Characterization of the Mitochondrial DNA Control Region of Cobia, *Rachycentron canadum*, from Mississippi Coastal Waters

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ABSTRACT

Limited research has been conducted on the biology and life history of the cobia, *Rachycentron canadum*, and no information on cobia molecular genetics has been published. This migratory, pelagic species is nearly circumglobally distributed. These fish are sought by offshore recreational anglers and are the basis of important commercial fisheries. Our preliminary research consisted of a characterization of the mtDNA control region and flanking tRNA genes. Whole DNA isolates were obtained from muscle tissue samples collected from cobia caught in Mississippi coastal waters. Universal primers were used in PCR amplifications to generate fragments of approximately 2,000 base pairs (bp). Subsequent direct sequencing produced sequences containing portions of cytochrome b and 12S rRNA, the entire tRNAs for proline (pro), threonine (thr), and phenylalanine (phe) and portions of the control region. Using these sequences, species specific primers were constructed in the tRNAs pro and phe flanking the control region and used in subsequent

sequence (TAS-I) and four conserved sequence blocks (CSB D, I, II, III) were identified and compared with other fish species. Sequence variability of the control region was low; only three transversions and seven indels were found between the six cobia. Additional sequence data is needed from geographically isolated regions to determine if sufficient sequence variability exists in the control region for it to serve as a useful molecular marker in population and stock enhancement studies.

KEY WORDS: *Rachycentron canadum*, genetics, mitochondrial DNA

INTRODUCTION

The cobia, *Rachycentron canadum*, is a pelagic species found worldwide in tropical and subtropical coastal waters, except off the eastern Pacific (Briggs 1960, Shaffer and Nakamura 1989). In the early spring off the southeastern U.S., cobia typically migrate from their wintering grounds off south Florida into the northeastern Gulf of Mexico, and from late March through October, they occur off northwest Florida, Alabama, Mississippi, and southeast Louisiana (Franks et al. 1991, Biesiot et al. 1994). This migration pattern has led scientists to speculate that cobia exist as a single population within the eastern Gulf of Mexico and perhaps over their entire range in U.S. waters. Tagging data from a study being conducted on northern Gulf cobia tends to support this hypothesis (Franks et al. 2000). Based on RFLP mitochondrial DNA (mtDNA) analyses of 90 fish collected off the Gulf states and Virginia, Biesiot et al. (1993) suggested cobia should be managed as a single unit. They found 86 haplotypes, but could not distinguish separate spawning stocks.

Increased fishing in the U.S. has put additional pressure on this species. For example, annual cobia commercial landings from the western central Atlantic, including the Gulf of Mexico, have increased from 58 metric tons in 1980 to 148 metric tons in 1998. The commercial catch peaked at 193 metric tons in 1996 (National Marine Fisheries Service 2000). Also, the price of cobia increased from approximately $0.38 per pound in 1980 to approximately $1.87 per pound in 1998 (National Marine Fisheries Service 2000). For reasons such as these, the U.S. Gulf of Mexico Marine Stock Enhancement Program listed cobia as a secondary species for the potential development of enhancement procedures.

The purpose of this study was to characterize the cobia mtDNA control region and preliminarily evaluate its suitability to assess cobia population structure. The mtDNA control region is typically a rapidly evolving portion of an already rapidly evolving genome (Brown et al. 1982, Vawter and Brown 1986), and can accumulate mutations two to five times faster than the rest of the mitochondrial genome (Meyer, 1993). The control region also contains conserved motifs and the sites of initiation of both heavy-strand replication, and heavy- and light-strand transcription (Chang and Clayton 1987 Clayton 1991a, 1991b, Digby et al. 1992). Because of its potentially high mutation rate, sequence data from the control region has been useful in assessing population structure of various species of marine fish (e.g. Garber 1999, Seyoum et al. 1999, Reeb et al. 2000).

In this initial study we obtained the sequence of the cobia mtDNA control region and its flanking tRNAs threonine (thr), proline (pro), and phenylalanine (phe). We then utilized this information to design species specific primers in the tRNAs pro and phe for direct sequencing and subsequent characterization of the control region.

