Ibn Al-Haitham Journal for Pure and Applied Sciences
Journal homepage: jih.uobaghdad.edu.iq
PISSN: 1609-4042, EISSN: 2521-3407
IHJPAS. 2025, 38 (1)

Morphological and Molecular Studies of *Acanthobrama marmid* Heckel, 1843 (Piscies, Cypriniformes, Leuciscidae) from the Middle of Iraq

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Received: 31 May 2023 Accepted: 12 July 2023 Published: 20 January 2025
doi.org/10.30526/38.1.3555

Abstract

Leuciscidae species are the abundant and widely distributed fish species in Iraq's inland waters. They are complex species, and morphology makes them difficult to identify. Molecular analysis achieved and confirmed the morphological characters. Twenty specimens of *Acanthobrama marmid* were collected from two localities at Tigris River, in the middle of Iraq; 15 specimens from the Al-Zubaydia sub-district and five specimens from Al-Tharthar Lake. We used the mitochondrial DNA cytochrome b (cytb) gene to sequence the DNA of *A. marmid*. The following analysis are compared the sequences with those of other fish genera and species found in the Gene Bank. The barcoding result (DNA sequencing) in fishes found in the same family (Leuciscidae) showed that it fit with *A. marmid*. We conclude, the results of the current study, by using the cytochrome b (*cytb*) gene, are a successful tool and confirmed the morphological characters for accurate identification of *A. marmid*.

Keywords: Cytochrome b, Fishes, Leuciscidae, Morphometric and Meristic characters, Tigris River.

1. Introduction

Acanthobrama marmid Heckel (1843) is a freshwater fish belonging to the family Leuciscidae, and this species is common and widely distributed in the Tigris and Euphrates River systems. It is one of the eight species in the genus *Acanthobrama*, which is endemic to Southwest Asia (1). Heckel (2) described Aleppo's *A. marmid* for the first time. Later, researchers recorded it from the Tigris River in Iraq (3, 4). Only Al-Nasirii and Shamsul (5) recorded fish belonging to the genus *Acanthobrama* Heckel, 1843, from the Shatt Al-Arab River, but they did not identify to species level. Al-Hassan and Al-Badri (6) recorded *A. marmid* for the first time from the Shatt

Al-Arab river near Ashar, Basrah, southern Iraq, and then recorded in the Little and Great Zab rivers, as well as in Northern Iraq (1, 7). There are many studies dealing with the taxonomic and distribution of this species in Syria, Iraq, and Turkey (3). On the other hand, there are some works that have dealt with some biological characteristics, such as reproduction and nutrition (8). *A. marmid* inhabits numerous clusters of shallow waters, and it can withstand water bodies such as lakes and rivers with medium pollution levels. Its length can reach up to 30 cm (9). There are numerous taxonomic methods available for recognizing fish species, such as the conventional morphological study. This method is helpful and fast, and it includes numerous morphometric and meristic characters for species identification (10, 11). The morphological similarities, particularly between species belonging to the same genus, have been one of the most common causes of confusion in recognizing the Iraqi fish fauna (12). In the past, it is primarily identified fish species by examining their external morphological characteristics. However, current taxonomic studies of the fishes have revealed numerous challenges in distinguishing between them, whether at the species or genus level. Consequently, we urgently need to base taxonomic work on diverse research directions, including molecular techniques that leverage information from the cell's molecular components to resolve these differences. Mitochondria cytochrome b (mt-cyb) gene is one of 11 components of a group of proteins called complex III. We generally use Cyt b as a mitochondrial DNA locus to identify evolutionary connections between different groups, and its sequence diversity makes it valuable both within and outside families (13). The aim of the current study is to confirm this classification of *A. marmid* by combining the morphological and molecular analysis.

2. Materials and Methods

2.1. Study area

Two localities at Tigris River were chosen to collect studied fish species as follows:

2.1.1. Al-Tharthar Lake

The largest natural lake in Iraq is located in Salahaddin Governorate; approximately 120 km northwest of Baghdad, situated between the Tigris and Euphrates rivers, within coordinates of 33°58′N 43°11′E. The Tigris River feeds the lake, which has an area between 1875 and 2710 km2 and a maximum depth of 68.4 m, and discharges its water into the Euphrates River (14, 15).

2.1.2. Al-Zubaydia sub-district

It is one of the sub-districts of the Al-Suwaira district in Wasit Governorate. Al-Zubaydia is located 50 km to the south of Al-Suwaira city and 85 km to the north of Kut city, the capital of Wasit Governorate, in south-eastern Baghdad, within coordinates 32°45'46.6"N 45°10'48.7"E. The sampling localities are illustrated in the map **Figure 1**.

