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SHORT COMMUNICATION

A Preliminary Investigation on the Population Genetic Structure of *Etroplus canarensis* Day, 1877 of the Western Ghats, India

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Abstract

The 'Canara pearl spot', *Etroplus canarensis* Day, 1877 is an endangered and endemic freshwater fish with a restricted distribution in the Western Ghats of India. In spite of the importance as an ornamental fish, no genetic and conservation studies are available for the species. In this work, mitochondrial 16S ribosomal gene sequences were used to infer the population genetic structure of three geographical populations. It is deduced from the study that all three populations in the study can be considered as a single population with a bottleneck or founder effect. Low haplotype and nucleotide diversity were observed among populations. These were supported by AMOVA analysis that showed the total variations are within the populations. Furthermore, data on the pairwise genetic distance (F_{ST}) and the rate of migration among populations (Nm) showed very weak genetic differentiation with low gene flow between populations. The phylogenetic tree with clustering of all haplotypes from three populations further supports the upshot of a single population. Meanwhile, the neutrality test results (Tajima's D, Fu's Fs, D*, and F*) provided evidence for the population bottleneck. Haplotype network analysis revealed a recent population expansion and the presence of a founder haplotype. Consolidated results of haplotype network and neutrality tests propose the scenario of recovery of the population from earlier bottleneck succeeded by very recent population expansion. The results provided in the study may serve as baseline data for future investigations.

Keywords: Canara pearl spot, cichlids, endemic, genetic structure, Western Ghats

Introduction

The family Cichlidae belonging to the order Perciformes is one of the most species-rich families of fishes which comprises approximately 3000 species (Nelson, 2006). The natural distribution of cichlids is centred on Africa, Latin America and Madagascar, with only a few species native to south India and Middle East (Genner et al., 2007). Indian cichlids are ancient, morphologically distinct lineage and represent the evolutionary sister group to all other cichlids (Sparks and Smith, 2004) with several unique, specialised characters and the only Gondwanan teleost forms in the whole India (Silas, 2010). The single endemic Indian Cichlid genus is *Etroplus* with three known species, i.e., *Etroplus suratensis* (Bloch, 1790), (green chromide) *Etroplus maculatus* (Bloch, 1795) (orange chromide), and *Etroplus Canarensis* Day, 1877 (Canara pearl spot).

The distribution of *E. canarensis* is restricted to a short stretch of Netravati and Kumaradhara rivers in Karnataka, India (Gopalakrishnan and Ponniah, 2000; Gururaja et al., 2007; Manickam et al., 2014). Day (1877) first described the species, and it was widely accepted that the fish was extinct until rediscovered in 1987 (Menon et al., 1993). Unlike the other two native cichlids, it is purely a freshwater fish. In 2011 the fish was classified as endangered by International Union for the Conservation of Nature (IUCN). Due to its distinctive colour and shape, *E. canarensis* has been used as an ornamental fish in the aquarium trade. Besides morphological description (Talwar and Jhigran, 1991; Jayaram, 1999), there are no reports on the life cycle, ecology, population genetics, and evolutionary history of *E. canarensis*.

Mitochondrial DNA regions have been well studied in fishes and can be effectively used to analyse the

relationship of recently diverged taxa such as population, species, genus, etc. The slow evolving 16S ribosomal gene can be successfully used to infer genetic structure and demographic history of freshwater fishes (Tabata et al., 2016; Camelier et al., 2018). Moreover, the rare occurrence of the base substitution in non-synonymous nucleotide sites in the 16S ribosomal gene may provide a higher signal for deep level comparisons used in population genetics (Li et al., 2008). In this study, mitochondrial 16S ribosomal gene sequences were used to collect preliminary information on the population genetic structure of *E. canarensis*.

Materials and Methods

Due to the current restricted geographic distribution and endangered status, we limited our sampling to 45 specimens (15 each from Kumaradhara, Netravati and the lower reaches of the merging point of the rivers at Uppinangadi (Fig. 1). Locality, longitude, and the latitude of the collection points were given in Table 1.

