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Sequence Variances and Phylogenetic Relationship among Seven River Nile Teleostei Species from Qena, Egypt using Partial Mitochondrial Cytochrome-*b* Gene Sequences

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Abstract: ***The PCR products from mitochondrial Cytochrome-b gene were sequenced for seven River Nile teleost species. A three-nucleotides cytochrome-b gene deletion existed in Siluformes catfishs sequences which may accounts for species adaptation and monophyletic evolution need. Analyzed Cytochrome-b gene sequences showed similar nucleotide composition to actinopterygian fishes, also high variation at nucleic level which supported the gene's suitability as DNA genetic marker in fish phylogentic studies. Results indicate* closer genetic relationship *between teleost actinopterygian River Nile fish species which reflected shared ancestry. Also, data confirm the close genetic and evolutionary relation of early divergent orders; Acipenseriforms (Sturgeons) and Semionotiformes (Alligator gars). These new sequence data perhaps is useful for successful fish managing, conservancy and aquaculture objectives. Additional DNA sequences from other River Nile telesot family representatives utilising the cytochrome-b gene primers used in this study, possibly provide contribution to future fish molecular phylogeny studies in this area.***

Keyword: Phylogeny, Actinopterygii, Genome, Variance , Cytochrome-*b*, River Nile, Qena

1. Introduction

In Egypt, the River Nile considers the superlative natural supply providing massive fertile deposits, fishes and diverse aquatic species. Nearly, total of 128 fish species inhabit the River Nile belonging to 27 families [1-3], from those families; Cichlidae, Cyprinidae, Mormyridae, Latidae, Schilbeidae, Bagaridae and Clariidae have some species with substantial economic and important aquaculture intentions [4-10].

The mitochondrial (mtDNA) genome spans about 16–18 kilobase pair with similar gene order from myxini to higher vertebrates [11-14]. The mtDNA encompass 37 genes which are likely to be conserved between vertebrates including 13 oxidative phosphorylation genes, 22 transfer RNAs, 2 ribosomal RNAs, L-strand replication origin and a control region [15-17]. mtDNA genome possess small size [18], rapid evolutionary rate at nucleotide level [19-20] and recombination deficiency [21]. Thus, the mtDNA has been widely used as constructive molecular genetic marker for evolutionary studies, genetic variations and taxonomic identifications among related families from population to species level.

The DNA variation using sequences of the Cytochrome-b gene has been widely used in population studies of teleostean fishes [24-26] also as discrimination tool for species identification and molecular evolution studies [27-29].

Molecular genetic tools are reliable and sensitive for assessment of the DNA sequence discrepancies [22-23]. Analysis of mtDNA sequence offers valuable genetic resource, for which the information can be beneficial for understanding evolutionary relations based on comparing DNA sequence among species. The aim of this work was evaluation of partial Cytochrome-*b* gene sequence regions for sequence variances and phylogenetic relationship among seven River Nile teleostei species. These species are *Schilbe mystus* (Linnaeus, 1758), *Barbus bynni* (Forskål, 1775), *Oreochromis niloticus* (Linnaeus, 1758) *Bagrus bajad* (Forsskål, 1775) *Mormyrus kannume* (Forsskål, 1775), *Lates niloticus* (Linnaeus, 1758) and *Clarias gariepinus* (Burchell, 1822).

2. Material and Methods

2.1 Fish Samples and DNA extraction.

Fish species (Figure 1) were acquired as previously reported [31] and classified into species level [32-34]. Genomic DNA from roughly 30mg fish muscle tissues was extracted by EZ-10 spin column genomic DNA kit (Bio Basic Inc., Canada). The DNA purity and concentration was estimated using UV spectrophotometry.

