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Application of genetic markers to provide species identification and define stock structure: Analyses of selected marine fishes of the Mid -Atlantic Bight

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APPLICATION OF GENETIC MARKERS TO PROVIDE SPECIES IDENTIFICATION AND DEFINE STOCK STRUCTURE: ANALYSES OF SELECTED MARINE FISHES OF THE MID-ATLANTIC BIGHT

A Dissertation Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Doctor of Philosophy

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by

Jan F. Cordes

2000

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APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

Jan F. Cordes

Approved, March 2000

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This dissertation is dedicated to my wife, Laura, and my son, Jakob. It might still have been possible to complete it without their constant patience, love, and understanding, but it would not have meant nearly as much.

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ABSTRACT

Molecular markers and techniques were employed to develop a genetic key for the forensic identification of 16 species of Chesapeake Bay sportfishes and to investigate the stock structure of one of those species, the weakfish *Cynoscion regalis*.

To develop the genetic key, regions within the ATP 6 synthetase (ATPase 6), cytochrome *b*, cytochrome *c* oxidase I, NADH dehydrogenase 4 (ND4), and 12S/16S ribosomal RNA mitochondrial genes were amplified using the polymerase chain reaction (PCR) and digested with a bank of restriction endonucleases to find a genetic marker that exhibited complete interspecific differentiation and low intraspecific variation. The final key was based on an approximately 1495 bp region of the mitochondrial genome encompassing part of the 12S and 16S rRNA genes. Complete separation of all 16 species was accomplished by restriction digestions with the single endonuclease *Rsa* I. Intraspecific variation in digestion patterns was minimal, with ten species exhibiting a single pattern, while the remaining six were dimorphic. This key should prove useful in enforcement of species-specific regulations when external morphological characters have been removed, and in the identification of early life history stages that lack distinguishing characteristics.

Analyses of four microsatellite loci and two nuclear intron regions were used to investigate the genetic basis of population structure of weakfish along the U.S. East

Coast. Samples of approximately fifty young-of-the-year (YOY) weakfish were collected at five locations (Georgia, North Carolina, Virginia, Delaware, and New York) in each of two years (1996 and 1997). Mean expected heterozygosities for the microsatellite loci ranged from a low of 8.5% for the SOCO14 marker to a high of 92.8% for the CNE612 locus. Mean expected heterozygosities for the CRESIA1 and RP2 intron regions were 5.1% and 24.0%, respectively. None of the sample genotype distributions differed significantly from Hardy-Weinberg expectations, and pairwise F_{ST} values were consistently low (0.000-0.087 for microsatellite loci, 0.000-0.050 for intron regions). Analyses of molecular variance (AMOVA) and exact *F* permutation tests of sample heterogeneity were nonsignificant for all loci; thus it was not possible to reject the null hypothesis that weakfish comprise a single, homogeneous stock.

Some individuals in the Georgia 1997 sample exhibited unusually small microsatellite allele sizes when compared to the rest of the sample locations. Evaluation of these specimens using the previously developed genetic key based on restriction fragment length polymorphism (RFLP) analysis of the 12S/16S rRNA region of the mitochondrial genome revealed that two other species of *Cynoscion*, the sand seatrout *C. arenarius* and the silver seatrout *C. nothus*, had been inadvertently included in the sample of YOY weakfish. Based on data from the mitochondrial marker and the SOC050 microsatellite locus, a number of the Georgia 1997 fish were identified as hybrid offspring of weakfish and sand seatrout.

APPLICATION OF GENETIC MARKERS TO PROVIDE SPECIES IDENTIFICATION AND DEFINE STOCK STRUCTURE: ANALYSES OF SELECTED MARINE FISHES IN THE MID-ATLANTIC BIGHT

GENERAL INTRODUCTION

Advances in molecular genetics over the last four decades have provided an increasing number of molecular markers available for use in fisheries science research. These markers have been applied to problems of taxonomy, species identification, stock structure, mixed-stock fishery composition, genetic interactions of hatchery and wild populations, hybrid zones, and the conservation of endangered stocks (Ryman and Utter 1987, Wirgin and Waldman 1994, O'Connell and Wright 1997). Molecular techniques offer a different perspective than traditional methodologies based on meristics, morphometrics, or life history traits because they directly assess the genetic differences, and not the phenotypic expression of genetic differences. To select which types of molecular markers and techniques are best suited to a particular problem in fisheries science, a researcher must consider the taxonomic unit of interest, the unique biological characteristics of each class of molecular marker, and the ability of different analytical techniques to reveal genetic variation in a given class of marker.

Classes of Molecular Markers

Most problems in fisheries science investigated with molecular genetic markers require discrimination between genetic units at some level of taxonomic hierarchy,

whether among individuals, populations, or species. This is true for investigations of parentage, inbreeding, genetic diversity in wild and hatchery-reared fish, population structure, species designations, and interspecific hybridization. Because molecular markers differ in mutation rates, modes of inheritance, and visibility to selection, they often reveal different levels of genetic variation within samples as well as genetic divergence between samples. No molecular markers is useful at all hierarchical levels, and it cannot be assumed that one class of marker is best at any given level, since the amount of genetic variation in a given marker can vary widely across taxa. The most common classes of molecular markers used in fisheries genetics studies are proteins (allozymes), mitochondrial (mt) DNA, and nuclear (n) DNA. Below is a brief description of each marker class and the analytical techniques used to assess the variation they contain.

Allozymes. Since the 1960s, allozyme starch gel electrophoresis has been the most commonly employed molecular method in fishery genetics (Hillis and Moritz 1996), and it is still in widespread use. Allozymes are allelic variants of proteins produced by a single gene locus. Amino acid differences in the polypeptide chains of the different allelic forms reflect changes in the underlying DNA sequence. Depending on the nature of the amino acid changes, the resulting protein products may migrate at different rates (due to charge and size differences) when run through a starch gel subjected to an electrical field. Differences in the presence/absence and relative frequencies of alleles are used to quantify genetic variation and distinguish among genetic units at the levels of populations, species, and higher taxonomic designations.

Allozyme electrophoresis is still one of the most cost-effective methods of genetic analysis available to researchers. The four primary methods of protein electrophoresis can be classified according to the gel medium: starch, polyacrylamide, cellulose acetate, and agarose. Starch gel electrophoresis (outlined above) is the most commonly used protocol to detect allelic variants in population genetic studies. Starch gels may be run horizontally or vertically. Although more costly in terms of supplies, sample quantities, and sample processing efficiency, vertical starch gel electrophoresis avoids electrodecantation, a drawback of horizontal gels in which high molecular weight proteins tend to settle toward the bottom of the gel as electrophoresis progresses (Murphy et al. 1996).

Proteins may also be separated by polyacrylamide gel electrophoresis (PAGE). The ability to accurately assess protein size by manipulating the acrylamide concentration in the gel makes this technique popular with laboratories involved in nucleic acid sequencing (Chrambach and Rodbard 1971). Cellulose acetate gel electrophoresis (CAGE) has also been used for the separation of proteins because it increases the repeatability of experiments (Harris and Hopkinson, 1976), but may not detect as much variation because the large pore size results in separation based on charge alone (Riley et al. 1992).

Agarose gel electrophoresis (AGE), also popular in mitochondrial and nuclear DNA studies, has its roots in protein analysis. Because of a relatively high concentration of acidic groups, however, AGE may result in electroendoosmosis, a 'backwash' of buffer caused by gel charge groups that accelerates the mobility of cationic enzymes while retarding or reversing the mobility of anionic enzymes (Murphy et al. 1996). The relative advantages and disadvantages of the four protein separation methods outlined above are

given in Table 1 of Murphy et al (1996). Other, less commonly used methods of protein electrophoresis include immunoelectrophoresis (Harris and Hopkins 1976), two-dimensional electrophoresis (Hames and Rickwood 1981), isoelectric focusing (Whitmore 1990), and paper electrophoresis (Freifelder 1992).

An advantage of allozymes markers is the relative ease with which a large number of loci and samples can be screened; at least 75 isozyme systems representing several hundred genetic loci are currently available (Murphy et al. 1990). Also, because the isozyme systems are coded for by nuclear loci, complications resulting from reduced effective population size and gender-biased migration inherent in mtDNA markers are avoided. Disadvantages associated with allozymes include the presence of null (enzymatically inactive) alleles that can produce heterozygote deficiencies, the effects of natural selection on protein-coding regions of DNA (most population structure analyses are based on assumptions that markers are selectively neutral), and the amount and quality of tissue samples required. In addition, most nucleotide changes do not produce electrophoretically distinct alleles, resulting in lower levels of detected variation. Some changes in nucleotide sequence do not change the encoded amino acid (synonymous substitutions), and some amino acid changes do not alter the mobility of the protein in an electrophoretic gel (silent substitutions). Low levels of genetic variation revealed in many allozyme studies of marine fish populations (e.g. striped bass, Siddell et al. 1980; Atlantic cod, Mork et al. 1985; weakfish, Crawford et al. 1989) have prompted continued search for markers with greater genetic resolution.

Mitochondrial DNA. Since the late 1970s, analyses of DNA have become increasingly prevalent in the field of fisheries genetics. The most frequently studied marker has been mitochondrial DNA, a small, circular molecule contained within the mitochondrion. In general, animal mtDNA is 15-20 kilobases (kb) in size and consists of about 37 genes coding for 22 transfer (t) RNAs, 13 messenger (m) RNAs, and 2 ribosomal (r) RNAs. Almost the entire mtDNA molecule is transcribed except for the approximately 1 kb control region (D-loop), where replication and transcription of the molecule is initiated. Studies in the early 1980s revealed that despite striking conservation of mtDNA function and gene arrangement in animals, the molecule showed high levels of sequence diversity at the species and population levels (Brown 1985). In general, noncoding segments like the D-loop exhibit elevated levels of variation relative to coding sequences such as the cytochrome *b* gene (Brown et al. 1993), presumably due to reduced functional constraints and relaxed selection pressure. Thus, analysis of the mtDNA molecule, through careful targeting of specific regions with different amounts of sequence conservation, can be used to investigate genetic variation and divergence at a number of taxonomic levels. Analyses of the mitochondrial D-loop region have been used to investigate stock structure in a variety of marine fishes (Cronin et al. 1993, Purcell et al. 1996). Higher-level taxonomic questions are often addressed with analyses of more conserved gene regions like cytochrome *b* (Bernardi and Crane 1999), 12S rRNA (Sarver et al. 1996), 16S rRNA (Birstein et al. 1997), NADH dehydrogenase subunit 3 (Mckay 1996), and ATP synthetase 6 (Domanico et al. 1997). The conservative nature of one gene region may differ among taxa, and all of the markers listed above have been used to investigate taxonomic questions both above and below the species level.

Studies of vertebrate species have generally shown that sequence divergences accumulate more rapidly in mtDNA than in nuclear DNA (Brown 1985). This has been attributed to a faster mutation rate in mtDNA that may result from a lack of repair mechanisms during replication (Wilson et al. 1985) and smaller effective population size due to the strictly maternal inheritance of the mitochondrial genome (Birky et al. 1989).

MtDNA can be analyzed using a variety of methods. Originally whole molecule mtDNA was isolated from individuals using cesium chloride density-gradient ultracentrifugation (Lansman et al. 1981) and variation was assessed using restriction fragment length polymorphism (RFLP) analysis. Restriction endonucleases are enzymes that recognize specific nucleotide sequences and cut DNA wherever these sequences are encountered. Each restriction enzyme recognizes a specific 4, 5, or 6 bp sequence, so that changes in the DNA due to mutation can result in the gain or loss of a restriction site. Because of differences in mtDNA sequences, digestion with restriction enzymes can result in mtDNA fragments whose number and size vary among individuals. MtDNA fragments for each individual are separated by using agarose gel electrophoresis and visualized with ethidium bromide (EtBr) staining or autoradiography (Sambrook et al. 1989). Alternatively, whole genomic DNA can be isolated (as opposed to purified), digested with restriction enzymes, and the fragments separated electrophoretically. The resulting mtDNA bands are visualized either immunologically or autoradiographically by hybridization with a labeled mtDNA probe on a nylon or nitrocellulose filter (Southern blotting; see Sambrook et al. 1989 for protocols). Differences in the banding patterns between individuals due to the gain or loss of a restriction site can be used to assess levels of genetic variation and relatedness at various hierarchical levels. Problems with

whole molecule mtDNA analyses include the amount and quality of tissue required for mtDNA isolation, the time involved in mtDNA isolation and development of a probe for Southern blotting, and the special requirements of working with radioisotopes (Sambrook et al. 1989).

The problems associated with traditional whole molecule mtDNA analysis can be alleviated using a system based on the polymerase chain reaction (PCR). With PCR, large amounts of DNA can be amplified from minute tissue samples preserved in a variety of manners and isolated with a standard phenol extraction protocol (Sambrook et al. 1989) that is relatively short compared to the cesium chloride procedure mentioned above. Also, with the large number of 'universal' primers available in the literature, a researcher can target regions of mtDNA that are either relatively conserved or rapidly evolving, depending on the amount of variation observed and the taxonomic level under examination. Finally, PCR products can be digested with restriction enzymes and visualized by simple staining with ethidium bromide due to the increased amount of DNA produced by the PCR method.

In those cases where RFLP analysis fails to uncover levels of variation suitable to test the hypothesis in question, techniques with finer resolution can be employed. Short fragments of PCR-amplified DNA can be compared using single-strand conformational polymorphism (SSCP) analysis (Orita et al. 1989). SSCP analysis is capable of detecting differences as little as a one base-pair substitution (Aguade et al. 1994, Orti et al. 1997), although this resolution can vary greatly for different regions of DNA (Moyret et al. 1994). SSCP analysis takes advantage of the conformational differences in single-stranded DNA (associated with changes in sequence) when run through a denaturing

polyacrylamide gel. Disadvantages of SSCP analysis include the increased cost and technical intricacy compared to traditional agarose gel/ethidium bromide staining techniques.

The finest level of genetic resolution can be achieved by sequencing regions of cloned or amplified mtDNA. Sequencing a large number of samples (as in population structure studies) can require considerable time and money, but more and more studies are using this approach. Population-level analyses of marine fishes have been performed based on sequences of the control (D-loop) region (Alvarado-Bremer et al. 1996, Stepien and Faber 1998, Duvernell and Turner 1998, Refseth et al. 1998), cytochrome *b* gene (Carr et al. 1995, Apostolidis et al. 1997, Grant et al. 1998), and ATP synthesase 6 gene (Quattro and Powers 1994). Sequencing as a tool in intraspecific studies will probably continue to grow in popularity, as the use of timesaving devices such as automated sequencers becomes more widespread.

An advantage of mtDNA markers over protein electrophoresis is the small amount of tissue required for mtDNA isolation, particularly when coupled with PCR protocols (discussed above). Two potential disadvantages of mtDNA as a molecular marker result from its mode of inheritance. Because all 37 genes contained within the mitochondrial genome are inherited as a single unit without recombination, the mtDNA molecule must be considered a single locus in genetic investigations (Avise 1994), compared to the 30-50 loci typically employed in protein electrophoresis studies. Also, because mtDNA is maternally inherited, the phylogenies and population structures derived from mtDNA data may not reflect those of the nuclear genome due to gender-biased migration (Birky et al. 1983) or introgression (Chow and Kishino 1995). Maternal inheritance of the

mtDNA molecule can be an asset, however. Studies of sunfish *Lepomis sp.* (Avise and Suanders 1984), chubs *Gila sp.* (Demarias et al. 1992), and char *Salvelinus sp.* (Bernatchez et al. 1995), have used a combination of nuclear and mitochondrial markers to reveal instances of introgressive hybridization between species. Similarly, a combination of nuclear and mitochondrial markers has been used to investigate gender-specific spawning site fidelity in marine turtles (Bowen et al. 1992, Karl et al. 1992) and whales (Palumbi and Baker 1994).

Nuclear DNA. In an effort to find molecular markers that combine the advantage of the fast rates of mutation and sequence divergence of mtDNA with the number of independent loci available for isozyme analysis, researchers have increasingly turned to studies of nuclear genes. In general, the eukaryotic genome can be divided into nonrepetitive and repetitive DNA. Nonrepetitive DNA may be coding or noncoding, and the two types often coexist within a single gene. Genes that code for proteins or RNA consist of nonrepetitive DNA, and noncoding segments (introns) often interrupt coding regions (exons) within the gene. Repetitive DNA is noncoding, and can account for up to 50% of the genome in higher animals (Lewin 1997). Repetitive DNA is often arranged into tandemly repeated units of short DNA sequences, with the size of the repeated unit ranging from as little as 2 base pairs (bp) up to 30 bp or more in length. Both introns and repetitive DNA have been shown to be highly variable compared to coding regions of DNA, a difference that may be due to relaxed selective constraints on non-coding regions, and, in the case of some repetitive DNA (discussed below), to unique characteristics of its mode of replication. Types of nuclear DNA markers that target

nonrepetitive DNA include randomly amplified polymorphic DNA (RAPDs), anonymous single copy nuclear DNA (ascnDNA), and noncoding regions (introns, transcribed, and nontranscribed spacers) of functional genes. Nuclear markers that exploit regions of repetitive DNA with a variable number of tandem repeats are known as VNTRs.

RAPD markers are developed by using the PCR to randomly amplify anonymous segments of nDNA with an identical pair of primers 8-10 bp in length. Because the primers are relatively short, the likelihood of amplifying multiple products is great, with each product (presumably) representing a different locus. Genetic variation and divergence within and between the taxa of interest are assessed by the presence or absence of each product, which is dictated by changes in the genetic sequence at each locus. Because most of the nuclear genome in vertebrates is noncoding (Wirgin and Waldman 1994), it is presumed that most of the amplified loci will be selectively neutral.

RAPDs have all the advantages of a PCR-based marker, with the added benefit that primers are commercially available and do not require prior knowledge of the target DNA sequence or gene organization. Multilocus amplifications can be separated electrophoretically on agarose gels and stained with ethidium bromide (Lasker et al. 1996), although higher resolution of bands has been achieved with discontinuous polyacrylamide gel electrophoresis (dPAGE) and silver staining (Dinesh et al. 1995), a somewhat costlier and more labor-intensive method.

Other advantages of RAPDs are the ease with which a large number of loci and individuals can be screened. RAPDs have been used to investigate genetic linkage maps, cryptic species, hybridization, and population structure in marine algae (Van Oppen et al. 1996), corals (Lasker et al. 1996), mollusks (Crossland et al. 1993), vascular plants

(Stiller and Denton 1995), mammals (Kappe et al. 1995), and fishes (Dinesh et al. 1995). Shortcomings of this type of marker include the difficulty of demonstrating Mendelian inheritance of the loci and the inability to distinguish between homozygotes and heterozygotes. In addition, the presence of paralogous PCR products (different DNA regions which have the same lengths and thus appear to be a single locus), limit the use of this marker to closely related taxa. These difficulties have so far conspired to limit the application of this marker within fisheries science (Wirgin and Waldman 1994).

Another method of exploiting the genetic variation found in nDNA involves the development of anonymous single-copy nuclear DNA (ascnDNA) markers. In this case nDNA from the target species is isolated, digested with restriction enzymes, and a selected size range of fragments is inserted into plasmid or viral vectors, creating a DNA library. Random fragments are cloned into a bacterial host, multiplied, and sequenced. PCR primers are then designed from the sequenced fragments and used to amplify putatively single-copy loci. As with all PCR-based markers, analysis of anonymous scnDNA requires small amounts of sample tissue and a simplified DNA isolation protocol. Development of the necessary primers can be time consuming (discussed above), but once the primers have been designed, screening of large numbers of samples is accomplished through RFLP analysis. Again, because of the relatively large amounts of DNA amplified by the PCR method, digestions are run out on an agarose gel and visualized by ethidium bromide staining. As with mtDNA markers, finer resolution of alleles can be obtained by SSCP analysis or sequencing (see above). AscnDNA markers suffer from some of the same limitations seen in many PCR-based systems such as the presence of null alleles (i.e. mutation in the primer binding site that results in a lack of

PCR product), paralogous PCR products, and non-Mendelian inheritance (see Hu and Foltz 1996, Foltz and Hu 1996, for discussion of these pitfalls in the American oyster, *Crassostrea virginica*). Although startup time in terms of creating a DNA library, sequencing, designing primers, and finding polymorphic loci can be daunting, once polymorphic loci have been developed, screening of individuals is relatively rapid (Wirgin and Waldman 1994). Wirgin and Maceda (1991) found that RFLP analysis of ascnDNA revealed approximately an order of magnitude more genetic variation than RFLP analysis of mtDNA in the striped bass *Morone saxatilis*, although Leclerc et al. (1996) found uniformly low variation at 13 randomly amplified loci in the same species. A comparison of markers used to elucidate population structure in the blue marlin *Makaira nigricans* found comparable levels of variation at allozyme and ascnDNA loci, in contrast to much higher levels for mtDNA (Buonaccorsi et al. 1999).

