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Sibship Reconstruction for Inferring the Number of Breeders of Gulf Sturgeon in the Apalachicola River

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by

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Abstract

The Gulf sturgeon is an anadromous fish that inhabits the Gulf of Mexico and its neighboring river drainages. The species is currently listed as threatened due to habitat alterations and overfishing. In this study, we focused on the Apalachicola River in Florida, which has had several historic spawning locations of the sturgeon blocked by the Jim Woodruff Lock and Dam. Age-1 juvenile sturgeon from the year 2013 (n=31) and 2014 (n=131) were genotyped using fourteen microsatellite loci. Sibship reconstruction and parentage assignment was performed in order to determine the effective number of breeders (N_b) and the total number of spawning adults (N_S). Genetic diversity measures in the two cohorts proved to be very similar. The 2013 sample had an N_b value of 38 and an N_S value of 28 while the 2014 sample had an N_b of 84 and an N_S of 79. Although there was a difference in the reproductive success between years, there wasn't much skew in terms of reproductive success of the parents contributing to a given cohort. It is not entirely clear why the 2014 age-1 cohort was larger, but it could reflect favorable environmental conditions increasing the number of spawning adults or increasing the survivorship of juveniles spawned that year. Overall, the genetic approach of inferring the number of breeders from sibship reconstruction proved to be an effective measure of reproduction in Gulf sturgeon and should be used in other river systems in future studies.

Key Terms: Conservation, Threatened Species, Gulf of Mexico, Management, Population Genetics, Spawning

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Introduction

The Gulf sturgeon (*Acipenser oxyrinchus desotoi*) is an anadromous species of fish that inhabits the north central coast of the Gulf of Mexico. The Gulf sturgeon is one of two subspecies of the Atlantic sturgeon (USFWS, GSMFC, & NMFS, 1995). Gulf sturgeon are distributed across major river systems that span from the Mississippi River to the Suwanee River in Florida and in marine waters that span from the Gulf of Mexico to the Florida Bay (Figure 1; USFWS, GSMFC, & NMFS, 1995).

As an anadromous species, sturgeon are vulnerable to threats in both the freshwater and marine environments including overharvest and habitat alteration, as well as natural stochastic events such as hurricanes and pollution events (Sulak et al., 2016). Exact population estimates for this range are currently unknown (USFWS, GSMFC, & NMFS, 1995). However, the comparison between historical and current data reveals that population levels have drastically dropped (USFWS, GSMFC, & NMFS, 1995). In 1991, under the U.S. Endangered Species Act (ESA), the Gulf sturgeon was officially listed as a threatened species (USFWS, GSMFC, & NMFS, 1995).

The Apalachicola River located in the state of Florida drains into the Gulf of Mexico (Figure 2) and was historically known to support populations of Gulf sturgeon. A report from the U.S. Commission on Fish and Fisheries revealed that at one time, the Apalachicola River was providing the largest commercial sturgeon fishery in the entire state of Florida (USFWS, GSMFC, & NMFS, 1995). A moratorium was placed on harvest in 1984, but the population has since struggled to replenish itself (Barkuloo, 1987). Furthermore, about 80 percent of the breeding habitat within the Apalachicola-Chattahoochee-Flint River basin is blocked by a dam known as the Jim Woodruff Lock

and Dam (USFWS, GSMFC, & NMFS, 1995). The passage of Gulf sturgeon through the lock has been previously investigated, and the results showed that they do not appear to enter the lock (USFWS and NMFS, 2009).

The recovery and management plan (USFWS, GSMFC, & NMFS, 1995) was developed in an effort to conserve the existing populations of Gulf sturgeon and prevent any further decrease across the range. Ongoing field surveys are currently being conducted in order to reveal information about the number of breeders contributing to a given reproductive class (USFWS and NMFS, 2009). These surveys include taking censuses of adult populations, but adult numbers in a river system don't always provide an accurate indication of the number of individuals who are spawning and contributing to the next generation. The number of breeding individuals is what ultimately factors into whether a population is on the path to demographic recovery or not. Recently, genetic approaches using microsatellite loci have been used to estimate the number of spawning adults in various species of sturgeon and salmonids by identifying related groups of individuals among juveniles spawned in a particular location (e.g., Duong et al., 2013; Jay et al., 2014; Kano et al., 2011; Ozerov et al., 2015).

Two important values to consider when dealing with an endangered species are the effective population size (N_e) and the effective breeding number (N_b), which, for a single cohort, are the same. Effective population size is the sum of all of the individuals in an ideal population that have the same degree of inbreeding, loss of heterozygosity, and random genetic drift as the actual population, which provides insight into the effects of evolutionary processes on the genetic diversity of a given population (Wright, 1931). The effective breeding number measures the effective population size for one particular

breeding season. This value can provide information about the effects on recruitment due to ecological fluctuations occurring during a given breeding season (Waples, 2002). Genetic data obtained from a particular age class can also be used to estimate the actual number of breeders contributing to a given reproductive season (N_S). This value can be difficult to calculate using traditional techniques like mark-recapture, but provides essential information in terms of assessing management efforts (Jay et al., 2014).

