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RESEARCH ARTICLE

Mitochondrial DNA based diversity studies reveal distinct and sub-structured populations of pearlspot, *Etroplus suratensis* (Bloch, 1790) in Indian waters

SIVAMANI BALASUBRAMANIAM*, MISHA SOMAN, VINAYA KUMAR KATNENI, SHERLY TOMY, GOPIKRISHNA GOPALAPILLAY and K. K. VIJAYAN

ICAR - Central Institute of Brackishwater Aquaculture, Chennai 600 028, India
*For correspondence. E-mail: drsivamani@gmail.com.

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Abstract. Pearlspot (*Etroplus suratensis*) is one of the most commercially important brackish water fish species widely found along the coastal regions of peninsular India and Sri Lanka. Pearlspot is known for its tender flesh, delectable taste, culinary tourism and high-yielding market value. Information on the genetic makeup of stocks/populations is extremely vital as it forms the basis for future genetic studies. For this, we utilized *ATPase6/8* genes of mtDNA of pearlspot populations collected from nine different locations ranging from Ratnagiri in Maharashtra state on the west coast to Chilika in Odisha on the east coast. Sequence analyses of these genes revealed 33 polymorphic sites, which include 17 singleton and 16 parsimony informative sites. Pair-wise genetic differentiation study (F_{ST} = 0.75) indicated significant ($P < 0.001$) differences among all the pairs of stocks except those from Chilika and Nagayalanka. The spatial analysis of molecular variance (SAMOVA) significantly delineated the population into four groups (F_{CT} = 0.69, P = 0.0001), namely northwest (Ratnagiri and Goa); southwest (Mangalore and lakes at Vembanad, Ashtamudi and Vellayani in Kerala); southeast (Pulicat in Tamil Nadu) and northeast (Chilika in Odisha and Nagayalanka in Andhra Pradesh). The above delineation is supported by clades of the phylogenetic tree and also the clusters of median joining haplotype network. The high haplotype diversity (0.84), low nucleotide diversity (0.003), and negative values of Tajima's D (–1.47) and Fu's Fs statistic (–14.89) are characteristic of populations having recently undergone demographic expansion. Mantel test revealed significant isolation by distance. The study identifies highly delineated structured populations with restricted gene flow. If such a stock is overfished, it is highly unlikely that it would recover through migration. For any future breeding programme in this species, it would be desirable to form a base population which incorporates the genetic material from all the locations so that we get a wide gene pool to select from.

Keywords. pearlspot; population diversity; mtDNA markers; demographic expansion; *Etroplus suratensis.*

Introduction

Etroplus suratensis (Bloch 1790) also commonly known as pearlspot or the green chromide, is an indigenous cichlid species of Asia with restricted natural distribution in lagoons and brackish water bodies of southern India and Sri Lanka (Ward and Wyman 1977). The fish has also been introduced into peninsular Malaysia and Singapore, where feral populations appear to be increasing (Welcomme 1988). Being a euryhaline species (Chandrasekar *et al.* 2014), it primarily inhabits brackish water bodies and has also been reported in fresh water habitats (Abraham 1995). In India, its occurrence in the scale of commercial catch extends from Goa on the west coast to Chilika lagoon on the east coast (Rattan 1994). Pearlspot is known for its tender flesh, delectable taste, culinary tourism and high-yielding market value. In Kerala, this fish is considered a delicacy through a culinary procedure specific to the state. The fish fetches about ₹400 per kg in Kerala. Besides its high demand as food source, this species is also gaining popularity as an ornamental fish due to its brilliant, attractive and appealing colouration.

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Total inland production of pearlspot in Kerala was around 4644 t in 2006–2007 which reduced to 4194 t in 2018–2019. At the same time, the total inland fish production in Kerala had increased from 79,647 t in 2006–2007 to 192,027 t in 2018–2019 (Kerala Inland Fisheries Statistics 2010; Fisheries Handbook 2020, Kerala). The annual landings of pearlpsot fish from Vembanad Lake of Kerala have also gone down from 458 t in 1989 (Kurup *et al*. 1995) to 135.28 t in 2012–2013 (Roshni *et al*. 2017). The decline is primarily caused by various anthropogenic activities including indiscriminate dredging of the lake bottom for lime shell deposits, developing ports and marinas, boating, overfishing and disposal of effluents and toxic sewages from industries and municipalities (Padmakumar *et al*. 2002; Bukola *et al*. 2015). The decline in the pearlspot population is also attributed to its low recruitment rate possibly due to the low fecundity and seasonal breeding behaviour of the species. In 2010, the Kerala state declared pearlspot as the 'State Fish' to recognize its overall importance and to protect it through various conservation measures. Recently, the fish has been gaining popularity as a candidate aquaculture species in inland saline regions too (Kumar *et al*. 2009).

The species is a euryhaline one and can be bred easily in captive conditions. Therefore, pearlspot appears to be a suitable species for a selective breeding programme. Before embarking on any studies in genetics of this species, it would initially be desirable to comprehend the genetic diversity of different stocks. Genetically diverse populations play a major role in conferring sustainability by adapting to the local climate and geographic changes and buffer the overall productivity (Ruzzante *et al*. 2006). It is well known that the base population in a selective breeding programme should contain fishes from different locations so that wide genetic diversity is incorporated to the maximum to realize substantial genetic gains.

Molecular markers are the most preferred tools for characterizing fish stocks due to their being independent of the environment and also polymorphic nature. They have been utilized to estimate effective population size, historical demography, gene flow and isolation by distance (IBD) (Habib *et al*. 2012). Among molecular markers, mitochondrial DNA (mtDNA) is much preferred because of its smaller genome size, high evolutionary mutation rate (Liu and Cordes 2004), neutral evolution (Galtier *et al*. 2009), nonrecombining nature (Wilson *et al*. 1985) and maternal inheritance (Birky *et al*. 1989). The *ATPase6* and *ATPase8* genes of mtDNA are generally variable in vertebrates, have a high evolutionary rate (1.3% per million years) in fishes (Habib *et al*. 2012) and have been utilized extensively to investigate the stock structure and phylogeny in several fish species including Bronze featherback (Gupta *et al*. 2013), Snow trout (Ali *et al*. 2014), Silver pomfret (Divya *et al*. 2015), Snakehead murrel (Baisvar *et al*. 2015), Catfish (Ponzetto *et al*. 2017) and Mrigal carp (Das *et al*. 2018). A few studies (Alex *et al*. 2013, 2016) have documented the molecular diversity of pearlspot populations of Kerala. However, there is another report by Chandrasekar *et al*. (2019) relating to the pearlspot populations of Karnataka, Kerala, Tamil Nadu and Andhra Pradesh.

Previously known demographic patterns of this indigenous cichlid are also not available. Absence of baseline data on evolutionary pattern, stock diversity and historical demography in its habitat are the major impediments in understanding the role of climate, geographic and environmental impact on dynamics of the population (Sukumaran *et al*. 2016). Further, comprehending the stock structure and its diversity pattern are essential towards generating the base population for initiating genetic improvement programme in any species. Hence, the present study was carried out to comprehend the subpopulation structure and diversity of pearlspot across the Indian coast extending from Maharashtra in the west to Odisha in the east, at nine different locations using the mtDNA marker-*ATPase 6/8* genes.

Materials and methods

Sample collection and DNA extraction

Two hundred and three specimens of pearlspot fish were collected from nine different locations of brackish and back-water lagoons (table 1; figure 1). The nine locations were further classified into four major geographical regions, namely near northeast coast (Chilika, Nagayalanka), southeast coast (Pulicat), southwest coast (Ullal in Mangalore and the lakes at Vellayani, Ashtamudi and Vembanad in Kerala state) and northwest coast (Goa and Ratnagiri). The codes for stocks are: CHL, Chilika; NGL, Nagayalanka; PUL, Pulicat; VEL, Vellayani; ASH, Ashtamudi; VMB, Vembanad; MAN, Mangalore; GOA, Goa; RAT, Ratnagiri. Mostly, the fishes were caught by gill net or cast net. Tissue samples (muscle and fin clips) were preserved and stored in 90% ethanol at room temperature until further use. Total DNA was extracted from 50 mg tissue using standard phenol-chloroform extraction method (Sambrook and Russel 2006). The DNA was confirmed by 1% agarose gel electrophoresis and quantification was carried out using Nanodrop 2000 (Thermo Fisher Scientific, USA).

