DETECTION OF INVASIVE LIONFISH (*PTEROIS VOLITANS AND PTEROIS MILES*) IN THE GULF OF MEXICO USING ENVIRONMENTAL DNA METHODS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Detection of Invasive Lionfish (*Pterois volitans* and *Pterois miles*) in the Gulf of Mexico Using Environmental DNA Methods

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Red Lionfish (*Pterois volitans*) and Devil Firefish (*Pterois miles*) are Indo-Pacific species introduced into the western North Atlantic Ocean in the 1980s. Their range currently extends from New York to Florida and adjacent waters of the Caribbean Sea and the Gulf of Mexico. Since the original sighting in 2010 off the Texas coast lionfish populations in the Gulf of Mexico have increased substantially and negative ecological impacts are expected. Lionfish detection relies on underwater visual surveys using divers and ROVs which are expensive and not always effective. This study seeks to employ real time polymerase chain reaction (RT PCR) to amplify aqueous environmental DNA (eDNA) as a highly sensitive alternative to detect and quantify lionfish. To minimize false positives and false negatives, highly specific primer sets targeting the mitochondrial DNA genome of lionfish were designed using ecoPrimers and ecoPCR. RT PCR on serial DNA dilutions indicated that the D-loop region was the best locus to amplify and quantify eDNA. Lionfish eDNA was successfully quantified in a series of mesocosms differing in volume at Moody Gardens Aquarium. Finally, the Red Lionfish presence was detected by assaying water samples collected from artificial reefs and the Flower Gardens Banks National Marine Sanctuary and adjacent reefs in the Gulf of Mexico.

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NOMENCLATURE

FGBNMS	Flower Garden Banks National Marine Sanctuary
eDNA	Environmental DNA
PCR	Polymerase Chain Reaction
RT PCR	Real Time Polymerase Chain Reaction
qPCR	Quantitative PCR
bp	Base Pair
Cq	Quantification Cycle
HRMA	High Resolution Melting Analysis

CHAPTER I

INTRODUCTION

Two lionfish species, the Red Lionfish (*Pterois volitans*) and the Devil Firefish (*Pterois miles*) (hereafter collectively referred to as lionfish) were introduced to the Northwest Atlantic Ocean in the 1980s most likely by releasing pets originally acquired through aquarium trade (Courtenay, 1995; Hamner, Freshwater, & Whitfield, 2007; Ruiz-Carus, Matheson, Roberts, & Whitfield, 2006). The initial release occurred after Hurricane Andrew when an estimated six individuals were released into Biscayne Bay, Florida (Courtenay, 1995; Ruiz-Carus et al., 2006). Since their initial introduction, lionfish have become prolific in the Northwest Atlantic Ocean and their range now extends from New York to Florida and adjacent waters including the Caribbean Sea and the Gulf of Mexico (Albins & Hixon, 2013; Evangelista, Young, Schofield, & Jarnevich, 2016; Morris & Whitfield, 2009; Schofield, 2009). The first sighting of lionfish in the Northern Gulf of Mexico was at the Flower Garden Banks National Marine Sanctuary (FGBNMS) in 2010 (Nuttall et al., 2014).

The high level of success of lionfish as invasive species in the Gulf of Mexico may be due to the absence of natural predators, the presence of fin spines with muscular toxins, and physiological adaptations that allow them to survive in deep waters, but also in shallow areas varying from nearly freshwater to hypersaline environments, and also capable of withstanding a wide range of temperatures (Albins & Hixon, 2013; K. A. Dahl, Patterson, & Snyder, 2016; Schofield, 2009). Lionfish feed on the larvae and juveniles of many species of fishes and have the potential to cause a decline of game fishes and other commercially important fishes such as flounder, grouper, and jacks (Kristen A. Dahl & Patterson, 2014). The hazards that lionfish

inflict on biodiversity can be detected at The FGBNMS and artificial reefs (e.g., decommissioned vessels, fabricated materials, and oil rig jackets and platforms) in the northwest Gulf of Mexico. Past studies determined fishes settling directly to reefs from plankton have the highest risk of lionfish predation, especially those settling to artificial reefs (Kristen A. Dahl & Patterson, 2014).

Lionfish detection has focused primarily on underwater visual surveys (UVS) using divers and remotely operated underwater vehicles (ROVs), but in many environments, such as deep marine waters, or in conditions of high turbidity, such methods are not practical or effective. Accordingly, the development of alternative methods to detect lionfish presence, is desirable (Anderson, Chapman, Escontrela, & Gough, 2017; Doi et al., 2015; Thomsen et al., 2012). The use of aqueous environmental DNA (eDNA) as a reporter of the presence of aquatic organisms is an efficient, cost effective method, which relies on the presence of epithelial cells and metabolic waste containing nucleotide oligomers suspended in the water column (Jerde, Mahon, Chadderton, & Lodge, 2011; Laramie, Pilliod, & Goldberg, 2015). Studies that have employed eDNA have focused on two different goals. One approach is to detect the presence of a particular species of concern (e.g., invasive, endangered, cryptic, etc.) by employing highly specific primers and or probes as reporters (Laramie et al. 2015). The alternative is to employ 'universal' primers that target a taxonomic group of interest (e.g., fishes) to amplify DNA, which is then sequenced massively to identify the presence and relative abundance of the species presence through metabarcoding analysis (Laramie et al., 2015; Valentini et al., 2016). The premise of the first approach, relies on specificity of primers that bind to eDNA contained in aqueous samples (Gargan et al., 2017; MacDonald & Sarre, 2017). By using bioinformatics it is

possible to select primer set with sufficient specificity *in silico* to guarantee the specificity of the assay *in vitro*.

Current studies that employ eDNA have the ability to determine the presence or absence of a species, but there are few studies that have demonstrated the ability to measure abundance in marine bodies of water (Stoeckle, Soboleva, & Charlop-Powers, 2017). Information on relative abundance is essential to determining areas of most concern for eradication efforts as opposed to others selected at random, or based solely on information of census derived from visual surveys (Anderson et al., 2017). Areas at highest risk for invasion by lionfish or other aquatic specimens can potentially be determined using eDNA methods.

In this project, novel molecular methods are used as tools to detect and quantify the presence of lionfish in the Gulf of Mexico. Specificity of the assay was attained through the bioinfomatic analysis of sequences stored in GenBank to design lionfish specific primer sets to be used in RT PCR. Relative quantification, based on calibrations using serial dilutions of eDNA, will be used to estimate relative abundance. Quantification of lionfish were carried out in predetermined locations at the FGBNMS and artificial reefs in the Gulf of Mexico. This information could be employed to prioritize areas of high lionfish abundance to optimize eradication programs.

CHAPTER II

METHODS

The experimental process was divided into four major phases: primer design with bioinformatics, development of quantification methods to set baseline results, testing methods in a controlled environment, and quantification of eDNA collected from the FGBNMS and artificial reefs. The systematic process of protocols can be visualized in Figure 1.

