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Original Article

First Molecular Identification and Phylogenetic Analysis of Three Unio Species (Bivalvia: Unionidae) from the Kurdistan Region, Iraq

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ABSTRACT

Unionidae Freshwater mussels are ecologically significant but little is known about them at a molecular level in Iraq. In this work, two freshwater sites of a lake close to Hizop River and the Greater Zab River close to Qandil village, Erbil Governorate, Kurdistan Region, Iraq were sampled from April to June 2025 and bivalves were collected. Morphological analysis with cytochrome oxidase subunit I (COI) sequence analysis of the mitochondrial genome was used to identify the species. Molecular studies indicated that the specimens were *Unio pictorum* (Linnaeus, 1758), *Unio bruguierianus* Bourguignat, 1853 and *Unio tumidus* Philipsson, 1788, with sequence similarity (99.8 to 100) to reference sequences that are available in GenBank. Neighbor-Joining, Maximum Likelihood and Bayesian Inference analyses provided phylogenetic support and well-supported clades with the highest bootstrap percentage (100%), thereby supporting the location of all three species in genus Unio (family Unionidae). The obtained COI sequences were deposited to NCBI GenBank database. *Unio pictorum* and *Unio tumidus* are the first reported in Iraq on both morphological and molecular evidence, and neither of the species has been recorded in the country before. *Unio bruguierianus*, the species which was previously known in Iraq only morphologically as *Unio ciconius* Kobelt, 1913, a name which has been synonymised in the *U. crassus* complex, is proven a separate species and described with the first molecular evidence. All these findings constitute the earliest morphological and molecular record of *U. pictorum* and *U. tumidus* in Iraq, and the earliest molecular record of *U. bruguierianus* in the Iraq. These findings are applicable to the distributions of all three species into the upper Tigris basin and will provide useful baseline information in future biodiversity studies and conservation strategies of freshwater mussels in the Kurdistan Region and Iraq.

Keywords: Unio pictorum, Unio bruguierianus, Unio tumidus, freshwater mussels.

1 Introduction

Freshwater bivalves of the family Unionidae represent one of the most evolutionarily diverse and ecologically significant groups of aquatic invertebrates in continental freshwater ecosystems [1-3]. Unionidae have an estimated total number of 800 species, which are distributed on all of the continents, with the exception of Antarctica and found in a wide range of freshwater environments such as rivers, lakes, streams and reservoirs [4-6]. These organisms play an important role in ecosystem functioning through biofiltration, nutrient cycling, bioturbation of sediments and tropic interactions and therefore play an important role in the critical structuring of freshwater communities and maintaining ecosystem stability [7]. Freshwater ecosystems in the Kurdistan Region of Iraq harbor diverse aquatic invertebrate communities, highlighting the biological richness of these water bodies [8].

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Although they have an established ecological importance, Unionidae are considered to be one of the most threatened groups of freshwater bivalves in the world, with more than 40% of assessed species threatened by the International Union of Conservation of Nature (IUCN) [1,9]. The traditional taxonomic approach to mussels of the unionid group has relied heavily on shell morphological features which have included shell geometry of the shell, shell size, surface sculpture, hinge dentition and periostracum features [4,5]. However, morphological definition of Unionidae is still full of problems as the shell characters highly respond to environmental parameters and ontogenetic differentiation during the stages of development and convergent evolution is witnessed between phylogenetically dissimilar taxa [10]. As a result, morphologically similar taxa could be genetically independent, the phenotypically divergent ones could be members of the same biological species [11,12]. With the advent of molecular systematics, the taxonomy of unionids has undergone radical changes to provide strong methodologies in solving taxonomic ambiguities and reorganizing the phylogenetic relationships within the family [13,14]. DNA barcoding, using the

