

**PRELIMINARY RESULTS FROM GENETIC ANALYSES OF NUCLEAR MARKERS IN  
SWORDFISH, *XIPHIAS GLADIUS*, REVEAL CONCORDANCE  
WITH MITOCHONDRIAL DNA ANALYSES**

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**SUMMARY**

This paper reports preliminary findings on the patterns of variation of two nuclear markers in samples of swordfish (*Xiphias gladius*). Intron variation in the aldolase C (aldC) and lactate dehydrogenase A (ldhA) genes was examined to test for concordance with geographic partitioning patterns of swordfish mitochondrial DNA (mtDNA). The mtDNA data have shown a high degree of nucleotide diversity and haplotype heterogeneity in swordfish worldwide. Since nuclear markers reflect contributions from both males and females, these data are less susceptible to possible female specific demographic biases that may affect mtDNA data. For this study, samples from the northwest Atlantic Ocean, the Mediterranean Sea and the north Pacific Ocean were analyzed. Comparisons across years for two of these regions (Mediterranean and the north Pacific) and across months for the third (northwest Atlantic) revealed no significant differences in allele frequencies for either aldC or ldhA, suggesting regional marker stability. Therefore, temporal samples were pooled per region for pairwise comparisons to test for genetic homogeneity. Among the three sampling regions, a high degree of heterogeneity ( $P < 0.01$ ) was observed for both loci. These data are concordant with mtDNA data for these regions and provide additional support that swordfish from these three areas are genetically distinct.

**RÉSUMÉ**

Le présent travail fait état des résultats préliminaires sur les modes de la variation de deux marqueurs nucléiques dans des échantillons d'espadon, *Xiphias gladius*. La variation des introns dans les gènes de l'aldolase (ALDC) et de la déshydrogénase lactate A (ldhA) a été examinée pour tester sa concordance avec les modes géographiques de division de l'ADN mitochondrial (ADNmt) de l'espadon. Les données sur l'ADNmt montraient un fort degré de diversité des nucléotides et d'hétérogénéité des haplotypes chez l'espadon à l'échelle globale. Du fait que les marqueurs nucléiques reflètent la contribution des mâles comme des femelles, ces données sont moins sensibles aux biais démographiques spécifiques des femelles susceptibles d'affecter les données sur l'ADNmt. Pour les besoins de cette étude, on a analysé des échantillons de l'Atlantique Nord-Ouest, de la Méditerranée et du Pacifique Nord. La comparaison entre années de deux de ces régions (Méditerranée et Pacifique Nord) et entre mois pour la troisième (Atlantique Nord-Ouest) n'a pas révélé de différences significatives de la fréquence des allèles dans l'aldC, ni dans la ldhA, ce qui suggère une stabilité des marqueurs à l'échelle régionale. Les échantillons temporels ont donc été regroupés par région en vue de la comparaison par paires pour tester l'homogénéité génétique. Un fort degré d'hétérogénéité ( $P < 0.01$ ) a été observé dans les deux loci entre les trois zones d'échantillonnage. Ces données concordent avec les données sur l'ADNmt pour ces régions, et fournissent une preuve de plus de la distinction génétique des espadons de ces trois zones.

**RESUMEN**

El documento informa sobre los hallazgos preliminares de los esquemas de variación de dos marcadores nucleares en las muestras de pez espada, *Xiphias gladius*. Se examinaron los genes de las variaciones en la aldolasa C (aldC) y lactasa dehidrogenasa A (ldhA) para comprobar la concordancia con los esquemas de partición geográfica del ADN mitocondrial (ADNmt) del pez espada. Los datos del ADNmt han mostrado un alto grado de diversidad nucleotídica y heterogeneidad de los haplotipos en el pez espada a nivel mundial. Dado que los marcadores nucleares reflejan las contribuciones de machos y hembras, estos datos son menos susceptibles de producir posibles sesgos demográficos específicos de las hembras que podrían afectar a los datos de ADNmt. Para este estudio, se analizaron muestras del océano Atlántico NW, mar Mediterráneo y Pacífico norte. Las comparaciones realizadas entre estas dos regiones (mediterránea y Pacífico norte) a lo largo de los años y a lo largo de los meses para la tercera (Atlántico NW) no revelaron diferencias significativas en las frecuencias de alelos para aldC ó ldhA, sugiriendo estabilidad en el marcador regional. Por tanto, se agruparon por regiones las muestras temporales para realizar comparaciones en cuanto a parejas para testar la homogeneidad genética. Entre las tres regiones de muestreo, se observó un alto grado de heterogeneidad ( $P < 0.01$ ) para ambos loci. Estos datos concuerdan con los datos de ADNmt para estas regiones, y adicionalmente respaldan que los peces espada de estas tres áreas son genéticamente distintos.

