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Development and Use of a Simple DNA Test to Distinguish Larval Redhorse Species in the Oconee River, Georgia

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Abstract.—The robust redhorse *Moxostoma robustum* is a rare catostomid species that was recently ''rediscovered'' in three Atlantic slope drainages in the southeastern United States, including the Oconee River, Georgia. Adult population size in the Oconee River is declining, and the population may be senescent due to recruitment failure. Evaluation of the environmental factors affecting the success of young life stages requires the ability to distinguish robust redhorse larvae from those of other redhorse species in the Oconee River. The use of morphological approaches, including size at collection date, have proven to be problematic in distinguishing larval robust redhorse from notchlip redhorse *M. collapsum* from the Oconee River. We developed a mitochondrial DNA (mtDNA) assay to distinguish between reference adults of these two redhorse species from the Oconee, Savannah, and Pee Dee rivers. This mtDNA assay was then applied to unknown larval redhorse collections from the Oconee River. In one collection, discordance was revealed in 40% of the individual larvae identified by both size at collection date and mtDNA. Of these, 75% of the fish thought to be notchlip redhorses based on size at date of collection exhibited robust redhorse mtDNA haplotypes. These results illustrate the utility of DNA techniques in characterizing the young life stages of fish and suggest that earlier surveys underestimated the abundance of larval robust redhorses in the Oconee River.

The robust redhorse *Moxostoma robustum* (see Nelson et al., in press) is a large (adult total length, 42–72 cm), long-lived (25–30 years), riverine, and rare catostomid species that was ''rediscovered'' in 1991 in the Oconee River, Georgia, which is one of two major tributaries of the Altamaha River (Bryant et al. 1996). It was originally described in 1869 from specimens collected by Edward Cope in the Yadkin River within the Pee Dee River drainage, North Carolina–South Carolina (Cope 1870). The robust redhorse was not reported again for more than 100 years until single specimens were collected in 1980 from the Savannah River, South Carolina–Georgia, and in 1985 from the Pee Dee River, North Carolina. Since then, small relict populations have been described in the Oconee River (600–3,000 adults) and populations of unknown census size in the Savannah River. Additionally, intensive sampling has located even smaller populations in the Pee Dee River and the Ocmulgee River, Georgia, the second major tributary of the Altamaha River.

Within the Oconee River, robust redhorses have been collected over a 113-km stretch of river in the fall line zone between Milledgeville and Dublin, Georgia. Annual electrofishing surveys have indicated a continuing decline in the abundance of spawning adults and an apparent senescence of the population. Despite intensive efforts, no juveniles and very few immature adults have been collected in the Oconee River. Recruitment failure has been hypothesized as the cause for this decline. In contrast, moderate numbers of spawning adults and immature robust redhorses have been collected from the Savannah River in the past 5 years.

Although not formally listed on the U.S. Endangered Species List, the robust redhorse is considered a federal species of concern and is listed as a state endangered species in Georgia. Federal agencies, state agencies, private electric utility industries, and conservation groups developed and implemented a memorandum of understanding to restore robust redhorses in the historic range of

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the species in North Carolina, South Carolina, and Georgia. Among the measures being used to conserve and restore populations is the development of captive breeding programs with broodstock presently being collected from the Oconee and Savannah rivers. Because of fixed differences in mitochondrial DNA (mtDNA) control region haplotypes and fixed and significant differences in the frequencies of microsatellite nuclear DNA genotypes, the Oconee River, Savannah River (Wirgin et al. 2001), and Pee Dee River (Wirgin, unpublished data) populations are currently being managed as separate evolutionarily significant units. They satisfy the criteria proposed by Waples (1991) of being reproductively isolated from each other and representing important components in the evolutionary legacy of the species.

Spawning of robust redhorses has been observed in the Oconee and Savannah rivers over shallow gravel bars immediately below the fall line in the spring. The prime spawning area in the Oconee River is near Oconee, Georgia, downstream of the Sinclair Hydroelectric Dam, which regulates water flow through the spawning area. Robust redhorse embryos incubate in gravel substrate at or near spawning sites prior to their emergence as larvae. In controlled laboratory experiments, sediment quality (i.e., the percentage of fine sediments in the gravel substrate) has been demonstrated to significantly affect the ability of larval robust redhorses to emerge (Dilts 1999). Therefore, river flow rates, coupled with sediment loading from the surrounding watershed, may significantly affect recruitment success and adult population size. Efforts to identify the causative environmental conditions in the Oconee River, such as flow rate and sediment loading events, that correlate with recruitment success are hindered by the absence of diagnostic morphological characters that can be used to distinguish young life stages of redhorse species in the Oconee River and elsewhere.

