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MICROBIOLOGY

High infestation and phylogenetic position of *Epistylis* sp. (Ciliophora, Peritrichia) on *Aegla serrana* Buckup & Rossi (Crustacea, Anomura) from southern Brazil

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Abstract: High infestations of epibiont ciliates on vertebrates or invertebrates are normally related to aquaculture tanks or similar environments, and the importance of this relationship in natural habitats is often disregarded. Here, we describe the first record of high infestation of ciliates on *Aegla serrana* in South America and conduct a brief morphological and phylogenetic characterization of these ciliates. Our findings confirm that cases of high infestation of ciliates on metazoans can indeed occur in natural environments.

Key words: Basibiont, Ciliates, Crustaceans, Epibiosis, Neotropical area.

INTRODUCTION

Epibiosis is an non obligatory ecological interaction between two organisms: the epibiont, which resides on another living organism, and the basibiont, which provides its habitat (Wahl 1989). Over the past 20 years, several occurrences of ciliates (Ciliophora) as epibionts on various vertebrate and invertebrate groups have been recorded (Azevedo et al. 2014, De Pádua et al. 2013, Cabral et al. 2017).

The Epistylididae family (Ciliophora: Peritrichia) is particularly important due to the damage that its members can cause to their basibionts when present in large quantities (Longshaw 2011, Martins et al. 2015). In aquaculture farms, high infestations of these ciliates have deleterious effects (Martins et al. 2015, Pala et al. 2018). However, we still know little about this type of relationship in natural environments. Some authors even question the relevance of this association in nature, suggesting that the high number of epibionts is directly related to the environmental conditions found in aquaculture settings (Longshaw 2011, Martins et al. 2015).

In this study, our primary objective was to document the initial occurrence of a significant *Epistylis* infestation on a specimen of *Aegla serrana* Bond-Buckup & Buckup 1994 (Crustacea: Decapoda) in its natural habitat. Additionally, we undertook phylogenetic investigations to offer a concise morphological characterization of these ciliates. These findings hold the potential to shed light on the ecological significance of this interaction in the natural environment of these crustaceans, providing valuable insights for future research.

MATERIALS AND METHODS

The *Aegla serrata* specimen was collected in Cambará do Sul (29°15'10"S, 50°15'45"W), Rio Grande do Sul, Brazil (Amato et al. 2003).

The sample was preserved in ethanol 70%. Photomicrographs of the crustacean infested by *Epistylis* sp. were taken using a Zeiss Axiolab microscope with phase contrast, and the ones from isolated peritrichids were taken using a Olympus BX51 microscope with differential interference contrast. Approximately 10 colonies were post-fixed in an alcoholic Bouin's solution and used for the protargol impregnation technique (Dieckmann 1995).

Twenty zooids were used for the molecular characterization. The DNeasy® Blood and Tissue kit (QIAGEN) was used to extract genomic material. Subsequently, the PCR method using the protocol described by Liao et al. (2021) and specific primers Peri_57F (5´ CATGCATGTGTAAGTATAAGTA) and Peri_1385R (5´ CGGTGTGTACATTTGC) were employed to amplify the 18S-rRNA gene. The PCR products were purified using the QIAquick PCR Purification Kit – Qiagen, and then sequenced by a specialized company.

The sequencing results were analyzed and edited using the SEQMAN® program from the Lasergene DNASTAR™ package (1989-1999). Subsequently, the sequence was compared with available peritrich sequences on the GenBank database using the BLAST algorithm (Altschul et al. 1990). The 10 sequences most similar to ours were added to a dataset containing 180 sequences of sessilids and some other ciliates (outgroup, n= 11), for phylogenetic investigation. This dataset was aligned using the MAFFT software (Katoh & Standley 2013) and then visually inspected and edited in the MEGA-X software (Kumar et al. 2018). After manual editing, the data were submitted to the GBLOCKS software (Castresana 2000) to remove poorly aligned sites, resulting in a matrix of 1075 base pairs. The generated curated dataset was subjected to the JModelTest software (Posada 2008) implemented in the MEGA-X platform to select the most suitable nucleotide substitution model. The RAxML software (Stamatakis 2014), using the previously selected GTR-GAMMA-I model, was used to construct the phylogeny based on Maximum Likelihood. Support values estimated for the phylogeny were based on 450 pseudoreplicates.

