Genetic Characterization of the Savannah and Pee Dee River Populations of Robust Redhorse (*Moxostoma robustum*) with Conservation Implications

Author(s): T. L. Darden and C. M. Tarpey
Source: Copeia, 2014(1):70-78. 2014.
Published By: The American Society of Ichthyologists and Herpetologists
DOI: http://dx.doi.org/10.1643/CI-12-089
URL: http://www.bioone.org/doi/full/10.1643/CI-12-089

Genetic Characterization of the Savannah and Pee Dee River Populations of Robust Redhorse (*Moxostoma robustum*) with Conservation Implications

T. L. Darden[1] and C. M. Tarpey[1]

Robust Redhorse (*Moxostoma robustum*) is a deep-dwelling catostomid that lives in southeastern rivers of the United States. After being lost to science for about 100 years, *M. robustum* was rediscovered in the 1980s by the Georgia DNR and is a federal species of special concern. Our goal was to genetically characterize populations of *M. robustum* in the Savannah and Pee Dee rivers based on genetic diversity and population structure using a suite of ten microsatellites. Substantial levels of population structure were detected between rivers ($R_{ST} = 0.308$), with high genetic diversity (0.81–0.87) and low inbreeding in both systems. Long-term estimates of effective population sizes were very high for both systems, but contemporary estimates were substantially lower, with the Pee Dee River estimate being of concern from a conservation standpoint. Their long life span and overlapping generations result in a high potential for across-year-class spawning and are likely contributing to the maintenance of high genetic diversity in light of their decreased effective population sizes. Although statistical modeling indicates a faster loss of allelic richness as compared to heterozygosity, results are congruent as the Savannah River population is forecast to maintain 90% of its genetic diversity for >200 years and the Pee Dee for ~65–120 years. These results support the current management view of each river as evolutionarily significant units, provide an important genetic baseline for future monitoring of populations of *M. robustum*, and build an excellent foundational tool for any future population restoration activities.

RIVER ecosystems in the southeastern United States have undergone vast environmental changes in the last century as a result of a variety of habitat alterations, having implications for the management of the fauna they support. The freshwater fish assemblages in the southeastern United States are the most diverse in North America, but this region exhibits some of the highest extinction rates in the world (Jelks et al., 2008). Because thorough knowledge of life history and population information are essential to the formation and implementation of an effective management plan, imperiled species for which little is known can be especially susceptible to the harmful effects of environmental changes. Often attention is focused on economically important fish with little incentive to study non-game fish until they have been identified as imperiled or endangered (Cooke et al., 2005). The family Catostomidae is one such group of fishes containing many species that are facing serious conservation challenges (Cooke et al., 2005).

The Robust Redhorse (*Moxostoma robustum*; Cope, 1870) is a large, deep water, long-lived catostomid, which reaches a maximum length of 760 mm (Wirgin et al., 2001) and lives up to 27 years (Hendricks, 1998). After being described, individuals of *M. robustum* escaped observation or mention in the literature for over 100 years. The fish was rediscovered when a specimen was collected in the Savannah River, SC–GA, in 1980, and identification as *M. robustum* was confirmed with the collection of another specimen from the Pee Dee River, NC in 1985 and five more from the Oconee River, GA in 1991 (Bryant et al., 1996; Hendricks, 1998). Little is known about the distribution and size of historical populations, though it is believed that the range historically included many of the rivers along the southeastern United States coast from the Pee Dee River, NC to the Altamaha River, GA (Bryant et al., 1996; Wirgin et al., 2001). Extensive sampling has since identified populations in the Oconee and Ocmulgee rivers of the Altamaha drainage, Savannah River, and Pee Dee River.

Because of their limited range and the low number of fish collected during extensive surveys, *M. robustum* is considered an imperiled species (Straight and Freeman, 2002). *Moxostoma robustum* is not a federally protected species, although it is recognized as a species of concern and listed as state endangered in Georgia (Wirgin et al., 2001; Straight and Freeman, 2002). To ensure proper monitoring and management of this species, the Robust Redhorse Conservation Committee (RRCC) was formed in 1995 to implement conservation measures, protect populations, and re-establish the species in its historic range within Georgia, South Carolina, and North Carolina (Bryant et al., 1996; Wirgin et al., 2001; Fiumera et al., 2004).

The RRCC recommends stock enhancement programs to restore and re-establish *M. robustum* in rivers within their historic range with suitable habitat (RRCC, 2002). Current efforts to re-establish populations include a stocking program in the Santee River, SC using broodstock from the Savannah River (L. Quattro, SCDNR, pers. comm.). The program employs intensive population rebuilding strategies where adults are captured in the Savannah River, strip-spawned in the field, and released. Fertilized eggs are transported to hatchery production facilities for grow out and offspring are later released into the Santee River. Genetic characterization of the population from which the broodstock are selected is crucial to the Responsible Approach of stocking as an enhancement tool (Blankenship and Leber, 1995). Although ensuring the viability and recovery of an endangered species like *M. robustum* involves a genetic requirement to assess recovery or re-establishment of a population, there is little information about prior effective population sizes or genetic composition of extant populations of *M. robustum* (Grabowski and Jennings, 2009). Past research on *M. robustum* has included evaluation of supplemental breeding programs (Fiumera et al., 2004) and genetic diversity using mitochondrial DNA (Wirgin et al., 2001).

The goal of our project was to develop baseline genetic data for the Savannah and Pee Dee river populations of *M.*

[1] South Carolina Department of Natural Resources, Marine Resources Research Institute, Hollings Marine Laboratory, 331 Fort Johnson Road, Charleston, South Carolina 29412; E-mail: (TLD) dardent@dnr.sc.gov. Send reprint requests to TLD.
Submitted: 20 July 2012. Accepted: 14 September 2013. Associate Editor: D. Buth.
 DOI: 10.1643/CI-12-089

Table 1. Number of samples of *M. robustum* genotyped from the Pee Dee and Savannah rivers by collection year. Samples represent all samples available from the SCDNR and North Carolina Museum of Natural Science collections during the study period.

Year	Pee Dee River	Savannah River
2001	2	–
2002	1	–
2003	–	1
2004	1	53
2005	6	75
2006	15	–
2007	9	–
2008	14	25
2009	8	21
2010	–	–
2011	–	20
Total	56	195

robustum based on archived genetic samples (2001–2010) using a suite of highly polymorphic microsatellite markers. Basic genetic characterization of each population included calculating allelic and genotypic diversities, estimating effective population size (N_e), evaluating the temporal stability and occurrence of recent bottlenecks, and simulating the retention of genetic diversity. We also evaluated the capabilities of the marker suite for future parentage and identity analyses. The resulting genetic data build the foundation for future evaluation of ongoing re-establishment of *M. robustum* within other river systems, such as the Santee River, provide the ability to monitor populations through time, and allow for the study of influences of environmental changes in either of these systems. Establishing baseline genetic data is one of the priorities of the RRCC and will fill a data gap with information necessary for understanding the genetic composition of populations of *M. robustum* within these two river systems (RRCC, 2002).