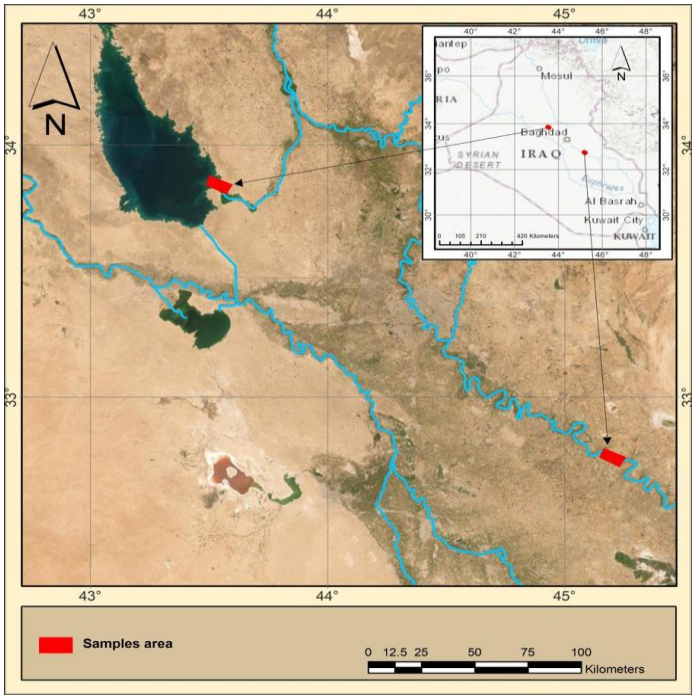

Figure 1. Sampling areas.

2.2. Samples collection

Fishermen used gill nets to collect a total of 20 fish specimens, 15 for morphology study and five for molecular study, from June 2022 to December 2022. The Iraq Natural History Research Centre and Museum, University of Baghdad, received the fish in a cool box filled with crushed ice.

2.3. Morphological study

Each specimen is measured using a 1-meter measuring board graduated in millimetres (mm) and a digital caliper, and the weight of each fish individual was done immediately by digital balance. The morphometric features are measured from the left side of each fish. The morphometric and meristic characters based on the guidelines provided by Hubbs and Lagler (16), as illustrated in **Table 1** and **Figures 2** and **3**.

Table 1. Morphometric and meristic characters and their abbreviations

No.	Characteristic name	Abbreviated name
1	Total Length	TL
2	Fork Length	FL
3	Standard Length	SL
4	Head Length	HL
5	Head Height	HH
6	Snout Length	SnL
7	Eye Diameter	ED
8	Interorbital Distance	IOD
9	Postorbital Length	POL
10	Maximum Body Height	MAXH
11	Minimum Body Height	MINH

No.	Characteristic name	Abbreviated name
12	Caudal Peduncle Length	CPL
13	Dorsal Fin Length	DFL
14	Dorsal Fin Height	DFH
15	Pectoral Fin Length	PFL
16	Pelvic Fin Length	PVL
17	Pectoral-Pelvic Distance	PPD
18	Pelvic-Anal Distance	PAD
19	Anal Fin Length	AFL
20	Anal Fin Height	AFH
21	Pre Dorsal Length	PDL
22	Post Back Distance	PBD
23	Upper Caudal Fin Length	UCFL
24	Lower Caudal Fin Length	LCFL
25	Centre of Caudal Fin Length	CCFL
26	Caudal Peduncle Height	CPH
27	Mouth Width	MW
28	Prepectoral Length	PPL
29	Prepelvic Length	PVL
30	Preanal Length	PAL
31	Preanus Length	PASL
	Meristic characteristics	
32	Scales of Lateral Line	SLL
33	Up Scales of Lateral Line	USLL
34	Down Scales of Lateral Line	DSLL
35	Dorsal Fin Spines	DFS
36	Dorsal Fin Rays	DFR
37	Anal Fin Spines	AFS
38	Anal Fin Rays	AFR
39	Pectoral Fin Rays	PFR
40	Pelvic Fin Rays	PVFR
41	Caudal Fin Rays	CFR
42	Number of scales around the least circumference of the caudal peduncle	SCP
43	Gill Rakers	GR

