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Indo-Pacific Population Structure of the Black Marlin, Makaira indica, Inferred from Molecular Markers

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Indo-Pacific Population Structure of the Black Marlin, *Makaira indica*, Inferred from Molecular Markers

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Master of Science

by
Brett Falterman

APPROVAL SHEET

This thesis is submitted in partial fulfillment of
the requirements of the degree of

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Brett Falterman

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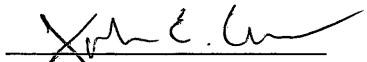

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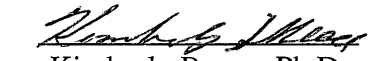

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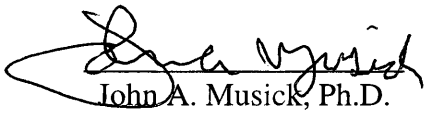

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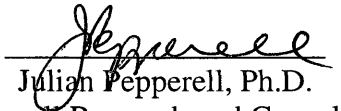

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ABSTRACT

The black marlin, *Makaira indica*, is the target of both commercial and recreational fisheries throughout its Indo-Pacific distribution, yet very little is known of the stock structure of this billfish. Restricted coastal distribution, isolated areas with high catch rates, and the seasonal occurrence of adults and larvae in specific areas have been used as evidence to support hypotheses of multiple stocks within the Indian and Pacific oceans. However, hypotheses of stock structure have not been rigorously tested. Molecular markers present an effective means for evaluating hypotheses of black marlin stock structure. This study employed different classes of molecular markers to determine if collections of black marlin from the Indian and Pacific oceans share a common gene pool and if the distribution of genetic variation within black marlin collections from a single location is stable over time.

Three classes of molecular markers previously found to reveal variation within istiophorid billfishes were employed to characterize the distribution of genetic variation within black marlin: mitochondrial DNA, single-copy nuclear DNA, and microsatellite DNA. High intraspecific variation was detected within the mtDNA control region (D-loop) and at five microsatellite loci. No variation was detected at seven single-copy nuclear loci screened in this study. MtDNA demonstrated temporal stability from two collection areas: Port Stephens, Australia ($p = 0.266$) and Taiwan ($p = 0.144$) and no significant differences were detected among geographic collections. Due to lack of temporal stability and poor conformance to the expectations for Hardy-Weinberg equilibrium, results were discounted for three microsatellite loci (GATA-52, GATA-08, and GATA-60). The other two microsatellite loci, GATA-10 and GATA-90, were temporally stable and conformed to expectations of Hardy-Weinberg equilibrium. No significant geographic heterogeneity was detected at either locus. These results are consistent with the hypothesis of gene flow across the range of the black marlin and a single genetic stock.

Indo-Pacific Population Structure of the Black Marlin, *Makaira indica*, Inferred from Molecular Markers

Chapter 1. INTRODUCTION

The black marlin, *Makaira indica*, is the target of both commercial and recreational fisheries throughout the tropical and subtropical waters of the Indian and Pacific oceans (Figure 1). Black marlin are caught as bycatch of the pelagic longline fishery for tunas and swordfish predominantly by Japanese, Chinese, Korean, and Taiwanese vessels. Black marlin are also taken in directed fisheries with nearshore drift-nets, harpoons, artisinal longlines, and rod and reel. Most recreational fisheries for black marlin have practiced catch and release over the past 10 years (Pepperell, pers. com). Catches of black marlin in the Pacific Ocean peaked at 6500 metric tons in 1956, fell to 3100 mt in 1959, and fluctuated between 2000 and 4000 mt between 1960 and 1975 (Wetherall, 1979). Indian Ocean catches of black marlin peaked at 2460 mt in 1968 and fell to 476 mt by 1976 (Yoshida, 1981). Substantial declines in catch were observed in both the Indian and Pacific oceans within less than 15 years of the inception of the pelagic longline fishery (Wetherall, 1979; Yoshida, 1981).

The status of pelagic fish stocks in the Indian and Pacific oceans is largely unknown. No agency is responsible for the management of these populations or the collection of fishery-dependent data over the entire region. Consequently, there are currently no management recommendations regarding the harvest of black marlin. Istiophorid species within the Atlantic Ocean have been fished to levels well below maximum sustainable yield (ICCAT 1996), indicating that billfishes are susceptible to overfishing. The same may be true for Indo-Pacific billfish populations.

Despite the importance of black marlin to commercial and recreational fisheries, little is known of its stock structure. Restricted coastal distributions, the presence of isolated areas with high catch rates, and seasonal occurrence of larvae and reproductively active adults in specific areas, have been cited as evidence for the existence of multiple stocks of black marlin within both the Pacific and Indian oceans (Figure 2). Shomura (1980) hypothesized a single eastern Pacific stock and either a single western Pacific stock, or separate northwestern and southwestern Pacific populations. Yoshida (1981) suggested the existence of multiple stocks within the Indian Ocean, although he did not delineate these units. Hypotheses of stock structuring for black marlin within and between oceans basins have not been rigorously tested.

Molecular markers present a powerful means for testing hypotheses of population structure (Avise, 1994). The application of genetic markers is a cost effective method to detect long term genetic differences that develop in the absence of gene flow. However, genetic analysis may not be appropriate for species with high gene flow (Waples, 1998). Results from genetic studies of continuously distributed pelagic fish species range from a lack of significant structuring on a global scale, to shallow but significant structuring within an ocean basin (Graves, 1998). Studies of the genetic population structure of istiophorid billfishes have uncovered intraspecific genetic heterogeneity using allozymes analysis (Morgan, 1992), restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA, (Graves and McDowell, 1994; Graves and McDowell, 1995; Graves et al., in review), RFLP analysis of single-copy nuclear DNA regions (Buonaccorsi et al., 1999), direct sequencing of mitochodrial DNA (Block et al., 1993; Finnerty and Block, 1992), and microsatellite DNA analyses (Buonaccorsi, 1998).

Species identification keys that include black marlin have been developed to distinguish between istiophorid billfishes (Innes et al., 1998; Chow et al., 1996, Graves and McDowell, unpub. data), but no previous studies have employed molecular markers to investigate the stock structure of black marlin. This study will examine the distribution of variation at specific microsatellite DNA markers, single-copy nuclear DNA (scnDNA) markers, and within the D-loop region of the mitochondrial genome.

Black Marlin Life History Characteristics

Black marlin are distributed throughout the tropical and sub-tropical waters of the Indian and Pacific oceans. The latitudinal range of black marlin as defined by Japanese commercial catches extends from 40° N to 45° S in the western Pacific, from 35° N to 30° S in the eastern Pacific, and throughout the Indian Ocean southward to 45° S (Nakamura, 1985). Individuals have been found to migrate into the Atlantic Ocean by way of the Cape of Good Hope, but there is no evidence to suggest the existence of a persistent Atlantic population (Nakamura, 1985).

Like other istiophorids, the black marlin is an epipelagic predator inhabiting surface waters between 15 and 30° C and is often found in nearshore areas adjacent to large land masses, islands, and coral reefs (Nakamura, 1985). Black marlin exhibit a higher degree of association with coastal and shelf waters than most other billfishes (Nakamura, 1985). Feeding habits vary between areas and analyses of stomach contents are consistent with results from studies of other epipelagic predators (Nakamura, 1985). Food items include skipjack tuna, yellowfin tuna, bigeye

tuna, and frigate tunas, as well as other scombrids, gempylids, coryphaenids, carangids, and various cephalopods (Nakamura, 1985).

Black marlin are strongly sexually dimorphic. Males rarely exceed 115kg, while females may attain weights of 900 kg and lengths of 4.5 meters (Cyr et al., 1990). The current IGFA world record is a 707.6 kg black marlin caught near Cabo Blanco, Peru in 1953 (IGFA, 1996) and larger fish have been reported from commercial catches (Cyr et al., 1990).

Spawning of black marlin is believed to occur in the East China and Coral seas (Nakamura, 1985). Spawning areas have been delineated based largely on the presence of mature females with well developed gonads. Larvae are poorly represented in ichtyoplankton samples. Only five black marlin larvae were reported from extensive Japanese icthyoplankton sampling between 1956 and 1984 (Nishikawa, 1985). Leis et al. (1987) reported the collection of 80 black marlin larvae from the seaward side of the Great Barrier Reef near Lizard Island, Australia between 1980 and 1985. These larvae were designated as belonging to the genus *Makaira* based on the vertebral formulae of 11+13=24 (Nakamura, 1985) and were distinguished from the congener, *Makaira nigricans*, by head profile, depth, and minor pigmentation differences (Leis et al. 1987).

Leis et al. (1987) noted that these larvae corresponded to the "unpigmented sailfish larvae" reported by Ueyanagi (1976a), suggesting that previous icthyoplankton studies may have misidentified black marlin larvae (e.g. Nishikawa, 1985). Leis et al. (1987) collected black marlin larvae during November along the Great Barrier Reef near Lizard Island, concurrent with the appearance of gravid female black marlin in the area. The timing of collections corresponded

with the southward seasonal shift of black marlin spawning activity along the eastern coast of Australia as reported by Shomura (1985). Spawning takes place in water temperatures between 27 and 28° C and begins in May and June near Hainan Island in the South China Sea, around Taiwan from August to November, and in the northwestern part of the Coral Sea between October and December (Nakamura, 1985). Larval duration is unknown, but is probably short, considering the high growth rate characteristic of other istiphorid billfishes (Prince, 1991).

Sex ratios of marlins have been observed to fluctuate on a seasonal basis. A predominance of mature males is considered indicative of spawning or reproductively active groups. Males typically remain on the spawning grounds throughout the spawning season while females come to spawn and then leave. High male to female ratios have been reported from known breeding grounds for blue marlin from Kona, Hawaii and Mauritius (Hopper, 1990; Cyr et al., 1990). Sexually inactive groups of white, striped, and blue marlins have been characterized by low male to female ratios (Baglin, 1977; Squire, 1987; Kume and Joseph, 1969).

Sex ratios for black marlin have been reported only from the Gulf of Thailand and for Mauritian waters. Takahashi et al. (1983) and Kanehara et al. (1985) both reported high male to female sex ratios from the Gulf of Thailand, where there was an abundance of immature males. The authors hypothesized these fish comprise a feeding school rather than a spawning assemblage, although gravid females were captured in June and November, 1982, as well as a male in breeding condition in June, 1982 (Takahashi et al., 1983). Equal numbers of males and females were observed in Mauritian waters, suggesting that spawning does not occur in this region (Cyr et al., 1990). In Australian

waters, males and females are equally represented in groups of juvenile fish less than 30 kg inside the Great Barrier Reef, while males are more common than females among adult fish found outside the reef (J. Pepperell, pers. com.).

Little is known about the stock structure of the black marlin. According to MacKenzie and Abaunza (1998), there are three main types of methods used to obtain information on stock structure: (1) natural methods, including morphometric and meristic analyses, use of parasites as biological tags, and genetic studies; (2) the use of externally and internally attached artificial tags; and (3) studies of biological parameters in relation to life cycle. Only parasite and streamer tags have been used to date as means of estimating stock structure of black marlin. Because of geographically restricted effort, neither method has been able to describe more than local movements (Speare, 1994; Pepperell,1990; Squire and Nielson, 1983).

Tagging efforts have provided only limited information regarding the movements of black marlin. Over 11,500 black marlin were tagged and released in the Pacific Ocean between 1961 and 1990. Tagging efforts supported by the Australian Game and Fish Commission and National Marine Fisheries Service (NMFS) Cooperative Tagging Program occurred primarily in eastern Australian waters where more than 97% of the releases took place. Only 309 fish were tagged elsewhere in the Pacific. Consequently, results from tagging studies have yielded some insight into the movement of fish within and from eastern Australian waters, but patterns in other areas of the Indian and Pacific oceans are poorly described. Studies by both Squire and Nielson (1983) and Pepperell

(1990) indicated that fish tagged in the Cairns-Lizard Island area show a tendency to move farther away from the site of tagging during the first year at large but many fish were recaptured near the site of tagging after approximately one year at large. This annual cycle was also noted by tag returns after multiple years at large. Studies of tag returns from other billfishes have also described annual migratory cycles whereby tagged fish are captured near the site of release after multiple years at large (Mather et al., 1974a; Mather et al., 1974b; Squire, 1974). The discrepancy between the 2.3% tag return rate reported by Squire and Nelson (1983), which is quite high compared to tag-return rates observed in other tagging programs for highly migratory species, and the tag-return rate of 0.5% reported by Pepperell (1990) was due to the cessation of the majority of the fishing effort in Australian waters in 1981 when the Australian Fishing Zone (AFZ) was closed to foreign longline fleets.

Although most black marlin tag returns describe movements within Australian waters, there is evidence for trans-oceanic movements. One fish tagged near Baja California, Mexico was recaptured NE of New Zealand, a distance of 5700 km, and another black marlin tagged at Christmas Island was recovered near the New South Wales coast of Australia (Pepperell, 1990). There are few data describing the movements of black marlin either within the Indian Ocean or between the Indian and Pacific oceans; however, tag returns from blue marlin suggest that transfer of black marlin might occur between the Indian and Pacific oceans (Pepperell, pers.com.).

Parasites have been used to investigate the recruitment of juvenile fishes to adult spawning grounds (Olson and Pratt, 1973; MacKenzie and Mehl, 1984; Speare, 1994), movements of adult fish (Kabata and Ho, 1981; Wood et al., 1989; Speare, 1994), and stock stucture of widely distributed marine fishes (Fennessy, 1998; Hogans and Brattey, 1982). By examining the permanent parasite fauna of black marlin in eastern Australian waters, Speare (1994) was able to distinguish between immature fish taken on the nearshore grounds and mature fish taken in the vicinity of Lizard Island. These observations indicate the existence of a juvenile assemblage present in nearshore waters and an aggregation of mature fish near Lizard Island during the spawning season. Weights of juvenile fish landed at coastal ports show an increase in average size towards the south, suggesting a southerly migration of juvenile fish beginning near Dunk Island and Cape Bowling Green, where they first appear around September, following the coast to Bermagui in March and then heading offshore, at which time they disappear from recreational and commercial catches (Pepperell, 1990).

While molecular genetic techniques have not been used to examine the stock structure of the black marlin, disparate patterns of genetic population structuring have emerged from previous investigations of the intraspecific genetic variation within other istiophorids species. A wide range of genetic variability was observed within istiophorid billfish species (Table 1). Blue marlin samples from both the Atlantic and Pacific oceans exhibited high levels of within-sample variation, with higher nucleotide sequence diversity being found in the Atlantic than in the Pacific (Finnerty and Block, 1992; Graves and McDowell, 1995; Graves, 1998). No significant genetic heterogeneity was

detected within ocean basins for blue marlin, although significant heterogeneity was reported between collections of blue marlin from the Atlantic and Pacific oceans (Finnerty and Block, 1992; Graves and McDowell, 1995; Buonaccorsi et al., 1998). Sailfish collections from the Pacific, Indian, and Atlantic oceans exhibited a wide range of within-sample diversity, although values were somewhat lower than those found for blue marlin (Graves and McDowell, 1995; Graves, 1998). Results from studies of the population genetic structure of the white marlin within the Atlantic Ocean revealed no population structuring and low overall sequence diversity (Graves et al., 1998). In contrast, shallow but significant population structuring was detected for the striped marlin in the Pacific Ocean (Graves and McDowell, 1994). Sequence diversity for striped marlin in the Pacific was higher than that reported for white marlin in the Atlantic, but lower than values for sailfish and blue marlin (Graves, 1998).

MOLECULAR GENETICS & INTRASPECIFIC VARIATION

Population structure is inferred from patterns of genetic variation that result from the interaction between the evolutionary forces of genetic drift, mutation, migration, and natural selection. Mutation, defined as a heritable change in genetic material, is the ultimate source of genetic variation, and tends to increase overall genetic diversity. Migration homogenizes genetic variation. Successful gene flow spreads new variants from one population to another, as migrating individuals reproduce. Genetic variation is

homogenized because changes that occur in one area are spread throughout the population via gene flow. In the absence of gene flow, random changes in allele frequencies due to sampling errors in finite populations accumulate. This phenomena is called genetic drift and the speed at which allele frequencies will "drift" is inversely proportional to the number of individuals in the population that actually contribute genes to the succeeding generation (effective population size). Genetic drift can result in the fixation of alleles within populations and the loss of genetic variation, although low levels of migration can prevent the fixation of allelic differences between population. Sewall Wright (1978) estimated that as few as one migrant per generation could preclude the fixation of alleles within a population; however, statistically significant differences in allele frequencies between populations can still result when there is a low level of migration (Allendorf and Phelps, 1981). Natural selection acts to change allele frequencies through differential reproduction of genotypes within a population. While migration and drift affect all loci simultaneously, natural selection acts on specific loci.

CLASSES OF MOLECULAR MARKERS

The field of molecular biology has expanded greatly in recent years, providing the population geneticist with a wide array of tools and techniques to infer the action of evolutionary forces. These include molecular markers that differ in evolutionary rate, mode of inheritance, and visibility to selective forces. The choice of an appropriate molecular marker depends on the phylogenetic resolution required, as well as the time

and funding available for the research project. A description of the general classes of molecular markers and their relative utility to investigations of intraspecific variation follows.

Allozymes

Protein electophoresis separates different allelic variants of the same gene locus on the basis of differences in protein size, shape, and charge under the influence of an electric field (Hillis et al., 1996). Allozyme analysis represents an efficient and cost effective means of surveying genetic information among individuals because reagents are relatively inexpensive and many loci can be screened simultaneously (Avise, 1994). Limitations of allozyme analysis include the need for relatively fresh or fresh frozen tissue, possible action of selection on genes coding for functional proteins, low levels of variation due in part to the redundancy of the genetic code, and a lack of charge change for many amino acid substitutions. Allozyme electrophoresis has been used for many investigations of intraspecific variation in marine fishes, including billfishes (Morgan, 1992; Shaklee et al., 1993), although low levels of allozyme variation have been reported for many marine species (Siddell, 1980; Crawford, 1989).

MtDNA

The mitochondrial genome of vertebrates is a haploid, maternally inherited molecule. In higher animals the gene composition and arrangement of mtDNA is highly conserved within taxonomic classes, coding for 13 mRNAs, 2 rRNAs, and 22 tRNAs

(Lewin, 1994). The "D-loop" region, typically about 1.0 kb, is the site of origin of DNA replication. Introns, pseudogenes, repetitive DNA, and sizeable spacer regions are absent from vertebrate mtDNA (Darnell et al., 1986). Point substitutions are the dominant form of mutation, although length heterogeneity has been reported. Evolution of mtDNA at the sequence level is rapid, perhaps due to the presence of free radicals within the mitochondrion or poor repair mechanisms (Brown et al., 1979; Vawter and Brown, 1986).

Analysis of mtDNA has become very popular for investigations of intraspecific variation due to the molecule's relatively rapid mutation rate, clonal inheritance, and small effective population size relative to nuclear DNA (Avise, 1987; Ovenden, 1990; Birky et al., 1989). Whole molecule mtDNA was originally purified using density gradient centrifugation, but the development of the polymerase chain reaction (PCR) has allowed researchers to quickly amplify specific regions of mtDNA. Because mtDNA is almost always clonally inherited, it must be treated as a single locus; however, differences in mutation rate exist among mtDNA regions, allowing this one marker to be used for investigations at different levels of taxonomic inference (Avise, 1994).

Nuclear DNA

Nuclear DNA comprises both protein coding and non-coding regions. Introns and other non-coding regions generally demonstrate higher genetic variability than protein coding regions because these areas produce no functional product on which selection might act, although these regions may affect the expression of genes. Linkage with

selected areas could confound this argument. As a result, intraspecific studies often target non-coding regions. Marker classes typically surveyed in analyses of non-repetitive nuclear regions are randomly amplified polymorphic DNA (RAPD), single-copy nuclear DNA (scnDNA), anonymous single-copy nuclear DNA (ascnDNA), and other non-coding regions, including introns and nontranscribed spacers.

Analysis of scnDNA has been used in a number of population studies (Palumbi and Baker, 1994; Corte-Real et al., 1994; Lessa and Applebaum, 1993; Karl and Avise, 1993; Reece et al., 1997; Buonaccorsi et al., 1999). In some studies, variation of exon-primed, intron-crossing regions was analyzed. Non-coding introns are flanked by coding regions (exons) that are typically more conserved because they code for a functional gene product, and are thus suitable regions for primer development. In other investigations of single copy DNA, variation at regions "anonymously" selected from a genomic library was surveyed. Because a high proportion of vertebrate DNA is composed of non-coding regions, anonymous single copy nuclear DNA (ascnDNA) loci are likely to be variable (Karl et al., 1992).

Regions of repetitive DNA known as variable number of tandem repeats (VNTRs) occur throughout the genome of higher vertebrates, comprising minisatellite and microsatellite DNA (Brooker, 1994). Minisatellite repeat motifs are typically 15-30 base pairs long (Wirgin and Waldman, 1994), although repetitive allele sequences as long as 50 kb have been documented (Bruford and Wayne, 1993). While minisatellite DNA has been used to identify individuals in human forensics (Jeffreys, 1985), attempts to

employ these markers in fisheries population studies have been complicated by the inability to assign alleles to specific loci and problems of consistency between gels (Bentzen, 1991; O'Connell and Wright, 1997; O'Reily and Wright, 1997).

Microsatellite DNA repeat motifs are 2-6 bp in length, and display considerable variation in repeat number among individuals (Tautz, 1986; Scribner et al., 1994; Patton, 1997). High variability of microsatellite DNA is ascribed to a high mutation rate and relaxed selection. While the variability of most other molecular markers is attributed to point mutations, microsatellite DNA polymorphisms typically result from differences in repeat number due to slipped-strand misparing (Levinson and Gutman, 1987). Microsatellite DNA is a useful genetic marker for studies of population differentiation and stock identification because it is widely distributed throughout the eukaryotic genome, exhibits high levels of allelic polymorphism, usually conforms with Mendelian inheritance, and is believed to be selectively neutral (Ruzzante, 1998; Jarne and Lagoda, 1996). Microsatellite loci are often flanked by unique conserved sequences (microsatellite flanking regions) for which primers can be designed, allowing for PCR amplification from small amounts of fresh or preserved tissue (Ruzzante, 1998).

