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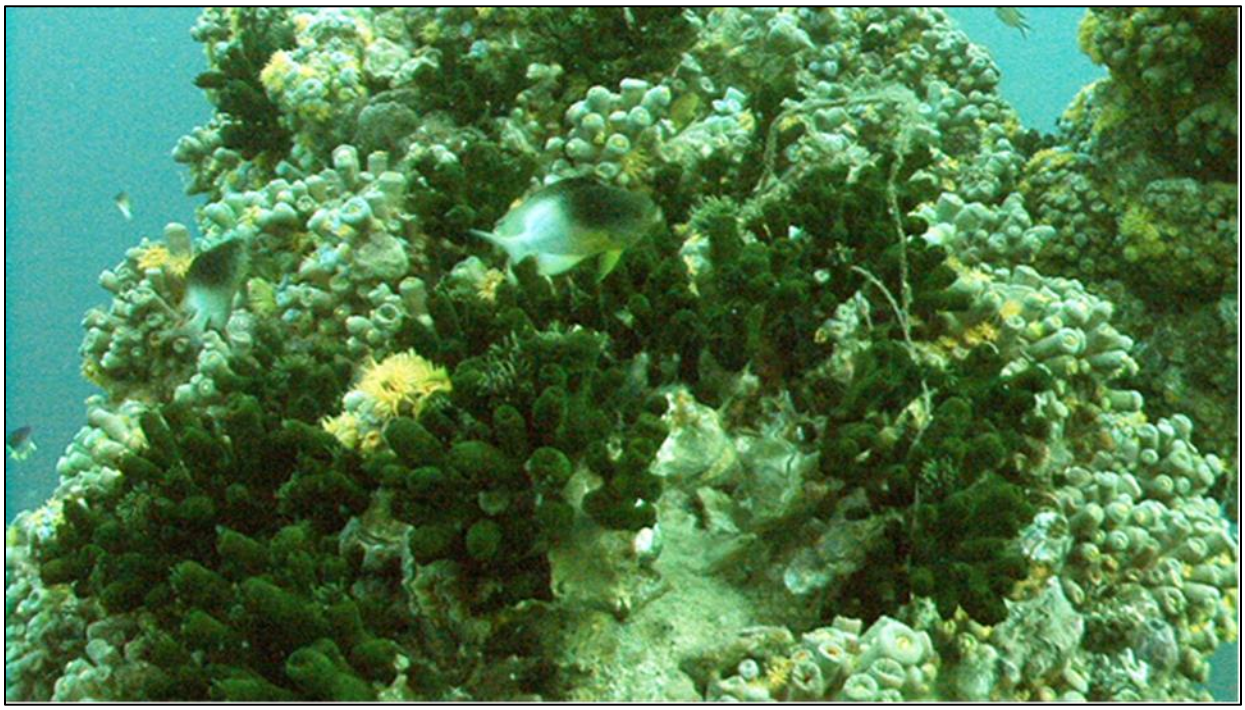
Genetic Affinities in Populations of the Invasive Indo-Pacific Coral *Tubastraea micranthus* on Northern Gulf of Mexico Platforms: Multiple Invasions?



U.S. Department of the Interior
Bureau of Ocean Energy Management
Gulf of Mexico OCS Region

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Genetic Affinities in Populations of the Invasive Indo-Pacific Coral *Tubastraea micranthus* on Northern Gulf of Mexico Platforms: Multiple Invasions?

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ABBREVIATIONS, ACRONYMS, AND SYMBOLS

AFLP	amplified fragment length polymorphism
BOEM	US Department of the Interior, Bureau of Ocean Energy Management
DI	de-ionized
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylene-diamine-tetra-acetic acid
GB	Garden Banks, offshore lease sector in the Gulf of Mexico
GI	Grand Isle, offshore lease sector in the Gulf of Mexico
GOM	Gulf of Mexico
LUMCON	Louisiana Universities Marine Consortium
MC	Mississippi Canyon, offshore lease sector in the Gulf of Mexico
MP	Main Pass, offshore lease sector in the Gulf of Mexico
M/V	motor vessel
PCR	polymerase chain reaction
R/V	research vessel
SED	Salt, EDTA, DMSO buffer
SP	South Pass, offshore lease sector in the Gulf of Mexico
ST	South Timbalier, offshore lease sector in the Gulf of Mexico
STRUCTURE	numerical analysis software for genetic population structure

1.0 INTRODUCTION

1.1 General Background

Before the 1940s, there was little hard substratum in shallow water in the Gulf of Mexico (GOM) between Texas and Florida (Curray, 1965a,b; Frost, 1977; Schroeder et al., 1995; Blum et al., 1998, 2001). Since the 1940s, the US has drilled at least 30,000 oil and gas wells in the northern GOM with 2,232 platforms currently remaining (Bureau of Ocean Energy Management, 2017). These platforms represent islands of hard substratum not previously present in these waters since the Holocene; 14,000–20,000 years ago. New substratum becoming available in the shallow euphotic zone created hard substratum for fauna and flora to settle on, grow, and establish new populations (Gallaway and Lewbel, 1981; Driessen, 1989; Bright et al., 1991; Adams, 1996; Boland, 2002). Among this new community of organisms were hermatypic (reef-building) Caribbean corals (Bright et al., 1991; Boland, 2002). Recent data has shown that as many as 12 coral species, both hermatypic and ahermatypic, native and invasive, have colonized oil and gas platforms in the northern GOM, extending west to east from the Matagorda Island (MI) to the Main Pass (MP) oil and gas lease blocks, and as far south as the Garden Banks (GB) (Sammarco et al., 2004, 2012a).

1.2 Invasive Coral Species in the Western Atlantic

Tubastraea coccinea (Cairns and Zibrowius, 1997) is a species which has invaded the western Atlantic from the Indo-Pacific. Here we define invasive species as any species reported to have become established outside of its native range (Richardson et al., 2000; Rejmánek et al., 2002; Molnar et al., 2008.) The successful establishment of an invasive species has been known to cause shifts in community structure and habitat as well as cause population declines of native plants and animals, because the non-indigenous species competes for the same limited resources as the native species. *T. coccinea* was first recorded in 1943 in Puerto Rico, then in 1948 in Curacao, where they were collected on ship's hulls (Cairns, 2000). By the late 1990s/early 2000s, the species was reported off Belize and Mexico (Fenner, 1999); northern GOM (Sammarco et al., 2004), and the Florida Keys (Shearer, 2008); Brazil (Figueira de Paula and Creed, 2004); Colombia, Panama, the Bahamas, and throughout the Lesser and Greater Antilles (Cairns, 2000; Humann and Deloach, 2002). It is one of the most successful species introductions in the western Atlantic, being the single most abundant scleractinian coral, hermatypic or ahermatypic, in the northern GOM on artificial substrata (Sammarco et al., 2004). Surveys performed on 42 platforms (Sammarco et al., 2012a) revealed that average densities of *T. coccinea* in the northern GOM had reached 28/m^2, with percent cover reaching an average of 50 percent per platform. *T. coccinea* occurs in abundances of hundreds of thousands of colonies per platform (Sammarco et al., 2012a, b). It commonly occurs in shallow-water down to ~35m (Sammarco et al., 2012a) but has been known to occur on deep banks in the northern GOM in lower abundance (Schmahl and Hickerson, 2006).

