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GENETIC ANALYSIS OF BLUE MARLIN (*MAKAIRA NIGRICANS)* STOCK STRUCTURE IN THE ATLANTIC OCEAN

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ABSTRACT The genetic basis of stock structure of blue marlin (*Makaira nigricans* Lacepède 1802) in the Atlantic ocean was inferred from analyses of mitochondrial control region sequences. Blue marlin were collected in 1998 from 4 major geographic locations: western North Atlantic, Caribbean Sea, western South Atlantic and eastern Atlantic. Haplotype diversity (*h*) ranged from 0.99–1.0 and nucleotide sequence diversity (π) ranged from 0.11–0.13 within samples indicating that the control region harbors a significant amount of genetic variation. However, no significant differences were found in the spatial partitioning of genetic variation among the 4 collections; all pairwise ϕ_{ST} values were negative and were therefore all taken as estimates around a true value of 0. As with previous studies of blue marlin, sequences were comprised of 2 distinct mitochondrial lineages separated by an average of 138 base pairs and ϕ_{ST} between clades of 0.799 ($P < 0.0001$). These 2 lineages were present in similar frequencies across sampling locations. Genetic data from this study support management of Atlantic blue marlin as a single, Atlantic-wide stock.

RESUMEN La base genética de la estructura de poblaciones del marlin azul (*Makaira nigricans* Lacepède 1802) en el Océano Atlántico fue inferido de analiza de sucesiones de región de control de mitochondrial (ADNm). El marlin azul fue reunido en 1998 de cuatro ubicaciones geográficas mayores: el norte occidental del Mar Atlántico, el Mar Caribe, el Atlántico Sur occidental y el Atlántico oriental. La diversidad de haplotipos (*h*) recorrió de 0,99–1,0, y la diversidad de la sucesión de nucleótido (π) recorrió de 0,11–0,13 dentro de las muestras, que indican que la región del control abriga una cantidad significativa de la variación genética. Sin embargo, ningunas diferencias significativas fueron encontradas en el dividir espacial de la variación genética entre las cuatro colecciones; todos valores de ϕ_{ST} fueron negativos y fueron por lo tanto todo tomado como estimaciones alrededor de un valor verdadero de 0. Al igual que con estudios previos del marlin azul, las sucesiones fueron comprendidas de dos linajes claros de mitochondrial separados por un promedio de 138 pares despreciables y ϕ_{ST} entre clades de 0,799 ($P < 0,0001$). Estos dos linajes fueron presentes en frecuencias semejantes a través de probar las ubicaciones. Los datos genéticos de esta investigación apoyo la administración del marlin azul Alántico como una sola población en Atlántico entero.

INTRODUCTION

The blue marlin, *Makaira nigricans* (Lacepède 1802), is a circumtropically distributed, highly migratory member of the family Istiophoridae that inhabits pelagic waters with surface temperatures above 22° C (Nakamura 1985). In the Atlantic Ocean, blue marlin are considered to be highly over-exploited; the stock was estimated to be at 40% of that needed for maximum sustainable yield at the last complete assessment (ICCAT 2000). Recent indices suggest a continuing decline of the stock abundance through 2004 while other indices suggest a leveling off of the decline (ICCAT 2006). Although more than half of the catch of blue marlin has historically come from the longline fishery, new artisinal fleets have recently harvested large catches of blue marlin, including a FAD fishery in the eastern Caribbean islands and a fleet of small longliners operating off Brazil between 20°S and 26°S (ICCAT 2006). These new fisheries have heightened concern about the status of the stock and highlighted the need for a better understanding of the stock structure of this species throughout the Atlantic Ocean for effective management.

Tagging studies have shown that blue marlin are capable of long distance migrations including trans-oceanic and trans-equatorial movements in both the Atlantic and Pacific oceans as well as inter-oceanic movements (Atlantic to Indian and Pacific to Indian oceans) (Ortiz et al. 2003). Although about 85% of recovered blue marlin were at large for 3 yr or less, analysis of the movement of blue marlin with longer times at liberty (up to 11 yr) found no indication of site fidelity or cyclical annual movements. However, there was evidence of strong seasonal movement patterns in the Atlantic, primarily from the US mid-Altlantic and Mexican Caribbean to Venezuela.