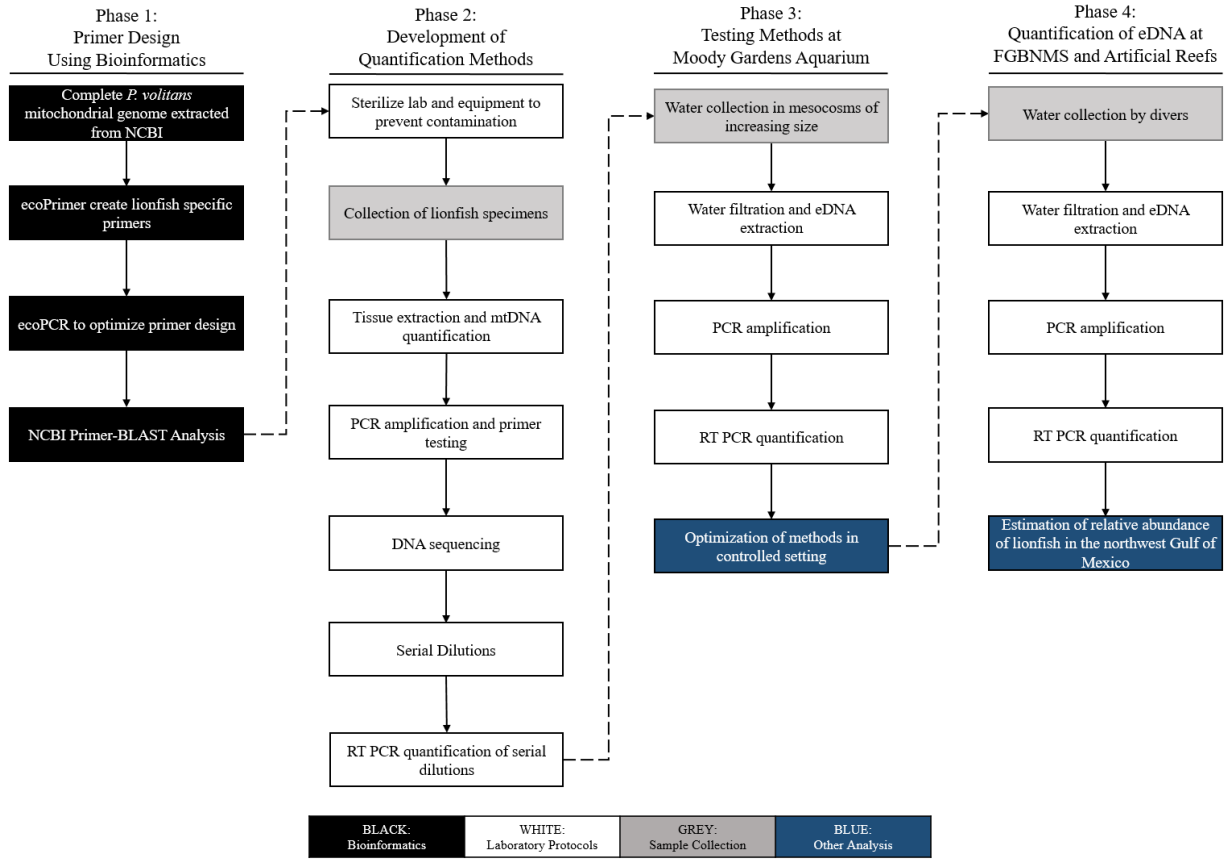

Figure 1. Protocols developed to detect lionfish in the Gulf of Mexico using eDNA.

Primer Design Using Bioinformatics

All mitochondrial genomes contained in the National Center for Biotechnology Information (NCBI) database were downloaded. The complete *P. volitans* mitochondrial genome (accession number: KJ739816) consisted of 16,500 base pairs was separately extracted from NCBI using the same system.

EcoPrimers, a Biolinux program, was used to create lionfish specific primers that would only detect lionfish DNA. To ensure that primers designed would only detect lionfish, but no other teleost species, an argument with the following settings l: 200, L: 250, r: 506672 E: 32443 was established. This argument was made possible by substituting the taxa identification number of *P. volitans* (185886) with that for *Metaphire sieboldi* (506672) such that lionfish would appear as a non-teleost species. EcoPrimers created over 12,980 primer pairs that were then optimized using ecoPCR. EcoPCR narrowed the search results by reporting the number of mismatched positions between the generated primer and the targeted sequence. All primer pairs containing a mismatch were discarded.

The number primer sets generated by ecoPCR was narrowed by removing all primer pairs that had a melting temperature difference greater than 2°C, and those that at the 3' end started with adenine or thymine. This filtering procedure resulted in 70 primer pairs that were then validated using NCBI Primer-BLAST. Primer pairs that amplified organisms other than *P. volitans* and *P. miles* were discarded. Primer pairs that had one base pair mismatch between *P. volitans* and *P. miles* were preferred to ensure both species of lionfish present in the Gulf of Mexico were detected.

Primer pairs only amplifying lionfish were analyzed in Geneious using the complete mitochondrial genome for *P. volitans* (accession number: KJ739816). The region of the

mitochondrial genome for each primer pair and the length of the amplicon was determined. Primers located in regions with poor amplification were discarded, and the primers with no major difference in region were chosen, and the corresponding oligonucleotide sequences were ordered.

Development of Detection and Quantification Methods

Preparation of Clean and DNA Free Workspace

To minimize the possibility of false positives due to contamination from other organisms the following measures were taken. All experimentation using lionfish DNA was done in a separate room that had no previous exposure to teleost DNA. The room was sanitized by whipping all surfaces with a 1:10 concentrated bleach solution prior to all laboratory work. All equipment used was soaked in 1:10 concentrated bleach solution or autoclaved, and gloves were worn at all times.

Specimen Collection

Four lionfish specimens were collected with a spear gun and put on ice after capture. The four collected specimens were from East Bank buoy 4 (93.81425, -93.81425), West Bank buoy 2 (27.875361, -93.81425), Stetson Bank buoy 2 (28.166417, -94.297361), and artificial reef HI-A-270 (28.42766, -93.81562). Specimens from East Bank, West Bank, and Stetson Bank were collected in 2016 and donated by NOAA, and the specimen from HI-A-270 was collected in 2017 and donated by Texas Parks and Wildlife Artificial Reef Program. All samples were stored in -80°C freezer.

DNA Extraction and Quantification

Tissue samples were taken from the caudal peduncle of all four samples. DNA extraction followed the manufacturer's instructions using the Qiagen Puregene Kit for Tissue. The quality of the extraction was tested using a 1% agarose gel and presence of bands in the gel ensured DNA was extracted from the tissue sample. Extractions that resulted in poor quality bands were discarded and DNA was re-isolated to ensure a sufficient amount of DNA was present.

The concentration of extracted DNA was determined using both Nanodrop and Qubit™ Assays. The Nanodrop determined the concentration of DNA and the presence of contaminants in the sample. Samples were required to have minimal contaminates in order to proceed. The concentration of the sample was reevaluated with the Qubit™ Assay that followed the manufacturer instructions if the concentration was greater than 0.01µg/mL. A 10:190 solution of sample to working solution was used for samples with concentrations less than 0.01µg/mL. The concentrations of the samples were recorded and used as a baseline for serial dilutions.

Tissue Amplification and Verification

Targeted segments of the mitochondrial DNA from the tissue extractions were amplified through polymerase chain reaction (PCR). PCR reactions were prepared with sterile water, Econotaq Plus Green, designated primer pairs, and template DNA. A negative control including the master mix and no template DNA was also tested. Thermocycling consisted of initial denaturing step at 94°C for 2 minutes, followed by 36 cycles of denaturing at 94°C for 25 seconds, annealing at 49°C (for D-loop primers) or 53°C (for ND2 and ATP6 primers) for 40 seconds, and extension at 72°C for 90 seconds, followed by a final extension step for 3 minutes at 72°C.

PCR products were inspected with 2% agarose gels using a 100 base pair (bp) ladder as a size marker. Primer pairs that produced fluorescent bands at the expected band length were counted as a positive result. Primer pairs with weak amplification or amplification not at the expected band length were discarded and not used in further laboratory protocols.

The PCR amplifications were validated as lionfish through Sanger sequencing PCR products. Amplification products were cleaned with the Zymo Research DNA Sequencing Clean-up Kit™ following manufacturer's instructions. Amplified fragments in both directions using the corresponding forward and reverse PCR primers, with reaction setups and thermocycling profiles as described in Cruscanti et al. (2015). The resulting sequences were input into Geneious where the DNA sequences were edited by eye and aligned. The sequences were compared against all other genomes using NCBI-BLAST. All results were ensured to have a 95% to 100% query cover with *P. volitans*. Primer pairs that produced sequences of DNA that had a query cover of over 80% with another species other than *P. miles* were discarded.

