FINAL REPORT

I. PROJECT INFORMATION

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

Author: John R. Gold

Organization: Texas Agricultural Experiment Station

Grant Number: NA17FD2371 (Saltonstall-Kennedy Program)

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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 $MgCl_2$. 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.

Molecular Ecology Notes (2005) 5, 84–86 doi: 10.1111/j.1471-8286.2004.00840.x

PRIMER NOTE

Microsatellite DNA markers for population genetic studies and parentage assignment in cobia, *Rachycentron canadum*

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Abstract

Twenty nuclear-encoded microsatellites from a genomic DNA library of cobia, *Rachycentron canadum*, were isolated and characterized. The microsatellites include two tetranucleotide, one trinucleotide, three combination tetranucleotide/dinucleotide, nine dinucleotide, and five imperfect (dinucleotide) repeat motifs. Gene diversity ranged between zero to 0.910; the number of alleles among a sample of 24 fish ranged from one to 15. Cobia support an important recreational fishery in the southeastern United States and recently have become of interest to aquaculture. The microsatellites developed will be useful tools for studying both population genetics (e.g. stock structure, effective population size) and inheritance of traits important to aquaculture.

Keywords: cobia, genomic library, microsatellites, polymerase chain reaction primers, *Rachycentron canadum*

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Cobia, *Rachycentron canadum*, is an economically important, pelagic fish distributed in tropical warm waters worldwide (Shaffer & Nakamura 1989). It is a highly prized food and recreational trophy fish, and is considered a prime candidate for aquaculture (Benetti *et al.* 2003). Because of the popularity of cobia as a 'game' fish, methods to identify or distinguish products harvested in cobia aquaculture from 'wild' stocks will be needed in order to ensure legal sale and alleviate potential conflicts. Nuclear-encoded microsatellites (Weber & May 1989) are especially well suited for this purpose because of their codominant, Mendelian inheritance and their high levels of polymorphism. Microsatellites also have many applications in breeding programs (Garcia de Leon *et al.* 1998) and for assessing population structure of 'wild' populations as a means to improve assessment and allocation of resources. In this note, we report the development from a genomic library of cobia DNA of polymerase chain-reaction (PCR) primers for 20, nuclear-encoded microsatellites.

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Whole genomic DNA was extracted from cobia muscle tissue using a standard phenol-chloroform method and digested with *Dpn*II (New England BioLabs). Fragments ranging in size from 500 to 2000 bp were size selected by extraction from a 1% agarose gel and purified using a gel extraction kit (Qiagen). Fragments were ligated into a *Bam*HI (New England BioLabs) digested and dephosphorylated (Calf Intestinal Alkaline Phosphatase, New England BioLabs) pBluescript vector using T4 DNA ligase (New England BioLabs) and transformed into XL10-Gold ultracompetent cells (Stratagene). Transformed cells were plated on X-Gal – IPTG Luria-Bertani (LB) agar with 50 μg/mL of ampicillin and grown overnight at 37 °C. Recombinant colonies were picked using a GENETIX QBOT, inoculated into 384 well plates that contained 50 μL of LB freezing media [36 mm K_2HPO_4, 13.2 mm KH_2PO_4, 1.7 mm sodium citrate, 0.4 mm $MgSO_4$, 6.8 mm $(NH_4)_2SO_4$, 4.4% (v/v) glycerol, 50 μg/mL ampicillin, LB], and incubated overnight at 37 °C before freezing at –80 °C.

A total of 19 200 clones (50 × 384-well plates) were spotted in a 4 × 4 array onto 22.5 cm × 22.5 cm Hybond nylon membranes (Amersham), with each clone being spotted twice to eliminate false positives. Membranes were placed

on LB agar plates with 50 μg/mL of ampicillin and incubated at 37 °C until colonies were approximately 1–2 mm (18–24 h). Membranes were placed on chromatography paper (3M) and soaked as follows to fix colonies: 10% SDS for 3 min, denaturing solution (1.5 mm NaCl, 1.5 Tris) for 5 min, and 2 × SSC for 5 min. Filters were incubated for 5 h at 65 °C.

Resulting colonies were probed with two cocktails of [gamma] ^{32}P-labelled oligonucleotides: (i) tetranucleotides $[GATA]_9$, $[CATA]_8$, $[GACA]_8$ and trinucleotide $[CAA]_8$, and (ii) trinucleotides $[GAA]_8$, $[TAA]_{13}$, and dinucleotides $[GA]_{13}$ and $[CA]_{13}$. A total of 164 positive clones were screened as follows. Frozen glycerol stocks arrayed in 96-well plates were used to inoculate 1 mL cultures of Luria Broth selective media (ampicillin) and incubated overnight at 37 °C. Plasmid DNA was isolated (alkaline lysis) with a BioRobot 8000 (QIAGEN, CA). Miniprep DNA was quantified, normalized, and both strands sequenced, using M13 forward and reverse sequencing primers and ABI BigDye Terminator v3.1. Products were purified and electrophoresed on an ABI 3100 DNA Analyser (Applied Biosystems, CA). Phred (CodonCode, MA) was used for DNA sequence base calling and vector trimming. A total of 54 complete sequences containing microsatellite arrays were obtained from the positive clones. Three of the clones contained two repeat sequences from which primer pairs were designed. A total of 45 primer pairs were developed using AMPLIFY 1.2 (Engels 1993) and Netprimer® (http://www.premierbiosoft.com/netprimer).

Unlabelled PCR primers were purchased from Invitrogen (Carlsbad, CA) and tested for amplification by screening DNA isolated from eight individuals obtained from offshore of Ocean Springs, Mississippi. PCR amplifications were performed in 10 μL reaction volumes containing 1 μL (100 ng) DNA, 1 μL 10× reaction buffer (500 mm KCl, 100 mm Tris, 10% Triton-X 100), 0.1 U of *Taq* DNA polymerase (Gibco-BRL), 0.5 μm of each primer, 200 μm of each dNTP, and 1 mm $MgCl_2$. PCR conditions consisted of an initial denaturation of 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C–65 °C for 45 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide. Once appropriate annealing temperatures for each primer were determined, microsatellite arrays were tested for polymorphisms by end-labelling (using T4 polynucleotide kinase) one primer from each pair with [γ^{32}P]-dATP and employing the same conditions as above. PCR products were electrophoresed in 6% polyacrylamide gels and visualized by autoradiography; 20 microsatellite repeats were chosen for further screening (Table 1). These microsatellites included one trinucleotide, two tetranucleotide, three combination tetranucleotide/dinucleotide, nine dinucleotide, and five imperfect dinucleotide repeats. Two of the microsatellites were on the same clone, *Rca*1B-E08A and *Rca*1B-E08B. Lengths of cloned alleles ranged in size from 122 to 308 base pairs. Optimal annealing temperatures ranged from 48 °C–60 °C.

Screening involved a total of 24 cobia obtained from offshore of Ocean Springs, Mississippi. One primer from each pair was fluorescently labelled with one fluorescent label of set D (Applied Biosystems) for run on an automated sequencer ABI-377. Alleles were sized using the GENESCAN® 400 HD Size Standard (Applied Biosystems); allele sizing and calling were performed using GENESCAN® 3.1.2 and GENOTYPER®, version 2.5 software. Genetic variability of the markers was measured by the number of alleles, gene diversity (expected heterozygosity), and the observed heterozygosity. Wright's F_{IS}, estimated as Weir and Cockerham's f in Genetic Data Analysis (GDA) (Lewis & Zaykin 2001), was used to measure departure of genotype proportions from Hardy–Weinberg expectations at each microsatellite. Fisher exact tests, as performed in GDA, were used to test significance of departures from Hardy–Weinberg equilibrium (genotype) expectations at each microsatellite and for departure from genotypic equilibrium at pairs of microsatellites. The effect of Hardy–Weinberg departures (within locus disequilibrium) on significance of between locus linkage disequilibrium tests was removed by preserving genotypes in GDA (Lewis & Zaykin 2001).

Summary data are presented in Table 1. The number of alleles detected per microsatellite ranged from one to 15. Expected heterozygosity ranged from zero to 0.910, while observed heterozygosity ranged from zero to 0.957. Genotypes at four microsatellites deviated significantly from Hardy–Weinberg expectations following sequential Bonferroni correction (Rice 1989). Three of the microsatellites exhibited heterozygote deficiency (*Rca* 1B-F06, $F_{IS} = 0.324$; *Rca* 1-B12, $F_{IS} = 0.578$; and *Rca* 1-E06, $F_{IS} = 0.468$), while one (*Rca* A-10) exhibited heterozygote excess ($F_{IS} = -0.079$). All pairwise comparisons of microsatellites did not deviate significantly from genotypic equilibrium following Bonferroni corrections (Rice 1989). The 20 microsatellites developed in this work will prove useful in future studies of population genetics and quantitative genetics of 'wild' and domesticated cobia, respectively.

Acknowledgements

We thank J. Franks of the Gulf Coast Research Laboratory and J. Graves of the Virginia Institute of Marine Science for providing the cobia tissues, and C. Bradfield for technical assistance in the laboratory. Funding was provided by the Saltonstall-Kennedy Program of U.S. Department of Commerce (Grant NA17FD2371) and by the Texas Agricultural Experiment Station (Project H-6703). This paper is number 42 in the series 'Genetic Studies in Marine Fishes' and Contribution no. 127 of the Center for Biosystematics and Biodiversity at Texas A & M University.

Table 1 Summary data for microsatellites developed from cobia, *Rachycentron canadum*

Microsatellite	Primer sequence (5′–3′)*	Repeat sequence†	T_A	N‡/N_A§	Size Range¶	H_E/H_O**	P_{HW}††
Rca 1B-A10	**GCAGCCCAATGCTAACAAGCC¶¶** CATGTAGTCAAGCGAGCCACG	$(GTT)_6$	60	24/4	174–186	0.735/0.792	**0.003**
Rca 1B-D09	**CAGCCTGCTTAGCCTATCA** GAAGGATGGACCACTTGTGAC	$(GT)_9(CTGT)_2(CT)_2(GT)_2$	60	23/1	169	0.000/0.000	1.000
Rca 1B-E02	**GTGTTGCAGCCAAATGCTA** CTCCCTAGTGCCACTACAGCTC	$(CT)_{18}$	60	24/7	298–314	0.598/0.667	0.483
Rca 1B-E08A§§	**CATATCAAGTCAATATCACAGACC** CCACGGAATAGCAGACTTTCTC	$(CA)_3GA(CA)_5A(CA)_{16}$	55	24/5	181–225	0.582/0.458	0.030
Rca 1B-E08B	**GCAGTTGATTCTGATTGCTACAC** CTAATGCCAGCTCATTATGTCC	$CA)_8GA(CA)_3$	60	24/2	120–122	0.496/0.417	0.672
Rca 1B-F06	**CAAGCAAATGCGTGGCCGA** CGTTAGCAACCACACGAGCTTG	$(CTAT)_{15}$	55	24/11	260–300	0.796/0.542	**0.000**
Rca 1B-F07	**GGAATCTGGTGGTGAGTCAT** CTGTGGCTGAAGCGTGTGTT	$(GACA)_6(CA)_{12}$	55	24/3	132–140	0.082/0.083	1.000
Rca 1B-G10	**GGAAACTCTATAACAGCATGTC** GTAGACAGAGCAACACATGAG	$(CT)_5TT(CT)_4$	55	23/2	154–156	0.043/0.043	1.000
Rca 1B-H09	**CATGTTATTCTCCAACTCATGG** GTGTATCCGCATACTTTCAG	$(GATA)_{31}$	48	23/12	176–224	0.910/0.957	0.351
Rca 1-A04	**CACGCACATGCACTACTTTAACC** GCTGTTGATGTGGCGAAGCAAC	$(CA)_9(CACT)_4$	60	24/6	196–206	0.722/0.625	0.095
Rca 1-A11	**CTACAGTGGTGTTCCCTGTTAG** CAGTACATAGAGAAACAGGAGG	$(GT)_{24}$	55	24/15	167–201	0.889/0.792	0.271
Rca 1-B12	**GCTTCAGGCAAGTGAGACC** GGGAGGTAATTATGTCCTGT	$(AC)_9$	55	24/7	177–193	0.780/0.333	**0.000**
Rca 1-C04	**GACATCAAGTGGCACTTTG** CACTAAACTTGTTCCTCCTG	$(GT)_{17}$	48	24/10	223–253	0.641/0.667	0.188
Rca 1-D04	**GCTGAACTTGTCGCCGCT** GGACTGAACCTCCCTATCCTC	$(TG)_9AC(TG)_5$	60	24/3	125–129	0.551/0.667	0.723
Rca 1-D11	**CGTAACACCTTTGGAAGACATC** CTCCATTGAGGCTGACTAGTG	$(GT)_8$	55	24/4	204–212	0.295/0.333	1.000
Rca 1-E04	**CCAAGAACAGGCGGGCAAC** GCCACCATTGTGTGTGGGTGA	$(CA)_8$8 bp$(CA)_5$	55	23/4	216–238	0.336/0.391	1.000
Rca 1-E06	**GGCACCAATCACTCACTACTG** TGTTGAGGTCTATCAGTGCC	$(CA)_{39}$	48	24/10	144–188	0.853/0.458	**0.000**
Rca 1-E11	**GTCCCAGCTCCAGCCCAAAC** GACACTGGCTGCGTGAGCA	$(CA)_{12}$	55	23/7	167–181	0.757/0.783	0.245
Rca 1-F01	**GCTCATTTCACTAAGTGTGTTGTAGC** CCATGAATCTACATTCACCTGCCA	$(TG)_{12}$	60	24/2	202–206	0.120/0.125	1.000
Rca 1-G05	**GGGCTGTCTGCTGGCTGTAA** GCATCTGTGTCCTGGTGAGAGTCCC	$(GT)_{17}$	60	24/7	274–282	0.697/0.667	0.148

*Primer sequences are forward (top) and reverse (bottom); †Repeat sequence indicates the repeat motif; T_A is annealing temperature in °C; ‡N is the number of individuals assayed; §NA is number of alleles detected; ¶Size range refers to alleles thus far uncovered; **H_E and H_O are expected and observed heterozygosity, respectively; ††PHW represents the probability of deviation from Hardy–Weinberg expectations (significant values after Bonferroni correction are in bold) and sequences of clones are listed in GenBank (Accession numbers AY721664–AY721682); §§Primers Rca 1B-E08A and Rca 1B-E08B were developed from the same clone; ¶¶The fluorescently labelled primer is in bold.

