Population structure of Atlantic swordfish (*Xiphias gladius* L. 1758) (Teleostea, Xiphiidae) using mitochondrial DNA analysis: implications for fisheries management

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Abstract

*Population structure of Atlantic swordfish (*Xiphias gladius *L. 1758) (Teleostea, Xiphiidae) using mitochondrial DNA analysis: implications for fisheries management.*— Recent studies on Atlantic swordfish (*Xiphias gladius* L. 1758) genetic structure have demonstrated significant heterogeneity but the precise boundary between populations remains to be identified. In this context, genetic diversity was investigated by PCR–RFLP analysis at the control region of mitochondrial DNA (D–loop) from 274 swordfish specimens collected from five different areas of the Atlantic Ocean. The analysis of molecular variance (AMOVA) showed that genetic variation was mainly due to differences within rather than between the studied areas. Additionally, the phylogenetic analysis did not show evident relationships among haplotypes from all areas. However, low but significant F_{ST} values were recorded when comparing Equatorial samples with those from the north central and north tropical Atlantic. These results do not support a need for changing the current management boundary for the Atlantic fishery.

Key words: Xiphiidae, Swordfish, *Xiphias gladius*, Mitochondrial DNA, Genetic variability, Atlantic Ocean.

Resumen

*Estructura poblacional del pez espada del Atlántico (*Xiphias gladius *L. 1758) (Teleostea, Xiphiidae) usando análisis de ADN mitocondrial: implicaciones para la gestión de pesquerías.*— Estudios recientes sobre la estructura genética del pez espada del Atlántico (*Xiphias gladius* L. 1758) han demostrado una heterogeneidad significativa, pero los límites precisos entre poblaciones no han sido identificados. En este contexto, la diversidad genética se ha investigado mediante análisis PCR–RFLP en la región control de ADN mitocondrial (bucle D) de 274 peces espada recolectados en cinco zonas diferentes del océano Atlántico. El análisis de la varianza molecular (AMOVA) mostró que la variación genética se debía a diferencias en cada zona y no entre las zonas estudiadas. Además, los análisis filogenéticos no muestran relaciones evidentes entre los haplotipos de todas las zonas. A pesar de ello, al comparar las muestras ecuatoriales con las de zonas más al norte, se obtienen valores de F_{ST} bajos pero significativos. Estos resultados indican que no es necesario cambiar los límites de las zonas de gestión para la pesquería del Atlántico.

Palabras clave: Xiphiidae, Pez espada, *Xiphias gladius*, ADN mitocondrial, Variabilidad genética, Océano Atlántico.

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Introduction

The swordfish (*Xiphias gladius* L. 1758) is a pelagic and highly migratory species inhabiting tropical and temperate waters in a geographical range extending from 45º N to 45º S (Nakamura, 1985). Up to two decades ago, the oceanic environment was considered free from pronounced barriers, suggesting a lack of population genetic structure in species with high migratory ability, such as swordfish (Waples, 1998). However, more recent studies on the genetic structure of swordfish populations (*i.e.* biological stocks) using both mitochondrial (mtDNA) and nuclear DNA (nDNA) have shown significant inter–oceanic differentiation of Atlantic, Indo–Pacific and Mediterranean populations (Alvarado Bremer et al., 1995, 1996, 2005; Kotoulas et al., 1995; Rosel & Block, 1996; Chow et al., 1997; Chow & Takeyama, 2000; Greig et al., 2000; Jean et al., 2006; Muths et al., 2009). Moreover, a significant northwest versus south Atlantic genetic distinction has been detected as sampling efforts increased both using mtDNA (Alvarado Bremer et al., 1996, 2005; Chow et al., 1997) and nDNA markers (Greig et al., 1999, 2000). In particular, significant heterogeneity in mtDNA diversity was found between the northwest and south Atlantic regions as well as by means of both nuclear *loci* aldolase *B* (*aldB*) and lactate dehydrogenase *A* (*IdhA*) (Greig et al., 1999, 2000). Also, a large survey based on nDNA calmodulin gene intron 4 (*CaM*) showed a sharp differentiation between northwest Atlantic swordfish and those from southern regions (Chow & Takeyama, 2000; Chow et al., 2007).