Serial Dilutions and Quantification of Diluted DNA

Serial ten-fold dilutions were conducted to establish the minimum threshold of DNA present for DNA amplification to occur. After extracting and quantified the DNA from the tissue as described above, it was PCR amplified. The amplification success was determined through ethidium bromide (EtBr) Tris-Acetate (TA) 2% agarose gel electrophoresis, and the resulting bands were compared against a 100 bp ladder. The minimum concentration of DNA required for amplification was determined by finding the amount at which amplified bands could no longer be visualized through a UV transiluminator.

Real Time Polymerase Chain Reaction (RT PCR) was used to quantify the amount of DNA present in each of the serial dilutions using a LightCycler® 96. RT PCR reactions used a master mix of 0.5µL per reaction of LCGreen+, 5 µL per reaction of Econotaq Plus (without Green loading dye), 0.5 µL per reaction of each specified primer pairs, and 2.5 µL per reaction of sterile water added to 1µL of diluted template DNA. Primers targeting different regions were tested separately. The reaction volumes for the master mix and template stayed constant over the entirety of the study.

RT PCR generated a fluorescence versus time curve, and samples that were more highly diluted were expected to amplify at greater cycle numbers than the less diluted samples, this corresponds to the quantification cycle (Cq). Primer pairs that did not follow this expected curve or showed the formation of primer-dimers in the form of amplification of the negative control were eliminated. The targeted region that followed the expected curve most closely was chosen for all further experimentation.

A standard curve was generated from the RT PCR curves of the chosen region using the LightCycler® 96 absolute quantification program. The standard curve gave a graph comparing the Cq mean with the transformed concentration mean. A linear regression was generated between all of the points from the serial dilutions. The output of the quantification curve produced in the LightCycler® 96's absolute quantification program was replicated in Microsoft Excel so it could be used as a standard when testing the unknown concentrations of eDNA from water samples.

Optimization of Methods at Moody Gardens Aquarium

The detection and quantification protocols developed in the laboratory setting were tested in a controlled environment at Moody Gardens Aquarium in Galveston, Texas. With help from the staff at Moody Gardens, lionfish were moved through a series of mesocosms of increasing size. Two bottles of 1000mL glass Wheaton containers were filled with water from each mesocosm sampled.

The first mesocosm tested had a volume of 1,700 gallons, included artificial structures and was rectangular shaped. The organisms in the mesocosms included three lionfish, several hermit crabs, and several other members of Family Scorpaenidae. Water was collected from the overflow tank. The second water collection was from a circularly shaped 4,500 gallon mesocosm with no artificial structures. The tank held a single lionfish and two Bonnethead Sharks (*Sphyrna tiburo*). Water samples were collected from the surface of the water at different areas around the tank. The third mesocosm tested was rectangular, had artificial structures, and a volume of 100,000 gallons. The mesocosm included a variety of teleost, including a single lionfish. Water was collected from the surface of the water across the mesocosm from the lionfish. Samples were filtered through a Nalgene® Filter Holder with Receiver and the DNA was captured on a 0.2µm filter.

The eDNA collected in the filter was extracted using Qiagen Puregene Kit and protocol followed manufacturer's instructions. Once the water was completely filtered, the filter paper was removed from the apparatus using forceps and cut into three equal sized pieces. Dissection scissors were used to cut each of the thirds of filter paper into many small pieces that were about 1cm x 1 cam squares. The small pieces of filter paper were then transferred into separate 1.5mL collection tubes that contained a liquid mixture of Lysis Solution and Purgene Proteinase K for

the first step of extraction. A plastic pestle was used to push all of the filter pieces into the liquid. Forceps, dissection scissors, and pestle were washed in 70% ethanol between each triplicate to prevent contamination between the three triplicate groups. Once extracted, PCR and RT PCR were completed following the protocols developed in the laboratory setting when testing serial dilutions. The number of cycles was increased to 99 cycles to ensure that all DNA in the samples had sufficient time to amplify and generate a detectable fluorescent signal. The relative abundance of lionfish was extrapolated using the LightCycler® 96 analysis package's test for absolute quantification. The analysis compared the known stock concentrations that were generated from the serial dilutions to the unknown concentration of eDNA samples.

Sequences were obtained from all samples that had strong fluorescence in the 2% agarose gel to ensure that only lionfish DNA was being amplified as described above. Generated sequences were cleaned and aligned in Geneious, and validated in NCBI-BLAST. All NCBI-BLAST results were required to have a percent cover of at least 95% with *P. volitans* in order to decisively conclude that the amplification was a result of lionfish DNA.

Sampling and Quantifying eDNA at FGBNMS and Artificial Reefs

Water Collection

Water samples were collected by scientific divers between June and July 2017. Sites sampled included East Flower Garden Bank and artificial reefs near to the FGBNMS (Figure 2). All water samples were taken at depth in a gallon Ziploc bags. Bags were put in freezer immediately after collection and stored in -40°C freezer for eight to ten months depending on collection date. Once on the surface the collection bag was double bagged to prevent leakage, and both the inner and outer bags were labeled.

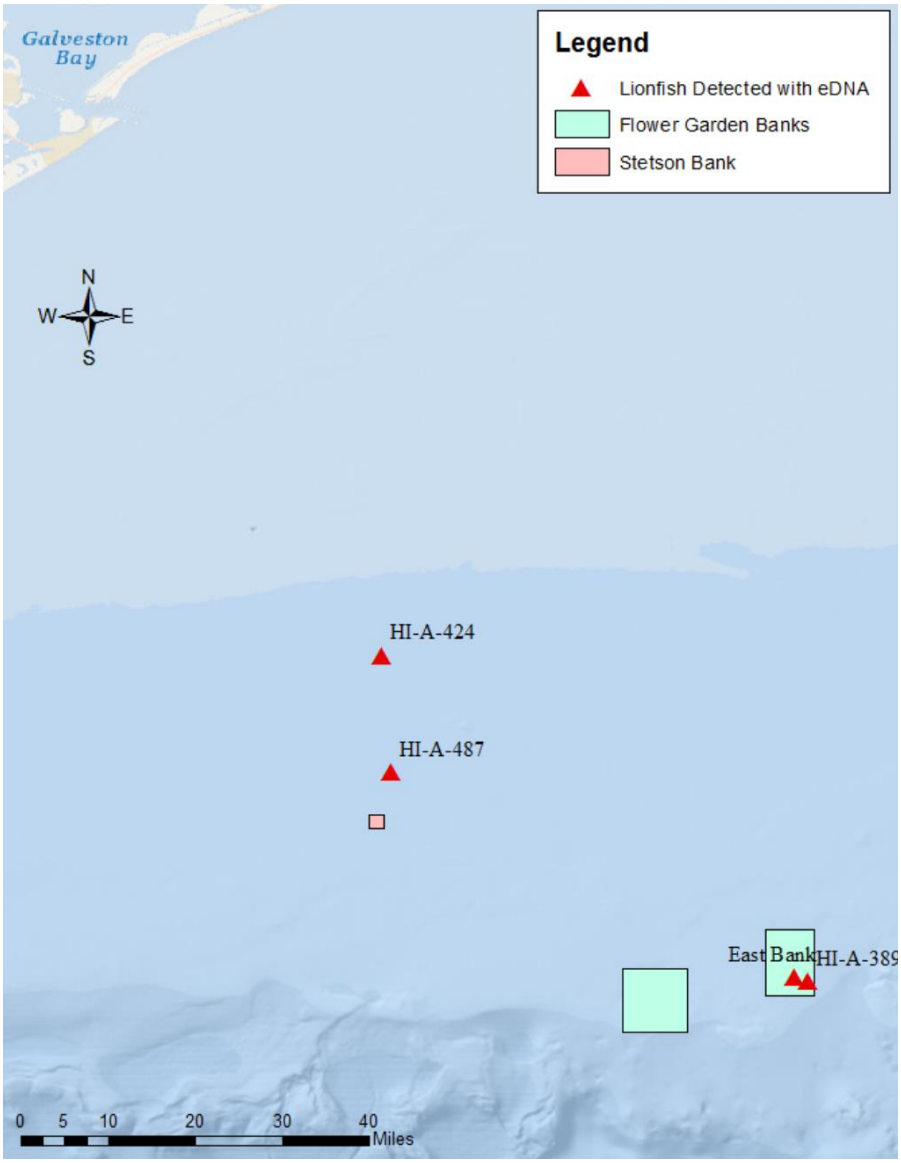

Figure 2. Locations off the Texas coast where water samples were taken.

eDNA Extraction and Quantification

Frozen bags of water were allowed to thaw slowly in coolers, and all bags were double bagged to prevent losing any of the water sample. After samples had thawed thoroughly, the entire 1.5 gallons of water collected was filtered using the same Nalgene® Filter Holder with Receiver and 0.2µm filter paper. Filter apparatuses that were used were autoclaved between each

use to prevent contamination. Filtering protocol was ensured to be identical to filtering protocol with Moody Gardens samples.

