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Evolution in Light of Mitonuclear Landscapes: An Examination of Mitochondrial Replacement in Killifish (Fundulus Spp.)

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EVOLUTION IN LIGHT OF MITONUCLEAR LANDSCAPES:

AN EXAMINATION OF MITOCHONDRIAL

REPLACEMENT IN KILLIFISH

(FUNDULUS SPP.)

by

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ABSTRACT

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The mitochondria are responsible for the bulk of energy production in eukaryotes. They possess their own genome that works in conjunction with the nuclear genome to accomplish the extraordinarily important task of energy conversion. When species hybridize there will be a mismatch in evolutionary histories between these two genomes. The deleterious interactions of these genomes have been studied in great detail (i.e. hybrid breakdown). However, little work has been conducted to understand the population genetic, and morphological consequences of wide-ranging replacement. The *Fundulus notatus* complex is comprised of 3 species: *F. notatus*, *F. olivaceus*, and *F. euryzonus*. Within the complex most pairs will hybridize with at least limited success. Unlike the other members of the species complex, *F. euryzonus* is restricted to two rivers in the Lake Pontchartrain drainage. In the Amite River *F. euryzonus* maintains its ancestral mtDNA, but in the Tangipahoa River there is some evidence that there is river-wide mitochondrial introgression (MI) with *F. olivaceus*. First I used restriction fragment length polymorphisms (RFLPs) and nuclear microsatellite markers to officially document river-wide MI between *F. olivaceus* and *F. euryzonus* in the Tangipahoa River along with frequency of hybridization in this system. I then assessed population structure using a

traditional microsatellite approach and a genomic scan of single nucleotide polymorphisms. Finally, I looked at morphological variations in body shape using geometric morphometrics. River-wide MI was confirmed in this system making it an interesting natural study system to examine the effect of MI on evolution. Population genetic and population genomic studies revealed species subdivision as expected (more subdivision in *F. olivaceus*, less subdivision in *F. euryzonus*), however in the Tangipahoa River *F. euryzonus* shows at least some subdivision. Morphologically, sexual dimorphism and species variation accounted for most of the variation in shape. The Fundulus notatus species complex is an emerging model for evolutionary study. The work in this manuscript adds to the knowledge base about locations of mitochondrial replacement, population subdivision, and shape variation.

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1.1 The Mitochondrion

Mitochondria (Greek: *mitos* meaning "thread" and *chondros* meaning "grain-like") are small cellular organelles that are paramount in the process of efficiently converting energy, when oxygen is present, to adenosine triphosphate (ATP). Each species, organism, and cell type contains variable numbers of mitochondria; however, mitochondria are nearly ubiquitous among all eukaryotes. Those eukaryotes that lack mitochondria produce proteins from nuclear genes that resemble mitochondrial proteins suggesting a former harboring and subsequent loss of mitochondria (Roger et al., 1998). Nevertheless, each mitochondrion maintains its own genome (mtDNA) (Nass & Nass 1963) and may contain multiple copies of the same genome (Wiesner et al., 1992). The mitochondria of animals are typically inherited maternally. Sperm cells may have one or more mitochondria, but the male mitochondria usually will not enter the zygote or may be otherwise destroyed. One such process of paternal mitochondrial destruction involves a ubiquitin (Coenzyme Q10) tag that the males attach to sperm mitochondria during spermatogenesis that labels sperm mitochondria for degradation by the lysosomes and proteasomes within the developing embryo (Sutovsky et al., 2000).

The genome of the mitochondria resembles that of Alphaproteobacteria in the Order *Rickettsiales* (Andersson et al., 1998) and more specifically the SAR11 clade (Thrash et al., 2011), a free-living and very abundant bacterium in the Earth's oceans. This resemblance suggests that mitochondria were likely free-living organisms related to bacteria that developed symbiotic relationships with early eukaryotes, called the

endosymbiotic theory (Wallin, 1923; Sagan, 1967). After the association, there was a massive amount of horizontal gene transfer (HGT) (reviewed by Blanchard & Lynch, 2000) leaving the genome of mitochondria reduced to the present day complement, and rendering them unable to survive without a host cell.

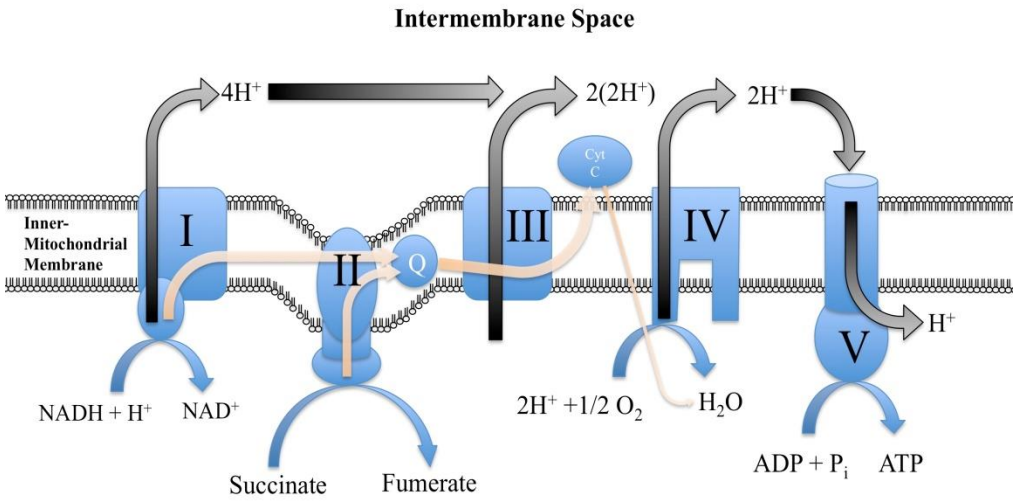
The amount and direction of HGT occurring between mitochondria and nuclear genomes depends on the taxonomic group in question. Insertion mutations, which occur in both genomes, are the driving mutations of HGT. This can happen bi-directionally however, insertions from the nuclear genome into the organelle genome are rare if not completely absent. Based on genome size alone there are more insertions in the larger nuclear genome than in the smaller organelle genomes. However, once a gene is inserted from the mitochondria into the nucleus the gene is then free to disappear from the mitochondrial genome. Once the gene is removed, the smaller genome has an advantage over larger genomes of other organelles (can replicate faster and more efficiently) and thus organelles are under selection to reduce genome size (Cavalier-Smith, 1987). This reduction in genome size is accompanied with a reduction in metabolic output resulting in a selfish view of mitochondria as opposed to symbiotic view. Also, the selective advantage associated with genome reduction lends itself to the apparent lack of bi-directional HGT between organelle and nuclear genomes. Additionally, considering that mitochondria are essentially asexual (maternally inherited) Muller's ratchet is applied and deleterious mutations can accumulate in these genomes (Hastings, 1992). Alternatively, the nuclear genomes allow for beneficial mutations to accumulate owing to a better environment (from a fitness perspective) for genes (Blanchard & Lynch, 2000).

1.2 Fitness Consequences of Cytonuclear Incompatibilities

The vertebrate mtDNA maintains ~37 total genes: two of these genes code for ribosomal ribonucleic acid (rRNA), ~22 mtDNA genes code for transfer ribonucleic acids (tRNA), and 13 genes code for proteins. All of the protein-coding genes in vertebrate mitochondrial genomes are associated with oxidative phosphorylation. Additionally, nearly all of the aforementioned genes are part of larger protein complexes that utilize gene products from both nuclear and mitochondrial sources (Figure 1.1). It follows that these sequences are well evolved to work together in these complexes (i.e. the coevolution of mitochondrial and nuclear genomes).

Coevolution is a mutual genetic change in interacting species due to natural selection that either species imposes on the other (Futuyma, 1998). In the case of mitochondria, genomes are the unit of interaction rather than species, but the definition still holds (reviewed by Rand et al., 2004). Also, protein complex functionality dictates individual fitness. Coevolution of mitochondrial and nuclear (cytonuclear) genes could then be defined as reciprocal genetic changes in interacting genomes owing to protein complex functionality imposed by interacting genomes.

All the genes of the mitochondrial genome that code for proteins are involved in a single extremely important biological process, oxidative phosphorylation. In studies of cybrid cells (cells designed with differential nuclear and mitochondrial evolutionary histories), only close evolutionary relationships between these two genomes allowed the cells to survive (Kenyon & Moraes, 1997). Similarly, complex breeding experiments of various lines of the copepod *Tigriopus califonicus* have shown reductions in the activity



Complex	I	II	III	IV	V
nuclear	43	4	10	10	14
mtDNA	7	0	1	3	2

Figure 1.1 The 13 genes encoded in the mitochondrial genome.

Note: All 13 genes produce proteins that are associated with protein complexes, which include products from both mitochondrial origin and nuclear origin. In Complex I, a key link between glycolysis which happens independent of the mitochondria 7 out of the 50 total proteins that make up this complex (14%) comes from the mitochondrial genome. Complex II is made entirely from proteins coded for in the nucleus of the cell. Complex III is made of 11 total proteins 1 of which is coded for in the mitochondrial genome (~9%) this is the Cytochrome-b gene was very commonly used in phylogenetic analyses. Complex IV of oxidative phosphorylation is made from 13 total genes, 3 of which are coded for in the mitochondria (~23%). Finally, Complex V, ATPsynthase, is made of 12.5%, or 2 out of 16 gene products, from mitochondrial-coded proteins.

of the cytonuclear gene complex cytochrome oxidase (reviewed by Burton et al., 1999). The process of oxidative phosphorylation relies on building a proton gradient on either side of the intermitochondrial membrane. This gradient is then used to power ATPase (a cytonuclear protein) to connect inorganic phosphate with an adenosine diphosphate

(ADP) molecule, making the energetic ATP. Protons are very small and in addition to the possibility of reduced functionality with an imperfect fit there is also the potential for protons to "leak" across the intermitochondrial membrane and produce excess reactive oxygen species (ROS) that are potentially damaging to the cell (Barrientos et al., 1998). A leaky membrane would mean reduced efficiency in converting energy from one form to the next, which could potentially manifest itself as a reduction in organismal fitness (Cruzan & Arnold, 1999; Blier et al., 2001; Rhode & Cruzan, 2005; Chase, 2007; Etterson et al., 2007; Sambatti et al., 2008; Burton et al., 2013). The result of an increase in ROS is immense stress put on the cell resulting in extreme environmental conditions with strong negative selection pressures. Additionally, mitochondrial genes are associated with signaling the nucleus that influences many activities of the cell (Ca^{2+} dynamics, nuclear gene expression, etc.) and by extension, the organism (aging, metabolic function, and disease) (reviewed by Butow & Avadhani, 2004).

Similar species coming together to make hybrid offspring would result in the potential for hybrids having proteins that have not coevolved. First generation hybrids will have mitochondria inherited maternally and an equal contribution of nuclear genomes from lineages or species. The interaction between different genes is termed epistasis (Bateson, 1909) and can be a strong driver in species evolution (reviewed by Wolf et al., 2000; Wade & Goodnight 2006). First generation hybrids can experience increased fitness (heterosis) due to dominance and overdominance (Lynch, 1991; Hedgecock et al., 1995; Burke & Arnold, 2001). However, it is common for successive generations to expeditiously accumulate genetic incompatibilities, resulting in a decrease

in fitness (Orr, 1995). The discordance in cytonuclear genes is one example of an epistatic relationship that has the potential to drive hybrid breakdown (Cruzan & Arnold, 1999; Blier et al., 2001; Rhode & Cruzan, 2005; Etterson et al., 2007; Sambatti et al., 2008; Burton et al., 2013). Extreme examples of cytonuclear incompatibilities exist using plant and yeast models where incompatibilities have been suggested to lead to sterility or death (reviewed by Chase, 2007). Reductions in fitness for hybrid individuals are known as hybrid breakdown. Fishman & Willis (2006) showed that male reproductive organs in monkey flowers (*Mimulus guttatus* complex) become deformed with conflicting cytoplasmic and nuclear backgrounds resulting in hybrid sterility. A model system for speciation, and specifically hybrid breakdown, is the intertidal harpacticoid copepod *Tigriopus californicus*. Interpopulation crosses of *T. californicus* show F2 hybrids losing fitness in respect to reduced survivorship (Burton, 1986), slower development (Burton, 1990) and lower fecundity (Edmands, 1999) compared to parental populations.

1.3 Mitochondrial Introgression

Despite the potential negative consequences of cytonuclear incompatibility, the phenomenon of mitochondrial introgression (MI) is not uncommon. In plants, organelle introgression (mitochondria and chloroplast) is well documented (Rieseberg & Wendel, 1993; Reboud & Zeyl, 1994; Senjo et al., 1999; Currat et al., 2008). As a marker for genetic studies of plants mitochondrial DNA (mtDNA) is avoided because plant mtDNA is more likely to have intramolecular recombination and low rates of base pair substitution (Newton, 1988; Palmer, 1992). This phenomenon has limited the ability to

detect natural MI events in plants. However, the effects on fitness due to introgression of the mitochondria, as observed in controlled experiments, are apparent (Chase, 2007; Sambatti et al., 2008; Leinonen et al., 2011).

Animals, examined in their natural environment, have provided many examples of MI (Besansky et al., 1994; Dabrowski et al., 2005; McGuire et al., 2007; Darling, 2011; Near et al., 2011). Introgression of mtDNA is usually detected in phylogeographic and systematic studies when there is discordance between trees generated by mitochondrial and nuclear gene data (Machado & Hey, 2003; Chan & Levin, 2005; Bachtrog et al., 2006; Bossu & Near, 2009). Freshwater fishes have provided ideal models to examine the mitochondrial and nuclear discordance. For example, Near et al. (2011) did a phylogenetic analysis of darters using 1 mtDNA marker (*cytb*) and 2 nuclear genes (*RAG1* and *S7*) and found that >12% of all darter species examined showed some form of heterospecific mtDNA. Interestingly, the mtDNA ranged from other extant species to introgression from phylogeneticaly distant extinct species. In an examination of the role of mitochondrial genes in adaptive radiation Nevado et al. (2009) showed that between two distinct species of cichlids one species had completely replaced its mitochondria with a single mitochondrial clade of the sister species. However, MI is more commonly found in populations associated with hybrid zones. There are many examples of mtDNA introgression into certain populations such as *Salvelinus fontinalis* and *S. namaycush* with *S. alpinus* mtDNA (Bernatchez et al., 1995; Wilson & Bernatchez, 1998, respectively) or *Fundulus notatus* mtDNA becoming fixed in certain populations of *F. olivaceus* (mitochondrial replacement) (Duvernell et al., 2007).

There are three species within the *Fundulus notatus* complex: *F. notatus* (Rafinesque), *F. olivaceus* (Storer) and *F. euryzonus* (Suttkus and Cashner). *Fundulus notatus* and *F. olivaceus* have broad overlapping ranges including areas with hybridization in sympatry (Duvernell et al., 2007; Schaefer et al., 2009; Schaefer et al., 2011; Duvernell & Schaefer, 2013). Duvernell et al. (2007) consulted museum records to find locations of sympatry and allopatry in Illinois and Missouri, the northern portion of the range. They used 4 microsatellite loci and 2 nuclear gene restriction fragment length polymorphism (RFLP) assays as well as sequencing for single nucleotide polymorphisms (SNPs) to establish nuclear genotypes and admixture between species. They also used an RFLP on a portion of the cytochrome *b* (cyt*b*) gene to establish mtDNA haplotypes. This study documented hybridization in areas where *F. notatus* and *F. olivaceus* co-occur. Additionally, unidirectional mtDNA introgression occurred in many of the documented transition zones and in one case (Brushy Creek), *F. olivaceus* mtDNA appeared to be fully replaced by *F. notatus* mtDNA.

The species of the *F. notatus* complex have similar ecological niches (Thomerson & Woolridge, 1970; Blanchard, 1996) but habitat transitions are important in the structure of the contact zone (Duvernell et al., 2007). In 2011, Schaefer et al. showed that many physical (stream size, temperature, substrate… etc.) and biological variables (fish assemblage) a correlated with dramatic effects on the size and shape of the contact zone as well as the rate at which hybridization occurs. Habitat effects are predictable and can be applied to other species pairs (*F. olivaceus* and *F. euryzonus*) within the *F. notatus* complex (Schaefer et al., 2009).

In contrast to the other two species, *F. euryzonus* has a narrow range, restricted to two rivers (the Amite and Tangipahoa Rivers) of the Lake Pontchartrain drainage (Suttkus & Cashner, 1981). *Fundulus euryzonus* is a sister species to *F. olivaceus* (Duvernell et al., 2013; Ghedotti & Davis, 2013) and overlaps with *F. olivaceus* throughout most of its range (Suttkus & Cashner, 1981). Hybridization in the West Fork of the Amite River has been reported between *F. euryzonus* and *F. olivaceus* at a rate of 2.9% (Schaefer et al., 2009; Schaefer et al., 2011). However, in the Tangipahoa River, there is far less known about the patterns of hybridization. In a few locations within the Tangipahoa River, all individuals morphologically identified as *F. euryzonus* possessed *F. olivaceus* mitochondrial DNA (Kreiser, unpublished data). Given what is known

about hybrid fertility, these observations are surprising. Hybrids of *F. notatus* and *F. olivaceus* are fertile, however, hybrids of *F. olivaceus* and *F. euryzonus* are sterile when the female parent is *F. olivaceus* (Vigueira et al., 2008). This pattern of sterility for female F1 hybrids with a *F. olivaceus* female parent suggests that mitochondrial introgression should proceed in the opposite direction than appears to be present in the Tangipahoa River.

1.4 Marker Types

Microsatellites have become widely employed in ecological populations genetics over the last two decades. These markers are easy and cheap to develop, selectively neutral, nuclear, and mutate at a very high rate that makes them ideal for population genetic studies (Queller et al., 1993; Jarne & Lagoda, 1996). However, there are many drawbacks to using these markers such as species specificity, complex mutation

processes that can be unclear, homoplasy can underestimate diversity, and can be very difficult to amplify (reviewed by Selkoe & Toonen, 2006). Recently microsatellite markers have been developed for *F. notatus* (Feldheim et al., 2014). Of these, seven loci amplify in both *F. olivaceus* and *F. euryzonus* (Feldheim et al., 2014). This small sampling of the nuclear genome will be sufficient to detect/measure the level of genetic variation (Koskinen et al., 2004).

Using new sequencing technologies to scan the genomes of individuals and find thousands of loci can ameliorate the drawbacks of microsatellites. Next generation sequencing technology, specifically that on the Illumina® platform, has dramatically increased the speed at which genomes can be sequenced. The technique is similar to capillary electrophoresis, utilizing fluorophore excitation as nucleotides are added. The difference is that instead of a single fragment, millions of fragments are built simultaneously. Genotype-by-sequencing (GBS) (Elshire et al., 2011) cleaves portions of the genome via restriction enzyme digest to several thousand to hundreds of thousands of small DNA fragments (100 base pairs). The fragments are produced at various locations in the genome and thus represent a variety of marker types. These are then assessed for single nucleotide polymorphisms (SNPs). A SNP is a location within the fragment where a single nucleotide varies among individuals, populations, or species. Contrary to microsatellites, SNPs may appear within protein coding regions as well as non-coding locations yielding an overall more conserved analysis of evolutionary history. However,

the dramatic increase in number of informative sites (~10 000 SNP loci vs. ~10-30 microsatellite loci) make this marker much more ideal for assessing evolutionary histories and demographic analyses.

The goal of this work is to examine the evolutionary history and potential consequences of mitochondrial replacement between *F. olivaceus* and *F. euryzonus* in the Lake Pontchartrain drainage. In doing so, this work represents a first step in studying the consequences of mitochondrial and nuclear coevolution in topminnows. I will first document mitochondrial replacement in the Tangipahoa River, along with frequency of hybridization in this system. I will then assess population structure using a traditional microsatellite approach and a GBS approach. I will use the *F. olivaceus* / *F. euryzonus* system to compare this new technique to microsatellite analyses. Then I will use GBS data to assess demographic changes over time in this drainage to gain historical understanding of the syntopic relationship of these species. Future work should focus on fitness related effects of mitochondrial replacement in these systems to better understand coevolution of the two genomes.

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CHAPTER II – WIDESPREAD INTROGRESSION OF *FUNDULUS OLIVACEUS* MITOCHONDRIAL DNA INTO *FUNDULUS EURYZONUS*

2.1 Abstract

Variation in mitochondrial genes has served as the principal data for analyses in phylogeography for the last several decades. More recently, the use of additional nuclear markers has elucidated a high frequency of mitochondrial introgression (MI). Individual fitness can be impacted by the ocurance of MI however the evolutionary significance of MI is only just beginning to be understood b. I examined two closely related, potentially interbreeding topminnows (*Fundulus olivaceus* and *F. euryzonus*) that only co-occur in two rivers of the Lake Pontchartrain drainage of Louisiana and Mississippi, USA. I examined mitochondrial cytochrome-b sequences and nuclear microsatellite loci to assess degrees of MI across the range. I resolved three different mtDNA fragment patterns across 225 individuals in the Tangipahoa River, all of which clustered with sequences associated with *F. olivaceus* from the literature. There were six *F. olivaceus* and two *F. euryzonus* with hybrid ancestry. Some hybrids were inferred however due to the low prevalence of hybridization in this system MI is probably the result of ancient evolutionary processes. This paper documents a previously unknown widespread MI event that can be used to analyze the effect of MI in evolutionary studies.

2.2 Introduction

Mitochondrial genes have long been the quintessential markers for phylogeography and systematics for a variety of reasons: mitochondrial genes lack recombination, exhibits a high mutation rate, reduced effective population size relative to the nuclear genome, and there is an abundance of universal primers (Avise, 2000; Lynch

et al., 2006). However, hybridization can facilitate introgression of entire mitochondrial genomes across species boundaries (Birky, 1995). The consequence of mitochondrial introgression (MI) is a potentially confounding factor for the use of mitochondrial genomes in phylogeography and systematics (reviewed by Zink & Barrowclough, 2008). This has become apparent as nuclear markers are increasingly being used to assess relationships and determine the arrangement of genetic clusters across a landscape. A byproduct of using a combination of marker types in these studies has revealed that MI is common, in both plants (Rieseberg & Wendel, 1993; Senjo et al., 1999) and animals (Besansky et al., 1994; Dabrowski et al., 2005; McGuire et al., 2007; Darling, 2011; Near et al., 2011). Typically, MI is detected by phylogeographic and systematic studies when there is discordance between trees generated by mitochondrial and nuclear gene data (Machado & Hey, 2003; Chan & Levin, 2005; Bachtrog et al., 2006; Bossu & Near, 2009).

The species that comprise the genus *Fundulus* have been proposed as a model system for genomics (Burnett et al., 2007), but they are also an emerging model system for evolutionary study. Specifically, topminnows in the *F. notatus* species complex have become an interesting system to examine the ecological and genetic interactions associated with hybridization in the field and in the laboratory (Duvernell et al., 2007; Vigueira et al., 2008; Earnest et al., 2014, Duvernell et al., 2013; Duvernell & Schaefer, 2013). There are three described species within the *F. notatus* complex: *F. notatus* (Rafinesque), *F. olivaceus* (Storer) and *F. euryzonus* (Suttkus & Cashner).

The species within the *F. notatus* complex are endemic to the southeastern United States. *Fundulus notatus* and *F. olivaceus* have broad overlapping ranges while the third

member, *F. euryzonus*, has a narrow range, existing in only two rivers of the Lake Pontchartrain drainage (Amite & Tangipahoa Rivers; Suttkus & Cashner, 1981). Additionally, *F. euryzonus* is a sister species to *F. olivaceus* (Duvernell et al., 2013; Ghedotti & Davis, 2013) which occurs in the same drainages. Occurrence of all three species of the *F. notatus* complex within the Amite River of Lake Pontchartrain drainage has been recorded with widespread co-occurrence of *F. euryzonus* and *F. olivaceus* (Suttkus & Cashner, 1981). The species segregate along the river continuum in an upstream-downstream manner where *F. olivaceus* occurs primarily in upstream locations and *F. euryzonus* occurs primarily downstream in larger rivers (Schaefer et al., 2009).

Hybridization in the West Fork of the Amite River is rare, but in this system females from both species appeared to be involved (Schaefer et al., 2009). In the other portion of the range for *F. euryzonus* (Tangipahoa River) there is far less known about the patterns of hybridization. One striking observation is that at the few locales examined individuals that were clearly *F. euryzonus* in appearance possessed *F. olivaceus* mitochondrial DNA (Kreiser, unpublished data). It is surprising to find mitochondrial replacement in this topminnow system given what is known about the fertility of hybrids generated in the laboratory. When *F. notatus* and *F. olivaceus* hybridize their offspring are fertile. However, this pattern is different for *F. olivaceus* and *F. euryzonus* offspring with sterility resulting from crosses where the female parent is *F. olivaceus* (Vigueira et al., 2008). The sterility of female F1 hybrids with a female *F. olivaceus* parent would suggest that MI should proceed in the opposite direction than appears to be present in the Tangipahoa River.

The goal of this research was to expand the knowledge about the contact zone between these two target species (*F. olivaceus* and *F. euryzonus*). Specifically, I examined the topminnows throughout the Tangipahoa River to determine if MI was a widespread phenomenon. I hypothesized that 1) MI would occur throughout the Tangipahoa River, where *F. olivaceus* mtDNA is found in both species and 2) current rates of hybridization between these species is minute and comparable to the Amite River. My aim was to determine if the pattern observed in my preliminary data was the product of ongoing hybridization or represented MI from a more ancient contact between the species. Based on the preliminary data and previous work I predict that a larger more robust study, such as this one, will show widespread historical MI and low rates of contemporary hybridization.

2.3 Methods

I sampled topminnows (*Fundulus* spp.) at 8 locations in the Tangipahoa River and associated tributaries (Table 2.1). The northernmost sampling location was at the headwaters of the Tangipahoa River in Mississippi (MS) 5 kilometers (km) northwest of McComb, MS. The southernmost sampling location was 6 km northeast of Amite City, Louisiana (LA) for a total distance of 97.9 river km (Figure 2.1). All sites were sampled between 20 May 2014 and 6 June 2014. At each location an approximately 100-meter reach of the stream was sampled for topminnows. The average stream width and depth with standard deviation were 14.57 ± 8.50 meters wide by 0.62 ± 0.17 meters deep. Typical characteristics at each site included a sand to gravel substrate, an average flow rate of 0.18 meters per second, and an average temperature of 22.9° Celsius (C).

Table 2.1

Locations sampled in the Tangipahoa River.

Site	Location Name	Latitude, Longitude	*F. oli.*	*F. eury.*	Hybrid ancestry
1	County Line Rd.	31.293, -90.553	30	0	0
2	Martin Rd.	31.226, -90.529	13	17	5
3	Hwy 51	31.113, -90.479	19	11	12
4	State Line Rd.	31.005, -90.449	8	22	7
5	Magnolia-Progress Rd	31.077, -90.370	15	0	0
6	Hwy 38	30.938, -90.490	15	15	6
7	Hwy 440	30.876, -90.495	26	4	2
8	Hayden Rd	30.793, -90.418	11	19	2

Note: Fish were collected at 8 different locations in the Tangipahoa River drainage. Site numbers match Figure 2.1 and site names were based on the nearest road crossing. Individuals were described as being from hybrid ancestry if the q-score from STRUCTURE was <0.90 for either species. The column "hybrid ancestry" includes individuals that were morphologically classified into either *Fundulus olivaceus* (*F. oli*) or *F. euryzonus* (*F. eury*).

I collected a total of 225 individuals of *Fundulus* spp., which included 15-30 at each location. I used dipnets to collect fish after visually locating them in the water. Each individual was tentatively identified by eye, to insure collection of target species, and euthanized in an overdose of tricaine mesylate (MS-222). A caudal fin clip was taken in the field for DNA extraction and stored in a preservation buffer (Seutin et al., 1991). Euthanized specimens were preserved in 10% formalin for morphological species identification and will be vouchered at the USM Museum Ichthyology Collection. Total genomic DNA was extracted from fin clips using a DNeasy Tissue Kit (Qiagen, Valencia, CA). The manufacturer's protocol was followed.

