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Genetic Indications for Anadromous Hilsa Shad (*Tenualosa ilisha*) in Shatt Al-Arab River Using mtDNA Cytochrome B Gene

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Abstract: Hilsa species are broadly described as largely anadromous fish with a large valuable population size. It is well-known as one of the most critical commercial and occurs in marine, estuarine and riverine environments Hilsa shad, *Tenualosa ilisha* locally known as Sbour, migrates to the freshwater environment of the Shatt Al-Arab River systems for breeding. It was determined the genetic variation in 70 specimens of *T. ilisha* from four sites at the Shatt Al-Arab River, the mtDNA cytochrome-b gene was selected as a molecular marker for detecting genetic diversity, origin, and divergence of the population through comparing them with 6 samples from different locations at Indo-Pacific. The AMOVA analysis showed that the variation between groups is 60.97% and 39.02% within population indicating statistically significant P value ($P<0.05$). Furthermore, the maximum likelihood phylogenetic tree showed two main clusters of all *T. ilisha* groups representing two stocks of separated breeding grounds have a common ancestor.

Keywords: Anadromous, Tenualosa ilisha, Shatt Al-Arab, AMOVA, Phylogenetic.

Introduction

The gathering of the two major rivers of Iraq (Tigris and Euphrates) at Qurnah city Southern Iraq form the Shatt al-Arab River. It has a length of about 204 km. It has share in its borderline between Iraq and Iran (Abbas & Mohammed, 2020), a width range between 250 at Qurnah to 1500 m at the estuarine zone and dissimilar in its depths ranging between 14 to 18 m. The downstream of Shatt al-Arab is located at Al-Fao city flowing towards southeastern Arabian Gulf for a distance 2 km describing as estuarine – deltaic ecosystem (Al-Hamad *et al*., 2017). Freshwater from Shatt Al-Arab River and the wintrily rainfall from January to May considered

as the principal supplier of nutrients which acting on the topic of seasonally dynamic of Arabian Gulf marine ecosystems (Al-Yamani, *et al*., 2007; Mohamed & Abood, 2020). These properties would be useful for water quality and biological productivity, however the estuarine and coastal environments are affected by flowing turbidity amounts, and nutrients arriving from the northern Gulf. Variety of marine commercially species migrate broadly to freshwater into Shatt Al-Arab River for breeding and nursery habitation such as shrimp, Hilsa shad, yellow-fin seabream, and pomfret (Resen, 2018; Lateef *et al*., 2020). Hilsa shad (*Tenualosa ilisha*) is the highest anadromous fish that overrun Arabian Gulf and Iraqi freshwater. They enter Shatt Al-Arab River as schooling migratory fish (Al-Noor, 1998) at different times from March to September (Mohamed *et al*., 2008; Nasir, 2016). *T. ilisha* is locally named Sbour, it ascends up Shatt Al-Arab River about 150-200 km north of Basrah city (Mohamed *et al*., 2009). Hilsa shad is considered the main resource of fish food in India, Pakistan, Malaysia, other Indo Pacific countries, and the Arabian Gulf (Al-Dubakel, 2011).

T. ilisha is described as a largely anadromous species; the adults travel to freshwater from the sea for spawning; but the hatching and young stages stay in the river, channels, and estuaries before returning them to the sea for feeding and growth (Hossain *et al*., 2016). The migration activities of Hilsa shad studied by tagging methods were accomplished first time by Pillay & Rosa (1963) in the Ganga River / India, their results disclosed that *T. ilisha* is observed in the lower Ganges arriving at estuarine areas across the main Padma River in Bangladesh. Islam & Talbot (1968) found that Hilsa shad assemblages migrate about 161 km from the Sea to Indus River in Pakistan, this statement indicates that Hilsa stock becomes as freshwater stock.

In Myanmar at Irrawady River, It is realized that Hilsa ascend into a distance of about 724 km from the Sea (Arai & Amalina, 2014). Coad (2010) indicated that *T. ilisha* reaches Qalaat Salih through Tigris River and Al-Fahod at Euphrates River nearly 150-180 km north of Basrah. Al-Hassan (1999) reported movements of *T. ilisha* along the Iranian side of the Arabian Gulf and in Iraqi waters through the year. This could be because of the diversity of ecological and biological conditions as well as the discharging of freshwater or estuary during monsoon.

