GENETIC DIVERSITY IN THE HYDROID *Janaria mirabilis:* POSSIBLE PRESENCE OF CRYPTIC SPECIES

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by

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ABSTRACT

Genetic Diversity in the Hydroid *Janaria mirabilis:* Possible Presence of Cryptic Species.
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Janaria mirabilis (Hydractiniidae, Hydrozoa, Cnidaria) is a calcareous hydrozoan that forms encrusting colonies on gastropod shells. Identifying Hydractiniids using morphology only has been shown to be misleading due to the possible presence of cryptic/sibling species that show little or no morphological diversification despite deep genetic divergence. We analyzed samples of *Janaria mirabilis* collected in Baja California to better characterize its species boundaries and test whether cryptic species are present within its geographic range. We used the 16S mitochondrial gene, which has been tested on other Hydractiniidae species and has been very useful in identifying cryptic and sibling species within Hydrozoa. Our phylogenetic hypothesis shows that the three populations of *Janaria mirabilis* (North Baja, Central Baja, and Pacific) despite morphological differences show no genetic diversity in the mitochondrial 16S gene. Our data show that the genetic differences display no clustering by geography and morphology.

ACKNOWLEDGMENTS

We would like to thank Dr. Sánchez-Ortiz for the collection and shipment of the samples, and Ramiro De Jesus (from Universidad Autónoma de Bajá California Sur) for information on life history, morphology, and ecology of *Janaria*. We would also like to thank Dr. Maria Pia Miglietta for overseeing the project and providing expertise and assistance. Lastly, we would like to thank Texas Sea Grant for generously providing funding.

CHAPTER I

INTRODUCTION

The Hydractiniidae are a family of globally distributed marine hydrozoans (phylum Cnidaria) that comprises 106 nominal species. Most Hydractiniidae live on a variety of living substrates such as crabs, hermit crabs, fishes, and algae (Miglietta & Cunningham, 2012). *Janaria mirabilis* is one of three hydractniid species able to build a calcareous skeleton (Figure 1). Due to its unique morphology, it has been frequently misidentified as a bryozoan or hydrocoral before being re-described and classified as a hydrozoan in 1984 (Cairns & Barnard, 1984). This species has been described from depths ranging from 17 to 65 meters in the marine waters of Panama, Fiji, and Baja California. It lives on gastropod shells inhabited by hermit crabs, and forms encrusting colonies that can invade the internal cavity, or can radiate as branches, elongating the shell (Cairns & Barnard, 1984).

Figure 1: Janaria mirabilis encrusting a gastropod shell inhabited by a hermit crab. Image obtained from Reef.org.

J. mirabilis, the only known species within the genus *Janaria*, has been described and studied from a morphological perspective only, and its genetic diversity has not been extensively studied. Only a handful of sequences belonging to this species have been produced so far (Miglietta and Cunningham, 2012; Miglietta et al. 2009). However, it is known that morphology

may not be sufficient to define species boundaries in Hydrozoa, mostly because genetically distinct species may look morphologically identical (Schuchert, 2014).

Samples of *Janaria mirabilis* from the Gulf of California and the Pacific Ocean were analyzed to understand its species boundaries and test whether cryptic species can be identified within its geographic range. Cryptic species were distinguished using a molecular approach. More specifically, the mitochondrial 16S gene was analyzed. Understanding the taxonomic status of this species provides more insight into the phylogeny of calcified hydroids, as *Janaria* is one of the few extant calcified hydrozoan genera (Miglietta et al. 2010).

The mitochondrial 16S gene has been widely used to determine species boundaries and it is considered the Hydrozoan barcoding molecule. However, the mitochondrial gene alone may not provide enough evidence to support distinct lineages among cryptic species. Support for the same lineage can be confirmed with the nuclear gene EF1α (Schuchert, 2014). This gene has been used in past studies on Hydractiniids and other hydroids to support the mitochondrial 16S phylogenetic hypothesis (Schuchert, 2014). The 16S mitochondrial gene has been used as the first exploratory tool and later we may sequence the EF1α gene to assist in determining whether divergent species exist within the encrusting species *Janaria mirabilis*.