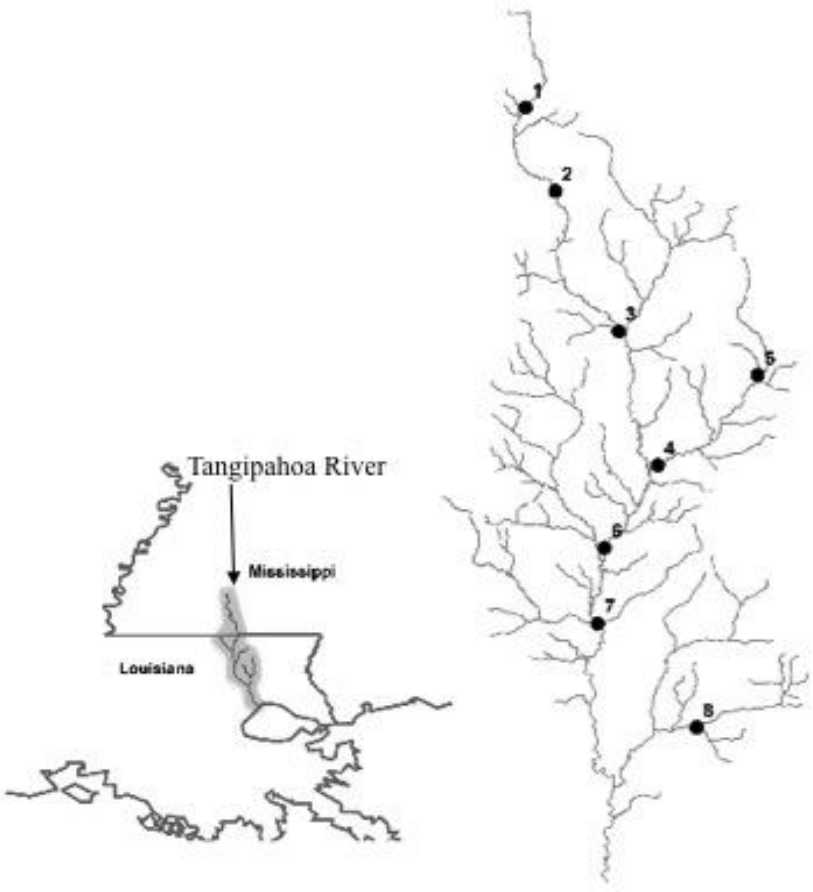

Figure 2.1 The eight collection localities for this study in the Tangipahoa River and associated tributaries of Mississippi and Louisiana.

Note: The numbers on the map correspond to the sites listed in Table 1.

Tentative morphological species identifications were made using relative band depth (RBD) following the methods of Schaefer et al. (2009). For each individual I used digital images to measure maximum band depth (MBAND) and maximum body depth (MBD) (Figure 2.2) with the ImageJ software (Schneider et al., 2012). To diagnose species differences I calculated RBD, which is a ratio of MBAND to MBD. RBD is not the most used or reliable among several diagnostic traits for these species (Suttkus &

Cashner, 1981; Ross, 2000). However, many of the meristic traits have significant overlap that does not relate to hybridization (Schaefer et al., 2009) therefore I did not count fin rays and relied upon RBD for tentative morphological identification.

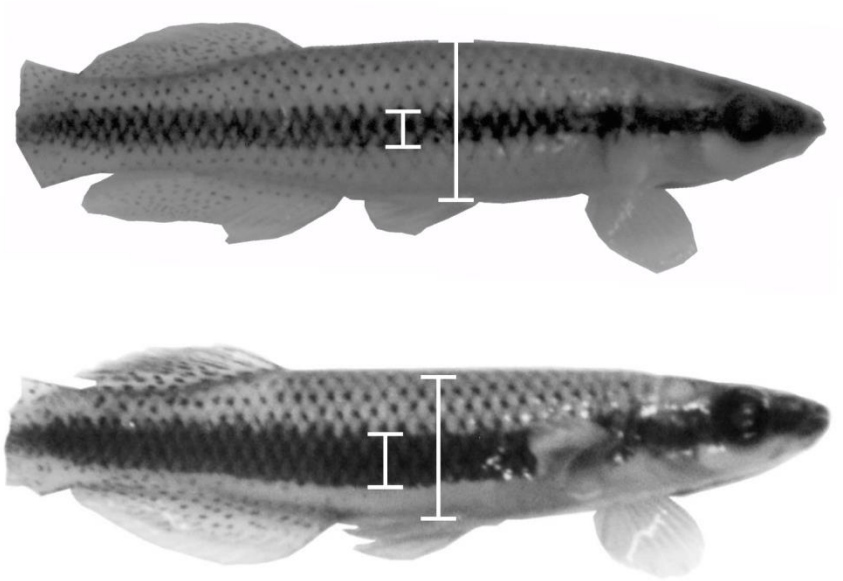

Figure 2.2 An example of fish sampled during this study.

Note: Both are males where *Fundulus olivaceus* is pictured on top and *F. euryzonus* is pictured on bottom. Caudal fins removed for genetic analyses). White lines represent the maximum band depth (MBAND), excluding vertical extensions of the band, and the maximum body depth (MBD).

I used published mitochondrial sequences of the cytochrome *b* gene (cyt*b*) for the target species within the Lake Pontchartrain Drainage (GenBank accession numbers: KF245749, KF245781-KF245786, KF245826, KF245827, KF245870, KF245871, KF245877, KF245878, KF245879, GQ119711, GQ119712) to identify species-specific base substitution that could be used in restriction fragment length polymorphism (RFLP) assays using Sequencher v. 4.10.1 (GeneCodes Co., Ann Arbor, MI, USA). I amplified a

truncated portion of the mitochondrial cyt*b* sequence, using the primers Amite1R (5'-GAGCCGGTTTCATGAAGGA-3') and L15269 (Song et al., 1998), which comprised a 547 base pair fragment containing the diagnostic restriction site within the cyt*b* gene. Polymerase chain reaction (PCR) amplifications were conducted in a total volume of 25 μL with an Applied Biosystems® Veriti® 96-Well Thermal Cycler. Our reagent concentrations were 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 2 mM $MgCl_2$, 200 μM dNTPs, 0.5 units *Taq* polymerase (New England Biolabs, Beverly, MA, USA), 0.3 μM of each primer, approximately 50 ng of template DNA and water to the final volume. PCR cycling conditions consisted of an initial 1 min denaturing step at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 50 °C and 1 min at 72 °C. A final elongation step of 7 min at 72 °C completed the cycle. I used the enzyme *HinP1*I (New England Biolabs) in our RFLP assay. Each digestion reaction was conducted following the manufacturers protocol in a 20 μL volume with 10 μL of the PCR product, 7.9 μL of water, 2 μL NEB supplied buffer, and 0.1 μL of the restriction enzyme (NEB). Digests were incubated at 37 °C for 4 hours and then visualized on a 2% agarose gel stained with ethidium bromide (0.5 μg/ml). Individuals were scored as having either *F. olivaceus* or *F. euryzonus* haplotypes.

A phylogenetic tree was then generated using the sequence data to verify the identity of the haplotypes resolved by the RFLP assays. I selected 16 individuals for sequencing that represented each haplotype in each species from genetically distinct portions of the drainage (Chapter IV) and an outgroup (*Profundulus sp.*: JQ254935). PCR products were cleaned with Exonuclease I - Shrimp Alkaline Phosphatase (Exo-SAP; USB Corp. Cleveland, Ohio), and sent to Eurofins Genomics (Louisville, KY) for Sanger

sequencing. Sequences were then edited and aligned using Sequencher v. 4.10.1 (GeneCodes Co., Ann Arbor, MI, USA). The Bayesian information criterion (BIC) from jModelTest 2.0 v0.1.1 (Posada, 2008; Darriba et al., 2012) was used to select the best substitution model. I then used the Bayesian tree building program implemented in MrBayes v. 3.2.5 (Ronquist & Huelsenbeck, 2003.) to build a phylogenetic tree. I used default settings in MrBayes with variable substation rates (HKY85 model) combined with gamma distribution with rate variation across sites with 5,000,000 Markov chain Monte Carlo (MCMC) iterations after 500,000 burn-in samples. The phylogenetic tree was then visualized using FigTree version 1.4 (Rambaut, 2012).

Each fish was also genotyped for seven microsatellite loci (*Fno*014, *Fno*091, *Fno*093, *Fno*112, *Fno*119, *Fno*242, and *Fno*261) (Feldheim et al., 2014). PCR amplifications for microsatellite loci were conducted in 12.5 μL total volume using 50 mM KCl, 10mM Tris-HCl (pH 8.3), 0.01% gelatin, 1.5 – 2.0 mM $MgCl_2$, 200 μM dNTPs, 0.188 units of *Taq* polymerase (New England Biolabs), 0.3 μM of the M13 tailed forward primer (Boutin-Ganache et al., 2001), 0.3 μM of the reverse primer, 0.1 μM of the M13 labeled primer (LI-COR), 20-100 ng of the template DNA and water to the final volume. Cycling conditions for PCR consisted of an initial denaturing step of 94°C for 2 minutes followed by 35 cycles of 30 seconds at 94°C, 1 minute at 56°C and 1 minute at 72°C. A final elongation step of 10 minutes at 72°C ended the cycle. I visualized the microsatellite alleles using a LI-COR 4300 DNA Analysis system and scored them using Gene Image IR v. 3.55 (LI-COR).

The identification of each individual as *F. euryzonus*, *F. olivaceus* or a hybrid was performed by analyzing the microsatellite genotype data with the program STRUCTURE

version 2.3.4 (Pritchard et al., 2000). Analyses were performed assuming two species (K=2) (Duvernell et al., 2007) using an admixture model with sampling location as a prior and using correlated allele frequencies. Other parameters used in STRUCTURE were 50,000 burn-in samples and 100,000 iterations of MCMC over 20 independent runs with all other parameters following default settings in STRUCTURE. A threshold q-score of 0.90 was used to determine species. Results of all 20 runs were compiled using CLUMPP v. 1.1.2 (Jakobsson & Rosenberg, 2007) and visualized using DISTRUCT version 1.1 (Rosenberg, 2004).

2.4 Results

Using a threshold RBD value of 0.28, 88 of the 225 fish sampled in the Tangipahoa River were morphologically identified as *F. euryzonus* and 137 were identified as *F. olivaceus*. The average RBD for *F. euryzonus* was 0.328 with a standard error of 0.0026 whereas the RBD for *F. olivaceus* was 0.223 with a standard error of 0.0023 (Figure 2.3). Sex ratios for both species were slightly female biased: 48 female and 40 male *F. euryzonus* and 77 female and 60 male *F. olivaceus*.

I resolved three different mtDNA fragment patterns across all 225 individuals in the Tangipahoa River: "haplotype 1" (290bp, 199bp, 58bp), "haplotype 2" (290bp and 257bp) and "haplotype 3" (a single uncut fragment of 547 bp). When compared with morphological identifications there were 91 *F. olivaceus* and seven *F. euryzonus* with haplotype 1, 40 *F. olivaceus* and zero *F. euryzonus* with haplotype 2, and six *F. olivaceus*

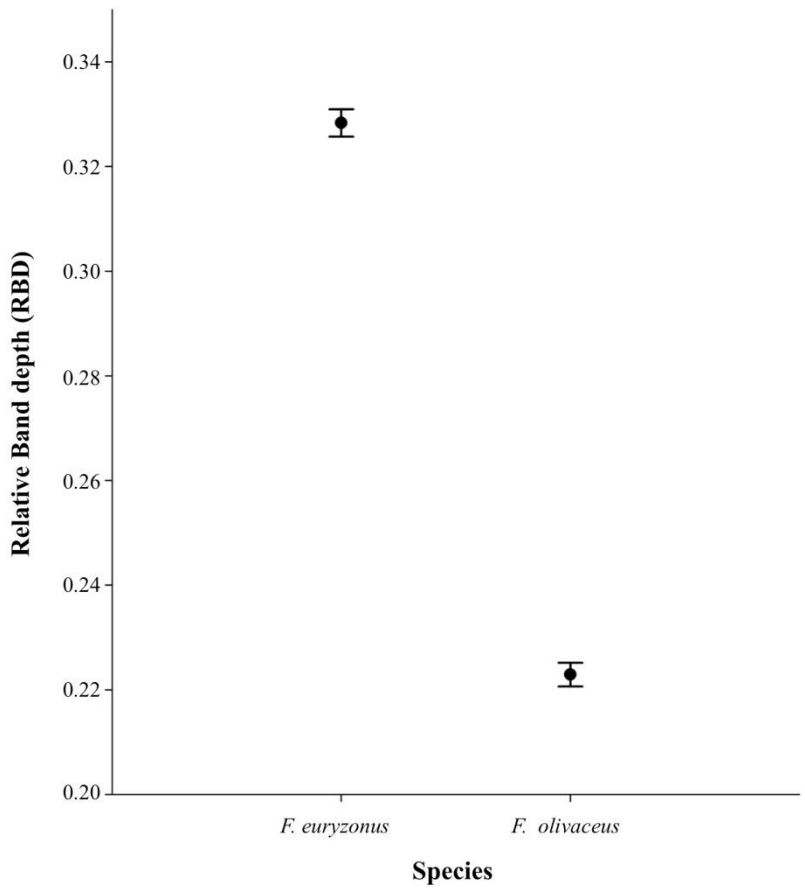

Figure 2.3 Mean relative band depth (RBD) of the two target species, *Fundulus olivaceus* and *F. euryzonus* (±1 SD).

Note: Species were tentatively morphologically identified using this criterion. A threshold value of 0.28 was used to place individuals into a species.

and 81 *F. euryzonus* with haplotype 3 (Table 2.2). There were six individuals of hybrid ancestry resolved by STRUCTURE (0.1<q-score>0.8) that were morphologically identified as *F. olivaceus* with haplotype 1, five with haplotype 2, and two with haplotype 3. Alternatively, there were two individuals morphologically identified as *F. euryzonus* with hybrid ancestry resolved by STRUCTURE (0.1<q-score>0.8) with haplotype 1 and six individuals with haplotype 3 (Table 2.2).

Table 2.2

Mitochondrial haplotypes resolved by an RFLP assay across sites and species.

Site	*Haplotype 1*	*Haplotype 2*	*Haplotype 3*
1	4/0/0	26/0/0	0/0/0
2	7/0/1	3/0/2	0/17/0
3	12/1/2	1/0/3	0/6/5
4	5/2/2	1/0/0	0/19/1
5	13/0/0	0/0/0	1/0/0
6	12/0/1	1/0/0	1/13/2
7	22/0/2	3/0/0	0/3/0
8	9/2/0	0/0/0	2/17/0

Note: Species were delineated via STRUCTURE analysis. Each column represents one of three resolved haplotypes. At each site data is represented in three numbers: number of *Fundulus olivaceus*, *F. euryzonus*, and hybrid ancestry, respectively. A threshold q-score of 0.90 was used to delineate species. Hybrid ancestry resolved by STRUCTURE (0.1<q-score>0.8).

The phylogenetic analysis of the internal mtDNA fragment amplified from the cytochrome b sequences revealed that all individuals from the Tangipahoa, regardless of their morphological and genetic identification, fell within a strongly supported monophyletic group of *F. olivaceus* sequences (Fig. 2.4).

I genetically identified 21 individuals as having hybrid ancestry, with a q-score between 0.8-0.2, comprising 9.3% of the population (Fig. 2.5A). The STRUCTURE analysis identified 124 *F. olivaceus* and 80 *F. euryzonus*. Sites exclusively inhabited by *F. olivaceus* had no evidence of hybrid ancestry (Fig. 2.5B; County Line Rd. and Magnolia Progress Rd.). There was a relatively even distribution of *F. euryzonus* and *F. olivaceus* across all other sites (Figure. 2.5B).

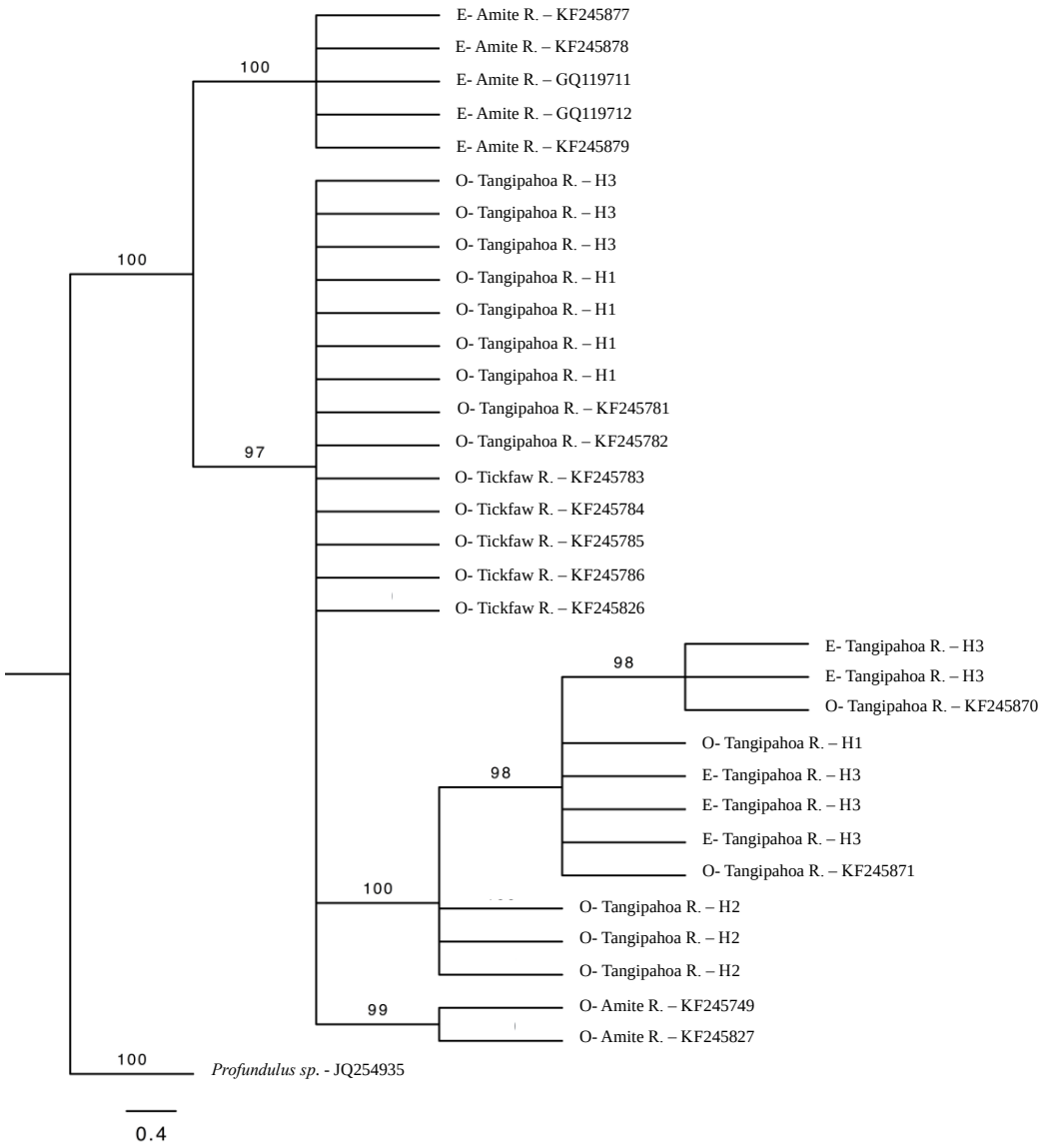

Figure 2.4 Inferred sequence relationship of *Fundulus* spp. cytochrome *b*.

Note: inferred using MrBayes from the internal mtDNA fragment of the cytochrome *b* gene of *Fundulus olivaceus* and *F. euryzonus* in the Lake Pontchartrain drainage. Mitochondrial sequences from either species in the Tangipahoa River appeared to be monophyletic with *F. olivaceus* mtDNA. Posterior probabilities reported in percentage on branches. Tree was rooted using an outgroup (*Profundulus sp.*) and visualized using Figtree.

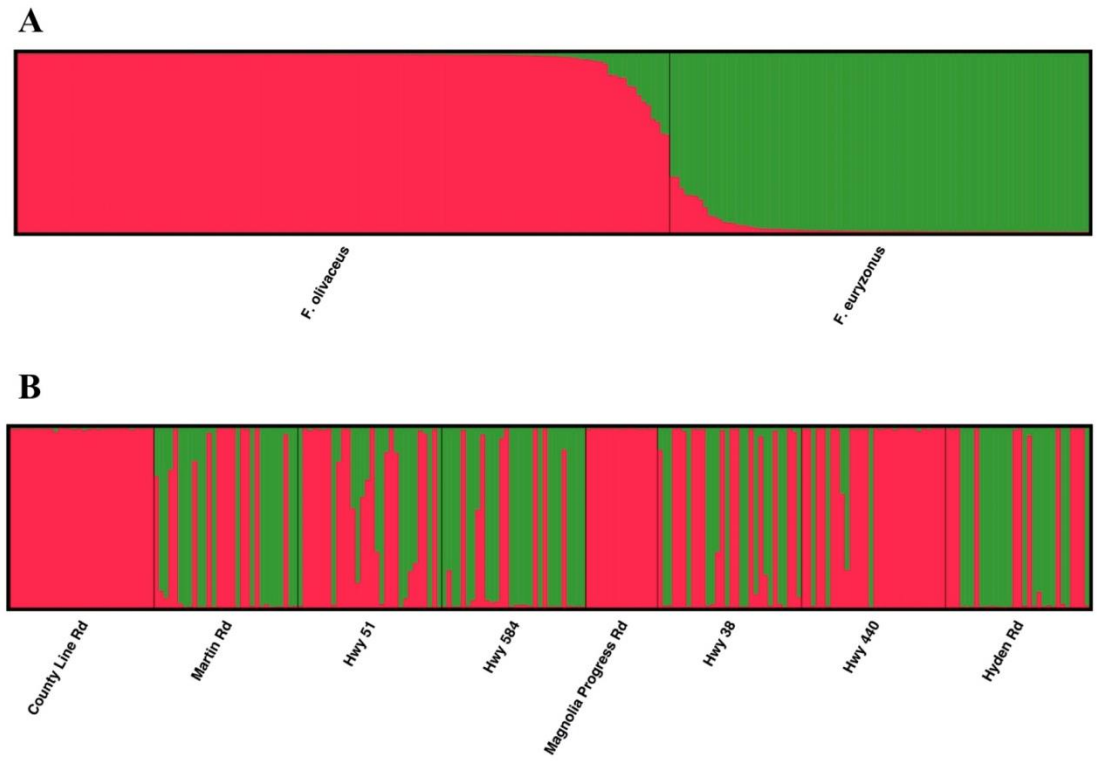

Figure 2.5 Bar plots for an analysis in STRUCTURE

Note: Plots organized by q-score (A) identified 21 hybrid individuals with a q-score between 0.8-0.2 (center), 124 *F. olivaceus* (red) and 80 *F. euryzonus* (green). Additionally, I organized by sampling location (B) to show areas (County Line Rd. and Magnolia Progress Rd) where *F. olivaceus* (red) occur without *F. euryzonus* (green) with very little nuclear introgression.

2.5 Discussion

In this study, I detected widespread mitochondrial introgression of *F. olivaceus* mtDNA into *F. euryzonus* over half the range of *F. euryzonus*. Various studies have been conducted on *F. euryzonus* from the Amite River (Duvernell et al., 2007; Vigueira et al., 2008; Schaefer et al., 2009; Schaefer & Walters 2010; Schaefer et al., 2011; Duvernell et al., 2013) but there are far fewer published records concerning *F. euryzonus* in the Tangipahoa River (Alford, 2014). However, there are currently no publications examining the differences between the genetically distinct groups of the two rivers where *F. euryzonus* occurs. I revealed higher rates of hybridization between these two species

than previously recorded in Schaefer et al. (2009) albeit with different markers. Another striking difference between this work and previously published research is that the hybridization was not recent. Duvernell et al. (2007) and Schaefer et al. (2009) both reported the appearance of F1 individuals. In the current study, fish appeared to have lower frequencies hybrid ancestry, and I did not recover any individuals that appeared to be first generation hybrids. There could be various explanations: 1) sampling error, 2) mitochondrial introgression has some effect on the rate of hybridization, or 3) using neutral microsatellites provided a different view of the genome than did RFLPs. The most likely of the aforementioned items is variation in marker types, but it is probably a combination of factors. More studies are warranted to further examine these systems for recent hybrids and common garden laboratory examinations of the nature of mitochondria origin as a determinant to hybridization rates.

Our morphological identification method is not a stand-alone technique for identifying these fish. Typically dorsal fin rays and gill rakers are counted along with RBD to delineate species within the *F. notatus* complex (Suttkus & Cashner, 1981; Ross, 2000). However, dorsal fin rays and gill raker counts can overlap dramatically (Schaefer et al., 2009) and I was conducting genetic analyses to corroborate RBD morphological identifications that are often cited as a distinct morphological character (Suttkus & Cashner, 1981; Ross, 2000; Schaefer et al., 2009).

I did not recover any haplotypes that fell into the *F. euryzonus* clade in the Tangipahoa River despite sampling 88 individuals across a large portion of the range (Suttkus & Cashner, 1981). In all 88 individuals I found haplotypes associated with *F. olivaceus*, revealing unidirectional mitochondrial introgression resulting in complete

fixation of the sister species mitochondria (i.e. mitochondrial capture). Haplotype 2 was not observed in any of our *F. euryzonus* samples, but was observed in high abundance in *F. olivaceus* only sites. Haplotype 1 was most commonly found in *F. olivaceus* and Haplotype 3 was found most commonly in *F. euryzonus*. Even though all haplotypes were of *F. olivaceus* ancestry there was a clear partitioning of haplotypes into different species. There is a rich literature documenting the effect of mtDNA haplotype on overall fitness (Cruzan & Arnold, 1999; Blier et al., 2001; Rhode & Cruzan, 2005; Etterson et al., 2007; Sambatti et al., 2008; Burton et al., 2013) and this could potentially be driving this sort of interaction. Further work would be needed to elucidate the role of the observed haplotypes in the different species to understand the role of these mitochondrial partitions.

Mitochondrial introgression is not rare in fish (Avise & Saunders, 1984; Wilson & Bernatchez, 1998; Yamada et al., 2001; McGuire et al., 2007; Genner & Turner, 2012; Laakkonen et al., 2015) or specifically in topminnows (Duvernell et al., 2007; Duvernell et al., 2013). Interestingly, the entire recorded range of *F. euryzonus* is a contact zone with *F. olivaceus* that includes only two rivers: the Tangipahoa River, a zone of mitochondrial capture, and the Amite River which is not an area of mitochondrial replacement (Schaefer et al., 2009). Thus, the current work outlines a simplified system to study the effects of mitochondrial capture on species evolution. However, there recent is evidence that *F. euryzonus* might also occur in the Pascagoula River (Schaefer, unpublished data).

When these two species hybridize, it has previously been shown that when the female is *F. olivaceus*, the F1 hybrids are sterile (Vigueira et al., 2008). This phenomenon leads to the hypothesis that introgression of *F. olivaceus* haplotypes into future generations would be limited. Not only was introgression not observed in the direction anticipated (*F. euryzonus* mtDNA into *F. olivaceus*) I observed the opposite result, which suggests some selective advantage overcame the apparent potential for loss of *F. olivaceus* mtDNA in this system and drove fixation. This introduces the need for future work to be done on fitness of hybrids in this system to elucidate the role of mitochondrial origin in the evolution of species.

2.6 Acknowledgments

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CHAPTER III – LANDSCAPE GENETICS OF FUNDULUS OLIVACEUS AND FUNDULUS EURYZONUS IN THE LAKE PONTCHARTRAIN DRANIAGE

3.1 Abstract

Understanding what drives and maintains biodiversity is one of the primary goals of evolutionary biology. Riverine systems containing closely related sympatric species provide an ideal system for understanding the role that different evolutionary processes play. I examined two sympatric species of the *Fundulus notatus* species complex (*Fundulus olivaceus* and *F. euryzonus*) across a narrow range within the Lake Pontchartrain Drainage, USA. One of these species, *F. euryzonus*, has a restricted range, being endemic to only two rivers in this drainage and has experienced widespread mitochondrial introgression with *F. olivaceus* within the Tangipahoa River. I sampled fish from 10 locations in the West Fork of the Amite River and examined how ecological factors and geographic distance influences genetic divergence. Then I replicated the study in the Tangipahoa River using 8 locations across a similar spatial scale. Populations were structured primarily based on species and river. When examining populations within rivers I found that *F. olivaceus* is heavily influenced by geographic location and environmental factors while *F. euryzonus* populations do not seem to differentiate within a river. These effects were stronger in the Amite River.

