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DNA BARCODING OF THE CYPRINIDAE FROM GREATER ZAB RIVER_GWER IN KURDISTAN REGION, IRAQ

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ABSTRACT

Cyprinidae species are most abundant fish species are distributed in many inland waters of Kurdistan Region/Iraq. Cyprinids are complex species and identification based on morphology is difficult. To accomplish the task there is a need to identify the species through DNA barcoding based on a fragment of cytochrome c oxidase subunit I (COI) gene in the mitochondrial genome is widely applied in species identification and biodiversity studies which is the ultimate tool for species identification, DNA barcoding can be helpful to distinguish species, and genetic relationship. The present investigation provides data on the genetic structure of some Cyprinidae species including *Carasobarbus kosswigi, Capoeta trutta, Cyprinion macrostomum, Luciobarbus barbulus, Garra rufa, Acanthobrama marmid, Alburnus mossulensis, Chondrostoma kinzelbachi, Leuciscus vorax, Squalius cephalus, Arabibarbus grypus, Carasobarbus luteus and Capoeta damascina* from Greater Zab River/ Gwer in Kurdistan Regin/ Iraq. *C. kinzelbachi* was recorded for the first time in Iraq. These species were sequenced for 635 base pair region of the COI gene. The genetic differences among them were 0.055-1.481% of 635 nucleotide bases of mitochondrial cytochrome. All species were identified 100% by COI gene sequence and these 13 different fishes were registered in NCBI GenBank. This study is one of the first steps towards enhancing genetic understanding of the relationship among species of Cyprinidae. There should also be further attempts in genetic research to fill data gaps among fish species in Kurdistan Region to help to establishing a global COI barcode database.

Keywords : Barcoding, Cyprinidae, Fish, Zab River_Gwer.

INTRODUCTION

The family Cyprinidae is one the largest family of freshwater fishes, with 376 genera and around 3160 species described (MOHSIN *et al*., 2021). Species of this family are most widespread in the world, its range occurring in freshwater and brackish water; North America (northern Canada to southern Mexico), Africa, and Eurasia (Durand *et al*., 2002). Many species of Cyprinidae are economically important in aquaculture, fisheries, ornamental fish trade and most of them serve as an essential source of animal protein for humans (Tan and Armbruster, 2018).

Cyprinidae is important species of most lakes and rivers in Iraq and Kurdistan Region. The main component of Iraqi fish species belongs to family Cyprinidae. This family contains by far the most species in Iraqi freshwaters, ca. 72% of native fishes 37 species belong to 19 genera (Brian, 2014).

The cyprinids include numerous species that are morphologically similar. Iraqi fish fauna was described by many authors, the 19th century only five works were published by (Coad, 1996; Günther, 1868). Among studies that focused on the classification of freshwater fishes like (Khalaf, 1961; Mahdi, 1962; Al-Hamed, 1966; Mahdi and Georg, 1969; Faddagh *et al*., 2012; Al Rawi *et al*., 1978; Daham, 1977; Banister, 1980; Al-Daham, 1982; Abdullah, 2002; Coad, 1991; Coad, 1996 and Brian, 2014). In 2010, Coad published a guide on freshwater fishes of Iraq (Coad, 1991). Most of these previous checklists were rejected by European authors Banister (1980) reason some of these records were synonyms to others, which means there is still considerable ambiguity in terms identifying species of Cyprinidae, is mostly based on limited data(Banister, 1980). Early studies of Iraqi fish fauna and identification of species has relied on morphological characteristics, such as body shape, number of scales, number of fin rays, number of gill rakers, pharyngeal dentition and color patterns, etc. (Karaman, 1971 and Almaca, 1989).

With the development of new genetic techniques, which use data obtained from molecular component of the cell. DNA barcoding was initiated recently as a tool for fast and accurate identification, taxonomy, phylogeny and population of fishes. The barcode system depends on sequence diversity in a portion of the mitochondrial gene encoding cytochrome c oxidase subunit 1 (CO1) has been used effectively to identify major animal species (Hebert *et al*., 2004; Hajibabaei *et al*., 2005; Ward *et al*., 2005 and Lakra *et al*., 2011) and the sequence diversity have been deposited in the GenBank

Due to the close morphological resemblance between the species of the family Cyprinidae inhabiting the freshwater system of Iraq. The present study aims to present a barcoding data as an additional tool separate those species inhabiting the Greater Zab River/Gwer in Kurdistan region.