MATERIALS AND METHODS

Sample collection.—We obtained a total of 283 fin clips from individuals of *M. robustum* representing all collections over the past ten years archived with the South Carolina Department of Natural Resources (SCDNR) and the North Carolina Museum of Natural Sciences. Samples were collected from May 2001 to June 2011 from the Savannah and Pee Dee rivers in South Carolina and North Carolina (Table 1). All fin clip samples were collected from adult *M. robustum* that were PIT-tagged to detect recaptures and eliminate duplicate analyses of samples. We genotyped a total of 251 samples, excluding 32 that were determined to be recaptures within the Savannah River based on PIT tags. Of the 251 genotyped individuals, 56 were collected in the Pee Dee River and 195 from the Savannah River.

Microsatellite genotyping.—Samples were stored in either 95% ethanol (EtOH) or sarcosyl-urea (1% sarcosyl, 8 M urea, 20 mM sodium phosphate, 1 mL EDTA of pH 6.8). Wizard SV Genomic DNA Purification System kits (Promega Corp., Madison, WI) were used to isolate DNA from the EtOH preserved fin clip tissues. A metal bead isolation protocol was used to isolate DNA from samples stored in sarcosyl-urea. We quantified a subset of samples using a Quant-iT dsDNA BR Assay Kit (Invitrogen Corp., Carlsbad, CA) and a Qubit fluorometer (Invitrogen Corp., Carlsbad, CA) to ensure that a sufficient quantity of DNA was being isolated.

Following DNA isolation, we amplified all samples at ten microsatellite loci in three multiplexed polymerase chain reactions (PCR; Table 2). The loci were originally developed by Lippe et al. (2004) for the Copper Redhorse (*Moxostoma hubbsi)* and were multiplexed by Moyer et al. (2009) for use with the undescribed ''Sicklefin Redhorse,'' *Moxostoma* sp. Our optimized multiplex reactions contained: dH_2O, 1X HotMaster PCR Buffer (5 Primer Inc., Gaithersburg, MD), 0.38 mM each dNTP, 2.0 mM $MgCl_2$, 0.25 μM forward primers, 0.25 μM reverse primers, 0.03 U HotMaster Taq DNA polymerase (5 Primer Inc., Gaithersburg, MD), and 1 μL sample DNA (isolations yielded between 8–250 ng/μL). Specific ratios and fluorescent Well-RED dye labels (SIGMA-Aldrich, St. Louis, MO) varied for each primer pair (Table 1). We performed all amplifications in 11 μL reaction volumes in BIORAD iCyclers (Bio-Rad Laboratories, Hercules, CA) using the following touchdown protocol: initial denaturing at 94°C for 10 min (1×); 33 cycles of denaturing at 94°C for 30 sec, annealing for 1 min, and extending at 68°C for 1 min; followed by a final extension at 68°C for 30 min (1×). The annealing temperature initiated at 56°C and decreased 0.2°C with every cycle reaching a final annealing temperature of 49.6°C.

PCR products were separated and visualized on a Beckman CEQ 8000 Genetic Analysis System (Beckman Coulter, Brea, CA) with a 400 bp size standard. We scored chromatograms using CEQ Fragment Analysis Software (Beckman Coulter, Brea, CA) with a frag 3/PA version 1 analysis algorithm to determine allele size. Two independent readers scored each

Table 2. Multiplex group, PCR primer ratio, fluorescent label (dye), and repeat motif for ten loci used to genotype *M. robustum.*

Multiplex group	Locus	Primer ratio	Well-RED dye	Motif
1	*Mohu-lav268*	1.5	D2	$[GACA]_5[GGCA]_2GGTATA[TAGA]_{23}$
	Mohu-lav294	3.0	D3	$[GATA]_{18}$
	Mohu-lav296	0.5	D4	$[ATCT]_{21}[TTCT]_{11}$
2	*Mohu-lav306*	1.2	D2	$[CAGA]_7[TAGA]_5[CAGA]_7[TAGA]_{19}$
	Mohu-lav321	3.0	D3	$[CTAT]_2CTGT[CTAT]_{10}CTGT[CTAT]_{12}[CTGTCTAT]_2CTGT[CTAT]_3$
	Mohu-lav336	0.8	D4	$[GATA]_{15}[GAGAGATA]_3$
3	*Mohu-lav212*	3.0	D2	$[GATA]_{19}$
	Mohu-lav213	3.0	D3	$[ATCT]_{17}$
	Mohu-lav286	1.5	D4	$[ATCT]_{17}$
	Mohu-lav203	0.3	D4	$[TGAG]_{15}[GATA]_{10}$

chromatogram for quality assurance. If agreement between scores could not be reconciled in person, scores were not included in further analyses.

Recapture analysis.—We determined the power of the loci suite to differentiate individuals by calculating an identity non-exclusion probability in CERVUS 3.0.3 (Kalinowski et al., 2007), which measures the probability that a set of markers will not be able to distinguish between individuals. The identity non-exclusion probability was calculated to be substantially less than 0.0001% for both the Pee Dee River (1.2×10^{-11}) and Savannah River (9.1×10^{-15}). Given the low probability that the markers would misidentify individuals, a recapture analysis was performed in CERVUS to verify no additional recaptured individuals were present in our sample collections.

Marker validation.—We tested all ten loci in each river for adherence to Hardy Weinberg equilibrium (HWE), linkage disequilibrium (LD, Savannah River population only), and the potential for null alleles. We evaluated departures from HWE and LD using the program GENEPOP 4.1 (Raymond and Rousset, 1995) with input parameters set to 100 batches of 5,000 iterations per batch with a 10,000 step burn-in. The frequency of null alleles was calculated for each locus using CERVUS. Additionally, we estimated the power of the loci suite to correctly identify parentage of fish based on allele frequencies of all samples within each river. A maximum likelihood parentage approach was used as implemented in CERVUS to provide a statistical evaluation of parentage capabilities.

