Global population structure of the swordfish (*Xiphias gladius* L.) as revealed by analysis of the mitochondrial DNA control region

Jaime R. Alvarado Bremer*, Jaime Mejuto*, Thomas W. Greig†, Bert Ely‡

*FISHTEC Genetics Laboratory, Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA

†Instituto Español de Oceanografía, Apdo. 130, 15080 La Coruña, Spain
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Jaime R. Alvarado Bremer*a, b, Jaime Mejutoa, Thomas W. Greig*, Bert Ely*

*FISHTEC Genetics Laboratory, Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA
Instituto Español de Oceanografía, Avenida de Pineda, 130, 15080 La Coruña, Spain

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Abstract

The global population structure of the swordfish (Xiphias gladius L.) was examined by analyzing the DNA sequence variation contained within the hypervariable left domain of the mitochondrial control region of 247 individuals. A total of 303 base pairs (bp) of sequence from 112 individuals collected in the Pacific (n = 26), the Atlantic (n = 47) and the Mediterranean (n = 39) revealed a total of 69 haplotypes. Extremely high values of haplotype diversity characterized all samples and all haplotypes occurred at low frequencies. The topology of a neighbor-joining tree was used to establish phylogenetic relationships among the mtDNA lineages. The genotypes could be assigned to 2 highly divergent clades, namely clade I and clade II (low nucleotide differences 3.9%). Furthermore, clade I could be divided into 2 groups designated alpha and beta. The presence of phylogenetically informative differences in the DNA sequence allowed the design of a Restriction Fragment Length Polymorphism (RFLP) assay which could discriminate members of the 2 clades.

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Keywords: Swordfish; mtDNA; D-loop; RFLP; Population genetics

Abbreviations: mtDNA: mitochondrial DNA; RFLP: restriction fragment length polymorphism; D-loop: D-loop region of the mitochondrial DNA; Haplotypes: the different forms of the mtDNA sequence; Population genetics: the study of the genetic composition of populations.
1. Introduction

Very little is known about the reproductive biology and population structure of the swordfish (Xiphias gladius L.). Spawning of this monotypic cosmopolitan species takes place year round in most circumtropical waters deeper than 200 m (Palko et al., 1981). Spawning apparently peaks in the winter (Grall et al., 1983). However, there are sampling biases in the collection of larvae in the Northwestern Atlantic (certain seasons gonadal maturity, which places the major breeding grounds for Lee, 1993). Spawning information for the rest of the Atlantic Ocean is extremely Atlantic between could be expected (Avise et al., 1987). Tagging probabilities of recapturing tagged fish were tagged have not been estimated (International Commission for the Conservation of Atlantic Tunas, 1993). Thus, the absence of trans-oceanic recaptures, which has been used as evidence favoring the existence of separate population stocks in the Atlantic, could be the result of this sampling bias. Tagging recaptures, however, have revealed that while swordfish are capable of journeys of several thousand kilometers (International Commission for the Conservation of Atlantic Tunas, 1993), females in the Northwest Atlantic display strong homing abilities towards feeding areas (Burnett et al., 1987).

Recently, the analysis of DNA sequences obtained directly from PCR (Polymerase Chain Reaction) amplified mtDNA has become the tool of choice for studies whose goal is to resolve intraspecific phylogenies or population structure (e.g., Quinn, 1992; Wenink et al., 1993; Brown et al., 1993). Many vertebrate species with life histories conducive to dispersal show no evidence of genetic differentiation among samples of individuals collected over vast geographical areas. Presumably this is because gene flow can act as a strong homogenizing force (Slatkin, 1987), which reduces the genetic differentiation of populations. However, high dispersal capabilities and the absence of physical barriers to gene flow do not necessarily translate into panmixia. Breeding populations of any species can be subdivided by strong maternal philopatry to breeding grounds, resulting in fixed differences being established as mutations accumulate over time. Examples of close phylogeographical association of maternal lineages to breeding areas include Canada Geese (Branta canadensis; Van Wagner and Baker, 1990), the Dunlin (Calidris alpina; Wenink et al., 1993), and the green turtle (Chelonia mydas; Bowen et al., 1992).

