Mitochondrial DNA Analysis of Cobia *Rachycentron canadum* Population Structure Uisng Restriction Fragment Length Polymorphisms and Cytochrome B Sequence Variation

Adam Wayne Hrincevich

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by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1.

Restriction Endonuclease Analysis of Cobia Mitochondrial DNA

INTRODUCTION

Cobia *Rachycentron canadum* represent the only species in the family Rachycentridae. They are large fish, known to reach 1.8 m in length and 68 kg in weight (Robins and Ray 1986). Cobia perform extensive seasonal migrations and inhabit most tropical, subtropical, and warm temperate seas of the world (reviewed in Shaffer and Nakamura 1989). They range from Massachusetts to Bermuda and Argentina and occur in the Gulf of Mexico (Richards 1967, Robins and Ray 1986). In the Gulf of Mexico, cobia range from Key West, Florida to Campeche, Mexico (Dawson 1971). Gulf of Mexico cobia are believed to overwinter in the Florida Keys and Caribbean and then travel northward in the spring to spawning/feeding grounds (Franks et al. 1992). Cobia first enter the region of the Florida panhandle in March and appear in Mississippi waters in April; they leave Mississippi waters by October (Franks et al. 1992). Other fish move northward along the Atlantic coast (Richards 1977, Robins and Ray 1986).

Cobia is a popular game fish and one of the most sought-after by sports fishermen in the Gulf of Mexico. Fishing pressure on cobia has increased in recent years, presumably due to regulatory measures placed on other popular coastal species such as grouper, king and Spanish mackerel, red drum, and snapper. The only regulatory measures currently placed on cobia are a minimum fork length of 83.8 cm and a catch limit of two fish per person per day. As a result of this increased fishing pressure, cobia are now considered a stressed fishery. Proper management of this species requires a detailed knowledge of the population structure. To date, this information has not been completely defined.

Despite the recreational value of cobia, the life history of the species is poorly understood. Aspects of the population biology which

have been examined include tag and release studies (Richards 1977, Franks et al. 1992), larval distribution and development (Ditty and Shaw 1992), and reproductive biology (Franks et al. 1992). Despite these studies, many aspects of the life history of the species remain unclear.

Richards (1977) investigated the migration habits of cobia in the Chesapeake Bay area. Cobia were tagged and released during fishing tournaments which were held during the early seasonal appearance (late to mid-June) of cobia in the Chesapeake Bay area. Over a two year period, twenty cobia were tagged and released. Six fish were recaptured, with times at-large ranging from one month to five years. The recaptures ranged from 12 to 36 nautical miles from the original tagging locations and were all reported during the summer months, July through August. These tagging returns suggest that distinct, repetitive summer habitation occurs and that Chesapeake Bay cobia may be a distinct group or subpopulation (Richards 1977).

Tagging studies by Franks et al. (1992) in the northern Gulf of Mexico demonstrated that some cobia do indeed tend to return to the same coastal region from which they were tagged. A total of 4,282 fish were tagged during the period 1988 through 1992; 239 of these fish were recaptured. Of these fish, 104 were recaptured in the same vicinity of the site they had been tagged, with at-large times ranging from one day to six months. Of particular interest were six fish recaptured at the identical location (the same buoy or wreck) where tagging had occurred the previous year. Five additional fish were recaptured two or more years later at the identical location of tagging. However, seasonal fidelity to original tagging locations is not always the case. Some fish are recaptured in areas very distant from the original tagging location. For example, a fish tagged off of South Marsh Island, LA was recaptured eight months later off of Daytona Beach, FL, traveling a distance of at least 1,300 nautical miles. Another long-distance recapture was a fish tagged off of the Chandeleur Islands, LA and recaptured near Charleston, SC, traveling approximately 1,200 nautical

miles. This recapture also represents the first known recapture that far north up the U.S. east coast of a fish tagged in the northern Gulf of Mexico. In another incident, a fish tagged off Cape Canaveral, FL was recaptured west off Grand Isle, LA. This example represents the first known recapture west of the Mississippi River of a cobia that was tagged off the U.S. east coast. Thus, cobia appear able to move freely between the Gulf of Mexico and the southwest Atlantic. The Franks et al. (1992) tagging returns suggest that cobia may consist of a heterogeneous breeding population, with reduced substructure.

The migratory routes for cobia within Gulf waters are still somewhat unclear. The tag and release data provided by Franks et al. (1992) have provided much of what is known about the trends in seasonal movement between spawning and wintering grounds. The strongest probability (at least for a large segment of the cobia population) is a spring migration from the south Florida region (where many cobia have over-wintered) northward off the Florida Gulf Coast, continuing along the Florida panhandle, then westward toward Alabama, Mississippi, and Louisiana. A reverse migration in late fall and early winter along a similar route is believed to occur for some segments of the population.

It appears, however, that not all cobia migrate from the northern to the southern Gulf of Mexico during winter (Franks et al. 1992). Some cobia tend to move into the deeper waters (100+ meters) of the northern Gulf during the colder months (where they may over-winter) and then move back toward shallower water and then on toward the Chandeleur Islands, LA during the spring. Four winter recaptures have been reported to date in the northern Gulf of Mexico and fish have been observed around oil rigs off of Louisiana in early February. Additionally, cobia are caught in the Keys and south Florida year-round. Due to the large number of fish tagged and released in the Franks et al. (1992) study, a clearer understanding of cobia migration patterns should become evident as more fish are recaptured.

Another interesting and unexplained aspect of the reproductive biology of cobia is the extremely low abundance (as compared to other species) of eggs, larvae, and juvenile fish which have been collected in the northern Gulf of Mexico. Cobia spawn sizes are extremely large, ranging between two and 191 million eggs per female per spawn (Richards 1967, Franks et al. 1992). The number of spawns females perform per season is uncertain. Studies by Richards (1967) and Franks et al. (1992) both suggest that cobia spawn more than once per season, but the exact number of times is unclear. While the fecundity rate of cobia appears to be extremely high, the only confirmed simultaneous collection of cobia eggs and yolk-sac larvae from the Gulf waters has been from the Crystal River estuary, FL (Ditty and Shaw 1992). These same authors reported only 70 larvae less than 20 mm in length collected and identified in plankton tows taken from the Gulf of Mexico during the period from 1967 to 1989. Additionally, juvenile cobia, 27 mm or greater in length, have rarely been observed or collected in the Gulf of Mexico or Atlantic waters (Dawson 1971).

Many pivotal questions about the life history of cobia remain unanswered, including whether the species exists as one breeding population or several distinct subpopulations. Past studies on fish population genetics have utilized protein electrophoresis to examine intraspecific population structure. Five of the common amino acids which make up proteins are charged: lysine, arginine, and histidine are positive, whereas aspartic acid and glutamic acid are negative (Martin et al. 1985). Thus, different proteins tend to have different net electrical charges. Electrophoresis uses this physical property to separate mixtures of proteins on the basis of charge. If allelic differences (i.e. different forms of a gene) occur at a protein coding locus, the net charge of the protein often changes. Gel electrophoresis takes advantages of these changes to identify such allelic differences, and thus, differences between individuals in a population.

Utter et al. (1989) examined the population genetic structure of chinook salmon *Oncorhynchus tshawytscha* using protein electrophoresis. These authors examined the variation at 25 polymorphic protein coding loci of fish representing 65 different locations. Sample sizes between 38 and 200 individuals were used when examining each of the 25 polymorphic protein coding loci. Mobilities of four proteins (dipeptidase, mannose-6-phosphate isomerase, phosphoglycerate kinase, and superoxide dismutase) defined nine genetically distinct populations. Similar studies have also identified intraspecific population structure in Greenland halibut *Reinhardtius hippoglossoides* (Fairburn 1981), capeline *Mallotus villosus* (Mork and Friis-Sörensen 1983), and bluegill sunfish *Lepomis macrochirus* (Chapman 1989b).

Protein electrophoresis is widely utilized for several reasons. It is relatively inexpensive, easily prepared, and many samples can be run on a single gel as compared to restriction analysis or sequencing techniques (Ryman and Utter 1988). However, there are limitations to the information that can be obtained by electrophoresis at protein coding loci. The information needed in population genetics relates to the base sequences of DNA, studied either directly or indirectly. The amino acid substitutions of proteins detected by electrophoresis are indirect reflections of the actual base substitutions in nucleotide sequences. All base substitutions do not necessarily result in changes of amino acids. The degeneracy of the mitochondrial genetic code accounts for the lack of many amino acid changes as a result of a third base substitution in a codon (Anderson et al. 1981). Furthermore, all amino acid substitutions do not result in protein changes that are electrophoretically detectable. These amino acid substitutions are usually ones which are not radical. For example, a neutral amino acid usually replaces a neutral amino acid (Grantham 1974).

Due to the limitations of protein electrophoresis, it is preferable to examine the DNA nucleotide sequence of organisms when investigating intra- or interspecific population relatedness. More genetic variation

can be detected at the DNA level. This allows for studies of nucleotide variations that do not affect gene products, i.e. silent mutations. Substitutions accumulate at different rates in genomic DNA and mitochondrial DNA (mtDNA) (summarized in Nei and Koehn 1983). MtDNA has been examined extensively and is well characterized for a variety of different species (reviewed in Ryman and Utter 1988). It also exhibits several unique qualities that make it particularly suitable for population genetics.

Ramsey and Wakeman (1987) utilized protein electrophoresis to survey allelic variation at four polymorphic loci among red drum *Sciaenops ocellatus* sampled from 12 locations in the northwestern Gulf of Mexico and two locations along the Atlantic coast. The results of their study determined that red fish essentially comprised a single, randomly mating population. Gold and Richardson (1991) performed a similar study, but instead of surveying allelic variation at polymorphic loci, these authors examined nucleotide variation in mtDNA utilizing restriction endonucleases. The results of their study contradicted those of Ramsey and Wakeman (1987) and indicated that the red drum population is subdivided, with semi-isolated subpopulations occurring along the southeastern Atlantic coast and in the northern Gulf of Mexico. This example, in addition to other recent studies in a variety of organisms, including fishes, demonstrates that restriction fragment or site analysis of mtDNA is more powerful than protein electrophoresis for differentiating subpopulations within species (Avise et al. 1987, Moritz et al. 1987, Nei and Koehn 1983).

It is generally accepted that animal mtDNA evolved from a larger, more complex symbiotic genome by progressive transfer of its genetic contents and functions to the nucleus (reviewed in Grivell 1983, Nei and Koehn 1983). The mtDNA of most multicellular animals ranges from 15.7 to 19.5 kilobases (kb) (Brown and Wright 1979, Nei and Koehn 1983). Cobia mtDNA is ~20.0 kb, one of the largest genomes among vertebrates observed to date. The animal mitochondrial genome consists of a single,

duplex, closed-circular DNA molecule (Brown et al. 1979, Grivell 1983). It does not undergo recombination and evolves more rapidly than genomic DNA (Nei and Koehn 1983). Therefore, a change in the nucleotide sequence is due to a base mutation and is not due to recombination events. Populations separated geographically will commonly exhibit extensive changes in their nucleotide sequence. The extent of these changes is used to identify distinct breeding populations. MtDNA is transmitted primarily by the female parent (Lansman et al. 1983, Gyllensten et al. 1985, Avise and Vrijenhoek 1987), though cytoplasmic inheritance in the ovum. Therefore, individuals with a particular mtDNA genotype are considered to be from the same maternal clone (Nei and Koehn 1983). Most of the data that suggest a maternal mode of mtDNA transmission have been based on single-generation mating experiments. Long-term mating experiments, employing repeated backcrossings to the same female, have demonstrated that mtDNA is transmitted by the male parent in low levels. Gyllensten et al. (1985, 1991) determined that in an average mouse approximately one mtDNA molecule in 1000 was contributed from the male parent. Similar results of a paternal mode of mtDNA transmission have also been observed in other organisms including mussels *Mytilus* (Hoeh et al. 1991, Zouros et al. 1992), insects *Heliothis* (Lansman et al. 1983), and fish *Poeciliopsis* (Avise and Vrijenhoek 1987).

Initial population studies utilizing the mitochondrial genome revealed virtually no intraspecific size heteroplasmy (variation in size of the mtDNA molecule between individuals) or sequence heteroplasmy (the possession by individuals of more than one type of mtDNA molecule). Most of the mutational changes were attributed to base substitutions either at silent sites or in the noncoding portions of the genome (reviewed in Nei and Koehn 1983). Insertions and/or deletions were rarely observed and the relative gene order was found to be highly conserved (reviewed in Nei and Koehn 1983). These observations led to

the perception that the animal mitochondrial genome was an extremely economical unit (Attardi 1985).

Recent studies of invertebrate and poikilotherm vertebrate mtDNAs have, however, have changed this perception. Mitochondrial genomes >20.0 kb have been found in several phylogenetically distant groups: nematodes (Powers et al. 1986), scallops (Snyder et al. 1987, La Roche et al. 1990, Gjetvaj et al. 1992), pine weevils (Boyce et al. 1989), frogs (Kessler and Avise 1985), newts (Wallis 1987), lizards (Densmore et al. 1985, Moritz and Brown 1986, 1987), and fish (Beckwitt 1985, Bermingham et al. 1986, Bentzen et al. 1988, Gach and Reimchen 1989). The length of animal mtDNA is now known to vary in size by a factor of nearly three, from 14.2 to 41.0 kb (Wolstenholme et al. 1987, Gjetvaj et al. 1992, reviewed in Nei and Koehn 1983). However, there appears to be no correlation between mtDNA size and taxonomic relationship (Gjetvaj et al. 1992).

Certain types of mtDNA size variation have been shown to result from large amplifications or deletions, which in many occasions include functional portions of the molecule (Moritz and Brown 1987, Wallace 1989). Heteroplasmy, when it occurs, is usually due to the differences in the copy number of tandemly repeated sequences among otherwise identical molecules. Heteroplasmy due to sequence differences is more rare and the differences are usually slight (<1%) (Hoeh et al. 1991). With heteroplasmy, the conditions for maintenance of a polymorphism are much less restrictive because selection in favor of heteroplasmic individuals can be invoked (Clarke 1988). The adaptive advantage of heteroplasmy might relate to somatic segregation with particular mtDNA genotypes favored and thus predominating in certain tissues (Fisher and Skibinski 1990).

Length polymorphisms have been documented in fruit fly (Solignac et al. 1986), cricket (Rand and Harrison 1986, 1989), scallop (Snyder et al. 1987, La Roche et al. 1990, Gjetvaj et al. 1992), newt (Wallis 1987), lizard (Densmore et al. 1985), fish (Bentzen et al. 1988,

Mulligan and Chapman 1989, Buroker et al. 1990), and avian (Avise and Zink 1988) mitochondrial genomes. The length of the individual repeat varied from <0.1 kb in lizards (Densmore et al. 1985) to >3.0 kb in nematodes (Hyman et al. 1988), although differences in copy number within the molecule generated multiple size classes that, in some cases, differed by as much as 10.0 kb (La Roche et al. 1990, Gjetvaj 1992). In the majority of the previously mentioned studies, the source of the length polymorphism was shown to lie within or close to the control region sequences, i.e. the D-loop. Hqwever, length polymorphisms have been shown to occur in several dispersed portions of the mitochondrial genome in a species of nematode (Beck and Hyman 1988) and two species of scallops (La Roche et al. 1990, Gjetvaj et al. 1992) as revealed by mapping and sequencing of the mitochondrial genome.

Direct sequencing of the mitochondrial genome can be a costly and time-consuming process. However, the mitochondrial genome of conspecific individuals may be screened for nucleotide sequence differences through the use of restriction endonucleases (reviewed in Ryman and Utter 1988). These endonucleases recognize specific nucleotide base pairs and consistently cleave the DNA within the recognition site. Multiple recognition sites result in a series of DNA fragments of various sizes. Nucleotide substitutions in the recognition sites prevent endonucleases from cleaving the DNA and thus reduce the number of mtDNA fragments. Similarly, base pair substitutions outside the recognition site may create new cleavage sites and additional mtDNA fragments. After digestion with a restriction enzyme, mtDNA fragments are separated by size electrophoretically in an agarose gel. Fragment sizes are estimated by comparing their electrophoretic mobilities (which are largely a function of their sizes) rather than their charge, as in protein electrophoresis, to the mobilities of known size standards. The differences in fragment sizes reflect single-point mutations in the base sequence recognized by the restriction endonucleases. The fragment patterns of individual mtDNAs digested with the same restriction enzyme

are then compared. The banding patterns which result are commonly referred to as restriction fragment length polymorphisms (RFLPs). The presence of characteristic RFLPs is used as the basis for determining intraspecific relatedness.

Restriction endonuclease analysis of mitochondrial DNA has become a powerful tool in the study of animal populations. Recently, it has been used successfully to identify genetically distinct populations of anadromous and nonanadromous Atlantic salmon *Salmo salar* (Palva et al. 1989, Birt et al. 1991). Palva et al. (1989) examined 23 nonanadromous and 9 anadromous fish. Fifteen restriction endonucleases recognizing four-, five-, or six-base sequences were used in a survey of restriction site variation. Of the 15 enzymes utilized, 13 produced the same RFLP pattern in all of the fish. Only the enzymes which recognized four and five base pair sequences, *Hae* III and *Hinf* I, respectively, revealed a sequence polymorphism in the salmon populations examined. Each enzyme revealed two genotypes that distinguished anadromous and nonanadromous stocks. The different genotypes persisted throughout each stock and could be clearly distinguished from each other. Birt et al. (1991) examined 71 salmon using 18 restriction endonucleases, all of which recognized six-base sequences. Of these 18 enzymes, 14 produced the same RFLP pattern in all fish. Four restriction endonucleases, *Bgl* I, *Bst*E II, *Dra* I, and *Hinc* II, revealed distinct RFLP patterns between the nonanadromous and anadromous populations. Similar studies examining RFLP patterns have identified discrete stocks of anadromous fishes including American shad *Alosa sapidissima* (Bentzen et al. 1988, 1989, Nolan et al. 1991), cisco *Coregonus artedii* (Bernatchez and Dodson 1990), striped bass *Morone saxitilis* (Chapman 1987, 1989a, Wirgin et al. 1990), and white perch *Morone americana* (Mulligan and Chapman 1989). Marine populations of haddock *Melanogrammus aeglefinus* (Zwanenburg et al. 1992), walleye pollock *Theragra chalcogramma* (Mulligan et al. 1992), and Atlantic and Gulf of Mexico populations of sea bass *Centropristis*

striata, menhaden *Brevoortia tyrannus* and *Brevoortia patronus*, and sturgeon *Acipenser oxyrhynchus* (Bowen and Avise 1990) have also been determined by RFLP studies.

Intraspecific size heteroplasmy has been used successfully to differentiate among discrete breeding populations. Wirgin et al. (1989) examined mitochondrial DNA diversity among populations of striped bass *Morone saxitilis* in the southeastern United States. A total of 206 fish was examined, representing five separate sampling localities. Ten restriction enzymes were utilized to examine nucleotide sequence similarities between the sampling areas. Based on the RFLP analysis, the nucleotide sequence divergence among the populations was extremely low. Two restriction enzymes, however, revealed RFLPs unique to populations of Gulf Coast striped bass. More importantly, four discrete mtDNA size classes were detected, differing by 0.2, 0.3, and 0.4 kb from the smallest mtDNA molecule observed. Based on these size polymorphisms, distinct breeding subpopulations were defined for the Apalachicola River system, FL and the Atlantic Coast regions, supporting the hypothesis that Gulf Coast and Atlantic Coast striped bass constitute separate populations.

Significant intraspecific variation of mtDNA sequences has been reported in several species of fish (reviewed in Ryman and Utter 1988) and has permitted high-resolution investigations of the genetic basis of population structure (Nei and Koehn 1983). However, studies that have compared results from protein electrophoresis and mtDNA restriction endonuclease analysis have demonstrated that the latter technique uncovers significantly more intraspecific variation and differentiation (Nei and Koehn 1983). Furthermore, because of the faster rate of mtDNA evolution, population-specific differences in mtDNA sequences accumulate more rapidly than electrophoretic differences, and significant genetic differentiation can be observed with smaller sample sizes (Nei and Koehn 1983).

This study reports the results of a RFLP analysis of cobia mtDNA. The objectives of this study were to examine the degree of nucleotide sequence divergence among cobia in the northern Gulf of Mexico and Chesapeake Bay area and determine whether the species constitutes a single breeding population or several discrete subpopulations. This information, in turn, will provide valuable biological data needed by fisheries biologists to properly manage and predict the long-term population status of this fishery.

MATERIALS AND METHODS

Sample Collection

Cobia tissue samples were obtained at fishing tournaments where anglers entered their fish in competition for prizes. The fish were well-iced from the time of capture until entry into the tournaments (from 2-24 h after landing) and all samples were in excellent condition. Cobia landed in Louisiana, Mississippi, and Florida waters were sampled from May to September of 1991 and 1992 whereas cobia from Texas and Alabama waters were sampled in 1992 only. Cobia from the Virginia region of the Chesapeake Bay were obtained in July and August of 1992.