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Gulf of Mexico Science (In press)

Microsatellite Markers for Cobia, *Rachycentron canadum*

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Polymerase chain reaction (PCR) primers are reported for 35 nuclear-encoded microsatellites 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. 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.

Cobia, *Rachycentron canadum*, is a migratory, coastal pelagic fish distributed in tropical and subtropical warm waters worldwide except for the eastern Pacific (Shaffer and Nakamura, 1989). The species constitutes an important recreational fishery in the Gulf of Mexico (Brown-Peterson *et al.*, 2001), and is caught incidentally in the commercial fishery (Shaffer and Nakamura, 1989). Interest in cobia aquaculture in the U.S. has been spiked recently by successes in captive spawning and larval rearing (Dodd, 2001), and it has been suggested (Bridger and Costa-Pierce, 2002) that cobia may be an ideal species for offshore cage culture.

In this note, we report optimized polymerase-chain-reaction (PCR) primers for 15 nuclear-encoded microsatellites developed from a cobia genomic library. Briefly, microsatellites are short stretches of nuclear DNA composed primarily of di-, tri-, and tetranucleotide repeats inherited in a codominant

(Mendelian) fashion and distributed throughout euchromatic regions of chromosomes (Weber and May, 1989; Weber, 1990; Wright and Bentzen, 1994). Microsatellites also accumulate mutations fairly rapidly (Shug et al., 1998), making them ideal genetic markers for a variety of applications ranging from stock-structure analysis of 'wild' populations (Gold and Turner, 2002; Zatcoff *et al.*, 2004) to parentage assignment and pedigree reconstruction in domesticated populations (Wilson and Ferguson, 2002; Jones and Arden, 2003). Included in this note are summary data for 20 other microsatellites for cobia developed in our laboratory by Pruett et al. (2005). The summary data for all 35 microsatellites include number and size of alleles detected, observed and expected heterozygosity, and results of tests of conformity to Hardy-Weinberg equilibrium expectations at each microsatellite. The summary data are published here to allow convenient access to all PCR primers and other data.

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 Pruett et al. (2005). 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 (Engels, 1993) and NETPRIMER® (http://www.premierbiosoft.com/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 $MgCl_2$. 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 (Table 1) for 45 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

The primer-pair sequences (forward on top, reverse on bottom), microsatellite motifs (repeat sequence), size of cloned alleles, and optimized annealing temperatures (AT) are given in Table

1. The suite of 35 microsatellites includes 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 are given in Table 1. Thirty-three of the microsatellites were found to be polymorphic; the average number of alleles per polymorphic microsatellites was 7.1 (range = 2-17). Estimates of observed (H_O) and expected (H_E) heterozygosity were computed using GDA (Lewis and Zaykin, 2001) and are given in Table 1. 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 are lower than averages reported previously by DeWoody and Avise (2000) for several species of marine fishes. Probabilities of departure from Hardy-Weinberg equilibrium expectations (P_{HW}) were computed using exact tests, as implemented in GDA (Lewis and Zaykin, 2001) and also are given in Table 1. Genotypes at seven of the microsatellites differed significantly from Hardy-Weinberg equilibrium expectations following (sequential) Bonferroni correction for multiple tests performed simultaneously (Rice, 1989). Results of analysis by MICROCHECKER (Van Oosterhout *et al.* 2004) 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. The 26 microsatellites whose genotypes were in HW equilibrium should prove extremely useful in future studies of cobia relating to both stock-assessment and aquaculture. The use of microsatellites as selectively neutral genetic markers to assess geographic boundaries and genetic diversity of 'wild' stocks is well reviewed in Wright and

Bentzen (1994) and Carvalho and Hauser (1995); the use of microsatellites in aquaculture includes parentage assignment, pedigree reconstruction, mapping of quantitative trait loci, and marker-assisted selection and is well reviewed in Liu and Cordes (2004).

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Table 1. Summary data for 35 microsatellites developed from a cobia (*Rachycentron canadum*) genomic library. PCR primer sequences are forward (top) and reverse (bottom). Primers *Rca* 1B-E08A and *Rca* 1B-E08B were developed from a single clone. Sequences of clones are listed under GenBank Accession Numbers AY721664-AY721682 and AY850008-AY850022. Significant deviations from Hardy-Weinberg expected proportions are in bold.

Microsatellite	PCR primer sequences (5'→3')	Repeat sequence of cloned allele	Size of cloned allele (base pairs)	AT	N	A_N	Range in allele size (base pairs)	H_O/H_E	P_{HW}
Rca 1B-A10	GCAGCCCAATGCTAACAAGCC CATGTAGTCAAGCGAGCCACG	$(GTT)_6$	180	60	24	6	169-187	0.417/0.723	**0.000**
Rca 1B-C06	CCAGCATATCTCCTCTCAAGA GGCTTGAACTTAACTACAGCTCCT	$(GATA)_{29}$	346	50	23	13	340-404	0.870/0.904	0.370
Rca 1B-D09	CAGCCTGCTTAGCCTATCA GAAGGATGGACCACTTGTGAC	$(GT)_9(CTGT)_2(CT)_2(GT)_2$	167	60	23	1	168	0.000/0.000	1.000
Rca 1B-D10	GCAACTGCCTCCACCAATCA CATGTGCATCGAAAGACAGAGA	$(CTAT)_{15}$	191	50	24	17	143-223	1.000/0.943	0.597
Rca 1B-E02	GTGTTGCAGCCAAATGCTA CTCCCTAGTGCCACTACAGCTC	$(CT)_{18}$	308	60	24	7	297-313	0.667/0.598	0.503
Rca 1B-E06	GGATCAGTGTGTTGCAGCCA CCCTAGTGCCACTACAGCTCCCT	$(TC)_{18}$	314	45	24	8	305-327	0.625/0.695	**0.000**
Rca 1B-E08A	CATATCAAGTCAATATCACAGACC CCACGGAATAGCAGACTTTCTC	$(CA)_3GA(CA)_5A(CA)_{16}$	227	55	24	5	181-225	0.458/0.582	0.028
Rca 1B-E08B	GCAGTTGATTCTGATTGCTACAC CTAATGCCAGCTCATTATGTCC	$(CA)_8GA(CA)_3$	122	60	24	2	121-123	0.458/0.510	0.692
Rca 1B-F06	CAAGCAAATGCGTGGCCGA CGTTAGCAACCACACGAGCTTG	$(CTAT)_{15}$	268	55	24	11	260-300	0.542/0.796	**0.000**
Rca 1B-F07	GGAATCTGGTGGTGAGTCAT CTGTGGCTGAAGCGTGTGTT	$(GACA)_6(CA)_{12}$	140	55	24	3	131-139	0.083/0.082	1.000
Rca 1B-G10	GGAAACTCTATAACAGCATGTC	$(CT)_5TT(CT)_4$	154	55	23	2	153-155	0.043/0.043	1.000

	GTAGACAGAGCAACACATGAG								
Rca 1B-H09	CATGTTATTCTCCAACTCATGG GTGTATCCGCATACTTTCAG	$(GATA)_{31}$	220	48	23	12	176-224	0.957/0.910	0.343
Rca 1-A04	CACGCACATGCACTACTTTAACC GCTGTTGATGTGGCGAAGCAAC	$(CA)_9(CACT)_4$	202	60	24	6	196-206	0.625/0.722	0.107
Rca 1-A08	GGATCATAAGGGATTGTGCTA CCTCGAGCCATATCATCAT	$(GT)_{13}GCAT(GT)_5$	289	48	24	8	287-321	0.208/0.575	**0.000**
Rca 1-A11	CTACAGTGGTGTTCCCTGTTAG CAGTACATAGAGAAACAGGAGG	$(GT)_{24}$	187	55	24	15	167-201	0.792/0.889	0.265
Rca 1-B12	GCTTCAGGCAAGTGAGACC GGGAGGTAATTATGTCCTGT	$(AC)_9$	181	55	24	9	176-196	0.500/0.808	**0.000**
Rca 1-C04	GACATCAAGTGGCACTTTG CACTAAACTTGTTCCTCCTG	$(GT)_{17}$	219	48	24	10	223-253	0.667/0.641	0.185
Rca 1-D04	GCTGAACTTGTCGCCGCT GGACTGAACCTCCCTATCCTC	$(TG)_9AC(TG)_5$	127	60	24	3	125-129	0.667/0.551	0.733
Rca 1-D07	CCATGGCTACAATCTGGTTCATC CGAATGCTGTGGAGAACAGG	$(GT)_9TTT(GT)_3$	157	60	23	4	154-162	0.130/0.128	1.000
Rca 1-D08	GCTTGACTCCAGCTCAAAC CACAAGGACGAGCCTCCA	$(CA)_{10}$	172	55	23	4	172-178	0.261/0.274	0.074
Rca 1-D11	CGTAACACCTTTGGAAGACATC CTCCATTGAGGCTGACTAGTG	$(GT)_8$	208	55	24	4	204-212	0.333/0.295	1.000
Rca 1-E04	CCAAGAACAGGCGGGCAAC GCCACCATTGTGTGTGGGTGA	$(CA)_88bp(CA)_5$	220	55	23	4	215-237	0.391/0.336	1.000
Rca 1-E05	GCAGTCGAGACGTGACTGAACGA CGTGGAGCTGCTCTGCAGGA	$(CA)_{20}(CGCA)_4(CA)_7(CGCA)_4$	248	55	24	8	241-259	0.542/0.768	**0.000**
Rca 1-E06	GGCACCAATCACTCACTACTG TGTTGAGGTCTATCAGTGCC	$(CA)_{39}$	180	48	24	9	144-186	0.458/0.826	**0.000**

Rca 1-E11	GTCCCAGCTCCAGCCCAAAC GACACTGGCTGCGTGAGCA	$(CA)_{12}$	173	55	23	7	167-181	0.783/0.757	0.236
Rca 1-F01	GCTCATTTCACTAAGTGTGTTGTAGC CCATGAATCTACATTCACCTGCCA	$(TG)_{12}$	198	60	24	2	201-205	0.125/0.120	1.000
Rca 1-F07	GCATCGGGTTGAGTTGTACT CGTTGCCTGTCAATCTGTGCT	$(CA)_6CG(CA)_3$	235	60	23	1	235	0.000/0.000	1.000
Rca 1-F10	CCGTTCTGTACAGACGTGAAC GCCTGTTGCTGTTTCCTGTCA	$(CA)_2CG(CA)_{12}CG(CA)_4$	287	55	23	5	287-297	0.261/0.423	0.004
Rca 1-F11	GTTGCCATGGCGACCGAGA GCCCCTATGTCTCGTTCCATC	$(GA)_8AA(GA)_5$	122	55	24	2	119-121	0.000/0.082	0.022
Rca 1-G02	GGGACCATGTGAACTCATGCT CCAGACATGGACTGGTACACCT	$(GT)_{14}$	238	60	23	2	240-244	0.043/0.043	1.000
Rca 1-G05	GGGCTGTCTGCTGGCTGTAA GCATCTGTGTCCTGGTGAGAGTC	$(GT)_{17}$	280	60	24	5	275-283	0.667/0.651	0.185
Rca 1-H01	GTCCCAAGGGAATAGCGAAG CCTCCAGACCAGACAGCAGA	$(CA)_{37}$	298	48	23	12	275-311	0.826/0.885	0.129
Rca 1-H04A	GGGAGCCATGTGGTACAGACT GGGCTTTACGAAAGATAGCTGA	$(GT)_{18}$	161	60	24	3	156-162	0.667/0.550	0.269
Rca 1-H08	GAGACCTACATGGCAGAAGGT GACCACTCCTTTGAGGTCTCT	$(GT)_{30}$	278	60	24	9	273-299	0.708/0.696	0.984
Rca 1-H10	GCACCGCACTGCACAACAC GCTGTGCATACTCACACTGCT	$(CA)_{16}$	121	60	24	8	119-139	0.583/0.777	0.145

AQUACULTURE (IN PRESS)

TECHNICAL PAPER

Microsatellite multiplex panels for genetic studies of three species of marine fishes: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*)

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Abstract

Multiplex panels of nuclear-encoded microsatellites were developed for three species of marine fishes of interest to both public and private aquaculture ventures: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*). The multiplex panels will be useful in a variety of applications, including strain and hybrid identification, parentage assignment, pedigree reconstruction, estimating genetic diversity and/or inbreeding, mapping of quantitative trait loci, and marker-assisted selection. The panels also will be useful in studies of stock-structure of 'wild' populations. Comparison of costs for expendable supplies revealed that four- and eight-panel multiplexes reduced expenditures four- and eight-fold, respectively, relative to single microsatellite gels. Personnel time also was reduced significantly.

