COMPARATIVE PHYLOGEOGRAPHY, HISTORICAL DEMOGRAPHY, AND POPULATION GENOMICS OF COLONIZING FAUNA IN RESTORED AND NATURAL *Spartina* SALT MARSHES ALONG THE NORTHERN GULF OF MEXICO

A Dissertation

by

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ABSTRACT

Salt marshes provide vital ecosystem services to nearby coastal communities and have suffered accelerated rates of loss, worldwide. A number of biotic and abiotic factors contribute to the success of restoration projects, but restoration focuses primarily on hydrology and plant cover. Gulf Killifish (*Fundulus grandis*), daggerblade grass shrimp (*Palaemon pugio*; previously *Palaemonetes pugio*), and phloem-feeding planthoppers (*Prokelisia marginata*) are three ecologically important and abundant species in salt marshes, for which there is a paucity of data on population genetics or the effects of restoration projects on levels of genetic diversity. In this study I: 1) developed a short amplicon high resolution melting assay (SA-HRMA) for molecular identification of two morphologically similar planthopper species, *Prokelisia marginata* and *Prokelisia dolus*; 2) compared population structure and historical demography for the Gulf Killifish, daggerblade grass shrimp, and *Prokelisia* planthopper in the north and west Gulf of Mexico (Gulf) using mitochondrial DNA (mtDNA) sequences; and 3) investigated levels of genetic diversity of Gulf Killifish inhabiting a reference marsh compared with restored marshes of differing ages and distances from the reference in Galveston Bay, Texas, using mtDNA sequences and nuclear single nucleotide polymorphism (SNP) data generated via double digest restriction site associated DNA (ddRAD) sequencing.

A 60 bp fragment of Cytochrome C Oxidase Subunit I (COI) was used in a SA-HRMA assay to differentiate *P. marginata* from *P. dolus* via a minimum separation of ~1.7°C between the melting peaks for each species. A high-throughput test (n=518) of the HRMA resulted in clearly diagnostic melting curves for species assignment of 213 *P.*

dolus individuals and 296 *P. marginata* individuals, with only 9 (1.7%) amplification failures. Mitochondrial sequence data revealed high levels of haplotypic diversity, evidence of isolation by distance (IBD), and population structure at regional levels, along with two distinct phylogroup associations and concordant distinct historical demography characteristics for Gulf Killifish in the Gulf. Grass shrimp and planthoppers displayed low levels of haplotypic diversity, and evidence of population structure, but both appear to contain snapshots of the total potential diversity for these species in the Gulf. Gulf Killifish inhabiting restored and reference marshes in Galveston Bay displayed no evidence of population structure or IBD, but SNP data showed significantly ($p < 0.05$) lower levels of heterozygosity in the two youngest restored marshes compared to the reference, and a higher degree of inbreeding in the two young marshes. Overall, it was determined that levels of genetic diversity in Gulf Killifish inhabiting restored marshes are similar to that in a reference marsh, suggesting Gulf Killifish have enough dispersal potential for adequate gene flow between marshes up to 10km distant from each other.

DEDICATION

To my husband, Joel, you have been the best possible partner for whom a person undertaking their Ph.D. could ever hope. In all these years, you have never once resented the time and energy this undertaking required from me, and have even dedicated a substantial amount of your own energy to it. You tromped through hip-deep, sulfur-smelling mud to help me get samples, carried heavy boxes full of sampling gear through miles of mosquito-infested marsh for me, spent what should have been your days off in the lab helping me sort samples, stayed up to the wee hours of the morning helping me practice presentations, spent your spare time fixing broken lab equipment, built entirely new computers so I could analyze my data, and spent countless hours running errands and doing chores around the house that I did not have time to do, myself. All of that does not even include the strength of your emotional support over the years, cheering for all of my wins, supporting me through my sadness, providing stability through my anger, and gently pushing me to continue when I thought I was not able. I am so very grateful to have your love and support as we go through life together.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Background, significance, and current knowledge

Coastal wetlands function as wildlife habitat and provide vital ecosystem services to nearby coastal communities (Engle 2011). Despite their importance, the worldwide rate of coastal wetland loss has continued to accelerate over the past 30 years (Li et al. 2018), and coastal wetland area has decreased throughout much of the continental United States (Moulton et al. 1997, Dahl 2011). Over a five year period from 2004-2009, Dahl and Stedman (2013) reported a decline in coastal wetland area in the contiguous U.S. of over 360 thousand acres, with an average loss of roughly 72 thousand acres per year. Many federal, state, and local authorities have implemented restoration programs to protect and restore the important ecological and hydrological functions, and ecosystem services that intact wetlands provide (Moulton et al. 1997, Kennish 2001). Current policy in the United States requires the replacement of any jurisdictional wetland that has been degraded or lost with restored or constructed wetlands of the same size and ecological value (USDA 2011).

While there is recognition that success in wetland restoration is highly variable and dependent on a variety of biotic and abiotic factors, salt marsh restoration efforts continue to focus primarily on hydrology and plant cover, likely due to the logistical challenges of focusing on other factors or predicting the outcome of different approaches (Zedler 2000, 2001). Studies have found that some ecological attributes of restored wetlands, such as habitat diversity, species diversity, soil characteristics, and secondary production, might take decades to reach the same levels as natural, undisturbed wetlands (Minello and Zimmerman 1992, Minello and Webb 1997, Craft et al. 1999, Craft and Sacco 2003). A

previous study incorporating long-term monitoring of a restoration site in San Diego Bay indicated that the desired mitigation outcomes for that site had not been achieved in 12 years, and seemed unlikely to be achieved in the near future (Zedler and Callaway 1999).

Evaluations of restored salt marshes have compared chronological series, or single time-fame levels of species diversity of faunal communities as indicators of health (Craft et al. 1999, Craft and Sacco 2003, Staszak and Armitage 2013), and recent advances have improved understanding in how changes in faunal communities and species diversity can influence ecosystem services (Finke 2004, Finke and Denno 2005, Deegan et al. 2007). For instance, decreases in the species diversity of spiders can alter herbivore density to levels that decrease productivity in *Spartina* ecosystems (Finke 2004, Finke and Denno 2005). Diversity at the level of communities has long been recognized as in important indicator of ecosystem health (Stevenson et al. 2000, Hooper et al. 2005, Richardson and Hussain 2006), but more recent research is beginning to highlight the influence of intraspecies genetic diversity on ecosystem health and resilience (i.e., stability in the face of natural and anthropogenic disturbances).

Genetic diversity and its relationship to ecosystem health and resilience

Diversity is a hierarchical system, with each level (e.g., ecosystem, habitat, population) dependent upon the levels below it, meaning genetic variability within a population can have ecological impacts at multiple levels (Hughes et al. 2008). Several studies have determined that increased genetic diversity of host plant species increases the mean fitness of the plants, significantly alters the structure of associated arthropod communities, and enhances ecosystem productivity and resilience (Wimp et al. 2005,

Johnson et al. 2006, Reusch and Hughes 2006). Reusch and Hughes (2006) note that seagrass and salt marsh systems are ideal for testing different hierarchical levels of diversity, and most genetic studies on restored near-shore systems to date have focused on the genetic diversity of the dominant, habitat-forming flora (Travis et al. 2002, Hughes and Stachowicz 2004, Reynolds et al. 2012). In eelgrass habitats, increased genetic diversity of the seagrass played a role in the system's resistance to community disturbances by grazing geese (Hughes and Stachowicz 2004). Reynolds et al. (2012) found that even a small increase in genetic diversity of seagrasses enhances the success of sea-grass bed restoration and augments ecosystem services, such as primary production, nutrient retention, and provision of invertebrate habitat. A study of genetic diversity of *Spartina alterniflora* in restored salt marshes by Travis et al. (2002) found similar levels of genetic diversity between restored and natural marshes. The only study comparing faunal genetic diversity in restored and natural near-shore habitats involves a recent study of genetic variation in oysters, which found levels of genetic diversity of oysters in restored reefs were similar to those in natural reefs (Arnaldi et al. 2018).

To date, no studies have attempted to characterize genetic variation in colonizing faunal populations of restored salt marshes. In a review of restoration ecology, Palmer et al. (1997) notes that restoration relies on an unverified assumption that species re-establish themselves in restored habitats. They go on to say that restoration science would benefit from research into the spatial scales necessary for restoring species diversity, and adequate knowledge of colonization sources, rates of migration, and how those factors influence restoration success (Palmer et al. 1997). Studies of genetic variation in colonizing fauna in restored marshes would fill these gaps by evaluating the levels of connectivity (i.e.,

migration and gene flow) between source populations and restored marshes as a function of distance, dispersal potential of colonizing fauna, and time since restoration. Estimating the levels of genetic variation of colonizing populations may provide information to evaluate resident fauna's genetic capacity for resilience, and by extension, the entire system's ability to adapt to and recover from stressful conditions.

High rates of coastal wetland loss and degradation combined with the logistical complications of incorporating spatial processes in the planning and selecting of locations for restoration efforts, result in substantial levels of fragmentation in salt marsh habitats (Britsch and Dunbar 1993, White and Tremblay 1995, Bell et al. 1997, Huxel and Hastings 1999). A comprehensive review of the genetic consequences of habitat fragmentation found considerable evidence that fragmentation can have negative impacts on population genetics (Keyghobadi 2007). Theoretically, fragmentation of habitats inhibits population connectivity and genetic outcrossing, which reduces adaptive fitness and the potential to adapt to stressful conditions for individuals and populations through four forces: (a) increased genetic drift, (b) elevated inbreeding, (c) reduced gene flow with other populations, and (d) increased probability of local loss of adaptive alleles (Templeton et al. 1990, Young et al. 1996, Reed and Frankham 2003, Avise 2004, Bijlsma and Loeschcke 2012, Palkovacs et al. 2012).

Since salt marshes and their resident fauna are subject to a multitude of stressors, including drought and dredging (Hartig et al. 2002), nutrient loading (Wigand et al. 2003, Deegan et al. 2007), tidal surges and fluctuations (Konisky and Burdick 2004), and climate change (Simas et al. 2001, Hartig et al. 2002), it is vital to evaluate the genetic diversity of faunal communities within restored marshes as a potential analog for species health and

resilience. To ensure a representative coverage of faunal communities present in a *Spartina* marsh, this study focuses on one aerial and two aquatic species, each with unique life history characteristics that lend well to illustrating patterns of colonization relative to mode of dispersal, habitat (aquatic versus aerial), and mode of reproduction, and thus are expected to display measureable differences in genetic variability.

Biological background for Gulf Killifish (*Fundulus grandis*)

Gulf Killifish are among the most abundant nekton in the marsh habitat along the Gulf of Mexico coast, often dominating fish assemblages in the marshes in the spring and fall (Rozas and Reed 1993, Rozas and Zimmerman 2000). They are an important link in the coastal marsh food web. As omnivores, they consume a variety of algae, vascular plants, and small animal prey, such as mosquito larvae and grass shrimp (Welsh 1975, Rozas and LaSalle 1990, Kennish 2001). They also act as important prey items for shore birds and for fisheries species that use coastal marshes as nursery habitat (Rozas and Reed 1993, Rozas and Zimmerman 2000).

Gulf Killifish reproduce frequently in their first year of life, and their average lifespan is approximately two years (Lipcius and Subrahmanyam 1986). Their breeding season lasts from March through August, with peak egg production occurring between April and May (Greeley and MacGregor 1983, Lipcius and Subrahmanyam 1986, Green et al. 2010). They are benthic spawners, attaching their eggs via filaments to substrate within the marsh (Nordlie 2000).

Gulf Killifish were found to have higher densities in vegetated areas, as they tend to stay in submerged marsh habitat whenever possible, only moving into deeper,

unvegetated waters at low tide, when the marsh surface is no longer inundated (Lipcius and Subrahmanyam 1986, Rozas and Zimmerman 2000). Mark-recapture studies along the Gulf of Mexico coast showed that Gulf Killifish exhibit high site fidelity, with most individuals travelling 100 m or less between connected marsh habitats (Nelson et al. 2014). Additionally, a study of population structure including eight nuclear microsatellites across ten populations in the northern Gulf of Mexico showed significant levels of isolation by distance (IBD) with limited dispersal occurring only between neighboring sites (Williams et al. 2008).

Their reproductive characteristics and limited movement as adults combine to make Gulf Killifish an excellent environmental indicator species in terms of genetic and physiological responses to exposure to a variety of industrial toxins. They tend to remain in their preferred marsh habitats, despite the presence of oil or toxins (Martin 2017). As a result, they have been used in studies of complex genetic responses to the Deepwater Horizon oil spill (Garcia et al. 2012), site-specific effects of crude oil contamination on biological function (Whitehead et al. 2012, Dubansky et al. 2013, Dubansky et al. 2014, Dubansky et al. 2017), and evolutionary genetic response to industrial pollutants in the Houston Ship Channel (Oziolor et al. 2014, Oziolor et al. 2016).

Biological background for Daggerblade grass shrimp (*Palaemon pugio*)

Grass shrimp are also among the most abundant nekton in marsh habitats along the Gulf of Mexico (Wood 1967, Rozas and Zimmerman 2000), with peak densities occurring in the fall (Welsh 1975). They are opportunistic feeders, consuming a variety of epiphytes, algae, meiofauna, detritus, and dead animal matter (Heard 1982). They in turn are used as

prey by fishes, crabs, and birds (Heard 1982, Anderson 1985). Grass Shrimp play a vital role in the food webs of coastal marsh habitats by breaking down organic materials and making those nutrients available at other trophic levels (Adams and Angelovic 1970, Odum and Heald 1972, Welsh 1975, Anderson 1985). They often remain in marsh habitat regardless of the presence of toxins, and they are frequently used as indicator species in studies of coastal habitat quality and toxicity response (Lewis and Foss 2000, Key et al. 2006, DeLorenzo et al. 2016, Gray and Weinstein 2017). Grass Shrimp have a life span of approximately 6-13 months and are sexually mature after 1.5-2 months (Anderson 1985, Cházaro-Olvera 2009b). Spawning season ranges from February-October and females may spawn multiple times in a season, with peak egg production occurring in June and July (Welsh 1975, Anderson 1985, Cházaro-Olvera 2009b). Upon fertilization, the eggs attach to the female abdomen until they hatch (Anderson 1985, Cházaro-Olvera 2009b).

Like Gulf Killifish, Grass Shrimp are most abundant around tidal marshes and submerged vegetation (Heard 1982). They are useful for this study because they possess reproductive and early life history traits that contrast with the Gulf Killifish. These traits include a shorter generation time (Wood 1967), and a potentially greater dispersal potential of the nauplii stage via currents, which may explain previous findings of high levels of gene flow between different populations on the southern Atlantic coast from Florida to South Carolina (Flowers 2004).

Biological background for phloem-feeding planthoppers (*Prokelisia marginata*)

Planthoppers are host-specific sap-feeders that, together with leafhoppers and mirid bugs, may reach combined densities that exceed 50,000 individuals/m^2 and account for

>90% of herbivore biomass (Denno and McClure 1983, Denno 1994, Denno et al. 2000). Accordingly, planthoppers are perhaps the most important browsers of *Spartina* and are thus closely associated to plant cover of native marshes. Previous studies have shown a negative association between abundance of planthoppers and primary productivity of *Spartina* marshes (Finke 2004, Finke and Denno 2005). Further, their mode of dispersal is relevant towards our understanding of colonization of restored or reconstructed marshes. Throughout its range, *P. marginata* is present in: (a) a wingless, more fecund brachypterous form; and (b) a less fecund, winged macropterous form capable of flight and with higher dispersal capabilities (Denno et al. 1989, Denno 1994). Wing form is determined by a developmental switch that responds to environmental cues, including host plant condition, temperature, photoperiod, level of habitat persistence, and intraspecific density, of which density is considered the most important (Denno and Roderick 1990, Denno et al. 1991, Denno 1994, Denno et al. 2001). The density that triggers the production of the winged form differs among species, and among populations of the same species (Denno et al. 1991). Previous studies suggest that the wingless form with limited dispersal capabilities is most common in salt marshes on the Gulf of Mexico coast (Denno and Roderick 1990, Denno et al. 1991), which would result in more limited dispersal potential.

Organization of chapters

The remainder of this dissertation is organized into the four following chapters. Chapter II outlines a molecular method for distinguishing the sympatric species, *Prokelisia marginata* and *Prokelisia dolus*, which are difficult to identify based solely on

morphological features. Fixed nucleotide differences in a segment of mitochondrial DNA (mtDNA) from the Cytochrome Oxidase Subunit 1 (COI) region are targeted for development of a short amplicon high resolution melting analysis, (SA) HRMA, resulting in a fast, cost-effective assay to differentiate these species. Chapter III is a comparative study of population genetics for three common salt marsh fauna along the Northern Gulf of Mexico Coast using mtDNA sequence data. Gulf Killifish are characterized using segments of the mitochondrial Control Region (CR1), Nitrogen Dehydrogenase Subunit 2 (ND2) and Nitrogen Dehydrogenase Subunit 5 (ND5). Daggerblade Grass Shrimp are characterized using a segment of mitochondrial 16sRNA. Phloem-feeding Planthoppers are characterized using a segment of COI. Due to the overall low levels of genetic diversity encountered at the regional level for both Daggerblade grass shrimp and Phloem-feeding planthoppers, the subsequent chapter that analyzes diversity at a local level will focus only on Gulf Killifish. Chapter IV explores the genetic and genomic diversity of Gulf Killifish in restored marshes in the Galveston Bay system that are in different age categories and are different distances from a natural, reference marsh. They are characterized in this chapter using two main marker types. The first set of markers are the same mtDNA sequence segments used in the previous chapter. The second set of markers are obtained via double digest restriction-site associated DNA (ddRAD) sequencing techniques, which allows us to characterize genetic variation across the nuclear genome using thousands of informative loci. Lastly, Chapter V will contain the general conclusions of this dissertation, including overall results, findings, implications, and recommendations.

CHAPTER II

GENETIC SPECIES IDENTIFICATION OF ECOLOGICALLY IMPORTANT PLANTHOPPERS (*Prokelisia spp.*) OF COASTAL *Spartina* SALT MARSHES USING HIGH RESOLUTION MELTING ANALYSIS (HRMA)*

Introduction

Members of *Prokelisia* are wing dimorphic, phloem-feeding planthoppers that, along with other species of leafhoppers and mirid bugs, may account for greater than 90% of herbivore biomass in salt marshes (Denno and McClure 1983, Denno 1994, Denno et al. 2000). Three of the five species of *Prokelisia*, namely *P. marginata, P. dolus,* and *P. crocea*, have overlapping geographic ranges along the east coast of North America, from the Gulf of Mexico to Canada, where they have a close association with *Spartina* salt marshes (Wilson 1982). They rank as the most important browsers, with the capacity to influence plant cover and, consequently, ecosystem services provided by *Spartina* grasses, which include such things as provision of complex habitat, primary production, and carbon sequestration (Olmstead et al. 1997, Finke 2004, Finke and Denno 2005). *P. crocea*, a species with ampler habitat preference than the other two, is easily distinguished by its larger size and unique orange markings on its face and thorax. By contrast, *P. marginata* and *P. dolus* are very similar in size and pigmentation patterns, and species identification is based on the frons shape, which in some individuals displays an intermediate condition (Denno et al. 1987, Heady and Wilson 1990). A more detailed microscopic examination of

* Reprinted under Creative Commons from: Espinoza J and JA Alvarado Bremer. 2019. Genetic species identification of ecologically important planthoppers (*Prokelisia spp.*) of coastal *Spartina* saltmarshes using High Resolution Melting Analysis (HRMA). Sci Rep **9** 20073.

the male genitalia of these species reveals differences in the shapes of the styles, aedeagus, and anal tube (Wilson 1982). Preliminary results from this study revealed that identification using this method is time consuming, and the character states of these traits can be easily misdiagnosed. Further, female sex organs of the two species differ from each other, but since they often display intermediate morphological features, species identification of females is unreliable (Heady and Wilson 1990). Also, while juveniles of both species can be assigned to the *Prokelisia* genus, their undeveloped sex organs make it impossible to distinguish their gender or identify them to species level.

Correct species identification is particularly relevant when conducting population studies, and when cryptic or hard-to-distinguish species occur in sympatry (Paterson 1991). Estimates of allele frequency and the corresponding diversity indices can be severely biased through the inclusion of misidentified specimens (Bickford et al. 2007). The use of molecular methodologies can facilitate the identification of cryptic species, and new technologies like high resolution melting analysis (HRMA) provide a fast, high-throughput alternative that is less expensive (e.g. ~ $0.10 per reaction for the SA-HRMA performed in this study) than other genotyping methodologies (Smith et al. 2010). HRMA is capable of detecting single nucleotide polymorphisms (SNPs) and small deletions present within amplified DNA fragments that are visualized as differences in the fluorescence of a saturating dye that is disassociated as the amplicons denature with increasing temperature (Erali and Wittwer 2010). A major advantage of HRMA over other genotyping methods is that the entire process, from amplification to scoring, can be completed in approximately 15-20 minutes using modern RT-PCR equipment. Since this occurs in a single, closed-tube assay, the potential for cross contamination is minimized, and there is no need for multiple

steps using different platforms to score alleles (Smith et al. 2010). HRMA has been successfully used to characterize wild populations (Smith et al. 2010), including the identification of marine fishes (Smith et al. 2010, Randall et al. 2015), as well as arthropods and spiders (Winder et al. 2011). This study seeks to use HRMA as a novel approach to rapidly distinguish between *P. marginata* and *P. dolus*.

Results and discussion

Preliminary HRMAs using the HR-1 High Resolution Melter and the primer pair Prok-HRMA4-F and Prok-HRMA4-R to characterize a small sample of validated males of *P. marginata* and *P. dolus* resulted in a separation (up to 3-4°C) between the melting peaks of the two species, with *P marginata* melting at a higher temperature (**Figure 1**). The Prok-HRMA4 primer-pair amplifies a short (60 bp) COI fragment, whose length is particularly well suited to diagnose SNPs using SA-HRMA, as previous studies have found that amplicons < 100 bp result in the highest resolution for genotyping using HRMA (Li et al. 2011).

The multiple sequence alignment of this short COI fragment consists of three representative haplotypes per species out of the 65 morphologically validated individuals sequenced, and reveals the presence of eight polymorphic sites. All polymorphic sites correspond to transitions at the third codon position (**Figure 2**), of which seven are synonymous. The exception is the A/G transition at the ninth nucleotide position that results in a change of Isoleucine in *P. dolus* for Methionine in *P. marginata*, both of which are hydrophobic amino acids. Four of the polymorphic sites fail to separate the two species, with the first, third, fifth and eight positions identified as plesiomorphies, as haplotypes of the two species share character states at any of these nucleotide sites.

Although these plesiomorphies produced variability in the melting profiles within two species (**Figure 1**), they did not affect the diagnostic power of this HRMA given that, on average, the haplotypes of the two species differ by 5.6 substitutions, with four of these fixed and responsible for the minimum (>1.7 °C) observed difference separating the melting peaks of these two species. The higher melting temperature of *P. marginata* (~79.4°C) compared to *P. dolus* (~77.0°C) is due to the presence in *P. marginata* of a G or a C in the majority of the polymorphic sites, compared to an A or a T at those sites in *P. dolus* (**Figure 2**).

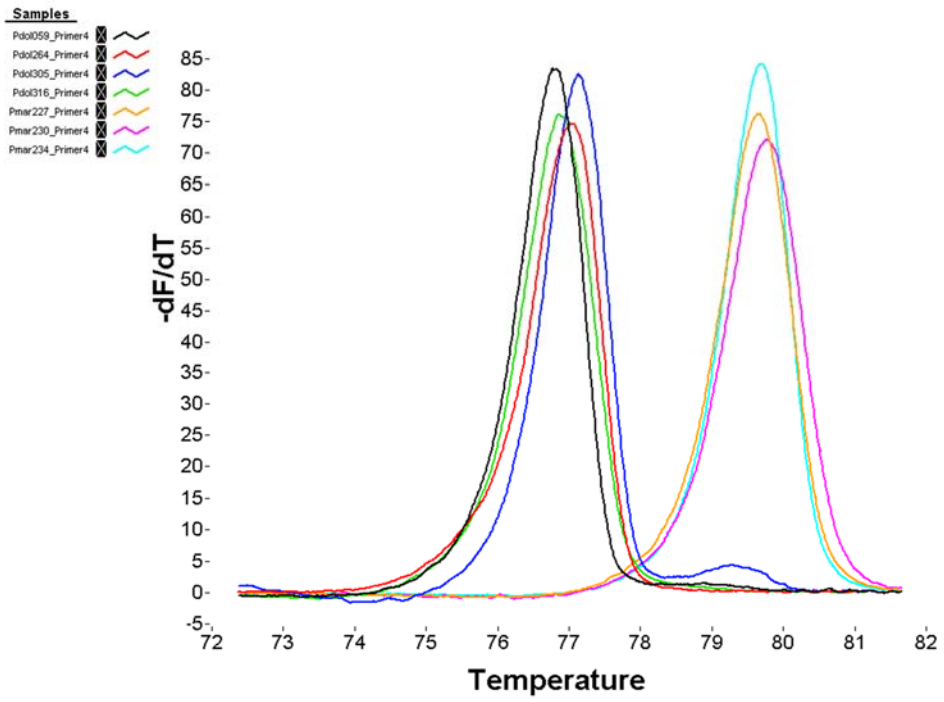

Figure 1. Preliminary results of SA-HRMA using Prok-HRMA4 primer pair. The graph shows normalized derivative plots of fluorescence with respect to temperature (-dF / dT) for a total of seven specimens of *P. marginata* (n=3) and *P. dolus* (n=4) each identified with a different line color. The specimen ID and the primer set employed is given in the inset. *P. marginata* haplotypes melted at a higher T_m than *P. dolus*. Reactions were carried out in a RapidCycler2 and scored in an HR-1 instrument.

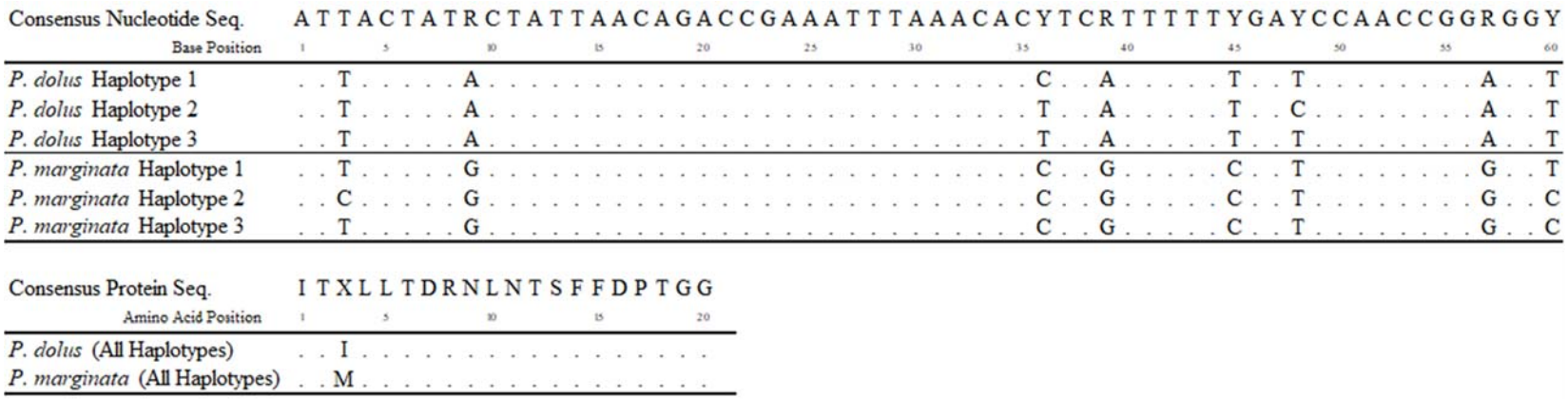

Figure 2. Multiple sequence alignment of the fragment amplified by Prok-HRMA4 primer pair. Alignment includes six planthopper haplotypes belonging to *P. marginata* and *P. dolus* defined by eight polymorphic sites, and translated amino acid sequences (20 amino acids). The corresponding consensus nucleotide and amino acid sequences are shown. All substitutions were transitions at the third codon position, and all but one (A/G at position 9) were synonymous. The haplotypes shown here correspond to individuals shown in Fig. 1.

Table 1. High-throughput HRMA results for the Prok-HRMA4 primer pair.

	Total	Males	Females
High-Throughput Samples	518	279	239
Failed Amps (%)	9 (1.7%)	9 (3.2%)	0
Ambiguous Curves (%)	0	0	0
Visually Mis-Identified (%)	N/A	30 (10.8%)	N/A

A high throughput analysis (n=518) using the Prok-HRMA4 primer pair was highly successful with only 1.7% (n=9) reported amplification failures (**Table 1**). The successful amplifications (n=509) produced clearly diagnostic melting curves for the unequivocal assignment of 213 individuals to *P. dolus* and 296 individuals to *P. marginata* (**Figure 3**) in spite of the latter displaying more variation in melting temperatures. Variations in melting temperatures can be attributed to differences in the eight variable sites that make up the six haplotypes, with higher temperatures corresponding to higher presence of G or C at those sites (**Figure 4**). A subset of *P. marginata* (n=124) were sequenced for a separate study of population structure in this species. The sequences of the segment targeted by the Prok-HRMA-4 primer set for all those individuals conformed to one of the three haplotypes for *P. marginata* shown in this study.

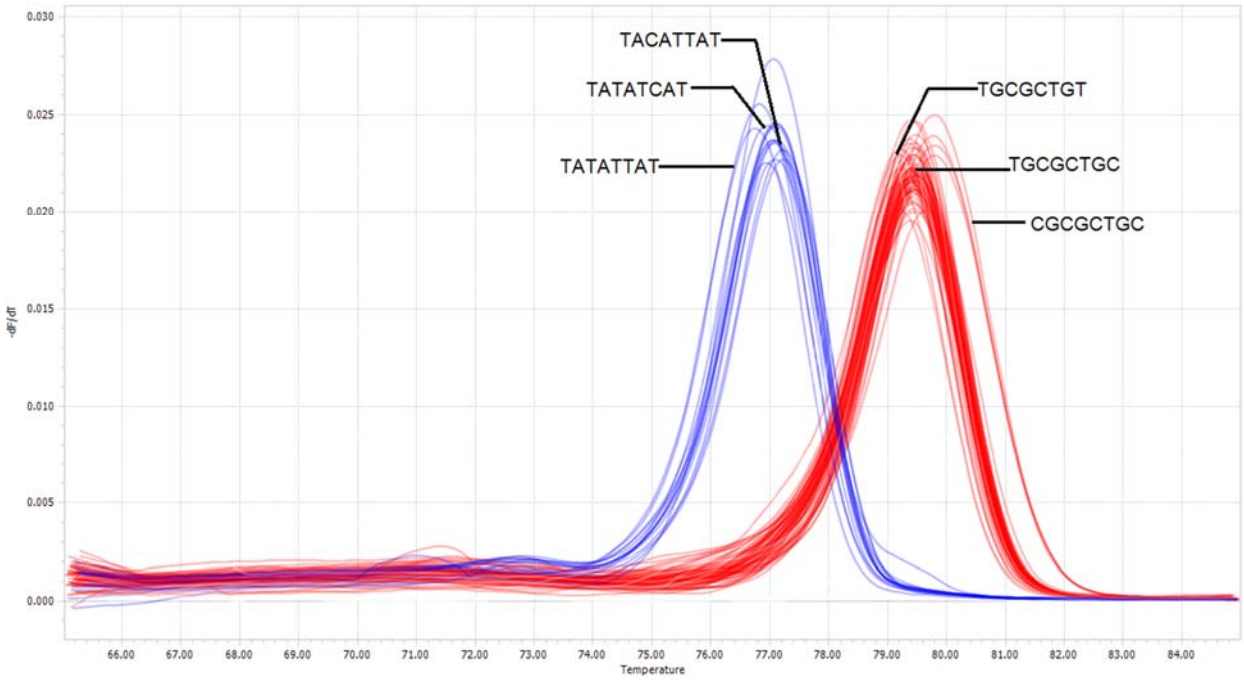

Figure 3. High-throughput SA-HRMA using Prok-HRMA4 primer pair. The graph shows normalized derivative plots of fluorescence with respect to temperature (-dF / dT) for a full plate (n=96) specimens. Nucleotide sequences correspond to the eight polymorphic sites for each haplotype. *P. marginata* haplotypes melted at a higher T_m than *P. dolus*. Reactions were carried out and scored with a LightCycler 96 RT-PCR instrument.

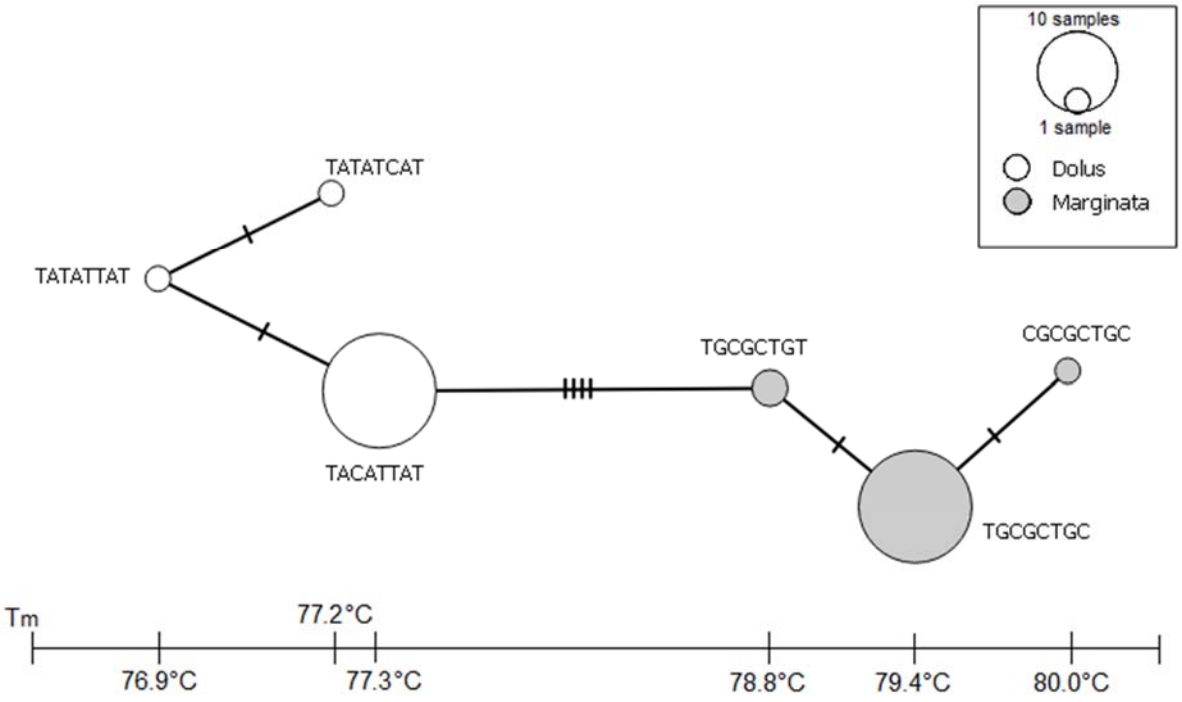

Figure 4. Minimum spanning network for the Prok-HRMA4 primer region for the sequences of the 65 morphologically validated specimens used to develop the HRM assay. Circles correspond to haplotypes, with circle size indicative of the number of individuals belonging to each haplotype. Nucleotide sequences correspond to the eight polymorphic sites for each haplotype. Bottom bar corresponds to the temperatures at which each haplotype melts.

Among the successful samples tested, 279 specimens, roughly half the sample, were males that had been pre-identified to species level based on morphology. Surprisingly, HRMA suggests that 30 individuals, or 10.75% of the male sample (15 *P. dolus* and 15 *P. marginata*), were misidentified based on putatively diagnostic morphological characters (Denno et al. 1987, Heady and Wilson 1990). An alternative explanation for the disagreement between morphological and molecular identifications is

mitochondrial introgression between *P. marginata* and *P. dolus*, a phenomenon that has not been reported to date in these insects. Demonstrating this alternative hypothesis would require an extensive analysis of the nuclear genomes and breeding experiments beyond the scope of this study.

The ability to rapidly and efficiently determine the species identity of planthoppers using HRMA, as described here, has important implications to population genetic studies on these species. If such a study were to be conducted relying solely on morphological identification of species, the resultant allele frequency counts would likely be biased due to the inclusion of a high percentage of heterospecifics that would also result in grossly overestimating the genetic diversity of the samples surveyed. Additionally, since the females of these two species are physically intermediate to each other (Heady and Wilson 1990) and juveniles do not display diagnostic characters, any population level study of either species would be limited to the characterization of adult male specimens, hindering the ability to distinguish gender effects from overall population effects. Also, while both species appear to occupy the same niche, and therefore might be grouped in ecological studies under the Ecological Species Concept (Valen 1976), that concept requires life histories of the grouped species to be the same, which is not always true in practice (Aldhebiani 2018). *P. dolus* and *P. marginata*, in particular, are capable of displaying different life histories even within their individual species, as evidenced by the existence of two wing morphs with different levels of fecundity and dispersal potential that each present under specific environmental conditions and stressors (Denno et al. 1989, Denno 1994). Therefore, ecological studies including these species would also benefit from

reliable species identification such that single species effects are not obfuscated by unintentional inclusion of multiple species.

Because of the very small size (2-3 mm) of the *Prokelisia* specimens, entire specimens were digested to provide sufficient DNA quantity (> 50ng/μL) and quality (≥ 10,000 bp length) to perform this SA-HRMA and subsequent sequencing experiments, including Sanger and next-generation sequencing (NGS). However, it should be noted that arthropods often contain gut endosymbionts that can contaminate massive parallel sequencing experiments (González et al. 2018). Therefore, investigators wishing to use NGS or RNA-seq techniques on these species should consider isolating nucleic acids from body parts, such as the legs or the head. Finally, it was determined that species identification using this SA-HRMA assay could be successfully carried on different RT-PCR platforms, including Idaho Technology's Rapidcycler-2 and HR-1 machine, Roche's Lightcycler 460 and Lightcycler 96, and in a Phoenix's MyGo Mini, all using the same chemistry and identical, or very similar thermocycling and melting profiles.

Methods

Field Collections and Sample Preparation

Arthropods, including *Prokelisia spp.* specimens, were captured from six *Spartina* marshes along the northern coast of the Gulf of Mexico using professional insect 15" muslin sweep nets (www.gemplers.com). Immediately after collection, samples were preserved in 70% ethanol (EtOH) and kept there until assayed in the lab. Preserved samples were examined under a dissecting microscope at 10x magnification, and those specimens with the characteristics of both *P. dolus* and *marginata* were separated from other arthropods. These specimens were further sorted by gender. The number of

Prokelisia specimens collected per marsh site ranged from 6-200 individuals. In total, 540 individuals, including males, females, and immature of *P. dolus* and *P. marginata* were collected. Males of the two species were further sorted to species level by examining the putative diagnostic features of their reproductive organs (Wilson 1982) under a compound microscope at 40x magnification. An example of the different styles shapes seen in *P. dolus* and *P. marginata* males viewed under a Nikon AZ100M microscope with motorized body and compiled via Nikon BR software is shown in Appendix A, Figure A-1.

HRMA Development

A sample consisting of 65 males were identified based on morphological characters, as described above, to belong to *P. marginata* (n=43) or *P. dolus* (n=22). To isolate the DNA, each specimen was individually ground using a sterile disposable pestle, followed by Proteinase K digestion without organic extractions as described by Greig (2000). A 450 bp fragment of the mitochondrial DNA (mtDNA) COI gene was amplified using primers C1-J-1751 and C1-N-2197 (Simon et al. 1994). COI was targeted because this segment has a proven record to distinguish con-generics of a variety of animal species, including arthropods (Caterino et al. 2000, Hebert et al. 2003, Barrett and Hebert 2005). PCR reactions were carried out in 12.5µL volumes, containing 1x Econotaq Plus Green Master Mix (Lucigen), 0.2 µM of each primer, and approximately 10 ng of isolated DNA as template. Thermocycling was performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with an initial denaturing step at 94°C for 2 min; followed by 35 cycles of denaturing at 94°C for 25 sec, annealing at 47°C for 40 sec, and extension at 72°C for 90 sec; and a final extension step at 72°C for 3 minutes. PCR products were then visualized for specificity and yield via electrophoresis on a 2% agarose gel pre-stained

with ethidium bromide (EtBr). PCR products that produced a single band were diluted 1:10 for post-PCR cleanup and sequenced in both directions, with reaction setups and thermocycling profiles as described in Cruscanti et al. (2015). Multiple sequence alignments were carried out in Geneious Pro v.9.1.8 (Biomatters Ltd., Aukland, NZ).

Design and Evaluation of HRMA

The multiple sequence alignment of 450 bp of COI obtained from the 65 morphologically validated male specimens was used to design a SA-HRMA. Fixed nucleotide differences between species along this segment were identified, and four sets of potential primers, each flanking a short segment (40-106 bp long) containing the diagnostic sites were chosen (Appendix A, Figure A-2) following the recommendations given by Smith et al. (2013). Preliminary analysis of specificity for each primer set was conducted via PCR amplification using the DNA from four voucher specimens per species from the pool of male *P. marginata* and *P. dolus* that were originally sequenced and employed to design the SA-HRMA assay. Initial PCR reactions to identify a diagnostic HRMA were performed in 10µL volumes in glass capillary tubes containing 1x Econotaq Plus Master Mix (Lucigen), 1x LCGreen (Idaho Technologies), 0.2µM of each primer, and approximately 10ng of template DNA. Each reaction was covered with ~10µL of mineral oil to prevent evaporation and ensure uniform melting (Erali and Wittwer 2010). A negative control was included in all reactions. Thermocycling was conducted in a RapidCycler2 (Idaho Technologies) with an initial denaturing step of 95°C for 5 minutes, followed by 45 cycles of denaturing at 94°C for 10 seconds, annealing at 48°C for 10 seconds, and extension at 72°C for 12 seconds, with a final step of melting at 94°C for 10 seconds and followed by cooling at 40°C and holding for 20 seconds prior to initiating

melting. Products were melted using HR-1 High Resolution Melter (Idaho Technologies), and data was acquired from 65-85°C with a melting ramp rate of 0.2 °C/s.

HRMA were carried out with the four primer sets. Five additional HRMAs were tested by combining forward and reverse primers from the original four sets. The primer pair Prok-HRMA4-F (5' CCA GTA CTT GCA GTT GCA 3') and Prok-HRMA4-R (5' GTT GAT ATA AGA TTG GAT CTC C 3') was identified as the most successful set of primers to use in HRMA, based on highest amplification efficiency and greatest differences in melting temperatures between species (**Figure 1**) compared to the other four sets (**Figure 5**). This HRMA was then used to diagnose a larger sample (n=518) consisting of 279 males that based upon morphological traits were putatively identified as either *P. marginata* (n=164) or *P. dolus* (n=115), and of 239 unidentified females and juveniles of these two species (**see Figure 3**).

PCR amplifications for high throughput HRMA were performed in 10µL volumes containing 1x Econotaq Plus Master Mix (Lucigen), 1x LCGreen (Idaho Technologies), 0.2µM of each primer, and approximately 10ng of template DNA. Negative controls were included in all reactions. Thermocycling, performed on a LightCycler 96 Real-Time PCR system (Roche Diagnostics), consisted of an initial denaturing step at 95°C for 2 min; followed by 55 cycles of denaturing at 95°C for 10 sec, annealing at 47°C for 10 sec, and extension at 72°C for 30 sec. Prior to melting curve analysis data acquisition, products were denatured at 95°C for 30 seconds, followed by rapid cooling (ramp rate = 2.2°C/sec) to 40°C and held there for 30 seconds. Products were then melted, and data was acquired at a rate of 20 acquisitions/°C from 65-85°C with a melting ramp rate of 0.05°C/s.

