Mol Biol Rep (2014) 41:3723–3731
DOI 10.1007/s11033-014-3237-z

Mitochondrial ATPase 6/8 genes reveal genetic divergence in the *Coilia dussumieri* (Valenciennes, 1848) populations of north east and northwest coasts of India

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Received: 28 March 2013 / Accepted: 6 February 2014 / Published online: 9 April 2014

Abstract The golden anchovy, *Coilia dussumieri,* though possessing discontinuous distribution along northeast and northwest coasts of India, it is being managed as unit stock for fishery assessment purposes. By considering the need for stock specific management of the species, mitochondrial ATP synthase 6 and 8 (ATPase 6/8) genes were analyzed for delineating genetic stock structure of the species. Sequence analysis revealed a total of 34 haplotypes across four populations from both the east and west coasts of India. Haplotype diversity (*h*) was found in the range of 0.7421–0.9368. Similarly, nucleotide diversity (π) varied from 0.0012 to 0.0025. AMOVA results indicated a high total variance of 72.66 % between east and west coast populations and less (1.34 %) among populations within the respective coast. Phylogenetic tree constructed using pair wise F_{ST} also indicated the genetic divergence of populations of east and west coasts of India. The findings of the present study will be helpful in developing stock specific management measures for conservation and sustainable utilization of the species.

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Keywords *Coilia dussumieri* · Mitochondrial DNA · ATPase 6/8 genes · Population genetic structure

Introduction

Coilia dussumieri (Teleostei: Clupeiformes: Engraulidae) commonly called as golden anchovy is an important fishery resource in the states of Maharashtra and Gujarat along northwest coast of India. The species exhibits discontinuous distribution and constitutes a fishery also in West Bengal, Orissa and Andhra Pradesh in the northeast coast. Though the distribution of the species exhibits a clear–cut geographical isolation, the populations of *C. dussumieri* from northeast and northwest coasts of India are considered as 'unit stock' for fishery stock assessment purposes and currently managed as a single stock in the Indian coastal waters [1]. Marine fish species are generally possess large population sizes, high dispersion capacity with wide biogeographical distribution. Lack of migrational barriers paves way to have high connectivity between distantly distributed populations and prohibits their allopatric subdivision [2]. Further, the effective dispersion in the sea is not fully understood [3] and the comprehension of how such genetic discontinuities arise and evolve requires more retrospective inference on historical biogeographical events and population genetic data [4]. Since the species possess definite isolated distribution, the information on population genetic structure is necessary for the formulation of effective fishery management measures for conservation purposes. Keeping this in the mind, the present study was carried out to ascertain if mixed stocks of *C. dussumieri* exist in Indian coastal waters.

Mitochondrial ATP synthase 6 and 8 (ATPase 6/8) genes have been reported to be useful for detecting intraspecific

Table 1 Details of sample collection sites of *Coilia dussumieri*

State	Sampling site	State code	Geographic location	Period of collection
Gujarat	Okha	GUJ	22° 46′N, 69° 06′E	Nov 2010–May 2011
	Veraval		20° 90′N, 70° 37′E	Nov 2010–May 2011
	Navabandar		21° 26′N, 69° 48′E	Nov 2010–May 2011
Maharashtra	Mumbai	MAH	19° 07′N, 72° 48′E	Nov 2010–May 2011
	Ratnagiri		16° 98′N, 73° 30′E	Nov 2010–May 2011
West Bengal	Kolkata	WB	22° 11′N, 88° 41′E	Mar 2011–Feb 2012
	Kakdwip		21° 52′N, 88° 11′E	Mar 2011–Feb 2012
Andhra Pradesh	Visakhapatnam	AP	17° 01′N, 83° 08′E	Mar 2011–Feb 2012
	Kakinada		16° 93′N, 82° 22′E	Mar 2011–Feb 2012

variation in several fish species across orders like Atheriniformes [5], Characiformes [6], Clupeiformes [7], Synbranchiformes [8], Petromyzontiformes [9], Tetraodontiformes [10], Orectolobiformes [11], Salmoniformes [12], Siluriformes [13], Cypriniformes [14] [15] and Perciformes [16] [17] [18]. The present study was aimed at demonstrating the potential of mitochondrial ATPase 6/8 genes to provide useful insights of genetic diversity, divergence and genealogy of *C. dussumieri* populations.

