Identification of northern and southern subspecies of largemouth bass for use as brood stock by the Mississippi Department of Wildlife, Fisheries, and Parks.

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Summary of major findings

In total, 46% (32 of 70 individuals) of individuals were categorized as backcrosses sharing a portion of their genome with *M. s. salmoides*. Only eight of 20 Alabama were identified as pure *M. s. floridanus*, with 12 individuals being potential backcrosses to *M. s salmoides.* All Louisiana samples were identified as backcrosses. In contrast, all Florida and Mississippi individuals were classified as *M. s. floridanus.*

IDENTIFICATION OF NORTHERN AND SOUTHERN SUBSPECIES OF LARGEMOUTH BASS FOR USE AS BROODSTOCK BY THE MISSISSIPPI DEPARTMENT OF WILDLIFE, FISHERIES, AND PARKS.

Justification

The Florida bass (*Micropterus salmoides floridanus*), which is endemic to peninsular Florida, is a subspecies of black bass in the freshwater fish family Centrarchidae. The northern largemouth bass, *M. s. salmoides*, is a native to the mid-west and northeast United States and the southern part of Canada. Florida bass and northern largemouth bass co-exist in a natural zone of introgression that extends through the southeastern and mid-Atlantic coastal states (Philipp et al. 1983). Stocking activities have extended the range of each subspecies and expanded the hybrid zone (Philipp et al. 1983). While genetic distinctiveness between northern and southern subspecies has been identified with several allozyme markers, subsequent introgression (i.e., repeated backcrossing of hybrid descendants with the parental population resulting in the infiltration of the genome of one species in to that of the other) makes the detection of backcrossed individuals difficult with these markers. This uncertainty poses problems for state hatcheries that assume purity of strain based on morphological comparisons for hatchery augmentation or reintroduction efforts.

Project Goal

The goal of this research was to assess the genetic purity *M. s. floridanus* individuals that will be used by the Mississippi Department of Wildlife, Fisheries, and Parks (herein termed State) for future hatchery brood stock in the state of Mississippi.

Project Objectives

Genetically screen n = 70 putative *M. s. floridanus* samples using four microsatellite markers known to be diagnostic between subspecies.

Methods

Largemouth bass tissue samples (n = 70) were provided by State biologists. These fish were a random sample of progeny from putative *M. s. floridanus* brood stock and intended for use by the State as future *M. s. floridanus* brood stock. The origin of these fish was as follows (progeny number in parentheses): Alabama (n = 20); Louisiana (n = 20); Florida (n = 20), and Mississippi (n = 10). A tissue clip from each sample was preserved in 95% non-denatured ethanol and sent to Warm Springs Conservation Genetics lab to be analyzed and archived.

Genomic DNA was extracted from a portion of the ethanol preserved fin clip using the DNeasy Blood and Tissue kit (QIAGEN, Inc., Valencia, CA) protocol. We used a suite of four (*Msa*-01, *Msa*-05, *Msa*-31, and *Msa*-32) microsatellite markers known to distinguish between largemouth bass subspecies (Tringali et al. 2010) except we had to redesign primers *Msa*-01 due to amplification issues (new primer sequence: *Msa*-01new_F: 5'-AGGTTGCAGGCTGGACGTGAC; *Msa*-01new_R: 5'-GTTTCTTGGCCGACACC AAGGCCAACA). Single polymerase chain reaction (PCR) amplifications were performed in 8 µL reactions containing 30-100ng/uL DNA. For *Msa*-01 and *Msa*-05 we used PCR reactions consisting of 0.31 × *Taq* reaction buffer (GoTaq Flexi, Promega, Madison, WI), 2.0 mM $MgCl_2$, 0.3 mM of each dNTP, 0.40 uM of each primer, and 0.12 U Taq DNA polymerase (GoTaq, Promega, Madison, WI). For *Msa*-31 PCR reagents were the same except we used 2.38 mM $MgCl_2$ and 0.14 U *Taq*; likewise, for *Msa*-32 we used 2.50 mM $MgCl_2$ and 0.14 U *Taq*.