The study of variation contained in the mitochondrial DNA offers an opportunity to unravel several aspects of the population structure of swordfish. The mitochondrial genome has many features that make it particularly suitable for genetic studies of intraspecific population structure, including its rapid rate of evolution, apparent lack of recombination, and predominantly maternal inheritance (Avise et al., 1987). In the swordfish, as in most vertebrate species (Brown et al., 1986; Wenink et al., 1993), most of the variation in the mitochondrial genome is confined to 2 hypervariable segments of the non-coding control (D-loop) region that are separated by a third highly conserved segment (Alvarado Bremer, 1994). Studies of patterns of sequence variation contained in the control region of a widespread sample of swordfish from the Pacific, the Atlantic and the Mediterranean yielded very high levels of diversity of mtDNA types (Alvarado Bremer, 1994; Alvarado Bremer et al., 1995). A phylogenetic analysis of the 630 bp DNA sequences obtained from these studies revealed that the mtDNA types could be assigned to 2 highly divergent clades with a corrected mean divergence of 2.7%. However, for the most part, no evidence of phylogeographic structuring was found between the Atlantic and the Pacific samples, due to small sample sizes (Alvarado Bremer, 1994; Alvarado Bremer et al., 1995). Both within the North Atlantic (Alvarado Bremer et al., 1995), and within the North Pacific (Grijalva-Chon et al., 1994), there is evidence of extensive mixing. In contrast, a phylogeographic association of swordfish mtDNA RFLP haplotypes appears to exist within the Mediterranean Sea (Magoulas et al., 1993), and historical isolation during Pleistocene regression was invoked to explain the present distribution of types (Alvarado Bremer et al., 1995). Thus, it is likely that in spite of the well known dispersal capabilities of swordfish (Burnett et al., 1987; International Commission for the Conservation of Atlantic Tunas, 1993), the Atlantic and the Mediterranean populations of swordfish are genetically distinct.

In this study, we have analyzed 246 individuals using a combination of DNA sequence analysis and RFLP assays. Phylogenetic and haplotypic frequency analyses of the resulting data demonstrated that geographic partitioning of haplotypes does occur and that significant differences could be observed among samples from the Pacific Ocean, the North Atlantic Ocean, the South Atlantic Ocean, and the Mediterranean Sea.

2. Materials and methods

2.1. Populations sampled

In a previous study (Alvarado Bremer, 1994; Alvarado Bremer et al., 1995), the DNA sequence of the mitochondrial control region was analyzed in a sample of 50 individuals including 8 from California, 7 from two localities in the Mediterranean Sea, 13 from the Northeastern Atlantic, 14 from the Northwestern Atlantic, and 8 from the South Atlantic. In this study, 330 bp of DNA sequence of the hypervariable left domain of the control region was determined from an additional 62 swordfish. The new samples included 18 swordfish captured off Hawaii during the 1992 and 1993 fishing seasons, 1 swordfish captured in the Gulf of Mexico in 1993, 10 swordfish caught off Puerto Rico in 1993, 1 swordfish captured in the Northeast Atlantic in 1992 and 32 swordfish caught in 1992 in the Ionian Sea (Table 1).

Mitochondrial types were also characterized by a hierarchical restriction analysis of PCR-amplified control regions from an additional 135 individuals (41 from 2 locations in the Northwestern North Atlantic, 40 from 4 locations in the Caribbean Sea and the
Table 1

<table>
<thead>
<tr>
<th>Region</th>
<th>n</th>
<th>Types no.</th>
<th>Diversity (h)</th>
<th>Probability of identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mediterranean</td>
<td>39</td>
<td>15</td>
<td>0.94</td>
<td>0.09</td>
</tr>
<tr>
<td>N.W. Atlantic</td>
<td>14</td>
<td>13</td>
<td>0.99</td>
<td>0.08</td>
</tr>
<tr>
<td>N.W. Atlantic</td>
<td>25</td>
<td>24</td>
<td>0.995</td>
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</tr>
<tr>
<td>S. Atlantic</td>
<td>8</td>
<td>8</td>
<td>1.00</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Diversity is given by \( h = \left( 1 - \sum x_i^2 \right) / (n - 1) \), where \( x_i \) is the frequency of a genotype and \( n \) is the sample size. The probability of identity is given by the sum of the squares of the frequencies (Vigilant et al., 1989).