Materials and methods

Sample collection and DNA isolation

Specimens of *C. dussumieri* ($n = 20$ each) were collected from selected landing centres of Gujarat and Maharashtra states in the west coast and West Bengal and Andhra Pradesh states in the east coast of India (Table 1). For DNA extraction, a piece of tissue (muscle tissue of approx. 5 g) was excised just below posterior portion of dorsal fin and preserved in 95 % alcohol. Total DNA was extracted from the tissue (muscle) samples following the procedure of Miller et al. [19] with minor modifications. DNA isolation of 20 samples each from the four states was carried out. The extracted DNA was checked through 0.7 % agarose gel (10 × 4 cm) electrophoresis with ethidium bromide incorporated in 1× TBE buffer. The quality and quantity of the extracted DNA was checked in UV spectrophotometer by taking the optical density (OD) at 260 nm and 280 nm. Concentrated samples were diluted with sterile double distilled water to reach appropriate concentrations (20 ng/μl) for PCR reactions.

PCR amplification

The whole ATP synthase 6 and 8 (ATPase 6/8) genes were amplified by PCR (Applied Biosystems) using universal primers-ATP8 2L8331: 5′-AAAGCRTYRGCCTTTTAAGC-3′ and COIII 2H9236: 5′-GTTAGTGGTCAKGGGCTTGGRTC-3′ [20]. Amplification was carried out in 25 μl volume containing 2.5 μl of 10 × PCR buffer (Fermentas), and 1.5 units of Taq DNA polymerase (Fermentas), 200 μM of each dNTPs (Fermentas), 20 pmol of each primer and 20 ng of genomic DNA. The PCR conditions were; initial denaturation of 95 °C for 5 min; followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. Electrophoresis of PCR products was carried out through 1.5 % agarose gels with ethidium bromide staining; and checked in the Gel-Doc system (BIO-RAD, Molecular Imager, Gel Doc™ XR). Molecular weights were determined using StepUp™100 bp DNA markers (GeNeiTM). Products were sequenced bidirectionally using ABI 3730 capillary sequencer (Applied Biosystems) at the sequencing facility. A total of 20 samples were sequenced for each state under study.

Population genetic structure analysis

The raw DNA sequences were edited using BioEdit sequence alignment editor version 7.0.5.2 [21]. DnaSP 5.0 [22] was used for calculating number of haplotypes and their frequency among different populations. Population genetic analysis was carried out from the sequence information of samples from all the four states which were grouped into two for further analysis based on the distribution of the species. Group 1 (*West coast*) included the populations from the distribution sites of *C. dussumieri* in the states of Gujarat and Maharashtra; and group 2 (*East coast*) consisted that of West Bengal and Andhra Pradesh. Intra population diversity was analysed by estimating gene diversity (h)—the probability that two randomly chosen haplotypes are different [23] and nucleotide diversity (π)—the probability that two randomly chosen homologous nucleotides are different [23, 24]. Hierarchical genetic differentiation and the significance of group and population structure were tested using analysis of molecular variance

(AMOVA) [25] and *F*-statistics [26] respectively. All the individuals collected from the different sampling sites of each state were treated as a single population. This analysis was performed for three hierarchical groupings of the data. The first level compared the variation among individuals within each population. The second level examined genetic structure among populations of each group. Finally, variation was determined among groups. This analysis provided insight into the proportion of genetic variation attributable to within-population (Φ_{ST}), within-group (Φ_{SC}) and among-group (Φ_{CT}) differences. Pair-wise F_{ST} values were also calculated between different populations. All population analyses were performed using Arlequin version 3.0 [27]. Pair-wise sequence divergence among populations according to Kimura two-parameter model [28] and number and rate of transitions/transversions were calculated using the program MEGA version 4.0 [29].

