CHARACTERIZATION OF GENETIC VARIATION OF THE GULF KILLIFISH (FUNDULUS GRANDIS) ALONG THE TEXAS COAST

An Undergraduate Research Scholars Thesis

by

AMANDA REYES

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ABSTRACT

Characterization of Genetic Variation of the Gulf Killifish (*Fundulus grandis*) Along the Texas Coast. (May 2015)

Amanda Reyes
Department of Marine Biology
Texas A&M University

Research Advisor: Dr. Jaime Alvarado-Bremer
Department of Marine Biology

The gulf killifish (*Fundulus grandis*) is a widely distributed cyprinodontiform of ecological importance in salt marshes of the Gulf of Mexico. Both the reproductive strategy and apparently limited dispersal capabilities should translate into strong phylogeographic association to natal sites. This study is the first to characterize the patterns of genetic differentiation among Texas populations using mitochondrial DNA sequence data. DNA was extracted from fin clips obtained from specimens sampled in two Texas locations, Galveston and Corpus Christi. Patterns of sequence variation and the phylogeographic association of haplotypes were characterized for 1,253 bp of sequence of the mitochondrial DNA genes ND2 and ND5, and for a segment of the D-loop region for 55 individuals. A strong phylogeographic signal was uncovered, with Galveston showing larger values of haplotypic and nucleotide diversities than Corpus Christi. AMOVA identified significant ($P < 0.05$) differentiation between Galveston and Corpus Christi samples using all loci, thus rejecting the null hypothesis of panmixia. The results of this study represent a valuable baseline for future assessments of *Fundulus grandis* variation along the Texas Gulf Coast, including a planned study of differences in genetic variation of colonizing fauna in natural and restored marshes.

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CHAPTER I

INTRODUCTION

The gulf killifish is a small cyprinodontiform found in estuaries from Florida to Laguna de Tampamachoco, Veracruz, Mexico (Hubbs et al. 2008). The gulf killifish is closely related to the mummichog (*F. heteroclitus*) –a model species widely used in toxicological, evolutionary and physiological studies. Although very little is known about the patterns of genetic differentiation of the gulf killifish, particularly along the coast of Texas, the egg stranding reproductive strategy displayed by this species (Greeley & MacGregor 1983), together with the limited dispersal capabilities of the members of the same genus (Sweeney et al. 1998; Skinner et al. 2005), appear to promote genetic differentiation among estuaries. A comparison of gulf killifish samples from Florida to Texas using microsatellite markers indicates a strong population subdivision between estuaries along the Gulf of Mexico coast (Williams et al. 2008).

With the exception of the characterization of the cytochrome b sequences employed to reconstruct the phylogeny of the genus *Fundulus* (Bernardi and Powers 1995), or the comparison of populations along the Florida coast (Gonzalez et al. 2009), there are no published studies assessing the patterns of genetic variation of gulf killifish using mtDNA data, and in particular along the Texas coast. Further, the rate of substitution for cytochrome b gene in *Fundulus* is slower than other mtDNA genes or the D-loop region (Whitehead 2009), making the characterization of faster evolving mtDNA segments desirable.

The purpose of this research is two-fold. First, to characterize the patterns of genetic variation in three mtDNA genes in the gulf killifish. Second, to establish the phylogeographic association of mtDNA haplotypes to estuaries located along the gulf coast of Texas. Genetic variation will be characterized at three mitochondrial genes, namely nitrogen dehydrogenase subunits 2 (ND2) and 5 (ND5), and the D-loop region because of their mutation rates (Whitehead 2009).

Objectives

Objective 1: To extract high quality DNA from previously obtained fin clips of *F. grandis* and use PCR and cycle sequencing techniques to isolate, amplify, and sequence three mtDNA genes, namely ND2 and ND5 and the d-loop region. These sequences will also be used to confirm the species identity.

Objective 2: To use patterns of variation to determine the population structure of *F. grandis* along the Texas gulf coast in order to make inferences about the phylogeography of this species.

Hypothesis 1: Nucleotide sequences of mtDNA should contain sufficient levels of inter-species differentiation in *Fundulus grandis*, to be diagnostic for species identification.

Hypothesis 2: Due to the expected strong association to estuaries concordant with their limited dispersal capabilities and reproductive strategy, it is expected that the patterns of variation in the mtDNA will reject the null hypothesis of panmixis of the *F. grandis* along the Texas coast.

