Genetic Comparison of Pacific and Mediterranean Swordfish, *Xiphias gladius*, by RFLP Analysis of the Mitochondrial D-loop Region

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ABSTRACT

The mitochondrial DNA D-loop region of swordfish, *Xiphias gladius*, was amplified by the polymerase chain reaction (PCR), and the amplified DNA fragments (1,950 base pairs) were subjected to restriction fragment length polymorphism (RFLP) analysis to investigate genetic differentiation among geographically distant samples. Four endonucleases (*Alu* I, *Dde* I, *Hha* I, and *Rsa* I) detected high levels of RFLP's in western (n=45) and eastern (n=35) North Pacific samples. Estimated diversity (h) was 59.9% and 56.1% (*Alu* I), 44.0% and 39.2% (*Dde* I), 42.1% and 21.4% (*Hha* I), and 60.4% and 64.0% (*Rsa* I) for western and eastern samples, respectively. In contrast, no RFLP was detected with *Hha* I and *Rsa* I in the Mediterranean sample (n=34). Frequency distributions of the restriction patterns in all endonuclease digestions were significantly different between the Mediterranean and Pacific samples, while no significant differences were observed between western and eastern Pacific samples. Much higher haplotypic diversity was observed in the Pacific (92.0%–94.4%) than in the Mediterranean (70.2%) samples, indicating that little genetic exchange has occurred between the two populations.

Introduction

Restriction fragment length polymorphism (RFLP) analysis of the whole mitochondrial DNA (mtDNA) molecule has been used to investigate genetic diversity within and between local samples of swordfish, *Xiphias gladius* (Grijalva-Chon et al. 1994; Katoulas et al., 1995). Katoulas et al. (1995) showed that genotype frequencies were significantly different in samples of swordfish from the Gulf of Guinea and from the eastern Atlantic (off Gibraltar) and Mediterranean (Greece, Italy, and Spain), suggesting the existence of genetically different stocks in the Atlantic. In contrast, no genetic heterogeneity was observed between swordfish samples from the eastern, central, and western North Pacific (Grijalva-Chon et al., 1994).

Recently, a direct nucleotide sequencing method based on the polymerase chain reaction (PCR) has been introduced and applied to further investigate genetic differentiation within and between swordfish populations (Finnerty and Block, 1992; Alvarado Bremer et al., 1996; Rosel and Block, 1996). In particular, Alvarado Bremer et al. (1996) and Rosel and Block (1996) reported that the left domain of the mtDNA D-loop region of the swordfish is hypervariable. They compared swordfish samples from the Atlantic, Mediterranean, and Pacific on the basis of sequence variation, and reported that mtDNA haplotype frequencies were significantly different.

This study reports on intraspecific RFLP in the D-loop region of swordfish mtDNA amplified by PCR. Genetic comparisons were made among two Pacific samples and one Mediterranean sample of swordfish.

Materials and Methods

All swordfish samples included both adults and juveniles. The western North Pacific sample (n=45) was caught by the Japanese commercial longline fleet and was collected at the landing site in Yaizu City, Japan, during November 1991–February 1992. Muscle dissected from the fresh fish was transferred on ice to the laboratory of the National Research Institute of Far Seas Fisheries (NRIFSF), where DNA extraction was carried out.

The eastern North Pacific swordfish sample (n=35) was caught by the Mexican commercial fleet using drift gillnets from November 1991 to February 1992. Crude DNA extracted from these fish was kindly provided by

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The Mediterranean sample (n=34) was collected by A. Di Natale of Aquastudio, in Italy, in 1994. A small piece of tissue dissected from frozen muscle was preserved in ethanol and transferred to the laboratory.

DNA extraction and PCR amplification procedures are described elsewhere (Chow and Inoue, 1993; Chow et al., 1993; Grijalva-Chon et al., 1994). Primer sequences for amplifying the mitochondrial D-loop region were from Palumbi et al.[1]; the nucleotide sequences were CB3R-L: 5'-CATATTAAACCCGAATGATATTT-3' and 12SAR-H: 5'-ATAGTGGGGTATCTAATCCCAGTT-3'. The PCR products were electrophoresed in 1% agarose gel to confirm amplification, whereafter the amplified samples were directly digested by the restriction endonucleases and electrophoresed through 2%–2.5% agarose gel (Biogel, Bio 101, La Jolla, CA[2]) followed by ethidium bromide staining and photography. Diversity (h) was calculated using the frequencies of restriction patterns and haplotypes (Nei, 1987). The G test of independence (Sokal and Rohlf, 1981) was employed to compare frequencies of restriction patterns and haplotypes between samples.

