EVALUATION OF THE CO-EVOLUTION AND GENETIC DIVERSITY OF THE STAG-HORNED HYDROCORAL AND ITS HERMIT CRAB HOST FROM THE PACIFIC AND GULF OF CALIFORNIA

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Evaluation of the Co-Evolution and Genetic Diversity of the Stag-Horned Hydrocoral and its Hermit Crab Host from the Pacific and gulf of California

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The aim of this project was to investigate the evolution and genetic diversity of populations of the hydrozoan *Janaria mirabilis* (Cnidaria, Hydrozoa: Hydractiniidae) and its hermit crab host *Manucomplanus varians* (Crustacea: Paguridae). Despite being popular ornamental species among marine aquarium enthusiasts, little is known about the general biology and ecology of the stag-horned hermit crab and its hydrozoan symbiont. In this study, samples of *J. mirabilis* and *M. varians* were collected from Pacific Baja California, Gulf of California (GoC), and acquired from the marine aquarium trade to be evaluated for species differentiation as well as species host specificity. The mitochondrial 16S ribosomal gene data was collected for both *M. varians* and *J. mirabilis*, with mitochondrial COI and nuclear Elongation Factor 1α data collected for *M. varians* and *J. mirabilis*, respectively. These genes were used to: a) assess the evolutionary relationships and genetic diversity among sampled populations of *J. mirabilis* and *M. varians*; b) evaluate for the presence of cryptic species; c) confirm if host specificity is consistently found between *J. mirabilis* and *M. varians*; and d) determine if observed

morphological differences between GoC and Pacific populations are consistent with genetic differentiation.

ACKNOWLEDGEMENTS

I would like to thank Dr. Sánche-Ortiz for the collection and shipment of the samples, and Ramiro De Jesus (from Universidad Autónoma de Bajá Californa Sur) for information on life history, morphology, and ecology of *Janaria* and *Manucomplanus*. I would also like to thank both Dr. Elizabeth Borda and Dr. Maria Pia Miglietta for overseeing the project and providing expertise and assistance and Yui Matsumoto for providing assistance and being there for me through the entirety of this project. I would also like to thank Katie Gillis, Iris Cato, and Taliese Laverne for their large contribution in the collection of molecular data at the beginning of the project. Lastly, I would like to thank Texas Sea Grant, LAUNCH, and Aggies Commit to Excellence Scholars programs for generously providing funding.

CHAPTER I

INTRODUCTION

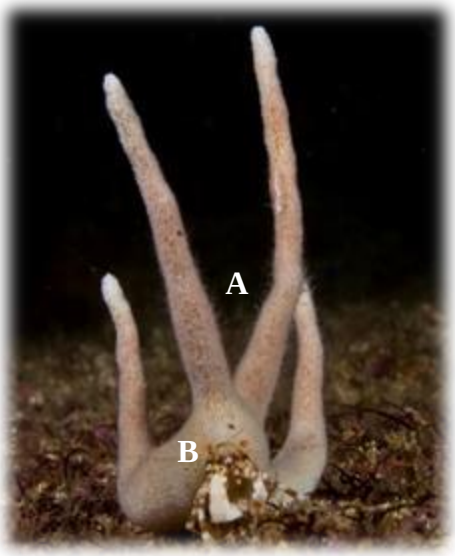


Figure 1. Stag-horned hermit crab, showing *J. miriabilis* (hydroid; A) and *M. varians* (hermit crab; B). Image. C. Sanchez

Hydractiniidae is one of three hydrozoan (Cnidaria) families that produce a calcium carbonate skeleton. *Janaria mirabilis* Stechow, 1921 (Fig. 1A) is monotypic and one of three hydractiniid species that are calcified (Miglietta, McNally, and Cunningham, 2010; Cairns and Barnard, 1984). This species is thought to only be associated with the hermit crab species, *Manucomplanus varians* Benedict, 1892 (Fig. 1B), and forms a calcareous pseudo shell that houses the crab (Miglietta and Cunningham, 2012). Hermit crabs are decapod crustaceans (Paguridae) that require protection from predators due to their non-calcified abdomens, and typically inhabit empty gastropod shells or hollowed-out substrates. Members of the genus *Manucomplanus* Lemaitre and McLaughlin, 1981 are unique in being reported to have strict associations with bryozoans and/or hydrozoans (Letmaire and McLaughlin, 1996). Together, *M. varians* and *J. mirabilis* have a distribution in the Gulf of California (GoC) and along the eastern