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## 1. INTRODUCTION

The genetic analysis of swordfish has been given a great deal of attention and has revealed a significant amount of genetic differentiation on both an inter- and intra-ocean basin level (Alvarado Bremer, 1992; Magoulas et al., 1993; Alvarado Bremer et al., 1995; 1996; Kotoulas et al., 1995; Rosel and Block, 1996; Chow et al., 1997). All of these studies have focused on the analysis of the mitochondrial genome, and a variety of techniques have been employed. Although the value of mtDNA in identifying population subdivision has been well documented (Avise, 1987; Avise et al., 1987; Ovenden, 1990), it has some potential limitations. For example, analyses based solely on mtDNA data only reflect the history of maternal lineages (Wilson et al., 1985; Avise et al., 1987). Differences in mtDNA haplotype frequencies could also reflect sex specific dispersal or migration patterns (Karl and Avise, 1992; Karl et al., 1992). Furthermore, the non-recombining nature of the mtDNA genome causes it to behave as a single genetic locus (Wilson et al., 1985). The use of a single locus potentially reduces the power to detect significant genetic differentiation (Palumbi and Baker, 1994). Therefore, the most informative analyses of population structure combine multiple loci to test for concordance of phylogenetic patterns across the geographic distribution of taxonomic units (Karl and Avise, 1992; Karl et al., 1992). In this study, we examined nuclear DNA introns at two loci, lactate dehydrogenase A (*ldhA*) and aldolase C (*aldC*), to test for concordant patterns of heterogeneity with published swordfish mtDNA data.

Since nuclear introns have a slower rate of mutation than mtDNA and four times the effective population size (Wilson et al., 1985), we decided to test the utility of these markers on samples taken from areas with the greatest geographic distance. Thus, samples were analyzed from the North Atlantic Ocean and the North Pacific Ocean. Samples from the Mediterranean Sea were also included because of the unique genetic composition of their mtDNA (Magoulas et al., 1993; Alvarado Bremer et al., 1995, 1996; Kotoulas et al., 1995; Rosel and Block, 1996; Chow et al., 1997).

## 2. METHODS

Tissue was obtained from commercial fishing vessels capturing swordfish with longlines. Tissue (heart or gonad) was either kept frozen or preserved in 70% alcohol (ethanol or isopropanol). To isolate DNA, approximately 0.05 g of swordfish tissue was placed in a 1.5 ul microfuge tube containing: 200 ul TENS solution (50mM Tris-HCl [pH 8.0], 100mM EDTA, 100 mM NaCl, 1% SDS) and 20 ul Proteinase K (10 mg/ml). Tubes were incubated overnight in a hybridization oven at 55°C with gentle rotation. After incubation, 20 ul of 5 M NaCl was added to the microfuge tubes, and the digested samples were centrifuged at full speed for 5-10 min. Sample supernatant was transferred to a second microfuge tube and mixed with two volumes 100% cold ethanol, and centrifuged at full speed for 10 min to precipitate DNA. The resulting DNA pellets were washed with 300 ul of cold 70% ethanol, and allowed to air dry. DNA was resuspended in 100 ul of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The quality of the extracted DNA was examined by electrophoresis of 3 ul of resuspended DNA through 1% agarose gels at 100 V for 30 min and visualized with ethidium bromide (EtBr).

Polymerase chain reactions (PCR) were performed using universal primers designed to amplify intron 6 in *ldhA* (Quattro and Jones, in press). Amplification of the *ldhA* locus required two PCR reactions substituting an internal forward primer in the second reaction (Quattro and Jones, in press). This internal primer was redesigned using swordfish nucleotide sequence information to allow amplification of this locus in a single PCR reaction (T.W. Greig, unpublished). To amplify the *aldC* intron, primers designed to target intron G were used (Lessa and Applebaum, 1993). All reactions contained approximately 20 ng of genomic DNA in a 50 ul reaction mixture containing 60 mM Tris-HCl, pH 9.0; 2 mM MgCl<sub>2</sub>; 0.2 uM of the indicated oligonucleotide primers each; 200 mM of each dNTP and 2.0 units of Ampli-taq DNA polymerase (Perkin Elmer Corp. Foster City, CA). PCR amplification conditions for all primer combinations and both markers were as follows: initial denaturation for 4 min at 94 °C followed by 35 cycles (denaturation, 1 min at 93 °C, annealing, 45 sec at 52 °C; extension, 45 sec at 72 °C) and a final extension for 5 min at 72 °C. PCR reaction products were electrophoresed through 1% agarose gels at 100 V for 30 min and visualized with EtBr to check for amplification quality and contamination.