mitochondrial cytochrome oxidase subunit I (COI), has emerged as a very effective technique in species identification, cryptic taxon detection, and inference of phylogeny in freshwater mollusks [15]. COI exhibits high interspecific divergence, low intraspecific variation, and is well represented in repositories such as GenBank and the Barcode of Life Data System[16]. Additional markers including mitochondrial 16S rRNA and nuclear 28S rRNA have further improved phylogenetic resolution and species delimitation in the Unionidae [17-19]. While COI remains the most widely used standard barcoding marker for freshwater mussels, the integration of multiple genetic markers such as 16S rRNA and 28S rRNA alongside COI has been increasingly recommended for more robust integrative taxonomic studies, as these markers provide complementary resolution at different phylogenetic levels [14]. In Iraq, and particularly in the Kurdistan Region, molecular studies on freshwater bivalves remain scarce, with previous records based predominantly on morphological identification and minimal phylogenetic data [20,21]. Despite the exceptional biodiversity of the Tigris-Euphrates river system, which represents one of the most biogeographically significant freshwater basins in the Middle East, the Unionidae fauna of Iraq remains critically understudied at the molecular level. Most existing records rely exclusively on shell morphological characters and lack genetic confirmation, making species-level identifications uncertain due to the high variability in shell shape and size among unionid species. A recent checklist of Iraqi Bivalvia further highlighted that molecular data remain absent for the majority of freshwater mussel species recorded from the country [22]. Many such records lack molecular confirmation. However, aquatic organisms inhabiting freshwater systems of the Kurdistan Region continue to receive increasing scientific attention [23]. The Kurdistan Region contains diverse freshwater habitats fed by the Zagros mountain range, supporting unionid lineages that appear unique and understudied [24,25]. Freshwater bodies in Erbil Governorate, including rivers and streams of the Kurdistan Region, have been the subject of increasing biological investigations in recent years[26]. Therefore, this study aims to provide the first integrative taxonomic characterization of unionid bivalves from selected sites in Erbil Governorate, Kurdistan Region of Iraq, using morphological analysis, COI DNA barcoding, phylogenetic reconstruction, and genetic distance analysis. By incorporating both morphological and molecular approaches, this study seeks to fill a critical gap in the taxonomic knowledge of Iraqi freshwater bivalves and to establish a reliable molecular baseline for the Unionidae of the Kurdistan Region.

2 Materials and Methods

2. 1 Sample Collection

Fresh water mussels were collected by hand from two sites in the Iraqi Kurdistan Region during April to June 2025. Site 1 was a lake near Hizop River, and Site 2 was along the banks of the Greater Zab River near Qandil village (Qasrok and Sarkawr), located in Erbil Governorate at 44° 11’ 04.0” E longitude and 36° 37’ 43.0” N latitude, 380 m above sea level as shown in **Error! Reference source not found.**. Approximately 80 specimens were collected during each sampling event, rinsed with river water in the field, and transported to the laboratory in containers filled with river water. At arrival, specimens were kept in aquaria under controlled conditions (25°C) and acclimated to laboratory conditions for seven days in glass containers (5 L aged tap water). Water was replaced every 24 hours with continuous aeration, and any mortalities were removed daily to avoid microbial contamination.