DNA was extracted from the filter using Qiagen Puregene Kit and followed manufacturer's instructions. Extraction of DNA from the filter paper was done in triplicate, and protocol was identical to what was done with the Moody Gardens Samples. It was noted if anything abnormal formed on the filter paper. After extraction, the DNA was amplified through PCR using the same reaction and program used during Moody Gardens testing. Medium 2% agarose gels were run to ensure that the targeted amplicon was what was actually being amplified.

RT PCR was run to determine the relative quantification of lionfish eDNA in the water samples. Conditions of PCR amplification were the same as those employed for Moody Gardens samples. RT PCR analyses were completed with the LightCycler® 96 to determine the absolute concentration by comparing the Cq value and concentration mean of each sample.

In some experiments the negative control generated a fluorescent signal. To determine if this was due to cross-contamination of the targeted segments the amplified products were subject to high resolution melting analysis (HRMA) to determine curves shapes and melting temperatures using the LightCycler® 96. The program used was set to 95°C for 30 seconds, 40°C for 30 seconds, 65°C for 1 second, and 95°C for 1 second. The results were analyzed in the LightCycler® 96 HRMA analysis package. A negative control that amplified in RT PCR, but which melted at a temperature below 70°C and with curves that were not consistent with those of the targeted product were assume to correspond to primer-dimers, and not due to cross-contamination.

Sequences were generated using the Zymo Research DNA Sequencing Clean-up Kit™ and followed manufacturer's instructions. Sequences were generated as stated above. All sequences were cleaned and annotated in Geneious, then validated with NCBI BLAST. Abnormalities in HRMA curves were compared to sequences.

Concentration values of lionfish eDNA were then compared against the relative quantification curve developed for the D-loop. Water samples that returned a positive result for lionfish presence were compared to visual diver surveys to determine which method would be best for future lionfish detection.

CHAPTER III

RESULTS

Primer Design using Bioinformatic Analysis

Table 1. Primers generated with bioinformatic programs.

Name	Predicted Gene	Sequence (5'→3')	Size (bp)	Tm (°C)	Targeted Fragment Size (bp)	Annealing Temperature (°C)
PV_D-loop_1F	D-loop	GGAAAACATAAGCGGGAG	18	50.7	217	50.0
PV_D-loop_2R	D-loop	CGAGAGAGGGACACTTAG	18	51.1		
PV_ATP6_1F	ATP6	CCGACGGCCCGATGATTA	18	57.3	244	57.0
PV_ATP6_2R	ATP6	CGAGAGCATGGGTGGGTT	18	57.6		
PV_ND2_1F	ND2	GCACCACCACCACATTTG	18	54.7	906	54.0
PV_ND2_4R	ND2	GTGTAGAGGTGGCAAGGG	18	55.3		

Bionformatic analyses using ecoPrimers and ecoPCR produced three sets of lionfish specific primers located in the mitochondrial D-loop, ND2, and ATP6 regions (Table 1). All of the primers selected for testing had one base pair mismatch with *P. miles.* The single mismatch should allow for *P. miles* also to be amplified. All of the primers resulted in a 96% to 100% query cover with *P. volitans* and a 95% query cover with *P. miles*. The reported query cover was the amount of the queried sequence that overlapped with the subjected sequence. Primers within these conditions were selected because the one base pair mismatch would allow for both species to be amplified, and future studies could be done to test for species differentiation.

Selection of Optimal Mitochondrial Region

Experiments conducted with serial DNA dilutions indicate that minimum concentration of DNA required for amplification of lionfish was 10,000X corresponding to about 0.27ng/mL to 0.31ng/mL. DNA concentrations below this threshold were not visible in 2% agarose gels, and DNA sequences could not be generated.

Amplified DNA obtained from ten-fold serial dilutions was sequenced to validate that only lionfish were amplified. All sequences returned a 95-100% query cover with lionfish sequences deposited in GenBank. The sequences had a higher cover with *P. volitans* than *P. miles*.

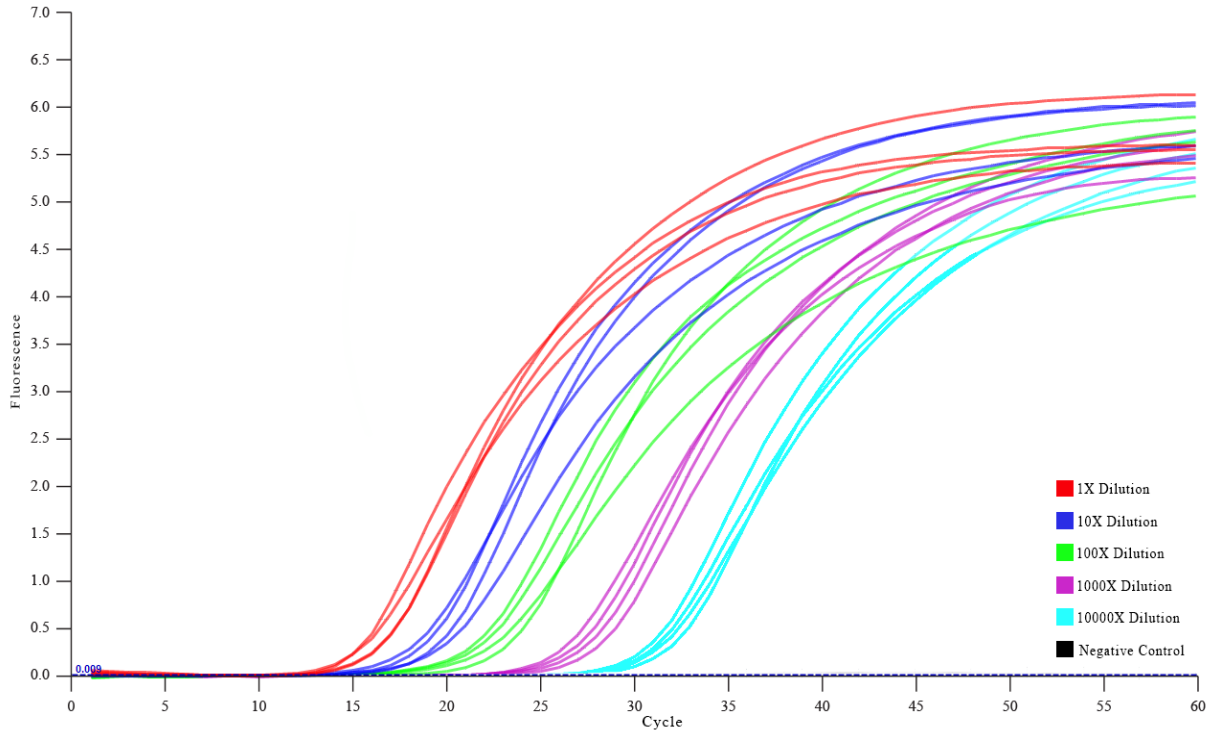

Figure 3. RT PCR amplification curve of D-loop region that was used to create a standard quantification curve for all eDNA quantification.