Microsatellites are by definition hypervariable markers, but studies have revealed that microsatellite variability in fishes is higher than in most other major vertebrate groups (Ruzzante, 1998; Bentzen et al, 1996; McConnell, 1997; Brooker et al, 1994; Ruzzante et al, 1996a). Accurate estimation of genetic distances and population structure requires knowledge of minimum sample size above which the influence of bias and sampling variance are minimized (Ruzzante, 1998). Because a single microsatellite locus

may exhibit more that 20 alleles, collection of sample sizes sufficiently large to estimate allelic frequencies is a concern. Ruzzante (1998) suggested using 50-100 individuals per sampling location for population studies using microsatellite markers.

This study employs three classes of molecular markers (mtDNA, scnDNA, and microsatellite DNA) to evaluate population structuring within the black marlin. Specifically, two hypotheses are addressed:

H_{o1}: Black marlin populations in the Indian and Pacific oceans share a common gene pool.

H_{o2}: The distribution of genetic variation within black marlin at a collection location is stable over time (multiple years).

Table 1. Intraspecific genetic variation observed in previous studies of istiophorid billfishes (Graves, 1998). Samples sizes (N), the number of geographically distant collections in the Atlantic (A), Pacific (P), and Indian (I) oceans, nucleon diversity (*h*), and nucleotide sequence diversity (π).

Species	Location	N	*h*	π
Striped marlin	4P	166	0.82 (0.69-0.84)	0.30% (0.20-0.32%)
White marlin	4A	235	0.78 (0.54-0.90)	0.15% (0.06-0.15%)
Blue marlin	3A, 3P	424	0.91 (0.58-0.97)	0.59% (0.14-0.80%)
Sailfish	2A, 1P, 1I	109	0.59 (0.28-0.73)	0.40% (0.22-0.66%)

Figure 1. Geographic distribution of the black marlin. Dark shading indicates areas of high abundance and arrows indicate regions of occasional distribution (Suzuki, 1976).

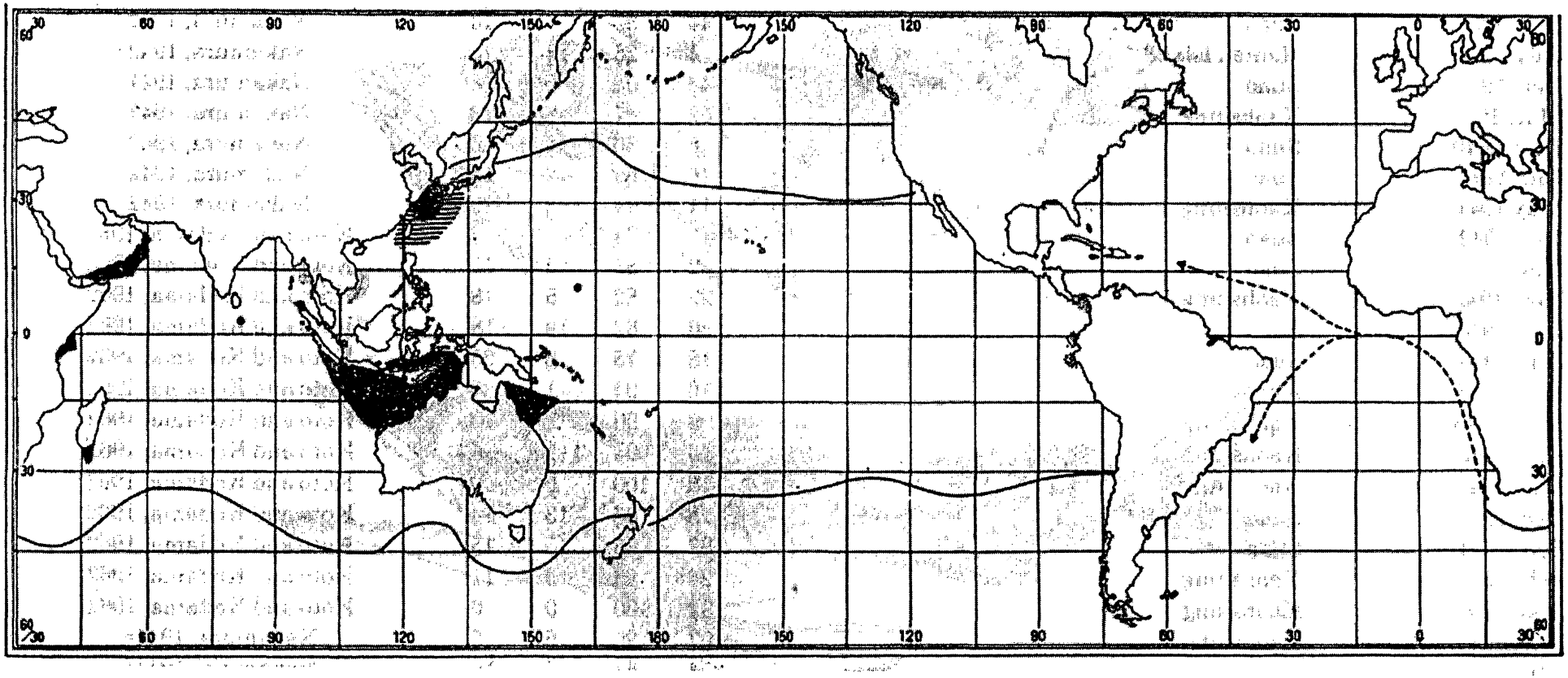
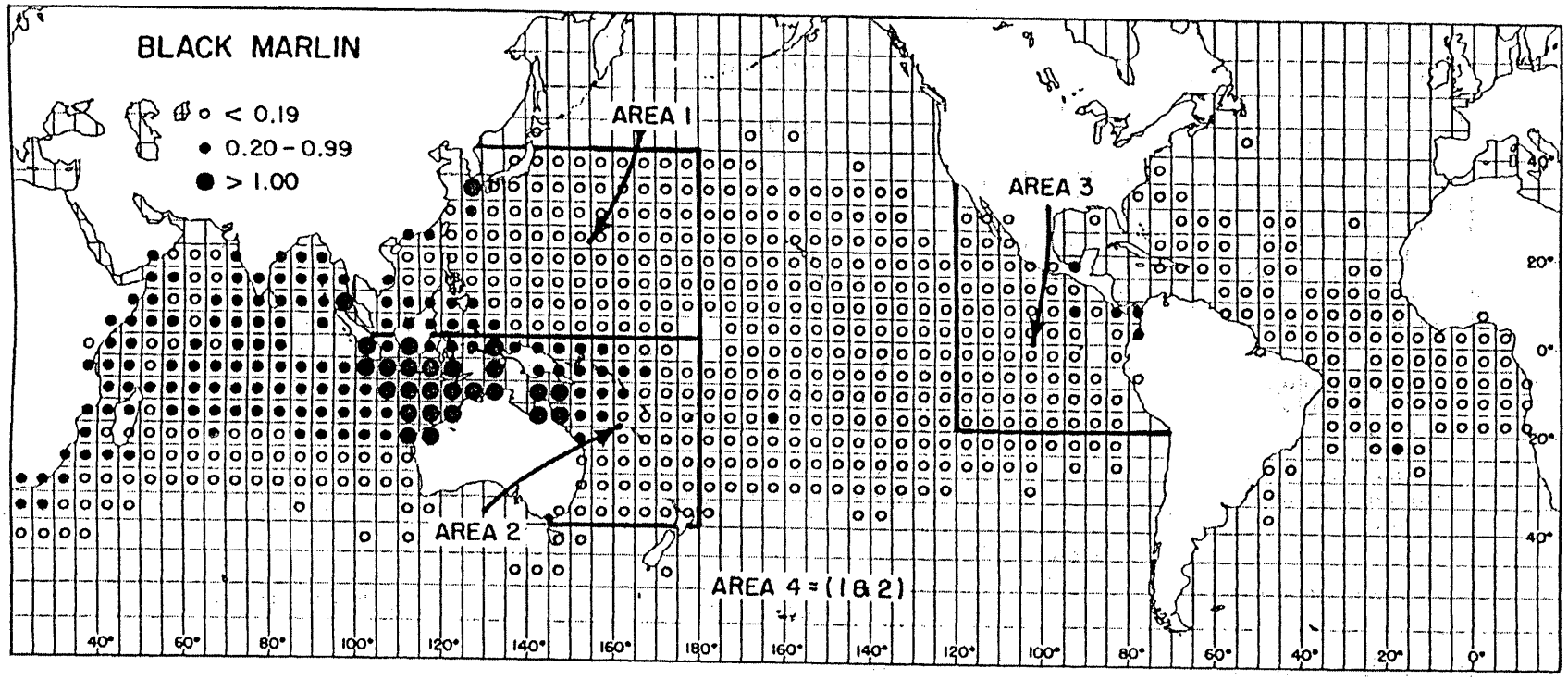

30
60
90
120
150
180
150
120
90
60
30
0
30
60
30
0
30

Figure 2. Black marlin CPUE (fish per 1000 hooks) and suggested stocks for the Indian and Pacific oceans (Shomura, 1980).


BLACK MARLIN
< 0.19
0.20 - 0.99
> 1.00
AREA 1
AREA 2
AREA 3
AREA 4 = (1 & 2)
40°
60°
80°
100°
120°
140°
160°
180°
160°
140°
120°
100°
80°
60°
40°
20°
0°
20°
40°

Chapter 2. METHODS

This study surveyed both retrospective and contemporary collections of black marlin tissue samples. Historical samples consisted of anal fin spines collected for aging studies by Julian Pepperell during the annual Port Stephens, Australia sportfishing tournament between 1984 and 1998 (PS85, PS86, PS94, and PS96). Sufficient muscle and fin membrane were attached to the frozen anal spines for DNA isolation and amplification, even though some samples were frozen for as long as 14 years in a conventional freezer. Contemporary samples were obtained from fish markets and recreational catches in South Africa (SA), Vietnam (VN), Taiwan (TW98 and TW99), Australia (PS97), Ecuador (EC), Mexico (MX), and Panama (PN; see Table 2 for sample details). These samples consisted of either frozen heart tissue or muscle tissue preserved in alcohol or tissue storage buffer (TSB; 0.25M EDTA, 20% DMSO, pH 8.0).

Black marlin hearts were frozen at -20°C within 8 hours of capture, shipped to the laboratory at VIMS on dry ice, and stored at -80°C until DNA could be isolated. DNA isolation from frozen heart tissue was performed using the cesium chloride (CsCl) density gradient centrifugation protocols of Lansman et al. (1981). In the process, a mitochondrially enriched fraction was prepared by tissue homogenization and differential centrifugation. Following mitochondrial lysis, samples were ultracentrifuged in a solution containing CsCl and ethidium bromide (EtBr) for at least 36 hours at 70,000 g in a Beckman Optima-TL ultracentrifuge. During ultracentrifugation, nuclear DNA and relaxed mtDNA were separated from molecules of supercoiled mtDNA due to the higher density of the supercoiled configuration. Following ultracentrifugation, samples were

illuminated with ultraviolet light, allowing for visualization and collection of the separate nuclear and mitochondrial bands. Ethidium bromide was removed with repeated butanol extractions and samples were desalted through dialysis in a Tris/EDTA buffer. DNA was then ethanol precipitated and rehydrated in sterile water.

Black marlin DNA was isolated from muscle tissues stored in either 95% ethanol or in tissue storage buffer following a modification of the high molecular weight isolation procedure of Sambrook et al. (1989). Briefly, a small piece of tissue only a few millimeters per dimension was homogenized and digested overnight in a solution containing 500 ul of isolation buffer (50 mM EDTA, 50 mM Tris, 150 mM NaCl, pH 8.0), 60 ul of 10% SDS, 10 ul of RNase (10 mg/ml) and 10 ul of proteinase K (25mg/ml). Following overnight digestion, samples were centrifuged in an Eppendorf 5415 C microcentifuge at 10,000 g for 30 minutes. The supernatant was then extracted with phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform: isoamyl alcohol (24:1). The DNA contained in the resulting supernate was precipitated with sodium acetate and 100% ethanol, centrifuged at 10,000 g for 15 minutes to collect all precipitate, dried in a Savant SC 100 speedvac, and rehydrated in 50 ul of sterile water.

MtDNA Analysis

In a preliminary analysis three mitochondrial loci, NADH dehydrogenase subunit 4 (ND4), cytochrome-*b* (cyt-b), and the displacement loop (D-loop), were amplified from a subsample of 12 black marlin from different sampling locations using primers designed

from published sequences (Rosel and Block, 1996; Kocher et al., 1989; Cronin et al., 1993; Palumbi et al., 1991; Bielawski and Gold, 1996). Amplification of PCR products was conducted in 50 ul reactions containing 42.25 ul sterile water, 5.0 ul 10X PCR buffer with 15 mM MgCl, 1.0 ul 10mM dNTP mix, 0.5 ul of each primer at 100 pmol/ul, and 0.25 ul *Taq* polymerase (5 U/ul; Gibco BRL) using a MJ PTC-200 thermocycler. After an initial denaturing period of 5 minutes at 95° C, samples were subjected to 35 cycles of 94°C for 1 min, primer-specific annealing temperature for 1 min, and 65 °C for 1 min, followed by a final extension at 65° C for 7 min. PCR products were stored at 4° C. PCR products were separated on 1.0% agarose gels and visualized by ethidium bromide (EtBr) staining and UV irradiation. PCR products were screened with 46 restriction enzymes for restriction fragment length polymorphisms (RFLPs) following the manufacturer's guidelines. Restriction digests of the ND4 and cyt-b regions did not produce RFLPs, although restriction site variation was detected within the D-loop region.

Universal D-loop primers "H1" and "L1," designed originally for salmon (Cronin et al., 1993; App. 1.1), produced the best PCR amplification products of four D-loop primer pairs tested in the pilot study. The D-loop was amplified with these primers for all samples at an annealing temperature of 42° C. Six restriction enzymes (*Dra* I, *Hha* I, *Sty* I, *Hpa* II, *Hinf* I, *Dde* I) revealed polymorphic restriction sites and were used to construct composite haplotypes. Fragment sizes were estimated by comparison to a standard 1 kb DNA ladder (Gibco-BRL) using either visual estimation or RFLP Scan (Scanalytics, CSPI).

Nuclear DNA Analysis

Seven single-copy nuclear loci, including three intron regions and four anonymous single-copy nuclear markers, were screened for intraspecific variation. The three intron regions surveyed were the short actin intron (Reece and Graves, 1997), creatine kinase intron 6 (Palumbi et al, 1991), and ribosomal protein intron 2 (Chow, 1998). Anonymous single-copy nuclear markers BM 32, BM 47, BM 81, and WM 08, developed by Buonoccorsi (1998) for blue marlin following the protocols of Karl and Avise (1993) were utilized in this study. All amplified products were screened with a suite of 43 restriction enzymes during the analysis across at least 20 individuals representing different geographic collections. In some cases (actin inton and BM32-3), as many as 100 individuals were screened. PCR amplification of nuclear loci followed the same protocols used for mtDNA loci, with the exception that nuclear DNA PCR protocols included 40 cycles and primer-specific annealing temperatures.

Six microsatellite DNA loci (GATA 1, GATA 8, GATA 10, GATA 52, GATA 60 and GATA 90) were screened for use as hypervariable molecular markers in this study. The loci were developed by Buonaccorsi (1998) using modifications of the protocols of Kijas et al. (1994) and Waldbieser (1995). Tetranucleotide repeats characterized by a "GATA" motif were selected based on their potential to hybridize to a $(GATA)_5$ probe, the presence of sufficient conserved flanking region to allow for primer development, and amplification during asymmetrical and exponential PCR under a variety of primer combinations. The use of these six microsatellite loci facilitated comparison with other billfish species (Bounaccorsi and Graves, 1999).

Six pairs of microsatellite primers were tested for amplification of hypervariable microsatellite markers in the black marlin. PCR cycling conditions began with an initial denaturation at 95°C, followed by 32 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, final extension at 72°C for 7 minutes, and storage at 4°C (Buonaccorsi, 1998). Annealing temperature was varied to optimize the amplification of microsatellite loci from black marlin. Annealing temperature and primer sequences are listed in Appendix 1.1. During PCR optimization, unlabeled products were separated on 3% agarose gels, stained with EtBr, and visualized under UV light. Once primer conditions were optimized, either the forward or reverse primer was labeled with a flourescent dye (IRD-800) to allow for size detection of amplified products on an automated DNA sequencer. Labeled PCR products were electrophoresed on 25cm, 8% Long-Ranger denaturing polyacrylaminde gels (FMC bioproducts) and detected using a LiCor scanner. Products were scored using RFLPSCAN software (Scanalytics, CSPI).

STATISTICAL ANALYSES

MtDNA

Composite haplotypes consisting of a letter designation for the fragment pattern produced by each enzyme were constructed for each individual and used to generate estimates of divergence, diversity, and population structure. The number of restriction site differences (site gains or losses) between haplotypes was inferred from completely additive changes in fragment patterns. The restriction site approach of Nei and Miller (1990) was used to estimate nucleotide sequence divergence (d) between composite haplotypes. Haplotype diversity (h), the probability that two haplotypes drawn

successively from a sample are different, was calculated according to Nei (1987). Mean nucleotide sequence diversity (π), the average nucleotide sequence divergence between haplotypes drawn randomly from a sample, was calculated for each collection and for pooled collections following Nei (1987). Uncorrected nucleotide sequence divergence was calculated and corrected for within-sample diversity, resulting in corrected nucleotide sequence divergence (δ; Nei, 1987). The Monte Carlo approach of Roff and Bentzen (1989) was used to determine differences in haplotype frequencies among temporal and geographic collections. The above calculations were performed with REAP 3.0 (McElroy et al., 1992). The sequential Bonferroni test was used to correct alpha-levels for comparisons involving multiple tests following Rice (1989). Hierarchical analysis of molecular variance (AMOVA) was performed with Arlequin v 1.1 (Schneider et al., 1997).

Nuclear DNA

Statistical analyses were performed to assess genetic distance between populations, genetic structure among populations, and conformance of populations to assumptions of Mendelian inheritance. θ and R estimators were used to evaluate genetic distance between populations for the microsatellite data. Both θ and R estimators relate to F-statistics, which are used to characterize population subdivision. Theta refers to F-statistics based only on allele frequency data (Weir and Cockerham, 1984), while R refers to F-statistics that take into account genetic distance between microsatellite alleles based on a stepwise mutation model (Mickalakis and Excoffier, 1996; Rousset, 1996). The θ and R estimators are more resistant to bias at small sample size than other measures of

microsatellite genetic distance (Ruzzante, 1998). Genetic structure was tested with an AMOVA by using Arlequin v 1.1 (Schneider et al., 1997). Conformance of microsatellite data to Hardy-Weinberg expectations was tested with a Markov-chain approach modified from Guo and Thompson (1993) by using Arlequin v 1.1 (Schneider et al., 1997).

Table 2. Restriction endonucleases used to screen black marlin mtDNA and nuclear DNA PCR products for intraspecific variation. Columns indicate the size of the enzyme recognition site in base pairs (BP).

4 BP	5 BP	6 BP
Alu I	Hinf I	Apa I
Hae III		Ava I
Hha I		BamHI
Hpa II		Ban II
Mbo I		Bcl I
Mse I		Bgl I
Rsa I		Bgl II
Taq I		Cla I
Tha I		Dde I
		Dra I
		EcoRII
		EcoRV
		EcrR I
		Hae II
		Hinc II
		Hind III
		Hpa I
		Kpn I
		Msp I
		Nci I
		Nde I
		Not I
		Nsi I
		Pst I
		Pvu
		Pvu II
		Sca I
		Sma I
		Spe I
		Sst I
		Sst II
		Sty I
		Xba I

Chapter 3. RESULTS

A total of 356 tissue samples was obtained for genetic analysis from 9 locations throughout the range of the black marlin (Table 3). Not all samples obtained for analysis were used in this study due to misidentification of specimens by collectors and the poor condition of some tissue.

Morphological similarity between blue and black marlins resulted in misidentification of specimens by collectors at some locations. Distinguishing between blue and black marlins is difficult, and the problem can be exacerbated by the disposition of fish at the time of sample collection. Fish collected from fish markets were usually headed and gutted, removing the distinguishing features and making species identification even more difficult. Putative species identifications were confirmed by using a molecular identification key developed in our lab based on RFLP analysis of the ND4 region of the mitochondrial genome and an anonymous nuclear locus, BM32-2, which exhibit fixed differences among billfishes (Figure 3A-D; Graves and McDowell, 1997). Based on the molecular key, 21 fish identified as black marlin were found to be blue marlin (Table 3). These samples were removed from the data base, resulting in smaller sample sizes from Malaysia, Seychelles, Vietnam, and Ecuador.

Some samples arrived in poor condition, preventing the isolation of high molecular weight DNA. No DNA was recovered from 15 individual tissue samples from 4 locations: Malaysia, Seychelles, South Africa, and Vietnam (Table 3). In addition, no molecular markers could not be amplified from 34 individuals. As a result of mis-identification (21), poor tissue quality (15), or lack of marker amplification (34), the original sample of 356 was reduced to 286 individuals.

MtDNA

RFLP analysis of the mtDNA D-loop resulted in 39 different composite haplotypes. All six enzymes revealed polymorphic restriction sites. *Hinf* I was the most variable enzyme, cutting the 1400 bp D-loop region at 12 different sites, producing 9 unique fragment patterns (Table 4). *Dra* I was the least variable enzyme, cutting the D-loop region in 4 locations, resulting in only two fragment patterns (Table 4). *Hha* I, *Dde* I, *Sty* I, and *Hpa* II produced from 3 to 6 different fragment patterns each, resulting from 5 to 10 individual restriction sites per enzyme (Table 4).

Considerable variation was present within each collection of black marlin (Table 5). Nucleon diversity (*h*) ranged from 0.73 for TW99 to 0.91 for PS94 and overall nucleon diversity was 0.82 (Table 6). Composite haplotypes differed by from 1 to 22 site changes (gains or losses), accounting for pairwise haplotype divergences (d) of 0.77% to 18.93% (Table 7). Nucleotide sequence diversities (π) ranged from 2.49% for the PS94 sample to 3.35% for the PS86 sample (Table 6) and overall sequence diversity was 2.84%.

