Phylogeny and molecular delineation of leiognathids in the Southeast coastal waters of India

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Abstract

DNA barcoding is very useful in identification of animal species and resolving taxonomic ambiguity of morphologically similar animals. The aim of the present study was to investigate molecular identification of like Leiognathid fishes based on cytochrome c oxidase I (COI) gene sequence. Molecular level identification and comparison of six species from four genera of Leiognatidae were done. Results showed that A + T content of Leiognathids ranged from 55.27 to 53.32%. Mean genetic diversity within the species and overall species diversity within the family were calculated to be 0.004 and 0.178, respectively. Maximum K2P genetic distance (0.255) was observed between *Gazza minuta* and *Leiognathus equulus*. We obtained a monophyletic phylogenetic tree for Leiognathids with two major clades. One major clade contained only *Gazza*, and *Eubleekeria, Karalla* and *Leiognathus*, were grouped under another clade. Genus Leiognathus, which is not sexually dimorphic, get clustered as a separate sub-clade. Thus COI gene sequence could be used for species identification and delineation of complex groups like Leiognathids.

Keywords: DNA Barcoding, COI, Genetic variation, Leiognathidae**,** Phylogeny

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Introduction

Leiognathids commonly called as ponyfishes, silverbellies or slipmouths are small sized, and laterally compressed fishes inhabiting shallow water in marine and estuarine water with bioluminescent characteristic and schooling behaviour. They constitute nine genera that contains approximately 50 species (Sparks and Dunlap, 2004; Chakrabarty *et al*., 2010; Seah *et al.*, 2012; Jawad *et al*., 2013; Eschmeyer *et al.,* 2016) . Ponyfishes are mainly caught in bottom trawls, mostly treated as trash fish and are converted into fishmeal, poultry feed or may be marketed as fresh or dry – salted (Woodland *et al*., 2001). In wild, they are the crucial components of food chain and food web of marine ecosystem (Seth *et al.,* 2016). They are internally and externally conservative in terms of morphology except at light organ system (LOS). The morphology based phylogenetic studies are less known in this family, and taxonomic comparison below family level is quiet difficult (Sparks *et al.,* 2005). Even though, classification and identification of ponyfishes are mainly based on the phenotypic characters with detailed inspection of specimen (Mazlan and Seah, 2006) and molecular studies such as DNA barcoding makes it easy (Azaz *et al*., 2021).

DNA barcoding is an omnipresent system of identification of species and their products, based on sequence variation in mitochondrial gene cytochrome c oxidase I (COI) and new specimens can be identified by analysing the gene sequence with barcoding reference library such as GenBank and BOLD Systems (Hajibabaei *et al.,* 2007; Lakra *et al.,* 2011). Molecular based taxon recognition by utilizing DNA sequence diversity among different species aid to identify organisms and to reduce morphology based taxonomic ambiguity even in the identification of new or cryptic species and also to intensify taxonomic revisions (Chakrabarty and Sparks, 2007; Candek and Kuntner, 2015). Inaccuracy in morphological identification often happens and it had reported by many researchers in several groups of organisms like dinoflagellates (Culverhouse *et al*., 2003) and some fish larvae (Ko *et al*., 2013). The Fish Barcode of Life campaign (FISH-BOL), an international research collaboration centre, is a DNA barcode library for reference and to monitor the DNA barcode project (Sachithanandam and Mohan, 2020). Taxonomical identification of different animals like fishes, birds and bats, through the COI gene sequence has been attested throughout the world and are used in the substantiation of species (Hebert *et al*., 2003, Ward *et al.,* 2005). Trustability of DNA barcoding relies on barcode gap, which is the discontinuity in values of inter and intra-specific divergence, and more barcode gap reflects more accurate results (Hebert *et al*., 2004; Meyer and Paulay, 2005; Dasmahapatra and Mallet, 2006; Meier *et al*., 2008).

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Molecular identification of several ponyfishes has been studied in the United Arab Emirates (Ludt *et al*., 2020) and Peninsular Malaysia (Seah *et al*., 2012) based on COI gene and in Perhent Islands of Malaysia based on 16S rRNA gene (Seah *et al*., 2008; Seth and Barik, 2021). In India, Banu *et al*. (2020) studied only three species of Leiognathids from Karaikal region including . This study is aimed at studying molecular analysis and phylogenetic characterisation of six species of Leiognathids including *Eubleekeria splendens, Gazza achlamys, G. minuta, Karalla daura, K. dussumieri* and *Leiognathus equulus* which are abundantly available in Tamil Nadu coast of India, using COI gene sequences.

