Nuclear and mitochondrial DNA markers for specific identification of istiophorid and xiphiid billfishes*

Jan R. McDowell
John E. Graves

School of Marine Science
Virginia Institute of Marine Science
College of William and Mary
Rt 1208 Greate Rd.
Gloucester Point, Virginia 23062
E-mail address (for J. McDowell): McDowell@VIMS.edu

Abstract—Independent molecular markers based on mitochondrial and nuclear DNA were developed to provide positive identification of istiophorid and xiphiid billfishes (marlins, spearfishes, sailfish, and swordfish). Both classes of markers were based on amplification of short segments (<1.7 kb) of DNA by the polymerase chain reaction and subsequent digestion with informative restriction endonucleases. Candidate markers were evaluated for their ability to discriminate among the different species and the level of intraspecific variation they exhibited. The selected markers require no more than two restriction digestions to allow unambiguous identification, although it was not possible to distinguish between white marlin and striped marlin with any of the genetic characters screened in our study. Individuals collected from throughout each species' range were surveyed with the selected markers demonstrating low levels of intraspecific character variation within species. The resulting keys provide two independent means for the forensic identification of fillets and for specific identification of early life history stages.

Manuscript accepted 18 September 2001.
Fish. Bull. 100:537–544 (2002).

Species-level identification of most marine fishes is typically based on adult characters. However, distinguishing characters may be removed from adults when they are processed for market or personal consumption, making such identification problematic. Furthermore, species identification from early life history stages of many marine fishes is not possible because diagnostic morphological characters at these stages are not currently known. Consequently, alternative means of identification are needed.

A variety of molecular genetic characters have been used to provide identifications of marine fishes. Marine fish eggs and larvae have been identified by using allozymes (Mork, et al., 1983; Graves et al., 1988), restriction analysis of whole mitochondrial (mt) DNA (Daniel and Graves, 1994), restriction analysis of specific mtDNA gene regions (Luczkovich et al.[1]), and specific amplification of mtDNA gene regions (Rocha-Olivares, 1998). A similar suite of molecular markers has been used to provide positive identification of adult marine fish tissues, with recent emphasis on restriction analysis of amplified regions of the mitochondrial genome (Chow et al., 1993; Chow 1994, Chow and Kishino, 1995; Heist and Gold, 1997; Innes et al., 1998; Cordes et al., 2001).

To be effective, a diagnostic molecular marker must demonstrate consistent differences among closely related species and exhibit very limited intraspecific variation. Restriction analyses of regions of the mitochondrial genome have met these criteria for several marine fishes. However, reliance on a single, maternally inherited character (mtDNA) can provide misleading results in situations where there is a possibility of hybridization or introgression, and analyses of both nuclear and mitochondrial markers are therefore desirable.

The istiophorid and xiphiid billfishes (marlins, spearfishes, sailfish and swordfish) represent an important commercial and recreational fisheries resource. Because of depleted stock levels, current regulations within the United States prohibit the sale of istiophorid billfish taken in the Atlantic Ocean. Although adult billfishes are easily identified on the basis of morphological characters, these characters are typically removed during processing, preventing morphological identification. In addition, the early life history stages of istiophorid billfishes are not well known, and specific identification is problematic (Nakamura, 1985).

Chow (1994) used 13 restriction enzymes in a restriction fragment length polymorphism (RFLP) analysis of a 350-bp region of the mtDNA cytochrome *b* gene to discriminate among ten nominal species of billfishes; however samples sizes were small for several species

* Contribution 2470 of the Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

[1] Luczkovich, J. J., H. J. Daniel, M. W. Sprague, S. E. Johnson, R. C. Pullinger, T. Jenkins, and M. Hutchinson. 1999. Characterization of critical spawning habitats of weakfish, spotted seatrout and red drum in Pamlico Sound using hydrophone surveys. Final report to the North Carolina Division of Marine Fisheries under grant numbers F-62-1 and F-62-2, 128 p. North Carolina Department of Environment and Natural Resources, Division of Marine Fisheries. Morehead City, NC 28557.

and banding patterns differed by as little as 15 base pairs, making alternate patterns difficult to distinguish. Innes et al. (1998) were able to discriminate among seven species of billfish found in Australian waters with RFLP analysis of a 1400-bp region of the mtDNA control region (D-loop). Their analysis, which employed four restriction enzymes, revealed relatively high levels of intraspecific variation of the diagnostic characters within some species, and there was some overlap of banding patterns between species. In neither study was an independent nuclear marker developed to corroborate specific identifications based on analyses of mtDNA.