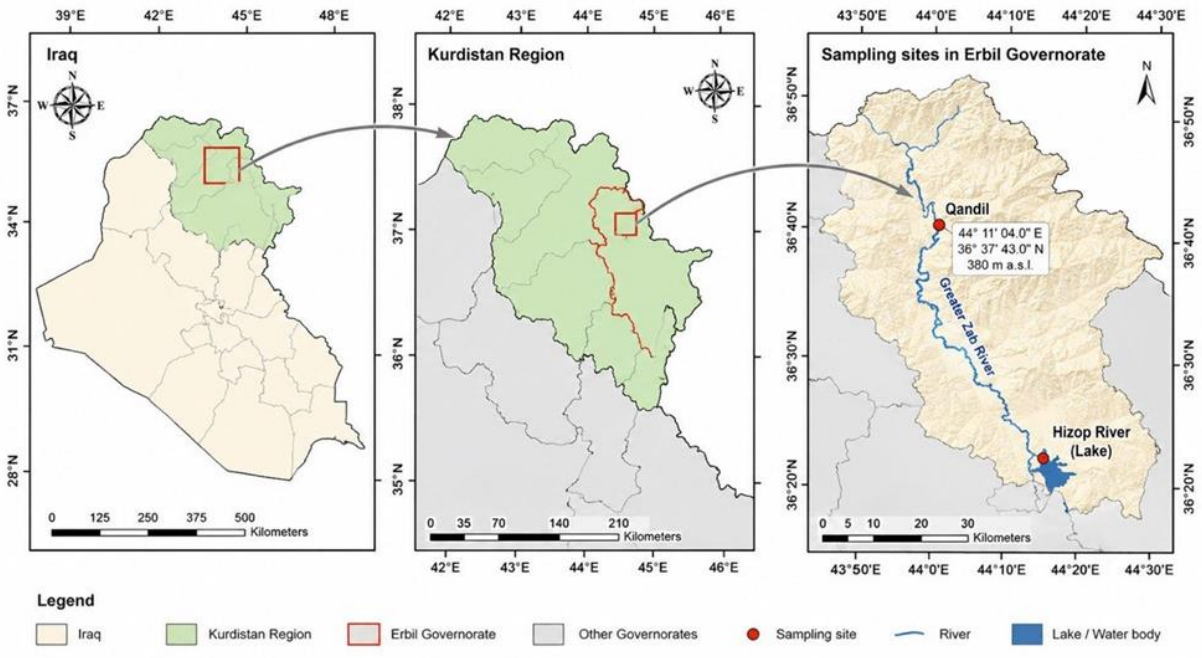

Figure 1: Map of the study area showing the sampling sites in Erbil Governorate, Kurdistan Region of Iraq.

2. 2 Morphological Identification of Bivalves

Morphological recognition of bivalve specimen was done using the basis of major diagnostic shell characters as generally accepted under taxonomic classification of family Unionidae. Those characters were shell shape and outline, shell inflation and thickness, periostracum colour and texture, growth lines and surface sculpture, umbo position and prominence, hinge dentition (pseudocardinal and lateral teeth), distribution of muscle scars, pallial line morphology, internal nacre colouration and shell dimensions (length, height, and width). The identification was done in accordance to[27-29].

2. 3 Molecular biological Study

2. 3. 1 DNA Extraction

Genomic DNA was extracted from fresh foot muscle tissue of each bivalve specimen using the Beta Bayern Tissue DNA Preparation Kit (Beta Bayern, Germany) according to the manufacturer's instructions with minor modifications adapted for bivalve tissue. Approximately 20-25 mg of foot tissue was used per sample. Purified DNA was stored at −20°C until further use [30].

2. 3. 2 Measurement of DNA Concentration and Purity Using NanoPhotometer Spectrophotometer

The extracted DNA from bivalve specimens was assessed for concentration and purity using Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, USA). All samples yielded DNA of acceptable quality with purity ratios (A260/A280) ranging from 1.7 to 2.0, confirming suitability for downstream PCR amplification [31,32].

2. 3. 3 Primers

The primers have been supplied by Macrogen, South Korea, as **Error! Reference source not found.** shows. Macrogen manufactured the primers in a lyophilized form. Primers were prepared to produce a stock concentration of 100 µM by mixing the lyophilized primer with the required volume of nuclease-free water according to the manufacturer's protocol. A working concentration of 10 µM was prepared by adding 10 µl of stock primer to 90 µl of nuclease-free water.

Table 1: Primers applied for the amplification and sequencing of the COI gene.

