Evolution of cytochrome *b* in the Scombroidei (Teleostei): molecular insights into billfish (Istiophoridae and Xiphiidae) relationships

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Abstract.—The suborder Scombroidei (Teleostei) has an extensive taxonomic history which traces its beginnings to at least 1832 (Cuvier and Valenciennes). However, a single well-corroborated phylogenetic hypothesis for the scombroid fishes does not exist. To date, efforts to define this suborder and determine the interrelationships of its constituent taxa have utilized morphological data almost exclusively. In this paper we present a molecular data set for addressing scombroid relationships: DNA sequences from the mitochondrial gene cytochrome *b*. These data provide valuable insights into scombroid relationships, especially regarding the long-standing debate over the placement of the billfishes (Istiophoridae and Xiphiidae). The cytochrome *b* data strongly refute a close relationship between Scombridae and billfishes and also support separation of the billfishes from the Scombroidei. In addition, these data suggest a new hypothesis on the evolutionary relationships among istiophorid billfishes.

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The suborder Scombroidei is a well studied assemblage of more than 100 marine teleosts. A consensus on the taxonomic limits and intrarelationships of this group remains elusive despite more than 150 years of study (Cuvier and Valenciennes, 1832; Regan, 1909; Gregory, 1933; Berg, 1940; Fraser-Brunner, 1950; Collette et al., 1984; Johnson, 1986; Potthoff et al., 1986; Block et al., 1993). In 1832, Cuvier and Valenciennes proposed the Scombroidei as a natural group containing the oilfishes and snake mackerels (family: Gempylidae), the cutlassfishes (Trichiuridae), and the "tunnies" (Scombridae). Regan (1909) expanded the Scombroidei by adding three families: Istiophoridae (marlins, sailfish, and spearfishes); Xiphiidae (the swordfish); and Luvaridae (the louvar). Most subsequent classifications agree that the monotypic Luvaridae is not a scombroid but an acanthuroid (Leis and Richards, 1984; Tyler et al., 1989). However, there is substantial disagreement over the relationships of the families Istiophoridae and Xiphiidae, collectively known as billfishes.

In the last ten years three morphological studies have proposed three different hypotheses on the relationships of billfishes. In 1984, Collette et al. published a scombroid phylogeny based on 40 morphological characters (Fig. 1). They proposed that billfishes are the sister group of the Scombridae. Their cladogram suggested that several synapomorphies unite billfishes and scombrids, including a pharyngeal toothplate stay, a pair of lateral keels on the caudal peduncle, and the extension of the caudal-fin rays to cover the hypural plate. However, the position of billfishes was not strongly defined in the Collette et al. study because of homoplasious character evolution. Of the twelve character-state transitions that occurred within the billfish lineage on their cladogram, five were reversals to the primitive state, and six occurred independently in other lineages. Collette et al. (1984) considered the placement of billfishes within the suborder Scombroidei to be uncertain and conditional upon additional evidence. They cited larval evidence (Potthoff et al., 1986) which indicates that the scombroid families Scombridae, Gempylidae, and Trichiuridae are closely related to each other and are distantly related to billfishes. We will refer to the Collette et al. hypothesis as the scombrid sister group hypothesis.

In 1986, Johnson published a scombroid phylogeny using many of the characters from the Collette et

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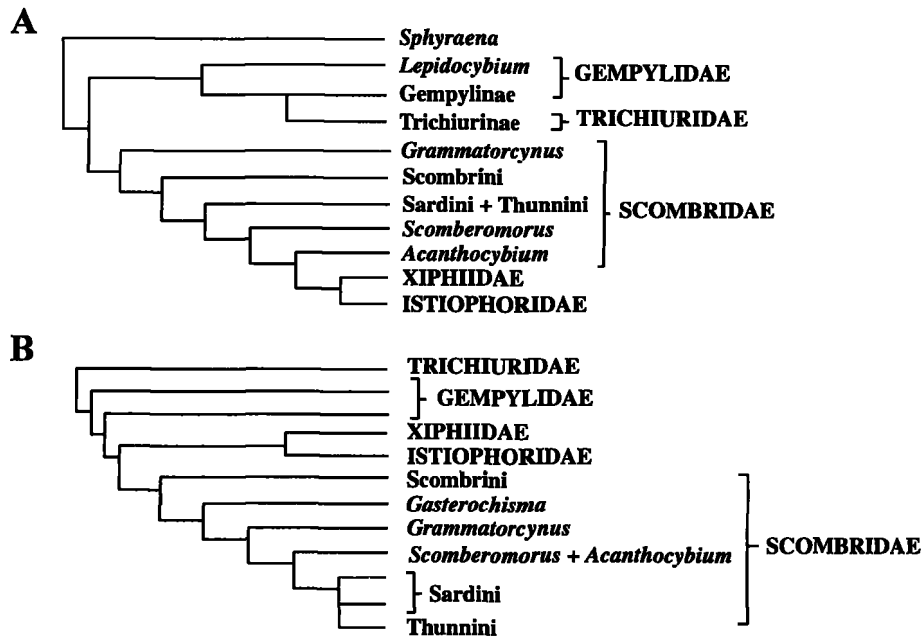

Figure 1

Two phylogenetic hypotheses for the scombroid fishes based on morphological evidence. The studies of (**A**) Johnson (1986) and (**B**) Collette et al. (1984) are examples of the scombrid-subgroup and the scombrid-sister group hypotheses of billfish (Istiophoridae and Xiphiidae) relationships. Johnson (1986) considered billfishes a subgroup of the family Scombridae most closely related to the wahoo, *Acanthocybium solandri*. Collette et al. (1984) placed the billfishes as a sister group to the Scombridae. Both hypotheses propose that billfishes and scombrids share a common ancestor to the exclusion of other scombroids. An alternative hypothesis for billfish relationships is that billfishes are not scombroids. This hypothesis has never been depicted explicitly in the form of a cladogram (Gosline, 1968; Nakamura, 1983; Potthoff et al., 1980; Potthoff et al., 1986).

al. (1984) study and several additional characters (Fig. 1). Like Collette et al., Johnson proposed that billfishes and scombrids compose a monophyletic group, but he regarded billfishes as a subgroup of the Scombridae. A critical piece of evidence supporting this hypothesis that billfishes are a derived group within scombrids is the presence of cartilaginous interconnections between gill filaments in billfishes and the scombrid *Acanthocybium solandri*. Based largely on this proposed synapomorphy, Johnson placed Istiophoridae and Xiphiidae as derived scombrids and *Acanthocybium* as their sister group. This association has been suggested by others (Lütken, 1880; Fraser-Brunner, 1950). However the position of billfishes in Johnson's study was only weakly supported because of homoplasy. For example, five of the ten character-state transitions that support billfish monophyly on Johnson's cladogram are reversals. We will refer to the Johnson hypothesis as the scombrid subgroup hypothesis.

Other workers have proposed that billfishes are not scombroids. In 1986, Potthoff et al. published a study of bone development in scombroids in which they discussed scombroid phylogeny. They concluded that billfishes are not scombroids because of their lack of resemblance to other scombroids in vertebral number and osteological development. They suggested that these characters indicate billfish affinities to the percoids. This hypothesis has been suggested in previous studies (Potthoff et al., 1980; Nakamura, 1983). We will refer to this hypothesis as the nonscombroid hypothesis.

It is evident from the morphological studies that there has been a great deal of homoplasious morphological evolution in billfishes. Therefore, it is difficult to reconstruct the evolutionary relationships of this group based on morphology alone. In an attempt to derive additional, independent data on scombroid intrarelationships and, in particular, to address the position of billfishes, we compiled a mo-

lecular data set that consists of DNA sequences from the mitochondrial gene cytochrome *b*. This gene codes for a functionally conserved protein that should facilitate sequence alignment over ancient divergences. Additionally, it has been used to examine both intraspecific genealogy (Finnerty and Block, 1992) and much deeper phylogenetic questions such as the origin of the mammalian orders (Irwin et al., 1991). The initial scombroid radiation probably occurred in the Paleocene epoch (Bannikov, 1985; Carroll, 1988). Therefore, cytochrome *b* sequence should be phylogenetically informative about divergences within the suborder.

The analysis presented in this paper builds on our earlier molecular study (Block et al., 1993). However, we have improved on the previous study in several ways which allow us to directly test the competing hypotheses of billfish relationships. First, we have obtained sequences from additional outgroups. The inclusion of presumably more distant outgroups permits us to address the question of scombroid monophyly. This is important because the nonscombroid hypothesis of billfish relationships argues that the Scombroidei is not a monophyletic group. Second, we include sequence information from the scombrid *Acanthocybium*, a taxon which is integral to the scombrid subgroup hypothesis. Third, we utilize statistical tests to directly compare different hypotheses of billfish relationships. Finally, we emphasize character-state changes that accrue relatively slowly in order to minimize the effects of phylogenetic noise.