Keywords: Microsatellites, multiplexes, aquaculture, red drum, *Sciaenops ocellatus*, red snapper, *Lutjanus campechanus*, cobia, *Rachycentron canadum*

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1. Introduction

Microsatellites are hypervariable, nuclear-encoded genetic markers that are widely used in a variety of aquaculture applications, including strain and hybrid identification, parentage assignment, pedigree reconstruction, estimating genetic diversity and/or inbreeding, mapping of quantitative trait loci, and marker-assisted selection (Liu and Cordes, 2004). The large number of microsatellites needed for many of these applications can often generate high costs due to materials and personnel time. Major cost reduction, however, can be achieved through multiplexing, the combination of polymerase-chain-reaction (PCR) amplification products from multiple microsatellites into a single lane of an electrophoretic gel (Olsen et al., 1996; Neff et al., 2000). Multiplexing can be accomplished through either co-amplification of multiple microsatellites in a single PCR cocktail (Chamberlain *et al.*, 1988) or combination of PCR products from multiple, single amplification reactions (Olsen *et al.*, 1996). A blend of the two approaches can optimize sample throughput (Devey *et al.*, 2002; Paterson *et al.*, 2004). Despite its cost-effectiveness multiplexing is often not employed, apparently because of a general apprehension that it increases complexity of microsatellite genotyping (Neff *et al.*, 2000).

In this technical report, we describe simple and straightforward protocols for multiplexing microsatellites in each of three species of marine fishes of interest to public and private (commercial) aquaculture: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*). Briefly, red drum is an estuarine-dependent sciaenid found primarily along the Atlantic and Gulf coasts of the southern United States (U.S.). Culture, including breeding, of red drum is relatively advanced (Lee and Ostrowski, 2001). Extensive use by resource management agencies of hatchery-raised red drum to enhance 'wild' stocks occurs in several southern states (Grimes, 1998; Smith et al., 2001), especially Texas (McEachron et al.,

1995). Commercial (private) aquaculture of red drum also occurs in the southern U.S. (Lee and Ostrowski, 2001), and in China, Israel, and Taiwan as well (Lutz, 1999; Hong and Zhang, 2003). Culture of red snapper, a structure- or reef-associated lutjanid found primarily along the continental shelf of the Gulf of Mexico (Hoese and Moore, 1977), is far less advanced. Wild-caught adults have been successfully strip-spawned in captivity following hormone inducement (Lee and Ostrowski, 2001; Riley et al., 2004), and efforts to optimize aquaculture protocols to mass produce fingerlings for stock enhancement and commercial aquaculture are underway (Chigbu et al., 2002; Riley et al., 2004). In addition, the grow-out and market potential for red snapper make it ideal for offshore cage culture (Bridger and Costa-Pierce, 2002). Cobia is a nearly cosmopolitan, pelagic species found in tropical and subtropical waters (Shaffer and Nakamura, 1989). Like red snapper, cobia is relatively new to aquaculture, although in Taiwan, cobia is now a very popular and profitable farmed marine fish (Liao et al., 2004). Recent success in captive spawning and progeny production in the U.S. (Dodd, 2001) has generated considerable interest in commercial aquaculture of cobia (Lee and Ostrowski, 2001; Smith et al., 2001).

PCR primer sets for microsatellites in all three species were developed previously for use in stock-structure analysis of 'wild' populations: red drum (Saillant et al., 2004); red snapper (Bagley and Geller, 1998; Gold et al., 2001), and cobia (Pruett et al., 2005; Renshaw et al., 2005). Subsets of these microsatellites were used to develop protocols for the microsatellite multiplex panels described in this report.

2. Materials and multiplex protocols

An alkaline-lysis method (Saillant et al., 2002) was used to purify DNA from tissue samples (fin clips or internal organs) that had been obtained for prior studies in our laboratory. All tissues had been fixed in 95% ethanol and stored at room temperature.

The initial step in designing multiplex protocols typically involves generating PCR sequences for individual microsatellites, optimizing annealing temperatures, and determining the approximate range in allele size that might be encountered for a given microsatellite. Because these data already were available for red drum, red snapper and cobia (Gold et al. 2001; Saillant et al. 2004; Renshaw et al. 2005), the initial effort was conceptual and involved combining individual microsatellites with similar, optimal annealing temperatures and assessing whether allele-size ranges overlapped and whether alternate ABI dyes could be employed when overlap occurred. Initial experiments in each species involved testing sets of eight to ten primers in 'mega-cocktails' where equimolar (5 picomol) amounts of each primer pair were run in the same PCR reaction. Other PCR reagents (buffer, magnesium, dNTPs, *Taq* polymerase) in the 'mega-cocktails' initially followed PCR procedures outlined in Saillant et al. (2004), Gold et al. (2001), and Renshaw et al. (2005) for red drum, red snapper, and cobia, respectively. One primer (either forward or reverse) from each pair was labeled with one of three different Applied Biosystems (ABI) fluorescent dyes of set D (6-FAM, HEX or NED). Microsatellites with non-overlapping allele-size ranges were labeled with the same fluorescent dye; whereas those with overlapping allele-size ranges were labeled with different dyes. Fragment analysis was carried out on an automated ABI-377 sequencer; fragments were sized using GENESCAN 3.1.2, and allele calling was performed using GENOTYPER® version 2.5.

Optimization of multiplex protocols began with screening of microsatellites that amplified in the 'mega-cocktails.' Primer pairs that failed to amplify were removed from the mix and further optimization focused on the remaining primer pairs; this led to six sets (hereafter multiplex panels or panels) of red drum microsatellites, three of red snapper microsatellites, and five of cobia microsatellites. Two 'touchdown' PCR protocols (I and II) were evaluated for each of the

14 panels. Touchdown protocols are used to amplify microsatellites with different, optimal annealing temperatures in the same PCR reaction via progressively reducing annealing temperature in successive annealing cycles (Rithidech et al., 1997; Fishback et al., 1999). The Touchdown I protocol featured a one-half degree (Celsius) reduction in annealing temperature at each of twelve cycles, as described by Fishback et al. (1999), followed by thirty cycles of amplification at a temperature (hereafter, 'bottom' temperature) six degrees below the starting annealing temperature. Initial evaluation started at the highest (optimal) annealing temperature for a given microsatellite included in each multiplex panel. The Touchdown II protocol was developed in our laboratory and involved a three-step reduction in annealing temperature. The initial step employed the highest optimal annealing temperature for a given microsatellite included in the panel, while the third step employed an annealing temperature slightly (1-2°C) below the 'bottom' temperature; the second step employed an annealing temperature intermediate between the other two. The Touchdown I protocol was composed of: (i) initial denaturation at 95°C for 3 min; (ii) 12 cycles of denaturation at 95°C for 30 sec, annealing (minus 0.5°C per cycle) for 1 min, and extension at 72°C for 4 min; (iii) 30 cycles of denaturation at 95°C for 30 sec, 'bottom' annealing temperature for 1 min, and extension at 72°C for 4 min; and (iv) final extension at 72°C for 10 min. The Touchdown II protocol was composed of three steps that followed an initial denaturation at 95°C for 3 min: Step - 1 seven cycles of denaturation at 95°C for 30 sec, annealing for 1 min, and extension at 72°C for 4 min; Step 2 - seven cycles of denaturation at 95°C for 30 sec, annealing for 1 min, and extension at 72°C for 4 min; and Step 3 - 28 cycles of denaturation at 95°C for 30 sec, annealing for 1 min, and extension at 72°C for 4 min. Final extension was at 72°C for 10 min. Annealing temperatures for both Touchdown I and II protocols are given in Tables 1-3.

The next step involved identifying the optimal touchdown protocol and corresponding annealing-temperature range for each of the 14 multiplex panels. Choice of touchdown protocol was based primarily on reliability of scoring PCR products at all microsatellites in a given panel. We also evaluated different concentrations of PCR reagents (including primers) to determine optimal conditions for each panel. Optimal PCR reactions across all panels were comprised of 1.5µl of DNA (approximately 50ng), 1µl of 10X reaction buffer [500mM KCl, 200mM Tris-HCl (pH 8.4)], 2mM $MgCl_2$, 2.5mM of each dNTP, 0.75 units *Taq* DNA polymerase (Gibco BRL), and various quantities of primers. Concentrations of different primers were adjusted relative to obtaining homogeneous amplification-product intensity at each microsatellite within a given panel, achieved by raising and lowering primer concentrations in response to PCR outcomes. Final volumes in all PCR 'cocktail' reactions were adjusted with double-distilled water to bring total cocktail volume to 11.5µl. Optimized PCR protocols for each of the 14 multiplex panels are given in Table 1 (red drum), Table 2 (red snapper), and Table 3 (cobia).

Development of the panels required evaluating an initial 'mega-cocktail' of PCR primer compatibility, reagents, and protocols, followed by the testing of primer concentrations to generate similar quantities of amplified products across all microsatellites. The latter allowed straightforward scoring of all the microsatellites included in the panels for all three species. The impetus for the work was the potential reduction in both personnel time (labor) and consumable supplies generally required for large genotyping projects (Neff et al., 2000). We assessed cost effectiveness by estimating expenses (in U.S. dollars) of supplies and labor required for running single microsatellite gels versus four- and eight-microsatellite panels. PCR supplies included *Taq* polymerase kits, primers and dyes, dNTPs, 96-well plates, tubes, pipet tips, and mineral oil; gel supplies included 44 HD Rox size standards, 96-well plates, long ranger singel packs,

sequencing combs, pipet tips, and formamide. 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. The estimates of personnel times were based on an experienced research assistant.

3. Synopsis

Multiplex panels of nuclear-encoded microsatellites were developed for three species of marine fishes of interest to aquaculture: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*). Optimization of each panel resulted in efficient assay and unambiguous scoring of microsatellites in all three species. The multiplex panels can be utilized in a variety of applications, including strain and hybrid identification, parentage assignment, pedigree reconstruction, estimating genetic diversity and/or inbreeding, mapping of quantitative trait loci, and marker-assisted selection. We presently are using the red drum panels drum to identify parentage in 'common-garden' experiments to estimate heritability of juvenile growth rates and thermal tolerance in red drum; the red snapper panels are being used to study stock-structure and genetic-effective size of 'wild' red snapper in the northern Gulf of Mexico.

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Table 1

Multiplex panels for red drum (*Sciaenops ocellatus*) developed from 31 microsatellite primers. Primer quantities (in picomols) and fluorescent labels (ABI dye) are given for finalized PCR cocktails. Allele size ranges are given for each microsatellite as previously described in Saillant et al. (2004). The multiplex PCR protocol and annealing temperatures (°C) for each panel are shown in the far right column; further description of the PCR protocols is given in the text. PCR primer sequences for each microsatellite may be found in Saillant et al. (2004).

Panel	Microsatellite	Quantity	ABI dye	Range in allele size	Touchdown protocol
1	*Soc*412	4.3	HEX	102-168	Touchdown I
	*Soc*416	3.2	NED	141-181	55° - 49.5°
	*Soc*417	0.7	6-FAM	86-112	49°
	*Soc*423	0.6	6-FAM	172-208	
	*Soc*428	2.7	HEX	172-242	
	*Soc*445	35	6-FAM	134-166	
2	*Soc*60	2.2	6-FAM	151-163	Touchdown I
	*Soc*140	0.5	NED	132-144	55° - 49.5°
	*Soc*201	9	HEX	224-243	49°
	*Soc*243	2	6-FAM	94-106	
	*Soc*419	1.4	6-FAM	238-260	
3	*Soc*19	5	6-FAM	195-267	Touchdown I
	*Soc*85	2.3	6-FAM	80-122	60° - 54.5°
	*Soc*138	4.5	HEX	77-123	54°
	*Soc*156	0.4	6-FAM		
	Soc 206	0.8	NED	246-265	
	*Soc*410	2.7	6-FAM	306-344	
4	*Soc*11	1.6	6-FAM	217-240	Touchdown II
	*Soc*83	9	HEX	114-142	62°
	*Soc*99	2.2	NED	131-209	56°
	*Soc*407	9	6-FAM	139-157	54°

	*Soc*424	10	HEX	204-230	
5	*Soc*400	3	6-FAM	245-266	Touchdown I
	*Soc*402	6	HEX	134-164	55° - 49.5°
	*Soc*404	4	6-FAM	150-212	49°
	*Soc*415	5	HEX	187-235	
	*Soc*432	0.8	HEX	98-118	
6	*Soc*44	7	HEX	211-271	Touchdown II
	*Soc*401	3.7	HEX	174-206	56°
	*Soc*433	0.7	6-FAM	84-102	52°
	*Soc*444	1	HEX	161-165	50°

Table 2

Multiplex panels for red snapper (*Lutjanus campechanus*) developed from 20 microsatellite primers. Primer quantities (in picomols) and fluorescent labels (ABI dyes) are given for finalized PCR cocktails. Range in allele size is based on work in our laboratory. The multiplex PCR protocol and annealing temperatures (°C) for each panel are shown in the far right column; further description of the PCR protocols is given in the text. PCR primer sequences for microsatellites with prefixes *Lca* and *Prs* may be found in Gold et al. (2001). PCR primer sequences for microsatellites *Ra*6, and *Ra*7 may be found in Bagley and Geller (1998).

