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DNA Barcoding and Biometric Investigation on the Invasive *Oreochromis niloticus* (Linnaeus, 1758) from the River Yamuna of Uttar Pradesh

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ABSTRACT

Oreochromis niloticus (Linnaeus, 1758), an invasive fish species is rapidly spreading in the Gangetic river system. The aim of the study was to identify and confirm its identity at species level by establishing a barcoding reference datasets in the river Yamuna and then to investigate its morphometric, meristic traits and length-weight relationship of *O. niloticus* from the Allahabad water of the river Yamuna by the examination of 341 fish specimens collected during October 2011 to September 2012. The taxonomic status of *O. niloticus* (Linnaeus, 1758), in the river Yamuna was assessed using DNA barcode marker COI gene sequences, have clearly identified the *O. niloticus* species with 100% similarity value with public database. The length-weight relationships were established as $W = 0.029486L^{2.881638}$ and $W = 0.058499L^{2.661735}$ for male and female. Differential growth in length-weight between male and female of *O. niloticus* were significant ($p < 0.05$), with a greater slope (b) value for male (2.88) than female (2.66). The t statistics estimated for the regression coefficients (b) were significant at 5% level of significance ($p < 0.05$), indicating an allometric growth pattern. The correlation was highest for the standard length and the total length (0.9878) and minimum for caudal peduncle length and total length (0.8022). Fin formula based on the meristic studies can be written as B_3, D 15-19/$_{11-15}$, P_{14}, V 1/$_5$, A 3/$_{8-11}$ and C_{16-22}.

Key words: Allometric, Fin Formula, Growth, Nile Tilapia, Regression coefficient.

INTRODUCTION

The river Yamuna is the largest right bank tributary of the river Ganga, known to be rich in aquatic biodiversity, harbours 112 fish species (Joshi *et al*., 2016) is being increasingly threatened by anthropogenic activities like damming, pollution and invasion of exotics (Sarkar *et al*., 2012, Joshi *et al*., 2014; Alam *et al*., 2015b). The river passes through the highly populated and industrialized cities like Yamunagar, Panipat, Delhi, Mathura, Agra and Etawah before meeting the river Ganga at Triveni Sangam, Pryagraj. The natural distribution of *O. niloticus* (Nile tilapia) mostly clustered around Central and East Africa (Trewavas, 1983), is now the most widely spread covering the countries of tropics, subtropics and temperate (Grammer *et al*., 2012; Lowe *et al*., 2012; Alam *et al*., 2015b). Ability to feed on a wide range of natural food items, tolerance to poor water quality, faster growth rate and successful reproduction strategies have extremely favoured Nile tilapia to be a successful invasive species (Lowe *et al*., 2012; Alam *et al*., 2015a; Alam *et al*., 2015b). Unauthorized introduction of the Nile tilapia into India is believed to have taken place, sometimes in the eighties (Jhingran, 1991) and have invaded the Ganga river and its tributaries (Joshi *et al*., 2014; Joshi *et al*., 2017). The earlier studies on the food and feeding habits of Nile tilapia in the river Yamuna revealed that it is an opportunistic feeder with omnivorous feeding habit, feeding at more than one trophic level in the food web (Alam *et al*., 2015b). Another investigation by Alam *et al*. (2015a) on its reproductive biology showed that breeding and recruitment takes place round the year in the river Yamuna. Feeding and reproductive plasticity adopted by the Nile tilapia might have actually favoured its adaptation and establishment of its population in the river Yamuna.

