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Research Article

ILLUSTRATIVE MORPHOLOGICAL TAXONOMY AND DNA BARCODING STUDY OF REBA CARP C*irrhinus reba* (HAMILTON) FROM LOWER ANICUT, TAMIL NADU, INDIA

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ABSTRACT

DNA barcoding are mainly focusing an analysis on a short standardized segment and 5' region of mitochondrial DNA cytochrome *c* oxidase subunit I (*COI*) gene is a standard marker of DNA barcode. Here, the photographic image for essentially helpful an identification guide for field collection. The mt*COI* gene has also been advocated for identification marker in many animal species. The present work discusses the mt*COI* gene to discriminate wild species *Cirrhinus reba*.

Key Words: Biometric characters, Mt*COI* gene, Phylogenetic tree, Species identification.

INTRODUCTION

Cirrhinus reba has drawn attention as one of the potential new candidate species for aquaculture and captive breeding (Ayyappan and Jena, 2001). In order to identify species, the exploited fish resource must be collected (Begg and Waldman, 1999). Resembling, analysis of morphometric and meristic characters has been widely used for identification of fish stocks to determine the taxonomic groups even to distinguish cohorts of a single species rather than sub-populations (Murta,

2000). In this context, the International Union of Conservation and Nature (IUCN, 2011) recommended complete research on taxonomical discrimination. Yet, the taxonomic ambiguity exists for several fish genera/ species and proper identification is imperative for fisheries management (Lakra *et al.,* 2011). Besides, identification is mega challenge in fishes prompted by an international Fish Barcoding of Life (FISH-BOL) initiative (http://www.fishbol.org) with the aim of barcoding for all fishes. Although, the mitochondrial cytochrome *c* oxidase subunit I (mt*COI*) gene divergence and species identification success has been previously assessed for some marine fishes, average divergence found among freshwater fish species is unknown. Given their higher diversity and dramatic phenotypic changes during developmental stages of fish, species identification is not easy task even for trained taxonomists (Ward *et al.,* 2009). In addition, species level identification is repeatedly complicated through lack of strong morphological and anatomical characters to discriminate species and degree of unpredictability which affects some diagnostic characters. Consequently, fish fauna provides an excellent opportunity to test the efficacy of barcode-based species delimitation and identification of freshwater fishes over a broad geographic range (Hebert *et al.,* 2008). Moreover, it is difficult to distinguish between *Labeo bata* and *C. reba* even in their adult stage using morphological characters. At the moment, accurate species identification system in fisheries research is a challenging one through the life cycle of fishes, from eggs and larvae to sibling adult stages. However, taxonomic and phylogenetic analyses using molecular markers are essential to test whether classification based morphological characters are corroborated (Mohanty *et al.,* 2013). In such cases wherever morphological information are insufficient to identify species, macromolecules such as DNA, RNA and Proteins may serve as unique identifiers to discriminate species. Similarly, DNA barcoding has emerged as a powerful molecular system for fast and accurate species identification because, the conserved sequence of the 5'- region in mt*COI* gene was proposed as a platform for the universal DNA barcoding of life (Hebert *et al.,* 2003). It has been recommended as a universal, standard and effective DNA barcoding marker of fishes (Hebert *et al.,* 2008). Further, in Tamil Nadu, the river of Cauvery has at least 106 freshwater species included in seventeen families (www.fishbase.com). Unfortunately, most of the species have not been barcoded yet. Moreover, the present mitochondrial DNA (mtDNA) barcode based classification of freshwater minor carp *C. reba* is to resolve the key areas of doubt arising from morphological taxonomy. It will also help further in easy identification of the studied species (Bhattacharjee *et al.,* 2012). The purpose of this study is to provide detailed morphological (photographical) image identification with special reference to DNA barcoding of this species, as a basis for further ontogenetic research as well as to contribute for taxonomic and phylogenetic inference.

MATERIALS AND METHODS

Study area and biometric sampling

Lower Anicut (Kollidam River) is one of the major freshwater fishery resources within the northern region of Tamil Nadu (11° 15' N latitude and 79° 30' E longitude) which was selected for the present study. Among the varied fish fauna landed, the family of Cyprinidae is one of the dominant fisheries in this region. Sampling focused the species *C. reba* collected from the landing centre at Lower Anicut, Tamil Nadu. Fifty individuals of fresh specimens were used for phenotypic-taxonomy and DNA barcoding. After collection, specimens were weighed in and total length (mm), standard length (mm), fork length (mm) was measured with the help of measuring board. Total weight (g) was measured with an electronic balance (DIGI' Arts maximum=1000 g to d=0.5 g). Specimens were identified morphologically using scientific literature relevant to the group with original descriptions by Talwar and Jhingran, 1991. After identification, fresh specimens were photographed in all morphological body parts alive (left-hand side) prior to tissue sampling. As a result, tissue samples (*i.e.*, caudal fin) were collected and stored in sterile eppendorf tubes containing absolute ethanol, sealed with parafilm kept at room temperature until further analysis.