Until recently, spawning areas of blue marlin were not well known. This lack of information stemmed from the difficulty in both collecting and identifying larval istiophorid billfishes to species based on pigmentation and morphometric characters (Richards 1974, Collette et al. 1984, Nishikawa and Rimmer 1987, Nishikawa 1991).

However, recent advances in species-level identification of larval istiophorids have allowed researchers to investigate the temporal and spatial patterns of billfish spawning. Spawning has recently been documented in several locations in the western North Atlantic during the summer months based on the presence of small blue marlin larvae. These areas include Exuma Sound, Bahamas, (Serafy et al. 2003), Straits of Florida (Luthy et al. 2005), Punta Cana, Dominican Republic (Prince et al. 2005), Bermuda (Luckhurst et al. 2006), and the Gulf of Mexico (Jay Rooker, Texas A&M University, pers. comm.).

Evidence of seasonal movement patterns from tagging data combined with new information on the habitat preference and spawning locations of blue marlin in the Atlantic have lead some researchers to hypothesize that blue marlin may exhibit fidelity to spawning grounds. A recent pop up satellite archival tag (PSAT) study off Punta Cana, Dominican Republic (Prince et al. 2005) suggested that reproductively active blue marlin may have a more constrained mean displacement per day in the spawning season than the range exhibited by blue marlin tagged in other localities and seasons (i.e., Graves et al. 2002, Kerstetter et al. 2003). It is not known whether blue marlin revisit the same spawning area in different years. However, taken with evidence from conventional tagging data that other members of the Istiophoridae, such as the striped marlin (Ortiz et al. 2003) and black marlin (Pepperell 1990) exhibit cyclical movements, it is conceivable that blue marlin could exhibit spawning site fidelity. If blue marlin exhibit site fidelity, one would expect this to result in population genetic structure. This issue needs to be examined further.

Previous genetic studies of blue marlin population structure have indicated that the species comprises a single Atlantic-wide population. However, these studies were primarily focused on differentiating Atlantic and Pacific populations of blue marlin, so sampling within the Atlantic was limited (Buonaccorsi et al. 1999, 2001, Graves and McDowell 2001). These studies used whole-molecule mitochondrial DNA (mtDNA) restriction fragment length polymorphism analysis (RFLP), which is less discriminatory than sequencing of individual loci; no comprehensive study based on mitochondrial DNA sequences has been conducted to date. The mitochondrial genome is an ideal candidate for population-level analyses because of its uniparental mode of inheritance and rapid rate of evolution (reviewed in Avise 2001, Hallerman 2003). The mitochondrial genome experiences a rate of nucleotide substitution that has been estimated to approach 5–10 times that of nuclear DNA (Brown et al. 1979, Saccone et al. 1999, Avise 2001) and the non-coding control region, evolves 3–5 times faster than the mitochondrial genome as a whole (Avise 2001). In addition, in the absence of gene flow and assuming an equal rate of mutation, selection and drift, genetic divergence accumulates more rapidly in the mitochondrial genome because it is haploid and uniparentally inherited (Hallerman 2003).