### 2.1 Nuclear marker screening

#### 2.1.1. Aldolase C (*aldC*)

Sequence analysis for the *aldC* locus revealed an imperfect polymorphic CA repeat contained within the amplified fragment. DNA from six individuals identified as heterozygous for this repeat was cloned and five clones from each reaction were sequenced to confirm that the observed size polymorphisms were contained within the imperfect repeat

region and not due to mutations elsewhere on the fragment. All observed size polymorphisms for the *aldC* locus were due to variation in the imperfect repeat. Therefore, in all subsequent analyses, amplified samples were run on 25 cm, 6% polyacrylamide gels at 25 V for 14 h and stained with EtBr so that size variation could be observed.

### 2.1.2. Lactate dehydrogenase A (*ldhA*)

The initial screening of 100 base pairs (bp) revealed four polymorphic sites that defined six alleles. Therefore, a nucleotide sequence analysis was performed on all amplified samples. To aid in the direct sequencing of PCR products, one primer was biotinylated (Salminen, 1992). Sequencing reactions were performed using a modified protocol (Alvarado Bremer et al., 1996) on 7  $\mu$ l of purified single stranded PCR products (Salminen, 1992). DNA fragments were separated on 40 cm, 5% Long Ranger (AT Biochem, Malvern, PA, USA), 7 M urea gels. Electrophoresis was done at 65 W of constant power for approximately 1.75 h. Gels were dried onto filter paper and the labeled DNA fragments were visualized with Kodak Biomax MR film.

## 2.2. Statistical Analysis

Genotypic frequencies for both loci, from each region were tested for deviations from Castle-Hardy-Weinberg (CHW) expectations with chi-square tests (Sokal and Rohlf, 1981). Temporal or among region heterogeneity in allelic frequencies were tested by the G-test statistic (Sokal and Rohlf, 1981). A significance level  $P < 0.05$  was used to reject the null hypothesis of temporal homogeneity. To more closely examine these differences, we analyzed these data through a Monte Carlo chi-square randomization as described by Roff and Bentzen (1989). A conservative standard Bonferroni correction for multiple tests was used to adjust the  $\alpha$  level (original  $\alpha=0.05$ ; corrected  $\alpha=0.016$ ). The computer programs REAP (McElory et al., 1992) and "Genes in Populations" (designed by B. May and C. Krueger and written by W. Eng, Cornell University) were used for genetic data analyses.

## 3. RESULTS

Sample sizes and approximate date and location of capture are presented in Table 1. Five size variants in a CA repeat identified within the *aldC* intron were used to detect genetic variation (Table 2, Fig. 1). Sequence analysis of the *ldhA* intron 6 revealed four polymorphic sites yielding six alleles (Table 2, Fig. 2). All samples conformed to CHW equilibrium for both loci ( $P > 0.05$ ). No significant differences in allele frequencies were detected for comparisons across years (North Pacific Ocean,  $P > 0.05$ ; Mediterranean Sea,  $P > 0.05$ ) or months (northwest Atlantic Ocean,  $P > 0.05$ ) within sampling regions for the *aldC* or *ldhA* introns. Therefore, regional samples were pooled. Allele frequency estimates for pooled regional samples and G-test statistics for the *aldC* and *ldhA* introns are presented in Table 2. Significant differences in pooled regional allele frequencies were detected for both the *aldC* and *ldhA* introns (Table 2). Monte Carlo randomization p-values and chi square values for *aldC* and *ldhA* are presented in Tables 3 and 4, respectively. For both loci, all pairwise comparisons between the northwest Atlantic Ocean, the Mediterranean Sea and the North Pacific Ocean were highly significant ( $P < 0.01$ ).

## 4. DISCUSSION

Significant differences in allele frequencies for a polymorphic imperfect AC repeat were observed in an intron of the aldolase C gene in swordfish samples collected from the northwest Atlantic Ocean, the Mediterranean Sea and the North Pacific Ocean (Table 2). Similarly, comparisons of allele frequencies determined by sequence analysis of the lactate dehydrogenase A intron 6 were also significant. No departures from CHW expectations were observed for individual or pooled samples for either the *aldC* or the *ldhA* data. Furthermore, within region comparisons across different collection years or across months revealed no significant differences for either locus. Taken together, these data provide strong evidence for the temporal stability of these markers and indicate that samples collected for the northwest Atlantic Ocean, the Mediterranean Sea and the North Pacific Ocean are displaying geographic heterogeneity.