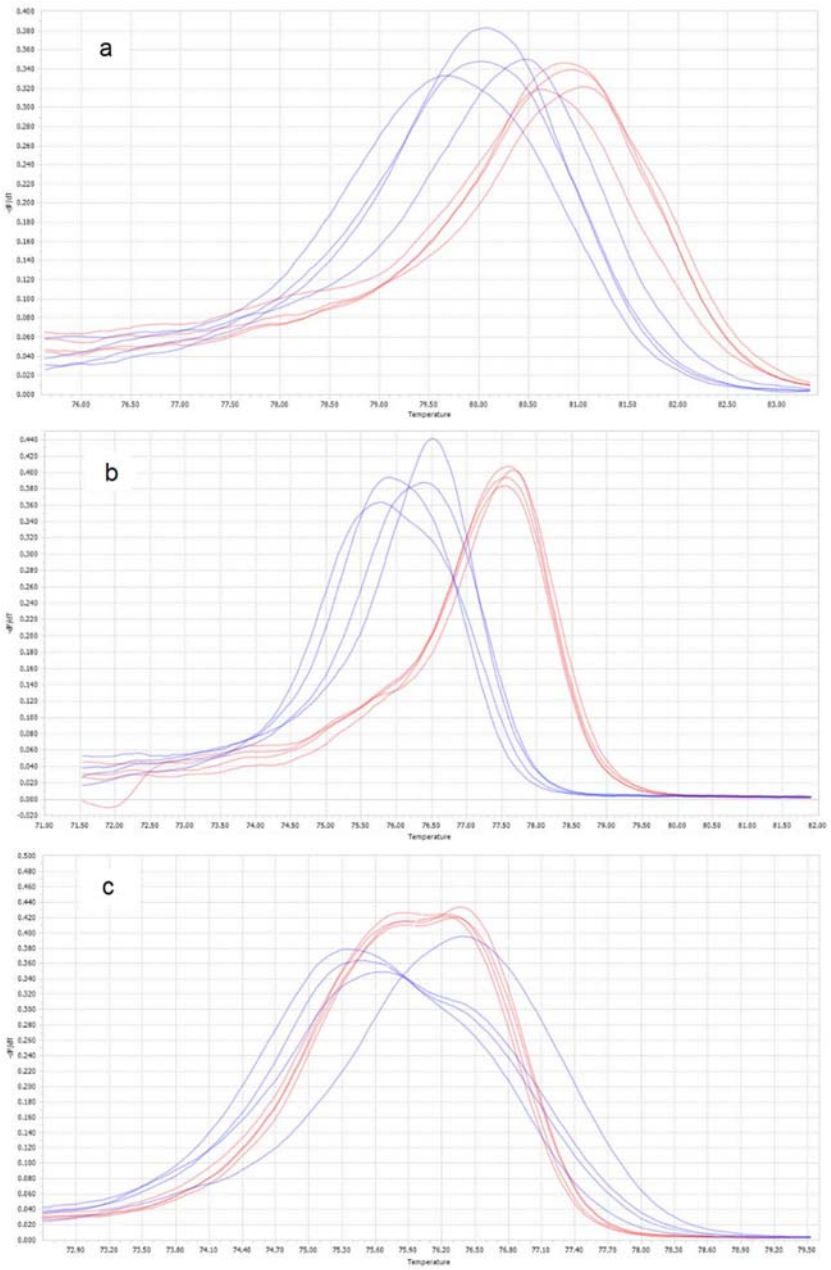

Figure 5. Preliminary HRMA results for four primer sets. The graphs show normalized derivative plots of fluorescence with respect to temperature (-dF/dT). In all panels, *P. dolus* is shown in blue while *P. marginata* is shown in red. Primer sets shown are (a) Prok-HRMA1, (b) Prok-HRMA2, and (c) Prok-HRMA3. Results for primer set Prok-HRMA4 are given in Figure 1.

Finally, to determine whether species identification could be carried on different RT-PCR platforms, we conducted this SA-HRMA assay successfully on Idaho Technology's Rapidcycler-2 and HR-1 machine, Roche's Lightcycler 460 and Lightcycler 96, and in a Phoenix MyGo Mini, all using the same chemistry and the same, or similar thermocycling and melting profiles (data available upon request).

CHAPTER III

COMPARATIVE PHYLOGEOGRAPHY, HISTORICAL DEMOGRAPHY, AND POPULATION GENETICS OF THREE COMMON COASTAL FAUNA IN Spartina MARSHES OF THE NORTHWESTERN GULF OF MEXICO

Introduction

Coastal wetlands function as essential wildlife habitat and provide vital ecosystem services to nearby coastal communities (Engle 2011). Despite their importance, coastal wetlands have decreased in area throughout much of the continental United States (Moulton et al. 1997, Dahl 2011). In a comprehensive review over a five-year period from 2004 to 2009, Dahl and Stedman (2013) reported a loss of over 360 thousand acres of coastal wetland area in the conterminous U.S., or roughly 72 thousand acres per year. Such high rates of habitat fragmentation and loss can be expected to have enormous negative impacts on biodiversity (Fahrig 2003, Ewers and Didham 2005), and the concomitant loss of faunal populations is expected to adversely affect the ecosystem services salt marshes provide (Finke 2004, Finke and Denno 2005, Deegan et al. 2007).

Habitat fragmentation reduces population connectivity and genetic outcrossing, and populations with a small effective population size (N_e) are prone to genetic erosion due to genetic drift, which in turn reduces fitness and adaptive responses to stressful conditions, thus increasing the risk of extinction (Avise 2004, Bijlsma and Loeschcke 2012, Palkovacs et al. 2012). Due to the hierarchical nature of diversity, genetic diversity can have important ecological consequences at the population, community and ecosystem levels, and in some cases, the effects are comparable in magnitude to the effects of species

diversity (Hughes et al. 2008). For example, genetic studies on restored sea grass beds found that increased genetic diversity of the dominant flora enhanced ecosystem services (Reynolds et al. 2012). Since salt marshes and their resident fauna are subject to a multitude of stressors, including drought and dredging (Hartig et al. 2002), nutrient loading (Wigand et al. 2003, Deegan et al. 2007), and tidal surges and fluctuations (Konisky and Burdick 2004), it is germane to investigate the genetic diversity, connectivity, and potential resilience of faunal communities within salt marsh habitats, particularly when facing the challenges associated with climate change (Simas et al. 2001, Hartig et al. 2002).

This study focuses on characterizing genetic variability in *Spartina* marsh fauna. To ensure a representative coverage of faunal salt marsh communities, we selected one aerial and two aquatic species known to be intimately associated with this habitat (Denno et al. 1987, Minello 1992, Denno 1994, Minello 1997, Olmstead et al. 1997). Each of the chosen species display distinct life history characteristics that uniquely illustrate patterns of gene-flow potential relative to their habitat (aquatic versus aerial), and modes of dispersal and reproduction, with the expectation that measurable differences in genetic variability, both among- and within-species, would exist. Comparative studies of species with similar geographic distributions can help reveal shared evolutionary events over both geography and time (Alvarado Bremer et al. 2005, Bowen et al. 2014). We chose Gulf Killifish, *Fundulus grandis* (Baird and Girard 1853), and daggerblade grass shrimp, *Palaemon pugio* (Holthius 1952), for the two aquatic species, because they are among the most abundant nekton in salt marsh habitats along the Gulf of Mexico (hereafter, Gulf) coast and represent vital links in coastal marsh food webs (Wood 1967, Rozas and Reed 1993, Rozas and Zimmerman 2000). For the aerial example, we selected the phloem-feeding planthopper,

Prokelisia marginata (Van Duzee 1897). Planthoppers are host-specific sap-feeders that, together with leafhoppers and mirid bugs, may reach combined densities that account for more than 90% of herbivore biomass in some habitats (Denno 1983, 1994, Denno et al. 2000).

The three species characterized in this study encompass a variety of life history traits and dispersal potentials. Gulf Killifish have relatively low fecundity and are benthic spawners that attach their eggs to substrate within the marsh (Greeley and MacGregor 1983, Lipcius and Subrahmanyam 1986, Nordlie 2000, Green et al. 2010), giving them low dispersal potential. Mark-recapture studies along the Gulf coast showed that Gulf Killifish exhibit high site fidelity, with individuals travelling 100m or less between connected marsh habitats (Nelson et al. 2014). Their reproductive characteristics and limited movement as adults combine to make Gulf Killifish an excellent indicator species in terms of genetic and physiological responses to exposure to a variety of industrial toxins, and they have been used extensively as such (Garcia et al. 2012, Whitehead et al. 2012, Dubansky et al. 2013, Dubansky et al. 2014, Oziolor et al. 2014, Oziolor et al. 2016, Dubansky et al. 2017).

Daggerblade grass shrimp (hereafter, grass shrimp), by contrast, reach sexual maturity quickly, have a short generation time, high fecundity, and high dispersal potential in their nauplii stages (Wood 1967, Welsh 1975, Anderson 1985, Cházaro-Olvera 2009a), although they appear to exhibit high levels of site fidelity as adults (Allen et al. 2015). They play a vital role in the food webs of coastal marsh habitats by breaking down organic materials and making those nutrients available at other trophic levels (Adams and Angelovic 1970, Odum and Heald 1972, Welsh 1975, Anderson 1985), and for these reasons, they are frequently used as indicator species in studies of coastal habitat quality

and toxicity response (Lewis and Foss 2000, DeLorenzo et al. 2016, Gray and Weinstein 2017).

Phloem-feeding planthoppers (hereafter, planthoppers), such as *P. marginata*, are closely associated to plant cover of native marshes. Previous studies have shown an association between abundance of planthoppers and ecosystem services of *Spartina* dominated salt marshes (Finke 2004, Finke and Denno 2005). Further, their mode of dispersal is relevant towards our understanding population connectivity between marshes. Planthoppers may present in a wingless, more fecund brachypterous form or a winged, less fecund macropterous form with higher dispersal capabilities, depending upon environmental conditions and stressors (Denno et al. 1989, Denno 1994). Along the Gulf coast, the wingless form with limited dispersal capabilities is most common (Denno et al. 1991).

Previous genetic studies of the Gulf Killifish in the Gulf indicate limited dispersal, primarily between adjacent estuaries, and significant levels of isolation by distance using both SNPs and microsatellites (Williams et al. 2008, Williams et al. 2010). Population genetics studies of grass shrimp are few, but include an allozyme study that compared nine collections on and around Galveston Island that reported lower levels of variation in isolated, recently formed ponds compared to those from larger populations occupying older bodies of water open to migration (Fuller 1977, Fuller and Lester 1980). By contrast, high levels of gene flow over a wide geographic range in marshes along the S. Atlantic coast were invoked to explain the reduced genetic partitioning among distant populations of grass shrimp using mitochondrial DNA (mtDNA) 16sRNA single strand conformation polymorphism (SSCP; Flowers 2004). In planthoppers, mtDNA Cytochrome Oxidase

Subunit I (COI) data revealed pronounced population structure at very large geographic scales (e.g., Gulf and S. Atlantic versus N. Atlantic), and the signature of the corresponding clades was used to identify the source of putatively introduced populations (Denno 2008).

Mitochondrial data for many individuals, particularly across a group of co-occurring species, can provide a baseline from which to generate questions for deeper investigation (Bowen et al. 2014). This study is part of a larger study aimed at characterizing genetic variation for both mtDNA and nuclear DNA, seeking to add to the current knowledge for these three important salt marsh residents. In here, we analyze mtDNA sequence data to investigate genetic diversity and connectivity of populations from estuaries along the north and west Gulf coast. We report very distinct patterns of population structure in these three species. In Gulf Killifish, there is evidence of regional population structure and isolation by distance (IBD), whereas in grass shrimp and planthoppers, a strong difference among locals were found, but these populations appear not to be at a migration-drift equilibrium, with local populations subject to local extinctions and recolonization or to the effects of variance in reproductive success. While the observed patterns fit well with the expectations of their respective life history patterns, comparative phylogeography and historical demography also reveal the influence of vicariance, population expansion, and the levels of variance in reproductive success on the regional phylogenies of the three species.

Materials and methods

Sampling

Gulf Killifish, grass shrimp, and planthoppers were captured in spring and summer months from 2014 to 2017, in *Spartina alterniflora* salt marshes along the northwestern Gulf of Mexico coastlines of Texas, Louisiana, and Mississippi (**Figure 6**). All specimens were immediately preserved in the field using 95% ethanol, and were transferred to 70% ethanol for long-term preservation within 24 hours of collection. Gulf Killifish were sampled using minnow traps baited with dog or cat food kibbles, placed in shallow (< 0.5m) water in low marsh habitat, and allowed to soak for up to 12 hours. Specimens were visually identified in the field and humanely sacrificed via immersion in MS-222 as per U.S. Federal policies on the use of laboratory animals as subjects (AUP# 2014-0111 and 2017-0105). Grass shrimp were collected using a dip net along marsh edge habitat. Morphological identification to species level was carried out on well-preserved, intact specimens using a dichotomous key for *Palaemon* spp. (Wood 1974, Anderson 1985), and verified via the mtDNA sequences generated in this study. Arthropods, including planthopper specimens, were obtained using professional insect 15" muslin sweep nets (www.gemplers.com). Preserved samples were examined under a dissecting microscope at 10x magnification, and specimens with the characteristics of *Prokelisia* spp. were separated from other arthropods. *P. marginata* and *P. dolus* are extremely similar morphologically, and distinguishing these two species is very difficult in males and impossible in females (Denno et al. 1987, Heady and Wilson 1990). Therefore, to avoid inflating the estimates of diversity via the inclusion of cryptic species (Paterson 1991, Bickford et al. 2007), planthoppers were identified to species level by characterizing their mtDNA via a High Resolution Melting Analysis (HRMA) assay developed specifically to

distinguish these two species (Espinoza and Alvarado Bremer 2019). Following identification, representative samples of each species from each location were sequenced and analyzed.

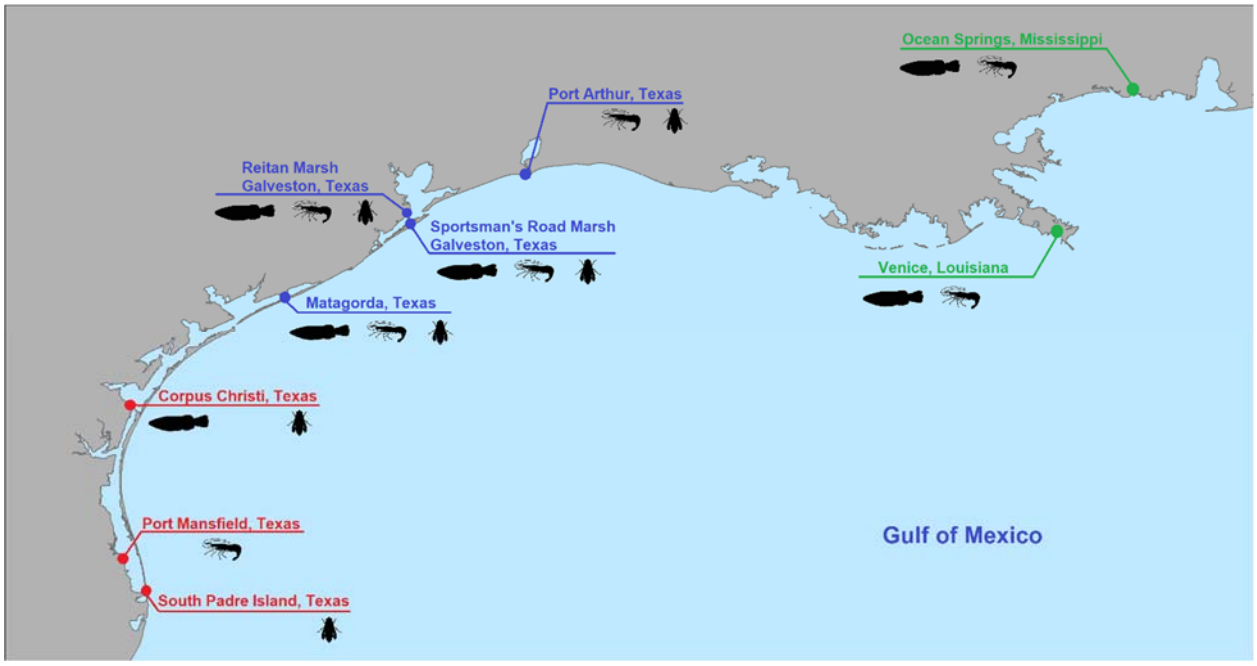

Figure 6. Sample map of the northwestern Gulf of Mexico coastline. Sampling locations are depicted with silhouettes indicative of species collected at each site: Gulf Killifish, Daggerblade grass shrimp, and Phloem-feeding planthoppers. Text color indicates geographic groupings used for analyses: red for South Texas (S.Tex), blue for East Texas (E.Tex), and green for North Gulf (N.Gulf).

DNA Isolation

DNA from Gulf Killifish and grass shrimp was isolated from axial muscle using Zymo Quick-DNA Universal Kit following the manufacturer's instructions for tissue (Zymo Research, Irvine, CA, U.S.A.). Because of their small size (< 5 mm), DNA isolation from planthoppers required individually grinding the entire specimen with a sterile disposable pestle. DNA was initially isolated using the Zymo Quick-DNA Universal Kit, but yields were low. However, since higher DNA yields were obtained using the Qiagen Pure-gene extraction kit (Qiagen, Hilden, Germany) with the addition of 1 µL glycogen solution, as recommended by the manufacturer, this kit was used with the majority of planthoppers.

Molecular Techniques

For Gulf Killifish, three sets of primers were designed to target a segment of the mitochondrial Control Region (CR1), and segments from Nitrogen Dehydrogenase Subunits 2 and 5 (ND2 and ND5) as their mutation rates are sufficient for investigating population structure (Whitehead 2009). For grass shrimp and planthoppers, we targeted segments of 16sRNA (16S) and Cytochrome C Oxidase Subunit I (COI), respectively, as previous studies have used these markers successfully in population studies of these species (Flowers 2004, Denno 2008). All primer sets used in this study are either primers from other studies altered to our target species, or were designed for this study using the Primer 3 software (Koressaar and Remm 2007, Untergasser et al. 2012) within Geneious v.9.1.8 (Biomatters Ltd., Aukland, NZ) as the most optimal among potential primer-pairs capable of amplifying an *in silico* fragment 400-600 bp in length for the targeted regions. The mitogenomes of Gulf Killifish (Accession # FJ445396) and grass shrimp (Accession #

EU868697) were used as reference in designing primers for those species. **Table 2** provides a summary of the primers used in this study.

PCR was carried out separately for each locus in 12.5 µL reactions containing 1x Econotaq Plus Green Master Mix, 0.2 µM of each primer, and 10-20 ng of isolated DNA as template. Thermocycling was performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with an initial denaturing step at 94°C for 2 minutes; followed by 35 cycles of denaturing 94°C for 25 seconds, annealing at the corresponding temperature for each primer pair (**See Table 2**) for 30 seconds, and extensions at 72°C for 90 seconds; and a final extension step at 72°C for 3 minutes. Negative controls were included in all reactions. PCR products were then visualized for specificity and yield via electrophoresis on a 2% agarose gel pre-stained with ethidium bromide (EtBR). PCR products that produced a single band were diluted 1:10 in ddH_2O for post-PCR cleanup and sequenced in both directions, with reaction setups and thermocycling profiles as described in Cruscanti et al. (2015).

Table 2. PCR primer summary for each species and marker sequenced in this study. Nucleotides that have been changed from the originally published primer sequence are denoted in bold, and nucleotides that have been inserted to the originally published sequence are underlined.

Locus	Primer Names	Primer Sequence	Fragment Size	Annealing Temp. (°C)
F. grandis CR-1 (Alvarado-Bremer et al. 1995, modified)	L15998-FG CSBD-H-FG	5' **CG**C CCC **TAG** CTC CCA AAG CTA 3' 5' A**A**T AGG AAC CA**A** ATG CCA G 3'	400 bp	50
F. grandis ND2 (This study)	L4173ND2-FG H4634ND2-FG	5' CAT CAT CCC CGA GCC GTT GA 3' 5' GGA AGG TTA AGG ATG GGA AG 3'	421 bp	50
F. grandis ND5 (This study)	L12137ND5-FG H12717ND5-FG	5' GCA GAA ACG GTA GTG TCC AC 3' 5' GTA CTT GAA TGC AGT AGG GC 3'	540 bp	50
P. pugio 16sRNA (Crandall and Fitzpatrick 1996, modified)	L-16sRNA-PP H-16sRNA-PP	5' **TCG** CCT GTT TA**T** CAA AAA CAT 3' 5' AGA TAG AAA CC**C** A**A**C **C**TG G 3'	470 bp	50
P. marginata COI (Simon et al. 1994)	C1-J-1751 C1-N-2197	5' GGA TCA CCT GAT ATA GCA TTC CC 3' 5' CCC GGT AAA ATT AAA ATA TAA ACT TC 3'	400 bp	57

Analyses

Multiple sequence alignments were carried out in Geneious Pro v.9.1.8 (Biomatters Ltd., Aukland, NZ). Haplotype data files were generated in DnaSP v6.12.03 (Rozas et al. 2017). Arlequin v.3.5.2.2 (Excoffier et al. 2005) was used to estimate genetic diversity within sampling locations, and to calculate sequence diversity indices, pairwise $F_{ST,}$ coancestry coefficients (Reynold's Distance), and Slatkin's Linearized F_{ST} (Reynolds et al. 1983, Weir and Cockerham 1984, Slatkin 1991, Raymond and Rousset 1995). Reynold's Distance was calculated in the event that genetic differentiation occurs only by genetic drift without mutations (Reynolds et al. 1983). A Mantel test of isolation by distance (IBD) was calculated using Slatkin's Linearized F_{ST} correlated against pairwise distances between sampling locations (Slatkin 1993, Raymond and Rousset 1995). P-values for pairwise comparisons of the six localities were corrected for multiple testing using Benjamini and Hochberg's (1995) method, which corrects for significance by controlling the false discovery rate (FDR) and produces fewer false negatives than Bonferroni corrections (Jafari and Ansari-Pour 2019). Since the distribution of diversity statistics falls on an asymptotic curve, rather than a normal curve, the Salicru et al. (1993) χ^2 method was used to test for pairwise significant differences in haplotypic diversity between sampling locations. POPart v.1.7 (Bandelt et al. 1999, Leigh and Bryant 2015) was used to build median joining networks (MJN) for Gulf Killifish markers. The MJN was chosen for Gulf Killifish sequences because of the large number of haplotypes separated by small genetic distances (Bandelt et al. 1999). Due to the small number of haplotypes in both grass shrimp and planthoppers*,* the respective relationship among lineages was reconstructed with minimum spanning networks (MSN) using POPart v.1.7. A representative of *F.*

heteroclitus (Accession #KT869378) was used as outgroup for the MJN of Gulf Killifish. Representatives of *P. vulgaris* and *P. dolus* sequenced in this study were used as outgroups for the MSNs of grass shrimp and planthoppers, respectively. Principle component analyses (PCAs) as implemented in R v3.6.1 (R Development Core Team) were also used to identify structure in the distribution of mtDNA variation of Gulf Killifish along the northwestern Gulf of Mexico coastlines of Texas, Louisiana, and Mississippi.

Spatial analysis of molecular variance (SAMOVA) was conducted to identify population structure (Dupanloup et al. 2002). SAMOVA is similar to AMOVA (Excoffier et al. 1992) except that it has the potential to identify genetic barriers between sampling groups without *a priori* constraints on the geographic composition of the groups. Since each species was sampled at six distinct locations, SAMOVAs were tested with two - five groups, and the grouping that produced the highest F_{CT} value was chosen as the best partitioning scheme.

In order to investigate patterns of historical demography and estimate female effective population sizes (N_e), Gulf Killifish samples were pooled into the most optimal hierarchical arrangement scheme based on SAMOVA results that was congruent with the phylogeographic association. For grass shrimp and planthoppers, due to overall low levels of haplotypic diversity, samples were pooled within species, to reconstruct their demographic history and estimate N_e. Female effective population sizes (N_e) were estimated using the method described in Roman and Palumbi (2003). Briefly, to determine the rate of divergence, each species was compared against the corresponding segments of the mitogenome of the closest relative in DnaSP v6.12.03 to obtain Tamura-Nei gamma-corrected distances (Da) between the species, and the times when the corresponding

speciation events happened from the literature. Accordingly, Gulf Killifish populations were compared against seven representatives of the Atlantic sister species, *F. heteroclitus* (Accession # KT869378, FJ445398, FJ445399, FJ445401, FJ445402, FJ445403, NC_012312). Grass shrimp were compared against the mitogenome of five representatives of their sympatric sister species, *P. vulgaris* (Accession # JQ042300, JN674358, KP178999, KT959473, KT959519). Planthoppers were compared against five representatives, each a different haplotype, of their sympatric sister species, *P. dolus*, that were sequenced by the authors for a previous study (see Espinoza and Alvarado Bremer 2019). Generation time was assumed to be one year for Gulf Killifish (Lipcius and Subrahmanyam 1986), 2 months for grass shrimp (Anderson 1985), and 1.5 months for planthoppers (Denno et al. 1989). For Gulf Killifish, mutation rates were calculated based on time since divergence from their Atlantic sister species. The times used were based on Avise (1992), who estimated divergence times between the Gulf and Atlantic Ocean for coastal fish species range from 0.5-4.8 million years (mean = 1.3 million years). For grass shrimp and planthoppers, comparisons with sympatric sister species prevent using geological events to estimate mutation rates. Therefore, mutation rates of 0.9% – 1.1% per MY for grass shrimp (Garcia-Merchan et al. 2012) and 2.7% per MY for planthoppers (Goodman 2010) were obtained from the literature for closely related taxa. Pairwise mismatch distributions (Rogers and Harpending 1992, Rogers 1995), the D statistic by Tajima (Tajima 1983), the R2 statistic by Ramos-Onsins and Rozas (Ramos-Onsins and Rozas 2002), and the estimated mutational time, tau (τ) since population expansion, (Beerli and Felsenstein 1999) were generated to estimate patterns of historical demography.

Results

Gulf Killifish

Sequences for three mtDNA segments, namely CR1 (336 bp), ND2 (344 bp), and ND5 (397 bp), were obtained from 166 Gulf Killifish specimens from six sampling locations. The sequences of all three loci were concatenated into one single segment 1077 bp long, containing 176 segregating sites (**Table 3**) resulting in 109 distinct haplotypes (Appendix B, Table B-1). For all loci, patterns of genetic variability within and among-localities were estimated. Nearly identical patterns of differentiation and diversity were obtained by analyzing each of these segments separately (Appendix B, Tables B2-B8); therefore, only the results for the concatenated segment are reported below.

High levels of haplotypic diversity ($h > 0.88$) were found in all sampling locations, but differences in levels of genetic variation among some of the samples were observed (**Table 3**). Values of haplotypic diversity were significantly lower in Venice than all other locations except Corpus Christi. Within Galveston Bay, Sportsman's Road was significantly less variable than the Reitan Marsh, which contained the highest overall value of haplotypic diversity (**Table 4**). Comparison of pairwise F_{ST} values for Gulf Killifish identify Corpus Christi as significantly different from all other locations ($p < 0.01$). Similarly, Venice and Ocean Springs differ significantly from all other locations ($p < 0.01$) and from each other ($p < 0.05$; **Table 4**). Calculations of Slatkin's Linearized F_{ST} and Reynold's distance (**Table 5**) yielded similar relationships. A Mantel test is consistent with IBD ($R^2 = 0.5385$; $p = 0.0129$) among the Gulf Killifish sampling locations characterized in this study (**Figure 7**).

Table 3. Molecular indices for 1077 bp of mtDNA concatenated sequences of CR1, ND2, and ND5 for Gulf Killifish by sample location. M, No. of haplotypes, *h,* haplotypic diversity; π, nucleotide diversity; SD, standard deviation; S, no. of segregating (polymorphic) sites; Ts, no. of transitions; Tv, no. of transversions; I/D, no. of insertions and/or deletions.

Location	N	M	*h* (SD)	π (SD)	S	Ts	Tv	I/D
Corpus Christi	26	15	0.945 (0.024)	0.013 (0.007)	64	55	7	2
Matagorda	18	16	0.980 (0.028)	0.019 (0.010)	64	55	9	2
Reitan Marsh	29	24	0.988 (0.012)	0.019 (0.010)	77	71	5	2
Sportsman's	24	23	0.956 (0.015)	0.020 (0.010)	80	70	10	3
Venice	36	18	0.886 (0.037)	0.004 (0.002)	37	30	8	0
Ocean Springs	33	26	0.983 (0.012)	0.007 (0.003)	54	43	13	1
All Samples	**166**	**109**	**0.987 (0.003)**	**0.018 (0.009)**	**176**	**142**	**34**	**4**

Table 4. Values for pairwise comparisons for 1077 bp of concatenated sequences of CR1, ND2, and ND5 for Gulf Killifish. Z-scores from Salicru χ^2 test for pairwise comparisons of haplotypic diversity are above the diagonal. Pairwise F_{ST} are below the diagonal. Significant values are in bold, with significance at $p < 0.05$ denoted by *, and significance at $p < 0.01$ denoted by **.

	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs
Corpus Christi		- 0.949	- 1.603	- 0.389	1.338	- 1.416
Matagorda	**0.18880** **		- 0.263	0.756	**2.026** *	- 0.098
Reitan Marsh	**0.13947** **	- 0.01239		**1.666** *	**2.622** **	0.295
Sportsman's	**0.20315** **	- 0.02873	- 0.00191		**1.753** *	- 1.406
Venice	**0.67054** **	**0.42053** **	**0.44186** **	**0.37772** **		**- 2.494** **
Ocean Springs	**0.63133** **	**0.37057** **	**0.40474** **	**0.34059** **	**0.03746** *	

Table 5. Values for pairwise population comparisons for 1077 bp of mtDNA concatenated sequences of CR1, ND2, and ND5 for Gulf Killifish. Slatkin's linearized F_{ST} is above the diagonal, and Reynold's Distance is below the diagonal.

	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs
Corpus Christi		0.03221	0.03032	0.03539	0.08943	0.03664
Matagorda	0.03274		0.00638	0.01289	0.07185	0.01846
Reitan Marsh	0.03079	0.00640		0.00000	0.06613	0.01480
Sportsman's	0.03602	0.01297	0.00000		0.06741	0.01578
Venice	0.09355	0.07449	0.06836	0.06973		0.03300
Ocean Springs	0.03732	0.01863	0.01491	0.01591	0.03355	

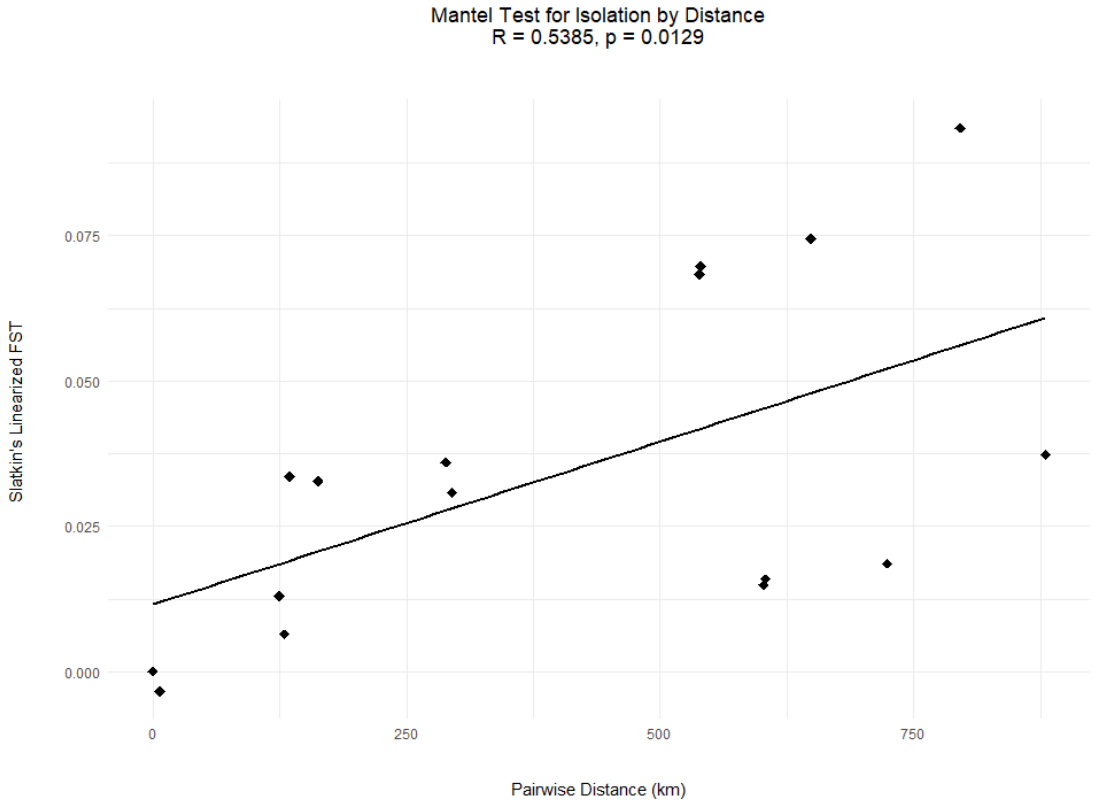

Figure 7. Mantel test of IBD for Gulf Killifish based on the RMA plot of geographic distances (km) between sampling locations against pairwise Slatkin's linearized F_{ST} values calculated from 1077 bp of mtDNA concatenated sequences of CR1, ND2, and ND5. The p-value is based on the number of random ≥ observed results in 10,000 permutations of the test.

SAMOVA results for Gulf Killifish produced the highest value of among-population variance (F_{CT}) when samples were allocated into three regional groups consisting of (1) Corpus Christi; (2) Matagorda, Reitan Marsh, and Sportsman's Road; and (3) Venice and Ocean Springs (**Table 6**), which correspond to the regional sampling carried in this study. Matagorda samples were originally expected to group with Corpus Christi samples, as the upper Laguna Madre and Matagorda Bay systems are contiguous; however, all analyses performed in this study indicate that Matagorda lineages are more accurately grouped with Galveston Bay samples, and will therefore be grouped together for the purposes of this study. Accordingly, hereafter these populations will be referred to as S.Tex, E.Tex, and N.Gulf, respectively.

The relationships among Gulf Killifish mtDNA haplotypes (n=109) can be visualized in a MJN that identifies two major phylogroups, separated from each other by at least 12 mutations, which display different levels of phylogeographic association (**Figure 8**). The first phylogroup appears in the MJN to be more closely related to *F. heteroclitus*, although it is important to note that the MJN fails to correctly place the root at the base of the Gulf Killifish tree, based on the construction of a phylogenetic tree (not shown). This phylogroup, referred to as Phylogroup I, includes at its baseline lineages primarily (87.1%) from E.Tex, although it also includes two haplotypes each from S.Tex and N.Gulf (Ocean Springs). In terms of the total number of E.Tex haplotypes, about half of these cluster within this portion of Phylogroup I. The second portion of Phylogroup I consists of lineages exclusively found in the N.Gulf, and includes two haplotypes whose frequency is higher than any other Gulf Killifish haplotype characterized in this study. From these two centroids a series of closely related haplotypes, one or two mutational steps apart emerge,

concordant with star-phylogenies. These two centroids have the highest frequency in Venice, the sampling location where the lowest value of haplotypic diversity was documented, but are also found in Ocean Springs, which is also significantly less variable than any locations sampled in Texas **(Table 3)**. Phylogroup II contains lineages found exclusively in S.Tex and E.Tex. About half of E.Tex lineages belong to this second phylogroup, which is the most divergent from the outgroup (i.e., *F. heteroclitus*). It should be noted that most (80.0%) Gulf Killifish from S.Tex (Corpus Christi) belong to Phylogroup II, and include eight haplotypes located at terminal branches, each separated by > 6 mutations from their respective nearest neighbor, most found in E.Tex. However, two haplotypes from S.Tex were also found in E.Tex (Matagorda and Reitan Marsh), and a third S.Tex haplotype clusters with other haplotypes from E.Tex. Lastly, intermediate to phylogroups I and II there are two haplotypes from E.Tex and one from S.Tex, that collectively are separated from these groups by 12 and eight mutations, respectively. It should be noted that this intermediate group is actually the most basal with regards to its relationship with *F. heteroclitus,* according to a phylogenetic tree (not shown).

Table 6. SAMOVA results for 1077 bp of mtDNA concatenated sequences of CR1, ND2, and ND5 for Gulf Killifish. The highest Among Groups (F_{CT}) value was obtained with three groups, as follows: Population 1 (S.Tex): Corpus Christi; Population 2 (E.Tex): Matagorda, Reitan Marsh, and Sportsman's; Population 3 (N.Gulf): Venice, LA and Ocean Springs, MS. P-values for fixation indices are based on significance tests with 1023 permutations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	2	485.23	4.5071 Va	39.45
Among Populations Within Groups	3	21.83	0.0135 Vb	0.12
Within Populations	163	1125.51	6.9050 Vc	60.43
Total	168	1632.57	11.42551	
Fixation Indices			**P-values (≥)**	
	F_{SC}:	0.00195	0.45064 +/- 0.01396	
	F_{ST}:	0.39565	0.00000 +/- 0.00000	
	F_{CT}:	0.39447	0.01271 +/- 0.00366	

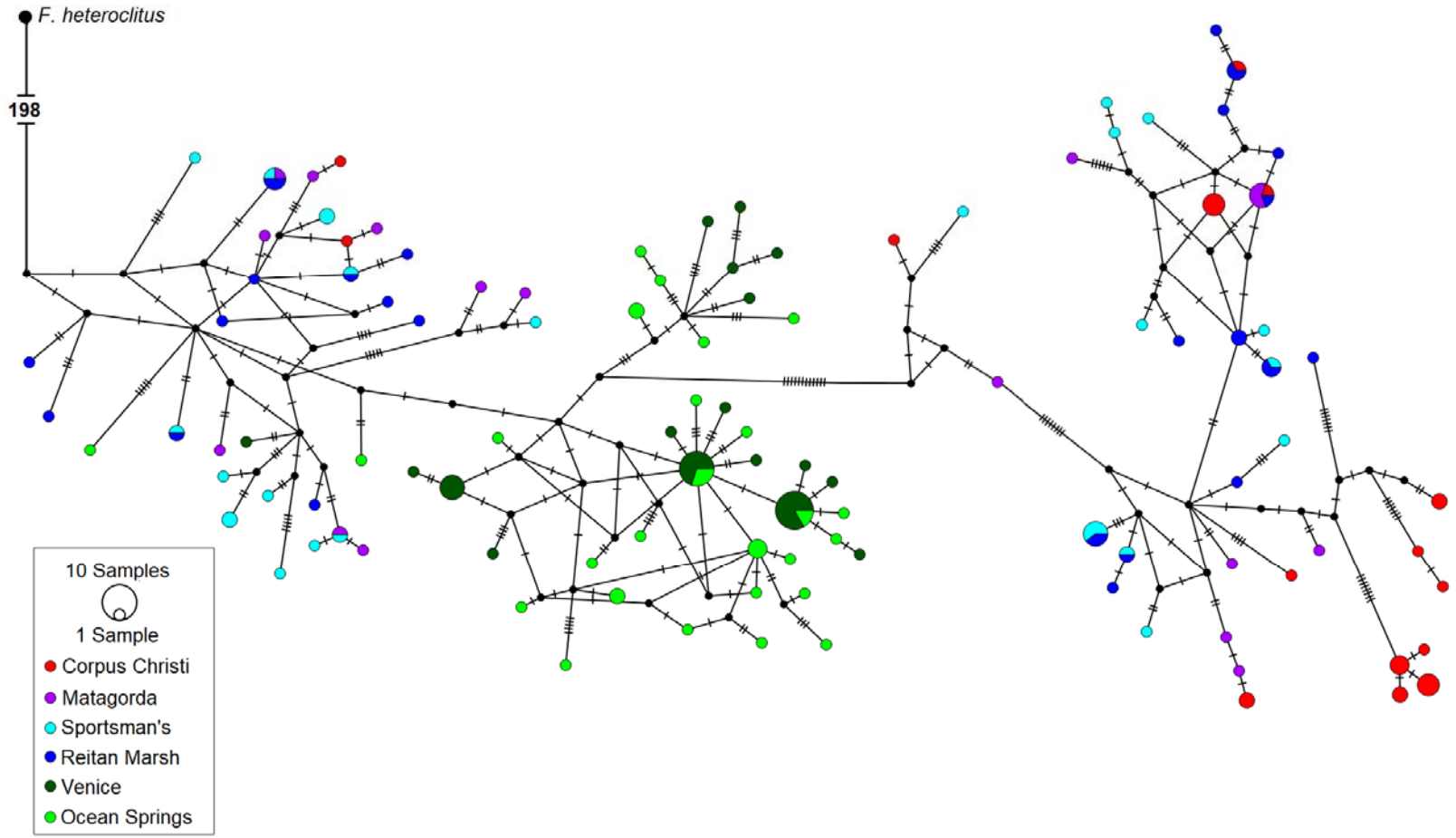

Figure 8. Median joining network (MJN) showing the relationship of Gulf Killifish mtDNA lineages based on 1077 bp of concatenated sequences of CR1, ND2, and ND5, with its sister species, *F. heteroclitus*, as the outgroup. Each circle represents a distinct haplotype, and its size, the number of times is repeated, with the fill colors representing sampling location (see inset). Hash marks indicate the number of segregating sites between each haplotype, with the black circles between them representing hypothetical haplotypes not found in the sample. This network fails to place the root at the base of the Gulf Killifish tree, which connects to the intermediate group, as indicated by a phylogenetic tree (not shown).

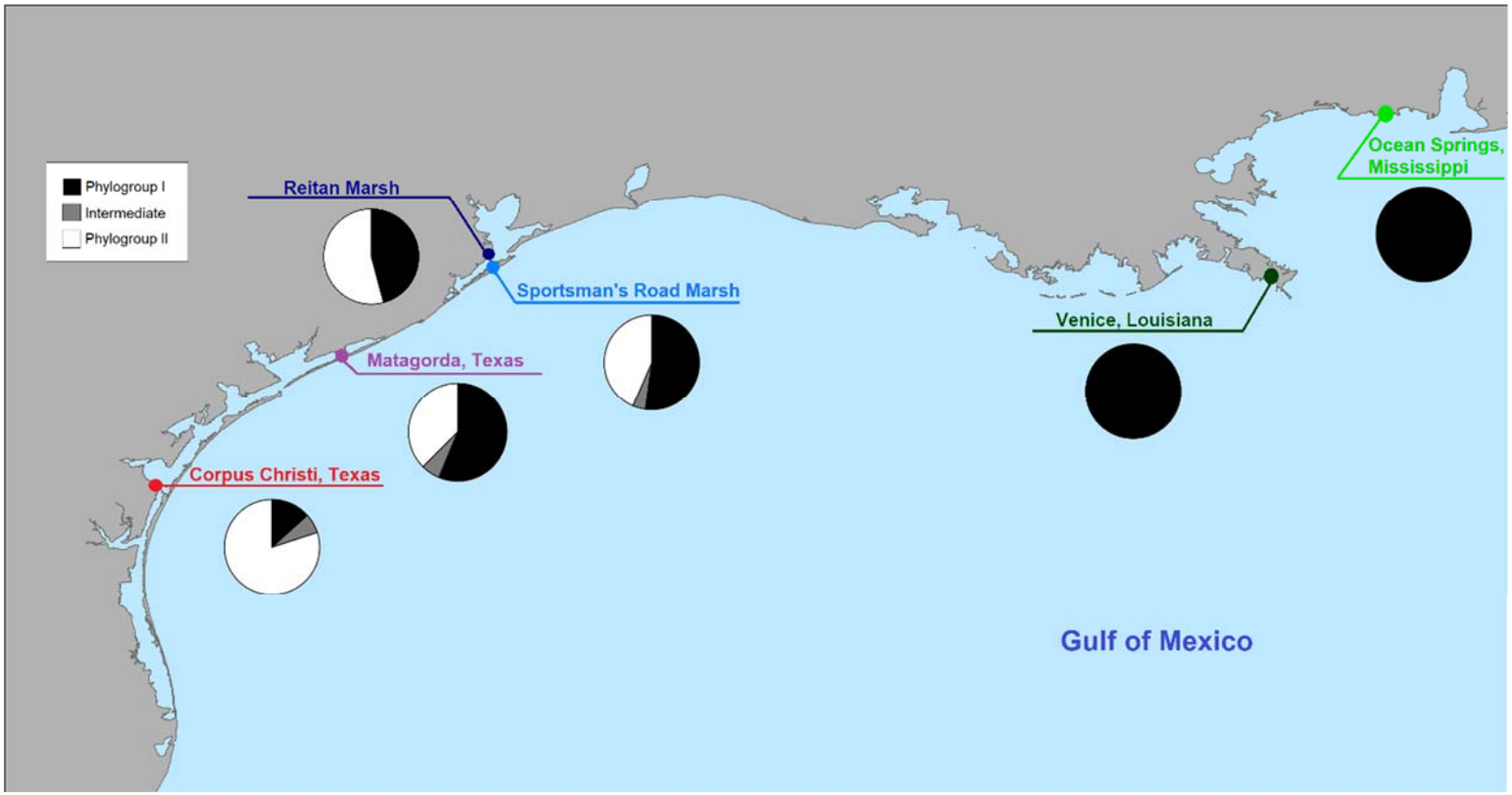

Figure 9. Pie charts depicting the frequency distribution of Gulf Killifish mtDNA phylogroups (see inset) along the northern Gulf of Mexico coast localities surveyed in this study. Haplotypes were assigned to the corresponding phylogroups identified with the MJN and PCA (Figs. 3 and 5, respectively).

