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The First Record of *Dina sivricensis* (Hirudinea: Erpobdellidae) from Duhok Province, Iraq, Based on COI Barcoding

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Abstract: Erpobdellid leeches are important freshwater predators that frequently pose major taxonomic problems due to their high morphological similarity. *Dina* Blanchard, 1892, is a genus distributed exclusively throughout Eurasia and has been differentiated from related genera by its mid-body annulation. This study presents the first molecular evidence for *Dina sivricensis* from Duhok Province, Iraq. Recently recorded as a new species from Türkiye, *D. sivricensis* is distinguished by its small cylindrical body and unique reproductive organs, including a small spherical atrium and an ellipsoidal atrial cornea. Species identification was verified using cytochrome c oxidase subunit I (COI) barcoding. The sequence obtained in this study (PZ144795) showed 95%–97% similarity to reference sequences of *D. sivricensis*, which were deposited under accession numbers MH013409 and MG949122 in GenBank. This study has recorded *D. sivricensis* from Iraq, thereby extending its known geographic range and contributing to the recorded diversity of erpobdellids in Iraq.

Keywords: Leech, molecular identification, phylogenetic analysis, biodiversity, genetic marker.

COI Barkodlama Temelinde *Dina sivricensis* (Hirudinea: Erpobdellidae)'in Irak'ın Duhok İlinden İlk Kaydı

Öz: Erpobdellid sülükleri, yüksek morfolojik benzerlikleri nedeniyle sıklıkla büyük taksonomik problemlere yol açan önemli tatlı su yırtıcılarıdır. Sadece Avrasya genelinde yayılış gösteren *Dina* Blanchard 1892 cinsi, vücut ortasındaki halka yapısı (anülasyon) ile akraba cinslerden ayırt edilmiştir. Bu çalışma, Irak'ın Duhok Valiliği'nden *Dina sivricensis* türüne ait ilk moleküler kanıtları sunmaktadır. Yakın zamanda Türkiye'den yeni bir tür olarak kaydedilen *D. Sivricensis* cinsinden küçük silindirik vücudu, küçük küresel atrium ve elipsoidal atrial korneayı içeren benzersiz üreme organları ile ayırt edilir. Tür teşhisi, sitokrom c oksidaz alt birim I (COI) barkodlaması kullanılarak doğrulanmıştır. Bu çalışmada elde edilen dizi (PZ144795), GenBank'ta MH013409 ve MG949122 erişim numaralarıyla kayıtlı *D. sivricensis* referans dizileri ile %95-%97 oranında benzerlik göstermiştir. Bu çalışma *D. sivricensis*'i Irak'tan kaydederek türün bilinen coğrafi dağılımını genişletmiş ve Irak'taki erpobdellid çeşitliliğine katkıda bulunmuştur.

Anahtar kelimeler: Sülük, moleküler tanımlama, filogenetik analiz, biyoçeşitlilik, genetik belirteç.

1. Introduction

Erpobdellidae leeches are an important component of the benthic fauna in freshwater habitats of the Northern Hemisphere, serving as key predators of various small invertebrates, such as insect larvae and oligochaetes (Kutschera, 2003; Sket & Trontelj, 2008). However, the species of this family, especially those belonging to the three most representative genera: *Dina* Blanchard, 1892, *Erpobdella* de Blainville, 1818, and *Trocheta* Dutrochet, 1817, often pose difficulties for their identification, especially because of their high degree of similarity (Saglam et al., 2025; Siddall, 2002). The genus *Dina* is endemic to Eurasia and differs from other related species in the particular pattern of mid-body annulation, in which the posterior annulus of each segment is expanded and divided once (Nesemann & Neubert, 1999; Pešić & Grosser, 2022).

The traditional morphological identification of members of this group is often difficult due to the variable nature of their external characters, including color patterns and eye positions, which are often lost during specimen preservation (Nesemann & Neubert, 1994; Saglam et al., 2025). Although there were earlier suggestions for the synonymization of the members of this group under the genus *Erpobdella* (Siddall, 2002), many researchers have continued to recognize *Dina* as a separate genus based on the unique morphological and evolutionary characteristics of the genus (Bilal et al., 2017; Saglam et al., 2025). Thus, molecular barcoding based on the mitochondrial cytochrome c oxidase subunit I (COI) gene has been standardized and proven to be an efficient method for identifying members of the Erpobdellidae family (Pešić & Grosser, 2022; Saglam et al., 2025; Zewayee & Ali, 2024).

Dina sivricensis Sağlam, Saunders & Shain, 2025, was recently described as a new species from the Kaniçi stream in Elazig, Türkiye (Saglam et al., 2025). Morphologically, the species is differentiated from other species by its

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relatively small, thin, cylindrical body, four pairs of eyes, and a small spherical atrium with medium-length ellipsoid atrial cornua (Saglam et al., 2025). Although other species, such as *Dina takasensis* Sağlam, Saunders & Shain, 2025 and *Erpobdella madenensis* Sağlam, Saunders & Shain, 2025, were recorded in the same area, *D. sivricensis* is distinguished from them by its unique reproductive system and a specific genetic marker at the COI locus (Saglam et al., 2025). In Iraq, the species diversity of erpobdellid leeches has recently been supplemented by the records of *Dina lineata* O.F. Müller, 1774 from the Greater Zab River (Ali & Jawair, 2013), and *Dina punctata* Johansson, 1927 from the Lesser Zab River (Bilal et al., 2017). Recently, *Dina prokletijaca* Grosser and Pešić, 2016 was recorded for the first time in the Bekhal stream of Erbil Province (Zewayee & Ali, 2024).

