THE HYBRIDIZATION BETWEEN THE ENDANGERED GAMBUSIA NOBILIS AND INTRODUCED GAMBUSIA GEISERI IN TEXAS

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A Thesis

Presented to

The Faculty of the Department of Biological Sciences

Sam Houston State University

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In Partial Fulfillment

of the Requirements for the Degree of

Master of Science

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by

Victoria Rodriguez

August, 2017

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DEDICATION

For my dad, Fred.

For my mom, Imelda.

ABSTRACT

Rodriguez, Victoria. *The hybridization between the endangered Gambusia nobilis and introduced Gambusia geiseri in Texas.* Master of Science (Biology), August, 2017, Sam Houston State University, Huntsville, Texas.

Introduced species are a major cause of biodiversity loss because of predation, competition for limited resources and space, and hybridization with native taxa. Hybridization poses the greatest risk for native taxa when the non-native and native taxa are closely related. This can compromise the genetic structure of native populations and drive those taxa to extinction. Moreover, the extinction risk to native taxa by hybridization with non-native is greatest when native taxa are rare (e.g., endangered or threatened) because rare taxa often lack the genetic variation necessary mitigate ongoing hybridization events. Herein, we provide morphological and genetic evidence to suggest that the introduced Largespring Gambusia (*Gambusia geiseri*) and endangered Pecos Gambusia (*Gambusia nobilis*) are hybridizing within the San Solomon Spring complex, Reeves, Co. Texas.

We inferred hybridization and gene flow from data collected on seven morphometric characters, nine meristic measurements, and five molecular markers (the mitochondrial gene Cytb; nuclear genes Rag 1, Rag 2, and RPS7; and one microsatellite) from the two species and the putative hybrid. The results support morphological intermediacy and mixed genetic heritage of *Gambusia nobilis* and *Gambusia geiseri* in some individuals. In addition, we were able to infer extensive hybridization and introgression over several generations. Thus, alternate conservation efforts may be needed to counteract the effects hybridization on the endangered Pecos Gambusia.

KEY WORDS: Hybridization, Gambusia, Morphology, Population Genetics.

ACKNOWLEDGEMENTS

First, I'd like to thank those who's help directly influenced the completion of this project. Thank you to Dr. Chad W. Hargrave and Dr. Chris P. Randle for providing me with the tools and support needed for completion. Thank you to Kelbi D. Deluene for helping collect and measure individuals for the morphological study. Thank you to Dr. Justin K. Williams for taking the gonopodia photos. Thank you to Dr. Juan D. Daza for allowing me to use his lab space when collecting morphological data.

Secondly, I'd like to thank my thesis committee, Dr. Chad Hargrave, Dr. Randle, and Dr. John B. Pascarella, for their suggestions on carrying out this project as well as comments and edits on this document.

Most importantly, I would not have completed this project without the help of my family, mentors, and friends. Thank you to Dr. Hargrave for entrusting me to help tie up this question proposed years ago. Thank you to Dr. Randle for the countless pep-talks and encouragement, incredible patience and guidance, and for going above and beyond the role of a committee member and mentor. Thank you to Dr. Daza for taking me in as a mentee, opening your lab to me, and for always considering me when networking. Thank you to Dr. Chris M. Schalk and Dr. Carmen G. Montaña for their continuous support, help, and advice in navigating through graduate school. Special thank you to Elizabeth Glynn, Maria C. Vajello-Pareja, and Melanie L. Velez for their help in keeping my mental sanity, helping me repair myself when I was broken, always saying exactly what I need to hear, and for always picking me up and helping me dust myself off. It is a direct result of the help and assistance of these individuals that I am now able to successfully close this chapter of my life and begin the next step in my academic career.

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CHAPTER I

Introduction

Introduced species can reduce diversity of native taxa by direct predation, competition for limited resources and space, and through hybridization (Rhymer and Simberloff 1996). Hybridization is particularly problematic when genetically distinct non-native and native taxa are closely related (Arnold 1997; Harrison 1993). In these cases, successful hybridization can compromise the genetic structure of the native species and drive native species extinction due to genetic introgression, outbreeding depression, or decreasing fecundity (Arnold 1997; Rhymer and Simberloff 1996). The risk of extinction is greater when hybridization occurs between a rare native species and an abundant introduced species (Rhymer and Simberloff 1996). The strong link between native species extinction and hybridization rates with non-native species has been well documented across various taxa in the literature (Allendorf *et al.* 2001; Dowling and Secor 1997).

The California Tiger Salamander, *Ambystoma californiense*, was once an abundant across California's Central Valley and Coast range (Fisher and Shaffer 1996). Declining populations due to habitat loss and fragmentation prompted US Fish and Wildlife Service to determine the California Tiger Salamander as a threatened status under the Endangered Species Act (USFWS 2004). This species is further threated due to competition and hybridization with the introduced Barred Tiger Salamanders, *Ambystoma tigrinum* (Riley et al. 2003; Fitzpatrick et al. 2009). Genetic analysis of the hybridization between these two species suggest the *A. californiense* may be genetically extinct and

protection of this introgressed species should be based on phenotypic and ecological authenticity (Fitzpatrick et al. 2009)

Mallard ducks, *Anas platyrhynchos*, have been widely introduced to stock hunting areas (Fowler et al. 2009; Simberloff 2013). The introduction of the mallards has contributed to the decline of native duck species via hybridization (Rhymer and Simberloff 1996). For example, the Hawaiian duck, *Anas wyvilliana*, endemic to the Hawaiian Islands, was once common but is now considered federally endangered due to habitat loss and hybridization with the mallards (Fowler et al. 2009; USDI 2005). Hybrids are currently found throughout the entire natural range of the Hawaiian duck and the persistence of mallard genes within the population increases introgression rates contributing to the decline of the Hawaiian duck species (Rhymer and Simberloff 1996; Uyehara et al. 2008).

The Apache trout, *Oncorhynchus apache*, was once native to streams of the White Mountains in eastern Arizona and has since been listed as a threatened species due to hybridization with the introduced Rainbow trout, *Oncorhynchus mykiss* (Rinne and Minckley 1985; Dowling and Childs 1992; Brown et al. 2004). The rainbow trout was introduced throughout Arizona as game fish by anglers and government agencies and has since extensively hybridized with native species, including the Apache trout (Rinne and Minckley 1985; Brown et al. 2004). As a result, populations of pure Apache trout were lost through hybridization and introgression (Dowling and Childs 1992; Rhymer and Simberloff 1996). Today, the Apache trout is limited to the Little Colorado River and part of the San Francisco River in the White Mountains and is extinct throughout most of its

native range where non-native trout thrive (Rinne and Minckley 1985; Dowling and Childs 1992).

Rare species often lack the genetic variation necessary to survive when faced with ongoing hybridization, resulting in a potential extinction of the rare taxa (Rhymer and Simberloff 1996). The protection of threatened species against genetic swamping by non-native species is important in maintaining biodiversity.

Background

The Pecos *Gambusia*, *Gambusia nobilis*, was historically widely distributed throughout the Pecos River in Texas and New Mexico; however, its range has declined due to river channelization created by the Civilian Conservation Corps in the 1930's and a reduction in spring flow due to irrigation practices (Fig. 1; Echelle and Echelle 1986; Echelle *et al.* 1989; Lewis *et al.* 2013; Winemiller and Anderson 1997). The Pecos *Gambusia* is currently restricted to four major areas of the Pecos river drainage, two in West Texas and two in Southeast New Mexico, and is listed as federally endangered by the U.S. Fish and Wildlife Service (Fig. 1; Echelle *et al.* 1989). Extant populations are under continued threat of habitat degradation and face being out competed by introduced congeners throughout their native range (C. Hargrave, pers. comm.; Echelle and Echelle 1986).

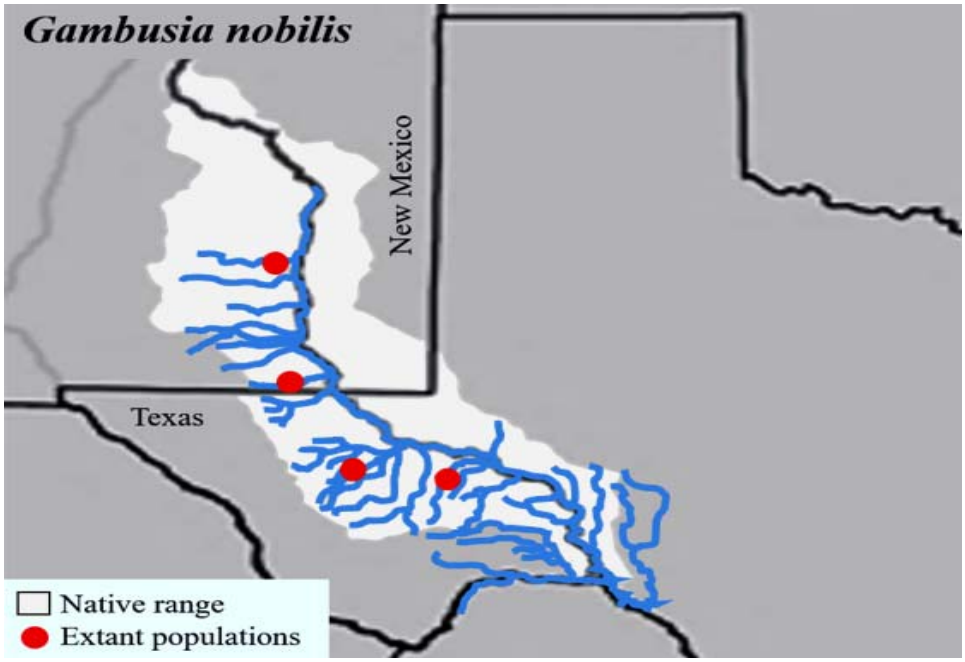

Fig. 1: The historical range and current location of *G. nobilis* within New Mexico and Texas as described by Echelle *et al.* 1989 within New Mexico and Texas.

The largespring *Gambusia*, *Gambusia geiseri*, was once restricted to the headwaters of San Marcos and Comal Springs in Texas; but was introduced throughout Texas in the 1930's to control mosquito populations via predation on larvae (Hubbs and Springer 1957; Cureton et al. 2010; Sanchez *et al.* 2013). As a result, populations of *G. geiseri* are disjunct, surviving only in stenothermal waters in central and west Texas, including spring habitats of *G. nobilis*: San Solomon Springs, Phantom Lake Springs, East Sandia Spring, and Diamond Y Spring (Hubbs and Springer 1957; Lewis et al. 2013; Sanchez *et al.* 2013). Where sympatric, *G. geiseri* has led to the decline of the *G. nobilis* due to competition and potential hybridization (Echelle *et al.* 1989; Hubbs *et al.* 2002; Sanchez *et al.* 2013). Potential hybrids between *Gambusia nobilis* and *Gambusia geiseri* have been suggested based on observed intermediacy of morphological characteristics in San Solomon Springs populations where *G. geiseri* have been noted to outnumber

individuals of *G. nobilis* twenty to one (Sanchez *et al.* 2013; C. Hargrave *et al.*, pers. comm.).

Evidence of habitat partitioning of the two congeners has been observed within the Balmorhea State Park refuge canal (Hubbs et al. 1995). However, based on gut content analysis, the diets of *G. nobilis* and *G. geiseri* overlap 100% suggesting competition for shared resources (Delaune 2015). These two congeners also exhibit similar mating system characteristics. *Gambusia* males possess a copulatory organ formed by a modified anal fin called the gonopodium (Greven 2011). The gonopodium is used to transfer sperm into the female gonopore, allowing internal fertilization (Langerhans 2011). In addition, the shape of the distal tip of the gonopodia varies between species of *Gambusia* and is typically analyzed when differentiating between close relatives (Langerhans 2011).

Courtship between males and females is rare with *Gambusia*, where sexual coercion occurs (Magurran 2011). Sexual coercion and harassment by males of *G. geiseri* has been reported (Plath et al. 2007) with no evidence of female choice (Espinedo et al. 2010). Males of *G. nobilis* show no evidence of male harassment towards unreceptive females in the wild (Leiser et al. 2011). Instead, female cooperation has been seen within *G. nobilis* in which only receptive females allow males to follow for copulation (Leiser et al. 2011). In addition, quality assessment of females by *G. nobilis* males is suggested in which most males briefly follow a receptive female but leave to assess others before choosing one to mate with (Leiser et al. 2011). Other characters, such as standard length and mean brood size, are similar between *G. geiseri* and *G. nobilis* individuals within San

Solomon Spring; however, there is evidence of differences in reproductive timing (Sanchez et al. 2014).