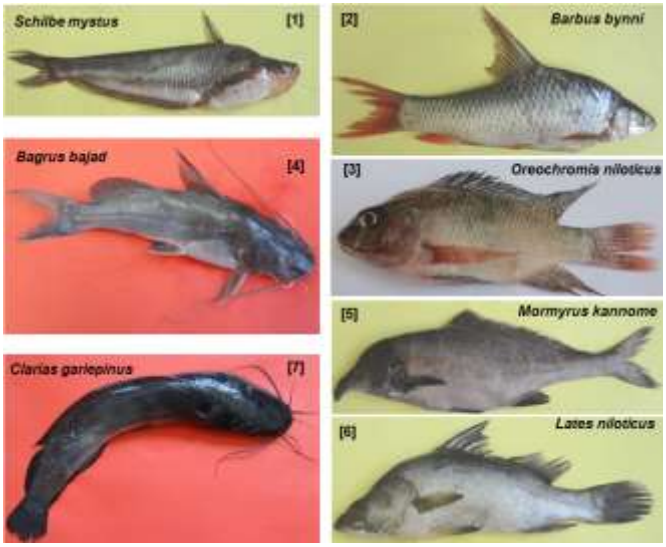

Figure 1: Photographs of the seven River Nile teleostei species in this study. Photos 1, 4 and 7 after EL-Mahdi, 2018 [30] while 2,3,5,6 after EL-Mahdi, 2018 [31].

2.2 PCR and sequencing of mtDNA Cytochrome-*b* gene.

The primers (GluFish; 5'AAC CAC CGT TGT TAT TCA ACT ACA A3' by Sevilla et al., 2007 and H15173; 5'CCC CTC AGA ATG ATA TTT GTC CTC A 3' by Parson et al., 2000) [35, 28] were used to amplify the mitochondrial cytochrome-b gene. The PCR reactions were carried out in 25 ml final volumes containing 1.0× premixed OnePCR™ 2X (GeneDireX Inc, USA), 10 pM of each primer and about 50ng of each DNA sample. PCRs were conducted using the cycling conditions: initial denaturation at 95°C for 2 min, 35 cycles (94°C for 1 min, 56°C for 1 min and 72°C for 2 min), and one cycle at 72°C for 10 min for final extinction in the Primus 25 advanced (PEQLAB Biotechnologie GmbH).

PCR products of 8 µl were electrophoresed on 1.5% (w/v) agarose/TAE/ethidium bromide (0.5 µg/ml) gel. PCR products sizes were estimated by comparison to 100 bp DNA ladder (0.1 µg/µl, Solis BioDyne, Estonia) which was approximately 460 base pairs. Gel images were taken under UV light by the Elttrofor M20 SaS Photo-Gel System (Italy). PCR fragments were bidirectionally sequenced (Macrogen Inc., Seoul, Republic of Korea) by the same primers used for PCR amplification.

2.3 DNA Sequence analysis

Sequences (sense/antisense reads) were manipulated using BIOEDIT version 7.0.5.3 [36] and free SnapGene Viewer v3.2.1 (GSL Biotech) and then aligned for entire targeted DNA fragment. The obtained partial sequences were compared to fish cytochrome-*b* gene DNA sequences in the GenBank nucleotide sequence database. For phylogenetic analysis, corresponding sequences of five species from five other families were recovered from the downloaded DNA sequences. Representatives from three families: *Nemachilichthys rueppelli* (Sykes, 1839) (Nemacheilidae); *Gymnarchus niloticus* (Cuvier, 1829) (Gymnarchidae) and *Synodontis serratus* (Rüppell, 1829) (Siluriformes; Mochokidae) were contained for use as in-group. The *Acipenser gueldenstaedtii* (Brandt/Ratzeburg, 1833) (Acipenseridae) and *Atractosteus spatula* (Lacépède, 1803) (Lepisosteidae) were selected as outgroup.

Sequence alignments were carried out using Muscle software [37] implemented in MEGA6 version 6 [38] under program's default. The MEGA6 software was also used for nucleotide compositions and phylogenetic analyses.

The appropriate nucleotide substitution model was chosen by ML fits of 24 different nucleotide substitution models [39]. Phylogentic trees were constructed using two approaches; the ML (Maximum likelihood) [40] and UPMGA (Unweighted pair group method with arithmetic mean) [41]. The robustness of the trees was approximated by performing 1000 bootstrap replicates [42] and branch length measured in number of substitutions per site.