Photographic image identification

The wild populations of *C. reba* were employed by invasive photographic techniques. The natural markings such as irregular shapes of dark greenish colour on the body surface with colour patterns of fins which remain unchanged during life stages. *C. reba* was selected individual, using a digital camera (SAMSUNG-PL 20 with 5x level; Lens focal length: 4.9-24.5; mega pixel 14.2 using Adobe Photoshop ver. CS3). Photographs were taken perpendicular to the subject were only used when the fish with all types of fins were fully extended. Additionally, the resulting of the high-resolution images was used to extrapolate the morphological identification.

Morphometrics and Meristics

Forty three morphometrics were measured to an accuracy of 0.1 mm respectively with all measurements (Fig. 1). Measurements and counts were done as per Hubbs and Lagler (1964) and made using point to point dial calipers and data were recorded to tenths of a millimeter. Measurements were made on the left side of the body whenever possible whereas the right side depending on state and shape of preservation. Measurements of the head are represented as a proportion of head length (HL); head length and body parts are given as proportions of standard length (SL). Statistical analyses were conducted on morphometrics and meristics, while meristics are discrete and fixed early in development (Turan *et al.*, 2006). Morphometrics were analyzed by SYSTAT, ver. 13.1 and SPSS, ver. 19.0. Linear regression and line parameter such as a (intercept) b (slope) and r^2 (regression) values were made with log-transformed measurement. Pearson's correlations co-efficient (r) were used between morphometrics at $P<0.05$ and $P<0.01$ level significance. Test of Homogeneity of Variances (Levene Statistic) by one-way Analysis of Variance (ANOVA) with Post Hoc Tuke's-b Alpha (0.01) multiple

comparison test using between morphometrics at P<0·001 level. 15 meristics (counts) were done using *C. reba* (Fig. 2). Fin rays and gill rakers were counted under a binocular microscope using transmitted light. Lateral line scales were counted from the anterior most (operculum) contact to centre of the body of last scale on the caudal fin. These were counted from dorsal-fin origin to lateral line oblique (above lateral line) and below lateral line scales were counted from lateral line oblique to anal-fin origin. Counts of caudal-fin rays include marginal unbranched and branched rays in both lobes by separately. Kruskal–Wallis non-parametric statistical test were employed by ANOVA (Hoaglin *et al.*, 1991) for significant differences among meristics.

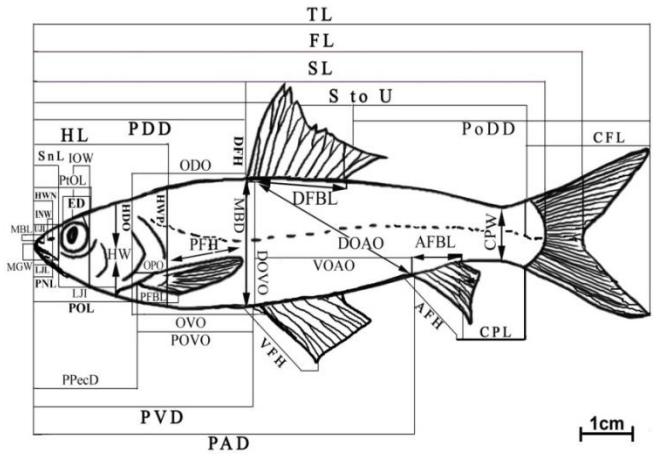

Figure 1. Morphometrics constructions of *Cirrhinus reba*.

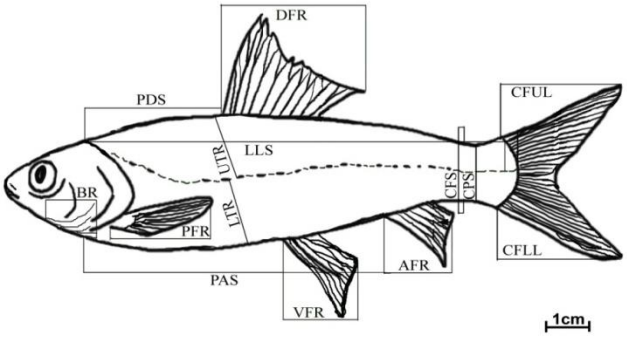

Figure 2. Meristics constructions of *Cirrhinus reba*.

Genomic DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted using standardized salting-out procedure with some modifications (Sambrook *et al.*, 1989). Polymerase chain reaction was performed to amplify 700 bp DNA fragment of mt*COI* gene using universal specific primer: Forward Fish F1 5'-TCAACCAACCACAAAGACATTGGCAC -3' and Reverse Fish R1 5'–AGACTTCTGGGTGGCCAAAGAATCA –3' (Ward *et al*,. 2005). A 25 µl PCR mixture contained 1-2µl of DNA template, 2.5µl 10x$Mgcl_2$ buffer, 1µl primer mix, 2µl dNTP, 0.5µl Taq DNA polymerase and 18µl deionised ultra-pure water. PCR reaction was performed in thermal cycling profile as: initial denaturation at 94ºC for 5 min, 35 cycles of denaturation at 94ºC for 30s, annealing at 54ºC for 30s, extension at 72º C for 1min and final extension at 72ºC for 10 min. Prior to sequencing, PCR products were checked on 1.5% agarose gel electrophoresis along with 100bp molecular weight marker.