Materials and methods

Samples

Partial cytochrome *b* sequences (590 base pairs) were obtained from 75 individuals representing 34 species of perciform fishes: 30 scombroid species and four putative outgroup taxa (*Sphyraena*, *Coryphaena*, *Mycteroperca*, and *Morone*; Table 1). We included *Sphyraena* based on the placement by Johnson (1986) of this taxon as the most primitive member of the Scombroidei. Several percoid taxa (*Coryphaena*, *Mycteroperca*, and *Morone*) were included because of the suggestion by some authors that billfishes are percoids (Gosline, 1968; Potthoff et al., 1980; Nakamura, 1983; Potthoff et al., 1986). Published cytochrome *b* sequences from two cypriniform fishes obtained from Genbank were used to root the phylogenetic analysis (*Crossostoma lacustre* [Tzeng et al., 1990] and *Cyprinus carpio* [Chang, 1994]). We verified the outgroup status of the cyprinids by first conducting a phylogenetic analysis using published sequence from the sturgeon *Acipenser transmontanus*, a holostean, to root a parsimony analysis. We attempted but were unable to obtain full length sequences (590 base pairs) from two fixed and preserved specimens of *Scombrolabrax heterolepis* possibly because of DNA degradation in these specimens.

DNA extraction

DNA was obtained from frozen tissue samples of the mitochondria-rich "heater tissue" (found in Istiophoridae, Xiphiidae, and *Gasterochisma melampus*; Block, 1986), red muscle, white muscle, or liver. Digestion of 0.1–0.6 g tissue was performed in ten volumes of extraction buffer containing 100 mM Tris Cl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 0.1% SDS, 50 mM DTT, and 0.7 mg/mL proteinase K. Digestion proceeded for 2–4 hours at 41°C. The homogenate was extracted twice with equal volumes of phenol (pH 8.0), once with 1:1 phenol/chloroform, and once with chloroform. The final extract was precipitated with 1/9 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol.

DNA amplification and sequencing

The polymerase chain reaction (PCR) was used to amplify a 700 base pair region of cytochrome *b*. A 305 base pair segment (not including primers) was generated by using published oligonucleotide sequences (Kocher et al., 1989). We amplified an overlapping, 425-bp region farther downstream with primers L15079 (5'-GAGGCCTCTACTATGGCTCTTACC-3') or L15080 (5'-CGAGGCCTTTACTACGGCTCTTACCT-3') and H15497 (5'-GCTAGGGTATAATT GTCTGGGTCGCC-3'). Double stranded amplification was performed in a 100-µL volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5–3.0 mM MgCl, 200 µM of each dNTP, each primer at 1 mM, 1 µg of template DNA, and 2 units of Amplitaq DNA polymerase (Perkin-Elmer/Cetus). Most templates were amplified through thirty cycles of PCR [1 minute denaturation (92–95°C), 1 minute annealing (40–50°C), and 3 minutes extension (72°C)] on an Ericomp thermal cycler. Alternatively, PCR was performed on a DNA Thermal Cycler 480 (Perkin-Elmer) with the following temperature cycling regime: 5 cycles of 1 minute denaturation at 95°C, 1 minute primer annealing at 40°C, 1:30 ramp to 72°C, and one minute extension at 72°C, followed by 25–35 cycles with an annealing temperature of 45°C. An 18-µL aliquot of the double stranded product was run by means of electrophoresis through a 1X TBE 1% agarose gel (Sea Plaque, FMC) at 5 V/cm for 45 minutes. A single stranded template was produced by asymmetric PCR (Gyl-

Table 1

Partial cytochrome b sequences (590 base pairs) were obtained from 34 perciform fishes, including 30 scombroid species. Published cytochrome *b* sequences were also obtained from Genbank for two cypriniform fishes.

Order and suborder[1]	Family and species	Common name	*n*	Locales[2]
Perciformes:Scombroidei	Istiophoridae			
	Istiophorus platypterus	sailfish	2	A, P
	Makaira indica	black marlin	2	I
	Makaira nigricans	blue marlin	8	A, P
	Tetrapturus albidus	white marlin	2	A
	Tetrapturus angustirostris	shortbill spearfish	2	P
	Tetrapturus audax	striped marlin	3	P
	Tetrapturus belone	Mediterranean spearfish	2	M
	Tetrapturus pfluegeri	longbill spearfish	1	A
	Xiphiidae			
	Xiphias gladius	broadbill swordfish	6	A, P
	Scombridae			
	Acanthocybium solandri	wahoo	3	A
	Scomberomorus cavalla	king mackerel	1	A
	Scomberomorus maculata	Spanish mackerel	2	A
	Gasterochisma melampus	butterfly mackerel	3	T
	Auxis thazard	frigate mackerel	2	P
	Euthynnus affinis	kawakawa	2	P
	Euthynnus alletteratus	little tunny	2	A
	Katsuwonus pelamis	skipjack tuna	2	P
	Thunnus alalunga	albacore tuna	2	P
	Thunnus albacares	yellowfin tuna	2	P
	Thunnus maccoyii	southern bluefin tuna	2	T
	Thunnus obesus	bigeye tuna	2	P
	Thunnus thynnus	northern bluefin tuna	2	A
	Sarda chiliensis	eastern Pacific bonito	1	P
	Sarda sarda	Atlantic bonito	2	A
	Scomber scombrus	Boston mackerel	2	A
	Scomber japonicus	chub mackerel	2	P
	Gempylidae			
	Gempylus serpens	snake mackerel	2	P
	Lepidocybium flavobrunneum	escolar	2	P
	Ruvettus pretiosus	oilfish	2	A
	Trichiuridae			
	Trichiurus lepturus	scabbard fish	3	A
Perciformes:Percoidei	Coryphaenidae			
	Coryphaena equiselis	pompano dolphin	2	P
	Serranidae			
	Mycteroperca interstitialis	yellowmouth grouper	1	A
	Percichthyidae			
	Morone saxatilis	striped bass	1	P
Perciformes:Sphyraenoidei	Sphyraenidae			
	Sphyraena sphyraena	Atlantic barracuda	1	A
Cypriniformes	Balitoridae			
	Crossostoma lacustre	hillstream loach	Tzeng et al., 1992	
	Cyprinidae			
	Cyprinus carpio	carp	Chang et al., 1994	

[1] Eschmeyer, 1990.

[2] A=Atlantic ocean; P=Pacific ocean; I=Indian ocean; T= Tasman sea; M=Mediterranean Sea.

lensten and Erlich, 1988) carried out in a 100-μL volume containing the same reactants as the initial PCR but using 10 μL of the dissolved gel band and reducing one primer concentration 100-fold. The product was washed by centrifugal dialysis with sterile water in Centricon microconcentrators (Amicon) to remove excess dNTP's. Sequencing was performed with the Sequenase kit (United States Biochemical, Cleveland, Ohio) by using the limiting primer from the asymmetric PCR reaction. Data from eight species were obtained by directly sequencing double-stranded PCR products. The template was purified prior to sequencing (either directly from the PCR reaction mix or following excision of the appropriate band from low-melt agarose) with Magic PCR Preps (Promega). Sequencing was performed with the Sequenase kit according to the specifications of Casanova et al. (1991). Sequences from *Mycteroperca* and *Morone* was obtained after first cloning the PCR products in pGEM t-vector (Promega) according to the manufacturer's instructions. Transformation was carried out by using XL–1 blue cells. Two positive clones were selected for each PCR product. Double-stranded sequencing (Sequenase 2.0) was performed following alkaline denaturation as recommended by the manufacturer. Sequence was obtained from both strands of the amplified fragment for all individuals.

Analysis

Sequences were aligned by using the MacVector program (IBI Biotechnologies). Maximum parsimony analysis was performed with PAUP 3.1. (Swofford, 1991). Neighbor-joining (Saitou and Nei, 1987) and UPGMA dendograms were constructed with Phylip 3.5 (Felsenstein, 1993). The strength of support for various nodes was assessed by using the bootstrap analysis (Felsenstein, 1985). Specific conditions for each analysis are contained in the figure legends.

Competing phylogenetic hypotheses were compared by using the "enforce topological constraints" option of PAUP 3.1. This option allowed us to determine the length difference between the most parsimonious trees that support each hypothesis. The cladistic permutation test for monophyly and nonmonophyly (Faith, 1991) was then used to ascertain whether the more parsimonious hypothesis is significantly better than the competing hypothesis according to the criterion of parsimony. The test was performed as follows. The actual length difference between trees supporting the two opposing hypotheses was obtained. Then 99 permuted data sets were constructed from the original data set by randomly shuffling the character states for each character. We then obtained the length difference between trees supporting the two opposing hypotheses for each permuted data set. If the actual length difference was matched or exceeded fewer than 5 times in all 100 data sets (the original data set plus 99 permuted data sets), then the more parsimonious hypothesis was considered to be significantly better than the less parsimonious hypothesis. This corresponds to a topology-dependent permutation tail probability, or T-PTP, of less than or equal to 0.05.

The effects of character weighting on parsimony analysis were assessed by EOR weighting (Thomas and Beckenbach, 1989; Knight and Mindell, 1993): each type of nucleotide substitution was weighted according to the ratio of its expected number of occurrences divided by its observed number of occurrences, or EOR. There are six types of nucleotide substitutions if we disregard the direction of change: A⇔G, C⇔T, G⇔T, G⇔C, A⇔T, and A⇔C. The observed number of each substitution type was obtained through pairwise sequence comparisons. Pairwise comparisons were performed between sets of sister species (sister species were identified through an initial unweighted phylogenetic analysis; see Fig. 2). Sister-species comparisons were used for two reasons. First, within a clade, sister species will tend to represent relatively recent speciation events. This recency lessens the chance that multiple substitutions have occurred at the same site and that more recent substitutions obscure older ones. Second, all comparisons between pairs of sister species are mutually independent. Therefore, if we restrict our comparisons to sister species, we cannot count the same base substitution twice.

