

FINAL REPORT

I. PROJECT INFORMATION

Report Title: Development of DNA Microsatellites for Genetic Applications in Cobia (*Rachycentron canadum*)

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II. ABSTRACT

Polymerase chain reaction (PCR) primers for 35 nuclear-encoded microsatellites were developed from a genomic library of cobia (*Rachycentron canadum*). All 35 microsatellites were tested for reproducibility and polymorphism, using 24 cobia sampled offshore off Ocean Springs, Mississippi. Thirty-three of the microsatellites were found to be polymorphic; genotypes at seven of these differed significantly from Hardy-Weinberg (HW) expectations, possibly due to the presence of null alleles. Levels of allele and gene diversity (expected heterozygosity) were lower, on average, than values reported previously for other marine fishes. All 35 microsatellites should provide useful tools in progeny tests to estimate genetic contributions to a variety of aquaculture production traits. The 26 microsatellites whose genotypes were in HW equilibrium should provide useful tools for future studies of cobia relating to both stock-assessment and aquaculture. Five multiplex panels were developed to facilitate and reduce expenses of using the microsatellites for both applications. Development of the multiplex panels was in addition to proposed objectives.

III. EXECUTIVE SUMMARY

Polymerase chain reaction (PCR) primers for 35 nuclear-encoded microsatellites were developed for application in quantitative- and population-genetic studies of cobia (*Rachycentron canadum*). Five multiplex panels also were developed and optimized. All 35 microsatellites will

prove useful in addressing legal constraints that might arise during marketing of cobia products cultured offshore and in genetic improvement of cobia broodstock. The 26 microsatellites whose genotypes were in HW equilibrium will prove useful in delineating geographic stocks of ‘wild’ cobia and in this way contribute to wise and effective management of cobia resources in U.S. waters. The multiplex panels developed will reduce costs of using the microsatellites.

IV. PURPOSE

A. *Description of problems:*

The specific priority to which the proposed activities responded was ‘Marine Aquaculture’ and implementation of aquaculture in the offshore (i.e., EEZ) environment. Issues addressed directly by the project included legal constraints that might be imposed in marketing products (cobia, in this case) cultured offshore, genetic selection for sustainable economic return through genetic improvement of cobia broodstock in either offshore or land-based aquaculture, and future efforts that relate to wise and effective management of wild cobia resources. Cobia, *Rachycentron canadum*, is a highly prized food and recreational-trophy fish and is considered a prime candidate for aquaculture development because of rapid juvenile growth and an expanding demand in the seafood marketplace. The project sought to develop species-specific genetic tools and background information that could be employed to address three issues, two of which impact both offshore and land-based aquaculture of cobia. The first issue was ‘forensics and the need for unequivocal genetic-based methods to identify or distinguish products harvested in aquaculture operations from wild stocks in order to ensure legal sale and alleviate potential conflicts. The second issue was future genetic improvement of cobia broodstock relative to any number of performance traits. The genetic markers that were to be developed could be employed in ‘common-garden-variety’ experiments to determine the additive genetic component (heritability) of variation in performance traits ranging from growth rate to various physiological/ecological parameters to marine survival to disease resistance. The third issue regarded assessment and allocation of ‘wild’ cobia resources in U.S. waters. The genetic markers to be developed are optimal tools for assessing population structure of marine fishes such as cobia.

B. *Objectives:*

The overall project objective is to develop 25-30 polymorphic microsatellite DNA markers that were specific for cobia and that could be utilized in forensic, quantitative genetic (broodstock enhancement), and stock-structure applications. Optimization of experimental conditions for assay of the microsatellites was a key experimental objective. Effective distribution/dissemination of project results was another key objective.

V. APPROACH

A. *Work performed:*

Complete details regarding genomic library construction, ligation of size-selected (500-2,000 base pair) fragments into cloning vectors, and transformation into competent *Escherichia coli* cells may be found in the first (Pruett et al. 2005) of the three papers published (or about to be published) based on the work done in the project. Copies of the three papers are appended to this report. Briefly, a total of 19,200 clones were hybridized with cocktails of oligonucleotide probes, and 164 positive clones were sequenced. A total of 54 clones containing microsatellite motifs were identified; 45 primer pairs were designed from sequences flanking the microsatellites by using the programs AMPLIFY 1.2 and NETPRIMER®. Optimization of PCR protocols was carried out on DNA from eight individuals. PCR amplifications were performed in 10µl reaction volumes, consisting of 1µl (~25 ng) DNA, 1µl of 10X reaction buffer (500mM KCl, 100mM Tris, 10% Triton-X 100), 0.1U of *Taq* DNA polymerase (GibcoBRL), 0.5µM of each primer, 200µM of each dNTP, and 1mM MgCl₂. PCR conditions consisted of an initial denaturation of 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at optimized temperature for 45 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Complete details of the development of the multiplex panels may be found in the paper by Renshaw et al, 2006 (appended).