Primer code	Sequence (5′–3′)	Amplicon size (bp)	Reference
LCO1490 Forward	5′-GGTCAACAAATCATTAAAGATATTGG-3′	720bp	[33]
HCO2198 Reverse	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′		

2. 3. 4 Identification of Bivalve Specimens via COI Gene Amplification

Conventional PCR was performed in 50 µl volume reactions that contained 25 µl of 2× Taq DNA Polymerase Master Mix (AMPLIQON A/S), along with 3 µl of each forward (COI-F) and reverse (COI-R) primer (20 pmol), 4 µl of DNA template (50 ng/µl), then completed by 15 µl of nuclease-free water, as shown in **Error! Reference source not found.**. The PCR cycling conditions were as follow: An initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 35 seconds, annealing at 59°C for 35 seconds and extension at 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes [34].

Table 2: COI gene PCR amplification reagents.

No.	PCR components	Concentration	Volume (µl)
1	Master Mix	2x	25
2	Forward Primer	20 pmol	3
3	Reverse Primer	20 pmol	3
4	DNase free Water	-	15
5	Template DNA	50 ng/µl	4
Total			50

2. 3. 5 Detection of PCR Products Using Gel Electrophoresis

Following PCR amplification, PCR products were loaded onto 1.0-1.5% agarose gel and stained with ethidium bromide, then visualized under UV trans-illuminator (Biobase, China) and stained with ethidium bromide [30,35]. A (1000 bp) DNA ladder

was used as a size marker. Results were documented using a gel documentation system[36].

2. 3. 6 DNA Sequencing Analysis, Alignment, Submission, Accession Numbers and Phylogenetic Analysis

Sanger sequencing technique was used to sequence PCR products by Macrogen in Seoul, South Korea. The resulting sequences were edited and analyzed using BioEdit version 7.2.5 [37] and the Basic Local Alignment Search Tool (BLAST), available on the NCBI (National Center for Biotechnology Information)[38]. Isolates were identified according to the percentage similarity with the sequences of identified species in the database. All sequences were submitted to GenBank and accession numbers were obtained as shown in **Error! Reference source not found.**. A phylogenetic tree was generated using MEGA 11 software [39] using Neighbor-Joining, Maximum Likelihood, and Bayesian Inference methods with bootstrap values calculated from 1000 replicates.

Table 3: Sample of bivalves of same species according blast of Genbank NCBI of partial COI gene percentage distribution.

Bivalve samples Accession Number	Query Cover %	Identic Number %	Genbank Accession Number	Genbank bivalve Species Identification
PX452484 PX452492 PX452493	100	100	JQ253874	*Unio pictorum*
	100	100	KX400130	
	100	100	AF15649	
	100	99.82	OQ564391	
	100	99.82	MZ165233	
	100	99.82	JX046581	
	100	99.82	MZ16524	
	100	99.82	MZ165231	
PX452488	100	100	OR841701	*Unio bruguierianus*
	100	100	OR841704	
	100	99.83	OR841700	
	100	99.66	MZ512290	
	100	99.66	MZ512246	
	100	99.66	MZ512276	
	100	99.49	OR841702	
PX452494	100	100	MZ165270	*Unio tumidus*
	100	100	AY074807	
	100	100	KY021076	
	100	100	JQ253872	
	100	100	MK034161	
	100	100	PP204156	

3 Results and Discussion

3. 1 Identification of the Collected bivalves

Morphological examination of the collected specimens revealed characteristics consistent with the family Unionidae, and three species were identified based on the diagnostic shell characters examined. *Unio pictorum* Linnaeus, 1758 is characterised by an elongated, relatively flattened shell with an elongate-oval outline (**Error! Reference source not found.**.A). The periostracum is smooth, olive-green to brownish in coloration, darkening progressively toward the umbo region and posterior end with advancing ontogeny, and displays prominent concentric growth lines across the entire outer surface. The umbones are depressed and located much in front of the midline. The anterior margin is widely rounded and the posterior one is gradually narrowed and slightly tapered. The dorsal margin is almost straight and the ventral strictly curved. The hinge apparatus is highly developed; the pseudocardinal, and lateral teeth are straight, thin, and compressed, in sharp contrast to the more robust, more rugged, and more often curved characteristics of the lateral teeth of *U. pictorum*, *U. tumidus*, *U. bruguierianus* [40]. The anterior and posterior adductor muscle scars are well impressed. The nacre is whitish to bluish-white internally. Shell length typically ranges from 50 to 100 mm, though considerable morphological variation exists among populations, likely attributable to differences in habitat conditions, water flow, and substrate composition [29,40]. These findings were similar to the findings of Araujo *et al.*, (2018)[28] and Lopes-Lima *et al.*, (2021)[29].

