Title: Unmasking the third sponge player in the Plakortis-Haplosclerida epizoic symbioses.

Investigators

Dennis Lavrov, Department of Ecology, Evolution and Organismal Biology, Iowa State University.
Jan Vicente, Institute of Marine Biology, University of Hawai'i at Manoa-Hawai'i.
Jaaziel E. Garcia-Hernandez, Department of Marine Sciences, University of Puerto Rico.
Nikolaos Schizas, Department of Marine Sciences, University of Puerto Rico.

Summary

Whole genome sequencing revealed the presence of the third sponge species ("The mystery sponge") in the *P. symbiotica – H. plakophila* association. The mitochondrial genome of this novel sponge is highly unusual and suggests that the species belongs to yet unknown lineage of sponges. The current projects aims to evaluate whether the third sponge species is an obligate part of the *P. symbiotica – H. plakophila*association and whether it is also present in the *Plakortis–Xestospongia deweerdtae* associations that occur in the same region. This will be accomplished by using molecular probes designed for each player in the association. If the third species is present in some but not all samples, we will investigate the effect of its presence on the other two sponges. Furthermore, we will attempt to identify spicules and cell types associated with the third species of sponges. Finally, using genome skimming techniques, we will start characterizing the genome of the "Mystery sponge".

Project Information

1 Background information

Sponges as communities of marine organisms Due to their porous body architecture and loose association of cells in the mesohyl, most sponges host a variety of other organisms including bacteria, algae, and other Metazoa [1]. Some of these organisms simply use sponges as a habitat or substrate, while other have important physiological relationships with their host. As an example, the majority of sponges on the Great Barrier Reef are net producers of oxygen due to their association with symbiotic algae [2].

Mutualism among sponges While cases of sponge-sponge epizoic interactions have long been reported in the literature (*e.g.,* Sollas 1888), our understanding of mutualistic relationships among sponges remains limited. Two main reasons behind such associations have been proposed. The first suggests that sponges form epizoic interactions with other sponges to cope with crowded environments and space limitation in reef caves [3, 4]. The second suggest that such association might allow protection of palatable sponges by chemically defended sponges from spongivorous predators [5]. In extreme cases, a sponge can be fully overgrown by its epibiont, but remained living in its mesohyl [3].

Homosleromorpha-Haplosclerida mutualism in Caribbean sponges Recently, three novel specialized sponge associations have been described in the Caribbean between two *Plakortis* species from the class Homoscleromorpha and two species from the demosponge order Haplosclerida [6, 7]. These sponge pairs were found living in reef caves and on the upper mesophotic reefs (30–36m) of the Bahamas and Puerto Rico. The basibiont *Plakortis deweerdtaephila* associates only with *Xestospongia deweerdtae*, while the second species, *P. symbiotica* associates with both *X. deweerdtae* and *Haliclona plakophila*. These are the only known *Plakortis* spp. in the world that associate with other sponges and this is a key taxonomic character in the description of these new species. Also, unlike previously described sponge pairs, Haplosclerida spp. not only grow over the *Plakortis* surface, but also form channels within the dense *Plakortis* mesohyl tissue. The observation that the associated Plakortis spp. and Haplosclerida spp. persist for long periods of time without the one species smothering the other, suggests that their relationship are mutualistic in which the *Plakortis* and that both species are receiving reciprocal benefits.

Mysterious third sponge species in the *Plakortis symbiotica* – *Haliclona plakophila* association. Recently, we generated genomic data from one of the samples of *H. plakophila* from the *P. symbiotica* – *H. plakophila* association. The resulting assembly contained complete mitohochondrial genomes (mt-genome) from both species involved in the association, but also the third mt-genome of unknown origin. While the third mt-genome was highly unusual in nucleotide composition and showed little similarity to other known species, a combination of features of this mt-genome indicates that it belongs to sponges and most likely demosponges [8]. Furthermore, the sequence coverage of the third genome was comparable to that of *H. plakophila* and much higher than that of the host species. Molecular probes designed for this "Mystery sponge" have shown its presence in one of the two other samples of *H. plakophila* that were available in the lab. However

it's prevalence in the *P. symbiotica – H. plakophila* association and it's presence in other *Plakortis* – Haplosclerida associations remains unknown.