Even including the case of *T. coccinea* (see above), there have been very few reports of successful introductions of non-indigenous coral species into the Atlantic. One is the introduction of *Fungia scutaria* to Discovery Bay, Jamaica, West Indies (P.W. Sammarco,

personal observation, 1973; Lajeunesse et al., 2005; J. Lang, personal communication, 2013). There are also reports of *T. tagusensis* and *T. coccinea* being introduced into Brazilian waters (Figueira de Paula and Creed, 2004; Silva et al., 2011).

1.3 A New Invasive Coral Species in the Gulf of Mexico: *Tubastraea micranthus*

Beginning in the late 1990s, a series of SCUBA and ROV surveys were completed assessing the distribution and abundance of scleractinian corals on 42 platforms throughout the northern GOM, from Corpus Christi, Texas (MI region) to Mobile, Alabama (MP region). These included standing production platforms and some deep structures redeployed as artificial reefs in accordance with the Bureau of Safety and Environmental Enforcement (BSEE) Rigs-to-Reefs policy (Sammarco et al., 2004, 2012a, 2014; BSEE, 2013). An independent parallel study surveyed an additional 53 platforms (S.A Porter, personal communication, 2009). In combination, these surveys covered 95 platforms in the High Island (HI), West Cameron (WC), East Cameron (EC), MI, Brazos (B), Garden Banks (GB), South Timbalier (ST), Ship Shoals (SS), and MP, and Grand Isle (GI) lease block areas. In addition, some platforms that had been transported onshore for salvage were examined (by S.A. Porter, personal communication, 2015).

In his surveys, Porter found a new invasive species in the GOM– the Indo-Pacific species *Tubastraea micranthus* (Sammarco et al., 2010) at 18-22 m depth on the platform GI-93C ($28^{o}32.96$'N, $90^{o}40.11$'W) near two major safety fairways SW of New Orleans, LA. *T. micranthus* colonies are easily distinguishable from those of *T. coccinea* in the field because of the black or dark olive-green color in the former (Sammarco et al., 2010), compared with bright orange-yellow for *T. coccinea* (Humann and DeLoach, 2002). Both species may be seen side-by-side on the cover photo of this report. In addition, their respective growth habits are different, with the former species being more erect and the latter more stout. Colonies of *T. micranthus* were observed to extend laterally up to linear distances of 3m to > 30 m, covering up to 80 percent of the substratum (S.A. Porter, personal communication, 2007). *T. micranthus* is a good competitor for space with observations of percent cover exceeding the average percent cover of *T. coccinea* by 30 percent on platforms. *T. coccinea* is also a good competitor for space (Hennessey and Sammarco, 2014; Sammarco et al., 2015). The addition of the recent *T. micranthus* invasion could well make it more difficult for native species to establish communities with such limited space, at least in the early stages of succession.

T. micranthus is a robust species and an effective disperser which may have the potential of spreading throughout the northern GOM and possibly the western Atlantic (Sammarco et al., 2012a, b). In a previous study, it was confirmed that this species had colonized 9 out of 14 platforms surveyed in the region south of the Mississippi River mouth - GI-93C, GI-116C, MC-109A, ST-206A, SP-87D, MC-311A, GI-115A, SP-89B, and GI-90, with average densities as high as $15/m^2$ (Sammarco et al., 2014). It is uncertain which platform served as the initial site of colonization. All surveyed platforms to the N, E, and S of GI-93C possessed populations, implying that this platform may have been the first point of invasion. On the other hand, colony sizes of the coral on MC-311A were the largest in size, suggesting that this population may be older and the center of colonization. This species also has a broad depth range. It may be found as shallow as 3–6 m and, in some cases, near the seafloor at 132 m (Sammarco et al., 2013).

Platforms have been perceived as potential "incubators" of, or receptacles for, invasive species (Robinson, 2005). A recent example of this was colonization of platforms by *Didemnum perlucidum*, a highly effective invasive ascidean from the Indo-Pacific (Culbertson and Harper, 2002; Sammarco, 2007). Other recent invasive species of concern in the western Atlantic include *T. coccinea* and *T. tagusensis* in Brazil (Figueira de Paula and Creed, 2004; Silva et al., 2011).

Would it be possible for *T. micranthus* to invade a new environment, such as the northern GOM, multiple times? This question is important because it can affect the control or eradication of the existing invasive populations. If a species such as *T. micranthus* is subject to multiple or repeated invasions from some source in the Indo-Pacific through time, this would make control or eradication difficult (Mack et al., 2000). The only mechanism by which to begin to approach the question of differentiation of two or more populations is through a molecular genetic population study. In recent decades, this approach has been able to assess genetic population differentiation among sites. There are many examples, some of which include differences between populations of deer (e.g., Coulon et al., 2006), fish (e.g., Santos et al., 2003), and echinoids (Hellberg, 2009). These techniques have also been successfully used to differentiate coral populations (Baco and Shank, 2005; Magalon et al., 2005; Underwood et al., 2007).

Thus, several questions arise regarding the expansion of *T. micranthus*. What is the genetic structure of the populations on these platforms? Is there any genetic evidence of within-population breeding occurring, and to what degree? Is the genetic differentiation strong? If so, is it possible that strong genetic differentiation could be indicative of a second introduction of larvae from the Indo-Pacific?

1.4 Molecular Genetic Techniques for Assessing Coral Populations

We have been successful in purifying and analyzing coral DNA without contamination by symbiotic zooxanthellae, in order to apply population genetic analyses to both juvenile and adult coral populations in the northern GOM and the Bahamas (Brazeau et al., 2005; Atchison et al., 2008; Sammarco et al., 2012b) using Amplified Fragment Length Polymorphisms (AFLPs). Zooxanthellae are symbiotic yellow-green or yellow–brown algae occurring in the cytoplasm of scleractinian corals, some radiolarians, and a variety of other marine invertebrates. In the case of hermatypic scleractinian corals, the symbiosis is obligate. AFLP is a DNA-"fingerprinting" technique (Sunnucks, 2000) that detects polymorphisms based upon the selective polymerase chain reaction (PCR) amplification of numerous restriction fragments. These fragments are generated by two different restriction enzymes (Vos et al., 1995; Mueller and Wolfenbarger, 1999) and a subsequent series of selective PCR amplifications that amplify a small subset of the total restriction fragment pool. AFLPs are highly polymorphic but not co-dominantly expressed. The significance of AFLPs <u>not</u> exhibiting co-dominance is that other genetic markers (e.g., microsatellites) that exhibit co-dominance contain approximately twice the information per marker as AFLPs. This advantage is offset by the ease of generating many more AFLP markers than other marker systems. In addition, data analysis may require a different approach when utilizing genetic markers that exhibit dominance.