CHAPTER II

METHODS

Sample Collection

Prior to the beginning of this study, fin clips were collected from gulf killifish in two sampling localities along the Texas gulf coast. Galveston Bay fish were captured at the Reitan Marsh. The sample from Corpus Christi Bay was collected near the Texas A&M Corpus Christi campus. Specimens were captured using cylindrical minnow traps baited with dog food. The fish were identified in the field and a small segment of the dorsal fin was clipped and preserved in 70% ethanol. Fishes were immediately released back as close as possible to their capture location.

DNA Extraction and Sequencing

In the laboratory, DNA was purified and extracted using proteinase K digestion of fin tissue followed by ethanol precipitation, as outlined in Grieg (2000). PCR included an initial denaturing step at 94^{o}C for 2 minutes followed by 35 cycles of denaturing at 94^{o}C for 20 seconds, annealing at 45^{o}C for 45 seconds, and extension at 72^{o}C for 30 seconds, with a final extension step at 72^{o}C for 1.5 minutes. Approximately 380 bp of D-loop were amplified using primers L15998-Pro-FG (5'-CGCCCCTAGCTCCCAAAGCTA- 3') and CSBDH-FG (5'-TGAAATAGGAACCAAATGCCAG-3') (Espinoza and Alvarado Bremer unpublished). The design of these two *F. grandis* specific primers was based on the piscine universal D-loop primers (Alvarado Bremer et al. 1995). In addition, approximately 400 bp of ND2 were amplified using primers L4173-ND2-FG (5'-CATCATCCCCGAGCCGTTGA-3') and H4634-ND2-FG (5'-GGAAGGTTAAGGATGGGAAG-3'); and approximately 500 bp of ND5 were

amplified using primers L12137-ND5-FG (5'-GCAGAAACGGTAGTGTCCAC-3') and H12717_ND5-FG (5'-GTACTTGAATGCAGTAGGGC-3') (Espinoza and Alvarado Bremer, unpublished). These ND2 and ND5 primers were based on the original primers targeting these two mtDNA genes in *F. heteroclitus* (Whitehead 2009).

PCR products were diluted 1:10 and sequenced using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA). Sequencing reactions were performed in 10µL reaction volumes containing 0.5µL of BigDye Terminator v.3.1, 2µL of BigDye 5x Sequencing Buffer, 1µL of 1X concentration light chain primer specific to each segment, and 5.5µL of sterile ddH_2O. The step-cycling program consisted of an initial denaturing step at 95ºC for 1 minute, followed by 14 cycles of denaturing at 95ºC for 10 seconds, annealing at 50ºC for 5 seconds, and extension at 60ºC for 1minute 15 seconds, 4 cycles of the same denaturing and annealing steps, but extension at 60ºC for 1 minute 30 seconds, and 4 more cycles of the same denaturing and annealing steps, with extension at 60ºC for 2 minutes. Reactions were then cleaned using the ZR DNA Sequencing Clean-up Kit (Zymo Research Corp., Irvine, CA). Samples were characterized in a 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA) following the manufacturer's instructions. Multiple sequence alignments were conducted in Geneious Pro v.6.1 (Biomatters Ltd., Aukland, NZ).

Data Analyses

Sequence alignments for were imported into MEGA v.6.06 (Tamura et. al 2013). A BLAST search was conducted for each sequence to determine if species identification was possible with any or all sequences characterized. MEGA v6.06 was used to select the best model of nucleotide substitution for each gene (Nei and Kumar 2000). Additionally, MEGA v6.06 was used to

construct Maximum Likelihood (ML) trees to identify phylogeographic association among localities. The full mitochondrial genome for an individual belonging to the sister taxa, *Fundulus heteroclitus*, was downloaded from the NCBI database (Accession FJ445398; Whitehead 2009), and orthologous segments were used as outgroup in the corresponding ML trees.

ARLEQUIN V.3.5 (Excoffier and Lischer 2010) was used to calculate Tajima's *D*, the number of haplotypes (M), haplotypic diversity (*h*), nucleotide diversity (π); number of segregating sites (S), and nucleotide composition for each loci. Analyses of molecular variance (AMOVA; Excoffier et al. 1992), were also conducted in ARLEQUIN.