DNA barcoding involves the use of a short DNA sequence or sequences from a standardized locus (or loci) as species identification tools. In animals, it is well established. Hebert (2003) demonstrated that a sequence of a 655 base fragment of 5' end of the mitochondrial cytochrome c oxidase subunit I (COI) is appropriate for

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discriminating closely related species across diverse animal Phyla. In recent years, the exotic fish species especially the Nile tilapia has appeared significantly in the landings along the lower and middle stretches of the river Yamuna (Alam *et al*., 2015b). Morphometric and meristic characters are found valid in the identification of species taxonomically, discrimination of fish stocks, separation of different morphotypes (Doherty and McCarthy, 2004; Mekkawy and Mohammad, 2011; Sangun and Guney, 2017) and in describing their spatial distribution (Costa *et al*., 2003). Length-weight relationship is essential in stock assessment models, population dynamic studies like growth estimation, length and age structure (Sen *et al*., 2018), in the estimation of weight from length observations (Froese, 2006; Panda *et al*., 2016; Borah *et al*., 2017), in determination of the well-being in fishes (Sen *et al*., 2018), in setting yield equations for estimating the number of fish landed and for comparing the population in space and time (Beverton and Holt, 1957).

Till date, there are no studies on the DNA barcoding, length-weight relationship, morphometric and meristic of the *O. niloticus* from the Ganga river system. Hence, an investigation was undertaken to characterize the invasive, Nile Tilapia using cytochrome c oxidase subunit I (COI), morphometric, meristic and length-weight relationship for strengthening the database in the Indian waters.

MATERIALS AND METHODS

DNA isolation, amplification and sequence analysis

Total Genomic DNA was isolated from fin tissue (four specimens) following the SDS- phenol/chloroform method described by Sambrook *et al.* (2001) with some modifications. The extracted DNA concentration was measured using a UV spectrophotometer and diluted up to 100 ng / µl. The mitochondrial partial COI gene was amplified with primers Fish F1 (5′-TCAACCAACCACAAAGACATTG GCAC-3′) and Fish R1 (5′-GGTCAACA AATCATA-AAGATATTGG-3′) (Ward *et al.,* 2005) in 25 µl reaction volume. The PCR amplification products were purified with a Gel Extraction Kit (Fermentas) and sequenced. These sequences were edited and aligned using the Clustal W program (Thompson *et al.,* 1997) with default settings implemented in MEGA V 6.0 software (Tamura *et al.,* 2011) and submitted to NCBI. To estimate the degree of haplotype diversity and increase in intraspecific divergence values, reported sequences from different geographical locations were downloaded from NCBI, GenBank (Table 1). The pairwise evolutionary distance values were estimated by the Kimura 2 parameter method employing MEGA V 5.0 software. The haplotype diversity and nucleotide diversity values were estimated by DnaSP v5.0 (Librado and Rozas, 2009).

Morphometric and meristic characteristics

A total of 341 specimens of *Oreochromis niloticus* represented by 167 males and 174 females were collected from the Sadiapur landing centre (N 25.42140° and E 081.82479°) located on the bank of the river Yamuna, Allahabad from October 2011 to September 2012 for examining its morphometric, length-weight relationship and meristic traits. The morphometric measurements, meristic counts and weight (g) were taken using the standard procedures described by Lagler *et al*. (1962). The following morphometric characters were measured on the left side of the fish : (1) Total length(TL) (2) Standard Length(SL), (3) Pre-dorsal length (PDL), (4) Caudal peduncle length(CL),(5) Pre-anal length (PAL), (6) Pre-pelvic length (PVL),(7) Pre-pectoral length (PPL), (8) Head length (HL), (9) Body depth(BD),(10) Caudal depth (CD),(11) Snout length (SNL), (12) Inter-orbital length (IOL), (13) Orbital length (OL) and (14) Scale length(SCL). All lengths were measured to the nearest 1mm using Vernier calliper. Meristic characters determined include the number of dorsal fin spines, dorsal fin rays, pectoral fin rays, pelvic fin spines, pelvic fin rays, anal fin spines, anal fin rays, caudal fin rays, gill rackers, scales on the upper and lower lateral lines and branchiostegal rays, respectively. Sexes were noted only after taking the morphometric measurement and meristic counts by dissection in the laboratory.

