

Mitochondrial DNA analysis reveals three stocks of yellowfin tuna *Thunnus albacares* (Bonnaterre, 1788) in 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 Pacific, Indian and Atlantic Ocean. It is commercially important fish and accounts for 19% of total tuna catches in Indian waters. In present study, population structure of yellowfin tuna was examined using sequence analysis of mitochondrial DNA from seven geographically distinct locations along the Indian coast. A 500 bp segment of D-loop region was sequenced and analysed for 321 yellowfin samples. Hierarchical analysis of molecular variance showed significant genetic differentiation among three groups (VE); (AG); (KO, TU, PO, VI, PB) analyzed ($\Phi_{ST} = 0.03844$, $P < 0.001$). In addition, spatial analysis of molecular variance identified three genetically heterogeneous groups of yellowfin tuna in Indian waters. Results were further corroborated by significant value of nearest neighbour statistic ($S_{nn} = 0.261$, $P \leq 0.001$). Thus finding of this study rejects the null hypothesis of single panmictic population of yellowfin tuna in Indian waters.

Keywords Yellowfin tuna, mtDNA, D-loop, Population structure, Indian waters.

Introduction

The tunas, belonging to family Scombridae, represent a group of highly commercial marine fisheries with an ever-growing demand world over. They are distributed circumglobally in tropical and subtropical waters of world oceans (Collete and Neuen 1983). The high commercial values of tuna and related products has resulted in unsustainable yield with many species are at the verge of collapse. According to FAO (2012) report, among the seven principal tuna species, one-third were estimated to be overexploited, 37.5% were fully exploited, and only 29% are underexploited. The Indian Ocean is one among the most productive area of tuna fishing (Anganuzzi et al. 1996). India with a coastline of over 8000 Km has the potential to help its economy by judiciously exploiting the tuna resources.

One of the most commercialised tuna fish is yellowfin tuna *Thunnus albacares* (YFT), distributed globally in areas of the Pacific, Atlantic and Indian Ocean having surface temperature above 18° C (Collette and Nauen 1983). Globally, YFT contributes approximately 27 % of all tuna catches, making it second largest among principal market tunas (FAO, 2012). Similarly, they are the most dominant species among larger pelagic fishes and contribute approximately 19% of all tuna catches in Indian waters (Vijayakumaran and Verghese 2010).

The key to sound management of commercially important stocks of fish species of any Ocean is the identification of the number, distribution and degree of reproductive independence of any subpopulation of the species that might occur. Stock structure information in species under intense fishing pressure is required for effective management and sustainable catch limit of the species. Knowing the exact stock structure is critical for management of fishery, as different stocks may possess novel genetic, physiological,

behavioural characteristics that may have an effect on life cycle traits such as growth rates, fecundity, abundance and disease resistance (Stepien 1995).

There are several methods available for stock delineation, based on morphology, spawning locations, tagging, parasite loads, microchemistry and genetics. Although deployment of one or more approaches may be appropriate for resolving stock structure issues, genetics approach is sensitive and reliable. In particular, analysis of mitochondrial DNA has proved to be useful in determining population stock of fish species due to its simple mode of transmission avoiding recombination, high mutation rate and predominantly maternal inheritance (Graves et al. 1984; Hoolihan et al. 2004). Most of the variation in mitochondrial genome is confined to non-coding control (D-loop) region in vertebrate species (Meyer 1993). The amplification and direct sequencing of highly polymorphic regions of mtDNA offers a potentially rich source of variation at the nucleotide level for determining population stock structure within species and the phylogeny of intraspecific lineages (Wenink et al. 1993). The control region of mtDNA has been analysed to define population structure in many highly migratory pelagic fishes (bigeye tuna: Alvarado Bremer et al. 1998; bluefin tuna: Alvarado Bremer et al. 1997; swordfish: Alvarado Bremer et al. 1995, 1996; Rosel and Block 1996; Reeb et al. 2000). Also, the direct sequencing of mtDNA D-loop region revealed population structure of skipjack tuna (Menezes et al. 2012) and frigate tuna (Kumar et al. 2012) in Indian waters.

