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Genetic Divergence of Robust Redhorse *Moxostoma robustum* (Cypriniformes Catostomidae) from the Oconee River and the Savannah River Based on Mitochondrial DNA Control Region Sequences

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A Memorandum of Understanding, which formed the Robust Redhorse Conservation Committee (RRCC), was developed and adopted by concerned stakeholders in Georgia, South Carolina, and North Carolina to attempt recovery of the recently "rediscovered," and rare, robust redhorse *Moxostoma robustum*. As part of the conservation program, genetic analyses of robust redhorse populations were needed to facilitate responsible hatchery programs that could be used to supplement natural reproduction in declining populations and to reestablish populations in rivers in which it may have historically been present. We analyzed mtDNA control region sequences in robust redhorse from the Oconee and Ocmulgee Rivers in the Altamaha River drainage and the Savannah River to determine the genetic relatedness of these populations to guide restoration efforts. Fixed differences in mtDNA haplotypes were found between robust redhorse from the two drainages that strongly argues for the designation of these populations as Evolutionarily Significant Units and against the interstock transfer of fish. Further studies will be needed to evaluate the full significance of these differences and associated management implications.

THE robust redhorse *Moxostoma robustum*, a large, riverine catostomid (maximum total length = 760 mm), was originally described by Edward Cope from specimens collected in 1869 from the Yadkin River in the Pee Dee River drainage in North Carolina (Fig. 1). Representatives of this species were not seen again for more than 100 years until single specimens were collected from the Savannah River, Georgia (GA)/South Carolina (SC) in 1980 and from the main stem of the Pee Dee River in 1985 (Bryant et al. 1996). The taxonomic relatedness of these specimens to Cope's 1869 collection was not established until a significant population was discovered in the Oconee River, GA, in 1991 (R. Jenkins, pers. comm.). It is believed that the historical range of the robust redhorse included many Atlantic coast slope rivers from the Pee Dee River drainage, North Carolina (NC)-SC, to the Altamaha River drainage, GA. However, recent targeted efforts to locate extant populations in some southeastern U.S. rivers with suitable habitat, including the Ogeechee River, GA, and the Pee Dee River, have failed.

The Oconee River and the Ocmulgee River merge to form the main stem of the Altamaha River. In August 1991, five robust redhorse specimens were collected by GA Department of Natural Resources biologists from the Oconee River near Toomsboro, GA, during routine sampling for Federal Energy Regulatory Commission relicensing of the Sinclair Dam (Bryant et al., 1996). Analysis of mark-recapture data acquired from broodstock collections conducted in the early and mid-1990s indicated that the Oconee River spawning population was comprised of approximately 1000–3000 adult fish from just below Milledgeville to Dublin, Georgia, a distance of approximately 85 km.

Spawning was observed to occur primarily over shallow gravel bars, and four geographically distinct spawning aggregations were identified (J. Evans, pers. comm.). Age and growth studies and length frequency analyses suggested that the Oconee River population may be approaching senescence (R. Jenkins, pers. comm.). In 1997, 90% of fish sampled from the Oconee River during broodstock acquisition were between 15 and 26 years of age. Over the past five years, little recruitment of younger age classes into the Oconee River spawning population has been detected, despite the observed occurrence of spawning activity and the presence of small numbers of larvae. It is thought that habitat degradation, perhaps resulting from high levels of fine sediments at spawning sites (Dilts, 1999), may adversely impact survivorship of young life stages by destroying or reducing emergence success of postyolk sac larvae from the gravel substrate. In the spring and fall of 1999, three robust redhorse, including two mature specimens, were collected from the Ocmulgee River.

In the past three years, a remnant robust red-

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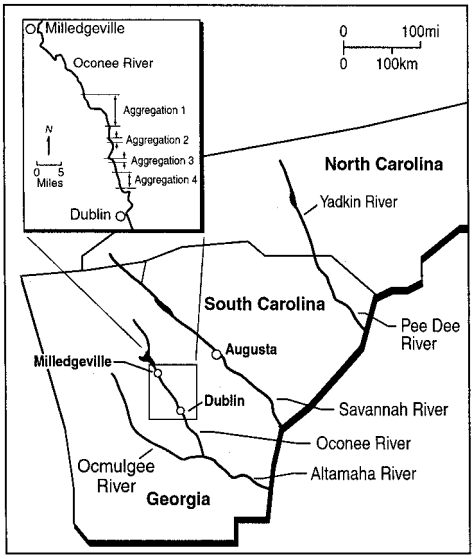

Fig. 1. A map showing rivers and aggregation sites at which robust redhorse were collected for this study and other locales at which collections of robust redhorse have been reported.