For this particular study, 162 juvenile (age-1) Gulf sturgeon were collected from the Apalachicola River during 2013 and 2014. The main objective is to use sibship analysis and parental reconstruction to estimate N_b and N_S for each year class found in our collections. These estimates will provide insight into the dynamics of recruitment in this important river system. Additionally, these results will provide important base-line information for the long-term monitoring of the status of Gulf sturgeon in the Apalachicola.

Methods

Sample Collection

Juvenile Gulf sturgeon were collected from the Apalachicola River by students working under the direction of Doug Peterson (University of Georgia) in June-July of 2013 and May-July of 2014. Sturgeon were captured via anchored monofilament gill nets that were set perpendicular to the current and checked every 30-90 minutes. Nets were set at two or three different sites per year. These sites were selected on the basis of previous collections and sonar surveys. After capture, sturgeon were measured to the nearest millimeter (fork length – FL) and injected with a passive integrated transponder (PIT) tag for future identification if recaptured. For some individuals, a portion of the pectoral fin ray was collected for use in a separate study to verify the ages of fish as estimated from the length-frequency histogram. This histogram was produced for the individuals of a given sampling year using the Fisheries Stock Assessment (FSA) package in R (Ogle, 2016). Tissue samples were collected from each individual in the form of a fin clip, which were stored in 100% ethanol.

Molecular Methods

Extraction of total genomic DNA from the fin clips was performed using the DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA). In order to ensure the integrity of the DNA obtained from the extractions, samples were visualized on 1% agarose gel. Amplification of the DNA samples were performed using the polymerase chain reaction (PCR) conditions outlined in Dugo et al. (2004). Briefly, PCR conditions were as follows: 12.5 μL reactions consisting of 1x *Taq* reaction buffer (New England Biolabs), 1.5-3 mM $MgCl_2$, 200 μM dNTPs, 0.25 units of *Taq* polymerase (New England Biolabs),

0.16 μM of the M13 tailed forward primer (Schuelke, 2000), 0.16 μM of the reverse primer, 0.08 μM of the M13 labeled primer (LI-COR Inc., Lincoln, NE), 20-100 ng of template DNA, and water to the final volume. The following cycling conditions were performed on a GeneAmp PCR system 9700 (Applied Biosystems): initial denaturation at 94° for 2 minutes, 35 cycles of denaturing at 94° for 30 seconds each, annealing for 30 seconds at 56-58°C, extension at 72°C for 1 minute, and one final extension for 10 minutes at 72°C. Microsatellite alleles were visualized using a LI-COR 4300 DNA sequencer and scored using a 50-350 bp size standard (LI-COR) with the Gene Image IR v. 3.55 software (LI-COR). Individuals were genotyped for a total of fourteen microsatellite loci including thirteen loci developed for Atlantic sturgeon (*Aox*B34, *Aox*D32, *Aox*D49, *Aox*D54, *Aox*D64, *Aox*D96, *Aox*D164, *Aox*D170, *Aox*D188, *Aox*D234, *Aox*D241, *Aox*D242, and *Aox*D297; Henderson-Arzapalo and King, 2002) and one locus developed for lake sturgeon (*LS*68; May et al., 1997).

Data Analyses

For each year of collection, individuals were tested for deviation from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium using GENEPOP on the web v. 4.1 (Raymond and Rousset, 1995; Rousset, 2008), with the alpha level of these tests adjusted by a sequential Bonferroni correction (Rice, 1989). Summary statistics of genetic variability at each locus (number of alleles, observed heterozygosity, and expected heterozygosity) were calculated using GenAlEx v. 6.5 (Peakall and Smouse, 2006; 2012). The power of the loci to perform kinship analyses was determined by calculating the probability of identity using GenAlEx v. 6.5. The probability of identity determined the average probability that two individuals would have an identical genotype

at one or more loci. Sibship analysis and parental reconstruction were conducted using COLONY v. 2.0.6.1 (Jones and Wang, 2010), which uses a maximum likelihood method to estimate pedigree relationships based on their multi-locus genotypes. Relationships between offspring were determined to be one of the following: full siblings sharing two parents, half siblings sharing one parent, or unrelated sharing no parents. I performed the analysis using the full-likelihood method, high precision, and maximum run length. A polygamous mating system without inbreeding was allowed for both males and females, which is consistent with what is known about the biology of Gulf sturgeon (Sulak et al., 2016). Allelic dropout was set to 0.001 and the false allele rate was 0.01. Analyses were conducted separately on the 2013 and 2014 collections from which estimates of N_S (the number of adults that contributed to the juveniles of that year class) and N_b (the effective number of breeding adults) were determined.