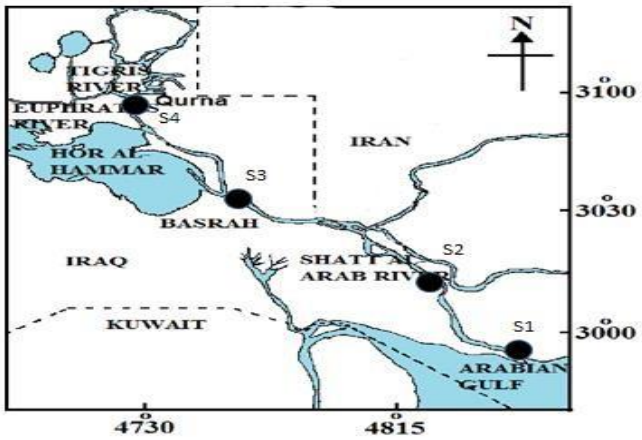

Fig. (1): The sites (black spot) for *T. ilisha* sampling in Shatt Al-Arab River, Al- Fao (S1/ site 1), Abul-khaseeb (S2/ site 2), Garmat Ali (S3/ site 3), Qurnah (S4/site 4).

The estimation of genetic structure and the number of genetic variations patterns among the different migratory of the Hilsa populations in the Shatt Al-Arab River still remain available to understanding their routes and ancestors. Molecular markers have been recognized to be guiding to noticeably of evolutional in fish genetics (Gkagkavouzis *et al*., 2021). These

markers were managed in genetic controlling, lineage assessment, and to determine practical markers for commercial traits (Tanya & Kumar, 2010).

Practically, we consider sequence data of the partial cytochrome b gene from mtDNA to target characterize the genetic structure and ancestors of 70 *T. ilisha* samples collected from four sites in Shatt Al-Arab River, by assessing the phylogenetic analysis during the spawning period.

Materials & Methods

Four sites along Shatt al-Arab River were chosen, namely: Al-Fao (S1/site 1), Abul-Khaseeb (S2/site 2), Garmat Ali (S3/site 3), and Qurnah (S4/site 4) (Fig. 1). 70 specimens of Hilsa shad (*T. ilisha*) were collected randomly during spawning migration season from March to September 2020.

A larger piece of the pectoral fin from each sample was amassed and immersed in 95% ethanol, each sample was placed in a labeling 1.5 Eppendorf tube; then they are incubated at -20 ˚C until extraction of DNA process. Approximately, 30-40 mg of the pectoral fin from each sample was placed in a 1.5 ml tube containing digestion buffer. After that, the sample was homogenized. It was then carfully dropped 5 µl RANase into each tube with gentle shaking then the mixture was incubated at 37˚C for 15 mints. Quietly, 25 µl of Proteinase K solution was added to the mixture with flipping manually, ultimately, the mixture was incubated at 55˚C for two hours with vortexing for every 15 minutes.

Genomic DNA was extracted according to the manufacturer protocol of kits (SinaPure TM DNA) by SinaClon Bioscience, Tehran, Iran. 400 µl Lysis buffer has been added into the samples and they were vortexed them, then 300 µl precipitation solution was added and vortexed at high speed. The solution was transferred to a spin column with a collection tube to finish washing stages, preheated (65oC) buffer solution was dropped to the column spin then centrifuged. The final product is the DNA which detected by electrophoresis using 1% agarose gels, and spectrophotometric was used to determine the quantity and quality of the DNA.

The mitochondrial DNA (mtDNA) cytochrome b gene of *T. ilisha* was used for genetic diversity analysis using polymerase chain reaction (PCR). The amplification reaction materials were shown in table 1. Primer Premier 5.0 software was used for designing a set of primers (forward and reverse -600 Pb, NC_016682.1, 5 pM)

F. 5'CTAACGACGCAGTAGTTGATCTCCCA3'

R. 5'CTGAGTTTAGCCCCGCAGGGTTGTT3'

Primer sequences were analyzed with BLAST software: www.ncbi.nlm.nih.gov/blast/Blast.cgi

Table (1): Display the reagents of polymerase chain reaction (PCR) used in *T. ilisha* cytochrome b gene amplification

No.	Reagents	Volume
1	Master mix	10 µl
2	Primer's mix (F & R)	1.5 µl
3	DNA template	2 µl
4	Nuclease free water	6.5 µl
Total Volumes		20 µl

The PCR cycling condition was started with one cycle for pre-denaturation at 95˚C for 5 minutes, followed by 35 cycles, including denaturation at 94˚C for 30 sec, annealing at 64˚C for 30sec, extension at 72˚C for 45 sec.

