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FRDC Project 97/112

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OBJECTIVES

- To carry out a pilot study into the genetic stock structure of bigeye tuna in the Indian Ocean aimed at determining whether fish from four locations (Western Australia, South Africa, Seychelles and Indonesia) are drawn from a common gene pool or whether they represent reproductively isolated spawning populations.
- To compare this Indian Ocean data with data already being collected for the Atlantic Ocean and western tropical Pacific Ocean to gain a broader understanding of the global population structure of bigeye tuna
- Should evidence of large scale stock structuring within the Indian Ocean be evident, then a more extensive study will be proposed to indicate the number and extent of the different stocks within the Indian Ocean.

1. NON-TECHNICAL SUMMARY

Bigeye tuna (*Thunnus obesus* Lowe) are large scombrid fish inhabiting tropical and subtropical waters of all oceans between 45^0N and 40^0S except the Mediterranean Sea. Its catch in the greater Indian Ocean in 1997 was approximately 72 000 tonnes. An important domestic bigeye tuna fishery is developing in the western Australian Fishing Zone (AFZ) off Western Australia.

While there have been several genetic and biological studies on bigeye tuna in the Pacific Ocean, there have been no comprehensive studies on stock structure or biological parameters in the Indian Ocean. In most instances where biological parameters for bigeye in the Indian Ocean were required, parameters have been estimated from Pacific and/or Atlantic Ocean studies.

The Western and Southern Tuna and Billfish Fishery (WSTBF) is contiguous with tuna and billfish fisheries in the greater Indian Ocean. Information regarding the Indian Ocean stock structure of bigeye tuna is therefore considered vital for the long-term sustainable management of the fishery within the WSTBF. The current genetic study was undertaken in response to a call from fisheries managers for research into the population structure of bigeye tuna stocks exploited in the west coast tuna fishery.

In this project, five collections of bigeye tuna from the Indian Ocean (Indonesia, Madagascar, Seychelles, west Indian Ocean and Western Australia, sample sizes ranging from 19 to 96 individuals) were examined for genetic variation in the mitochondrial DNA genome (two regions) and the nuclear DNA genome (seven microsatellite loci). Initial statistical tests examined whether there was any overall evidence of heterogeneity among the five collections; no such evidence was forthcoming from either the mtDNA (the conserved ATCO fragment and the more variable D-loop region) or the seven microsatellite loci. The null hypothesis that Indian Ocean bigeye tuna are drawn from a common gene pool could not be rejected. A second set of tests examined whether there was any east-west differentiation (by pooling Indonesia and Western Australia into an eastern collection, and Madagascar, Seychelles and west Indian Ocean into a western collection). The ATCO region and one microsatellite locus showed evidence of differentiation, the D-loop region and six microsatellite loci showed no such evidence.

We suggest that it would be premature to accept the hypothesis that Indian Ocean bigeye comprise a single panmictic population, and that there is a possibility of separate eastern and western components. This needs to be confirmed (or refuted). There was no evidence that Indian Ocean bigeye are genetically distinct from Pacific Ocean bigeye, although not all markers could be compared between this Indian Ocean study and an earlier Pacific Ocean study.

In contrast, bigeye tuna from the Atlantic Ocean show striking mtDNA haplotype frequency differences from Indian Ocean bigeye, although microsatellite allele frequencies are similar. MtDNA is maternally inherited, and mtDNA differentiation with little nuclear DNA differentiation can be explained by females homing to natal areas with males being less philopatric, or by the greater sensitivity of mtDNA to population size variation over time. Alternatively, it may be that there is nuclear DNA differentiation between these two oceans, but that the dinucleotide microsatellite markers used lacked the resolving power to detect it.

Further studies of bigeye tuna population structure in the Indian Ocean are required. We recommend sample sizes of 200 fish per site, with additional sites being examined. Genetic analyses should use, in addition to the markers deployed here, tri and tetranucleotide microsatellite markers and markers with a lower mutation rate.

KEYWORDS: bigeye tuna, *Thunnus obesus*, stock structure, population structure, microsatellite, mitochondrial DNA

2. BACKGROUND

Bigeye tuna (*Thunnus obesus* Lowe) are large scombrid fish inhabiting tropical and subtropical waters of all oceans between 45^{0}N and 40^{0}S except the Mediterranean Sea (Calkins 1980; Collette and Nauen 1983). They are a relatively fast-growing species, reaching approximately 45cm after one year and up to 100cm by the third year (Larcombe *et al.* 1997).

Based on tagging data, bigeye tuna in the Atlantic Ocean are believed to display philopatric behaviour to the only known breeding area in this ocean around the Gulf of Guinea between 15^{0}N and 15^{0}S (Cayre and Diouf 1984; ICCAT 1997). In the Pacific Ocean, spawning occurs between 10^{0}N and 10^{0}S in the eastern Pacific. In the Indian Ocean, bigeye tuna are found throughout the area north of 35^{0}S (Alvarado-Bremer *et al.* 1998). Two areas of concentration have been observed in the Indian Ocean; an equatorial concentration where spawning is observed throughout the year and a winter concentration of sexually inactive bigeye tuna around 30^{0}S (Kume *et al.* 1971).

Bigeye tuna are multiple spawners capable of spawning every 1-2 days over several months (Nikaido *et al.* 1992). Their eggs are epipelagic and juveniles and adults pelagic; otherwise rather little is known of bigeye tuna life history (Nishikawa *et al.* 1985). In general, warm surface waters are the preferred habitat for young juveniles while adults have wider distributions as they can tolerate oxygen depleted and cooler waters (Hanamoto 1976).

Bigeye are considered a valuable sashimi tuna. In the greater Indian Ocean basin they are targeted by deep longline gear on Japanese, Korean and Taiwanese distant-water fleets (Larcombe *et al.* 1997). The total bigeye tuna catch in the Indian Ocean has increased in the last ten years with up to 97 000 tonnes caught in 1997 (FAO 1999).

While there have been several genetic studies on bigeye in the Pacific Ocean (Suzuki 1962; Alvarado-Bremer *et al.* 1998; Grewe and Hampton 1998), to date there have been no comprehensive studies on stock structure or biological parameters of bigeye tuna in the Indian Ocean (Larcombe *et al.* 1997). On a wider scale, Suzuki (1962) examined Tg blood type and found no difference between Indian and Pacific Ocean bigeye tuna samples. Alvarado-Bremer *et al.* (1998) and Chow *et al.* (2000) found no

difference in mtDNA haplotype frequencies between Indian and Pacific Ocean bigeye tuna, but did find significant differentiation between Indian/Pacific and Atlantic Ocean bigeye tuna samples. Grewe and Hampton (1998) also generally found no differences between mtDNA haplotypes of samples of bigeye tuna from different regions of the Pacific Ocean.

In most instances where biological parameters for bigeye in the Indian Ocean are required, parameters are estimated from Pacific and/or Atlantic Ocean studies (Larcombe *et al.* 1997). It is assumed that a single stock exists in each ocean basin including the Indian Ocean (Kume *et al.* 1971; Larcombe *et al.* 1997; reviews by Miyabe and Bayliff 1998; Pallares *et al.* 1998; Stobberup *et al.* 1998).

Kume *et al.* (1971) concluded that bigeye were widely distributed in the Indian Ocean north of the northern limit of the west wind drift (approximately 35^0S), but catch data indicated that there were two distinct groups. Running east to west, the equatorial bigeye group is distributed throughout the equatorial region and the southern group occurs between 25^0S and 35^0S during the southern winter months (Kume *et al.* 1971). Kume *et al.* (1971) observed that fish in the equatorial group were sexually active (based on gonad indices) but that fish of the southern group were not. The size structure of fish in both groups was however similar, with both small and medium size classes observed in both groups (small size=<120cm, medium size=120-150cm) (Kume *et al.* 1971). Kume *et al.* (1971) concluded that the southern and equatorial groups must mix at some undetermined time during the life cycle. Due to the lack of stock structure data, bigeye tuna caught within the WAFZ are assumed to be part of the single Indian Ocean stock with fish caught south of 25^0S part of the "southern" group as opposed to the "equatorial" group (Larcombe *et al.* 1997).

Examination of mtDNA markers is now an established technique for determining population genetic structure. MtDNA is a small closed-circular genome found in the mitochondria of cells. It shows variation between individuals on both an intra and inter-species level, and has proven to be an effective genetic marker for population structure analysis (Avise *et al.* 1987; Ovenden 1990; Billington and Hebert 1991). In addition, and owing to its haploid and maternal mode of inheritance, the effective population size of mtDNA is one quarter that of nuclear DNA (given equal numbers of males and females).

MtDNA is therefore more sensitive to bottlenecks in population size than nuclear genes, and more subject to genetic drift and population differentiation (Nei and Tajima 1981; Wilson *et al.* 1985; Moritz *et al.* 1987; Billington and Hebert 1991). In bigeye tuna, there are specific mtDNA polymorphisms known to differentiate bigeye from the Indo-Pacific and Atlantic Oceans (Alvarado-Bremer *et al.* 1998; Chow *et al.* 2000). These polymorphisms were used in the current study for both species identification and for stock structure analysis of Indian Ocean bigeye tuna.

As outlined above, genetic variation in bigeye tuna has previously been assessed primarily through mtDNA examination. Since these studies, new DNA techniques have become available; some of which may be more powerful than allozyme and mtDNA methods for quantifying levels of genetic variation within and among populations. Foremost among these are microsatellite analyses.

Microsatellites are tandem repeats of short sequence motifs that are distributed throughout the nuclear DNA genome and believed to be abundant in all eukaryotes (Tautz 1989). Polymorphisms at microsatellite loci were first demonstrated by Tautz (1989) and Weber and May (1989). Microsatellite allelic variation is assessed from the different electrophoretic mobility of PCR products which display length polymorphisms (Weber and May 1989; Brooker *et al.*, 1994). Microsatellites are usually 20-300 base pairs in length, fast evolving, and a high proportion of microsatellite loci surveyed in fish are polymorphic (Brooker *et al.* 1994; Carvalho and Hauser 1994; Prodohl *et al.*, 1994; O'Reilly and Wright 1995; Jarne and Lagoda 1996; Nielsen *et al.*, 1997; Rico *et al.*, 1997; Takagi *et al.*, 1997; Bagley *et al.*, 1999; Nugroho and Taniguchi 1999; Takagi *et al.*, 1999; DeWoody and Avise 2000). Microsatellites show high levels of genetic variation and high mutation rates; meaning that populations are likely to diverge not only by genetic drift but by mutation as well.

The use of microsatellites as genetic markers in fisheries science has increased rapidly (Wright and Bentzen 1994; O'Connell and Wright 1997; O'Reilly and Wright 1995; O'Reilly *et al.*, 1996; DeWoody and Avise 2000). They are frequently hypervariable and, since they reflect variation in non-coding sequences, are thought to be neutral markers (unaffected by selection).

To date, there have been only four microsatellite studies in tuna species. Two (Broughton and Gold 1997; Takagi *et al.* 1999) examined population structure in small samples of Pacific northern bluefin tuna (*T. thynnus orientalis*) and Atlantic northern bluefin tuna (*T. thynnus thynnus*), and the third (Grewe and Hampton 1998) examined bigeye tuna (*T. obesus*) within the Pacific Ocean. Grewe and Hampton (1998) found no evidence of significant differentiation among nine collections of Pacific Ocean bigeye tuna at eight microsatellite loci. Appleyard *et al.* (submitted) also found no significant spatial heterogeneity at five microsatellite loci in Pacific Ocean yellowfin tuna (*T. albacares*).

3. NEED

The current study was developed in response to a call from the West Coast Tuna and Billfish Management Advisory Committee (WCTBFMAC) for research into the population structure of bigeye tuna stocks exploited in the west coast tuna fishery. The longline fishery for bigeye tuna off the western coast of Australia is a valuable fishery due to the fish's high export value.

Bigeye tuna was the second most valuable tuna targeted by the Japanese fishing fleet in the western Australian fishing zone (WAFZ) (now known as the Western and Southern Tuna and Billfish Fishery), the southern bluefin tuna being number one (Larcombe *et al.* 1997). These catches were taken by longliners operating in the AFZ under bilateral agreements but, since November 1997, Japanese longliners have been excluded from Australian waters (Fishery Status Reports 1999). The annual catch of bigeye in the WAFZ peaked at 1000 tonnes in 1987 (due to increased fishing effort by the Japanese fishing fleet). In 1998, the combined domestic longline and handline catch of bigeye in the WSTBF was 146 tonne (Fishery Status Reports 1999).

The WSTBF is contiguous with tuna and billfish fisheries in the greater Indian Ocean. As such, Australia is a member of the Indian Ocean Tuna Commission. The IOTC encourages its members to comply with high sea tuna conservation and management measures (Fishery Status Reports 1999). Information regarding the stock structure of bigeye tuna is therefore vital for the long-term sustainability of the fishery. Uncertainty regarding bigeye stock structure seriously restricts the confidence that scientists and fisheries managers can place in the regional assessments that have been carried out to date. In the Indian Ocean, despite improvements of production models, it has still not been possible to make firm statements on the bigeye tuna stocks as a whole (Larcombe *et al.* 1997, Fishery Status Reports 1999).