3. Results

3.1 Amplification of mitochonderial Cytochrome-b gene

PCR primers targeting mitochondrial cytochrome *b* gene were successfully amplified the expected DNA fragments from the seven fish species and yielded amplicons of approximately ~460 bp (Figure 2).

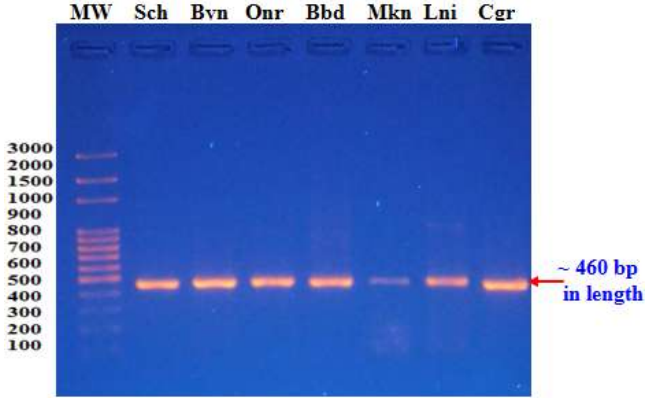

Figure 2: Gel electrophoresis of amplified PCR products from the seven investigated River Nile fish species. It shows a single band at 460 bp for part of mitochondrial cytochrome-*b* gene. Sch: *Schilbe mystus*; Byn: *Barbus bynni*; Orn: *Oreochromis niloticus*; Bbd: *Bagrus bajad*; Mkn: *Mormyrus kannume*; Lni: *Lates niloticus*; Cgr: *Clarias gariepinus* ; MW: DNA ladder (100-3000 bp).

3.2 DNA sequence analysis

The partial Cytchrome-*b* gene sequences were verified as being derived from studied species using similarity searches of GenBank DNA sequences. Determined DNA sequences from this study have been deposited in GeneBank database with accession numbers: MH133960, MH133961, MH133962, MH133963, MH133964, MH133965, and MH133966 (Table 1).

After excluding the forward and reverse primers regions, sequence analysis of partial Cytochrome-*b* gene based on a total of 402 base pair (Figure 3) .

Alignments of partial Cytochrome-*b* gene sequences from species under studies showed a deletion of three nucleotides in sequences derived from catfish species (*Schilbe mystus*, *Bagrus bajad* and *Clarias gariepinus*) since their partial sequences contained only 399 base pairs (Figure 3 Top). The three-nucleotide deletion was not seen in the remaining aligned partial sequences. When partial sequences from the seven River Nile fish species under study aligned with 5 retrieved sequences from GeneBank database (Figure 3 Below), the three-nucleotide deletion was also observed in the nucleotide sequence of the catfish *Synodontis serratus* (Siluriformes; Mochokidae).

Table 1: The Gene Bank accession numbers for nucleotide sequences in this study.

Name of species	Order	Family	Accession No.
Present study, the River Nile species partial Sequences			
Schilbe mystus (Linnaeus, 1758)	Siluriformes	Schilbeidae	MH133960
Barbus bynni (Forskål, 1775)	Cypriniformes	Cyprinidae	MH133961
Oreochromis niloticus (Linnaeus, 1758)	Perciformes	Cichlidae	MH133962
Bagrus bajad (Forsskål, 1775)	Siluriformes	Bagaridae	MH133963
Mormyrus kannume (Forsskål, 1775)	Osteoglossiformes	Mormyridae	MH133964
Lates niloticus (Linnaeus, 1758)	Perciformes	Latidae	MH133965
Clarias gariepinus (Burchell, 1822)	Siluriformes	Clariidae	MH133966
The Genebank/NCBI Sequences used			
Nemachilichthys rueppelli (Sykes, 1839)	Cypriniformes	Nemacheilidae	AP011305.1
Gymnarchus niloticus (Cuvier, 1829)	Osteoglossiformes	Gymnarchidae	AP008930.1
Synodontis serratus (Rüppell, 1829)	Siluriformes	Mochokidae	HF566064.1
Acipenser gueldenstaedtii (Brandt/Ratzeburg, 1833)	Acipenseriformes	Acipenseridae	KJ789859.1
Atractosteus spatula Lacépède, 1803	Semionotiformes	Lepisosteidae	JF912044.1