The robust redhorse is closely related to the notchlip redhorse *M. collapsum* (recently elevated from synonymy with the silver redhorse *M. anisurum*; see Nelson et al., in press), and their co-occurring larvae in the Oconee River are difficult to distinguish by means of morphological characters. Because the spawning of notchlip redhorses was thought to precede that of robust redhorses by 4–6 weeks, larval size at a given sampling date was used to distinguish between the two species. Recent evidence suggests that that may not always be the case, however; in fact, there may be considerable overlap in spawning times between the two species during some years, thereby invalidating the use of larval size at sampling date as a reliable character for distinguishing these species.

Our objectives in this study were (1) to isolate DNA markers that could be used to unequivocally distinguish between reference adult robust and notchlip redhorses from the three rivers in which they are known to co-occur and (2) to validate these markers for use on field collections of larval redhorse species from the Oconee River. Because mtDNA is almost exclusively maternally inherited and does not undergo recombination, we focused our efforts on identifying mtDNA sequences that exhibit fixed differences between species but that are monomorphic within the two species. The development of such markers would allow for the unequivocal identification of the two larval redhorse species collected in the Oconee River and perhaps elsewhere.

Methods

Sample collections and processing.—In total, 45 adult robust redhorses and 52 adult notchlip redhorses were used as reference specimens. Twenty adult robust redhorses were sampled from the Oconee River in April 1996 and May 1997 (total length [TL], 545–700 mm), and 20 adult notchlip redhorses were collected from the Oconee River in early December 1999 (TL, 299–482 mm). Twenty adult robust redhorses were collected from the Savannah River in June 1998 and May 1999 (TL, 501–655 mm) and 20 adult notchlip redhorses from the Savannah River in June 1999 (TL, 336–475 mm). Five adult robust redhorses were collected from the Pee Dee River between April 2000 and October 2002 (TL, 375–692 mm) and 12 adult notchlip redhorses from the Pee Dee River in April 2000 and May 2001 (TL, 227–522 mm).

Larval robust and notchlip redhorses were sampled weekly from the Oconee River from May 16 through July 27, 2001. Larval redhorses from the Oconee River were randomly divided into three collections and subjected to mtDNA analysis. Larvae were sampled with light traps, 0.5-m-diameter conical plankton nets (505-μm mesh), and beach seines (10 m in length × 2 m in width, with 0.8-mm mesh). Samples were preserved in 95% solutions of ethanol and transported to the University of Georgia for processing. In the laboratory, all larval fish, including redhorse species, were extracted from the samples, enumerated, and assigned a tentative species identification based on standard taxonomic references (e.g., Kay et al. 1994). Each redhorse larva was placed in a sep-

arate vial with ethanol, labeled with a unique sample number, and shipped to the New York University School of Medicine for independent genetics-based species identification.

DNA isolation and amplification.—Total DNA was isolated from adult fin clips or whole individual larval redhorse specimens using hexadecyltrimethylammonium bromide buffer (Saghai-Maroof et al. 1984) with standard proteinase K digestions, phenol-chloroform extractions, and alcohol precipitations. Initially, two mtDNA primers specific to robust redhorses, RRDloop1 and RR1275 (Wirgin et al. 2001), were used to amplify and sequence most of the mtDNA control region from two adults from both *Moxostoma* species. The derived DNA sequence was then used to design nested polymerase chain reaction (PCR) primers that were better conserved between both species. The forward primer, SRD LOOP1 (5′-ATGGTGTAGTACATAATATGC-3′), is located just upstream of the 5′ prime portion of the mtDNA control region sequence reported for the robust redhorse (GenBank accession number AF217565). The reverse primer, RobSilver (5′-ATTTCTGTTGATCTTTGC-3′), is located at the nucleotides 182–199 reported for the robust redhorse.

