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Preliminary Assessment of Hybridization and Introgression of Morone Species in the Toledo Bend Reservoir, Louisiana

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by

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Abstract

Hybridization occurs when two genetically distinct populations interbreed and produce offspring. Fisheries managers are often interested in identifying hybrids and estimating the extent of hybridization caused by the introduction of non-native stocked fish in order to maintain specific fisheries for recreational fishing. I used *M. chrysops* and *M. mississippiensis* blood samples (n = 26) from the Toledo Bend Reservoir and the Sabine River, Louisiana, to determine individual genotypes and estimate the extent of hybridization in the reservoir. Published microsatellite primers developed for *M. chrysops* and *M. saxatilis* were used to identify species and hybrids. Genotypes showed that five fish had alleles from both the *M. chrysops* and *M. mississippiensis/M. saxatilis* allele size ranges, confirming that *Morone* species hybridize in the Reservoir. Future work will include a larger sample size and a third species, *M. saxatilis* to better estimate the extent of hybridization.

Introduction

Hybridization occurs when two genetically distinct populations interbreed and produce offspring (Allendorf, 2007). While it is a widespread evolutionary genetic process, it may also have negative consequences that cause populations to decline, eventually driving them to extinction (Allendorf, 2001). Hybridization can cause population declines in two different ways: through loss of reproductive potential or through introgression. If sterile hybrids are produced, there is a loss of reproductive potential, which may decrease population growth rates. The second, and arguably the most detrimental consequence of hybridization is introgression, defined as the incorporation of genes from one population into another population, a process that occurs when fertile hybrids backcross with parental populations. Introgression results in genetic mixing leading to a hybrid swarm, which may cause the loss of distinct evolutionary lineages (Allendorf 2007).

Increases in hybridization rates among populations have been correlated with an increase in human activity. Most anthropogenically-caused hybridization occurs when human changes such as habitat modification or intentional introductions eliminate barriers isolating distinct populations, causing populations to spread beyond their normal range and come into contact with each other (Allendorf, 2001). While habitat destruction is the number one cause for the loss of fish diversity, intentional introductions have not left fisheries unscathed. Although, thousands of stocking programs occur throughout the United States without any damaging repercussions, and many are done to protect native species. (Bartley, 2001), stocking programs for fish and game are clear examples of intentional introductions that may lead to hybridization. For instance, the introduction of hatchery-bred fish into waterways outside their range has caused hybridization in native North Atlantic salmon populations in the Northwest region of America (Fraser et. al. 2008). Another example is the introduction of the Florida subspecies of largemouth bass into populations of the northern largemouth bass subspecies. Geographical barriers isolated these two genetically distinct subspecies populations until stocking programs began (Lutz-Carillo et al., 2006). The Florida subspecies, arguably a distinct species from the northern subspecies, was brought into Texas, within the range of the northern largemouth bass subspecies, to enhance fisheries for newly created reservoirs throughout the region (Forshage and Fries 1995). The hybrids of these two species have resulted in the desired outcome of a larger fish recreationally, but the effect on the parental species is unknown. A third case of hybridization caused by fish

introductions is the hybridization of westslope cutthroat trout (*Oncorhynchus clarki lewisi*) with introduced rainbow trout (*Oncorhynchus mykiss*) where the introduction of rainbow trout has caused a drastic decline in the native cutthroat trout populations due to introgression.

Fisheries managers are often interested in identifying hybrids and estimating the extent of hybridization caused by the introduction of non-native stocked fish. This information is important to assess the effect on native fish and useful for propagating the desired introduced species rather than hybrids when brood stock is obtained for hatchery programs. Striped bass, (*Morone saxatilis*) is an example of a hatchery-raised species, anthropogenically-introduced for sport fishing to the Toledo Bend Reservoir, a cross-boundary waterway that is managed by the Louisiana Department of Wildlife and Fisheries (LDWF) and the Texas Parks and Wildlife Department (TPWD). The Reservoir is outside of the *M. saxatilis* range, which is generally in the cooler Northeast. *M. saxatilis* is hatchery-raised because it is not well adapted to reservoir habitat because it requires stream flow to keep fertilized eggs oxygenated, making it less successful in reservoir habitats (Texas Parks and Wildlife Department 2009). According to the LDWF's records, *M. saxatilis* have been stocked in the reservoir since 1967. Between 1967 and 2009, 9,372,330 striped bass have been released into the Reservoir. This total includes stocking numbers from the TPWD. Lack of interest in striped bass by anglers led to the closure of the Louisiana *M. saxatilis* hatchery in 2003, although approximately 5,000 fish were released in 2007 (LDWF 2010). The Texas hatchery is also no longer releasing *M. saxatilis* in the Toledo Bend Reservoir. White bass (*Morone chrysops*) and yellow bass (*Morone mississippiensis*) both occur naturally in the Toledo Bend Reservoir.