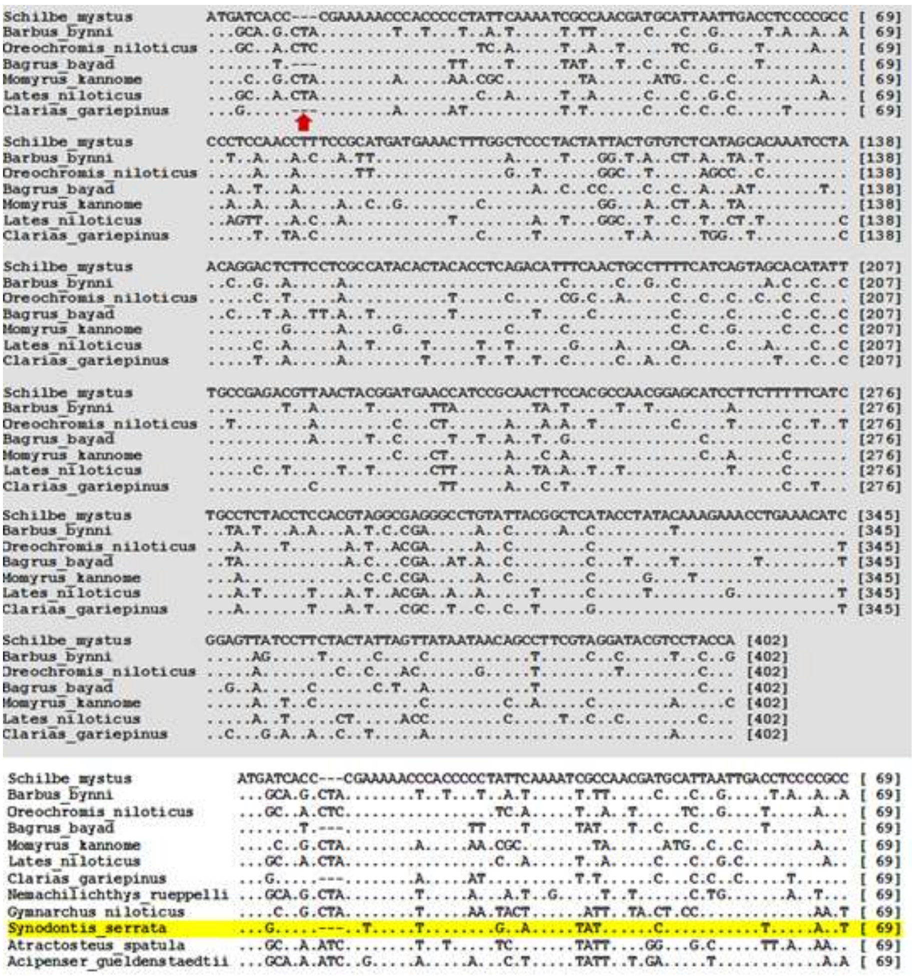

Figure 3: Top) Aligned partial sequences of cytochromeb gene among the seven River Nile teleostei fishes under study. Sequences are from the forward strand. Identities among sequences are designated by dots. There is a deletion of three base pair arrowed and marked by datchs. **Below)** Part from the multiple alignment (1-60 bp) for total of 12 sequences (including current study partial sequences) corresponding to 12 actinopetrygian families. The three-nucleotides deletion is clearly visible in the siluriforme *synodontis serrtus* sequence (Accession no: HF566064.1, marked)

3.3 Nucleotide composition bias

In average, nucleotides composition (Table 2) was T(U) =27.6, C=30.4, A=27.8 and G= 14.2. The G+C=44.59% and A+T=55.40% displayed nucleotides preference towards AT contents. The 402 sites that represents at least on third (1/3) of the cytochrome *b* gene coding region revealed 234 (58.20%) conserved nucleotides, and 168 (41.79%) variable nucleotides that included 103 (25.62%) parsimony informative, and 65 (16.17%) singleton sites. A greater part of cytochrome-*b* gene being analysed was conserved (58%), however sequence divergence of 33.33% was observed.

