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GEOGRAPHIC DISTRIBUTION OF MOLECULAR VARIANCE WITHIN THE BLUE MARLIN (*MAKAIRA NIGRICANS*): A HIERARCHICAL ANALYSIS OF ALLOZYME, SINGLE-COPY NUCLEAR DNA, AND MITOCHONDRIAL DNA MARKERS

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Abstract.—This study presents a comparative hierarchical analysis of variance applied to three classes of molecular markers within the blue marlin (*Makaira nigricans*). Results are reported from analyses of four polymorphic allozyme loci, four polymorphic anonymously chosen single-copy nuclear DNA (scnDNA) loci, and previously reported restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA (mtDNA). Samples were collected within and among the Atlantic and Pacific Oceans over a period of several years. Although moderate levels of genetic variation were detected at both polymorphic allozyme (H = 0.30) and scnDNA loci (H = 0.37), mtDNA markers were much more diverse (h = 0.85). Allele frequencies were significantly different between Atlantic and Pacific Ocean samples at three of four allozyme loci and three of four scnDNA loci. Estimates of allozyme genetic differentiation (θ_O) ranged from 0.00 to 0.15, with a mean of 0.08. The θ_O values for scnDNA loci were similar to those of allozymes, ranging from 0.00 to 0.12 with a mean of 0.09. MtDNA RFLP divergence between oceans (θ_O = 0.39) was significantly greater than divergence detected at nuclear loci (95% nuclear confidence interval = 0.04–0.11). The fourfold smaller effective population size of mtDNA and male-mediated gene flow may account for the difference observed between nuclear and mitochondrial divergence estimates.

Key words.—Allozyme, billfish, blue marlin, comparative molecular evolution, genetic drift, Istiophoridae, *Makaira nigricans*, mitochondrial DNA, population genetics, single-copy nuclear DNA.

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The influence of selection, mutation rate, sex-biased migration, and effective population size on patterns of genetic variation within a species must be considered to reconcile data from various molecular markers. Notably different distributions of nuclear and mitochondrial DNA variation were detected within the American oyster (*Crassostrea virginica*), Atlantic cod (*Gadus morhua*), and green sea turtle (*Chelonia mydas*). In the American oyster, the geographic partitioning of genetic variation along the U.S. mid-Atlantic coast was much less pronounced in allozymes (Buroker 1983) relative to single-copy nuclear DNA (scnDNA; Karl and Avise 1992) or mitochondrial DNA markers (mtDNA; Reeb and Avise 1990), which suggests that balancing selection affected the allozyme polymorphisms (Karl and Avise 1992). However, a subsequent scnDNA study detected no difference in genetic divergence between scnDNA and allozyme loci in oysters also taken from mid-Atlantic and Gulf locations (McDonald et al. 1996). The discrepancy between the two scnDNA studies has led some authors to speculate that large variance in scnDNA population divergence estimates resulted from linkage of scnDNA loci to regions influenced by selective forces (FitzSimmons et al. 1997a).

In Atlantic cod, highly variable nuclear restriction fragment length polymorphisms (RFLPs; Pogson et al. 1995), single-locus minisatellite (Galvin et al. 1995), and microsatellite DNA markers (Bentzen et al. 1996) exhibited significant heterogeneity among populations across the North Atlantic and across the Scotian shelf. These findings contrast to those based on less polymorphic allożyme (Mork et al. 1985) and whole molecule mtDNA RFLP studies (Arnason et al. 1992; but see Dahle 1991) in the same species. Differences in the amount of population structure detected within and among marker classes in Atlantic cod have been attributed alternatively to balancing selection on allozyme polymorphisms (Pogson et al. 1995), directional selection on allozyme polymorphisms (Mork et al. 1985), directional selection on sites linked to scnDNA loci (Pogson et al. 1995), and increased discriminatory power in markers with higher mutation rates (Galvin et al. 1995; Bentzen et al. 1996).

In the green sea turtle, global analysis of scnDNA polymorphisms suggested moderate gene flow, whereas analysis of mtDNA indicated very restricted female gene flow. It was suggested that enhanced male-mediated gene flow accounted for observed differences between the two classes of markers (Karl et al. 1992). Regional analyses of gene flow among northern and southern Great Barrier Reef populations of green turtles similarly revealed highly significant heterogeneity in mtDNA RFLPs but nonsignificant divergence in nuclear DNA markers. However, direct testing of differential natal homing tendencies among sexes revealed that males and females were equally philopatric to their natal sites. Marker discrepancy was thus attributed to opportunistic mating along migrational pathways (Fitzsimmons et al. 1997b).

In this study, the influence of population-level processes on different molecular markers is investigated within blue marlin (*Makaira nigricans*). This species is circumtropically distributed and inhabits pelagic waters with surface temperatures greater than 22°C (Rivas 1975; Nakamura 1985). Tagging studies have revealed long-distance movements of individuals across the Atlantic Ocean (Scott et al. 1990; Anonymous 1994), from the Pacific to Indian Oceans (J. Pepperrell, pers. comm. 1997), and from Atlantic to Indian Oceans (Anonymous 1994; see Fig. 1). Although long-distance movements are notable, the majority of reported tag recaptures occurred in the general vicinity of release, even after

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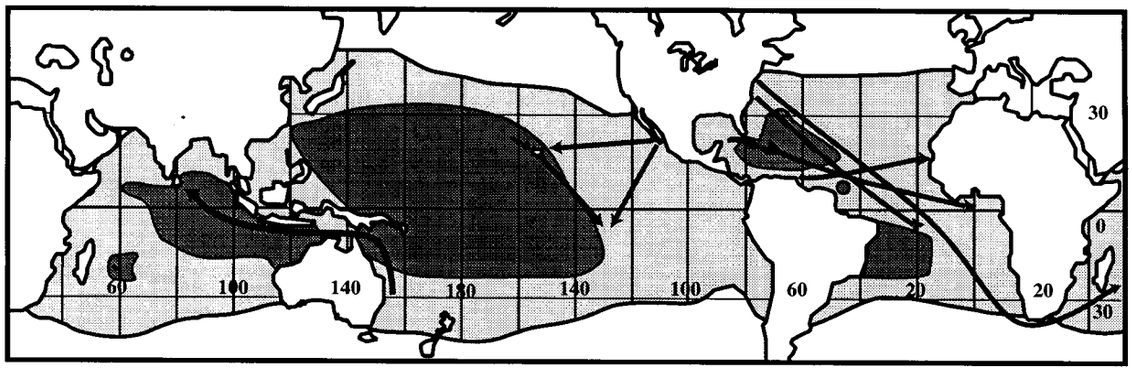

FIG. 1. Range and dispersal capability of blue marlin. Light shaded areas designate species' range (Nakamura 1974). Darker shaded areas designate larval distribution in the Atlantic (Bartlett and Haedrich 1968; Eschmeyer 1968; Ueyanagi et al. 1970) and Indo-Pacific (Howard and Ueyanagi 1965; Matsumoto and Kazama 1974; Nishikawa et al. 1985). Arrows indicate selected long-distance migration routes inferred from tag- and recapture-data in the Atlantic (Scott et al. 1990; Anonymous 1994; Bayley and Prince 1994) and Indo-Pacific Oceans (Anonymous 1996; J. Pepperrell, pers. comm. 1997).

years at liberty (Anonymous 1996). Spawning appears to occur over extensive geographic regions, based upon the distribution of larvae and adults with mature gonads (Strasburg 1969; Matsumoto and Kazama 1974). Evidence for discrete spawning cycles near island chains also has been reported (Hopper 1990).