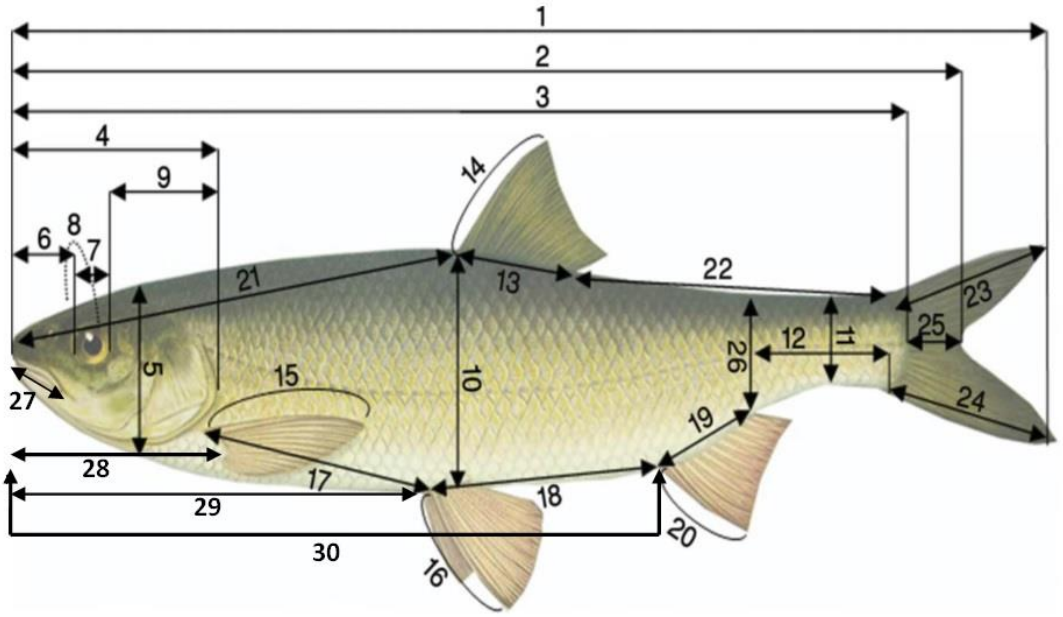

Figure 1. The morphometric measurements carried out of *Acanthobrama marmid* Heckel, 1843(17 with personal modification)

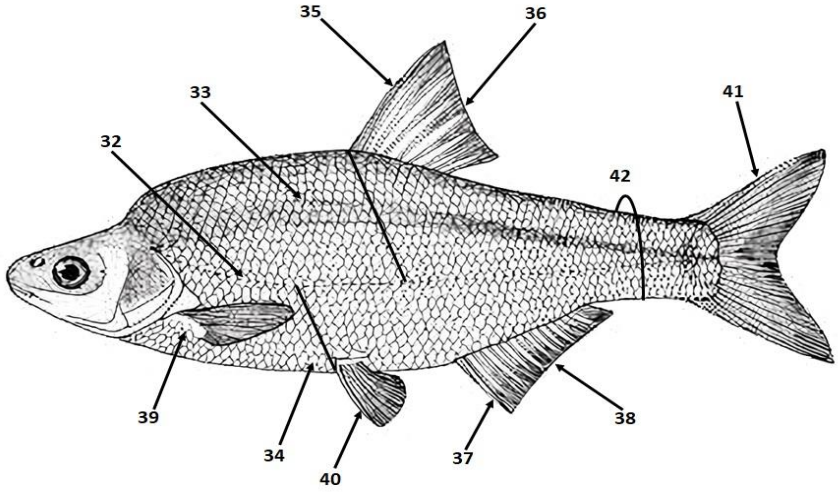

Figure 2. The meristic measurement carried out on *Acanthobrama marmid* Heckel, 1843(From Coad (1) with personal modification)

2.4. DNA Extraction

Using the Genomic DNA Extraction Kit (addbio/Korea, Cat. no. 10023), the total DNA is extracted from the muscle dorsal side of each fish individual and preserved the samples in absolute ethanol (100%). Genomic DNA was taken out according to Sambrook *et al.* (18), and it was tested by electrophoresis on a 1% agarose gel that had been stained with ethidium bromide.