Material and methods

Sample collection

Six morphotypes of Leiognathids were collected from Parangipettai landing centre, Tamil Nadu, India (11.4831° N, 79.7729° E) from January to June, 2018. The morphotypes were identified by FAO sheet (Bookstein *et al*., 1985). After collection, the caudal fin of ten fishes from each morphotypes were dissected out and preserved in 95% ethanol for DNA isolation.

DNA isolation and PCR

The isolation of DNA was achieved by the standard phenol-chloroform ethanol method (Sambrook *et al*., 1989). The absorbance ratio was taken at 260 nm and 280 nm (260/280) to estimate the quality of DNA. This DNA was diluted with TAE buffer to a concentration of 100 ng/ml. PCR amplification of the COI gene was brought off in a 50 µl volume PCR mix with 5 µL of 10X Taq polymerase $MgCl_2$ (25 mM) buffer, 1 µL of each dNTP (0.05 mM), 1 µL of each primer (0.01 mM), 0.6 U of Taq polymerase, 2 µL of genomic DNA and 36 µL of double distilled water. *Fish F1*-5'TCAACCAACCACAAAGACATTGGCAC3' and *FishR1*-5'TAGACTTCTGGGTGGCCAAAGAATCA3' (Ward *et al*., 2005) were the universal primers employed in the amplification of the COI gene. The thermal cycle regime consisted of an initiation stage of 4 min at 95°C and subsequent variation of thermal exposure of 40 s at 94°C, 45 s at 54°C and 50 s at 72°C for 30 cycles and a final extension for 10 min at 72°C. The PCR product was run on 1.5% agarose gel after mixed with 2 µL Ethidiumbrimode. The purified PCR product was sequenced by Sangers sequencing method.

Sequence analysis

The obtained sequences were edited with BioEdit v 7.2.6.1 and BLAST searched for similarity. All the sequences were submitted to NCBI through BankIt submission portal and got the accession numbers. Nucleotide composition, nucleotide diversity and genetic distance were calculated with the help of MEGA v 10.1.7 (Kumar, 2018) and the phylogenetic tree was constructed based on Neighbour-

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Joining (NJ) method (Saitou and Nei, 1987).

Results and discussion

All the COI sequences were about 600 bp in length, without insertions, deletion or stop codon. In BLAST search, all the sequences showed more than 98% of similarity to specific species with more than 90% query coverage. Based on the BLAST results, the six morphotypes were identified as *E. splendens, G. achlamys, G. minuta, K. daura, K. dussumieri* and *L. equulus*and the accession numbers were MW843003, MW843014, MW843015, MW843018, MW843019, MW843034, MW843574, MW843598, MW844034, MW844035, MW844036, MW844041, MW844042, MW844046, MW846619, MW846620, MW850500 and MW856658.

Among the six species, the percentage of A+T content was ranged from 55.27 (*L. equulus*) to 53.32 (*G. minuta*) and the G+C content ranged from 46.68 to 44.73. The percentage values of nucleotides are displayed in Figure 1. These values are in accordance with the findings of Ward *et al.* (2005) where the G+C content in COI of fishes was ranged from 42.2 to 47.1%. The mean values of G+C content in this study resemble with previous observations of Lakra *et al.* (2011) and Seah *et al.* (2012) for Leiognathids and those values were 44.7% and 45.25% respectively. But a slight deviation from the observations of Banu *et al.* (2020), where the G+C content was 48.26%.

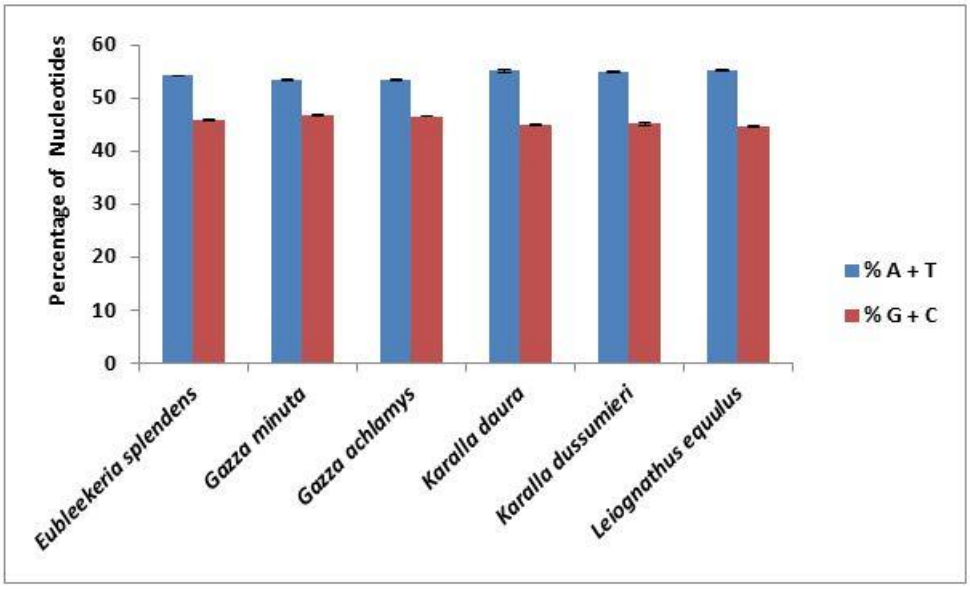

Figure 1: Percentage of nucleotide compositionof COI gene in Leiognathids.