Niche partitioning and differences in reproductive characteristics, both physical and behavior, may be preventing the species from successful introgression. However, similarities in diet, habitat, mating systems, and most importantly morphological intermediacy support the possibility of hybrid presence. Herein, I propose to examine potential hybridization leading to pattern of introgression between these two closely related taxa.

The objective of this study was to identify whether admixture is occurring between the endangered Pecos *Gambusia*, *Gambusia nobilis*, and the largespring *Gambusia*, *Gambusia geiseri*, by (1) quantifying morphological differences between *G. nobilis* and *G. geiseri* to infer intermediacy within putative hybrids, (2) collecting genetic evidence to estimate the degree of admixture, and (3) measuring the extent of introgression, if occurring, between *G. nobilis* and *G. geiseri* within San Solomon Springs at Balmorhea State Park in Toyahvale Texas. We predict that morphological and molecular analysis of *Gambusia nobilis* and *Gambusia geiseri* will reveal extensive hybridization and introgression over several generations.

CHAPTER II

Methods

Study area and sampling

Whole body samples of *Gambusia nobilis*, *Gambusia geiseri*, and putative hybrids were collected in San Solomon Springs within Balmorhea State Park in Reeves County, Texas. Individuals were examined by eye before morphological identification as either *Gambusia nobilis*, *Gambusia geiseri*, or putative hybrid. Traits used to identify individuals as *G. nobilis* included the lack of body spots, presence of a tear drop, and a deep body, head, and caudal peduncle. The body type of *G. geiseri* was considered streamlined with spots on the body and fins. Individuals considered putative hybrids were classified based on the presence of body and fin spots, similar to *G. geiseri*, on an individual with the deep body, head, and caudal peduncle similar to *G. nobilis*.

Individuals were collected seasonally (spring – March/April, summer – July/August, and winter – December/January) for 6 years, totaling 350 individuals to be used for the morphological study. The collection included 114 individuals (64 females, 50 males) identified as *G. nobilis*, 100 individuals (50 females, 50 males) identified as *G. geiseri*, and 136 individuals (86 female, 50 male) identified as putative hybrids. Collected specimen were preserved in 10% formalin for morphometric and meristic measurements.

We collected 110 individuals for molecular analysis during the summer seasons of 2015 and 2016. Sampling was performed using a 9.14 meter seines and individuals collected were placed in 90% EtOH for later morphological and genetic analysis. The collected 110 individuals were identified based on morphology, 42 individuals (27 females, 15 males) identified as *G. geiseri*, 26 individuals (21 females, 5 males)

identified as *G. nobilis*, and 42 individuals (25 females, 17 males) identified as putative hybrids.

Additionally, we obtained 13 EtOH preserved *Gambusia geiseri* individuals, from the Biodiversity Research and Teaching Collections (BRTC) at Texas A&M University, that were collected in the headwaters of San Marcos River in September 2016. This San Marcos River population of *G. geiseri* is roughly 400 miles away from the *Gambusia* individuals of San Solomon Springs and connection between these two locations is unlikely due to the lack of persistence beyond spring environments by *G. geiseri*. Therefore, *G. geiseri* individuals from San Marcos River served as 'pure' *G. geiseri* individuals during analysis as they are presumably unaffected by the proposed interspecific gene flow between *G. nobilis* and *G. geiseri*. Although this population is unaffected by *G. nobilis*, one *Gambusia affinis* male was found within the collection.

Morphological measurements

Morphological counts and measurements were made of individuals identified as *Gambusia nobilis*, *Gambusia geiseri*, or putative hybrid. Morphometric measurements were made using a Mitutoyo caliper and were estimated to the nearest 0.01 mm. Meristic characteristics were ranked on a scale of 3 or 6 depending on the characteristic (Table 1). The following characteristics were ranked on a scale of 0 to 3, where 0 equaled no pigment and 3 equaled dark pigment: the dorsal streak, post anal-streak, mouth pigment and tear drop. Characteristics ranked on a scale of 0 to 6, where 0 equaled no pigment and 6 equaled thick and dark pigment, included the lateral band and anal pigment.

Gonopodia were removed and placed in a VWR International oven set at 54°C for one week within individually labeled 1.5 ml microcentrifuged tubes. Images of the distal

tip of the gonopodium were taken using a Scanning Electron Microscope located at Sam Houston State University (Fig. 2). Gonopodial counts and numerical codes follow Greenfield (1983) and Langerhans (2012). Measurements of relative length of serrae following Peden (1973) and were performed in the image processing program ImageJ (Rasband 1997). The gonopodium representative of *G. nobilis* provided in Rivas (1963) was also examined and used as a paratype to compare results. Samples provided by the BRTC at TAMU were used as gonopodial paratype representation of *G. geiseri*.

Morphological analysis

Measurements collected for each individual were converted into relative measures (per unit of standard length, SL^{-1}) for analysis, to account for body size variation within and among the groups. To analyze the intermediacy of the putative hybrids, a discriminate functional analysis (DFA) was performed on a total of three datasets using IBM SPSS Statistics Version 22.0 (IBM Corp., Armonk, NY). The datasets used contained the following: (1) morphometric (SL^{-1}) and meristic measurements of females, (2) morphometric (SL^{-1}) and meristic measurements of males, and (3) gonopodial-tip morphology. The DFA performed a Bootstrapping of 1000 number of samples, with a Mersenne Twister seed of 2,000,000 and a confidence interval level set to 95.0%, on all five datasets independently.

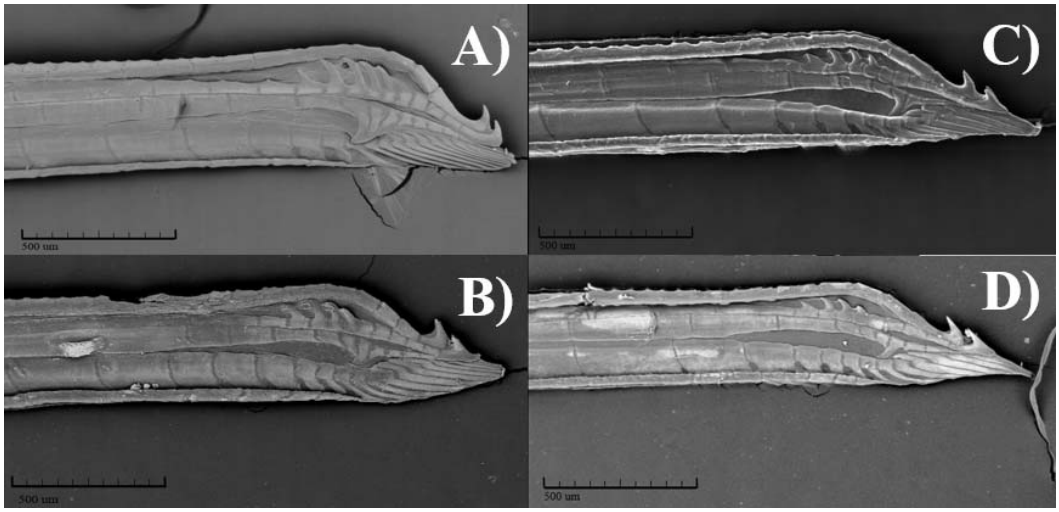

Fig. 2: Gonopodial scans: A) Individual GH116, morphologically classified as *Gambusia nobilis*. B) Individual GH121 identified as a putative hybrid. C) Individual GG110 identified as *Gambusia geiseri*. D) Individual TCWC105 used as a pure *Gambusia geiseri.*

Table 1

Character coding of recorded meristic characteristics.

Character	0	1	2	3	4	5	6
Dorsal Streak	None	Light	Dusky	Dark	-	-	-
Lateral Band	None	Light, Thin	Dusky, Thin	Dark, Thin	Light, Thick	Dusky, Thick	Dark, Thick
Post Anal Streak	None	Light	Dusky	Dark	-	-	-
Anal Pigment	None	Small, Light	Small, Dusky	Small, Dark	Large, Light	Large, Dusky	Large, Dark
Mouth Pigment	None	Light	Dusky	Dark	-	-	-
Tear Drop	None	Light	Dusky	Dark	-	-	-
Color	-	Grey	Taupe	Tan	-	-	-
Dorsal Spot Alignment	No Pattern	Very messy	Half Aligned	In a line	Perfectly Aligned	-	-
Caudal Spot Alignment	No Pattern	Very messy	Half Aligned	In a line	Perfectly Aligned	-	-

Note: Dashes represent the lack of numeral use for the character.

DNA extractions and sequencing

Molecular analysis was performed on all EtOH preserved individuals, the 110 individuals collected from San Solomon Spring and the 13 *Gambusia geiseri* individuals from San Marcos River. The caudal peduncle from each individual was cut off to extract genomic DNA from the tissue and caudal fin using QIAGEN DNEasy Kit (QIAGEN, Valencia, CA). Samples were placed 95% EtOH for storage after tissue samples were collected. Once DNA was extracted, we obtained sequences from 3 nuclear genes (RAG1, RAG2, S7RP), the mitochondrial gene (Cybt), and genotypes from a single microsatellite locus to test the hypothesis of introgression between *G. geiseri* and *G. nobilis* (Table 2).

The single microsatellite locus, GG2B, was developed by Cureton et al. (2010) for *Gambusia geiseri*, but was also noted to cross-amplify in *Gambusia nobilis*. Although Cureton et al. (2010) developed eight other microsatellite loci, we were unable to obtain reliable cross-amplification. PCR reactions were performed in 20ul total volume containing 4 µl 5X PCR flexi buffer (Promega, Sigma–Aldrich, Inc), 0.5 mM dNTPs, 3.125 mM MgCl2, 1.25 mg/ml bovine serum albumin (BSA), 0.8 µM Forward primer, 0.8 µM reverse, 2.0 µl of DNA, and 0.15 U of Taq polymerase. We were unable to amplify the microsatellite loci using the protocol used by Cureton et al. (2010), and so the following PCR cycling conditions were used: Initial denaturation at 96°C for 2 min, followed by 40 cycles of 96°C for 1 min, 65°C for 1 min, 72°C for 2 min, and a final extension of 72°C for 10 min. The PCR products were visualized under ultraviolet light on pre-stained ethidium bromide 2% agarose gels for amplification verification. Electrophoreses of PCR products were conducted on Beckman Coulter CEQ 8000

sequencer (Beckman Coulter, Inc., Fullerton, CA). Allelic scoring was performed by hand on Beckman Coulter CEQTM 8000 Genetic Analysis software.

Primers used to amplify RAG1 were described in Lopez et al. (2004). Amplification was performed using reaction and thermal cycling conditions modified from Whitehead (2010). PCR reactions were performed in 20 µl volumes of 4 µl 5X PCR flexi buffer (Promega, Sigma–Aldrich, Inc), 0.3 mM dNTPs, 2.5 mM MgCl2, 1.0 mg/ml bovine serum albumin (BSA), 0.8 µM forward primer, 0.8 µM reverse primer, 2.0 µl of genomic DNA, and 0.15 U of Taq polymerase. The PCR cycling conditions were as followed: the Initial denature at 94°C for 2.5 min, at 55°C for 1 min, at 72°C for 2 min, followed by 38 cycles of 94°C for 45 sec, 55°C for 1 min, 72°C for 2 min, and a final extension of 72°C for 8 min.

Amplification of RAG2 was performed using protocols described by Heinen-Kay et al. (2014a) with the following modifications to the PCR reaction: 6 µl 5X PCR flexi buffer (Promega, Sigma–Aldrich, Inc), 0.2 mM dNTPs, 2.0 mM MgCl2, 0.67 mg/ml bovine serum albumin (BSA), 0.2 µM forward primer, 0.2 µM reverse primer, 2.0 µl of genomic DNA, and 0.12 U of Taq polymerase in 30.0 µl volume reactions.

The first intron S7 ribosomal protein gene was amplified following protocols described in Chow and Hazama (1998), using the S7RPEX1F forward primer and S7RPEX3R reverse primer. The PCR reaction outlined has a final volume of 10 µl, in which we increased to 30 µl by increasing the mixture content threefold.

We amplified the mitochondrial gene Cytb following the protocol described in Vidal et al. (2010) with minor changes. The PCR reaction had a final volume of 30 ul containing 6 µl 5X PCR reaction buffer (Promega, Sigma–Aldrich, Inc), 0.2 mM dNTPs,

0.4 µM of forward primer, 0.4 µM of reverse primer, 2.0 µl of DNA, and 0.1 U of Taq polymerase. PCR thermal conditions given by Vidal et al. (2010) were followed. The products were visualized on pre-stained ethidium bromide 2% agarose gels for amplification verification.