Pacific from the Baja California Peninsula to Panama and the Galapagos Islands, with collection records from depths ranging 7-717m (Cairns and Bernard, 1984; Lemaitre and McLaughlin, 1996). Distribution ranges reported in live aquaria sites report a distribution as distant as the IndoPacific (www.bluezooaquatics.com), however this is not verified by the literature.

The stag-horned hermit crab, and its symbiont, is valued by aquarium enthusiasts as an ornamental species selling at an average of $30.00 per crab (Calado et al., 2003). Like many other decapods that receive the ornamental status, they are valued due to their hardiness in captivity and by being suitable tank habitants for other aquatic organisms. Traditionally, the main focus of conservation and management efforts of decapod crustaceans have entirely focused on crustacean fisheries for human consumption. However, species in the pet trade are fetching high market prices in the aquarium industry. Because of this, it is vital that the fishing pressure caused by the harvest of wild populations be regulated to a sustainable level in order to develop proper culture technologies (Calado et al., 2003). Thus, the stag-horned hermit crab is a target species in need of study to provide insights into their genetic population structure, as well as their general biology and ecology to enable better management and conservation efforts. The association between the two species is poorly understood due to limited research focusing on the biology and ecology of both species, which has strong potential in providing insights to promote proper conservation efforts. The association between the two species is poorly understood due to limited research focusing on the biology and ecology, which has strong potential in providing insights to promote proper conservation efforts.

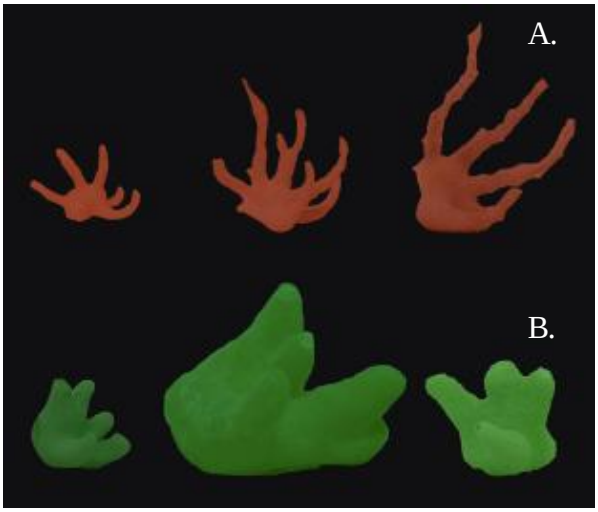


Figure. 2. Morphological variation in *J. miriabilis*. A. Central Gulf of California; B. Pacific, off Baja California. Image. R. de Jesus

Both *J. mirabilis* and *M. varians* have mainly been evaluated from a morphological perspective (Cairns and Bernard, 1984; Lemaitre and McLaughlin, 1996), with only one higher-level molecular phylogenetic study each including a representative of *J. mirabilis* (Miglietta, Schuchert, and Cunningham, 2009) and a single representative of the genus *Manucomplanus* (Bracken-Grissom, et al., 2013); with no molecular studies that have evaluated their association together from a genetic perspective. Morphological variation in *J. mirabilis* (e.g., Fig. 2) and *M. varians* has been observed between populations in the GoC and eastern Pacific Ocean (de Jesus Arcos Aguilar, et al., In prep). However, it is unclear whether these differences are due to intraspecific diversity due to variation in ecological and environmental factors or interspecific diversity due to speciation. Some hydroids demonstrate strict host specificity while others are generalists (Miglietta and Cunningham, 2012). Thus the discovery of cryptic *Manucomplanus* and/or *Janaria* species could provide new insights into the evolutionary association of these symbiotic species and characterization of species populations. Therefore, goals of this study were to: a) assess the evolutionary relationships and genetic diversity among sampled populations of *J. mirabilis* and *M. varians*; b) evaluate for the presence of cryptic species; c) confirm if host specificity is consistently found between *J. mirabilis* and *M. varians*; and d) determine if

observed morphological differences between GoC and Pacific populations are consistent with genetic differentiation.