4. OBJECTIVES

- To carry out a pilot study into the genetic stock structure of bigeye tuna in the Indian Ocean aimed at determining whether fish from four locations (Western Australia, South Africa, Seychelles and Indonesia) are drawn from a common gene pool or whether they represent reproductively isolated spawning populations.
- To compare this Indian Ocean data with data already being collected for the Atlantic Ocean and western tropical Pacific Ocean to gain a broader understanding of the global population structure of bigeye tuna
- Should evidence of large scale stock structuring within the Indian Ocean be evident, then a more extensive study will be proposed to indicate the number and extent of the different stocks within the Indian Ocean.

5. METHODS

5.1. Tuna Population Sampling

Samples were collected around the western and eastern Indian Ocean from 1997 to 1999. Fish from South Africa were not available; instead fish from Madagascar were used. Samples from the Atlantic Ocean (for comparative study) were collected in 1993.

Table 1 Thunnus obesus collections, lengths and sampling locations (approximate longitude and latitude). A is total number of genetically confirmed bigeye, B is number of measured bigeye, and C is total sample size.

Collection	Sample size			Average length	Date of sampling	Location
	A	B	C	cm (s.e)[a]		
Indonesia	91	88	94	86.8 (2.4)	Aug 1998	9-12^{0}S, 108-115^{0}E
Madagascar	53	37	96	121.8 (2.9)	Feb 1999	21-26^{0}S, 47-55^{0}E
Seychelles	89	83	95	53.9 (1.1)	Dec 1998-Jan 1999	0-11^{0}S, 50-88^{0}E
west Indian Ocean	19	19	19	76.1 (3.5)	Nov-Dec 1995	0^{0}S-7^{0}N, 56-67^{0}E
Western Australia	96	96	96	119.9 (1.5)	June-July 1997	31-33^{0}S, 111-113^{0}E

[a]average lengths based on length to caudal fork measurements of confirmed bigeye tuna

Fig 1. Approximate locations of the bigeye samples used in this report.

Samples consisted of pieces of white muscle dissected from whole fish and stored frozen at –20^{0}C or frozen in alcohol preserving solutions and stored at –20^{0}C until DNA extraction.

Genetic analysis involved the use of mitochondrial DNA for species identification (identifying individuals as bigeye/non-bigeye tuna) and population haplotype differentiation using restriction fragment length polymorphism (RFLP) analyses. Nuclear DNA was used in microsatellite analysis for population discrimination. For both approaches, total genomic DNA was extracted from approximately 50mg of tissue from each individual using a modified CTAB (hexadecyltrimethylammonium bromide) extraction protocol described in Grewe *et al.* (1993). After precipitation with isopropanol and ethanol, genomic DNA pellets were resuspended in 150-200μl of deionized H_2O and stored at 4^{0}C. Stock DNA from Western Australia, Atlantic and west Indian Ocean collections were diluted 1:5 with distilled water for both mtDNA and microsatellite applications while genomic DNA from the remaining collections was used undiluted.

5.2. mtDNA Haplotype Analysis

MtDNA variation for the identification and population haplotype differentiation of bigeye tuna was examined through restriction digestion of the amplified ATCO fragment and the amplified control region (D-loop).

The ATCO fragment contains the flanking region between the ATPase-6 and cytochrome oxidase subunit III genes and was amplified using primers (LAT6 (L8562) 5'- CTTCGACCAATTTATGAGCCC-3' and HCOIII (H9432) 5'-GCCATATCGTAGCCCTTTTTG-3') described by Chow and Inoue (1993).

The more variable DL19-12S fragment contains the control region or D-loop and is flanked by the transfer RNA proline gene and the 12S rRNA gene. This fragment was amplified using primers L-19 5'-CCACTAGCTCCCAAAGCTA-3' (Bernatchez *et al.* 1992) and 12SAR-H (H1067) 5'-ATAGTGGGGTATCTAATCCCAGTT-3' (Palumbi *et al.* 1991).

The same PCR conditions were used for both fragments. PCR amplifications were performed in a PE-Applied Biosystems 9600 thermocycler in a total volume of 50μl. Individual amplifications consisted of 200μM dNTP's, 10mM Tris HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.2μM of forward and reverse primer, 0.025U/μl Amplitaq Gold (Perkin Elmer, USA) and 10μl of genomic DNA. After an initial cycle of 93^0C × 10 minutes, 55^0C × 45 seconds and 72^0C × 2 minutes, samples were subjected to 93^0C × 30 seconds, 50^0C × 1 minute and 72^0C × 2 minutes for 40 cycles with a final extension step of 72^0C × 10 minutes.

Resulting ATCO PCR products were directly subjected to restriction endonuclease digestion with the enzymes *MseI* (New England Biolabs, 4000 U/mL) to confirm species identity and *RsaI* (New England Biolabs, 10 000U/ml) to determine haplotype differences between collections. For *MseI*, 10μl of PCR product was added to 1.5μl buffer, 1.5μl BSA (bovine serum albumin), 1.4μl water and 0.4μl of the enzyme and incubated at 37^0C for 90 minutes. For *RsaI*, 10μl of PCR product was added to 1.5 μl buffer, 3.2μl water and 0.2μl of the enzyme and incubated at 37^0C for 90 minutes.

Resulting DL19-12S PCR products were subjected to a double digest with *HinfI* and *BglI* (New England Biolabs, each 10 000 U/mL). 10μl of PCR product was added to

buffer, BSA, water and 0.3μl of each enzyme and incubated at 37^{0}C for 120 minutes. In addition, a *TaqI* (New England Biolabs, 20 000 U/mL) digest was undertaken on the DL19-12S fragment. 10μl of PCR product was added to buffer, BSA, water and 0.2μl of the enzyme and incubated at 65^{0}C for 120 minutes. These two sets of digests were used to examine haplotype variation within and among the bigeye tuna collections.

The products from each restriction digest were run separately on a 2% 1X TBE agarose gel (containing ethidium bromide) at 120 volts for 60-80 minutes. A Promega 100 base pair ladder was loaded on each gel to enable sizing of various fragments. Resulting fragments were visualised under U.V. light and photographed using a digital camera.

Individuals that were identified as bigeye tuna were used in the microsatellite analysis; others were excluded from further analysis.

5.3. DNA Microsatellite Markers

The eight DNA microsatellite loci initially examined were previously isolated from yellowfin tuna (Grewe, unpublished data). PCR primers were designed for flanking regions around the microsatellite repeats (Table 2). Oligonucleotides were synthesised by Bresatec Pty Ltd (Adelaide, South Australia) with one of the primer pairs end-labelled with a fluorescent tag; FAM, TET or HEX. The eight loci are designated *cmrTa-102*, *cmrTa-113*, *cmrTa-117*, *cmrTa-121*, *cmrTa-125*, *cmrTa-144*, *cmrTa-161* and *cmrTa-208* (*cmr*=CSIRO Marine Research, *Ta*=*Thunnus albacares*, *T. albacares* or yellowfin tuna being the species used in the isolation of these microsatellite sequences), but are referred to hereafter as *102*, *113*, *117*, *121*, *125*, *144*, *166* and *208*. Due to the large number of alleles present and a difficulty in consistently scoring samples, *117* was not used in the final analyses.

Table 2 Microsatellite motif, primer sequences, number of alleles observed and allele size of bigeye microsatellite loci. The motif listed is that obtained from the original yellowfin sequence used to generate amplification primers.

Locus	Motif	Primer sequences	Number of alleles	Allele size range[a]
102	$(GA)_2(CA)_{32}$	5'-GAC CAC ATC CCT GCT CCT TTA-3'	21	132-172
		5'-TCC CAC ATC GCA CCC ACA G-3'		
113	$(CA)_{12}$	5'-CAT ATT GTC TGC ATC TGA AAA CTG-3'	31	105-167
		5'-CAT CCT CCT GCT TGA ACT GA-3'		
117	$(CA)_{12}$	5'-TCA CAG CAT GGG ACA AC-3'	35[b]	170[b]
		5'-ATA GTG AAA TGA TTA GAA CAG TGC-3'		
121	$(CA)_4(TA)$	5'-CCC TCC CTC TTT GCC ACT T-3'	22	258-306
	$(CA)_7$	5'-ATG CGC CAC CGA AAT CTG C-3'		
125	$(CA)_{10}$	5'-TTG GGC TGC CTG ATG AAG-3'	4	158-166
		5'-GTG TCT CTG AAA TGA TGG AAA CA-3'		
144	$(CA)_6$	5'-TCC TCA TTT AGA AAG CCA CTG TA-3'	8	162-178
		5'-ACC TGT TGA TTA TTG CTT TTA TGT-3'		
161	$(CA)_{19}$	5'-CAG TAT TTT CTC ATG GAT ACC AGC AC-3'	24	170-220
		5'-GAT TTC GTG CAG CCT TGT GCA G-3'		
208	$(CA)_{10}$	5'-CAC AAA CTT CCT CTT AAA CCG ATC ATG-3'	19	138-176
		5'-GAT GTA TGG AAA GCA GGG GAC TG-3'		

[a]sizing in base pairs
[b]minimum of 35 alleles, minimum allele size

5.4. Microsatellite Analysis

In the development phase, individual microsatellite loci were amplified separately in a subsample of individuals. These were then run on an ABI Prism 377 DNA sequencer (PE Applied Biosystems) for three hours to check for overlapping allele sizes and to select colours. Four loci (*125*, *144*, *161* and *208*) were optimised for use in the first multiplex reaction where all four pairs of primers were added to a single PCR reaction. The remaining loci (*102*, *113*, *117* and *121*) were optimised for use in another PCR reaction. PCR amplifications were performed in a PE-Applied Biosystems 9600 thermocycler in a total volume of 25μl. Individual amplifications for samples identified as bigeye tuna consisted of 100μM dNTP's, 10mM Tris HCl pH 8.3, 50mM KCl, 2.5mM

$MgCl_2$, 0.23μM for each forward and reverse primer (first multiplex reaction), 0.36μM for each forward and reverse primer (second multiplex reaction), 0.05 U/μl Amplitaq Gold (Perkin Elmer) and 10μl of genomic DNA. After an initial cycle of 93^0C × 10 minutes, 55^0C × 15 seconds and 72^0C × 2 minutes, samples were subjected to 93^0C × 15 seconds, 54^0C × 15 seconds and 72^0C × 2 minutes for 35 cycles with a final extension step of 72^0C × 10 minutes.

Amplified products were used undiluted and mixed with a formamide loading dye containing ABI Prism GeneScan350 Tamra internal lane size standards (PE Applied Biosystems) and blue dextran loading dye, denatured at 94^0C × 2 minutes, and immediately placed on ice. 1.3μl of sample was stagger loaded into a 4.8% 6M Urea denaturing polyacrylamide gel and run using 1X TBE buffer on the ABI Prism 377 DNA sequencer (PE Applied Biosystems) for three hours. PCR products for each locus were analysed using GENESCAN™ 3.1 collection software (PE Applied Biosystems) and local southern size calling method. Genotyping at each locus was completed using GENOTYPER™ 1.1.1 software (PE Applied Biosystems) which enabled the formation of approximately two base pair bins for each locus. Bin widths generally corresponded to a repeat unit.

5.5. Statistical Analysis

5.5.1. mtDNA haplotypes

Variation in mtDNA haplotype frequencies among bigeye tuna collections was assessed using standard Monte-Carlo chi-square approaches (Roff and Bentzen 1989) in the program CHIRXC (Zaykin and Pudovkin 1993), with 5000 randomisations of the data used to estimate *P* values. Analysis of variance (AMOVA) (see below) was also used to determine the level of population differentiation attributable to haplotype frequency differences among the bigeye tuna collections.

5.5.2. Microsatellite genotypes

Genetic diversity for each locus per collection was estimated by the number of alleles per locus and by the observed (H_{obs}) and Hardy-Weinberg expected (H_{exp}) heterozygosity. H_{obs}, H_{exp} and tests for deviations from Hardy-Weinberg Equilibrium (HWE) within samples were estimated using ARLEQUIN vers. 2.00 (Schneider *et al.* 2000). An index of heterozygote deficiency or excess (D), where $D=[H_{obs}-H_{exp}]/H_{exp}$. (Koehn *et al.* 1973) was also calculated from the heterozygosity estimates. Significance levels for deviations from HWE were based on 100 000 steps of a Markov chain procedure.

Linkage disequilibrium was assessed using exact tests in GENEPOP vers. 3.2 (Raymond and Roussett 2000). The null hypothesis of genotypes at one locus being independent from genotypes at another locus was tested using contingency tables and probability tests between loci. Significance of departure from equilibrium levels was tested by a Markov chain procedure, with significance levels determined after 400 batches of 4000 iterations each.

