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A single origin of bivalve-inhabiting hydrozoans (Cnidaria, Hydrozoa, Leptomedusae) in the family Eirenidae based on an analysis of 16S rRNA gene

Shin Kubota[1] and Allen Collins[2]

[1] Seto Marine Biological Laboratory, Kyoto University, Shirahama Town, Wakayama Prefecture, P.O.Box 649-2211, Japan
[2] NMFS, National Systematics Laboratory, National Museum of Natural History, MRC-153, Smithsonian Institution, P.O.Box 37012, Washington, DC 20013-7012, USA

Abstract. The benthic eirenid polypoid phases of species in *Eugymnanthea* and *Eutima* inhabit the mantle cavity of bivalve molluscs. Whereas the polyps of two known species of *Eugymnanthea* live in this habitat, only a subset of *Eutima* species (three species) is associated with bivalves in the Eirenidae. Using 16S rRNA gene sequence data, we conducted phylogenetic analysis of eirenid and its allied hydrozoans (*Eutonina* and *Blackfordia*), and found that all of the bivalve-inhabiting species presently analyzed (four distinct species) form a well-supported clade in Eirenidae. This implies that this unique life habit evolved in the most recent common ancestor (MRCA) of *Eugymnanthea japonica*, *Eugymananthea inquilina*, *Eutima saphinoa* and *Eutima japonica*.

Key words: bivalve, Eirenidae, *Eugymnanthea*, *Eutima*, evolution, hydrozoa, molecular analysis, 16S rRNA

Introduction

The only known cases where polyps live on the soft parts of bivalve molluscs are species within the hydrozoan family Eirenidae in the order Leptomedusae, and this family was recently reviewed by Lerner and Giribet (2014). Rather than being widely distributed, this association with bivalves is limited to species in just two of the ten genera of Eirenidae, *Eugymnanthea* Palombi, 1935 and *Eutima* McCrady, 1859. Even within *Eutima*, not all species have polyps that live on the soft parts of bivalves. Whereas both known species of *Eugymnanthea* have polyps that live in this habitat, only a subset of *Eutima* species (three species) is associated with bivalves.

Species of *Eugymnanthea* produce eumedusoids rather than fully developed, ordinary medusae (Govindarajyan et al. 2005), and it is mainly this feature that differentiates *Eugymnanthea* from *Eutima*. It has been hypothesized that species of the genus *Eugymnanthea* have a progenetic origin from a *Eutima*-like ancestor with long-living, fully developed medusae, and that this evolution has taken place in parallel in the Pacific and the Atlantic Oceans (Kubota 2000). Analyses by Leclère et al. (2007) and Maronna et al. (2016) raise doubts about whether Eirenidae is monophyletic. Their analyses showed that the eirenid taxa are closely related to those of the families Aequoreidae, Blackfordiidae, Malagazziidae, Sugiuridae and some species of Lovenellidae. In order to assess whether there is one or more origins of hydrozoans inhabiting the mantle cavities of bivalves, we conducted a phylogenetic analysis of Eirenidae and closely allied taxa based on a region of the 16S rRNA gene.

*Corresponding author: kubota.shin.5e@kyoto-u.ac.jp

Materials and Methods

Materials of DNA samples of bivalve-inhabiting hydrozoans and one species of outgroup (*Eutonina indicans*) prepared in the present study are marked (*) in addition to *Eutima sapinhoa* from Brazil (**) as shown in the analyzed tree (Fig. 1). All of the materials, both polyps and their medusae obtained in the laboratory by culture (food: *Artemia* nauplii) were preserved in 100% ethanol after starvation. Used samples are as follows: *Eutima japonica* (northern form) from Qingtao, China collected in September 2007 associated with *Mytilus galloprovincialis* (Kubota 2008); *Eutima sapinhoa* from Florida, USA associated with *Crassostrea virginica* collected on November 18, 2011 (Kubota 2012a) and the same species associated with *Tivela mactroides* from Brazil (** in Fig. 1: Migotto et al. 2004); *Eutima japonica* (northern form) from Souma and Hisanohama, Fukushima Prefecture, Japan collected in 2012, associated with *Mytilus galloprovincialis* (Kubota 2012b); *Eutima japonica* (intermediate form: *Eucheilota/Lovenella*–like) from Takesiki and Toyo, Tsushima Island, Nagasaki Prefecture, Japan associated with *Mytilus galloprovincialis* collected in 2012 (Kubota 2012c). As one of the outgroups, aquarium cultured medusae of *Eutonina indicans* from Kamo, Yamagata Prefecture, Japan in 2011 were used.