COI sequence divergence play a vital role in species identification. A fish species which shows a deep intra-species divergence can be rejected by a taxonomic expert, from the same species (Bhattacharya *et al.*, 2016). According to Zhang and Hanner (2011), the mean intra-specific K2P distance for

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marine fishes was 0.003 and the present study also displaysthe same intra-specific distance. Mean genetic distance within the species and the overall species distance were calculated to be 0.004 and 0.178, respectively, that show slight deviations from earlier observation by Banu *et al*. (2020). The mean intra-specific and intra-generic K2P distance of Leiognathids in this study is similar to the previous reports where it was 0.003 and 0.066 (Lakra *et al*., 2011) and 0.014 and 0.056 (Seah *et al*., (2017).

Among the six species of Leiognathids, the maximum inter-specific K2P distance between *L. equulus* and *G. minuta* was 0.255 and minimum between *K. daura* and *K. dussumieri* was 0.126 (Table 1). The observed low K2P distance between these two species is due to their taxonomic location in a same genus. Interestingly, a high K2P distance was observed between *G. achlamys* and *G. minuta* even though they are also coming under same genus. Maximum inter-generic K2P distance observed between *Leiognathus* and *Gazza* (0.228) and minimum was between *Karalla* and *Eubleekeria* (0.187). Maximum intra-generic distance was 0.118 in *Gazza* and minimum in both *Eubleekeria* and *Leiognathus* that was 0.007 (Table 2).

Table 1: K2P Genetic distance between and within species (bold).

	E. splendens	***G. minuta***	***G. achlamys***	***K. daura***	***K. dussumieri***	***L. equulus***
E. splendens	**0.007**					
G. minuta	0.249	**0.003**				
G. achlamys	0.202	0.196	**0.000**			
K. daura	0.191	0.236	0.199	**0.004**		
K. dussumieri	0.183	0.227	0.189	0.126	**0.001**	
L. equulus	0.211	0.255	0.201	0.210	0.175	**0.007**

Table 2: K2P Genetic distance between and within genera.

	Eubleekeria	***Gazza***	***Karalla***	***Leiognathus***
Eubleekeria	**0.007**			
Gazza	0.226	**0.118**		
Karalla	0.187	0.213	**0.077**	
Leiognathus	0.211	0.228	0.192	**0.007**

The mean genetic distance within the genus is around 18 fold higher than the mean genetic distance within the species and this observation reflects the earlier reports on genetic variation studies of family Nemipteridae from Malasyian water by En *et al.* (2019). Increase in genetic diversity as we go from lower to higher taxonomic categories and it was supported by the findings of Habert *et al*. (2004) and Lakra *et al*. (2011).Intra-specific variation ranged from 0.007 (*E. splendens*) to 0.00 (*G. achlamys*) and these K2P values may less and this should be confirmed by using more number of individuals. Similar low intra-specific genetic variation was observed by Banu *et al.* (2020) in Indian Leiognathids, Ward *et al.* (2005)

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in Australian fishes and En *et al.* (2019) in fishes of Nemipteridae. Barcoding gap is the difference in the distribution of pairwise distance among intra-specific and inter-specific individuals, and the branch length among inter-species individuals serves to be much deeper than the intra-specific individuals (Meyer and Pauly, 2005).

Neighbour-Joining tree was constructed based on K2P boot strap method and *Caranx sexfasciatus* (Family: Carangidae) was selected as out group according to the postulates of Sparks *et al*. (2005) that Gerreids and Carangids are close relatives of ponyfishes. Figure 2 shows that family Leiognathidae is monophyletic and the individuals belong to same genus and species are clustered as a definite clade with high bootstrap values. Phylogenetic tree of Leiognathids consists mainly of two clades, first major clade comprises of genus *Gazza* which include *G. achlamys* and *G. minuta*. Second major clade includes genus *Karalla*, *Leiognathus* and *Eubleekeria*. Genera *Leiognathus* do not exhibit sexual dimorphism even in the case of LOS (Sparks *et al*., 2005; Chakrabarty *et al*., 2011; Seah *et al*., 2012) and it gets clustered as a separate group in the second major clade.