Immediately after each fish was weighed and measured, whole gonads were removed, placed in separate resealable plastic bags, and stored on ice for 4-12 h until weights could be recorded and aliquots for DNA isolation taken. Data including location of capture, sex, total weight, and measurements (total and fork lengths) were also maintained. Separate aliquots (~10 g) from each gonad were stored at 4°C in MSB-C-E buffer (210 mM mannitol, 70 mM sucrose, 50 mM Tris, pH 7.5 and 3 mM calcium chloride and 10 mM EDTA) (Lansman et al. 1981) for one to five days before mtDNA extraction. Duplicate aliquots were also archived in buffer and stored at -80°C.

Ninety cobia were examined for this study. These fish included representatives of both sexes, capture locations in waters of six different states, different months and years of capture, and different stages of gonadal maturity (Table 1). Almost all of the males were running ripe; i.e., the testes yielded milt when they were sliced open and gently squeezed. Samples of ovary were fixed in 10% buffered formalin, processed using a modification of Humason (1979) in an automatic tissue processor, embedded in paraffin, sectioned at 5 μm, stained using Delafield's hematoxylin and eosin, and examined microscopically.

Table 1. *R. canadum* catch statistics and haplotypes. Numerals (females) or letters (males) under Gonad Stage indicate stage of gonad development: 1, previtellogenesis; 2, vitellogenesis; 3, final maturation; 4, post-ovulation; 1', 2nd previtellogenesis; 2', 2nd vitellogenesis; M, sexually mature male fish; n/d, not determined. Letter sequences under Haplotype refer to the digestion profiles as listed in Table 3 for endonucleases from left to right: *Hind* III, *Sac* II, *Kpn* I, *Bcl* I, *Sac* I, *Ban* I, *Nde* I, *Hinc* II, *Eco*R I, *Ava* I, *Eco*R V.

Fish #	Sex	Capture Location	Month	Year	Gonad Stage	Haplotype
1	♀	MS	Mar	91	n/d	AABAABCCCDE
2	♀	LA	May	91	3	AABCCDFHILP
3	♀	MS	May	91	3	AAAAACEDFHK
4	♀	LA	May	91	1	AABAAACCCCC
5	♀	LA	May	91	1	AABBAADCCFF
6	♂	LA	Jul	91	M	AAAAABBDDCC
7	♀	FL	Jul	91	1'	AABBACDECFF
8	♂	FL	Jul	91	M	AAAAAAABBAN
9	♂	FL	Jul	91	M	AAAAABBDBCC
10	♂	FL	Jul	91	M	AAABADEFHIN
11	♀	FL	Jul	91	3	AABACCDECFR
12	♀	FL	Jul	91	1'	AABBACDECFI
13	♀	FL	Jul	91	1'	AABBBCEEEHK
14	♀	MS	Aug	91	n/d	AAAAABCBDDD
15	♀	MS	Aug	91	n/d	AAAAACDDFGH
16	♂	LA	Aug	91	M	AAAAABCCDDE
17	♂	LA	Aug	91	M	AAAAADEFHIM
18	♀	LA	Aug	91	n/d	AABAACDCEHK
19	♀	MS	Aug	91	1	AABBADEEGKN
20	♀	LA	Aug	91	1	AABABAGAAAC
21	♀	MS	Sep	91	1	AABAABCCCFG
22	♀	MS	Sep	91	2	AABABABAABB
23	♂	MS	Sep	91	M	AAAAAABBBBC
24	♀	MS	Sep	91	1	AABBBCEEEHJ
25	♀	MS	Apr	92	4	AABBADEFEHK
26	♀	MS	Apr	92	3	AAAAAABBBBB
27	♀	MS	Apr	92	3	AAABACDDDFG
28	♀	MS	Apr	92	4	AABAABDCCEF
29	♀	MS	Apr	92	4	AABBADEFFHL
30	♀	MS	Apr	92	3	AAAAAACBBCC
31	♀	MS	Apr	92	3	AAAAAACBBCC
32	♀	MS	Apr	92	3	AAABADFFHKN
33	♀	MS	Apr	92	1'	AABAABDCCEF
34	♀	MS	Apr	92	4	AAAAABCBDOD
35	♂	MS	Apr	92	M	AAAAABCDDEE
36	♀	MS	Apr	92	3	AAABADDDFGJ
37	♀	MS	Apr	92	3	AAAAADDDDFG
38	♀	MS	Apr	92	2	AAAAACCBDDE
39	♀	LA	Apr	92	3	AAAAADCBDDE
40	♀	MS	May	92	2	AAAAACBBBCC
41	♀	MS	May	92	2'	AAAAABGBBCC
42	♂	MS	May	92	M	AAABACCBDDG
43	♀	MS	May	92	4	AABBADDDDFG
44	♀	MS	May	92	4	AAABABCBDCF
45	♀	MS	May	92	3	AAAAABCBDBE
46	♀	MS	May	92	3	AAAAABCBDCF
47	♀	LA	May	92	3	AAAAABCBDCF
48	♀	MS	May	92	3	AAAAABDDDEJ

Table 1. *Continued*

49	♀	MS	May	92	3	AAAAABCBDCE
50	♀	MS	May	92	3	AAACACEDFHN
51	♀	MS	May	92	4	AAAAABCBDDG
52	♀	MS	May	92	1	AABAABBACBE
53	♀	MS	May	92	3	AAABABCBDMF
54	♀	MS	May	92	4	AAAAABCBDCE
55	♀	MS	May	92	3	AAABACEDFHN
56	♀	MS	May	92	4	AAACADFFJHO
57	♀	MS	May	92	3	AAABABDDDEI
58	♀	MS	May	92	3	AAABABCDDCF
59	♀	AL	Jul	92	4	AAAABBBABAA
60	♀	AL	Jul	92	2	AAACADFFHKN
61	♀	AL	Jul	92	2	AAABACDDFFJ
62	♀	AL	Jul	92	3	AABBADEEGIN
63	♀	AL	Jul	92	. 2	AABBBCDCKEH
64	♀	AL	Jul	92	1'	AABBACEEEHL
65	♂	FL	Jul	92	M	AAABADEFHHM
66	♀	FL	Jul	92	3	AAAAABBBBBB
67	♂	FL	Jul	92	M	AAAAABABBAA
68	♂	TX	Jul	92	M	AAAAABBBBBB
69	♂	TX	Jul	92	M	AAABACCDDDE
70	♀	TX	Jul	92	4	AABABBCCACC
71	♂	TX	Jul	92	M	AAABACDFDFH
72	♂	TX	Jul	92	M	AAABACDDDEG
73	♂	TX	Jul	92	M	AAABACDFFFI
74	♂	TX	Jul	92	M	AAABACCBDDE
75	♀	TX	Jul	92	3	AAACADEFHJL
76	♀	VA	Jul	92	n/d	AAAAABDDDFG
77	♀	VA	Jul	92	n/d	AAABACFGHKN
78	♀	VA	Jul	92	n/d	AAAAAACBDCD
79	♀	VA	Jul	92	n/d	AAABABDDDFG
80	♀	VA	Jul	92	n/d	AAABABDDDFH
81	♀	VA	Aug	92	3	AAABACEDFGJ
82	♀	VA	Aug	92	1'	AABABCECEHK
83	♀	VA	Aug	92	1	AABABBCAKCD
84	♀	VA	Aug	92	2	AABABDFEAKN
85	♀	VA	Aug	92	4	AABABBCCCDE
86	♀	VA	Aug	92	2	AAAAAEHBLNQ
87	♀	VA	Aug	92	2	AABBACDDCEF
88	♂	VA	Aug	92	M	AAABACDDDEF
89	♂	TX	Aug	92	M	AAACADFFHIM
90	♀	TX	Aug	92	4	AAABACDDDEH

Ovaries were assigned to one of six different stages of ovarian development including: Stage 1, previtellogenesis (primary oocytes dominate); Stage 2, vitellogenesis (yolk is being produced); Stage 3, final maturation or ripeness (large, yolky oocytes are present); Stage 4, post-ovulation (post-ovulatory follicles are present and unspent Stage 3 oocytes are being resorbed); Stage 1', subsequent previtellogenic stage after a spawning episode (Stage 1 primary oocytes as well as post-ovulatory follicles and unspent ripe oocytes occur); and Stage 2', subsequent vitellogenic stage after a spawning episode (Stage 2 vitellogenic oocytes occur along with post-ovulatory follicles and resorbing unspent ripe oocytes). For further details of cobia ovarian development, see Caylor (1992).

Extraction of Mitochondrial DNA

MtDNA was routinely isolated from both ovary and testis giving a high yield of relatively undegraded mtDNA. The protocol for mtDNA isolation was based on modifications of Lansman et al. (1981), Chapman and Powers (1984), and Wallis (1987). Ten grams of gonadal tissue were thoroughly minced using scissors and then homogenized using a motor-driven glass-teflon homogenizer in ice-cold MSB-C buffer (210 mM mannitol, 70 mM sucrose, 50 mM Tris, pH 7.5, and 3 mM calcium chloride) (Lansman et al. 1981). Ethylenediamine tetraacetic acid (EDTA) was added to the homogenate for a final concentration of 10 mM. The homogenate was centrifuged at 2,500 x g for 10 min at 4°C until no pellet formed; usually four rounds of centrifugation were necessary. Mitochondria were pelleted by centrifuging at 20,000 x g for 20 min at 4°C. The pellet was resuspended in ice-cold MSB-E (210 mM mannitol, 70 mM sucrose, 50 mM Tris, pH 7.5 and 10 mM EDTA); the high speed centrifugation step was repeated and the pellet was resuspended in STE buffer (100 mM NaCl, 50 mM Tris, pH 8.0, and 10 mM EDTA) (Lansman et al. 1981). Mitochondria were lysed with 80 μl of 25% sodium dodecylsulfate (SDS), treated with 250 μg RNAse A and 250 units T_1 RNAse for 30 min at 37°C, and then

treated with 250 μg Proteinase K for two h at 37°C. MtDNA was extracted with an equal volume of phenol:isoamyl alcohol:chloroform (25:1:24, v/v). Extractions were typically performed two to three times, until no interface formed. The aqueous phase was transferred to a fresh tube and 2.5 volumes of 100% ethanol (EtOH) and 0.5 volumes of 7.5 M ammonium acetate were added and stored overnight at 4°C. Precipitated mtDNA was obtained by centrifugation at 12,000 rpm in an Eppendorf microcentrifuge for 45 min at 4°C. The pellet was washed once with ice-cold 70% EtOH, once with 100% EtOH, dried briefly under vacuum to remove residual EtOH, resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0), and stored at -20°C. Samples were run in 0.8% agarose gels to estimate quality and quantity of mtDNA. These preparations were of sufficient purity for digestion by restriction enzymes.

Quantification of Mitochondrial DNA

Mitochondrial DNA was quantified by spectrophotometric methods using the fluorescent dye bisbenzimidazole (2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol•3HCl), also known as Hoëchst dye 33258 (Labarca and Paigen 1980). The working concentration is 100 ng Hoëchst dye per ml of TNE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4). A Perkin Elmer 650-10S Fluorescence Spectrophotometer was used to measure fluorescence levels.

To quantitate mtDNA, a standard curve was first constructed using mtDNA of known concentration, as determined by A_{260} measurements. The spectrophotometer was first zeroed using 1 ml of Hoëchst buffer. One μl, equivalent to 100 ng of mtDNA, was added to a quartz cuvette which contained 1 ml of Hoëchst buffer. Fluorescence measurements were read at an excitation level of 352 nm and an emissions level of 448 nm. This process was repeated using additional mtDNA to construct a standard curve which ranged from 0 to 500 ng mtDNA. For samples of unknown concentrations, 1 μl of mtDNA was added to 1 ml of Hoëchst buffer and

fluorescence levels were measured. It was sometimes necessary to use up to 5 μl of purified mtDNA in cases where concentrations were low.

All fish mtDNA preparations were initially quantified in the above manner. However, since the preparations included varying amounts of contaminating genomic DNA, quantifications were fine tuned by using photographs of gels which depicted the uncut samples run after initial isolation and purification of mtDNA. The concentration of Lambda DNA used for the size standard was known and could be used to estimate concentrations of the uncut mtDNAs. Samples quantified in this manner sometimes yielded mtDNA concentrations ~10 times lower than those of the fluorometric methods.

Ultimately, the actual amount of mtDNA sufficient to visualize all restriction fragments was determined empirically. If fragments were faint, the volume of mtDNA was increased. It is estimated that ~100 to 200 ng were used per digest as determined by comparisons to the molecular weight size standard.

Initial Screening of Cobia Mitochondrial DNA

In 1991, an initial battery of 22 restriction endonucleases (Table 2) was selected to characterize the mtDNA from 24 fish. All of the enzymes tested were commercially available at reasonable prices, ranging from $0.10 to $0.88 per digest. Primarily six-base recognition endonucleases were selected; only *Rsa* I and *Hinf* I are four and five-base cutters, respectively. In general, four-base cutters generate three to six times as many fragments as six-base cutters (reviewed in Ryman and Utter 1988). The 22 restriction endonucleases chosen represented a variety of cleavage sequences, an important consideration since nothing was known about the nucleotide composition of cobia mtDNA. For example, the enzyme *Dra* I recognizes and cleaves a sequence which is AT-rich (5'-TTT▾AAA-3') whereas the enzyme *Sac* II recognizes and cleaves a sequence which is GC-rich (5'-CCGC▾GG-3'). The 24 fish used in the initial survey included both sexes, capture locations from three states (MS, LA,

Table 2. Restriction enzymes used for initial characterization of *R. canadum* mtDNA. Asterisks (*) indicate enzyme was used throughout duration of study. Numbers under Proficiency of Cutting mtDNA indicate: 1, enzyme cut to completion and produced fragments which could be accurately sized; 2, enzyme produced the same genotype in all fish; 3, enzyme did not always cut mtDNA to completion; 4, enzyme produced too many small fragments to accurately size.

Restriction Endonuclease	Recognition Sequence (5'----3')	Proficiency of Cutting mtDNA
*Ava I	C▾PyCGPuG	1
*Ban I	G▾GPyPuCC	1
*Bcl I	T▾GATCA	1
*EcoR I	G▾AATTC	1
*EcoR V	GAT▾ATC	1
*Hinc II	GTPy▾PuAC	1
*Kpn I	GGTAC▾C	1
*Nde I	CA▾TATG	1
*Sac I	GAGCT▾C	1
*Hind III	A▾AGCTT	2
*Sac II	CCGC▾GG	2
BamH I	G▾GATCC	3
Bgl I	GCCNNNN▾NGGC	3
Mlu I	A▾CGCGT	3
Pst I	CTGCA▾G	3
Sal I	G▾TCGAC	3
Xba I	T▾CTAGA	3
Xho I	C▾TCGAG	3
Dra I	TTT▾AAA	4
Hinf I	G▾ANTC	4
Pvu II	CAG▾CTG	4
Rsa I	GT▾AC	4

FL), different months of capture, and various stages of gonadal maturity in order to represent an unbiased sample.

The manufacturer's recommendations (New England BioLabs, New York BioLabs, or Promega) were followed and approximately 150 to 200 ng of mtDNA were used in each digest. Of the original 22 restriction endonucleases, 11 cut cobia mtDNA to completion and generated fragments which could be accurately sized. These 11 enzymes were used for the duration of the study to examine 66 additional fish. The remaining 11 enzymes did not cut to completion in all fish or produced too many small fragments which were not easily sized or which ran off the gels.

Restriction Endonuclease Analysis of Mitochondrial DNA

MtDNAs from the 90 fish were digested using 11 six-base recognition endonucleases: *Ava* I, *Ban* I, *Bcl* I, *Eco*R I, *Eco*R V, *Hinc* II, *Hind* III, *Kpn* I, *Nde* I, *Sac* I and *Sac* II. Digests were performed according to manufacturer's recommendations (New England BioLabs, New York BioLabs, or Promega). In general, 10 to 20 units of each enzyme were used to digest ~200 ng mtDNA. Digestion times generally ranged from 8 to 12 h at the recommended temperatures. The resulting fragments were separated via horizontal electrophoresis on 0.8% agarose gels run overnight in 1X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) at approximately 70 volts. Gels were stained for approximately 30 min with ethidium bromide (2 μg/ml), examined under 302 nm UV illumination and photographed with Polaroid 667 film using an orange filter.

Data Analysis

Size estimates of restriction fragments were made by using Lambda DNA digested with *Hind* III (New England BioLabs) as a molecular weight size standard. In cases where incomplete digests resulted or gels became distorted and fragments could not be accurately sized, the digests were repeated. Restriction fragments larger than 0.5 kb were generally visible and easily sized. Fragments less than 0.5 kb could not be visualized in this study, although they should not be regarded as

absent. Though not performed, increasing the amount of mtDNA in each digest, using acrylamide gels, or employing end-labeling procedures would have enabled visualization of these small fragments.

Each fish was assigned a genotype, denoted by a single letter. These genotypes were based on the fragment patterns generated within individual endonucleases. A multi-letter composite mtDNA haplotype, consisting of the letters representing the fragment patterns generated by each restriction endonuclease, was then constructed for each individual (see Table 1).

To ensure consistent scoring of genotypes among gels, representative mtDNA samples from 1991 were included in all 1992 gels. Running conditions of the gels (voltage, temperature, and time) were also kept as constant as possible in order to assure reproducibility. However, slight differences did occur when sizing restriction fragments from the same fish when digests were run on separate gels. These slight differences were unavoidable and did not pose a significant problem in assigning genotypes.

To estimate phylogenetic relationships among the 90 cobia examined, fragments of coincident mobility were assumed to be products of single nucleotide substitutions within a restriction site and were considered to be homologous within each restriction endonuclease. Fragments were treated as characters, each fragment defined by the flanking restriction sites. Character states were coded as either the presence or absence of the fragment. The overall proportion of shared fragments to the total number of possible fragments across all restriction endonucleases was calculated pairwise for all fish. This method of estimating the degree of similarity between pairs of fish was a modification of the method developed by Nei and Li (1979). The pairwise comparisons were used to generate a similarity coefficient matrix. The matrix values were then clustered by UPGMA (unweighted pair-group method using arithmetic averages) (Sneath and Sokal 1973), using the average linkage algorithm of the SPSS-X™ statistical package (SPSS, Inc.). The result was a

phenogram which consisted of a branching network which related the overall similarity of the 90 fish. The phenogram was then examined to determine whether there was a correlation between any of the catch statistics and the fish which clustered close together. The similarity matrix values in this study were also clustered using the average linkage within groups, the single linkage, and the complete linkage algorithms of the SPSS-X™ statistical package (SPSS, Inc.) to determine whether the same fish repeatedly clustered together.

RESULTS

Quantification of Mitochondrial DNA

The Hoëchst method was chosen to quantitate mtDNA levels for several reasons. In theory it is a simple, reliable, and sensitive method for quantitative determinations of DNA. It is capable of detecting DNA concentrations down to 10 ng/ml and the presence of RNA in the samples does not interfere significantly in the determination of DNA levels (Labarca and Paigen 1980).

For the purposes of this study, however, this method of DNA quantification was quite variable. The Hoëchst dye method typically reported mtDNA concentrations which were much higher than actual levels. This elevated reading was attributed to contamination by genomic DNA. For example, samples which were determined to be identical in concentration by the Hoëchst dye method would be used in equal amounts when performing a restriction digest. In many cases, there was an extreme variability in the intensity of the fragments. It was for this reason that mtDNA concentrations were ultimately estimated by visual inspection of gel photographs of uncut mtDNA.

Initial Screening of Cobia Mitochondrial DNA

Of the initial 22 restriction endonucleases which were tested, 11 were selected to use for the duration of the study. The enzymes *Ava* I, *Ban* I, *Bcl* I, *Eco*R I, *Eco*R V, *Hinc* II, *Kpn* I, *Nde* I, and *Sac* I all cleaved cobia mtDNA completely and resulted in fragments which could be easily sized. The number of restriction fragments which resulted after digestion by these enzymes ranged from one fragment, as in genotype A of *Kpn* I, to eight fragments, as in genotype E of *Ban* I (see Table 3). Only one genotype was found using the enzymes *Hind* III and *Sac* II in the original survey. However, these enzymes continued to be used for the duration of the study in hope that additional genotypes would be found in fish taken from other states. None were subsequently found. The

Table 3. Molecular weight sizes (kb) and digestion profiles (genotypes) for restriction fragments of *R. canadum* mtDNA. The sample sizes of males and females representative of each genotype are given below the sum of the fragments. Superscripts represent common fragments.

