Mitochondrial Control Region of Striped Mullet, *Mugil cephalus*: A Tool to Restore Marine Fisheries Resources

NIKOLA M. GARBER, AMBER F. GARBER, KENNETH C. STUCK and WALTER D. GRATER

The University of Southern Mississippi, Institute of Marine Sciences
Gulf Coast Research Laboratory
P.O. Box 7000
Ocean Springs, Mississippi 39566-7000 USA

ABSTRACT

Mitochondrial DNA (mtDNA) has been frequently used as a molecular marker in fisheries stock assessment and has potential applications in stock enhancement. In some fish species, sequence data from the mtDNA control region exhibits enough intraspecific variability that the likelihood of two unrelated individuals being identical is very low to nil. Efforts to restore the depleted natural stocks of the striped mullet, *Mugil cephalus*, have been undertaken in the Pacific, through cultured fish release. In such enhancement programs the use of molecular markers, particularly during the initial pilot-scale releases, is a reliable method to estimate contributions of cultured fish to wild stocks. The purpose of this study was to create primers in the flanking tRNAs to amplify the mtDNA control region in *M. cephalus* as a prerequisite to then conduct population genetic studies supporting stock enhancement activities. Further study of the control region of *M. cephalus* will allow separation of individual stocks and the possible utilization of molecular tags to identify genetic contributions of hatchery-reared individuals to the wild population in stock enhancement activities in the Gulf of Mexico and Caribbean.

KEY WORDS: *Mugil cephalus*, control region, mitochondrial DNA

INTRODUCTION

The steadily increasing demand for seafood in the United States, together with technological improvements in harvesting methods and natural events have resulted in depletion of marine fisheries resources. About half of the commercially important marine fishes in the United States are currently being harvested beyond their maximum sustainable yield, leading to a steady decline in abundance (National Marine Fisheries Service 1997, Blankenship and Leber 1995). This trend will likely continue into the future as a result of projected worldwide increases in human population, particularly in developing countries (National Marine Fisheries Service 1997).

Restoration of depleted stocks is possible through effective traditional management procedures designed to reduce fish mortality, reduce fishing effort, and restore habitat. Since overfishing of most commercial stocks is inevitable

(Grimes 1998), traditional management procedures, along with non-traditional methods, such as stock enhancement, should be considered before stocks are significantly depleted.

Stock enhancement has been utilized in varying degrees from the mid-1880s to present time in the United States, Canada, France, Australia, United Kingdom, New Zealand, and Norway (Grimes 1998). Early attempts at marine stock enhancement often involved the indiscriminate release of eggs and newly hatched larvae; most of which were considered failures and in some instances were thought to have caused deleterious effects on existing stocks (Grimes 1998). Blankenship and Leber (1995) proposed a more responsible approach to marine stock enhancement which prioritizes and selects a target species, develops stock rebuilding goals, and uses genetic resource management to avoid deleterious genetic effects. Baseline genetic data is necessary to direct restocking efforts and minimize the negative genetic impacts caused by accidental or intentional inbreeding (Hinder et al. 1991, Blankenship and Leber 1995).

The primary objective of this study is to develop the necessary technical procedures for using mtDNA, specifically the control region, as a molecular marker to support stock enhancement activities through the United States Gulf of Mexico Marine Stock Enhancement Consortium Program (Consortium). The goals of this program are the development, refinement, field testing, and demonstration of a successful marine fishery stock management program for the U.S. coastal Gulf of Mexico that blends stock enhancement technology with traditional fishery management practices. When the identified constraints are resolved, the technology will be transferred to local and regional resource management agencies for their consideration as an additional management tool to supplement, and help replenish certain high-priority, declining stocks of marine fishes. The striped mullet, *Mugil cephalus*, was used as a test species in this study because of its initial designation as a prototype target species by the Consortium. These procedures will be used in future papers to discuss the characterization of the control region, as well as facilitation of a population genetic study of *M. cephalus* from three ocean basins. The procedures described in this study will then be adapted to other target species as designated by the Consortium.

MATERIALS AND METHODS

Mugil cephalus was collected from Biloxi Bay, Mississippi. White muscle tissue was excised and placed in SED buffer (250 mM EDTA, pH 7.5, 20% DMSO, 3.42 M NaCl; weight to volume ~ 2 - 3 grams to 16 ml). *M. cephalus* was identified using fish keys by Hoese and Moore (1977), Murdy (1983), and Robins and Ray (1986), and taxonomic specialists (Jim Franks, Stuart Poss, and Richard Waller) at the Gulf Coast Research Laboratory, Ocean Springs, MS.