PCR amplifications were conducted using a GeneAMP PCR System 9700 (Applied Biosystem, Inc., Foster City, CA). *Msa*-01 and *Msa*-05 PCR conditions were an initial denaturation at 94 °C (10 min), followed by a touchdown procedure involving 33 cycles and consisting of denaturing (94 °C, 30 s), annealing, and extension (74 °C, 30 s) cycles, where the initial annealing temperature was initiated at 56 °C and decreased by 0.2 °C per cycle for 30 s. In contrast, PCR conditions for *Msa*-31 and *Msa*-32 were an initial denaturation at 94 °C (2 min), then 33 cycles each at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, followed by an extension step at 72°C for 7 min.

Prior to electrophoresis, 2 µL of a 1:100 dilution of PCR product was mixed with a 8 µL solution containing 97% formamide and 3% Genescan LIZ 500 size standard (Applied Biosystems, Inc.). Microsatellite reactions were visualized with an ABI 3130 genetic analyzer (Applied Biosystems, Inc.) using fluorescently labeled forward primers and analyzed using GeneMapper software v3.7 (Applied Biosystems, Inc.).

Subspecies identification was determined by binning each individual for each microsatellite marker in to one of three categories, *M. s. floridanus*, *M. s. salmoides,* or hybrid. For example, *Msa*-01 allele 256 was found only in *M. s. floridanus* whereas allele 332 was found only in *M. s. salmoides* (Tringali et al 2010). Thus if an individual in question was scored as alleles 256/256, then it was assigned an identification of *M. s. salmoides* at this locus. In contrast, a 256/332 individual would be scored as a hybrid. After summarizing individual score calls across all four microsatellite markers, a pure *M. s. floridanus* sample would have all diagnostic alleles for this subspecies for all four markers. Hybrid taxa should have a hybrid score for all loci, and backcrossed individuals should have a combination of hybrid, *M. s.*

floridanus and/or *M. s. salmoides* scores. Tringali et al. (2010) showed that the combination of these markers could assign pure individuals to their respective taxa 99.2% of the time.

Results

A summary of individual allele scores and identifications can be found in Appendix. Our new Msa-01 primers yielded a product 128bp larger than that of Tringali et al 2010. In total, 46% (32 of 70 individuals) of individuals were categorized as backcrosses sharing a portion of their genome with *M. s. salmoides*. Only eight of 20 Alabama were identified as pure *M. s. floridanus*, with 12 individuals being potential backcrosses to *M. s salmoides.* All Louisiana samples were identified as backcrosses. In contrast, all Florida and Mississippi individuals were classified as *M. s. floridanus.*

Discussion

The large number of putative backcrosses in progeny of what were thought to be pure *M. s. floridanus* was alarming and highlights the importance of validating the genetic makeup of brood stock via genetic analysis. The identification of Alabama samples was conservative because the identification at microsatellite marker *Msa*-032 was arduous. The allele 300 at this locus was not reported by Tringalli et al 2010), but allele 302, which was reported by these authors, was found at low frequencies in both subspecies. Thus, as a cautionary approach, we chose to score all individuals having allele 300, as hybrids at this locus instead of M. s. floridanus and hence the designation of backcross (denoted ad backcross? in Appendix). To actually discern the frequency of *Msa*-32 allele 300 would require more extensive sampling of *M. salmoides* -- an effort outside the scope of this study.

Finally, we recommend that any individual labeled as a backcross in this document, regardless if it is questionable, be withdrawn from any future *M. s. floridanus* brood stock plans because the risk of introducing known *M. s. salmoides* alleles into areas where only *M. s. floridanus* occurs is too great to warrant inclusion.

Literature cited

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Tringali, M., Porak, W., Bellotti, P., Seyoum, S., Trippel, N., Strickland, A., Knight, J., Paxton, C., Puchulutegui, C., and Davis, M. 2010. Population Genetics of Freshwater Fishes for Fish Management and Stock Enhancement. Completion Report for the Federal Aid in Sport Fish

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Appendix. Allele score calls for putative *M. s. floridanus* samples at four microsatellite markers known to be diagnostic between *M. salmoides* subspecies. Subspecies identification was performed for each locus (allele a and b) separately and summed to provide an overall ID. Florida or Northern represent identification of an individual as *M. s. floridanus* or *M. s. salmoides,* respectively. Backcross? denotes a questionable ID because *Msa*-32 allele 300 was not reported by Tringali et al. (2010) but allele 302 was found in both subspecies (see Discussion for details).

