

Genetic Variation in Yellowfin Tuna *Thunnus Albacares* (Bonnaterre, 1788) Along Indian Coast Using Pcr-Rflp Analysis of Mitochondrial Dna D-Loop Region



Biology

KEYWORDS : Yellowfin tuna, mtDNA, D-loop, PCR-RFLP, Mitotype, Population structure, Indian waters

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ABSTRACT

Yellowfin tuna Thunnus albacares is an epipelagic, oceanic species of family Scombridae, found in tropical and subtropical region of the Pacific, Atlantic and the Indian Ocean. It is a commercially important fish species.

In present study, population structure of yellowfin tuna was examined using PCR-RFLP analyses of mitochondrial DNA D-loop region from eight geographically distinct locations along the Indian coast. A 500 bp segment of D-loop region was analysed for 370 yellowfin samples using six restriction enzymes (Alu I, Hae III, Hinf I, Hha I, Msp I and Rsa I), resulting in 14 composite mitotype. Analysis of molecular variance (AMOVA) showed no significant genetic differentiation among sampling localities ($\Phi_{ST} = 0.00185$; $P = 0.327$). Thus finding of this study suggests single panmictic population of yellowfin tuna in Indian waters.

Introduction

Yellowfin tuna *Thunnus albacares* Bonnaterre, 1788 is globally distributed throughout tropical and subtropical waters except Mediterranean Sea (Collette and Nauen, 1983). It is a commercially important fishery worldwide and is said to be potential replacement for other endangered tuna species such as bluefin tuna (Wu et al., 2010). Though ubiquitous in presence across world Ocean, it is the Indian Ocean which is most productive areas for tuna fishing (Anganuzzi et al., 1996). In Indian Ocean, it is distributed north of latitude 30°S and is the most dominant species in Indian waters (Vijaykumar and Verghese, 2010). Despite high catch and increasing commercial values of yellowfin tuna, limited studies had been done to find the stock structure of this species in Indian waters. There are various methods available for determining genetic stock structure of fish species, among them genetic approach is more effective.

Genetic analyses can provide important insights into problem of inter and intraspecific population structuring (Avisé, 1994). Molecular biology techniques have become important in determining fish stock structure. There is considerable increase in the number of molecular markers available for genetic analyses of population structure for last three decades with allozyme, mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) have been used for this purpose. Each marker has its own advantages and have been successfully used for population genetic analyses of several tuna species (allozyme: Ward et al., 1994b; mtDNA: Menezes et al., 2006 and nDNA: Menezes et al., 2008). The mtDNA is haploid, maternally inherited and has a rapid evolution rate in comparison to nDNA and thus provides higher level of genetic resolution than allozyme (Birky et al., 1983 and Brown et al., 1992). In particular with the advent of polymerase chain reaction (PCR), an analysis of restriction site polymorphism of mtDNA has become a widely used population genetics tool in fisheries (Ravago-Gotanco et al., 2004; Hoolihan et al., 2006; Menezes et al., 2006; Turan et al., 2009; Kumar et al., 2012b).

The mtDNA D-loop region is highly polymorphic in tunas (Alvarado Bremer et al., 1998, 1999; Chow et al., 2000). These variations have been detected using conventional PCR-RFLP analysis in several tuna species such as skipjack tuna, frigate tuna from Indian region (Menezes et al., 2006, Kumar et al., 2012b).

An understanding of population genetic variation is important for successful conservation and management of fisheries. Yellowfin tuna is one of the most studied tuna species across the globe and has been studied for the last 50 years with the earliest genetic investigation was carried out in samples from the Pacific and the Indian Ocean by Suzuki (1962). However, to the best of our knowledge, there is no report from Indian waters on genetic

population structure of yellowfin tuna. The present study aims to find genetic variation in yellowfin tuna along Indian coast while testing the null hypothesis of population panmixia.

Materials and Methods

Sample collection

A total of 370 yellowfin tuna fin clip samples were collected from eight distinct geographical localities along east and west coast of India including two island samples (Fig. 1). The samples were immediately preserved in absolute alcohol and brought back to laboratory for further processing.

