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Note

Preliminary stock structure assessment of the ladyfish *Elops machnata* (Forsskal, 1775) in south-east and south-west coasts of India by mitochondrial DNA control region sequence analysis

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ABSTRACT

Elops machnata (Forsskal, 1775) is considered as least concern (LC) in the IUCN red list and the population trends is unknown in India. Mitochondrial DNA control region (CR) sequences were used to study the genetic status of this fish in Muthupettai, Parangipettai, Marakanam and Cochin estuaries in south India. The K2P genetic distance was high (0.22) between Cochin and Muthupettai populations. Parangipettai and Muthupettai populations are homogeneous and showed more F_{ST} value (1.000). There was a significant positive correlation of genetic distance in relation to the geographic distance (R^2=0.235). There were a total of 8 haplotypes and Tajima's *D* test showed a mean value of 0.2579±0.4468 in the four populations. Analysis of molecular variance showed very less percentage of variation within population (5.30%) than variation among populations (15.02%; p = 1.000±0.000). Both Neighbour joining (NJ) tree and the minimum spanning network of haplotypes, clearly indicated two separate estuarine populations of the species in Tamil Nadu and Kerala. Mitochondrial CR here provides a contemporary evidence of partial restricted gene flow in *E. machnata* among the four populations. The population genetic structure of this species could further be studied using other molecular markers to provide additional data for effective management and conservation of the resource.

Keywords: *Elops machnata*, Genetic structure, Gene flow, Ladyfish, Mitochondrial control region

Elops machnata (Forsskal, 1775) also called as ladyfish occur in marine or brackishwater with wide distribution particularly in the tropical regions mainly in the Indo-West Pacific. This species is considered as least concern (LC) in the International Union for Conservation of Nature (IUCN) red list (Pal, 2012). *E. machnata* population trends are currently stable in Eastern Africa, Indian Ocean but are unknown throughout the rest of its expansive range. However, a number of questions remain regarding their taxonomic uncertainty, population status, fisheries interactions and potential threats in the eastern part of its range (Adams *et al*., 2014).

Mitochondrial genome has been widely used in population and phylogenetic studies in various fish species. This is due to its dynamics in evolutionary rates of different genes and among different position within genes (Kondo *et al*., 1993). Particularly, control region (CR) or D-loop is a hypervariable region with a size of approximately 1,100 bp in case of fishes which has evolved rapidly because of the lack of coding constraints, especially in vertebrates and has high efficiency to reveal the population history (Edwards, 1993). The evolution of CR is not only based on substitutions but involves insertions or deletions of various lengths and differences in number of copies of tandem repeats (Sbisa *et al.,* 1997). Several studies utilised CR sequences for resolving population divergences such as population genetic variation and phylogenetic studies in rainbow fishes (Zhu *et al*., 1994) and phylogenetic analysis of East Asian cyprinids (Liu and Chen, 2003). This CR has been suggested to evolve 2-5 times more quickly than the protein coding genes of the same mitochondrial genome (Meyer, 1993). Due to its elevated evolutionary rate, CR has been considered the appropriate candidate marker for a variety of intra-specific genetic investigations in a vast majority of taxa (Taberlet, 1996). So, in the present study, mitochondrial control region was selected for population genetic study to discriminate genetic variation among different populations of *E. machnata* from selected estuaries along the east and west coasts of India.

Four estuaries *viz*, Marakanam (12°12′0″N; 079°57′0″E), Parangipettai (11°30′33″N; 079°43′13″E), Muthupettai (10°23′0″N; 079°30′19″E) and Cochin (09°58′4.8″N; 076°14′38.4″E) in south India were selected for this study (Fig. 1). *E. machnata* specimens were collected by engaging local fishermen in the respective estuarine region. Fin clips from 15 specimens each in the four stations were dissected out and preserved in 95% ethanol for DNA isolation.