Table 2: Nucleotide constitution for part of cytochrome *b* gene sequences analyzed for seven River Nile teleosts species. C= conserved; V= variable; PI= parsimony informative; S= singleton sites.

Species/Nucleotide constitution	T(U)%	C %	A%	G%	Total	G+C %	A+T%	C	V	PI	S
Schilbe mystus	26.1	32.3	27.6	14.0	399.0	46.36	53.63	234	168	103	65
Barbus bynni	29.1	26.6	29.9	14.4	402.0	41.04	58.96				
Oreochromis niloticus	27.4	32.3	25.6	14.7	402.0	47.01	52.99				
Bagrus bajad	29.8	28.8	28.3	13.0	399.0	41.85	58.15				
Momyrus kannume	23.4	33.8	27.9	14.9	402.0	48.76	51.24				
Lates niloticus	28.9	29.6	27.9	13.7	402.0	43.28	56.72				
Clarias gariepinus	28.3	29.6	27.8	14.3	399.0	43.86	56.14				
Average.	27.6	30.4	27.8	14.2		44.59	55.40				

3.4 Molecular phylogentic Analysis.

For phylogentic analysis, total of twelve sequences (seven analysed in the current study, and five retrieved from Genbank) were used. Trees were rooted with *Acipenser gueldenstaedtii* and *Atractosteus spatula* as an outgrop. The GTR+G best-fit model of nucleotide substitution (BIC= 5553.773; AIC= 5359.948; *lnL*=-2649.778; transition/transversion bias (R)= 2.89; (+G) = 0.29; Nucleotide frequencies f(A), f(T), f(C), and f(G) were 0.280; 0.278; 0.298; and 0.144 receptively) was selected using the MEGA6 software.

The genetic distances between twelve sequences from actinpetrygian species calculated by the ML/GTR+G model with rate variation among sites modelled with a gamma distribution (shape parameter = 0.29) is shown in Table 3.

The distance values among species ranged from 0.323 (*schilbe mystus* with *clarias gariepinus*) to 0.761 (clarias *gariepinus* with *Acipenser gueldenstaedtii*) as shown in Table 3. Among the seven studied River Nile teleostei, the highest genetic distance is between *Barbus bynni* and *Clarias gariepinus* (0.639) whilst the lowest was between *Schilbe mystus* with both *Clarias gariepinus* (0.323) and *Bagrus bajad* (0.375).

The ML/GTR+G model formed a tree (Figure 4) with highest log likelihood -2650.2693 based on evolutionary distances (Table 3). This tree showed three major clades/groups. Clade A demonstrated a clear separation of siluformes catfish species of families; Schilbidae, Mockedae, Bagridae, and Claridae with high bootstrap support value of 83. While perciformes species; *Oreochromis niloticus* (cichlidae) and *lates niloticus* (Lattidae) grouped together on well-supported branch (value of 87).

Clade B contains the cyprinformes *Barbus bynni* (cyprinidae) and *Nemachilichthys rueppelli* (Nemacheilidae) with high branch support of 97. The Osteoglossiformes *Mormyrus kannume* (Mormyridae) and *Gymnarchus niloticus* (Gymnarchidae) clustered together with branch support of 46. In clade/group C, the outgroup representatives

separated together with branch support value of 69. Also, the tree revealed three evolutionary lineages, the 7 species from this study, *Nemachilichthys rueppelli*, *Gymnarchus niloticus*, and *Synodontis serratus* clustered in accordance with their genetic proximity as supported by pairwise distances (Table 2). The UPMGA -based GTR+R model (Figure 5) with sum of branch length 2.74173698 based on pairwise distance estimated ML resulted in a tree with comparable topology to the ML tree. The twelve analyzed sequences were placed in three main groups and the bootstrap confidence levels were reasonably high for majority of nodes within the tree.

The clear groupings between similar/related species were clearly demonstrated.

Table 3: Pair wise genetic distance involving 12 nucleotide sequences (Seven studied River Nile teleost species + 5 retrieved sequences) and conducted using the Maximum Composite Likelihood model. The rate variation among sites was modelled with a gamma distribution (shape parameter = 0.29).