2.5. Amplification and polymerase chain reaction PCR

This step carried out by amplified approximately 508 bp from the 5' region of the Cytb gene using universal fish primers. The forward primer is Fish F1 5' (CAAGCCTACGAAAAACMCAC) 3, and the reverse primer is 5' Fish R1 (TCTACTGAGAAKCCRCCTCA) 3'. Polymerase Chain

Reaction (PCR) reactions are 50 µl in size and have 25 µl of 2xTaq DNA Polymerase Master Mix, 2 µl of each primer (Fish F1 and Fish R1), 17 µl of free water, and 4 µl of DNA template. They are run on an Optimus 96G thermal cycler. The PCR thermal cycling conditions were: 94ºC for five min; 35 cycles of 94ºC for 30s; 52ºC for 30s; 72ºC for 30s; final extension of 72ºC for 5 min; and hold at 4ºC. We then electrophoresed the PCR products on a 2% agarose gel, stained with ethidium bromide dye. For this test, we used Promega's 100-bp ladder. We tested the profiles on a UV light transilluminator and documented them using a Canon camera and a gel documentation tool.The PCR product for sequencing sent to Macrogen/Korea using a forward primer. The results are evaluated using Bioedit software, version 7; 2013; https://bioedit.software.informer.com/7.2/ . Then aligned and compared the sequences generated from the sequencing findings with data from the same organism genes found in the gene bank at the National Center for Biotechnology (NCBI), which had previously undergone investigations in various nations worldwide using the Basic Local Alignment Search Tool (BLAST). The results of sequencing showed 99–100% agreement with reference sequences. The phylogenetic tree is drawn of the species from the sequence data of the studied samples and compared them with the previously studied species using MEGA X: Molecular Evolutionary Genetics Analysis (19). The nucleotide location value of variance is calculated using the maximum likelihood (ML) and generated a molecular phylogenetic tree. The bootstrapping method with 500 repeats is used to assess the accuracy of the estimated phylogenies.

3. Results

A total of 20 specimens of *A. marmid* are collected from Al-Zubaydia sub-district (15 specimens) and Tharthar Lake (five specimens), both at the Tigris River in the middle of Iraq.

3.1. Morphological study

The morphological and meristic characters of *A. marmid* are indicated in **Tables (2** and **3),** and **Figures (3A** and **3B**) to show the general morphology of *A. marmid***.**

Table 2. Morphological characters proportional measurements as expressed as percentage of standard length or of head length for *Acanthobrama marmid*.

Character	min-max (Mean ±SD)	Proportional measurements as expressed as percentage of Standard Length or of Head length*	
Weight	22.4- 38.1 (30.84± 9.35)	-	
Total Length TL	114-157 (141.4±14.1)	-	
Fork Length FL	102-145 (129±13.5)	-	
Standard Length SL	93-131 (117.2±12.2)	-	
Maximum Body Height MAXH	27-43 (37.2 ± 5.2)	27.83-35.92 (31.65 ± 2.29)	
Minimum Body Height MINH	10-14 (11.6 ± 1.0)	8.39-11.65 (9.94 ± 0.86)	
Caudal Peduncle Length CPL	11-21 (17.2 ± 2.8)	11.53-17.47 (14.67 ± 2.00)	
		11.53- 16.50 (13.32 ±1.30)	
Dorsal Fin Length DFL	12-20(15.6± 2.0)		
			19.23-25.77
Dorsal Fin Height DFH	22-31 (26.1 ± 2.4)	19.08-26.27 (22.26±3.27[a])	(22.52±1.81[b])

Character	min-max (Mean ±SD)	Proportional measurements as expressed as percentage of Standard Length or of Head length*	
Pectoral Fin Length PFL	16-23 (20.9 ± 2.2)	15.70-20.38(18.44 ± 1.84[a])	16.92-17.96 (17.60±0.39[b])
Pelvic Fin Length VFL	13-21 (17.8 ± 2.3)	12.09-16.49 (15.24 ±1.20)	
Pectoral-pelvic Distance PV	18-33(26.2 ± 3.9)	19.35-26.21 (22.34 ± 1.85)	
Predorsal distance	46-72 (61.6 ± 7.6)	49.46- 55.38 (52.52±1.71)	
Prepelvic Distance PVD	41-59 (52.6 ± 5.2)	43.63-49.51 (44.92±1.55)	
Prepectoral Distance PPD	22-35 (28.4 ± 3.5)	21.53-27.34 (24.30±1.81)	
Preanal Distance PAD	58-88 (75.0 ± 8.2)	61.15-67.69 (64.03±1.96)	
Pelvic-Anal Distance VA	18-32 (24.8 ± 3.6)	19.60-27.11 (21.20±2.12)	
Anal Fin Length AFL	19-29 (23.5±2.9)	17.94-22.88 (20.12±1.89)	
Anal Fin Height AFH	11-18 (14.2±2.2)	9.16-16.21 (12.17 ±1.91)	
Preanus Distance	54-84 (61.6±8.1)	53.71-64.61 (59.38±3.00)	
Post Back Distance POB	31- 47 (39.6±5.3)	30-38.01(33.76±2.48)	
Upper Caudal Fin Length UCFL	21- 32 (27.6±3.0)	22.72-26.27 (24.22±1.52[a])	20.76-25.77 (23.26±1.60[b])
lower Caudal Fin Length DCFL	20-33 (27.4±3.7)	20.51-25.78 (23.35±1.61)	
Center Caudal Fin Length CCFL	10-17 (12.5±2.3)	7.69-10.31 (11.69±1.03)	
Caudal Peduncle Height	10-18 (14.0±2.0)	10.74-13.84 (11.98±1.08)	
Head Length	21-30 (25.8±2.6)	(HL/SL) ×100 20.72-24.27(22.10±1.14)	
Head Height	14-19(16.1±1.5)	56.00-73.91 (62.68±6.05)	
Snout Length	4.4-7.2(6.0±0.8)	17.6-28.57 (23.30±3.01)	
Orbit diameter	5-7 (6.0±0.6)	19.25-26.92 (23.51±2.30)	
Eye Diameter	3-4 (3.3±0.3)	11.11-15.38 (13.10±1.40)	
Post Orbit Distance	10-14 (12.0±1.1)	41.37-56.00 (46.81±3.91)	
Between Eye Distance	6-9.6 (8.0±1.0)	26.92-34.8 (31.65±2.67)	
Mouth width	5-8.5 (6.8±1.1)	22.22-30.4 (26.31±2.49)	