MATERIALS AND METHODS

Study Area

Greater Zab River is a large river (392km), located to the east of Tigris in Kurdistan region, north of Iraq. The sampling area during this study was located near Gwer district (36º-37º north latitude, 43º- 44º east longitude) southwest of Erbil city (Fig. 1).

Fig. 1 : Map showing the collection site of specimens of fish

Sample Collection

Eight five specimen of fishes were collected by fisherman using gillnets. Specimens were kept in a cool box and they were initially identified based on morphology using Coad (2010) key(Brian, 2014). Approximately 80-100 mg of white muscle tissue was excised from the left body side of each specimen. The tissue was preserved in 95% ethanol. The cytochrome c oxidase subunit one sequences of 13 species were examined. Among these one species is a new record in Iraq (Table 1).

DNA Extraction

Total genomic DNA was extracted from muscle samples where was preserved in 95% ethanol by using Jena Bioscience blood, animal and plant DNA preparation kit (Jena Bioscience GmbH. 07749 Jena Germany). Approximately, 635 bp were amplified from 5' region of the COI gene using universal fish barcoding primers described by (Ward *et al*., 2005):

FishF1(5'-TCAACCAACCACAAAGACATTGGCAC-3')

FishR(5'-TAGACTTCTGGGTGGCCAAAGAATCA-3')

Polymerase Chain Reaction PCR reactions were administered as 50 µl total volumes containing 25 µl of 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S Stenhuggervej 22); 18 µl free water; 2 µl of every COI primer (FishF1 and FishR1) and 3µl of DNA template by Bioresearch PTC-200Gradient thermo-cycler.

Table 1: Cyprinid Species Analyzed in Present Study

Subfamily	GenBank Species name	Accession no.	Length of CO1 (bp)	Identity (%)
Barbinae	*Capoeta damascina*	MW250386MW250393MW250394	631	100
			632	100
			633	100
	Capoeta trutta	MW251738MW250395	631	100
			632	100
	Cyprinion macrostomum	MW250387	629	100
	Luciobarbus barbulus	MW250381	634	100
Labeoninae	*Garra rufa*	MW250391	633	100
Leuciscinae	*Acanthobrama marmid*	MW250389	610	100
	Alburnus mossulensis	MW250392	628	100
	Chondrostoma kinzelbachi	MW250385	610	100
	Leuciscus vorax	MW250382	615	100
	Squalius cephalus	MW250384	625	100
Torinae	*Arabibarbus grypus*	MW250383	632	100
	Carasobarbus luteus	MW250388	632	100
	Carasobarbus kosswigi	MW250390	633	100

The PCR thermal cycling conditions was: 95ºC for five min; 35 cycles of 95ºC for 40s, 62ºC for 35 s, 72ºC for 1 min; final extension of 72ºC for 10 min; and hold at 4ºC. Amplification products were visualized on 1.2% agarose gel stained with ethidium bromide. The PCR products were run on 1.5% agarose gel stained with ethidium bromide (0.5 µg/mL), in TAE buffer, and were visualized under UV Quantum-Capt ST4 system (Vilber Lourmat, France). The concentrations of purified PCR products were estimated by NanoDrop 2000C UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). Amplicons were sequenced bi-directionally using sequencing primers FishF1 or FishR1 (Ward *et al*., 2005)and therefore the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.).

Sequences were manually checked for correction with ABI Prism Terminator Sequencing Kit (Applied Biosystem) at Macrogene Company of Korea. Chromatograms of COI

were edited and base calls checked using Finch broadcast program software. After sequence alignment, sequence divergences were calculated using the Kimura two-parameter (K2P) distance model (Kimura, 1980). The molecular phylogenetic tree was constructed using Mega5, a distance-based method as Neighbour-joining (NJ) and a cladistics phylogenetic tree as maximum parsimony (MP) criterion were used. The reliability of the inferred phylogenies was evaluated using the bootstrap method with 1000 replicates.

RESULTS

The mitochondrial cytoc total 16 COI sequences were obtained from 13 fish species belonging to 11 genera and four subfamilies (Table 1). *C. kinzelbachi* Krupp, 1985 of the 13 species collected has not been reported in Iraq. Among the 13 species sampled, all species are native.