Results

A total of 162 individuals were collected over 2013 (n=31) and 2014 (n=131). In 2013, individuals ranged in size from 371-537 mm FL (average = 453.5; standard deviation = 44.6) while in 2014, individuals ranged in size between 372-531 mm FL (average = 456.0; standard deviation = 40.3). The distribution of individual fork length around the mean was bimodal in 2013 (Figure 3), but unimodal in 2014 (Figure 4). However, in both years the vast majority of the individuals collected were smaller than the 510-540 mm FL and none exceeded 540 mm FL. This suggests that our samples represent age-1 juveniles (A. Kaeser, USFWS, personal communication) and were spawned in the year prior to their collection.

I genotyped the 162 individuals for fourteen loci (Appendix I). For the 2013 samples, none of the loci deviated from HWE and only one pair of loci (*Aox*B34 and *Aox*D241) demonstrated linkage disequilibrium after a sequential Bonferroni correction. Two loci deviated (*Aox*D188 and *Aox*D241) significantly from HWE in the 2014 samples, and a total of five pairs of loci (*Aox*B34/*Aox*D44; *Aox*D188/*Aox*D170; *Aox*D170/*Aox*D241; *Aox*D241/*LS*68; & *Aox*D170/*Aox*D297) were not in linkage equilibrium after a sequential Bonferroni correction. None of these loci were out of HWE or were in linkage disequilibrium in adults collected from this river (B. Kreiser, unpublished data), so I chose to use all loci in subsequent analyses.

Measures of genetic diversity were generally similar for the 2013 and 2014 collections (Tables 1 & 2). The average number of alleles per locus (N_A) was 7.214 (range of 4-16) in 2013 and 9.143 (range of 4-21) in 2014. Mean observed heterozygosity per locus (H_o) was 0.636 and 0.635, while mean expected heterozygosity

per locus (H_e) was 0.641 and 0.657 for the 2013 and 2014 collections, respectively. The average probability of identity (PI) across loci was 0.190 for 2013 and 0.180 for 2014, and the average probability of identity of siblings (PI_{sib}) across loci was 0.48 for 2013 and 0.47 for 2014. For both years, the PI and PI_{sib} for increasing combinations of loci rapidly approached zero (Figure 5).

The COLONY analysis of the 2013 collection detected no full or half-sib dyads with a probability greater than 0.9. The effective number of breeders (N_b) was estimated at 38 with a 95% confidence interval (CI) that ranged from 23-70. The total number of spawning adults (N_S) from this cohort was estimated as 28 with an equal number of individuals inferred to represent either parent (Table 3). In the 2014 collection, there were multiple full and half-sib dyads detected when using a probability threshold of 0.9. Four dyads were found to represent full sibs (range of 0.92-0.97), and 18 dyads represented half-sibs (range of 0.90-0.96). Many of the individuals within these dyads were collected at different sites. The 2014 collection had a higher N_b (84; 62-118, 95% CI) and higher N_S (79 total; 38 parent #1, 41 parent #2) (Table 3).

Discussion

The Apalachicola River supported a very large population of Gulf sturgeon at one time, but due to fishery exploitation and habitat alterations, the population quickly began to collapse (USFWS, GSMFC, & NMFS, 1995). In addition, the building of the Jim Woodruff Lock and Dam located at the Apalachicola-Chattahoochee-Flint River basin further decreased the size of the population because the sturgeon could no longer travel to their breeding locations (USFWS, GSMFC, & NMFS, 1995). There are now only three known locations below the dam where the sturgeon are able to spawn (Sulak et al., 2016). All of these events have consequently led to recovery and management plans being put into effect in order to preserve the current population and encourage new population growth.

Sibship reconstruction has been proposed as a tool to monitor the spawning activity of a given population since many of the more traditional monitoring approaches involve labor-intensive sampling and have high degrees of uncertainty. The use of genetic data has proven to be an effective method to avoid these complications (Jay et al., 2014). In a study performed by Jay et al. (2014), microsatellite loci obtained from eggs and larvae successfully provided estimates of both N_b and N_S for the White sturgeon. My study is the first time this approach has been applied to Gulf sturgeon.