While the hatchery operated, *M. saxatilis* brood stock were originally taken out of the Reservoir and used for propagation. If introgression was present, it would have been possible to collect individuals that were not pure *M. saxatilis* as brood stock, which could eventually lead to a hybrid swarm in the Reservoir. Uncertainty on having a pure stock of *M. saxatilis* with which to stock the Reservoir was the primary reason that LDWF funded a study of hybridization in the Toledo Bend area.

Hybridization appears to occur between *M. chrysops* and *M. saxatilis* in the Toledo Bend Reservoir and the Sabine River. Hybrids can sometimes be determined phenotypically by comparing physical characteristics from the parent populations. However, a hybrid may show characteristics of one parent species only, which makes identification difficult without using genetic analyses. Previous genetic work for the LDWF, which confirmed the existence of *M. chrysops* x *M. saxatilis* hybrids in the reservoir, used allozyme analysis on a small number of samples (Deb Kelly pers. comm.). The two isozymes used were glycerol-3-phosphate dehydrogenase (G3PD), found in muscle tissue, and isocitrate dehydrogenase (IDH), found in liver tissue. *M. saxatilis* is homozygous at both loci (allele B3 and allele B3) and *M. chrysops* is homozygous for both loci (allele B1 and allele B1) making a hybrid distinguishable if it is heterozygous. These two loci could reliably identify hybrids if all hybrids were F1 hybrids, however, two loci are insufficient for identifying hybrids in a hybrid swarm. If introgression was occurring, the backcrosses have the potential to be homozygous like the parental generation. *M. saxatilis* also appears to have become smaller over time (William Kelso pers. comm.), suggesting that hybridization may be widespread. Therefore, LDWF is interested in identifying the extent of hybridization occurring in the Toledo Bend area between *M. chrysops*, *M. mississippiensis* and *M. saxatilis*. I examine herein hybridization between *M. chrysops* and *M. mississippiensis* using a sufficient number of microsatellite loci to identify hybrids in a hybrid swarm.

Methods

Sampling

26 *Morone spp.* blood samples were collected to determine individual genotypes and estimate the extent of hybridization in the Toledo Bend Reservoir. The 26 individuals collected were given the following field identifications by LDWF staff: three *M. mississippiensis*, one potential *M. chrysops*/*mississippiensis* hybrid, and 22 *M. chrysops*. The samples were collected outside of Logansport, LA (coordinates: N 31.99207, W 94.01820) on the upper part of the Sabine River, above the Toledo Bend Reservoir. LDWF fisheries biologists conducted boat-based electro-fishing in order to catch the specimens. Blood was drawn from the ventral side of the backbone by entering behind the caudal fin of the collected fish using 18 gauge needles. The collected blood was then stored in two 1.5 ml tubes, one containing ethanol and one containing Queen's lysis buffer (Seutin et al. 1991). LDWF fisheries biologists have not collected any *M. saxatilis* to date.

Genetic methods

DNA from each of the 26 specimens was extracted from blood using a Chelex resin protocol. Approximately 0.5 ml of Chelex resin (50 mg/ml) and 2 µl Prot K enzyme (10mg/ml) were added to 800 µl milli Q water in a 1.5 ml tube, along with about 5 µl blood stored in Queen's lysis buffer. The mixture was incubated at 65°C for two hours, followed by another incubation period at 95°C for 10 min. The mixture was then centrifuged for 10 min at 13,000 rpm at room temperature. DNA suspended in the supernatant was drawn off and used for amplification.