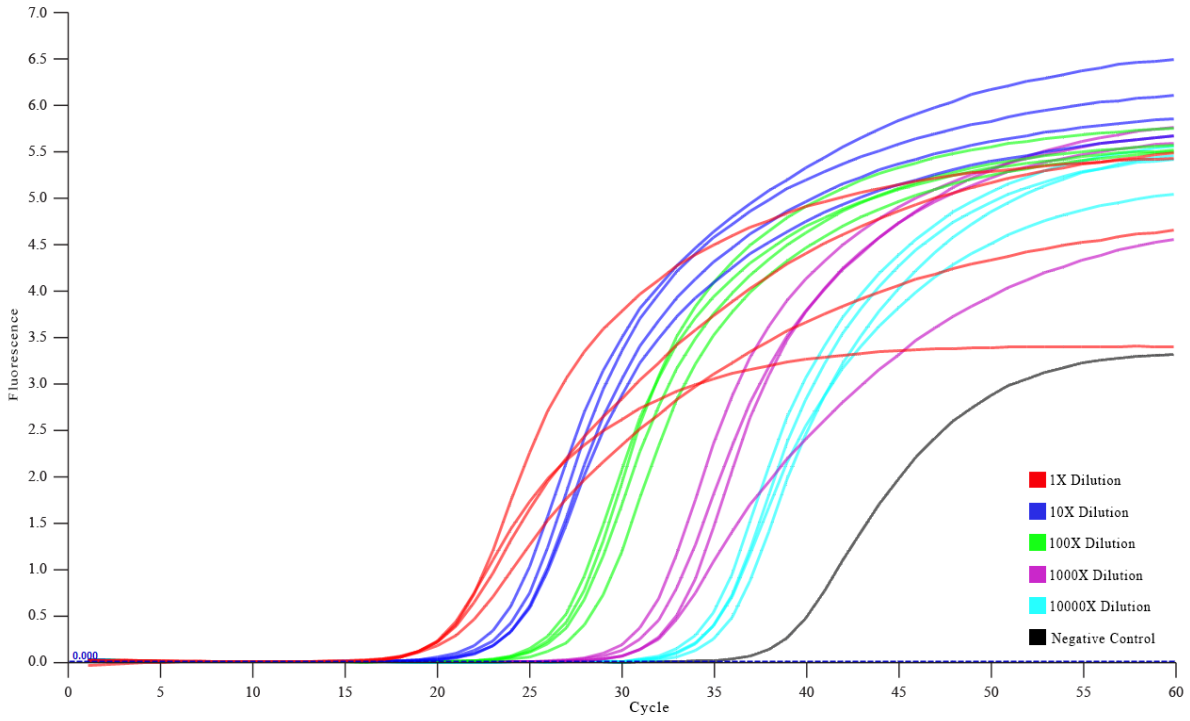

Figure 4. RT PCR amplification curve of ATP6 region.

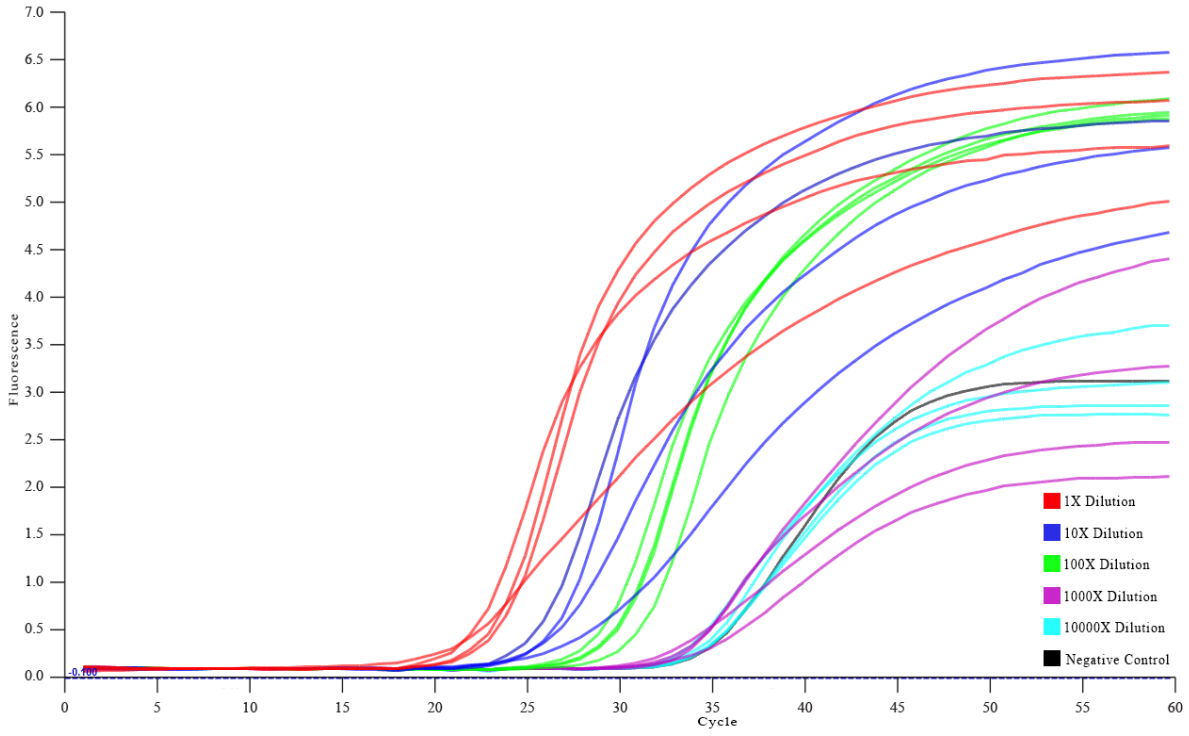

Figure 5. RT PCR amplification curve of ND2 region.

All serial dilutions were amplified in PCR and RT PCR. PCR was used to ensure the targeted DNA was being amplified, and RT PCR detected the relative concentrations of DNA from the sample. An amplification curve was generated based on the amount of fluorescent DNA relative to the cycle number. The ND2, ATP6, and D-loop region were amplified with RT PCR and the curves were analyzed. The D-loop produced the highest similarity in the amplification curves between replicates at given dilution, and also by producing a fluorescent signal at a certain number of cycles corresponding to DNA concentration (Figure 3). Further, the negative control of the D-loop region did not produced fluorescence. By contrast, the negative controls of the ND2 and ATP6 loci produced primer-dimers formed around cycle 35 when targeting the ND2 region and around cycle 40 when targeting the ATP6 region (Figure 4 and 5). Regions that formed of primer-dimers were not selected for further testing to prevent skewing results. For these reasons, the D-loop region was chosen for all further testing for eDNA.

