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J. R. Alvarado Bremer · B. Stequert
N. W. Robertson · B. Ely

Genetic evidence for inter-oceanic subdivision of bigeye tuna (*Thunnus obesus*) populations

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Abstract Bigeye tuna (*Thunnus obesus* Lowe, 1839) are a commercially important species of tuna found in the Atlantic, Indian, and Pacific oceans. To initiate an analysis of bigeye tuna population-structure, three PCR–RFLP assays were developed based on the published mtDNA control-region sequences of four bigeye tuna. Population analyses using these three restriction assays on a total of 248 individuals resulted in an array of 13 composite haplotypes. A total of 347 nucleotides of mtDNA control-region sequence was characterized for 11 of the 13 composite haplotypes. Phylogenetic analyses demonstrated that the DNA sequences belong to two monophyletic clades. However, only one of the three restriction assays was able to discriminate between the two clades. The other two assays were confounded by excessive homoplasy. Both parallel (independent occurrences of the same nucleotide change) and convergent (different nucleotide changes within the same restriction site) changes of restriction sites were observed. These results emphasize the importance of DNA sequence-analysis for the interpretation of restriction-site polymorphism data. Analyses of the frequency distribution indicated that samples of bigeye tuna from the Atlantic Ocean were genetically distinct from those found in the Indian and Pacific oceans. Thus, these results reject the null hypothesis of a single global population of bigeye tuna.

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J.R. Alvarado Bremer (✉) · N.W. Robertson · B. Ely
FISHTEC Genetics Laboratory,
Department of Biological Sciences,
University of South Carolina,
Columbia, South Carolina 29208, USA
Fax: +803 777 4002
e-mail: jaimeab@biol.sc.edu

B. Stequert
ORSTOM
Centre de Recherches Océanologiques,
P.O. Box V18,
Abidjan, Ivory Coast, Africa

Introduction

Bigeye tuna (*Thunnus obesus* Lowe) are large epi- and mesopelagic fish. They are found from the surface of the ocean to depths ranging in temperature from 13 to 29 °C (Collette and Nauen 1983). However, major concentrations coincide with the temperature range of the permanent thermocline, between 17 and 22 °C. Accordingly, temperature and thermocline depth appear to be important environmental factors governing the vertical and horizontal distribution of bigeye tuna.

Based on tagging data, bigeye tuna in the Atlantic Ocean are believed to display strong philopatric behavior towards the only known breeding area, a region including the Gulf of Guinea located between 15°N and 15°S (Cayre and Diouf 1984; ICCAT 1997). Bigeye larvae were also reported in the Gulf of Mexico (Richards et al. 1990). However, the authors of that report now believe that the larvae were misidentified (B. Richards personal communication). The Gulf of Guinea is the only known nursery area for young bigeye in the Atlantic. Elsewhere, spawning has been recorded between 10°N and 10°S in the eastern Pacific, with a peak from April through September in the northern hemisphere, and between January and March in the southern hemisphere. In the Indian Ocean, bigeye tuna are found throughout the area north of 35°S. However, two areas of concentration have been observed, an equatorial concentration where spawning is observed throughout the year, and a winter concentration of sexually inactive fish in a zone at 30°S (Kume et al. 1971; Suda 1973). The highest concentrations of larvae in the Indian Ocean have been found south of Indonesia from November to April (Stequert and Marsac 1986).

Very little is known about the population structure of bigeye tuna. On the basis of fisheries data, geographic distribution, tagging results, and the location of spawning and nursery areas, a single population is assumed to inhabit the Atlantic Ocean (ICCAT 1997). For management purposes, both the Indian Ocean and Pa-

cific Ocean populations also are considered to be single units. The amount of gene flow between ocean basins is not known.

The only published genetic population study of bigeye tuna is that of Suzuki (1962), who examined the Tg blood-group system in samples from the eastern Pacific and the Indian Ocean and found no significant differences. In contrast, mitochondrial DNA (mtDNA) studies have demonstrated significant levels of interoceanic differentiation for other highly migratory fishes, including Atlantic and Indo-Pacific samples of blue marlin (*Makaira nigricans*) (Finnerty and Block 1992), albacore tuna (*Thunnus alalunga*) (Chow and Ushiama 1995), swordfish (*Xiphias gladius*) (Alvarado Bremer et al. 1996; Rosel and Block 1996; Chow et al. 1997), and sailfish (*Istiophorus platypterus*) (Graves and McDowell 1995). Considerable genetic variation occurs among bigeye tuna. Bartlett and Davidson (1991) surveyed the variation contained in the mtDNA cytochrome b gene of several tuna species, and concluded that bigeye tuna was the most variable. Similarly, appreciable levels of polymorphism were reported for five allozyme loci in bigeye tuna from the Pacific Ocean (Elliot and Ward 1995). Recently, Alvarado Bremer et al. (1997) examined about 400 base pairs (bp) of nucleotide sequence from the mtDNA control-region in four individuals collected in the Northwest Atlantic, and found extremely high values of nucleotide diversity ($> 7.0\%$). In addition, they demonstrated that the nucleotide sequences corresponded to two highly divergent mtDNA lineages.

In this study, we analyzed 248 individuals using a combination of nucleotide-sequence analysis and RFLP assays of the mtDNA control region. The resulting data demonstrated a geographic association of haplotypes, and significant differences were observed among samples from the Atlantic and Indo-Pacific oceans.

Materials and methods

Samples of *Thunnus obesus* Lowe, 1839 were obtained from commercial fishing operations and ORSTOM research vessels. Sample collection information is presented in Table 1, including dates and locations of capture and sample sizes. A small piece of tissue, usually heart muscle, was fixed in ethanol and shipped by airmail to the FISHTEC Genetics Laboratory in Columbia. DNA extraction and PCR amplification conditions followed those of Alvarado Bremer et al. (1996). Briefly, mtDNA Control Region I was amplified using two primers designed specifically for fish (Alvarado Bremer 1994). The L-strand primer L15998-PRO (5′- TAC CCC AAA CTC CCA AAG CTA –3′) was used in combination with the H-strand primer CSBDH (5′-TGA ATT AGG AAC CAG ATG CCA G-3′) to generate a fragment $\simeq 450$ bp long. The amplification was carried out in 50 µl volumes containing: 67 m*M* Tris, pH 8.8, 2 m*M* $MgCl_2$, 200 µ*M* dNTPs, 1 U of Amplitaq (PE Applied Biosystems, Foster City, California), and 16 pmol of each primer. The amplification profile was: an initial denaturing step of 5 min at 94 °C, followed by 36 cycles of the profile: denaturing at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min. The final extension was at 72 °C for 5 min. PCR products were purified prior to sequencing using the QIA quick PCR Purification Kit (Qiagen Inc., Chatsworth, California), following the manufacturer's recommendations.