CHAPTER II

METHODOLGY

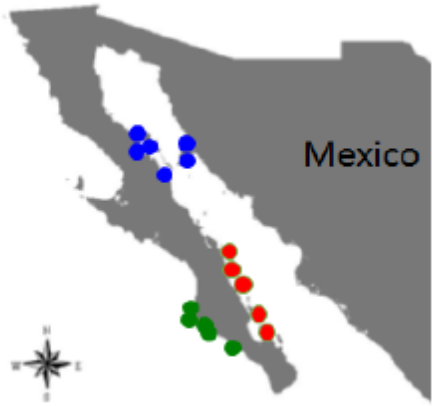


Figure 3. Map of collection sites. Color coded by location. Blue: North, Red: Central, Green: Pacific

A total of 31 *M. varians* samples and 24 *Janaria* plus *Manucomplanus* samples were collected from the north and central GoC and the eastern Pacific Ocean, off the coast of the Baja California (Figure 3), by Dr. Carlos Sánchez-Ortiz (Universidad Autónoma de Bajá California Sur) and preserved in ethanol. Additional representatives were purchased from liveaquaira.com, which indicated a distribution from the IndoPacific. These samples were used for DNA extraction and amplification of the 16S and EF1 α genes. Five additional samples were purchased online from liveaquaria.com for sampling.

Genetic Analysis

DNA was extracted from samples of both species following the protocols in Miglietta et al. (2009; 2010). For *Janaria*, mitochondrial 16S (~611 bp) and the nuclear Elongation Factor 1α (~231 bp; EF 1α) genes and for *Manucomplanus*, mitochondrial COI (~700 bp) and 16S (~450 bp) were collected for genetic analyses. Primers and annealing temperatures used for

amplification the four genes can be found in Table 1. The PCR protocol for 16S and EF1α can be found in Miglietta et. al 2009 and the PCR protocol for CO1 can be found in the reference in the table.

Table 1. PCR primers, annealing temperatures, and PCR protocols for the four genes being tested.

Gene	Primer	Annealing Temperature	Reference
Janaria			
16S	SHA 5'- TCGACTGTTACCAAAAACATA-3' SHB 5'- ACGGAATGAACTCAAATCATGT-3'	50°C	Cunningham and Buss, 1993
EF1α	EF1B 5'-TGGTATGGTTGCCTCTGACA-3' EF2B 5'-ACAGCGAAACGTCCTAATGG-3'	50°C	Linder et al., 2008
Manucomplanus			
16S	arL 5' - CGCCTGTTTATCAAAAACAT-3' brH 5'- CCGGTCTGAACTCAGATCACGT-3'	50°C	Linder et al., 2008
CO1	LCO1 5'-GGTCAACAATCATAAAGATATTGG-3' HCO2 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	72°C	Folmer et al., 1994

Prior to PCR, DNA purity and concentration were measured using a NanoDrop™ spectrophotometer. An A260/280 value of around 1.8 ± 0.6 and a A260/230 value of around 2.0 to 2.2 ± 0.6 were considered suitable for PCR analysis. Values closer to the 1.8 and 2.0 were preferred but samples with values with a 0.6 difference still yielded results. Lower A260/280 values could indicate protein contamination and lower A260/230 values could indicate a general contamination of salts or other solvents. If DNA concentration exceeded 100 ng/µl, the extract was diluted using nuclease free sterile water before performing PCR.

A standard PCR master mix was used for both genes and consisted of 12.5µl GreenTaq® (Promega), 9.5µl nuclease free sterile water, and 1µl each of forward and reverse primer and 1µl of DNA. PCR products were imaged on a 1% TBE agarose gel pre-stained with SYBR® Safe DNA to assay the quantity and quality of the product. The PCR product was then purified using ExoSAP-It (Affymetrix) and used as a template for Sanger Sequencing at the Texas A&M University Corpus Christi Genomics Core Lab.