Table 2 : Summary of nucleotide from 16 COI sequences of 13 Cyprinidae species collected from Greater Zab River / Gwer

	Mean	Minimum	Maximum	Stander error
T%	27.96	26.54	29.54	0.2479
A%	26.01	23.57	27.33	0.2336
G%	17.51	16.59	18.79	0.1821
C%	28.52	26.54	29.91	0.2939
GC%	46.02	43.1	47.9	0.3044

In each COI sequence obtained there were not found stop codons, insertions, and deletions. The COI barcodes obtained ranged from 610 to 634 bp, with an average of 627 bp. The average nucleotide composition was 25.97% A, 27.94% T, 17.53% G and 28.56%. Overall nucleotide content is presented in Table (2). *Alburnus mosseulensis* Heckel,1843 from subfamily Leuciscinae showed the highest overall GC content at 47.9% while *C. kosswigi* (Ladiges, 1960) form subfamily Torinae had the lowest at 43.1%. The mean percentage base composition of each species is presented in Table (3).

Table 3 : Mean percent base composition

Species	n	T	C	A	G	GC
Acanthobrama marmid	1	27.54 ±0	29.02 ±0	25.90 ±0	17.54 ±0	46.6±0
Alburnus mossulensis	1	28.50 ±0	29.14 ±0	23.57 ±0	18.79 ±0	47.9±0
Arabibarbus grypus	1	28.32 ±0	27.85 ±0	26.58 ±0	17.25 ±0	45.1±0
Capoeta damascina	3	26.58±0.02	29.85±0.03	26.58±0.02	16.98±0.04	46.83±0.03
Capoeta trutta	2	27.40 ±0.02	29.30 ±0.02	26.44± 0.02	16.86 ±0.06	46.15±0.05
Carasobarbus kosswigi	1	29.54 ±0	26.54 ±0	27.33 ±0	16.59 ±0	43.1±0
Carasobarbus luteus	1	29.43 ±0	26.74 ±0	26.74 ±0	17.09 ±0	43.8±0
Chondrostoma kinzelbachi	1	28.20 ±0	28.69 ±0	25.74 ±0	17.38 ±0	46.1±0
Cyprinion macrostomum	1	28.14 ±0	28.62 ±0	24.96 ±0	18.28 ±0	46.9±0
Garra rufa	1	28.75 ±0	26.54 ±0	26.07 ±0	18.64 ±0	45.2±0
Leuciscus vorax	1	28.29 ±0	28.29 ±0	25.04 ±0	18.37 ±0	46.7±0
Luciobarbus barbulus	1	26.97 ±0	29.34 ±0	26.50 ±0	17.19 ±0	46.3±0
Squalius cephalus	1	29.12 ±0	27.36 ±0	25.12 ±0	18.40 ±0	45.8±0

n: sequencing number of specimen

Pairwise genetic distances were qualified according to kimura 2-parameter (K2P) distance moedel (Kimura, 1980) for interspecific variations (Kimura, 1980). Mean genetic diversity between species was calculated as 1.481 and 0.055 respectively. Pairwise distance between species is shown in Table (4). The lowest genetic distance is marked between *C. damascina* and *C. trutta* (0.055) at the sime time the highest one is marked between *C. macrostomum* and *C. luteus* (1.481).

Table 4 : Distance between species

	1	2	3	4	5	6	7	8	9	10	11	12
Luciobarbus barbulus												
Leuciscus vorax	0.170											
Arabibarbus grypus	0.135	0.189										
Squalius cephalus	1.382	1.316	1.320									
Chondrostoma kinzelbachi	1.345	1.364	1.338	1.199								
Cyprinion macrostomum	1.102	1.070	1.126	1.228	1.416							
Carasobarbus luteus	1.245	1.223	1.234	1.120	1.423	1.481						
Acanthobrama marmid	1.383	1.416	1.377	1.226	0.098	1.465	1.410					
Carasobarbus kosswigi	0.137	0.172	0.062	1.332	1.390	1.121	1.223	1.423				
Garra rufa	0.153	0.183	0.184	1.363	1.345	1.088	1.229	1.390	0.163			
Alburnus mossulensis	1.355	1.378	1.349	1.212	0.113	1.406	1.361	0.104	1.386	1.367		
Capoeta damascina	0.062	0.164	0.130	1.356	1.390	1.102	1.239	1.430	0.132	0.164	1.399	
Capoeta trutta	0.065	0.170	0.130	1.363	1.370	1.078	1.234	1.410	0.141	0.168	1.380	0.055

Neigbour Joining (NJ) tree shows that distinct nodes for each species were formed, in NJ phylogenetic tree (Figure 2), two phylogenetic nodes were detected; in the first node two branches were detected; in the first *C. macrostomum*, *L. barbulus*, *C. trutta* and *C. damascina* are grouped with sister *C. kossiwigi*, *C. tluteus* and *A. grypus* with *G. rufa* in the second branch. At the second nod *A. mossulensis*, *L. vorax*, *A. marmid*, *S. cephalus* and *C. kinzelbachi* grouped together. In addition, the result presented in Figure (2) shows some morphology characters of species [e.g., number of anal and dorsal fin rays, presence/absence of barbels and lateral line scales.