2 Research Goals

The overall goal of this project is to determine the prevalence and specificity of the "Mystery sponge" in Homoslceromorpha/Haplosclerida associations as well as its effect on this association. Specific aims include:

Aim 1: To conduct survey of *Plakortis*/Haplosclerida associations to determine the pattern of the presence/absence of the "Mystery sponge".

- Collect three known association of Plakortis/Haplosclerida species
- Use molecular markers to determine prevalence and specificity of the "Mystery sponge" in these associations
- Determine wether the "Mystery sponge" is primarily associated with the basibiont *Plakortis* or the epibiont haplosclerid.

Aim 2: To compare structural differences between sponge association with/without the third sponge

- Determine whether morphology of Plakortis/Haplosclerida association is influenced by the presence/absence of the third species
- Identify and analyze any additional spicules and cell types present in the three-sponges association

Aim 3: To compare genomic differences between sponge associations with/without the "Mysery sponge"

- Conduct exploratory DNA Illumina sequencing for associations with/without the "Mystery sponge
- Compare assembled sequences to identify those belonging to the "Mystery sponge"

The innovative, novel, and intellectual merits of the proposed work. The novelty of the proposed research is two-fold. First, it will provide additional insights into the *Plakortis*–Haplosclerida association. *Plakortis deweerdtaephila* and *P. symbiotica* are the only two known species in the genus that form mutualistic associations with other species. However, it is still unclear whether these associations are mutually beneficial to both species. Second, the project will provide insight into the "Mystery sponge", putatively a new lineage of sponges.

3 Research Activities

3.1 Aim 1: To conduct survey of *Plakortis*/Haplosclerida associations to determine the pattern of the presence/absence of the "Mystery sponge".

Rationale So far the "Mystery sponge" was found in two of the tree analyzed samples of *Haliclona plakophila*. However, these samples were collected back in 2009 and their DNA was partially

degraded. Thus, the first task of our research team will be to conduct a more comprehensive sampling of *Plakortis* – Haplosclerida associations. Several steps will be taken to accomplish this task:

1.1. Collect three known association of Plakortis/Haplosclerida species Samples will be collected from the three known associations of *Plakortis*–Haplosclerida species and saved in 95% ethanol as well as RNA later. *Haliclona plakophila* will be sampled from the same location where the "Mystery sponge" was found as well as from more distant areas. When possible, the basibiont and the epibiont will be sampled and preserved separately.

1.2. Use molecular markers to determine prevalence and specificity of the "Mystery sponge" in these associations We have designed and tested primers for mitochondrial sequences from all three sponges in the *Haliclona plakophila* association. These primers will be used to test for the prevalence of the "Mystery sponge" in the newly collected samples. Primers for the basibiont and epibiont will be used as positive controls. New primers will be designed for *Plakortis deweerdtaephila* and *Xestospongia deweerdtae* and, together with the "Mystery sponge" primers will be used to test specificity of the latter species.

1.3. Determine wether the "Mystery sponge" is primarily associated with the basibiont *Plakortis* or the epibiont haplosclerid. We plan to use qPCR approach to answer this question. Basibiont and epibiont samples will be separated as well as possible and the amount of "Mystery sponge" DNA in these samples will be compared by qPCR to those of single copy genes.

3.2 Aim 2: To compare structural differences between sponge association with/without the third sponge

Rationale Our preliminary data suggest that the "Mystery sponge" is present in some but not all *Plakortis symbiotica – Haliclona plakophila* associations. If confirmed by experiments proposed for Aim #1, this pattern would allow us to investigate the effects of the "Mystery sponge" on the other two sponges as well as to attempt to identify cellular and extra-cellular components of the unknown sponge.

2.1. Determine whether morphology of Plakortis/Haplosclerida association is influenced by the presence/absence of the third species We will compare the changes in morphologies of both epibiont and basibiont in the presence of the "Mystery sponge" by analyzing thick sections of these sponges. Furthermore, we will extract their spicules by boiling small sponge pieces in nitric acid and washing them with distilled water. Spicules will be analyzed under the light microscope for measurements and photography as well as imaged with Scanning Electron Microscope (SEM).

