

DNA barcoding of seven cyprinid fish species in the Iraqi Inland waters using mitochondrial *COI* gene sequence

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Abstract

Family Cyprinidae is the largest fish family in the Iraqi inland waters. The cyprinid fish species were described by traditional biometry. Family Cyprinidae fish species in Iraq are important because of ecological and economic aspects. While the morphological similarity among the cyprinid species made the identification not easy. DNA barcoding was chosen to confirm the taxonomy and ensure genetic diversity. Seven cyprinid fish species, *Luciobarbus barbulus*, *L. xanthopterus*, *L. kersin*, *L. esocinus*, *Arabibarbus grypus*, *Cyprinus carpio*, and *Acanthobrama marmaid* were collected from the Shatt Al-Arab River, the Marshes, and the Mosul Dam reservoir. The mitochondrial Cytochrome C Oxidase gene of the specimens was amplified and sequenced. Universal primers were chosen for this purpose. Chromas software was used for processing the sequences. The result showed that the sequence ranged from 600-657 bp. While the neighbor-joining tree created by Clustal Omega software revealed the four *Luciobarbus* species clustering into two central branches, while the other three diverged. Nucleotide distribution statistically for the studied fish species was compared. The results of DNA barcoding using COI gene sequence proved the four independent *Luciobarbus* fish species. The COI gene sequence was successful as a DNA barcode which is accurate in species identification. The sequences were deposited in the gene bank under OM669701, OM669699, OM669702, OM669705, OM669700, OM669703 and OM669704. This study represents the starting line for the DNA barcoding project to detect all fish fauna sequences in the Iraqi inland waters. In addition, it will be very useful in the conservation program of native species in Iraqi inland waters.

Keywords: Cytochrome C Oxidase, Cyprinidae, DNA barcode, Iraq, *Luciobarbus*.

Introduction

Fish represents significant economic value in the lives of the people of Iraq. While freshwater fisheries play a central role in the life of society due to the water bodies that cover the Iraqi land. The Euphrates and Tigris Rivers, lakes, reservoirs, canals, and marshes were suitable habitats for the Iraqi fish fauna. The family Cyprinidae belongs to the order Cypriniformes. It is considered the largest fish family in Iraqi inland waters. It includes the most important genus and species in Iraqi inland waters.

Traditionally, the Iraqi fish species were described using biometry¹. These morphological and meristic characteristics confused the similarity between nearby species, causing overlapping of the morphological and meristic ranges². Therefore, the biochemical composition is utilized as a biomarker to differentiate among similar species. allozyme was the alternative method to differentiate inter-species³. Nevertheless, allozyme cannot discriminate all intra-species⁴. Recently, Polymerase Chain Reaction

(PCR) has been developed and become available in most laboratories. The Cytochrome C Oxidase subunit1 gene was chosen to be the barcode for species⁵.

DNA barcoding is a fast, accurate taxonomy method⁶. It's a DNA sequence that uniquely identifies each species of living organism by comparing them with known barcodes in Blast databases of NCBI⁷. DNA barcoding in fish studies is used to identify and differentiate fish species in any life stage, particularly fish larvae, because of the complexity of morphological taxonomy⁸ and monitoring the fish diversity in the water bodies⁹. Mitochondrial Cytochrome C Oxidase was the most preferred gene for that purpose in fish¹⁰. The sequence of this gene is called DNA barcodes.

Materials and Methods

Thirty-eight specimens of *Luciobarbus barbulus*, *L. xanthopterus*, *L. kersin*, *L. esocinus*, *Arabibarbus grypus*, *Cyprinus carpio*, and *Acanthobrama marmid* were collected from the Shatt Al-Arab River, the Marshes, and the Mosul Dam reservoir. They transferred in a cool box filled with ice to the Marine Biotechnology laboratory in the Marine Science Centre- University of Basrah. The specimens were classified using morphological characters according to reference¹³. Tissue clips were cut from the dorsal muscle and preserved in 95% ethanol alcohol under -20° C until PCR experiments.

Genomic DNA extracted by Genomic DNA Mini Kit, [(Cat. No. GT100/ Lot. No. TJ35501) Geneaid Biotech. Ltd Kit]. The method of the manufacturer was followed. The genomic DNA was electrophoresed on %0.8 agarose gel with ethidium bromide dye, for 25 minutes at 70 volts, then the product was tested for integration by a UV light illustrator. The mitochondrial gene COI was selected to be amplified. Polymerase Chain Reaction (PCR) was conducted with primers FishF2_t1-5'-TGTA AACGACGGCCAGTCGACTAATCATA AAGATATCGGCAC-3' and FishR2_t1-5'-

Results and Discussion

The results of COI barcoding (cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial) of, *Luciobarbus barbulus*, *L. xanthopterus*, *L. kersin*, *L. esocinus* *Arabibarbus*

Ecologically, Iraqi fish species, particularly the family Cyprinidae, play a central role in the freshwater aquatic environment. The similarity among some taxa that belong to the same genus makes the species identification a complex trail. Besides, molecular studies on the Iraqi Cyprinids are rare. Faddagh *et al.*¹¹ used RAPD markers to differentiate some cyprinid species. Also, ribosomal RNA was used to distinguish some species belonging to the subfamily Cyprininae¹². So, Iraqi cyprinid fish species need genetic barcoding using the mitochondrial COI gene. That would be beneficial in the taxonomy, conservation programs, and propagation activities. The present study aimed to investigate DNA barcodes of seven native cyprinid fish species inhabiting the Iraqi inland waters using COI gene sequence, genetic relationship, and genetic variation and recording them in NCBI.