Hind III	*Sac* II	*Kpn* I		*Bcl* I		
A	A	A	B	A	B	C
13.4	14.3	14.9[a]	14.9[a]	12.5	12.8	12.9
4.5	1.4		9.7	4.1[a]	4.1[a]	4.1[a]
0.5	1.2	14.9	24.6	3.6[b]	3.6[b]	3.6[b]
18.4	1.0	19♂ 42♀	0♂ 29♀	20.2	20.5	20.6
19♂ 71♀	0.9			9♂ 39♀	9♂ 27♀	1♂ 5♀
	18.8					
	19♂ 72♀					

Sac I			*Ban* I				
A	B	C	A	B	C	D	E
14.5[a]	14.5[a]	13.8	10.9[b]	11.2[a]	11.5	11.7	11.2[a]
	0.8	3.2	3.8[c]	3.8[c]	3.8[c]	3.8[c]	10.9[b]
14.5	15.3	17.0	2.0[d]	2.0[d]	2.0[d]	2.0[d]	3.8[c]
19♂ 58♀	0♂ 11♀	0♂ 2♀	1.5[e]	1.5[e]	1.5[e]	1.5[e]	2.0[d]
			1.1[f]	1.1[f]	1.1[f]	1.1[f]	1.5[e]
			0.8[g]	0.8[g]	0.8[g]	0.8[g]	1.1[f]
			0.6[h]	0.6[h]	0.6[h]	0.6[h]	0.8[g]
							0.6[h]
			20.7	21.0	21.3	21.5	31.9
			2♂ 8♀	6♂ 27♀	7♂ 21♀	4♂ 14♀	0♂ 1♀

Nde I							
A	B	C	D	E	F	G	H
9.9[a]	9.9[a]	9.9[a]	9.9[a]	9.9[a]	9.9[a]	9.9[a]	9.9[a]
7.2	7.6[c]	8.0[b]	8.3	8.6	9.0	3.7	8.0[b]
2.5[d]	2.5[d]	2.5[d]	2.5[d]	2.5[d]	2.5[d]	3.0	7.6[c]
1.3[e]	1.3[e]	1.3[e]	1.3[e]	1.3[e]	1.3[e]	2.5[d]	2.5[d]
						1.3[e]	1.3[e]
20.9	21.3	21.7	22.0	22.3	22.7	20.4	29.3
2♂ 0♀	4♂ 6♀	5♂ 22♀	4♂ 21♀	3♂ 13♀	1♂ 6♀	0♂ 2♀	0♂ 1♀

Table 3. *Continued.*

Hinc II

A	B	C	D	E	F	G	H
11.2[c]	11.2[c]	11.5[b]	11.5[b]	11.8[a]	11.8[a]	11.9	12.1
3.8[d]	3.8[d]	3.8[d]	3.8[d]	3.8[d]	3.8[d]	3.8[d]	3.8[d]
2.2[e]	1.3[f]	2.2[e]	1.3[f]	2.2[e]	1.3[f]	1.3[f]	2.2[e]
1.3[f]	1.2[g]	1.3[f]	1.2[g]	1.3[f]	1.2[g]	1.2[g]	1.3[f]
1.2[g]	0.9[h]	1.2[g]	0.9[h]	1.2[g]	0.9[h]	0.9[h]	1.2[g]
0.9[h]		0.9[h]		0.9[h]			0.9[h]
20.6	18.4	20.9	18.7	21.2	19.0	19.1	21.5
0♂ 5♀	6♂ 20♀	1♂ 11♀	6♂ 18♀	0♂ 9♀	6♂ 6♀	0♂ 1♀	0♂ 1♀

*Eco*R I

A	B	C	D	E	F	G	H
9.4[a]	9.4[a]	9.4[a]	9.4[a]	9.4[a]	9.4[a]	9.4[a]	9.4[a]
7.8[e]	7.8[e]	8.2[d]	8.2[d]	8.7[c]	8.7[c]	9.1[b]	9.1[b]
6.1[f]	5.3[g]	6.1[f]	5.3[g]	6.1[f]	5.3[g]	6.1[f]	5.3[g]
5.3[g]		5.3[g]		5.3[g]		5.3[g]	
28.6	22.5	29.0	22.9	29.5	23.4	29.9	23.8
0♂ 4♀	5♂ 7♀	0♂ 12♀	9♂ 23♀	0♂ 6♀	1♂ 8♀	0♂ 2♀	4♂ 4♀

I	J	K	L
9.4[a]	9.4[a]	12.3	9.4[a]
6.1[f]	5.3[g]	8.7[c]	8.2[d]
5.3[g]		8.2[d]	7.8[e]
			5.3[g]
20.8	14.7	29.2	30.7
0♂ 1♀	0♂ 1♀	0♂ 2♀	0♂ 1♀

Table 3. *Continued.*

Ava I

A	B	C	D	E	F	G	H	I	J	K
7.5[a]	7.5[a]	7.5[a]	7.5[a]	7.5[a]	7.5[a]	7.5[a]	7.5[a]	7.5[a]	7.5[a]	7.5[a]
7.0[b]	7.0[b]	7.0[b]	7.0[b]	7.0[b]	7.0[b]	7.0[b]	7.0[b]	7.1[b]	7.2[b]	7.4[b]
5.5[f]	5.8	6.1[e]	6.2[d]	6.5	6.6[c]	6.8	1.1[g]	7.0[b]	7.0[b]	7.0[b]
1.1[g]	1.1[g]	1.1[g]	1.1[g]	1.1[g]	1.1[g]	1.1[g]	1.0[h]	1.1[g]	1.1[g]	1.1[g]
1.0[h]	1.0[h]	1.0[h]	1.0[h]	1.0[h]	1.0[h]	1.0[h]	0.8[i]	1.0[h]	1.0[h]	1.0[h]
0.8[i]	0.8[i]	0.8[i]	0.8[i]	0.8[i]	0.8[i]	0.8[i]		0.8[i]	0.8[i]	0.8[i]
22.9	23.2	23.5	23.6	23.9	24.0	24.2	17.4	24.5	24.6	24.8
2♂ 2♀	2♂ 5♀	2♂ 14♀	4♂ 6♀	3♂ 7♀	2♂ 12♀	0♂ 3♀	1♂ 11♀	3♂ 1♀	0♂ 1♀	0♂ 5♀

L	M	N	O
7.7	7.5[a]	7.5[a]	7.5[a]
7.5[a]	6.6[c]	7.0[b]	7.0[b]
7.0[b]	6.2[d]	6.1[e]	6.2[d]
1.1[g]	1.1[g]	5.5[f]	6.1[e]
1.0[h]	1.0[h]	1.1[g]	1.1[g]
0.8[i]	0.8[i]	1.0[h]	1.0[h]
		0.8[i]	0.8[i]
25.1	23.2	29.0	29.7
0♂ 1♀	0♂ 1♀	0♂ 1♀	0♂ 1♀

*Eco*R V

A	B	C	D	E	F	G	H	I	J	K
13.6[a]	13.6[a]	13.6[a]	13.6[a]	13.6[a]	13.6[a]	13.6[a]	13.6[a]	13.6[a]	13.6[a]	13.6[a]
4.5[e]	4.7	5.0[d]	5.1[c]	5.2	5.4[b]	5.5	5.6	5.7	5.8	5.9
18.1	18.3	18.6	18.7	18.8	19.0	19.1	19.2	19.3	19.4	19.5
1♂ 1♀	1♂ 3♀	3♂ 7♀	0♂ 4♀	4♂ 8♀	1♂ 10♀	2♂ 7♀	1♂ 4♀	1♂ 2♀	0♂ 5♀	0♂ 5♀

L	M	N	O	P	Q	R
13.6[a]	13.6[a]	13.6[a]	13.6[a]	13.6[a]	13.6[a]	13.6[a]
6.1	6.2	6.4	7.0	7.3	5.1[c]	5.4[b]
					4.5[e]	5.0[d]
19.7	19.8	20.0	20.6	20.9	23.2	24.0
0♂ 3♀	3♂ 0♀	2♂ 8♀	0♂ 1♀	0♂ 1♀	0♂ 1♀	0♂ 1♀

size values listed for each fragment in Table 3 are mean values calculated from homologous fragments on different gels.

The restriction enzymes *BamH* I, *Bgl* I, *Mlu* I, *Pst* I, *Sal* I, *Xba* I, and *Xho* I did not always cut cobia mtDNA to completion. It was difficult to determine which fragments were the result of incomplete digestions in many of the cases. In several of the digests utilizing *Xho* I, *Xba* I, and *BamH*, cobia mtDNA was not cleaved at all. Digestions which employed both excess enzyme (up to 40 units in some cases) and digestion times were not successful in cleaving mtDNA.

Cleavage by the restriction enzymes *Dra* I, *Hinf* I, *Rsa* I, and *Pvu* II resulted in numerous restriction fragments, many of which were too small to accurately size. This was expected of the enzymes *Hinf* I and *Rsa* I because 4- and 5-base cutters generate three to six times as many fragments as six-base cutters (reviewed in Ryman and Utter 1988). In the case of *Dra* I and *Pvu* II an explanation is not as easily justified. The most likely possibilities are that either the sequences recognized by *Dra* I (5'-TTT▾AAA-3') and *Pvu* II (5'-CAG▾CTG-3') are quite frequent in cobia mtDNA or else these enzymes may have exhibited star activity.

Restriction Site Variation

The 11 enzymes generated a total of 91 unique restriction fragments (3 *Hind* III, 5 *Sac* II, 2 *Kpn* I, 5 *Bcl* I, 4 *Sac* I, 10 *Ban* I, 11 *Nde* I, 10 *Hinc* II, 8 *Eco*R I, 16 *Ava* I, and 17 *Eco*R V) (Table 3). Restriction fragments were sized by comparing them to the Lambda/*Hind* III molecular weight standard. Within each restriction enzyme, each fish was assigned a genotype based on the size and number of restriction fragments. To ensure consistent scoring of genotypes between samples run on different gels, at least one representative fish from 1991 was rerun as a size comparison with all digests performed on fish in 1992. This control lane proved to be very helpful due to unavoidable variations in running conditions between gels.

The sizes of the fragments representative of each genotype were calculated by averaging fragments of coincident mobility across all fish. Due to slight differences in running conditions of the gels, actual fragment sizes were not the ultimate factor when assigning genotypes. The relative position of other fragments in the digest was also taken into consideration. For example, in the *Ava* I digests, the estimated size of the largest common fragment varied from 6.9 to 7.7 kb. The size of this particular fragment did vary between gels, but within a gel it was fairly consistent in size. .Therefore, this fragment was treated as identical in all digests. *Ava* I also had four additional common fragments and one variable fragment. The four common fragments were used as references in determining the variable fragment if there was any doubt as to its identity.

Considerable polymorphism was observed among cobia. Only *Hin*d III and *Sac* II did not generate RFLPs. Within the remaining nine restriction enzyme, 2 to 18 genotypes were detected: two *Kpn* I, three *Bcl* I, three *Sac* I, five *Ban* I, eight *Nde* I, eight *Hinc* II, 12 *Eco*R I, 15 *Ava* I, and 18 *Eco*R V (Table 3). The pairwise differences among genotypes were attributed to the gain or loss of one or more restriction sites. For example, *Eco*R I genotype A had fragment sizes of 9.4, 7.8, 6.1, and 5.3 kb. The *Eco*R I genotype B lacked the 6.1 kb fragment. It was determined that 86 haplotypes (composite genotypes) defined the 90 fish examined in this study (Table 1). Only four haplotypes were repeated: AAAAAACBCBC, AAAAABCBCDF, AAAAABCBCDE, and AAAAABBBBBB shared by fish pairs 30/31, 46/47, 49/54, and 66/68, respectively.

An average of 41 fragments per fish was generated. Only the flanking ends of these fragments were of known sequence. Some enzymes cleave mtDNA in the center of the recognition sequence, creating blunt ends; i.e., *Eco*R V which recognizes the sequence 5'-GAT▾ATC-3'. Others cleave mtDNA at the flanking ends of the sequence, creating sticky ends; i.e., *Eco*R I which recognizes the sequence 5'-G▾AATTC-3'. All of the enzymes

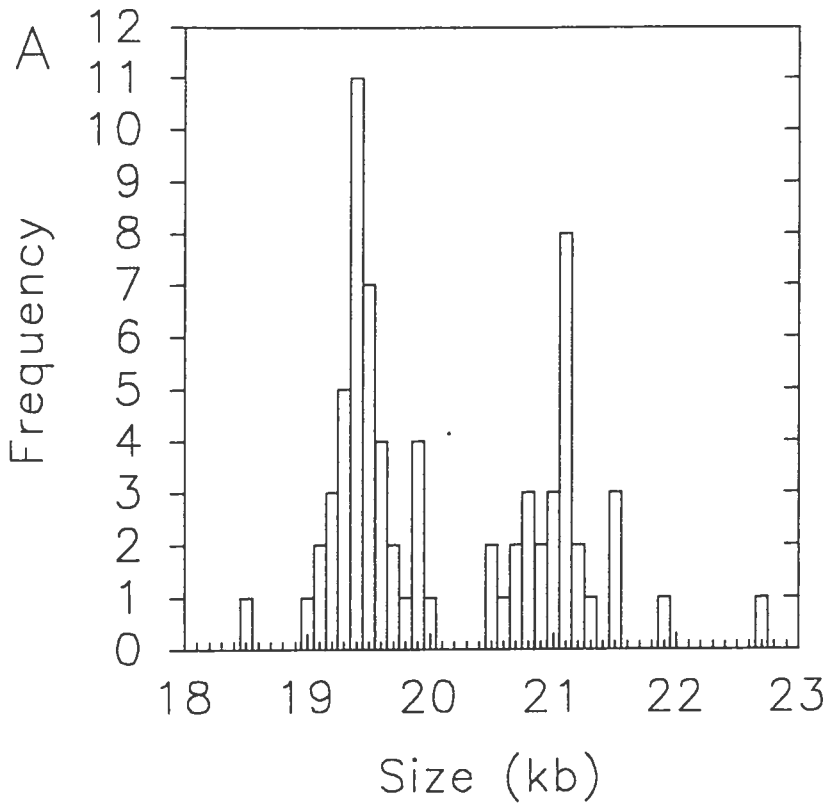
utilized in this study were six-base cutters. Therefore, a total of 246 nucleotides (6 nucleotides x 41 fragments), or 1.23% of the ~20.0 kb cobia mitochondrial genome, was determined.

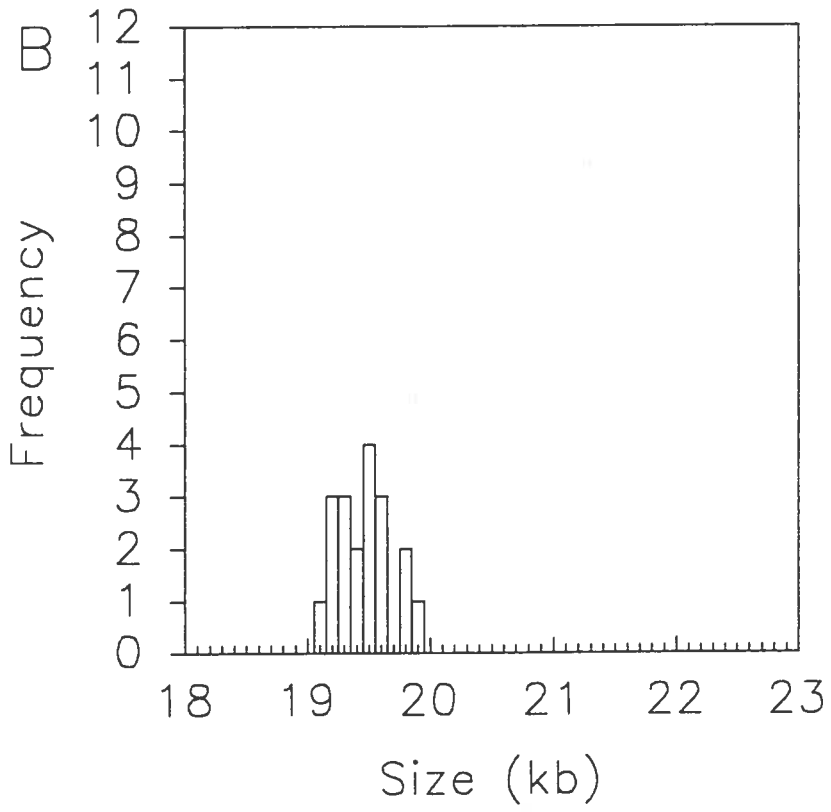
Calculation of Cobia Mitochondrial Genome Size

Size estimates of restriction fragments were made by using Lambda DNA digested with *Hind* III. The size (mean ± standard deviation) of the cobia mitochondrial genome inclusive of all fish was calculated by averaging the sum of restriction fragments of each genotype for each enzyme. This value was 20.0 ± 0.8 kb, with a range of 18.5 to 22.7 kb. This value compares well with an estimated 20.0 kb based on the size of the uncut mtDNA. The average genome size across all enzymes was calculated separately for males and females and resulted in sizes of 19.5 ± 0.2 kb and 20.1 ± 0.9 kb, respectively. The frequency of average genome sizes is plotted separately for females and males in Figures 1A and 1B. Females show a bimodal distribution of sizes: 19.5 ± 0.3 kb (X ± SD, N=42) in one cluster and 21.1 ± 0.4 kb (X ± SD, N=29) in the other. These two values are quite disparate and do not overlap until greater than two standard deviations from the mean.

The number of male and female fish characteristic of each genotype within restriction endonucleases was also examined (Table 3). In general, male fish tended to have the smaller genotypes (based on the sum of fragments) or lacked additional restriction fragments when compared to the rest of the genotypes for a particular restriction enzyme. This trend was noted even though almost four times as many females as males were sampled, 71 and 19, respectively. For example, *Kpn* I has two genotypes, A and B, which have restriction fragments totaling 14.9 and 24.6 kb, respectively. All of the male fish were classified as genotype A (the smallest), whereas only 59% of the females were genotype A. *Sac* I has genotypes A, B, and C with fragments totaling 14.5, 15.3, and 17.0 kb, respectively. Eighty-one percent of the females (58 of 71) were genotype A whereas all of the males are

Figure 1. Distribution of average mitochondrial genome sizes for female (A) and male (B) fish based on the sum of all restriction fragments.


A
Frequency
0
1
2
3
4
5
6
7
8
9
10
11
12
18
19
20
21
22
23
Size (kb)


B
Frequency
0
1
2
3
4
5
6
7
8
9
10
11
12
18
19
20
21
22
23
Size (kb)

classified as genotype A; one would expect ~three genotype B males if the proportion of A genotypes is the same in males and females. The genotypes A through I of *Eco*R I all have two common fragments (9.4 and 5.3 kb), one variable-sized fragment, and a fragment (6.1 kb) which is either present or absent. All of the male fish lack the 6.1 kb fragment. This distribution of male fish is consistent throughout all restriction endonucleases where there is more than one genotype, with one exception. All of the genotypes of *Hinc* II have three common fragments (1.3, 1.2, and 0.9 kb), one variable fragment, and fragment which is either present or absent (2.2 kb). All but one of the males are classified as genotypes which lack the 2.2 kb fragment except fish 16 (genotype C). Upon reexamination of the photos of this digest, it is somewhat unclear whether the 2.2 kb fragment is actually present or not, due to the incomplete digestion of the mtDNA in this particular fish. The digestion was repeated several times but the results were consistently ambiguous.

For the enzymes *Ava* I, *Ban* I, *Eco*R I, *Eco*R V, *Hinc* II, *Kpn* I, and *Nde* I, female fish tended to belong to genotypes which were exceptionally larger (based on the sum of fragments) and/or which possessed additional restriction fragments when compared to the rest of the genotypes. For example, most *Ban* I genotypes had six common fragments and one variable-sized fragment. Genotype E (a female), however, had two variable-sized fragments so that this genotype was much larger than the rest. The enzyme *Eco*R V also follows this pattern; the largest-sized genotypes within an enzyme are characteristically females. In *Eco*R I, genotypes A, C, E, G, K, and L are all ~6.0 kb larger than the other genotypes and are only comprised of females; the remaining 44 females are distributed among the other eight genotypes (B, D, F, H, I, and J), of which genotype J is the smallest (14.7 kb). The same is true for *Ava* I where 11 females are classified as genotype H, which is smaller that the other genotypes for that enzyme. It is clear, however, that some restriction

enzymes reveal two distinct classes of females based on average mitochondrial genome size.

The fish numbered 86 was a special case; it had an extremely large genome size and constituted its own unique genotype for five restriction enzymes: genotypes E, H, N, L, and Q of enzymes *Ban* I, *Nde* I, *Ava* I, *Eco*R I, and *Eco*R V, respectively. A common feature of each of these genotypes is that they are usually a combination of two genotypes. That is, *Ban* I genotype E is a combination of genotypes A and B, *Nde* I genotype H is a combination of genotypes B and C, *Eco*R I genotype L is a combination of genotypes B and D, *Ava* I genotype N is a combination of genotypes A and C, *Eco*R V genotype Q is a combination of genotypes A and D, and *Kpn* I genotype B has a 9.8 kb fragment not seen in genotype A.

Data Analysis of RFLPs

Similarity coefficients were computed based on the number of fragments shared in common by pairs of fish computed across all the enzymes. In other words, a 90 fish by 90 fish matrix was constructed and pairwise comparisons were made of the number of shared fragments divided by the total number of fragments. Cluster analysis of the similarity coefficient matrix was performed using UPGMA; the phenogram generated is shown in Figure 2. UPGMA (Sneath and Sokal 1973) defines the distance between two clusters as the average of the differences between all pairs of cases in which one member of the pair is from each of the clusters. For example, if individuals V and W form cluster 1 and individuals X, Y and Z form cluster 2, the distance between clusters 1 and 2 is taken to be the average of the following pairs of cases: (V,X) (V,Y) (V,Z) (W,X) (W,Y) (W,Z). The larger the similarity coefficients are, the more similar or related each pair of fish is assumed to be. The similarity values ranged from a low of 0.536 between fish pairs 63/88 to a high of 1.000 between the fish pairs 30/31, 46/47, 49/54, and 66/68. The four latter pairs each had the same haplotype.