Another type of single-copy nuclear marker takes advantage of the unique sequence properties of some functionally described genes. These genes contain highly conserved, coding (exon) regions that flank highly variable, noncoding (intron) regions. The conservative flanking regions have been used to design primers that will amplify introns across a wide range of taxa (Slade et al. 1993). These exon-primed, intron-crossing (EPIC) amplifications reveal considerable polymorphism at the population and species levels. By using universal primers from the literature to amplify, sequence, and design species-specific primers, researchers can dispense with genomic library construction and move relatively quickly to the screening of samples (Slade et al. 1993).

ScnDNA markers developed from genes with known functions share many of the same advantages and disadvantages of anonymous scnDNA. Assuming that primers

bracketing a sequence of interest are already available from another organism, the initial screening and sequencing of clones to design primers is eliminated, although the DNA produced by PCR amplification with 'universal' primers should be cloned and sequenced to validate that the correct gene has been amplified. In the case of multiple products, the primers have to be redesigned to produce a single product. Also, care should be taken to anchor primers in conserved regions to minimize the problem of null alleles. As with anonymous scnDNA markers, allele discrimination is accomplished through RFLP analysis (in conjunction with agarose or SSCP gels) or sequencing.

Slade et al. (1993) investigated the usefulness of introns within the histone H2AF, myoglobin, major histocompatibility complex (MHC) DQA, and aldolase (ALD) genes in assessing population-level variation across a diverse set of taxa. This type of nuclear marker has been used in only a few population-level studies of marine organisms, including cetaceans (Palumbi and Baker 1994), bivalves (Corte-Real et al. 1994), Pacific salmon (Moran et al. 1997), and four species of the bass genus *Morone* (Wirgin et al. 1992).

The last decade has seen the emergence of a new type of nuclear marker, satellite DNA, in investigations of genetic variation and divergence. Also known as VNTRs, these loci consist of short, tandemly repeated DNA sequences randomly scattered throughout the genome of most higher vertebrates (Brooker et al. 1994). Satellite DNA can be split into two types (minisatellites and microsatellites) based on the length of the repeat unit.

Minisatellite loci consist of repeat units that are generally 15-30 bp in length (Wirgin and Waldman 1994), although they may reach lengths up to 200 bp, with alleles as large as 50 kilobases (Bruford and Wayne 1993). Variation in minisatellite loci can be

extremely high, with heterozygosities greater than 90% and mutation rates exceeding 10^{-2} per generation (Bruford and Wayne 1993). Minisatellite loci have been identified in fish through hybridization of labeled human minisatellite DNA with fish nDNA. When the fish nDNA is digested with restriction enzymes and then hybridized to the labeled human minisatellite probe, the resulting multilocus band pattern can be highly variable at the individual and population levels. Variation is usually due to alleles at a given locus differing in their number of repeat units. Minisatellites were originally developed (as DNA fingerprinting) for forensic applications and paternity tests in humans (Jeffreys et al. 1985), although the methods were soon applied to problems in fisheries science (see O'Reilly and Wright 1995 for review). Unfortunately, the inability to assign alleles to specific loci, and problems with reproducibility between gels (Bentzen et al. 1991) limit the applicability of multilocus fingerprinting to problems in fisheries science that do not require testing of Hardy-Weinberg expectations (O'Connell and Wright 1997). Also prohibitive is the amount of high-quality purified target DNA needed for hybridization (see techniques below), although these problems have been offset with the development of single-locus minisatellite primers that can be used to amplify target DNA via PCR (Galvin et al. 1995a, b). This method allows for the amplification of alleles from a single locus using minute amounts of template DNA, and eliminates the ambiguity between loci and alleles inherent in Southern blot analysis.

The second class of VNTR markers is microsatellite loci, or simple sequence repeats (SSRs). In contrast to minisatellites, microsatellite loci consist of short di-, tri-, or tetranucleotide repeat units. Wright (1993) estimated that minisatellite loci occur approximately once every 1500 kb in fishes, while microsatellites may occur as often as

once every 10 kb. It has been reported that microsatellite loci show a high incidence of polymorphism relative to other classes of molecular markers such as mitochondrial genes (Patton et al. 1997, Brunner et al. 1998), although this has not proven to be universal (see O'Connell and Wright 1997, Table 2). The elevated levels of microsatellite polymorphism are believed to result from a high mutation rate and relaxed selective pressure at these noncoding loci. Mutation is currently believed to occur through polymerase slippage during DNA replication (Levinson and Gutman 1987), which increases or decreases the number of repeats, by one or more units. Direct studies of human families have shown that new microsatellite mutations usually differed from the parental allele by only one or two repeats (Weber and Wong 1993).

Identification and utilization of microsatellite loci can be a lengthy process, although methods for accelerating initial marker development have been published (Kijas et al. 1994, Waldbeiser 1995). Very briefly, purified nDNA from the target species is digested with a restriction enzyme, and fragments of DNA ranging from 300-1500 base pairs (bp) in size are ligated into plasmid vectors. Ligated fragments are amplified by asymmetrical PCR, resulting in a predominantly single-stranded DNA product. Fragments containing microsatellite regions are extracted from the single-stranded PCR product by filtering the product past streptavidin-coated magnetic beads complexed with an oligonucleotide probe made up of a small VNTR such as $(ATA)_n$ (Kijas et al. 1994). The microsatellite-enriched asymmetrical PCR product is again amplified using standard PCR protocols to yield double-stranded product. The microsatellite-enriched, double-stranded PCR product is ligated into a plasmid vector, cloned, and sequenced. PCR primers are then designed from the more conserved regions flanking the microsatellite, and these primers are used

to amplify the microsatellite locus in all samples. As in minisatellites, the number of repeat units at a given microsatellite locus can vary between individuals of a species, giving rise to length polymorphisms (different alleles) that can be used in studies of genome mapping, parentage, kinships, and stock structure (see O'Connell and Wright 1997 for review). In the last five years, microsatellite markers have been used extensively in population structure analyses of a wide variety of marine fishes, including sandbar sharks *Carcharhinus plumbeus* (Heist and Gold 1999), Pacific herring *Clupea pallasi* (O'Connell et al. 1998), Atlantic cod *Gadus morhua* (Bentzen et al. 1996), and many salmonids (Scribner et al. 1996, Small et al. 1998, Ostberg and Thorgaard 1999).

Satellite DNA can be analyzed using a variety of techniques. Minisatellites in fishes were originally analyzed by digesting DNA with a restriction enzyme, separating the fragments by agarose gel electrophoresis, and visualizing the DNA by Southern blotting with a minisatellite probe made from human DNA. More recently, PCR primers have been developed for at least one minisatellite locus in fishes (Galvin et al. 1995a, b), so that amplified alleles from a single locus can be separated and visualized by simple agarose gel electrophoresis and staining with ethidium bromide.

Due to polymerase slippage during replication, small size differences between alleles of a given microsatellite locus (as little as 2 bp in a locus comprised of dinucleotide repeats) are possible. Because of this, PCR-amplified microsatellite DNA was traditionally labeled radioactively, separated on a sequencing gel, and then exposed on X-ray film overnight (Sambrook et al. 1989). Significant increases in the number of samples which can be typed in a day have been achieved by using automated fluorescent sequencers coupled with computer imaging systems (O'Reilly and Wright 1995).

Study Goals

Much of the work done by fisheries scientists is dependent on properly identifying the management unit of interest at both the inter- and intraspecific levels. Many management regulations are species-specific, and require proper species identification for enforcement purposes. In addition, the accuracy of stock management models based on egg production or larvae surveys hinges on proper species identification of early life history stages. At the intraspecific level, population subdivisions must be accurately assessed to determine whether a species is best managed as a single unit or as multiple independent stocks. The aim of this dissertation was to investigate two interrelated problems in fisheries science, focused at different taxonomic levels (species and populations), using a comparative approach to find the best combination of molecular markers and techniques to address each question.

In Chapter 1 of this study, molecular markers were used to construct a genetic key for identification of 16 species of marine fishes commonly harvested in the Chesapeake Bay and throughout the mid-Atlantic Bight. This key should aid state and federal officials in the enforcement of species-specific regulations in cases when identification of fishes by external characteristics is no longer possible. The key should also prove useful for identifying early life history stages in some of the closely related species that lack distinguishing morphological characteristics.

Chapter 2 of this study focused on the population structure of one of these species, the weakfish *Cynoscion regalis*, along the U.S. East Coast. Traditional studies using

morphology, life history, and growth characteristics have produced conflicting evidence of population structure. Investigations using allozyme and whole molecule mtDNA markers revealed no population structure, but were limited by low levels of genetic variation. The results suggested that a more sensitive marker might be needed to better test the null hypothesis that this species comprises a single, genetically homogeneous stock. In the present study a number of new molecular markers was used to search for genetic variation within the species, and to test for the presence of genetically distinct stocks. Through the course of this investigation it was necessary to use the genetic marker key developed in Chapter 1 to provide species identification of anomalous individuals within some samples. At least three species other than weakfish *Cynoscion regalis* were inadvertently included in the samples, including the sand seatrout *C. arenarius* and the silver seatrout *C. nothus*. Finally, a combination of markers from both chapters was used to demonstrate hybridization among these three *Cynoscion* species.

CHAPTER 1

Forensic Identification of Sixteen Species of Chesapeake Bay Sportfishes Using Restriction Fragment Length Polymorphism (RFLP) Analysis of Mitochondrial DNA

Introduction

Fisheries scientists and managers are faced with the challenge of properly regulating finfish species under often intense harvesting pressure from both commercial and recreational fishers. In order to maintain healthy stocks and insure long-term sustainable yields, management strategies for a given fishery may include gear restrictions as well as seasonal, size, and bag limits. In most instances these regulations are species-specific, and identifications are based on morphological characters. These distinguishing traits are often lost if the catch is filleted or otherwise processd before inspection by enforcement personnel, requiring some other method of identification if regulations are to be effectively implemeted.

The goal of this study was to develop a molecular genetic key for a number of important sportfishes found in Virginia marine waters and common throughout much of the mid-Atlantic Bight. The intent was to develop a key using a molecular marker that was easily surveyed from typical field samples using standard laboratory equipment. To accomplish this, it was decided that identifications would be based on restriction fragment length polymorphism (RFLP) analysis of a small section of mitochondrial DNA (mtDNA) amplified using the polymerase chain reaction (PCR) for the following reasons: (1) The maternal mode of inheritance of the mitochondrial genome simplified analysis by eliminating within-individual variation due to multiple alleles (heterozygosity); (2) the

amount of tissue required for PCR amplification is minimal (a single egg or fin clip, frozen or preserved in a variety of buffers, is sufficient), and the limitations on tissue condition are not as stringent as those for other methods of genetic analysis; (3) universal PCR primers that amplify specific regions of the mitochondrial genome from a wide taxonomic range of species are readily available in the literature; and (4) RFLP analysis was chosen over nucleotide sequencing because it is faster, less expensive, requires equipment common to most molecular laboratories, and has proven its utility in interspecific identification of marine fishes including billfishes (Chow 1994; Innes et al. 1998), snappers (Chow et al. 1993), and various sciaenids (Daniel and Graves 1994). The resulting key should prove useful not only as an enforcement tool, but also in the identification of eggs and larvae in ichthyoplankton studies, in cases of suspected hybridization between species, and in other studies that rely on the correct identification of sampled species.

Materials and Methods

Sample Collection and Storage. Approximately 20 individuals from each of the species listed in Table 1 were collected in 1995 and in 1996. The majority of fishes used in this study were collected in the Chesapeake Bay by the Virginia Institute of Marine Science (VIMS) trawl survey. Species not commonly taken in the trawl survey were obtained through a variety of sources. Samples of *Menticirrhus americanus* and *Menticirrhus saxatilis* from 1995-96 were supplemented with fish from Trey Knott of the NMFS Southeast Fisheries Center in Charleston, South Carolina. *Menticirrhus americanus* from 1996 were supplemented by fish from the VIMS seine survey. Samples of *Tautoga onitis* were supplied by VIMS graduate student Geoff White in 1995 and George's Seafood of Norfolk, Virginia in 1996. *Scomberomorus maculatus* samples were obtained through VIMS graduate students Sarah Gaichas (1995) and Vincent Buonaccorsi (1996). Samples of *Rachycentron canadum* for both years were donated by local fishermen with the cooperation of Wallace's Marina in Fox Hill, Virginia. VIMS graduate student Jan McDowell supplied *Pomatomus saltatrix* samples from 1996. Because no local *Sciaenops ocellata* samples could be secured in 1995, we used archived Chesapeake Bay samples from 1986 provided by Dr. John Gold and Linda Richardson at Texas A&M University. In 1996 samples of *Sciaenops ocellata* were collected off the coast of Louisiana by VIMS graduate student Brett Falterman.

Fish were maintained on ice until transported to the laboratory, where tissue samples (mostly muscle) were dissected and stored at -80° C. Some tissue samples were preserved in 95% ethanol or DMSO storage buffer (25 mM EDTA, 20% DMSO, saturated NaCl), while blood samples of *Menticirrhus americanus* and *Menticirrhus saxatilis* from South Carolina were preserved in SDS/urea (1% urea, 8 M SDS, 180 mM $NaPO_4$, 4 mM EDTA). All samples stored in preservation buffers were maintained at room temperature.

Whole Genomic DNA Isolation. A high molecular weight DNA extraction protocol modified from Sambrook et al. (1989) was used to isolate DNA from frozen samples and those stored in 95% ethanol or DMSO buffer. A cube of tissue, no more than two millimeters on a side, was diced with a razor blade and placed in a 1.5 ml microfuge tube on ice. To each tube 500 µl isolation buffer (50 mM EDTA, 50 mM Tris, 150 mM NaCl, pH 8.0), 60 µl 10% SDS, 10 µl RNAse (10 mg ml^{-1}), and 10 µl proteinase K (25 mg ml^{-1}) were added, and the tubes were left to incubate overnight in a water bath at 37° C. Samples were then extracted once with equilibrated phenol, once with phenol: chloroform:isoamyl alcohol (25:24:1), and once with chloroform:isoamyl alcohol (24:1). Precipitated DNA was collected by the addition of ethanol at –80° C for 1 hour. DNA was pelleted by centrifugation, washed with 70% ethanol, dried in a Savant SC100 Speed Vac, and resuspended in 50 µl sterile 0.1X TE (10 mM Tris, 1 mM EDTA, pH 8.0).

DNA was isolated from blood samples stored in SDS/urea using a modified version of the protocol in White and Densmore (1992). 400 µl samples were incubated at 65° C overnight, extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol

(25:24:1), and then extracted twice more with an equal volume of chloroform:isoamyl alcohol (24:1). DNA was precipitated, pelleted, dried, and resuspended as described above. All DNA samples were stored at -20° C.

DNA Amplification. Primers for five mitochondrial gene regions obtained from the literature were used to amplify DNA from the 16 species (Table 2). Of the five primer sets, only the mitochondrial NADH dehydrogenase 4 (ND4) and 12S/16S ribosomal RNA (rRNA) regions amplified consistently across all 16 species. Both of these mitochondrial regions proved useful in distinguishing among the 16 species tested here, although levels of within species polymorphism was markedly higher in the amplified ND4 enzyme digestions (Cordes et al. submitted). As a result, the 12S/16S rRNA region was chosen as the primary marker for the genetic key.

A 1495 bp region of the mitochondrial genome encompassing part of the 12S and 16s ribosomal RNA (rRNA) genes was amplified using the primers of Palumbi et al. (1991). DNA was amplified using the PCR Reagent System (Gibco BRL, Gaithersburg, MD, USA) in either a Perkin Elmer Cetus DNA Thermal Cycler or an MJ Research PTC-200 Peltier Thermal Cycler. The 25 µl amplification reactions consisted of 12.675 µl sterile distilled water, 2.5 µl 10X PCR buffer with 15 mM $MgCl_2$, 0.5 µl 10 mM dNTP mixture, 0.25 µl primers (100 pm· μl^{-1}), 0.125 µl *Taq* I polymerase (5 U· μl^{-1}), and 0.25 µl sample DNA (approximately 50 ng). Samples were first denatured for 5 min at 95° C, followed by 35 cycles of PCR amplification performed under the following conditions: 1 min at 95° C, 1 min at 45° C, and 3 min at 65° C. Reactions were given a final 10 min extension at 65° C and the resultant products were stored at 4° C. The length of the amplified

product was confirmed by running 4 µl on a 1% agarose (Gibco BRL) gel in 1X TBE buffer (45 mM Tris, 45 mM Boric acid, 1 mM EDTA, pH8.3) against 1KB DNA Mass Ladder (Gibco BRL).

Digestion of Amplified DNA with Restriction Enzymes. 12S/16S rRNA PCR products from a subset of each species were digested with four enzymes (*Alu* I, *Rsa* I, *Hinf* I, and *Msp* I) to screen for variation. Based on the abilities of the different enzymes to distinguish between species while minimizing intraspecific variation, the enzyme *Rsa* I (Gibco BRL) was chosen for use in the genetic key. Restriction digests were carried out according to manufacturer's instructions in 15 µl reactions containing 5-8 µl PCR product DNA, 1.5 µl of the appropriate reaction buffer, 0.3 ul of enzyme, and enough sterile distilled water to bring the volume to 15 µl. Digests were terminated with 3 µl stop solution (40% glycerol, 60% 1X TBE, 0.5% weight/volume SDS, 0.02% w/v bromophenol blue), and the resulting fragments separated on 2.5% agarose gels using 1% NuSieve (FMC BioProducts, Rockland, ME) and 1.5% agarose (Gibco BRL) in 1X TBE buffer. The 1KB DNA Mass Ladder was included in one or more lanes on each gel. Gels were stained in 1X TBE buffer containing 30 µl (5 mg/ml) ethidium bromide (EtBr), visualized on a Spectroline Model TR-302 Transilluminator, and photographed using a Polaroid CU-5 Land Camera.

Data Analysis. Restriction digestion patterns were analyzed using the software program RFLPScan Plus 3.0 (Scanalytics, Billerica, MA, USA) on an IBM-compatible computer equipped with a scanner. Gel photographs were scanned into the program, and sample fragment sizes were estimated using a calibration curve generated by plotting

migration distances of the size-standard fragments against their known lengths.

Results

DNA Amplification. Initial amplifications with primers for the five mitochondrial regions listed in Table 2 yielded a range of success rates across the 16 species tested. Cytochrome *b* amplifications were non-existent, weak, or exhibited multiple bands in seven of the 16 species. The same was true for eight of the 16 species tested with the cytochrome *c* oxidase I primers. ATPase amplifications were unsuccessful in three species and unacceptably weak in a fourth, although further refinement of the PCR protocol for this region probably could have improved the results. Both the ND4 and 12S/16S rRNA regions amplified well in all 16 species.

Digestion Patterns. Restriction enzyme digestion patterns of the amplified 12S/16S rRNA region digested with *Rsa* I were determined from 20 individuals of each species for each of two years (total of 40 individuals per species), with the exception of *Menticirrhus saxatilis*. This species was added to the study only after it was found to have been inadvertently included in some of the *Menticirrhus americanus* collections. Fragment sizes for each species are listed in Table 3, and patterns are illustrated in Fig. 1. The number of bands in a given pattern ranged from three in *Pomatomus saltatrix* to five in *Micropogonias undulatus* (mean = 4.4). In most cases band sizes below 100 bp were

inferred through comparison of patterns, since bands in this size range are poorly resolved by agarose gel electrophoresis.

The size of the amplified 12S/16S rRNA region for each species was estimated by summing the fragment sizes of each *Rsa* I digestion pattern. Sizes ranged from 939 bp (*Leiostomus xanthurus*) to 1514 bp (*Sciaenops ocellata*). Since all sixteen species showed no discernable size difference in the undigested 12S/16S rRNA amplifications, it was assumed this discrepancy was due to the presence of smaller bands not resolved on the gel, inaccuracies in the size estimations of individual bands (see Discussion below), or the presence of unresolved doublets. This is illustrated in the case of *Leiostomus xanthurus*, whose estimated size based on two other enzymes (*Hinf* I and *Msp* I) is approximately 1550 bp (Figure 2), a figure more in keeping with the published sizes given for amplifications in other species with these primers (Palumbi et al. 1991).

No 12S/16S rRNA RFLP patterns were shared among any of the 16 species digested with *Rsa* I. Ten of the species were monomorphic over the 40 individuals screened; each of the remaining six species were dimorphic (Table 3, Fig. 1). The addition or deletion of a single restriction site could be inferred to explain differences between the two patterns in all six of the dimorphic species.

Comparison of intraspecific variation between the 12S/16S rRNA and ND4 regions led to the selection of 12S/16S as the preferred marker for the genetic key. Although no ND4 RFLP patterns were shared by any species digested with the endonuclease *Bst*O I, nine of the 16 species surveyed were polymorphic (Table 4, from Cordes et al. submitted). The number of patterns in a given species ranged from one in the seven monomorphic species to five in *Sciaenops ocellata*. A second enzyme was employed in

the ND4 analysis because the relatively high degree of polymorphism within a number of species suggested that unknown variants might be found in the future. Because the two enzymes recognize different combinations of nucleotides in the genetic code, a mutational change effecting the digestion pattern produced by one of the enzymes would not effect the digestion pattern of the second. Digestions with the endonuclease *Ava* II produced eight polymorphic species, with the number of digestion patterns in a given species ranging from one in the 8 monomorphic species to five in *Leiostomus xanthurus* (Table 4). Four species were polymorphic for both ND4 enzymes. Although the ND4 marker was able to unambiguously distinguish between the 16 species tested, the increased intraspecific variation and concomitant increase in complexity of analysis made it a less efficient choice for identification than the 12S/16S rRNA marker.