Total genomic DNA was extracted from each sample, under sterile conditions, using a procedure modified from Taggart et al. (1992). DNA was then quantified using fluorescence spectrophotometry, as described in Gallagher (1994). Samples were adjusted to a concentration of 100 ng/μl in TE (10 mM Tris, 1 mM EDTA, pH 8.0) prior to storage at -20°C.

Portions of cytochrome b, 12S rRNA, and the control region, and the entire tRNA threonine (tRNA-T) and proline (tRNA-P) were amplified by PCR using universal primers CB3R (5' CACATTCAACCAGAATGATATTT 3'; Palumbi, 1996; Fig. 1) and 12SA-H1067, referred to in the present study as 12SAR, (5' ATAATAGGGTATCTAATCCTAGTT 3'; Martin et al., 1992; Figure 1). PCR amplification was performed in replicate 25 μl reactions containing 100 ng template DNA, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates (Promega, Inc.), 0.3 μM of each primer, and 1.75 units *Taq* DNA polymerase with 10X PCR buffer (Amersham Life Science). PCR cycling parameters were 3 min at 94°C, followed by 35 cycles consisting of a denaturing of 0.75 min at 94°C, an annealing of 1 min at 55°C, and an extension of 2 min at 72°C, with a final elongation of 7 min at 72°C. After visualization on an agarose gel, the appropriate PCR product was excised, purified using the QIAquick Gel Extraction Kit (Qiagen, Inc.), quantified, and direct sequenced. Species-specific primers in the genes coding for tRNA-P (MulPro, 5' CCAAGGCCAGGATTTTTACGTT 3'; Figure 1) and 12S rRNA (Mul12S, 5' CACGAGATTTACCGGCCCTATTAG 3'; Figure 1) were then designed.

These primers, MulPro and Mul12S, were then utilized to produce a PCR fragment using the previously described cycling conditions. Purified PCR products were cloned using the pGEM®-T Easy Vector System (Promega, Inc.). Ligated vector DNA was transformed into competent JM109 cells that were then cultured on Luria-Bertani (LB)/ampicillin plates with x-gal and IPTG. Colonies containing inserts were identified by blue/white selection and used to inoculate 5 ml minipreps. The cloned plasmid DNA was isolated using the Wizard® *Plus* DNA Purification System (Promega, Inc.). Plasmid DNA was then purified using the PEG method (Nicoletti and Condorelli 1993), quantified, and sequenced.

A species-specific primer in the tRNA-phenylalanine (tRNA-F; MulPhe, 5' TCTTGACATCTTCAGCGTCGC 3'; Figure 1) was subsequently designed and used with the primer MulPro to amplify the entire control region with the above PCR cycling parameters. The appropriate PCR-product was gel-purified, quantified, and direct sequenced. All DNA sequencing was done with an ABI model 373A automated sequencer at the University of Maine DNA Sequencing Facility.

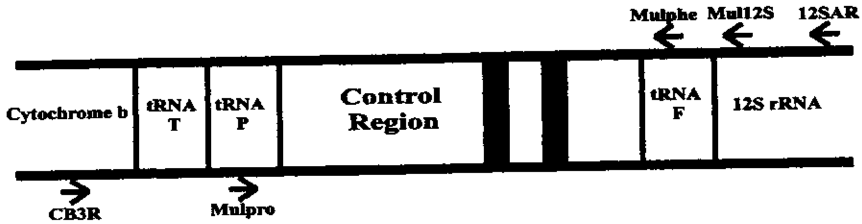

Figure 1. Relative positions of primers used to amplify the mitochondrial DNA control region and flanking genes of *Mugil cephalus*. Abbreviations of the tRNAs are threonine (tRNA-T), proline (tRNA-P, and phenylalanine (tRNA-F).

RESULTS

Total genomic DNA was extracted, and a piece of DNA approximately 2000 base pairs (bp) in length was produced by amplification with the universal primers CB3R and 12SAR (Figure 2a). Approximately 640 bp of sequence was obtained from the CB3R priming site and 400 bp of sequence from the 12SAR site. Results using the NCBI's BLAST WWW Server (Basic Local Alignment Search Tool; Altschul et al. 1990) on the resulting sequences indicated the products obtained closely matched portions of the 12S rRNA, control region, and cytochrome b, as well as tRNA-T and tRNA-P. This sequence information was used to design species-specific primers located in the tRNA-P (MulPro) and in the 12S rRNA (Mul12S) located approximately 180 bp downstream of the 12SAR site. The species-specific primers, MulPro and Mul12S, produced a 1,300 bp PCR-product (Figure 2b). This DNA fragment was subsequently gel purified, T/A cloned (Figure 2c), and completely sequenced, producing the entire control region, tRNA-F, and a partial 12S rRNA. A species-specific primer located in the tRNA-F (MulPhe) was designed from this available sequence data. Finally, MulPro and MulPhe primers produced a DNA-fragment of approximately 880 bp (Figure 2d), which contained the entire control region. BLAST searches identified these sequences as the mtDNA control region and it was most homologous with *Xiphias gladius* (swordfish). The entire 1694 bp sequence is deposited in GenBank, accession number AF108270.