ID	**Origin**	**ID for *Msa*-01**	***Msa*-01a**	***Msa*-01b**	**ID for *Msa*-05**	***Msa*-05a**	***Msa*-05b**	**ID for *Msa*-31**	***Msa*-31a**	***Msa*-31b**	**ID for *Msa*-032**	***Msa*-32a**	***Msa*-32b**	**Overall ID**
7009	Alabama	Florida	264	300	Florida	127	147	Florida	236	236	hybrid/Florida	288	302	**backcross?**
7010	Alabama	Florida	264	300	Florida	127	127	Florida	236	236	hybrid/Florida	288	302	**backcross?**
7011	Alabama	Florida	264	300	Florida	127	127	Florida	236	236	hybrid	264	290	**backcross**
7012	Alabama	Florida	238	300	Florida	127	127	Florida	236	236	Florida	290	298	**Florida**
7013	Alabama	Florida	238	244	hybrid	121	127	Florida	236	236	Florida	288	288	**backcross**
7014	Alabama	Florida	264	300	hybrid	125	127	Florida	236	236	hybrid/Florida	296	300	**backcross**
7015	Alabama	Florida	298	302	Florida	127	127	Florida	236	236	Florida	290	296	**Florida**
7016	Alabama	Florida	298	300	Florida	127	127	Florida	236	236	hybrid	264	292	**backcross**
7017	Alabama	Florida	264	300	Florida	127	127	Florida	236	236	hybrid/Florida	296	300	**backcross?**
7018	Alabama	Florida	234	298	Florida	127	129	Florida	236	236	hybrid/Florida	282	300	**backcross?**
7019	Alabama	Florida	238	264	Florida	127	137	Florida	236	236	Florida	292	298	**Florida**
7020	Alabama	Florida	236	264	Florida	127	127	Florida	236	236	Florida	290	292	**Florida**
7021	Alabama	Florida	238	264	Florida	127	127	Florida	236	236	hybrid/Florida	296	300	**backcross?**
7022	Alabama	Florida	264	300	Florida	127	127	Florida	236	236	Florida	284	294	**Florida**
7023	Alabama	Florida	236	238	Florida	127	137	Florida	236	236	Florida	292	296	**Florida**
7024	Alabama	Florida	238	238	hybrid	121	127	Florida	236	236	Florida	282	290	**backcross**
7025	Alabama	Florida	238	300	Florida	127	127	Florida	236	236	hybrid/Florida	282	300	**backcross?**
7026	Alabama	Florida	300	300	Northern	121	125	Florida	236	236	hybrid/Florida	298	300	**backcross**
7027	Alabama	Florida	244	264	Florida	127	127	Florida	236	236	Florida	288	296	**Florida**
7028	Alabama	Florida	238	300	Florida	127	137	Florida	236	236	Florida	290	292	**Florida**
7029	Louisiana	hybrid	256	332	Northern	121	121	Florida	236	236	hybrid/Northern	256	314	**backcross**
7030	Louisiana	Florida	256	256	Northern	121	121	Florida	236	236	Northern	256	264	**backcross**
7031	Louisiana	Florida	256	256	Northern	121	121	Florida	236	236	Northern	256	264	**backcross**
7032	Louisiana	Florida	256	256	Northern	121	121	Florida	236	236	hybrid/Northern	264	316	**backcross**