Discussion

The utility of a molecular marker for species identification is based on its ability to reveal consistent interspecific differences while minimizing intraspecific variation. The combination of complete interspecific separation and low intraspecific variation shown by the 12S/16S rRNA marker makes it ideal for species-level discrimination. No overlap of RFLP digestion patterns was seen among 40 individuals of 16 species when screened with a single enzyme, and the low incidence of intraspecific variation suggests that unrecognized variants in future studies should not be a major concern.

Other factors affecting the utility of a molecular marker for species identification are its temporal and geographic stability, variables that are sometimes ignored when developing species identification systems. Although the low intraspecific variability characteristic of a good species identification marker should minimize both temporal and spatial variation within each species, this is not always tested. Restriction patterns for the 12S/16S rRNA marker did not differ for 15 of the 16 species over two consecutive years (1995 and 1996); restriction patterns also proved stable in the last species *Sciaenops ocellata* between samples from 1986 and 1996. In addition, geographically distant samples of *Menticirrhus americanus*, *Menticirrhus saxatilis*, and *Sciaenops ocellata* shared the same restriction patterns within each species.

One concern involving the 12S/16S rRNA marker is the apparent discrepancy between

the size of the undigested PCR products and the size estimated by summing digestion pattern bands. There were no apparent differences between species in the size of the undigested PCR products when viewed on a 1% agarose gel. When the bands of individual digest patterns were summed, however, the totals varied between species by as much as 575 bp (*Leiostomus xanthurus* and *Sciaenops ocellata*, Table 3). Some of this variation may be explained by the inability of low percentage agarose gels to clearly show bands less than approximately 100 bp in length. In addition, unresolved doublets can lead to underestimation of total fragment size (see Results above). Another source of variation concerns the accuracy of the measurements produced by the RFLPScan program. Because larger bands tend to be compressed towards the top of an agarose gel, the accuracy of the standard in predicting the size of a given band decreases as band size increases, with the size of larger bands tending to be over-estimated. Taking all of this into account, it is probably wise to emphasize relative rather than absolute band size when making comparisons between patterns.

The ability of different molecular systems to discriminate among species varies widely. Although allozyme electrophoresis has been routinely used to discriminate between fish species in the U.S. food industry (AOAC 1984), it may fail to distinguish between closely related species (Bartlett and Davidson, 1991). In contrast, other systems may find so much variation within species that they may be of more use in intraspecific population studies (Withler et al. 1997; Innes et al. 1998). Other criteria for determining useful species identification systems include ease of use, cost effectiveness, and the quality and quantity of sample tissue required. Both allozyme electrophoresis and RFLP analysis of whole molecule mtDNA have been performed on samples as small as a single

fish egg (Mork et al. 1983; Graves et al. 1990; Daniel and Graves 1994). Although traditional allozyme techniques are fast and relatively inexpensive, they usually require fresh or freshly frozen tissue. In contrast, PCR-based genetic markers can be used on small samples that are fresh or frozen, or preserved in alcohol, formalin, or a variety of storage buffers. In this study samples which were fresh, frozen, or stored in alcohol, SDS/urea, or DMSO storage buffer all worked equally well. The PCR/RFLP technique used here is less time-consuming than traditional whole-molecule methods such as southern blotting, and unlike DNA sequencing, the equipment is readily available in most molecular laboratories. In addition, PCR/RFLP analysis is still faster (despite automated sequencers and direct sequencing methods) and less expensive than sequencing when processing large numbers of samples. Rocha-Olivares (1998) developed a haplotype-specific PCR system that eliminates the need for RFLP analysis; species identification is based on the presence or absence of amplified cytochrome *b* PCR products. Successful amplification is controlled by point mutations in the genetic code at the place of primer attachment. Under high stringency conditions any change in the code will cause a failure of the primers to anneal and no amplification will occur. Although this does eliminate the need for restriction enzymes, it requires known cytochrome *b* sequences for each species in question so appropriate primers can be designed. In addition, lack of amplification can also result from a number of other causes including variations in sample quality or laboratory conditions, resulting in misidentifications. In contrast, the 12S/16S rRNA primers used in the present study are based on well-conserved regions (Palumbi et al. 1991) and require no previous knowledge of specific DNA sequences, which may make them more practical when screening a large number of species.

In this study a genetic marker was developed as an efficient and cost-effective means of discrimination between 16 species of Chesapeake Bay sportfishes. The 12S/16S rRNA marker proved its utility by unambiguously distinguishing all 16 species by RFLP analysis with a minimal number of enzymes. Although the original intent of this study was to provide an efficient means of sportfish species identification to a state agency (Virginia Marine Resources Commission), it is hoped that it may have more general application. The 12S/16S rRNA marker has already proved useful in distinguishing between eggs of closely related sciaenid species (Luczkovich et al. 1999) and reevaluating misidentified samples in a study of *Cynoscion regalis* population structure (Chapter 2). In addition, it has been used in conjunction with microsatellite markers to identify suspected hybrids off the coast of Georgia between *Cynoscion regalis* and sand seatrout, *Cynoscion arenarius*, a species thought to be limited in distribution to the Gulf of Mexico (Chapter 2). Such supplementation with a nuclear DNA marker can be used to overcome one of the drawbacks of mtDNA-based markers, i.e. their inability to recognize hybrids. A number of nuclear DNA markers have recently been developed in salmonid species identification studies (Pendas et al. 1995, Withler et al. 1997). As more become available, the joint use of mitochondrial and nuclear DNA-based markers in species identification studies, particularly where hybridization may play a role, should become routine.

Table 1. Chesapeake Bay marine and anadromous sportfishes for which a genetic key based on mitochondrial DNA was developed.

Atlantic croaker (*Micropogonias undulatus*)	southern kingfish (*Menticirrhus americanus*)
black drum (*Pogonias cromis*)	Spanish mackerel (*Scomberomorus maculatus*)
black sea bass (*Centropristis striata*)	spot (*Leiostomus xanthurus*)
bluefish (*Pomatomus saltatrix*)	spotted seatrout (*Cynoscion nebulosus*)
cobia (*Rachycentron canadum*)	striped bass (*Morone saxatilis*)
northern kingfish (*Menticirrhus saxatilis*)	summer flounder (*Paralichthys dentatus*)
red drum (*Sciaenops ocellata*)	tautog (*Tautoga onitis*)
silver perch (*Bairdiella chrysoura*)	weakfish (*Cynoscion regalis*)

Table 2. Primer pairs used to amplify 5 mitochondrial gene regions (ATPase 6, cytochrome *b*, cytochrome oxidase I, ND4, and 12S/16S rRNA).

Primer Sequences (5'-3')	Approximate Size (bp)	Source
ATPase 6		Joseph
ATPase L8331: TAAGCRNYAGCCTTTTAAG	750	Quattro[a]
ATPase H8969: GGGGNCGRATRAANAGRCT		
Cytochrome b		Joseph
CTYB-F: TGGGSNCARATGTCNTWYTG	340	Quattro[a]
CYTOB-R: GCRAANAGRAARTACCAYTC		
Cytochrome C Oxidase I		Folmer 1994
LCO1490: GGTCAACAAATCATAAAGATATTGG	710	
HCO2198: TAAACTTCAGGGTGACCAAAAAATCA		
12S/16S rRNA		Palumbi et al.
12SA-L: AAACTGGGATTAGATACCCCACTAT	1495	1991
16SA-H: ATGTTTTTGATAAACAGGCG		
ND4		Bielawski and
ND4 ARG-BL: CAAGACCCTTGATTTCGGCTCA	1700	Gold 1996
ND4 LEU: CCAGAGTTTCAGGCTCCTAAGACCA		

[a] Personal communication. FISHTEC Genetics Laboratory, Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA

Table 3. Restriction digest patterns of the 12S/16S mitochondrial region for 16 species of Chesapeake Bay sportfishes digested with the enzyme *Rsa* I. n = number of individuals exhibiting the adjacent pattern.

Species	Enzyme	n	Pattern	Band Size (bp)						Total size (bp)
Atlantic Croaker	*Rsa* I	18	a	461	344	288	205	169		1467
		22	b	461	288	252	205	169	92	
Black Drum		40	a	451	288	257	246	197		1439
Black Sea Bass		39	a	464	437	295	206			1402
		1	b	464	437	273	206	22		
Bluefish		40	a	740	392	243				1375
Cobia		39	a	523	266	241	185	40		1255
		1	b	563	266	241	185			
Northern Kingfish		31	a	512	312	271	197			1293
		1	b	512	312	197	182	89		
Red Drum		40	a	455	341	292	252	174		1514
Silver Perch		40	a	510	448	296	245			1499
Southern Kingfish		40	a	517	299	192	162			1170

Table 3. Continued.

Species	Enzyme	n	Pattern	Band Size (bp)					Total size (bp)
Spanish Mackerel		39	a	465	305	245	220		1266
		1	b	305	245	245	220	220	
Spot		40	a	303	262	201	173		939
Spotted Seatrout		39	a	457	448	305	202		1412
		1	b	457	305	269	202	168	
Striped Bass		40	a	470	308	240	186		1204
Summer flounder		40	a	513	294	238	172		1217
Tautog		40	a	350	296	271	253		1170
Weakfish		40	a	461	300	200	167		1128

Table 4. Restriction digestion patterns of the ND4 mitochondrial region for 16 species of Chesapeake Bay sportfishes digested with the enzymes *BstO* I and *Ava* II. n = number of individuals exhibiting the adjacent pattern. From Cordes et al. (submitted).

Species	Enzyme	n	Pattern					Band Size (bp)	Total size (bp)
Atlantic Croaker	*BstO* I	17	a	669	426	410	395		1900
		23	b	669	426	410	245	150	
Black Drum		37	a	691	691	536			1918
		3	b	1382	536				
Black Sea Bass		40	a	707	392	386	226	184	1901
Bluefish		6	a	1506	230	148			1884
		9	b	1056	273	230	177	148	
		25	c	1056	450	230	148		
Cobia		36	a	805	660	492			1957
		4	b	1465	492				
Northern Kingfish		31	a	457	457	391	284	209	1872
		1	b	741	457	391	209		

Table 4. Continued.

Red Drum		6	a	1065	796				1861
		24	b	1065	590	206			
		8	c	1065	349	241	206		
		1	d	741	590	324	206		
		1	e	590	590	475	206		
Silver Perch		40	a	480	480	435	223	217	1835
Southern Kingfish		40	a	531	400	400	293	214	1838
Spanish Mackerel		37	a	749	401	396	274	78	1898
		2	b	1150	396	274	78		
		1	c	827	401	396	274		
Spot		37	a	538	445	374	334	86	1777
		1	b	905	538	334			
		1	c	538	445	420	374		
		1	d	538	374	334	246	199	86
Spotted Seatrout		40	a	1756	243				1999
Striped Bass		40	a	1331	343	213			1887
Summer Flounder		38	a	961	492	462			1915
		2	b	743	492	462	218		
Tautog		40	a	1534	490				2024
Weakfish		40	a	599	330	304	247	217	193	1890
Atlantic Croaker	*Ava* II	40	a	1157	608	196			1961

Table 4. Continued.

Black Drum	40	a	1592	379					1971
Black Sea Bass	40	a	855	277	223	107	154	124	1800
Bluefish	40	a	1736	308					2044
Cobia	40	a	980	961					1941
Northern Kingfish	29	a	842	746	322				1910
	3	b	746	501	341	322			
Red Drum	39	a	1064	860					1924
	1	b	1064	450	410				
Silver Perch	37	a	1239	306	248	210			2003
	3	b	1123	306	248	210	116		
Southern Kingfish	40	a	1457	581					2038
Spanish Mackerel	39	a	750	683	330	125	92		1980
	1	b	750	683	422	125			
Spot	16	a	748	493	407	394			2042
	15	b	1142	900					
	3	c	1142	493	326	81			
	5	d	1142	493	407				
	1	e	900	748	407				
Spotted Seatrout	37	a	1021	586	388				1995
	2	b	819	586	388	202			
	1	c	927	586	388	94			

Table 4. Continued.

Striped Bass	39	a	1601	204	200			2005
	1	b	1601	404				
Summer Flounder	39	a	510	510	332	239	184	78	1853
	1	b	510	510	332	317	184	
Tautog	38	a	486	415	349	332	204	182	1968
	1	b	536	486	415	349	182	
	1	c	531	486	415	332	204	
Weakfish	40	a	550	523	495	367			1935

Figure 1. Restriction endonuclease digestion patterns of the 12S/16S rRNA gene region in 16 species of Chesapeake Bay sportfishes using *Rsa* I. All profiles observed for each species are shown. Fragments were separated on a 1.5% agarose/1% NuSieve gel and stained with ethidium bromide. Lanes 1, 15, and 25 = size standards, 2 and 3 = *Micropogon undulatus* (patterns a and b, respectively), 4 and 5 = *Rachycentron canadum* (patterns a and b, respectively), 6 = *Pogonias cromis*, 7 and 8 = *Centropristis striata* (patterns a and b, respectively), 9 = *Pomatomus saltatrix*, 10 = *Menticirrhus americanus*, 11 = *Paralichthys dentatus*, 12 = *Bairdiella chrysoura*, 13 and 14 = *Scomberomorus maculatus* (patterns a and b, respectively), 16 and 17 = *Cynoscion nebulosus* (patterns a and b, respectively), 18 = *Leiostomus xanthurus*, 19 = *Morone saxatilis*, 20 = *Cynoscion regalis*, 21= *Tautoga onitis*, 22 = *Sciaenops ocellata*, and 23 and 24 = *Menticirrhus saxatilis* (patterns a and b, respectively).

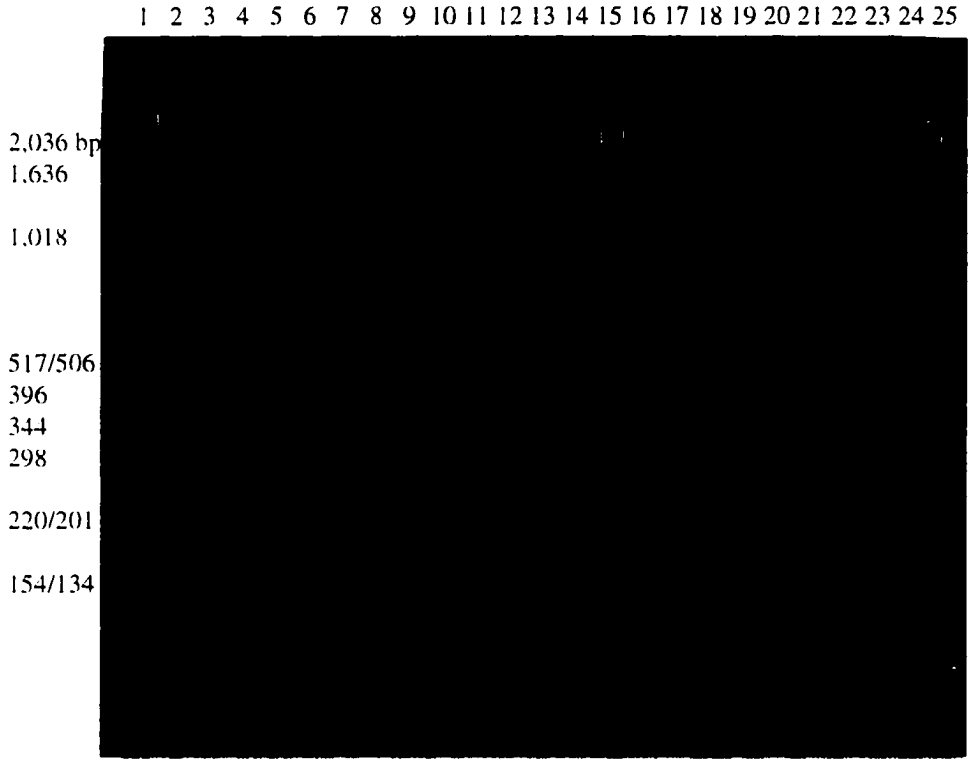
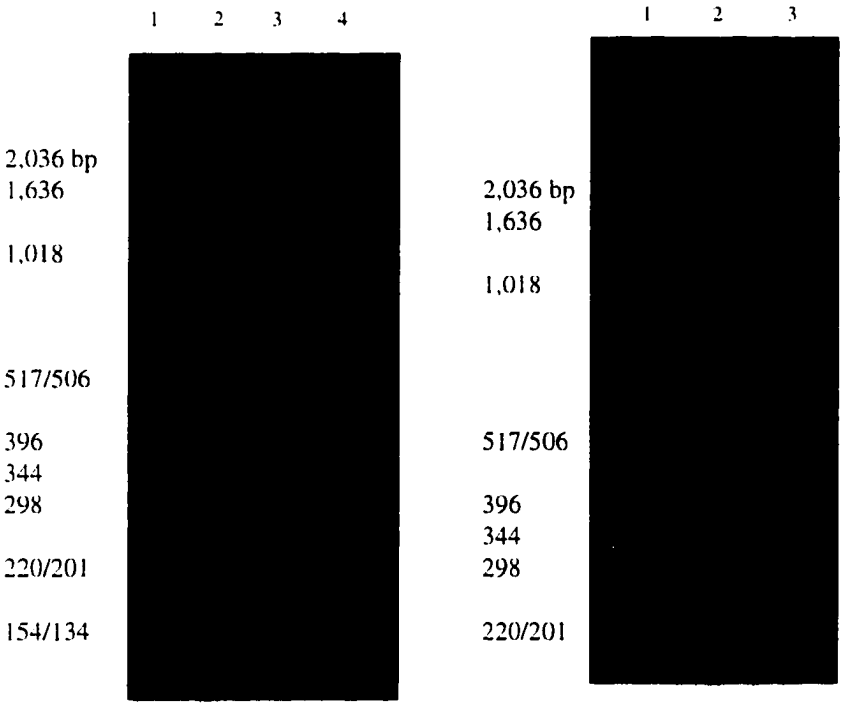


Figure 2. Restriction endonuclease digestion patterns of the 12S/16S gene region in spot *Leiostomus xanthurus* using (a) *Hinf* I: Lane 1= size standard, lane 2 = empty, 3 and 4 = spot; and (b) *Msp* I: Lane 1 = size standard, lanes 2 and 3 = spot.



(a) *Hinf* I (b) *Msp* I

Species	Enzyme		Band Sizes	(bp)		Total Size
Spot	*Hinf* I	781	336	288	144	1549
Spot	*Msp* I	1018	510			1528

CHAPTER 2

Stock Structure Analysis of Weakfish *Cynoscion regalis* Using Nuclear Microsatellite and Intron Markers

Introduction

The weakfish *Cynoscion regalis* is distributed along the U.S. East Coast from Massachusetts to eastern Florida, with greatest abundance occurring from New York to North Carolina (Bigelow and Schroeder 1953). The species undergoes substantial seasonal migrations. In the spring, fish move north and inshore into estuaries to spawn. In the fall, juveniles move south to overwinter off the coast of North Carolina, while older fish are thought to migrate south and offshore (Fig. 3; Wilk 1976).

Weakfish support substantial commercial and recreational fisheries along the eastern seaboard. Precipitous drops in total annual catches between 1980 and 1994 (from 80 million lb to 8 million lb) led to a temporary ban on commercial fishing in federal waters in 1995 (Anonymous 1995), and there is growing concern that bycatch of juvenile weakfish by shrimp trawlers at the southern end of the species range is adversely impacting abundance (Vaughan et al. 1991).

There is no clear consensus on the stock structure of weakfish. Traditional studies based on tag and recapture data (Nesbit 1954), scale morphology (Perlmutter et al. 1956), morphological data (Scoles 1990), and various life history characters (Shepherd and Grimes 1983, 1984) suggest two or more independent stocks. Unfortunately, differences based on these kinds of data could be due to environmental effects (Shepherd and Grimes

1983, Vaughan et al. 1991), and may not reflect genetically distinct (reproductively isolated) stocks.

Most modern management strategies are based on stocks defined as "a group of organisms whose demographic/genetic trajectory is largely independent from other such groups" (Waples 1998). Recent molecular population studies employing allozyme analysis (Crawford et al. 1989) and restriction fragment length polymorphism (RFLP) analysis of whole molecule mtDNA (Graves et al. 1992) were unable to falsify the null hypothesis that weakfish comprise a single, genetically homogeneous stock. Unfortunately, the low overall genetic variation revealed by both techniques reduced the power of the analyses. Much larger sample sizes or a more sensitive marker are required to detect differences between weakfish populations if they do indeed exist.