Molecular genetic data can provide information on the relationship among existent populations and also reveal information on recent evolutionary history such as past population size [30]. Phylogenetic and molecular evolutionary analysis was conducted using MEGA 4.0 software. A neighbour joining tree was constructed using pair wise F_{ST} values. A mtDNA parsimony cladogram of haplotypes was constructed (at 95 % level connectivity) using TCS software version 1.18 [31]. Haplotype networks reconstruct the genealogical history of haplotypic variation and illustrate the evolutionary relationship among unique haplotypes. Demographic history was investigated by analyzing mismatch distributions of pair wise differences between all the individuals of each population using the Arlequin software.

Results

Sequence variation

The size of ATPase 6/8 genes was found to be 842 bp with 10 bp overlapping between the ATPase 6 and 8 genes. Out of the 842 characters obtained, 806 (95.72 %) were constant and 36 (4.28 %) were variable, in which 22 (61.11 %) were informative for parsimony and 14 (38.89 %) were singleton. The empirical percentages of the different nucleotides were A = 30.9 %, C = 27.4 %, G = 12.2 % and T = 29.5 %. The transition-to-transversion rate (Ti/Tv) estimate for the ingroup was 5.83.

Population variability

Out of 20 samples analysed from each state under study, a total of 34 haplotypes were found (Accession Nos: JX944184-JX944217); Gujarat had the maximum number of haplotypes (11) followed by West Bengal (10). No haplotype was found to be shared between any populations of group 1 (*West coast*) and group 2 (*East coast*), which indicated significant genetic separation between these populations. There was sharing of haplotypes observed within the groups (Table 2). Nevertheless, statistical parsimony network revealed all the haplotypes originated through mutations in haplotype WB1 of West Bengal population of east coast connected by three mutational events with GUJ7 of Gujarat population of the west coast which possibly represents ancestral lineages.

Haplotype diversity (*h*), within the four populations was found to be high (0.9368) in the case of Gujarat and low (0.7421) in Andhra Pradesh. Similarly, nucleotide diversity (π) was varied for the four populations from 0.0012 (Andhra Pradesh) to 0.0025 (Gujarat). The details are given in Table 3.

Population differentiation

The analysis of molecular variance (Table 4) for *C. dussumieri* indicated that a high proportion of the total variance (72.66 %) was attributed to differences between the groups ($\Phi_{CT} = 0.726$), which was significant with less variation among populations within groups (1.34). Estimates of genetic differentiation among all the four populations using *F*-statistics and mean K2P genetic distances are given in Table 5. Low level of genetic differentiation was observed among populations within the groups with values ranging between 0.041 and 0.061. The level of genetic differentiation among groups was high with values ranging between 0.697 and 0.783; similar results were obtained for mean pair wise K2P distances indicated genetic divergence among the *C. dussumieri* populations of both the coasts of India.

Phylogenetic analysis

A neighbour joining tree constructed using mean pair wise F_{ST} revealed two separate clusters consisting of West Bengal and Andhra Pradesh populations of east coast in one cluster and Gujarat and Maharashtra populations of west coast in other one. The results indicated coast wise genetic stock structure of *C. dussumieri* in Indian waters. (Fig. 1). In haplotype network, WB1 (West Bengal) was shown to be ancestral to all the haplotypes of *C. dussumieri*. In the west coast, GUJ7 (Gujarat) was found to be the common one from which all other haplotypes unique to the coast originated. It was observed that the clusters consisted of both the populations of respective coasts of India. It was noted that all the haplotypes were connected by one to three mutational events explaining that there was no deep branching among haplotypes of *C. dussumieri* (Fig. 2).