In this article we present a molecular key to the identification of istiophorid and xiphiid billfishes using RFLP analyses of independent mitochondrial and nuclear DNA regions. We demonstrate low intraspecific variation of the characters within large collections of individuals sampled from throughout each species' range and show the utility of the markers for the identification of fillets and early life history stages.

Materials and methods

Collections of striped marlin (*Tetrapturus audax*), white marlin (*Tetrapturus albidus),* blue marlin (*Makaira nigricans*), and sailfish (*Istiophorus platypterus*) were available from previous analyses of stock structure (Graves and McDowell 1994, 1995; Graves, 1998), and individuals from locations throughout each species' range were selected for the present study (Table 1). These DNA samples consisted of the nuclear and mitochondrial bands resulting from mtDNA purifications with the equilibrium density gradient centrifugation protocols of Lansman et al. (1981). Samples of black marlin (*Makaira indica*), longbill spearfish (*Tetrapturus pfluergeri*), shortbill spearfish (*Tetrapturus angustirostris*), and swordfish (*Xiphias gladius)*, were obtained from recreational and commercial fishermen (Table 1) and consisted of either frozen heart tissue or white muscle tissue preserved in DMSO storage buffer (Seutin et al., 1991). DNA was extracted from these tissues following the protocols of Winnepenninckx et al. (1993).

Evaluation of candidate mitochondrial and nuclear loci involved a two-step process. The first was to ensure consistent amplification by the polymerase chain reaction (PCR) of a similar-size product across all taxa. The second step was to screen those loci that successfully amplified across all billfish species with a panel of restriction endonucleases to identify enzymes that discriminated among species and revealed limited intraspecific variation.

Several candidate mitochondrial and nuclear gene regions were amplified by PCR (Table 2). The 25 μL PCR reactions consisted of 0.25 μL template DNA, 2.5 μL 10X PCR buffer plus magnesium, 0.5 μL dNTP mix, 0.25 μL forward primer, 0.25 μL reverse primer, 0.125 μL *Taq* DNA polymerase, and 21.125 μL PCR grade water. Primers were ordered from either Life Technologies (Gaithersburg, MD) or Genosys Biotechnologies Inc. (The Woodlands, TX), and PCR reactions were carried out in an MJ Research Corporation PTC-200 Peltier thermal cycler (Watertown, MA) by using the Life Technologies PCR reagent system (Gaithersburg, MD). Initial screening demonstrated that the mitochondrial ND4 gene region and the nuclear MN32-2 locus produced the most reliable amplifications across taxa and PCR conditions were optimized for these loci. The cycling parameters for the ND4 gene region were an initial denaturation at 95°C for 5 min., followed by 35 cycles of 94°C for 1 min., 47°C for 1 min., 65°C for 3 min., and a final extension at 65°C for 7 min. Amplification of MN32-2 proceeded with an initial denaturation at 95°C for 5 min., 40 cycles of 94°C for 1 min., 57°C for 1 min., 65°C for 3 min., and a final extension at 72°C for 7 min. Amplified products were held at 4°C until use. The size of each amplification product was determined on a 1% agarose gel run in TBE (45 mM Tris, 45 mM boric acid, I mM EDTA) at 100 volts for 1 hour. ND4 amplification resulted in a product of approximately 1.7 kb and MN32-2 amplification resulted in a product of approximately 1.2 kb.

Amplified products were screened with a panel of restriction endonucleases to identify those that discriminated among species, and revealed a minimum level of variation within species. All enzymes were purchased from Gibco/BRL Life Technologies Inc. (Bethesda, MD) with the exception of *Ban*I, which was purchased from Promega (Madison, WI). All were used according to the manufacturers' instructions.

Restriction fragments were separated on 2.5% horizontal agarose gels made from 1.25% UltraPure agarose (Life Technologies Inc., Bethesda, MD) and 1.25% NuSeive

Table 1
Collection information for billfish samples surveyed.