horse population was also ''rediscovered'' in the Savannah River. In the fall of 1997, one adult fish was collected approximately 150 miles from the Atlantic coast during routine sampling. A concerted effort was launched in the spring of 1998 to specifically target adults at habitats in the Savannah River similar to those at which spawning occurs in the Oconee River. This effort resulted in the collection of four adult fish from the area of Augusta, GA. In the spring of 1999, a similar effort was undertaken which focused on the shoals between the City of Augusta Diversion Dam and the New Savannah River Bluff Lock and Dam (NSRBLD) and also included the tailwaters immediately below the NSRBLD. This effort resulted in the collection of an additional 23 mature adults. Because of their imperiled status and the unwillingness to sacrifice specimens, thorough internal morphological comparisons of robust redhorse from the two rivers have not been attempted. However, cursory external examinations failed to reveal statistically significant morphological differences between fish from the Oconee and Savannah River populations (R. Jenkins, pers. comm.).

The robust redhorse was formerly considered a Category 2 candidate for federal listing under the Endangered Species Act, but with the elimination of that category, robust redhorse is now considered a ''species of special concern.'' It is listed in the Endangered category under the state of Georgia's list of protected species. In 1995, various stakeholder groups, including federal and state agencies, private industries, and conservation groups drafted and signed a Memorandum of Understanding forming the Robust Redhorse Conservation Committee (RRCC). The RRCC was charged ''to develop and coordinate the implementation of a conservation program for the robust redhorse, consisting of implementation of conservation measures that will focus on protection and management of the remaining population of robust redhorse, establishment of captive-breeding populations, and reestablishment of the species within a significant portion of its historic range in Georgia, North Carolina, and South Carolina.'' One immediate objective of this approach was to develop hatchery programs and culture techniques for robust redhorse with the ultimate objective of establishing refugial populations, which could be tapped in the event of a catastrophic decline in wild populations, and to reestablish populations in three rivers with suitable habitat in which robust redhorse may have historically been present.

We sought to determine the genetic relatedness of robust redhorse (1) from four geographically distinct spawning aggregations within the Oconee River, (2) between the Oconee River and the Ocmulgee River populations both located within the Altamaha River drainage, and (3) between the Oconee River and the Savannah River populations. This information could be used to identify the genetic diversity and genetic structure of robust redhorse populations and to guide hatchery efforts to restore or reestablish populations with fish best suited for these habitats. We used direct sequencing of the mtDNA control region to address these questions because of its relatively rapid rate of evolutionary change (Brown et al., 1996; Wirgin et al., 2000) and ease in the interpretation of results.

Materials and Methods

Sample collections.—All robust redhorse specimens from the Oconee River were mature adults (545–700 mm total length) collected by electrofishing and were obtained from four major spawning aggregations in the Oconee River during broodstock acquisition. In total, 34 specimens were analyzed from the Oconee River of which nine fish were from aggregation 1, 10 from aggregation 2, three from aggregation 3, and 11 from aggregation 4 (collection site of one fish is unknown). In 1996, samples were collected from 29 April to 15 May and in 1997

on 12 May and 13 May. Three additional specimens were collected from the Ocmulgee River on 23 June 1999.

In total, 22 fish were analyzed from the Savannah River of which 21 were acquired during spring broodstock collections. One specimen was collected from the Savannah River on 14 October 1997 at river mile 149. In 1998, four additional specimens were collected from the Savannah River in the area of Augusta Shoals between the City of Augusta Diversion Dam and the New Savannah River Bluff Lock and Dam on 3 June and 4 June. In 1999, 17 additional specimens were collected on 25 May from the Augusta Shoals. Fin clips were excised from all specimens and stored in 95% ethanol until processing. All fish that were collected during broodstock collections from both rivers were double tagged prior to spawning with PIT or Floy dart tags and released back into the environment.

DNA isolations.—Total DNAs were isolated from fin clips using the CTAB method (Saghai-Maroof et al., 1984) followed by standard phenol-chloroform extractions and alcohol precipitations. DNAs were resuspended in TE buffer and DNA concentrations and purity were determined spectrophotometrically at 260 and 280 nm.