Despite the circumtropical distribution and capacity for extensive gene flow within blue marlin, mtDNA evidence suggests limited exchange between ocean basins. Finnerty and Block (1992) sequenced a 612-bp region of the mitochondrial cytochrome *b* gene of 26 blue marlin and detected two divergent clades of haplotypes. These clades occurred at significantly different frequencies in Atlantic and Pacific collections. Similar findings were reported in an analysis of whole-molecule mtDNA RFLPs (n = 114; Graves and McDowell 1995). The RFLP data were characterized by high levels of within-ocean variation and significant divergence in haplotype frequencies between ocean samples. Haplotypes comprised two divergent clades, one of which was present only in the Atlantic at a frequency of approximately 40%.

The present study characterizes genetic variation within blue marlin at scnDNA and allozyme loci and examines the degree to which pronounced interocean mtDNA divergence was corroborated by nuclear loci. Independent genetic loci respond differently to species' history due to stochastic genetic drift, variation in mode of inheritance, and variation in mutation rate among marker classes. Allozyme and scnDNA nuclear loci are diploid and biparentally inherited. In contrast, mtDNA is effectively a single locus, with haploid, maternal inheritance resulting in a fourfold lower effective population size than at nuclear markers. Due to lower effective population size, the influence of genetic drift is enhanced at mtDNA loci. Due to maternal inheritance, mtDNA provides information only on female gene flow. Allozyme, scnDNA, and mtDNA loci may also exhibit a broad range in heterozygosity levels within a single species (Angers et al. 1995; Jorde et al. 1995; Bentzen et al. 1996), presumably due to variation in mutation rate. It is commonly assumed that polymorphism at most molecular markers is selectively neutral. However, selection may influence polymorphism at mtDNA (William et al. 1995), allozyme (Simmons et al. 1989), or scnDNA loci if the polymorphism is subject to functional constraints or tightly linked to regions influenced by selection (Pogson et al. 1995; Fitzsimmons et al. 1997a). Because selection tends to act independently on individual loci, the influence of selection on an estimate of gene flow is minimized when the estimate is based on several loci. The markers used to estimate gene flow in this study may, therefore, differ in apparent or realized mutation rate and susceptibility to selective pressures, as well as effective population size and mode of inheritance. From comparison of the geographic distribution of genetic variation at mtDNA and a series of nuclear markers, one may identify the effects of selection, maternal philopatry, effective population size, and mutation rate on each marker class.

MATERIALS AND METHODS

Blue Marlin Tissue Samples.—Blue marlin eye, liver, and heart tissues were obtained over several years in the Atlantic (U.S. mid-Atlantic coast and Jamaica) and Pacific Oceans (Hawaii, Mexico, Ecuador, and eastern Australia; Table 1). Samples were obtained from fish landed at sport fishing tournaments, artisinal fisheries, and research cruises. Tissues were dissected within eight hours of death, chilled on ice, and subsequently frozen at −20°C. Samples were transported to the laboratory frozen on dry ice or a commercial ice substitute and maintained at −70°C until analysis.

Allozymes.—Horizontal starch gel electrophoresis was conducted following the protocols of Murphy et al. (1990) and Shaklee et al. (1990a). After initial screening, 33 enzyme systems were run on one of seven buffer systems resulting in 44 presumptive loci. Enzyme nomenclature follows Shaklee et al. (1990b). The following loci were surveyed: *Aat-1, Aat-2, Aatm, Acoh-1, Adh, Ada-1, Cbp-1, Cbp-2, Cbp-3, Ck-A, Ck-B, Ddh, Est-1, Est-2, Est-D, Eno, Fum, Gapdh-1, Gapdh-2, G3pdh–1, Gcdh, G6pdh–1, Gpi-1, Gpi-2, Idh, Iddh, Ldh-1, Ldh-2, αMan, Mdh-1, Mdh-2, Mdhp-1, Mdhp-2, Mpi, Pgk, Ala-Met, Gly-Leu, Gly-Leu-Leu, Leu-Pep, Leu-Tyr, Pgdh, Pgm, Sod,* and *Xdh.* Details on the buffer system used for each

TABLE 1. Blue marlin samples. For single-copy nuclear DNA (ScnDNA) markers ocean, location, and year form components of a hierarchical analysis of molecular variance (AMOVA). *N* refers to the number of individuals sampled.

Ocean	Location	Year	Abbreviation	Scn-DNA *N*	Allozyme *N*	Mt-DNA[1] *N*
Atlantic	Puerto Rico	1990	PR90		12	
		1991	PR91		12	31
	Jamaica	1991	JM91	48	20	25
		1992	JM92	55		
		1993	JM93	42		
		1994	JM94	45		
		1995	JM95	24		
	Eastern USA					
	New Jersey	1994	NJ94	11		
	North Carolina	1992	NC92	12		
Pacific	Hawaii	1990	HI90		54	26
		1992	HI92	30		32
		1993	HI93	48		
		1994	HI94	25		
	Eastern Pacific					
	Mexico	1994	MX94	49		
		1995	MX95	24		
	Ecuador	1995	EC95	19		
	Australia	1991	AU91	10		
		1994	AU94	15		

[1] Mitochondrial DNA samples from Graves and McDowell (1995).

enzyme and tissue type surveyed are available from Morgan (1992).