Panel	Microsatellite	Quantity	ABI dye	Range in allele size	Touchdown protocol
1	*Lca*43	5	6-FAM	162-192	Touchdown II
	*Prs*260	0.9	6-FAM	111-129	56°
	*Prs*55	2.7	HEX	180-208	53°
	*Prs*229	0.9	HEX	123-137	50°
	*Prs*248	1.3	NED	212-260	
	*Prs*303	0.6	NED	124-150	
	*Lca*20	5	6-FAM	203-223	
2	*Prs*275	4	6-FAM	133-150	Touchdown II
	*Prs*137	7	6-FAM	155-185	54°
	*Prs*328	0.9	6-FAM	196-214	52°
	*Prs*282	5	HEX	113-143	50°
	*Prs*221	4	HEX	220-266	
	*Ra*6	2	NED	112-130	
	*Lca*64	5.2	HEX	151-181	
3	*Lca*91	5	6-FAM	130-144	Touchdown I
	*Lca*22	4	6-FAM	228-258	56° - 50.5°
	*Prs*333	0.7	HEX	156-157	50°
	*Prs*240	1.4	HEX	193-223	
	*Lca*107	4	HEX	96-120	
	*Ra*7	1.6	NED	145-167	

Table 3

Multiplex panels for cobia (*Rachycentron canadum*) developed from 35 microsatellite primers. Primer quantities (in picomols) and fluorescent labels (ABI dye) are given for finalized PCR cocktails. Allele size ranges are given for each microsatellite as previously described in Renshaw et al. (2005). The multiplex PCR protocol and annealing temperatures (°C) for each panel are shown in the far right column; further description of the PCR protocols is given in the text. PCR primer sequences for each microsatellite may be found in Renshaw et al. (2005).

Panel	Microsatellite	Quantity	ABI dye	Range in Allele size	Touchdown protocol
1	*Rca*1B-E08A	1.1	6-FAM	181-229	Touchdown II
	*Rca*1-E05	8	NED	221-261	55°
	*Rca*1-E04	0.7	HEX	215-237	53°
	*Rca*1-A11	2.5	NED	165-201	51°
	*Rca*1-E11	4.5	HEX	167-183	
	*Rca*1B-G10	1.1	6-FAM	153-155	
2	*Rca*1-D11	0.9	6-FAM	204-212	Touchdown II
	*Rca*1B-F06	2.2	HEX	260-308	55°
	*Rca*1-B12	1.2	NED	176-196	53°
	*Rca*1-D08	1.1	6-FAM	172-178	51°
	*Rca*1-F11	0.5	6-FAM	119-123	
	*Rca*1B-F07	0.8	HEX	131-143	
	*Rca*1-F10	1.6	NED	287-351	
3	*Rca*1B-A10	0.4	6-FAM	169-187	Touchdown II
	*Rca*1B-E02	4	6-FAM	297-315	60°
	*Rca*1-H10	1.2	HEX	119-139	57°
	*Rca*1-A04	1.8	HEX	196-206	54°
	*Rca*1-G05	1.9	HEX	269-285	
	*Rca*1-D07	1.5	NED	154-162	
	*Rca*1-F07	1.9	NED	235	
4	*Rca*1B-E08B	0.8	6-FAM	117-123	Touchdown II

	*Rca*1-F01	1.2	6-FAM	199-205	60°
	*Rca*1-D04	1.2	HEX	125-131	57°
	*Rca*1-H04A	2.5	HEX	156-162	54°
	*Rca*1-G02	2	HEX	240-244	
	*Rca*1B-D09	5	NED	168	
	*Rca*1-H08	10	NED	273-299	
5	*Rca*1B-C06	4	6-FAM	336-404	Touchdown I
	*Rca*1B-D10	1.4	6-FAM	143-223	50° - 44.5°
	*Rca*1B-E06	1	HEX	305-327	44°
	*Rca*1B-H09	10	HEX	168-224	
	*Rca*1-A08	4	6-FAM	287-321	
	*Rca*1-C04	3	NED	217-253	
	*Rca*1-E06	3.2	NED	144-186	
	*Rca*1-H01	3	NED	275-311	

Subject: Progress Report NA17FD2371
Date: 14-Aug-2005 11:52.22
From: "Scot Plank" <Scot.Plank@noaa.gov>
To: Scot Plank <scot.plank@noaa.gov>

This message is for the Federal Program Officer ONLY.
GMD no longer wishes to receive email certifications for uploads.

PROGRAM PROGRESS REPORT CERTIFICATION
FOR GRANTS AND COOPERATIVE AGREEMENTS

THIS CERTIFICATION WAS UPLOADED TO NGS FROM FISHERIES GRANTS

AWARD NUMBER:	NA17FD2371
RECIPIENT NAME:	Texas A&M University - College Station
REPORTING PERIOD:	12/01/04 to 05/31/05
DATE RECEIVED:	06/24/05
COMMENT:	
ACCEPTABLE REPORT:	Yes
TYPE OF REPORT:	Interim

ort 1.

Subject: Re: TM Review of TXAM Award NA17FD2371 Report 12/01/04 to 05/31/05
Date: Wed, 03 Aug 2005 09:48:48 -0400
From: Scot Plank <Scot.Plank@noaa.gov>
To: Patricia Rosel <Patricia.Rosel@noaa.gov>

Hello Patty,

Is the report acceptable? thx

Scot Plank wrote:

> *Hello Dr. Rosel,*
> *Attached is a copy of the above referenced report which was received in our office for your review and comments as to the acceptability of the work performed. Please indicate whether you believe the report demonstrates satisfactory progress toward achieving study objectives as described in project documents.*
> *Please provide one of the following responses by July 29, 2005.*
> *This report is acceptable as written.*
> *This report is acceptable, supplemental comments attached.*
> *This report is unacceptable, comments attached.*
> *Thank you,*
> *Scot*
> *727 551 5734*
> *Name: 2371SA.doc*
> *2371SA.doc Type: Microsoft Word (application/vnd.msword)*
> *Encoding: base64*
> *Name: 2371A.pdf*
> *2371A.pdf Type: Acrobat (application/pdf)*
> *Encoding: base64*
> *Name: 2371B.doc*
> *2371B.doc Type: Microsoft Word (application/vnd.msword)*
> *Encoding: base64*
> *Name: 2371C.doc*
> *2371C.doc Type: Microsoft Word (application/vnd.msword)*
> *Encoding: base64*
> *Name: 2371D.doc*
> *2371D.doc Type: Microsoft Word (application/vnd.msword)*
> *Encoding: base64*

Subject: Re: Reminder of Upcoming Progress Report Due
Date: Fri, 24 Jun 2005 15:33:10 -0500
From: "John Gold" <goldfish@tamu.edu>
To: <Scot.Plank@noaa.gov>

Scot,

Report and papers/manuscripts attached.

John

>>> "Scot Plank" <Scot.Plank@noaa.gov> 6/24/2005 11:38:51 AM >>>
Dear Dr. Gold:

Ref: Development of DNA Microsatellites for Genetic Applications in Cobia (Rachycentron canadum)

This is a reminder that the Semi-Annual
progress report for Award Number NA17FD2371
is due 06/30/2005.
The time period covered by this report is
12/01/2004 to 05/31/2005

You may email your report to me at
scot.plank@noaa.gov

Thank you for your timely attention to this matter.

Scot Plank

Report for December '04 to May '05.DOC	**Name:** Report for December '04 to May '05.DOC **Type:** Microsoft Word (application/msword) **Encoding:** base64

Cobia primers (MEN).pdf	**Name:** Cobia primers (MEN).pdf **Type:** Acrobat (application/pdf) **Encoding:** base64

Cobia primer note (GoM Sci).doc	**Name:** Cobia primer note (GoM Sci).doc **Type:** Microsoft Word (application/msword) **Encoding:** base64

Renshaw et al. (Aquaculture Manuscript).doc	**Name:** Renshaw et al. (Aquaculture Manuscript).doc **Type:** Microsoft Word (application/msword) **Encoding:** base64

Semi-Annual Progress Report

A. Grant Number: NA17FD2371

B. Amount of Grant: Federal $120,627 Match $40,542 Total$161,169

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

C. Grantee: John R. Gold

D. Award Period: From 6/1/02 To 5/31/05

E. Period Covered by this Report: From 12/01/2004 to 5/31/2005

F. Summary of Progress and Expenditures to Date:

1. Work Accomplishments:

a. Describe tasks scheduled for this period (from proposal and amendments, if appropriate).

(i) Finish optimizing20-25polymerase-chain-reaction (PCR) primer pairs designed from 150 putative microsatellite sequences obtained from a genomic library of cobia DNA.
(ii) Screen sample of cobia for polymorphism and fit of genotypes to Hardy-Weinberg equilibrium
(iii) Submit manuscript on first set of PCR primers.
(iv) Prepare manuscript on second set of PCR primers.

b. Describe tasks accomplished this period.

(i) A total of 35PCR primers pairs for microsatellites isolated from a genomic library of cobia DNA were optimized for amplification quality.
(ii) A sample of 24 cobia,obtained from offshore of Ocean Springs, Mississippi, was genotyped for allelic variation at all 35 microsatellites. 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.
(iii) Two manuscripts were submitted and accepted. One has been published and one is 'in 'press' (copies are attached to this report). A third manuscript, describing methods developed to 'multiplex' the microsatellites is in review. A copy of this manuscript also is attached.

c. Explain special problems, differences between scheduled and accomplished work, etc.

No problems were encountered.

2. Expenditures:

a. Describe expenditures scheduled for this period.

Salary	$10,000.00
Fringe	368.78
Dom Travel	0
Supplies	1,000.00
Other Direct	0

Reserve	0
Indirect	4,215.00
TOTAL	$15,583.78

b. Describe actual expenditures this period.

	ACTUAL
Salary	$13,037.94
Fringe	811.12
Dom Travel	0
Supplies	0
Other Direct	0
Reserve	0
Indirect	0
TOTAL	$13,849.06

c. Explain special problems, differences between scheduled and actual expenditures, etc.

No problems were experienced.

Prepared By: *John R. Gold* 6/24/05
Signature of Principal Investigator Date

Molecular Ecology Notes (2005) 5, 84–86

doi: 10.1111/j.1471-8286.2004.00840.x

PRIMER NOTE

Microsatellite DNA markers for population genetic studies and parentage assignment in cobia, *Rachycentron canadum*

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*Center for Biosystematics and Biodiversity, Department of Wildlife and Fisheries Sciences, Texas A and M University, College Station, TX 77843–2258, USA, †USDA/ARS National Center for Cold and Cool Water Aquaculture, 11861 Leetown Road, Kearneysville, West Virginia 25430, USA

Abstract

Twenty nuclear-encoded microsatellites from a genomic DNA library of cobia, *Rachycentron canadum*, were isolated and characterized. The microsatellites include two tetranucleotide, one trinucleotide, three combination tetranucleotide/dinucleotide, nine dinucleotide, and five imperfect (dinucleotide) repeat motifs. Gene diversity ranged between zero to 0.910; the number of alleles among a sample of 24 fish ranged from one to 15. Cobia support an important recreational fishery in the southeastern United States and recently have become of interest to aquaculture. The microsatellites developed will be useful tools for studying both population genetics (e.g. stock structure, effective population size) and inheritance of traits important to aquaculture.

Keywords: cobia, genomic library, microsatellites, polymerase chain reaction primers, *Rachycentron canadum*

Received 01 September 2004; revision accepted 06 October 2004

Cobia, *Rachycentron canadum*, is an economically important, pelagic fish distributed in tropical warm waters worldwide (Shaffer & Nakamura 1989). It is a highly prized food and recreational trophy fish, and is considered a prime candidate for aquaculture (Benetti *et al.* 2003). Because of the popularity of cobia as a 'game' fish, methods to identify or distinguish products harvested in cobia aquaculture from 'wild' stocks will be needed in order to ensure legal sale and alleviate potential conflicts. Nuclear-encoded microsatellites (Weber & May 1989) are especially well suited for this purpose because of their codominant, Mendelian inheritance and their high levels of polymorphism. Microsatellites also have many applications in breeding programs (Garcia de Leon *et al.* 1998) and for assessing population structure of 'wild' populations as a means to improve assessment and allocation of resources. In this note, we report the development from a genomic library of cobia DNA of polymerase chain-reaction (PCR) primers for 20, nuclear-encoded microsatellites.

Correspondence: J. R. Gold, Fax: + 1979 8454096; E-mail: goldfish@tamu.edu

Whole genomic DNA was extracted from cobia muscle tissue using a standard phenol-chloroform method and digested with *Dpn*II (New England BioLabs). Fragments ranging in size from 500 to 2000 bp were size selected by extraction from a 1% agarose gel and purified using a gel extraction kit (Qiagen). Fragments were ligated into a *Bam*HI (New England BioLabs) digested and dephosphorylated (Calf Intestinal Alkaline Phosphatase, New England BioLabs) pBluescript vector using T4 DNA ligase (New England BioLabs) and transformed into XL10-Gold ultracompetent cells (Stratagene). Transformed cells were plated on X-Gal – IPTG Luria-Bertani (LB) agar with 50 μg/mL of ampicillin and grown overnight at 37 °C. Recombinant colonies were picked using a GENETIX QBOT, inoculated into 384 well plates that contained 50 μL of LB freezing media [36 mm K_2HPO_4, 13.2 mm KH_2PO_4, 1.7 mm sodium citrate, 0.4 mm $MgSO_4$, 6.8 mm $(NH_4)_2SO_4$, 4.4% (v/v) glycerol, 50 μg/mL ampicillin, LB], and incubated overnight at 37 °C before freezing at –80 °C.