DNA Isolation

The fin clip samples were used for isolating high molecular weight (HMW) genomic DNA following the standard TNES-Urea-Phenol-Chloroform protocol (Asahida et al., 1996). Further the DNA pellet was suspended in 50 µl of Tris-EDTA buffer (pH 8.0). Qualitative and quantitative measurement of DNA was carried out using UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan). Each sample was estimated to have 20-30 ng of DNA per micro litre of solution. The DNA samples were stored at 4°C prior to PCR analysis.

DNA Amplification

The D-loop region of mtDNA was amplified using the primer set designed by Menezes et al. (2006) (Fig. 2a). The primer sequences were as follows: 5' CCGACGTCGGAGGTTAAAAT 3' (forward) and 5'AGGAACCAATGCCAGGAATA 3' (reverse). DNA samples were amplified in Eppendorf Thermocycler (EP Gradient S). Amplification was carried out in 50 µl reaction mixture containing 2 µl of template DNA; 5 µl of 10X buffer (100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 500 mM KCl); 1.0 µl of each primer (100 pmol); 5 µl of a 2.5 mM solution of each deoxyribonucleoside triphosphate (dNTP); 2.5 units of Taq DNA polymerase and milliQ water. PCR parameters consisted of 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. Final extension was done at 72°C for 5 minutes.

RFLP

PCR products were digested with six restriction enzymes (*Rsa I*, *Alu I*, *Hinf I*, *Hha I*, *Msp I* and *Hae III*). Restriction digestion was carried out in a 10 µl volume containing 2 µl of PCR product, 2 units of restriction enzyme, 1 µl of the appropriate buffer, and 7 µl of ultrapure water, at 37°C for overnight. Restriction fragments were resolved on a 2.5% agarose gel buffered with 1X Tris-Borate-EDTA (TBE), and stained with ethidium bromide. Bands on the gels were visualized by UV transilluminator and photographed for further analysis.

Data Analyses

The mtDNA haplotypes (mitotypes) generated by each restriction endonuclease were designated by a capital letter in order of detection. Composite mitotypes were constructed from four alphabets corresponding to the mitotypes of *Rsa* I, *Alu* I, *Hinf* I and *Hha* I, respectively. Since *Hae* III and *Msp* I did not show polymorphism, restriction pattern of these two enzymes were not used for constructing composite mitotypes. A matrix data was created, which includes presence or absence of different restriction fragment pattern with respect to each endonuclease. The presence or absence of a given fragment was indicated by 1 and 0 respectively.

The restriction site matrix was used to calculate nucleotide diversity (π) and composite mitotype diversity (h) using program ARLEQUIN version 3.11 (Excoffier *et al.*, 2005). The extent of genetic differentiation between samples was estimated using the fixation index Φ_{ST} (Wright, 1951). Estimates of expected number of migrant females between populations per generation (Nfm) were calculated using the formula $2Nfm = ((1/\Phi_{ST}) - 1)$ (Takahata and Palumbi, 1985). The significance of Φ_{ST} was tested by 1,000 permutations for each pairwise comparison. Hierarchical analysis of molecular variance was performed to partition variance components attributable to (1) variation among groups; (2) variation among populations within groups; and (3) variation within populations, to evaluate hypothesized patterns of spatial genetic structure. The null hypothesis of population panmixia was also tested using an exact test of composite mitotype homogeneity among samples. The exact test of population differentiation of composite mitotypes tests the hypothesis that the observed distribution of frequencies is less likely than the distribution expected under panmixia. Statistical significance was estimated via 1,000 Markov Chain Monte Carlo simulations as proposed by Raymond and Rousset (1995). The population parameters θ and τ were also estimated for the yellowfin tuna samples. Tau (τ) is a relative measure of time since population expansion, but can be transformed to estimate the actual time (T) since a population expansion using formula $T = \tau/2\mu$ where μ is the mutation rate per site per generation. In the present study, the mutation rate of 3.6×10^{-8} mutations per site per year was applied for the control region sequence of yellowfin tuna as this rate has been reported for the mtDNA control region in teleosts (Donaldson and Wilson, 1999). Historical demographic/spatial expansions were investigated using Tajima's D test (Tajima, 1989) and Fu's FS test (Fu, 1997) with 1,000 permutations. ARLEQUIN was also used to estimate both Harpending's raggedness index (Hri ; Harpending, 1994) and mismatch distributions (SDD), to test the goodness-of-fit of observed mismatch distributions to the theoretical distribution under a sudden expansion model (Rogers and Harpending, 1992). To examine genealogical relationships among composite mitotypes, a minimum spanning network was constructed using HapStar Version 0.5 (Teacher and Griffiths, 2011).