Figure 2. Phenogram generated by UPGMA clustering of cobia mtDNA RFLP similarity coefficient values. Columns indicate: A, fish identification number; B, sex; C, capture location; D, month of capture; E, stage of gonad development, 1 (previtellogenesis), 2 (vitellogenesis), 3 (final maturation), 4 (post-ovulation), 1' (2nd previtellogenesis), 2' (2nd vitellogenesis), M (sexually mature male fish), and N (not determined); F, average genome size: A (19.5 kb ♀), B (21.1 kb ♀), and C (19.5 kb ♂). Asterisks (*) indicate pairs of fish which had identical haplotypes and, therefore, similarity coefficients of 1.000. The scale of 0-25 is arbitrary; node lengths do not represent actual genetic distances.

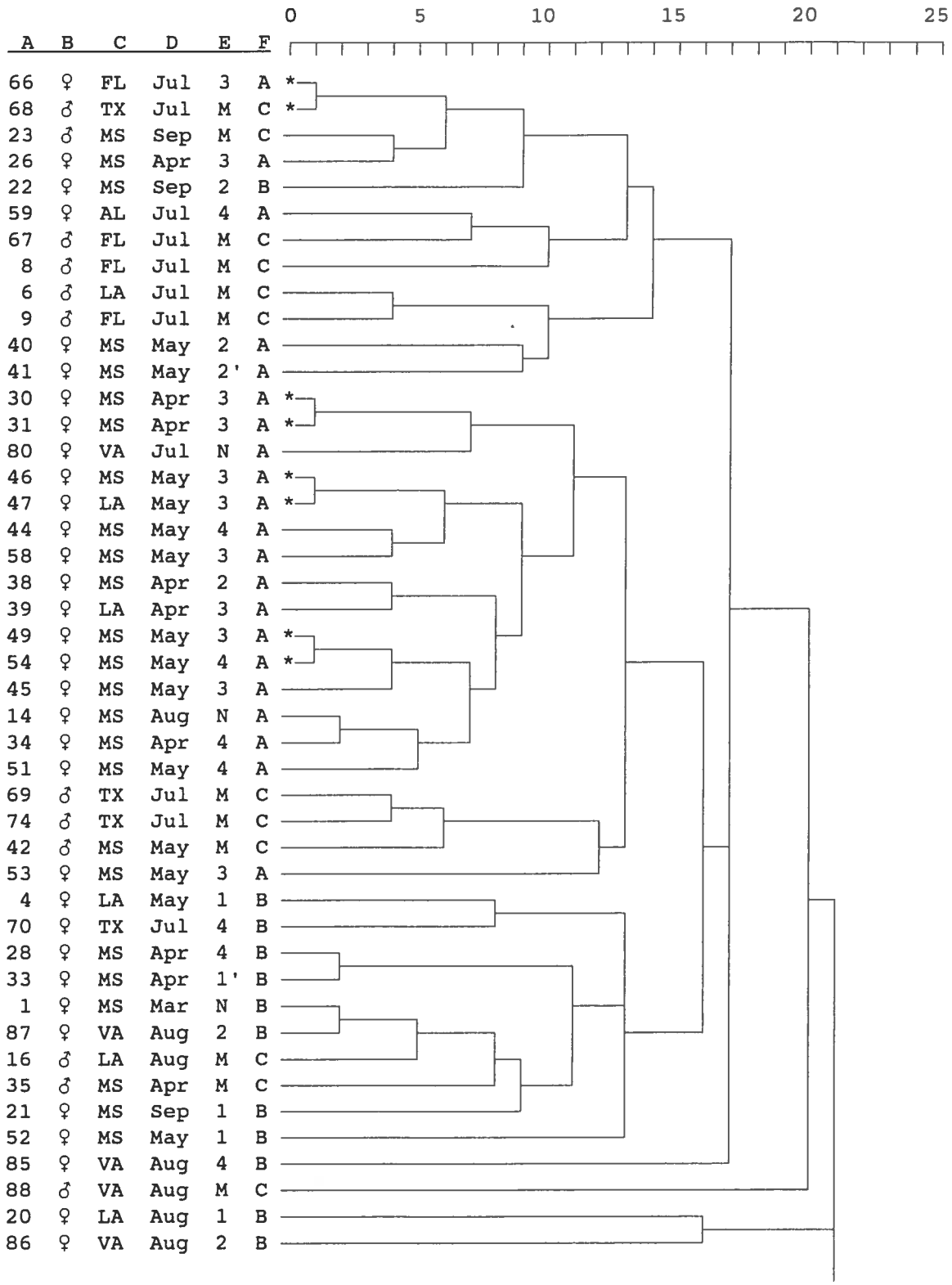
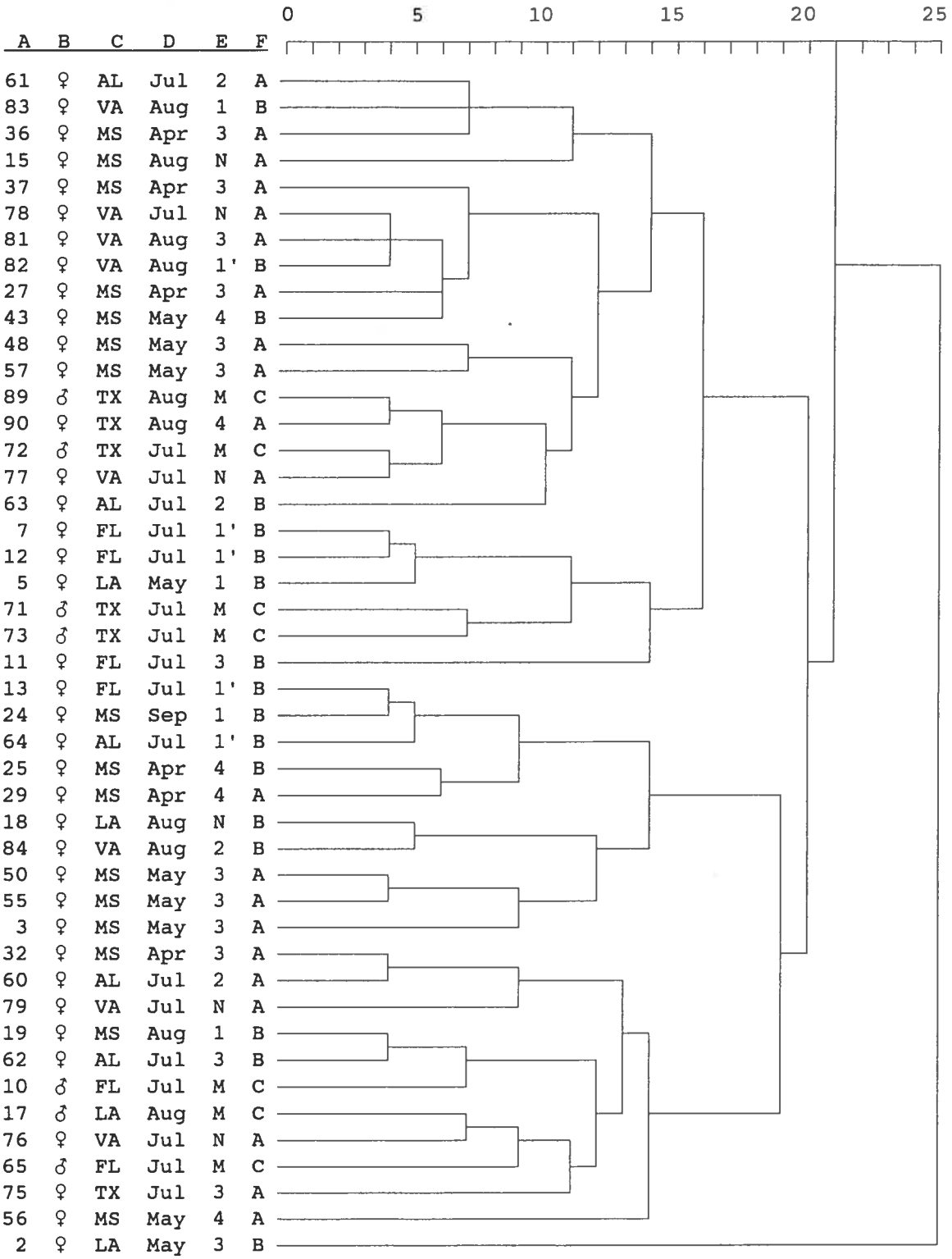

0 5 10 15 20 25
A B C D E F
66 ♀ FL Jul 3 A *
68 ♂ TX Jul M C *
23 ♂ MS Sep M C
26 ♀ MS Apr 3 A
22 ♀ MS Sep 2 B
59 ♀ AL Jul 4 A
67 ♂ FL Jul M C
8 ♂ FL Jul M C
6 ♂ LA Jul M C
9 ♂ FL Jul M C
40 ♀ MS May 2 A
41 ♀ MS May 2' A
30 ♀ MS Apr 3 A *
31 ♀ MS Apr 3 A *
80 ♀ VA Jul N A
46 ♀ MS May 3 A *
47 ♀ LA May 3 A *
44 ♀ MS May 4 A
58 ♀ MS May 3 A
38 ♀ MS Apr 2 A
39 ♀ LA Apr 3 A
49 ♀ MS May 3 A *
54 ♀ MS May 4 A *
45 ♀ MS May 3 A
14 ♀ MS Aug N A
34 ♀ MS Apr 4 A
51 ♀ MS May 4 A
69 ♂ TX Jul M C
74 ♂ TX Jul M C
42 ♂ MS May M C
53 ♀ MS May 3 A
4 ♀ LA May 1 B
70 ♀ TX Jul 4 B
28 ♀ MS Apr 4 B
33 ♀ MS Apr 1' B
1 ♀ MS Mar N B
87 ♀ VA Aug 2 B
16 ♂ LA Aug M C
35 ♂ MS Apr M C
21 ♀ MS Sep 1 B
52 ♀ MS May 1 B
85 ♀ VA Aug 4 B
88 ♂ VA Aug M C
20 ♀ LA Aug 1 B
86 ♀ VA Aug 2 B

Figure 2. *Continued*



The phenogram demonstrates two major clusters, one of which consists solely of fish number 2. The other major cluster consists of the remaining 89 fish. This cluster was further divided into three subclusters, all of which were further subdivided a number of times. As expected, the four pairs of fish which had similarity coefficients of 1.000 (marked by asterisks on the phenogram) clustered together. One pair (30/31) had identical capture locations, months of capture, and stages of gonad development. However, the other three pairs (46/47, 49/54 and 66/68) differed by at least one variable. Fish pairs 46/47 and 49/54 differed in location of capture and stage of gonad development, respectively, whereas the pair 66/68 differed in location of capture, stage of gonad development, and sex of the fish.

Fish representing each of the variables were distributed randomly throughout the clusters. For example, males were randomly distributed throughout the clusters as were each of the gonad stages characteristic of females. Ripe females clustered with spent and developing females and/or males. The other catch statistics, including month, location, and year of capture were also randomly distributed throughout the clusters. Even the fish caught in Virginia do not form a separate cluster. If cobia exist as distinct breeding subpopulations in the northern Gulf of Mexico, fish caught in the Chesapeake Bay would most likely cluster together in the phenogram since these two areas represent separate ocean basins which are semi-geographically isolated from each other by Florida. Nonetheless, individual fish from Virginia cluster with individuals from all other states.

Female fish representative of the two distinct average mitochondrial genome sizes, 19.5 and 21.1 kb, clustered together more so than any of the other of the previously examined variables. For example, the cluster in the phenogram which consisted of fish 30 through 51 was composed entirely of females with an average genome size of 19.5 kb. Additionally, the cluster which consisted of fish 4 through 52 was composed primarily of females with an average genome size of 21.1 kb,

with the exception of two male fish (16 and 35). However, this type of clustering was expected because females with larger genome sizes would naturally share more fragments than would females with a smaller genome size. This pattern of distribution was not, however, consistent throughout the entire phenogram. The three major subclusters (fish 66 through 88, 20 through 86, and 61 through 56) consisted of females representative of both genome sizes, in addition to male fish. However, as the clusters were further subdivided, females of the same genome size do cluster together. For example, the subcluster including fish 13 through 3 includes females of both genome sizes and is further subdivided down into several smaller clusters. The subclusters inclusive of fish 13 through 64, 18 through 84, and 50 through 3 are all representative of females with the same genome sizes.

There was not a consistent correlation among any of the catch variables which could be used to delineate the various clusters from each other. Thus, there is no evidence from the RFLP analysis to support the hypothesis that cobia exist as distinct breeding subpopulations.

DISCUSSION

The initial screening of cobia mtDNA proved helpful in determining which endonucleases would be informative and generate restriction fragments which were easily sized. The enzyme *Pvu* II was atypical in that it usually cleaved cobia mtDNA in excess of 11 times and generated numerous small fragments which were not easily sized. Nei and Li (1979) determined that it is possible to calculate the expected frequency of restriction sites within a segment of DNA based on a known G + C content, *g*. If all the nucleotides are randomly distributed in the DNA sequence, the expected frequency of restriction sites, *a*, with *r* nucleotide pairs is

$$a = (g/2)^{r1}[(1-g/2)]^{r2}$$

in which r1 and r2 are the number of G + C and the number of A + T nucleotides in the restriction site, respectively, and *r*1 + *r*2 = r. The restriction site recognized by *Pvu* II is 5'-CAG▾CTG-3', yielding *r*1 and *r*2 values of 4 and 2, respectively. Although the exact G + C content was not determined for the cobia mitochondrial genome, the value is 42.5% for the cobia cytochrome *b* gene (see Chapter 2). If the G + C content of this gene is hypothetically representative of the entire mitochondrial genome, and is used for *g* in the above equation (assuming a mitochondrial genome size of 20.0 kb), the number of expected restriction sites for the enzyme *Pvu* II is 3.37. If hypothetical values of 30%, 50%, 60%, and 70% G + C are substituted, the expected frequency of restriction sites is 1.24, 4.88, 6.47, and 6.75, respectively. Even though the exact G + C value of the cobia mitochondrial genome is not known, the range of 30% to 70% should be inclusive of the actual value for cobia. The number of fragments in a typical *Pvu* II digest are considerably more than the calculated values by a factor of 1.6 to 8.9. A possible explanation for this may be attributed to altered activity of the enzyme.

It has been demonstrated that under extreme non-standard conditions, restriction endonucleases are capable of cleaving sequences which are similar to, but not identical to, their defined recognition sequence. This altered specificity has been termed star activity and was first observed in the restriction endonuclease *Eco*R I (Polisky et al. 1975). Star activity has also been observed in the restriction endonucleases *Ase* I, *Bam*H I, *Bss*H II, *Eco*R I, *Eco*R V, *Hin*d III, *Hin*f I, *Kpn* I, *Pst*, I, *Pvu* II, *Sal* I, *Sca* I, *Taq* I, and *Xmn* I. It has been suggested that star activity may be a general property of restriction endonucleases (Nasri and Thomas 1986) and that any restriction endonuclease can be made to cleave noncanonical sites under certain extreme conditions. These conditions include glycerol concentrations >5% (v/v), a high units of restriction enzymes to μg of DNA ratio (usually >100 units/μg), low ionic strength (<25 mm), high pH (> pH 8.0), presence of organic solvents (i.e. ethanol), and/or the substitution of Mg^{++} with other divalent cations (i.e. Mn^{++}, Cu^{++}, Co^{++}, or Zn^{++}) (Polisky et al. 1975, summarized in Nasri and Thomas 1986, 1987).

The restriction endonuclease *Pvu* II has been demonstrated to exhibit star activity as demonstrated by Nasri and Thomas (1987). In the present study, as many as 20 units of enzyme were used to digest ~150 to 200 ng DNA, a concentration which exceeds the 100 units/1 μg recommended limit. This high concentration of enzyme may have contributed star activity which generated the large number of restriction fragments.

In cases where the genome sizes are exceptionally large, for example, females with an average genome size of 21.1 kb in digests using *Eco*R I, the additional fragments are most likely not the result of star activity. The number of fragments generated using this enzyme falls into the expected range using the Nei and Li (1979) formula. The fragments in these digests are very distinct and do not appear to be partial digestions.

The Nei and Li (1979) method of calculating the degree of similarity in a group of individuals typically calculates the degree of nucleotide

sequence divergence among populations of individuals. These values are then clustered by programs such as UPGMA to examine the genetic relationship of the individuals. This study did not calculate the degree of nucleotide sequence divergence between individuals, although it would be interesting to determine if the value would be significantly larger than most vertebrate species. Typical ranges of intraspecific sequence divergence are ~0.75% (summarized in Ryman and Utter 1988) with a low of nearly 0% in populations of striped bass *Morone saxitilis* (Wirgin et al. 1990) to greater than 12% in several species of trout of the genus *Salmo* (reviewed in Ryman and Utter 1988).

The similarity matrix values in this study were clustered using the average linkage within groups, the single linkage, and the complete linkage algorithms of the SPSS-X™ statistical package (SPSS, Inc.). Each method is a variant of UPGMA. The average linkage within groups method combines clusters so that the average distance between all cases in the resulting cluster is as small as possible. Thus, the distance between two clusters is taken to be the average of all the distances between all possible pairs of cases in the resulting cluster. Single linkage, sometimes called "nearest neighbor" technique, differs from UPGMA in that the first two cases combined are those with the smallest distance, or greatest similarity, between them. The distance between the new cluster and individual cases is then computed as the minimum distance between an individual case and a case in the cluster. The distances between cases that have not been joined do not change. At every step the distance between two clusters is taken to be the distance between their two closest points. Using complete linkage, or the "furthest neighbor" technique, the distance between two clusters is calculated as the distance between their two furthest points. UPGMA was initially selected for use because this method uses information about all pairs of distances, not just the nearest or the furthest. It is for this reason that it is usually preferred to the single and complete linkage methods for cluster analysis.

The phenograms generated by each of the described methods were similar to the phenogram generated by UPGMA. Fish representing each of the catch statistics were distributed randomly throughout the clusters. Based on the cluster analyses of the RFLPs in cobia and the correlation of catch statistics, there is no evidence to support the hypothesis that the species exists as separate breeding subpopulations.

It must be noted that there were certain assumptions which were taken into consideration at the beginning of this study. The first one was that restriction sites were randomly distributed throughout the mitochondrial genome. The genome size was also assumed to be constant. In fact, the female mtDNA genome size was found to vary. The clustering methods utilized in this study did not, however, detect subpopulations based on these size polymorphisms.

Size differences in the mitochondrial genome between closely related vertebrate species have been documented in diverse groups and is not unusual (reviewed in Nei and Koehn 1983). However, significant intraspecific mtDNA size heteroplasmy (insertions or deletions greater than 0.1 kb) is less common among the vertebrates, although it has been documented in several species of fish (Bentzen et al. 1988), frogs (Bermingham et al. 1986), and lizards (Densmore et al. 1985). The size differences are usually the result of tandem insertions of varying copy number which is located in or close to the D-loop region of the mitochondrial genome.

Sex-biased intraspecific population structure based on size heteroplasmy of the mitochondrial genome has been documented in few instances. The investigation of striped bass *Morone saxitilis* populations by Wirgin et al. (1989) in the Atlantic and Gulf of Mexico provides a classic example of the case where size heteroplasmy was used to define population substructure. Many attempts had been made to identify discrete stocks of striped bass in the Gulf of Mexico, along the Atlantic coast, and within the Chesapeake Bay. Studies using meristic and morphometric techniques (summarized in Waldeman et al.

1988) to distinguish striped bass stocks provided low to moderate levels of resolution. Electrophoretic studies found only limited allozyme variation (Sidell et al. 1980, Rogier et al. 1985). Studies examining mtDNA RFLPs (Wirgin et al. 1989) have determined that the base sequence divergence among populations in the Chesapeake Bay, along the Atlantic coast, and in the Gulf of Mexico is extremely low, indicating little population substructure. Based on these methods, discrimination of stocks has been problematic. However, distinct populations of female striped bass have been identified based on size differences of the mitochondrial genome as identified by Wirgin et al. (1989). Four discrete mtDNA size classes, each differing by ~0.2, 0.3, and 0.4 kb from the smallest mtDNA molecule were observed. These size differences were determined to lie within a confined area of the mitochondrial genome as revealed by mapping studies. Overall, these size differences defined two discrete populations which previous methods had not identified.

In cobia, there appears to be two distinct populations of female fish based on the mitochondrial genome size. The mechanism for these size differences is not known. A possibility, although not likely, could be incorrect scoring of restriction fragments. One group of females had a 1.6 kb smaller average genome size based on the sum of restriction fragments. In the case of *Ava* I, genotype H is exceptionally smaller than the rest of the genotypes; it lacks an extra ~7.0 kb fragment characteristic of the other genotypes. If, however, the 7.0 kb fragment which is present is actually a doublet, the sum of the fragments would then equal a sum more characteristic of the other genotypes. This doublet could possibly be the result of a tandemly repeated sequence for which a restriction site exists within the repeated sequence. Digestion by this enzyme would produce a band whose position on the gel corresponds to the size of the repeated sequence and whose intensity varies in relation to the copy number of the sequence. In some of the fish classified as genotype H, this does not appear to be the case. The

intensity of the 7.0 kb fragment is not any stronger than the 7.5 kb fragment in all of the fish, essentially ruling out the possibility of a doublet.