Single-Copy Nuclear Loci.—The protocols of Karl and Avise (1993) were used for selection of scnDNA loci amplified by the polymerase chain reaction (PCR) with the following modifications and details. Total genomic DNA was isolated following Sambrook et al. (1989) and digested with *Pst I* (Gibco BRL, Gaithersburd, MD). Fragments in the 500–2000-base pair (bp) size range were transferred to DE-81 paper by electrophoresis (Whatman International Ltd., Maidstone, U.K.), ligated into the plasmid vector Bluescript KS$^+$ (Stratagene, La Jolla, CA) and transformed into *Escherichia coli* DH5 α competent cells following the protocol of Sambrook et al. (1989). Copy number was determined through genomic Southern blot analysis (Southern 1975). In this procedure, genomic DNA was digested with three restriction enzymes (*Pst I, EcoR I, BamH I*) and probed with plasmid vectors containing cloned DNA fragments that were labeled using the BioNick labeling system (Gibco BRL). Hybridization and detection reactions were performed following the manufacturer's protocols for BluGene Nonradioactive Nucleic Acid Detection System kits (Gibco BRL). The number of bands that appeared for each enzyme approximated the copy number of the cloned fragment (Southern 1975). The flanking 200–400 bp of single- and low-copy clones were sequenced (Sanger et al. 1977), with Sequenase Version 2.0 kits (U.S. Biochemical, Cleveland, OH). Primers were designed with the assistance of the computer program PC/GENE (Bairoch 1989). GenBank was searched for matches between published sequences and nuclear sequences obtained in the study. Open reading frames were searched for with PC/GENE (Bairoch 1989), using the method of Fickett (1982).

TABLE 2. Single-copy nuclear DNA restriction endonuclease survey. Complete sequences were obtained for two blue marlin, a striped and white marlin at WM13. Percent base pairs polymorphic was calculated as (100[number of sites polymorphic]/[number of base pairs surveyed]), assuming one base-pair polymorphism per polymorphic restriction site. For BM32, the *Nci I* polymorphism was screened in population analyses.

Locus	Length (base pairs)	# base pairs surveyed[1]	Polymorphic enzymes	# sites polymorphic	% base pairs polymorphic
BM47	1900	134	*Bcl I*	1	0.75
BM32	1900	140	*Msp I/Dra I Dde I/Hinc II Hinf I/Nci I*	8	5.71
BM81	1700	98	*Dra I*	1	1.02
WM08	1200	114	*Ban II*	1	0.88
WM13	380	380		0	0.00
WM84	900	74		0	0.00
Overall	7980	940		11	1.17

[1] Based on restriction digestion with a battery of 20 to 50 restriction enzymes.

The polymerase chain reaction (PCR) was used to amplify target fragments from genomic DNA. Template nuclear DNA was obtained from the nuclear band of cesium chloride/ethidium bromide density gradient mtDNA purifications (Lansman et al. 1981). After dialysis, PCR was performed using the BRL PCR Reagent Systems (Gibco BRL), with a final concentration of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 μM of each primer, approximately 50 ng template DNA, and 1.125 units *Taq* polymerase in 50 μl total volume. Typical cycling conditions included an initial denaturation of 5 min at 95°C, followed by 40 cycles of 1 min at 55°C, 1 min at 68°C, and 1 min at 95°C. Final extension was carried out for 7 min at 72°C. PCR primers were designed to amplify genomic fragments less than 2000 bp in length.

Ten to 20 blue marlin from different collections were screened for polymorphism by digestion of five to 10 microliters of amplified DNA with 20 to 50 randomly selected restriction enzymes. Digested products were electrophoresed on a 2.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. DNA samples were subsequently digested with a single polymorphic restriction enzyme at each locus to generate allele frequencies (Table 2). Genotypes were scored for presence or absence of a restriction site.

Statistical Analyses.—Nonparametric, exact-significance testing methods (exact θ significance tests and exact probability tests) were used to evaluate conformance to linkage and Hardy-Weinberg genotypic equilibria and homogeneity of spatial and temporal distributions of allele frequencies. Unbiased estimators of exact significance probabilities were calculated using the Markov chain algorithm of Guo and Thompson (1993) for tests of linkage equilibria, Hardy-Weinberg equilibria, and population divergence. Tests were performed using the computer program GENEPOP, with a Markov chain length of 500,000 steps (Raymond and Rousset 1995). Type I error was controlled using the sequential Bonferroni method (Rice 1989).

The Weir and Cockerham (1984) method of generating unbiased, hierarchical *F*-statistics was chosen to analyze patterns of genetic diversity within and between populations. This method incorporates explicit corrections for sample size and number of populations surveyed, which facilitates comparison of different marker classes (Excoffier et al. 1992). A hierarchical analysis of variance was performed as described in Weir (1996), using the computer program Genetic Data Analysis (GDA; Lewis and Zaykin 1997). Hierarchical levels are outlined in Table 1. Subscripts for variance components (σ^2) and *F*-statistics (θ) were designated as follows: *O,* oceans; *L,* geographic locations within oceans; *Y,* years within geographic locations; *I,* individuals within years; and *A,* alleles within individuals. F_{IS} or *f,* the local inbreeding coefficient (Weir and Cockerham 1984), was calculated with the program GENEPOP; *f*-values represent the quantity $(1 - [Ho/He])$ with correction for sample bias following Weir and Cockerham (1984), where *Ho* and *He* refer to observed and expected heterozygosities, respectively, measuring the deviation of each sample from conformance to Hardy-Weinberg expectations ($f = 0$). The contribution to the overall hierarchical analysis of years within each geographic location and geographic locations within each ocean was subsequently examined. The 95% confidence intervals (CIs) of θ values were estimated from 15,000 bootstrap replicates over loci, performed using the computer program FSTAT (Goudet 1995). Exact probability tests (Raymond and Rousset 1995) and exact θ permutation procedures (Goudet 1995) were used to determine significance of population subdivision within a hierarchical level. Pairwise tests for population differentiation were performed using the exact probability test.

Estimates of the number of migrants (N_em) between oceans were calculated from ($\theta = 1/[4N_em + 1]$), assuming an island model of migration (Wright 1969). Neighbor-joining dendrograms (Saitou and Nei 1987) were constructed for each polymorphic locus and over all four polymorphic loci (0.05 criterion) using Nei's (1978) unbiased genetic distance from GDA. Nei's (1972) genetic distance (*D*) also was calculated for comparison to previous works. Allele-frequency CI values were estimated as binomial variables assuming a normal distribution of allelic counts, taking into consideration deviations from Hardy-Weinberg equilibrium (Weir 1996).

To relate the mitochondrial variation reported in Graves and McDowell (1995) to the nuclear data from this study, an analysis of molecular variance (AMOVA) was performed (Excoffier et al. 1992), which generates *F*-statistics comparable to the θ values of Weir and Cockerham (1984). Hierarchical *F*-statistics were calculated: (1) with haplotype distances input as squared Euclidean distances, calculated as the number of restriction site differences between haplotypes (Φ); (2) without haplotype distance information considered (θ), and (3) with haplotypes defined as belonging to either Atlantic or ubiquitous clades (binned θ). Data were analyzed in hierarchical fashion, with Atlantic and Pacific populations consisting of two subpopulations each, as shown in Table 1 (Graves and McDowell 1995). Significance of mitochondrial *F*-statistics was evaluated as described in Excoffier et al. (1992) using exact *F* permutation procedures. Because the intraocean levels for allozyme and mtDNA RFLP data represent a mix of geographic and temporal samples (Table 1), the focus of the analysis for these markers was on the interocean level of variance. Estimates of the effective number of female migrants (N_em_f) between oceans were calculated from mitochondrial data using ($F = 1/[2N_em_f + 1]$). The influence of selection on mtDNA diversity was evaluated using Tajima's (1989) test of selective neutrality, as implemented by Arlequin version 1.1 (Schneider et al. 1997). This test compares estimates of the parameter $2N_eu$ calculated from the number of segregating sites and the average number of pairwise differences among mtDNA haplotypes, where N_e refers to effective population size and u to mutation rate.