Linear regression equations were obtained for the morphometric traits using the least square method described by Snedecor and Cochran (1967) and the scattergram was plotted. This is illustrated by equation

$$Y = a + bX$$

Where,

"Y"- dependent variable, "X"- independent variable, "a"- intercept and "b"- allometric coefficient. The" a" and "b" values were estimated as follows:

$$a = \bar{y} - b\bar{x} \text{ and } b = [n\Sigma xy - \Sigma x\Sigma y] / [n\Sigma x^2 - (\Sigma x)^2]$$

The correlation coefficient(r) that expresses the linear association of the two variables was determined as follows-

$$r = [n\Sigma xy - \Sigma x\Sigma y] / \sqrt{[n\Sigma x^2 - (\Sigma x)^2][n\Sigma y^2 - (\Sigma y)^2]}$$

Length-weight relationship

The length-weight relationship was established as per the Le Cern (1951) separately for male and female as-

$$W = a L^b$$

If we take the logarithm of the length and weight data, the above model will become linear as:

$$Ln (W) = ln (a) + b Ln (L)$$

Where,

L = total length (cm), W = total weight of the fish samples (g), a = the intercept and b = the regression coefficient.

To identify whether there were any statistically significant differences at 5% level in "b" values between sexes, analysis of covariance (ANCOVA) was performed (Snedecor and Cochran, 1967) and to test whether the length-weight relationship followed isometric growth at 5% level of significance, t-test by Pauly (1984) was used.

$$t = \frac{sd\ ln\ L}{sd\ ln\ W} * \frac{|\ b-3\ |}{\sqrt{(1-r\text{^}2)}} * \sqrt{(n-2)}$$

Where,
n = sample size, sd ln L and sd ln W are standard deviations in total length and body weight, b = regression coefficient and r^2 = coefficient of determination

RESULTS AND DISCUSSION

Sequence analysis

Around 57 samples (4 samples from India, 53 reported sequences) were included for DNA barcoding investigation. Around 10 haplotypes were found (Table 1) and the haplotype diversity (H_d) and nucleotide diversity were 0.638 and 0.03691, respectively. The overall K2P divergence values at COI locus were 0.040±0.002 and this value increased from one population to another as distance increases between them. However, this distance was not consistent especially between the USA and other populations and it was substantiated by similar haplotype sharing across these populations (Table 2).

Morphometric and meristic characteristics

The population means for length and weight of males were 25.1 cm and 335.3 g and that of females was 25.43 cm and 334.7 g, respectively. The mean lengths and mean weights of males and females were statistically insignificant (t-test; $p > 0.05$). Overall morphometric traits of *O. niloticus* with their range, mean, standard deviation and coefficient of variation are illustrated in Table 3. Eye diameter showed a minimum coefficient of variation (10.7%), while caudal peduncle length depicted maximum variation (16%). The correlation coefficient (r^2) between morphometric parameters against total length ranged from 0.8022 to 0.9878, showing significantly high positive correlation ($p<0.05$), being maximum for standard length and minimum for caudal peduncle length (Fig 1). Highly significant relationships were found between body depth and caudal depth ($r^2 = 0.8265$, $p<0.05$) (Fig 2). Again the relationship between compared traits *viz*. post-orbital length, snout length, orbital length, scale length against head length were also highly significant ($r^2 = 0.8041$ to 0.9116, $p<0.05$), being maximum for postorbital length and minimum for orbital length (Fig 1 and 2). All length-length relationships were linear. The allometric coefficients of morphometric characters that serve as criteria for the degree of a differential increase in length relative to total length, head length and body depth showed positive allometry (Mekkawy and Mohammad, 2011).

Branchiostegal rays (3), pectoral fin rays (14), pelvic fin rays (5), pelvic fin spine (1) and anal fin spines (3) were observed to be constant. Anal fin rays showed maximum coefficient of variation (6.5%) followed by caudal fin rays

Table 1: Global Haplotype distribution in Nile Tilapia.