The most recent estimate for the Apalachicola is from 2014, which suggested that the subadult and adult population was comprised of 785 with a confidence interval of 631-1037 (Sulak et al., 2016). I can compare this population estimate with my genetic estimates if I make the following assumptions. If approximately 60% of this number represents sexually mature adults, this would mean that the current spawning population

of the Apalachicola is around 471 individuals (A. Kaeser, USFWS, personal communication). If we then assume that there is an equal sex ratio (Pine et al., 2001), then there should be around 230 sexually mature females. In a given year somewhere between 5-25% of the females spawn (Sulak and Clugston, 1999), although Pine et al. (2001) suggests that 5% is probably the better estimate. This would mean that we should expect somewhere between 12 (5% rate) to 58 (25% rate) females spawning in a given year. The number of spawners calculated for 2013 ($N_S = 28$) approximates the 5% estimate if we follow the inferred number of parents being split roughly equally among the two sexes (i.e., about 14 of each). However, in 2014 N_S was calculated to be 79, which more closely approximates a rate of 25% of the females spawning.

Both the N_b and N_S values were higher for the 2014 cohort. Perhaps this is simply a function of a much larger sample size in 2014 (n=131) than in 2013 (n=31). However, the larger number of juveniles collected may reflect real differences in spawning success in the two years. There were distinct differences in the hydrograph of the Apalachicola River between 2012 and 2013, the years that the 2013 and 2014 age-1 cohorts were spawned. The USGS gaging station (02358000) on the river at Chattahoochee, FL recorded a major spring flood in 2013 (above flood stage), as well as similarly high flows between July and September. The spring flood is a cue for the onset of the migration run and may have attracted more individuals to spawn that year (Sulak et al., 2016). Another possibility is that the higher summer flows enhanced juvenile survival during a period of time when temperatures normally become stressful or allowed better access to feeding areas in the floodplain (A. Kaeser, USFWS, personal communication). Admittedly the confidence interval values for the N_b estimates overlap between the 2013 and 2014

cohorts, but it is not unreasonable to think that there are real differences in spawning success and recruitment between the two years.

Across both years, there did not appear to be much skew in terms of reproductive success as measured by N_b. Similarly, for both cohorts, the number of spawning adults appeared to be equally distributed between parent 1 and parent 2, meaning that no single pair of parents produced the bulk of the cohort. In the study performed by Saarinen et al. (2011), a very high degree of relatedness among individuals was established. The discrepancy between my work and this study could lie in the fact that eggs were sampled instead of juveniles. Egg sampling measured the immediate outcome of the spawn, but by the time the sturgeon reached age-1, there didn't seem to be that same degree of relatedness among the individuals sampled.

This study documents a successful application of sibship reconstruction as a monitoring tool for Gulf sturgeon. Estimates of the effective number of breeders and the overall number of spawning adults provided with this study provide some insight into biology of Gulf sturgeon and its current status in the Apalachicola River. Going forward, this tool can be applied to other systems in order to better manage the Gulf sturgeon.

Figures

Figure 1. Map of the southeastern United States depicting the range of the Gulf sturgeon. The blue lines show designated critical habitats and the grey shading shows the historic range of the species (Map from USFWS, 2017).

Figure 2. Map showing the location of the Jim Woodruff Lock and Dam on the Apalachicola River in Florida (Nature Conservancy, 2010).

Figure 3. Length-Frequency Histogram for the 2013 Population. Fork length on the x-axis displays the length of the sturgeon measured in millimeters. Frequency on the y-axis displays the number of individual sturgeon recorded to have that measurement (n=31).

