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DNA barcoding identification of Perciform fishes of the Arabian Gulf commercially harvested in Qatar

Md Samsul Alam[1]*; Aisha Dasmal Alkuwari[2]; Noura Alhashimi[2]; Ameena Almalki[2]

1: Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
2: Genetic Engineering Section, Agricultural Research Department, Ministry of Municipality and Environment, PO Box. 200022, Doha, Qatar
*Corresponding Author: samsul.alam@bau.edu.bd

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ABSTRACT

Species identification through DNA Barcoding method has frequently been used in taxonomic studies and has proved its effectiveness. The present study was conducted to identify five commercially harvested fish species from the Arabian Gulf in Qatar. Approximately, 650 bp fragment of the mitochondrial COI gene; five specimens of each species, was sequenced and amplified using universal LCO1490/HCO2198 primer set. A remarkable high level of similarities, ranging from 99 to 100% , with the sequence of known specimens of the five species available in the NCBI database and the BOLD system, was observed. The identifications have been supported by the phylogeny tree where the samples of the same species formed an individual clade. Therefore, the DNA barcoding technique could be used as an effective tool in the identification of adult, larvae or even eggs of *Lethrinus lentjan*, *Lethrinus nebulosus*, *Epenephelus coioides*, *Argyrops spinifer*, and *Acanthopagrus bifasciatus*. The present study detected a low intraspecific divergence (average 0.46%, range 0.2 – 1.4%) with a relatively high genetic diversity in *L. nebulosus*. Consequently, the DNA barcoding sequences submitted to the database would help to identify larvae and processed products of the five Perciform fishes from Qatar waters and throughout the Gulf region.

INTRODUCTION

Fishes are the largest group of vertebrates with a current global estimation of 33, 932 species **(Froese & Pauly, 2020).** This assessment is obviously increasing due to the discovery of new species. To study the patterns and underlying causes of biodiversity, it is essential to know what species are available in an aquatic environment. A diverse group identification of fish has always been a difficult task that requires a high level expertise and experience in taxonomy. Notably, fishes undergo metamorphosis in the larval development phases, which, in turn, makes the task of identifying even more

problematic in the early life stages. Alternative to conventional morphological methods, DNA sequence based identification systems are promising, and, evidently, gaining tremendous popularity. Considerably, DNA based identification does not require great taxonomic experience and fishes can be identified at any stage of their life cycle **(Teletchea, 2009; Bingpeng et al., 2018).**

In order to assist the acquisition, storage, analysis and publication of DNA barcode records the Barcode of Life Data System (BOLD), an informatics workbench has been established and has evolved into a resource for the DNA Barcoding community **(Ratnasingham & Hebert, 2007).** As of May 18, 2020, BOLD included 341714 DNA barcode sequences from 21, 854 fin fish species (http://www.boldsystems.org/).

In DNA barcoding, short sequences of standard target genes are used to build sequence profiles of known species that can be compared with sequences of unknown samples, and, subsequently identified. The hypothesis of identifying species by DNA barcode is based on the difference between the intra-specific variation and inter-specific diversity. The higher the difference the more effective is the species identification **(Hebert *et al.*, 2003, 2004)**. A fragment of approximately 650 bp from the 5′ end of the mitochondrial cytochrome oxidase c I (CO1) gene has been found to be an efficient barcode for identification of most of fish and animals with a high discriminatory power **(Ivanova *et al.*, 2007; Radulovici *et al*., 2010; Jinboet al., 2011; Lyra *et al.*, 2017**). The most important implication of DNA barcoding technique is the ability to identify the eggs, embryos, and larvae of a fish species as well as the prey of predators, processed fish, fish product and cryptic species.