Phylogenetic Analysis

Phylogenetic analyses were performed according to the protocols conducted by Miglietta et. al. 2009. Sequences were assembled, edited, and aligned using MUSCLE as implemented Geneious 6.1.6 (Biomatters). Phylogenetic analyses of the aligned sequences were performed using the maximum likelihood optimality criterion in GARLI v0.951.OsX-GUI (Zwickl 2006). Clade stability was assessed by the ML bootstrap analyses (Felsenstein 1985) in GARLI (100 bootstrap replicates). The ML analyses in GARLI were performed using random starting trees and default termination conditions. Pairwise genetic distances were calculated in MEGA 5.2 (Tamura et al. 2011) using the Kimura 2 Parameter model of evolution.

CHAPTER III

RESULTS

For *Manucomplanus*, 61 samples were used for genetic analysis for COI and 16S, 39 of which had a *Janaria* counterpart (Appendix A). Extracts and/or dilutions were present for all specimens. Successful amplifications were denoted with a green box and failed amplifications were denoted with a red box. Successful amplifications were sent off for sequencing. Results for 16S were not reported in this study due to a protocol error by the TAMUCC Genomics Lab.

For *Janaria*, 41 samples were used for genetic analysis for 16S and EF1α, 39 of which had a *Manucomplanus* counterpart (Appendix B). Extracts and/or dilutions were present for all specimens. PCR was performed on all samples. Those with successful amplifications were denoted with a green box and failed amplifications were denoted with a red box. Those samples that showed successful amplification were sent off for sequencing. Currently EF1 α amplifications have not yielded results for the sequencing step due to possible protocol error by TAMUCC Genomics Core. Trouble-shooting is still underway.

The phylogenetic relationships of *Manucomplanus* (Appendix C) was assessed based on COI data alone. All representatives of *Manucomplanus* were found to be monophyletic (boot=100%), with a subset of the Pacific samples all formed a "Pacific" clade, with the exception of one outlier (P-M13-3). All GoC samples were found in a sister clade with the exception of 3 outliers, which were nested in the "Pacific" clade. Within the Pacific clade, tow GoC individuals (SC11-01-02; SC09-0729) were highly supported (boot=84%) as nested among the majority of the Pacific only representatives. Pairwise genetic distances among the different

localities for the *Manucomplanus* estimated for COI (Table 2) indicated that genetic distances between the two clades and among groups did not exceed 3%.

Table 2: Pairwise genetic (K2P) distances for COI among the *Manucomplanus* groups based on general collection localities.

	COI Gene	1	2	3	4
1.	Manucomplanus (Pacific)	--			
2.	Manucomplanus (Pacific Outliers)	3.0%			
3.	Manucomplanus (GoC)	2.9%	0.8%		
4.	Manucomplanus (GoC Outliers)	0.5%	3.0%	2.9%	--

The phylogenetic relationships of *Janaria* representatives were based on 16S (Appendix D). All *Janaria* were monophyletic (boot=100%), with a subset of Pacific representatives forming a low supported clade (boot=60%), however, three of the "IndoPacific" represented were strongly supported as a subclade (boot=98%), and nested among GoC and Pacific outliers.. Pairwise genetic distances among the different localities for the *Janaria* estimated for16S (Table 2) indicated that genetic distances among populations groups did not exceed 1%.

Table 3: Pairwise genetic (K2P) distances for 16S among the *Janaria* groups based on general collection localities.

	16S Gene	1	2	3	4	5
1.	Janaria (IndoPacific)	--				
2.	Janaria (Pacific)	1.0%	--			
3.	Janaria (Pacific Outliers)	0.7%	0.6%	--		
4.	Janaria (GoC)	0.8%	0.6%	0.4%	--	
5.	Outgroup	8.9%	9.2%	8.7%	8.9%	--

CHAPTER IV

CONCLUSION

The overall goal of this project was to compare the genetic diversity and population structure of both *Manucomplanus varians* and *Janaria mirabilis* in an effort to improve our knowledge on these unique and poorly studied symbiotic invertebrates. Both have been evaluated separately in the literature (REFS), however, together to infer their co-evolutionary relationship. A striking feature are the morphological differences observed in the general morphology of *Janaria* and *Manucomplanus* populations collected from the Pacific Baja California Peninsula and those from the GoC. The analysis of genetic data now provides new insights to better understanding the evolutionary patterns associated with both species.