Design of PCR primers.—Initially, mtDNA PCR primers were designed from genes (tRNA Proline and 12 S rRNA) that flank the mtDNA control region and that are conserved across taxa. Amplification and sequencing with $tPro^2$ (5′-ACCCTTAACTCCCAAAGC-3′; Brown et al., 1996) and MT 12S (5′-TAGAACAGGCTCCTCT-AG-3′) were used to obtain robust redhorse-specific mtDNA sequences within the tRNA Proline gene, control region, tRNA Phenylalanine gene, and 12 S rRNA gene. Reactions were performed in 25 μl total volumes containing 2.5 μl of 10 × reaction buffer, 0.5 μl of each dNTP (100 μM), 0.83 μl of each primer ($tPro^2$ and MT 12S) (30 μM), 1 μl of template DNA (approximately 100 ng), 0.2 μl (1 unit) of *Taq* I DNA polymerase (Boehringer Mannheim), and 17.6 μl of ddH_2O. Amplification conditions were 94 C for 5 min; 35 cycles of 94 C for 1 min, 56 C for 1 min, and 72 C for 1 min, followed by a final extension at 72 C for 7 min. PCR products were electrophoresed in 1.0% low melt agarose (Amresco) minigels made in 0.5 × TBE buffer, DNA fragments were visualized by ethidium bromide staining and photographed. A product of the expected size (1.6 kb) was obtained; this was excised in a gel slice and used for direct cycle sequencing (Stratagene Cycle Sequencing Kit) with ^{35}S as described by Kretz and O'Brien (1993) with the $tPro^2$ and MT 12S primers. Sequencing reactions were electrophoretically separated in denaturing 6.0% polyacrylamide gels, gels were air-dried onto one glass plate, and autoradiography was used to visualize DNA sequencing ladders. The two robust redhorse sequences exhibited similarity to consensus $tRNA^{pro}$ and 12S rRNA sequences from other teleosts and these sequences were then used to design robust redhorse-specific PCR primers, RR D1 and RR 1275, whose product was believed to span the complete mtDNA control region.

PCR and sequence analysis of populations.—The two robust redhorse-specific primers, RR D1 loop (5′-GTACTGAACTGCCTATATGG-3′) and RR 1275 (5′-CCTCTAACCACCCTTTACG-3′) were used in PCR exactly as described above to amplify a 1.3 kb amplicon from robust redhorse total DNAs. Amplicons were electrophoretically purified in low melt agarose gels and RR D1 loop and RR 1275 primers were used as described above to manually sequence approximately 350 nucleotides from each end of the amplicons from three fish each from the Oconee River and the Savannah River. Polymorphisms were seen with RR D1 loop but not with RR 1275. RR D1 and RR 1275 were used for PCR amplification and RR D1 for cycle sequencing of all remaining specimens for population analyses with *Taq* DNA sequencing kits (Boheringer Mannheim) and ^{32}P.

Data analysis.—Population structure and genetic differentiation between the Oconee River aggregations and the Savannah River population were evaluated using the Analysis of Molecular Variance (AMOVA) software package (Excoffier et al., 1992). AMOVA tests whether the differences among populations and within populations are significant.

Results and Discussion

Six polymorphic nucleotide sites were observed among the 305 nucleotides surveyed, of which five were transitions and one was a transversion (sequences deposited in GenBank; see Materials Examined). Three of these sites [polymorphic sites (PS) PS2, PS4, and PS5] were informative in distinguishing fish from the two drainages. Fixed differences at all three of these polymorphic sites were observed between fish from the Oconee-Ocmulgee River and Savannah River drainages. All fish from the Oconee-

TABLE 1. FREQUENCIES OF MTDNA CONTROL REGION HAPLOTYPES IN ROBUST REDHORSE FROM THE OCONEE RIVER, OCMULGEE RIVER, AND SAVANNAH RIVER.

River/-Aggregation	mtDNA haplotypes					
	n	A	B	C	D	D′
Oconee						
1	9	5	4	0	0	0
2	10	5	5	0	0	0
3	3	3	0	0	0	0
4	11	3	8	0	0	0
Unknown	1	0	1	0	0	0
Ocmulgee	3	1	2	0	0	0
Savannah	22	0	0	10	11	1

Ocmulgee River exhibited mtDNA haplotypes A and B. These haplotypes were absent in fish from the Savannah River (Table 1). In contrast, all fish from the Savannah River exhibited mtDNA haplotypes C, D, D′ and these were absent in fish from the Oconee River and the Ocmulgee River. Thus, all fish from the Oconee River and Ocmulgee River populations were unequivocally distinguishable from all fish from the Savannah River based on mtDNA control region haplotypes. Approximately 1.0% sequence divergence was observed between all fish from the Oconee-Ocmulgee and Savannah Rivers. AMOVA analysis indicated that the greatest component of variance was between the Oconee-Ocmulgee River and Savannah River populations (86.5%), whereas only 13.5% of variance was observed within these populations.

In contrast, no differences in mtDNA haplotype frequencies were observed among the four major spawning aggregations within the Oconee River or between the Oconee River and Ocmulgee River samples. Maximum divergence among fish from the Oconee River was one nucleotide and two nucleotides among fish from the Savannah River.