Table 2 Relative haplotype frequencies for ATPase 6/8 region of *Coilia dussumieri*

Population/haplotype	WB	AP	GUJ	MAH	Population/haplotype	WB	AP	GUJ	MAH
					GUJ1	–	–	0.15	0.10
WB1	0.40	0.50	–	–	GUJ2	–	–	0.15	0.10
WB2	0.20		–	–	GUJ3	–	–	0.10	–
WB3	0.05		–	–	GUJ4	–	–	0.15	–
WB4	0.05	0.15	–	–	GUJ5	–	–	0.05	–
WB5	0.05	–	–	–	GUJ6	–	–	0.10	–
WB6	0.05	–	–	–	GUJ7	–	–	0.10	0.20
WB7	0.05	–	–	–	GUJ8	–	–	0.05	0.10
WB8	0.05	–	–	–	GUJ9	–	–	0.05	–
WB9	0.05	–	–	–	GUJ10	–	–	0.05	–
WB10	0.05	–	–	–	GUJ11	–	–	0.05	–
AP1	–	0.10	–	–	MAH1	–	–	–	0.10
AP2	–	0.05	–	–	MAH2	–	–	–	0.15
AP3	–	0.05	–	–	MAH3	–	–	–	0.05
AP4	–	0.05	–	–	MAH4	–	–	–	0.05
AP5	–	0.05	–	–	MAH5	–	–	–	0.05
AP6	–	0.05	–	–	MAH6	–	–	–	0.05
					MAH7	–	–	–	0.05

Table 3 Intra-population haplotype diversities (*h*), Nucleotide diversities (π) and Tajma's D for ATPase 6/8 region of *Coilia dussumieri*

Population	Haplotype diversity	Nucleotide diversity	Tajma's D
WB	0.8211 ± 0.0746	0.0022 ± 0.0015	−1.21158
AP	0.7421 ± 0.0961	0.0012 ± 0.0001	−1.41159
GUJ	0.9368 ± 0.0288	0.0025 ± 0.0016	−1.12645
MAH	0.8368 ± 0.0766	0.0022 ± 0.0015	−1.59003

Table 4 Hierarchical analysis of molecular variance (AMOVA) of populations of *C. dussumieri* based on mitochondrial ATPase 6/8 region

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among groups (East coast and West coast)	1	96.550	2.37062 Va	72.66
Among populations within groups	2	3.450	0.04385 Vb	1.34
Within populations	76	64.450	0.84803 Vc	25.99
Total	79	164.450	3.26250	

Fixation indices

ΦSC : 0.04916

ΦST : 0.74007

ΦCT : 0.72663

Table 5 Pair wise mean K2P genetic distances (above diagonal) and F_{ST} values (below diagonal) between populations of *C. dussumieri* based on *ATPase* 6/8 genes sequences

	Populations			
	WB	AP	GUJ	MAH
Populations				
WB	0.0000	0.0020	0.0080	0.0090
AP	0.0610	0.0000	0.0090	0.0100
GUJ	0.6970*	0.7718*	0.0000	0.0030
MAH	0.7075*	0.7826*	0.0408	0.0000

* $P < 0.01$

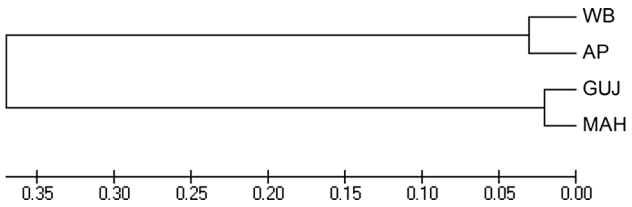


Fig. 1 Neighbour joining tree of different populations of *C. dussumieri* based on pair wise F_{ST} values

Inference on past demography

The pair wise mismatch distribution plots for all the four populations exhibited smooth unimodal curves matching