Temporal comparisons.—To determine the validity of combining samples across collection years, we evaluated the temporal genetic stability within the Savannah River. Only the 2004 and 2005 collection years had sufficient samples for a comparison of temporal genetic difference. To assess the degree of temporal genetic variation within the Savannah River, pair-wise comparisons of R_{ST} were performed in ARLEQUIN 3.11 (Excoffier et al., 2005). Exact tests for genic distributions in the same collection years were also performed using GENEPOP.

Genetic characterization of populations.—To estimate the degree of gene flow and population structure between the Savannah and Pee Dee rivers, we evaluated the spatial genetic variation between the rivers with pair-wise comparisons of R_{ST} in ARLEQUIN and exact tests for genic distribution performed in GENEPOP as described for the temporal comparisons. We calculated basic molecular diversity indices for each locus using ARLEQUIN, including allelic size range (R), allelic richness (A), observed heterozygosity (H_O), and gene diversity (H_E, Nei, 1987). Private allelic richness ($_pA$) was calculated using the program HP-Rare (Kalinowski, 2005). Inbreeding coefficients (F_{IS}, Weir and Cockerham, 1984) were calculated using GENEPOP.

We estimated effective population size (N_e) from the Pee Dee and the Savannah rivers using both long-term as well as contemporary methods. Long-term estimates were calculated using heterozygosity-based methods (Ohta and Kimura, 1973) with a step-wise mutation model (SMM) as it is likely the most appropriate algorithm for microsatellite data (Luikart and Cornuet, 1998). The SMM predicts that at mutation-drift equilibrium, N_e is represented by $[(1/1-H_E)^2-1]/8\mu$, and in these model estimates the most commonly used mutation rate in fishes was used ($\mu = 5\times10^{-4}$; Estoup and Angers, 1998). Contemporary (parental generation) estimates of N_{eb} (effective number of breeding adults) were estimated using the single-sample programs LDNe 1.31 (Waples and Do, 2008) and COLONY 2.0.0.1 (Wang, 2004; Wang and Santure, 2009). As data from multiple cohorts were evaluated, in reality these contemporary estimates represent something in between N_e and N_{eb}. Genetic drift generates non-random associations among unlinked loci; LDNe analyzes this linkage disequilibrium between a set of loci to determine contemporary N_{eb} for a single time point. Minimal allele frequencies for inclusion were set at default values (0.01, 0.02, and 0.05) and a random mating model was assumed. COLONY also calculates N_{eb} for a single time point based on sibship relationships among individuals. A polygamous breeding system was assumed for both males and females. One medium-length run was performed using the pair-likelihood method with no priors. Default settings were used for the other input parameters (core/CPU = 1, random seed = 1234). Marker error rates were set at 0 for the allelic dropout rate and 0.0005 for the mutation rate, and allele frequencies were considered ''unknown.'' All other input parameters (# mothers/fathers, etc.) were set at 0.

We used the software program BOTTLENECK 1.2.02 (Cornuet and Luikart, 1996; Piry et al., 1999) to test for recent ($2N_e$–$4N_e$ generations) reductions in population size in *M. robustum* in the Pee Dee and Savannah rivers through the evaluation of a population's heterozygosity excess based on that expected at mutation-drift equilibrium. Microsatellite markers generally evolve under a model more similar to SMM than an infinite alleles model (Luikart and Cornuet, 1998), with a two phase mutational model (TPM) with 95% single step mutations and 12% variance among mutational steps recommended for dinucleotide repeats (Piry et al., 1999). However, as all loci evaluated for these samples of *M. robustum* are tetranucleotide repeats, which are expected to more strictly follow the SMM (Shriver et al., 1993), only the TPM and the SMM were evaluated. Although all three tests of population bottlenecks were performed, interpretation was based on the Wilcoxon signed rank test as the sign test suffers from low statistical power and the standardized differences test requires at least 20 polymorphic loci for robust results (Cornuet and Luikart, 1996). The M ratio test of Garza and Williamson (2001) was also used to identify population reduction(s) with M < critical M being indicative of populations that have undergone a recent bottleneck. Critical_M software (http://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298) was used to determine a study-specific critical M (Mc) using the Pee Dee River sample size to provide a conservative estimate. A pre-bottleneck theta of 12.786, 2.8 average size of large mutations (Garza and Williamson, 2001), and 5% of mutations introducing changes greater than one repeat unit (Piry et al., 1999) produced Mc = 0.80.

Recommended conservation goals often include maintaining 90% of current genetic diversity over a 100-year period (Frankham et al., 2002). We used BOTTLESIM v2.6 (Kuo and Janzen, 2003) to simulate the evolution of genetic diversity over time for *M. robustum* taking into account their long life span and overlapping generations to estimate the sustainable population size needed to meet this conservation goal. All simulations were conducted for a 200-year period with randomly assigned initial age structures, stable

population sizes, dioecious reproduction, random mating, 1:1 sex ratio, 27-year life span, and an age at maturity of five years, with 1000 iterations. Sex ratios of *M. robustum* are not known; however, exploratory simulations with ratios from 1:1 to 1:4 only showed minor influence on results (H_o: <5% and ${}_oA$: <12% after 100 years). Multiple population sizes were used for the simulations in each river system, but were based on the calculated contemporary N_e estimates. As the contemporary estimates are realistically somewhere in between the effective number of breeders and the true effective population size, we modeled population sizes ranging from the worst case scenario (N_{eb}s are the true population sizes) to $\sim 5N_{eb}$.

RESULTS

Recapture analysis.—The recapture analysis identified six additional recaptures beyond those detected in the field via PIT tags, one from the Pee Dee River and five from the Savannah River. Along with matching genotypes, recaptures were verified with PIT tag identification numbers when available, as well as collection locations and other identification parameters. Length was consistent with expected growth based on time between captures and all six recaptures were male. All identified recaptures were removed from further analyses, and one sample from the Savannah River was excluded due to contamination, resulting in 55 individuals from the Pee Dee River and 189 from the Savannah River.

Marker validation.—After Bonferroni correction for multiple comparisons (Rice, 1989), all microsatellite markers were found to be in HWE ($P \geq 0.005$) except for *Mohu-lav306* in both systems and *Mohu-lav286* in the Pee Dee River (Table 3). Linkage disequilibrium examinations for the Savannah River detected three pairs (*Mohu-lav294*×*Mohu-lav306*, *Mohu-lav294*×*Mohu-lav212*, and *Mohu-lav203*×*Mohu-lav286*) to be linked ($P \leq 0.0011$). The potential frequency of null alleles was low for all loci except *Mohu-lav306* (Table 3). Due to consistent issues observed with HWE and LD, locus *Mohu-lav306* was removed from all further analysis. The average parent-pair non-exclusion probabilities for the loci suite were 4.3×10^{-10} and 2.1×10^{-7} for the Savannah and Pee Dee rivers, respectively. These estimations indicate very low probabilities of incorrectly identifying parentage throughout the South Carolina–North Carolina range of *M. robustum*.