Since it was developed by **Hebert *et al.* (2003)** the DNA, barcoding technique has been used for the identification of both marine and freshwater fish species in many countries and regions across the world **(Ardura *et al*., 2010; Zhang, 2011; Mabragana *et al.*, 2011; Abbas *et al.*, 2017; Popa *et al.*, 2017; Bingpeng *et al*., 2018; Wang *et al*., 2018; Kundu *et al*., 2019; Panprominet al., 2019).** The success of identifying fishes with mtDNA CO1 gene barcode ranged from 93 to 100% **(Ivanova *et al.*, 2007; Hubert *et al.*, 2008; Steinke *et al*., 2009)**. In addition to the adult fishes, the DNA barcode technique was successfully applied to identify marine fish larvae in Australia **(Pegg *et al*., 2006; Victor, 2007)** and Antarctic **(Webb *et al*., 2006). Rabaoui *et al*.(2019)** identified 117 fish species from the Saudi Arabian waters using the COI DNA barcoding systems. DNA barcoding has successfully been used not only for the teleost fishes **(Ward *et al.*, 2005)** but also for the Chondrichthyan fishes **(Ward & Holmes, 2007).**

DNA Barcoding technique can be applied to authenticate species in markets in order to monitor commercial landings and assess the fishing targets which would help conserve fish resources of a particular region **(Ardura *et al.*, 2010; Zhang & Hanner, 2011)**. Among the 350 fish species of the Arabian Gulf, 35 species are commercially harvested in Qatar. *Lethrinus nebulosus, Lethrinus lentjan Epinephelus coioides Argyrops spinifer* were among the top 11 found fish in Qatar in 2014 **(Stamatopoulos & Abdallah, 2015).** According to **FAO (2016)** *Lethrnus nebulosus* and *Epinephelus coioides* belong to over exploited and *Argyrops spinifer*, while *Lethrinus lentjan* belong to underexploited category **(FAO, 2016).** The key goal of the current study was to develop a mitochondrial CO1 sequence-based barcodes for the five most common

commercial marine fishes of Qatar ,namely; *Lethrinus lentjan, Lethrinus nebulosus, Epenephelus coioides, Argyrops spinifer,* and *Acanthopagrus bifasciatus*.

MATERIALS AND METHODS

A total of 25 specimens, five specimens for each of the five species such as *Epinephelus coioides, Lethrinus lentjan, Lethrinus nebulosus, Acanthopagrus bifasciatus, Argyrops spinifer* were collected from the fish landing center located in Al Khor, Qatar.

The specimens were identified using taxonomic keys documented by **Carpenter *et al.* (1997)** and tissue samples of five specimens of each species (trunk muscle/fin) were collected and preserved in 95% ethanol. The specimens were preserved in 10% formalin for future reference materials. DNA was extracted from small pieces of muscle tissue using a DNeasy blood and tissue kit of Qiagen (Germany) following manufacturers' instructions. PCR was conducted for the mitochondrial CO1 barcode region using the universal primer set (LCO1490: 5'-ggtcaacaaatcataaagatattgg-3', HC02198: 5'-taaacttcagggtgaccaaaaaatca-3') for animals developed by **Folmer *et al*. (1994)**. The 50 µl reaction contained 5 µl of 10X buffer, 1.5mM MgCl2, 0.2µM each of forward and reverse primer and 1 unit Taq DNA polymerase. The PCR products were purified using ExoSap-ITPCR purification kit (Thermo Fisher Scientific) before being used for sequencing. DNA sequencing reactions were carried out with forward as well as reverse primer using a Veriti Thermal Cycler (Applied Biosystems) according to the standard protocol with a Big Dye Terminator kit (Applied Biosystems) using an ABI 3130 Genetic Analyzer.

The chromatograms of individual sequences of each specimen were manualy checked, the reading errors were edited (if any) by using the software Bioedit 5.0.**9 (Hall, 1999),** and a consensus sequence was acquired for each specimen. The molecular identification of the species was achieved through comparing the consensus sequences with those of respective species available in the GenBankby BLAST **(Altschulet al., 1997).** To identify the specimens, The BOLD (Barcode of Life Data Systems) was used **(Ratnasingham and Hebert, 2007)**. The Barcode data have been submitted to the NCBI GenBank.

To conduct a phylogenetic analysis, the highest matching sequence for each species was downloaded from GenBank. For interspecific comparison, the sequences of all the species were aligned using the ClustalW method in MEGA 7.0 **(Kumar et al., 2016)** and both ends were trimmed to equalize the total lengths which was 636bp. Various parameters such as GC content, number of transitions and tranversions, polymorphic sites and parsimony informative sites were calculated.The within species and between species distances were calculated using the Kimura-2-parameter (K2P) model **(Kimura, 1980)** constructing a neighbor-joining (NJ) **(Saitou & Nei, 1987)** phylogenetic tree. The integrity of the tree was tested by bootstrapping for 1000 repeated sampling.