Primers (n = 15; Table 1) were chosen from among 149 microsatellite markers developed by Couch et al. (2006) for *M. chrysops* and *M. saxatilis* (*M. mississippiensis* was not screened in this study). Primers were selected based on easily distinguished allele size differences between *M. chrysops* and *M. saxatilis*, and included loci that were monomorphic for different alleles in one or both species to ensure that the species were easily distinguishable from each other. One primer in each pair was tagged with a fluorescent M13 marker to make the alleles visible on Li-COR gels, which were used for genotyping (Table 1). This adds 19 or 20 base pairs (depending on whether an M13 forward or reverse primer was used) to the allele size reported by Couch et al. (2006). Each locus was initially tested separately for amplification on agarose gels with four different DNA samples. Amplification reactions (10 µl) consisted of 1 X buffer (Promega), 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphate set (dNTPs; Qiagen), 0.05µM of forward and reverse primers (IDT), 0.25 U GoTaq (Promega), 1 µl DNA, and nanopure water. All loci and individuals were tested and run using the following PCR protocol: 95º for 2 minutes, 35 cycles of 94º for 30 seconds, 50°C for 30 seconds and 72º for 1 minute, followed by a final extension step of 72º for 7 minutes. Polymerase chain reaction (PCR) product (2 µl) and 2 µl diluted EZ vision dye were combined and loaded on 1.2% 1 x TBE agarose gels and visualized under UV light to check for amplification.

Table 1. Primer sequence and PCR characteristics for *Morone saxatilis* and *Morone chrysops* as developed by Couch et al. (2006).

Locus	Primer sequences (5′-3′) Forward / Reverse	M13 700 Forward [a] / Reverse [b]	T_a (°C)	A^e	*M. chrysops* allele range Published [c] / Observed [d]	*M. mississippiensis* allele range Observed [d]	*M. saxatilis* allele range Published [c]
MSM 1134	ACTTTTCTGTCAGGACACAGC		52°C	1	167		206-242
	CGATGGCAGCTTACATAGG [2]	Reverse			168-170	180-182	
MSM 1142	TGCCAGGAGATTAGATAGCTTGCAC		52°C	1	142		178-182
	CGAAATTGGACTTGGCGAAATC [2]	Forward			140	132-140	
MSM 1076	TTTCTCTCGGAACATCATTGCTATCTG	Reverse	52°C	2	225-227		205
	CTGAGGCTACAATACAGGCACTGGTAA [2]				226	204	
MSM 1085	TCTTTTATTTTTAGCCTCATTCAGACTGAT	Reverse	52°C	2	129-131		164-209
	CAGCAACAGATGATGGTCAAGTATG [2]				129	No amplification	
MSM 1165	TCGGTCAGAGTGAGCTCAGAGT		52°C	2	180-182		223-251
	CAGGTTACAACGACCACGACA [2]	Reverse			183	173	
MSM 1102	AGAGAGACTGGATGATACGG		52°C	2	138-142		162
	GACTAAGCAGGATTACTCAAGG [2]	Reverse			143	160	
MSM 1079	CGCCGAAAAGACACAGTTTAC	Reverse	52°C	1	257		270
	CTCCTGCTGGAAAAACTGATG [2]				259-263	289	
MSM 1081	TGTGTGTAAATGTCAAGGGTGTAA		52°C	1	184		162
	TGCACCATTTTAATTTAGTGTGAG [2]	Forward			180-184	No amplification	
MSM 1107	GATAACCTATAGGCCACGTTG	Reverse	52°C	1	149		164-245
	TTCACAAGACTGCACGTACA [2]				149	151-153	
MSM 1157	TGTCTGAGCAGGATGCTTACC		52°C	1 [3]	160		185-220
	GCCCATTAGCTTTTGTAGCAAC [2]	Reverse			160	170	
MSM 1137	GCAGGCAGGTTTTATCTAGGTTAG	Forward	52°C	1 [3]	145		173-260
	ACACTCTCTGCCCTTTGAGTTC [2]				141-143	149-151	
MSM 1078	GCAGGACTCCCGTGAAATACAACC	Forward	52°C	2 [3]	152-161		145
	AATTGAGAGGCCTTGGCTAGCATC [2]				158-162	170	
MSM 1149	GAAAAGCACTCAGAGGAACACACGC	Reverse	52°C	1 [3]	198		208-214
	GTCAACACTCACAGCAAGACACTGACA [2]				196	198	

[a] Forward M13 sequence: CACGACGTTGTAAAACGAC (5′-3′)

[b] Reverse M13 sequence: GGATAACAATTTCACACAGG (5′-3′); these labels are adjacent to the M13 tagged primer

[c] 19 or 20 base pairs have been added to the published range numbers to account for the addition of the M13 primer tags.

[d] Based on the analysis laid out in the methods section.

