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DNA Barcoding Resolves Taxonomic Ambiguity in Mugilidae of Parangipettai Waters (Southeast Coast of India)

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Abstract

Species delineation among three genera and ten species of mullets (Family : Mugilidae) (*Liza macrolepis, Liza parsia, Liza planiceps, Liza subviridis, Liza tade, Liza vaigiensis, Mugil cephalus, Valamugil cunnesius, Valamugil seheli, Valamugil speigleri*) occurring in Parangipettai coastal waters was attempted using morphological characters and by sequencing the partial mitochondrial Cytochrome C Oxidase subunit I gene. The first three principal components (PCA) explained 90.82 % of the total variation in the 25 morphometric characters. Characters with high loadings (≥ 0.8) in the first component such as snout to I dorsal, snout to II dorsal, snout to anal, standard length, fork length and total length were selected for delineating all the 10 species using Canonical Analysis of Principal Coordinates (CAP). In spite of its robustness CAP failed to separate the species and there was high overlapping. On the contrary, all the 10 species could be clearly differentiated with COI gene sequences. While COI was found to be good for species level identification, other markers have to be used in conjunction with COI to study the phylogeny and to know the evolutionary history of mugilids.

Keywords: Mullets, CAP, COI, morphometry, species delineation.

Introduction

The grey mullets are most common in the coastal waters and estuaries of the tropical and subtropical zones of world seas. The grey mullets are of considerable importance in the capture and culture fisheries in many parts of the world. Parangipettai waters abound in mullets and serve as very good nursery ground as evidenced by a large number of species occurring here supporting a good fishery throughout the year (Reddy, 1977). The family Mugilidae was previously placed in the order Perciformes but is now considered the sole representative of the order Mugiliformes (Nelson, 1994). According to the latest taxonomic revision made by Thomson (1997), this family includes 14 genera and a total of 64 valid species, most of them under the genera *Liza*, *Mugil* and *Valamugil*. The taxonomic status of some species and genera of this family is still confused (Rossi *et al.*, 1998).

Morphological characters have been commonly used in fisheries biology to measure discreteness and relationships among various taxonomic categories. There are many morphometric studies which provide evidence for stock and species discreteness (Corti, 1988; Shepherd, 1991; Avsar, 1994; Bembo, 1996). Mugilids are one of the most difficult taxonomic groups. Mugilid species have a highly conservative morphology, and identifying them using classical morphometric characters has proven to be complex and difficult (Menezes, 1983; Gilbert, 1993; Thomson, 1997). Unraveling the mystery behind the taxonomic status of Mugilidae family has been endeavored by several workers at various levels based on the morphological characters (Schultz, 1946; Trewavas and Ingham, 1972; Thomson, 1981; Harrison and Howes, 1991) and the results obtained were mostly contentious and failed to prove the identity of species of mugilids. Cryptic species complexes could not easily be differentiated with classical morphology as most members of the family display a general morphological uniformity, which as a consequence restricts the number of suitable characters that can be used to identify species unambiguously. Many of the characters considered to be of taxonomic value of mullets undergo marked changes during growth and makes them difficult to identify. Absence of adipose eyelids in younger stages and development of extensive adipose tissue covering the eyes when it becomes adult (*M. cephalus*) and presence of cycloid scales in younger stages and development of ctenoid scales during growth (*M.*

cephalus, L. parsia and *L. tade*) lends credence to this fact (Sarojini, 1953; Pillay, 1954; Thomson, 1954). The identification of species belonging to genus *Liza* involves quite a lot of subjectivity as the species are identified based on the position of dorsal fin in relation to snout and caudal fin. In the case of species belonging to the genus *Valamugil*, it is based on the extension of pectoral fin in relation to the dorsal fin spines. As a result, the phylogenetic status of the Mugilidae family remains particularly obscure, especially at the interspecific level (Stiassny, 1993; Rossi *et al.*, 1998).

More recently the phylogenetic relationships of grey mullets have been investigated with the use of non-morphological characters, employing biochemical and nucleic acid markers (Delgado *et al.*, 1992; Rossi *et al.*, 1996, 1997, 2000; Gornung *et al.*, 2001, 2004; and Nirchio *et al.*, 2003). Cryptic species are also identifiable by genetic methods (Price, 1996; Fontdevila and Moya, 2003). In Vellar estuary situated at Parangipettai (lat. 11°30'N, long. 79°46'E), 10 species of mullets **(**six congeneric species of *Liza- L. macrolepis, L. parsia, L. planiceps, L. subviridis, L. tade, L. vaigiensis*), three congeneric species of *Valamugil - V. cunnesius, V. seheli, V. speigleri* and a non-congeneric species (*M. cephalus*) occur commonly. In view of the difficulties in the identification of the above species, the present study was undertaken to delineate species using both classical morphometry and partial sequencing of COI (Cytochrome C Oxidase subunit I) of mtDNA.