RESULTS

A total of 25 mitochondrial COI sequences was obtained from five species (five from each species). The consensus COI sequence length varied from 647 bp (*Lethrinus nebulosus*) to 669 bp (*Argyrops spinifer*).The sequences longer than 600 bp were analyzed with an observation of a non stop codons. Thus, insertions or deletions in any of the sequences were determined. The sequences of each species were aligned and the polymorphisms were analyzed using DNAsp software, and a total of 16 haplotypes were identified. The GenBank Accession numbers of the haplotype sequences with their sizes are presented in Table 1. The complete barcode sequences of the species were used to calculate the number of haplotypes and nucleotide diversity within the species. Four haplotypes were identified in each of *Argyrops spinifer*, *Epinephelus coioides* and *Lethrinus nebulosus* and two haplotypes were detected in each of *Acanthopagrus bifasciatus* and *Lethrinus lentjan* (Table 1).

Table 1. Classification of species analyzed for COI sequences, the sizes (bp) of the consensus sequence and their GenBank Accession numbers

Order	Family	Species	Common Name	GenBank Accession Number			
Perciformes	Sparidae	*Argyrops Spinifer*	King solder bream	669 MT325506	669 MT325507	669 MT325508	669 MT325509
Perciformes	Sparidae	*Acanthopagrus Bifasciatus*	Two-bar seabream	660 MT325510	660 MT325512		
Perciformes	Serranidae	*Epinephelus coioides*	Orange-spotted grouper	655 MT328984	655 MT328985	655 MT328986	655 MT328987
Perciformes	Lethrinidae	*Lethrinus Nebulosus*	Spangled emperor	647 MT328988	647 MT328989	647 MT328990	647 MT328991
Perciformes	Lethrinidae	*Lethrinus Lentjan*	Pink ear Emperor	665 MT324685	665 MT324686		

Molecular identification showed 99.37 to 100% identity with the sequences of the GenBank and BOLD system (Table 2). The NJ tree constructed from the haplotypes detected in the present study along with one sequence of each of the five species retrieved from the GenBank is shown in Fig 1. The sequences (haplotypes) of all specimens of the same species clustered together with the sequences retrieved from the GenBank of the same species, indicating the correct morphological identification based on taxonomic keys.

For comparisons in nucleotide compositions, the 16 sequences were aligned by ClustalW in MEGA and both ends were clipped which resulted in a homologous sequence lengths of 636 bp. The nucleotide pair frequency analysis of all the 16 haplotypes showed that 437 of 636 (68.71%) sites were conserved, 199 (31.29%) sites were variable, 197 (30.97%) sites were parsimony informative, and 2 sites were singleton.

Table 2. Molecular identification of five Perciform fish species of the Arabian Gulf collected from Qatar. The consensus COI haplotypes sequences of each species were subjected to identification by BLAST in GenBank and the BOLD system. The identity/similarity percentage and the Accession numbers of the source sequences are shown along with the length of the query sequences.N/A: Not applicable