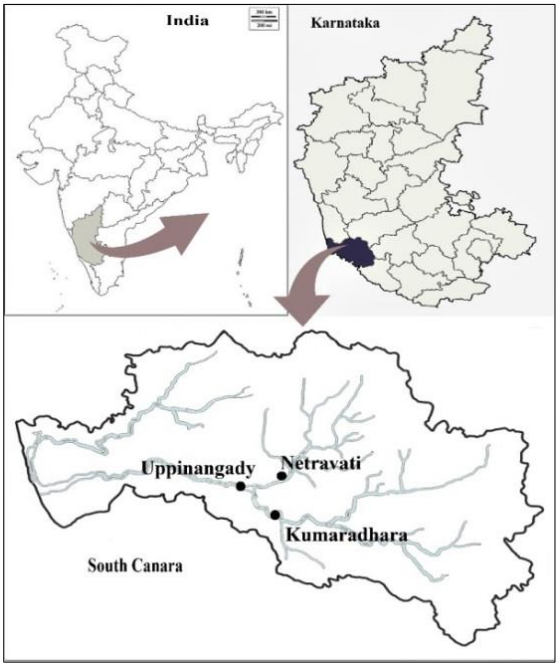

Fig. 1. Sampling locations of *Etroplus canarensis* in South Canara region of Karnataka, India.

Table 1. Sampling locations of *Etroplus canarensis* and the number of individuals (n) used for 16S analysis.

Locality	Latitude	Longitude	No. of Individuals (n)
Kumaradhara	12^{0}50′26.1″N	75^{0}15′23.5″E	15
Netravati	12^{0}44′03.1″N	75^{0}18′58.2″E	15
Uppinangadi	12^{0}50′24.2″N	75^{0}14′23.1″E	15

The caudal fin clips were cut out from each fish and stored in absolute alcohol for DNA isolation. Total genomic DNA was isolated from the fin clips using the DNeasy blood and tissue kit (Qiagen, India) according to the manufacturer's instructions. A partial fragment of the mitochondrial 16S ribosomal RNA (16S) was amplified via polymerase chain reaction using a universal primer (Palumbi et al., 1991). The PCR amplification was performed with a thermal cycler (Eppendorf) in a total volume of 10 µL, containing 2 µL of 5× PCR buffer, 0.2 µL of dNTP (2 mM), 0.5 µL of each primer (10 mM), 0.2 µL of Phire Taq DNA polymerase (Applied Biosystems, Foster City, CA), 3.5–5.5 µL of ddH_2O and 1–3 µL of template DNA (10–20 ng). The cycling condition as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 40 s, 72 °C for 90 s, and then followed by final extension step at 72 °C for 5 min. There was a negative control for each round of PCR to check for contamination. PCR products were purified by using ExoSap IT (USB Corporation, Cleveland, OH) and sequenced in an ABI 3730 capillary sequencer using Big Dye Terminator V.3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA).

Sequences were aligned using Bioedit (Hall, 1999). Taxonomy was confirmed by the 16S sequence similarity using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Haplotypes for 16S sequences were identified, and haplotype/nucleotide diversity was calculated using the program DnaSP 5.10 (Librado and Rozas, 2009). Population diversity indices such as numbers of segregating site (S), haplotype number (h), haplotype diversity (Hd) and nucleotide diversity (π) and average number pairwise nucleotide differences within the population (K) were estimated using DnaSP 5.10. The neutrality indices (Tajima's D, Fu and Li's D, F, D*, and F*) for each population were also calculated (Tajima, 1989; Fu, 1997). A median-joining network was constructed using NETWORK version 4.1.0.9 (Rohl, 2004) to visualize clustering, connectivity, and haplotype history. The pairwise genetic difference was estimated for all populations by calculating Wright's F-statistics, and gene flow (Nm) was calculated by using Arlequin v 3.5.x (Excoffier and Lisher, 2010). Besides, the average number of pairwise nucleotide differences (Kxy) and nucleotide substitution per site (Dxy) were calculated by DnaSP. The evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the Kimura 2 Parameter (K2P) model implemented in MEGA 7 (Kumar et al., 2016). The analysis was performed on 16 haplotypes obtained in the study along with the 16S sequences of *E. canarensis*, *E. suratensis*, and *E. maculatus* retrieved from the GenBank.