MATERIALS AND METHODS

Cobia were collected off the coast of Mississippi, USA. White muscle tissue was excised and fixed in SED buffer (250 mM EDTA, pH 7.5, 20% DMSO, 3.42 M NaCl). Total genomic DNA was extracted from each sample using a procedure modified from Taggart et al. (1992), quantified using fluorescence spectrophotometry (Gallagher 1994), and adjusted to 100 ng/µL with 1 mM Tris, pH 8.5.

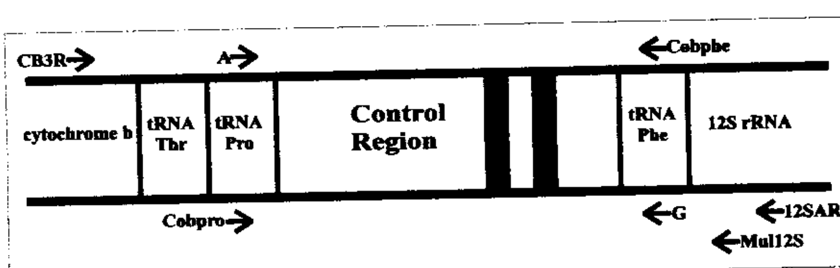
Two primer sets were initially employed in PCR (Fig.1): CB3R (5' CACAT TCAAC CAGAA TGATA TTT 3'; Palumbi, 1996) and 12SA-H1067, referred to in this work as 12SAR, (5' ATAAT AGGGT ATCTA ATCCT AGTT 3'; Martin et al. 1992), and CB3R and MUL12S (5'-CACGA GATTT ACCGG CCCTA TTAG-3'; Garber 1999). Appropriate PCR products were gel-purified using the QIAquick™ Gel Extraction Kit (Qiagen, Inc.), quantified as above, and direct sequenced at the University of Maine DNA Sequencing Facility on an ABI Model 373A Sequencer. One set of sequences from one cobia was produced from each primer pair. Species specific primers in pro (CobPro, 5' ACCTG TACCT CTGGC TCCCA A 3') and phe (CobPhe, 5' CCGGG TTAGT GGCCA TCTTA A 3') were then designed from the resulting sequence information and used to amplify the entire control region from the previously sequenced sample and five additional samples (Figure 1). Appropriate PCR products were gel-purified, quantified, and sequenced as above.

A nested PCR using control region primers from a previously published study ("A" 5' TTCCA CCTCT AACTC CCAAA GCTAG 3' and "G" 5' CGTCG GATCC CATCT TCAGT GTTAT GCTT 3'; Lee et al. 1995; Figure 1) was conducted to verify we had sequenced mtDNA and not a nuclear pseudogene. In this procedure, the gel-purified product from the CB3R/12SAR amplification was used as template in a second reaction with the aforementioned primers.

All PCR amplifications were conducted in replicate 50 µL reactions containing 200 ng template, 1.5 mM $MgCl_2$, 200 µM each dNTP (Promega, Inc.), 0.4 µM of each primer, and 3.5 units of *Taq* DNA polymerase with 10X PCR buffer supplied by the manufacturer (Amersham Pharmacia & Biotech). PCR conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 55°C for 1 min (universal and mullet primers)/58°C for 30 sec (cobia primers)/52°C for 1 min (nested primers), 72°C for 2 min, and a final elongation of 72°C for 7 min.

DNA sequences were imported into OMIGA, ver. 1.1 (Oxford Molecular Ltd., Oxford, England), a multiple sequence editor, aligned using CLUSTALW (Higgins and Sharp, 1988) with the default settings, and adjusted by eye. Transfer RNA secondary structures were elucidated using Sean Eddy's Lab tRNAscan-SE Search Server (Lowe and Eddy 1997). Sequences were compared with those deposited at the National Center for Biotechnology Information (NCBI) using NCBI's BLAST WWWServer (Basic Local Alignment Tool; Altschul et al. 1990).

Figure 1. The control region and flanking areas of the piscine mitochondrial genome. Arrows indicate the relative position of primers employed to amplify the control region of the cobia, *Rachycentron canadum*.