Results

Genetic Diversity

The primers consistently amplified a 500bp DNA fragment of mtDNA D-loop in all individual, without apparent size differences among or within individuals from different sampled populations (Fig. 2). Restriction endonuclease *Rsa* I and *Alu* I produced four mitotypes (A, B, C and D), *Hinf* I and *Hha* I produced three mitotypes (A, B and C) while *Msp* I and *Hae* III produced a single mitotype (A) giving a total of 14 composite mitotypes across the total sample size of 370 yellowfin tuna samples (Table 1). Furthermore, the polymorphic band patterns of restriction enzymes (*Rsa* I, *Alu* I, *Hinf* I, *Hha* I) are presented in Figure 3a-3h.

The composite mitotype $m1$ was most common and observed in all sampling sites followed by $m2$ (five sampling sites), $m5$ (four sampling sites), $m3$ (three sampling sites), $m6$, $m11$ and $m12$ (two sampling sites). Composite mitotype $m4$, $m7$, $m8$, $m9$, $m10$, $m13$ and $m14$ were unique and present in VE, KO, PO, PO, PB, TU and VI respectively (Table 2). Mitotype diversity (h) and nucleotide diversity (π) were low, ranged from 0.0721 to 0.2287 and 0.1739 to 0.4875 respectively (Table 1). All the pairwise comparisons were insignificant and most of the pairwise

Φ_{ST} values were negative (Table 3).

Population Genetic Structure

Analysis of molecular variance (AMOVA) performed on mtDNA RFLP data set revealed no significant genetic heterogeneity among the eight sampling sites ($\Phi_{ST} = 0.00185$; $P = 0.32747$) (Table 4). The estimated value of female migrants per generation (using Φ_{ST} values) was 270 among the eight sampling sites. Hierarchical AMOVA was performed to test the significance of the partitioning of genetic variance resulting from different groupings of the populations into geographical groups. Results revealed that variation attributed to among groups composite mitotype frequency differences was very low (<1%), with almost all of the variation (99.81%) found in samples within populations (Table 4). The exact test of population differentiation (non-differentiation exact P values) showed no significant differences among eight sampling sites ($P = 0.35977$). A minimum spanning network (Figure 3) indicates that composite mitotypes are closely related and there is no clear pattern of composite mitotypes and geographic location among sampling sites.

Historic Demography

The ARLEQUIN analyses of mtDNA showed large differences in θ_0 (population before expansion) and θ_1 (population after expansion) within all sampling sites, suggesting rapid population expansion (Table 5). In addition, all Harpending's raggedness indices and mismatch distributions were non-significant. Tajima's D (-1.579) values were mostly negative and significant ($P < 0.05$) while Fu's F values were negative but insignificant. The overall tau (τ) value was estimated to be 3.000 (95% confidence interval), which reflects the time of founding of yellowfin tuna in Indian waters of 83,333 years ago.

Discussion

The genetic variability and differentiation in yellowfin tuna samples has been evaluated using PCR-RFLP analysis of mtDNA D-loop region. The data exhibited very little divergence among the samples of eight localities analyzed, suggesting the existence of single panmictic population of yellowfin tuna in Indian waters. Hence, the null hypothesis of population panmixia cannot be rejected. The outcome is well supported by non-significant values of pairwise Φ_{ST} (Table 3) and AMOVA analyses, which did not revealed geographically meaningful group along Indian coast (Table 4). The null hypothesis is further corroborated by exact test of population differentiation which gave non-significant P value, indicating that the mitotypes were distributed randomly with respect to locality.

It has been observed that marine species have low genetic sub-population differentiation than the fresh water ones. For instance, PCR-RFLP analysis studies have shown no genetic differentiation among Pacific bigeye tuna samples (Alvarado-Bremer *et al.*, 1998; Chow *et al.*, 2000; Durand *et al.*, 2005). Similarly population genetic involving mtDNA revealed low level of genetic differentiation in Pacific yellowfin tuna samples (Scoles and Graves, 1993; Ward *et al.*, 1994, 1997; Appleyard *et al.*, 2001).