We modified the method of Knight and Mindell (1993) to derive the expected number of substitutions in each class. This method accounts for differences in the frequencies of the four nucleotides that greatly influence the expected frequency of each substitution type. For instance, if guanine residues are very rare, then substitutions of other nucleotides for guanine will also be rare. The L-strand base composition of cytochrome *b* in scombroid fishes is strongly skewed (Table 2), as it is in other groups examined (for example, Irwin et al., 1991). Cytosines and thymidines each compose nearly 30% of the total nucleotide population whereas guanines compose less than 16%. In order to incorporate knowledge of the base composition into our derivation of the expected number of each substitution type, we proceeded as follows. First, the average frequency of each nucleotide (f) was obtained for all species used in the pairwise sequence comparisons. Second, the observed number of each substitution type ($S_{O[i \Leftrightarrow j]}$), where i and j represent two different nucleotides, was obtained by summing the results from all pairwise comparisons of

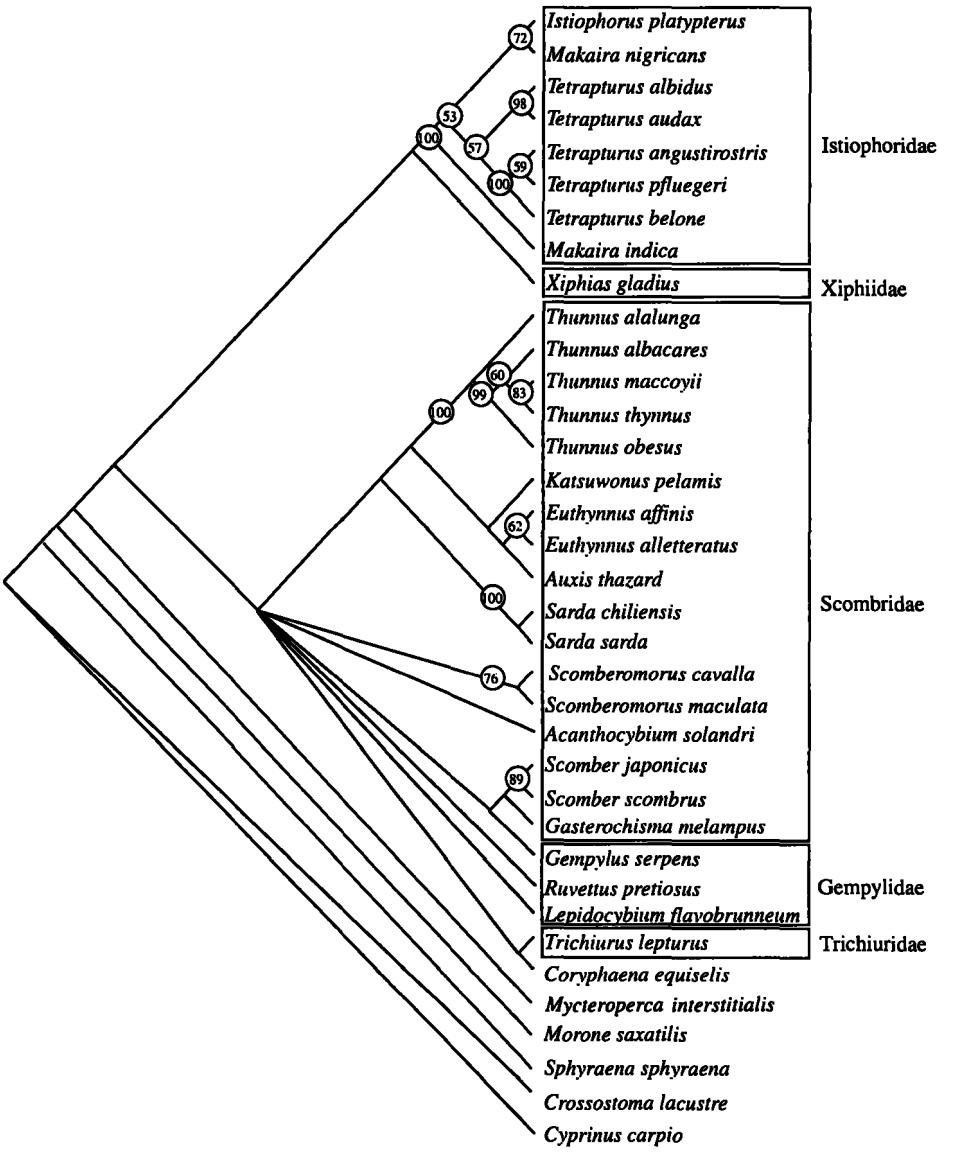

Figure 2

Phylogeny of the Scombroidei based on an unweighted analysis of 248 phylogenetically informative nucleotide sites. The cladogram depicted is a strict consensus of four equally parsimonious trees identified by using a heuristic search procedure on the program PAUP 3.1. (Swofford, 1991): TBR (tree bissection and reconnection) branch swapping was performed on 10 starting trees generated through random stepwise addition of taxa. *Crossostoma* and *Carpio* were specified as the outgroup. Length, consistency index, and retention index are the following: L=1595, CI=0.317, RI=0.539. Circled numbers at nodes indicated the percentage of trials in which a given partition between taxa is supported in 1,000 replications of the bootstrap analysis (Felsenstein, 1985). Only nodes supported in >50% of bootstrap replications are indicated.

sister species. The expected number of each of the six substitution types ($S_{E[i\Leftrightarrow j]}$)was then derived as follows:

$$S_{E[i\Leftrightarrow j]} = \left(f_i + f_j\right)\left(S_{0[total]}\right)/3.$$

We divide by three because three types of base substitutions are possible for each base, and we are interested in obtaining an expectation for one of them. For example, the expected number of A⇔T substitutions equals the average frequency of A's (0.23) plus

Table 2

Nucleotide substitutions by type determined through pairwise alignments. The observed substitutions for each type, $S_{O[total]}$, where i and j represent two different nucleotides, were calculated by summing the results from 8 pairwise comparisons of sister taxa. The expected substitutions for each type, $S_{E[total]}$, were calculated according to the formula $S_{E[total]}=(f_i+f_j)(S_{O[total]})/3$, where f_i and f_j are the frequency of nucleotides i and j. Average base frequencies for the 16 species are as follows: G=0.16, A=0.23, T=0.29, C=0.32.

	Substitution Types						
	TRANSITIONS				TRANSVERSIONS		
Pairwise comparison	A⇔G	C⇔T	G⇔T	G⇔C	A⇔T	A⇔C	Total
Tetrapturus audax vs. *Tetrapturus albidus*	0	0	0	1	1	0	2
Tetrapturus angustirostris vs. *Tetrapturus pfluegeri*	7	1	0	0	0	0	8
Makaira nigricans vs. *Istiophorus platypterus*	1	21	0	0	1	0	23
Euthynnus affinis vs. *Euthynnus alletteratus*	5	27	0	0	3	3	38
Thunnus thynnus vs. *Thunnus maccoyii*	4	3	0	0	1	0	8
Scomberomorus maculata vs. *Scomberomorus cavalla*	13	38	1	1	8	11	72
Sarda sarda vs. *Sarda chiliensis*	6	15	0	3	0	2	26
Scomber japonicus vs. *Scomber scombrus*	25	35	2	5	6	6	79
Total observed substitutions	61	140	3	10	20	22	256
Expected substitutions (see Methods section)	33.28	52.05	38.40	40.96	44.37	46.92	256
Expected/observed ratio (EOR)	0.55	0.37	12.80	4.10	2.22	2.13	

the average frequency of T's (0.29) multiplied by the total number of substitutions (256) divided by three, or 44.37 (Table 2).

The weights used for each substitution type (Table 2) are the ratios of expected substitutions divided by observed substitutions for that substitution type, rounded to the nearest integer (expected divided by observed ratios, or EOR's). All EOR's less than one were rounded to one. Weights were entered into PAUP 3.1. (Swofford, 1991) in the form of a step matrix.

Results

Sequence evolution and interfamilial relationships

Molecular data sets, such as the cytochrome *b* sequences presented in this study, are known to encompass subsets of characters that evolve at different rates. Subsets of data that differ in their evolutionary rates will also differ in their phylogenetic utility. Character state changes that accrue very rapidly should permit resolution of very recent divergences. However, these rapid character state changes can provide false inferences about distant relationships because of homoplasy. The likelihood of reversals and independent acquisitions is high if a particular site is evolving rapidly because there are only four possible character states (G, A, T, and C) and only six possible types of character state change (A⇔G, C⇔T, G⇔T, G⇔C, A⇔T, and A⇔C). Therefore, in order to make an accurate reconstruction of the earliest branching events in scombroid history, we should emphasize slowly evolving character state changes.

