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RESEARCH NOTE

Occurrence of a bivalve-inhabiting marine hydrozoan (Hydrozoa: Hydroidolina: Leptothecata) in the amber pen-shell *Pinna carnea* GMELIN, 1791 (Bivalvia: Pteriomorphia: Pinnidae) from Bocas del Toro

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Hydroidomedusan polyps are involved in associations ranging from simple epibiosis to strict symbiosis (mutualism and parasitism) with organisms belonging to many animal phyla, including sponges, cnidarians, molluscs, annelids, arthropods (crustaceans), bryozoans, echinoderms, tunicates and vertebrates (fishes) (Gili & Hughes, 1995). Many known symbiotic hydroids are found on mollusc shells, as are the cases of Neoturris pileata (Forsskål, 1775) on Nucula spp. (see Edwards, 1965); Hydractinia angusta Hartlaub, 1904 on Adamussium colbecki (E. A. Smith, 1902) (Cerrano et al., 2000, 2001); Zanclea costata Gegenbaur, 1857 on the three bivalve species Chamelea gallina (Linnaeus, 1758), Cardium sp. and Spisula subtruncata (da Costa, 1778) (Gravili, Boero & Bouillon, 1996; Cerrano, Amoretti & Bavestrello, 1997) and Monobrachium parasitum Mereschkowsky, 1877 on the shells of some Antarctic bivalves (Jarms & Mühlenhardt-Siegel, 1998).

Two genera of hydroids have been observed inhabiting the mantle cavity of bivalves during their benthic phase, *Eutima* McCrady, 1859 and *Eugymnanthea* Palombi, 1935; both in Leptothecata, Eirenidae (Brinckmann-voss, 1973; Bouillon, 1985; Kubota, 1985, 1992). Polyps of these hydroids attach to soft body parts of the host bivalves (mainly the mantle) and reproduce asexually by budding (Kubota, 1983, 2012; Piraino *et al.*, 1994). The polyp of *Eutima* releases a medusa with immature gonads, but with a well-developed feeding apparatus comprising long tentacles and a well-differentiated manubrium (Kubota, 1983, 2012). In contrast, the polyp of *Eugymnanthea* releases a reproductively mature medusoid with a reduced feeding apparatus that has no tentacles and a reduced manubrium (Kubota, 1979).

As far as it is known these hydroids do not show strict host specificity, but they do display a certain host preference like Mytilus galloprovincialis Lamarck, 1819, Crenomytilus grayanus (Dunker, 1853) (Cerruti, 1941; Crowell, 1957; Kubota, 1983, 1992; Kubota & Larson, 1990; Rayyan, Photis & Chintiroglou, 2004), Crassostrea gigas (Thunberg, 1793), C. virginica (Gmelin, 1791), C. rhizophorae (Guilding, 1828) (Kubota, 1979, 1983, 2012; Kubota & Larson, 1990), Tivela mactroides (Born, 1778) (Narchi & Hebling, 1975; Migotto, Caobelli & Kubota, 2004), Acanthocardia tuberculata (Linnaeus, 1758), Cerastoderma glaucum (Bruguière, 1789), Cardites antiquata (Linnaeus, 1758), Ruditapes decussatus (Linnaeus, 1758), Venus verrucosa Linnaeus, 1758 and Arca noae Linnaeus, 1758 (Kubota, 1979, 1983; Piraino et al., 1994). Details of these

hydroid-bivalve associations have been reviewed in Kubota (1983, 1987) and Piraino et al. (1994).

Although the hydroid-bivalve interaction has been described as parasitic (Cerruti, 1941) it is now thought to be commensalistic (Mattox & Crowell, 1951; Kubota, 1983) or even mutualistic (Rees, 1967; Piraino et al., 1994). The hydroids inhabiting the bivalve mantle cavity benefit from a sheltered environment and from the food transported by the mollusc inner current in exchange for protection against intruders (Rees, 1967). In fact, Piraino et al. (1994) suggest that polyps can feed on trematode sporocysts that infest the bivalve host, causing parasitic castration. However, some species have been associated with deleterious or harmful consequences on commercial shellfish species, i.e. decreased growth and fertility and reduced condition index (i.e. measurement of shellfish overall quality) induced by Eutima japonica Uchida, 1925 in Mizuhopecten yessoensis (Jay, 1857) (Baba et al., 2007) and by Eugymnanthea inquilina Palombi, 1935 in Mytilus galloprovincialis (Rayyan et al., 2004; Mladineo et al., 2012). As a consequence, the occurrence, life cycle and evolution of these symbiotic hydroids have been studied in detail (Kubota, 2000; Bouillon et al., 2004; Migotto et al., 2004; Govindarajan et al., 2005; Cartwright et al., 2008). Nevertheless, to our knowledge, there is no report of these hydroids occurring in the large bivalves of the family Pinnidae.

