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Direct comparison of mitochondrial markers for the analysis of swordfish population structure

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ABSTRACT

When examining the population structure of swordfish (*Xiphias gladius*; Linnaeus, 1758) for fisheries management, the traditional marker of choice has been the mitochondrial control region. However, homoplasy within the control region may render it an unsuitable marker. We compared the effectiveness of two mitochondrial markers for examining the population structure between four Indian and one Pacific Ocean sample sites, collected between 2006 and 2009. The control region analysis was unable to detect structure among populations, while ND2 analysis revealed structure between the Seychelles and two other populations, Timor Sea (Φ_{ST} = 0.012, P = 0.024) and Coral Sea (Φ_{ST} = 0.011, P = 0.029). The level of homoplasy observed in the control region (HI = 0.736) was considerably higher than that of ND2 (HI = 0.290). These results suggest that use of the control region has likely led in an underestimation of genetic differentiation between populations. Genetic markers should be chosen that are appropriate to the scale of subdivision under investigation.

1. Introduction

The broadbill swordfish, *Xiphias gladius* (Linnaeus, 1958), is a large pelagic fish with a broad global distribution ranging from temperate to tropical waters. Molecular analysis of populations has led to the recognition of four distinct stocks: Mediterranean, North Atlantic, South Atlantic and the Indo-Pacific (Alvarado Bremer et al., 1996, 2005; Chow et al., 1997; Chow and Takeyama, 2000; Rosel and Block, 1996).

Swordfish are of great commercial value and are fished extensively worldwide. Within the Indian Ocean, mean catch of swordfish between 2004 and 2008 was 29,900 tonnes (IOTC, 2009). While it has been estimated that the maximum sustainable yield ranges from 32,000 to 34,000 tonnes, by-catch rates reported by the Japanese fleet suggest that localised depletion has occurred in the south west of the Indian Ocean (IOTC, 2009).

The genetic marker most commonly used to examine swordfish populations has been the mitochondrial control region (D-loop). Although involved in replication and transcription of the mitochondrial genome, the control region does not code for a gene. Within swordfish, the control region is approximately 835 base pairs in length and consists of two hypervariable regions, separated by a conserved region (Rosel and Block, 1996).Only one study has used the entire length of the control region when examining stock structure of swordfish (Lu et al., 2006). Other studies have used either the first hypervariable region (~330 bp; Alvarado Bremer et al., 1996, 2005; Rosel and Block, 1996; Viñas et al., 2010), excluded the conserved region (~629 bp; Reeb et al., 2000; Ward et al., 2001), or used the first 517 bp (Muths et al., 2009).

Phylogenetic analysis of the mitochondrial control region has led researchers to propose that swordfish can be divided into two divergent clades; an abundant Clade I that is present in all stocks, and Clade II which decreases in abundance with distance from the Mediterranean Sea (Alvarado Bremer et al., 1996, 2005; Rosel and Block, 1996). Although a small proportion of Clade II haplotypes has been observed in the Indian Ocean, none have been identified from the Pacific Ocean (Lu et al., 2006; Muths et al., 2009; Reeb et al., 2000; Ward et al., 2001).

Swordfish within the Atlantic Ocean consist of a northern and southern stock, each of which show no apparent substructure (Alvarado Bremer et al., 2005; Chow et al., 1997; Chow and Takeyama, 2000). However, there are hints of differentiation within each of the Mediterranean and Indo-Pacific stocks (Alvarado Bremer et al., 2006; Lu et al., 2006; Reeb et al., 2000; Viñas et al., 2010; Ward et al., 2001). Within the Indian Ocean, analysis of control region sequences failed to distinguish western populations surrounding Madagascar (Muths et al., 2009). Broader sampling has suggested that some structure exists within the Indian Ocean (Lu et al., 2006), though stronger statistical evidence would require larger sample sizes. Use of microsatellite markers revealed a small

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Table 1
Collection information of samples used in this study.