In recent years a number of new molecular markers and analytical techniques have been used to study stock structure in a variety of marine fishes (see General Introduction). The purpose of this study was to utilize some of these molecular markers and techniques to investigate the stock structure of weakfish along the U.S. East Coast, and to compare results generated by each marker. More specifically, the objectives of this study were to: 1) find or develop primers to amplify microsatellite and other nuclear regions using the polymerase chain reaction (PCR), 2) assess the genetic variation contained in these markers in young-of-the-year (YOY) weakfish from different nursery areas along the eastern seaboard, 3) repeat the above assessment for a second year of samples from the same locations to test for the temporal stability of allele frequencies, and 4) compare the results of the different markers to infer population structuring in the weakfish.

Microsatellite loci and gene intron regions were chosen for this study because both have shown elevated levels of genetic variation compared to other marker classes (i.e. allozymes and mtDNA) in marine fishes. Microsatellites have proved useful to reveal variation in species with low genetic diversity (based on allozyme and mtDNA analyses) such as northern pike *Esox lucius* (Miller and Kapuscinski 1996) and Arctic char *Salvelinus alpinus* (Brunner et al. 1998). Although higher genetic variation does not necessarily translate into higher resolution stock delineations (Seeb et al. 1998), microsatellite loci have been able to distinguish between populations in such species as broad whitefish *Coregonus nasus* (Patton et al. 1997) and Atlantic cod *Gadus morhua* (Bentzen et al. 1996, Ruzzanti et al. 1996) when more traditional markers have failed.

Although relatively few gene introns have been used in stock structure analyses (Palumbi and Baker 1994, Moran et al. 1997), they may quickly become a standard molecular marker in these investigations. One advantage of these exon-primed, intron-crossing (EPIC) markers is the conserved nature of their primer sequences. Designed in the coding (exon) regions of the gene, they often amplify across a wide variety of taxa (Palumbi and Baker, 1994, Moran et al. 1997). A number of analytical techniques are available for assessing variation in single-copy nuclear (scn) DNA such as gene introns (see General Introduction). RFLP analysis was chosen for assessment of genetic variation in the scnDNA markers employed in this study because of its cost effectiveness, ease of use, and ability to quickly process a large number of samples (Leclerc et al. 1996).

Materials and Methods

Sample Collections. If genetically discrete weakfish populations do indeed exist, adults would have to return to and spawn in their natal waters in order to maintain genetic distinctiveness. To avoid the confounding effects of mixing between stocks by non-mating individuals, sample collection can either be restricted to spawning adults (Graves et al. 1992) or to recently spawned larvae and juveniles that are believed to remain in their natal estuaries during the first months of growth (Wilk 1976, Rowe and Epifanio 1994). For this reason only YOY weakfish were used in the present study.

Fish were collected in the summers of 1996 and 1997 from five sites along the U.S. East Coast (Fig. 4) as part of a larger project combining microchemical analysis of otoliths and genetic markers to investigate the stock structure of weakfish. Samples were provided by Patrick Geer (Virginia Institute of Marine Science), Simon Thorrold (Old Dominion University), Louis Barbieri (University of Georgia), Susan Lowerre-Barbieri (University of Georgia), C. Grahn (New York Deptment of Environmental Conservation), and M. Greene (NOAA/NMFS Beaufort Lab). Trey Knott (NOAA/NMFS Southeast Fisheries Center) supplied samples of banded drum *Larimus fasciatus*, Gulf kingfish *Menticirrhus littoralis*, and star drum *Stellifer lanceolatus* from the south Atlantic Bight. Samples of silver seatrout *Cynoscion nothus* and sand seatrout *C. arenarius* from the Gulf of Mexico were provided by Bill Karel (Texas Parks and Wildlife Department). Fish were

maintained on ice until transported to the laboratory, where samples were stored at -80° C. In the laboratory, muscle tissue was excised from each weakfish sample and either stored at -80° C or placed in DMSO buffer (25 mM EDTA, 20% DMSO, saturated NaCl) prior to otolith removal.

Whole Genomic DNA Isolation. Whole genomic DNA was isolated using the protocol modified from Sambrook et al. (1989) outlined in Chapter 1. All isolated DNA samples were stored at -20° C.

Development of Novel Microsatellite Loci. A microsatellite-enriched weakfish genomic library was produced following the protocol of Kijas et al. (1994). Whole genomic DNA was isolated from a single weakfish specimen and digested using the restriction endonuclease *Mbo* I (Gibco BRL). Fragments of DNA ranging from 300-1500 base pairs (bp) in size were collected from a 1.5% agarose gel electrophoresed in 1X TAE buffer (40 mM tris, 40 mM sodium acetate, 1 mM EDTA, pH 8.2) following Karl and Avise (1993). DNA fragments were ligated into BlueScript SK^+ plasmid vectors using T4 DNA ligase (Stratagene, La Jolla, CA, USA) following manufacturer's protocols. Ligated weakfish DNA fragments were amplified by asymmetrical PCR using the T7 Promoter (Gibco BRL) and M13 Reverse Primer (New England Biolabs, Beverly, MA, USA) that recognize the plasmid vector sequences flanking the weakfish DNA inserts. By using an excess of one primer, asymmetrical PCR results in a predominantly single-stranded DNA product. Two amplifications, each with an excess of one primer, were preformed following manufacturer's instructions (PCR Reagent System, Gibco BRL).

Each 50 µl reaction consisted of 37.75 µl sterile dH_2O, 5.0 µl 10X PCR buffer with 15 mM $MgCl_2$, 1.0 µl 10 mM dNTP mixture, 5.0 µl excess primer (100 pm· μl^{-1}), 0.5 µl second primer (100 pm· μl^{-1}), 0.25 µl *Taq* I polymerase (5 U· μl^{-1}), and 0.5 µl DNA (approximately 50 ng). Samples were first denatured for 4 min at 95° C, followed by 32 cycles of PCR amplification performed under the following conditions: 1 min at 94° C, 1 min at 37° C, and 1 min at 72° C. Reactions were given a final 4 min extension at 72° C and amplified products were stored at 4° C.

DNA fragments containing variable number of tandem repeats (VNTRs or microsatellites) were extracted from the single-stranded PCR product by filtering the product past streptavidin-coated magnetic beads (Promega) complexed with an $(ATA)_6$ oligonucleotide probe. A total of 1.0 µg biotinylated probe was attached to 50 µl magnetic beads in 100 µl 5X SSC (1X SSC= 150 mM NaCl, 15 mM sodium citrate) for 15 min at room temperature. Unbound probe was removed with three washes of 100 µl 5X SSC and the beads were resuspended in 35 µl 10X SSC. Next 10 µl of the asymmetrical PCR product was diluted with 55 µl sterile dH_2O, denatured for 10 min at 98° C, and hybridized to the probe/bead complex for 20 min at 30° C (total volume 100 µl). The beads were repeatedly sedimented in a magnetic field and washed, first with four 5 min washes in 100 µl low-stringency solution (2X SSC, 0.5 ng μl^{-1} each universal primer), and then with four 5 min washes in 100 µl high-stringency solution (1X SSC, 0.5 ng μl^{-1} each universal primer). To remove unbound DNA, beads were resuspended in 20 µl of 0.15 M NaOH and incubated for 20 min at room temperature. The beads were then sedimented and the supernatant neutralized with 2.2 µl 10X TE (100 mM Tris, 10 mM

EDTA, pH 7.3) and 1.3 µl 1.25 M acetic acid. Finally, each supernatant was desalted using Microcon ®-30 spin columns (Amicon, Beverly, MA, USA) following manufacturer's instructions.

The microsatellite-enriched asymmetrical PCR product was again amplified using standard PCR to yield double-stranded product. Each 50 µl reaction consisted of 37.75 µl sterile dH_2O, 5.0 µl 10X PCR buffer with 15 mM $MgCl_2$, 1.0 µl 10 mM dNTP mixture, 0.5 µl T7 promoter and M13 reverse primer (100 pm· μl^{-1}), 0.25 µl *Taq* I polymerase (5 U· μl^{-1}), and 5.0 µl microsatellite-enriched asymmetrical PCR product. Samples were amplified using the same PCR program outlined above for the asymmetrical PCR step.

In order to obtain clones for DNA sequencing, the microsatellite-enriched, double-stranded PCR product was ligated into BlueScript KS^+ plasmid vectors and transformed into competent *E. coli* cells using INVαF' One Shot™ Competent Cells (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. Transformed cells were incubated overnight at 37° C on LB/ ampicillin/ X-GAL plates using standard protocols (Sambrook et al. 1989). White transformed colonies were restreaked on a fresh plate, incubated overnight as before, and screened for weakfish DNA inserts. Transformed colonies were scraped from the plate, resuspended in 35 µl STE (100 mM NaCl, 20 mM Tris-HCL, 10 mM EDTA, pH 7.5) and extracted once with an equal volume of phenol/chloroform (24:1). The resulting supernatant was treated with 1 µl RNAase (1mg/ml) and electrophoresed against a standard of non-transformed plasmid DNA on a 1.5% agarose gel in 1X TBE buffer. Plasmid DNA from clones potentially containing inserts was then extracted using the PERFECT®prep kit (5 prime→ 3 Prime, Inc.,

Boulder, CO, USA), digested with the enzyme *BssH* II (Stratagene) and electrophoresed on a 1.5% agarose gel in 1X TBE to confirm the presence of an insert.

Clones with weakfish DNA inserts were sequenced using the T7 promoter and M13 reverse primer according to the Sanger et al. (1977) dideoxy chain-termination method using the Sequenase® Sequencing Kit (United States Biochemical, Cleveland, OH, USA) and visualized by autoradiography using S^{32} labeling (Sambrook et al. 1989).
PCR primers were designed for those clones containing microsatellite sequences using the program PC/Gene (Intelligenetics Inc., Geneva, Switzerland).

Adaptation of Existing Microsatellite Loci Primers. Two sets of microsatellite primers developed for red drum *Sciaenops ocellata* (Turner et al. 1998), and two sets of primers developed for red drum and spotted seatrout *Cynoscion nebulosus* (Robert Chapman, Marine Resources Research Institute, Department of Natural Resources, Charleston, South Carolina, unpublished data) were used to amplify weakfish DNA in 25 µl reactions containing 21.125 µl sterile dH_2O, 2.5 µl 10X PCR buffer with 15 mM $MgCl_2$, 0.5 µl 10 mM dNTP mixture, 0.25 µl forward and reverse primers (100 pm· μl^{-1}), 0.125 µl of *Taq* I polymerase (5 U· μl^{-1}), and 0.25 µl weakfish DNA. Samples were denatured for 4 min at 95° C, followed by 25 cycles of PCR amplification performed under the following conditions: 1 min at 94° C, 1 min at 50° C, and 3 min at 65° C. Reactions were given a final 10 min extension at 65° C. Single products in the correct size range for each primer set were then cloned using the Original TA Cloning® Kit (Invitrogen) following manufacturer's instructions. Clones were screened for inserts as described previously, and inserts were sequenced according to manufacturer's protocols on a Model 4000

Automated DNA Infrared Sequencer from Li-Cor (Lincoln, NE, USA). Sequences were run on 4% Long Ranger™ (FMC Bioproducts) polyacrylamide gels using The Thermoseqenase Kit (Amersham, Cleveland, OH, USA) to confirm the presence of microsatellites.

Development of Actin and Ribosomal Protein 2 Introns. Universal actin gene primers developed by G. Warr and M. Wilson (cited in Reece et al. 1997) were used to amplify actin gene regions in weakfish DNA. The PCR reaction mix and program were the same as for the red drum microsatellite loci given in the *Adaptation of Existing Microsatellite Loci Primers* section above, with the exception that the annealing temperature was lowered to 45° C. Three fragments of different sizes (800, 1200, and 1300 bp) were amplified, presumably representing three different loci in the actin gene family. Fragments were cloned and sequenced using the procedure outlined in the previous section. Sequences of the 800 bp fragment were aligned using GeneJockey II software from Biosoft (Cambridge, UK) with actin gene sequences from bluefin tuna (Kimberly Reece, Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, unpublished data) to confirm their identity and then used to design weakfish primers for an actin intron (CRESIA1) using PC/Gene (Intelligenetics Inc., Geneva, Switzerland).

PCR amplification using S7 ribosomal protein intron 2 primers originally developed from swordfish *Xiphius gladius* (RP2; Chow and Hazama 1998) yielded a single product in weakfish. PCR was performed with the same reaction mix as used for the red drum loci given in the *Adaptation of Existing Microsatellite Loci Primers* section above. Samples

were denatured for 4 min at 95° C, followed by 34 cycles of PCR amplification performed under the following conditions: 1 min at 94° C, 1 min at 60° C, and 3 min at 72° C. Reactions were given a final 10 min extension at 72° C. The product was cloned and sequenced as described in the *Adaptation of Existing Microsatellite Loci Primers* section, and the sequence was matched to published sequences in Genbank to confirm its identity as a ribosomal protein gene intron. The original primers were then used without modification in subsequent population analysis.

Weakfish Stock Structure Analysis. Microsatellite, actin, and RP2 loci were amplified from YOY weakfish DNA collected at the five locations in Fig. 4. Primer sequences are given in Table 4. Amplifications of all microsatellite loci were carried out in 10 µl reactions containing 8.30 µl sterile dH_2O, 1.0 µl 10X PCR buffer with 15 mM $MgCl_2$, 0.20 µl 10 mM dNTP mixture, 0.05 µl forward primer (100 pm· μl^{-1}) labeled with a fluorescent dye (Licor), 0.20 µl reverse primer (100 pm· μl^{-1}), 0.05 µl *Taq* I polymerase (5 U·μl^{-1}), and 0.20 µl weakfish DNA. Samples were first denatured for 4 min at 95° C, followed by 32 cycles of PCR amplification performed under the following conditions: 1 min at 94° C, 1 min at 50° C, and 1 min at 72° C. Reactions were given a final 7 min extension at 72° C. PCR product alleles were separated electrophoretically on a 6% Long Ranger™ polyacrylamide gel using the Li-Cor automated sequencer. A fluorescent-labeled sequence of nuclear DNA derived from a major histocompatibility complex (MHC) locus in blue marlin *Makaira nigricans* was used as a size standard (Vincent Buonaccorsi, National Marine Fisheries Service, La Jolla, CA; unpublished data).

CRESIA1 and RP2 PCR amplifications used in the stock structure analysis were

performed under the same conditions as those outlined for the actin and ribosomal protein 2 introns. Amplified CRESIA1 products were digested with a panel of restriction endonucleases (*Alu* I, *Bgl* I, *Hae* II, *Hinf* I, *Msp* I, *Rsa* I, *Ava* I, *Ava* II, *Ban* II, *Bcl* I, *Dra* I, *Dde* I, *EcoR* I, *EcoR* II, H*ha* II, *Hpa* II, *Mbo* I, *Mse* I) and separated on 2.5% agarose gels using 1% NuSieve and 1.5% agarose in 1X TBE buffer. Gels were stained and photographed as described in Chapter I Materials and Methods and a subset of each population was screened for enzymes that revealed polymorphism. Amplified RP2 products were also digested with a series of restriction endonucleases (*Alu* I, *Bgl* I, *Hae* II, *Hinf* I, *Msp* I, *Rsa* I) and screened for polymorphisms in the same manner. Only the enzyme *Dra* I revealed polymorphism in the CRESIA1 marker. Both *Alu* I and *Hinf* I revealed polymorphism in the RP2 marker, but the large number of small bands (< 150 bp) produced by digestion with *Alu* I made these gels difficult to score. Due to these results, the enzymes *Dra* I (CRESIA1) and *Hinf* I (RP2) were chosen for use in the RFLP analyses.

Data Analysis. Microsatellite gel images were stored on an IBM-compatible computer directly from the Li-Cor automated sequencer and analyzed using the software program RFLPScan Plus 3.0 (Scanalytics, Billerica, MA, USA), where allele sizes (in bp) were estimated as outlined in Chapter 1 Materials and Methods. A single loading on a gel typically comprised 51 lanes, three of which contained size ladders and the other 48 containing the PCR-amplified microsatellite of individual fish (Fig 5). Each sample lane represented the genotype of the individual as either one (homozygote) or two (heterozygote) bands.

Restriction enzyme digestion patterns for CRESIA1 and RP2 were also analyzed using RFLPScan on an IBM-compatible computer equipped with a scanner as outlined in Chapter 1 Materials and Methods.

Statistical analyses for all loci were performed in the Arlequin 1.1 software program of Schneider et al. (1997). Nonparametric, exact–significance tests (exact θ significance tests and exact probability tests) were used to evaluate sample genotype distributions for departures from Hardy-Weinberg expectations. Unbiased estimators of exact significance probabilities for the Hardy-Weinberg equilibrium tests were calculated using the Markov chain algorithm of Guo and Thompson (1992) with a Markov chain length of 100,000 steps. Patterns of genetic diversity and divergence within and between populations were evaluated using the analysis of molecular variance (AMOVA) of Excoffier et al. (1992), which generates *F*-statistics analogous to the θ values of Wier and Cockerham (1984). Significance of *F*-statistics was evaluated using exact *F* permutation procedures (Excoffier et al. 1992). Type I error was controlled for all multiple testing using the sequential Bonferroni method of Rice (1989).

Results

Sample Collections. Length-frequency distributions of YOY weakfish sampled in the summers of 1996 and 1997 are presented in Figures 6 and 7, respectively. Only fish <140mm in length were used in the study, since individuals in this size ranged are presumed to have hatched in the current spawning season (Wilk 1976). Because fish in this size class are not believed to move out of their natal spawning areas (Wilk 1976, Rowe and Epifanio 1994), exchange of YOY fish between sampled locations was assumed to be negligible.

Novel Microsatellite Loci. Approximately 200 transformed colonies were screened for the presence of a weakfish DNA insert. Sixteen inserts in a size range of 200-500 bp were chosen randomly and sequenced to determine if they contained a microsatellite locus (multiple tandem repeats). Five of the 16 did not contain microsatellite motifs. Three of the remaining 11 inserts had a mix of tetra- and dinucleotide repeats and were rejected for use, since alleles of the same length could not be assumed to have identical sequences (i.e. a loss of one tetranucleotide repeat would result in the same allele length as the loss of two dinucleotide units). Three of the remaining eight inserts had regions flanking the microsatellite repeats that were suitable for designing primers. Primers that successfully amplified the microsatellite regions in two of these inserts (CRE66 and CRE80, Table 5)

were designed and used to screen a subset of 24 fish from each 1996 weakfish population for length polymorphisms. Both loci revealed a single allele across all populations and were not considered further.

Red Drum and Spotted Seatrout Microsatellite Loci. Microsatellite motifs for the three red drum (SOC050, SOC044, and SOC014) and a single spotted seatrout (CNE 612) loci are given in Table 5. All four loci were polymorphic in all weakfish samples from both years. Allele frequency distributions for each locus are shown in Figure 8. Sample allele frequencies for each locus are given in Table 6, while sample sizes, number of alleles, expected heterozygosities (gene diversities), and significance test results for Hardy-Weinberg equilibrium are given in Table 7.

The smallest number of alleles (2) was seen in the SOC014 locus. Allele frequencies for the most common form (114 bp) in all sample locations ranged from 90.7-97.7% (mean = 95.1%) in 1996 and 92.3-98.2% (mean = 96.1%) in 1997 (Table 6). Expected heterozygosities for this locus ranged from 0.038 in the Delaware Bay 1996 sample to 0.170 in the Georgia 1997 sample (Table 7). None of the SOC014 sample genotype distributions differed significantly from Hardy-Weinberg expectations after correcting for multiple tests (Rice 1989).

The SOC044 locus exhibited three alleles, although the rarest form (200 bp) appeared only once in a single population, North Carolina 1996 (Table 6). Frequencies of the 202 bp allele, the most common form in all sample locations, ranged from 69.6-85.5% (mean = 75.6%) in 1996 and 70.5-88.5% (mean = 78.1%) in 1997. Expected heterozygosities ranged from 0.203 in the Delaware Bay 1997 sample to 0.434 in the North Carolina 1996

sample (Table 7). Again, none of the sample genotype distributions differed significantly from Hardy-Weinberg expectations after correcting for multiple tests.

The seven alleles exhibited by the SOC050 locus were arranged in a roughly unimodal distribution (Fig. 8) that was discontinuous due to the absence of any samples with the 199 bp allele. Overall the 193 bp allele was the most common, with frequencies ranging from 33.7-44.9% (mean = 41.1%) in 1996 and 27.3-38.5% (mean = 35.9%) in 1997 (Table 6). The 191 bp allele was also common, with frequency means of 27.1% in 1996 and 32.1% in 1997, and displaced the 193 bp allele as the dominant form in the Georgia 1997 sample (34.8% vs. 27.3%). Expected heterozygosities for this locus ranged from a low of 0.694 in the Chesapeake Bay 1996 sample to a high of 0.758 in the Georgia 1997 sample. None of the SOC050 sample genotype distributions differed significantly from Hardy-Weinberg expectations after correcting for multiple tests.