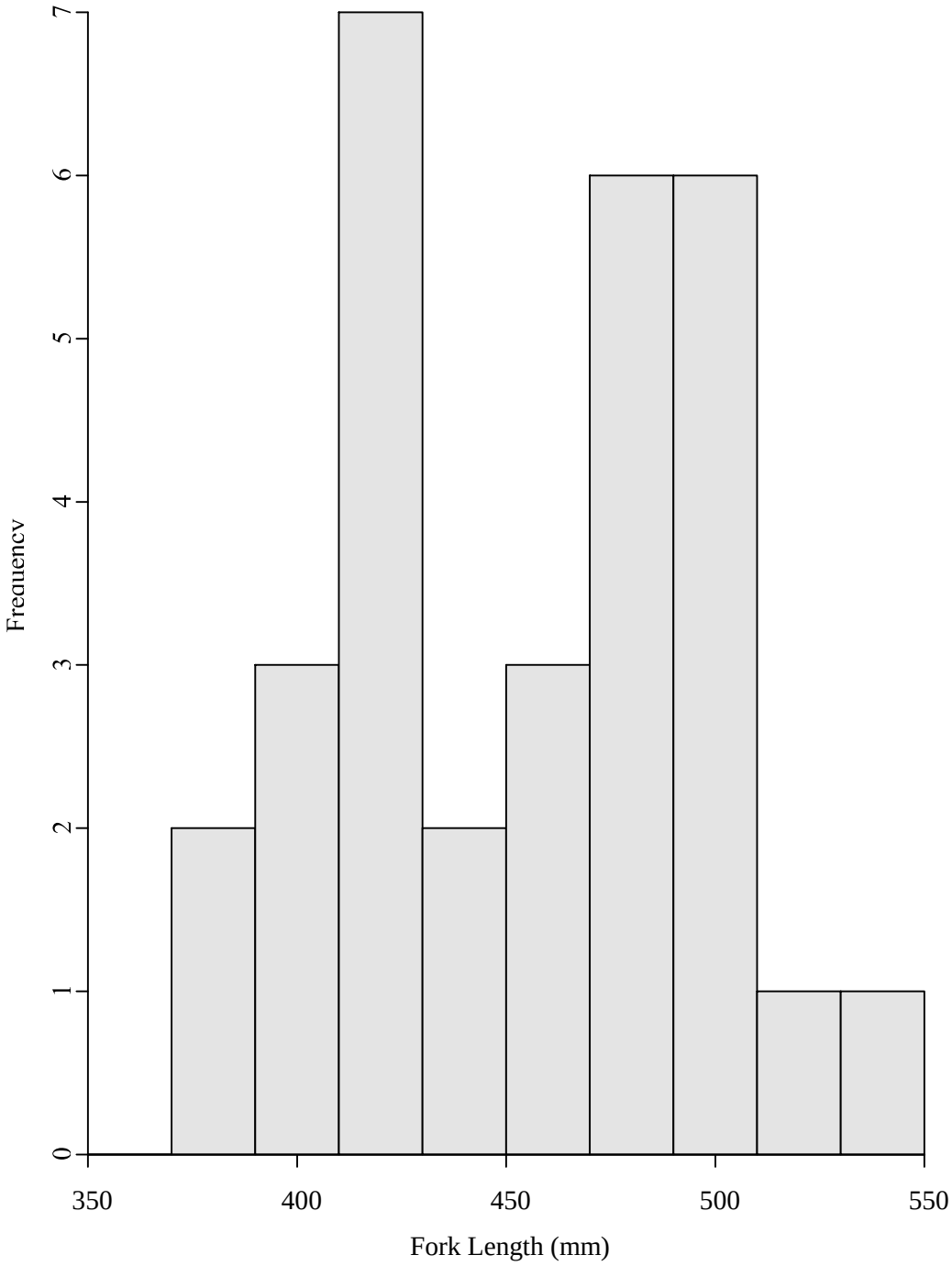


Figure 4. Length-Frequency Histogram for the 2014 Population. Fork length on the x-axis displays the length of the sturgeon measured in millimeters. Frequency on the y-axis displays the number of individual sturgeon recorded to have that measurement (n=131).

