Abstract—The identification of larval istiophorid billfishes from the western North Atlantic Ocean has long been problematic. In the present study, a molecular technique was used to positively identify 27 larval white marlin (*Tetrapturus albidus*), 96 larval blue marlin (*Makaira nigricans*), and 591 larval sailfish (*Istiophorus platypterus*) from the Straits of Florida and the Bahamas. Nine morphometric measurements were taken for a subset of larvae (species known), and lower jaw pigment patterns were recorded on a grid. Canonical variates analysis (CVA) was used to reveal the extent to which the combination of morphometric, pigment pattern, and month of capture information was diagnostic to species level. Linear regression revealed species-specific relationships between the ratio of snout length to eye orbit diameter and standard length (SL). Confidence limits about these relationships served as defining characters for sailfish >10 mm SL and for blue and white marlin >17 mm SL. Pigment pattern analysis indicated that 40% of the preflexion blue marlin examined possessed a characteristic lower jaw pigment pattern and that 62% of sailfish larvae were identifiable by lower jaw pigments alone. An identification key was constructed based on pigment patterns, month of capture, and relationships between SL and the ratio of snout length to eye orbit diameter. The key yielded identifications for 69.4% of 304 (blind sample) larvae used to test it; only one of these identifications was incorrect. Of the 93 larvae that could not be identified by the key, 71 (76.3%) were correctly identified with CVA. Although identification of certain larval specimens may always require molecular techniques, it is encouraging that the majority (92.4%) of istiophorid larvae examined were ultimately identifiable from external characteristics alone.

Toward identification of larval sailfish (*Istiophorus platypterus*), white marlin (*Tetrapturus albidus*), and blue marlin (*Makaira nigricans*) in the western North Atlantic Ocean*

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Research on the early life history of exploited fishes benefits management efforts by elucidating the temporal and spatial distribution of spawning, cohort strength, and biological and physical factors affecting recruitment (Lasker, 1987). The ability to confidently identify specimens to species is necessary in any early life history study (Collette and Vecchione, 1995). This has not yet been achieved for larval billfishes of the family Istiophoridae from the Atlantic Ocean: sailfish (*Istiophorus platypterus*), blue marlin (*Makaira nigricans*), white marlin (*Tetrapturus albidus*), and longbill spearfish (*Tetrapturus pfluegeri*).

Larval istiophorids are easily distinguished from larval swordfish (*Xiphias gladius,* family Xiphiidae). However, larval istiophorids are difficult to identify below the family level. Full fin-ray complements are not present until a larva reaches 20 mm in length, and even then, meristic counts are of limited use for identification because of significant overlap in counts among species. At best, species possibilities can be eliminated only for specimens with counts in the extremes of their ranges (Richards, 1974). The only definitively diagnostic count is the vertebral formula for *Makaira* (11 precaudal and 13 caudal) versus that of the other istiophorids (12 precaudal and 12 caudal) (Richards, 1974). Larger blue marlin lar-

vae may also be identified by the presence of a complex lateral line. Ueyanagi (1964) found this character in Pacific blue marlin of 20 mm standard length (SL), but the smallest SL of an Atlantic blue marlin from a recent collection in which a complex lateral line was visible was 26.9 mm. At lengths <20 mm, specific identification of istiophorids is even more uncertain. Ueyanagi (1963; 1964) based the identification of Indo-Pacific istiophorids <5 mm SL on four characters: 1) anterior projection of the eye orbit; 2) the position of the tip of the snout in relation to the middle of the eye; 3) presence of pigments on the branchiostegal and gular membranes; and 4) whether the pectoral fins are rigid—a character that applies to larval black marlin (*Makaira indica*), a species not known to spawn in the Atlantic Ocean. For fish >5 mm SL, the characters of relative snout length and eye size are used. Ueyanagi (1964) described sailfish, striped marlin (*Tetrapturus audax*, the Pacific counterpart to white marlin), and shortbill spearfish (*Tetrapturus angustirostris*) between 10 and 20 mm SL as having long snouts. The short snout group comprised blue marlin and black marlin. The angles at which the pterotic and preopercular spines protrude from the body have also been useful in identifying Indo-Pacific specimens (Ueyanagi, 1974a).

A troubling aspect of current larval istiophorid identification methods is the difficulty in using some of the above characters. If a specimen is fixed with its mouth open, snout position with respect to eye is an unreadable character (Richards, 1974), and misidentifications can occur (Ueyanagi, 1974a). Evaluation of certain characters (e.g., whether the eye orbit projects anteriorly) can be highly subjective. The lack of confirming identification characters compounds the problem; if just one character cannot be assessed, identification may not be possible (Richards, 1974). An additional problem is the apparently high variability in characters such as pigment locations and head spine angles in Atlantic istiophorids (Richards, 1974).

Most of the larval specimens examined by Ueyanagi came from the Indo-Pacific; he assumed that the same identification characters would apply to their Atlantic counterparts (Ueyanagi, 1963, 1974a). Although recent genetic evidence supports Morrow and Harbo's (1969) opinion that Atlantic and Indo-Pacific sailfish are actually populations of a global species (Finnerty and Block, 1995; Graves and McDowell, 1995), morphological differences have been noted in sailfish, especially at 90 cm. Specifically, the pectoral fin is longer, in relation to the body, in Atlantic sailfish than in Indo-Pacific sailfish. Differences in the spread of the caudal fin and maximum total length have also been observed. These characters were the impetus behind the separation of sailfish, at least to subspecies, by ocean basin (Nakamura, 1974). Regardless of the taxonomic status of the Atlantic and Indo-Pacific billfishes, physical attributes of istiophorid species may vary by region. Therefore, the assumption that the larvae of Atlantic istiophorids can be identified by using the same characters attributed to Indo-Pacific istiophorids may not be valid.

Billfishes are not the only group whose larval identification has proven difficult. Species of the genus *Sebastes*, the rockfishes, have some morphological and pigmentation differences as larvae, but identification was difficult and uncertain until genetic methods were employed (Rocha-Olivares et al., 2000). Fulford and Rutherford (2000) solved a similar problem by combining allozyme analysis of larval tissues with landmark-based morphometrics to distinguish between species of the genus *Morone*. In each study, a molecular technique was used to confirm larval species identity, facilitating the development of morphometric identification techniques.

