Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 04/04/2022;
Date Revised: 18/10/2022;
Date Published Online: 31/03/2023;

Authors' Affiliation:
1. Faculty of Marine Sciences, Lasbella University of Agriculture, Water, and marine Sciences, Uthal Baluchistan - Pakistan
2. Faculty of Veterinary and Animal Sciences, Lasbella University of Agriculture, Water, and marine Sciences, Uthal Baluchistan - Pakistan
3. Institute of Biochemistry, Faculty of Life Sciences, University of Baluchistan - Pakistan

***Corresponding Author:**
Khalil Hasni
Email: Khalil_hasni1@yahoo.com

How to Cite:
Rahim S, Hasni K, Baloch AH, Nawaz A, Wajid C, Tariq S, Hasni MS, Jan M (2023). Variation Analysis of *Acanthopagrus latus* found in the costal belt of Lasbela by using Mitochondrial DNA, D- Loop region. Adv. Life Sci. 10(1): 42-47.

Keywords:
Acanthopagrus latus; Mitochondrial DNA; Fish; Polymerase Chain Reaction

Open Access

Variation Analysis of *Acanthopagrus latus* found in the costal belt of Lasbella by using Mitochondrial DNA, D- Loop region

Shazia Rahim[1], Khalil Hasni*[1], Abdul Hameed Baloch[2], Ahmed Nawaz[2], Chandni Wajid[2], Sonia Tariq[1], Muhammad Sharif Hasni[3], Muhammad Jan[2]

Abstract

Background: Fish is a major source of food and plays major role in the blue economy of Baluchistan, Pakistan. The Yellow fin Sea Bream (*A. latus*) is found in coastal belts and locally known as "Dhandya". Variation analysis between fish species were studied, using mitochondrial DNA. To understand genetic diversity of fish, mitochondrial DNA D-loop provides a good source of information for the management as well as the selective breeding of the sea fauna.

Methods: The current study was focus on variation analysis, phenotypic and genetic variation in *A. latus* fish found in different region of coastal belt of Baluchistan. A total of forty-five fish *A. latus* from three different areas of Baluchistan (Gaddani, Dam, and Kund Malir) were collected aseptically. The Phenotypic study was done on the bases of their body shape, body color and color of the fins. Four different kinds of *A. latus* were observed and named as type A, B, C and D. The genetic characteristics were studied by observing the mitochondrial DNA D-loop region.

Results: For Mitochondrial DNA analysis, the blood samples were collected from the selected samples and processed for DNA extraction. Primer was designed and PCR was performed. PCR products were sequenced and analyzed for observing genetic variation in mitochondrial DNA of *A. latus.*

Conclusion: The analysis revealed three genetic variations; two heterozygous variations at 32 and 172 nucleotide positions (Adenine substituted by Thymine and Thymine substituted by Guanine) and one homozygous variation at 72 nucleotide position (an insertion of a Thymine).

Introduction

Acanthopagrus latus (A. latus), the Yellow fin Sea Bream locally known as "Dhandya" is found in coastal belts of Baluchistan and Sindh, Pakistan. *A. latus* is very tasty and liked by human population Pakistan [1]. It is an important sea food specie and contributes to the food industry of the world including Indo-Pacific regions. On daily basis, million tons of sea fauna is overfished in the coastal belts of Baluchistan and Sindh. Because of huge involvement of commercial trawlers and local fishermen, the local species including yellow sea bream has declined in the coastal regions.

From anatomical and morphological point of view, *A. latus* has 11 dorsal spines, 8-9 anal spines, 10-11 dorsal soft rays and 24 vertebrae and fine congeners, that differentiate it from other types of fish. *A. latus* belongs to Sparidae family [2]. About 14 species and 8 genera in this family are found in Pakistan.5 species of *A. latus* (*Acanthopagrus*. *arabicus*, *Acanthopagrus sheim*, *Acanthopagrus catenula*, *Diplodus capensis and Rhabdosargus haffara*) were reported in coastal regions of Pakistan. Recently yellow fin Arabian Sea bream (*A. arabicus*) and spotted yellowfin sea bream (*A. sheim*) species were also reported in coastal of Baluchistan. *A. latus* is a yellow sea bream fish which is widely distributed throughout the word. *A. latus* mainly obtains food from different, mollusks and crustacean. In fish products, species replacements are frequent in aquaculture. Intentional mishandling and alteration of fish species to gain profit has caused problems. In such a situation, for the determination of fish species and their likely alteration, mitochondrial DNA sequencing is a useful tool.