Frequencies of the 34 alleles exhibited by the CNE612 locus were arranged in a continuous, roughly unimodal distribution (Fig. 8). The most common allele overall (123 bp) ranged in frequency from 11.6-20.6% (mean = 16.3%) in 1996 to 10.6-16.6% (mean = 13.5%) in 1997 (Table 6). Two other common alleles exhibited higher frequencies in three of the sample locations (Table 6). The 133 bp allele was more frequent in the North Carolina 1997 sample (13% vs. 12%), while the 119 bp allele was more common in the Delaware Bay 1996 sample (19% vs. 14%), the North Carolina 1997 sample (13.9% vs. 12%), and the Chesapeake Bay 1997 sample (14.4% vs. 10.6%). Expected heterozygosities for this locus ranged from 0.912 in the Delaware Bay 1996 sample to 0.943 in the North Carolina 1996 sample. None of the sample genotype distributions for

this locus differed significantly from Hardy-Weinberg expectations after correcting for multiple tests (Rice 1989).

To test for population structuring microsatellite loci were analyzed individually and as a combined data set. AMOVA test results (Table 8) did not reveal significant differences between sample locations or years for any of the four loci or for the combined data (but see results for the Georgia 1997 SOC050 locus in the *Inclusion of Non-target Species in Weakfish Samples* section below). At least 98% of the variation was contained in the within population component of the AMOVA analyses for all loci.

Single-locus population pairwise F_{ST} values were relatively low, with values of $F_{ST} \leq$ 0.000 for many of the comparisons across all four loci (Table 9). Mean F_{ST} for SOC014 was 0.005, with the highest value ($F_{ST} = 0.040$) occurring in the comparison between New York 1996 and Delaware Bay 1997. Mean F_{ST} for SOC044 was 0.018, with a high of $F_{ST} = 0.089$ between North Carolina 1996 and Delaware Bay 1997. The highest F_{ST} value for SOC050 (mean = 0.002) was $F_{ST} = 0.018$ between Georgia 1997 and both North Carolina 1996 and New York 1996.). Mean F_{ST} for CNE612 was 0.002, with the highest value ($F_{ST} = 0.007$) occurring between Chesapeake Bay 1996 and Delaware Bay 1996. Exact *F* permutation tests were not significant for any of the four loci or the combined data set after correction for multiple testing (Table 9).

Actin and Ribosomal Protein 2 Introns. Digestion of actin intron amplifications with the restriction endonuclease *Rsa* I revealed a single polymorphic restriction site that produced two alleles (Fig. 9, Table 10). The most common allele (A) contained the restriction site and consisted of two bands 419 bp and 135 bp in length. The second allele

(B) did not have the restriction site and was left undigested, resulting in a single band 554 bp in length. Sample allele frequencies are given in Table 11, while sample sizes, number of alleles, expected heterozygosities, and significance test results for Hardy-Weinberg equilibrium are given in Table 12. Expected heterozygosities ranged from 0.000 for the monomorphic Georgia 1997 sample to 0.096 for the Chesapeake Bay 1996 sample. None of the sample genotype distributions differed significantly from Hardy-Weinberg expectations after correcting for multiple tests (Rice 1989).

AMOVA test results for the actin locus detected no significant differences between sample locations or years (Table 13). Effectively all of the variation was contained in the within population component of the AMOVA analysis. Single-locus population pairwise F_{ST} values were consistently low (mean = 0.005), ranging from $F_{ST} < 0.000$ for most of the comparisons to an F_{ST} of 0.035 between Georgia 1996 and Georgia 1997 and between Chesapeake Bay 1996 and Georgia 1997 (Table 14). A single exact F permutation test, between Delaware 1996 and Georgia 1997, was significant after correction for multiple testing ($P < 0.001$; Table 14).

Digestion of the 731 bp RP2 amplifications with the restriction endonuclease *Hinf* I resulted in two alleles (Fig. 9, Table 10). The most common allele (A) displayed three restriction sites, yielding four bands 224 bp, 224 bp, 158 bp, and 125 bp in length. The second allele (B) lacked one of these restriction sites, resulting in three bands 382 bp, 224 bp, fragment and 125 bp in length. Sample allele frequencies are given in Table 11, while sample sizes, number of alleles, expected heterozygosities, and significance test results for Hardy-Weinberg equilibrium are given in Table 12. Expected heterozygosities ranged from 0.194 in the Delaware Bay 1997 sample to 0.370 in the Georgia 1997 sample. None

of the sample genotype distributions differed significantly from Hardy-Weinberg expectations after correcting for multiple tests (Rice 1989).

AMOVA test results for the RP2 locus revealed no significant differences between sample locations or years (Table 13). As with the CRESIA1 locus, effectively all of the variation was contained in the within population component of the AMOVA analysis. Single-locus population pairwise F_{ST} values were low (mean = 0.006), ranging from F_{ST} < 0.000 for most of the comparisons to a high of 0.050 between Georgia 1997 and Delaware Bay 1997 (Table 14). Exact *F* permutation tests were not significant after correction for multiple testing (Table 14).

Inclusion of Non-target Species in Weakfish Samples. During initial analysis of the 1996 and 1997 SOC050 microsatellite data a significant departure of genotypic frequencies from expectations of Hardy-Weinberg equilibrium was seen in the Georgia 1997 sample (P= 0.005), even after correction for multiple tests (Rice 1989). Initial SOC050 AMOVA results indicated a significant within-population variance (Table 15), and exact *F* permutation tests of population pairwise F_{ST} values resulted in a number of near-significant *P* values after correction for multiple tests, all involving the Georgia 1997 sample (Table 16). Close inspection of the Georgia 1997 SOC050 allele frequencies revealed a bimodal distribution of weakfish allele sizes that was not evident in the other samples in either year (Fig. 10). It was suspected that fish with one or both of their alleles in the smaller mode (< 187 bp) might represent misidentified species, F_1 hybrids, or carriers of introgressed alleles.

To further investigate this discrepancy, these samples were analyzed using the 12S/16S rRNA mitochondrial marker developed in Chapter I. RFLP analysis of the 12S/16S region of these fish yielded three distinct digestion patterns (Fig. 11, Table 17), including the weakfish pattern and two others (unknowns A and B) that did not match any of the 16 species tested previously. To determine the identity of the unknown patterns, voucher samples of five additional sciaenid species occurring in the western Atlantic and/or the Gulf of Mexico (sand seatrout *Cynoscion arenarius*, silver seatrout *Cynoscion nothus*, banded drum *Larimus fasciatus*, Gulf kingfish *Menticirrhus littoralis*, and star drum *Stellifer lanceolatus*) were obtained and analyzed using the 12S/16S mitochondrial marker. Unknown digestion pattern A matched the pattern exhibited by silver seatrout *Cynoscion nothus*, while unknown digestion pattern B matched the pattern exhibited by sand seatrout *Cynoscion arenarius* (Fig. 11, Table 17).

The voucher silver seatrout *Cynoscion nothus* and sand seatrout *Cynoscion arenarius* samples were amplified with the SOC050 primers to characterize the range of alleles in these species, at least as far as the small sample sizes allowed (Table 18). *Cynoscion nothus* samples exhibited alleles ranging in size from 175-181 bp, while *Cynoscion arenarius* samples exhibited alleles ranging in size from 175-193 bp.

By combining the nuclear and mitochondrial data, anomalous individuals from the Georgia 1997 sample were divided into three general classes: individuals with weakfish *Cynoscion regalis* mtDNA and a single aberrant nuclear allele (n= 3), individuals with sand seatrout *Cynoscion arenarius* mtDNA and a single aberrant nuclear allele (n= 2), and individuals with either sand seatrout *Cynoscion arenarius* or silver seatrout *Cynoscion nothus* mtDNA and two aberrant nuclear alleles (n= 3 and 7, respectively;

Table 19). Presumably pure silver seatrout *Cynoscion nothus* (n = 7) from the Georgia 1997 sample exhibited silver seatrout mtDNA and two alleles ranging in size from 175-179 bp. Presumably pure sand seatrout *Cynoscion arenarius* (n = 3) from the Georgia 1997 sample exhibited sand seatrout mtDNA and two alleles ranging in size from 171-177 bp. Reevaluation of the remaining 1996-97 SOC050 data revealed occasional occurrences of anomalous alleles in all but the New York samples (Table 19), although frequencies were apparently low enough (Table 20) not to disturb Hardy-Weinberg equilibrium tests or produce significant AMOVA or exact *F* permutation test values. A single silver perch *Bairdiella chrysoura* was found in the Chesapeake Bay 1997 sample (silver perch mtDNA and two alleles 171 bp in size). All other questionable individuals were putative hybrids with weakfish *Cynoscion regalis* mtDNA and a single aberrant allelecharacteristic of silver and sand seatrout. Analysis of subsamples of 20 weakfish taken from each of the four locations outside of Georgia with the 12S/16S marker revealed only weakfish mtDNA.

As a result of these findings all individuals in the 1996-97 data exhibiting a least one anomalous allele (< 183 bp) were eliminated from the analyses reported above.

Discussion

Previous Studies. Weakfish population studies based on allozymes (Crawford et al. 1989) and restriction fragment-length polymorphism (RFLP) analysis of whole molecule mtDNA (Graves et al. 1992) were unable to reject the null hypothesis of a single genetic stock. The low overall genetic variation revealed by both techniques reduced the power of the analyses, and suggested that larger sample sizes or a more sensitive marker would be needed to detect small genetic differences between populations if they did indeed exist. The purpose of the present study was to develop a set of nuclear DNA markers that would exhibit enough variation (as indicated by higher levels of heterozygosities) to adequately investigate stock structure in the weakfish.

Microsatellite Data. The number of alleles per locus and levels of expected heterozygosity (H_{exp}) seen in the four microsatellite loci presented here followed the general expectations that greater variation is found in microsatellite loci comprised of smaller repeat units (Schlötterer and Tautz 1992) and larger numbers of repeat units per loci (Weber 1990). Both loci based on tetranucleotide repeats developed directly from weakfish DNA proved monomorphic, and the dinucleotide loci with the smallest number of repeats (SOC014) also had the smallest number of alleles (2) and the lowest range of H_{exp}. Expected heterozygosities for the four microsatellite loci ranged from a low of 3.8-

17% for SOCO14 to a high of 91.2-94.3% for CNE612 (Table 7). These values are consistent with heterozygosity ranges reported in other multi-locus microsatellite studies on species including Atlantic cod (Bentzen et al. 1996), northern pike (Miller and Kapuscinski 1996), pink and sockeye salmon (Seeb et al. 1998), and arctic charr (Brunner at al. 1998). In contrast, Crawford et al. (1989) found very low levels of genetic variation in weakfish allozymes, and Graves et al. (1992) reported nucleon diversities (a measure used in mtDNA analyses analogous to heterozygosity) ranging from 7.9-23%. This range is similar to the range of H_{exp} found in SOC014, but is substantially lower than ranges for the other three loci, suggesting that the more variable microsatellites loci employed in this study may be more sensitive markers and better able to detect population differences.

Nuclear Intron Data. Levels of genetic variation within the two intron regions developed here fell within the limits reported for allozyme (Crawford et al. 1989) and whole molecule mtDNA (Graves et al. 1992) markers used in weakfish stock structure analyses, and were low compared to the heterozygosities found in the microsatellite loci discussed above. Each locus exhibited two alleles, and expected heterozygosities ranged from 0-9.6% for CRESIA1 and 19.4-37.0% for RP2. Another study utilizing nuclear intron RFLP analysis showed similar levels of heterozygosity in Pacific salmon (Moran et al. 1997), as did RFLP studies of anonymous single copy nuclear (ascn) DNA loci in Atlantic cod *Gadus morhua* (Pogson et al. 1995) and blue marlin *Makaira nigricans* (Buonaccorsi et al. 1999). In contrast, higher heterozygosities (44-58%) were reported in an ascnDNA/RFLP analysis of striped bass *Morone saxatilis* by Leclerc et al. (1996). Better detection of variation using RFLP analysis of nuclear intron markers may be

achieved by utilizing larger introns, which are more likely to contain polymorphic restriction endonuclease recognition sites. Alternatively, the increased time and cost involved in finer-resolution analyses such as sequencing may be justified by the increase in detected polymorphisms when dealing with species traditionally exhibiting low levels of variation.

Management Implications. The results of the stock structure analyses based on the four microsatellite loci and two nuclear intron regions reported above were unable to reject the null hypothesis that weakfish *Cynoscion regalis* comprise a single, genetically homogeneous stock in the U.S. western Atlantic. Temporal stability of allele frequencies is often assumed in stock structure analyses, and researchers will sometimes compare or combine samples from different years without first testing the validity of this practice. No significant differences in allele frequencies occurred among any of the sampled locations or between sample years, indicating both geographic and temporal stability of allele frequencies in weakfish, at least over the two years of this study.

Characterization of weakfish as a single, homogeneous stock has important implications for fisheries managers. Considering the drastic fluctuations in weakfish catches exhibited over the past century (see Lowerre-Barbieri 1994 for review), a management plan aimed at wisely harvesting the standing stock is clearly needed. If weakfish do indeed comprise a single stock, then development of such a plan would have to include fisherman, managers, and scientists from New York to Florida. A single stock model would also lend credence to the idea that bycatch of YOY weakfish in the southern shrimp fishery is impacting adult weakfish catches in more northern waters (Mercer 1983,

Vaughan et al. 1991). Unfortunately, the amount of genetic exchange between locations necessary to eliminate evidence of stock subdivision based on genetic markers may be as little as a few individuals per generation (Allendorf and Phelps 1981), a rate of mixing negligible in terms of fisheries management. In order to better assess the exchange of weakfish between locations, traditional methods such as tagging studies could be employed, although the scale of the project needed to insure sufficient returns would be prohibitive in terms of time and expense.

A better means of estimating the contribution of various regions to the weakfish stock may be the use of otolith microchemistry. Thorrold et al. (1998), using the same samples as the present study, were able to correctly assign YOY weakfish to their natal waters using unique elemental concentrations in their otoliths. This same technique could be used to identify the natal origin of adult weakfish by sampling their otolith cores. By comparing the place of birth with the place of capture, an estimate of the amount of mixing between locations could be possible (Thorrold et al. 1998). This assumes that either the chemical signature of specific locations is stable between years, or that baseline data derived from YOY weakfish is available for each adult cohort studied. It is hoped that the combined use of genetic and non-genetic techniques such as this will become more commonplace, and that such studies will help present clearer pictures of the genetic structure and mixing dynamics underlying fishery stocks.

Polyspecific Samples and Putative Hybrids. The bimodal distribution of SOC050 alleles and a significant departure of allele distributions from Hardy-Weinberg expectations in the Georgia 1997 collection suggested that non-target species might have

been included in this sample. MtDNA digestion patterns of these questionable individuals were generated using the 12S/16S marker developed in Chapter 1 and compared to the 16 species characterized in that study, as well as five other species of sciaenids common to the U.S. Atlantic and Gulf of Mexico. Application of this marker in conjunction with the results of the SOC050 microsatellite locus clearly showed that two different species other than weakfish were inadvertently included in the Georgia 1997 sample. In addition, these markers revealed a misidentified individual in the Chesapeake Bay 1997 collection, and suggested that introgression of non-weakfish DNA had occurred in all of the sample locations except New York.

A single individual from the Chesapeake Bay 1997 sample exhibited two SOC050 alleles (171 bp) smaller than the typical size range found in weakfish. Analysis of this individual with the 12S/16S marker identified it as a silver perch *Bairdiella chrysoura*. Inclusion of this specimen in the weakfish collection was probably due to a simple oversight while sorting YOY fish of closely related species.

More intriguing is the situation found in the Georgia 1997 sample. Analysis of individuals with the smaller SOC050 alleles using the 12S/16S marker revealed two distinct mtDNA digestion patterns other than weakfish. Comparison with known samples clearly identified seven of these individuals as silver seatrout *Cynoscion nothus*. The inclusion of these individuals in the collection may not be surprising, since both weakfish and silver seatrout are common in the southern Atlantic Bight (Bigelow and Schroeder 1953, Hildebrand 1955) and are difficult to distinguish during their early life history stages. Although the later species is known to inhabit deeper waters as adults (Ginsburg

1931), both species are inshore summer spawners (Devries and Chittenden 1982, Shepherd and Grimes 1984).

Data also suggest the presence of a third species of Cynoscion in the Georgia 1997 collection. Three fish with SOC050 alleles clearly smaller than the range seen in weakfish had a mtDNA haplotype that matched the haplotype found in sand seatrout *Cynoscion arenarius* (Figure 11). There is some question as to the taxonomic relationship between weakfish and sand seatrout. Some suggest they may be separate populations of a single species (Moshin 1973, Weinstein and Yerger 1976, Cowan 1985, Ditty 1989), while others treat them as separate species (Schlossman and Chittenden 1981) with distributions confined to the western Atlantic (weakfish) and the Gulf of Mexico (sand seatrout). Paschall (1986) was unable to distinguish between the two species using allozyme electrophoresis. In contrast, results presented here are consistent with the existence of two distinct species and that sand seatrout co-occur off the east coast of the United States at least as far north as Doboy Sound, Georgia.

Current distributions of weakfish and sand seatrout may be explained in the context of biogeographic patterns exhibited by other species common to the Atlantic and Gulf regions. Avise (1992) reviewed the phylogeographic patterns of 19 freshwater, coastal, and marine species distributed along the U.S. East Coast and the Gulf of Mexico that exhibited geographically concordant forks in their intra- and interspecific mtDNA phylogenies. Patterns in a number of the marine species seemed to repesent a continuum of scenarios based on historical separations and subsequent contact around the Florida peninsula. A number of species including hardhead catfish *artius felis* (Avise et al. 1987) and American eel *Anguilla rostrata* (Avise et al. 1996) showed no mitochondrial

divergence between Atlantic and Gulf populations. In the case of toadfishes *Opsanus tau* and *O. beta* (Avise et al. 1987), sister species were separated by the Florida peninsula into Gulf and Atlantic distributions. Menhaden, traditionally divided into Atlantic (*Brevoortia tyrannus*) and Gulf (*B. patronus*) species, appeared to represent once isolated populations of a single species that reestablished contact through movement of the Gulf population into the Atlantic (Bowen and Avise 1990). In the present situation, apparently distinct Gulf (sand seatrout) and Atlantic (weakfish) species have reestablished contact in a hybrid zone (see below) through movement of the Gulf species into the Atlantic, providing yet another variation in the phylogeographic continuum mentioned above.

To further complicate matters, five individuals from the Georgia 1997 collection showed evidence of being hybrid offspring. Three of the individuals had weakfish mtDNA and one SOC050 allele that fell into the size range found in both silver seatrout (175-181 bp) and sand seatrout (175-193 bp, Table 19). Similarly, two individuals exhibited the sand seatrout mtDNA haplotype, one SOC050 allele that matched the range for sand seatrout or silver seatrout, and a larger allele consistent with the size range for weakfish. These data suggest that hybridization occurs between weakfish and sand seatrout, and that the genetic exchange is not gender biased. Because of the overlap in microsatellite allele sizes seen between silver seatrout and sand seatrout (Table 18), hybridization between weakfish and silver seatrout could not be excluded. The lack of suspected hybrids with silver seatrout mtDNA, however, suggests that hybridization did not involve this species. Interestingly, low frequencies of aberrant SOC050 alleles (range 0.008-0.034, mean = 0.013; Table 20) were found in putative hybrids from all the more northern samples except New York, although non-weakfish mtDNA was not. This may

indicate that introgressive hybridization is responsible for the migration of the smaller alleles into more northern weakfish populations, although the northward movement of hybrid fish out of the contact zone cannot be ruled out.

The possibility exists that the putative hybrids are in fact weakfish with rare mtDNA haplotypes common to the three *Cynoscion* species studied here. This seems unlikely, however, since only one weakfish pattern was noted in the species identification study reported in Chapter I. Furthermore, analysis of subsamples of 20 weakfish taken from each of the four locations outside of Georgia with the 12S/16S marker revealed no new mtDNA patterns. Also, the mtDNA haplotypes seen in sand seatrout and silver seatrout seem to be polymorphic in size and can not be clearly related to the weakfish haplotype by the addition or deletion of presumed restriction sites (Table 16, Fig. 10), conditions more in keeping with mtDNA of different species.

An argument could also be made that the smaller SOC050 alleles found in the more northern weakfish samples could be forms rare in weakfish but shared with other members of the genus (ancestral alleles). This could not be corroborated by the other three microsatellite loci used in this study because they were unable to distinguish between the three species based on allele sizes. A shift in sand seatrout and silver seatrout allele frequencies (relative to the weakfish data) towards the smaller alleles in CNE612 and the more rare allele in SOC044 was evident, however, at least based on the small number of sand seatrout and silver seatrout samples (Table 18). Because neither sand seatrout or silver seatrout were successfully amplified via PCR using the CRESIA1 and RP2 primers, corroborative evidence of introgressive hybridization will require further research.

Table 5. Primer sequences for amplifying microsatellite, actin gene intron, and ribosomal protein 2 gene intron loci in weakfish *Cynoscion regalis*.