Results and Discussion

Forty-five sequences of the mitochondrial 16S region were generated from three geographical populations (Netravati, Uppinangadi, and Kumaradhara; Fig. 1) of *E. canarensis*. The final alignment had 532 bases with 88 polymorphic sites and 59 parsimony informative sites. The composition of each nucleotide was as follows: C 25.4 %; G 22.1 %; T 22.8 %; and A 29.7 %. Of the 45 sequences, 16 haplotypes were identified (Table 2) and submitted to GenBank (Acc. No. MN176485-MN176500).

Table 2. Diversity indices of *Etroplus canarensis* population calculated from the nucleotide sequence of mitochondrial 16S gene.

Population	n	K	H	Hd ± SD	π ± S.D
Kumaradhara	15	12.8	4	0.371 ± 0.153	0.024 ± 0.011
Netravati	15	6.5	5	0.476 ± 0.155	0.012 ± 0.009
Uppinangadi	15	25.7	9	0.848 ± 0.088	0.048 ± 0.008
All population	45	15.5	16	0.589 ± 0.089	0.029 ± 0.006

n: number of sequences examined; S: Number of segregating (polymorphic/variable) sites; K: Average number of pairwise nucleotide differences; H: Number of haplotypes; Hd: Haplotype diversity; π: nucleotide diversity.

There were four unique haplotypes found in Netravati and three in Kumaradhara populations. Samples from the merging site of both the rivers (Fig.1; Uppinangadi) resulted in 8 unique haplotypes. Mutation steps connect all the haplotypes as revealed from haplotype network analysis (Fig. 2), which indicated a recent population expansion. The haplotype network observed in the present study, which also supports the recent population expansion hypothesis, as observed by Yodsiri et al. (2017) in twisted-jaw fish populations. The analysis further revealed the presence of a founder haplotype (hap1), which is distributed in all three geographical populations. A similar finding was reported for the *E. suratensis* population, where two founder haplotypes were identified (Chandrasekar et al., 2019). The presence of a single founder haplotype than many indicates that the migration of species from the native land is accompanied by a population bottleneck (Bailliet et al., 1994). All the other haplotypes are private, which indicates local adaptation (Sjostrand et al., 2014) and may result from the local environmental pressures. The presence of a large number of private haplotypes in the Uppinangadi population may be due to the development of intense physical and chemical gradients in the confluence of two rivers that have resulted in particular environmental conditions that facilitate biological growth (Kiffney et al., 2006). Haplotype diversity (Hd) for all the 45 sequences was found low (0.589 ± 0.089). The average number of pairwise nucleotide differences (K) was found to be 15.5 and nucleotide diversity (π) was 0.029 ± 0.006 (Table 2). Haplotype and nucleotide diversity indices were the highest in Uppinangadi population, followed by the Netravati population and the lowest in the Kumaradhara population. On the combinations of low and high haplotype diversities and nucleotide diversities based on mtDNA, marine fishes have been categorized into four groups (Grant and Bowen, 1998). Such a condition is also applicable to freshwater fishes, as reported by Bermingham and Avise (1986). The *E. canarensis* populations fall into the third category with low haplotype diversity and high nucleotide diversity, which suggests the presence of a highly divergent haplotype that has resulted from secondary contact between isolated populations or by a substantial bottleneck in a formerly large stable population. Low level of haplotype and nucleotide diversities resulted from population bottlenecks, or founder events were also reported in *E. suratensis* distributed in South India (Chandrasekar et al., 2019). Neutrality indices for all the samples were found to be negative for Tajima's D indicated that they had undergone a sudden population expansion. The positive values obtained for Fu's Fs analysis showed the presence of neutral mutations in the populations under study (Table 3) which is an evidence for a deficiency of alleles as would expect from a population bottleneck or from over a dominant selection (Ramos-Onsins and Rozaas, 2002; Fu, 1997).