Several molecular methods for identifying adult billfishes have been developed (Chow, 1993; Innes et al., 1998; McDowell and Graves, 2002). In the present study, larval istiophorids from Atlantic waters were identified to species using restriction fragment length polymorphism (RFLP) analysis of a 1.2-kb segment of nuclear DNA, as described for adult billfishes by McDowell and Graves (2002). In this article we present data for genetically identified istiophorid larvae, analyses of morphometric and qualitative characters, and a key for the identification of larval istiophorids of the Straits of Florida and the Bahamas.
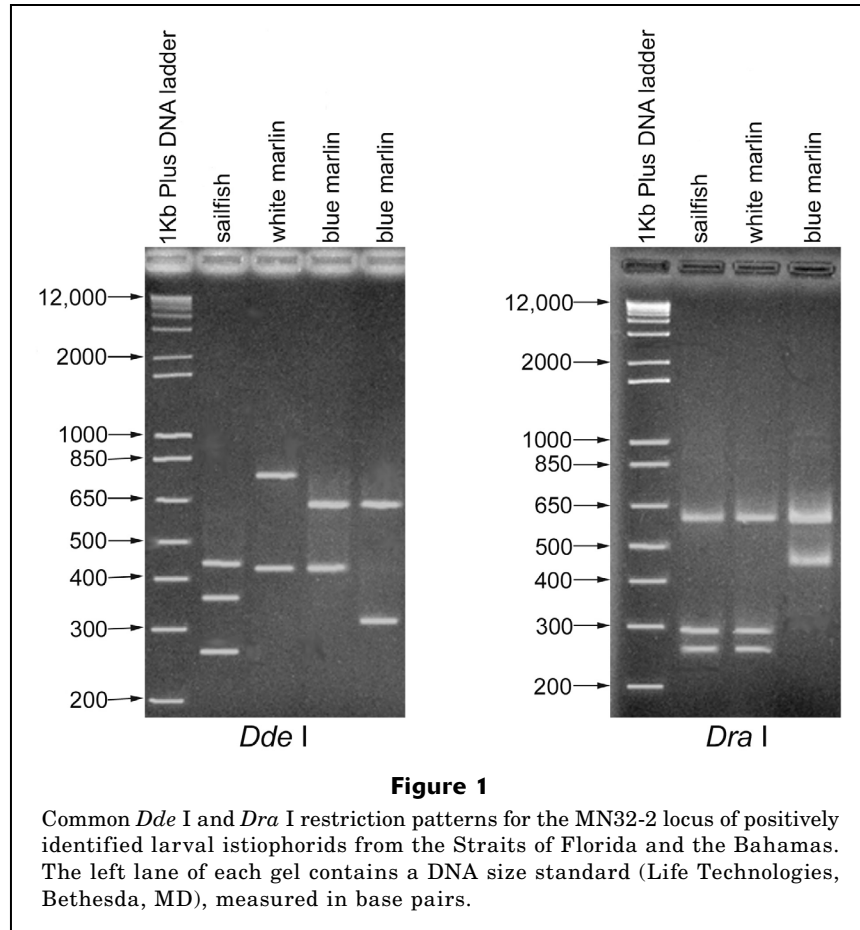
Materials and methods

Larval material

Larval istiophorids were collected between June 1998 and April 2002 from the Straits of Florida and Exuma Sound, Bahamas. Several preservation fluids were used, but the majority of the larvae (~1000) were preserved in 70–95% ethanol. Butylated hydroxytoluene (BHT) saturated ethanol was used to preserve 150 larvae. Approximately 300 larvae were fixed in 10% unbuffered formalin and then transferred to 70% ethanol. In the laboratory, each fish was assigned a unique identification number and stored separately.

Molecular identification

Total DNA was extracted from the right eyeball of each larva, using either a quick-digest method (Ruzzante et al., 1996) or a standard high-molecular weight DNA extraction protocol (Sambrook et al., 1989). Larval identification was achieved by PCR amplification of the nuclear locus MN32-2 (Buonaccorsi et al., 1999), and subsequent RFLP analysis (restriction endonucleases *Dra* I and *Dde* I, Life Technologies, Bethesda, MD). If the restriction fragment pattern (Fig. 1) of a larva matched one of those described for a known-identity adult, the larva was assigned to that species. See McDowell and Graves (2002) for detailed protocols and reaction parameters. Preliminary attempts to amplify DNA from formalin-fixed larvae failed; only ethanol-preserved specimens were used in subsequent molecular work.

Figure 1

Common *Dde* I and *Dra* I restriction patterns for the MN32-2 locus of positively identified larval istiophorids from the Straits of Florida and the Bahamas. The left lane of each gel contains a DNA size standard (Life Technologies, Bethesda, MD), measured in base pairs.
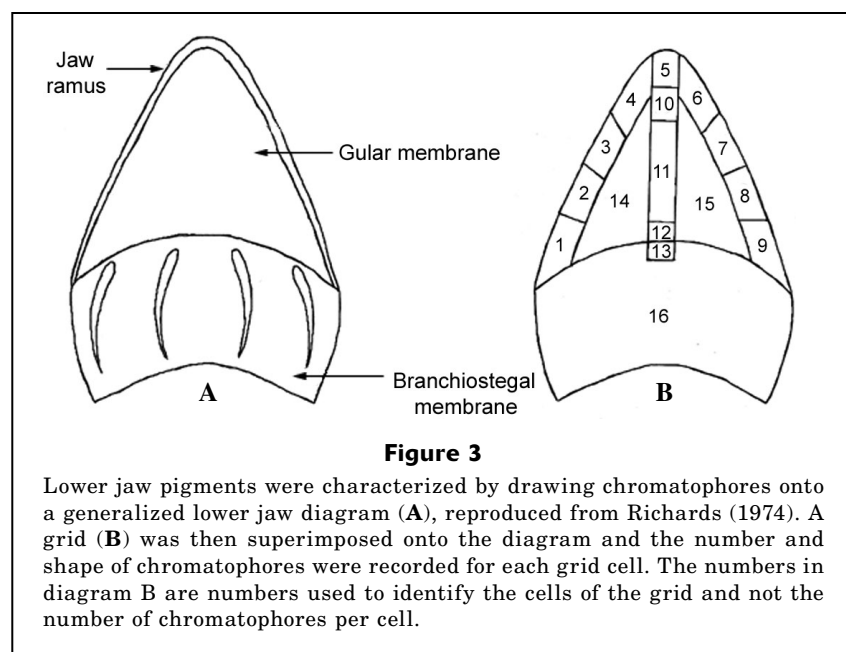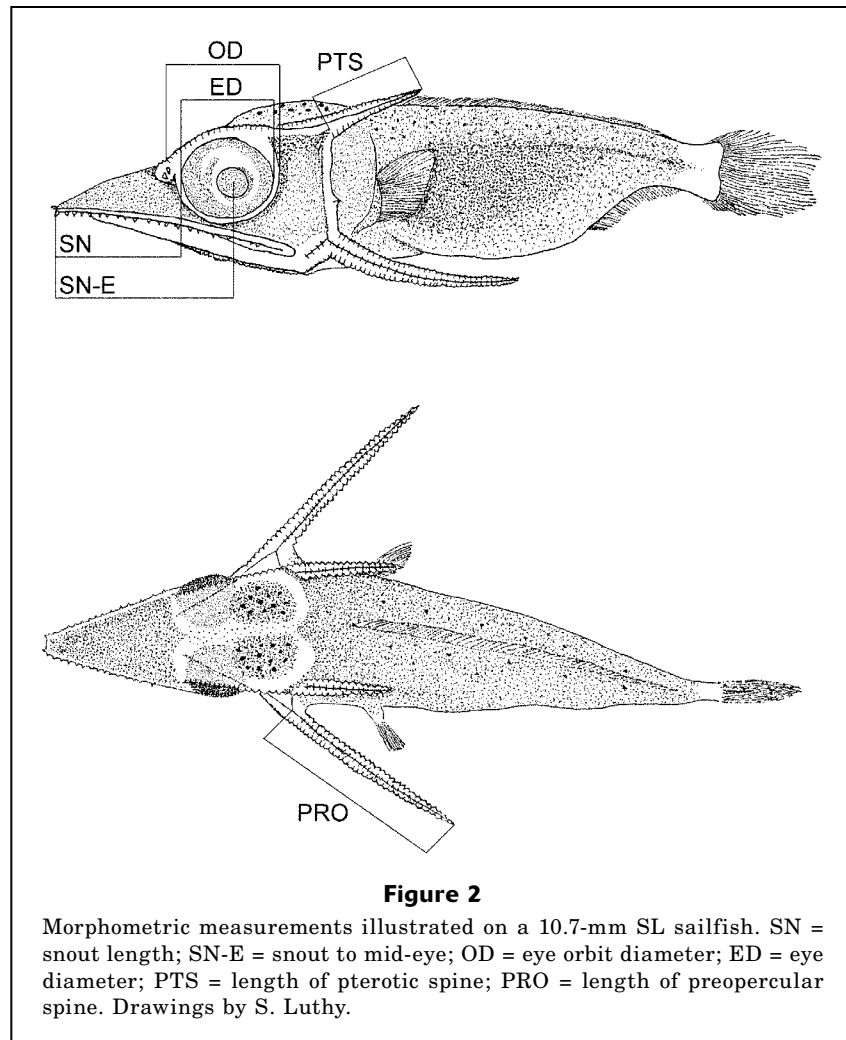
Characters