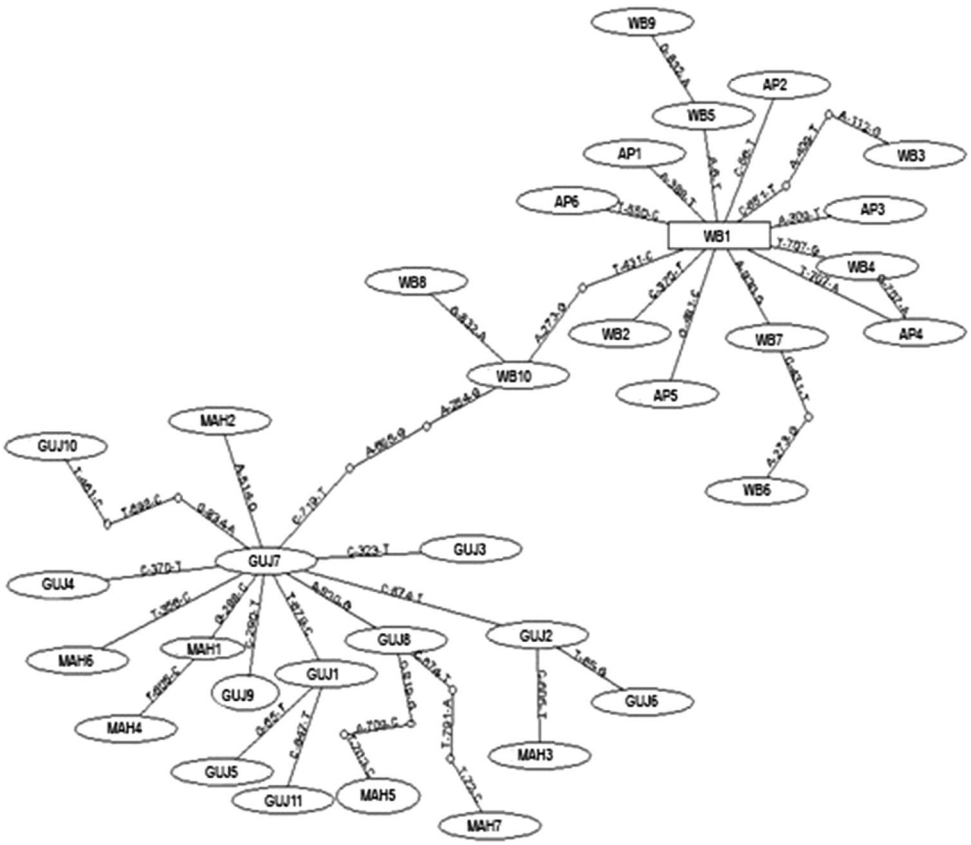


Fig. 2 Haplotype network from four populations of *C. dussumieri*

the expected distributions under the sudden expansion model which explains the history of occurrence of rapid expansion in the populations of *C. dussumieri* (Fig. 3). The position of the highest peak in mismatch distribution ranged from one to eight in all the populations. Highest peak position was found at around two differences for West Bengal, Andhra Pradesh and Maharashtra populations where as it was at around three differences in the case of Gujarat population.