Locus	Primer Sequence (5'-3')	Length (bp)	Repeat Sequence in Weakfish	Annealing Temp. (°C)	Original Reference
			Microsatellites		
CRE66	CRE66F: TGGTCTGTTAGTCCACAGTGTTG	251	$[GATA]_{25}$	40	This study
	CRE66R: CGTTGCCTTCATTACAGGAGAC				
CRE80	CRE80F: ACAGCATGTGAGGGTTAAGGAT	136	$[GATA]_9$	40	This study
	CRE80R: TACAGCTCTCTGACTGATGTAGTTGA				
SOC050	SOC050F: CCCGTGATTTTAGGCTCATCAGATA	193	$[GT]_4n_3[GT]_{10}n_7[GT]_9$	50	Turner et al. 1998
	SOC050R: CCTTTAGAGTGCAGTAAGTGATTT				
SOC044	SOC044F: GAGGGTGACGCTAACAGTTGA	202	$[CA]_3n_{39}[GT]_3n_5[GT]_2n_2[GT]_2$	50	Turner et al. 1998
	SOC044R: CACAGCTCCACTCTGATATG				
SOC014	SOC014F: GTATGTATTAAGGGCACAAGGTG	114	$[CA]_5$	50	Robert Chapman[a],
	SOC014R: GATTGCTGCTGGACAGACTG				unpublished data

Table 5. Continued.

CNE612	CNE612F: CAAGTGCACGGTATGTGATG	131	$[GT]_5n_{10}[GT]_{11}$	50	Chapman et al. 1999
	CNE612R: AGGAACCTGACCAATCCAAA				
			Nuclear gene introns		
CRESIA1	CRESIA1F: ATGCCTCTGGTCGTACCACTGG	545		52	This study
	CRESIA1R: CAGGTCCTTACGGATGTCG		------		
RP2	RP2F: AGCGCCAAAATAGTGAAGCC	731		60	Chow and Hazama 1998
	RP2R: GCCTTCAGGTCAGAGTTCAT		------		

[a] Marine Resources Research Institute, Department of Natural Resources, Charleston, South Carolina, unpublished data

Table 6. Allele frequencies of four microsatellite loci used to screen five populations of weakfish *Cynoscion regalis* for two consecutive years. GA= Georgia, NC= North Carolina, CB= Chesapeake Bay, DB= Delaware Bay, NY= New York.

Alleles (bp)	GA1996	NC1996	CB1996	DB1996	NY1996	GA1997	NC 1997	CB 1997	DB 1997	NY 1997
					SOC050					
187	0.167	0.133	0.210	0.152	0.206	0.212	0.221	0.182	0.128	0.194
189	0.078	0.092	0.055	0.163	0.087	0.091	0.067	0.081	0.077	0.046
191	0.255	0.275	0.281	0.337	0.206	0.348	0.298	0.291	0.346	0.324
193	0.402	0.449	0.430	0.337	0.435	0.273	0.385	0.373	0.385	0.380
195	0.030	0.031	0.016	0.000	0.011	0.000	0.019	0.055	0.013	0.019
197	0.040	0.020	0.008	0.011	0.044	0.061	0.000	0.018	0.051	0.037
201	0.010	0.000	0.000	0.000	0.011	0.015	0.010	0.000	0.000	0.000
					SOC044					
200	0.000	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
202	0.766	0.696	0.754	0.855	0.709	0.722	0.817	0.777	0.885	0.705
204	0.234	0.293	0.246	0.145	0.291	0.278	0.183	0.223	0.115	0.295

Table 6. Continued.

Alleles (bp)	GA1996	NC1996	CB1996	DB1996	NY1996	GA1997	NC 1997	CB 1997	DB 1997	NY 1997
					SOC014					
114	0.953	0.958	0.977	0.962	0.907	0.923	0.982	0.955	0.981	0.965
116	0.047	0.042	0.023	0.038	0.093	0.077	0.018	0.045	0.019	0.035
					CNE612					
99	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000
105	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.000
107	0.013	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.009
109	0.000	0.024	0.016	0.000	0.022	0.000	0.019	0.047	0.000	0.017
111	0.051	0.058	0.000	0.040	0.054	0.077	0.036	0.029	0.020	0.063
113	0.013	0.012	0.008	0.050	0.022	0.000	0.019	0.029	0.020	0.000
115	0.000	0.012	0.000	0.000	0.000	0.000	0.019	0.000	0.020	0.000
117	0.013	0.012	0.024	0.010	0.000	0.030	0.046	0.029	0.030	0.027

Table 6. Continued.

Alleles (bp)	GA1996	NC1996	CB1996	DB1996	NY1996	GA1997	NC 1997	CB 1997	DB 1997	NY 1997
119	0.141	0.093	0.097	0.190	0.076	0.136	0.139	0.144	0.120	0.080
121	0.038	0.000	0.040	0.040	0.043	0.045	0.028	0.058	0.020	0.009
123	0.193	0.116	0.161	0.140	0.206	0.136	0.120	0.106	0.160	0.151
125	0.090	0.058	0.065	0.070	0.109	0.061	0.065	0.096	0.060	0.054
127	0.038	0.058	0.033	0.050	0.043	0.015	0.028	0.047	0.040	0.054
129	0.038	0.082	0.105	0.030	0.043	0.030	0.046	0.029	0.110	0.054
131	0.064	0.105	0.040	0.070	0.065	0.092	0.046	0.096	0.050	0.071
133	0.116	0.082	0.097	0.130	0.098	0.045	0.130	0.096	0.130	0.107
135	0.064	0.047	0.056	0.000	0.033	0.061	0.028	0.029	0.050	0.036
137	0.051	0.047	0.049	0.040	0.043	0.045	0.009	0.019	0.020	0.045
139	0.026	0.036	0.024	0.030	0.011	0.000	0.019	0.029	0.010	0.080
141	0.013	0.047	0.024	0.020	0.022	0.000	0.000	0.038	0.010	0.027

Table 6. Continued.

Alleles (bp)	GA1996	NC1996	CB1996	DB1996	NY1996	GA1997	NC 1997	CB 1997	DB 1997	NY 1997
143	0.038	0.047	0.073	0.020	0.000	0.092	0.046	0.029	0.020	0.036
145	0.000	0.000	0.024	0.000	0.011	0.015	0.000	0.010	0.010	0.017
147	0.000	0.012	0.040	0.020	0.033	0.015	0.019	0.000	0.050	0.027
149	0.000	0.012	0.000	0.020	0.022	0.000	0.073	0.010	0.020	0.009
151	0.000	0.000	0.016	0.010	0.000	0.045	0.019	0.010	0.020	0.000
153	0.000	0.000	0.000	0.000	0.011	0.015	0.019	0.000	0.000	0.000
155	0.000	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.009
157	0.000	0.012	0.000	0.000	0.000	0.000	0.009	0.000	0.010	0.000
159	0.000	0.012	0.000	0.010	0.000	0.000	0.009	0.010	0.000	0.009
161	0.000	0.000	0.000	0.000	0.011	0.000	0.009	0.000	0.000	0.000
163	0.000	0.000	0.000	0.000	0.011	0.000	0.000	0.000	0.000	0.009
165	0.000	0.000	0.008	0.000	0.011	0.000	0.000	0.000	0.000	0.000
167	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.010	0.000	0.000
169	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.000

Table 7. Sample sizes (N), number of alleles (n), expected heterozygosities (H_{exp}), and *P* values for tests of Hardy-Weinberg equilibrium for four microsatellite loci. GA= Georgia, NC= North Carolina, CB= Chesapeake Bay, DB= Delaware Bay, NY= New York.

	GA 1996	NC 1996	CB 1996	DB 1996	NY 1996	GA 1997	NC 1997	CB 1997	DB 1997	NY 1997
					SOC050					
N	51	49	64	46	46	33	52	55	42	54
n	7	6	6	5	7	6	6	6	8	7
H_{exp}	0.741	0.702	0.694	0.731	0.724	0.758	0.712	0.740	0.737	0.722
P^a	0.067	0.542	0.507	0.130	0.959	0.566	0.577	0.721	0.349	0.174
					SOC044					
N	47	46	63	55	55	36	60	56	52	56
n	2	3	2	2	2	2	2	2	2	2
H_{exp}	0.362	0.434	0.374	0.251	0.416	0.407	0.302	0.350	0.203	0.419
P^a	1.000	0.019	0.496	0.303	0.512	0.010	0.669	0.116	0.0.512	0.198

Table 7. Continued.

	GA 1996	NC 1996	CB 1996	DB 1996	NY 1996	GA 1997	NC 1997	CB 1997	DB 1997	NY 1997
					SOC014					
N	43	48	64	52	54	39	56	55	52	57
n	2	2	2	2	2	2	2	2	2	2
H_{exp}	0.090	0.081	0.046	0.075	0.170	0.144	0.053	0.088	0.038	0.068
P^a	1.000	1.000	1.000	1.000	1.000	1.000	0.027	1.000	1.000	1.000
					CNE612					
N	39	43	62	50	46	33	54	52	50	56
n	17	23	20	20	22	19	25	23	22	23
H_{exp}	0.916	0.943	0.928	0.912	0.916	0.935	0.934	0.934	0.923	0.936
P^a	0.050	0.113	0.898	0.530	0.238	0.752	0.522	0.419	0.060	0.290

[a] None of the samples differed significantly from Hardy-Weinberg expectations after sequential Bonferroni corrections (α=0.005).

Table 8. Analysis of molecular variance (AMOVA) results for four microsatellite loci used to test population heterogeneity in weakfish *Cynoscion regalis*.

Source of Variation	Degrees of Freedom	Sum of Squares	Variance Components	Percentage of Variation	Significance Tests (P)[a]
			SOC050		
Among Years	1	0.530	0.00046	0.13	0.110
Among Populations/ Within Years	8	2.460	-0.00056	-0.15	0.633
Within Populations	974	352.839	0.36226	100.03	0.551
Total	983	355.829	0.36215	-	-
			SOC044		
Among Years	1	0.181	-0.00056	-0.32	0.553
Among Populations/ Within Years	8	3.755	0.00281	1.59	0.022
Within Populations	1042	182.082	0.17674	98.73	0.016
Total	1051	186.018	0.17699	-	-

Table 8. Continued.

Source of Variation	Degrees of Freedom	Sum of Squares	Variance Components	Percentage of Variation	Significance Tests (P)[a]
			SOC014		
Among Years	1	0.022	-0.000007	-0.18	0.581
Among Populations/ Within Years	8	0.481	0.00018	0.44	0.167
Within Populations	1030	42.549	0.04131	99.74	0.216
Total	1039	43.053	0.04142	-	-
			CNE612		
Among Years	1	0.32	-0.00041	-0.09	0.858
Among Populations/ Within Years	8	3.981	0.00035	0.08	0.285
Within Populations	960	445.281	0.46383	100.01	0.370
Total	969	449.564	0.46378	-	-

Table 8. Continued.

Source of Variation	Degrees of Freedom	Sum of Squares	Variance Components	Percentage of Variation	Significance Tests (P)[a]
			All Loci		
Among Years	1	0.885	-0.001	-0.080	0.611
Among Populations/ Within Years	8	9.851	0.002	0.200	0.144
Within Populations	851	897.349	1.056	99.880	0.166
Total	859	908.085	1.057	-	-

[a] None of the sample variances differed significantly from expectations after sequential Bonferroni corrections (α= 0.01).

Table 9. Population pairwise F_{ST} values (above diagonal) and exact F permutation test P [a] values (below diagonal) for four microsatellite loci. GA= Georgia, NC= North Carolina, CB= Chesapeake Bay, DB= Delaware Bay, NY= New York.

	GA 1996	NC 1996	CB 1996	DB 1996	NY 1996	GA 1997	NC 1997	CB 1997	DB 1997	NY 1997
					SOC050					
GA 1996	-	-0.004	-0.006	0.004	-0.007	0.004	-0.007	-0.007	-0.002	-0.006
NC 1996	0.851	-	-0.003	0.005	-0.002	0.018	-0.001	-0.003	-0.004	-0.001
CB 1996	0.970	0.723	-	0.010	-0.004	0.012	-0.007	-0.004	0.001	-0.005
DB 1996	0.535	0.455	0.426	-	0.014	-0.002	0.002	-0.001	-0.001	0.003
NY 1996	0.911	0.782	0.891	0.257	-	0.018	-0.001	-0.001	0.006	0.002
GA 1997	0.505	0.327	0.406	0.762	0.257	-	0.001	0.001	0.002	-0.001
NC 1997	0.911	0.713	0.950	0.614	0.634	0.663	-	-0.007	-0.001	-0.007
CB 1997	0.970	0.823	0.832	0.742	0.673	0.653	0.940	-	-0.004	-0.007
DB 1997	0.733	0.842	0.594	0.723	0.386	0.673	0.624	0.861	-	-0.005
NY 1997	0.960	0.683	0.960	0.574	0.584	0.733	0.990	0.930	0.901	-

Table 9. Continued.

	GA 1996	NC 1996	CB 1996	DB 1996	NY 1996	GA 1997	NC 1997	CB 1997	DB 1997	NY 1997
					SOC044					
GA 1996	-	0.000	-0.001	0.016	-0.002	-0.007	-0.002	-0.010	0.038	-0.001
NC 1996	0.386	-	-0.002	0.057	-0.010	-0.011	0.027	0.005	0.089	-0.010
CB 1996	0.931	0.465	-	0.023	-0.003	-0.008	0.003	-0.007	0.046	-0.002
DB 1996	0.218	0.059	0.198	-	0.051	0.043	-0.004	0.011	-0.005	0.054
NY 1996	0.564	0.990	0.604	0.109	-	-0.011	0.023	0.003	0.081	-0.009
GA 1997	0.723	0.921	0.723	0.188	0.990	-	0.015	-0.003	0.074	-0.011
NC 1997	0.465	0.119	0.465	0.653	0.109	0.337	-	-0.004	0.009	0.025
CB 1997	0.861	0.356	0.792	0.406	0.426	0.693	0.624	-	0.031	0.004
DB 1997	0.168	0.030	0.089	0.742	0.050	0.119	0.346	0.198	-	0.084
NY 1997	0.455	0.931	0.663	0.109	0.990	0.931	0.109	0.297	0.020	-

Table 9. Continued.

	GA 1996	NC 1996	CB 1996	DB 1996	NY 1996	GA 1997	NC 1997	CB 1997	DB 1997	NY 1997
					SOC014					
GA 1996	-	-0.011	-0.001	-0.010	0.005	-0.004	-0.005	-0.010	0.001	-0.008
NC 1996	0.990	-	-0.004	-0.010	0.010	0.000	-0.006	-0.010	-0.001	-0.009
CB 1996	0.366	0.792	-	-0.005	0.037	0.023	-0.008	-0.001	-0.008	-0.006
DB 1996	0.990	0.990	0.822	-	0.014	0.003	-0.007	-0.009	-0.003	-0.009
NY 1996	0.386	0.267	0.119	0.248	-	-0.010	0.029	0.008	0.040	0.019
GA 1997	0.376	0.346	0.139	0.346	0.990	-	0.017	-0.002	0.028	0.007
NC 1997	0.812	0.802	0.990	0.644	0.059	0.257	-	-0.004	-0.008	-0.008
CB 1997	0.990	0.990	0.624	0.990	0.277	0.673	0.465	-	0.001	-0.08
DB 1997	0.584	0.564	0.990	0.990	0.079	0.317	0.990	0.673	-	-0.004
NY 1997	0.990	0.990	0.713	0.990	0.099	0.455	0.802	0.752	0.990	-

Table 9. Continued.

	GA 1996	NC 1996	CB 1996	DB 1996	NY 1996	GA 1997	NC 1997	CB 1997	DB 1997	NY 1997
					CNE612					
GA 1996	-	-0.002	-0.002	-0.004	-0.004	-0.004	-0.002	-0.004	-0.003	-0.002
NC 1996	0.921	-	-0.001	0.003	0.003	-0.002	0.000	-0.002	-0.001	-0.006
CB 1996	0.960	0.931	-	0.007	0.003	0.001	0.002	0.004	-0.004	0.000
DB 1996	0.990	0.703	0.386	-	0.006	0.004	-0.002	-0.004	0.002	0.004
NY 1996	0.950	0.742	0.733	0.574	-	0.006	0.005	0.004	0.001	0.000
GA 1997	0.980	0.940	0.832	0.742	0.554	-	0.000	-0.001	0.004	0.002
NC 1997	0.990	0.931	0.802	0.970	0.574	0.901	-	-0.003	-0.003	0.001
CB 1997	0.990	0.960	0.624	0.970	0.634	0.931	0.980	-	0.002	0.001
DB 1997	0.980	0.931	0.990	0.871	0.792	0.723	0.980	0.752	-	0.000
NY 1997	0.960	0.990	0.931	0.693	0.871	0.832	0.861	0.842	0.921	-

Table 9. Continued.

	GA 1996	NC 1996	CB 1996	DB 1996	NY 1996	GA 1997	NC 1997	CB 1997	DB 1997	NY 1997
					All	**Loci**				
GA 1996	-	-0.004	-0.006	-0.002	-0.003	-0.003	-0.003	-0.006	0.000	-0.003
NC 1996	0.832	-	-0.005	0.010	-0.003	-0.004	0.003	-0.001	0.012	-0.008
CB 1996	0.960	0.980	-	0.008	0.001	0.001	-0.001	-0.001	0.006	-0.004
DB 1996	0.802	0.168	0.208	-	0.017	0.010	-0.001	-0.003	-0.004	0.009
NY 1996	0.832	0.772	0.554	0.030	-	0.000	0.012	0.006	0.021	0.000
GA 1997	0.663	0.832	0.505	0.188	0.544	-	0.008	0.003	0.017	-0.003
NC 1997	0.832	0.485	0.812	0.832	0.099	0.168	-	-0.004	-0.002	0.002
CB 1997	0.921	0.772	0.762	0.871	0.307	0.416	0.980	-	-0.001	0.000
DB 1997	0.594	0.059	0.346	0.901	0.010	0.040	0.802	0.673	-	0.011
NY 1997	0.851	0.990	0.990	0.158	0.663	0.792	0.426	0.614	0.139	-

[a] None of the exact F permutation test P values were significant after sequential Bonferroni corrections (α=0.005).

Table 10. Restriction digestion patterns of the actin intron (CRESIA1) and ribosomal protein 2 intron (RP2) gene regions in weakfish *Cynoscion regalis*.

Locus	Enzyme	Pattern	Band Size (bp)				Total Size (bp)
CRESIA1	*Rsa* I	A	414	131			545
		B	545				
RP2	*Hinf* I	A	224	224	158	125	731
		B	382	224	125		

Table 11. Allele frequencies of the actin intron (CRESIA1) and ribosomal protein 2 intron (RP2) gene regions in five geographic samples of weakfish *Cynoscion regalis* for 1996 and 1997. GA= Georgia, NC= North Carolina, CB= Chesapeake Bay, DB= Delaware Bay, NY= New York.

Alleles (bp)	GA1996	NC1996	CB1996	DB1996	NY1996	GA1997	NC 1997	CB 1997	DB 1997	NY 1997
					CRESIA1					
A	0.950	0.975	0.950	0.988	0.988	1.000	0.990	0.954	0.967	0.973
B	0.050	0.025	0.050	0.012	0.012	0.000	0.010	0.046	0.033	0.027
					RP2					
A	0.864	0.889	0.878	0.869	0.854	0.759	0.865	0.888	0.893	0.854
B	0.136	0.111	0.122	0.131	0.146	0.241	0.135	0.112	0.107	0.146

Table 12. Sample sizes (N), number of alleles (n), expected heterozygosities (H_{exp}), and *P* values for tests of Hardy-Weinberg equilibrium for the actin intron (CRESIA1) and ribosomal protein 2 intron (RP2) gene regions in weakfish *Cynoscion regalis*. GA= Georgia, NC= North Carolina, CB= Chesapeake Bay, DB= Delaware Bay, NY= New York. NT= monomorphic sample not tested.

	GA 1996	NC 1996	CB 1996	DB 1996	NY 1996	GA 1997	NC 1997	CB 1997	DB 1997	NY 1997
					CRESIA1					
N	40	42	40	42	40	36	51	54	45	55
n	2	2	2	2	2	1	2	2	2	2
H_{exp}	0.096	0.089	0.031	0.055	0.096	0.000	0.025	0.053	0.047	0.020
P^a	0.076	0.091	1.000	0.036	0.078	NT	1.000	1.000	1.000	1.000
					RP2					
N	48	45	45	42	41	29	48	49	42	41
n	2	2	2	2	2	2	2	2	2	2
H_{exp}	0.237	0.200	0.217	0.230	0.253	0.373	0.237	0.201	0.194	0.253
P^a	0.184	0.432	0.104	0.120	0.180	0.298	0.189	0.465	0.052	0.179

[a] None of the samples differed significantly from Hardy-Weinberg expectations after sequential Bonferroni corrections (α=0.005).

Table 13. Analysis of molecular variance (AMOVA) results for the actin intron (CRESIA1) and ribosomal protein 2 intron (RP2) gene regions in weakfish *Cynoscion regalis*.