A preliminary analysis of the four bigeye tuna control-region nucleotide sequences reported by Alvarado Bremer et al.(1997) revealed the presence of two highly divergent clades, designated Clade I and Clade II. Inspection of the DNA sequences suggested that restriction sites for the restriction endonucleases *Acc*I, *Mnl*I and *Rsa*I might be diagnostic for the two clades. Restriction digests were carried out in 25 µl reactions containing 7 µl of the purified amplification products and 5 U of restriction endonuclease in the buffer, under conditions recommended by the manufacturer (New England Biolabs, Beverly, Massachusetts). Restriction digests were subjected to electrophoresis in 2% agarose. The extent of geographic heterogeneity in allele-frequency distribution was analyzed through Monte Carlo randomization as described by Roff and Bentzen (1989), using the program MONTE in REAP (Version 4) (McElroy et al. 1992). The sequential Bonferroni method (Holm 1979) was used to correct for multiple testing.

Control-region nucleotide sequences were obtained using a LICOR automated DNA-sequencer at the Institute for Biological Research and Technology (IBRT) facilities of the University of

Table 1 *Thunnus obesus*. Sample-collection data [*NMFS* National Marine Fisheries Service (USA); *ORSTOM* L'Institut Français de Recherche Scientifique pour le Développement en Coopération (France); *IFREMER* Institut Français de Recherche pour L'Exploitation de la Mer (France); *VIMS* Virginia Institute of Marine Science; *LL* longline; *PS* purse seine; *FL* lower jaw fork length; *na* not available]

Region, Locality		Latitude; Longitude	Collection dates	(*n*)	Collecting institution	Gear	FL ($\bar{x} \pm$ SD)
Atlantic Ocean							
NW Atlantic	1993	35–44°N; 39–74°W	17 June–2 Aug.	(15)	NMFS	LL	128.7 ± 16.2
Gulf of Mexico and Caribbean	1995	16–25°N; 64–90°W	5 Aug.–6 Nov.	(28)	NMFS	LL	na
Gulf of Guinea	1995	0–1°S; 3–7°E	27 June–22 July	(18)	ORSTOM	PS	129.2 ± 24.8
	1996	4°N–3°S; 8°E–9°W	15–20 June	(23)	ORSTOM	PS	55.6 ± 5.9
Indian Ocean							
Reunion Island	1995	23°S–9°N; 54–67°E	3 Nov.–24 Dec.	(22)	IFREMER	LL	81.4 ± 20.3
Pacific Ocean							
North Pacific	1994	1–12°N; 165°W	10–20 Oct.	(17)	VIMS	LL	125.1 ± 26.7
South Pacific	1995	8–16°S; 142–151°W	27 July–25 Aug.	(70)	ORSTOM	LL	104.3 ± 15.3
	1996	5–19°S; 134–150°W	12 Jan.–2 Mar.	(55)	ORSTOM	LL	81.7 ± 32.7

Table 2 *Thunnus obesus*. Haplotype assignment from electrophoretic patterns generated by digesting PCR-amplified control region of bigeye tuna mtDNA with three restriction endonucleases. Haplotype frequency was estimated for total pooled sample of 248 individuals (*bp* base pairs; – no corresponding restriction fragment for that haplotype; *spaces* no corresponding fragment in restriction pattern generated by that restriction endonuclease)

Enzyme	Estimated fragment sizes (bp)							Haplotype	Frequency
*Rsa*I	–	330	–	–	60	60	–	A	0.74
	390	–	–	–	60	–	–	B	0.20
	–	330	–	120	–	–	–	C	0.03
	–	–	300	–	60	60	30	D	0.03
*Mnl*I	–	275	–	–	90	60		A	0.62
	390	–	–	–	–	60		B	0.36
	–	–	230	160	–	60		C	0.02
*Acc*I	450	–	–					A	0.69
	–	400	50					B	0.31

Table 3 *Thunnus obesus*. Geographic distribution of bigeye composite haplotypes. Each letter of composite haplotypes corresponds to digestion patterns observed with restriction enzymes *Rsa*I, *Mnl*I, and *Acc*I, respectively. Haplotype BAB could not be assigned unequivocally to either clade (see "Results – RFLP analysis"). Northwest Atlantic sample consisted of NW Atlantic, Gulf of Mexico and Caribbean samples listed in Table 1

Composite haplotypes *Rsa*I/*Mnl*I/*Acc*I	Northwest Atlantic	Gulf of Guinea		Indian Ocean	North Pacific	South Pacific		Totals
		1995	1996			1995	1996	
AAA	7	4	8	17	13	48	31	128
ABA	1	1		1		12	8	23
AAB	1			1	3	1	2	8
DAA						1	6	7
BBA	1			1		3	2	7
CAA		1				2	2	5
CBA	1							1
Subtotal Clade I	11	6	8	20	16	67	51	179
BBB	25	6	7			2	1	41
ABB	6	5	5	2	1	1	1	21
ACB	1	1	2					4
BCB			1					1
CAB							1	1
Subtotal Clade II	32	12	15	2	1	3	3	68
BAB							1	1
Total	43	18	23	22	17	70	55	248

Table 4 *Thunnus obesus*. Analysis of geographic heterogeneity in frequency distributions using Monte Carlo randomization procedure (Roff and Bentzen 1989) [*Below diagonal* probability values estimated from frequency of 13 composite haplotypes identified in this study; *above diagonal* probabilities obtained from comparison of frequencies of Clade I and Clade II lineages (see Table 3)] Probabiliites were estimated after 10 000 replicates and include ties (* table–wide significance at 0.05 level using sequential Bonferroni method)