Evolutionary relationships and genetic diversity of J. mirabilis

The results from the 16S gene data (Appendix D), highlighted two main cluster of Pacific and "IndoPacific" representatives, with several outliers from both groups found nested among GoC representatives. The currently available data does not support there to be clear differentiation among populations, other than the sub clades, to indicate separate species either based on morphology or locality. From an ecological standpoint, Pacific representatives have a more robust morphology likely adapted to the harsher environment (e.g. stronger currents, wave impact) in the Pacific side of the Baja California Peninsula.

Evolutionary relationships and genetic diversity of M. varians

Among the *Manucomplanus* samples there is support for some genetic differentiation, with Pacific samples forming clade that is distinct from their GoC counterparts (Appendix C).

However, there was one Pacific outlier nested among the GoC populations and at least three GoC representatives nested with those from the Pacific. This subtle difference in genetic sequences between the Pacific populations and the GoC populations for the *Manucomplanus* slightly coincides with their *Janaria* counterparts; through a more defined populations structure was observed, with strong support for two *M. varians* subclades roughly defined geographically. Several outliers were observed in both clades possibly indicating translocation associated with the aquarium clade (morphologically of Janaria symbiont was not recorded for GoC outliers). Additionally, data and specimens are needed to further explore the genetic connectivity between the two regions.

Presence or absence of cryptic species

Based on the results of Manucomplanus and *Janaria*, it is clear that there are no cryptic species present within and among populations. However, COI genetic distances for *Manucomplanus* indicate that some genetic differentiation, which will need additional exploration with the 16S and nuclear gene data. The differentiation was not as clearly observed in the 16S data for *Janaria*, with overall genetic distances being less than 1% among populations.

Host specificity between J. mirabilis and M. varians

All representatives of *Janaria* and *Manucomplanus*, each were found to be genetically similar and monophyletic, thus there was no evidence to support the presence of other species of *Manucomplanus*, or other pagurid, to be associated with *Janaria*; and vice versa.

Consistency between observed morphological differences between GoC and Pacific populations with genetic differentiation

The general morphology of the Pacific, including IndoPacific, and GoC populations, indicated that the Pacific representatives were generally larger and stockier in build for both *Manucomplanus* and *Janaria*, while those from the GoC tend to be smaller, slender build (de Jesus Arcos Aguilar, et al., In prep). There was some correspondence for this in *Manucomplanus* with the genetic data because the representatives from the Pacific and GoC generally formed their own clade, however this was less clear for *Janaria* because all of the representatives fell within the same clade indicating little genetic differentiation.

Future work

The future aim for this project will be to publish these results for this study in conjunction with a broader ecological study of the stag-horned hermit crab (de Jesus Arcos Aguilar, et al., In prep), including demographic and morphological data. The next step for the current genetic project will be to finalize the data collection of nuclear EF1α (*Janaria*) and Internal Transcribed Spacer (*Manucomplanus*) and mitochondrial 16S data for *Manucomplanus*. Additionally, the next steps for the Manucomplanus will be to collect COI data for the samples with the corresponding Janaria individuals, re-sequence the failed 16S gene, and target the ITS gene for comparison. The next step for the Janaria would be to re-sequence the EF1α and adding missing 16S data for all samples. Once all genes are sequenced, new analyses will be conducted, in order to provide more definitive conclusions in association with the ecological data.

REFERENCES

Benedict, J. E. (1892). Preliminary description of thirty-seven new species of hermit crabs of the genus Eupagurus in the U.S National Museum.