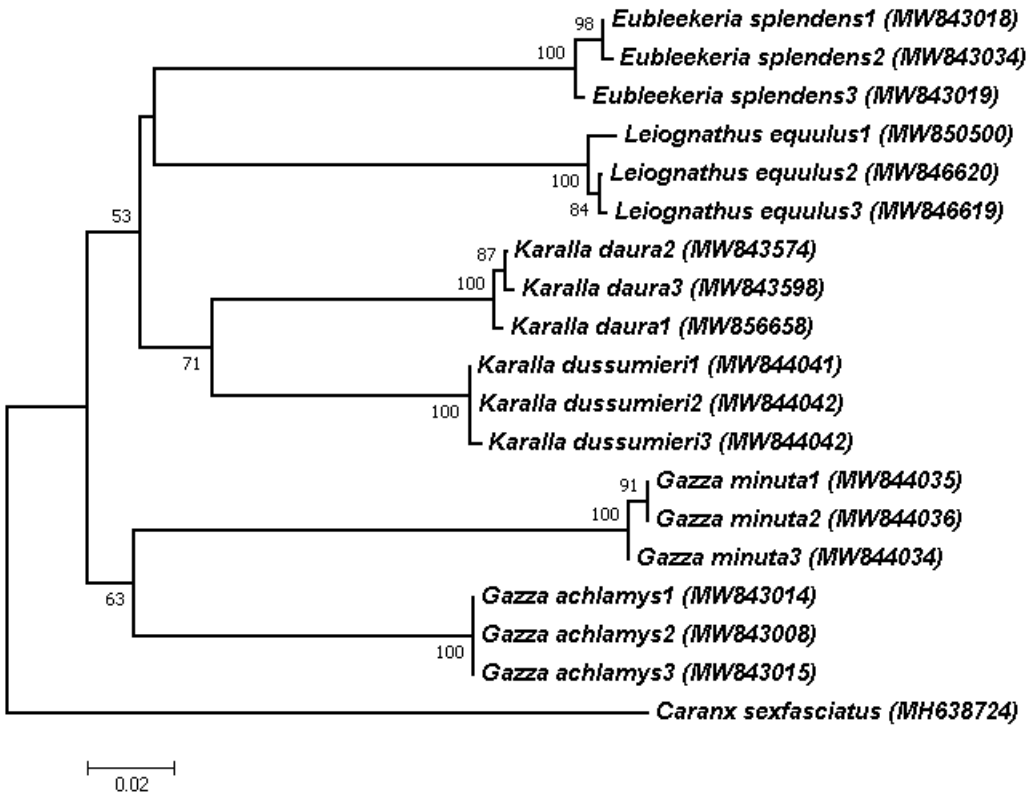

Figure 2: Neighbour joining tree of Leiognathidae based on COI sequences.

Anatomical changes of LOS in sexually dimorphic ponyfishes, may result in reproductive isolation and thus to sexual selection. Sexual selection can cause continuous diversification irrespective of habitat or environmental

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factors. Diversification rate in ponyfishes are higher in sexually dimorphic taxa than in non-sexually dimorphic (Chakrabarty *et al*., 2011) and it reflects the clustering pattern of *Leiognathus* in this study. *Karalla* seems to be a valid genus in the NJ tree and it supports earlier findings of Seah *et al*. (2012) with the 16S rRNA sequences. *Leiognathus equulus* and *E. splendens* are found to be close relatives in the present study and which is not in accordance with the result obtained by Banu *et al*. (2020) by COI sequences as well as Seah *et al*. (2012) by 16S rRNA sequencesand this may be due to the quality of sequences used in these studies. Monophyly of *Gazza* is very evident from the phylogenetic tree and which is similar to the previous findings of Spark and Dunalp (2004) and Ikejima *et al*. (2004). The paraphyly of Leiognathus was reported based on COI sequences (Spark and Dunalp, 2004; Ikejima *et al.*,2004) and 16S rRNA (Seah *et al*., 2008) but the same pattern is not observed in the present study. This may be due to the consideration of *E. splendens* as *L. splendens* in their study.

Conclusion

Misidentification exists in Leiognathids because of the morphological similarities among the species. Molecular identification is an inevitable technique and sufficient data that should be added in the BOLD system. Present study on Leiognathids from Parangipettai coast of Tamil Nadu provides taxonomists with new information to the molecular identification of Leiognathids. Grouping of non-sexually dimorphic species, mean genetic distance values within and between groups, G+C ratio of Leiognathids and monophylystatus of genera in this study are very relevant in terms of their molecular identification and comparison. Even though the study is based on COI gene sequences, the findings are correlated with COI, and 16S rRNA sequences of previous reports. In short, it is understood that molecular phylogeny of Leiognathids is in compatible with their morphological delineation.

Conflict of interest

All the authors declare that, there is no conflict of interest.

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