Temporal comparisons.—No significant temporal genetic differences were found within the Savannah River between 2004 and 2005 using nine microsatellite loci. Exact tests for genic divergence ($\chi^2 = 10.58$, $P = 0.911$) and pair-wise comparisons of R_{ST} ($R_{ST} = 0.007$, $P = 0.083$) were not significant. Therefore, the lack of significant genetic divergence between years validates combining samples across capture years within rivers for the spatial comparison.

Genetic characterization of populations.—Significant spatial genetic divergence was detected between the Savannah and Pee Dee rivers. Pair-wise comparisons of R_{ST} were significant ($R_{ST} = 0.308$, $P < 0.001$) and exact tests indicated that the allelic frequency distributions significantly differed between the two river systems ($\chi^2 = \infty$, $P < 0.001$), indicating a high level of genetic divergence and reproductive isolation between the two rivers. Allele frequency distribution differences occurred in allelic range as well as the presence of private alleles in both systems. The degree of divergence varied among loci; differences in the frequency distribution of the allelic ranges and private alleles present between the two rivers was most evident in loci *Mohu-lav296, Mohu-lav321, Mohu-lav212*, and *Mohu-lav286* (Fig. 1). All project allele frequency data is available from the authors by request.

Basic molecular diversity indices showed high levels of genetic diversity for both individual loci and all loci taken together. The average allelic range was 12.6 (±5.2) for the Pee Dee River with an average allelic richness of 9.38 (±2.16), and an average private allelic richness of 2.81 (±1.59). The average allelic range was 15.7 (±5.1) for the Savannah River with an allelic richness of 13.0 (±2.85), and average private allelic richness of 6.46 (±3.73). Allelic statistics for both rivers were based on 46 samples following the rarified approach. The overall average observed heterozygosity was high for both the Savannah River (0.891±0.046) and Pee Dee River (0.789±0.083). Overall inbreeding coefficients were low for both the Pee Dee River (0.0212) and Savannah River (−0.0247).

Estimates of long term N_e based on the SMM were approximately 2–2.5 orders of magnitude higher than contemporary estimates of N_{eb} for both river systems (Table 4). In all cases, estimates were lower in the Pee Dee River as compared to the Savannah River. All estimates of contemporary N_{eb} resulted in small confidence intervals and similarity among methods. All minimal inclusion allele frequencies produced similar estimates of N_{eb} in LDNe with small overlapping confidence intervals, so only results from the intermediate frequency criteria are reported. The resulting LDNe estimates are not expected to be underestimated due to bias of low sample size as all estimates are lower than the sample size (England et al., 2006) and LDNe incorporates a bias correction from Waples (2006). However, to evaluate the potential influence of sample size on estimation of N_{eb}, a random subsampling of the Savannah River samples (equal to the Pee Dee River size) was used for a second estimation. The adjusted Savannah River estimated N_{eb} was the same as the original COLONY estimate, and similar to the original LDNe estimate (134; range 90.8–238.6). Therefore, substantial differences across all estimates of N_e are evident between the Savannah and Pee Dee river populations of *M. robustum*.

No evidence of recent population bottlenecks was detected in either the Savannah or Pee Dee rivers. Wilcoxon signed rank tests for heterozygosity excess were not significant under either the SMM or TPM (Savannah: SMM $P = 0.632$, TPM $P = 0.285$; Pee Dee: SMM $P = 0.990$, TPM $P = 0.752$), and results were consistent across all three tests. No evidence of a population bottleneck was detected from the M ratio test for the Savannah River, with the average ratio (M = 0.88) above the critical threshold of 0.80. However, a recent population bottleneck was detected with this test in the Pee Dee River, with the average ratio (M = 0.77) below the critical value.

Simulations of populations of *M. robustum* suggest that declines in allelic richness will occur much more rapidly than declines in heterozygosity (H_o; Fig. 2) as expected, since recombination during reproductive events is required for losses in diversity to occur. For the Savannah River population, even with the worst case scenario of N_{eb} representing the approximate true population size, 97.3% of the observed heterozygosity would be retained after 100 years; however, only 81.5% of allelic richness would be retained. The Savannah River population would need to remain above ~300 individuals to meet the recommended

Table 3. Microsatellite locus statistics for sample collections of *M. robustum* from the Savannah River, SC, and Pee Dee River, NC.

Locus		Savannah	Pee Dee	Locus		Savannah	Pee Dee
Mohu-lav268	*n*	187	55	*Mohu-lav336*	*n*	183	51
	R	169–213	173–209		*R*	183–243	167–239
	A	10.6	8.9		*A*	13.6	12.8
	$_PA$	3.1	1.4		$_PA$	3.8	3.0
	H_O	0.904	0.745		H_O	0.902	0.882
	H_E	0.873	0.804		H_E	0.905	0.859
	F_{IS}	−0.035	0.073		F_{IS}	0.004	−0.027
	P	0.720	0.234		*P*	0.537	0.681
	Null	−0.019	0.031		Null	0.000	−0.018
Mohu-lav294	*n*	167	46	*Mohu-lav203*	*n*	170	54
	R	220–292	228–272		*R*	86–130	94–130
	A	15.0	11.0		A	9.0	8.0
	$_PA$	6.2	2.2		$_PA$	2.4	1.4
	H_O	0.916	0.870		H_O	0.841	0.759
	H_E	0.8627	0.882		H_E	0.834	0.7383
	F_{IS}	−0.063	0.013		F_{IS}	−0.009	−0.029
	P	0.563	0.045		*P*	0.009	0.680
	Null	−0.034	0.004		Null	−0.006	−0.013
Mohu-lav296	*n*	147	54	*Mohu-lav212*	*n*	175	55
	R	166–246	170–214		*R*	153–233	125–213
	A	14.9	10.7		*A*	13.9	11.0
	$_PA$	6.9	2.6		$_PA$	8.9	6.0
	H_O	0.898	0.759		H_O	0.909	0.927
	H_E	0.876	0.767		H_E	0.883	0.856
	F_{IS}	−0.025	0.010		F_{IS}	−0.028	−0.085
	P	0.406	0.109		*P*	0.640	0.209
	Null	−0.014	0.005		Null	−0.017	−0.044
Mohu-lav306	*n*	154	55	*Mohu-lav213*	*n*	169	52
	R	160–252	156–212		*R*	136–172	136–172
	A	14.3	7.0		*A*	8.2	7.8
	$_PA$	10.1	2.7		$_PA$	1.5	1.1
	H_O	0.545	0.764		H_O	0.793	0.750
	H_E	0.883	0.764		H_E	0.764	0.764
	F_{IS}	0.383	0.001		F_{IS}	−0.038	0.019
	P	0.000	0.001		*P*	0.427	0.184
	Null	0.235	0.007		Null	−0.032	0.012
Mohu-lav321	*n*	187	55	*Mohu-lav286*	*n*	178	53
	R	180–232	156–184		*R*	184–280	192–260
	A	13.8	6.0		*A*	17.1	10.7
	$_PA$	12.8	5.0		$_PA$	9.0	2.6
	H_O	0.914	0.709		H_O	0.944	0.698
	H_E	0.906	0.730		H_E	0.923	0.854
	F_{IS}	−0.009	0.029		F_{IS}	−0.023	0.184
	P	0.123	0.009		*P*	0.285	0.000
	Null	−0.007	0.008		Null	−0.013	0.092