Bracken-Grissom, H. D., Cannon, M. E., Cabezas, P., Feldman, R. M., Schweitzer, C. E., Ahyong, S. T., Felder, D. L.; Lemaitre, R., Crandall, K. A. (2013). A comprehensive and integrative reconstruction of evolutionary history for Anomura (Crustacea: Decapoda). *Evolutionary Biology 13(128).*

Cairns, S. D., and Barnard, J. L. (1984). Redescription of *Janaria mirabilis*, a Calcified Hydroid from the Eastern Pacific. *Southern California Academy of Sciences*, 1-11.

Calado, R., Lin, J., Rhyne, A. L., Araugo, R., and Narciso, L. (2003). Marine Ornamental Decapods – Popular, Pricey, and Poorly Studied. *Journal of Crustacean Biology 23(4)*, 963-973.

Chu, K. H., Li, C. P., & Ho, H. Y. (2001). The first internal transcribed spacer (ITS-1) of ribosomal DNA as a molecular marker for phylogenetic and population analyses in Crustacea. *Marine Biotechnology*, *3*(4), 355-361.

Folmer 0., Black M., Hoeh W., Lutz R., and Vrijenhoek R. (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* (1994) 3(5), 294-299.

Letmaitre, R., and McLaughlin, P. A. (1996). Revision of *Pylopagurus* and *Tomopagurus* (Crustacea: Decapoda: Paguridae), with the Descriptions of New Genera and Species. Part V. *Anisopagurus* McLaughlin, *Manucomplanus* McLaughlin, and *Protoniopagurus* New Genus. *Bulletin of Marine Science 59(1)*, 89-141.

Lindner A., Cairns S. D., Cunningham C. W. (2008). From Offshore to Onshore: Multiple Origins of Shallow-Water Corals from Deep-Sea Ancestors. *Pols One 3(5)*, 1-6.

Miglietta, M. P., & Cunningham, C. W. (2012). Evolution of Life Cycle, Colony Morphology, and Host Specificity in the Family Hydractiniidae (Hydrozoa, Cnidaria). *Evolution 66 (12)*, 3876-3901.

Miglietta, M. P., McNally, L., & Cunningham, C. W. (2010). Evolution of Calcium-carbonate Skeletons in the Hydractiniidae. *Intergatie and Comparative Biology 50(3)*, 428-435.

Miglietta, M. P., Schuchert, P., & Cunningham, C. W. (2009). Reconciling genealogical and morphological speceis in the Hydractiniidae (Cnidaria, Hydrozoa). *Zoologica Scripta 38*, 403-430.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28:2731-2739.

APPENDIX

APPENDIX A

Appendix A: Samples for *Manucomplanus* CO1 and 16S genes with corresponding ID, region of sample, locality of sample, PCR success, and sequencing. Blank spaces indicate that the information is unavailable. The green box denotes success/ presence of, and red box denotes failure/ absence of.

Sample ID	Region	Locality	PCR		Sequencing	
			CO1	16S	CO1	16S
22 SC09-07-29	North					
23 SC09-12-01	Central					
24 SC09-12-01	Central					
25 SC09-12-01	Central					
26 SC10-06-03		La Poma				
27 SC10-06-03		La Poma				
28 SC10-06-04	North					
29 SC10-06-04	North					
30 M-11-1	Pacific					
31 M-12-1	Pacific					
32 M-12-2	Pacific					
33 M-12-3	Pacific					
34 M-13-1	Pacific					
35 M-13-2	Pacific					
36 M-13-3	Pacific					
37 M-14-1	Pacific					
38 M-14-2	Pacific					
39 SC10-06-18	North	Isla Patos				
40 SC10-06-18	North	Isla Patos				
41 SC10-06-18	North	Isla Patos				
42 SC10-07-02	Central	Isla San Jose				
43 SC10-07-02	Central	Isla San Jose				
44 SC10-07-02	Central	Isla San Jose				
45 SC10-07-18	North					
46 SC11-01-02	Central	Is. Coronado				
47 SC11-01-02	Central	Is. Coronado				
48 SC11-01-02	Central	Is. Coronado				
49 SC09-07-10	North					
50 SC09-07-15	North					
51 SC09-07-15	North					
52 SC09-07-29	North					
53 SC09-07-29	North					
54 SC10-06-04	North					
55 SC09-07-37	North					
56 SC09-07-37	North					
57 SC09-07-37	North					
58 SC09-07-37	North					
59 SC09-07-37	North					
60 SC09-07-42	North	Danzante				
61 SC09-07-47	North	Las Animas				
62 M-6-1	Central	Isla Coronado				
63 M-6-2	Central	Isla Coronado				