RESULTS

Many *Epistylis* sp. colonies were found in high numbers throughout the body of the basibiont, *Aegla serrana*, mainly between the locomotor appendages, pereiopods, and in the intersegmental unions of the cephalothorax and abdomen (Figure 1).

The *Epistylis* sp. recorded on *A. serrana* have colonies with 7-40 zooids, supported by a large and rigid stalk, more than four times the size of the zooid, longitudinally striated, and appearing to have a gelatinous coating. Colonies were dichotomously branched, with the main branches ramifying, at the same level, close to the zooids. The cytoplasm is clear or grayish. The macronucleus has a 'C' shape and is in the central part of the body. The micronucleus is spherical, positioned near the macronucleus. In *Epistylis* sp., polykinety 1 (P1) and polykinety 3 (P3) originate from the same point (Figure 1), while polykinety 2 (P2) originates slightly above the others. P1 has 3 rows and is the only one that extends from the peristome to the cytostome (Figure 1). P2 has three rows and is slightly shorter than P1, ending just before the cytostome (Figure 1). On the other hand, P3 has only two rows and is much shorter than the others.

The phylogenetic analysis (Figure 2) demonstrated that *Epistylis* sp. (OR166805) found on *A. serrana* is closely related to four unidentified sequences, one from the epibiont *Epistylis plicatilis* Ehrenberg, 1831 (HM627236),