AFLPs were originally used primarily in agricultural or for commercially valuable species, and until recently, not commonly on animals (Bensch and Akesson, 2005). They have become more

popular, however, within the past decade and applied to many animal species. They have been used successfully to estimate migration rates (He et al., 2004), species boundaries (Lopez et al., 1999; Fukami et al., 2004), and degree of parental contributions to populations (Van Toai et al., 1997). AFLPs perform quite well for population assignment or allocation studies (Mueller and Wolfenbarger, 1999; Blears et al., 1998; Brazeau et al., 2005, 2008, 2011), where the number of polymorphic loci surpasses allelic diversity in importance (Duchesne and Bernatchez, 2002).

1.5 Objectives

The objectives of this study are

1) To examine the possibility of whether a single or multiple introduction of this species may have occurred on the study platforms; and,
2) To determine how strongly differentiated the study populations are on the sampled target platforms; that is, how closely or distantly related they are to each other.
3) To examine the genetic structure of the sampled populations with respect to sexual and asexual reproduction.

2.0 MATERIALS AND METHODS

2.1 Study Sites

Six offshore platforms in the vicinity of the Mississippi River mouth were visited to collect colonies of *T. micranthus*. These were GI-93C (28.548886N; -90.068677W), GI-116A (28.30920000N; -90.07054334W), MC-280A (28.39760740N; -89.09465600W), MC-109A (28.86467N; -88.93079667W), SP-87D (28.720222N; -89.430861W), and ST-206A (28.429881N; -90.308155W); (Figure 1). MC-109A only yielded one sample, which was insufficient for statistical analysis. Platforms in the study area that were previously documented to possess *T. micranthus* were, along with their depths of occurrence, derived from earlier studies (Sammarco et al., 2013). Both GI-93C and MC-280A had substantial populations within SCUBA diving depths ($\leq$ 33m), primarily between 14 and 23 m. Populations occurred down to 66 m on GI-93C. Depth-distribution data were not available for MC-280A.

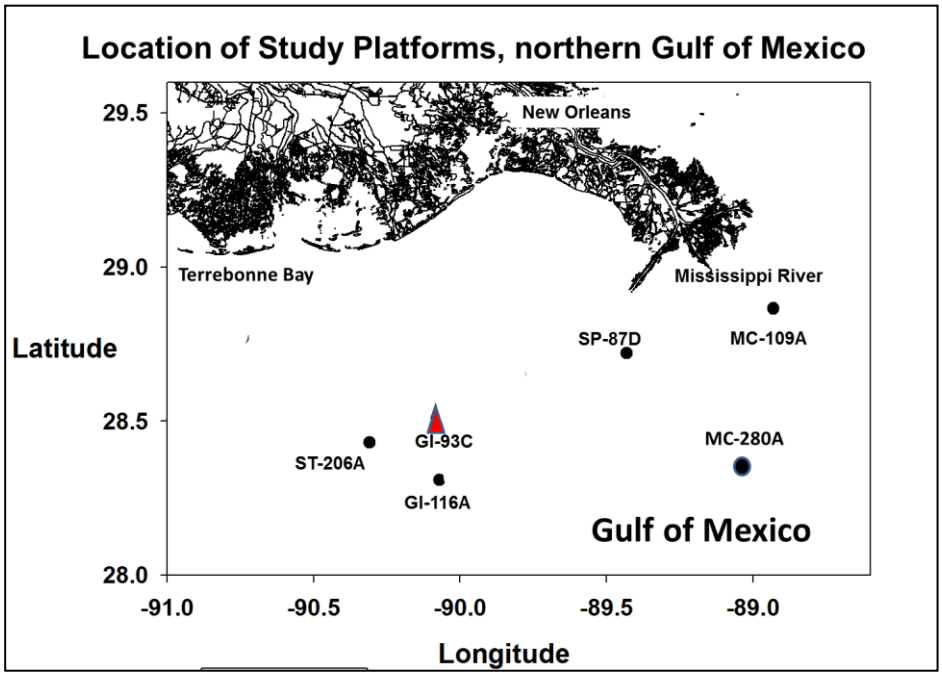

Figure 1. Map of the north-central Gulf of Mexico showing locations of the six offshore oil and gas platforms studied here.
GI-93C is the site where *Tubastraea micranthus* was first sighted (Sammarco et al., 2010).

2.2 Sample Collection and Pre-Processing

2.2.1 Duration

This study ran from 2012 through 2016. A variety of issues such as poor weather conditions and severe weather resulted in cruise delays, and fewer sampling locations extended the study beyond the original projected date of completion. The first one to two years were spent planning field and laboratory operations. The next two years were spent collecting samples, conducting molecular analyses, conducting statistical analyses on the data, and drafting a report and manuscript for publication.

2.2.2 Support Vessel and Dive Operations

Most of the samples were collected using the R/V *Pelican*, a University National Oceanographic Laboratories System vessel, owned and operated by the Louisiana Universities Marine Consortium (LUMCON). This vessel is 35.2 m in length, 8.0 m in the beam, and is capable of supporting a scientific crew of 14 and a ship's crew of 7. Additional samples were collected using the M/V *Fling* (33 m), Freeport, Texas.

2.2.3 Initial Sample Preservation and Processing, SED (Salt, EDTA, DMSO) Buffer Solution

In a 10 L polyethylene container, 4.1L of deionized (DI) water, 357g of sodium chloride (NaCl), and 581g of ethylene-diamine-tetra-acetic acid (EDTA) were mixed together. The volume was then brought up to 5 L using DI water, and 1.25 L of dimethyl sulfoxide (DMSO) was subsequently added. This procedure was repeated for a second container, and both containers of Salt, EDTA, DMSO (SED) salt buffer solution were stored at room temperature. Containers were thoroughly shaken and mixed before dispensing the solution onto any sample.

2.2.4 Storage and Additional Pre-Processing

Small tissue samples (2.5-5.0 cm^2) were collected from *T. micranthus* from colonies occurring at depths accessible by SCUBA divers ($\leq$ 37 m). Samples were collected by hand using latex gloves, a geological hammer, and small chisel. Samples were placed in ZipLoc© freezer bags. Tissue samples were returned to the ship and covered in high-salt SED buffer to arrest DNA-ase activity and preserve the DNA for molecular genetic analysis. Tissue was then frozen on ship-board laboratory freezers and once the ship reached port, transferred to LUMCON laboratories and stored at -20^{o}C. Tissue was later separated from the skeletons in Sammarco's laboratory at LUMCON. DNA was extracted and isolated from the coral tissue, then cleaned in a pre-analysis procedure. DNA was isolated from the coral tissue utilizing the Promega Wizard® Genomic DNA Purification Kit (Promega Corporation, 2014). The protocol described in Promega's technical manual, which was relevant to the analysis of animal tissue, was followed.