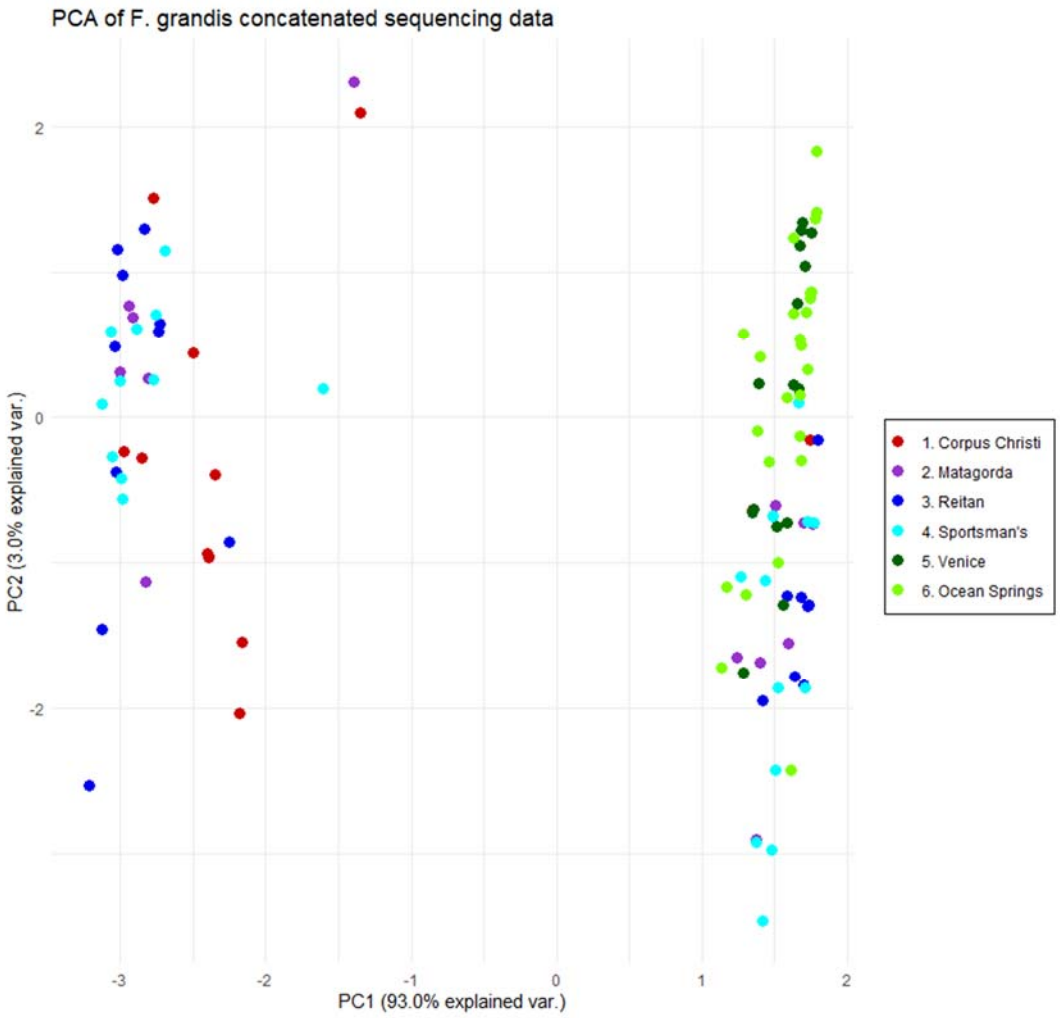

Figure 10. PCA for 1077 bp of mtDNA concatenated sequences of CR1, ND2, and ND5 for Gulf Killifish. Points correspond to individual sequences and colors correspond to sampling location as noted in the legend.

An investigation of the frequency distribution of phylogroups by sampling location (**Figure 9**) clearly depicts the shift in phylogroup association from west to east, with the majority of S.Tex lineages belonging to Phylogroup II, all N.Gulf lineages belonging to Phylogroup I, and E.Tex lineages being split nearly evenly between the two Phylogroups. The first two axes of the PCA of Gulf Killifish mtDNA sequence data explained 96.0% of observed variation (**Figure 10**). Congruent with the MJN, PCA loadings along PC1 separate most haplotypes into two major clusters. The first cluster, characterized by strong positive loadings along PC1, contains individuals from all sampling localities, including S.Tex, albeit at a much lower frequency. The second cluster, characterized by negative loadings along the first axis, groups individuals sampled exclusively along the Texas coast, from E.Tex to S.Tex.

The historical demographic signature of Gulf Killifish differed among the regions of the Gulf of Mexico surveyed. Both D and R2 statistics suggest population expansion ($\alpha < 0.05$) in in the N.Gulf population **(Table 7)**. Mismatch distributions of pairwise differences d S.Tex and E.Tex yielded multiple peaks over wide range of pairwise differences (0-40). By contrast, the N.Gulf distribution is unimodal, with pairwise differences ranging between 0-15 (**Figure 11**).

Table 7. Historical population demography parameters and estimates of female effective population size (N_e) for populations of Gulf Killifish. Populations are based on SAMOVA results. Da, Tamura-Nei gamma corrected genetic distance between the population and sister species, *F. heteroclitus*; τ, estimated mutational time since population expansion; D, Tajima's D with probability value (P); R2, Ramos-Onsins and Rozas's R2 with probability value (P); T, time since divergence in millions of years used for mutation rate estimations; N_e, estimated female effective population size, in millions of individuals.

Population	Da	τ	D (P)	R2 (P)	T	N_e
S.Tex	0.076	6.50	-0.483 (0.346)	0.109 (0.368)	0.50	50.48
					1.25	126.21
					4.8	484.65
E.Tex	0.072	8.62	-0.343 (0.424)	0.888 (0.417)	0.50	80.74
					1.25	201.84
					4.8	775.07
N.Gulf	0.078	2.95	**-2.058 (0.003)**	**0.035 (0.001)**	0.50	18.89
					1.25	47.23
					4.8	181.35

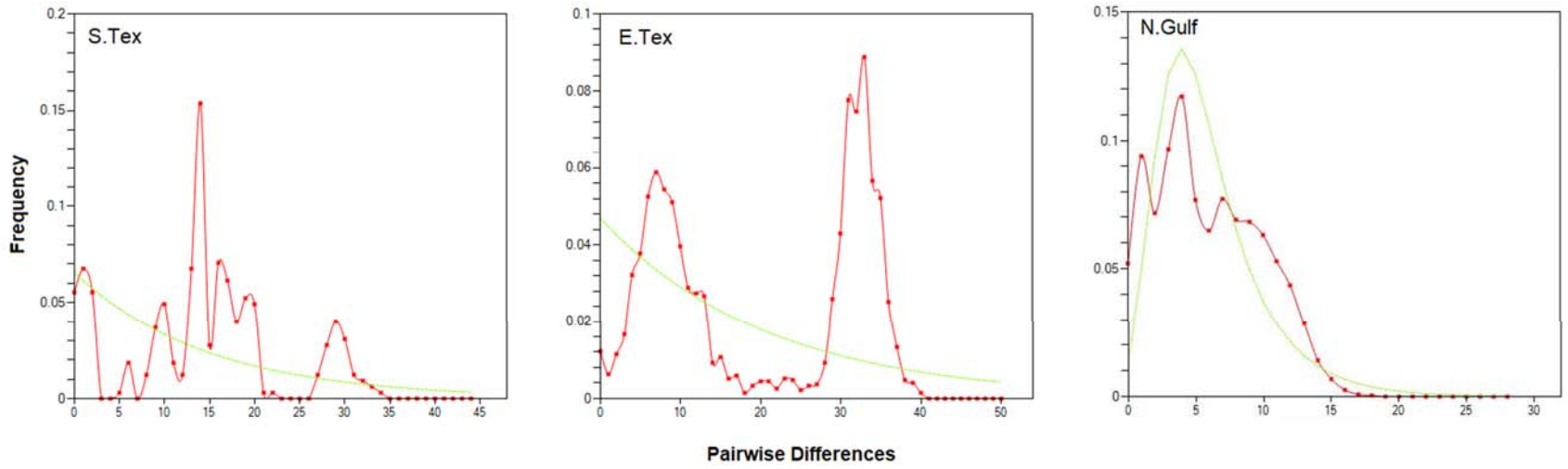

Figure 11. Frequency distribution of observed (red line) and expected (green line) pairwise differences for 1077 bp of mtDNA concatenated sequences of CR1, ND2, and ND5 for Gulf Killifish populations. Frequency, on the y-axis, refers to the relative frequency of pairs of individuals that differ by the number of pairwise differences, on the x-axis. Groups are based on population structure found in this study, and consist of: S.Tex: Corpus Christi; E.Tex: Matagorda, Reitan Marsh, and Sportsman's; N.Gulf: Venice and Ocean Springs.

Table 8. Historical population demography parameters and estimates of female effective population size (N_e) for populations of Gulf Killifish. Groupings are based on phylogroups depicted in the median joining network. Da, Tamura-Nei gamma corrected genetic distance between the population and sister species, *F. heteroclitus*; τ, estimated mutational time since population expansion; D, Tajima's D with probability value (P); R2, Ramos-Onsins and Rozas's R2 with probability value (P); T, time since divergence in millions of years used for mutation rate estimations; N_e, estimated female effective population size, in millions of individuals.

Grouping	Da	τ	D (P)	R2 (P)	T	N_e
Phylogroup I	0.076	5.63	**-2.019 (0.003)**	**0.034 (0.005)**	0.50	17.76
All lineages					1.25	44.39
					4.8	170.43
Phylogroup I	0.074	6.34	**-1.578 (0.043)**	**0.059 (0.011)**	0.50	9.41
E.Tex lineages					1.25	23.51
					4.8	90.29
Phylogroup I	0.078	2.95	**-2.058 (0.003)**	**0.035 (0.001)**	0.50	18.89
N.Gulf lineages					1.25	47.23
					4.8	181.35
Phylogroup II	0.079	6.19	-1.106 (0.127)	0.069 (0.127)	0.50	26.10
All lineages					1.25	65.25
					4.8	250.57
Phylogroup II	0.081	5.16	-1.312 (0.076)	0.071 (0.064)	0.50	16.14
E.Tex lineages					1.25	40.34

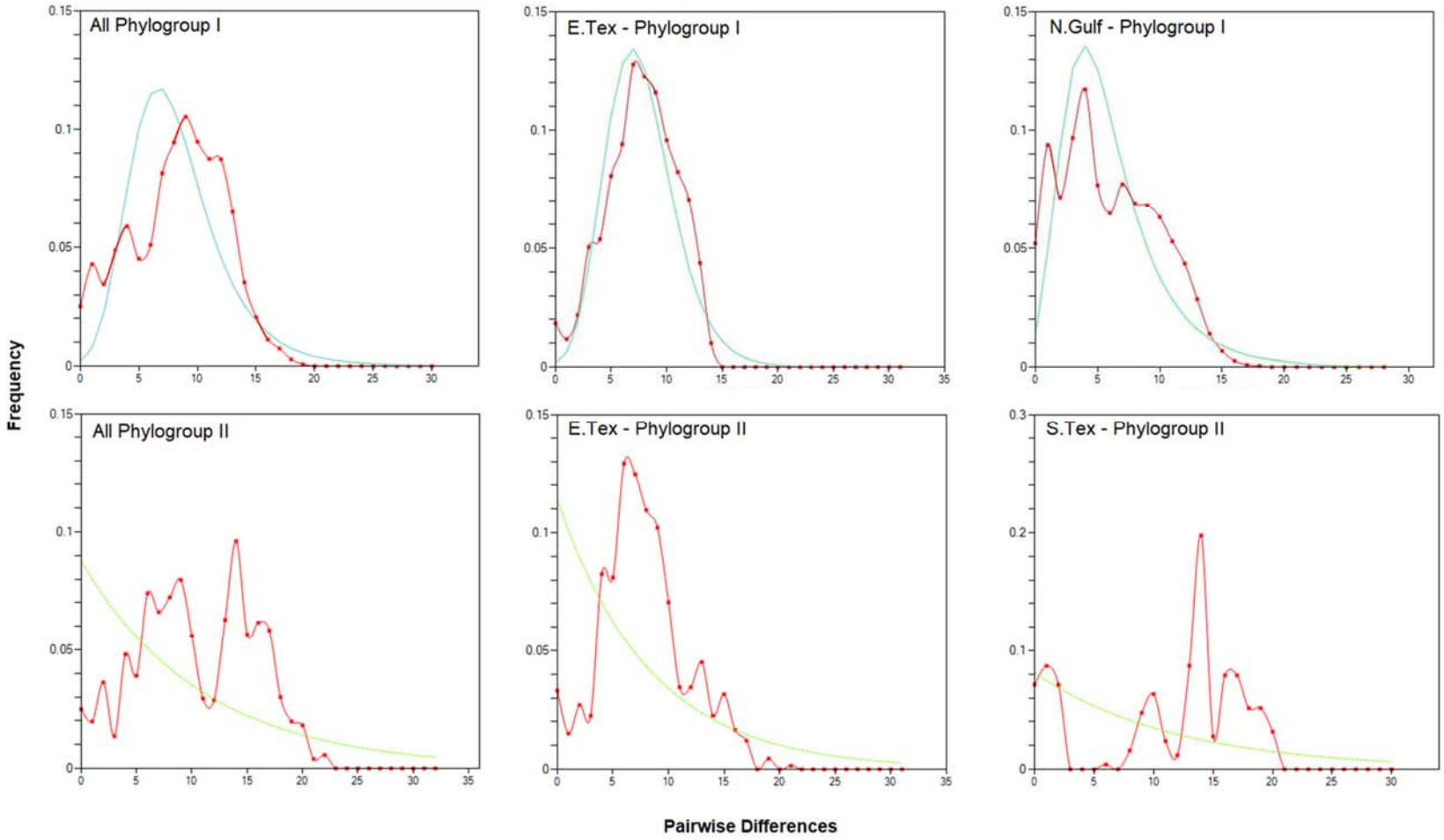

Figure 12. Frequency distribution of observed (red line) pairwise differences for 1077 bp of mtDNA concatenated sequences of CR1, ND2, and ND5 for Gulf Killifish Phylogroups found in this study. Expected differences are shown for the expansion model (blue line) and the neutral model (green line). Frequency, on the y-axis, refers to the relative frequency of pairs of individuals that differ by the number of pairwise differences, on the x-axis.

The corresponding values of τ suggest that the populations E.Tex and S.Tex had long and stable histories, which are two to three times older than the N.Gulf population. Estimates of female N_e ranged from 19 million to 775 million, depending on the estimated time since divergence used in the calculation (**Table 7**). However, since the MJN identifies two phylogroups that have strong phylogeographic associations, the estimates of N_e may be upward biased, as they would be the product of two separate evolutionary histories (see Alvarado Bremer et al. 2005), specifically for the E. Texas and S. Texas populations of Gulf Killifish. Accordingly, mismatch distributions and historical demography statistics, and consequently female N_e, were recalculated separately for phylogroups I and II for E.Tex (**Table 8**), whereas for S.Tex the historical demography estimates were only recalculated for Phylogroup II, due to the paucity of Phylogroup I lineages. For the N.Gulf, these estimates remained the same as the original calculations (**Table 7**), since all the lineages found in Louisiana and Mississippi belong to Phylogroup I (**Figures 8-9**). Recalculated D and R2 test for Phylogroup I lineages in E.Tex was significant, congruent with the population expansion recorded for this Phylogroup in the N.Gulf, although the estimates N_e for this latter region is twice as large as E.Tex. The separate mismatch distributions of each phylogroup in E.Tex were considerably less variable and unimodal for Phylogroup I. However, these tests were not significant in Phylogroup II, both in E.Tex and S.Tex. Overall, the number of pairwise differences was reduced by half compared to the analysis with all lineages together (**Table 8**). The number of pairwise differences for S.Tex distribution by phylogroup translated into a reduction from 35 pairwise differences in the original analysis to 21 pairwise differences (**Figure 12**). Values of τ indicate that Phylogroup II has a slightly longer and stable history than Phylogroup I, and that the two

subgroups of Phylogroup II underwent concurrent expansions. By contrast, comparisons τ values within Phylogroup I indicate that E.Tex lineages are twice as old as the N.Gulf lineages. The new estimates of female N_e for E.Tex based on the sum of the two phylogroups, is about four times smaller than when all lineages are included. For S.Tex, N_e dropped by about 15% (see **Tables 7-8**).

Grass Shrimp

Grass shrimp specimens (n=119) from six sampling locations were successfully sequenced for 466 bp of the mtDNA 16sRNA gene containing 9 variable sites, which define 13 haplotypes throughout the region studied (Appendix B, Table B-1). Except for Ocean Springs, the values of *h* obtained for most sampling locations were extremely low, (**Table 9**), and included two localities in E.Tex (Pt. Mansfield and Sportsman's Road) where only one haplotype (*h*=0) distinct to that locality was detected for each (**Figure 13**). The Sportsman's Road haplotype was private to that locality, but its two nearest neighbors came from Venice and Ocean Springs, whereas the Pt. Mansfield haplotype was also present in those two N.Gulf localities, which collectively reflect all the variability summarized in the MSN, extending from the closest relative to the outgroup, *P. vulgaris*, to the lineage most divergent from that outgroup. It is also worth noting that, in the N.Gulf localities, there is no single haplotype whose frequency exceeds 50%. The two remaining localities, Matagorda and Port Arthur, both in E.Tex, share a common haplotype also found in Ocean Springs. The relationship of that common haplotype to other lineages in these two E. Texas localities resembles a star phylogeny, with daughter lineages one or two mutational steps apart from the centroid (**Figure 13**).

The levels of haplotypic diversity varied along the coastal range sampled. The Salicru χ^2 test indicate significant differences in the levels of variability among samples (**Table 10**). Notably the N.Gulf localities (Ocean Springs and Venice) are more variable ($h \geq 0.667$) than any of the four localities in Texas, where Matagorda ($h = 0.284$) was more variable, with the rest devoid (h=0.000) or nearly devoid ($h \leq 0.100$) of variation (**Table 9**). In spite of the overall low levels of genetic variability, pairwise F_{ST} in grass shrimp revealed significant differences ($p < 0.01$) among the majority of pairwise comparisons, except between Matagorda and Pt. Arthur (**Table 10**).

SAMOVA results (**Table 11**) returned the highest F_{CT} value (non-significant) for five distinct populations in agreement with the significant F_{ST} values. However, due to the low haplotypic diversity in the localities from E.Tex, it was necessary to pool those samples in order to obtain meaningful estimates of both historical demography and female N_e. The mismatch distribution of pairwise differences for grass shrimp (**Figure 14**) indicate that about 22% of the shrimp share haplotypes (i.e., zero differences), with an additional 50% of the individuals differing by 1-3 mutations, and the rest by 4-5 pairwise differences. Both D and R2 tests were non-significant for ($p > 0.05$) as reflected by the multimodal shape of the curve. Female N_e was estimated at 1.9-2.3 million individuals (**Table 12**). A Mantel test could not be calculated for grass shrimp due to the lack of variability in the two E.Tex sampling locations mentioned above, and PCA is not shown for grass shrimp, as there was not enough variation to generate meaningful plots.

Table 9. Molecular indices for 466 bp of 16sRNA sequences for grass shrimp by sample location. M, No. of haplotypes, *h,* haplotypic diversity; π, nucleotide diversity; SD, standard deviation; S, no. of segregating (polymorphic) sites; Ts, no. of transitions; Tv, no. of transversions; I/D, no. of insertions and/or deletions.

Location	N	M	*h*(SD)	π (SD)	S	Ts	Tv	I/D
Pt. Mansfield, TX	20	1	0.000 (0.000)	0.000 (0.000)	0	0	0	0
Matagorda, TX	20	4	0.284 (0.128)	0.001 (0.000)	3	1	1	0
Sportsman's Road	20	1	0.000 (0.000)	0.000 (0.000)	0	0	0	0
Pt. Arthur, TX	20	2	0.100 (0.088)	0.001 (0.000)	1	0	0	0
Venice, LA	19	4	0.667 (0.086)	0.002 (0.002)	3	1	1	0
Ocean Springs, MS	20	6	0.811 (0.047)	0.004 (0.003)	5	2	2	0
All Samples	**119**	**13**	**0.779 (0.021)**	**0.005 (0.003)**	**9**	**6**	**3**	**0**

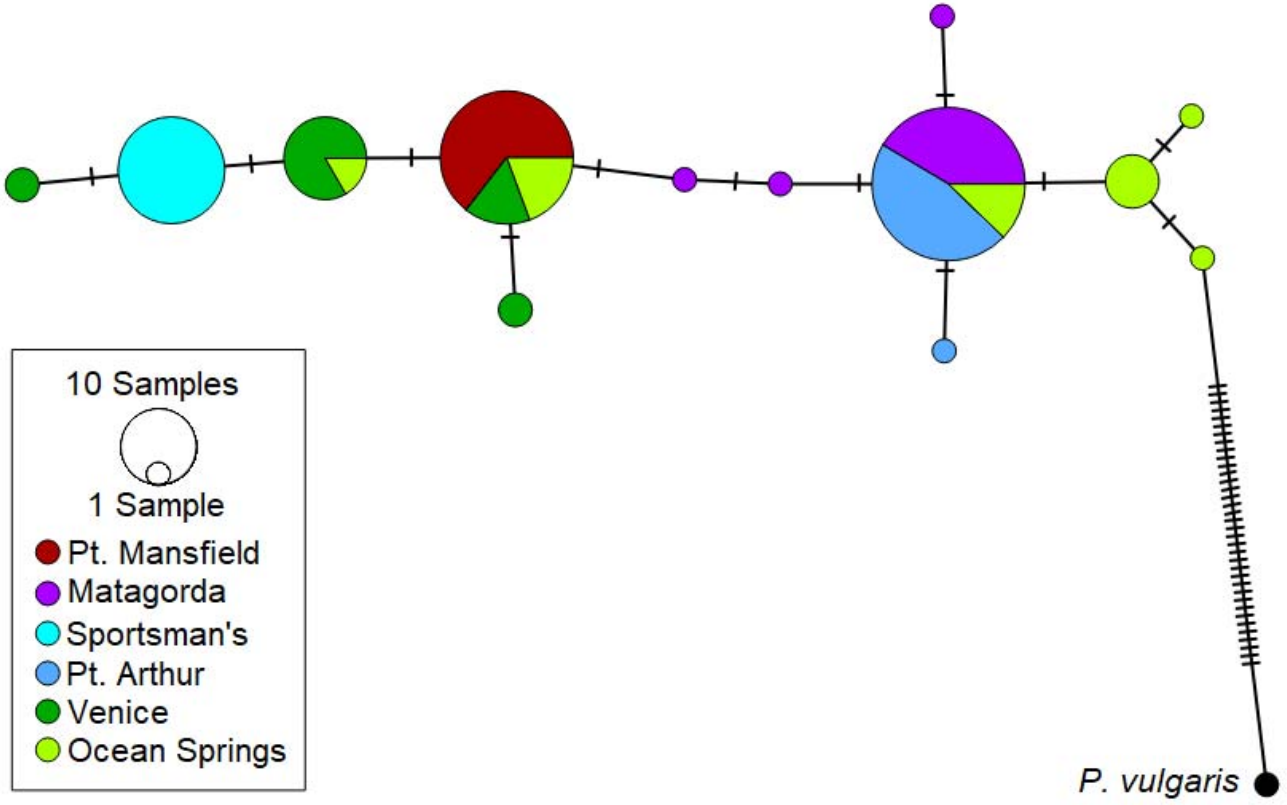

Figure 13. Minimum spanning network (MSN) for 466 bp of 16sRNA sequences for grass shrimp, *P. pugio* rooted with sister species, *P. vulgaris* (black circle). Each circle represents a distinct haplotype, and its size, the number of times is repeated, with the fill colors representing sampling location (see inset). Hash marks indicate the number of segregating sites between each haplotype.

Table 10. Values of pairwise comparisons for 466 bp of 16sRNA sequences for grass shrimp. Z-scores from Salicru χ^2 test for pairwise comparisons of haplotypic diversity are above the diagonal. Pairwise F_{ST} are below the diagonal. Significant values are in bold, with significance at $p < 0.05$ denoted by *, and significance at $p < 0.01$ denoted by **.

	Pt. Mansfield	Matagorda	Sportsman's	Pt. Arthur	Venice	Ocean Springs
Pt. Mansfield		**- 2.219 ***	0.000	- 1.136	**- 7.756 ****	**- 17.255 ****
Matagorda	**0.93285 ****		**2.219 ***	1.185	**- 2.484 ****	**- 3.865 ****
Sportsman's	**1.00000 ****	**0.96026 ****		- 1.136	**- 7.756 ****	**- 17.255 ****
Pt. Arthur	**0.98361 ****	0.02105	**0.99010 ****		**- 4.608 ****	**- 7.127 ****
Venice	**0.45823 ****	**0.81601 ****	**0.65339 ****	**0.86007 ****		- 1.469
Ocean Springs	**0.46252 ****	**0.40897 ****	**0.73527 ****	**0.47953 ****	**0.41360 ****	

Table 11. SAMOVA results for 466 bp of 16sRNA sequences for grass shrimp. The highest Among Groups (F_{CT}) value was obtained with three groups, as follows: Population 1: Sportsman's; Population 2: Port Mansfield; Population 3: Matagorda and Port Arthur; Population 4: Ocean Springs, MS; and Population 5: Venice, LA. P-values for Fixation Indices are based on Significance Tests with 1023 permutations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	4	104.55	1.1235 Va	80.72
Among Populations Within Groups	1	0.18	-0.0049 Vb	-0.35
Within Populations	113	30.87	0.2732 Vc	19.63
Total	118	135.60	1.31703	
Fixation Indices			**P-values (≥)**	
	F_{SC}:	-0.01830	0.49365 +/- 0.01428	
	F_{ST}:	0.80372	0.00000 +/- 0.00000	
	F_{CT}:	0.80724	0.06940 +/- 0.00845	

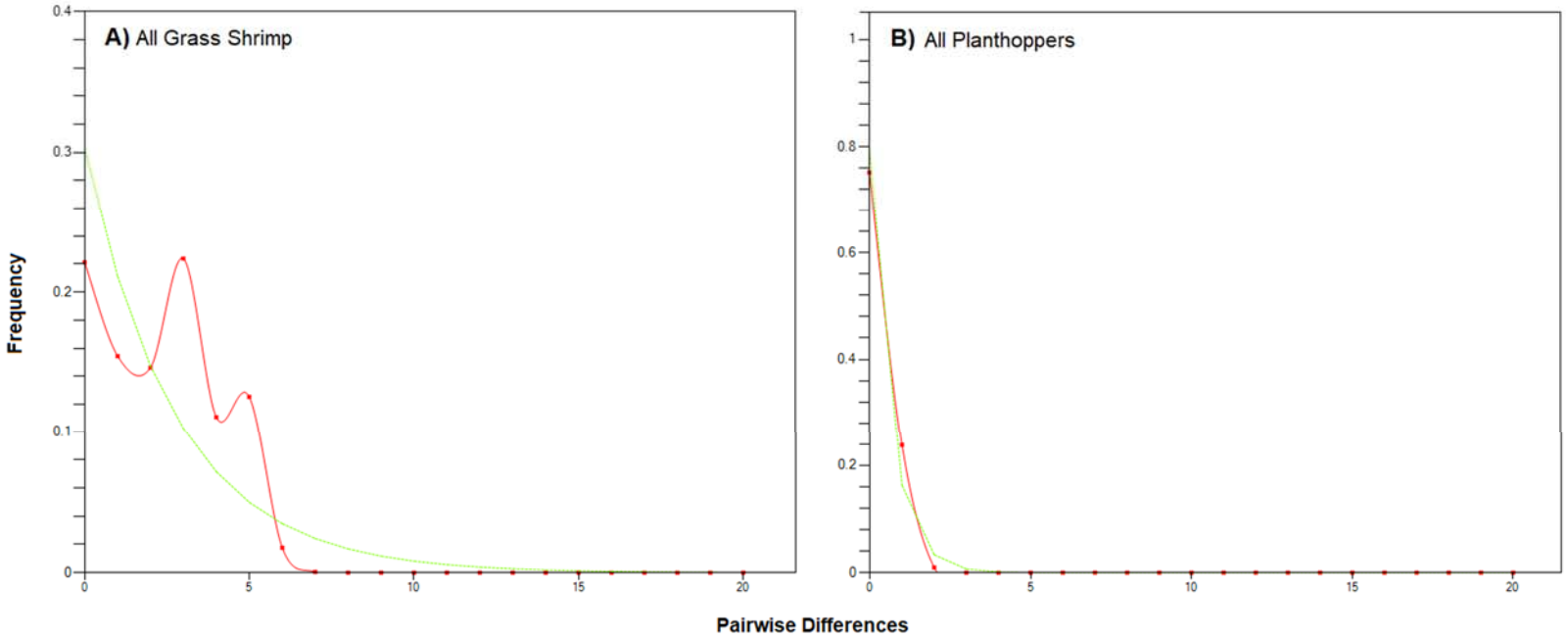

Figure 14. Frequency distribution of observed (red line) and expected (green line) pairwise differences for A) 466 bp of 16sRNA sequences for all samples of grass shrimp, and B) 372 bp of COI sequences for all samples of planthoppers. Frequency, on the y-axis, refers to the relative frequency of pairs of individuals that differ by the number of pairwise differences, on the x-axis.

Table 12. Historical population demography parameters and estimates of female effective population size (N_e) for populations of grass shrimp and planthoppers. Da, Tamura-Nei gamma corrected genetic distance between the population and sister species, *P. vuglaris* for grass shrimp and *P. dolus* for planthoppers; τ, estimated mutational time since population expansion; μ, mutational rate per million years; D, Tajima's D with probability value (P); R2, Ramos-Onsins and Rozas's R2 with probability value (P); N_e, estimated female effective population size, in millions of individuals.

Species	Da	τ	μ	D (P)	R2 (P)	N_e
Grass shrimp	0.0695	1.435	0.009	0.893 (0.824)	0.126 (0.832)	2.359
			0.011			1.930
Planthoppers	0.0470	0.258	0.027	-0.927 (0.214)	0.043 (0.168)	0.444

Planthoppers

Planthopper specimens (n=124) from six sampling locations were successfully sequenced for 372bp of COI, and although this species displayed less diversity ($h < 0.45$) and fewer haplotypes (n=4; **Table 13**) than both Gulf Killifish and Grass Shrimp, the levels of variation and the phylogeographic association were sufficient to reveal significant differences in haplotypic diversity among samples with more than 26% of the variance explained among-groups (**Table 14**). Corpus Christi and Matagorda, which showed similar values of *h*, were more variable than the majority of planthopper localities sampled, whereas South Padre and Reitan Marsh were less variable (h=0) than any other localities. In addition, these two geographically distant sampling locations shared the same haplotype (**Figure 15**), and thus were not different from each other (**Table 15**). In each of the six locations sampled, a common haplotype accounted for > 85% of the individuals, with three additional haplotypes, one mutational step away from the main haplotype, accounting for the remaining individuals in four localities (**Figure 15**). The F_{ST} between Corpus Christi and Matagorda was not significant after corrections for multiple testing, but these two samples differed respectively from South Padre, Reitan Marsh, Sportsman's Road, and Pt. Arthur (**Table 15**). Slatkin's linearized F_{ST} and Reynold's distance for planthoppers could not be calculated due to sampling locations with h =0.

Table 13. Molecular indices for 372 bp of COI sequences for planthoppers by sample location. M, No. of haplotypes, *h,* haplotypic diversity; π, nucleotide diversity; SD, standard deviation; S, no. of segregating (polymorphic) sites; Ts, no. of transitions; Tv, no. of transversions; I/D, no. of insertions and/or deletions.

Location	N	M	*h*(SD)	π (SD)	S	Ts	Tv	I/D
South Padre	20	1	0.000 (0.000)	0.000 (0.000)	0	0	0	0
Corpus Christi	19	2	0.409 (0.100)	0.001 (0.001)	1	1	0	0
Matagorda	20	2	0.442 (0.088)	0.001 (0.001)	1	1	0	0
Reitan Marsh	24	1	0.000 (0.000)	0.000 (0.000)	0	0	0	0
Sportsman's Road	21	3	0.267 (0.120)	0.001 (0.001)	2	2	0	0
Pt. Arthur	20	3	0.279 (0.012)	0.001 (0.001)	2	2	0	0
All Samples	**124**	**4**	**0.248 (0.049)**	**0.001 (0.001)**	**3**	**3**	**0**	**0**

Table 14. SAMOVA results for 372 bp of COI sequences for planthoppers. The highest Among Groups (F_{CT}) value was obtained with three groups, as follows: Population 1: Corpus Christi and Matagorda; Population 2: South Padre, Reitan Marsh, Sportsman's, and Port Arthur. P-values for Fixation Indices are based on Significance Tests with 1023 permutations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	1	2.20	0.0399 Va	26.39
Among Populations Within Groups	4	0.27	-0.0023 Vb	-1.50
Within Populations	118	13.40	0.1135 Vc	75.11
Total	123	135.60	1.31703	
Fixation Indices			**P-values (≥)**	
	F_{SC}:	-0.02034	0.64027 +/- 0.01649	
	F_{ST}:	0.24892	0.00000 +/- 0.00000	
	F_{CT}:	0.26389	0.07136 +/- 0.00884	

Table 15. Values of pairwise comparisons for 372 bp of COI sequences for planthoppers. Z-scores from Salicru χ^2 test for pairwise comparisons of haplotypic diversity are above the diagonal. Pairwise F_{ST} are below the diagonal. Significant values are in bold, with significance at $p < 0.05$ denoted by *, and significance at $p < 0.01$ denoted by **.

	South Padre	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Pt. Arthur
South Padre		**- 4.090 ****	**- 5.023 ****	0.000	**- 2.225 ***	**- 23.250 ****
Corpus Christi	**0.93285 ****		- 0.248	**4.090 ****	0.909	1.291
Matagorda	**1.00000 ****	**0.96026 ****		**5.023 ****	1.176	**1.835 ***
Reitan Marsh	**0.98361 ****	0.02105	**0.99010 ****		**- 2.225 ***	**- 23.250 ****
Sportsman's	**0.45823 ****	**0.81601 ****	**0.65339 ****	**0.86007 ****		- 0.099
Pt. Arthur	**0.46252 ****	**0.40897 ****	**0.73527 ****	**0.47953 ****	**0.41360 ****	

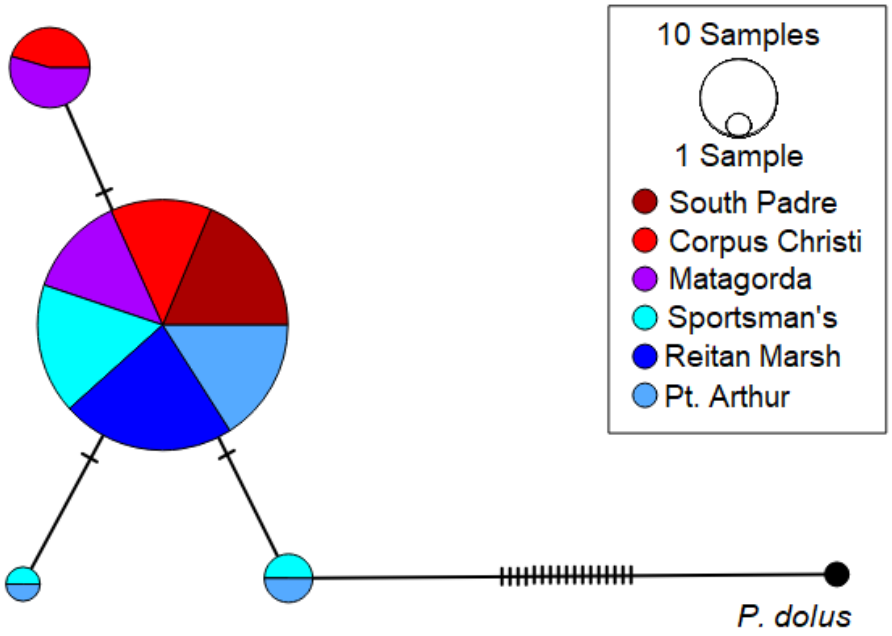

Figure 15. Minimum spanning network (MSN) for 372 bp of COI sequences for planthoppers, *P. marginata*, rooted against sister species, *P. dolus* (black circle). Each circle represents a distinct haplotype, and its size, the number of times is repeated, with the fill colors representing sampling location (see inset). Hash marks indicate the number of segregating sites between each haplotype.

The highest F_{CT} value for planthoppers in SAMOVA was obtained by placing S. Padre and E.Tex in the same group (**Table 14**). However, this result has to be questioned since the index was not significant and there are no biological rationale for grouping these two geographically discreet samples, which are nearly equidistant from the intermediate locality of Corpus Christi. Although no temporal samples were obtained to verify the stability in haplotype frequency, the observed patchiness of the genetic signature among locals may be due to sweepstakes in female reproductive success within demes

(Hedgecock and Pudovkin 2011) that may be subject dramatic changes in population size and that may include local extinction and recolonization events. As such, the demographic estimates at the local level may not be very informative given that four out of six localities had < 2 haplotypes, and the remaining two had only three haplotypes each. Accordingly, to obtain a regional reconstruction of the historical demography and female N_e of planthoppers, samples were pooled together and a pairwise mismatch distribution for planthoppers was obtained (**Figure 14**). Both D and R2 tests were not significant ($p > 0.05$) suggesting that these populations were not subject to a population bottleneck followed by expansion, and the curve fit was concordant with a stable population at mutation drift equilibrium. Long-term Female N_e was estimated at 444,000 individuals (**Table 12**). A Mantel test could not be calculated for planthoppers due to low levels of variation across sampling locations. Similarly, a PCA is not shown for planthoppers, as there was not enough variation to generate meaningful plots.

Discussion

Gulf Killifish

This study was aimed at comparing the levels of genetic variation among the populations of three ecologically important residents of the *Spartina* salt marshes found in estuaries along the northern central and west coast of the Gulf of Mexico. These species were selected because they display contrasting life histories, and therefore substantially different genetic signatures could be expected by analyzing the respective patterns of mtDNA sequence variation. In addition to adding to the current knowledge about the patterns of connectivity of these species, this information was expected to shed light on the

corresponding historical demographic signatures and the timing of events that may have influenced the populations of these species in the same region.

Our results show that Gulf Killifish are highly variable in all the localities sampled except in Venice, LA, near the mouth of the Mississippi River. Gulf Killifish showed high levels of genetic structuring, with nearly 40% of the total variance explained by differences among the three regions of the northern Gulf surveyed (**Table 6**). Gulf Killifish displays high site fidelity, with individuals typically only moving ~100 m between connected salt marsh sites through their lifetimes (Nelson et al. 2014). The sedentary nature of Gulf Killifish, in conjunction with self-adhesive and demersal eggs, high predation rates, and a limited (expected) life span, limits overall movements, reducing gene flow (Gricius 1994). Williams et al. (2008) concluded from the patterns of isolation by distance, spatial autocorrelation, and assignment tests derived from microsatellite data that dispersal is limited, occurring primarily between neighboring sites. However, our mtDNA data indicates that dispersal over long periods of time is sufficient to overcome the genetic separation among adjacent marshes within regions, such that there are three populations that are at migration-drift equilibria, separated from each other by some barriers to gene flow.

This was evidenced by strong phylogeographic associations of mtDNA lineages and phylogroups, and by the distinct historical demographic signatures that exist within-region. Previous investigations of Gulf Killifish population structure using allozymes and restriction fragment length polymorphisms (RFLP) of total mtDNA indicate that that Florida, Louisiana, and Texas populations are more similar to each other than Mobile Bay, Alabama, which stands as an outlier (Gricius 1994). The distinctiveness of Mobile Bay

population relative to Florida was corroborated in a more recent study using microsatellites (Williams et al. 2008), although principal component analysis and Bayesian clustering revealed that Mobile Bay has a closer affinity to the samples of Louisiana and Texas. In here, while we did not sample Mobile Bay, we did characterize Ocean Springs, MS, which lies about 40 miles away. This sample, along with Venice, LA, contained a subset of private lineages belonging to Phylogroup I. Unfortunately, it is not possible to determine the relationship of this phylogroup to those characterized by Gricius (1994) for two reasons. First, in that study, total mtDNA was digested with six-base cutter restriction enzymes and the fragments were separated through agarose gels subjected to Southern Blots hybridization using a *F. heteroclitus* probe. In consequence, the number of fragments characterized was low, resulting in a reduced number of haplotypes. For instance, Gricius (1994) only reported two haplotypes among 16 individuals, and 15 of these shared the same haplotype (h=0.125) in Corpus Christi, whereas in here for that locality, mtDNA sequence data rendered 15 haplotypes among 26 individuals (h= 0.945). Secondly, the parsimony network presented by Gricius (1994) was not rooted against the outgroup (i.e., the Mummichog, *F. heteroclitus*), and consequently the relative position of lineages relative to our MJN cannot be determined. The characterization of mtDNA sequences of additional samples, from Mobile Bay and from Florida, but also from northern Mexico, as suggested by Williams et al. (2008), is needed to unravel the sequence of events that gave rise to the phylogeny of Gulf Killifish along the Gulf coast as it diverged from its sister species, the Mummichog.

By using mtDNA sequence data, the current study was able to further subdivide the Gulf Killifish population to the west of Mobile Bay into three units, with phylogeographic

breaks placed roughly at the Mississippi River and the upper Laguna Madre region of the Texas coast, north of Corpus Christi. These findings are concordant with the Mississippi River acting as a barrier to gene flow that results in a phylogeographic break for many coastal marine species in the Gulf, including fish and elasmobranchs (Neigel 2009, Portnoy and Gold 2012, Portnoy et al. 2014), and studies that demonstrate a phylo-geographic break in the hypersaline Laguna Madre system of Texas for oysters (King et al. 1994) and fiddler crabs (Barnwell and Thurman 2008).

The high levels of haplotypic diversity of Gulf Killifish samples are indicative of large population sizes (McCusker and Bentzen 2010), congruent with previous studies on this species (Williams et al. 2008), and estimates of contemporary female N_e in this study ranged from 10s to 100s of millions. To gain an understanding of how Gulf Killifish reached such high numbers, the historical demographic data was analyzed in two different ways. The first approach was to obtain estimates for the groups that yielded the highest amount of among-group variance (F_{CT}) in SAMOVA (**Table 6**), which consisted of three populations: 1) S.Tex (Corpus Christi), 2) E.Tex (Matagorda, Reitan Marsh and Sportsman's Road), and 3) N.Gulf (Venice and Ocean Springs). Mismatch distributions (Rogers and Harpending 1992, Harpending 1994, Rogers 1995, Rogers et al. 1996) were used to provide insight into historical demography for each group, irrespective of the phylogeographic association of the two distinct mtDNA phylogroups (**Figure 11**). The corresponding mismatch distributions for S.Tex and E.Tex were multimodal (Rogers and Harpending 1992, Harpending 1994), and the results of D and R2 tests (**Table 7**) were non-significant ($p > 0.05$) indicative of large populations at equilibrium (Tajima 1983, Ramos-Onsins and Rozas 2002). Despite a large number of major tropical storms and oil

spills in the Gulf in recent years, these populations appear not to have suffered recent bottlenecks, suggesting that the effect catastrophic storms, such as Katrina and Ike, may not be as important on coastal fish assemblages as previously thought (Greenwood et al. 2006). Such resilience may be partially associated to the Gulf Killifish benthic feeding behavior shared with other estuarine fish, which keeps them closely associated to their preferred habitat rather than moving to avoid the presence of oil (Martin 2017). By contrast, the shape of the mismatch distribution and the associated significance to D and R2 ($p = 0.003$, 0.001, respectively) for N.Gulf suggest a population bottleneck followed by sudden expansion (Rogers and Harpending 1992, Harpending 1994).