Although the population genetic study of YFT can be traced back to 1960s, when Tg2 blood group antigens were used for stock delineation in samples from equatorial Pacific and Indian Ocean (Suzuki 1962), there appears to be a lack of information on genetic stock of this species in Indian waters. Thus, this study attempts to investigate the genetic differentiation among samples of YFT from seven different regions along the Indian coast using mtDNA control region sequence data.

Material and methods :

DNA isolation and amplification

YFT (n=321) fin clip samples were collected from the seven fishing jetty all along the Indian coast including Lakshadweep and Andaman Sea (Table 1; Fig. 1) and were preserved in absolute ethanol. Whole genomic DNA were isolated from fin clip samples using TNES-Urea-Phenol Chloroform protocol (Asahida et al. 1996) and stored at -20 °C till further processing. A 500 bp fragment containing the first half of mtDNA D-loop (control region) was subjected to PCR amplification using the primer set and procedure as described in Menezes et al. (2006).

DNA Sequencing

PCR products were purified using PCR Cleanup Kits (Axygen Biosciences, California, USA) following the steps recommended by the manufacturer. All samples were sequenced in forward direction by the same primer as used for PCR amplification. Sequences were obtained at the DNA sequencing facility of CSIR-NIO, Goa, India, using Big Dye Terminator cycle sequencing kit (V3.1, Applied Biosystems, USA) following the manufacturers protocol and analyzed in Genetic analyzer (3130xl Genetic Analyzer, Applied Biosystems, USA). The representative sequences have been deposited in GenBank, with accession numbers KC165847-KC166134.

Data analysis

The sequences were edited in BioEdit version 7.0.1 (Hall 1999) and aligned with program ClustalW (Thompson et al. 1994) in MEGA5 (Tamura et al. 2011). Also MEGA5 was used for calculating nucleotide composition and DnaSP 4.0 (Rozas et al. 2003) to estimate the number of polymorphic sites and number of haplotypes.

ARLEQUIN version 3.11 (Excoffier et al. 2005) was used for calculating molecular diversity indices, such as transitions, transversions and also for estimating the relative population size (θ) and relative time since population expansion (τ). The estimated τ value was transformed to estimate time since expansion using the formula $\tau = 2\mu t$, where μ is the mutation rate per site per generation and t is the time since population expansion (Slatkin and Hudson 1991). In present study, the mutation rate of 3.6×10^{-8} mutations per site per year was applied for the control region sequence of YFT, as this rate has been reported for the mtDNA control region in teleosts (Donaldson and Wilson 1999).

Genetic diversity in each sampling region was measured as haplotype diversity (h), and nucleotide diversity (π) (Nei 1987). Analysis of molecular variance (AMOVA) was performed using Φ statistic (Φ_{ST}), to examine the amount of genetic variability partitioned among, between and within YFT populations (Excoffier et al. 1992). The significance of Φ_{ST} was tested by 10,000 permutations for each pairwise comparison. For all Φ_{ST} analysis, Tamura and Nei (1993) distance method was used. Spatial analysis of molecular variance (SAMOVA) (Dupanloup et al. 2002) was performed to identify groups that are geographically homogenous and maximally differentiated from each other. It considers geographical information on sampling sites along with statistics derived from AMOVA. It incorporates a simulated annealing approach to maximise the Φ_{CT} among groups of population and also identifying possible genetic barriers between them without pre-defining population.

The nearest-neighbour statistics S_{nn} (Hudson 2000) were used to measure the extent of population differentiation by testing if the sequences with low divergence are geographically proximate. S_{nn} is a measure to find out how often closely matched sequences are from the same locality in geographical location. Significance of S_{nn} was tested using 1,000 permutations.

Estimates of expected number of migrant females between populations per generation (N_{jm}) were calculated using the formula $2N_{jm} = ((1/\Phi_{ST})-1)$. For examining whether the population from different regions are at genetic equilibrium, Tajima's D statistical test (Tajima 1989) and F_s test of Fu (Fu 1997) were carried out. DnaSP was used (under the assumption of selective neutrality) for mismatch distribution analysis of pair-wise differences of all YFT samples to evaluate possible historical events of population growth and decline. Furthermore, Harpending's raggedness index (H_{ri}) (Harpending 1994) and sum of squared deviations (SSD) was calculated using ARLEQUIN to test whether the sequence data deviate significantly from the expectations of population expansion model.