In order to isolate the DNA, we macerated samples lightly in SED buffer and spun them at 16 times gravity for 5 min to pellet the zooxanthellae from the homogenate. We then purified the

DNA using the Wizard® SV Genomic DNA Purification System (Promega Corporation, 2014), following the manufacturer's instructions for animal tissue.

DNA samples were then shipped overnight in insulated containers filled with dry ice (-78.5°C) to Brazeau's laboratory at the University of New England for detailed molecular analysis.

2.3 Amplified Fragment Length Polymorphisms (AFLPs)

We used AFLP DNA assays to generate numerous polymorphic markers, which provided population-specific markers for the corals (Atchison, 2005; Brazeau et al., 2005, 2008; Atchison et al., 2008; Sammarco et al., 2012b). We chose the AFLP technique because: (1) It uses a PCR, and thus only requires a small amount of tissue (0.1 to 100 μg) for analysis; (2) it can potentially yield a limitless number of polymorphic markers; and (3) AFLPs employ two PCR amplifications under high stringency conditions, using adapter-specific primers that provide polymorphism markers without prior sequence information.

Like other similar molecular genetic techniques, AFLPs draw a subset of markers from a large population of markers. A portion of the subset obtained from a given AFLP experiment is often sensitive to specific reaction conditions. Extra caution is therefore required in the processing of samples through all procedural steps in order to maximize the repeatability of results. We processed samples drawn from large lots containing members from all populations. Thus, if there was any error potentially introduced by reaction conditions, it would be distributed uniformly between populations in an unbiased fashion. Also, we performed all PCR reactions on the same thermocycler using the same thermal cycle profile.

2.4 Genomic Coral DNA digestion and adapter ligation

We prepared a restriction-ligation "master mix" with the following reagents (measures are per sample): 1.1 μl T_4 DNA ligase 10X buffer (10 millimoles [mM] magnesium chloride [$MgCl_2$] / 30 mM Tris-HCl solution, pH 7.8 / 10 mM dithiothreitol [DTT] / 1 mM adenosine 5'-triphosphate [ATP]), 0.5 μl bovine serum albumin (BSA; 1 mg/ml), 1.1 μl of 0.5 M sodium chloride (NaCl), 1.0 μl Mse I adapters (50 μM), 0.25 μl Mse I (4 units of activity [U]/μl; New England BioLabs, Beverly, Massachusetts), 0.25 μl of EcoRI (20U/μL; New England BioLabs), 1.0 μl EcoRI adapter (5 μM), and 0.33 μl of T_4 ligase (3U/μl; 10 mM Tris-HCl, pH 7.0 / 1mM DTT / 50 mM potassium chloride [KCl] / 0.1 mM EDTA / 50% glycerol). See Table 1 for sequences for the Mse I and EcoRI adapters and PCR primers.

We then added 5.5 μl of the restriction-ligation mixture plus 5.5 μl (500 ng genomic DNA) of the purified genomic to each new 1.7 ml tube. The tubes were then centrifuged for 15 s, and incubated at room temperature overnight. At the end of the restriction-ligation reaction, 189.0 μl of TE buffer (10 mM Tris-HCl, pH 8.0 / 0.1 mM EDTA) was added (10-fold dilution) serving as the template for the next-step, pre-selective amplification.

Table 1. Nucleic acid sequences.
A list of nucleic acid sequences, which are associated various adaptors, pre-selective primers, and selective primers used during the DNA isolation mode of the genetic analysis.

Name		Sequence
Adapters *Eco* RI	*EcoF*	5'-CTCGTAGACTGCGTACC
	EcoR	5'-AATTGGTACGCAGTCTAC
Adapters *Mse* I	*MseF*	5'-GACGATGAGTCCTGAG
	MseR	5'-TACTCAGGACTCAT
Pre-selective primer	*Eco* RI **A**	5'-GACTGCGTACC AATTC **A**
Pre-selective primer	*Mse* I **C**	5'-GATGAGTCCTGAG TAA **C**
Selective primers	*Eco* RI	5'-GACTGCGTACCAATTC **ACT**
(Set 1)	*Mse* I	5'-GATGAGTCCTGAGTAA **CAG**
Selective primers	*Eco* RI	5'-GACTGCGTACCAATTC **ACC**
(Set 2)	*Mse* I	5'-GATGAGTCCTGAGTAA **CTT**

2.4.1 Pre-selective (PS) Amplification of Coral DNA

We made a second "master mix" for pre-selection (PS) amplification, using the following reagents per sample measure given: 8.1 µl of nuclease-free water, 2.0 µl of 10X PCR buffer (15 mM Mg^{++} in buffer), 2.0 µl of EcoRI PS primer (2.75 µM), 0.8 µl of 5 mM dNTP's, 0.1 µl of Thermostable (Taq) DNA polymerase (5U/µl), and 2.0 µl of Mse I PS primer (2.75 µM), for a volume (total) of 15.0 µl. We added 15 µl of the PS amplification master mix to each 0.5 ml tube. We then measured 5 µl of each of the diluted restriction ligation reaction samples to be vortexed and centrifuged for 15 s. We amplified the samples with a 2-min initial incubation at 72ºC. This was followed by 20 cycles of denaturation for 20 s at 94ºC, then a 30 s annealing process at 56ºC, and finally a 2 min extension at 72ºC. These last steps were final extensions at 72ºC, and 30 min final incubations at 60ºC for 2 min. After the cycling was completed 180.0 µl of TE buffer was added to each tube. This created the templates for the final step, called selective amplification.

2.4.2 Selective Amplification of Coral DNA

A selective amplification "master mix" was made, containing the following components: 8.1 µl of nuclease-free water, 0.8 µl of 5mM dNTP's, 2.0 µl of 10X PCR buffer (with Mg^{++} at 15mM), 2.0 µl of Mse I selective primer (2.75 µM), 2.0 µl of EcoRI selective primer with 6FAM dye tag

(0.46 µM), and 0.1 µl of Taq DNA polymerase (5U/µl) for a total volume of 15.0 µl. 5 µl of the diluted pre-selection PCR reaction was added to each 0.5 ml micro-centrifuge tube, mixed, and centrifuged for 15 s. We then placed samples in the thermocycler. The cycle profile was performed as follows: 2 min initial denaturation at 94ºC, then one cycle of 20 s denaturation at 94ºC, followed by 30 s annealing at 66ºC and 2 min extension at 72ºC. This was followed by 9 repetitive cycles of - 20 s at 94ºC, initial 30 s at 66ºC (reduced 1ºC/cycle), and 2 min at 72ºC. The final step consisted of 20 cycles of the following: 20 s at 94ºC, 30 s at 56ºC, and 2 min at 72ºC, followed by a 30 min final incubation at 60ºC.