Species	Location	Number	Total
Sailfish	Brazil	34	99
	Mexico	24	
	Ecuador	17	
	Australia	24	
White marlin	Brazil	38	99
	Morocco	36	
	Venezuela	25	
Striped marlin	Mexico	28	96
	Ecuador	38	
	Australia	30	
Black marlin	Ecuador	12	60
	Australia	48	
Spearfish	Venezuela	12	16
	Hawaii	4	
Blue marlin	Mexico	24	150
	Australia	4	
	Ecuador	20	
	Hawaii	63	
	Jamaica	39	
Swordfish	Hawaii	20	20

Table 2

Primer pairs used to amplify regions evaluated in this study.

Locus	Primer sequence (5′–3′)	Source
Cytochrome b:		
CYTB-F	TGGGSNCARATGTCNTWYTG	Joseph Quattro, personal commun.[1]
CYTB-R	GCRAANAGRAARTACCAYTC	
ATPase 6:		
ATPase L8331	TAAGCRNYAGCCTTTTAAG	Joseph Quattro, personal commun.[1]
ATPase H8969	GGGGNCGRATRAANAGRCT	
D-Loop:		
CB3R-L	CATATTAAACCCGAATGATATTT	Palumbi et al., 1991
12SAR-H	ATAGTGGGGTATCTAATCCCAGTT	
ND4:		
ND4 ARG-BL	CAAGACCCTTGATTTCGGCTCA	Bielawski and Gold, 1996
ND4 LEU	CCAGAGTTTCAGGCTCCTAAGACCA	
ITS:		
ITS-3	TATGCTTAAATTCAGCGGGT	Goggin, C.L, 1994
ITS-5	CGTAGGTGAACCTGCGGAAGG	
SACTIN:		
SACSMSF-F	CGGACGCCCCCGTCACCAGGTAC	This study
SACIN-R	CCAGAGGCATACAGGGACAGCACAGC	
MN32-2:		
MN32-2F	GTAGCAAGGGGCTGTTGCATAG	Buonaccorsi et al., 1999
MN32-2R	GAGTCAGTGGTTCGGGATTTTATC	
MN47:		
MN47-F	GCTGTTGACCCAAACAATCCGG	Buonaccorsi et al., 1999
MN47-R	GGGCATAAATGCTCAGGACACTT	
MN81:		
MN81-F	CACTCAAACAGGTGAATCCTGGC	Buonaccorsi et al., 1999
MN81-R	CAAAACAACAGATGCCGCTAAGG	
WM08:		
WM08F	AGCAGCTAGGGACACACGATTCC	Buonaccorsi et al., 1999
WM08R	GGCAAACCTTACACTGAGGGGATG	

[1] Quattrro, J. 1995. Personal commun. Department of Biological Sciences, University of South Carolina College of Science and Mathematics, Columbia, SC, 29208.

GTG agarose (FMC BioProducts, Rockland, ME), and visualized under UV light after having been stained with ethidium bromide. Fragment sizes were estimated by comparison with a 1-kb size standard (Life Technologies Inc., Bethesda, MD) using RFLPScan Plus 3.0 (Scanalytics, Billerica, MA).

Results

Mitochondrial marker

Four mtDNA regions (cytochrome *b*, D-loop, ND4, and ATPase) were included in the initial screening (Table 2). The ATPase region was tested with eight potentially useful enzymes based on published sequences and was found to have an extremely low level of interspecific variation (many species exhibiting identical banding patterns). The cytochrome *b* region was screened with four enzymes based on published sequences, but because of the small size of the amplification product (350bp), differences in banding patterns were small and difficult to distinguish. The D-loop region was screened with a total of 40 enzymes. Of these, *Bcl* I, *Alu* I, *Rsa* I, and *Hinf* I were tested with up to 50 individuals from each species. Banding patterns that were initially thought to be diagnostic for blue marlin based on *Rsa* I were found to occur at low frequency in sailfish. This overlap combined with the large amount of intraspecific variation in some species made this region unsuitable for use as a forensic marker. Finally, the ND4 region was screened with a total of 47 restriction enzymes. Of these, 17 were tested more extensively, and the combi-