Table 1
Polymorphisms and genetic diversity, h, in mtDNA from western Pacific swordfish (n=45).

Endonuclease	Sequence at site	Variation[1]	No. individuals	h^2(%)
Alu I	AG˅CT	P	45	59.9
Bsa JI	C˅CNNGG	M	14	0.0
Bsl I	CCN_5˅N_2GG	M	22	0.0
Bst UI	CG˅CG	M	14	0.0
Dde I	C˅TNAG	P	45	44.0
Hae III	GG˅CC	M	22	0.0
Hha I	GCG˅C	P	45	42.1
Hinf I	G˅ANTC	M	22	0.0
Mbo I	˅GATC	M	14	0.0
Mse I	T˅TAA	P	18	9.1
Nla III	CATG˅	P	45	38.0
Rsa I	GT˅AC	P	45	60.4
Sau 96I	G˅GNCC	M	22	0.0
Scr FI	CC˅NGG	M	10	0.0
Taq I	T˅CGA	P	18	24.7

[1] P = polymorphic; M = monomorphic.
[2] After Nei (1987).

Results

RFLP Analysis

The western North Pacific sample was tried with 15 endonucleases, of which 7 detected polymorphisms (Table 1). Since 4 of the 7 (*Alu* I, *Dde* I, *Hha* I, and *Rsa* I) detected relatively high polymorphisms in this sample, these 4 endonucleases were used to analyze the other 2 samples. The electrophoretic profiles of all detected restriction patterns (alphabetically labeled) are shown in Figure 1. The observed number of restriction patterns was 4 in *Alu* I and *Dde* I, 3 in *Hha* I, and 6 in *Rsa* I digestions.

Comparisons between the Samples

Frequencies of the restriction patterns in each endonuclease digestion of swordfish mtDNA are shown in Table 2. In the *Alu* I digestions, pattern *A* was common and occurred at a similar frequency in all samples. Frequencies of patterns *B* and *C* were similar in western and eastern North Pacific samples, but the Mediterranean sample had a much lower frequency of *B* and a higher frequency of *C* than the Pacific samples. In the *Dde* I digestion, *C* was common in all samples; *A* was not observed in the Mediterranean sample; and *D* was much more frequent in the Mediterranean than in the Pacific sample. Similarly, for *Hha* I digestion the Mediterranean sample was monomorphic (h = 0), while a moderate level of variation was observed in the Pacific samples (h = 42.1% for the western and 21.4% for the eastern sample). The largest difference between the Pacific and Mediterranean samples was observed in the *Rsa* I digestion. The Mediterranean sample was monomorphic for this endonuclease digestion, as all individuals examined possessed pattern *C*. In contrast, the Pacific samples were highly polymorphic (h = 60.4% and 64.0% for the western and eastern samples, respectively).

A G test of independence indicated that the frequency distributions of the restriction patterns were significantly different between the Pacific and Mediterranean samples for all digestions (P<<0.05). In contrast, no significant difference was observed between the western and eastern North Pacific samples.

An analysis of the composite haplotypes derived from the restriction patterns of all 4 endonuclease digestions also demonstrated differences between the samples. All in all, 24 haplotypes were observed among 113 individuals (Table 3). Twenty and thirteen haplotypes were found in the western and eastern Pacific samples, respectively, while only five were observed in the Mediterranean sample.

[1] Palumbi, S., et. al. 1991. The simple fool's guide to PCR, version 2.0. Dep. of Zoology, Univ. Hawaii, Honolulu.