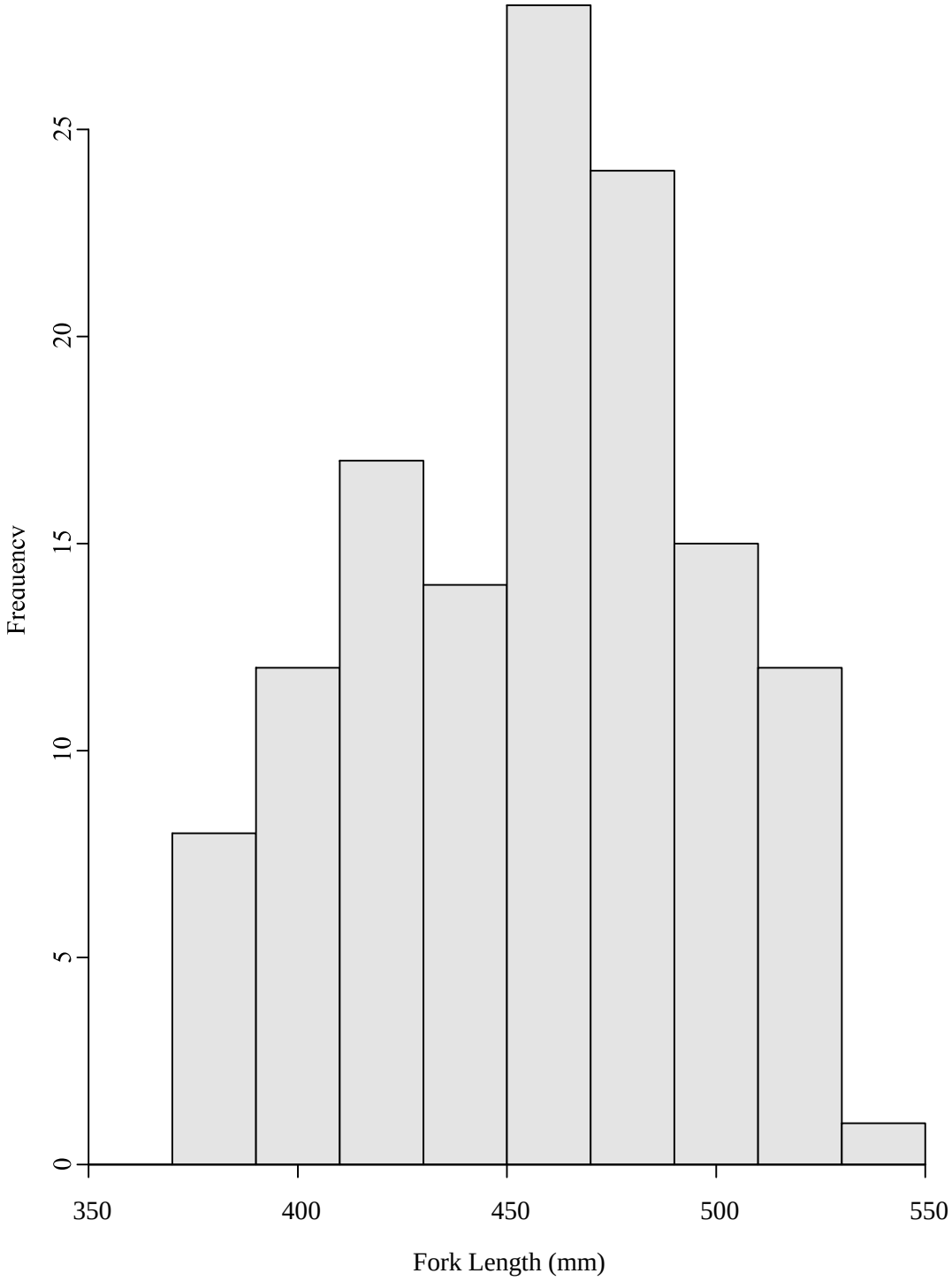
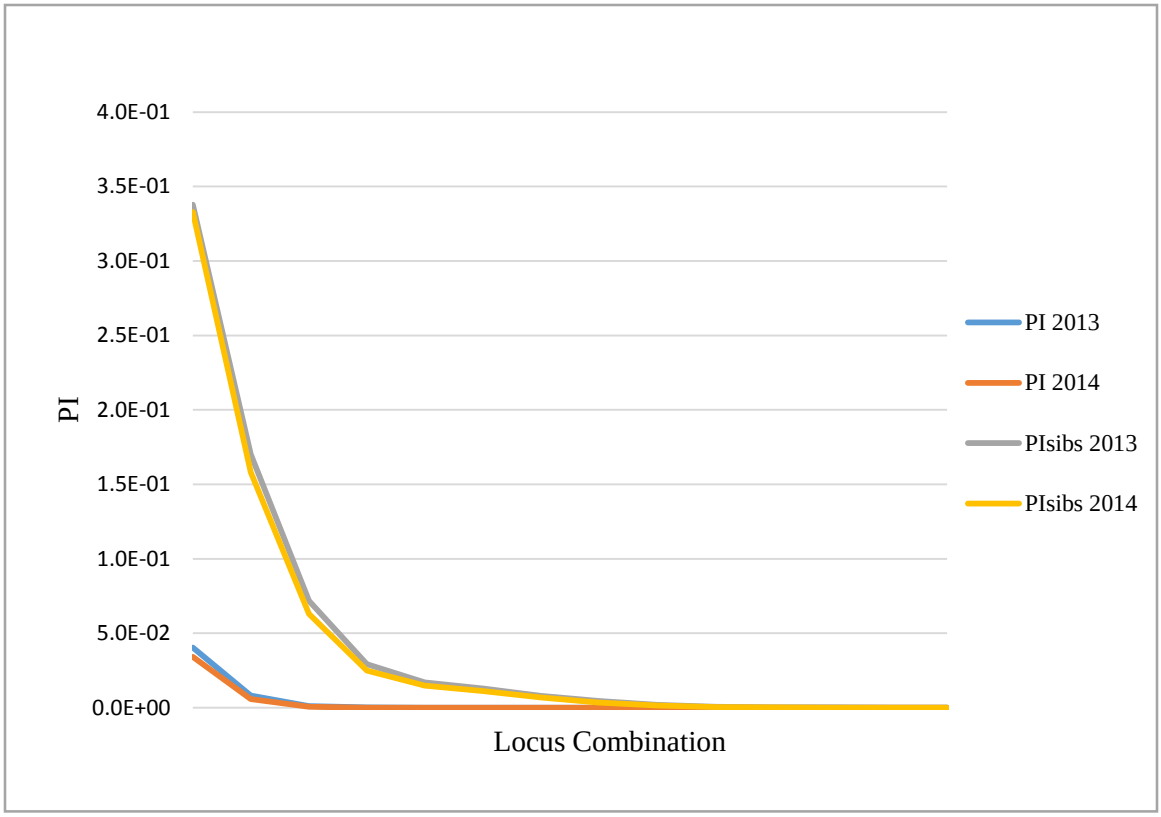


Figure 5. Graph displaying Probability of Identity (*PI*) and Probability of Identity Siblings (PI_{sib}) for the 2013 and 2014 populations. The x-axis displays the locus combinations while the y-axis displays the probability of identity values. The colored lines on the graph correspond to the probability of identity for the 2013 (blue) and 2014 (red) populations and the probability of identity siblings for the 2013 (gray) and 2014 (yellow) populations.



