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Project Title: Are Cryptic Millepore Species Prevalent in the Caribbean?

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INTRODUCTION

For centuries, biologists have attempted to group organisms based on shared characteristics in order to understand the evolutionary relationships of the tremendous diversity of life. This construction of evolutionary relationships (phylogenies) is called systematics and allows biologists to ascertain patterns of relationships among species.

Traditionally, phylogenies are constructed based on similarities. The more similar organisms are (more shared characters), the more closely related they are presumed to be. The opposite is also true. There are numerous problems associated with constructing phylogenies based on similarities. Phylogenetic relationships are estimated using morphological, behavioral, and other phenotypic characters. However, these characters may not accurately represent evolutionary relationships because evolution is not always divergent (Hendry, 2009; Schluter, 2009). Two species can independently evolve the same features due to similar habitats and favored adaptations. Therefore, two species that are not closely related may end up more phenotypically similar to each other than to their closest relatives.

The problems associated with understanding evolutionary relationships between organisms are stiking in reef-building coral assemblages that serve as the foundation of complex reef ecosystems. Coral taxonomic classification (as well as our current understanding of coral evolution) is based upon morphological characters of the calcareous skeleton (Wallace and Willis, 1994). Unfortunately, the calcareous skeleton of many marine organisms shows considerable phenotypic plasticity (the ability of an organism with a given genotype to change its phenotype in response to environmental changes). The architecture of the coral skeleton is affected by environmental factors such as underwater irradiance, water motion, water temperature and sedimentation (Barnes and Chalker, 1990). Additionally, calcification rates are affected by lunar, diurnal and seasonal fluctuations (Barnes and Lough, 1989). Hence, the evolutionary history and current speciation in this diverse class of animals remain paradoxical.

The coral phylogenetic issue is particularly problematic in the calcareous hydrozoan coral, *Millepora*, which is one of the most common skeleton-forming animals on reefs. This group of corals, known as “fire-coral” because of its painful sting, is represented by multiple species and is nearly ubiquitous on reefs in the Atlantic, Indian and Pacific Oceans. Millepores are important reef framework builders, second only to the scleractinia (stony or hard) corals (Lewis, 2006). The morphology of the millepores is highly variable and shows phenotypic plasticity (Stearn and Riding, 1973; Lewis, 2006, Tepper et al. 2012). The various growth forms of *Millepora* in the Caribbean range from thinly encrusting sheets and delicate dendroid branches for *M. alcicornis*, to thicker, rigid bladed forms for *M. complanata* (Stearn and Riding, 1973). It is this variation in morphology that has led to constant controversy about millepore classification. Currently, species designation within the genus is mainly based on growth form,

geographical distribution and morphological differences such as surface texture, nematocyst structure, and the size and shape of pores (Lewis, 2006).

Tepper et al. (2012) examined the evolutionary relationship of the two commonly found millepores (*M. complanata* and *M. alcicornis*) in the northern Caribbean off the coast of San Salvador, Bahamas. In addition to these two recognizable species, they reported the existence of numerous intermediate forms, of which the specific species status was unknown. Because of the wide range of growth forms, the question arises whether the blade (*M. complanata*) and branching (*M. alcicornis*) forms are separate species or represent phenotypic variations of one highly variable species.

The purpose of our research is to determine whether the colony morphologies represented by the described species of *Millepora* are matched by genetic isolation. We hope to distinguish among four hypotheses: 1) Millepores of the Bahamas are heterogeneous assemblages of genetically distinct forms. 2) The described "species" are a spectrum of colony growth forms reflecting ecological conditions rather than genetic isolation. 3) The range of growth forms observed is the result of extensive hybridization. 4) Millepores are reproductively isolated cryptic species and that traditional macro- and microskeletal features used for classification cannot distinguish them.

Abundance surveys were conducted on various patch reefs off the coast of San Salvador, Bahamas in order to distinguish between our proposed hypotheses (Tepper et al., 2012). Millepore abundance surveys at some reefs suggested that the two standard morphologies utilized different habitats since the blade form (*M. complanata*) was found predominately in shallow waters and the branch form (*M. alcicornis*) was found in deeper waters. At other reef locations, they reported the occurrence of all growth forms in mutual proximity. Because both morphs resided in the same location on the reef, they concluded that morphological variation in the millepores is not primarily a response of a single species to environmental differences and that genetic differences exist between the growth forms.

The use of molecular genetics has provided the tools to establish a more accurate assessment of evolutionary relatedness. The inherent uncertainty in using morphological characters as a way to establish phylogenetic relationships can be aided by using genetic markers to distinguish between closely related growth forms. Among the most widely used genetic markers are the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). Ribosomal RNA genes are organized in clusters of hundreds to thousands of tandem repeats per chromosome, each of which consists of three coding regions (18S, 5.8S and 28S), two internal transcribed spacer regions (ITS -1 and ITS-2), one external transcribed spacer (ETS), and one non-transcribed spacer (NTS) region (Prokopowich et al., 2003).

The two ITS regions have been used for detecting differences between conspecifics and are useful in studying closely related individuals due to their higher rate of mutation compared to the conserved rRNA genes (Hillis and Dixon, 1991). Meroz-Fine et al. (2003) utilized DNA sequence information from the ITS region of the Red Sea fire-coral, *Millepora dichotoma*, to show that the currently recognized single species with two growth forms (blade and branching) was in fact two distinct species. Forsman et al. (2009) reported that they were unable to use morphological characters to identify species diversity in the coral genus *Porites*. Using rDNA ITS regions and mitochondria gene markers, they revealed numerous cryptic patterns of species diversity in *Porites*.