Finally, one cycle for Final extension at 72˚C for 10 minutes. After that, the purified PCR products were examined in 1.0 % agarose gel to determine its quality, imagining was carried out by ultraviolet light box Gel Documentation system. PCR products were sequenced utilizing the same set of primers subjected to the management of Pishgam Biotech Company, Tehran. Iran.

The ethanol precipitation method described by Abdullah *et al*. (2017) was used for purification of PCR products. PCR products were sequenced using both amplifying primers bidirectional (Sanger *et al*., 1977). All haplotype sequences for *T. ilisha* samples from four sites were deposited in GenBank under accession numbers (LC 619669-LC619672).

Data analysis

The DNA sequences were assembled with the consensus sequences of four site samples (by http://emboss.open-bio.org/wiki/Appdocs) with all specimens for Indo Pacific regions collected from NCBI/GeneBank (Table 2) then aligned using employing BioEdit software. DNA v5.10.01 software program (Librado & Rozas, 2009; Stecher *et al*., 2020) was used to estimate the sequence variation sites, nucleotide diversity,

Table (2): Accession numbers of *cytochrome b* gene for *T. ilisha* specimens from different countries used in the current study.

Country		Accession No.
Bangladesh		KX859109.1
India	1	MK993293.1
	2	KC816468.1
	3	MN101849.1
Malaysia		KU888658.1
Thailand		AP011610.1

and haplotype diversity within the population. After that, a Maximum composite Likelihood tree was structured for all haplotypes depending on MEGA X (Stecher *et al.*, 2020).

To consider the branching confidence of the dendrogram, a completed bootstrap analysis (1000 data sets) was achieved. For statistical analysis of the genetic structure of all *T. ilisha* population assembled ,Arlequin 3.5 was done (Excoffier & Lischer, 2010).

Results

Partial mtDNA of Cytochrome b (600 bp) sequences for 4 consensuses taken from 70 *T. ilisha* specimens in four sites in the Shatt Al-Arab River were aligned with six samples of *T. ilisha* from 6 countries in the southwest Indian Ocean. The sequence of *T. ilisha* for southern West Indian Ocean specimens was obtained from the accession numbers in GenBank (perc. of similarities/ Identity100%) (Table2). All sequences were reconstructed using maximum likelihood (ML) phylogeny through the MEGA X software program. Branch nodes are designated as the values of bootstrap support, which was generated with 1000 replicates. The ML tree appeared as a summary of bootstrap analysis. The phylogeny dendrogram is constructed on genetic distance resulted in two main branch clusters and more closely related (Fig. 2). The first cluster was subsequently divided into two sub-groups formed by *T. ilisha* from Malaysia, Thailand, India 3, Bangladesh, and India 1. The second cluster included all sites taxa from the Shatt Al-Arab River with India 2. The phylogenetic tree revealed that *T. ilisha* collected from the Shatt Al-Arab River are more close to India 2, and all of them being as sister taxa with the same root, in addition, there was also no apparent haplotype branch from them, the low bootstrap value was