Polymerase chain reactions were carried out in 10-μL volumes and contained 1 μL of template DNA of unknown concentrations, 1 μL of 10× polycarbonate buffer (PC2; Ab Peptides, Inc., St. Louis, Missouri), 0.8 μL of deoxynucleotide triphosphate (dNTP) mix (25 mm of each dNTP; Ab Peptides, Inc.), 0.4 μL of each primer (30 μM), 0.2 μL of KlenTaq (25 units/μL; AB Peptides), and 6.6 μL of double-distilled H_2O. Polymerase chain reaction conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of 94° for 30 s, 50° for 30 s, and 72° for 60 s; and final extension at 72°C for 7 min. The success of the PCR amplifications was monitored by electorphoresis of the PCR products in 1.2% agarose gels with ethidium bromide staining. The expected size of the PCR product was 212 base pairs.

Three μL of the PCR products were digested individually with *Hin*f I and *Ssp* I for 2–4 h following the manufacturer's recommendations (New England Biolabs). Digests were electrophoretically separated in 1.8% 15-cm × 15-cm agarose gels for 3 h at 80 V, and DNA fragments were visualized by ethidium bromide staining and photographed.

Results and Discussion

Initially, the robust-redhorse-specific primers RR1275 and RRDloop1, described in Wirgin et al. (2001), were used to PCR-amplify and sequence mtDNA control region fragments from reference adult robust and notchlip redhorse specimens. Of the 304 nucleotides compared between the two species, 17 fixed differences were observed between adult robust and notchlip redhorses. We then screened these polymorphic nucleotide sequences with the MAP program in the GCG package (Wisconsin Package Version 10.3, Accelrys Inc., San Diego, California) to determine which should be recognized by commercially available restriction enzymes and found that an *Ssp* I site was present in mtDNA from the robust redhorse but not the notchlip redhorse. Conversely, a *Hin*f I site was present in mtDNA from the notchlip redhorse but not the robust redhorse.

The restriction enzymes *Hin*f I and *Ssp* I were then applied to a training set of mtDNA control region PCR products amplified from 45 reference adult robust redhorses and 52 notchlip redhorses from the Oconee, Savannah, and Pee Dee rivers. As expected, all mtDNA amplicons from adult robust redhorses from all three rivers digested with the restriction enzyme *Ssp* I but not *Hin*f I (Figure 1A). Conversely, all mtDNA from adult notchlip redhorses from all three rivers digested with the restriction enzyme *Hin*f I but not *Ssp* I (Figure 1B). These results confirmed the accuracy of the assay in distinguishing mtDNA from adult robust and notchlip redhorses from all three of the rivers in which they are known to co-occur. By using two enzymes, one that digested each species' mtDNA but failed to digest the second species' mtDNA, the assay excluded the possibility of species misidentification due to the presence of inhibitors in the DNA isolations or PCR amplifications.

We then validated the use of the assay on three samples of redhorse larvae that were selected from larger collections of young-of-the-year fishes from the Oconee River. All specimens from all three samples provided results that were consistent with those obtained from the adult training set, that is, each DNA digested with either *Hin*f I or *Ssp* I but not both. All larval redhorses in the first two samples (n = 56 and 10, respectively) exhibited mtDNA fragment patterns consistent with those of adult notchlip redhorses. Larvae from the third sample (n = 41) showed mtDNA restriction fragment length polymorphisms (RFLP) haplotypes representative of both species. Of the 41 larvae in the third sample characterized by PCR–RFLP analysis, 38 exhibited robust redhorse mtDNA haplotypes and 3 notchlip redhorse haplotypes. Of the 40 larvae in this collection that were characterized