Region		1	2	3	4	5	6
NW Atlantic	(1)		0.3415	< 0.0001*	0.0003*	< 0.0001*	< 0.0001*
Gulf of Guinea	(2)	0.1282		< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*
Indian Ocean	(3)	< 0.0001*	0.0007*		1.0000	0.5875	0.6188
North Pacific	(4)	< 0.0001*	0.0002*	0.5904		1.0000	1.0000
South Pacific 1995	(5)	< 0.0001*	< 0.0001*	0.3833	0.0695		1.0000
South Pacific 1996	(6)	< 0.0001*	< 0.0001*	0.4558	0.0983	0.4424	

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                  10         20         30         40         50         60         70         80
                   .          .          .          .          .          .          .          .
                AccI       RsaI
AAA-1      TATGTCTGCG CATGTACATA TATGTAATTA CACCATATTT ATATATGAAC CATATACAGT AATGTTCTAG GACATATATG
AAA-2      ........T. ..C..... .......... .......... .......... .......... .......... ..........
AAA-3      .......... ..C......? A......... ........C. .......G.. -......... ....?..... ..........
AAA-8      .......... ..C......? .?.?...... ........C. .......G.. .......... .......... ..........
ABA-1      .........A ..C......C .?........ ........C. ......AG.. ........A. ...??..... ..........
ABA-2      .......... ..C......? .......... .......C.. .......G.. .......... .......... ..........
ABA-3      .........A ..C..... .......... ........C. .......... .......... .......... ..........
CAA-1      .......... ..CA.....? ?......... ........C. .......G.. .......... .......... ..........
CAA-2      .......... ..?A..... ?.???..... .......... .......G?. ........A. .......... .....G....
CBA        .......... ..CA....?? ???..... ........C. .......... ........A. ....?..... ..........
DAA        .......... ..C..... .......... ........C. .......G.. .......... ?......... ..........
BBA-1      ........T. ..C.....?? .......... .......... ......AG.. ........A. ...??..... ..........
BBA-2      .......... ..C..... .......... .......... .......G.. ........A. ....C..... ..........
AAB        .......A.. ..C..... .......... ........C. .......... .......... .......... ..........
BBB-1      .......A.. T.C..... .......... .......CC. ......-... ........A. ....C.T... ..........
BBB-2      .......A.. ..C..... .......... .......C.. ......T... ......T.A. .......... ..........
ABB-1      .......A.. ..C..... .......... .......C.. ......T... ......T.A. .......... ..........
ABB-2      .......A.. ..C..... .......... .......C.. ......T... ......T.A. .......... ......C...
ABB-3      .......A.. ..C..... .......... .......C.. ......T... ......T.A. .......... ..........
ACB        .......A.. ..C..... .......... .......CC. ......TG.. ........A. ....C..... ..........
BAB        .......A.. ..C..... ?......... .........C ......AG.. ......T.A? ......T... ..........
BFT#617    .......... .......... .......... .........C ......AG.. ......T.A. ...A..T... ..........
SBT162     .......... .......... .......... .......C.. ......TG.. ....C.T.A. ....C.T... ......C...

                  90         100        110        120        130        140        150        160
                   .          .          .          .          .          .          .          .
                            RsaI                                                          MnlI
AAA-1      TATTAAAACC ATTACTAGTA CTAAACCATT CATATGT?AA CAAATAATGA AGACTTACAT AAACCATACA GACATATTCC
AAA-2      .......... .......... .......... .......?.. ...?C..... .......... .......... A.T.......
AAA-3      .......... .......... .......... .....A.C.. ....C..... .......... .......... ..T....C..
AAA-8      .......... .......... .......... .....A.C.. ....C..... .......... .......... ..T.......
ABA-1      .......... .......... .......... .......C.. ....CG..T. ...T...... .......... AGT.......
ABA-2      .......... .......... .......... .....A.C.. ....C..... .......... .......... ..T....C.?
ABA-3      .....??... .......... .......... .....A.C.. ....C..... .......... .......... A.T.......
CAA-1      .......... .......... .......... .......C.. ...-C..... .......-.. .........G A.T.......
CAA-2      .......... .......... .....?.... .....A.C.. ....C..... .......... .......... ..........
CBA        .......... .......... .........? .......C.. ....C..... .......... .........G A.T.......
DAA        .......... .......... .......... .....A.C.. ....C..... .......... .......... ..T.......
BBA-1      .......... .......... T..G...... .......C.. ....C..... ...T...... .......... AGT.......
BBA-2      .......... .......... T......... .......C.. ....C..... ...T...... .......... AGT.......
AAB        .......... .......... .......... .....A.C.. ....C..... .......... .......... ..T.......
BBB-1      .......... ........?. T......... .......C.. ...?C..... .......... .......... .......CT.
BBB-2      .......... .......... T......... .....A.C.. .......... ..GT...... .......... ..T....CT.
ABB-1      .......... .......... .......... .....A.C.. ....C..... ..GT...... .......... ..T....CT.
ABB-2      .......... .......... .......... .....A.C.. ....C..... ..GT...... .......... ..T....CT.
ABB-3      .......... .......... .......... .....A.C.. ....C..... ..GT...... .......... ..T....CT.
ACB        .......... .......... .......... .......C.. ....C..... .......... .......... ......CCT.
BAB        .......... ..A..?.... T......... .......C.. ....C..... ...T...... .........G A.T.......
BFT#617    .......... .......... T......... .......C.. T......... ...T...... .......... A.T.A.CCT.
SBT162     .......... .......... T......... .......C.. ....C..C.. ...T...... .........G ..T.A.C...

                  170        180        190        200        210        220        230        240
                   .          .          .          .          .          .          .          .