Despite the considerable advances in our understanding of Atlantic swordfish population structure that suggest the existence of genetic heterogeneity within the Atlantic, the data so far acquired still leave two questions unanswered. The first concerns the genetic homogeneity within the North Atlantic (West *versus* East), which has not yet been thoroughly assessed because of the limited sampling effort in the Eastern region. As a further consequence, the degree of differentiation among swordfish from the South and NE Atlantic has been poorly evaluated. The second question regards the geographic boundary and the possible mixing of North and South Atlantic populations. ICCAT sets the management boundary between the Atlantic populations at 5° N but scientists and managers are still questioning the validity of this hypothesis (Chow et al., 2007; Viñas et al., 2007; Smith & Alvarado Bremer, 2010). This is due to the fact that the genetic studies carried out so far share several limitations concerning the sampling scheme or the marker used (Alvarado Bremer et al., 2006). For example, Kasapidis et al. (2007) increased the sampling effort in the North Atlantic region and, by means of microsatellites, suggested a north–south reduction in gene flow within the Atlantic but they recommended further studies using a higher number of microsatellites. More recently, Smith & Alvarado Bremer (2010) applied different nuclear markers (SNPs and RFLPs of nuclear genes *CaM, ARP, Mlc2, ActA2*) to assess the extent of population admixture along the north–south boundary: the markers were promising but the low number of specimens assayed made the results preliminary.

The present study deals with this latter question, concerning the location and the extent of admixture of Northwestern and Southern Atlantic swordfish stocks. Management implications regarding this question are obvious since the Atlantic swordfish is subject to intense harvest and, to date, it is managed as two stocks that are regulated by quota assignments. Thus, unequivocal data supporting a sustainable management of the Atlantic swordfish are urgently needed as this is widely acknowledged as crucial for their conservation and management (Hilborn et al., 2003). To this end, the sampling effort in the Eastern Atlantic region and within the boundary location between the Atlantic populations were increased in order to give a more comprehensive analysis of the Atlantic swordfish stock structure trying to fill the gaps that so far exist. For this purpose, the highly polymorphic control region of the mtDNA (D–loop) was used to characterise the genetic diversity within and among swordfishes from different Atlantic areas and to examine their level of genetic divergence through: i) investigating the population structure of North and Central Atlantic swordfish; and ii) testing the heterogeneity among swordfish specimens collected from distinct geographic areas.

Material and methods

Sampling

Muscle tissue samples were collected from 274 swordfish specimens onboard commercial long line fishing vessels between May 2003 and November 2007 in five different areas throughout the Atlantic Ocean, namely: Northwest (NW, N = 39, July 2006), North Central (NC, N = 53, May 2003), Northeast (NE, N = 51, May 2003), Northern Tropical (TR, N = 48, June–August 2007) and Equatorial (EQ, N = 83, June–November 2007) (fig. 1). A small portion of muscle tissue was taken from each specimen using a sterilized scalpel, stored in an Eppendorf tube and kept frozen at –80ºC until assayed.

DNA extraction, PCR amplification and RFLP analysis

DNA extraction was based on the CTAB (Cetyltrimethyl Ammonium Bromide) method slightly adapted from Murray & Thompson's (1980) protocol. The dried DNA pellet was re–suspended in 100 µl of TE–buffer (EDTA 1 mM and tris 10 mM, pH 8). DNA was then diluted (1:50) with distilled water and so suitable for polymerase chain reaction (PCR).

