelihood study for the nodes gaining more than 50% support (values below 50% are indicated by dashes). The scale bar indicates the number of changes per site.

Natural History Museum with associated collection number (HNHM 70455). Also, small subunit ribosomal DNA sequence was deposited in GenBank (accession number KJ200360).

Etymology. The specific name refers to the common name of the host in Saudi Arabia and other Arabian countries.

DISCUSSION

Over 280 *Ceratomyxa* species have so far been described in fish (Heiniger and Adlard 2013), of them nine species were described from members of the family Serranidae. None, however, have previously been described from the orange-spotted grouper *E. coioides*. On the basis of a morphological and dimensional comparison of the spores, several species of *Ceratomyxa* are found to resemble the present form. These are: *Ceratomyxa bassoni* Abdel-Ghaffar et al. 2008 from *Plectorhynchus gaterinus* (Haemulidae) in the Red Sea; *C. buri* Yokoyama and Fukuda 2001 from cultured yellowtail *Seriola quinqueradiata* (Carangidae) in Japan; *Ceratomyxa flexa* Meglitsch 1960 from *Plagiogenion rubiginosus* (Emmelichthyidae) in the Pacific Ocean; *Ceratomyxa gibba* Meglitsch 1960 from *Congiopodus leucopaecilis* (Congiopodidae) in the Pacific Ocean; *Ceratomyxa honckenii* Reed et al. 2007 from *Amblyrhynchotes*

honckenii (Tetraodontidae) in South Africa; *Ceratomyxa ireneae* Heiniger and Aldlard, 2013 from *Archamia fucata* (Apogonidae) in the Great Barrier Reef in Australia; *Ceratomyxa recta* Meglitsch 1960 from *Genypterus blacodes* (Ophidiidae) in the Pacific Ocean in New Zealand; *Ceratomyxa sparusaurati* SitjÀ-Bobadilla et al. 1995 from *Sparus aurata* (Sparidae) in the Mediterranean Sea, and *Ceratomyxa vepallida* Meglitsch 1960 from *Caulopsetta scapha* (Bothidae) in the Pacific Ocean in New Zealand (Table 3). The spores of these species differ from those of our new one in the following ways.

Ceratomyxa bassoni differs in having two distinct patterns of spores, one with a flat and the other with a bent posterior end, and in having a higher number of polar filament turns. Although *C. buri* exhibits some similarity both in morphology and in dimensions, the measurement ranges never in fact overlapped (5.5–7.5 × 11–16.5 vs. 6–8 × 15–18). In addition, *C. buri* differs in having rounded smaller polar capsules compared to the larger pyriform ones in the present species (2–3 vs. 3–5). As well as *C. buri* described from a fish in a very different family. The spores of *C. flexa*, meanwhile, are arcuate in shape with unequal valves and spherical polar capsules with a higher number of polar filament turns (4–5 vs. 3). Spores of *C. gibba* can be distinguished by their unequal valves and unequal polar capsules. *Ceratomyxa honckenii* have thicker spores (18–21 vs. 15–18) with spherical polar capsules. *Ceratomyxa ireneae* can be differentiated by their smaller polar capsules, which are nearly half the size of those in our form (1.6–2.3 vs. 3–5). *Ceratomyxa recta* and *C. sparusaurati* differ in having a spherical polar capsule with a higher number of polar filament turns (4–5; 6 respectively vs. 3). *Ceratomyxa vepallida* have longer and thicker spores (8.6 × 18.7 vs. 7 × 16.5) with slightly unequal valves.

The infection occurred throughout the year with the highest prevalence occurring in winter and the lowest prevalence in autumn, similar to the pattern reported for *Ceratomyxa shasta* (Hendrickson et al. 1989), for *C. buri and Ceratomyxa seriolae* (Yokoyama & Fukuda, 2000), for *Ceratomyxa puntazzi* (Alama-Bermejo et al. 2013), and for *Henneguya ghaffari* (Abdel-Baki et al. 2014). Some other myxosporean species, however, have showed a higher prevalence in summer and a lower prevalence during other seasons (Abdel-Baki et al. 2011; Yemmen et al. 2013; Yokoyama et al. 2005). Authors have variously suggested that the seasonal and annual patterns of myxosporean infection may be due to the endogenous cycles of the parasites, the availability of susceptible hosts, or the effects of environmental factors (Foott and Hedrick 1987; Yokoyama and Fukuda 2001). The seasonal variation in the prevalence of the *Ceratomyxa* may be also, influenced by the presence of an intermediate host in the life cycle (Bartholomew et al. 1997). Moreover, Yokoyama and Fukuda (2001) suggested that the seasonal variation in prevalence of *Ceratomyxa* may be explained by the variable condition of bile secretion at each sampling period.