Tables

Table 1. Summary Statistics of Genetic Diversity for the 2013 Population. The 14 different loci are listed along with the corresponding sample size (***N***), number of different alleles at each locus (N_A), observed heterozygosity (H_o), expected heterozygosity (H_e), probability of identity (***PI***), and probability of identity siblings (PI_{sib}). The bottom two rows of the table display the mean and standard error (**SE**) for each column of measured values.

Locus	N	N_A	H_o	H_e	PI	PI_{sib}
*Aox*B34	31	10	0.806	0.844	0.040	0.340
*Aox*D188	31	6	0.484	0.594	0.200	0.500
*Aox*D32	29	6	0.759	0.721	0.130	0.420
*Aox*D44	28	7	0.607	0.739	0.110	0.410
*Aox*D64	22	6	0.455	0.481	0.290	0.580
*Aox*D54	31	4	0.290	0.260	0.560	0.760
*Aox*D96	29	5	0.483	0.446	0.340	0.610
*Aox*D165	27	4	0.519	0.524	0.280	0.560
*Aox*D170	29	5	0.793	0.703	0.130	0.430
*Aox*D234	31	16	0.935	0.904	0.017	0.300
*Aox*D241	29	13	1.000	0.889	0.022	0.310
*Aox*D242	31	8	0.581	0.660	0.150	0.460
*LS*68	29	5	0.483	0.515	0.280	0.560
*Aox*D297	28	6	0.714	0.694	0.130	0.440
Mean	28.929	7.214	0.636	0.641	0.190	0.480
SE	0.642	0.939	0.054	0.049	0.038	0.033

Table 2. **Summary Statistics of Genetic Diversity for the 2014 Population.** The 14 different loci are listed along with the corresponding sample size (***N***), number of different alleles at each locus (N_A), observed heterozygosity (H_o), expected heterozygosity (H_e), probability of identity (***PI***), and probability of identity siblings (PI_{sib}). The bottom two rows of the table display the mean and standard error (**SE**) for each column of measured values.

Locus	N	N_A	H_o	H_e	PI	PI_{sib}
*Aox*B34	123	16	0.846	0.851	0.034	0.330
*Aox*D188	127	8	0.630	0.635	0.170	0.480
*Aox*D32	130	11	0.731	0.755	0.100	0.400
*Aox*D44	127	10	0.717	0.754	0.098	0.400
*Aox*D64	126	8	0.452	0.466	0.300	0.590
*Aox*D54	129	5	0.271	0.276	0.540	0.750
*Aox*D96	130	5	0.423	0.429	0.350	0.620
*Aox*D165	123	4	0.610	0.619	0.210	0.490
*Aox*D170	128	5	0.602	0.672	0.150	0.450
*Aox*D234	131	21	0.863	0.897	0.019	0.310
*Aox*D241	128	17	0.883	0.911	0.015	0.300
*Aox*D242	131	7	0.550	0.570	0.230	0.520
*LS*68	129	5	0.581	0.595	0.210	0.500
*Aox*D297	129	6	0.729	0.771	0.082	0.390
Mean	127.929	9.143	0.635	0.657	0.180	0.470
SE	0.683	1.418	0.047	0.049	0.038	0.033

Table 3. Summary of COLONY Results for the 2013 and 2014 Populations. The sample size (***N***) for each year is shown along with the effective number of breeders (N_b) calculated at a 95% confidence interval (***CI***). The CI column displays the range for the number of effective breeders. The total number of spawning adults (N_S) is displayed in the last column.

Year	***N***	N_b	**95% *CI***	N_S
2013	31	38	23-70	28
2014	131	84	62-118	79

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Appendices

Appendix I. Allele Frequencies and Sample Size by Population. Each locus is displayed along with its various different alleles (**n**). The number of individuals in the 2013 and 2014 populations expressing each locus (**N**) is shown in the first row of each locus listed. The values listed below the year displays the frequency of each allele for a given locus expressed in the population.