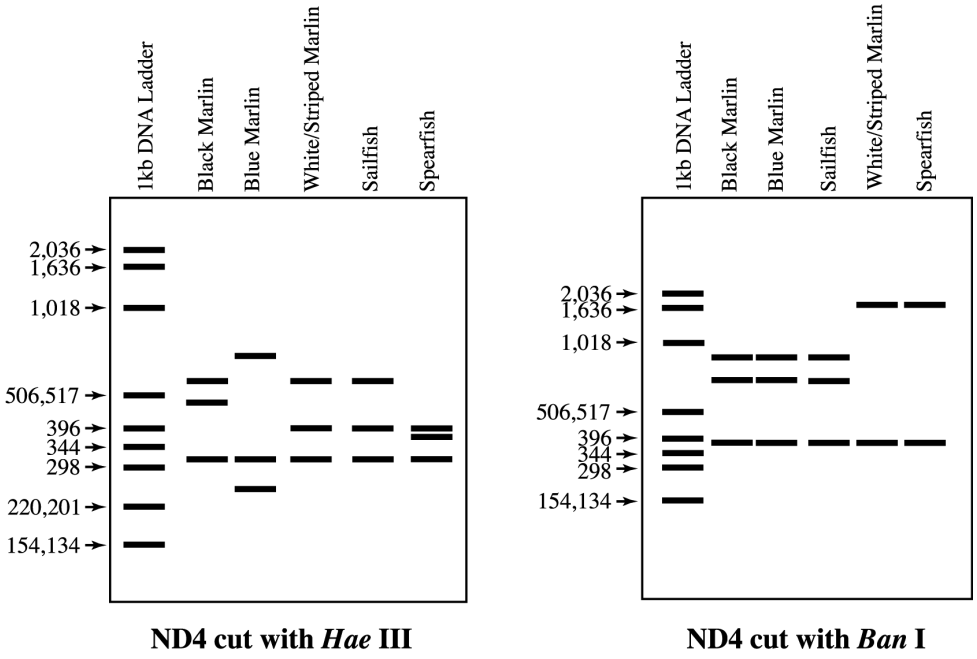

Figure 1

ND4 gel. Most common restriction fragment patterns of the ND4 mitochondrial gene region of istiophorid billfishes. (**A**) Digestion with *Hae* III. From left to right, 1-kb DNA ladder, black marlin (pattern D), blue marlin (pattern A), white and striped marlin (pattern B), sailfish (pattern B), shortbill spearfish (pattern E), and longbill spearfish (pattern E). (**B**) Digestion with *Ban* I. From left to right, 1-kb DNA ladder, black marlin (pattern A), blue marlin (pattern A), white and striped marlin (pattern B), sailfish (pattern A), shortbill spearfish (pattern B), and longbill spearfish (pattern B).

Table 3

Restriction fragment patterns of the mitochondrial ND4 region of istiophorid and xiphiid billfishes. Only diagnostic bands are shown.

(**A**) Digestions with *Hae* III. A = blue marlin; B = striped marlin, white marlin, sailfish; C = white marlin, sailfish; D = black marlin; E = spearfish; F = white marlin; G = spearfish; H = swordfish.

A	B	C	D	E	F	G	H
800	570	800	570	405	570	405	950
320	405	405	530	380	405	320	300
270	320	320	320	320	380	270	290

(**B**) Digestions with *Ban* I. A = blue marlin, sailfish, black marlin, spearfish; B = striped marlin, white marlin, spearfish; C = swordfish.

A	B	C
850	1500	700
650	400	650
400		400

nation of *Ban* I and *Hae* III was found to be diagnostic and to reveal a low level of intraspecific variation.

After finding diagnostic enzymes for use with the ND4 region, a total of 540 billfish samples was screened (Table 1) to evaluate the accuracy of the marker. Samples from a broad geographic range, including both the Atlantic and Indo-Pacific, were used for each species whenever possible. Of these, the white marlin, spearfishes and sailfish each exhibited one alternative restriction pattern for *Hae* III at low frequency (3.6%, 20.0% and 7.4% respectively) but in no case was the alternate pattern the same as a pattern seen in another species (Table 3). In addition, spearfishes exhibited an alternate pattern for the enzyme *Ban* I at a frequency of 40%; however, because *Ban* I was used only to discriminate white and striped marlin from sailfish in the ND4 identification key, this pattern did not affect the results (Figs. 1 and 2).