Gulf of Mexico, 37 from the Mediterranean, 13 from 3 locations in the South Atlantic and 4 from the Northeast Atlantic).

2.2. DNA extraction

Fresh solid tissue was obtained from swordfish captured with longlines by commercial fishing vessels. Tissue (heart, liver or gonads) was either kept frozen until assayed or preserved in 70% alcohol (ethanol or isopropanol). Total DNA extractions followed the protocol described in Sambrook et al. (1989). Briefly, approximately 0.04 g of tissue were washed with cold distilled water to remove traces of blood. The samples were placed in 1.5 ml microfuge tubes containing: 350 \( \mu \)l STE buffer (100 mM NaCl, 20 mM Tris–HCl (pH 8.0), 2.5 mM EDTA), 40 \( \mu \)l 10% SDS and 30 \( \mu \)l Proteinase K (20 mg/ml). The tubes were incubated overnight at 55°C, with gentle rotation inside a hybridization oven. The lysates were extracted once with an equal volume of buffer-saturated phenol, and twice with 500 \( \mu \)l chloroform–isoamyl alcohol (24:1), followed by ethanol precipitation. The DNA pellet was dissolved in 50–100 \( \mu \)l water. A 200-fold dilution was obtained by combining 5 \( \mu \)l sample with 1000 \( \mu \)l water, and 1 \( \mu \)l aliquots were used as the template in 50 \( \mu \)l PCR reactions. Some samples required further dilutions (e.g., \( 10^{-3} \) final dilution) to obtain satisfactory results.

2.3. PCR amplification and sequencing

Two primers specifically designed for the control region of fish (Alvarado Bremer, 1994) were used to amplify about 440 bp using PCR (Saiki et al., 1985, 1988). The L-strand primer L15998-PRO (5'-TAC CCC AAA CTC CCA AAG GTA TCA-3'), which corresponds to a region in the adjacent rRNApro gene, was used in combination with the H-strand primer CSBDH (5'-TGA ATT AGG AAC CAG ATG CCA G-3'), which corresponds to the conserved sequence block D (Southern et al., 1988) in the central domain of the control region. The PCR cycling parameters used to amplify double-stranded DNA were as follows: an initial denaturing step of 5 min at 94°C, followed by 36 cycles of the profile: denaturing at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 1 min. The final extension was at 72°C for 5 min. The amplified product was purified with low-melting-point agarose using a Gene Clean Kit (BIO 101, La Jolla, CA, USA) and resuspended in 15 \( \mu \)l water. Alternatively, a 5 \( \mu \)l sample of the PCR reaction was electrophoresed to verify the quality of the amplification. Subsequently, the primers, dNTPs and Taq DNA polymerase were removed from the remaining PCR product by using 30 000 NMMW Ultra-free-MC filters units (Millipore, Bedford, MA, USA) according to the manufacturer’s recommendations. Double-stranded DNA sequencing reactions were performed with the dyeoxy chain termination method (Sanger et al., 1977) using Sequenase kits (version 2.0; US Biochemical, Cleveland, OH, USA) and [\( \alpha^{-32}P \)]dATP (NEN/Dupont, Boston, MA, USA) on 7 \( \mu \)l purified DNA from the PCR reactions, with the following modifications of the above protocol: DNA was denatured in boiling water for 10 min in the presence of 1 \( \mu \)l (8 pmol) of one of the amplification primers. After snap-freezing in a dry ice/ethanol bath, the reaction was thawed by holding it between the fingers, and 2 \( \mu \)l annealing reaction buffer from the kit was added. The tubes were kept on ice for 5 min, and then annealing was allowed to proceed for 2 min at room temperature. The labelling mix was diluted 1:50, to obtain a sequence close to the primer. Labelling was carried out for 8 min on ice and then 2 min at room temperature. After labelling, a 2–5 min termination reaction was carried out in a titration plate (Nunc, Denmark) which had been pre-heated for 5 min at 42–45°C. The resulting DNA fragments were separated in 40 cm 5% Long Ranger (AT Biochem, Malvern, PA, USA), 7 M urea gels. Electrophoresis was done at 65 W of constant power, approximately 1500 V, for 1.5 h for short runs and 3.0 h for long runs. The gels were dried onto filter paper and autoradiographed with Kodak X-AR film or Kodak Biomax MR film.