With divergent mtDNA lineages with strong phylogeographic associations, the risk exists to misinterpret the local multimodal signature as evidence of a large stable population (Alvarado Bremer et al. 2005). It is thus recommended to analyze the signatures separately by phylogroups or clade. Accordingly, pairwise distributions were estimated separately by phylogroup, and then regionally by phylogroup. For Phylogroup I, only E.Tex was reanalyzed in this way, since the N.Gulf only contains members of a subgroup of Phylogroup I (**Figures 8-9**), and those results have been presented (**Table 7**). For Phylogroup II estimates were calculated for E.Tex and S.Tex separately. Regional estimates of Female N_e by phylogroup were four times smaller than with all the data combined; still estimates of 10s to 100s of millions of females for each population were obtained (**Table 8**). Mismatch distributions for Phylogroup I for E.Tex and N.Gulf, or for the analysis of all the lineages from all localities (**Figure 12**), combined are congruent with an historical bottlenecks followed by population expansion (D and R2 tests, both $p < 0.05$) that occurred approximately 170 – 180 thousand years bp. By contrast, mismatch

distributions for Phylogroup II, whether conducted for all lineages and localities together or separately for E.Tex and S.Tex, display multiple peaks, and D and R2 tests that are non-significant, indicating long demographic histories at equilibrium. Williams et al. (2008) also concluded that their microsatellite data suggest that populations in the western Gulf may be at or near migration–drift equilibrium at a regional scale, but that dispersal barriers and potential historical signatures on population structure will need to be taken into consideration at larger spatial scales.

Several hypotheses have been advanced on how Pleistocene and Holocene climatic events effected patterns of phylogeography in coastal species in the Gulf, many of which are discussed in great detail by Barnwell and Thurman (2008). Delcourt and Delcourt (1979) hypothesized that at the peak of the Wisconsin glaciation (~18,000 years ago), a eustatic sea level drop of about 120 m below its current level occurred, causing most salt marsh habitat to disappear from the northern Gulf except along isolated patches of the Texas continental shelf and southwestern parts of Florida. Additionally, Florida's land mass expanded, and created a cooler, more arid environment around the southern tip of the peninsula, which effectively separated Gulf and Atlantic populations (Reeb and Avise 1990, Avise 1992). The resulting isolated patches in Florida and Texas became refugia that eventually served as sources to colonize new marsh habitat formed when the continental glaciers retreated and the sea level rose during the Holocene. The longer demographic history of Phylogroup II, as indicated by higher τ values and the stability of the S.Tex and E.Tex regions could be explained by the resilience of isolated patches of salt marsh habitat throughout the Last Glacial Maxima (LGM), but perhaps extending to the glacial maxima and minima over the past 1.6 million years. The sudden expansion of the N.Gulf

population after a being subject to a bottleneck could be explained by a founder event, as individuals from refugia elsewhere arrived in this region.

These hypotheses only partially explain the relationships of haplotypes revealed by the MJN and the historical demography patterns of the two phylogroups of Gulf Killifish. The lineages belonging to Phylogroup I appear basal relative to *F. heteroclitus,* but a phylogeographic tree (not shown) found the three lineages of the intermediate Phylogroup to be basal, relative to the sister species. This indicates that the oldest lineages from the Gulf are associated with S.Tex and E.Tex. This would agree with some models of Pleistocene coastal features and currents, which indicate net movement starting in Mexico then moving north and east along the coast (Barnwell and Thurman 2008). Williams et al. (2008) found a significant negative relationship between genetic diversity and latitude, a pattern consistent with the presence of hypothesized refugia in the southern Gulf regions during the Pleistocene that later recolonized the northern Gulf. Mitochondrial sequences, however, do not support this pattern, as no correlation (positive or negative) was found between haplotypic diversity and latitude (not shown). An alternative explanation centers on the oceanographic properties of the upper Laguna Madre. Studies of other coastal species in the Gulf show strong phylogeographic breaks at the Laguna Madre, leading to the hypothesis that this hypersaline system is a barrier to gene flow (Reeb and Avise 1990, Avise 1992, King et al. 1994, Barnwell and Thurman 2008). Additional sampling to include locations in Florida and Mexico are necessary to resolve the patterns of historical gene flow for Gulf Killifish.

Grass Shrimp

According to F_{ST} values and SAMOVA, grass shrimp populations are genetically structured in the north and west regions of the Gulf of Mexico (**Tables 10-11**). However, at a regional level, the groupings that explain the largest proportion of variance make little sense geographically or biologically, as they do not correspond to a migration-drift equilibrium model. Flowers (2004) characterized the distribution of genetic variation in grass shrimp along U.S. Atlantic Coast, from S. Carolina to northeast Florida, contained in 16sRNA using SSCP, and identified six haplotypes. The dominant haplotype A was present at frequencies between 66-100% across nine of the ten localities sampled. The exception was St. Mary's River, GA, where it only accounted 8% with most grass shrimp containing haplotype B (62%) or haplotype F (31%). Haplotype B was present only in the southern portion of the 350 km of coastline sampled, and its frequency increased from the Ogeechee River in the north towards St. Mary's River in the south. Conversely, the frequency of haplotype A, increased towards the north, and included two localities where that haplotype was fixed. As a result of this cline, a significant Mantel test, concordant with IBD was reported along the east coast by Flowers (2004), a pattern not present along the more than 1200 km of Gulf coastline surveyed in here. Further, twice as many haplotypes were found in the Gulf than in the east coast, and while this may be a function of the higher resolution of direct sequencing compared to SSCP, or by the much longer stretches of sequence and or coastline surveyed in here, the geographic manner by which this variation is distributed, cannot. Specifically, no single locality along the east coast contained as much of the overall mtDNA variation as that recorded in Gulf locality of Ocean Springs, MS (see below). In the Gulf, there is no single dominant haplotype, as

exemplified by haplotype A along the east coast. However, there are certain similarities; in both the Gulf and the east coast there were two localities in each that registered no mtDNA variation (h=0), although in the east coast, the two northern Georgia localities of Moon River and Lazarretto Creek are less than 30 km apart, whereas Pt. Mansfield and Sportsman's Road, on Galveston Island, are more than 500 km apart, with Matagorda Bay (h=0.284), the most variable location in Texas, between them. At the local level, the patterns of gene flow need to be explored. Using allozymes, Fuller (1977) described population structuring at a small spatial scale when comparing grass shrimp living in ponds on Galveston Island to semi-open and open systems connected to Galveston Bay. Specifically, grass shrimp living in closed systems (ponds) were less diverse and displayed stronger signals of population differentiation compared to channels connected to the Bay, with the largest diversity in localities open to the Bay, where gene flow was expected to be more substantial. In light of these findings, it is important to note that the overall low levels of haplotypic diversity reported here and by Flowers (2004), may be largely due to the low levels of variability contained in 16sRNA, which may be similar to Penaeid shrimp where low levels of genetic variation at both COI and 16sRNA loci have been reported (Quan et al. 2001), and that variation at the nuclear level needs to be investigated.

In spite of reduced levels of genetic variation (τ = 1.435) in grass shrimp, the data suggest the presence of multiple demes along the northern and western portions of the Gulf of Mexico, each with a very distinct historical demographic signature. Ocean Springs, for instance, is the most variable locality surveyed (h=0.811), with Venice, the second most variable (h=0.667). When these two localities are pooled together, they virtually encompass all the variation documented by the MSN (**Figure 13**) along the entire region of

the Gulf surveyed, from Mississippi to Texas. Such depauperate levels of variation in Texas described in here, collectively represent a snapshot of the total mtDNA variation observed for the North Gulf, and could be explained by either founder events (Mayr 1978, Barton and Charlesworth 1984), or sweepstakes in reproductive success (i.e., SRS or the Hedgecock effect; Hedgecock and Pudovkin 2011). Grass shrimp females are highly fecund, capable of producing 100s of eggs per spawning event, which are repeated multiple times in a season (Welsh 1975, Anderson 1985, Cházaro-Olvera 2009a). Additionally, while actual population densities of grass shrimp have been reported to be as high as 1.2 million individuals per 0.01 km^2 in a single marsh (Welsh 1975), this study estimated female N_e to be 1.9 – 2.4 million for the entire range sampled, from Mississippi to Texas. This dramatic reduction of N_e in comparison to actual population numbers, combined with high fecundity and Type III life history of grass shrimp is concordant with expected outcomes based on the SRS hypothesis. Accordingly, the results of this study indicate that grass shrimp in Texas have not reached migration-drift equilibrium as that displayed by Gulf Killifish in the same region.

Planthoppers

We found that among 124 COI sequences, only four very closely related (τ = 0.258) haplotypes were discovered, that included a common haplotype found at frequencies of 85% or higher. In spite of such low levels of variation, planthoppers displayed higher levels of genetic population structure along the north and west Gulf of Mexico than Gulf Killifish, although not as pronounced as grass shrimp. Within E.Tex, none of the localities differed from each other, but individually they differed from the

S.Tex localities of Corpus Christi and Matagorda, but not from South Padre, which lies farther to the south, thus explaining why SAMOVA groups South Padre with E.Tex, a grouping that makes little sense geographically or biologically. Using the same primers for COI, Denno (2008) characterized a sample of 53 *P. marginata* individuals and 15 populations, seven from the Atlantic US Coast and four from the Gulf, in addition to putatively introduced populations from California and Portugal, with *P. dolus* and *Toya venilia* as outgroups. Their phylogenetic analysis unraveled extensive geographic structure among native North American populations of *P. marginata* characterized by strong phylogeographic associations. *P. marginata* haplotypes clustered into two well-supported sister clades: one comprising mid-Atlantic coast (Virginia to New York) lineages, and another of south-Atlantic (South Carolina to northern Florida) and Gulf Coast lineages (**Figure 16**). Within the south Atlantic clade, there is only one well-supported subgroup (bootstrap > 87) of haplotypes private to the western Gulf (Louisiana and Texas). The rest of this Clade consists of three closely related haplotypes, one found in South Carolina, Florida, and Mississippi, another one from Florida, and a third haplotype found in Mississippi, but also in Virginia Beach, VA, where the Mid-Atlantic Clade dominates. Accordingly, the variation characterized in here apparently matches the well-supported groups of western Gulf *P. marginata* haplotypes. This is relevant, because rather than concluding that COI is depauperate of genetic variation in *P. marginata,* it illustrates that the Northern Gulf from Ocean Springs, MS to Pt. Mansfield, TX, contains a small cross-section of the total mtDNA variation. Such paucity in regional genetic variation must be the effect of past losses associated with founder events and the establishment of new variants that colonized the western portion of the Gulf of Mexico from the east, and with

time, became isolated from the eastern portion of this basin. This explains the bootstrap support for that group, which forms part of the South Atlantic - Gulf Coast Clade that separated from the Mid-Atlantic population during the Pleistocene.

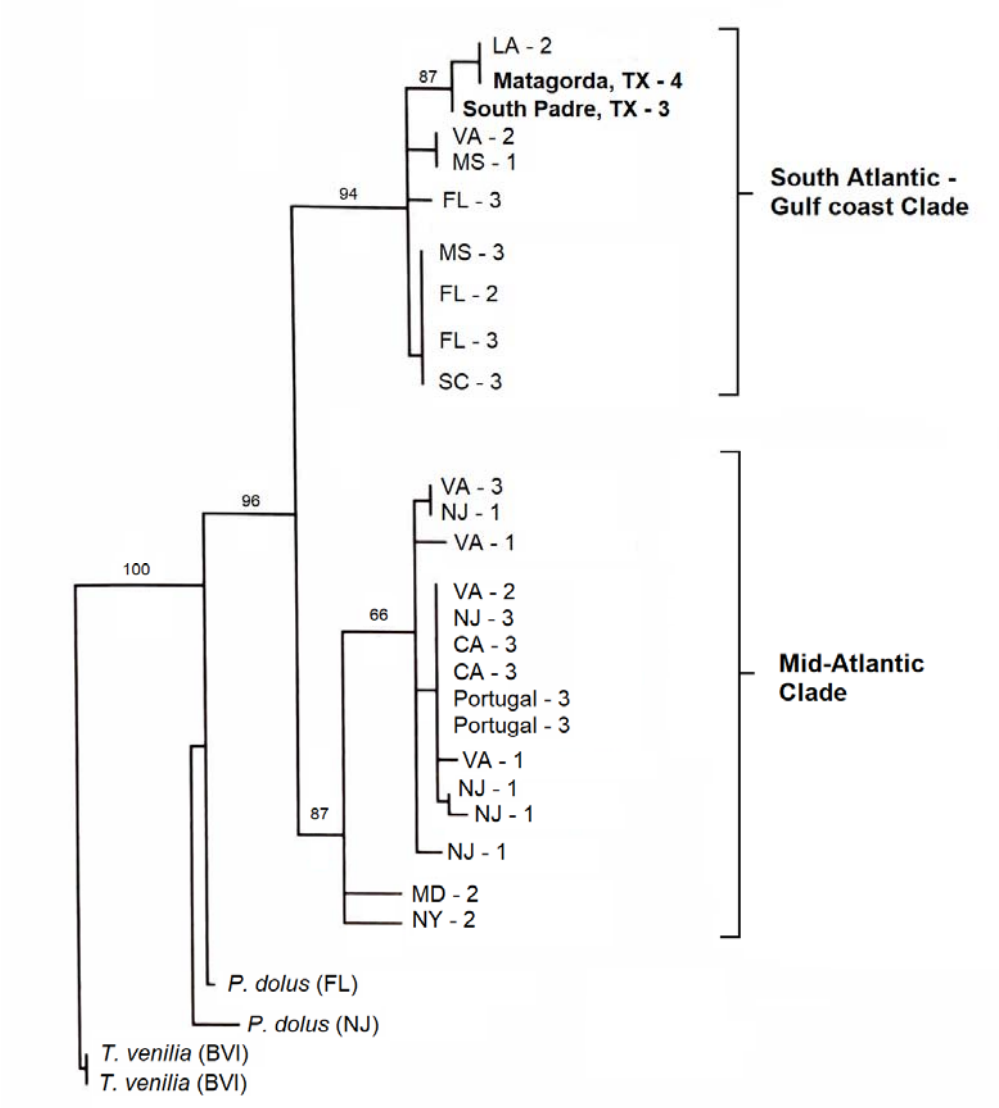

Figure 16. Estimated phylogeny of mtDNA COI haplotypes of *P. marginata,* adapted from Denno (2008). Haplotypes group into two clades consisting of a south Atlantic – Gulf coast Clade and a mid-Atlantic Clade. Branch labels correspond to sampling locality and number of individuals sharing each haplotype. Bootstrap support for nodes are shown above the branches. Each of the Texas localities (bold) consist of a single haplotype per locality sampled.

While the above phylogeographic reconstruction of events may explain the establishment of planthopper mtDNA lineages found in the western portion of the northern Gulf of Mexico, it does not account for the patchiness in the geographic distribution of variation recorded in here. Population structure of planthoppers may be strongly influenced by the fluidity of their life histories, as planthoppers can switch between a fecund, wingless morph (brachypterous) and a less fecund winged morph (macropterous) with high dispersal potential (Denno et al. 1989, Denno 1994). While previous studies suggest that the brachypterous form is most common in salt marshes along the Gulf coast (Denno and Roderick 1990, Denno et al. 1991), our collections consisted almost exclusively of the macropterous form. The higher dispersal potential of macropterous forms may increase gene flow between neighboring marshes and weaken genetic signals of population structure (Waples 1998, Bohonak 1999). While the presence of a dominant allele could be the result of high levels of gene flow, it does not necessarily account for the low levels of genetic diversity observed, which could be explained instead by the behavior of the COI gene. A study of COI sequences in 344 species of the order Hemiptera found low levels of intra-specific sequence divergence (< 2%) in the majority of the species sampled (Park et al. 2011). While a low mutation rate may explain the reduced number of haplotypes found in planthoppers along the Texas coast, the local absence of genetic variation in two out of six localities surveyed, suggest that, similar to grass shrimp, local extinctions followed by recolonization may be responsible for observed genetic patchiness in the overall distribution of variation.

Conclusions

The comparison of the patterns of genetic variation of three species inhabiting *Spartina* salt marshes in the northern Gulf of Mexico provide insight into the forces that modeled such patterns individually and differentially for each of the species. Gulf Killifish mtDNA sequence data generated for this study found evidence of IBD, and of population structure in the Gulf, west of Mobile Bay, Alabama, that had not been found in previous studies of this species. The E.Tex population, in particular, can be further subdivided by the phylogroup associations found in this study, and results in two subgroups within E.Tex with distinct patterns of historical demography. These different signatures may be attributed to multiple historical breaks between the Gulf and Atlantic during periods of glaciation in the Pleistocene. An alternative hypothesis is a historic phylogeographic break between S.Tex and E.Tex, during which time the E.Tex population underwent expansion and colonized the N.Gulf. Following this colonization, contact between S.Tex and E.Tex was reestablished, generating the two subgroups now seen in the E.Tex population. Different theories on how the Pleistocene climate affected phylogeography in the Gulf could support either hypothesis. Therefore, further sampling to include populations from Mexico and Florida are necessary to resolve the patterns of historical demography found in this study.

The mtDNA sequencing data generated for both grass shrimp and planthoppers revealed relatively low levels of haplotypic diversity. In both, analyses of the hierarchical distribution of variation indicated population structure, although the suggested groupings that maximize such variance did not make geographic or biological sense. For grass shrimp, the levels of variation in the eastern portion of the sampled range contained most

of the variation found in the western regions (E.Tex and S.Tex), but the localities in these regions appear as individual snap shots of all the potential genetic variation present along the sampling region. This suggests that the localities in Texas may be subject to extinction and colonization events, or to random fluctuations in population size, that when combined with variance in reproductive success, result in a mosaic representation of the overall variation. Considering the life history details of the species, the observed patterns are consistent with the SRS hypothesis. For planthoppers, haplotypic diversity is extremely low, with most individuals sharing a single haplotype. However, such depauperate levels of variation found in the western portion of the Gulf represents only a small fraction of the overall mtDNA variation contained in planthoppers, since towards the east Gulf and along the Atlantic coast, substantial levels of genetic variation exist. Further investigations targeting nuclear markers with a greater degree of polymorphism would be beneficial in resolving finer scale population structure in this geographic range.

CHAPTER IV

COMPARISON OF GENETIC AND GENOMIC DIVERSITY OF GULF KILLIFISH INHABITING DIFFERENTLY AGED AND SPACED RESTORED *Spartina* MARSHES IN GALVESTON BAY, TEXAS

Introduction

The worldwide rate of coastal wetland loss has continued to accelerate over the past 30 years (Li et al. 2018), including throughout most of the continental United States (Moulton et al. 1997, Dahl 2011), where over the five year period between 2004-2009 roughly 72 thousand acres per year were lost, for a total loss of over 360 thousand acres (Dahl and Stedman 2013). Coastal wetlands function as wildlife habitat and provide vital ecosystem services to coastal communities (Engle 2011), and federal, state, and local authorities have implemented restoration and protection programs (Moulton et al. 1997, Kennish 2001), and United States policies require the replacement of any jurisdictional wetland that has been degraded or lost with restored or constructed wetlands of the same size and ecological value (USDA 2011). Wetland restoration success is dependent on a variety of biotic and abiotic factors, but salt marsh restoration efforts continue to focus primarily on hydrology and plant cover, likely due to the logistical challenges of focusing on other factors, or predicting the outcome of different approaches (Zedler 2000, 2001). Studies show that some ecological attributes of restored wetlands, such as habitat diversity, species diversity, soil characteristics, and secondary production, might take decades to reach the same levels as natural, undisturbed wetlands (Minello and Zimmerman 1992, Minello and Webb 1997, Craft et al. 1999, Craft and Sacco 2003). Long-term monitoring of a restoration site in San Diego Bay indicated that the desired mitigation outcomes for

that site had not been achieved in 12 years, and seemed unlikely to be achieved in the near future (Zedler and Callaway 1999).

Evaluations of restored salt marshes have compared chronological series, or single time-frame levels of species diversity of faunal communities as indicators of health (Craft et al. 1999, Craft and Sacco 2003, Staszak and Armitage 2013), and recent advances have improved our understanding in how changes in faunal communities and species diversity can influence ecosystem services (Finke 2004, Finke and Denno 2005, Deegan et al. 2007). For instance, decreases in the species diversity of spiders can alter herbivore density to levels that decrease productivity in *Spartina* ecosystems (Finke 2004, Finke and Denno 2005). Diversity at the level of communities has long been recognized as in important indicator of ecosystem health (Stevenson et al. 2000, Hooper et al. 2005, Richardson and Hussain 2006), but more recent research is beginning to highlight the influence of intraspecies genetic diversity on ecosystem health and resilience (i.e., stability in the face of natural and anthropogenic disturbances).

Diversity is a hierarchical system, with each level (e.g., ecosystem, habitat, population) dependent upon the levels below it, such that genetic variability within populations can have ecological impacts at multiple levels (Hughes et al. 2008). Increased genetic diversity of host plant species have been found to increase the mean fitness of the plants, significantly alter the structure of associated arthropod communities, and enhance ecosystem productivity and resilience (Wimp et al. 2005, Johnson et al. 2006, Reusch and Hughes 2006). Reusch and Hughes (2006) note that seagrass and salt marsh systems are ideal for testing different hierarchical levels of diversity, but most genetic studies on restored near-shore systems to date have focused on the genetic diversity of the dominant,

habitat-forming flora (Travis et al. 2002, Hughes and Stachowicz 2004, Reynolds et al. 2012). In eelgrass habitats, increased genetic diversity of the seagrass played a role in the system's resistance to community disturbances by grazing geese (Hughes and Stachowicz 2004). Reynolds et al. (2012) found that even a small increase in genetic diversity of seagrasses enhances the success of sea-grass bed restoration and augments ecosystem services, such as primary production, nutrient retention, and provision of invertebrate habitat. A study of genetic diversity of *Spartina alterniflora* in restored salt marshes by Travis et al. (2002) found similar levels of genetic diversity between restored and natural marshes. The only study comparing faunal genetic diversity in restored and natural near-shore habitats involves a recent study that found levels of genetic diversity of oysters in restored reefs to be similar to those in natural reefs (Arnaldi et al. 2018).

To date, no studies have attempted to characterize genetic variation in colonizing faunal populations of restored salt marshes. In a review of restoration ecology, Palmer et al. (1997) notes that restoration relies on an unverified assumption that species re-establish themselves in restored habitats. They go on to say that restoration science would benefit from research into the spatial scales necessary for restoring species diversity, and adequate knowledge of colonization sources, rates of migration, and how those factors influence restoration success (Palmer et al. 1997). Studies of genetic variation in colonizing fauna in restored marshes would fill these gaps by evaluating the levels of connectivity (i.e., migration and gene flow) between source populations and restored marshes as a function of distance, dispersal potential of colonizing fauna, and time since restoration. Estimating the levels of genetic variation of colonizing populations may provide information to

evaluate resident fauna's genetic capacity for resilience, and by extension, the entire system's ability to adapt to and recover from stressful conditions.

High rates of coastal wetland loss and degradation combined with the logistical complications of incorporating spatial processes in the planning and selecting of locations for restoration efforts, result in substantial levels of fragmentation in salt marsh habitats (Britsch and Dunbar 1993, White and Tremblay 1995, Bell et al. 1997, Huxel and Hastings 1999). A comprehensive review of the genetic consequences of habitat fragmentation found considerable evidence that fragmentation can have negative impacts on population genetics (Keyghobadi 2007). Theoretically, fragmentation of habitats inhibits population connectivity and genetic outcrossing, which reduces adaptive fitness and the potential to adapt to stressful conditions for individuals and populations through four forces: (a) increased genetic drift, (b) elevated inbreeding, (c) reduced gene flow with other populations, and (d) increased probability of local loss of adaptive alleles (Templeton et al. 1990, Young et al. 1996, Reed and Frankham 2003, Avise 2004, Bijlsma and Loeschcke 2012, Palkovacs et al. 2012). Since salt marshes and their resident fauna are subject to a multitude of stressors, including drought and dredging (Hartig et al. 2002), nutrient loading (Wigand et al. 2003, Deegan et al. 2007), tidal surges and fluctuations (Konisky and Burdick 2004), and climate change (Simas et al. 2001, Hartig et al. 2002), it is vital to evaluate the genetic diversity of faunal communities within restored marshes as a potential analog for species health and resilience. This study seeks to evaluate levels of faunal genetic diversity in restored marshes of differing ages and distances from a natural marsh using the Gulf Killifish (*Fundulus grandis*).

Materials and methods

Species Selection

Gulf Killifish are among the most abundant nekton in the marsh habitat along the Gulf of Mexico coast, often dominating coastal salt marsh fish assemblages in the spring and fall (Rozas and Reed 1993, Rozas and Zimmerman 2000). They are an important link in the coastal marsh food web, as they consume a variety of algae, vascular plants, and small animal prey, such as mosquito larvae and grass shrimp (Welsh 1975, Rozas and LaSalle 1990, Kennish 2001), and act as important prey items for shore birds and fisheries species that use coastal marshes as nursery habitat (Rozas and Reed 1993, Rozas and Zimmerman 2000). Gulf Killifish reproduce frequently in their first year of life, with peak egg production occurring between April and May (Greeley and MacGregor 1983, Lipcius and Subrahmanyam 1986, Green et al. 2010), and their average lifespan is approximately two years (Lipcius and Subrahmanyam 1986). They are benthic spawners, attaching their eggs via filaments to substrate within the marsh (Nordlie 2000).

Gulf Killifish were found to have higher densities in vegetated areas, as they tend to stay in submerged marsh habitat except when the marsh surface is no longer inundated during low tides (Lipcius and Subrahmanyam 1986, Rozas and Zimmerman 2000). Mark-recapture studies along the Gulf of Mexico coast revealed that Gulf Killifish exhibit high site fidelity, with most individuals travelling 100 m or less between connected marsh habitats (Nelson et al. 2014). Additionally, previous studies of the Gulf Killifish indicate limited dispersal, primarily between adjacent estuaries, and significant levels of isolation by distance using both SNPs and microsatellites (Williams et al. 2008, Williams et al. 2010).

Their reproductive characteristics and limited movement as adults combine to make Gulf Killifish an excellent environmental indicator species in terms of genetic and physiological responses to exposure to a variety of industrial toxins. They tend to remain in their preferred marsh habitats, despite the presence of oil or toxins (Martin 2017). As a result, they have been used in studies of complex genetic responses to the Deepwater Horizon oil spill (Garcia et al. 2012), site-specific effects of crude oil contamination on biological function (Whitehead et al. 2012, Dubansky et al. 2013, Dubansky et al. 2014, Dubansky et al. 2017), and evolutionary genetic response to industrial pollutants in the Houston Ship Channel (Oziolor et al. 2014, Oziolor et al. 2016). The Gulf Killifish was chosen for this study because their abundance, strong association with marsh habitat, and low dispersal potential make them an excellent species for investigating patterns of genetic diversity and gene flow between natural and restored marshes on a small geographic scale.

Sampling and DNA Isolation

Gulf Killifish were captured in spring and summer months from 2014 to 2019, in restored and natural *Spartina alterniflora* salt marshes in Galveston Bay, Texas (**Figure 17**). The Sportsman's Road marsh (SMR) was used as the natural, reference marsh for this study, and the restored marshes are clustered into two geographically distinct groups of three differently aged marshes. The marshes near 11-Mile Road (11M marshes) are located 2.4 - 3.6 km from the reference, and the marshes near Jamaica Beach (JB marshes) are located 7.3 - 9.5 km from the reference. The young marshes (11MY, JBY) in each group were restored since 2010, the medium marshes (11MM, JBM) in each group were restored between 2004-2010, and the old marshes (11MO, JBO) in each group were restored before

2004. Gulf Killifish were sampled using minnow traps baited with dog or cat food kibbles, placed in shallow (< 0.5m) water in low marsh habitat, and allowed to soak for up to 12 hours. Specimens were visually identified in the field and humanely sacrificed via immersion in MS-222 as per U.S. Federal policies on the use of laboratory animals as subjects (AUP# 2014-0111 and 2017-0105). All specimens were immediately preserved in the field using 95% ethanol, and were transferred to 70% ethanol for long-term preservation within 24 hours of collection. Gulf Killifish DNA was isolated from axial muscle tissue using Zymo Quick-DNA Universal Kit following the manufacturer's instructions for tissue (Zymo Research, Irvine, CA, U.S.A.).

Mitochondrial DNA (mtDNA) Sanger Sequencing

Three sets of primers based on the mitogenome of Gulf Killifish (Accession # FJ445396) were designed to target the mtDNA Control Region I (CR1), and segments from Nitrogen Dehydrogenase Subunits 2 and 5 (ND2 and ND5) as their mutation rates are sufficient for investigating population structure (Whitehead 2009). The primer sets used in this study are either primers from other studies altered to our target species, or were designed for this study using the Primer 3 software (Koressaar and Remm 2007, Untergasser et al. 2012) within Geneious v.9.1.8 (Biomatters Ltd., Aukland, NZ) as the most optimal among potential primer-pairs capable of amplifying an *in silico* fragment 400-600 bp in length for the targeted regions. **Table 16** provides a summary of the primers used in this study.

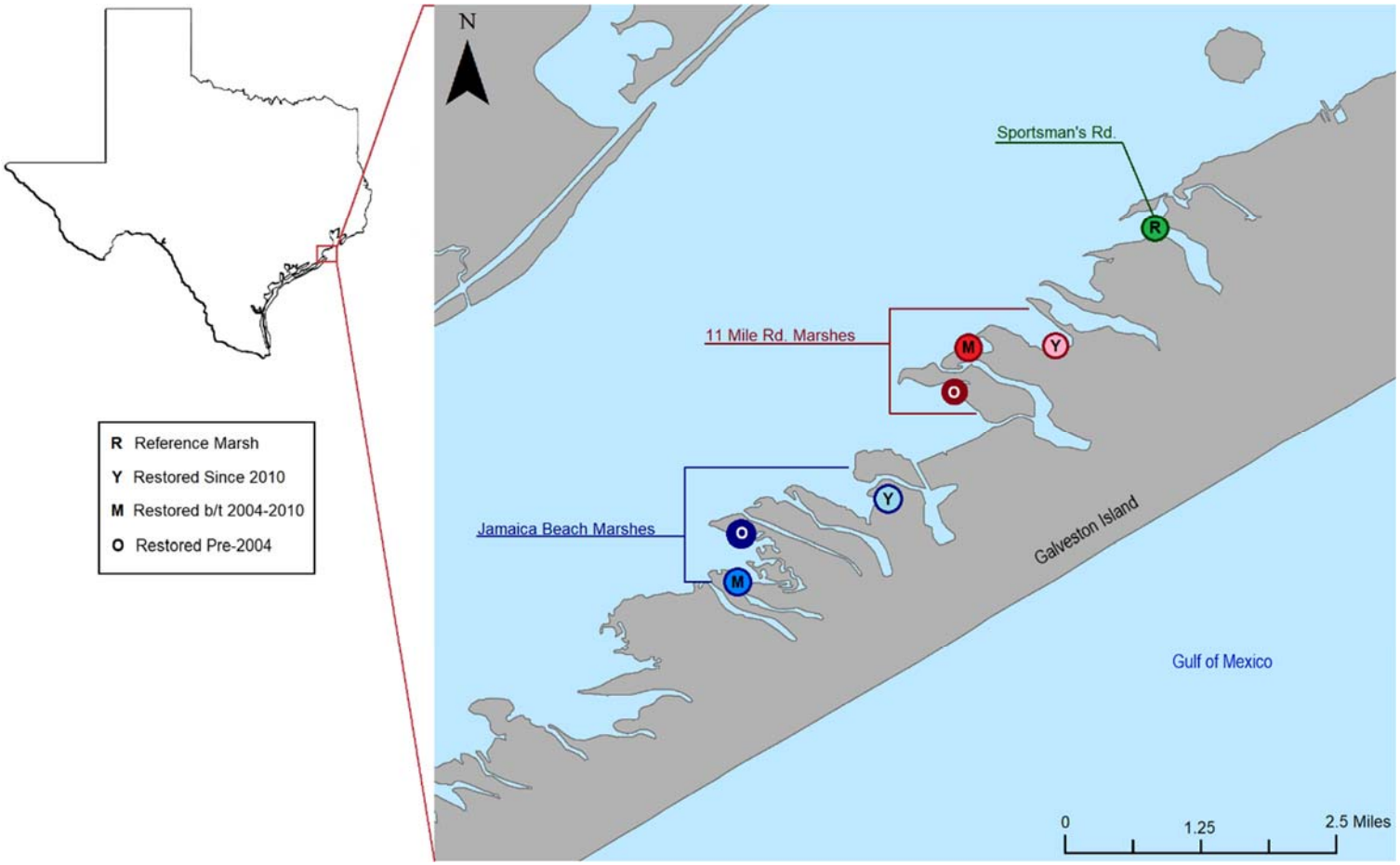

Figure 17. Map of sampling sites for Gulf Killifish on Galveston Island, Texas. Geographic position of marshes is denoted by color: Green for the reference marsh at Sportsman's Road (SMR), Red for near-reference marshes at 11 Mile Road (11M-Y, 11M-M, 11M-O), and Blue for distant marshes at Jamaica Beach (JB-Y, JB-M, JB-O). Age of marshes are denoted via alphabetic character: R for reference, Y for marshes restored since 2010, M for marshes restored between 2004 and 2010, and O for marshes restored before 2004.

Table 16. PCR primer summary for markers sequenced in this study. Nucleotides that have been modified from the originally published sequence are denoted in bold, and nucleotides that have been inserted to the originally published sequence underlined.

Locus	Primer Names	Primer Sequence	Fragment Size	Annealing Temp. (°C)
F. grandis CR-1 (Alvarado-Bremer et al. 1995, modified)	L15998-FG CSBD-H-FG	5' **CG**C CCC **TAG** CTC CCA AAG CTA 3' 5' A**A**T AGG AAC CA**A** ATG CCA G 3'	400 bp	50
F. grandis ND2 (This study)	L4173ND2-FG H4634ND2-FG	5' CAT CAT CCC CGA GCC GTT GA 3' 5' GGA AGG TTA AGG ATG GGA AG 3'	421 bp	50
F. grandis ND5 (This study)	L12137ND5-FG H12717ND5-FG	5' GCA GAA ACG GTA GTG TCC AC 3' 5' GTA CTT GAA TGC AGT AGG GC 3'	540 bp	50

PCR was carried out separately for each locus in 12.5 µL reactions containing 1x EconoTaq Plus Green Master Mix (Lucigen, Middleton, WI), 0.2 µM of each primer, and 10-20 ng of isolated DNA as template. Thermocycling was performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with an initial denaturing step at 94°C for 2 minutes; followed by 35 cycles of denaturing 94°C for 25 seconds, annealing at the corresponding temperature for each primer pair (**See Table 16**) for 30 seconds, and extension at 72°C for 90 seconds; and a final extension step at 72°C for 3 minutes. Negative controls were included in all reactions. PCR products were then visualized for specificity and yield via electrophoresis on a 2% agarose gel pre-stained with ethidium bromide (EtBR). PCR products that produced a single band were diluted 1:10 in ddH_2O for post-PCR cleanup and sequenced in both directions, with reaction setups and thermocycling profiles as described in Cruscanti et al. (2015).

mtDNA Sequence Analysis

Multiple sequence alignments were carried out in Geneious Pro v.9.1.8 (Biomatters Ltd., Aukland, NZ). Haplotype data files were generated in DnaSP v6.12.03 (Rozas et al. 2017). Arlequin v.3.5.2.2 (Excoffier et al. 2005) was used to estimate genetic diversity within sampling locations, and to calculate sequence diversity indices, pairwise $F_{ST,}$ coancestry coefficients (Reynolds Distance), and Slatkin's Linearized F_{ST} (Reynolds et al. 1983, Weir and Cockerham 1984, Slatkin 1991, Raymond and Rousset 1995). Reynold's Distance was calculated in the event that genetic differentiation occurs only by genetic drift without mutations (Reynolds et al. 1983). P-values for pairwise comparisons of the six localities were corrected for multiple testing using Benjamini and Hochberg's (1995)

method, which corrects for significance by controlling the false discovery rate (FDR) and produces fewer false negatives than Bonferroni corrections (Jafari and Ansari-Pour 2019). Since the distribution of diversity statistics falls on an asymptotic curve, rather than a normal curve, the Salicru et al. (1993) χ^2 method was used to test for pairwise significant differences in haplotypic diversity between sampling locations. POPart v.1.7 (Bandelt et al. 1999, Leigh and Bryant 2015) was used to build median joining networks (MJN) for Gulf Killifish markers, using a representative of *F. heteroclitus* (Accession #KT869378) as an outgroup. The MJN was chosen for Gulf Killifish sequences because of the large number of haplotypes separated by small genetic distances (Bandelt et al. 1999). Principle component analyses (PCAs) as implemented in R v3.6.1 (R Development Core Team) were used to investigate structure in the distribution of mtDNA variation of Gulf Killifish among the natural and restored marsh sites.

Analyses of molecular variance (AMOVAs; Excoffier et al. 1992) were conducted to test the following alternative hypotheses to the null hypotheses of no difference. The first alternative hypothesis is that restored marshes differ from the reference marsh and each other by distance from the reference; AMOVAs were conducted on the following groupings: (1) SMR; (2) restored marshes 2.4 – 3.6 km from the reference: 11M-Y, 11M-M, and 11M-O; and (3) restored marshes 7.3 – 9.5 km from the reference: JB-Y, JB-M, and JB-O. The second alternative hypothesis is that restored marshes differ from the reference marsh and each other by age; AMOVAs were conducted using the following groupings: (1) SMR, (2) marshes restored since 2010: 11M-Y and JB-Y, (3) marshes restored between 2004 – 2010: 11M-M and JB-M, and (4) marshes restored before 2004: 11M-O and JB-O.

Double-Digested Restriction Site Associated DNA (ddRAD) Sequencing

For all samples, DNA isolate concentrations and nucleic acid purity were measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA), a Qubit Fluorometer (Thermo Scientific, Waltham, MA), and visualized for molecular weight and quality in 1% Tris-acetate (TA) agarose gels pre-stained with ethidium bromide (EtBr). A subset (n=10) of the most successful isolations from each sampling location was chosen for ddRAD sequencing based on DNA isolate concentration (> 50 ng/uL) and molecular weight (> 10,000 bp). Samples were sent to the Texas A&M University AgriLife Genomics and Bioinformatics Service Center (College Station, TX) for ddRAD sequencing. Based on in-silico surveys on the efficacy of commonly used restriction enzymes across a wide range of taxa by Herrera et al. (2015), we chose MspI (5' CTGCAG 3') and PstI (5' CCGG 3') as enzymes likely to correspond to a sufficient number of cut sites in a teleost genome. In the interest of brevity, a detailed description of the ddRAD sequencing protocol provided by the sequencing center is included in Appendix C (Figure C-1). Raw Illumina reads were demultiplexed, had adapters removed, and were sorted by barcode by the sequencing center before the data was released to the investigators.

ddRAD Bioinformatic Analyses

Paired-end Illumina reads received from the sequencing center were re-sorted and filtered for quality (Phred > 30) using Process_Radtags from the STACKS v.2.4 package (Catchen et al. 2013). These reads were then aligned using BWA-MEM (Li 2013) to a reference genome for *F. heteroclitus*, the Atlantic sister species to Gulf Killifish (Project Accession JXMV00000000; NCBI Resource Coordinators 2016). The resulting aligned

reads were input to the ref_map pipeline in STACKS to call variant sites, which were then filtered through the POPULATIONS module within STACKS. Four sets of loci were generated using different filtering parameters (**Table 17**). The "initial" dataset allowed 20% missing data per locus within populations (-r 0.80), and a minimum minor allele frequency at 10% (--min_maf 0.10). A minimum minor allele frequency of 10% is thought to help remove sequencing errors and reduce inclusion of uninformative rare alleles without being so strict as to unrealistically skew downstream analyses (Hendricks et al. 2018). The "main" dataset of single nucleotide polymorphism (SNP) markers was generated using the filtering parameters for the initial dataset, with an additional parameter allowing only 10% missing data per locus over all individuals (-R 0.90) to reduce potential bias in population level estimates due to missing data. To reduce bias due to linkage disequilibrium between markers, downstream analyses were conducted on only the first SNP in each locus. Two subsets of markers were generated from the main set. "Subset A" was generated via the "blacklist" option in the POPULATIONS module of STACKS to exclude loci that deviate significantly from Hardy Weinberg Equilibrium (HWE) in at least one of our sampling locations, and "Subset B" was generated via the "whitelist" option include only those loci that deviate from HWE expectations in at least one sampling location. **Table 17** briefly summarizes the datasets generated for this study and the analyses conducted on each.

Loci of the initial dataset was blasted against the NCBI non-redundant (–nr) protein database with an e-value threshold of e^{-15} using BlastX as implemented through DIAMOND (Buchfink et al. 2015). The top hit for each loci was kept, and gene ontology (GO) Terms for the Blast results were investigated and visualized using Blast-2-GO software (Gotz et al. 2008). To assess rates of genotyping errors and whether the dependence of those rates

Table 17. Brief summary of datasets of ddRAD loci for Gulf Killifish generated with different filtering parameters, and the downstream analyses conducted on each.

Name of Dataset	Filtering Parameters In POPULATIONS	Downstream Analyses	No. of Loci	Mean Length (bp)
Initial	-r 0.80 --min_maf 0.10	BlastX and Blast2Go Pipeline	205,157	266
Main	-r 0.80 --min_maf 0.10 - R 0.90	Heterozygote Miscall Rate Molecular Indices All population-level analyses AMOVA to test hypotheses	13,397	281
Subset A	Same as "Main" --blacklist loci out of HWE	Molecular Indices All population-level analyses	12,463	281
Subset B	--whitelist loci out of HWE	BlastX and Blast2Go Pipeline Molecular Indices All population-level analyses	934	309

on read depth resulted in an erroneous excess of homozygotes, the whoa package v.0.0.1 (Anderson 2019) in R v.3.6.1 was used to visualize expected versus observed genotype frequencies by locus and calculate estimated heterozygote miscall rate in the main dataset (Hendricks et al. 2018). For the main dataset and both subsets, molecular indices for each marsh, including expected and observed heterozygosity, mean nucleotide diversity, variant sites that deviate significantly from HWE, the mean inbreeding coefficient (F_{IS}) of individuals relative to the population (Hartl and Clark 2006), and pairwise F_{ST} (Weir and Cockerham 1984) were included in the POPULATIONS module output. The Salicru et al. (1993) χ^2 method was used to test for pairwise significant differences in heterozygosity between sampling locations. Population structure was investigated by estimating the number of underlying populations (K) through the cross-validation method in ADMIXTURE v.1.3.0 (Alexander and Lange 2011).

For the main dataset, analyses of molecular variance (AMOVA) as described by Excoffier et al. (1992) were conducted using the poppr v.2.8.5 package (Kamvar et al. 2014, Kamvar et al. 2015) in R to test the hypotheses in this study. To determine if restored marshes differ from the reference marsh and each other according to distance from the reference, AMOVA was conducted on the following groupings: (1) SMR; (2) restored marshes 2.4 – 3.6 km from the reference: 11M-Y, 11M-M, and 11M-O; and (3) restored marshes 7.3 – 9.5 km from the reference: JB-Y, JB-M, and JB-O. To determine if restored marshes differ from the reference marsh each other by age, AMOVA was conducted using the following groupings: (1) SMR, (2) marshes restored since 2010: 11M-Y and JB-Y, (3) marshes restored between 2004 – 2010: 11M-M and JB-M, and (4) marshes restored before 2004: 11M-O and JB-O. PCA was conducted using the adegenet v.2.1.3 package

(Jombart 2008, Jombart and Ahmed 2011) in R to investigate structure in the distribution of SNP variation among the natural and restored marsh sites. The adegenet package was also used to investigate the relative abundance of shared alleles among samples and generate a heatmap to visualize the degree of similarity between individuals and an accompanying dendrogram of relatedness between individuals based on genetic distances.