Appendix A Continued						
64 M-6-3	Central	Isla Coronado				
65 M-7-1	Central	Isla Danzante				
66 M-8-1	Central	Isla Monserrat				
67 M-9-1	Central	Los Islotes				
68 M-9-2	Central	Los Islotes				
69 M-10-1	Central	Isla la Bellena				
70 M-10-2	Central	Isla la Bellena				
71 M-10-3	Central	Isla la Bellena				
72 M-1-1	North	Isla Patos				
73 M-1-2	North	Isla Patos				
74 M-1-3	North	Isla Patos				
75 M-2-1	North	Isla Tiburon				
76 M-2-2	North	Isla Tiburon				
77 M-2-3	North	Isla Tiburon				
78 M-3-1	North	Angel de la Guarda				
79 M-3-2	North	Angel de la Guarda				
80 M-3-3	North	Angel de la Guarda				
81 M-4-1	North	Bahia de los Angeles				
82 M-4-2	North	Bahia de los Angeles				
83 M-4-3	North	Bahia de los Angeles				
84 M-5-1	North	San Francisquito				
85 M-5-2	North	San Francisquito				
86 M-5-3	North	San Francisquito				
89 JmP1	IndoPacific					
90 JmP2	IndoPacific					
91 JmP3	IndoPacific					
92 JmP4	IndoPacific					
93 JmP5	IndoPacific					