The DNA amplification was achieved using the primers specifically designed for the control region of swordfish (Alvarado Bremer et al., 1995). The L–strand primer L15998 (5'–TAC CCC AAA CTC CCA AAG CTA–3') was used in combination with the H–strand primer H235 (5'–CGT GTG CAC TCT GAA ATG TCA–3') to amplify a DNA fragment composed by *c.* 525 bp using a Perkin Elmer thermal cycler apparatus (GeneAmp 2400). The PCR reaction was then cycled

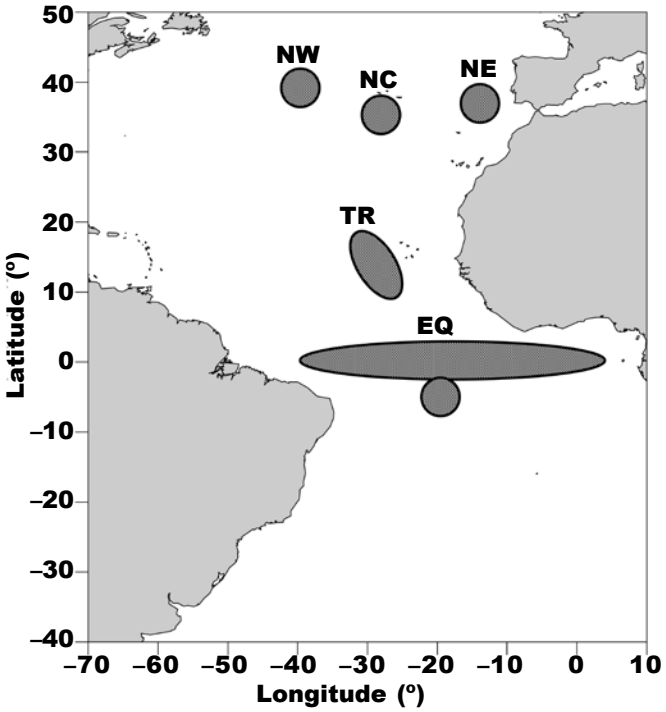

Fig. 1. Swordfish sampling areas in the Atlantic Ocean: Northwest (NW), North Central (NC), Northeast (NE), Northern Tropical (TR) and Equatorial (EQ).

Fig. 1. Áreas de muestreo del pez espada en el océano Atlántico: noroeste (NW), norte central (NC), nordeste (NE), norte tropical (TR) y ecuatorial (EQ).

for 4 min at 94ºC followed by 35 cycles of 1 sec. at 94ºC, 1 sec at 50ºC, 1 sec at 72ºC and the final cycle at 72ºC for 10 sec. Negative controls without DNA template were prepared in every series of amplification to exclude the possibility of contamination of reagents or reaction buffers.

Five restriction enzymes (*Alu I*, *Dra I*, *Vsp I*, *Hpa II* and *Dde I*) were used to digest the amplified fragments. Digests were performed in 7.5 µl final reaction volume. After centrifuging, the reaction mixture digestions were incubated for 3 h at 37ºC for all the enzymes used in the study. The resulting restricted fragments were analysed by electrophoresis on 3.0% agarose gel using TBE–buffer (0.045M tris–borate; 0.001M EDTA, pH 8), stained with 0.01% ethidium bromide and finally photographed. A molecular weight marker and a reference PCR product was run along with the digested PCR products to estimate the sizes of the resulting mtDNA fragments.

Restriction patterns generated from each restriction endonuclease were labelled with letters (A, B, C and D) indicating variant digestion patterns (table 1). Composite mtDNA haplotypes were constructed from all the enzymes used and arranged in the following order: *Alu I*, *Dra I*, *Vsp I*, *Hpa II* and *Dde I*. Thus, each fish was assigned a code of five letters that described its composition in terms of multi–enzyme haplotype.