The temporal stability of mtDNA haplotype frequencies was evaluated for four separate years from Port Stephens, Australia (1985, 1986, 1996, 1997) and two years from Taiwan (1998 and 1999). RFLP analysis of 137 individuals from Port Stephens produced 25 composite haplotypes, of which only 8 occurred at frequencies greater than 1% (Table 5). Haplotype BAAAAA occurred at an overall frequency of 35% (range among years = 27.7% - 41.2%). Haplotypes ABAABB and BBBBAA were common,

occurring at frequencies of 22% and 17%, respectively (ranges among years = 14.7%-30.5% and 13.0%-19.4%). All other haplotypes were rare, occurring in less than 5% of the individuals. Uncorrected nucleotide sequence divergence ranged from 2.7% between PS94 and PS96 to 3.1% between PS86 and PS97. Corrected nucleotide sequence divergence ranged from –0.08% between both PS86 and PS96 and between PS86 and PS96, to 0.13% between PS94 and PS96. Following the chi-square analysis described by Roff and Bentzen (1989), no differences in allele frequencies among years were detected. The overall comparison was not significant ($p = 0.582$) nor were any of the 6 possible pairwise comparisons involving the Australian temporal samples. Exact p-values ranged from $p = 0.08$ for the pairwise comparison between PS94 and PS96 to $p = 0.958$ for the pairwise comparison between PS94 and PS96 (Table 9). The pairwise comparison between Taiwan 1998 and Taiwan 1999 was also non-significant ($p = 0.144$).

Analysis of molecular variance (AMOVA) among years at Port Stephens, Australia revealed that all variance was attributable to within-sample variation (100.85%) and that variance among samples was negligible (-0.85%). The overall Φ_{ST} was not significantly different from zero (Table 11). Because no significant differences were detected among temporal samples from Australia and Taiwan, pooling among years at geographic locations was justified for the mtDNA D-loop data.

Geographic analysis of mtDNA haplotypes involved comparison among five geographic locations: eastern Australia, Taiwan, South Africa, Vietnam, and a pooled eastern Pacific collection (EPO) comprising samples from Ecuador, Mexico, and Panama. Haplotype BAAAAA was most common, occurring at a frequency of 37.4% in the pooled collection, and ranging from 27.2% to 48.0% within individual collections.

Haplotypes ABAABB and BBBBAA were also common, occurring at frequencies of 20.6% (range = 4.0% to 34.6%) and 13.3% (range = 6.25% to 19.2%) in the pooled collection, respectively (Table 5). Uncorrected nucleotide sequence divergences among collections ranged from 2.39% to 3.19% and corrected sequence divergences ranged from –0.098% to 0.22% (Table 8). When temporal samples were pooled, corrected nucleotide sequence divergences ranged from –0.00911% to 0.923% (Table 10). When chi-square tests for heterogeneity were performed for the geographic analysis (Roff and Bentzen, 1989), the overall comparison was not significant ($p = 0.266$). Of the 10 possible pairwise comparisons, none was significant after correction for multiple tests ($\alpha \leq 0.005$; Rice, 1989).

A hierarchical analysis of molecular variance for the mtDNA D-loop data was performed on three different population structuring models: an overall comparison with no assigned structure, a comparison between ocean collections where a structure was assigned in which individuals were grouped by ocean basin, and a comparison between eastern and western Pacific Ocean collections. All tests indicated that within-sample variation represented the vast majority of the total variance. Φ_{ST} was not significantly different from zero, and no tests were significant at the 0.05 level (Table 11).

ScnDNA

Seven single-copy nulcear DNA loci were screened with 43 different restriction endonucleases (Table 2). While many restriction enzymes cleaved the PCR products from

these regions at many sites, no intraspecific variation was detected at any of the seven loci.

Microsatellite DNA

Five of the six microsatellite loci screened in this study consistently amplified for black marlin DNA: GATA-10, GATA-90, GATA-52, GATA-08, and GATA-60. GATA-01 did not amplify consistently, producing a detectable PCR product for only 1 of 5 individuals tested. Among the five loci that consistently amplified, there was a marked difference in heterozygosity, resulting in three distinct classes of microsatellite markers: those with low, medium, and high variability.

GATA-10 was the least variable microsatellite locus surveyed in this study (Figure 4). GATA-10 was characterized as exhibiting low variability with $H_{obs} = 34.8\%$ overall with heterozygosities of individual collections ranging from 22.9% (TW99) to 46.2% (VN; Table 13). Six alleles were detected at GATA-10. The allelic distribution of GATA-10 was dominated by a single allele, which occurred at a frequency of 80.2%. The distribution of genotypes in collections conformed to Hardy-Weinberg expectations for GATA-10 (Table 12).

GATA-90 was also characterized by low variation ($H_{obs} = 42.0\%$; Table 13, Figure 5). Heterozygosities for individual collections ranged from 31.7% (VN) to 51.6% (PS96). Seven alleles were observed at GATA-90, and the allelic distribution was dominated by a single allele which occurred at a high frequency (74.8%). The distribution of genotypes conformed to the expectations of Hardy-Weinberg equilibrium (Table 12).

Moderate variation was detected at GATA-52. The overall heterozygosity was 90.1% and within-collection values ranged from 80.0% (SA) to 94.4% (EPO; Table 13). GATA-52 displayed 11 alleles and the allelic distribution was bell-shaped (Figure 6). The most common allele occurred at a frequency of 24.2%. The distribution of genotypes in two collections, VN and EPO, failed to conform to expectations of Hardy-Weinberg equilibrium (Table 12).

GATA-08 exhibited intermediate to high variability. Heterozygosities ranged from 88.6 to 100.0% and the overall heterozygosity was 93.9% (Table 13). The allelic distribution at GATA-08 was slightly bimodal and encompassed 21 alleles (Figure 7). Four alleles occurred at frequencies near 10%. The distribution of genotypes at three collections, TW98, VN, and EPO, failed to conform to Hardy-Weinberg equilibrium (Table 12).

GATA-60 was the most variable microsatellite locus surveyed in this analysis, exhibiting 27 different alleles and an observed heterozygosity of 97.1% (range = 93.8%-100.0%; Table 13). The allelic distribution was strongly bimodal, with peaks separated by 14 repeats and a range in allele size of 31 repeats (Figure 8). Significant deviations of genotypic frequencies from Hardy-Weinberg equilibrium were detected at four collections: TW99, SA, VN, and EPO (Table 12).

All microsatellite loci were used in subsequent temporal and spatial analyses; however, many tests were compromised by failure of loci to conform to the assumption of Hardy-Weinberg equilibrium or temporal instability.

Divergence: Temporal Stability. The majority of the microsatellite alleles were homogeneously distributed using either the infinite alleles model (IAM) or the step-wise mutation model (SMM). Significant results occurred with the infinite allele model (IAM; θ_{ST}) for GATA-52 and GATA-08 where $\theta_{ST\text{-}52}$=0.01494 (p=0.02) and $\theta_{ST\text{-}08}$=0.0198 (p<0.0001; Table 10). Significant results occurred for the stepwise mutation model (SMM; R_{ST}) at GATA-08 and GATA-60, where $R_{ST\text{-}08}$=0.178 (p<0.0001) and $R_{ST\text{-}60}$=0.0225 (p=0.0489; Table 10). Hierarchical analysis of variance indicated that the distribution of alleles at 3 of 5 microsatellite loci was not heterogeneous among years for at least either θ_{ST} or R_{ST}. Consequently, pooling among years at a given location was justified only for GATA-10 and GATA-90.

Divergence: Geographic Heterogeneity. Analysis of molecular variance (AMOVA) was used to test for geographic partitioning of allele frequencies at all 5 microsatellite loci. As with the mtDNA D-loop data, three tests were performed: overall, between oceans, and within the Pacific Ocean. Again, each AMOVA was performed twice in order to incorporate the use of both the IAM and SMM. Significant results occurred for the overall comparison at three loci, GATA-52, GATA-08, and GATA-60, the same loci with significant deviations from Hardy-Weinberg equilibrium. For GATA-52, significant results were noted for the SMM where R_{ST}=0.05949 (p=0.00149). Both the IAM and the SMM produced significant values for GATA-08 and GATA-60, where $\theta_{ST\text{-}08}$=0.01289 (p=0.0003) and $R_{ST\text{-}08}$=0.1219 (p<0.0001). For GATA-60, $\theta_{ST\text{-}60}$=0.01005 (p=0.00327) and $R_{ST\text{-}60}$=0.04293 (p=0.00485).

When AMOVA was used to test for differences between ocean basins, significant results were observed for the same three loci: GATA-52, GATA-08, and GATA-60. Only the SMM produced significant results at GATA-52, where R_{ST}=0.1245 (p=0.00059). Both the IAM and the SMM were again significant for GATA-08 and GATA-60. For GATA-08, θ_{ST}=0.01332 (p=0.00089) and R_{ST}=0.1393 (p<0.0001). For GATA-60, θ_{ST}=0.01141 (p=0.00376) and R_{ST}=0.05495 (p=0.00574).

Hierarchical analysis of variance was also used to test for differences in the partitioning of variance among collections within the Pacific Ocean. Significant differences were observed for three loci. According to the IAM, significant results occurred in the Pacific Ocean for GATA-52 (θ_{ST}=0.00948, p= 0.0291), GATA-08 (θ_{ST}= 0.0137, p<0.0001), and GATA-60 (θ_{ST}=0.00894, p=0.007). Following the SMM for the Pacific, significant results were observed for GATA-52 (R_{ST}=0.06047, p=0.00059), GATA-08 (R_{ST}=0.10085, p<0.0001), and GATA-60 (R_{ST}=0.04317, p=0.01).

Table 3. Black marlin sample information, including location and year, original sample size (N), prevalence of species mis-identification (number of blue marlin), sample quality (no DNA), and effective sample size. The effective sample size is given in parenthesis in the final column and indicates individual black marlin from which DNA was both isolated and amplified (mtDNA D-loop analysis).

Location	Year	N	blue marlin	no DNA	black marlin
Eastern Australia	1997	35	0	0	35 (35)
	1996	39	0	0	39 (26)
	1994	10	0	0	10 (10)
	1986	43	0	0	43 (41)
	1985	43	0	0	43 (39)
Malaysia	1997	8	1	7	0
Seychelles	1998	8	6	2	0
Mexico	1999	7	0	0	7 (6)
Panama	1999	10	0	0	10 (10)
South Africa	1996	7	0	4	3 (0)
	1998	12	0	0	12(9)
	1999	20	0	0	20 (18)
Taiwan	1998	39	1	0	38 (37)
	1999	34	0	0	34 (31)
Vietnam	1996	5	0	2	3 (3)
	1998	19	7	0	12 (12)
Ecuador	1995	12	6	0	6 (6)
	1998	5	0	0	5(5)
TOTAL		356	21	15	320 (286)

Table 4. Restriction endonuclease digestion patterns from RFLP analysis of black marlin mtDNA D-loop. Fragment sizes are in base pairs and were estimated by comparion to molecular size standards with RFLP Scan (Scanalytics, CSPI).

Dra **I**

A	B
-	1050
765	-
428	428
322	-

Hha I

A	B	C	D	E	F
-	-	715	-	-	-
515	515	515	515	515	515
485	-	-	-	-	-
-	-	-	455	-	455
-	-	-	420	-	-
-	-	-	-	390	-
-	340	-	-	340	340
205	205	-	-	-	-
125	125	-	-	-	-
-	115	-	-	115	-
	-	100	-	-	-

Hinf I

A	B	C	D	E	F	G	H	I
-	-	-	-	1150	-	-	-	-
-	-	-	1065	-	-	-	-	-
-	850	850	-	-	-	-	850	850
715	-	-	-	-	715	715	-	-
-	-	450	-	-	450	-	450	-
375	375	-	-	-	-	-	-	375
-	-	-	-	-	-	320	-	-
-	-	-	278	-	-	-	-	-
-	-	-	-	155	-	-	-	-
-	-	-	-	-	-	150	-	-
140	-	-	-	-	140	140	-	140
100	100	100	100	100	100	-	-	-
75	75	-	-	-	-	-	75	75

(Table 4 cont.)

Dde I

A	B	C
1121	-	-
-	-	755
-	667	-
-	383	-
-	-	332
311	311	311

Sty I

A	B	C	D
-	-	1400	-
-	-	-	1150
795	-	-	-
625	625	-	-
-	495	-	-
-	295	-	-
	-	-	250

Hpa II

A	B	C
925	-	-
-	690	-
-	-	575
370	370	370
-	-	-
-	-	350
	235	-

Table 5. Distribution and occurrence of mtDNA D-loop composite haplotypes.

	PS97	PS96	PS94	PS86	PS85	PS00	VN	SA	TW98	TW99	EPO	Total
AAAAAA	0	0	0	0	0	0	0	1	0	0	0	1
AAAAAB	0	0	0	0	0	0	0	0	0	0	1	1
AAAABA	0	0	0	0	0	0	0	0	0	0	1	1
AAAABB	0	0	0	0	0	0	0	0	5	1	1	7
AABABA	0	0	0	0	0	0	0	1	0	0	0	1
AABAIA	0	1	0	0	0	0	0	0	0	0	0	1
AABBAA	2	0	0	0	0	0	0	0	0	0	0	2
AABBBA	0	0	0	1	0	0	0	0	0	0	0	1
ABAAAB	1	0	0	0	0	0	0	1	0	0	0	2
ABAABA	0	0	2	2	1	1	1	0	0	1	0	8
ABAABB	5	9	2	3	11	1	3	6	7	11	1	59
ABAABC	0	0	1	0	0	0	0	0	0	0	0	1
ABBAAA	0	0	1	0	0	0	0	0	0	0	0	1
ABBABA	1	0	1	1	3	1	2	4	1	0	1	15
ABBABC	1	0	0	0	0	0	0	0	0	0	0	1
ABBACA	0	0	0	0	0	0	0	1	0	0	0	1
ABBAGA	0	0	0	1	0	0	0	0	0	0	0	1
ABBBAA	1	1	0	1	0	0	0	2	0	1	1	7
ABBBBA	0	0	0	0	0	0	0	0	3	0	1	4
ABEABA	0	0	0	0	0	0	0	0	2	0	0	2
ACAABB	0	0	0	0	1	0	0	0	0	0	0	1
ADBABA	1	0	0	0	0	0	0	0	0	0	0	1
BAAAAA	14	8	3	5	19	3	4	12	13	14	12	107
BAAAAC	2	0	0	1	2	1	0	0	0	0	0	6
BAAABA	0	0	0	0	0	0	0	0	0	0	1	1
BAAACA	0	0	0	0	1	0	0	0	0	0	0	1
BAAAFA	0	0	0	0	0	0	0	1	0	0	0	1
BAAAHA	0	0	0	0	0	0	0	0	0	0	1	1
BAACDA	1	0	0	0	0	0	0	0	0	0	0	1
BACAAA	1	1	0	0	0	0	0	0	0	1	0	3
BADCDA	0	0	0	0	0	0	0	0	1	0	0	1
BADCEA	0	0	0	0	0	0	0	1	0	0	0	1
BBAABA	0	0	0	0	0	0	0	0	1	0	0	1
BBABAA	0	0	0	0	0	0	0	0	0	1	0	1
BBBAAA	0	0	0	0	1	0	0	0	0	0	0	1
BBBABA	0	0	0	0	1	0	0	0	0	0	0	1
BBBBAA	5	5	1	3	8	0	2	2	4	4	4	38
BBBBGA	0	0	0	0	1	0	0	0	0	0	0	1
BDBABA	0	1	0	0	0	0	0	0	0	0	0	1
Total	35	26	11	18	49	7	12	32	37	34	25	286

Table 6. Nucleon diversity (h) and nucleotide sequence diversity (π) of black marlin collections based on mtDNA D-loop RFLP analysis.

	h	π (%)
PS97	0.8101	2.97
PS96	0.7723	2.99
PS94	0.9091	2.49
PS86	0.8889	3.35
PS85	0.7806	2.97
VN	0.8571	2.52
SA	0.8333	2.64
TW98	0.8206	3.16
TW99	0.7291	3.29
EPO	0.7600	2.35
Mean	0.8161	2.87

Table 7. Nucleotide sequence divergence (d) between mtDNA D-loop composite haplotypes below diagonal and minimum number of site changes between haplotype above diagonal.

	AAAAAA	AAAAAB	AAAABA	AAAABB	AABABA	AABAIA	AABBAA	AABBBA	ABAAAB	ABAABA	ABAABB	ABAABC	ABBAAA	ABBABA	ABBABC
AAAAAA	X	1	1	2	2	2	2	3	2	2	3	3	2	3	4
AAAAAB	0.0086	X	2	1	3	3	3	4	1	3	2	4	3	4	5
AAAABA	0.0095	0.0191	X	1	1	4	3	2	3	1	2	2	3	2	3
AAAABB	0.0191	0.0086	0.0095	X	2	5	4	3	2	2	1	3	4	3	4
AABABA	0.0195	0.0283	0.0109	0.0195	X	2	2	1	4	2	3	3	2	1	2
AABAIA	0.0195	0.0283	0.0314	0.0416	0.0196	X	2	3	4	5	6	6	2	3	4
AABBAA	0.0181	0.0254	0.0276	0.0358	0.0168	0.0168	X	1	4	4	5	5	2	3	4
AABBBA	0.0276	0.0358	0.0196	0.0276	0.0087	0.0277	0.0081	X	5	3	4	4	3	2	3
ABAAAB	0.0168	0.0081	0.0273	0.0168	0.0358	0.0358	0.0333	0.0437	X	2	1	3	2	3	4
ABAABA	0.0183	0.0273	0.0096	0.0183	0.0196	0.0395	0.0361	0.0287	0.0179	X	1	1	2	1	2
ABAABB	0.0273	0.0168	0.0183	0.0088	0.0276	0.0490	0.0437	0.0361	0.0081	0.0089	X	2	3	2	3
ABAABC	0.0273	0.0363	0.0183	0.0273	0.0276	0.0490	0.0437	0.0361	0.0270	0.0089	0.0179	X	3	2	1
ABBAAA	0.0181	0.0254	0.0276	0.0358	0.0168	0.0168	0.0165	0.0253	0.0168	0.0182	0.0265	0.0265	X	1	2
ABBABA	0.0276	0.0358	0.0196	0.0276	0.0087	0.0277	0.0253	0.0178	0.0265	0.0101	0.0182	0.0182	0.0081	X	1
ABBABC	0.0358	0.0441	0.0276	0.0358	0.0168	0.0373	0.0330	0.0253	0.0350	0.0182	0.0265	0.0094	0.0165	0.0081	X
ABBACA	0.0395	0.0490	0.0302	0.0395	0.0184	0.0428	0.0362	0.0275	0.0389	0.0200	0.0293	0.0293	0.0184	0.0090	0.0184
ABBAGA	0.0674	0.0791	0.0995	0.1152	0.0845	0.0428	0.0620	0.0914	0.0681	0.0868	0.1026	0.1026	0.0442	0.0729	0.0885
ABBBAA	0.0265	0.0333	0.0361	0.0437	0.0253	0.0253	0.0075	0.0157	0.0238	0.0258	0.0336	0.0336	0.0075	0.0157	0.0235
ABBBBA	0.0361	0.0437	0.0287	0.0361	0.0178	0.0362	0.0157	0.0081	0.0336	0.0183	0.0258	0.0258	0.0157	0.0081	0.0157
ABEABA	0.0506	0.0582	0.0433	0.0506	0.0196	0.0395	0.0361	0.0287	0.0478	0.0326	0.0401	0.0401	0.0182	0.0101	0.0182
ACAABB	0.0546	0.0422	0.0462	0.0348	0.0548	0.0777	0.0737	0.0669	0.0542	0.0592	0.0475	0.0667	0.0737	0.0669	0.0737
ADBABA	0.0297	0.0384	0.0213	0.0297	0.0095	0.0298	0.0275	0.0196	0.0471	0.0314	0.0391	0.0391	0.0275	0.0196	0.0275
BAAAAA	0.0110	0.0196	0.0215	0.0312	0.0315	0.0315	0.0291	0.0396	0.0278	0.0304	0.0393	0.0393	0.0291	0.0396	0.0477
BAAAAC	0.0196	0.0282	0.0312	0.0408	0.0402	0.0402	0.0363	0.0477	0.0358	0.0393	0.0483	0.0278	0.0363	0.0477	0.0363
BAAABA	0.0215	0.0312	0.0121	0.0215	0.0230	0.0447	0.0396	0.0318	0.0393	0.0217	0.0304	0.0304	0.0396	0.0318	0.0396
BAAACA	0.0349	0.0460	0.0240	0.0349	0.0348	0.0629	0.0527	0.0435	0.0540	0.0337	0.0437	0.0437	0.0527	0.0435	0.0527
BAAAFA	0.0215	0.0312	0.0349	0.0460	0.0447	0.0447	0.0396	0.0527	0.0393	0.0437	0.0540	0.0540	0.0396	0.0527	0.0621
BAAAHA	0.0349	0.0460	0.0240	0.0349	0.0348	0.0348	0.0527	0.0435	0.0540	0.0337	0.0437	0.0437	0.0527	0.0435	0.0527
BAACDA	0.0747	0.0873	0.0621	0.0747	0.0706	0.0706	0.0896	0.0787	0.0945	0.0710	0.0828	0.0828	0.0896	0.0787	0.0896
BACAAA	0.0293	0.0374	0.0413	0.0505	0.0569	0.0569	0.0524	0.0645	0.0454	0.0499	0.0583	0.0583	0.0524	0.0645	0.0719
BADCDA	0.1104	0.1214	0.0996	0.1104	0.1123	0.1123	0.1280	0.1189	0.1274	0.1071	0.1171	0.1171	0.1280	0.1189	0.1280
BADCEA	0.1104	0.1214	0.0996	0.1104	0.1123	0.1123	0.1280	0.1189	0.1274	0.1071	0.1171	0.1171	0.1280	0.1189	0.1280
BBAABA	0.0304	0.0393	0.0217	0.0304	0.0318	0.0527	0.0481	0.0409	0.0291	0.0112	0.0200	0.0200	0.0294	0.0213	0.0294
BBABAA	0.0291	0.0364	0.0397	0.0479	0.0481	0.0481	0.0272	0.0369	0.0260	0.0283	0.0367	0.0367	0.0272	0.0369	0.0446
BBBAAA	0.0291	0.0363	0.0396	0.0477	0.0277	0.0277	0.0267	0.0363	0.0270	0.0294	0.0376	0.0376	0.0094	0.0183	0.0267
BBBABA	0.0396	0.0477	0.0318	0.0396	0.0197	0.0397	0.0363	0.0289	0.0376	0.0213	0.0294	0.0294	0.0183	0.0102	0.0183
BBBBAA	0.0376	0.0443	0.0481	0.0556	0.0363	0.0363	0.0170	0.0259	0.0341	0.0369	0.0446	0.0446	0.0170	0.0259	0.0337
BBBBGA	0.0896	0.1005	0.1232	0.1382	0.1069	0.0644	0.0633	0.0932	0.0876	0.1084	0.1235	0.1235	0.0633	0.0932	0.1083
BDBABA	0.0427	0.0513	0.0344	0.0427	0.0214	0.0428	0.0394	0.0316	0.0600	0.0447	0.0522	0.0522	0.0394	0.0316	0.0394