In an effort to best utilize the phylogenetic information from both slowly and rapidly evolving character state changes, our phylogenetic analysis proceeds in several discrete steps. We begin with an unweighted analysis of all informative nucleotide sites. This analysis is strongly influenced by nucleotide substitutions that accrue rapidly and should be most informative concerning recent speciation events. We then attempt to improve our resolution of more ancient divergences by giving greater weight to less frequent types of nucleotide substitutions. We conclude with a phylogenetic analysis based on the inferred amino acid sequences. The amino acid sequences evolve very slowly and should provide our most reliable estimates of the earliest splits between lineages. In each instance, the phylogenetic analysis is preceded by a discussion of the evolutionary variation in the character subset under consideration.

Unweighted nucleotide analysis

A 590-base pair fragment of the cytochrome *b* gene, representing positions 134 through 723 of the human cytochrome *b* sequence, was aligned across all

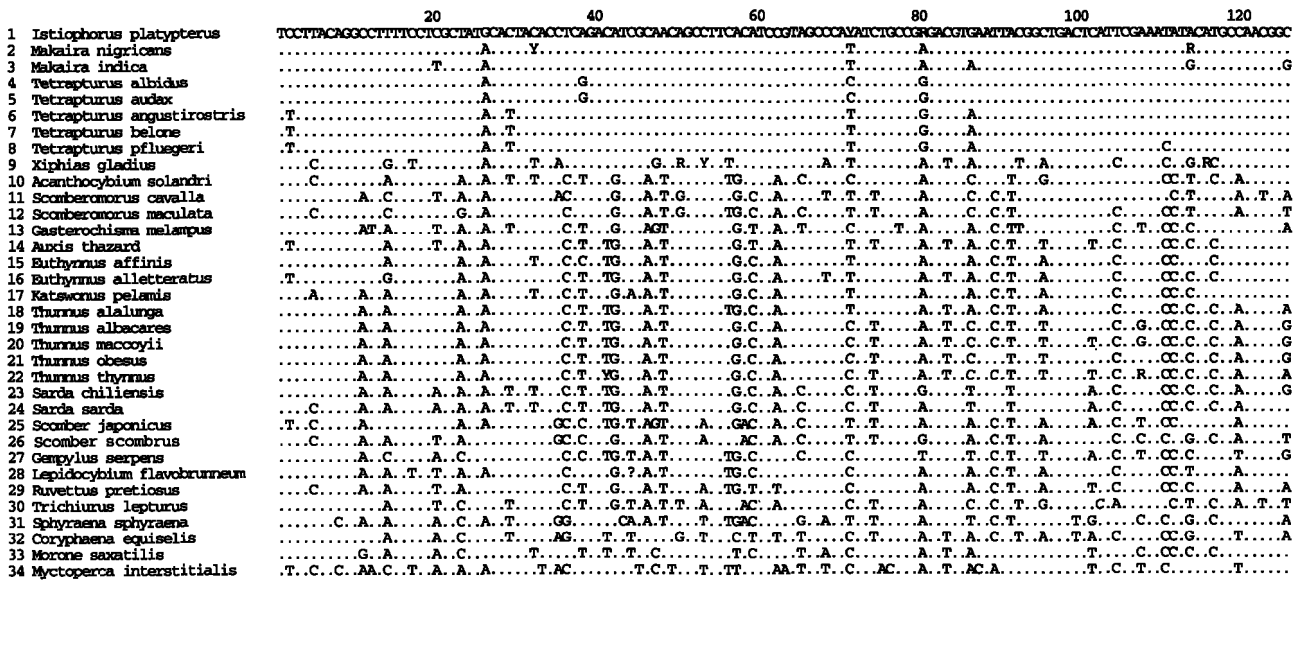

Figure 3

Alignment of partial cytochrome *b* sequence (590 base pairs) across 34 species of perciform fishes and two species of Cypriniformes. Nucleotide position 1 is equivalent to position 134 of the human cytochrome *b* gene. Intraspecific polymorphism is indicated as follows: R=A/G, Y=C/T, M=A/C, S=C/G, K=G/T, W=A/T, H=A/T/C, D=A/G/T. Ambiguities are indicated by '?'

thirty-six species included in the analysis (Fig. 3). No deletions or insertions were detected. Overall, 293 nucleotide positions are variable; 248 were potentially phylogenetically informative. As expected for a protein coding sequence, the degree of nucleotide variability differs according to codon position (Table 3). The third position is most variable and the second position is least variable. Differences in nucleotide variability at the three codon positions are due to the fact that many third position substitutions are silent, whereas many second position substitutions result in nonconservative amino acid replacements.

The differences in substitution rates between codon positions becomes more apparent when we compare

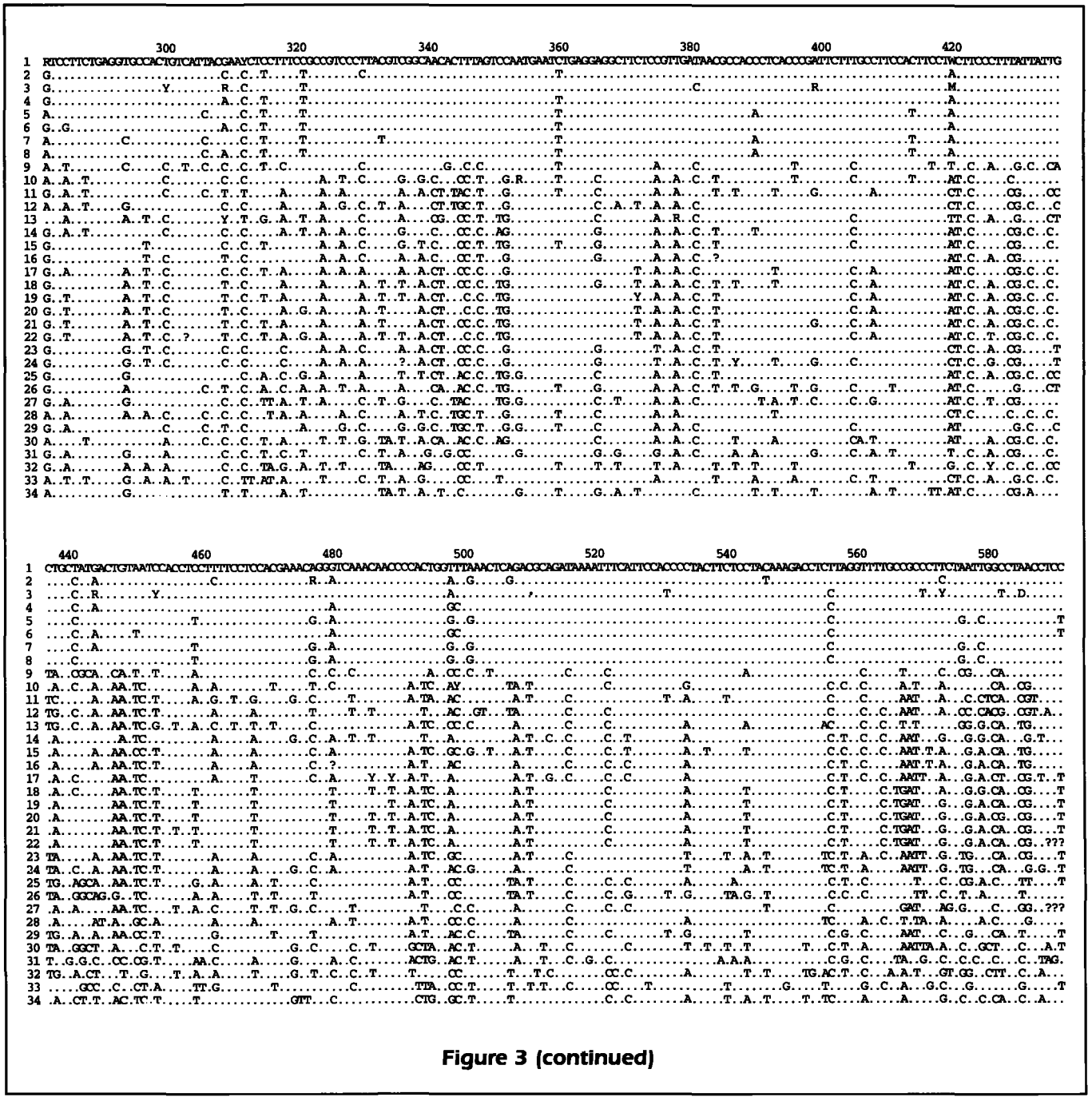

Figure 3 (continued)

the inferred number of substitutions (Table 3). For example, if a nucleotide site is twofold variable, i.e. if two bases occur at that position in an alignment of all species, then at least one base substitution has occurred at that position during the evolutionary history of the species concerned. Likewise, if a position is threefold variable, at least two substitutions have occurred, and so on. By this approximation, the 293 variable positions have experienced at least 521 substitutions, and substitutions at the third position outnumber substitutions at the first and second positions by nearly 4 to 1 and by more than 12 to 1, respectively.

Figure 2 presents a phylogeny of the Scombroidei based on an unweighted parsimony analysis of all informative nucleotide sites. In this cladogram, only the relationships among recently diverged taxa are strongly supported. There is support for the monophyly of genera within the family Scombridae (*Thunnus*, *Euthynnus*, *Sarda*, *Scomber*, and *Scom-*

Table 3

Variable sites in the cytochrome *b* nucleotide alignment (Fig. 2) according to codon position. Variable sites are further characterized according to how many nucleotide states are present: 2 states=twofold variable, 3 states=threefold variable, fourstates=fourfold variable.