CHAPTER III

RESULTS

Aligned sequences were inspected to identify possible ambiguities and misalignments and, if present, were corrected by hand. Only those sequences whose ambiguities could be resolved in this manner were kept for analysis. A total of 144 sequences were determined (**Table 1)**, corresponding to 46 D-loop sequences 376bp long (23 Corpus Christi, 23 Galveston), 48 ND2 sequences 392bp long (24 Corpus Christi, 24 Galveston), and 50 ND5 sequences 485bp long (20 Corpus Christi, 30 Galveston). Concatenated sequences (1,253 bp long) were constructed only for individuals that had high quality sequences for all three loci (n=35, 16 Corpus Christi, 20 Galveston). BLAST searches for D-loop, ND2, and ND5 sequences all came back with *F. grandis* mtDNA as the top two best matches. The next nearest match in all instances was *grandis's* sister species, *F. heteroclitus*.

The best models of nucleotide substitution based on Bayesian Information Criterion (BIC) were the Tamura 3-parameter model (Tamura 1992) with a gamma distribution of 0.05 for the D-loop, and the same model with a uniform distribution for ND5. For ND2, the best model was the Kimura 2-parameter model (Kimura 1980). The HKY model (Hasegawa, Kishino, and Yano 1985) with a gamma distribution of 0.05 was the best model for the concatenated sequences. Each of these four models were used accordingly to reconstruct ML gene trees and all other calculations performed in MEGA. Since the Tamura 3-parameter and the HKY models are unavailable in ARLEQUIN, D-loop, ND5, and concatenated sequences were analyzed in that program using Tamura and Nei (1993) distances, with gamma parameters where appropriate.

The ML trees for *F. grandis* D-loop (**Figure 1**), ND2 (**Figure 2**), ND5 (**Figure 3**) and for concatenated sequence data (**Figure 4**) all reveal a certain degree of genetic differentiation between the two sampling locations. D-loop reveals the strongest pattern of differentiation, with Galveston containing more haplotypes, and with many of these basal, and thus ancestral to the Corpus Christi haplotypes.