Although multiple classes of molecular markers have been used to look for evidence of population structure in blue marlin, mtDNA was found to be more sensitive to population structuring in this species than either allozymes, single-copy nuclear DNA or microsatellites (Buonaccorsi et al. 2001). Analyses of mtDNA sequence variation have been successfully used to address phylogeographic questions in a number of fish taxa. These include studies pertinent to stock management of pelagic marine fishes such as swordfish, *Xiphias gladius* (Reeb et al. 2000, Alvarado-Bremer et al. 2005), blue marlin, *M. nigricans* (Buonaccorsi et al. 1999), black marlin, *Istiompax indica* (Falterman 1999), sailfish, *Istiophorus platypterus* (Graves and McDowell 1995, McDowell and Graves 2002), white marlin, *Kajikia albida* (Graves and McDowell 2001, 2003, 2006), striped marlin, *K. audax* (Graves and McDowell 1994, 2003), bigeye tuna, *Thunnus obesus* (Chow et al. 2000, Durand et al. 2005, Martinez et al. 2006), bluefin tuna, *T. thynnus* (Carlsson et al. 2004, Alvarado-Bremer et al. 2005), albacore tuna, *T. alalunga* (Vinas et al. 2004), and wahoo, *Acanthocybium solandri* (Garber et al. 2005). In the present study, we analyze mitochondrial control region samples of blue marlin taken throughout their Atlantic range to test the null hypothesis that blue marlin do not exhibit genetic stock structure.

Materials and Methods

In this study, 57 blue marlin samples were collected from four major geographic regions: the western North Atlantic (WNA; US mid-Atlantic; $n = 15$), the Caribbean Sea (CAR; Jamacia; $n = 11$), the western South Atlantic (WSA; southern Brazil; $n = 13$) and the eastern Atlantic (EA; Ghana; $n = 18$). All samples were collected in the same calendar year (1997–1998) with the exception of 9 of the WNA samples, which were collected in 1995. Samples consisted of either heart tissue removed after capture, cooled on ice, and stored at –80° C, or white muscle preserved in 0.25 mM EDTA pH 8.0, 20% DMSO, and saturated NaCl (Seutin 1991) at room temperature.

Total genomic DNA was extracted from 0.05–0.5g skeletal and/or heart muscle following the methods of Sambrook et al. (2001). Briefly, tissue was digested at 37° C over night with 15 µl proteinase K (25 mg/ml), 15 µl RNAse (10mg/ml), 60µl 10% sodium dodecyl sulfate

(SDS) and 500 µl isolation buffer (50 mM EDTA, 50mM Tris, 150 mM NaCl, pH 8.0). DNA was isolated using a standard phenol extraction procedure and precipitated using an equal volume of isopropanol and 0.04x volume 5 M NaCl. Double-stranded nucleotide sequences from the hypervariable mitochondrial control region were amplified using *Taq* PCR Core reagents (Qiagen Corp. Valencia, CA) with published universal PCR primers DloopK (5' AGCTCAGCGCCAGAGCGC CGGTC TTGTAAA 3'; Lee et al. 1995), DloopL (5' AGTAAGAGCCCACCATCAGT 3'; Lee et al. 1995), 1CD-Loop(H1) (5' TTGGGTTTCTCGTATGACCG 3'; Cronin et al. 1993). Each 25 µl PCR reaction contained the following: approximately 5–25 ng purified DNA, 2.5 µl 10X PCR reaction buffer (Tris·Cl, KCl, $(NH4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7), 0.5 µl 10 mM dNTP mix (dATP, dCTP, dGTP, dTTP, 10 mM each), 0.125µl *Taq* DNA polymerase (5 units/µl), 0.5 µl of 10 mg/ml bovine serum albumin (BSA), and 10 pmoles of each primer. PCR amplification conditions consisted of an initial denaturation of 4 min at 94º C, followed by 35 cycles of 1 min at 94º C, 1 min at 48º C, and 1 min at 72º C, followed by a final extension of 3 min at 72º C. Following electrophoresis, products were visualized using a UV transilluminator. Amplified products were purified by column filtration (QIAquick PCR Purification, Qiagen Corp., Valencia, CA), or by using EXOSAP (USB Scientific, Cleveland, OH) prior to DNA sequencing.