PCR amplification of ATPase 6/8 gene and sequencing

The *ATPase 6/8* genes were PCR amplified using in-house designed primers which are specific to the genus *Etroplus* (E-ATPF-7922: 5′-AGCCTTTTAAGCTAAAGACTGGTG-3′ and E-ATPR-8841: 5′-TACTATGTGATATGCGT GTGCTTG-3′). Amplification was carried out in 25 μL volume using Taq DNA polymerase 2× master mix Red containing final concentration of 1.5 mM $MgCl_2$ (Ampliqon,

Table 1. Sampling details and diversity indices.

Region	Population/stock	Number of samples	Month and year of collection	Total haplotypes	Variable sites	Haplo type diversity	Nucleotide diversity	Per cent of private haplotype	GenBank accession number
Northeast coast	Chilika	19	February, 2019	2	1	0.105	0.00013	3.45	MH197304–322
	Nagayalanka	10	February, 2019	1	0	0	0	0	MH197323–332
Southeast	Pulicat	32	January, 2019	3	2	0.333	0.00044	6.90	MH197333–352; MK953764–775
	East coast/east	61		4	3	0.567	0.0008	10.34	
Southwest coast	Vellayani	17	July, 2019	5	5	0.728	0.00180	10.34	MK953776–792
	Ashtamudi	24	July, 2019	4	3	0.308	0.00050	10.34	MK953793–800; MK956146–161
	Vembanad	40	January, 2019	9	10	0.691	0.00138	20.69	MH219931–970
	Mangalore	19	March, 2019	5	7	0.673	0.00232	13.79	MH219971–989
	Southwest	100		19	22	0.681	0.00163	48.28	
Northwest coast	Goa	19	March, 2019	7	6	0.667	0.00114	20.69	MH220013–031
	Ratnagiri	23	March, 2019	1	0	0	0	3.45	MH219990–MH220012
	Northwest	42		7	6	0.587	0.00097	24.14	
	West coast	142		26	31	0.807	0.00326	86.21	
Overall		203		29	33	0.843	0.00317		

Denmark), 50 ng DNA and 0.6 μM of each primers. The thermal profile used to amplify *ATPase 6/8* genes consisted of an initial denaturation of 95°C for 2 min followed by 30 cycles of 95°C for 25 s, 56°C for 30 s, 72°C for 1 min and a final extension at 72°C for 5 min. The amplified PCR products were resolved in 1% agarose gel electrophoresis and visualized using ethidium bromide staining under UV light. The PCR products were purified and sequenced bidirectionally using ABI 3730 capillary sequencer (Applied Biosystems) following the manufacturer's instructions. Sequencing was outsourced.

Generation of consensus sequences

The consensus sequences were generated using Geneious-R11.1. They were aligned using ClustalW application and edited by Bioedit7.2.6 (Hall 1999) for ensuring equal length. The sequences were verified by NCBI-blastn for the concerned species and gene fragment. The default parameters for blastn were utilized to search the database of nucleotide collection (nr/nt) using Megablast which was optimized to hit highly similar sequences (>95% identity). The query sequence is expected to match around 99% with the available *ATPase 6/8* sequences of the complete mtDNA of pearlspot.

Population genetic structure

The number of polymorphic sites (S), nucleotide diversity (π), number of haplotypes, haplotype diversity (H), private haplotypes, nucleotide divergence (D_{xy}), net genetic distance (D_a), coefficient of differentiation (G_{ST}), gene flow (N_m) and transition transversion count were assessed for each population and region using Dnasp v6 (Rozas *et al.* 2017). Fixation indices (F_{ST}) were calculated to assess overall genetic divergence and also between paired populations. The F_{ST} was estimated by conventional F statistics using haplotype frequencies with 1023 nonparametric permutations. The significance of the F_{ST} was determined by the P value generated in Arlequin v3.5.1.3 (Excoffier and Lischer 2010). To determine the population genetic structure within and between populations, a hierarchical analysis of molecular variance (AMOVA) was computed using Arlequin v3.5.1.3. Spatial analysis of molecular variance (SAMOVA) by SAMOVA 2.0 was carried out (Dupanloup *et al.* 2002) to identify groups of populations that were geographically homogeneous and maximally differentiated from each other based on K2P molecular distance (Kimura 1980). A range of values of k from $k = 2$ to $k =$ maximum collected populations (9) was tested using 10000 simulated annealing processes to determine the optimal clades for these nine populations. Haplotype network was constructed using PopART v. 1.7 (Bandelt *et al.* 1999).

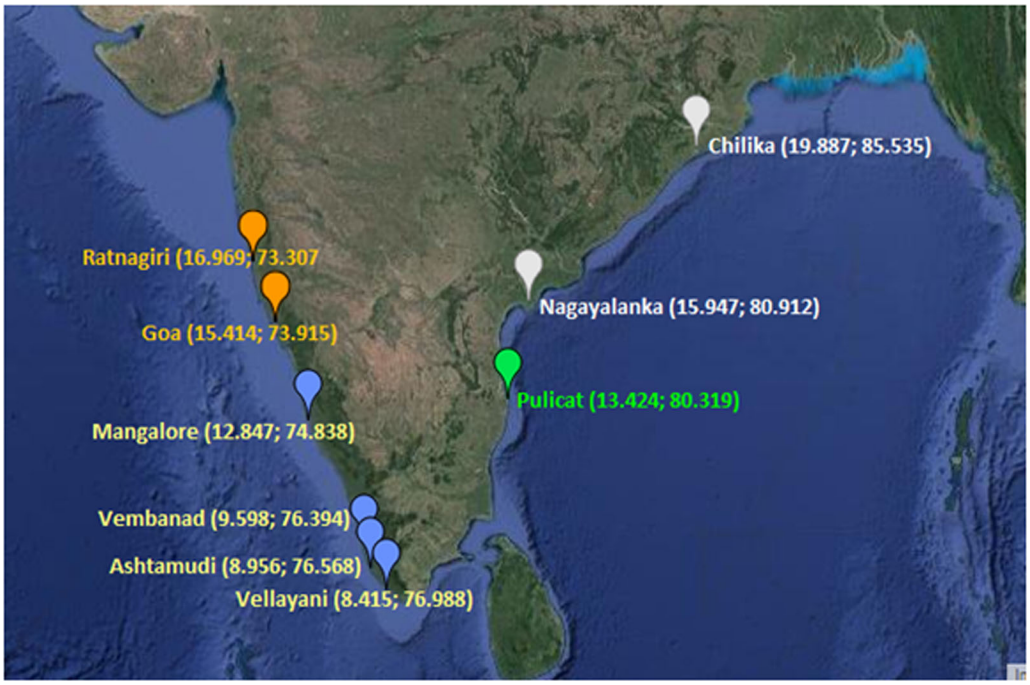

Figure 1. Sampling locations.

Phylogenetic tree construction

The phylogenetic tree was constructed by Mega-v7.0 (Kumar *et al*. 2016) using maximum likelihood method. The best evolutionary model for nucleotide substitution was selected using the corrected Akaike's information criterion (AICc) implemented in Mega-v7.0 (file 1 in electronic supplementary material at http://www.ias.ac.in/jgenet). Robustness of the inferred tree was evaluated using bootstrap analysis on 1000 replications. A Bayesian phylogenetic tree was also constructed using Mr.Bayes-v3.2.2 (Ronquist *et al*. 2012). The best fit partitioning schemes and evolutionary model were selected for the sequences using AICc implemented in PartitionFinder 2 (Lanfear *et al*. 2016).

Population demographics

Mismatch distribution (Rogers and Harpending 1992; Schneider and Excoffier 1999) was estimated on the entire population and on each major geographical region using Arlequin3.5.1.2 and DnaSP6.12.01.