During a field trip conducted to the archipelago of Bocas del Toro, Panama, in March 2012, we observed prevalent occurrence of a hydroid on the gills of an amber penshell, *Pinna carnea* Gmelin, 1791 (from a total sample of 25 P. carnea; Fig. 1A, B), a common bivalve species in the bays of the archipelago of Bocas del Toro, the Caribbean and nearby Atlantic. These large bivalves are commonly found half or completely buried in mud or sand in seagrass beds (Turner & Rosewater, 1958; Rosewater, 1961; Schultz & Huber, 2013) where they are often encountered with the valves open while they filter feed. Our infected specimen had a thick and large shell, typical of an old individual and it was found semi-buried in coarse sand in a seagrass meadow at a depth of 3 m in Managuar Cay (9.29032°-82.18983°). The particular large shell of these bivalves allows them to often be used as a substrate for epibionts, including algae, cnidarians, tunicates, sponges, corals, sedentary polychaetes, or other molluscs (bivalves, chitons, gastropods). Many species in the family Pinnidae are also known to host symbionts within their pallial

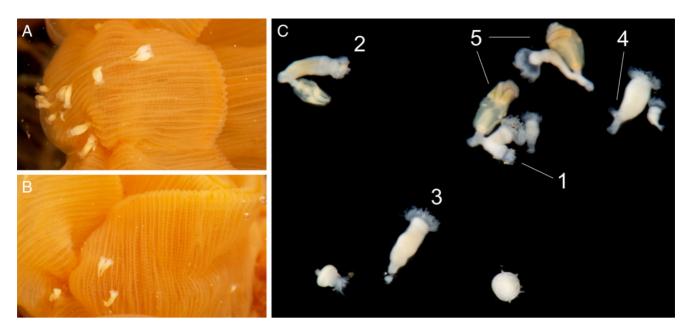


Figure 1. Bivalve-inhabiting Leptothecata observed in *Pinna carnea* in the archipelago of Bocas del Toro, Panama; live polyps photographed on the gills of *Pinna carnea* (**A**, **B**); observed different growth stages (**C**): 1. polyps with a budding daughter; 2. polyp with a medusa bud; 3. young polyps; 4. polyps with a primordial medusa; 5. polyps with a developed medusa.

cavity, mainly crustaceans, including shrimps in the family Pontoniidae and pea crabs in the genus *Pinnotheres* (Hipeau-Jacquotte, 1971; Fransen, 2002; Baeza *et al.*, 2011), but no symbiotic hydroids have been reported so far living in the mantle cavity. Here, we report the occurrence of hydroid symbionts probably of the genus *Eutima* on the gills of a specimen of *Pinna carnea* from the archipelago of Bocas del Toro, Panama.

The gills of the infected *P. carnea* were dissected out and preserved in 96% EtOH for genetic analyses. The shell and a tissue sample of the pinnid specimens are deposited in the Museum of Comparative Zoology Harvard University, in the Malacology collection (MCZ MAL 381160) and the hydrozoans in the Inver-tebrate Zoology collection (MCZ 25220). Additional specimen data and images can be obtained from MCZbase (http://mczbase.mcz.harvard.edu).