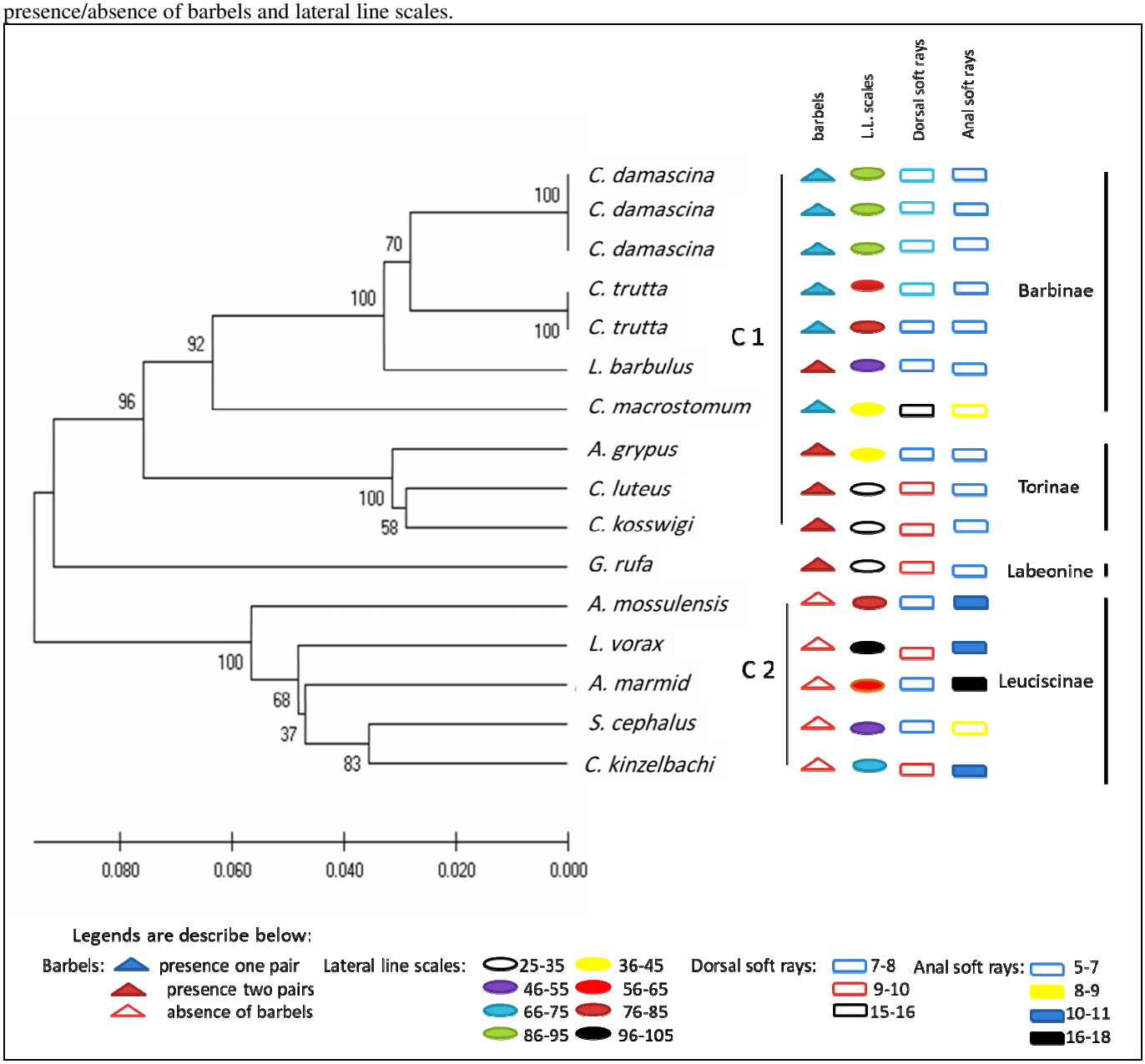

Fig. 2 : Neighbour joining phylogenetic tree based on COI sequences, and showing some morphological characters.

