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Phylogenetic analysis among Cyprinidae family using 16SrRNA

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Abstract
In the present study, the cyprinid fishes (*Labeo rohita, Catla catla, Cirrihinus mrigala*) of Agra region were selected for resolving their phylogeny. Sequencing of fishes were done using mitochondrial 16S rRNA gene and it was compared with other cyprind fishes on the basis of similarity. 17 sequences of cyprinid fishes were downloaded from NCBI along with one out group of family Balitoridae as a root of tree. They were examined to construct the phylogenetic tree within the most diverse family Cyprinidae. The present study reveals the high rate of mutation in the fishes. The overall transition/ transversion bias R shows the high deviation from the neutral evolution where R= 0.5. In the phylogenetic tree the neutrality test is conducted which rejected the neutral variation. The negative values of Tajima's D test shows the bottle neck effect whereas values of Li and Fu'S D* and Li and Fu's F* test show excess of external mutation. The maximum parsimony analysis method shows the relation of *Catla catla* with subfamily Labeoninae and these are indicated through the bootstrap values. The family Cyprinidae was resolved as a paraphyletic group which shows the divergence may occur, but they share the same common ancestor and the value of consistency index and retention index supports that they have shared the common ancestor.

Keywords: Cyprinidae, Phylogenetic, Neutral evolution.

1. Introduction
The family Cyprinidae of order Cypriniformes is one of the most diverse family of freshwater fishes in the world consisting of atleast 220 genera and 2420 species [1]. They are cosmopolitan in nature, but they are not found in South America, Australia and Antarctica [2]. These fishes are present in large quantity and are good source of proteins and therefore they are economically important. Due to tremendous diversity within this family becomes a problem to establish a phylogenetic relationship. The systematic history of Cyprinidae fishes is the most debated part and only by considering the morphological characters it is impossible to solve the phylogeny. There were many hypothesized subfamilies formed within the family Cyprinidae due to which their phylogenetic relationship is yet disputed. Previously, the phylogeny was done on the basis of morphological characters like the structures of lips and associated characters in a pre buccal cavity of Labeoninae fishes [3]. The Cyprinidae family was classified into 10 subfamilies on the basis of osteological characters and in two tribes Leuciscini and Barbini [4]. Later on Cyprinid was classified into Labeoninae, Barbinae and Cyprininae [5] and in many tribes and subtribes by Rainboth [6]. During the last two decades the mitochondrial and nuclear genes were used to solve the problem of phylogeny among this group. The mitochondrial genome is the entirety of hereditary information. Sequence similarities serve as the proof for structural and functional conservation.

The first molecular phylogeny of family Cyprinidae was presented by Brioly *et al.* [7] using mitochondrial gene cytochrome b while Gilles *et al*. [8] threw light on phylogenetic picture using 16 ribosomal DNA along with cytochrome b and Simons *et al.* in same year used 12S rRNA and 16S rRNA to resolve phylogenetic relationship within cyprinids. The first molecular study of phylogeny within labeoninae was performed by Li *et al*. [9] using 16S rRNA sequences. In 2008 Li *et al*. [10] reconstructed the phylogenetic relationship using mitochondrial 16S rRNA gene with secondary structure constraints in 93 cyprinid fishes. Recently, many researchers have investigated new phylogenetic interrelationships and tested the reliability of earlier established phylogenetic relationships using nuclear DNA [11]. Mayden *et al.* [2] selected eleven cyprinid

fishes as out groups due to their unambiguous relationship with subfamilies Labeoninae. For the current study on cyprinids mitochondrial 16S rRNA was used. These genes are conserved and non-coding in nature which played vital role in determination of new phylogenetic relationships and in checking reliability of earlier established phyletic classification. In eukaryotic mitochondria these genes evolve slowly and these genes are present in all fishes used in the study. Thus pursued our study on Cyprinidae family in Agra region using molecular markers and the samples were collected from Yamuna river of Agra region and the major carps are *Labeo rohita, Catla catla* and *Cirrhinus mirgala*. The aim of this study is to: determine the Cyprinine phylogeny based on mitochondrial 16S rRNA genes, and to provide the phylogeny within the family Cyprinidae.

2. Materials and Methods

2.1 Specimen's Collection

The cyprinid fishes i.e. *Labeo rohita*, *Catla catla*, *Cirrhinus mrigala* were collected from river Yamuna near Malpura village, Agra (Uttar Pradesh), India with the help of local fisherman. The fishes were in adult stage. The DNA was isolated from fins.