A total of 19 200 clones (50 × 384-well plates) were spotted in a 4 × 4 array onto 22.5 cm × 22.5 cm Hybond nylon membranes (Amersham), with each clone being spotted twice to eliminate false positives. Membranes were placed

on LB agar plates with 50 μg/mL of ampicillin and incubated at 37 °C until colonies were approximately 1–2 mm (18–24 h). Membranes were placed on chromatography paper (3M) and soaked as follows to fix colonies: 10% SDS for 3 min, denaturing solution (1.5 mm NaCl, 1.5 Tris) for 5 min, and 2 × SSC for 5 min. Filters were incubated for 5 h at 65 °C.

Resulting colonies were probed with two cocktails of [gamma] ^{32}P-labelled oligonucleotides: (i) tetranucleotides $[GATA]_9$, $[CATA]_8$, $[GACA]_8$ and trinucleotide $[CAA]_8$, and (ii) trinucleotides $[GAA]_8$, $[TAA]_{13}$, and dinucleotides $[GA]_{13}$ and $[CA]_{13}$. A total of 164 positive clones were screened as follows. Frozen glycerol stocks arrayed in 96-well plates were used to inoculate 1 mL cultures of Luria Broth selective media (ampicillin) and incubated overnight at 37 °C. Plasmid DNA was isolated (alkaline lysis) with a BioRobot 8000 (QIAGEN, CA). Miniprep DNA was quantified, normalized, and both strands sequenced, using M13 forward and reverse sequencing primers and ABI BigDye Terminator v3.1. Products were purified and electrophoresed on an ABI 3100 DNA Analyser (Applied Biosystems, CA). Phred (CodonCode, MA) was used for DNA sequence base calling and vector trimming. A total of 54 complete sequences containing microsatellite arrays were obtained from the positive clones. Three of the clones contained two repeat sequences from which primer pairs were designed. A total of 45 primer pairs were developed using AMPLIFY 1.2 (Engels 1993) and Netprimer® (http://www.premierbiosoft.com/netprimer).

Unlabelled PCR primers were purchased from Invitrogen (Carlsbad, CA) and tested for amplification by screening DNA isolated from eight individuals obtained from offshore of Ocean Springs, Mississippi. PCR amplifications were performed in 10 μL reaction volumes containing 1 μL (100 ng) DNA, 1 μL 10× reaction buffer (500 mm KCl, 100 mm Tris, 10% Triton-X 100), 0.1 U of *Taq* DNA polymerase (Gibco-BRL), 0.5 μm of each primer, 200 μm of each dNTP, and 1 mm $MgCl_2$. PCR conditions consisted of an initial denaturation of 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C–65 °C for 45 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide. Once appropriate annealing temperatures for each primer were determined, microsatellite arrays were tested for polymorphisms by end-labelling (using T4 polynucleotide kinase) one primer from each pair with [γ^{32}P]-dATP and employing the same conditions as above. PCR products were electrophoresed in 6% polyacrylamide gels and visualized by autoradiography; 20 microsatellite repeats were chosen for further screening (Table 1). These microsatellites included one trinucleotide, two tetranucleotide, three combination tetranucleotide/dinucleotide, nine dinucleotide, and five imperfect dinucleotide repeats. Two of the microsatellites were on the same clone, *Rca*1B-E08A and *Rca*1B-E08B. Lengths of cloned alleles ranged in size from 122 to 308 base pairs. Optimal annealing temperatures ranged from 48 °C–60 °C.

Screening involved a total of 24 cobia obtained from offshore of Ocean Springs, Mississippi. One primer from each pair was fluorescently labelled with one fluorescent label of set D (Applied Biosystems) for run on an automated sequencer ABI-377. Alleles were sized using the GENESCAN® 400 HD Size Standard (Applied Biosystems); allele sizing and calling were performed using GENESCAN® 3.1.2 and GENOTYPER®, version 2.5 software. Genetic variability of the markers was measured by the number of alleles, gene diversity (expected heterozygosity), and the observed heterozygosity. Wright's F_{IS}, estimated as Weir and Cockerham's f in Genetic Data Analysis (GDA) (Lewis & Zaykin 2001), was used to measure departure of genotype proportions from Hardy–Weinberg expectations at each microsatellite. Fisher exact tests, as performed in GDA, were used to test significance of departures from Hardy–Weinberg equilibrium (genotype) expectations at each microsatellite and for departure from genotypic equilibrium at pairs of microsatellites. The effect of Hardy–Weinberg departures (within locus disequilibrium) on significance of between locus linkage disequilibrium tests was removed by preserving genotypes in GDA (Lewis & Zaykin 2001).

Summary data are presented in Table 1. The number of alleles detected per microsatellite ranged from one to 15. Expected heterozygosity ranged from zero to 0.910, while observed heterozygosity ranged from zero to 0.957. Genotypes at four microsatellites deviated significantly from Hardy–Weinberg expectations following sequential Bonferroni correction (Rice 1989). Three of the microsatellites exhibited heterozygote deficiency (*Rca* 1B-F06, $F_{IS} = 0.324$; *Rca* 1-B12, $F_{IS} = 0.578$; and *Rca* 1-E06, $F_{IS} = 0.468$), while one (*Rca* A-10) exhibited heterozygote excess ($F_{IS} = -0.079$). All pairwise comparisons of microsatellites did not deviate significantly from genotypic equilibrium following Bonferroni corrections (Rice 1989). The 20 microsatellites developed in this work will prove useful in future studies of population genetics and quantitative genetics of 'wild' and domesticated cobia, respectively.

Acknowledgements

We thank J. Franks of the Gulf Coast Research Laboratory and J. Graves of the Virginia Institute of Marine Science for providing the cobia tissues, and C. Bradfield for technical assistance in the laboratory. Funding was provided by the Saltonstall-Kennedy Program of U.S. Department of Commerce (Grant NA17FD2371) and by the Texas Agricultural Experiment Station (Project H-6703). This paper is number 42 in the series 'Genetic Studies in Marine Fishes' and Contribution no. 127 of the Center for Biosystematics and Biodiversity at Texas A & M University.

Table 1 Summary data for microsatellites developed from cobia, *Rachycentron canadum*

Microsatellite	Primer sequence (5′–3′)*	Repeat sequence†	T_A	N‡/N_A§	Size Range¶	H_E/H_O**	P_{HW}††
Rca 1B-A10	**GCAGCCCAATGCTAACAAGCC¶¶** CATGTAGTCAAGCGAGCCACG	$(GTT)_6$	60	24/4	174–186	0.735/0.792	**0.003**
Rca 1B-D09	**CAGCCTGCTTAGCCTATCA** GAAGGATGGACCACTTGTGAC	$(GT)_9(CTGT)_2(CT)_2(GT)_2$	60	23/1	169	0.000/0.000	1.000
Rca 1B-E02	**GTGTTGCAGCCAAATGCTA** CTCCCTAGTGCCACTACAGCTC	$(CT)_{18}$	60	24/7	298–314	0.598/0.667	0.483
Rca 1B-E08A§§	**CATATCAAGTCAATATCACAGACC** CCACGGAATAGCAGACTTTCTC	$(CA)_3GA(CA)_5A(CA)_{16}$	55	24/5	181–225	0.582/0.458	0.030
Rca 1B-E08B	**GCAGTTGATTCTGATTGCTACAC** CTAATGCCAGCTCATTATGTCC	$CA)_8GA(CA)_3$	60	24/2	120–122	0.496/0.417	0.672
Rca 1B-F06	**CAAGCAAATGCGTGGCCGA** CGTTAGCAACCACACGAGCTTG	$(CTAT)_{15}$	55	24/11	260–300	0.796/0.542	**0.000**
Rca 1B-F07	**GGAATCTGGTGGTGAGTCAT** CTGTGGCTGAAGCGTGTGTT	$(GACA)_6(CA)_{12}$	55	24/3	132–140	0.082/0.083	1.000
Rca 1B-G10	**GGAAACTCTATAACAGCATGTC** GTAGACAGAGCAACACATGAG	$(CT)_5TT(CT)_4$	55	23/2	154–156	0.043/0.043	1.000
Rca 1B-H09	**CATGTTATTCTCCAACTCATGG** GTGTATCCGCATACTTTCAG	$(GATA)_{31}$	48	23/12	176–224	0.910/0.957	0.351
Rca 1-A04	**CACGCACATGCACTACTTTAACC** GCTGTTGATGTGGCGAAGCAAC	$(CA)_9(CACT)_4$	60	24/6	196–206	0.722/0.625	0.095
Rca 1-A11	**CTACAGTGGTGTTCCCTGTTAG** CAGTACATAGAGAAACAGGAGG	$(GT)_{24}$	55	24/15	167–201	0.889/0.792	0.271
Rca 1-B12	**GCTTCAGGCAAGTGAGACC** GGGAGGTAATTATGTCCTGT	$(AC)_9$	55	24/7	177–193	0.780/0.333	**0.000**
Rca 1-C04	**GACATCAAGTGGCACTTTG** CACTAAACTTGTTCCTCCTG	$(GT)_{17}$	48	24/10	223–253	0.641/0.667	0.188
Rca 1-D04	**GCTGAACTTGTCGCCGCT** GGACTGAACCTCCCTATCCTC	$(TG)_9AC(TG)_5$	60	24/3	125–129	0.551/0.667	0.723
Rca 1-D11	**CGTAACACCTTTGGAAGACATC** CTCCATTGAGGCTGACTAGTG	$(GT)_8$	55	24/4	204–212	0.295/0.333	1.000
Rca 1-E04	**CCAAGAACAGGCGGGCAAC** GCCACCATTGTGTGTGGGTGA	$(CA)_8$8 bp$(CA)_5$	55	23/4	216–238	0.336/0.391	1.000
Rca 1-E06	**GGCACCAATCACTCACTACTG** TGTTGAGGTCTATCAGTGCC	$(CA)_{39}$	48	24/10	144–188	0.853/0.458	**0.000**
Rca 1-E11	**GTCCCAGCTCCAGCCCAAAC** GACACTGGCTGCGTGAGCA	$(CA)_{12}$	55	23/7	167–181	0.757/0.783	0.245
Rca 1-F01	**GCTCATTTCACTAAGTGTGTTGTAGC** CCATGAATCTACATTCACCTGCCA	$(TG)_{12}$	60	24/2	202–206	0.120/0.125	1.000
Rca 1-G05	**GGGCTGTCTGCTGGCTGTAA** GCATCTGTGTCCTGGTGAGAGTCCC	$(GT)_{17}$	60	24/7	274–282	0.697/0.667	0.148

*Primer sequences are forward (top) and reverse (bottom); †Repeat sequence indicates the repeat motif; T_A is annealing temperature in °C; ‡N is the number of individuals assayed; §NA is number of alleles detected; ¶Size range refers to alleles thus far uncovered; **H_E and H_O are expected and observed heterozygosity, respectively; ††PHW represents the probability of deviation from Hardy–Weinberg expectations (significant values after Bonferroni correction are in bold) and sequences of clones are listed in GenBank (Accession numbers AY721664–AY721682); §§Primers Rca 1B-E08A and Rca 1B-E08B were developed from the same clone; ¶¶The fluorescently labelled primer is in bold.

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Microsatellite Markers for Cobia, *Rachycentron canadum*

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Polymerase chain reaction (PCR) primers are reported for 35 nuclear-encoded microsatellites 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. The 26 microsatelliteswhose genotypes were in HW equilibrium should provide usefultools for future studies of cobia relating to both stock-assessment and aquaculture.

Cobia, *Rachycentron canadum*, is a migratory, coastal pelagic fish distributed in tropical and subtropical warm waters worldwide exceptfor the eastern Pacific (Shaffer and Nakamura, 1989). The species constitutes an important recreational fishery in the Gulf of Mexico (Brown-Peterson *et al.*, 2001), and is caught incidentally in the commercial fishery (Shaffer and Nakamura, 1989). Interest in cobia aquaculture in the U.S. has been spiked recently by successes in captive spawning and larval rearing (Dodd, 2001), and it has been suggested (Bridger and Costa-Pierce, 2002) that cobia may be an ideal species for offshore cage culture.

In this note, we report optimized polymerase-chain-reaction (PCR) primers for 15nuclear-encoded microsatellites developed from a cobia genomic library. Briefly, microsatellites are short stretches of nuclear DNA composed primarily of di-, tri-, and tetranucleotide repeats inherited in a codominant (Mendelian) fashion and distributed throughout euchromatic regions of chromosomes (Weber and May, 1989; Weber, 1990; Wright and Bentzen, 1994). Microsatellites also accumulate mutations fairly rapidly (Shug et al., 1998), making them ideal genetic markers for a variety of applications ranging from stock-structure analysis of 'wild' populations (Gold and Turner, 2002; Zatcoff *et al.*, 2004) to parentage assignment and pedigree reconstruction in domesticated populations (Wilson and Ferguson, 2002; Jones and Arden, 2003). Included in this note are summary data for 20 other microsatellites for cobia developed in our laboratory by Pruett et al. (2005). The summary data for all 35 microsatellites include number and size of alleles detected, observed and expected heterozygosity, and results of tests of conformity to Hardy-Weinberg equilibrium expectations at each microsatellite. The summary data are published here to allow convenient access to all PCR primers and other data.