DISCUSSIONS

DNA barcoding using COI gene as a symbol for identifying animal species (Hebert *et al*., 2004) especially fish species, has recently attracted consideration (McCusker *et al*., 2013; Knebelsberger *et al*., 2014 and Shen *et al*., 2016). This study has validated the efficacy of COI gene for identifying species of Cyprinidae family. The primes used in the study were able to target and amplify the COI gene region in all 16 specimens.100% of all species was amplified with DNA barcoding primer. NO stop codons, insertion, or deletions were detected in any sequences, supporting the hypothesis that all amplified sequences drive from functional mitochondrial COI sequences.

For the family Cyprinidae, morphology options were unable to determine any of the specimens on the far side of the family whereas DNA barcoding was able to identify these specimens to species. Morphology conjointly often miss-assigned members of this family to others like *Carasobarbus* and *Chondrostoma*. Traditionally taxonomists learning morphological variation during this cluster additional or less accepted taxonomic group classifications, this study provides biological group sampling of the various taxonomic group of Cyprinidae and a survey of representative species of the taxonomic group like Barbinae, Labeoninae, Leuciscinae and Torinae these results give any confirmation of hypotheses for these subfamilies as presented by He *et al*. (2008).

The COI gene nucleotide composition of the present study was (T= 27.96%, A =26.01%, G= 17.51% and C= 28.52%), T and C content were highest in the COI region the present finding is by several studies. The value of GC

content (46.02%) was lower than AT content (53.98%), which is the result similar to the result found (Ward *et al*., 2005).

The performance of species identification through DNA barcoding relies on both interspecific divergence and intraspecific divergence (Lara *et al*., 2010). The comparison of mean interspecific and intraspecific genetic distance in barcode studies is usually accustomed to delimit species. However, there is still no universal standard threshold defined for interspecies demarcation. A variation between mean interspecific and mean intraspecific is that the commonplace. COI threshold for identification of animal species that a 10-fold sequences was recommended by (Hebert *et al*., 2004). Interspecific nucleotide sequences variation was ranging from 0.055 to 1.481(K2P) whereas variations between within genus *Capoeta* was 0.055%, and within genus, *Carasobarbus* was 1.223%. In the present, the genetic distance value between *C. tutta* and *C. damascina* is very low and the genetic distance between *Cyprinion macrostomum* and *Carasobarbus luteus* is higher than other distance genetic.

The generated NJ tree shows that distinct nodes for each species were found most clustered together, *C. damascina* and *C. trutta* formed a single clade, and this clade forming a sister group with *L. barbus* and also this group were a monophyletic with *C. macrostomum*, on the other hand, *G. rufa*, forming a larger clad (1) this phylogenetic analysis is agreement with results of (Durand *et al*., 2002)and (Ko *et al*., 2013). *A. grypus* was forming a sister group with *C. luteus* and *C. kosswigi* two species were in a single this agree with (Durand *et al*., 2002)

Yang *et al*. (2012). At clad (2) *C. kinzelbachi*, *S. cephalus* and *A. marmid* formed a single clade and this clade forming a sister group with *L. vorax* also this group were monophyletic with *A. mossulensis* this analysis agree with (Durand *et al*., 2002; Ko *et al*., 2013 and Ko *et al*., 2013).

DNA barcoding has been demonstrated as it has formerly been stated that this approach is a powerful and usefulness tool to identify salt and freshwater fish species in different geographic regions (Ward *et al*., 2005 and Hubert *et al*., 2008). Nonetheless, this study is one of the first steps towards enhancing genetic understanding of relationships among Cyprinidae species in Kurdistan Region/Iraq. According to this study, the mitochondrial cytochrome gene differs from the 17 types of fishes have tested regarding nucleotide sequences. The sequences previously have recorded in the gene bank, it turned out that we found 13 different strains among the 17 fishes. And these 13 different fishes were registered in NCBI GenBank. This research is an important expression for all fish trainers and researchers who need to be effective in conserving and genetically improving the native fish of the region.

In conclusion, this study has successfully identified species of Cyprinidae by using robust molecular techniques. The data of present study have led to conclude that COI barcodes could be helpful to identify species of complex freshwater fish family. *C. kizelbachi* is recorded for first time in Iraq. There is a scarcity of information about fish barcoding in our region. Further genetic researches most focus on filling data gaps existing among fish species in water bodies in Kurdistan Region, Iraq to benefit from establishing a global COI barcode database.

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