In the case of *Hin*d III, all fish are classified as the same genotype, with a genome size of ~18.4 kb; it is expected that one or more small fragments (<0.5 kb) may not have been detected. It is also possible that secondary structure or a bending of the *Hin*d III fragment may have contributed to a smaller than actual estimated size. The average *Ava* I genome size is ~24.0 kb, 5.6 kb larger than determined by the *Hin*d III digests. If there were two distinct populations of female fish based on genome size, why was this size difference not revealed in the *Hin*d III digests? The existence of ~11 fragments less than 0.5 kb is not very likely.

There is the possibility that the female fish 86 carries two distinct populations of mtDNA in its cells. It was shown to have a much larger than average genome size and it usually had two fragments that were present singly in other genotypes. Each of the genotypes which combine to form the fish 86 genotype have both male and female representatives. Fish 86 would have to retain the two types of mtDNA molecules in about the same proportion because the intensities of the fragments were all about equal. The source of this extraneous mtDNA could be paternal, though it is highly unlikely. There have been several studies performed utilizing backcross strains to detect a possible low level of paternal leakage of mtDNA. Using mice, Gyllensten et al. (1985) analyzed the backcross progeny of matings originally between *Mus musculus* and *Mus spretus*. They observed an extremely low level of paternally derived mtDNA and calculated that at least 99.8% of the mtDNA molecules were inherited from the female parent each generation. The mouse sperm delivers about 50 molecules of paternal mtDNA into the egg, which contains ~10^5 mtDNA copies of maternal mtDNA molecules (Michaels et al. 1982, Hecht et al. 1984), for a total of 0.05% of paternally contributed

mtDNA molecules (Gyllensten et al. 1985). Therefore, the possibility of a paternal contribution to mtDNA inheritance does exist.

Zouros et al. (1992) examined the mode of inheritance of mtDNA in the marine mussel *Mytilus*. Interspecific backcrossings were performed using closely related species of mussels, *Mytilus edulis* and *Mytilus trossulus*. Intraspecific crossings were also carried out for each of the species. Extensive contribution of paternal mtDNA, amounting to several orders of magnitude higher than that inferred for *Drosophila* or mice, was observed in both inter-and intraspecific crosses. This study was also the first direct observation of paternal mtDNA transmission over the span of one generation. The degree of paternal transmission was ~0.1%, roughly 1000 times larger than that for mice (Gyllensten et al. 1985).

In the case of cobia, the possibility that fish 86 has two different types of mtDNA, one possibly contributed by a paternal mode of transmission, is theoretically possible but extremely improbable. The two types of mtDNA molecules would have to be retained in about equal magnitudes in order to be able to detect the fragments at the level observed in this study. In order to determine whether this size discrepancy is a true characteristic of fish 86, sequencing of the mitochondrial genome could be performed. Although sequencing of the entire genome of fish 86 would be quite cumbersome, it would very informative. Tandemly repeated sequences in the genome, if present, would be detected. If, however, it has two distinct populations of mtDNA molecules, detection of this characteristic would be more difficult. It is possible that a very few nucleotide differences exist between the two mtDNAs. The loss or gain of a singular restriction site would result in RFPLs between the two molecules. It is possible that some of the fragments would overlap and the portion of the genome where the restriction site was altered would result in two fragments of different mobilities. However, these two fragments would be less intense of the gel because the fragments which were overlapping should

be approximately twice as intense due to double the amount of mtDNA at that fragment position. This was not consistently apparent throughout the digests of fish 86.

The conservatism in gene arrangement, length, and length variation observed in early studies of animal mtDNA had led to the notion that this molecule has been under strong selection for small size and invariable structure over the course of evolution (reviewed in Nei and Koehn 1983, Attardi 1985). However, recent discoveries of large-scale size variation in several diverse species have indicated that functional or structural constraints do not keep the mtDNA molecule within a narrowly varying size of less than 20.0 kb. A common feature of enlarged animal mtDNA molecules (i.e. greater than 20.0 kb) is the presence of repeated sequences.

The selective advantage of maintaining two types of mitochondrial genomes is not exactly known. A biparental mode of inheritance of mtDNA would provide a mechanism for generating mtDNA divergence within an individual. A consequence of biparental mtDNA transmission would mean that phylogenies are not exclusively matriarchal. Consequently, the effective population size for a species will therefore be larger than for a strictly maternally inherited genome (Gyllensten et al. 1991). If this is the case, the mtDNA of many organisms, including humans, should be reexamined to accurately define evolutionary lineages.

Evidence for the ability of RFLP analysis of mtDNA to detect subtle population differentiation is well-documented in the scientific literature for a number of diverse organisms. The unusual size distribution of female mitochondrial genome sizes does merit further examination. It is possible that two distinct populations of females exist which are defined by their mitochondrial genome size.

A lack of genetic divergence has been observed in RFLP studies of mtDNA in other marine teleosts. Avise et al. (1986) examined the restriction site polymorphism in the mtDNA of American eels *Anguilla rostrata* sampled from over a 4,000 km stretch of North American

coastline. The lack of genetic differentiation is most likely attributed to the extraordinary life history of these catadromous fishes, which involves perhaps a single spawning population in the western mid-Atlantic Ocean and subsequent widespread dispersal of larvae by ocean currents. A lack of genetic differentiation over such a large area contrasts sharply with results for terrestrial and freshwater vertebrates (Avise et al. 1987).

Populations of the same species inhabiting different oceans have been observed to have little or no intraspecific division in the population structure. For example, Graves et al. (1984) examined mtDNA restriction site variation in Atlantic and Pacific populations of skipjack tuna *Katsuwonus pelamis*. There was no significant genetic difference between these two populations which was distinguishable by RFLPs. Similarly, Graves and Dizon (1989) found no significant differences between Atlantic and Pacific albacore tuna *Thunnus alalunga*. The lack of significant intraspecific genetic differentiation between Atlantic and Pacific tuna indicates that the two groups have either been separated for a very short time or that they maintain some form of genetic contact, probably around the Cape of Good Hope.

Cobia, on the other hand, exhibit an extremely high degree of heterogeneity in mtDNA genotypes, larger than the heterogeneity observed in walleye pollock *Theragra chalcogramma* (Mulligan et al. 1992). The degree of heterogeneity in cobia is one of the largest observed to date for marine fish. However, this heterogeneity did not support the hypothesis that discrete stocks of cobia exist in the northern Gulf of Mexico.

The Franks et al. (1992) tag and release study has demonstrated that there is interchange between fish in the Chesapeake Bay area and the Gulf of Mexico. The migratory life history apparently allows for significant mixing of the population such that there is no barrier to gene flow. The number of fish required to migrate between ocean basins to contribute to genetic differentiation is very small (Ryman and Utter

1988). Theoretical models of mtDNA differentiation (Takahata and Palumbi 1985) indicate that migration on the order of individuals per generation is sufficient to contribute to genetic heterogeneity. The information generated from the present study will be useful to fishery biologists in defining proper management of procedures of the species; cobia in the Gulf of Mexico and Atlantic should be managed as a single stock.

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CHAPTER 2.

Amplification and Sequencing of the Mitochondrial Cytochrome *b* Gene

INTRODUCTION

Cobia *Rachycentron canadum* represent the only species in the family Rachycentridae. They are large fish, known to reach 1.8 m in length and 68 kg in weight (Robins and Ray 1986). Cobia perform extensive seasonal migrations and inhabit most tropical, subtropical, and warm temperate seas of the world (reviewed in Shaffer and Nakamura 1989). They range from Massachusetts to Bermuda and Argentina and are present in the Gulf of Mexico (Richards 1967, Robins and Ray 1986). In the Gulf of Mexico, cobia range from Key West, Florida to Campeche, Mexico (Dawson 1971). Gulf of Mexico cobia are believed to overwinter in the Florida Keys and Caribbean and then travel northward in the Spring to spawning/feeding grounds (Franks et al. 1992). Cobia first enter the region of the Florida panhandle in March and appear in Mississippi waters in April; they leave Mississippi waters by October (Franks et al. 1992). Other fish move northward into the Atlantic Ocean (Richards 1977, Robins and Ray 1986).

Despite the recreational value of cobia, the life history of the species is poorly understood. There are conflicting views concerning the population structure of the species based on tag and release statistics. Tagging studies performed by Richards (1977) investigated migration habits of cobia in the Chesapeake Bay area. Twenty fish were tagged and released during fishing tournaments over a two year period. All fish were tagged early during the spawning season, early to mid-June, as cobia returned to the Chesapeake Bay waters. These fish were thought to be returning from overwintering grounds in the southwestern Atlantic. Six fish were recaptured between one month and five years later at locations ranging from 12 to 36 nautical miles from the original tagging location. Richards' (1977) tagging returns suggest that distinct, repetitive habitation occurs and that Chesapeake Bay

cobia may be a distinct group or subpopulation. Tagging studies by Franks et al. (1992) demonstrated that some cobia do indeed return to the same coastal region in which they were tagged. However, other fish are recaptured in areas very distant from the original tagging location. A fish tagged in the northern Gulf of Mexico near the Chandeleur Islands, LA was recaptured in the Atlantic Ocean off Charleston, SC, 1200 nautical miles away from the original tagging location. In another case, a fish tagged off of Cape Canaveral, FL was recaptured west of the Mississippi River in Louisiana waters. Distances as far as 1300 nautical miles from the original tagging location have been documented, with fish moving freely between the Gulf of Mexico and the Atlantic Ocean, including the Chesapeake Bay area. Franks et al. (1992) tagging returns suggest that cobia may consist of a heterogeneous breeding population, with reduced substructure.

Another interesting and unexplained aspect of the reproductive biology of the cobia is the extremely low abundance of eggs, larvae, and juvenile fish which have been collected in the northern Gulf of Mexico as compared to other species. Cobia spawn sizes are extremely large, ranging between two and 191 million eggs per female per spawn (Richards 1967, Franks et al. 1992). Reproductive biology studies by Richards (1967) and Franks et al. (1992) both suggest that cobia spawn more than once per season, but the exact number of times is unclear. While the fecundity rate of cobia appears to be extremely high, the only confirmed simultaneous collection of cobia eggs and yolk-sac larvae from the Gulf waters has been from the Crystal River estuary, FL (Ditty and Shaw 1992). Ditty and Shaw (1992) reported only 70 larvae less than 20 mm in length collected and identified in plankton tows taken from the Gulf of Mexico during the period from 1967 to 1989. Additionally, juvenile cobia, 27 mm or greater in length (Dawson 1971), have rarely been observed or collected in the Gulf of Mexico or Atlantic waters.

The paucity of information available about cobia life history has limited the understanding of the population biology of the species.

Studies examining various aspects of cobia biology in the Chesapeake Bay (Richards 1967, 1977) and northern Gulf of Mexico (Ditty and Shaw 1992, Franks et al. 1992) have contributed much of what we do know about the species. However, many questions remain unanswered. This study represents the first molecular analysis of cobia and addresses the question of population structure of the species in U.S. waters.

Conventional studies using protein variation have been useful in demonstrating the intraspecific genetic structure of many marine fishes (reviewed in Ryman and Utter 1988). For species which display little intraspecific variation, sample sizes must be very large in order to detect significant differentiation between putative stocks. In such cases, analysis of a more rapidly evolving set of molecular characters provides a better estimate of population structure with a more manageable number of samples.

It is preferable to examine the nucleotide sequence of an organism when studying the population biology of a species because the amount of information increases as one goes from protein to nucleotide sequence (summarized in Ryman and Utter 1988). Many changes which occur in a protein may be masked due to the degeneracy of the genetic code. A third position nucleotide substitution in a codon does not always result in a change in the amino acid at that particular codon. Additionally, amino acid substitutions are not always detectable unless there is a change in the overall charge of a protein when using protein electrophoresis.

Advances in molecular biology have now made it possible to circumvent many of the obstacles and limitations associated with examining protein variation. The nucleotide sequence of individuals is easily examined utilizing the polymerase chain reaction (PCR) (Saiki et al. 1988) in conjunction with restriction fragment length polymorphism (RFLP) analysis or direct nucleotide sequencing. These techniques have been extremely useful in resolving stock structure within species which exhibit little protein variation (reviewed in Ryman and Utter 1988).

Gene amplification by means of the PCR (Saiki et al. 1988) was used to selectively amplify a segment of the mitochondrial genome, the cytochrome *b* gene. The PCR process is an enzymatic technique used to amplify the number of copies of a single target DNA (or RNA) sequence by 10^5 to 10^7 times. Copies of the DNA sequence are prepared by shuttling a thermal-stable DNA polymerase between two primers. The basis of this amplification technique is multiple cycles (typically 30 to 60) of temperature changes to denature, then re-anneal primers, followed by extension to synthesize new DNA strands in the region located between the flanking primers. The number of copies of the target segment grows exponentially because each newly made copy can serve as a template for further replication. Specific sequences of DNA can be cloned *in vitro* in a matter of hours and the single-copy sequences can be amplified with very high specificity (Saiki et al. 1988). The nucleotide sequence of the amplified region can then be examined using restriction endonucleases or sequenced directly.

Depending on the type of organism being examined and by appropriate choice of gene segments, it is possible to study DNA sequence variation among individuals, local populations, or species (Kocher et al. 1989, Vigilant et al. 1989). Mutational events tend to occur at different rates along the mitochondrial genome as revealed by fine-scale mapping and comparative sequencing studies (Brown et al. 1982, Nei and Koehn 1983, summarized in Ryman and Utter 1988). Of the genes which are highly conserved along the mitochondrial genome, the greatest rate of mutations tend to occur in the D-loop region and the least in the tRNA genes (Brown and Simpson 1981, Cann and Wilson 1983, Greenberg et al. 1983, Cann et al. 1984). The rates of mutations vary significantly between different organisms. The mitochondrial genes of fishes and invertebrates are hypothesized to have a five-fold slower rate of amino acid substitution as compared to birds and mammal lineages (Kocher et al. 1989).

Carr and Marshall (1991) determined the nucleotide sequence of a portion of the mitochondrial cytochrome *b* gene of Atlantic cod *Gadus morhua* from Norway and from ten locations within the Northern Cod complex and adjacent stocks off Newfoundland. Eleven variable nucleotide positions defined 12 distinct genotypes. These genotypes differed significantly between Newfoundland and Norwegian populations, and supported the hypothesis that these fish exist as several distinct breeding subpopulations (Carr and Marshall 1991). Finnerty and Block (1992) sequenced a portion of the cytochrome *b* gene of blue marlin *Makaira nigricans* from Atlantic and Pacific waters. Thirteen variable nucleotide positions defined seven distinct genotypes. These genotypes differed significantly between Atlantic and Pacific populations and defined two distinct breeding subpopulations. In the present study, the mitochondrial cytochrome *b* gene was examined for two fish, numbered 39 (a female, caught in April in LA waters, in a final stage of gonad maturation) and 42 (a male, caught in May in MS waters, which was reproductively mature) (refer to Chapter 1). Variations in the nucleotide sequence of this gene were used to determine the degree of intraspecific sequence divergence of cobia inhabiting the northern Gulf of Mexico.

The mitochondrial cytochrome *b* gene was chosen for several reasons. This gene is highly conserved and contains four invariant amino acids which are considered necessary for cytochrome *b* function (Kocher et al. 1989). These four amino acids are thought to occupy active sites which are located at fixed codon positions on the cytochrome *b* gene. The identities of these amino acids and positions along the gene, with respect to the human sequence are arginine, histidine, arginine, and histidine at codons 80, 83, 97, and 100, respectively. All vertebrate species sequenced by Kocher et al. (1989), Finnerty and Block (1992), and Block et al. (1993) support this claim. Therefore, this characteristic of the cytochrome *b* gene serves as a guide to verify that

the nucleotide sequence obtained from a forensic experiment is actually that of the cytochrome *b* gene. There are, however, other regions on the mtDNA which are also highly conserved, such as the D-loop region and the 12S rRNA subunit (Kocher et al. 1989). Universal primers have been constructed which consistently and accurately amplify these regions which can also be used for population genetic studies (Kocher et al. 1989).

The mtDNA of most multicellular animals ranges from 15.7 to 19.5 kilobases (kb) (Brown and Wright 1979, .Nei and Koehn 1983). Cobia mtDNA is ~20.0 kb, one of the largest genomes among vertebrates observed to date. MtDNA, as opposed to genomic DNA, has several unique qualities that make it particularly suitable for population genetics. The animal mitochondrial genome consists of a single, duplex, closed-circular DNA molecule (Brown et al. 1979, Grivell 1983). It does not undergo recombination and evolves more rapidly than genomic DNA (Nei and Koehn 1983). Therefore, a change in the nucleotide sequence is due to a mutation in a base and is not due to recombination. Populations separated geographically will commonly exhibit extensive changes in their nucleotide sequence. The extent of these changes is used to identify distinct breeding populations. MtDNA is transmitted primarily by the female parent (Lansman et al. 1983, Gyllensten et al. 1985, Avise and Vrijenhoek 1987) though cytoplasmic inheritance in the ovum. Therefore, individuals with a particular mtDNA genotype are considered to be from the same maternal clone (Nei and Koehn 1983). Most of the data that suggest a maternal mode of mtDNA transmission have been based on single-generation mating experiments. Long-term mating experiments, employing repeated backcrossings to the same female, have demonstrated that mtDNA is transmitted by the male parent in very low levels. Gyllensten et al. (1985, 1991) determined that in an average mouse, approximately one mtDNA molecule in 1000 was contributed from the male parent. Similar results of a paternal mode of mtDNA transmission have also been observed in selected genera of mussels, *Mytilus* (Hoeh et al.

1991, Zouros et al. 1992), fish, *Poeciliopsis* (Avise and Vrijenhoek 1987), and insects, *Heliothis* (Lansman et al. 1983).

A few years ago it was impractical for fishery biologists to use nucleotide sequencing to assess the genetic variation within a species (Ryman and Utter 1988). Before the advent of PCR, it was necessary to use conventional cloning procedures in order to generate sufficient amounts of DNA for examination by restriction endonucleases or nucleotide sequencing. PCR circumvents the necessity for cloning and many of the associated obstacles (Saiki et al. 1988).

PCR and nucleotide sequencing have made it practical to determine the sequence of a gene from a large number of individuals of the same species in a short period of time (Gyllensten and Erlich 1988, Saiki et al. 1988, Kocher et al. 1989). The information obtained is pertinent since entire segments of specific genes or regions of the mitochondrial DNA are sequenced rather than a random survey of the entire genome being made using restriction endonucleases and RFLP analysis. There are usually problems associated with comparing results from different labs when performing a RFLP analysis. When different restriction endonucleases are used, it is difficult to normalize the results since each enzyme recognizes and cleaves at a different site. There are also difficulties determining fragment size similarities for the same endonucleases when samples are not run side by side for comparison purposes. PCR and sequence analysis overcome many of these problems. The cytochrome *b* gene is well characterized in many vertebrate species, including several migratory marine fish (Graves and Dizon 1989, Kocher et al. 1989, Johansen et al. 1990, Bartlett and Davidson 1991, Carr and Marshall 1991, McVeigh et al. 1991, Nolan et al. 1991). Therefore it is possible to align sequences and determine levels of divergence with relative ease. Additionally, PCR and nucleotide sequencing are applicable to any stretch of DNA.

The objectives of this study are to analyze the population structure of cobia in U.S. waters and to determine whether distinct breeding

subpopulations exist. The identification of such subpopulations is important in understanding the basic ecology of the species. It will also provide valuable biological data needed by fisheries biologists to properly manage and predict the long-term population status of this stressed fishery. This study presents the results of a preliminary examination of DNA sequence polymorphism in the mitochondrial cytochrome *b* region.

MATERIALS AND METHODS

Sample Collection

Cobia tissue samples were obtained at fishing tournaments where anglers entered their fish in competition for prizes. The fish were well-iced from the time of capture until entry into the tournaments (from 2-24 h after landing) and all samples were in excellent condition. Tissue samples were available from cobia landed in Louisiana, Mississippi, and Florida waters were sampled from May to September of 1991 and 1992 whereas cobia from Texas and Alabama waters were sampled in 1992 only. Cobia from the Virginia region of the Chesapeake Bay were obtained in July and August of 1992.

Immediately after each fish was weighed and measured, whole gonads were removed, placed in separate resealable plastic bags, and stored on ice for 4-12 h until weights could be recorded and aliquots for DNA isolation taken. Data including location of capture, sex, total weight, and measurements (total and fork lengths) were also maintained. Separate aliquots (~10 g) from each gonad were stored at 4°C in MSB-C-E buffer (210 mM mannitol, 70 mM sucrose, 50 mM Tris, pH 7.5 and 3 mM calcium chloride and 10 mM EDTA) (Lansman et al. 1981) for one to five days before mtDNA extraction. Duplicate aliquots were also archived in buffer and stored at -80°C.