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 Pruett et al. (2005). 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 programsAMPLIFY 1.2 (Engels, 1993) and NETPRIMER® (http://www.premierbiosoft.com/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 $MgCl_2$. 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 (Table 1) for 45 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

The primer-pair sequences (forward on top, reverse on bottom), microsatellite motifs (repeat sequence), size of cloned alleles, and optimized annealing temperatures (AT) are given in Table 1.The suite of 35 microsatellites includes 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 sampledoffshore of Ocean Springs,Mississippi. The number of assayed individuals (N), the number of alleles (A_N), and the range in size of detected allelesfor each microsatellite also are given in Table 1. Thirty-three of the microsatellites were found to be polymorphic; the average number of alleles per polymorphic microsatellites was 7.1 (range = 2-17). Estimates of observed (H_O) and expected (H_E) heterozygosity were computed using GDA (Lewis and Zaykin, 2001) and are given in Table 1. 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 are lower than averages reported previously by DeWoody and Avise (2000) for several species of marine fishes. Probabilities of departure from Hardy-Weinberg equilibrium expectations (P_{HW}) were computed using exact tests, as implemented in GDA (Lewis and Zaykin, 2001) and also aregiven in Table 1. Genotypes at seven of the microsatellites differed significantly from Hardy-Weinberg equilibrium expectations following (sequential) Bonferroni correction for multiple tests performed simultaneously (Rice, 1989). Results of analysis by MICROCHECKER (Van Oosterhout *et al.* 2004) 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. The 26 microsatellites whose genotypes were in HW equilibrium should prove extremely useful in future studies of cobia relating to both stock-assessment and aquaculture. The use of microsatellites as selectively neutral genetic markers to assess geographic boundaries and genetic diversity of 'wild' stocks is well reviewed in Wright and Bentzen (1994) and Carvalho and Hauser (1995); the use of microsatellites in aquaculture includes parentage assignment, pedigree reconstruction, mapping of quantitative trait loci, and marker-assisted selection and is well reviewed in Liu and Cordes (2004).

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Table 1. Summary data for 35 microsatellites developed from a cobia (*Rachycentron canadum*) genomic library. PCR primer sequences are forward (top) and reverse (bottom). Primers *Rca* 1B-E08A and *Rca* 1B-E08B were developed from a single clone. Sequences of clones are listed under GenBank Accession Numbers AY721664-AY721682 and AY850008-AY850022. Significant deviations from Hardy-Weinberg expected proportions are in bold.

Microsatellite	PCR primer sequences (5'?3')	Repeat sequence of cloned allele	Size of cloned allele (base pairs)	AT	N	A_N	Range in allele size (base pairs)	H_O/H_E	P_{HW}
Rca 1B-A10	GCAGCCCAATGCTAACAAGCC CATGTAGTCAAGCGAGCCACG	$(GTT)_6$	180	60	24	6	169-187	0.417/0.723	**0.000**
Rca 1B-C06	CCAGCATATCTCCTCTCAAGA GGCTTGAACTTAACTACAGCTCCT	$(GATA)_{29}$	346	50	23	13	340-404	0.870/0.904	0.370
Rca 1B-D09	CAGCCTGCTTAGCCTATGA(GA)$_9$(CTGT)$_2$(CT)$_2$(GT)$_2$ GAAGGATGGACCACTTGTGAC		167	60	23	1	168	0.000/0.000	1.000
Rca 1B-D10	GCAACTGCCTCCACCAATCA CATGTGCATCGAAAGACAGAGA	$(CTAT)_{15}$	191	50	24	17	143-223	1.000/0.943	0.597
Rca 1B-E02	GTGTTGCAGCCAAATGCTA CTCCCTAGTGCCACTACAGCTC	$(CT)_{18}$	308	60	24	7	297-313	0.667/0.598	0.503
Rca 1B-E06	GGATCAGTGTGTTGCAGCCA CCCTAGTGCCACTACAGCTCCCT	$(TC)_{18}$	314	45	24	8	305-327	0.625/0.695	**0.000**
Rca 1B-E08A	CATATCAAGTCAATATCACAGA(CA)$_3$GA(CA)$_5$A(CA)$_{16}$ CCACGGAATAGCAGACTTTCTC		227	55	24	5	181-225	0.458/0.582	0.028
Rca 1B-E08B	GCAGTTGATTCTGATTGCTACAC CTAATGCCAGCTCATTATGTCC	$(CA)_8GA(CA)_3$	122	60	24	2	121-123	0.458/0.510	0.692
Rca 1B-F06	CAAGCAAATGCGTGGCCGA CGTTAGCAACCACACGAGCTTG	$(CTAT)_{15}$	268	55	24	11	260-300	0.542/0.796	**0.000**
Rca 1B-F07	GGAATCTGGTGGTGAGTCAT CTGTGGCTGAAGCGTGTGTT	$(GACA)_6(CA)_{12}$	140	55	24	3	131-139	0.083/0.082	1.000

Rca 1-E05	GCAGTCGAGACGTGACTGAACCA CGTGGAGCTGCTCTGCAGGA	$(CA)_{20}(CGCA)_4(CA)_7(CGCA)_4$	248	55	24	8	241-259	0.542/0.768	**0.000**
Rca 1-E06	GGCACCAATCACTCACTACTG TGTTGAGGTCTATCAGTGCC	$(CA)_{39}$	180	48	24	9	144-186	0.458/0.826	**0.000**
Rca 1-E11	GTCCCAGCTCCAGCCCAAAC GACACTGGCTGCGTGAGCA	$(CA)_{12}$	173	55	23	7	167-181	0.783/0.757	0.236
Rca 1-F01	GCTCATTTCACTAAGTGTGTTGTAGC CCATGAATCTACATTCACCTGCCA	$(TG)_{12}$	198	60	24	2	201-205	0.125/0.120	1.000
Rca 1-F07	GCATCGGGTTGAGTTGTACT CGTTGCCTGTCAATCTGTGCT	$(CA)_6CG(CA)_3$	235	60	23	1	235	0.000/0.000	1.000
Rca 1-F10	CCGTTCTGTACAGACGTGAA GCCTGTTGCTGTTTCCTGTCA	$(CA)_2CG(CA)_{12}CG(CA)_4$	287	55	23	5	287-297	0.261/0.423	0.004
Rca 1-F11	GTTGCCATGGCGACCGAGA GCCCCTATGTCTCGTTCCATC	$(GA)_8AA(GA)_5$	122	55	24	2	119-121	0.000/0.082	0.022
Rca 1-G02	GGGACCATGTGAACTCATGCT CCAGACATGGACTGGTACACCT	$(GT)_{14}$	238	60	23	2	240-244	0.043/0.043	1.000
Rca 1-G05	GGGCTGTCTGCTGGCTGTAA GCATCTGTGTCCTGGTGAGAGTC	$(GT)_{17}$	280	60	24	5	275-283	0.667/0.651	0.185
Rca 1-H01	GTCCCAAGGGAATAGCGAAG CCTCCAGACCAGACAGCAGA	$(CA)_{37}$	298	48	23	12	275-311	0.826/0.885	0.129
Rca 1-H04A	GGGAGCCATGTGGTACAGACT GGGCTTTACGAAAGATAGCTGA	$(GT)_{18}$	161	60	24	3	156-162	0.667/0.550	0.269
Rca 1-H08	GAGACCTACATGGCAGAAGGT GACCACTCCTTTGAGGTCTCT	$(GT)_{30}$	278	60	24	9	273-299	0.708/0.696	0.984
Rca 1-H10	GCACCGCACTGCACAACAC	$(CA)_{16}$	121	60	24	8	119-139	0.583/0.777	0.145

GCTGTGCATACTCACACTGCT

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Rca 1B-G10	GGAAACTCTATAACAGCATGTC GTAGACAGAGCAACACATGAG	$(CT)_5TT(CT)_4$	154	55	23	2	153-155	0.043/0.043	1.000
Rca 1B-H09	CATGTTATTCTCCAACTCATGG GTGTATCCGCATACTTTCAG	$(GATA)_{31}$	220	48	23	12	176-224	0.957/0.910	0.343
Rca 1-A04	CACGCACATGCACTACTTTAACC GCTGTTGATGTGGCGAAGCAAC	$(CA)_9(CACT)_4$	202	60	24	6	196-206	0.625/0.722	0.107
Rca 1-A08	GGATCATAAGGGATTGTGCTA CCTCGAGCCATATCATCAT	$(GT)_{13}GCAT(GT)_5$	289	48	24	8	287-321	0.208/0.575	**0.000**
Rca 1-A11	CTACAGTGGTGTTCCCTGTTAG CAGTACATAGAGAAACAGGAGG	$(GT)_{24}$	187	55	24	15	167-201	0.792/0.889	0.265
Rca 1-B12	GCTTCAGGCAAGTGAGACC GGGAGGTAATTATGTCCTGT	$(AC)_9$	181	55	24	9	176-196	0.500/0.808	**0.000**
Rca 1-C04	GACATCAAGTGGCACTTTG CACTAAACTTGTTCCTCCTG	$(GT)_{17}$	219	48	24	10	223-253	0.667/0.641	0.185
Rca 1-D04	GCTGAACTTGTCGCCGCT GGACTGAACCTCCCTATCCTC	$(TG)_9AC(TG)_5$	127	60	24	3	125-129	0.667/0.551	0.733
Rca 1-D07	CCATGGCTACAATCTGGTTCATC CGAATGCTGTGGAGAACAGG	$(GT)_9TTT(GT)_3$	157	60	23	4	154-162	0.130/0.128	1.000
Rca 1-D08	GCTTGACTCCAGCTCAAAC CACAAGGACGAGCCTCCA	$(CA)_{10}$	172	55	23	4	172-178	0.261/0.274	0.074
Rca 1-D11	CGTAACACCTTTGGAAGACATC CTCCATTGAGGCTGACTAGTG	$(GT)_8$	208	55	24	4	204-212	0.333/0.295	1.000
Rca 1-E04	CCAAGAACAGGCGGGCAAC GCCACCATTGTGTGTGGGTGA	$(CA)_88bp(CA)_5$	220	55	23	4	215-237	0.391/0.336	1.000

In this note, we report optimized polymerase-chain-reaction (PCR) primers for 15nuclear-encoded microsatellites developed from a cobia genomic library. Briefly, microsatellites are short stretches of nuclear DNA composed primarily of di-, tri-, and tetranucleotide repeats inherited in a codominant (Mendelian) fashion and distributed throughout euchromatic regions of chromosomes (Weber and May, 1989; Weber, 1990; Wright and Bentzen, 1994). Microsatellites also accumulate mutations fairly rapidly (Shug et al., 1998), making them ideal genetic markers for a variety of applications ranging from stock-structure analysis of 'wild' populations (Gold and Turner, 2002; Zatcoff *et al.*, 2004) to parentage assignment and pedigree reconstruction in domesticated populations (Wilson and Ferguson, 2002; Jones and Arden, 2003). Included in this note are summary data for 20 other microsatellites for cobia developed in our laboratory by Pruett et al. (2005). The summary data for all 35 microsatellites include number and size of alleles detected, observed and expected heterozygosity, and results of tests of conformity to Hardy-Weinberg equilibrium expectations at each microsatellite. The summary data are published here to allow convenient access to all PCR primers and other data.

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 Pruett et al. (2005). 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 programsAMPLIFY 1.2 (Engels, 1993) and NETPRIMER® (http://www.premierbiosoft.com/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 $MgCl_2$. 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 (Table 1) for 45 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

The primer-pair sequences (forward on top, reverse on bottom), microsatellite motifs (repeat sequence), size of cloned alleles, and optimized annealing temperatures (AT) are given in Table 1.The suite of 35 microsatellites includes 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 sampledoffshore of Ocean Springs,Mississippi. The number of assayed individuals (N), the number of alleles (A_N), and the range in size of detected allelesfor each microsatellite also are given in Table 1. Thirty-three of the microsatellites were found to be polymorphic; the average number of alleles per polymorphic microsatellites was 7.1 (range = 2-17). Estimates of observed (H_O) and expected (H_E) heterozygosity were computed using GDA (Lewis and Zaykin, 2001) and are given in Table 1. 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 are lower than averages reported previously by DeWoody and Avise (2000) for several species of marine fishes. Probabilities of departure from Hardy-Weinberg equilibrium expectations (P_{HW}) were computed using exact tests, as implemented in GDA (Lewis and Zaykin, 2001) and also aregiven in Table 1. Genotypes at seven of the microsatellites differed significantly from Hardy-Weinberg equilibrium expectations following (sequential) Bonferroni correction for multiple tests performed simultaneously (Rice, 1989). Results of analysis by MICROCHECKER (Van Oosterhout *et al.* 2004) 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. The 26 microsatellites whose genotypes were in HW equilibrium should prove extremely useful in future studies of cobia relating to both stock-assessment and aquaculture. The use of microsatellites as selectively neutral genetic markers to assess geographic boundaries and genetic diversity of 'wild' stocks is well reviewed in Wright and Bentzen (1994) and Carvalho and Hauser (1995); the use of microsatellites in aquaculture includes parentage assignment, pedigree reconstruction, mapping of quantitative trait loci, and marker-assisted selection and is well reviewed in Liu and Cordes (2004).

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Table 1. Summary data for 35 microsatellites developed from a cobia (*Rachycentron canadum*) genomic library. PCR primer sequences are forward (top) and reverse (bottom). Primers *Rca* 1B-E08A and *Rca* 1B-E08B were developed from a single clone. Sequences of clones are listed under GenBank Accession Numbers AY721664-AY721682 and AY850008-AY850022. Significant deviations from Hardy-Weinberg expected proportions are in bold.