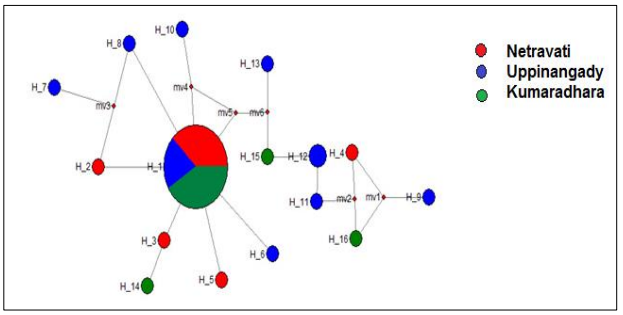

Fig. 2. Median-joining haplotype network constructed for 16S haplotypes found in *the Etroplus canarensis* population. Each circle represents individual haplotype with its size proportional to its frequency. Colour represents different populations from different sites.

Table 3. Neutrality indices of *Etroplus canarensis* population calculated from the nucleotide sequence of mitochondrial 16S gene.

Population	Tajima' D	Fu's Fs	Fu's Li's D	Fu's Li's F	Fu's Li's D*	Fu's Li's F*
Kumaradhara	-1.326^{ns}	10.012^{ns}	0.050^{ns}	-0.481^{ns}	0.150^{ns}	-0.302^{ns}
Netravati	-2.437***	4.048^{ns}	-4.540**	-4.747**	-3.385**	-3.596**
Uppinangadi	0.193^{ns}	4.262^{ns}	-0.542^{ns}	-0.374^{ns}	-0.298^{ns}	-0.185^{ns}
Total	-0.91322^{ns}	3.702^{ns}	-	-	-1.228^{ns}	-1.328^{ns}

$^{**}P < 0.02$, $^{***}P < 0.001$, ns = $P > 0.10$

A negative value for Fu's Fs and Tajima D is expected for a population under bottleneck. Contrary to this, positive Fu's Fs obtained in the present study may be an indication of a recent recovery from a founder

effect or bottleneck (Tchouassi et al., 2014). For the Netravati population, significantly negative values for Tajima's D was obtained which may be due to an excess of low-frequency polymorphism indicating a population expansion after a bottleneck or selective sweep. Significantly negative values for Tajima's D, Fu and Li's D, F, D*, and F* propound a theory of positive selection in the Netravati population by which new advantageous genetic variants sweep a population. A Positive Tajima D is obtained for the Uppinangady population suggesting, low levels of both high and low-frequency polymorphism, stipulating a decrease in population size or a balancing selection. Positive Fu Fs values are obtained for all three populations, which provide evidence for a deficiency of alleles, as would be expected from a recent population bottleneck. Fu's Li's D* and Fu's Li's F* neutrality test are more sensitive to background selection, hence nonsignificant values in D* and F*, disclosing an absence of background selection further support indication of demographic expansion. In a nutshell, the neutrality test conducted for *E. canarensis* suggests a scenario of recovery of the population from earlier bottleneck succeeded by very recent population expansion. However, analysis with an increased sample size conveys more information on the population of *E. canarensis*.

Inter-population nucleotide differences (Kxy) and the average number of nucleotide substitutions per site between all these populations (Dxy) varied from 9.2933 (Kumaradhara) and 19.8133 (Netravati) to 0.0174 (Kumaradhara) and 0.0372 (Netravati) respectively (Table 4). Pairwise genetic distance (F_{ST}) in all the three populations showed very weak genetic differentiation (0.02 to 0.1; Table 5). The G_{ST} value between Kumaradhara and Netavati was found to be negative, indicating no differentiation (Weir and Cockerham, 1984) at these loci and a lack of gene flow between these populations as revealed from Nm. However, weak differentiation with gene flow (N_m) was observed between Kumaradhara/Uppinagadi and Netravati/Uppinadi populations (Table 5) Pairwise F_{ST} estimate between Netravati and Uppinagadi population indicating that within-population variation was higher than that between populations as reported (Liu et al., 2019). The finding was further confirmed with AMOVA analysis as 94.18 % (Table 6) of the total variations are within the populations, which may indicate the presence of an unstructured population of *E. canarensis* in the Western Ghats. The phylogenetic tree based on 16 S sequences (Fig. 3) showed that *E. canarensis* from three populations are clustered together with a sister-group relationship with *E. maculatus* and *E. suratensis*. Since the among-population difference was meagre, the three geographical populations used in the present study can be considered as a single population.

