Original Research Article

Creation of Species Specific Molecular Signatures of Schilbeid Fishes from River Ganga by Integrative Taxonomy

Abstract

The digital revolution has driven the society to the age where all required information should be available on tablets or android phones. Creation of molecular signature is to facilitate the accurate identification of biological species by end user, particularly when morphological characters among congeners overlap. Also the phylogenetic analysis of Asian schilbeids could not be clearly defined due to lack of data, so the present study generated morphological and DNA barcode data for five commercially important schielbid species namely *Clupisoma garua*, *Eutropiichthys murius*, *Eutropiichthys vacha*, *Ailia coila* and *Silonia silondia* from the River Ganga, India. Additionally, 31 sequences of Schielbid species available in GenBank were also included in analysis to present a clear picture of the phylogenetic relationship among Schilbeids. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model with Gamma distribution. The average Kimura two parameter (K2P) distance between the species and within the species, show the sufficient separation of species. The neighbour-joining tree revealed distinct clusters in concurrence with the taxonomic status of the species. Our study established sister group relationship between genera of Asian schilbeids as (*Clupisoma-Laides) Eutropicthys) Silonia) Ailia* and *(Horabagrus, Pseudeutropius, Neotropius or Pachypterus*) and also suggest placement of *C. sinensis* to the genus *Laides*.

Keywords: Cluster analysis, DNA Barcoding, integrative taxonomy, principal component analysis, phylogeny, Schielbidae.

Introduction: The family Schilbeidae consists of five African and five Asian genera [4] and are morphologically distinguished by the laterally compressed body with two to four pairs of barbels on the snout; anal fin very long and pectoral fin always have a strong spine. The schilbeid catfishes, commonly called glass catfishes [2] are exploited for food, angling sports and aquariums [1]. The congeners of *Eutropiichthys* are differentiable based on length of maxillary barbells and numbers of fin rays. Since all these characters are present only in adults hence the specimens at the early stage are hard to identify [27] in juvenile stage. Very early stages of *Clupisoma garua* closely resemble to those of *Silonia silondia* and *Bagarius bagarius* in general appearance and body contour. *C. garua* and its allied species *Eutropiichthys vacha* closely resemble in general appearance that they are often mistaken as the same species and thus both considered by name of 'Bacha' in commercial landings and market. This species identification based on morphological characters and meristic count is very difficult in early life stages and can be addressed by the DNA marker. Hebert et al., [9] proposed DNA barcoding based on mitochondrial gene cytochrome c oxidase I (COI). Since then, this has been successfully tested as species identification tool in a large variety of organisms and found a suitable marker for discriminating between closely related species as well as cryptic species of marine and freshwater fishes [7, 30, 17, 23]. This study utilizes COI markers to fix the molecular signature for Schilbeid species to provide a suitable tool for species identification as well as infer the phylogenetic relationship.

Based on morphological characters, interrelationships among Schilbeid catfishes were studied by Mo [18] including *Clupisoma* and *Platytropius*, but could not place the genus *Clupisoma* in phylogenetic tree. The Schilbeidae was not monophyletic, as the African genera formed a distantly related monophyletic group as studied by Peng et al. [21] and Hardman [8] based on mitochondrial gene cytochrome b and Sullivan et al., [24] based on nuclear genes RAG1 and RAG2. Karinthanyakit and Jondeung [14] studied six schilbids of Thailand based on the mitochondrial genes and *E. vacha* was established as a sister group of *Clupisoma*. Wang et al., [29] using the concatenated mitochondrial genes COI, cytb, and 16S rRNA, as well as the nuclear genes RAG1 and RAG2, established a sister-group relationship for (*Ailia* (*Laides*, *Clupisoma*)) and the Sisoroidea and a sister taxon association of (*Horabagrus, Pseudeutropius*) and the Bagridae. In

contrast, analyses of the combined nuclear data indicate (*Ailia* (*Laides*, *Clupisoma*)) to be the sister group to (*Horabagrus, Pseudeutropius*). The interrelationship among Schilbeidae genera vis-a-vis other catfish families remained unclear due to absence of the Asian genera *Clupisoma, Ailia, Eutropiichthyes* and *Silonia.* In present study, COI sequences of these genera were generated to clarify the exact relationship among Schilbeid catfishes.