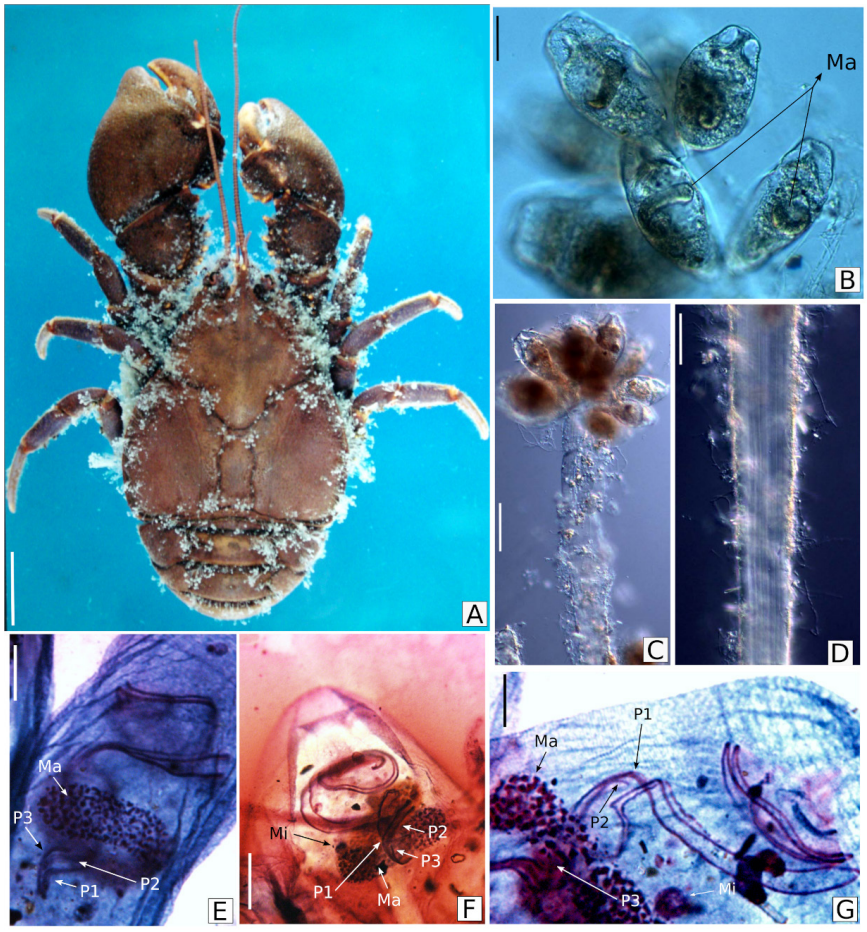

Figure 1. Peritrich ciliate *Epistylis* sp. colonizing crustaceans of the species *Aegla serrana*. **a:** Colonies of *Epistylis* sp. attached to *A. serrana* (high infestation). **b-d:** *Epistylis* sp. fixed in alcohol, with details of zooids (b), colony (c) and stalk (d). **e-g:** Ciliary pattern after protargol impregnation, showing peniculi P1, P2 and P3. Ma = Macronucleus; Mi= Micronucleus; P1= Polykinety 1; P2= Polykinety 2; P3= Polykinety 3. Reference bar length for figures: 1a = 1 cm; 1b = 35 µm; 1c = 70 µm: 1d = 30 µm; 1e, f = 10 µm; 1g = 5 µm.

and one from *Epistylis vaginula* Stokes, 1884 (MW172840), with a high support value (97%).

DISCUSSION

The large number of *Epistylis* sp. colonies found on the body of *Aegla serrana* indicate that some of its capabilities may be impaired. Studies conducted in aquaculture facilities have shown that high concentrations of epistylid ciliates can cause reproductive and physiological problems, and even lead to the death of their basibionts (Longshaw 2011, Martins et al. 2015). However, we still know little about the effects of these relationships in nature. Currently, there have been few cases of high infestation by epistylids reported in natural conditions (Utz 2007), which has led to the disregard of the occurrence and significance of this relationship in a natural environment, while the association between high infestations of epistylids and the conditions found in aquaculture settings has been established (Longshaw 2011, Martins et al. 2015). However, the record presented here demonstrates that cases of superinfestation can indeed occur in nature. Nevertheless, the low number of similar case records raises doubts about the importance of this relationship for the basibiont community and highlights the need for further studies focusing on the influence of peritrichous epibionts on aquatic invertebrates under natural conditions.

Due to the lack of knowledge regarding the diversity of epistylid epibionts of crustaceans in Brazil, especially in endemic species like *A. serrana*, we attempted to perform a