Nuclear marker

Six nuclear markers were screened in the preliminary analysis (Table 2). These included the short actin intron, the internal transcribed spacer (ITS) region (Goggin, 1994), and four anonymous single copy nuclear (scnDNA) markers. The scnDNA markers MN32-2, BM47, BM81, and WM08 were originally developed for analyses of popu-

lation structure in blue marlin (Buonaccorsi et al., 1999). The short actin intron primers were modified from "universal" actin gene primers "480" and "483" (Siddall et al., 2001).

Both the short actin and the ITS marker were rejected because neither marker amplified reliably across species. For the scnDNA markers, the program GeneJockey (Taylor, 1996) was used to search for the presence of restriction sites in sequences previously generated for blue marlin. The WM08 marker was screened with a total of ten enzymes, each of which produced identical patterns across species. BM47 and BM81 were also screened with ten enzymes each. For BM47, the combination of enzymes *Bcl* I and *Dde* I appeared to be diagnostic in a preliminary screening. However, upon further analysis, it was discovered that this combination of locus and enzymes produced confounding patterns for blue marlin and sailfish; the most common pattern for blue marlin was seen as a rare pattern for sailfish. Likewise, the BM81 locus did not distinguish between blue marlin and sailfish or between white and striped marlin and spearfish with any of the enzymes used. The MN32-2 locus was screened with a total of nine restriction endonucleases. The combination of *Dra* I and *Dde* I was found to allow for unambiguous identification of billfish species (Figs. 3 and 4).

As with the ND4 locus, after determining a diagnostic enzyme-locus combination, we screened a total of 540 billfish samples from a broad geographic range to evaluate the accuracy of the marker (Table 1). The enzyme *Dra* I was found to have two alternate alleles: "D" and "E" for blue marlin at a frequency of 19% and 5.5%, respectively. All other species appeared to be fixed for different (homozygous) alleles with respect to this enzyme. For *Dde* I, spearfishes had an alternate allele, "E," at a frequency of 36%. In addition, blue marlin had two alternate alleles "H" and "I" at frequencies of 16% and 40%, respectively (Table 4). Although the "H" allele in blue marlin was the only allele seen in black marlin, use of *Dde* I was not necessary to distinguish the two species since they are easily differentiated by *Dra* I (Figs. 3 and 4). All other species appeared to be homozygous for different alleles.

Figure 2

Key to distinguish species of billfishes based on the mitochondrial locus ND4.

Table 4

Restriction fragment patterns of the nuclear gene region BM32-2 of istiophorid billfishes. This locus did not amplify in the swordfish.

(**A**) Digestions with *Dra* I. A = blue marlin; B = striped marlin, white marlin, sailfish, black marlin; C = spearfish; D = blue marlin; E = blue marlin. A/D and A/E heterozygotes were seen in blue marlin cut with *Dra* I. Individuals of all other species were fixed homozygous.

A	B	C	D	E
650	640	640	650	1200
450	280	280	400	
	260	220	50	

(**B**) Digestions with *Dde* I. A = blue marlin; B = striped marlin, white marlin; C = spearfish; D = sailfish; E = spearfish; H = blue marlin; I = blue marlin. A/I A/H and H/I heterozygotes were seen in blue marlin cut with *Dde* I. C/E heterozygotes were seen in spearfish cut with *Dde* I. Individuals of all other species were fixed homozygous.

A	B	C	D	E	H	I
700	850	700	475	475	775	700
300	425	475	370	370	425	425
125		125	280	205		125
			130	125		

Discussion

The purpose of this study was to develop a key to the identification of billfish species based on independent mitochondrial and nuclear markers. Our goal was to make the process streamlined and capable of being performed in a modestly equipped genetics laboratory. Specific identification can be accomplished with a single PCR amplification of either the mitochondrial ND4 locus or nuclear MN32-2 locus and two restriction digestions. Previous methods with other mitochondrial gene regions have required the use of either four or 13 restriction digestions (Innes et al., 1998 and Chow 1994, respectively).