3.2 Introduction

One of the main goals in evolutionary biology is to understand the processes that shape and maintain biodiversity. Sympatric populations of closely related organisms offer an ideal perspective when trying to characterize evolutionary processes. Cohabitation of similar species has been attributed to adaptive radiation or competition. Adaptive

radiation in the honeycreepers of Hawaii (subfamily Drepanidinae) and competition in *Anolis* lizards in the Caribbean (family Dactyloidae) have aided in understanding the process of speciation and cohabitation (Losos et al., 1998; Pratt, 2001; Reding et al., 2009). Conversely, divergent evolution can occur solely due to ecological factors in allopatry where contemporary syntopic populations are due to secondary contact (Schluter, 2009).

Rivers are useful systems in which to examine ecological forces impacting diversification of genetic lineages, and provide a natural way to replicate these studies. Also, distinct and predictable ecological gradients exist along the river continuum (Vannote et al., 1980). Populations of aquatic species are known to demonstrate genetic differentiation based on the ecological gradients found along the river continuum (Wise et al., 1995; Langerhans et al., 2004; Dunithan et al., 2012; Jackrel & Wooten, 2014). Along with heterogeneity of habitat, distance itself can limit gene flow between populations of fishes in rivers where stochastic processes lead to diversification (Carlsson et al., 1999; Gomez-Uchida et al., 2009; Kanno et al., 2011; Stelkens et al., 2012; Earnest et al., 2014). Comparing evidence for isolation-by-distance (IBD) versus genetic associations to specific ecological conditions can elucidate the importance of ecological and adaptive factors on differentiation (Lowe et al., 2012; Espírito- Santo et al., 2013).

The topminnows in the *Fundulus notatus* complex are an emerging model system for natural hybridization studies (Duvernell et al., 2007; Schaefer et al., 2009; Schaefer et al., 2011; Duvernell et al., 2013; Duvernell & Schaefer, 2014). Within the *F. notatus* complex the broad-striped topminnow (*F. euryzonus*) is only recorded to exist in two rivers, the Amite River and Tangipahoa River (Suttkus & Cashner 1981). These species

are not ecological equivalents: *F. olivaceus* appears to specialize in smaller headwater streams, and *F. notatus* and *F. euryzonus* tend to be found in larger order streams (Blanchard, 1996; Schaefer et al., 2009; Earnest et al., 2014). This suggests that increased isolation of habitats preferred by *F. olivaceus* can lead to higher degrees of genetic differentiation compared to downstream species (Earnest et al., 2014).

The goal of this work is to use a fish model, *Fundulus* spp., to examine the patterns of genetic differentiation along a river continuum in the Amite and Tangipahoa Rivers. The framework of this study is similar to the one employed by Earnest et al. (2014) for their comparison of *F. notatus* and *F. olivaceus*. I will be comparing patterns of population structure/genetic differentiation between species in the two rivers. With rivers serving as replicates, I examined the underlying ecological and genetic factors at play in shaping genetic differentiation. I hypothesize that 1) *F. euryzonus,* like *F. notatus*, will exhibit characteristics of larger river fish, such as little to no genetic divergence within a river, whereas *F. olivaceus* will show patterns of greater genetic divergence along the same geographic scale and 2) relative abundance of these species will follow predictable biotic and abiotic factors (as per Schaefer et al., 2009). This work provides insight into the general evolutionary histories of this emerging model system.

3.3 Methods

3.3.1 Sampling Locations

Fish were sampled at 18 locations in the northern Amite and Tangipahoa Rivers of the Lake Pontchartrain Drainage in Mississippi and Louisiana, USA (Figure 3.1). Eight locations were sampled along the Tangipahoa River and tributaries (locations 1-8, Chapter II) from May, 2014-August, 2014, and 10 locations were sampled on the west

fork of the Amite River and tributaries therein (locations 9-18) from May, 2010-August, 2010. The sites sampled within the Amite River were identical to those sampled by Schaefer et al. (2009) in 2008.

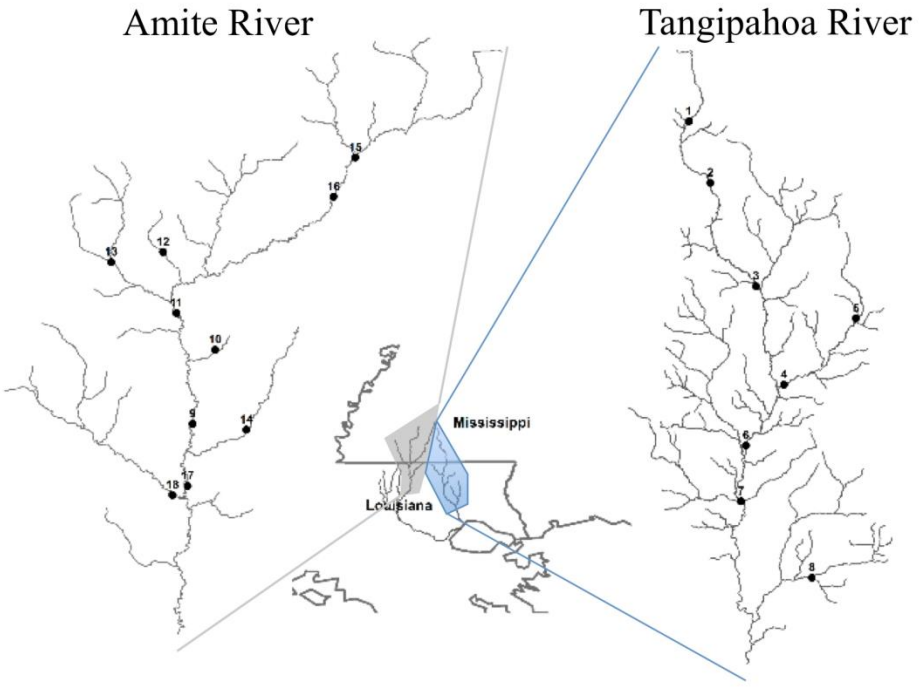

Figure 3.1 A map of the study sites in the Amite and Tangipahoa Rivers.

Note: Rivers are not to scale but are instead magnified to show sampling locations. This map shows the following sites within our study site: Tangipahoa River: 1-County Line Rd., 2-Martin Rd., 3- Hwy 51, 4- Hwy 584, 5- Magnolia-Progress Rd., 6- Hwy 38, 7- Hwy 440, 8- Hayden Rd.; Amite River: 9- Hwy 24, 10- Lazy Creek, 11- Clyde Graves Rd., 12- Newman Branch, 13- Days Creek, 14- Speculation Creek, 15- Rollinson Rd., 16- Coleman Rd., 17- Hwy 48, 18- Wagoner Creek.

3.3.2 Environmental data

Environmental data collection followed Schaefer et al. (2009) where data were collected and averaged across three transects at each site (upstream, middle, and downstream). At each transect, variables were measured at 3 places across the width of the river (25%, 50%, and 75%). Variables taken along each transect and each location included 9 total measurements each of the following 7 variables: temperature (temp)

(°C), conductivity (spc), total dissolved solids (tds) (YSI Professional Plus Series), substrate particle size (substrate) (modified Wentworth scale; Bain et al., 1985), surface current velocity (flow) (Marsh–McBirney Flowmate 2000, HACH Company, Loveland, Colorado, USA), and depth. All 9 measurements for each aforementioned environmental variable were averaged for a location. For each of the three transects I took a single measurement of wetted stream width, which was averaged for each location. Then, I also took a single measurement at each location of dissolved oxygen content (DO), pH (YSI Professional Plus Series), and turbidity (nephelometric turbitiy units, NTU; HACH 2100 turbidity meter, HACH Company, Loveland, Colorado, USA). The total suite of environmental parameters included 10 variables (temp, spc, tds, substrate, flow, depth, width, DO, pH, and turbidity).

3.3.3 Fish collection and preservation

I collected fish at each location using a dip net. Amite River sampling was conducted June-August, 2010 whereas Tangipahoa River sampling was conducted June – August, 2014. All fish were euthanized using an overdose of tricaine methanesulfonate (MS-222). Caudal fin clips were taken from euthanized fish and placed into a salt saturated (SED) preservation buffer (Seutin et al., 1991). The fish were then placed in an individually labeled 50 ml centrifuge tube containing 10% buffered formalin.

I extracted total genomic DNA from dorsal fin clips using the Qiagen DNeasy extraction kit (QIAGEN Inc., Valencia, CA), and genotyped each individual for seven microsatellite loci developed for *F. notatus* by Feldheim et al. (2014; *Fno*014, *Fno*091, *Fno*093, *Fno*112, *Fno*119, *Fno*242, *Fno*261). Polymerase chain reaction (PCR)

amplifications for microsatellite loci were conducted in 12.5 μL total volume using 50 mM KCl, 10mM Tris-HCl (pH 8.3), 0.01% gelatin, 1.5 – 2.0 mM $MgCl_2$, 200 μM dNTPs, 0.188 units of *Taq* polymerase (New England Biolabs), 0.3 μM of the M13 tailed forward primer (Boutin-Ganache et al., 2001), 0.3 μM of the reverse primer, 0.1 μM of the M13 labeled primer (LI-COR), 20-100 ng of the template DNA and water to the final volume. Cycling conditions for PCR consisted of an initial denaturing step of 94°C for 2 minutes followed by 35 cycles of 30 seconds at 94°C, 1 minute at 56°C and 1 minute at 72°C. A final elongation step of 10 minutes at 72°C ended the cycle. I visualized the microsatellite alleles using a LI-COR 4300 DNA Analysis system and scored them using Gene Image IR v. 3.55 (LI-COR).

3.3.4 Analyses

Species were defined in this study using Q-scores (proportion of an individual's ancestry assigned to a particular group) obtained using a Bayesian approach found in the software STRUCTURE v. 2.3.4 (Pritchard et al., 2000) analysis (Figure 3.2). Because fish are restricted to waterways and differentiation between waterways could confound species diagnosis (Chapter II), I therefore assumed 4 distinct genetic groups (*K*), 2 species in 2 different rivers where individuals with Q-score for any group greater than 0.90 were considered to belong to a species (see Chapter II). In the analysis I used the admixture model with sampling location as a prior (Hubisz et al., 2009) and a burn-in period of 100,000 generations with 200,000 steps of Markov Chain Monte Carlo (MCMC) simulations to estimate ln Pr(X|*K*). Results were then visualized using the program DISTRUCT v. 1.1 (Rosenberg, 2004). Individuals of hybrid ancestry were excluded from future analyses. If the removal of admixed individuals decreased the

sample size for a species at a given site to fewer than 4 individuals then the site was eliminated from future analyses (following McClintock & Waterway, 1993; Jump et al., 2003). Then, each species was examined for further population subdivision.

The number of genetic groups for each species was then determined from the reduced data set using identical parameters in STRUCTURE. Subsequent STRUCTURE runs were then performed in a hierarchical fashion until recovering K=1 for individual clusters. The average log likelihood of twenty iterations (Gilbert et al., 2012) for each value of K was obtained using the program CLUMPP (Jakobsson & Rosenberg, 2007). The best value of K was then determined by examination of the log likelihood scores (Pritchard et al., 2000) and the ΔK analysis of Evanno et al. (2005) as implemented by STRUCTURE HARVESTER (Earl & vonHoldt, 2012). Results for the best value of K were visualized using the program DISTRUCT.

For each species within each drainage, allele frequencies, number of alleles (N_A), observed heterozygosity (H_O), and expected heterozygosity (H_E) were calculated using GeneAlEx v. 6.5 (Peakall & Smouse, 2006, 2012). Tests for Hardy-Weinberg equilibrium and linkage disequilibrium were performed with Genepop v. 4.2 (Raymond & Rousset, 1995; Rousset, 2008) with a post-hoc Bonferroni correction applied. I used ARLEQUIN (Excoffier et al., 2005) to calculate F_{ST} values for population pairs using the full data set. To assess IBD I used a Mantel's test implemented in the Isolation by Distance Web Service (IBDWS) version 2 (Jensen et al., 2005). River distance (km) among sites was calculated using GOOGLE EARTH (Google, Mountain View, CA). Environmental variation was visualized using a principal component analysis (PCA) in R (R Development Core Team, 2013).

3.4 Results

The average number of alleles per locus at a site ranged between 6.143 and 16.143 (Table 3.1). Average observed heterozygosity estimates ranged between 0.519 and 0.827 (Table 3.1). All loci were within Hardy-Weinberg equilibrium (HWE) and among all locus pairs there was no evidence for linkage disequilibrium. In terms of occurrence, *F. olivaceus* in both streams dominated the headwater/tributary locations (County Line Rd. and Magnolia-Progress Rd. in the Tangipahoa River and Rollinson Rd., Coleman Rd., Days Creek, and Speculation Creek in the Amite River) whereas downstream locations were often sites of co-occurrence (Table 3.1).

Table 3.1

Average number of alleles (N_A), average observed heterozygosity (H_O) and average expected heterozygosities (H_E) for the different species and hybrids at each location.

	Site	Species	N	N_A	H_O	H_E
Tangipahoa River	1 – County Line Rd	*F. olivaceus*	30	14.000	0.551	0.756
		F. euryzonus	0	-	-	-
		Hybrids	0	-	-	-
	2 – Martin Rd	*F. olivaceus*	10	8.857	0.643	0.727
		F. euryzonus	17	9.286	0.529	0.630
		Hybrids	3	-	-	-
	3 – Hwy 51	*F. olivaceus*	13	12.000	0.633	0.775
		F. euryzonus	7	6.143	0.612	0.636
		Hybrids	10	10.429	0.600	0.826
	4 – Hwy 584	*F. olivaceus*	5	6.429	0.550	0.746
		F. euryzonus	22	12.000	0.598	0.744

		Hybrids	3	-	-	-
	5 – Magnolia Progress Rd	*F. olivaceus*	15	10.857	0.571	0.749
		F. euryzonus	0	-	-	-
		Hybrids	0	-	-	-
	6 – Hwy 38	*F. olivaceus*	14	11.857	0.592	0.751
		F. euryzonus	13	8.143	0.593	0.640
		Hybrids	3	-	-	-
	7 – Hwy 440	*F. olivaceus*	25	16.143	0.640	0.783
		F. euryzonus	3	-	-	-
		Hybrids	2	-	-	-
	8 – Hayden Rd	*F. olivaceus*	11	9.857	0.519	0.751
		F. euryzonus	19	9.000	0.579	0.578
		Hybrids	0	-	-	-
Amite River	9 – Hwy 24	*F. olivaceus*	9	8.571	0.587	0.708
		F. euryzonus	20	10.000	0.681	0.744
		Hybrids	1	-	-	-
	10 – Lazy Creek	*F. olivaceus*	28	15.857	0.727	0.769
		F. euryzonus	1	-	-	-
		Hybrids	1	-	-	-
	11 – Clyde Graves Rd	*F. olivaceus*	15	11.286	0.698	0.756
		F. euryzonus	13	7.286	0.766	0.714
		Hybrids	2	-	-	-
	12 – Newman Branch	*F. olivaceus*	14	9.857	0.712	0.735
		F. euryzonus	0	-	-	-
		Hybrids	0	-	-	-

13 – Days Creek	*F. olivaceus*	30	16.000	0.723	0.759
	F. euryzonus	0	-	-	-
	Hybrids	0	-	-	-
14 – Speculation Creek	*F. olivaceus*	17	10.286	0.667	0.731
	F. euryzonus	0	-	-	-
	Hybrids	0	-	-	-
15 – Rollinson Rd	*F. olivaceus*	29	14.286	0.670	0.729
	F. euryzonus	1	-	-	-
	Hybrids	1	-	-	-
16 – Coleman Rd	*F. olivaceus*	12	10.286	0.702	0.725
	F. euryzonus	9	6.143	0.743	0.731
	Hybrids	4	-	-	-
17 – Hwy 48	*F. olivaceus*	0	-	-	-
	F. euryzonus	19	9.143	0.659	0.714
	Hybrids	1			
18 – Wagoner Creek	*F. olivaceus*	2	-	-	-
	F. euryzonus	21	8.286	0.682	0.781
	Hybrids	8	6.143	0.827	0.743

Note: Seven microsatellite loci (Feldheim et al., 2014) were amplified for both *Fundulus euryzonus* and *Fundulus olivaceus*. Species determinations were based on a Q-score >0.90 for either species individuals with a q-score between 0.10 and 0.90 were assumed to have hybrid ancestry and were reported as "Hybrids" in the following table. Sites are labeled with a number that corresponds to Figure 3.1. Population metrics for populations with fewer than 5 individuals were not calculated.

3.4.2 Species identification and hybrids

Bayesian clustering analyses in STRUCTURE of 4 populations when examining all 483 individuals across 18 locations, separated the 2 species into 2 rivers (Figure 3.2). Before examining further subdivision, I separated analyses by species and then

eliminated admixed individuals with a Q-score less than 0.90 for either species. Admixed individuals represented 8.1% of the complete dataset, leaving 444 individuals in the dataset. In the Amite River, I observed 7.0% admixed individuals whereas the Tangipahoa had 9.3% admixed individuals. Both rivers had ~1% putative F1 with Q-scores between 0.4-0.6. The average Amite River *F. olivaceus* Q-score was 0.994 (standard deviation, SD = 0.013) while the average Tangipahoa River *F. olivaceus* q-score was 0.992 (SD = 0.009). Similarly, the average Amite River *F. euryzonus* Q-score was 0.987 (SD = 0.017), and the average Tangipahoa River *F. euryzonus* Q-score was 0.974 (SD = 0.110). After removing admixed individuals and separating species, 3 *F. euryzonus* locations (Hwy 440, Lazy Creek, and Rollinson Rd.) and 1 *F. olivaceus* location (Wagoner Creek) had fewer than 4 individuals (n=3, n=1, n=1 and n=2 respectively) and were subsequently removed from future analyses but still considered syntopic locations.

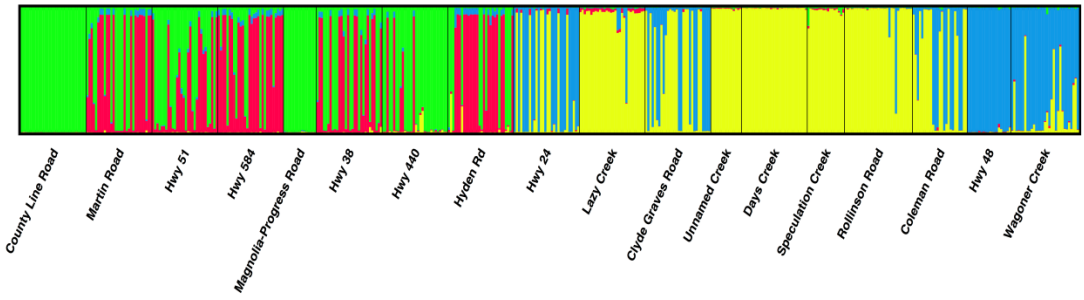

Figure 3.2 A bar plot of ancestry coefficients of all fish sampled together shows a clear distinction between species and rivers.

Note: The Tangipahoa River sites are represented in the above figure as the first 8 sites (County Line Road-Hayden Road) and the Amite River sites are the following 10 locations (Hwy 24-Wagoner Creek). In the Tangipahoa River *F. olivaceus* are represented by the color green and *F. euryzonus* are represented with the color red. In the Amite River *F. olivaceus* are represented with yellow and *F. euryzonus* are represented with blue.

3.4.3 Population differentiation

The Amite River, despite having a shorter sampling range, had far more genetic clusters across species (6 total groups) compared to the Tangipahoa River (3 total groups). *Fundulus olivaceus* had far more genetic clusters across the sampling range (7 total groups) compared to *F. euryzonus* (2 total groups). In the Tangipahoa River, *F. olivaceus* appeared as two genetic groups (Figures 3.3 & 3.4) representing upstream (sites 1 & 2) and downstream (site 8) portions with various degree of admixture across sites in the middle. It is important to note that site 3 does not fully represent this pattern but is also a hotspot for hybridization between species. Similarly, in the Amite River *F. olivaceus* demonstrated some degree of differentiation along predictable geographic groupings (Figures 3.5 & 3.6). The Hwy 24 and Lazy Creek locations cluster together genetically, are close together geographically, the Clyde Graves Road, Newman Branch, and Days Creek locations are genetically similar and geographically close together, the Speculation Creek location exists on a secluded larger tributary, and the Rollinson and Coleman Road are genetically similar locations in the northeastern portion of the sampling range. The Tangipahoa River *F. euryzonus* analysis suggested $K = 3$ (Figure 3.7) however that clustering scheme did not work well, leaving clusters without representative members (Figure 3.8) suggesting a highly variable single group ($K = 1$) is more likely. Similarly, the Amite River *F. euryzonus* analysis suggested many potential values of K (Figure 3.9) with high error, but when consensus bar plots were examined clusters were without representative members, and did not conform to a reasonable biological scenario (Figure 3.10) and was therefore considered to be a single variable group ($K = 1$).

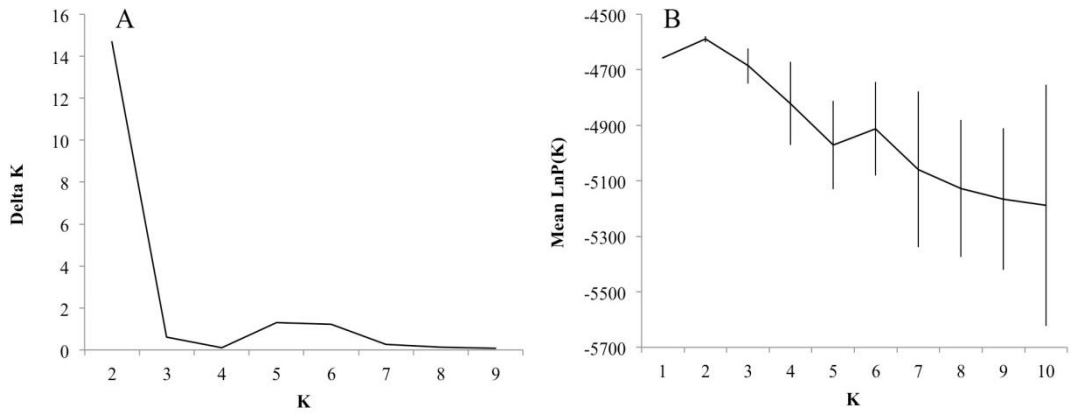

Figure 3.3 Delta *K* plot (A) and plot of mean likelihood values at each *K* (B) with error represented in standard deviation of *F. olivaceus* populations in the Tangipahoa River.

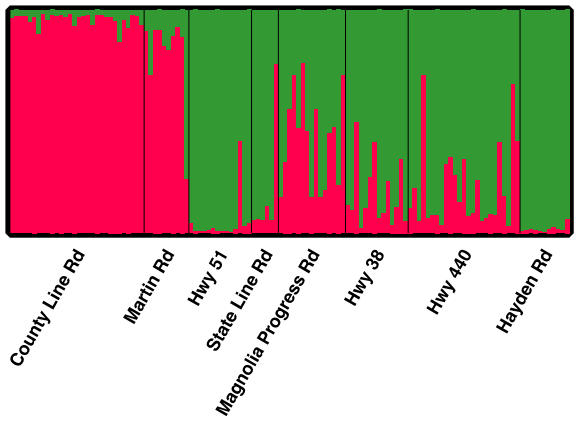

Figure 3.4 Bar plot from STRUCTURE representing *K* = 2 averaged across 20 iterations for *F. olivaceus* in the Tangipahoa River.

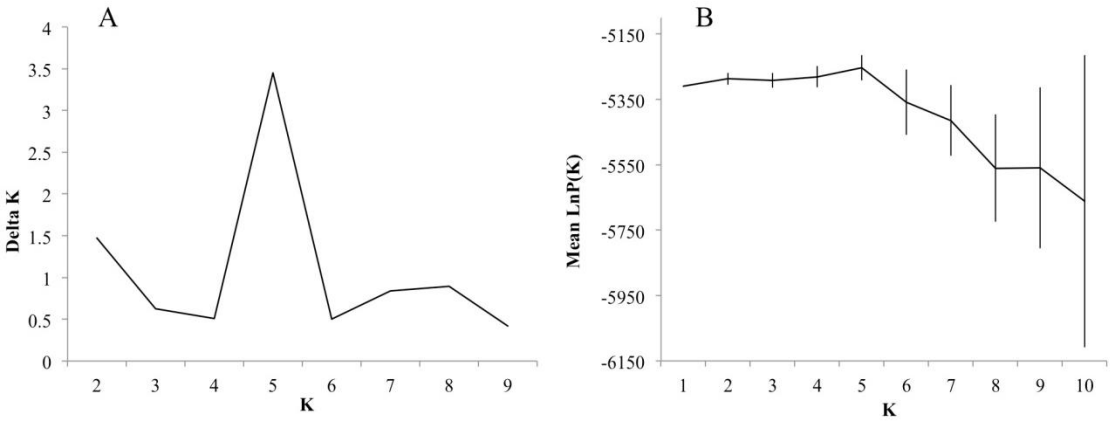

Figure 3.5 Delta *K* plot (A) and plot of mean likelihood values at each *K* (B) with error represented in standard deviation of *F. olivaceus* populations in the Amite River.

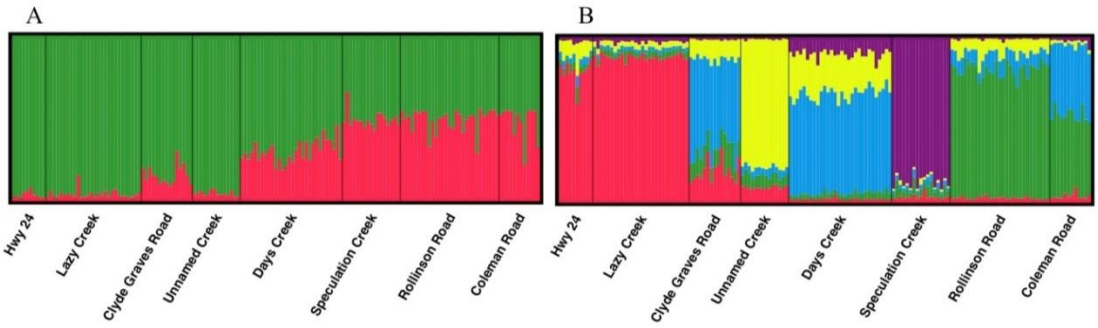

Figure 3.6 Bar plots from STRUCTURE representing two different *K* values; K=2 (A) and K=5 (B), values were averaged across 20 iterations for *F. olivaceus* in the Amite River.

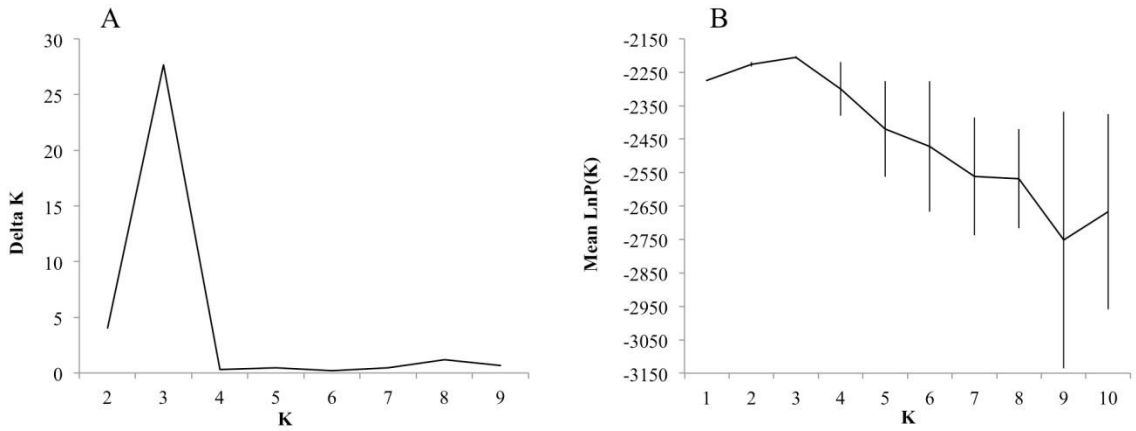

Figure 3.7 Delta *K* plot (A) and plot of mean likelihood values at each *K* (B) with error represented in standard deviation of *F. euryzonus* populations in the Tangipahoa River.