Material and Methods

Sample collection: Five species of Schilbedie family were collected from the Middle stretch of Ganga River at Allahabad, India. The species were identified based on existing information in "The Freshwater Fishes of the Indian Region" [12], "Catfishes of India" [13] and "Fishbase" [6]. The dichotomous keys of Talwar and Jhingran [27] and Jayaram [12] were also followed to confirm the species identification. All the fish voucher specimens were tagged with an alphanumeric code and deposited in the Department of Zoology, University of Allahabad. Muscle and fin tissues were removed from fresh samples acquired during netting. Approximately 100 mg of white muscle tissue from six individuals of each species were preserved in 95% ethanol until used. Specimen details and Gene accession numbers are given in Table1.

Principal component analysis: The morphometric characters analyzed for five Schilbeid species included Total Length: TL, Standard Length: SL, Fork Length: FL, Body Depth/Maximum Body Depth: MBD, Eye Diameter: ED, Post-orbital Length: PostOL, Snout Length: SnL, Prepectoral Length: PrePecL, Prepelvic Length: PrePeL, Preanal Length: PreAL, Caudal Length: CL and Caudal Depth: CD. Principal component analysis (PCA) and cluster analysis (CA) were carried out to discriminate the five fish species of Schilbeidae family (Fig 1 and 2).

DNA isolation: Approximately 50 mg of fin or muscle tissue was used for DNA isolation following standard phenol: chloroform: isoamyl alcohol method [20]. Precipitated DNA was re-suspended in TE buffer (10mM Tris –HCl, 0.1 mM EDTA, pH 8) and concentration was determined using Nanodrop 2000 (Thermo Scientific, USA).

PCR amplification and Sequencing: The COI gene was amplified in a 50µL volume with 5µL of 10X Taq polymerase buffer, 2µL of $MgCl_2$ (50mM), 0.25µL of each dNTP (0.05mM), 1µL of each primer (0.01mM), 2 U of Taq polymerase and 150 ng of genomic DNA. The primers used for amplification of COI gene were FishF1-5'TCAACCAACCACAAAGACATTGGCAC3' and Fish R1-5'TAGACTTCTGGGTGGCCAAAGAATCA3' [30]. The thermal regime consisted of an initial step of 2 min at 95℃ followed by 35 cycles of 40s at 94℃, 40 s at 54℃ and 1 min s at 72℃ followed in turn by final extension of 10 min at 72℃. The PCR products were visualized on 1.2% agarose gels, the good quality PCR products were selected for sequencing. Products were labeled using the Big Dye Terminator V.3.1 Cycle sequencing kit (Applied Biosystems, Inc) and sequencing bidirectinally using an ABI 3730 capillary sequencer following manufacturer's instructions.

Sequence analysis:

In present study 30 COI sequences of five commercially important Schielbid species namely *Clupisoma garua*, *E. murius*, *E. vacha*, *A. coila* and *Silonia silondia* were used for analysis. In addition, 31 sequences of Schielbid species and 2 outgroups available in NCBI GenBank were also included to make a comprehensive overview and elucidate phylogenetic relationship among Schilbeids (Table 1). Sequences were aligned using CLUSTALW integrated in MEGA 6 (Molecular Evolutionary Genetics Analysis) software [28]. The discrepancies were referred against electropherograms, sequences were blasted in NCBI (http://www.ncbi.nlm.nih.gov) for the nearest matches and submitted to GenBank (Table 1). To analyse the evolutionary isolation and the level of divergence within species, K2P distance was calculated by averaging pairwise comparisons of sequence across all individuals by the Kimura 2-Parameter method [15] under Gamma distribution in MEGA 6 software. The maximum likelihood (ML) phylogenetic tree were constructed by using the best fit substitution model HKY+G+I (Hasegawa-Kishino-Yano + Gamma distribution + some invariable sites) with 1000 bootstraps (Fig. 3) using MEGA6 software.