A neighbour-joining (NJ) tree (Satiou and Nei 1987) was constructed based on Kimura two-parameter model (Kimura 1980) using MEGA5 (Tamura et al. 2011). The robustness of NJ phylogenetic hypothesis was tested by 10,000 bootstrap replicates (Felsenstein 1985). Only nodes with bootstrap support of greater than 50% are shown in the final tree.

Results:

Genetic Diversity

Sequence analysis of D-loop region revealed 196 polymorphic sites (120, parsimony informative) defining 288 haplotypes, of which 262 were unique (Supplementary data).

Haplotype diversity (h) of samples ranged from 0.9967(VI) to 1.0000(AG), while nucleotide diversity differed greatly among samples ranging from 0.053801(AG) to 0.10110(VE) (Table 1). The nucleotide transitions outnumbered transversion in mtDNA control region of YFT across all sampling sites (Table 2).

Population genetic structure

Pairwise comparison of Φ_{ST} after Bonferroni correction showed no significant genetic structure of YFT along the Indian waters (Table 3). AMOVA conducted for all the seven sampling sites in a single group revealed significant genetic variation among (3.84%, $P \leq 0.001$) and within (96.16%, $P \leq 0.001$) population (Table 3). The SAMOVA analysis showed one geographically meaningful group among the five groups analyzed ($\Phi_{CT} = 0.08124$, $P = 0.04790$; Table 4). Additionally, hierarchical analysis of molecular variance showed significant differentiation among three groups ($\Phi_{CT} = 0.08965$, $P < 0.05$; Table 5). Similarly, the S_{nn} analysis showed significant differentiation among sampling sites ($S_{nn} = 0.261$, $P \leq 0.001$). The expected number of female migrants between sampling sites per generation (using $\Phi_{ST} = 0.03844$; Table 3) was 13. The phylogenetic analysis using neighbor-joining method resulted in shallow branching with no clear partition between samples of different geographic locations (Fig. 2).

Historical demography

Tajima's D (-1.422) and Fu's F_s (-23.382) were negative and significant ($P < 0.001$; Table 6). The overall tau (τ) value of sequence data was 12.970, which reflects the population expansion of YFT in Indian Ocean occurred about 360,289 years ago. Large differences were observed between θ_0 (population before expansion) and θ_1 (population after expansion) suggesting the past population expansion of YFT in Indian waters (Table 6). In addition, the

unimodal mismatch distribution of pair-wise differences (Fig. 3), as well as non-significant deviations for the sum of squared deviations (*SSD*) and Harpending's raggedness index (*H_{ri}*) were also consistent in supporting the occurrence of population expansions of YFT in the Indian Ocean basin (Table 6).

Discussion

Most of the earlier assumptions about YFT stock structure in Indian Ocean were based on analysis carried out in other oceans, claiming single stock of YFT in Indian Ocean. However, a recent study based on mtDNA (ATPase 6 and 8 region) and nuclear DNA (microsatellites) markers suggested presence of more than one stock of YFT in Indian Ocean (Dammannagoda et al. 2008). Results of present study further strengthen the presence of more than one stock of YFT in Indian Ocean. The present study is the first genetic evaluation of YFT samples from the Indian region using mtDNA sequence data.

The analysis of population structure of YFT rejects the null hypothesis of a single panmictic population in the Indian region. The outcome is further supported by significant value of S_{nn} and AMOVA analysis. Furthermore, SAMOVA analysis of tested samples of YFT recognized VE and AG as discrete populations, and all remaining sites (KO, PB, VI, TU, and PO) together as a third discrete population.

The heterogeneity observed among YFT samples from Indian region could be a result of sampling error or alternatively biological and physical processes that promote reproductive isolation. In general, small sample size limit the statistical power of AMOVA and so it would possibly result in failure to detect population structure (type I error) rather than falsely indicating population structure. Further, a recent study carried out by Dammannagoda et al observed fine scale genetic heterogeneity in YFT samples around Sri Lanka (2008). Also, there have been reports of multiple highly divergent mtDNA lineages in other tuna and bill

fish species both between and within ocean basins (Menezes et al. 2012; Kumar et al. 2012; Martinez et al. 2006). The mtDNA heterogeneity observed in these studies and the current study may have been caused by common vicariance events during Pleistocene glacial maxima that resulted in isolation of populations by reduction in availability of tropical marine habitats due to decrease in ocean water temperature (Alvarado Bremer et al. 2005; Dammannagoda et al. 2008, 2011).