n = locus sample size, *R* = allelic range, *A* = allelic richness, $_PA$ = private allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = inbreeding coefficient, *P* = HWE *P*-value, and Null = null allele probability.

goal of 90% of genetic diversity retained for 100 years, in which case 89.4% of allelic richness and 98.7% of H_o would be preserved. For the Pee Dee River, the worst case scenario (n∼20) would only retain 52.1% of allelic richness and 82.0% of H_o after 100 years. A population size of ∼100 individuals would allow for the retention of 79.2% of allelic richness and 96.0% of H_o for 100 years.

DISCUSSION

Overall, our genetic evaluation indicates substantial levels of genetic structure between the Savannah and Pee Dee rivers as indicated by the high R_{ST} value and distinct allele frequency distributions, with a high number of private alleles in each system. The detection of significant genetic divergence between the Savannah and Pee Dee rivers is congruent with both Wirgin's estimation that genetic divergence between these systems occurred 1.5 million years ago (DeMeo, 2001) based on mitochondrial control region sequence data (Wirgin et al., 2001) and his unpublished preliminary microsatellite evaluation of these systems. Collectively, these results support the continuation of current management of rivers as distinct population segments, as suggested by Wirgin et al. (2004), due to the

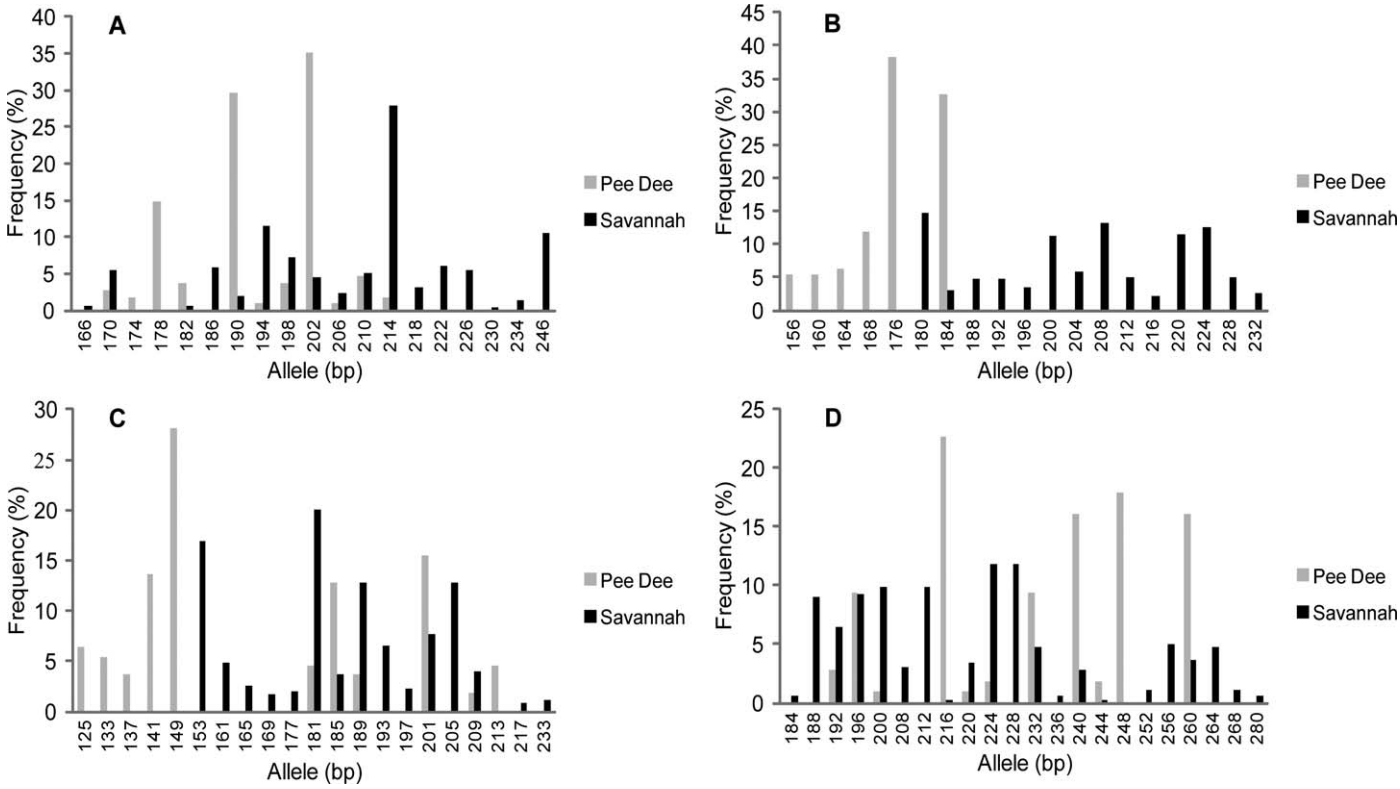

Fig. 1. Allele frequency distributions for loci *Mohu-lav296* (A), *Mohu-lav321* (B), *Mohu-lav212* (C), and *Mohu-lav286* (D) in the Pee Dee and Savannah river populations of *M. robustum*. The degree of divergence varied among all loci, but was most evident in these four. All project allele frequency data is available from the authors by request.

importance of genetic composition for future evolutionary growth of the species.

