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Genetic characterisation of silurid species based on mtDNA marker- cytochrome B gene

Mohd Imran
Department of Zoology, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Abstract
The work deals with the divergence and substitution pattern in the cytochrome b gene and its primary protein structure in siluriformes species by tracing the variabilities at all three positions of triplet nucleotide codons of cytochrome b gene (cyt. b) on the basis of the nucleotide substitutions and their location in the triplet codons of the resulting amino acid sequence. The maximum Pairwise divergence was observed in between *S. Seenghala* and *H. fossilis* (0.2203) and the minimum was found between *C. gariepinus* and *H. fossilis* (0.1602). The 3rd nucleotide codon position exhibits the highest substitution rate where the evolutionary divergence followed as maximum at 3rd nucleotide codon position trailed by the 1st nucleotide position and the minimum on 2nd nucleotide codon position. At protein level there were twelve such amino acid locations where happened the amino acid substitutions as a result of nucleotide substitutions at triplet codons. Three diverse variabilities were noticed on three different codon positions of cyt. b gene triplet codons- Nucleotide substitutions: 3rd (87) >1st (32) >2nd (03); amino acid substitution: 1st (11) >3rd (04) >2nd (02); and non-conservative amino acid replacements: 2nd (1:2) >1st (5:11) >3rd (1:4). Hence, the study has provided an in-depth understanding of the relative variability and substitution trends in the mitochondrial cytochrome b gene in catfish species at both DNA and protein level.

Keywords: Amino acid, cytochrome b, divergence

Introduction
Different Mitochondrial DNA markers has long been used for genetic characterization of wide range of taxa (Near *et al*. 2003; An *et al*. 2005) [1, 14, 19] but cytochrome b gene is the most widely investigated marker in molecular Phylogenetics and population studies of wide range of organisms (Peng *et al*. 2004, Ketmaier *et al*. 2004) [9, 15]. The nucleotide sequence of cytochrome b gene changes at a sufficient pace to resolve the genetic relatedness among any closely related species (Merritt *et al*., 1998) [18].
Universal primers for cyt.b gene created by Kocher *et al*., (1989) [11] has become the basis of a number of studies on fish phylogenetics. Siluriformes is a distinct order of ray finned fishes comprises approx. 3,895 valid species from 39 families of catfishes (Eschmeyer and Fong 2018) [3]. In this study four families- Bagridae, Clariidae, Heteropneustidae and Siluridae have been included.
The present study was undertaken with the aim to estimate of variation in partial sequence of cyt.b gene to determine the genetic relatedness between the individual of family Bagridae, Claridae, Siluidae and Heteropneustidae.

Materials and Methods
Specimens were collected from different water sampling site fed with the Ganges riverine system. Approximately 0.5-1ml of the blood extracted from the heart in EDTA coated vial. High Salt Method was used for genomic DNA extraction. The amount of DNA was quantified in a Spectrophotometer (Specord 50). The selected DNA samples were then subjected to 'cytochrome b gene' specific PCR amplification using primers set of forward: 5'AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A 3') and reverse: 5'AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TCA AA 3'), (Kocher *et al*. 1989) [11]. It amplified a segment of approx. 350 bps from all six species (figure 1). PCR amplification was done in Eppendorf thermocycler with the initial denaturation at 95°C for 5 min, followed by 35 cycles as: Template denaturation at 95°C for 1min, Primer annealing, varied with species within a range of 47-53°C and Extension at 72°C for 1 min. The amplified product was run on 1.5% agarose gel for qualitative analysis of amplified product on horizontal Bio-Rad gel assembly at 70mV for 1.5 hours followed by Sanger sequencing. Sequence alignment was done using MEGA7 software (Kumar *et al*. 2016) [13]. After alignment sequences were analysed for the percent nucleotide variability using DnaSP software- Ver. 6.12.01 (Rozas *et al*. 2017) [20].

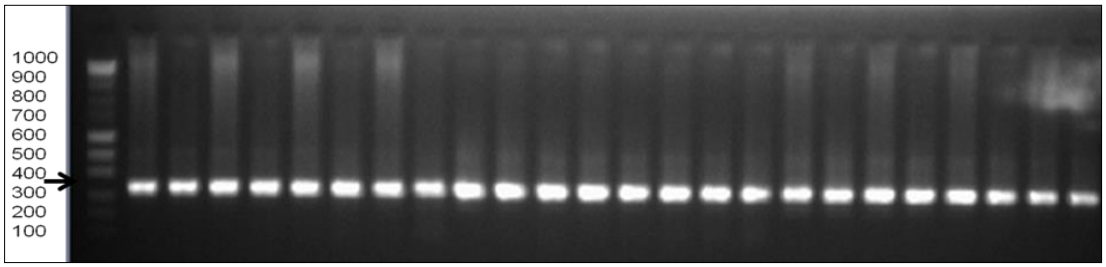

Fig 1: Agarose Gel electrophoresis showing the cytochrome b gene specific amplification of 350 bp.