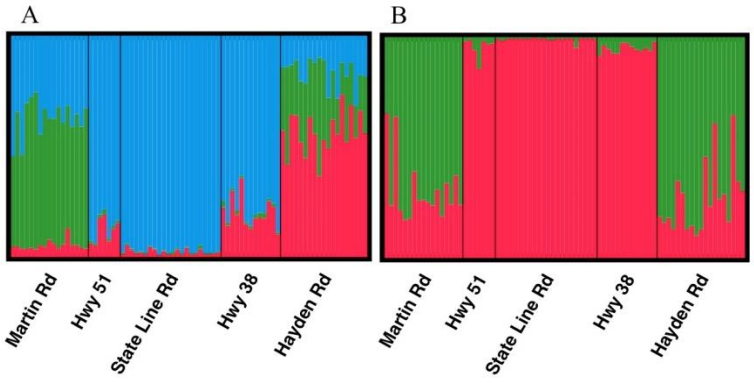

Figure 3.8 Bar plots from STRUCTURE representing two different *K* values: K=3 (A) and K=2 (B), values were averaged across 20 iterations for *F. euryzonus* in the Tangipahoa River.

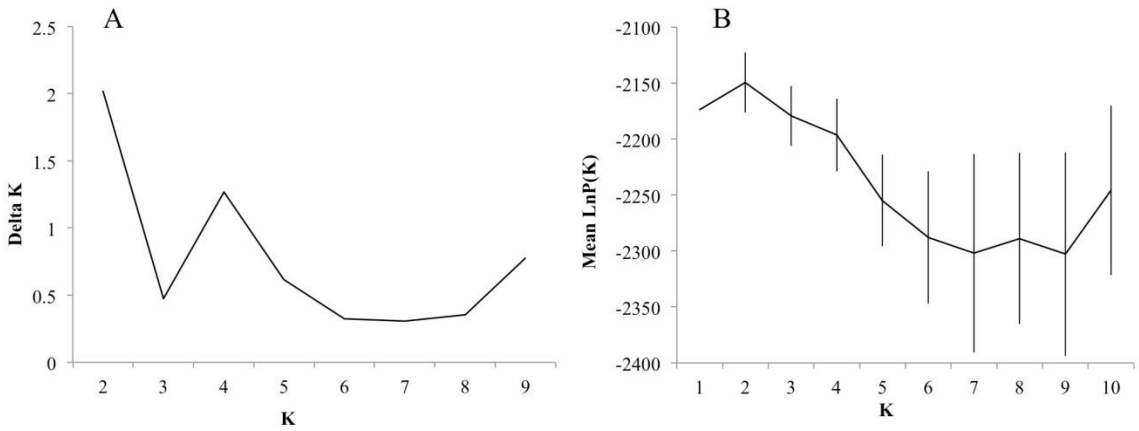

Figure 3.9 Delta *K* plot (A) and plot of mean likelihood values at each *K* (B) with error represented in standard deviation of *F. euryzonus* populations in the Amite River.

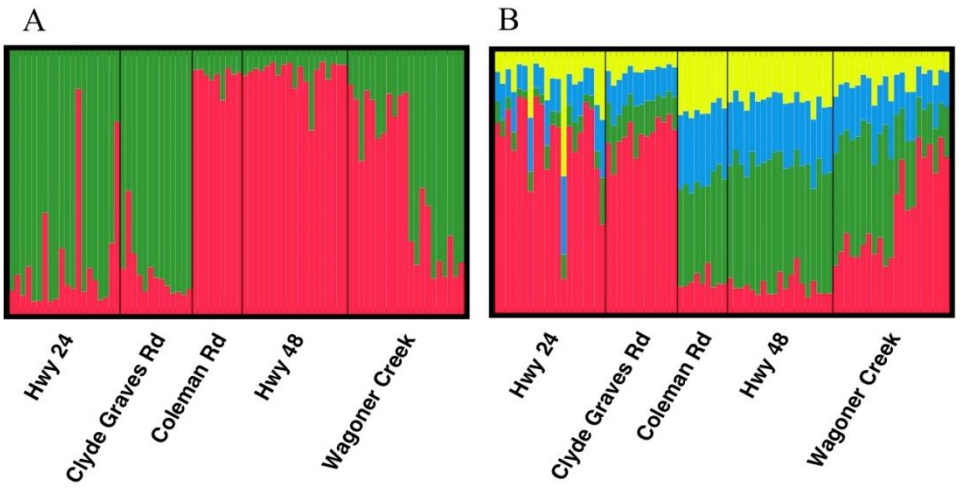

Figure 3.10 Bar plots from STRUCTURE representing two different *K* values; K=2 (A) and K=4 (B), values were averaged across 20 iterations for *F. euryzonus* in the Amite River.

Both species in both rivers showed no sign of IBD. Tangipahoa River *F. olivaceus* (R^2=0.001; p = 0.512; Table 3.2; Figure 3.11), Amite River *F. olivaceus* (R^2=0.039; p = 0.208; Table 3.3; Figure 3.12), Tangipahoa River *F. euryzonus*

(R^2=0.136; p = 0.125; Table 3.4; Figure 3.13), and Amite River *F. euryzonus* (R^2=0.026; p = 0.320; Table 3.5; Figure 3.14) all lacked evidence of IBD.

Table 3.2

Genetic and geographic distances among sites of *Fundulus olivaceus* in the Tangipahoa River.

Location	1	2	3	4	5	6	7	8
1	-	12.98	33.15	73.37	55.050	66.770	77.367	113.607
2	0.032	-	20.170	60.390	42.070	53.791	64.387	100.627
3	0.034	0.050	-	40.220	21.900	33.621	44.217	80.457
4	0.024	0.034	0.027	-	18.320	34.760	41.776	78.016
5	0.058	0.065	0.068	0.040	-	12.860	23.456	59.696
6	0.034	0.042	0.035	0.022	0.047	-	10.596	46.836
7	0.022	0.029	0.028	0.019	0.047	0.025	-	36.240
8	0.039	0.054	0.043	0.031	0.062	0.036	0.027	-

Note: Below the diagonal are the pairwise F_{ST} values from Arlequin and above the diagonal are river distances in kilometers (km). Among sites Location numbers correspond with Figure 3.1 where: 1-County Line Rd., 2-Martin Rd., 3- Hwy 51, 4- Hwy 584, 5-Magnolia-Progress Rd., 6- Hwy 38, 7- Hwy 440, 8- Hayden Rd.

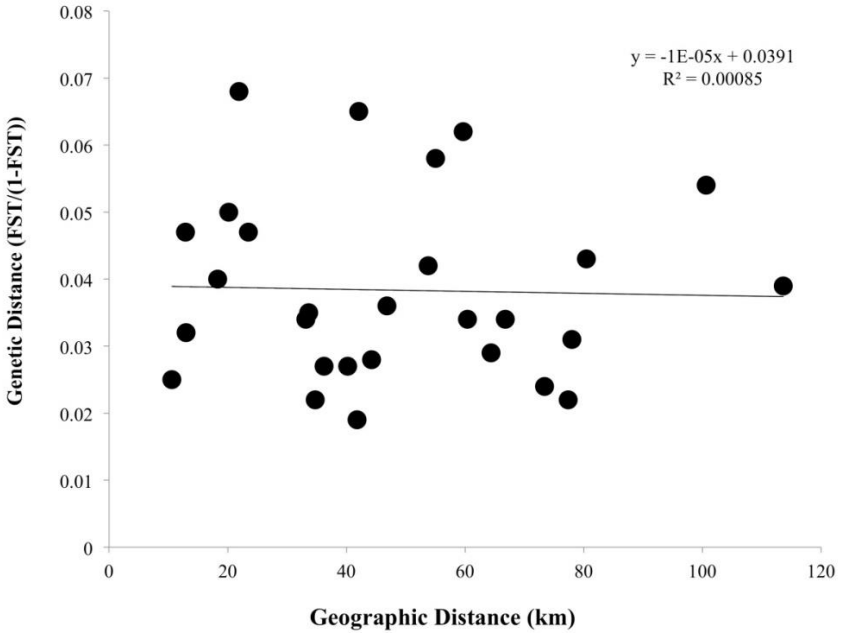

Figure 3.11 Relationship between genetic distance (FST/(1-FST)) and geographic distance (km) for *F. olivaceus* in the Tangipahoa River (p=0.512).

Table 3.3

Isolation by Distance matrix for *Fundulus olivaceus* in the Amite River.

Location	9	10	11	12	13	14	15	16	18
9	-	8.320	10.700	16.300	18.290	8.500	30.370	26.390	9.800
10	0.040	-	6.960	12.560	14.550	16.820	26.630	22.650	18.120
11	0.043	0.026	-	5.600	7.590	19.200	19.670	15.690	20.500
12	0.050	0.023	0.040	-	10.210	24.800	19.090	15.110	24.610
13	0.058	0.020	0.025	0.028	-	26.790	24.280	20.300	28.090
14	0.071	0.030	0.046	0.050	0.027	-	38.870	34.890	11.220
15	0.067	0.023	0.034	0.034	0.025	0.041	-	3.980	34.010
16	0.061	0.027	0.030	0.042	0.028	0.043	0.020	-	30.030
18	0.112	0.085	0.073	0.101	0.085	0.098	0.092	0.077	-

Note: The lower matrix is the pairwise F_{ST} from Arlequin, the upper matrix is river distance in kilometers (km). Location numbers correspond with Figure 3.1 where: 9- Hwy 24, 10- Lazy Creek, 11- Clyde Graves Rd., 12- Newman Branch ,13- Days Creek, 14- Speculation Creek, 15- Rollinson Rd., 16- Coleman Rd., 18- Wagoner Creek.

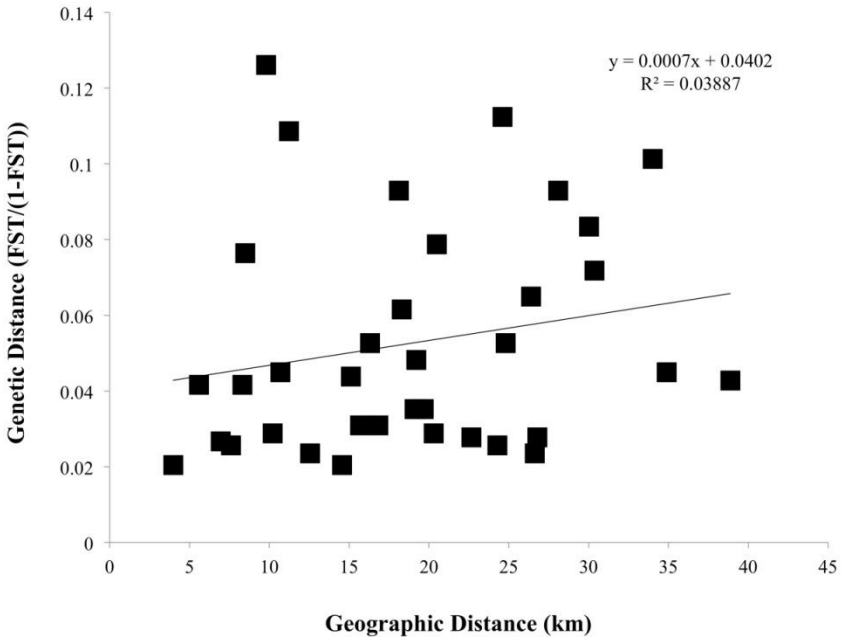

Figure 3.12 Relationship between genetic distance (FST/(1-FST)) and geographic distance (km) for *F. olivaceus* in the Amite River (p=0.208).

Table 3.4

Isolation by Distance matrix for *Fundulus euryzonus* in the Tangipahoa River.

Location	2	3	4	6	7	8
2	-	20.170	42.070	53.791	64.387	100.627
3	0.076	-	21.900	33.621	44.217	80.457
4	0.051	0.038	-	12.860	23.456	59.696
6	0.060	0.032	0.030	-	10.596	46.836
7	0.106	0.066	0.062	0.047	-	36.240
8	0.064	0.054	0.052	0.040	0.050	-

Note: The lower matrix is the pairwise F_{ST} from Arlequin, the upper matrix is river distance in kilometers (km). Location numbers correspond with Figure 3.1 where: 2-Martin Rd., 3- Hwy 51, 4- Hwy 584, 6- Hwy 38, 7- Hwy 440, 8- Hayden Rd.

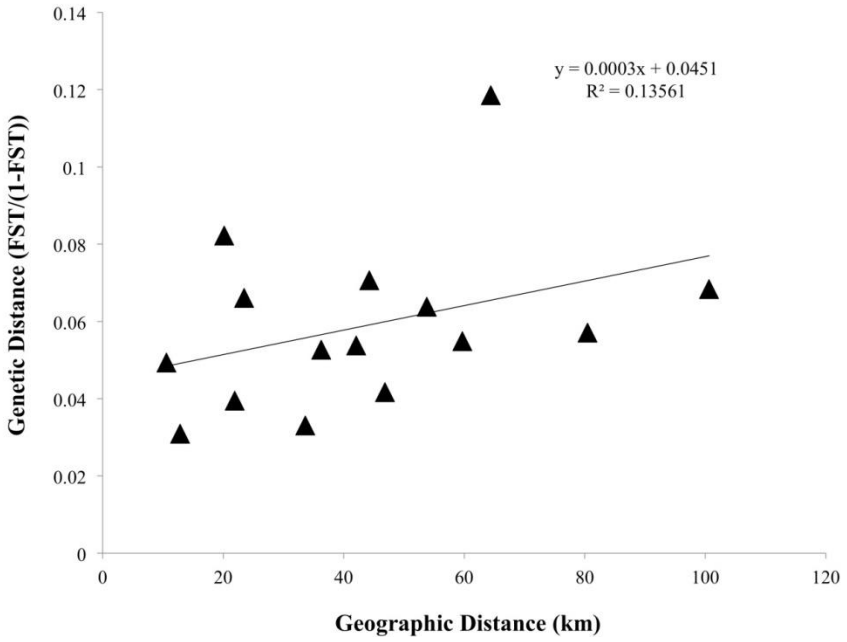

Figure 3.13 Relationship between genetic distance (FST/(1-FST)) and geographic distance (km) for *F. euryzonus* in the Tangipahoa River (p=0.125).

Table 3.5

Isolation by Distance matrix for *F. euryzonus* in the Amite River.

Location	9	11	16	17	18
9	-	8.320	10.700	26.390	9.800
11	0.013	-	6.960	22.650	18.120
16	0.043	0.043	-	15.690	20.500
17	0.039	0.042	0.025	-	30.030
18	0.020	0.022	0.029	0.028	-

Note: The lower matrix is the pairwise F_{ST} from Arlequin, the upper matrix is river distance in kilometers (km). Location numbers correspond with Figure 3.1 where: 9- Hwy 24, 11- Clyde Graves Rd., 16- Coleman Rd., 17- Hwy 48, 18- Wagoner Creek.

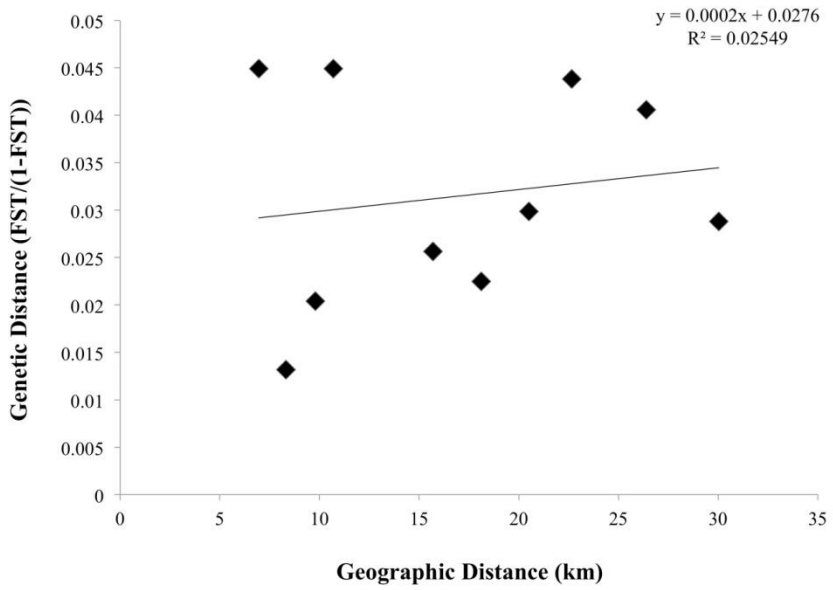

Figure 3.14 Relationship between genetic distance (FST/(1-FST)) and geographic distance (km) for *F. euryzonus* in the Amite River (p=0. 320).

The principal components analyses (PCA) summarizing the variation among environmental variables examined along the Amite River in ordination space suggest differentiation in locations containing one species (Figure 3.15). Three axes, from the broken stick model, were used to describe 77.02% of the variation in the data. PCI (40.42%) best described species only locations where PCI is most closely related to flow rate, DO, and width (lower scores) and spc and tds (higher scores). The PCA describing the arrangement of the Tangipahoa locations in ordination space also suggest differentiation in environmental parameters associated with locations where only one species is found (Figure 3.16). There was, however, a single site (Site 4 – Hwy 584) with both species that did not conform to the observed differentiation. The genetic groupings within the *F. euryzonus* of the Tangipahoa showed no clear distinction in environmental variables between groups. The final model used 2 axes describing 71.31% of the

variation in the data. PCI explains 45.83% of the variation in the data. Lower scores on PCI are associated with increased width, and substrate particle size, and higher scores are associated with an increase in turbidity. PCII explains 27.48 % of the variation. Higher scores on PCII are associated with increased DO content whereas lower scores are associated total dissolved solids (tds) and conductivity (spc). Locations where only *F. olivaceus* were recovered (red) and syntopic locations (blue) are plotted in different colors for visualization purposes. Sites with *F. olivaceus* only tend to have higher turbidity, and are smaller, slower, and warmer than sites with both species. Additionally, the circled location had a higher level of hybridization. Both streams had a single site with abnormally high admixture: Wagoner Creek in the Amite River, and the Hwy 51 location in the Tangipahoa River (circled in the figures).

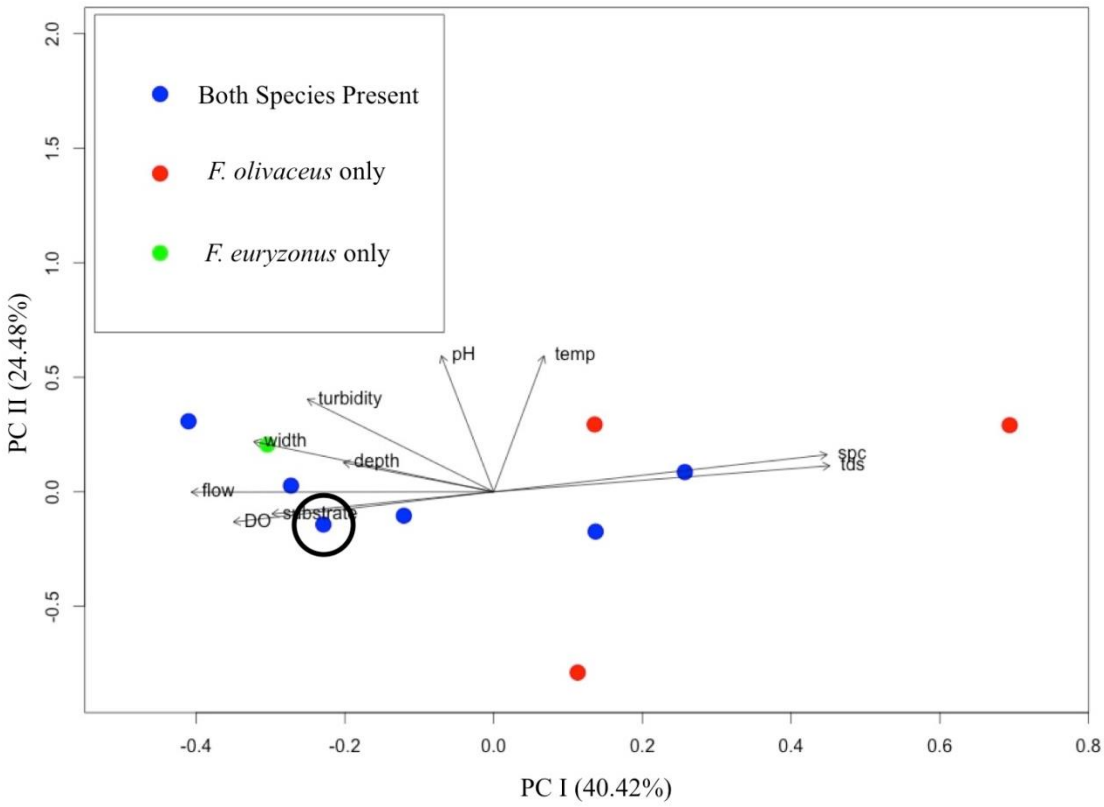

Figure 3.15 A principal components analyses (PCA) describing the arrangement of the Amite River locations in environmental ordination space.

Note: Blue points are locations that had both species, red points are sampling locations where only *Fundulus olivaceus* were recovered and the green point is the location where only *F. euryzonus* were recovered. Three axes were used to describe the whole model that accounted for 77.02 % of the variation in the data. Plotted above are the first two axes describing 40.42% of the data and 24.48% of the data respectively. Both PC axes describe typical headwater to larger river variation where *F. olivaceus* is found in headwater streams and *F. euryzonus* is located in the largest streams. Circled is the location with the highest amount of hybridization.

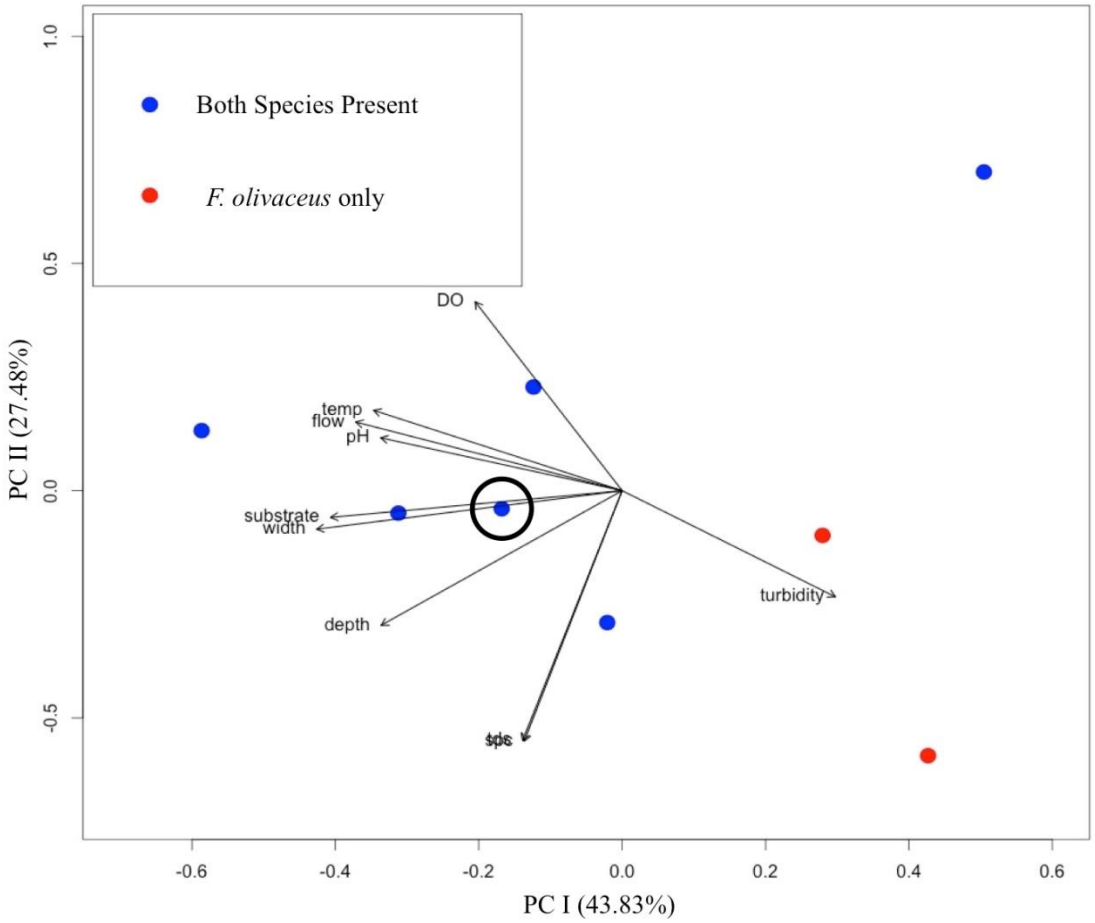

Figure 3.16 A principal components analysis (PCA) plot describing the variation in the locations in environmental ordination space.

3.5 Discussion

The data supported the prediction that species which tend to be found in headwater streams experience more population subdivision than species that tend to be located downstream. In both rivers, *F. olivaceus* showed more pronounced genetic differentiation, including the presence of multiple genetically distinct groups, compared to *F. euryzonus*. Pronounced genetic differentiation of *F. olivaceus* is consistent with Earnest et al. (2014). However, in the present study I did not detect significant differences in IBD or any indications of patterns in F_{ST} values among populations for

either species. However, R-squared values suggest a trend to IBD for *F. euryzonus* in the Tangipahoa River (R^2=0.136). And, considering that STRUCTURE results for that particular group were inconclusive there may be underlying IBD even though the results of the current study were not significant. Further studies with more locations analyzed in this system might resolve IBD.

As in other studies examining these species (Blanchard, 1996, Schaefer et al., 2009) and this species complex (Earnest et al., 2014) I showed there is an ecological difference between headwater and downstream species that is typical of the river continuum. Environmental analyses, for both rivers, were generally consistent with the river continuum concept (Vannote et al., 1980) where smaller streams are quantitatively different from larger downstream habitats. In the Amite River, I sampled sites with both species present and sites where each species exists alone (Figure 3.16). Locations where the only species recovered was *F. olivaceus* were indicative of smaller streams (more narrow, shallow, less turbid, and slower moving). Locations where the only species recovered was *F. euryzonus* were indicative of larger streams (wider, deeper, more turbid, and faster moving). Locations where both species were recovered were more intermediate streams. In the Tangipahoa River high turbidity measures, which should be associated with large streams, were associated with the smallest streams. Both of the smallest streams are within agricultural areas that could have affected the turbidity readings where Site 1 (State Line Rd.) had the higher scores. Additionally, there were no *F. euryzonus* only locations recovered in the Tangipahoa River. Similar to the Amite River, locations with both species were more intermediate and locations with *F. olivaceus* only were indicative of smaller streams (more narrow, lower DO).

Using different markers, Schaefer et al. (2009) observed slightly higher levels of hybridization (2.8%) at analogous locations to the present study (7.0%). However, hybrid individuals in the Schaefer et al. (2009) study came from 5 of the 10 locations each with similar proportions of hybridization. Earnest et al. (2014) observed a 7.6% hybridization rate between *F. olivaceus* and *F. notatus*, which more closely resembles the hybridization rate observed in the current study; however, hybridization was spread across multiple locations. In the present study, the Tangipahoa River had higher rates of hybridization (9.3%) potentially indicating that the two sister species within this species complex (*F. olivaceus* and *F. euryzonus*) more readily interbreed in the wild. However, laboratory studies are needed to corroborate this observation.

Both streams had a single site with high levels of admixture: Wagoner Creek in the Amite River, and the Hwy 51 location in the Tangipahoa River (circled in Figures 3.16 and 3.17). Both of these sites were in tributaries either at the confluence of two large tributaries (site 3) or just before the interface with the main channel (site 18) (Figure 3.1). These results corroborate the results of Schaefer et al. (2011) observing that hybrids are found near confluences of tributaries and rivers. The strength of the ecological gradient was also important as both of these locations were among the most typical environmental locations in the data set being closely placed in ordination space to the origin. This suggests that hybridization hotspots may be closely tied to locations with the most shared environmental conditions for both species.

With the recent description of microsatellite loci by Feldheim et al. (2014), this work represents an early representation of the power of these loci to differentiate populations in an ecological model (Earnest et al., 2014). Contact zones, such as the

system described in this work, offer a natural laboratory to study evolution (Bridle et al., 2001; Schaefer et al., 2011). This particular contact zone is interesting because of the limited range of *F. euryzonus* to these two river systems and that one of the two river systems shows evidence of a mitochondrial replacement event. In future studies this system can provide novel insight, not only into the evolution of these two species, but also the effect of mitochondrial replacement on various ecological and evolutionary phenomenon.