Materials and Methods

Sampling

A total of 50 individuals each of all ten species belonging to genera *Liza, Mugil* and *Valamugil* were collected from Parangipettai waters during November 2010 - December 2011. In addition to that tissue samples were also collected and preserved in 95% ethanol for DNA barcoding. Species identification was done based on Thomson (1997) and FAO species identification sheets for fishery purposes (Fishing area 57- Eastern Indian Ocean).

Morphometric Analysis

The morphometric measurements were taken following the methodology of Thomson (1954). All the measurements were recorded to the nearest 0.5mm with fine draftsman dividers using fresh fishes in a near to relaxed live condition as possible (Holden and Raitt, 1974). A total of 25 morphometric characters were recorded namely, body depth at ventral origin, head length, maximum head width, minimum height of head at pre orbital, maximum height of head, inter orbital distance, eye diameter, snout length, post orbital length, snout to first dorsal fin, snout to second dorsal fin, snout to ventral fin, snout to anal fin, height of second dorsal fin, length of pectoral fin, height of anal, width of first dorsal base, width of second dorsal base, width of anal base, body depth at first dorsal origin, depth of caudal peduncle, length of caudal peduncle, standard length, fork length and total length. The percentage of overlapping ratio between the species of *Liza* and *Valamugil* were calculated using the following formula:

$$\text{Percentage of overlapping} = \frac{\text{Overlapping ratio}}{\text{Extreme overlapping ratio}} \times 100$$

where, Overlapping ratio = Maximum value of species A – Minimum value of species B and
Extreme overlapping ratio = Maximum value of species B – Minimum value of species A

Statistical Analysis

To separate the mugilids based on morphological characters Principal Component Analysis (PCA) was performed using the statistical package PAST (version 2.14) and to delineate the species Canonical Analysis of Principal Coordinates (CAP) was performed using PRIMER (version 6.1).

DNA Extraction, Amplification and Sequencing

DNA was isolated as per the standard protocol (Ward *et al.*, 2005). Briefly, muscle cubes were digested in lysis buffer in the presence of protease K and salted out using high molar sodium chloride solution. The DNA was precipitated in cent percent ethanol and washed with 70% ethanol before air drying (at room temperature). DNA dissolved in double distilled water acted as template for PCR reactions. COI amplification was carried out using primer pair FishF1-
5′TCAACCAACCACAAAGACATTGGCAC-3′ and FishR1-
5′TAGACTTCTGGGTGGCCAAAGAATCA-3′
(Ward *et al.*, 2005). The following PCR conditions were adopted; 95°C for 2 minutes, 5 cycles of 94°C for 30 seconds, 45°C for 40 seconds, 72°C for 30 seconds and 35cycles of 94°C for 30 seconds, 54°C for 40 seconds, 72°C for 30 seconds and final extension was carried out at 72°C for 10 minutes. Amplicons were agarose gel checked and sequenced using ABI high throughput sequencer (Bioserve Biotechnologies, Hyderabad, India).

Sequence Data Analysis

The electrophenerogram generated by automated DNA sequencer was read by Chromas Pro v1.42 and the sequences were carefully checked for mis-calls and base spacing. ClustalX 2.0.6 was used to align the nucleotide sequences (Thomson, 1997). MEGA 4.1 was used to construct phylogenetic trees via

Neighbourhood joining method using Kimura 2-parameter and to calculate genetic distance of the given set of sequences (Tamura *et al.*, 2007). Barcode sequence of *Lates calcarifer* (JF919828) sampled in addition to the mullet samples from Parangipettai coastal waters was used as an outgroup in constructing the phylogenetic tree.

Accession Numbers

The COI sequences of Mugilids produced in the present study were submitted in the GenBank (NCBI) and the accession numbers are as follows: JQ045776 (*Liza tade*), JQ045777 (*Valamugil cunnesius*), JQ045778 (*Valamugil speigleri*), JQ045779 (*Liza parsia*), JQ045780 (*Liza vaigiensis*), JQ045781 (*Valamugil seheli*), JQ045782 (*Liza subviridis*), JQ045783 (*Mugil cephalus*), JQ045784 (*Liza planiceps*), JQ045785 (*Liza macrolepis*).

Results

Analysis of Morphometric Characters

In Principal Component Analysis (PCA) done after log transforming, the first three components with higher eigenvalues explained 90.82 % of the total variation (Table 1). The first axis alone explained about 68.51% of the variation. The second and third components explained 15.45 % and 6.85 % respectively. To select the most useful morphometric characters which will be helpful in separating the species, the eigenvectors (coefficients) associated with the first three components were used. Characters with high loadings (≥ 0.8) in the first component were snout to I dorsal, snout to II dorsal, snout to anal, standard length, fork length and total length (Figure 1). In the second and third components no character showed high loading. These characters were selected for further analysis.