Extraction of Mitochondrial DNA

MtDNA was routinely isolated from both ovary and testis giving a high yield of relatively undegraded mtDNA. The protocol for mtDNA isolation was based on modifications of Lansman et al. (1981), Chapman and Powers (1984), and Wallis (1987). Ten grams of gonadal tissue were thoroughly minced using scissors and then homogenized using a motor-driven glass-teflon homogenizer in ice-cold MSB-C buffer (210 mM mannitol, 70 mM sucrose, 50 mM Tris, pH 7.5, and 3 mM calcium chloride) (Lansman et al. 1981). Ethylenediamine tetraacetic acid (EDTA) was added to the homogenate for a final concentration of 10 mM. The homogenate was

centrifuged at 2,500 x g for 10 min at 4°C until no pellet formed; usually four rounds of centrifugation were necessary. Mitochondria were pelleted by centrifuging at 20,000 x g for 20 min at 4°C. The pellet was resuspended in ice-cold MSB-E (210 mM mannitol, 70 mM sucrose, 50 mM Tris, pH 7.5 and 10 mM EDTA); the high speed centrifugation step was repeated and the pellet was resuspended in STE buffer (100 mM NaCl, 50 mM Tris, pH 8.0, and 10 mM EDTA) (Lansman et al. 1981). Mitochondria were lysed with 80 μl of 25% sodium dodecylsulfate (SDS), treated with 250 μg RNAse A and 250 units T_1 RNAse for 30 min at 37°C, and then treated with 250 μg Proteinase K for two h at 37°C. MtDNA was extracted with an equal volume of phenol:isoamyl alcohol:chloroform (25:1:24, v/v). Extractions were typically performed two to three times, until no interface formed. The aqueous phase was transferred to a fresh tube and 2.5 volumes of 100% ethanol (EtOH) and 0.5 volumes of 7.5 M ammonium acetate were added and stored overnight at 4°C. Precipitated mtDNA was obtained by centrifugation at 12,000 rpm in an Eppendorf microcentrifuge for 45 min at 4°C. The pellet was washed once with ice-cold 70% EtOH, once with 100% EtOH, dried briefly under vacuum to remove residual EtOH, resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0), and stored at -20°C. Samples were run in 0.8% agarose gels to estimate quality and quantity of mtDNA. These preparations were of sufficient purity for DNA amplification.

Polymerase Chain Reaction

Amplification of the cobia cytochrome *b* gene was accomplished utilizing PCR (Saiki et al. 1988). An Ericomp SingleBlock™ System thermal cycler was used. This system had the advantage of the "in sample" temperature monitor which allows the instrument to accurately adjust for the lag-time it takes for the actual samples to reach the correct temperature. The thermal-stable *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus) was utilized due to its low error rate as compared

to many of the other thermal-stable DNA polymerases (Innis et al. 1988, Saiki et al. 1988, Tindall and Kunkel 1988, Innis et al. 1990).

Double-Stranded (Symmetric) DNA Amplification of the Mitochondrial Cytochrome *b* Gene

The procedure for amplifying the mitochondrial cytochrome *b* gene was adapted from Saiki et al. (1988) and Kocher et al. (1989). The primers used for amplification were the "universal cytochrome *b* primers" designed by Kocher et al. (1989). To design these primers, the authors searched for highly conserved regions on the mtDNA genome and took advantage of the evolutionary stability of regions of rRNA, the anticodon loops of tRNAs, and the active sites of enzymes. The 3' ends of the primers are located on the first or second base of codons from amino acids that are evolutionary conserved (e.g. tryptophan). The nucleotide sequence of each primer is as follows:

L14841 5'-CCATCCAACATCTCAGCATGATGAAA-3'

H15149 5'-CCCCTCAGAATGATATTTGTCCTCA-3'

The H and L refer to heavy or light strand, respectively, and the numbers correspond to the location of the primer sequence in the human mitochondrial genome sequence (Anderson et al. 1981, Kocher et al. 1989). These primers amplify a 358 base pair region of the cobia mtDNA genome, including the primers. Oligonucleotides were synthesized on a MilliGen/Biosearch cyclone DNA synthesizer in the DMT-off mode and were not purified prior to use.

Symmetric (double-stranded) amplifications were carried out in a final volume of 25 μl in reactions containing 25 mM Tris/HCl, pH 8.3, 50 mM potassium chloride, 0.001% gelatin, 100 μm each of dATP, dTTP, dGTP and dCTP (U.S. Biochemical), 7.5 pmol of each primer, 2mM magnesium chloride, and 1 μl of the purified mtDNA as template (Saiki et al. 1988). Negative control reactions which contained all amplification components except template were also run in order to verify that contamination by extraneous DNA did not occur. A drop of mineral oil was added to cover the reaction mixture and the tube was preheated to

70°C for 10 min to denature the DNA and minimize "primer-dimer" or primer self-annealing (D'Aquila et al. 1991). After preliminary denaturation, 0.5 unit of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus) was added and 30 cycles of amplification (94°C for 45 sec, 60°C for 45 sec and 70°C for 90 sec) were carried out. A final cycle of 70°C for 10 min extended incompletely amplified molecules.

Electrophoresis of a 10 μl portion of the symmetric amplification product was carried out in a 2% NuSieve gel (FMC BioProducts). A molecular weight size standard of Lambda DNA (New England BioLabs) digested with *Hind* III was run simultaneously in order to accurately size amplification products. DNA fragments were visualized by staining with ethidium bromide and examining under ultraviolet illumination. The 358 base pair product was excised, added to 50 μl of distilled H_2O, and remelted at 65°C. A second amplification was carried out using 1 μl of the remelted material as template under the same conditions as above except that the total reaction volume was increased to 100 μl. A 10 μl portion of this symmetric amplification product was run in a 2% NuSieve gel (FMC BioProducts) in order to verify a single product. Residual nucleotides, primers, and buffer salts were removed from the remaining 90 μl using the Prep-A-Gene DNA purification kit™ (Bio-Rad) according to manufacturer's instructions. The resulting purified cytochrome *b* was resuspended in TE buffer and stored at -20°C.

Single-Stranded (Asymmetric) DNA Amplification of the Mitochondrial Cytochrome *b* Gene

The procedure for asymmetric (single-stranded) DNA amplification was taken from Gyllensten and Erlich (1988) and Innis et al. (1988). By using unequal molar amounts of the two amplification primers, it is theoretically possible in a single step to amplify a single-copy gene and produce an excess of single-stranded DNA of a chosen strand. This DNA can then be purified and used directly for sequencing, thus avoiding the need to clone. Asymmetric amplifications were carried out on 1 μl of the purified symmetric amplification product under the same

conditions as the symmetric amplifications except that one primer was diluted. Dilutions of each primer ranging from 1:10 to $1:1x10^6$ were unsuccessful in generating an asymmetric amplification product. Asymmetric protocols developed by Kocher et al. (1989) and Kaltenboeck et al. (1992) were also unsuccessful. Due to the inability to produce an asymmetric amplification product, it was necessary to utilize conventional cloning procedures.

Cloning

Cloning of the symmetric amplification product was performed utilizing a TA Cloning System™ (version 1.0: Invitrogen). This system provides a quick one-step cloning procedure for direct insertion of a PCR product into a plasmid vector. Ligation and transformation procedures were performed according to manufacturer's instructions. Colonies containing a positive insert were selected for small-scale alkaline lysis DNA extraction (Maniatis et al. 1987). Plasmid DNA was purified using a Magic Minipreps Purification System (Promega), chemically denatured, and used directly for sequencing reactions.

Sequencing Reactions

Sequencing reactions were carried out using a Sequenase kit (version 2.0: U.S. Biochemical) with α-^{32}P-dATP (DuPont) as label. For each sequencing reaction, ~2.0 μg of purified plasmid and 1 picomole of M13mp18 (-40) primer (U.S. Biochemical) or T7 promoter primer (Promega) were used. The sequence of each primer is as follows:

T7 Promoter	5'-TAATACGACTCACTATAGGG-3'
M13mp18 (-40)	5'-GTTTTCCCAGTCACGAC-3'

Each clone was sequenced using both primers in order to verify complementary strand nucleotide sequence. The manufacturer's instructions were followed except for the additional use of terminal deoxynucleotidyl transferase (TdT) (Kho and Zarbl 1992). The DNA polymerase supplied in the Sequenase sequencing kit has a tendency to pause at regions on the DNA where secondary structure is exceptional.

This pausing of the polymerase can result in the detection of bands at the same position in all four dideoxy sequencing gel lanes. The TdT enzyme catalyzes the rapid, repetitive addition of deoxyribonucleotides to the terminal 3' OH of the DNA. TdT effectively moves the spurious bands out of their pre-terminated positions, thereby revealing the correct sequences that were generated by the dideoxy sequencing reactions, but which were hidden by the polymerase premature termination artifacts. One unit of TdT (Promega) was added prior to the addition of the Stop Solution (95% foramide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and incubated at 37°C for 15 min. Sequencing products were separated on 60 cm, 7% polyacrylamide (19:1 acrylamide:bis-acrylamide), 7M urea gels. Electrophoresis was done at 45 W constant power (~2000 V). The gels were dried onto filter paper and autoradiographed using Fugi RX film.

These procedures routinely allowed the sequence of 358 nucleotides of the cytochrome *b* gene to be determined. Amino acid sequences were determined utilizing the Pustell Sequence Analysis Software System (International Biotechnologies, Inc.). Nucleotide sequences were entered into the system and protein coding regions were identified, taking into consideration the unique genetic code utilized by mitochondrial DNA (Anderson et al. 1981). This process identified the corresponding 119 amino acids based on the 358 nucleotides. All sequences are given as their coding strand equivalents.

RESULTS

Single-Stranded (Asymmetric) DNA Amplification of the Mitochondrial Cytochrome *b* Gene

Generation of an asymmetric PCR product was unsuccessful following the protocols of Gyllensten and Erlich (1988), Kocher et al. (1989), and Kaltenboeck et al. (1992). Cross contamination by an extraneous DNA source may have been a contributing factor to this problem. Negative controls, which purposely lacked added template DNA, yielded amplification products midway through this study. Empirically, it was determined that both primers had become contaminated. The source of this contamination can be contributed to several factors, the most likely being carryover introduced by pipetors, which were not positive-displacement instruments. A fresh pair of noncontaminated primers were employed using the previous protocols. Nonetheless, all of these procedures were unsuccessful in generating an asymmetric PCR product which yielded satisfactory sequencing results. It was for these reasons that it was necessary to utilize conventional cloning procedures.

Mitochondrial Cytochrome *b* Gene Sequence

The nucleotide sequence of a 358 base pair region of the mitochondrial cytochrome *b* gene was obtained from two cobia, fish numbered 39 (female) and 42 (male); refer to Chapter 1 for additional information. A consensus sequence and inferred amino acid sequence of the coding strand of this region were constructed by comparing the nucleotide sequences from both fish and are shown in Figure 3. In mitochondrial DNA there are none or very few noncoding bases, or introns, between adjacent genes, except for the D-loop region (Anderson et al. 1981). The absence of introns makes it easy to assign amino acids to the nucleotide triplets using the mitochondrial genetic code. The numbering system in Figure 3 is arbitrary but representative of results which could be obtained with ease from two loadings on a wedged sequencing gel 60 cm in length. The corresponding segment of the human

Figure 3. Consensus nucleotide sequence and inferred amino acid sequence of a 358 base pair region of the *R. canadum* mitochondrial cytochrome *b* gene (top 2 lines). The corresponding information for the human sequence (Anderson et al. 1981) is shown in the bottom 2 lines. In the *R. canadum* sequence, the variable nucleotide is marked with an asterisk; Y indicates a pyrimidine. Deviations in the *R. canadum* cytochrome *b* gene from the human sequence are indicated in bold lower case letters for nucleotide changes or underlined for amino acid changes.

```
CCA  TCC  AAC  ATC  TCa  GCA  TGA  TGA  AAt  TTt  GGC  TCA  CTC        39
 P    S    N    I    S    A    W    W    N    F    G    S    L         13
CCA  TCC  AAC  ATC  TCC  GCA  TGA  TGA  AAC  TTC  GGC  TCA  CTC
 P    S    N    I    S    A    W    W    N    F    G    S    L

CTc  GGC  ctt  TGt  CTt  ATC  acC  CAA  ATt  cta  ACA  GGc  CTg        78
 L    G    L    C    L    I    T    Q    I    L    T    G    L         26
CTT  GGC  GCC  TGC  CTG  ATC  CTC  CAA  ATC  ACC  ACA  GGA  CTA
 L    G    A    C    L    I    L    Q    I    T    T    G    L

TTt  CTt  GCC  ATa  CAt  TAC  aCA  tCA  GAC  atC  gCA  ACg  GCt        117
 F    L    A    M    H    Y    T    S    D    I    A    T    A         39
TTC  CTA  GCC  ATG  CAC  TAC  TCA  CCA  GAC  GCC  TCA  ACC  GCC
 F    L    A    M    H    Y    S    P    D    A    S    T    A

TTc  TCA  TCc  gTC  GCC  CAC  ATt  tgT  CGA  GAt  GTt  AAT  TAT        156
 F    S    S    V    A    H    I    C    R    D    V    N    Y         52
TTT  TCA  TCA  ATC  GCC  CAC  ATC  ACT  CGA  GAC  GTA  AAT  TAT
 F    S    S    I    A    H    I    T    R    D    V    N    Y

GGC  TGg  cTt  cTt  CGC  aAC  CTa  CAY*  GCt  AAT  GGC  GCC  TCc        195
 G    W    L    L    R    N    L    H    A    N    G    A    S         65
GGC  TGA  ATC  ATC  CGC  TAC  CTT  CAC  GCC  AAT  GGC  GCC  TCA
 G    W    I    I    R    Y    L    H    A    N    G    A    S

tTc  TTC  TTT  ATC  TGt  gTt  Tat  CTt  CAC  ATt  GGt  CGA  GGC        234
 F    F    F    I    C    V    Y    L    H    I    G    R    G         78
ATA  TTC  TTT  ATC  TGC  CTC  TTC  CTA  CAC  ATC  GGG  CGA  GGC
 M    F    F    I    C    L    F    L    H    I    G    R    G

CTt  TAc  TAC  GGc  TCt  Tac  tTa  TAt  aaA  GAA  ACt  TGA  AAt        273
 L    Y    Y    G    S    Y    L    Y    K    E    T    W    N         91
CTA  TAT  TAC  GGA  TCA  TTT  CTC  TAC  TCA  GAA  ACC  TGA  AAC
 L    Y    Y    G    S    F    L    Y    S    E    T    W    N

AcC  GGa  gTT  ATt  CTt  CTt  CTa  ctc  ctT  ATA  GgA  ACt  GCt        312
 T    G    V    I    L    L    L    L    L    M    G    T    A        104
ATC  GGC  ATT  ATC  CTC  CTG  CTT  GCA  ACT  ATA  GCA  ACA  GCC
 I    G    I    I    L    L    L    A    T    M    A    T    A

TTt  gTt  GGa  TAT  GTa  CTt  CCa  TGA  GGa  CAA  ATA  TCA  TTC        351
 F    V    G    Y    V    L    P    W    G    Q    M    S    F        117
TTC  ATA  GGC  TAT  GTC  CTC  CCG  TGA  GGC  CAA  ATA  TCA  TTC
 F    M    G    Y    V    L    P    W    G    Q    M    S    F

TGA  GGG  G                                                          358
 W    G                                                              119
TGA  GGG  G
 W    G
```

cytochrome *b* sequence, equivalent to positions 14,816 to 15,173 of the human mitochondrial genome, is shown below the cobia sequence for comparative purposes.

Both strands of five separate clones from each fish were sequenced in order to verify any sequence ambiguities. There were six nucleotide positions at which inconsistencies were observed. The positions of these sites and the specific nucleotides occupying them are summarized in Table 4. For example, the clones from fish 39 all had a T at position 228. All but one of the clones sequenced for fish 42 had a T at this position; clone 6 was atypical in that it had a C. In cases such as this, it was reasoned that the correct nucleotide identity of a position for a particular fish was the nucleotide that appeared the majority of the time among the clones of that same fish. Following this reasoning, it was determined that the nucleotide identities were G, A, T, and T at positions 143, 152, 156, and 213, respectively, for both fish. The sequence differences between the individual clones can be most likely attributed to mutations introduced during PCR or cloning procedures. When a particular clone was resequenced, the identical nucleotide sequence resulted. For example, when clone 45 from fish 39 was resequenced, an A consistently resulted at position 143. Upon sequencing the complementary strand of this same clone, T consistently resulted at the site complementary to position 143. Mutations introduced during the sequencing reactions were therefore ruled out.

At position 186, each clone for fish 39 had a T whereas each clone for fish 42 had a C. Sequencing the complementary strands for each fish also confirmed this. This singular, third-position nucleotide substitution at base 186 on the coding strand defined a unique genotype for each fish. The substitution was, however, a silent mutation since there was no change in the amino acid sequence of the cytochrome *b* gene.

Table 4. Nucleotide variation at specific sites when sequencing separate clones of the same fish.

		Nucleotide position					
Fish #	Clone #	143	152	156	186	213	228
39	10	G	A	T	T	T	T
	16	G	A	T	T	T	T
	20	G	A	T	T	T	T
	27	G	A	T	T	T	T
	45	A	A	T	T	T	T
42	5	G	A	T	C	T	T
	6	G	G	C	C	T	C
	7	G	G	C	C	T	T
	9	G	A	T	C	T	T
	68	G	A	T	C	C	T

Patterns of Mitochondrial DNA Sequence Changes

When comparing the cobia and human cytochrome *b* gene sequences (Figure 3), 75% of the nucleotide positions were invariant. Of the nucleotides that differed, 23%, 14%, and 63% were first, second, and third position changes, respectively. Transitions and transversions occurred at about the same frequency, 51% and 49%, respectively. The amino acids of the cobia and human sequence were invariant at 81% of the positions. At positions where the amino acids were different between the two sequences, 82% of the changes were not drastic, as defined by Grantham (1974). This author devised a formula to determine what type of substitutions were preferentially avoided. Characteristics such as the atomic weight ratio of noncarbon elements in end groups or rings to the carbons in the side chains, polarity, and molecular volume of amino acids were examined in deriving this formula. When comparing the cobia and human sequence, neutral amino acids replaced neutral amino acids 95% of time. A single substitution, which accounted for 5% of the total variation, was a more nonconventional change from a basic to a neutral amino acid (Grantham 1974).

In order to investigate the types of nucleotide changes observed in other fish within the class Osteichthyes, the cytochrome *b* sequences from 41 different species were compared to the cobia cytochrome *b* nucleotide sequence. The results of these comparisons are shown in Figure 4. These 41 species represent three different orders and 11 different families of fishes. This information was gathered from the National Institute of Health GenBank (version 75) and several recent studies on freshwater (Kocher et al. 1989, McVeigh et al. 1991), anadromous (McVeigh et al. 1991), and marine fish (Bartlett and Davidson 1991, Carr and Marshall 1991, Hartley et al. 1992, Block et al. 1993). The information in Figure 4 represents the consensus nucleotide sequence for each species. This information was obtained by sequencing of a cloned product for cobia and direct nucleotide sequencing of a PCR amplified product for the remaining 41 species. When comparing the

Figure 4. Consensus nucleotide sequence of the 358 base pair region of the *R. canadum* mitochondrial cytochrome *b* gene compared to the consensus cytochrome *b* gene nucleotide sequences from 41 different species of fish within the class Osteichthyes. Numbers (1, 2, or 3) above nucleotides of *R. canadum* represent positions in the cobia nucleotide triplet that are different from the other species. Asterisks above the *R. canadum* sequence represent nucleotide positions unique to cobia. Y is a pyrimidine, R a purine, and M is the nucleotide A or C. Dashes (-) indicate nucleotides that were not determined.