[2] Reference to trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

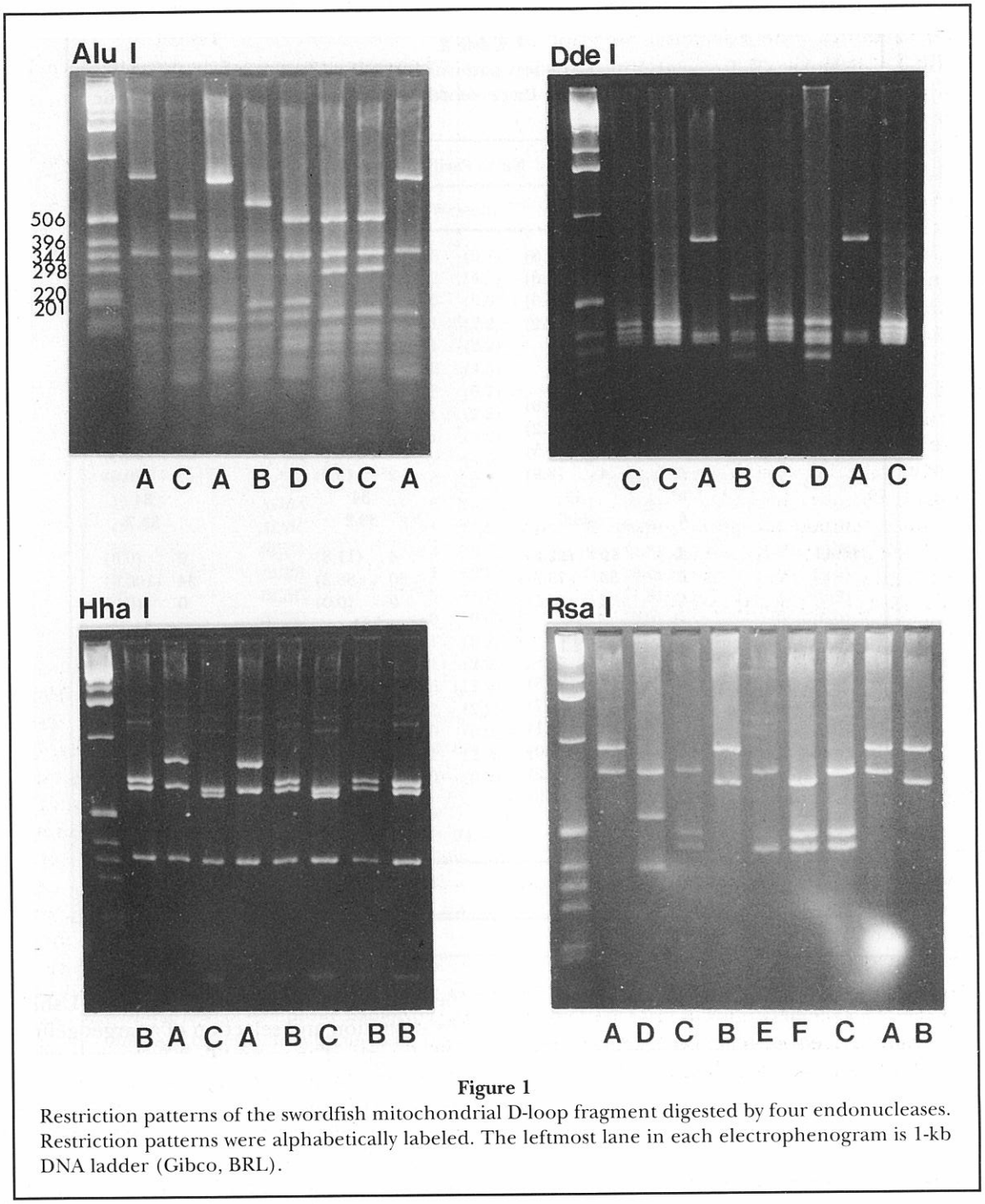

Figure 1
Restriction patterns of the swordfish mitochondrial D-loop fragment digested by four endonucleases. Restriction patterns were alphabetically labeled. The leftmost lane in each electrophenogram is 1-kb DNA ladder (Gibco, BRL).

Haplotypic diversity estimates (H; Nei, 1987) reflected the skewed number of haplotypes in the Mediterranean sample. Thus, the eastern and western Pacific samples had much higher diversity (94.4% and 92.0%, respectively) than the Mediterranean sample (70.2%).

This difference was amplified when only *Hha* I and *Rsa* I restriction fragment patterns were considered (Table 4). Eight and five haplotypes were found in the western and eastern Pacific samples, respectively, and only one (*BC*) was observed in the Mediterranean sample. The haplotypic diversities were 77.6% and 71.5% for the western and eastern Pacific samples, respectively, while that of the Mediterranean was 0. The difference in haplotype distribution between the Pacific and Mediterranean samples was highly significant ($P<<0.001$). These results indicate that there is little gene flow between the swordfish populations of the Pacific Ocean and the Mediterranean Sea.

Table 2

Number (% frequency) of restriction patterns in each of four restriction endonuclease digestions of mtDNA from three swordfish samples, and calculated genetic diversity, *h* (after Nei, 1987).