Distribution of pair-wise nucleotide differences generated by the stepwise expansion model (demographic and spatial) was validated by parametric bootstrapping which compares the fit of the observed and 1000 simulated mismatch distributions with the expected mismatch distribution. To test the goodness-of-fit of observed distributions with those expected, the sum of squared deviations (SSD) and Harpending's raggedness index (r) were calculated using demographic and spatial model of population expansion. To find the neutrality of evolution of sequences, Tajima's D statistics (Tajima 1989) and Fu's F_S (Fu 1997) were tested using Arlequin 3.5.1.2. The significance of the test was carried out with over 1000 permutations.

The parameters of the demographic expansion τ, θ_0, and θ_1 were derived using generalized nonlinear least-squares approach (Schneider and Excoffier 1999) with bootstrap confidence intervals as implemented in Arlequin, v. 3.5.1.2. The values of τ were transformed to estimate of real time since expansion with the equation $\tau = 2ut$, where τ is the mode of the mismatch distribution. The parameter '2u' was estimated with the equation $2u = \mu k$ (Nei and Tajima 1981); where μ is the mutation rate per nucleotide and $k = 792$ which is the number of nucleotides of the sequence analysed. Here, t in years ($t = 1$) is the generation length of the species studied. A mutation rate of 1.3% per million years (Myr) was used for *ATPase* which is reported as the mean rate for vertebrate mtDNA (Bermingham *et al*. 1997). Effective population size was estimated for initial population N_0 and expanded population N_1 by substituting $\theta_0 = 2uN_0$ ($N_0 = \theta_0/2u$) and $\theta_1 = 2uN_1$ ($N_1 = \theta_1/2u$) where $2u = \mu k$ is the mutational rate of mitochondrial DNA considering a generation length of about a year for pearlspot (Sukumaran *et al.* 2017).

The past population dynamics in terms of female effective population size (N_e) through time was assessed using Bayesian skyline analysis and the skyline plots (Barido-Sottani *et al.* 2018) were constructed using BEAST v1.8. The *ATPase 6/8* sequences were converted to xml file using BEAUti-V2.6.1 and the parameters were set for GTR site model under strict clock with 1.3% Myr mutation rate, one year generation time and 9×10^6 chain length. The xml file

was run using standard Markov chain Monte Carlo (MCMC) sampling procedure in Beast-v2.6.1 to generate log and tree file. The Beast run was visualized using Tracer 1.7.1.

The relationship between genetic dissimilarity and geographic distance was determined by Mantel test (Mantel 1967) using ade4 library of R. The F_{ST} values represented genetic distance matrix whereas geographic distance matrix was represented by the shortest water route (in nautical miles) between the sampling sites estimated by Google Earth.

Results

Sequence analysis

Of the 920 bp fragment of *ATPase 6/8* genes obtained from 203 pearlspot fish samples, only 792 bp was utilized for the diversity studies as the consensus sequences were edited at both the ends after the alignment to obtain equal length. The edited consensus sequences had initial 136 nucleotides from *ATPase8* gene and the rest 656 were from the *ATPase6* gene including an overlapping region of 10 bp from 127 to 136 bp. Three highly conserved regions were observed and all of them were from *ATPase6* gene region, i.e. 159–250; 399–496 and 666–739. The nucleotide composition of all the sequences were 26.2% A, 27.6% T, 34.4% C, 11.8% G, 53.8% A+T, and 46.2% G+C. The presence of A+T was comparatively more than G+C. Total variable sites were 33 (including 17 singleton and 16 parsimony informative sites) and accounted for 4.17% of the nucleotide length utilized in this study. Further, it had more transition (32) substitutions than transversions (2).

Genetic statistics of populations

The analysis identified 29 haplotypes. The overall haplotype and nucleotide diversity values were 0.843 and 0.003, respectively (table 1). The most common haplotype was found among 54 samples and it belonged to southwest region only. The southwest group is the largest haplotype group which consists of 19 haplotypes with a diversity value of 0.681. The next bigger one is northwest group with seven haplotypes and the diversity of 0.587. This was followed by east-coast group having four haplotypes with a diversity of 0.567. Haplotype diversity ranged from 0 to 0.728 across the stocks. Between the stocks, Vellayani exhibited highest haplotype diversity (0.758) followed by Vembanad (0.691). The stocks from Ratnagiri and Nagayalanka were monomorphic and each harboured a single haplotype. Nucleotide diversity among the stocks ranged from 0.00013 (Chilika) to 0.00232 (Mangalore). Among the stocks, Goa and Vembanad had more unique haplotypes (6) followed by Mangalore (4), Vellayani (3) and Ashtamudi (3). The stocks with high number of nucleotide variable sites were Vembanad (10), Mangalore (7) and Goa (6). Pearlspot from the southwest coast region exhibited highest nucleotide diversity (0.00163) followed by northwest (0.00097) and east-coast (0.0008). Both nucleotides and haplotypes demonstrated a high level of genetic diversity among the southwest region stocks.

The average number of nucleotide difference values (table 2) within populations ranged from 0 (Ratnagiri and Nagayalanka) to 1.836 (Mangalore) and 0.053 to 6.000 between populations. The average number of nucleotide differences between populations was maximum between Pulicat and Ratnagiri populations and minimum between Chilika and Nagayalanka populations. Goa and Ratnagiri populations exhibited a higher average number of nucleotide differences values compared to other populations, however, among them, it remained minimum.

The values of D_{xy}, D_a were also calculated among the fish populations (table 3). The least D_{xy} (0.00007) and D_a (0.0000) values were observed between Chilika and Nagayalanka stocks. The highest D_{xy} (0.00758) and D_a (0.00735) were observed between Pulicat and Ratnagiri stocks. The above findings reveal that the D_{xy} and D_a values were substantially consistent in the present study to define genetic differentiation between the populations.

Table 2. Average number of nucleotide differences and N_m estimates.

	CHL	NGL	PUL	VEL	ASH	VMB	MAN	GOA	RAT
CHL	**0.105**	∞	0.181	0.340	0.138	1.179	0.318	0.314	0.025
NGL	0.053	**0.000**	0.177	0.375	0.132	1.231	0.346	0.342	0.000
PUL	1.053	1.000	**0.351**	0.492	0.237	0.592	0.456	0.452	0.117
VEL	1.347	1.294	2.239	**1.426**	2.566	3.616	6.683	1.148	0.239
ASH	1.261	1.208	2.208	1.091	**0.395**	1.674	3.197	0.452	0.093
VMB	0.903	0.850	1.845	1.353	0.958	**1.092**	3.189	1.065	0.341
MAN	2.158	2.105	3.095	1.957	1.314	1.853	**1.836**	1.013	0.227
GOA	4.526	4.474	5.474	4.356	3.682	4.224	4.579	**0.901**	0.266
RAT	5.053	5.000	6.000	4.882	4.208	4.750	5.105	1.158	**0.000**

Above diagonal, N_m estimates; diagonal (bold), within average number of nucleotide differences; below diagonal, between average number of nucleotide differences.

Table 3. D_{xy} D_a values for *E. suratensis* populations (below diagonal, D_{xy}; above diagonal, D_a).

	CHL	NGL	PUL	VEL	ASH	VMB	MAN	GOA	RAT
CHL	*****	0.00000	0.00104	0.00073	0.00128	0.00038	0.00150	0.00508	0.00631
NGL	0.00007	*****	0.00104	0.00073	0.00128	0.00038	0.00150	0.00508	0.00631
PUL	0.00133	0.00126	*****	0.00170	0.00232	0.00142	0.00253	0.00612	0.00735
VEL	0.00170	0.00163	0.00283	*****	0.00023	0.00012	0.00041	0.00403	0.00526
ASH	0.00159	0.00153	0.00279	0.00138	*****	0.00027	0.00025	0.00383	0.00506
VMB	0.00114	0.00107	0.00233	0.00171	0.00121	*****	0.00049	0.00407	0.00531
MAN	0.00272	0.00266	0.00391	0.00247	0.00166	0.00234	*****	0.00405	0.00529
GOA	0.00572	0.00565	0.00691	0.00550	0.00465	0.00533	0.00578	*****	0.00089
RAT	0.00638	0.00631	0.00758	0.00616	0.00531	0.006	0.00645	0.00146	*****

Table 4. Estimates of F_{ST} and G_{ST} (F_{ST}, below diagonal; G_{ST}, above diagonal) for *E. suratensis* populations.