Results and Discussion

Out of 697 positions in the COI gene sequences analysed, 273 (39.1%) were variable, and 236 positions (33.8%) were parsimoniously informative. The average base composition [Thymine (T); Cytosine (C); Adenine (A) and Guanine (G)] were A=26.3%, C=26.5%, G=17.7%, T=29.5% which showed COI gene were A+T rich (55.8%). Intra species pairwise distances of Schilbeidae species were highlighted in the Table 2. The Maximum Likelihood (ML) phylogenetic tree was constructed and 1000 bootstrap re-sampling strategy was used to assess the reliability of the phylogenetic tree. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (-3828.9686) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.4935)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 57.1794% sites).

The present study shows that Schilbeidae has polyphyletic origin as also indicated by Mo [18] based on morphological data and (Hardman [8]; [24]; Wang et al., [29] based on molecular data and form two distantly related monophyletic groups of Asian and African schilbids. Mo [18] concluded that the Asian schilbids comprised two distinct groups: Ailia and the genera Horabagrus, Pseudeutropius and *Platytropius* using morphological data. Hardman [8] resolved the relationships as (*Pseudeutropius* (*Horabagrus, Clupisoma*)) and assigned these genera to the Horabagridae (erected by de Pinna), but analysis did not include *Ailia* and *Laides* genus. Sullivan et al., [25 and Sullivan et al., [24] suggested that Asian group consist of (*Ailia, Ladies*), and (*Horabagrus, Pseudeutropius*). Wang et al., [29] gave similar phylogenetic relationship among five representatives Asian schilbid genera with two monophyletic groups (*Ailia* (*Laides, Clupisoma*)) and (*Horabagrus, Pseudeutropius*) and formally erected a new family, Ailiidae fam. nov. for a monophyletic Asian group having three genera *Ailia, Laides* and *Clupisoma* and our result also supports these findings. However these studies does not present the clear picture as Mo (1991) did not clearly commented on the relationship of *Clupisoma* with other genera and Wang et al., [29] and Sullivan et al., [24] did not include the genus *Eutropicthys* and *Silonia*. The anomalies in phylogenetic classification of this family might be due to the absence of critical taxa in the study. Our study is the first to feature

the phylogenetic relationships for all nine recognized genera of Asian Schilbidei and also supports the finding that Asian Schilbidei appears to be a sister group of (*Horabagrus, Pseudeutropius*).

In our study, groups (*Ailia* (Laides, *Clupisoma*)) and (*Horabagrus, Pseudeutropius*) does not support the monophyly of the “Big Asia” as proposed by Sulliva et al., [24] and Sullivan et al., [25] . The present phylogenetic analysis also established sister group relationship between recognized genera of Asian Schilbeids as (((*Clupisoma-Laides)Eutropicthys) Silonia) Ailia and (Horabagrus, Pseudeutropius, Neotropius or Pachypterus*) while Big African Schilbeids include (*Schilbe, Paraila, Pareutropius*).

Ng [20] renamed chinese Schilbeid *Platytropius sinensis* (Huang, 1981) as *Clupisoma sinensis* and Chen et al., [3] considered *C. sinensis* and *C. longianalis* as congeners. In our study, *C. sinensis* is closer to *Laides hexanema* than *C. garua* and *C. prateri*. *Clupisoma sinensis* and *Laides hexanema* claded together with strong bootstrap value of 99 percent. *Clupisoma sinensis* and *Laides hexanema* jointly form a distinct sister clade with *C. prateri* and *C. garua,* but with suboptimal bootstrap value of 41 percent. Hence the phylogenetic position of *Clupisoma sinensis* is still questionable. The *C. sinensis* may be placed in *Laides* genus instead of *Clupisoma* as also suggested by Wang et al., [29]. The enigmatic *Clupisoma sinensis* was recognized as more closely related to *Laides hexanema* (pairwise distance between *Clupisoma sinensis* and *Laides hexanema* is 0.056) than to *Clupisoma prateri* (pairwise distance between *Clupisoma sinenses* and *Clupisoma prateri* is 0.102). So, there is a probability that *Clupisoma sinensis* may be placed in *Laides* genus instead of *Clupisoma* (Table 2, Fig 4). Thus, based on the COI genetic distances, a recategorization of *C. sinensis* to the genus *Laides* is suggested.

