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Abstract

Florida bass (*Micropterus floridanus*) have been introduced throughout much of the southern U.S. over the past 50 years. This species readily hybridizes with the extant largemouth bass (*M. salmoides*). Within Arkansas, the Florida bass is currently stocked in the southern half of the state. Previous studies of a northern Arkansas hatchery and a reservoir revealed the existence of Florida bass alleles in each. Other studies in Oklahoma and Texas have revealed the presence of Florida bass alleles in stream systems proximal to lakes stocked. Our goal was to investigate, using microsatellite analysis of 7 diagnostic loci, the presence of Florida bass alleles in 8 northeastern Arkansas streams to determine if Florida bass or hybrids had escaped from private farm ponds as compared to stocked reservoirs. We found rare instances of Florida bass alleles in most drainages, consistent with previous studies demonstrating a lack of containment of Florida bass once stocked. In Cane Creek, which flows adjacent to privately stocked farm ponds, one-third of the individuals had Florida bass alleles.

Introduction

State and federal agencies in the U.S. began stocking exotic fish species in the 1800's with the introduction of trout. Other sportfish species such as sunfishes and percids were eventually stocked into new and existing bodies of water across the U.S. The potential for negative consequences of these exotic stocking events were not initially considered, and their effects have forever changed the biological landscape of many North American watersheds.

One fish species regularly stocked over the past 50 years through much of the southern U.S. is the Florida bass (FB, *Micropterus floridanus*), which is stocked in reservoirs containing extant populations of Largemouth Bass (LMB, *Micropterus salmoides*). The FB has a reputation of greater maximal growth than the LMB (Addison and Spencer 1971, Wright and Wigtil 1980, Horton and Gilliland 1993, Hobbs et al. 2002), with several state records of bass in southern US states comprised of exotic FB rather than native LMB (Oklahoma, Horton and Gilliland 1993; Texas, Lutz-Carrillo et al. 2006; Louisiana, Hughes and Wood 1995; and Arkansas, Lamothe and Johnson 2013). The Arkansas Game and Fish Commission (AGFC) stocks FB in the southern half of the state based upon thermal criteria established for stocking FB in Oklahoma (Gilliland 1992).

The native range of the FB is limited to peninsular Florida (Boschung and Mayden 2004), yet hybridization among FB and LMB occurred naturally in areas where the native ranges overlap. Hybridization also occurs readily in waters where FB are stocked in waters with extant LMB populations. Further, bass stocked in reservoirs can escape from whence they are stocked. Researchers have identified non-native Florida bass alleles in streams adjacent to reservoirs stocked with FB (Gelwick et al. 1995, Ray et al. 2012).

Additionally, private landowners often stock FB rather than LMB in Arkansas ponds, including in areas that are north of the limits of stocking practices by the AGFC. If individuals from that pond escape to a stream, FB alleles may be introduced. However, it is unlawful to release native or non-native aquatic wildlife into any waters of the state without the written permission of the Commission (AGFC 2014).

In an effort to determine whether FB were escaping from farm ponds into neighboring stream systems, bass were sampled from several streams (n = 8) in northeast Arkansas (Table 1). Northeast Arkansas was selected because only two waterbodies in this region, lakes Ashbaugh and Greenlee, have been historically stocked with FB by the AGFC. Lake Ashbaugh, was renovated with a fish kill using rotenone in 1996 and re-stocked with LMB (Johnson and Fulton 1999). Lake Greenlee was renovated in 2000 and stocked with FB thereafter. Streams were

Table 1. Fin clip samples collected from Largemouth bass and potential Florida bass in various locations in northeast Arkansas.

Location	No. Samples	Latitude, Longitude	Source	Stream Mouth
Martin's Creek near Ravenden	9	36.279306, -91.328056	Natural Spring	Spring River
Higginbotham Creek south of Jonesboro at Ingels Road	5	35.791972, -90.652806	Jonesboro City Drainage	Ditch Number 103
Des Arc Bayou near mouth at White River	3	35.009861, -91.520944	Ozark Mountains	White River
Bull Creek near Vinity Rd	23	35.074667, -91.829333	Ozark Mountains	Des Arc Bayou
Cane Creek near US 67/167	18	35.139833, -91.809111	Ozark Mountains	Des Arc Bayou
Spring River south of Hardy	5	36.292331, -91.438892	Mammoth Springs	Black River
Big Creek at AR Hwy 349	7	35.840617, -90.801826	Lake Frierson	Bayou DeView
Swan Pond Ditch near Raybourn Rd in Poinsett County	3	35.637518, -90.803378	Agricultural Drainage Ditch	Claypool Reservoir

chosen to represent a variety of smaller watersheds distant from reservoirs stocked with bass by the AGFC; each stream flows without dams or other obstructive barriers.

