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Research article

Genetic distance of anadromous *Tenualosa ilisha* (Hamilton, 1882) in Shatt al-Arab River based on partial sequencing of mitochondrial DNA

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Abstract

The genetic similarity of anadromous Hilsa shad (*Tenualosa ilisha*) samples from four stations along the Shatt Al-Arab River was assessed using partial sequencing of the mtDNA *cytochrome b* gene to describe population structures and to compare samples. Two-way sequencing was conducted on 70 samples from DNA extracted from fin samples. A phylogenetic tree was structured depending on the consensus of sample sequences using the MEGA X software. Based on all the *T. ilisha* samples, there were two major clades on one branch that were considered monophyletic (100% bootstrap value) and the genetic distance values were in the range 0.01–0.14. The *T. ilisha* samples from the Shatt al-Arab stations and an India2 sample were in one clade and the other clade consisted of unrelated pairs of *T. ilisha*, while *T. thibaudeaui* was the out group. The genetic differentiation between the clades was not significant ($p > 0.05$) showing the two different stocks with minor genetic differences belonged to a common genetic ancestor.

Introduction

Hilsa shad (*Tenualosa ilisha*) is the most important clupeid species, with largely anadromous pelagic characteristics (Al-Dubakel, 2011) It is recognized as the most commercial fish of the Indo-Pacific region and locally in Iraq it is known as 'sbour', where it is an important migratory species in the north west Arabian Gulf (Al-Dubakel, 2011). This euryhaline fish is distributed in marine, coastal and freshwater environments and regularly appears adopting schooling behavior in shoreline waters (Mohindra et al., 2019). The fish is present in the Arabian Gulf, Arabian Sea, Red Sea, Bay of Bengal, Vietnam Sea and China Sea (Mohindra et al., 2019) The riverine habitat covers the Shatt al-Arab river in Iran, Tigris and Euphrates river in Pakistan, the Irrawaddy River in Myanmar, Indian rivers and the coastal rivers of Bangladesh (Bhaumik, 2013). Estuaries are the major sites where Hilsa shad migrate for breeding and nurturing young ones (Mandal et al., 2018).

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It feeds and grows generally in the sea, migrates to rivers for spawning and thereafter, juveniles develop and grow in fresh water, before migrating to the ocean, where they spend the majority of their lives (Asaduzzaman et al., 2020).

Tenualosa ilisha migrate into the Shatt al-Arab River from March to September and spawning begins as early as February with a peak at the end of March and the beginning of April (Al-mukhtar et al., 2016). *T. ilisha* moving through the Shatt al-Arab Channel may travel as far as 150–180 km north of Basrah (Al-Noor, 1998).

The movement of an anadromous fish between marine and freshwater requires a wide-range of life-history strategies to acclimate within heterogenic environmental conditions and complex aquatic systems (Bernatchez, 2016) to achieve a specific genetic composition and native adaptation of genetically based phenotypic traits for peak fitness (Tamario et al., 2019; Asaduzzaman et al., 2020). However, it is not well-known if the fish populations combine during the migration or whether they cross each other and this remains a subject of controversy (Asaduzzaman et al., 2020).

The aim of the current study was to analyze the genetic distance of samples collected from four stations in the Shatt al-Arab River where there was inter migratory activity and to systematically compare them with *Tenualosa* sequences from Indo-Pacific countries available in the NCBI GenBank. The results should provide better understanding of the genetic similarity of this commercially important species and offer insight into the origin of the strain by using mitochondrial DNA *cytochrome b* (Cytb) gene sequencing.

Materials and Methods

In total, 70 specimens of *Tenualosa ilisha* were collected from four stations in the Shatt al-Arab River with the help of artisanal fishers during the spawning migration season from March to September 2020 (Fig. 1). Stations 1 and 2 each had a sample size of 20 and stations 3 and 4 each had a sample size of 15.

A large piece (1–2 cm) of pectoral fin from each sample was carefully collected and stored at -20°C until next use for the extraction of DNA.