Table 2

Primer sequences used for molecular analysis.

Gene	Primer	Sequence	Size/Length
GG2B	GG2BF	5' - TCTGCTGCTTCTCTCCTCC - 3'	252-258
	GG2BR	5' - GTCCGTCAAAGACTGTCCC - 3'	
Rag1	RAG1F1	5' - CTGAGCTGCAGTCAGTACCATAAGATGT - 3'	1398
	RAG1R2	5' - TGAGCCTCCATGAACTTCTGAAGRTAYTT - 3'	
Rag2	RAG2F	5' - GACCCCGAGYGYTACCTCATCC - 3'	652
	RAG2R	5' - TCGGTGGAGTAGTAAGGCTCCCA - 3'	
S7RP	S7RPEX1F	5' - TGGCCTCTTCCTTGGCCGTC - 3'	628
	S7RPEX3R	5' - GCCTTCAGGTCAGAGTTC - 3'	
Cytb	CytBF1	5' - ATGGCCAACCTACGAAAAAC - 3'	396
	CytBR1	5' – GGGTAGRACATAACCTACGAAG - 3'	

PCR products of the nuclear and mitochondrial genes were purified on polyethylene glycol following be precipitation on 85% and 100% EtOH sequentially. Purified PCR products were sent to the University of Arizona Genomic Center where both forward and reverse strands were sequenced. Gene fragments were assembled and checked by eye using Geneious v8 (http://www.geneious.com, Kearse et al. 2012).

Molecular Analysis

Polymorphic nucleotides sites from nuclear genes were recorded for each individual while sites that were identical or uninformative were ignored. Polymorphism occurring in forward and reverse chromatograms of a single individual was interpreted as evidence heterozygosity. To standardize the coding of alleles, alleles of *G. geiseri* individuals collected from San Marcos coded first, followed by *G. geiseri* individuals from San Marcos Spring, *G. nobilis*, then putative hybrids. Nucleotide data collected from individuals were converted into a binary code, with '001001' representing the homozygosity of allele 1, '002002' representing the homozygosity of allele 2, and '001002' representing heterozygosity of both alleles, for all recorded sites (Fig. 3). The microsatellite loci was coded in a similar fashion to avoid program bias and added to the dataset. Due to the haplotypic nature of mitochondrial DNA and the uniformity of species-specific site classification, Cytb was evaluated separately.

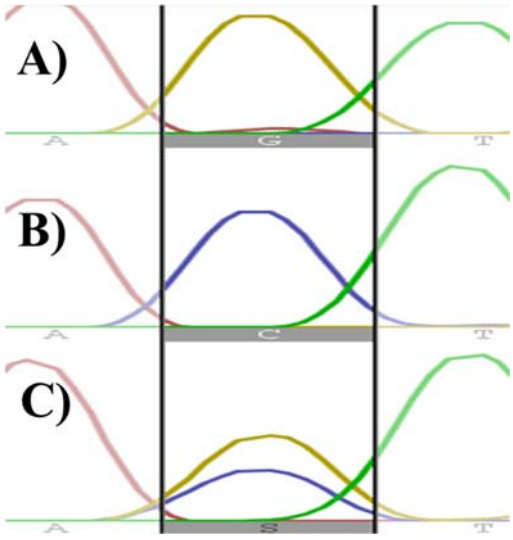

Fig. 3: Polymorphic site of Rag1 at bp862. A) Homozygosity of allele 1 nucleotide, coded as 001001. B) Homozygosity of allele 2 nucleotide, coded as 002002. C) Heterozygosity of allele 1 and 2 nucleotide from both A&B, coded as 001002.

In the creation of data-matrices, individuals were grouped into hypothetical populations corresponding to their phenotypic classification: ‘populations’ included pure *G. geiseri* (San Marcos River), putative *G. geiseri* (San Solomon Springs), putative *G. nobilis* (San Solomon Springs), and putative hybrid (San Solomon Springs). The dataset was formatted to run in the population genetics software GENEPOP version 4.6 (Raymond and Rousset 1995) and converted to other data formats using CREATE (Coombs et al. 2008).

Basic diversity statistics of the population, including allelic richness, expected heterozygosity (He) and observed heterozygosity (Ho), were estimated using FSTAT version 2.9.3.2 (Goudet 1995). Deviation from Hardy-Weinberg equilibrium and test of linkage disequilibrium between loci, and among all loci, and among populations were tested in GENEPOP. Population pairwise Fsts were estimated in Arlequin version 3.5 (Excoffier et al. 2015). Within-group statistics including estimation of heterozygosity, assessment of Hardy-Weinberg deviation, and test of loci linkage disequilibrium, were also performed in Arlequin.

To assess population admixture, the Bayesian admixture model in STRUCTURE v 2.3.4 (Pritchard et al. 2000) was used. Using the admixture model, the program was run for 50,000 Markov chain Monte Carlo (MCMC) replicates after a burn-in period of 100,000 iterations. The number of genepools were set to K=2, as a test of the hypothesis of introgression between two species. As a complimentary approach, hybrid status of individuals was also inferred by NewHybrid (Anderson and Thompson 2002) using a uniform prior for a 100,000 MCMC burn-in and 100,000 steps.

Simulation of a hybrid event was performed in HYBRIDLAB 1.0 (Neilsen et al. 2001) to assess the performance of the two programs and infer the correct Q-value threshold acceptance. Ideally, pure parental genotypes should be used during the simulation to create a list of possible F1 and backcross genotypes. To overcome the lack of pure *G. nobilis*, individuals were selected as representatives of pure *G, nobilis* if they bore nucleotides at polymorphic sites that were not found in pure *G. geiseri* individuals. Using the putative parental genotypes, a dataset with simulated hybrid genotypes (F1, F2, and F1 backcrosses) were created and used to test the inferred admixture and generation in STRUCTURE, with regards to the Q-value, and NewHybrid. The use of Q-values from STRUCTURE to infer hybrids and introgressed individuals can be supported by the computational framework of the admixture model within STRUCTURE. The admixture model estimates mixed ancestry by considering the origin of the allele copy to infer the proportion of the individual's genome originating from given population(s) (Pritchard 2000). The MCMC algorithm of the admixture model estimates the probability distribution of admixture proportions in which possible proportions of an individual are sampled with regards to their genotype. The Q-value is used to denote the estimated mean value of admixture proportions of each individual. Note that prior population information was not considered by the program, therefore the value does not imply the probability of assigning the individual to a given population.

A population with a known ancestry, such as the ideal admixed population simulated, should provide a range of Q-values that we can attribute to each of the six pedigree classes considered. These Q-value thresholds can be applied to our San

Solomon Spring population as the genotypes simulated should theoretically mirror the existing population if admixture if occurring.

The inferred pedigree class in NewHybrid is accepted based on the highest probability of an individual belonging to a given class. The program predicts the origin of an allele on a locus and calculates a joint probability of the multi-locus genotype belonging to a class. The MCMC algorithm of the program estimates posterior probability of an individual belonging to each of the classes. Bayes' law then obtains the full conditional distribution of the individuals placement within a class based on the locus origin.

CHAPTER III

Results

Morphology

Female analysis. Differences between female *Gambusia nobilis* and *Gambusia geiseri* can be inferred by the mean and standard deviation of morphological characteristics (Table 3). Notable characteristics typical of *G. nobilis* include tan coloration and a deeper length in body, head, and caudal peduncle. Notable characteristics typical of *G. geiseri* include dark mouth pigmentation and post-anal streak with a many of spots on the body and fins. Means of putative hybrids typically fall between the two phenotypes.

The results of the univariate ANOVA suggest that the means of the characteristics used differ between the three groups at a significance level of $p<0.001$ with the exceptions of: the relative pre-dorsal length ($p=0.156$), relative post-dorsal length ($p=0.047$), relative head length ($p=0.002$), and lateral band ($p=0.012$). Any characteristic with a significance level $p>0.05$ may not be a reliable determinant based on similarities in means. The significance of 21 out of 22 characteristics suggests a good distinction of characteristics between the groups.

Function 1 of the DFA accounted for 86.9% more of the among-group variance for the three classifications in the dependent variable than the second discriminant function (13.1%). The canonical correlations were both large (0.942 and 0.737) indicating a good association between the individual functions and the dependent variables. The significant Wilk's Lambda ($p=0.000$) suggests that both functions explain the classification results well.

The structure matrix provided the loading scores of each characteristic for both functions used to discriminate against the three groups (Table 4). The loading scores of the structure matrix give meaningful labels to the function, where the correlation between the variables and the discriminant function can be inferred. Characteristics with loading scores ± 0.3 for Function 1 included: dorsal spot alignment (0.497), post-anal streak (0.496), caudal spot alignment (0.433), and tear drop (-0.460). The positive loading scores suggest that the dark post anal streak and the alignment of the dorsal and caudal spots increases the chances that an individual will be classified as *G. geiseri*, while negative loadings suggest the possession of the tear drop increases the chances of an individual being classified as *G. nobilis*. The characteristic with loading scores ± 0.3 correlated with Function 2 was coloration (0.510). The positive loading scores of Function 2 suggests that a taupe coloration increases the chances of being classified as a hybrid individual.

Plotting the observed scores of individuals from the two discriminant functions showed a clear separation of *Gambusia nobilis*, *Gambusia geiseri*, and putative hybrid congruent with our original classification (Fig. 4). The plot implies the importance of Function 2 in discriminating groups based on the overlap that occurs between the individuals of the putative hybrids and *G. geiseri* along Function 1 alone. If placed linearly along Function 2, there would be a complete overlap between individuals clustering near all three group centroids. The power of Function 1 was likely to be due to the distinct discrimination of individuals identified as *G. nobilis* from individuals identified as *G. geiseri* and putative hybrids.

The classification of individuals identified *a prior* were compared to the predicted group classifications inferred by discriminate functions. Despite the discrepancies seen in the results, individuals can be accurately classified with 90.9% accuracy (Table 5).

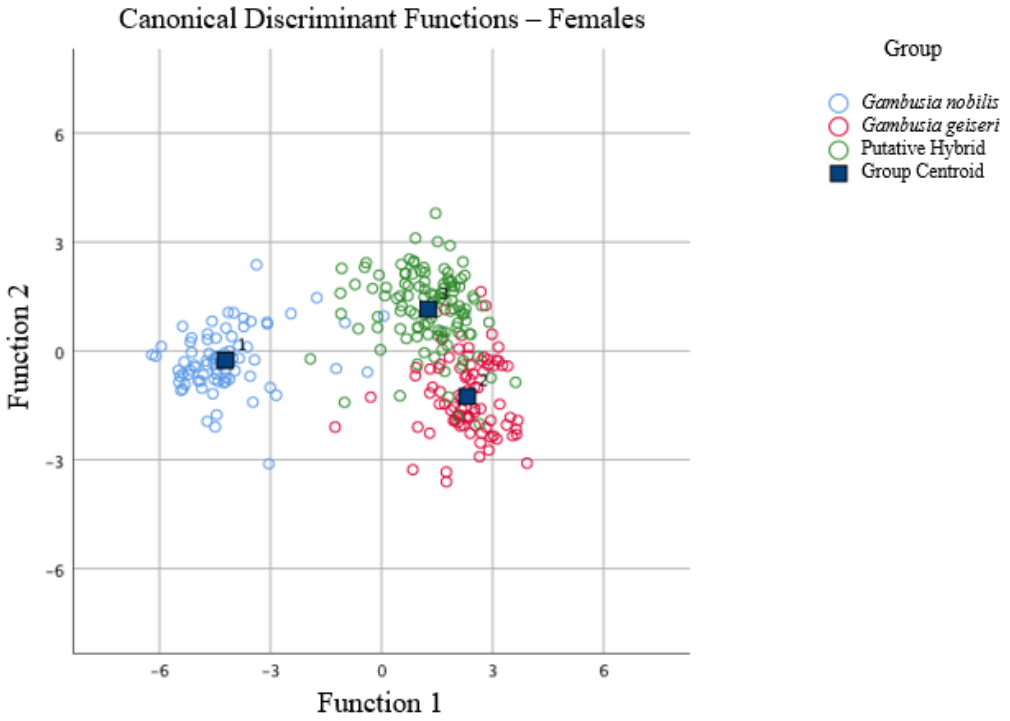

Fig. 4: Scatterplot of female grouping from the Discriminant Function Analysis.