Purified PCR products were sequenced in forward and reverse directions using BigDye Terminator v3.1 Cycle Sequencing reagents (Applied Biosystems, Warrington, UK). Sequencing reactions were composed of 10–50 ng template DNA, 0.25 µl sequencing primer, 0.25 µl BigDye master mix, 1 µl 5x reaction mix and water to a final volume of 5 µl. Cycle sequencing conditions consisted of an initial denaturation of 1 min at 96º C, followed by 25 cycles of 10s at 96º C, 5s at 50º C, and 4 min at 60º C. Primers used for cycle sequencing were identical to primers used in original PCR amplification reactions. Amplification products were electrophoresed on an ABI 3130 sequencer equipped with an 80 cm capillary. Results were analyzed using Sequencing Analysis v. 5.1.1 software (Applied Biosystems, Warrington, UK). Standard chromatogram format (SCF) curves were exported for subsequent analyses and consensus sequences of forward and reverse reactions were created using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI). All sequences were subsequently aligned using the Clustal W algorithm (Thompson 1997) in MacVector 7.2 (Accelrys Inc., San Diego CA), with default parameters and adjusted by eye.

Sequence characteristics including base composition and number of substitutions (as well as relative contribution by transitions, transversions, indels) were calculated in Arlequin v 3.1 (Excoffier et al. 2005). A different haplotype designation was given to each unique DNA sequence. Haplotype diversity (h), nucleotide diversity (π) (Nei 1987), and pairwise nucleotide sequence divergence were calculated using Arlequin 3.1 (Excoffier et al. 2005). PAUP* 4.0b10 (Swofford 1999) was used to generate an unweighted pair group method with arithmetic mean (UPGMA, Sneath and Sokal 1973) tree to visualize phylogeographic patterns within this species. In addition, hierarchical analyses of molecular variance (AMOVA) were performed in Arlequin 3.1 to evaluate geographic structuring of molecular variance. Population pairwise ϕ_{ST} values were calculated and used as a proxy of gene flow. All analyses were performed using the Tamura-Nei model (Tamura 1993). For AMOVA and ϕ_{ST} calculations, the probability of significance was assessed using 10000 permutations of the data.

RESULTS

Mitochondrial control region sequences were collected from all 57 blue marlin samples and were deposited in Genbank under accession numbers EF607795-607852. Sequence length ranged from 878 to 891 base pairs (bp) and comprised most of the control region. The final alignment of 904 bp, including indels, exhibited 301 polymorphic sites, 237 of which were parsimony informative. There were 282 transitions, 22 transversions and 39 indels and the transition:transversion ratio was estimated to be 12.8. The relative contribution of each nucleotide was about 32.2% A, 22.8% C, 16.2% G, and 28.9% T; the mean nucleotide composition was biased towards adenine and thymine. Fifty-four of the 57 samples examined had unique haplotypes ($h = 0.9981$, $\pi = 0.1034 \pm 0.050$). Three haplotypes were represented by 2 individuals. These were shared between EA and WNA, between WNA and WSA and within EA. No haplotype was found more than twice. The absolute number of differences between haplotypes ranged from 2 to 166 (mean = 93.45 ±40.8).

Diversity was high in all collections and the mean number of pairwise differences between individuals randomly drawn from a collection ranged from 99.8 (WNA) to 110.2 (WSA) and nucleotide diversity ranged from 0.115 (WNA) to 0.129 (CAR) (Table1). These values were similar to those between randomly selected individuals from different collections. The corrected mean pairwise differences between collections were all negative (essentially zero). The global ϕ_{ST} based on Tamura-Nei (1993) distances of –4.61 (essentially zero) was not significantly different from 0 ($P = 0.95624$). Pairwise ϕ_{ST} values were

TABLE 1

Measures of diversity for Atlantic samples of blue marlin, and for groups of alleles comprising the Atlantic (ATL) and ubiquitous (UCL) clades. *N* refers to the total number of samples. No. Haps refers to the number of distinct haplotypes. *h* refers to haplotypic diversity, π refers to the nucleotide sequence diversity i.e., the mean number of base pair differences between 2 randomly chosen haplotypes within the sample. $s_{\bar{x}}$ refers to standard error associated with the sampling process.