Our results strongly argue against the interstock transfer of robust redhorse between the Oconee and the Savannah Rivers. Fixation of mtDNA haplotypes between conspecific populations of fishes suggests that the reproductive isolation of these two riverine populations is complete and historically these populations may have been small. However, the lack of extensive nucleotide divergence between the two populations indicates that they shared a common ancestry in the not too distant evolutionary past. Although our sample size is small, we believe that transfer of offspring from Oconee River broodstock to the Ocmulgee River is acceptable based on their sharing of identical haplotypes and the fact that both rivers are part of the Altamaha River drainage and the lack of physical barriers to prevent natural movement of fish between the two rivers.

Recent attention in conservation biology has focused on the concept of Evolutionary Significant Units (ESUs; Nielsen, 1995). The concept of ESUs was developed to provide a rationale basis for prioritizing taxa for conservation efforts based on the realization of limited resources and that not all populations may equally reflect underlying historical genetic diversity within species (Moritz, 1994). However, there has been considerable debate in defining what constitutes an ESU. Waples (1995) suggests that ESUs should be defined as a population or group of populations that (1) is substantially reproductively isolated from other conspecific population units and (2) represents an important component in the evolutionary legacy of the species (Waples, 1995).

Despite their geographic proximity, the Oconee River and the Savannah River populations are genetically distinct and have apparently been reproductively isolated for some time. The Oconee River and Savannah River populations represent the only remaining gene pools of robust redhorse presently known, and because they represent the limits of the species' known current distribution, they must be considered as important components in the evolutionary legacy of the species. Thus, both the Oconee River and Savannah River populations may each warrant ESU designations despite their proximity.

Mortiz (1994) provided operational genetic criteria for defining ESUs with an emphasis on historical population structure rather than current adaptation. Thus, he suggests two genetic criteria for recognizing ESUs: (1) reciprocal monophyly for mtDNA haplotypes; and (2) significant diversity of allelic frequencies at nuclear DNA loci. Our preliminary results with microsatellite analyses of nuclear DNA are concordant with mtDNA analysis in that fixed differences in alleles were observed at a single locus (*RR*37) between the Oconee River and Savannah River samples. In addition, highly significant differences in allelic frequencies were detected at three microsatellite loci (*RR*13, *RR*38, and *RR*55) between fish from the two populations ($n \geq 60$ Oconee River; $n = 22$ Savannah River; I. Wirgin and L. Maceda, unpubl. data). In contrast, no differences were observed in microsatellite allelic frequencies between fish from the Oconee and Ocmulgee Rivers or among spawning aggregations within the Oconee River. Thus, based on two conservative genetic crite-

ria, these two robust redhorse populations warrant ESA designation.

Materials Examined

GenBank accession numbers for mtDNA control region sequences are AF217565 (haplotype A) to AF217569 (haplotype D1). Total DNAs from specimens used in this study are available from IW.

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Literature Cited

Brown, J. R., K. Beckenbach, A. T. Beckenbach, and M. J. Smith. 1996. Length variation, heteroplasmy and sequence divergence in the mitochondrial DNA of four species of sturgeon (*Acipenser*). Genetics 142:525–535.

Bryant, R. T., J. W. Evans, R. E. Jenkins, and B. J. Freeman. 1996. "The Mystery Fish." Southern Wildl. 1:26–35.

Dilts, W. W. 1999. Effects of fine sediment and gravel quality on survival to emergence of larval robust redhorses. Unpubl. master's thesis, Univ. of Georgia, Athens.

Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491.

Kretz, K. A., and J. S. O'Brien. 1993. Direct sequencing of polymerase chain reaction products from low melting temperature agarose. Methods Enzymol. 218:72–79.

Mortiz, C. 1994. Defining "Evolutionarily Significant Units" for conservation. Trends Ecol. Evol. 9: 373–375.

Nielsen, J. L. 1995. Evolution and the aquatic ecosystem: defining unique units in population conservation. American Fisheries Society Symposium 17, Bethesda, MD.

Saghai-Maroof, M. A., K. M. Soliman, R. A. Jorgensen, and R. W. Allard. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81:8014–8018.

Waples, R. S. 1995. Evolutionarily significant units and the conservation of biological diversity under the Endangered Species Act, p. 8–27. *In:* Evolution and the aquatic ecosystem: defining unique units in population conservation. J. L. Nielsen (ed.). American Fisheries Society Symposium 17, Bethesda, MD.

Wirgin, I., J. R. Waldman, J. Rosko, R. Gross, M. R. Collins, S. G. Rogers, and J. Stabile. 2000. Genetic stock structure of Atlantic sturgeon populations based on mitochondrial DNA control region sequences. Trans. Am. Fish. Soc. 129:476–486.

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