Microsatellite	PCR primer sequences (5'?3')	Repeat sequence of cloned allele	Size of cloned allele (base pairs)	AT	N	A_N	Range in allele size (base pairs)	H_O/H_E	P_{HW}
Rca 1B-A10	GCAGCCCAATGCTAACAAGCC CATGTAGTCAAGCGAGCCACG	$(GTT)_6$	180	60	24	6	169-187	0.417/0.723	**0.000**
Rca 1B-C06	CCAGCATATCTCCTCTCAAGA GGCTTGAACTTAACTACAGCTCCT	$(GATA)_{29}$	346	50	23	13	340-404	0.870/0.904	0.370
Rca 1B-D09	CAGCCTGCTTAGCCTATCA$(TA)_9(CTGT)_2(CT)_2(GT)_2$ GAAGGATGGACCACTTGTGAC		167	60	23	1	168	0.000/0.000	1.000
Rca 1B-D10	GCAACTGCCTCCACCAATCA CATGTGCATCGAAAGACAGAGA	$(CTAT)_{15}$	191	50	24	17	143-223	1.000/0.943	0.597
Rca 1B-E02	GTGTTGCAGCCAAATGCTA CTCCCTAGTGCCACTACAGCTC	$(CT)_{18}$	308	60	24	7	297-313	0.667/0.598	0.503
Rca 1B-E06	GGATCAGTGTGTTGCAGCCA CCCTAGTGCCACTACAGCTCCCT	$(TC)_{18}$	314	45	24	8	305-327	0.625/0.695	**0.000**
Rca 1B-E08A	CATATCAAGTCAATATCACAGA$(CA)_3GA(CA)_5A(CA)_{16}$ CCACGGAATAGCAGACTTTCTC		227	55	24	5	181-225	0.458/0.582	0.028
Rca 1B-E08B	GCAGTTGATTCTGATTGCTACAC CTAATGCCAGCTCATTATGTCC	$(CA)_8GA(CA)_3$	122	60	24	2	121-123	0.458/0.510	0.692
Rca 1B-F06	CAAGCAAATGCGTGGCCGA CGTTAGCAACCACACGAGCTTG	$(CTAT)_{15}$	268	55	24	11	260-300	0.542/0.796	**0.000**
Rca 1B-F07	GGAATCTGGTGGTGAGTCAT CTGTGGCTGAAGCGTGTGTT	$(GACA)_6(CA)_{12}$	140	55	24	3	131-139	0.083/0.082	1.000

Rca 1B-G10	GGAAACTCTATAACAGCATGTC GTAGACAGAGCAACACATGAG	$(CT)_5TT(CT)_4$	154	55	23	2	153-155	0.043/0.043	1.000
Rca 1B-H09	CATGTTATTCTCCAACTCATGG GTGTATCCGCATACTTTCAG	$(GATA)_{31}$	220	48	23	12	176-224	0.957/0.910	0.343
Rca 1-A04	CACGCACATGCACTACTTTAACC GCTGTTGATGTGGCGAAGCAAC	$(CA)_9(CACT)_4$	202	60	24	6	196-206	0.625/0.722	0.107
Rca 1-A08	GGATCATAAGGGATTGTGCTA CCTCGAGCCATATCATCAT	$(GT)_{13}GCAT(GT)_5$	289	48	24	8	287-321	0.208/0.575	**0.000**
Rca 1-A11	CTACAGTGGTGTTCCCTGTTAG CAGTACATAGAGAAACAGGAGG	$(GT)_{24}$	187	55	24	15	167-201	0.792/0.889	0.265
Rca 1-B12	GCTTCAGGCAAGTGAGACC GGGAGGTAATTATGTCCTGT	$(AC)_9$	181	55	24	9	176-196	0.500/0.808	**0.000**
Rca 1-C04	GACATCAAGTGGCACTTTG CACTAAACTTGTTCCTCCTG	$(GT)_{17}$	219	48	24	10	223-253	0.667/0.641	0.185
Rca 1-D04	GCTGAACTTGTCGCCGCT GGACTGAACCTCCCTATCCTC	$(TG)_9AC(TG)_5$	127	60	24	3	125-129	0.667/0.551	0.733
Rca 1-D07	CCATGGCTACAATCTGGTTCATC CGAATGCTGTGGAGAACAGG	$(GT)_9TTT(GT)_3$	157	60	23	4	154-162	0.130/0.128	1.000
Rca 1-D08	GCTTGACTCCAGCTCAAAC CACAAGGACGAGCCTCCA	$(CA)_{10}$	172	55	23	4	172-178	0.261/0.274	0.074
Rca 1-D11	CGTAACACCTTTGGAAGACATC CTCCATTGAGGCTGACTAGTG	$(GT)_8$	208	55	24	4	204-212	0.333/0.295	1.000
Rca 1-E04	CCAAGAACAGGCGGGCAAC GCCACCATTGTGTGTGGGTGA	$(CA)_88bp(CA)_5$	220	55	23	4	215-237	0.391/0.336	1.000

Rca 1-E05	GCAGTCGAGACGTGACTGAACCA	$(CA)_{20}(CGCA)_4(CA)_7(CGCA)_4$	248	55	24	8	241-259	0.542/0.768	**0.000**
	CGTGGAGCTGCTCTGCAGGA								
Rca 1-E06	GGCACCAATCACTCACTACTG TGTTGAGGTCTATCAGTGCC	$(CA)_{39}$	180	48	24	9	144-186	0.458/0.826	**0.000**
Rca 1-E11	GTCCCAGCTCCAGCCCAAAC GACACTGGCTGCGTGAGCA	$(CA)_{12}$	173	55	23	7	167-181	0.783/0.757	0.236
Rca 1-F01	GCTCATTTCACTAAGTGTGTTGTAGC CCATGAATCTACATTCACCTGCCA	$(TG)_{12}$	198	60	24	2	201-205	0.125/0.120	1.000
Rca 1-F07	GCATCGGGTTGAGTTGTACT CGTTGCCTGTCAATCTGTGCT	$(CA)_6CG(CA)_3$	235	60	23	1	235	0.000/0.000	1.000
Rca 1-F10	CCGTTCTGTACAGACGTGAA GCCTGTTGCTGTTTCCTGTCA	$(CA)_2CG(CA)_{12}CG(CA)_4$	287	55	23	5	287-297	0.261/0.423	0.004
Rca 1-F11	GTTGCCATGGCGACCGAGA GCCCCTATGTCTCGTTCCATC	$(GA)_8AA(GA)_5$	122	55	24	2	119-121	0.000/0.082	0.022
Rca 1-G02	GGGACCATGTGAACTCATGCT CCAGACATGGACTGGTACACCT	$(GT)_{14}$	238	60	23	2	240-244	0.043/0.043	1.000
Rca 1-G05	GGGCTGTCTGCTGGCTGTAA GCATCTGTGTCCTGGTGAGAGTC	$(GT)_{17}$	280	60	24	5	275-283	0.667/0.651	0.185
Rca 1-H01	GTCCCAAGGGAATAGCGAAG CCTCCAGACCAGACAGCAGA	$(CA)_{37}$	298	48	23	12	275-311	0.826/0.885	0.129
Rca 1-H04A	GGGAGCCATGTGGTACAGACT GGGCTTTACGAAAGATAGCTGA	$(GT)_{18}$	161	60	24	3	156-162	0.667/0.550	0.269
Rca 1-H08	GAGACCTACATGGCAGAAGGT GACCACTCCTTTGAGGTCTCT	$(GT)_{30}$	278	60	24	9	273-299	0.708/0.696	0.984
Rca 1-H10	GCACCGCACTGCACAACAC	$(CA)_{16}$	121	60	24	8	119-139	0.583/0.777	0.145

GCTGTGCATACTCACACTGCT

TECHNICAL PAPER (*Submitted*)

Microsatellite multiplex panels for genetic studies of three species of marine fishes: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*)

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Abstract

Multiplex panels of nuclear-encoded microsatellites were developed for three species of marine fishes of interest to both public and private aquaculture ventures: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*). The multiplex panels will be useful in a variety of applications, including strain and hybrid identification, parentage assignment, pedigree reconstruction, estimating genetic diversity and/or inbreeding, mapping of quantitative trait loci, and marker-assisted selection. The panels also will be useful in studies of stock-structure of 'wild' populations. Comparison of costs for expendable supplies revealed that four- and eight-panel multiplexes reduced expenditures four- and eight-fold, respectively, relative to single microsatellite gels. Personnel time also was reduced significantly.

Keywords Microsatellites, multiplexes, aquaculture, red drum, red snapper, cobia

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1. Introduction

Microsatellites are hypervariable, nuclear-encoded genetic markers that are widely used in a variety of aquaculture applications, including strain and hybrid identification, parentage assignment, pedigree reconstruction, estimating genetic diversity and/or inbreeding, mapping of quantitative trait loci, and marker-assisted selection (Liu and Cordes, 2004). The large number of microsatellites needed for many of these applicationscan often generate high costs dueto materials and personnel time. Major cost reduction, however, can be achieved through multiplexing, the combination of polymerase-chain-reaction (PCR)amplification products from multiple microsatellitesinto a single lane of an electrophoretic gel (Olsen *et al.*, 1996;Neff *et al.*, 2000). Multiplexing can be accomplished through either co-amplification of multiple microsatellites in a single PCR cocktail(Chamberlain *et al.*, 1988) orcombination of PCR products from multiple, single amplification reactions (Olsen *et al.*, 1996). Ablendof the two approachescan optimize sample throughput (Devey *et al.*, 2002; Paterson *et al.*, 2004). Despite itscost-effective advantages, however,multiplexing is often not employed, apparently because of a general apprehension that itincreases complexity of microsatellite genotyping (Neff *et al.*, 2000).

In this technical report, we describe simple and straightforward protocols for multiplexing microsatellitesin each of three species of marine fishes of interest to public and private (commercial) aquaculture: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*). Briefly, red drum is an estuarine-dependent sciaenid found primarily along the Atlantic and Gulf coasts of the southern United States (U.S.). Culture, including breeding, of red drum is relatively advanced (Lee and Ostrowski, 2001). Extensive use by resource management agencies of hatchery-raised red drum to enhance 'wild' stocks occurs in several southern states (Grimes, 1998; Smith *et al.*, 2001), especially Texas (McEachron *et al.*, 1995).Commercial(private) aquaculture of red drum also occurs in the southern U.S. (Lee and Ostrowski, 2001), and in China, Israel, and Taiwanas well (Lutz, 1999; Hong and Zhang, 2003).Culture of red snapper, a structure- or reef-associated lutjanid found primarily along the continental shelf of the Gulf of Mexico (Hoese and Moore, 1977),is far less advanced. Wild-caught adults have been successfully strip-spawned in captivityfollowing hormone inducement (Lee and Ostrowski, 2001; Riley *et al.*, 2004), and efforts to optimize aquaculture protocols to mass produce fingerlings for stock enhancement and commercial aquacultureare underway (Chigbu et al., 2002; Riley *et al.*, 2004). In addition, the grow-out and market potential for red snapper make it ideal for offshore cage culture (Bridger and Costa-Pierce,

2002). Cobia is a nearly cosmopolitan, pelagic species found in tropical and subtropical waters (Shaffer and Nakamura, 1989). Like red snapper, cobia is relatively new to aquaculture, although in Taiwan, cobia is now averypopular and profitablefarmed marine fish (Liao *et al.*, 2004). Recent success in captive spawning and progeny production in the U.S. (Dodd, 2001) has generated considerable interest in commercial aquaculture of cobia (Lee and Ostrowski, 2001; Smith *et al.*, 2001).

PCR primer sets for microsatellites in all three species were developed previously for use in stock-structure analysis of 'wild' populations: red drum (Saillant et al., 2004); red snapper (Bagley and Geller, 1998; Gold et al., 2001), and cobia (Pruett *et al.*, 2005; Renshaw *et al.*, 2005). Subsets of these microsatellites were used to develop protocols for the microsatellite multiplex panels described in this report.

2. Materials and multiplex protocols

An alkaline-lysis method (Saillant *et al.*, 2002) was used to purify DNA from tissue samples (fin clips or internal organs) that had been obtained for prior studies in our laboratory. All tissueshad been fixed in 95% ethanol and stored at room temperature.

The initial step in designing multiplex protocolstypically involves generating PCR sequences for individual microsatellites, optimizing annealing temperatures,and determining the approximate range in allele size that might be encountered for a given microsatellite. Because these data already were available for red drum, red snapper and cobia (Gold *et al.* 2001; Saillant *et al.* 2004; Renshaw *etal.* 2005), our initial effort was conceptual and involved combining individual microsatellites with similar, optimal annealing temperatures andassessing whether allele-size ranges overlappedand whether alternate ABI dyes could be employed whenoverlap occurred. Initial experiments in each species involved testing sets of eight to ten primersin 'mega-cocktails' where equimolar (5 picomol) amounts of each primer pair were run in the same PCR reaction. Other PCR reagents (buffer, magnesium, dNTPs, *Taq* polymerase) in the 'mega-cocktails' initially followed PCR procedures outlined in Saillant *et al.* (2004), Gold *et al.* (2001), and Renshaw *et al.* (2005) for red drum, red snapper, and cobia, respectively. One primer (either forward or reverse) from each pair was labeled with one of three different Applied Biosystems (ABI) fluorescent dyes of set D (6-FAM, HEX or NED). Microsatellites with non-overlapping allele-size ranges were labeled with the same fluorescent dye; whereas those with overlapping allele-size ranges were labeled with different dyes. Fragment

analysis was carried out on an automated ABI-377 sequencer; fragments were sized using GENESCAN 3.1.2, and allele calling was performed using GENOTYPER® version 2.5.