In principle, for marine organisms the degree of gene flow is determined by the dispersal abilities of organisms and the presence/absence of geologic or oceanographic barriers. The evolution of genetically distinct populations (stock) is attributed to the disjunct geographical distribution. However, the apparent lack of barriers to dispersal in the marine environment effectively reduces heterogeneity among populations, often making it difficult to differentiate discrete populations (Palumbi, 1992; Shulman and Birmingham 1995; Menezes *et al.*, 2008). Also, low level of intraspecific gene flow and reduced population structure had been reported in marine fisheries compared to freshwater fishes, likely due to fewer barriers to dispersal in the marine environment (Ward *et al.*, 1994). Oceanic pelagic fishes such as yellowfin tuna in present study conform to this pattern.

In particular, genetic differentiation is low among tuna populations within and between the Pacific and the Indian Ocean (Alvarado Bremer *et al.*, 1998; Grew and Hampton, 1998; Chow *et*

al., 2000; Appleyard et al., 2002; Durand et al., 2005; Chiang et al., 2008; Wu et al., 2010) because of the occurrence of continuous, circumtropical pelagic environment and a wide range of suitable spawning grounds.

There were close relatedness among composite mitotypes and insignificant differences in their frequencies among the sampled populations. This homogeneity indicates gene flow between the sampled populations in Indian waters, since even small rates of migration between populations should eventually homogenize any neutral genetic variation (Crow, 1986; Schultz and Cowen, 1994; Menezes et al., 2008 and Kumar et al., 2012).

A recent population bottleneck or founder event by single or few mtDNA lineages may cause low genetic diversity at both composite mitotypes and nucleotide level (Grant and Bowen, 1998). There were large difference between θ_0 (population before expansion) and θ_1 (population after expansion) within all the sampling localities in present study, as expected during rapid population expansion.

Rates of gene flow as low as a few fish per generation are sufficient to prevent genetic differentiation by genetic drift (Wright, 1931; Hauser and Ward, 1998). Insignificant genetic differentiation and high rates of gene flow ($Nfm = 270$) observed in present study supports single genetic stock yellowfin tuna in Indian waters. Similarly, Wu et al (2010) observed low and insignificant Φ_{ST} values, indicating low genetic differentiation and suggested the presence of possible gene flow between the Western Pacific and the Western Indian Ocean. A recent study carried out in Indian waters also revealed low level of Φ_{ST} value and high rates of gene flow in little tuna, concluding single stock around Indian waters (Kumar et al., 2012a).

The tau (τ) values for mtDNA data set were same for all sampling sites ($\tau = 3.000$), which further corresponds that the estimated time for population expansion of yellowfin tuna in Indian waters is 83,333 years ago(95% confidence interval). Thus, recent founding and insufficient time to attain the migration-drift equilibrium among populations of yellowfin tuna could be the possible reason for lack of genetic structure observed in present study.

The resolution of stock structure is important for effective and sustainable management of fisheries. However failure to reject null hypothesis does not necessarily means lack of stock structure, but only that it was not observed. This may be owing to the marker selected or the presence of sufficient gene flow to prevent accumulation of significant genetic divergence or insufficient time to attain the significant genetic differences (Graves and McDowell, 2003). Ward (2000) observed that by increasing sample size or using different class of marker with different population dynamics may reveal heterogeneity and restrictions on gene flow among regions once classed as homogeneous. Interestingly, a recent study involving mtDNA and nuclear DNA markers showed fine scale genetic heterogeneity in yellowfin tuna samples around Srilanka (Dammangodda et al., 2008). This finding warrants the need to carryout studies in Indian waters taking into consideration of both mtDNA as well as nuclear DNA.