The significance of allele frequency differences at each locus among bigeye collections was assessed using exact tests in GENEPOP vers. 3.2 (Raymond and Roussett 2000). The null hypothesis of allele distributions being identical across collections was estimated with an unbiased estimate of the *P* value; significance levels were determined after 400 batches of 4000 iterations each of a Markov chain. The significance of genotype differences at each locus among collections was tested in GENEPOP vers. 3.2, with an unbiased estimate of the *P* value of a log-likelihood (G) based exact test (Goudet *et al.* 1996). Significance levels were again determined after 400 batches of 4000 iterations each.

In all cases with multiple tests, significant levels were adjusted using a standard Bonferroni procedure (Miller 1980, Lessios 1992). *P* values had to be equal to or less than this adjusted value (0.05 divided by number of tests) to be deemed significant.

The computation of estimates of F-statistics (F_{IS}, F_{IT} and F_{ST}) was done in GENEPOP vers. 3.2 (Raymond and Roussett 2000). These hierarchical values are all types of inbreeding coefficients but differ in respect to their reference population (Hartl

1988). F_{IS} values estimate the reduction in heterozygosity of an individual due to non-random mating within its subpopulation (Hartl 1988). Typically, F_{IS} values close to zero indicate random mating within subpopulations. The overall inbreeding coefficient of an individual, F_{IT}, measures the reduction of heterozygosity of an individual relative to the total population. The effects of population subdivision are measured by the fixation index, F_{ST}, which is the reduction in heterozygosity of a subpopulation relative to the total population of which they are a part, due to random genetic drift (Hartl 1988). F_{ST} values can be used to estimate overall population differentiation. F_{ST} values in the current study were estimated by a weighted analysis of variance (Cockerham 1973; Weir and Cockerham 1984) and multilocus estimates were computed as in Weir and Cockerham (1984). These F estimates are related by: $1- F_{IS} = (1- F_{IT})(1- F_{ST})$.

The AMOVA (Analysis of Molecular Variance) procedure developed by Excoffier *et al.* (1992) in the program ARLEQUIN vers. 2.00 (Schneider *et al.* 2000) was also used to measure the genetic variance of population structure. The proportion of gene diversity within and among bigeye collections was estimated using ϕ_{ST}, an analogue of F_{ST}. ϕ_{ST} is obtained as the estimated variance components resulting from differences among collections divided by the estimated total variance (Michalakis and Excoffier 1996). For the current study, ϕ_{ST} values are equivalent to F_{ST} values, as equal genetic distances among alleles or haplotypes were assumed. The significance of the variance component associated with F_{ST} was tested using non-parametric permutation procedures (Excoffier *et al.* 1992). The F_{ST} value was tested by comparison with a null distribution of random sampling from the global population and was based on 16 000 re-sampling trials.

An overall exact test of population differentiation based on the seven microsatellite loci was also undertaken in ARLEQUIN vers. 2.00 (Schneider *et al.* 2000) to test the hypothesis of random distribution of individuals between pairs of collections (Raymond and Rousset 1995; Goudet *et al.* 1996). Significance levels were based on 100 000 steps of a Markov chain procedure.

Finally, genetic distances between collections were assessed with Nei's (1978) unbiased genetic distance measure, converted to a dendrogram by UPGMA (unweighted pair-group arithmetic averaging). The program POPGENE vers 1.31 (Yeh *et al.* 1999)

was used, modified from the NEIGHBOR procedure of PHYLIP vers 3.5 (Felsenstein 1995).

6. RESULTS

6.1. MtDNA for species identification based on the ATCO fragment

All tuna sampled for this study were examined for variation of the ATCO fragment to determine species identity. The typical ATCO *MseI* mtDNA bigeye patterns were as in Chow and Inoue (1993) and consisted of two different haplotypes; BET1 (present mainly in Indian Ocean collections) with major bands of approximately 300, 230 and 195 base pairs and BET2 (present mainly in Atlantic Ocean) with major bands of approximately 250, 230, 195 and 120 base pairs (Figure 2). The yellowfin haplotype consisted of major bands of approximately 270, 230, 195 and 120 base pairs.

Table 3 Identification of species in the six collection samples. The mtDNA test is based on *MseI* haplotypes from the ATCO mtDNA fragment; where this fragment could not be amplified a microsatellite test was used (see text).

Test	Species	Atlantic	Indonesia	Madagascar	Seychelles	west Indian Ocean	Western Australia
mtDNA	BET	15	57	19	85	19	96
	YFT	0	0	5	6	0	0
Microsatellite	BET	0	34	34	4	0	0
	uncertain	0	3	38	0	0	0
Totals	BET	0	91	53	89	19	96
	YFT + uncertain	0	3	43	6	0	0
Grand Totals		15	94	96	95	19	96

From the bigeye collections, 96 individuals from Western Australia, 85 individuals from Seychelles, and 19 individuals from the west Indian Ocean were identified successfully as bigeye tuna using the *MseI* digest. Only 57 individuals from Indonesia and 19 from Madagascar were identified as bigeye using this digest. Five and six individuals were identified as yellowfin tuna in the Madagascar and Seychelles collections (Table 3). As the results above indicate, the ATCO fragment was only amplified in a limited number of individuals from Indonesia and Madagascar collections. The DNA in these collections did not amplify successfully for the 930 base pair ATCO

fragment. However, microsatellites have been amplified in these collections; their fragment sizes were nearly a third smaller than that of the ATCO fragment. Smaller DNA fragments generally amplify better than large fragments, especially when the DNA is partially degraded. Several different modifications were made to the PCR reactions in attempts to obtain successful PCR products from the ATCO fragment including increasing the amount of template used in reactions, lowering annealing temperatures and re-extracting genomic DNA from non-amplifying individuals.

For those individuals in which the ATCO fragment amplification was not successful (77 individuals in Madagascar, 37 in Indonesia and 4 individuals in Seychelles), composite microsatellite genotypes (at loci *144* and *208*) were used to identify individuals as either bigeye or uncertain. Those classified as uncertain were most probably yellowfin tuna. In yellowfin tuna, the 174/174 homozygote at microsatellite locus *144* is the dominant genotype (0.723, allele frequency 0.850), and at locus *208*, the 142 allele is the most common (0.665) and the 150 allele not common (0.016) (Appleyard *et al.* submitted). In bigeye tuna, 144*174/174 genotypes are uncommon (0.063), the 208*142 allele is rare (0.028), and the 208*150 allele is common (0.284). Therefore individuals in the current study in which the ATCO fragment did not amplify, but which had the 174/174 genotype (at *144*) together with either a 142/142 genotype or a heterozygote 142 with another allele other than 150 (at *208*) were identified as of uncertain origin (most probably yellowfin); they were not used in any subsequent analysis.

The numbers of individuals identified subsequently as bigeye tuna or non-bigeye tuna (uncertain plus yellowfin tuna) can be seen in Table 3. Generally non-bigeye tuna comprised less than 5% of the samples sent to us as bigeye tuna; the exception was the Madagascar collection where 45% of individuals classified as non-bigeye.

6.2. MtDNA for bigeye tuna population differentiation - ATCO fragment

Two haplotypes can be clearly resolved following *MseI* digestion of the ATCO fragment from bigeye tuna (BET1 and BET2), and two haplotypes can be clearly resolved following *RsaI* digestion of the ATCO fragment (α and β) (Figure 2).

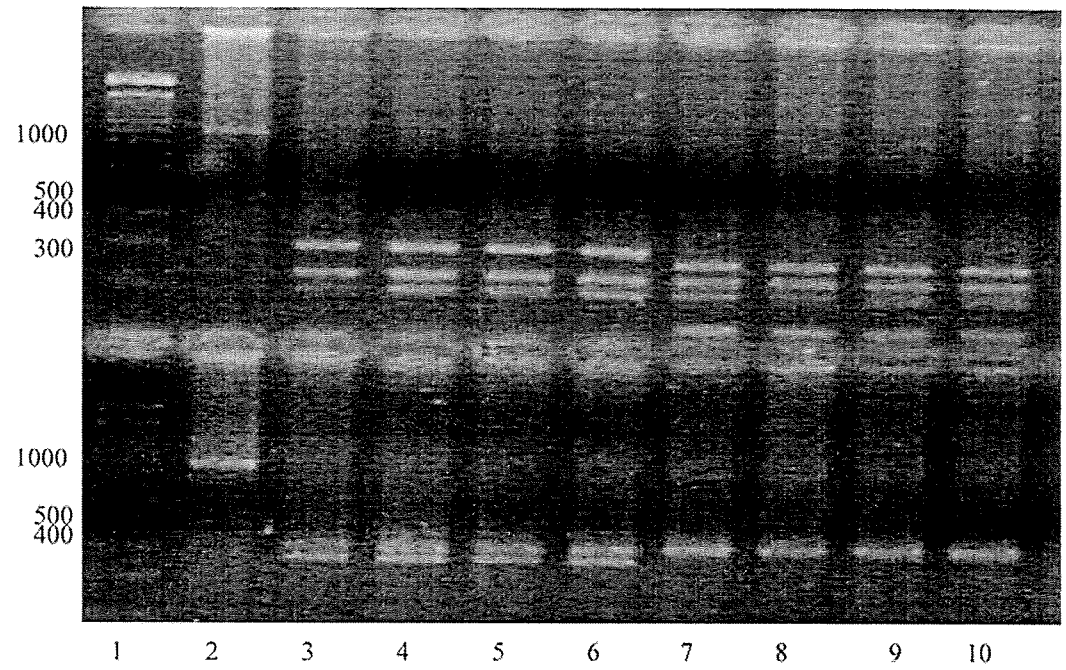

Figure 2 1×TBE 2.5% agarose gel of ATCO mtDNA fragment from Indian and Atlantic Ocean bigeye tuna. *Top line*: Lane 1, 100 bp ladder; Lane 2, uncut amplified ATCO fragment; Lanes 3-6, Indian Ocean bigeye digested with *MseI* - haplotype BET1; Lanes 7-10, Atlantic Ocean bigeye digested with *MseI* - haplotype BET2. *Lower line*: Lane 1, 100 bp ladder; Lane 2, uncut amplified ATCO fragment; Lanes 3-6, Indian Ocean bigeye digested with *RsaI* - haplotype β; Lanes 7-10, Atlantic Ocean bigeye digested with *RsaI* - haplotype α.

6.2.1. MseI digestion

Numbers of BET1 and BET2 haplotypes in the Indian Ocean and Atlantic collections were assessed (Table 4).

Table 4 Distribution of *MseI* haplotypes from the ATCO mtDNA fragment in five Indian Ocean bigeye collections and one Atlantic collection.

Haplotype	Indonesia	Madagascar	Seychelles	west Indian Ocean	Western Australia	*Indian Ocean Total*	Atlantic Ocean
BET1	57	18	82	19	96	*272*	1
BET2	0	1	3	0	0	*4*	14
Total	57	19	85	19	96	*276*	15

6.2.1.1. Indian Ocean heterogeneity

Haplotype differentiation (BET1 and BET2, Table 4) analysis among Indonesia, Madagascar, Seychelles, west Indian Ocean and Western Australia bigeye individuals was not significant (χ^2=7.039, *P*=0.119) (frequency of mtDNA haplotypes - Indonesia BET1=1.000; Madagascar BET1=0.947; Seychelles BET1=0.965; west Indian Ocean BET1=1.000; Western Australia BET1=1.000). In addition, analysis of variance among the Indian Ocean collections was not significant (F_{ST}=0.0151, *P*=0.122) (Table 5).

Table 5 AMOVA of *MseI* haplotypes among Indian Ocean bigeye tuna collections

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
among pops	4	0.101	0.0002	1.50
within pops	271	3.841	0.0142	98.5
Total	275	3.942	0.01439	

6.2.1.2. Indian and Atlantic Ocean heterogeneity

In the Atlantic Ocean, only 1 individual was identified as BET1 and 14 individuals were identified as BET2 (Table 4).

When bigeye individuals from the Atlantic Ocean collection were considered with those from the Indian Ocean, chi-square analysis unsurprisingly, given the data, detected highly significant heterogeneity (χ^2=208.717, P<0.001) among the oceans. All pairwise comparisons between the Atlantic collection and each Indian Ocean collection gave significant results (χ^2=26.319-102.532, P<0.001). This significant heterogeneity reflects the high frequency of the BET2 haplotype in the Atlantic Ocean samples (BET2=0.9333) while BET1 is the most frequent haplotype in the Indian Ocean collections (mean frequency BET1=0.985). AMOVA (Table 6) between the Indian Ocean collections and the Atlantic Ocean collection demonstrated that most of the variance is attributable to among ocean differences (96.14%, P<0.001) (Table 6) with a consequently very high F_{ST} value (0.962).