RESULTS

The primer pair CB3R/12SAR produced a ,2000 base pair (bp) fragment, and the primer pair CB3R/MUL12S produced a 1,900 bp fragment (Figure 2a). Sequencing produced approximately 550 bp at both the 5' and 3' ends. When compared with sequences deposited at the NCBI, the 5' end was identified as a portion of cytochrome b and the complete tRNAs thr and pro (Figure 3a, 3b). A 27 bp noncoding region between thr and pro was also identified (Figure 4). The 3' end was identified as the complete tRNA phe (Fig. 3c) and a portion of 12S rRNA. The sequence fragments were aligned and deposited in Genbank (accession number AF311947).

The sequence information obtained was utilized to design species specific primers located in pro (CobPro) and phe (CobPhe). These primers produced a DNA fragment of approximately 1100 bp from six fish (Fig. 2b). NCBI searches indicated these sequences were the mtDNA control region. The sequences were deposited in Genbank (accession numbers AF311945-950). Nested PCR of the gel-purified CB3R/12SAR product with the primers A and G produced a DNA fragment of approximately 1100 bp (Fig. 2c), as anticipated.

The control region of six fish ranged from 1067-1068 bp with a consensus length of 1069 bp (Figure 4). The consensus sequence contained three transversions, seven indels (insertions/deletions), and one unresolvable sequence ambiguity (N) in a single individual. One termination associated sequence (TAS) was identified: TAS-I located in the tRNA pro. Four conserved sequence blocks (CSB), CSB-D, CSB-I, CSB-II, and CSB-III, were also identified. A pyrimidine block of 21 bp, containing one purine, was identified between CSB-D and CSB-I (Fig. 4).

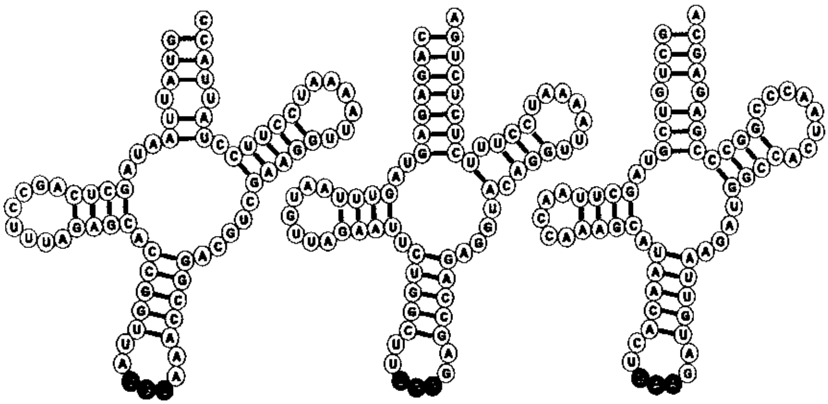
DISCUSSION

The primer pairs, CB3R/12SAR and CB3R/MUL12S, produced fragments that had significant similarity to the mtDNA cytochrome b and 12S rRNA genes, and flanking tRNAs of numerous fish. A 27 bp noncoding region, located between thr and pro, was also identified in these fragments (Figure 4). Although this region is not common in other fish species, Johansen *et al.* (1990) identified a 74 bp gap in the Atlantic cod. Species specific primers developed in the flanking tRNAs, pro and phe, produced an 1100 bp fragment showing significant similarity to both the corresponding sequence of the original fragment and the mtDNA control region. The fact that this product was the predicted length was a direct indication the primers were amplifying the desired portion of the mtDNA and not a nuclear pseudogene (Palumbi 1996). Nuclear pseudogenes have been observed in other species of marine organisms such as sea urchins, crabs, and corals (Jacobs et al. 1983, Palumbi 1996). An additional precaution was undertaken to exclude this possibility. Following the concept of "long PCR" (Cheng et al. 1994a, 1994b), the PCR product produced with the universal primers CB3R/12SAR was used as template in a nested PCR with previously published fish mtDNA primers located in pro and phe (Lee et al. 1995). The resulting PCR product was the expected size, thus confirming the mtDNA control region was amplified.

A. B. C.

Figure 2. Agarose gels of the mitochondrial DNA control region of the cobia, *Rachycentron canadum*, amplified with the following primer pairs: (A) Lanes 2-4: CB3R/12SAR, Lanes 5-6: CB3R/MUL12S (lanes are numbered left to right), (B) CobPro/CobPhe, and (C) A/G (see text for details). Agarose Gel Ladder sizes (top to bottom): 2000, 1500, 1000, 750, 500, 300, 150, and 50 base pairs (Amresco, Inc.).