Table 3

Mean ± Standard Deviation (mm) of morphometric measurements and meristic characteristic for female *Gambusia nobilis*, *Gambusia geiseri*, and putative hybrid.

Character	*Gambusia nobilis*	*Gambusia geiseri*	Putative Hybrid
Standard Length	24.0883 ± 6.4888	23.7348 ± 4.0820	23.7333 ± 3.8624
Body Depth (SL^{-1})	0.2521 ± 0.0294	0.2092 ± 0.0242	0.2307 ± 0.0362
Pre-dorsal Length (SL^{-1})	0.6367 ± 0.0274	0.6236 ± 0.0419	0.6286 ± 0.0536
Post-dorsal Length (SL^{-1})	0.3823 ± 0.0331	0.3959 ± 0.0327	0.3924 ± 0.0411
Head Length (SL^{-1})	0.2354 ± 0.0300	0.2320 ± 0.0267	0.2215 ± 0.0296
Head Depth (SL^{-1})	0.1768 ± 0.0201	0.1508 ± 0.0171	0.1569 ± 0.0181
Caudal Peduncle Depth (SL^{-1})	0.1455 ± 0.0109	0.1206 ± 0.0121	0.1286 ± 0.0142
Body spots (SL^{-1})	0.1628 ± 0.2016	1.0087 ± 0.6020	0.5644 ± 0.4970
Basidorsal spots (SL^{-1})	0.0056 ± 0.0271	0.0844 ± 0.0790	0.1017 ± 0.0929
Middorsal spots (SL^{-1})	0.0086 ± 0.0337	0.1379 ± 0.0745	0.1249 ± 0.0825
Terminal dorsal spots (SL^{-1})	0.0000 ± 0.000	0.0251 ± 0.0562	0.0059 ± 0.0195
Basicaudal spots (SL^{-1})	0.0221 ± 0.0361	0.1486 ± 0.0931	0.0982 ± 0.0588
Midcadual spots (SL^{-1})	0.0067 ± 0.0410	0.2556 ± 0.1454	0.2263 ± 0.1640
Terminal caudal spots (SL^{-1})	0.0000 ± 0.0000	0.0802 ± 0.1622	0.0094 ± 0.0315
Dorsal Streak	2.2437 ± 0.6314	2.7000 ± 0.3105	2.6081 ± 0.3955
Lateral Band	2.1875 ± 1.6311	2.4294 ± 1.5720	1.8243 ± 1.1132
Post Anal Streak	0.9687 ± 0.6231	2.6706 ± 0.3582	2.4955 ± 0.5580
Anal Pigment	4.4687 ± 2.1544	2.2588 ± 1.5917	1.6712 ± 1.8297
Mouth Pigment	1.3000 ± 0.9795	2.1823 ± 0.3846	1.6712 ± 0.5897
Tear Drop	1.4562 ± 0.7639	0.0118 ± 0.0763	0.1081 ± 0.4123
Color	2.2250 ± 0.4493	1.3353 ± 0.5845	2.0766 ± 0.4327
Dorsal Spot Alignment	0.1500 ± 0.7647	3.2176 ± 1.1058	3.3964 ± 1.0936
Caudal Spot Alignment	0.4812 ± 0.9725	3.1294 ± 0.9134	3.2207 ± 1.0696

Note: SL^{-1} accounts for body size variation; where SL represents Standard Length and -1 represents the Character/SL.

Table 4

Discriminant loading scores of female characteristics from the Discriminant Function Analysis.

Character	Function 1	Function 2
Body Depth (SL^{-1})	-0.176	0.196*
Pre-dorsal Length (SL^{-1})	-0.040*	0.028
Post-dorsal Length (SL^{-1})	0.053*	-0.015
Head Length (SL^{-1})	-0.043	-0.160*
Head Depth (SL^{-1})	-0.205*	0.042
Caudal Peduncle Depth (SL^{-1})	-0.273*	0.130
Body spots (SL^{-1})	0.225	-0.272*
Basidorsal spots (SL^{-1})	0.184*	0.168
Middorsal spots (SL^{-1})	0.287*	0.048
Terminal dorsal spots (SL^{-1})	0.082	-0.188*
Basicaudal spots (SL^{-1})	0.254*	-0.188
Midcadual spots (SL^{-1})	0.284*	0.035
Terminal caudal spots (SL^{-1})	0.088	-0.262*
Dorsal Streak	0.147*	-0.016
Lateral Band	-0.001	-0.166*
Post Anal Streak	0.496*	0.080
Anal Pigment	-0.211	-0.212*
Mouth Pigment	0.158	-0.226*
Tear Drop	-0.460*	-0.118
Color	-0.191	0.510*
Dorsal Spot Alignment	0.497*	0.279
Caudal Spot Alignment	0.433*	0.218

Note: * Reported absolute correlation between each variable and any discriminant function.

Table 5

Classification results of female specimen.

		Predicted Group Membership of Females			
		G. nobilis	*G. geiseri*	Hybrid	Total
Original Classification	*G. nobilis*	75	0	5	80
	G. geiseri	1	77	7	85
	Hybrid	2	10	99	111

Note: 90.9% of the original grouped cases correctly classified.

Male analysis. Differences between male *Gambusia nobilis* and *Gambusia geiseri* can be inferred by the mean and standard deviation of morphological characteristics (Table 6). Notable characteristics typical of *G. nobilis* males include dark anal pigmentation, possession of a tear drop, and a tan coloration. Notable characteristics typical of *G. geiseri* males include having spots on the body and fins, a dark post-anal streak, dark mouth pigmentation, and a gray coloration. Putative hybrids are typically intermediate between the two phenotypes.

The results of the univariate ANOVA suggest that the means of the characteristics used differ between the three groups at a significance level of $p<0.001$; with the exception of the relative post-dorsal length ($p=0.010$), relative head length ($p=0.803$), terminal dorsal spots ($p=0.014$), terminal caudal spots ($p=0.019$), dorsal streak ($p=0.057$), anal pigment ($p=0.020$). The six insignificant characteristics ($p>0.05$) should be avoided when classifying male individuals, while the remaining 16 characteristics may be significant discriminants of classification.

To interpret the DFA scatterplot of individuals (Fig. 5), loading scores (Table 7) ± 0.3 correlated with Function 1 suggest that the dark post anal streak (0.592), the alignment of the dorsal spots (0.445), the presence of basal dorsal (0.314) and mid-dorsal spots (0.385), and a dark mouth pigment (0.309) increases the chances that an individual will be classified as *G. geiseri* or putative hybrid. The negative loading score correlated with Function 1 suggests the tear drop (-0.456) increases the chances of an individual being classified as *G. nobilis*. Characteristics with a positive loading score of ± 0.3 correlated with Function 2 include coloration (0.644) suggesting that a taupe coloration increases the chances of being classified as a hybrid individual. The negative loading score correlated with Function 2 suggests that a smaller relative head depth (-0.321) increases the chances of an individual being classified as *G. geiseri*. Function 1 of the DFA accounted for 90.6% more of the among-group variance for the three classifications in the dependent variable than the second discriminant function (9.4%). The canonical correlations were both large (0.939 and 0.662) indicating a good association between the individual functions and the dependent variables but the association were significant in Function 1. Based on the significance of Wilk's Lambda (p=0.000), both functions were important determinants in the analysis. This is made evident when looking at the separation of *Gambusia nobilis*, *Gambusia geiseri*, and putative hybrid on the scatter plot. As seen in the female results, if examined linearly there would be a complete overlap between individuals clustering around putative hybrids and *G. geiseri* centroids along Function 1; and a complete overlap between individuals clustering near all three group centroids along Function 2. The power of Function 1 was likely to be due to the distinct discrimination of individuals identified as *G. nobilis* from individuals identified

as *G. geiseri* and putative hybrids. The plot infers the importance of Function 2 in discriminating groups based on the overlap that occurs between the individuals of the putative hybrids and *G. geiseri* along Function 1 alone.

The classification of individuals identified *a prior* were compared to the predicted group classifications inferred based on the discriminate functions. The results suggest that the original grouped cases correctly classified 86.8% of the time (Table 8).

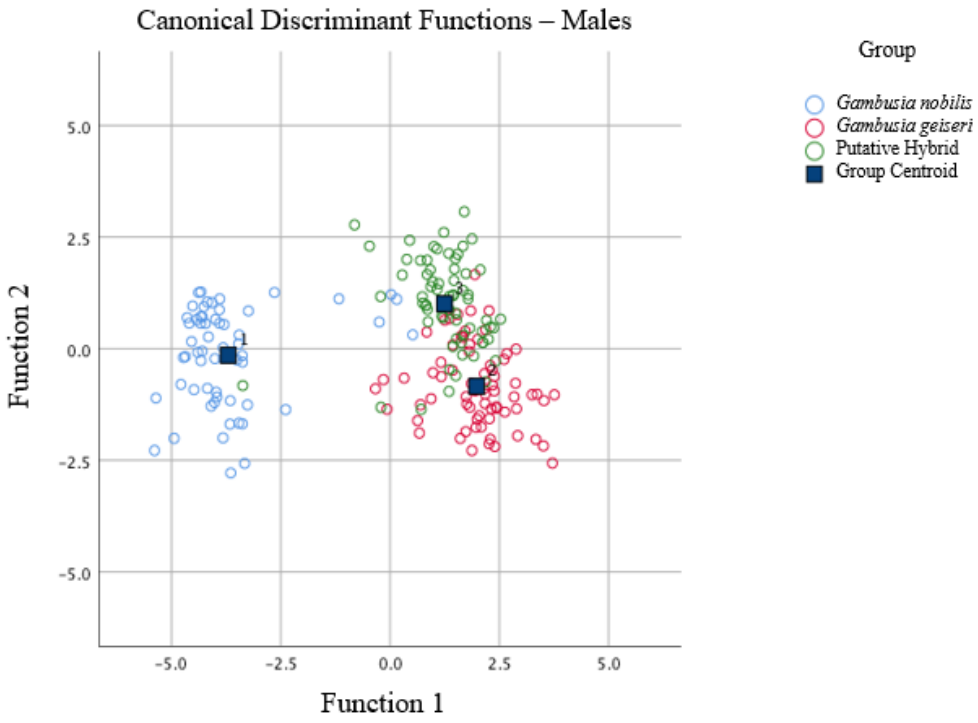

Fig. 5: Scatterplot of male grouping from the Discriminant Function Analysis.

Table 6

Mean ± Standard Deviation (mm) of morphometric measurements and meristic characteristic for male *Gambusia nobilis*, *Gambusia geiseri*, and putative hybrid.

Character	*Gambusia nobilis*	*Gambusia geiseri*	Putative Hybrid
Standard Length	23.7142 ± 2.2886	20.5650 ± 2.1365	20.8316 ± 2.5161
Body Depth (SL^{-1})	0.2476 ± 0.0159	0.2082 ± 0.0272	0.2166 ± 0.0279
Pre-dorsal Length (SL^{-1})	0.5929 ± 0.0250	0.5652 ± 0.0280	0.5767 ± 0.0319
Post-dorsal Length (SL^{-1})	0.4217 ± 0.0255	0.4357 ± 0.0285	0.4358 ± 0.0330
Head Length (SL^{-1})	0.2432 ± 0.0326	0.2402 ± 0.0185	0.2420 ± 0.0256
Head Depth (SL^{-1})	0.1664 ± 0.0171	0.1524 ± 0.0113	0.1443 ± 0.0174
Caudal Peduncle Depth (SL^{-1})	0.1569 ± 0.0121	0.1390 ± 0.0123	0.1408 ± 0.0110
Body spots (SL^{-1})	0.2642 ± 0.3178	0.9331 ± 0.4802	0.7831 ± 0.4186
Basidorsal spots (SL^{-1})	0.0148 ± 0.0486	0.1683 ± 0.1036	0.1635 ± 0.0775
Middorsal spots (SL^{-1})	0.0054 ± 0.0351	0.2053 ± 0.1080	0.1837 ± 0.0855
Terminal dorsal spots (SL^{-1})	0.0000 ± 0.0000	0.0227 ± 0.0527	0.0169 ± 0.0548
Basicaudal spots (SL^{-1})	0.0203 ± 0.0411	0.1135 ± 0.0778	0.0918 ± 0.0533
Midcadual spots (SL^{-1})	0.0023 ± 0.0132	0.2409 ± 0.1826	0.2224 ± 0.1892
Terminal caudal spots (SL^{-1})	0.0000 ± 0.0000	0.0201 ± 0.0637	0.0068 ± 0.0274
Dorsal Streak	2.6250 ± 0.4750	2.7714 ± 0.2649	2.6791 ± 0.2977
Lateral Band	3.6833 ± 1.5622	2.1857 ± 1.0221	2.1716 ± 1.1728
Post Anal Streak	1.0833 ± 0.5459	2.6786 ± 0.3301	2.6791 ± 0.4743
Anal Pigment	1.4167 ± 1.9769	0.6143 ± 1.3437	0.9254 ± 1.5281
Mouth Pigment	1.4333 ± 0.8050	2.6357 ± 0.4810	2.4179 ± 0.5193
Tear Drop	1.9000 ± 0.7237	0.1714 ± 0.4807	0.3507 ± 0.6277
Color	2.4583 ± 0.5231	1.3714 ± 0.5226	2.2836 ± 0.7448
Dorsal Spot Alignment	0.2667 ± 1.0062	2.9071 ± 1.0401	2.8358 ± 0.9103
Caudal Spot Alignment	0.7417 ± 1.3545	2.8500 ± 1.1078	2.5075 ± 1.3885

Table 7

Discriminant loading scores of the male Discriminant Function Analysis.