Principal component analysis (PCA) and cluster analysis (CA) were carried out to discriminate the five fish species of Schilbeidae family (Fig 1 and 2). The morphometric characters analyzed for five Schilbeid species included Total Length: TL, Standard Length: SL, Fork Length: FL, Body Depth/Maximum Body Depth: MBD, Eye Diameter: ED, Post-orbital Length: PostOL, Snout Length: SnL, Prepectoral Length: PrePecL, Prepelvic Length: PrePeL, Preanal Length: PreAL, Caudal Length: CL and Caudal

Depth: CD. The two multivariate analyses (Cluster and PCA), Phylogenetic tree and Pairwise distances among Indian Schilbeids indicates that *C. garua* is more closely related to *E. vacha* and *E. murius* is more closely related to *A. coila* while *S. silondia* has a separate cluster / group.

Hypophthalmus goongwaree was described by Sykes [26] based on material collected in the Mota Mola River near Poona, Maharashtra, in peninsular India. However, Ferraris Jr and Vari [5] concluded that Hora [10] without mentioning location of sampled species and catalog number reported that his specimens was conspecific to *H. goongwaree* and concluded that the specimen belongs to genera *Eutropiichthys*. Because of these discrepancies, Ferraris Jr and Vari [5] tentatively concluded that while Hora's specimen(s) may represent a species of *Eutropiichthys*, that species is not conspecific with *H. goongwaree* should not be included in *Eutropiichthys* and renamed it as *Proeutropiichthys goongwaree* (Sykes, 1839) with question. According to our study *H. goongwaree* should be placed in *Eutropiichthys* as it form a clade with *Eutropiichthys* species in contrast to other *Hypophthalmus* species (Fig 4). In our study also, this species could not find an undisputed taxonomic position and there is need for high resolution molecular markers like cytochrome b, whole mitochondrial genome etc. to confirm its position.

The genus *Horabagrus* has been placed in 3 different families namely Bagridae [18], Schilbeidae [19], Horabagridae [13,16]. Present study supported Family Horabagridae with genera *Horabagrus, Pseudeutropius* and *Pachypterus*. In summary, our studies suggested that the group *Clupisoma, Laides*, *Eutropicthys*, *Silonia* and *Ailia* is monophyletic and (*Horabagrus, Pseudeutropius, Neotropius* or *Pachypterus*) is its sister-group.

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Figure captions

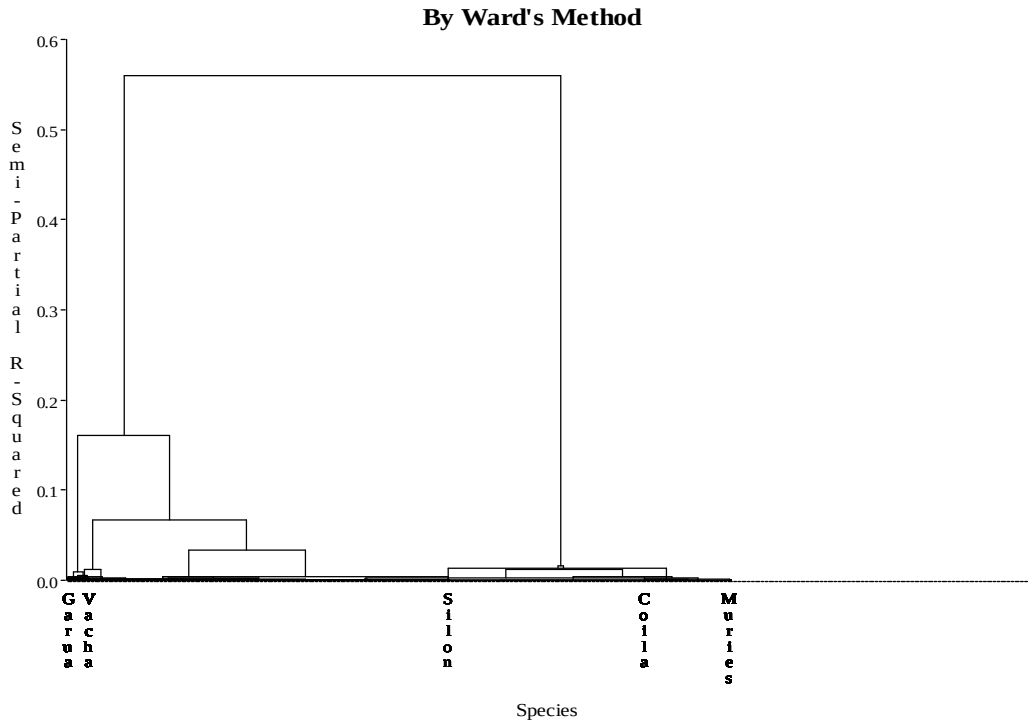
Fig.1 Cluster analysis among Indian freshwater fishes of Family Schilbeidae