2.4. Phylogenetic analysis

Genetic divergence between haplotypes was estimated using either the Kimura 2-parameter model (Kimura, 1980) or the Tamura–Nei model (Tamura and Nei, 1993) in MEGA (Kumar et al., 1993). For the Tamura–Nei model, we assumed that the rate of nucleotide substitution followed a gamma distribution specified by parameter \( a \) which was set at 0.5. Tree topology was constructed using the neighbor-joining method (Saitou and Nei, 1987).

2.5. Hierarchical restriction analysis

A hierarchical PCR-RFLP analysis was used to classify an additional 135 individuals into 3 subgroups: alpha, beta and theta. The amplified control regions were digested separately with 3 restriction endonucleases, RsaI, DraI and MseI. These 3 enzymes were selected because inspection of the DNA sequence profiles of the 68 distinct haplotypes indicated that they would allow us to classify individuals to one of the 3 major mitochondrial subgroups identified in this study (alpha, beta, or theta) with greater than 99% accuracy. Based on this analysis, we assumed that these restriction assays could be used to classify a larger sample with equal accuracy even though a larger sample was likely to contain previously undetected haplotypes. Restriction digests were carried out in 25 \( \mu \)l reactions containing 5 \( \mu \)l of amplified products and 5 units of restriction endonuclease in the buffer recommended by the manufacturer (New England Biolabs, Beverly, MA, USA), and subjected to electrophoresis in a 1% agarose gel (RsaI), a 2%
Table 2

Digestion patterns of swordfish amplification products using restriction endonucleases Rsal and Dral

<table>
<thead>
<tr>
<th></th>
<th>Rsal</th>
<th></th>
<th>Dral</th>
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<td></td>
<td>50</td>
<td>50</td>
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<td></td>
</tr>
</tbody>
</table>

*Fragment sizes in bp are listed in base pairs underneath each genotype.

agarose gel (MseI) or a 6% polyacrylamide gel (DraI). Table 2 shows the restriction fragments produced by digestion with either Rsal or DraI. Samples were classified as alpha if the 260 bp Rsal fragment was present (Rsal haplotypes C or D). Those samples that were not classified as members of the alpha subgroup were digested with restriction endonuclease DraI so that the number of the TACA repeats could be detected. Members of clade II (theta) always had a single copy of the TACA motif (DraI haplotype C or F); whereas, members of clade I (alpha or beta) had 2 or 3 tandem copies of this motif (DraI haplotypes D, E or G). In some cases (DraI haplotypes A or B), a missing DraI site made it impossible to discriminate the 4 base differences caused by the presence or absence of a copy of the repeat. Analysis of the DNA sequence data from these individuals showed that they could be distinguished on the basis of the presence or absence of an MseI site embedded within the variable DraI site. Members of clade II (theta) with mutations in the DraI site also lacked the MseI site and gave rise to a 77 bp fragment that was missing from those in the beta subgroup.

2.6. Monte Carlo distributions

The extent of geographic heterogeneity in population frequency distributions was analyzed through a Monte Carlo simulation as described by Roff and Bentzen (1989), using the program MONTE in REAP (version 2.0) (McElroy et al., 1992). The level of heterogeneity in the original data matrix was assessed through chi-square analysis and compared to that estimated from 10000 randomizations of the original matrix. By repeatedly randomizing the original matrix, a mean $X^2$ value based on chance alone was estimated, and the probability of encountering a $X^2$ value equal or larger than the original was determined.

2.7. Gamma

The degree of population subdivision when mutation rates are high also can be estimated by a measure of mutational divergence ($\gamma$) (Latter, 1973). We calculated $\gamma$ using the program RAND-GST written in PASCAL by Dr. Alejandro Lynch (Department of Zoology, University of Toronto, Toronto, Canada). Gamma is based on the probability of identity of sequence variants among populations relative to the probability of identity within populations. Meaningful values of $\gamma$ range from zero to unity. Unity values reflect the fixation of different sequence variants in each population. Under an island model of population structure, $\gamma$ approximates the ratio between mutation and migration rates. Thus, it provides evidence of gene flow among mtDNA gene pools. A total of 1000 permutations were conducted for each calculation of $\gamma$.