3.6 Acknowledgments

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CHAPTER IV – LANDSCAPE GENOMICS OF FUNDULUS SPP. IN THE LAKE PONTCHARTRAIN DRAINAGE

4.1 Abstract

Evolutionary biology aims to elucidate the driving factors of biodiversity. Closely related syntopic species provide the primer condition for understanding the role of different evolutionary processes. I examined populations of two species of topminnows, *Fundulus olivaceus* and *F. euryzonus*, in the Lake Pontchartrain drainage. I sampled northern and southern syntopic locations in the Amite and Tangipahoa rivers, and then sampled comparable latitudes in the Tickfaw River. Using genotype-by-sequencing (GBS) technology to scan the genomes for single nucleotide polymorphisms (SNPs) I developed hierarchical populations genomic models of differentiation in the Lake Pontchartrain drainage to 1) better understand the population structure in a emerging model system and 2) examine evidence for cohabitation of all three species in the *F. notatus* complex in a single location, As expected, *F. olivaceus* were more genetically subdivided within rivers than were *F. euryzonus*. However *F. olivaceus*, in the Tickfaw River, are not subdivided and *F. euryzonus* in the Tangipahoa may have underlying subdivision. There was no evidence of cohabitation for all three species in the Tangipahoa River. *Fundulus olivaceus* has population subdivision patters similar to downstream fish such as *F. euryzonus*.

4.2 Introduction

Understanding the processes that drive and maintain patterns of biodiversity is an essential aspect of evolutionary biology. Species complexes, groups of species that are morphologically and ecologically very similar but have pronounced genetic divergence,

offer a unique perspective to examine the factors that influence biodiversity. Typically, these organisms are closely related arising from adaptive radiation (Losos et al., 1998; Filchak et al., 2000; Xie et al., 2007; Reding et al., 2009) or ecological speciation (Schluter, 2009). However, members of species complexes are not always completely reproductively isolated and may interbreed (Vigueira et al. 2008). Understanding the patterns of differentiation for the members of a species complex can shed light on the evolutionary processes that led to divergence.

The topminnows in the *Fundulus notatus* complex are an emerging model system for hybridization studies (Duvernell et al., 2007; Schaefer et al., 2009; Schaefer et al., 2011; Duvernell & Schaefer, 2014). Within the *F. notatus* complex the broad-striped topminnow (*F. euryzonus*) is only recorded in two rivers, the Amite River and Tangipahoa River (Suttkus & Cashner, 1981) whereas the other two species (*F. olivaceus* and *F. notatus*) are widespread covering most of the eastern United States. Chappepeela Creek (tributary toTangipahoa River) is a rare case where all the species of the *F. notatus* complex are suspected to occur (Tulane University Museum of Natural History; Accessed through the Fishnet2 Portal, www.fishnet2.org, 2016-09-14). Previously, reciprocal monophyly has described for all the species of this complex (Duvernell et al., 2013) and all the members of this complex have been shown to interbreed (Vigueira et al., 2008) and hybridize in the wild (Duvernell et al, 2007; Schaefer et al., 2009; Chapter II & III this manuscript).

The advancing technologies in DNA sequencing have made powerful population genomic assessments of non-model species possible. Specifically genotype-by-sequencing (GBS) improves resolution in analyzing population structure by covering a

larger portion of the genome and provides a more refined picture of genetic structure and divergence (reviewed by Luikart et al., 2003). Previous work with topminnows using traditional techniques have provided evidence that *F. olivaceus* demonstrates more population subdivision than the downstream members of the *F. notatus* species complex, *F. notatus* (Earnest et al. 2014) and *F. euryzonus* (Chapter III this manuscript). However, neither of the aforementioned studies detected subdivision in the downstream representatives of the complex.

The goal of this work is to examine and corroborate the extent of population structure for the *F. euryzonus*/*F. olivaceus* system and to determine if museum records have accurately identified a location of where all three members of the *F. notatus* species complex are sympatric. I previously showed more subdivision for *F. olivaceus* relative to *F. euryzonus* (Chapter III) the pattern was not straight forward and the better resolution provided by a GBS approach should clarify the population structure. I hypothesize that there will be more population structure for *F. olivaceus* as this species specializes in isolated headwater streams (Suttkus & Cashner, 1981; Schaefer et al., 2009; Chapter II and III) and that I will recover all three species of the *F. notatus* complex coexisting in the southern Tangipahoa.

4.3 Methods

The goal was to genotype a total of 130 fish, 20 fish (10 of each target species) from 2 locations (upstream and downstream) on the Amite (31.221, -90.854; 30.888, -90.848; respectively) and Tangipahoa (31.226, -90.529; 30.557, -90.348; respectively) Rivers (Figure 4.1), an additional 20 *F. olivaceus* from 2 locations (10 upstream and 10 downstream) from the Tickfaw River (31.078, -90.608; 30.504, -90.677; respectively)

and an extra 10 putative *F. notatus* from the southern Tangipahoa River (30.557, -90.348). To accomplish this task I doubly oversampled each location to account for field misidentification, errors in DNA extraction or sequencing, and subsequent SNP filtering. Locations were determined based on historical data of northern and southern syntopic locations (Suttkus & Cashner, 1981). Collections were made by visually spotting fish at the surface and using dipnets to capture. Fish were euthanized in an overdose of tricaine mesylate (MS-222). Caudal fins were clipped on-site, and samples were stored in salt saturated (SED) preservation buffer (Seutin et al., 1991), and stored on ice. Samples were immediately moved into a -20°C incubator and DNA extractions took place within 120 hours after euthanasia. Euthanized specimens were preserved in 10 percent (%) buffered formalin for morphological species identification (as in Chapter II).

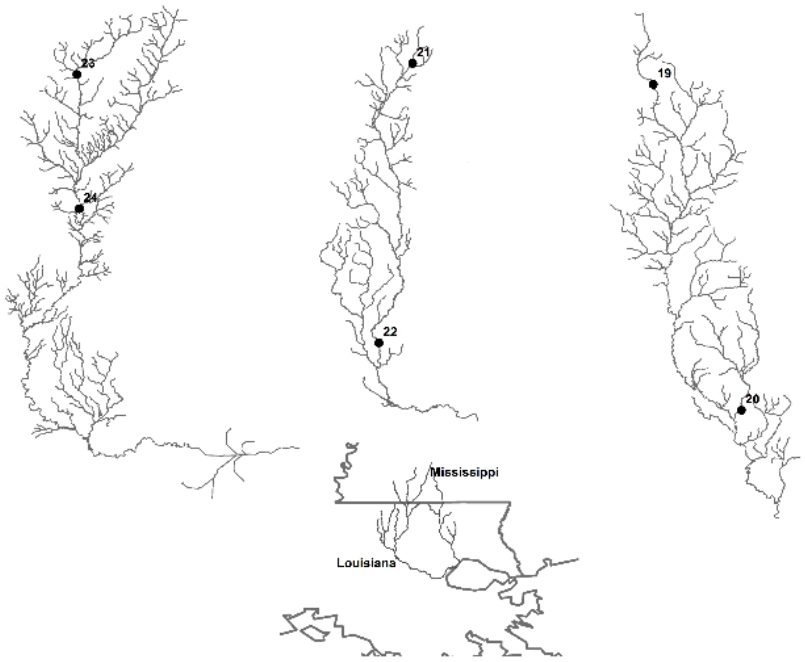

Figure 4.1 A map of sampling locations in the Amite, Tickfaw, and Tangipahoa Rivers.

Note: I selected sites based on historical data northern and southern maxima of cohabitation between the target species, *Fundulus olivaceus* and *F. euryzonus*. I sampled similar latitudes in the Tickfaw River even though *F. euryzonus* does not occur there

Genotype-by-sequencing (GBS) libraries were constructed at the Biotechnologies Resource Center (BRC) at Cornell University (http://www.biotech.cornell.edu) following a modified protocol from Elshire et al. (2011). Total genomic DNA was extracted from fish fin clips using DNeasy Tissue Kit (Qiagen, Valencia, CA). The manufacturer's protocol was followed with the addition of RNase A (100mg/ml) to achieve total RNA free genomic DNA for sequencing. Genomic DNA concentrations were assessed using an intercalating dye and was digested using the enzyme *Eco*T22I. Then, single-end, short (100 bp) read sequences were produced on an Illumina HiSeq® 2000/2500. Sequence reads were processed with the pipeline TASSEL 5.0 referencing the *Fundulus heteroclitus* genome (https://my.mdibl.org/display/FGP/Home) using Bowtie 2.0 (Langmead & Salzberg, 2012) to call SNPs (Bradbury et al., 2007). Only biallelic loci without indels were included in analyses. All reads were reduced from the 3' end to 64 bp to reduce errors in sequence calls associated with the ends of 100 bp sequences. Loci were removed from analyses if more than 10% of the data were missing or if more than 70% of the individuals were heterozyogotic for a locus (Schaefer et al., 2016). In addition, loci were filtered if there were other loci within 50 bp of one another. Individuals were eliminated from analyses if more than 20% of the loci were missing. All analyses were conducted in the R statistical computing environment (R Development Core Team, 2013) using the "hapmap" package (Schaefer, unpublished data).

I then hierarchically assessed the genetic substructure in the data using the Bayesian clustering algorithm implemented in the program STRUCTURE v. 2.3.4 (Pritchard et al., 2000; Pritchard et al., 2007) and by ordination of genotypes via principal coordinate analysis. Using STRUCTURE a burn-in of 100 000 steps followed by

2,000,000 MCMC steps were used assuming an admixture model, correlated allele frequencies, and no prior information on taxon identity. I varied the number of groups (*K*) from 1 to 10, with 20 independent runs for each value of *K*. The support for different values of *K* were assessed using the Δ*K* method (Evanno et al., 2005) implemented in STRUCTURE HARVESTER Web v. 0.6.94 (Earl & vonHoldt, 2012), visual inspection of the STRUCTURE barplots, and by examining genotypes in ordination space. Results were averaged across replicates using CLUMPP v. 1.1.2 (Jakobsson & Rosenberg, 2007) and graphically displayed with DISTRUCT v. 1.1 (Rosenberg, 2004). Then, within each group I re-ran the Bayesian clustering using the same parameters in structure with more inclusive groups (species-river of origin-position in the river) until finding clusters where *K*=1.

4.4 Results

A total of 107 individuals and 6,977 SNPs passed filtering procedures. Putative populations are coded first with the river of origin (Amite River as A, Tangipahoa River as Ta, and Tickfaw River as Ti), then by location within the river (Northern as N and southern as S), and finally by putative morphological species identification (*F. olivaceus* as O, *F. notatus* as N, and *F. euryzonus* as E). The final complement of 11 putative populations included was as follows: ASE, ASO, ANO, ANE, TaNE, TaNO, TaSE, TaSO, TaSN, TiNO, and TiSO. Ten individuals were included for ASE, 9 individuals for ASO, 9 individuals for ANO, 9 individuals for ANE, 9 individuals for TaNE, 8 individuals for TaNO, 11 individuals for TaSE, 12 individuals for TaSO, 10 individuals for TaSN, 10 individuals for TiNO, and 10 individuals for TiSO. A delta *K* plot (Figure 4.2 A) and mean likelihood plot (Figure 4.2 B) both suggest two genetic clusters where

the highest Δ*K* value and mean likelihood asymptote with lowest error both appear at *K*=2. A bar plot of *K*=2 also revealed two clear groups (Figure 4.3) separating morphological species and clumping putative *F. notatus* with *F. olivaceus* (TaSO & TaSN). As these two species can be very difficult to identify morphologically often genetic analyses are used to separate species. When separated by species, ordination suggests that each known species within the drainage is likely separated in to two groups (Figure 4.4) and no *F. notatus* samples were collected (Figure 4.3). Important to note of the ordination, is that PC1 explained 80.77% of the data so groupings along that axis are more explanatory.

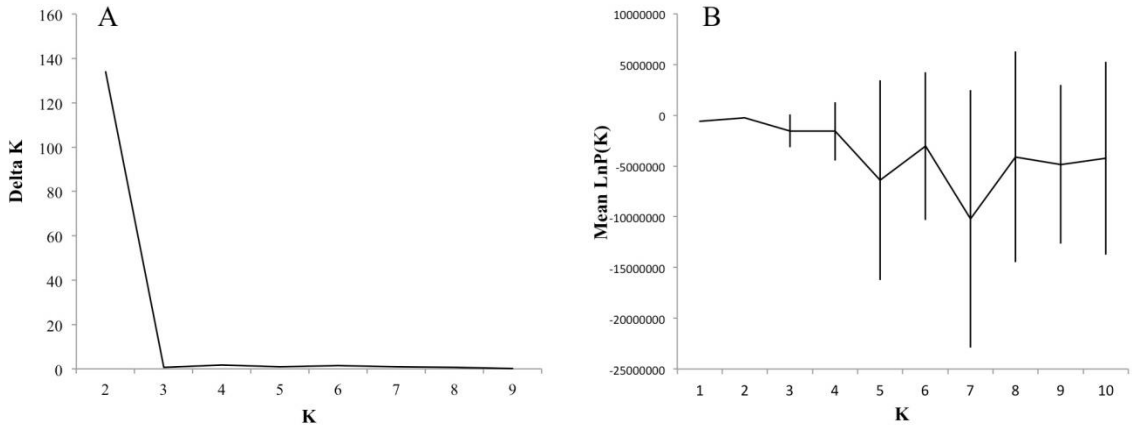

Figure 4.2 Delta K plot (A) and mean likelihood plot (B) at each value of K.

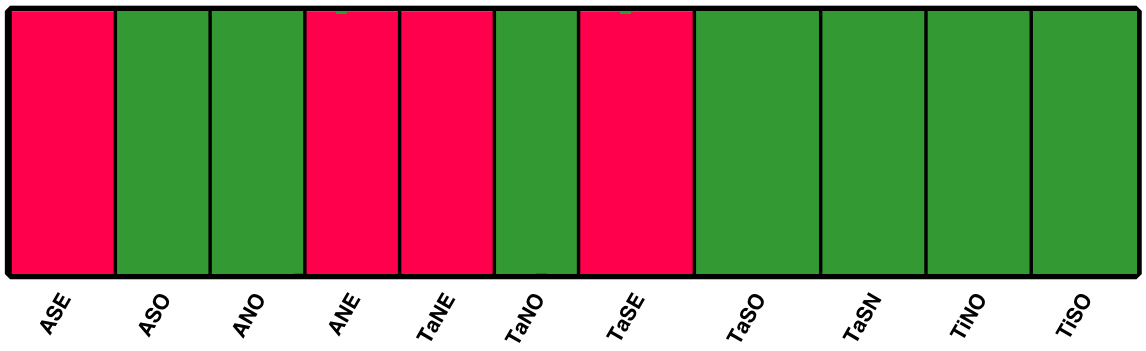

Figure 4.3 Bar plot from STRUCTURE representing two genetic clusters (*K*=2) averaged across 20 iterations for *Fundulus spp.* in the Lake Pontchartrain Drainage.

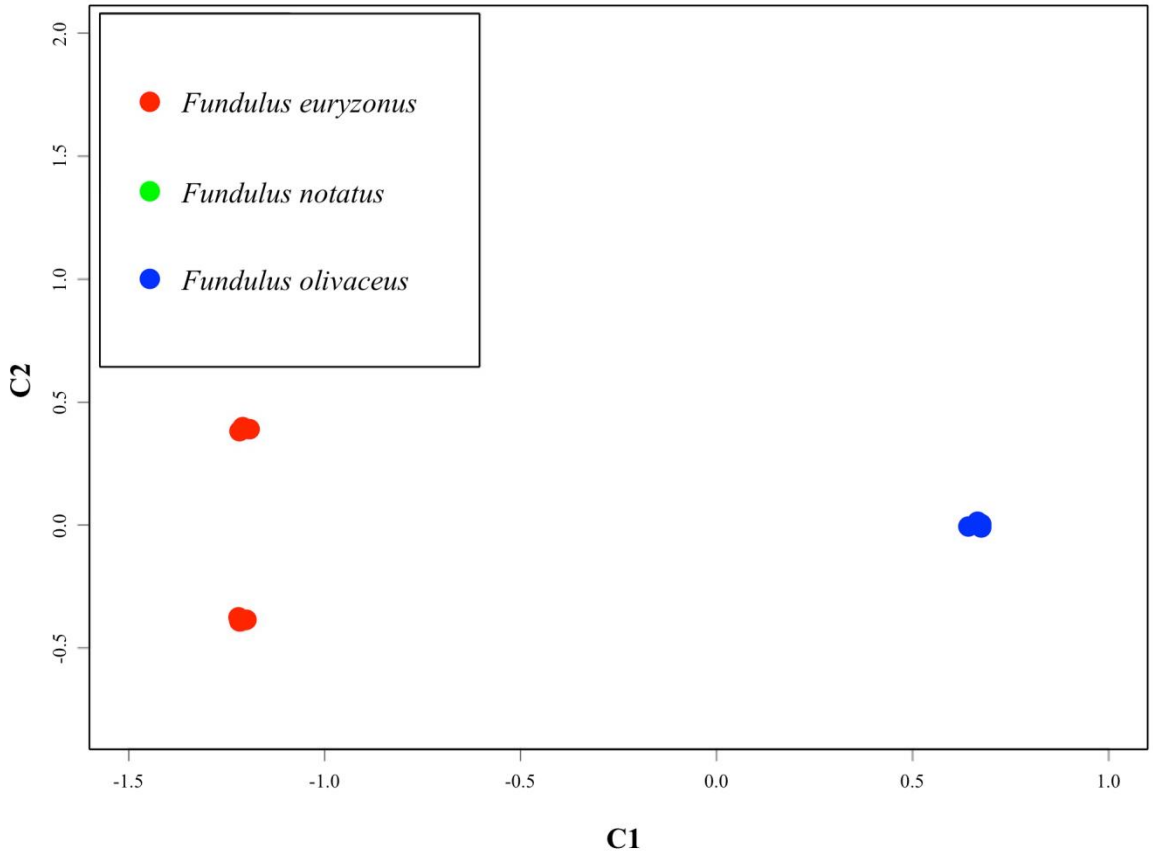

Figure 4.4 Principal coordinates analysis of all species from all locations.

When *F. olivaceus* samples were analyzed separately, for variation in and among rivers, 69 individuals were assessed after filtering with 3,151 loci. The analysis consisted of 9 individuals for ASO, 9 for ANO, 7 for TaNO, 24 for TaSO, 10 for TiNO, and 10 for TiSO individuals. A delta *K* plot (Figure 4.5 A) suggests *K*=2 as the most likely number of clusters for *F. olivaceus* in the Lake Pontchartrain Drainage. The peak at *K*=2 is much higher than any other *K* value. However there is also a substantial peak at *K*=3. The plot of mean likelihood values (Figure 4.5 B) at each *K* also suggests *K*=2 as the most likely number of clusters as this is where the plot shows the least amount of error and the least negative value. However, *K*=3 is also reasonable having low error and a similar value. Two bar plots for *K*=2 (Figure 4.6 A) and *K*=3 (Figure 4.6 B) definitively show that *K*=3 is the more reasonable solution where each river is an independent genetic cluster. The PCA (Figure 4.7) corroborates a *K*=3 solution where each group appears to separate by northern and southern locations.

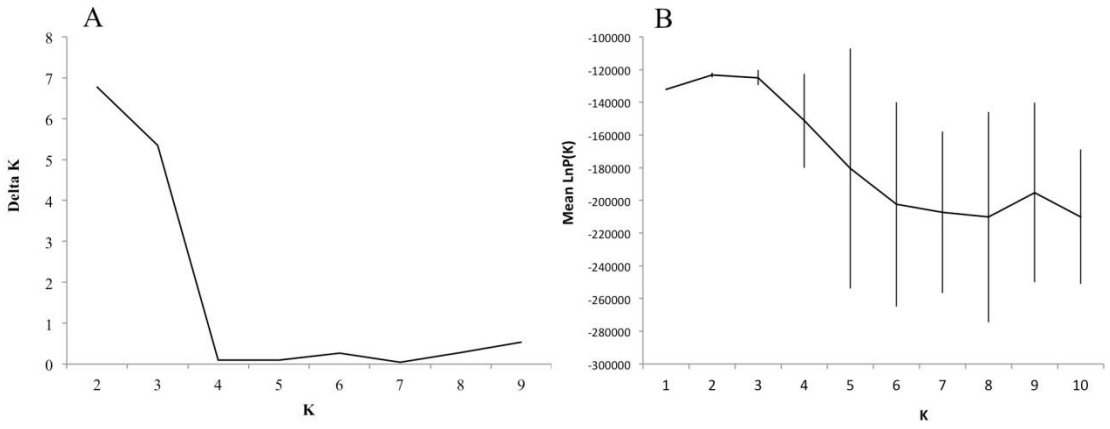

Figure 4.5 Delta *K* plot (A) and mean likelihood plot (B) for each value of *K* for *F olivaceus*.

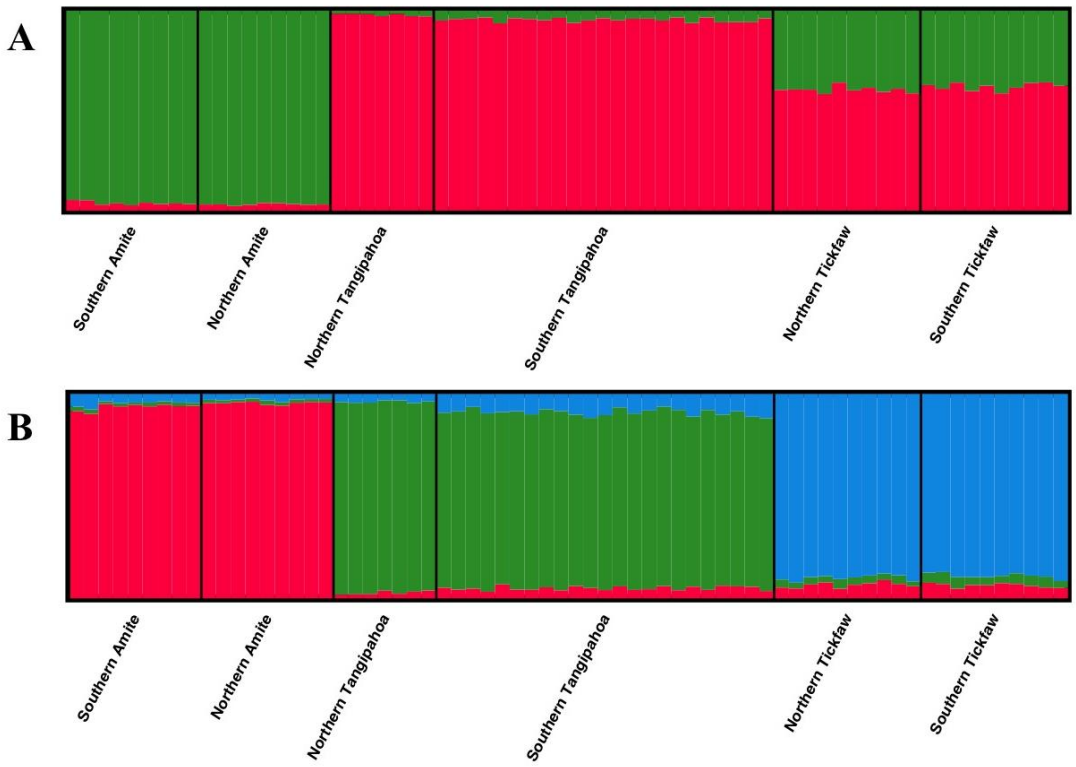

Figure 4.6 Bar plots from STRUCTURE representing two different values of *K*: A (*K*=2) & B (K=3). Values were averaged across 20 iterations for *F. olivaceus* in the Lake Pontchartrain Drainage.

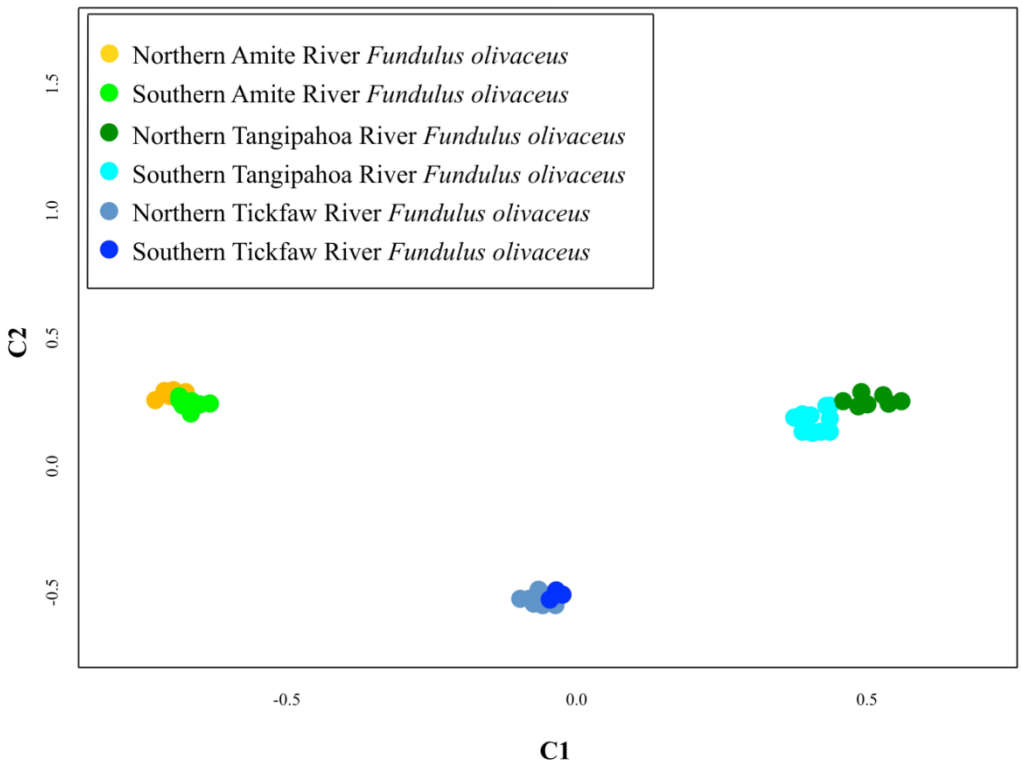

Figure 4.7 Principal coordinates analysis for genotypes of all *Fundulus olivaceus* collected in the Lake Pontchartrain drainage.

In the Amite River *F. olivaceus* samples, when assessed alone, 19 individuals passed filtering procedures with 1,256 loci. Of the individuals that passed filtering procedures 10 were ASO and 9 were ANO. Delta K (Figure 4.8 A) and mean likelihood plots (Figure 4.8 B) both suggest *K*=3 as the best number of genetic groups. The bar plot for *K*=3 (Figure 4.9 A) shows a single individual in the northern samples that is divergent. Since two locations were sampled a bar plot of *K*=2 (Figure 4.9 B) shows a clear genetic divergence that is biologically relevant, based on sampling location. The PCA plot shows a similar result where northern and southern locations are clearly divergent (Figure 4.10). However, the northern location has an outlier sample (the same individual that confounded STRUCTURE results) that appears to be more closely associated with the north, which was corroborated by STRUCTURE *K*=2 (Figure 4.9 B).