Table 7. (cont.)															
ABBACA	ABBAGA	ABBBAA	ABBBBA	ABEABA	ACAABB	ADBABA	BAAAAA	BAAAAC	BAAABA	BAAACA	BAAAFA	BAAAHA	BAACDA	BACAAA	BADCDA
5	8	3	4	6	6	3	1	2	3	4	3	4	9	3	13
6	9	4	5	7	5	4	2	3	4	5	4	5	10	4	14
4	12	4	3	5	5	2	3	4	1	3	4	3	7	5	12
5	13	5	4	6	4	3	4	5	3	4	5	4	9	6	13
2	10	3	2	2	6	1	4	5	3	4	5	4	8	7	13
5	5	3	4	5	9	3	4	5	5	7	5	4	8	7	13
4	7	1	2	4	9	3	3	4	5	6	5	6	10	6	15
3	11	2	1	3	8	2	5	6	4	5	6	5	9	7	14
5	8	3	4	6	6	5	3	4	5	6	5	6	11	5	15
2	10	3	2	4	7	4	4	5	3	4	5	4	8	6	12
3	12	4	3	5	6	5	5	6	4	5	6	5	10	7	14
3	12	4	3	5	8	5	5	3	4	5	6	5	10	7	14
2	5	1	2	2	9	3	3	4	5	6	5	6	10	6	15
1	8	2	1	1	8	2	5	6	4	5	6	5	9	7	14
2	10	3	2	2	9	3	6	4	5	6	7	6	10	8	15
X	7	3	3	2	9	3	6	7	5	4	5	7	13	9	17
0.0572	X	6	9	10	18	11	12	11	14	12	8	12	13	13	17
0.0259	0.0510	X	1	3	10	4	4	5	6	7	6	7	11	7	16
0.0171	0.0790	0.0077	X	2	9	3	6	6	5	6	7	6	10	8	15
0.0200	0.0868	0.0258	0.0183	X	11	4	7	8	7	8	9	8	12	6	12
0.0809	0.1594	0.0853	0.0791	0.0926	X	5	8	9	7	9	10	9	14	11	18
0.0301	0.0986	0.0373	0.0301	0.0314	0.0432	X	5	6	4	5	7	5	10	8	15
0.0527	0.0822	0.0376	0.0481	0.0645	0.0690	0.0427	X	1	1	3	1	3	8	2	12
0.0621	0.0938	0.0443	0.0556	0.0719	0.0773	0.0513	0.0096	X	2	4	2	4	9	3	14
0.0435	0.1168	0.0481	0.0409	0.0574	0.0609	0.0344	0.0106	0.0213	X	1	3	1	6	4	11
0.0349	0.1008	0.0611	0.0527	0.0711	0.0769	0.0473	0.0243	0.0366	0.0120	X	1	3	10	5	15
0.0435	0.0706	0.0481	0.0611	0.0794	0.0864	0.0570	0.0106	0.0213	0.0243	0.0120	X	5	12	4	17
0.0593	0.1008	0.0611	0.0527	0.0711	0.0769	0.0473	0.0243	0.0366	0.0120	0.0287	0.0437	X	5	5	9
0.1082	0.1082	0.0973	0.0872	0.1071	0.1223	0.0857	0.0669	0.0806	0.0531	0.0863	0.1049	0.0394	X	11	5
0.0794	0.1104	0.0605	0.0725	0.0499	0.0911	0.0697	0.0188	0.0279	0.0310	0.0467	0.0310	0.0467	0.0908	X	11
0.1504	0.1504	0.1344	0.1260	0.1071	0.1593	0.1291	0.1048	0.1166	0.0930	0.1284	0.1448	0.0815	0.0403	0.0908	X
0.1504	0.1504	0.1344	0.1260	0.1071	0.1593	0.1291	0.1048	0.1166	0.0930	0.1284	0.1448	0.0815	0.0665	0.0908	0.0263
0.0322	0.1028	0.0369	0.0295	0.0455	0.0740	0.0447	0.0205	0.0303	0.0107	0.0228	0.0341	0.0228	0.0630	0.0405	0.1012
0.0490	0.0765	0.0184	0.0275	0.0600	0.0937	0.0621	0.0198	0.0279	0.0306	0.0442	0.0306	0.0442	0.0848	0.0382	0.1198
0.0295	0.0567	0.0170	0.0259	0.0294	0.0880	0.0394	0.0200	0.0279	0.0306	0.0440	0.0306	0.0440	0.0833	0.0441	0.1239
0.0201	0.0873	0.0259	0.0184	0.0213	0.0814	0.0316	0.0306	0.0395	0.0219	0.0339	0.0440	0.0339	0.0715	0.0565	0.1141
0.0370	0.0633	0.0089	0.0172	0.0369	0.0997	0.0492	0.0292	0.0366	0.0399	0.0532	0.0399	0.0532	0.0916	0.0529	0.1308
0.0781	0.0200	0.0531	0.0816	0.1084	0.1893	0.1236	0.0833	0.0951	0.1186	0.1024	0.0715	0.1024	0.1109	0.1121	0.1539
0.0432	0.1156	0.0492	0.0422	0.0447	0.0575	0.0118	0.0332	0.0427	0.0239	0.0371	0.0479	0.0371	0.0783	0.0614	0.1244

Table 7. (cont.)

BADCEA	BBAABA	BBABAA	BBBAAA	BBBABA	BBBBAA	BBBBGA	BDBABA
13	4	3	3	5	4	10	5
14	5	4	4	6	5	12	6
12	3	5	5	4	6	14	4
13	4	6	6	5	6	16	5
13	4	6	3	2	4	12	2
13	6	6	3	5	4	7	5
15	6	3	3	4	2	7	5
14	5	4	4	3	3	11	4
15	3	3	3	4	4	10	7
12	1	3	3	2	4	13	5
14	2	4	4	3	5	14	6
14	2	4	4	3	5	14	6
15	3	3	1	2	2	7	5
14	2	4	2	1	3	11	4
15	3	5	3	2	4	13	5
17	4	6	3	2	4	9	5
17	12	9	7	10	7	2	13
16	4	2	2	3	1	6	6
15	3	3	3	2	2	9	5
12	5	7	3	2	4	13	5
18	9	11	10	9	12	22	7
15	5	7	5	4	6	14	1
12	2	2	2	4	3	10	4
14	4	3	3	5	4	11	5
11	1	4	4	3	5	14	3
15	3	5	5	4	6	12	4
17	4	4	4	5	5	8	6
9	3	5	5	4	6	12	4
8	7	10	10	8	11	13	9
11	5	4	5	7	6	13	7
3	12	14	14	13	15	18	14
X	12	14	14	13	15	18	14
0.1012	X	2	2	1	3	12	4
0.1198	0.0189	X	2	3	1	7	6
0.1239	0.0201	0.0186	X	1	1	7	4
0.1141	0.0113	0.0284	0.0090	X	2	10	3
0.1308	0.0284	0.0097	0.0082	0.0172	X	5	5
0.1539	0.1031	0.0583	0.0562	0.0874	0.0457	X	14
0.1244	0.0352	0.0544	0.0305	0.0218	0.0412	0.1190	X

Table 8. Uncorrected nucleotide sequence divergence (above diagonal) and corrected nucleotide sequence divergence (below diagonal) among samples based on RFLP analysis of the mtDNA D-loop.

	PS97	PS96	PS94	PS86	PS85	VN	SA	TW98	TW99	EPO
PS97		0.02965	0.02858	0.03121	0.02933	0.02821	0.03051	0.03187	0.02778	0.02594
PS96	-0.0001		0.02709	0.03091	0.02924	0.0269	0.03009	0.03082	0.02712	0.02708
PS94	0.00131	-0.0003		0.02845	0.02770	0.02387	0.02765	0.02854	0.02533	0.02639
PS86	-0.0004	-0.0008	-0.0008		0.03109	0.02861	0.03166	0.03310	0.02963	0.02878
PS85	-0.0003	-0.0005	0.00041	-0.0005		0.02743	0.03027	0.03151	0.02735	0.02649
VN	0.00016	-0.0013	-0.0018	-0.0014	-0.0006		0.02787	0.02866	0.02530	0.02571
SA	-0.0001	-0.0006	-0.0006	-0.0009	-0.0004	-0.0011		0.03174	0.02824	0.02779
TW98	0.00054	-0.0006	-0.0004	-0.0002	0.00017	-0.0011	-0.0005		0.02900	0.02927
TW99	0.00027	-0.0006	0.00021	0.00017	-0.0002	-0.0006	-0.0002	-0.0002		0.02498
EPO	-0.0006	0.0004	0.0022	0.00028	-0.00098	0.00075	0.00027	0.00104	0.00056	

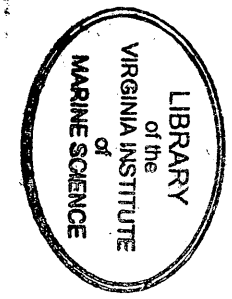


Table 9. Probability of homogeneous distributions of mtDNA D-loop composite haplotypes among temporal samples of black marlin from Port Stephens, Australia (Roff and Bentzen, 1989). Values given here have not been corrected for multiple tests.

	PS85	PS86	PS94	PS96	PS97
PS85	X	0.452	0.227	0.391	0.485
PS86		X	0.958	0.284	0.653
PS94			X	0.088	0.226
PS96				X	0.545
PS97					X

Table 10. Probability of homogeneous distributions of mtDNA D-loop composite haplotypes among geographic samples of black marlin from Australia (AUST), Taiwan (TW), Vietnam (VN), South Africa (SA), and the eastern Pacific Ocean (EPO) are given above the diagonal and corrected nucleotide sequence divergences (δ) for the pooled geographic collections are given below the diagonal (Roff and Bentzen, 1989). Values given here have not been corrected for multiple tests.

	AUST	VN	SA	TW	EPO
AUST		0.957	0.144	0.008	0.057
VN	-0.000632		0.918	0.461	0.547
SA	-0.000338	-0.001135		0.017	0.219
TW	0.00012	-0.000797	-0.000341		0.147
EPO	0.000181	0.000750	0.000269	0.000852	

Table 11. Exact Φ_{ST} (mtDNA) and θ_{ST} and R_{ST} (microsatellite DNA) values from analysis of molecular variance at the mtDNA D-loop and five microsatellite loci for all four tests: temporal, overall, between oceans, and within the Pacific Oceans. Bold print indicates significant p-values ($\alpha \leq 0.05$).

	MtDNA	Nuclear DNA									
	D-loop	**GATA-10**		**GATA-90**		**GATA-52**		**GATA-08**		**GATA-60**	
	Φst	θ_{ST}	R_{ST}	θ_{ST}	R_{ST}	θ_{ST}	R_{ST}	θ_{ST}	R_{ST}	θ_{ST}	R_{ST}
temporal	-0.0085	-0.01209	-0.00749	0.00583	0.00544	**0.01494**	0.01164	**0.01983**	**0.17830**	0.00088	**0.02252**
overall	-0.00099	-0.00406	0.00447	0.01360	-0.00862	0.00790	**0.05949**	**0.01289**	**0.12189**	**0.01005**	**0.04293**
oceans	-0.00769	0.00771	-0.00685	0.01362	-0.00097	0.00679	**0.12450**	**0.01332**	**0.13943**	**0.01141**	**0.05495**
w/in Pac	-0.00524	0.01354	0.01370	0.02150	-0.01138	0.00948	**0.06047**	**0.01373**	**0.10085**	**0.00894**	**0.04317**

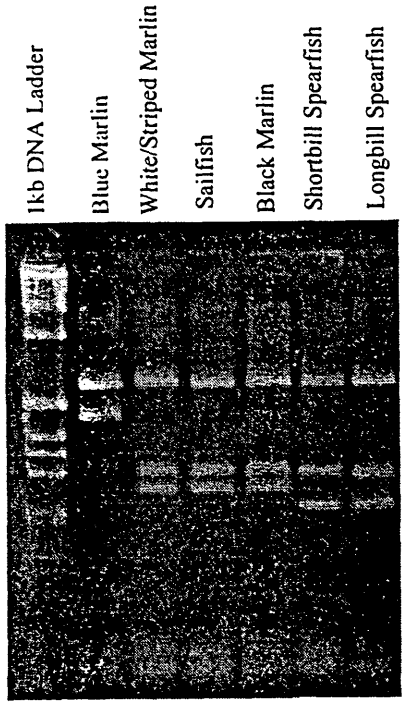
Table 12. Tests for conformance of microsatellite loci genotypic distributions to expectations of Hardy-Weinberg equilibrium. Significant deviations from the expectations of Hardy-Weinberg equilibrium for a collection at a locus are indicated by "-," while "+" indicates tests of Hardy-Weinberg equilibrium where $p<0.05$. Sample sizes are given in parentheses next to collections and number of overall alleles at each locus are given in parentheses next to each locus heading.

	GATA-10 (6)	GATA-90 (7)	GATA-52 (11)	GATA-08 (21)	GATA-60 (27)
PS97 (33)	+	+	+	+	+
PS96 (26)	+	+	+	+	+
PS86 (41)	+	+	+	+	+
PS85 (35)	+	+	+	+	+
TW98 (37)	+	+	+	-	+
TW99 (34)	+	+	+	+	-
SA (32)	+	+	+	+	-
VN (12)	+	+	-	-	-
EPO (25)	+	+	-	-	-

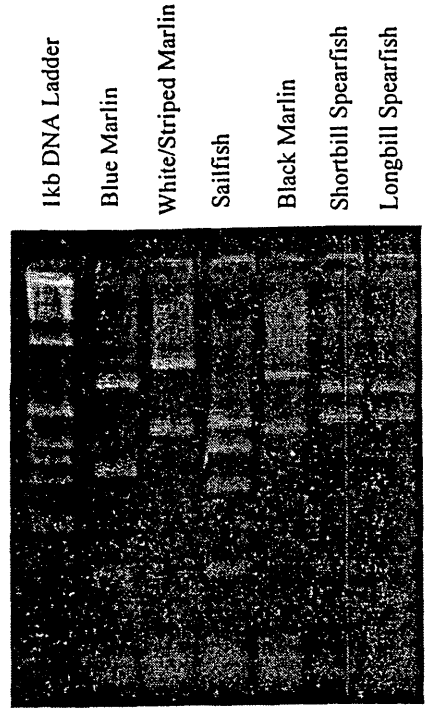
Table 13. Observed heterozygosity (H), number of unique alleles (# All), and individual sample size (N) for microsatellite loci.

	GATA-10			GATA-90			GATA-52			GATA-08			GATA-60		
	H	# All	N	H	# All	N	H	# All	N	H	# All	N	H	# All	N
PS97	45.7	5	70	42.9	5	56	93.3	8	60	94.1	14	68	96.3	14	54
PS96	32.4	2	68	51.6	4	62	87.5	7	32	88.6	13	70	94.9	18	78
PS86	35.1	3	74	51.4	3	70	93.1	7	58	97.1	14	68	94.9	21	78
PS85	44.1	3	86	44.2	3	86	82.3	8	82	89.5	14	76	97.6	19	82
TW98	22.9	2	70	35.9	4	78	93.1	7	58	93.2	13	48	93.8	18	64
TW99	25.8	2	62	33.3	4	42	92.3	8	56	100.0	14	48	100.0	15	48
EPO	30.8	3	52	38.1	4	42	94.4	9	36	95.2	16	42	100.0	16	44
VN	46.2	3	26	31.7	2	26	93.3	8	30	95.0	16	40	94.3	15	30
SA	31.6	3	38	42.1	4	38	80.0	9	32	96.0	15	50	100.0	16	36
Total	34.8	6	546	42.0	7	500	90.1	11	444	93.9	21	510	97.1	27	514

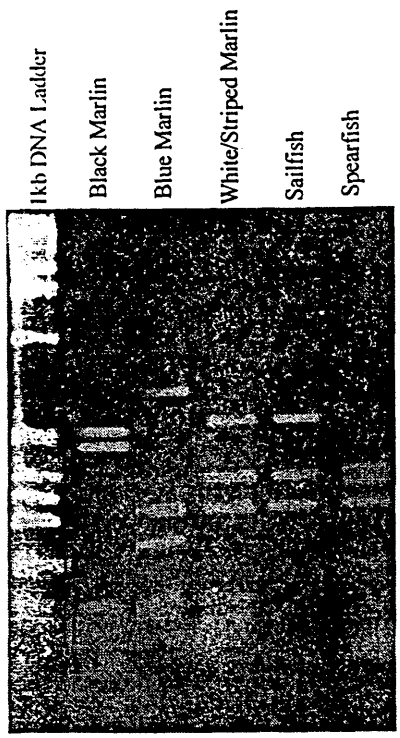
Figure 3. Billfish species identification key based on the mitochondrial ND4 region and the anonymous single-copy nuclear locus BM32-2.



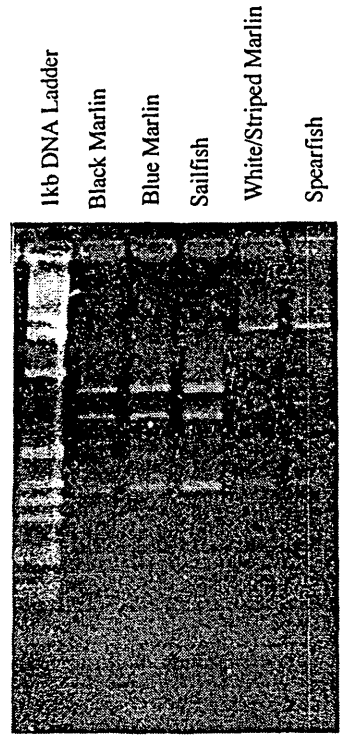
Bm32-2 cut with *Dra* I



Bm32-2 cut with *Dde* I



ND4 cut with *Hae* III



ND4 cut with *Ban* I

Figure 4. Distribution of alleles at the microsatellite DNA locus GATA-10.

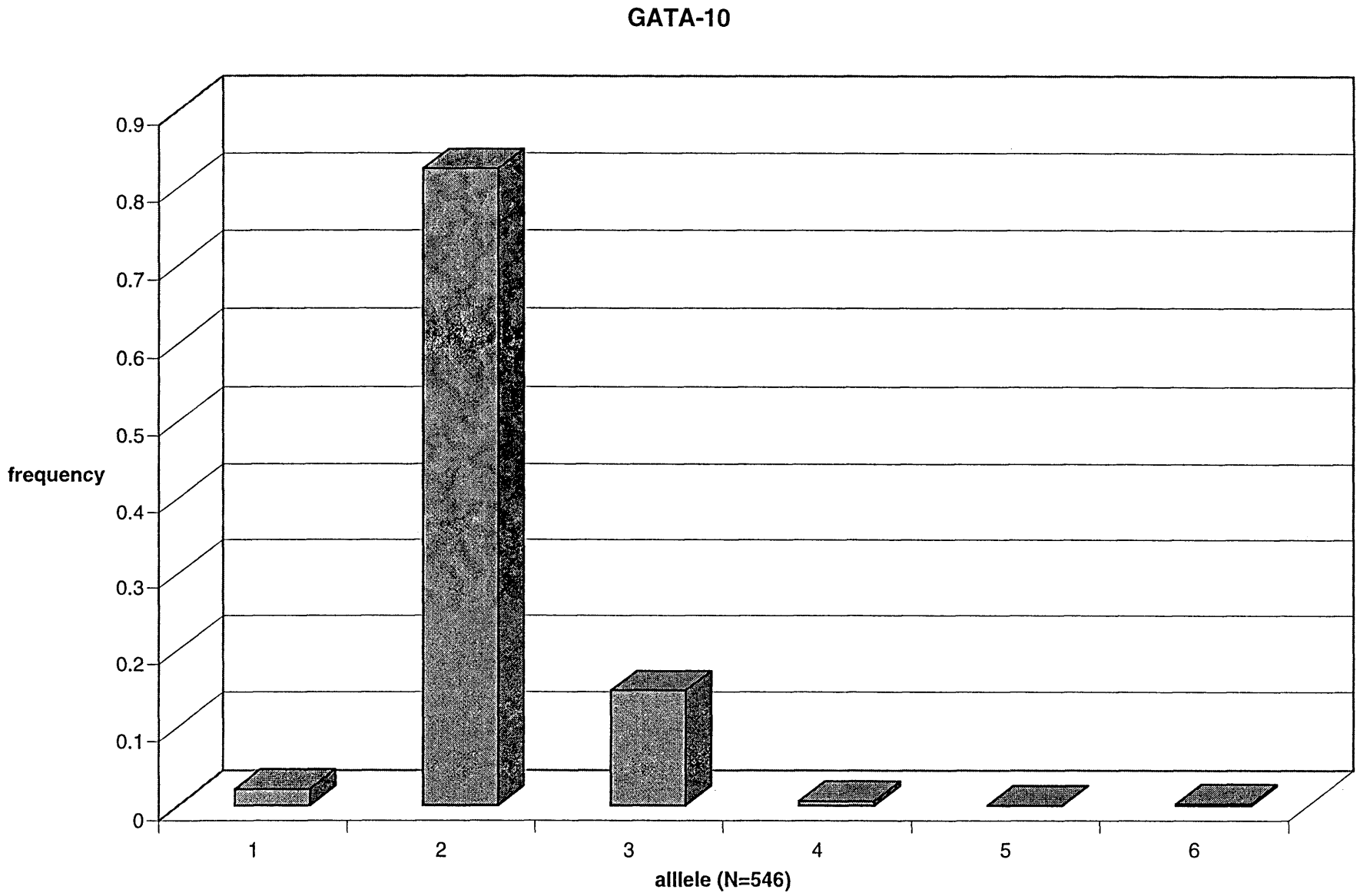

GATA-10
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
frequency
1
2
3
4
5
6
alllele (N=546)

Figure 5. Distribution of alleles at the microsatellite DNA locus GATA-90.