AAA-1      AGCATTCAAA TTAAG-TCAG GTAATTACAC GAGATTTAAG ACCTACCACA AACACTAAAT CGTCTAAGCC ATACCAAGTC
AAA-2      .A.......G ....A-..GA .......... .......... .......... .......... .......... ..........
AAA-3      .A........ .....-.... .......A.. .......... .......... .......... .......... ..........
AAA-8      .A.......G .....-.... .......A.. .......... .......... .G........ .......... ..........
ABA-1      .A.......G .....-...A .G.....A.. .......... .......... ..A....... .......... ..........
ABA-2      .A........ .....-.... .......A.. .......... .......... .......... .......... .........T
ABA-3      .AT......G .....-...A .......A.. .......... .......... .......... .......... ..........
CAA-1      .A......G. .....-...A ..C....A.. .......... .......... .......... .......... ..........
CAA-2      .A........ .....-...A .......A.. .......... .......... .......... .......... ..........
CBA        .A......G. .....-...A .......A.. .......... .......... .......... .........T ..........
DAA        .A.......G .....-.... .......A.. .......... .......... .......... .......... ..........
BBA-1      .A.......G .....-...A .......A.. .........? .......... .......... .......... ..........
BBA-2      .A.......G .....-...A .......A.. .......... .......... .......... .......... ..........
AAB        .A....A... .....-...A .......A.. .......... .......... .......... .......... ..........
BBB-1      .A...C..T. C....-...A .......A.. ..A.C..... .......... ....T..... .......... .A........
BBB-2      .A...C..T. C....-...A .......A.. ..A.C..... .......... .......... .......... .A........
ABB-1      .A...C..TC C....-...A .......A.. ..A.C..... .......... .......... .......... .A........
ABB-2      .A...C..TT C....-...A .......A.. ..A.C..... .......... .......... .......... .A........
ABB-3      .....C..T. C....-...A .......A.. ..A.C..... .......... .......... .......... .A........
ACB        .A...C..TG C....-...A .......A.. ..A.C..... .........T. ...GT..... .......... .A........
BAB        .A........ .....-...A .......A.. .......... ........T. .......... ....?..... ..........
BFT#617    .A......TC ..G.AT..G. ..G....A.. .......... .....A..T. ..T-...... .......... ..........
SBT162     .A.....-CC ....AT...A .......A.. .......... ...C.A.... ...-...... .......... ..........
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Fig. 1

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                250        260        270        280        290        300        310        320
                 .          .          .          .          .          .          .          .
           MnlI
AAA-1      TCCTCATCCC TGAGATCAAG TAAATTTAAG CGCAGTAAGA GCCTACCATC CAGTCCATAT CTTAATGCAT ACGGTTATTG
AAA-2      .......T.. ...A...... .......... .......... .......... .......... .......... ..........
AAA-3      C......T.. ...A...... C......... .......... A......... .......... .......... ..........
AAA-8      .......... ...A...G.. .......... .......... .......... .......... .......... ..........
ABA-1      C..A...... ...A..-.G. .......... .......... .......... .......... .......... ..........
ABA-2      C..C...... ...A...G.. .......... .......... .......... .......... .......... ..........
ABA-3      ...C...... ...A...... .......... .......... .......... .......... .......... ..........
CAA-1      .......... ...A...... .......... .......... A......... .......... .......... ..........
CAA-2      .......T.. ...AG..G.. ....C..... .......... .......... .......... .......... ..........
CBA        ...C...... ...A...... .......... .......... A......... .......... .......... ..........
DAA        .......... ...A...G.. .......... .......... A......... .......... .......... ..........
BBA-1      C..A...... ...A..-... .......... .......... .......... .......... .......... ..........
BBA-2      C..A...... ...A..?... .......... .......... .......... .......... .......... ..........
AAB        C......... ...A...... .......... .......... A......... .......... .......... ..........
BBB-1      ..AC....T. .A.A...... .......... .......... .......... .......... .......... ..........
BBB-2      C.A....T. .A.A...... .......... .......... .......... .......... .......... ..........
ABB-1      C.A....T. .A.A...... .......... .......... .......... .......... .......... ..........
ABB-2      C.A....T. .A.A...... .......... .......... .......... .......... .......... ..........
ABB-3      C.A....T. .A.A...... .......... .......... .......... .......... .......... ..........
ACB        C.A....T. .A.A...... .......... .......... .......... .......... .......... ..........
BAB        C.......T. ...A...... .......... .......... .......... .......... .......... ..........
BFT#617    ...C....T. ...C...TC. ....C..... .......... A......... .......T. .......... ..........
SBT162     C......... .......T.. .......... .......... A......... .......T. .........C ..........

                330        340
                 .          .
           MnlI
AAA-1      AAGGTGAGGA ACAATAACTG TGGGGG?
AAA-2      .......... .......?.. ......?
AAA-3      .........G .......T.. ......T
AAA-8      .........G .......T.. ......T
ABA-1      .........G .......T.. ......T
ABA-2      .........G .......T.. ......T
ABA-3      .........G .......T.. ......T
CAA-1      .........G .......T.. ......T
CAA-2      .........G .......T.. ......T
CBA        .........G .......T.. ......T
DAA        .........G .......TC. ......T
BBA-1      .........G .......T.. ......T
BBA-2      .........G .......T.. ......T
AAB        .........G .......T.. ......T
BBB-1      .........G .......T.. .....??
BBB-2      .........G .......T.. ......T
ABB-1      .........G .......T.. ......T
ABB-2      .........G .......T.. ......T
ABB-3      .........G .......T.. ......T
ACB        .........G .......T.. ......T
BAB        .........G .....G.TC. ......T
BFT#617    .........G .......T.. ......T
SBT162     .........? .......T.. ......?
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Fig. 1 *Thunnus obesus*. Nucleotide sequence of 347 base pair (bp)-long segment of mtDNA control region of 21 distinct bigeye tuna haplotypes [*Numbers above sites* arbitrary nucleotide positions on L-strand of bigeye control-region aligned against control-region sequences of northern bluefin tuna *T. thynnus thynnus* (*BFT*) and southern bluefin tuna *T. maccoyii* (*SBT*) included in Alvarado Bremer et al. (1997); *dots* indicate identity with reference sequence; – nucleotide deletion] Locations of *Rsa*I, *Mnl*I, and *Acc*I sites are underlined in reference sequence (*AAA*-1) (Designations *AAA*-1, *AAA*-2 etc. refer to different haplotypes with same restriction pattern)

South Carolina. Sequencing reactions were performed using a ThermoSequenase, fluorescently-labeled primer, cycle-sequencing kit with 7-deaza-dGTP (Amersham Life Science, Arlington Heights, Illinois) and 1 μl purified PCR template. Reaction conditions were: an initial denaturing step at 95 °C for 2 min, followed by 20 cycles of denaturing at 95 °C for 30 s, annealing at 65 °C for 30 s, and extension at 70 °C for 1 min.