Region	Sampling date	*N*	Location
Coral Sea	March, October 06, April 08	50	25–26°S, 156–159°E
Timor Sea	May 08, May–June 09	45	9–14°S, 110–120°E
Fremantle	Feb–Mar 08	37	Off Fremantle coast
Central Indian Ocean	May 07, July–December 08	25	16–32°S, 76–93°E
Seychelles	September–December 08	48	Off Seychelles coast
Total		205	

degree of structure between Western Australia and Reunion Island (F_{ST} = 0.0113; P = 0.001), though this structure was not observed when the same samples were sequenced for the control region (Ward et al., 2001).

While the mitochondrial control region appears to be a suitable marker for distinguishing between the four known stocks of swordfish (Alvarado Bremer et al., 1996, 2005; Chow et al., 1997; Rosel and Block, 1996), concern has been raised regarding its suitability as a marker when focusing on differentiation at the intra-ocean basin level (Rosel and Block, 1996). The high evolutionary rate that has made the control region an appealing marker for researchers may be obscuring the true relationships between populations due to high haplotype diversity as well as homoplasy. Homoplasy is sequence similarity between individuals that is not due to inheritance but has arisen through parallel, convergent, or reverse evolution. Reeb et al. (2000) reported that the hypervariable regions of the control region has a large homoplasy index (HI) of 0.699 in swordfish. An alternative mitochondrial marker with a slower evolutionary rate may more accurately reveal the patterns of variation when compared to the control region.

The mitochondrial gene, nicotinamine dehydrogenase subunit 2 (ND2), has a slower rate of evolution than the control region and may provide more suitable levels of differentiation for examining swordfish population structure within the Indo-Pacific stock. The ND2 gene is 1047 base pairs in length and encodes a subunit of NADH dehydrogenase (GenBank accession number NC_012677.1). This study investigates the application of ND2 for use in defining Indo-Pacific swordfish population structure. We make a comparison of homoplasy levels and population structure to that of the control region to help determine if the control region is a suitable marker.

2. Materials and methods

2.1. Sampling

Swordfish tissue samples (gonad or muscle) were obtained from 205 fish captured by commercial longline fishing vessels between 2006 and 2009. Samples were collected from five locations (Table 1): Coral Sea (n = 50), Timor Sea (n = 45), Fremantle (n = 37), Central Indian Ocean (n = 25), and Seychelles (n = 48). Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions.

To confirm the species identity of samples collected, control region sequences were compared to GenBank sequences using a nucleotide megablast (NCBI; http://blast.ncbi.nlm.nih.gov/). All specimens used in this study contained 96–100% identity to swordfish sequences on the database.

2.2. Marker amplification and sequencing

The mitochondrial control region was amplified using the primers Pro-L and Phe-ABT. These primers are located in the flanking genes, $tRNA^{Pro}$ and $tRNA^{Phe}$ respectively. ND2 was amplified using the novel primers Swo-MetL and Swo-TrpH, which are located in the gene $tRNA^{Met}$ and $tRNA^{Trp}$ respectively. Primer sequences and their corresponding references are listed in Table 2.

PCRs were performed in 50 μL volumes, containing 40 ng of template DNA, 0.8× reaction buffer, 2.4 mM $MgCl_2$, 0.16 mM dNTPs, 0.16 μM of each primer, and 4U Taq polymerase (GoTaq Flexi; Promega). Cycling parameters consisted of an initial denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. Purification of DNA fragments was performed using the Wizard SV Gel and PCR Clean-Up System (Promega), following the manufacturer's instructions. The purified products were sequenced on an ABI3730XL capillary machine by Macrogen Inc (South Korea).

2.3. Analysis

Sequences were aligned using Sequencher v 4.6 (Gene Codes Corporation) and were checked by eye to ensure the accuracy of variable sites identified by the program. Haplotype diversity (Hd) and nucleotide diversity (π), as well as the neutrality tests Tajima's D (Tajima, 1989) and Fu and Li's F^* (Fu and Li, 1993) statistics were calculated using DnaSP v 5 (Librado and Rozas, 2009). The homoplasy index (HI) was determined for the different markers using PAUP* v 4.0b10 (Swofford, 2002), by first generating an unrooted maximum parsimony (MP) tree. Heuristic MP searches were performed using the program's default settings. For the control region, gaps were treated as a new character-state. The appropriate model of substitution was calculated using ModelTest v 3.7 (Posada and Crandall, 1998). The optimal model for the control region was HKY+I+G with a gamma shape parameter α = 0.513. The optimal model for ND2 was TrN+G with a gamma shape parameter α = 0.284.