Both the Pee Dee and Savannah river populations show high within-population diversity as measured by heterozygosity as compared to the average genetic diversity measured for freshwater fishes (0.54, DeWoody and Avise, 2000), as well as low levels of inbreeding. Additionally, diversity estimates of populations of *M. robustum* from both rivers are in the upper range of those reported for other species of *Moxostoma* (0.63 in Black Redhorse, *M. duquesnei*, Reid et al., 2008a; 0.72 in River Redhorse, *M. carinatum*, Reid et al., 2008b; 0.76 in Shorthead Redhorse, *M. macrolepidotum*, Reid et al., 2008b; 0.77 in Copper Redhorse, *M. hubbsi*, Lippe et al., 2006; 0.85 in ''Sicklefin Redhorse'', *M.* sp., Moyer et al., 2009).

All estimates of N_e differed between rivers with consistently lower estimates in the Pee Dee River, which is consistent with the suggestion of a smaller population size in the Pee Dee River (Wirgin et al., 2004). However, even the higher contemporary N_{eb} estimates for the Savannah River were lower than those estimated for the endangered *M. hubbsi* ($N_{eb(LD)}$ = 480, Lippe et al., 2006). Additionally, the estimates of contemporary N_{eb} are well below the goals identified in the conservation strategy for *M. robustum* (Nichols, 2003). Similar evolutionary population trends were detected in the Pee Dee and Savannah rivers. As a recent population bottleneck was not detected in the Savannah River, the substantial reduction from long-term to contemporary estimates likely indicates a gradual decrease in the population of *M. robustum* over a very long time period. Although bottleneck detection capability is dependent on the severity of the event, the tests utilized here are highly robust with population reductions to $N_e <$ 10 (Luikart and Cornuet, 1998); therefore, the inconsistent results among tests for the Pee Dee River population suggest the low contemporary N_{eb} are the result of both a long term gradual population decline as well as a recent moderate population bottleneck. Although interpretation of genetic data suggested a similar long term trend for *M. hubbsi* (Lippe et al., 2006), it is interesting that the long term estimates of N_e were higher in both the Pee Dee and Savannah populations of *M. robustum* (*M. hubbsi* N_e: 4,476).

Similar to other species of *Moxostoma*, the life history strategy of *M. robustum* is characterized by both a long life span and overlapping generations. The maximum age

Table 4. Effective population size estimates for *M. robustum* from the Savannah and Pee Dee rivers.

	Long term N_e (range)	Contemporary N_{eb} (95% CI)		
Population	SMM	COLONY	LDNe	*n*
Savannah River	14,318 (4,236–41,788)	71 (54–98)	156.3 (133.1–186.7)	189
Pee Dee River	6,393 (3,382–17,556)	17 (9–34)	9.7 (8.3–11.1)	55

Range for long term N_e is based on estimates from individual loci; *n* = sample size.

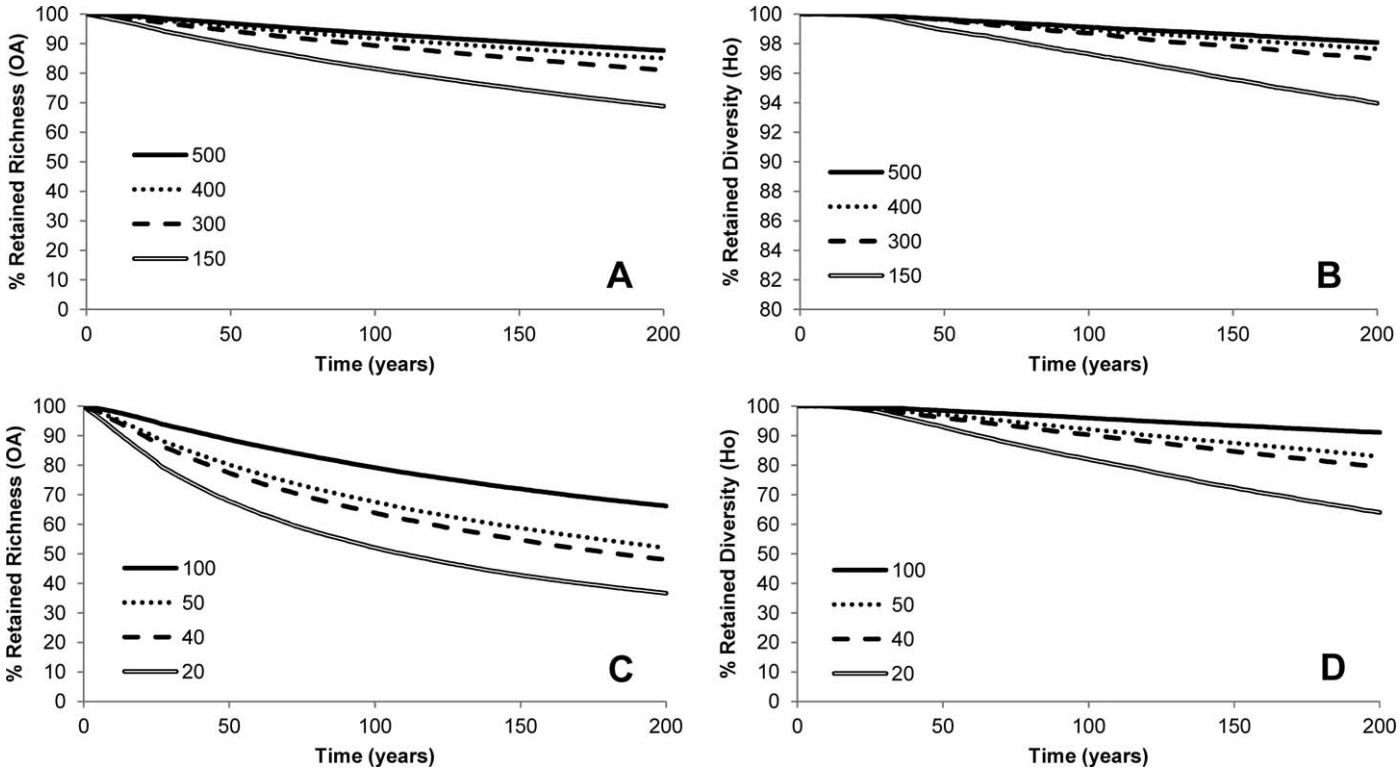

Fig. 2. Simulated loss of genetic diversity in Savannah River (top, A and B panels) and Pee Dee River (bottom, C and D panels) populations of *M. robustum* over a 200-year time period with various population sizes kept constant over time. Initial genetic diversity was the same for all within-river simulations. Evolution of allelic richness (OA) is shown on the left for each river (A and C panels) and observed heterozygosity (H_o) is shown on the right (B and D panels). Note contracted y-axis scale in panel B.

reported for *M. robustum* is 27 years with reproductive maturity occurring at 4–5 years in males and 5–6 years for females, leaving up to a 22-year reproductive window for any individuals that manage to realize the maximum possible life span (RRCC, 2002). Therefore, although the very low N_{eb} estimate in the Pee Dee River population is cause for concern from a genetic management standpoint, their long life span and overlapping generations appear to result in a high potential for across-year-class spawning and is likely contributing to the maintenance of high genetic diversity in light of their low effective population sizes. Although the high genetic diversity and lack of inbreeding indicators observed within both the Pee Dee and Savannah river populations of *M. robustum* would normally be indicative of populations in good genetic health with sufficient adaptive potential, it is unknown if the life history characteristics and current high genetic diversity of *M. robustum* will be capable of overcoming the negative effects of low effective population sizes in the long term. Therefore, conservative management approaches and continued monitoring of the populations are warranted as Kuo and Janzen (2004) have suggested that long life and overlapping generations could potentially mask accelerated rates of drift in small populations.