	CHL	NGL	PUL	VEL	ASH	VMB	MAN	GOA	RAT
CHL	0.00000	0.00366	0.56646	0.42141	0.64185	0.16402	0.44000	0.44304	0.90790
NGL	-0.0382	0.00000	0.51648	0.39390	0.61860	0.13686	0.40629	0.40945	1.00000
PUL	**0.73465**	**0.73853**	0.00000	0.31279	0.50835	0.29512	0.33674	0.33911	0.67705
VEL	**0.5951**	**0.57114**	**0.50427**	0.00000	0.08361	0.05740	0.03591	0.17830	0.50134
ASH	**0.78345**	**0.7908**	**0.6784**	0.16308**	0.00000	0.12602	0.06990	0.35167	0.72890
VMB	**0.29786**	0.28884**	**0.45783**	0.12148**	**0.22996**	0.00000	0.06639	0.17200	0.40926
MAN	**0.61111**	**0.59077**	**0.52275**	0.06961*	0.13526**	0.13555**	0.00000	0.19790	0.51950
GOA	**0.61404**	**0.59402**	**0.52534**	**0.30338**	**0.52512**	**0.31949**	**0.33041**	0.00000	0.47985
RAT	**0.9524**	**1.000**	**0.81039**	**0.67678**	**0.84308**	**0.5946**	**0.68786**	**0.65299**	0.00000

*Significant at $P<0.05$; **significant at $P<0.01$; bold values below diagonal are significant at $P<0.001$.

Genetic structure of populations

The genetic differentiation between nine fish populations was studied using Wright's pair-wise F-statistics (F_{ST}) and genetic differentiation coefficient (G_{ST}). There were 36 possible stock comparisons and estimates for the pairwise F_{ST} values (below diagonal) and G_{ST} values (above diagonal), all of which are presented in table 4.

From the estimate of F_{ST}, the level of gene flow as measured by the product N_m can be computed from Wright's (1951) result for haploid organisms in an island model of population structure. In our study, the number of migrants was calculated using F_{ST} values and is presented in table 2. As evident, the number of migrants between Chilika and Nagayalanka was very high and no significant F_{ST} difference between the two regions was detected. Next highest value was observed between Vellayani and Mangalore populations. Southwest populations have comparatively higher N_m among them. Other populations have comparatively less N_m among them. The least N_m was observed with Ratnagiri and east populations (Nagayalanka, Chilika and Pulicat). This is as expected because Ratnagiri is in the west coast and Nagayalanka, Chilika and Pulicat are in the east.

Hierarchical AMOVA test (table 5) was carried out among and within populations using partitioned variance. The populations were analysed as gene pool-1 (considering all the stocks as a single gene pool); genepool-2 (1. Chilika, Nagayalanka, Pulicat; 2. Vellayani, Ashtamudi, Vembanad, Mangalore, Goa, Ratnagiri) and genepool-3 (1. Chilika, Nagayalanka, Pulicat; 2. Vellayani, Ashtamudi, Vemband, Mangalore; 3. Goa, Ratnagiri). The F_{ST} values were significant ($P<0.001$) in all comparisons with the highest value observed in genepool-3 comparison (0.79) followed by genepool-2 (0.77) and genepool-1 (0.75). Under genepool-1, the percentage of variation among populations (74.55%) was greater than within populations (25.45%). In genepool-3, maximum percentage of variation was observed among groups (66.57%). These results indicate that the genetic divergence both between populations as well as groups was significant. However genepool-2 exhibited the highest percentage of variation (54.5%) within groups, the F_{ST} being 0.776 ($P<0.000$).

SAMOVA (table 6) was applied to identify k genetically differentiated populations, where the proportion of total genetic variance (F_{CT}) get maximized due to between group population differences. The stocks were classified into K = 4 clusters with the highest and highly significant F_{CT} value (F_{CT} = 0.69515, P = 0.000). It had perfect accordance with the haplotype network also. The populations from Chilika and Nagayalanka formed the first cluster, Pulicat the second, Ratnagiri and Goa the third and southwest stocks (i.e. Mangalore, Vembanad, Ashtamudi, Vellayani) the fourth. The F_{CT} values of K = 2 and K = 8 were nonsignificant.

Table 5. AMOVA analysis of genetic structure of *E. suratensis.*

	Variance	% Variation	*F* statistics	*P* value
One gene pool (Chilika, Nagayalanka, Pulicat, Vellayani, Ashtamudi, Vembanad, Mangalore, Goa and Ratnagiri)				
Among populations	1.04	74.55	F_{ST} = 0.745	<0.001
Within populations	0.35	25.45		
Two gene pool (Chilika, Nagayalanka, Pulicat) (Vellayani, Ashtamudi, Vembanad, Mangalore, Goa and Ratnagiri)				
Among groups	0.365	23.13	F_{SC} = 0.709	<0.001
Within groups	0.859	54.49	F_{ST} = 0.776	<0.001
Within populations	0.353	22.38	F_{CT} = 0.231	0.089
Three gene pool (Chilika, Nagayalanka, Pulicat) (Vellayani, Ashtamudi, Vembanad, Mangalore) (Goa and Ratnagiri)				
Among groups	1.142	66.6	F_{SC} = 0.384	<0.001
Within groups	0.22	12.8	F_{ST} = 0.794	<0.001
Within populations	0.353	20.6	F_{CT} = 0.666	<0.001

Table 6. SAMOVA analysis.

K	F_{ST} (*P* value)	F_{SC} (*P* value)	F_{CT} (*P* value)	Structure of populations
2	0.52015 (0.000)	0.85696 (0.000)	0.70191 (0.020)	(1234567) (89)
3	0.39922 (0.000)	0.81414 (0.000)	0.69063 (0.005)	(124567) (3) (89)
4	0.29672 (0.000)	0.78561 (0.000)	0.69515 (0.000)	(12) (3) (4567) (89)
5	0.27190 (0.000)	0.76976 (0.000)	0.68381 (0.000)	(12) (3) (456) (7) (89)
6	0.31319 (0.000)	0.76711 (0.000)	0.66091 (0.004)	(1) (2) (3) (456) (7) (89)
7	0.07690 (0.000)	0.75287 (0.026)	0.73228 (0.002)	(1) (2) (3) (456) (7) (8) (9)
8	0.14443 (0.000)	0.75063 (0.024)	0.70854 (0.065)	(1) (2) (3) (46) (5) (7) (8) (9)

Sub-populations: 1, Chilika; 2, Nagayalanka; 3, Pulicat; 4, Vellayani; 5, Ashtamudi; 6, Vembanad; 7, Mangalore; 8, Goa; 9, Ratnagiri.

Phylogenetic relationship

The best evolutionary model for nucleotide substitution in the *ATPase* gene sequences was HKY and TN93 based on AICc value. The phylogenetic tree was constructed using maximum likelihood and Bayesian methods (figure 2) based on the *ATPase 6/8* sequences of 203 individuals. The phylogenetic tree topologies constructed by these methods were similar and yielded nearly consistent topological structures and similar support levels. Both the methods identified three lineages, namely Pulicat lineage; southwestern-Chilika-Nagayalanka lineage and northwestern lineage. Among them, northwestern lineage consisted of specifically Ratnagiri and Goa samples only.

Median joining haplotype network (figure 3) too complies with the phylogenetic tree but revealed four distinct clades. All the four clades represented three lineages of phylogenetic tree. The first clade consists of Pulicat only while the second clade consists mostly of Chilika and Nagayalanka haplotypes. However, Vembanad and Vellayani also were present. The third clade, which represents the southwestern region has a star shaped network linking. The fourth clade (northwestern) consists of seven haplotypes and they did not share any haplotype with other regions. It is distanced from the third clade by three mutational events.