Unio bruguierianus Bourguignat, 1853, a species recently recognised as distinct from the *Unio crassus* complex based on molecular and morphological evidence [28,29,41], is characterised by a broadly oval to subrectangular shell, moderately to strongly inflated, equivalve, and inequilateral (**Error! Reference source not found.**.B). The periostracum is smooth, olive-green to yellowish-brown in coloration, darkening progressively to dark brown with advancing ontogeny, and displays regular concentric growth lines across the entire outer surface. The umbones are low and positioned well anterior to the midline. The anterior margin is broadly rounded, while the posterior margin is bluntly truncated with a broadly rounded outline, distinguishing this species from the more tapered posterior of *U. pictorum*. The dorsal margin is slightly arched and the ventral margin is broadly and evenly curved. The hinge plate is robust, bearing well-defined pseudocardinal and lateral teeth that are thicker and more rugged than those of *U. pictorum* [40]. The anterior and posterior adductor muscle scars are well-impressed and ovate to subcircular. The nacre ranges from whitish to pale salmon internally. Shell length typically ranges from 40 to 80 mm, though considerable morphological variation exists among populations across the species range [28,29,41]. These findings were consistent with the findings of Araujo *et al.*, (2018)[28] and Lopes-Lima *et al.*, (2021, 2024)[29,41].

Unio tumidus **Philipsson, 1788** is characterised by a broadly oval to wedge-shaped shell, moderately to strongly inflated, with thick and solid valves, equivalve, and inequilateral (**Error! Reference source not found.**.C). It is quite inflated as compared to either *U. pictorum* or *U. bruguierianus*, and this can easily be seen in lateral view. The periostracum is smooth, olive-green to dark brownish-green in coloration, darkening progressively with advancing ontogeny, and displays prominent concentric growth lines across the entire outer surface. The umbones are broad, inflated, and positioned well anterior to the midline, bearing characteristic double-looped sculpture consisting of multiple rows of bilobed folds, a diagnostic feature that distinguishes *U. tumidus* from *U. pictorum* and *U. bruguierianus* [40]. The anterior margin is broadly rounded, while the posterior margin gradually tapers to a somewhat pointed end, imparting the characteristic wedge-shaped outline of the species. The dorsal margin is slightly convex and the ventral margin is broadly and evenly curved. The hinge apparatus is well-developed; pseudocardinal and lateral teeth are thick, rugged, and peg-like, and the lateral teeth are frequently curved, clearly distinguishing this species from *U. pictorum* in which the teeth are straight, thin, and compressed [40]. The anterior and posterior adductor muscle scars are well-impressed. The nacre is white to bluish-white internally. Shell length typically ranges from 60 to 100 mm, though considerable morphological variation exists among populations across the species range, likely attributable to differences in habitat conditions, water flow, and substrate composition [29,40]. These findings were similar to the findings of Klishko *et al.*, (2017)[40], Araujo *et al.*, (2018)[28] and Lopes-Lima et al. (2024)[41].

Figure 2: Shows A. *Unio pictorum* , B. *Unio bruguierianus* , C. *Unio tumidus* .