The amount of variation among populations was examined using an Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992) in the program Arlequin v 3.1 (Excoffier et al., 2005). The HKY+I+G model identified by Modeltest as the optimal model for the control regions is not available in Arlequin so both markers were analysed using the TrN+G model (Tamura and Nei, 1993), with gamma shape parameters α = 0.513 and 0.284 for the control region and ND2 respectively. Pairwise population Φ_{ST} was also calculated, using the same settings. Multiple tests off significance were corrected using the sequential Bonferroni method (Rice, 1989).

Table 2
List of PCR primers used in this study.

Primer	Marker	Sequence	Reference
Pro-L	Control region	5′-CTACCCCTAACTCCCAAAGC	Reeb et al. (2000)
Phe-ABT	Control region	5′-CCATCTTAACATCTTCAGTG	P. Grewe (unpublished)
Swo-MetL	ND2	5′-TCCACTACACCACTTCCTAGTAAAGTCAGC	This study
Swo-TrpH	ND2	5′-GGCCCTTGGTCTTGTCTTATCCTAAGTCCC	This study

Table 3
AMOVA of control region sequences, testing genetic variation between Coral Sea, Fremantle, Timor Sea, Central Indian Ocean and Seychelles.

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation	Fixation index	*P*-value[a]
Among groups	4	24.156	−0.01391	−0.21	−0.002	0.70088 ± 0.01165
Within populations	200	1320.428	6.60214	100.21		

[a] Based on 1023 permutations.

Table 4
Pairwise comparisons of Φ_{ST}, comparing control region sequences between Coral Sea, Fremantle, Timor Sea, Central Indian Ocean and Seychelles. Φ_{ST} values are below the diagonal and *P* values above the diagonal.

Name	Coral Sea	Fremantle	Timor Sea	C. Indian Ocean	Seychelles
Coral Sea		0.3682	0.4712	0.6621	0.5215
Fremantle	0.0007		0.4648	0.8887	0.5645
Timor Sea	−0.0002	−0.0005		0.3262	0.4922
Central Indian Ocean	−0.0048	−0.0116	0.0023		0.9307
Seychelles	−0.0015	−0.0020	−0.0006	−0.0105	

3. Results

3.1. Sequence analysis

The complete length of the control region (~835 bp) was sequenced. Of the sequences obtained from the 205 individuals examined (GenBank accession numbers HQ218055–HQ218223), 131 variable sites (excluding indels) were observed, including 33 singletons, that defined 169 haplotypes. Haplotype diversity (Hd) was 0.997 and nucleotide diversity (π) was 0.015. Although neutrality tests showed negative values, both tests were not significant (Fu and Li's $F^* = -1.7$, $P > 0.10$; Tajima's $D = -1.5$, $P > 0.10$).

The complete length of the ND2 gene was sequenced (1047 bp; GenBank accession numbers HQ218224–HQ218304). A total of 88 variable sites were observed, including 40 singletons, that defined 76 haplotypes. Haplotype diversity (Hd) was 0.911 and nucleotide diversity (π) was 0.002. No stop codons or indels were present within the gene, although 26 amino acid substitutions were observed. Both neutrality tests were negative and significant (Fu and Li's $F^* = -4.2$, $P < 0.02$; Tajima's $D = -2.6$, $P < 0.001$).

A homoplasy index (HI) of 0.7476 (0.7007 if uninformative characters are included) was calculated for the control region sequences (excluding indels). In contrast, HI for the ND2 sequences was 0.2899 (0.1802 if uninformative characters are included). HI was also determined for individual variable sites. Of those values, the control region had 67 sites (excluding indels) that had an HI above 0.500, whereas the ND2 sequence possessed only two.