Location	*N*	No. Haps	*h*	± $s_{\bar{x}}$	π	± $s_{\bar{x}}$
EA	18	17	0.993	± 0.021	0.121	± 0.061
WNA	15	15	1.000	± 0.024	0.115	± 0.059
CAR	11	11	1.000	± 0.039	0.129	± 0.068
WSA	13	13	1.000	± 0.030	0.124	± 0.064
CLADES						
ATL	27	25	0.994	± 0.012	0.0619	± 0.031
UCL	30	29	1.000	± 0.009	0.0170	± 0.009

also all negative and were therefore all taken as estimates around a true value of 0. A UPGMA clustering of the 57 mtDNA control region sequences (Figure 1) did not indicate a significant association of haplotypes with geographic area.

Clade Distribution

Evidence of 2 distinct clades was noted in the UPGMA tree generated from pairwise nucleotide sequence divergence values based on a Tamura-Nei model (Figure 1). Sequences were designated as either Atlantic, referring to sequences found only in the Atlantic, or ubiquitous, composed of sequences found in both Atlantic and Pacific individuals. This designation was based on alignment of sequences with control region sequences taken from 2 individuals from the Pacific, which were known to belong to the ubiquitous clade based on previous RFLP data and by comparison of the RFLP profiles of Atlantic samples known to belong to the Atlantic and ubiquitous clades based on previous studies to their control region sequences. The clades were present in similar frequencies, 47.4% (25 haplotypes) of individuals belonged to the Atlantic clade while 52.6% (29 haplotypes) belonged to the ubiquitous clade. The relative frequencies of Atlantic and ubiquitous clade haplotypes at each sampling location were as follows: WNA 40% Altantic, 60% ubiquitous, CAR; 45.5% Atlantic, 54.5% ubiquitous; WSA 53.8% Atlantic, 46.2% Ubiquitous and EA; 50% Atlantic and 50% Ubiquitous.

Although the distribution of the clades was relatively homogenous, they had very different diversity indices. The ubiquitous clade was generally much less diverse, having 141 polymorphic sites including 124 transitions and a single tranversion, while the Atlantic clade sequences had 210 polymorphic sites with 190 transitions and 9 transversions. Nucleotide sequence diversity within the ubiqitous clade was 1.7% with a mean of 15.18 ± 6.98 $s_{\bar{x}}$ pairwise differences between sequences while nucleotide sequence within the Atlantic clade was 6.2% with a mean of 54.97 ± 24.5 $s_{\bar{x}}$ pairwise differences between sequences. The Atlantic and ubiquitous clades were very divergent from one another. The mean corrected nucleotide sequence divergence between clades was 173.66 (corrected value = 138.01) and the ϕ_{ST} between clades was 0.799 ($P < 0.0001$).

Discussion

Analysis of mtDNA control region sequences of blue marlin taken from throughout their Atlantic range showed no evidence of structuring among locations. This corroborates the results of previous studies of blue marlin demonstrating a lack of detectable structure within the Atlantic. Buonaccorsi et al. (1999) compared three classes of molecular markers with a wide range of mutation rates to look for evidence of population structure within blue marlin taken from three Atlantic and four Pacific locations over five years; although not all collection locations were used for all marker classes. The 2 slower evolving marker classes, scnDNA loci, which were surveyed at 2 Altantic and four Pacific locations, and allozymes, which were surveyed from 2 Atlantic and a single Pacific location, revealed significant differences in the distribution of allele frequencies between collections from different oceans. Data based on allozymes were insufficient to allow analysis of intra-ocean divergence, but analysis of the scnDNA loci did not reveal significant intra-ocean diver-