Phylogenetic inference and statistical analyses

Bidirectional sequences of the amplified *COI* gene products were purified by automated capillary sequencer (ABI 3100 PE). Nucleotide sequences were aligned and edited based on chromatogram inspection using FinchTV ver. 1.4. Sequences were trimmed according to the translated vertebrate mitochondrial amino acid code program namely MEGA ver. 5.0 (Kumar *et al.*, 2011). The result obtained was blasted with similar sequences from National Center for Biotechnology Information (NCBI), Barcode of Life Database (BOLD) and Basic Local Alignment Search Tool (BLAST) program. Based on the percentage of similarity, the species was confirmed within and between species sequences with high similarity phylogenetic analysis. Sequences have been deposited to NCBI GenBank and accession numbers for barcodes. Phylogenetic trees were constructed to provide species divergence through Neighbor Joining (NJ) and Minimum Evolution (ME) algorithm, were implemented by MEGA ver. 5.0. Sequence were conducted using the Kimura 2-parameter (K2P) model (Kimura, 1980) includes all nucleotide sequences with codon positions (1^{st} + 2^{nd} +3^{rd}+ Non-coding). Herein, Evolutionary divergence, Codon-based Z test for neutrality and Fisher's exact test for neutrality was carried out. Probability of rejecting null hypothesis of strict-neutrality position in synonymous and non-synonymous substitutions (d_N = d_S) codon-based test of neutrality were also analyzed.

RESULTS AND DISCUSSION

Major Keys	Description
Key to family	One or two pairs or no barbels. Body laterally compressed.
Key to subfamily	No knob at symphysis or junction of two arms of lower jaw. Dorsal fin 7 to 30 branched rays inserted before or opposite origin of pelvic fins and with or without an osseous simple ray. Lateral line complete or incomplete running along middle of the caudal peduncle (Talwar and Jhingran, 1991).
Key to genus	Dorsal fin 8 to 16 branched rays. Upper lip and lower lip not continuous.
Key to subgenus	Dorsal fin 11 to 15 branched rays
Key to species	Lateral line scales 34 to 38. Dorsal fin less than body height.

Morphological identification

Body fairly elongates its depth much more than head length. Snout slightly projecting beyond mouth, more pronounced in juveniles often with pores. Mouth broad; upper lip entire, often fringed in juveniles; a thin cartilaginous covering inside of lower jaw. Barbels of one pair of short rostrals generally present. Dorsal fin height less than depth of body. Pectoral fins as long as head. Caudal fin deeply forked. Scales hexagonal and moderate, lateral line with 34 to 38

scales; lateral transverse scale-rows 7/5-6 for the present study. Following this, D. ii-iii 8; A. iii 5; P. i 15; V. i 8 (Talwar and Jhingran, 1991).

Colour and valid scientific name

Anterior-dorsally grayish in both side of the body, silvery on flanks and distended belly; scales are generally darkest at their edges. Adults and Juveniles with light green-color irregular shapes along the side in above lateral line region from operculum to caudal peduncle with dusky tip on the dorsal fin. Anal fin and pelvic fin-orange-tipped; caudal fin base pinkish and tip light grayish (Talwar and Jhingran, 1991). This species were globally accepted scientific and valid name *Cirrhinus reba*.

Fishery information

C. reba grows upto 30cm in natural waters (Talwar and Jhingran, 1991). In the present study, the maximum size were recorded in 23.5cm by two year continuous collection which is the first record in Lower Anicut (Kollidam river), Tamil Nadu. This species were cultivated in ponds along with Indian major carps in India and Bangladesh. It is a bottom feeder particularly plankton feeder. Breeding takes place in flooded shallows during June-September with peak in July (Gupta, 1975; Mathialagan, 2013). Males are smaller than female. However, males are always cylindrical body shape even fully matured whereas the females are breadth in nature and soft distended belly outer visible at the time well matured (Mathialagan, 2013). Sexual maturity (Lm_{50}) was estimated in males (132 mm) and females (148 mm). Following, sex ratio of *C. reba* males are significantly outnumbered of the females. Juveniles are available in abundance during June-September in Cauvery and Bhavani waters (Gupta, 1975). However, the fecundity ranged between 91, 321-3, 37, 389. In addition, the maximum age was noticed to be 6^+ years for males and 7^+ years for females (Mathialagan, 2013). *C. reba* was assessed at Least Concern (LC ver.3.1.) level in world waters (IUCN, 2011).

Photographical (morphological) image identification

C. reba can be distinguished by photographical identification for the present study. Based on this image the dorsal fin rays, ventral fin rays, pectoral fin rays, anal fin rays, caudal fin rays were clearly photographed and explained in Fig. 3. Barbels are covered only for maxillary region with very small size (notch like structure) nearly in 0.2mm. Five pairs of branchiostegal rays with four pairs of gill arches in each side of the operculum. Each gill arch contains 56 to 59 gill rakers (Fig. 4). Pair of external nostrils is clearly visible. Lateral lines with scales are arranged in the middle region of the body starting from operculum to caudal peduncle with flank cycloid silvery scales. In the present taxonomical observation, no more spines are covered for all types of fins.