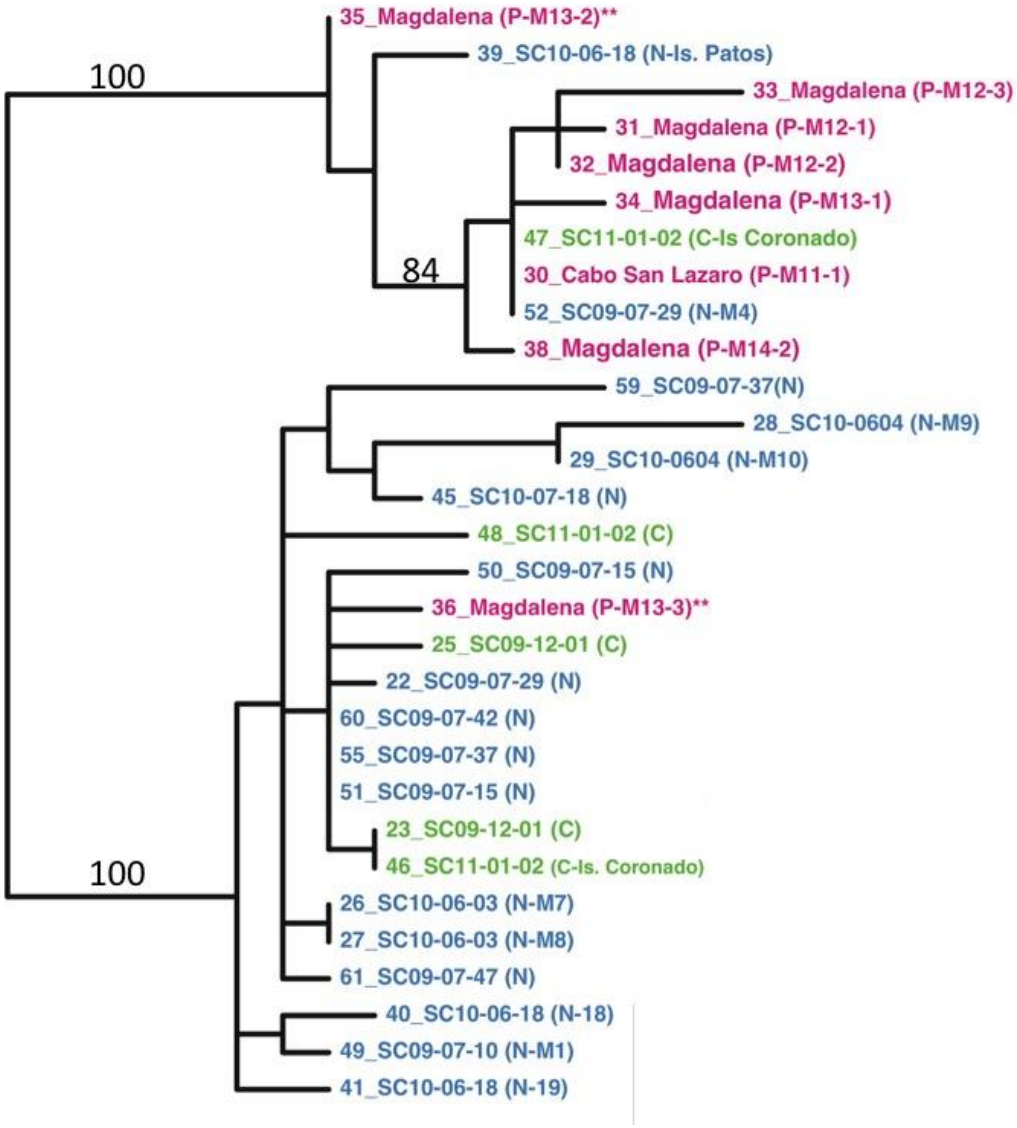
APPENDIX B

Appendix B: Samples for *Janaria* EF1α gene and the 16S gene with corresponding ID, region of sample, locality of sample, PCR success, and sequencing. Blank spaces indicate that the information is unavailable. The green box denotes success/ presence of, and red box denotes failure/ absence of

			PCR		Sequencing	
Sample ID	**Region**	**Locality**	**EF1α**	**16S**	**EF1α**	**16S**
1 J-1-1	Golfo Norte	Punta Suroeste				
2 J-1-2	Golfo Norte	Punta Suroeste				
3 J-1-3	Golfo Norte	Punta Suroeste				
4 J-2-1	Golfo Norte	Punta Oeste				
5 J-2-2	Golfo Norte	Punta Oeste				
6 J-2-3	Golfo Norte	Punta Oeste				
7 J-3-1	Golfo Norte	Islote la Muela				
8 J-3-2	Golfo Norte	Islote la Muela				
9 J-3-3	Golfo Norte	Islote la Muela				
10 J-4-1	Golfo Norte					
11 J-4-2	Golfo Norte					
12 J-4-3	Golfo Norte					
13 J-5-1	Golfo Norte					
14 J-5-2	Golfo Norte					
15 J-5-3	Golfo Norte					
16 J-6-1	Golfo Centro					
17 J-6-2	Golfo Centro					
18 J-6-3	Golfo Centro	Punta Norte				
19 J-7-1	Golfo Centro	El Submarino				
20 J-8-1	Golfo Centro	Punta Sureste				
21 J-9-1	Golfo Centro	Espiritu Santo				
22 J-9-2	Golfo Centro	Espiritu Santo				
23 J-9-3	Golfo Centro	Espiritu Santo				
24 J-10-1	Golfo Centro					
25 J-10-2	Golfo Centro					
26 J-10-3	Golfo Centro					
27 J-11-1	Pacifico					
28 J-12-1	Pacifico	La Bocana				
29 J-12-2	Pacifico	La Bocana				
30 J-12-3	Pacifico	La Bocana				
31 J-13-1	Pacifico	Punta Prieta				
32 J-13-2	Pacifico	Punta Prieta				
33 J-13-3	Pacifico	Punta Prieta				
34 J-14-1	Pacifico	La Ilusion				
35 J-14-2	Pacifico	La Ilusion				
36 J-14-3	Pacifico	La Ilusion				
37 JmP1	IndoPacific					
38 JmP2	IndoPacific					
39 JmP3	IndoPacific					
40 JmP4	IndoPacific					
41 JmP5	IndoPacific					

APPENDIX C

MANUCOMPLANUS CO1 TREE WITH BOOTSTRAP REPICALTES



APPENDIX D

JANARIA 16S TREE WITH BOOTSTRAP REPLICATES