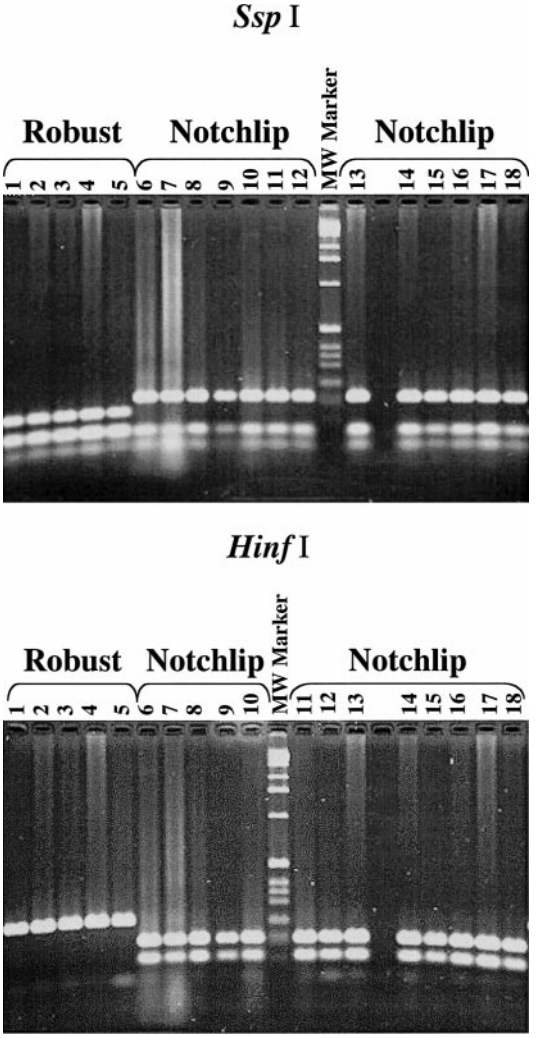

FIGURE 1.—Top panel shows mitochondrial DNA control region polymerase chain reaction (PCR) products from adult robust and notchlip redhorses digested with the restriction enzyme *Ssp* I that were run in a 1.8% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. Lane MW contains DNA from a 1-kilobase (kb) molecular weight marker. Bottom panel shows mitochondrial DNA control region PCR products from adult robust and notchlip redhorses digested with the restriction enzyme *Hin*f I that were treated in the same fashion.

by both morphological and mtDNA approaches, discordance was observed for 16 individuals (40%). Of those larvae identified as robust redhorses by mtDNA, 40% were misidentified as notchlip redhorses by the size-at-date method. Of those larvae identified as notchlip redhorses, 75% were misidentified by the size-at-date method (Figure 2). Of all misidentified larvae, 13 (81%) that were morphologically identified as notchlip redhorses exhibited robust redhorse mtDNA haplotypes and 3 larvae (19%) that were morphologically identified as robust redhorses exhibited notchlip redhorse mtDNA haplotypes.

The size range of larvae misidentified as notchlip redhorses was 15.7–21.6 mm, whereas the size range of those misidentified as robust redhorses was 13.9–14.3 mm (Figure 2). Early laboratory taxonomic identification attempts to discriminate between the two species depended on larval size at collection date because notchlip redhorses were thought to spawn at least 4 (and up to 6) weeks before robust redhorses. This assumption was obviously incorrect, as was evident in the pattern of misidentifications among these species. All of the notchlip redhorses misidentified as robust redhorses were smaller than notchlip redhorses were expected to be at that time of the year. The converse was also true for the misidentified robust redhorses (Figure 2). This suggests that in earlier studies the abundance of robust redhorse larvae was underestimated due to their misidentification as notchlip redhorses.

Our success in distinguishing between robust redhorse and notchlip redhorse larvae from the Oconee River must be tempered by a caveat. No other redhorse species are known to exist in the Oconee River, although two related catostomid species do occur, namely, the brassy jumprock *Scartomyzon* sp. (undescribed) and the striped jumprock *M. rupiscartes*. Other redhorse species are known to exist in other rivers in the southeastern United States (for example, the shorthead redhorse *M. macrolepidotum*), and one or more redhorse species may share the mtDNA RFLP digest patterns for *Hin*f I or *Ssp* I that are consistent with those of either of robust or notchlip redhorses. Thus, before its application to other rivers, this assay should be expanded to include other catostomid species.

The results of this study illustrate the utility of DNA, and particularly mtDNA analysis, in the identification of early life stages of ecologically and commercially important aquatic species. The planktonic larval distributions of bivalves are particularly refractory to successful morphological identification, and many efforts have been initiated to use DNA techniques in species identification (reviewed in Garland and Zimmer 2002). Other studies have attempted to use mtDNA polymorphisms to identify the young life stages of sympatric fish species such as Hawaiian amphidrom-