[e] Number of alleles as published by Couch et al. 2006

All loci successfully amplified on agarose and so were used to develop multiplex PCR reactions to run on Li-COR gels for genotyping. Five panels of three loci were developed using the Qiagen Multiplex PCR kit. PCR reactions for multiplex sets 1, 3, 4, and 5 (10 µl) consisted of 1 X Qiagen Multiplex Mix, 0.5 µM of each primer, 0.03 µM of M13 700 forward and reverse tags, 2.4 µl RNase free water and 1 µl DNA extracted from each of the samples. The PCR reactions for multiplex set 2 (10 µl) consisted of 1 X Qiagen Multiplex Mix, 0.5 µM each primer, 0.03 µM M13 700 reverse tags, 2.7 µl RNase free water and 1 µl DNA extracted from each of the samples. Multiplex set 2 differed from the other four sets because the primers were only

tagged with M13 reverse and therefore did not require an M13 700 forward tag. PCR thermocycling parameters were 95º for 15 minutes, 40 cycles of 94º for 30 seconds, 52º for 1:30 minutes, and 72º for 1:30 minutes, followed by a final extension step of 72º for 10 minutes. 3µl of stop dye was added to the PCR product following the completion of the reaction. The annealing temperature of 52°C was determined with a gradient PCR where all loci panels were amplified for four individuals (yb #6, wb #7, wb #8, and wb #9) as follows: 95º for 15 minutes, 35 cycles of 94º for 30 seconds, a gradient from 48°C to 60°C for 1:30 minutes and 72º for 1:30 minutes, followed by a final extension step of 72º for 10 minutes. These parameters were based on those given by the Qiagen Multiplex PCR kit. 52° C was selected as the annealing temperature given the clarity of the amplifications at that temperature.

Li-COR gels for genotyping were poured using gel mix consisting of 16 ml Sequagel XR, 4 ml Ureagel Complete, and 160 µl 10% ammonium persulfate (APS) with bind silane rubbed onto the glass plates in the comb (64-well) area prior to pouring. Bind silane consisted of 50 µl bind silane and 50 µl 10% acetic acid. Prior to loading, PCR product was denatured at 95°C for 8 minutes. Gels were run on Li-COR 4200 machines using 1000 ml of 1xTBE buffer and 0.8 µl of PCR product loaded into individual wells. In total, three gels were used to run all individuals under all primer sets with 700 low molecular weight standards (4 lanes) to establish allele sizes.

Analysis

Gels were scored using Li-COR Saga Generation 2 software, which identifies allele sizes for each individual at each locus. Once individual genotypes were obtained, individuals were separated into two groups, *M. chrysops* (including the putative hybrid) and *M. mississippiensis*. Linkage disequilibrium, allele frequencies, and overall F_{IS} (a measure of inbreeding) and F_{ST} (a measure of population differentiation) were calculated using GENETIX v. 4.05 and FSTAT v. 2.9.3 program. Hardy-Weinberg Equilibrium was assessed with GENEPOP v. 3.4.

Results

Of the 15 microsatellite loci that were originally selected, 13 amplified (see Table 2 for details) with no evidence of linkage disequilibrium. The analysis of these 13 loci by GENETIX v. 4.05 yielded allele frequencies (Table 2), expected heterozygosity (the percentage of heterozygotes expected under Hardy-Weinberg Equilibrium) and observed heterozygosity (the percentage of heterozygotes that is actually observed) for both groups (*M. chrysops* and *M. mississippiensis*). For *M. chrysops*, H_o was 0.1484 and H_E was 0.2158. For *M. mississippiensis*, H_o was 0.3542 and H_E was 0.3542. Overall F_{IS} was 0.19029 and overall F_{ST} was 0.72146. The F_{IS} value compares the heterozygote proportions within the two species to those expected under Hardy-Weinberg Equilibrium (Allendorf, 2007). If the value is positive there is a deficit of heterozygotes in the population, which may be caused by inbreeding. If the value is negative, it indicates that inbreeding is absent (Allendorf, 2007). The F_{ST} is known also as the fixation index and refers to how fixed particular alleles are in the population and the populations' genetic divergence from one another. The closer the value is to 1, the more alleles are fixed within the populations (Allendorf 2007).