AMOVA revealed high levels of haplotype diversity among the sampling sites. Most of the observed haplotype appeared just once in different sampling sites, which is in accordance to the pattern classified for Scombrid fishes (Zardoya et al. 2004). High haplotype diversity within population may be due to the large population size, environmental heterogeneity and life history traits that favour rapid population growth (Nei 1987). In marine fishes, the high level of haplotype diversity is primarily due large population sizes (Avisé 1998). YFT is the second largest tuna fishery globally as well as in Indian waters, thus, large population size of YFT could be the reason for high haplotypes diversity observed in present study. However, pairwise comparisons among seven samples resulted in non significant Φ_{ST} values. The non significant Φ_{ST} value indicates lack of reproductive isolation between different populations (Boustany et al. 2008).

SAMOVA of mtDNA data rejects the null hypothesis of panmixia; with the identification of three genetically heterogeneous groups i.e. north-western (VE), south-western (AG) and rest of Indian seas (KO, PB, VI, TU, and PO). However, isolation by distance was not observed, hence strong philopatry and homing to specific natal spawning grounds would be needed to maintain the genetic differentiation observed.

Many life history traits such as homing behaviour (for spawning and feeding) play a significant role in determining genetic variability and population structure of marine fish

species. The observed population structure of VE and AG could be related to specific spatial feeding regimes.

In Indian Ocean, spawning occurs mainly from December to March in the equatorial area (0- 10°S) with the main spawning grounds west of 75°E (IOTC 2006). Secondary spawning grounds exist off India, in the Mozambique Channel and in the eastern Indian Ocean off Australia. A well recognised feeding ground has also been reported in the Arabian Sea, closer to AG site (IOTC 2006). It is quite possible that the samples of AG site might have originated from western Indian Ocean spawning ground and migrated towards feeding ground which is close to AG site.

VE is rich in coral reef fisheries (Deshmukhe et al. 2000). Tunas are known to feed on coral fishes, thus coral reef fisheries of VE making it as an important feeding ground for the tuna species. Although YFT spawning region is not yet not found in Arabian Sea near VE site, but a spawning region for kingfish near Oman Sea has been reported (Claereboudt et al. 2005). It is highly probable that somewhere near that region YFT spawning region may also exist. And the fish may have migrated towards Indian waters, where food availability is in bulk due to coral reef fisheries (Deshmukhe et al. 2000). Thus, the presence of specific feeding areas at AG and VE may have caused segregation of stocks within the geographical distribution of the YFT samples. The individual at remaining sampled sites (KO, TU, PO, VI, and PB) may have come from locally spawned fish.

Alternatively, the three different stocks (VE, AG and rest all) may have there spawning region in distant areas of the Indian Ocean, following which juveniles might disperse towards Indian water, which is highly fertile feeding ground. This high fertile water are result of the coastal upwelling along the coast of Arabia, Somalia and Indian subcontinent during south-west monsoon periods leading to high primary and secondary productivity

(Wyrcki 1973; Qasim 1982). This high primary productivity is responsible for large schools of baitfish upon which tuna species including YFT feed. YFT is capable of extensive movement, can seek out a convivial habitat and may exploit total areas of ocean productivity. Thus, in present study the three genetically different populations of YFT may spawn in different areas of the Indian Ocean and physically mix during their extended duration of feeding migration as observed in case of skipjack tuna (Fujino 1996).

Phylogenetic analysis of mtDNA D-loop control region sequences revealed no obvious phylogeographic pattern separating the sampling sites of YFT (Fig. 3). Haplotypes from all the samples are interspersed throughout the tree. The presence of single clade among seven sampling sites of YFT is due to frequent contact and interbreeding among samples subsequent to their geographical isolation for long period. The asymmetrical distribution of haplotypes can be attributed to the potential unidirectional gene flow of formerly allopatric populations during interglacial periods providing secondary contacts (Alvarado Bremer et al. 2005).

