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Variation Analysis of *Acanthopagrus Latus* found in the Coastal Belt of Lasbela by using Mitochondrial DNA, D- Loop Region

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Abstract

Background: Fish is a major source of food and plays a 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 was 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 focused on variation analysis, phenotypic and genetic variation in *A. latus* fish found in different regions 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 basis 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. Primers were designed and PCR was performed. PCR products were sequenced and analyzed for observing genetic variation in the 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).

Keywords:

Acanthopagrus latus;
Mitochondrial DNA; Fish;
Polymerase Chain Reaction

Editorial Note:

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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 the human population of Pakistan [1]. It is an important seafood species and contributes to the food industry of the world including Indo-Pacific regions. On daily basis, million tons of sea fauna are overfished in the coastal belts of Baluchistan and Sindh. Because of huge involvement of commercial trawlers and local fishermen, the local species including yellowfin seabream 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 fin congeners, which 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. Five 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 world. *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 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 (male and female sexes are separate) [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 world. It obtains its food from different mollusks, crustacean and helminths (worm). In 2013, 541,050 tons of family Sparidae were obtained in the Persian Gulf of the coastal area. Different types 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 °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 performed at 25 °C. All the samples centrifuged for 12 minutes at 4,000 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. Pellets 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 placed on 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 precipitate 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. 2 mL of 70% ethanol was used to wash DNA. DNA was centrifuged at 4,000 rpm for 10 minutes and ethanol was discarded. DNA pellet was dried in air at 37°C for 24 hours. The next day, 400 µL 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 1x 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 1X TAE buffer. The level of TAE buffer was kept 1cm above the gel. 3 µL of tracing agent: 3X Bromophenol blue was added in 2µL DNA 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, fluorescent 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 thermocycler. 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, Distilled 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 100°C 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 an equalized place. Then gel was taken from the gel casting tray and kept in gel electrophoresis. The samples and ladder were loaded in corresponding wells. Then power supply was provided to the gel for electrophoresis. 100 V, 200 mA 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	CCTGGATAGAATGTCTCTTG	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 genetic variation.

Phenotypic Characterization

The fishes observed in current study were classified phenotypically on the basis 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 elongated yellow and white 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 with slight yellow at the dorsal end (Fig. 4, 5).



Figure 1 : A photograph of *Acanthopagrus latus*

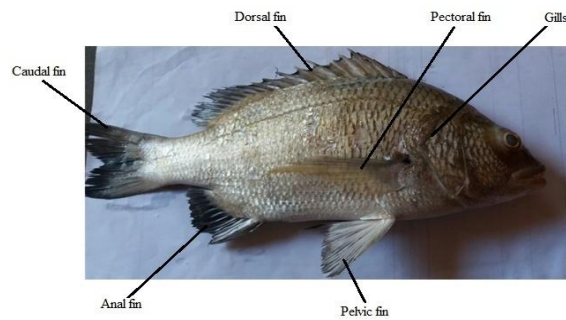


Figure 2: A Labeled photograph of an *Acanthopagrus latus* showing its different parts observed for the classification.



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

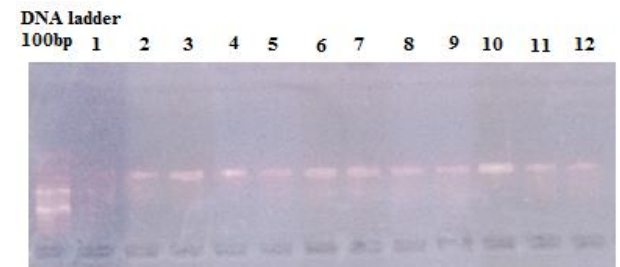


Figure 8: Gel photograph showing amplified DNA products

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

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TTTTGAGGAACCAGGAATGCTTCACCATACAAGCAAG
GACATATACATATATTTATAACAATATATTATTAGG
TATATAGGACTACAATTGACTATGTACATACATCTAT
ATGTGTACATATACTAATGTATATAACACATATATTT
GTAATAACAGCATTTCATCTATATTCACCATACAAGCA
AGGACATTCTATCCAGGTATTTACCAGCAAAAGTGAT
TTCAAGCTACCTCAGAATGTTTAAGGACAAATACTCT
AGGACCTAGTAAACTTTGAATTCAATAAATATACAC
CAAGTATCTAGCATTCCCTGGTTCCTCAAAA
  
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TTTGGAGGAACCGGAATGCTTCAACATACAAAGGACATATACATATATTTATAACAATATATTATGG
 TATATAGGACTACAATTGACTATGTACATACATCTATATGTGTACATATACTAATGTATATAACACATATATTTGTAA
 TAACAGCATTCTATATTTGACCATACAAGCAAGGACATTCTATCCAGGTATTACCAAGCAAAAGTATTCAA
 GCTACCTCAGAATGTTAAGGACAAATCTCTAGGACCTAGTAACTTTTGAATTCATAAATATACCAAGTATC
 TAGCATTCTGGTCTCTCAAAA

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

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

G C T T C A C C A T A C A A G C A A G G

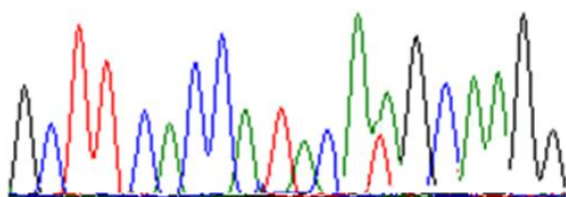


Figure 10: Heterozygous variation showing substitution of an A into T.

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

A T T A T T A T G G T A T A T A G G A C T A C

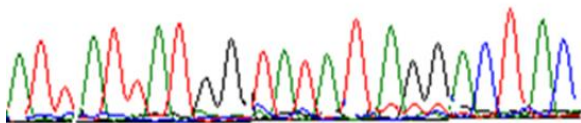


Figure 11: Sequencing result showing an insertion of T.

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

C T A T A T T C A C C A T A C A A G C A

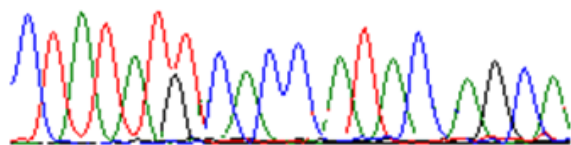


Figure 12: Heterozygous variation showing substitution of a T by a G.

Discussion

Acanthopagrus latus (Yellowfin sea bream) is one of the most important and common species 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 widely used 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 Oman has resulted in severe decline in the population of *A.latus* [8]. During the current study, it was observed that *A. latus* is being exploited in the coastal 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 extinct due to overfishing [11]. Current study was performed on *A. latus* found in the coastal belt of Baluchistan. 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 as its diverse morphological characteristics have been previously classified in various species including *A.arabicus*, a novel species found in Middle East, coastal belts of Iran and Pakistan [12]. Study carried out by Orrel et al reported species with variable color formation ranging from red or pink to yellow or gray. It was also found with golden or silver reflection with dark spots, stripes or bars [10,12]. It has been also reported 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 populations [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 were reclassified 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 Hasni identified species, performed analysis. All authors commented and edited the manuscript.

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