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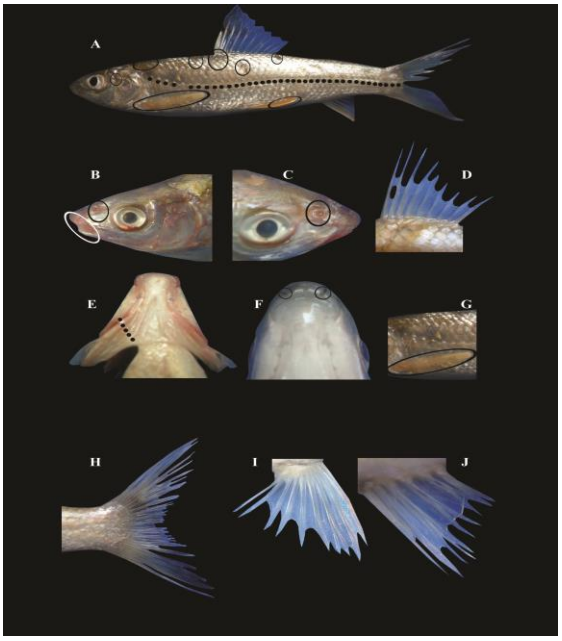


Figure 3. Photographical (morphological) image identification of *Cirrhinus reba*. (A) Colour in live specimen *C. reba* 13.7 mm SL. Note: oval shape ring are covered by pale green in pectoral and vertical fins. Circular shape ring are covered by dark green patches in body surface. (B) Structure of the mouth (sub-terminal shape). (C) Nasal hole. (D) Dorsal fin. (E) Branchiostegal rays. (F) Structure of the barbel (very small size). (G) Pectoral fin. (H) Caudal fin. (I)Ventralfin.(J)Analfin.

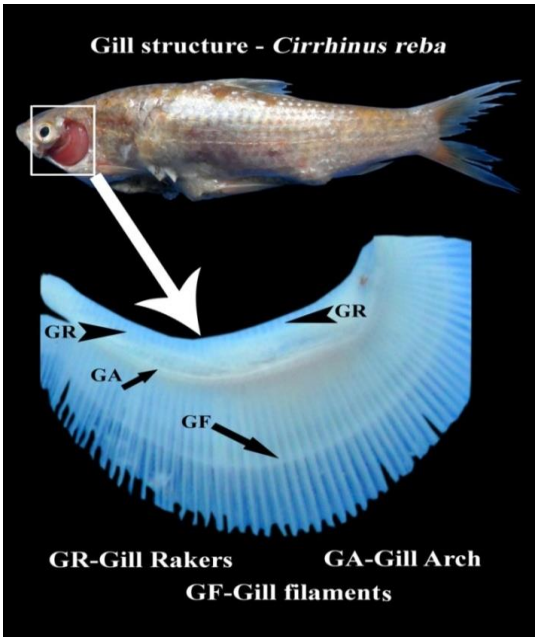


Figure 4. Structure of gill rakers of *Cirrhinus reba*.

Morphometrics and Meristics

Statistically analyses of the descriptive and morphometric characters were detailed in Table 1. Regression analysis were ranging from (r^2 -0.628-0.994) inter nostril width and standard length, whereas, the r = (r-0.761*-0.997**) at *P<0.05 and **P<0.01 level significant. Significance homogeneity of variances (0.000-0.990) at **P<0.01 level with the significant differences for homogeneous subsets (Table 3a) were found only two morphometrics namely anal fin base length and inter nostril width at ***P<0.001 level.

Consequently, the post hoc tuke's-b alpha test shows significant differences for all morphometrics at **P<0.01 level. In addition, statistical analyses as proportions of HL were represented in Table 2. As a result, the r^2 ranging from (r^2 - 0.539-0.921) inter nostril width to post orbital length while, the r = (r - 0.720*- 0.966**) at *P<0.05 and **P<0.01 level significant. Similarly above, the significant homogeneity of variances ranged between

Table 1. Biometric data of the standard length with proportional measurements of C. *reba*.