The family Sparidae normally identified as sea bream, is effected both by temperature and tropical coastal water [3]. They have been identified to be monoecious i.e. both male and female sex are present in an individual but some have different sex that are known as dioecious (both male and female sex are separated)[4]. Many of the sea bream fishes are excellent as a source of food and are great for economy [5]. *A. latus* is a yellow sea bream fish which is widely distributed throughout the word. It obtains its food from different mollusks, crustacean and helminths (worm). In 2013,541050 tons of family Sparidae were obtained in the Persian gull of the coastal area. Different type of studies were conducted to elucidate dynamic and biological population of the family of Sparidae [6].

This study was conducted to observe several kinds of *A. latus* based on its body type, body color and color of fins. The genetic characteristics such as variation in genes were studied by observing the mitochondrial DNA D-loop region.

Methods

The present study is a cross-sectional descriptive study. Chemicals were bought from Sigma Aldrich. BIO-RAD T100 thermal cycler was used for gene sequencing. Results were analyzed by the help of software "Bioedit".

Sampling

A total of forty-five fish (*Acanthopagrus latus)* from three different areas of Baluchistan (Gaddani, Dam and Kund Malir) were collected aseptically. Blood sample (1mL) was collected from caudal vein by using vacuum tubes with EDTA (ethylenediaminetetraacetic acid) as an anticoagulant. The samples were stored in ice container at -20^{o}C and brought to the laboratory of molecular Genetics, Faculty of Veterinary and Animal Sciences, Lasbella University of Agriculture, Water and Marine Sciences, Uthal, for further analysis.

DNA Extraction

DNA (deoxyribonucleic acid) was extracted from samples by using inorganic method as suggested by previous study [7]. Before the DNA extraction, blood samples were frozen at temperature of -70°C for 20 to 30 minutes and at -20°C for twenty -four hours. The blood samples were thawed before extraction of DNA. Tris EDTA buffer (Tris HCL 10mM, EDTA 2mM) was added in 1ml blood samples and mixed inverting three to four times. All centrifugation in further stages were done at 25°C. All the samples centrifuged for the 12 minutes at 4000 rpm and after that discarded the supernatants. Pellets were produced at the bottom and dissolved smoothly. Once again TE buffer was added. All the supernatant was discarded leaving 0.5ml.Pallets were added in 3000 µL TSE Buffer (Buffer A1) (Tris HCL, EDTA, NaCl) and 200µL of 10% SDS & 20µL proteinase K. Proteinase K was used for protein digestion in the blood. After that , samples were incubated in water bath at 40°C for 24 hours. After 24 hours, the incubation was checked for the total digestion of the pellets. The resultant tubes were places in ice and 500µL NaCl was added . After mixing, tubes were placed again placed on ice for 10-15 minutes, centrifuged at 4000 rpm for 10 minutes to shot down the protein sample and salt. The supernatant was poured in a 15mL falcon tube and isopropanol with the same volume was added and the tubes were slightly overturned until the DNA became observable. The tubes were stored at room temperature for 10 minutes. The tubes were spun to attach the DNA on tube and the supernatant was carefully discarded. 2mL of 70% ethanol was used to wash DNA. DNA was centrifuged at 4000 rpm for 10 minutes and ethanol was discarded. DNA pellet was dried in air at 37°C for 24 hours. The next day, 400µl TE buffer was added to dissolve DNA. To inactivate nucleases, the tubes were placed in a water bath at 70°C for 1 hour. Tubes were left to cool off at room temperature. DNA was shifted into cap of 2.0 ml autoclaved tubes and stored at -20 °C.

Conformation and quantifying of DNA

For the conformation, quality and quantity of DNA, gel electrophoresis was used. 0.6 g of agarose gel powder was weighed and transferred into a beaker having 100ml IX TAE buffer. The solution was heated in oven for two minutes. After 2 minutes, 0.6µL ethidium bromide was added in the solution for DNA staining. The solution was left on gel plate for 30 minutes at room temperature for solidification. When the gel got solidified, it was transferred into electrophoresis tank which was filled with IX TAE buffer. The level of TAE buffer was kept 1cm above the gel. 3µL of tracing agent: 3X Bromophenol blue was added in 2µLDNA and was loaded in the wells carefully. Finally, the gel was observed under UV trans illuminator. The quality and quantity of DNA sample was analyzed and single compact pink florescent bands were observed.