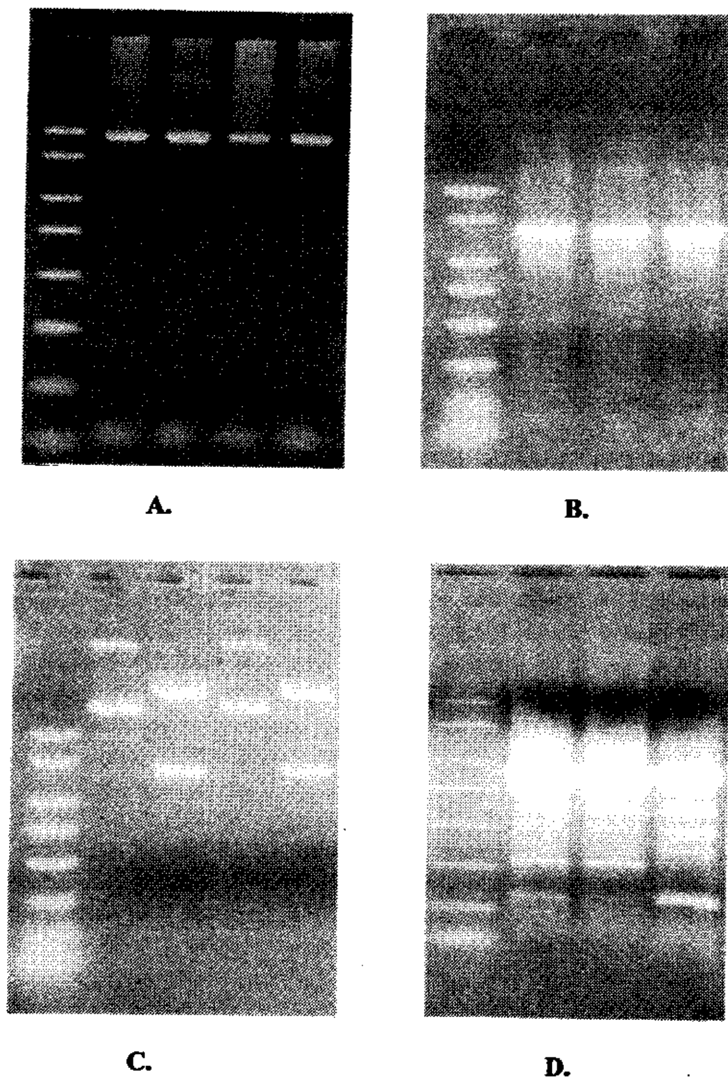

Figure 2. PCR products of *Mugil cephalus* mitochondrial control region and flanking genes produced, using the primers: (A) CB3R/12SAR; (B) MulPro/Mul12S; (C) MulPro/Mul12S, Lanes 2 and 4 contain uncut clones, Lanes 3 and 5 contain clones from which the insert was cut out with EcoRI enzyme; (D) MulPro/MulPhe (see Materials and Methods for details). Lane 1 in all gels is the ladder of sizes 2000, 1500, 1000, 750, 500, 300, 150, and 50 base pairs (Amresco PCR marker, Amresco, Inc.).

DISCUSSION

The purpose of this study, providing the needed technical background for the eventual development of genetic tags to support stock enhancement activities in the Gulf of Mexico and Caribbean, was accomplished by sequencing the portion of the mtDNA between the cytochrome b and 12S rRNA. It was necessary to create species-specific primers in the flanking tRNAs because tRNAs are conserved within species and amplification of the smallest sequence, including the entire control region, is needed to obtain the greatest overlap in the 5' and 3' sequences. Further utilization of these primers with an increased number of samples allowed for characterization of the control region and a population genetic study of *M. cephalus* between three geographically distant populations: Gulf of Mexico, Atlantic, and Pacific Basins (Garber 1999). This thesis research determined the genetic differences within the Gulf of Mexico and between the three Basins, as well as determining if these differences constituted genetically distinct populations. The sequences could then also be utilized to evaluate whether or not a partial or entire control region can be used as a genetic tag.