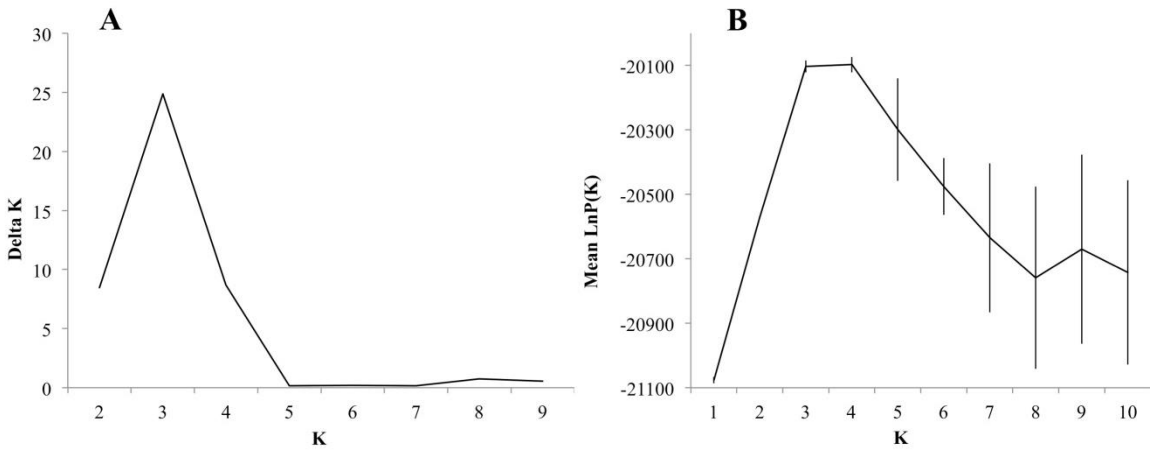

Figure 4.8 Delta *K* plot (A) and mean likelihood plot (B) for each value of *K* for Amite River *F olivaceus*.

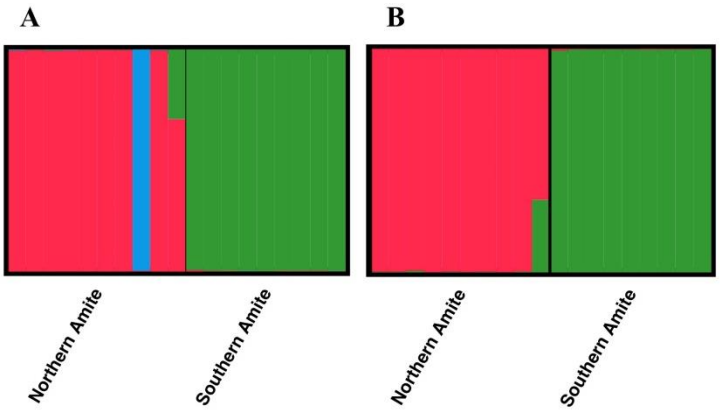

Figure 4.9 Two bar plots from STRUCTURE representing *K*=3 (A) and *K*=2 (B) averaged across 20 iterations for *F. olivaceus* in the Amite River.

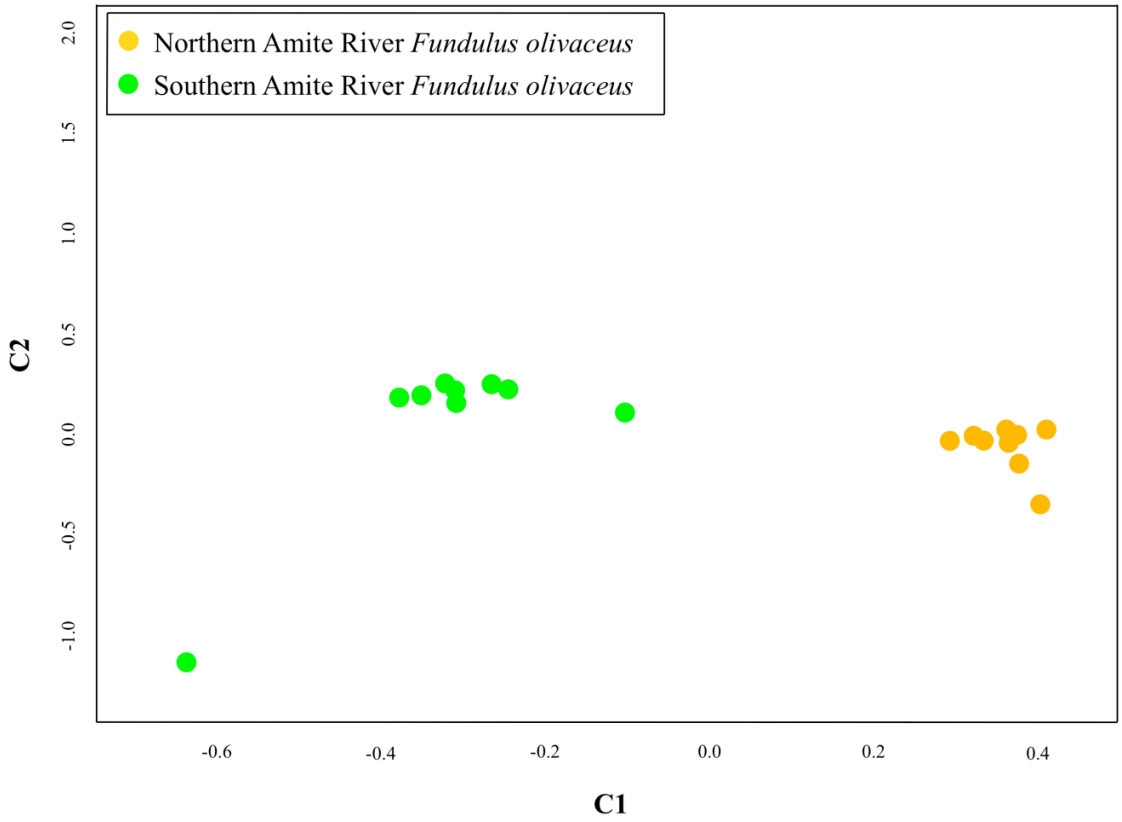

Figure 4.10 Principal coordinates analysis for genotypes of all *Fundulus olivaceus* collected in the Amite River.

In the Tangipahoa *F. olivaceus* samples, when assessed alone, 31 individuals passed filtering procedures with 2,772 loci. Of the individuals that passed filtering procedures 24 were TaSO and 7 were TaNO. Delta K (Figure 4.11 A) and mean likelihood plot (Figure 4.11 B) both show strong evidence for K=2 as the best solution. The bar plot (Figure 4.12) and the PCA plot (Figure 4.13) clearly differentiate northern and southern locations. However, both plots show three individuals with high degrees of admixture with the northern group captured at the southern location.

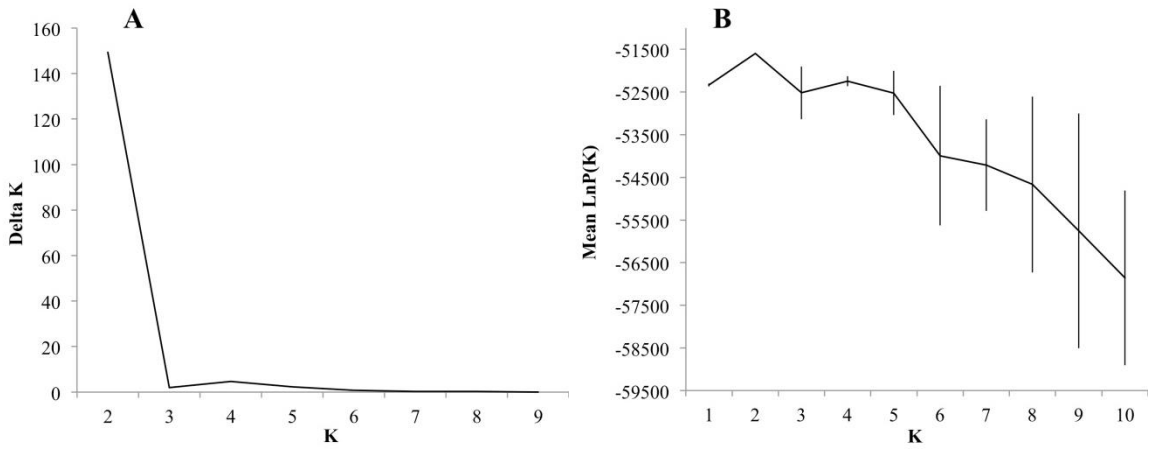

Figure 4.11 Delta K (A) and mean likelihood plot (B) for *F. olivaceus* in the Tangipahoa River.

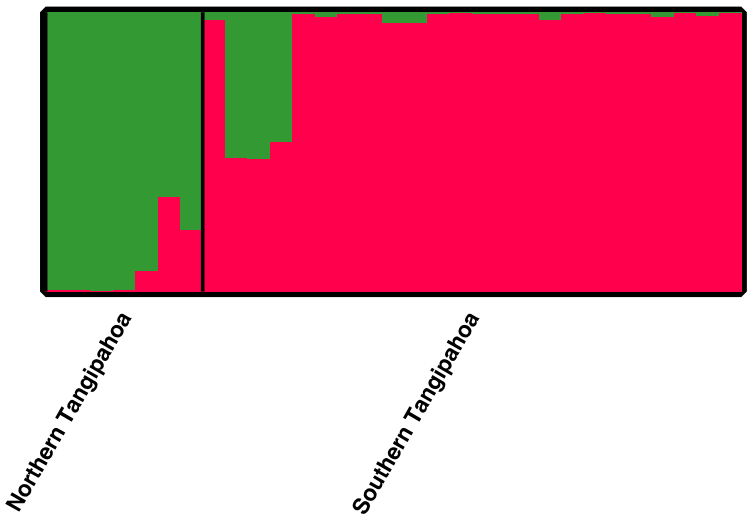

Figure 4.12 Bar plot of genetic groups for *F. olivaceus* in the Tangipahoa River.

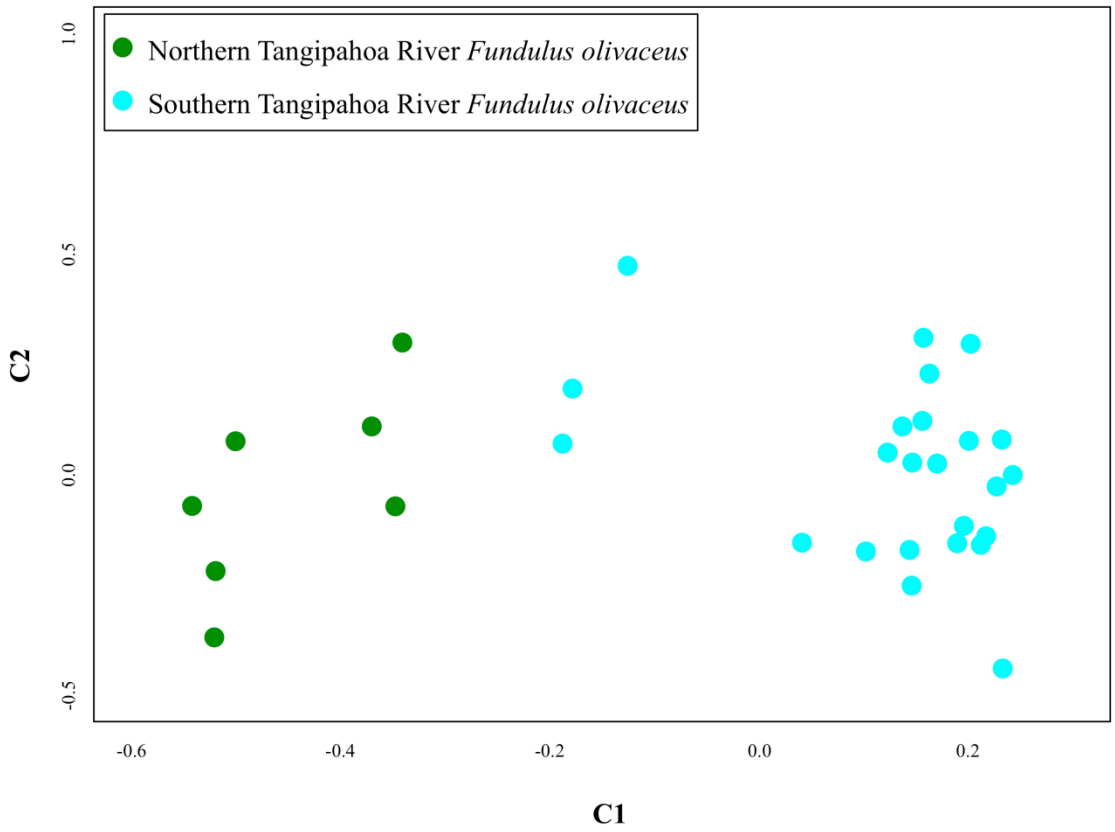

Figure 4.13 Principal coordinates analysis for genotypes of all *Fundulus olivaceus* collected in the Tangipahoa River.

In the Tickfaw *F. olivaceus* samples, when assessed alone, all 20 individuals passed filtering procedures with 2,701 loci. Of the individuals that passed filtering procedures 10 were TiSO and 10 were TiNO. Delta K (Figure 4.14 A) suggests K=3 or K=9 as the most likely solution. However, mean likelihood scores decrease with increasing *K* (Figure 4.14 B) suggesting *K*=1 as the most likely number of distinct genetic groups. A bar plot of K=3 (Figure 4.15) revels that two individuals sampled from the northern locations were considered a distinct group an outcome that supports *K*=1. The PCA plot confirms that two individuals in the northern sampling location are distinct (Figure 4.15) and that northern and southern locations are distinct genetic entities.

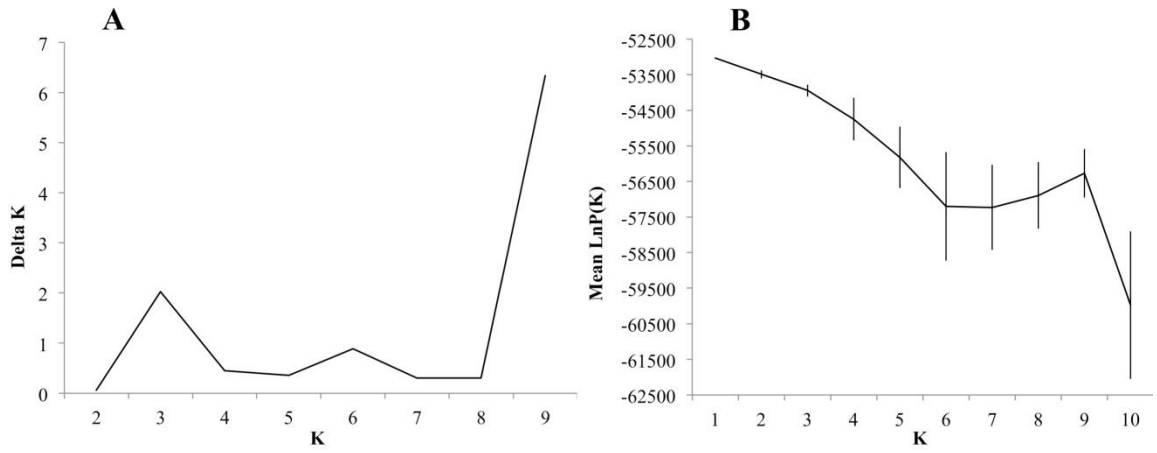

Figure 4.14 Delta K (A) and mean likelihood plot (B) for *F. olivaceus* in the Tickfaw River.

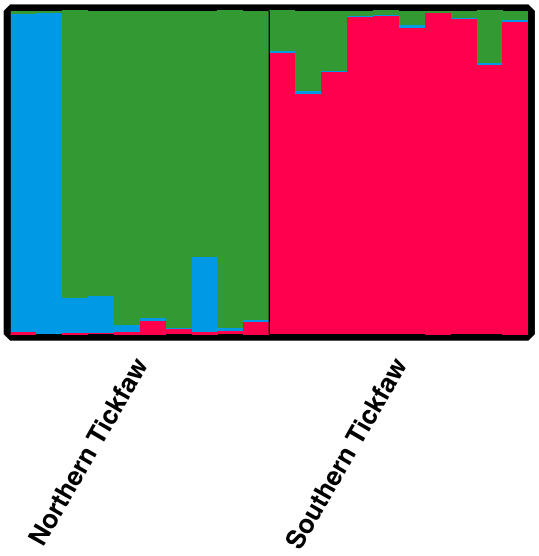

Figure 4.15 Bar plot of K=3, averaged across 20 iterations in STRUCTURE for *F. olivaceus* in the Tickfaw River.

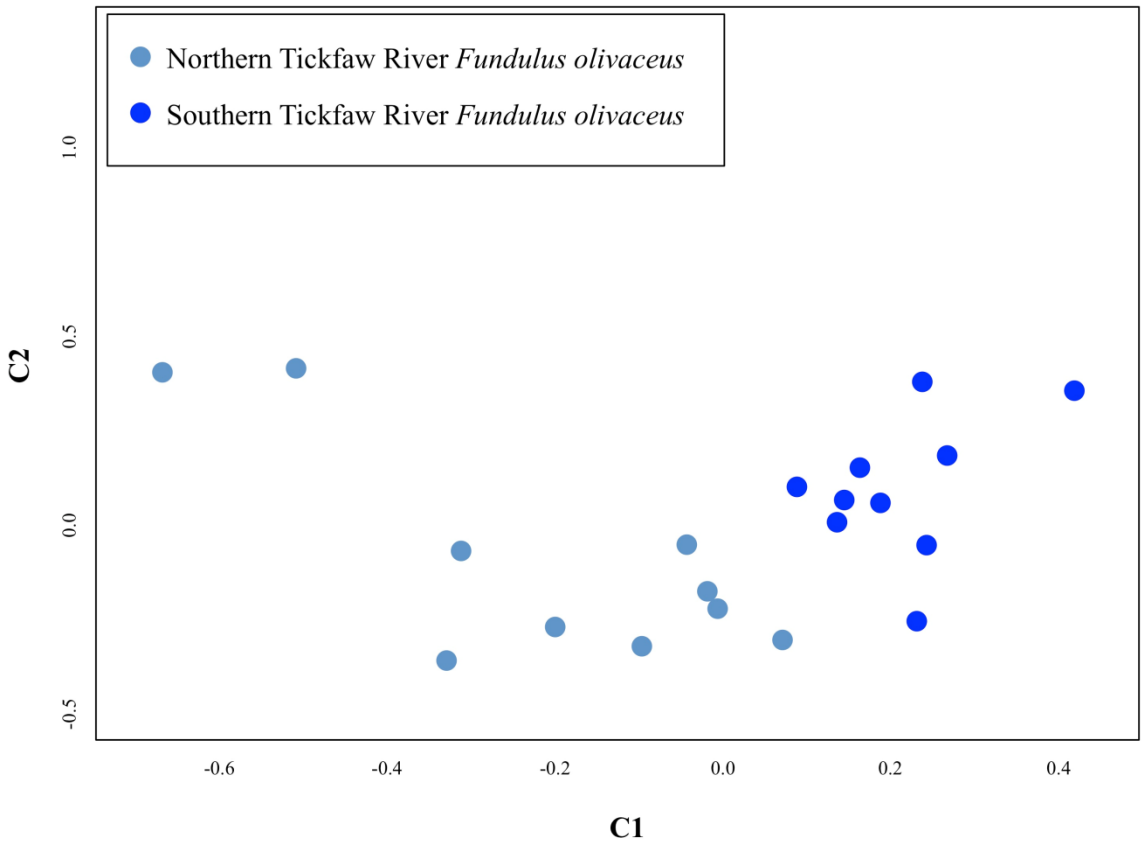

Figure 4.16 Principal coordinates analysis for genotypes of all *Fundulus olivaceus* collected in the Tickfaw River.

There were 38 *F. euryzonus* not filtered out of the analysis for the entire Lake Pontchartrain Drainage with 6,163 loci. Of the 38, there were 19 each from the Amite and Tangipahoa Rivers. A delta *K* plot (Figure 4.17 A) suggests K=2 as the most likely number of clusters for *F. euryzonus* in the Lake Pontchartrain Drainage. The peak at *K*=2 is much higher than any other *K* value. The plot of mean likelihood values at each *K* (Figure 4.17 B) also suggests *K*=2 as the most likely number of clusters as this is where the plot shows the least amount of error (represented in standard deviation) and the least negative value. The result of *K*=2 in this system was corroborated with a bar plot (Figure 4.18), where genetic clusters are associated with river differentiation, and by PCA analysis (Figure 4.19), where rivers represent clear genetic differentiation.

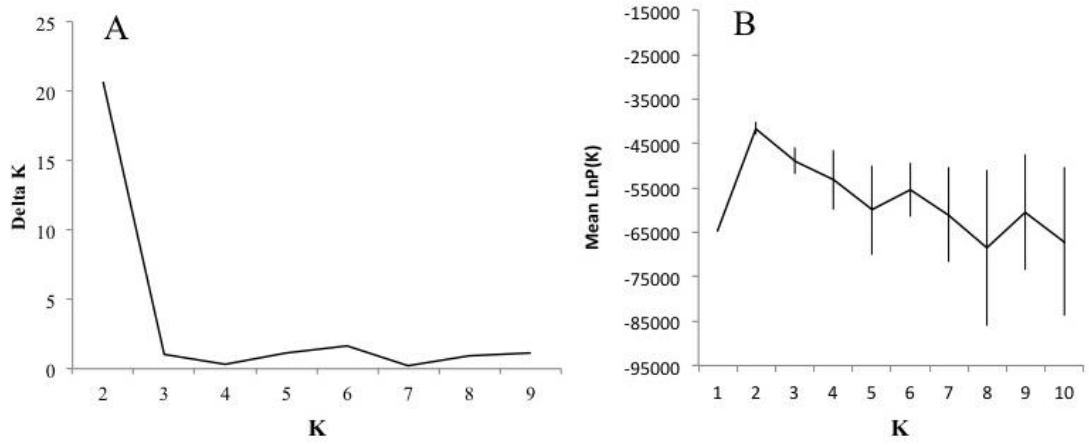

Figure 4.17 Delta K (A) and mean likelihood plot (B) for each value of *K* for *F. euryzonus* in the Lake Pontchartrain Drainage.

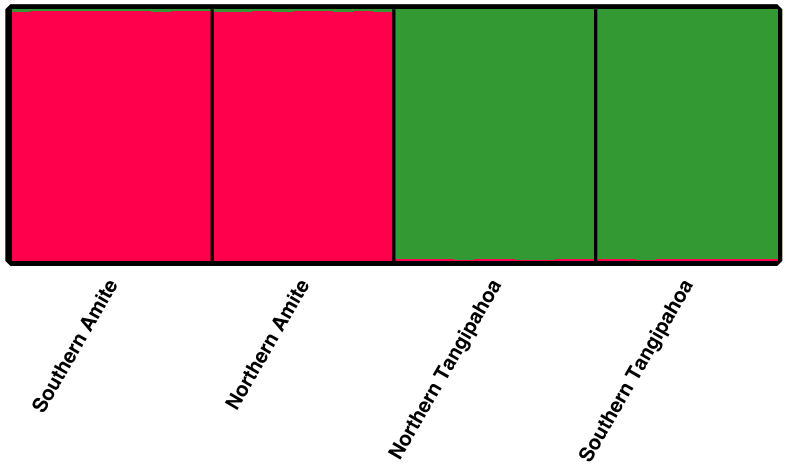

Figure 4.18 Bar plot from STRUCTURE representing two genetic clusters (K=2) averaged across 20 iterations for *F. euryzonus* in the Lake Pontchartrain Drainage.

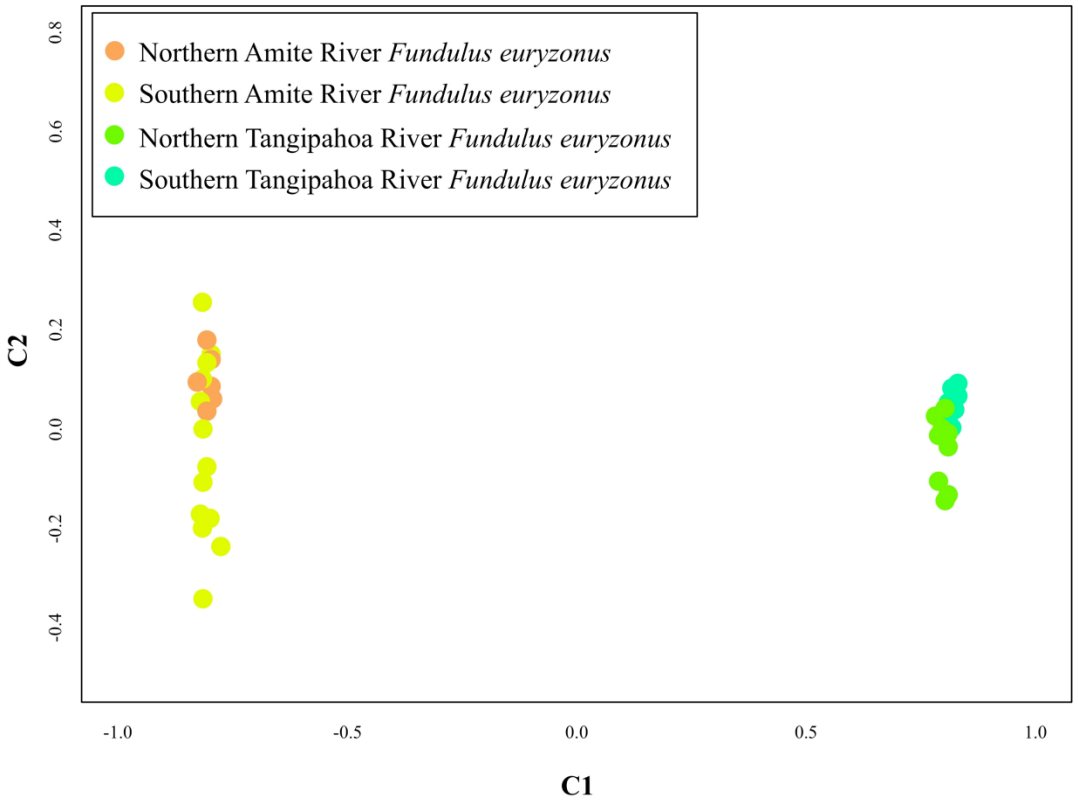

Figure 4.19 Principal coordinates analysis for genotypes of all *Fundulus euryzonus* collected in the Lake Pontchartrain drainage.

Nineteen *F. euryzonus* from the Amite River were left after filtering procedures with 5,341 loci, including 13 ANE and 6 ASE. The peak at *K*=2 on the delta *K* plot (Figure 4.20 A) is more positive than any other *K* value for *F. euryzonus* in the Amite River. The plot of mean likelihood values at each *K* (Figure 4.20 B) show *K*=1 or *K*=2 as the most likely number of clusters. The bar plot (Figure 21) of *K*=2 does not assign any population or individual to a specific genetic cluster and the PCA (Figure 22) does not suggest any differentiation associated with putative north-south populations.

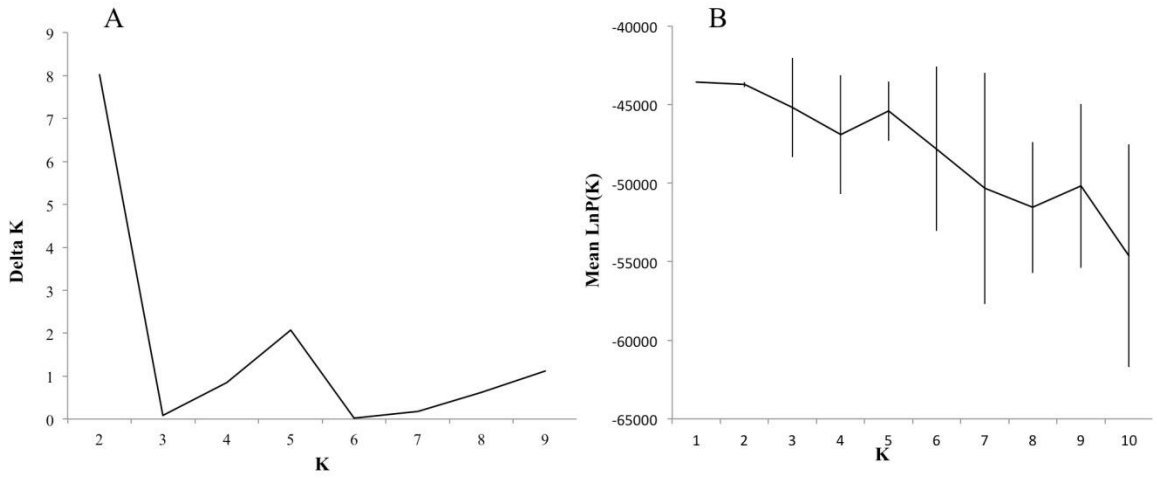

Figure 4.20 Delta *K* plot (A) and mean likelihood plot (B) values at each *K* for *F. euryzonus* in the Amite River.