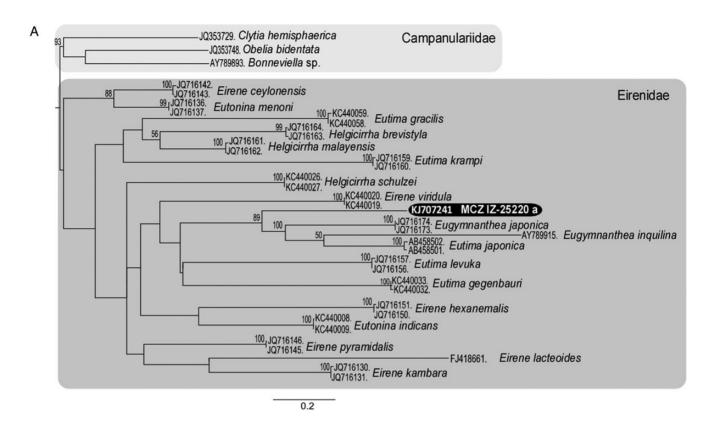
Total DNA was extracted from two polyps using Qiagen's DNEasy® Tissue kit (Valencia, CA, USA), following the manufacturer's instructions. Molecular markers consisted of one mitochondrial protein-encoding gene, cytochrome c oxidase subunit I (650 bp; COI hereafter) and one nuclear ribosomal gene, 18S rRNA (1.7 kb), as information for related species was available for these markers in GenBank. COI was amplified using primer pair LCO1490-HCO2198 (Folmer et al., 1994) and the complete fragment of 18S rRNA was amplified in three overlapping fragments, using primer pairs 1F-4R, 3F-18Sbi and 18Sa2.0-9R (Giribet et al., 1996; Whiting et al., 1997). Polymerase chain reactions (PCR) and DNA sequencing are as in Lemer et al. (2014). Sequence data were visualized and edited in GENEIOUS PRO 5.3.4 (Drummond et al., 2010). All new sequences have been deposited in GenBank under the accession numbers (KJ707239-KJ707241).

For COI and 18S rRNA one to two sequences were retrieved from GenBank per available species in the family Eirenidae (Fig. 2). We also retrieved sequences from three species of Campanulariidae, the sister family of Eirenidae, to use as outgroups. In total 34 COI and five 18S rRNA sequences of Eirenidae, five COI and six 18S rRNA outgroup sequences and our two samples were aligned in GENEIOUS PRO 5.3.4. using MUSCLE v.3.6 (Edgar, 2004). Independent maximum

likelihood (ML) searches were conducted for each marker using RAxML v. 7.2.7 (Stamatakis, 2006). A General Time Reversible model of sequence evolution with a correction for a discrete gamma distribution for rate heterogeneity (GTR + Γ) was specified. Nodal support was estimated via 1000 replicates of a rapid bootstrap algorithm (Stamatakis, Hoover & Rougemont, 2008) using the GTR + Γ model, through the CIPRES V. 3 gateway (Miller, Pfeiffer & Schwartz, 2010). In addition, we re-run these analyses with unpublished COI data from A. Collins, which included a sample of *E. sapinhoa* from Brazil collected by A. E. Migotto.

The solitary hydrozoan polyps found in P. carnea aggregated densely on the external surface of the gills. They were attached by a flattened pedal disc, exhibited numerous tentacles (c. 40) and varied in size, assuming that multiple age classes were represented. Some polyps displayed a budding daughter polyp attached to the pedal disc (Fig. 1C-1) whereas a significant amount displayed a medusa bud attached laterally on the lower third of their column (Fig. 1C-2). We generally observed four different growth stages: (1) young polyps with no budding (Fig. 1C-3); (2) polyps with a primordial medusa bud where the medusa structures are not yet recognizable (Fig. 1C-4); (3) polyps with a more developed medusa bud with several recognizable structures such as a subumbrellar cavity, tentacles and statocysts (Fig. 1C-5); (4) polyps with no recognizable tentacles and medusa with umbrella almost completely expanded. No free medusa stage was observed.

The phylogenetic analyses conducted for each of the two markers clustered the pinnid hydrozoans within a clade of Eirenidae, closely related to species of *Eutima* and *Eugymnanthea* (Fig. 2), although neither genus is monophyletic in the denser COI tree (Fig. 2A). For the more conserved 18S rRNA, the pinnid hydrozoan sequences clustered with *Eutima sapinhoa* Narchi & Hebling, 1975 from Brazil (A. Collin, pers. comm.; Cartwright *et al.*, 2008); and differed from *E. sapinhoa* by only 2 nucleotides out of a total of 1679 (Fig. 2B), suggesting a close relationship of these two species. The COI marker placed our specimen as the sister group to a clade containing the sequences of *Eugymnanthea japonica* (Kubota, 1979), *E. inquilina* and *Eutima*