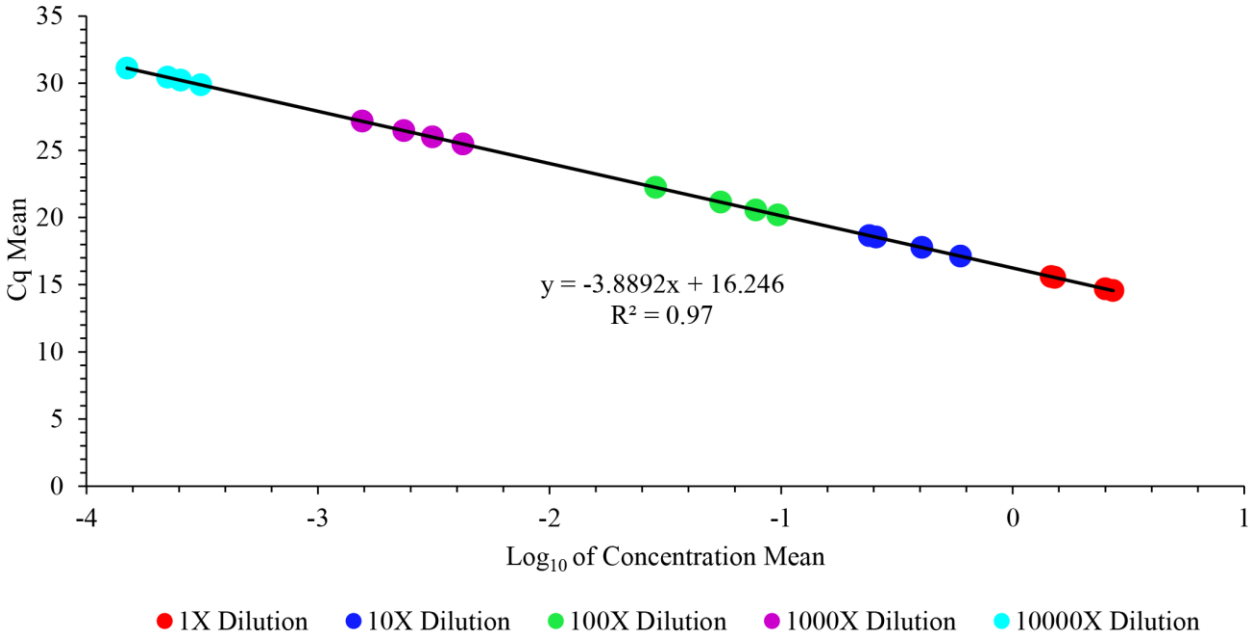

Figure 6. Quantification curve based on the D-loop serial dilution concentrations.

A standard curve for DNA concentrations was generated based off the results from the D-loop amplification curve. The standard quantification curve was used to determine the relative concentration of lionfish eDNA for all water samples tested in this study. Each dilution included four samples (East Bank, West Bank, Stetson Bank, and HI-A-270). Figure 6 shows that, as expected, the concentration of DNA present decreased as the sample became more dilute.

Testing Genetic Assay in Controlled Environment at Moody Gardens Aquarium

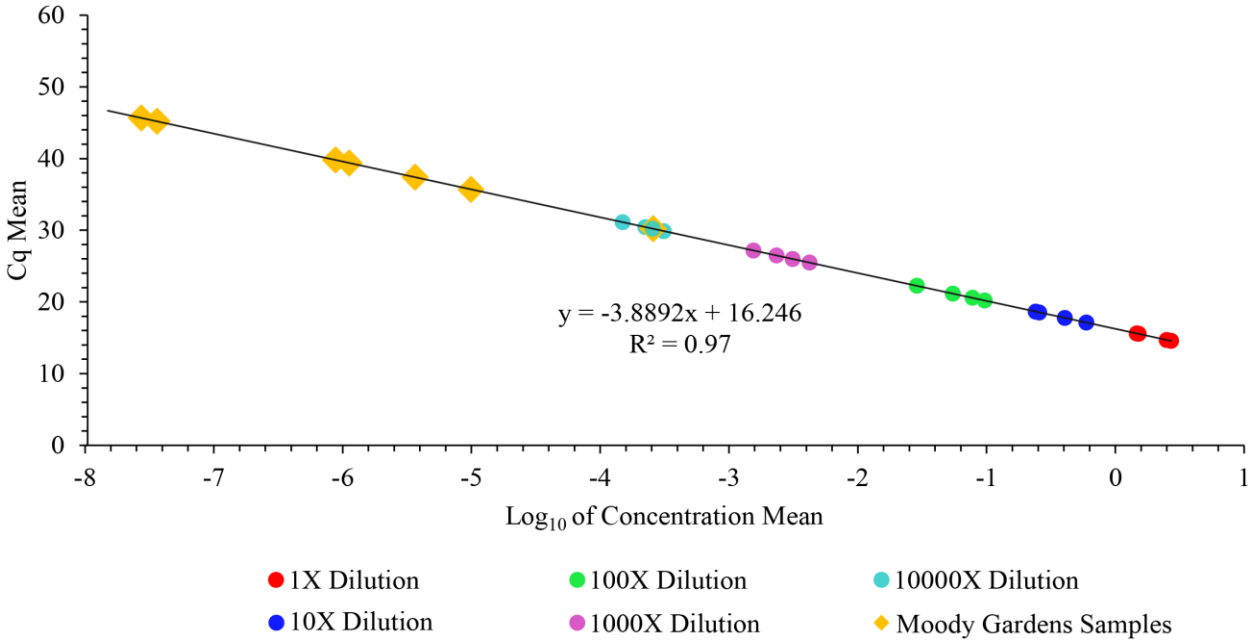

Figure 7. The log of the mean concentration of lionfish eDNA collected from different sized mesocosms compared to the standards generated from ten-fold serial dilutions.

Samples were collected from three different mesocosms at Moody Gardens Aquarium. The first mesocosm was 1,700 gallons and three lionfish were in the mesocosm. Lionfish DNA was amplified in PCR, and the PCR products were sequenced. The sequences from all three

mesocosm sampled were matched *P. volitans* with a 98% or greater cover and 95% *P. miles*. No matches with any other species were returned.

The mean concentration of lionfish eDNA was determined using RT PCR. The number of cycles in RT PCR was increased to 99 cycles because of delayed amplification due to more dilute samples. The relative quantity of eDNA was determined using the standard curve that was generated from the serial dilution standards. Figure 7 shows that samples collected from mesocosms at Moody Gardens required more amplification cycles than the serial dilutions, and had less DNA per microliter tested. The volume of the mesocosm had no effect on the relationship between the Cq value and the concentration mean. The concentrations of the samples generated in RT PCR were compared to concentration values generated from Nanodrop and Qubit. The samples were similar and no major differences were noted.

Quantification of Lionfish eDNA at FGBNMS and Artificial Reefs

Water samples collected from artificial reefs and one location in the FGBNMS were filtered, extracted, and amplified through PCR and RT PCR. The samples that were tested were from East Flower Garden Bank (EFGB), HI-A-424, HI-A-389, and HI-A-487.

DNA extractions had small pellets that formed at the bottoms of all tubes as artifacts of extraction. Samples filtered from EFGB had a large amount of salt formation on the filter prior to extraction, and after extraction the pellet was white.

The extracted samples were amplified in PCR and viewed in a 2% agarose gel. The gel showed strong amplification around 200 bp from all samples, and the strongest fluorescence was in samples from HI-A-389 and HI-A-424. No amplification was seen in the negative control.