Species	Reference				
				3	3 3 31
(1). *Rachycentron canadum*		CCATCCAACA	TCTCAGCATG	ATGAAATTTT	GGCTCACTCC
(2). *Salmo salar*	McVeigh et al. (1991)	----------	----------	------CTTT	GGCTCACTCT
(3). *Salmo trutta*	McVeigh et al. (1991)	----------	----------	------CTTT	GGCTCACTCT
(4). *Salvelinus alpinus*	Hartley et al. (1992)	----------	----------	-------TTT	GGTTCACTCT
(5). Serranidae	Block et al. (1993)	----------	----------	----------	----------
(6). *Coryphaena equiselis*	Block et al. (1993)	----------	----------	----------	----------
(7). *Chichlasoma citrinellum*	Kocher at al. (1989)	----------	----------	----------	----------
(8). *Cichlasoma labiatum*	Kocher at al. (1989)	----------	----------	----------	----------
(9). *Cichlasoma centrarchus*	Kocher at al. (1989)	----------	----------	----------	----------
(10). *Cichlasoma nicaraguense*	Kocher at al. (1989)	----------	----------	----------	----------
(11). *Gadus morhua*	Carr and Marshall (1991)	----------	----------	----------	GGCTCTCTTC
(12). *Julidochromis regani*	Kocher at al. (1989)	----------	----------	----------	----------
(13). *Sphyraena sphyraena*	Block et al. (1993)	----------	----------	----------	----------
(14). *Gempylus serpens*	Block et al. (1993)	----------	----------	----------	----------
(15). *Lepidocybium flavobrunneum*	Block et al. (1993)	----------	----------	----------	----------
(16). *Ruvettus pretiosus*	Block et al. (1993)	----------	----------	----------	----------
(17). *Trichiurus lepturus*	Block et al. (1993)	----------	----------	----------	----------
(18). *Gasterochisma melampus*	Block et al. (1993)	----------	----------	----------	----------
(19). *Scomber japonicus*	Block et al. (1993)	----------	----------	----------	----------
(20). *Scomber scombrus*	Block et al. (1993)	----------	----------	----------	----------
(21). *Scomberomorus cavalla*	Block et al. (1993)	----------	----------	----------	----------
(22). *Scomberomorus maculata*	Block et al. (1993)	----------	----------	----------	----------
(23). *Sarda chiliensis*	Block et al. (1993)	----------	----------	----------	----------
(24). *Sarda sarda*	Block et al. (1993)	----------	----------	----------	----------
(25). *Auxis thazard*	Block et al. (1993)	----------	----------	----------	----------
(26). *Euthynnus affinis*	Block et al. (1993)	----------	----------	----------	----------
(27). *Euthynnus alletteratus*	Block et al. (1993)	----------	----------	----------	----------
(28). *Katsuwonus pelamis*	Block et al. (1993)	----------	----------	----------	----------
(29). *Thunnus alalunga*	Bartlett and Davidson (1991)	----------	----------	---AAACTTT	GGCTCACTAC
(30). *Thunnus albacares*	Bartlett and Davidson (1991)	----------	----------	---AAACTTT	GGCTCACTAC
(31). *Thunnus maccoyii*	Block et al. (1993)	----------	----------	----------	----------
(32). *Thunnus obesus*	Bartlett and Davidson (1991)	----------	----------	---AAACTTT	GGCTCACTAC
(33). *Thunnus thynnus*	Bartlett and Davidson (1991)	----------	----------	---AAACTTT	GGCTCACTAC
(34). *Xiphias gladius*	Block et al. (1993)	----------	----------	----------	----------
(35). *Isiophorus platypterus*	Block et al. (1993)	----------	----------	----------	----------
(36). *Makaira indica*	Block et al. (1993)	----------	----------	----------	----------
(37). *Makaira nigricans*	Block et al. (1993)	----------	----------	----------	----------
(38). *Tetrapturus albidus*	Block et al. (1993)	----------	----------	----------	----------
(39). *Tetrapturus angustirostris*	Block et al. (1993)	----------	----------	----------	----------
(40). *Tetrapturus audax*	Block et al. (1993)	----------	----------	----------	----------
(41). *Tetrapturus belone*	Block et al. (1993)	----------	----------	----------	----------
(42). *Tetrapturus pfluegeri*	Block et al. (1993)	----------	----------	----------	----------

Figure 4. *Continued*

```
      3   1 3   31 31231 3   31 3  3   3  31 3   3  3  3  3   3  3  31  3  31 312 31 3  3  3 123  3  31
(1).  TCGGCCTTTG TCTTATCACC CAAATTCTAA CAGGCCTGTT TCTTGCCATA CATTACACAT CAGACATCGC AACGGCTTTC TCATCCGTCG
(2).  TAGGCCTATG TCTAGCCACC CAAATCCTTA CCGGGCTCTT CCTAGCCATA CACTACACCT CCGATATCTC AACAGCTTTT TCCTCTGTTT
(3).  TAGGCTTATG TCTAGCCACC CAAATTCTTA CCGGACTCTT CCTAGCCATA CACTACACCT CCGATATCTC AACAGCCTTT TCCTCTGTTT
(4).  TAGGCCTATG TTTGGCCACC CAAATTCTTA CCGGACTCTT CCTAGCCATA CACTACACCT CCGATATTTC AACAGCTTTT TCCTCTGTGT
(5).  ---------- ---------- ----TTCTTA CAGGCCTATT TCTAGCCATA CACTACACAT CAGACATCGC CACCGCCTTC TCGTCCGTTG
(6).  ---------- ---------- ----TCCTTA CAGGCCTATT CCTAGCCATG CACTACACAG CAGATATTGC AACGGCTTTC CCTTCTGTTG
(7).  ---------- ---------- ---------A CAGGCCTTTT CCTTGCAATA CACTACACTT CCGATATCGC AACAGCCTTT TCATCCGTCG
(8).  ---------- ---------- ---------A CAGGCCTTTT CCTTGCAATA CACTACACTT CCGATATCGC AACAGCCTTT TCATCCGTCG
(9).  ---------- ---------- ---------A CAGGCCTATT CCTTGCAATA CACTACACTT CCGATATCGC AACAGCCTTT TCATCCGTTG
(10). ---------- ---------- ---------A CAGGCATTTT CCTTGCAATA CATTACACTT CTGACATCGC AACAGCCTTT TCATCCGTTG
(11). TAGGCCTTTG CTTAATTACT CAACTTCTAA CAGGACTATT TCTAGCCATA CACTATACCT CAGACATCGA GACAGCCTTC TCATCCGTAG
(12). ---------- ---------- ---------A CAGGCCTTTT CCTAGCCATA CACTATACCT CCGACATCGC CACCGCCTTT TCCTCCGTCG
(13). ---------- ---------- ----TCCTTA CAGGACTATT CCTAGCCATA CATTACACCG CAGACATAGA ATCAGCTTTT GCCTCCGTGG
(14). ---------- ---------- ----TCCTTA CAGGACTCTT CCTAGCCATG CACTACACCC CCGATGTTGA ATCAGCCTTT GCCTCCGTCG
(15). ---------- ---------- ----TCCTTA CAGGACTATT TCTTGCAATA CACTACACCC CAGACGTNGA ATCAGCCTTT GCCTCCGTAG
(16). ---------- ---------- ----TCCTCA CAGGACTATT CCTTGCAATG CACTACACCC CTGACGTCGA ATCAGCATTT GCTTCTGTAG
(17). ---------- ---------- ----TCCTCA CAGGTCTTTT CCTTGCCATG CACTACACCG CTGACATCGA ATCTGCATTC AACTCAGTTG
(18). ---------- ---------- ----TCCTTA CAGGACTATT CCTTGCAATA CATTACACCC CTGACATCGA GTCAGCCTTC GCTTCAGTTG
(19). ---------- ---------- ----TTCTCA CAGGACTTTT CCTCGCAATA CACTACACGC CCGATGTTGA GTCAGCATTC GACTCAGTCG
(20). ---------- ---------- ----TCCTCA CAGGACTATT CCTTGCAATG CACTACACGC CCGACGTCGA ATCAGCATTC AACTCAGTCG
(21). ---------- ---------- ----TCCTTA CAGGACTCTT CCTTGCAATA CACTACACAG CAGACGTCGC CACGGCCTTC CCCTCAGTAG
(22). ---------- ---------- ----TCCTTA CAGGACTCTT CCTAGCGATA CACTACACAG CAGACATCGC CACRGCYTTC CCCTCAGTAG
(23). ---------- ---------- ----TCCTTA CAGGACTATT CCTAGCAATA CATTATACCC CTGATGTCGA ATCAGCCTTC GCCTCAGTCG
(24). ---------- ---------- ----TTCTTA CAGGCCTATT CCTAGCAATA CACTACACCC CTGATGTCGA ATCAGCCTTC GCTTCAGTAG
(25). ---------- ---------- ----TTCTTA CAGGCCTATT CCTTGCAATA CACTACACCC CTGATGTCGA ATCAGCCTTC GCTTCAGTAG
(26). ---------- ---------- ----TCCTTA CAGGCCTATT CCTCGCAATA CACTATACCC CCGATGTCGA ATCAGCCTTC GCCTCAGTAG
(27). ---------- ---------- ----TTCTTA CAGGCCTGTT CCTCGCAATA CACTACACCC CTGACGTCGA ATCAGCCTTC GCCTCAGTAG
(28). ---------- ---------- ----TCCTAA CAGGACTATT CCTCGCAATA CACTATACCC CTGACGTAGA ATCAGCCTTC GCCTCAGTAG
(29). TTGGCCTTTG CCTTATTTCT CAAATCCTTA CAGGACTATT CCTCGCAATA CACTATACCC CTGATGTCGA ATCAGCCTTC GCCTCAGTAG
(30). TTGGCCTTTG CCTTATTTCT CAAATCCTTA CAGGACTATT CCTCGCAATA CACTACACCC CTGATGTCGA ATCAGCCTTC GCCTCAGTAG
(31). ---------- ---------- ----TCCTTA CAGGACTATT CCTCGCAATA CACTACACCC CTGATGTCGA ATCAGCCTTC GCCTCAGTAG
(32). TTGGCCTTTG CCTTATTTCT CAAATCCTTA CAGGACTATT CCTCGCAATA CACTACACCC CTGATGTCGA ATCAGCCTTC GCCTCAGTAG
(33). TTGGCCTTTG CCTTATTTCT CAGATCCTTA CAGGACTATT TCTCGCTATA CACTATACAT CAGACATCGC GACGGCCTTT ACATCCGTAG
(34). ---------- ---------- ----TCCTCA CAGGCCTGTT TCTCGCTATA CACTATACAT CAGACATCGC GACGGCCTTT ACATCCGTAG
(35). ---------- ---------- ----TCCTTA CAGGCCTTTT CCTCGCTATG CACTACACCT CAGACATCGC AACAGCCTTC ACATCCGTAG
(36). ---------- ---------- ----TCCTTA CAGGCCTTTT CCTTGCTATA CACTACACCT CAGACATCGC AACAGCCTTC ACATCCGTAG
(37). ---------- ---------- ----TCCTTA CAGGCCTTTT CCTCGCTATA CACTAYACCT CAGACATCGC AACAGCCTTC ACATCCGTAG
(38). ---------- ---------- ----TCCTTA CAGGCCTTTT CCTCGCTATA CACTACACCT CGGACATCGC AACAGCCTTC ACATCCGTAG
(39). ---------- ---------- ----TTCTTA CAGGCCTTTT CCTCGCTATA CATTACACCT CAGACATCGC AACAGCCTTC ACATCCGTAG
(40). ---------- ---------- ----TCCTTA CAGGCCTTTT CCTCGCTATA CACTACACCT CGGACATCGC AACAGCCTTC ACATCCGTAG
(41). ---------- ---------- ----TTCTTA CAGGCCTTTT CCTCGCTATA CATTACACCT CAGACATCGC AACAGCCTTC ACATCCGTAG
(42). ---------- ---------- ----TTCTTA CAGGCCTTTT CCTCGCTATA CATTACACCT CAGACATCGC AACAGCCTTC ACATCCGTAG
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Figure 4. *Continued*

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                                               *   *
        23  3  3    3  3  3  3    23 23  3    3  31 3 2  3  31 3  3    3  3  3     3  3  31   3  31 3  3 1 3  3123
 (1).  CCCACATTTG TCGAGATGTT AATTATGGCT GGCTTCTTCG CAACCTACAY GCTAATGGCG CCTCCTTCTT CTTTATCTGT GTTTATCTTC
 (2).  GCCACATTTG CCGAGATGTT AGCTATGGCT GACTCATCCG TAACATTCAC GCTAACGGAG CATCTTTCTT CTTTATCTGT ATTTATATAC
 (3).  GCCACATTTG CCGAGATGTT AGCTACGGCT GACTCATCCG AAACATTCAC GCTAACGGAG CATCTTTCTT CTTTATCTGT ATTTATATAC
 (4).  GCCATATCTG CCGAGATGTA AGTTACGGCT GACTCATCCA GAATATCCAC GCTAACGGAG CATCTTTCTT CTTTATCTGT ATTTACATAC
 (5).  CCCACATTTG CCGAGACGTA AACTACGGCT GACTTATCCG CAACATGCAC GCCAACGGAG CCTCCTTCTT CTTTATCTGC ATTTATGCTC
 (6).  CCCACATTTG CCGAGATGTA AACTATGGAT GACTAATCCG AAACCTGCAT GCTAACGGAG CCTCATTCTT CTTTATCTGT GTTTACTTCC
 (7).  CCCACATCTG CCGAGATGTA AACTATGGCT GACTAATCCG TAATATACAC GCCAACGGCG CATCCTTTTT CTTTATTTGC ATCTACCTTC
 (8).  CCCACATCTG CCGAGATGTA AACTATGGCT GACTAATCCG TAATATACAC GCCAACGGCG CATCCTTTTT CTTTATTTGC ATCTACCTTC
 (9).  CCCACATCTG CCGAGATGTA AACTATGGCT GACTAATCCG TAATATACAT GCCAACGGCG CATCCTTTTT CTTTATTTGC ATTTACCTTC
(10).  CCCACATCTG TCGAGATGTA AATTATGGCT GACTCATCCG CAATATACAC GCCAATGGCG CATCCTTCTT CTTCATCTGC ATCTATCTTC
(11).  TCCACATCTG TCGTGATGTA AACTACGGCT GACTAATTCG GAATATACAT GCTAATGGTG CCTCTTTCTT TTTCATTTGT CTTTATATGC
(12).  CCCACATCTG TCGTGACGTC AACTACGGTT GACTCATCCG AAATATGCAC GCCAACGGCG CATCCTTCCT CTTCATTTGT ATTTACCTCC
(13).  CACATATTTG CCGAGACGTT AACTTCGGCT GACTGATTCG CAACATGCAC GCCAACGGAG CATCATTCTT CTTTGTATGT ATCTACCTTC
(14).  CCCACATCTG CCGTGACGTT AACTTCGGTT GACTAATCCG TAACCTCCAT GCTAACGGGG CCTCCTTCTT CTTCATTTGC ATCTATTTCC
(15).  CCCACATCTG CCGAGACGTA AACTTCGGAT GACTCATCCG AAACCTTCAT GCAAACGGCG CATCTTTCTT CTTCATCTGT ATTTACTTCC
(16).  CCCACATCTG CCGAGACGTA AACTTCGGAT GACTTATCCG AAACCTCCAT GCAAACGGAG CATCATTCTT CTTCATCTGC ATTTATTTCC
(17).  CCCATATATG CCGAGACGTC AATTTTGGGT GACTCATCCG AAACTTCCAC GCAAACGGGG CTTCCTTCTT CTTCTTCTGC CTCTACCTTC
(18).  CCCACATCTG CCGGGACGTA AACTTTGGCT GACTCATCCG TAACCTCCAT GCCAACGGAG CATCCTTCTT CTTCGTCTGC ATTTATTCCC
(19).  CCCATATTTG CCGGGACGTA AACTTCGGAT GACTAATCCG TAACCTACAT GCAAACGGCG CCTCCTTCTT CTTCATTTGC ATTTATCTCC
(20).  CCCATATTTG CCGAGACGTA AACTTCGGAT GACTCATCCG CAACATGCAC GCAAACGGTG CCTCTTTCTT CTTCATTTGC CTTTACCTCC
(21).  CCCATATTTG CCGAGACGTC AACTTCGGCT GACTCATCCG AAACCTTCAT GCAAACGGAG CATCCTTCTT CTTCATCTGT ATCTACCTTC
(22).  CCCATATTTG CCGAGACGTA AACTACGGAT GACTTATCCG TAACCTGCAT GCCAACGGAG CCTCCTTCTT CTTTATCTGT GTCTACGTTC
(23).  CCCACATTTG CCGAGACGTT AATTTCGGCT GACTAATCCG AAACCTCCAC GCAAACGGGG CCTCCTTCTT CTTCATCTGT ATCTACTTCC
(24).  CCCATATTTG CCGAGATGTA AACTTCGGTT GACTTATCCG AAACCTCCAC GCAAACGGCG CCTCCTTCTT CTTCATCTGC ATCTACTCAC
(25).  CCCATATTTG CCGAGATGTA AACTTCGGTT GACTTATCCG AAACCTCCAC GCCAACGGCG CCTCCTTCTT CTTCATCTGC ATCTACTCAC
(26).  CCCATATCTG CCGAGACGTA AACTTCGGAT GACTCATCCG AAACCTACAC GCCAACGGCG CTTCCTTCTT CTTCATCTGC ATTTACTCAC
(27).  CTCATATCTG CCGAGATGTA AACTTCGGAT GACTCATCCG AAACCTCCAC GCCAACGGCG CTTCCTTCTT CTTCATTTGC ATTTACTCAC
(28).  CCCATATCTG CCGAGACGTA AACTTCGGAT GACTCATCCG AAACCTCCAT GCCAACGGCG CTTCCTTCTT CTTCATCTGC ATTTACTCCC
(29).  CCCACATTTG CCGAGATGTC AACTTCGGTT GACTCATCCG GAACCTCCAC GCAAACGGGG CCTCTTTCTT CTTTATCTGC ATCTACTTCC
(30).  CCCACATTTG CCGAGATGTC AACTTCGGTT GACTCATCCG GAACCTCCAC GCAAACGGGG CCTCTTTCTT CTTTATCTGC ATCTACTTCC
(31).  CCCACATTTG CCGAGATGTC AACTTCGGTT GACTTATCCG GAACCTCCAC GCAAACGGGG CCTCTTTCTT CTTTATCTGT ATCTACTTCC
(32).  CCCACATTTG CCGAGATGTC AATTTCGGTT GACTCATCCG GAACCTCCAC GCAAACGGGG CCTCTTTCTT CTTTATCTGT ATCTACTTCC
(33).  CCCACATTTG CCGAGATGTC AACTTCGGTT GACTTATCCG GAACCTCCAC GCAAACGGGG CCTCTTTCTT CTTTATCTGT ATCTACTTCC
(34).  CACATATCTG CCGAGATGTA AATTATGGAT GACTCATCCG AAACATGCAC GCCAACGGCG CATCCTTCTT CTTCATCTGC ATTTACCTCC
(35).  CCCACATCTG CCGGGACGTG AATTACGGCT GACTCATTCG AAATATACAT GCCAACGGCG CATCTTTCTT CTTTATCTGC ATTTACCTCC
(36).  CCCATATCTG CCGAGACGTA AATTACGGCT GACTCATTCG AAATATGCAT GCCAACGGGG CATCTTTCTT CTTCATCTGC ATTTACCTCC
(37).  CCCATATCTG CCGAGACGTG AATTACGGCT GACTCATTCG AAATATRCAT GCCAACGGCG CATCTTTCTT CTTTATCTGC ATCTACCTCC
(38).  CCCACATCTG CCGGGACGTG AATTACGGCT GACTCATTCG AAATATACAT GCCAACGGCG CATCTTTCTT CTTCATCTGC ATTTACCTCC
(39).  CCCATATCTG CCGGGACGTA AATTACGGCT GACTCATTCG AAATATACAT GCCAACGGCG CATCTTTCTT CTTCATCTGC ATCTACCTCC
(40).  CCCACATCTG CCGGGACGTG AATTACGGCT GACTCATTCG AAATATACAT GCCAACGGCG CATCTTTCTT CTTCATCTGC ATTTACCTCC
(41).  CCCATATCTG CCGAGACGTA AATTACGGCT GACTCATTCG AAATATACAT GCCAACGGCG CATCTTTCTT CTTCATCTGC ATCTACCTCC
(42).  CCCATATCTG CCGGGACGTA AATTACGGCT GACTCATTCG AAATCTACAT GCCAACGGCG CATCTTTCTT CTTCATCTGC ATCTACCTCC
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Figure 4. *Continued*

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                                                                    *                                   *         **
         3  3 23    3  31 3  3   31 3 23    31 3 2312  3  3  3  3  3123   3  31 31 31  3  31 3123  3123   31
(1).  ACATTGGTCG AGGCCTTTAC TACGGCTCTT ACTTATATAA AGAAACTTGA AATACCGGAG TTATTCTTCT TCTACTCCTT ATAGGAACTG
(2).  ACATCGCCCG AGGACTTTAT TATGGTTCCT ATCTATATAA AGAAACCTGA AATATCGGAG TTGTACTTCT ACTTCTCACT ATAATAACTG
(3).  ATATCGCCCG AGGACTCTAC TATGGTTCCT ACCTATATAA AGAAACCTGA AATATCGGAG TCGTACTGCT ACTTCTCACT ATAATAACCG
(4).  ATATCGCCCG AGGACTCTAC TACCGGTGCT ACCTATATAA AGAAACCTGA AATATTGGAG TAGTATTACT ACTTCTAACT ATAATGACTG
(5).  ACATTGGACG AGGCCTTTAC TACGGCTCTT ACCTCTATAA AGAAACCTGA AACATTGGAG TTATTCTCCT CCTACTAGTG ATGATAACAG
(6).  ATATCGGTCG AGGCCTGTAC TATGGGTCTT ACCTCTACAA AGAGACTTGA AATATTGGAG TAGTCCTTCT TCTACTCGTT ATAATCACTG
(7).  ACATTGGCCG AGGACTATAC TACGGCTCCT ACCTTTATAA AGAAACATGA AATGTAGGAG TTGTTCTCCT CCTCCTAACC ATAATAAC--
(8).  ACATTGGCCG AGGACTATAC TACGGCTCCT ACCTTTATAA AGAAACATGA AATGTAGGAG TTGTTCTCCT CCTCCTAACC ATAATAAC--
(9).  ACATTGGCCG AGGACTATAC TACGGCTCCT ACCTTTATAA AGAAACATGA AATGTAGGAG TTGTTCTACT CCTCCTAACC ATAATAAC--
(10). ACATTGGCCG AGGATTATAC TATGGCTCCT ACCTTTACAA AGAAACATGA AACGTCGGAG TTATCCTCCT CCTCCTAACC ATAATAAC--
(11). ACATTGCCCG AGGTCTCTAT TATGGTTCCT ATCTTTTTGT AGAGACATGA AACATCGGGG TTGTCCTTTT CCTTTTAGTA ATAATAACCT
(12). ACATTGGCCG AGGGTTATAC TATGGCTCCT ATTTATATAA AGAAACCTGA AATATTGGAG TTATCCTCCT CCTTTTAACT ATAATAAC--
(13). ACATCGGCCG TGGCCTTTAC TACGGCTCCT ACCTTTACAA GGAAACATGG AACATTGGCG TAATTCTTCT TCTGCTAGTT ATAATGACTG
(14). ACATCGGACG AGGTCTTTAC TACGGCTCAT ACCTTTTTAA AGAAACATGG AATATTGGCG TAGTACTCCT CCTCCTAGTA ATGATAACAG
(15). ACATTGGACG AGGCCTTTAC TACGGCTCTT ACCTCTATAA AGAAACATGA AACATCGGTG TAGTCCTCCT CCTTCTAGTA ATAATAACTG
(16). ACATCGGACG AGGCCTTTAC TACGGCTCCT ACCTCTATAA AGAAACATGA AACATTGGCG TAGTCCTTCT ACTGCTCGTT ATAATAACAG
(17). ACATCGGCCG AGGACTTTAC TACGGCTCCT ACCTATATAA AGAAACATGA AACATTGGAG TAGTTCTCCT CCTCCTCGTA ATAATAACTG
(18). ACATTGGACG AGGCCTCTAC TACGGCTCCT ACCTCTACAA AGAAACATGA AACATCGGGG TAGTCCTACT ACTCCTAGTT ATGATRACCG
(19). ACATCGGGCG AGGTCTTTAC TACGGTTCCT ACCTTTTTAT GGAGACATGA AACATTGGTG TGGTTCTTCT CCTCCTCGTA ATAATAACCG
(20). ACATCGGACG AGGCCTGTAC TACGGCTCAT ACCTTTTTAT AGAGACATGA AACGTCGGTG TAGTCCTCCT CCTCCTCGTA ATGATGACTG
(21). ACATTGGCCG AGGTCTCTAC TACGGCTCCT ACCTCTACAA AGAAACCTGA AACATTGGAG TTGTTCTTCT ACTCCTGGTA ATAATGACAG
(22). ACATTGGCCG AGGCCTGTAC TAYGGCTCYT ACCTCTACAA AGAAACCTGA AACMTYGGCG TTATTCTTCT ACTCCTAGTT ATAATAACAG
(23). ACATTGGCCG AGGCCTTTAC TACGGCTCCT ACCTCTACAA AGAAACATGA AACATTGGAG TAGTCCTTCT ACTTCTAGTA ATGATGACCG
(24). ACATCGGACG AGGCCTTTAC TACGGCTCCT ACCTCTACAA AGAAACATGA AACATTGGAG TAGTTCTTCT ACTGCTAGTC ATGATGACTG
(25). ACATCGGACG AGGCCTTTAC TACGGCTCCT ACCTCTACAA AGAAACATGA AACATTGGCG TAGTTCTTCT ACTGCTAGTC ATGATGACTG
(26). ACATCGGCCG AGGTCTTTAC TACGGCTCTT ACCTCTACAA AGAAACATGA AACATCGGTG TAGTTCTACT ACTTCTAGTG ATGATAACTG
(27). ACATCGGCCG AGGTCTTTAC TACGGCTCTT ACCTTTACAA AGAAACATGA AACATTGGTG TAGTACTTCT ACTCCTAGTA ATGATAACCG
(28). ATATTGGCCG AGGTCTCTAC TACGGTTCCT ACCTCTACAA AGAAACATGA AACATCGGTG TAGTCCTACT TCTCCTAGTA ATGATGACTG
(29). ACATCGGCCG AGGCCTTTAC TACGGCTCTT ACCTCTACAA AGAAACATGA AACATCGGAG TAGTACTCCT ACTTCTAGTT ATGATGACCG
(30). ACATCGGCCG AGGACTTTAC TACGGCTCTT ACCTATACAA GGAAACATGA AACATCGGAG TAGTACTCCT ACTCCTAGTT ATGATGACCG
(31). ACATCGGCCG AGGACTTTAC TACGGCTCTT ACCTATACAA AGAAACATGA AACATCGGAG TAGTACTCCT ACTCCTAGTT ATGATAACCG
(32). ACATCGGCCG AGGACTTTAC TACGGCTCTT ACCTATACAA AGAAACATGA AACATCGGAG TAGTACTACT ACTCCTAGTT ATGATGACCG
(33). ACATCGGCCG AGGACTTTAC TACGGCTCTT ACCTATACAA AGAAACATGA AACATCGGAG TAGTACTCCT ACTCCTAGTT ATGATGACCG
(34). ACATTGGCCG AGGTCTTTAC TATGGCTCCT ATCTATACAA AGAAACATGG AATATTGGAG TTGTTCTCCT TCTCCTAGTA ATGATGACCG
(35). ATATTGGCCG AGGCCTCTAC TACGGCTCTT ACCTATATAA AGAAACATGA AACGTGGGTG TCGTCCTCCT ACTTCTAGTC ATGATAACCG
(36). ATATTGGTCG AGGCCTCTAC TATGGCTCTT ACCTATATAA AGARACATGA AACGTAGGTG TCGTCCTCCT ACTCCTAGTC ATGATAACCG
(37). ATATCGGTCG AGGCCTCTAC TATGGCTCTT ACCTATATAA AGAAACATGA AACGTGGGYG TCGTCCTTCT ACTCCTAGTC ATGATAACCG
(38). ATATCGGTCG AGGCCTCTAC TATGGCTCTT ACCTGTATAA AGAGACATGA AACGTAGGTG TAGTCCTCCT ACTTCTAGTC ATGATAACCG
(39). ACATCGGTCG GGGCCTCTAC TATGGCTCTT ACCTGTATAA AGAGACATGA AACGTGGGTG TCGTCCTCCT ACTCCTAGTC ATGATAACCG
(40). ATATCGGTCG AGGCCTCTAC TATGGCTCTT ACCTGTATAA AGAGACATGA AACGTAGGTG TAGTCCTCCT ACTTCTAGTC ATGATAACCG
(41). ACATCGGTCG GGGCCTCTAC TATGGCTCTT ACCTGTATAA AGAGACATGA AACGTGGGTG TCGTCCTCCT ACTCCTAGTC ATGATAACCG
(42). ACATCGGTCG GGGCCTCTAC TATGGCTCTT ACCTGTATAA AGAGACATGA AACGTGGGTG TCGTCCTCCT ACTCCTAGTC ATGATAACCG
```