Fig.2 Principal component analysis of all Indian Fresh water species of Family Schilbeidae

Symbol: 1- *C.garua,* 2-*E. vacha*, 3- *E. murius*, 4-*S. silondia* and 5-*A. coila*

Fig 3 Evolutionary relationships of taxa

Fig 4 Molecular Phylogenetic analysis by Maximum Likelihood method



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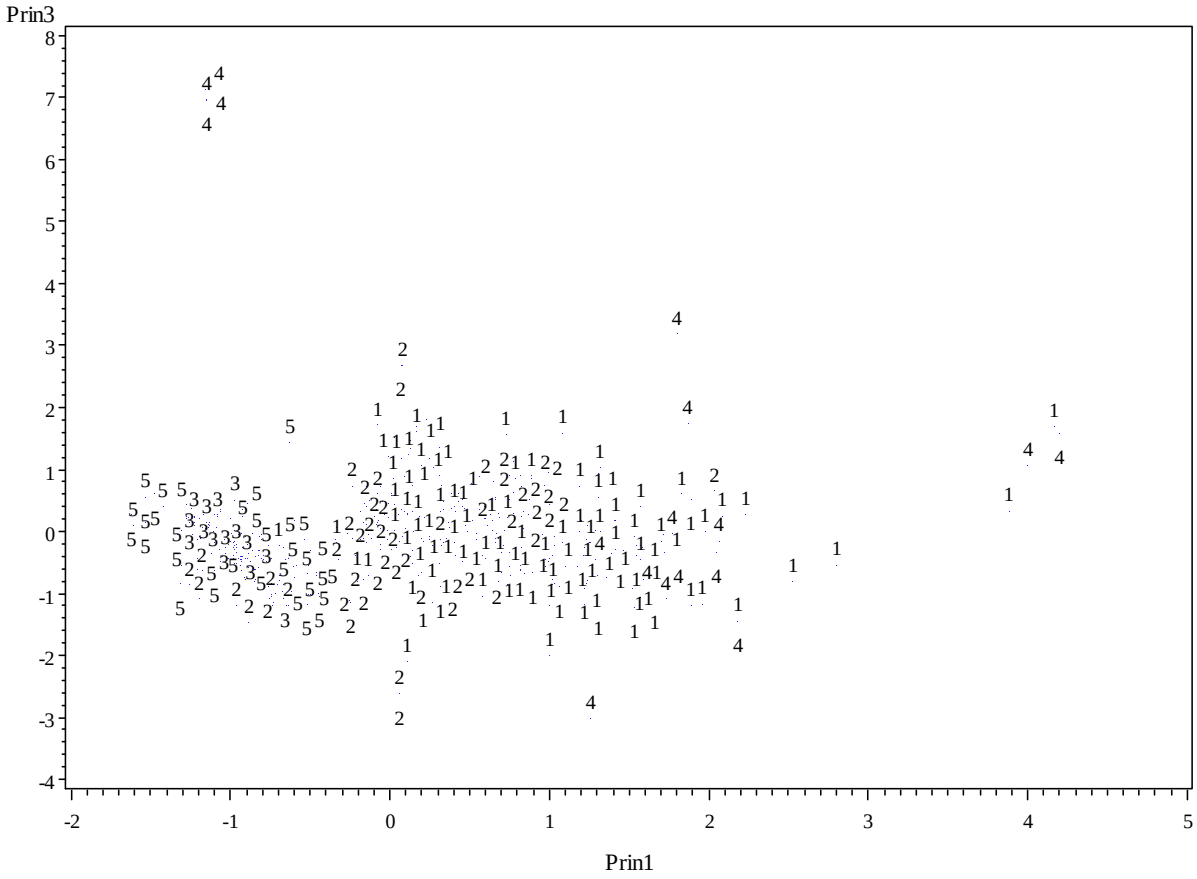