The percentage of overlapping of the above 6 morphometric characters between species belonging to *Liza* and *Valamugil* is given in Table 2. The overlapping percentage of Snout to I dorsal among the species of *Liza* species was in the range of 8.536 - 90.736 with an average of 59.8%. Among the species of *Valamugil* the overlapping ranged from 78.181 to 94.736 with an average of 86.86%. With respect to snout to II dorsal, the percentage of overlapping among the species of *Liza* was in the range of 9.322 - 87.368 with an average of 56% and among the species *Valamugil* 76.92 - 93.93 with an average of 86.17%. Overlapping ratio for Snout to anal within *Liza* species ranged from 11.111 to 94.68 with an average of 51.3% and within *Valamugil* species between 90.384 and 97.56 with an average of 94.532%.

For standard length, the percentage of overlapping within *Liza* species was in the range of 29.807 – 97.777 with the average of 69.975% and among the species of *Valamugil* in the range of 44.696 -72.64 with an average of 62.66%. The percentages of overlapping for fork length among the species of *Liza* and *Valamugil* were in the ranges of 32.46 – 99.24 and 40.5 – 67.63 with averages of 73.774 and 58% respectively. The percentage of overlapping for total length among species of *Liza* was in the range of 30.256 – 99.29 with an average of 68.43% and among species of *Valamugil* 37.579 - 66.666 with an average of 56.58%. Overall the overlapping ratio between morphometric characters of *Liza macrolepis* and *L. parsia* was on the higher side whereas it was lower between *Liza subviridis* and *Liza vaigiensis*. Among the species of *Valamugil* the overlapping ratio was on the higher side.

In view of high overlapping between many species, Canonical Analysis of Principal Coordinates (CAP) was performed separately for the species of *Liza* and *Valamugil* to delineate their congeners (Figs. 2 & 3). The results of CAP analysis are given in Tables 3-6. The percentage of variation explained by the subset of axes (*m*) in the data cloud of *Liza* species was on the higher side (94.88%-100%). However the percentage of samples correctly allocated to their own group was only 62%. The details of cross validation given in Table 3 showed that the allocation success of morphometric characters to the right species was maximum for *L. vaigiensis* (98%) and minimum (36%) in both *L. tade* and *L. planiceps*. In the CAP plot (Figure 2) *L. tade* fell on the left side and *L. vaigiensis* on the right side with the other species falling in between. The data cloud of *L. tade* overlapped with that of *L. subviridis* which in turn overlapped with that of *L. macrolepis* and *L. planiceps*. The latter overlapped with *L. parsia*. That way CAP analysis could not separate the species of *Liza* clearly.

The percentage of variation explained by the subset of axes (*m*) in the data cloud of *Valamugil* species was also on the higher side (85.73%-100%). The percentage of variation described by the first subset of axis (85.73%) was lower than that of *Liza* species. However the percentage of samples correctly allocated to their own group was more than that of *Liza* species (70%). The details of cross validation given in Table 3 showed that the allocation success of morphometric characters to the right species was maximum for *V. cunnesisus* (82%) and minimum (64%) in *V. seheli* with *V. speigleri* coming inbetween (70%). In the CAP plot (Figure 3) for the species of *Valamugil*, data cloud of *V. cunnesius* fell on the left side and that of *V. speigleri* on the right side with *V. seheli* falling in between. Eventhough the pattern was comparatively better, overlapping was seen among the three species. That way here also CAP analysis could not separate the three species of *Valamugil* clearly.

Analysis of Molecular Characteristics

The genetic distance was calculated between the species belonging to the three genera of Mugilidae

Table 1. Eigenvalue and percentage of variance among the members of Mugilidae based on 6 characters

PC	Eigenvalue	% of variation	Total variation
1	4.1108	68.513	68.513
2	0.927051	15.451	83.964
3	0.411491	6.858	90.822
4	0.400211	6.670	97.492
5	0.150447	2.508	100.000