The forward simulations support the suggested influences of the life history characteristics of *M. robustum* on genetic diversity evolution. Similar to patterns observed in *M. hubbsi* (Lippe et al., 2006), the masking of influences is apparent by the increased loss rate of allelic richness as compared to overall diversity (i.e., heterozygosity) as well as the low loss rates of overall diversity even with small population sizes. Although a continual loss of diversity is projected for both populations, in the absence of future bottleneck(s) the Savannah River population appears to be genetically secure over a typical conservation time line, with more than 90% of its overall diversity retained over a 200-year period even in the lowest modeled population size. Although we modeled a range of population sizes for the Pee Dee River population, current (2006–2009) population size has been estimated at 38–55 (95% CI: 23–118) adults (RRCC, 2009). Using the census estimates as a guide, 90–92% of the Pee Dee River population's overall diversity will be retained over a 100-year period even with this extremely small population size, but with a 64–69% loss in allelic richness. However, with the lowest end of the confidence intervals of the census estimates (n~20), the simulations indicate a much steeper loss trajectory for the Pee Dee river population. The lack of demographic population history for *M. robustum* makes it challenging to ground truth interpretation of genetic results, and monitoring and management recommendations would greatly benefit from current (Savannah River) and ongoing (Pee Dee River) demographic population estimates. Although both populations of *M. robustum* appear (in the absence of major population perturbation) to be on a successful conservation trajectory based on standard criteria, we recommend that more aggressive criteria would be appropriate for *M. robustum* as all modeling indicates an onset of declines in allelic richness within the next 20–40 years.

The contemporary LD-based estimates of N_e for two populations of *M. robustum* provide an important benchmark for future detection of bottlenecks as it has recently been shown that LD-based estimates have the sensitivity to comparatively detect population reductions within a single

generation (Antao et al., 2011). A substantial level of knowledge and intensive effort is necessary for successful recovery of protected species, and the genetic data generated during this study provide information on an important aspect of the biology of *M. robustum* that will be valuable in the continued monitoring and management of this species.

The microsatellites used to evaluate these populations of *M. robustum* have proved to be a valuable genetic tool. In addition to genetic characterization and monitoring of these populations, the marker suite provides a statistically robust mechanism of parentage analysis and individual identification in both rivers. Evaluation of the success of any restoration effort is dependent on the ability to identify stocked individuals, which necessitates the use of a tag or mark to distinguish hatchery from wild produced fish (Blakenship and Leber, 1995). The use of molecular markers as genetic tags avoids some of the constraints and pitfalls associated with conventional tags, in that molecular markers require no additional tagging, the mark is never lost, and tag recovery is non-lethal. The archiving of production and genetic information for *M. robustum* will allow for offspring identification of future recaptures within the Santee River during re-establishment efforts. As genotyping of Santee River offspring progresses, genetic characterization of the new population can be assessed and compared to the Pee Dee and Savannah river populations.

ACKNOWLEDGMENTS

We would like to thank SCDNR freshwater fish team, particularly F. Sessions and S. Lamprecht, for access to the fin clips from *M. robustum* from the Savannah River and the North Carolina Museum of Natural Sciences, particularly W. Starnes, M. Raley, and G. Hogue, for providing access to additional Savannah River samples as well as the Pee Dee River samples. We also acknowledge the Robust Redhorse Conservation Committee Pee Dee Technical Working Group for its role in acquiring and carefully archiving tissues of *M. robustum*. We thank I. Stern for his immense lab assistance genotyping samples as well as E. Cushman, M. Jamison, D. Farrae, and M. Walker for providing invaluable assistance and comments on the project. Funding for this work was provided through SC State Wildlife Grants Program and partially supported by the U.S. National Science Foundation under REU Grant DBI-1062990. This is publication number 690 from the Marine Resources Division, South Carolina Department of Natural Resources.

LITERATURE CITED

Antao, T., A. Perez-Figueroa, and G. Luikart. 2011. Early detection of population declines: high power of genetic monitoring using effective population size estimators. Evolutionary Applications 4:144–154.

Blankenship, H., and K. Leber. 1995. A responsible approach to marine stock enhancement. American Fisheries Society Symposium 15:167–175.

Bryant, R., J. Evans, R. Jenkins, and B. Freeman. 1996. The mystery fish. Southern Wildlife 1:26–35.

Cooke, S., C. Bunt, S. Hamilton, C. Jennings, M. Pearson, M. Cooperman, and D. Markle. 2005. Threats, conservation strategies, and prognosis for suckers (Catostomidae) in North America: insights from regional case studies of a diverse family of non-game fishes. Biological Conservation 121:317–331.

Cornuet, J., and G. Lukiart. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144:2001–2014.

DeMeo, T. 2001. Report of the Robust Redhorse Conservation Committee Annual Meeting. October 3–5, 2001. South Carolina Aquarium, Charleston, South Carolina. http://www.robustredhorse.com/h/reportpubs.html

DeWoody, J., and J. Avise. 2000. Microsatellite variation in marine, freshwater, and anadromous fishes compared to other animals. Journal of Fish Biology 56:461–473.

England, P., J. Cornuet, P. Berthier, D. Tallmon, and G. Luikart. 2006. Estimating effective population size from linkage disequilibrium: severe bias in small samples. Conservation Genetics 7:303–308.

Estoup, P., and B. Angers. 1998. Microsatellites and minisatellites for molecular ecology: theoretical and empirical considerations, p. 55–86. *In*: Advances in Molecular Ecology. G. Carvalho (ed.). NATO Science Series, IOS Press, Amsterdam.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1:47–50. http://cmpg.unibe.ch/software/arlequin3/

Fiumera, A., B. Porter, G. Looney, M. Asmussen, and J. Avise. 2004. Maximizing offspring production while maintaining genetic diversity in supplemental breeding programs of highly fecund managed species. Conservation Biology 18:94–101.