A subset of the molecularly identified istiophorid larvae were examined to ascertain which morphological characters might aid in specific identification and possibly obviate the need for future molecular work. The measurements made by Richards (1974) served as a starting point for quantitative larval descriptions: standard length (SL); snout length (SN); tip of the snout to the center of the eyeball (SN-E); diameter of the eye (ED); diameter of the eye orbit (OD); head length (HL); and difference in length between the upper and lower jaws (JD). To this suite were added measurements of the preopercular (PRO) and pterotic (PTS) head spines. All measurements were taken with Image-Pro Plus software (version 4.5, Media Cybernetics, Silver Spring, MD), and each specimen was viewed through a CoolSNAP-PROcf monochrome digital camera (Media Cybernetics, Silver Spring, MD) which was connected to a Leica MZ12 dissecting microscope (at magnifications 0.8–10.0×). Each larva was soaked in tap water for one minute before measurements were taken, to rehydrate the fish and facilitate handling. SL and PRO measurements were made from the dorsal view, JD measurements were made from the ventral view, and all other measurements were made from the left lateral view (Fig. 2). Because the preopercular spine often prevents an istiophorid larva from lying on its side, a side view was obtained by using the surface tension of the still-wet larva to adhere it to the side wall of a Petri dish. Care was taken to maintain the two points of measurement on a plane parallel to the microscope lens.

Pigments observed on the ventral surface of the lower jaw rami, gular membrane, and branchiostegal membranes of each larva were drawn onto a generalized diagram of the larval istiophorid lower jaw (Fig. 3). A grid was then superimposed on the diagram, and the shape (pointate or stellate) and number of chromatophores in each grid cell were recorded. Pigment data were also recorded as binary presence or absence per grid cell. Two other categorical variables assessed were flexion stage (i.e., preflexion, flexing, postflexion) and the position of the tip of the snout with regard to a plane passing through the center of the eye and the mid-line of the body (i.e., below, even, above). Although the latter character is useful for identifying Indo-Pacific istiophorids (Ueyanagi, 1963, 1964), in our collection it was highly variable within species, and therefore it was not analyzed further.

Month of capture was considered a partially discriminating character based on differences in the length and timing of spawning seasons of local populations. Spawning seasons were determined by de Sylva and Breder (1997) by gonad histology studies.

Figure 2

Morphometric measurements illustrated on a 10.7-mm SL sailfish. SN = snout length; SN-E = snout to mid-eye; OD = eye orbit diameter; ED = eye diameter; PTS = length of pterotic spine; PRO = length of preopercular spine. Drawings by S. Luthy.

Figure 3

Lower jaw pigments were characterized by drawing chromatophores onto a generalized lower jaw diagram (**A**), reproduced from Richards (1974). A grid (**B**) was then superimposed onto the diagram and the number and shape of chromatophores were recorded for each grid cell. The numbers in diagram B are numbers used to identify the cells of the grid and not the number of chromatophores per cell.

Data analyses

Canonical variates analysis (CVA) was used to visualize the separation between species and the relative importance of all variables (morphometric characters, pigment patterns, and month of capture) in that separation. Results from the CVA were used to help drive character selection for subsequent analyses. The significance of the canonical axes was obtained with a Monte Carlo permutation test (499 iterations). The canonical analyses were performed with the software CANOCO (version 4.5, Microcomputer Power, Ithaca, NY), and plotted with the associated software CANODRAW.

In the CVA, all the molecularly identified white marlin (21) and blue marlin (68) with full measurement sets (i.e., no missing values) and a subset of sailfish (135) with full measurement sets were compared. Every attempt was made to include fish from different locations, different years and months of collection, and across the full available size range of each species, in order to capture as much intra- and inter-species variation as possible. Forward selection was used as a guide for the creation of a reduced set of variables by retaining those that were significant for discrimination at $\alpha=0.05$ in a Monte Carlo permutation test (499 iterations). Months that were excluded by selection were restored to the variable set to insure that the entire spawning season was represented. It was assumed that pigment on the right lower jaw ramus was of equal importance as pigment in the corresponding location on the left lower jaw ramus; thus if a pigment grid from only one side of the jaw was selected, the corresponding grid from the other side of the jaw was added back to the reduced set.

In addition to its function as an exploratory tool for character selection, CVA with the reduced set of variables was used to identify unknown larvae to species. Ordination coordinates of an unknown larva were obtained by summing the products of the canonical coefficients and the character values for the unknown (standardized to mean 0, standard deviation 1). The identity of an unknown larva was determined by its placement in the ordination with respect to the reference larvae.

The CVA provided clues as to which individual pigment grid cells were important for species discrimination, but cluster analysis was employed to examine overall lower jaw pigment patterns. Simple average link cluster analysis of Jaccard similarity indices was executed on pigment grid cell presence (binary coding) in the suite of lower jaw grid cells with BioDiversity Pro[1] software for the 26 white marlin with undamaged lower jaws and for equal numbers of randomly chosen blue marlin and sailfish. Analyses were conducted on all larvae together, and separately by flexion stage. Pigment drawings of the individual larvae within single-

species clusters were examined visually for commonalities. If a pattern was detected, the entire database of pigment position, number, and shape of all molecularly identified larvae was searched for that pattern. Lower jaw pigment patterns that were confined to one species only were deemed diagnostic characters.