Haplotypes	Number	Populations	Accession numbers
H1	9	Nigeria, China, Mexico	HM882927,HM882898,HM882899,GU477627EU751880-82
H2	33	India, China, Israel, USA, Egypt, Thailand, Indonesia	KJ920135-38*, HQ219152, HQ219154DQ426667,GU277625, GU477626, 628, GU370126, FJ348105-114, HQ024985-88, EU752146, KJ553958, KJ554049, JQ742041-43, HM345941
H3	1	India	JX173759
H4	2	China, USA	GU238433, NC013663
H5	2	Israel	FJ348103-04
H6	1	Indonesia	HM345942
H7	1	Philippines	HQ654742
H8	1	Philippines	HQ654744
H9	2	Philippines, China	HQ654747, GU477624
H10	5	Brazil, Mexico	GU702135-137, EU751881,883

*Sequenced in the present study.

Table 2: K2P divergence values of mitochondrial COI gene among different populations.

Location	Nigeria	India	China	Israel	USA	Thailand	Indonesia	Philippines	Egypt	Brazil	Mexico
Nigeria		0.002	0.0013	0.002	0.01	0.02	0.002	0.001	0.02	0.003	0.002
India	0.072		0.02	0.02	0.03	0.001	0.01	0.02	0.006	0.002	0.03
China	0.070	0.036		0.03	0.02	0.002	0.02	0.03	0.005	0.001	0.01
Israel	0.082	0.014	0.029		0.01	0.003	0.03	0.03	0.003	0.003	0.02
USA	0.083	0.021	0.031	0.010		0.004	0.01	0.002	0.02	0.04	0.03
Thailand	0.083	0.013	0.028	0.001	0.010		0.002	0.003	0.008	0.03	0.01
Indonesia	0.084	0.014	0.029	0.002	0.010	0.001		0.001	0.006	0.01	0.002
Philippines	0.044	0.070	0.062	0.072	0.069	0.073	0.074		0.007	0.001	0.0012
Egypt	0.083	0.013	0.028	0.001	0.010	0.000	0.001	0.073		0.02	0.002
Brazil	0.003	0.076	0.074	0.087	0.087	0.088	0.089	0.048	0.088		
Mexico	0.018	0.062	0.063	0.068	0.070	0.068	0.069	0.051	0.068	0.019	0.000