3.2. Population analysis

The AMOVA did not reveal any population structure for the full length of the control region (Table 3). Although values fluctuated when different sequence lengths were analysed (data not shown), the percentage variation among populations remained below zero. The Φ_{ST} values between populations also did not reveal any genetic structure (Table 4). Of all the sequences, only two individuals were identified as belonging to Clade II. The two specimens had been collected from the Central Indian Ocean and Fremantle sites.

While the majority of variation described by AMOVA for ND2 sequences was within populations, 0.56% of the variation was due to variation among populations ($P = 0.049$; Table 5). Pairwise Φ_{ST} values (Table 6) reveal a small distinction between Seychelles and Coral Sea ($\Phi_{ST} = 0.011$, $P = 0.029$), as well as Seychelles and Timor Sea ($\Phi_{ST} = 0.012$, $P = 0.024$). These values were not significant after Bonferroni correction. No other significant differences were observed between populations. When the Timor Sea and Fremantle populations were pooled, the Φ_{ST} between the pooled Western Australian population and Seychelles was 0.009 ($P = 0.037$).

The two samples identified as belonging to Clade II using the control region were also distinguishable from other samples using ND2 sequences, with seven fixed differences (all transition) between Clade I and Clade II.

4. Discussion

4.1. Suitability of the control region in assessing population structure

We have obtained sequences of the traditionally used control region and the slower evolving ND2 gene to compare their utility in resolving population structure of swordfish. Given that all markers on the mitochondrial genome are essentially linked, there could be

Table 5
AMOVA of ND2 sequences, testing genetic variation between Coral Sea, Fremantle, Timor Sea, Central Indian Ocean and Seychelles.

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation	Fixation index	*P*-value[a]
Among groups	4	5.655	0.00626	0.54	0.00536	0.04888 ± 0.00766
Within populations	200	232.087	1.16043	99.46		

[a] Based on 1023 permutations.

Table 6
Pairwise comparisons of Φ_{ST}, comparing ND2 sequences between Coral Sea, Fremantle, Timor Sea, Central Indian Ocean and Seychelles. Φ_{ST} values are below the diagonal and *P* values above the diagonal.

Name	Coral Sea	Fremantle	Timor Sea	C. Indian Ocean	Seychelles
Coral Sea		0.0820	0.1992	0.1221	0.0293[a]
Fremantle	0.0083		0.4092	0.6836	0.2295
Timor Sea	0.0042	0.0007		0.4502	0.0244[a]
Central Indian Ocean	0.0075	−0.0063	0.0002		0.4023
Seychelles	0.0112	0.0042	0.0122	0.0006	

[a] *P* value not significant after Bonferroni correction.

the expectation that the control region and ND2 would show similar patterns of variation among swordfish populations, with the higher variable marker better able to resolve structure. While both markers can segregate swordfish into the two clades previously identified (Alvarado Bremer et al., 1996; Rosel and Block, 1996), only the analysis of ND2 sequences revealed structure between Seychelles and two populations, Timor Sea and Coral Sea. Although the observed structure was not significant after Bonferroni correction, it is likely that this calculation is too stringent for this level of differentiation (Reeb et al., 2000).

The hypervariable regions of the swordfish control region have previously been reported to have a homoplasy index (HI) of 0.699 (Reeb et al., 2000). This is slightly lower than the HI we observed using the entire length of the marker (HI = 0.748). Compared with both of these values, the HI of ND2 was considerably lower (HI = 0.290). Both markers have high haplotype diversity, however the control region has more singleton haplotypes (94%) compared to ND2 (67%). Given that population analysis of ND2 revealed structure between populations that analysis of control region sequences could not detect, this suggests that the large number of unique haplotypes and the level of homoplasy within the control region leads to an underestimation of genetic relationships. Similar observations have been made in yellowfin tuna, where analysis of the control region between Atlantic and Indo-Pacific populations was unable to detect structure but PCR-RFLP data of the ATPase 6 and cytochrome oxidase III segment did (Ely et al., 2005).