B. *Project management:*

Several people participated variously in the project. These included S. C. Bradfield, J. C. Patton, C. L. Pruett, M. E. Renshaw, and E. Saillant of PI Gold's laboratory and C. E. Rexroad III of the USDA/ARS National Center for Cold and Cool Water Aquaculture. Personnel in {I

Gold's laboratory executed virtually all of the laboratory effort; Dr. Rexroad contributed critical advice involving sequencing.

VI. FINDINGS

A. *Actual accomplishments and findings:*

Forward and reverse PCR primer-pair sequences for 35 microsatellites were developed from a genomic library of cobia DNA and optimized according to standard procedures. The primer sequences, microsatellite motifs (repeat sequence), size of cloned alleles, and optimized annealing temperatures (AT) may be found in the appended publications. The 35 microsatellites included 26 di-, one tri-, and four tetranucleotide repeat motifs; four of the microsatellites contain complex repeats (i.e., a combination of different repeat motifs). Genotypes for all 35 microsatellites were acquired from 24 cobia sampled offshore of Ocean Springs, Mississippi. The number of assayed individuals (N), the number of alleles (A_N), and the range in size of detected alleles for each microsatellite also may be found in the appended publications. Thirty-three of the microsatellites were found to be polymorphic; the average number of alleles per polymorphic microsatellites was 7.1 (range = 2-17). For the polymorphic microsatellites, average observed heterozygosity was 0.496 (range = 0.000 – 1.000), while the average expected heterozygosity was 0.563 (range = 0.043 - 0.943). The average number of alleles and average expected heterozygosity (also called gene diversity) per microsatellite were lower than averages reported previously for several species of marine fishes. Genotypes at seven of the microsatellites differed significantly from Hardy-Weinberg equilibrium expectations following (sequential) Bonferroni correction for multiple tests performed simultaneously. Results of analysis by MICROCHECKER indicated that six of these seven microsatellites (all but *Rca* 1B-E06) had a general excess of homozygotes for most allele-size classes, suggesting the presence of null alleles. Development of multiplex included evaluation of 'mega-cocktail' PCR primer compatibility, reagents, and protocols, followed by the testing of primer concentrations to generate similar quantities of amplified products across all microsatellites. Once developed, cost effectiveness of the multiplex panels was estimated in terms of supplies and labor required for running single microsatellite gels versus four- and eight-microsatellite panels. The total costs per microsatellite for 96-well reactions (= 96 samples) was ~\$64.00 (single microsatellite gels)

versus ~\$18.25 (four-microsatellite panel) versus ~\$9.50 (eight-microsatellite panels). Personnel time per microsatellite (also estimated for 96 samples) was reduced as well: single microsatellite gels involved ~2.5 hours, whereas four- and eight-microsatellite panels involved ~45 minutes and ~30 minutes, respectively. Estimates of personnel times were based on an experienced research assistant.

B. *Problems encountered*

No problems that affected final results were encountered.

VII. EVALUATION

A. *Attainment of project goals:*

All project goals were attained with no modification(s) to goals and objectives.

Development of the multiplex panels was 'extra' in that development of the panels was not proposed initially.

B. *Dissemination of project results:*

A total of three manuscripts were prepared based on project accomplishments; one (Pruett et al. 2005) is already published in a scientific journals, while the other two (Renshaw et al, 2005, 2006) are 'in press' at two different scientific journals. These publications are listed below.

Complete copies are appended.

Pruett, C.L., Saillant, E., Renshaw, M. A., Patton, J.C., Rexroad, C.E. III, and Gold, J.R. (2005). Microsatellite DNA markers for parentage assignment and population-genetic studies in cobia, *Rachycentron canadum*. *Molecular Ecology Notes* 5: 84-86.

Renshaw, M. A., Pruett, C. L, Saillant, E., Patton, J. C., Rexroad, C. E. III, and Gold, J. R. (2005). Microsatellite markers for cobia, *Rachycentron canadum*. *Gulf of Mexico Science*. In press.

Renshaw, M. A., Saillant, E., and Gold, J. R. (20__). Microsatellite multiplex panels for genetic studies of three species of marine fishes: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*). *Aquaculture*. In press.