Our research goal is to clarify the phylogenetic relationship between the Caribbean Millepores using DNA sequence analysis of the rDNA ITS regions. Results from ITS rDNA sequence comparisons of all three growth forms of Bahamian millepores show that the three morphologies are very closely related genetically (Tepper et al., 2012). However, the rDNA ITS region exhibits considerable divergence when compared to the reproductively isolated *M. exaesa*

found in the Red Sea. Our results indicated that regardless of the growth form examined, all millepores had a rDNA fragment length of approximately 824 base pairs. However, when sequence data was used to construct a phylogenetic tree (Maximum Likelihood) the resulting tree showed the existence of two independent clades. Each clade contained a fixed nucleotide sequence at five identical polymorphic sites in the 824 base pair rDNA region. These sites, called single nucleotide polymorphisms (SNPs), were responsible for separating the millepores into the two independent clades. The independent clades do not share DNA at the five SNPs. The only explanation for maintenance of these two independent clades is reproductive isolation. Hence, the different clades represent different species. Our results indicate that the two species that exist off the coast of San Salvador, Bahamas are cryptic and independent of growth form, depth and reef location.

Although, phylogenetic analysis based on DNA has helped untangle evolutionary relationships, one of the major concerns with using rDNA is the existence of variability among the repeated rDNA units, which may cause extensive differentiation even within single individuals leading to false phylogenetic relationships (Vollmer and Palumbi, 2004). The problem concerns the assumption that these repeated sequences have undergone concerted evolution, a process involving homogenization of individual repeats of a multigene family (Elder and Turner, 1995). We have analyzed the variability within rDNA repeats in order to ascertain the level of intragenomic variability within the millepores.

We analyzed twenty rDNA clones isolated from a single *Millepora complanata* colony and found low intragenomic sequence divergence ranging from 0-0.087% (Gaynor et al. 2013). When these 20 clones were compared to the geographically isolated *M. exaesa*, the rDNA sequence divergence ranged from 0.649-0.655%. Our results indicate that millepore intraspecific rDNA sequence divergence is consistently lower than interspecific sequence divergence. This implies that ribosomal DNA repeats have been homogenized in the millepores, and rDNA regions have the potential to be used as a species-specific molecular marker capable of distinguishing millepore species. These results substantiate our conclusions that the millepores in the northern Caribbean are represented by two cryptic species.

Project Goals

In February of 2013, Cornell students collected approximately 40 specimens of *M. complanata*, *M. alcicornis* and intermediate *Millepora* growth forms from various reefs surrounding South Water Caye, Belize located in the western Caribbean. Students subsequently isolated DNA from the coral samples

The purpose of this proposal and our research is to determine whether the independent clades do indeed constitute different reproductively isolated species throughout the Caribbean. Specifically, we propose to clone and sequence the rDNA ITS region of the coral samples collected in Belize. The current representation of coral DNA sequence in our data set is solely from the Bahamas and hence lacks representation of other Caribbean locations. Once we obtain rDNA sequence from the various samples we plan to use a phylogenetic tool called MEGA. MEGA is an integrated tool for conducting automatic and manual sequence alignment, inferring phylogenetic trees, estimating rates of molecular evolution, and testing evolutionary hypotheses. We hope our efforts lead to a more comprehensive understanding of the phylogenetic relationship of the Caribbean Millepores and allow us to address more fundamental questions concerning their evolution.

Methods and Means to be Utilized

We are using a coral specific primer set described by Takabayashi et al. (1998). The polymerase chain reaction (PCR) conditions for successfully amplifying the ITS regions have been described by Takabayashi et al. (1998), Meroz-Fine et al. (2003) and Tepper et al. (2013). We plan to sequence the rDNA ITS region of the collected coral, compare the sequences to our

existing DNA sequence data set and construct phylogenetic trees. DNA sequence analysis will be conducted on our LI-COR 4300S DNA Sequencer. My lab has successfully developed the sequencing protocol and its use is now standard protocol. In addition to requesting a student to carry out the DNA work, I am requesting funds to purchase the reagents needed to carry out DNA amplification, cloning and DNA sequence analysis.

STUDENT RESPONSIBILITIES AND DESIRED OUTCOMES

Yasmin Karim Bou Karim has impressed me with her academic potential and scientific interest. She has the background to accomplish the goals explained in this proposal. Yasmin will conduct all aspects of the proposed research.

Student-faculty research provides our undergraduates with a personalized education that promotes intellectual maturation. This interaction fosters discovery-based and active learning and prepares students for the independence required to succeed in today's scientific community. Students who participating in research have a meaningful exposure to the primary literature, have the opportunity to articulate and test hypotheses, and to design experiments to test their hypotheses. Research encourages students to conceptualize and communicate objectives, approaches, analyses, and conclusions. It should also be noted that faculty mentors also stand to benefit from the interaction. The student-faculty interaction is a direct expression of what the mentor values in undergraduate education.

Teaching students that research projects are not complete until they are presented and reviewed by other scientists is an important lesson. This past year, my lab group had one paper published in the journal of Marine Science (Tepper et al., 2013) and another paper accepted in the Proceedings of the 15th Symposium on the Natural History of the Bahamas (Gaynor et al., 2013). My students and I will present our research at the 2013 Cornell College Student Symposium, the McElroy Student/Faculty Research Symposium (May, 2013) and the 15th Symposium of the Natural History of the Bahamas Conference in June, 2013. Yasmin will be expected to present her research findings at the 2014 Cornell College Student Symposium and another relevant molecular biology meeting.

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