Results

Allozymes.—A total of 107 individuals from one Pacific and three Atlantic samples was screened for allozyme variation, 20 of which were included in scnDNA analyses (Table 1). Four of 44 (9%) allozyme loci were polymorphic at the 0.05 criterion. The resulting average heterozygosity for the four polymorphic loci was 0.30, and over all 44 loci, an average heterozygosity of 0.0285 was calculated. Significant deviations from Hardy-Weinberg equilibrium were not detected at any polymorphic allozyme locus ($P > 0.08$). The θ_O-values for polymorphic allozyme loci averaged 0.077 with a 95% bootstrap CI ranging from 0.011 to 0.138. Significant allele frequency differences were detected at three of four loci (Tables 3, 4). Based on the four polymorphic loci, an average genetic distance of 0.038 separated Atlantic and Pacific samples (Table 4), and over all 44 loci an interocean *D* of 0.003 was calculated.

Single-Copy Nuclear DNA.—A total of 145 plasmid DNA clones with insert sizes ranging from 500 to 2000 bp was generated. Eighteen randomly selected clones were screened for copy number by using genomic Southern blot analysis. Of these, 13 were estimated to be single-copy, three low-copy, and two high-copy clones. Six of 13 single-copy clones were chosen for partial sequencing. Primers were developed from five of the sequenced clones, and those producing a single, reliable, polymorphic amplification product were selected for further analysis (see Appendix). In addition, three primer pairs (WM08, WM13, WM84) from a white marlin (*Tetrapturus albidus*) mini-DNA library of plasmid clones also were tested on blue marlin DNA. Six of these eight primer pairs reliably produced a single PCR product in blue marlin (Table 2). Four of these loci proved sufficiently polymorphic (0.05 criterion) for population genetic analysis (BM47, BM81, BM32, WM08). A total of 457 individuals from eight Pacific and seven Atlantic samples was screened for polymorphism at these four loci. A single restriction site polymorphism (RSP) was surveyed at each locus (Table 3). Blast searches of the GenBank database for a match of the partial scnDNA sequences to published sequences were nonsignificant (smallest sum probability $P > 0.084$; Altschul et al. 1990). No open reading frame larger than 200 bp was detected.

One of four scnDNA markers (BM32) exhibited a significant deviation from Hardy-Weinberg equilibrium for three of the 15 populations surveyed. Genotypic scoring did not vary over a broad range of PCR annealing temperatures (45–65°C), but did vary upon primer redesign. Amplifications

TABLE 3. Allele frequencies (A), confidence intervals (CI), and sample size per locus (2*N*) of Atlantic and Pacific blue marlin. Allele frequencies refer to frequency of the most common allele. All loci were diallelic. Confidence intervals were calculated following Weir (1996), not assuming Hardy-Weinberg equilibrium. 2*N* refers to the number of alleles surveyed (twice the number of individuals surveyed). Sample abbreviations follow Table 1.

Single-copy nuclear DNA:	BM47				BM81				BM32-2				WM08			
Sample	A		CI	2*N*	A		CI	2*N*	A		CI	2*N*	A		CI	2*N*
HI92	0.91	±	0.07	(58)	0.54	±	0.16	(48)	0.67	±	0.12	(64)	0.45	±	0.13	(60)
HI93	0.94	±	0.05	(96)	0.61	±	0.11	(88)	0.57	±	0.10	(90)	0.50	±	0.10	(86)
HI94	0.82	±	0.13	(50)	0.68	±	0.13	(50)	0.48	±	0.11	(50)	0.56	±	0.15	(48)
Hawaii	0.90	±	0.04	(204)	0.61	±	0.08	(186)	0.58	±	0.07	(204)	0.50	±	0.07	(194)
MX94	0.95	±	0.04	(98)	0.60	±	0.08	(98)	0.58	±	0.10	(98)	0.42	±	0.09	(90)
MX95	0.92	±	0.08	(48)	0.72	±	0.10	(46)	0.60	±	0.14	(48)	0.52	±	0.16	(44)
EC95	0.92	±	0.09	(38)	0.50	±	0.17	(38)	0.53	±	0.18	(38)	0.50	±	0.16	(36)
E. Pacific	0.94	±	0.04	(184)	0.61	±	0.06	(182)	0.58	±	0.07	(184)	0.47	±	0.07	(170)
AU91	1.00	±	0.00	(18)	0.71	±	0.20	(14)	0.50	±	0.24	(18)	0.42	±	0.16	(12)
AU94	0.97	±	0.07	(30)	0.57	±	0.24	(28)	0.59	±	0.22	(32)	0.43	±	0.17	(30)
Australia	0.98	±	0.04	(48)	0.62	±	0.17	(42)	0.56	±	0.15	(50)	0.43	±	0.12	(42)
JM91	0.91	±	0.06	(96)	0.81	±	0.10	(84)	0.35	±	0.10	(92)	0.79	±	0.09	(66)
JM92	0.94	±	0.04	(108)	0.81	±	0.07	(110)	0.39	±	0.10	(108)	0.67	±	0.09	(92)
JM93	0.89	±	0.07	(84)	0.72	±	0.10	(82)	0.38	±	0.10	(84)	0.83	±	0.08	(78)
JM94	0.96	±	0.04	(90)	0.67	±	0.10	(90)	0.32	±	0.10	(92)	0.62	±	0.11	(82)
JM95	0.85	±	0.10	(48)	0.81	±	0.10	(48)	0.31	±	0.13	(48)	0.79	±	0.10	(48)
Jamaica	0.92	±	0.03	(426)	0.76	±	0.04	(414)	0.35	±	0.05	(424)	0.73	±	0.05	(366)
NC92	0.96	±	0.09	(24)	0.73	±	0.17	(22)	0.25	±	0.16	(24)	0.58	±	0.22	(24)
NJ94	0.91	±	0.13	(22)	0.73	±	0.22	(22)	0.23	±	0.22	(22)	0.80	±	0.17	(20)
U.S.	0.94	±	0.08	(46)	0.73	±	0.14	(44)	0.24	±	0.13	(46)	0.68	±	0.15	(44)
Allozymes:	*Acoh*				*Adh*				*G3pd*				*Iddh*			
Sample	A		CI	2*N*	A		CI	2*N*	A		CI	2*N*	A		CI	2*N*
Atlantic	0.89	±	0.06	(106)	0.85	±	0.08	(106)	0.77	±	0.09	(102)	0.86	±	0.07	(100)
Pacific	1.00	±	0.00	(108)	0.59	±	0.11	(102)	0.62	±	0.10	(102)	0.86	±	0.09	(86)

were performed with a redesigned reverse primer BM32R–2 in combination with the initial BM32F primer. There was a substantial shift in deviation from Hardy-Weinberg equilibrium upon reamplification of all samples, from a strong heterozygote deficiency (mean $f = 0.37$) toward equilibrium (mean $f = 0.01$). After adjustment, significant deviations from the null hypothesis ($f = 0$) were not detected, which is consistent with Mendelian inheritance at these loci. Deviations from linkage equilibrium were nonsignificant after corrections for multiple tests ($P > 0.002$; initial $\alpha = 0.00055$). In summary, the four polymorphic scnDNA loci used in population screening were statistically independent, noncoding DNA regions whose terminal sequences did not match any known sequences.