The cobia control region ranged in length from 1,067 to 1,068 bp which is comparable to the control region length in other fish species. Lee et al. (1995) reported that the control region length of 27 species of fish ranged from 856 to 1,500 bp. Cichlids and gadids contained the shortest control regions and pleuronectids contained the longest. The nucleotide sequences and locations of the TAS and the CSBs, elements that are highly conserved in vertebrate control regions, were similar to those of other fish species (Table 1). We identified a TAS-I, as well as, a CSB-D, a CSB-I, a CSB-II, and a CSB-III (Fig. 4). TAS-I was located in pro as in the white sturgeon (Buroker et al. 1990), rainbow trout (Digby et al. 1992), and wahoo (Garber et al. in press). We also identified a pyrimidine block of 21 bp, that contained one purine, between CSB-D and CSB-I (Figure 4). A 17 bp pyrimidine block was identified in the Atlantic cod (Johansen et al. 1990) and a 26 bp block in the rainbow trout (Digby et al. 1992). This site may provide a point of interaction with mtDNA single-strand-binding protein (Digby et al. 1992).



A. B. C.

Figure 3. Sequence and structure of the mitochondrial tRNAs (A) threonine, (B) proline, and (C) phenylalanine from the cobia, *Rachycentron canadum*. Black circles represent the anticodons.

Pairwise comparisons revealed that each set of cobia sequences shared 99% sequence similarity. Only three transversions, one unresolvable ambiguity, and seven indels were observed (Figure 4). Seven of the 10 mutations and the unresolvable ambiguity were located near the ends of the samples and could possibly have resulted from direct sequencing artifacts. Regardless of their origin, variability was low and a central conserved region, flanked by more variable segments, could not be identified as in other fish species (Buroker et al. 1990, Johansen et al. 1990, Digby et al. 1992, Lee et al. 1995).

Mitochondrial DNA control region sequences have proven useful in population genetic studies and fisheries management. Although, their value has been contingent on the presence of a substantial amount of variability. Rosel and Block (1996) found there was enough variation in a 300 bp segment of the control region to indicate swordfish populations were structured on a global scale, but the variation was too high to detect subdivision within ocean basins. Therefore, by sequencing a 629 bp portion of the control region and increasing sample size, Reeb et al. (2000) was able to identify population structuring of swordfish in the Pacific. Seyoum et al. (1999) also found sufficient variability in a 369 bp portion of the red drum control region to suggest fisheries in the Atlantic and Gulf continue to be managed separately. To assess whether or not the cobia control region generally lacks variation or if this lack of variation is related to its migratory patterns and its possible utility as a molecular marker, it would be necessary to sequence more individuals from different localities.