To facilitate specific identification, an objective of this study, was to develop diagnostic markers that exhibited limited intraspecific variation. Analysis of large sample sizes (60–100 or more) of sailfish, white marlin, striped marlin, blue marlin, and black marlin from throughout each species' range revealed minimal variation of the species-specific characters. Most species displayed a single genotype for digestions with the two enzymes used to cleave either the mitochondrial or nuclear amplification products, and no species exhibited more than three genotypes for any locus-restriction enzyme combination. In

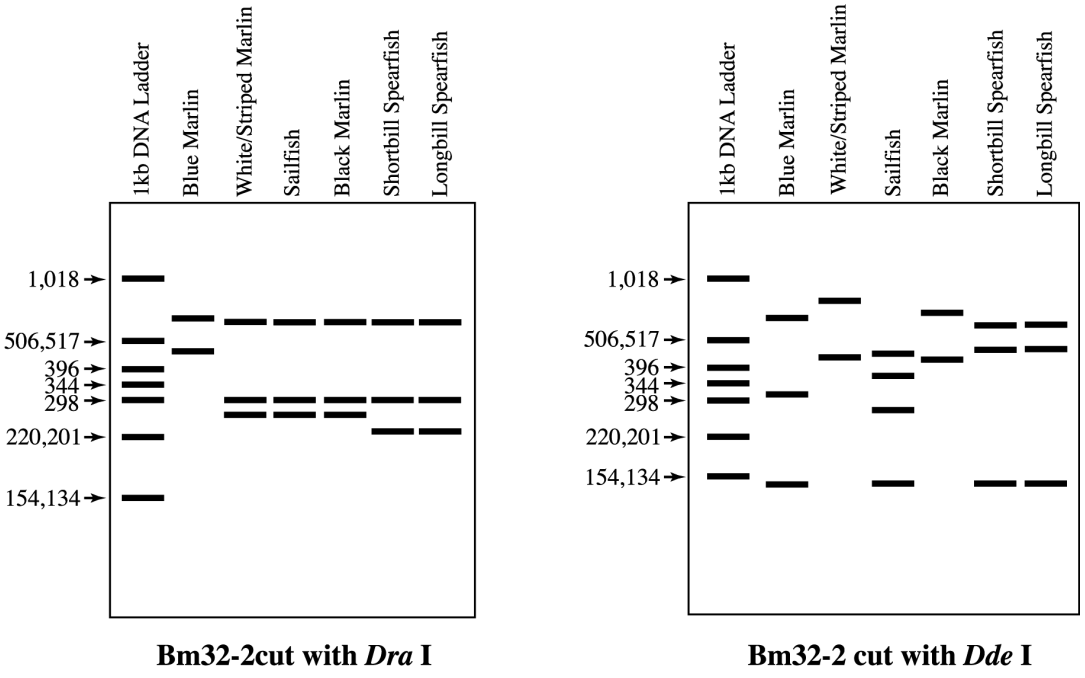

Figure 3

BM32-2 gel. Most common restriction fragment patterns of the nuclear gene region BM32-2 of istiophorid billfishes. This locus did not amplify in the swordfish. (**A**) Digestions with *Dra* I. From left to right, 1-kb DNA ladder, blue marlin (pattern A), white and striped marlin (pattern B), sailfish (pattern B), black marlin (pattern B), shortbill spearfish (pattern C), and longbill spearfish (pattern C). (**B**) Digestions with *Dde* I. From left to right, 1-kb DNA ladder, blue marlin (pattern A), white and striped marlin (pattern B), sailfish (pattern D), black marlin (pattern H), shortbill spearfish (pattern C), and longbill spearfish (pattern C).

Figure 4

Key to distinguish species of billfishes based on the single copy nuclear locus MN32-2.

contrast, Innes et al. (1998) reported ten composite haplotypes among 47 black marlin, six composite haplotypes among 26 blue marlin, six composite haplotypes among 46 striped marlin, and six haplotypes among 21 swordfish, all from the southwest Pacific. From the level of intraspecific variation in relation to the sample sizes and the regional nature of their collections, it is reasonable to assume that Innes et al. (1998) may have missed several composite genotypes characteristic of the different species. In fact, from the level of variation exhibited by black marlin, striped marlin, and swordfish, Innes et al. (1998) suggested that their diagnostic species markers could be of potential use in population structure analyses. It occurs to us that if a genetic character exhibits sufficient intraspecific variation to be useful for analyses of stock structure, it is probably not a good candidate for species identification.

A high degree of genetic similarity was noted among white marlin and striped marlin in our study. None of the molecular markers evaluated in this study was able to unambiguously distinguish between the two species.