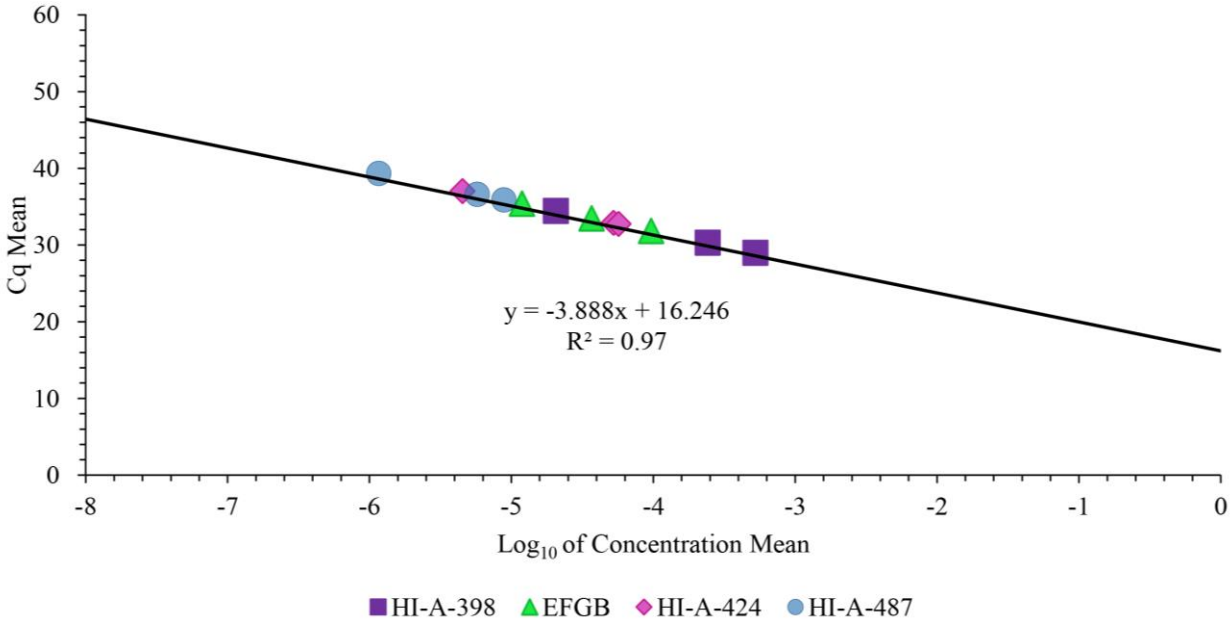

Figure 8. Concentrations of Lionfish eDNA at East Flower Garden Bank and Artificial Reefs.

RT PCR was run and the fluorescence curve was recorded. A fluorescence versus time curve was generated, and the relative concentration of DNA was extrapolated. Figure 8 shows the concentration of each of the triplicates at all of the locations sampled. The location with the greatest concentration of lionfish eDNA was HI-A-487 (Figure 8). The second greatest was EFGB, then HI-A-424, and lastly HI-A-389, as shown in Figure 8. It should be noted that no lionfish were sighted by divers at EFGB and HI-A-424, but lionfish were detected with eDNA at these locations as shown in Figure 9.

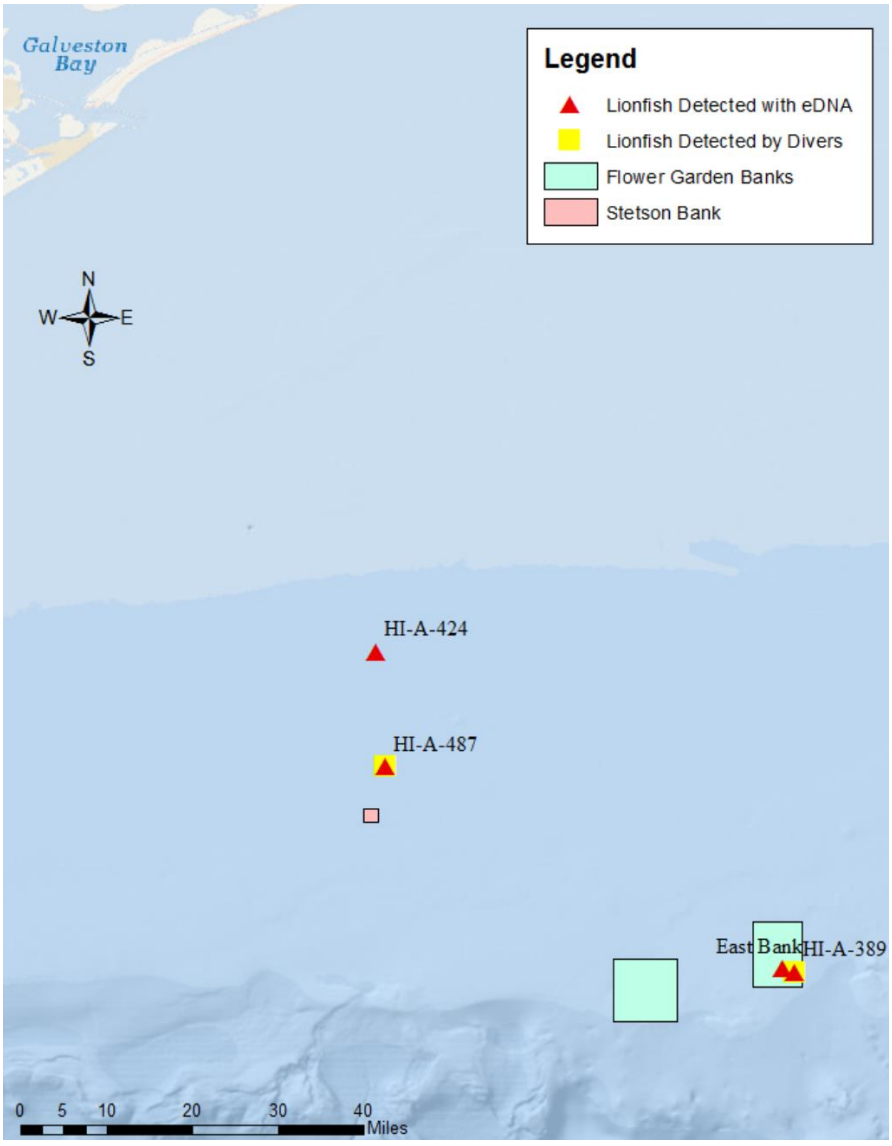

Figure 9. Locations were lionfish were detected with visual diver surveys compared to detection with eDNA.

The negative control in the RT PCR amplified, so HRMA was done to determine if amplification was corresponded to primer-dimer formation or cross-contamination. The results of HRMA showed that the amplification was likely the result of primer-dimers since the melting profile differed substantially in shape and melting temperature compared to the positives. In those instances, amplified products produced two peaks at about 79°C and at 81.5°C, perhaps

corresponding to two melting domains along the D-loop region. Sequences from these products matched *P. volitans* (97%) with a lower match to *P. miles* (95%), confirming that the amplification seen in PCR and RT PCR was due to lionfish eDNA. A gene-tree was produced in Geneious to determine if different haplotypes were present in the samples. The output showed the presence of two different haplotypes. The sequences that were generated were compared against the melting profiles for the corresponding amplicon obtained with HRMA.

CHAPTER IV

DISCUSSION

Since their initial introduction to the northwest Atlantic Ocean in the 1980s, lionfish have become one of the most successful marine invasive species, and have extended their range to the northwest Gulf of Mexico (Carballo-Cardenas, 2015; Courtenay, 1995; Nuttall et al., 2014). Lionfish's cryptic coloration, physiological abilities to survive in a broad range of depths and salinities, and lack of natural predators make lionfish such a successful marine invasive species (Albins & Hixon, 2013; K. A. Dahl et al., 2016; Schofield, 2009). These characteristics in combination with current methods of lionfish detection make getting an accurate measure of relative abundance nearly impossible (Anderson et al., 2017; Bacheler, Geraldi, Burton, Munoz, & Kellison, 2017; Johnston et al., 2016).

Eradication efforts, including lionfish derbies and traps, have shown to be effective at reducing the lionfish population (Johnston et al., 2016; Malpica-Cruz, Chaves, & Cote, 2016). Culling efforts target locations with larger lionfish populations, which are reported by visual diver surveys (Barbour, Montgomery, Adamson, Diaz-Ferguson, & Silliman, 2010; Malpica-Cruz et al., 2016). The results of this study show that extracting eDNA from water samples using lionfish specific primers to detect and quantify the abundance of lionfish is more effective than visual diver detection. The ability to effectively and affordably detect lionfish using the genetic assay developed in this study will give a more accurate assessment of the relative abundance of lionfish which will allow for a more streamlined effort in culling efforts by eliminating of diver bias.

The study was separated into four major phases in order to develop a genetic assay that prevented false negative and false positives. The four major phases were: development of lionfish specific primers with bioinformatic programs, serial dilutions to determine the optimal region for amplification, testing methods in a controlled environment, and detection and quantification of lionfish eDNA at FGBNMS and artificial reefs in the Gulf of Mexico.