The data indicates that the *E. canarensis* population, restricted to individual pockets of the Netravati-Kumaradhara river system, suffers a low genetic diversity with weak genetic differentiation and has critical implications for conservation. Being edible and an ornamental fish, *E. canarensis* experiences a substantial loss due to aquarium trade (personal observation) and human consumption. Aquarium trade in wild-caught, endangered freshwater fishes, are a severe threat to freshwater biodiversity of the Western Ghats (Raghavan et al., 2013). The results obtained in the present study, even though preliminary, employing a limited number of samples and a single molecular marker (16S), may provide a piece of first-hand information on the genetic variability of an endangered fish species of the Western Ghats and may provide baseline data for designing conservation management plans for endemic freshwater fish populations in the Western Ghats, India.

Table 4. Population genetics indices between different populations of *Etroplus canarensis* calculated from nucleotide sequences of the mitochondrial 16S gene.

Population 1	Population 2	K_{xy}	D_{xy}	G_{ST}
Kumaradhara	Netravati	9.2933	0.0174	-0.0125
Netravati	Uppinangadi	19.8133	0.0372	0.0546
Uppinangadi	Kumaradhara	18.1733	0.0342	0.0327

K_{xy}: Average proportion of nucleotide differences between populations; D_{xy}: The average number of nucleotide substitutions per site between populations; GST: Genetic differentiation index based on the frequency of haplotypes.

Table 5. Pairwise genetic distance (F_{ST} in lower diagonal) and gene flow (N_m in upper diagonal between different populations of *Etroplus canarensis* calculated from nucleotide sequences of mitochondrial 16S gene.

	Kumaradhara	Netravati	Uppinangadi
Kumaradhara		-12.95	17.01
Netravati	0.0401^{ns}		3.91
Uppinangadi	0.0286^{ns}	0.1133*	

ns = $P > 0.10$; *$P < 0.05$.

Table 6. Analysis of molecular variation (AMOVA) performed for the partial 16S sequences of *Etroplus canarensis* populations.

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among population	2	1.089	0.01746va	5.82
Within population	42	11.867	0.28254vb	94.18
Overall	44	12.956	0.30000	

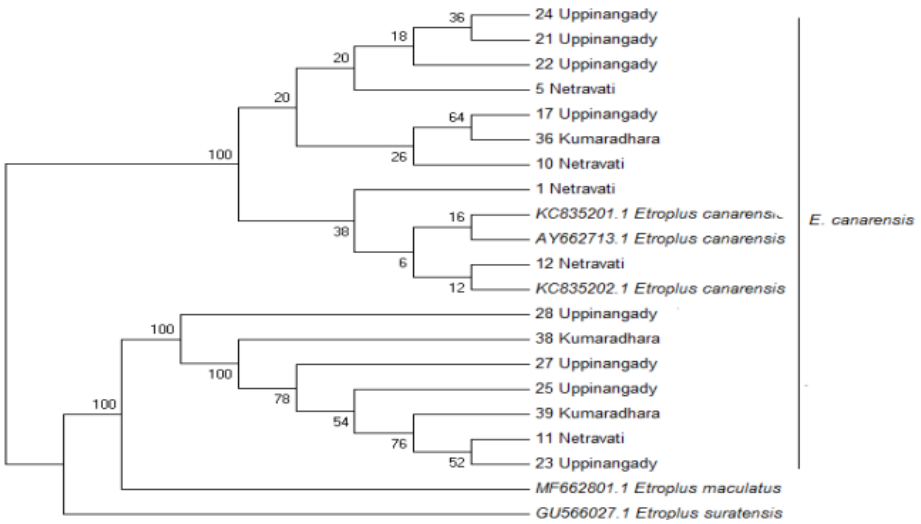

Fig. 3. Maximum likelihood tree of 16S sequences based on the Kimura 2 parameter method.

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