The present study, in addition to provide insight into population structure also aimed at revealing evolutionary history of YFT in Indian waters incorporating several statistical analyses such as, Tajima's D , Fu's F_s , τ value, and estimation of theta (θ). If populations deviate from the neutral expectations, Tajima's D test gives low significant value. In present study, Tajima's D is negative and highly significant suggesting historical population expansion which is consistent with the study carried out by Ely et al. (2005). The Fu's F_s tends to be negative when there is an excess of recent mutations, indicating deviation caused by population expansion and/or selection (Su et al. 2001). Rapid historical population expansion is well supported by large differences observed between θ_o (6.904) and θ_i (99999). The global YFT population has experiences past population expansion as observed in previous studies (Ward et al. 1997; Ely et al. 2005). The tau value (τ) provided a rough

estimate of the time when rapid population expansion started in Indian Ocean in general and Indian waters in particular. The overall tau (τ) value observed in this study (12.970) was greater than global YFT population ($\tau=8.516$; Ely et al. 2005). Interestingly, higher tau (τ) value had been observed in samples of skipjack tuna from Indian waters, which is proposed to be due to the origin of this species in Indian Ocean and thus also being oldest population (Menezes et al. 2012). Furthermore, the historical population expansion is also consistent with unimodal mismatch distribution of pair-wise differences in all YFT samples as well as non-significant deviations for the sum of squared deviations (*SSD*) and Harpending's raggedness index (*Hri*).

The present study concludes low but significant genetic differentiation among seven sampling sites of YFT in Indian EEZ region. Findings of this study support at least three genetic stocks of YFT in Indian waters i.e. one around north-western coast (VE), a second around south-western coast (AG) and a third around rest of Indian Seas. The presence of genetically distinct YFT populations in Indian region raises important management considerations for this species. However further work is needed to confirm the natal homing of three YFT populations to identify potential spawning sites in the Indian Ocean.

The genetic marker used in the present study is mtDNA which is maternally inherited. The genetic effect of male dispersal cannot be addressed by mtDNA. In addition, it is also non-recombining and often treated as a single character. Therefore nuclear DNA studies (microsatellite markers) should also be taken in to account to study genetic stocks of YFT in future studies. In addition to above molecular techniques, electronic tagging studies will certainly help to identify possible migratory pattern and hence direct evidence of population structure and forces that maintain that structure.

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Fig. 1 Map showing the sampling locations of YFT along Indian coast. Verava(VE), Agatti(AG), Kochi (KO), Port-Blair (PB), Tuticorin (TU), Pondicherry (PO), Vizag (VI).