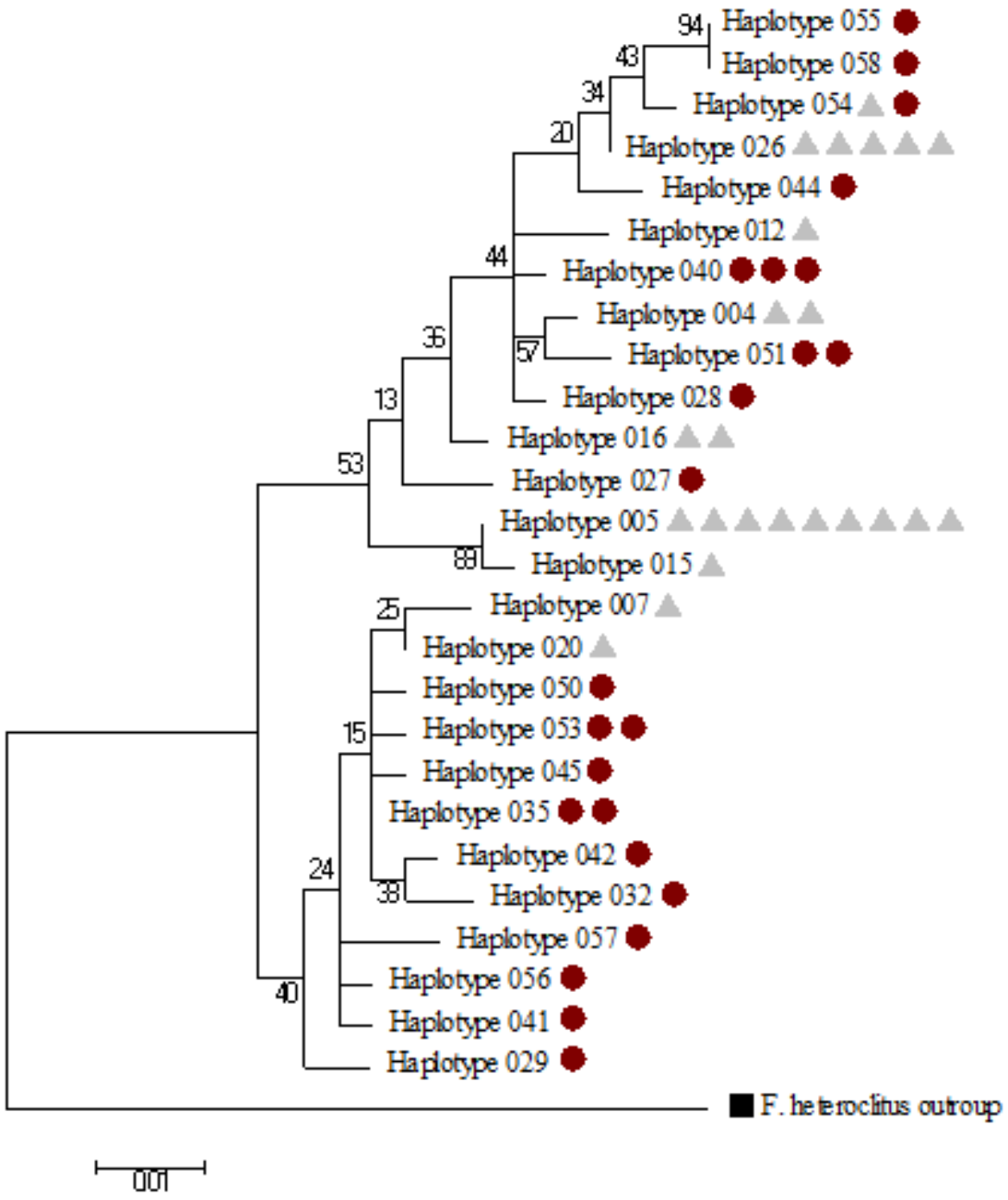

Figure 1: ML consensus tree showing the relationship of *F. grandis* D-loop haplotypes. Symbols at the tips of terminal branches represent locality of capture as follows: ▲ Corpus Christi, ● Galveston, and ■ the outgroup, *F. heteroclitus*. The number of times each symbol is repeated represents number of individuals sharing a particular haplotype. Bootstrap support is denoted at the nodes of the corresponding branches.