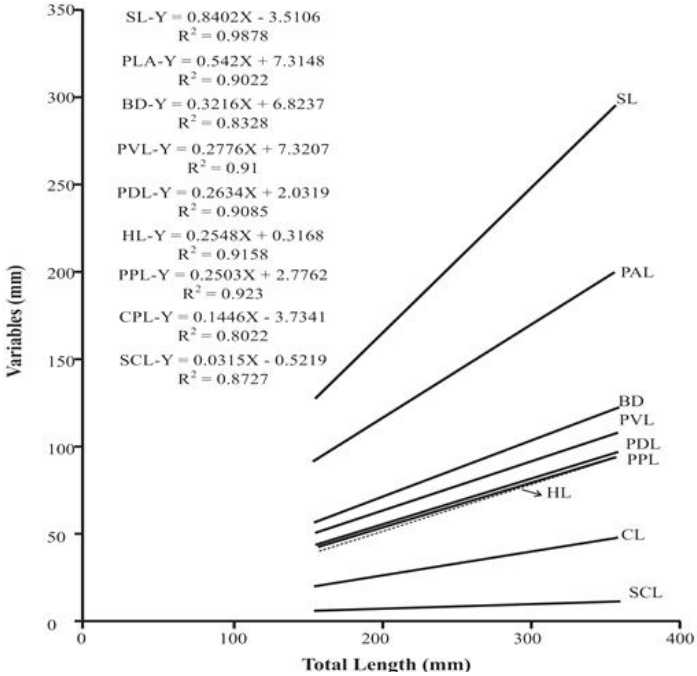

Fig 1: Relationships of morphometric traits with total length in *O. niloticus.*

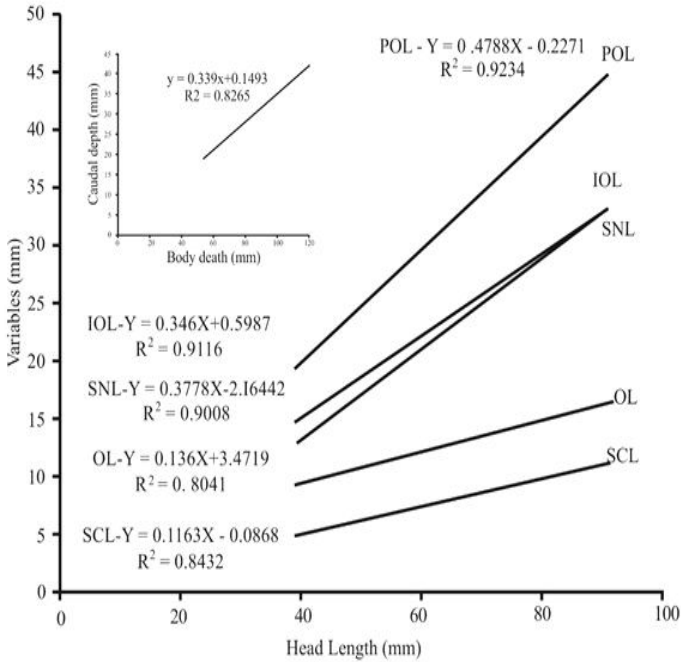

Fig 2: Relationships of morphometric traits with head length and caudal depth with body depth in *O. niloticus*.

(6.3%), gill rackers (6.19%), scales on the lower lateral line (6.05%), dorsal fin rays (4.72%), dorsal fin spines (3.48%) and upper lateral line scales (3.59%). Analysis of meristic traits revealed that *O. niloticus* has a dorsal fin with 15-19 spines and 11-15 soft rays, pectoral fins with 14 soft rays, the pelvic fin has 1 spine and 5 soft rays, anal fin with 3 spines followed by 8 -11 soft rays. Scales on the upper and lower lateral line ranged from 20 to 24 and 16 to 22, respectively (Table 4).

Length-weight relationship

The length-weight relationship for the *Oreochromis niloticus* was established as-

$W=0.029486L^{2.881638}$ and $W= 0.058499L^{2.661735}$

for male and female respectively.

The same is represented in logarithmic form as-

Ln (W) = -3.52383+ 2.881638l Ln (L) for male (R^2= 0.935215**)** and Ln (W) = -2.83875 + 2.661735 ln (L) (R^2=0.906204) for female.

Table 3: Statistical estimates of various morphometric characters of *O. niloticus* collected from the river Yamuna, Allahabad.

Morphometric traits	Range		Mean	Mode	Median	Standard error	Standard deviation	Coefficient of Variation (%)
	Min	Max						
Total length	155	350	252.6	260	255	1.8	32.6	12.9
Standard length	125	295	208.7	215	210	1.5	27.5	13.2
Pre-anal length	85	205	144.2	150	145	0.1	18.6	12.9
Pre-dorsal length	44.5	97.7	68.5	73.3	68.6	0.5	9.0	13.1
Pre-pectoral length	42.8	93.5	66.0	67.5	65.8	0.5	8.5	12.9
Pre-pelvic length	51.8	105.7	77.4	86.8	77.3	0.5	9.5	12.2
Mid-body depth	54.7	121.1	88.0	84.4	87.5	0.6	11.5	13.0
Caudal peduncle length	18.6	47.1	32.8	27.4	32.1	0.3	5.3	16.0
Head length	40.1	93.2	64.7	63.4	64.5	0.5	8.7	13.4
Caudal depth	18.0	41.7	30.0	31.6	30.1	0.2	4.3	14.3
Post-orbital length	18.7	44.0	30.7	32.0	31.1	0.2	4.3	14.1
Inter-orbital length	14.7	32.3	23.0	24.0	22.9	0.2	3.1	13.7
Eye diameter	8.9	16.2	12.3	12.2	12.2	0.1	1.3	10.7
Snout length	13.0	33.7	21.8	23	21.6	0.2	3.5	15.8
Scale length	4.7	10.7	7.4	7.8	7.4	0.1	1.1	14.8

Table 4: Statistical estimates of various meristic characters of *O. niloticus* collected during October 2011 to September 2012 from the river Yamuna, Allahabad.