CAGGAAACAGCTATGACACTTCAGGGTGAC CGAAGAATCAGAA-3' recovered from reference¹⁴.

The thermocycler is programmed as the first stage: initial denaturation 95°C for 5 min., second stage for 30 cycles; denaturation: 95°C for 1 min., annealing: 58°C for 30 Sec., extension: 72°C for 1 min. and third stage final extension 72°C for 6 min. The PCR product was electrophoresed on agarose gel stained with ethidium bromide with a 100 bp DNA ladder. Then the PCR products were tested on a UV light plate. Nanodrope was used to check the product's purity. The amplified COI genes were sequenced. Chromas software was used to process the sequences. The sequence results were checked with the NCBI blast to confirm the fish species' taxonomy. Nucleotide distribution among sequences was assessed using CLC bio software (trial version). The sequences were tested with translation into amino acid sequences to check the stop codon. The neighbor-joining tree was generated using online Clustal omega. *Danio rario* COI of NCBI⁷ was used as an outgroup. The sequences are deposited in the gene bank.

grypus, *Cyprinus carpio*, and *Acanthobrama marmid*. Were ranged from 600 bp. in *A. marmid* to 657 bp in *C. carpio*. The sequences were deposited in the gene bank under OM669701, OM669699,

OM669702, OM669705, OM669700, OM669703, and OM669704. E value and % identity of the seven sequences recovered from Blast as in (Table 1).

Table 1. Molecular identification of seven freshwater fish species belonging to the family Cyprinidae recorded in NCBI blast.

Scientific name	NCBI accession No.	NCBI (E value)	NCBI Identity %	Length of COI seq. (bp)
<i>Luciobarbus barbulus</i>	OM669701	0.0	100.00	655
<i>Luciobarbus xanthopterus</i>	OM669699	0.0	100.00	642
<i>Luciobarbus kersin</i>	OM669702	0.0	100.00	622
<i>Luciobarbus esocinus</i>	OM669705	0.0	100.00	612
<i>Arabibarbus grypus</i>	OM669700	0.0	100.00	644
<i>Cyprinus carpio</i>	OM669703	0.0	100.00	657
<i>Acanthobrama marmid</i>	OM669704	0.0	100.00	600

The sequences of the seven sequences were recorded in the gene bank revealing an E value equal to 0.0 for the whole above sequences and the identity percentage was 100% (Table 1). The statistics of nucleotide content in the seven sequences showed that the frequency of A+T was higher than G+C (Table 2). while the different nucleotides were almost similar in the seven species. The dendrogram of the seven species revealed by the Neighbour-joining tree showed the *Luciobarbus* species clustered into two branches in the middle of the tree, whereas

Acanthobrama marmid clustered with *Danio rerio* despite their being of different genera (Fig. 1). While the two other cyprinid species diverged in different branches. The sequences can be compared to reference sequences in NCBI assisting in species identification and contributing to broader studies on the genetic diversity and conservation of fish species in Iraqi waters. The nucleotide distribution was relatively similar (Table 2) because the species belong to the same family.

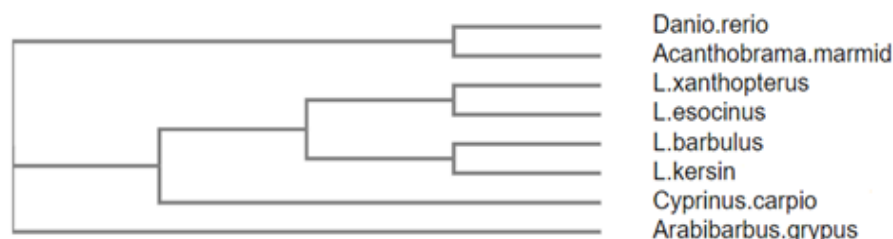


Figure 1. Phylogenetic relationship among the seven fish species revealed by Neighbour-joining tree using Clustal Omega.

Table 2. Nucleotide distribution of COI gene sequence in seven fish species.