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|Threonine
GTATTAATAG CTCAGCCTTT AGAGCACCGG TTATGTAAAC     40
                                 |Noncoding Region
CGGACGTCGA AGGTTAAAAT CCTTCCTATT ACCAAACTTT     80
                      |Proline
TTAACCAAGC TCTGCCACAC TCAGAGAGAA AGGATTTTAA    120
                                    TAS-I
CCTGTACCTC TGGCTCCCAA AGCCAGAATT CTAACATTAA    160
           |Control Region
ACTACTCTCT GGTATAACAC ATGTACTCCA AGTATAGTAC    200
ATATNTGTAT ATACCCCATA CATCTATATG TACGCATTCA    240
TGTAGTCTTC TAGGACATAA TTTATTATTC ACCTAACTTT    280
CCTTTCAACC ATAAAATCGA GGACAACCTT CCGACATAGG    320
TATAACATAT CAATGAATAT TCAATTTCAC ATGATAACGC    360
TTTTTAGACA TTTAWGTAAA ACTTCTAGTT TCACCATACT    400
ATTAAAATCC AAAGATATAC CAGGACTCAA CAATCTATTA    440
TCTCTAAACA GTTTAATGTA GTAAGAGCCC ACCATCAGTT    480
GATTCCTTTA CGCTAACGGT TCTTGATGGT CAAGGACAGT    520
                                    CSB-D
AACCGTGGGG GTCACATAAC TTGAATTATT CCTGGCATTT    560
GGTTCCTACC TCAGGGTCAC TAATCGCGTT ACTCCCCTCA    600
CTTTCATTGA CGCTCGCATA AGTTAATGCT TTTTATACAT    640
ACTCCTCGTT ACCCAGCATG CCGAGCGTTC TCTCCACAGG    680
               Pyrimidine Block
GGCCAGGGGT ATTTTTTTAT TTTTTCCTTT TCACTCGCAT    720
TTCACAGTGC AGAGCTAAGA CAGTTGAGCC AAGGTGGAGC    760
ATTTTCTTGC TTACACGTAC TACCGTTTAA TTATGAAGGA    800
                 CSB-I
CATTTACTGA TAAGTTACAT AACTGATATC AAGAGCATAA    840
TGCATGTAAT ATCCCCCTGG GATCTCTAAG AACCTTAATT    880
TCTCAGAACT TCCAGGATTA AACTAAAGGT AGGTGGGCGA    920
      CSB-II
TAAACCCCCC TACCCCCCTA AACTCCTAAG ATCAATGTGA    960
        CSB-III
CTCCTGCAAA CCCCCCGGAA ACAGGAAAAT CTTAGGGTTA   1000
GAATCAAACT AAACGTCCCA AAATTAAATG CTAAGAGACA   1040
AACAAGGCCC TGACACACCC CCCCCCTTTT TTAGCATACG   1080
CAAAACACGC CCTCTTGTAC TATAGTGCCC CTAAAATCAA   1120
AATATTGAGG CATCATAAAA ATTTATATTA TCATATTATT   1160
TTTTATAATA ATATTTTAAT TTTATACAGG GTAACCAAAW   1200
                                          |Phenylalanine
TCWCGTAACA ATACTATTTG AATTTTATGT AACATCACCG   1240
CTGTCGTAGC TTAACCGAAG CATATCACTG AAGATGTTAA   1280
GATGGCCACT AACCCGGCCC GAGAGCA                 1320
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Figure 4. Consensus sequence of the mtDNA control region and flanking tRNAs of the cobia, *Rachycentron canadum*. The termination associated sequence (TAS-I), conserved sequence blocks (CSB-D, CSB-I, CSB-II, CSB-III), and pyrimidine block are underlined. The transversions (W) and unresolved sequence ambiguity (N) are in bold text and double underlined.

Due to rising importance in commercial and recreational fisheries, increased pressure has been placed on the cobia. Therefore, information on the biology, life history, and genetics of the cobia is fundamental to preventing a decrease in stocks beyond a sustainable level and the associated loss of genetic diversity. This study provides initial molecular genetic data that will prove useful in future research.

Table 1. Comparative alignment of termination associated sequence (TAS) and conserved sequence blocks (CSB) identified in the cobia, *Rachycentron canadum*, and other fish species.

TAS-I		
	Cobia	ACATTAAACTACT
	Wahoo[1]	AAATTAAACTATT
	Trout[2]	AAATTAAACTACC
	Sturgeon[3]	ACATTAAACTATT
CSB-D		
	Cobia	GG-CATTTGG-TTCCTACCT-CAGGGTCACT
	Wahoo	GGGCATTkGGGTTCCTAATTTCAGG-TCCAT
	Swordfish[4]	GG-CATTTGG-TTCCTACTT-CAGGG-CCAT
	Sturgeon	GG-CATCTGG-TTCCTA-TTTCAGG-TCCAT
CSB-I		
	Cobia	ACATA
	Wahoo	ACATA
	Trout	ACATA
	Snook[5]	ACATA
CSB-II		
	Cobia	TAAACCCCCCTACCCCC
	Wahoo	TAAACCCCCCTACCCCC
	Trout	TAAACCCCCCTACCCCC
	Sturgeon	-AAACCCCC-TACCCCC
CSB-III		
	Cobia	TGCAAA-CCCCCCGGAA-C-A
	Wahoo	TG-AAAACCCCCCGTAAAC-A
	Trout	TGTTAAA-CCCC—TAAACCA
	Swordfish	TG-AAAA-CCCCC

[1]Garber et al. in press; [2]Digby et al. 1992; [3]Buroker et al. 1990; [4]Rosel and Block 1996; [5]Wilson et al. 1997

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