EVIDENCE OF SPATIAL GENETIC HETEROGENEITY IN PACIFIC SWORDFISH (*XIPHIAS GLADIUS*) REVEALED BY THE ANALYSIS OF *LDH*-A SEQUENCES

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ABSTRACT

DNA sequence polymorphisms contained in intron 6 of the lactate dehydrogenase-A (*ldh*-A) gene were used to examine the genetic population structure of Pacific swordfish (*Xiphias gladius* Linnaeus, 1758). Seven alleles defined by five polymorphic sites were identified among 305 swordfish. Comparisons of allele frequency were conducted for 11 samples, including Chile (multiple years), Ecuador (multiple years), Mexico, Hawaii (multiple years), eastern Australia, and western Australia. Although there was evidence of genic differentiation, global differentiation was low (F_{st} = 0.001). To increase the power of the tests of differentiation, samples within each region were pooled into four regional samples. No deviations from Hardy-Weinberg equilibrium were observed, and the global fixation index increased more than tenfold (F_{st} = 0.013). Global exact tests of genic and genotypic differentiation were significant, and so were the pair-wise comparisons between the south-eastern Pacific Ocean (SEPO) sample from Chile, and all other regions. In addition, the north-eastern Pacific Ocean (NEPO; Ecuador to Mexico) was different from the north-central Pacific Ocean (NCPO; Hawaii), which in turn was different from the south-western Pacific Ocean (SWPO; pooled eastern and western Australia). These results may have important implications for the fishery management of Pacific swordfish, particularly because of the heterogeneity observed between SEPO and NEPO.

Studies of the genetic structure of swordfish populations using mitochondrial DNA (mtDNA) and single copy nuclear DNA (scnDNA) markers have indicated significant inter-oceanic differentiation of Atlantic, Indo-Pacific, and Mediterranean populations (Alvarado Bremer et al., 1995, 1996, 2005a; Kotoulas et al., 1995; Rosel and Block, 1996; Chow et al., 1997; Chow and Takeyama, 2000; Greig, 2000; Nohara et al., 2003). Within the Atlantic Ocean, independent research using mtDNA and scnDNA markers have also shown differences between NW Atlantic and South Atlantic populations (Alvarado Bremer et al., 1996; Greig et al., 1999; Chow and Takeyama, 2000; Nohara et al., 2003; Alvarado Bremer et al., 2005b). Genetic results support evidence from fisheries data used by ICCAT to separate swordfish resources into north and south Atlantic stocks for fisheries management.

Such general agreement has not been reported previously for swordfish resources in the Pacific Ocean, where fisheries managers have provided evidence for various numbers of stocks (e.g., Sakagawa and Bell, 1980–3 stocks; Bartoo and Coan, 1989–1 or 3 stocks). Furthermore, recent analyses of more detailed data for the eastern Pacific Ocean (EPO; east of 150°W) (Hinton and Deriso 1998; Hinton 2003) identified the presence of two stocks, a north and south, just within this region. Unfortunately, the results from previous genetic studies on Pacific swordfish have failed to supply comprehensive understanding of swordfish population structure. For example, Grijalva-Chon et al. (1994) using restriction fragment length polymorphism (RFLP) of the entire mtDNA molecule, found no differences between an EPO sample from waters off Baja California, Mexico, and a north-central Pacific Ocean (NCPO) sample from Hawaii. However, a subsequent allozyme study (Grijalva-Chon et al., 1996) revealed

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significant allele frequency differences between these two regions at three allozyme loci. The lack of concordance between the nuclear DNA (nDNA) and the mtDNA data may reflect differences in the mode of inheritance or demographic factors affecting these genomes (Grijalva-Chon et al., 1996). Two subsequent PCR-RFLP studies with a comprehensive sampling coverage of the Pacific Ocean did not help resolve this conflict. Both the analysis of mtDNA control region (Chow et al., 1997) and the analysis of calmodulin gene intron 4 (*CaM*) (Chow and Takeyama, 2000) revealed no genetic heterogeneity in the Pacific. However, Reeb et al. (2000) reported very shallow but significant differentiation with mtDNA control region sequence data on a large geographical scale in the Pacific that could not be detected with RFLP data or using smaller sample sizes and coverage (c.f., Rosel and Block, 1996).