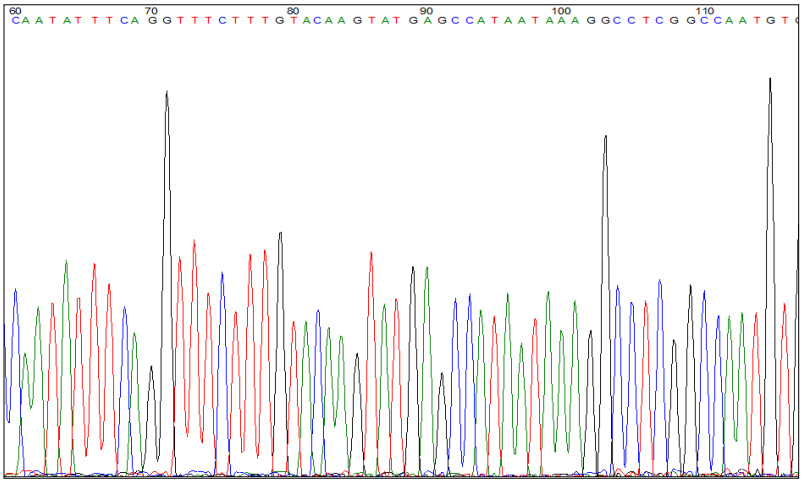

Fig 2: Each sequence was analyzed for the sequence peaks and background noise to avoid any kind of sequencing error. The diagram showing the differently colored peak corresponding to four different nucleotide (Red: T; Green: A; Black: G; Blue: C).

Amino acid variability

Before translating the nucleotide sequences into amino acid sequences, the correct codon positions were identified in the terminally trimmed sequences in the "select and edit gene/domain" option in the sequence data explorer window in Mega 7. The partial cytochrome b gene segment from each species were then translated into amino acid sequences and analysed for the possible substitutions. To locate their actual positions, the partial amino acid sequences were aligned with complete amino acid sequences of cyt. b gene (accession no. AF416888.1) on NCBI.

Results

The nucleotide sequences of all six species were found A+T rich. The average frequencies of four nucleotides in terminally trimmed Sequence for all the catfish species are- A: 28.6%; T: 29.3%; G: 13.6%; C: 28.5% with guanine is in the lowest percentage because of anti-guanine bias at 3rd and 2nd codon position (Table 1). This bias is found strongest in *R. rita* where the contribution of guanine is zero within this segment of cyt. b gene. This has been further confirmed by aligning the *R. rita* sequences with its complete cyt. b sequence form NCBI (accession no. DQ119457.1). The rate of transition (ts) is found higher than transversion (tv) with ts/tv ratio is 1.17, the highest transition present at position 3rd followed by position 1st and 2nd with a ts/tv ratio in the order of 3 (2.81) >1 (1.76) >2 (0.67) (Kimura 1980) [10]. The transitions are higher than transversions at 1st and 3rd codon positions, and vice versa at 2nd codon position. Beside this, 190 nucleotide sites were found monomorphic; Parsimony informative sites were 104, Singleton variable sites: 18, 2-fold and 4-fold sites were found 40 each.

Table 1: Percent nucleotide composition at three codon positions of cytochrome b gene segment estimated by MEGA 7 (T- Thymine, C- Cytosine, A- Adenine, G- Guanine)

Species	Codon Position no. 1				Codon Position no. 2				Codon Position no. 3			
	T-1	C-1	A-1	G-1	T-2	C-2	A-2	G-2	T-3	C-3	A-3	G-3
H. fossilis	36.0	15.4	25.0	24.0	40.0	22.1	21.2	16.3	24.2	33.6	40.4	1.0
R. rita	33.0	19.2	25.0	23.1	40.0	22.1	21.2	16.3	14.0	41.3	44.2	0.0
C. gariepinus	30.2	21.9	24.0	24.0	40.0	21.2	21.2	17.3	13.0	45.6	40.0	1.9
S. seenghala	30.0	21.2	27.9	21.2	40.0	22.1	21.2	16.3	09.0	55.8	32.7	2.9
W. attu	30.5	22.1	26.4	21.2	40.0	22.1	21.2	16.3	15.5	45.7	37.0	1.4
C. batrachus	30.8	21.7	23.4	24.2	40.6	22.3	19.8	16.9	14.4	39.4	40.4	3.6
Average	31.7	20.2	25.3	23.0	40.1	22.0	21.0	16.5	15.0	43.6	39.1	1.8

When evolutionary divergences were calculated at all three codon positions separately (Table 2), the 2nd codon position found more conserved than 1st followed by 3rd position among all species. So, the contribution of 3rd codon position is highest in the overall divergence between species (table 3).