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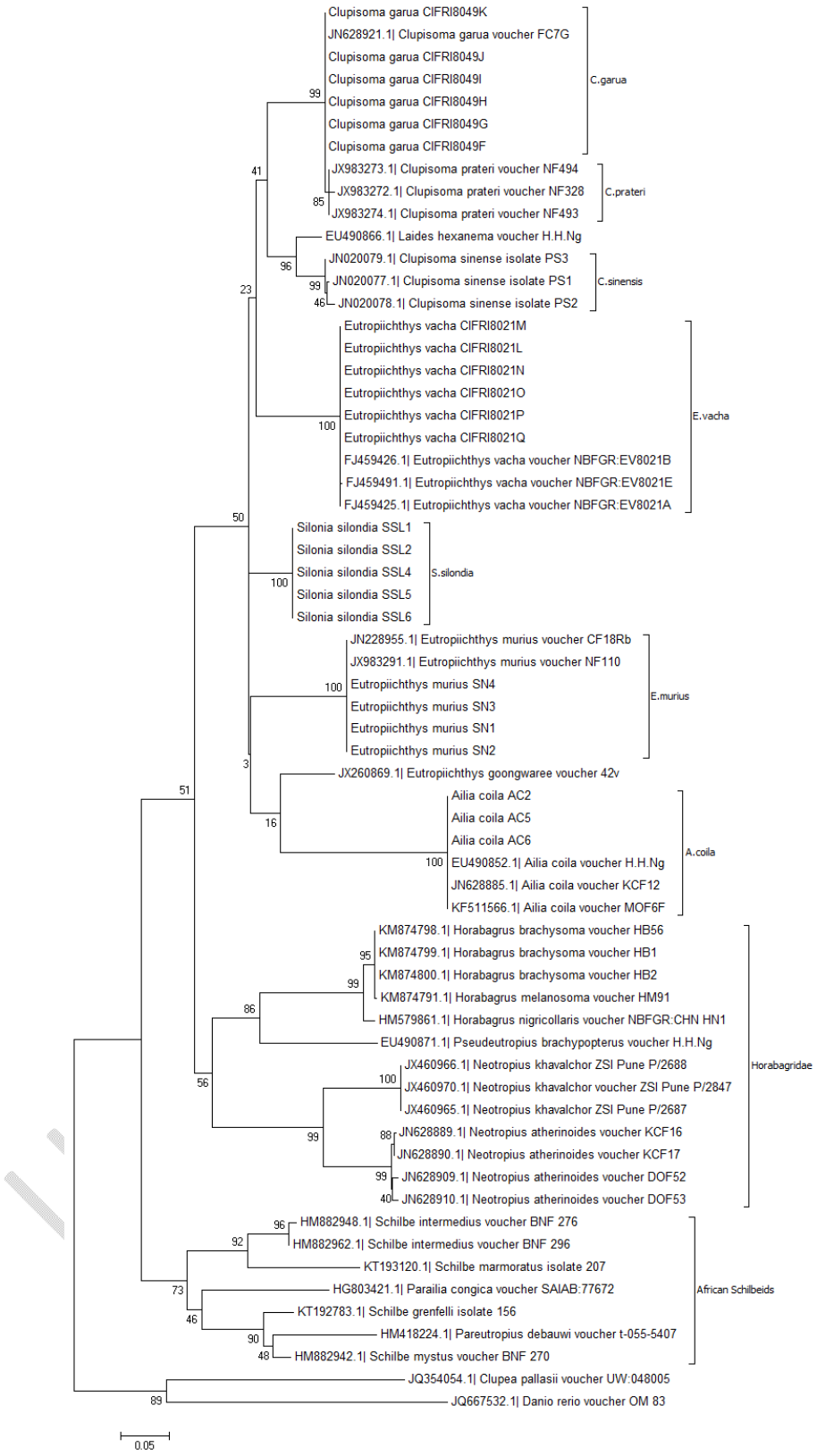