We used GeneWiz (Fredrick, MD) and Applied Biosystems 3730xi Genetic Analyzers to sequence products of the selective PCR. Electropherograms were analyzed using PeakScanner software V.1 (Applied Biosystems, ThermoFisher Scientific, Grand Island, NY; 2015) which also generated and exported fragment sizes. TinyFLP (ver 1.3) generated binary allelic files for each sample.

2.5 Statistical Analyses

STRUCTURE V2.3.4 (Pritchard et al., 2000) software was used to assess population differentiation within and between populations. This routine uses Bayesian techniques and Monte Carlo simulations to allocate samples to different populations. STRUCTURE made assignments that minimized deviations from the Hardy-Weinberg equilibrium. This is unlike more basic assignments algorithms, where assignment is based upon marker frequencies which are characterized by the assumption that the population giving rise to the recruits constitutes a large, randomly mating one. STRUCTURE, using this approach, calculates not only probabilities of individual assignment and estimates of the fixation index (F_{ST}; the mean reduction in heterozygosity of a sub-population relative to the total population due to genetic drift among sub-populations), but also probable generational (e.g., paternity, grand-paternity, etc.) relationships. It is comparable to the AFLP technique in that it can accommodate similar dominant marker data. In this case, we found STRUCTURE to be a more powerful analysis, providing greater insight into the data, than Analysis of Molecular Variance; thus, we preferred to use it in this study.

Parameter estimates were calculated by subjecting the entire data set to several preliminary runs using Markov Chain Monte Carlo (MCMC) iterations to determine points of stability. We also defined a level for the parameter of migration rate (MIGPRIOR) before running STRUCTURE. This parameter represented the prior probability of a spat being derived from an external (compared with internal) source. For comparative purposes, we ran the procedure at two levels – 0.05 and 0.50, which accounted for different potential estimated migration rates. Data were analyzed using a burn-in count of 500,000 iterations once these parameters were set. This was followed by another 100,000 MCMC repetitions. Each run to assess the likely number of groups was run 20 times beginning with one group out to ten groups. These data were then used to calculate L'(K), defined as the mean difference between successive likelihood values of K from each simulation run present in populations of the coral *T. micranthus*, where K is the true number of clusters in a sample of individuals when population patterns are not homogeneous (Evanno et.al, 2005). The real value of K is indicated by the K value with the greatest drop in L'(K).

3.0 RESULTS

We visited a number of platforms for this study in order to sample them. Only two platforms had enough colonies to be accessed in shallow water by SCUBA divers, and to be sufficient in number to be analyzed for genetic structure. These were GI-93C and MC-280A; the latter was added to the survey group.

The STRUCTURE analysis for all samples from GI-93C and MC-280A identified four as the most probable value for K or the number of genetic clusters. Figure 2 shows a clear drop in L'(K) in going from 3 to 4 groups, indicating that *Tubastraea micranthus* on the two platforms examined clearly fell into four clusters. Figure 3 indicates that colonies from platform MC-280A can be assigned almost entirely to a single group (arbitrarily called Group 1). By contrast, individuals from GI-93C were assigned to each of the four groups, with two groups accounting for over 77 percent of the individual samples. STRUCTURE was used to analyze these data and make the assignments shown below. Note that this program assigns colors to its groups arbitrarily, and colors which repeat in different sites (e.g., GI-93C and MC-280A, do not necessarily designate the same group or cluster). The differences in levels of genetic heterogeneity between the coral populations on the two platforms may easily be seen in Figure 4. This graph shows the percent attribution into each of the four groups of corals for each colony. Colonies from MC-280A were largely assigned to Group 1, whereas colonies collected from GI-93C were more heterogeneous, with many colonies showing possible assignment to more than a single group. When STRUCTURE analyzed the data using four clusters as the basis for comparison of individual colonies and their associated genetic affinities, the results were quite clear. GI-93C was characterized by a wide array and mixture of genetic associations (Figure 5). All four clusters were represented there. Some colonies had complete affinity with only one cluster, while others had affinities to all four clusters. On MC-280A, however, all clusters were represented in the colonies, all except one group of colonies accounted for a very small proportion of the population. All colonies showed $\geq$95 percent affinity with Cluster 2, and most exhibited levels of 98-99 percent.

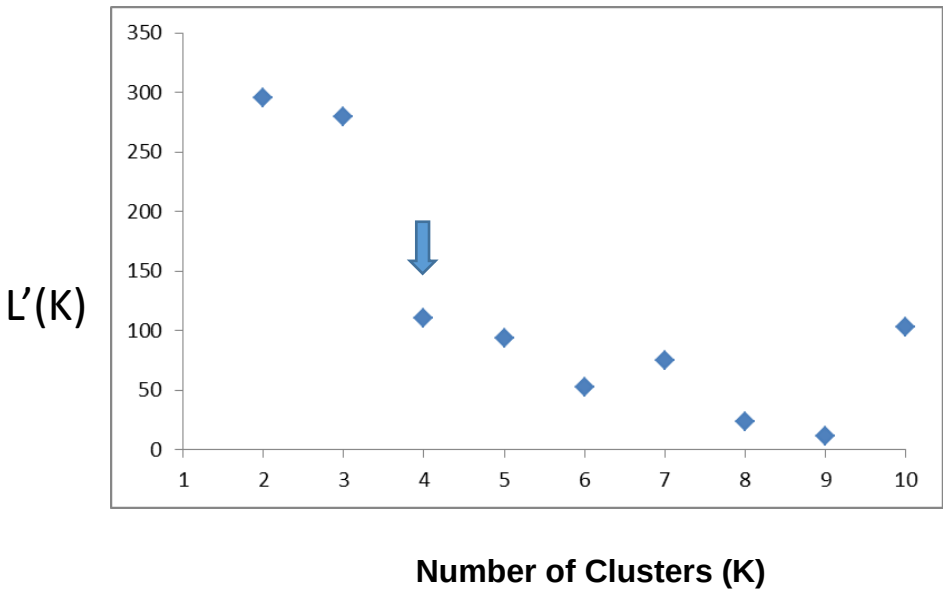

Figure 2. Plot of K, number of estimated clusters, and L'(K), mean difference between successive likelihood values of K, from each simulation run present in populations of the coral *Tubastraea micranthus* using STRUCTURE.
The two populations were derived from the oil and gas platforms GI-93C and MC-280A in the northern Gulf of Mexico.