The orthology of mtDNA Control Region I sequences with other species of tuna and swordfish was established previously (Alvarado Bremer et al. 1997). Nucleotide sequences were edited using the Eyeball Sequence Editor (ESEE Version 3.0; Cabot and Beckenbach 1989). Estimates of nucleotide diversity (c) and nucleotide divergence (d) (Nei and Jin 1989) were obtained with the program SEND (Version 1.0) written by Dr. L. Jin, using Jukes–Cantor distances (Jukes and Cantor 1969).

Haplotype phylogenies were determined with several algorithms to ensure accuracy, using Test Version 4.0d61 of PAUP (phylogenetic analysis using parsimony), written by D.L. Swofford. Neighbor-joining (NJ) analyses (Saitou and Nei 1987) were performed using Tamura–Nei's distances. Missing data and insertions and deletions (indels) were treated by ignoring the affected pairwise comparison. Maximum-parsimony analyses (Kluge and Farris 1969; Fitch 1971) were carried out with heuristic searches. Indels were excluded from the analyses. Bootstrap resampling (Felsenstein 1985) was employed with the MULPARS option in PAUP, while keeping four trees at each replicate. In all phylogenetic analyses, the control-region DNA sequences of Atlantic northern bluefin tuna (*Thunnus thynnus thynnus* L.) and southern bluefin tuna (*T. maccoyii* Castelnau) reported in Alvarado Bremer et al. (1997) were used as the outgroup to root the trees.

Results

RFLP analysis

Digests of amplified control-region DNA with restriction enzymes *Rsa*I, *Mnl*I and *Acc*I generated two, three and four restriction patterns, respectively, in *Thunnus obesus*. For each enzyme, two Restriction Patterns (A and B) accounted for $> 97\%$ of the observations (Table 2). Additional restriction patterns were identified, but occurred at very low frequencies. A total of 13 composite haplotypes was identified (Table 3). The majority (68%) of the haplotypes could be classified either as AAA or BBB. However, Haplotypes ABA and ABB each were present at frequencies of $\simeq 9\%$. A strong geographic association of composite haplotypes was observed. The majority of AAA and ABA haplotypes were found in the Indo-Pacific samples, while the majority of the BBB and ABB haplotypes were found in the Atlantic Ocean samples. The Monte Carlo randomization analysis of haplotype frequencies for the Northwest Atlantic, Gulf of Guinea, Indian Ocean, North Pacific and South Pacific samples is shown in Table 4. The overall distribution of the 13 composite haplotypes was highly heterogeneous ($P < 0.0001$; 10 000 permutations). We were able to test the temporal stability of haplotype distribution in two sampling locations. No difference ($P = 0.7340$; 10 000 permutations) was found among the two Gulf of Guinea samples (Table 3). These two samples, which differed significantly in body length ($F_s = 33.96 \gg F_{0.01[1,38]}$), were collected in consecutive years and represent two distinct cohorts. In subsequent analyses, these two samples were pooled to increase the size of the Gulf of Guinea sample. In addition, no heterogeneity in haplotype distribution was found among summer (1995) and winter (1996) South Pacific samples (see Tables 3 and 4). In contrast, the North and South Atlantic Ocean samples were each significantly different from the Indian Ocean, the North Pacific and the South Pacific samples. No significant differences were found among the Indian Ocean, the North Pacific and the South Pacific samples, or between the Northwest Atlantic and the Gulf of Guinea samples.

Nucleotide-sequence analysis

The battery of three enzymes used in this study allowed us to assign the composite haplotypes AAA and BBB, respectively, to the two mtDNA clades identified by Alvarado Bremer et al. (1997). However, the 11 additional combinations of the A and B alleles could not be unequivocally assigned to either clade. Therefore, we determined the control-region nucleotide sequence for representatives of 11 of the 13 composite haplotypes observed in our PCR–RFLP analysis (Fig. 1). Phylogenetic analyses of the nucleotide sequences demonstrated that all but one of the nucleotide sequences could be placed within one of the two clades identified originally (Fig. 2). The exception was a rare haplotype (BAB) placed at the base of the bigeye sub-tree. Each clade displayed high levels of within-group variation. The observed nucleotide diversity values were 5.0 ± 0.5 and $2.7 \pm 0.6\%$ for Clades I and II, respectively. The divergence estimate between the two clades was estimated at $\simeq 4.9 \pm 0.1\%$ when corrected for within-group divergence. Thus, the majority of bigeye composite mtDNA haplotypes belong to one of two highly divergent clades.

The nucleotide-sequence analysis revealed that the character states of restriction enzymes *Rsa*I and *Mnl*I were homoplasious (Fig. 1). The *Rsa*I site at Positions 98 to 101 that is characteristic of Clade I was absent in two rare Clade I haplotypes (BBA-1 and BBA-2) and present in four rare Clade II haplotypes (ABB-1 through-3, and ACB). In addition, a second *Rsa*I site at Nucleotide Position 14 to 17 was lost in three of the Clade I haplotypes (CAA-1, CAA-2, and CBA) and a third *Rsa*I site was present in one Clade I haplotype (DAA). Similarly, both clades contained independent mutations that caused the loss of the *Mnl*I site at Positions 242 to 245 (Figs. 1; 3a). This *Mnl*I site is present in all species of the genus *Thunnus* (Alvarado Bremer et al. 1997), but is missing in members of bigeye Clade II due to a C to A transversion (CCTC to CATC). In addition, the first branch emerging from Clade I includes a T to A transversion at Nucleotide Position 244 (CCTC to CCAC). Transitions also affect the *Mnl*I site. At least three independent occurrences of a T to C transition were found at Nucleotide Position 244, two in Clade I and one in Clade II. Thus, several mutations have caused the loss of the same *Mnl*I restriction site.