Table 2. Allele frequencies of *Morone chrysops* and *Morone mississippiensis* subpopulations in the Toledo Bend Reservoir

Locus	N *M. chrysops* *M. mississippiensis*	Alleles per locus	Alleles	Allele Frequencies *M. chrysops*	Allele Frequencies *M. mississippiensis*
MSM 1134	7	4	168	0.7143	0
	1		170	0.1429	0
			180	0	0.5000
			182	0.1429	0.5000
MSM 1142	16	2	132	0.0625	0.1667
	3		140	0.9375	0.8333
MSM 1076	7	2	204	0.2143	1.0000
	2		226	0.7857	0
MSM 1085	13	1	129	1.0000	
	0				
MSM 1165	15	2	173	0	1
	2		183	1	0
MSM 1102	22	3	143	0.9545	0
	1		160	0	1.0000
			166	0.0455	0
MSM 1079	7	2	259	0.3571	
	0		263	0.6429	
MSM 1081	8	2	180	0.3125	
	0		184	0.6875	
MSM 1107	8	3	149	1.0000	0
	2		151	0	0.7500
			153	0	0.2500
MSM 1157	7	3	160	0.9286	0
	2		169	0.0714	0
			170	0	1.0000
MSM 1137	9	4	141	0.5000	0
	1		143	0.5000	0
			149	0	0.5000
			151	0	0.5000

MSM 1078	8 0	2	158 162	0.0625 0.9375
MSM 1149	9 0	1	196	1.0000

From analysis by GENETIX v. 4.05

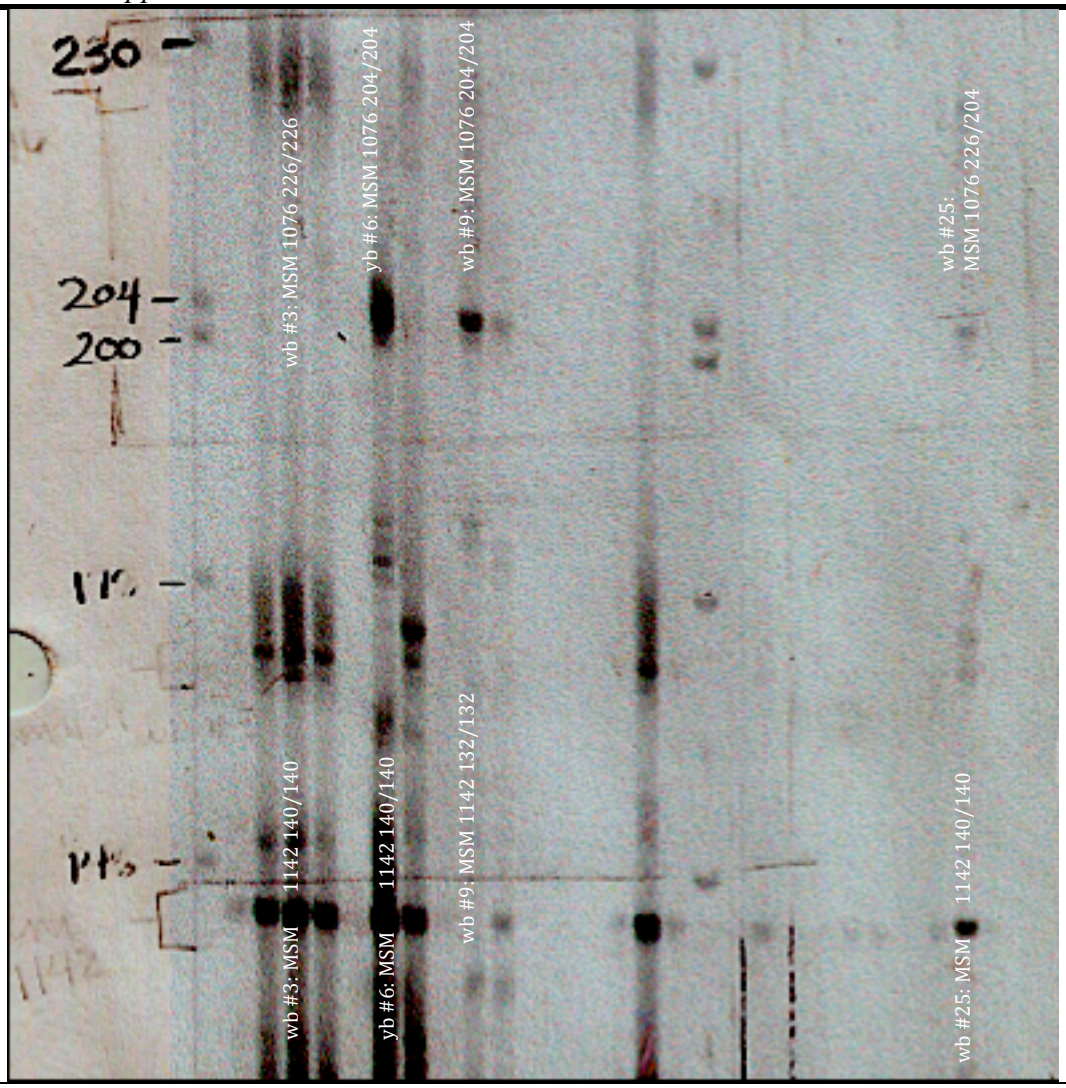
Based on published allele size ranges for *M. chrysops* and an examination of the genotypes, there are several potential hybrids within the sampled fish.