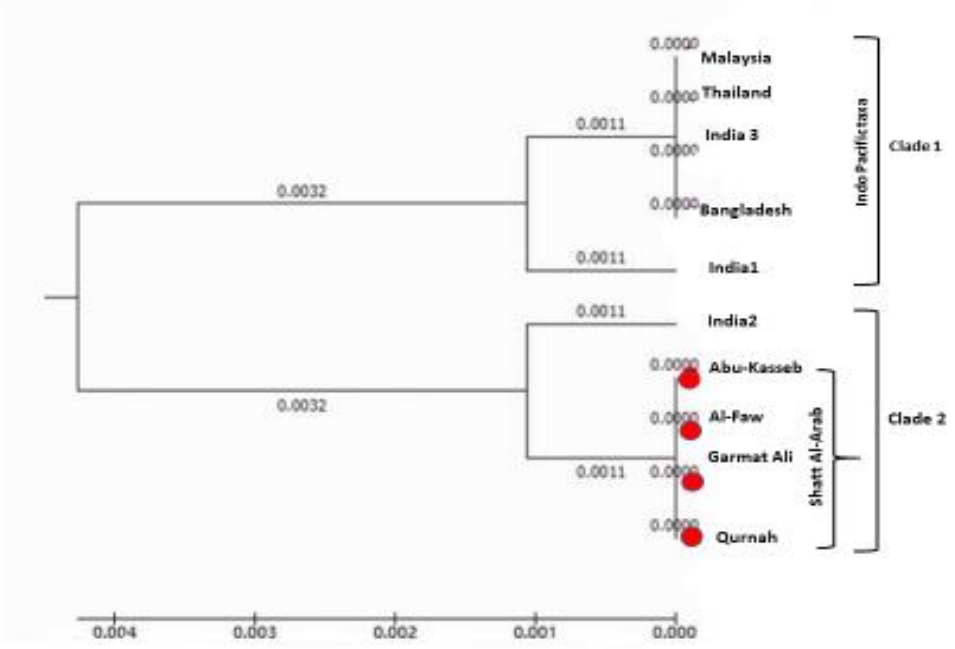

Fig. (2): Maximum Composite Likelihood tree representing phylogenetic relationships among the specimens of *T. ilisha* applied for four sites at Shatt Al-Arab River (red spots) and 6 samples from Indo Pacific regions. The allocation of genetic distance estimates presented at the branch

Table (3): The Pairwise population differentces of genetic structure tested by fixation index (FST) among populations according to MEGA X software.

	1	2	3	4	5	6	7	8	9	10
1 Bangladish										
2 Abu Kasseb	0.009									
3 Al-Faw	0.009	0.000								
4 Garmat Ali	0.009	0.000	0.000							
5 Qurnah	0.009	0.000	0.000	0.000						
6 India 1	0.002	0.011	0.011	0.011	0.011					
7 India 2	0.006	0.002	0.002	0,002	o.oo2	0.009				
8 India 3	0.000	0.009	0.009	0.009	0.009	0.002	0.006			
9 Malaysia	0.000	0.009	0.009	0.009	0.009	0.002	0.006	0.000		
10 Thailand	0.000	0.009	0.009	0.009	0.009	0.002	0.006	0.000	0.000	

Table (4): AMOVA testing of the genetic structure of *T. ilisha* groups.

Source of variation	Sum of squares	Variance components	Percentage variation
Among population	8.133	0.83333	60.97561
Within population	2.667	0.53333	39.02439
Total	10.800	1.366	
P value = (0.00098) (*P*<0.05)			

noticed in the tree (Bootstrap 0.0000). Pairwise differences among populations were 0.000-0.011measred by fixation index (Table 3).

Analysis of AMOVA signified the percentage of variation among populations (60.97%) and within the population (39.02%). P-value was (0.00098) (P<0.05) (Table 4).

Discussion

Understanding the population genetic structure grants us important information for the management of species. The maintainance of populations is related to knowledge the genetic diversity (Luhariya *et al*., 2012; Asaduzzaman *et al*., 2020b). Generally, the Spatio-temporal interactivity of ecological and heterogenic environmental progression systems are influenced by the phenotypic and the genetic situations of anadromous fish (Tamario *et al*., 2019).

Our finding on the maximum likelihood phylogeny revealed two main clades forming a monophyletic group indicating two different stocks. The two main clusters of all *T. ilisha* represents two separated breeding grounds with changeable environmental conditions. The first clade includes specimens from Malaysia, Thailand, India 3 and Bangladesh attached with India 1 as sister taxa; they were dominant at the coastline of the Indian Ocean. The second clade included all specimens from the Shatt Al-Arab River with Indian 2. The small distance among them denotes that they are belonging to the same stock and share a common ancestor. This result matches with Asaduzzaman, *et al*. (2020 a, b), it can represent native adaptations and genetically conserved at various ecological habitations and divergence migratories.

The bootstrap of each branch read lower value (0, 0000-0, and 0011). The low bootstrap of the Hilsa specimens in Shatt Al- Arab River suggests that they do not have significant differences, this may due to the conformity of the environmental conditions and the suitable spawning area for them (Asaduzzaman, et al. 2020b). The affinity between the *T. ilisha* group from Shatt Al-Arab and Indian 2 proved that they have a single population. The examination of discernment of population displayed significant *p*-values ($p < 0.05$), showing that amalgamated haplotypes were not distributed randomly, but due to preference for spawning areas (Mazumder & Alam, 2009).