Meristic traits	Range		Mean	Median	Mode	Standard error	Standard deviation	Coefficient of Variation (%)
	Min	Max						
Dorsal fin spines	15	19	17	17	17	0.03	0.60	3.48
Dorsal fin rays	11	15	13	13	13	0.03	0.61	4.72
Pectoral fin rays	14	14	14	14	14	-	-	-
Pelvic fin spine	1	1	1	1	1	-	-	-
Pelvic fin rays	5	5	5	5	5	-	-	-
Anal fin spines	3	3	3	3	3	-	-	-
Anal fin rays	8	11	10	10	10	0.03	0.64	6.5
Caudal fin rays	16	22	19	20	20	0.07	1.21	6.32
Upper lateral line scales	20	24	22	22	23	0.11	1.94	3.59
Lower lateral line scales	16	22	18	18	18	0.06	1.10	6.05
Gill rackers	28	36	31	30	31	0.11	1.94	6.19
Branchiostegal rays	3	3	3	3	3	-	-	-

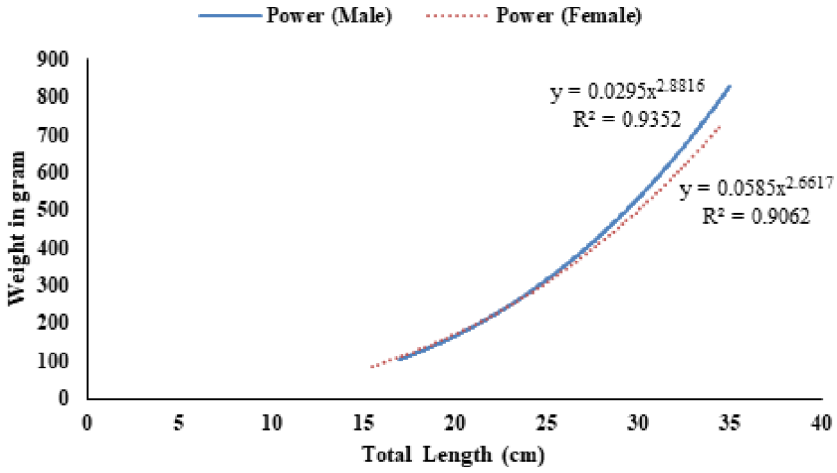

Fig 3: Power length-weight relationship of *O. niloticus*.

Table 5: Comparison of meristic characters of *O. niloticus* with earlier studies.

Authors	Location	Dorsal fin spine	Dorsal fin rays	Pectoral fin rays	Pelvic fin spine	Pelvic fin rays	Anal fin spine	Anal fin rays	Caudal fin rays	Gill rackers	Branchi -ostegal rays
Siddique *et al.* (2007)	Bangladesh	16-17	11-15	15	1	5	3	8-11	-	-	-
Nazrul *et al.* (2011)	Bangladesh	16-17	11-13	13-14	1	5	3	9-11	16	-	3
Bakhoum (2002)	Egypt	16-18	12-14	12-15	-	-	-	9-10	-	24-35	-
Present study	India	15-19	11-15	14	1	5	3	8-11	16-22	28-36	3

Table 6: Comparison of length-weight relationships of O. *niloticus* with earlier studies.

Authors	Location	Sex	Sample size (n)	Intercept (a)	Slope (b)	Correlation (r^2)
Nazrul *et al.* (2011)	Bangladesh	Pooled (GIFT strain)	100	-4.0895	2.6932	0.994
Nazrul *et al.* (2011)	Bangladesh	Pooled (GIFU strain)	100	-4.1421	2.7221	0.99
Bernard (2010)	Nigeria	Male	9	0.0149	3.3632	0.959
		Female	21	0.0302	3.033	0.991
		pooled	30	0.0300	3.0412	0.986
Olurin and Aderibigbe	Nigeria	Male	-	-2.03	3.14	-
(2006)		Female	-	-1.96	2.90	-
		pooled	100	-2.0	3.1	-
Grammer *et al.* (2012)	USA	pooled	259	0.0000215	2.992	0.994
Khallaf *et al.* (2003)	Egypt	Male	-	-	2.65052	-
		Female	-	-	2.70562	-
		pooled	162	-	2.70308	-
Present study	India	Male	3	-3.52383	2.881638	0.935215
		Female	41	-2.83875	2.661375	0.906204
		pooled		-3.20144	2.777197	0.920788