Table 3. Potential hybrids

ID	Initial identification	Loci indicating hybridization
wb #2	*M. chrysops*	MSM 1157
wb #7	*M. chrysops*	MSM 1134
wb #9	*M. chrysops*	MSM 1134, 1142, 1076
yb #10	*M. mississippiensis*	MSM 1142
wb #25	*M. chrysops*	MSM 1076, 1102

The potential hybrid individuals (Table 3) contain a mixture of alleles from both parental species. For example, individual wb #9 has alleles from both parental species at three loci. At locus MSM 1076, wb #9, originally identified phenotypically as *M. chrysops*, expresses a homozygous allele size of 204, which is consistent with the sizes expressed by individuals identified as *M. mississippiensis*. All other individuals identified as *M. chrysops* expressed a homozygous allele size of 226, which is considerably larger than 204. The one individual, wb #16, that was identified through physical characteristics as a potential *M. chrysops* X *M. mississippiensis* hybrid did not exhibit any genotypic hybridization (three loci amplified).

Figure 1. Li-COR gel illustrating a *M. chrysops*, a *M. mississippiensis*, and a *M. chrysops* X *M. mississippiensis*



Individual 6 is representative of *M. mississippiensis*; individuals 9 &25 are representative of a potential *M. chrysops* X *M. mississippiensis;* individual 3 is representative of *M. chrysops*

After identifying the hybrids, the data was re-analyzed excluding the putative hybrids. This caused a shift in the allele frequencies (Table 4), expected heterozygosity and observed heterozygosity for both groups (*M. chrysops* and *M. mississippiensis*). Only two *M. mississippiensis* and 19 *M. chrysops* individuals remained, reducing the sample size particularly for *M. mississippiensis*, which already had a small sample size. After re-analysis, $H_o = 0.1115$ and $H_E = 0.132$ for *M. chrysops*, and $H_o = 0.429$ and $H_E = 0.429$, for *M. mississippiensis*. Overall F_{IS} was -1.00 and overall F_{ST} was 0.876. I elected to remove all individuals who exhibited any hybridization. However, to reduce the effects of potential genotyping errors and conservatively estimate the number of hybrids, just individuals showing hybridization at more than a single locus could be used.

Table 4. Allele frequencies of *Morone chrysops* and *Morone mississippiensis* after hybrids were removed from the analysis.

Locus	N *M. chrysops* *M. mississippiensis*	Alleles per locus	Alleles	Allele Frequencies *M. chrysops*	Allele Frequencies *M. mississippiensis*
MSM 1134	3	3	168	1.0000	0
	1		180	0	0.5000
			182	0	0.5000
MSM 1142	12	1	140	1.0000	1.0000
	2				
MSM 1076	3	2	204	0	1.0000
	1		226	1.0000	0
MSM 1085	9	1	129	1.0000	
	0				
MSM 1165	11	2	173	0	1
	1		183	1	0
MSM 1102	18	1	143	1.0000	
	0				
MSM 1079	4	2	259	0.2500	
	0		263	0.7500	
MSM 1081	4	2	180	0.3750	
	0		184	0.6250	
MSM 1107	5	3	149	1.0000	0
	1		151	0	0.5000
			153	0	0.5000
MSM 1157	4	2	160	1.0000	0
	1		170	0	1.0000
MSM 1137	6	4	141	0.5000	0
	1		143	0.5000	0
			149	0	0.5000
			151	0	0.5000
MSM 1078	5	2	158	0.1000	
	0		162	0.9000	

MSM 1149	60	1	196	1.0000

From analysis by GENETIX v. 4.05

With the removal of potential hybrids, several of the loci showed a change in their allele frequencies. An example of this is locus MSM 1134, which had three alleles for *M. chrysops* when all individuals were considered. After the hybrids were removed, only one allele remained. Changes in allele frequencies also occurred at MSM 1078, 1157, 1107, 1081, 1079, 1102, 1076, and 1142.

White bass were not in Hardy-Weinberg Equilibrium when all individuals were considered ($p = 0.0001$), but when the hybrids were removed, there was no departure from Hardy-Weinberg expectations ($p = 0.1095$). Hardy-Weinberg analyses were not possible for yellow bass because the sample size was too small.