Extraction of DNA was as follows: using AutoGenPrep 965 high-throughput DNA extraction robotic system (AutoGen) in accordance with the manufacturer's instructions for Whole Blood. Polymerase chain reaction (PCR): in 10 µl aliquots and comprised final concentrations of the following: 0.5 units Taq (Biolase DNA polymerase [Bioline USA Inc., Taunton, MA], 0.3 mM of each primer, 0.5 mM dNTPs (Bioline), 1.5 mM magnesium chloride, 2.5

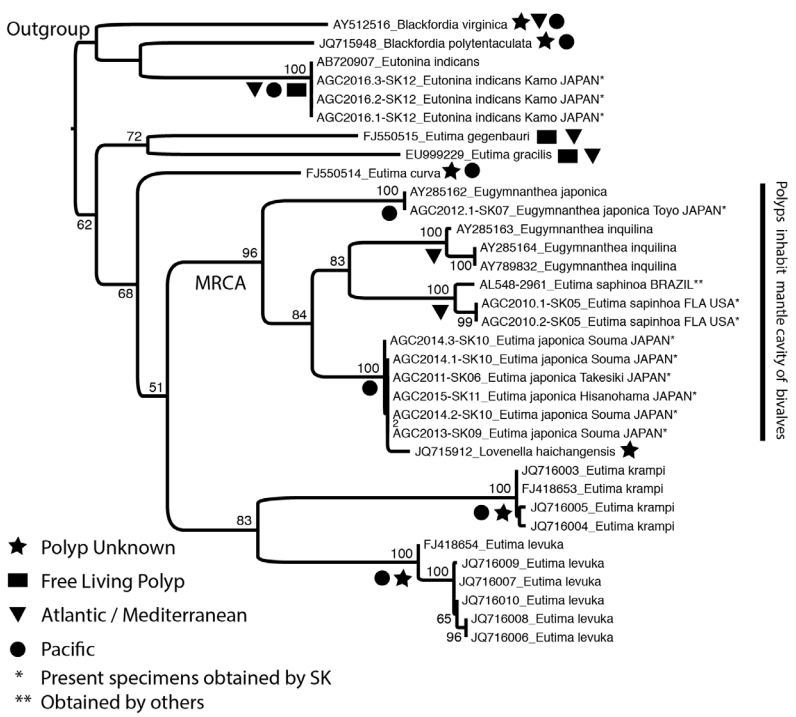

Fig. 1. Phylogenetic hypothesis for Eirenidae based on ML analysis of 16S rRNA gene sequences. MRCA: the most recent common ancestor. SK: Shin Kubota.

μl Bovine serum albumin (BSA) (New England BioLabs Inc., Ipswich, MA), and 1μl Buffer, DNAase-free Water to bring the volume to 10 μl]. The thermocycling protocol was an initial denaturation step of 95oC for 5 min, 35 cycles of 95oC for 30 s, 50oC for 30 s, 72oC for 45 s, followed by a final extension step of 72 oC for 5 min. PCR products were then cleaned, used for cycle sequencing and sequenced on an Applied Biosystems 3730xl DNA Analyzer. 16S rRNA gene was amplified and sequenced using newly developed primers (Lawley et al. 2016). All forward and reverse sequence reads were processed in Geneious (Drummond et al. 2011), which was used for assessing quality, trimming read ends and assembling contigs. Datasets were assembled using newly sequenced samples, as well as sequences obtained from Genebank. The multiple sequence alignment program MAFFT (version 7) (Katoh and Standley 2013) was used for alignment.