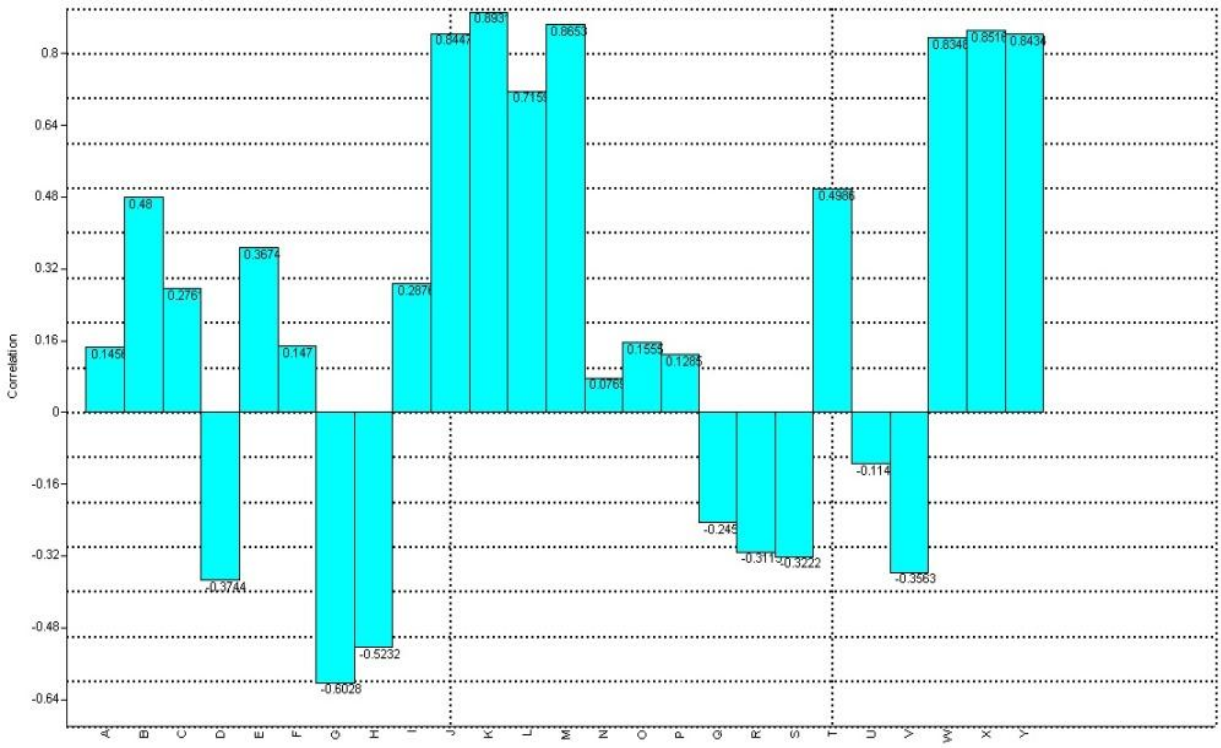

Figure 1. Loadings for factors in first component based on 25 characters.

Table 2. Percentage of overlapping of body proportions between the species of *Liza* and *Valamugil*

1	Species	Percentage of overlapping ratio					
		Sn. to I dorsal	Sn. to II dorsal	Sn. to anal	Standard length	Fork length	Total length
Liza	*L. mac. vs L. par.*	78.873	74.725	78.666	94.202	93.43	92.7
	L. mac. vs L. pla.	77.941	84.415	75.362	71.929	99.242	99.29
	L. mac. vs L. sub.	68.181	50	38.947	90.769	89.473	77.702
	L. mac. vs L. tad.	90.14	75.257	49.523	89.051	81.578	74.404
	L. mac. vs L. vai.	25	34.065	38.666	37.579	45.283	38.69
	L. par. vs L. pla.	63.855	65.346	63.736	77.9	93.288	92.993
	L. par. vs L. sub.	55.555	40.677	36.752	97.142	96.453	73.78
	L. par. vs L. tad.	74.418	61.157	45.669	95.238	88.125	71.195
	L. par. vs L. vai.	45.833	59.782	62.962	39.428	39.772	46.551
	L. pla. vs L. sub.	90.476	61.956	56.382	78.698	89.655	80.838
	L. pla. vs L. tad.	89.473	87.368	65.384	80.924	82.317	77.54
	L. pla. vs L. vai.	17.857	28.712	30.769	29.807	43.529	44.68
	L. sub. vs L. tad.	81.081	77	94.68	97.777	90.728	93.827
	L. sub. vs L. vai.	8.536	9.322	11.111	34.131	41.279	30.256
	L. tad. vs L. vai.	29.885	30.578	22.047	35.057	32.46	32.093
Valamugil	*V. cun. vs V. se.*	78.181	76.923	90.384	44.696	40.506	37.579
	V. cun. vs V. se.	87.671	93.939	97.56	70.666	67.63	65.517
	V. se. vs V. spe.	94.736	87.671	95.652	72.641	66.071	66.666

Table 3. DIAGNOSTICS done by CAP for the morphometric data of *Liza species* of Parangipettai waters

m	prop.G	ssres	d_1^2	d_2^2	d_3^2	d_4^2	d_5^2	%correct
1	0.9416	4.703	0.321	0	0	0	0	30.667
2	0.9718	4.6581	0.3817	0.0317	0	0	0	34
3	0.9893	4.2561	0.4164	0.3816	0.0297	0	0	49.333
4	0.9941	4.163	0.4366	0.3999	0.0727	0.0291	0	55.333
5	0.9979	4.1672	0.4572	0.4003	0.0901	0.0322	0	56.333
6	1	3.9266	0.5773	0.4141	0.1791	0.0843	0.0123	62

m=Subset of axes, prop.G=proportion of variation in the data cloud described, ssres=the leave-one-out residual sum of squares, d_1^2=the size of the first squared canonical correlation and %correct =the percentage of the left-out samples that were correctly allocated to their own group.