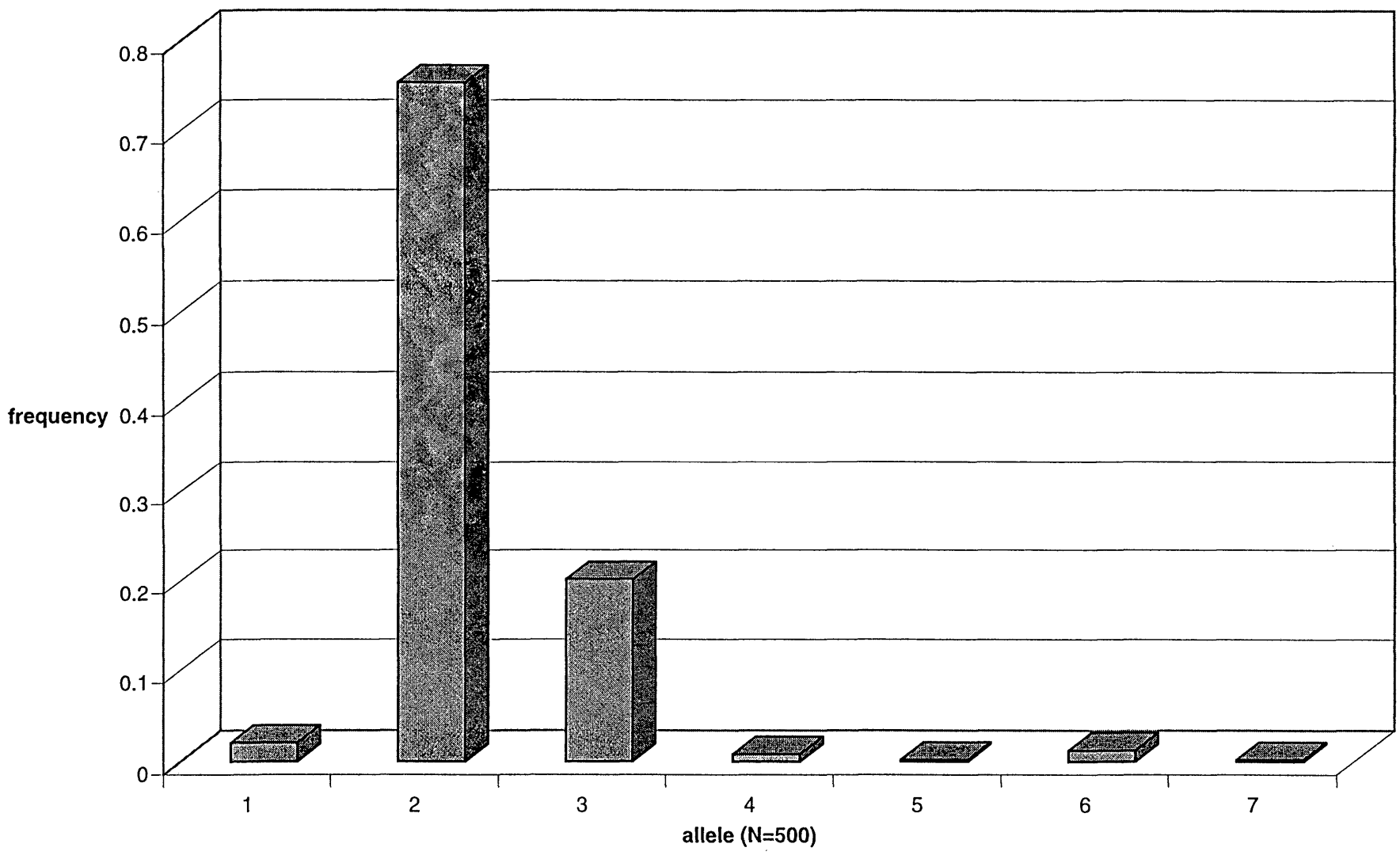

GATA-90
frequency
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
allele (N=500)

Figure 6. Distribution of alleles at the microsatellite DNA locus GATA-52.

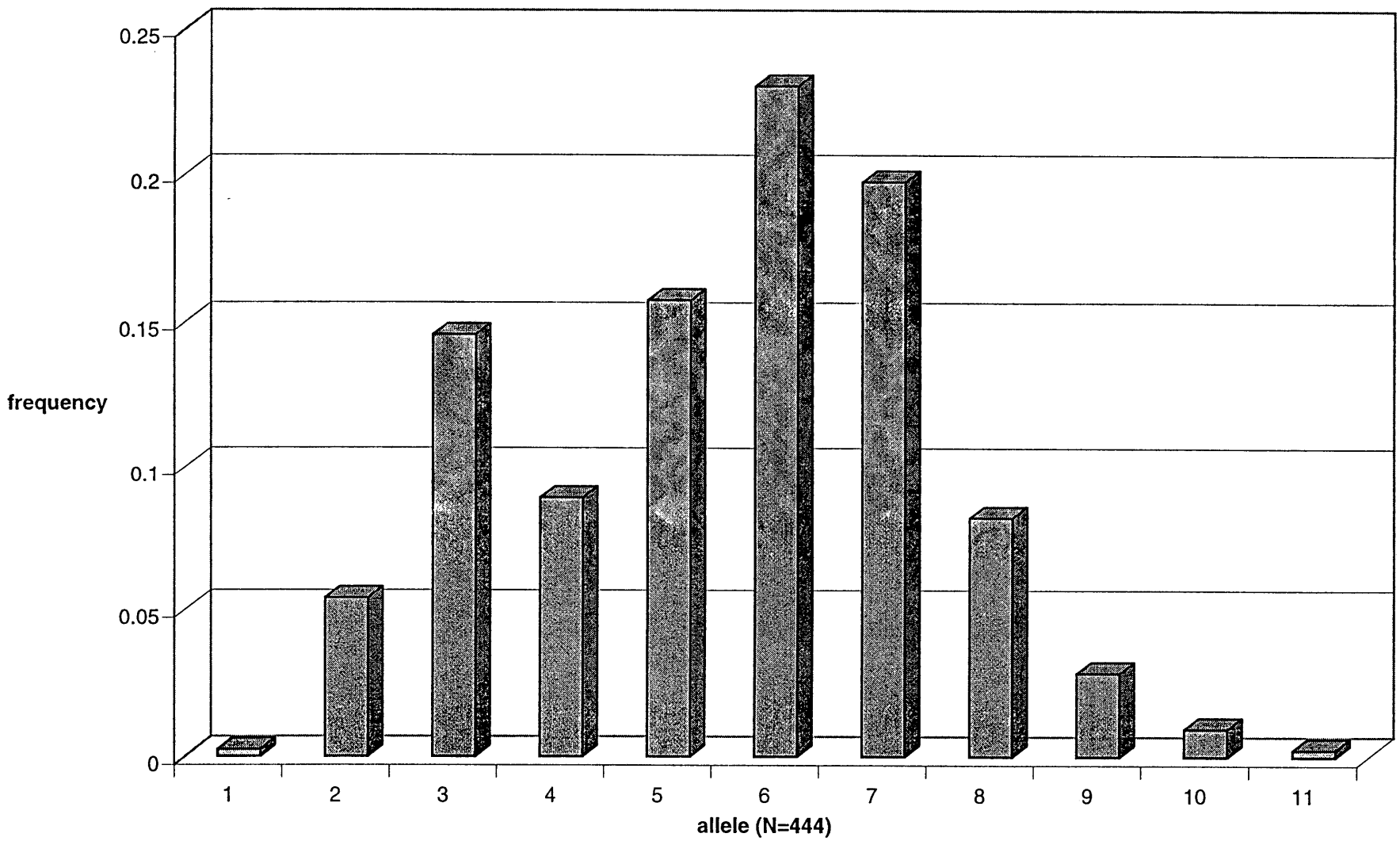

GATA-52
0.25
0.2
0.15
0.1
0.05
0
frequency
1
2
3
4
5
6
7
8
9
10
11
allele (N=444)

Figure 7. Distribution of alleles at the microsatellite DNA locus GATA-08.

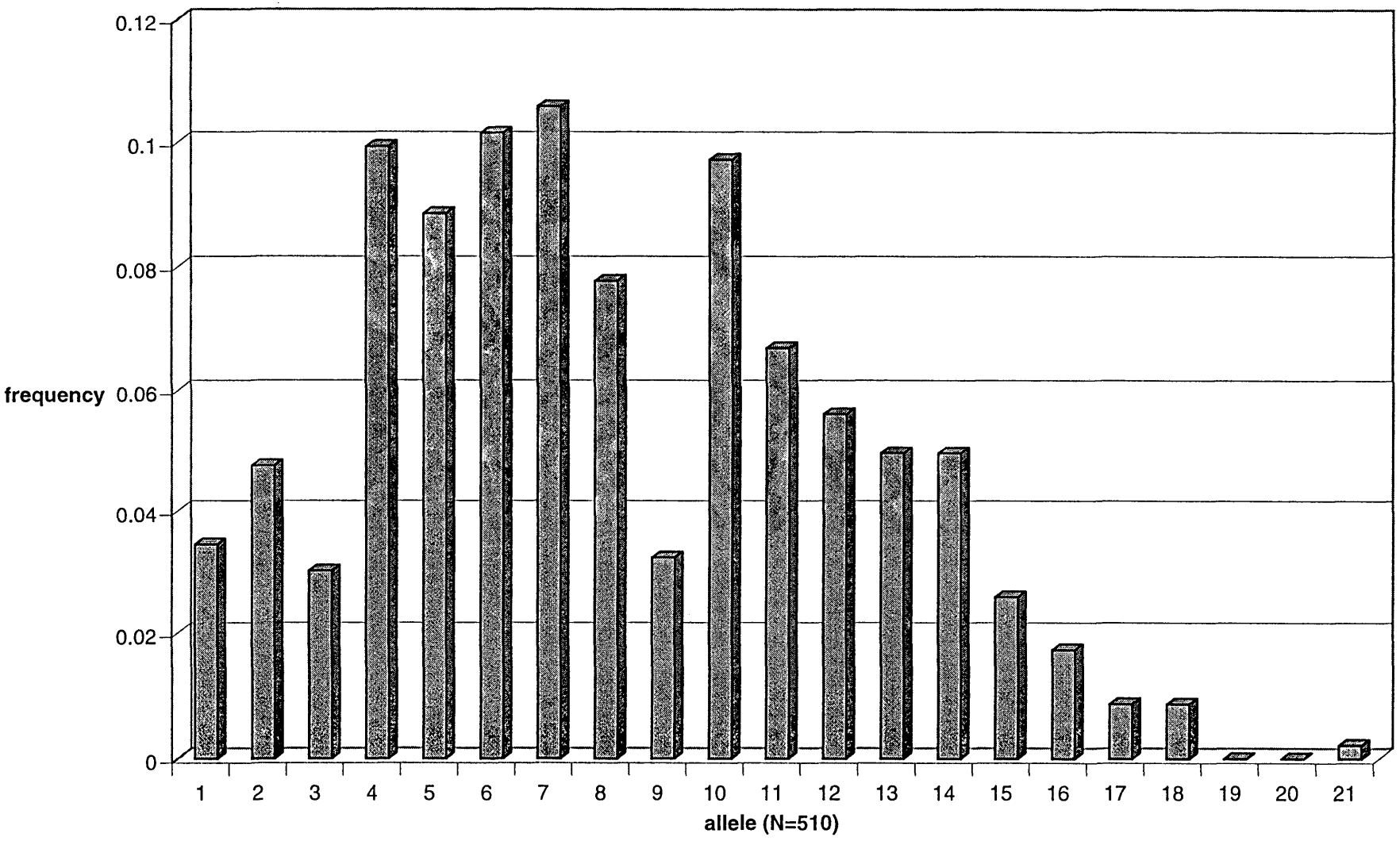
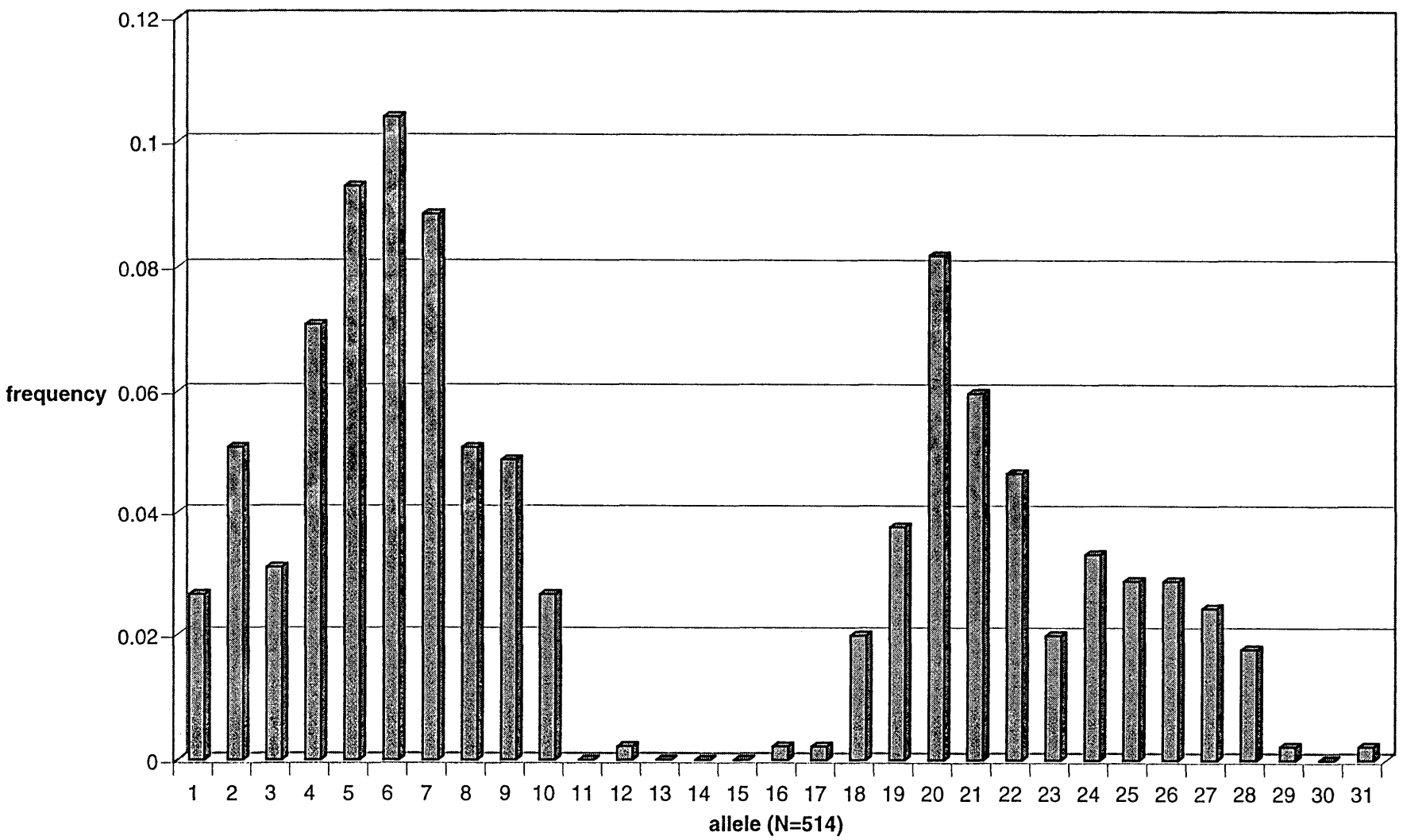

GATA-08
frequency
0.12
0.1
0.08
0.06
0.04
0.02
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
allele (N=510)

Figure 8. Distribution of alleles at the microsatellite DNA locus GATA-60.


GATA-60
frequency
0.12
0.1
0.08
0.06
0.04
0.02
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
allele (N=514)

Chapter 4. DISCUSSION

Three classes of molecular markers, mtDNA, scnDNA, and microsatellite DNA, were successfully amplified from black marlin DNA. Consistent amplifications were obtained for the mtDNA D-loop, seven scnDNA loci, and five of the six microsatellite loci surveyed. Intraspecific variation was detected with RFLP analysis of the mtDNA D-loop, but no intraspecific variation was detected at any of the seven scnDNA loci. Consequently, the scnDNA loci were excluded from the analysis. The five microsatellite loci that amplified in the black marlin demonstrated high intraspecific variation.

Genotypic distributions for two of the five microsatellite loci conformed to expectations of Hardy-Weinberg equilibrium. Genotypic frequencies at GATA-52, GATA-08, and GATA-60 deviated significantly from Hardy-Weinberg expectations. Lack of conformance of these loci to Hardy-Weinberg expectations precludes their use in the temporal and geographic analyses. Other studies have attributed lack of conformance to Hardy-Weinberg equilibrium to null alleles (e.g. GATA-10 for the blue marlin; Bounaccorsi, 1998), but lack of conformance in this study appears to be more a function of sample size than locus-specific amplification problems. The collections that were most problematic were those with the smallest sample sizes and the largest number alleles (Table 11). Specifically, VN and EPO deviated from Hardy-Weinberg expectation for the three most variable loci, GATA-52, GATA-08, and GATA-60 (Table 11). The SA collection, which had slightly more samples, deviated only for GATA-08 and GATA-60 (Table 11).

Genetic Variation

In order to discriminate between populations, a molecular marker must exhibit intraspecific variation. Black marlin exhibited high levels of intraspecific genetic variation in a 1400 bp fragment of the of the mtDNA D-loop region when surveyed with polymorphic enzymes. MtDNA nucleon diversities (h) of black marlin collections (0.81, 0.73-0.91) was comparable to that reported in other studies of istiophorid billfishes employing RFLP analysis of mtDNA. Based on results from RFLP analyses of whole molecule mtDNA with 11 enzymes, nucleon diversities in other istiophorid billfishes ranged from 0.59 (0.28-0.85) for sailfish from the Atlantic, Pacific, and Indian oceans to 0.91 (0.58-0.97) for blue marlin from the Atlantic and Pacific oceans (Graves, 1998). Striped marlin and white marlin also fell within this range (Graves, 1998). Sequence diversities (π) from the same whole molecule mtDNA studies (0.15% for white marlin to 0.58% for blue marlin) tended to be lower than that observed in the D-loop region of the black marlin (2.87%), probably because of the higher genetic variation characteristic of the D-loop region when compared to the entire mitochondrial genome. A 300 bp region of the D-loop from the swordfish, *Xiphias gladius*, demonstrated a mean nucleotide sequence diversity of 3.45% (Rosel and Block, 1996), comparable to that observed in this study for RFLPs of the entire black marlin D-loop region (2.87%). Sufficient variation was revealed with RFLPs from the black marlin D-loop region to provide a high resolution molecular marker with which to test for population structure.

ScnDNA. No intra-specific genetic variation was revealed within black marlin by any of the seven single copy nuclear loci examined in this study. The four anonymous

single copy loci that were invariant in this study demonstrated genetic variation within blue marlin, white marlin, and striped marlin (Bounaccorsi et al., 1999). The three invariant intron regions screened in this study were previously used to demonstrate intraspecific variation in related species, such as blue marlin and several species of tunas (Graves and McDowell, 1997; Reece, 1997). ScnDNA markers have been used successfully in many studies of population genetic structure for a wide range of organisms, including Atlantic cod, *Gadus morhua* (Pogson et al., 1995), green sea turtle *Chelonia mydas* (Karl et al., 1992), and American oysters *Crassostrea virginica* (Karl and Avise, 1992). The lack of genetic variation at these scnDNA loci within the black marlin was unexpected, and preclude their use in population structure analysis.

Microsatellite DNA. Genetic variation observed at the five microsatellite markers employed in this study was relatively high and comparable to levels observed for the same five loci in four other species of istiophorid billfish: blue marlin, white marlin, striped marlin, and sailfish. Interspecific comparisons are presented below based on data from the blue marlin (N=465; Bounaccorsi, 1998), white marlin (N=115; Graves et al., unpub. data), striped marlin (N=220; Graves et al., unpub. data), and sailfish (N=456; McDowell, unpub. data).

Variation in black marlin was lower than that seen in other istiophorids for two microsatellite loci. The allelic distributions at GATA-10 and GATA-90 for black marlin were dominated by a single allele that occurred at a frequency greater than 75% for each locus. The total number of alleles was low for these two loci (6 and 7, respectively; Figures 4 and 5). In contrast to black marlin, GATA-10 exhibited a much wider range of

alleles in white marlin, striped marlin and blue marlin (Figure 9). GATA-10 was most variable in the blue marlin, which exhibited 27 different alleles that differed in size from 8 to 40 repeats. White and striped marlin also displayed a wide range of repeats at this locus, with 15 and 17 alleles that spanned a repeat range from 27 to 45 repeats, respectively. In sailfish, 19 different alleles were detected that spanned from 13 to 33 repeats. A similar trend was seen for GATA-90 at which only 7 alleles were detected in the black marlin. In contrast, 37 alleles were detected at GATA-90 in the blue marlin, ranging in repeat number from 8 to 42, and 45 alleles were detected in sailfish, ranging from 21 to 67 repeats (Figure 10).

Allelic distributions at GATA-08 and GATA-60 in black marlin were similar to those found in other billfish species in terms of diversity and number of alleles (Figures 6, 7, 11, and 12). For black marlin, 19 alleles occurred at GATA-08 that differed by 21 repeats and formed a slightly bimodal distribution. Blue marlin were quite variable at GATA-08, with 48 alleles ranging in size from 1 to 58 repeats. White marlin and striped marlin were more variable than black marlin but less variable than blue marlin, with 24 and 30 alleles, respectively, ranging from 8 to 33 repeats for the white marlin and 4 to 39 repeats for the striped marlin. Bimodal distributions were also observed at GATA-08 for white marlin and Atlantic blue marlin.

GATA-60 was the most variable microsatellite locus surveyed in black marlin, with 27 alleles spanning a range of 31 repeats and a strongly bimodal distribution. Striped marlin exhibited 31 alleles at GATA-60, ranging from 21 to 53 repeats. Twenty-four alleles occurred at GATA-60 for white marlin, ranging from 12 to 30 repeats. Thirty-six alleles were detected at this locus for the blue marlin, where alleles ranged from 5 to 53

repeats. Slight bimodal distributions were noted for GATA-60 within blue marlin in both the Atlantic and Pacific oceans as well as in striped marlin.