Genetic data analysis

Chromatographic curves of forward and reverse sequences were edited in Chromas v.1.6. All sequences were then aligned by eye using Clustal X v.1.83 (Thompson et al., 1997). The amount of sequence divergence for each geographical population was assessed by estimating the number of polymorphic sites (S), haplotype diversity (*h*; Nei, 1987), nucleotide diversity (π, Nei, 1987) and average number of pairwise nucleotide differences (*k*; Tajima, 1983) all performed using Arlequin v.3.1 (Excoffier et al., 2005).

The level of genetic diversity within and among sampled areas was hierarchically evaluated by analysis of molecular variance (AMOVA, Excoffier et al., 1992). Significance of pairwise comparison was tested with 10,000 permutations. Samples were hierarchically divided into two groups (1: NW, NC, NE and TR; 2: EQ) to test the accuracy of the current management boundary set at 5º N (see fig. 1). Significance of the pairwise *p*–values achieved for the F_{ST} comparisons between the sampled areas was corrected applying a B–Y FDR method (Narum, 2006). As mentioned by Narum (2006), this method provides the most important critical value for evaluating significance of population differentiation in conservation genetics. Tajima (1989) *D*–test and Fu

Table 1. Restriction patterns of the mtDNA D–loop haplotypes recorded in 274 swordfishes from the Atlantic Ocean (values show restriction fragments length in bp): RE. Restriction enzymes.

Tabla 1. Patrones de restricción de los haplotipos del bucle D del ADN mitocondrial, registrados en 274 peces espada del océano Atlántico (los valores muestran la longitud de los fragmentos de restricción en pares de bases): RE. Enzimas de restricción.

		Haplotype				
RE	Recognition sequence	A	B	C	D	E
Alu I	AG–CT	525	273	177	94	241
			252	348	177	
					254	
Dra I	TTT–AAA	25	227	25	525	
		294	298	500		
		206				
Vsp I	AT–TAAT	112	230	111		
		291	295	414		
		122				
Hpa II	C–CGG	490	262	525		
		35	224			
			39			
Dde I	C–TNAG	275	192	525	274	
		250	81		155	
			252		96	

(1997) F_S–test were used to test the deviation from neutral molecular evolution in relation to mtDNA sequences. Significance was assessed in both tests by generating random samples (1,000 simulated samples) under the hypothesis of selective neutrality and population equilibrium. Both AMOVA and neutrality tests were performed with Arlequin v.3.1 (Excoffier et al., 2005).

Table 2. Genetic variability of Atlantic swordfish within the five sampled areas: SA. Sampling area; N. Sample size; H. Number of haplotypes; S. Number of polymorphic sites; *h*. Haplotype diversity; *k*. Mean pair–wise nucleotide differences; π. Nucleotide diversity.

Tabla 2. Variabilidad genética del pez espada del Atlántico dentro de las cinco zonas muestreadas: SA. Área de muestreo; N. Tamaño de la muestra; H. Número de haplotipos; S. Número de sitios polimórficos; h. *Diversidad del haplotipo;* k. *Media de las diferencias de los pares de nucleótidos;* π. *Diversidad nucleotídica.*

SA	N	H	S	*h* ± s.d.	*k* ± s.d.	π ± s.d.	Tajima's *D* test (*p*–value)	Fu's F_S test (*p*–value)
NW	39	21	58	0.852 ± 0.056	11.178 ± 5.187	0.021 ± 0.011	–0.742 (0.234)	–1.988 (0.244)
NC	53	19	63	0.755 ± 0.063	11.144 ± 5.142	0.021 ± 0.011	–0.702 (0.255)	0.700 (0.663)
NE	51	23	65	0.868 ± 0.043	10.534 ± 4.880	0.020 ± 0.010	–0.914 (0.195)	–1.877 (0.282)
TR	48	20	61	0.781 ± 0.060	8.843 ± 4.150	0.017 ± 0.009	–1.288 (0.068)	–1.456 (0.336)
EQ	83	39	77	0.935 ± 0.017	13.748 ± 6.236	0.026 ± 0.013	–0.394 (0.412)	–6.050 (0.090)

Table 3. Hierarchical AMOVA analysis on the Atlantic swordfish molecular data: Group 1 (NW, NC, NE and TR), and Group 2 (EQ): SV. Source of variation; VC. Variance components: P. Percentage of variation; Fi. Fixation indices. (* significant at 0.05 level).