2.2 Isolation of genomic DNA and PCR amplification

The genomic DNA was extracted from fins of fishes using standard phenol-chloroform isoamyl alcohol method [12]. The mitochondrial 16S rRNA was amplified using universal 16S rRNA primer [13] and standard PCR techniques. All PCRs contained 1 µl of template DNA (150 ng), 5 µl 10X Taq buffer. 1 µl 2.5 mM dNTP mixture,1µl of 10 mM dilution of each primers. 0.5 µl Taq DNA polymerase. $MgCl_2$ 4 µl and milli Q water to find volume of 50 µl. The PCR reaction consists of initial denaturation step (95 ºC for 3 min) 35 cycles of denaturation (95 ºC for 30 sec) annealing for 30 sec at primer specific temperature and extension 72 ºC for 30 sec and post cycling extension 72 ºC for 10 min, amplified product were run on 1.5% agarose gel stained with ethidium bromide. PCR products were custom sequenced and the sequences were submitted to NCBI database.

3. Analyzing Phylogenetic Tree

3.1 Sequence alignment and nucleotide diversity

The sequences were aligned using CLUSTAL X2 multiple sequence alignment program. Variation in the sequences within each population was estimated through nucleotide diversity (π), average number of nucleotide differences (k) and transition/transversion bias R. Nucleotide diversity (π) and average numbers of nucleotide differences (k) were calculated using DnaSP 5.0 program (14) and transition/transversion bias R was calculated using MEGA 5.0.

3.2 Phylogenetic analysis

The phylogeny was established using the maximum parsimony (MP method) using MEGA 5.0 program. The evolutionary distances were calculated using the maximum composite likelihood method which was shown by the units of the number of base substitutions per site. All position containing gaps and missing data were eliminated from data set (complete deletion). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. The maximum parsimony tree was obtained using the Subtree Pruning Regrafting algorithm (SPR) with search level 1 in which the initial trees were obtained with the random addition of sequence (10 replicates). All position containing gaps and missing data were eliminated. One outgroup *Nemacheilus montana* was included in alignment as a root of tree. Phylogenetic analyses were conducted in MEGA 5.0.

3.3 Population differentiation

To determine the strength of population substructure several nonparametric methods were used to test genetic differentiation among the cyprinidae family. Hudson's *Ks* is a sequence-based data that is useful for small sample sizes, a high rate of mutation, and equal sample sizes [15]. Hudson's nearest neighbour statistic (*Snn*), is powerful when analyzing samples of variable size and mutation rate (15, 16). All these calculations were calculated using DnaSP 5.0.

3.4 Test of neutral evolution

Three different neutrality test were examined for the combined DNA sequence alignment: Tajima's D [17], Fu and Li's D* [18], Fu and Li's F* [18]. The DnaSP 5.0 program was used to calculate level of polymorphism and divergence for pairwise comparision of sequence both within and between population and to test expected neutral evolution of the sequence. The null hypothesis of neutral evolution of the marker was tested by using Tajima's D, Fu and Li's D*, Fu and Li's F*.

4. Results

4.1 PCR results

The amplified products that are run on agarose gel electrophoresis are visualized by UV in gel doc and the picture of gel obtained is given below in figure 1:

4.2 Results of sequencing

Permanent Accession numbers has been allotted by NCBI for the sequences submitted to them which are *Labeo rohita (*KF641860), *Catla catla* (KF641859), *Cirrhinus mrigala (*KF641861).

4.3 Blast search

For the study of cyprinidae, mitochondria 16S rRNA gene was used. The partial sequence of *Labeo rohita* (614bp), *Catla catla* (613 bp) and *cirrhinus mrigala* (600bp) were obtained after sequencing and submitted to NCBI and their Accession number are shown in Table 2 . The 17 sequences were taken from public domain on the basis of percentage similarity with cyprinidae and one out group *Nemacheilus montana* was taken and accession number are shown in Table 3 . The sequences were downloaded from GenBank (NCBI, EMBL). *Rectoris posehensis* (DQ845891), *Pseudogyrinocheilus prochilus* (DQ845894), *Lefua sps.*(HQ123460), *Schizothorax waltoni* (GQ406266), *Lobocheilos melanotaenia* (HM536759), *Discogobio tetra batrachus* (DQ845888), *Parasinilabeo assimilis* (DQ845887) *Sinilabeo dero* (JX074078) *Henicorhynchus lineatus* (GQ406263), *Pseudo Crossocheilus siamensis* (DQ845895), *Bangana sps.* (JX074074), *Labeo rajasthanicus* (DQ520913), *Labeo gonius* (HQ645097), *Cirrhinus cirrhosus* (JX074066) *Incisilabeo behri* (JX074091) *Cyprinus carpio* (AB741890) *Semilabeo notabilis* (DQ845886) *Nemacheilus Montana* (GQ478539).