Source of Variation	Degrees of Freedom	Sum of Squares	Variance Components	Percentage of Variation	Significance Tests (P)[a]
			CRESIA1		
Among Years	1	0.008	-0.001	-0.180	0.569
Among Populations/ Within Years	8	0.229	<0.001	0.080	0.628
Within Populations	860	23.101	0.269	100.100	0.672
Total	869		0.268	-	-
			RP2		
Among Years	1	0.032	<0.001	-0.14	0.635
Among Populations/ Within Years	8	0.829	<0.001	-0.13	0.682
Within Populations	850	99.493	0.117	100.27	0.747
Total	859	100.353	0.117	-	-

Table 14. Population pairwise F_{ST} values (above diagonal) and exact F permutation test P values (below diagonal) for the actin intron (CRESIA1) and ribosomal protein 2 intron (RP2) gene regions in weakfish *Cynoscion regalis*. GA= Georgia, NC= North Carolina, CB= Chesapeake Bay, DB= Delaware Bay, NY= New York.

	GA 1996	NC 1996	CB 1996	DB 1996	NY 1996	GA 1997	NC 1997	CB 1997	DB 1997	NY 1997
					CRESIA1					
GA 1996	-	-0.002	-0.013	0.003	0.010	0.035	0.019	-0.011	-0.008	-0.004
NC 1996	0.248	-	-0.002	-0.012	-0.009	0.009	-0.005	-0.003	-0.010	-0.010
CB 1996	0.990	0.267	-	0.003	0.010	0.035	0.019	-0.011	-0.008	-0.004
DB 1996	0.496	0.663	0.426	-	-0.014	0.002	-0.011	0.001	-0.007	-0.009
NY 1996	0.455	0.990	0.535	0.990	-	-0.001	-0.011	0.008	-0.002	-0.006
GA 1997	0.426	0.990	0.485	**<0.001**[a]	0.990	-	-0.004	0.028	0.018	0.011
NC 1997	0.416	0.782	0.327	0.990	0.990	0.465	-	0.014	0.003	-0.001
CB 1997	0.990	0.584	0.990	0.495	0.366	0.307	0.307	-	-0.008	-0.004
DB 1997	0.871	0.990	0.782	0.772	0.723	0.475	0.218	0.861	-	-0.010
NY 1997	0.544	0.990	0.564	0.990	0.752	0.505	0.515	0.337	0.990	-

Table 14. Continued.

	GA 1996	NC 1996	CB 1996	DB 1996	NY 1996	GA 1997	NC 1997	CB 1997	DB 1997	NY 1997
					RP2					
GA 1996	-	-0.008	-0.010	-0.011	-0.011	0.024	-0.010	-0.008	-0/008	-0.011
NC 1996	0.733	-	-0.011	-0.010	-0.006	0.047	-0.008	-0.011	-0.012	-0.006
CB 1996	0.842	0.990	-	-0.011	-0.009	0.036	-0.010	-0.010	-0.010	-0.009
DB 1996	0.990	0.822	0.990	-	-0.011	0.027	-0.011	-0.010	-0.009	-0.011
NY 1996	0.891	0.495	0.703	0.891	-	0.015	-0.011	-0.006	-0.005	-0.012
GA 1997	0.287	0.218	0.198	0.267	0.376	-	0.025	0.047	0.050	0.015
NC 1997	0.990	0.673	0.891	0.990	0.871	0.267	-	-0.008	-0.008	-0.011
CB 1997	0.733	0.990	0.990	0.752	0.713	0.119	0.752	-	-0.011	-0.006
DB 1997	0.683	0.990	0.891	0.644	0.604	0.198	0.772	0.990	-	-0.005
NY 1997	0.861	0.485	0.713	0.861	0.733	0.346	0.871	0.614	0.604	-

[a] Significant exact F permutation test P values after sequential Bonferroni corrections (α=0.005).

Table 15. Analysis of molecular variance (AMOVA) results for the microsatellite locus SOC050 in 1996 and 1997 samples suspected of containing species other than weakfish *Cynoscion regalis*. GA= Georgia, NC= North Carolina, CB= Chesapeake Bay, DB= Delaware Bay, NY= New York.

Source of Variation	Degrees of Freedom	Sum of Squares	Variance Components	Percentage of Variation	Significance Tests (*P*)
			SOC050		
Among Years	1	0.808	<0.001	0.15	0.097
Among Populations/ Within Years	8	4.105	0.001	0.37	0.078
Within Populations	1022	378.705	0.370	99.48	0.031
Total	1031	383.618	0.372	-	-

Table 16. Population pairwise F_{ST} values (above diagonal) and exact F permutation test P [a] values (below diagonal) for the microsatellite locus SOC050 in 1996 and 1997 samples suspected of containing species other than weakfish *Cynoscion regalis*. GA= Georgia, NC= North Carolina, CB= Chesapeake Bay, DB= Delaware Bay, NY= New York.

	GA 1996	NC 1996	CB 1996	DB 1996	NY 1996	GA 1997	NC 1997	CB 1997	DB 1997	NY 1997
					SOC050					
GA 1996	-	-0.004	-0.004	-0.005	-0.006	0.020	-0.005	-0.006	-0.002	-0.005
NC 1996	0.861	-	-0.003	0.005	-0.002	0.040	0.000	-0.001	-0.003	-0.001
CB 1996	0.871	0.792	-	0.008	-0.003	0.038	-0.007	-0.001	0.001	-0.006
DB 1996	0.644	0.574	0.416	-	0.014	0.026	0.002	0.000	-0.002	0.003
NY 1996	0.891	0.822	0.792	0.327	-	0.039	0.000	0.001	0.007	0.002
GA 1997	0.129	**0.010**[a]	**0.020**[a]	0.129	0.059	-	0.027	0.019	0.023	0.027
NC 1997	0.911	0.644	0.960	0.594	0.614	**0.040**[a]	-	-0.005	-0.001	-0.008
CB 1997	0.960	0.723	0.683	0.713	0.634	0.148	0.901	-	-0.003	-0.005
DB 1997	0.792	0.772	0.574	0.782	0.515	0.089	0.812	0.861	-	-0.005
NY 1997	0.901	0.733	0.891	0.574	0.545	0.089	0.970	0.931	0.861	-

[a] Nearly significant exact F permutation test P values after sequential Bonferroni corrections (α=0.005) involving the Georgia 1997 sample.

Table 17. Restriction digestion patterns of the 12S/16S mitochondrial region for putative weakfish *Cynoscion regalis* individuals in the Georgia 1997 sample, sand seatrout *C. arenarius*, and silver seatrout *C. nothus* digested with the enzyme *Rsa* I. n = number of individuals exhibiting the adjacent pattern.

Species	Pattern	n	Band Sizes(bp)				Total Size (bp)
Georgia 1997 Sample							
Weakfish		3	461	300	200	167	1128
Unknown A		7	413	300	200	167	1080
Unknown B		5	461	300	256	167	1184
Known Standards							
Cynoscion arenarius	A	15	461	300	256	167	1184
Cynoscion nothus	A	13	413	300	200	167	1080

Table 18. Sand seatrout *Cynoscion arenarius* and silver seatrout *C. nothus* allele sizes for four microsatellite loci. Neither of the species successfully amplified for the SOC014 locus.

Sample	Allele Sizes (bp)				Sample	Allele Sizes (bp)			
	SOC050	**SOC044**	**SOC014**	**CNE612**		**SOC050**	**SOC044**	**SOC014**	**CNE612**
Cynoscion arenarius					*Cynoscion nothus*				
1	177, 185	202, 204	-	121, 125	1	-	204, 204	-	107, 109
2	175, 177	202, 202	-	113, 127	2	177, 177	204, 204	-	113, 123
3	177, 183	-	-	111, 115	3	177, 179	204, 204	-	111, 123
4	181, 193	202,204	-	121, 123	4	175, 177	204, 204	-	113, 113
5	177, 179	202.202	-	111, 119	5	177, 181	204, 204	-	111, 123
6	177, 179	202, 202	-	129, 131	6	177, 177	202, 204	-	125, 131
7	179, 181	202, 204	-	121, 121	7	-	204, 204	-	105, 107
8	177, 177	202, 204	-	-	8	175, 175	204, 204	-	111, 123
9	177, 179	204, 204	-	-	9	-	204, 204	-	111, 113
10	179, 187	202, 204	-	-	10	-	-	-	-
11	179, 179	202, 204	-	-	11	175, 177	204, 204	-	115, 115

Table 18. Continued.

Sample	Allele Sizes (bp)			
	SOC050	**SOC044**	**SOC014**	**CNE612**
Cynoscion nothus				
12	177, 177	204, 204	-	109, 115
13	-	204, 204	-	109, 115
14	177, 177	204, 204	-	109, 115
15	179, 181	204, 204	-	115, 117

Table 19. Mitochondrial DNA haplotype and SOC050 alleles for anomalous weakfish *Cynoscion regalis* specimens taken from four sampling locations in 1996 and 1997. * = Suspected hybrids.

Individual	MtDNA Type	Alleles (bp)	Individual	MtDNA Type	Alleles (bp)
Georgia	**1997**		**Chesapeake**	**Bay 1997**	
1-2	Cynoscion nothus	177, 177	1-6*	Cynoscion regalis	179, 187
1-4	Cynoscion arenarius	175, 177	2-3	Bairdiella chrysoura	171, 171
1-5	Cynoscion nothus	175, 177	2-20*	Cynoscion regalis	177, 189
1-9*	Cynoscion arenarius	175, 193	**Delaware**	**Bay 1997**	
1-18*	Cynoscion regalis	173, 191	1-7*	Cynoscion regalis	181, 193
1-19*	Cynoscion regalis	175, 193	1-30*	Cynoscion regalis	177, 187
1-22	Cynoscion arenarius	171, 175	3-2*	Cynoscion regalis	181, 193
1-24*	Cynoscion regalis	175, 187	3-9*	Cynoscion regalis	181, 193
1-27*	Cynoscion arenarius	175, 193	3-11*	Cynoscion regalis	177, 193
1-47	Cynoscion nothus	177, 177	**Georgia**	**1996**	
1-48	Cynoscion nothus	175, 175	1-24*	Cynoscion regalis	177, 193
1-49	Cynoscion nothus	177, 177	2-25*	Cynoscion regalis	175, 191
1-50	Cynoscion arenarius	171, 175	2-37*	Cynoscion regalis	175, 193
1-55	Cynoscion nothus	175, 175	3-17*	Cynoscion regalis	179, 187
1-56	Cynoscion nothus	175, 179	**North**	**Carolina 1996**	
North	**Carolina 1997**		1-4*	Cynoscion regalis	177, 195
2-14	Cynoscion regalis*	177, 191			

Table 19. Continued.

Individual	MtDNA Type	Alleles (bp)	Individual	MtDNA Type	Alleles (bp)
Chesapeake	**Bay 1996**		**Delaware**	**Bay 1996**	
2-21*	Cynoscion regalis	177, 187	3-17*	Cynoscion regalis	181, 187
			3-18*	Cynoscion regalis	179, 193

Table 20. Frequencies of unusual alleles in four geographical samples of weakfish *Cynoscion regalis* taken in 1996 and 1997.

Sample	Allele (bp)	Frequency	Sample	Allele (bp)	Frequency
Georgia 1996	175	0.018		175	0.125
	177	0.009		177	0.094
	179	0.009		179	0.010
North Carolina 1996	177	0.010	North Carolina 1997	177	0.009
Chesapeake Bay 1996	177	0.008	Chesapeake Bay 1997	171	0.009
Delaware Bay 1996	179	0.011		177	0.009
	181	0.011		179	0.009
Georgia 1997	171	0.021	Delaware Bay 1997	177	0.023
	173	0.010		181	0.034

Figure 3. Migration patterns of weakfish *Cynoscion regalis* in (a) spring and summer, and (b) fall and winter (from Wilk 1976).

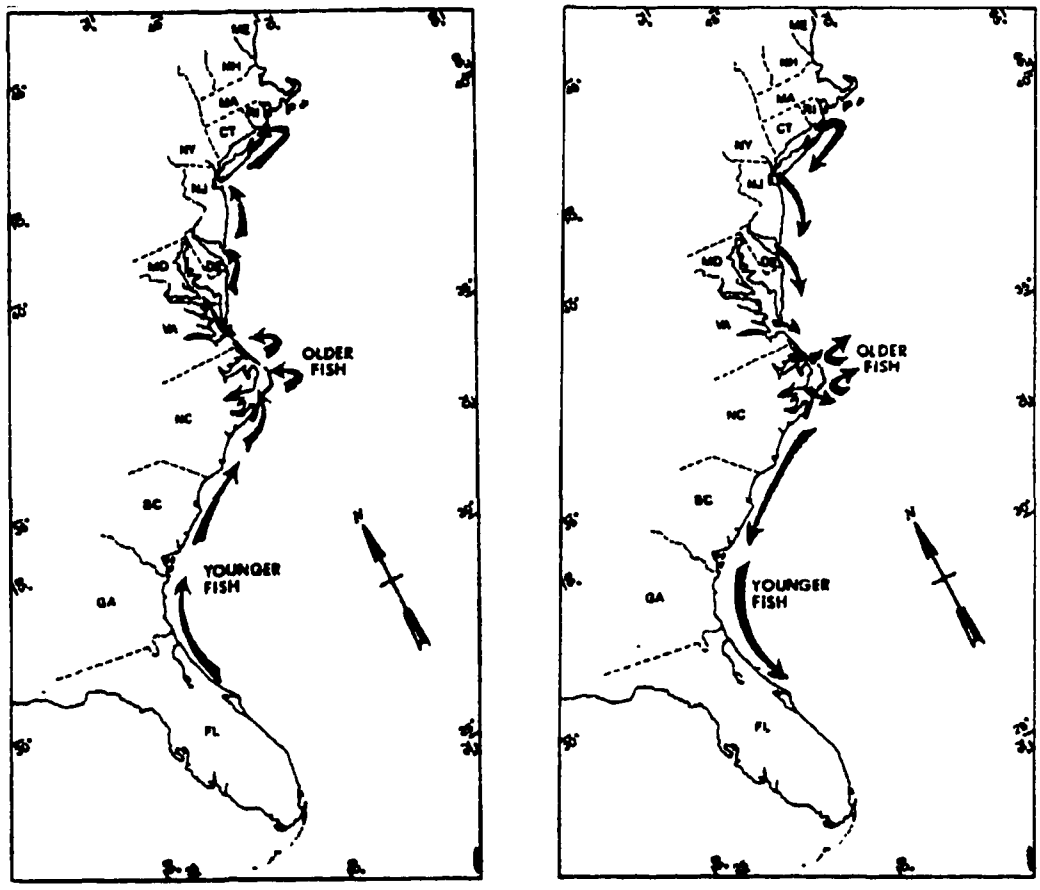


Figure 4. Sampling locations for young-of-the-year (YOY) weakfish *Cynoscion regalis* in the summers of 1996 and 1997. Sites are Peconic Bay, New York (NY), Delaware Bay, Delaware (DB), Chesapeake Bay, Virginia (CB), Pamlico Sound, North Carolina (NC), and Doboy Sound, Georgia (GA).

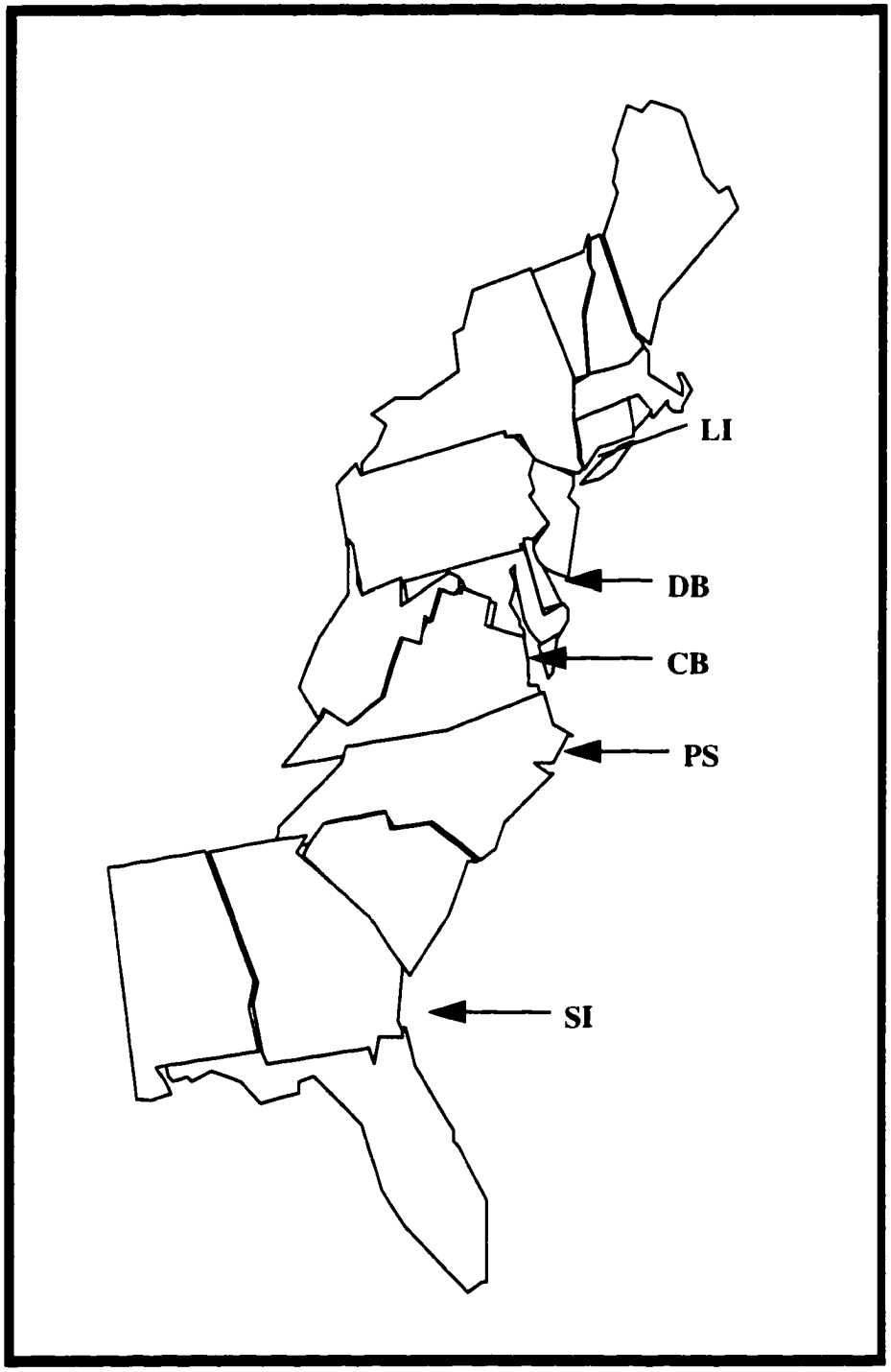


Figure 5. Gel image of microsatellite locus SOC050 alleles for 48 YOY weakfish *Cynoscion regalis* from the Delaware Bay 1996 sample. Lane 1-3, 5, 28-31, 54-56, 58 = size ladder. A= individual homozygous for the 191 bp allele. B = individual heterozygous for the 189 bp and 193 bp alleles.

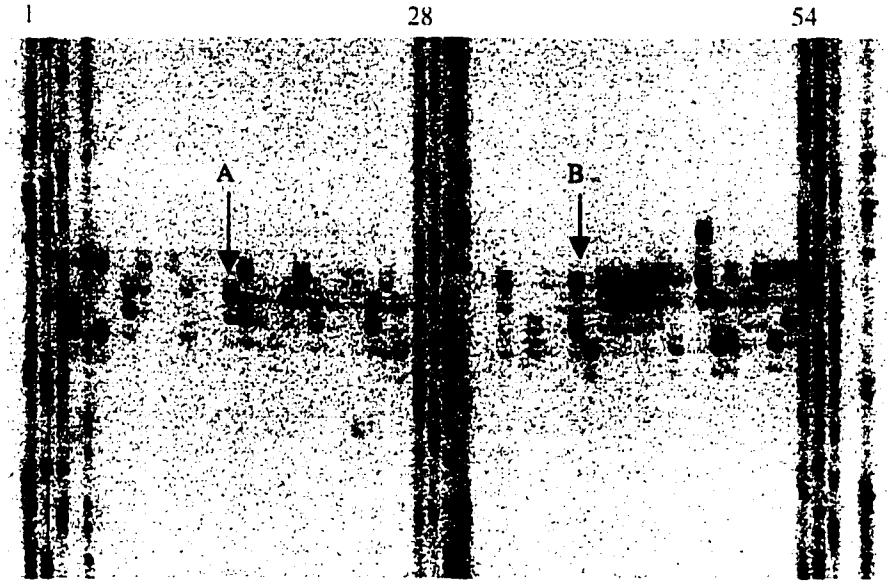


Figure 6. Length-frequency distributions for young-of-the-year (YOY) weakfish *Cynoscion regalis* collected from five locations in the summer of 1996. GA= Georgia, NC= North Carolina, CB= Chesapeake Bay, DB= Delaware Bay, NY= New York.