This study is the preliminary step toward assessing calcareous hydroid interactions with their host hermit crabs. Some sessile hydroids show strict host specificity, while others are generalists with regard to their substrate. While it is known that *Janaria* only lives on gastropod shells inhabited by hermit crabs, it is unknown if *Janaria* is specialized to a single host species. The

discovery of cryptic species within *Janaria* would allow for a better understanding of symbiotic relationships between calcareous hydroids and their hosts.

This study focused on obtaining the mitochondrial 16S gene sequences from *Janaria mirabilis* specimens collected in the Baja California region. The sequences were used to build a phylogenetic hypothesis to determine if cryptic species are present within the samples. This is the first step in an extensive study that will help to further understand the evolution of this calcified genus.

CHAPTER II

METHODS

Collection of Samples

A total of 464 samples of *Janaria* were collected from the Gulf of California and the Pacific Ocean by Dr. Sánchez-Ortiz from Universidad Autónoma de Bajá California Sur (Figure 2). The samples were preserved in ethanol and thirty-six were shipped from Mexico to Texas A&M University at Galveston to be analyzed.

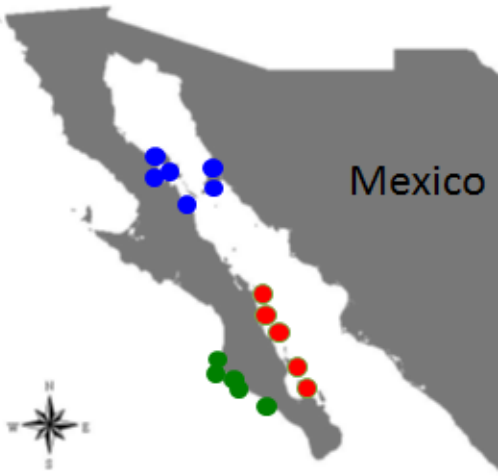

Figure 2: Map of collection sites. Color coded by location. Blue: North, Red: Central, Green: Pacific

Genetic Analysis

Genetic analysis of samples followed the sequencing protocols as used in Miglietta et al. (2009). Total DNA was extracted following the protocol in Miglietta et. al (2009 and 2010). We measured DNA purity and concentration using a NanoDrop™ spectrophotometer. A260/280 and A260/230 values greater than 1.8 were suitable for PCR analysis. Lower A260/280 values may indicate protein contamination and lower A260/230 values indicate contamination with salts or solvents (e.g. phenol). When good quality and quantity DNA was obtained, a 611-bp fragment of the mitochondrial 16S gene was amplified using the polymerase chain reaction (PCR) and primers SHA 5′-ACGGAATGAACTCAAATCATGT-3′ and SHB 5′-TCGACTGTTTACCAAAAACATA-3′(Cunningham & Buss 1993). Amplification took place under the PCR conditions specified in Miglietta et al. (2009). The PCR product was run on a 2%

agarose gel stained with SYBR® Safe DNA to assay the quantity and quality of the product. The PCR product was purified using a mixture of exonuclease I and shrimp alkaline phosphatase (ExoSAP; USB). The purified PCR product was used as a template for double stranded sequencing carried out at the genomics center at Texas A&M University, Corpus Christi.

Phylogenetic Analysis

Phylogenetic analysis was performed according to the protocols conducted by Miglietta et al. (2009). The sequences were first assembled and edited using the software Geneious 6.1.6 (Biomatters). They were then aligned using MUSCLE as implemented in Geneious 6.1.6. Phylogenetic analysis of the aligned sequences was performed using the maximum parsimony optimality criterion in PAUP*4b10-x86-macosx (Swofford 2001) and the maximum likelihood (ML) optimality criterion in GARLI v0.951.OsX-GUI (Zwickl 2006) and PhyML as implemented in TOPALi (Version 2.5 Biomathematics and Statistics Scotland). Bayesian analysis was performed in MrBayes as implemented in TOPALi2 (version 2.5). Clade stability was assessed by ML bootstrap analyses (Felsenstein 1985) in GARLI (100 bootstrap replicates) and by Mr Bayes. The ML analysis in GARLI was performed using random starting trees and default termination conditions. Best fit models in Mr. Bayes and PhyML were calculated in TOPALi 2.5. Genetic distances (p distances and base pair distances) were measured using the software MEGA 5.2.2.