	Species name	1	2	3	4	5	6	7	8	9	10	11	12
1	*Schilbe mystus*	---											
2	*Barbus bynni*	0.591	---										
3	*Oreochromis niloticus*	0.519	0.547	----									
4	*Bagrus bajad*	0.375	0.523	0.529	-----								
5	*Momyrus kannume*	0.440	0.468	0.487	0.521	----							
6	*Lates niloticus*	0.628	0.538	0.375	0.584	0.599	----						
7	*Clarias gariepinus*	0.323	0.639	0.603	0.453	0.529	0.561	----					
8	*Nemachilichthys rueppelli*	0.499	0.254	0.587	0.646	0.472	0.657	0.579	----				
9	*Gymnarchus niloticus*	0.687	0.469	0.623	0.495	0.380	0.639	0.576	0.447	----			
10	*Synodontis serrata*	0.371	0.522	0.491	0.379	0.491	0.543	0.314	0.664	0.601	-----		
11	*Atractosteus spatula,*	0.674	0.504	0.582	0.589	0.551	0.721	0.681	0.573	0.561	0.625	----	
12	*Acipenser gueldenstaedtii*	0.752	0.751	0.668	0.696	0.654	0.610	0.761	0.576	0.613	0.841	0.522	---

Figure 4: Molecular phylogenetic analysis using Maximum likelihood method/GTR+G model based on partial cytochrome-*b* sequences of seven River Nile teleost species and other related actinopterygians species. The tree is drawn to scale where branch lengths measured in the number of substitutions per site (below the branches) and a discrete Gamma distribution was used to model evolutionary rate differences among sites (+G, parameter = 0.29).

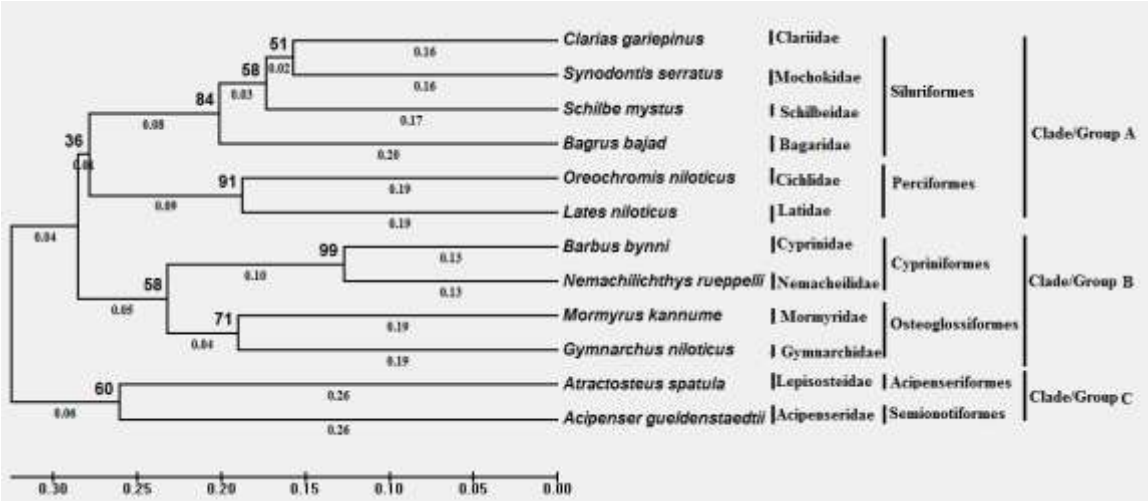

Figure 5: Molecular phylogenetic analysis by UPGMA method using partial cytochrome b sequences of seven fish species and other related actinopterygians based on evolutionary distance estimated using the maximum likelihood method. Branch lengths (above the branches) in the same units of the number of base substitutions per site and the bootstrap support of 1000 replicates is shown next to the branches.