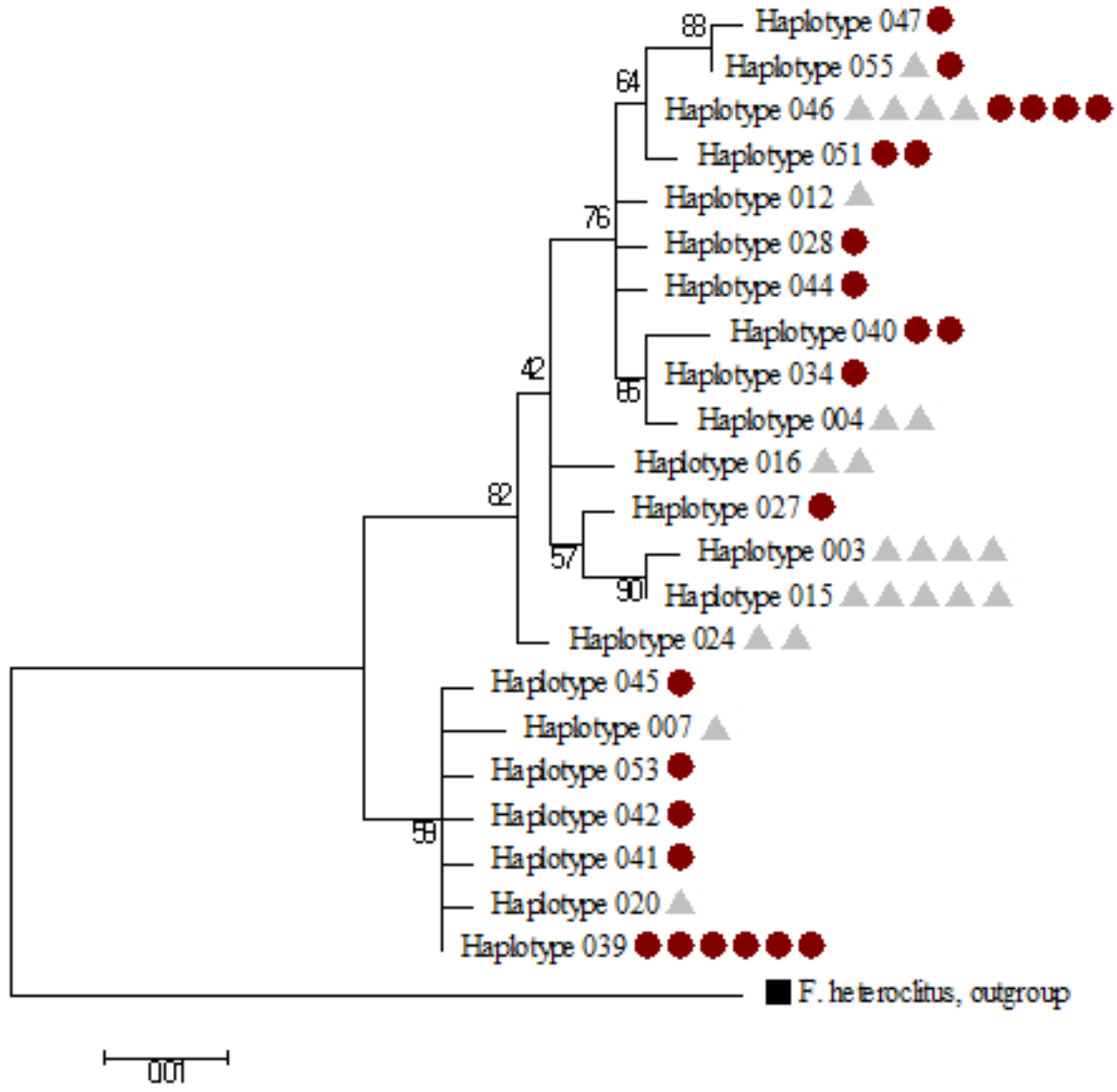

Figure 2: ML consensus tree showing the relationship of *F. grandis* ND2 haplotypes. Symbols at the tips of terminal branches represent locality of capture as follows: ▲ Corpus Christi, ● Galveston, and ■ the outgroup, *F. heteroclitus*. The number of times each symbol is repeated represents number of individuals sharing a particular haplotype. Bootstrap support is denoted at the nodes of the corresponding branches.