Power relationships *O. niloticus* (male and female) are illustrated in Fig 3. The analysis of covariance (ANCOVA test) showed a significant difference between the b values of males and females at 5% level of significance ($F=6.14322$, $p < 0.05$) suggesting the use of one power model for male and another for female. The b values were tested against the exponent in isometric growth showed that b values were significantly lower than 3 in male ($t= 2.005$, $p<0.05$) and female ($t= 5.18$, $p<0.05$), which indicated a negative allometric growth pattern (t-test, $p<0.05$). The values of the correlation coefficient were highly significant ($p < 0.001$). The rate of increase in weight in relation to length was slightly higher in males (b=2.88) than females (b=2.66).

The mitochondrial cytochrome c oxidase subunit I gene has been successfully used for species discrimination (Herbert, 2003; Kim *et al*., 2017; Hanan, 2018; Lee and Kim, 2019). In this study also, DNA barcodes (COI gene sequences) have clearly identified the *O. niloticus* species with 100% similarity value when compared with the public database. Even though DNA barcodes are used for species delimitation, some of the studies used DNA barcodes for population characterization (Arruda *et al.,* 2009). In the current study, most of the haplotypes were observed as per their populations; however, some of the haplotypes were shared by different populations. The results showed that the populations might have adapted to their respective ecology and accumulated mutations within the COI gene. However, since this gene is a protein-coding gene, mutations were limited to codon 3rd base position and high divergence values could not be identified. The increase in intraspecific divergence value was also in similar lines of morphometric differences between different populations. A further detail study at population level is required for understanding the genetic structure and differentiation of the Nile Tilapia populations employing the mitochondrial DNA genes for the inference of its adaptability in the river Yamuna.

A comparison of the meristic traits of *O. niloticus* with earlier studies is presented in Table 5. Meristic characters described by Siddique *et al*. (2007) are almost similar to the present findings. However, the only difference is the presence of 15 and 19 dorsal spines and 14 pectoral fin rays. Dorsal spines ranged from 16 and 18 in most specimens except in one specimen where it was 15 and two had 19 spines. Based on the above observations, the fin formula for the *O. niloticus* can be represented as- B_3, D $15\text{-}19/_{11\text{-}15}$, P_{14}, V $1_{/5}$, A $3/_{8\text{-}11}$ and $C_{16\text{-}22}$.

Earlier studies on the length-weight relationships carried out in different parts of the world (Table 6) include those of Khallaf *et al*. (2003) from Egypt, Olurin and Aderibigbe (2006) and Bernard (2010) from Nigeria, Nazrul *et al*. (2011) from Bangladesh and Grammer *et al*. (2012) from temperate Mississippi, USA. Present findings compare favourably with those obtained from Bangladesh, Egypt and the USA. The values of the regression coefficient (b) were found to be

between 2.661735 and 2.881638 which were within the expected range of 2.5 to 3.5, reported by Froese (2006). The values of the regression coefficient, b = 3 show isometric or symmetric growth pattern and deviation in the b values from three, suggested allometric growth. The values of b > 3 are reported from Nigeria by Olurin and Aderibigbe (2006) and Bernard (2010). A variation in b values may occur due to species variation, strain variation, stock variation, differences in ecological factors of the habitats or variation in the physiology of the animal, or both (Bhattacharya and Acharya, 1984; Verma *et al*., 2018), etc. Hence the variation noticed in the length-weight relationship might be due to the geographical and ecological differences which ultimately determine the water quality parameters and food availability, are responsible for the growth of the fishes (Mommsen, 1998). The higher values of 'b' in the males may be due to their higher feeding intensity and lesser energy being invested in reproductive effort than the females. This study would form the baseline information for the management and undertaking further research of this invasive fish species in the Gangetic River System.

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