Bioinformatic analysis was used to create of lionfish specific primers that would only amplify *P. volitans* but allowing a one base pair mismatch with *P. miles* so both species could be detected. Pervious lionfish genetic studies (Freshwater et al., 2009; Johnson, Bird, Johnston, Fogg, & Hogan, 2016; Meyer, Morrissey, & Schartl, 1994) have utilized 'universal' primers which were determined in here to co-amplify other species of teleost, and not only lionfish. The primers used in those studies are called 'lionfish specific', but after analyzing these primers in Primer-BLAST, the primers targeted ray finned fishes and wrasses (Figure 10). Due to the required specificity of primers in this project, new primers needed to be generated in order to prevent amplifying an array of teleost species. The primers generated and optimized in ecoPrimers and ecoPCR were analyzed in both Geneious and NCBI Primer-BLAST to prevent the amplification of any other species when testing water samples that was collected with other teleost species in the area.

Serial dilutions in the laboratory setting using different regions of the mitochondrial genome were tested to determine which region should be targeted for amplification. The three regions of the mitochondrial genome tested were: ND2, ATP6, and D-loop. Results from RT PCR showed the amplification curves, and the region with expected amplification pattern as well as lack of amplification in the negative control was determined to be optimal (Figures 3-5). The optimal region was determined to be the D-loop center region (Figure 3). Past studies have not

determined the optimal region for lionfish amplification (Freshwater et al., 2009; Hamner et al., 2007). These studies have assumed the cytochrome b to be the optimal region for amplification. This study is unique in determining the optimal region that can prevent any bias of amplification.

DNA extracted from filtered water samples that contained eDNA, followed by PCR and RT PCR that utilized lionfish specific primers, allowed for the detection and quantification of only lionfish regardless of the sample location (e.g., Moody Gardens Aquarium, artificial reefs, or FGBNMS). Water samples were collected from mesocosms of increasing size at Moody Gardens Aquarium, and all samples collected showed amplification of lionfish eDNA regardless of volume sampled. It should be noted that the volume of the mesocosm was not correlated with the concentration of eDNA detected. This may be due to the different shapes of the mesocosms compared and or the presence or absence of artificial structures, and the location where the water samples were obtained (e.g., water column versus surface). Nevertheless, the protocol developed in the laboratory setting, when applied to samples taken from Moody Gardens Aquarium, had positive results.

The protocols that were developed and tested at Moody Gardens Aquarium were applied to field samples taken from East Flower Garden Bank, HI-A-389, HI-A-424, and HI-A-487 as shown in Figure 2. These samples were taken by scientific divers, and they noted whether lionfish were seen during the dive. Lionfish were detected at all four sites that were sampled, but visual diver surveys only detected lionfish at two of the sites (HI-A-389 and HI-A-487) as shown in Figure 9. Studies have shown that divers are prone to under or over report the actual population of fishes (Murphy & Jenkins, 2010). Divers may not have seen lionfish at all these location because of diver bias from sources including depth limits, limited visibility, and inability to view lionfish due to cryptic coloration (Anderson et al., 2017; Malpica-Cruz et al.,

2016; Murphy & Jenkins, 2010). This study shows that capturing eDNA removes the chances of under or over reporting lionfish population due to diver bias, and gives a more accurate representation of the relative abundance.

The detection of lionfish at HI-A-424 is important to note because the artificial reef (a 371 foot long decommissioned vessel called the Kraken) sampled in this OCS (outer continental shelf) block was reefed in January 2017, six months prior to water collection. No lionfish sightings were reported at this location by divers, and lionfish were not expected to be present because of how little time had passed since the initial introduction of the reefed vessel. The detection of lionfish at HI-A-424 shows that lionfish, like other fishes including Red Snapper (*Lutjanus campechanus*), Mackerel Scad (*Decapterus macarellus*), and 46 other fish species reported by divers, were some of the first fishes to recruit to the newly introduced artificial reef.

This study was successful in developing a novel genetic assay based on RT PCR to detect and quantify lionfish in the field. Future studies should further assess the lionfish population across Stetson Bank, East Flower Garden Bank, and West Flower Garden Bank as well as both inshore and offshore artificial reefs to determine the extent of lionfish population in the northwest Gulf of Mexico using the genetic assay developed in the study. Other studies should be done to more accurately test if both lionfish species (*P. volitans* and *P. miles*) are present in the northwest Gulf of Mexico. One previous study reported that only *P. volitans* is present in the Gulf of Mexico (Johnson et al., 2016), but after preliminary HRMA analysis, *P. miles* may also be present. Using the primers developed in this study which have a one base pair mismatch between *P. miles* and *P. volitans*, the use of HRMA on eDNA samples targeting lionfish will allow a more efficient and accurate assessment of lionfish species in the northwest Gulf of

Mexico. Future studies should take note that water samples should be either filtered on site or collected and frozen in plastic bottles to prevent losing part of the sample during filtering.

CHAPTER V

CONCLUSION

The genetic assay developed in this study uses RT PCR to detect and quantify the relative abundance of lionfish by capturing eDNA in water samples. This method has proven to be a more accurate and efficient alternative to current methods of lionfish detection including ROVs and underwater visual surveys by divers. The genetic assay was developed in four major steps: bioinformatic analysis to create lionfish specific primers, laboratory methods to select the optimal region of the mitochondrial genome that should be targeted, testing methods in a controlled environment at Moody Gardens Aquarium, and applying methods at artificial reefs and the FGBNMS. The optimal region of the mitochondrial genome was determined to be the D-loop region and the primers used were PV_D-loop_1F (5'-GGAAAACATAAGCGGGAG-3') and PV_D-loop_2R (5'-CGAGAGAGGGACACTTAG-3'). The methods were tested in a controlled environment at Moody Gardens, and sources of false positives and false negatives were removed. These methods were then tested at East Flower Garden Bank, HI-A-424, HI-A-389, and HI-A-487, and all samples had detection and quantification of eDNA. The results of the study proved that utilizing eDNA is a more effective method for estimating the relative abundance of lionfish in the northwest Gulf of Mexico. The findings in this study can determine locations where culling activities should be focused. The novel protocols developed in the study can be modified for other species of aquatic organisms that may be cryptic, endangered, or invasive.

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APPENDIX

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CCATCTTAACATCTTCAGTG	20	51.28	40.00	6.00	6.00
Reverse primer	CATATCAATATGATCTCAGTAC	22	49.25	31.82	8.00	4.00

Products on target templates

>XM_016490554.1 PREDICTED: Sinocyclocheilus anshuiensis sugar phosphate exchanger 2-like (LOC107691751), transcript variant X3, mRNA

```
product length = 76
Reverse primer  1     CATATCAATATGATCTCAGTAC  22
Template        2617  ..C..G.......C........T  2596

Reverse primer  1     CATATCAATATGATCTCAGTAC  22
Template        2542  ..C..G.......C........T  2563
```

>XM_016490553.1 PREDICTED: Sinocyclocheilus anshuiensis sugar phosphate exchanger 2-like (LOC107691751), transcript variant X1, mRNA

```
product length = 76
Reverse primer  1     CATATCAATATGATCTCAGTAC  22
Template        2592  ..C..G.......C........T  2571

Reverse primer  1     CATATCAATATGATCTCAGTAC  22
Template        2517  ..C..G.......C........T  2538
```

>XM_020645823.1 PREDICTED: Labrus bergylta lysine demethylase 2A (kdm2a), mRNA

```
product length = 254
Forward primer  1     CCATCTTAACATCTTCAGTG  20
Template        1122  G..G.........A....A  1141

Forward primer  1     CCATCTTAACATCTTCAGTG  20
Template        1375  TT....C.....G.......  1356
```

Figure 10. NCBI Primer-BLAST results of primers used in past lionfish genomic studied. Note that none of the species targeted is *P. volitans* or *P. miles*.