Table 4. Cross validation of CAP results for morphometric data of *Liza species* of Parangipettai waters Leave-one-out Allocation of Observations to Groups (for the choice of m: 6) Classified

Orig. group	*L. macrolepis*	*L. parsia*	*L. planiceps*	*L. subviridis*	*L. tade*	*L. vaigiensis*	Total	%correct
L. macrolepis	37	3	5	1	1	3	50	74
L. parsia	1	24	17	2	0	6	50	48
L. planiceps	6	11	18	5	0	10	50	36
L. subviridis	5	0	2	40	3	0	50	80
L. tade	11	1	0	14	18	6	50	36
L. vaigiensis	1	0	0	0	0	49	50	98

Total correct: 186/300 (62%)
Mis-classification error: 38%

Table 5. DIAGNOSTICS done by CAP for the morphometric data of *Valamugil species* of Parangipettai waters

m	prop.G	ssres	d_1^2	d_2^2	%correct
1	0.8573	1.8354	0.1859	0	51.333
2	0.9815	1.7923	0.1876	0.0633	50.667
3	0.992	1.8193	0.1922	0.1018	52.667
4	0.997	1.6865	0.3648	0.1132	64.667
5	0.9993	1.5534	0.4952	0.1218	71.333
6	1	1.5494	0.4975	0.1425	72

m=Subset of axes, prop.G=proportion of variation in the data cloud described, ssres=the leave-one-out residual sum of squares, d_1^2=the size of the first squared canonical correlation and %correct =the percentage of the left-out samples that were correctly allocated to their own group.

Table 6. Cross validation of CAP results for morphometric data of *Valamugil species* of Parangipettai waters Leave-one-out Allocation of Observations to Groups (for the choice of m: 6) Classified

Orig. group	*V. cunnesius*	*V. seheli*	*V. speigleri*	Total	%correct
V. cunnesius	41	4	5	50	82
V. seheli	2	32	16	50	64
V. speigleri	7	8	35	50	70

Total correct: 108/150 (72%)
Mis-classification error: 28%

occurring in Parangipettai waters. *Valamugil seheli* showed more genetic distance from the other members of family Mugilidae. The number of base substitutions per site from pairwise analysis between the species (Table 7) showed the maximum value of 0.259 between *Liza planiceps* and *Liza parsia* and minimum of 0.002 between *Liza subviridis* and *Liza vaigiensis.* The average AT content of the Mugilidae family was found to be 53.4 % and the GC content 46.50 % (Table 8). The maximum AT content was found in *Valamugil cunnesius* (55.93%) and the minimum in *Liza subviridis* (51.31%). The maximum and minimum GC contents were observed in *Liza subviridis* (48.69%) and *Valamugil cunnesius* (44.07%) respectively. The pairwise distance variation at different codon positions (Table 9) showed maximum variations in the 2nd codon position and minimum in 1st codon position.

The phylogenetic tree constructed (Figure 4) showed two major clades. *Valamugil cunnesius* and