Analyses of mtDNA from swordfish collected within these three areas also have shown among region differentiation (Alvarado Bremer et al., 1995, 1996; Rosel and Block, 1996; Chow et al., 1997), that remains stable over time (Alvarado Bremer et al., SCRS/98/128). Taken together, the results for the mtDNA analyses are concordant with our nuclear data suggesting that swordfish sampled from these areas have unique genetic signals with regard to the allele frequency differences observed.

This study demonstrates the power of analyzing nuclear introns to study the genetic population structure of swordfish. Significant genetic heterogeneity was observed among samples from the northwest Atlantic Ocean, the Mediterranean Sea and the north Pacific Ocean. The observed concordance in regional heterogeneity for both the nuclear and the mtDNA data make a strong argument 1) for the utility of these markers to examine genetic differentiation and 2) that the heterogeneity observed with mtDNA is valid and not the result of sample bias relative to mtDNA inheritance. It is likely that further studies using these or similar nuclear markers (e.g. Chow and Takeyama, 1998) will provide further insight into the genetic structure of swordfish and other pelagic fish populations. Additional studies are in progress to examine intra-ocean genetic differentiation of swordfish samples using these markers.

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**Table 1. Approximate samplings dates(month/year), locales. Sample sizes are also given.**

Location	Sampling Date	Lat/Long	Sample size
Mediterranean Sea	11/92	37° N 17° W	38
	02/94	37° N 17° W	42
North Pacific Ocean	04/92	26° N 157° W	34
	12/93	33° N 157° W	32
Northwest Atlantic	07/90	40° N 68° W	99
	10/90	40° N 68° W	74

**Table 2. Pooled sample allele frequencies per basin for *aldC* and *ldhA* introns.**

Locus		North Pacific Ocean	Mediterranean Sea	Northwest Atlantic Ocean
Allele				
<i>aldC</i>	1	0.073	0.000	0.039
	2	0.618	0.685	0.890
	3	0.164	0.110	0.018
	4	0.136	0.205	0.038
	5	0.009	0.000	0.015
	n	55	73	169
<i>ldhA</i>	1	0.395	0.527	0.561
	2	0.088	0.020	0.031
	3	0.482	0.433	0.299
	4	0.000	0.000	0.076
	6	0.000	0.020	0.009
	7	0.035	0.000	0.024
	n	57	75	173
	G	df		
<i>aldC</i>	90.092*	10		
<i>ldhA</i>	65.594*	12		

\* significant at  $P < 0.05$

**Table 3. Pairwise comparisons of *aldC* allele frequencies ( $P$ -value below the diagonal, chi square above).**

	NWA1990	Med92&93	N. Pacific
NWA1990	-	63.91	55.45
Med92&93	<0.001*	-	15.46
N. Pacific	<0.001*	0.002*	-

\* denotes significance after Bonferroni correction for multiple comparisons

Abbreviations: NWA1990 = northwest Atlantic Ocean; Med92&93 =Mediterranean Sea;  
N. Pacific = north Pacific Ocean.

**Table 4. Pairwise comparisons of *ldhA* allele frequencies ( $P$ -value below the diagonal, chi square above).**

	NWA1990	Med92&93	N. Pacific
NWA1990	-	22.56	30.68
Med92&93	0.001*	-	16.32
N. Pacific	<0.001*	0.0012*	-

\* denotes significance after Bonferroni correction for multiple comparisons

Abbreviations: NWA1990 = northwest Atlantic Ocean; Med92&93 = Mediterranean Sea;  
N. Pacific= North Pacific Ocean.

Allele 2 AAAATAAAAACAAACCAACACACACACACACACACCTGTTG  
 Allele 3 AAAATAAAAACAAACCAACACACAAACACA----CCTGTTG  
 Allele 4 AAAATAAAAACAAA-----CACACACACACCTGTTG  
 Allele 5 AAAATAAAAACAAGCCAACACACAAAC-----CTGTTG

Figure 1. Partial sequence alignment of four *aldC* alleles.

Allele 1 gctgatattcatccttgttgattagtttacaaaacataactgtacattctaaagat  
 Allele 2 gctgatattcatccttgttgattagtttacaaaacataatgtacattctaaagat  
 Allele 3 gctgatattcatccttgttgattagtttacaaaacataactgtacattctaaagat  
 Allele 4 gctgagattcatccttgttgattagtttacaaaacataactgtacattctaaagat  
 Allele 6 gctgatattcatcatgttgattagtttacaaaacataactgtacattctaaagat  
 Allele 7 gctgatattcatccttgttgattagtttacaaaacataactgtacattctagagat

Figure 2. Partial sequence alignment of six *ldhA* alleles (\* donotes polymorphic sites).