a, b mean significant differences in characters between two populations of *A. marmid* from two localities (a Tharthar Lake, and b Al-Zubaydia sub-district)

* mean Proportional measurements as expressed as percentage of Head length

Table 3. Meristic characters for *Acanthobrama marmid.*

Character	min-max (Mean ±SD)
Lateral Line Scales (LLS)	53-55 (54 ±0.67)
Above Scales of Lateral Line (ASLL)	12-13 (12.5 ± 0.51)
Below Scales of Lateral Line (BSLL)	5-6 (5.2± 0.45)
Unbranched Dorsal Fin rays (UDFR)	3-3 (3±0)
Branched Dorsal Fin Rays (BDFR)	8 (8± 0)
Pectoral Fin Rays (PFR)	12-14 (13± 0.63)
Unbranched Anal Fin Ray (UAFR)	3-3 (3±0)
Branched Anal Fin Rays (BAFR)	13-15 (14± 0.89)
Caudal Fin Rays (CFR)	16-18 (17±0.70)
Pelvic Fin Spine (VFS)	1-1 (1±0)
Pelvic Fin Rays (VFR)	8-8 (8±0)

Character	min-max (Mean ±SD)
Scales around the least circumference of the caudal peduncle (SCP)	10-12 (11±0.97)
Gill Rakers (GR)	12-14 (13± 0.59)

Figure 3 A. *Acanthobrama marmid* (Whole body) from Al-Zubaydia sub-district, Wasit Governorate.

Figure 3 B. *Acanthobrama marmid* (Whole body) from Al-Tharthar Lake, Salahaddin Governorate.

3.2. Statistical analysis

The SPSS program version 26 was used to find out the significant differences in characters between two populations of *A. marmid* from two localities: Al-Zubaydia sub-district and Tharthar Lake.

The results of this study show that there were minor differences between the studied populations in some morphological characteristics, including dorsal fin height, pectoral fin length, and upper caudal fin length, but that there were no significant differences in meristic characters.

3.3. Molecular study

PCR primers of the cytochrome b gene (*cyto b*), and specific primers der are successfully amplified, and gel electrophoresis is performed to show PCR amplification of the *cyto b*, which yielded a 508 bp product. These amplifications are used for diagnosis the species and give comparative data for developmental taxonomy studies and family development research at the

species level. Sequencing of these genes is performed in order to determine the genotype of *A. marmid*, which is collected from the Al-Zubaydia sub-district. DNA sequencing is one of the most important methods that contributed to the rapid diagnosis of species (20). The sequence of one gene examined forward and reverse primer is performed, this is part of the sequence process requirements and the use of PCR technology in the context of the method of genetic analysis. The results of nucleotide alignment with the sequences in the gene bank showed that the identities ranged 99%. BLAST results are shown in **Table 4**.

Table 4. The BLAST output of *Acanthobrama marmid* fish for cytochrome b partial sequence gene.