No comparison with other billfish is possible for GATA-52. In black marlin, GATA-52 exhibited moderate levels of variation with 11 alleles that spanned 11 repeats. This locus was developed for use in blue marlin, but was not utilized in previous studies. It has been successfully amplified in the other istiophorid species (Bounaccorsi, unpub. data) and should be useful in future studies that incorporate large sample sizes.

Overall, the microsatellite DNA markers developed by Buonaccorsi (1998) for blue marlin have been useful for population structure analyses throughout the Istiophoridae, indicating interspecific conservation of microsatellite flanking regions. One exception to this trend was found within the black marlin at the locus GATA-01, which amplified well in all billfishes surveyed to date except the black marlin.

Interspecific conservation of microsatellite DNA loci priming sites was also noted by Broughton and Gold (1997) who described microsatellite DNA markers from bluefin tuna (*Thunnus thynnus*) that were subsequently amplified in six other scombrids from three genera: yellowfin tuna (*T. albacares*), blackfin tuna (*T. atlanticus*), bigeye tuna (*T. obesus*), albacore tuna (*T. albacares*), skipjack tuna (*Katsuwonis pelamis*), and king mackerel (*Scomberomorus cavalla*). Broughton and Gold (1997) noted that the pattern of successful amplification among these fishes was consistent with molecular phylogenetic relationships among scombrids (Block et al., 1993; Chow and Kishino, 1995).

Interspecific variation in the amplification of microsatellite loci among istiophorid billfishes may corroborate phylogenetic implications of previous molecular investigations of this group. Recent genetic studies of istiophorids have questioned current taxonomy,

especially the relationship between the blue and black marlins and the validity of the genus *Makaira* (Morgan, 1992; Block et al., 1993). In fact, it has been hypothesized that the black marlin may be the outgroup to a monophyletic clade containing the other istiophorids (Block et al., 1993).

In general, microsatellite DNA variation reported for billfishes is slightly higher than that reported for other marine teleosts (H_{obs}>90%; Brooker et al., 1994), although marked differences exist among the Istiophoridae (Buonaccorsi, 1998). Levels of diversity and heterogeneity for two microsatellite DNA markers, GATA-10 and GATA-90, were lower in black marlin than in other billfishes examined to date (H_{obs} = 34.8 and 42.0%, respectively). The patterns of allelic distribution and heterozygosity at these loci are similar to those reported for microsatellite loci from other closely related pelagic fishes, such as the northern bluefin tuna and king mackerel. Five microsatellite loci described from bluefin tuna had an average H_{obs} of 43.4% and two loci from king mackerel exhibited H_{obs}=50.5% and 60.6%, respectively (Broughton and Gold, 1997; Broughton et al., unpub. data). Allelic distributions were similar between the two black marlin microsatellite loci, GATA-10 and GATA-90, and three bluefin tuna microsatellite loci: 15, 16, and 38 (Broughton and Gold, 1997). The distributions were characterized by a single common allele with a few other alleles at low frequency. The patterns observed for black marlin at GATA-10 and GATA-90, although different from patterns seen at the same loci within the Istiophoridae, exhibit allelic distributions similar to those observed in other confamilial scombrid fishes.

Temporal Genetic Stability

MtDNA. In order to evaluate the significance of geographic genetic heterogeneity among samples collected in different years, one must first demonstrate the temporal stability of molecular markers over years at a location. Results from analysis of black marlin mtDNA D-loop composite haplotypes among samples collected at Port Stephens, Australia in four different years and from two temporal collections from Taiwan support the null hypothesis that there is no difference in allele frequencies between years at collection locations. This is an important result for it justifies comparisons among samples from different locations collected in different years and allows pooling of temporal collections, thereby increasing the power of subsequent population structure analyses. Temporal stability of mtDNA haplotypes has been reported in many other studies of genetic heterogeneity (Goodbred and Graves, 1995; Crosetti et al., 1994, Graves and McDowell, 1995; Graves and McDowell, 1994).

Microsatellite DNA. Temporal stability of allele frequencies was noted for two microsatellite DNA markers. Significant differences among years at Port Stephens, Australia were observed with indices of population subdivision at the three most variable loci: GATA-52, GATA-08, and GATA-60; however, these loci were excluded from population structure analysis because collections exhibited significant deviations from Hardy-Weinberg (Table 11). In three of five cases where the microsatellite markers were not temporally stable (Table 10), the magnitude of the F-statistic for the temporal comparison was greater than any of the values for the geographic comparisons. This phenomena has been noted in other studies (Broughton et al., unpub. data; Hedgecock, 1994; Smolenski et al., 1993; Purcell et al, 1996). Hedgecock (1994) attributed the

discrepancy between temporal and geographic F-statistics to genetic drift brought about by differential reproductive success of a given cohort. The cause for the discrepancy between temporal and geographic measures of population structure in the present study is unclear.

Lack of concordance among F_{ST} analogs (θ_{ST} and R_{ST}) was documented at GATA-52, GATA-08, and GATA-60 (Table 10). The most obvious example is GATA-08, where R_{ST} estimates exceed θ_{ST} by nearly an order of magnitude. Such differences stem from the differences between the models used to calculate these statistics. When data fit the IAM, alleles identical in state are identical in descent and θ_{ST} is an appropriate measure of subdivision; however, when data fit the SMM, θ_{ST} may tend to underestimate the degree of subdivision and indices that account for allele relationships like R_{ST} (length) may provide better estimates (Slatkin, 1995; Rousset, 1996; Broughton and Gold, 1997). In addition, the largest discrepancies between θ_{ST} and R_{ST} come from GATA-08 and GATA-60, which exhibit bimodal distributions. Bimodal distributions are believed to be formed when a mutation event occurs that results in the gain or loss of multiple repeat units (Levinson and Gutman, 1987). Such a mutation would result in two peaks around which single-steps in both directions yield bell-shaped curves around each mode. R_{ST} will overestimate the difference among alleles in this case because the distance between alleles is not described by a single step relationship. This may explain the high R_{ST} values observed for GATA-08 and GATA-60.

Repeat modes were stable over time. Pairwise θ_{ST}s and R_{ST}s indicate that there was no significant difference between the allelic distributions through the time period sampled for this study. As shown in Figure 13, the repeat modes were very similar

between the two most temporally distant samples, PS85 and PS97. The stability of the allelic distributions implies that genetic drift due to differential cohort success is not occurring at detectable levels over the time period represented in this study (Figure 13).

Geographic Genetic Heterogeneity

MtDNA. No significant geographic heterogeneity was detected with the RFLP analysis of the mtDNA D-loop. The overall chi-square test indicated that none of the pairwise tests was significant after correction for multiple testing and none of the Φ_{ST} values were significantly different from zero. Although nucleon diversity was high, the three most common haplotypes occurred at relatively similar frequencies in all collections.

Microsatellite DNA. Once loci that deviated from Hardy-Weinberg equilibrium were removed from the analysis, no significant differences among collections was detected. Neither θ_{ST} nor R_{ST} was significant in any of the three geographic comparisons (overall, between oceans, within Pacific) for GATA-10 and GATA-90 (Table 10).

Based on results from the mtDNA D-loop and two microsatellite loci from the black marlin, one cannot reject the null hypothesis of no genetic difference among geographic sample locations. These results are consistent with gene flow across the range of the black marlin. The level of genetic divergence among collections is similar to that observed for blue marlin, in that neither mtDNA nor microsatellite DNA markers detect differences within the Pacific Ocean ($\theta_{ST\text{-}overall}$=0.00088; Bounaccorsi, 1998). Greater

divergence among collections was noted for striped marlin, which exhibited significant differences with both mtDNA RFLPs and across all microsatellite DNA markers ($\theta_{ST\text{-}overall}$=0.016; Buonaccorsi, unpub. data). Because the present study is the first billfish population genetic study that includes Indian Ocean sample locations, no comparisons are available for between ocean comparisons with other billfishes. Bremer et al. (1998) were able to obtain samples for bigeye tuna, *Thunnus obesus*, from the Indian Ocean, and based on RFLP and sequence analysis of the mtDNA D-loop, the authors detected distinct Atlantic and Indo-Pacific clades similar to those reported for other highly migratory species; however, the authors were unable to reject the null hypothesis of genetic homogeneity between the Indian and Pacific oceans.

General Discussion

The conclusion that sufficient gene flow occurs across the range of the black marlin to prevent significant geographic genetic heterogeneity is consistent with tag returns from this species. A large tag-recapture data base is available for waters east of Australia due in part to the large recreational fisheries supported by black marlin in Australian waters. Tag-recapture data suggest two major themes in black marlin movements (Figure 14). The first is an annual cycle, whereby tagged animals return to the region of capture on a yearly basis. This pattern has been documented for times at liberty as long as five years (J. Pepperell, pers. com). The second theme is easily summarized: dispersal. For a large number of tagged animals, net displacement increases linearly with time at liberty. As can be seen from Figure 14, there is a stark difference between the behavior of black marlin and striped marlin after release. Both tagging

studies (Squire et al, 1984; Pepperell, pers. com.) and genetics (Graves and McDowell, 1994) suggest that stock structure exists in the Pacific for striped marlin. Figure 10 illustrates this point well, indicating that striped marlin typically do not travel far from the point of capture, rarely venturing more than 500 NM in periods as long as two years. Black marlin movements are not nearly so localized and based on tag recaptures 4000 NM from the point of tagging, it would seem that black marlin movements are limited only by the size of the ocean basin.

Black marlin larvae are poorly know. With the exception of larvae caught near the Great Barrier Reef reported by Leis (1987), there are very few collections of black marlin larvae and even when they have been reported (Nishikawa, 1986), the morphological characters used to identify the larvae are suspect. Consequently, spawning areas have usually been inferred from the distribution of individuals with high gonadosomatic indices, rather than the presence of larvae and spawning adults (Nakamura, 1985). The only well documented spawning area for black marlin is the Coral Sea, where spawning occurs from October to December. The Australian recreational fishery in Cairns targets this annual spawning aggregation.

During the early 1970s, a fishery for large female black marlin characteristic of the Cairns fishery existed near Cabo Blanco, Peru. While the presence of large females caused conjecture that spawning might occur in the eastern Pacific, records from the Cabo Blanco Fishing Club indicate that the fishery was dominated by females and that a high male to female ratio indicative of a spawning aggregation was not observed (Figure 16). It is unlikely that the Cabo Blanco fishery targeted a spawning aggregation.

These observations generate two unanswered questions: how restricted is the spawning range of the black marlin and where are the adults during the rest of the year? Japanese catch statistics pre-dating the 200 mile Australian Fishing Zone document the disappearance of black marlin from commercial catches after the spawning season in the waters east of Australia between 10 and 20° S longitude (Nakamura, 1969), consistent with the dispersal of the spawning aggregation. Estimates of the area over which black marlin spawn range from the entire Indonesian archipelago and adjacent seas to a restricted region in the Coral and South China seas. More detailed analyses of larval abundance and GSIs from adult black marlin is needed to better define the spawning area.

Why is defining the spawning range of black marlin important for genetic studies? If we fail to reject our null hypothesis of genetic homogeneity, then there should be an explanation for the cause of gene flow. In this case, the question is 'which life stage is responsible for genetic homogenization?' This question might seem trivial since adult black marlin exceed 4m lower-jaw fork length and 1500 pounds (i.e. they are good dispersers). However, despite several hundred tag-recaptures of adult black marlin, no recaptures have been reported between the Indian and Pacific oceans, suggesting a possible barrier to mixing.

Recent tag returns offer an alternate hypothesis that may explain how genetic mixing occurs between oceans without records of dispersal between the two oceans by large adults. During 1996 and 1997, large numbers of juvenile black marlin were tagged off New South Wales, Australia, and recaptures of fish from these year classes were reported over the following years. The majority of recaptures have come from the nearshore recreational fishery for juvenile black marlin which typically begins early in

the year and moves down the coast until the fish disappear offshore near Bermagui. In recent years, several recaptures have come from regions within the Indonesian archipelago. In particular, a group of 8 recaptures came from waters just south of Irian Java, a region almost directly adjacent to the Indian Ocean (Figure 15). These tag results provide an alternate explanation for gene flow that does not depend on the poorly know movements of adult black marlin. Juvenile black marlin could easily disperse throughout the Indonesian archipelago, a likely occurrence given that these small fish seem to prefer the shallow coastal seas characteristic of this region. Additionally, large numbers of black marlin would not have to move between the two oceans to maintain genetic homogeneity. Wright (1978) estimated that only one migrant per generation was required to prevent the occurrence of fixed differences between isolated populations and that statistically different allele frequencies could be prevented by serveral migrants per generation. Thus, dispersal of juveniles may facilitate the genetic homogeneity observed in this study.

The black marlin is a large pelagic predator with a nearly global, genetically homogeneous population and a relatively restricted spawning distribution. The only other pelagic fish that demonstrates a similar pattern is the southern bluefin tuna, *Thunnus maccoyii*. The range of the southern bluefin tuna encompasses the southern reaches of all three world oceans, yet there is only one known spawning ground south of Java (Caton, 1991). Both genetic (Grewe et al., 1997) and otolith microconstituent studies (Proctor et al., 1995) have been unable to reject the hypothesis of a single panmictic population of

southern bluefin tuna. The similarity between the spawning distributions of the southern bluefin tuna and black marlin is noteworthy.

Black marlin are know to demonstrate a strong preference for 27-28°C water for spawning (Nakamura, 1985; J. Pepperell, pers. com.). Current spawning distribution may be a reflection of historical biogeographical patterns. During the Pleistocene, sea surface temperatures were much colder and water temperatures conducive to black marlin spawning were restricted to waters east of southeast Asia (CLIMAP, 1976). It is conceivable that the contemporay spawning distribution is a reflection of a historical pattern linked to Pleistocene sea surface temperatures. Furthemore, coastal seas separated Australia and Indonesia even during the early Pleistocene, facilitating movement of juveniles and adults between the Indian and Pacific oceans. Water temperatures were cold enough south of Australia to deter migration via the southerly route, but transfer across the top of Australia was indeed possible. Thus, Australia and the Indonesian land mass do not seem to have functioned as barriers to gene flow for black marlin within recent geological time.

Future Work

In order to increase the discriminatory power of the microsatellite analysis, future population studies of black marlin should utilize larger sample sizes and better sample representation from the Indian Ocean. Specifically, larger samples from South Africa and

the eastern Pacific Ocean would have been very useful. Better representation from the eastern Indian Ocean and from the south of Japan would also be valuable.

Genetic applications should also be utilized for aspects of black marlin biology other than population structure. Black marlin larvae are poorly described, and the use of the forensic molecular key constructed by Innes et al. (1998) or Graves and McDowell (1997) would both confirm the morphology of black marlin larvae as well as substantiate the hypothesized spawning areas. Also, sequencing of nuclear DNA regions should be employed to resolve the phylogeny of the Istiophorid billfishes.

There is a strong need for further studies of the basic biology for the black marlin. Spawning periodicity, age and growth, and behavioral biology are all areas where future research is needed. Also, catch reporting and fisheries monitoring must be improved in the Pacific and Indian oceans, both for the black marlin as well as other large pelagic fishes. Without confident estimates of catch statistics, the condition of the stock continues to be unknown and there exists no scientific basis for management recommendations.

Figure 9. Distribution of alleles ay the microsatellite locus GATA-10 for sailfish, white marlin, striped marlin, and blue marlin.

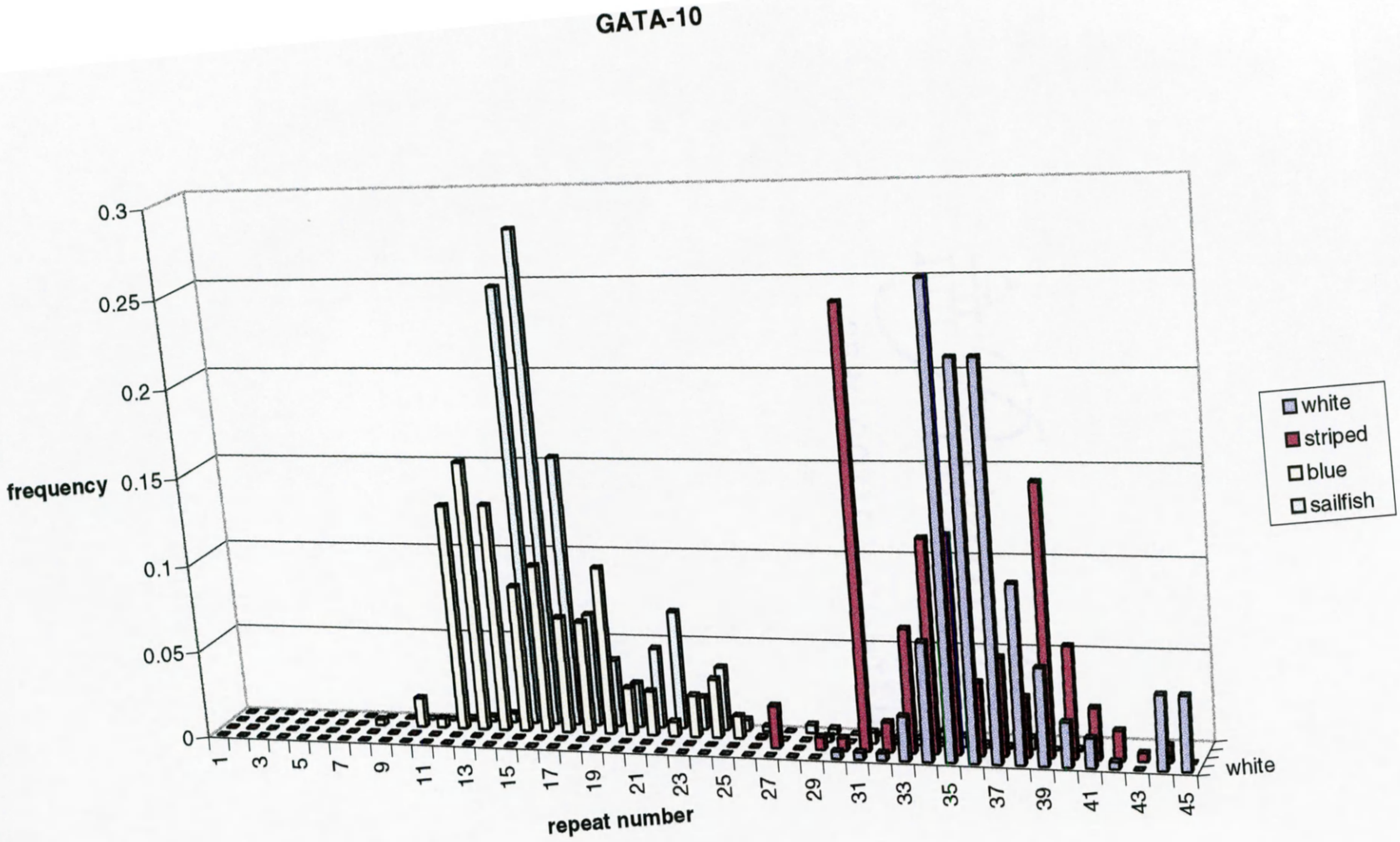

GATA-10
0.3
0.25
0.2
0.15
0.1
0.05
0
frequency
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41
43
45
repeat number
white
white
striped
blue
sailfish

Figure 10. Distribution of alleles ay the microsatellite DNA locus GATA-90 for sailfish and blue marlin.

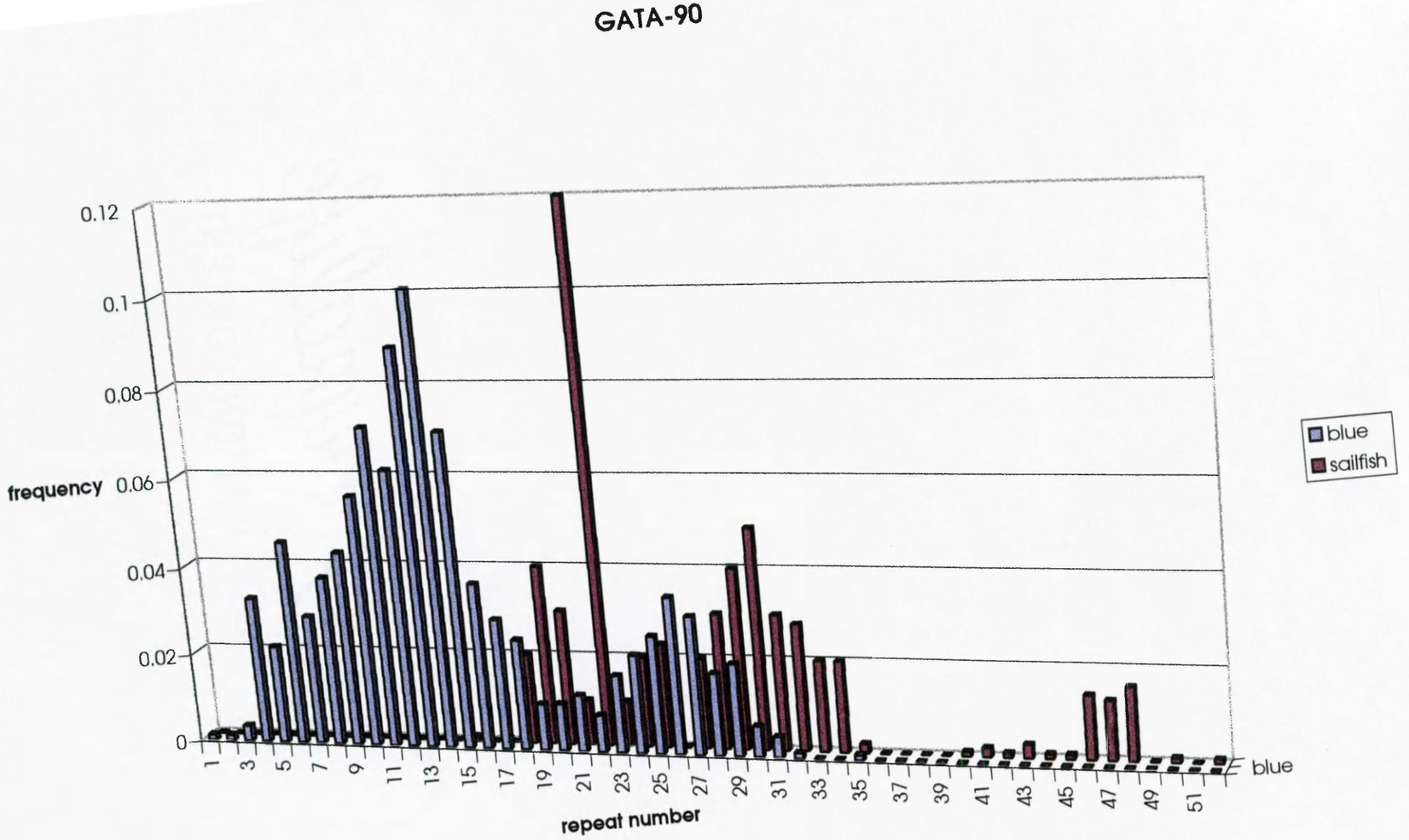

GATA-90
0.12
0.1
0.08
0.06
0.04
0.02
0
frequency
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41
43
45
47
49
51
repeat number
blue
sailfish
blue

Figure 11. Distribution of alleles at the microsatellite DNA locus GATA-08 for white, striped, and blue marlin.