The objectives of this study were as follows: 1) to estimate the frequency of FB alleles in various northeastern Arkansas streams; and 2), if FB alleles are present, determine the level of intergradation of fish containing those alleles.

Materials and Methods

Sampling and DNA Processing

Fin clips were collected from angled fish and stored in 95% v/v ethanol solution. DNA extraction was performed using a modified version of the Saghai-Maroof *et al.* (1984) CTAB (chloroform tris-acetate-borate) method. The nucleic acid concentrations of the stock solutions were determined using a Thermo Scientific Nanodrop 8000c Spectrophotometer (Wilmington, DE) and standardized to working solution concentrations of 50 ng/µl.

The polymerase chain reaction (PCR) was performed using a modified version of the Lutz-Carrillo *et al*. (2006) protocol. Seven microsatellite primer sets were divided into two multiplex reactions, MPX1 and MPX2, based on their annealing temperatures. MPX1 reactions consisted of the forward and reverse primers needed to amplify the loci *Lma*12, *Mdo*7, and *Msa*21. MPX2 reactions consisted of the forward and reverse primers to amplify *Mdo*3, *Mdo*6, *Msa*13, and *Msa*29 (Colbourne *et al*. 1996, DeWoody et al. 2000, Malloy et al. 2000).

Forward primers of each set were tagged using fluorescent markers (Integrated DNA Technologies©, Coralville, IA). Each multiplex reaction included 50 ng DNA, 0.4 uM each of upstream and downstream microsatellite primers, 0.2 mM of dNTPs, 1X REDTaq® PCR reaction buffer, and 0.5 U REDTaq® polymerase (Sigma-Aldrich, St. Louis, MO), and sterile water to a total volume of 10 µl. Each multiplex PCR reaction was performed using a Bio-Rad iCycler© Version 4.006 (Hercules, CA) with the conditions as follows: for MPX1, denature at 94 °C for 1.5 min, then 31 cycles of 94 °C for 30 s to denature, 47 °C for 30 s to anneal and 72 °C for 30 s for extension; for MPX2, denature at 94 °C for 1.5 min, then 32 cycles of 94 °C for 30 s to denature, 60 °C for 30 s to anneal and 72 °C

for 1 min for extension.

The size of amplicons from the PCR reactions was determined by capillary electrophoresis using a Beckman-Coulter CEQ8000 system (Beckman-Coulter, Inc., Fullerton CA), and the CEQ fragment analysis software version 7.0. The results were then manually verified and recorded.

Statistical Analysis

Control samples from 3 Arkansas fish hatcheries (FB, Andrew H. Hulsey State Fish Hatchery, n = 83; LMB, Joe Hogan State Fish Hatchery, n = 32; and LMB, William H. Donham State Fish Hatchery, n = 43) were previously scored for each microsatellite locus (Allen et al. 2009); alleles were designated as exclusively LMB, exclusively FB, or shared between species. The program GeneAlEx (Peakall and Smouse 2006) was used to determine allelic frequencies for each locus for two bass populations, Cane Creek and Bull Creek, and for the entire data set.

The software program STRUCTURE 2.3 (Pritchard et al. 2000) was first used with an admixture model with correlated allele frequencies and default settings to establish pure species and their intergrades (50,000 burn-in steps; 500,000 Monte Carlo/Monte Carlo steps). The result of this analysis was a statistical value for the individual admixture proportion (q) of each individual and for the population as a whole. Consistent with Allen et al. (2009), the number of clusters (k) was identified as 2, with values ranging from 0.0 (FLMB) to 1.0 (NLMB. The degree of admixture for Cane Creek (n = 18) and Bull Creek (n = 23) bass, the entire data set, and the hatchery controls were identified during a single run. The resultant output provided an individual admixture proportion (*q*) on a scale, 0.000 - 1.000, where 1.000 corresponds with LMB and 0.000 corresponds to FB for each individual. The *q*-value was used to classify individuals as to species following the 0.050 thresholds given by Schwartz and Beheregaray (2008). Pure LMB had *q*-values greater than or equal to 0.950, whereas pure FB had *q*-values less than or equal to 0.050. All broodstock controls were within this threshold and distinguished as pure species (NLMB: Joe Hogan Hatchery, n = 32; q = 0.996; William Donham Hatchery, n = 42; q = 0.989; FLMB: Andrew Hulsey Hatchery, n = 83; q = 0.002).