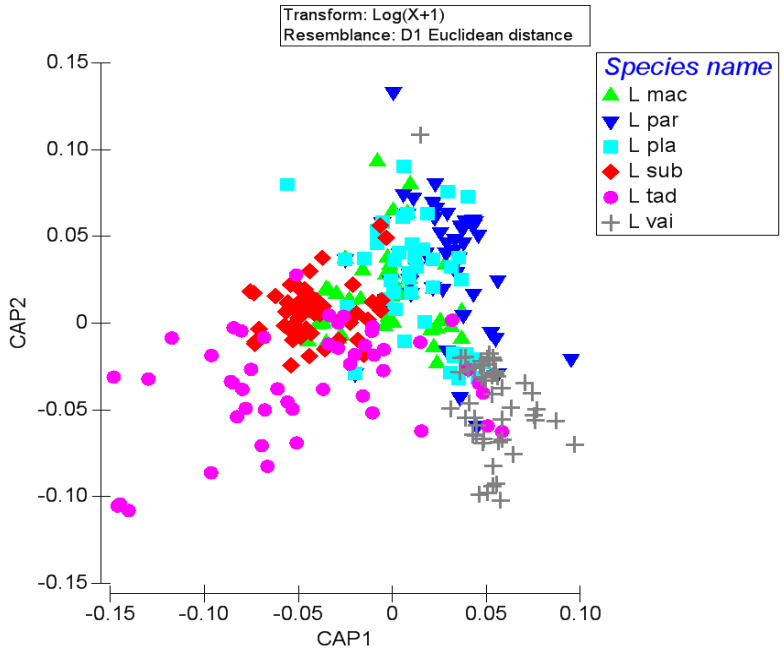

Figure 2. Scatter diagram of Canonical Analysis of Principal Coordinates (CAP) for morphometric data of species of *Liza*.

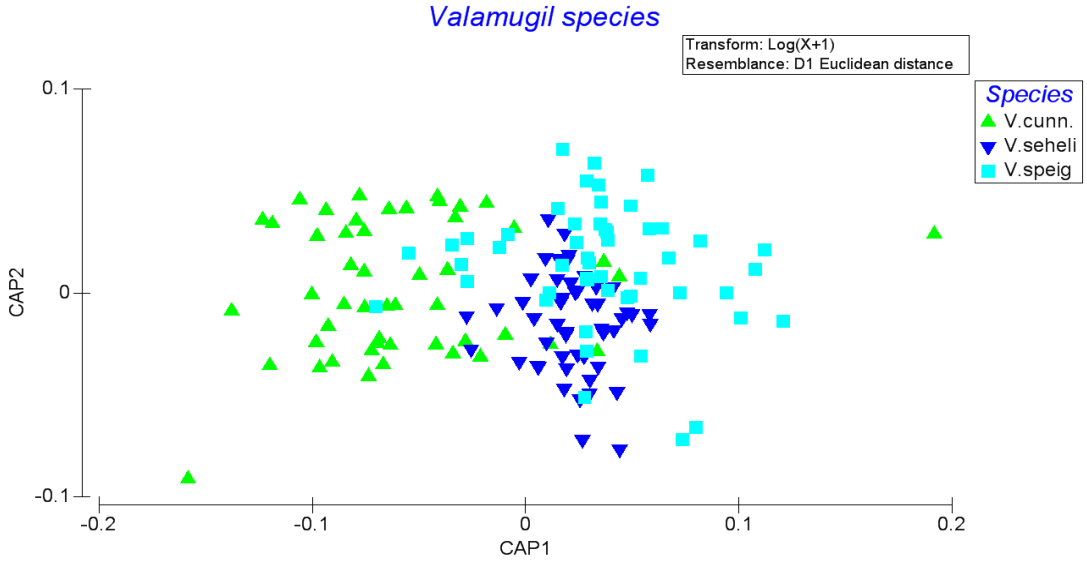

Figure 3. Scatter diagram of CAnonical Principal coordinates (CAP) analysis showing the congeners of *Valamugil.*

Table 7. Pairwise analysis table constructed using Kimura 2-parameter method for Mugilid species (Lower left diagonal shows the pairwise distance between the species and the upper right diagonal shows the standard error).

Species	1	2	3	4	5	6	7	8	9	10
Liza_tade(1)		0.014	0.022	0.027	0.033	0.033	0.033	0.037	0.029	0.029
Liza_parsia(2)	0.096		0.018	0.022	0.034	0.035	0.033	0.039	0.034	0.027
Valamugil_cunnesius(3)	0.164	0.124		0.009	0.030	0.031	0.029	0.036	0.035	0.024
Valamugil_speigleri(4)	0.212	0.161	0.039		0.035	0.035	0.033	0.037	0.036	0.028
Liza_vaigiensis(5)	0.226	0.229	0.209	0.235		0.001	0.006	0.009	0.035	0.027
Liza_subviridis(6)	0.229	0.232	0.211	0.238	0.002		0.006	0.009	0.035	0.028
Liza_macrolepis(7)	0.232	0.228	0.200	0.226	0.021	0.022		0.011	0.034	0.027
Liza_planiceps(8)	0.249	0.259	0.236	0.247	0.048	0.047	0.064		0.034	0.032
Mugil_cephalus(9)	0.211	0.239	0.228	0.238	0.243	0.245	0.246	0.237		0.034
Valamugil_seheli(10)	0.222	0.205	0.169	0.191	0.205	0.208	0.209	0.239	0.234	

Table 8. Percentage composition of nucleotides A, T, G, C, AT and GC in Mullet species