Figure 5

Larval billfish and its corresponding specific identification as a sailfish based on the BM32-2 locus. Lane 1 *Dra* I, lane 2, 1-kb plus DNA ladder (Gibco/BRL Life Technologies Inc., Bethesda MD), lane 3, *Dde I*.

Chow (1994) was also unable to distinguish between the two species based on RFLP analysis of the cytochrome *b* gene, and Innes et al. (1998) did not consider white marlin in their investigation because it does not occur in Australian waters. RFLP analysis of the whole mtDNA molecule indicated that white and striped marlin share composite haplotypes, although there are highly significant frequency differences between the species (Graves and McDowell, 1995; Graves, 1998). Sequence analysis of the mtDNA cytochrome *b* gene also demonstrated a lack of genetic divergence among white and striped marlin (Finnerty and Block, 1995), and a further genetic analysis of the species' relationships is warranted.

To evaluate the utility of the methods outlined in our study with those of other investigators, detailed protocols and six unknown billfish samples were sent to the Southeast Fisheries Science Center's (now National Ocean Survey's) Charleston, SC, laboratory. Scientists at the Charleston Laboratory analyzed both mitochondrial and nuclear DNA markers for each sample and arrived at consistent, correct identifications for each of the samples of unknown billfish. In addition, samples of juvenile billfish collected by investigators at the University of Miami were analyzed in our laboratory with these molecular markers. Samples consisting of one eye taken from a 3-mm juvenile billfish provided sufficient DNA to amplify the mtDNA and nuclear markers, allowing specific identification (Fig. 5). The technique is currently being used to determine the temporal occurrence of istiophorid larvae in the Florida Straits (Luthy and McDowell[2]).

Although the methods presented in our study allow the specific identification of billfish species, more sensitive molecular markers are required to distinguish among ocean populations of some istiophorid species. Amendment I to the Fishery Management Plan for Atlantic Billfishes prohibits the sale of blue marlin, white marlin, and sailfish taken in the Atlantic Ocean, although it is legal to market blue marlin, striped marlin, and sailfish from the Indian or Pacific oceans. Enforcement of this regulation requires the ability to discriminate between Atlantic and Indo-Pacific individuals of blue marlin, sailfish, and white and striped marlin. Examination of our results suggests that there are several other molecular markers, which while not used in this study, occur at relatively high frequencies in Atlantic blue marlin, sailfish, and white marlin but do not occur in their Pacific conspecifics. These molecular markers could potentially be used to identify some Atlantic individuals without misclassifying a Pacific fish, thereby allowing the enforcement of the management plan. Additional work will be required to develop a database that would support such analyses.

[2] Luthy, S., and J. McDowell. 2001. A molecular approach to the identification of larval billfishes. Submitted to the Australian Journal of Marine and Freshwater Research as part of the 3rd international billfish symposium. CSIRO Publishing, 150 Oxford St. Collingwood, Vic. 3066, Australia.

Acknowledgments

We thank all those who so generously helped with collections including Julian Pepperell, Guy Harvey, Ed Everett, Eric Prince, and Mike Judge, as well as the anglers at Hotel Spa Buenavista, Baja California Sur, Mexico, and The Mid-Atlantic 500,000 Tournament, Cape May, New Jersey. We also thank Cheryl Woodley and the National Ocean Services' Charleston Laboratory for helping to validate our results. This project was funded by the National Oceanic and Atmospheric Association's Saltonstall-Kennedy Program grant number NA67FD00038.

Literature cited

Bielawski, J. P., and J. R. Gold.
1996. Unequal synonymous substitution rates within and

between two protein-coding mitochondrial genes. Mol. Biol Evol. 13:889–892

Buonaccorsi, V. P., K. S., Reece, L.W. Morgan, and J. E. Graves.
1999. Geographic distribution of molecular variance within the blue marlin (*Makaira nigricans*): a hierarchical analysis of allozyme, single-copy nuclear DNA, and mitochondrial DNA markers. Evolution 53:568–579.

Chow, S.
1994. Identification of billfish species using mitochondrial cytochrome *b* gene fragment amplified by polymerase chain reaction. Int. Comm. Conserv. Atl. Tunas. Report of the 2nd ICCAT billfish workshop. ICCAT 41:549–56.