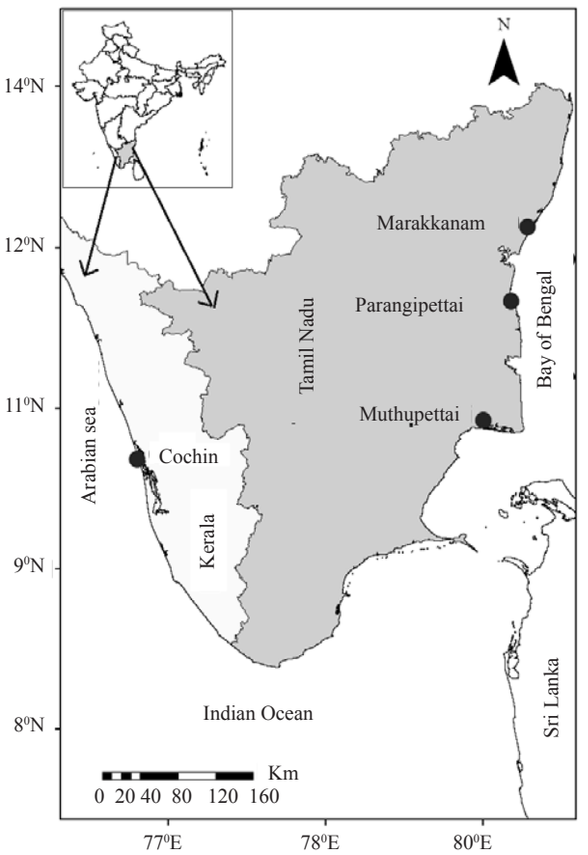

Fig. 1. Map showing the collection sites of *E. machnata*

DNA was isolated from the fin clip samples following the standard salting-out procedure (Sambrook *et al*., 1989). The hypervariable control region was amplified using primers *LBSS88* 5′-TTAACTCCCACCCC TAACTCC-3′ and *BSSH13* 5′-GGGCCCATCTTAACATCTTC-3′ (Santos and Quilang, 2012). The amplification reaction was carried out in a 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM of each primer, 1 U of *Taq* DNA polymerase and 50 ng of genomic DNA. The reaction was performed in a thermalcycler (Techgene, UK) for 30 cycles comprising denaturation at 94°C for 30 s, annealing at 50°C for 60 s, extension at 72°C for 60 s followed by a final extension for 5 min. The resulting PCR products were visualised in 1.5% agarose gel and the size of the amplified product was determined using 100 bp DNA ladder. The cleaned up PCR product was sequenced at the sequencing facility (Eurofins, Bangalore).

The mitochondrial CR partial sequences of twelve individuals were edited using MEGA 6.0 (Tamura *et al*., 2013) and aligned with Clustal W 1.6, in the same software and the haplotype definitions were submitted to the NCBI GenBank. Nucleotide diversity, genetic variation and pairwise evolutionary distance among haplotypes were determined by the Kimura 2 Parameter method (Kimura, 1980). neighbour-joining (NJ) tree was constructed (Saitou and Nei, 1987) and to verify the robustness of the internal nodes of the tree, bootstrap analysis was carried out using 1000 pseudo replications. Tajima's (*D*) and Fu's (*F*) tests were conducted for each population using Arlequin Ver. 3.1 (Excoffier *et al*., 2005). Analysis of molecular variance (AMOVA) was performed to estimate molecular variances within and among populations using this software. The haplotype network between the four populations was drawn by Network 6.1 software (Bandelt *et al*., 1999).

The aligned motpchondrial control region sequences were submitted to GenBank with the accession numbers KF918408 to KF918411 and KF939635 to KF939642. The K2P genetic distance between the four populations is given in Table 1. The K2P genetic distance was high (0.22) between Cochin and Muthupettai populations. Parangipettai and Muthupettai populations are homogeneous and genetic distance was zero. The genetic distance between Marakanam and Cochin population was 0.012. Fig. 2 shows the correlation of genetic distance in relation to the geographic distance, which explains that there is a significant positive correlation ($R^2 = 0.235$).