Results

Mitochondrial Sequence Data

Sequences for three mtDNA segments, namely CR1 (336 bp), ND2 (344 bp), and ND5 (397 bp), were obtained from 161 Gulf Killifish specimens from the seven marshes targeted in this study. The sequences of all three loci were concatenated into one single segment 1077 bp long that defined 89 haplotypes (Appendix C, Table C-1), and the patterns of genetic variability within and among-localities were estimated. Nearly identical patterns of differentiation and diversity were obtained by analyzing each of these segments separately; therefore, the results for the concatenated segments are reported below, while the results for individual markers can be found in Appendix C (Tables C-2 – C-9). High levels of haplotypic diversity ($h > 0.978$) were found in all sampling locations (**Table 18**). The JB-Y marsh had the lowest value of h, while the JB-O marsh had the highest, but these values were not significantly different between sampling locations (**Table 19**). Comparison of pairwise F_{ST} values identify the greatest differences between JBO with all other marshes, with the highest values associated with JBO compared against SMR and 11MO, but no pairwise F_{ST} values for any markers or locations in this study were statistically significant (**Table 19**). Calculations of Reynold's distance (Appendix C, Table C-3) yielded similar relationships.

Table 18. Molecular indices for 1077 bp of concatenated mtDNA sequence for 161 individuals of Gulf Killifish. M, No. of haplotypes, *h,* haplotypic diversity; π, nucleotide diversity; SD, standard deviation; S, no. of segregating (polymorphic) sites; Ts, no. of transitions; Tv, no. of transversions; I/D, no. of insertions and/or deletions.

Location	N	M	*h*(SD)	π (SD)	S	Ts	Tv	I/D
SMR	27	23	0.986 (0.015)	0.020 (0.010)	82	70	10	3
11MY	24	21	0.986 (0.018)	0.020 (0.010)	85	75	8	2
11MM	21	18	0.981 (0.023)	0.020 (0.010)	77	65	9	2
11MO	24	20	0.986 (0.016)	0.021 (0.011)	81	73	7	2
JBY	24	20	0.978 (0.021)	0.020 (0.010)	78	68	8	2
JBM	21	18	0.981 (0.023)	0.020 (0.010)	83	74	6	2
JBO	20	18	0.990 (0.019)	0.017 (0.009)	66	57	6	2
TOTAL	**161**	**89***	**0.983 (0.004)**	**0.021 (0.010)**	**140**	**124**	**16**	**5**

* Total M excludes shared haplotypes among localities.

Table 19. Table of pairwise comparisons for 1077 bp of mtDNA concatenated sequences for 161 individuals of Gulf Killifish. Z-scores from Salicru χ^2 test for pairwise comparisons of haplotypic diversity are above the diagonal, Pairwise F_{ST} are below the diagonal. No significant ($p < 0.05$) comparisons for either index were obtained.

	SMR	11MY	11MM	11MO	JBY	JBM	JBO
SMR		0.0000	0.1821	0.0000	0.3010	0.1821	-0.1652
11MY	-0.0192		0.1712	0.0000	0.2892	0.1712	-0.1528
11MM	-0.0188	-0.0337		-0.1785	0.0963	0.0000	-0.3017
11MO	-0.0164	-0.0241	-0.0344		0.3030	0.1785	-0.1610
JBY	-0.0181	-0.0323	-0.0387	-0.0273		-0.0963	-0.4237
JBM	-0.0188	-0.0219	-0.0192	0.0036	-0.0173		-0.3017
JBO	0.0522	0.0138	0.0121	0.0515	0.0081	-0.0081	

Table 20. AMOVA by distance for 1077 bp of mtDNA concatenated sequences for 161 individuals of Gulf Killifish. Variation was assessed via grouping samples by distance from reference marsh. Groups consist of 1) SMR, 2) all three 11M marshes, and 3) all three JB marshes. P-values for fixation indices are based on significance tests with 1023 permutations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	2	24.493	0.13577 Va	1.29
Among Populations Within Groups	4	23.396	-0.21216 Vb	-2.02
Within Populations	154	1628.633	10.57554 Vc	100.73
Total	160	1676.522		
Fixation Indices			**P-values (≥)**	
	F_{ST}:	-0.00728	0.71065 +/- 0.017	
	F_{SC}:	-0.02470	0.90909 +/- 0.009	
	F_{CT}:	0.01293	0.08113 +/- 0.008	

Table 21. AMOVA by age for 1077 bp of mtDNA concatenated sequences for 161 individuals of Gulf Killifish. Variation was assessed via grouping samples by age since restoration. Groups consist of 1) SMR, 2) 11MY and JBY, 3) 11MM and JBM, and 4) 11MO and JBO. P-values for fixation indices are based on significance tests with 1023 permutations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	3	17.927	-0.10007 Va	-0.96
Among Populations Within Groups	3	29.962	-0.02641 Vb	-0.25
Within Populations	154	1628.633	10.57554 Vc	101.21
Total	160	1676.522	10.44906	
Fixation Indices			**P-values (≥)**	
	F_{ST}:	-0.01210	0.75171 +/- 0.016	
	F_{SC}:	-0.00250	0.43011 +/- 0.019	
	F_{CT}:	-0.00958	0.81623 +/- 0.014	

AMOVA based on distance from the reference marsh explained a very small proportion (1.23%, $p > 0.05$) of the total genetic variation contained in our mtDNA sequence data (**Table 20**). Similarly, AMOVA based on age since restoration returned negative values for the percentage of genetic variation explained by this grouping (**Table 21**). For both analyses, little-to-no genetic variation is explained by the hypothesis-based groupings or by differences between individual marshes within those groupings; in both cases, 100% of the variation is attributed to differences between individuals.

The relationships among concatenated mtDNA sequence haplotypes ($n = 89$) is summarized with a MJN (**Figure 18**) that identifies two major phylogroups, separated from each other by at least 37 mutations. The first phylogroup (Phylogroup A) is more closely related to *F. heteroclitus*, and contains 48 haplotypes, comprising more than half (52.6% - 66.7%) of the lineages from every marsh except 11MM, where it comprises less than half (44.4%). Lineages belonging to Phylogroup B are also found in every marsh, but at a lower frequency, overall. Intermediate to the two phylogroups and separated from these by a range of 10-35 mutations, several haplotypes exist; two haplotypes in JBO, and one haplotype each in SMR and 11MM. Among all haplotypes, 24 are shared by two or more marshes, and the majority of these shared haplotypes (62.5%) are associated with phylogroup A. At terminal branches of both phylogroups, and in the intermediate group, there are three lineages from the JBO marsh that diverge by ≥ 6 mutational steps from their nearest neighbor.

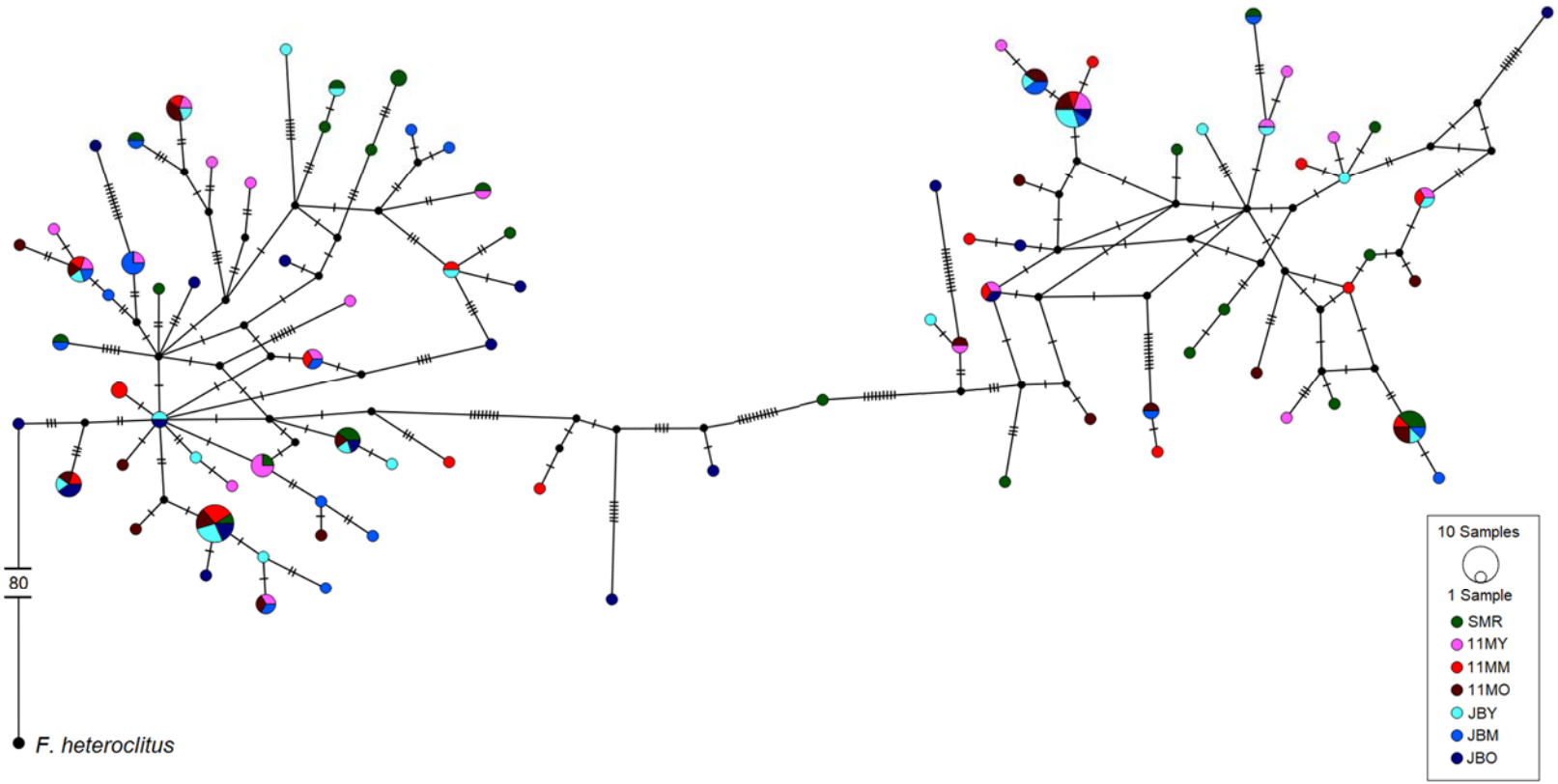

Figure 18. Median Joining Network (MJN) showing the relationship of Gulf Killifish mtDNA lineages based on 1077 bp of concatenated sequences for CR1, ND2, and ND5, with its sister species, *F. heteroclitus,* as the outgroup. Each circle represents a distinct haplotype, and its size, the number of times it is repeated, with the fill colors representing sampling location (see inset). Hash marks indicate the number of segregating sites between each haplotype, with the black circles between them representing hypothetical haplotypes not found in the sample.

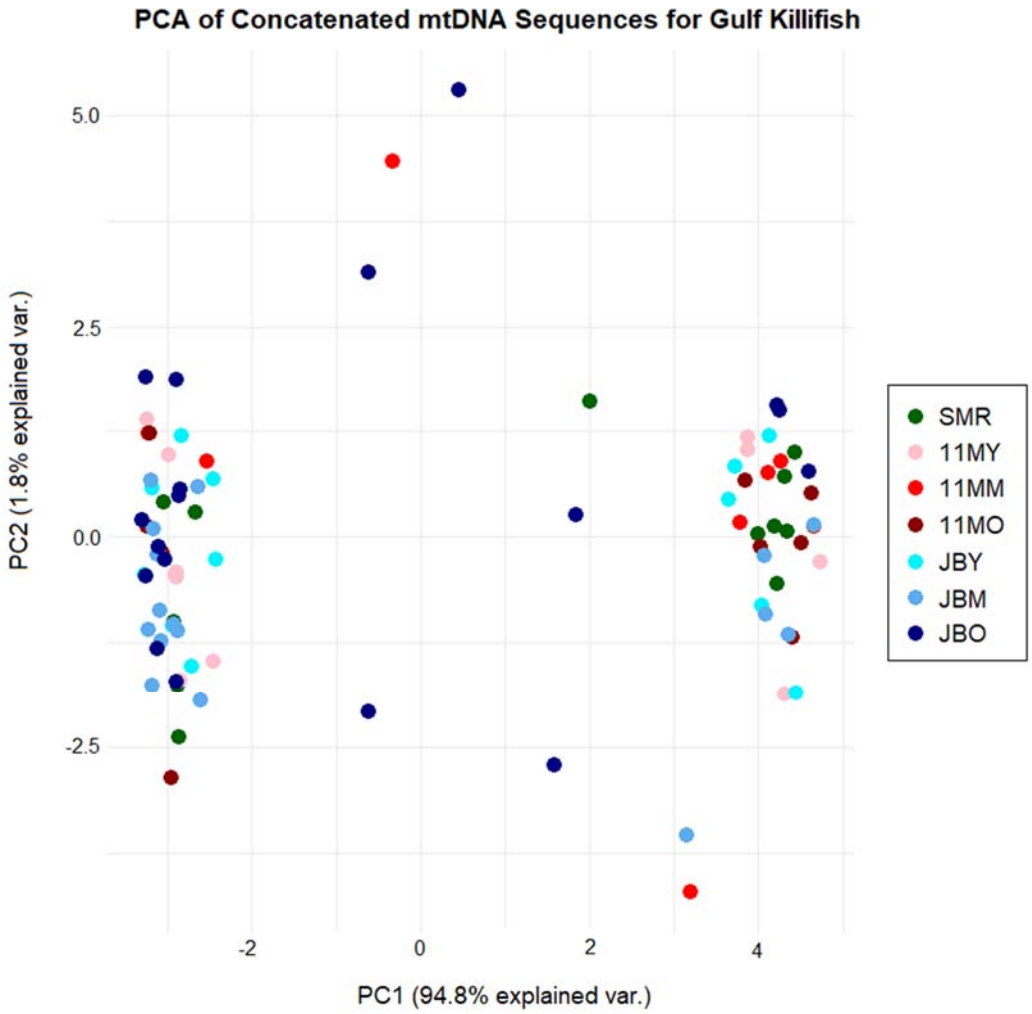

Figure 19. PCA for 1077 bp of mtDNA concatenated sequences of CR1, ND2, and ND5 for 161 individuals of Gulf Killifish. Points correspond to individual sequences and colors correspond to sampling location, as noted in the legend.

PCA of sequence data explained 96.6% of observed variation on the first two axes (**Figure 19**). Concordant with the MJN, most haplotypes are separated into two major clusters by their loading on PC1. The first cluster, characterized by negative loadings along PC1, is the larger of the two clusters, and contains individuals from all sampling locations

in this study. The second, smaller cluster, characterized by positive loadings along PC1 also contains individuals from all sampling locations. Several individuals from SMR, 11MM, JBM, and JBO are intermediate to the two clusters along PC1.

ddRAD Sequence Data

Paired-end ddRAD sequence data was obtained for ten specimens from each of the seven marshes targeted in this study (n=70). The raw reads used in this analysis are available in the Sequence Read Archive (SRA) through NCBI via the BioProject number PRJNA641155. After quality filtering, an average of 3.65 million reads per individual were retained to be aligned to the *F. heteroclitus* reference genome, and approximately 66.5% - 69.4% reads per individual, corresponding to 598,118 loci, were kept through clustering in STACKS. After filtering through the POPULATIONS module, the initial dataset retained 205,157 loci with an average length of 266 bp (**Table 17**). The Blast2Go pipeline on these loci resulted in 21,325 sequences with hits from BlastX (e-value = e^{-15}) and 5,407 mapped sequences, resulting in 4,613 GO annotated sequences (annotation cutoff value = e^{-03}). The majority of sequences (approx. 13,000) had a top BLAST hit corresponding to the Atlantic sister species, *F. heteroclitus* (**Figure 20**). Of the 29 named species comprising our top BLAST hits, all except *E. coli* were species of fish. Successful GO annotations associated with Molecular Functions and Biological Processes are dominated by the "others" category, but are largely associated with binding functions, and oxidation-reduction or DNA transcription processes, respectively

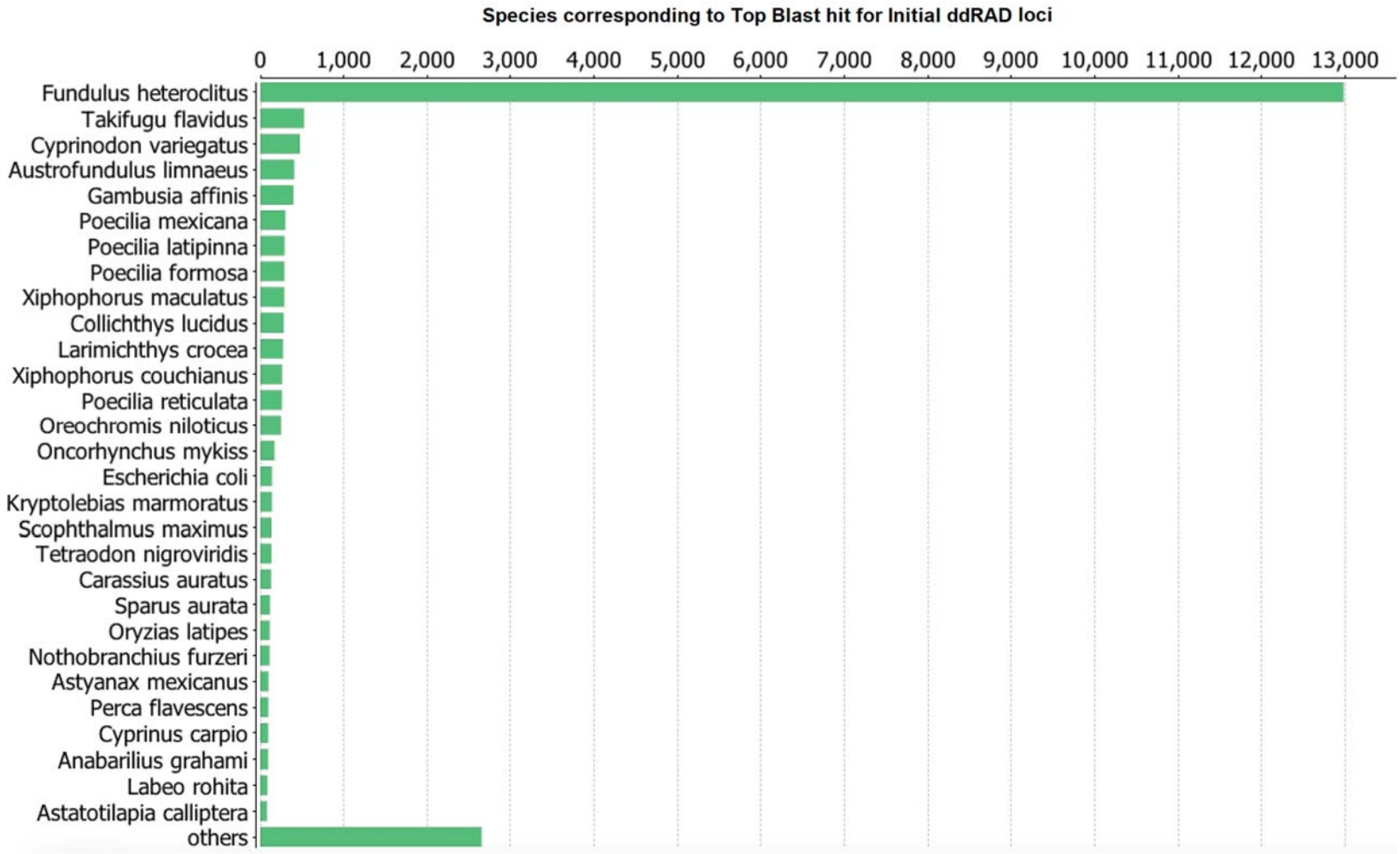

Figure 20. Species correspondences for 21,325 BLAST hits obtained via BlastX (e-value e^{-15}) for initial dataset of ddRAD loci.

(Appendix C, Figures C-1 and C-2). Annotations associated with Cellular Components are dominated by cell membrane components (Appendix C, Figure C-3).

By increasing filtering parameters in the POPULATIONS module, the main dataset contained 13,397 loci with an average length of 281 bp (**Table 17**). Investigations of observed versus expected genotypes for these loci showed low levels of distortions from HWE (**Figure 21**) and returned an average heterozygote miscall rate of 0.000018.

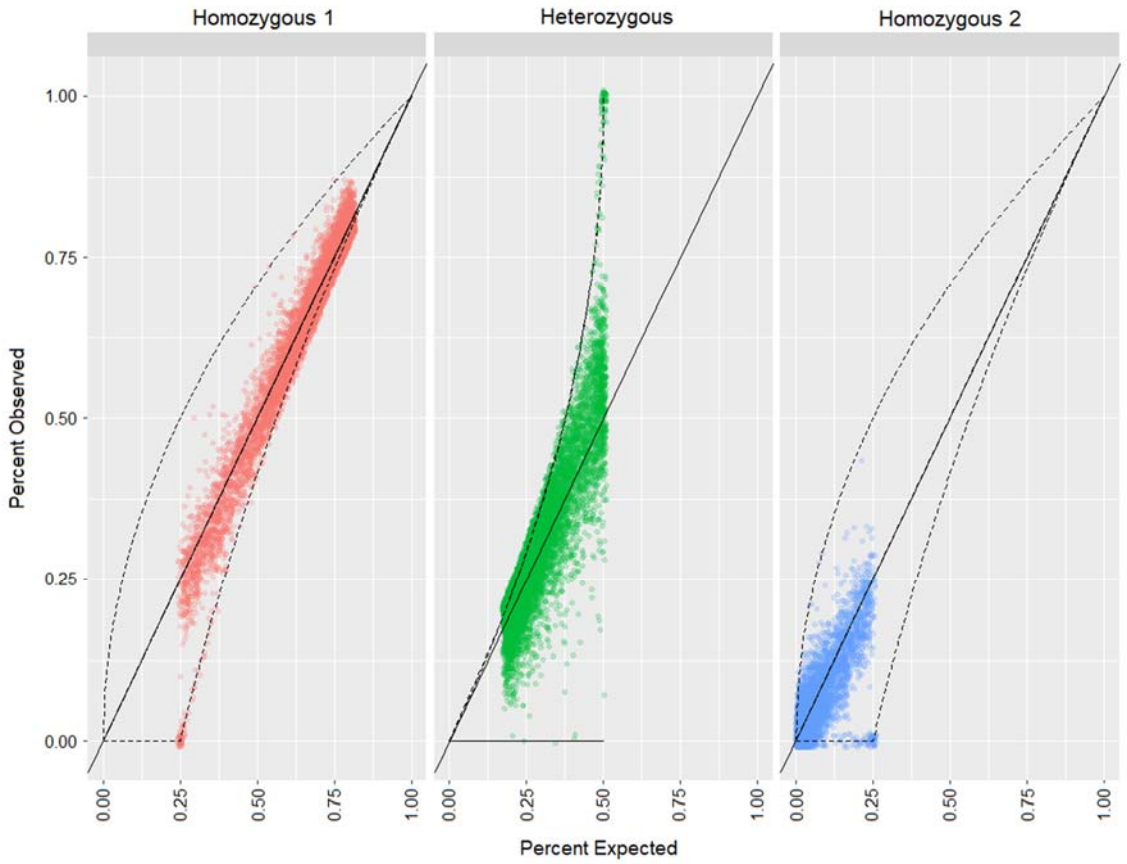

Figure 21. Scatterplot of percent observed vs. expected genotype frequencies for main dataset of ddRAD SNP markers. Solid lines denote the points where observed genotypes = expected genotypes. Dotted lines correspond to theoretical maximum and minimum observed values given the expected values computed from the dataset.

Levels of observed heterozygosity (H_O) for the main dataset ranged from 0.286 – 0.300, with the highest values found in the reference marsh, SMR (**Table 22**). Both of the youngest marshes, 11MY and JBY, had the lowest values for H_O, and differed significantly ($p < 0.05$) from H_O in SMR (**Table 23**). All sampling locations have near-zero values for the inbreeding coefficient, F_{IS}, with SMR having the lowest value (**Table 22**). The two youngest marshes differed from all other sites by having positive values for F_{IS}. All of the marshes had fewer than 2.3% of variant sites that deviated from HWE, and the two young marshes had the highest number of variant sites that deviated significantly from HWE, while JBO had the fewest (**Table 22**). Pairwise F_{ST} was greatest (non-significant) for comparisons of SMR against JBY, and comparisons of 11MY against 11MM and JBO (**Table 23**).

ADMIXTURE returned the lowest value of cross validation error for K=1, signifying that all marshes in the study can likely be grouped into a single population (**Figure 22**). PCA of variant sites associated the majority of SMR, 11MM, 11MO, JBM, and JBO individuals into a loose cluster characterized by negative loadings on PC1 and near-zero loadings on PC2 (**Figure 23**). One individual each from SMR and 11MM are removed from the main cluster by positive loadings on PC2, and one individual from 11MO is removed by highly positive loadings on PC1 combined with highly negative loadings on PC2. The majority of individuals from 11MY and JBY deviate from the main cluster, largely by positive loadings on PC1; however, some individuals of both marshes are also distinguished from the main cluster by greater positive or negative loadings on PC2.

Table 22. Molecular indices for main dataset of ddRAD SNP markers. M, Mean no. of individuals per locus in the population, H_O, Mean observed heterozygosity; π, mean nucleotide diversity; SD, standard deviation; P, mean major allele frequency over all loci, F_{IS}, inbreeding coefficient; HWE, no. of variant sites out of Hardy Weinberg Equilibrium.

Location	N	M	H_O (SD)	π (SD)	P	F_{IS}	HWE
SMR	10	9.26	0.300 (0.005)	0.282 (0.063)	0.820	-0.0376	243
11MY	10	9.47	0.286 (0.005)	0.288 (0.061)	0.816	0.0120	289
11MM	10	9.21	0.292 (0.005)	0.279 (0.034)	0.823	-0.0229	230
11MO	10	9.40	0.291 (0.005)	0.283 (0.061)	0.820	-0.0142	263
JBY	10	9.56	0.286 (0.005)	0.287 (0.061)	0.816	0.0107	307
JBM	10	9.41	0.294 (0.005)	0.285 (0.062)	0.819	-0.0167	271
JBO	10	9.11	0.293 (0.005)	0.278 (0.064)	0.823	-0.0308	219

Table 23. Table of pairwise comparisons for main dataset of ddRAD SNP markers. Above the diagonal are z-scores from Salicru χ^2 test for pairwise comparisons of levels of heterozygosity, with comparisons significant at $p < 0.05$ in bold. Below the diagonal are pairwise weighted F_{ST}. No comparisons for this index were significant at $p < 0.05$.

	SMR	11MY	11MM	11MO	JBY	JBM	JBO
SMR		**1.872 ***	1.078	1.137	**1.855 ***	0.787	0.876
11MY	0.029		-0.772	-0.728	-0.023	-1.096	-0.975
11MM	0.028	0.030		0.050	0.752	-0.306	-0.200
11MO	0.027	0.029	0.028		0.708	-0.360	-0.252
JBY	0.029	0.027	0.029	0.028		-1.077	-0.956
JBM	0.028	0.029	0.028	0.027	0.028		0.103
JBO	0.028	0.029	0.028	0.027	0.029	0.028	

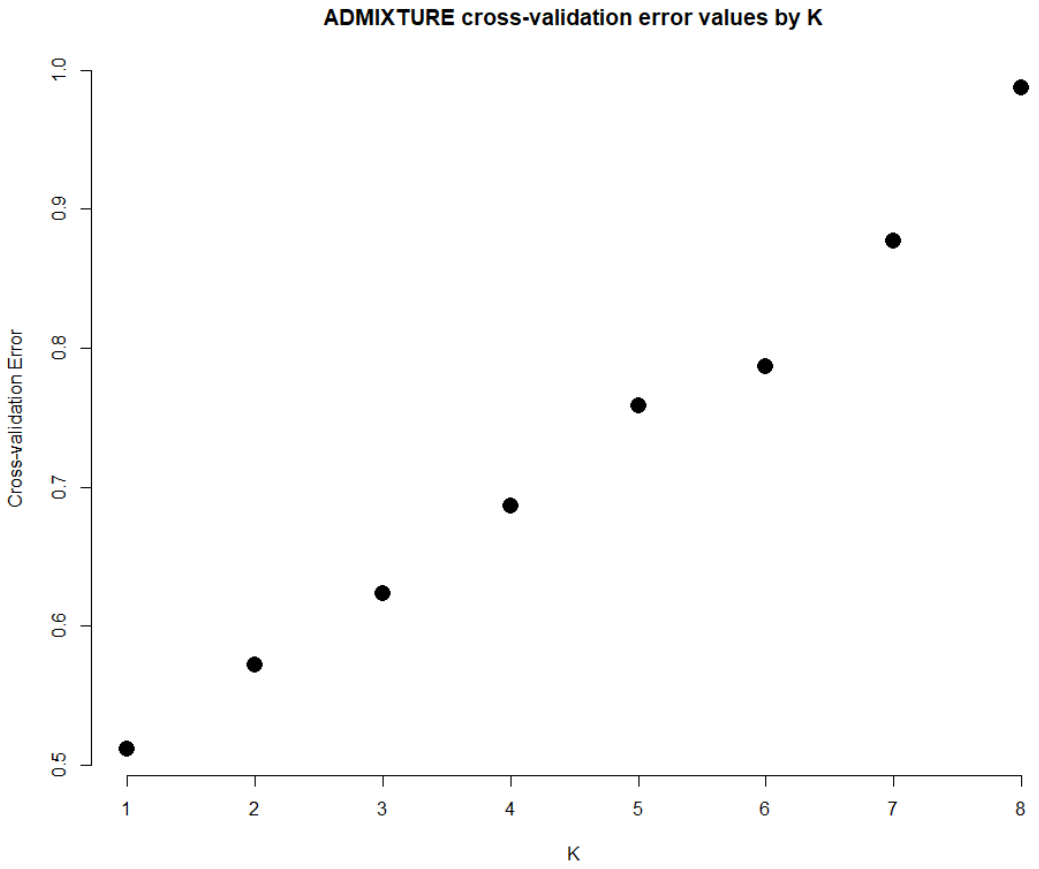

Figure 22. ADMIXTURE cross validation error values by putative number of populations, K, based on main dataset of ddRAD SNP markers.

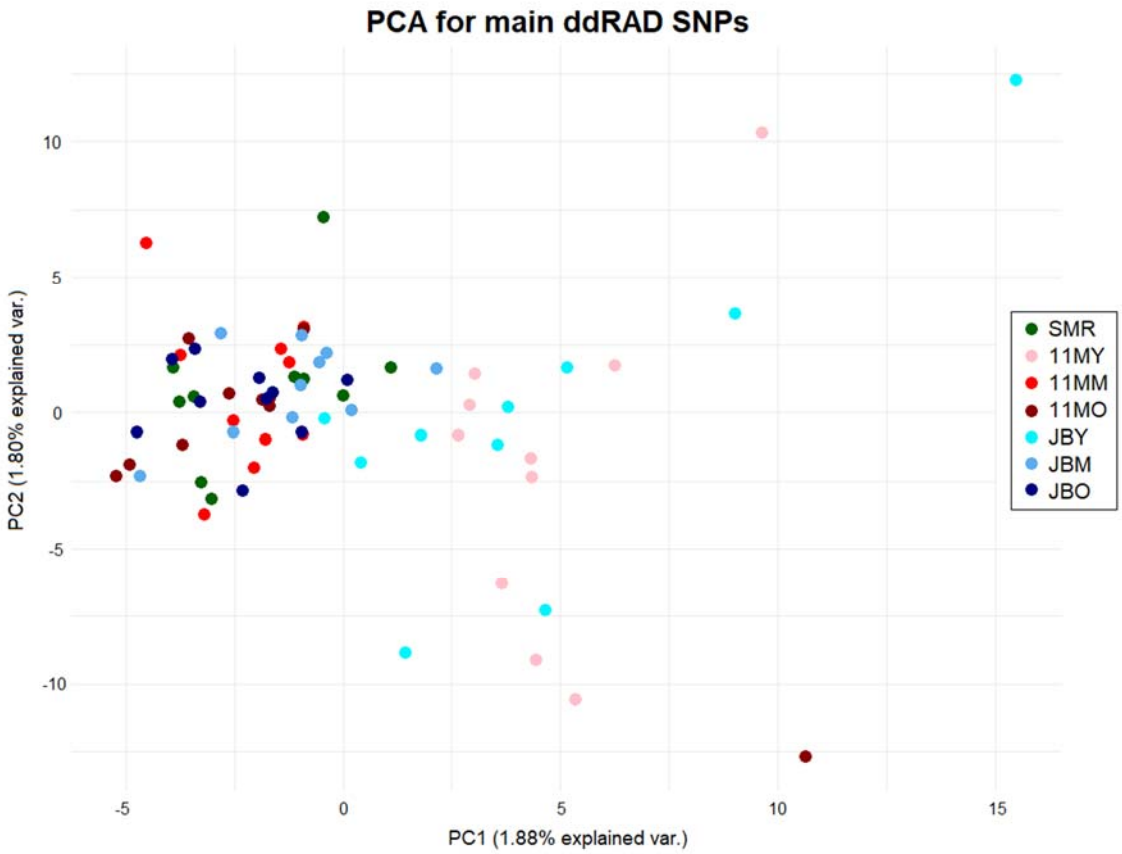

Figure 23. PCA for main dataset of ddRAD SNP markers. Points correspond to individual samples, and colors correspond to sampling location, as noted in the legend.

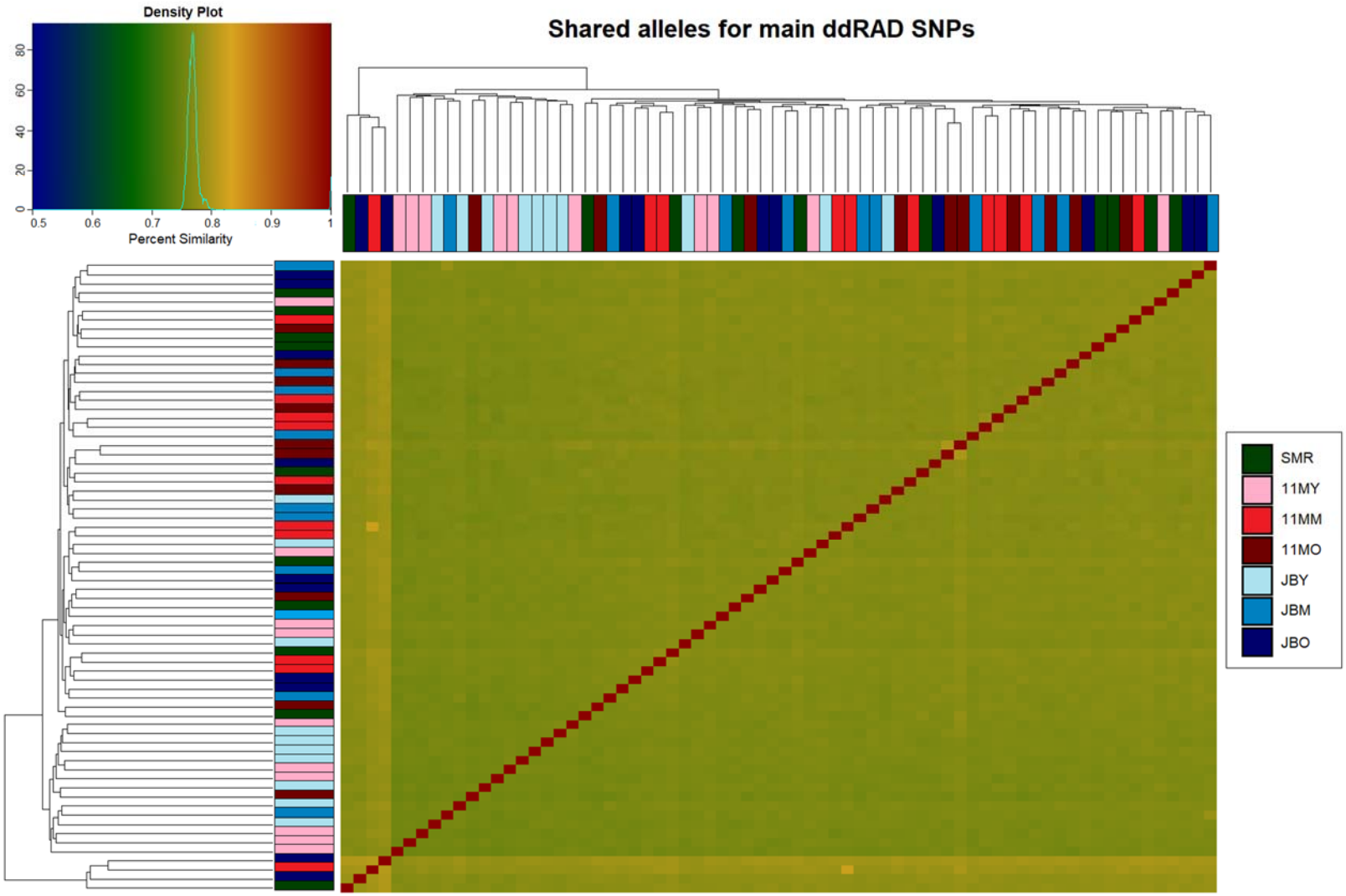

Figure 24. Heatmap of shared alleles based on the main dataset of ddRAD SNP markers. Dendrograms represent inferred relationships between samples based on genetic distances. Colored bars at the terminal branches of dendorgrams correspond to sampling location of the individual, as noted in the legend.

Table 24. AMOVA by distance for main dataset of ddRAD SNP markers. Variance was assessed via grouping samples by distance from the reference marsh. Groups consist of 1) SMR, 2) all three 11M marshes, and 3) all three JB marshes.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	2	3724.81	-3.72	-0.20
Among Populations Within Groups	4	7768.46	12.26	0.67
Within Populations	63	114630.53	1819.53	99.53
Total	69	126123.80	1828.07	
Φ_{ST}		0.00467		
Φ_{SC}		0.00669		
Φ_{CT}		-0.00203		

Table 25. AMOVA by age for main dataset of ddRAD SNP markers. Variance was assessed by grouping samples by age since restoration. Groups consist of 1) SMR, 2) 11MY and JBY, 3) 11MM and JBM, and 4) 11MO and JBO.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	3	5916.07	6.59	0.36
Among Populations Within Groups	3	5577.20	3.95	0.22
Within Populations	63	114630.53	1818.53	99.42
Total	69	126123.80	1830.07	
Φ_{ST}		0.00576		
Φ_{SC}		0.00217		
Φ_{CT}		0.00360		

A heatmap of shared alleles indicates 70% - 80% of alleles in the main dataset are shared across all individuals (**Figure 24**). The dendrogram associated with the heatmap shows three distinct clades. The largest clade (74% of samples) contains no less than 80% of individuals from each of SMR, 11MM, 11MO, JBM, and JBO. The medium clade (20% of samples) contains the majority of 11MY (60%) and JBY (70%) individuals. The final clade includes four individuals, two from JBO and one each from 11MM and SMR. AMOVAs based on either distance from the reference marsh (**Table 24**) or age since restoration (**Table 25**) resulted in less than 1% of the genetic variation in our data explained by hypothesis-based groupings. Concordant with the AMOVA results for mtDNA data, > 99% of the variation is attributed to differences between individuals, rather than between hypothesis-based groupings or subpopulations.

The final two rounds of filtering through the POPULATIONS module resulted in two datasets: Subset A, which removed all loci that deviated from HWE in at least one population and resulted in 12,463 loci with an average length of 281 bp, and Subset B, which kept only those loci that deviated from HWE in at least one population and resulted in 934 loci with an average length of 309 bp (**Table 17**). The Blast2Go pipeline on Subset B loci resulted in 227 sequences with hits from BlastX (e-value = e^{-15}) and 82 mapped sequences, resulting in 64 GO annotated sequences (annotation cutoff value = e^{-03}). The subsequent findings are congruent with those found in the initial dataset; therefore, GO Term data for Subset B is presented as supplemental information. The majority of sequences (n > 105) had a top BLAST hit corresponding to the Atlantic sister species, *F. heteroclitus*, and all except one of the 29 named species comprising our top BLAST hits were species of fish (Appendix C, Figure C-4). When broken down into Molecular

Functions, Biological Processes, and Cellular Components, the highest number of successful GO annotations for subset B were associated with metal ion binding and nucleic acid binding, DNA integration, and integral membrane components, respectively (Appendix C, Figures C-5 - C-7).

Levels of observed heterozygosity (*Ho*) for Subset A ranged from 0.291 – 0.280, with the highest values found in SMR (**Table 26**), with 11MY and JBY both having significantly lower values for *Ho* when compared with SMR (**Table 27**). Subset A markers resulted in zero values for the inbreeding coefficient, F_{IS} (**Table 26**). Pairwise F_{ST} values were similar and non-significant between all marshes (0.027 – 0.029), with the highest values corresponding to comparisons of SMR and JBO against 11MY and JBY, and 11MY against 11MM (**Table 27**).

Levels of observed heterozygosity (*Ho*) for subset B ranged from 0.231 – 0.242, with the highest values found in SMR followed closely by JBO, with JBY having the lowest value for *Ho* (**Table 28**). The differences in *Ho* for subset B, however, were not significantly different between any of the marshes (**Table 29**). The HWE markers resulted negative values for the inbreeding coefficient in all marshes, F_{IS}, with JBO having the lowest value, followed by SMR (**Table 28**). Pairwise F_{ST} were non-significant for all comparisons and returned very similar values between all marshes, ranging from 0.023 – 0.029. Pairwise F_{ST} were greatest for comparisons of 11MY against 11MY, and JBY against both JBM and JBO (**Table 29**).

Table 26. Molecular indices Subset A of ddRAD SNP markers, where no deviations from HWE were allowed. M, Mean no. of individuals per locus in the population, *Ho*, Mean observed heterozygosity; π, mean nucleotide diversity; SD, standard deviation; P, mean major allele frequency over all loci, F_{IS}, inbreeding coefficient.

Location	N	M	H_O (SD)	π (SD)	P	F_{IS}
SMR	10	9.252	0.291 (0.002)	0.277 (0.004)	0.827	0.000
11MY	10	9.468	0.280 (0.01)	0.282 (0.004)	0.823	0.000
11MM	10	9.210	0.284 (0.02)	0.274 (0.004)	0.829	0.000
11MO	10	9.402	0.284 (0.002)	0.277 (0.004)	0.826	0.000
JBY	10	9.566	0.280 (0.001)	0.282 (0.004)	0.823	0.000
JBM	10	9.412	0.287 (0.002)	0.279 (0.004)	0.825	0.000
JBO	10	9.110	0.286 (0.002)	0.273 (0.004)	0.829	0.000

Table 27. Table of pairwise comparisons for Subset A of ddRAD SNP markers, where no deviations from HWE were allowed. Below the diagonal are z-scores from Salicru χ^2 test for pairwise comparisons of levels of heterozygosity, with comparisons significant at $p < 0.05$ in bold. Above the diagonal are pairwise weighted F_{ST}. No comparisons for this index were significant at $p < 0.05$.

	SMR	11MY	11MM	11MO	JBY	JBM	JBO
SMR		0.029	0.028	0.027	0.029	0.028	0.028
11MY	**1.753**		0.029	0.028	0.027	0.029	0.029
11MM	1.034	-0.688		0.028	0.029	0.028	0.028
11MO	1.143	-0.610	0.089		0.028	0.028	0.027
JBY	**1.737**	-0.027	0.665	0.587		0.028	0.029
JBM	0.692	-1.072	-0.360	-0.457	-1.051		0.028
JBO	0.841	-0.888	-0.194	-0.287	-0.867	0.163	

Table 28. Molecular indices Subset B of ddRAD SNP markers, in which every locus is out of HWE in at least one marsh. M, Mean no. of individuals per locus in the population, H_O, Mean observed heterozygosity; π, mean nucleotide diversity; SD, standard deviation; P, mean major allele frequency over all loci, F_{IS}, inbreeding coefficient.