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Figure

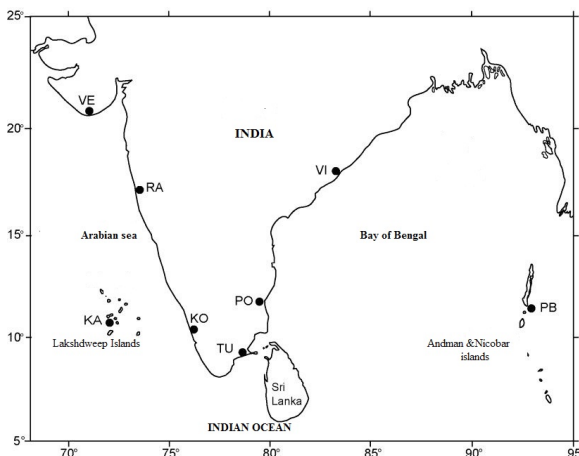


Figure 1. Map showing location of sampling sites along Indian coast VE, Veraval; RA, Ratnagiri; KO, Kochi; KA, Kavaratti; PB, Port-Blair; TU, Tuticorin; PO, Pondicherry; VI, Vizag.

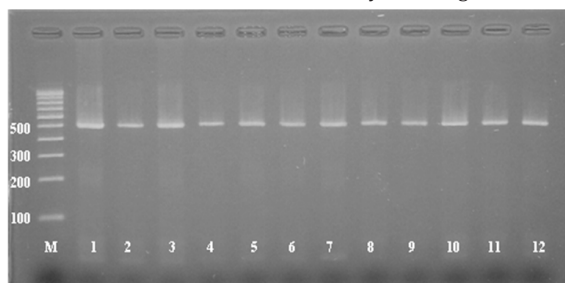


Figure 2. 500bp PCR amplified product of yellowfin tuna. Where M is the 100bp DNA marker and 1-12 is the amplified DNA product.

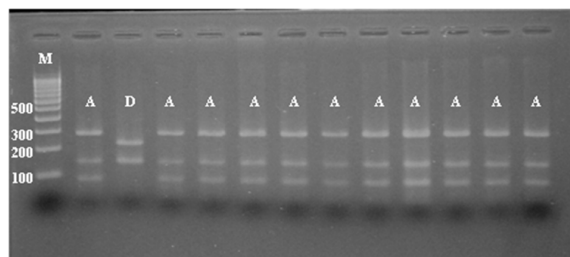
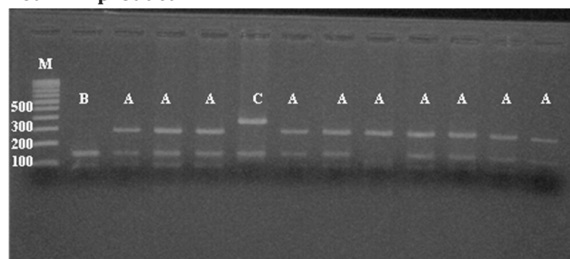


Figure 3 a, b. The restriction digestion of mtDNA (D-loop) of yellowfin tuna with enzyme *Rsa* I. Where M is the 100bp DNA marker and A, B, C and D are different restriction patterns.

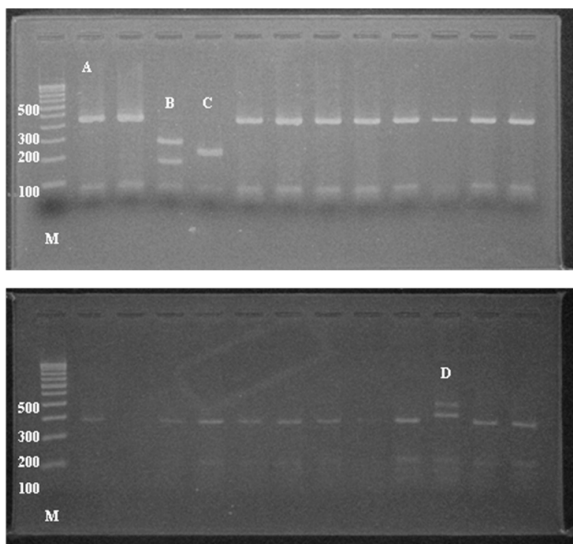


Figure 3 d, e. The restriction digestion of mtDNA (D-loop) of yellowfin tuna with enzyme *Alu* I. Where M is the 100bp DNA marker and A, B, C and D are different restriction patterns.