Sample	Organism	Sequence ID	Score	Expect	Identities	No. nucleotide
Isolate T 4, T5 and T6	*A. marmid*	AY026411.1	826	0.0	99 %	44 - 499

Partial CDs and *cytochrome b* (*cytb*) gene mitochondria are compatible with the same sequence fragment marker, which is available at the Gene Bank in the National Center for Biotechnology Information (NCBI). **Figure 4** shows the fish specimens' partial sequence and pair-wise analysis.

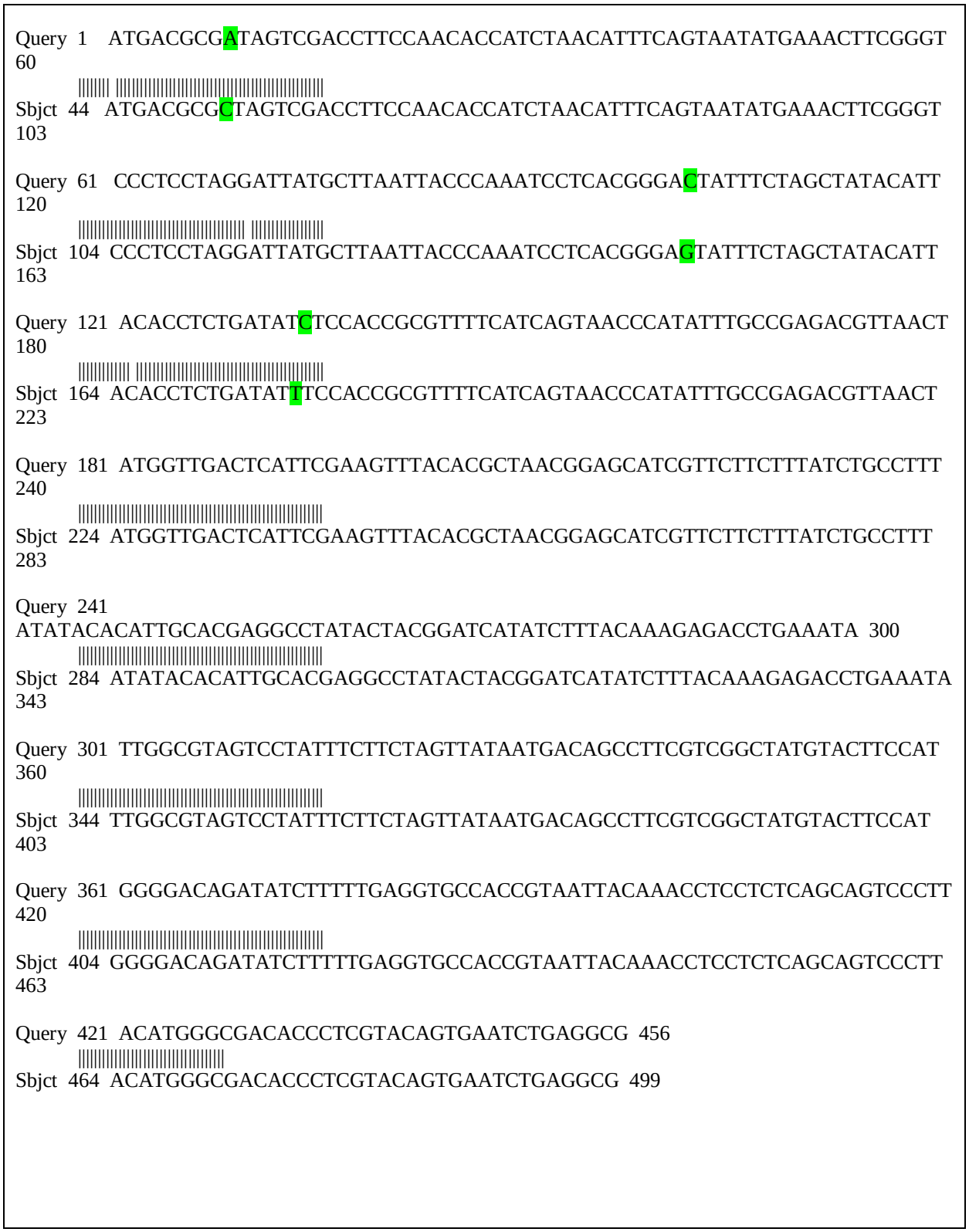

Figure 4. Pair wise alignment of partial cds, cytochrome b (*cyt b*) gene of *Acanthobrama marmid* Query is the study or sample sequence and subject is the Gene Bank sequence