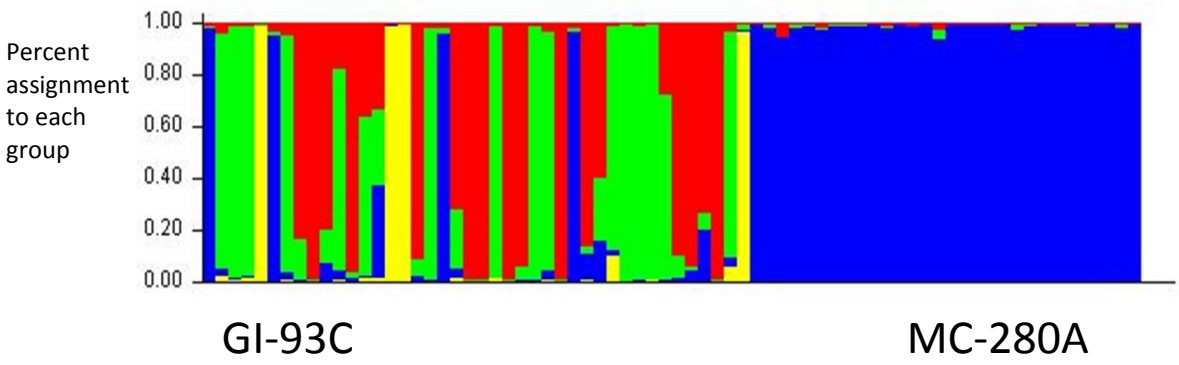

Figure 3. A graph depicting the proportion of membership of the platform populations occurring on the oil and gas platforms GI-93C and MC-280A.

The proportion of colonies in each of the four clusters and groups or genetic signatures are shown by different color bars (red, green, yellow and blue). Color bars are assigned arbitrarily by STRUCTURE; i.e., blue in GI-93C is not the same as blue in MC-280A.

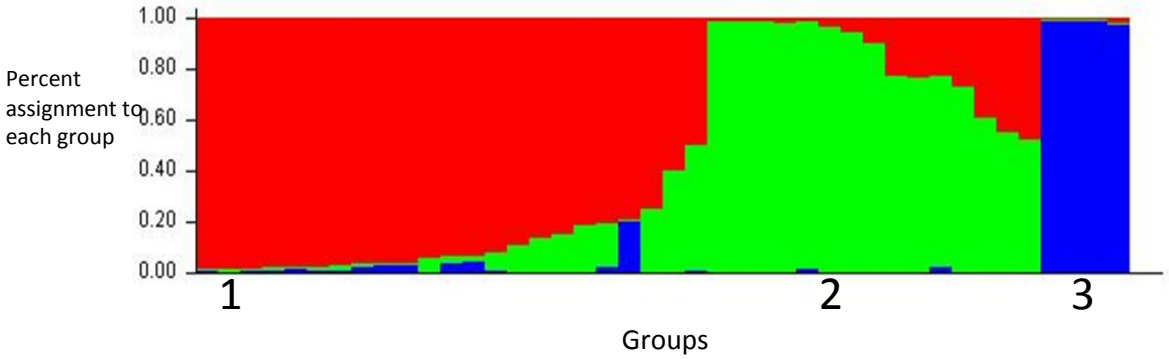

Figure 4. A graph depicting individual colonies of the coral *Tubastraea micranthus* and their genetic affinities as percent assignment to a given cluster or genetic signature.
Samples were collected from oil and gas platforms in the northern Gulf of Mexico – namely, GI-93C and MC-280A. Each column represents a sample from a single colony. Data generated by STRUCTURE. Individual colonies may show affinity for more than one cluster.

To assess the structure within just GI-93C alone, the STRUCTURE analysis was repeated using only individuals from GI-93C. Without the inclusion of samples from MC-280A, the analysis indicates the most likely number of groups defining the genetic structure of the corals on this platform is three. That is, the L'(K) values show the largest drop at K=3 (Figure 5). Individuals from GI-93C show some mixing among groups 1 and 2, with the third group showing little admixture (Figure 6).

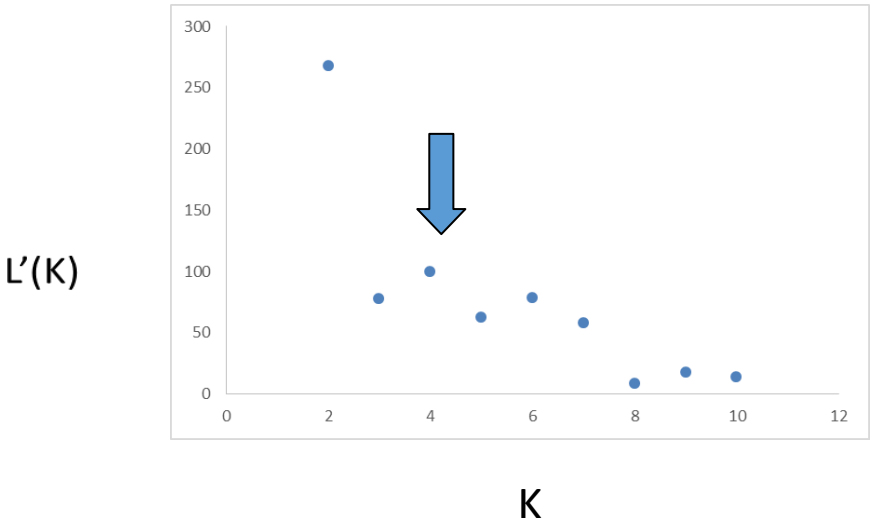

Figure 5. A graph depicting the relationship between K and L'(K), defining the number of probable genetic clusters or signatures present in populations of the coral *Tubastraea micranthus,* using STRUCTURE.
The population analyzed here was derived from the oil and gas platform, GI-93C, in the northern Gulf of Mexico.