Tajima's *D* test and Fu's *F* test indicate that mutations in the mitochondrial control region sequences in Cochin, Parangipettai and Muthupettai population are under negative selection (*D* = 0.000), whereas, Marakanam population showed higher *D* value (>1.000) (Table 2). Estimates from Tajima's *D* test showed 0.2579±0.4468 in overall mean calculations. Analysis of molecular variance showed very low percentage of variation within population (5.30%) than variation among populations (15.02%; p = 1.000±0.000). Based on AMOVA analysis, no statistically significant geographical structure was detected among the populations studied (Table 3). When the samples were pooled into groups, AMOVA analysis indicated that the variation among groups contributed 79.67% of the total variation. Measures of F_{ST} have often been used for making inferences about such phenomena as population structure, migration patterns and range expansions. In this study, the F_{ST} value is more (1.000) between Parangipettai and Muthupettai populations based on the control region sequence data. Whereas, the pairwise F_{ST} was very less (0.1219) between Cochin and Marakanam populations (Table 4).

There were a total of 8 haplotypes in all these four populations of *E. machnata* ranging from 1-3 and represented by different coloured circles in Fig. 3. The most common and widespread haplotype, MU1 was shared by 6 individuals representing about 50%

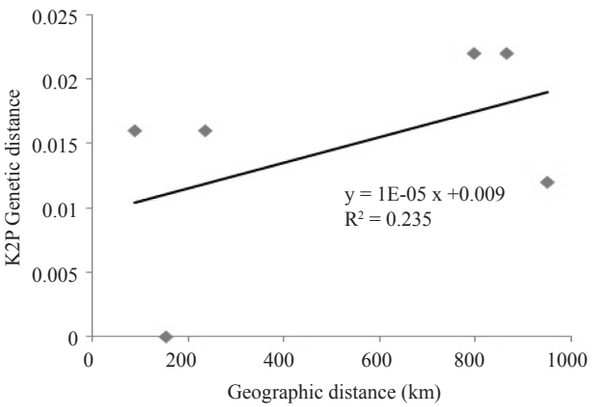

Fig. 2. Genetic distance in relation to geographic distance based on mitochondrial CR sequence data

Table 1. K2P genetic distance between the four *E. machnata* populations

Population	Muthupettai	Cochin	Marakanam	Parangipettai
Muthupettai	*****			
Cochin	0.022	*****		
Marakanam	0.016	0.012	*****	
Parangipettai	0.000	0.022	0.016	******
Overall mean	0.012			

of the sample. All the three haplotypes of Cochin and Marakanam populations were not shared with any other populations. In the dendrogram analysis, individuals of four populations clustered in two major clades. But the Cochin and Marakanam individuals were clustered further into two clades as shown in Fig. 4. Parangipettai and Muthupettai populations are arising from a common ancestral node. From the dendrogram analysis, it is predicted that populations are under neutral condition *i.e.,* genetically homogenous with no expansion or deterioration. All these sampled populations possess better node stability with bootstrap values ranging down from 11% to the best of 100% for branch support. However, individuals of each population branched into different clusters forming a seperate homogenous population. The neighbour joining tree separated two haplotype groups in such a way that all the individuals of the four populations being clustered into separate clades. These two groups were also distinguished by the minimum spanning network generated. Both the NJ tree and the minimum spanning network could clearly separate haplotypes of specimens from different areas of coastal Tamil Nadu and Kerala.