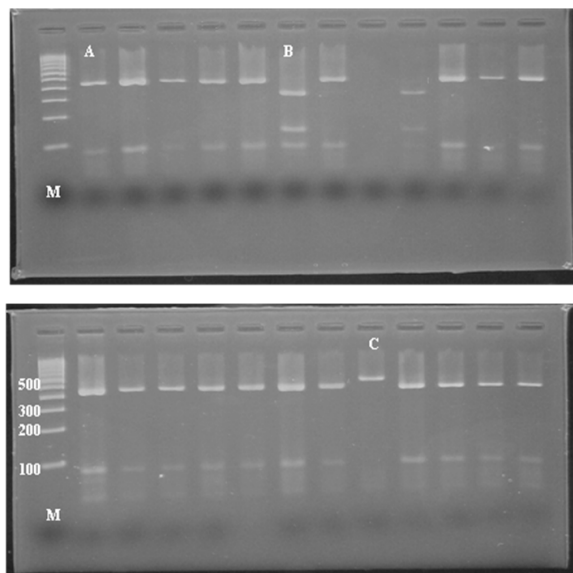


Figure 3 f, g. The restriction digestion of mtDNA (D-loop) of yellowfin tuna with enzyme *Hinf* I. Where M is the 100bp DNA marker and A, B and C are different restriction patterns.

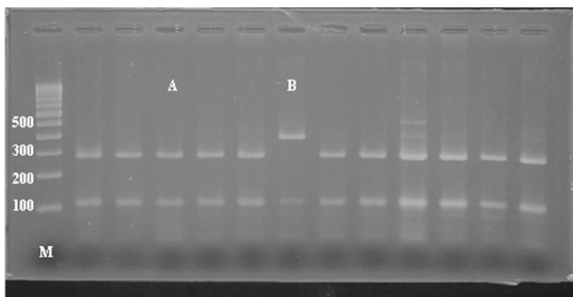


Figure 3 h. The restriction digestion of mtDNA (D-loop) of yellowfin tuna with enzyme *Hha* I. Where M is the 100bp DNA marker and A, B are different restriction patterns.

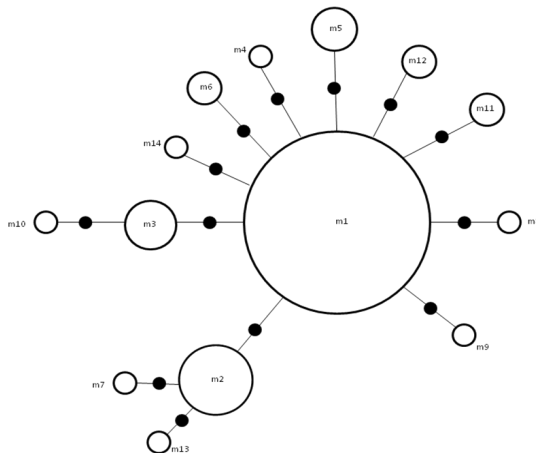


Figure 4. Minimum spanning network showing relationships among 14 mitochondrial DNA D-loop region composite mitotype. Each circle represents a unique composite mitotype in the sample. The size of each circle represents relative frequency of each composite mitotypes.

Table 1. Estimates of genetic diversity within populations based on RFLP: number of samples (n); number of mitotypes (nh); mitotype diversity (h) and nucleotide diversity (π).

Population	Location	Date of collection	n	nh	h	π
Veraval (VE)	20.54°N 70.22°E	October, 2007	58	5	0.2263	0.4670
Ratnagiri (RA)	16.98°N 73.3°E	January, 2008	12	2	0.1667	0.3333
Kochi (KO)	9.58°N 76.16°E	February, 2008	50	5	0.1551	0.3902
Pondicherry (PO)	11.56°N 79.50°E	July, 2008	60	6	0.2203	0.4587
Port-Blair (PB)	11.40°N 92.46°E	July, 2008	55	3	0.0721	0.2154
Tuticorin (TU)	8.49°N 78.08°E	September, 2009	55	5	0.2061	0.4875
Vizag (VI)	17.42°N 83.15°E	February, 2009	57	5	0.2287	0.4699
Agatti (AG)	10.50°N 72.12°E	November, 2008	23	2	0.0870	0.1739
Total			370	14	0.1704	0.3745

Table 2. Relative frequencies of 14 mitotypes from RFLP data. Letters reflects individual composite mitotypes for restriction enzymes *Rsa*I, *Alu*I, *Hinf*I, and *Hha*I (left to right)