Analysis of molecular variance (AMOVA) showed a very low variation within the population in *T. ilisha* (39.02%) and high

variation among the population (60.97%), indicating that there is significant genetic variation between the two main clades.

The mean genetic distance F-statitics among 10 *T. ilisha* samples (Table 3) ranged from 0.000-0.011; the lowest pairwise genetic distance was observed between Al-Fao and Garmat Ali, while the maximum divergences were observed between India 1 and Qurnah. These data indicating to two different stocks and they are associated with similar geographical distribution and same drainage system. It is also indicating to the least genetic diversity of the Hilsa population in the Shatt Al- Arab River. It is expected that the *T. ilisha* group from Shatt Al-Arab has acclimated to the freshwater environment and established its spawning stock in the Shatt Al-Arab River, similarly to the *T. ilisha* group in another clade in the Indian rivers. Parallel searches indicated the presence of different races of Hilsa shad in Indo pacific regions.

Mohindra *et al*. (2019) employed the *cytochrome b* gene from mtDNA data to contemplate migratory routes among the environmental regions for *T. ilisha* populations, the study was remarked homogeneiticaly and significantly genetic levels of migratory strains. Behera *et al.* (2015) estimated genetic variation of Indian shad, *T. ilisha* from the Bay of Bengal and the Arabian Sea based on partial mtDNA *Cytochrome b* gene , the result guided to two separated populations. Verma *et al*. (2016) used mitochondrial genomic for study population structure, they noticed a wide structure of Hilsa population between the Bay of Bengal and the Arabian Sea.

Conclusions

The current work would help to assess sites divergence (among or within) and the extent of similarity at genomic levels between the Hilsa shad populations residing in diversified habitats based on the *cytochrome b* region of mtDNA which displayed useful insight into genetic diversity for fish genetic diversity and adaptability experiments.

Monitoring the genetic variety is critical for developing self-controlled reproduction, stock enhancement, and management strategies. Besides the phylogenetic results, the morphometric, genetic, physical, and ecological criteria may answer the question of Hilsa shad survival population variation.

Practically, the knowledge of genetic diversity of fish species will support taxonomic programs and regulate species individualism. Variation at an individual can provide us with concept about the different genetic modules, genetic diversity within or between them, and the adaptation in the locality habitants.

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Conflict of interest

The authors declared that they have no conflict of interest.

Ethical approval

All applicable national and international guidelines for the care and use of animals were followed.

Conflict of interests

The authors declare no conflict of interest.

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الدلالات الوراثية للصبور *Tenualosa ilisha* المهاجرة في شط العرب باستخدام mtDNA cyt B

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المستخلص: الصبور تعتبر من الاسماك البحرية واسعة الهجرة والتي تتوزع في شكل مجاميع ,محليا تعرف يأسم سمكة (الصبور) وتهاجرهذه ألاسماك الى المياه العذبة لنهر شط العرب لغرض التكاثر. ومن أجل تحديد التنوع الوراثي تم فحص 70 عينة من اسماك الصبور جمعت من أربع محطات على شط العرب أعتمادا على جين mtDNA cytochrome−b كدلالة جينية معتمدة يمكن أن تكشف التنوع الجيني وتحدد أصول هذه الماميع من سمكة الصبور عن طريق مقارنتها ب 6 عينات منتخبة من دول مختلفة تقع ضمن منطقة المحيطين الهندي والهادي. أظهرت نتيجة تحليل AMOVA أن التباين بين المجموعات هو 60.97% وبين السكان هو 39.02% وقيمة P<0,05 تدل على وجود فروقات معنوية قليلة بين العينات. علاوة على ذلك. أظهرت شجرة النشوء والتطور أحتمالية وجود مجموعتين رئيسيتين يمثلان مخزونين منفصلين, مع مناطق تكاثر منفصلة ومشتركين بسلف مشترك (أصل مشترك).

الكلمات المفتاحية: الهجرة من البحر الى النهر, الصبور، شط العرب، أختبار الاموفا، شجرة النسب.