Under Mantel test, the geographic distance and F_{ST} (figure 4) was found to have a significant positive correlation (r = 0.4497; P = 0.02) which indicates the firm existence of isolation by distance.

Population demographics

Population demography was studied using mismatch distribution plot, raggedness index, sum of squared deviations (SSD) and neutrality tests (table 7). Mismatch distribution (figure 5) for all the individual stocks revealed unimodal plot except for Mangalore which had a bimodal pattern. The distribution plots indicate the individual stocks had undergone a recent population expansion after a bottleneck. The estimated raggedness (Harpending) index and *SSD* were nonsignificant which indicates that the data has a relatively good fit between the observed and expected distributions and hence supports the recent population expansion of individual stocks. However, Mangalore stock was found to have stable population with equilibrium. Negative and nonsignificant Fu's F_S and Tajima's D value for all individual stocks indicate presence of excessive rare nucleotide variants and rare haplotypes respectively than what would be expected under neutrality and also implies that the stocks at individual capacity had undergone population expansion after a bottleneck. Hence, the results of pairwise distribution are well supported by neutrality tests.

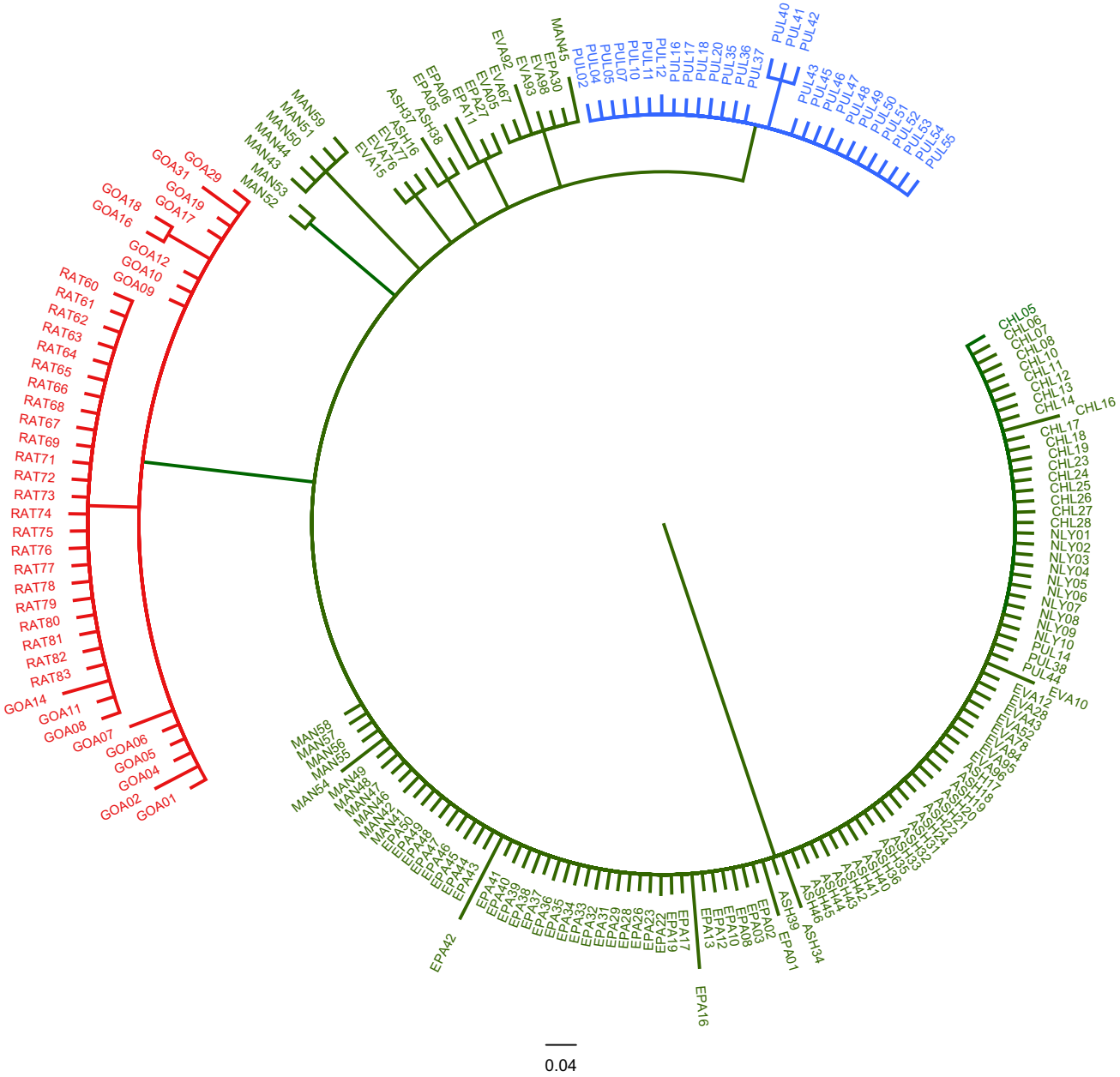

Figure 2. Bayesian phylogenetic tree distinctly separate the stocks (clades in red, northwest stocks; clades in blue, Pulicat stocks; clades in green: southwest, Nagayalanka and Chilika stocks). Here EVA and EPA codes represent Vellayani and Vembanad stocks, respectively.

Mismatch distribution of all the samples as a single population generated a bimodal plot which indicates stable population size throughout the history and has now achieved equilibrium. The estimated raggedness (Harpending) index and SSD under demographic and spatial expansion models were nonsignificant which supports the good fit of the pair-wise distributions. On the contrary, negative and significant neutral tests (Tajima's D (P = 0.03) and Fu's F_S (P = 0.00)) indicate a recent population expansion after a bottleneck.

The region-wise mismatch distribution (K = 4) plots for southwest, northwest, northeast and southeast revealed a unimodal one. The nonsignificance of raggedness index and SSD both support the plot and hence the K=4 structured populations are considered to have undergone a recent expansion. Negative values of the neutrality tests also confirm the same.

Using the evolutionary rate of 1.3% per Myr time, since expansion was estimated by demographic and spatial expansion models for the entire population, and it was found to be between 5.691×10^4 and 5.695×10^4 years before the present respectively, possibly indicating that spatial expansion took place along with demographic expansion. The expansion time for the subpopulations was estimated to