Family: Mugilidae	A %	T %	G %	C%	AT %	GC %
Liza_tade	23.21	31.15	18.93	26.72	54.35	45.65
Liza_parsia	23.21	32.52	18.93	25.34	55.73	44.27
Liza_vaigiensis	22.91	28.48	19.04	29.57	51.39	48.61
Liza_subviridis	22.73	28.57	19.2	29.49	51.31	48.69
Liza_macrolepis	23.16	28.53	19.02	29.29	51.69	48.31
Liza_planiceps	23.11	28.35	19.11	29.43	51.46	48.54
Valamugil_cunnesius	22.96	32.97	18.64	25.42	55.93	44.07
Valamugil_seheli	23.7	29.51	18.2	28.59	53.21	46.79
Valamugil_speigleri	22.31	33.39	19.34	24.96	55.69	44.31
Mugil_cephalus	24.23	29.94	18.21	27.62	54.17	45.83
Mean					53.493	46.507

Table 9. Pairwise distance variation at codon positions 1, 2 and 3 among the members of Mugilidae

Position of codon	1st	2nd	3rd
Liza_tade	0.004	1.293	0.03
Liza_parsia	0.008	1.259	0.028
Valamugil_cunnesius	0.004	1.103	0.024
Valamugil_speigleri	0.004	1.327	0.024
Liza_vaigiensis	0.004	1.004	0.028
Liza_subviridis	0.004	1.008	0.028
Liza_macrolepis	0.007	1.011	0.033
Liza_planiceps	0.004	1.248	0.033
Mugil_cephalus	0.007	1.664	0.038
Valamugil_seheli	0.004	1.346	0.038

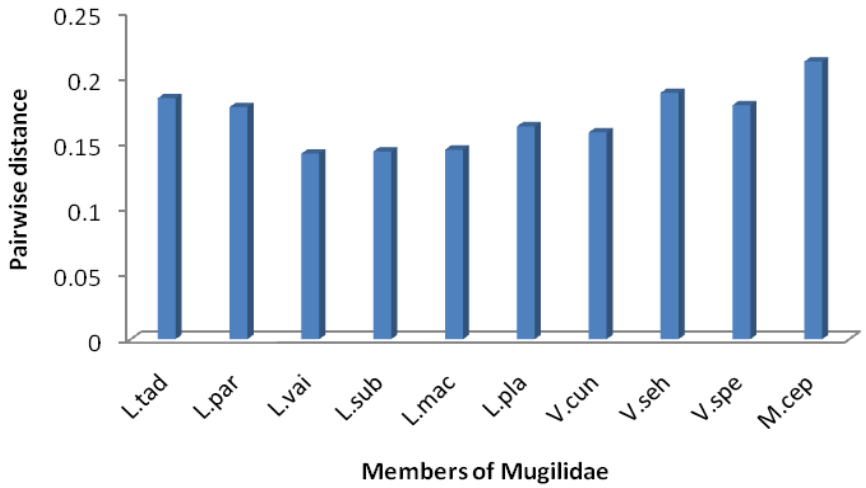

Figure 4. Inter-species variations within the barcode sequences of various species of Mugilidae.

Valamugil speigleri showed more genetic relatedness to *Liza tade* and *Liza parsia*, while the rest of the members from genus *Liza* were grouped in a separate major clade. *Mugil cephalus* fell in between the above clades as it is the most divergent species within the family (Figure 5).

Discussion

In the present study after the identification of the mullets, efforts were taken to validate the species both through morphometrics and molecular taxonomy. The morphometric data of the mugilid species showed higher overlapping as reported earlier by Stiassny (1993), Rossi *et al.* (1998) particularly, among the species belonging to genera *Liza* and *Valamugil.* Therefore CAP analysis was done. CAP is a routine for performing canonical analysis of principal coordinates. The purpose of CAP is to find axes through the multivariate cloud of points that either are the best at discriminating among *a priori* groups or have the strongest correlation with some other set of