Table 2: Estimates of Evolutionary Divergence over Sequence Pairs between Groups at codon position 1st, 2nd and 3rd (The number of base differences per site from averaging over all sequence pairs between groups are shown. The rate variation among sites was modeled with a gamma distribution)

Sequence Divergence between species at 1st codon position					
	H. fossilis	*R. rita*	*C. gariepinus*	*S. seenghala*	*W. attu*
H. fossilis					
R. rita	0.1209				
C. gariepinus	0.0705	0.1122			
S. seenghala	0.1195	0.1209	0.0751		
W. attu	0.1128	0.1139	0.0747	0.0889	
C. batrachus	0.1436	0.1199	0.0834	0.1006	0.1198
Sequence Divergence between species at 2nd codon position					

	H. fossilis	*R. rita*	*C. gariepinus*	*S. seenghala*	*W. attu*
H. fossilis					
R. rita	0.0197				
C. gariepinus	0.0097	0.0196			
S. seenghala	0.0197	0.0000	0.0196		
W. attu	0.0197	0.0197	0.0196	0.0197	
C. batrachus	0.0327	0.0327	0.0326	0.0327	0.0300
Sequence Divergence between species at 3rd codon position					
	H. fossilis	*R. rita*	*C. gariepinus*	*S. seenghala*	*W. attu*
H. fossilis					
R. rita	0.5487				
C. gariepinus	0.5586	0.4894			
S. seenghala	0.7295	0.5982	0.6616		
W. attu	0.5132	0.5080	0.4895	0.5708	
C. batrachus	0.5006	0.5464	0.4795	0.7508	0.5307

Table 3: Estimates of Evolutionary Divergence over Sequence Pairs between Groups. The number of base substitutions per site from averaging over all sequence pairs between groups are shown. The rate variation among sites was modelled with gamma distribution.

	H. fossilis	***R. rita***	***C. gariepinus***	***S. seenghala***	***W. attu***
H. fossilis					
R. rita	0.1905				
C. gariepinus	0.1602	0.1764			
S. seenghala	0.2203	0.1895	0.1946		
W. attu	0.1810	0.1812	0.1730	0.1820	
C. batrachus	0.1949	0.1935	0.1668	0.2189	0.1926

The maximum Pairwise divergence was observed in between *S. Seenghala* and *H. fossilis* (0.2203). The minimum Pairwise divergence was found between *C. gariepinus* and *H. fossilis* (0.1602) (table 3).

Amino acid variability

When the amino acid sequences were compared between six species, only 12 such sites were found where happened the amino acid substitutions with total 29 amino acid substitutions including multiple individuals of six catfish species (Table 4). When the positions were compared with the 8-helix structure of cyt. b protein (Howell, 1989) [6], these were found located within the first three helix of its tertiary structure constituting the first 127 amino acids (Barrientos-Villalobos & Monteros 2008) [2].

At 3rd codon position of all the triplet codons, there were total 87 nucleotide variabilities, but only one change was found non-conservative in the final protein product i.e., leucine/phenylalanine (L/F) at position no. 62 of amino acid sequence, where it is phenylalanine (F) in all six species and both leucine and phenylalanine (L & F) in the individuals of *C. gariepinus*. Three changes were found conservative at position no. 41, 45, 77 of the amino acid sequence. Rest of the changes were found synonymous i.e. the changes at the nucleotide positions not translated into any variation in the amino acids of the cyt b protein.

Similarly, at 1st codon position, out of total 32 nucleotide variabilities in all six species with their multiple individuals 11 changes were found responsible for amino acid substitutions with 5 non-conservative amino acid changes at amino acid position no. 23, 66, 74, 81, 122 as serine/ proline (S/P), alanine/threonine (A/T), tyrosine/asparagine (Y/N), leucine/phenylalanine (L/F) and valine/threonine (V/T), respectively. The rest 6 of the 11 changes were resulted into conservative amino acid substitutions at position no. 23, 42, 45 as serine/threonine (S/T), valine/methionine (V/M), methionine/leucine (M/L), and isoleucine/valine (I/V) at position no. 58, 77 and 117. All the remaining 21 nucleotide variations out of total 32 resulted into synonymous substitutions.