Attempting to resolve these conflicting findings, a preliminary study of swordfish within the Pacific using both mtDNA control region I (CR-I) sequence data and nDNA data from samples collected in the north-central Pacific Ocean (NCPO; Hawaii), the eastern Pacific ocean (EPO; Ecuador and Mexico), and the south-western Pacific (SWPO: Australia) was conducted (J. R. Alvarado Bremer, Texas A&M University, unpubl. data). Three nuclear loci were examined (1) Beta tubulin, (2) aldolase-B (*ald*-B), and (3) lactate dehydrogenase-A (*ldh*-A) intron 6 (Greig, 2000; and references therein), but only *ldh*-A was polymorphic in the Pacific. Although insufficient, these preliminary results of *ldh*-A together with CR-I data offered some insight to resolve the genetic population structure of Pacific swordfish. First, the results of CR-I were in general agreement with those of Reeb et al. (2000) regarding the differentiation of the NCPO and the SWPO, but also in failing to reveal differences among the samples collected in Ecuador, Mexico, and Hawaii. Thus, the lack of differentiation between Mexico and the NCPO is in partial agreement with the mitochondrial study of Grijalva-Chon et al. (1994). However, the allele frequency of the *ldh*-A locus suggested differentiation among Pacific regions, since it was noted that a single allele (allele 5) was absent from the NCPO samples, but was present in the EPO and the SWPO samples at a frequency ranging between 4%–8%.

This study builds on preliminary analyses to test the null hypothesis of panmixia in Pacific swordfish by comparing the allele frequencies of the *ldh*-A locus throughout the Pacific Ocean both temporally and on a regional scale using larger sample sizes.

Materials and Methods

Sample Collection.—Axial muscle tissues were obtained from 305 adult swordfish landed by the commercial longline fishery corresponding to 11 samples from the Pacific Ocean and from one sample from the eastern Indian Ocean (Table 1). The Pacific samples correspond to five regions, (1) NCPO (Hawaii-multiple years), (2) NEPO (Mexico and Ecuador-multiple years), (3) SEPO (Chile–multiple years), and (4) SWPO (eastern Australia). Tissue samples were preserved in 70% ethanol and kept at room temperature until assayed in the laboratory.

DNA Extraction, PCR Amplification, Sequencing, and Allele Scoring.—DNA extraction, amplification, and sequencing were carried as described by Greig (2000). The computer program FACTURA (Applied Biosystems, Foster City, California) was used to conduct a preliminary identification of heterozygote individuals directly from the nucleotide sequence data. Nucleotide sequences were aligned in

Table 1. Details of Pacific swordfish samples assayed in this study. Abbreviations: NCPO: central North Pacific Ocean; NEPO: north-eastern Pacific; SCPO: central South Pacific; SEPO: south-eastern Pacific; SWPO: south-western Pacific, and EIO: eastern Indian Ocean.

Sample name	Region	Dates sampled	Number	Location
HAW92	NCPO	4/26/1992 to 5/04/1992	28	24°N, 157°W, Hawaii
HAW93	NCPO	11/27/1993 to 12/04/1993	29	33°N, 157–159°W, Hawaii
HAW98	NCPO	5/27/1998 to 6/19/1998	20	27–29°N, 172–174°W, Hawaii
HAW99	NCPO	2/10/1999 to 4/19/1999	48	29–30°N, 168–172°W, Hawaii
ECU9798	NEPO	4/15/1997 to 3/23/1998	34	1°S, 81°W, Ecuador
ECU94	NEPO	1994	25	0–10°S, 91°W, Ecuador
MEX97	NEPO	7/14/1997 to 8/23/1997	19	26°N, 116°W, Mexico
CHL97	SEPO	12/15/1997	28	26–31°S, 80–81°W, Chile
CHL99	SEPO	1999	29	26–31°S, 80–81°W, Chile
EAUS95	SWPO	2/11/1995 to 2/27/1995	13	Off eastern Australian coast
WAUS95	EIO	7/3/1995 to 7/17/1995	32	Off western Australian coast
Total			305	