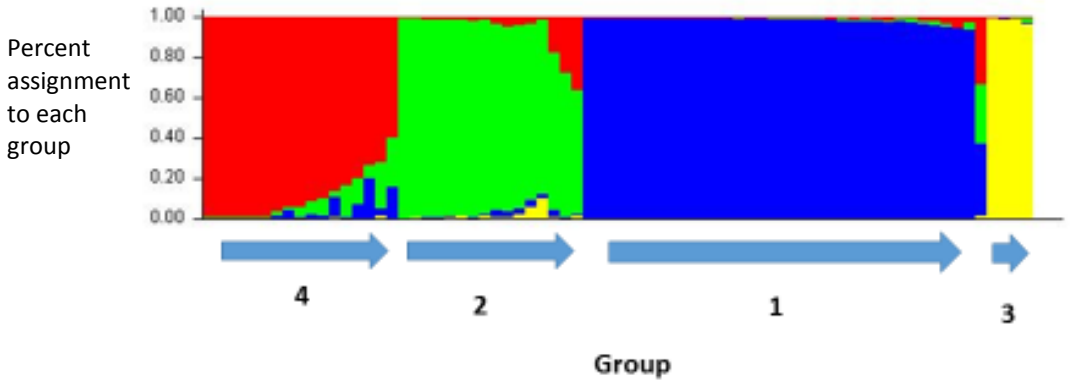

Figure 6. A graph depicting individual colonies of the coral *Tubastraea micranthus* and their genetic affinities as percent assignment to a given cluster or genetic signature.
Samples are ordered from least diverse to most diverse, for a given cluster or genetic signature, as shown by the arrows. Data generated by STRUCTURE. Samples were taken from the oil and gas platforms GI-93C and MC-280A in the northern Gulf of Mexico. Each column represents a sample from a single colony. Clusters 2, 3 and 4 are found in the GI-93C population, and cluster 1 is found almost exclusively in the MC-280A population. Color bars are assigned arbitrarily by STRUCTURE; i.e., blue in GI-93C is not the same as blue in MC-280A.

When STRUCTURE ranked the colonies according to their Cluster affiliations, using a four-cluster basis for analysis, the patterns arising were informative. When all colonies were considered, the first 7 colonies were derived primarily from Cluster 1 at the 99 percent level, with the remaining 1 percent being derived from Cluster 2 (Figure 6). The next 12 colonies had representations from Clusters 1, 2, and 3, varying from 67-90 percent for Cluster 1, 5–25 percent for Cluster 2, and 1-20 percent for Cluster 3. Cluster 2 dominated the remaining colonies, at levels of 1—83 percent, being associated with Clusters 1, 3, and 4, with additional representation at much lower levels. Within the MC-280A population, Cluster 3 dominated at least half of all of the colonies. Cluster 3 also appeared in another 30 percent of the samples in small proportions, later joined by Cluster 2. One colony shows equal affiliation with Clusters 1, 2, and 3, while the last four colonies are almost 100 percent Cluster 4.

Figure 7 summarizes data regarding the proportion of colonies assigned to genetic clusters 1 through 4, respectively, on GI-93C compared with MC-280A.

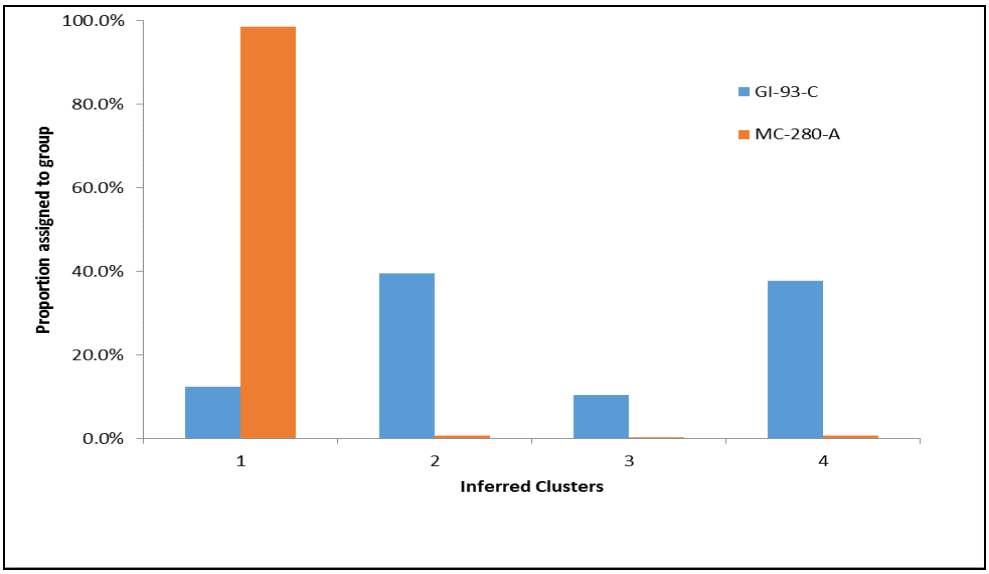

Figure 7. A graph depicting colonies of the coral *Tubastraea micranthus* and their genetic affinities as percent assignment to a given cluster or genetic signature.

Samples are ordered from least diverse to most diverse, for a given cluster or genetic signature. Data generated by STRUCTURE. Samples were taken from the oil and gas platforms GI-93C (blue bars) and MC-280A (orange bars) in the northern Gulf of Mexico. Each column represents a sample from a single colony.

4.0 Discussion

The STRUCTURE analysis showed a substantial drop in the value of L'(K) between trials of three and four clusters, respectively. This indicated that the two populations of *Tubastraea micranthus* resident on the platforms GI-93-C and MC-280A were characterized best by four genetic clusters or signatures. This result was because the genetic structures of the populations analyzed were identified as being statistically significantly different, yielding four clearly identifiable groups. The origin of these signatures is unknown, but the results allow us to conjecture about how the observed patterns may have arisen. For example, this would be particularly true if a single species introduction arose from recruits derived from a single colony or set of colonies carrying a single genetic signature.

Use of four clusters by STRUCTURE yielded detailed information regarding genetic relatedness of coral colonies on the two platforms. The fact that all four clusters were represented in the GI-93C population indicates high genetic diversity of coral colonies there. This was evident in a variety of analyses, covering various scales of resolution. The result was the same in each case – high genetic diversity in the GI-93C population, with widely varying percentages of representation. The MC-280A population, on the other hand, was strongly dominated by a single group (Table 1). This occurred with analyses performed at the lowest level of resolution to the highest. The indicator of a lack of genetic diversity was strong and consistent in the MC-280A population. Thus, with a four-cluster analysis, the dichotomy between the two platform populations became clearer. That is, the two platforms are clearly genetically distinct from one another. The STRUCTURE analysis assigned samples back to the original source platform with ease and with high probability.

This suggests that the founding individuals were likely also genetically distinct. Strong differences were found in genetic heterogeneity between the two platforms. That is, one was highly heterogeneous and the other highly homogeneous. This implies that the two platforms were derived from different sets of recruitment and different larval sources. STRUCTURE identified four probable groups within the samples; one dominated MC-280A, and an additional three others dominated GI-93C. It is possible that the MC-280A population is in an early stage of development and may become more genetically diverse, as it receives additional recruits from other platforms through time. At this point, however, the focus is on the disparity between the two populations and its implications for past recruitment sources.

In general, the data suggest that recruitment to both platforms is of a random "lottery" nature (Sale, 1993). That is, the genetic structure is not a set of gradual, smoothed yearly recruitment events; rather there have been limited isolated or perhaps punctuated recruitment events.