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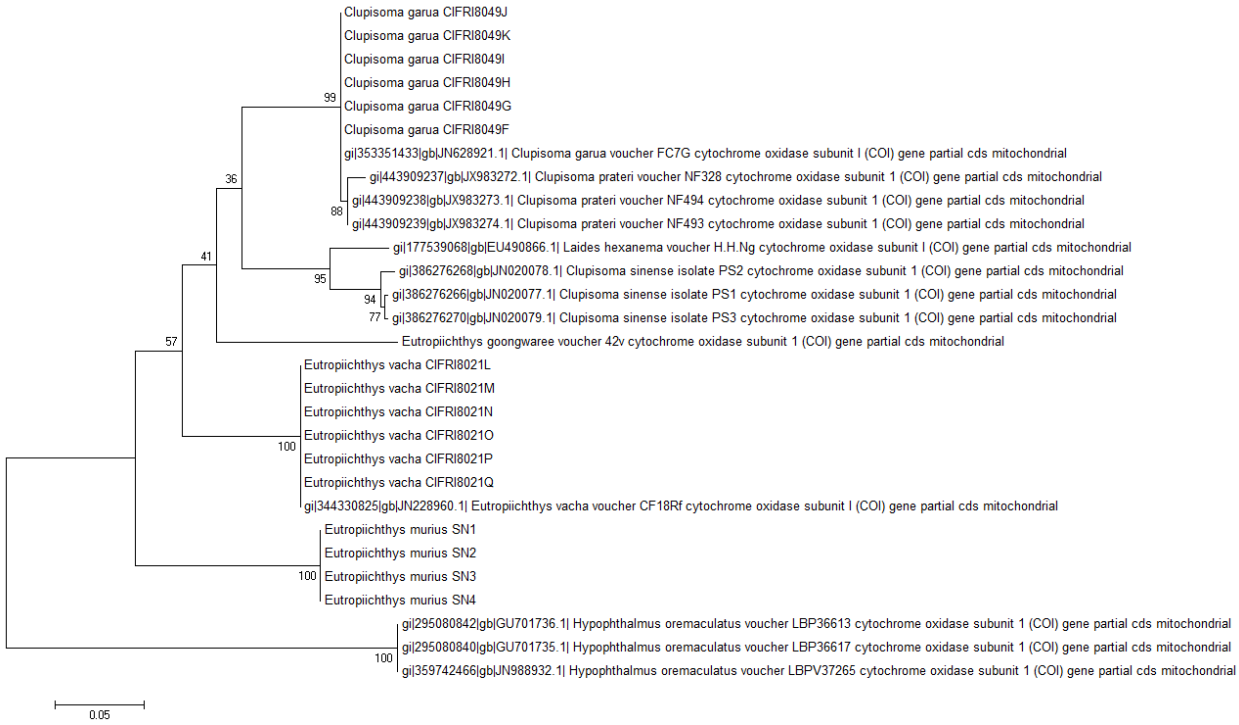

Fig 4 Molecular Phylogenetic analysis by Maximum Likelihood method

Table 1: Detail of fish samplings and GenBank accession numbers**.**

S.N.	Collection Site	Sample size		Latitude/ longitude	GenBank accession numbers	
		Cyt b	ATPase 8/6		ATPase 8/6	Cyt b
1.	Hoogly Feeder Canal, Farraka, West Bengal	10	9	24.48N/ 87.55E	KF475255-63	KC816486-95
2.	Ganga River, below Farraka Barrage, Malda, West Bengal	7	7	24.47N/ 87.55E	KF475281-87	KC816514-20
3.	Hooghly River at Kotghat, Kolkata, West Bengal	1	1	22.51N/ 88.22E	KF475246	KC816485
4.	Diamond Harbour, West Bengal	11	8	22.10N/ 88.10E	KF475247-54	KC816475-84, KC816521
5.	Paradip Port, Odisha	10	9	20.19N/ 86.36E	KF475264-72	KC816496-505
6.	Godavari River,	8	8	16.56N/	KF475273-80	KC816506-

	Rajahmundry, Andhra Pradesh.			81.44E		13
7.	Narmada River, Barkal, Gujarat	12	13	21.55N/ 73.25E	KF475288-KF475300	KC816522-33
8.	Tapti River, Ukai Dam, Surat, Gujarat	10	8	21.15N/ 73.35E	KF475238-45	KC816465-KC816474