In contrast to the other two restriction sites, only one instance of homoplasy was observed at the *Acc*I site. The rare haplotype AAB, a member of Clade I, has a transition from G to A at Nucleotide Position 8 which results in the loss of the *Acc*I site (Fig. 1). Since this PCR–RFLP haplotype was observed only eight times (Haplotype AAB) among 248 individuals (Table 3), the presence of the *Acc*I site can be used to identify members of Clade I with 97% accuracy. When the PCR–RFLP data were re-analyzed using only the *Acc*I information, the differences between the Atlantic and Indo-Pacific samples became more obvious. The Atlantic samples contained mostly (73%) Clade II individuals and the Indo-Pacific samples contained mostly (90%) Clade I individuals (Fig. 4). Table 4 shows the Monte Carlo randomization estimated from the frequency distribution of Clades I and II. This additional chi-square analysis corroborated the pattern of interoceanic differentiation obtained from the frequency of composite haplotypes. Similarly, no evidence of intraoceanic heterogeneity was detected at the 0.05 level of significance.

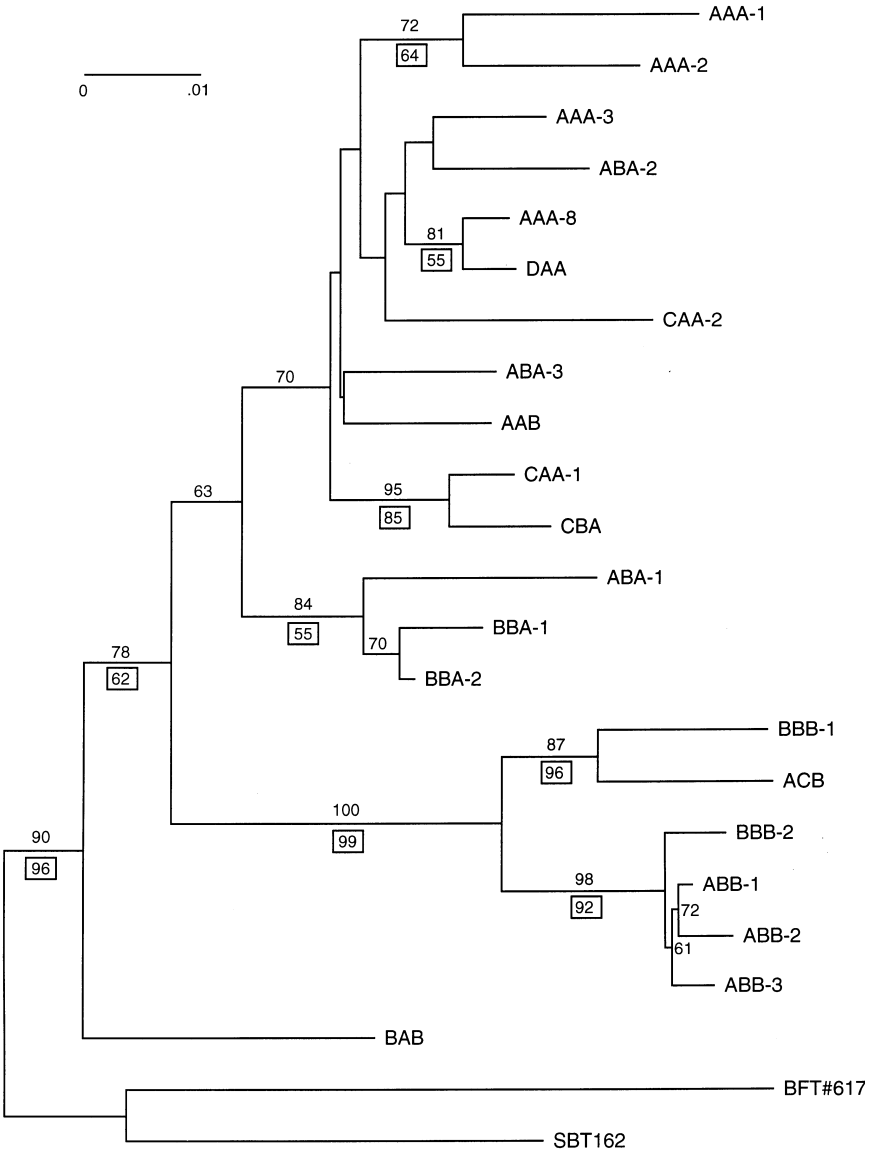

Fig. 2 *Thunnus obesus*. Neighbor-joining tree estimated from Tamura-Nei's distances among mtDNA lineages (Fig. 1). All characters were given equal weight. Positions 19 through 24 excluded because of missing data. Other instances of missing data and deletions were treated with pair-deletion option in PAUP. Bootstrap values above branches of tree were obtained with 500 replications. Tree is rooted with respect to monophyletic outgroup formed by northern bluefin tuna and southern bluefin tuna. Tree groups most of bigeye haplotypes into two groups, namely Clade I (*top*) and Clade II (*bottom*), supported by bootstrap values $>63\%$. Similar tree topology was supported by maximum-parsimony bootstrap analysis (*boxed bootstrap values below branches of tree*)

Discussion

The control-region sequence for *Thunnus obesus* is extremely variable. The DNA sequence of the control region from 21 individuals revealed that 75 out of 347 nucleotide positions were variable. A neighbor-joining analysis of these sequences demonstrated that the control-region nucleotide sequences could be grouped into two previously identified clades (Alvarado Bremer et al. 1997) which are $\simeq 5\%$ divergent (Fig. 2 of present study). This divergence value is similar in magnitude to the values of nucleotide divergence reported for the binary comparisons between the same segment of the mtDNA control regions of yellowfin, blackfin, and longtail tunas (Alvarado Bremer et al. 1997).