4. Discussion

4.1 Cytochrome-*b* gene amplification and DNA sequence analyses

Herein, the mitochondrial Cytochrome-*b* gene coding portions were sequenced for seven River Nile teleost species. The Cytochrome-b primers were productively amplified the expected DNA fragments from each species. Analysis of total 402 base pairs from cytochrome-*b* coding sequences revealed a deletion of three nucleotides in Siluformes catfish sequences which may occurs as demands for species monophyletic evolution needs. This confirmed by the absence of this deletion in species sequences under studies which may represent the Siluformes catfishes monophely. A study reported the existing of three nucleotide deletion in Bagrid catfishes cytochrome-*b* gene when compared to characiformes and cyprinformes fishes [43]. Genetically related species have more similar DNA base constitutions than those who are distantly related. Data here show high level of nucleotide sequence identity in the cytochrome-*b* gene portion analysed from species under study (Figure 2) which suggests their genetic relatedness highlighting their close evolutionary relationship. Studies reported that, comparable cytochrome-*b* sequences among different species considered informative for measuring levels of genetic variation, evolutionary relation and sequence divergence [27, 44-45].

Nucleotide composition confirmed an anti-Guanine and preference in bias of thymine, cytocine and adenine as reported for Actinopterygian fishes and other mitochondrial coding genes [45-49]. Nucleotides preference towards adenine raised the AT content, that may reflect more transcriptional activities and less methylation. Studies reported that bias towards AT contents rather G+C contents considers common phenomenon in fish mitochondrial genome [50-52].

Alignment of a total of 402 nucleotide of Cytochrome-*b* gene coding region reveals variations at nucleic level (conserved, variable, parsimony informative and singleton sites). This suggests that the studied region of Cytochrome-*b* gene is a valuable having more useful phylogenetic information. A study reported usefulness of sequences with high number of informative sites in differentiation and genetic structure among populations [53]. Also, the favouritism towards conservations by 58% with probable sequence divergence of ~ 33.44 % may suggest hidden evolutionary functional constraints required for internal genetic modification to the evolutionary and sudden environmental changes.

4.2 Molecular phylogenetic relationships

Species assorted into same cluster would have similar features and likely to share common sequences from previous ancestors. Here, the phylogenetic analysis that based on cytochrome-*b* partial sequences (Figures 4, 5) demonstrated trees with similar topologies and presented groups of genetically related species allocated closely; those would be genetically descent from common ancestor. But degree of the genetic closeness varies depending on shared genetic formations as indicated by high nucleotide identities. Mostly, trees clarified that species possessing high sequence identities are grouped together or closely assigned. The ML and UPMGA tree constructions support the evolutionary distances estimated based on mitochondrial cytochrome-*b* partial sequence information (Table 3). Some studies reported that, the genetic divergence and phylogentic relations among related species groups can be figured out from informational gene DNA contents [54-55]. Also, both trees showed separating of out-group species from other analysed sequences, which suggested that these two species are from a single descent clade and likely to have common ancestor with other studied teleosts species. As reported by Inoue et al., 2003 [56] and Venkatesh et al., 2001[57] the acipenseriforms (*Acipenser gueldenstaedtii*), and gars (*Atractosteus spatula*) are related to teleosts fishes. Several studies reported usefulness of cytochrome-*b* gene for evolutionary and relationship analysis between recently-diverged taxa [58] and widely been used in fish systematics and phylogeography [59-62].

5. Conclusion

In this study, portions mitochondrial of Cytochrome-*b* from seven River Nile teleost species were sequenced and evaluated for sequence variances and phylogenetic relationship. A three-nucleotide deletion existed in Siluformes catfishs Cytochrome b gene sequences which may accounts for species adaptation and evolution monophyletic need. Cytochrome b gene nucleotide compositions were similar to actinopterygian fishes, also to other mitochondrial coding genes. The studied Cytochrome b gene region displayed high variations at nucleic level, which supported the gene‘s importance as a DNA genetic marker in fish phylogentic studies. DNA sequences obtained are consistent and convincing which reflected the sequence differences, evolutionary and phylogenetic outlines among fish species under study. Results of this study indicate closer genetic relationship between the twelve actinopterygian fish (including those in this study) which may be useful for successful fish managing, conservation and aquaculture objectives. Data obtained builds a helpful contribution to future actinopterygian molecular phylogeny studies in this area.

6. Acknowledgment

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