BioEdit (Hall, 1999) and the polymorphic sites identified by FACTURA were re-inspected directly in the electropherograms. Ambiguities were resolved by sequencing the PCR product in both directions. Swordfish *ldh*-A alleles (Table 2) were identified using the system described by Greig (2000) as expanded by Marques (2001). Two new alleles were discovered in this study.

Population Genetics Analysis.—Descriptive statistics of population genetic variability, including allele frequencies, number of alleles per locus, private alleles, observed heterozygosity (H_O), expected heterozygosity (H_E), and conformity to Hardy-Weinberg equilibrium (HWE), were estimated with GENEPOP (Ver. 3.1, Raymond and Rousset, 1995a). Exact HWE probability tests were conducted using the Markov Chain method (Guo and Thompson, 1993). The alternative test of heterozygote deficiency (Rousset and Raymond, 1995) was also conducted. Exact tests for genic (Goudet et al., 1996) and genotypic (Raymond and Rousset, 1995b) differentiation among-populations were performed. These two tests estimate the P-value of a G-based exact test, and thus are in principle equivalent to the Fisher exact probability test. The lengths of Markov chains employed in differentiation and HWE tests were obtained with 100 batches and 1000 iterations, and using a dememorization

Table 2. System of *ldh*-A intron-6 alleles in swordfish. Seven alleles (1–3, 5, 7, and 14–15) were found in Pacific swordfish. The nucleotide positions of sites polymorphisms along the segment sequenced correspond to the number of nucleotides towards the 5′-end (negative) or the 3′-end (positive) away from the reference polymorphic site (*), as described in Figure 1.

	Nucleotide position				
Allele	–51	–8	*	+24	+36
1	G	T	T	C	A
2	.	.	C	A	.
3	.	.	C	.	.
4	.	G	.	.	.
5	A	.	C	.	.
6	.	.	A	.	.
7	.	.	.	.	G
14	.	.	C	.	G
15	.	.	C	.	T

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      -51                              - 31                              -8
CCCTGGGAGCAAGCCCTGAACTTCAATDTTGGGCAGATCCTAGCTGCTGAKA

        *                                +24              +36
TTCATCHTGTTGATTAGTTTACAAAACATAMTGTACATTCTAD
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Figure 1. Nucleotide sequence of the *ldh*-A intron-6 locus of Pacific swordfish. Polymorphic sites are identified with the corresponding IUB code (see Table 2). The position of each polymorphic site along the 97base pair (bp) stretch of sequence is identified with the number of nucleotides towards the 5-end (negative) or 3-end (positive) relative to the reference polymorphic site identified with an asterisk (*).

value of 1000. When multiple tests were conducted, the levels of significance were adjusted with the sequential Bonferroni technique (Rice, 1989). The effective number of migrants (N_m) among populations was estimated using the private alleles method (Slatkin, 1985; Barton and Slatkin, 1986) as implemented in GENEPOP.