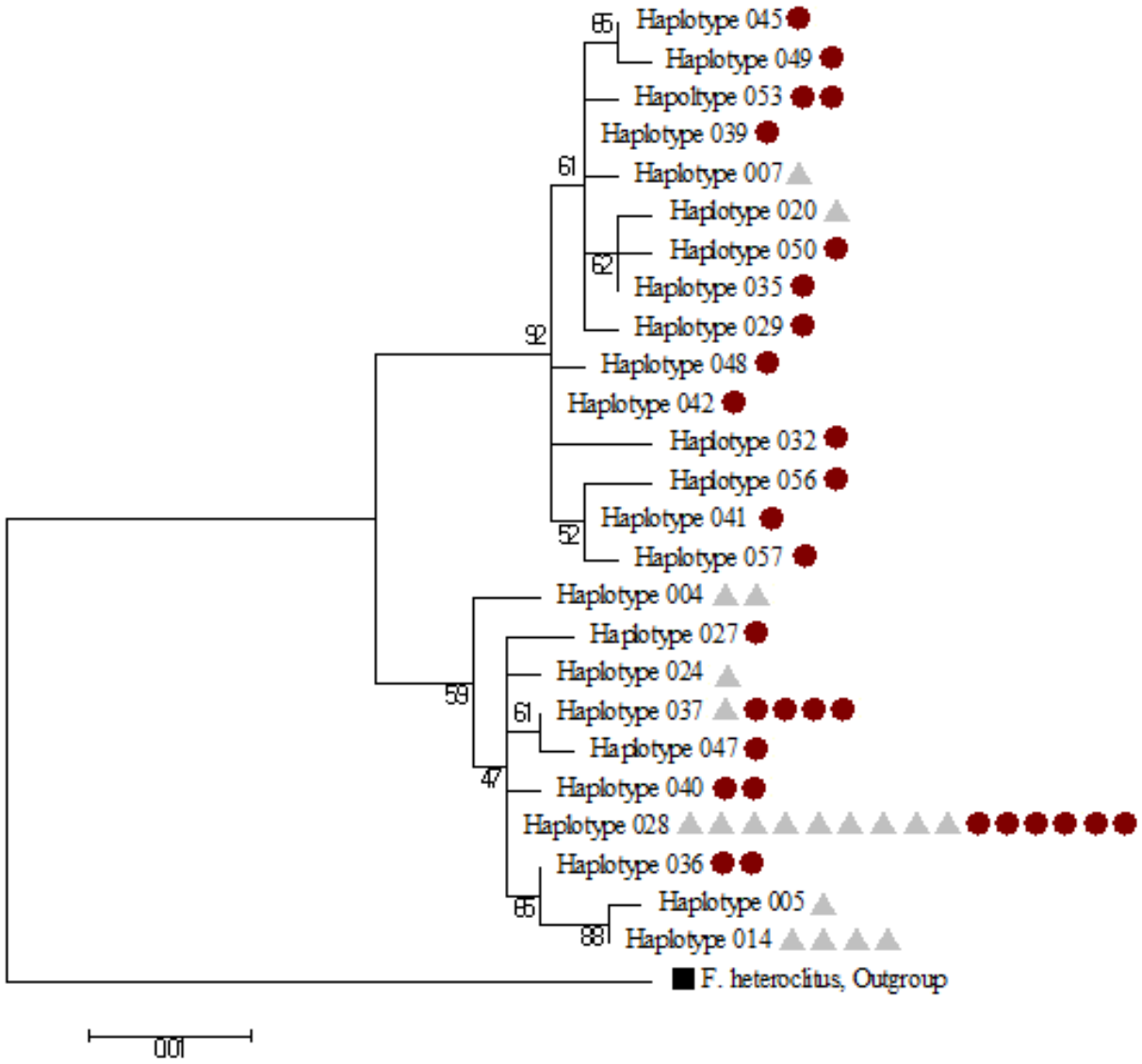

Figure 3: ML consensus tree showing the relationship of *F. grandis* ND5 haplotypes. Symbols at the tips of terminal branches represent locality of capture as follows: ▲ Corpus Christi, ● Galveston, and ■ the outgroup, *F. heteroclitus*. The number of times each symbol is repeated represents number of individuals sharing a particular haplotype. Bootstrap support is denoted at the nodes of the corresponding branches.