Discussion

This study shows that some hybridization occurs between *M. chrysops* and *M. mississippiensis* in the Toledo Bend Reservoir: five individuals had alleles that appeared to come from both parental species. However, the high F_{ST} value obtained here (all individuals), and expected in a comparison between two distinct species (*M. chrysops* and *M. mississippiensis*), suggests that introgression is not widespread. Additional evidence for introgression includes comparisons of results between analyses with and without hybrids. After removing the effect of hybrids from the analysis, F_{ST} increased, as expected. F_{IS} was moderately low (all individuals) or -1 (hybrids excluded), suggesting that inbreeding is relatively minor, which is consistent with hybridization. The F_{IS} value of -1 that I obtained is unusual particularly because it signifies less inbreeding when the hybrids were removed from analysis but this may be due to small sample sizes. Finally, *M. chrysops* was not in Hardy-Weinberg Equilibrium when hybrids were included in the analysis but showed no departures from Hardy-Weinberg Equilibrium when the hybrids were excluded. This result is consistent with introgression, which is a known cause for deviations in Hardy-Weinberg Equilibrium

This preliminary assessment requires additional work. For instance, a complete study should include larger samples sizes and *M. saxatilis* samples. A large sample size improves precision (Raudys 1990) and is especially important for *M. mississippiensis* (n = 3, this study) because it would allow *M. mississippiensis* allele size ranges to be generated more precisely. Currently, there are no published data on allele size ranges for this species and the ones that I observed show some overlap with the published allele size ranges for *M. saxatilis*. Therefore, the hybrids that I identified may be *M. chrysops* X *M. mississippiensis* or *M. chrysops* X *M. saxatilis*. Overlap between allele size ranges for *M. mississippiensis* and *M. saxatilis* suggests that it may be necessary to choose different primers whose allele size ranges do not overlap. Additional primers could be selected from Couch et al. (2006). In addition to catching more fish, sample size could be increased by improving amplification. Approximately half of my samples did not amplify, therefore, DNA may need to be re-extracted with a different protocol or more DNA may need to be added to PCR reactions. Finally, it may be beneficial to run loci singly rather than multiplexed to improve clarity when scoring gels. It would take longer to collect the necessary data but there was some difficulty in creating the multiplex panels. While panels could be created without overlap of the allele ranges between *M. chrysops* and *M.*

mississippiensis, maintaining that separation and preventing overlap of the allele ranges for *M. saxatilis* would be difficult.

To improve estimates of allele size ranges in each species, it would be helpful to obtain samples of all three *Morone* species from areas where only one species is present. This would give allele size ranges without the possibility of hybrids and should give a clear picture of which individuals are hybrids in the Toledo Bend Reservoir. In addition, because I do not know where Couch et al. (2006) obtained their samples for primer development, using local populations may also avoid problems of allele size range differences among populations of the same species. However, there do not appear to be any local *Morone spp.* (all three species of interest) that exist in isolation.

Most *M. saxatilis* hatcheries began with a captive generation and have since propagated further generations without clearly knowing whether the brood stock was entirely purebred *M. saxatilis* (i.e. without alleles representative of other *Morone* species). There are no records of pure *M. chrysops* and *M. mississippiensis* in any study that could be used as a base standard to compare allele sizes when determining genotypes. Woods et al. (1995) was able to obtain a genetic analysis of *M. saxatilis* stocks in the Crane Aquaculture Facility, the largest facility stocking the Chesapeake Bay area, which showed that *M. saxatilis* in the facility were purebred. While this is helpful and could potentially be used for comparison in our further analysis of additional individuals within the Toledo Bend Reservoir, this would not account for any differences in alleles due to differences in locations of the populations.

Although I was primarily interested in hybridization between *M. saxatilis* and *M. chrysops*/*M. mississippiensis*, *M. saxatilis* samples were not available, which restricted my analysis to hybridization between *M. chrysops* and *M. mississippiensis*. These two species occur naturally in the Reservoir, so the reasons for hybridization are unclear. *M. chrysops* and *M. mississippiensis* deposit eggs on the substrate without the creation of a nest, and these are fertilized when males release sperm into the water above the egg deposition (TPDW 2009). Although both species breed in the same area, they usually breed at different times of the year, which may create a temporal reproductive barrier. *M. mississippiensis* generally breed from April to early May while *M. chrysops* breed from May through early June. However, this reproductive barrier may not be complete because there is some overlap in breeding seasons, which would allow for some naturally occurring hybridization between these two species (Illinois Department of Natural Resources 2011).