	Codon position			
	1	2	3	Total
Total sites	196	197	197	590
Variable sites	72	29	192	293
A) twofold variable sites	50	27	69	146
B) threefold variable sites	15	2	49	66
C) fourfold variable sites	7	0	74	81
Minimum inferred substitutions = [(A) + 2(B) + 3(C)]	101	31	389	521
Phylogenetically informative variable sites	42	18	188	248

beromorus), and for the monophyly of the family Istiophoridae. Interrelationships within the family Istiophoridae and the genus *Thunnus* are well resolved. No other nodes are supported by more than fifty percent of bootstrap replicates (Felsenstein, 1985). Furthermore, there is a substantial polychotomy.

Weighted nucleotide analysis

The lack of resolution in the unweighted nucleotide analysis is not entirely unexpected. From Table 3, we can surmise that many nucleotide sites have incurred multiple substitutions and therefore the likelihood of convergent substitutions or reversals is high. In order to minimize the confounding effects of these homoplasious base substitutions, we have weighted infrequent substitution types more heavily using a modification of the method of Knight and Mindell (1993). If we disregard the direction of character change, we can place all nucleotide substitutions into six classes : A⇔G, C⇔T, G⇔T, G⇔C, A⇔T, and A⇔C. Through pairwise sequence comparisons we obtained observed counts for each of these substitution types (Table 2; also see Methods section). We observe a nearly 50-fold difference between the most common (C⇔T) and the least common (G⇔T) substitution types. Then, from the total number of observed substitutions and the observed frequency of each base, we derived the expected number of occurrences for each substitution type. The ratios of expected occurrences to observed occurrences for each substitution type (EOR's) were then used to weight the six types of base substitutions. The result of this weighting scheme is that substitution types that occur less frequently than expected are weighted more heavily.

A phylogeny based on EOR weighting of nucleotide substitutions is presented in Figure 4. It retains all of the strongly supported nodes that appear in the unweighted topology. In addition, the weighted topology contains three more basal nodes that are strongly supported by the bootstrap analysis (>50%): the node uniting Gempylidae, Scombridae, and Trichiuridae, the node uniting Xiphiidae and Istiophoridae, and the node uniting *Auxis* and *Euthynnus*. This suggests that the character weighting scheme has accomplished its goal to some extent: we have retained the phylogenetic signal from rapidly evolving substitutions while emphasizing the phylogenetic signal from slowly evolving substitutions.

According to the weighted cladogram (Fig. 4), all scombroids fall into two clades. The billfishes comprise one clade consisting of a monophyletic Istiophoridae and its sister group, Xiphiidae. All other scombroids (Gempylidae, Scombridae, and Trichiuridae) fall into a separate clade. This major split within the suborder Scombroidei is in agreement with our previous study (Block et al., 1993). However, in contrast with our previous study, the use of character weighting and the inclusion of more distant outgroups leads to the result that the suborder Scombroidei is not monophyletic. On the most parsimonious tree, *Sphyraena* and *Coryphaena* share a common ancestor with the gempylid-scombrid-trichiurid clade to the exclusion of billfishes, though this node does not receive particularly strong support from the bootstrap analysis. This result indicates some support for the hypothesis that billfishes are not scombroids. More importantly, the cladogram excludes the possibility that billfishes and scombrids comprise a monophyletic group within the Scombroidei, as required by the scombrid subgroup and scombrid sister group hypotheses. In summary, the weighted analysis agrees with the nonscombroid hypothesis and conflicts with the scombroid subgroup and scombroid sister group hypotheses.

Amino acid analysis

Amino acid substitutions occur far less frequently than nucleotide substitutions owing to the strong functional constraints on many regions of the molecule. Cytochrome *b* is a component of the electron transport chain and spans the inner mitochondrial membrane. The portion of the gene sequenced in this study encodes 195 amino acids corresponding to residues 46 through 240 of the human cytochrome *b* (Fig. 5).

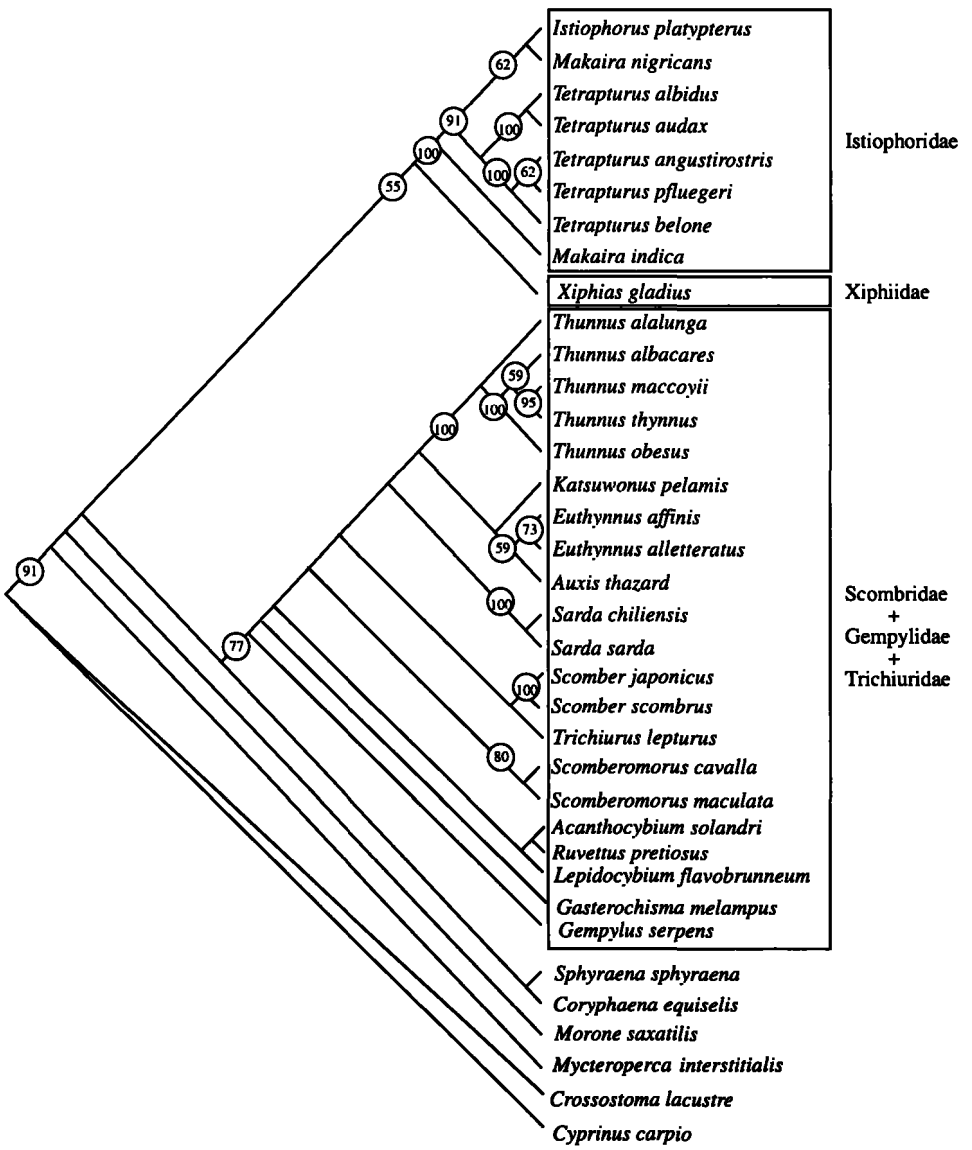

Figure 4

Phylogeny of the Scombroidei based on a weighted, maximum parsimony analysis of informative nucleotide sites. The six types of nucleotide substitutions are weighted according to the ratio of their expected occurrence to their observed occurrence (see Table 3). Weights used for each substitution type are the following: A⇔G=1, C⇔T=1, G⇔T=13, G⇔C=4, A⇔T=2, and A⇔C=2. *Crossostoma* and *Carpio* were specified as the outgroup. The tree depicted is the single most parsimonious topology identified in a heuristic search: TBR branch swapping was performed on 10 starting trees generated through random stepwise addition of taxa. Tree length is 2,348 steps. PAUP 3.1. was unable to derive consistency and retention indices for the cladogram that incorporated the weighting scheme. Circled numbers at nodes indicate the percentage of trials in which a given partition between taxa is supported in 1,000 replications of the bootstrap analysis (Felsenstein, 1985).

This fragment spans four transmembrane domains and includes part of the region implicated as the outer membrane redox reaction center (Howell and Gilbert, 1988; Howell, 1989; Fig. 6). In a comparison of the inferred peptide sequences across the 36 species included in this study, 134 (69%) of the 195 amino acid residues are invariant, 34 (17%) occur in 2 amino acid states, and 27 (14%) occur in 3 or more states.