Character	Function 1	Function 2
Body Depth (SL^{-1})	-0.247*	0.073
Pre-dorsal Length (SL^{-1})	-0.137	0.142*
Post-dorsal Length (SL^{-1})	0.080*	0.031
Head Length (SL^{-1})	-0.015	0.027*
Head Depth (SL^{-1})	-0.187	-0.321*
Caudal Peduncle Depth (SL^{-1})	-0.245*	-0.016
Body spots (SL^{-1})	0.248*	-0.082
Basidorsal spots (SL^{-1})	0.314*	0.087
Middorsal spots (SL^{-1})	0.385*	0.018
Terminal dorsal spots (SL^{-1})	0.077*	-0.033
Basicaudal spots (SL^{-1})	0.237*	-0.086
Midcadual spots (SL^{-1})	0.251*	0.035
Terminal caudal spots (SL^{-1})	0.061	-0.132*
Dorsal Streak	0.053	-0.106*
Lateral Band	-0.201*	-0.079
Post Anal Streak	0.592*	0.217
Anal Pigment	-0.071*	0.066
Mouth Pigment	0.309*	-0.058
Tear Drop	-0.456*	-0.027
Color	-0.204	0.644*
Dorsal Spot Alignment	0.445*	0.128
Caudal Spot Alignment	0.259*	-0.033

Note: * Reported absolute correlation between each variable and any discriminant function.

Table 8

Classification results of study male specimen.

		Predicted Group Membership of Males			
		G. nobilis	*G. geiseri*	Hybrid	Total
Original Classification	*G. nobilis*	57	0	3	60
	G. geiseri	0	60	10	70
	Hybrid	2	11	54	67

Note: 86.8% of the original grouped cases correctly classified.

Gonopodial analysis. Based on the means and standard deviations, differences of *Gambusia nobilis* and *Gambusia geiseri* can be seen (Table 9). Notable characteristics of *G. nobilis* include more segments distal to elbow on Ray 4a, a larger number of segments making up the elbow, overlap between the elbow and Ray 4p serrae, and longer relative length of Ray 4p serrae. Notable characteristic of *G. geiseri* include more segments distal to serrae on Ray 4p and longer relative length of the hook located on Ray 4p.

The results of the univariate ANOVA suggest that the means of the characteristics used differ between the three groups at a significance level of $p<0.001$; with the exception of the segments distal serrae on 4p ($p=0.001$) and relative length of hook on ray 4p ($p=0.040$). The relative length of hook on ray 4p may be the only gonopodia characteristic not good for discriminating, as it does not have a significant difference of means ($p<0.05$).

The Discriminant Function Analysis provided the loading scores, for each characteristic, as two functions used to discriminate against the three groups (Table 10). Loading scores ± 0.3 from Function 1 suggests that an increased number of segments

distal to the elbow on Ray 4a (0.606), a longer relative length of serrae on Ray 4p (0.473), the increased number of segment used to form the elbow on Ray 4a (0.451), and a large number of serrae on Ray 4p (0.404) increases the chances of being classified as a *G. nobilis* gonopodia. The only characteristic associated with an increased chance of being classified as *G. geiseri* was the larger number of segments between the elbow position and the serrae of Ray 4p (-0.651). Along Function 2, a small number of serrae on Ray 4p (0.317) and a small distance between the elbow and the serrae of Ray 4p (0.475) increases the chances of being classified as a gonopodia of *G. geiseri*. A low number of segments used to form the elbow on Ray 4a (-0.401) and a smaller relative length of serrae on Ray 4p (-0.386) increases the chances of being classified as a gonopodia of a putative hybrid.

Function 1 of the DFA accounts for 95.6% more of the among-group variance for the three classifications in the dependent variable than the second discriminant function (4.4%). The canonical correlations of Function 1 (0.959) and Function 2 (0.588) suggests a good association between Function 1 and the dependent variables but the association were weak in Function 2. The observed Wilk's Lambda confirms the insignificance of Function 2 ($p=0.085$) while confirming the significant power of Function 1 ($p=0.000$). Based on the eigenvalue components and the insignificance of Wilk's Lambda, Function 2 was not an important determinant and may be ignored from the model if chosen to. Looking at the scatter plot (Fig. 6), however, Function 2 can help discriminate individuals around group centroid 2 and 3.

As seen with the results from male body characteristics, the power of Function 1 was likely to be due to variation in gonopodia between these species. The scatter plot

differs from the others in that the position of the *G. geiseri* gonopodia centroid was slightly closer to the *G. nobilis* gonopodia centroid than the putative hybrid. The centroids were close enough to each other to imply that classification of males based on their gonopodia due to the similarities of *G. geiseri* and putative hybrid gonopodial characteristics. The scatterplot suggests that, like other morphological characteristics, that gonopodia of hybrid individuals favor characteristic of *G. geiseri*, with vague separation between the two. An increase in sample size may give clearer discrimination among the *G. geiseri* and putative hybrid gonopodia characteristics.

The classification of individuals identified were given from the DFA classification based on their respective morphological traits were congruent 86.1% of the time (Table 11). The paratype gonopodia for *G. nobilis* and *G. geiseri* both were properly classified by the DFA.

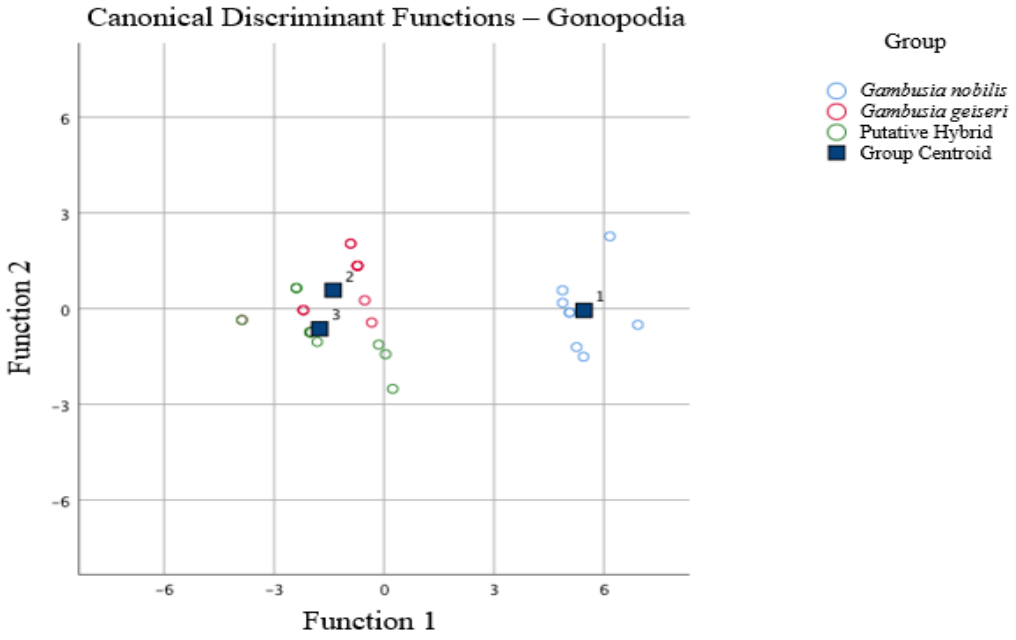

Fig. 6: Scatterplot of gonopodia grouping from the Discriminant Function Analysis.

Table 9

Mean ± Standard Deviation of male gonopodial characteristics for *Gambusia nobilis*, *Gambusia geiseri*, and putative hybrid.

Character	*Gambusia nobilis*	*Gambusia geiseri*	Putative Hybrid
Number of spines on Ray 3	8.500 ± 0.756	6.733 ± 0.594	6.769 ± 1.092
Segments distal to 4a elbow	5.500 ± 0.534	3.067 ± 0.458	3 ± 0.577
Fused elbow elements	3.750 ± 0.463	1.6 ± 0.507	1.846 ± 0.689
Elbow relative to 4p serrae	-2.937 ± 0.776	1.2 ± 0.368	0.846 ± 1.068
Number of serrae on Ray 4p	4.750 ± 0.462	3.467 ± 0.516	3.154 ± 0.375
Segments distal to 4p serrae	5.000 ± 0.534	5.8 ± 0.414	5.923 ± 0.64
Relative length of 4p serrae	2.711 ± 0.462	1.574 ± 0.225	1.689 ± 0.219
Relative length of 4p hook	0.756 ± 0.122	0.956 ± 0.173	0.893 ± 0.233

Note: Relative length taken by the length/width of the character following Peden (1973).

Table 10

Loading scores of gonopodia characterisics in Discriminant Function Analysis

Character	Function 1	Function 2
Number of spines on Ray 3	0.266*	-0.103
Segments distal to 4a elbow	0.606*	-0.092
Fused elbow elements	0.451*	-0.401
Elbow relative to 4p serrae	-0.651*	0.475
Number of serrae on Ray 4p	0.404*	0.317
Segments distal to 4p serrae	-0.208*	-0.087
Relative length of 4p serrae	0.473*	-0.386
Relative length of 4p hook	-0.112	0.243*

Note: * Reported absolute correlation between each variable and any discriminant function.

Table 11

Classification results of male gonopodia.

		Predicted Group Membership of Males based on Gonopodial data			
		G. nobilis	*G. geiseri*	Hybrid	Total
Discriminant Classification	*G. nobilis*	8	0	0	8
	G. geiseri	0	10	5	15
	Hybrid	0	0	13	13

Note: 86.1% of the original grouped cases correctly classified.

Molecular Analysis

Gene classification and information. Sequences for the mitochondrial Cytb produced a 347 bp fragment. Two different haplotypes were found within the San Solomon Spring population, differing at 20 nucleotide sites. Individuals of *G. geiseri* from San Marcos River were monomorphic for one of the haplotypes observed, here referred to as haplotype 1, and completely lacked the other. The mitochondrial haplotype of individuals originally identified as *Gambusia geiseri* from San Solomon Springs also bore haplotype 1, with only a single individual bearing haplotype 2. Individuals originally identified as *G. nobilis* bore mitochondrial haplotype 2 with one individual containing haplotype 1. Of the 48 individuals originally identified as putative hybrids, 33 individuals bore haplotype 1 while the other 15 individuals bore haplotype 2.

Rag1, Rag2 and S7 sequences were 1398 bps, 652 bps, and 628 bps respectively (Table 12). Individuals of *G. geiseri* from San Marcos River were treated as a pure population and served as a reference for differentiating *G. nobilis* and *G, geiseri* alleles.

This study uses the following terminology: alleles labeled “1” are associated with the pure *G. geiseri* population. Alleles labeled “2” were assumed to be ancestral typical of *G. nobilis* morphotypes. This distinction is not relevant for analysis, but does allow consistency in the presentation of genotypic results.