TABLE 4. Nuclear marker comparison of diversity and interocean divergence. *F*-statistics (θ_O, Weir and Cockerham 1984), genetic distances (D_1, Nei 1972; D_2, Nei 1978), and heterozygosity (*H*, Nei 1978) were based on polymorphic loci. *P*-values represent the probability that θ_0 is not greater than zero, permuting alleles among populations; 95% CI, 95% confidence intervals calculated from bootstrap replicates over loci; N_em, the effective number of migrants inferred from θ_O-values; inf, infinite numbers of migrants.

Class	Locus	*H*	θ_O	*P*	N_em	D_1	D_2
Single-copy nuclear DNA:	BM47	0.14	−0.003	0.892	inf		
	BM81	0.43	0.052	< 0.001	4.6		
	BM32-2	0.47	0.102	< 0.001	2.2		
	WM08	0.45	0.123	< 0.001	1.8		
	Overall	0.37	0.086	< 0.001	2.7	0.068	0.054
	95% CI		(0.038–0.110)		(6.3–2.0)		
Allozyme:	*Acoh*	0.11	0.107	< 0.001	2.09		
	Adh	0.40	0.146	< 0.001	1.46		
	G3pdh	0.43	0.050	0.011	4.78		
	Iddh	0.24	−0.012	0.999	inf		
	Overall	0.30	0.077	< 0.001	3.00	0.038	0.034
	95% CI		(0.011–0.138)		(22.0–1.6)		
Nuclear:	Overall	0.33	0.08	< 0.001	2.875		
	95% CI		(0.042–0.111)		(5.7–2.0)		

TABLE 5. Hierarchical analysis of molecular variance based on single-copy nuclear DNA loci. Values represent percentage of total variance. Source of variance components are: σ_A^2, alleles within individuals; σ_I^2, individuals within years; σ_Y^2, years within geographic locations; σ_L^2, locations within oceans; and σ_O^2, between oceans. Negative values result from bias corrections when the true percentage is near zero.

Variance component	BM47	BM81	BM32-2	WM08	Overall
σ_O^2	−0.305	5.164	10.244	12.261	8.554
σ_L^2	0.000	−1.138	−0.010	−0.811	−0.557
σ_Y^2	0.555	0.842	−0.387	1.157	0.520
σ_I^2	3.471	−1.366	−0.483	−7.808	−2.350
σ_A^2	96.279	96.497	90.646	95.201	93.833

Within-population variation (among alleles), designated σ_A^2, represented the largest proportion of overall variation at each locus (Table 5). A negligible fraction of overall variation was attributed to temporal (σ_Y^2) and intraocean (σ_L^2) components of variance. Significant temporal variation was not detected at any scnDNA locus among samples from Hawaii, Mexico, Australia, Jamaica, or the continental United States (Table 3). Tests for frequency differences using the exact θ and exact probability tests were in close agreement. A minor inconsistency between the two tests occurred at a borderline P-value in the interannual comparisons of Jamaican samples at the WM08 locus. All 95% θ_Y CIs included negative values, which indicates nonsignificance. Significant intraocean heterogeneity was not detected among samples within Pacific or Atlantic Oceans. Overall and pairwise θ_L estimates were nonsignificant at all loci and θ_L CIs included negative values. Neighbor-joining trees generated from each locus and over all scnDNA loci supported a lack of structure within ocean basins because temporal population replicates were intermingled among geographic locations (see Fig. 2).

Interocean divergence (σ_O^2) formed a substantial component (up to 12%) of overall variance (Table 5). Three of four scnDNA loci demonstrated significant allele frequency differences between samples from the Atlantic and Pacific Oceans (Table 4). The θ_O values between the two oceans for scnDNA loci averaged 0.086 with a 95% CI ranging from 0.038 to 0.110 (Table 4). Atlantic and Pacific populations were separated by an average D of 0.055 (Table 4; Fig. 2). An average nuclear θ_O of 0.08 was calculated over all polymorphic allozyme and scnDNA markers, with 95% bootstrap CI values ranging from 0.042 to 0.111. Corresponding N_em values were 2.0 to 5.7 migrant individuals per generation (Table 4). In phenogram analysis, two loci (BM32-2 and WM08) separated Atlantic and Pacific samples (Fig. 2), which illustrates cohesiveness of samples within oceans. BM81 was less discriminatory, with mixing of three Atlantic and two Pacific populations. At the 0.01 level, zero, nine, 24, and 25 of 56 interocean pairwise comparisons of allele frequencies were significant for BM47, BM81, BM32-2, and WM08, respectively. Sixteen of 224 interocean pairwise comparisons were significant after corrections for multiple tests over all loci (see Fig. 3).

Mitochondrial DNA.—Reanalysis of the blue marlin mtDNA RFLP data from Graves and McDowell (1995) included 114 individuals from two Pacific and two Atlantic samples, 67 of which were included in allozyme analysis and 55 of which were included in scnDNA analysis (Table 1). When haplotype distances were input as squared Euclidean distances, the mtDNA AMOVA Φ_O was calculated as 0.25 ($P < 0.001$), which corresponds to $N_em_f = 1.5$. Without haplotype distance information considered, an AMOVA θ_O of 0.06 ($P < 0.001$) was calculated ($N_em_f = 7.8$), and when haplotypes were binned into either ubiquitous or Atlantic clades (Graves and McDowell 1995), a θ_O of 0.39 ($P < 0.001$) was calculated ($N_em_f = 0.78$). Tajima's (1989) D-statistic was not significantly different from zero for any of the four mtDNA samples (range −1.57–1.19; $P > 0.05$), which indicates that the null hypothesis of selective neutrality could not be rejected.