ID based on morphology	GenBank Accession No.	GenBank/BOLD ID	GenBank Identity (%)	GenBank E Value	BOLD Similarity (%)	Length of COI
Acanthopagrus bifasciatus	MT076867.1	*Acanthopagrus bifasciatus*	100.00	0.0	100.00	660/660
Acanthopagrus bifasciatus	MT076867.1	*Acanthopagrus bifasciatus*	99.85	0.0	99.85	659/660
Argyrops spinifer	KU499786.1	*Argyrops spinifer*	99.70	0.0	N/A	667/669
Argyrops spinifer	KU499786.1	*Argyrops spinifer*	99.85	0.0	N/A	669
Argyrops spinifer	KU499786.1	*Argyrops spinifer*	100	0.0	N/A	669
Argyrops spinifer	KU499786.1	*Argyrops spinifer*	99.85	0.0	N/A	669
Epinephelus coioides	MT076846.1	*Epinephelus coioides*	99.85	0.0	99.85	655/654
Epinephelus coioides	MT076846.1	*Epinephelus coioides*	100	0.0	100.00	655/655
Epinephelus coioides	MT076847.1	*Epinephelus coioides*	99.85	0.0	99.85	655/654
Epinephelus coioides	MT076847.1	*Epinephelus coioides*	99.69	0.0	99.69	655/652
Lethrinus lentjan	KU317872.1	*Lethrinus lentjan*	100	0.0	100.00	665/665
Lethrinus lentjan	KU317872.1	*Lethrinus lentjan*	99.85	0.0	100.00	665/664
Lethrinus nebulosus	MF123938.1	*Lethrinus nebulosus*	99.85	0.0	100.00	647/646
Lethrinus nebulosus	HQ149872.1	*Lethrinus nebulosus*	99.37	0.0	99.37	636
Lethrinus nebulosus	HQ149872.1	*Lethrinus nebulosus*	99.85	0.0	99.84	636
Lethrinus nebulosus	HQ149872.1	*Lethrinus nebulosus*	100	0.0	100.00	636

Comparing the 636 nucleotides, an average of 539 identical pairs (ii) were obsereved of which 205 were found at the first codon position, 211 were at the second codon position and 122 were found at the third codon position. In the 16 COI sequences, a number of transitional pairs (si=59) was found greater than transversional pairs (sv = 38), with a si/sv (R) ratio of 1.56. The overall mean nucleotide frequencies in these sequences were 28.80, 28.50, 23.80 and 18.90%for T, C, A, and G, respectively; the AT content (52.60%) was higher than the GC content (47.40%). Significantly, different usage frequencies were observed among the four bases. At the first codon position, the usage of

T, A, C and G were 16.0%, 26.20%, 26.20%, and 31.40%, respectively. At the second codon position, the content of T, A, C, and G were 41.0%, 15.10%, 28.90% and 15.10%,respectively. At the third codon position, the base usage was 29.00, 30.50, 29.90 and 10.10% for T, C, A, and G, respectively.The highest variability in the 3rd codon position with a standard error of 1.3923 and least variation in the 2nd position with a standard error of only 0.0618 were observed. The variation in the 1st codon position with a standard error of 0.1735 was intermediate between the 3rd and 2nd positions.

The within species K2P distances of the COI sequence ranged from 0.002 to 0.014 with an average distance of 0.0046, whereas the between species distances ranged from 0.163 to 0.254 with an average of 0.2137 (Table 3). The average between species genetic distance was 46.45 times of the average within species genetic distance.

Table 3. Kimura-2-parameter genetic divergence (K2P) values within (in bold faced figures) and between (below diagonal) the species of the five Perciform fish species of the Arabian Gulf. .

	AB	AS	EC	LL	LN
AB	**0.002**				
AS	0.202	**0.002**			
EC	0.207	0.201	**0.003**		
LL	0.234	0.211	0.254	**0.002**	
LN	0.220	0.209	0.230	0.163	**0.014**

AB= *Acanthopagrus bifasciatus*; AS = *Argyrops spinifer*; EC = *Epinephelus coioides*; LL=*Lethrinus lentjan; LN= Lethrinus nebulosus*

DISCUSSION

The use of DNA barcodes to identify marine and freshwater fishes has become a well-accepted concept **(Ward *et al.*, 2005; Ardura *et al.*, 2010; Mabragana *et al*., 2011; Zhang, 2011)**. We have successfully identified five finfish species belonging to four genera and three families of the order Perciformes by using the barcode sequence of the mitochondrial cytochrome c oxidase I (COI) gene without any ambiguity. A number of primer sets have been developed to amplify the barcode sequence of the COI gene and identify all the fishes as fish constitute a very diverse group of vertebrates **(Ward *et al.*, 2005; Ivanova *et al.*, 2007)**. In the present study,LCO1490/HCO2198 primer set developed by **Folmer *et al.* (1994)** was used, and a very clear amplification in all the analyzed five fish species was obtained. **Folmer *et al.* (1994)** developed the universal primer set using mtDNA sequences of all major classes of animals including fish and mammals. According to **Ward *et al.* (2005),** an efficient primer set allows identification of numerous taxa with a relatively few specimens of each.