DNA extraction

Approximately 100 mg of tissue was placed in a 1.5 mL tube containing 300 µL digestion buffer (Abdullah et al., 2019) and 15 (3mm) glass beads. Homogenization was completed using Bioprep-24 (Allsheng, China) in two cycles of 3,000 revolutions per minute for 30 sec. Genomic DNA was isolated using a SinaPure TM DNA extraction kit (Sinaclon, Iran) according to manufacturer's instruction. Analysis on 1% agarose gels electrophoresis and spectrophotometric methods were used to determine DNA quality and quantity.

Primers and polymerase chain reaction

The specific primers were designed using Primer Premier 5 (PRIMER Biosoft-USA) to amplify a 600 base pair (bp) fragment from the mitochondrial Cytb gene. Polymerase chain reactions (PCRs) were conducted using a pair of primers (Table 1).

Amplification of a fragment from the mitochondrial Cytb gene was carried out at a final volume of 20 µL according to the procedure described in Table 2. The thermal cycling conditions are shown in Table 2.

The PCR products were assessed using electrophoresis on a 1% agarose gel to determine the specificity of amplification.

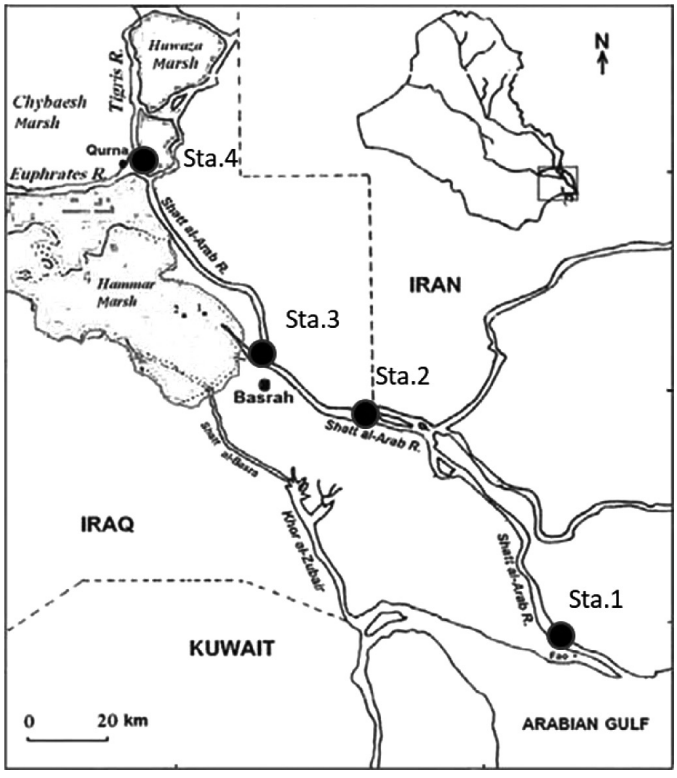

Fig. 1 Site locations of *Tenualosa ilisha* from Shatt al-Arab River, where Sta. 1 = AlFaw (n = 20), Sta. 2 = Abu al-Khassib (n = 20), Sta. 3 = Garmat Ali (n = 15) and Sta. 4 = Al-Qurnah (n = 15)

Table 1 Primers and procedure used for amplification and sequencing of mtDNA

Reagent	Volume
Master mix (2X)	10 µL
Primer mix (NC_016682.1)	1.5 µL
F: 5'-CTAACGACGCAGTAGTTGATCTCCCA-3'	(10 pMol)
R: 5'-CTGAGTTTAGCCCCGCAGGGTTGTT-3'	
DNA template (50 ng/µL)	2 µL (50 ng)
Nuclease free water	6.5 µL
Total Volume	20 µL

Sequencing

The purification of PCR products was done using the ethanol precipitation method described by Abdullah et al. (2019). Sequencing was performed on all samples. The amplicons were sequenced using both amplifying primers, bidirectional (Sanger et al., 1977). All haplotype sequences for *T. ilisha* samples were deposited in the NCBI GenBank with accession numbers: LC619669–LC619672.

Analysis of DNA sequences

The Cytb sequences obtained were imported into the BioEdit software (Version 7.2 available from https://bioedit.software.informer.com/7.2/) to check and correct any possible errors. The DNA sequence polymorphism software (DNASP 5.10.01) (Librado Rozas, 2009) was used to align all sequences obtained from the NCBI database in addition to the sequences from the studied samples.