Location	N	M	H_O (SD)	π (SD)	P	F_{IS}
SMR	10	9.460	0.242 (0.036)	0.186 (0.022)	0.857	-0.095
11MY	10	9.547	0.231 (0.035)	0.187 (0.022)	0.855	-0.065
11MM	10	9.425	0.237 (0.036)	0.182 (0.022)	0.858	-0.092
11MO	10	9.589	0.237 (0.036)	0.182 (0.022)	0.859	-0.092
JBY	10	9.630	0.237 (0.036)	0.187 (0.022)	0.856	-0.080
JBM	10	9.548	0.237 (0.035)	0.184 (0.022)	0.859	-0.089
JBO	10	9.375	0.241 (0.036)	0.177 (0.022)	0.863	-0.114

Table 29. Table of pairwise comparisons for Subset B of ddRAD SNP markers, in which every locus is out of HWE in at least one marsh. Below the diagonal are z-scores from Salicru χ^2 test for pairwise comparisons of levels of heterozygosity. Above the diagonal are pairwise weighted F_{ST}. No comparisons for either index were significant at $p < 0.05$.

	SMR	11MY	11MM	11MO	JBY	JBM	JBO
SMR		0.027	0.026	0.023	0.026	0.026	0.025
11MY	0.224		0.029	0.025	0.026	0.028	0.026
11MM	0.015	-0.016		0.026	0.028	0.026	0.026
11MO	0.100	-0.123	-0.001		0.026	0.023	0.023
JBY	0.102	-0.121	-0.001	0.002		0.028	0.028
JBM	0.107	-0.118	0.000	0.006	0.004		0.024
JBO	0.028	-0.195	-0.011	-0.072	-0.075	-0.079	

Concordant with the results of the main dataset, ADMIXTURE returned the lowest value of cross validation error for K=1 for both subsets A and B (Appendix C, Figures C-8 and C-9). PCA results for Subset A (**Figure 25**) showed similar patterns of variation to the PCA of the main dataset, with the majority of individuals from all except the youngest marshes forming a single, loose cluster with neutral loadings on both PC1 and PC2. Individuals from both 11MY and JBY were distinct from the main cluster by positive loadings on PC1, and several individuals are also distinct via positive or negative loadings on PC2. One individual each from SMR and 11MM deviate slightly from the main cluster by negative loadings on PC2, and one individual from 11MO differs substantially from others by positive loadings on both PC1 and PC2. PCA of Subset B markers differed slightly from those for the main dataset and subset A, and associated the majority of individuals in a single, loose cluster characterized by near-zero loadings on both PC1 and PC2 (**Figure 26**). Three individuals from 11MY, one from 11MO and one from JBM deviate from the main cluster by negative loadings on PC1 and neutral-to-negative loadings on PC2. One individual from 11MO deviates from the main cluster by very positive loadings on PC2, and one individual from JBY deviates by very positive loadings on PC1 combined with negative loadings on PC2.

A heatmap of shared alleles shows that individuals across the entire subset A marker set share 75% - 85% of alleles (**Figure 27**). The associated dendrogram splits the individuals into three clades that strongly agree with those produced in the heatmap for the main dataset. The heatmap for Subset B markers indicate that all individuals share 90% - 95% of alleles (**Figure 28**). The dendrogram for Subset B shows four distinct clades: who larger clades that each contain 32 individuals, and two smaller clades that each contain

three individuals. The first large clade contains the majority of individuals from the reference and old marshes (50-80%), while the second large clade contains the majority of individuals from both young marshes and JBM (60-70%). Of the small clades, one has 100% membership from the young marshes, and the other consists of 1 individual each from 11MM, 11MO, and JBO.

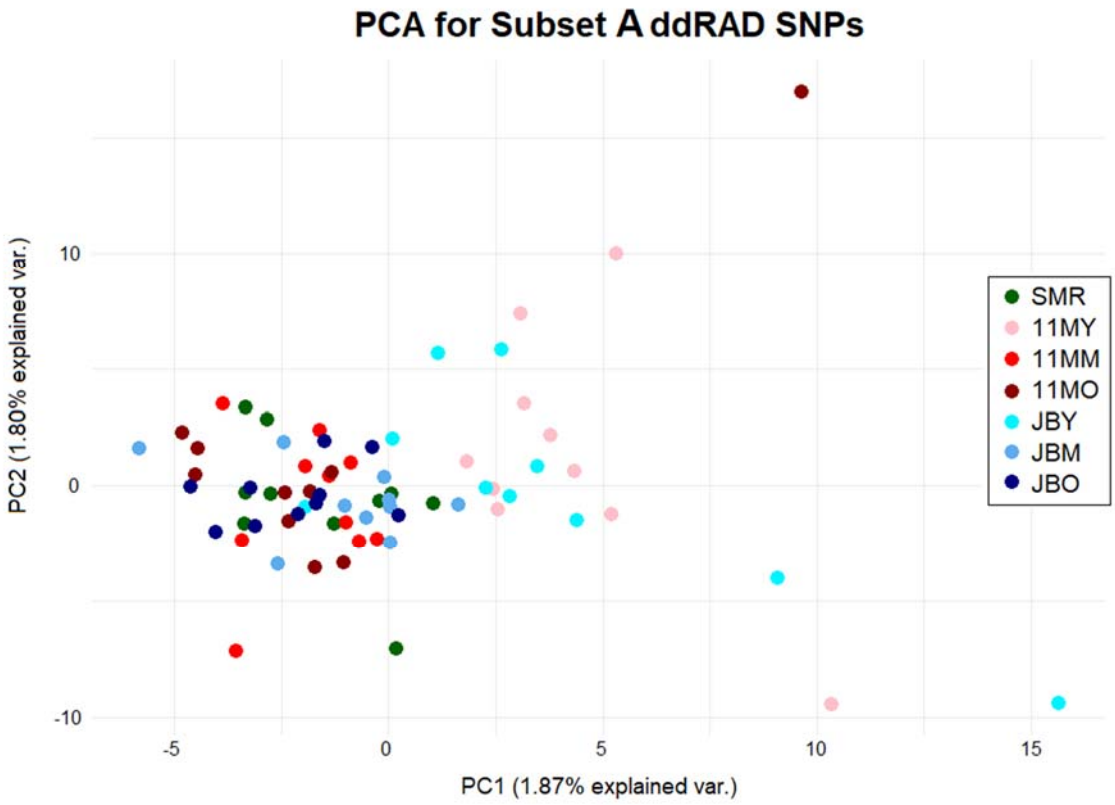

Figure 25. PCA for Subset A of ddRAD SNPs, where deviations from HWE were not allowed. Points correspond to individual samples, and colors correspond to sampling location, as noted in the legend.

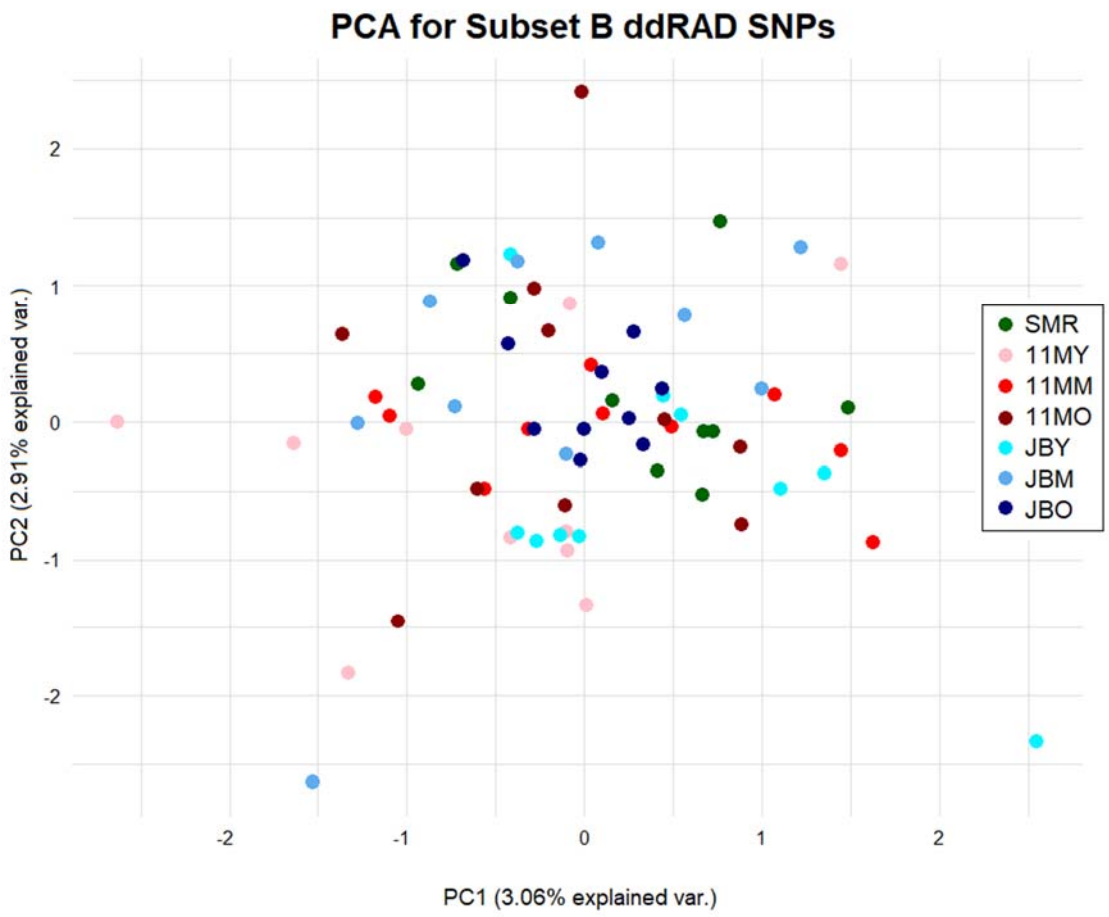

Figure 26. PCA for Subset B of ddRAD SNP markers, in which every locus is out of HWE in at least one marsh. Points correspond to individual samples, and colors correspond to sampling location, as noted in the legend.

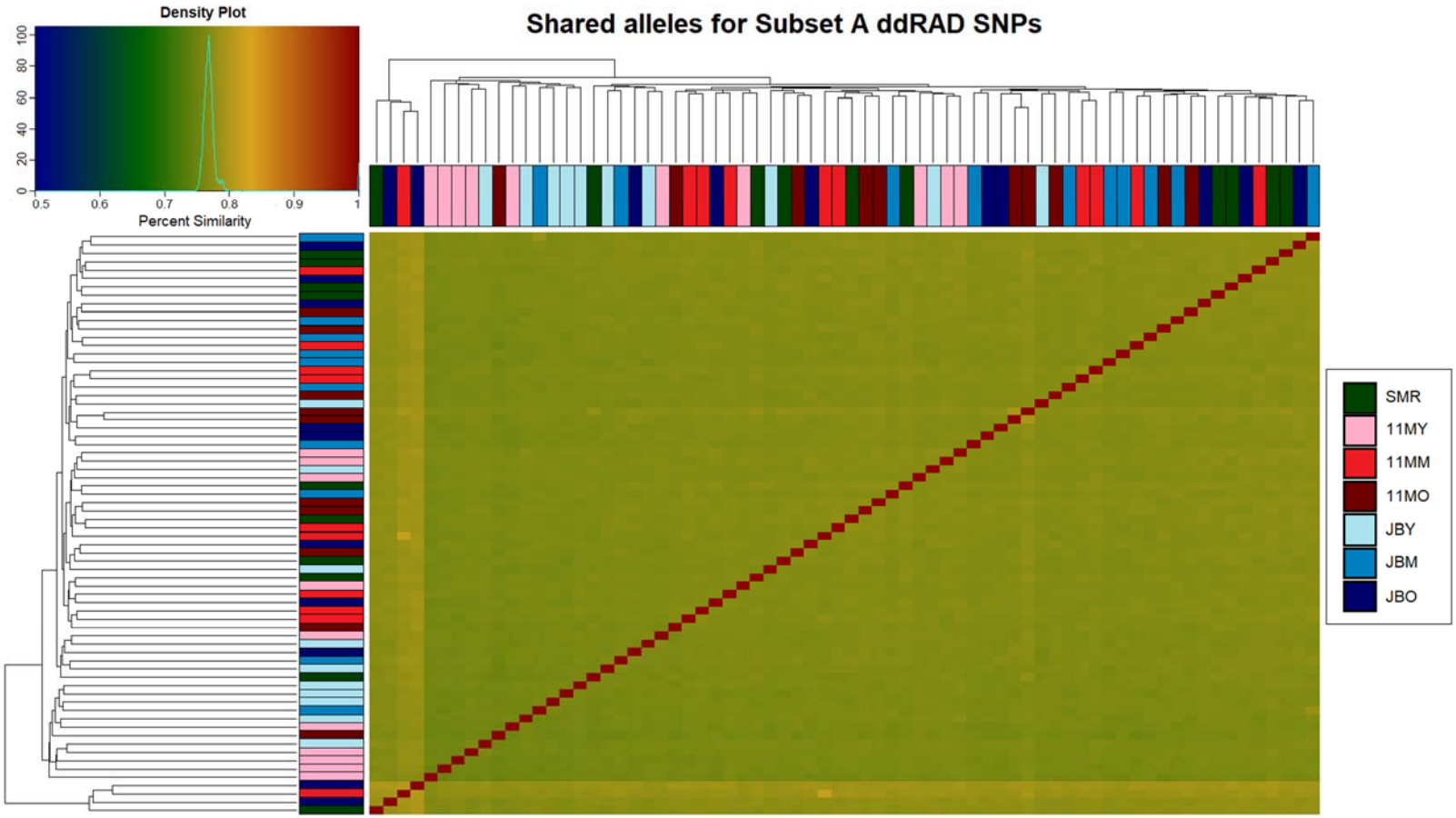

Figure 27. Heatmap of shared alleles Subset A of ddRAD SNPs, where deviations from HWE were not allowed. Dendrograms represent inferred relationships between samples based on genetic distances. Colored bars at the terminal branches of dendorgrams correspond to sampling location of the individual, as noted in the legend.

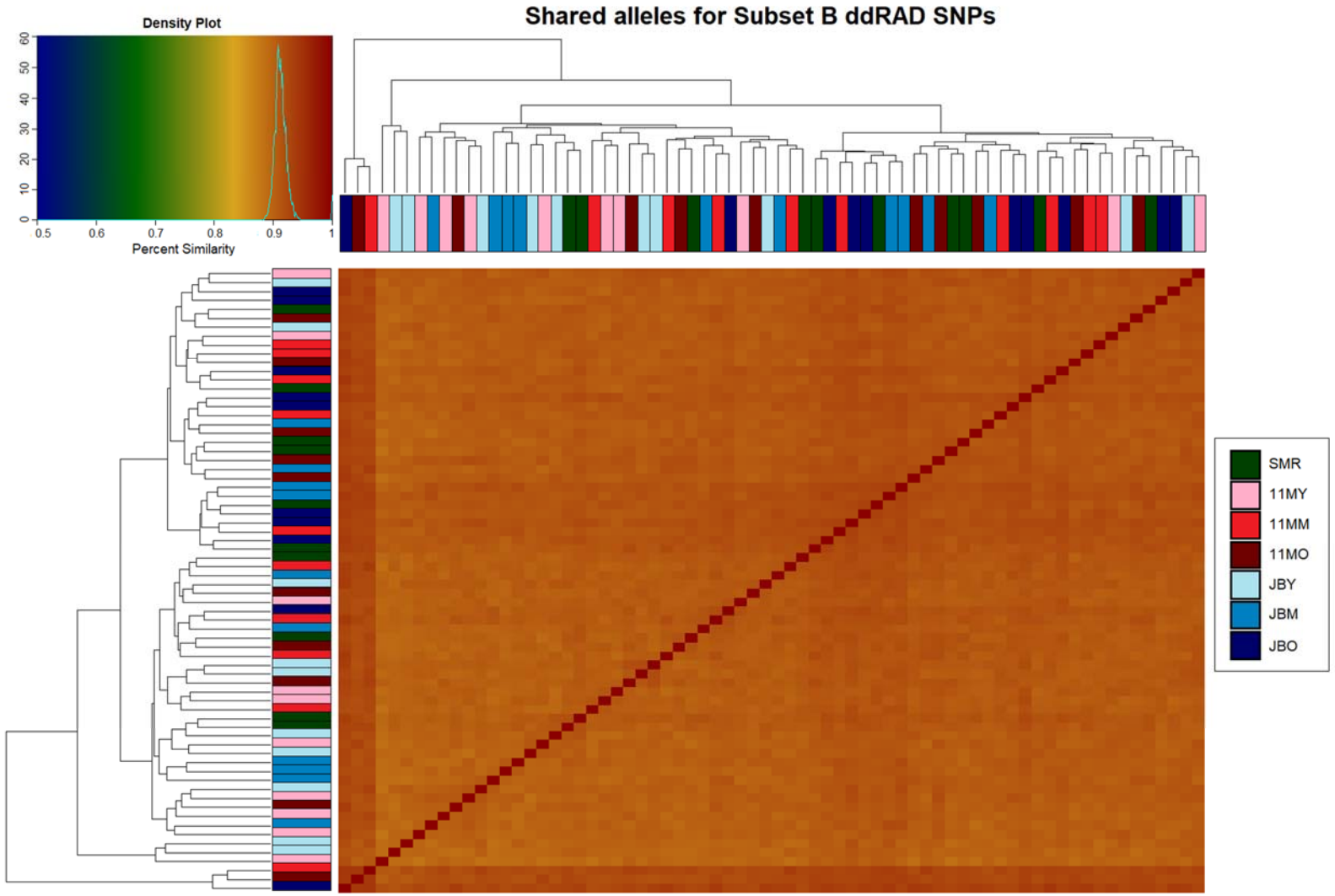

Figure 28. Heatmap of shared alleles in Subset B of ddRAD SNP markers, in which every locus is out of HWE in at least one marsh. Dendrograms represent inferred relationships between samples based on genetic distances. Colored bars at the terminal branches of dendorgrams correspond to sampling location of the individual, as noted in the legend.

Discussion

This study sought to add to the existing body of salt marsh restoration knowledge by investigating levels of genetic diversity of an important *Spartina* salt marsh resident in a natural, reference marsh as compared to restored marshes. We compared levels of genetic diversity in Gulf Killifish using both mtDNA sequencing data and nuclear SNPs derived from ddRAD sequencing to determine if there are differences based on the age of restored marshes, or the distance of restored marshes from the reference marsh. According to the mtDNA results, no signal of population structuring or differences in levels of haplotypic diversity for Gulf Killifish inhabiting natural and restored *Spartina* salt marsh habitat in Galveston, Texas. Congruent with previous studies of Gulf Killifish using microsatellites (Williams et al. 2008) and mtDNA data (See Chapter 3), all marshes surveyed contained high levels of haplotypic diversity, *h*. While one of the young marshes (JBY) had the lowest *h*, the highest *h* was found, not in the reference marsh (SMR), but in one of the oldest restored marshes (JBO), and levels of *h* were statistically similar between all the marshes sampled (**Tables 18-19**). Partitioning the genetic variation for mtDNA sequence data either by age or by distance resulted in very low, non-significant values of among-group variance (F_{CT}), and little-to-no variation being explained by the partitioning scheme. In both cases, nearly all the genetic variation in the mtDNA sequence data was explained by differences between individuals, rather than by the marsh from which the individual originated (**Tables 20-21**). Interestingly, the MJN (**Figure 18**) and the PCA (**Figure 19**) for mtDNA sequence data divide individuals into two distinct phylogroups, both of which contain lineages from all the marshes sampled in this study, which is consistent with

previous findings for Gulf Killifish on the east Texas coast using mtDNA sequence data (See Chapter 3 for data and full discussion).

To determine if population structure or differences in genetic diversity could exist at a finer scale than that provided by mtDNA sequence data, we also investigated > 13,000 SNPs derived from ddRAD sequencing. Similar to the mtDNA sequencing results, SNP data did not reveal enough differences to subdivide marshes into separate populations. Greater than 70% of alleles for the main dataset were shared among all individuals (**Figure 24)**, and ADMIXTURE results showed the lowest cross-validation error value for K=1, indicating all samples in this study can be grouped into a single population (**Figure 22**). Like mtDNA, partitioning the genetic variation for the main SNP dataset by age since restoration or distance to reference marsh returned small, non-significant values for F_{CT}. No variation in our SNPs was explained by partitioning schemes, and nearly all the genetic variation was attributed to differences among individuals (**Tables 24-25**).

While SNP data did not reveal population structure, it did reveal differences in levels of genetic diversity among marshes. Both of the youngest marshes (11MY and JBY) were characterized by significantly ($p < 0.05$) lower levels of observed heterozygosity *Ho* when compared to the reference marsh (**Tables 22-23**). Concordantly, the two youngest marshes also had the highest values for F_{IS}, indicating a greater degree of inbreeding compared to the other marshes, and they contained the greatest number of loci that deviate significantly from HWE expectations. The youngest marshes were also differentiated from all other marshes by their association along both axes of the PCA for the main dataset (**Figure 23**), and the dendrogram based on genetic distance between samples grouped the

most individuals from the young marshes in a separate clade from the majority of individuals from all other marshes (**Figure 24**).

Because the two marshes that displayed significant differences in in levels of genetic diversity as compared to the reference were also the ones that contained the highest number of loci that deviate from HWE, we tested two subsets of the main dataset: one that excluded all loci out of HWE (Subset A), and one that included only loci out of HWE (Subset B) to determine what role, if any, those loci played in the differences we found. The results for Subset A were consistent with those obtained with the main dataset, including significantly lower *Ho* for the two young marshes as compared with the reference marsh, a greater degree of inbreeding, and clear separation of individuals from the young marshes from the other group on both axes of the PCA. The results for Subset B showed no significant differences in *Ho* between any of the marshes, all individuals shared ~ 90% alleles, and no specific group of individuals deviate from the others in the PCA. The outcomes for Subset A and Subset B lead us to conclude that our analyses of the main dataset are not skewed by the inclusion of loci out of HWE, nor are the uncovered patterns of genetic diversity influenced by those loci.

Our reference marsh, assumed to provide a source population for recolonization of nearby restored marsh sites, is located 2.4 km – 9.5 km distant from the restored sites used in this study, and Nelson et al. (2014) found that Gulf Killifish display high site fidelity, with individuals only moving ~100m between connected salt marsh sites. This high level of site fidelity, combined with adhesive, demersal eggs, high predation rates and limited (expected) life spans, are thought to limit overall movements of Gulf Killifish and reduce gene flow (Gricius 1994). Patterns of isolation by distance (IBD), spatial autocorrelation,

and assignment tests derived from microsatellite data for this species in the northern Gulf of Mexico also suggest limited dispersal (Williams et al. 2008). The data in this study, however, indicates that the Gulf Killifish populations are mobile enough to supply sufficient colonizing individuals to prevent a paucity of genetic diversity due to founder effects in restored marshes. This is congruent with studies that describe Gulf Killifish from the northern Gulf of Mexico, except for Mobile Bay, Alabama, as a single population using microsatellites (Williams et al. 2008), using allozymes and restriction fragment length polymorphism (RFLP) of mtDNA (Gricius 1994), and findings by this author (see Chapter 3) that describe Gulf Killifish on the east Texas coast as a single population using mtDNA sequences.

Overall, our results agree with a variety of studies comparing restored and natural, pristine, or reference habitats. A non-molecular comparison of restored and reference marshes, using an ecosystem index score found that the urbanized reference marshes in Galveston, Texas scored, on average, 81%, while restored marshes averaged 75%, leading those authors to conclude that restored marshes in Galveston Bay are relatively well developed (Staszak and Armitage 2013). Using 94 markers of amplified fragment length polymorphisms (AFLPs), restored populations of *Spartina alterniflora* (smooth cordgrass) displayed comparable levels of genetic diversity as compared with nearby, natural populations (Travis et al. 2002), and restored *Banksia* woodlands in Australia also contained comparable levels of *Ho* and allelic diversity to natural stands when analyzed via seven microsatellite loci (Ritchie and Krauss 2012). A very relevant study with regards to our young marshes compared microsatellite markers in oregano plants growing in natural and restored grassland patches, finding similar levels of genetic diversity among all

populations, but with significantly higher inbreeding coefficients in the youngest populations (Helsen et al. 2013). Williams and Davis (1996) used four polymorphic allozymes and found significantly reduced genetic diversity in terms of *Ho,* polymorphic loci, and allele richness in transplanted sites of eelgrass when compared to pristine sites; however, a more recent study using seven allozymes compared eelgrass beds based on age and size and found no differences in genetic structure and diversity with regards to either age or size (Rhode and Duffy 2004). Based on nine microsatellite markers, restored oyster beds developed comparable levels of genetic diversity to natural beds in as little as a single month post-restoration (Arnaldi et al. 2018). Far more long-term effects of introduction of fish species to new habitat were studied by Planes and Lecaillon (1998), who looked at allozyme markers in two fish species that were introduced to coral reefs in Hawaii from French Polynesia source populations in the mid-late 1950s, and found that Hawaiian populations had no major differences in polymorphism and heterozygosity for the two species studied when compared with the source populations, despite Hawaiian populations likely being isolated from gene flow with other reefs.

One factor that may contribute to equivalent levels of genetic variation found in restored sites as compared to reference sites, and which is of vital importance to restoration science, is mode of colonization. Salt marsh restoration efforts rely on the assumption that species re-establish themselves once the habitat is made available (Palmer et al. 1997), and therefore no effort is made to intentionally transplant important faunal residents. This may actually be beneficial to the colonizing organisms, as multiple studies have found that natural colonization of both flora and fauna in restored habitats resulted in levels of genetic diversity comparable to, or even higher than, natural habitat (Travis et al. 2002, Helsen et

al. 2013, Arnaldi et al. 2018; but see Williams and Davis 1996). Additionally, the study of Polynesian fish introduced to Hawaii led those authors to conclude that the success of species introductions into new habitat is highly dependent on the number of individuals able to colonize (Planes and Lecaillon 1998), highlighting the importance of both nearby source populations to restoration projects, and accurate information regarding the dispersal potential of ecologically valuable species.

Our results suggest that Gulf Killifish have sufficient dispersal potential to colonize restored marsh habitats located < 10 km from a natural, reference marsh, and that gene flow between the marshes is adequate to maintain comparable levels of genetic diversity between natural and restored sites, and that allowing for natural colonization of restored sites for this species does not appear to result in reduced genetic diversity due to founder effects. This level of dispersal is contrary to earlier studies that suggest the Gulf Killifish displays high site fidelity and does not move more than ~100m within adjacent marshes (Nelson 2014). However, it is important to note that the mark recapture study only studied movement of Gulf Killifish over a six week period in the summer, the middle of the Gulf Killifish breeding period, in marshes on the coast of Alabama. This does not take into account potential larger scale movements during winter months, or possible regional differences in the behavior of this species. Also, while there were storm events during the mark-recapture study, they were not tropical storm or hurricane intensity (Nelson 2014); such large storms, which are common in the Gulf of Mexico, may also contribute to dispersal of small aquatic species, like the Gulf Killifish.

While the two young marshes had reduced levels of *Ho* and showed some evidence of elevated inbreeding, the results of the medium and old marshes, combined with the

findings of genetic diversity in oregano plants in young grasslands by Helsen et al. (2013), suggest that levels of *Ho* will catch up to those in older marshes and inbreeding will be reduced within a few years. This study sought to improve our knowledge of how salt marsh restoration practices impact the genetic diversity of colonizing marsh fauna using restored marshes located < 10 km from the reference marsh; therefore, future studies comparing marshes located farther from reference marsh populations may help determine if there is an upper limit to the distance from a natural marsh that should be considered in planning restoration projects. Additionally, Gulf Killifish represent only one important faunal marsh resident out of many that are vital to estuarine food webs and nutrient cycling processes; therefore, it would be beneficial to conduct similar, comparative studies on other ecologically important fauna.

CHAPTER V

GENERAL CONCLUSIONS AND SUMMARY

This dissertation consisted of the following studies: 1) development of a short amplicon high resolution melting assay (SA-HRMA) for molecular identification of two morphologically similar planthopper species, *Prokelisia marginata* and *Prokelisia dolus*; 2) comparison of population structure and historical demography for the Gulf Killifish, daggerblade grass shrimp, and phloem-feed planthopper in the north and west Gulf of Mexico (Gulf) using mitochondrial DNA (mtDNA) sequence data; and 3) investigation into levels of genetic diversity of Gulf Killifish inhabiting a reference marsh compared with restored marshes of differing ages and distances from the reference in Galveston Bay, Texas, using mtDNA sequence data and nuclear single nucleotide polymorphism (SNP) data generated via double digest restriction site associated DNA (ddRAD) sequencing. The general results and conclusions for these three studies are as follows:

High resolution melting assay for species identification

The accidental inclusion of heterospecifics when conducting population genetic studies has the potential of introducing bias to genetic indices such as allele frequency counts and estimates of genetic diversity. Therefore, development of a reliable method for identification of these species has important implications for population genetic studies of these species, such as those conducted in Chapter III. To that end in Chapter II, I describe how a 60 bp fragment of the Cytochrome C Oxidase Subunit I (COI) gene was used to develop a SA-HRMA assay to differentiate *P. marginata* from *P. dolus*, two species of planthopper that are difficult to identify via morphology. The targeted fragment contains

eight polymorphic sites, four of which are fixed differences that result in a minimum > 1.7 °C separation between the melting peaks for each species. The other four polymorphic sites are plesiomorphies that produce variability in the melting profiles within the two species, but do not reduce the diagnostic power of the HRMA. *P. marginata* melts at a higher temperature (~79.4°C) than *P. dolus* (~77.0°C) due to a higher concentration of G or C nucleotides in the majority of polymorphic sites for *P. marginata*. A test of the HRMA was conducted using high throughput (n=518), and results in clearly diagnostic melting curves for species assignment of 213 *P. dolus* individuals and 296 *P. marginata* individuals, with only 9 (1.7%) amplification failures.

Population structure and historical demography for three common salt marsh fauna

Comparative phylogeography has the potential to reveal differences and similarities in sympatric species. When comparing fauna characterized by pronounced differences in their life history patterns, predictions of the expected levels of variability, and of the geographic distribution of such variability can be made, as well as estimates of how variation in each of the compared species was accrued, or lost, over time. Accordingly, three species whose life history is intimately tied to *Spartina* salt marshes were investigated, namely the Gulf Killifish, the daggerblade grass shrimp, and a *Prokelisia* planthopper. In Chapter III, 1077 bp of concatenated mtDNA sequence data, including 331 bp of Control Region 1 (CR1), 344 bp of nitrogen dehydrogenase subunit 2 (ND2), and 397 bp of nitrogen dehydrogenase subunit 5 (ND5), for Gulf Killifish in the north and west Gulf of Mexico revealed 109 haplotypes over the region sampled. Gulf Killifish data includes high levels of haplotypic diversity, evidence of isolation by distance (IBD), and

population structure at regional levels. The population structure for Gulf Killifish generates phylogeographic breaks roughly at the Mississippi River and the Upper Laguna Madre system, which is the first time a study of this species has found population structure in the north Gulf, west of Mobile Bay, Alabama (Gricius 1994, Williams et al. 2008). The east Texas population is further subdivided into two phylogroups with distinct patterns of historical demography, which may be attributed either to historic breaks between the Gulf and Atlantic during periods of glaciation in the Pleistocene, or a historic phylogeographic break between east and south Texas, followed by population expansion from east Texas into the north Gulf and subsequent reestablishment of contact between the two Texas populations. Members of Phylogroup I, including all north Gulf lineages and some east Texas lineages show evidence of recent bottlenecks, followed by expansion, while east and south Texas lineages belonging to Phylogroup II show evidence of large, stable populations, and estimates of female effective population size (N_e) range from the 10s to 100s of millions.

Sequence data for grass shrimp (466 bp of 16sRNA) and planthoppers (372 bp of COI) displayed relatively low levels of haplotypic diversity, with 13 closely-related haplotypes in grass shrimp, and four haplotypes only one mutational step from each other in planthoppers. Both species show evidence of population structure; although, the suggested groupings to explain maximum variance in both cases makes little biological or geographic sense. For grass shrimp, lineages from the north Gulf contain most of the variation found throughout the sampling range of this study, which is much higher than the level of mtDNA variation found in previous studies of this species in the south Atlantic (Flowers 2004). East and south Texas sampling locations, on the other hand, are each

characterized by few haplotypes that collectively represent all the potential variation in the sampling range. The reduced variation in Texas localities may be explained by extinction and colonization events resulting in founder effects, or random fluctuations in population size combined with sweepstakes reproductive success (i.e., SRS or the Hedgecock effect).

For planthoppers, the majority of individuals in the sampling range sharing a single haplotype. A previous study of the same mtDNA segment in this species of planthopper along the Atlantic U.S. and north Gulf coastlines found greater levels of genetic variation (Denno 2008), and the variation characterized in here appears to correspond to *P. marginata* haplotypes from the western Gulf in that study. Thus, the variation characterized in Chapter III likely represents a small subset of the total mtDNA variation in this species, rather than signifying that this species is depauperate of genetic variation, or that the choice of genetic marker was inappropriate. Similar to grass shrimp, reduced genetic variation in planthoppers throughout this range may be attributed to historic losses followed by founder events with new variants from the east, followed by subsequent isolation from the east, or to the fluidity of planthopper life history, which allows this species to switch between a fecund wingless morph with low dispersal potential, or a less fecund winged morph with high dispersal potential.

Further investigations with an expanded sampling range to include localities in Mexico and Florida for all three species, and investigations targeting markers with a greater degree of polymorphism for grass shrimp and planthoppers, would be beneficial in resolving patterns of historical demography and finer scale population structure for Gulf Killifish, daggerblade grass shrimp, and phloem-feed planthoppers in this geographic range.

Levels of genetic diversity in Gulf Killifish inhabiting reference and restored marshes

In Chapter IV, comparisons were made for mtDNA and nuclear SNP diversity in Gulf Killifish between a reference marsh and restored marshes of differing ages or distance from the reference marsh in Galveston Bay, Texas. For mtDNA, 1077 bp of concatenated sequence data, including 331 bp of Control Region 1 (CR1), 344 bp of nitrogen dehydrogenase subunit 2 (ND2), and 397 bp of nitrogen dehydrogenase subunit 5 (ND5), revealed 89 haplotypes. Gulf Killifish display similar high levels of haplotypic diversity in all marshes and no evidence of population structure or IBD at the scale of this study was observed. Individuals are subdivided into two phylogroups, similar to those generated by the east Texas samples from Chapter 3, but with each phylogroup containing lineages from every marsh in this study.

For nuclear SNPs, 13,397 SNPs were generated using ddRAD sequencing data. Based on SNP data, the two youngest restored marshes display significantly lower levels of heterozygosity than samples from the reference marsh. The two young marshes are also distinguished by a greater degree of inbreeding, and more loci out of Hardy Weinberg Equilibrium (HWE) expectations. A PCA of SNP data show individuals of the two youngest marshes deviating from the main cluster along both axes, and a dendrogram based on genetic distance between the samples grouped the majority of individuals from the two youngest marshes in a separate clade from the majority of individuals from all the other marshes. The findings regarding the two youngest marshes are similar to findings in oregano plants that revealed similar levels of genetic diversity, but a greater degree of inbreeding in the youngest restored habitats sampled (Helsen et al. 2013). Investigations of

the SNP data without the loci that deviate from HWE, and data containing only those loci that deviate from HWE indicate that our results are not likely skewed by the inclusion of those loci.

The two main hypotheses of Chapter IV require testing for differences in levels of genetic variation in relation to the age of the restored marsh, or the distance of the restored marsh from a reference marsh. Hierarchical variance partitioning for both mtDNA and nuclear SNP data attributes nearly 100% of genetic variation in this study to differences between individuals, rather than differences between subpopulations, or differences between hypothesis-generated groups based on age or distance. Despite the assumed low dispersal potential of Gulf Killifish, the data from this study indicates that they are mobile enough to supply sufficient colonizing individuals to prevent founder events in restored habitats. Additionally, the results of this study combined with other studies that investigated levels of genetic diversity in flora and fauna allowed to naturally colonize restored habitat (Travis et al. 2002, Helsen et al. 2013, Arnaldi et al. 2018) indicate that allowing natural colonization, as opposed to intentional transplanting, of fauna from nearby source populations may contribute to comparable levels of genetic diversity between restored and natural habitats. Investigations of genetic diversity for fauna in restored marsh habitat that is more distant from potential source populations than the < 10 km investigated in Chapter IV, or comparative investigations of genetic diversity in other ecologically important marsh residents would be beneficial additions to this study.

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APPENDIX A

SUPPLEMENTAL MATERIAL FOR CHAPTER II

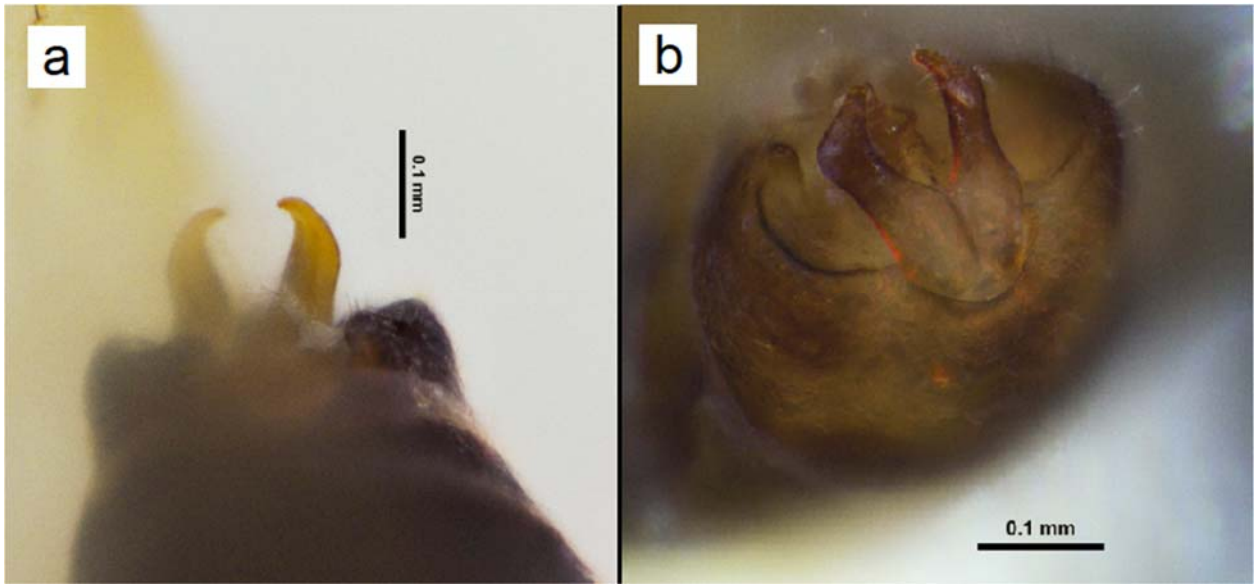

Figure A-1. Magnified view of the styles of male specimens of (a) *P. marginata* and (b) *P. dolus*. The difference in shape, i.e. curved in *P. marginata* versus angular in *P. dolus*, was the main diagnostic feature used to identify male individuals to species level for this study. These images were taken with a Nikon AZ100M microscope with motorized body and compiled via Nikon BR software.

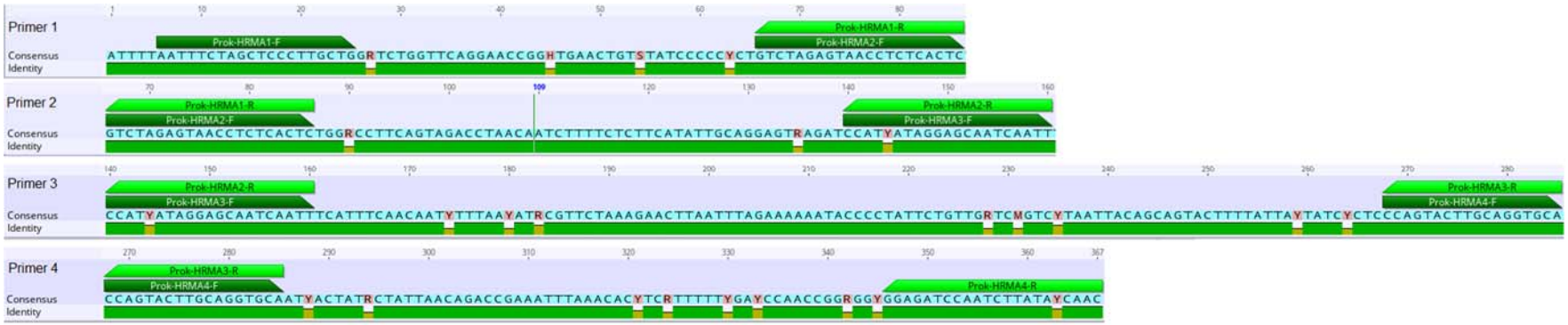

Figure A-2. Consensus sequence from multiple sequence alignment of COI segment for 65 morphologically validated male specimens of *P. marginata* (n=43) and *P. dolus* (n=22). Forward and reverse primer locations for the four primer sets designed in this study are annotated on the sequence. Ambiguities in the consensus sequence correspond to polymorphic sites within and between the species, and are highlighted in pink and coded in standard IUPAC notation.

APPENDIX B

SUPPLEMENTAL MATERIAL FOR CHAPTER III

Table B-1 Haplotype frequencies by sampling location for Gulf Killifish (*F. grandis*), Daggerblade grass shrimp (*P. pugio*), and Phloem-feeding planthoppers (*P. marginata*). Haplotype data for Gulf Killifish is shown for 366 bp of Control Region 1 (CR1), 344 bp of Nitrogen Dehydrogenase Subunit 2 (ND-2), 397 bp of Nitrogen Dehydrogenase Subunit 5 (ND-5) and 1077 bp of Concatenated sequence. Haplotype data for grass shrimp and planthoppers is shown for 466 bp of 16s RNA, and for 372 bp of Cytochrome Oxidase Subunit I (COI), respectively.