The mitochondrial control region has traditionally been the marker of choice when examining population structure and has shown separation of the four major stocks of swordfish. Previous studies using the control region have revealed some level of differentiation within the Indo-Pacific and Mediterranean stocks (Lu et al., 2006; Reeb et al., 2000; Viñas et al., 2010). Data presented in this study suggests that there is a superior marker for studying the populations of swordfish within stocks. ND2 provides greater resolution for detecting subdivision; therefore conclusions of population structure should not be based solely on the mitochondrial control region. However, given the maternal inheritance of mitochondrial DNA and the linkage of the markers, additional markers such as microsatellites or single nucleotide polymorphisms (SNPs), such as the markers characterised within Smith et al. (2010), should be considered in conjunction with ND2.

Regardless of the species under study, a genetic marker should be selected based upon its ability to resolve the scale of subdivision that is under investigation. Homoplasy is of particular concern as this can result in misleading interpretation of data and the development of unsuitable management actions. Preliminary analysis should be conducted to determine the suitability of the marker before investing in large-scale population analysis.

4.2. Swordfish population structure in sampled populations

The limited number of studies investigating the Indian Ocean provide some support that population structure is present within that ocean basin. Analysis of microsatellite loci revealed structure between Western Australia and Reunion (Ward et al., 2001). On the basis of our analysis, we have divided Western Australia into Timor Sea and Fremantle populations. By contrast, Ward et al. (2001) combined samples collected in the Timor Sea and off the central coast of Western Australia, which may have led to an underestimate of structure between the samples. Comparison of populations using the complete control region has also suggested that structure is present within the Indian Ocean, though the authors were more conservative in their interpretation of the data and concluded that only the North Malagasy and Bay of Bengal populations were distinct (Lu et al., 2006).

Philopatric behaviour in the form of fidelity to discrete spawning sites has often been ascribed as the driving force behind genetic heterogeneity of swordfish populations (Alvarado Bremer et al., 2005, 2006; Viñas et al., 2010). Regions of spawning included in this study are the Coral Sea, Timor Sea, and Seychelles Islands. While various lines of evidence suggest that the Coral Sea and in the waters near Seychelles are spawning grounds (García-Cortés and Mejuto, 2003; Young et al., 2003), in the Timor Sea a larval survey suggests that this may also be a region of spawning (Nishikawa et al., 1985). Further evidence is required in order to be certain that the Timor Sea is a spawning ground (Alvarado Bremer et al., 2005; Poisson and Fauvel, 2009). Our Seychelles sample lacks precise location information, but if these samples were collected from a spawning ground, then philopatry could explain the structure observed among the two eastern populations and that of Seychelles.

Philopatry alone cannot account for the lack of observed genetic structure between the Coral Sea and Timor Sea. Lack of structure between these seas has been previously observed through analysis of both mitochondrial and nuclear markers (Alvarado Bremer et al., 2006; Lu et al., 2006; Reeb et al., 2000; Ward et al., 2001). This is in contrast to the expectation that fidelity to these separate spawning grounds would lead to genetic structure between the different regions. An alternative hypothesis that previous researchers have proposed is that the Indonesian throughflow ocean current provides an opportunity for larvae and juveniles to be transported from the Pacific to Indian Ocean (Chow and Takeyama, 2000; Lu et al., 2006; Ward et al., 2001); however, the throughflow enters through the Makassar Strait into the Banda Sea and does not directly link the Coral Sea to the Indian Ocean (Kuhnt et al., 2004).

While our analysis has revealed population subdivision within the Indian Ocean, no structure was observed between the putative spawning grounds (Coral Sea, Timor Sea, and Seychelles) and non spawning grounds (Fremantle and Central Indian Ocean). The Fremantle and Central Indian Ocean sampling areas are believed to represent transition zones, where fish are migrating between spawning and feeding grounds (Poisson and Fauvel, 2009). These transition zones may comprise fish from any putative spawning grounds within the Indian Ocean, including around Reunion Island and in the Bay of Bengal (Poisson and Fauvel, 2009). Within the Indian Ocean there is a need to gain a more comprehensive understanding of population structure of swordfish in order to develop effective management strategies. This can be achieved by sampling from all potential contributing spawning areas, as well as from additional transition and feeding zones.

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