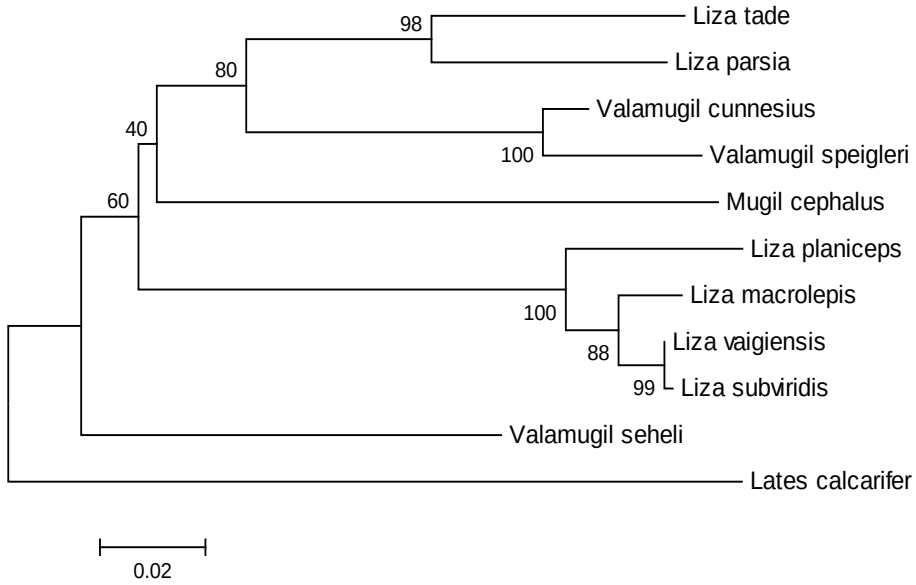

Figure 5. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.73976740 is shown. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site (given as bar scale at the bottom of the phylogram). There were a total of 639 positions in the final dataset which is a near full length barcode region (~650bp).

variables. This tool (constrained) is advantageous in that when there are real differences among *a priori* group that cannot be seen in unconstrained ordination such as PCA, MDS and PCO, this constrained ordination is helpful. Another advantage of this tool is once a CAP model has been developed, it can be used to classify new points into existing groups. When it has been done for various species of fishes, given a new fish that has values for each of these same measures, it can allocate or classify that fish into one of the groups using this routine (Anderson *et al.*, 2008). In view of this capability it was used in the present study. However it spite of its robustness in delineating group of data/species, it was not useful due to overlapping of morphometric data.

But DNA barcoding using COI clearly delineated all the 10 species of mugilids occurring in Parangipettai waters as could be seen in the phylogram. Among the 10 species the sequence for *Valamugil speigleri* is not available in the GenBank and this is the first sequence for this species in GenBank. It will help the investigators with identification of this species in future. This species should also be barcoded from other regions and it will be helpful in knowing the genetic diversity of this species. The present study demonstrated to efficacy of DNA barcoding in delineating species in a taxonomically difficult group as mullets. However it has thrown up several questions regarding the interrelationship of various species of mugilids barcoded. The *Valamugil seheli* falls away from all the species of mugilids instead of sharing the clade with its kins *V. cunnesius* and *V. speigleri*. Similarly species of *Liza* fall in two distant clades. The subclade of *L. parsia* and *L. tade* is closely related to *V. cunnesius* and *V. speigleri*, than with their congeners (*L. macrolepis*, *L. planiceps*, *L. subviridis* and *L. vaigiensis*) which form a separate major clade. *M. cephalus* falls in the middle of the phylogram showing its relationship to the members of *Liza* and *Valamugil.* Eventhough it is indicating the efficacy of DNA barcoding in delineation of species within taxonomically difficult group as mugilids, the mix-up among the species in the phylogram may be due to the evolutionary history.

The phylogram strongly questioned the monophyletic origin of *Liza* and *Valamugil*. The monophyly of *Liza* has been questioned previously by several authors (Caldara *et al.,* 1996; Papasotiropoulos *et al.,* 2001, 2002, 2007; Rossi *et al.,* 2004; Fraga *et al.,* 2007; Turan *et al.,* 2005, 2011). Semina *et al.* (2007) recommended synonymising *Liza* with *Chelon.* The monophyly of *Liza* is not clearly supported when considered along with relevant information on chromosomes, morphology, allozymes, RFLPs and mtDNA sequences. *Mugil cephalus* was found to be most divergent species in the present investigation as evidenced from the pairwise distance data (Turan *et al.,* 2011). In case of *Liza* and *Valamugil*, more research is needed to address taxonomic issues at the infra-generic level as suggested by Durand *et al.* (2012).

Currently, the emphasis is on the development of a pluralistic system (Hendry *et al.,* 2000) involving variations in mtDNA, nuclear DNA and morphological traits, within and among groups above the species level (Avise and Walker, 2000) which

could improve the classical biological classification of Mugilidae. Additional markers and other data are also very useful in groups where the ratio of divergence within and between species is unknown (Locke *et al.*, 2010; Kumar *et al.*, 2011). Delimiting species on the basis of any single molecular marker is also controversial (Kunz, 2002; Hickerson *et al.*, 2006; Poulin and Keeney, 2007; Fre´zal and Leblois, 2008).

In the present study specimens of all the species were collected from Vellar estuary in Parangipettai (Southeast coast of India) and COI is found to be efficient in identifying all the species. To make sound inferences regarding evolutionary history of these species what is required is extensive sampling from different geological locations covering the entire range of distribution of species and the use of multiple mitochondrial markers such as Cytochrome b and 16S rRNA genes or any other marker which will be of immense use in revealing the evolutionary history of mugilids. Therefore efforts have to be taken at regional and global levels to investigate the above aspect in mugilids.

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