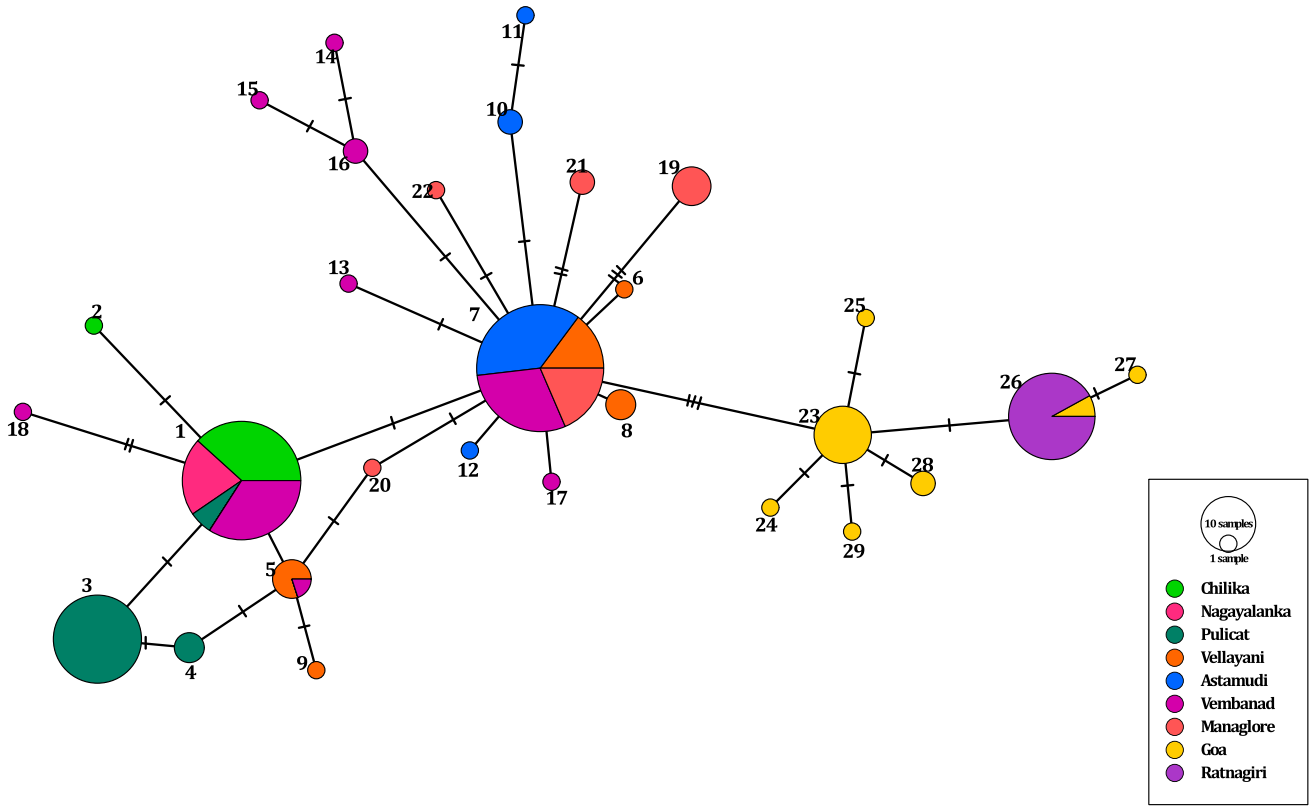

Figure 3. Median joining haplotype network of *E. suratensis* populations.

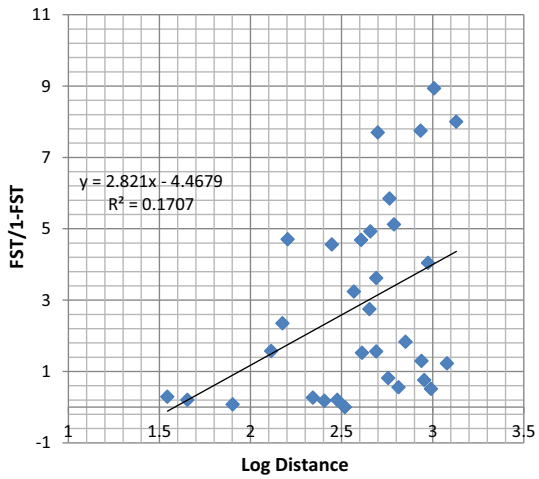

Figure 4. Relationship between transformed F_{ST} and logarithm of geographic distance.

range between 0.1Ma and 0.361Ma under demographic model and between 0.1Ma and 0.279 Ma under spatial model. Analysis of the prehistoric population size dynamics in Indian subcontinent through Bayesian skyline plot (figure 6) also showed increasing population number ~15 to 20 Ka before present after the last glacial maximum. Above 20 Ka years before present, the skyline plot was flat which indicates constant population size over time before expansion.

Effective female population size before demographic expansion ranged from zero (founder events) to 511 and after demographic expansion ranged from 1.2×10^4 to 9.7×10^{10} at different locations. Regarding molecular diversity indices, Vellayani, Goa, Vembanad and Mangalore populations had higher θ_H, θ_K, θ_S and θ_π values, respectively.

Discussion

Our study aimed to unravel the population genetic structure of pearlspot fish *E. suratensis* across the Indian coast using *ATPase 6/8* an mtDNA molecular marker on 203 individuals from nine different populations. The results revealed high genetic diversity and high level of genetic structuring among the populations. The 10 bp overlapping region observed in *ATPase 6/8* is a common feature in several fish species (Divya *et al*. 2015; Gopalakrishnan *et al*. 2018). The order of most represented bases (C>A>T>G) and the AT rich (53.8%) composition are similar to those reported in many fish species (Johns and Avise 1998; Baisvar *et al*. 2015). In some species, the AT content was reported to be higher than the present one, i.e. 60% (Divya *et al*. 2015) and 60.8% (Gupta *et al*. 2013) and it could be attributed to the differences between the codon usage pattern between the species. Anti-G bias, the presence of low guanine (11.8%) is a special feature of mitochondrial genome (Cantatore *et al*. 1994; Zhu

et al. 2017). In our study, a total of 29 haplotypes could be detected. Other *CO1* mt DNA marker based studies identified nine haplotypes from 11 geographical locations in Kerala state of India (Alex *et al*. 2016) and seven haplotypes in four Indian populations (Chandrasekar *et al*. 2013). A recent study on D loop gene revealed 21 haplotypes in five south-Indian pearlspot populations (Chandrasekar *et al*. 2019). The estimates of overall haplotype and nucleotide diversity of the populations in our study were 0.843 and 0.003, respectively which were consistent with those reported by Chandrasekar *et al*. (2019) but are higher than those reported by Alex *et al*. (2016). The occurrence of numerous unique haplotypes was reflected in the high haplotype diversity observed here. Further, it could also be attributed to a larger sample size and wider distribution of sample collection area. The higher haplotype diversity (> 0.5) and lower nucleotide diversity (< 0.5%) in our study explains a recent population expansion after a period of low effective population size (Grant and Bowen 1998) and therefore the rapid population growth retains the newly established mutations (Avise *et al*. 1984). This is further supported by the star-shaped haplotype network observed among southwestern region and northwestern region stocks. Haplotypes 1 and 7 that were widely shared by the population also explains the same. Walsh (2000) had opined that the concept of phylogenetic studies in species while defining conservation units is based on the assumption that the fixation of a particular character state in a population is diagnostic of a long history of reproductive isolation. It would be pertinent to mention here that the Vellayani stock found in the largest freshwater lake of the capital city of Kerala is a land-locked one. Being a landlocked water body, the existing fish would have adapted fairly well to the prevailing ecosystem. This could possibly be the reason as to why the Vellayani stock is exhibiting the highest haplotype diversity (0.758).

The southwest region harboured the largest haplo group consisting of 19 haplotypes with a diversity of 0.681. The next were from the stocks of the northwest region with seven haplotypes and a haplotype diversity of 0.587. Stocks from the east coast harboured four haplotypes with a haplotype diversity of 0.567. The stocks from Goa and Vembanad lake harboured about six unique haplotypes, Mangalore stock had four and Vellayani and Ashtamudi lakes had three each. These unique haplotypes would possibly have evolved due to the environmental changes at different locations. The highest nucleotide variable sites were observed in the stock from Vembanad lake (10) followed by that from Mangalore (seven) and Goa (six). It is important to note that the highest nucleotide diversity of 0.00163 was observed in the stocks from southwest group followed by those from northwest (0.00097), the least being from the stocks of east (0.0008). It would be pertinent to note here that the western coast receives rains from two monsoon seasons, i.e. the southwest (June to September) and the northeast (late October to December) each year whereas the east coast is affected mainly by the northeast monsoon. Consequently, the fish stocks in the western coast are subjected to a large variation in environment, especially in terms of temperature and salinity compared to their counterparts in the eastern coast. Similarly, Teacher *et al*. (2013) while studying the genetic differentiation of Atlantic herring (*Clupea harengus*) in the Baltic Sea, reported that genetic differentiation was associated with site differences in temperature and salinity and population structuring was influenced by oceanography as well as environmental factors. Various factors of diversity including number of variable sites, percentage of private haplotypes, haplotype diversity, nucleotide diversity and average number of nucleotide differences were higher among the populations of western region than the eastern and therefore western region acts as diversity hotspot for pearlspot populations (Alex *et al*. 2013; Chandrasekar *et al*. 2019).

Table 7. Mismatch distribution and neutrality tests for *E. suratensis*.