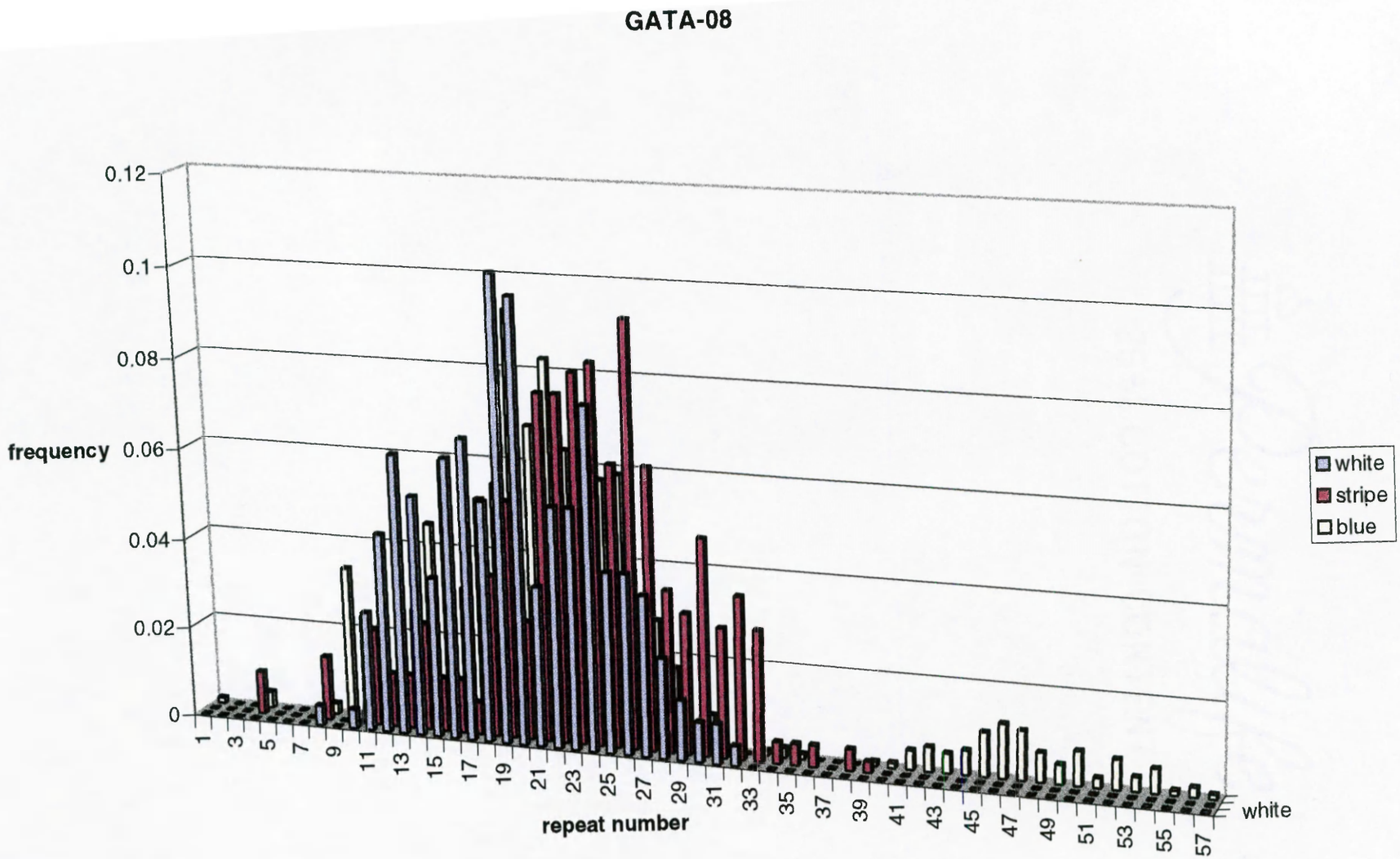

GATA-08
frequency
0.12
0.1
0.08
0.06
0.04
0.02
0
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41
43
45
47
49
51
53
55
57
repeat number
white
white
stripe
blue

Figure 12. Distribution of alleles at the microsatellite DNA locus GATA-60 for white, striped, and blue marlin.

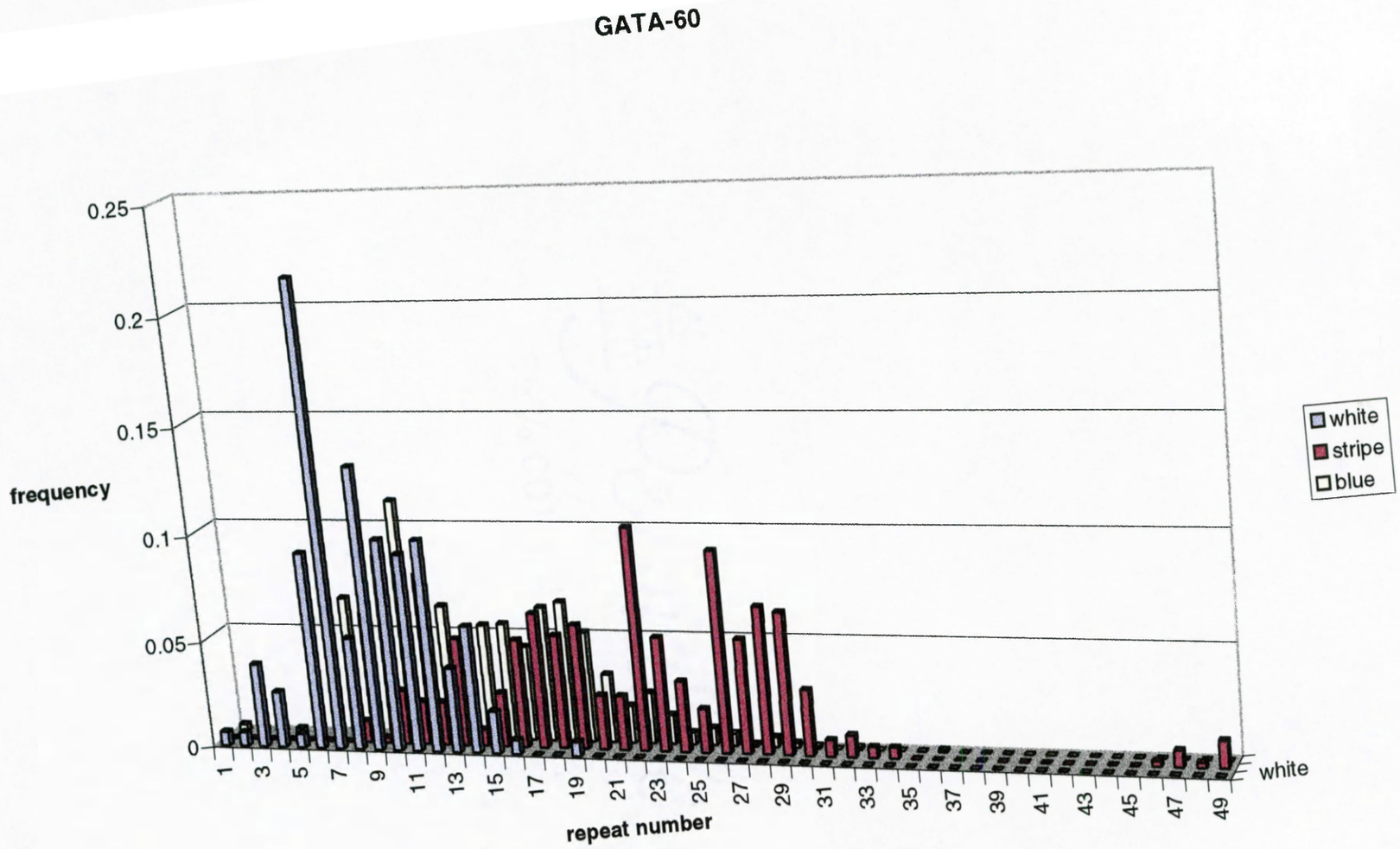

GATA-60
0.25
0.2
0.15
0.1
0.05
0
frequency
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41
43
45
47
49
repeat number
white
white
stripe
blue

Figure 13. Distribution of alleles at GATA-60 for two temporal collections: PS85 and PS97.

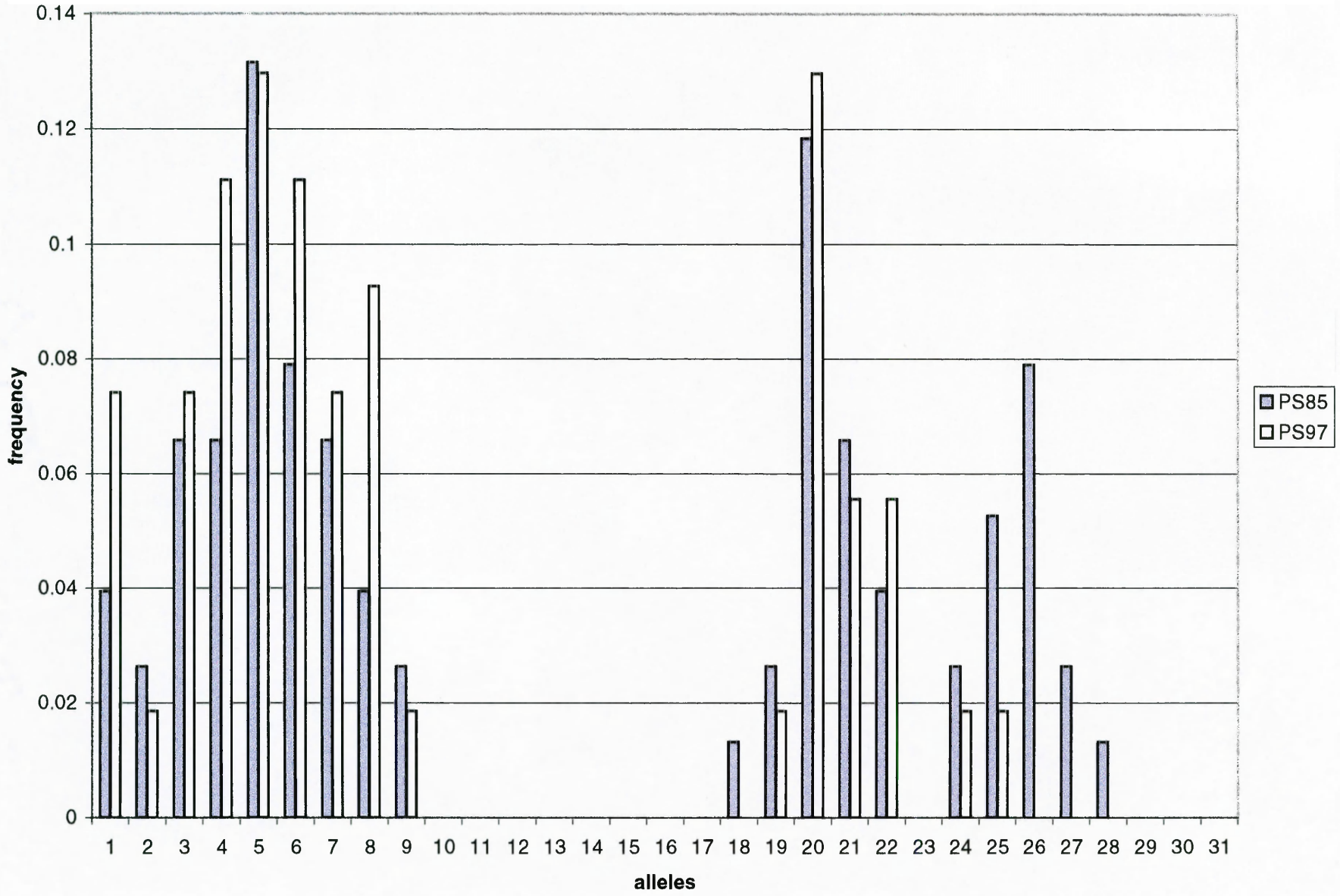

0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
frequency
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
alleles
PS85
PS97

Figure 14. Days at liberty versus net displacement for black and striped marlin (J. Pepperell, unpub. data).

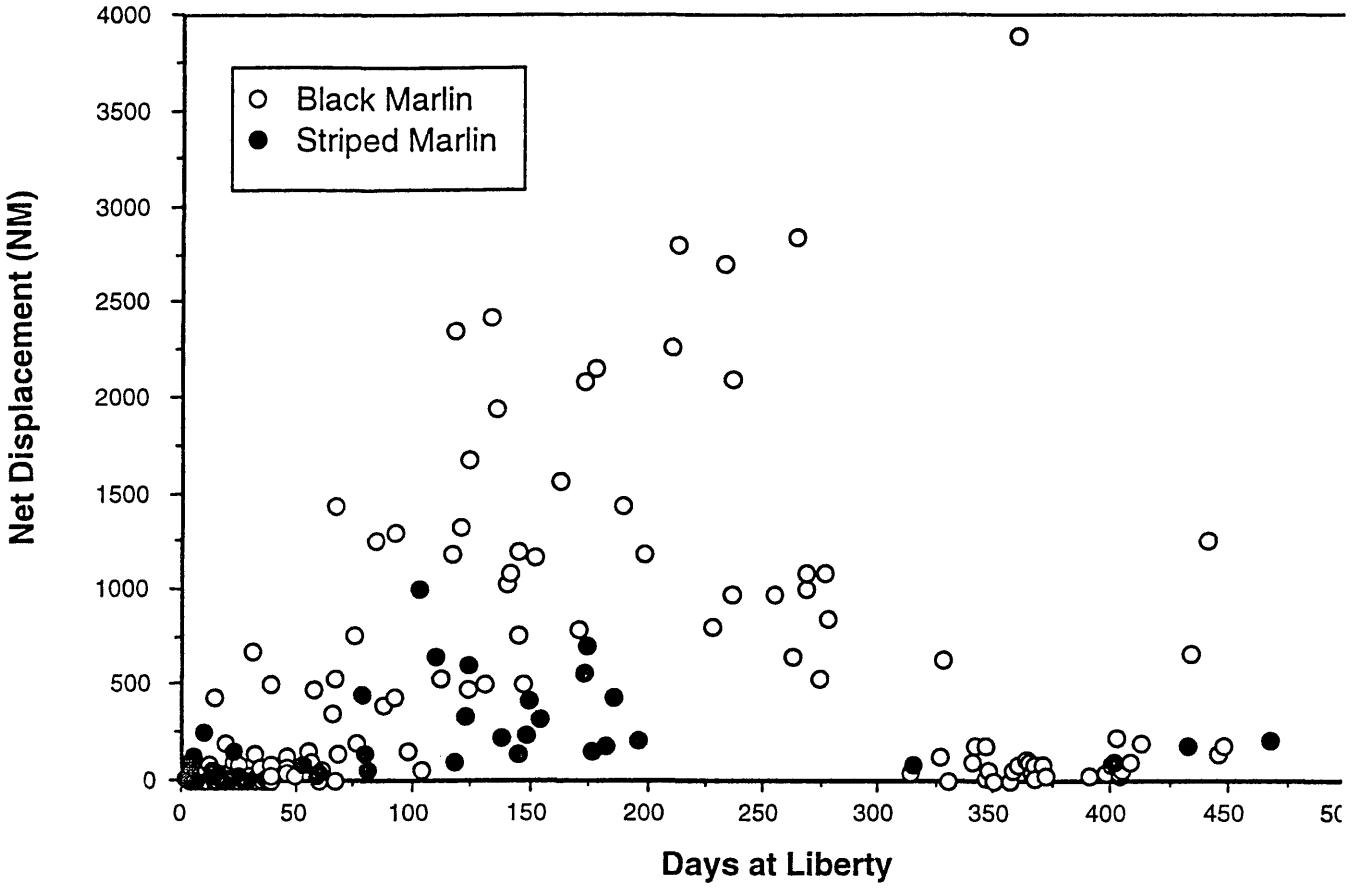

Black Marlin
Striped Marlin
Net Displacement (NM)
Days at Liberty
4000
3500
3000
2500
2000
1500
1000
500
0
0
50
100
150
200
250
300
350
400
450

Figure 15. Selected tag-recaptures from black marlin from the Pacific Ocean (J. Pepperell, unpub. data).

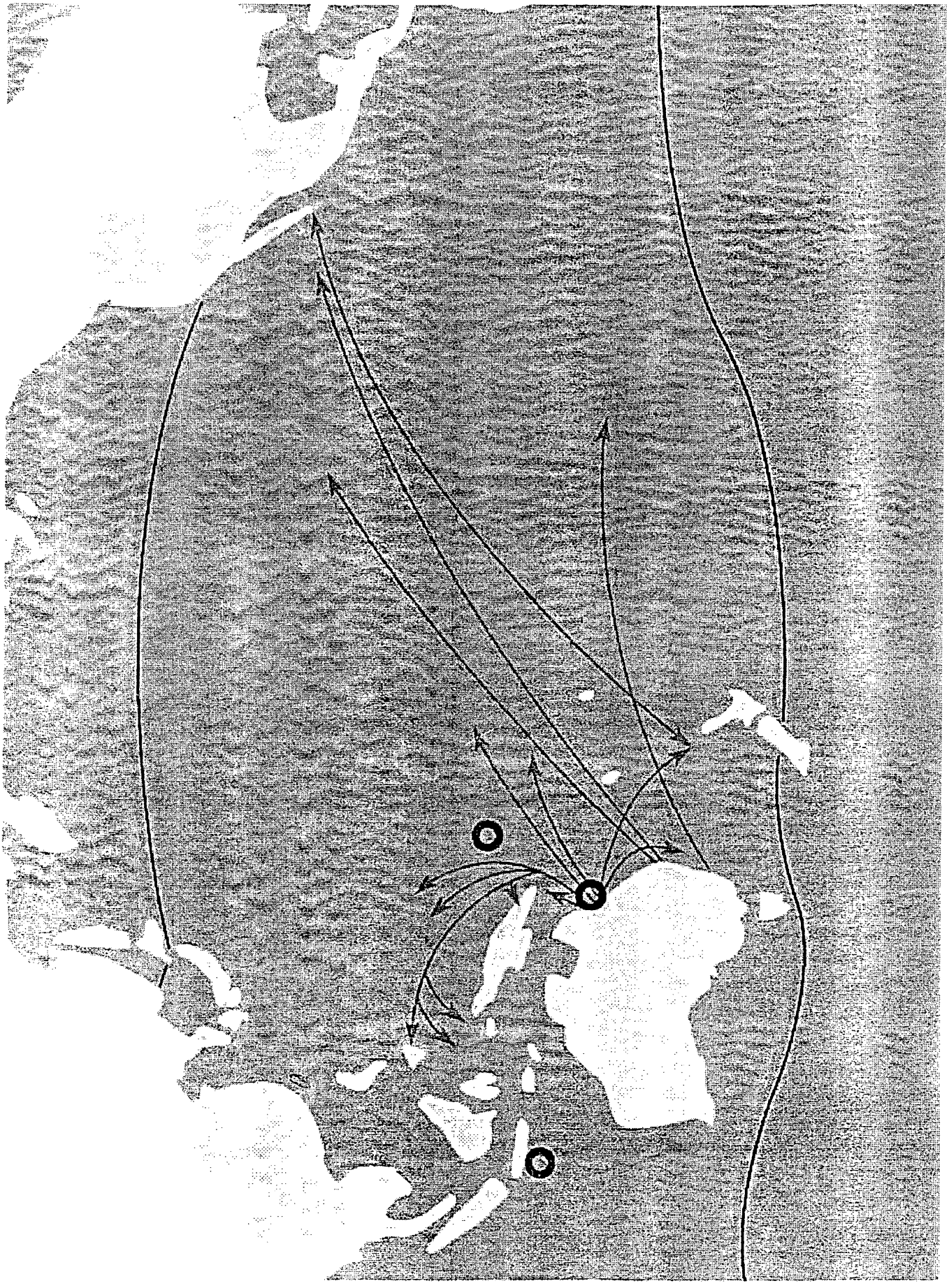
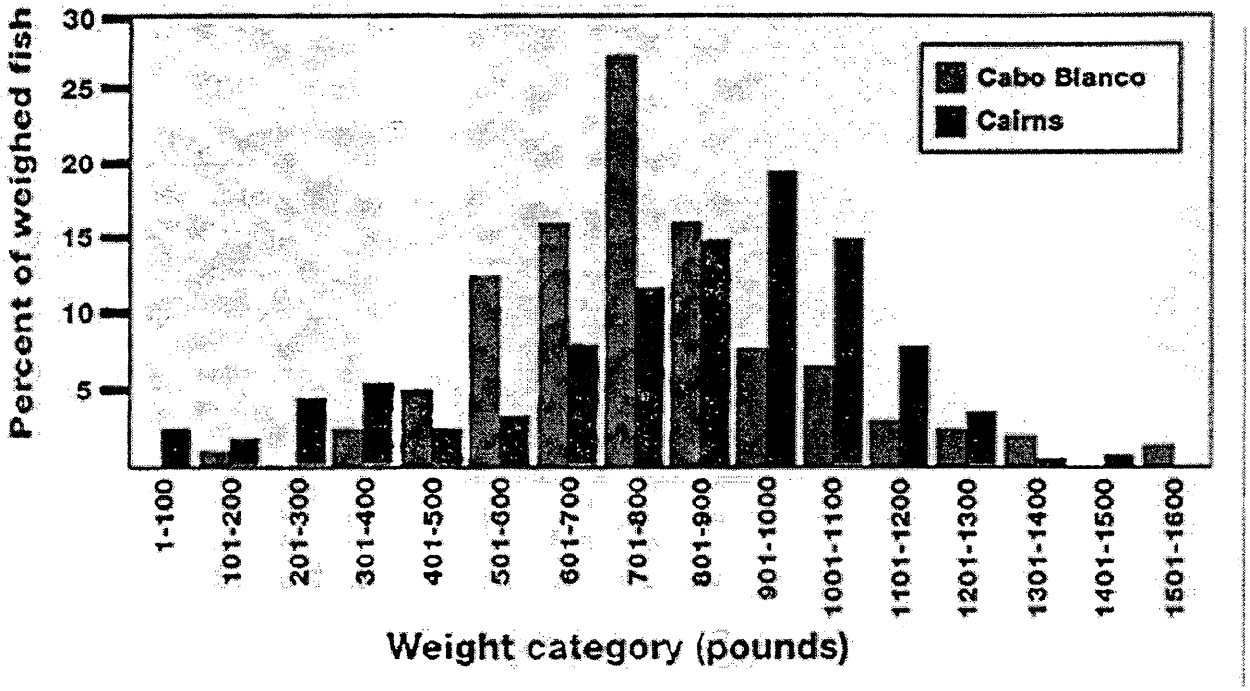


Figure 16. Weight distribution of black marlin caught near Cairns, Australia and Cabo Blanco, Peru during 1973 and 1974.