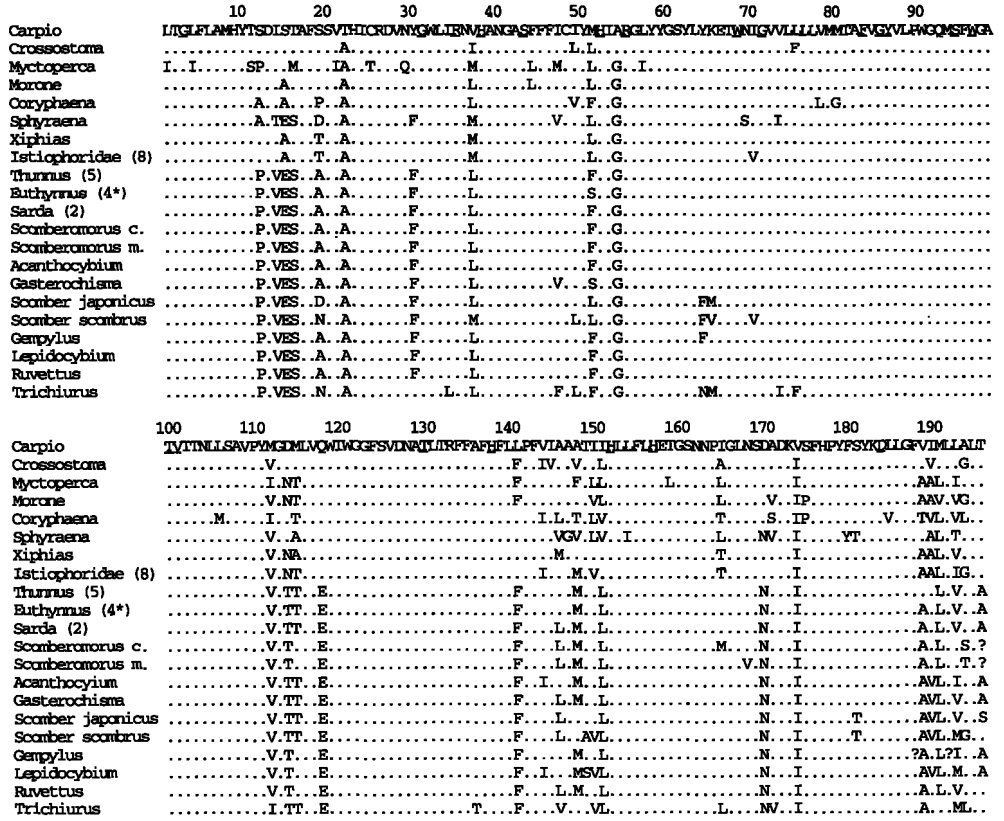

Figure 5

Alignment of inferred amino acid sequences. The amino acid sequences were inferred from the nucleotide sequences presented in Figure 3 by using the translation option of MacVector (IBI technologies) and the animal mitochondrial genetic code. There was no variation in inferred amino acid sequence within the family Istiophoridae, within the genus *Thunnus*, within the genus *Sarda*, nor among the genera *Auxis*, *Euthynnus*, and *Katsuwonus*. Conserved positions identified by previous studies are underlined in the query sequence, *Carpio* (Howell and Gilbert, 1988; Esposti et al., 1993).

This level of variability in amino acid sequence is very similar to that reported in a study of placental mammals, a group whose divergence times are probably comparable to scombroids (Irwin et al., 1991). Much of the variation in scombroid cytochrome *b* occurs in the transmembrane portion of the molecule and represents substitutions between hydrophobic residues (leucine, isoleucine, and valine). The largest stretches of invariant residues (21 and 17) occur in a region implicated as part of the Q_o redox reaction center (Howell and Gilbert, 1988; Howell, 1989; Fig. 6). All of the functionally constrained sites identified by previous studies are conserved throughout the fishes included in this study (see Fig. 5; Howell and Gilbert, 1988; Esposti et al., 1993).

Figure 7 presents a parsimony analysis based on 38 informative amino acid sites. The amino acid sequences do not provide information about more recent speciation events because they evolve very slowly, but they contain important evidence about the relationship of billfishes to other scombroids. The amino acid analysis shares two important similarities with the weighted nucleotide analysis: first, Scombridae, Gempylidae, and Trichiuridae comprise a clade, and second, *Sphyraena* and *Coryphaena* share a common ancestry with this Scombridae-Gempylidae-Trichiuridae assemblage to the exclusion of the billfishes (Xiphiidae and Istiophoridae). The node uniting *Sphyraena* with the scombrid-gempylid-trichiurid clade is one of the more strongly supported nodes according to the bootstrap analysis. Therefore, cytochrome *b* amino acid substitutions support the non-scombroid hypothesis and conflict with the scombrid subgroup and scombrid sister group hypotheses.

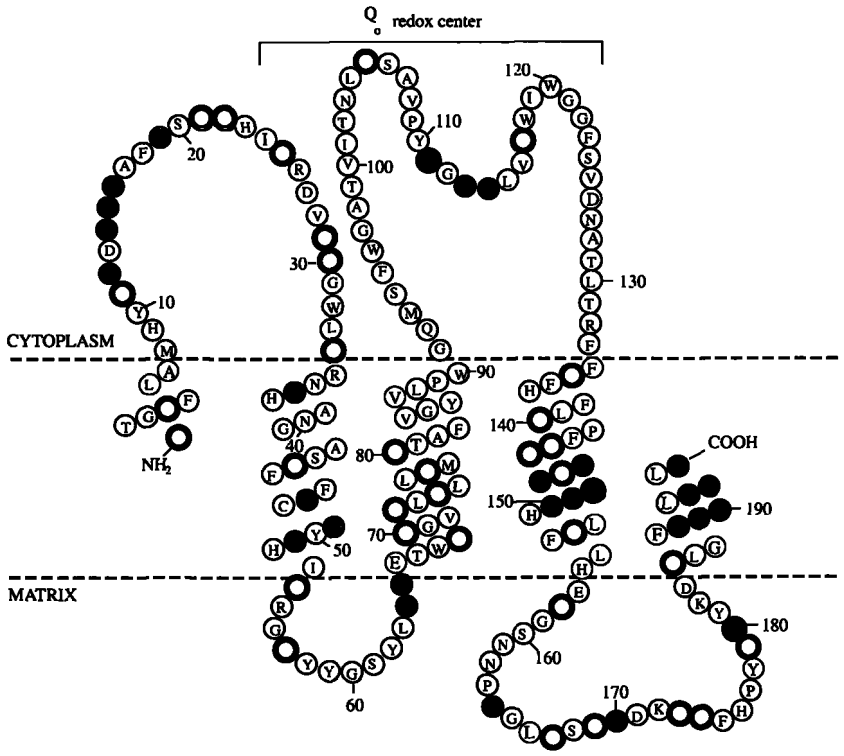

Figure 6

Variability in amino acid sequence superimposed over a structural model for cytochrome *b* (Howell, 1989). Hypervariable residues, present in three or more amino acid states, are indicated by solid circles. Variable residues, present in two states, are indicated by open circles. The amino acids present at invariant residues are specified on the diagram. Residue 1 of this fragment is equivalent to the forty-sixth residue from the amino terminal of the protein in humans.

The strength of the evidence that billfishes are not scombroids can be emphasized by directly examining the amino acid characters that are informative about this issue. Of the 38 informative amino acid sites, no sites unite billfishes and other scombroids to the exclusion of other perciforms, whereas eight sites unambiguously separate billfishes from all other scombroids, i.e. sites where all billfishes possess one character state and all other scombroids possess some other character state (characters 12, 14, 15, 16, 113, 117, 140, and 169; Fig. 5). At all of these sites, billfishes share the same character state as one or more of the percoid fishes. Furthermore, at three of these eight sites (15, 16, and 169), Gempylidae, Scombridae, and Trichiuridae share a common state with *Sphyraena* to the exclusion of all other species in the study. As this character analysis emphasizes, the amino acids are consistent with the hypothesis that billfishes are not scombroids and that *Sphyraena* is the sister group of a clade consisting of Gempylidae, Scombridae, and Trichiuridae.

Intrafamilial relationships

Within the family Istiophoridae (*Istiophorus*, *Makaira*, and *Tetrapturus*), cytochrome *b* nucleotide sequence provides a particularly well resolved and strongly supported phylogenetic signal. This is probably due to the recency of the istiophorid radiation. The maximum sequence divergence between any two species within this clade is less than five percent. We have performed a more in depth analysis of the interrelationships of istiophorids using the exhaustive search option of PAUP 3.1 (Swofford, 1991). Use of the exhaustive search option guarantees identification of the most parsimonious tree. The topology of this tree is identical to the topology of the istiophorid clade within the more inclusive scombroid phylogeny (Fig. 8, cf. Fig. 2). Neighbor-joining and UPGMA analyses produce an identical topology. Computer simulations suggest that agreement between these three methods should increase our confidence in a phylogenetic hypothesis (Kim, 1993).