Primer Designing

For the amplification of mtDNA D-loop the specific primers were designed for this study by using the software (Primer3 Input-0.4.0) following the sequence of D-loop region Mt DNA (>AB916800.1) available on NCBI (http://www.ncbi. nlm.nih.gov. Sequence and product size of the specific primers are given in (Table 1). For successful result of the PCR, both primers were amplified(64°C to 54°C) and were used in thermo cycler. The temperature at which primer showed best results was selected. The subsequent PCR were carried out at optimized annealing temperatures. Polymerase chain reactions were carried out in a 25 µL reaction mixture containing, PCR Buffer(5x) 2.5 µL, Taq Polymerase 0.5 µL, dNTPs 0.5 µL, MgCl2 2 µL, Primer Forward 1 µL, Primer Reverse 1 µL, DNA 3 µL, Distal Water 14.2 µL. The amplification conditions for primers of the mitochondrial DNA D-loop were as follows: denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 sec; annealing at 57°C for 30 sec; and extension at 72°C for 45 sec; with a final extension at 72°C for 10 min, on a Thermocycler. The PCR product was subjected to electrophoresis for detection amplified DNA.

Gel Electrophoresis and UV Trans-illumination

The Agarose gel electrophoresis method was used for the visualization of DNA and PCR product. This method was performed for the confirmation of the presence of PCR product. For the making 1.8% gel, 1.2gm agarose gel was weighed on weighing balance and mixed with 60ml of 1X TAE buffer into a sterilized beaker and transferred into microwave oven for two minutes at 100ºC for fully dissolving the agarose gel. Then taken out the beaker from oven and 0.6µL of Ethidium bromide was added in the beaker for the staining of DNA in the agarose gel. To make wells, combs were carefully fixed in the gel casting tray and then mixture was poured carefully and gently into it. For the solidification of gel casting tray was kept in a equalized place. Then gel was taken from the gel casting tray and kept in gel electrophoresis. The samples and ladder was loaded in corresponding wells. Then power supply was provided to the gel for electrophoresis. 200 Amperes 100 Volts were used to run the sample for 30 minutes. At the end, for the observation of DNA bands, gel was visualized in UV illuminator. The DNA bands of the samples were differentiated with base pair standard marker for the identification.

Sequencing of PCR Products

For the sequencing of the products, after preparation products the samples were sent to CAMB (Center of Applied and Molecular Biology) University of Punjab Lahore for Sequencing of the samples.

Analysis of Results

DNA sequencing was analyzed by the help of software manually using Bioedit software version [8]. Blast2 software was used for the sequences against the normal sequences.

Primers	primers Sequence (5_3)	Length Annealing	Product
Left	TTCACCATACAAGCAAGGAC	53.9	285
Right	TTTGAGGAACCAGGAATGCT	53.4	
Left	GAACCAGGAATGCTTCACCA	55.4	
Right	CCTGGATAGAATGTGTCCTTG	55.4	

Table 1: List of the primers were used in this study

Results

Acanthopagrus latus (Yellowfin Sea bream) locally called Dhandya sampled from Lasbella costal belt was studied for its phenotypic and genetical variation.

Phenotypic Characterization

The fishes observed in current study were classified phenotypically on the bases of their body formation, body color and color of the fins (Fig. 4). These types were named A, B, C and D. Type A was observed with a flat dark body and dark gray fins (Fig. 4). Type B had a slightly long (predator) white and yellow body. The pectoral fin was slightly yellow and light gray, whereas other fins were light gray (Fig. 4). Type C had a flat and predator body. The color of body was white, yellow and red. The dorsal fins were light gray, pectoral fins were yellow and light gray, pelvic and anal fins were yellow whereas the caudal fin was gray (Fig. 4). Type D had a flat body. The color of body was light gray, dorsal, and pectoral fins were light gray, the pelvic and anal fins were yellow, whereas the caudal fin was gray and slightly yellow at the dorsal end (Fig. 4, 5).