3. Results

3.1. Variation in the control region

We determined the DNA sequence of 330 bp of the left domain of the mitochondrial control region for 62 individuals. These data were combined with the sequence data of 50 individuals reported by Alvarado Bremer et al. (1995). A total of 94 variable sites were identified within this stretch of sequence, resulting in 69 distinct mtDNA types among the 112 individuals (Fig. 1). Very large values of haplotypic diversity, $h$ (Nei and Tajima, 1981), were estimated for all populations (Table 1). The probability that any 2 randomly drawn specimens among the 47 Atlantic swordfish were different was 0.99. Similarly, the haplotypic diversity for the Pacific sample was 0.99. Lower diversity values were obtained for the Mediterranean, but the values for this region were still high ($h = 0.92$).

3.2. Phylogenetic analysis

Phylogenetic analysis of the 69 distinct mtDNA haplotypes resulted in two highly divergent clades (Clades I and II) (Fig. 2), previously identified by Alvarado Bremer et al. (1995) using both parsimony and neighbor-joining analyses. Ten fixed differences (transversion, 2 insertions or deletions (indels) and 7 transitions) distinguish these 2 clades. One obvious feature of the members of Clade I is the presence of the indel repeat 5'-TACA-3' at nucleotide position 18 (Fig. 1). The mean divergence between the 2 clades is 6.6% (SE 0.011), whereas the net nucleotide difference (Nei, 1987) between the two clades is 3.9%. Clade I includes 59 haplotypes corresponding to 88 individuals that were collected in all ocean basins sampled (Fig. 2). The mean divergence within Clade I was 2.9%. Although the branches at the base of Clade I are shallow (most nodes are supported by only a few shared derived changes), several major nodes can be identified. The branches bifurcating from these nodes appear to have some level of phylogeographic structuring. For instance, the branches comprising the alpha subgroup (Fig. 2) include 23 distinct types. Twenty-two of these types corresponded to 23 Atlantic and 14 Mediterranean individuals. The remaining branch represents 1 individual captured in the Pacific. The balance of clade I, designated the beta subgroup, is polyphyletic in origin and contains the other 25 Pacific samples. However, the beta subgroup also includes individuals from the Atlantic and the Mediterranean. Conversely, Clade II (designated theta) includes 24 individuals with 10 haplotypes, numbers 11, 19,
Fig. 1. Variable sites in the control-region segment of swordfish mtDNA. Numbers above sites refer to nucleotide position on the L-strand of the control region of the swordfish (Alvarado Bremer et al., 1995). Identity with sequence 1 is indicated by dots, and deletions of 1 residue with dashes. Types of mtDNA are numbered 1–69, and the same designations are used throughout the text and figures in this study. The mtDNA types have been sorted according to groups with beta first, followed by alpha and theta.

20, 24, 25 and 63–67 (Fig. 2). Members of this clade were found exclusively in the Atlantic Ocean and the Mediterranean Sea (Fig. 3). Approximately 71% of these individuals came from the Mediterranean (n = 17) and the Northeastern Atlantic (n = 3);
The remaining 4 individuals, corresponding to haplotypes 11, 63, 64 and 65 came from the Western North Atlantic and the Caribbean. The mean divergence within Clade II was 1.7% (SD 0.2).

3. Monte Carlo distributions

The extent of geographic heterogeneity in population frequency distributions was analyzed through a Monte Carlo simulation as described by Roff and Bentzen (1989). The pooled haplotype frequencies for the Pacific, the North Atlantic and the Mediterranean were examined. The probability (P = 0.0000; 10 000 permutations) of obtaining by chance a mean chi-square value ($\chi^2 = 123.43$) (including ties) larger than the original ($\chi^2 = 176.32$), suggested that the inter-oceanic frequency distribution of haplotypes is heterogeneous. The analysis of heterogeneity between several population pairs was also estimated. Highly significant differences exist between the Pacific and the North Atlantic ($P = 0.0145$), the Pacific and the Mediterranean ($P = 0.0001$), and the North Atlantic and the Mediterranean ($P = 0.0001$).