Table 6 AMOVA of *MseI* haplotypes between Indian and Atlantic Ocean bigeye tuna collections

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
among oceans	1	12.011	0.4214	96.14
among pops within oceans	4	0.101	0.0001	0.04
within pops	285	4.775	0.0167	3.82
Total	290	16.887	0.4383	

6.2.2. RsaI digestion

Within the Indian Ocean, the same collections as above, excluding Madagascar individuals which had poor PCR amplification and some individuals in the Indonesia and Seychelles collections which did not amplify or haplotypes were not visible, were also examined for *RsaI* diversity within the ATCO fragment. This analysis aimed to determine if significant population heterogeneity existed within this fragment. Two mtDNA haplotypes were observed; the α haplotype consisted of major bands of approximately 430 and 405 base pairs and the β haplotype consisted of major bands of approximately 405 and 370 base pairs (Chow and Inoue 1993; Chow *et al.* 2000) (Table 7) (Figure 2).

Table 7 Distribution of *RsaI* haplotypes from the ATCO mtDNA fragment in four Indian Ocean bigeye collections and one Atlantic collection. The haplotypes of the Madagascar collection could not be reliably resolved

Haplotype	Indonesia	Seychelles	west Indian Ocean	Western Australia	*Indian Ocean Total*	Atlantic Ocean
α	0	3	1	0	*4*	13
β	52	74	18	96	*240*	2
Total	52	77	19	96	*244*	15

6.2.2.1. Indian Ocean heterogeneity

As with the *MseI* haplotype analysis, no significant differences were detected in haplotype distribution among the Indian Ocean bigeye collections (χ^2=6.446, *P*=0.084) (frequency of mtDNA haplotypes - Indonesia *β*=1.000; Seychelles *β*=0.961; west Indian Ocean *β*=0.947; Western Australia *β*=1.000). In addition, analysis of variance among the Indian Ocean collections was not significant (F_{ST}=0.0203, *P*=0.070) (Table 8).

Table 8 AMOVA of *MseI* haplotypes among Indian Ocean bigeye tuna collections

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
among pops	3	0.104	0.0003	2.03
within pops	240	3.830	0.0159	97.97
Total	243	3.934	0.01439	

6.2.2.2. Indian and Atlantic Ocean heterogeneity

In the Atlantic Ocean, out of 15 individuals, 13 displayed the *α* haplotype while only 2 displayed the *β* haplotype (Table 7).

Very significant heterogeneity (χ^2=168.279, *P*<0.001) was observed once the Atlantic Ocean collection was introduced into the chi-square analysis. Pairwise comparisons between the Atlantic collection and each tested Indian Ocean collection gave significant results (χ^2=22.932-94.236, *P*<0.001). The *α* haplotype is most commonly

observed in the Atlantic Ocean (α=0.8670 while the β haplotype is observed in high frequencies in the Indian Ocean collections (β=0.947-1.000).

In the *RsaI* haplotype AMOVA between the Indian Ocean collections and the Atlantic Ocean collection, a very large and significant proportion of variance was attributable to among ocean differences (94.2%, P<0.001) (Table 9), with a very large F_{ST} value (0.943) observed among the oceans.

Table 9 AMOVA of *RsaI* haplotypes between Indian and Atlantic Ocean bigeye tuna collections

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
among oceans	1	10.216	0.3606	94.22
among pops within oceans	3	0.104	0.0002	0.06
within pops	254	5.564	0.0219	5.72
Total	290	16.887	0.4383	

6.2.3. Association between RsaI and MseI haplotypes

Most individuals with the BET1 haplotype (produced in the *MseI* digest) also displayed the β haplotype (produced by the *RsaI* digestion) and most BET2 individuals displayed the α haplotype. In two cases however, this association was not observed (west Indian Ocean where one individual displayed the BET1 and α haplotype; Atlantic Ocean where one individual displayed the BET2 and β haplotype). The two *MseI* haplotypes are however highly correlated with the two *RsaI* haplotypes as outlined above (r=0.937, P<0.05).

6.3. MtDNA for bigeye tuna population differentiation - D-loop fragment

The DL19-12S fragment (1.4kb in size) containing the variable D-loop was only amplified successfully in individuals from Western Australia, Seychelles, west Indian Ocean and Atlantic Ocean collections. Digestion of this fragment with the double digest produced six different banding patterns and the single *TaqI* digest resulted in eight

different banding patterns. In combination, this resulted in 16 different composite haplotypes (Table 10). Amplification of this large fragment from individuals in the Indonesia and Madagascar collections was unsuccessful even after several PCR attempts.

Table 10 mtDNA composite haplotype frequencies among bigeye tuna collections based on examination of the DL19-12s fragment using *HinfI & BglI* and *TaqI*

Composite haplotype*	Seychelles	west Indian Ocean	Western Australia	*Indian Ocean Total*	Atlantic Ocean
AP	23	3	34	*60*	3
AR	3	2	8	*13*	4
AS	14	6	19	*39*	2
BP	18	1	12	*31*	1
CP	4	-	2	*6*	-
BR	6	1	5	*12*	2
AN	1	-	1	*2*	-
BS	2	-	3	*5*	1
AQ	9	2	7	*18*	1
DP	-	3	1	*4*	-
BO	-	-	1	*1*	-
BN	-	-	1	*1*	-
AT	-	-	1	*1*	-
EP	-	-	1	*1*	1
BU	2	-	-	*2*	-
FT	1	1	-	*1*	-
Total fish	83	19	96	*198*	15

*composite haplotype=first haplotype produced by double digest of *HinfI & BglI* e.g., "A", second haplotype produced by *TaqI* digest e.g., "P".

Analysis of overall differentiation of the composite haplotypes was not significant among the three Indian Ocean collections (χ^2=44.850, P=0.052). Differentiation was also not significant between the three Indian Ocean collections and the Atlantic Ocean collection (χ^2=64.374, P=0.0564) nor between the pooled Indian Ocean collections compared with the Atlantic collection (χ^2=17.740, P=0.314).

Chi-square analysis was also undertaken on combined *MseI/RsaI* haplotypes with DL19-12S composite haplotypes in the Atlantic Ocean, Seychelles, west Indian Ocean and Western Australia collections to determine if the D-loop haplotypes were randomly distributed with respect to the ATCO haplotypes. The composite DL19-12S haplotypes were not significantly associated with the ATCO haplotypes (χ^2=35.483, P=0.093).

6.4. Microsatellite loci

The seven microsatellite loci used were a mixture of perfect (CA) and imperfect or mixed repeat motifs. Allele frequencies at the seven microsatellite loci for confirmed

bigeye tuna are given in Appendix 3. Four (locus *125*) to 31 (locus *113*) alleles were detected at the seven loci. Loci *125* and *144* produced relatively "clean" banding patterns, generally free of subbanding or "stuttering". Locus *208* was characterised by a slight stuttering in the allele banding pattern while loci *102, 113, 121* and *161* produced quite severe stutter bands. Dinucleotide repeats, as used in the current study, are often characterised by stuttering. Stuttering may be caused by slipped strand mispairing during PCR (Tautz 1989). This laddering of bands can result in difficulties in allele scoring and while minimised using fluorescent-labelled primers and analysis on the ABI377 DNA sequencer, was still sometimes a problem in the current study. Of the seven loci, loci *102, 113* and *121* produced the largest number of band stutters that could have led to inaccurate allele scoring in some instances.

Genetic diversity statistics for the Indian Ocean collections were estimated by the numbers of alleles per loci and observed and expected heterozygosity per locus and per collection (Tables 11 and 12). The Atlantic Ocean diversity statistics are also given, but the overall Indian Ocean diversity estimates exclude the Atlantic Ocean data. All seven microsatellite loci examined were highly polymorphic in all collections.

Locus *113* had the highest number of alleles present in all collections (mean of 25.4 alleles per collection) while locus *125* showed the lowest number of alleles (mean of 3.8 alleles). Total numbers of alleles per locus per collection ranged from 3 to 29 (Table 11) with an average of 14.9 alleles/locus across the five collections. The less variable loci, *125* and *144* (3-8 alleles), had more common alleles of higher frequencies, while the remaining more variable loci did not have any alleles with frequencies greater than 0.300 (Appendix 3).

Not unexpectedly, loci *102* (number of alleles=21), *113* (number of alleles=31), *121* (number of alleles=22) and *161* (number of alleles=24) demonstrated the highest mean observed heterozygosities across all collections (0.961, 0.906, 0.937 and 0.982 respectively) while locus *125* had the lowest mean observed heterozygosity (0.420).

Table 11 Summary of genetic variability data per locus in each bigeye tuna collection

Collection		Loci *102*	*113*	*121*	*125*	*144*	*161*	*208*
Atlantic Ocean	N	15	14	15	15	15	15	15
	Nallele	15	17	13	3	5	14	9
	H_{obs}	0.933	0.929	0.933	0.400	0.867	1.000	1.000
	H_{exp}	0.938	0.963	0.926	0.402	0.637	0.947	0.876
	D[a]	-0.005	-0.035	0.008	-0.005	0.361	0.056	0.142
Indonesia	N	89	89	91	91	91	90	91
	Nallele	20	28	19	4	8	21	14
	H_{obs}	0.978	0.899*	0.945	0.385	0.626	0.944	0.802
	H_{exp}	0.942	0.940	0.910	0.343	0.608	0.925	0.797
	D[a]	0.038	-0.044	0.038	0.119	0.030	0.021	0.008
Madagascar	N	52	51	53	53	53	32	53
	Nallele	18	24	18	4	6	19	14
	H_{obs}	0.981*	0.784*	0.981	0.396	0.755	1.000	0.925
	H_{exp}	0.934	0.926	0.922	0.353	0.674	0.945	0.804
	D[a]	0.050	-0.143	0.064	0.122	0.120	0.058	0.150
Seychelles	N	88	88	87	88	86	87	87
	Nallele	20	29	20	3	6	22	11
	H_{obs}	0.932	0.977*	0.989	0.500	0.744	1.000	0.874
	H_{exp}	0.940	0.952	0.924	0.400	0.633	0.927	0.774
	D[a]	-0.009	0.026	0.070	0.250	0.175	0.079	0.129
west Indian Ocean	N	19	19	19	19	19	19	19
	Nallele	14	18	14	4	5	14	11
	H_{obs}	0.947	0.947	0.842	0.474	0.842	1.000	1.000*
	H_{exp}	0.943	0.954	0.935	0.431	0.716	0.937	0.848
	D[a]	0.004	-0.007	-0.010	0.100	0.176	0.067	0.179
Western Australia	N	96	94	96	96	96	92	96
	Nallele	20	28	19	4	6	24	14
	H_{obs}	0.969	0.926	0.927	0.344	0.708	0.967	0.813
	H_{exp}	0.940	0.952	0.921	0.311	0.665	0.944	0.789
	D[a]	0.031	-0.027	0.007	0.106	0.065	0.024	0.034
Mean[b]	N	68.8	68.2	69.2	69.4	69.0	64.0	69.2
	Nallele	18.4	25.4	18.0	3.8	6.2	20.0	12.8
	H_{obs}	0.961	0.906	0.937	0.420	0.735	0.982	0.883
	H_{exp}	0.940	0.945	0.922	0.368	0.660	0.936	0.802

N=total number of fish, Nallele=number of alleles

H_{obs}=observed heterozygosity, H_{exp}= expected heterozygosity under Hardy-Weinberg expectations (Nei 1978)

[a]Selanders index of heterozygote deficiency/excess $D=(H_o-H_e)/H_e$ (Koehn *et al.* 1973), is similar to Wright (1978) fixation index F_{IS} where negative values indicate a heterozygote deficiency and positive values a heterozygote excess

[b]Indian Ocean collections only

*significant deviation from Hardy-Weinberg equilibrium after Bonferroni correction

Nei's (1978) unbiased estimate of expected heterozygosity per locus (under Hardy-Weinberg expectations) for each of the collections ranged from 0.311 (locus *125*) to 0.954 (locus *113*) (within the Western Australia and west Indian Ocean collections

respectively) (Table 11). An average Hardy-Weinberg expected heterozygosity level of between 0.368 (locus *125*) to 0.945 (locus *113*) was demonstrated across the five Indian Ocean collections.

Genotype proportions in each Indian Ocean collection for each locus were tested for goodness-of-fit to Hardy-Weinberg expectations (Table 11). There were five tests (five collections) of goodness-of-fit for each locus (seven loci). Five of the 35 tests showed a significant deviation after Bonferroni correction for multiple tests (corrected α_b significance value of 0.05/35=0.0014). These were locus *113* in Indonesia, loci *102* and *113* in Madagascar, locus *113* in Seychelles and locus *208* in the west Indian Ocean.

Overall, there was some evidence of a small heterozygote excess. Of the total of 35 tests, 29 showed a heterozygote excess and 6 a homozygote excess (χ^2=7.84, df=1, *P*=0.01). Three of the five significant deviations from Hardy Weinberg showed a heterozygote excess, two a homozygote excess. In general, collections accorded well with Hardy-Weinberg expectations.