3. 2 Molecular Identification and Species Confirmation

To confirm the morphological identification of samples, molecular analysis was accomplished by amplification of the COI gene of bivalves. The mitochondrial COI gene PCR amplification generated distinct amplicons with a length of approximately 720 bp in all examined specimens [33,42]. The integrity of the amplified products was also confirmed by Agarose gel electrophoresis and displayed clear and specific bands with no nonspecific amplification and primer-dimer artefacts as shown in **Error! Reference source not found.** [30,35]. Universal Folmer primers (LCO1490/HCO2198) were also found to amplify the COI barcode region of the COI gene in a wide variety of molluscan taxa including freshwater bivalves [43,44]. It is worth mentioning, COI gene has become the molecular marker of choice to identify the species at the species level in the Unionidae because it is highly interspecifically divergent and minimally intraspecifically variable [40,45]. This confirms that all of the specimens were successfully amplified to confirm that the extracted DNA is of the required quality and that the PCR process was successfully optimised to unionid bivalve tissue, as is the case with similar molecular studies of freshwater mussels [28].

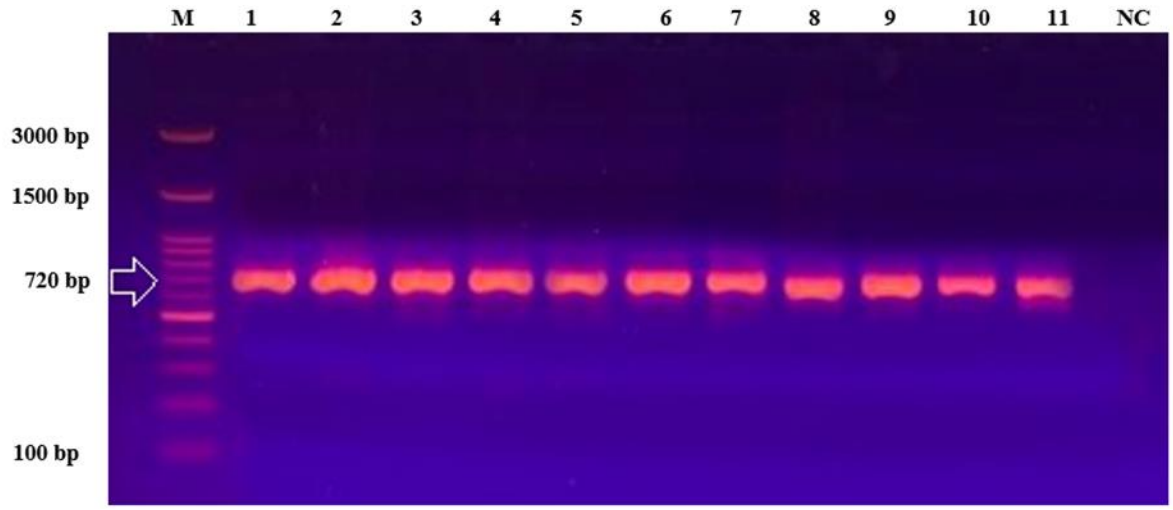

Figure 3: PCR amplification of partial COI gene of bivalves wells, wells start with the Ladder (3000-100 bp), lane1-11, amplification of the gene bands with size of 720 bp, NC control of negative positions without the band.

