First records of epibiont ciliates (Ciliophora) in methane enriched sediments with species redescriptions

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Abstract
The article is dedicated to re-discovery of ciliates Loricophrya bosporica (Suctorea) and Cothurnia sp. (Peritrichia) found at nematodes from methane seep of the Ria Formosa lagoon (Southern Portugal). Based on authors’ data the lagoon sediments might be attributing to the hypoxic (periodically anoxic) methane seepage environment. The morphological characterizations of found ciliates with emphasis on poorly studied stylotheca structure in suctorian ciliate and macronucleus morphology in peritrich are presented.

Key words: ciliate, epibiont, commensal, nematode, host, methane seep, Ria Formosa lagoon.

Introduction

Methane seeps referred to chemosynthetic extreme ecological communities, the main producers in which are methane bacteria and archaea (Pasulka et al., 2017). However, unicellular eukaryotes (protists) of methane seeps are still poorly understood components.

The first finds of protists, especially ciliates in chemosynthetic communities have been in the hydrothermal vents (Van Dover et al., 1988), where, in particular, the abundant colonies of ciliates (so-called blue mats) from family Folliculinidae Dons, 1914 (Folliculinopsis sp.), attached to inanimate substrates were observed (Kories et al., 2010).

In addition, the commensal suctorian ciliate Corynophrya abissalis Bartsch et Dovgal, 2010, which, apparently, is a specialized inhabitant of this community was found in hydrothermal vent (Bartsch & Dovgal, 2010, Dovgal & Sergeeva, 2016).

As for the methane seeps, the colonies of family Folliculinidae representatives (named indigo mats) were found in the Gulf of California (Lobban et al., 2007). In addition, the genomes of several ciliate classes’ representatives were identified in methane seeps of the coast of Oregon (Pasulka et al., 2016).

Recently folliculinid ciliates on various inanimate substrates were discovered and studied in detail from methane seeps at the Pacific coasts of North and Central America (Pasulka et al., 2017).
In the latter work two new species: *Eufolliculina methanicola* Pasulka et al., 2017 and *E. caerulea* Pasulka et al., 2017 were proposed on the basis of molecular studies. However, the morphological features of the new species do not discuss in the article.

It should be mentioned such specific habitat of marine protists as hydrogen-sulfide zone of the Black Sea where combined effect of extremal factors – lack of oxygen and the presence of H$_2$S is characteristic here (Dovgal & Sergeeva, 2016). However, representatives of many groups of eukaryotic organisms were found in these presumably hypoxic habitats (Sergeeva et al., 2014, Sergeeva & Dovgal, 2014, Dovgal & Sergeeva, 2016).

It was assumed that the eukaryotic life is absent both in anoxic Black Sea waters and in oxygen-free sediments enriched by sulfides (Kiseleva, 1979, Azovskii & Mazei, 2005, Zaitsev et al., 2007, Zaitsev & Polikarpov, 2008, Gulin, 2012, 2013). So it is still an open question whether these found eukaryotes are obligate components of any chemosynthetic community in above mentioned conditions or observed species are only surviving in extreme conditions.

In turn, Wylezich & Jürgens (2011) used molecular tools, reported high diversity of ciliates, and flagellates in hypoxic, and particularly in sulfidic water layers of the Black Sea.

In this regard, particular interest is a godsend in the hydrogen sulphide zone a new species of commensal suctorian *Loricophrya bosporica* Sergeeva et Dovgal, 2016 (Sergeeva & Dovgal, 2016).

The present article is dedicated to re-discovery of the latter suctorian species that were found at nematodes in methane seepage sediments of the Ria Formosa lagoon (Southern Portugal) along with the representative of subclass Peritrichia *Cothurnia* sp. The redescriptions of these ciliates with attention to poorly studied stylotheca structure in suctorian ciliate and macronucleus morphology in peritrich are presented. The systematic position of suctorian ciliate have been given after Dovgal (2002, 2013) whereas systematics of peritrich ciliate have been shown after Lynn (2008).

**Materials and Methods**

The Ria Formosa mesotidal lagoon as an example of critical sea bottom habitats where both the natural and human induced stressors accelerated an eutrophication, hypoxia and hydrogen sulfide contamination. Besides anthropogenic loads of pollution, the natural seepage of different gases through the sea bottom may be a significant source of organic input on the Ria Formosa lagoon sediments.

Coastal zones are commonly known areas of gas emissions from the sea bottom. Data on tidal-cycle fluxes of CO$_2$, methane (CH$_4$) and a range of volatile organic compounds (VOCs) are characterizing the Ria Formosa lagoon sediments as gas enriched. Averaged CH$_4$ flux above lagoon sediments was reported the 3.0 (μmol m$^{-2}$ h$^{-1}$) (Bahlmann et al., 2015). Thus, at the methane seepage sediments of the Ria Formosa lagoon, the high level of H$_2$S contamination is, probably, caused due to microbial chemotrophic activity.