The fish tissues are sequenced a total of 508 bp at the 5 ends of the cytb mtDNA region for three samples, and the best likelihood tree from the partitioned maximum likelihood analysis revealed two sub-branches in Fig. 5. One of the branches revealed that *A. marmid*, found in local samples T5 and T6, formed a sister group with a bootstrap value (BP) of 83%, while another sample, local sample T4, formed a sister group with a bootstrap value (BP) of 63%. There was a similarity between local samples (T4, T5, and T2) and standard sequences of *A. marmid* of the same gene from France (AY026411.1), with a bootstrap value (BP) of 99%. The other branch demonstrated a close clustering of the genus *Acanthobrama* with the genera *Abramis* Cuvier, 1816, *Vimba* Fitzinger, 1873, *Acanthalburnus* Berg, 1916, *Blicca* Heckel, 1843, and *Petroleuciscus* Bogutskaya, 2002.

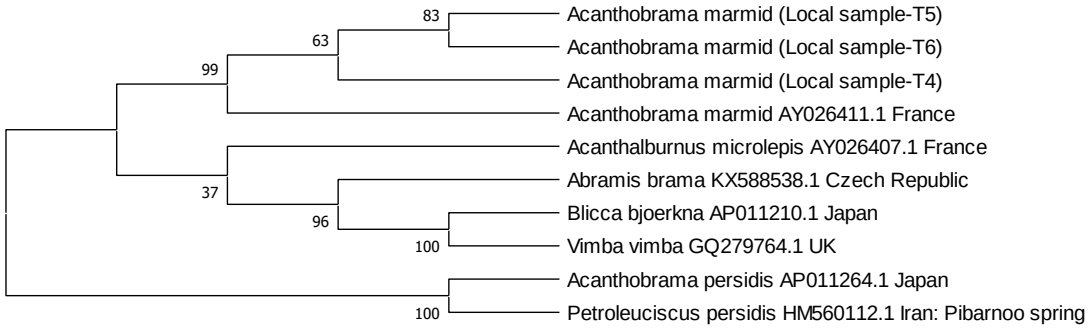

Figure 5. Phylogenetic tree analysis of *Acanthobrama marmid* local samples resemble for others in different countries; draw by MEGA X using Maximum like hood (ML) method with bootstrap value 500 repeats.

4. Discussion

4.1. Morphological study

Morphometric and meristic traits are practical tools in fish morphology for new species definition (21) and species identification (22). In addition, fish morphology can influence their swimming performance, reproductive, camouflage, feeding activities, etc. (23). The fish species in the current study previously belonged to the Cyprinidae, but now belong to the Leuciscidae, according to the global research of Stout *et al*. (24), which provides robust resolution for the relationships among Cypriniformes using anchored enrichment. The descriptions of the current *A. marmid* specimens are similar to those provided by Coad (1). The number of dorsal fin rays in this study is similar to that in Al-Hassan *et al*. (6), but the number of lateral line scales and the anal fin ray of present specimens are smaller (53-55 vs. 66 and 16-18 vs. 19), respectively. Krupp *et al.* (25) found a specimen that exhibited characters intermediate between *A. marmid* and *Alburnus mossulensis* (= *A. sellal*). However, these specimens are similar to the hybrid species described by Krupp *et al*. (25). For example, both they have 53–55 lateral line scales above and below, as well as the same number of dorsal fin rays, anal fin rays, gill rackers, and lateral line scales. at the current results have a few differences in characteristics of *A. marmid* compared to the results of Coad (1). These differences were 12–18 for the number of pectoral-fin rays, 16–22 for the number of anal-fin rays, 11–8 for the number of dorsal-fin rays, 12–17 for the number of pelvic-fin rays, and 53–72 for the number of gill rackers. When made comparison in some features of *A. marmid* in the current study, such as the number of branched dorsal-fin rays, the number of pelvic-fin rays, and the number of anal-fin rays,