BLAST analysis of the obtained COI sequences revealed high sequence similarity (99.8–100%) with reference sequences deposited in GenBank as shown in **Error! Reference source not found.**. Based on this molecular comparison, three unionid species were identified: *Unio pictorum*, *Unio bruguierianus* and *Unio tumidus*. The specimens corresponding to *U. pictorum* (accession numbers PX452484, PX452492, PX452493) showed 100% identity with the GenBank reference sequence JQ253874 and 99.82% similarity with several additional reference sequences including OQ564391, MZ165233, and JX046581. The specimen identified as *U. bruguierianus* (PX452488) showed 100% identity with OR841701 and OR841704, while the *U. tumidus* specimen (PX452494) matched at 100% identity with multiple reference sequences including MZ165270, AY074807, KY021076, JQ253872, MK034161, and PP204156. The generated sequences were deposited in the NCBI GenBank database under accession numbers PX452484, PX452492, and PX452493 (*U. pictorum*), PX452488 (*U. bruguierianus*), and PX452494 (*U. tumidus*). *Unio pictorum* and *Unio tumidus* are hereby reported for the first time from Iraq based on both morphological and molecular evidence, with no prior records of either species in the country [22,46]. *Unio bruguierianus*, previously recorded in Iraq only morphologically as *Unio ciconius* Kobelt, 1913 (Annandale, 1918) [47], a taxon now synonymised within the *U. crassus* complex, is here confirmed as a distinct species and documented for the first time based on molecular evidence, consistent with recent taxonomic revisions that recognised it as a valid species [28,29,41]. All of these observations are the first morphological and molecular evidence of *U. pictorum* and *U. tumidus* in Iraq, and the first molecular evidence of *U. bruguierianus* in the country. Although earlier records of the Iraqi bivalve fauna indicated some of the unionid species in terms of morphological characters only [46], the current paper presents the molecular data which would help to confirm these taxonomic placements with a higher degree of confidence. Clear high values of the sequence similarity (99.8 or more) between Iraqi samples and the reference sequences of European and Anatolian populations indicate that COI DNA barcoding can be used to identify species classification in the Unionidae, as has been seen in other geographical locations[29,48,49]. The current research thus complements the molecularly reported unionid fauna of Iraq with three species that have COI sequence information, therefore giving background reference sequences to future biodiversity and conservation researches in the Kurdistan Region and Iraq.

3. 3 Phylogenetic Relationships

Phylogenetic studies involving COI nucleotide sequences were done using Neighbor-Joining, Maximum Likelihood, and the Bayesian Inference technique. The group of specimens of the Iraqi bivalves depicted as a Neighbor-Joining tree formed with MEGA 11, **Error! Reference source not found.** showed that the group divided into three well-supported (100 -maximal support) clades of *U. pictorum*, *U. bruguierianus*, and *U. tumidus*. Even different genetic distances between the three species distinctly separated them. Topologies generated with the help of Maximum Likelihood and Bayesian Inference were concordant, which further supported positioning of all three species, being members of a genus Unio (family Unionidae). *U. pictorum* (PX452484, PX452492, PX452493) were then put into a well-established clade along with reference sequences in the GenBank, an attempt that confirmed the identity of this species at the molecular level. Neither *U. pictorum* nor *U. tumidus* has been previously recorded from Iraq in any published morphological or molecular survey [22,50], and the present study therefore represents the first morphological and molecular documentation of both species in the Iraq.

U. bruguierianus (PX452488) was placed within a well-supported clade with reference sequences available in GenBank. This identification is consistent with recent taxonomic revisions that recognized *U. bruguierianus* as a valid species distinct from the *U. crassus* complex [28,29,51]. Given that the only previous record of this species group from Iraq was documented as *Unio*

ciconius Kobelt, 1913 by Annandale (1918)[52], a taxon now synonymised within the *U. crassus* complex under current taxonomy, it is likely that this earlier Iraqi record corresponds to *U. bruguierianus* sensu Araujo et al. (2018)[28]. The present study therefore provides the first molecular confirmation of *U. bruguierianus* from Iraq, and represents the first documented record of this species from Erbil Province, Kurdistan Region.

Unio tumidus (PX452494) was placed within a strongly supported clade with reference sequences available in GenBank, confirming the presence of this species in Iraq for the first time based on both morphological and molecular evidence. The finding of this species, which is largely of European origin, extends the range of the species south eastwards into the Middle East upper Tigris basin, a major extension of the range [29].

These results support the usefulness of COI-based barcoding to delimit a species' taxonomic boundaries across morphologically diverse freshwater mussels as well as the significance of integrative taxonomic methods to identify a species accurately across poorly studied areas in Iraq.