To investigate whether individuals belonging to the two highly divergent mtDNA clades comprised separate nuclear gene pools, blue marlin were tested for scnDNA allele frequency divergence between Atlantic individuals belonging to the two mtDNA clades. A total of 226 individuals from the Atlantic Ocean was assigned to either Atlantic or ubiquitous clades based on whole molecule RFLP data (J. Graves, unpubl. data). Allele frequency differences were not detected at any of the four scnDNA loci in pairwise comparisons between Atlantic blue marlin belonging to the two mitochondrial clades ($P > 0.12$).

DISCUSSION

Allozymes.—The level of allozyme variation and interpopulational divergence revealed by this investigation falls within range of previous studies of blue marlin and other marine fishes. Shaklee et al. (1983) surveyed 35 allozyme loci in 95 blue marlin from Hawaii and detected a level of variation somewhat higher than that exhibited in this study when considering heterozygosity over all loci ($H = 0.061$ vs. $H = 0.029$) and percent polymorphic loci ($P = 20\%$ vs. $P = 9\%$). However, heterozygosity over polymorphic loci ($H = 0.29$ vs. $H = 0.30$) was similar, and the current study included a different set of allozyme loci. Similar to this study, Rosenblatt and Waples (1986) reported low levels of divergence between widely dispersed populations of 12 marine fish species using allozyme analysis. Nonsignificant trans-Pacific and interoceanic Ds ranged from less than 0.01 to 0.06. A trans-Pacific F_{ST} of 0.04 for milkfish (*Chanos chanos*) populations was revealed in another study (Winans 1980).

Single-Copy Nuclear DNA.—The majority of randomly selected genomic DNA fragments were single copy and demonstrated suitable levels of polymorphism for assessment of nuclear gene flow between distant geographic areas. Relative to the other scnDNA loci, BM32 demonstrated a high level of polymorphism, and it likely contains an appreciable level of variation undetected by RFLP analysis. In initial analysis of the BM32 locus, several populations demonstrated significant deviations from Hardy-Weinberg equilibrium. The isolated nature of this phenomenon, high variability of this locus, and correction of f-values upon primer redesign suggest that intraspecific variation within the BM32R priming site initially resulted in preferential amplification of alleles (Hare et al. 1996).

Assessment of temporal and intraocean homogeneity of

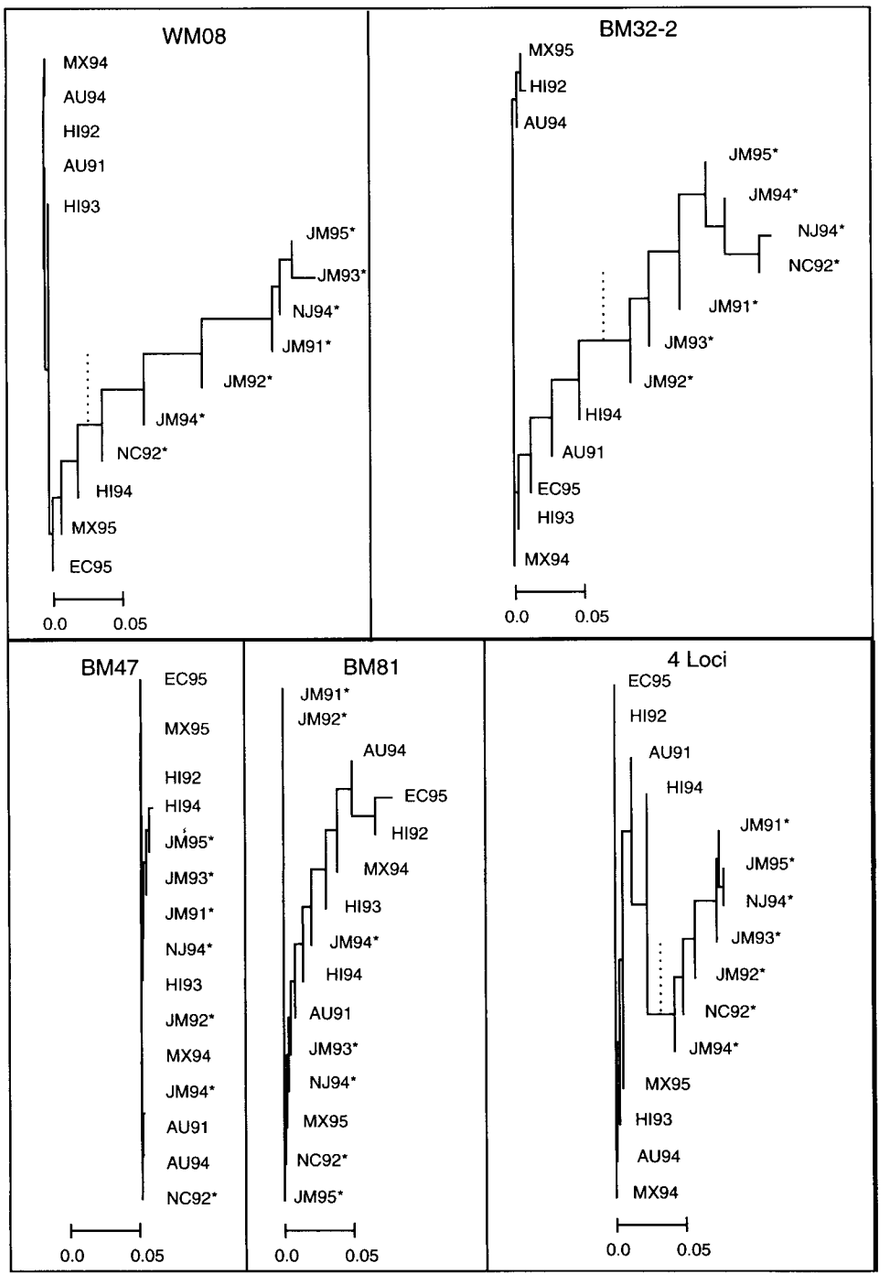

FIG. 2. Neighbor-joining trees generated from four polymorphic single-copy nuclear DNA markers shown individually and over all loci. Nei's (1978) unbiased genetic distance was used as a measure of divergence. Populations abbreviations follow Table 1. Dashed line indicates assumed midpoint root. Asterisks indicate Atlantic origin of sample.

allele frequencies established the independence of sampled frequencies on sampling year and location within an ocean from which the sample was obtained. Significant temporal heterogeneity was not detected among samples at any scnDNA locus. Tests included five consecutive years of samples from Jamaica and three years of samples from Hawaii. Both are areas of intense spawning activity (De Sylva 1974; Hopper 1990). Each blue marlin sample likely comprised a number of year classes because individuals reach maturity within two to three years and can survive for longer than 20 (Rivas 1975; Cyr et al. 1990; Hopper 1990; Prince and Brown 1994). Temporal stability indicates that frequencies are not heavily skewed by random sampling associated with either the formation of gametes among individuals or the