Percent of weighed fish
30
25
20
15
10
5
Cabo Blanco
Cairns
1-100
101-200
201-300
301-400
401-500
501-600
601-700
701-800
801-900
901-1000
1001-1100
1101-1200
1201-1300
1301-1400
1401-1500
1501-1600
Weight category (pounds)

APPENDIX I. Primer sequences and annealing temperatures. The D-loop primers were taken from Cronin et al (1993) and the microsatellite primers were developed by Bounacorrsi (1998).

Locus	primer	T (C)	sequence
D-loop	H1	42	5'-TTGGGTTTCTCTATGACCG
	L1		5'-AGAGCGTCGGTCTTGTAAACC
GATA-01	1F	60	5'-TGCTAACATTTGCTCCATTTGGC
	1R		5'-GGACAGAACACATCCGTCACCC
GATA-08	8F	55	5'-CTAACTTTGTGACTAGCTTGA
	8R-2		5'-GACTGACCAAGACATTGTTTCTGG
GATA-10	10F	60	5'-GCCACAACATTAAAACCAGTTACTG
	10R		5'-GTGCATCACTCAGGACATAAGGTCG
GATA-52	52F	55	5'-ATCCACTGTCGTTCAAGTTAGCG
	52R-2		5'-GCTGAGCGGAGCAGGATGATGT
GATA-60	60F	57	5'-AGCTCTTAATGGAGCCTGATGTT
	60R		5'-AGCCAAAGACACCCAAATCATCT
GATA-90	90F-2	50	5'-TGTGAACTCTATGTGTGAGGAAGTGT
	90R-2		5'-TAACATCAAAGGCTTAGACCCAGACT

APPENDIX II. Frequency of alleles at microsatellite loci according to sample collection. Colmun headers indicate the allele number and row headers indicate individual collections. The locus is spcified in the top right cell.

GATA-10	1	2	3	4	5	6
PS85	2	62	12	2	0	0
PS86	2	63	13	0	0	0
PS96	2	55	13	0	0	0
PS97	4	54	8	0	0	0
TW98	0	61	7	0	0	0
TW99	0	51	7	0	0	0
SA	1	32	5	0	0	0
VN	0	19	6	0	0	1
EPO	0	45	6	1	0	0

GATA-90	1	2	3	4	5	6	7
PS85	3	64	19	0	0	0	0
PS86	2	43	25	0	0	0	0
PS96	1	46	14	0	0	1	0
PS97	0	40	13	2	0	1	0
TW98	2	64	9	0	0	3	0
TW99	0	35	5	0	1	1	0
SA	2	27	5	2	0	0	0
VN	0	21	5	0	0	0	0
EPO	1	32	8	0	0	0	1

GATA-52	1	2	3	4	5	6	7	8	9	10	11
PS85	0	1	5	13	6	9	12	18	8	1	0
PS86	0	0	5	6	4	12	10	15	5	0	0
PS96	0	0	1	6	4	7	8	5	1	0	0
PS97	0	0	4	9	8	13	19	3	1	0	0
TW98	0	0	3	8	4	11	12	11	4	0	0
TW99	0	0	7	6	1	6	12	12	5	3	0
SA	0	0	0	3	3	3	6	7	3	1	1
VN	0	0	0	4	1	3	8	3	6	2	1
EPO	1	0	1	6	1	3	4	6	5	5	1

GATA-08	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
PS85	2	13	7	8	1	7	5	5	13	4	1	0	0	0	2	1	1	0	0	0	0
PS86	2	0	0	2	4	6	6	6	2	6	5	3	13	4	2	2	0	2	0	0	0
PS96	2	4	1	11	5	6	4	7	11	7	5	4	2	0	0	0	0	0	0	0	0
PS97	2	1	5	11	6	8	6	6	5	7	2	2	1	1	0	0	0	0	0	0	0
TW98	1	0	3	5	4	8	2	6	2	3	2	4	1	1	1	0	1	0	0	0	0
TW99	2	1	1	2	5	5	4	1	4	6	5	3	0	3	1	0	0	0	0	0	0
SA	1	0	1	1	11	3	5	5	3	5	4	1	3	2	3	2	0	0	0	0	0
VN	1	1	0	0	0	1	5	1	3	2	3	2	1	6	2	2	2	3	0	0	1
EPO	2	1	0	4	1	1	6	2	3	3	1	2	3	4	2	1	0	0	0	0	0

GATA-60	1	2	3	4	5	6	7	8	9	10	12	16	17	18	19	20	21	22	23	24	25	26	27	28	29	31
PS85	3	2	5	5	10	6	5	3	2	0	0	0	0	1	2	9	5	3	0	2	4	6	2	1	0	0
PS86	1	4	1	5	4	10	4	2	6	2	0	0	0	2	2	7	6	1	1	2	2	0	5	6	1	0
PS96	1	4	1	7	6	14	6	1	3	2	1	0	0	0	1	7	3	3	0	1	1	0	0	0	0	0
PS97	4	1	4	6	7	6	4	5	1	0	0	0	0	0	2	1	3	2	2	4	1	0	0	0	0	0
TW98	2	10	3	7	6	7	3	2	0	1	0	0	1	3	1	3	3	2	3	2	1	1	0	0	0	0
TW99	1	0	1	0	2	1	10	5	6	1	0	0	0	0	2	1	3	2	2	4	1	0	0	0	0	0
SA	0	2	0	0	4	3	6	1	3	2	0	0	0	0	1	3	2	0	2	1	1	1	2	1	0	0
VN	0	0	0	0	1	1	1	4	1	2	0	2	0	3	5	2	0	4	0	0	2	0	1	0	0	1
EPO	0	0	0	1	4	3	8	1	2	1	0	0	0	0	1	2	0	4	4	1	4	1	3	2	0	0

APPENDIX III. MtDNA D-loop composite haplotype and alleles for GATA-10 and GATA-90 for individual black marlin from this study. For the microsatellite loci, '?' indicates that the alleles are unknown due to poor amplification.

Sample	Comp. Hap.	GATA-10			GATA-90	
Port Stephens 1985 (PS85)						
PS85-01	ABAABB	2	2		2	2
PS85-02	ABAABB	2	2		2	2
PS85-03	BAAAAA	2	3		2	2
PS85-04	BAAAAA	?	?		2	2
PS85-05	BAAAAA	2	2		3	3
PS85-06	BBBBGA	2	2		2	2
PS85-07	BAAACA	2	3		1	2
PS85-08	BBBBAA	2	3		2	2
PS85-09	BBBBAA	2	2		3	3
PS85-10	ABAABA	2	3		2	2
PS85-11	ABAABB	2	2		2	3
PS85-12	ABAABB	2	3		3	3
PS85-13	ABAABB	?	?		1	2
PS85-14	BAAAAA	2	2		2	3
PS85-15	BAAAAC	2	2		2	3
PS85-16	ABAABB	2	2		2	3
PS85-17	ABBABA	?	?		2	2
PS85-18	BAAAAA	2	2		2	3
PS85-19	BAAAAA	2	3		2	2
PS85-20	BBBBAA	2	3		2	2
PS85-21	BBBABA	2	2		2	3
PS85-22	BAAAAA	2	2		2	2
PS85-23	BBBBAA	1	2		2	3
PS85-24	BAAAAA	2	2		2	3
PS85-25	ACAABB	2	2		2	2
PS85-26	ABAABB	2	2		2	2
PS85-27	BAAAAA	2	2		1	2
PS85-28	ABAABB	2	2		2	2
PS85-29	BAAAAA	3	3		2	2
PS85-30	BAAAAA	2	2		2	3
PS85-31	BAAAAA	2	3		2	2
PS85-32	BAAAAA	2	2		2	2
PS85-33	BBBBAA	2	2		2	2
PS85-34	BBBBAA	2	2		2	2
PS85-35	ABBABA	1	2		2	2
PS85-36	BAAAAA	2	4		2	3
PS85-37	BAAAAA	?	?		2	3
PS85-38	BAAAAA	2	3		2	3
PS85-39	BAAAAA	2	3		2	3
PS85-40	ABBABA	2	2		2	2
PS85-41	BBBAAA	2	2		2	2
PS85-42	BBBBAA	2	4		2	2
PS85-43	ABAABB	2	2		2	2
Port Stephens 1986 (PS86)						

PS86-01	ABAABB	2	2		2	2
PS86-03	BBBBAA	2	2		2	3
PS86-04	ABBAGA	2	2		2	2
PS86-05	BAAAAA	2	2		3	3
PS86-06	ABAABB	2	2		2	2
PS86-07	BAAAAA	2	2		1	2
PS86-08	ABAABA	3	3		2	2
PS86-09	ABBBAA	2	2		3	3
PS86-10	ABBABA	2	2		2	2
PS86-12	ABAABA	2	2		3	3
PS86-13	AABBBA	2	2		1	2
PS86-14	BBBBAA	2	2		3	3
PS86-16	BAAAAA	2	2		2	3
PS86-17	BBBBAA	2	3		2	2
PS86-18	BAAAAA	2	3		2	3
PS92-01	BAAAAA	?	?		?	?
PS92-04	ABBABA	?	?		?	?
Port Stephens 1994 (PS94)						
PS94-01	ABAABA	2	2		2	3
PS94-02	BAAAAA	2	3		2	2
PS94-03	ABAABA	2	3		2	2
PS94-04	BAAABA	2	2		2	3
PS94-05	ABAABB	2	3		2	2
PS94-06	ABBAAA	2	3		2	2
PS94-07	ABAABC	?	?		?	?
PS94-09	BBBBAA	?	?		?	?
PS94-10	ABAABB	?	?		?	?
PS94-11	BBBBAA	?	?		?	?
PS94-12	BAAAAA	?	?		?	?
PS95-01	ABAABA	?	?		?	?
PS95-02	BAAAAA	?	?		?	?
PS95-03	ABAABB	?	?		?	?
PS95-04	BAAAAA	?	?		?	?
PS95-06	BAAACA	?	?		?	?
Port Stephens 1996 (PS96)						
PS96-01	BAAAAA	2	2		2	2
PS96-02	BAAAAA	1	2		2	3
PS96-03	ABAABB	2	2		2	3
PS96-04	AABAIA	2	2		2	3
PS96-05	BAAAAA	2	2		2	2
PS96-06	BBBBAA	2	2		2	3
PS96-07	ABAABB	2	3		2	3
PS96-08	BAAAAA	3	3		2	3
PS96-09	BAAAAA	2	2		2	3
PS96-10	BBBBAA	2	2		2	3
PS96-11	ABAABB	1	2		2	2
PS96-12	BAAAAA	2	2		2	6
PS96-13	ABAABB	2	2		2	2
PS96-14	ABBBAA	2	2		2	3
PS96-15	ABAABB	2	2		2	3

PS96-16	BBBBAA	2	2		?	?
PS96-17	BBBBAA	2	3		2	2
PS96-18	ABAABB	2	3		2	2
PS96-19	BAAAAA	2	2		2	2
PS96-20	BDBABA	2	2		?	?
PS96-21	BAAAAA	2	2		?	?
PS96-22	ABAABB	2	2		2	2
PS96-23	BACAAA	2	3		2	3
PS96-24	ABAABB	2	3		2	2
PS96-25	ABAABB	2	2		2	2
PS96-26	BBBBAA	2	2		?	?
Port Stephens 1997 (PS97)						
PS97-01	ABBABC	2	2		2	3
PS97-02	ABAABB	2	2		2	2
PS97-03	ABBBAA	2	3		?	?
PS97-04	BAAAAA	2	2		?	?
PS97-05	BBBBAA	?	?		?	?
PS97-06	ABAABB	2	2		3	3
PS97-07	BAAAAA	2	2		2	2
PS97-08	BAAAAA	2	3		2	3
PS97-09	BBBBAA	2	2		2	2
PS97-10	ABAABB	2	2		2	2
PS97-11	BAAAAA	2	3		2	3
PS97-12	AABBAA	1	2		2	4
PS97-13	ABBABA	2	2		?	?
PS97-14	BAAAAA	2	2		3	4
PS97-15	BAAAAC	2	2		2	2
PS97-16	BAAAAA	2	2		2	3
PS97-17	BAAAAA	2	2		2	2
PS97-18	BAAAAA	2	3		3	3
PS97-19	AABBAA	1	2		2	2
PS97-20	BAAAAA	2	3		2	2
PS97-21	BAAAAA	2	3		2	3
PS97-22	ADBABA	1	3		2	3
PS97-23	BBBBAA	2	2		2	3
PS97-24	ABAABB	2	2		2	3
PS97-25	BAAAAA	2	2		2	2
PS97-26	BBBBAA	2	2		?	?
PS97-27	ABAABB	2	2		2	2
SW97-01	ABAAAB	1	3		2	2
SW97-02	BAAAAA	2	2		2	6
SW97-03	BAACDA	2	2		2	2
SW97-04	BAAAAC	2	2		2	2
BB97-01	BAAAAA	2	2		?	?
Berm86-01	BAAAAC	2	2		2	2
Berm86-02	BAAAAA	?	?		?	?
Berm97-01	BBBBAA	?	?		2	2
Berm97-02	BAAAAA	2	3		2	3
Berm97-03	BACAAA	2	2		2	3
South Africa (SA)						

SA96-01	BAAAAA	?	?		?	?
SA96-02	BADCEA	?	?		?	?
SA96-03	ABAABB	?	?		?	?
SA98-01	ABBACA	?	?		?	?
SA98-02	BAAAAA	?	?		?	?
SA98-04	BAAAAA	?	?		?	?
SA98-06	ABAABB	?	?		?	?
SA98-08	BAAAAA	?	?		?	?
SA98-09	ABAAAB	2	2		2	2
SA98-10	BAAAAA	2	2		2	2
SA98-11	BBBBAA	2	3		2	3
SA98-12	BAAAAA	2	3		2	2
SA99-01	AAAAAA	2	3		2	2
SA99-02	ABAABB	2	3		2	2
SA99-03	BAAAAA	2	2		2	2
SA99-04	BAAAAA	2	3		2	3
SA99-05	BAAAFA	2	2		1	2
SA99-06	BAAAAA	2	3		2	2
SA99-07	ABAABB	2	2		2	2
SA99-08	ABBBAA	2	3		2	3
SA99-09	ABBABA	2	2		1	2
SA99-10	BAAAAA	2	2		2	2
SA99-11	BAAAAA	1	2		2	2
SA99-12	ABAABB	2	2		3	3
SA99-13	BBBBAA	2	2		2	2
SA99-14	ABBABA	2	3		2	4
SA99-15	ABBABA	2	2		2	2
SA99-16	ABAABB	2	2		1	2
SA99-17	BAAAAA	2	2		2	4
SA99-18	ABBABA	2	2		2	2
SA99-19	ABBBAA	2	2		2	3
SA99-20	AABABA	2	2		2	3
TV84-02	BBBBAA	?	?		?	?
TV84-07	ABAABB	?	?		?	?
Taiwan 1998 (TW98)						
TW98-01	ABAABB	2	2		2	3
TW98-02	AAAABB	2	2		2	3
TW98-03	AAAABB	2	2		2	6
TW98-04	ABAABB	2	2		2	2
TW98-06	ABAABB	?	?		1	2
TW98-08	BAAAAA	2	2		2	2
TW98-09	ABBABA	2	2		2	2
TW98-10	BBBBAA	2	3		2	2
TW98-11	ABAABB	2	2		2	2
TW98-12	BAAAAA	?	?		2	2
TW98-13	BAAAAA	2	2		2	6
TW98-14	BAAAAA	2	3		2	3
TW98-15	BAAAAA	?	?		2	2
TW98-16	AAAABB	2	2		2	2
TW98-17	BAAAAA	2	3		2	2

TW98-18	AAAABB	2	2		2	2
TW98-19	BBBBAA	2	2		3	6
TW98-20	ABBBBA	2	2		2	2
TW98-21	ABEABA	2	2		2	3
TW98-22	ABAABB	2	2		2	2
TW98-23	BAAAAA	2	2		2	2
TW98-24	ABAABB	2	2		2	2
TW98-25	BAAAAA	2	2		2	3
TW98-26	ABBBBA	2	3		2	2
TW98-27	BAAAAA	2	3		2	2
TW98-28	BAAAAA	2	2		2	2
TW98-29	AAAABB	2	2		2	2
TW98-30	ABBBBA	2	2		2	2
TW98-31	ABAABB	2	2		2	2
TW98-32	BADCDA	?	?		?	?
TW98-33	BAAAAA	2	2		2	2
TW98-34	BBAABA	2	2		2	2
TW98-35	ABEABA	2	2		2	3
TW98-36	BAAAAA	2	3		2	2
TW98-37	BAAAAA	2	2		2	2
TW98-38	BBBBAA	2	2		1	2
TW98-39	BBBBAA	2	3		2	2
Taiwan 1999 (TW99)						
TW99-01	ABAABA	2	2		2	3
TW99-02	ABAABB	2	2		2	2
TW99-03	BAAAAA	2	3		2	2
TW99-04	BACAAA	2	2		2	2
TW99-05	ABBBAA	?	?		2	2
TW99-06	BAAAAA	2	2		2	3
TW99-07	BBBBAA	2	3		2	2
TW99-08	ABAABB	2	2		2	2
TW99-09	BAAAAA	2	2		2	2
TW99-10	BAAAAA	?	?		2	2
TW99-11	BAAAAA	?	?		2	2
TW99-12	ABAABB	2	2		2	3
TW99-13	BAAAAA	?	?		2	2
TW99-14	ABAABB	2	2		2	2
TW99-15	ABAABB	2	2		2	2
TW99-16	BAAAAA	?	?		2	2
TW99-17	ABAABB	2	3		2	3
TW99-18	BAAAAA	3	3		2	2
TW99-19	BAAAAA	?	?		?	?
TW99-20	BAAAAA	?	?		?	?
TW99-21	ABAABB	?	?		2	2
TW99-22	BAAAAA	2	3		2	3
TW99-23	BBBBAA	3	3		2	2
TW99-24	ABAABB	?	?		?	?
TW99-25	BAAAAA	?	?		?	?
TW99-26	BBABAA	?	?		?	?
TW99-27	BBBBAA	2	2		2	5

TW99-28	BAAAAA	2	2		2	2
TW99-29	ABAABB	2	3		2	3
TW99-30	BAAAAA	?	?		?	?
TW99-31	ABAABB	?	?		?	?
TW99-32	BBBBAA	?	?		?	?
TW99-33	ABAABB	?	?		?	?
TW99-34	AAAABB	?	?		?	?
Vietnam (VN)						
VN96-02	BBBBAA	?	?		?	?
VN98-01	BAAAAA	3	3		2	2
VN98-02	BAAAAA	2	2		2	2
VN98-04	ABBABA	2	3		2	2
VN98-06	ABAABA	2	3		2	2
VN98-07	ABAABB	2	2		2	2
VN98-10	BAAAAA	2	2		2	2
VN98-11	ABAABB	2	2		2	2
VN98-12	BAAAAA	2	2		2	3
VN98-13	BBBBAA	2	2		2	2
VN98-14	ABBABA	2	3		2	3
VN98-15	ABAABB	2	3		3	3
VN98-18	ABBBAA	2	6		2	3
Eastern Pacific Ocean (EPO)						
Cabo91	BAAAHA	?	?		?	?
EC95-02	ABAABB	?	?		?	?
EC95-03	BAAAAA	?	?		?	?
EC95-05	BAAAAA	?	?		?	?
EC95-06	AAAAAB	2	2		2	3
EC95-09	BBBBAA	2	3		2	2
EC95-10	BAAAAA	2	2		2	2
EC97-01	AAAABA	?	?		2	2
EC97-02	ABBBBA	?	?		?	?
EC97-03	AAAABB	2	2		?	?
EC97-04	BAAAAA	2	2		?	?
EC97-05	ABBBAA	2	2		2	3
MX98-01	BAAABA	2	2		2	2
MX98-02	BAAAAA	2	2		2	3
MX98-03	BAAAAA	2	2		2	2
MX98-04	BAAAAA	2	2		2	2
MX98-05	BBBBAA	2	4		2	3
MX98-06	BAAAAA	2	3		2	2
PN99-01	BAAAAA	2	2		2	2
PN99-02	ABAAAB	2	2		2	3
PN99-03	BAAAAA	2	2		2	2
PN99-04	BBBBAA	2	3		2	2
PN99-05	BBBBAA	2	2		2	3
PN99-06	ABAAAB	?	?		?	?
PN99-07	ABBAAA	2	2		2	2
PN99-08	BAAAAA	?	?		2	3
PN99-09	ABBABA	2	3		3	3
PN99-10	BAAAAA	2	2		2	2

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