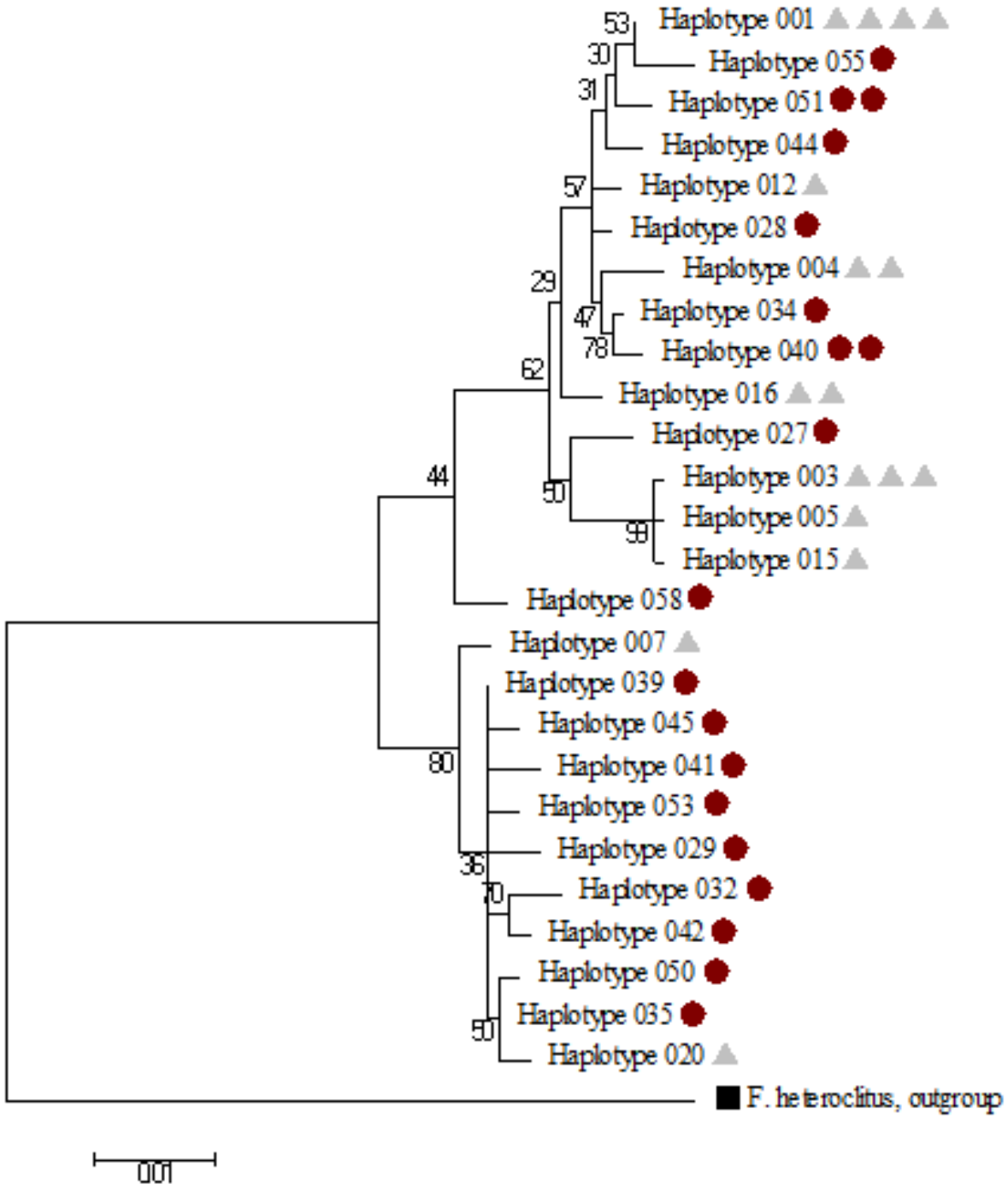

Figure 4: ML consensus tree showing the relationship of *F. grandis* concatenated sequences. Symbols at the tips of terminal branches represent locality of capture as follows: ▲ Corpus Christi, ● Galveston, and ■ the outgroup, *F. heteroclitus*. The number of times each symbol is repeated represents number of individuals sharing a particular haplotype. Bootstrap support is denoted at the nodes of the corresponding branches.

Tajima's *D* for all loci and populations tested was near 0 ($-2 < D < 2$), and none of the values were considered significant ($P > 0.05$), indicating that the loci used in these analyses are not under selection (Nei and Kumar 2000; Tajima 1989). For all loci tested, Galveston samples contained a greater number of polymorphic sites (S), haplotypes (M), haplotypic (*h*) diversity, and nucleotide (π) diversity than Corpus Christi samples. Among the single loci samples, D-loop showed the greatest variation, followed by ND2, then ND5. All loci sequenced had a greater A and T content (57.8% - 68.3%) than G and C (31.7% - 42.2%) (**Table 1**).

Table 1: Summary of statistics for D-loop, ND2, ND5, and concatenated sequences of *Fundulus grandis*

Sample	*N*	M	*h* (SD)	π (SD)	S	*D* (*P*)	% AT	% GC
D-loop (pooled)	46	26	0.947 (0.021)	0.023 (0.012)	35	-0.161 (0.528)	68.3	31.7
Corpus Christi	23	9	0.810 (0.065)	0.016 (0.009)	21	0.048 (0.541)		
Galveston	23	18	0.977 (0.020)	0.024 (0.013)	31	-0.140 (0.503)		
ND2 (pooled)	48	22	0.938 (0.018)	0.018 (0.009)	35	-0.394 (0.399)	57.8	42.2
Corpus Christi	24	10	0.891 (0.037)	0.014 (0.008)	24	-0.495 (0.357)		
Galveston	24	14	0.917 (0.040)	0.019 (0.010)	26	0.199 (0.645)		
ND5 (pooled)	50	25	0.898 (0.035)	0.013 (0.007)	37	-0.73 (0.237)	59.7	40.3
Corpus Christi	20	8	0.774 (0.083)	0.009 (0.005)	21	-1.105 (0.143)		
Galveston	30	19	0.945 (0.027)	0.014 (0.008)	29	-0.149 (0.485)		
Concatenated (pooled)	35	26	0.978 (0.013)	0.018 (0.009)	100	-0.339 (0.403)	61.7	38.3
Corpus Christi	16	9	0.908 (0.048)	0.014 (0.007)	60	-0.163 (0.458)		
Galveston	19	17	0.988 (0.021)	0.019 (0.010)	80	0.050 (0.571)		

N, No. of sequences; M, No. of haplotypes; *h*, Haplotypic diversity; π, Nucleotide diversity; *S*, No. of segregating (polymorphic) sites; SD, standard deviation; *D*, Tajima's *D* neutrality test with probability value (*P*)

In all AMOVAs, the majority of variation for each loci was found within populations (81.98% - 86.49%). However, a relatively large amount of significant among-group variation was uncovered. Differences among groups was highest for D-loops (18.02%), followed by ND5 (16.68%), and then ND2 (13.51%). The AMOVA results were significant ($P < 0.05$) for all loci tested (**Table 2, A-D**).