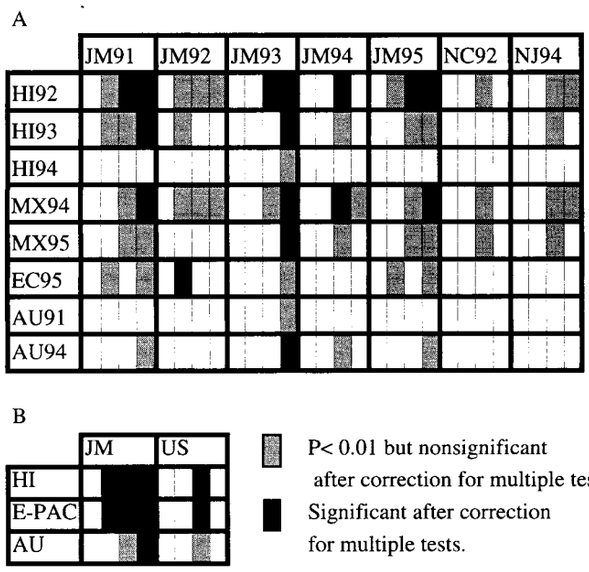

FIG. 3. Results of interocean pairwise tests for population differentiation. Tests were performed among all populations (A) and among geographic locations pooled over years (B). For each population comparison, boxes indicate the significance level for BM47, BM81, BM32–2, and WM08, respectively, as indicated in the legend. Within-ocean probability levels were greater than 0.01 except at WM08 for JM93 versus JM94 ($P = 0.005$; initial $\alpha = 0.00045$). Population abbreviations follow Table 1.

physical sampling of a population by an investigator (Weir 1996).

Analysis of scnDNA loci revealed nonsignificant intraocean divergence. The hypothesis of a single nuclear gene pool within either the Pacific or Atlantic Oceans was supported by nonsignificant θ_L-values, nonsignificant intraocean pairwise comparisons, and mixing of temporally replicated samples among geographic locations upon phenetic clustering of populations by genetic distance.

Concordance of Single-Copy Nuclear DNA and Allozyme Markers.—ScnDNA and allozyme markers revealed similar levels of genetic variation and population structure within and between Atlantic and Pacific samples of blue marlin, although a larger percentage of scnDNA loci were polymorphic. Considering polymorphic loci, the percentage of loci exhibiting significant interocean divergence and average θ_O-values were similar for allozyme and scnDNA marker classes. Furthermore, θ_O 95% bootstrap CIs were broadly overlapping. Further support for interocean structure at scnDNA loci included significant interocean pairwise comparisons of allele frequencies and phenetic separation of samples into ocean of origin at BM32-2, WM08, and overall neighbor-joining trees. Although significant, the level of nuclear divergence was low, which suggests some gene flow between oceans. A nuclear average N_em of 2.875 between oceans was estimated (range = 2.0 to 5.7 effective migrants). These values are sufficiently greater than the theoretical number of migrants per generation ($N_em = 1$) sufficient to prevent accumulation of fixed genetic differentiation between populations (Allendorf and Phelps 1981).

The range in levels of interoceanic divergence exhibited by allozyme and scnDNA loci presumably illustrates the stochastic and independent process of genetic drift and underscores the need for examination of a number of loci for estimating population parameters. Goudet (1995) has suggested that bootstrap CI values are most accurate with at least five informative loci. Although the combined nuclear estimate substantially increased the precision of the allozyme bootstrap CI values, the scnDNA estimate was only slightly improved.

Other comparative studies have suggested widespread balancing selection on allozymes, based on substantially lower allozyme divergence levels relative to nDNA values (Pogson et al. 1995) or both nDNA and mtDNA markers (Karl and Avise 1992). The broad overlap of divergence levels from allozyme and scnDNA nuclear markers in this study suggests that both sets of allele frequency distributions represent neutral markers in blue marlin. A similar concordance of polymorphic allozyme and scnDNA molecular markers was observed in one study of American oyster (*C. virginica*; McDonald et al. 1996).

The absence of haplotypes from the Atlantic mtDNA clade in the Pacific suggests a predominantly one-way migratory route of individuals from the Pacific into the Atlantic. If such one-way migration occurs, there could be a detectable difference in nuclear allele frequency within the Atlantic between individuals belonging to the two mitochondrial clades. However, tests at each of the four scnDNA loci failed to detect significant differences between these two groups, which indicates that random mating in the Atlantic has mixed nuclear genes of individuals belonging to the two mitochondrial clades.

Comparison of Nuclear and Mitochondrial Markers.—The degree of variation and interocean divergence revealed by both classes of nuclear markers was significantly less than that revealed by mtDNA (Graves and McDowell 1995). RFLP analysis of mtDNA (employing 11 enzymes) revealed 38 alleles in 114 individuals and a haplotype diversity (h) of 0.85. In contrast, only a single restriction site polymorphism was detected for three of four scnDNA loci when surveyed with 20 to 50 restriction enzymes. Phylogenetic analysis of the mtDNA RFLP data divided haplotypes into two highly divergent clades, one of which was present exclusively in the Atlantic at a frequency of 40%. It has been suggested that clades were formed during the Pleistocene when tropical zones were compressed, restricting interocean gene flow around South Africa for a number of tropical species (Graves and McDowell 1995). Mitochondrial interocean divergence was substantially larger when calculated with restriction site relatedness information between haplotypes ($\Phi_O = 0.25$) than without molecular information ($\theta_O = 0.06$). However, the former estimate may be affected by accumulation of historical genetic distance between oceans, which is irrelevant to current gene flow. The latter value underestimates interocean divergence, based on the average of a large number of small, phylogenetically unordered, haplotype frequency shifts. We suggest that the most appropriate estimator of interocean mitochondrial divergence is based on clade frequency alone ($\theta_O = 0.39$). Historical divergence between haplotypes is not considered in this estimate. Also, assignment of haplotypes

into clade is unambiguous, which reduces mtDNA to a diallelic system. This divergence estimate describes the sharing of clades between oceans, thus reflecting current gene flow under the assumption that the distribution of clades reflects migration-drift equilibrium. Mitochondrial DNA divergence is larger than average nuclear interocean divergence (θ_O = 0.08) and falls outside the 95% CI for nuclear loci (θ_O = 0.04–0.11).

Evolutionary Processes.—Mutation rate differences, natural selection, sex-biased migration, and effective population size could each contribute to contrasting results for mitochondrial and nuclear estimates of genetic divergence. Under an infinite-alleles model of evolution, markers with higher mutation rates generate greater diversity and thus allow rapid divergence to accumulate between isolated populations. The high mtDNA mutation rate and more rapid lineage sorting (relative to nuclear loci) presumably generated distinct clades during a historic condition of oceanic isolation. However, a significant correlation was not detected between interocean divergence and heterozygosity, considering polymorphic loci from all marker classes (Spearman's rank correlation test, $P > 0.1$; Abacus Concepts 1996). Furthermore, the mtDNA θ_O based on clade frequency was larger than the Φ_O estimate based on the entire restriction site dataset. These results suggest that the degree of clade sharing between oceans contributed more to the observed Φ_O than historical divergence between clades or more recent mutational events.