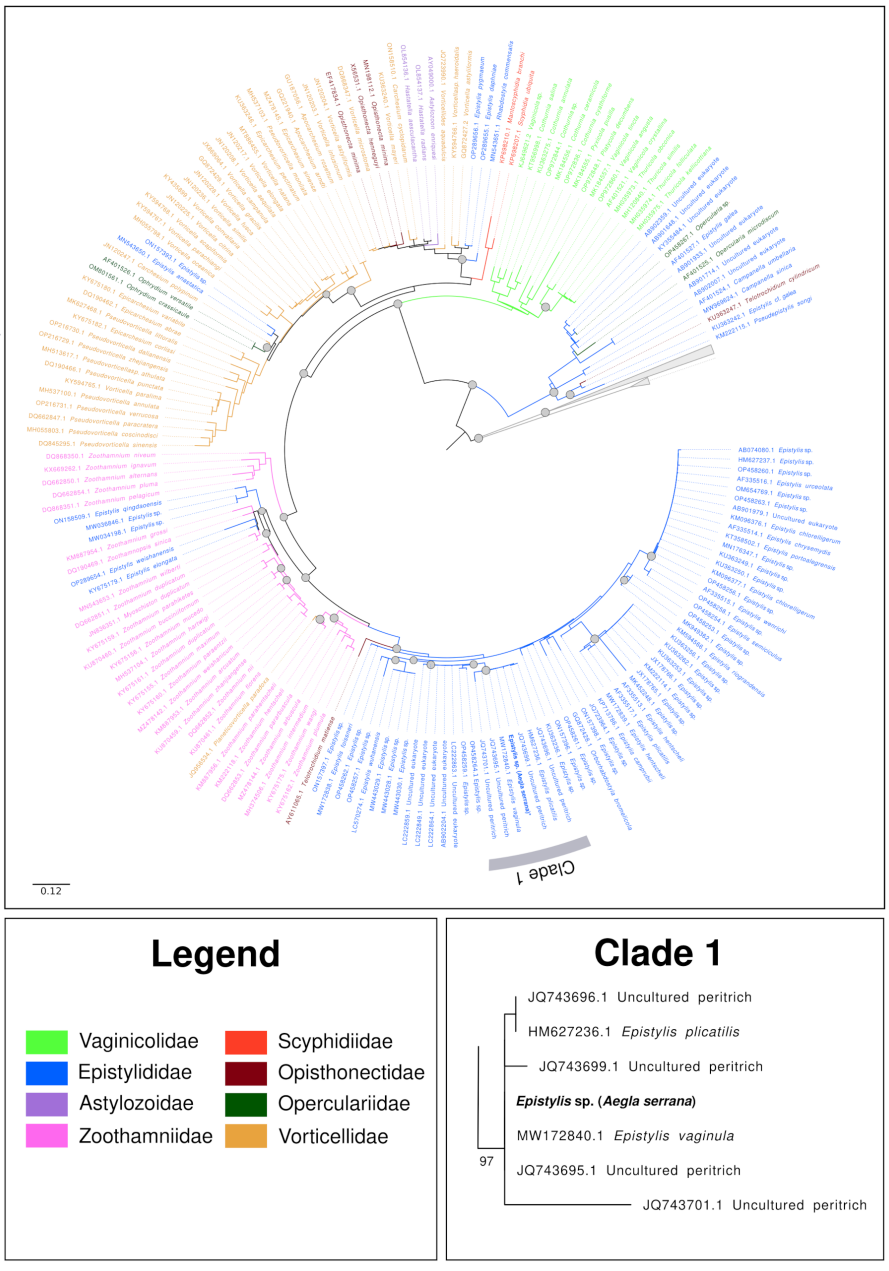

Figure 2. Phylogenetic tree based on Maximum likelihood method of peritrich ciliates (Ciliophora, Oligohymenophora, Peritrichia) based on 18S rDNA data. Other oligohymenophorids were chosen as out-groups. The gray circle indicates nodes with bootstrap values bigger than 70. The scale bar corresponds to 12 substitutions per 100 nucleotide positions. Clade where *Epistylis* sp. from *Aegla serrana* is positioned in evidence, Clade 1. Families colored with different colors.

morphological and molecular characterization of these organisms, despite the limitations imposed by the fixation method used in the analyzed samples. The species of *Epistylis* found here are closely related to *Epistylis plicatilis* and *Epistylis vaginula*, both in morphological and molecular characteristics, although differences among the three species are still noticeable. Among the most important differences, we can mention the peduncle, which in *Epistylis* sp. is large, longitudinally striated, with a gelatinous coating and not distinctly dichotomous between zooids. On *E. plicatilis*, it is long, smooth, without a gelatinous coating, and with easily observable dichotomous separation. On the other hand, *E. vaginula* also presents a smooth and clearly dichotomous but much longer peduncle, reaching up to 1.5 cm in length (Wu et al. 2021).

Another difference among the three species is found in the oral infraciliature. In *Epistylis* sp., P1 and P3 originate from the same point, while P2 originates slightly above them. P1 and P2 of this species have three rows, while P3 has only two. *E. plicatilis* presents a very similar pattern in its oral infraciliature, with the only variation being the number of rows in P3, which is three (Utz 2007). On the other hand, the oral infraciliature of *E. vaginula* shows more variation. All the polykineties have three rows, similarly to *E. plicatilis*, but P1 starts first and continues up to the peristomial disc, and P2 starts above P1 and extends only to the end of the cytostome, where one of the three rows separates from the others and joins the rows of P1. Finally, P3 starts between P1 and P2 and is extremely short (Wu et al. 2021).

The results obtained here suggest that the species found on *Aegla serrana* is possibly *Epistylis plicatilis*. However, due to the discrepancies found, the inability to compare *in vivo* basic morphological characters due to the sample fixation method, and the current discussions on the relevance of infraciliature characteristics for the comparison of closely related species (Lu et al. 2023), we have decided not to designate the epistylid found here as *Epistylis plicatilis*.

Our findings confirm that cases of high infestation of *Epistylis* in metazoans can indeed occur in natural environments and demonstrate that studies focusing on understanding the effect of this relationship in natural communities can be extremely important for the conservation of both the basibiont and the ciliate epibiont.

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