There were six polymorphic sites found within Rag1 with two different nucleotides at each of the six sites among the collected individuals. Pure *Gambusia geiseri* individuals from San Marcos River, were homozygous at all six nucleotide sites. Congruent with the pure population, individuals identified as *G. geiseri* from San Solomon Springs were homozygous for allele 1 at all six nucleotide sites with the exception of one individual missing data from two sites. Individuals originally identified as *G. nobilis* contained a mix of genotypes. Of the 26 individuals originally classified as *G. nobilis*, three of them were homozygous for allele 1 at all six nucleotide sites and five individuals were homozygous for allele 2 at all six polymorphic sites. Heterozygosity was inferred by the presence of overlapping peaks for alternative alleles at the six polymorphic sites. One individual was heterozygous at all six sites, eleven individuals were homozygous for allele 2 at four nucleotide sites (240, 586, 657, 1332) and heterozygous two sites (252, 862), five individuals were homozygous for allele 2 at four sites (240, 586, 657, 1332) and homozygous for allele 1 at two sites (252, 862), and one individual was heterozygous at four sites (240, 586, 657, 1332) and homozygous for allele 1 at two sites (252, 862). Of the 42 individuals originally classified as a putative hybrid, 28 individuals were homozygous for allele 1, one individual was homozygous for allele 2, six individuals were heterozygous at all six sites, five individuals were heterozygous at four sites (240, 586, 657, 1332) and homozygous for allele 1 at two sites

(252, 862), one individual was heterozygous at one site (240) and homozygous for allele 1 at five sites although the lack of the compliment pair for this site could not confirm the heterozygosity, one individual was heterozygous at five sites with data from the sixth site (1332) missing. There seemed to be a linkage of the four nucleotide sites 240, 586, 657, and 1332, and the two sites 252 and 862. Based on this, only two nucleotide sites were used for analysis: site 252 and site 586, labeled as Rag1-2 and Rag1-3 respectively; these sites were used to assign a genotype to each individual. For Rag1-2, allelic richness was reported at 1.892, expected heterozygosity was 0.221 while observed heterozygosity was 0.248. For Rag1-3, allelic richness was reported at 1.988, expected heterozygosity was 0.363 while observed heterozygosity was 0.114 (Table 13).

For S7RP, there were two alternate nucleotides at each of five polymorphic nucleotide sites among the collected individuals (Table 12); nomenclature follows that presented for RAG-1 in the preceding paragraph. Individuals of *G. geiseri* from San Marcos River were homozygous for allele 1 at all five sites, with the exception of one individual in which was heterozygous for allele 1 and 2 at site 554. Of the 42 individuals identified as *G. geiseri*, 41 individuals were homozygous for allele 1 at all sites while one individual was heterozygous. Of the 26 individuals identified as *G. nobilis*, three individuals were homozygous for allele 1, 22 individuals were homozygous for allele 2, and one individual was heterozygous. Of the 42 individuals identified as putative hybrids, 27 individuals were homozygous for allele 1, one individual was homozygous allele 2, and fourteen individuals were heterozygous. Due to the observed linkage among all five sites, only one nucleotide site, 407, was used during analysis as S7RP-3 to assign a

genotype to each individual. The allelic richness for S7RP-3 was reported at 1.992, expected heterozygosity was 0.379 while observed heterozygosity was 0.130 (Table 13).

There were four polymorphic nucleotide sites found within Rag2 (Table 12) with two different nucleotides at a single site among the collected individuals. Pure *G. geiseri* individuals collected from San Marcos River were homozygous for allele1 among all four sites. Similarly, individuals from San Solomon Springs identified as *G. geiseri* were all homozygous for allele 1. Of the 26 individuals identified as *G. nobilis*, three individuals were homozygous for allele 1, 21 individuals were homozygous for allele 2, one individual was heterozygous for allele 1 and 2, and one was unable to properly sequence. Of the 42 individuals identified as putative hybrids, 29 individuals were homozygous for allele 1, one individual was homozygous for allele 2, and twelve individuals were heterozygous for allele 1 and 2. Due to the observed linkage among all four sites, only one nucleotide site, 151, was used during analysis as Rag2-1 to assign a genotype to each individual. Allelic richness for Rag2-1 was reported at 1.988, expected heterozygosity was 0.361 while observed heterozygosity was 0.107 (Table 13). The *G. nobilis* individual with an unsuccessful sequence created 0.81% missing data.

The microsatellite loci 2B was successfully sequenced for 109 individuals, out of the 123, with a total of four different alleles seen throughout the population. Of the eight *G. geiseri* individuals collected from Sam Marcos River successfully sequenced, five individuals were homozygous for an allele 1, and three individuals were heterozygous for alleles 1 and 2. Of the 42 individuals identified as *G. geiseri*, 41 individuals were homozygous for allele 1 and one individual was heterozygous for alleles 1 and 3. Of the 20 individuals identified as *G. nobilis* successfully sequenced, three individuals were

homozygous for allele 1, one individual was homozygous for allele 3, four individuals were homozygous for allele 4, two individuals were heterozygous for alleles 1 and 3, two individuals were heterozygous for alleles 1 and 4, one individual was heterozygous allele 2 and 4; and seven individuals were heterozygous for allele 3 and 4. Of the 39 individuals identified as putative hybrids successfully sequenced, 26 individuals were homozygous for allele 1, two individuals were homozygous for allele 4, three individuals were heterozygous for alleles 1 and 3; and eight individuals were heterozygous for allele 1 and 4. Allelic richness for 2B was reported at 2.854, expected heterozygosity was 0.377 while observed heterozygosity was 0.248 (Table 13). The 14 individuals with unsuccessful sequencing created 11.38% missing data.

Table 12

List of all polymorphic sites observed in the nuclear genes Rag1, S7RP, and Rag2.

	Polymorphic Sites						Total bps
Rag1	240	252	586	657	862	1332	1398
Gambusia geiseri	G	C	G	G	G	T	
Gambusia nobilis	A	T	A	T	C	C	
S7RP	6	124	407	486	554		628
Gambusia geiseri	A	C	T	T	G		
Gambusia nobilis	C	G	C	C	A		
Rag2	151	291	387	466			652
Gambusia geiseri	G	C	T	T			
Gambusia nobilis	A	T	A	C			

Table 13

Loci specific information among the population.

Loci	N	A	Ho	He	Percent missing
2B	4	2.854	0.248	0.377	11.38%
RAG1-2	2	1.892	0.154	0.221	0.00%
RAG1-3	2	1.988	0.114	0.363	0.00%
S7RP-3	2	1.992	0.130	0.379	0.00%
RAG2-1	2	1.988	0.107	0.361	0.81%

Note: N notes the total number of individual alleles observed. A stands for Allelic richness among the population. Ho stands for observed heterozygosity. He stands for expected heterozygosity.

Simulation and inferred ancestry. HybridLab was used to simulate the genotypes of an admixed population that would result from the mating putative pure individuals, pure *G. geiseri* x putative pure *G. nobilis*. The resulting population contained 50 of the F1 genotype, 50 F2 genotypes (F1xF1), 50 *G. geiseri* backcross genotypes (GGxF1), and 50 putative *G. nobilis* backcross genotypes (GNxF1). All six genotypes, including the two putative pure genotypes, were run in STRUCTURE to infer the proper Q-value thresholds. Q-values thresholds were calculated using the simulated population for *G. geiseri* ($0.939 \leq 1$), *G. geiseri* backcross ($0.939 \leq 0.860$), F2 with more *G. geiseri* genotypes ($0.860 \leq 0.591$), F1 individuals ($0.591 \leq 0.409$), F2 with more putative *G. nobilis* genotypes ($0.409 \leq 0.146$), backcross of putative *G. nobilis* ($0.146 \leq 0.074$), putative *G. nobilis* ($0.074 \leq 0$).

Individuals collected were run on STRUCTURE; resulting Q-values and inferred pedigree class can be seen in Tables 14-17. NEWHYBRID was used to generate the probability of belonging to each pedigree class (pure *G. geiseri*, pure *G. nobilis*, F1, F2,

G. geiseri backcross, or *G. nobilis* backcross). Results were largely congruent with results from STRUCTURE. Admixture was detected within three *G. nobilis* individuals in both analyses (Table 16). Of the putative hybrids, the analyses were congruent when estimating the level of admixture in thirteen individuals (Table 17).

There were some disagreements between the analyses. Based on the Q-value threshold, the STRUCTURE analysis suggested admixture within two *G. geiseri* individuals while the probability given by NEWHYBRID suggested no admixture in the *G. geiseri* genotype (Table 15). Similarly, there was discrepancies in the levels of admixture between the two analyses for three putative hybrids (Table 17).

With consideration to Mitochondrial DNA. When examining mitochondrial inheritance and pedigree classification together, we see differences of maternal lineages within individuals with admixture. For example, individuals GG028 and GG031 both have identical nuclear diagnoses, favoring *G. geiseri* genotypes, yet have different haplotypes with GG028 having the mitochondrial haplotype of *G. geiseri* and GG031 having the mitochondrial haplotype of *G. nobilis*. Both GN120 and GN121 have mitochondrial haplotypes associated with *G. nobilis*; however, the nuclear DNA of GN120 is typical of *G. geiseri* and GN121 nuclear DNA is typical *G. nobilis*. Five individuals exhibited the mitochondrial haplotype of *G. nobilis* but admixed nuclear DNA favoring *G. geiseri*. Of these, two exhibit genotypes matching a pure *G. geiseri*, with no admixture. However, no individual exhibited the *G. geiseri* mitochondrial haplotype and had nuclear DNA favoring the *G. nobilis* genotype. A single individual classified as an F1 bore the *G. geiseri* mitochondrial haplotype while 10 F1 individuals that have mitochondria associated with *G. nobilis*.

Reclassification of individuals. Evidence for the need of reclassification can be seen with the 25 individuals within putative hybrids that are homozygous for allele 1 across all loci and have mitochondria associated with *G. geiseri*, inferring that these individuals are *G. geiseri* that were misclassified as hybrids (Table 17). The morphological analysis also shows the discrepancies between the original classification and the DFA classification in some individuals. Using the DFA, STRUCTURE, and NewHybrid results, reclassification of individuals was based on the agreement of at least 2 analyses. The mitochondrial information was not considered due to the nature of inheritance. A comparison of bar plots generated, by STRUCTURE, of population before (Fig. 7) and after (Fig. 8) reclassification is provided as visual support.

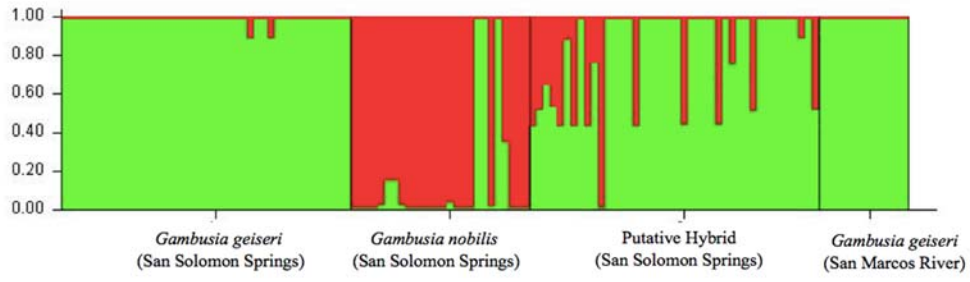

Fig. 7: Bar plot showing the results of the admixture STRUCTURE analysis before reclassification.

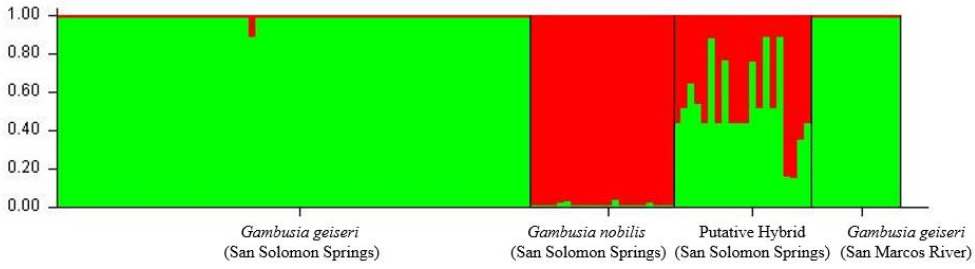

Fig. 8: Bar plot showing the results of the admixture STRUCTURE analysis after reclassification.

Population Genetics. The results of the heterozygosity statistics for each classification after reclassification supports heterozygosity seen within putative hybrid but also heterozygosity still found *G. nobilis* individuals even after reclassification (Table 18). As expected, Rag1-3, S7RP-3, and Rag2-1 suggested that there was no heterozygosity present within *G. geiseri* and *G. nobilis* individuals based on an allelic richness of 1.000 and an observed and expected heterozygosity of 0.000. Despite being homozygous in these three sites, the observed heterozygosity of Rag1-2 within reclassified *G. nobilis* individuals is 0.476 but the allelic richness is 2.000. Considering equal representation of allele1 and 2 based on allelic richness, about half of the individuals of *G. nobilis* are heterozygous, leaving a fourth of the individuals to be homozygous for allele 1 and a fourth of individuals to be homozygous for allele 2. Meaning that of the 21 individuals, only ~5 are homozygous for allele 1 and ~5 are homozygous for allele 2 at all four nuclear sites.