Table 2 (A-D): AMOVA results for each loci for *Fundulus grandis*

Table 2A: D-loops

Source of Variation	d.f.	SS	% of Variation
Among Populations	1	45.385	18.02
Within Populations	44	329.757	81.98
	Fst: 0.180		**P:** 0.002 (0.001)

Table 2B: ND2

Source of Variation	d.f.	SS	% of Variation
Among Populations	1	15.665	13.51
Within Populations	46	151.749	86.49
	Fst: 0.135		**P:** 0.005 (0.002)

Table 2C: ND5

Source of Variation	d.f.	SS	% of Variation
Among Populations	1	17.526	16.68
Within Populations	48	144.901	83.32
	Fst: 0.167		**P:** 0.004 (0.002)

Table 2D: Concatenated Sequences

Source of Variation	d.f.	SS	% of Variation
Among Populations	1	65.199	13.68
Within Populations	48	573.37	86.21
	Fst: 0.137		**P:** 0.026 (0.005)

CHAPTER IV

CONCLUSIONS

In all instances, sequences submitted to GenBank using BLAST searches returned matches to *F. grandis* mitochondrial genome. Accordingly, sequences of the three segments of mtDNA used in this study provide sequences diagnostic for the identification of *F. grandis*, as hypothesized. The variation between populations, as obtained through AMOVA, ranged from 13.5% for ND2 to 18.0% for D-loop sequences. The differentiation between populations was highly significant for all loci ($P < 0.005$) (**Table 2**). Accordingly, the null hypothesis of panmixia along Texas Gulf Coast is rejected for *F. grandis*. These results are concordant with the limited dispersal capabilities and high site fidelity reported for other members of *Fundulus* (Sweeney et al. 1998; Skinner et al. 2005) and assumed to operated also in *F. grandis*. Further, this result is in agreement with the high degree of population subdivision of the Gulf killifish among estuaries along the entire Gulf Coast using microsatellites (Williams et al. 2008).

The characterization of genetic variation within each sample revealed that there is much higher levels of haplotypic (h) and nucleotide (π) diversities in Galveston than Corpus Christi (**Table 1**). ML trees showed that Corpus Christi contains a smaller number of haplotypes, with some of these repeated several times (e.g., Haplotype 028). Each of the individual ML gene trees identifies two very well supported clades. One clade contains a nearly equal representation of Galveston and Corpus haplotypes, whereas the second clade contains mostly Galveston haplotypes, with only two haplotypes (007 and 020) detected in two specimens collected in

Corpus Christi. In all ML tree reconstructions, except with ND5, this second clade occupies a more ancestral position (**Figures 1-4**) as indicted by shorter, and thus basal, branches with respect to the outgroup. The lower genetic diversity in mtDNA may indicate that the Corpus Christi population has undergone a recent bottleneck event (Bernatchez et al. 1989).

Values of nucleotide diversity indicate that the highest variation was contained in the D-loop (9.3% of sites variable), followed by the protein coding regions for ND2 and ND5 (8.9% and 7.6% of sites variable, respectively). These findings conflict with a previous study by Whitehead (2009), who reported greater variability in protein coding regions of the mtDNA of *Fundulus* spp. than the D-loop. It should be noted, however, that Whitehead (2009) found a greater proportion of variable sites, overall, than in this study (13% versus 9.3% for D-loops; 21% versus 7.6-8.9% for protein coding regions). The differences between these studies may be due to the fact that in Whitehead's (2009), the entire mitochondrial genome was used, while this study analyzed segments of the D-loop region, and the ND2, and ND5 genes. However, the most likely explanation of the disparity is that in Whitehead's (2009) study, five species of *Fundulus* were compared, whereas in here, only on *F. grandis* was characterized

Finally, the results of this study provide a baseline for the levels of genetic variation in mtDNA of *F. grandis* found within and between different localities along the Texas Gulf Coast. This baseline will be an integral part of a planned study involving comparisons of genetic diversity of colonizing fauna in natural and restored marshes on the Texas Gulf Coast. A stronger sampling scheme, including more locations to the east of Galveston Bay, between Galveston and Corpus Christi, and farther south of Corpus Christi along the Texas Coast could greatly enhance the data

provided in this study. Additional studies should focus on increased sampling coverage, and possibly including other common marsh fauna with differing life histories for comparison purposes.

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