Sediment samples were taken from the Western part of the Ria Formosa mesotidal lagoon at southern Portugal on two transects (13 stations) extending away from two sewage outfalls in Faro city area in August 2009 in order to indicate the pollution gradient using meiothens community parameters. Nematodes infected with ciliates were found at two stations named A and B (Fig. 1, Table 2) in this work.

![Figure 1. Sampling stations A and B located at Western part of the Ria Formosa lagoon (Southern Portugal).](image-url)
Sediment samples for meiofauna analyses were collected during low tide with plastic tube (diameter 2 cm) with three replicates on each station. Then samples were fixed with 4% neutralized formalin with addition of stain Rosa Bengal, after the samples was centrifuged with LUDOX as described by Giere (2009). Supernatant was sieved through 63 mm mesh size and sorted using a light microscope. For identification to species level, nematodes were extracted and mounted on microscope slides following Higgins and Thiel (1988).

At the same time with meiofauna investigations, the determination of hydrogen sulfide concentrations of bottom water were made by photometric method based on the reaction of hydrogen sulfide and N, N-dimethyl-para-Phenylenediamine (DMP) in an acidic medium.

The sediment samples for granulometry analyses by sieving according to Wentworth (1922) were taken from each study site.

The photomicrographs and measurements were made using a microscope Nikon Eclipse E200. Four individuals of *L. bosphorica* and two individuals of *Cothurnia* sp. were measured.

**Results and Discussion**

**Ciliate species descriptions**

Class Suctorea Claparède et Lachmann, 1859
Subclass Exogenia Collin, 1912
Order Metacinetida Jankowski, 1978
Family Paracinetidae Jankowski, 1975
Genus *Loricophrya* Matthes, 1956

*loricophrya bosphorica* Sergeeva et Dovgal, 2016 (Fig. 2, 3)

**Improved diagnosis.** Marine loricate suctorian with elongate slightly asymmetric cell body completely covered with a lorica. Lorica (stylotheca), conical. The stylotheca characterized by a gradual thickening of the walls toward the mouth. There is a transverse wrinkle in the lower part of stylotheca in some individuals. The lower part of stylotheca, which separated from the upper part by a well-visible septum, slightly longitudinally striated, have thinner walls, finishes by widening (basal disc) that use for the attachment to the host's body surface. The cell body is attached to the lorica in the mouth with the help of its edges folded inwards. A few clavate tentacles are located at the apical surface of the body. Macronucleus rounded. Reproduction was not observed.

**Dimensions** (in µm): Stylotheca length 20-56, lower part of stylotheca length 15-21, maximal stylotheca width 14-26, minimal stylotheca width 5-8, wall thickness of the lorica 1, body length 10-35, body width 12-20, tentacle length 4-21, dimensions of macronucleus 5-10X7-11 (Table 1).

**Localities:** Istanbul Strait's area, Bosporus region, the Black Sea (type locality), the Ria Formosa lagoon (Southern Portugal).

**Hosts:** Desmoscolex cf. minutus Claparède, 1863 (type host), Metachromadoroides remanei (Gerlach, 1951).

**Remark.** The observed specimens are differing from found of Sergeeva and Dovgal (2016) by presence of additional lower part of stylotheca, which separated from the upper part by septum. It is our opinion that in the Sergeeva’s and Dovgal’s materials, this structure was hidden beyond the edge of the host's body, whereas in our material is clearly visible. All other features and dimensions are almost identical with those of the Sergeeva and Dovgal (2016) diagnosis (Table 1).

Class Oligohymenophorea de Puytorac et al., 1974
Subclass Peritrichia Stein, 1859
Order Sessilida Kahl, 1933
Family Vaginicolidae de Fromentel, 1874
Genus *Cothurnia* Ehrenberg, 1831

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**Table 1.** Comparison of the measurements (in μm) of *Loricophrya bosporica* from Istanbul Strait's area (the Black Sea) and the Ria Formosa lagoon.

| Location                        | Stylotheta length | Minimal | Maximal | Lorica wall thickness | Body length | Body width | Tentacle length | Dimensions of macronucleus |
|---------------------------------|-------------------|---------|---------|-----------------------|-------------|------------|-----------------|-----------------------------|
| The Ria Formosa lagoon          | 20-56             | 5-8     | 14-26   | 1                     | 10-35       | 12-20      | 4-21            | 5-10 X 7-11                 |
| Istanbul Strait's area           | 43-60             | 7       | 18-26   | 1-2                   | 17-30       | 16-23      | 7-20            | 6-9 X 6-9                  |

Figures 2-3. 2 *Loricophrya bosporica* on nematode *Metachromadoroides remanei*. 3 Group of *Loricophrya bosporica* individuals on nematode *Metachromadoroides remanei*. Scale bar 10 μm.