Ward *et al.* (2005) reported an average GC content of 47.1% in the 655 bp region of the COI gene of 143 species of Osteichthyes. **Saccone *et al.* (1999)** reported an average GC content of 43.2% in the complete mitochondrial genome of nine bony fishes. An overall GC content of 47.6% was attained in 636bp sequence of the five Perciform fishes which corresponds well with the findings of **Ward *et al*. (2005)** for the GC content

of the teleosts and of **Rabaoui *et al*. (2019)** for the GC content of Perciform fish of the Saudi Arabian waters. However, thepresent findings of GC contents of the 1st, 2nd and the 3rd codon position differ from those reported by **Rabaoui *et al*. (2019)** for Perciform fish. Varied rates of nucleotide changes in the three codon positions were observed. The 3rd codon position showed more changes in nucleotide than the 1st codon position, and the 1st codon position showed more changes than the 2nd codon position. The standard errors of the GC percentages of the 2nd, 1st and 3rd bases of the five Perciform fishes were 0.174, 0.0618 and 1.392, respectively, which indicates that most synonymous mutations occur at the 3rd position and least at the 2nd position **(Bingpeng *et al.*, 2018).**

In a review of nucleotide composition of mitochondrial genome of 248 bony fishes **Satoh *et al.* (2016)** reported that, in average, the COI gene contained T, C, A, and G with values: 29.7, 27.2, 24.7 and 18.4%, respectively. The content of T was the highest at the 2nd position (40.5%) and the lowest at the first position (22.0%), C content was the highest at the 3rd position (33.2%) and the lowest at the first position (22.2%), A content was the highest at the third position (30.8%) and the lowest at the 2nd position (18.1%), and the G content was the highest at the first position (30.9%) and the lowest at the third position (9.3%).

Although barcode analysis is basically used to delineate species boundaries, however, the cox1 sequence data have stuffs that can also be used for phylogenetic analysis **(Khan *et al.*, 2011)**. Neighbor-joining analysis of COI sequences displayed solid units of the species having little sequence variation (Fig. 1) testifying the correct taxonomic identification using the COI barcode sequence. In the present study, the NJ tree has formed three clades which have not necessarily corresponded to the three families. For example, *Argyrops spinifer* belonging to the family Sparidae has not formed subclades with *Acanthopagrus bifasciatus* of the same family rather formed a separate subclade; *Acanthopagrus bifasciatus*has formed one subclade with *Epinephelus coioides* of Serranidae family. On the other hand, the two species of the genus *Lethrinus* have formed a single clade as expected (Fig. 1).

The COI barcoding sequences obtained in the present study have unambiguously identified the five perciform fish species which indicate their potential to clearly identify the eggs, larvae, and even processed products of these species, and hence, will act as a reference data for identifying respective species around the world. Since the barcoding sequences of the same species obtained in the present study as well as the sequence retrieved from the gene bank consistently clustered in the same clade, it is clear that across geography barcodes of the same species do not contain a lot of variations; the CO1 sequences as a universal DNA markers for identification of fishes.

The mean intraspecific distance observed in the present study (0.46%) was similar to that reported for marine (0.25–0.39%) **(Ward *et al*., 2005; Steinke *et al*., 2009)** and freshwater species (0.3–0.45%) **(Hubert *et al.*, 2008; Valdez-Moreno *et al.*, 2009**). The ratio between mean inter- and intraspecific divergences was 46.35%, which fall within the range of 10.4- and 66.7-fold, as reported by **Asgharian *et al.* (2011). Popa *et al*. (2017)** observed a maximum genetic distance value of 0.08 for the Acipenseriformes and a maximum value of 0.2 for the Salmoniformes fishes while the value was 0.27 when estimated between the species of the orders Acipenseriformes and Salmoniformes.