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Control Region 1**							
CR1 – 01	5	0	0	0	0	0	MT622370
CR1 – 02	2	3	0	0	0	0	MT622371
CR1 – 03	9	0	0	0	0	0	MT622372
CR1 – 04	2	0	0	0	0	0	MT622373
CR1 – 05	1	0	0	0	0	0	MT622374
CR1 – 06	1	0	3	1	0	0	MT622375
CR1 – 07	1	0	0	0	0	0	MT622376
CR1 – 08	2	0	0	0	0	0	MT622377
CR1 – 09	1	0	0	0	0	0	MT622378
CR1 – 10	1	1	0	2	0	0	MT622379
CR1 – 11	1	3	2	0	0	0	MT622380
CR1 – 12	0	1	2	3	0	0	MT622381
CR1 – 13	0	1	0	0	0	0	MT622382
CR1 – 14	0	1	0	0	0	0	MT622383

Table B-1 Continued

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Control Region 1 Continued**							
CR1 – 15	0	1	0	1	0	0	MT622384
CR1 – 16	0	2	1	1	0	0	MT622385
CR1 – 17	0	1	0	0	0	0	MT622386
CR1 – 18	0	1	2	1	0	0	MT622387
CR1 – 19	0	1	0	0	0	0	MT622388
CR1 – 20	0	1	5	2	0	0	MT622389
CR1 – 21	0	1	0	0	0	0	MT622390
CR1 – 22	0	0	0	0	17	13	MT622391
CR1 – 23	0	0	0	0	0	1	MT622392
CR1 – 24	0	0	0	0	0	2	MT622393
CR1 – 25	0	0	0	0	0	2	MT622394
CR1 – 26	0	0	0	0	6	3	MT622395
CR1 – 27	0	0	0	0	2	1	MT622396
CR1 – 28	0	0	0	0	0	1	MT622397
CR1 – 29	0	0	0	0	0	1	MT622398
CR1 – 30	0	0	0	0	0	1	MT622399
CR1 – 31	0	0	0	0	0	1	MT622400
CR1 – 32	0	0	0	0	0	1	MT622401
CR1 – 33	0	0	0	0	0	1	MT622402
CR1 – 34	0	0	0	0	0	1	MT622403
CR1 – 35	0	0	0	0	0	1	MT622404
CR1 – 36	0	0	0	0	0	1	MT622405
CR1 – 37	0	0	0	0	0	1	MT622406
CR1 – 38	0	0	0	0	0	1	MT622407
CR1 – 39	0	0	1	0	0	0	MT622408
CR1 – 40	0	0	1	0	0	0	MT622409
CR1 – 41	0	0	1	0	0	0	MT622410

Table B-1 Continued

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Control Region 1 Continued**							
CR1 – 42	0	0	2	0	0	0	MT622411
CR1 – 43	0	0	1	0	0	0	MT622412
CR1 – 44	0	0	1	1	0	0	MT622413
CR1 – 45	0	0	1	0	0	0	MT622414
CR1 – 46	0	0	2	1	0	0	MT622415
CR1 – 47	0	0	1	0	0	0	MT622416
CR1 – 48	0	0	1	0	0	0	MT622417
CR1 – 49	0	0	1	0	0	0	MT622418
CR1 – 50	0	0	1	0	0	0	MT622419
CR1 – 51	0	0	0	3	0	0	MT622420
CR1 – 52	0	0	0	1	0	0	MT622421
CR1 – 53	0	0	0	1	0	0	MT622422
CR1 – 54	0	0	0	1	0	0	MT622423
CR1 – 55	0	0	0	1	0	0	MT622424
CR1 – 56	0	0	0	1	0	0	MT622425
CR1 – 57	0	0	0	1	0	0	MT622426
CR1 – 58	0	0	0	1	0	0	MT622427
CR1 – 59	0	0	0	1	0	0	MT622428
CR1 – 60	0	0	0	1	0	0	MT622429
CR1 – 61	0	0	0	1	0	0	MT622430
CR1 – 62	0	0	0	1	0	0	MT622431
CR1 – 63	0	0	0	0	2	0	MT622432
CR1 – 64	0	0	0	0	1	0	MT622433
CR1 – 65	0	0	0	0	1	0	MT622434
CR1 – 66	0	0	0	0	1	0	MT622435
CR1 – 67	0	0	0	0	1	0	MT622436

Table B-1 Continued

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Control Region 1 Continued**							
CR1 – 68	0	0	0	0	1	0	MT622437
CR1 – 69	0	0	0	0	1	0	MT622438
CR1 – 70	0	0	0	0	1	0	MT622439
CR1 – 71	0	0	0	0	1	0	MT622440
CR1 – 72	0	0	0	0	1	0	MT622441
***F. grandis* Nitrogen Dehydrogenase Subunit 2**							
ND2 – 01	5	3	5	1	0	0	MT622442
ND2 – 02	2	2	0	0	0	0	MT622443
ND2 – 03	4	0	0	0	0	0	MT622444
ND2 – 04	6	0	0	0	0	0	MT622445
ND2 – 05	2	0	0	0	0	0	MT622446
ND2 – 06	1	1	0	0	0	0	MT622447
ND2 – 07	1	0	3	0	0	0	MT622448
ND2 – 08	1	0	1	0	0	0	MT622449
ND2 – 09	1	0	0	0	0	0	MT622450
ND2 – 10	2	0	0	0	0	0	MT622451
ND2 – 11	1	3	5	3	1	5	MT622452
ND2 – 12	0	1	0	0	0	0	MT622453
ND2 – 13	0	1	0	0	0	0	MT622454
ND2 – 14	0	1	0	1	0	0	MT622455
ND2 – 15	0	1	0	0	0	0	MT622456
ND2 – 16	0	1	3	2	0	0	MT622457
ND2 – 17	0	1	0	0	0	0	MT622458
ND2 – 18	0	1	0	0	0	0	MT622459
ND2 – 19	0	1	0	0	0	0	MT622460
ND2 – 20	0	1	1	1	0	0	MT622461
ND2 – 21	0	0	0	0	13	4	MT622462

Table B-1 Continued

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Nitrogen Dehydrogenase Subunit 2 – Continued**							
ND2 – 22	0	0	0	0	0	1	MT622463
ND2 – 23	0	0	0	0	0	6	MT622464
ND2 – 24	0	0	0	0	0	1	MT622465
ND2 – 25	0	0	0	0	0	2	MT622466
ND2 – 26	0	0	0	0	0	1	MT622467
ND2 – 27	0	0	0	0	0	1	MT622468
ND2 – 28	0	0	0	0	11	4	MT622469
ND2 – 29	0	0	0	0	0	1	MT622470
ND2 – 30	0	0	0	0	0	1	MT622471
ND2 – 31	0	0	0	0	0	1	MT622472
ND2 – 32	0	0	0	0	0	1	MT622473
ND2 – 33	0	0	0	0	5	1	MT622474
ND2 – 34	0	0	0	0	0	1	MT622475
ND2 – 35	0	0	0	0	0	1	MT622476
ND2 – 36	0	0	0	0	0	1	MT622477
ND2 – 37	0	0	1	0	0	0	MT622478
ND2 – 38	0	0	2	3	0	0	MT622479
ND2 – 39	0	0	2	1	0	0	MT622480
ND2 – 40	0	0	2	1	0	0	MT622481
ND2 – 41	0	0	1	0	0	0	MT622482
ND2 – 42	0	0	1	0	0	0	MT622483
ND2 – 43	0	0	1	1	0	0	MT622484
ND2 – 44	0	0	1	1	0	0	MT622485
ND2 – 45	0	0	0	3	0	0	MT622486
ND2 – 46	0	0	0	1	0	0	MT622487

Table B-1 Continued

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Nitrogen Dehydrogenase Subunit 2 – Continued**							
ND2 – 47	0	0	0	3	0	0	MT622488
ND2 – 48	0	0	0	2	0	0	MT622489
ND2 – 49	0	0	0	1	0	0	MT622490
ND2 – 50	0	0	0	1	0	0	MT622491
ND2 – 51	0	0	0	1	0	0	MT622492
ND2 – 52	0	0	0	0	3	0	MT622493
ND2 – 53	0	0	0	0	1	0	MT622494
ND2 – 54	0	0	0	0	1	0	MT622495
ND2 – 55	0	0	0	0	1	0	MT622496
***F. grandis* Nitrogen Dehydrogenase Subunit 5**							
ND5 – 01	10	5	8	5	0	0	MT635859
ND5 – 02	2	0	0	0	0	0	MT635860
ND5 – 03	8	0	0	0	0	0	MT635861
ND5 – 04	2	0	0	0	0	0	MT635862
ND5 – 05	1	1	1	2	0	0	MT635863
ND5 – 06	1	0	4	1	0	0	MT635864
ND5 – 07	1	1	1	1	0	0	MT635865
ND5 – 08	1	0	0	0	0	0	MT635866
ND5 – 09	0	1	0	2	0	0	MT635867
ND5 – 10	0	1	0	0	0	0	MT635868
ND5 – 11	0	1	0	0	0	0	MT635869
ND5 – 12	0	1	0	0	0	0	MT635870
ND5 – 13	0	1	0	0	0	0	MT635871
ND5 – 14	0	1	2	1	0	0	MT635872
ND5 – 15	0	1	1	1	0	0	MT635873
ND5 – 16	0	1	0	0	0	0	MT635874
ND5 – 17	0	1	0	0	0	0	MT635875

Table B-1 Continued

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Nitrogen Dehydrogenase Subunit 5 – Continued**							
ND5 – 18	0	1	0	0	0	0	MT635876
ND5 – 19	0	1	0	2	0	0	MT635877
ND5 – 20	0	0	0	0	22	17	MT635878
ND5 – 21	0	0	0	0	0	1	MT635879
ND5 – 22	0	0	0	0	2	5	MT635880
ND5 – 23	0	0	0	0	0	1	MT635881
ND5 – 24	0	0	0	0	0	1	MT635882
ND5 – 25	0	0	0	0	0	1	MT635883
ND5 – 26	0	0	0	0	7	2	MT635884
ND5 – 27	0	0	0	0	0	1	MT635885
ND5 – 28	0	0	0	0	0	1	MT635886
ND5 – 29	0	0	0	0	0	1	MT635887
ND5 – 30	0	0	0	0	0	1	MT635888
ND5 – 31	0	0	0	0	1	1	MT635889
ND5 – 32	0	0	1	0	0	0	MT635890
ND5 – 33	0	0	1	0	0	0	MT635891
ND5 – 34	0	0	2	3	0	0	MT635892
ND5 – 35	0	0	1	0	0	0	MT635893
ND5 – 36	0	0	1	0	0	0	MT635894
ND5 – 37	0	0	1	0	0	0	MT635895
ND5 – 38	0	0	1	1	0	0	MT635896
ND5 – 39	0	0	1	0	0	0	MT635897
ND5 – 40	0	0	2	0	0	0	MT635898
ND5 – 41	0	0	1	2	0	0	MT635899
ND5 – 42	0	0	0	2	0	0	MT635900
ND5 – 43	0	0	0	1	0	0	MT635901
ND5 – 44	0	0	0	1	0	0	MT635902

Table B-1 Continued

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Nitrogen Dehydrogenase Subunit 5 – Continued**							
ND5 – 45	0	0	0	1	0	0	MT635903
ND5 – 46	0	0	0	1	0	0	MT635904
ND5 – 47	0	0	0	0	1	0	MT635905
ND5 – 48	0	0	0	0	1	0	MT635906
ND5 – 49	0	0	0	0	1	0	MT635907
ND5 – 50	0	0	0	0	1	0	MT635908
***F. grandis* Concatenated Sequences**							
Con – 001	4	0	0	0	0	0	MT622370, MT622442, MT635859
Con – 002	2	0	0	0	0	0	MT622371, MT622443, MT635860
Con – 003	4	0	0	0	0	0	MT622372, MT622444, MT635861
Con – 004	2	0	0	0	0	0	MT622372, MT622445, MT635862
Con – 005	2	0	0	0	0	0	MT622373, MT622445, MT635862
Con – 006	1	0	0	0	0	0	MT622374, MT622447, MT635863
Con – 007	3	0	0	0	0	0	MT622372, MT622445, MT635861
Con – 008	1	0	2	0	0	0	MT622375, MT622448, MT635864
Con – 009	1	0	0	0	0	0	MT622370, MT622449, MT635859
Con – 010	1	0	0	0	0	0	MT622376, MT622450, MT635859
Con – 011	1	0	0	0	0	0	MT622377, MT622451, MT635859
Con – 012	1	0	0	0	0	0	MT622378, MT622445, MT635861
Con – 013	1	0	0	0	0	0	MT622379, MT622452, MT635865
Con – 014	1	3	1	0	0	0	MT622380, MT622442, MT635859
Con – 015	1	0	0	0	0	0	MT622377, MT622451, MT635866
Con – 016	0	0	1	0	0	0	MT622408, MT622478, MT635890
Con – 017	0	0	1	0	0	0	MT622409, MT622461, MT635859
Con – 018	0	0	1	0	0	0	MT622410, MT622452, MT635891
Con – 019	0	0	2	0	0	0	MT622411, MT622442, MT635859
Con – 020	0	0	2	3	0	0	MT622381, MT622479, MT635892

Table B-1 Continued

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Concatenated Sequences – Continued**							
Con – 021	0	0	1	0	0	0	MT622412, MT622452, MT635893
Con – 022	0	0	1	0	0	0	MT622375, MT622442, MT635864
Con – 023	0	0	1	1	0	0	MT622413, MT622480, MT635859
Con – 024	0	0	1	1	0	0	MT622389, MT622452, MT635865
Con – 025	0	0	1	0	0	0	MT622414, MT622480, MT635859
Con – 026	0	0	2	1	0	0	MT622415, MT622481, MT635859
Con – 027	0	0	1	0	0	0	MT622389, MT622452, MT635863
Con – 028	0	0	1	0	0	0	MT622385, MT622482, MT635894
Con – 029	0	0	1	0	0	0	MT622416, MT622449, MT635873
Con – 030	0	0	1	0	0	0	MT622417, MT622483, MT635859
Con – 031	0	1	2	1	0	0	MT622387, MT622457, MT635873
Con – 032	0	0	1	0	0	0	MT622418, MT622448, MT635895
Con – 033	0	0	1	1	0	0	MT622389, MT622484, MT635896
Con – 034	0	0	1	0	0	0	MT622419, MT622452, MT635897
Con – 035	0	0	1	0	0	0	MT622389, MT622457, MT635898
Con – 036	0	0	1	0	0	0	MT622389, MT622485, MT635898
Con – 037	0	0	1	0	0	0	MT622380, MT622442, MT635864
Con – 038	0	0	0	1	0	0	MT622420, MT622485, MT635899
Con – 039	0	0	0	1	0	0	MT622421, MT622486, MT635900
Con – 040	0	0	0	1	0	0	MT622375, MT622487, MT635864
Con – 041	0	1	0	0	0	0	MT622381, MT622452, MT635859
Con – 042	0	1	0	0	0	0	MT622371, MT622443, MT635867
Con – 043	0	0	0	0	1	0	MT622432, MT622493, MT635880
Con – 044	0	0	0	0	4	0	MT622395, MT622474, MT635884
Con – 045	0	0	0	0	10	2	MT622391, MT622462, MT635878
Con – 046	0	0	0	0	1	0	MT622433, MT622493, MT635880
Con – 047	0	0	0	0	1	0	MT622396, MT622494, MT635905

Table B-1 Continued

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Concatenated Sequences – Continued**							
Con – 048	0	0	0	0	1	0	MT622432, MT622493, MT635884
Con – 049	0	0	0	0	7	3	MT622391, MT622469, MT635878
Con – 050	0	0	0	0	1	0	MT622396, MT622495, MT635878
Con – 051	0	0	0	0	1	0	MT622434, MT622469, MT635878
Con – 052	0	0	0	0	1	0	MT622435, MT622452, MT635906
Con – 053	0	0	0	0	1	0	MT622436, MT622474, MT635884
Con – 054	0	0	0	0	1	0	MT622437, MT622469, MT635878
Con – 055	0	0	0	0	1	0	MT622395, MT622462, MT635889
Con – 056	0	0	0	0	1	0	MT622438, MT622469, MT635907
Con – 057	0	0	0	0	1	0	MT622439, MT622462, MT635878
Con – 058	0	0	0	0	1	0	MT622440, MT622469, MT635908
Con – 059	0	0	0	0	1	0	MT622395, MT622496, MT635884
Con – 060	0	0	0	0	1	0	MT622441, MT622462, MT635878
Con – 061	0	0	0	0	0	1	MT622392, MT622463, MT635879
Con – 062	0	0	0	0	0	3	MT622391, MT622464, MT635878
Con – 063	0	0	0	0	0	1	MT622393, MT622465, MT635880
Con – 064	0	0	0	0	0	2	MT622394, MT622452, MT635880
Con – 065	0	0	0	0	0	2	MT622395, MT622466, MT635878
Con – 066	0	0	0	0	0	1	MT622396, MT622452, MT635881
Con – 067	0	0	0	0	0	1	MT622397, MT622467, MT635878
Con – 068	0	0	0	0	0	1	MT622398, MT622468, MT635880
Con – 069	0	0	0	0	0	1	MT622399, MT622469, MT635882
Con – 070	0	0	0	0	0	1	MT622400, MT622470, MT635878
Con – 071	0	0	0	0	0	1	MT622401, MT622471, MT635878
Con – 072	0	0	0	0	0	1	MT622402, MT622464, MT635878
Con – 073	0	0	0	0	0	1	MT622403, MT622452, MT635883
Con – 074	0	0	0	0	0	1	MT622404, MT622464, MT635878

Table B-1 Continued

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Concatenated Sequences – Continued**							
Con – 075	0	0	0	0	0	1	MT622405, MT622464, MT635884
Con – 076	0	0	0	0	0	1	MT622393, MT622452, MT635880
Con – 077	0	0	0	0	0	1	MT622391, MT622472, MT635885
Con – 078	0	0	0	0	0	1	MT622391, MT622473, MT635886
Con – 079	0	0	0	0	0	1	MT622391, MT622474, MT635887
Con – 080	0	0	0	0	0	1	MT622406, MT622475, MT635888
Con – 081	0	0	0	0	0	1	MT622391, MT622462, MT635889
Con – 082	0	0	0	0	0	1	MT622407, MT622462, MT635878
Con – 083	0	0	0	0	0	1	MT622391, MT622476, MT635878
Con – 084	0	0	0	0	0	1	MT622395, MT622477, MT635884
Con – 085	0	1	0	0	0	0	MT622382, MT622453, MT635868
Con – 086	0	1	0	0	0	0	MT622383, MT622454, MT635869
Con – 087	0	1	0	0	0	0	MT622384, MT622455, MT635870
Con – 088	0	1	0	0	0	0	MT622385, MT622452, MT635871
Con – 089	0	1	0	0	0	0	MT622379, MT622456, MT635865
Con – 090	0	1	0	0	0	0	MT622386, MT622447, MT635872
Con – 091	0	1	0	0	0	0	MT622388, MT622458, MT635873
Con – 092	0	1	0	0	0	0	MT622389, MT622459, MT635874
Con – 093	0	1	0	0	0	0	MT622371, MT622443, MT635875
Con – 094	0	1	0	0	0	0	MT622371, MT622460, MT635876
Con – 095	0	1	0	1	0	0	MT622385, MT622452, MT635877
Con – 096	0	1	0	0	0	0	MT622390, MT622461, MT635859
Con – 097	0	0	0	1	0	0	MT622422, MT622488, MT635867
Con – 098	0	0	0	2	0	0	MT622420, MT622486, MT635899
Con – 099	0	0	0	2	0	0	MT622379, MT622489, MT635863
Con – 100	0	0	0	1	0	0	MT622423, MT622490, MT635859
Con – 101	0	0	0	1	0	0	MT622424, MT622457, MT635873

Table B-1 Continued

Haplotype	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs	Accession
***F. grandis* Concatenated Sequences – Continued**							
Con – 102	0	0	0	1	0	0	MT622425, MT622491, MT635901
Con – 103	0	0	0	1	0	0	MT622426, MT622488, MT635902
Con – 104	0	0	0	1	0	0	MT622427, MT622442, MT635859
Con – 105	0	0	0	1	0	0	MT622428, MT622461, MT635903
Con – 106	0	0	0	1	0	0	MT622429, MT622452, MT635877
Con – 107	0	0	0	1	0	0	MT622430, MT622488, MT635867
Con – 108	0	0	0	1	0	0	MT622431, MT622492, MT635900
Con – 109	0	0	0	1	0	0	MT622384, MT622455, MT635904

Haplotype	Pt. Mansfield	Matagorda	Sportsman's	Pt. Arthur	Venice	Ocean Springs	Accession
***P. pugio* 16sRNA**							
Ppug – 01	0	0	20	0	0	0	MT629892
Ppug – 02	20	0	0	0	5	6	MT629893
Ppug – 03	0	17	0	19	0	5	MT629894
Ppug – 04	0	1	0	0	0	0	MT629895
Ppug – 05	0	1	0	0	0	0	MT629896
Ppug – 06	0	1	0	0	0	0	MT629897
Ppug – 07	0	0	0	1	0	0	MT629898
Ppug – 08	0	0	0	0	2	0	MT629899
Ppug – 09	0	0	0	0	10	2	MT629900
Ppug – 10	0	0	0	0	2	0	MT629901
Ppug – 11	0	0	0	0	0	5	MT629902
Ppug – 12	0	0	0	0	0	1	MT629903
Ppug – 13	0	0	0	0	0	1	MT629904

Table B-1 Continued

Haplotype	South Padre Isle.	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Pt. Arthur	Accession
***P.marginata* COI**							
Pmar – 01	20	14	14	24	18	17	MT602510
Pmar – 02	0	5	6	0	0	0	MT602511
Pmar – 03	0	0	0	0	2	2	MT602512
Pmar – 04	0	0	0	0	1	1	MT602513

Table B-2 Molecular indices for individual markers of *F. grandis* by sample site. *N,* No. of Sequences; M, No. of haplotypes, *h,* haplotypic diversity; π, nucleotide diversity; SD, standard deviation; S, No. of segregating (polymorphic) sites; D, Tajima's D neutrality test with probability value (P).

Locus	Site	M	*h*(SD)	π (SD)	S	Ts	Tv	I/D
CR-1								
	Corpus Christi	11	0.849 (0.050)	0.0193 (0.011)	25	20	3	2
	Matagorda	13	0.954 (0.034)	0.0209 (0.012)	19	16	3	2
	Reitan Marsh	18	0.956 (0.022)	0.0242 (0.013)	31	28	2	2
	Sportsman's	21	0.977 (0.017)	0.0220 (0.012)	29	24	5	2
	Venice	13	0.757 (0.069)	0.0068 (0.004)	18	12	6	0
	Ocean Springs	17	0.843 (0.061)	0.0097 (0.006)	23	17	6	1
ND-2								
	Corpus Christi	11	0.895 (0.034)	0.0153 (0.009)	22	19	3	0
	Matagorda	13	0.954 (0.034)	0.0199 (0.011)	24	20	4	0
	Reitan Marsh	14	0.929 (0.025)	0.0187 (0.010)	23	22	1	0
	Sportsman's	17	0.960 (0.019)	0.0199 (0.011)	24	22	2	1
	Venice	8	0.768 (0.044)	0.0042 (0.003)	8	7	2	0
	Ocean Springs	17	0.928 (0.024)	0.0064 (0.004)	16	11	7	0
ND-5								
	Corpus Christi	14	0.935 (0.052)	0.017 (0.009)	21	19	1	1
	Matagorda	16	0.909 (0.040)	0.016 (0.009)	23	22	1	0
	Reitan Marsh	16	0.949 (0.025)	0.017 (0.009)	25	23	2	0
	Sportsman's	8	0.598 (0.084)	0.003 (0.002)	11	11	0	0
	Venice	12	0.722 (0.080)	0.004 (0.003)	15	15	0	0
	Ocean Springs	8	0.769 (0.059)	0.007 (0.004)	17	16	1	0

Table B-3. Values for pairwise F_{ST} for 336 bp of CR1 for Gulf Killifish. Significant values are in bold, with significance at $p < 0.05$ denoted by *, and significance at $p < 0.01$ denoted by **.

	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs
Corpus Christi						
Matagorda	**0.13048 ****					
Reitan Marsh	**0.12630 ****	- 0.02397				
Sportsman's	**0.16789 ****	- 0.28040	- 0.01095			
Venice	**0.55378 ****	**0.41388 ****	**0.37837 ****	**0.37176 ****		
Ocean Springs	**0.51352 ****	**0.36350 ****	**0.34385 ****	**0.33616 ****	- 0.00321	

Table B-4. Values for pairwise population comparisons for 336 bp of CR1 for Gulf Killifish. Slatkin's linearized F_{ST} is above the diagonal, and Reynold's Distance is below the diagonal.

	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs
Corpus Christi		0.15006	0.14456	0.20176	1.24103	1.05560
Matagorda	0.13981		0.00000	0.00000	0.70614	0.57108
Reitan Marsh	0.13502	0.00000		0.00000	0.60868	0.52404
Sportsman's	0.18379	0.00000	0.00000		0.59174	0.50638
Venice	0.80694	0.53423	0.47541	0.46483		0.00000
Ocean Springs	0.72057	0.45176	0.42136	0.40971	0.00000	

Table B-5. Values for pairwise F_{ST} for 344 bp of ND2 for Gulf Killifish. Significant values are in bold, with significance at $p < 0.05$ denoted by *, and significance at $p < 0.01$ denoted by **.

	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs
Corpus Christi						
Matagorda	**0.18510 ****					
Reitan Marsh	**0.11354 ****	0.00436				
Sportsman's	**0.16658 ****	- 0.01954	- 0.00196			
Venice	**0.67035 ****	**0.41758 ****	**0.49739 ****	**0.41995 ****		
Ocean Springs	**0.63384 ****	**0.36811 ****	**0.46049 ****	**0.38327 ****	**0.10165 ****	

Table B-6. Values for pairwise population comparisons for 344 bp of ND2 for Gulf Killifish. Slatkin's linearized F_{ST} is above the diagonal, and Reynold's Distance is below the diagonal.

	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs
Corpus Christi		0.22714	0.12808	0.19988	2.03356	1.73107
Matagorda	0.20469		0.00000	0.00000	0.71699	0.58256
Reitan Marsh	0.12052	0.00000		0.00000	0.98963	0.85352
Sportsman's	0.18222	0.00000	0.00000		0.72400	0.62147
Venice	1.10974	0.54057	0.68795	0.54465		0.11315
Ocean Springs	1.00469	0.45905	0.61709	0.48333	0.10719	

Table B-7. Values for pairwise F_{ST} for 397 bp of ND5 for Gulf Killifish. Significant values are in bold, with significance at $p < 0.05$ denoted by *, and significance at $p < 0.01$ denoted by **.

	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs
Corpus Christi						
Matagorda	**0.26557 ****					
Reitan Marsh	**0.21357 ****	- 0.02293				
Sportsman's	**0.23144 ****	- 0.35870	- 0.02046			
Venice	**0.78959 ****	**0.43453 ****	**0.42580 ****	**0.40329 ****		
Ocean Springs	**0.75483 ****	**0.38376 ****	**0.38567 ****	**0.36170 ****	0.02557	

Table B-8. Values for pairwise population comparisons for 397 bp of ND5 for Gulf Killifish. Slatkin's linearized F_{ST} is above the diagonal, and Reynold's Distance is below the diagonal.

	Corpus Christi	Matagorda	Reitan Marsh	Sportsman's	Venice	Ocean Springs
Corpus Christi		0.36160	0.27157	0.30114	3.75272	3.07884
Matagorda	0.30866		0.00000	0.00000	0.76843	0.62275
Reitan Marsh	0.24025	0.00000		0.00000	0.74154	0.62779
Sportsman's	0.26324	0.00000	0.00000		0.67586	0.56666
Venice	1.55872	0.57009	0.55477	0.51633		0.02625
Ocean Springs	1.40581	0.48412	0.48723	0.44894	0.02591	

APPENDIX C

SUPPLEMENTAL MATERIAL FOR CHAPTER IV

Table C-1 Haplotype frequencies by sampling location for Gulf Killifish (*F. grandis*) for 366 bp of Control Region 1 (CR1), 344 bp of Nitrogen Dehydrogenase Subunit 2 (ND-2), 397 bp of Nitrogen Dehydrogenase Subunit 5 (ND-5) and 1077 bp of Concatenated sequence. SMR: Sportsman's Road, 11MY: 11 Mile – Young, 11MM: 11 Mile – Medium, 11MO: 11 Mile – Old, JBY: Jamaica Beach – Young, JBM: Jamaica Beach – Medium, JBO: Jamaica Beach – Old.

Haplotype	SMR	11MY	11MM	11MO	JBY	JBM	JBO	Accession
***F. grandis* Control Region 1**								
CR1 – 01	0	0	1	0	0	0	0	MT655753
CR1 – 02	3	0	2	2	1	2	0	MT655754
CR1 – 03	0	0	1	1	1	0	2	MT655755
CR1 – 04	0	1	1	0	0	0	1	MT655756
CR1 – 05	0	0	1	0	1	0	0	MT655757
CR1 – 06	2	4	3	1	2	1	4	MT655758
CR1 – 07	1	0	3	3	3	0	2	MT655759
CR1 – 08	0	0	1	0	0	0	0	MT655760
CR1 – 09	0	0	1	0	0	0	0	MT655761
CR1 – 10	0	0	1	0	0	0	2	MT655762
CR1 – 11	1	2	1	3	4	3	0	MT655763
CR1 – 12	0	1	1	2	1	0	0	MT655764
CR1 – 13	0	0	1	0	0	0	0	MT655765
CR1 – 14	0	1	1	0	1	0	0	MT655766
CR1 – 15	0	0	1	0	0	0	0	MT655767
CR1 – 16	0	0	0	1	0	0	0	MT655768
CR1 – 17	0	0	0	1	0	0	0	MT655769
CR1 – 18	0	1	0	2	1	1	0	MT655770
CR1 – 19	1	1	0	1	0	0	2	MT655771

Table C-1 Continued

Haplotype	SMR	11MY	11MM	11MO	JBY	JBM	JBO	Accession
***F. grandis* Control Region 1 Continued**								
CR1 – 20	0	0	0	1	0	0	0	MT655772
CR1 – 21	0	0	0	1	0	0	0	MT655773
CR1 – 22	0	0	0	1	0	0	0	MT655774
CR1 – 23	0	1	0	1	1	1	0	MT655775
CR1 – 24	2	0	0	1	2	0	0	MT655776
CR1 – 25	0	0	0	1	0	0	0	MT655777
CR1 – 26	0	0	0	1	0	0	0	MT655778
CR1 – 27	0	1	0	0	0	0	0	MT655779
CR1 – 28	0	1	0	0	0	0	0	MT655780
CR1 – 29	0	1	0	0	0	0	0	MT655781
CR1 – 30	0	1	0	0	1	0	0	MT655782
CR1 – 31	0	1	0	0	0	0	0	MT655783
CR1 – 32	0	1	0	0	0	0	0	MT655784
CR1 – 33	1	1	0	0	0	0	0	MT655785
CR1 – 34	0	1	0	0	0	3	0	MT655786
CR1 – 35	0	1	0	0	0	0	0	MT655787
CR1 – 36	0	1	0	0	0	0	0	MT655788
CR1 – 37	0	1	0	0	0	0	0	MT655789
CR1 – 38	0	1	0	0	0	0	0	MT655790
CR1 – 39	0	0	0	0	0	1	0	MT655791
CR1 – 40	0	0	0	0	0	1	0	MT655792
CR1 – 41	1	0	0	0	0	1	0	MT655793
CR1 – 42	0	0	0	0	0	1	0	MT655794
CR1 – 43	0	0	0	0	0	1	0	MT655795
CR1 – 44	0	0	0	0	0	1	0	MT655796
CR1 – 45	0	0	0	0	0	2	0	MT655797
CR1 – 46	0	0	0	0	0	1	0	MT655798
CR1 – 47	1	0	0	0	0	1	0	MT655799

Table C-1 Continued

Haplotype	SMR	11MY	11MM	11MO	JBY	JBM	JBO	Accession
***F. grandis* Control Region 1**								
CR1 – 48	1	0	0	0	1	0	2	MT655800
CR1 – 49	0	0	0	0	0	0	1	MT655801
CR1 – 50	0	0	0	0	0	0	1	MT655802
CR1 – 51	0	0	0	0	0	0	1	MT655803
CR1 – 52	0	0	0	0	0	0	1	MT655804
CR1 – 53	0	0	0	0	0	0	1	MT655805
CR1 – 54	1	0	0	0	1	0	0	MT655806
CR1 – 55	0	0	0	0	1	0	0	MT655807
CR1 – 56	0	0	0	0	1	0	0	MT655808
CR1 – 57	0	0	0	0	1	0	0	MT655809
CR1 – 58	3	0	0	0	0	0	0	MT655810
CR1 – 59	1	0	0	0	0	0	0	MT655811
CR1 – 60	1	0	0	0	0	0	0	MT655812
CR1 – 61	1	0	0	0	0	0	0	MT655813
CR1 – 62	1	0	0	0	0	0	0	MT655814
CR1 – 63	1	0	0	0	0	0	0	MT655815
CR1 – 64	1	0	0	0	0	0	0	MT655816
CR1 – 65	1	0	0	0	0	0	0	MT655817
CR1 – 66	1	0	0	0	0	0	0	MT655818
CR1 – 67	1	0	0	0	0	0	0	MT655819
***F. grandis* Nitrogen Dehydrogenase Subunit 2**								
ND2 – 01	0	0	1	1	0	1	0	MT655820
ND2 – 02	1	1	2	1	1	0	1	MT655821
ND2 – 03	3	6	6	6	5	5	7	MT655822
ND2 – 04	1	1	2	2	0	0	2	MT655823
ND2 – 05	1	3	1	0	2	0	0	MT655824
ND2 – 06	2	0	3	2	4	2	3	MT655825
ND2 – 07	0	0	1	0	0	0	0	MT655826

Table C-1 Continued

Haplotype	SMR	11MY	11MM	11MO	JBY	JBM	JBO	Accession
F. grandis **Nitrogen Dehydrogenase Subunit 2 Continued**								
ND2 – 08	1	1	1	0	0	1	2	MT655827
ND2 – 09	3	0	1	2	1	1	0	MT655828
ND2 – 10	1	2	2	2	3	1	1	MT655829
ND2 – 11	0	1	1	2	1	0	0	MT655830
ND2 – 12	0	1	0	1	1	0	1	MT655831
ND2 – 13	0	1	0	2	1	2	0	MT655832
ND2 – 14	0	0	0	1	0	0	0	MT655833
ND2 – 15	0	1	0	1	0	1	0	MT655834
ND2 – 16	2	0	0	1	2	0	1	MT655835
ND2 – 17	1	1	0	0	0	0	0	MT655836
ND2 – 18	0	1	0	0	0	0	0	MT655837
ND2 – 19	1	1	0	0	0	0	0	MT655838
ND2 – 20	0	1	0	0	0	3	1	MT655839
ND2 – 21	0	1	0	0	0	0	0	MT655840
ND2 – 22	0	1	0	0	1	0	0	MT655841
ND2 – 23	0	0	0	0	0	0	1	MT655842
ND2 – 24	1	0	0	0	0	1	0	MT655843
ND2 – 25	0	0	0	0	0	1	0	MT655844
ND2 – 26	3	0	0	0	0	1	0	MT655845
ND2 – 27	0	0	0	0	0	1	0	MT655846
ND2 – 28	0	0	0	0	1	0	0	MT655847
ND2 – 29	0	0	0	0	1	0	0	MT655848
ND2 – 30	3	0	0	0	0	0	0	MT655849
ND2 – 31	1	0	0	0	0	0	0	MT655850
ND2 – 32	1	0	0	0	0	0	0	MT655851
ND2 – 33	1	0	0	0	0	0	0	MT655852

Table C-1 Continued

Haplotype	SMR	11MY	11MM	11MO	JBY	JBM	JBO	Accession
***F. grandis* Nitrogen Dehydrogenase Subunit 5**								
ND5 – 01	0	0	1	1	0	1	1	MT655853
ND5 – 02	4	7	6	5	6	3	3	MT655854
ND5 – 03	0	0	1	1	1	0	3	MT655855
ND5 – 04	0	0	2	0	0	0	0	MT655856
ND5 – 05	1	1	3	3	4	1	4	MT655857
ND5 – 06	2	1	1	0	1	0	2	MT655858
ND5 – 07	0	0	1	0	0	0	0	MT655859
ND5 – 08	0	1	1	0	0	1	1	MT655860
ND5 – 09	3	0	1	2	1	2	0	MT655861
ND5 – 10	1	3	1	0	0	0	0	MT655862
ND5 – 11	1	3	1	2	1	4	1	MT655863
ND5 – 12	0	1	1	0	1	0	1	MT655864
ND5 – 13	0	2	1	1	1	2	0	MT655865
ND5 – 14	0	0	0	1	0	0	0	MT655866
ND5 – 15	0	0	0	1	0	1	0	MT655867
ND5 – 16	1	1	0	1	1	0	1	MT655868
ND5 – 17	0	0	0	1	0	0	0	MT655869
ND5 – 18	0	0	0	1	0	0	0	MT655870
ND5 – 19	2	0	0	1	2	0	2	MT655871
ND5 – 20	0	0	0	1	0	0	0	MT655872
ND5 – 21	0	0	0	1	0	0	0	MT655873
ND5 – 22	0	0	0	1	0	0	0	MT655874
ND5 – 23	0	1	0	0	0	0	0	MT655875
ND5 – 24	0	1	0	0	0	0	0	MT655876
ND5 – 25	0	1	0	0	0	0	0	MT655877
ND5 – 26	0	1	0	0	1	0	0	MT655878
ND5 – 27	0	0	0	0	0	1	0	MT655879
ND5 – 28	1	0	0	0	0	1	0	MT655880

Table C-1 Continued

Haplotype	SMR	11MY	11MM	11MO	JBY	JBM	JBO	Accession
***F. grandis* Nitrogen Dehydrogenase Subunit 5 Continued**								
ND5 – 29	0	0	0	0	0	1	0	MT655881
ND5 – 30	0	0	0	0	0	1	0	MT655882
ND5 – 31	0	0	0	0	0	1	0	MT655883
ND5 – 32	1	0	0	0	0	1	0	MT655884
ND5 – 33	0	0	0	0	0	0	1	MT655885
ND5 – 34	0	0	0	0	0	0	1	MT655886
ND5 – 35	2	0	0	0	1	0	0	MT655887
ND5 – 36	0	0	0	0	1	0	0	MT655888
ND5 – 37	0	0	0	0	1	0	0	MT655889
ND5 – 38	0	0	0	0	1	0	0	MT655890
ND5 – 39	3	0	0	0	0	0	0	MT655891
ND5 – 40	2	0	0	0	0	0	0	MT655892
ND5 – 41	1	0	0	0	0	0	0	MT655893
ND5 – 42	1	0	0	0	0	0	0	MT655894
ND5 – 43	1	0	0	0	0	0	0	MT655895
***F. grandis* Concatenated Sequences**								
Concat – 01	0	0	1	0	0	0	0	MT655753, MT655820, MT655853
Concat – 02	0	0	1	0	0	0	0	MT655754, MT655821, MT655854
Concat – 03	0	0	1	1	1	0	2	MT655755, MT655822, MT655855
Concat – 04	0	1	1	0	0	0	1	MT655756, MT655823, MT655854
Concat – 05	0	0	1	0	0	0	0	MT655757, MT655824, MT655854
Concat – 06	0	0	2	0	0	0	0	MT655758, MT655822, MT655856
Concat – 07	1	0	3	2	3	0	2	MT655759, MT655825, MT655857
Concat – 08	0	0	1	0	1	0	1	MT655760, MT655822, MT655858
Concat – 09	0	0	1	0	0	0	0	MT655761, MT655826, MT655859
Concat – 10	0	1	1	0	0	1	0	MT655758, MT655827, MT655860
Concat – 11	0	0	1	0	0	0	0	MT655762, MT655823, MT655854
Concat – 12	3	0	1	2	1	1	0	MT655754, MT655828, MT655861

Table C-1 Continued

Haplotype	SMR	11MY	11MM	11MO	JBY	JBM	JBO	Accession
F. grandis **Concatenated Sequences Continued**								
Concat – 13	1	2	1	2	3	1	1	MT655763, MT655829, MT655854
Concat – 14	0	0	1	0	0	0	0	MT655763, MT655822, MT655862
Concat – 15	0	1	1	2	1	0	0	MT655764, MT655830, MT655863
Concat – 16	0	0	1	0	0	0	0	MT655765, MT655829, MT655854
Concat – 17	0	1	1	0	1	0	0	MT655766, MT655821, MT655864
Concat – 18	0	1	1	1	1	1	0	MT655767, MT655822, MT655865
Concat – 19	0	0	0	1	0	0	0	MT655767, MT655822, MT655865
Concat – 20	0	0	0	1	0	0	0	MT655759, MT655822, MT655866
Concat – 21	0	0	0	1	0	1	0	MT655768, MT655822, MT655867
Concat – 22	0	1	0	1	0	0	0	MT655769, MT655822, MT655867
Concat – 23	0	0	0	1	0	0	0	MT655772, MT655832, MT655854
Concat – 24	0	0	0	1	0	0	0	MT655773, MT655823, MT655869
Concat – 25	0	0	0	1	0	0	0	MT655774, MT655833, MT655870
Concat – 26	0	1	0	1	0	1	0	MT655775, MT655834, MT655857
Concat – 27	2	0	0	1	1	0	1	MT655776, MT655835, MT655871
Concat – 28	0	0	0	1	1	2	0	MT655763, MT655832, MT655854
Concat – 29	0	0	0	1	0	0	0	MT655758, MT655822, MT655872
Concat – 30	0	0	0	1	0	0	0	MT655777, MT655823, MT655873
Concat – 31	0	0	0	1	0	0	0	MT655770, MT655822, MT655874
Concat – 32	0	0	0	1	0	0	0	MT655778, MT655821, MT655854
Concat – 33	0	1	0	0	0	0	0	MT655779, MT655824, MT655854
Concat – 34	0	1	0	0	0	0	0	MT655780, MT655836, MT655875
Concat – 35	0	1	0	0	0	0	0	MT655781, MT655837, MT655863
Concat – 36	0	1	0	0	1	0	0	MT655782, MT655824, MT655854
Concat – 37	0	1	0	0	0	0	0	MT655783, MT655832, MT655854
Concat – 38	0	1	0	0	0	0	0	MT655784, MT655824, MT655854
Concat – 39	1	1	0	0	0	0	0	MT655785, MT655838, MT655858
Concat – 40	1	3	0	0	0	0	0	MT655758, MT655822, MT655862

Table C-1 Continued

Haplotype	SMR	11MY	11MM	11MO	JBY	JBM	JBO	Accession
F. grandis **Concatenated Sequences Continued**								
Concat – 41	0	1	0	0	0	3	0	MT655786, MT655839, MT655863
Concat – 42	0	1	0	0	0	0	0	MT655787, MT655822, MT655876
Concat – 43	0	1	0	0	0	0	0	MT655788, MT655840, MT655877
Concat – 44	0	1	0	0	0	0	0	MT655789, MT655822, MT655865
Concat – 45	0	1	0	0	0	0	0	MT655790, MT655841, MT655878
Concat – 46	1	0	0	0	0	1	0	MT655791, MT655825, MT655863
Concat – 47	0	0	0	0	0	1	0	MT655792, MT655825, MT655879
Concat – 48	1	0	0	0	0	1	0	MT655793, MT655843, MT655880
Concat – 49	0	0	0	0	0	1	0	MT655794, MT655822, MT655881
Concat – 50	0	0	0	0	0	1	0	MT655795, MT655822, MT655865
Concat – 51	0	0	0	0	0	1	0	MT655797, MT655822, MT655867
Concat – 52	0	0	0	0	0	1	0	MT655798, MT655822, MT655882
Concat – 53	0	0	0	0	0	1	0	MT655797, MT655844, MT655883
Concat – 54	1	0	0	0	0	1	0	MT655799, MT655845, MT655884
Concat – 55	0	0	0	0	0	1	0	MT655754, MT655846, MT655861
Concat – 56	0	0	0	0	1	0	1	MT655758, MT655822, MT655871
Concat – 57	0	0	0	0	0	0	1	MT655762, MT655822, MT655885
Concat – 58	0	0	0	0	0	0	1	MT655758, MT655821, MT655864
Concat – 59	0	0	0	0	0	0	1	MT655758, MT655827, MT655858
Concat – 60	0	0	0	0	0	0	1	MT655771, MT655831, MT655855
Concat – 61	0	0	0	0	0	0	1	MT655771, MT655827, MT655857
Concat – 62	0	0	0	0	0	0	1	MT655800, MT655822, MT655860
Concat – 63	0	0	0	0	0	0	1	MT655801, MT655825, MT655857
Concat – 64	0	0	0	0	0	0	1	MT655762, MT655823, MT655854
Concat – 65	0	0	0	0	0	0	1	MT655802, MT655839, MT655868
Concat – 66	0	0	0	0	0	0	1	MT655803, MT655842, MT655863
Concat – 67	0	0	0	0	0	0	1	MT655800, MT655822, MT655858
Concat – 68	0	0	0	0	0	0	1	MT655804, MT655822, MT655886

Table C-1 Continued

Haplotype	SMR	11MY	11MM	11MO	JBY	JBM	JBO	Accession
F. grandis **Concatenated Sequences Continued**								
Concat – 69	0	0	0	0	1	0	0	MT655758, MT655841, MT655878
Concat – 70	1	0	0	0	1	0	0	MT655806, MT655822, MT655887
Concat – 71	0	0	0	0	1	0	0	MT655807, MT655847, MT655888
Concat – 72	0	0	0	0	1	0	0	MT655808, MT655831, MT655868
Concat – 73	0	0	0	0	1	0	0	MT655776, MT655835, MT655889
Concat – 74	0	0	0	0	1	0	0	MT655757, MT655824, MT655854
Concat – 75	0	0	0	0	1	0	0	MT655775, MT655825, MT655857
Concat – 76	0	0	0	0	1	0	0	MT655809, MT655848, MT655890
Concat – 77	1	0	0	0	0	0	0	MT655810, MT655827, MT655891
Concat – 78	1	0	0	0	0	0	0	MT655800, MT655849, MT655858
Concat – 79	1	0	0	0	0	0	0	MT655771, MT655850, MT655868
Concat – 80	1	0	0	0	0	0	0	MT655811, MT655845, MT655892
Concat – 81	1	0	0	0	0	0	0	MT655812, MT655821, MT655854
Concat – 82	2	0	0	0	0	0	0	MT655810, MT655849, MT655891
Concat – 83	1	0	0	0	0	0	0	MT655813, MT655836, MT655854
Concat – 84	1	0	0	0	0	0	0	MT655815, MT655834, MT655893
Concat – 85	1	0	0	0	0	0	0	MT655816, MT655823, MT655854
Concat – 86	1	0	0	0	0	0	0	MT655758, MT655852, MT655894
Concat – 87	1	0	0	0	0	0	0	MT655817, MT655824, MT655895
Concat – 88	1	0	0	0	0	0	0	MT655818, MT655845, MT655892
Concat – 89	1	0	0	0	0	0	0	MT655819, MT655822, MT655887

Table C-2. Molecular indices for Gulf Killifish mtDNA sequences by marker and sample location. M, No. of haplotypes, *h,* haplotypic diversity; π, nucleotide diversity; SD, standard deviation; S, no. of segregating (polymorphic) sites; Ts, no. of transitions; Tv, no. of transversions; I/D, no. of insertions and/or deletions.