		North Pacific		
Endonuclease	Patterns	Western	Eastern	Mediterranean
Alu I	*A*	29 (57.8)	21 (60.0)	18 (53.0)
	B	9 (20.0)	10 (28.6)	1 (2.9)
	C	9 (20.0)	4 (11.4)	14 (41.2)
	D	1 (2.2)	0 (0.0)	1 (2.9)
	n[1]	45	35	34
	h	59.9	56.1	56.4
Dde I	*A*	7 (15.6)	6 (17.6)	0 (0.0)
	B	1 (2.2)	0 (0.0)	0 (0.0)
	C	33 (73.3)	26 (76.5)	27 (79.4)
	D	4 (8.9)	2 (5.9)	7 (20.6)
	n[1]	45	34	34
	h	44.0	39.2	33.7
Hha I	*A*	10 (22.2)	4 (11.8)	0 (0.0)
	B	33 (73.3)	30 (88.2)	34 (100.0)
	C	2 (4.5)	0 (0.0)	0 (0.0)
	n[1]	45	34	34
	h	42.1	21.4	0.0
Rsa I	*A*	17 (37.8)	15 (42.9)	0 (0.0)
	B	3 (6.7)	3 (8.5)	0 (0.0)
	C	23 (51.1)	15 (42.9)	34 (100.0)
	D	0 (0.0)	2 (5.7)	0 (0.0)
	E	1 (2.2)	0 (0.0)	0 (0.0)
	F	1 (2.2)	0 (0.0)	0 (0.0)
	n[1]	45	35	34
	h	60.4	64.0	0.0

[1] n = number of individuals examined.

Discussion

Evaluation of intraspecific haplotypic diversity via RFLP analysis is very sensitive to the numbers of endonucleases used and individuals sampled (Nei, 1987), and RFLP analyses appear to miss many nucleotide substitutions (Beckenbach, 1991). Therefore, comparisons of haplotypic diversity between species via RFLP analysis are much less meaningful than spatio-temporal comparison of values within species. Especially for genetic stock analysis, it is much more important to find a way to detect diagnostic variation.

PCR-RFLP analysis is well suited for analyzing large numbers of specimens because it is quite simple and less costly than conventional restriction analysis of mtDNA via Southern blotting or with direct nucleotide sequencing. This technique has been used to detect genetic polymorphisms in the mitochondrial ATPase gene of albacore, *Thunnus alalunga*, using a large number of individuals (n=620; Chow and Ushiama, 1995). Examination and selection of a target gene region and the length of the amplified fragments are critical for PCR-RFLP analysis (Chow and Inoue, 1993; Chow et al., 1993). Alvarado Bremer et al. (1995, 1996) demonstrated that the D-loop region of swordfish mtDNA is extremely polymorphic, using nucleotide sequence analysis. In the present study, RFLP analysis of the amplified swordfish D-loop region was able to detect a considerable amount of genetic variation. In addition, the haplotypic diversity obtained by using a limited number of endonucleases was higher than that obtained by restriction analysis of the entire mtDNA molecule (see Grijalva-Chon et al., 1994; Katoulas et al., 1995).

The present results coincide with those of previous studies. Samples from the Pacific were found to be genetically homogeneous (Grijalva-Chon et al., 1994) using RFLP analysis on the entire mtDNA molecule, whereas large differences in haplotypic distributions

Table 3

Number of composite haplotypes (% frequency) in mtDNA from three swordfish samples. Four columns of the haplotype represent four endonucleases: *Alu* I, *Dde* I, *Hha* I, and *Rsa* I, from left to right.

		North Pacific		
Clone	Haplotype	Western	Eastern	Mediterranean
1	*AAAA*	3 (6.7)	3 (8.8)	0 (0.0)
2	*AAAC*	2 (4.5)	0 (0.0)	0 (0.0)
3	*AABC*	0 (0.0)	3 (8.8)	0 (0.0)
4	*AABE*	1 (2.2)	0 (0.0)	0 (0.0)
5	*ABBA*	1 (2.2)	0 (0.0)	0 (0.0)
6	*ACAA*	2 (4.5)	1 (2.9)	0 (0.0)
7	*ACAC*	3 (6.7)	0 (0.0)	0 (0.0)
8	*ACAF*	1 (2.2)	0 (0.0)	0 (0.0)
9	*ACBA*	1 (2.2)	3 (8.8)	0 (0.0)
10	*ACBB*	2 (4.5)	2 (5.9)	0 (0.0)
11	*ACBC*	6 (13.3)	7 (20.6)	11 (32.4)
12	*ADBA*	3 (6.7)	1 (2.9)	0 (0.0)
13	*ADBC*	1 (2.2)	0 (0.00)	7 (20.6)
14	*BCBA*	3 (6.7)	4 (11.8)	0 (0.0)
15	*BCBB*	1 (2.2)	0 (0.0)	0 (0.0)
16	*BCBC*	5 (11.1)	4 (11.8)	1 (2.9)
17	*BCBD*	0 (0.0)	2 (5.9)	0 (0.0)
18	*CACA*	1 (2.2)	0 (0.0)	0 (0.0)
19	*CCBA*	1 (2.2)	2 (5.9)	0 (0.0)
20	*CCBC*	6 (13.3)	1 (2.9)	14 (41.2)
21	*CCCA*	1 (2.2)	0 (0.0)	0 (0.0)
22	*CDBB*	0 (0.0)	1 (2.9)	0 (0.0)
23	*DCBA*	1 (2.2)	0 (0.0)	0 (0.0)
24	*DCBC*	0 (0.0)	0 (0.0)	1 (2.9)
Total		45	34	34
H^1(%)		94.4	92.0	70.2

[1] Haplotypic diversity (Nei, 1987).