we found that the scales above of the lateral line and the scales below of the lateral line are 8, 8, 16-18, 12-13, and 5-6, respectively, which are in agreement with the results of Agha (26). However, the number of lateral line and gill rakers in this study is lower (53-55 vs 57-69) and 12-14 vs 14-16, respectively. The lateral line scales and numbers of the anal-fin rays 53-55 and 16-18, respectively, are inconsistent with the results of Al-Moussawi and Afrasiab (27), which are 66 and 19, respectively. The number of branched dorsal fin rays, pelvic fin rays, and pectoral fin rays in this study is consistent with Kaya *et al.*'s results (28), but the lateral scale line and number of gill rakers of current specimens are lower than those of Turkish specimens (53-55 vs 61-70 and 12-14 vs. 14-17 respectively). The total length of the specimens ranges from 11.4-15.7, which is in line with the results of Eagderi *et al*. (29), which show a range of 4.85-15.49. The current study compares several features of *A. marmid*, including the number of dorsal fin rays, the number of pelvic fin rays, the number of pectoral fin rays, the number of anal-fin rays, the number of lateral line scales, the number of above and below lateral line scales, and the number of lateral line scales. These results align with those of Coad (30), which are 11, 7-9, 12-18, 16-22, 53-77, 10-14, and 4-7, respectively. The phenotype of an organism constantly interacts with its environment and biological factors, either directly or indirectly influencing its body shape and the selection of preferred features. It increases the likelihood of survival and reproduction in an environment. This can lead to the creation of a new population (31). The results of the current study showed that there were differences between the studied populations in some morphological characteristics, including dorsal fin height, pectoral fin length, and upper caudal fin length. There are many studies that showed these differences in morphometric and meristic characters of some species that belong to the Cypriniformes, such as Keivany *et al*. (32) Looked at 29 groups of *G. rufa* from six river basins and systems in Iran. They discovered big differences ($p<0.05$) in the ratios of the dorsal fin base to standard length and the pectoral fin to standard length. This matches what Zamani-Faradonbe *et al*. is found. (33). The latter study conducted on *G. rufa* from three distinct populations, revealing significant differences in 14 morphometric measures and several metrics, including lateral line scales, predorsal scales, and circamucaudal scales.

4.2. Molecular study

The present study classified and identified conventionally on the basis of external morphological traits. However, the various developmental stages of the fishes are difficult to identify by morphological characteristics alone. Therefore, the traditional identification and classification results were supported by mitochondrial DNA sequencing, and, fortunately, the results matched and there was no confusion in the scientific nomenclature, the *cyto b* gene as a code for animal species identification (34); especially the fish species, which have been attracting attention lately. This study demonstrated the effectiveness of the *cyto b* gene in identifying Leuciscidae species. Aziz (35) used the same gene *cyto b* to identify nine species of Cyprinidae. The DNA sequencing results showed that all species belong to Cyprinidae, and the phylogenetic relationship degree with this family for *Cyprinion macrostomum*, *Luciobarbus esocinus*, *Capoeta trutta*, and *L. xanthopterus* was a BP of 90%, for *C. luteus* was a BP of 87%, for *B. grypus* (=*A. grypus*) was a BP of 76%, and *C. regium*, *C. carpio*, and *C. carassius* was a BP of 75%. In this study, the results

are in agreement with Durand *et al.* (36), who used mitochondrial cytochrome b DNA sequences with strong statistical support to study the phylogenetic of *A. marmid* in the Middle East region and indicated that the genus *Acanthobrama* closely clusters with the genera *Abramis*, *Vimba,* and *Acanthalburnus*. Gaffaroglu *et al*. (37), examined the karyotype of *A. marmid*, and discovered that it closely resembles the karyotype pattern of several leuciscine genera, such as *Alburnus* Rafinesque, 1820, *Alburnoides* Jeitteles, 1861, *Abramis*, Blicca, *Leucaspisus* Heckel & Kner, 1857, *Leuciscus* Cuvier, 1816, *Petroleuciscus* Bogutskaya, 2002, *Pseudaspius* Dybowski, 1869, *Rutilus* Rafinesque, 1820, *Scardinius* Bonaparte, 1837, and *Vimba*, among others. This finding is consistent with the current study, which includes DNA sequencing results indicating that there is a genetically close relationship among them, and these species belong to the Leucisidae, which in turn belongs to the order Cypriniformes. Behrens-Chapuis *et al.* (38) used the mitochondrial COI gene for species identification. Still, it concluded this gene failed to identify closely related species of Leucisidae such as *Acanthobrama, Alburnus, Mirogrex, Phoxinus, Scardinius, Chondrostoma, Gobio,* and *Squalius*. Still, the cytochrome b (*cytb*) gene used in the current study is successful in the identification of *Acanthobrama* belonging to *Leucisidae*.

5. Conclusions

The current study's results confirm the morphological identification of *A. marmid*. The DNA sequencing results validated the validity of the fish species and demonstrated the identification of correctly sequenced species using the cytob gene.

Acknowledgement

We extend our sincere thanks to Prof. Dr. Hula Y. F. Al-Sadi, College of Sciences, University of Baghdad, for her invaluable contribution and advice about molecular work.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Funding

There is no funding for the article.

Ethical Clearance

There is no ethical clearance for the used animals.

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