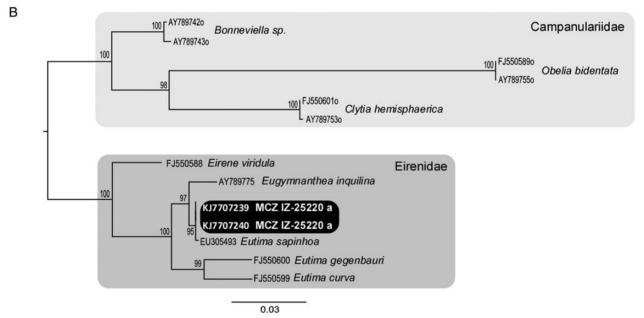


Figure 2. Phylogenetic relationships of species in the family Eirenidae inferred from maximum likelihood analysis of two genetic markers: **A.** COI and **B.** 18S rRNA. Bootstrap values shown above branches. GenBank accession numbers and species are indicated at the tips of the trees. The hydrozoans sampled in *Pinna carnea* are highlighted.

japonica. Although no COI sequence is available for Eutima sapinhoa in GenBank, the unpublished sequence from Brazil formed a well-supported clade (100% bootstrap support) with our sequence, both differing by only 25 nucleotides out of a total of 354 (7% divergence; data not shown). Although the monophyly of Leptothecata is well supported (Collins et al., 2006; Leclère, Schuchert & Manuel, 2007; Leclère et al., 2009; Cantero, Sentandreu & Latorre, 2010), no molecular phylogenetic

analysis has been conducted on the family Eirenidae. As a result, it is still unclear whether Eirenidae is monophyletic and the relationships among the 9 to 10 recognized genera (Bouillon et al., 2006; Daly et al., 2007) and the 65 species (Bouillon et al., 2006) remain unstudied. Both phylogenetic analyses of 18S rRNA and COI show however that the Eutima is not monophyletic, and the COI tree also shows Eirene Eschscholtz, 1829, Eugymnanthea and Eutonina Hartlaub, 1897 not monophyletic

and a lack of support in the deepest nodes. In this context we can only deduce from our phylogenetic analyses that the sampled hydrozoan specimens are closely related to species of the genera *Eutima* and *Eugymnanthea*, most particularly to *Eutima sapinhoa*.

The morphology of the different polyp stages observed in our specimens is concordant with the description of species in the genera Eutima and Eugymnanthea (Narchi & Hebling, 1975; Migotto et al., 2004). However, the only two other species known of Eugymnanthea have not been recorded in the Western Atlantic: Eugymnanthea inquilina is Mediterranean and Eugymnanthea japonica is Pacific. Within Eutima, the only other species known to inhabit bivalves in the Atlantic are Eutima satinhoa and Eutima ostrearum (Mattox & Crowell, 1951; Kubota, 2012), the former having been described from the mantle cavity of Tivela mactroides from the coast of Brazil. Specimens from Florida reported as Eutima sp. by Kubota & Larson (1990) have been recently assigned to Eutima sapinhoa by Migotto et al. (2004) and confirmed by a study of the life cycle by Kubota (2012), suggesting that this species could have a wide western Atlantic distribution. Eutima ostrearum was described based on material from Puerto Rico, and to our knowledge it has not been observed in any other locality, but the lack of molecular data from this species and our morphological examination do not allow for a conclusive identification of our material. Life cycle studies are essential to accurately identify and characterise species of hydrozoans because their classification relies mostly on characteristics of the medusa stage; this is particularly true for the species in the family Eirenidae. Unfortunately we were not able to study this due to the low infection rates (polyps were preferably preserved for genetic analysis) and our limited expertise in rearing medusae.

In conclusion, both the observed morphology of our samples and the phylogenetic analyses support the conclusion that the hydrozoans found on the gills of one specimen of *Pinna carnea* from the archipelago of Bocas del Toro, Panama, belong to the family Eirenidae, and are closely related to *Eutima sapinhoa*. Since no species of Eirenidae has been previously reported to inhabit the mantle cavity or the gills of bivalves in the family Pinnidae, the report of *Eutima* in this new host constitutes a notable finding.

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