Locus	**Allele/n**	**2013**	**2014**
***Aox*B34**	**N**	31	123
	174	0.113	0.102
	183	0.290	0.313
	186	0.000	0.024
	192	0.000	0.020
	222	0.065	0.041
	225	0.016	0.000
	231	0.000	0.028
	234	0.113	0.081
	237	0.097	0.098
	240	0.065	0.053
	243	0.081	0.118
	246	0.145	0.041
	249	0.000	0.041
	252	0.000	0.004
	255	0.000	0.028
	258	0.000	0.004
	270	0.000	0.004
	273	0.016	0.000
***Aox*D188**	**N**	31	127
	330	0.000	0.004
	334	0.597	0.551
	338	0.065	0.055
	346	0.032	0.047
	350	0.000	0.004
	354	0.194	0.201
	358	0.081	0.122
	362	0.032	0.016
***Aox*D32**	**N**	29	130
	214	0.000	0.004
	218	0.000	0.004
	226	0.362	0.285
	230	0.172	0.288
	234	0.000	0.012
	236	0.000	0.004
	242	0.103	0.081

	246	0.017	0.031
	250	0.017	0.004
	258	0.328	0.269
	262	0.000	0.019
***Aox*D44**	**N**	28	127
	145	0.321	0.335
	181	0.161	0.161
	185	0.125	0.094
	193	0.000	0.024
	197	0.000	0.012
	201	0.018	0.020
	205	0.339	0.311
	207	0.000	0.020
	208	0.018	0.000
	209	0.018	0.020
	213	0.000	0.004
***Aox*D64**	**N**	22	126
	232	0.068	0.063
	236	0.114	0.079
	240	0.045	0.004
	244	0.045	0.056
	248	0.023	0.067
	256	0.705	0.718
	260	0.000	0.004
	264	0.000	0.008
***Aox*D54**	**N**	31	129
	196	0.081	0.093
	212	0.016	0.012
	216	0.000	0.016
	224	0.855	0.845
	228	0.048	0.035
***Aox*D96**	**N**	29	130
	192	0.155	0.127
	198	0.052	0.092
	200	0.724	0.738
	202	0.052	0.023
	204	0.017	0.019
***Aox*D165**	**N**	27	123
	152	0.167	0.199
	164	0.019	0.024
	168	0.648	0.528
	172	0.167	0.248
***Aox*D170**	**N**	29	128
	208	0.086	0.039
	216	0.190	0.215

	220	0.172	0.133
	228	0.466	0.500
	232	0.086	0.113
***Aox*D234**	**N**	31	131
	220	0.000	0.019
	228	0.145	0.198
	232	0.032	0.011
	236	0.065	0.038
	240	0.145	0.160
	260	0.016	0.076
	264	0.000	0.004
	272	0.065	0.042
	276	0.000	0.004
	280	0.065	0.076
	284	0.016	0.004
	288	0.048	0.011
	296	0.016	0.034
	300	0.032	0.080
	304	0.065	0.034
	308	0.048	0.023
	312	0.032	0.035
	316	0.161	0.103
	320	0.000	0.004
	324	0.000	0.004
	336	0.048	0.038
***Aox*D241**	**N**	29	128
	204	0.000	0.008
	212	0.017	0.020
	220	0.000	0.023
	236	0.086	0.090
	240	0.086	0.035
	244	0.207	0.172
	248	0.138	0.102
	252	0.086	0.074
	256	0.086	0.063
	264	0.034	0.074
	268	0.017	0.047
	270	0.017	0.000
	272	0.068	0.074
	276	0.069	0.098
	278	0.000	0.004
	280	0.000	0.031
	284	0.000	0.008
***Aox*D242**	**N**	31	131
	188	0.048	0.015

	192	0.048	0.019
	200	0.032	0.046
	204	0.532	0.615
	216	0.016	0.004
	220	0.016	0.000
***LS*68**	**N**	29	129
	162	0.017	0.047
	166	0.052	0.050
	170	0.224	0.217
	174	0.655	0.585
	178	0.052	0.101
***Aox*D297**	**N**	28	129
	216	0.143	0.163
	220	0.107	0.101
	224	0.482	0.384
	232	0.054	0.116
	240	0.018	0.074
	244	0.196	0.163

Appendix II. The Institutional Animal Care and Use Committee (IACUC) Letter of Approval.

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.6791 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

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 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 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:	**17101202 (Renewal of 11092206)**
PROJECT TITLE:	**Population Genetics & Systematics of Freshwater Fishes and Herps**
PROPOSED PROJECT DATES:	**10/2017 – 09/2020**
PROJECT TYPE:	**New**
PRINCIPAL INVESTIGATOR(S):	**Brian Kreiser**
DEPARTMENT:	**Biological Sciences**
FUNDING AGENCY/SPONSOR:	**N/A**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2020**

Jake Schaefer, PhD
IACUC Chair

10/18/2017
Date