Characters	Holotyp	% SL (mm)	A	b	r^2	R	THV (LS)	PHT'bA
Standard Length	53-113	91.2	- 0.084	1.047	0.994	0.997**	0.000	0·000***
Snout to urocentrum	48-104	93.1	- 0.056	1.019	0.989	0.991**	0.912	0.000***
Head Length	12-25	22.8	- 0.676	1.038	0.967	0.987**	0.990	0.000***
Head Width	06-16	13.4	- 0.968	1.101	0.926	0.962**	0.000	0.002*
Head Width at Nostril	06-12	11.3	- 0.743	0.774	0.856	0.916**	0.000	0.000***
Head Width at Pupil	05-10	8.86	- 0.761	0.680	0.806	0.868**	0.641	0.000***
Head Depth at Occiput	09-18	16.9	- 0.476	0.677	0.838	0.914**	0.000	0.000***
Occiput to Dorsal Origin	13-22	22.5	- 0.275	0.591	0.947	0.974**	0.000	0.001*
Occiput to Pectoral Origin	08-14	13.8	- 0.463	0.566	0.855	0.911**	0.000	0.005*
Occiput to Ventral Origin	17-27	27.2	- 0.140	0.533	0.923	0.951**	0.000	0·176
Pre Orbital Length (or) Snout Length	05-10	8.87	- 0.977	0.918	0.967	0.975**	0.806	0·000***
Post Orbital Length	04-11	8.98	- 1.304	1.278	0.944	0.966**	0.000	0·001*
Eye Diameter	03-06	5.99	- 1.034	0.793	0.861	0.943**	0.595	0·000***
Inter Orbital Width	03-10	8.73	- 1.269	1.219	0.714	0.930**	0.838	0·000***
Pre Nasal Length	02-05	4.61	- 1.515	1.193	0.871	0.948**	0.677	0·000***
Inter Nostril Width	03-05	4.99	- 0.820	0.469	0.628	0.761*	0.025	0·040**
Pre Occipital Length	12-22	19.7	- 0.555	0.836	0.889	0.929**	0.000	0·089**
Upper Jaw Length	05-10	9.11	- 0.896	0.838	0.686	0.798**	0.275	0·000***
Lower Jaw Length	04-06	5.86	- 0.876	0.609	0.829	0.908**	0.501	0·000***
Mouth Gap Width	06-10	10.4	- 0.488	0.488	0.998	0.963**	0.474	0·000***
Lower Jaw to Isthmus	05-10	8.61	- 0.915	0.835	0.880	0.926**	0.201	0·004*
Maxillary Barbel Length	01-02	1.87	- 1.782	1.051	0.689	0.832**	0.237	0·003*
Maximum Body Depth	17-35	30.6	- 0.531	1.017	0.950	0.979**	0.000	0·052**
Dorsal Fin Height	14-26	25.1	- 0.320	0.690	0.658	0.861**	0.000	0·037**
Pectoral Fin Height	12-21	20.2	- 0.494	0.781	0.945	0.982**	0.000	0·003*
Ventral Fin Height	11-20	18.9	- 0.597	0.863	0.894	0.960**	0.095	0·001*
Anal Fin Height	11-19	18.4	- 0.537	0.781	0.908	0.965**	0.095	0·001*
Pre-Dorsal Distance	23-47	41.1	- 0.332	0.940	0.942	0.975**	0.000	0·073
Post Dorsal Distance	28-49	46.7	- 0.027	0.668	0.955	0.977**	0.000	0·241
Pre Pectoral Distance	13-24	22.7	- 0.425	0.760	0.894	0.964**	0.000	0·029
Pre Ventral Distance	29-53	48.8	- 0.205	0.883	0.967	0.987**	0.000	0·036
Pre Anal Distance	40-82	74.9	- 0.130	1.005	0.994	0.996**	0.000	0·022
Dorsal Fin Base Length	09-19	15.6	- 0.663	0.842	0.889	0.946**	0.000	0·010
Pectoral Fin Base Length	02-06	4.24	- 1.939	1.605	0.928	0.966**	0.206	0·000***
Ventral Fin Base Length	03-08	6.23	- 1.246	1.044	0.947	0.980**	0.294	0·000***
Anal Fin Base Length	04-09	7.24	- 1.231	1.096	0.915	0.972**	0.205	0·000***
Dorsal Origin to Anal Origin	19-33	31.1	- 0.278	0.749	0.975	0.982**	0.000	0·026
Dorsal Origin to Ventral Origin	12-31	25.5	- 0.831	1.256	0.962	0.979**	0.000	0·176
Pectoral Origin to Ventral Origin	18-30	29.2	- 0.215	0.650	0.895	0.958**	0.000	0·007*
Ventral Origin to Anal Origin	18-32	29.7	- 0.414	0.876	0.964	0.985**	0.000	0·007*
Caudal Peduncle Length	15-26	26.5	- 0.096	0.471	0.804	0.903**	0.014	0·000***
Caudal Peduncle Width	05-19	11.1	- 1.056	1.110	0.961	0.988**	0.788	0·000***
Caudal Fin Length (both lobe)	16-27	27.9	- 0.072	0.471	0.804	0.907**	0.173	0·000***

SL-Standard Length; SD= standard deviation; Regression line parameters; a-intercept; b-slope, r^2-regression co-efficient, Pearson's correlation co-efficient (r) significant at **P<0.05 and highly significant at *P<0.01 level, THV-Test of Homogeneity of Variances (Levene Statistic) and Post Hoc Tukey's-b Alpha (0.01) test very highly significant at ***P<0·001.

0.000 - 0.381 at **P<0.01 level while, the significant differences for homogeneous subsets (Table 3b) were found only two morphometrics such as inter nostril width and inter orbital width at ***P<0.001 level. Besides, the post hoc tuke's-b alpha test shows a significant difference for all morphometrics at **P<0.01 level. Moreover, the Kruskall–Wallis test shows significant differences for all meristic characters, except the caudal fin rays (Table 4) at *P>0·05 whereas, Dorsal fin rays, Upper transverse rows, Lower transverse rows and Circum-peduncle scales shows very significant at **P<0·01 and other characters shows highly significant at ***P<0·001**.**

DNA barcoding and phylogenetic tree with statistical analyses

Isolated genomic DNA (Fig. 5a) and PCR amplified *COI* gene bands was displayed in Fig. 5b.