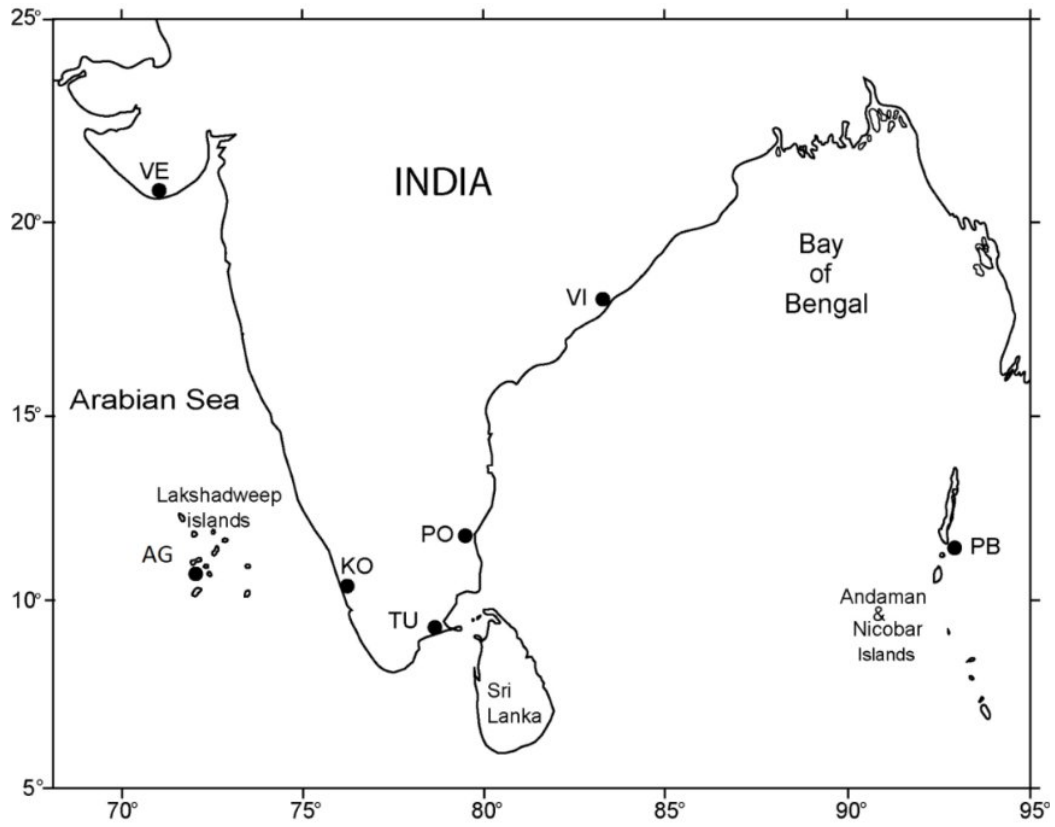


Fig. 2 Mismatch distributions of YFT (*Thunnus albacores*) mtDNA D-loop region. Exp: expected distribution, Obs: observed distribution under the sudden expansion model.

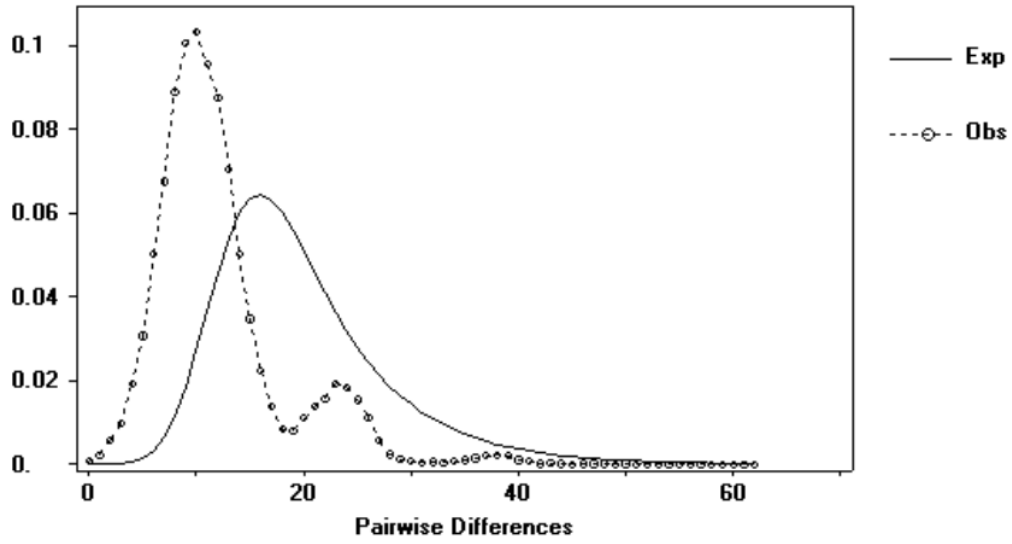


Fig. 3 Neighbour-Joining tree estimated from Kimura two-parameter distances among mtDNA haplotypes of yellowfin tuna. Numbers along side the nodes indicates bootstrap value, and only values >50% are shown.