Table 4

Comparison of composite haplotype frequencies between three swordfish samples on the basis of restriction patterns in digestions by *Hha* I and *Rsa* I endonucleases.

		North Pacific		
Clone	Haplotype	Western	Eastern	Mediterranean
1	*AA*	5 (11.1)	4 (11.8)	0 (0.0)
2	*AC*	5 (11.1)	0 (0.0)	0 (0.0)
3	*BC*	18 (40.0)	15 (44.1)	34 (100)
4	*BE*	1 (2.2)	0 (0.0)	0 (0.0)
5	*BA*	10 (22.2)	10 (29.4)	0 (0.0)
6	*AF*	1 (2.2)	0 (0.0)	0 (0.0)
7	*BB*	3 (6.7)	3 (8.8)	0 (0.0)
8	*BD*	0 (0.0)	2 (5.9)	0 (0.0)
9	*CA*	2 (4.4)	0 (0.0)	0 (0.0)
Total		45	34	34
H^1(%)		77.6	71.5	0

[1] Haplotypic diversity (Nei, 1987).

were observed between Pacific and Mediterranean samples using nucleotide sequence analysis, as reported by Alvarado Bremer et al. (1996) and Rosel and Block (1996). Thus, regardless of the methods employed, mtDNA analysis may be quite powerful for the detection of genetic differentiation among geographically distinct populations. Further PCR-RFLP analysis is underway on swordfish samples from other locations in the Pacific as well as the North and South Atlantic and Indian Oceans. The results may be useful in clarifying the global population structure of this highly migratory and cosmopolitan fish.

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Literature Cited

Alvarado Bremer, J. R., A. J. Baker, and J. Mejuto.
1995. Mitochondrial DNA control region sequences indicate extensive mixing of swordfish (*Xiphias gladius*) populations in the Atlantic Ocean. Can. J. Fish. Aquat. Sci. 52:1,720–1,732.

Alvarado Bremer, J. R., J. Mejuto, T. W. Greig, and B. Ely.
1996. Global population structure of the swordfish (*Xiphias gladius* L.) as revealed by analysis of the mitochondrial DNA control region. J. Mar. Biol. Ecol. 197:295–310.

Beckenbach, A. T.
1991. Rapid mtDNA sequence analysis of fish populations using the polymerase chain reaction (PCR). Can. J. Fish. Aquat. Sci. 48:95–98.

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

Chow, S., and S. Inoue.
1993. Intra- and interspecific restriction fragment length polymorphism in mitochondrial genes of *Thunnus* tuna species. Bull. Far Seas Fish. Res. Lab. 30:207–225.

Chow, S., and H. Ushiama.
1995. Global population structure of albacore (*Thunnus alalunga*) inferred by RFLP analysis of the mitochondrial ATPase gene. Mar. Biol. 123:39–45.

Finnerty, J. R., and B. A. Block.
1992. Direct sequencing of mitochondrial DNA detects highly divergent haplotypes in blue marlin (*Makaira nigricans*). Mol. Mar. Biol. Biotech. 1:1–9.

Grijalva-Chon, J. M., K. Numachi, O. Sosa-Nishizaki, and J. de la Rosa-Velez.
1994. Mitochondrial DNA analysis of North Pacific swordfish (*Xiphias gladius*) population structure. Mar. Ecol. Prog. Ser. 115:15–19.

Kotoulas, G., A. Magoulas, N. Tsimenides, and E. Zouros.
1995. Marked mitochondrial DNA differences between Mediterranean and Atlantic populations of the swordfish, *Xiphias gladius*. Mol. Ecol. 4:473–481.

Nei, M.
1987. Molecular evolutionary genetics. Columbia Univ. Press, New York.

Rosel, P. E., and B. A. Block.
1996. Mitochondrial control region variability and global population structure in the swordfish, *Xiphias gladius*. Mar. Biol. 125:11–22.

Sokal, R. R., and F. J. Rohlf.
1981. Biometry. W. H. Freeman, New York.