Results

Sample Diversity and HWE Equilibrium Tests.—Nucleotide sequence analysis of 97 base pairs (bp) of the *ldh*-A intron-6 revealed the presence of 7 alleles (1–3, 5, 7, and 14–15) among the 305 Pacific swordfish surveyed, for a total of 607 alleles scored (Figure 1; Table 2). Alleles 1–7 were validated by cloning and sequencing as described by Greig (2000). Alleles 4 and 6, identified by Greig (2000) respectively in the Atlantic and Mediterranean, were not found in the Pacific, nor were alleles 8–12, which were identified among western South Atlantic swordfish by Marques (2001). Conversely, two new alleles (alleles 14 and 15), not previously described for the Atlantic and Mediterranean (Greig, 2000; Marques, 2001) were found in the Pacific. These two new alleles were inferred from the corresponding site polymorphism(s) with respect to the reference alleles, and differ from the common allele 3 by one mutation at nucleotide position +36 (Table 2). Allele 15 is a rare allele found once in the NEPO, whereas, allele 14 is private to the SEPO. Observed heterozygosities ranged between 0.333 (CHL97) and 0.789 (MEX97), and although some localities displayed positive F_{is} values, indicative of heterozygote deficits, none of the probability tests for HWE were significant (Table 3). HWE tests for the alternative hypothesis of heterozygote deficit suggested deviations only for the two samples from Ecuador, but these tests were not significant after Bonferroni corrections. Conversely, since the F_{is} for the sample MEX97 was negative, tests for heterozygote excess were conducted, but no deviation from HW could be detected (P = 0.200).

Allele Frequency of *ldh*-A.—The distribution of allele frequencies across populations is summarized in Table 3. Alleles 1 and 3 accounted for the majority (> 88%) of the observations, with evidence of heterogeneity in their distribution. Most of this variability was associated with comparisons involving Chile, where allele 3 was more common than in any other sample. As a result, evidence of global differentiation among the Pacific samples assayed was obtained for genic data (P = 0.042 ± 0.008), although this test was not significant after Bonferroni correction. In addition, the global test of genotypic differentiation was not significant (P = 0.074 ± 0.011), and the global fixation index of differentiation was very low (F_{st} = 0.001). However, it should be noted that the F_{st} values obtained from the pairwise comparison of CHL97 against samples from other regions, were at least one order of magnitude larger

Table 3. Allele frequencies, number of alleles (n), and descriptive statistics for each of the samples of Pacific swordfish. Probabilities (P-val) for HWE probability test (HWE-P) and heterozygote deficit test (HWE-D) are included. No significant probabilities were observed after sequential Bonferroni corrections for multiple tests (initial $\alpha = 0.05/11 = 0.0045$). Sample locations in Table 1.

	Allele Number								Heterozygosity			HWE-P	HWE-D
Sample name	1	2	3	5	7	14	15	n	H_E	H_O	F_{is}	P-val	P-val
CHL97	0.259 (14)	0.056 (3)	0.667 (36)			0.019 (1)		54	0.497	0.333	0.330	0.145	0.072
CHL99	0.386 (22)	0.018 (1)	0.596 (34)					57	0.502	0.357	0.289	0.112	0.086
ECU9798	0.368 (25)	0.059 (4)	0.515 (35)	0.059 (4)				68	0.600	0.441	0.270	0.103	0.018
ECU94	0.400 (20)	0.080 (4)	0.480 (24)	0.040 (2)				50	0.614	0.440	0.299	0.121	0.030
MEX97	0.368 (14)	0.105 (4)	0.421 (16)	0.053 (2)	0.026 (1)		0.026 (1)	38	0.689	0.789	–0.149	0.340	0.881
HAW98	0.350 (14)	0.100 (4)	0.550 (22)					40	0.582	0.450	0.228	0.233	0.053
HAW99	0.417 (40)	0.115 (11)	0.458 (44)		0.010 (1)			96	0.596	0.610	0.024	0.584	0.346
HAW92	0.429 (24)	0.054 (3)	0.482 (27)		0.036 (2)			56	0.590	0.571	0.032	0.945	0.510
HAW93	0.379 (22)	0.103 (6)	0.483 (28)		0.034 (2)			58	0.622	0.621	0.002	0.709	0.617
EAUS95	0.500 (13)	0.038 (1)	0.346 (9)	0.038 (1)	0.077 (2)			26	0.647	0.615	0.050	1.000	0.438
WAUS95	0.453 (29)	0.109 (7)	0.391 (25)	0.047 (3)				64	0.637	0.688	–0.079	0.982	0.809
Total	237	48	300	12	8	1	1	607				0.008	0.008

(0.013–0.110), whereas the pairwise F_{st} value between the two samples from Chile was not significantly different from zero (F_{st} = –0.0051). The estimate of the number of migrants across populations using the private alleles method (Barton and Slatkin, 1986) was roughly 8 individuals per generation when corrected for sample size. This value is sufficiently large to prevent the fixation of alleles across the areas sampled.