	CHL	PUL/southeast	VEL	ASH	VMB	MAN	GOA	Overall	Southwest	Northwest	Northeast
Demographic											
Raggedness index (RI)	0.63	0.21	0.03	0.25	0.09	0.15	0.12	0.03	0.03	0.09	0.75
P-value of RI	0.84	0.21	0.95	0.57	0.27	0.37	0.31	1.00	0.91	0.45	0.90
SSD	0.00	0.23	0.00	0.00	0.00	0.05	0.01	0.21	0.00	0.00	0.00
P-value of SSD	0.31	0.13	0.78	0.72	0.44	0.33	0.31	0.00	0.80	0.42	0.16
Spatial											
Raggedness index (RI)	0.63	0.21	0.03	0.25	0.09	0.15	0.12	0.03	0.03	0.09	0.75
P-value of RI	0.74	0.44	0.95	0.53	0.28	0.63	0.33	0.56	0.90	0.47	0.79
SSD	0.0001	0.00	0.00	0.00	0.00	0.03	0.01	0.01	0.00	0.00	0.00
P-value of SSD	0.40	0.18	0.80	0.68	0.23	0.53	0.23	0.28	0.72	0.25	0.23
Tajima's *D*	−1.16	−0.59	−0.11	−1.28	−1.60	−0.28	−1.54	−1.58	−2.04	−1.19	−1.15
P- value of Tajima's *D*	0.14	0.30	0.50	0.07	0.05	0.44	0.06	0.03	0.00	0.11	0.13
Fu's F_S	−0.84	−0.57	−0.44	−1.94	−4.18	−0.37	−4.07	−14.80	−14.30	−3.20	−1.18
P-value of Fu's F_S	0.09	0.22	0.41	0.04	0.01	0.61	0.00	0.00	0.00	0.03	0.05

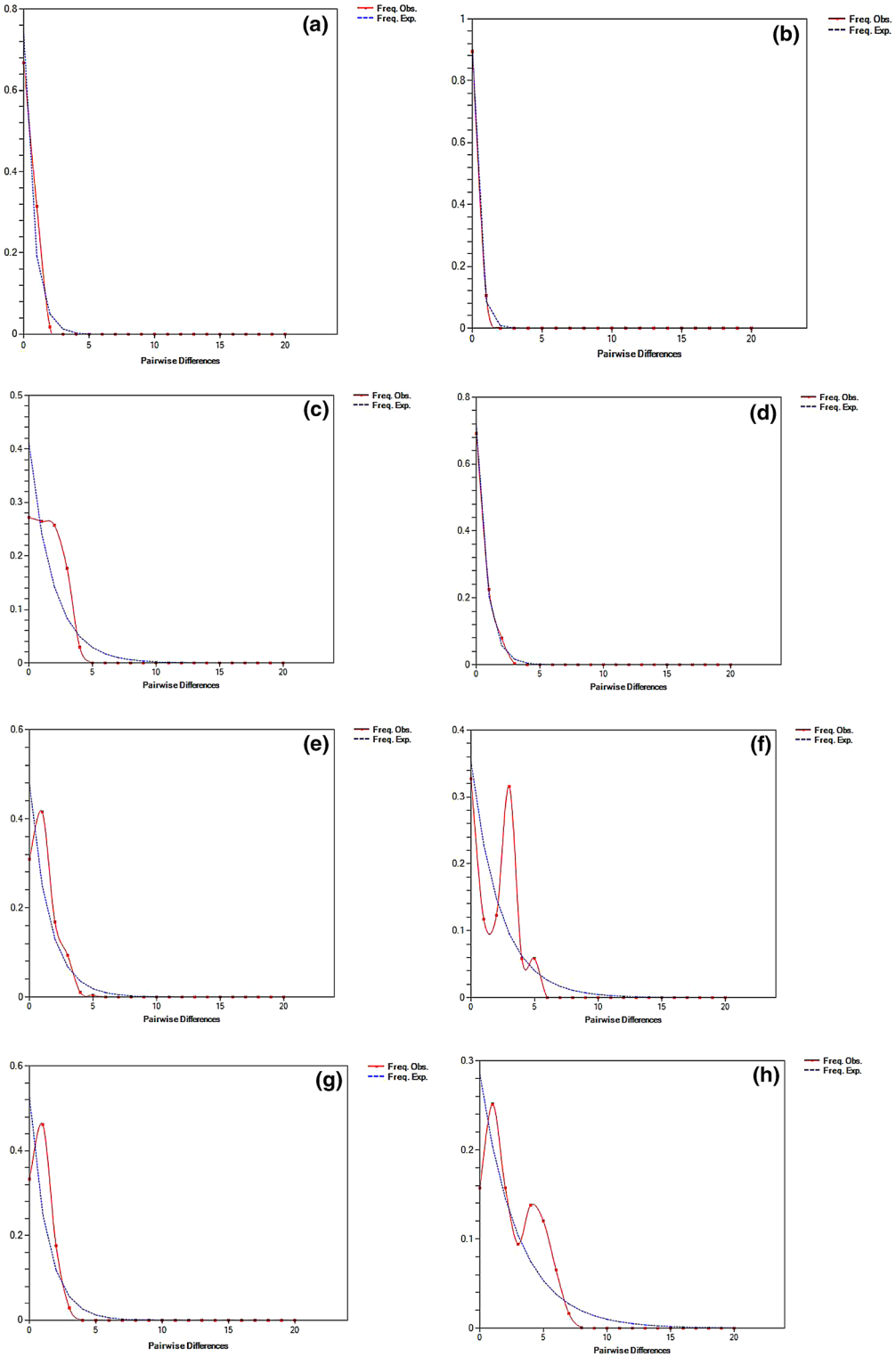

Figure 5. Mismatch distribution plots of pearlspot populations (a) Pulicat, (b) Chilika, (c) Vellayani, (d) Ashtamudi, (e) Vembanad, (f) Mangalore, (g) Goa, (h) Whole Pearlpost population.

Population differentiation and stock structure

The F_{ST} and G_{ST} values ranged from –0.03 to 1.000 and 0.0037 to 1.0. The Chilika and Nagayalanka pair of stocks exhibited the least F_{ST} and G_{ST} values ($F_{ST} = -0.038$; $G_{ST} = 0.00366$) whereas Nagayalanka and Ratnagiri pairs had the highest F_{ST} and G_{ST} values ($F_{ST} = 1.0$; GST = 1.0). This clearly indicates that there is not much difference between

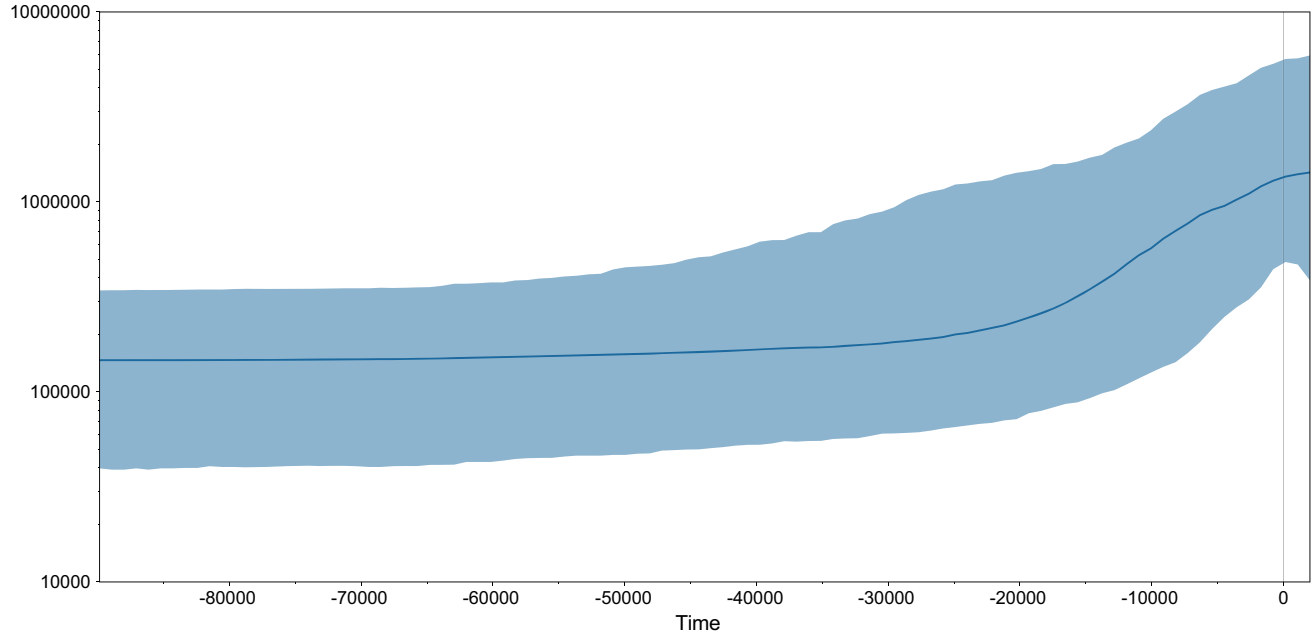

Figure 6. Bayeisan skyline plot constructed for *E. suratensis* populations.