Lower jaw pigment patterns alone did not resolve the differences among the species sufficiently for identification of all larvae. Therefore, for each species, continuous variables related linearly to SL were regressed against SL by using SAS (version 8.02, SAS Institute, Cary, NC) software. Two ratios were also examined in this way—snout length divided by eye orbit diameter, and snout length divided by eye diameter. Both ratios were suggested by the results of the full-model CVA because the influence of snout length was large and opposite in sign to the large and similar vectors of orbit diameter and eye diameter. The former ratio was also considered by Ueyanagi (1963, 1964, 1974b) to be an important distinguishing character for istiophorid larvae. The same larvae that were used in the CVA analyses were used for the regressions, plus three white marlin, two sailfish, and two blue marlin that were excluded from CVA because of a missing measurement. Suitability of the characters for linear regression was assessed visually. Confidence intervals of 95%, 99%, and 99.9% were constructed around the regressions. Intersections of the three levels of confidence intervals for the three species were examined for maximum discrimination at the smallest standard length. The relationships that provided the best separation were included in the identification key.

The identification key was constructed from the various characters that showed differences among the three species. All of the larvae used in developing the key were tested with it, as well as 12 blue marlin and 61 sailfish that were previously excluded from the analyses. A set of 50 larvae were independently identified by two observers unfamiliar with the key (naïve observers). The only information about the fish provided to them was month of capture, so that each made his own measurements and pigment evaluations. The percent accuracy of their identifications was taken as a measure of the utility of the key.

Results

Molecular identification

The molecular identification technique was applied to 1044 larvae. Amplification success rates appear to have been negatively affected by the addition of BHT to ethanol and by the use of the Ruzzante et al. (1996) DNA extraction protocol. Overall, 714 (68.4%) istiophorids were successfully identified to the species level. Sailfish represented 82.8% of this group (591 larvae), whereas 96 blue marlin (13.4%) and 27 white marlin (3.8%) were identified. No longbill spearfish were identified. Sailfish larvae (2.9 mm–18.3 mm SL) were collected from April

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[1] McAleece, N., P. J. D. Lambshead, G. L. J. Paterson, and J. D. Gage. 1997. The National History Museum and The Scottish Association for Marine Science. Website: http://www.sams.ac.uk/. [Accessed 5 February 2003.]
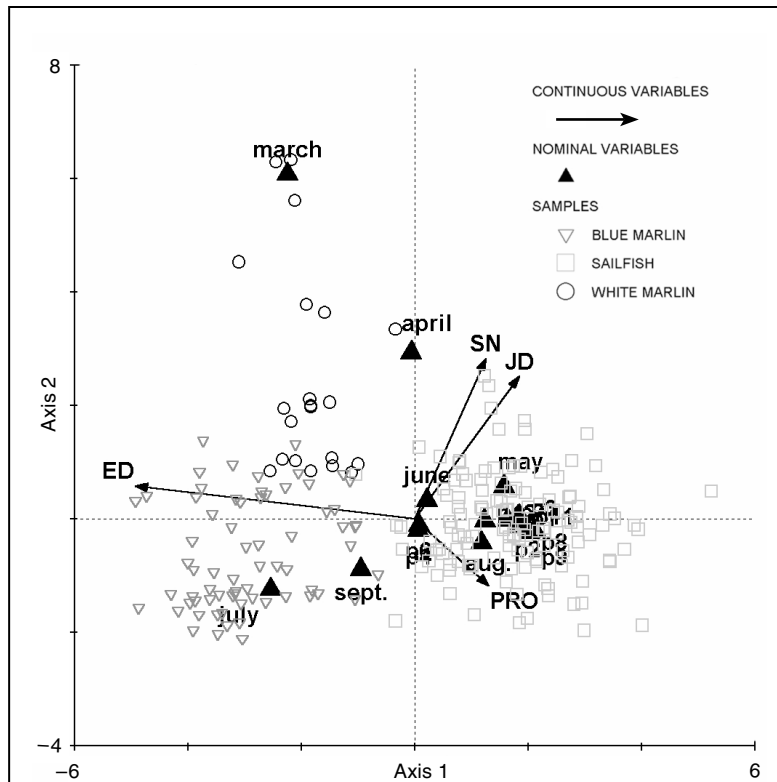
Figure 4

Canonical variates analysis with the reduced set of variables. Arrows indicate the direction of increase in continuous variables and may be extended backward through the origin of the graph to show a decrease in the value of the character. Variables that extend farthest from the origin are most useful in the separation. SN = snout length; JD = difference in the lengths of the jaws; ED = eye diameter; PRO = length of preopercular spine; p (number) = presence of pigment in lower jaw grid cell (number).
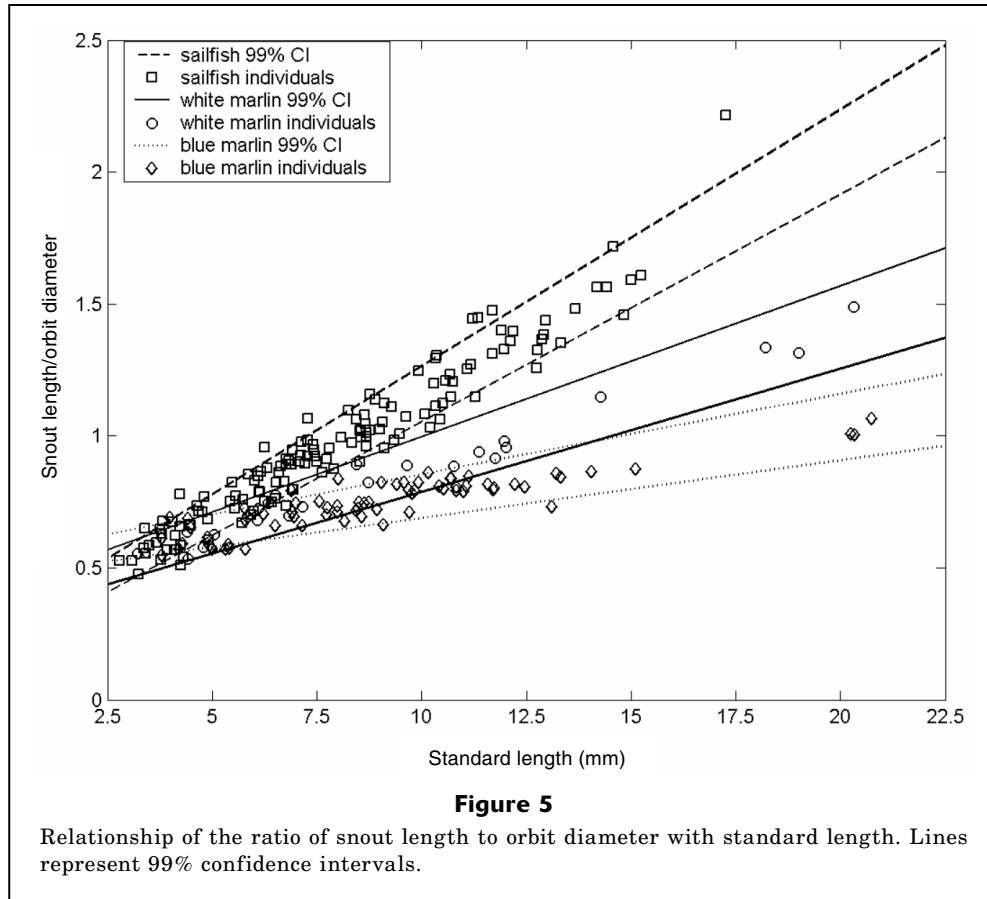
through September, white marlin (4.5 mm–20.3 mm SL) were collected from March through June, and larval blue marlin (3.8 mm–22.1 mm SL) were collected from June through September. Month of capture closely matched the reported spawning seasons for these species in the western North Atlantic: April through October for sailfish, March through June for white marlin, and July through October for blue marlin (de Sylva and Breder, 1997). Because blue marlin larvae were also caught in June, the blue marlin spawning season was expanded to include that month for the purposes of the identification key.