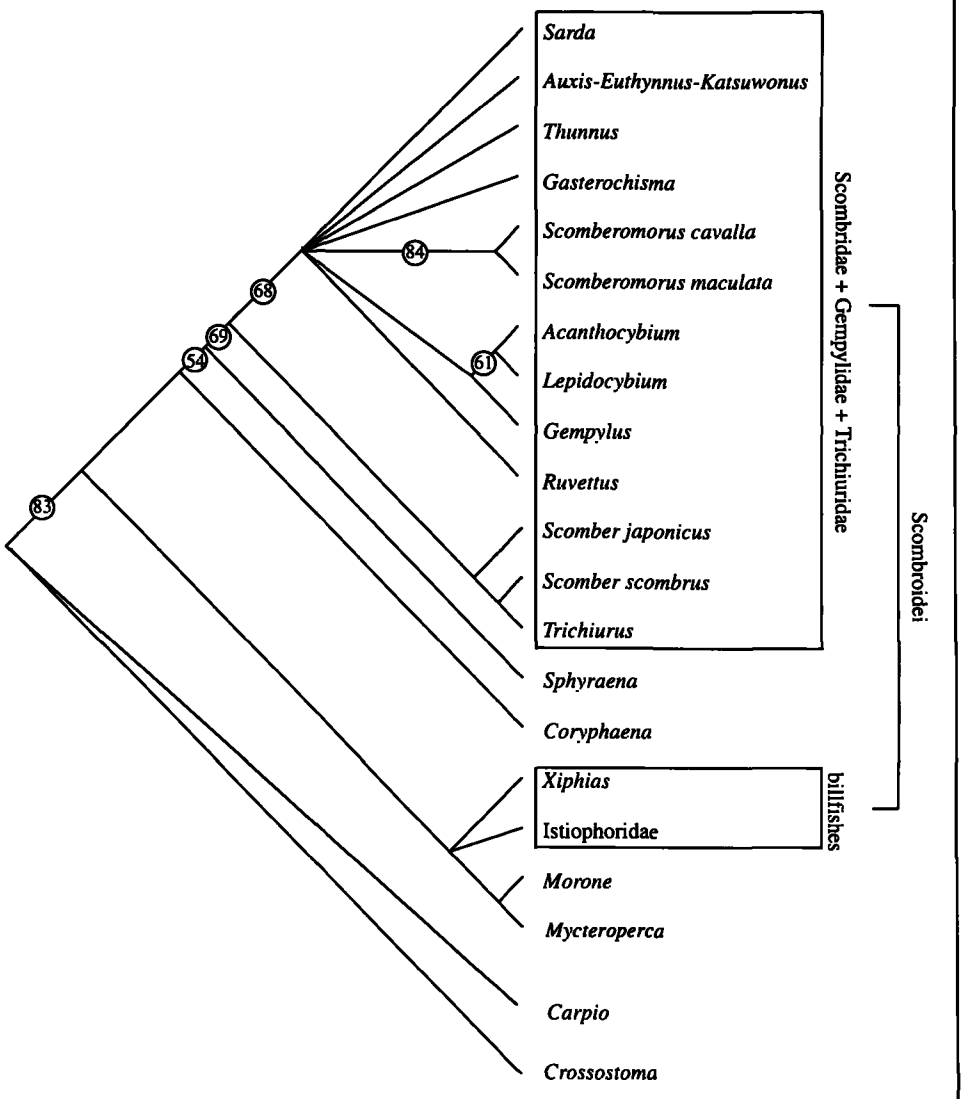

Figure 7

Phylogeny of the Scombroidei based on maximum parsimony analysis of inferred amino acid sequences. The cladogram depicted is a strict consensus of 68 equally parsimonious trees identified through a heuristic search procedure: TBR branch swapping was performed on 10 starting trees generated through random stepwise addition of taxa. *Crossostoma* and *Carpio* were specified as the outgroup. Length, consistency index, and retention index are the following: L=141, CI=0.567, RI=0.619. Circled numbers at nodes indicate the percentage of trials in which a given partition between taxa is supported in 1,000 replications of the bootstrap analysis (Felsenstein, 1985).

Cytochrome *b* sequence strongly supports the monophyly of the Istiophoridae. The extant members of this family have experienced a long period of common ancestry as indicated by numerous istiophorid synapomorphies (20 transitions and 35 transversions, Fig. 8). The monophyly of *Tetrapturus*, and within this genus, clades consisting of *audax* + *albidus* and *pfluegeri* + *angustirostris* + *belone*, are also supported. The number of substitutions separating *Tetrapturus audax* from *T. albidus*, and *T. angustirostris* from *T. belone* and *T. pfluegeri* indicates that these mitochondrial lineages share a recent common ancestry (Table 3). The sequence differences between these species (<0.5%) are small compared to the maximum intraspecific sequence difference (1.8%) detected among blue marlin, *M. nigricans*, over a similar region of cytochrome *b* (Finnerty and Block, 1992). These data call into question the status of *T. audax*

and *T. albidus* as separate species as well as *T. pfluegeri*, *T. angustirostris*, and *T. belone*.

Our data conflict with other aspects of current istiophorid taxonomy at the generic level. Analysis of cytochrome *b* does not support the monophyly of the genus *Makaira*. The black marlin, *Makaira indica*, appears to be the sister group of a clade containing all other istiophorids, while the blue marlin, *M. nigricans*, is sister group of the sailfish, *I. platypterus*. The most parsimonious tree that contains a monophyletic *Makaira* is six steps longer than the shortest tree overall (158 versus 152), and on the most parsimonious tree, the *M. nigricans-I. platypterus* node is strongly supported by bootstrap analysis (85%).

Cytochrome *b* provides good resolution of the relationships of the genera of the tribe Thunnini (*Auxis, Euthynnus, Katsuwonus,* and *Thunnus*). According to the nucleotide data the nine Thunnini species sequenced in this study comprise two clades, one consisting of the genus *Thunnus* and one containing the other genera: *Auxis*, *Euthynnus*, and *Katsuwonus*. This distinct split in the Thunnini was proposed by Kishinouye in 1923 and is consistent with the morphological hypothesis of Collette et al. (1984). Support for the monophyly of the *Thunnus* clade is particularly robust; however, the relationships within the genus cannot be resolved without the inclusion of both *Thunnus tonggol* and *Thunnus atlanticus* which were not sequenced in this study. The number of substitutions separating *T. thynnus* from *T. maccoyii* (<0.5% sequence divergence) are small considering their status as separate species.

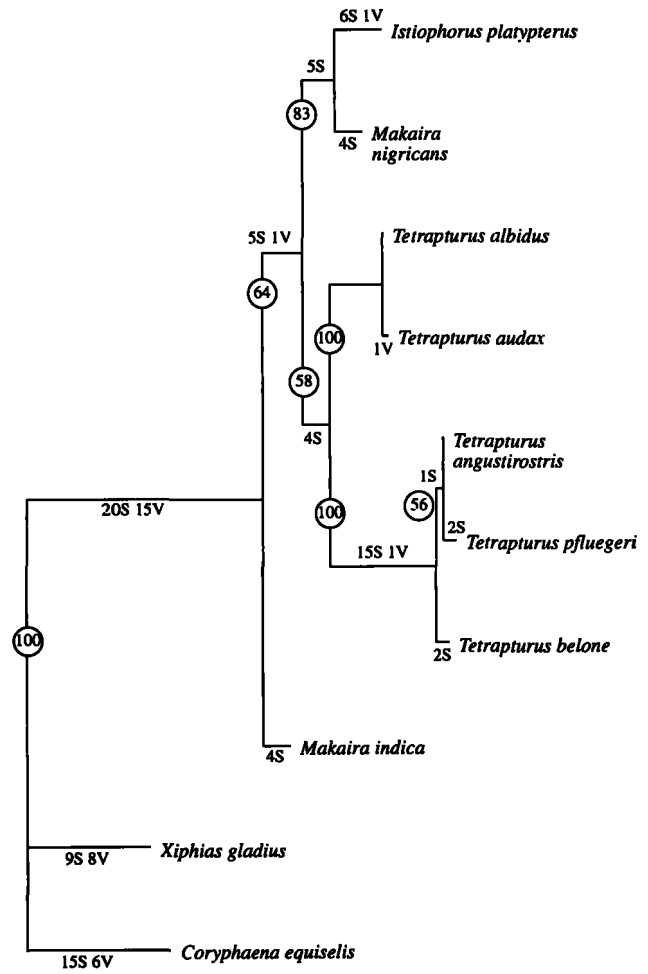

Figure 8

Phylogeny of the Istiophoridae based on 78 informative nucleotide sites. The phylogram depicted is the single most parsimonious tree identified by the exhaustive search option of PAUP 3.1 (Swofford, 1991). *Xiphias gladius* and *Coryphaena equiselis* were specified as the outgroup. Length, consistency index, and retention index are the following: L=152, CI=0.776, RI=0.721. Circled numbers at nodes indicate the percentage of trials in which a given partition between taxa is supported in 2,000 replications of the bootstrap analysis (Felsenstein, 1985). Character state transformations were inferred by using the accelerated transformation (ACCTRAN) option of PAUP: S=nucleotide transitions (A⇔G or C⇔T); V=nucleotide transversions (C/T⇔A/G). Within the Istiophoridae transitions outnumber transversions 54 to 6. Neighbor-joining (Saitou and Nei, 1987) and UPGMA dendrograms produced with Phylip 3.5 (Felsenstein, 1993) have the same topology. Distance trees were constructed by using Kimura's (1980) two parameter genetic distance, and by assuming a transition to transversion bias of 9:1.