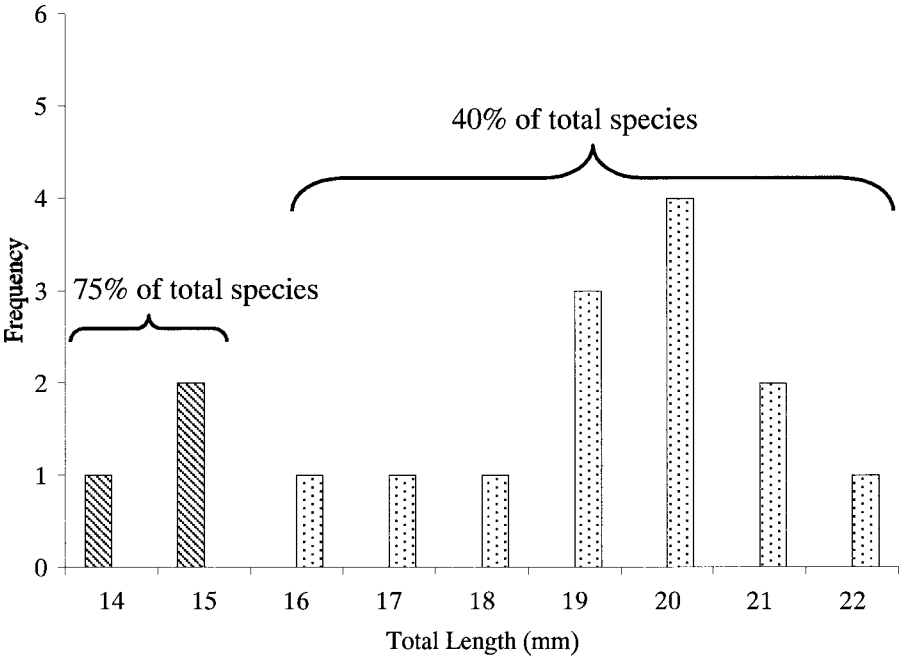

FIGURE 2.—Length frequency histogram of misidentified redhorse larvae from the Oconee River, Georgia, whose identification was based on size at date. Notchlip redhorses that were misidentified as robust redhorses are indicated by the crosshatched bars. Robust redhorses that were misidentified as notchlip redhorses are identified by stippled bars.

ous gobioids (Lindstrom 1999), diadromous galaxiids from New Zealand (Charteris and Ritchie 2002), and species within the genus *Centropomus* (Tringali et al. 1999). Often, it is the early life stages that are most important in determining recruitment success, and larval identification to the species level has been problematic in the past, often depending on size, pigmentation, and other morphological attributes. In some cases, such as that of the redhorse species discussed here, there is an absence of definitive diagnostic tools that can be used to distinguish larval life stages. Using a DNA-based identification approach in conjunction with classical ecological studies may become critical in evaluating the environmental factors that affect early-life-stage survival success. Additionally, in stock identification studies the use of embryo or larval samples may be preferred because of the increased assurance that the fish were spawned in close proximity to their collection sites. Finally, DNA-based approaches may be needed to ground-truth species identifications of early life stages made on the basis of morphological characters.

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References

Bryant, R. T., J. W. Evans, R. E. Jenkins, and B. J. Freeman. 1996. ‘‘The mystery fish.’’ Southern Wildlife 1:26–35.

Charteris, S. C., and P. A. Ritchie. 2002. Identification of galaxiid nests, emigrating larvae, and whitebait using mitochondrial DNA control region sequences. New Zealand Journal of Marine and Freshwater Research 36:789–795.

Cope, E. D. 1870. Partial synopsis of the fishes of the fresh waters of North Carolina. Proceedings of the American Philosophical Society 448–495.

Dilts, W. W. 1999. Effects of fine sediment and gravel quality on survival to emergence of larval robust redhorses. Master’s thesis. University of Georgia, Athens.

Garland, E. D., and C. A. Zimmer. 2002. Techniques for the identification of bivalve larvae. Marine Ecology Progress Series 225:299–310.

Kay, L. K., R. Wallus, and B. L. Yeager. 1994. Reproductive biology and early history of fishes in the Ohio drainage, volume 2, Catostomidae. Tennessee Valley Authority, Chattanooga.

Lindstrom, D. P. 1999. Molecular species identification of newly hatched Hawaiian amphidromous gobioid larvae. Marine Biotechnology 1:167–174.

Nelson, J. S., E. J. Crossman, H. Espinosa-Pérez, L. T. Findley, C. R. Gilbert, R. N. Lea, and J. D. Williams. In press. Common and scientific names of fishes in the United States, Canada, and Mexico. American Fisheries Society, Special Publication 29, Bethesda, Maryland.

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. Proceedings of the National Academy of Sciences of the United States of America 81:8014–8018.

Tringali, M. D., T. M. Bert, and S. Seyoum. 1999. Genetic identification of centropomine fishes. Transactions of the American Fisheries Society 128:446–458.

Waples, R. 1991. Pacific salmon, *Oncorhynchus* spp., and the definition of ''species'' under the Endangered Species Act. Marine Fisheries Review 53:11–21.

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