Canonical variates analysis

In the CVA with all variables included, separation of the three species was achieved with little overlap. Sailfish larvae were separated from the marlins along canonical axis 1 (eigenvalue=5.45). The separation was driven mainly by ED, OD, and lower jaw pigmentation. White marlin larvae separated from blue marlin primarily

along canonical axis 2 (eigenvalue=0.79), largely by month of capture, as well as SN, SN–E, and JD . The overall ordination was significant at P=0.002.

The forward selection process, along with the re-addition of counterpart pigment grids and the full spawning season, yielded the following 21 out of 32 variables: March, April, May, June, July, August, September, SN, JD, ED, PRO, and pigment grids 1–4, 6–9, 11, and 12. The following variables were ultimately excluded from the data set: SL, SN-E, OD, HL, PTS, and pigment grids 5, 10, and 13–16. The degree of species overlap was similar to that in the full model (Fig. 4). This overall ordination was also significant at P=0.002. The eigenvalue of the first canonical axis was 4.71, whereas the eigenvalue of the second canonical axis was 0.71. Coordinates obtained from the canonical coefficients and character values, standardized by reference set character means and standard deviations (Table 1), accurately placed test "unknowns" in the ordination of the reference larvae.

Figure 5

Relationship of the ratio of snout length to orbit diameter with standard length. Lines represent 99% confidence intervals.

Lower jaw pigment patterns

Sailfish of all flexion stages with chromatophores on one or both sides of the lower jaw rami and sometimes in the middle of the gular membrane comprised single-species clusters. Examination of all molecularly identified larvae showed that many sailfish had pigment on the posterior ¾ of the lower jaw, but a few marlins also had stray pigments in that region. The minimum criterion to identify sailfish by lower jaw pigment without misidentifying other species was pigment in at least three of lower jaw pigment grids 1, 2, 3, 7, 8, 9, and 11. The shape and number of chromatophores within the grids was inconsequential. Not all sailfish larvae possessed the putative sailfish pattern, but 61.8% of molecularly identified sailfish (353 of 571 with intact lower jaws) could be identified by their lower jaw pigments alone.

Preflexion and flexing blue marlin also formed single-species clusters owing to the pattern of a single, pointate chromatophore in each of lower jaw grid cells 4 and 6, but without any other pigment (except occasionally in grid cell 12 or 13). However, not all small blue marlin exhibited this pattern. Eight of the 20 (40%) preflexion, molecularly identified blue marlin with intact lower jaws could be accurately identified by lower jaw pigments. Although some postflexion white marlin had a similar pattern, no preflexion or flexing larvae of other species were misidentified as blue marlin by virtue of this pigment pattern.

Linear regressions

Residual plots showed no deviations from homogeneity of variance. Snout length, snout to mid-eye, ratio of snout length to eye diameter, and ratio of snout length to orbit diameter were all linearly related to SL. Jaw difference was linear and appeared to be helpful for discriminating istiophorids >12 mm SL, but too few larvae of this size were available for meaningful regressions. The ratio of snout length to orbit diameter provided the most separation between the species as indicated by the full model CVA. The 99% upper limit of the regression of this ratio against SL for white marlin was used to separate sailfish from both marlin species at 10 mm SL. If white marlin is ruled out as a possibility by month of capture, sailfish can be separated from blue marlin by the blue marlin upper 99% confidence limit for the regression of the ratio of snout length to orbit diameter at 8 mm SL. The lower 99% confidence limit for the regression of the ratio of white marlin snout length to orbit diameter separated them from blue marlin at 17 mm SL (Fig. 5, Table 2).

Table 1

Canonical coefficients, mean, and standard deviation of each character from the canonical variates analysis (reduced set of characters). The coordinate of a larva on canonical axis 1 (x) can be found by $x=\sum_{i=1}^{21}c_{1i}z_i$, where c = canonical coefficient and z = (character value – character mean)/character standard deviation. The coordinate of a larva on canonical axis 2 (y) can be found by $x=\sum_{i=1}^{21}c_{2i}z_i$. PRO = pre-opercular; SN = snout length; ED = eye diameter; and JD = difference in length between upper and lower jaws.

i (iterative count)	Character	Canonical coefficient, c_1, for canonical axis 1	Canonical coefficient, c_2, for canonical axis 2	Character mean (reference set)	Character standard deviation (reference set)
1	March	−0.0963	0.7538	0.0134	0.1149
2	April	0.0772	0.7354	0.0357	0.1856
3	May	0.1961	0.7347	0.1786	0.3830
4	June	0.1267	0.6460	0.3036	0.4598
5	July	−0.0369	−0.2988	0.2054	0.4040
6	August	0.3465	0.2116	0.2143	0.4103
7	September	0.0000	0.0000	0.0491	0.2161
8	PRO	0.6697	−0.6728	2.0781	0.7076
9	SN	3.1678	0.9640	1.4978	0.8711
10	ED	−2.8386	0.0739	1.2011	0.4426
11	JD	−0.9464	−0.4947	0.1806	0.2222
12	Pigment 1	0.1450	−0.1156	0.2366	0.4250
13	Pigment 2	0.3483	−0.0953	0.2366	0.4250
14	Pigment 3	0.3564	0.1262	0.3036	0.4598
15	Pigment 4	0.0887	−0.2251	0.7768	0.4164
16	Pigment 6	−0.0263	−0.1084	0.8214	0.3830
17	Pigment 7	−0.0375	−0.1584	0.3259	0.4687
18	Pigment 8	0.2684	−0.0507	0.2098	0.4072
19	Pigment 9	0.3262	−0.0603	0.2545	0.4356
20	Pigment 11	0.4757	−0.1622	0.4241	0.4942
21	Pigment 12	0.2250	−0.1191	0.3438	0.4750

Table 2

Regression of the ratio of snout length to orbit diameter against standard length. r^2 = coefficient of determination and n = number of fish in sample.