Figure 1 : A photograph of *Acanthopagrus latus*

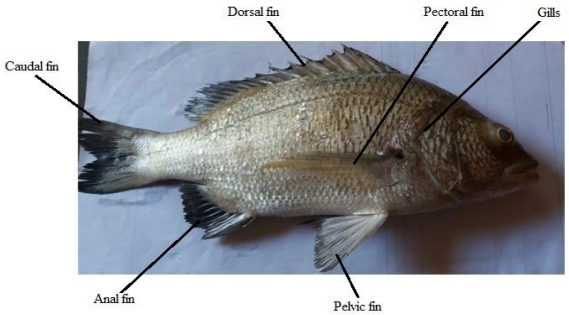

Figure 2: A Labeled photograph of an *Acanthopagrus latus* showing its different parts observed for the classification.

Figure 3: A photograph of type A (A flat dark body)

Figure 4: A photograph of Type B (Long predator type yellow and white body)

Figure 5: A photograph of Type C (flat and predator type of white, yellow and red body)

Figure 6: A photograph of Type D(A flat light gray body)

Genetic Characterization

Genetic characterization reveals different types of fishes and their variations. For Genetic Characterization, 2-3 ml of venous blood samples were collected from caudal vein and processed for DNA extraction. Genomic DNA was extracted from blood samples and run on gel electrophoresis for confirmation and quantification. DNA samples were amplified by Polymerase Chain Reaction (PCR) using specific primers for D-Loop region mitochondrial DNA of *A. latus* (Figure 7 and 8).

Figure 7: Gel electrophoresis photograph showing bands of Genomic DNA

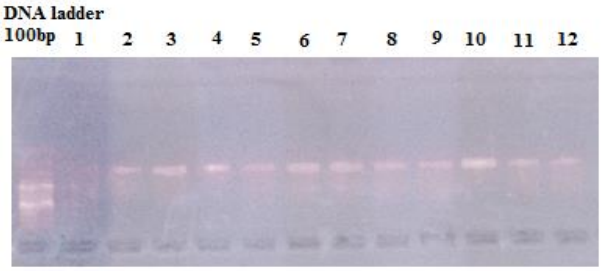

Figure 8: Gel photograph showing amplified DNA products

Amplified DNA samples were sequenced commercially and analyzed for variation analysis. The sequencing results revealed 3 variations (Figure 9).

TTTTGAGGAACCAGGAATGCTTCACCATACAAGCAAG
GACATATACATATATTTATAAACAATATATTATTAGG
TATATAGGACTACAATTGACTATGTACATACATCTAT
ATGTGTACATATACTAATGTATATAACACATATATTT
GTAATAACAGCATTCATCTATATTCACCATACAAGCA
AGGACATTCTATCCAGGTATTTACCAGCAAAAGTGAT
TTCAAGCTACCTCAGAATGTTTAAGGACAAATACTCT
AGGACCTAGTAAACTTTTGAATTCAATAAATATACAC
CAAGTATCTAGCATTCCTGGTTCCTCAAAA

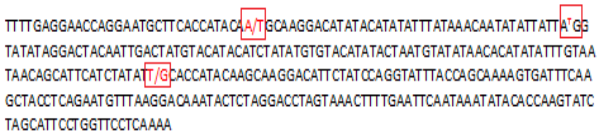

Figure 9: FASTA sequence (GenBank: AB916800.1) showing the regions with variation identified.

A heterozygous variation was observed at 32 nucleotide position where an adenine (A) substituted by a thymine (T) (Figure 10).

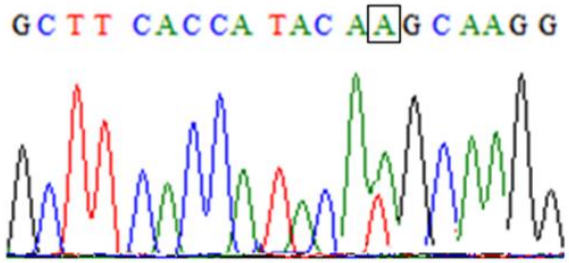

Figure 10: Heterozygous variation showing substitution of an A into T.

A variation was observed with an insertion of a thymine (T) after 72ndnucleotide (Figure 11).

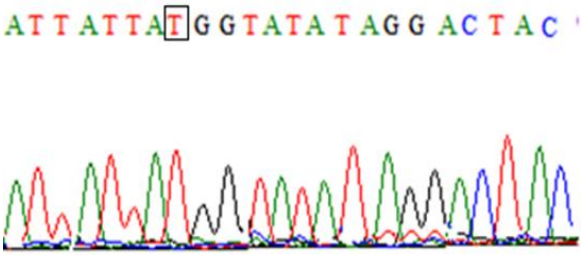

Figure 11: Sequencing result showing an insertion of T.