Nucleotide-sequence analysis revealed that the interpretation of all three restriction endonuclease assays was complicated by homoplasy. The assays with *Rsa*I and *Mnl*I were subject to independent mutations within the recognition site. Both parallel (independent occurrences of the same nucleotide change) and convergent (different nucleotide changes within the same restriction site) changes of restriction sites were observed. Some of these changes were also observed in other species of tuna (present Fig. 3a and Alvarado Bremer et al. 1997). Thus, both the *Rsa*I and the *Mnl*I sites appear to mutate at a relatively high frequency and are not reliable indicators of identity by common descent. In contrast, the *Acc*I PCR–RFLP assay was reliable $>97\%$ of the time for the discrimination of the two bigeye mtDNA clades. These results emphasize the importance of DNA-sequence analysis for the interpretation of RFLP data. The two bigeye mtDNA clades would not have been identified if we had relied solely on the PCR–RFLP data.

When the geographic association of bigeye control-region lineages was examined, the null hypothesis of a

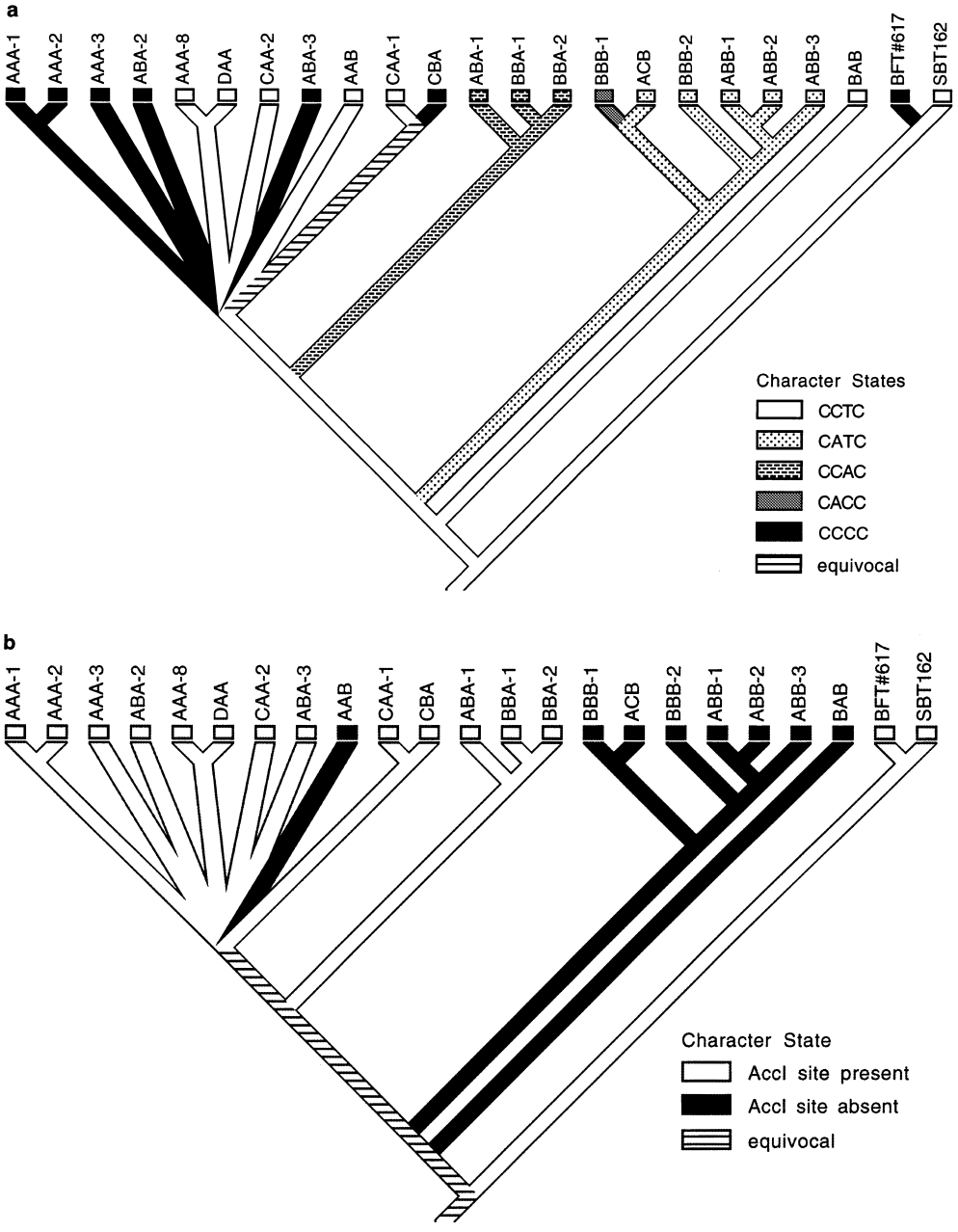

Fig. 3 *Thunnus obsesus*. Loss and gain of restriction sites traced on topology of a neighbor-joining tree implemented with MacClade (Version 3.04; Maddison and Maddison 1992). Branches with bootstrap values $< 50\%$ were collapsed. **a** Loss of *Mnl*I restriction site (5′-CCTC-3′) located between Nucleotide Positions 242 to 245 due to four altered motifs (see *Character States*); **b** loss of *Acc*I restriction site (*black branches in* **b** loss due to transition from G to A at Nucleotide Position 8) (*Halotype designations* as in Fig. 1)

single panmictic unit for all oceans was rejected. Comparisons between the Atlantic and Indo-Pacific bigeye tuna samples indicated that the populations in these two regions are genetically distinct. A major ubiquitous mtDNA clade is observed in both the Atlantic and Indo-Pacific samples, whereas a second clade occurs almost entirely in the Atlantic sample. This pattern of distribution of two highly divergent mtDNA lineages occurs in several other large pelagic scombroid fishes including blue marlin (Finnerty and Block 1992; Graves and McDowell 1995), swordfish (Alvarado Bremer 1994; Alvarado Bremer et al. 1995, 1996), and sailfish (Graves and McDowell 1995). In each case, the observed unbalanced distribution has been hypothesized to be the result of uni-directional gene flow from the Indo-Pacific to the Atlantic. However, the presence of a low level of Atlantic clade lineages in the Pacific in many of these species suggests that some gene flow from the Atlantic to the Indo-Pacific must have occurred as well.