Species	Regression equation	r^2	n
Sailfish (*Istiophorus platypterus*)	*SN:OD* = 0.092SL + 0.242	0.94	137
White marlin (*Tetrapturus albidus*)	*SN:OD* = 0.052SL + 0.373	0.95	24
Blue marlin (*Makaira nigricans*)	*SN:OD* = 0.026SL + 0.510	0.74	70

Identification methods

Combination of species diagnostic lower jaw pigment patterns, regression equations, and month of capture resulted in the identification key found in Table 3. Of the 304 larvae that were examined with the key by the authors, only one was misidentified. This was an 8.02-mm blue marlin that was mistakenly identified as a sailfish by question 6a in part I of the key. Of the remaining fish, 31 larvae, all between 4 mm and 10 mm SL could not be identified with the key. An additional 62 larvae, again mostly less than 10 mm SL, could be narrowed down to only two species possibilities. Overall, 69.1% of the fish were correctly identified to species. Accuracy improved with size. Eighty-five of the 93 larvae that could not be identified by the key were plotted as unknowns on the ordination (reduced set of variables), at which time correct identification was obtained for 71 of them. Seven larvae could not be identified at all, and seven were incorrectly identified because they were plotted at the interface of two species groupings. The remaining eight were incompatible with CVA because

Table 3

Key for ethanol-preserved larvae and postlarval specimens of Istiophoridae caught in the Straits of Florida and the Bahamas.

Part I: for larvae <10 mm standard length (SL)

1a Preflexion or flexing; a single, pointate chromatophore in each of lower jaw pigment grids 4 and 6; with or without a single pigment in either grid 12 or 13; no other lower jaw pigments . . . . ***Makaira nigricans***

1b Not as above. **2**

2a Any flexion stage; chromatophores of any number or shape in 3 or more of lower jaw pigment grids 1, 2, 3, 7, 8, 9, 11. . . . . ***Istiophorus platypterus***

2b Not as above. **3**

3a Larva caught in March, April, or May. either ***Istiophorus platypterus*** or ***Tetrapturus albidus***

3b Larva caught in June or later. **4**

4a Larva caught in June . . . . either ***Istiophorus platypterus***, ***Tetrapturus albidus***, or ***Makaira nigricans***

4b Larva caught in July, August, September, or October. **5**

5a Standard length ≥8 mm **6**

5b Standard length <8 mm . . . . either ***Istiophorus platypterus*** or ***Makaira nigricans***

6a Snout length / orbit diameter >0.030*SL* + 0.551. . . . . ***Istiophorus platypterus***

6b Snout length / orbit diameter ≤0.030*SL* + 0.551. . . . . ***Makaira nigricans***

Part II: for larvae ≥10 mm SL

1a Chromatophores of any number or shape in 3 or more of lower jaw pigment grids 1, 2, 3, 7, 8, 9, 11. . . . . ***Istiophorus platypterus***

1b Without the above lower jaw pigment pattern **2**

2a Snout length / orbit diameter >0.057*SL* + 0.427. . . . . ***Istiophorus platypterus***

2b Snout length / orbit diameter ≤0.057*SL* + 0.427. . . . . **3**

3a Larva caught in March, April, or May. . . . . ***Tetrapturus albidus***

3b Larva caught in June or later. **4**

4a Larva caught in July, August, September, or October. . . . . ***Makaira nigricans***

4b Larva caught in June **5**

5a Standard length ≥17 mm **6**

5b Standard length <17 mm . . . . either ***Makaira nigricans*** or ***Tetrapturus albidus***

6a Snout length / orbit diameter ≥0.047*SL* + 0.319. . . . . ***Tetrapturus albidus***

6b Snout length / orbit diameter <0.047*SL* + 0.319. . . . . ***Makaira nigricans***

a measurement was missing. Thus, when the key and CVA analyses were combined, 92.4% of the tested larvae were correctly identified.

One of the two naïve observers found that one larva out of the test set of 50 was too damaged to be evaluated. He correctly identified 35 larvae and found 14 to be unidentifiable with the key. Overall, his success rate was 71.4%. The other observer correctly identified 30 larvae, misidentified one (the larva not evaluated by the other observer and the same larva misidentified by the authors), and found 19 to be unidentifiable by the key. His overall success rate was 60%. The difference in the number of larvae that could not be identified with the key was the result of differences in interpretation of the lower jaw pigment position for larvae less than 10 mm SL.

Discussion

Because adults of four istiophorid species are found in the Straits of Florida and Bahamian waters, a reliable larval identification technique for these species is necessary (Voss, 1953). Incorrect species identifications can have serious ramifications on other areas of istiophorid early life history research. For example, studies on early growth would suffer if a larval blue marlin, which is thought to reach 174 cm lower jaw fork length (LJFL) by age one (Prince et al., 1991), were to be confused with a larval sailfish, which reportedly grows to only 108.9 cm LJFL (Hedgepeth and Jolley, 1983; Prager et al., 1995) by age one.

Few characters are available to separate the species of larval istiophorids (Richards, 1974). Although

a single character may be used to separate fish into groups, early work has lacked a means to confirm the identity of the groups. Molecular techniques provided a solution to this problem. A limitation of the molecular identification technique that we used was that only those larvae preserved in ethanol could be identified. Formalin fixation does not always preclude the use of PCR-based methods, but work is usually limited to small fragments; 570 bp is considered large for successful amplification (Shedlock et al., 1997). In the present study, DNA quality was too low in the formalin-fixed istiophorid larvae for PCR to amplify the 1.2-kb MN32-2. Consequently, only ethanol-preserved larvae could be used for key development and testing. Because of likely differences in length shrinkage between larvae preserved only in ethanol and those fixed in formalin, it is possible that the regressions presented in the present study are not valid for the latter.

No longbill spearfish were among the molecularly identified larvae; thus this species could not be included in the key. Very little is known about the longbill spearfish, but it is reported that larvae are found offshore (Ueyanagi et al., 1970), and that even adults are quite rare in United States and Bahamian waters (Robins, 1975). The longbill spearfish spawning season appears to range from late November to early May and peaks in February (Robins, 1975; de Sylva and Breder, 1997). Although there is some overlap in the spawning season of longbill spearfish with the spawning seasons of other Atlantic istiophorids, because of the rarity and predominantly offshore occurrence of the longbill spearfish, its absence from the key may not pose major problems for the identification of istiophorid larvae from our study area.

The larval istiophorids used to create and test the identification key were all captured either in the Straits of Florida or in Bahamian waters and were all smaller than 22 mm SL. Caution must be used when applying the key to larvae from other parts of the world or to larger sizes. Ueyanagi (1963) assumed that species pairs from different oceans (white marlin and striped marlin [*Tetrapturus audax*], longbill spearfish and shortbill spearfish [*Tetrapturus angustirostris*], Atlantic and Pacific blue marlin, Atlantic and Pacific sailfish]) would be identifiable by the same characters. Although these pairs exhibit the same RFLP patterns at the MN32-2 locus (McDowell and Graves, 2002), we have not tested the key with Pacific larvae and cannot be certain that their measurements would fall within the same regression limits or that they would have the same lower jaw pigment patterns. Even within the Atlantic Ocean, spawning seasons vary with location (e.g., Bartlett and Haedrich [1968] collected larval blue marlin off the coast of Brazil in February and March). Month of capture was crucial in our analyses for discriminating between small marlins when spawning season overlap is minimal; therefore our key may need adjustment to reflect local spawning seasons when applied to other locations.