Stock enhancement is considered a non-traditional option for restoration of marine fisheries, but it may become an extremely useful alternative management tool in the near future. Genetic considerations need to be defined and maintained, such as: identifying the genetic risks and consequences of enhancement, defining an enhancement strategy with adequate genetic controls, implementing those genetic controls in the hatchery, and monitoring and evaluating effects/ impacts of hatchery-reared fish on wild stocks. Utilizing mtDNA, specifically the control region, may be extremely useful in assessing adverse impacts of releases and possibly provide solutions necessary to circumvent many of these problems before they occur. A population-genetics study using control region sequence data (Rocha-Olivares et al. submitted) as well as a complete characterization of the control region (Garber et al. in preparation) will be presented elsewhere. Appropriate genetic monitoring will result in preservation of fisheries resources and will protect the natural genetic population structure of marine species worldwide. If our fishery stocks are to ever recover, fisheries management entities must work together to combine the available biological, technological, economical, and political information in an effort to identify the best possible solutions.

ACKNOWLEDGMENTS

A special appreciation is extended to Jim Franks for his extra time, effort, and direction in everyday questions and problems encountered during this research. Immense gratitude for editing assistance is given to Axayacatl Rocha-Olivares of Louisiana State University, Baton Rouge, Louisiana. Identifications

were confirmed by Jim Franks, Stuart Poss, and Dick Waller to whom we are grateful. A personal thanks is given to David Geter for his technical computer expertise.

Funding was provided by National Marine Fisheries Service in cooperative agreement with the National Oceanic and Atmospheric Administration, U.S. Department of Commerce grant, number NA76FL0446. Research was conducted under the U.S. Gulf of Mexico Marine Stock Enhancement Consortium, a cooperative effort of the Gulf Coast Research Laboratory (Ocean Springs, Mississippi), the Mote Marine Laboratory (Sarasota, Florida), and the Oceanic Institute (Honolulu, Hawaii).

LITERATURE CITED

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403-410.

Blankenship, H.L. and K.M. Leber. 1995. The responsible approach to marine stock enhancement. *Am. Fish. Soc. Symp.* **15**:167-175.

Gallagher, S.R. 1994. Commonly used biochemical techniques, Appendix 3. Pages A.3.10-A.3.11 in: K. Janssen, (series ed.) *Current Protocols in Molecular Biology*. John Wiley and Sons, Inc., USA.

Garber, N.M. 1999. Application of the mitochondrial DNA control region in population structure studies of *Mugil cephalus* (striped mullet) in North America. M.S. Thesis. The University of Southern Mississippi, Hattiesburg, MS. 86 pp.

Garber, N.M., A. Rocha-Olivares, A.F. Garber and K.C. Stuck. Complete characterization of *Mugil cephalus* (striped mullet) mitochondrial DNA control region in the Atlantic, Gulf of Mexico, and Pacific Ocean Basins. In prep.

Grimes, C.B. 1998. Marine stock enhancement: sound management or techno-arrogance? *Fisheries* **23**(9):18-23.

Hinder, K., N. Ryman and F. Utter. 1991. Genetic effects of cultured fish on natural fish populations. *Can. J. Fish. Aquat. Sci.* **48**:945-957.

Hoese, H.D. and R.H. Moore. 1977. *Fishes of the Gulf of Mexico: Texas, Louisiana, and adjacent waters*. Texas A & M University Press, College Station, TX. 327 pp.

Lowe, T. and S.R. Eddy. 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequences. *Nucl. Acids Res.* **25**:955-964.

Martin, A.P., R. Humphreys and S.R. Palumbi. 1992. Population genetic structure of the armorhead, *Pseudopentaceros wheeleri*, in the North Pacific ocean: application of the polymerase chain reaction to fisheries problems. *Can. J. Fish. Aquat. Sci.* **49**:2386-2391.

Murdy, E.O. 1983. *Saltwater fishes of Texas: a dichotomous key.* Texas A&M University Sea Grant College Program, College Station, TX. 220 pp.

National Marine Fisheries Service. 1997. *Fisheries of the United States, 1996.* Current Fishery Statistics No. 9500.

Nicoletti, V.G. and D.F. Condorelli. 1993. Optimized PEG method for rapid plasmid DNA purification: high yield from "midi-prep." *Biotechniques* **14**(4):535-536.

Palumbi, S.R. 1996. Nucleic acids II: the polymerase chain reaction. Pages 205-247 in: D.M. Hillis, C. Moritz and B.K. Mable, (eds.) Molecular Systematics, 2nd ed., Sinauer Associates, Inc., Sunderland, Massachusetts, USA.

Robins, C.R. and G.C. Ray. 1986. *The Peterson field guide series: a field guide to Atlantic coast fishes North America.* Houghton Mifflin Company, Boston, MA. 354 pp.

Rocha-Olivares, A., N.M. Garber and K.C. Stuck High genetic diversity, deep inter-oceanic divergence and historical demography in the striped mullet *Mugil cephalus. Mol. Ecol.* Submitted.

Taggart, J.B., R.A. Hynes, P.A. Prodohl and A. Ferguson. 1992. A simplified protocol for routine total DNA isolation from salmonid fishes. *J. Fish. Biol.* **40**:963-965.