7033	Louisiana	hybrid	256	332	Northern	121	121	Florida	236	236	hybrid/Northern	256	314	**backcross**
7034	Louisiana	hybrid	306	332	Northern	121	121	Florida	236	236	Northern	256	264	**backcross**
7035	Louisiana	Florida	256	256	Northern	121	121	Florida	236	236	Northern	256	264	**backcross**
7036	Louisiana	Florida	256	256	Northern	121	121	Florida	236	236	hybrid/Northern	264	316	**backcross**
7037	Louisiana	Florida	256	306	Northern	121	121	Florida	236	236	hybrid/Northern	264	316	**backcross**
7038	Louisiana	hybrid	256	332	Northern	121	121	Florida	236	236	hybrid/Northern	264	316	**backcross**
7039	Louisiana	Northern	304	332	Northern	121	121	Florida	236	236	Northern	256	264	**backcross**
7040	Louisiana	Florida	256	306	Northern	121	121	Florida	236	236	hybrid/Northern	264	316	**backcross**
7041	Louisiana	Florida	256	256	Northern	121	121	Florida	236	236	hybrid/Northern	264	316	**backcross**
7042	Louisiana	Florida	256	306	Northern	121	121	Florida	236	236	hybrid/Northern	264	316	**backcross**
7043	Louisiana	hybrid	256	332	Northern	121	121	Florida	236	236	Northern	256	264	**backcross**
7044	Louisiana	hybrid	256	332	Northern	121	121	Florida	236	236	Northern	256	264	**backcross**
7045	Louisiana	hybrid	256	332	Northern	121	121	Florida	236	236	Northern	256	264	**backcross**
7046	Louisiana	Florida	256	256	Northern	121	121	Florida	236	236	hybrid/Northern	264	316	**backcross**
7047	Louisiana	Florida	256	306	Northern	121	121	Florida	236	236	Northern	256	264	**backcross**
7048	Louisiana	hybrid	256	332	Northern	121	121	Florida	236	236	hybrid/Northern	264	316	**backcross**
7049	Florida	Florida	236	238	Florida	127	127	Florida	236	236	Florida	292	296	**Florida**
7050	Florida	Florida	238	240	Florida	127	147	Florida	236	236	Florida	292	296	**Florida**
7051	Florida	Florida	238	238	Florida	127	147	Florida	236	236	Florida	288	296	**Florida**
7052	Florida	Florida	238	238	Florida	127	127	Florida	236	236	Florida	292	292	**Florida**
7053	Florida	Florida	238	264	Florida	127	129	Florida	236	236	Florida	288	296	**Florida**
7054	Florida	Florida	236	238	Florida	127	127	Florida	236	236	Florida	292	292	**Florida**
7055	Florida	Florida	238	238	Florida	127	147	Florida	236	236	Florida	282	284	**Florida**
7056	Florida	Florida	238	238	Florida	127	127	Florida	236	236	Florida	292	296	**Florida**
7057	Florida	Florida	238	238	Florida	127	147	Florida	236	236	Florida	296	296	**Florida**
7058	Florida	Florida	238	238	Florida	127	147	Florida	236	236	Florida	296	296	**Florida**
7059	Florida	Florida	238	238	Florida	127	127	Florida	236	236	Florida	292	296	**Florida**
7060	Florida	Florida	236	244	Florida	129	147	Florida	236	236	Florida	296	296	**Florida**
7061	Florida	Florida	236	238	Florida	129	147	Florida	236	236	Florida	282	296	**Florida**
7062	Florida	Florida	244	302	Florida	137	147	Florida	236	236	Florida	282	296	**Florida**
7063	Florida	Florida	238	238	Florida	127	147	Florida	236	236	Florida	282	294	**Florida**

7064	Florida	Florida	236	238	Florida	127	127	Florida	236	236	Florida	292	296	**Florida**
7065	Florida	Florida	238	244	Florida	127	147	Florida	236	236	Florida	282	292	**Florida**
7066	Florida	Florida	238	238	Florida	127	129	Florida	236	236	Florida	292	296	**Florida**
7067	Florida	Florida	236	238	Florida	127	147	Florida	236	236	Florida	292	292	**Florida**
7068	Florida	Florida	238	238	Florida	127	147	Florida	236	236	Florida	282	284	**Florida**
7069	Mississippi	Florida	238	264	Florida	127	171	Florida	236	236	Florida	298	298	**Florida**
7070	Mississippi	Florida	244	264	Florida	137	147	Florida	236	236	Florida	288	298	**Florida**
7071	Mississippi	Florida	238	238	Florida	127	129	Florida	236	236	Florida	284	288	**Florida**
7072	Mississippi	Florida	238	264	Florida	127	171	Florida	236	236	Florida	288	290	**Florida**
7073	Mississippi	Florida	298	302	Florida	127	127	Florida	236	236	Florida	290	296	**Florida**
7074	Mississippi	Florida	244	302	Florida	127	147	Florida	236	236	Florida	292	298	**Florida**
7075	Mississippi	Florida	238	264	Florida	127	171	Florida	236	236	Florida	288	298	**Florida**
7076	Mississippi	Florida	244	264	Florida	137	147	Florida	236	236	Florida	288	288	**Florida**
7077	Mississippi	Florida	238	264	Florida	127	171	Florida	236	236	Florida	288	298	**Florida**
7078	Mississippi	Florida	238	244	Florida	127	137	Florida	236	236	Florida	288	288	**Florida**