4.4 Nucleotide diversity

The sequence length varied from 600 bp to 614bp. Here 628 characters were included out of which 497 (79.14%) were conserved sites (monomorphic) and 129 (20.54%) were

variable sites (polymorphic) out of 129 variable sites 54 were parsimony informative sites. The nucleotide diversity was π = 0.02667. The average number of nucleotide difference kt = 14.989. The nucleotide frequencies are 31.21% (A), 21.80% (T), 22.71% (C), and 24.28% (G). The transition/transversion rate ratios are k_1 = 4.885 (purines) and k_2 = 8.155 (pyrimidines). The overall transition/transversion bias is R = 3.131, where $R = [A*G*k_1 + T*C*k_2]/ [(A+G)*(T+C)]$ and the bias was towards transitional mutation (Table 1) (19).

Table 1: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

		A		T		C		G
A		-		*2.6*		*2.89*		**13.22**
T		*3.72*		-		**23.6**		*2.71*
C		*3.72*		**21.18**		-		*2.71*
G		**18.17**		*2.6*		*2.89*		-

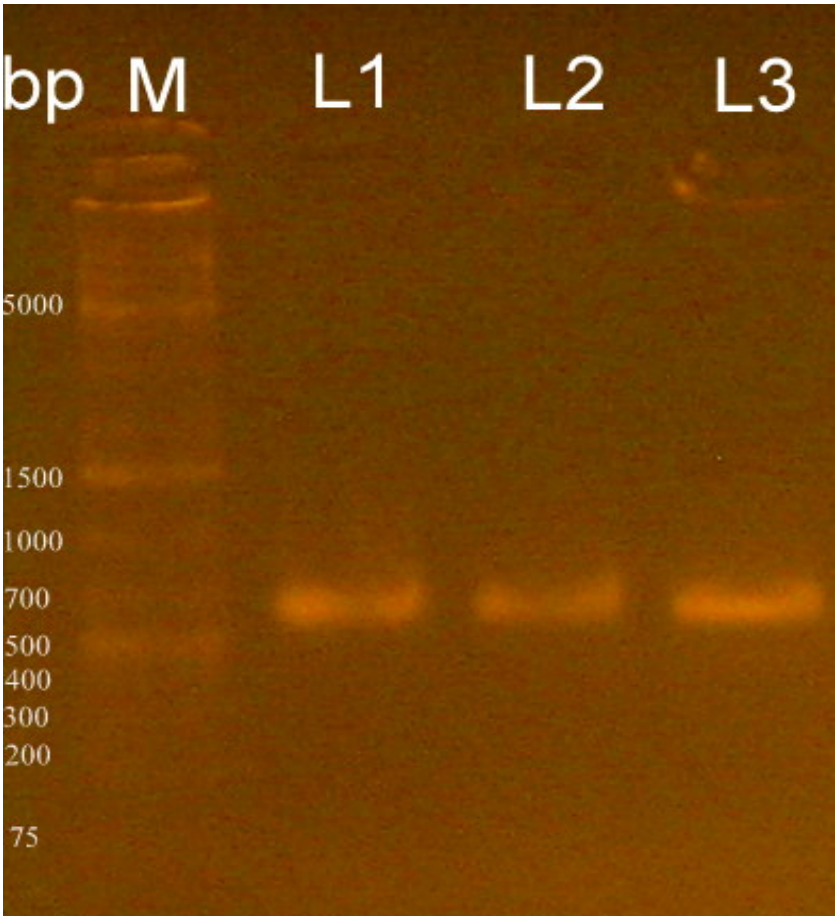

Fig 1: Shows the amplified product of 16S rRNA gene

4.5 Phylogenetic analysis

A phylogenetic tree (Figure 2) was constructed from the combined dataset of 16S rRNA consisting 21 sequences aligned with one outgroup as a root (*Nemacheilus montana*). The consensus tree inferred from 2 most parsimonious trees. Branches corresponding to partitions reproduced in less than 95% trees are collapsed. The consistency index is (0.517241), the retention index is (0.710345), and the composite index is 0.507389 (0.367420) for all sites and parsimony-informative sites (in parentheses) by Nei and Kumar [20]. The maximum parsimony tree was differentiated into 5 clusters with *N. montana* as a root of the tree. From dendrogram it is clear that all the genus of Cyprininae were clustered according to their