Addressing problematic cases. Although heterozygosity was apparent for Rag1 through the presence of overlapping peaks at polymorphic sites, not all of those sites showed overlapping peaks in heterozygotes, indicating a history of recombination in heterozygotes of previous generations. The most reasonable approach was to distinguish two sets of linked loci as separate loci; these separate loci were not in linkage disequilibrium. Nonetheless, if Rag1-2 and Rag1-3 are removed from the analyses, we still see admixture within the population to support our hypothesis.

Ancestral origin is questioned in one of the polymorphic sites based on a pure *G. geiseri* individual collected from San Marcos River. This individual was heterozygous at site 554 of S7RP, one of the five polymorphic sites within the gene. Using S7RP-3 (site

407) in the analysis, we interpreted the results under the assumption that allele 1 was characteristic of the pure *G. geiseri* individuals from San Marcos River and any allele not found within the pure *G. geiseri* population is characteristic of *G. nobilis*. The heterozygosity of the pure *G. geiseri* individual at site 554 questions the origin of the allele within individuals of San Solomon Springs; whether the presence of the second allele is characteristic of *G. nobilis* or introduced from the San Marcos River population. Although it is likely that variation at the other four polymorphic sites are ancestral divergence of these two species, site 554 serves as a reminder that polymorphism can arise in even pure populations of *G. geiseri*, producing heterozygosity.

Table 14

Summary of test results used to infer ancestry of each individual morphologically identified as *Gambusia geiseri* from San Marcos River.

ID#	Gonopodia	DFA Assignment	MtDNA haplotype	NewHybrid Assignment	STRUCTURE Assignment	Q-value
TCWC101	-	G	G	G	G	0.988
TCWC102	-	G	G	G	G	0.987
TCWC103	-	G	G	G	G	0.987
TCWC104	-	G	G	G	G	0.988
TCWC105	G	G	G	G	G	0.988
TCWC006		G	G	G	G	0.991
TCWC007		G	G	G	G	0.991
TCWC008		G	G	G	G	0.988
TCWC009		G	G	G	G	0.991
TCWC010		G	G	G	G	0.991
TCWC011		G	G	G	G	0.987
XTCWC002		G	G	G	G	0.989
XTCWC003		G	G	G	G	0.991

Note: 'G' represent *G. geiseri*; Gonopodia: '-' represent inability to collect gonopodia data, Cells were left empty for female specimen.

Table 15

Summary of test results used to infer ancestry of each individual originally identified as *Gambusia geiseri* from San Solomon Springs.

ID#	Gonopodia	DFA Assignment	MtDNA haplotype	NewHybrid Assignment	STRUCTURE Assignment	Q-value
GG001		H	G	G	G	0.991
GG002		G	G	G	G	0.991
GG003		H	G	G	G	0.991
GG004		G	G	G	G	0.991
GG005		G	G	G	G	0.991
GG006		G	G	G	G	0.991
GG007		G	G	G	G	0.991
GG008		G	G	G	G	0.991
GG009		H	G	G	G	0.991
GG110	G	G	G	G	G	0.991
GG111	G	G	G	G	G	0.991
GG112	G	G	G	G	G	0.991
GG113	G	G	G	G	G	0.991
GG114	G	H	G	G	G	0.991
GG015		G	G	G	G	0.991
GG116	G	G	G	G	G	0.991
GG117	H	G	G	G	G	0.991
GG118	H	G	G	G	G	0.991
GG119	G	G	G	G	G	0.991
GG120	I	H	G	G	G	0.991
GG121	I	H	G	G	G	0.991
GG122	G	G	G	G	G	0.991
GG123	H	H	G	G	G	0.991
GG124	H	G	G	G	G	0.991
GG125	I	G	G	G	G	0.991
GG026		G	G	G	G	0.991
GG027		H	G	G	G	0.991
GG028*		H	G	G	Gbx	0.887
GG029		H	G	G	G	0.991
GG030		G	G	G	G	0.991
GG031		G	N	G	Gbx	0.889
GG032		G	G	G	G	0.991
GG033		G	G	G	G	0.991
GG034		G	G	G	G	0.991
GG035		G	G	G	G	0.991
GG036		G	G	G	G	0.991
GG079		G	G	G	G	0.991
GG080		G	G	G	G	0.990
GG081		G	G	G	G	0.991
GG082		G	G	G	G	0.991
GG083		G	G	G	G	0.991
GG084		G	G	G	G	0.991

Note: '*' notes individual reclassified for analysis; 'G' represents *G. geiseri*, 'N' represents *G. nobilis*, 'H' represents hybrid, 'Gbx' represents *G. geiseri* backcross; Gonopodia: 'I' represents immature gonopodia, Cells were left empty for female specimen.

Table 16

Summary of test results used to infer ancestry of each individual originally identified as *Gambusia nobilis* from San Solomon Springs.

ID#	Gonopodia	DFA Assignment	MtDNA haplotype	NewHybrid Assignment	STRUCTURE Assignment	Q-value
GN001		N	N	N	N	0.008
GN002		N	N	N	N	0.010
GN003		N	N	N	N	0.012
GN004		N	N	N	N	0.008
GN005		N	N	N	N	0.028
GN006*		N	N	0.5Nbx+0.4N	Nbx	0.161
GN107*	N	N	N	0.4N+0.3Nbx	Nbx	0.158
GN108	N	N	N	N	N	0.028
GN109	N	N	N	N	N	0.010
GN110	N	N	N	N	N	0.013
GN111	N	N	N	N	N	0.008
GN012		N	N	N	N	0.010
GN013		N	N	N	N	0.013
GN014		N	N	N	N	0.013
GN015		N	N	N	N	0.039
GN016		N	N	N	N	0.010
GN017		N	N	N	N	0.010
GN018		N	N	N	N	0.010
GN119*	H	H	N	G	G	0.991
GN120*	H	H	N	G	G	0.991
GN121	N	N	N	N	N	0.021
GN122*	I	H	G	G	G	0.991
GN123*	I	N	N	0.4F1+0.3Nbx	Nbx	0.352
GN024		N	N	N	N	0.010
GN025		N	N	N	N	0.013
GN026		N	N	N	N	0.010

Note: '*' notes individual reclassified for analysis; 'G' represents *G. geiseri*, 'N' represents *G. nobilis*, 'H' represents hybrid, 'Nbx' represents *G. nobilis* backcross, 'F1' represents first generation hybrids; Gonopodia: 'I' represents immature gonopodia, Cells were left empty for female specimen.

Table 17

Summary of test results used to infer ancestry of each individual originally identified as Putative hybrids from San Solomon Springs.

ID#	Gonopodia	DFA Assignment	MtDNA haplotype	NewHybrid Assignment	STRUCTURE Assignment	Q-value
GH001		H	N	F1	F1	0.434
GH002		H	N	F1	F1	0.515
GH003		H	N	F2	F2G	0.646
GH004		H	N	F1	F1	0.533
GH005		H	N	F1	F1	0.430
GH007		H	G	G	Gbx	0.882
GH008		H	N	F1	F1	0.435
GH010*		H	G	G	G	0.991
GH011		H	G	F1	F1	0.434
GH015		H	N	0.7G+0.3Gbx	F2G	0.764
GH116*	N	N	N	N	N	0.008
GH117*	H	H	G	G	G	0.991
GH118*	H	H	G	G	G	0.991
GH119*	H	H	G	G	G	0.988
GH120*	H	G	G	G	G	0.991
GH121	H	H	N	F1	F1	0.435
GH122*	H	G	G	G	G	0.991
GH123*	H	H	G	G	G	0.990
GH124*	I	H	G	G	G	0.991
GH125*	H	H	G	G	G	0.991
GH126*	H	H	G	G	G	0.991
GH127*	H	H	G	G	G	0.991
GH128	I	H	N	F1	F1	0.436
GH129*	G	G	G	G	G	0.991
GH130*	I	G	G	G	G	0.991
GH131*	H	H	G	G	G	0.991
GH132*	H	H	G	G	G	0.991
GH033		H	N	F1	F1	0.436
GH034*		H	G	G	G	0.991
GH035*		H	N	0.6Gbx+0.2F2	Gbx	0.757
GH036*		H	G	G	G	0.991
GH037*		G	G	G	G	0.991
GH038		H	N	F1	F1	0.514
GH039*		H	G	G	G	0.991
GH040*		G	G	G	G	0.991
GH042*		G	G	G	G	0.991
GH043*		G	G	G	G	0.991
GH044*		G	G	G	G	0.991
GH045*		G	G	G	G	0.991
GH046		H	N	G	Gbx	0.888
GH047*		H	G	G	G	0.991
GH048		G	N	F1	F1	0.515

Note: '*' notes individual reclassified for analysis; 'G' represents *G. geiseri*, 'N' represents *G. nobilis*, 'H' represents hybrid, 'Gbx' represents *G. geiseri* backcross, 'F1' represents first generation hybrids, 'F2' represents second generation hybrids (F1xF1); Gonopodia: 'I' represents immature gonopodia, Cells were left empty for female specimen.

Table 18

Population specific information with regards to individual loci.

	Gambusia geiseri (San Solomon Springs)				*Gambusia nobilis* (San Solomon Springs)				Putative Hybrid (San Solomon Springs)				Pure *Gambusia geiseri* (San Marcos River)			
Loci	N	A	Ho	He	N	A	Ho	He	N	A	Ho	He	N	A	Ho	He
2B	2	1.118	0.015	0.015	4	3.334	0.647	0.573	3	2.864	0.706	0.553	2	2.000	0.375	0.321
R1-2	1	1.000	0.000	0.000	2	2.000	0.476	0.512	2	1.993	0.429	0.343	1	1.000	0.000	0.000
R1-3	1	1.000	0.000	0.000	1	1.000	0.000	0.000	2	2.000	0.667	0.479	1	1.000	0.000	0.000
S73	1	1.000	0.000	0.000	1	1.000	0.000	0.000	2	2.000	0.762	0.505	1	1.000	0.000	0.000
R2-1	1	1.000	0.000	0.000	1	1.000	0.000	0.000	2	2.000	0.650	0.476	1	1.000	0.000	0.000

Note: N represents the number of alleles observed. A stands for Allele Richness. Ho stands for observed Heterozygosity. He stands for expected Heterozygosity.

CHAPTER IV

Discussion

Hybridization within San Solomon Springs

The predictions of this study are supported: the presence of morphological intermediacy and mixed genetic heritage of *Gambusia nobilis* and *Gambusia geiseri* in some individuals within San Solomon Springs. In addition, we were able to infer extensive hybridization and introgression over several generations.

Morphological intermediacy. The DFA statistically supports the observed presence of phenotypes intermediate to those of *G. nobilis* and *G. geiseri* in both males and females. However, the putative hybrids are more morphologically similar to *G. geiseri* than *G. nobilis*. Considering this, misidentification of hybrids as *G. geiseri* and vis-versa is not surprising. Despite the putative hybrid morphology favoring *G. geiseri* and error in identification that it caused, the original classifications were still fairly accurate with 90.9% of females and 86.8% of males correctly identified. Overall this supports the presence of the three morphological groups, *Gambusia nobilis*, *Gambusia geiseri,* and hybrids.

Gonopodial analysis. Changes in gonopodial structure within other species of *Gambusia* are suggested to be due to environmental factors. Populations of the Bahamas mosquitofish, *Gambusia hubbsi*, are located within isolated caves. A study performed by Heinen-Kay and Langerhans (2013) suggests that the presence of predators within environments can cause a rapid divergence in gonopodial characteristics by sexual selection to increase the probability of successful fertilization in face of predation. Isolation due to human-induced habitat fragmentation has also been suggested to cause

rapid changes in gonopodial charateristics (Heinen-Kay et al. 2014b). Individuals morphologically classified as *G. nobilis* or hybrids had corresponding gonopodial characteristics. Five *G. geiseri* individuals possessed intermediate gonopodial characteristics, suggesting that the gonopodium displays intermediacy faster than that of the overall phenotype.

Genetic Admixture. The STRUCTURE and NewHybrid analysis suggest introgression over several generations with individuals represented in each examined pedigree class (*G. geiseri*, *G. nobilis*, F1, F2, *G. geiseri* backcross, or *G. nobilis* backcross). Maternal lineages inferred by the mitochondrial data also gave insight to the extent of introgression. Two individuals exhibit genotypes matching a pure *G. geiseri* yet bear the *G. nobilis* haplotype, representing the highest degree of genetic swamping measurable by this study. No individual sampled exhibited nuclear DNA favoring the *G. nobilis* genotype accompanied by the *G. geiseri* mitochondrial haplotype. The closest we come to this ideal is in a single individual classified as an F1 bearing the *G. geiseri* mitochondrial haplotype. In comparison, there are 10 F1 individuals that have mitochondria associated with *G. nobilis*.