As in Indo-Pacific istiophorid larvae (Ueyanagi, 1964, 1974b), snout length, eye orbit diameter, and lower jaw

pigmentation are important characters for identifying larval istiophorids of the western Atlantic. However, white marlin differ markedly from their Indo-Pacific counterpart, striped marlin. White marlin larvae, long-held as members of the "long-snout group" of istiophorids, actually more closely resemble the short-snouted blue marlin until 17 mm standard length (Fig. 6). After they reach this size, snout length is intermediate between that of blue marlin and sailfish. This result cautions against the assumption that even large larvae with short snouts are blue marlin. Snout length may be useful as a character in phylogeny studies.

The identification methods presented in the present study reduce subjectivity in the evaluation of characters. This study also brings to light the caveats of using lower jaw pigment patterns as a means of identification and limits which pigment patterns qualify as diagnostic. Although there is a family of lower jaw pigment patterns that appears to mark sailfish only, if this character were the only means of identifying sailfish, nearly 40% of our sailfish (as confirmed by RFLP analysis) would have been misidentified or escaped classification. Likewise, the preflexion blue marlin pigment pattern will not lead to misidentifications, but too many preflexion blue marlin lack the pattern to justify its use as a stand-alone identification character. Lower jaw pigment patterns have also been suggested as potentially useful characters for separation of subspecific populations of both sailfish (Ueyanagi, 1974a, 1974b) and striped marlin in the Indo-Pacific (Nishikawa, 1991). The hypothesis of pigment-delineated sailfish populations was not borne out (Leis et al., 1987), and the high variability of lower jaw pigments among larvae of each species from our study area casts further doubt on the notion of using pigments alone to distinguish populations.

Our identification key does not enable separation of species for certain classes of istiophorid larvae. For example, larvae that are caught in June, are less than 10 mm SL, and possess none of the diagnostic lower jaw pigment patterns are especially problematic. In these "dead end" cases, discriminant analysis (CVA) is useful. Although a few larvae were misidentified with the CVA, these larvae were plotted near the interface of two species groupings; this position alerts the user to the fact that misidentification is a possibility. One disadvantage of using CVA (or any discriminant analysis) for identification is that all of the variables must have a value, meaning that a larva with broken preopercular spines, for example, cannot be entered into the analysis. When the species possibilities are narrowed down to blue marlin and either sailfish or white marlin, it may be feasible to identify larvae by vertebral formula. Richards (1974) suggests that this is difficult with larvae less than 20 mm SL, but it is the method that Prince et al. (1991) used to identify blue marlin that were 5–10 mm SL. Molecular identification is always an option for resolving dead ends.

The identification of larval istiophorids has never been an easy task. Molecular identification is reliable, but can be relatively more labor intensive and expensive
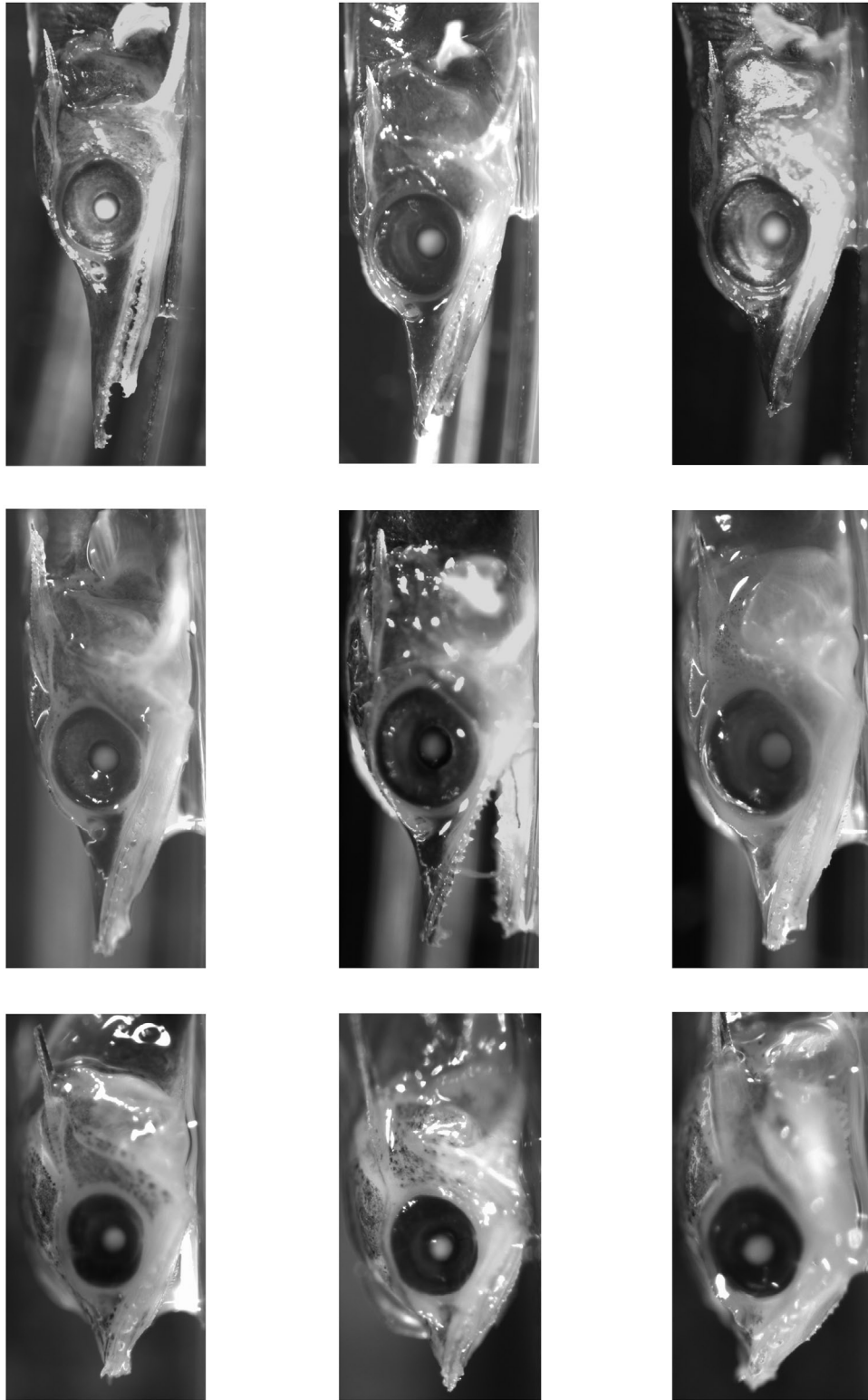
Figure 6

Size series of genetically identified representatives of each species. Top row: sailfish. Middle row: white marlin. Bottom row: blue marlin. Left column: ~5 mm SL. Middle column: ~10 mm SL. Right column: ~15 mm SL.

than traditional methods. The creation of a key based on characters developed from molecularly identified Atlantic larvae makes it possible to use more traditional methods to make reliable identifications. Despite the limitations of the key, it works well for larvae caught in our area. We recommend further testing with istiophorid larvae from other waters, and the inclusion of longbill spearfish larvae.