CHAPTER III

RESULTS

Table 1: Samples and corresponding locality for which DNA extraction was performed. Column 5 and 6 show whether PCR and/or sequencing were successful.

Sample ID	Region	Locality	Extraction	PCR	Sequencing
J-1-1	North Baja California	Isla patos	Yes	X	X
J-1-1	North Baja California	Isla patos	Yes	X	X
J-1-2	North Baja California	Isla patos	Yes	X	X
J-1-2	North Baja California	Isla patos	Yes	X	X
J-1-3	North Baja California	Isla patos	Yes	X	X
J-1-3	North Baja California	Isla patos	Yes	X	X
J-2-1	North Baja California	Isla Tiburon	Yes	X	X
J-2-1	North Baja California	Isla Tiburon	Yes	X	X
J-2-2	North Baja California	Isla Tiburon	Yes	X	X
J-2-2	North Baja California	Isla Tiburon	Yes	X	X
J-2-3	North Baja California	Isla Tiburon	Yes	X	X
J-2-3	North Baja California	Isla Tiburon	Yes	X	X
J-3-1	North Baja California	Angel de la Guarda	Yes	X	X
J-3-1	North Baja California	Angel de la Guarda	Yes	X	X
J-3-2	North Baja California	Angel de la Guarda	Yes	X	X
J-3-2	North Baja California	Angel de la Guarda	Yes	X	X
J-3-3	North Baja California	Angel de la Guarda	Yes	X	X
J-3-3	North Baja California	Angel de la Guarda	Yes	X	X
J-4-1	North Baja California	Bahia de los Angeles	Yes	✓	X
J-4-1	North Baja California	Bahia de los Angeles	Yes	✓	X
J-4-2	North Baja California	Bahia de los Angeles	Yes	✓	X
J-4-2	North Baja California	Bahia de los Angeles	Yes	✓	X
J-4-3	North Baja California	Bahia de los Angeles	Yes	✓	X
J-4-3	North Baja California	Bahia de los Angeles	Yes	✓	X
J-5-1	North Baja California	San francisquito	Yes	✓	X
J-5-1	North Baja California	San francisquito	Yes	✓	X
J-5-2	North Baja California	San francisquito	Yes	X	X
J-5-3	North Baja California	San francisquito	Yes	X	X
J-6-1	Central Baja California	Isla Coronado	Yes	✓	✓
J-6-2	Central Baja California	Isla Coronado	Yes	✓	✓
J-6-3	Central Baja California	Isla Coronado	Yes	✓	✓

Table 1: Continued

J-7-1	Central Baja California	Isla Danzante	Yes	✓	✓
J-8-1	Central Baja California	Isla Monserrat	Yes	X	X
J-8-1	Central Baja California	Isla Monserrat	Yes	X	X
J-9-1	Central Baja California	Los Islotes	Yes	✓	✓
J-9-2	Central Baja California	Los Islotes	Yes	X	X
J-9-2	Central Baja California	Los Islotes	Yes	X	X
J-9-3	Central Baja California	Los Islotes	Yes	✓	✓
J-10-1	Central Baja California	Isla la Ballena	Yes	✓	✓
J-10-2	Central Baja California	Isla la Ballena	Yes	✓	✓
J-10-3	Central Baja California	Isla la Ballena	Yes	✓	✓
J-11-1	Pacific Baja California	Cabo san Lazaro	Yes	X	X
J-11-1	Pacific Baja California	Cabo san Lazaro	Yes	✓	X
J-12-1	Pacific Baja California	Magdalena- La bocana	Yes	✓	✓
J-12-2	Pacific Baja California	Magdalena- La bocana	Yes	✓	✓
J-12-3	Pacific Baja California	Magdalena- La bocana	Yes	✓	✓
J-13-1	Pacific Baja California	Magdalena- Punta prieta	Yes	✓	✓
J-13-2	Pacific Baja California	Magdalena- Punta prieta	Yes	✓	✓
J-13-3	Pacific Baja California	Magdalena- Punta prieta	Yes	✓	✓
J-14-1	Pacific Baja California	Conquista agraria	Yes	✓	✓
J-14-2	Pacific Baja California	Conquista agraria	Yes	X	X
J-14-2	Pacific Baja California	Conquista agraria	Yes	X	X
J-14-2	Pacific Baja California	Conquista agraria	Yes	✓	✓
J-14-3	Pacific Baja California	Conquista agraria	Yes	✓	✓

We analyzed 24 sequences (22 belonging to *Janaria mirabilis*, and two outgroups *Hydrissa sodalis* and *Hydractinia mirabilis*) (Table 1). All new sequences will be deposited in Genbank and made available to the public at the completion of the project.