2.2. Identify and analyze any additional spicules and cell types present in the three-sponges association We will also analyze both unexpected spicules as cell types in the associations containing the mystery sponge that may help reveal identity of the latter species.

3.3 Aim 3: To compare genomic differences between sponge associations with/without the "Mysery sponge"

Rationale The putative involvement of the third sponge in the *Plakortis symbiotica – Haliclona plakophila* association was revealed by the presence of unusual mitochondrial DNA in the total DNA assembly. Identification of nuclear contigs from that species is less straightforward because of their lower sequence coverage, fewer reference sequences, and presence of contamination. One possible way to overcome some of these problems is to perform sequencing on samples with and without the "Mystery sponge." This approach would allow selective elimination of sequences from both *Plakortis symbiotica* and *Haliclona plakophila* and retaining the sequences of interest.

3.1. Conduct exploratory DNA Illumina sequencing for associations with/without the "Mystery sponge We will utilize Illumina sequencing to obtain genomic data from both *P. symbiotica* and *H. plakophila* as well as the third "Mystery" species, when present.

3.2. Compare assembled sequences to identify those belonging to the "Mystery sponge" The sequences obtained in Specific Aim 3.1 will be assembled and the assemblies compared in order to identify nuclear contigs belonging to the "Mystery sponge".

3.4 Challenges & Alternative Approaches

There are two main presuppositions that will determine the success of the proposed research. First, we assume that the three-sponge association found in 2009 is biologically meaningful, persistent, and relatively widespread. Second, several proposed experiments depend on the assumption that the presence of the "Mystery sponge" is not obligatory. Both of these assumptions will be tested in Specific Aim 1 of the proposal. Several alternative methods can be used to identify both cells and genomic sequences from the Mystery sponge. For example, flow cytometry and/or single cell sequencing can be used to obtain genomic sequences from the Mystery sponge. Alternatively, long read sequencing can be utilized to simplify assembly of the genomes of interest.

References Cited

[1] Janie L. Wulff. Ecological interactions of marine sponges. *Canadian Journal of Zoology*, 84(2):146–166, feb 2006.

[2] Clive R. Wilkinson. Net primary productivity in coral reef sponges. *Science*, 219(4583):410–412, jan 1983.

[3] Klaus Rtzler. Spatial competition among porifera: Solution by epizoism. *Oecologia*, 5(2):85–95, jun 1970.

[4] M. Sar. Competition and cooperation in sponge populations. In W. G. Fry, editor, *The Biology of the Porifera.*, volume 25, pages 273–284. 1970. Proceedings of the 1st International Sponge Conference.

[5] Blake Ramsby, Andrew Massaro, Emily Marshall, Thomas Wilcox, and Malcolm Hill. Epibiont–basibiont interactions: examination of ecological factors that influence specialization in a two-sponge association between *Geodia vosmaeri* (sollas, 1886) and *Amphimedon erina* (de laubenfels, 1936). *Hydrobiologia*, 687(1):331–340, oct 2011.

[6] Jan Vicente, Sven Zea, Ryan J. Powell, Joseph R. Pawlik, and Russell T. Hill. New epizooic symbioses between sponges of the genera *Plakortis* and *Xestospongia* in cryptic habitats of the Caribbean. *Marine Biology*, 161(12):2803–2818, oct 2014.

[7] Jan. Vicente, Zea. Sven, and Russell T. Hill. Sponge epizoism in the Caribbean and the discovery of new *Plakortis* and *Haliclona* species, and polymorphism of *Xestospongia deweerdtae* (Porifera). *Zootaxa*, 4178(2):209, oct 2016.

[8] Dennis V. Lavrov, Lise Forget, Michelle Kelly, and B. Franz Lang. Mitochondrial genomes of two demosponges provide insights into an early stage of animal evolution. *Molecular Biology and Evolution*, 22(5):1231–1239, feb 2005.