similarity with each other. In cluster A5 the two genus were clustered with 100% bootstrap value and associated with the same subfamily labeoninae. In cluster A4 sequences of *Semilabeo notabilis* and *P. prochilus* clustered together with bootstrap value of 100%. In cluster A3 the genus from different subfamilies i.e. Cyprininae, Schizothoracine, Labeoninae and Nemachellidae were clustered with different bootstrap values at different nodes such that members of Cyprininae and Schizothoracine clustered together with the bootstrap value of 100% and members of labeoninae were clustered with 100% bootstrap value but as a whole in main cluster they clustered with the bootstrap value of 50%. In cluster A2 *Cirrhinus mrigala* clustered with *Labeo gonius* and

Labeo rohita clustered with the bootstrap value of 100% as they were associated with the same subfamily Labeoninae but both these sub clusters together have the bootstrap value of the 50%. In cluster A1 *Catla catla* was clustered with a bootstrap value of 100% with *Cirrhinus cirrhosus* which is the member of labeoninae but when clustered with the member of Cyprininae it shows the bootstrap value of 50%.

Here, M: Ladder; L1: Lane 1 (*Labeo rohita*); L2: Lane2 (*Catla catla*); L3: Lane 3 (*Cirrhinus mrigala*)

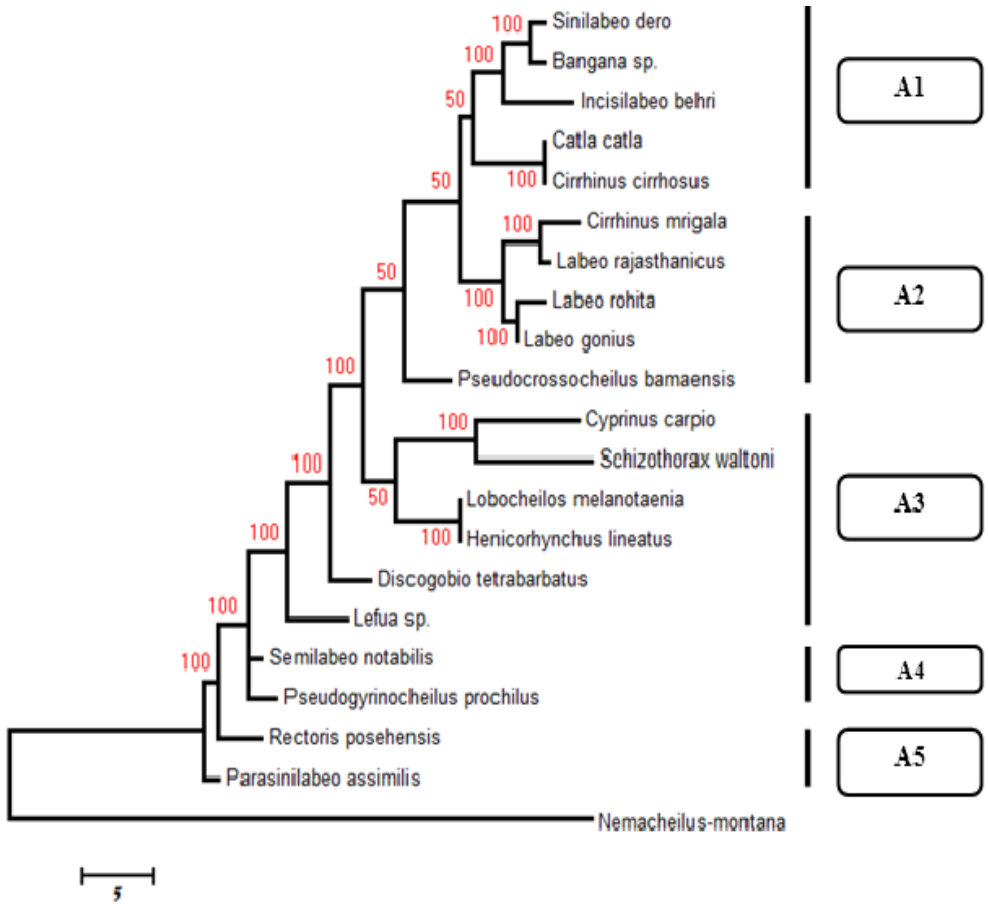

Fig 2: Phylogenetic tree obtained by maximum parsimony analysis among cyprinidae family showing phylogenetic relationships. The tree is rooted with *Nemacheilus montana.*

4.6 Population differentiation

For the estimation of genetic differentiation of population sub division at nucleotide level different methods i.e. Hudson's *Ks*, Hudson's *Snn.* For different tests the value obtained were Hudson's *Ks* =0.36455, and Hudson's *Snn* = 0.79167.