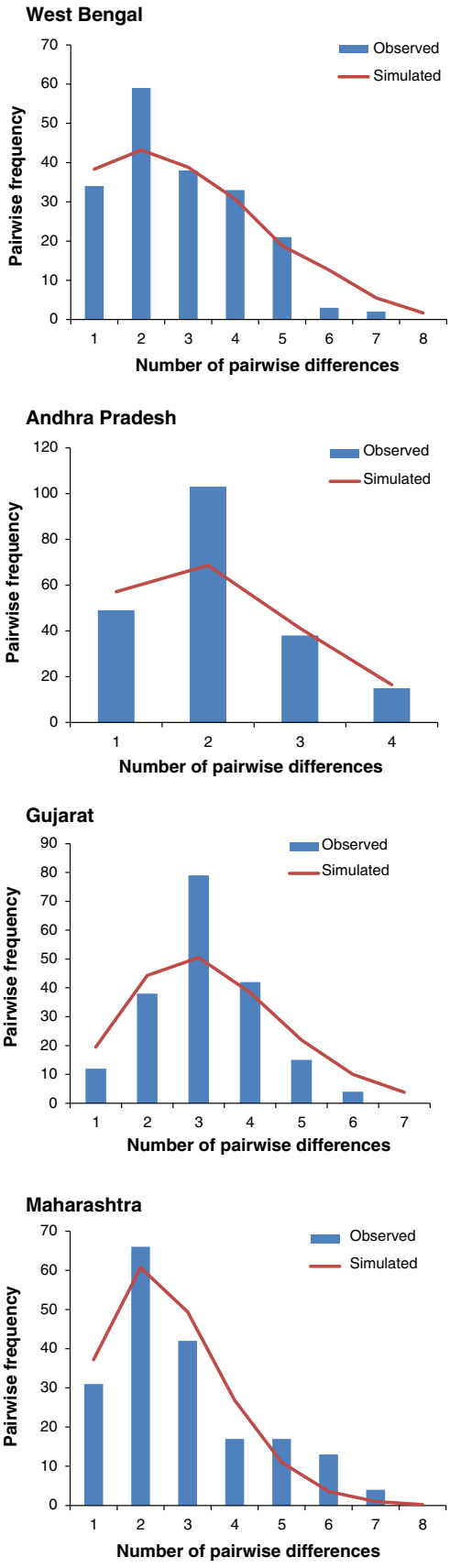
Tajima D value was found to be negative and significant for all the four populations. A significant negative value implies that there were more nucleotide site variants than would be expected under neutral model of evolution [32]. Significant negative value of the statistic in this study indicated that *C. dussumieri* population of both the coasts had experienced population expansion. The unimodal mismatch frequency distribution pattern based on the mtDNA sequence accorded well the predicted distribution under a model of population expansion.

Discussion

The size and nature of amplified products of ATPase 6/8 genes under study are similar to that reported in other fishes like *Andinoacara coeruleopunctatus*. [33]; *Amphilophus rhytisma* [34]; *Channa marulius* [35] and *Engraulis japonicus* [36] Similiarly, nucleotide composition is in line with the results obtained in cyprinids [37], tuna [38], murrel [35] and genus *Rhamdia* [8]. The low number of G may be due to anti-G bias which is characteristic for the mitochondrial genome [39].

The genetic variation among populations of both the groups (72.66 %) revealed in the present study is higher than that reported (32.4 %) for non-migratory species [40]. It is hypothesized that high level of genetic differentiation of east and west coast populations of *C. dussumieri* in Indian waters can be largely explained by the limited dispersal of the species which consequently leads to the restricted gene flow due to discontinuous distribution of the species. The degree of differentiation between populations is obviously associated with geographical distance. Space is not the only parameter that determines genetic population structure and gene flow. Instead, landscape features between populations can influence dispersal rates and migration success. Therefore, the divergence and genetic differentiation between the populations of *C. dussumieri* of both the coasts can be explained with a model of isolation by distance [41].

High haplotype diversity and low nucleotide diversity observed in the present study is in similar line with the



◀**Fig. 3** Pair wise mismatch distributions for each population of *C. dussumieri*

results obtained in *Craterocephalus stercusmuscarum* [5] and in *Luciobarbus* sp [42]. It has been proposed that marine fishes can be classified into four categories based on different combinations of small and large values for haplotype diversity (h) and nucleotide diversity (π) of mtDNA sequences to interpret different scenarios of population history [43]. *C. dussumieri* falls in high haplotype diversity and low nucleotide diversity possibly indicating that the population of *C. dussumieri* could have undergone a population bottleneck followed by sudden expansion which leads to the formation of new haplotypes which are found in low frequencies. This is also supported by fixing of haplotypes in each coast due to restricted gene flow across the coasts of India.

Distribution of pair wise mismatch distributions is used to explore demographic historic events [44]. In this approach, a population that has grown rapidly is expected to have a smooth and unimodal mismatch distribution while those with a constant population size display ragged multimodal distribution. In the present study, the observed pair wise mismatch distributions for all the four populations were not significantly different from the expectations predicted under a sudden population expansion model as observed in other species such as *Lutjanus campechanus* [45]; fat snook *Centropomus parallelus* [46]; reef damselfish *Chromis multilineata* [47]; eel species, *Gymnothorax undulatus* and *G. flavimarginatus* [48] and Atlantic cod [49].