Frankham, R., J. Ballou, and D. Broscoe. 2002. Introduction to Conservation Genetics. Cambridge University, Cambridge, UK.

Garza, J., and E. Williamson. 2001. Detection of reduction in population size using data from microsatellite loci. Molecular Ecology 10:305–318.

Grabowski, T., and C. Jennings. 2009. Post-release movements and habitat use of robust redhorse transplanted to the Ocmulgee River, Georgia. Aquatic Conservation: Marine and Freshwater Ecosystems 19:170–177.

Hendricks, A. 1998. The conservation and restoration of the robust redhorse, *Moxostoma robustum*. Volume 1. Report to the Federal Energy Regulatory Commission prepared by Georgia Power Company, Environmental Laboratory, Atlanta, Georgia. http://www.robustredhorse.com/h/reportpubs.html

Jelks, H., S. Walsh, N. Burkhead, S. Contreras-Balderas, E. Díaz-Pardo, D. Hendrickson, J. Lyons, N. Mandrak, F. McCormick, J. Nelson, S. Platania, B. Porter, C. Renaud, J. Schmitter-Soto, E. Taylor, and M. Warren, Jr. 2008. Conservation status of imperiled North American freshwater and diadromous fishes. Fisheries 33:372–407.

Kalinowski, S. 2005. HP-Rare: a computer program for performing rarefaction on measures of allelic diversity. Molecular Ecology Notes 5:187–189. http://www.montana.edu/kalinowski/Software/HPRare.htm

Kalinowski, S., M. Taper, and T. Marshall. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16:1099–1006. http://www.fieldgenetics.com

Kuo, C., and F. Janzen. 2003. BOTTLESIM: a bottleneck simulation program for long-lived species with overlapping generations. Molecular Ecology Notes 3:669–673. http://chkuo.name/software/BottleSim.html

Kuo, C., and F. Janzen. 2004. Genetic effects of a persistent bottleneck on a natural population of ornate box turtles (*Terrapene ornata*). Conservation Genetics 5:425–437.

Lippe, C., P. Dumont, and L. Bernatchez. 2004. Isolation and identification of 21 microsatellite loci in copper redhorse (*Moxostoma hubbsi*; Catostomidae) and their variability in other catostomids. Molecular Ecology Notes 4:638–641.

Lippe, C., P. Dumont, and L. Bernatchez. 2006. High genetic diversity and no inbreeding in the endangered copper redhorse, *Moxostoma hubbsi* (Catostomidae, Pisces): the positive sides of a long generation time. Molecular Ecology 15:1769–1780.

Luikart, G., and J. Cornuet. 1998. Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. Conservation Biology 12:228–237.

Moyer, G., J. Rousey, and M. Cantrell. 2009. Genetic evaluation of a conservation hatchery program for reintroduction of sicklefin redhorse *Moxostoma* sp. in the Tuckasegee River, North Carolina. North American Journal of Fisheries Management 29:1438–1443.

Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York, New York.

Nichols, M. 2003. Conservation strategy for robust redhorse (*Moxostoma robustum*), p. 17. *In*: Report to the Robust Redhorse Conservation Committee prepared by Georgia Power Company, Environmental Laboratory, Atlanta, Georgia. http://www.robustredhorse.com/h/reportpubs.html

Ohta, J., and M. Kimura. 1973. A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. Genetical Research 22:201–204.

Piry, S., G. Luikart, and J. Cornuet. 1999. BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. Journal of Heredity 90:502–503. http://www1.montpellier.inra.fr/URLB/bottleneck/bottleneck.html

Raymond, M., and F. Rousset. 1995. GENEPOP 1.2: population genetics software for exact tests and ecumenicism. Journal of Heredity 86:248–249. http://genepop.curtin.edu.au/

Reid, S., C. Wilson, L. Carl, and T. Zorn. 2008b. Species traits influence the genetic consequences of river fragmentation on co-occurring redhorse (*Moxostoma*) species. Canadian Journal of Fisheries and Aquatic Sciences 65:1892–1904.

Reid, S., C. Wilson, N. Mandrak, and L. Carl. 2008a. Population structure and genetic diversity of black redhorse (*Moxostoma duquesnei*) in a highly fragmented watershed. Conservation Genetics 9:531–546.

Rice, W. 1989. Analyzing tables of statistical tests. Evolution 43:223–225.

RRCC (Robust Redhorse Conservation Committee). 2002. Robust redhorse conservation committee policies. http://www.robustredhorse.com/h/reportpubs.html

RRCC (Robust Redhorse Conservation Committee). 2009. Electrofishing surveys for robust redhorse on the Pee Dee River, North and South Carolina, p. 4. *In*: Report by the RRCC Yadkin-Pee Dee Technical Working Group. http://www.robustredhorse.com/h/reportpubs.html

Shriver, M., L. Jin, R. Chakraborty, and E. Boerwinkle. 1993. VNTR allele frequency distributions under the stepwise mutation model: a computer simulation approach. Genetics 134:983–993.

Straight, C., and B. Freeman. 2002. Status of robust redhorse (*Moxostoma robustum)*: reintroduction efforts and preliminary results of sonic-tracking in the Ocmulgee River. *In*: Proceedings of the 2002 Georgia Water Resources Conference, April 23–24, 2003, University of Georgia. K. J. Hatcher (ed.). Institute of Ecology, University of Georgia, Athens, Georgia.

Wang, J. 2004. Sibship reconstruction for genetic data with typing errors. Genetics 166:1963–1979.

Wang, J., and A. Santure. 2009. Parentage and sibship inference from multilocus genotype data under polygamy. Genetics 181:1579–1594.

Waples, R. 2006. A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. Conservation Genetics 7:167–184.

Waples, R., and C. Do. 2008. LDNE: a program for estimating effective population size from data on linkage disequilibrium. Molecular Ecology Resources 8:753–756.

Weir, B., and C. Cockerham. 1984. Estimating *F*-statistics for the analysis of population structure. Evolution 38:1358–1370.

Wirgin, I., D. Currie, J. Stabile, and C. Jennings. 2004. Development and use of a simple DNA test to distinguish larval redhorse species in the Oconee River, Georgia. North American Journal of Fisheries Management 24:293–298.

Wirgin, I., T. Oppermann, and J. Stabile. 2001. Genetic divergence of robust redhorse *Moxostoma robustum* (Cypriniformes Catostomidae) from the Oconee River and the Savannah River based on mitochondrial DNA control region sequences. Copeia 2001:526–530.