At 2nd codon position of the triplet codons, contrasting to 1st and 3rd codon positions, only 3 nucleotide sites have shown variability and two of them resulted into amino acid changes with conservative substitution at amino acid position no. 41 as alanine/glycine (A/G) and second is the non-conservative change at position no. 122 along with codon position 1st as valine /threonine (V/T).

Three different variability trends are observed in the cyt. b gene which run simultaneously on three different codon positions. This shows that although the frequency of nucleotide substitutions at three codon positions follows the order 3rd (87) >1st (32) >2nd (03) the frequency of amino acid substitution follows the order 1st (11) >3rd (04) >2nd (02); and the frequency of non-conservative amino acid changes found as 2nd (1:2) >1st (5:11) >3rd (1:4).

Table 4: Amino acid Positions and their Locations in tertiary structure of cytochrome b protein (based on 8- helix model of Howell 1989)6v. Amino acids are represented in single letter abbreviation: A= Alanine, F= Phenylalanine, G= Glycine, I= Isoleucine, L= Leucine, M= Methionine, N= Asparagine, P=Proline, S= Serine, T= Threonine, V= Valine, Y= Tyrosine. Fish species abbreviated as: *Hf (H. fossilis), Rr (Rita rita), Cg (C. gariepinus), Ss (S. seenghala), Wa (W. attu), Cb (C. batrachus).* All the variable codon positions are highlighted in yellow.

Amino Acid position no.#	**Amino acid variations**						**Location in tertiary structure#**
	Hf	*Rr*	*Cg*	*Ss*	*Wa*	*Cb*	
23	S (TCA)	S (TCA)	P (CCA)	S (TCA)	S(TCA)/ T(ACA)	P (CCA)	N-terminus segment
41	A (GCA)	M (ATA)	G(GGA)	M(ATA)	I (ATT)	M (ATA)	1st transmembrane α-helix
42	V (GTA)	V (GTA)	V (GTA)	M(ATA)	M (ATA)	V (GTA)	1st transmembrane α-helix

45	M (ATA)	L (CTT)	L (CTC)	L (TTA)	L (CTA)	I (ATC)	1st transmembrane α-helix
58	I (ATC)	I (ATT)	I (ATC)	I (ATC)	I(ATC)	V (GTC)	1st inter-membrane loop
62	F (TTT)	F (TTC)	L(TTA)/ F (TTC)	F (TTC)	F (TTC)	F (TTT)	1st inter-membrane loop
66	V (GTT)	A (GCC)	V (GTA)	T (ACC)	V (GTT)	V (GTA)	1st inter-membrane loop
74	Y (TAC)	Y (TAC)	Y (TAC)	N(AAC)	Y (TAC)	Y (TAT)	1st inter-membrane loop
77	I (ATC)	V (GTT)*	I (ATT/ ATC)*	I (ATC)	L (CTA)	V (GTC)	1st inter-membrane loop
81	L (CTA)	L (CTC)	L (CTT)	L (CTT)	L (CTC)	F (TTC)	2nd transmembrane α-helix
117	V (GTA)	I (ATC)	V (GTA)	V (GTC)	V (GTA)	V (GTA)	3rd transmembrane α-helix
122	V (GTT)	V (GTA)	V (GTA)	V (GTA)*	T (ACA)*	V (GTT)	3rd transmembrane α-helix

*Two codon positions are involved in amino acid variation.

#Amino acid positions and locations in tertiary structure of cyt b gene as per the Howell, 1989 [6].

1st codon variations; 2nd codon variations; 3rd codon variations

Percent genetic similarity in the individuals within a species, which follows a decreasing trend as: R. *rita* >*C. gariepinus* >*S. seenghala* >*H. fossilis* >*W. attu* >*C. batrachus. Rira rita* show the highest genetic identity (99.37%) hence possess less genetic variability while *C. batrachus* has minimum overall identity (95.59%) (table 5). Multiple individual of each species were included in the analysis to avoid any deviation from existing relationship due to likely sequencing errors.