Samples from platform GI-93C were assigned to four genetic groups. Most (87.8%) were assigned to three groups largely absent on MC-280A (1.5%, ~ 4 individuals). The groups identified on GI-93C are quite distinct from one another, suggesting that these individuals represent separate recruitment events. The representation of different genetic clusters within single colonies commonly occurring in corals on GI-93C implies that there may be some interbreeding occurring, particularly among at least two of the groups. This also implies that these groups are the "oldest" on the platform.

The high genetic diversity observed on GI-93C may have been derived from a different set of source populations in the region, indicating separate sub-samples of populations from different sources–local or distant. Alternatively, the high genetic diversity may imply that the GI-93C population is older than the MC-280A population, allowing for higher genetic diversity to be represented over a greater time period.

The low genetic diversity exhibited by the MC-280A population, however, may be the result of one or combination of several events: a single recruitment event; small number of similarly-related corals recruiting; younger population age; and/or a single recruitment from a secondary invasion. Samples from platform MC-280A were assigned to a single group by STRUCTURE. Firstly, this could suggest a single recruitment event. If many individuals were involved in this recruitment, they were genetically homogeneous, suggesting a single source population. 'For example, if a few coral colonies were all located in one place and reproduced asexually, and they were sufficiently isolated to prevent interbreeding with other colonies, this could produce a low genetic diversity in the population.

Recruitment may have been from a small number of similarly related corals. In addition, the population may be much younger than the GI-93C population, not yet having had the opportunity to recruit from a broad set of sources.

Lastly, it is possible that this population is derived from a single recruitment event derived from a secondary invasion of *T. micranthus*. In this case, new planula larvae may have been derived from adult colonies attached to the hull of a ship traversing from the western Indo-Pacific, or from planulae traveling within, or released by adult coral colonies within, the hold of the ship. The likelihood of either of these events is not known, and may well be low. Ships do return to ports repeatedly, so multiple exposures to a given region are possible. Average population densities of *T. micranthus* were $15/m^2$, which is generally high for corals. Population abundances for MC-280A were similar, but data on average densities were not available. Whether the population densities were high or low, the recruits could have been derived from a foreign or local source.

If it is the latter case, we are dealing with the introduction of new larvae from the Indo-Pacific due to incoming vessels, and this would be a case of multiple invasions of this introduced species. Multiple invasions of this type would make this or any other type of invasive species very difficult to control or eradicate. This is because any removal of this species from a region would be ineffective due to the possibility of replacement through additional recruitment derived from external sources.

Future research on *T. micranthus* might include continuing reconnaissance of the expansion of this species, the genetic structure of newly established populations if it continues to expand its range, and its potential to compete with native GOM and Caribbean reef fauna and flora if it reaches native coral reefs in the western Atlantic. The genetic structure of this coral species will be added to the genome database.

4.1 Relationship of Reproduction in *Tubastraea micranthus* to Genetic Structure Results

The genetic data from GI-93C indicate that there is great genetic variability in this population of *T. micranthus.* One of the major ways that this can occur is through sexual reproduction occurring frequently, with local recruitment. Another is for this platform to have received multiple cohorts of recruits from different populations over a number of years. On the other hand, the population of this coral on MC-280A exhibited a surprisingly low level of genetic variation. This is an indication that the recruits that colonized this platform were homogeneous, most likely being derived from a single source and possibly from a single recruitment event. This is what opens the possibility that they may have been derived from an independent, second invasion event because this set of recruits is by and large unrelated to the colonies in the GI-93C population.

Tubastraea spp. are brooders (Figueira de Paula et al., 2014). That is, their polyps have eggs which are fertilized internally. Generally, gametes are derived from another colony, causing sexual reproduction to occur. Nonetheless, fertilization can also be derived from "selfing" or self-fertilization (Glynn et al., 2008). They can produce and release asexual planulae. This would decrease genetic variability in the population. These planulae are released over multiple months during the year and for several weeks per month.

Coral planulae tend to spend minimally hours but usually days or weeks and sometimes months dispersing in the water column (Harrison and Wallace, 1990). It is unlikely that they would settle on the same platform that they were spawned from because of the open structure of the platform. There is little hard structure to act as an obstacle to far-field flow, causing eddies to occur in the lee of the current (Sammarco and Andrews, 1988, 1989; Andrews et al., 1989). Such eddies are known to retain larvae for later settlement on the same reef. It is more likely that these planulae are carried away from the platform. Thus, it is more likely that planulae colonizing the platforms are from external sources. This can be seen in reviewing the data of Sammarco et al. (2012b), where coral planulae colonizing the platforms are clearly derived from the Flower Garden Banks National Marine Sanctuary.

5.0 CONCLUSIONS

Of the two populations of *T. micranthus* that were sampled and analyzed, the population on the platform GI-93C was found to be highly genetically diverse, with colonies exhibiting association with four different genetic clusters in widely varying proportions. The genetic variability in the GI-93C population may have resulted from one or more of the following: different recruitment events, recruitment from different populations in the region, or the population simply being older than that on MC-280A. This result also suggests that different bouts of sexual recruits have colonized GI-93C.

The *T. micranthus* population on platform MC-280A exhibited an overwhelming association with only one genetic cluster. Although all four genetic signatures were represented on MC-280A, three of these signatures were only present in small proportions of colonies (e.g., 1–5%) while 95-99 percent of the colonies exhibited association with a single genetic cluster. The lack of genetic variability on MC-280A could have resulted from one or more of the following; a single recruitment event due to greater geographic isolation, recruitment from a smaller set of closely related corals, a much younger population, a higher level of asexual reproduction, or a single recruitment event derived from a second invasion of this species. The last of these remains a possibility and cannot be ruled out. Such a case would thwart any attempts to control or eradicate this species.

The genetic structure of *Tubastraea micranthus*, as determined through this study, will be added to a publicly accessible genome database.

5.1 Future Considerations Questions

. What are the temperature and salinity tolerances of *T. micranthus* which may help define the geographic limits of its spread throughout this region and the wider GOM? This can be determined through controlled laboratory experimentation.

. How would these tolerances compare to those of *T. coccinea*, which can also be established by similar means?

. We know generally what the modes of reproduction are for *Tubastraea*, particularly for *T. coccinea*, but what are the specific modes of reproduction in *T. micranthus*? Sexual planular reproduction? Asexual planular production? Selfing? Asexual budding? Asexual stolon production? All of the above?

. What is the mode of introduction or invasion of this and similar species colonizing the platforms? Planulae carried and released in the ballast water of ships coming in from the Indo-Pacific? Coral colonies attached to and growing on the hulls of incoming vessels, "hitch-hiking," and surviving despite exposure for days of freshwater in Gatun Lake in the Panama Canal.

. In terms of control or eradication, what are the weaknesses and sensitivities of this coral which can result in its mortality? How can they be reasonably controlled? Bleach? Manual crushing? Asphyxiation?

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