Pooled Analysis.—Since there was no evidence of genetic heterogeneity among temporal samples within regions, they were pooled to increase the power of the test of differentiation. The global HWE test was significant (P = 0.009 ± 0.002). By contrast, none of the pooled regional samples deviated from HWE after correcting for multiple tests (Table 4). The global comparison of pooled samples was highly significant for both the genic (P = 0.0007 ± 0.0007) and genotypic (P = 0.0001 ± 0.0001) differentiation even after Bonferroni corrections (P < 0.008).

Pair-wise exact tests of genic differentiation and pair-wise F_{st} values indicate that the SEPO is the most distinct region among the Pacific regions surveyed, being significantly different from all other regions (Table 5). In addition, the NCPO is significantly different from the NEPO and from the SWPO. In turn, the NEPO and the SWPO are not different from each other.

Discussion

Patterns of Diversity of *ldh*-A in Pacific Swordfish.—We found that the *ldh*-A locus is polymorphic (H_o = 0.59) and is characterized by significant genic and genotypic differentiation among Pacific swordfish samples. None of the samples deviated from HWE after corrections for multiple tests. Two alleles (alleles 14 and 15), not previously described for the Atlantic and Mediterranean (Greig, 2000; Marques, 2001) were found in the Pacific. Conversely, allele 4, which occurs at a frequency of 8% in the Atlantic, and allele 6, which has only been detected at low frequency (2%) in the Mediterranean, were not found in the Pacific. The level of polymorphism that characterizes the *ldh*-A locus contrasts with loci Beta tubulin (Greig et al., 1999; Greig, 2000; Alvarado Bremer et al., 2001) and *CaM* (Chow and Takeyama, 2000), both reportedly monomorphic among Pacific swordfish.

Genetic Differentiation of the SEPO.—One of the most relevant individual findings of this study is the significant differentiation of the SEPO compared to all other Pacific regions surveyed. Chile displays a higher frequency of allele 3, accounting for about 63% of the observations, compared to all other regions surveyed, where it never exceeded 51%. The genetic differentiation of the SEPO from the NEPO is consistent with fishery stock structure analysis that indicates two stocks in the eastern Pacific, a north and south with a boundary at about 5°S (Hinton, 2003).

The separation of NCPO and NEPO was examined by Hinton and Deriso (1998) and Hinton (2003), but not clearly resolved. The analysis of pooled genetic data allowed us to examine the hypothesis of separation of these two putative stocks. In establishing the setup for this analysis, we noted that there was temporal stability in allele distributions at individual sample locations; that *ldh*-A allele 5 was found in samples from Ecuador and Mexico at a frequency of about 5%, but not in any of the NCPO samples; and that there was no evidence of differentiation between Mexico and Ecuador in the pair-wise comparison of regional samples. When samples from Ecuador and Mexico were pooled into a common NEPO sample, the two Chilean samples into a SEPO sample, the four samples from Hawaii into a NCPO sample,

Table 4. Allele frequencies, number of alleles (n), and descriptive statistics for pooled regional samples of Pacific swordfish. Probability values (P-val) for Hardy-Weinberg Equilibirum (HWE) probability test (HWE-P) and HWE heterozygote deficit test (HWE-D) are included. Deviation from HWE after sequential Bonferroni corrections for multiple tests (initial $\alpha = 0.05/4 = 0.0125$) is indicated ***. Region abbreviations as in Table 1.