Table 5: Percent Intraspecific similarity in the cyt.b gene segment

S. No.	Species	Overall identity
	C. batrachus	95.59 %
	C. gariepinus	98.77%
	H. fossilis	98.22 %
	R. rita	99.37 %
	S. seenghala	98.50%
	W. attu	98.19%

Discussion

The frequency of four nucleotide found following a non-uniform trend at the three codon positions due to the different biases which are reported even in higher vertebrates like anti-guanine bias at 3rd codon position (Johns and Avise, 1998) [8], comparatively lower guanine even at 2nd codon position (Irwin *et al.*, 1991; Farias *et al.*, 2001) [4, 7] a higher proportion of thymine at second codon position (Krajewski and King, 1989) [12] which reaches up to 40%, and an unbiased composition at first codon position. These biasing hence, in a way, seems controlling the random mutation in mtDNA.

Anti-guanine bias in not the only thing at third codon position, overall, the fluctuations in the base frequencies are considerable unlike more or less uniformity at first and second codon positions. Out of 122 variable sites 87 were found at third codon positions. There is an inclination towards cytosine (C) over Adenine (A) in *C. gariepinus*, *S. seenghala and W. attu;* thymine (T) found in considerably lower frequency in *S. seenghala* i.e., just 9%, and it ranges from 9 to maximum 25% in *H. fossilis* at this position. Cytosine ranges from 33.7 in *H. fossilis* up to 55.8% in *S. seenghala.* No such trends were observed at first and second codon positions. This is because there is no functional constrains on third codon position and it behave as a silent site. Hence its mutation rate is highest among the three codon position (Foighil *et al.*, 1998; Song *et al.*, 1998; Near *et al.*, 2003; Maggio *et al.*, 2005; Ward and Holmes, 2007) [5, 14, 16, 19, 21, 22]. The nucleotide changes here results into the synonymous substitution, an outcome of degenerate nature of genetic code i.e., no change in final amino acid product. Unlike this, the first and second bears functional constrain imposed basically by the functional role of the final protein product (Meyer, 1994) [17]. This is clearly indicated by the divergence values of three codon positions as the 2nd codon position found least diverged followed by 1st and then maximum divergence at 3rd codon position. (Table 2).

When the amino acid sequences were compared between six species, only 12 such sites were found where happened the amino acid substitution (Table 4). When the positions were compared with the 8-helix structure of cyt. b protein (Howell, 1989) [6], these were found located within the first three helix of its tertiary structure constituting the first 127 amino acids (Barrientos-Villalobos & Monteros 2008) [2].

At 3rd codon position the maximum nucleotide variability of 87 with least rate of non-conservative substitution of 1:4 clearly indicate its wobble nature with majority of the changes are synonymous and confirming that the 3rd codon position is functionally less important and contributing least to the overall substitution trend in comparison to the contribution from 1st and 2nd codon positions. The maximum number of amino acid substitutions are coming from 1st codon position which is contributing both the conservative and non-conservative amino acid substitutions which shows that it is more liable for nucleotide and corresponding amino acid substitutions than 2nd codon position which is contributing the least. This least variability at 2nd codon position showing its tendency as a conserved codon position or more robust codon position which seems not get easily substituted and also indicating its major role in maintaining the amino acid composition of cytb protein and its functionality. Hence, the resultant of these three diverse variability trends seems playing a major role in the overall divergence between difference catfish species.

Conclusively, this study has provided an in-depth understanding of the relative variability and substitution trends in the mitochondrial cytochrome b gene in catfish species at both DNA and protein level. The different substitution trends observed have clearly indicated the evolutionary behaviour of cyt b protein coding gene and its relevance in understanding species divergence. This pattern of exploring the role of individual nucleotide on the basis of its codon position found significantly informative in understanding the pattern of amino acid substitutions in cyt.b protein in catfish species, emphasizing that it is the conservation of functional aspect of cytochrome b protein which imposes three different substitution trends running parallel at three codon positions, simultaneously. And, it is this substitution trend which forms the basis of nucleotide divergence, which in turn induces divergence in gene or coding DNA as a whole, and which is the basis of molecular divergence at the species level.

The study implies that the group catfishes could be well understood in terms of their evolutionary relationships/molecular divergence using this approach by

working on complete cytochrome b gene along with its complete protein product rather than emphasizing only on DNA sequences as the protein product aids in better understanding of the variability trends in DNA. Since, cytochrome b gene is a marker significant to resolve the questions pertaining to biodiversity- phylogenetics, stock identification, species delimitation; the use of the relative variability trends between nucleotide sequences and corresponding amino acid sequences in the aforementioned catfish species could provide important insights in understanding the applications of cytochrome b gene in molecular biodiversity studies.

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