The amount of divergence between clades in each of the pelagic fish studies described above is consistent with a vicariant isolation event. One such event could be the rise of the Isthmus of Panamá. However, the closing of the Isthmus cannot account for the observed patterns of differentiation by itself, since there is evidence of present-day contact between populations of these species around the Cape of Good Hope (Talbot and Penrith 1962). This contact is facilitated by the unidirectional flow of warm water from the Indian Ocean to the Southeast Atlantic carried along the west coast of Africa

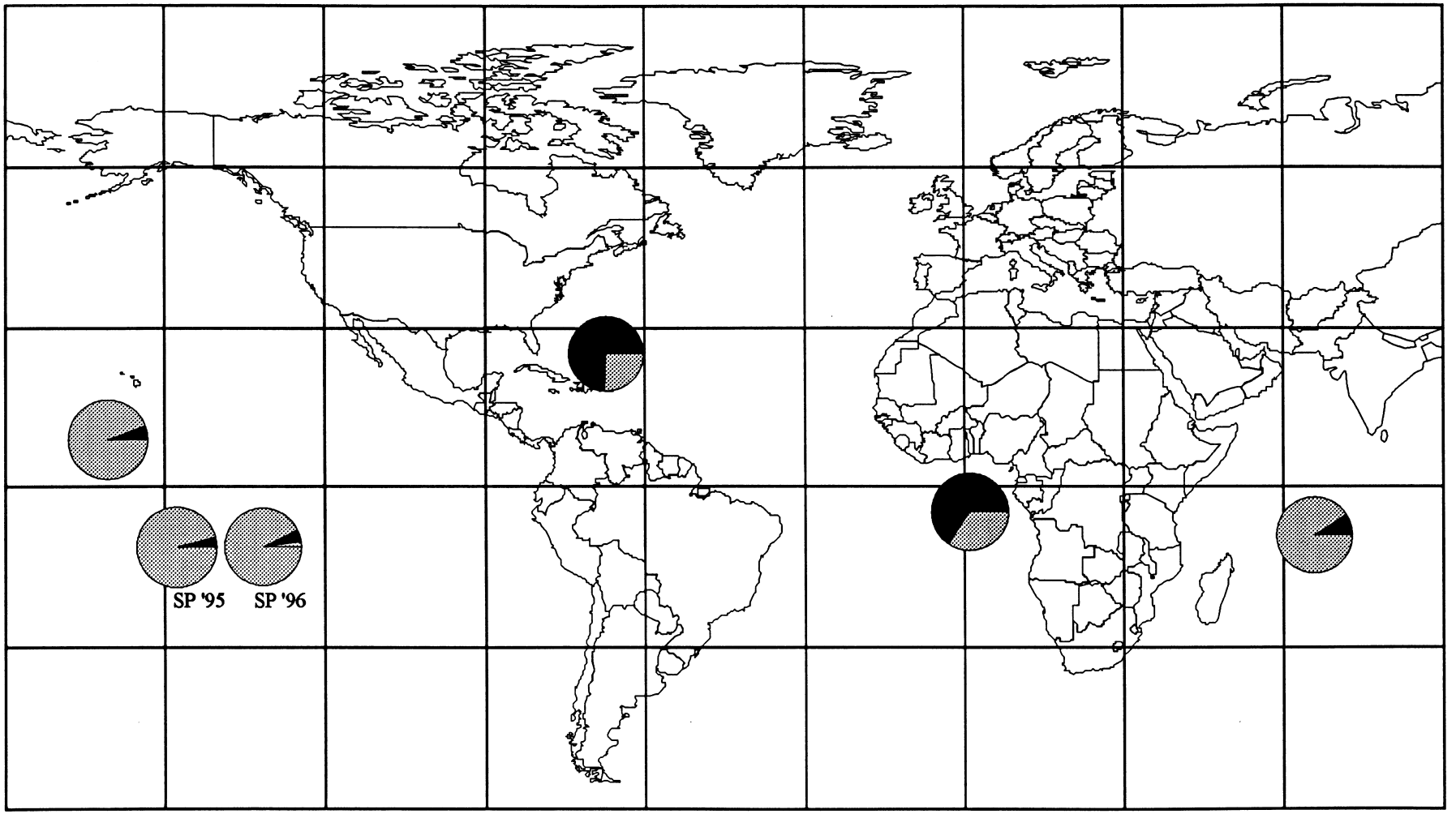

Fig. 4 *Thunnus obesus*. Frequency distribution of bigeye mtDNA lineages among sampling regions. Composite haplotypes in Table 3 were grouped into their respective clades based on phylogenetic analysis of DNA sequences as described in "Results – Nucleotide-sequence analysis" (see also Fig. 2). Slices of pie graphs represent frequencies of Clade I (*stippled*), Clade II (*black*) or rare (*white*) lineages (*SP*'95, *SP*'96 South Pacific spring 1995 and winter 1996 samples, respectively)

by the Agulhas current (Shannon et al. 1990). However, at the peak of the major glacial periods, water temperatures may have been considerably lower than those observed today (Schmiedl and Mackensen 1997), perhaps preventing movement of fish between ocean basins and allowing the mtDNA of each species to diverge into two distinct clades.

Based on the frequency distribution of mitochondrial haplotypes, we were unable to reject the null hypothesis of homogeneity within the Atlantic or within the Indo-Pacific oceans. In the Atlantic Ocean, juveniles have been observed only in the Gulf of Guinea (ICCAT 1997). Tagging studies indicate trans-Atlantic movements of bigeye from the Gulf of Guinea to the central Atlantic north of Brazil, and northerly migration from the Gulf of Guinea to the eastern Atlantic (Pereira 1995). The results presented here are consistent with a single spawning stock of bigeye in the Atlantic. However, since sampling was limited to only two areas of the Atlantic, additional analyses are needed to test this hypothesis.

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