Chow, S., M. E. Clarke, and P. J. Walsh.
1993. PCR-RFLP analysis on thirteen western Atlantic snappers (subfamily Lutjaninae): a simple method for species and stock identification. Fish. Bull. 91:619–627.

Chow, S., and K. Hazama.
1998. Universal PCR primers for S7 ribosomal protein gene introns in fish. Mol. Ecol. 7:1247–1263.

Chow, S., and H. Kishino.
1995. Phylogenetic relationships between tuna species of the genus *Thunnus* (Scombridae: Teleostei): Inconsistent implications from morphology, nuclear and mitochondrial genomes. J. Mol. Evol. 41:741–748.

Cordes, J. F. A., S. L. Armknecht, E. A. Starkey, and J. E. Graves.
2001. Forensic identification of sixteen species of Chesapeake Bay sportfishes using mitochondrial DNA restriction fragment–length polymorphism (RFLP) analysis. Estuaries. 24:49–58.

Daniel, L. B., III, and J. E. Graves.
1994. Morphometric and genetic identification of eggs of spring-spawning sciaenids in lower Chesapeake Bay. Fish. Bull. 92:254–261.

Finnerty, J. R., and B. A. Block.
1995. Evolution of cytochrome *b* in the Scombroidei (Teleostei): molecular insights into billfish (Istiophoridae and Xiphiidae) relationships. Fish. Bull. 9:78–96.

Goggin, C. L.
1994. Variation in the two internal transcribed spacers and 5.8S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). Mol. Biochem. Parasitology 65 (1994)179–182.

Graves, J. E.
1998. Molecular insights into the population structures of cosmopolitan marine fishes. J. Heredity 89:427–437.

Graves, J. E., and J. R. McDowell.
1994. Genetic analysis of striped marlin *Tetrapturus audax* population structure in the Pacific Ocean Can. J. Fish. Aquat. Sci., 51:1762–1768.
1995. Inter-ocean genetic divergence of istiophorid billfishes. Mar. Biol. 122:193–204.

Graves, J. E., M. A. Simovich, and K. M. Schaefer.
1988. Electrophoretic identification of early juvenile yellowfin tuna. Fish. Bull. 86:835–838

Heist, E. J., and J. R. Gold.
1997. Genetic identification of sharks in the U.S. Atlantic large-coastal fishery. Fish. Bull. 97:53–61.

Innes, B. H. , P. M. Grewe, and R. D. Ward.
1998. PCR-based genetic identification of marlin and other billfish. Mar. Freshwater Res. 49:383–388.

Lansman, R. A., R. O. Shade, J. F. Shapira, and J. C. Avise.
1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. J. Mol. Evol. 17:214–226.

Mork, J., P. Solemdal, and G. Sundnes.
1983. Identification of marine fish eggs: a biochemical genetics approach. Can. J. Fish. Aquat. Sci. 40: 361–369.

Nakamura, I.
1985. FAO species catalogue. Vol. 5: Billfishes of the world: an annotated and illustrated catalogue of marlins, sailfishes, spearfishes and swordfishes known to date. FAO Fish Synop 125. FAO, Rome.

Palumbi, S., A. Martin, S. Romano, W.O. McMillan, L. Stice, et al.,
1991. The simple fool's guide to PCR, 47 p. Department of Zoology and Kewalo Marine Laboratory, Univ. of Hawaii, Honolulu, HI.

Rocha-Olivares, A.
1998. Multiplex haplotype-specific PCR: a new approach for species identification of the early life stages of rockfishes of the species-rich genus *Sebastes* Cuvier. J. Exp. Mar. Biol. Ecol. 231:279–290.

Seutin, G., B. N. White, and P. T. Boag.
1991. Preservation of avian blood and tissue samples for DNA analysis. Can. J. Zool. 69:82–90.

Siddall, M. E., K. S. Reece, T. A. Nerad, and E. M. Burreson.
2001. Molecular determiniation of the phylogenetic position of a species in the genus *Colpodella* (Alveolata). American Museum Novitiates 3314:1–10.

Taylor, P. L.
1996. GeneJockey-II sequence processor. Software distributed by BIOSOFT, Cambridge, UK.

Winnepenninckx, B., T. Backeljau, and R. De Wachter.
1993. Extraction of high molecular weight DNA from mollusks. Trends Genet. 9:407.