**Cothurnia sp. (Fig. 4)**

**Diagnosis.** Marine loricate peritrich ciliate with lengthy lorica. There is an external stalk, short and broad, attached to substrate via basal disc, endostyle, present, mesostyle, absent. Contracted zooid occupies nearly the bulk of lorica. Macronucleus rounded, pellicle is only weakly striated. Reproduction was not observed.

Dimensions (in μm): Lorica length 50-80, minimal lorica width 7-8, maximal lorica width 21-30, wall thickness of the lorica 2-4, external stalk length 10-17, contracted zooid length 45-50, width 19-20, dimensions of macronucleus 8X12.

Remarks. On such characters as lorica morphology, the presence of an endostyle, absence of mesostyle and dimensions the found peritrich species is closest to *Cothurnia membranoloricata* Stiller, 1968, previously found on marine algae (Stiller, 1968, Viljoen & van As, 1983). However, in our materials ciliates supplied with rounded macronucleus. In turn in the original description of *C. membranoloricata* Stiller (1968) does not mentioned the shape of the macronucleus, whereas in description of Viljoen and As (1983) *C. membranoloricata* showed a horseshoe-shaped macronucleus.

It should be mentioned that in ciliate described of Stiller (1968) the peristomial lip is conspicuously thickened (Warren & Paynter, 1991) whereas that illustrated by Viljoen & As (1983) is very thin thus, it is
not unlikely that in mentioned works two different species were described. At the same time, we have only fixed materials, in which the characters as peristome morphology, the degree to which the zooid extends beyond the lorica aperture, etc were not visible. As a result, now we cannot reliably identify the peritrich species.

Figure 4. *Cothurnia* sp. on nematode *Spirinia* sp. Scale bar 10 μm.

The ecological peculiarities of found ciliates

At the methane seepage sediments of the Ria Formosa lagoon (Bahlmann *et al*., 2015), the H$_2$S rising due to microbial chemotrophic activity. We measured the H$_2$S concentration at the bottom water, which was a 0.024 μmol/l at the station B (Table 2). Any traces of H$_2$S were no detected at the station A (Table 2).

Granulometry sediment composition of both areas was classified as gravely muddy sand (using Wenthwort, 1922). Mean values of redox potential (Eh) across the lagoon sediments ranged from −135 mV at the natural reserved areas up to −323 mV at the city sewage outfall (location of maritime terminal “Cais Comercial”).

Sediment properties analyzed here along with previous study results (Bahlmann *et al*., 2015), allow attributing the lagoon sediments to hypoxic (and even periodically anoxic) methane seepage shallow waters environment.

Table 2. Characteristics of two stations in the Ria Formosa lagoon.

| Location | Coordinates | Sediment granulometry composition | H$_2$S, μmol/l, bottom water | Ciliate species | Host species |
|----------|-------------|-----------------------------------|------------------------------|----------------|--------------|
| Station A | N 37°0'42" E 7°56'34" | Gravely Muddy Sand | 0 | *Cothurnia* sp. (2 individuals) | *Spirinia* sp. |
| Station B | N 37°0'24" E 7°54'59" | Gravely Muddy Sand | 0.024 | *Loricophrya bosporica* (4 individuals) | *Metachromadoroides remanei* |
Host basibionts for both ciliate species from hypoxic and gas enriched sediments of the Ria Formosa lagoon were a nematodes. The nematode *Spirinia* sp. is dominated the Nematoda community by their abundances (25%) from 20 species reported at the station A. At the same time the nematode *Metachromadoroides remanei* Gerlach, 1951 also dominated the community by their densities (32%) from the 22 species of Nematoda recorded at the station B.

As folliculinid ciliates living on inanimate substrates were previously found in methane seeps the finds of commensal ciliates in the community is of certain interest. It is believed that the commensal ciliates especially specific commensals or parasites of extremophile hosts are possible specific inhabitants of extreme environments (Dovgal et al., 2015, Dovgal & Sergeeva, 2016). However, the hosts of ciliates found at Ria Formosa lagoon are not specific inhabitants of methane seeps. As for ciliate specificity to the nematode hosts only *L. bosporica*, the species that was identified can be discussed.

*L. bosporica* from the Black Sea hydrogen sulfide zone settle on *Desmoscolex cf. minutus*. In our case, the species was attached to the bodies of *M. remanei*. It appears that the suctorian species is not specific to a particular species of nematodes, but prefers the host species with the well-sculptured cuticle. However, the re-discovery of this suctorian in habitat with deficiency of oxygen and the presence of methane and hydrogen sulfide may be indicative about prevalence of *L. bosporica* to extreme conditions.

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