The position of highest peak in mismatch distribution curve can provide information as to when population expansion began. A single major peak at two differences in West Bengal, Andhra Pradesh and Maharashtra populations implies that expansion have first taken place about two mutational time limits ago, with the presence of some divergent haplotypes that have entered the population at a difference time or have not taken part in expansion [50]. Significant negative value of Tajima D value in this study, along with high haplotype and low nucleotide diversity and smooth unimodal mismatch distribution curves obtained for all the populations matching the expected distributions under the sudden expansion model which explains the history of rapid expansion after genetic bottleneck event in the populations of *C. dussumieri*. Similar results of population expansions were observed in tassel fish (*Polynemus sheridani*) [51]; West African estuarine fishes (*Sarotheradon melanotheron* and *Ethmalosa fimbriata*) [52]; tuna *Euthynnus affinis* [53].

The genetic differentiation exhibited by the neighbor joining tree constructed using pair wise F_{ST} was in

concordance with the similar pattern revealed in other fishes such as bigeye tuna [54–56], Atlantic mackerel [57], swordfish [58–60], blue marlin [61] and sailfish [62]. The genetic differentiation revealed between the populations of east and west coasts of India may represent very rare migrants between these localities, the overall level of genetic exchange may be below that required to homogenize populations. This suggests some degree of reproductive isolation of *C. dussumieri* populations leading geographical structuring with limited or no gene flow between populations of east and west coast of Indian waters.

Haplotype network generated by TCS software has been used to infer population level genealogies when divergences are low [63–65]. Statistical parsimony method emphasizes what is shared among haplotypes that differ minimally rather than the differences among the haplotypes and provide an empirical assessment of deviations from parsimony [66]. Under coalescent principles, internal haplotypes in a network are assumed to be ancestral, while tip haplotypes are considered younger and more recently derived ones [67–69]. The haplotype network based on statistical parsimony clearly showed that there were two separate clusters consisting east and west coast of India, which is connected by a link of three mutational events between haplotypes of West Bengal and Gujarat populations.

In the marine environment, currents can be circuitous and oceanographic features like eddies and fronts can prevent mixing and diffusion of pelagic larvae, decoupling pelagic larval dispersal from Euclidean distance [70]. Two adjacent sites may rarely exchange migrants if located on different sides of an oceanographic front [71]. This concept is well suited for explaining the discontinuous distribution of *C. dussumieri* in Indian waters. Similar results were obtained in wreck fish *Polyprion americanus* populations of north and south Atlantic oceans [72]. In anchovies, two separate stocks of Japanese anchovy *E. Japonicus*, were identified in the northern and southern parts of Taiwan strait in the Pacific Ocean [73].

The late Pleistocene period was characterized by a series of large glacial-interglacial changes [74]. This climatic oscillation produced great changes in the sea levels. For example, decline in sea levels of 120–140 metres was noted during glacial maxima [75]. Such a sea level induced environment signal was amplified in the marginal seas of the world, giving rise to drastic changes in areas and configurations of these seas during the late quaternary glacial cycles [76, 77]. The range contractions and expansions for marine biota during the periodic climatic oscillations over the Pleistocene were generally much more dramatic in marginal seas than in open sea systems; due to the shallow shelf in marginal seas. The consequence was that extinctions and population expansions of marine biota might have occurred within marginal seas during the Pleistocene ice ages. Such changes were considered to have a great influence on the amount and distribution of intra specific genetic variation in some marine fishes [77–81]. This might also be another reason for separation of populations of *C. dussumieri* in west and east coast of India.

In conclusion, the analysis of mitochondrial ATPase 6/8 region in *C. dussumieri* revealed coast specific haplotypes which can be used for identification of specific populations in India. The stock genetic structure revealed by the above study will be helpful for conservation and management of stocks of *C. dussumieri* in Indian waters.

Acknowledgments The authors acknowledge the research scholars of NBFGR Kochi Unit, Kochi for their help in carrying out the above work.

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