Table 2. Biometric data of the head length with proportional measurements of *C. reba*.

Characters	% HL (mm)	a	B	r^2	r	THV (LS)	PHT'bA
Head Width	58.4	- 0.243	1.032	0.907	0.963**	0.000	0.006*
Head depth at nostril	49.2	- 0.230	0.711	0.805	0.905**	0.000	0.004*
Head depth at pupil	38.7	- 0.310	0.623	0.754	0.862**	0.042	0.001*
Head depth at occiput	74.3	- 0.023	0.609	0.755	0.886**	0.000	0.008*
Pre Orbital Length or Snout Length	38.7	- 0.368	0.841	0.904	0.951**	0.152	0.001*
Post Orbital Length	39.3	- 0.462	1.196	0.921	0.966**	0.000	0.000***
Eye Diameter	26.2	- 0.505	0.718	0.786	0.908**	0.106	0.000***
Inter Orbital Width	38.2	- 0.480	1.201	0.772	0.945**	0.000	0.000***
Pre Nasal Length	20.2	- 0.717	1.068	0.778	0.909**	0.019	0.000***
Inter Nostril Width	21.8	- 0.504	0.412	0.539	0.720*	0.055	0.077**
Pre Occipital Length	86.3	- 0.009	0.800	0.909	0.947**	0.000	0.000***
Upper Jaw Length	39.8	- 0.363	0.861	0.807	0.866**	0.030	0.344
Lower Jaw Length	25.7	- 0.474	0.570	0.808	0.904**	0.381	0.000***
Mouth Gape Width	45.4	- 0.232	0.593	0.886	0.958**	0.001	0.000***
Lower jaw to isthmus	37.7	- 0.369	0.800	0.902	0.940**	0.301	0.000***
Maxillary Barbel Length	8.2	- 1.074	0.919	0.587	0.777**	0.240	0.008*

HL-Head Length; SD= standard deviation; Regression line parameters; a-intercept; b-slope, r^2-regression co-efficient, Pearson's correlation co-efficient (r) is significant at **P<0.05 and highly significant at *P<0.01 level, THV-Test of Homogeneity of Variances (Levene Statistic).

Table 3a. Homogeneous subsets for SL between AFBL and INW for test of homogeneity variances (thv-levene statistic) and post hoc tukey's-b alpha (0.01; pht'ba) test.

Inter Nostril Width (INW)	Inter OrbitalWidth (IOW)	Subset alpha for AFBL		Subset alpha for INW	
		1	2	1	2
0.3	0.5	1.250**	0.000***	1.300**	0.000***
0.4	0.3	1.817**	0.000***	1.350**	0.000***
0.5	0.6	2.450	0.000***	1.600	0.000***
0.0	1.0	0.000***	0.000***	0.000***	2.450

Post Hoc Tukey's-b Alpha (0.01) test significant at **P<0.05 and highly significant at ***P<0·001.

Table 3b. Homogeneous subsets for hl between inw and iow for test of homogeneity variances (thv-levene statistic) and post hoc tukey's-b alpha (0.01; pht'ba) test.

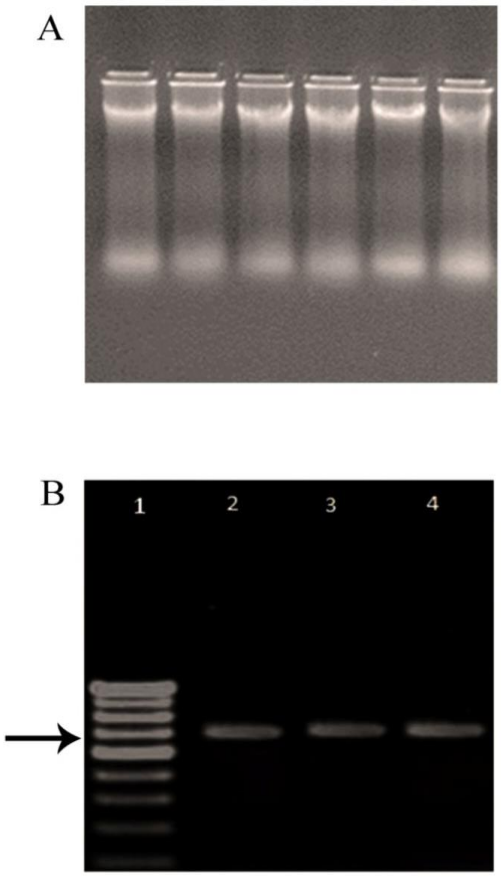
Anal Fin Base Length (AFBL)	Inter Nostril Width (INW)	Subset alpha for AFBL		Subset alpha for INW	
		1	2	1	2
0.4	0.3	6.050**	0.000***	5.450	0.000***
0.5	0.4	6.700	0.000***	7.917	0.000***
0.8	0.5	0.000***	10.625	10.850	0.000***

Tukey's-b Alpha (0.01) test significant at**P<0.05 and highly significant at***P<0·

Table 4. Kruskal–wallis test for meristic characters of *C. reba*.