The alignment length was 631bp, and the number of phylogenetic informative sites was 88 (13.95%). Trees built with RaxML, PhyML, and MrBayes showed the same topology. ML phylogenetic hypothesis is shown in Figure 3. *Janaria mirabilis* resulted monophyletic with a bootstrap support of 100 (and Bayesian Posterior Probability of 100). Phylogenetic analysis

shows no phylogenetic clustering by geography with respect to the three main collecting zones in the Gulf of California and the Pacific Ocean (North, Central and Pacific), or the sampling site. *J. mirabilis* 4-2N from Baha de los Angeles shows a long branch. Also, specimens 11-1P, 12-1P, 12-2P, 12-3P and 13-3P form a monophyletic clade albeit with very low ML bootstrap support and Bayesian posterior probability. Genetic distances between these two groups and all the rest of the sequences were calculated in Mega and are shown in Table 2.

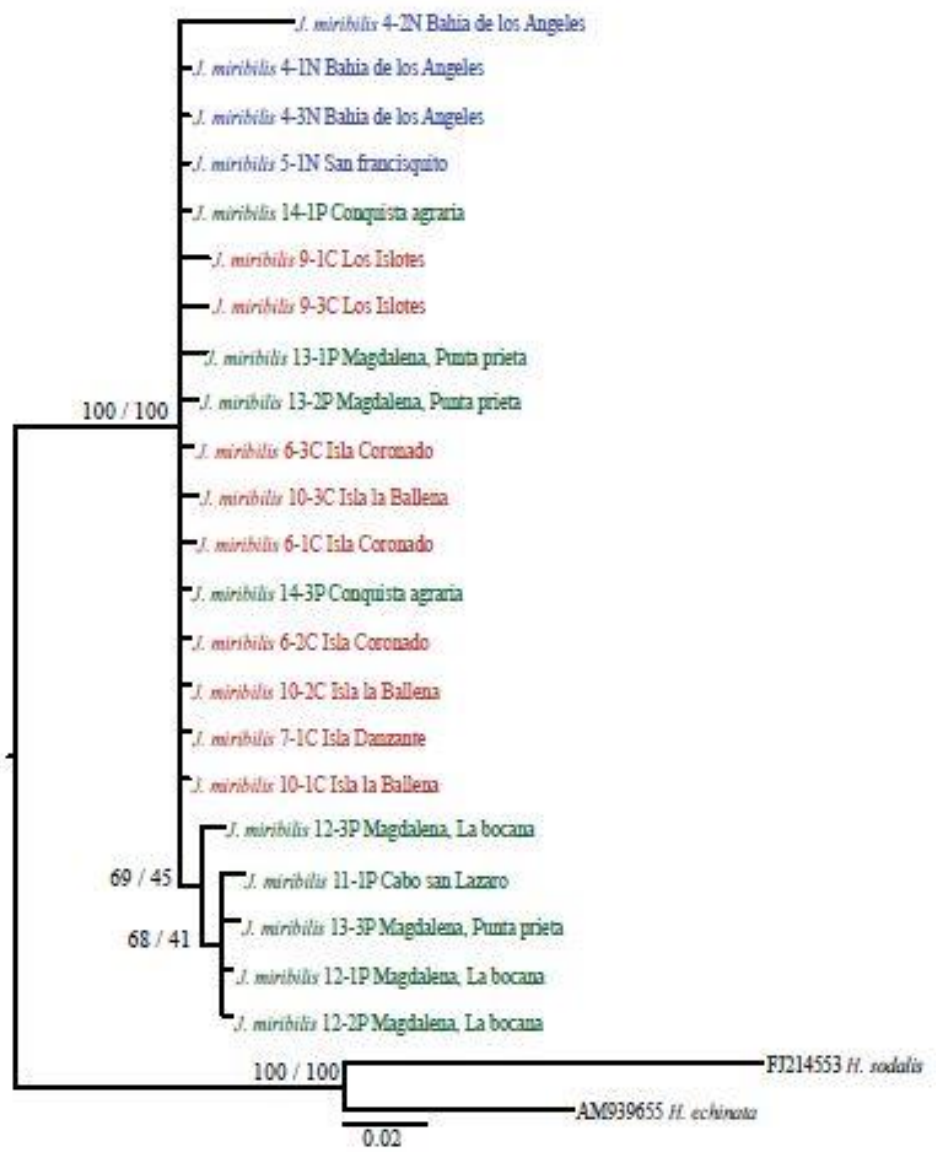

Figure 3: Maximum likelihood of phylogenetic hypothesis based on partial 16S mitochondrial gene (c. 600 bp) containing all the sequences. The branch length indicator represents 0.1 substitutions per site. Members of the family Hydractiniidae (Hydractinia echinata and Hydrissa sodalis) that were used as outgroup. Geographic regions are color coded (North Baja California, Central Baja California and Pacific). Bayesian posterior probability/ML bootstrap values are indicated at each node.

Table 2: p distances (and base pairs distances) between divergent lineages as identified in Figure 3.

Groups	[1]	[2]	[3]
[1] *J. mirabilis* (4-2N)	---	---	---
[2] *J. mirabilis* (all other samples)	1.935 % (11.625)	---	---
[3] *J. mirabilis* (11-1P, 12-1P, 12-2P, 12-3P, 13-3P)	2.060% (12.2)	.484% (2.875)	---

CHAPTER IV

DISCUSSION

As described in the Introduction, gene phylogenies using the mitochondrial 16S gene have been shown to reliably identify reciprocally monophyletic clades in the Hydrozoa (Miglietta et al. 2007; Miglietta & Lessios 2009; Moura et al. 2008). Although there seems to be some genetic structure, more data is necessary to test whether well supported monophyletic clades are present within *J. mirabilis*. Interestingly, one individual colony from Bahia de los Angeles in the Northern Gulf of California (*Janaria* 4-2N) displays a long branch (11.625 bp difference and 1.935% difference) (Figure 3, Table 2). Unfortunately, the small sample size does not allow speculation on whether this represents a separate monophyletic clade. More sequences are necessary to draw any conclusion regarding the specific status of this individual colony.