Results

Genetic Diversity

Over the entire data set, all 7 loci were polymorphic, though not for each population. The total number of alleles per locus ranged from 2 (Msa21) to

Table 2. Number of alleles per locus with allele sizes (base pairs) in parentheses for hatchery controls, the entire data set and Bull Creek and Cane Creek collection sites. The other sites are combined due to small sample sizes.

Locus	**Andrew Hulsey** *n* = 83	**Joe Hogan** *n* = 32	**William Donham** *n* = 43	**Bull Creek** *n* = 23	**Cane Creek** *n* = 18	**Other stream samples** *n* = 32	**Total NE AR** *n* = 73
*Lma*12	4	4	5	3	2	4	4 (103-121)
*Mdo*7	3	6	6	8	5	6	8 (164-183)
*Msa*21	5	1	1	1	2	2	2 (199-203)
*Mdo*3	4	9	5	7	7	9	10 (101-123)
*Mdo*6	2	2	2	2	2	4	4 (142-154)
*Msa*13	3	4	4	4	4	5	5 (188-196)
*Msa*29	3	5	6	5	6	8	9 (260-279)

10 (Mdo3), with a total of 42 alleles for all loci from the collected samples (Table 2). The fish from Bull Creek and Cane Creek had a total of 30 and 28 alleles for the 7 loci, respectively. The remaining stream samples had a total of 38 alleles and each of the 7 loci were polymorphic (Table 2). Cane Creek was polymorphic over all 7 loci, whereas Bull Creek was monomorphic at the Msa21 locus. The control samples from the Joe Hogan and William Donham Fish Hatcheries were monomorphic at the Msa21 locus, whereas the Andrew Hulsey Fish Hatchery samples were polymorphic at all 7 loci.

Nineteen alleles were classified as only FB alleles and another four alleles were designated as shared between FB and LMB. At the *Mdo*3 and *Mdo*6 loci, there were two alleles that were not found in any of the hatchery control samples, nor previously found in more than 5000 reservoir bass studied (Johnson *unpublished*).

Bass Genotypes

FB alleles were common in bass of Cane Creek (x = 0.158) and Des Arc (x = 0.119), yet were rare for other stream samples (x = 0.032; Table 3). Most stream bass populations had 1-2 percent FB alleles, although it must be reiterated that sample sizes are small. Trends were similar for alleles shared between species. Greater than one-fourth of the alleles for bass from Cane Creek were either exclusive to FB or shared between species. Most alleles contributing to the totals for other stream bass were shared between species rather than being exclusively FB alleles. Consistent with allele frequency data, bass from streams sampled had high average *q*-values, whereas Cane Creek had a lower *q*-value. The *q*-values of all bass ranged between 0.585 and 0.998, with an overall average *q*-value of 0.971 (Table 3). Of the 73 samples, there were only 7 fish that were not classified as pure LMB (*q*-value < 0.950). Six of these hybrid individuals were collected from Cane Creek, and had *q*-values ranging from 0.585 to 0.907. The other hybrid fish was collected from Des Arc Bayou and had a *q*-value of 0.932 (Table 3).

Table 3. Average allele frequencies and admixture proportions (q-values) and 1-q for bass from stream samples. The Mixed column represents alleles shared by both species.

Population	**LMB**	**FB**	**Mixed**	**FB + Mixed**	**LMB *q***	**FB 1-*q***
Collection sites:						
Martins Creek	0.930	0.016	0.054	0.070	0.992	0.008
Higgin. Creek	0.875	0.018	0.107	0.125	0.991	0.009
Des Arc Bayou	0.810	0.119	0.071	0.190	0.970	0.030
Bull Creek	0.903	0.022	0.075	0.097	0.996	0.004
Cane Creek	0.737	0.158	0.105	0.263	0.907	0.093
Spring River	0.871	0.029	0.100	0.129	0.991	0.009
Big Creek	0.909	0.020	0.071	0.091	0.992	0.008
Swan Pond	0.977	0.000	0.023	0.023	0.998	0.002
Totals	0.876	0.048	0.076	0.124	0.971	0.029

Discussion

Florida bass alleles were present, yet uncommon, in bass of northeast Arkansas stream systems other than for Cane Creek, The presence of FB alleles was unexpected, as the AGFC does not currently stock FB in most northeast Arkansas waters. The high incidence of FB alleles in Cane Creek is alarming. This creek flows proximal to several privately-owned farm ponds. One landowner adjacent to Cane Creek stated that he regularly pumps water into and out of the creek from his farm pond (*pers. commun.*). Further, overland flooding of this creek regularly occurs, creating linkages of pond and stream waters. Flooding events contributing to the contamination of bass stocks has been demonstrated in other systems (Maceina et al. 1988, Gelwick et al. 1995, Allen et al. 2009). Immigration of fish into streams would readily occur during those times. The nearest AGFC public lake stocked with FB is Lake Greenlee located east of Brinkley and is 65 km linearly from Cane Creek, and each is part of the White River Drainage. Lake Greenlee serves as a second potential source of FB alleles for bass in Cane Creek, yet has no connection to a stream system.