Phylogenetic tree

The maximum likelihood tree of haplotypes of *Tenualosa* species was assembled using the Tamura-nei model and the third codon position with scaled branches to draw the phylogeny using the MEGA X software program (Kumar et al., 2018). The phylogenetic tree was based on 600 bp *CytB* gene sequences for consensus sequences of *T. ilisha* samples from four stations along the Shatt al-Arab River (Al-Faw, Abu al-Khassib, Qarmat Ali and Al-Qurnah) compared with six samples of *T. ilisha* having 100% Blast similarity, and *T. thibaudeaui* as the out group. Samples from the stations along the Shatt al-Arab River were merged into four consensus samples due to the large sample size in order to make the tree compact and simple without affecting tree branching. Their accession numbers are listed in Table 3.

Results

The PCR products were checked on 1 % agarose gels using spectrophotometry (Fig. 2). Alignment of all Cytb sequences was obtained using the BioEdit software. The molecular phylogenetic tree located in Fig. 3, showed two major branches; one involved all the *T. ilisha* samples and

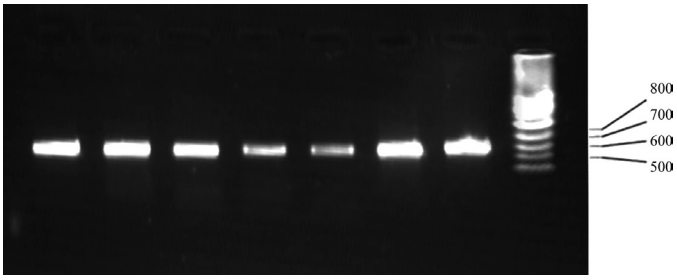

Fig. 2 Agarose electrophoresis of polymerase chain reaction products (600 bp) on 1% gels

Table 3 Samples of *Tenualosa ilisha* from Indo-Pacific regions by country and Accession number based on Cytb gene used to generate the phylogeny tree and compared with *T. ilisha* samples from Shatt Al-Arab River

Country	Species name	Accession No.
India1	*T. ilisha*	AP011610.1
India2	*T. ilisha*	KC816530.1
Malaysia	*T. ilisha*	KU888658.1
Bangladesh	*T. ilisha*	KX859109.1
Thailand	*T. ilisha*	AP011611.1
Laos	*T. thibaudea*ui	AP011604.1

Table 2 Thermal cycling steps for amplification of mt DNA from *Tenualosa ilisha* samples

Step	Temperature	Time	Number of cycles
Pre-denaturation	95°C	5 min	1
Denaturation	94°C	45 sec	
Annealing	64°C	30 sec	35
Extension	72°C	45 sec	
Final extension	72°C	10 min	1