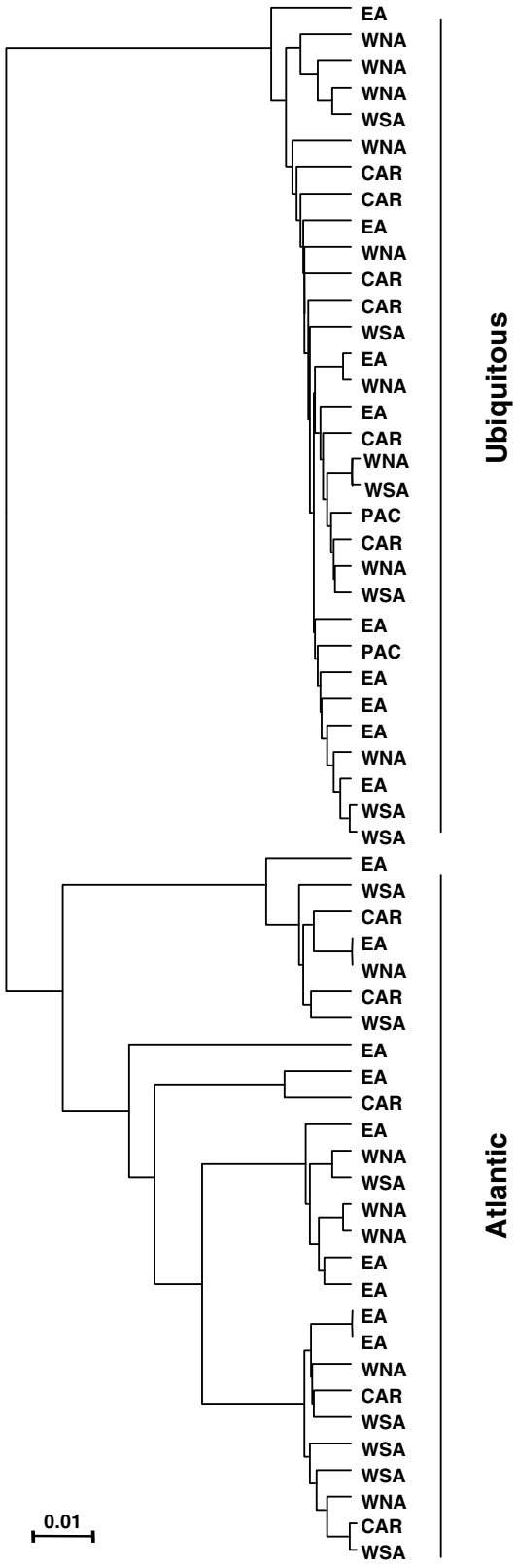

Figure 1. UPGMA tree of blue marlin haplotypes based on a Tamura-Nei distance. Abbreviations are the western North Atlantic (WNA), the Caribbean Sea (CAR), the western South Atlantic (WSA), the eastern Atlantic (EA), and Pacific (PAC).

gence within either ocean. Likewise, RFLP data based on the more rapidly evolving mitochondrial DNA molecule revealed significant inter-ocean divergence. A subsequent study by Buonaccorsi et al. (2001) compared results of previous data with data from five highly polymorphic tetranucleotide nuclear microsatellite markers. Results showed a level of inter-ocean divergence similar to that seen using mtDNA RFLP data, but no effort was made to look at intra-ocean collections; Atlantic collections in the study were limited to samples taken in the Caribbean (Puerto Rico and Jamaica). A later study by Graves and McDowell (2001) used the same loci as Buonaccorsi et al. (1999) and Buonaccorsi et al. (2001) and also included RFLP analysis of the mitochondrial cytochrome *b* and the nuclear RP2 intron gene regions. In addition, the study included samples taken from the eastern Atlantic (Ghana) and western South Atlantic (Brazil). As in previous studies, there was no evidence of significant spatial heterogeneity among Atlantic blue marlin collections. The results of these studies agree that there is a significant barrier to gene flow between Atlantic and Pacific blue marlin populations, but none show evidence of genetic structure within ocean basins.