Acknowledgments

Literature cited

Bartlett, M. R., and R. L. Haedrich.
  1968. Neuston nets and South Atlantic larval blue marlin (*Makaira nigricans*). Copeia 1968:469–474.
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.
  1993. Identification of billfish species using mitochondrial cytochrome *b* gene fragment amplified by polymerase chain reaction. Collect. Vol. Sci. Pap. ICCAT 41:549–556.
Collette, B. B., and M. Vecchione.
  1995. Interactions between fisheries and systematics. Fisheries 20:20–25.
de Sylva, D. P., and P. R. Breder.
  1997. Reproduction, gonad histology, and spawning cycles of North Atlantic billfishes (Istiophoridae). Bull. Mar. Sci. 60:668–697.

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. 93:78–96.
Fulford, R. S., and D. A. Rutherford.
  2000. Discrimination of larval *Morone* geometric shape differences with landmark-based morphometrics. Copeia 2000:965–972.
Graves, J. E., and J. R. McDowell.
  1995. Inter-ocean genetic divergence of istiophorid billfishes. Mar. Biol. 122:193–203.
Hedgepeth, M. Y., and J. W. Jolley.
  1983. Age and growth of sailfish, *Istiophorus platypterus*, using cross sections from the fourth dorsal fin spine. *In* Proceedings of the international workshop on age determination of oceanic pelagic fishes: tunas, billfishes, and sharks (E. D. Prince and L. M. Pulos, eds.), p. 131–135. NOAA Tech. Rep. 8.
Innes, B. H., P. M. Grewe, and R. D. Ward.
  1998. PCR-based genetic identification of marlin and other billfish. Mar. Freshw. Res. 49:383–388.
Lasker, R.
  1987. Use of fish eggs and larvae in probing some major problems in fisheries and aquaculture. Am. Fish. Soc. Symp. 2:1–16.
Leis, J. M., B. Goldman, and S. Ueyanagi.
  1987. Distribution and abundance of billfish larvae (Pisces, Istiophoridae) in the Great Barrier Reef Lagoon and Coral Sea near Lizard Island, Australia. Fish. Bull. 85:757–765.
McDowell, J. R., and J. E. Graves.
  2002. Nuclear and mitochondrial DNA markers for specific identification of istiophorid and xiphiid billfishes. Fish. Bull. 100:537–544.
Morrow, J. E., and S. J. Harbo.
  1969. A revision of the sailfish genus *Istiophorus*. Copeia 1969:34–44.
Nakamura, I.
  1974. Some aspects of the systematics and distribution of billfishes. *In* Proceedings of the international billfish symposium; Kailua-Kona, Hawaii, 9–12 August 1972. Part 2: Review and contributed papers (R. S. Shomura and F. Williams, eds.), p. 45–53. NOAA Tech. Rep. NMFS SSRF-675.
Nishikawa, Y.
  1991. On the melanophore patterns on lower jaw of the larvae of striped marlin. Bull. Natl. Res. Inst. Far Seas Fish. 28:15–19.
Prager, M. H., E. D. Prince, and D. W. Lee.
  1995. Empirical length and weight conversion equations for blue marlin, white marlin, and sailfish from the North-Atlantic Ocean. Bull. Mar. Sci. 56:201–210.
Prince, E. D., D. W. Lee, J. R. Zweifel, and E. B. Brothers.
  1991. Estimating age and growth of young Atlantic blue marlin *Makaira nigricans* from otolith microstructure. Fish. Bull. 89:441–459.
Richards, W. J.
  1974. Evaluation of identification methods for young billfishes. *In* Proceedings of the international billfish symposium; Kailua-Kona, Hawaii, 9–12 August 1972. Part 2: Review and contributed papers (R. S. Shomura and F. Williams, eds.), p. 62–72. NOAA Tech. Rep. NMFS SSRF-675.
Robins, C. R.
  1975. Synopsis of biological data on the longbill spearfish, *Tetrapturus pfluegeri*, Robins and de Sylva. *In*

Proceedings of the international billfish symposium; Kailua-Kona, Hawaii, 9–12 August 1972. Part 3: Species synopsis (R. S. Shomura and F. Williams, eds.), p. 28–37.   NOAA Tech. Rep. NMFS SSRF-675.

Rocha-Olivares, A., H. G. Moser, and J. Stannard.
    2000.   Molecular identification and description of pelagic young of the rockfishes *Sebastes constellatus* and *Sebastes ensifer*.   Fish. Bull. 98:353–363.

Ruzzante, D. E., C. T. Taggart, and D. Cook.
    1996.   Spatial and temporal variation in the genetic composition of a larval cod (*Gadus morhua*) aggregation: Cohort contribution and genetic stability.   Can. J. Fish. Aquat. Sci. 53:2695–2705.

Sambrook, J., E. F. Fritsch, and T. Maniatus.
    1989.   Molecular cloning: a laboratory manual, p. 9.17–9.19.   Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shedlock, A. M., M. G. Haygood, T. W. Pietsch and P. Bentzen.
    1997.   Enhanced DNA extraction and PCR amplification of mitochondrial genes from formalin-fixed museum specimens.   Biotechniques 22:394–400.

Ueyanagi, S.
    1963.   Methods for identification and discrimination of the larvae of five istiophorid species distributing in the Indo-Pacific.   Rep. Nankai Reg. Fish. Res. Lab. 17:137–150.   [In Jap., Engl. sum.]
    1964.   Description and distribution of larvae of five istiophorid species in the Indo-Pacific.   Mar. Biol. Assoc. India. Proc. Symp. Scombroid Fish. Mandapam Camp, Part I:499–528.
    1974a.   On an additional diagnostic character for the identification of billfish larvae with some notes on the variations in pigmentation.   *In* Proceedings of the international billfish symposium, Kailua-Kona, Hawaii, 9–12 August 1972. Part 2: Review and contributed papers (R. S. Shomura and F. Williams, eds.) p. 73–78.   NOAA Tech. Rep. NMFS SSRF-675.
    1974b.   Present state of billfish larval taxonomy.   *In* The early life history of fish (J. H. S. Blaxter, ed.), p. 649–658.   Springer-Verlag, New York, NY.

Ueyanagi, S., S. Kikawa, M. Uto, and Y. Nishikawa.
    1970.   Distribution, spawning, and relative abundance of billfishes in the Atlantic Ocean.   Bull. Far Seas Fish. Res. Lab. 3:15–55.

Voss, G. L.
    1953.   A contribution to the life history and biology of the sailfish, *Istiophorus americanus* Cuv. and Val., in Florida waters.   Bull. Mar. Sci. Gulf Carib. 3:206–240.