A heterozygous variation was observed at 172nd nucleotide position where a thymine (T) substituted by a Guanine (G) (Figure 12).

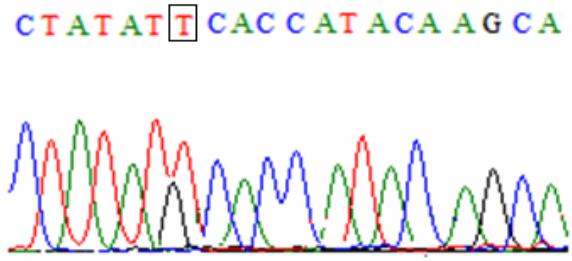

Figure 12: Heterozygous variation showing substitution of a T by a G.

Discussion

Acanthopagrus latus (Yellowfin sea bream) is one of the most important and common specie found in Indian and pacific warm coastal belts [8,9]. It is found in coastal belts of Baluchistan and Sindh, Pakistan with diverse phenotypic characteristics. *Acanthopagrus latus* which is locally called as Dandhia is available throughout the year in the fish markets of Balochistan and Sindh with reasonable prices and widelyused as a sea food by the local population. It is a commercially significant specie and a high protein source [3,10]. *A.latus* is also used and processed in the feed mills as feed for the poultry industry. It is a high concern that overfishing in the gulfs of Persian and Omanhas resulted insevere decline in the population of *A.latus* [8]. During the current study,it was observed that *A.latus*is being exploited in the costal belts of Baluchistan which may cause a decline in the population of the *A.latus* in the region. This concern has been raised by International Union for Conservation of Nature (IUCN) in Pakistan. Food and Agriculture Organization (FAO) of America suggests that *A.latus* and related species that belong to family Sparidae are threatened or near-threatened to be extinctdue to overfishing [11]. Current study was performed on *A. latus* found in the costal belt of Balochistan. The prime objective of the study was to analyze the morphological (phenotypic) and genetic characteristics using the mitochondrial DNA D-loop region.A total of forty-five fish *A. latus* from three different areas of Baluchistan (Gaddani, Dam, and Kundmalir) were collected aseptically.

The phenotypic characteristics revealed four types of *Acanthopagrus*named as type A with flat dark body and gray fins, type B with slightly long body with yellow and white color, the color of fins was observed light gray except pectoral fins which were yellow and light gray. Type C was flat and had a predator type of body. The color of the body was white, yellow and red. The dorsal fins were light gray, pectoral fin was yellow and light gray, pelvic and anal fins were yellow and caudal fin was gray. Type D had a flat body with light gray color. Dorsal and pectoral fins were light gray, pelvic and anal fins were yellow and the caudal fin was gray with slightly yellow color at the dorsal end.Having such a diverse variety of the *Acanthopagrus*is not surprising asits diverse morphological characteristics have been previously classified in various species including *A.arabicus,* a novel specie found in Middle East, costal belts of Iran and Pakistan [12]. Study carried out by Orrel et al reported species with variable color formation ranging from red or pinkto yellowor gray. It was also found with golden or silver reflection with dark spots, stripes or bars [10,12]. It has been also repotrted diverse color formation including white, yellow to strong vivid, black streaks at the base of anal fin. In their study they also reported high diversity among individuals and moderate diversity among population [2].

The genetic characterization was done by observing the mitochondrial DNA D-loop region in the current study. Mitochondrial DNA D-loop provides a good source of information for the management as well as the selective breeding or diversity of the sea fauna.

Displacement region (D-loop) of mitochondrial DNA is a noncoding, hypervariable region controlling the replication and transcription of mitochondrial DNA [13]. It has widely been used in various species from human to fish for genetics characterization and variation analysis among the species. Mitochondrial DNA D-loop region is divided into three domains including left, center and right domains with high variable regions in the left and right domains. Few scientists also have reported 64 variable sites at mitochondrial DNA, D-loop region of *A.latus* including 60 variations at 5' end, 3 at 3' end and 1 variable at $tRNA^{Phe}$ gene [14]. The current study revealed three variations;two heterozygous variations at 32 and 172 nucleotide positions (Adenine substituted by Thymine and Thymine substituted by Guanine) and one homozygous variation at 72 nucleotide position (an insertion of a Thymine).