Tabla 3. Análisis AMOVA jerárquico sobre los datos moleculares del pez espada del Atlántico: Grupo 1 (NW, NC, NE y TR) y Grupo 2 (EQ): SV. Fuente de variación; VC. Componentes de la Varianza; P. Porcentaje de variación; Fi. Índices de fijación (* significativo al nivel 0,05).

SV	VC	P	Fi
Among groups	0.012	2.95	F_{CT} = 0.030
Among areas			
Within groups	0.000	0.02	F_{SC} = 0.000
Within areas	0.424	97.03	F_{ST} = 0.030*

Table 4. Pairwise F_{ST} values among the five sampled swordfish areas in the Atlantic Ocean. F_{ST} values were calculated with 110 permutations (significant by B–Y FDR method). (For areas abbreviations, see figure 1)

Tabla 4. Valores F_{ST} *representados por parejas, entre las cinco áreas del océano Atlántico muestreadas para el estudio del pez espada. Los valores de* F_{ST} *se calcularon con 110 permutaciones (significativas mediante el método B–Y FDR). (Para las abreviaturas de las áreas, ver figura 1).*

	NW	NC	NE	TR	EQ
NW					
NC	–0.00329				
NE	–0.00213	0.00714			
TR	–0.00183	–0.00484	0.00938		
EQ	0.01689	0.04195*	0.01633	0.03336*	

The phylogenetic relationships among haplotypes were graphically arranged in Mega v.4 (Tamura et al., 2007) with unrooted neighbour–joining dendrogram using the gamma corrected Tamura–Nei distance matrix. The statistical robustness of neighbour–joining distances was determined by 1,000 bootstrap replicates (Felsenstein, 1985).

Results

Molecular attributes

A single fragment of approximately 525 bp was amplified from each specimen and no apparent size differences among them were observed. Restriction profiles obtained by each of the five endonucleases showed five patterns in *Alu I*, four in *Dra I*, three in *Vsp I*, three in *Hpa II* and four in *Dde I* (table 1). Most samples shared the most common restriction pattern for each endonuclease digestion, despite the difference in their frequencies. Pattern A in *Alu I* digestion was the most common in EQ, while pattern B prevailed in the remaining samples. Pattern A in *Dra I*, *Vsp I*, *Hpa II* and *Dde I* was the most frequent in all the sampled areas.

A total of 68 composite haplotypes were recorded but only one (BAAAA) was distributed across the five areas with relatively high frequency (NW = 38.5%; NC = 49.1%; NE = 35.3%; TR = 45.8%; EQ = 20.5%) and 38 were limited to one area, namely: 8 in NW, 2 in NC, 6 in NE, 7 in TR and 15 in EQ. However, it must be noted that a larger number of individuals were sampled in the Equatorial region, which could influence the observed results. A total of 84 polymorphic nucleotide sites were observed, of which 19 were singleton variable sites and 65 were parsimony informative. The substitution bias favoured transitions over transversions, with their ratio being 10.9. The overall relative nucleotide frequencies were: C = 20.7%, T = 31.2%, A = 31.7% and G = 16.5%.

Genetic variability was high for the pooled samples, displaying a value of 0.860 ± 0.019 for haplotype diversity (h) and 0.022 ± 0.011 for nucleotide diversity (π). The h ranged between 0.935 ± 0.017 in EQ and 0.755 ± 0.063 in NC, while π from 0.026 ± 0.013 (EQ) to 0.017 ± 0.009 (TR) (table 2).