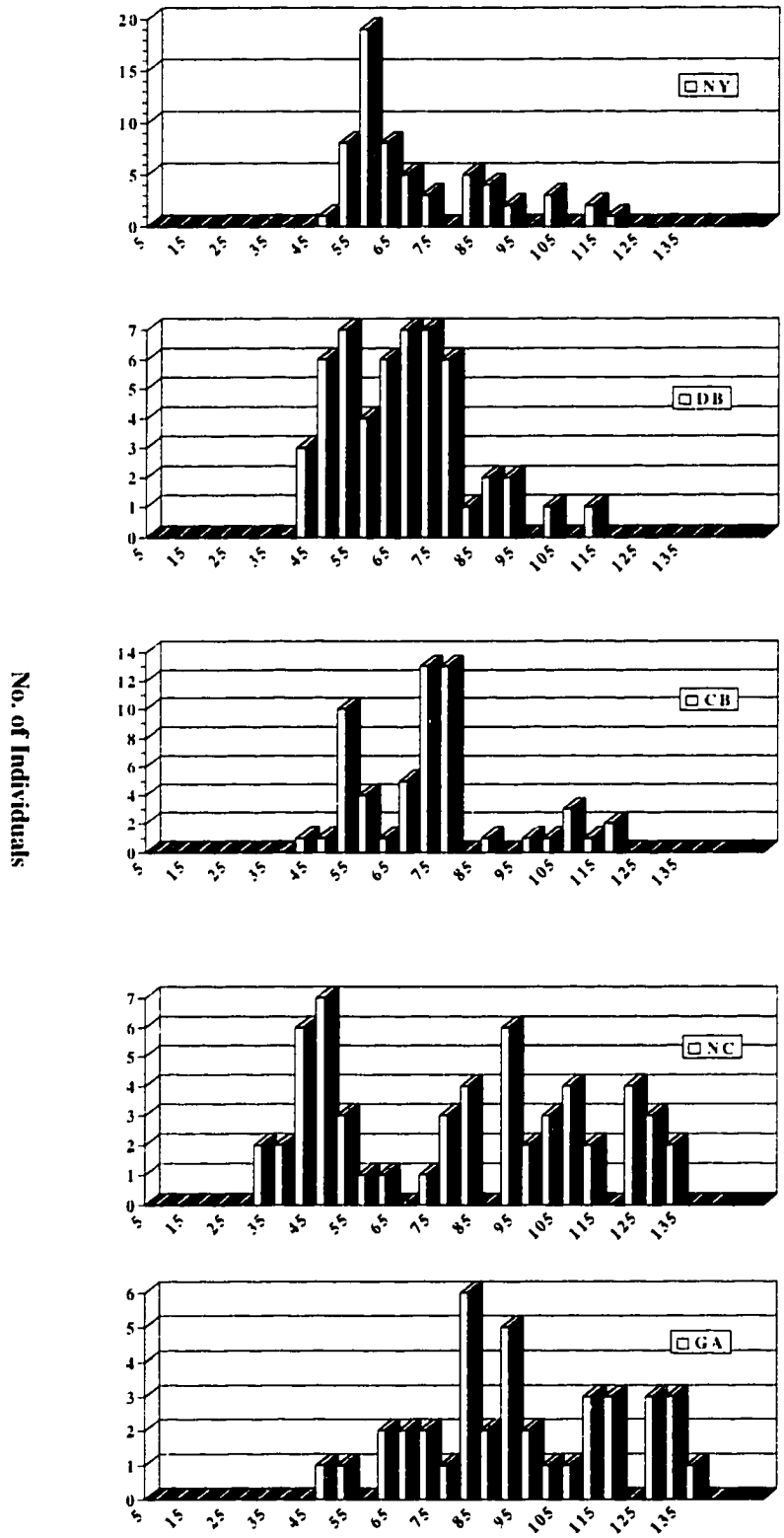


Figure 7. Length-frequency distributions for young-of-the-year (YOY) weakfish *Cynoscion regalis* collected from five locations in the summer of 1997. GA= Georgia, NC= North Carolina, CB= Chesapeake Bay, DB= Delaware Bay, NY= New York.

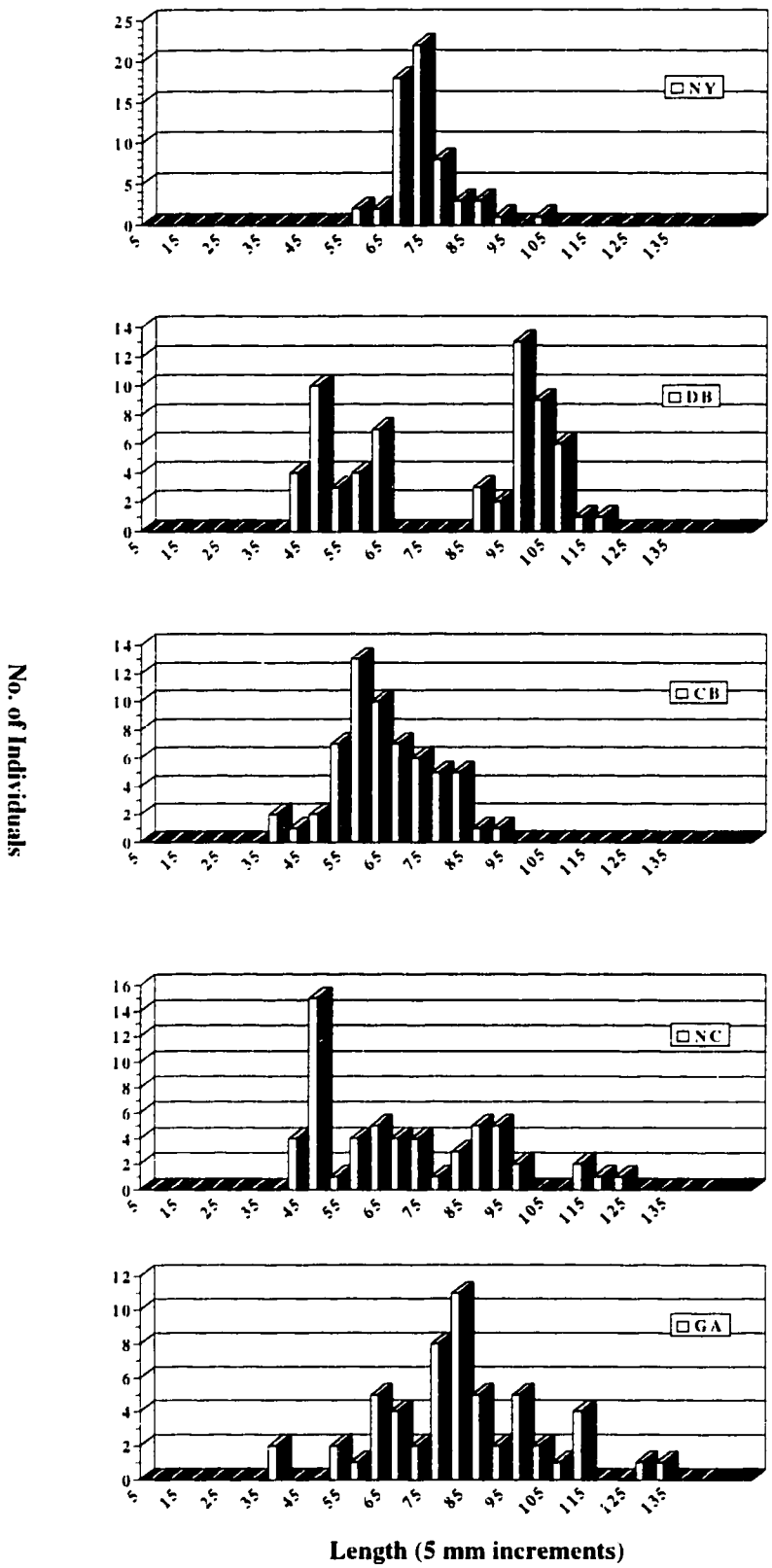


Figure 8. Microsatellite allele frequency distributions in weakfish *Cynoscion regalis*.

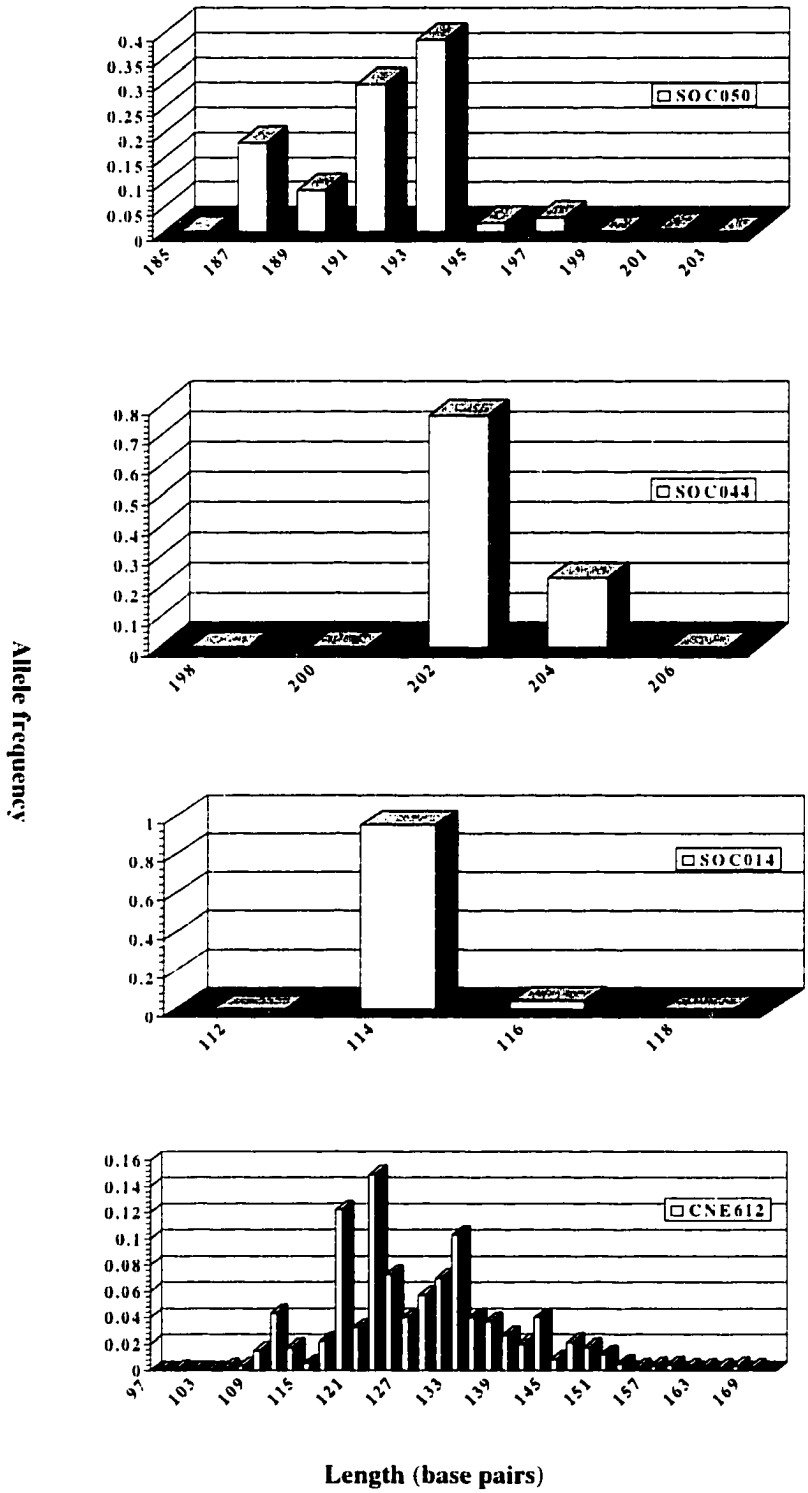
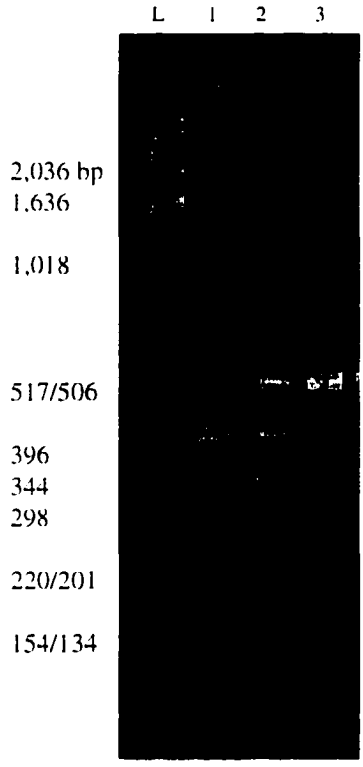
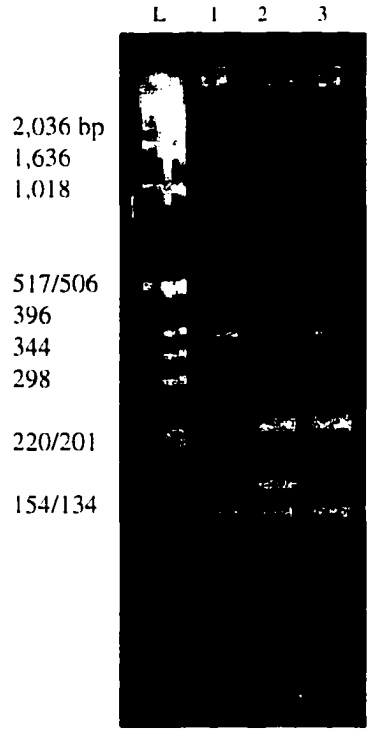


Figure 9. Restriction endonuclease digestion patterns of (a) CRESIA1 and (b) RP2 nuclear intron regions in weakfish *Cynoscion regalis*. (a) Lane 1 = size standard, 2 = homozygote pattern A, 3 = heterozygote pattern AB, and 4 = homozygote pattern B. (b) Lane 1 = size standard, 2 = heterozygote pattern AB, 3 = homozygote pattern A, and 4 = homozygote pattern B.



(a)



(b)

Figure 10. Microsatellite locus SOC050 allele frequency distribution for the Georgia 1997 sample of YOY weakfish *Cynoscion regalis*.

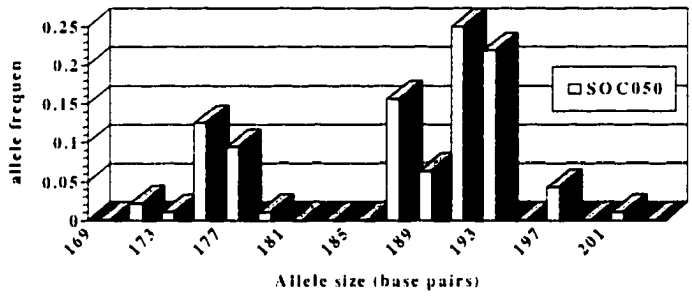
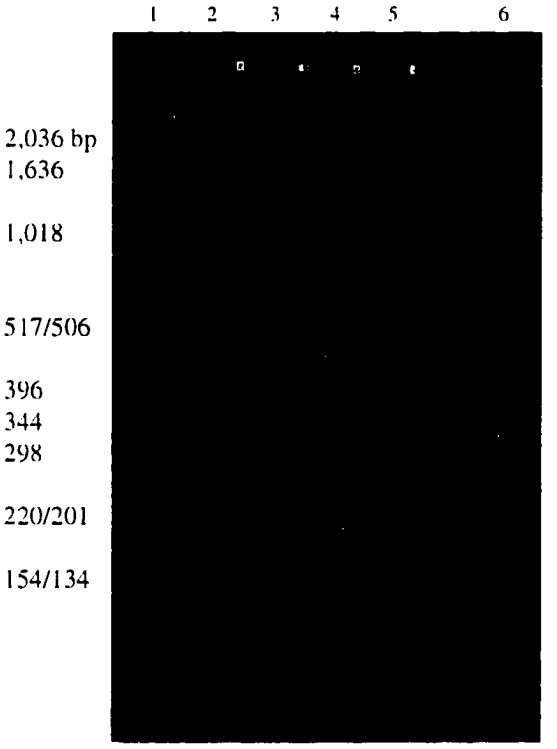


Figure 11. *Rsa* I restriction endonuclease digestion patterns of the 12S/16S mitochondrial region for weakfish *Cynoscion regalis*, two unknown mtDNA types, sand seatrout *C. arenarius*, and silver seatrout *C. nothus*. Lane 1 = size standard, lane 2 = *Cynoscion regalis*, lane 3 = unknown A, lane 4 = unknown B, lane 5 = *Cynoscion nothus*, lane 6 = *Cynoscion arenarius*.



GENERAL SUMMARY

The recent proliferation of molecular genetics in fisheries science research can make it difficult to decide which combination of markers and techniques is best suited for a given problem. Although some generalities have been drawn regarding the appropriateness of particular markers and techniques for inter- and intraspecific investigations, these guidelines are by no means absolute. In the present species identification study the preferred marker was one that amplified across a wide range of species, distinguished between all species concerned, and demonstrated a minimal amount of intraspecific variation. These criteria were met by the 12S/16S mtDNA marker. Other regions of the mtDNA genome met some of the criteria, but not all. Some regions such as the ATPase, cytochrome oxidase I, and cytochrome *b* genes showed promise but did not amplify across all species. In contrast, the ND4 gene region amplified well but showed higher levels of intraspecific variation compared to the 12S/16S marker, resulting in up to five different digestion patterns in a single species (Cordes et al. submitted). Thus, different regions within a molecule (mtDNA) considered to be rapidly evolving relative to nuclear loci may still be applicable to a wide variety of studies (depending on the chosen method of analysis) both above and below the species level, as is evidenced by its use in questions of higher taxonomy as well as population structure.

The use of a mtDNA marker for the genetic key was chosen to eliminate the added

complication of heterozygotes when scoring gels for species identification. Unfortunately, this means the resulting key is of little or no use in the identification of hybridization between species. A number of nuclear regions including the growth hormone gene were tested for use in the study, but amplification was inconsistent with the primers available in the literature (J. Cordes, unpublished data). With more and more 'universal' primers being published (see recent issues of *Molecular Ecology*), this limitation will not persist for long, and a genetic identification key based on a combination of mtDNA and nuclear markers that can identify hybrids should not be far off. Such a key has already been developed for the istiophorid billfishes (Graves and McDowell 1997). The low variability seen in the CRESIA and RP2 intron markers would seem to recommend these regions as candidates for such a multiple-marker key, but the primers would have to be redesigned to amplify a wider variety of species.

In general microsatellite loci show a high incidence of polymorphism relative to other classes of molecular markers such as mitochondrial genes (Scribner et al. 1994, Patton et al 1997), although this has not proven to be universal (O'Connell and Wright 1997). Microsatellite loci used in this study exhibited a wide range of variation from two monomorphic tetranucleotide loci to the highly variable CNE612 locus, with 34 alleles and expected heterozygosities in the range of 90-95%. Interestingly, this highly polymorphic locus was no better at discriminating between sand seatrout *Cynoscion arenarius* and silver seatrout *C. nothus* specimens in the weakfish samples than was either of the two less variable loci, SOC014 and SOC044. This provides cautionary evidence that the most variable loci are not automatically the best markers for either inter- or intraspecific investigations. Unfortunately a comparison of the microsatellite

and nuclear intron markers as tools in species identification was not possible due to the lack of intron amplification in the sand seatrout and silver seatrout samples.

One of the advantages of the large number of genetic markers and techniques now available to researchers is the increasing practicality of multiple-marker studies. By using a variety of loci from two or more classes of molecular markers such as allozymes, mtDNA, and nuclear DNA, it is possible to better substantiate conclusions through congruence of results between marker types. Some doubt as to the existence of a single genetic stock of weakfish in the U.S. western Atlantic remained after earlier works on allozymes (Crawford et al. 1989) and whole molecule mtDNA (Graves et al. 1992). However, their data, taken in conjunction with the microsatellite and nuclear intron data presented here, make a strong case in support of the single genetic stock hypothesis. Recent examples of stock structure analyses based on two or more classes of molecular markers are common (Nielsen et al. 1994, Sanchez et al. 1996, Patton et al. 1997, Estoup et al. 1998, Seeb et al. 1998, Buonaccorsi et al. 1999), and should continue to increase in popularity.

The presence of non-target species in the weakfish stock structure analysis presented an unexpected opportunity to incorporate the use of molecular genetic techniques at both the inter- and intraspecific levels in a single study, and illustrates the advantages in using multiple marker systems. If only a single microsatellite locus had been used, or if the study had been restricted to nuclear intron markers alone, it is very likely that the sand seatrout and silver seatrout specimens would have gone unnoticed. This could easily have resulted in a type II error (rejecting the null hypothesis of a single stock when in fact it is true). Instead, it was possible not only to recognize the individuals as anomalous, but also

to identify them to species and provide evidence of hybridization between at least two of the congeners. It is hoped that further refinement of the inter- and intraspecific molecular markers developed here and in other studies will eventually be helpful in further clarifying the taxonomic status, population dynamics, and possible hybridization within the genus *Cynoscion*.

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