Marker	Location	N	M	*h*(SD)	π (SD)	S	Ts	Tv	I/D
CR-1	SMR	27	21	0.977 (0.017)	0.022 (0.012)	29	24	5	2
	11M-Y	24	20	0.975 (0.024)	0.025 (0.013)	33	28	5	2
	11M-M	21	15	0.962 (0.026)	0.023 (0.012)	24	18	5	2
	11M-O	24	17	0.967 (0.021)	0.025 (0.013)	29	25	4	2
	JB-Y	24	17	0.960 (0.025)	0.024 (0.013)	32	26	5	2
	JB-M	21	15	0.962 (0.026)	0.023 (0.013)	28	24	3	2
	JB-O	20	12	0.942 (0.032)	0.022 (0.012)	25	21	2	2
ND2	SMR	27	17	0.960 (0.019)	0.020 (0.011)	25	23	2	1
	11M-Y	24	16	0.931 (0.040)	0.019 (0.010)	25	24	1	0
	11M-M	21	11	0.900 (0.047)	0.018 (0.010)	20	19	1	0
	11M-O	24	13	0.924 (0.037)	0.021 (0.011)	23	23	1	0
	JB-Y	24	13	0.924 (0.032)	0.019 (0.010)	22	22	1	0
	JB-M	21	13	0.927 (0.039)	0.019 (0.010)	24	23	1	0
	JB-O	20	10	0.863 (0.063)	0.014 (0.008)	18	17	2	0
ND5	SMR	27	16	0.954 (0.021)	0.018 (0.010)	26	23	3	0
	11M-Y	24	13	0.899 (0.046)	0.017 (0.010)	25	23	2	0
	11M-M	21	13	0.910 (0.049)	0.017 (0.009)	23	21	2	0
	11M-O	24	16	0.946 (0.031)	0.018 (0.010)	25	23	2	0
	JB-Y	24	15	0.920 (0.040)	0.017 (0.009)	22	20	2	0
	JB-M	21	14	0.948 (0.031)	0.018 (0.010)	27	25	2	0
	JB-O	20	11	0.926 (0.034)	0.015 (0.008)	18	16	2	0

Table C-3. Table of pairwise comparisons for mtDNA sequences of CR1, ND2, and ND5 for Gulf Killifish. Pairwise FST is above the diagonal, and z-scores from Salicru χ^2 test for pairwise comparisons of haplotypic diversity are below the diagonal. No values were significant at $p < 0.05$.

		SMR	11M-Y	11M-M	11M-O	JB-Y	JB-M	JB-O
CR-1	SMR		-0.0202	-0.0195	-0.0126	-0.0188	0.0307	0.0428
	11M-Y	0.0680		-0.0298	-0.0248	-0.0341	-0.0171	0.0062
	11M-M	0.4829	0.3674		-0.0376	-0.0387	0.0002	0.0144
	11M-O	0.3701	0.2509	-0.1496		-0.0303	-0.0051	0.0354
	JB-Y	0.5623	0.4328	0.0554	0.2144		-0.0095	0.0073
	JB-M	0.4829	0.3674	0.0000	0.1496	-0.0554		0.0025
	JB-O	0.9659	0.8250	0.4851	0.6532	0.4433	0.4851	
ND2	SMR		-0.0245	-0.0299	-0.0222	-0.0234	-0.0077	0.0305
	11M-Y	0.6549		-0.0377	-0.0249	-0.0037	-0.0226	0.0036
	11M-M	1.1835	0.5023		-0.0313	-0.0371	-0.0243	0.0056
	11M-O	0.8655	0.1285	-0.4012		-0.0246	0.0061	0.0537
	JB-Y	0.9673	0.1367	-0.4221	0.0000		-0.0227	0.0017
	JB-M	0.7607	0.0716	-0.4421	-0.0058	-0.0595		-0.0257
	JB-O	1.4741	0.9112	0.4707	0.8349	0.8633	0.8638	
ND5	SMR		-0.0127	-0.0083	-0.0152	-0.0120	0.0035	0.0691
	11M-Y	1.0877		-0.0329	-0.0229	-0.0287	-0.0273	0.0193
	11M-M	0.8254	-0.1637		-0.0317	-0.0402	-0.0226	-0.0006
	11M-O	0.2137	-0.8473	-0.6209		-0.0267	0.0010	0.0473
	JB-Y	0.7526	-0.3445	-0.1581	0.5138		-0.0214	0.0007
	JB-M	0.1602	-0.8833	-0.6554	-0.0456	-0.5533		-0.0095
	JB-O	0.7007	-0.4720	-0.2683	0.4347	-0.1143	0.4781	

Table C-4. Pairwise values of Reynold's distance for mtDNA sequences of CR1, ND2, ND5 and Concatenated sequences for Gulf Killifish.

		SMR	11M-Y	11M-M	11M-O	JB-Y	JB-M
CR-1	11M-Y	0.00000					
	11M-M	0.00000	0.00000				
	11M-O	0.00000	0.00000	0.00000			
	JB-Y	0.03113	0.00000	0.00000	0.00020		
	JB-M	0.00000	0.00000	0.00000	0.00000	0.00513	
	JB-O	0.04375	0.00621	0.00735	0.01450	0.00252	0.03606
ND-2	11M-Y	0.00000					
	11M-M	0.00000	0.00000				
	11M-O	0.00000	0.00000	0.00000			
	JB-Y	0.00000	0.00000	0.00000	0.00000		
	JB-M	0.00000	0.00000	0.00000	0.00000	0.00609	
	JB-O	0.03096	0.00362	0.00186	0.00565	0.00000	0.05521
ND-5	11M-Y	0.00000					
	11M-M	0.00000	0.00000				
	11M-O	0.00000	0.00000	0.00000			
	JB-Y	0.00352	0.00000	0.00000	0.00000		
	JB-M	0.00000	0.00000	0.00000	0.00000	0.00102	
	JB-O	0.07158	0.01947	0.00072	0.00000	0.00000	0.04846
Concatenated	11M-Y	0.00000					
	11M-M	0.00000	0.00000				
	11M-O	0.00000	0.00000	0.00000			
	JB-Y	0.00957	0.00000	0.00000	0.00000		
	JB-M	0.00000	0.00000	0.00000	0.00000	0.00359	
	JB-O	0.5361	0.01386	0.00814	0.01213	0.00000	0.05283

Table C-5. AMOVA for CR1 sequences of Gulf Killifish, grouped by distance from reference. Groups consist of 1) SMR, 2) all three 11M marshes, and 3) all three JB marshes. P-values for fixation indices are based on significance tests with 1023 permutations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	2	9.026	0.04018 Va	1.02
Among Populations Within Groups	4	10.456	-0.06021 Vb	-1.53
Within Populations	154	609.144	3.95548 Vc	100.51
Total	160	628.626	3.93545	
Fixation Indices			**P-values (≥)**	
	F_{ST}:	-0.00509	0.67644 +/- 0.016	
	F_{SC}:	-0.01546	0.82991 +/- 0.010	
	F_{CT}:	0.010210	0.13587 +/- 0.011	

Table C-6. AMOVA for ND2 sequences, grouped by distance from reference. Groups consist of 1) SMR, 2) all three 11M marshes, and 3) all three JB marshes. P-values for fixation indices are based on significance tests with 1023 permutations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	2	6.661	0.03911 Va	1.24
Among Populations Within Groups	4	6.062	-0.07493 Vb	-2.38
Within Populations	154	490.479	3.18493 Vc	101.14
Total	160	503.202		
Fixation Indices			**P-values (≥)**	
	F_{ST}:	-0.01137	0.81329 +/- 0.014	
	F_{SC}:	-0.02409	0.90811 +/- 0.009	
	F_{CT}:	0.01242	0.04497 +/- 0.007	

Table C-7. AMOVA for ND5 sequences, grouped by distance from reference. Groups consist of 1) SMR, 2) all three 11M marshes, and 3) all three JB marshes. P-values for fixation indices are based on significance tests with 1023 permutations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	2	9.002	0.05504 Va	1.63
Among Populations Within Groups	4	7.481	-0.06795 Vb	-2.02
Within Populations	154	521.14	3.38403 Vc	100.38
Total	160	537.623	3.37112	
Fixation Indices			**P-values (≥)**	
	F_{ST}:	-0.00383	0.61877 +/- 0.016	
	F_{SC}:	-0.02049	0.84066 +/- 0.013	
	F_{CT}:	0.01633	0.07136 +/- 0.008	

Table C-8. AMOVA for CR1 sequences, grouped by age since restoration. Groups consist of 1) SMR, 2) 11M-Y and JB-Y, 3) 11M-M and JB-M, and 4) 11M-O and JB-O. P-values for fixation indices are based on significance tests with 1023 permutations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	3	7.557	-0.3668 Va	-0.94
Among Populations Within Groups	3	11.925	0.00088 Vb	0.02
Within Populations	154	609.144	3.95548 Vb	100.91
Total	160	628.626	3.91968	
Fixation Indices			**P-values (≥)**	
	F_{ST}:	-0.00913	0.64027 +/- 0.013	
	F_{SC}:	0.00022	0.42717 +/- 0.016	
	F_{CT}:	-0.00936	0.78495 +/- 0.013	

Table C-9. AMOVA for ND2 sequences, grouped by age since restoration. Groups consist of 1) SMR, 2) 11M-Y and JB-Y, 3) 11M-M and JB-M, and 4) 11M-O and JB-O. P-values for fixation indices are based on significance tests with 1023 permutations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	3	3.616	-0.04583 Va	-1.46
Among Populations Within Groups	3	9.107	-0.00671 Vb	-0.21
Within Populations	154	490.479	3.18493 Vc	101.68
Total	160	503.202	3.13239	
Fixation Indices			**P-values (≥)**	
	F_{ST}:	-0.01677	0.81329 +/- 0.014	
	F_{SC}:	-0.00211	0.41349 +/- 0.016	
	F_{CT}:	-0.01463	0.87195 +/- 0.009	

Table C-10. AMOVA for ND5 sequences, grouped by age since restoration. Groups consist of 1) SMR, 2) 11M-Y and JB-Y, 3) 11M-M and JB-M, and 4) 11M-O and JB-O. P-values for fixation indices are based on significance tests with 1023 permutations.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Among Groups	3	6.796	-0.02402 Va	-0.72
Among Populations Within Groups	3	9.687	-0.00696 Vb	-0.21
Within Populations	154	521.14	3.38403 Vc	100.92
Total	160	537.623	3.35305	
Fixation Indices			**P-values (≥)**	
	F_{ST}:	-0.00924	0.64321 +/- 0.013	
	F_{SC}:	-0.00206	0.43011 +/- 0.017	
	F_{CT}:	-0.00716	0.74389 +/- 0.016	

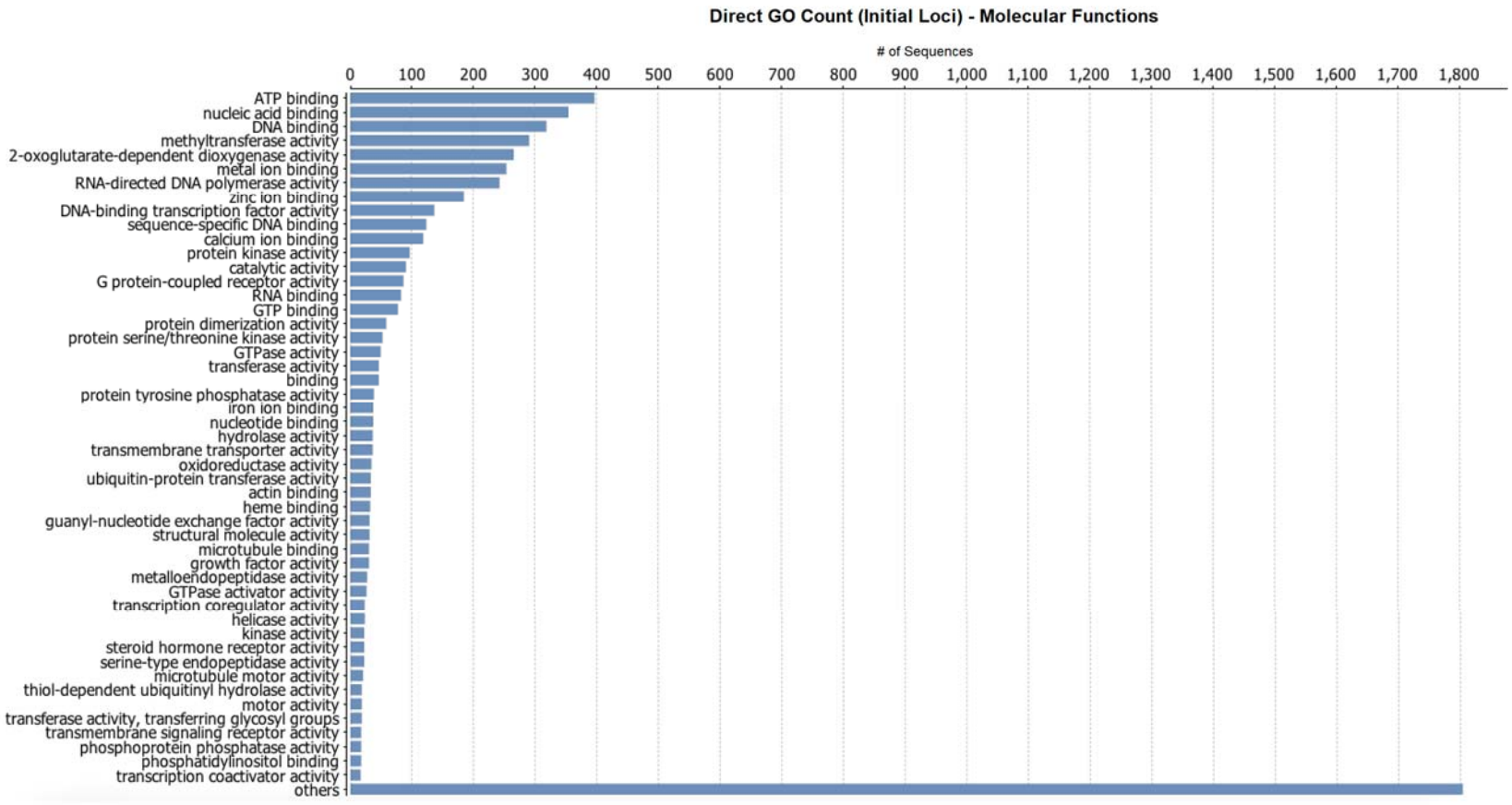

Figure C-1. Gene Ontology (GO) Terms related to Molecular Functions for 4,613 successfully annotated sequences from the initial ddRAD loci.

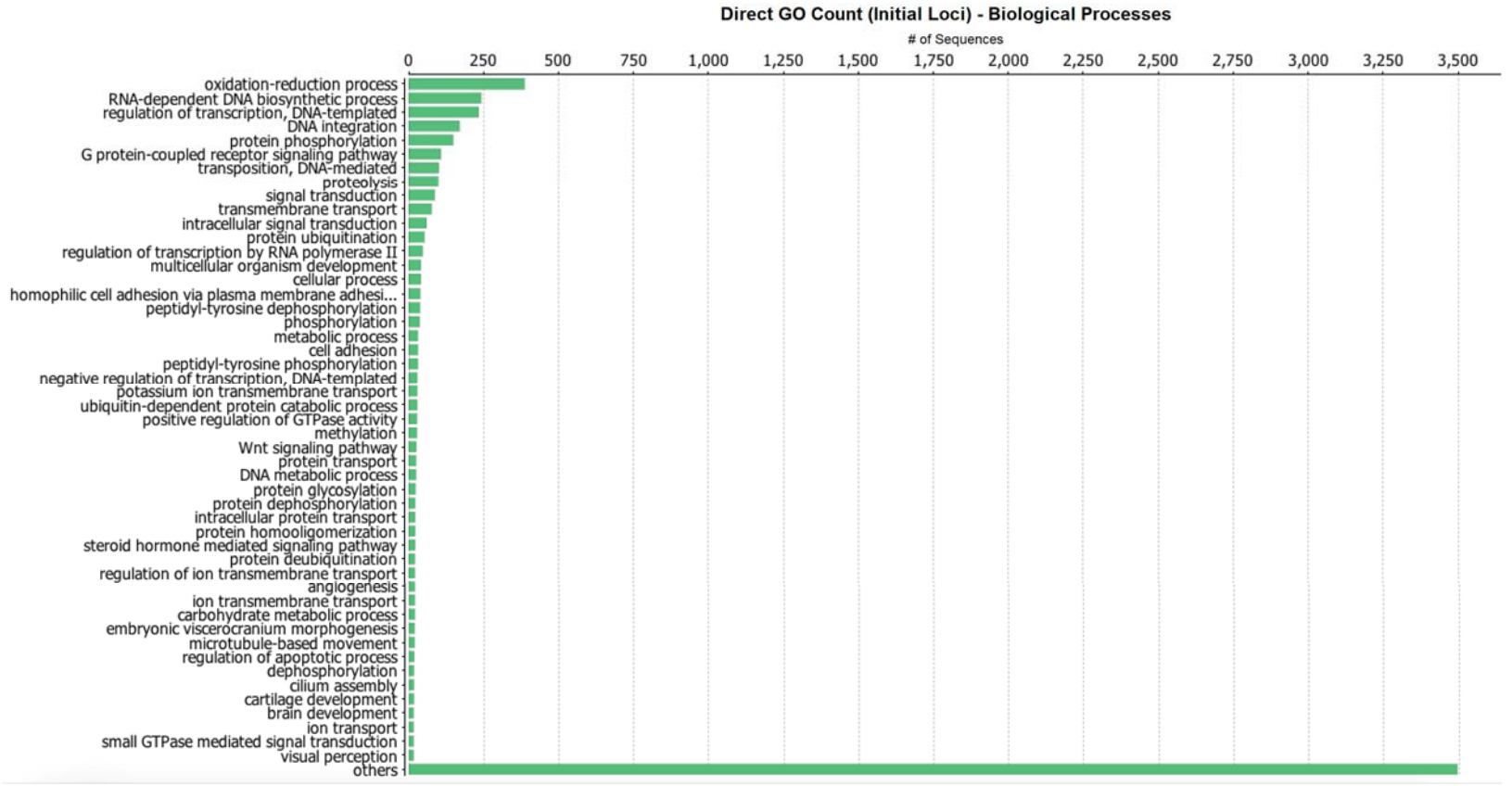

Figure C-2. Gene Ontology (GO) Terms related to Biological Processes for 4,613 successfully annotated sequences from the initial ddRAD loci.

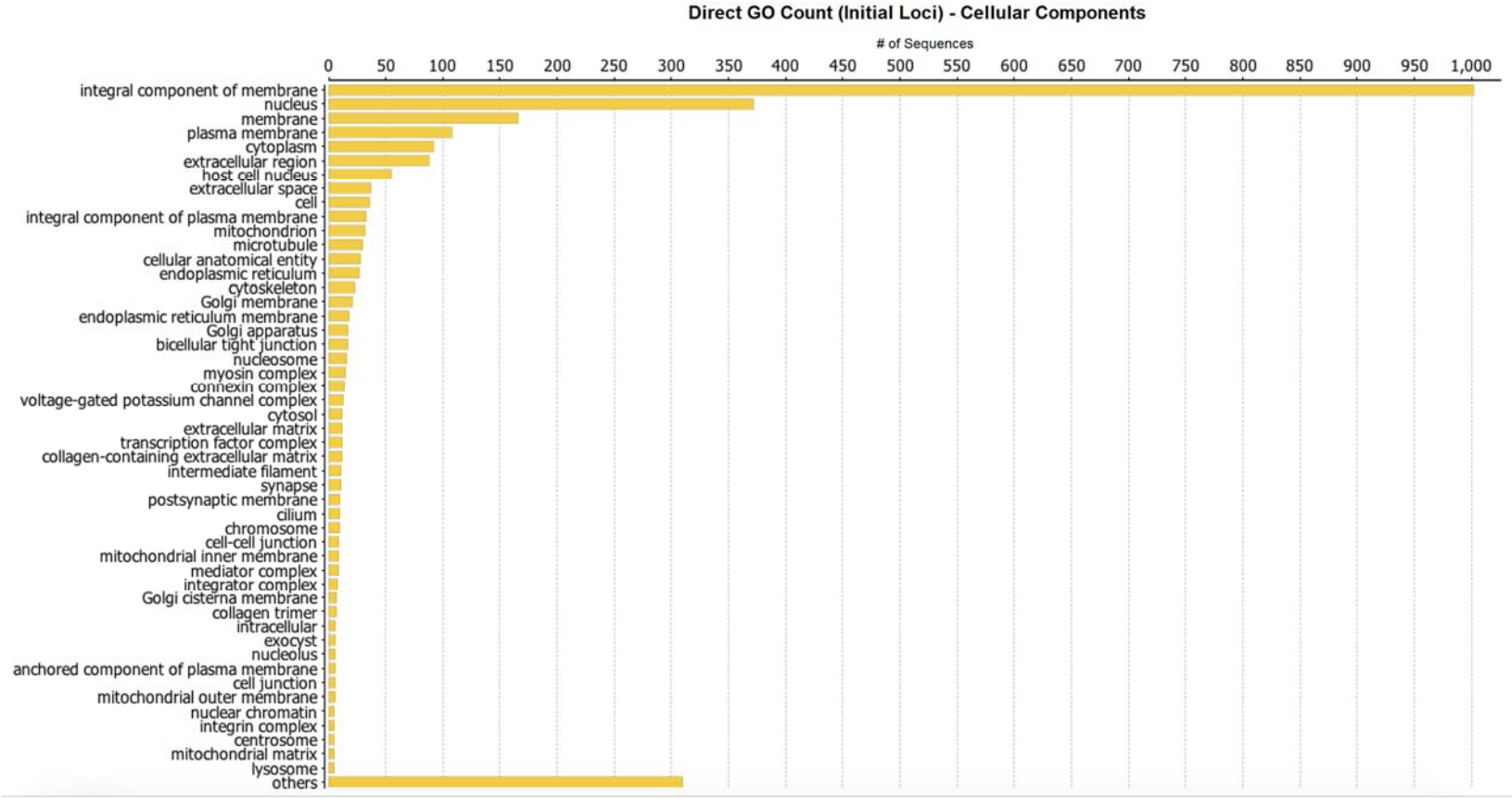

Figure C-3. Gene Ontology (GO) Terms related to Cellular Components for 4,613 successfully annotated sequences from the initial ddRAD loci.

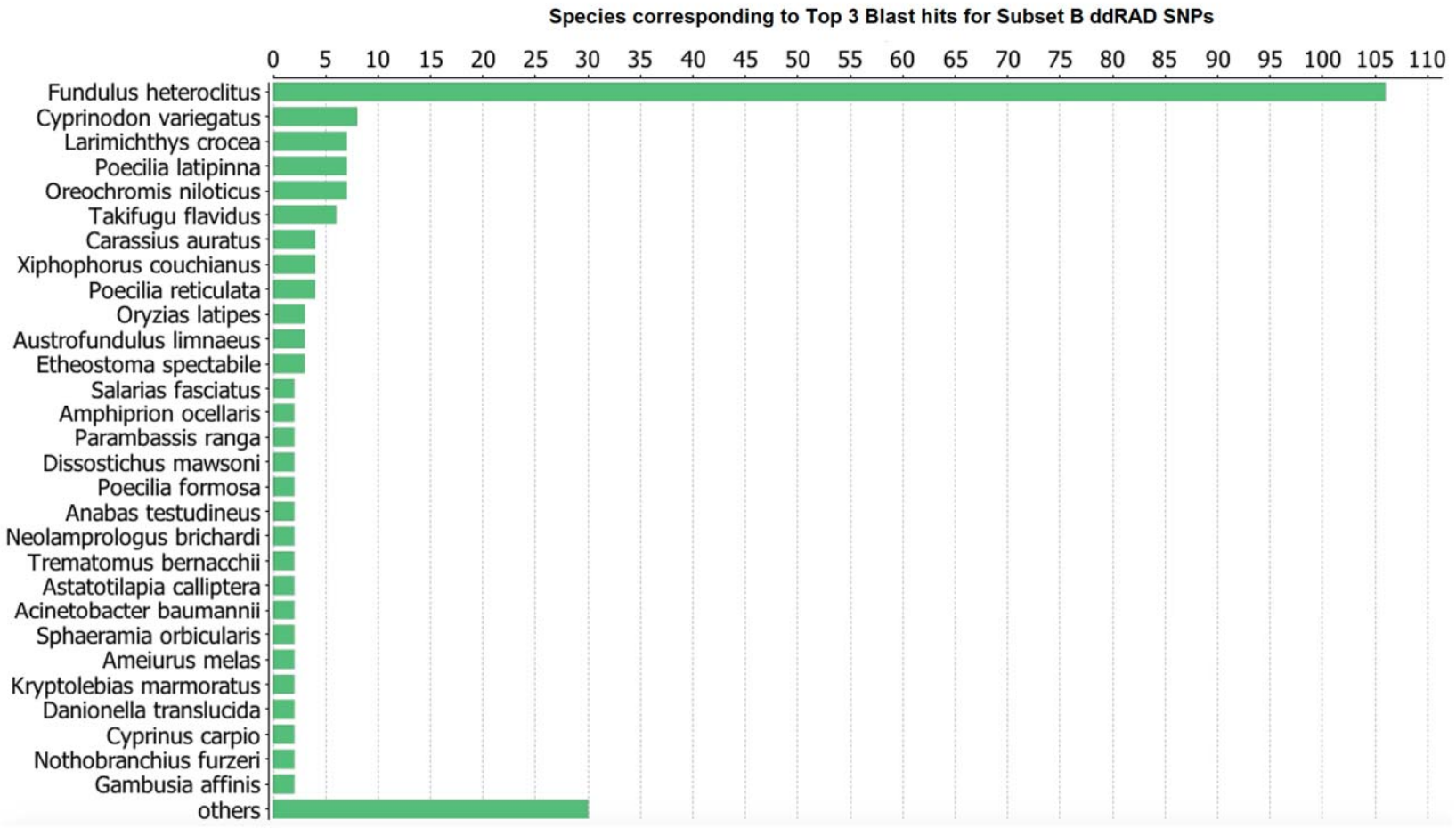

Figure C-4. Species correspondences for 227 BLAST hits obtained via BlastX (e-value e^{-15}) for Subset B of ddRAD loci

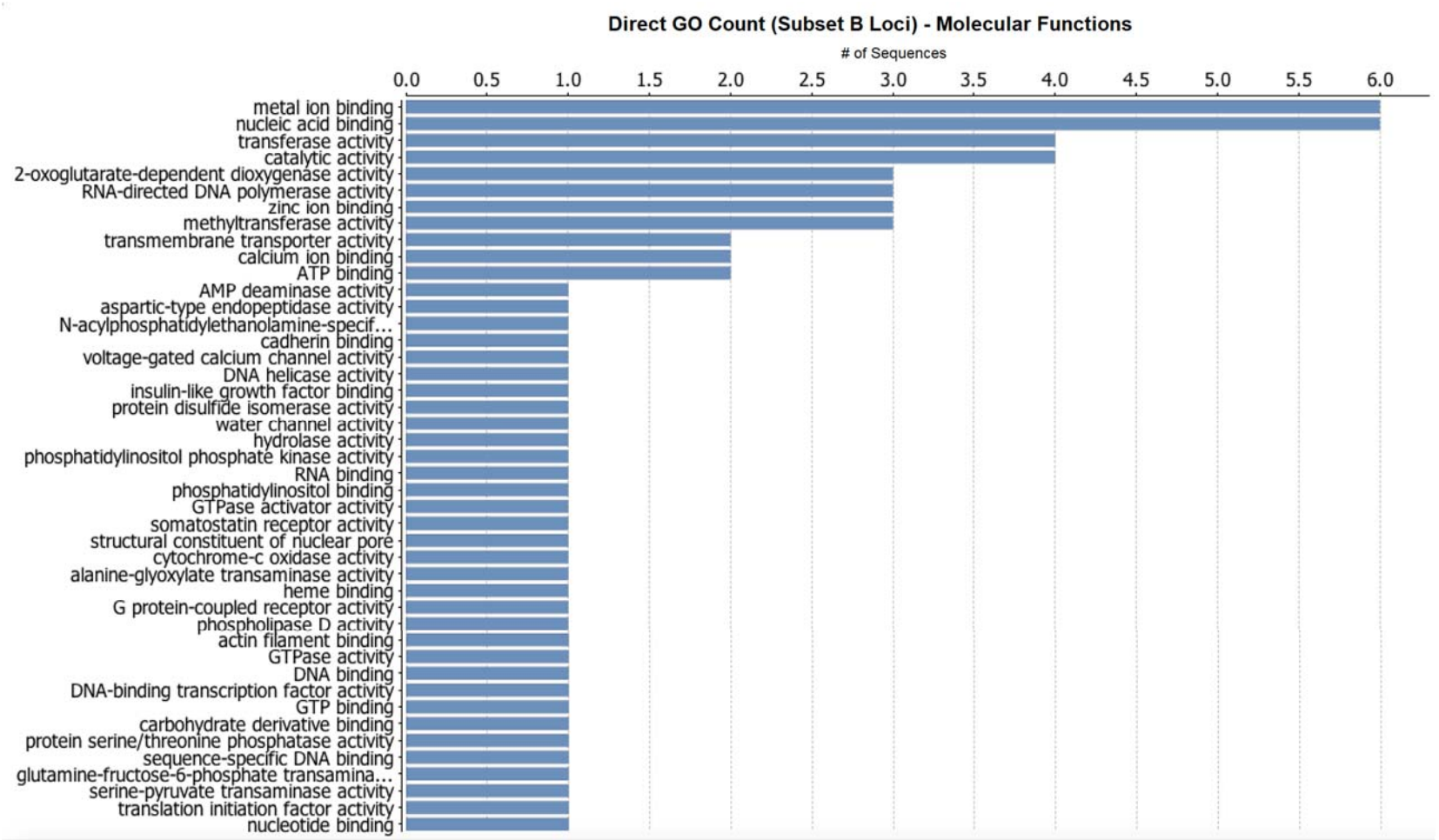

Figure C-5. Gene Ontology (GO) Terms related to Molecular Functions for XXX successfully annotated sequences from Subset B ddRAD loci.

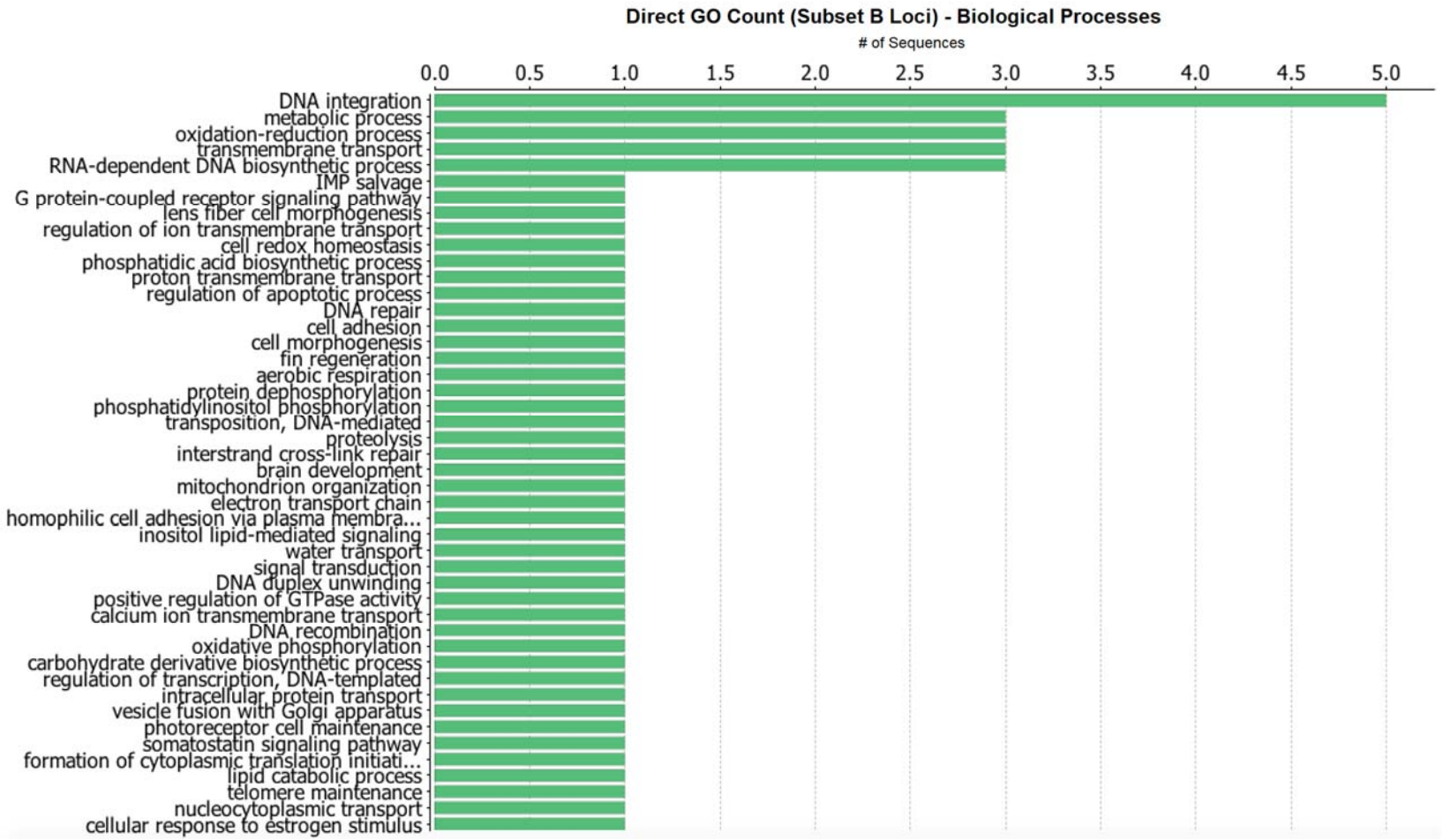

Figure C-6. Gene Ontology (GO) Terms related to Biological Processes for XXX successfully annotated sequences from Subset B ddRAD loci.

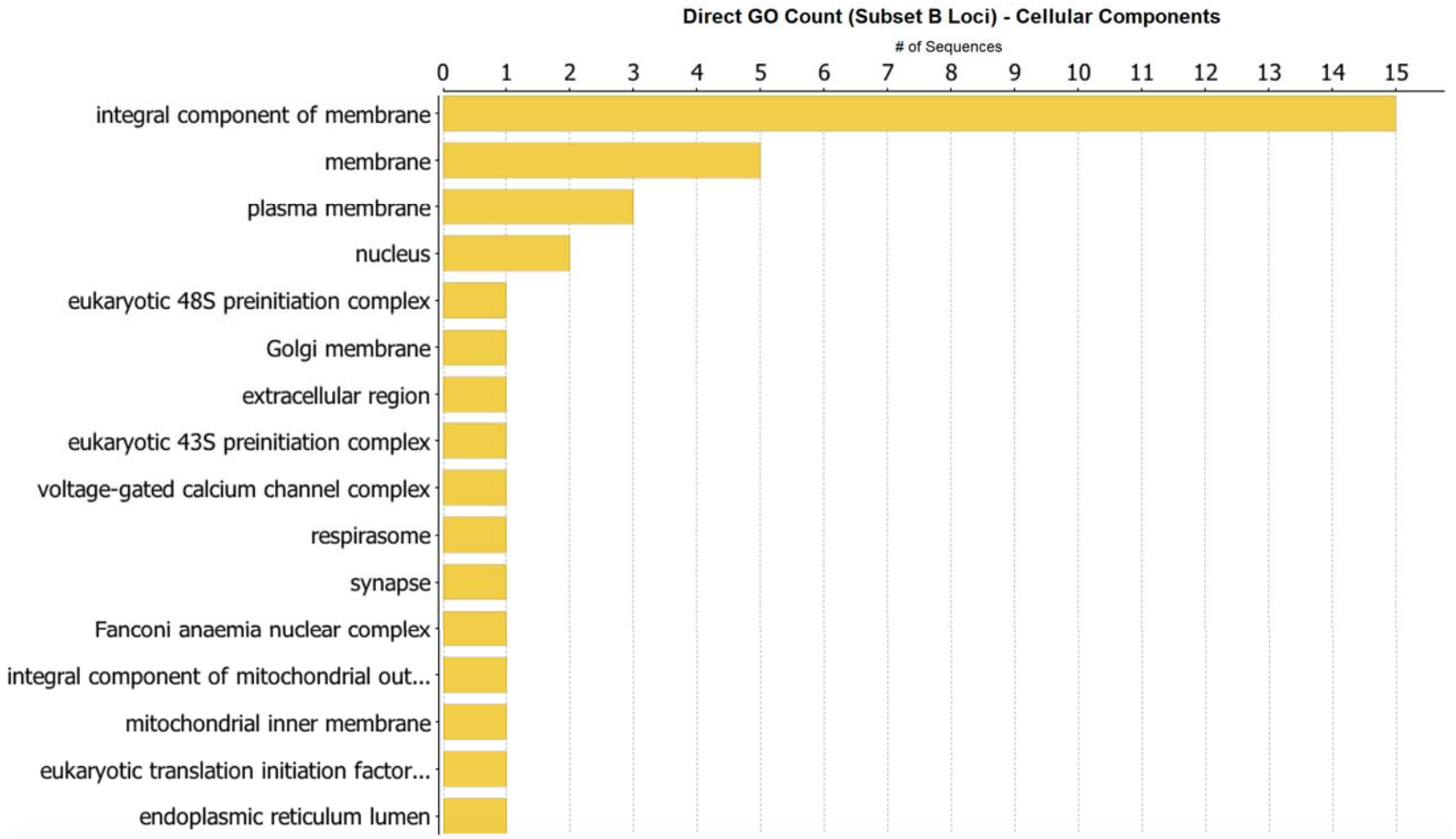

Figure C-7. Gene Ontology (GO) Terms related to Cellular Components for XXX successfully annotated sequences from Subset B ddRAD loci.

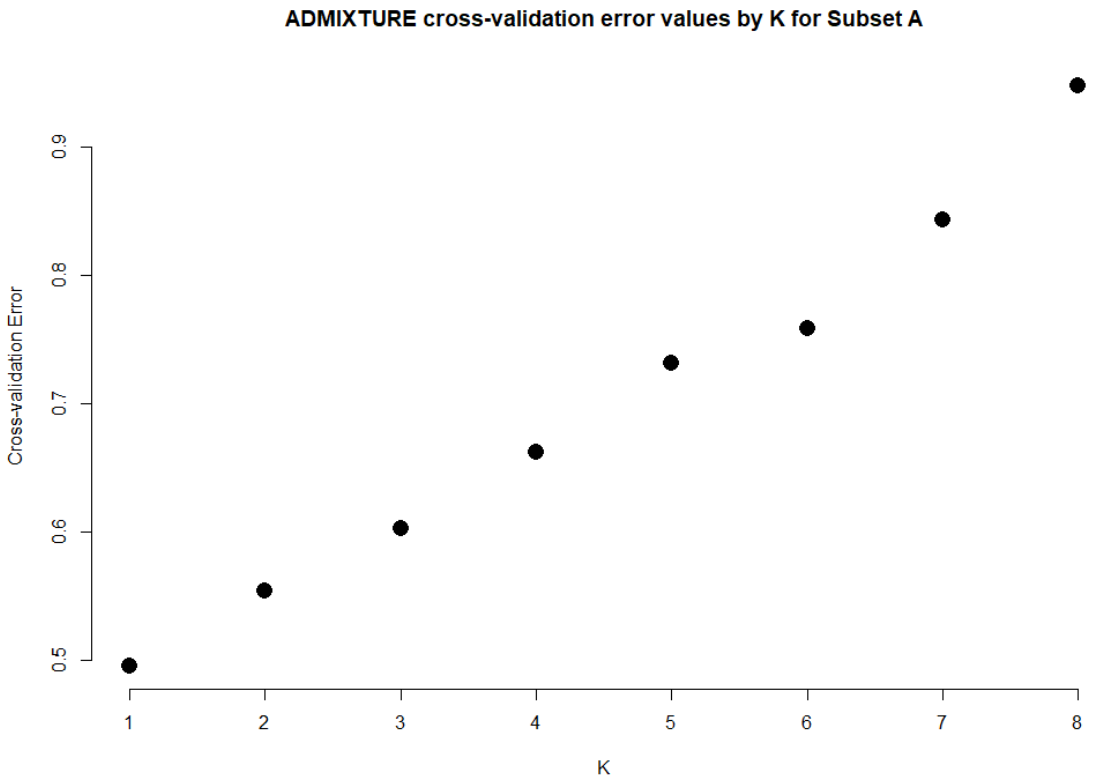

Figure C-8. ADMIXTURE cross validation error values by putative number of populations, K, based on Subset A of ddRAD SNPs.

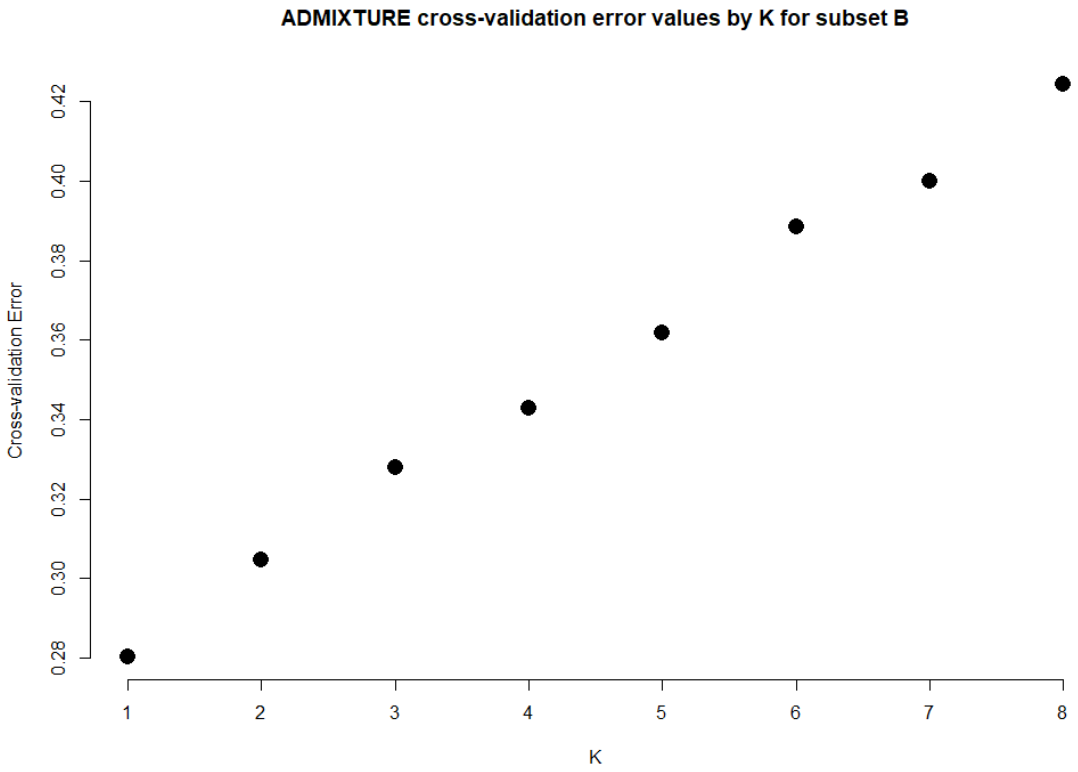

Figure C-9. ADMIXTURE cross validation error values by putative number of populations, K, based on Subset B of ddRAD SNPs.