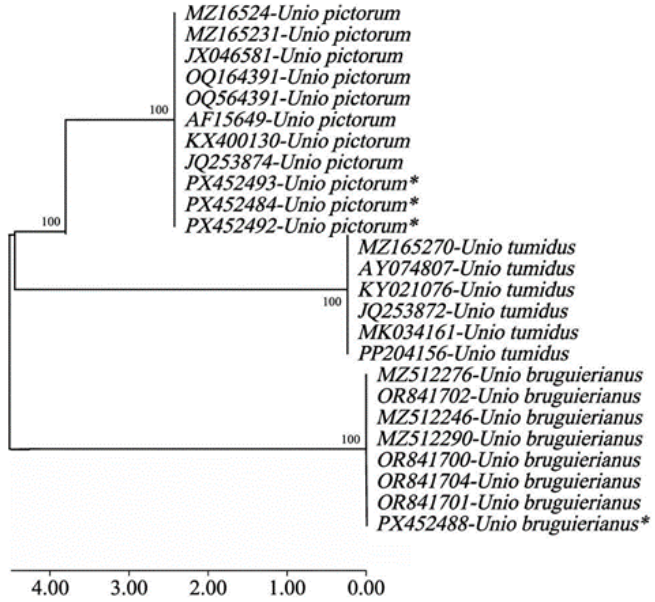

Figure 4: Neighbor-Joining phylogenetic tree constructed using MEGA 11, showing the relationships between the studied bivalve samples (*) and reference COI sequences retrieved from GenBank.

4 Conclusions

This paper presents the initial molecular characterisation of three species of Unio in Iraq through DNA barcoding based on COI and morphological analysis. The findings support the existence of *Unio pictorum*, *Unio bruguierianus*, and *Unio tumidus* on freshwater of the Erbil Governorate, Kurdistan Region, Iraq. *Unio pictorum* and *Unio tumidus* are first recorded in Iraq and both morphologically and molecularly, neither species has been previously recorded in the country. *Unio bruguierianus*, which had not previously been reported in the country other than morphologically as the *Unio ciconius* Kobelt, 1913, a taxon currently synonymised within the *U. crassus* complex, is confirmed as a species here and described based on molecular data, and in agreement with much more recent taxonomic treatments that have considered it a valid species. The taxonomic position of all three species within the genus Unio was confirmed as the results (Neighbor-Joining, Maximum Likelihood, and Bayesian Inference) of phylogenetic analysis generated three well supported clades with the highest bootstrap values (100%). The observed high sequence similarity (99.8-100) between Iraqi specimens and available reference sequences in GenBank indicates the efficacy and success of the COI barcoding in identifying species at the species level in Unionidae, as seen in its previous use in other geographic areas. The COI sequences obtained in this study and submitted to NCBI GenBank database with accession numbers PX452484, PX452492, PX452493 (*U. pictorum*), PX452488 (*U. bruguierianus*), and PX452494 (*U. tumidus*) are a useful molecular baseline for future research on the biodiversity, conservation, and evolutionary history of freshwater mussels in Iraq. These discoveries extrapolate known distributions of all the three species into the upper Tigris basin, which is a major contribution to biogeography. It is acknowledged that the present study is limited to two sampling sites within Erbil Governorate and a single collection period. Future studies would benefit from broader spatial coverage across multiple river systems in Iraq, including the Tigris and Euphrates basins, and from seasonal sampling to better capture the full diversity of unionid bivalves in the region. It is highly suggested that further molecular surveys on more freshwater ecosystems in Iraq should be conducted to learn more about the entire diversity, distribution, and conservation.

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Conflict of interests

The authors declare they have no conflict of interest.

Authors contribution

Luay Abdul-Qadir Ali and Jannah Nasraldeen Saber both participated in the development of the idea of the research. Each author played an important role in contributing ideas. Both authors contributed equally to the design and execution of the study. Their collaborative efforts and complementary expertise informed every stage of the work, and the findings presented herein reflect their shared commitment to advancing this field of research.

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