Species	L. barbustus		L. xanthopterus		L. kersin		L. esocinus		A. grypus		C. carpio		Acantho. marmid	
Seq. length (bp)	655		642		622		612		644		657		600	
Nucleotide	count	Freq.	count	Freq.	count	Freq.	count	Freq.	count	Freq.	count	Freq.	count	Freq.
Adenine (A)	165	0.267	172	0.270	154	0.270	166	0.271	154	0.272	173	0.268	155	0.258
Cytosine (C)	180	0.291	184	0.288	166	0.291	178	0.291	156	0.275	184	0.285	172	0.287
Guanine (G)	105	0.170	113	0.177	95	0.167	103	0.168	96	0.169	111	0.172	106	0.177
Thymine (T)	169	0.273	169	0.265	155	0.272	165	0.270	161	0.284	178	0.276	167	0.278
C + G	285	0.460	297	0.466	261	0.458	281	0.459	252	0.444	295	0.457	278	0.463
A + T	334	0.540	341	0.534	309	0.542	331	0.541	315	0.556	351	0.543	322	0.537

The COI gene sequence showed that the DNA barcode is a useful tool to identify the family Cyprinidae species, which includes the most fish fauna in Iraqi freshwater¹⁵. Also, the COI barcode proved that the four *Luciobarbus* species were distinctive from each other despite the morphological similarity⁸. This result ensured morphological identification among close species. In addition, the COI barcode would uncover the morphological overlapping of the most similar species, as in the first three *Luciobarbus* species in this study (Fig. 1). Moreover, the COI barcode showed the interspecific genetic diversity of this studied group and phylogenetic relationships among them¹⁶. The resulting data would be beneficial to

species conservation programs and their management¹⁷. Also, by sequence data analysis, the evolutionary history and classification of Cyprinidae fish species will be revealed¹⁸. Geiger *et al.*¹⁹ used a COI gene barcode to detect the freshwater fishes of the most diverse point of the Mediterranean Sea. In Egypt, COI was used to detect the barcodes of freshwater fishes²⁰. Finally, the use of DNA barcoding in Cyprinidae fish species has several practical applications, such as assisting in monitoring and managing fish populations, and facilitating conservation efforts. The present study represents the first step of DNA barcoding the Cyprinid fish species in Iraqi inland waters. These details would be significant as a baseline of fish fauna in Iraqi waters.

Conclusion

The employment of COI gene barcode for DNA barcoding of Cyprinidae fish species offers a valuable tool for species identification, conservation, and management. It enhances our understanding of fish biodiversity, assists in the detection of illegal

activities, and contributes to the overall protection of aquatic ecosystems. Continued research and collaboration are crucial for maximizing the potential of DNA barcoding in understanding the diversity and conservation needs of Cyprinidae fish species.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- The author has signed an animal welfare statement.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University of Basrah.

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تحديد الشفرة الجينية لسبعة أنواع من أسماك الشبوطيات في المياه الداخلية العراقية باستخدام جين COI

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الخلاصة

إن عائلة Cyprinidae هي أكبر عائلة سمكية في المياه الداخلية العراقية. تاريخياً وصفت أنواع الأسماك الشبوطيات بواسطة القياسات المظهرية التقليدية. تعتبر أنواع أسماك عائلة Cyprinidae في العراق مهمة من النواحي البيئية والاقتصادية. في حين أن التشابه المورفولوجي بين أنواع أسماك الشبوطيات جعل تحديد الأنواع ليس أمراً سهلاً. اختيرت شفرة الحامض النووي لتأكيد التصنيف وإيضاح التنوع الجيني بين الأنواع المتشابهة العائدة للجنس نفسه. جمعت سبعة أنواع من أسماك الشبوطيات *L. Luciobarbus barbatus*، *L. Acanthobrama marmid* و *Cyprinus carpio*، *Arabibarbus grypus*، *L. esocinus*، *L. kersin* و *xanthopterus* من نهر شط العرب والأهوار وخزان سد الموصل. تم تضخيم جين الميتوكوندريا Cytochrome C Oxidase للجين المستهدف وتحليل النتائج النيوكليوتيدي. اختيرت بادئات مخصصة لهذا الغرض. أستخدم برنامج Chromas لمعالجة نتائج التسلسل النيوكليوتيدي. وأظهرت النتيجة أن التسلسل النيوكليوتيدي تراوح بين 657-600 زوج قاعدي. في حين كشفت الشجرة الوراثية neighbor joining tree المتحصلة من موقع Clustal Omega عن أنواع *Luciobarbus* الأربعة التي تتجمع في فرعين مركزيين، بينما تباعدت الأنواع الثلاثة الأخرى. وتمت مقارنة توزيع النيوكليوتيدات إحصائياً لأنواع الأسماك المدروسة باستخدام برنامج CLC bio. أثبتت نتائج ترميز الحامض النووي باستخدام تسلسل الجينات COI وجود أربعة أنواع من أسماك *Luciobarbus* المستقلة. أثبتت الدراسة إن استخدام جين COI كشفرة وراثية للحامض النووي ناجحاً ودقيقاً في تحديد الأنواع. أودعت التسلسلات في البنك الجيني تحت رموز OM669701 و OM669699 و OM669702 و OM669705 و OM669703 و OM669700 و OM669704. إن هذه الدراسة هي نقطة الانطلاق لمشروع ترميز الحامض النووي لجميع الشفرات الوراثية للأنواع السمكية في المياه الداخلية العراقية. بالإضافة إلى ذلك، ستكون نتائج الدراسة مفيدة جداً في برنامج الحفاظ على الأنواع المحلية في المياه الداخلية العراقية.

الكلمات المفتاحية: سايتوكروم سي أوكسيداز، الشبوطيات، شفرة دنا، العراق، لوشيو باربص.