Meristic counts		Range		*H*-statistics	Probability
		Minimum	Maximum		
Branchiostegal Rays		0	5	4.721	P<0·001***
Gill Rakers		56	59	2.633	P<0·001***
Dorsal Fin Rays	U	2	3	31.013	P<0·01**
	B	0	8		
Ventral Fin Rays	U	0	1	28.034	P<0·001***
	B	0	8		
Pectoral Fin Rays	U	0	1	11.355	P<0·001***
	B	0	15		
Anal Fin Rays	U	0	3	25.942	P<0·001***
	B	0	5		
Pre Dorsal Scales		12	14	8.458	P<0·001***
Pre Anal Scales		23	25	14.612	P<0·001***
Lateral Line Scales		35	38	19.562	P<0·001***
Upper Transverse Rows		6	8	9.713	P<0·01**
Lower Transverse Rows		5	7	9.311	P<0·01**
Circumpeduncle Scales		17	19	7.215	P<0·01**
Circumference scales		30	32	5.349	P<0·001***
Caudal Fin Upper Lobe	U	0	3	38.893	P>0·05*
	B	0	9		
Caudal Fin Lower Lobe	U	0	3		
	B	0	8		

B=Branched, UB=Unbranched, *H* - Kruskal–Wallis statistical value; *P>0·05 - significant; **P<0.01 - very significant; ***P<0·001 – highly significant.



Black arrow mark referred 700bp of mtCOI gene.

Figure 5A and 5B. Isolated genomic DNA and PCR amplified mt*COI* gene product.

All individuals of *C. reba* showed good amplification. Present *COI* sequences showed 99% identity (96% query coverage) compared to other *Cirrhinus* species through NCBI databases. Based on the similarity sequence analysis the present sequences confirmed with compared to NCBI gene sequences as the species confirmed *C. reba.* The mt*COI* gene sequences were submitted to GenBank with unique voucher name and accession number (KF110672, KF110673 and KF110674). The NJ tree (Saitou and Nei, 1987) using the K2P distances and branch length = 0.46096992 were illustrated *COI*-based genetic divergence among intra and inter-specific hierarchical units. All the individuals were clustered together with *COI* gene and correctly identified for all individuals. The bootstrap analysis consensus the phylogenetic trees with 1000 replicates (Felsenstein, 1985) showed slightly varied genetic relationships among species of this genus (Fig. 6). In the NJ tree, evolutionary distances were computed using the (*P*) probability (Nei and Kumar, 2000) and base differences per site concerning 16 nucleotide sequences with codon positions were included $1^{st}+2^{nd}+3^{rd}$. Totally 482 positions in the final dataset with three major

clades were observed. First clade includes *C.reba*, *C.moliterella* second clade *C.microlepis*, *C.mrigala*, *C.cirrhosus* and third one out-group clade, namely *Amblypharyngodon ckakeiensis*, *Megalobrama terminalis* and there were no distances were found between *C.reba*. Like, the first clade referred to candidate and GenBank species *C.reba* were very closely joined with *C.moliterella.* Second clade, among the three species, *C.mrigala* and *C.cirrhosus* were closely arranged with *C.microlepis* showed as a deviated branch. Analysis showed 1% genetic distance between species of *Cirrhinus* and no distance was found within individuals of particular species of *Cirrhinus*. Except the only species *C. molitorella* showed 1% distance with the out-group species *M. terminalis*. Finally, all other *Cirrhinus* species showed 2% distances from the out-group species. Resembling, the Z-test neutrality for probability of rejecting null hypothesis of the strict-neutrality ($d_N = d_S$) were represented in below diagonal. Values of *P* are considered as significant at $P<0.05$ level and highlighted in bold with italicized. However, the two *C.reba* individuals for the present study significantly varied from the GenBank data base. The selection analyses of the present species showed no significant differences with other *C.reba* individuals. Herein, DNA barcoding provides taxonomical identification of species; the accuracy of such as an assignment depends upon whether the species are mono or polyphyletic with respect to sequence variations of mt*COI* gene. Specifically, individuals of the given species are more closely related to all other conspecific than any member of the other species (Funk and Omland, 2003).