	Allele number								Heterozygosity			HWE-P	HWE-D
Region	1	2	3	5	7	14	15	n	H_E	H_O	F_{is}	P-val	P-val
NCPO	0.400	0.096	0.484		0.028			250	0.599	0.573	0.043	0.297	0.245
	(100)	(24)	(121)		(7)								
NEPO	0.378	0.077	0.481	0.051	0.006		0.006	156	0.621	0.526	0.155	0.020	0.017
	(59)	(12)	(75)	(8)	(1)		(1)						
SWPO	0.467	0.089	0.378	0.044	0.022			90	0.636	0.667	–0.048	0.983	0.731
	(42)	(8)	(34)	(4)	(2)								
SEPO	0.324	0.036	0.631			0.009		111	0.498	0.345	0.301	0.055	0.081
	(36)	(4)	(70)			(1)							
Total	237	48	300	12	10	1	1	607	0.562	0.482	0.176	0.009***	

and the two Australian samples into SWPO sample, the larger regional sample sizes resulted in a substantial increase in the levels of differentiation and the associated probabilities, and increase in power for tests of differentiation. After pooling, the overall F_{st} value increased ten-fold from 0.001 to 0.0130. These results indicate a significant difference between the NEPO and the NCPO stocks, in agreement with the fishery evidence presented by Hinton (2003) and Hinton and Deriso (1998). Further, the results are inconsistent with fisheries stock assessments that have placed the southern boundary of swordfish North Pacific stocks in the NEPO north of the equator (e.g., Nakano, 1998) or that have combined southern and northern stocks in the EPO (Bartoo and Coan, 1989).

Previous comparisons of Hawaii and the EPO using mtDNA data in the form of RFLP analysis of the entire genome (Grijalva-Chon et al., 1994), and sequence data of the control region (Reeb et al., 2000), failed to support differences among these two regions. However, the significant differences in allele frequency of *ldh*-A obtained in this study between NCPO and the NEPO, corroborate the differences reported by Grijalva-Chon et al. (1996) between Hawaii and the EPO (Baja California, Mexico) at three allozyme loci (*PROT-3**, *ODH**, and *PROT-2**), as well as the differentiation between the NCPO and the SWPO using mtDNA control region data reported by Reeb et al. (2000). These findings of genetic differentiation in Pacific swordfish suggest that this species regardless of its high migratory potential must display philopatric behavior towards separate breeding grounds as has been suggested for Altantic swordfish (Alvarado Bremer et al., 2005b).

Future work on Pacific swordfish should focus on further resolution of stock boundaries in this basin based on a more ample coverage. Accordingly, samples from the northwestern Pacific and from the south-central Pacific should be surveyed. Ideally, samples analyzed should include multiple years and also samples taken during El Niño years, and samples before and after, providing the opportunity to contrast in a hierarchical analysis the potential influence of such oceanographic changes on the distribution of swordfish populations. Recently, several microsatellite loci have been developed for swordfish (Kotoulas et al., 2003; Reeb et al., 2003) that have been used to examine the heterogeneity of swordfish collections from eastern and western Australian fisheries (Ward et al., 2001). The inclusion of such additional markers, as well as the development of additional exon-primed amplified introns, would be desirable to confirm the signal of differentiation revealed here by the *ldh*-A data.

In summary, we report that the allele distribution of the *ldh*-A gene in Pacific swordfish is homogeneous among temporal samples within-regions but that significant differences exist among pooled regional samples from the NCPO (Hawaii-multiple years), the north-eastern Pacific Ocean (NEPO: Mexico and Ecuador-multiple years), the south-eastern Pacific Ocean (SEPO: off Chile), and the SWPO (Australia).

Acknowledgments

We thank J. Turner, D. English, and K. Stroud for help in the laboratory. We thank C. Patnode for help with figures. Additionally, we thank the following individuals and organizations for assistance with obtaining samples at various locations: S. Chow, NRIFSF Japan; P. Ward and P. Grewe, CSIRO Australia; M. Donoso, IFOP Chile; R. Dollar, T. Kazama, and R. Humphreys, NMFS Honolulu; O. Sosa-Nishisaki, CISESE Mexico. This manuscript was improved

by comments from L. Adams, NOAA-NOS, and two anonymous referees. This project was financially supported by the IATTC and startup funds from TIO and TAMUG to JRAB.

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