The phenotypic characteristics revealed four different types of the *A. latus* depending on the color of the body and fins. They wereclassified as type A, B, C and D.All types show variation in the color of the body and color of dorsal, pectoral, pelvic, caudal, and anal fins. The genetic sequencing of the study revealed three genetic variations at mt DNA D-loop region. The heterozygous variations at 32 and 172 nucleotide positions were observed as Adenine was substituted by Thymine and Thymine was substituted by Guanine. Whereas one homozygous variation at nucleotide position :72 was observed as an insertion of Thymine.

Competing Interest

The authors declare that there is no conflict of interest.

Author Contributions

Khalil Hasni and Abdul Hameed Baloch designed the experiments and performed experiments.Shazia Rahim and Muhammad Jan did sampling, performed the experiments, and wrote the initial draft of manuscript. Ahmed Nawaz performed experiments,Chandni Wajid did sampling and did phenotypic studies, Sonia Tariq did sampling, analyzed the data and wrote the manuscript, Muhammad Sharif Hasniidentified species, performed analysis. All authors commented and edited the manuscript.

References

1. Abou-Seedo F, Dadzie S, Al-Kanaan K. Sexuality, sex change and maturation patterns in the yellowfin seabream, Acanthopagrus latus (Teleostei: Sparidae)(Houttuyn, 1782). Journal of Applied Ichthyology, (2003); 19(2): 65-73.
2. Iwatsuki Y, Carpenter KE. Acanthopagrus randalli (Perciformes: Sparidae), a new black seabream from the Persian Gulf. Zootaxa, (2009); 2267(1): 43–54-43–54.
3. Iwatsuki Y, Carpenter KE. Acanthopagrus taiwanensis, a new sparid fish (Perciformes), with comparisons to Acanthopagrus berda (Forsskål, 1775) and other nominal species of Acanthopagrus. Zootaxa, (2006); 1202(1): 1–19-11–19.
4. Haley RW, Culver DH, White JW, Morgan WM, Emori TG, et al. The efficacy of infection surveillance and control programs in preventing nosocomial infections in US hospitals. American journal of epidemiology, (1985); 121(2): 182-205.
5. Vahabnezhad A, Taghavimotlagh S. Growth pattern and reproductive biology of Acanthopagrus latus from the Persian Gulf. Journal of Survey in Fisheries Sciences, (2023); 18-28.
6. Hoseini H, Savari A. Some aspects of reproductive biology of *A. latus* in Boushehr water (Persian Gulf). Journal of Iranian Fisheries Science, (2004); 3(1): 45-52.
7. Sambrook J, Russel D. Extraction and purification of plasmid; screening of bacterial colonies by hybridization. Molecular Cloning: a laboratory manual, 3rd Ed Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, (2001).
8. Zhu K-c, Zhang N, Liu B-S, Guo L, Guo H-Y, et al. A chromosome-level genome assembly of the yellowfin seabream (Acanthopagrus latus; Hottuyn, 1782) provides insights into its osmoregulation and sex reversal. Genomics, (2021); 113(4): 1617-1627.
9. Giusti A, Tinacci L, Sotelo CG, Acutis PL, Ielasi N, et al. Authentication of ready-to-eat anchovy products sold on the Italian market by BLAST analysis of a highly informative cytochrome b gene fragment. Food control, (2019); 9750-57.
10. Chuen-Tan Jean C-FH, Lee S-C, Chen C-T. Variation in mitochondrial DNA and phylogenetic relationships of fishes of the subfamily Sparinae (Perciformes: Sparidae) in the coastal waters of Taiwan. Zoological Studies, (1995); 34(4): 270-280.
11. Sharma H, Singh A, Sharma C, Jain SK, Singh N. Mutations in the mitochondrial DNA D-loop region are frequent in cervical cancer. Cancer cell international, (2005); 51-6.
12. Saccone C, Attimonelli M, Sbisa E. Structural elements highly preserved during the evolution of the D-loop-containing region in vertebrate mitochondrial DNA. Journal of molecular evolution, (1987); 26205-211.
13. Ghasemi A, Shadi A. Population Structure of Acanthopagrus latus from the Northern Persian Gulf and Gulf of Oman Based on Microsatellite Markers. Turkish Journal of Fisheries and Aquatic Sciences, (2018); 18(8): 983-990.
14. Jean F Ökonomie der Bürgerkriege. Chapter: Book Name. 1999 of publication; Hamburger Ed.

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License. To read the copy of this license please visit: https://creativecommons.org/licenses/by-nc/4.0/