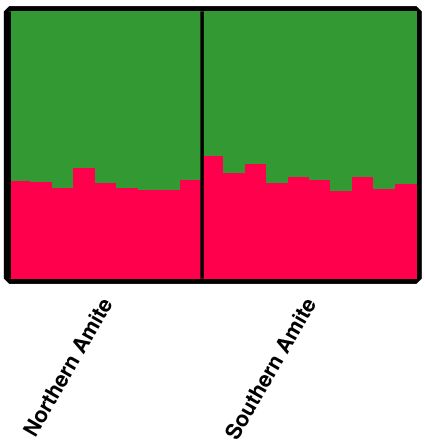

Figure 4.21 Bar plot from STRUCTURE representing two genetic clusters (K=2) averaged across 20 iterations for *F. euryzonus* in the Tangipahoa River

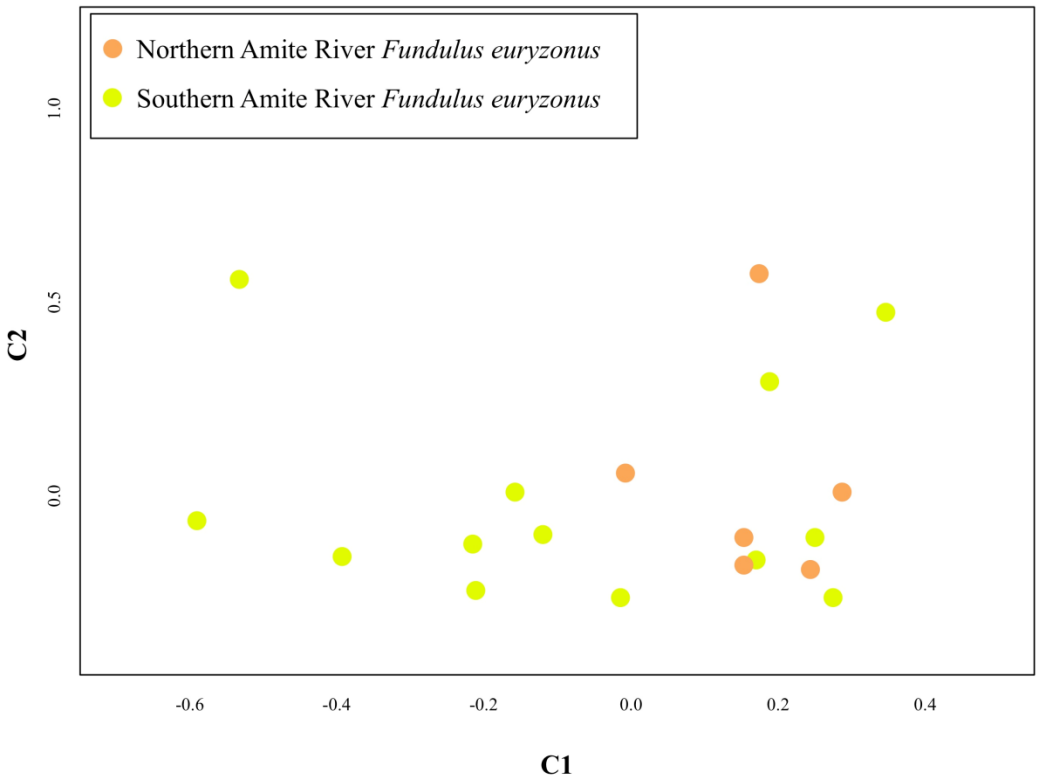

Figure 4.22 Principal coordinates analysis for genotypes of all *Fundulus euryzonus* collected in the Amite River.

In the Tangipahoa River 19 *F. euryzonus* passed filtering procedures, 10 in the northern location and 9 in the southern location, with 5,607 loci. Delta *K* plot (Figure 4.23 A) suggests *K*=2 as the most likely number of clusters for *F. euryzonus* in the Tangipahoa River. The peak at *K*=2 is more positive than any other *K* value. The plot of mean likelihood values at each *K* (Figure 4.23 B) also suggests *K*=2 as the most likely number of clusters. A bar plot (Figure 4.24) constructed form the structure analysis indicates that two clusters are unlikely because no putative population is assigned to a single cluster. The PCA plot (Figure 4.25) however indicates that the northern and southern locations are genetically distinct

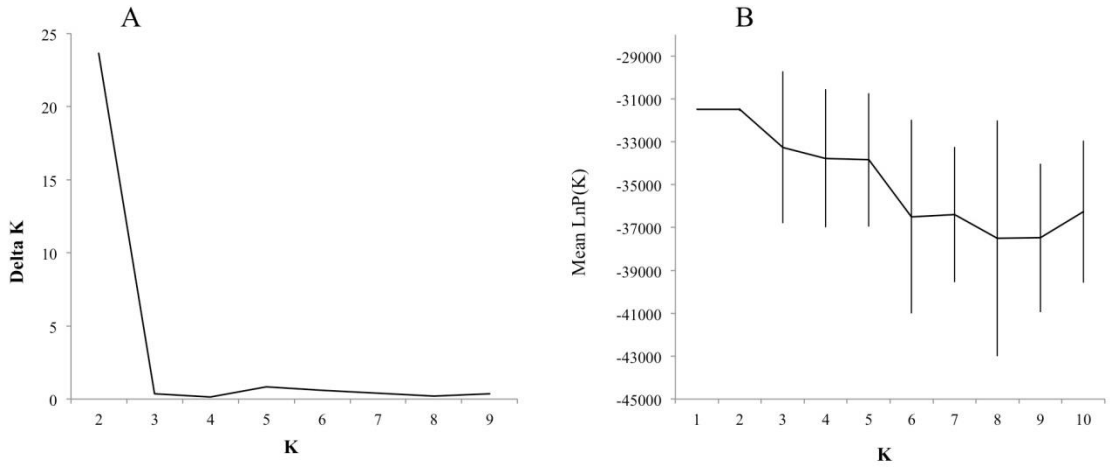

Figure 4.23 Delta K plot (A) and plot of mean likelihood values at each K (B) for *F. euryzonus* in the Tangipahoa River.

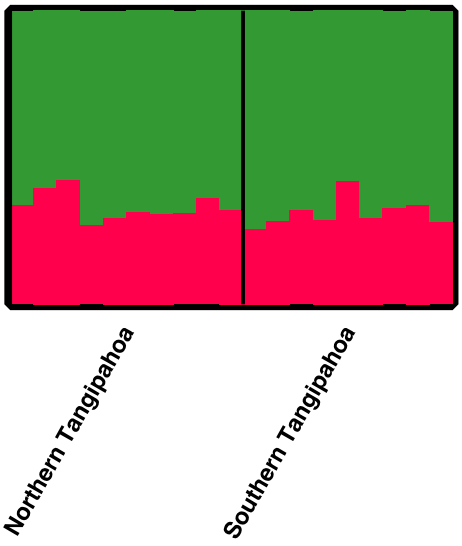

Figure 4.24 Bar plot from STRUCTURE representing two genetic clusters (K=2) averaged across 20 iterations for *F. euryzonus* in the Tangipahoa River.

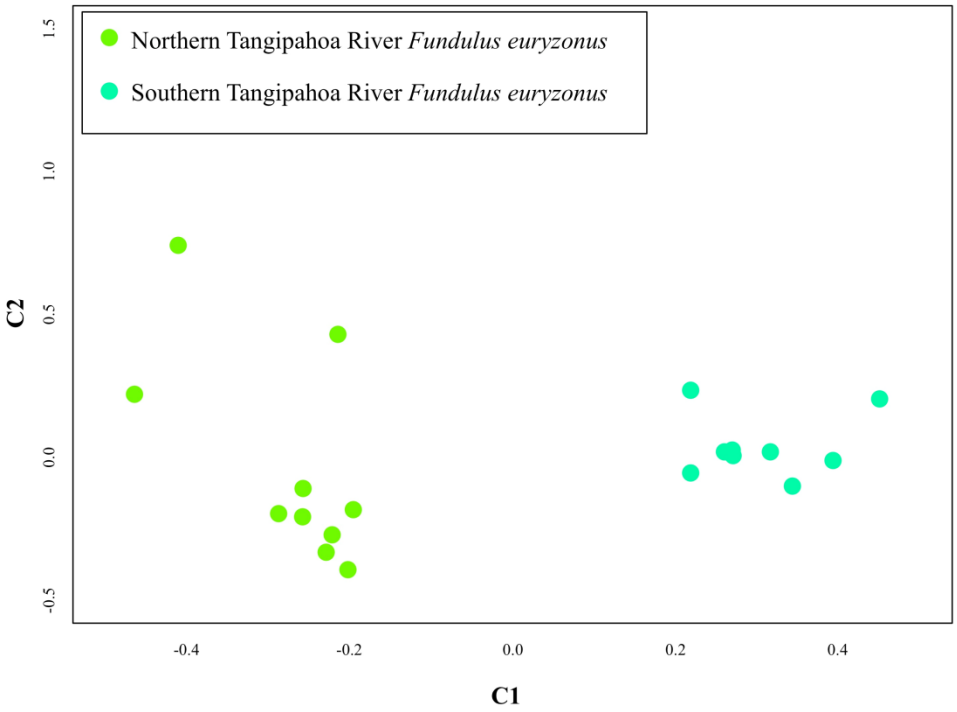

Figure 4.25 Principal coordinates analysis for genotypes of all *Fundulus euryzonus* collected in the Amite River.

4.5 Discussion

As predicted *F. olivaceus* demonstrated more population structure than *F. euryzonus* along the river continuum. *Fundulus olivaceus* is often more abundant in upstream locations and habitat types (Braasch & Smith 1965; Blanchard 1996; Smiley et al. 2005; Schaefer et al. 2009; Duvernell & Schaefer, 2014; Chapter II; Chapter III). Considering the dendritic nature of riverine systems, a species inhabiting upstream locations can become subdivided (Earnest et al. 2014). Specifically, the findings of Earnest et al. (2014) and Chapter III (this manuscript) are directly corroborated by the results of this study where *F. olivaceus* has diverged into more genetically distinct populations than its downstream counterpart (*F. euryzonus* in this system). In the Tickfaw

River however, *F. olivaceus* populations are not subdivided and therefore appear more like a downstream fish (*F. notatus* or *F. euryzonus*). And, in the Tangipahoa River there is evidence of underlying population structure for *F. euryzonus*.

Data suggests that both the Amite and Tangipahoa River *F. euryzonus* had a single population that spanned the entire sampling range. However, there may be underlying population structure for *F. euryzonus* in the Tangipahoa River. The STRUCTURE (delta *K* and mean likelihood plots) and PCA results are contradictory to the bar plot result which might indicate isolation by distance (IBD).

Seemingly absent hybridization is inconsistent with published analyses of similar systems within this species complex. Considering the present study did not find any F1s or any other level of admixture is potentially indicative that hybridization beyond the F1 level is rare. Schaefer et al. (2009) found hybridization rates for these organisms in the same drainage at 3% admixed individuals using gene restriction fragment length polymorphisms. Earnest et al. (2014) using microsatellite markers found hybridization rates for this species complex in Illinois, USA at 7% admixed individuals. Recently, I showed that within this system hybridization rates are as high as 7-9% admixed individuals (Chapter II & III). Schaefer et al. (2011) found that hybrids are typically found in tributaries near the confluence with the main channel. Additionally, I have shown that specific tributary localities that are environmentally typical of both species can have higher rates of hybridization than less typical habitats (Chapter III). Schaefer et al. (2016) reported much higher rates of hybridization (49-55%) when sampling targeted locations of increased hybridization (hybrid hotspots) in the *F. notatus* species complex. It is possible that the sampling design, via locations, in the present study was insufficient

to recover admixed individuals. However, all of the southern sites (especially site 20) fit the Schaefer et al 2011 and Chapter III description of potential hybridization locations (Figure 4.1). If F1s had reproduced then some individuals representing generations of backcrossing might demonstrate an admixed genome, which was not detected in any amount in the current study. When all species were assessed together there were no average Q-scores (probability of assignment to one genetic group) below 99.7% for either species (Figure 4.3).

Putative *F. notatus*, identified in the field using spot density, in this study clumped with *F. olivaceus* (TaSO & TaSN) in the same genetic cluster. These findings are in direct contrast to historical museum records. *Fundulus notatus* may either recently been extirpated from Chappepeela Creek or *F. olivaceus* may take on characteristics in this part of the range that makes it morphologically indistinguishable from *F. notatus*. Notably, the appearance of spots and overall spot density for *F. olivaceus*, and presumably other species in this complex, is highly variable based on sex, geographic location, or river system and can be problematic as a diagnostic tool (Duvernell et al. 2007; Schaefer et al. 2009, 2011, 2012). The Tickfaw River also has evidence of *F. notatus* samples in tributaries (Louisiana State University Museum of Zoology, Accessed through the Fishnet2 Portal, www.fishnet2.org, 2016-09-29). The samples collected in this study did not confirm the cohabitation of *F. notatus* with *F. olivaceus* in the southern Tickfaw River. The southern Tickfaw River sample site included in the current work is south of the current *F. notatus* records and an ideal location for hybridization, and cohabitation (Schaefer et al. 2011; Chapter III) therefore should have been a location where collecting *F. notatus* was possible. The current work shows that the species *F.*

olivaceus subdivides like a downstream fish in the Tickfaw River (*K*=1) that might indicate the absence of other species in this river. Future genetic studies in the southern Tickfaw River should be conducted to confirm the occurrence of *F. notatus*.

It is likely that *F. notatus* is not present in the Tangipahoa or Tickfaw Rivers as previously thought. As expected, *F. olivaceus* populations have diverged between northern and southern locations in all rivers. *Fundulus euryzonus* do not differentiate along rivers, but in the Tangipahoa River it shows at least some degree of divergence potentially indicative of IBD. Future studies in this system should focus on sampling individuals from more locations along each river channel to detect hybridizations either historical or contemporary, assess IBD, and examine outlier loci for functional differences between populations.

4.6 Acknowledgments

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CHAPTER V – BODY SHAPE VARIATION ASSOCIATED WITH SIZE, SEX, AND RIVER OF ORIGIN IN THE *FUNDULUS NOTATUS* SPECIES COMPLEX

5.1 Abstract

Body shape in aquatic organisms is closely tied to energetics and locomotion. In addition, body shape, which can vary as organism's age, can be important to sexual selection and can reveal differences in populations and habitat preferences. I examined body shape variation in *Fundulus olivaceus* and *F. euryzonus* in the Lake Pontchartrain Drainage. I sampled fish from a northern and southern location in 3 rivers in the drainage. Then I did geometric morphometric analyses of body shape with looking at each potential factor that can influence body shape (size, species differentiation, sexual dimorphism habitat/position along the river continuum, and river of origin). The two species differed in shape from each other and among populations when compared across drainages. Within a given drainage *Fundulus euryzonus* demonstrated divergence along the river continuum while *F. olivaceus* did not. Size, measured as centroid size, also significantly affected body shape in almost all cases and interacted with sexual dimorphism. Sexual dimorphism was prevalent in both species and mostly associated with the anal fin. Size interacted with sexual dimorphism suggesting that body shape changes as individuals increase in size.

5.2 Introduction

One of the most consistent themes in nature is form relates to function. In the ecological sciences this theme typically takes shape in the relationship between morphology and performance. Hydrodynamic effects in the aquatic environment can dramatically affect swimming performance and maneuverability of fish (Drucker &

Lauder, 2000). Thus, body shape, especially in connection with muscle activity, has likely been shaped by natural selection with regard to the influence of hydrodynamics (Drucker & Lauder, 2000; Tokić & Yue, 2012). Optimal body shape in specific situations can result in augmented fitness by improving predator avoidance, prey capture and locomotion energetics (Langerhans et al., 2004; Langerhans & Reznick, 2010). Highly mobile fish have a torpedo shaped profile whereas fish that live in and around the benthos have deeper bodies to help maneuver in unpredictable flow patterns (Webb, 1984) and those that rely on habitat near the surface of the water have yet another body shape (Barlow, 1972). Body shape is not only the product of genetics but can also vary due to extrinsic factors (Langerhans & DeWitt, 2002; Langerhans et al., 2003).

Body shape in organisms can be important to sexual selection and can be diagnostic in differentiating between sexes. For example, shape sexual dimorphism is widespread in lizards (Vitt, 1983; Olson & Madsen, 1998). Often male lizards have larger heads, for male-male competition, and female lizards have longer bodies, added space for egg development (Olsson et al., 2002). In fish, the shape of the head (Herler et al., 2010) or the anal fin (Carranza & Winn, 1954; Foster, 1967) can be important in sexual reproductive success.

Body shape can be a plastic trait that is heavily influenced by the surrounding environment (Wimberger, 1992; Vences et al., 2002; Schaefer et al., 2011). As organisms become isolated they can adapt to local habitats, genetic divergence can occur further differentiating between groups and adding to shape differences (Zinetti et al., 2013). For example, fish in lotic environments compared to fish in lentic reservoirs can have dramatic differences in body shape (Haas et al. 2010; Franssen et al. 2013). To

further complicate analyses of shape variation allometry, changes as individuals increase in size, can also play a role in determining body shape (Klingenberg, 1998; Welsh et al., 2013).

The species in the genus *Fundulus* are an emerging model system for evolutionary study. Ecological and genetic interactions associated with hybridization among the various species of the *Fundulus notatus* complex have been a rich area of evolutionary research (Duvernell et al., 2007; Vigueira et al., 2008; Earnest et al., 2014; Duvernell et al., 2013; Duvernell & Schaefer, 2013; Schaefer et al., 2016). One species in the complex, *F. euryzonus*, only exists in two rivers within the Lake Pontchartrain Drainage (Suttkus & Cashner, 1981) Throughout the entire restricted range, *F. euryzonus* coexists with *F. olivaceus*. The range of *F. olivaceus* is much more broad covering much of the southeastern United States (Duvernell et al., 2007). In the overlapping portion of the range these 2 species hybridize in the wild (Schaefer et al., 2009; Schaefer et al., 2016; & see Chapter II & III).

Shape variation is limited within streams for the *Fundulus notatus* complex and there are limited differences between the species (Schaefer et al., 2011). The most dramatic differences occurred in these species along the river continuum suggesting that habitat use has very dramatic effects on the shape of these fish (Schaefer et al., 2011). While Schaefer et al. (2011) did not assess sexual dimorphisms in the species complex. Welsh et al. (2013) found evidence for it in *Fundulus notatus*.

The goal of this study is to examine the effect of size, sex, species and river of origin on shape variation. There is a gap in the understanding of shape in the *F. notatus* species complex; Schaefer et al. (2011) did not examine sexual dimorphism, Welsh et al.

(2013) only looked at variation in a single species, and Welsh & Fuller (2015) examined variations in fin shapes. In the present study, members of the *Fundulus notatus* species complex were examined to expand the knowledge of this system and provide a complete picture of shape variation where similar outcomes were expected. I hypothesize that allometry, sex, species, position along the river continuum (north vs. south), and river of origin will all have distinct body shape categories. I expect that sexual dimorphism, as in Welsh et al. (2013), will have a significant effect on body shape, as in Schaefer et al. (2011) differences in species will have a nominal effect on body shape variation, position along the river continuum (henceforth, position) and river of origin (henceforth origin) will also effect the shape of the individuals (Schaefer et al., 2011).

5.3 Methods

I used geometric morphometric (GM) analyses of body shape (Zelditch et al., 2004; Claude, 2008; Mitteroecker & Gunz, 2009) to compare each sex and species, as well as position, and origin. I collected fish from May-August 2015. I selected sites based on historical data to sample northern and southern locations of cohabitation between the target species, *Fundulus olivaceus* and *F. euryzonus*. Additionally I sampled similar latitudes in the Tickfaw River even though *F. euryzonus* does not occur there (Figure 5.1, Table 5.1). Collections were made using dipnets. Fish were euthanized using MS-222 and then the caudal fin was clipped, which was stored in SED buffer (Seutin et al., 1991) for genetic analysis. Voucher specimens to be used for shape analyses were fixed in 10% buffered formalin in the field. Individuals that were noticeably affected during the preservations process were eliminated from future analyses.

Pictures were taken of the right side of each individual with an 8-megapixel iSight camera (Apple). Seventeen landmarks (henceforth, LM) were digitized for each individual based on LM locations used in Schaefer et al. (2011) (Figure 5.2). I digitized photographs using the Geomorph v. 2.0 package (Adams & Otarola-Castillo, 2013) and analyzed data with the package shapes v. 1.1 (Dryden & Mardia, 1998) in R v 3.2.3 (R Development Core Team, 2013). Centroid size, the square root of the summed squared distances of each landmark to the centroid, was calculated for each individual as a measure of individual size (henceforth, CS). I then performed a generalized Procrustes analysis (GPA) to rotate and scale images for a pooled analysis containing all individuals regardless of sex and species, an analysis with sex and species as factors, an analysis with position (northern or southern) as a factor for each sex and species combination (4 separate analyses), and origin analyses for each sex and species combination (4 separate analyses). Then, I used a principal components analysis (PCA) to visualize and summarize LMs in ordination space. Then with the final complement of shapes for sex and species and for origin variation, both pooled and individual sex and species variation, followed by a multivariate analyses of variance (MANOVA; base package; Wilks' Lambda distribution) to test for difference along PCA axis scores

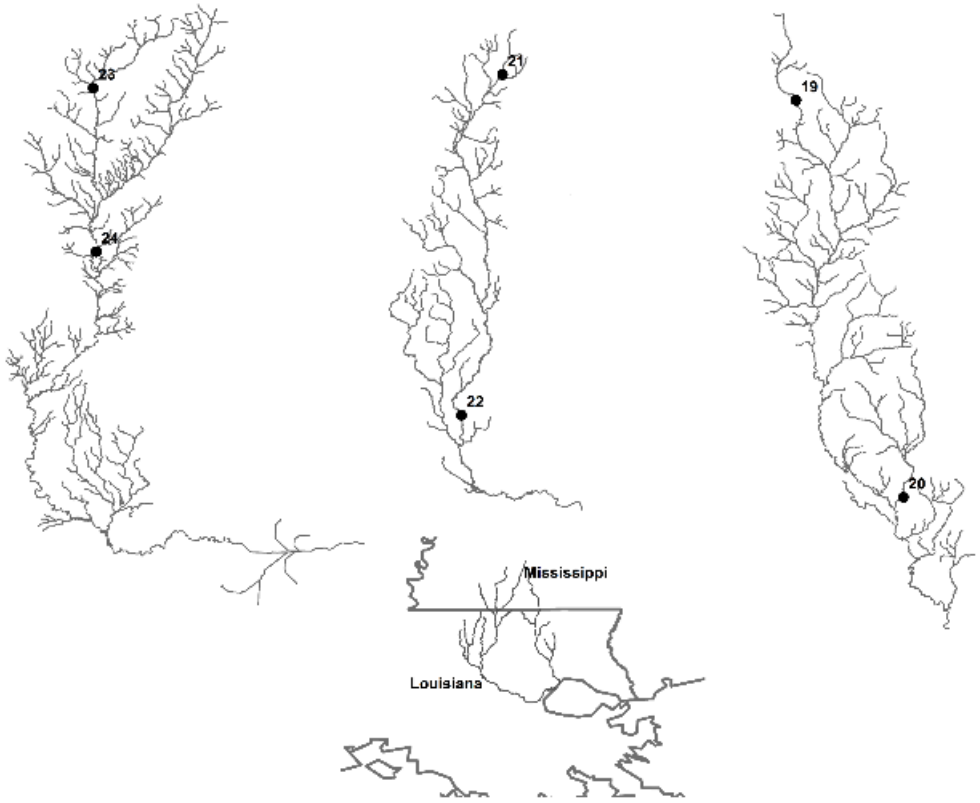

Figure 5.1 Sampling locations in the Amite, Tickfaw, and Tangipahoa Rivers.

Table 5.1

Site names and location of each of the sampling locations.

Site	Location Name	latitude/longitude
19	Martin Rd.	31.225717, -90.529317
20	Chappepeela Creek	30.556814, -90.348289
21	Hwy 568	31.078047, -90.607689
22	Hwy 190	30.503769, -90.676689
23	Clyde Graves Road	31.220900, -90.853700
24	Hwy 10	30.888056, -90.847500

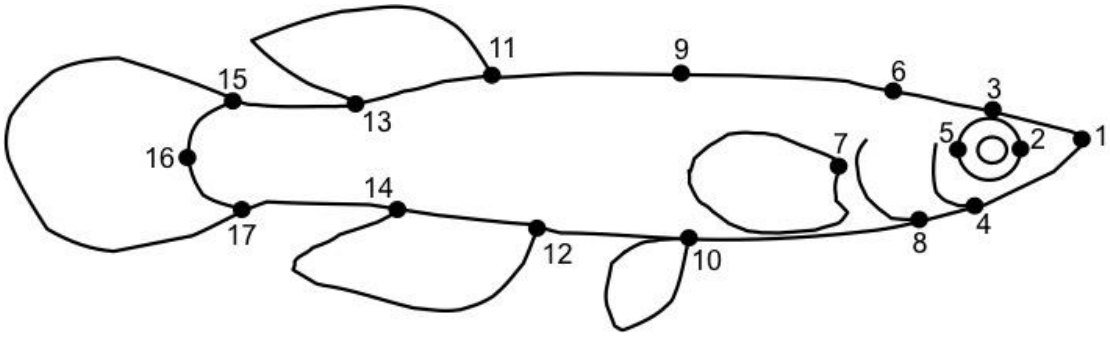

Figure 5.2 Landmarks digitized for all specimens in geometric morphometric analyses.

5.4 Results

5.4.1 Sexual dimorphism and species differentiation

I examined the shapes of a total of 201 individuals for differences among species and sexual dimorphism including 16 female and 12 male *F. olivaceus* and 24 female and 24 male *F. euryzonus* from the Amite River, 39 female and 23 male *F. olivaceus* and 12 female and 16 male *F. euryzonus* from the Tangipahoa River, and 17 female and 18 male *F. olivaceus* from the Tickfaw River. Six individuals (one female and two male *F. olivaceus* and one female and one male *F. euryzonus* from the Amite River; one female *F. olivaceus* from the Tangipahoa River) were eliminated due to contortions during the preservation process leaving 195 individuals in the final analyses. CSs are presented with one standard error (SE) in parenthesis following the CS. The mean female CS was 72.66 (±0.92) and the mean male CS was 76.49 (±1.36) when all sampling locations and species are pooled together. Female CS ranged between 49.68 and 94.97 and male CS ranged from 52.27 and 109.13 when all sampling locations and species are pooled together. The mean CS of the *F. olivaceus* pooled together was 74.88 (± 1.04) ranging between 49.68 and 109.12. The mean CS of the *F. euryzonus* pooled together was 73.66 (±1.29) ranging between 55.25 and 99.90.

The mean female *F. olivaceus* CS was 73.55 (± 1.17) and the mean male *F. olivaceus* CS was 76.73 (± 1.85). Female *F. olivaceus* CS ranged between 49.68 and 94.97 and male *F. olivaceus* CS ranged from 52.27 and 109.13 when all sampling locations are pooled together. The mean female *F. euryzonus* CS was 70.86 (±1.43) and the mean male *F. euryzonus* CS was 76.18 (±2.01). Female *F. euryzonus* CS ranged between 57.58 and 85.24 and male *F. euryzonus* CS ranged from 55.25 and 99.90 when all sampling locations are pooled together. In general, for both species, differences between the consensus shapes for females compared to consensus shape for males differed in the location of landmarks around the anal fin (Figure 5.3).

Using a broken stick model the first 3 axes explained 57.0 % of the variation in the shape coordinates for sexual dimorphism and species differentiation (Figure 5.4). Axis one explained 23.4 % of the variation, which most clearly separated females (lower scores) and males (higher scores). However, axis one (PCI) is also associated with species variation where higher scores, within a sex, are associated with *F. olivaceus* and lower scores are associated with *F. euryzonus*. Higher scores on axis one are associated with more anteriorly located anal fin origin (LM 12) and more posteriorly located anal fin insertion (LM 14). Additionally higher scores on PCI were associated with an upturned snout (LM 1), lengthening of the caudal peduncle (LM 16, 17, 18), a more anteriorly located dorsal fin insertion (LM 13), and a deeper body (LM 9, 10). Axis two explained 22.4 % of the variation in shape data and is mostly associated with species differentiation where higher scores, within a sex, are associated with *F. olivaceus* and lower scores are associated with *F. euryzonus*. However, axis two is also associated with variation

between sexes, within a species, where females have higher scores than males. Higher scores on axis two (PCII), in contrast to axis one, are associated with a more anteriorly positioned anal fin (LM 12 & 14) and are indicative of female fish.