The lack of observed of genetic stock structure in Atlantic blue marlin in the current study is also consistent with results of tagging studies demonstrating that blue marlin are capable of trans-oceanic and trans-equatorial migrations in the Atlantic as well as fisheries data showing that blue marlin are continuously distributed across the 5°N latitude throughout the year. In addition, data from Nakamura (1985) suggests that blue marlin spawn over a wide area and have a protracted spawning period. This high migratory capability highlights the potential for gene flow. These results are consistent with the results of the recent study of the white marlin by Graves and McDowell (2006), which also failed to detect stock structure based on analysis of genetic data.

The current study was able to overcome some of the limitations of previous investigations, such as a lack sufficient geographic coverage to allow for a robust test of the null hypothesis that blue marlin do not exhibit stock structure within the Atlantic Ocean. In addition, unlike previous studies, this study includes a comparison of samples of blue marlin taken in the same calendar year to eliminate sampling error associated with sampling across multiple years, although each sample in the current study likely included animals spanning several year classes. The current study does have several limitations, including the relatively low samples sizes from each location (n = 11–18). It is possible that samples sizes were insufficient to detect genetic structure, although neither the distribution

of haplotypes nor the relationship among haplotypes was significantly associated with collection location. Another potentially more serious problem involves the age of the fish examined. It is possible that blue marlin exhibit genetic stock structure but that this structure may not be evident if fish were sampled from a mixed stock (i.e., on feeding grounds or on migratory routes; see Bowen et al. 2005 for an in-depth discussion). To overcome this limitation, future samples either need to be taken when the stocks are separated i.e., during spawning, or need to be comprised of young animals before they are capable of extensive dispersal. No evidence of sex-biased dispersal was found in a previous study of blue marlin (Buonaccorsi et al. 2001), however the possibility that genetically distinct stocks may mix at certain times has not been examined, and many studies have shown that highly migratory marine species have complex life histories that can confound the presence of underlying structure (Carlsson et al. 2004, Hueter et al. 2004).

As in the present study, previous studies of blue marlin using mitochondrial RFLP analysis revealed the presence of 2 distinct mitochondrial clades, one present only in the samples taken from the Atlantic and one present in both Atlantic and Pacific samples. This pattern of cladogensis has also been observed in other large pelagics including sailfish (Graves and McDowell 1995, McDowell and Graves 2002), swordfish (Alvarado-Bremer et al. 1996, 2005, Rosel and Block 1996) and bigeye tuna (Chow et al. 2000, Durand et al. 2005, Martinez et al. 2006). The most common explanation for this recurring pattern involves vicariant isolation during the Pleistocene followed by secondary contact via unidirectional migration (Chow et al. 2000, Buonaccorsi et al. 2001, Graves and McDowell 2003, Alvarado-Bremer et al. 2005, Durand et al. 2005).

Although the distribution of clades in this study was relatively even overall (47.4% vs 52.6%), it is interesting to note that the Atlantic clade increased in frequency from 40% to 45.5% to 53.8% moving from north to south in the western Atlantic. Sample sizes were not sufficient to assess whether this trend was statistically significant, however if this pattern remains when larger sample sizes are examined, it would suggest that gene flow is limited. Future studies should examine larval and young-of-year fish before they are capable of dispersing for long distances. However, the current study provides a good baseline to elucidate the amount of genetic variation present and will be invaluable for comparison with larval samples in future studies.

As with previous methods, this study could not disprove the null hypothesis that geographic collections of Atlantic blue marlin were sampled from a single genetic stock. Based on the data in this study, there is no evidence to suggest that there are discrete stocks of blue marlin in the Atlantic or that blue marlin exhibit spawning site fidelity. This result is consistent with ICCATs current management of blue marlin as a single Atlantic-wide stock. However, it is important to note that although limited gene flow may be sufficient to prevent the accumulation of genetic divergence, this does not necessarily mean that interactions among blue marlin from different geographic areas are sufficient to prevent regional over-fishing from a management perspective. In other words, although there is little danger that over-harvesting of blue marlin from a single area will remove unique genetic variation, it is not possible to predict the rate at which migrants would supplement a locally depleted stock from this genetic analysis.

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