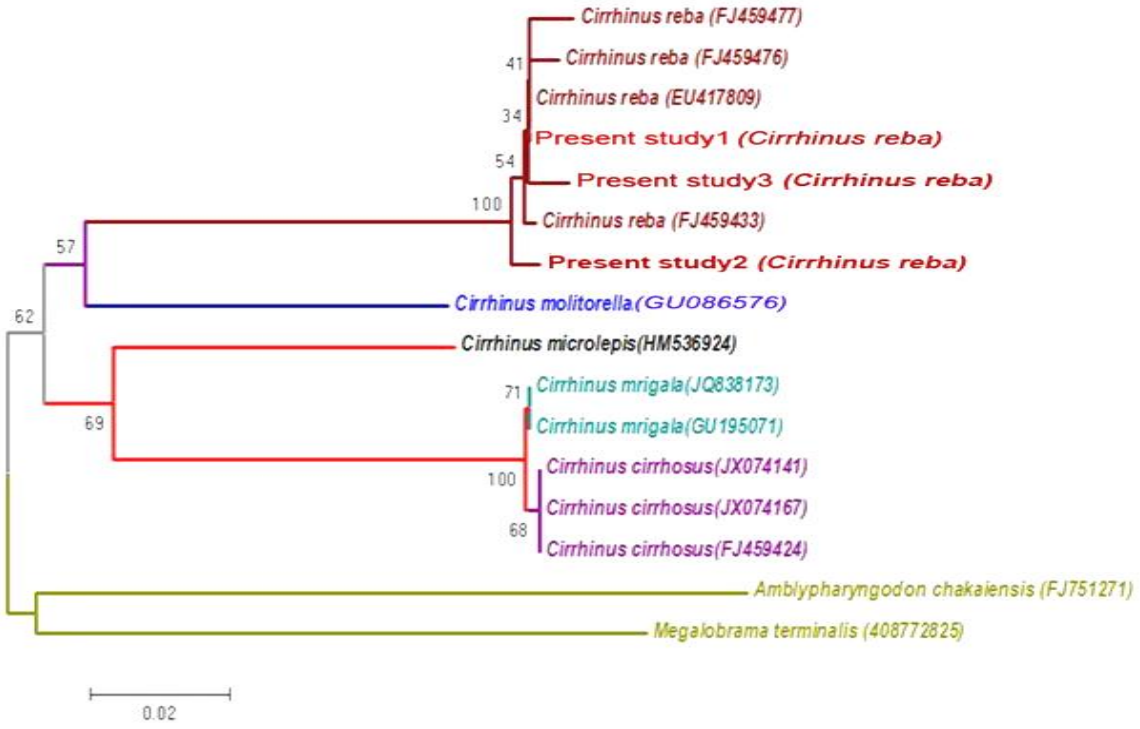


Figure 6: Phylogenetic analysis by Neighbor Joining algorithm method from mt*COI* gene sequences of *C.reba.*

Thus, the DNA barcode approach provides an additional important data for the precise identification of this species in this sampling region. Ward *et al.* (2005) reported the phylogenetic analysis of the mt*COI* gene sequences could be effectively most cluster in congeneric and confamilial species. Herein, some of them freshwater work were encoded in the previous studies such as Australian fishes (Ward *et al.,* 2005), Canadian freshwater fishes (Hebert *et al.,* 2008), freshwater fishes from Mexico and Guatemala (Valdez-Moreno *et al.,* 2009) and Cuban freshwater fishes (Lara *et al.,* 2009). In fact, more than 98% of the species pairs showed greater than 2% sequence divergence (Hebert *et al.,* 2003). Sequencing revealed that mt*COI* gene length varied from 600bp-700bp for all individuals of *C.reba* by DNA barcoding study. The results indicated the significant level of nucleotide variation in the amplified region of *COI* gene compared to past research work. So, this percentage is appreciably higher for freshwater species, around 20% on average (Ward *et al.,* 1994). The present results indicate an identification system of *Cirrhinus* genus based on the mt*COI* gene will be highly effective. Once a global *COI* barcode database has been established for fishes, anyone with direct or indirect access to a DNA sequence will be able to identify a high degree of certainty any fish eggs and larva or carcass fragment whereas, the scientific and practical benefits for fish barcoding are manifold one (Collins *et al.,* 2012). Hence, the present phylogenetic analysis is unclear by species barcode. However, this has needed to be done the species *C.reba* was treated with remaining entire mitochondrial genome, *16S rRNA* and *D-loop* control region. Moreover, the *C.reba* was genetically farthest different branch from other carp species (Mohanty *et al.,* 2013). As a result, the phylogenetic tree to give an outcome the species *Labeo bata* and *C.reba* was phenotypic very similar, but it was genetically dissimilar from each other with this information were strongly occur through this study this reservoir. Following this, mtDNA markers to confirm whether, these two types of genus are most likely different but possibly it should be assigned them same or different ancestors. In this context, the present study area is the first assess judgment of DNA barcoding for this species and which contain a variety of major fish groups. Further, it is widely associated the division of rivers from the continental freshwater network leads to be a more pronounced genetic structure among populations and deeper divergence between haplotypes.

CONCLUSION

Species assignment is most important background in fisheries research and consumer protection. Herein, the present species morphological taxonomic approach provided unique and ecologically important views of fish diversity patterns across the streams in this region. So far, we are following the DNA barcoding was helpful to standardizing disparate levels of taxonomic identification with species-level. Presently the results suggested that mitochondrial DNA COI gene sequence analysis will become a useful means for genetic level stock identification of the species *C.reba* and moreover, the significant development

of streams in this region. So far, we are following the DNA barcoding was helpful to standardizing disparate levels of taxonomic identification with species-level. Presently the results suggested that mitochondrial DNA COI gene sequence analysis will become a useful means for genetic level stock identification of the species *C.reba*. Moreover, the significant development of our knowledge concerning about systematic of the freshwater fishes in this region and also facilitate to monitoring changes in the geographic distribution that will almost certainly occur in the future.

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