Evidence of introgression is seen within most *G. nobilis* individuals sampled. Only six of the 21 *G. nobilis* individuals were homozygous for allele 2, presumably characteristic of *G. nobilis*, at Rag1-2. The other 16 individuals bore at least one copy of allele 1, characteristic of *G. geiseri*. The addition of locus 2B is difficult to interpret but regardless, heterozygosity is higher than expected and at least three alleles were well represented; suggesting the presence of *G. geiseri* alleles and furthering the idea of an admixture event in the lineage of some *G. nobilis* individuals. At this point, it may be rare

to find a *G. nobilis* individual without some degree of admixture from *G. geiseri* within its genome.

Inferences of mating system. Evaluating the pedigree classification and mitochondrial haplotype gives a better insight into the direction of introgression and hybrid matings. The lack of sampled individuals favoring the *G. nobilis* genotype and the *G. geiseri* mitochondrial haplotype implies that *G. nobilis* males are not likely to mate successfully with females from a *G. geiseri* mitochondrial lineage. In contrast, the two individuals exhibiting genotypes matching a pure *G. geiseri* and the *G. nobilis* haplotype implies *G. geiseri* males mate with females from a *G. nobilis* maternal lineage. It is important to note that we are speaking of successful matings. Although we recovered one F1 female with a *G. geiseri* mitochondrial haplotype, we cannot be certain that this female can successfully produce offspring if mated with a *G. nobilis* male. With a higher proportion of *G. geiseri* individuals in the population, the mating between *G. geiseri* females and *G. nobilis* males should occur more frequently than *G. nobilis* female x *G. nobilis* male matings, if no behavioral barriers preventing cross-species matings are in place. But the lack of individuals with *G. geiseri* mitochondria and favored *G. nobilis* nuclear DNA suggests that successful mating is not equal among individuals.

A study performed by Swenton (2011) between a *G. nobilis* population in New Mexico and *Gambusia affinis* suggested that males of both species preferred conspecific females but will also attempt mating with heterospecific females. Female preference was also suggested with a higher success rate of conspecific copulation when males of both species compete. Similarly, males in a study performed by Espinedo et al. (2010) between *G. geiseri* and *G. affinis* preferred conspecific females but also attempted mating

with heterospecific females and produced sperm for both female species at equal amounts. However, in this study, no female preference for either male species was observed.

The preference for conspecific females likely contributes to the persistence of both *G. geiseri* and *G. nobilis* within San Solomon Spring since the 1930's despite admixture occurring. Although the two prefer conspecific females, it is the promiscuity and tendency to also mate with heterospecific females that sustains the hybrid community. The cross-species mating inequality is likely due to differences in pre-copulatory behaviors in *G. geiseri* (Plath et al. 2007) and *G. nobilis* (Leiser et al. 2010) males. The successful mating of *G. geiseri* males to females of both species is likely due to harassment, lack of female choice, and forced copulations performed by *G. geiseri* males. Assuming that the lack of evidence of successful *G. geiseri* female x *G. nobilis* male matings is indicative of the wild, the quality assessment of potential mates and lack of harassment towards unreceptive females by *G. nobilis* males may create behavioral barriers. In total, *G. nobilis* males tend mate with conspecific females despite a higher abundance of *G. geiseri* female. The persistence of the *G. nobilis* at San Solomon Springs, despite underrepresentation, may be attributed to this behavior. The evidence of introgression is seen within *G. nobilis* individuals likely occurred with F1 males, resulting from a *G. geiseri* male x *G. nobilis* female, backcrossing with *G. nobilis* females and offspring continue to backcross with *G. nobilis* individuals.

Consensus of Data. Evidence of admixture within the population is supported both morphologically and molecularly. Evaluating the individuals collected for molecular analysis suggest morphological classification of *G. nobilis* or *G. geiseri* is a good

predictor of genetic identify. The morphological hybrids, however, tend to favor high levels of *G. geiseri* genotypes, with a few individuals exhibiting no evidence of genetic admixture. The favoring of *G. geiseri* genotypes by intermediate individuals is congruent with the morphology results of being more morphologically similar to *G. geiseri* than *G. nobilis*. The errors in identification due to morphological similarities likely results from the different levels of genetic admixture within the population. Discriminant Function Analysis exhibited a strong separation between *G. nobilis* and a cluster including both *G. geiseri* and putative hybrids. This is likely because *G. nobilis* males select females with attractive *G. nobilis* characteristics, while *G. geiseri* exhibit less preferential mate selection. Of the 26 individuals identified *a priori* as *G. nobilis*, 23 individuals favor *G. nobilis* genotypes. Using the phenotype of *G. nobilis* as a predictor of favored *G. nobilis* genetic identity can be helpful in the conservation of this endangered species.

Conservation of *Gambusia nobilis*. Natural hybridization is a common occurrence in the evolutionary history of some taxonomic groups (Arnold 1997). Although hybridization is often linked with outbreeding depression and a reduction in fitness, it may lead to adaptive characteristics that allow displacement of parental species or expansion into habitats unoccupied by parental species. Hybridization due to human intervention creates unnecessary loss in biodiversity and species effected should be protected.

Hybrid individuals are not directly considered under the Endangered Species Act (ESA), in which *G. nobilis* was listed by the US Fish and Wildlife Service (USFWS) in 1970 (Echelle and Echelle 1986; Haig and Allendorf 2006). The USFWS occasionally places admixture threshold values on hybrid populations, consisting of both endangered

and invasive genes, to consider individuals for ESA protection. For example, the endangered westslope cutthroat trout, *Oncorhynchus clarkia lewisi*, is known to hybridize with the invasive rainbow trout, *Oncorhynchus mykiss*. The USFWS determined that hybrids should be morphologically identical to westslope cutthroat trout with no more than 20% rainbow trout admixture to be included for ESA protection (USFWS 2003). However, this allows rainbow trout alleles to actively remain within the population, contributing to the decline of pure westslope cutthroat trout (Haig and Allendorf 2006).

In this study, the highest percent admixture of individuals with *G. nobilis* phenotypes was 35%, with most backcrosses having a percent admixture of ~15%. Considering that the phenotype of *G. nobilis* is a good indicator of favored *G. nobilis* genotypes, preservation of this endangered species may be possible simply by the eradication of *G. geiseri* and hybrid individuals from San Solomon Spring to prevent further introgression. With most individuals phenotypically identifiable as *G. nobilis* still bearing evidence of admixture, the eradication of *G. geiseri* individuals will not eradicate *G. geiseri* alleles from the population. Therefore, it may not be feasible to expect the population to revert back to a genetically pure population over time. Introduction of *G. nobilis* from a location unaffected by *G. geiseri* may help reverse the effects of this genetic pollution. However, while *G. nobilis* populations in Texas are sympatric with *G. geiseri*, *G. nobilis* co-exist with *G. affinis* in New Mexico populations (Bednarz 1979). Hybridization between *G. nobilis* and *G. affinis* have been proposed to occur in low frequencies based on gonopodial morphology (Bednarz 1979); however, it has been reported that offspring displayed deformities and may not be fertile (Swenton 2011).

In closing, the results support our hypothesis of morphological and molecular admixture of *G. nobilis* and *G. geiseri* within San Solomon Spring. As *G. nobilis* co-occurs with similar cogeners in disjunct spring-fed waters of the Pecos River, at five sites in Texas and six in New Mexico, our hypothesis is supported at this location and may not represent the status of *G. nobilis* species as a whole. As this location has one of the highest abundance of *G. geiseri* individuals relative to *G. nobilis* individuals, the observed levels of admixture may have been driven by differences in species abundance. With the presence of *G. geiseri* alleles within individuals displaying no signs of phenotypic admixture, investigation of other localities is needed to test for introgression and infer the genetic status of *G. nobilis*.

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APPENDIX

Table A1

Pairwise Fst

	G. nobilis	Putative hybrid	*G. geiseri*	Pure *G. geiseri*
G. nobilis	0.000	0.492	0.965	0.911
Putative hybrid	**	0.000	0.573	0.324
G. geiseri	**	**	0.000	0.000
Pure *G. geiseri*	**	**	NS	0.000

Note: Top diagonal are Fst values for population comparisons. The bottom diagonal represents significance of Fst pairwise difference where ** represents significance at P = 0.00, and NS stands for Not Significant (P = 0.99 at NS location).

VITA

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EDUCATION

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RESEARCH EXPERIENCE

Graduate Research Student: Department of Biological Sciences, SHSU
Spring 2014 – Summer 2017
Research advisor**:** Dr. Chad Hargrave

- Quantified morphological differences between *G. nobilis* and *G. geiseri* to infer intermediacy within putative hybrids
- Collected genetic evidence to estimate the degree of admixture and measured the extent of introgression

Visiting Research Scholar: Department of Biology, TAMU
Summer 2015
Immediate Supervisor**:** Dr. Gil Rosenthal

- Assisted in running experiments investigating the mate choice of two naturally hybridizing fish species of *Xiphophorus*
- Performed DNA extractions on *Xiphophorus* species to explore genetic diversity

Undergraduate Research Assistant: Department of Wildlife and Fisheries, TAMU
Summer 2012 – Summer 2013
Research advisor**:** Dr. Gary Voelker; Mentor: Maura Palacios

- Sequenced samples from the freshwater fish *Poeciliopsis gracilis* complex to reconstruct evolutionary relationships and challenge current recognized species
- Collected morphological data using museum samples from the Biodiversity Research Teaching Collections to compare to molecular findings and clarify taxonomical structure

TEACHING EXPERIENCE

Teaching Assistantship: Department of Biological Sciences, SHSU
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-Responsible for teaching laboratory courses including:

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INTERNSHIPS

Houston Toad Conservation Intern: Houston Zoo Inc
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-Practiced biosecurity and performed basic husbandry to care for the endangered Houston Toad, *Bufo houstonensis*

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PRESENTATIONS

V. Rodriguez, C.W. Hargrave. The potential hybridization between the endangered *Gambusia nobilis* and invasive *Gambusia geiseri*, in Texas. 17th annual Ecological Integration Symposium, College Station, Texas, USA, April 1, 2016. Oral Presentation.

V. Rodriguez, C.W. Hargrave. Genetic analysis of potential hybridization between the endangered *Gambusia nobilis* and invasive *Gambusia geiseri*, in Texas. 18th Annual Graduate Research Exchange, Huntsville, Texas, USA, February 18, 2015. Oral Presentation.

V. Rodriguez, C.W. Hargrave. Genetic analysis of potential hybridization between the endangered *Gambusia nobilis* and invasive *Gambusia geiseri*, in Texas. 2014 Biological Sciences Graduate Research Symposium, Huntsville, Texas, USA, December 10, 2014. Poster Presentation.

V. Rodriguez, M. Palacios, G. Voelker, K. Conway. A genetic and morphological approach to resolving taxonomic confusion in the freshwater fish species of the *Poeciliopsis gracilis* complex. Evolution 2013 Conference, Snowbird, Utah, USA, June 22, 2013 ID: P21020. Poster Presentation.

V. Rodriguez, M. Palacios, G. Voelker, K. Conway. Rediscovering the past of Freshwater Poeciliid fish in Middle America. Minorities in Agriculture, Natural Resources, and Related Sciences Career Fair and Training Conference, Sacramento, California, USA, March 22, 2013. Oral Presentation.

MANUSCRIPTS IN PREPARATION

Rodriguez, V.X., Randle, C.P., and Hargrave, C.W. Hybridization between the endangered *Gambusia nobilis* and invasive *Gambusia geiseri* in Texas.

Rodriguez, V.X. and Daza, J.D. Molecular and Morphological Incongruences among Squamates: A Review.

Palacios, M., **Rodriguez, V.X.**, Voelker, G., and Conway, K. A genetic and morphological approach to resolving taxonomic confusion in the freshwater fish species of the *Poeciliopsis gracilis* complex (*Poeciliopsis,* Poeciliidae: Cyprinodontiformes). *(To be submitted to Zootaxa).*

HONORS/AWARDS

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American Society of Ichthyologists and Herpetologists	2016-Present
Texas Academy of Science	2014-Present
The Texas Chapter of the American Fisheries Society	2014-Present
League of United Latin American Citizens, SHSU Chapter	2014-Present
Biological Sciences Graduate Student Organization, SHSU Chapter	2014-Present