Population structure and phylogeny

The overall hierarchical AMOVA showed that the greatest genetic differentiation (97.03%, p = 0.005) in the swordfish control region was found within distinct geographical areas, and a very small amount (0.02%, p = 0.354) was due to divergence among distinct geographical areas within groups (table 3). The amount of variance among groups was 2.95% and did not display statistically significant differences (p = 0.191). If a single AMOVA group was considered in the analysis, the results showed again that most variance was explained by differences within areas (98.10%, p = 0.001) rather than among them (1.90%). The highest pairwise F_{ST} values were found between EQ and NC (0.042, $p < 0.05$) and the lowest between TR and NW (–0.002, $p > 0.05$) (table 4). It is worth noting that statistically significant differences among pairwise F_{ST} values occurred between EQ *vs.* NC and EQ *vs.* TR (table 4).

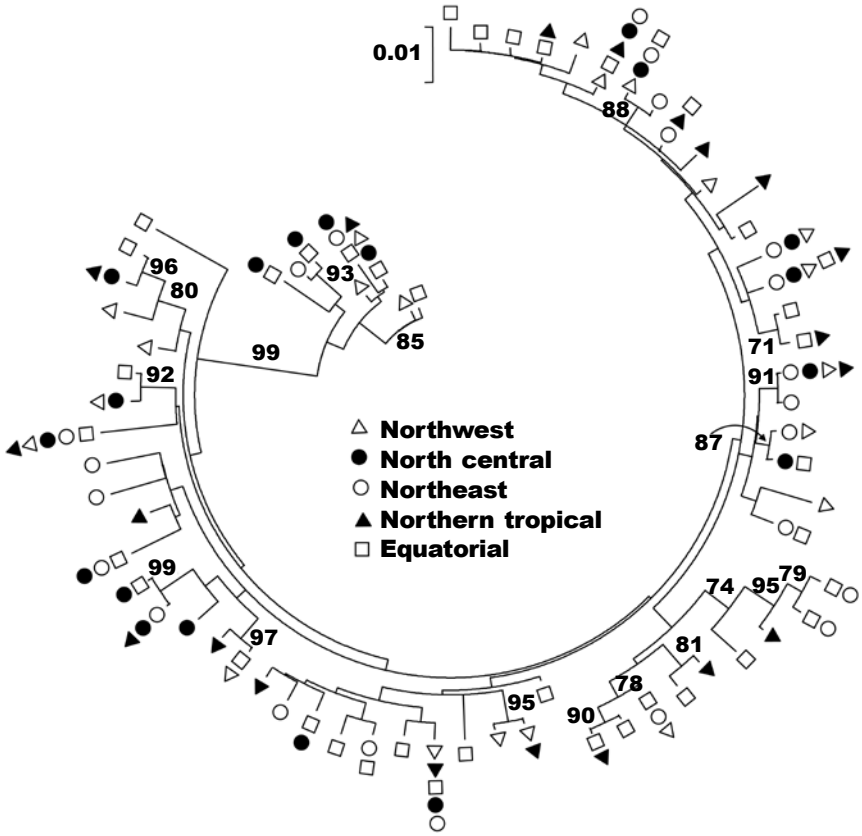

Fig. 2. Neighbor–joining tree of the 68 haplotypes from Atlantic swordfish. Bootstrap value support above 70% is shown by the branches. Symbols are related with the geographic origin of each haplotype.

Fig. 2. Árbol por agrupación de vecinos de los 68 haplotipos del pez espada del Atlántico. El soporte del valor bootstrap por encima del 70% se muestra mediante las ramas. Los símbolos están relacionados con el origen geográfico de cada haplotipo.

The indices of neutral evolution (Tajima's *D* and Fu's F_S tests) yielded moderately low negative values in the large part of the sampled areas, but failed to detect statistically significant differences (table 2).