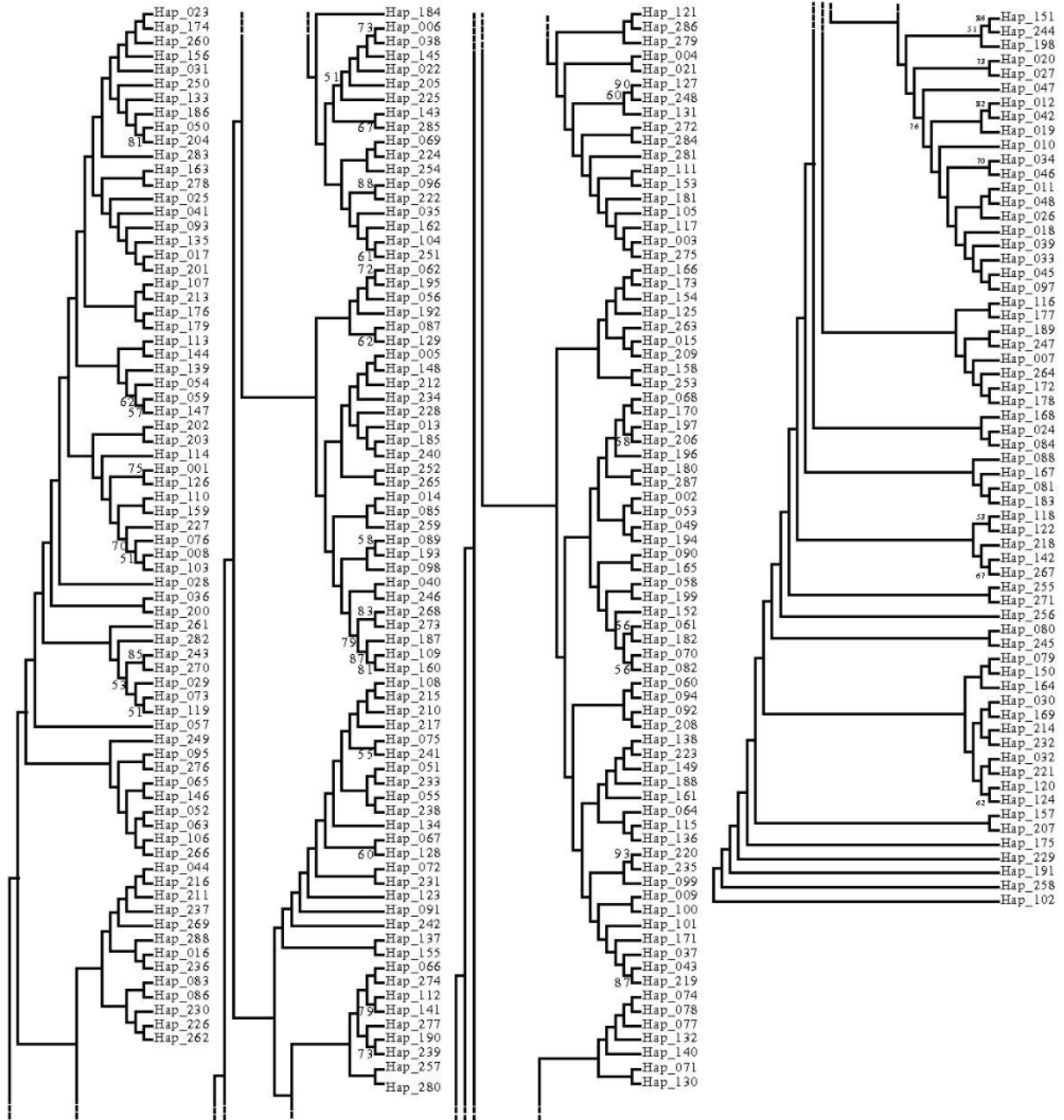


Table 1 Details of sampling

Estimates of genetic diversity within populations for mitochondrial DNA sequence data:

number of samples (*n*); number of haplotypes (*nh*); and haplotype diversity (*h*).

Population	Location	n	Date of collection	nh	h
Veraval (VE)	20.54°N 70.22°E	50	October, 2007	48	0.9984
Kochi (KO)	9.58°N 76.16°E	48	February, 2008	44	0.9991
Port-Blair (PB)	11.40°N 92.46°E	50	July, 2008	42	0.9991
Vizag (VI)	17.42°N 83.15°E	50	February, 2009	44	0.9967
Pondicherry (PO)	11.56°N 79.50°E	50	July, 2008	44	0.9984
Agatti (AG)	10.50°N 72.12°E	23	November, 2008	19	1.0000
Tuticorin (TU)	8.49°N 78.08°E	50	September, 2009	47	0.9992
Total		321		288	0.9987

Table 2 Pairwise Φ_{ST} (below) and p (above) values among populations of YFT after Bonferroni correction ($\alpha = 0.05/21 = 0.0023$).