3.4. Gamma

As mutation rates increase, $\gamma$ will increase because most haplotypes will be private to single populations or to a subset of them unless migration rates are exceptionally high. A null value of $\gamma$ was obtained for the haplotypic frequencies comparison between the Mediterranean and the Pacific ($P = 0.001$). This value represents the fixation of different sequence variants in each population, and is indicative of the absence of gene flow among the mtDNA gene pools. A high $\gamma$ value of 0.927 ($P = 0.025$) was obtained for the comparison between the North Atlantic sample and the Pacific sample, again suggesting limited levels of gene flow. The gamma estimates for the comparison of the North Atlantic and the Mediterranean samples were lower ($\gamma = 0.772; P = 0.044$), indicating that although gene flow is limited some shared haplotypes occur.

3.5. Geographic distribution of the alpha, beta and theta subgroups

Inspection of the DNA sequences of members of the alpha, beta, and theta subgroups revealed that samples could be classified to the 3 subgroups on the basis of fragment sizes produced by digests with the restriction endonucleases Rsal, DraI and MseI. Using this approach, we classified an additional 135 samples obtained from the Mediterranean Sea and the Atlantic Ocean. These data were combined with the data from the DNA sequence analysis, and the geographic distribution of the 3 subgroups was analyzed using an $R \times C$ G-test of independence (Sokal and Rohlf, 1981). Samples from 5 locations within the North Atlantic were tested and no significant differences were found ($P \geq 0.1$; Table 3). Therefore, these samples were combined to form one North Atlantic sample. Similar analyses verified that both the Pacific and the Mediterranean samples could be treated as single samples (data not shown). In contrast, when the North Atlantic samples were compared to the South Atlantic samples, significant differences were observed ($P \leq 0.005$; Table 4). Thus, the samples appear to be drawn from 2 genetically
distinct populations of swordfish in the Atlantic. This result is in agreement with the heterogeneity between the Atlantic and the Mediterranean swordfish reported by Magoulas et al. (1993). Comparisons were also made with the Pacific and the Mediterranean samples, in all pairwise combinations. In every case; the 4 populations were significantly different from each other (P < 0.025). These results were confirmed by Monte Carlo simulations (Roff and Bentzen, 1989). No significant difference was observed between the Eastern and the Western Atlantic samples (P = 1.0), but the North Atlantic, the South Atlantic, the Mediterranean and the Pacific samples were significantly different from each other in all pairwise combinations (P < 0.03). There are a minimum of 4 genetically distinct populations of swordfish, 2 in the Atlantic, 1 in the Mediterranean, and 1 in the Pacific. The frequencies of the alpha, beta, and theta subgroups in these 4 geographic populations are summarized in Fig. 3.

4. Discussion

4.1. Patterns of sequence variation

Approximately 30% (94) of the 330 nucleotides in the hypervariable left domain of the mitochondrial control region were variable among the 112 individuals surveyed (Fig.

Table 4

<table>
<thead>
<tr>
<th>Location</th>
<th>Haplotypes</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td>β</td>
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<tr>
<td>N. Atlantic</td>
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<td>36</td>
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<tr>
<td>S. Atlantic</td>
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<td>13</td>
</tr>
<tr>
<td>Mediterranean</td>
<td>32</td>
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</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>88</td>
</tr>
</tbody>
</table>

* Specimen from Azores (Alvarado Bremer, 1994) not included.

Table 3

<table>
<thead>
<tr>
<th>Location</th>
<th>Haplotypes</th>
<th>Total</th>
</tr>
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<tr>
<td>Total</td>
<td>63</td>
<td>35</td>
</tr>
</tbody>
</table>

2) This represents an increase of 30 variable sites compared to those reported by Alvarado Bremer et al. (1995). A total of 69 distinct mtDNA types were identified, compared to the 43 types reported by Alvarado Bremer et al. (1995) for a sample of 50 individuals. All regions of capture had extremely high values of haplotypic diversity. The majority of the haplotypes occurred at extremely low frequencies both within and among regions. This contrasts with the findings of studies on other highly vagile and broadly distributed species, where the sequences of the hypervariable portions of the control region were surveyed (Wenink et al., 1993, 1995). In each of these studies some haplotypes occurred at high frequencies.