Among the morphologically similar species, the 18S sequences of only *C. ireneae* and *C. buri* are available.

Table 3. Comparative description of *Ceratomyxa hamour* n. sp. with morphologically similar species (measurements in µm)

Species	Host	Locality	Spore size	Polar capsule size	References
C. buri Yokoyama & Fukuda, 2001	*Seriola quinqueradiata*	Japan	6.5 (5.5–7.5) × 14.3 (11.0–16.5)	2.4 (2.0–3.0)	Yokoyama and Fukuda (2001)
C. bassooni Abdel-Ghaffar, Ali, Al-Quraishy, Al Rasheid, Al Farraj, Abdel-Baki, Bashtar, 2008	*Plectorhynchus gaterinus*	Egypt (Red Sea)	6.1 (5–7) × 18 (15–20)	3.2 (3–4) × 2.4 (2–3)	Abdel-Ghaffar et al. (2008)
C. flexa Meglitsch, 1960	*Plagiogenion rubiginosus*	New Zealand (Pacific Ocean)	5.6 (5.6–7.9) × 15.9 (13.6–16.9)	2.6 (2.3–3.4)	Eiras (2006)
C. gibba Meglitsch, 1960	*Congiopodius leucopaecilis*	New Zealand (Pacific Ocean)	6.9 (5.6–8) × 17(14.2–18.9)	2.8 (2.5–3.3) × 2.3 (1.5–2.2)	Eiras (2006)
C. honckenii Reed, Basson, Van As, Dykova, 2007	*Amblyrhynchotes honckenii*	South cost of south Africa	7.5–8 × 18–21	3–3.2 × 3–3.1	Reed et al. (2007)
C. ireneae Heiniger and Adlard, 2013	*Archamia fucata*	Australia (Lizard Island)	5.1(4.5–6.2) × 14.5 (12.2–17.3)	2 (1.6–2.3) × 1.7 (1.5–2)	Heiniger and Adlard (2013)
C. recta Meglitsch, 1960	*Genypterus blacodes*	New Zealand (Pacific Ocean)	7.8 (6.8–8.8) × 15.6 (14.7–16.7)	2.6 (2–3.4)	Eiras (2006)
C. sparusaurati Sitjabobadilla, Palenzuela, Alvarez-Pellitero, 1995	*Sparus aurata*	Spain (Mediterranean sea)	5.6 (4.5–7.5) × 15.7 (14–17.5)	2.2 (2.2–3.4)	Eiras (2006)
C. vepallida Meglitsch, 1960	*Caulopsetta scapha*	New Zealand (Pacific Ocean)	8.6 (7.8–9.6) × 18.7 (16–21.4)	3.2 (2.9–3.6) × 2.9 (2.5–3.2)	Eiras (2006)
Ceratomyxa hamour n. sp. (Present study)	*Epinephelus coioides*	Saudi Arabia (Arabian Gulf)	7 (6–8) × 16.5 (15–18)	4 (3–5) × 3 (2–4)	Present study

Molecular analysis based on partial sequence of the 18S gene shows that the highest percentage of similarity (97.8%) was observed with *C. buri*. This similarity was also confirmed in the phylogenetic tree, where *C. buri* and the new species form an individual cluster supported by bootstrap values of 100%. The genetic distance between the two *Ceratomyxa* is, however, sufficient to separate them into two different species. The two sequences differ by 97 nucleotide substitutions and 34 insertion/deletion events. In this regard we noticed that, of the sequences we analyzed, we tended to observe quite a high percentage of similarity between different species, as for example between *C. ireneae* and *Ceratomyxa diamenti* (99.6%), between *Ceratomyxa dennisi* and *Ceratomyxa moseri* (99.8%), etc. This tends to support the contention that *C. buri* and the present *Ceratomyxa* species from hamour are different.

Accordingly, it seems that the present species does not conform with the characters of any of the previously described species. Therefore, it is suggested that the investigated material is considered distinct and should be designated as a new species. The name *C. hamour* n. sp. is proposed after the host Arabic local name, Hamour.

ACKNOWLEDGMENTS

We extend our appreciation to the Dean of Scientific Research, King Saud University, for funding the work through the research group project number RGP-002.

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