Phylogenetic hypotheses were assessed using the criterion of Maximum Likelihood (ML) as implemented by PhyML (Guindon et al. 2010), which was also used to assess node support by bootstrap values (400 replicate searches). Resulting topologies were rooted on the relatively closely related taxa *Eutonina* and *Blackfordia* (outgroup).

The 16S rRNA dataset consisted of 187 sequences, with an aligned length of 670 basepairs. Jmodeltest identified GTR+gamma as the most appropriate model of nucleotide evolution. When the dataset was trimmed to include only single exemplars from different taxa, the number of taxa decreased to 34, including six definitive outgroups in the genera *Blackfordia* and *Eutonina*. Sequences will be registered in Genebank.

Results

Present phylogenetic analysis of the eirenid bivalve-inhabiting hydrozoans from 16S rRNA gene (Fig. 1) shows that (1) Independent evolution and appearance of solitary, nude, bivalve-dwelling polyps within Eirenidae is not detected; (2) The two Atlantic bivalve-inhabiting taxa, *Eutima sapinhoa* and *Eugymnanthea inquilina*, are grouped together; (3) The group with bivalve-inhabiting polyps is derived from *Eutima*-like ancestors with a life cycle involving a free-living polyp. Further, *Eutonina* outgroup is valid; (4) *Eutima gegenbauri* and *E. gracilis* have free-living polyps as demonstrated by culture by Russel (1970) and these two species form a genetically separate group from the bivalve-inhabiting eirenid hydrozoans; (5) *Lovenella haichangenesis* Xu and Huang, 1983 from China is grouped together with both intermediate and northern forms of *Eutima japonica* from Japan.

Discussion

In the 16S rRNA gene-based tree (Fig. 1), bivalve-inhabiting hydrozoans are one distinct group separated from other *Eutima* group that includes species with free-living, colonial polyp such as *Eutima gegenbauri* and *Eutima gracils*. Still being unknown, the life cycles of *Eutima krampi*, *E. levuka* and *E. curva* should be resolved in the future. We predict that the polyp of *Lovenella haichangensis* Xu and Huang, 1983 from China, which resembles an intermediate form of *Eutima japonica* medusa from Tsushima, Japan is a bivalve-inhabiting hydrozoan, and the scientific name should be emended. It should be mentioned here that similar mature medusae have been collected from Okinawa Island, as well as from Tsushima Island, Nagasaki Prefecture and Ago Bay, Mie Prefecture, Japan (Kubota 2003).

Earlier work based on morphological and life history observations suggested that an Atlantic *Eutima sapinhoa* is distributed both in the southern and northern Atlantic Ocean (Kubota 2012a), and we confirm this result with genetic data (Fig. 1). In

the bivalve-inhabiting group, the Atlantic species including the Mediterranean Sea (*Eugymnanthea inquilina* and *Eutima saphinoa*) are closely related genetically despite medusa morphology being very different. The present analysis cannot tell us about the specific ancestors of both *Eugymnanthea inquilina* and *Eugymnanthea japonica*. There may be many additional species left to analyze including the most complicated form of Indian species, *Eutima commensalis* Santhakumari, 1970 which has a mature medusa resembling that of *Eutima japonica*, (Shin Kubota tried to collect this very unique polyp, but failed: Kubota and Santhakumari 2005) and there may also be additional species that have become extinct.

Independent (parallel) evolution from *Eutima* to *Eugymnanthea* could have taken place in the Pacific and the Atlantic Oceans as inferred earlier by Kubota (2000). The present analysis does not support a single origin of the reduced medusa (medusoid) of *Eugymnanthea*, and the Pacific *Eugymnanthea japonica* appears as the earliest diverging lineage of bivalve-inhabiting hydrozoans. Therefore, it is not consistent with the assumption of progenetic origin of *Eugymnanthea* in the Pacific (Kubota 2000), but this could be due to limited taxon sampling.

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