The genetic phylogenetic analysis relating the 69 mtDNA types corroborates the findings of Alvarado Bremer et al. (1995) that there are 2 highly divergent mitochondrial lineages. The genetic distance difference between these 2 clades was about 3.9%. Most of the local differences separating these 2 clades were transitions, but they also included 1 transition and 2 indels. Among the 112 individuals whose DNA sequence was determined, 75% had mtDNA sequences belonging to Clade I.

Significant results were obtained from Monte Carlo simulations used to test the genetic heterogeneity in frequency distributions of mtDNA haplotypes among the pooled Pacific, Atlantic, and Mediterranean samples. When testing sequence data, the x² values obtained in 10,000 replicates never exceeded the observed x² value when the Pacific was compared with either the North Atlantic or the Mediterranean. Chi-square values also indicated a high level of diversity among the Mediterranean and the Atlantic samples.

In support of Monte Carlo simulations, high estimates of gamma, which are indicative of fixation of different sequence variants within populations, were obtained for the comparison of the Atlantic and the Pacific samples. This result suggests that there are low levels of inter-oceanic gene flow. In fact, a value of unity, indicative of no gene flow, was estimated for the comparison between the haplotype frequencies of the Pacific and the Mediterranean. Lower values of gamma were obtained for the comparison of the Atlantic and the Mediterranean samples, and even lower values of y were obtained by comparing the Mediterranean with the Northeastern Atlantic haplotype frequencies. This latter result was expected since these 2 areas share several mtDNA types. These results are in agreement with observations by de la Serna et al. (1992) which suggest that seasonal migratory movements take place in and out of the Mediterranean Sea.

The estimates of gamma and the Monte Carlo distributions for x² values are subject to distortion due to the high levels of haplotypic diversity and the low probability of identity of genotypes. However, an analysis of the phylogeographic association of alpha, beta, and theta haplotypes does not support this view. The distribution of the 3 haplotypes is clearly different among the Pacific, North Atlantic, South Atlantic and Mediterranean samples as demonstrated by both the G-tests of independence and the Monte Carlo distributions. Analysis of the 69 haplotypes identified by DNA sequencing allowed us to develop an RFLP assay that had a phylogenetic basis. When the haplotype frequencies obtained by the RFLP analysis were compared to those obtained by the DNA sequence analysis, no significant differences were obtained for any of the regions tested (data not shown). Therefore, we were able to pool the data obtained by the 2 methods and analyze the data as a traditional 3 allele system. This approach allowed us...
to double the overall sample size, and the analysis of allele frequencies provided an independent confirmation of the conclusions obtained from the phylogenetic analysis. Furthermore, the increased sample size allowed us to show that the South Atlantic samples were genetically distinct from those obtained in the North Atlantic. These results demonstrate that the combination of an RFLP analysis with DNA sequence information is an extremely powerful approach. When an RFLP analysis is based on DNA sequence information, one is assured of phylogenetically meaningful data from individual restriction sites without sacrificing the ease and simplicity of an RFLP assay. A similar approach has been used by Quinn (1992) for the analysis of snow goose populations.

The heterogeneous distribution of mtDNA types in the swordfish and in most species of istiophorid billfishes (Finnerty and Block, 1992; Graves and McDowell, 1995) suggests the presence of population structure. This contrasts with the findings for several other highly mobile species of scrombroid fishes. The null hypotheses of a common gene pool for the Atlantic and the Pacific populations of skipjack, albacore or yellowfin tuna, could not be rejected (Graves et al., 1984; Graves and Dixon, 1989; Scoles and Graves, 1993). Aspects of behavior, demography, mutation rates and historical phylogeography may account for the differences and similarities in population structuring of these species (Alvarado Bremer, 1994; Graves and McDowell, 1995). Thus, it is very important to avoid generalizations of the patterns observed in one species to other species, based on superficial similarities. The relative importance of different factors affecting the current geographic distribution of genotypes needs to be investigated.

In summary, we have demonstrated that the control region of swordfish mtDNA is extremely valuable for resolving their stock structure. We have shown that the Pacific Ocean, the North Atlantic Ocean, the South Atlantic Ocean and the Mediterranean Sea all contain significant differences in haplotype frequencies. Thus, there are at least 4 genetically distinct populations of swordfish.

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