Optimization of multiplex protocols began with screening of microsatellites that amplified in the 'mega-cocktails.' Primer pairs that failed to amplify were removed from the mix and further optimization focused on the remaining primer pairs; this led to six sets (hereafter multiplex panels or panels) of red drum microsatellites, three of red snapper microsatellites, and five ofcobia microsatellites. Two 'touchdown'PCR protocols(I and II) were evaluated for each of the 14 panels. Touchdown protocols are used to amplify microsatellites with different, optimal annealing temperatures in the same PCR reaction via progressively reducing annealing temperature in successive annealing cycles (Rithidech *et al.*, 1997; Fishback *et al.*, 1999). The Touchdown Iprotocol featured a one-half degree (Celsius) reduction in annealing temperature at each of twelve cycles, as described by Fishback*et al.* (1999),followed by thirty cycles of amplification at a temperature (hereafter, 'bottom' temperature) six degrees below the starting annealing temperature.Initial evaluationstarted at the highest (optimal) annealing temperature for a given microsatellite included in eachmultiplex panel.The Touchdown II protocol was developed in our laboratory and involved reduction in temperature at each of three separate annealing steps; the initial step employed the highest optimal annealing temperature for a given microsatellite included in each panel, while the second and third steps employed temperatures that flankedthe lowest optimal annealing temperature among the microsatellites in a given multiplex panel. The Touchdown Iprotocol ultimately resolved involved the following:(i) initial denaturationat 95^{o} C for 3 min; (ii) 12 cycles of denaturation at 95^{o}C for 30 sec, annealing (minus 0.5^{o}C per cycle) for 1 min, and extension at 72^{o}C for 4 min;(iii) 30 cycles of denaturation at 95^{o}C for 30 sec,'bottom' annealing temperature for 1 min, and extension at 72^{o}C for 4 min; and (iv) final extension at 72^{o}C for 10 min. The Touchdown II protocol ultimately resolved involved three steps following initial denaturation at 95^{o}C for 3 min:Step - 1 seven cyclesof denaturation at 95^{o}C for 30 sec, annealing for 1 min, and extension at 72^{o}C for 4 min; Step 2 - seven cycles of denaturation at 95^{o}C for 30 sec, annealing for 1 min, and extension at 72^{o}C for 4 min; and Step 3 - 28 cycles of denaturation at 95^{o}C for 30 sec, annealing for 1 min, and extension at 72^{o}C for 4 min. Final extension was at 72^{o}C for 10 min. Annealing temperatures for both Touchdown I and II protocols are given below.

The next step involved identifying the optimal touchdown protocol and correspondingannealing-temperature range for each of the 14 multiplex panels. Choice of touchdown protocol was based primarily on reliability of scoring PCR products at all microsatellites in a givenpanel. We also evaluated different concentrations of PCR reagents (including primers) to determine optimal conditions for each panel. Optimal PCR reactions across all panels were comprised of 1.5µl of DNA (approximately 50ng), 1µl of 10X reaction buffer [500mM KCl, 200mM Tris-HCl (pH 8.4)], 2mM $MgCl_2$, 2.5mM of each dNTP, 0.75 units *Taq* DNA polymerase (GibcoBRL), and various quantities of primers. Concentrations of different primers were adjusted relative to obtaining homogeneous amplification-product intensity at each microsatellite within a given panel, achieved by raising and lowering primer concentrations in response to PCR outcomes. Final volumes in all PCR 'cocktail' reactions were adjusted with double-distilled water to bring total cocktail volume to 11.5µl.

Optimized PCR protocols for each of the 14 multiplex panels are given in Table 1 (red drum), Table 2 (red snapper), and Table 3 (cobia). Optimal primer concentrations (in picomols) for each primer within each multiplex panel are given, as are the ABI dye employed and the range in allele size (based on our prior studies in each species). Appropriate touchdown protocols also are given for each panel. For Touchdown I protocols, the range of temperatures for the first 12 cycles (with a 0.5°C reduction at each cycle) is listed on the first line; the 'bottom'annealing temperature islisted on the second line. For Touchdown II protocols, annealing temperatures forSteps1-3 are listed in order, i.e., the temperature for Step 1 (seven cycles) is on the first line, the temperature for Step 2 (seven cycles) is on the second line, and the temperature for Step 3 (28 cycles) is on the third line. A gel image of multiplex panels 1 - 6 for red drum is shown in Figure 1.

Development of the panels required evaluating an initial 'mega-cocktail' of PCR primer compatibility, reagents, and protocols, followed by the testing of primer concentrations to generate similar quantities of amplified products across all microsatellites. The impetus for the work was the projected reduction in both personnel time (labor) and consumable supplies generally required for large genotyping projects (Neff *et al.*, 2000). To assess whether, and to what extent, using multiplex panels was cost effective, we estimated expenses (as of January 2005 and in U.S. dollars) of PCR and gel supplies and labor for running single microsatellite gels versus four- and eight-microsatellite panels. PCR supplies included *Taq* polymerase kits, primers and dyes, dNTPs, 96-well plates,

tubes, pipet tips, and mineral oil; gel supplies included 44 HD Rox size standards, 96-well plates, long ranger singel packs, sequencing combs, pipet tips, and formamide. 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. The estimates of personnel times were based on an experienced research assistant.

3. Synopsis

Multiplex panels of nuclear-encoded microsatellites were developed for three species of marine fishes of interest to both public and private aquaculture concerns: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*). The panelscan be utilized in a variety of applications, including strain and hybrid identification, parentage assignment, pedigree reconstruction, estimating genetic diversity and/or inbreeding, mapping of quantitative trait loci, and marker-assisted selection. The panels also will be useful in studies of stock-structure of 'wild' populations.

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Table 1

Multiplex panelsfor red drum (*Sciaenops ocellatus*) developed from31 microsatellite primers. Primer quantities (in picomols) and fluorescent labels (ABI dye) are given for finalized PCR cocktails. Allele size ranges are given for each microsatellite as previously described in Saillant *et al.* (2004). The multiplex PCR protocol and annealing temperatures for each panel are shown in the far right column; further description of the PCR protocols is given in the text. PCR primer sequences for each microsatellite may be found in Saillant *et al.* (2004).

Panel	Microsatellite	Quantity	ABI dye	Range in allele size	Touchdown protocol
1	*Soc*412	4.3	HEX	102-168	Touchdown I
	*Soc*416	3.2	NED	55° 141-181	
	*Soc*417	0.7	6-FAM	86-112	49°
	*Soc*423	0.6	6-FAM	172-208	
	*Soc*428	2.7	HEX	172-242	
	*Soc*445	35	6-FAM	134-166	
2	*Soc*60	2.2	6-FAM	151-163	Touchdown I
	*Soc*140	0.5	NED	55° 122-144	
	*Soc*201	9	HEX	224-243	49°
	*Soc*243	2	6-FAM	94-106	
	*Soc*419	1.4	6-FAM	238-260	
3	*Soc*19	5	6-FAM	195-267	Touchdown I
	*Soc*85	2.3	6-FAM	60° 80-132	
	*Soc*138	4.5	HEX	77-123	54°
	*Soc*156	0.4	6-FAM		
	Soc 206	0.8	NED	246-265	
	*Soc*410	2.7	6-FAM	306-344	
4	*Soc*11	1.6	6-FAM	217-240	Touchdown II
	*Soc*83	9	HEX	114-142	62°
	*Soc*99	2.2	NED	131-209	56°
	*Soc*407	9	6-FAM	139-157	54°
	*Soc*424	10	HEX	204-230	
5	*Soc*400	3	6-FAM	245-266	Touchdown I

	*Soc*402	6	HEX	550124-164	
	*Soc*404	4	6-FAM	150-212	49°
	*Soc*415	5	HEX	187-235	
	*Soc*432	0.8	HEX	98-118	
6	*Soc*44	7	HEX	211-271	Touchdown II
	*Soc*401	3.7	HEX	174-206	56°
	*Soc*433	0.7	6-FAM	84-102	52°
	*Soc*444	1	HEX	161-165	50°

Table 2

Multiplex panels for red snapper (*Lutjanus campechanus*) developed from 20microsatellite primers. Primer quantities (in picomols) and fluorescent labels (ABI dyes) are given for finalized PCR cocktails. Range in allele size is based on work in our laboratory. The multiplex PCR protocol and annealing temperatures for each panel are shown in the far right column; further description of the PCR protocols is given in the text. PCR primer sequences for microsatellites with prefixes *Lca* and *Prs* may be found in Gold*et al.* (2001). PCR primer sequences for microsatellites*Ra*6,and *Ra*7may be found in Bagley and Geller (1998).

Panel	Microsatellite	Quantity	ABI dye	Range in allele size	Touchdown protocol
1	*Lca*43	5	6-FAM	162-192	Touchdown II
	*Prs*260	0.9	6-FAM	111-129	56°
	*Prs*55	2.7	HEX	180-208	53°
	*Prs*229	0.9	HEX	123-137	50°
	*Prs*248	1.3	NED	212-260	
	*Prs*303	0.6	NED	124-150	
	*Lca*20	5	6-FAM	203-223	
2	*Prs*275	4	6-FAM	133-150	Touchdown II
	*Prs*137	7	6-FAM	155-185	54°
	*Prs*328	0.9	6-FAM	196-214	52°
	*Prs*282	5	HEX	113-143	50°
	*Prs*221	4	HEX	220-266	
	*Ra*6	2	NED	112-130	
	*Lca*64	5.2	HEX	151-181	
3	*Lca*91	5	6-FAM	130-144	Touchdown I
	*Lca*22	4	6-FAM	228-258	56-50.5°
	*Prs*333	0.7	HEX	156-157	50°
	*Prs*240	1.4	HEX	193-223	
	*Lca*107	4	HEX	96-120	
	*Ra*7	1.6	NED	145-167	

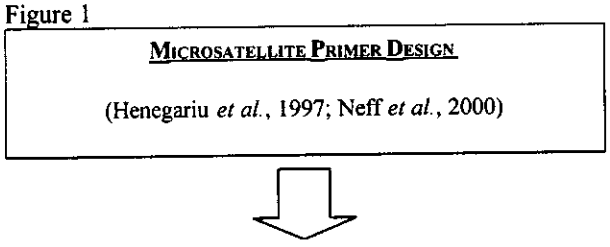
Table 3

Multiplex panels for cobia (*Rachycentron canadum*) developed from 35 microsatellite primers. Primer quantities (in picomols) and fluorescent labels (ABI dye) are given for finalized PCR cocktails. Allele size ranges are given for each microsatellite as previously described in Renshaw *et al.* (2005). The multiplex PCR protocol and annealing temperatures for each panel are shown in the far right column; further description of the PCR protocols is given in the text. PCR primer sequences for each microsatellite may be found in Renshaw et al. (2005).

Panel	Microsatellite	Quantity	ABI dye	Range in Allele size	Touchdown protocol
1	*Rca*1B-E08A	1.1	6-FAM	181-229	Touchdown II
	*Rca*1-E05	8	NED	221-261	55°
	*Rca*1-E04	0.7	HEX	215-237	53°
	*Rca*1-A11	2.5	NED	165-201	51°
	*Rca*1-E11	4.5	HEX	167-183	
	*Rca*1B-G10	1.1	6-FAM	153-155	
2	*Rca*1-D11	0.9	6-FAM	204-212	Touchdown II
	*Rca*1B-F06	2.2	HEX	260-308	55°
	*Rca*1-B12	1.2	NED	176-196	53°
	*Rca*1-D08	1.1	6-FAM	172-178	51°
	*Rca*1-F11	0.5	6-FAM	119-123	
	*Rca*1B-F07	0.8	HEX	131-143	
	*Rca*1-F10	1.6	NED	287-351	
3	*Rca*1B-A10	0.4	6-FAM	169-187	Touchdown II
	*Rca*1B-E02	4	6-FAM	297-315	60°
	*Rca*1-H10	1.2	HEX	119-139	57°
	*Rca*1-A04	1.8	HEX	196-206	54°
	*Rca*1-G05	1.9	HEX	269-285	
	*Rca*1-D07	1.5	NED	154-162	
	*Rca*1-F07	1.9	NED	235	
4	*Rca*1B-E08B	0.8	6-FAM	117-123	Touchdown II
	*Rca*1-F01	1.2	6-FAM	199-205	60°
	*Rca*1-D04	1.2	HEX	125-131	57°
	*Rca*1-H04A	2.5	HEX	156-162	54°
	*Rca*1-G02	2	HEX	240-244	
	*Rca*1B-D09	5	NED	168	
	*Rca*1-H08	10	NED	273-299	

5	*Rca*1B-C06	4	6-FAM	336-404	Touchdown I
	*Rca*1B-D10	1.4	6-FAM	163-223	50°-44.5°
	*Rca*1B-E06	1	HEX	305-327	44°
	*Rca*1B-H09	10	HEX	168-224	
	*Rca*1-A08	4	6-FAM	287-321	
	*Rca*1-C04	3	NED	217-253	
	*Rca*1-E06	3.2	NED	144-186	
	*Rca*1-H01	3	NED	275-311	

Figure 1



Gel image for six multiplex panelsof red drum (*Sciaenops ocellatus*) microsatellites. Panels 1-6 are oriented left to right);microsatellite *Soc*445 is absent from Panel 1. Colors correspond to ABI fluorescent dye-labels as such: blue = 6-FAM, green = HEX, and yellow = NED. Red bands correspond to the 400 HD Rox Size Standard (Applied Biosystems). Size orientation of microsatellite fragments is large (top) to small (bottom).