Figure 4. *Continued*

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        3  3  3 2 3  31 31 3   3     3   3  3  3   3     3
 (1). CTTTTGTTGG ATATGTACTT CCATGAGGAC AAATATCATT CTGAGGGG
 (2). CCTTCGTAGG C--------- ---------- ---------- --------
 (3). CCTTCGTGGG C--------- ---------- ---------- --------
 (4). CCTTT----- ---------- ---------- ---------- --------
 (5). CCTTTGTTGC CTACGTTCTC CCTTGAGGAC AAATATCTTT TTGAGGGG
 (6). CCTTTGTCGC CTATGTCTTT CCATGAGGAC AAATGTCATT CTGAGGAG
 (7). ---------- ---------- ---------- ---------- --------
 (8). ---------- ---------- ---------- ---------- --------
 (9). ---------- ---------- ---------- ---------- --------
(10). ---------- ---------- ---------- ---------- --------
(11). CTTTCGTAGG TTATGTCC-- ---------- ---------- --------
(12). ---------- ---------- ---------- ---------- --------
(13). CCTTTGTTGC GTACGTTCTC CCTTGAGGAC AAATGTCATT CTGAGGGG
(14). CATTCGTCGC CTACGTTCTT CCCTGAGGAC AAATGTCATT CTGAGGGG
(15). CCTTTGTTGC CTACGTACTT CCTTGAGGAC AAATATCATT CTGAGGAG
(16). CCTTCGTCGC CTATGTACTT CCATGAGGAC AAATGTCATT CTGAGGTG
(17). CCTTCGTAGC ATACGTCCTT CCCTGAGGAC AAATATCATT CTGAGGCG
(18). CATTCGTGGC CTACGTACTC CCCTGAGGAC AAATRTCATT CTGAGGAG
(19). CATTCGTCGC CTACGTCCTT CCCTGAGGTC AAATGTCCTT CTGAGGGG
(20). CCTTCGTTGC CTACGTCCTT CCCTGAGGAC AAATGTCCTT CTGAGGAG
(21). CCTTCGTAGC CTACGTTCTC CCCTGAGGAC AAATGTCATT TTGAGGTG
(22). CCTTCGTTGC CTACGTCCTT CCCTGAGGAC AGATATCATT TTGAGGGG
(23). CTTTCGTCGC GTACGTTCTT CCCTGAGGAC AGATGTCCTT CTGAGGGG
(24). CATTCGTCGC CTACGTTCTT CCATGAGGAC AAATGTCATT TTGAGGTG
(25). CATTCGTCGC CTACGTACTT CCATGAGGAC AAATGTCATT TTGAGGTG
(26). CCTTTGTAGC CTACTTACTC CCCTGAGGAC AAATGTCCTT CTGAGGTG
(27). CCTTCGTCGC CTACTTACTY CCYTGAGGAC AAATGTCMTT CTGAGGTG
(28). CCTTCGTCGC CTACGTACTT CCCTGAGGGC AAATGTCTTT CTGAGGAG
(29). CCTTCGTTGG CT-------- ---------- ---------- --------
(30). CCTTCGTTGG CT-------- ---------- ---------- --------
(31). CCTTCGTTGC CTACGTTCTT CCCTGAGGAC AAATGTCTTT CTGAGGAG
(32). CCTTCGTTGG CT-------- ---------- ---------- --------
(33). CCTTCGTTGG CT-------- ---------- ---------- --------
(34). CTTTCGTAGG TTATGTATTA CCCTGAGGCC AAATATCTTT CTGAGGCG
(35). CCTTCGTCGG CTATGTCCTC CCCTGAGGAC AAATATCCTT CTGAGGTG
(36). CCTTCGTCGC CTACGTCCTC CCCTGAGGAC AAATGTCCTT CTGAGGTG
(37). CCTTCGTCGC CTACGTCCTC CCCTGAGGAC AAATGTCCTT CTGAGGTG
(38). CCTTCGTCGC CTACGTCCTC CCCTGAGGAC AAATGTCCTT CTGAGGTG
(39). CCTTCGTCGC CTACGTCCTT CCCTGAGGAC AAATATCCTT CTGAGGTG
(40). CCTTCGTCGC CTACGTCCTC CCCTGAGGAC AAATGTCGTT CTGAGGTG
(41). CCTTCGTCGC CTACGTCCTT CCCTGAGGAC AAATATCCTT CTGAGGCG
(42). CCTTCGTCGC CTACGTCCTT CCCTGAGGAC AAATATCCTT CTGAGGTG
```

cobia cytochrome *b* with the 41 species, all of the sequences were invariant at 56% of the nucleotide positions. The differences were all base substitutions and represented 21%, 10%, and 69% first, second, and third position changes, respectively. There were no insertions or deletions in the cytochrome *b* gene among any of the species. Transversions occurred at a moderately higher frequency than transitions, 58% versus 42%, respectively. Transitions between pyrimidines occurred at a much higher frequency than transitions between purines, 75% versus 25%, respectively.

There were six nucleotide positions at which cobia were unique when compared to all other species (Table 5). For example, the cobia sequence was unique at position 162 in that it had a **G**, whereas all the others had an **A**. The presence of either A or G at this position did not alter the amino acid which coded for the corresponding codon. The nucleotide triplets TGA/TGG both code for tryptophan. However, position 166 is a different case. The cobia sequence was unique in that it had a **C** at this position, whereas all the others had an **A**. This first position change resulted in an amino acid change at the corresponding codon. The cobia nucleotide triplet **CTT** codes for leucine while the other species have the triplets **ATT/ATC**, both of which code for isoleucine. The cobia sequence was also unique at position 225 in that it had a **C**, whereas all the others had a **T**. The cobia triplet was **ACC**, which codes for threonine, while the other species were **ATC/ATT**, **GTA/GTG/GTC**, or **CTC/CTT**, which code for isoleucine, valine, or leucine, respectively. Similar cases are observed at positions 298 and 304. Cobia had a unique nucleotide at both of these positions when compared to the rest of the species. The amino acids at these positions were also unique when compared to the other species. Dashes under the amino acid column for position 305 are shown because positions 304 and 305 are associated with the same nucleotide triplet.

The cobia cytochrome *b* sequence is the most similar (83% & 92%), nucleotide and amino acid, respectively, to the consensus sequence of

Table 5. Nucleotides at positions which were unique to *R. canadum* when compared to the other 41 species of fish. The inferred amino acids for these positions are also shown.

	Position					
	162	166	225	298	304	305
	Nucleotides					
Cobia	G	C	C	C	G	G
Other species	A	A	T	A/G	A	T
	Corresponding Amino Acids					
Cobia	Trp	Leu	Thr	Leu	Gly	-
Other species	Trp	Ile	Ile Val Leu	Thr Val	Met Ile	- -

the family Serranidae (sea basses). The pompano dolphin *Coryphaena equiselis* and black cichlid *Cichlasoma nicaraguense* consensus sequences are the next most similar, 81% & 90% and 80% & 91%, respectively. The species most dissimilar to cobia are the Atlantic mackerel (*Scomber scombrus*) and skipjack tuna (*Katsuwonus pelamis*), 72% & 84% and 72% & 86% similar to the cobia sequence, respectively.

DISCUSSION

A 358 base pair region of the mitochondrial cytochrome *b* gene of two fish was sequenced. The singular nucleotide substitution, a transition located at base 186, was a third-position silent change and did not result in a change in the amino acid sequence. This pattern of nucleotide substitution has been seen in other fish cytochrome *b* genes. Bartlett and Davidson (1991) examined nucleotide sequence differences between four species of tunas. Of the differences observed, 95% were third position changes, none of which resulted in a change in the amino acid sequence. The same types of changes were also seen in Atlantic cod (Carr and Marshall 1991). Third position substitutions accounted for 82% of the nucleotide changes. Of these changes, 78% were silent mutations and did not change the amino acid sequence. The occurrence of third-position, silent nucleotide substitutions over second and first position substitutions, as observed here, agrees with the expected substitutional pattern for a protein-coding mitochondrial gene (Kocher et al. 1989, Irwin et al. 1991) and has been seen in other fish cytochrome *b* genes (Kocher et al. 1989, McVeigh et al. 1991, Finnerty and Block 1992, Hartley et al. 1992, Block et al. 1993).

Beckenbach et al. (1990) examined the intraspecific sequence variation in the mtDNA of rainbow trout *Oncorhynchus mykiss* and found that transitions between purines (67%) predominated over transitions between pyrimidines (33%). In deer of the genus *Odocoileus*, however, transitions between pyrimidines (92%) were much more common than transitions between purines (8%) (unpublished data cited in Carr and Marshall 1991). If there is a bias toward transitions between purines in fish and transitions between pyrimidines in mammals, cobia do not support this supposition. In the cobia sequence, 75% of the transitions were between pyrimidines. Since the cobia cytochrome *b* sequence is comprised of 60% pyrimidines and 40% purines, this bias may account for predominant transitional changes.

Bartlett and Davidson (1991) used PCR and direct nucleotide sequencing to examine a segment of the mitochondrial cytochrome *b* gene from four species of tuna: bluefin *Thunnus thynnus*, bigeye *Thunnus obesus*, yellowfin *Thunnus albacares*, and albacore *Thunnus alalunga*. They detected intraspecific sequence variation within each of the species. More importantly, they observed specific sequence differences between the four species which could be used to determine the identity of an individual tuna with a high degree of confidence. For example, bigeye tuna had a T at position 35 whereas the other three species all had a G. There were six positions along the cytochrome *b* sequence at which nucleotide differences were used to identify a particular species from the other three species. A similar, but unconfirmed, condition may also be present in cobia. There were six nucleotide positions at which the cobia sequence had a unique nucleotide present when compared to the other 41 species. These positions have the potential to be used as species specific genetic markers for cobia. However, it must first be demonstrated that these positions are indeed unique to cobia and are not being obscured as a result of only consensus sequences shown for each species in Figure 4.

The singular base substitution which was observed at position 186 can not be used categorically to imply that these two fish represent different stocks. Although Carr and Marshall (1991) and Finnerty and Block (1992) used single base substitutions to identify possible distinct stocks of Atlantic cod *Gadus morhua* and blue marlin *Makaira nigricans*, respectively, the sample sizes used in those studies, 55 and 26 fish, respectively, were much larger than in the present study. Additionally, rather than using a singular nucleotide at a particular site to determine separate stocks, those studies used all the information available over the entire sequence of the gene to support the probability that separate stocks existed. The present study would need to examine more fish in order to determine whether these two fish represent two separate stocks. Variables such as sex, catch location,

month of capture, and reproductive stage should also be examined in order to determine if there is correlation between any of these variables and the cytochrome *b* gene nucleotide sequence.

The life history and evolutionary relationship of cobia with other groups of fishes has not been thoroughly investigated. The family Rachycentridae has been placed close to the dolphins (family Coryphaenidae), jacks and pompanos (family Carangidae), and remoras (family Echeneididae) based on larval morphology (Nelson 1976, Moser et al. 1984). There is a stronger argument for a relationship between Rachycentridae and Coryphaenidae based on morphology of larvae (Moser et al. 1984); both families have identical patterns of larval head spination. The results of the present study indicate that cobia and dolphin have similar cytochrome *b* nucleotide sequences. There was an 81% similarity in nucleotide sequence and a 90% similarity in amino acid sequence between these two species. The amino acid substitutions were not radical (Grantham 1974); they were amino acids which were similar in physical and chemical characteristics. For example, neutral amino acids replaced neutral amino acids 100% of the time. However, the Serranid sequence is the most similar to the cobia cytochrome *b* nucleotide sequence. There was an 83% similarity in nucleotide sequence and a 92% similarity in amino acid sequence between these two species. Again, the amino acid substitutions were not radical. Based on this information, it can be proposed that there is a closer evolutionarily relationship between cobia and the sea basses. While the pompano dolphin, sea bass, and cobia are all included in the suborder Percoidei, the similarity in larval morphology between cobia and dolphin supports a much stronger relationship between these two species than between cobia and sea basses. Additionally, the cytochrome *b* gene represents only a very small portion (~2.0%) of the mitochondrial genome. A more accurate estimate of the evolutionary relationship of cobia with other species within the suborder Percoidei should encompass sequencing other regions of the mitochondrial genome such as the 12S rRNA or D-loop regions.

Nucleotide substitutions in these regions, in addition to the cytochrome *b* gene, would provide a more complete description of the degree of nucleotide similarity, and therefore evolutionary relatedness, between cobia and other species of fish within the suborder Percoidei.

Sequence variation observed in this study may be an authentic polymorphism, or may be an artifact introduced by errors in the PCR, cloning, or sequencing processes. The *AmpliTaq* DNA polymerase used in PCR amplification is known to have single base substitution error rate between 1.1×10^{-4} to 2.0×10^{-4} (Innis et al. 1988, Saiki et al. 1988, Tindall and Kunkel 1988, Innis et al. 1990). This error rate is due to several factors. *AmpliTaq* DNA polymerase lacks a 3'→ 5' exonuclease proofreading activity (Innis et al. 1988). Damage to the template DNA and degradation of the *AmpliTaq* DNA polymerase may also occur during the repeated cycling to high temperatures (Tindall and Kunkel 1988, Innis et al. 1990, Rychlik et al. 1990). These effects may result in artifactual sequence variation if replication errors occur in early rounds of amplification. Thermal effects were minimized by reducing the time of the denaturing step and reducing the number of thermal cycles. Mutations introduced during the cloning and sequencing processes could also contribute to sequence variation and have a cumulative effect (Pääbo and Wilson 1988). The absolute authenticity of sequence variants can only be verified by reamplifying, recloning, and resequencing both strands from the original DNA source.

The preliminary results from this study indicate that there is a degree of intraspecific variation within cobia. In order to thoroughly investigate this degree of intraspecific variation, a much larger sample size must be examined. An even more complete picture of genetic variation in cobia should also include an examination of the nuclear genome. PCR and nucleotide sequencing is applicable to any stretch of DNA. This technology has the potential to increase our understanding of the population structure of cobia and ultimately determine whether distinct breeding subpopulations exist in the northern Gulf of Mexico.

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