In the Sabine River/Toledo Bend reservoir, the speciesmost in danger from introgression are the native *M. chrysops* and *M. mississippiensis*. Because *M. saxatilis* is not as common in the Reservoir, if endemic at all, its loss would not be important. However, the loss of *M. chrysops*, or *M. mississippiensis*, could be. In North Carolina, problems with introgression in *M. saxatilis* are of greater concern because it is native to the area. For instance, the Cape Fear River Estuary in North Carolina, one of the southern regions of the *M. saxatilis* endemic range, has been experiencing problems with an increase in the abundance of *M. saxatilis* X *M. americana* hybrids into the native *M. saxatilis* population. Hybrids were stocked in the area in order to establish a recreational fishery during a time when the native population was experiencing a decline (Patrick 2001). Over a nine-year period, the native population declined, while the hybrid population thrived (Patrick 2001). As a result, it has been suggested that the stocking program be terminated and all hybrids removed (Patrick 2001).

Although hybridization and potential introgression between *M. saxatilis* and *M. chrysops* may be of concern to the preservation of the two parental populations, these hybrids are propagated as a desired recreational fish and stocked as such in waterways throughout Texas, North Carolina, and other states within the *Morone spp.* range (Bartley 2001). Furthermore, the aquaculture industry propagates hybrid *M. saxatilis* X *M. chrysops* to produce fish that exhibit hybrid vigor and outperform both parental species (Bartley 2001). The *M. saxatilis* X *M. chrysops* fishery makes-up the fourth largest fish-farming sector (McGinty 2008). The Texas Freshwater Fisheries Center hatchery in Athens, TX continues to produce 5.8 million *M. saxatilis* X *M. chrysops* hybrids annually for stocking purposes elsewhere than the Toledo Bend Reservoir (TPWD 2009).

Stocking may not always have detrimental effects, and in the Toledo Bend Reservoir, there are stocking alternatives besides *M. saxatilis* that may not cause introgression in native species. For instance, several different hybrids have been produced that yield infertile offspring, which eliminates the potential for introgression to occur. This type of hybrid would require restocking from year to year but would not affect the native populations within the region being stocked through hybridization/introgression. One of these nonviable hybrid options is a tiger muskie, a cross between a northern pike (*Esox lucius*) and a muskellunge (*Esox masquinongy*). This cross has lower hatchery costs than its parent species, which is an excellent trait for a stocking program (Wahl and Stein, 1993). While these are northern species like *M. saxatilis*, they are more tolerant to higher temperatures than *M. saxatilis*, which is one of the main reasons that *M. saxatilis* did not reproduce naturally in the Toledo Bend Reservoir. As with any hybrid there may be ecological problems besides hybridization. Tiger muskies, for example, have voracious appetites like pike, one of the parental species, which may cause problems for native species (Wahl and Stein, 1993). There are several ecological standpoints that should be considered before stocking any species, hybrid or natural, into a non-native region.

While much conflict is generated between aquaculturists, who develop and maintain productive fisheries, and ecologists who strive to maintain ecosystem biodiversity, it is possible for them to work together and even benefit from each other (Cowx et al., 2010). With the halt on the hatchery in the Toledo Bend Reservoir, I am hopeful that the future of native *M. chrysops* and *M. mississippiensis* is ensured when the hybrids eventually die out depending on the amount of introgression that has already occurred. Perhaps another recreational, non-fertile, more carefully selected hybrid could be introduced if the desire to have more recreational fish persists, though that is not in the plans thus far for the area.

Effective September 1, 2011, a plan to have consistent recreational fishing regulations between Louisiana and Texas was established because the Toledo Bend Reservoir is shared between the two states (LDWF Title 76). These regulations include bag limits for *M. saxatilis, M. chrysops,* and *M. mississippiensis* but not for hybrids (LDWF Title 76) because the size of their population is not yet known and because there is no concern for hybrids. Other than these bag limits, LDWF and the TPWD do not manage for hybrids in the Toledo Bend Reservoir. Hopefully these regulations can eventually be extended to take into account the existence of hybrids, potentially by encouraging their removal within the Toledo Bend Reservoir once further analysis of the populations has occurred. It is also important that all aspects of the biological community in the Toledo Bend reservoir that may be affected by future stocking programs be taken into consideration so as to maximize the recreational fishery while still maintaining the native populations of *M. chrysops* and *M. mississippiensis*.

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