Discussion

Interfamilial relationships and the limits of the Scombroidei

Throughout this analysis, we have focused on the long-standing controversy over the limits of the Scombroidei and, particularly, whether billfishes are scombroids. Cytochrome *b* appears to be informative on this issue. In the two phylogenetic analyses that emphasize the more slowly evolving characters (see Figs. 4 and 7), the most parsimonious tree topology is clearly most consistent with the hypothesis that billfish are not scombroids: in each case, one or more nonscombroids share a common ancestry with the scombrid-gempylid-trichiurid clade to the exclusion of billfishes (Table 4). Therefore, according to the criterion of parsimony, the nonscombroid hypothesis is superior to the scombrid subgroup and to the scombrid sister group hypotheses. However, in our opinion, the

most parsimonious trees alone do not constitute sufficient evidence to reject these unfavored hypotheses. The question we must ask is the following: How unparsimonious are these hypotheses?

In comparing the tree topologies that support each competing hypotheses (Table 4), it is clear that our data refute the notion that billfishes share a common ancestor with the Scombridae to the exclusion of other scombroids (Gregory and Conrad, 1937; Berg, 1940; Fraser-Brunner, 1950; Collette et al., 1984; Johnson, 1986). For example, the shortest trees supporting a billfish-scombrid clade are 13% longer than the minimum-length tree based on inferred amino acid sequence (Table 4). According to the cladistic permutation test for nonmonophyly (Faith, 1991), this length difference constitutes significant evidence against the monophyly of scombrids plus billfishes. The condition that billfishes and scombrids comprise a monophyletic group is a requirement of both the scombrid subgroup and scombrid sister group hypotheses. Therefore, according to the cytochrome *b* data, we reject these two hypotheses.

The cytochrome *b* data clearly support the third hypothesis, that billfishes are not scombroids, though not as strongly as they refute the first two hypotheses. According to the inferred amino acid sequences, the shortest tree that supports scombroid monophyly places billfishes as sister group to all other scombroids and is nearly 3% longer than the most parsimonious tree overall (145 versus 141 steps). This length difference alone does not constitute significant evidence against the monophyly of the Scombroidei according to a cladistic permutation test for nonmonophyly (see Methods section; Faith, 1991). However, as previously mentioned, there are three amino acid characters that unite scombrids, gempylids, and trichiurids with *Sphyraena* to the exclusion of billfishes (amino acids 15, 16, and 169). There are no characters that unite scombrids, gempylids, and trichiurids with billfishes to the exclusion of the putative outgroups. Our study is consistent with the hypothesis that billfishes are most closely related to some percoid lineage (Nakamura, 1983; Potthoff et al., 1986). The question of which taxon is most closely related to billfishes remains unanswered. On the basis of this evidence, we support a conservative definition of the Scombroidei, including only the families Scombridae, Gempylidae,

Table 4

Comparison of three competing hypotheses of billfish (Istiophoridae and Xiphiidae) relationships based on inferred amino acid sequences from cytochrome *b*. 'na' = not applicable.

	Hypothesis		
	I: Scombrid subgroup	II: Scombrid sister group	III: Nonscombroid
Characteristics of tree which would support hypothesis	Scombridae + billfishes are a monophyletic group *Acanthocybium* is the sister group of billfishes	Billfishes are sister group of a monophyletic Scombridae	Billfishes do not compose part of a monophyletic group with any other scombroid taxon or taxa
Does the most parsimonious tree support the hypothesis?	No	No	Yes
If the answer to B is no, how much longer is the shortest tree which does support the hypothesis?	13.5% (160 steps vs. 141 steps)	13.0% (159 steps vs. 141 steps)	na
Based on the increase in tree length, can we reject the underlying hypothesis with statistical significance?	Yes (*T-PTP* = 0.01)	Yes (*T-PTP* = 0.01)	na

[1] The topology dependent permutation tail probability (*T-PTP;* Faith, 1991) was used to determine the significance of the length difference. Values of *T-PTP*≤0.05 were considered significant. See Methods section.

and Trichiuridae (Cuvier and Valenciennes, 1832; Gosline, 1968; Potthoff et al., 1986).

How can these inferences from molecular data be reconciled with the morphological data? We believe that this is an instance where molecular data complement morphological data well. Cytochrome *b* provides an unambiguous phylogenetic signal that billfishes are genetically distant from other scombroids. In contrast, the existing morphological data does not clearly discriminate between a number of hypotheses. The number of character reversals in morphological phylogenies that classify billfishes as scombroids indicates that there have been many homoplastic changes in the billfish lineage. According to the morphological evidence, either billfishes are scombroids and have undergone several reversals to the primitive state, such as their low number of vertebrae, or billfishes are not scombroids but have evolved many convergent similarities to scombroids, such as their paired lateral caudal keels.

Many of the morphological characters that unite billfishes to other scombroids, particularly to Scombridae, may be adaptations for continuous swimming, and are therefore of questionable phylogenetic value. These include hypurostegy, the projection of the caudal fin-ray bases anteriorly to cover the hypurals (Collette et al., 1984; Johnson, 1986), fusion of the hypurals (Collette et al., 1984; Johnson, 1986), and interfilamentar gill fusion (Johnson, 1986). Hypurostegy and interfilamentary gill fusion are known to have evolved convergently in nonscombroid taxa (*Luvarus imperialis* [Leis and Richards, 1984]; and *Amia calva* [Bevelander, 1934]). The molecular data presented here provide a phylogenetic signal that is independent of convergent morphological adaptations that might confound phylogenetic analysis. There has been convergent evolution in the molecular characters, but unlike many of the morphological characters mentioned, this convergent evolution does not appear to be the result of strong selection: most amino acid substitutions exchange amino acids with similar size, charge, and degree of polarity. Therefore, when compared with the existing morphological data, the phylogenetic signal in the molecular data is less likely to have been obscured by similar selective pressures acting upon distantly related lineages.

Istiophorid phylogeny

Historically, there have been numerous disagreements over the number of species within the Istiophoridae and their interrelationships (Goode, 1882; Jordan and Evermann, 1926; LaMonte and Marcy, 1941; Nakamura, 1983). This is evidenced by the synonymies for many istiophorids, e.g. the Mediterranean spearfish, *Tetrapturus belone*, has also been assigned to *Istiophorus* (Ben-Tuvia, 1953) and *Makaira* (Tortonese, 1958). The most thorough treatment of billfish systematics to date is a phenetic analysis conducted by Nakamura (1983). Nakamura recognized 11 species of istiophorid billfishes in three genera, including the designation of separate Atlantic and Indo-Pacific species for blue marlin (*Makaira nigricans* and *M. mazara*) and sailfish (*Istiophorus albicans* and *I. platypterus*).

The molecular evidence presented here agrees with Nakamura (1983) in supporting the monophyly of the genus *Tetrapturus*, and within this genus, clades consisting of *audax* + *albidus* and *pfluegeri* + *angustirostris* + *belone*. Cytochrome *b* does not support the recognition of separate Atlantic and Pacific species of blue marlin and sailfish. Previous results (Finnerty and Block, 1992) identified substantial overlap in the cytochrome *b* haplotypes found among Atlantic and Pacific populations of blue marlin. The sailfish sample in this study includes one Atlantic specimen and one Pacific specimen that differ at only two sites among 590 (0.3%). We infer from the cytochrome *b* data (Block et al., 1993; and this study) a nonmonophyletic *Makaira* and support for a clade consisting of the blue marlin (*Makaira nigricans*) and the sailfish (*Istiophorus platypterus*).

Based on the cytochrome *b* data, istiophorid taxonomy at the generic level is not concordant with phylogeny. It is premature to suggest taxonomic revision of istiophorid genera, but we believe it is imperative to obtain more molecular data, particularly from nuclear genes, to determine whether the inferences presented here can be corroborated. Furthermore, we recognize the need for an extensive cladistic analysis of istiophorid relationships based on additional morphological data. Another taxonomic issue raised by this study concerns the number of valid *Tetrapturus* species. An extensive genetic survey of several populations from each species is required to determine the number of evolutionarily independent or reproductively isolated lineages within this genus.

Relationships within the genus *Thunnus*

The systematics of the genus *Thunnus* have been well studied owing to the commercial importance of tunas and interest in physiological specializations associated with the evolution of endothermy. Collette (1978) suggested a taxonomic subdivision of the genus reflecting a split between tropical species (subgenus *Neothunnus*: blackfin tuna, *Thunnus atlanticus*, longtail tuna, *Thunnus tonggol*, and yellowfin tuna, *Thunnus albacares*) and species that inhabit cooler waters (subgenus *Thunnus*: bluefin tuna,

Thunnus thynnus, southern bluefin tuna, *Thunnus maccoyii*, albacore, *Thunnus alalunga*, and bigeye tuna, *Thunnus obesus*). According to this hypothesis, the primitive condition for the genus *Thunnus* is a tropical distribution, and the cold water tunas compose a monophyletic group united by specializations that allowed them to exploit cooler temperate or deep waters. The nucleotide analyses presented in Figures 2 and 4 are not consistent with this hypothesis. The cytochrome *b* phylogeny groups a tropical species, the yellowfin tuna, *Thunnus albacares*, with two species adapted for extremely cold water, the bluefin tuna and southern bluefin tuna (*Thunnus thynnus* and *Thunnus maccoyii*). However, it is premature to draw conclusions about relationships within the genus *Thunnus* until data are obtained from two tropical species not included in this study, *Thunnus atlanticus* and *Thunnus tonggol*.

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