Several other studies have identified FB alleles in waters adjacent to locations where state agencies stock FB. For example, in Oklahoma Gelwick et al. (1995) used allozyme analysis at 5 diagnostic loci in order to determine the extent of introgression of FB in 21 stream populations through much of the state. Four percent of individuals sampled and 11% of the sites sampled showed the presence of FB alleles. They also found FB alleles in bass of the Arkansas River basin, which could have resulted in FB alleles being introduced to Arkansas bass; none of the streams of this study are part of the Arkansas River drainage system, however. Gelwick et al. (1995) noted that the highest concentration of FB alleles, up to 18% in one stream, was in southeastern Oklahoma where the Oklahoma Department of Wildlife Conservation regularly stocks FB. They also reported FB allele frequencies up to 2.5% in the northwest part of the state where there were historical stockings of FB. Similar to this study, they hypothesized that events of overland flooding could likely be the cause for FB introductions from farm ponds.

Ray et al. (2012) conducted a more focused study of bass populations in Texas streams using mitochondrial DNA analysis. They found high levels of fish (26%) possessing FB haplotypes. They identified FB alleles in their farthest sampling location 80 km away from the closest documented stocking site, indicating high dispersal potential of stocked bass and their progeny.

Johnson and Fulton (1999) noted FB allele persistence in Lake Ashbaugh of northeast Arkansas following regular stockings of LMB. Lake Ashbaugh was initially stocked with FB when the lake was constructed in 1981 and thereafter with LMB. Using allozyme analysis, Johnson and Fulton (1999) determined that 62% of the bass sampled from Lake Ashbaugh contained FB alleles and that roughly one-fourth of the alleles were FB alleles.

It is also possible that low levels of FB alleles are being unintentionally directly introduced throughout the state by the AGFC. Although the AGFC regularly screens their FB hatchery to maintain genetic purity of broodstock, they do not screen their LMB hatcheries for the presence of FB alleles. Historically, FB alleles were found in two LMB hatcheries (Hogan and Donham) by Johnson and Staley (2001), although, more recent analysis did not identify FB alleles in those hatcheries (Allen et al. 2009); researchers have identified contamination of FB broodstock prior to establishing genetic testing of those broodstock (Maceina et al. 1988, Gilliland and Whittaker 1989, Barthel et al. 2010). Periodic testing of native broodstock should be performed in order to reduce the chances of unintentionally introducing exotic alleles.

Lastly, anglers have been identified as transporting fish from one waterbody to another (Rahel 2004, 2010). Locally, in 2012, the AGFC had to disqualify what would have been the state record LMB (7.4 kg), a record held for 37 years, due to a lack of angler licensure. This bass was genetically confirmed using microsatellite analysis to be a FLMB in a reservoir not previously stocked with FLMB, but was within 2 km of a reservoir that had been stocked with FLMB (Lamothe and Johnson 2013).

The introduction of non-native FB into extant LMB populations offers the possibility of introducing maladaptive genes into those populations. These introductions could have long term consequences for generations due to the persistence of those alleles (Philipp 1991). Although outbreeding depression has not been observed in Arkansas bass populations resulting from hybridization (Johnson and Fulton 2004 Allen et al. 2009, Lamothe 2013), it has been observed for bass in more northern latitudes (Philipp and Claussen 1995, Philipp et al. 2002) and in laboratory settings (Cooke et al. 2001, Cooke and Philipp 2006, Goldberg et al. 2005).

Summary

While this represents a small sample of northeast Arkansas streams, the presence of FB alleles in bass remote from AGFC stockings should pose concern. Introductions of FB, whether intentional or accidental, into existing native LMB populations may persist for long periods of time. As it is unknown at this time whether fitness is lowered in Arkansas bass as a result of FB alleles, caution should prevail. Stream systems are open and continuous, so that alleles introduced in one area can be moved within these systems. Short-term goals of providing larger bass to anglers must be tempered with the potential of long-term consequences of introducing alleles of unknown impact. Further, we recommend that a larger analysis of bass within Arkansas stream systems be conducted to identify the extent of introductions of FB alleles into those systems.

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