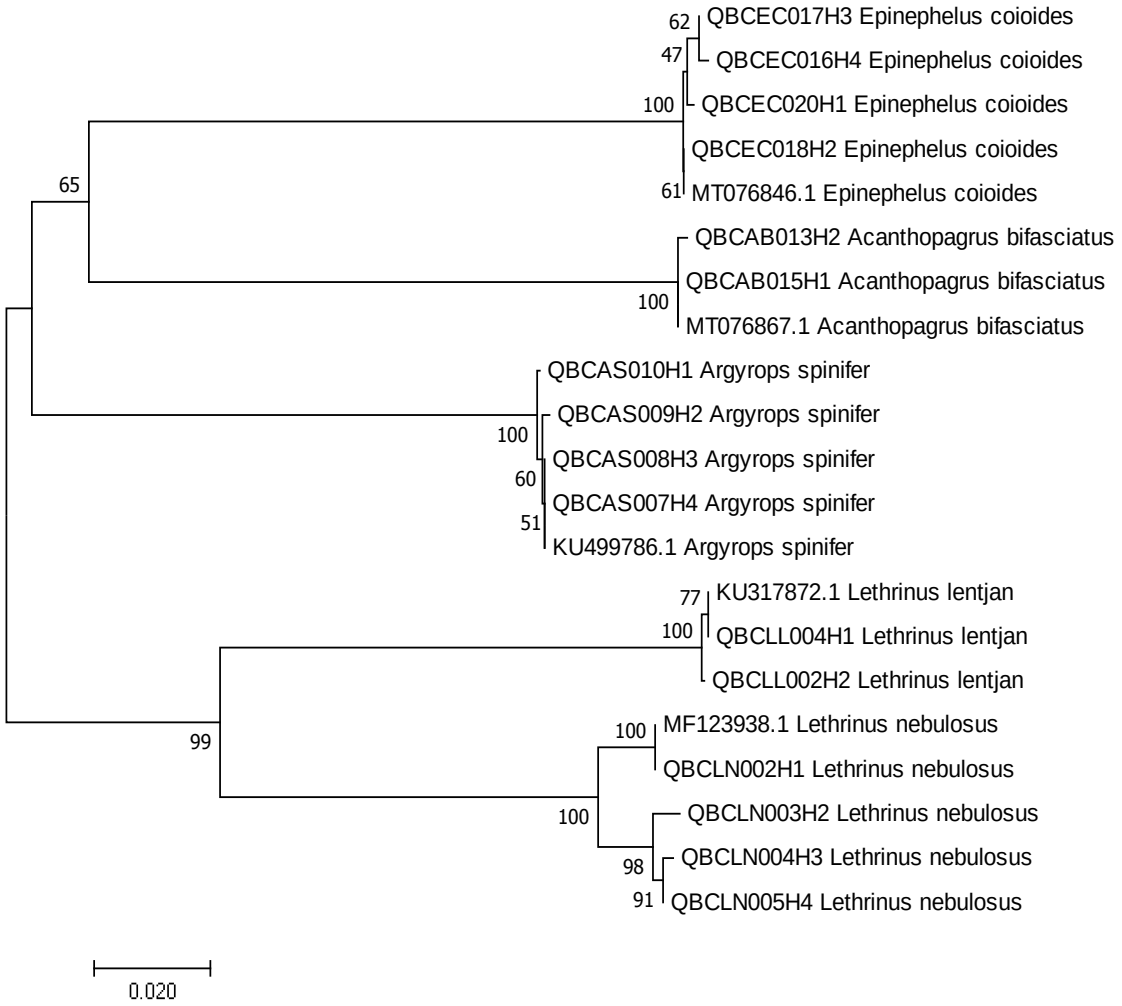

Fig. 1. Neighbor-joining tree based on evolutionary distances computed from the COI sequences using the Kimura 2-parameter **(Kimura, 1980).** The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved a total of 21 nucleotide sequences, 16 from the present study and five collected from the GenBank as references. There were a total of 636 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 **(Kumar et al., 2016).** The experimental sequences correctly matched with the GenBank reference sequences and clustered together.

As expected, a more prominent gap was observed between mean intraspecific and interspecific distances (0.46–21.31%) than that obtained between mean interspecific and intergeneric distances (21.31–21.36%). **Asgharian *et al.* (2011)** also reported that the genetic variation got lesser with increasing taxonomic levels and above the species level, moreover, there would be overlaps between maximum K2P distance at one taxonomic level and minimum distance at adjacent levels. Furthermore, no taxonomic deviation at the species level were detected, indicating that the five perciform species could be validated by the barcode approach.

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