Linkage disequilibrium tests using a Markov chain to determine significance between pairs of microsatellite loci demonstrated no significant linkage disequilibrium (after Bonferroni correction) in any collection. We conclude that the seven loci are not linked and that they represent independent genetic markers.

Overall, the mean numbers of alleles per locus among the collections ranged from 11.4 (west Indian Ocean) to 16.5 (West Australia) (Table 12). Mean number of alleles per locus was highly correlated with sample size (r=0.968, *P*<0.01). This is unsurprising given that many alleles are rare and the larger the sample size the more rare alleles are expected to be detected. Average observed heterozygosity per locus in all collections was high (0.797-0.865).

Table 12 Genetic variability at seven loci in all bigeye tuna collections (standard errors in parenthesis)

Collection	Mean sample size per locus	Mean number alleles per locus	Heterozygosity observed	Heterozygosity expected[b]
Atlantic Ocean	14.9 (0.1)	10.9 (2.0)	0.866 (0.08)	0.813 (0.08
Indonesia	90.3 (0.4)	16.3 (3.1)	0.797 (0.08)	0.781 (0.09)
Madagascar	49.6 (2.9)	14.7 (2.8)	0.832 (0.08)	0.794 (0.07)
Seychelles	87.3 (0.3)	15.9 (3.6	0.859 (0.07)	0.793 (0.07)
west Indian Ocean	19.0 (0.0)	11.4 (2.0)	0.865 (0.07)	0.823 (0.06)
Western Australia	95.1 (0.6)	16.5 (3.4)	0.808 (0.09)	0.789 (0.08)

[b]unbiased Nei (1978) estimate of Hardy-Weinberg expectations

6.4.1. Indian Ocean heterogeneity

Exact tests of heterogeneity of allele frequencies at the seven loci in the five Indian Ocean collections were undertaken. There was no evidence for spatial heterogeneity of allele frequencies at any locus (Table 13). Additionally, genotypic distribution at each of the seven loci among the five collections was assessed in a G-like test using Markov chain resampling (4000 iterations per batch). After Bonferroni correction for multiple tests (0.05/7=0.007), no significant differences were detected between genotypic frequencies in the Indian Ocean collections (Table 13).

An overall exact test of collection differentiation based on total allele frequencies (Markov chain, significance determined after 100 000 steps) similarly showed no significant allele frequency differences among the Indian Ocean collections (exact P=1.000). Likewise, F_{ST} values indicated very low levels of genetic differentiation across the seven microsatellite loci over different geographic locations (Table 13). F_{ST} values per locus ranged from essentially zero to 0.006 with an average of only 0.002. Pairwise comparisons based on collection F_{ST} values produced no significant comparisons between any of the Indian Ocean collections. F_{IS} values were also generally small, indicating random mating within each of the five bigeye tuna collections.

Table 13 Exact tests of analysis for allelic and genotypic differentiation and F statistics at seven microsatellite loci in five Indian Ocean bigeye tuna collections, *P* values based on Markov chain resampling with 4000 iterations per batch

Locus	No. of fish	Total no. alleles	Allele P^a	GenotypeP^b	F_{IS}^c	F_{ST}^c	F_{IT}^c
102	344	20	0.385	0.310	0.024	0.000	-0.024
113	341	32	0.071	0.159	0.035	0.002	0.038
121	346	22	0.423	0.034	-0.034	0.000	-0.035
125	347	4	0.122	0.087	-0.186	0.006	-0.179
144	345	8	0.124	0.137	-0.099	0.000	-0.099
161	320	24	0.386	0.319	-0.045	0.002	-0.043
208	346	18	0.315	0.232	-0.077	-0.003	-0.081

[a] *P* value of allelic differentiation
[b] *P* value of genotypic differentiation
[c] F statistics are estimated as in Weir and Cockerham (1984)

An analysis of variance among the Indian Ocean collections showed all variation observed could be attributable to within collection differences with an overall non-significant F_{ST} value of –0.0038 (*P*=1.000) (significance determined after 16 000 permutations) (Table 14). If the Indian Ocean collections are divided into two groups to reflect bigeye occurring in the equatorial group (approximately 0^0) and the southern group (between 25^0S and 35^0S) (Kume *et al.* 1971), all observed variance differences can still be attributed to within collection differences; no significant differences were detected between the groups (F_{ST}= –0.0036, *P*=1.000).

Table 14 AMOVA of Indian Ocean bigeye tuna collections across seven microsatellite loci

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
among pops	4	5.210	-0.01014	0
within pops	691	1831.280	2.65019	100
total	695	1836.490	2.64005	

When each locus was treated individually within the AMOVA analysis, all F_{ST} values at the seven loci were non-significant among the Indian Ocean bigeye tuna collections; hence the overall AMOVA among bigeye tuna collections in the Indian Ocean was non-significant.

Genetic distances (estimated using Nei (1978) unbiased genetic distance) between the five Indian Ocean collections were all, as expected from their allele similarities, very small (Table 15); the average Nei pairwise distance was 0.018 (standard error 0.054). A derived dendrogram of bigeye tuna collection relationships was produced from Nei's unbiased genetic distance estimates using cluster analysis and the unweighted pair-group method with arithmetic averaging (UPGMA). Very close relationships between all bigeye tuna collections were observed (Figure 3).

Table 15 Matrix of genetic distance coefficients between bigeye collections in the Indian Ocean, below diagonal = Nei (1978) unbiased genetic distance.

Collection	Indonesia	Madagascar	Seychelles	west Indian Ocean	Western Australia
Indonesia	*****				
Madagascar	0.0165	*****			
Seychelles	0.0118	0.0167	*****		
west Indian Ocean	0.0219	0.0184	0.0217	*****	
Western Australia	0.0159	0.0179	0.0161	0.0225	*****

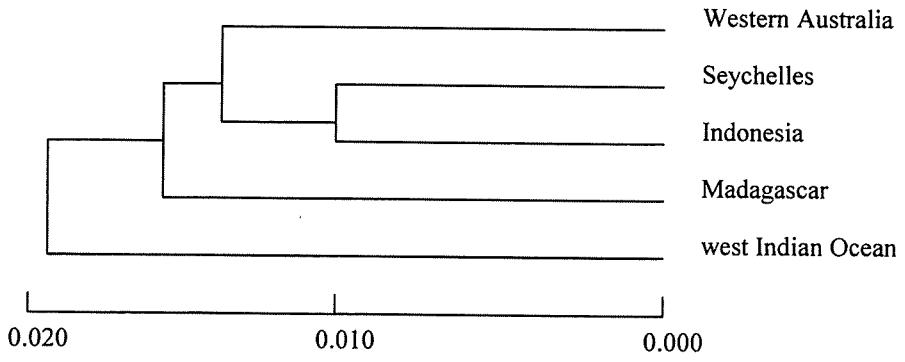

Figure 3 Genetic relationships between Indian Ocean bigeye tuna collections based on Nei's (1978) unbiased genetic distance and clustered using UPGMA.

6.4.2. Indian and Atlantic Ocean heterogeneity

Exact tests of heterogeneity of allele frequencies at the seven loci among the six bigeye collections were undertaken. The aim was to determine if the significant

population heterogeneity observed in the mtDNA haplotypes between the Indian and Atlantic Ocean collections was reflected in nuclear DNA microsatellite markers. There was significant evidence for spatial heterogeneity of allele frequencies at one locus, locus *208* (Table 17). This remained so after Bonferroni correction for multiple tests (α=0.05/7=0./007). This locus was also the only locus to show significant differences in genotype frequencies across these six collections (Table 17).

Table 17 Exact tests of analysis for allelic and genotypic differentiation and F statistics of seven microsatellite loci in six bigeye tuna collections, *P* values based on Markov chain resampling with 4000 iterations per batch.

Locus	No. of fish	Total no. alleles	Allelic P^a	Genotypic P^a	F_{IS}	F_{ST}	F_{IT}
102	357	20	0.437	0.349	-0.023	0.000	-0.023
113	352	32	0.109	0.242	0.035	0.002	0.037
121	361	22	0.461	0.384	-0.033	0.000	-0.034
125	362	4	0.142	0.103	-0.184	0.004	-0.178
144	360	8	0.222	0.242	-0.110	-0.001	-0.111
161	327	24	0.627	0.541	-0.046	0.001	-0.045
208	361	19	**0.001**	**0.001**	-0.081	0.003	-0.078

[a]significant *P* value of allelic differentiation after Bonferroni correction for multiple tests shown in bold
[b]significant *P* value of genotypic differentiation after Bonferroni correction for multiple tests shown in bold

A test of the pooled Indian Ocean collections at locus *208* and the Atlantic Ocean collection was also significant (χ^2=87.999, *P*=0.0006). Furthermore, exact tests of pairwise comparisons between each of the collections at locus *208* demonstrated that the Atlantic Ocean collection was significantly different to all Indian Ocean collections except the small west Indian Ocean sample (n=19) at this locus (Table 18).

Table 18 Exact test of allelic frequency differentiation at locus *208* between Indian and Atlantic Ocean bigeye collections, bolded *P* values are significant after probability of H_0 calculated from 4000 iterations of a Markov chain (and following Bonferroni correction for 15 multiple tests, α becoming 0.05/15=0.0033)

Collection	Atlantic	Indonesia	Madagascar	Seychelles	west Indian Ocean	Western Australia
Atlantic	*****					
Indonesia	**0.00023**	*****				
Madagascar	**0.00018**	0.580	*****			
Seychelles	**0.00001**	0.301	0.732	*****		
west Indian Ocean	0.040	0.344	0.418	0.178	*****	
Western Australia	**0.00017**	0.645	0.335	0.068	0.330	*****

An overall exact test of population differentiation based on total allele frequencies among the five Indian Ocean collections and the Atlantic Ocean collection (Markov chain, significance determined after 100 000 steps) showed, however, no significant allele frequency differences (exact P=1.000) when the seven loci are considered together. Additionally, pairwise collection comparisons based on overall F_{ST} values for each collection did not produce any significant comparisons (after Bonferroni correction) between the Atlantic Ocean and the other six Indian Ocean collections.

An analysis of variance among two bigeye groups (Indian Ocean collections versus the Atlantic Ocean collection) showed nearly all variation observed could be attributable to within collection differences with an overall non-significant F_{ST} value between oceans of 0.0021 (P=1.000) (significance determined after 16 000 permutations) (Table 19).

Table 19 AMOVA of Indian and Atlantic Ocean bigeye tuna collections across seven microsatellite loci

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
among oceans	1	3.194	0.01567	0.59
among pops within oceans	4	5.210	-0.01017	-0.38
within pops within oceans	720	1911.180	2.65442	99.79
Total	725	1919.584	2.65991	

Nei's (1978) unbiased genetic distances between the six bigeye collections were again small; the average Nei pairwise distance was 0.038 (standard error 0.071) (data not shown). A UPGMA dendrogram (Figure 4) showed that the Indian Ocean collections clustered together with the same topology as Figure 3; the Indian Ocean cluster branched as quite a tight cluster off from the Atlantic Ocean collection. The structure of this dendrogram supports the suggestion from locus 208 that there are some small differences between the Indian Ocean collections and that from the Atlantic.

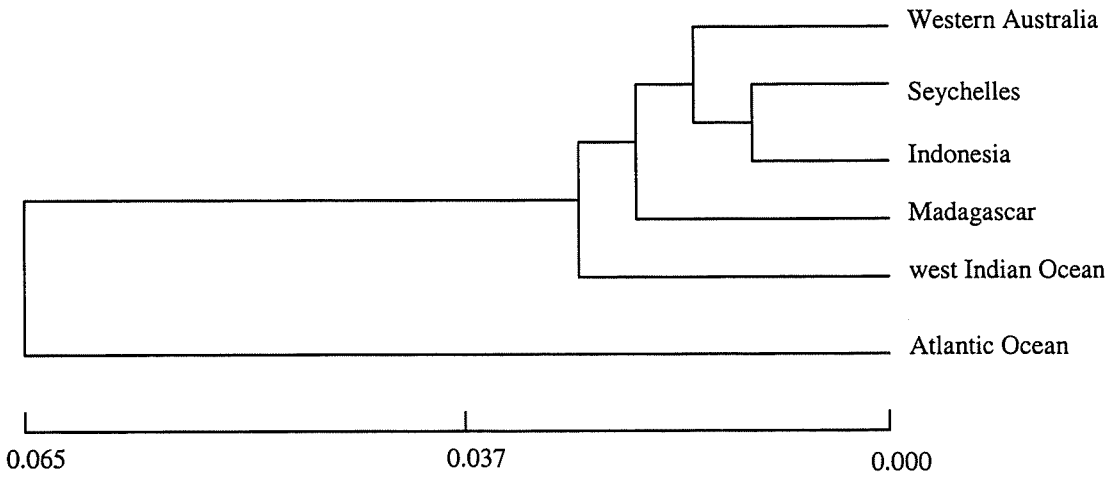

Figure 4 Genetic relationships among Indian and Atlantic Ocean bigeye tuna collections based on Nei's (1978) unbiased genetic distance and clustered using UPGMA

6.4.3. Testing for east-west heterogeneity in the Indian Ocean

The foregoing tests of genetic heterogeneity failed to reveal any overall heterogeneity among the collections from the Indian Ocean. The null hypothesis of panmixia could not be rejected. However, reading the admittedly limited information available on distribution of bigeye tuna together with tagging returns in this ocean, suggested a different null hypothesis should be tested, that of an eastern and western population.