the Chilika and Nagayalanka stocks and Ratnagiri stock was totally different from the Nagayalanka stock as they are on either sides of the Indian peninsula. Nevertheless, Ratnagiri stock had higher F_{ST} value compared to the rest of the stocks and hence was more distantly related to other stocks which is further evident from haplotype network and phylogenetic tree where Ratnagiri stock formed a distinctly separate clade. The similarity observed between Chilika and Nagayalanka stocks could probably due to the transport of seed of pearlspot from one locality to another for culture by farmers, which then found a way into the ecosystem. Although, Chilika and Nagayalanka pair had no difference, F_{ST} has significantly ($P < 0.01$) differentiated other 35 pair-wise fish stock comparisons and thus our study confirms distinct pearlspot populations in Indian waters. In population genetics, genetic differentiation based on G_{ST} values is classified as low (< 0.05), medium (0.05–0.15), or high (> 0.15) (Li *et al*. 2018). Medium G_{ST} exhibited among the Vellayani, Vembanad, Ashtamudi and Mangalore stocks further supports their structure as southwest group. Nevertheless, the southwest stocks exhibited high G_{ST} values compared to those from stocks of other locations. The D_{xy} and D_a values were also substantially consistent with the genetic differentiation observed in our study. Several studies had also reported distinct populations in a few fish species (Alves *et al*. 2001; Knight *et al*. 2009; Nwafili and Gao 2016; Chandrasekar *et al*. 2019).

Apart from the pair-wise differences between the populations, our study established distinct population structure also. The AMOVA results revealed that most of the genetic variation observed in the populations is due to variation between the populations (74.5%) which is further corroborated by the restricted gene flow between populations. Further, to bring accuracy in structuring the population SAMOVA was attempted which grouped the population into four and it was supported by the clusters formed in median joining haplotype network and the lineages of phylogenetic tree. The N_m estimates also support the geneflow to be greater between the nearby stocks rather than the farthest ones. Isolation by distance estimated by the Mantel test also supports the fine scale genetic structure established in our study which seems to be a common phenomenon in poor dispersing fish species (Reusch *et al*. 2001), thus, IBD scenarios are a common phenomenon in these species as well (Reusch *et al*. 2001; Taylor *et al*. 2001). Primmer *et al*. (2006) studied the population genetic structure of Atlantic Salmon across 11 different locations within or nearby the Varzuga river tributary systems in Russia using 17 microsatellites. After carrying out detailed analysis involving Mantel tests and spatial autocorrelation, the authors inferred that dispersion is less likely to occur to Salmon populations found deep in the tributary system. The relatively high level of genetic structuring and significant isolation-by-distance signal observed in our study are concordant with the predictions of the member-vagrant evolutionary model as elucidated by Primmer *et al*. (2006).

The distinct, differentiated and structured populations observed in the present study may be well explained by geological, ecological and behavioural factors (Allan and Flecker 1993). Vandamme *et al*. (2014), while unraveling the population structure of turbot (*Scophthalmus maximus*) in different geographical locations throughout the northeast

Atlantic Ocean, opined that stable environmental selection pressure contributes to relatively strong local adaptation of the species in the Baltic sea. Teacher *et al*. (2013) investigated the genetic differentiation of Atlantic herring (*Clupea harengus*) in the Baltic Sea and observed that genetic differentiation was associated with site differences in temperature and salinity as evidenced by a particular gene locus Her14 which appeared to be under directional selection. The authors inferred from their results that oceanography and environmental factors contributed a major role in population structuring. It has already been demonstrated that ecological factors like salinity, temperature and availability of micro (iron, boron, cobalt, copper and molybdenum) and macro (carbon, nitrogen and phosphorus) nutrients contribute to the local adaptation of populations (Teacher *et al*. 2013; Vandamme *et al*. 2014). The behavioural traits of pearlspot fish like adhesive nature of eggs, nonmigratory adults, natal homing nature of spawn, exceptionally good parental care and geographical factors like topography and land locked nature of lakes possibly lead to restricted gene flow between the stocks resulting in high genetic differentiation. Studies on potamodromous fish species reported genetic structure in hydrographic systems without apparent physical barriers, resulting from behaviours related to IBD and homing (Paixao *et al*. 2018). Studies by Martinez *et al.* (2018) emphasized that both life-history characteristics and habitat too play a role in shaping patterns of genetic diversity in fishes.

Demographic changes

Demographic and spatial model of mismatch parameters indicated that the expansion of pearlspot populations took place around 5.69×10^4 years before present which corresponds to the upper Pleistocene epoch. Several studies have also reported the same time of expansion; 9.4×10^4 years before present for Indian oil sardine (Sukumaran *et al*. 2016) and 6.8×10^4 years before present for silver catfish (Nwafili and Gao 2016). Bayesian skyline plots indicated that a huge expansion of pearlspot populations took place during 15,000 to 20,000 years before present and thereafter it followed a stable population history. The variation observed in the time of population expansion through Bayesian skyline plots and molecular mismatch parameters might be due to the inability of mismatch and neutrality tests to make full use of historical demographic signals present in DNA genealogies (Grant 2015). The mismatch analysis parameters under spatial expansion for various regions too had similar range.

Considering all the subpopulations as a single population, a bimodal distribution plot was observed which indicated a stable population throughout history. However, the high values of haplotype diversity, low values of nucleotide diversity and significant negative values of Tajima's *D* and Fu's F_S demonstrated a recent population expansion after a period of bottleneck or low effective population size. All the individual populations except Mangalore and region-wise populations too demonstrated a uni-modal distribution plot. In Mangalore stock, the distributions tended to be bimodal which may be due to a colonization event consisting of random haplotype lineages (Lecomte *et al*. 2004).

Our study which followed Walsh's principles (Walsh 2000) is more consistent and reliable compared to previous studies (Chandrasekar *et al*. 2013, 2019; Alex *et al*. 2016) in terms of distribution range and sample size. Apart from that, in our study, *ATPase 6/8* genes have been used for the first time as a molecular marker in this species compared to all previous studies (Alex *et al*. 2013, 2016; Chandrasekar *et al*. 2013, 2019).

In conclusion, the study utilized *ATPase 6/8* region of mtDNA and identified distinct differentiated sub-structured pearlspot populations across Indian waters. However, the monoallelic population identified from Nagayalanka and Ratnagiri may be due to the fixation of alleles as a consequence of genetic drift possibly due to the small effective population size. The west coast of India has highly diversified pearlspot populations compared to the east which could possibly be due to the wide fluctuation in environmental conditions that exist especially with monsoon occurring twice a year. It may be mentioned here that the sample size ($n = 142$) of sub-populations from the west coast was also quite substantial. The private haplotypes identified in the present study could find utility in differentiating the stocks. Nevertheless, since the study includes lesser coverage of the mito-genome, it is also recommended to explore more robust genomewide approaches that could utilize next-generation sequencing to assess genetic demography. Our study area encompassed numerous estuaries and backwaters which form the breeding ground for this species and hence represent locations where the pearlspot is readily available. Looking into the importance being given to this species, there is focus to initiate a genetic improvement programme in an effort to realize maximum genetic gain. While doing so, it would be desirable to incorporate the stocks from all the sampled areas to form a synthetic population which can then form the base for long-term selection.

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