Control region genetic variation is now a well-documented phenomenon and in most cases this variation arises due to slippage errors or false termination/ elongation during mitochondrial DNA replication. This study was done for an appraisal of population relationships among the four populations of *E. machnata* in Tamil Nadu and Kerala estuaries using control region sequence as a population assessment marker. The length of the *E. machnata* control region in this study (~900 bp) fell within the range of sizes found for many other fishes such as common snook *Centropomus undecimalis* (804 bp) (Wilson *et al*., 1997); swordfish *Xiphias gladius* (842 bp) (Rosel and Block, 1996) and Atlantic cod *Gadus*

Table 4. Pairwise F_{ST} among the four *E. machnata* populations

Population	Cochin	Marakanam	Parangipettai	Muthupettai
Cochin	0.0000			
Marakanam	0.1219	0.0000		
Parangipettai	0.7567*	0.6697*	0.0000	
Muthupettai	0.8048*	0.7176*	1.000*	0.0000

(*p>0.05)

Table 2. Tajima's (*D*) and Fu's (*F*) tests value in four *E. machnata* populations

Populations	Tajima's *D*		Fu's *F*	
	D	p	*F*	p
Cochin	0.0000	0.7140	1.2719	0.4910
Marakanam	1.0319	0.8460	-0.08161	0.2770
Parangipettai	0.0000	1.0000	0.0000	NA
Muthupettai	0.000	1.0000	0.0000	NA
Overall mean±SD	0.2579±0.4468	0.8965±0.1237	0.0000±0.0000	0.6927±0.3168

Table 3. Analysis of molecular variance (AMOVA) within and between the four *E. machnata* populations

Source of variation	Degrees of freedom	Summation of mean squares	Contribution of variation	Percentage of variation
Among groups	1	112.778	8.094	79.670
Among populations	2	22.889	1.526	15.028
Within populations	26	14.000	0.538	5.300
Total	29	149.667	10.159	
p value = 1.000±0.000				

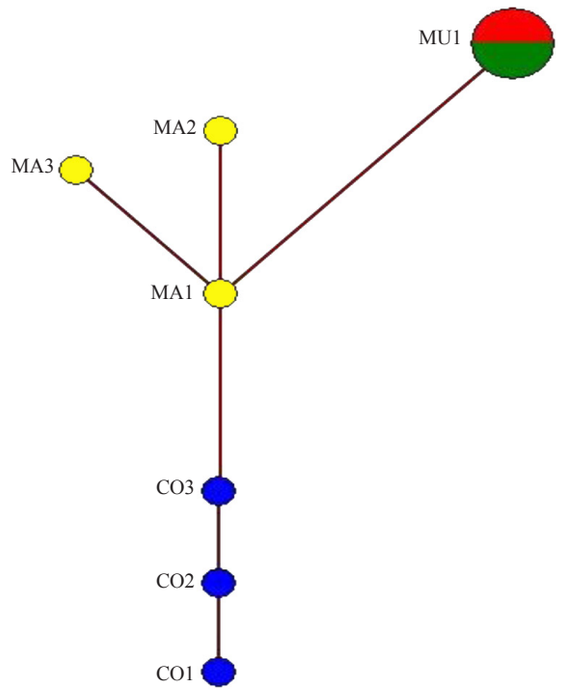

Fig. 3. The mitochondrial CR haplotype network of the four *E. machnata* populations (Yellow: Marakanam; Red: Parangipettai; Blue: Cochin; Green: Muthupettai)

morhua (997 bp) (Johansen *et al*., 1990). Some flatfish species have larger control regions (~1,500 bp) due to longer repetitive sequences at the 3′ end (Lee *et al*., 1995). The nucleotide content for the control region in all the four populations averaged 63.35% (A+T), 36.65% (G+C) and this present study is consistent with the bias reported in other vertebrates (Alvarado Bremer *et al*., 1997).

The overall mean genetic distance estimated in this study is 0.012 which seems to be very low in order to define all these populations as stable genetic units representing low heterozygous genetic structure. Estimates of net evolutionary divergence values displayed between populations' revoked isolation by distance criteria with more divergence between Parangipettai and Cochin . The simulations of Ray *et al*. (2003) as well as Santos and Quilang (2012) show that lower class modes have higher frequencies for spatial range expansions that occurred more recently. This may not be true for *E. machnata* that had not recently underwent a population decline showing moderate frequencies against the pairwise differences in lower class modes which makes the expected and observed values unparallel.