Catch data show that while bigeye tuna are broadly distributed in the Indian Ocean between latitudes 25^0N and 40^0S; the highest catches tend to be in the eastern and western tropical regions and, to a lesser extent, in the southern high latitude regions (Kume *et al.* 1971; Mohri *et al.* 1991; Larcombe *et al.* 1997). Catches are certainly not uniform throughout the Indian Ocean, indicating that bigeye tuna are more abundant in some areas than others. There appears to be some evidence of a separation into east and west components, at least in the tropical regions, although catch rate (catch per 1000 hooks) appears to be relatively uniform (Mohri *et al.* 1991). Gonad indices show that it is this equatorial group that is sexually active, rather than the more southerly, feeding, group, and there is some evidence sexual activity is concentrated in western and central parts of the Indian Ocean, and an eastern region off Indonesia (Kume *et al.* 1971). Limited tagging data (6,000 releases, 45 recaptures) focused on the equatorial west and east Indian Ocean showed some long-distance (as expected for these highly mobile large tuna) but no trans-ocean movements (Nishida *et al.* 2000). It would be a mistake to place too much faith on recaptures of so few fish – a larger-scale tagging program is clearly required – but both the tagging and catch data suggest the possibility of some restriction on gene flow between bigeye tuna of the eastern and western regions of the Indian Ocean. This then appeared to us to be a possibility worth testing.

With this in mind, it is interesting that the rare ATCO mtDNA haplotypes, BET2 for *MseI* and α for *RsaI*, were only observed in collections from the western Indian Ocean; the eastern collections being monomorphic for BET1 and β. Pooling collections into western (Seychelles, west Indian Ocean, Madagascar) and eastern (Indonesia, Western Australia) regions reveals significant differentiation for both sets of digests (P=0.038 for *MseI* and P= 0.010 for *RsaI*). While the very pronounced association between the haplotypes produced by these two enzymes means that these are not independent tests, this is an indication of some trans-Indian Ocean difference. Chow *et al.* (2000) also examined ATCO/*RsaI* haplotypes in bigeye from one area of the Indian Ocean, an eastern region, and all 51 individuals showed the β haplotype. Adding these to our eastern data set gives marginally more significant east-west differentiation (P falling from 0.010 to 0.008). This apparent east-west differentiation is not, however, reflected in the D-loop region of mtDNA (P=0.550).

Tests of microsatellite variation show some evidence of east-west differentiation in allele frequencies (locus *125*, P=0.005; locus *144*, P=0.313; locus *161*, P=0.324; locus *208*, P=0.053; locus *102*, P=0.373, locus *113*, P=0.452, locus *121*, P=0.651; over all loci, P=0.029), although an AMOVA test was non-significant. Locus *125* showed the most pronounced differentiation and was the only one to retain significance after Bonferroni correction for multiple tests. At this locus, allele 158 was about twice as frequent in western collections (frequencies 0.142 to 0.188) as in eastern collections (0.082, 0.094; see Appendix).

To summarise: genetic variants in the ATCO region of mtDNA and one microsatellite locus support the hypothesis of a restriction on gene flow between western and eastern parts of the Indian Ocean, while variants in the d-loop region of mtDNA and six other microsatellite do not support this hypothesis. Clearly it would be premature at this time to state unequivocally that there are two population groups within the Indian Ocean, but it would be equally wrong to state unequivocally that the Indian Ocean population is a single panmictic population. More data are urgently required to resolve these issues; we recommend that sample sizes be increased to 200 fish per sample and that additional sites are sampled within the Indian Ocean.

7. DISCUSSION

In the current study, we used genetic variation in two mtDNA gene fragments and seven dinucleotide microsatellite loci to investigate the population structure of bigeye tuna within the Indian Ocean basin. The five Indian Ocean collections were almost fixed for a single mtDNA haplotype in the ATCO fragment but greater diversity was identified in the DL19-12S fragment. There was considerable microsatellite variation in all collections with an overall mean number of alleles per locus of 14.9 and overall mean observed heterozygosity of 0.832.

7.1. Bigeye tuna stock structure within the Indian Ocean

Restriction fragment length polymorphism (using both *MseI* and *RsaI*) of the ATCO fragment of the mitochondrial genome in bigeye from the Indian Ocean revealed low levels of variation within the region; only two haplotypes were detected with either of the restriction enzymes. BET1 and BET2 haplotypes were detected with the *MseI* enzyme and α and β haplotypes with the *RsaI* enzyme. There was no significant haplotype differentiation among the Indian Ocean collections; the five collections showed an average of 98% of BET1 and 97% of β haplotypes.

Variation found within the other mtDNA fragment (containing the typically highly variable D-loop) was much higher than that found within the ATCO fragment. Sixteen composite haplotypes were detected, although again there was no significant heterogeneity among the Indian Ocean collections.

High levels of microsatellite variation were detected in all Indian Ocean bigeye tuna collections, but results from the seven loci did not support population sub-structuring within the Indian Ocean. These analyses did not permit us to reject the null hypothesis of a single panmictic population in this ocean.

A few deviations from Hardy-Weinberg expectations were observed, with some evidence of a small overall excess of heterozygotes. We consider that these excesses are more likely to reflect band-scoring difficulties of loci that produce stutter bands (a few homozygotes being mistakenly scored as heterozygotes) than reflecting any real

biological phenomenon. No linkage disequilibrium was detected between pairs of microsatellite loci in each of the five bigeye collections, and it was considered that the microsatellite loci represented independent markers.

For future applications in fisheries stock structure, development and analysis of tri and tetranucleotide repeat microsatellite loci should help to reduce the levels of band stuttering observed and therefore reduce possible mis-identification of alleles and genotypes. Tetranucleotide repeats are also known to amplify more faithfully than dinucleotide repeats (Hughes and Queller 1993) although they are more difficult to isolate and clone than GT or CT repeats (O'Reilly and Wright 1995; Paetkau and Strobeck 1995).

As in the mtDNA analysis, individuals from the Western Australian collection were not different genetically from those caught in the greater Indian Ocean. Statistical analysis of neither the Indian Ocean mtDNA haplotype or microsatellite allele frequencies showed any overall significant differentiation among collections.

The statistical analyses discussed above indicate that we are unable to reject the hypothesis of panmixia of bigeye tuna within the Indian Ocean. However, inspection of catch, gonad indices and tagging data (Kume *et al.* 1971; Mohri *et al.* 1991; Larcombe *et al.* 1997) suggested that another hypothesis would be worth testing, that of a possible east-west separation. Subsequent pooling of collections into eastern and western regions did produce evidence of differentiation for the ATCO region of mtDNA and for one microsatellite locus, but not for the D-loop region of mtDNA nor for six other microsatellite loci.

In conclusion, overall tests of spatial heterogeneity among five Indian Ocean collections of bigeye tuna gave no evidence for significant differentiation. However, pooling collections into eastern and western regions did produce some evidence for east-west population differentiation. A larger study, with sample sizes of around 200 per collection and with more collections, is required to confirm (or refute) the suggestion of east-west differentiation of the present study, and to clarify genetic connections between Indian Ocean and Atlantic Ocean bigeye tuna. Further work with other methodologies, especially tagging, is needed to confirm stock structure and management units.

7.2. Indian and Atlantic Ocean heterogeneity

Atlantic Ocean bigeye are known to be well-differentiated from Indo-Pacific bigeye for several mtDNA markers (Alvarado-Bremer *et al.* 1998, Chow *et al.* 2000). The α haplotype for *RsaI*, rare in the western Indian ocean and (currently) unrecorded from the eastern Indian ocean, is in fact more abundant than the β haplotype in Atlantic bigeye (α frequency of 0.64 to 0.78, Chow *et al.* 2000). There is genetic evidence that the Atlantic and Indian Ocean stocks mix around the Cape of Good Hope (Alvarado-Bremer *et al.* 1998, Chow *et al.* 2000). The rare α haplotype individuals we have observed in the western Indian Ocean may represent migrants from the Atlantic into a panmictic Indian Ocean population or remnants of past genetic exchange between the two oceans coupled with very limited exchange between east and west Indian ocean bigeye populations. These two hypotheses could be differentiated if that were an effective nuclear DNA (nDNA) marker that permitted separation of Atlantic and Indo-Pacific bigeye in the same way as the ATCO mtDNA region. Association of the mtDNA and nDNA markers in western Indian Ocean fish would argue for mixing without inter-mating of Atlantic and Indian Ocean derived fish, the Atlantic-derived fish returning to the Atlantic to spawn; no association would argue for historical gene flow between bigeye tuna of the two oceans.

Variation found within the other mtDNA fragment, that containing the D-loop, was much higher than that found within the ATCO fragment. Significant heterogeneity between the Indian and Atlantic Ocean collections was, however, not detected in this fragment. In contrast, Chow *et al.* (2000) did detect very significant heterogeneity between the Atlantic and Indo-Pacific samples (but not among either the Atlantic or Indo-Pacific Ocean samples) in the D-loop. However, we used different restriction enzymes (*HinfI*, *BglI* and *TaqI*) to those used by Chow *et al.* (2000) (*DpnII* and *RsaI*) and therefore detected different cut site variation. Chow *et al.* (2000) found that the ATCO segment was a more powerful discriminator of Atlantic and Indo-Pacific fish than the D-loop, which we certainly also find.

With respect to the microsatellite loci, only one locus of the seven showed significant heterogeneity of allele frequencies between the Indian and Atlantic Oceans (locus *208*). While the dendrogram of genetic collection relationships did suggest that the

Atlantic collection was distinguishable from the Indian Ocean collections, an overall AMOVA based on all seven microsatellite loci does not support significant differentiation among these collections. This is in direct contrast with the results of the mtDNA analyses using the ATCO fragment (and Chow *et. al*'s (2000) data from the D-loop) which indicate very restricted gene flow between the Indian and Atlantic Ocean bigeye tuna populations.

The studies of Chow *et al.* (2000) and Alvarado-Bremer *et al.* (1998) coupled with the current study demonstrate that, at least as far as mitochondrial DNA is concerned, there is significantly restricted gene flow between bigeye tuna found within the Indian (Indo-Pacific) Ocean and the Atlantic Ocean. The current study did not investigate possible areas of mixing between the bigeye populations, but Chow *et al.* (2000) have concluded that fish from these distinct stocks are mixing around South Africa. In the Atlantic Ocean collection, the predominantly "Indian Ocean " haplotypes of BET1 and β were found at a rate of 6.6% and 13.3% respectively. Additionally, six of the seven microsatellites detected no significant heterogeneity among the Indian and Atlantic Ocean collections, while significant heterogeneity was detected at locus *208*. These findings suggest that gene flow between populations of bigeye found in the Indian and Atlantic Oceans is small but very limited. Similarly Alvarado-Bremer *et al.* (1998) demonstrated that a major mtDNA clade is observed in both the Indo-Pacific and Atlantic Ocean samples but that a second clade only occurs almost entirely in the Atlantic samples.

Although there are no physical barriers to bigeye mixing between the Indian and Atlantic Oceans (water transport between the Indian and Atlantic Oceans is summarised in Tomczak and Godfrey (1994)), Chow *et al.* (2000) suggest that the currents around South Africa must affect bigeye within each ocean basin. Fish migration from the Indian to Atlantic Oceans maybe facilitated by the unidirectional warm water from the Indian Ocean to the southeast Atlantic Ocean along the west coast of Africa by the Agulhas current (Shannon *et al.* 1990; Alvarado-Bremer *et al.* 1998).

Differentiation observed in (maternally inherited) mtDNA haplotypes but not in (biparentally inherited) nuclear DNA markers such as microsatellites may reflect females returning to their place of origin for reproduction. This could be an explanation of our data. Based on tagging data, bigeye tuna in the Atlantic Ocean are known to display strong philopatric behaviour towards their only known natal breeding grounds (Alvarado-

Bremer *et al.* 1998) in an area between 15^0N and 15^0S (ICCAT 1997). However, this explanation of the contrast between our mtDNA and nDNA data requires that females are strongly more philopatric than males, and we are not aware of any data that suggests this might be so. However, sex biased dispersal in marine mammals has been used to explain significant mtDNA heterogeneity observed between ocean basins. In harbour porpoises (*Phocoena phocoena*), strong female philopatry results in significant geographical heterogeneity in the north-west Atlantic Ocean in mtDNA sequences, while male mediated gene flow may maintain microsatellite homogeneity (Rosel *et al.* 1999). Likewise, significant differences in mtDNA sequences but not microsatellite loci exist among sperm whales (*Physeter macrocephalus*) of different oceans, suggesting sex differential interoceanic movements (Lyrholm *et al.* 1999). Sex biased dispersal has also been argued to account for differences in patterns of mtDNA and nuclear markers in humpback whales in the north Pacific (Palumbi and Baker 1994) and fin whales (*Balaenoptera physalus*) in the north Atlantic Ocean and Mediterranean Sea (Berube *et al.* 1998).