Table 2: Haplotype and nucleotide diversities in different populations of *T. ilisha.*

	ATPase 8/6			**Cyt b**		
Populations	**No. of haplotypes**	**Haplotype diversity(h)**	**Nucleotide diversity(π)**	**No. of haplotypes**	**Haplotype diversity(h)**	**Nucleotide diversity(π)**
Diamond Harbour	6	0.929±0.084	0.0025±0.0005	5	0.709±0.137	0.00129±0.00040
Hoogly Feeder Canal	4	0.694±0.147	0.0066±0.0030	5	0.844±0.080	0.00154±0.00029
Paradip Port	4	0.583±0.183	0.0010±0.0003	7	0.911± 0.077	0.00243± 0.00032
Godavari	4	0.750±0.139	0.0016±0.0004	4	0.786±0.113	0.00158±0.00034
Ganga	3	0.667±0.160	0.0012±0.0003	5	0.905±0.103	0.00190±0.00036
Narmada	3	0.410±0.154	0.0020±0.0010	5	0.66667± 0.141	0.00208±0.00072
Tapti	2	0.571± 0.094	0.0040±0.0006	3	0.64444±0.101	0.00307±0.00041

Table3: Hierarchal analysis of molecular variance (AMOVA) for *T. ilisha.*

Source of variation	**Variance**	**% Total**	**Fixation indices**	**p-value**

One gene pool (Tapti, Narmada, Diamond Harbour, Hooghly, Hooghly Feeder Canal, Paradip Port, Godavari, Ganga)				
ATPase 8/6				
Among populations	0.0918	22.45	0.2245	p<0.001
Within population	0.3172	77.55	-	-
Cyt b				
Among population	0.04244	9.93	0.09932	p<0.01
Within population	0.38486	90.07	-	-
Two gene pool (Tapti, Narmada) and (Diamond Harbour,Hooghly, Hooghly Feeder Canal, Paradip Port, Godavari, Ganga)				
ATPase 8/6				
Among groups	0.1430	29.94	0.299	p<0.05
Among populations within group	0.0175	3.67	0.052	NS
Within population	0.3172	66.39	0.336	p<0.001
Cyt b				
Among groups	0.06546	14.24	0.14243	p<0.05
Among population within groups	0.00928	2.02	0.02354	NS
Within population	0.38486	83.74	0.16261	p<0.01
Three gene pool (Tapti, Narmada), (Diamond Harbour, Hooghly, Hooghly Feeder Canal, Paradip Port, Ganga) and (Godavari)				
ATPase 8/6				
Among groups	0.1309	29.03	0.290	p<0.05
Among populations within group	0.0028	0.63	0.008	NS
Within population	0.3172	70.34	0.296	p<0.001
Cyt b				
Among groups	0.06012	13.43	0.13427	p<0.01

Among population within groups	0.00279	0.62	0.00720	NS
Within population	0.38486	85.95	0.14050	p<0.01
Four gene pool (Tapti, Narmada), (Diamond Harbour, Hooghly, Hooghly Feeder Canal, Ganga), (Godavari) and (Paradip Port)				
ATPase 8/6				
Among groups	0.1097	25.54	0.255	p<0.05
Among populations within group	0.0027	0.64	0.008	NS
Within population	0.3172	73.83	0.261	p<0.001

Table 4: Pairwise F_{ST} (below diagonal) ATPase 8/6 and Pairwise F_{ST} (above diagonal) Cyt b among *T. ilisha* population.

Populations	Sampling sites							
	Hooghly	**Diamond Harbour**	**Hooghly Feeder Canal**	**Paradip Port**	**Godavari**	**Ganga**	**Narmada**	**Tapti**
Hooghly	0	-0.5600	-0.2063	-0.0123	-0.2571	-0.2666	0.2000	-0.2888
Diamond Harbour	-0.2381	0	-0.0044	0.0922	0.0348	0.0208	0.2437*	0.0688
Hooghly Feeder Canal	0.1071	-0.0230	0	-0.0449	0.0539	-0.0542	0.2079*	0.1241