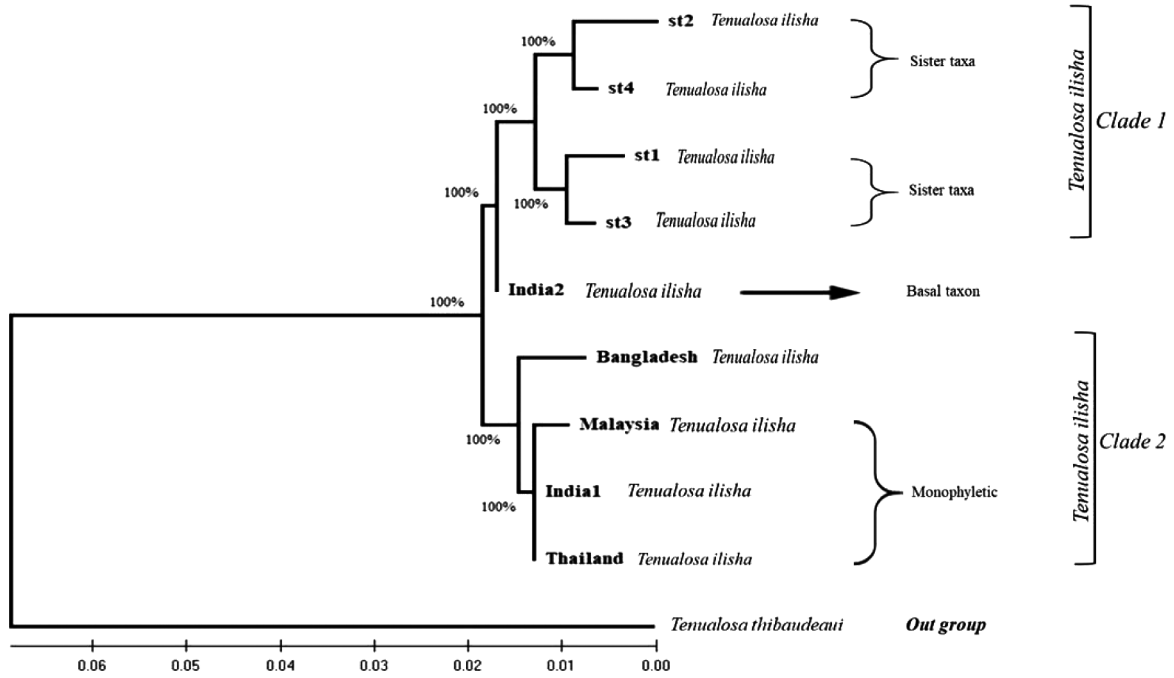

Fig. 3 Dendrogram of molecular phylogenetic analysis of 10 haplotypes of *Tenualosa ilisha* and *T. thibaudeaui* constructed using maximum likelihood method

the other was limited to the out group sample *(T. thibaudeaui).* These branches had 100% bootstrap values. The positioning of *T. ilisha* revealed two major clades for all *T. ilisha* specimens. The first clade gathered all the samples of *T. ilisha* collected from the four stations along the Shatt al-Arab River; they were clustered within the *T. ilisha* from India2 (KC816530.1) (Fig. 3) and were monophyletic (100% bootstrap value). The second clade consisted of *the T. ilisha* samples from Bangladesh, India1, Malaysia and Thailand that were also monophyletic with a 100% bootstrap value. *T. thibaudeaui* was used as the out group.

The pairwise *Fst* values based on mtDNA ranged from 0.0000 (*T. ilisha* Thailand and India2) to 0.1410 (*T. thibaudeaui* and *T. ilisha* from AbuAl-kasseeb); the overall mean distance was 0.0369 (Table 4) and Tajima's D value was -1.64755 ($0.10 > p > 0.05$).

Discussion

The mitochondrial markers are very popular genetic marker due to their high richness and their ability to easily capture locus-specific inheritance (Javanmanesh et al., 2019). These markers have been used in many species including animals and humans (Ingman et al., 2003; Azghandi et al., 2017; Afsharian et al., 2018; Hamadalahmad et al., 2020). The Cytb gene has often been used as a marker for considerations of evolution,

Table 4 Pairwise genetic distance between individuals of *Tenualosa ilisha* from different sites

Location	Abulkhaseeb	AlFaw	Bangladesh	Garmat	India1	India2	Laos	Malaysia	Qurnah	Thailand
Abulkhaseeb										
AlFaw	0.0161									
Bangladesh	0.0233	0.0142								
Garmat	0.0197	0.0088	0.0142							
India1	0.0251	0.0196	0.0053	0.0160						
India2	0.0178	0.0124	0.0088	0.0088	0.0070					
Laos	0.1410	0.1368	0.1276	0.1277	0.1253	0.1211				
Malaysia	0.0269	0.0215	0.0070	0.0178	0.0018	0.0088	0.1274			
Qurnah	0.0125	0.0124	0.0160	0.0124	0.0178	0.0106	0.1340	0.0196		
Thailand	0.0251	0.0196	0.0053	0.0160	0.0000	0.0070	0.1253	0.0018	0.0178	

genetic population structure and to determine taxonomic variances in many fish species (Behera et al., 2015).

The phylogenetic maximum likelihood based on neighbor-joining analyses was structured with high bootstrap scores (100%), revealing no significant genealogical branches or clusters based on the sampling regions. The two main branches of the tree revealed that the *Tenualosa* genus (specifically *T. ilisha* and *T. thibaudeaui*) had descended from a common evolutionary ancestor and all the major clusters were attendant on a central ancestral haplotype.