Other mtDNA studies in which significant levels of haplotype differentiation between Indo/Pacific and Atlantic populations have been detected in large pelagic fishes include blue marlin (*Makaira nigricans*) (Finnerty and Block 1992), swordfish (*Xiphias gladius*) (Alvarado-Bremer *et al.* 1996; Rosel and Block 1996; Chow *et al.* 1997), albacore (*T alalunga*) (Chow and Ushiama 1995) and sailfish (*Istiophorus platypertus*) (Graves and McDowell 1995). Female fidelity to spawning grounds is one hypothesis proposed to account for the haplotype differentiation in several of these studies (Graves and McDowell 1995; Rosel and Block 1996).

7.3. Indian and Pacific Ocean heterogeneity

An objective of the current study was to investigate the wider population structure of bigeye tuna. As such, previous studies in the literature were examined and genetic data on Pacific Ocean bigeye tuna were examined in parallel with data obtained in the current study.

In earlier catch rate studies by Kume *et al.* (1971), very little relationship between bigeye tuna found within the Indian and Pacific Oceans was assumed. Subsequently, Alvarado-Bremer *et al.* (1998) and Chow *et al.* (2000) detected no mtDNA genetic differences between samples from the Indian and Pacific Oceans. No mtDNA or microsatellite differences were detected between east and west Pacific bigeye collections (Grewe and Hampton 1998).

Examination of the ATCO fragment in Pacific Ocean bigeye tuna collections using *MseI* digests also revealed two restriction patterns (Grewe and Hampton 1998), plus a third diagnostic pattern for yellowfin tuna. One of the two bigeye patterns was rare, being present in only 2.1% and 1% of individuals from Hawaii and Philippines respectively. This rare haplotype in the Pacific Ocean bigeye individuals is BET2, similarly rare in the Indian Ocean but dominant in Atlantic Ocean bigeye tunas.

Grewe and Hampton (1998) also undertook a mtDNA assessment of the DL19-12S fragment in Pacific Ocean bigeye using the same double digest and *TaqI* enzyme as in the current study. They found up to 33 composite haplotypes and analysis of overall diversity among the Pacific Ocean collections bordered on significance (P=0.046). Likewise in the current study, the diversity in this fragment among the Indian Ocean collections approached significance (P=0.052) and was close to significance between the Indian and Atlantic Ocean populations (P=0.056). As in the current study, the three most frequent composite haplotypes in Pacific bigeye (frequencies of at least 0.05 in all collections) were AS, AP and BP. A chi-square test of heterogeneity between the Indian and Pacific Ocean bigeye collections based on these most frequent haplotypes was not-significant (χ^2=27.329, P=0.194) and not significant among the Indian, Pacific and Atlantic Ocean bigeye collections (χ^2=27.699, P=0.276).

Although direct microsatellite loci comparisons between the current study and that of Grewe and Hampton (1998) is difficult (due to slightly different allele scoring and the use of modified primers for loci *125* and *208*), the same trends in the data were obtained in both bigeye studies. Generally, the same sets of primers and loci were used in both studies (primers used in the current study had previously been designed in the CSIRO Marine Research laboratory, Grewe unpublished data). Slight differences in allele sizes can be attributed to differences in determining the actual bin size of each allele class but

generally the same numbers of alleles (sample size dependent) were identified in both studies (Indian Ocean: Pacific Ocean; *102* 21:24, *113* 31:30, *121* 22:21, *125* 4:4, *144* 8:5, *161* 24:24, *208* 19:19). As with the Indian Ocean collections, chi-square analysis of differentiation among nine Pacific Ocean bigeye collections was not significant at four loci (125a, *144*, *161*, *208a*) and not significant at eight loci (*102*, *113*, *117*, *121*, *125a*, *144*, *161*, *208a*) between the Philippines and Ecuador bigeye collections.

Chow *et al.* (2000) propose that a water pathway between the Indian and Pacific Oceans (via the Indonesian throughflow) transports the north and south Pacific waters to the Indian Ocean through the Australasian Mediterranean Seas. Bigeye larvae and juveniles from the western tropical Pacific may be transported to the Indian Ocean (Chow *et al.* 2000) via this pathway. This is the likely explanation of the genetic similarities between Indian Ocean and Pacific Ocean collections.

7.4. Use of different genetic techniques

Stock structure in the current study was assessed using both nuclear and mitochondrial DNA markers. The variability of these markers differed markedly with only two haplotypes per restriction enzymes observed in the conserved mtDNA fragment yet up to 31 alleles at microsatellite loci. The issue of the number of alleles observed at a locus is important. Ferguson and Danzmann (1998) suggest that genetic marker systems such as microsatellites which are characterised by large numbers of alleles may not be suitable for detecting significant differences between genetically similar populations, at least with the sample sizes typically employed in such studies. Several loci in bigeye tuna have more than twenty alleles segregating with some very low allelic frequencies. The large number of alleles at these microsatellite loci suggests that a larger sample size (>200) may be needed to confirm or identify any small but significant levels of genetic differentiation. While a high proportion of microsatellite loci screened in fish are polymorphic (O'Connell and Wright 1997; Nielsen *et al.* 1997; Rico *et al.* 1997; Bagley *et al.* 1999; Takagi *et al.* 1999), loci with only a few alleles tend to be more suitable for population studies while those with greater numbers of alleles are best suited for parentage and linkage studies (Carvalho and Hauser 1994; O'Reilly and Wright 1995).

The very limited differentiation in the Indian Ocean bigeye tuna could, in principle, also be a product of the type of genetic marker used in the current study. Carvalho and Hauser (1994) suggest stabilizing selection arising from exposure to similar environments may result in populations not being genetically differentiated even if gene flow is restricted. However, it is accepted that microsatellites are generally selectively neutral (see references in Introduction); stabilising selection is unlikely to account for the lack of differentiation observed.

One concern with microsatellites is that mutation rate may be so high (Goldstein *et al.* 1995; Slatkin 1995; Estoup *et al.* 1998; Shaw *et al.* 1999) that population differences brought about by restricted gene flow are obscured.

However, it should be pointed out that while the ATCO mtDNA markers did not detect any overall significant differentiation in Indian Ocean collections (although possible east-west differentiation was indicated), pronounced differentiation using the same mtDNA markers was identified among Atlantic and Indian Ocean collections. At least for these markers, lack of differentiation in the Indian Ocean bigeye tuna does not appear to be a product of the marker type.

It would therefore seem that the genetic data at both the mtDNA and microsatellite loci among the Indian Ocean collections reflect genetic exchange within this ocean basin, albeit with a possible restriction on east-west gene flow. In contrast, there is major reproductive isolation between populations in the Atlantic and Indian Oceans. MtDNA shows striking differentiation of the Atlantic collection from the Indo-Pacific collections, which appear to be genetically similar for mtDNA. On the other hand, microsatellite analyses reveal very little evidence of population differentiation. This may reflect sex-biased dispersal, as discussed earlier, or population bottlenecks. MtDNA is more sensitive to genetic drift and population bottlenecks than nuclear DNA, as it has an effective population size of only 1/4 that of nuclear DNA due to its maternal inheritance (Wilson *et al.* 1985). If the Atlantic bigeye population has ever been reduced to a small number of individuals (or indeed had been founded from a small number of individuals), then drift would have accentuated the mtDNA differences more than the nuclear DNA differences. At this stage, this bottlenecking explanation cannot be accepted or rejected over the biased sex dispersal model - more data, especially sex specific tagging data, are needed.

The higher mutation rates of microsatellites (and other nuclear non-coding markers) than mtDNA markers have sometimes been proposed to result in increased powers of microsatellites for testing population differentiation (Rousset and Raymond 1995; Goudet *et al.* 1996); however, we found this not to be the case in the current study.

A study undertaken on nuclear intron allele size differences in the CK gene also detected no significant heterogeneity between individuals from Western Australia and Seychelles collections (Appleyard, unpublished), neither was heterogeneity detected between these individuals and those from the Atlantic Ocean collection (Appleyard, unpublished). These results parallel the overall microsatellite results given above.

The combined use of mitochondrial and DNA analyses is more powerful than using either type of analysis alone. Without mtDNA analyses, for example, the striking genetic differentiation of the Atlantic collection from the Indo-Pacific collections would not have been evident. We are, however, concerned that the high mutation rate of the dinucleotide microsatellite markers decrease the resolving power of this type of marker. We therefore recommend that any further study into bigeye tuna population structure use, in addition to the mtDNA and dinucleotide microsatellite markers deployed here, tri and tetranucleotide microsatellite markers and nuclear DNA markers with a lower mutation rate.

7.5. Species identification, sampling and storage

In addition, we wish to comment on the importance of sampling, obtaining morphological data for each sample, and the importance of using different genetic analyses. In only two out of the five Indian Ocean collections were we provided with details regarding individual sex, catch locations, lengths and weights. This restricts our ability to undertake even the most basic morphological analyses on the whole data set with which we could combine our genetic data. While we are extremely grateful to our sample collectors, it can sometimes be difficult to identify the required species when individuals of different tuna species are caught together. In particular, in the Madagascar collection we were unable to be confident about the identity of about half the samples; most of these uncertain samples are likely to be yellowfin tuna. Without genetically

based species identification, these individuals would not have been identified correctly and might have led to incorrect conclusions (such as a discrete Madagascar population). It is therefore suggested that for future population studies, genetic species identification be carried out routinely prior to population analysis unless it is absolutely certain that all specimens have been correctly identified and labelled.

Furthermore, we found that poor tissue storage can have a major impact on the quality and quantity of genomic (and subsequently mtDNA) DNA extracted from tissues. A large proportion of individuals from the Madagascar collection and at least a third of the individuals from the Indonesia collection did not amplify successfully for the ATCO fragment. These samples had been stored in DMSO saturated with sodium chloride and stored at room temperature for a period of time. Genomic DNA was however successfully obtained from individuals in those collections in which tissues had been stored in 95% ethanol and then maintained at -20^0C (Western Australia, west Indian Ocean, Atlantic Ocean). The tissue storage and extraction of DNA for microsatellite analysis did not have as much effect as that for mtDNA. This is probably due to the smaller amplification product size required for microsatellites as compared to mtDNA markers. We would therefore recommend that for DNA extraction purposes, tissues are either stored at ultra low temperatures (although this is not always possible) or in 95% ethanol and then maintained at a minimum of -20^0C for medium to long term storage.

8. BENEFITS

The major achievements of this research are:-

- a number of Indian Ocean bigeye tuna collections were examined with both mitochondrial DNA and nuclear DNA markers, and there was a suggestion of east-west differences.
- based on mtDNA haplotype analysis, Indian Ocean bigeye tuna are significantly different from bigeye tuna found in the Atlantic Ocean.
- microsatellites showed only small differences between the Indian and Atlantic Ocean bigeye collections.
- on a global scale, bigeye tuna appear to be divided into two genetically well-separated stocks, one within the Indo-Pacific Oceans and the other within the Atlantic Ocean

These data can now be used to refine conservation and management plans for this area. Uncertainty regarding bigeye stock structure has until now seriously restricted the ability of fisheries managers to make confident statements about the stock structure of bigeye in the Indian Ocean and its future sustainability. Our data suggest that there may be two stocks of bigeye tuna in the Indian Ocean, an eastern and a western stock. Bigeye tuna caught within the WSTBF appear to be part of the putative eastern bigeye stock. However, much of the data points toward a single panmictic Indian Ocean stock. Final resolution of the stock structure in the Indian Ocean will require further genetic analyses and further tagging studies. Data from other studies suggest that bigeye populations within the Indian Ocean are contiguous with those found within the Pacific Ocean with possible larval/juvenile mixing achieved through the waterways of the Indonesia throughflow. Additionally, longer term benefits for this fishery will be achieved when other biological parameters such as estimation of age data (via otolith analysis) and more detailed catch and tagging data are obtained and used in combination with the genetic data analysis.

The routine use of a combination of genetic analysis tools is recommended, particularly combining data from both nuclear and mitochondrial genomes. While microsatellites provide the researcher with highly variable loci, problems with scoring

from dinucleotide repeats and the large number of alleles observed at loci may limit the ability of these markers to detect small but perhaps significant differences between genetically similar populations. In addition, the combining of genetic data with catch statistics and morphological measurements will enhance the power of stock structure investigations.