The topology of the gene tree obtained using neighbour–joining analysis showed that there is no evident phylogeographic relationship, as they are randomly allocated across the tree diagram (fig. 2). No apparent latitudinal gradient of the proportion of each haplotype was observed, excepting the most common haplotype (BAAAA) that decreased in the Equatorial area. Indeed, statistically significant differences (χ^2–test: $p < 0.05$) were observed when comparing EQ with NC and TR areas. Moreover, only 19 branches received values higher than 70%.

Discussion

The mitochondrial control region sequence of swordfish showed moderately high levels of variation, with 16% of the nucleotide positions being polymorphic. However, the substitution bias ratio (r = 10.9) herein achieved for swordfish was higher than those recorded by Alvarado Bremer et al. (1997) in albacore (r = 9.0) mitochondrial genome. All the samples were characterized by high levels of haplotype diversity, with 56% of the haplotypes as private of a single area. Such a pattern has been previously reported for other scombrid species, such as albacore (Chow & Ushiama, 1995; Viñas et al., 2004). The nucleotide diversity found was within the range reported for Atlantic swordfish by Alvarado Bremer et al. (2005), but lower than the values found for other highly migratory species, such as albacore (Viñas et al., 2004) bigeye tuna (Martinez et al., 2006) and bluefin tuna (Carlsson et al., 2004). High genetic diversity within geographic areas and low genetic differentiation among areas within the same ocean basin are commonly observed in large pelagic marine fishes and could be explained by their wide distribution range that favour gene flow, and their large population sizes (Avise, 1998).

In the present study, no appreciable variance was attributable to variation among groups (north *versus* south, divided at 5º N), despite the significant heterogeneity found within the sampling areas. The lack of evident structure among the North Atlantic samples (NW, NC, NE and TR) was also confirmed by the moderately low F_{ST} statistics, which pointed out that the genetic exchange rate between them is sufficient to prevent genetic divergence. However, it is worth noting that F_{ST} values were significantly different when the Equatorial samples (EQ) were compared with NC and TR samples. This observation could reinforce the findings that the Equatorial area (between 5º N and 10º S) may represent a zone of intergradation within the Atlantic Ocean. Such results may also be supported by the finding that the higher levels of genetic divergence were recorded exactly within the Equatorial samples. However, these results must be interpreted with caution due to the higher sample size in the Equatorial area that could influence the higher number of haplotypes recovered there. Moreover, the reduced sampling effort in the South Atlantic area probably did not allow the detection of north–south stock differences. Therefore, the present results do not support a need for changing the current management boundary at 5º N.

The absence of an evident genetic differentiation among the NW, NC, NE and TR samples did not exclude the two–stock hypothesis (east to west) considered for managing the Atlantic swordfish fisheries (Miyake & Rey, 1989), as the sample coverage was limited to the East of 40ºW. Kasapidis et al. (2007) also failed to detect a east–west difference. However, as in the present study, the west area was limited to longitude 47º W. Moreover, for the same reason, the possible subdivision between NW Atlantic (west of 40º W) and South Atlantic swordfish could not be excluded. In fact, such a division was strongly supported by concordant results for both mtDNA and scnDNA data (Alvarado Bremer et al., 2005). Furthermore, the extent of mixing between Atlantic swordfish samples should cover a large area southern to the current boundary location, but the present study does not suggest evidence for stock mixing north of 5º N. Indeed, the results presented here did not reveal significant heterogeneity in mtDNA diversity between the samples collected more southern and northern than 30º N, as suggested by Chow & Takeyama (2000) analysing CaM locus. Kasapidis et al. (2007) also failed to detect genetic divergences among northern and mid–Atlantic areas.

At present, the main swordfish fisheries in the Atlantic Ocean are managed as two different units, North and South. The management unit definition recognizes genetically structured populations connected by limited gene flow. The results achieved with the present study did not provide evidence to change the current management boundary for the Atlantic swordfish fishery, as slight genetic structuring was observed only for the Equatorial area. Further examination of a larger number of samples from the Southern Atlantic is needed to confirm and possibly quantify the extent of genetic differentiation reported herein.

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