4.7 Test of neutral evolution

Three neutrality test Tajima's D, Fu and Li's D*, Fu and Li's F* were conducted to test the neutral evolution among the Cyprinidae family. Values obtained for different test obtained were Tajima's D = -0.73881 (P>0.10); Fu and Li's D* = -0.60838 (P>0.10); and Fu and Li's F* = -0.75531 (P>0.01).

5. Discussion

The fishes of Cyprinidae family are cosmopolitan but their phyletic classification is ambiguous. The great diversity in family Cyprinidae causes an obstacle for clearing the phylogeny the arbitrary rules and characters were creating a hurdle for the resolution of phylogeny of Cyprinidae. [7, 21] The present study provides the phylogenetic relationship among the members of Cyprnidae family of Agra region and it questions the monophyletic origin of Cyprinidae family. Phylogenetic analysis is conducted to identify the monophyletic analysis. The members of which share a common ancestors. To align a monophyletic group one or more morphological characters have to be studied due to which the natural classification of taxa were obtained but the molecular information provided an impartial characters on which the natural classification may be done. This correlates with the study of Li *et al*., [22] Maximum parsimony analysis of 21 sequences including outgroup were clustered at different subfamily level. All the three species of interest i.e. *Labeo rohita, Cirrhinus mrigala* and *Catla catla* were clustered in two different groups. In which *Catla catla* were placed in cluster A1 with the members of labeoninae and shows the higher rate of similarity with *Cirrhinus cirrhosus* and the other two fishes i.e. the *Labeo rohita, Cirrhinus mrigala* were clustered in A2 cluster and L. rohita shows maximum similarity with the *L. gonius* while *C. mrigala* shows the higher rate of similarity with *L. rajthanicus* similar studies was also done by He *et al.* [11] in Cyprinidae.

The maximum parsimony tree is paraphyletic in nature as also

reported by Li *et al.* [15] and cluster A1 and A2 consist of fishes of interest which shows that these fishes are from same lineage and may share a common ancestors. A higher value for nucleotide diversity $\pi = 0.02667$ and the nucleotide difference Kt = 14.989 indicate high genetic diversity among the sequences. The transition/transversion bias R deviate from the neutral evolution (R = 0.5) Li *et al.* [15] also reported similar results. The reason behind this deviation may be due to the structure of nucleotide bases and the complementary base pairing as discussed by Topal and Fresco [23] also this was established by deviation of bias towards transitional mutation from table 4. The higher value of transitional mutations reflect that the fishes are under selection procedure as observed by Rosenberg *et al*., [24]. The maximum parsimonious tree was differentiated into main 5 clusters and they have the bootstrap value of 100% which shows that genus of family cyprinidae were clustered with their similar neighbors reflects the specific evolutionary nature. The molecular studies done by earlier workers He *et al.,* [11]; Li *et al*., [22] showed the evolutionary trend of Cyprinidae. Probably, the present study offered first significant information on genetic diversity among the Cyprinidae family in North India.

The maximum parsimony tree suggesting that the *Catla catla* whose subfamily is unknown may be a member of labeoninae as it clusture with *Cirrhinus cirrhosus* along with the bootstrap value of 100%. *Cirrhinus mrigala* and *Labeo rohita* remain in subfamily Labeoninae as mentioned in Fish base ver (10/2013). Present study of neutrality test rejected the neutral variation. The negative value of Tajima's D test shows population expansion or bottleneck effect or purifying selection [17]. It causes the bottleneck effect resulting the selection pressure on fishes. The negative value of D* and F* is indicating that the test were insignificant thus reflecting excess of external mutation.

For all test a significant P value is showing subdivision, would be equal or less than to critical value of 0.05. Hudson's *Ks* value was 0.14938 and Hudson's *Snn* value was 0.79167 [15, 16]. All these test shows significant subdivision among cyprinidae. Phyletic classification based on morphological characters was not clear representative but genetic differentiation was clearly reflected by molecular method. The data gathered is showing that all fishes in this group had a common ancestor but now they have separated into distinct evolutionary lineages. The current study shows that the *Catla catla* may be a member of subfamily Labeoninae as it is clustering with member of these family and shows the 100% bootstrap value of *Cirrhinus cirrhosus* as neighbour. Identical work has been conducted by Saitoh *et al*. [25] in order Cypriniformes.

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