9. FURTHER DEVELOPMENT

Copies of this Report will be forwarded to the Southern and Western Tuna MAC, the Eastern Tuna MAC and the SBT MAC. While the majority of benefits of this study will be applicable to the Western Tuna and Billfish Fishery, the lack of clear genetic heterogeneity between bigeye tuna found within the Pacific and Indian Oceans (this study and others) will also be of interest to the managers of the Eastern and Southern Tuna and Billfish Fisheries.

The research reported here suggests the possibility of an eastern and western stock of bigeye tuna within the Indian Ocean. However, much of the data was consistent with the null hypothesis of a panmictic population of bigeye tuna in the Indian Ocean. A more powerful test of stock structure in the Indian Ocean would be achieved if sample sizes were at least double the ones used here and additional collection sites were sampled (Table 20) and if additional microsatellites including tri and tetra-repeat loci were examined along with nuclear DNA markers with a lower mutation rate.

Table 20 Current and proposed sampling sites and sample sizes for further investigation into bigeye tuna in the Indian Ocean. Proposed sites and additional sample sizes are in bold

Collection sites	Sample sizes
Indonesia	91 **(100)**
Madagascar	53 **(150)**
Seychelles	89 **(110)**
west Indian Ocean	19 **(180)**
Western Australia	96 **(100)**
Atlantic Ocean	15 **(185)**
off Cape of Good Hope	**200**
off Kenya/Somali Republic	**200**
off Sri Lanka	**200**
middle of Indian Ocean ≡15^{0}S	**200**

10. CONCLUSION

The current genetic data (mtDNA and microsatellites) do not enable us to reject unequivocally the null hypothesis of a single panmictic bigeye tuna population in the Indian Ocean; there is an indication of a possible east-west separation. Waples (1998) contends that if components of a stock complex exhibit high gene flow, then management should not be based on genetic data alone. We would also suggest that the relative lack of genetic differentiation observed among the bigeye tuna collections be approached carefully. Further genetic data are required in order to confirm or refute the suggestion of east-west differences, and other biological data such as tagging (present information is extremely limited), morphology and otolith chemistry are required to help to determine management units. If the bigeye tuna within the Indian Ocean are truly panmictic, then managing the fishery as a single stock will not affect recruitment from overfished areas. If, however, different bigeye tuna populations do exist, management as a single stock will mean that over-exploitation in certain areas will lead to reductions in effective population size and yield in these areas.

On a wider scale however, our mtDNA data give strong support to the hypothesis of restricted gene flow between bigeye tuna individuals in the Indian Ocean and the Atlantic Ocean. This finding has been outlined in the literature previously (Alvarado-Bremer *et al.* 1998; Chow *et al.* 2000).

A more refined and more powerful analysis of genetic population structure within the Indo-Pacific would require larger sample sizes (of at least 200 per site) plus, ideally, the development and deployment of tri or tetranucleotide microsatellites developed specifically for bigeye tuna. An assessment of the extent if any of temporal variation should also be made, although the relative lack of spatial differentiation observed here suggests that this is likely to be a minor component. Non-genetic future research on bigeye tuna within the Indian Ocean should concentrate on life history studies, catch rates, tagging and other biological parameters so as to confirm or refute the findings of the genetic analyses presented here.

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APPENDIX 1

INTELLECTUAL PROPERTY

The intellectual data arising from this research are:

1. Genotype and allele frequency data.
2. Copyright in this report.

APPENDIX 2

Staff

Dr Robert Ward	CSOF8	Principal investigator, report preparation
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Dr Sharon Appleyard	CSOF3	collection and analysis of all mtDNA and microsatellite data, report preparation

APPENDIX 3 Allele frequencies at seven microsatellite loci in bigeye tuna populations from the Indian and Atlantic Oceans

Locus *102*

Allele	Atlantic Ocean	Indonesia	Madagascar	Seychelles	west Indian Ocean	Western Australia
132	0.000	0.000	0.000	0.006	0.000	0.000
134	0.000	0.056	0.029	0.045	0.079	0.052
136	0.033	0.073	0.067	0.080	0.053	0.057
138	0.000	0.034	0.067	0.040	0.000	0.005
140	0.067	0.056	0.000	0.051	0.000	0.063
142	0.200	0.062	0.029	0.085	0.000	0.083
144	0.067	0.056	0.115	0.045	0.105	0.042
146	0.100	0.045	0.058	0.074	0.079	0.099
148	0.100	0.096	0.106	0.074	0.079	0.078
150	0.033	0.039	0.038	0.045	0.026	0.068
152	0.033	0.079	0.087	0.051	0.132	0.047
154	0.067	0.107	0.087	0.074	0.053	0.083
156	0.067	0.056	0.096	0.108	0.079	0.073
158	0.067	0.073	0.067	0.068	0.105	0.073
160	0.067	0.028	0.048	0.034	0.079	0.052
162	0.000	0.056	0.058	0.057	0.000	0.047
164	0.033	0.022	0.010	0.017	0.026	0.021
166	0.000	0.017	0.019	0.023	0.026	0.026
168	0.000	0.017	0.000	0.006	0.000	0.021
170	0.033	0.011	0.010	0.011	0.079	0.005
172	0.033	0.017	0.010	0.006	0.000	0.005
Total	15	89	52	88	19	96

Locus *113*

Allele	Atlantic Ocean	Indonesia	Madagascar	Seychelles	west Indian Ocean	Western Australia
105	0.000	0.000	0.000	0.006	0.000	0.016
109	0.000	0.000	0.010	0.000	0.000	0.000
111	0.036	0.000	0.000	0.000	0.000	0.016
113	0.000	0.011	0.020	0.023	0.000	0.016
115	0.000	0.006	0.020	0.011	0.026	0.011
117	0.071	0.051	0.088	0.051	0.132	0.032
119	0.000	0.157	0.196	0.068	0.105	0.080
121	0.036	0.034	0.039	0.028	0.053	0.037
123	0.036	0.056	0.059	0.034	0.079	0.037
125	0.107	0.084	0.108	0.080	0.026	0.053
127	0.107	0.067	0.029	0.102	0.000	0.074
129	0.071	0.045	0.078	0.068	0.026	0.080
131	0.071	0.045	0.059	0.080	0.000	0.069
133	0.107	0.073	0.039	0.045	0.053	0.074
135	0.036	0.062	0.020	0.040	0.079	0.074
137	0.000	0.051	0.010	0.034	0.000	0.048
139	0.036	0.017	0.020	0.051	0.053	0.053
141	0.036	0.006	0.029	0.011	0.000	0.021
143	0.071	0.056	0.059	0.057	0.053	0.053
145	0.000	0.028	0.020	0.023	0.053	0.021
147	0.036	0.006	0.010	0.034	0.053	0.043
149	0.000	0.017	0.020	0.034	0.000	0.011
151	0.071	0.011	0.029	0.034	0.000	0.011
153	0.000	0.017	0.000	0.006	0.026	0.016
155	0.036	0.034	0.010	0.006	0.079	0.016
157	0.000	0.017	0.020	0.023	0.053	0.000
159	0.000	0.011	0.010	0.006	0.026	0.005
161	0.036	0.022	0.000	0.017	0.000	0.027
163	0.000	0.006	0.000	0.017	0.026	0.005
165	0.000	0.006	0.000	0.006	0.000	0.000
167	0.000	0.006	0.000	0.006	0.000	0.000
Total	14	89	51	88	19	94

Locus *121*

Allele	Atlantic Ocean	Indonesia	Madagascar	Seychelles	west Indian Ocean	Western Australia
258	0.000	0.000	0.009	0.006	0.000	0.005
260	0.033	0.005	0.000	0.006	0.026	0.005
262	0.000	0.005	0.000	0.011	0.000	0.000
264	0.067	0.016	0.019	0.040	0.026	0.026
266	0.100	0.082	0.057	0.069	0.053	0.099
268	0.167	0.055	0.094	0.069	0.053	0.109
270	0.000	0.044	0.047	0.063	0.053	0.036
272	0.033	0.132	0.066	0.103	0.105	0.057
274	0.133	0.148	0.170	0.121	0.132	0.141
276	0.133	0.071	0.057	0.057	0.132	0.089
278	0.033	0.093	0.047	0.080	0.105	0.073
280	0.100	0.143	0.104	0.138	0.158	0.109
282	0.100	0.055	0.113	0.052	0.079	0.083
284	0.000	0.066	0.047	0.034	0.026	0.031
286	0.033	0.038	0.057	0.080	0.026	0.042
288	0.033	0.016	0.009	0.006	0.026	0.036
290	0.033	0.005	0.019	0.006	0.000	0.016
292	0.000	0.011	0.038	0.029	0.000	0.000
294	0.000	0.005	0.000	0.011	0.000	0.000
296	0.000	0.005	0.038	0.000	0.000	0.021
298	0.000	0.000	0.009	0.000	0.000	0.010
306	0.000	0.000	0.000	0.017	0.000	0.010
Total	15	91	53	87	19	96

Locus *125*

Allele	Atlantic Ocean	Indonesia	Madagascar	Seychelles	west Indian Ocean	Western Australia
158	0.067	0.082	0.142	0.188	0.184	0.094
162	0.800	0.808	0.802	0.750	0.763	0.833
164	0.133	0.093	0.047	0.063	0.053	0.063
166	0.000	0.016	0.009	0.000	0.000	0.010
Total	15	92	53	88	19	96

Locus *144*

Allele	Atlantic Ocean	Indonesia	Madagascar	Seychelles	west Indian Ocean	Western Australia
162	0.000	0.005	0.000	0.000	0.000	0.005
166	0.067	0.016	0.057	0.023	0.053	0.063
168	0.067	0.104	0.047	0.091	0.158	0.115
170	0.033	0.038	0.104	0.108	0.079	0.073
172	0.533	0.571	0.500	0.523	0.447	0.505
174	0.300	0.236	0.274	0.227	0.263	0.240
176	0.000	0.005	0.000	0.000	0.000	0.000
178	0.000	0.022	0.019	0.006	0.000	0.000
Total	15	91	53	86	19	96

Locus *161*

Allele	Atlantic Ocean	Indonesia	Madagascar	Seychelles	west Indian Ocean	Western Australia
170	0.000	0.000	0.000	0.000	0.000	0.005
176	0.000	0.000	0.000	0.011	0.000	0.005
178	0.000	0.011	0.063	0.034	0.000	0.016
180	0.000	0.022	0.000	0.029	0.000	0.022
182	0.033	0.028	0.031	0.023	0.000	0.065
184	0.000	0.017	0.016	0.006	0.000	0.016
186	0.100	0.194	0.109	0.178	0.132	0.076
188	0.000	0.050	0.031	0.046	0.079	0.033
190	0.000	0.044	0.047	0.011	0.000	0.022
192	0.067	0.078	0.078	0.063	0.132	0.087
194	0.033	0.044	0.031	0.080	0.105	0.054
196	0.067	0.078	0.094	0.075	0.053	0.082
198	0.133	0.050	0.078	0.086	0.053	0.109
200	0.100	0.050	0.063	0.046	0.079	0.033
202	0.067	0.028	0.031	0.057	0.053	0.043
204	0.100	0.033	0.078	0.029	0.053	0.087
206	0.100	0.050	0.109	0.069	0.053	0.043
208	0.067	0.094	0.047	0.075	0.105	0.065
210	0.067	0.050	0.016	0.034	0.026	0.043
212	0.000	0.006	0.031	0.017	0.053	0.022
214	0.033	0.022	0.031	0.006	0.000	0.038
216	0.033	0.022	0.016	0.017	0.026	0.016
218	0.000	0.028	0.000	0.006	0.000	0.011
220	0.000	0.000	0.000	0.000	0.000	0.005
Total	15	90	32	87	19	92

Locus *208*

Allele	Atlantic Ocean	Indonesia	Madagascar	Seychelles	west Indian Ocean	Western Australia
138	0.000	0.000	0.009	0.000	0.000	0.016
140	0.000	0.016	0.000	0.000	0.000	0.000
142	0.000	0.033	0.075	0.029	0.000	0.016
144	0.000	0.000	0.019	0.023	0.026	0.010
146	0.000	0.055	0.066	0.063	0.079	0.041
148	0.100	0.286	0.264	0.299	0.263	0.290
150	0.167	0.308	0.321	0.322	0.263	0.337
152	0.200	0.148	0.132	0.172	0.132	0.119
154	0.200	0.055	0.038	0.029	0.053	0.073
156	0.167	0.022	0.019	0.029	0.026	0.010
158	0.033	0.022	0.019	0.017	0.079	0.031
160	0.000	0.011	0.009	0.006	0.000	0.016
164	0.067	0.011	0.009	0.000	0.000	0.016
166	0.000	0.022	0.000	0.000	0.026	0.016
168	0.000	0.000	0.009	0.000	0.000	0.000
170	0.000	0.005	0.000	0.000	0.000	0.010
172	0.033	0.000	0.000	0.000	0.026	0.000
174	0.000	0.005	0.009	0.011	0.026	0.000
176	0.033	0.000	0.000	0.000	0.000	0.000
Total	15	91	53	87	19	96