A morphological analysis of the colonies is ongoing at the Universidad Autónoma de Bajá California Sur and has shown great morphological variation within the species (De Jesus unpublished). Specifically, there is a distinct morphotype associated with each of the three regions. The samples from the Pacific have consistently larger colonies with stronger, wider septa than the colonies collected from the Gulf of California (De Jesus unpublished). Our data supports the hypothesis that the morphological differences associated to the three regions are due to intraspecific diversity and not to the presence of cryptic species. A morphological and genetic analysis of the hermit crab host (genus *Manucomplanus)* is ongoing and may better clarify the nature of the interaction between *Janaria* and its host.

Some problems were encountered during this study. DNA extraction of some specimens yielded low quality and low quantity DNA that was not successfully amplified by PCR (Table 1). On these problematic samples, DNA extraction was repeated twice, yielding consistently low quality DNA. These specimens were lighter in color and their calcified skeleton was softer, which may indicate that these colonies where not healthy, or were already dead with no or little live tissue.

We hope to obtain additional samples from Dr. Sánchez-Ortiz in the close future and obtain more 16S sequences. The nuclear EF1α gene may be amplified and sequenced if necessary. Once enough samples have been sequenced, a comparison between the *Janaria* phylogenetic hypothesis and the one obtained for the hermit crab will be conducted. This will better clarify the interaction between these two symbiotic species and the specificity of their interaction.

Finally, the Miglietta laboratory will try to obtain further specimens from the other localities in which *Janaria* has been reported, such as Panama and Fiji. This will establish a more complete picture of the phylogenetic status of *Janaria mirabilis* from all its geographic range.

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