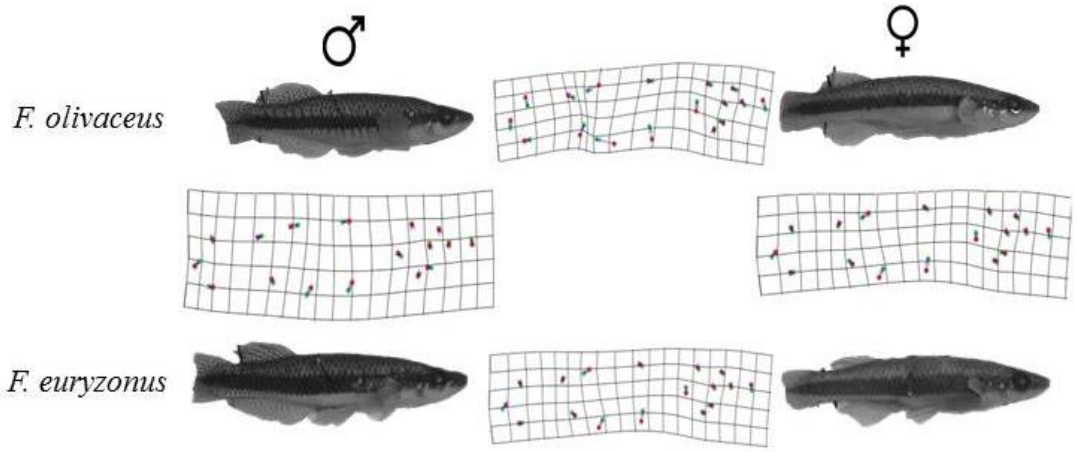

Figure 5.3 Reading clockwise, thinspline grid array (vectors magnified 3 times) showing variation between sexes and species.

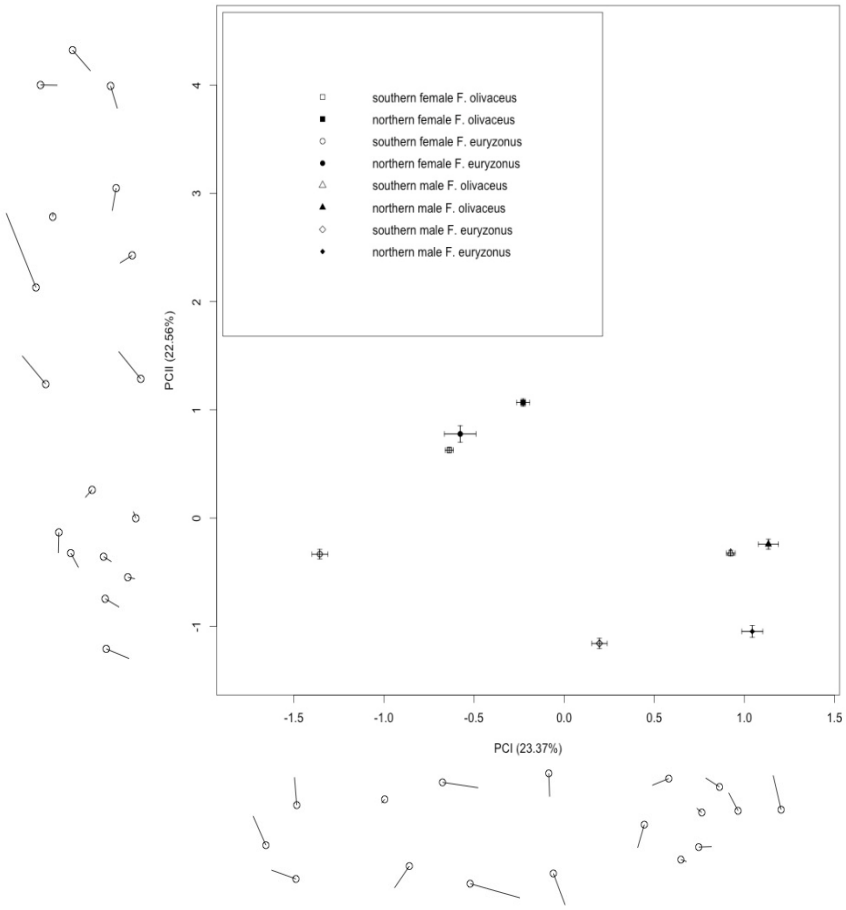

Figure 5.4 For visualization purposes, means of PCA (± 1 SE) for species differentiation, sexual dimorphism, and drainage position analyses are presented in a single figure.

There were significant differences in shape (Table 5.2) between CS (Df=1, F=9.484, P< 0.001), sex (Df=1, F=179.396, P< 0.001), and species (Df= 1, F= 10.760, P< 0.001) of *Fundulus* spp. There was a significant interaction effect between CS and sex (Df= 1, F=11.205, P< 0.001). However, there were no significant interaction effects between CS and species (Df= 1, F=0.826, P=0.481), sex and species (Df= 1, F=1.122, P=0.342), or size, sex, and species (Df= 1, F=0.186, P=0.9058).

Table 5.2

Results of MANOVA comparing the first 3 PCA axes scores for size, sexual dimorphism, and species differentiation.

	Df	Wilks	Approx. F	num Df	den Df	eta^2	Pr(>F)
CS	1	0.867	9.484	3	186	0.077	< 0.001
Sex	1	0.257	179.396	3	186	0.738	< 0.001
Species	1	0.852	10.760	3	186	0.141	< 0.001
CS * Sex	1	0.847	11.205	3	186	0.165	< 0.001
CS * Species	1	0.987	0.826	3	186	0.019	0.481
Sex * Species	1	0.982	1.122	3	186	0.018	0.342
CS * Sex * Species	1	0.997	0.186	3	186	0.002	0.9058
Residuals	188						

5.4.2 Position Along the River Continuum

Groups, when separated into northern and southern locations had CSs that were comparable to other analyses performed on the data where, mean CS for northern female *F. olivaceus* was 74.201 (±1.289), and southern female *F. olivaceus* was 73.132 (±1.104), northern female *F. euryzonus* was 72.746 (±1.201), and southern female *F. euryzonus* was 69.871 (±1.539), northern male *F. olivaceus* was 84.289 (±2.047), and southern male *F. olivaceus* was 72.947 (±1.514), northern female *F. euryzonus* was 80.974 (±1.654), and southern female *F. euryzonus* was 72.845 (±2.089). To visualize structure in the dataset, groups (female *F. olivaceus*, female *F. euryzonus*, male *F. olivaceus*, and male *F.*

euryzonus) were split into northern and southern positions (pooled regardless of origin) and placed in ordination space (Figure 5.4). Higher scores on both axes are associated with an upturned snout shape conformation and a larger anal fin indicative of more "male" shape. Both female and male *F. olivaceus* appear to be undifferentiated in the ordination in regards to position. Whereas, both female and male *F. euryzonus* appeared to differentiate along the river continuum where northern fish had higher scores along axis 1. Females of *F. euryzonus* were also strongly differentiated along axis 2.

There were significant differences in shape between northern and southern groups of female (Df=1, F=7.407, P< 0.001) and male (Df=1, F=7.070, P< 0.001) *F. euryzonus* (Table 5.3). Neither test (females or males) for differences in position was significant for *F. olivaceus* (Df=1, F=0.979, P=0.711; Df=1, F=0.987, P=0.895, respectively). Using position groups, there were two significant CS tests, male *F. olivaceus* (Df=1, F=2.882, P=0.046), and female *F. euryzonus* (Df=1, F=0.620, P< 0.001) and one interaction effect between size and position for male *F. euryzonus* (Df=1, F=0.760, P=0.027).

Table 5.3

Results of MANOVA comparing the first 3 PCA axes scores for position differentiation.

	Df	Wilks	Approx. F	num Df	den Df	eta^2	Pr(>F)
F. olivaceus females							
CS	1	0.937	1.457	3	65	0.064	0.235
Position	1	0.979	0.460	3	65	0.021	0.711
CS * Position	1	0.928	1.692	3	65	0.072	0.177
Residuals	67						
F. olivaceus males							
CS	1	0.839	2.882	3	45	0.113	0.046
Position	1	0.987	0.201	3	45	0.013	0.895
CS * Position	1	0.923	1.244	3	45	0.077	0.305
Residuals	47						
F. euryzonus females							
CS	1	0.620	5.930	3	29	0.329	< 0.001
Position	1	0.566	7.407	3	29	0.434	< 0.001
CS * Position	1	0.985	0.145	3	29	0.015	0.932
Residuals	31						
F. euryzonus males							
CS	1	0.936	0.750	3	33	0.105	0.530
Position	1	0.609	7.070	3	33	0.391	< 0.001
CS * Position	1	0.760	3.473	3	33	0.240	0.027
Residuals	35						

5.4.3 River of Origin Effect

I examined the shapes of a total of 71 *F. olivaceus* females for differentiation based on origin: 16 from the Amite, 38 from the Tangipahoa River, and 17 from the Tickfaw River. The mean Amite River CS was 75.55 (±1.43), the mean Tangipahoa River CS was 75.48 (±1.66) and the Tickfaw River CS was 67.37 (±2.44). Amite CS ranged between 68.21 and 90.58, Tangipahoa River CS ranged from 57.44 to 94.97, and the Tickfaw River CS ranged between 49.68 and 82.03. The first 4 axes explained 65.7 % of the variation in the shape coordinates for origin differentiation (Figure 5.5). Axis one explained 26.1% of the variation, which did not separate the shapes of *F. olivaceus* females among river of origin. Axis two explained 19.4 % of the variation in shape among origin and more clearly differentiated shapes associated with origin for *F. olivaceus* females. Shapes from the Amite and Tangipahoa Rivers (higher scores on axis two) were associated with a longer snout (LM 1), a greater distance between the pectoral fin origin (LM 10) and the anal fin origin (LM 12), and a shorter caudal peduncle (LMs 16, 17, and 18) relative to the shapes from the Tickfaw River.

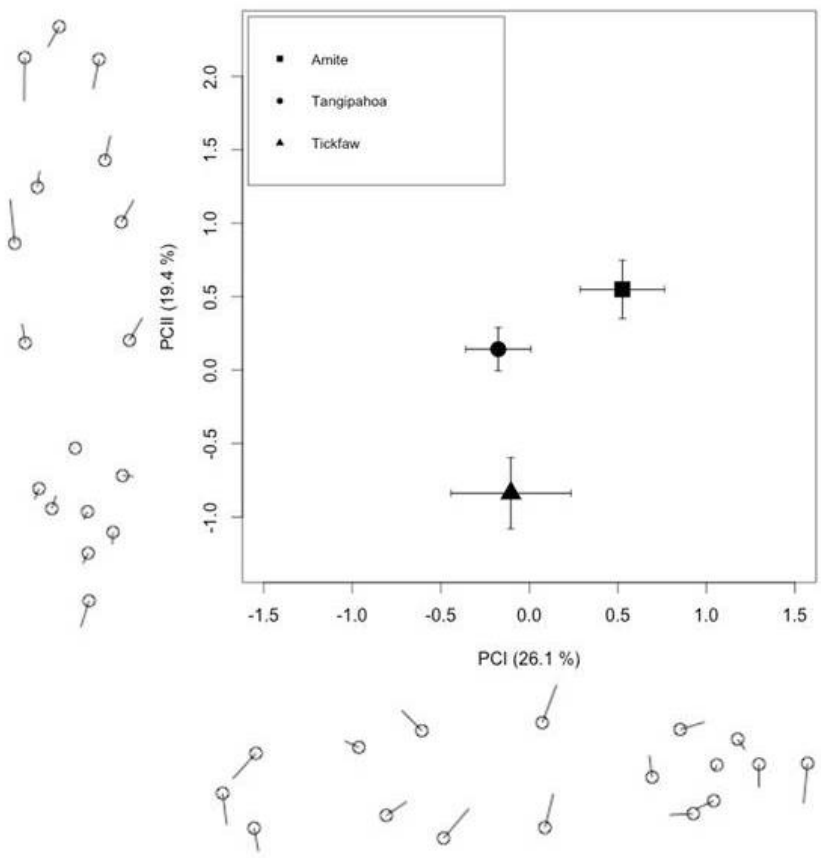

Figure 5.5 Mean (± 1 SE) scores of the first three PCA axes for female *F. olivaceus* only.

I examined the shapes of a total of 51 *F. olivaceus* males for differentiation based on origin; 10 from the Amite, 23 from the Tangipahoa River, and 18 from the Tickfaw River. The mean Amite River CS was 73.18 (±2.94), the mean Tangipahoa River CS was 76.74 (±2.27) and the Tickfaw River CS was 78.68 (±4.11). Amite CS ranged between 52.71 and 82.71, Tangipahoa River CS ranged from 61.03 to 97.85, and the Tickfaw River CS ranged between 52.27 and 109.13. The first six axes explained 76.6 % of the variation in the shape coordinates for origin differentiation in male *F. olivaceus*. Axis one explained 21.8 % of the variation however there does not appear to be any separation in origin from the ordination (Figure 5.6). Axis two explains 17.4 % of the variation and

appears to separate the Amite River (higher scores) from the Tangipahoa River (lower scores) where the Tickfaw River shapes are similar to the other two rivers. Higher scores on axis two are associated with an up turned snout where LMs 1, 2, 15, 16 and 17 are more superior and LMs 9, 10, 11, and 12 are more inferior relative to the consensus shape.

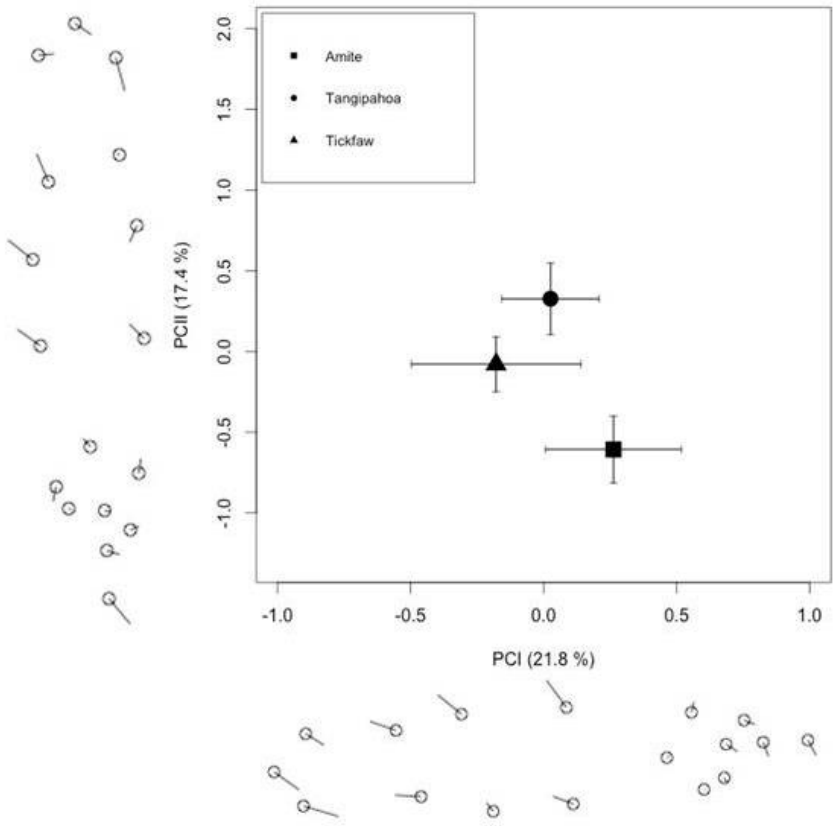

Figure 5.6 Mean (± 1 SE) scores of the first three PCA axes for male *F. olivaceus* only.

I examined the shapes of a total of 35 *F. euryzonus* females for origin differentiation; 23 from the Amite, and 12 from the Tangipahoa River. The mean Amite River CS was 71.38 (±1.81), and the mean Tangipahoa River CS was 69.87 (±2.43). Amite CS ranged between 57.58 and 85.24, and the Tangipahoa River CS ranged from 57.94 to 83.80. The first three axes explained 63.9 % of the variation in the shape

coordinates for origin differentiation in female *F. euryzonus*. Axis one explained 34.4 % of the variation and most clearly separated origin (Figure 5.7). Higher scores on axis one are associated with an upturned snout and a "u-shaped" conformation and reflect the shape observed in the Tangipahoa River. Axis two explains 18.0 % of the variation but does not distinguish origin.

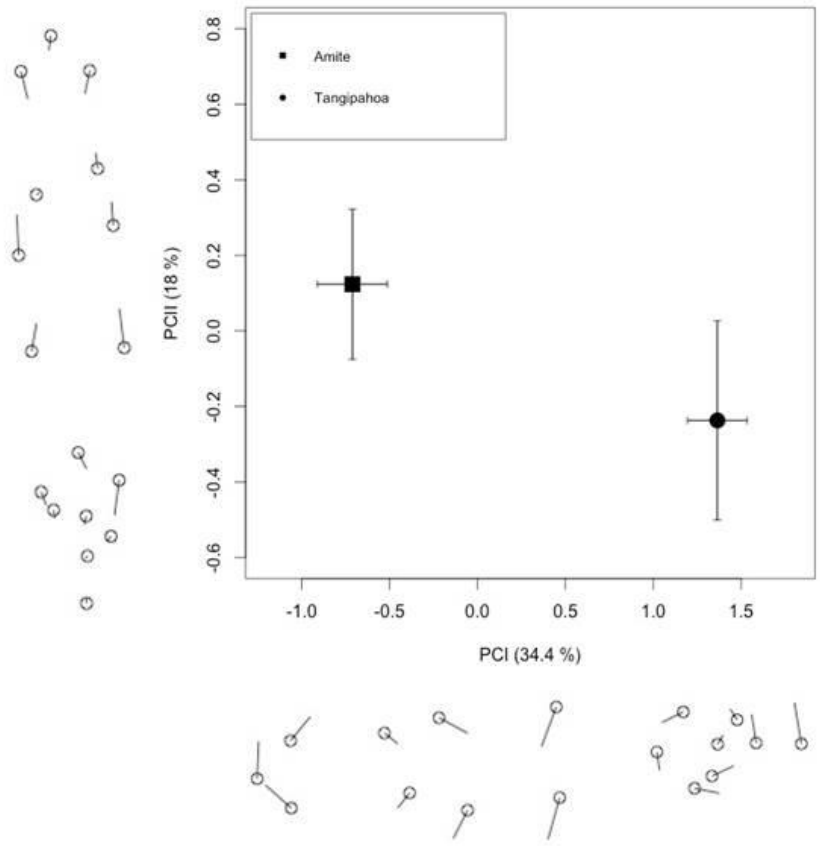

Figure 5.7 Mean (± 1 SE) scores of the first three PCA axes for female *F. euryzonus* only.

I examined the shapes of a total of 37 *F. euryzonus* males for origin differentiation; 21 from the Amite, and 16 from the Tangipahoa River. The mean Amite River CS was 73.61 (±2.82), and the mean Tangipahoa River CS was 79.63 (±3.03). Amite CS ranged between 55.25 and 94.34, and the Tangipahoa River CS ranged from

61.79 to 99.90. The first three axes explained 59.3 % of the variation in the shape coordinates for origin differentiation in male *F. euryzonus*. Axis one explained 29.3 % of the variation in the shape data and most clearly separated origin (Figure 5.8). Higher scores on axis one are associated with an upturned snout and a "u-shaped" conformation and reflect the shape observed in the Tangipahoa River. Axis two explains 17.3 % of the variation in the shape data but does not distinguish origin.

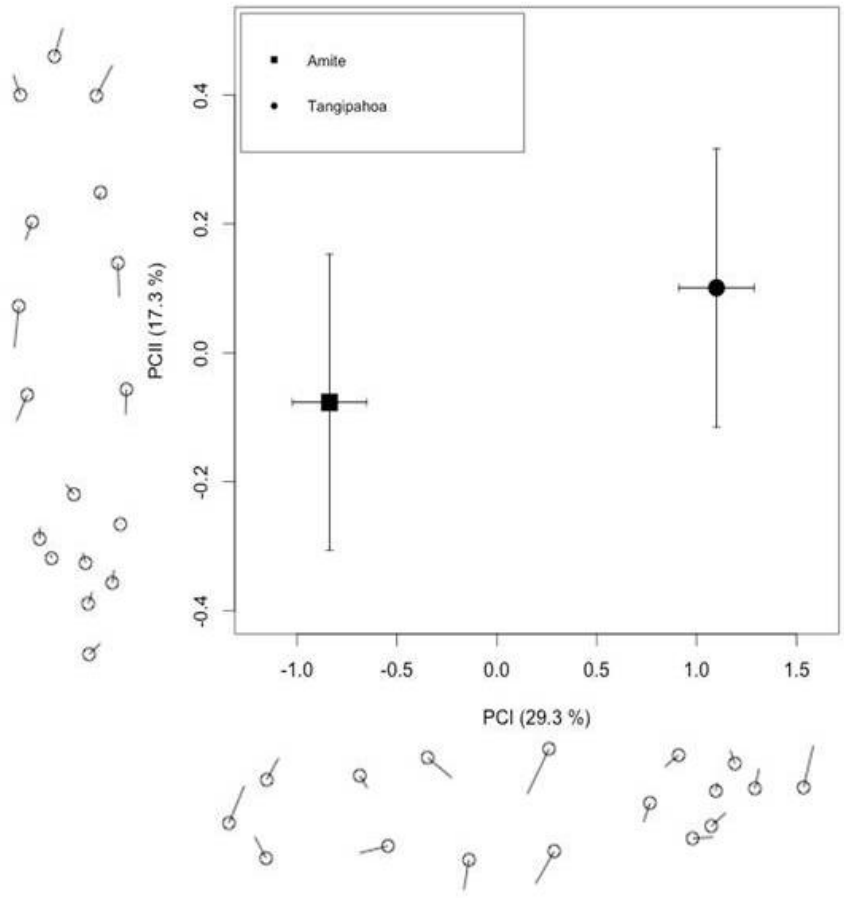

Figure 5.8 Mean (± 1 SE) scores of the first three PCA axes for male *F. euryzonus* only.

There were significant differences in shape (Table 5.4) between origin for *F. olivaceus* females (Df=2, F=4.697, P< 0.001), *F. olivaceus* males (Df=2, F=0.206, P< 0.001), *F. euryzonus* females (Df=2, F=23.230, P< 0.001), *F. euryzonus* males (Df=1,

F=35.872, P< 0.001). There was a significant effect of CS on shape in all cases (Df=1, F=0.8.725, P< 0.001; Df=1, F=13.183, P< 0.001; Df=1, F=7.850, P< 0.001; Df=1, F=9.066, P< 0.001; respectively). However, there were no significant interaction effects between CS and origin (Df=2, F=0.853, P=0.256; Df=2, F=0.716, P=0.288; Df=1, F=1.772, P=0.175; Df=1, F=2.449, P=0.082; respectively).

Table 5.4

Results of MANOVAs for the first four PCA axes for the river of origin effect

	Df	Wilks	Approx. F	num Df	den Df	eta^2	Pr(>F)
F. olivaceus females							
CS	1	0.640	8.725	4	62	0.277	< 0.001
Origin	2	0.589	4.697	8	124	0.229	< 0.001
CS * Origin	2	0.853	1.287	8	124	0.075	0.256
Residuals	65						
F. olivaceus males							
CS	1	0.336	13.183	6	40	0.679	< 0.001
Origin	2	0.206	0.206	12	80	0.529	< 0.001
CS * Origin	2	0.716	0.716	12	80	0.151	0.288
Residuals	45						
F. euryzonus females							
CS	1	0.552	7.850	3	29	0.397	< 0.001
Origin	1	0.294	23.230	3	29	0.706	< 0.001
CS * Origin	1	0.845	1.772	3	29	0.155	0.175
Residuals	31						
F. euryzonus males							
CS	1	0.533	9.066	3	31	0.618	< 0.001
Origin	1	0.224	35.872	3	31	0.776	< 0.001
CS * Origin	1	0.810	2.449	3	31	0.192	0.082
Residuals	33						

5.5 Discussion

Most of the variability in body shape observed in the present study was accounted for by sexual dimorphism. Sexual dimorphism is common in fish (Parker, 1992) and has been described in this species complex (Welsh et al., 2013). However, this is the first use of GM to quantify sexual dimorphism in the remaining two species of this complex (*F. olivaceus* and *F. euryzonus*). More specifically, in the current work the variation in the anal fin origin point (LM 12) was the diagnostic LM that differentiated the sexes. The location of the urogenital opening in females and the lengthening of the anal fin in males for courtship displays are likely responsible for the movement of this LM. The result is that the first two PC axes of the sexual dimorphism/species shape variation analyses both reflect LM 12 movement to a more inferior position, further from the body for females (Figure 5.4). Variation in the shape and size of the anal fin is also sex specific and has been attributed to courtship rituals and spawning in this genus (Carranza & Winn, 1954). Welsh & Fuller (2015) described the variation in anal fin shape and size between sexes in detail where fins were excised and spread flat for digitization. This allowed Welsh & Fuller (2015) to accurately measure the base length, the length of the attachment of the fin to the body as well as differences in angle at the fin apex. In the current study, I did not excise fins but could approximate variation in base length between groups using the whole body LM technique (variation in LM 12 & 14). Additionally, Welsh et al. (2013) and Welsh & Fuller (2015) assessed age and standard length of individuals; in this study, I assumed age and standard length as a function of centroid size to streamline analyses.

Allometry (CS) was also a significant factor in determining the shape of individuals (Tables 5.2, 5.3 and 5.4). These findings are consistent with Schaefer et al. (2011), and Welsh et al. (2013). CS did not have a significant interaction effect with respect to species or between species and sex. Interactions between size and sex have also been observed in the cichlid genus *Tropheus* (Herler et al., 2010). These fishes are mouthbrooders and incubate their eggs in the buccal cavity, a trait that leads to very specific shape variation in cranial anatomy (Oliveira & Almada, 1995; Kocher, 2004). In *Tropheus* fishes, the cranial anatomy of females drive sexual selection in much the same way male anal fin anatomy likely drives sexual selection in *Fundulus* fish (Carranza & Winn, 1954; Foster, 1967). Many of the studies relating to sexual dimorphism are associated with size (reviewed by Blanckenhorn, 2005) but studies such as this one improve understanding about the prevalence of shape sexual dimorphism.

Perhaps not surprisingly species differed in body shape (Table 5.1, Figure 5.3). In the present study, *F. olivaceus* had a more upturned snout body conformation relative to *F. euryzonus* (Figure 5.3). These results are consistent with previous studies concerning shape variation among species in *Fundulus*. Schaefer et al. (2011) showed that differentiation among species, although much less distinct than variation due to river of origin, was more evident between *F. euryzonus* and other members of the species complex. Additionally, Schaefer et al. (2011) found that the shape variation was associated with a more terminal mouth. The findings of this study corroborate that of Schaefer et al. (2011) and add that the entire body shape of *F. olivaceus* conforms to a more u-shape with upturned caudal fin and a more inferior body relative to the *F. euryzonus* as well as a more superior mouth position (Figures 5.3 and 5.4).

River of origin had an effect on the shape of the sexes within each species. Female and male *F. olivaceus* appeared to have downturned snout shape conformation in the Amite River relative to the consensus shape of all rivers combined (Figures 5.6 and 5.7). Additionally, a shorter caudal peduncle was detected in females from the Tangipahoa and Amite Rivers relative to the consensus shape (Figure 5.6) and for males from the Amite River relative to the consensus shape (Figure 5.7). Both female and male *F. euryzonus* exhibit upturned snout body shape conformation in the Tangipahoa River (Figures 5.8 and 5.9) relative to the consensus shape for the respective sex and species groupings. Also, the upturned snout body shape was apparent in all northern fish, even if the MANOVA was not always significant (Figure 5.4). These results, similar to Schaefer et al. (2011) suggest that habitat variation can have profound effects on the shape of an organism.

5.6 Acknowledgments

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APPENDIX A – IACUC Letter

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.4063 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**11092206**
PROJECT TITLE:	**Population Genetics & Systematics of Freshwater Fishes and Herps**
PROPOSED PROJECT DATES:	**9/2014 – 9/ 2017**
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Brian Kreiser**
DEPARTMENT:	**Biological Sciences**
FUNDING AGENCY/SPONSOR:	**na**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2017**

Frank Moore, Ph.D.
IACUC Chair

Date 10-15-14