Strong selection on mtDNA polymorphism acting differentially between oceans could contribute to enhanced mtDNA divergence. However, failure to reject the null hypothesis of selective neutrality based on Tajima's (1989) *D*-statistic provides evidence that the influence of selection for common or rare mtDNA alleles is not pronounced within the areas surveyed. This observation is in accordance with the apparently homogeneous circumtropical pelagic environment. Selection also could have prevented divergence of nuclear allele frequencies during a period of allopatry. However, it is difficult to imagine the same selective pressures acting on both the presumably independent, noncoding, randomly selected nuclear DNA loci and the protein encoded allozyme loci.

Another factor contributing to lower divergence in nuclear markers relative to mtDNA could be preferential (and occasional) exchange of males between oceans. For blue marlin, sexual dimorphism in maximum size (females attain a maximum size three to four times larger than males; De Sylva 1974) invokes the possibility for sex-specific migrational activity. Evidence for sex-dependent migratory behavior includes predictable sex ratio shifts in spawning regions (Nakamura 1949; Nakamura et al. 1953; Erdman 1968; Hopper 1990) and differences in sex composition between regions in the Pacific Ocean (Kume and Joseph 1969). A paucity of tag returns and lack of sex identification in tagged blue marlin precludes inferences regarding relative magnitudes of male or female dispersal. It is worth noting, however, that many migratory blue marlin appear to be large females (E. Prince, pers. comm. 1997).

In a number of studies, greater divergence in mtDNA markers (relative to nuclear DNA) has been explained by invoking complex behavioral patterns, including male-mediated dispersal. These include studies of humpback whales in the North Pacific (Palumbi and Baker 1994) and North Atlantic (Larsen et al. 1996), three sympatric forms of brown trout (*Salmo trutta*; Ferguson et al. 1995), residential and anadromous forms of brown trout (Ferguson et al. 1995), global populations of the green turtle (*Chelonia mydas*; Karl et al. 1992), and green turtle populations on a regional scale (FitzSimmons et al. 1997a,b). The hypothesis of male-mediated gene flow between populations is supported by higher mitochondrial than nuclear divergence in allele frequencies and in some cases by behavioral observations. Although greater partitioning of mitochondrial diversity suggests the possibility of male-mediated gene flow, the unknown magnitude of genetic drift confounds the ability to identify sexually dimorphic behavioral patterns from such genetic data alone.

Among stable populations at equilibrium, one would expect mitochondrial markers, on average, to reveal fourfold greater divergence than nuclear markers due to the enhanced effect of genetic drift accompanying the smaller mtDNA effective population size. The mtDNA interocean divergence estimates incorporating allelic relatedness were greater than the nuclear average by a factor of three to five, which suggests that effective population size considerations alone are sufficient to explain the observed difference. When converted into numbers of migrants and corrected for mode of inheritance, a mitochondrial $N_e m_f$ of 1.50 and average nuclear $N_e m$ of 2.87 were obtained. These gene flow estimates are similar when one considers that the number of migrating females is expected to be half the total number of migrants.

Historical biogeographic factors may affect the distribution of genetic variation if equilibrium has not been reached under current conditions. Migration-drift equilibrium is approached at the rate $1/m$, where m represents migration rate in units of migrants per generation (Hartl and Clark 1989). For a given $N_e m$, as N_e decreases, m increases and time to equilibrium decreases. Population census sizes for blue marlin are poorly known. Adult population sizes of 40,000 and 400,000 individuals were inferred from Atlantic (Cramer and Prager 1994) and Pacific (Skillman 1989) stock production analyses, respectively. Based on these estimates, 7000 and 70,000 generations are necessary, respectively, for the blue marlin in the two oceans to reach equilibrium under one-way migration and an $N_e m$ of 2.8 (eq. 6.17 of Hartl and Clark 1989). For mitochondrial genes, equilibrium would be obtained between 1700 and 17,000 generations. If secondary contact were established in the late Pleistocene, that is, 10,000 years ago (at most 3,000 generations), the nuclear genome may not yet have reached equilibrium. If not, interocean divergence should be less pronounced at mtDNA than at the nuclear genome, assuming both genomes had diverged to a comparable degree during isolation, and are in the process of homogenizing. The larger mtDNA than nuclearDNA divergence detected in this study is consistent with migration-drift equilibrium in both genomes, which suggests that the lower population size in the Atlantic has affected a more rapid approach to equilibrium.

Contrary to the expectation of larger mitochondrial divergence resulting from enhanced genetic drift, greater population divergence was reported at nuclear loci among samples of broad whitefish (*Coregonus nasus*) in North Alaska (Patton

et al. 1997), cod (*Gadus morhua*) in the North Atlantic (Arnason et al. 1992; Galvin et al. 1995; Pogson et al. 1995; Bentzen et al. 1996), and brook trout (*Salvelinus fontinalis*) in eastern Canada (Angers et al. 1995; Jones et al. 1996). Greater nuclear divergence was attributed largely to the high mutation rate and sensitivity of microsatellite markers used relative to those of the mitochondrial markers.

Polymorphic allozyme and scnDNA markers yielded concordant estimates of average heterozygosity and population subdivision, which is consistent with the hypothesis that variation at both markers is selectively neutral. The genetic variation detected at nuclear and mtDNA genomes is consistent with a historical period of allopatry followed by recent mixing. The rapid mutation rate of mtDNA appears of little consequence in determining current mtDNA divergence relative to the degree of clade sharing. Migration-drift equilibrium expectations are sufficient to explain the lower nuclear than mitochondrial interocean divergence among ocean populations of blue marlin.

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APPENDIX

Locus	Primer sequence 5′–3′
BM47 F	GCTGTTGACCCAAACAATCCGG
BM47 R	GGGCATAAATGCTCAGGACACTT
BM32 F	TTGCGTCCTGTGGTACATAGTGG
BM32 R	ATTGGAGCAGTGGGTGCTGTC
BM32-2 F	GTAGCAAGGGGCTGTTGCATAG
BM32-2 R	GAGTCAGTGGTTCGGGATTTTATC
BM81 F	CACTCAAACAGGTGAATCCTGGC
BM81 R	CAAAACAACAGATGCCGCTAAGG
WM08 F	AGCAGCTAGGGACACACGATTCC
WM08 R	GGCAAACCTTACACTGAGGGGATG
WM13 F	CGTGAGCATGTGGCAAAGTAATG
WM13 R	CAGTTTATGGTTCATCTGGTGAGAGTG
WM84 F	TTTCTCTCGGTTAGGGACATCAAG
WM84 R	TCCTCTGGCAGATTAGGATAAG