	VE	KO	PB	VI	PO	AG	TU
VE		0.24915	0.12090	0.01275	0.18175	0.25625	0.40145
KO	0.11783		0.95500	0.04065	0.34065	0.51465	0.69140
PB	0.07823	0.00853		0.08605	0.54270	0.75990	0.44640
VI	0.09218	0.00586	-0.00767		0.02480	0.11740	0.23285
PO	0.09478	-0.00365	-0.00251	-0.00547		0.85045	0.60130
AG	0.09499	0.00761	0.01041	0.01321	0.00267		0.57135
TU	0.10902	0.00324	-0.00560	-0.00526	-0.00688	0.01613	

Table 3 Results of analysis of molecular variance (AMOVA) testing genetic structure of YFT based on mitochondrial D-loop region sequence data.

Structure tested (sampling sites)	Variance	Percentage of Variation	Φ Statistic	<i>p</i> -values
One group (VE, (KO, PB, VI, PO, AG, TU)				
Among population	0.25686	3.84	$\Phi = 0.03844^{***}$	<0.001
Within Population	6.42450	96.16		

*** Significant at $P < 0.001$.

Table 4 Population structure based on mtDNA differentiation of YFT (in Spatial analysis of molecular variance, SAMOVA)

No. of Groups(K)	Structure	Variation among groups	Percentage of variation	Φ_{CT}	p-values
2	(VE); (KO, PB, VI, PO, AG, TU)	0.70370	10.74	0.10744	0.14467
3	(KO, PB, VI, PO, TU); (VE); (AG)	0.51615	8.12	0.08124	0.04790
4	(KO); (PB, VI, PO, TU); (AG); (VE)	0.37081	5.99	0.05990	0.02248
5	(PB); (VE); (KO); (AG); (VI, PO, TU)	0.30100	4.92	0.04923	0.02444
6	(TU); (AG); (KO); (PO);(VE); (PB,VI)	0.26459	4.35	0.04352	0.00978

*, Significant at $P < 0.05$

Table 5 Results of analysis of molecular variance (AMOVA) testing genetic structure of YFT based on mitochondrial D-loop region sequence data.

Structure tested (sampling sites)	Variance	Percentage of Variation	Φ Statistic	<i>p</i> -values
Three groups (KO, PB, VI, PO, TU), (VE), (AG)				
Among groups	0.63066	8.97	Φ_{CT} = 0.08965*	<i>P</i> = 0.0439
Among population within groups	-0.02069	-0.29	Φ_{SC} = -0.00323	<i>P</i> = 0.7341
Within Population	6.42450	91.33	Φ_{ST} = 0.08671	<i>P</i> < 0.001

*, Significant at *P* < 0.05

Table 6 Genetic diversity indices and demographic parameters of YFT based on mitochondrial D-loop region sequence data. t_i , number of transitions; t_v , number of transversions; π , nucleotide diversity. Mismatch distribution parameters τ , θ_0 , θ_1 , Tajima's D test and Fu's F_s values, Harpending's Raggedness index (Hri), and sum of squared differences (SDD).

Population	t_i	t_v	π	τ	θ_0	θ_1	Tajima's D	Fu's F_s	Hri	SDD
VE	75	20	0.101	20.523	29.121	99999	-0.409*	-24.241***	0.0082	0.0143
KO	81	20	0.062	12.656	9.066	99999	-1.721*	-24.585***	0.0025	0.0003
PB	80	27	0.073	12.667	2.228	99999	-1.585*	-24.449***	0.0047	0.0015
VI	76	26	0.069	12.755	2.420	99999	-1.605*	-24.480***	0.0063	0.0016
PO	80	25	0.066	12.205	2.144	99999	-1.678*	-24.515***	0.0077	0.0022
AG	46	08	0.053	9.324	1.028	99999	-1.321*	-16.761***	0.0129	0.0103
TU	71	15	0.058	10.660	2.323	99999	-1.636*	-24.646***	0.0058	0.0006
Mean	72.71	20.14	0.069	12.970	6.904	99999	-1.422*	-23.382***	0.0068	0.0044

*, *** Significant at $P < 0.05$ and $P < 0.001$ respectively.