The relationships between *T. ilisha* haplotypes were symbolized using a minimum straddling tree (Fig. 3), which showed that there were two main clades with many individuals linked by a few mutations. Clades had clear haplotype branches corresponding to geographical populations. *T. ilisha* specimens from the Shatt Al-Arab River and Indo-Pacific regions were the most closely correlated species, Although *T. thibaudeaui* was an out group, its similar overall structure and appearance have resulted in confusion in the morphological classification of the species to date. Clade 1 included all *T. ilish* specimens from the Shatt Al-Arab River and the India2 sample (KC816530.1), suggesting that *T. ilisha* which migrated to the Shatt al-Arab River belonged to the same Hilsa stock dominating Indian regions (as indicated by the 100% bootstrap value). Clade 2 implied all *T. ilisha* samples are endemic species to the Indo-Pacific seashore and closely related by the same ancestry based on the clade pairwise genetic distance between them (Table 4), indicating that all individuals share high genetic similarities and belong to a common genetic ancestor, despite being from two different stocks.

The genetic differentiation between the two groups from the same locality was not significant according to the DNA polymorphism analysis that resulted in low haplotype diversity (0.978) at a confidence level (95%) with little mutation (83), while Tajima's D result was not significant ($0.10 > p > 0.05$), as shown in Table 5.

Table 5 DNA polymorphism analysis for consensus sequences of *Tenualosa ilisha* samples

Type of analysis	Result
Average of pairwise differences (K)	19.556
Stander diffusion of haplotype diversity	0.054
Variance of haplotype diversity	0.00292
Total number of mutations	83
Haplotype diversity	0.978
Confidence level of haplotype diversity (%)	95

The nucleotide diversity, genetic distance and phylogenetic relationship data, showing different groups with a similar geographical distribution that indicated an aligned phylogenetic relationship, was consistent with Barat et al. (2012). A monophyletic group (Al-Faw and Garmat Ali) was a sister taxon of individuals within clade 1. This divergence might indicate that the two individuals came from other *T. ilisha* populations due to migratory behavior or gene flow to adapt to a heterogeneous environment during its life cycle, so that they may have higher rates of divergence. This suggested that these haplotypes include unique individuals belonging to the same species (Blaber et al., 1996; Mohindra et al., 2019). Verma et al. (2016), utilizing the mitochondrial control region, established the broad structuring of Hilsa populations between the Bay of Bengal and the Arabian Sea, but did not comment on the arrangement between riverine populations. Behera et al. (2015) noted two different stocks of Hilsa shad fitting the Arabian Sea and the Bay of Bengal sources depending on the Cytb gene amplified. Arjunaidi et al. (2016) reported that *T. ilisha* had apparent native adaptation to various environmental effects to enable successful long-distance migration.. Lal et al. (2004) and Puvanasundram et al. (2018) suggested that anadromous Hilsa shad from the same gene pool migrated to estuaries or rivers with high distribution ability due to a broadly anadromous life strategy that had caused significant genetic variations within individuals and a high-level of diversification to adaptation to a heterogeneous environment and varying environmental conditions. The low genetic differentiation noted in the current study might have been due to an inbreeding population and other evolutionary influences such as selection, genetic drift, migration and topographical barriers.

The current results indicated a high level of gene flow within the Hilsa shad *T. ilisha* population but a lower variation across their migratory distribution range. This could be due to the wide distribution and great dispersal ability of *T. ilisha* due to the large population size. Also since this anadromous fish that is found in diverse migratory habitats during their life-history stages, the effects of environmental pressures influences on *T. ilisha* natural population genetic diversity, therefore from species conservation point of view, there would be less concerns for loosing diversity (Asaduzzaman et al., 2020).

The current study indicated that it is essential to maintain the genetic diversity of Hilsa shad *T. ilisha* in its different habitats. The current study successfully identified the close genetic relationship of India2 individuals of *T. ilisha* to individuals from the Shatt al-Arab River rather than to the samples India-1, Malaysia, Taiwan and Bangladesh.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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