The undergoing mutations that have generated the different haplotypes were under purifying selection in all these four populations based on Tajima's *D* test and Fu's *F* test. Significantly negative estimates from Tajima's *D* and Fu's *F* testing, as well as the haplotype network, give support to the population expansion hypothesis. There was a significant difference between the Tamil Nadu and Kerala populations which was evident from AMOVA analysis. This becomes clearly apparent from the branching of minimum spanning network construction, in which there is an evident group formation between the sampled sites, since a given sample should be identified from the area of collection to know its genetic relationship with the other haplotypes analysed.

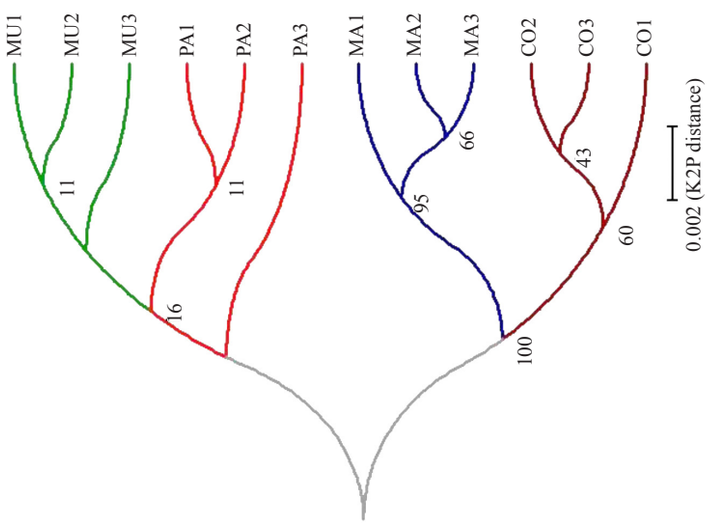

Fig. 4. Neighbour joining tree of the four *E. machnata* populations based on CR sequence data (MA: Marakanam; PA: Parangipettai; MU: Muthupettai; CO: Cochin)

There were less striking variations of nucleotide and haplotype diversity values in all the four *E. machnata* populations. Among these, Cochin and Marakanam showed haplotype diversity (0.333). Overall, there are low nucleotide diversity and high haplotype diversity values. Results from this study indicate that *E. machnata* populations in the sampled sites are under genetic bottle neck with rapid population growth leading to formation of new haplotypes. Genetic distance based neighbour joining method displayed two main clades and several sub-clades with better bootstrap support. But there is no distinct population-wise clade formation based on location of fish sampling sites. The results show that gene flow exists among the four sites despite their geographic distances. As such, specimens from all the four sites cannot be unambiguously distinguished based on mitochondrial control region sequences. The neighbour joining tree and minimum spanning network showed little geographic separation among the haplotypes. This was also obvious in the pairwise genetic distance analysis, where populations grouped in chaos with the evidence of geographical distance. Such a lack of population differentiation also occurs in some penaeid species (McMillan and Bert, 2003; Valles Jimenez *et al*., 2006).

Genetic diversity analysis of *E. machnata* populations revealed little genetic differentiation among the specimens from the four sampling sites despite its long coastline (about 950 km) from Marakanam to Cochin and change of estuary environment in Bay of Bengal and Arabian Sea. Although the haplotype diversity was high, the nucleotide diversity observed was low which indicates that genetic bottleneck may have occurred in *E. machnata* populations. Evolutionary forces that act on *E. machnata* in the Cochin population are expected to have the same effects on the other three populations but with minor genetic changes in case of frequencies of mismatch pairwise differences and neighbour joining tree clades. Molecular data from this study using mitochondrial CR provides contemporary evidence of partial restricted gene flow in *E. machnata*. The population genetic structure of *E. machnata* could further be studied using other molecular markers such as microsatellites to provide additional data for the proper management and conservation of this species.

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