S.No	Mitotype	VE	RA	KO	PO	PB	TU	VI	KA
1	AAAA	0.879	0.917	0.92	0.883	0.964	0.891	0.877	0.957
2	BAAA	0.0172	0.0833	0.02	0	0	0.0545	0.0702	0
3	AAAB	0.0345	0	0	0.0333	0.0182	0	0	0
4	AAAC	0.0172	0	0	0	0	0	0	0
5	CAAA	0.0517	0	0.02	0.0333	0	0	0.0175	0
6	AABA	0	0	0.02	0.0167	0	0	0	0
7	BABA	0	0	0.02	0	0	0	0	0
8	ACAA	0	0	0	0.0167	0	0	0	0
9	DAAA	0	0	0	0.0167	0	0	0	0
10	CAAB	0	0	0	0	0.0182	0	0	0
11	ACAA	0	0	0	0	0	0.0182	0.0175	0
12	ABAA	0	0	0	0	0	0.0182	0	0.0435
13	BBAA	0	0	0	0	0	0.0182	0	0
14	ADAA	0	0	0	0	0	0	0.0175	0

Table 3. Pairwise Φ_{ST} (below diagonal) and p (above diagonal) values among populations of yellowfin

Sample	VE	RA	KO	PO	PB	TU	VI	KA
VE		0.71171	0.31532	0.82883	0.61261	0.11712	0.24324	0.18919
RA	-0.02529		0.99099	0.65766	0.31532	0.99099	0.99099	0.50450
KO	0.00215	-0.03714		0.70270	0.16216	0.22523	0.39640	0.21622
PO	-0.00931	-0.02002	-0.00611		0.81081	0.03604	0.12613	0.39640
PB	-0.00457	0.00932	0.01157	-0.00813		0.01802	0.03604	0.31532
TU	0.01265	-0.04193	0.00235	0.01558	0.02625		0.82883	0.51351
VI	0.00464	-0.04733	-0.00323	0.00996	0.02291	-0.01270		0.32432
KA	0.01020	0.01421	0.00714	0.00023	0.00399	-0.00291	0.00952	

Table 4. Results of analysis of molecular variance (AMOVA) testing genetic structure of yellowfin tuna based on mitochondrial D-loop region sequence data.

Structure tested (sampling sites)	Variance	Percentage of Variation	Φ Statistic	p-values
One groups (VE, RA, KO, PO, PB, TU, VI, KA)				
Among populations	0.00037	0.19	$\Phi_{ST} = 0.00185$	P=0.32747
Within populations	0.19970	99.81		
Three groups (VE, RA, KO, PO, TU, VI); (KA) and (PB)				
Among groups	0.00030	0.15	$\Phi_{CT} = 0.00147$	P=0.40567
Among populations within groups	0.00025	0.12	$\Phi_{SC} = 0.00125$	P=0.51906
Within populations	0.19970	99.73	$\Phi_{SC} = 0.00272$	P=0.33431

Table 5. Demographic parameters of yellowfin tuna based on mitochondrial D-loop region RFLP: τ , θ_0 , θ_1 , Tajima's D test and Fu's F_s values, Harpending's Raggedness index (Hr_i), and sum of squared differences (SDD)

Population	τ	θ_0	θ_1	Tajima's D	Fu's F_s	Hr_i	SDD
VE	3.000	0.00176	99999	-1.581*	-2.066	0.6947	0.0610
RA	3.000	0.00000	99999	-1.451	0.431	0.7500	0.0370
KO	3.000	0.00000	99999	-1.576*	-2.711*	0.7435	0.0239*
PO	3.000	0.00000	99999	-1.933**	-3.366**	0.6973	0.0576*
PB	3.000	0.00000	0.44990	-1.674*	-1.190	0.8662	0.0049
TU	3.000	0.00176	99999	-1.316	-1.990	0.6898	0.0430
VI	3.000	0.00000	99999	-1.584*	-2.063	0.6939	0.0625*
KA	3.000	0.00000	0.09927	-1.514*	-0.153	0.8487	0.0107*
Mean	3.000	0.00044	99999	-1.579*	-1.638	0.7480	0.0376

*, ** Significant at $P < 0.05$ and $P < 0.01$ respectively.

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