Despite these developments, the erpobdellid leeches are considered a neglected taxonomic group in the regional scientific literature (Saglam et al., 2025). This study presents the first molecular record of the species *D. sivricensis* in the Duhok Province of Iraq. This study confirms the presence of the species beyond its type locality in Türkiye using the COI barcoding method, thereby expanding its distribution.

2. Material and Method

2.1. Study Area and Sample Collection

The study area is Duhok Province in Iraq. This province is mostly mountainous, sharing borders with Türkiye and Syria (Fig. 1). Its latitudes and longitudes fall between 36°40' to 37°20'N and 43°20' to 44°10'E, respectively. Duhok Province is divided into seven districts: Zakho, Sumel, Duhok, Amedi, Shekhan, Akre, and Bardarash. In the present study, 12 leech specimens were collected between July and October 2025 from Safyoke Spring, located in a deep valley near Nordina village, Zakho District (37°13'44.6"N 42°48'06.1"E). Nordina is an uninhabited village with no permanent residents and contains a single natural spring together with two small buildings used seasonally as sheep shelters. A water well has been constructed directly over the spring and excess water overflows to form a small stream. The inner concrete surfaces of the well were covered with algae and all specimens were collected attached to these submerged algal-covered concrete blocks. The specimens were preserved in 95% ethanol and kept at -20°C for molecular study.

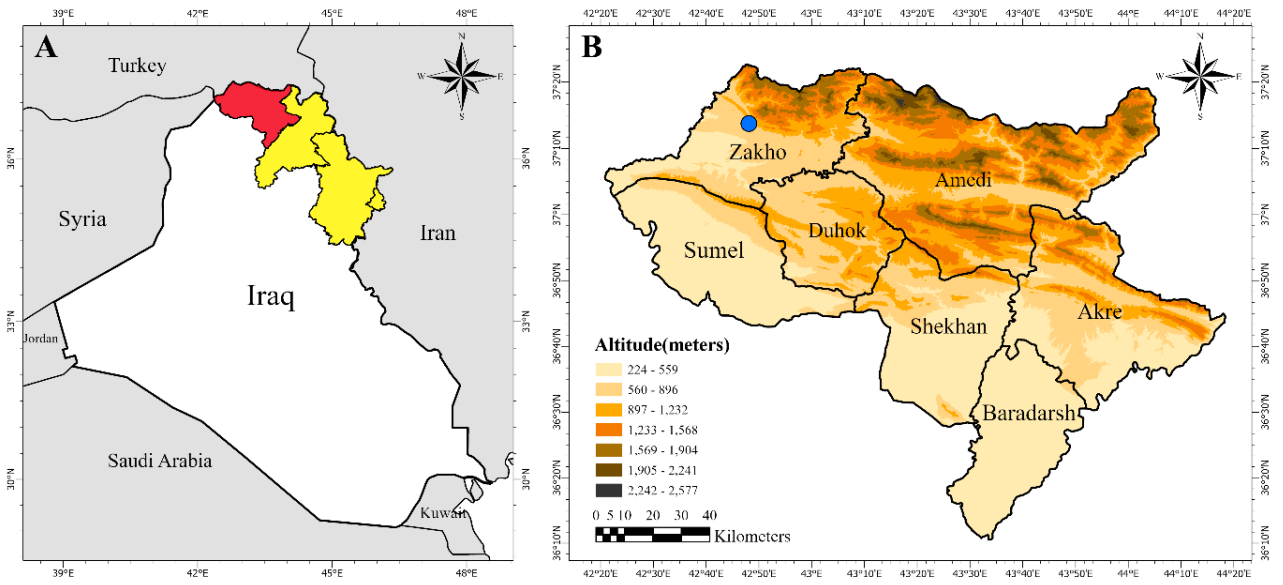

Figure 1. (A) Map of Iraq showing the location of Duhok Province (red), where *Dina sivricensis* was collected. (B) Topographic map of Duhok Province showing the sampling locality in Zakho District (blue dot).

2.2. Molecular Analysis

2.2.1. DNA Extraction

Genomic DNA was isolated from the posterior sucker tissue using the AddPrep Genomic DNA Extraction Kit (Addbio, South Korea). Before extraction, the tissue samples were soaked in physiological saline solution (PiONEER, Iraq) for 15–20 min to eliminate residual ethanol and then carefully dried on sterile filter paper. Each sample was transferred to a sterile 1.5 mL Eppendorf tube and mechanically homogenized with a polypropylene micropestle (Shenzhen Baimai Life Science, China). The homogenized tissue was then digested with the lysis buffer and Proteinase K supplied in the kit, followed by incubation at 56°C for 1–3 h until the tissue was completely lysed. The remaining extraction steps were performed strictly according to the manufacturer's protocol. The purified DNA was finally stored at −20°C for subsequent molecular analyses.

2.2.2. Mitochondrial COI Gene Fragment Amplification

Polymerase chain reaction (PCR) targeting the mitochondrial COI barcoding region was performed using a GeneAmp® PCR System 9700 (Applied Biosystems, USA). Universal primers described by Folmer et al. (1994) and listed in Table 1 were used for amplification. These primers are widely employed to amplify an approximately 710 bp fragment of the COI gene, which serves as a standard DNA barcode for the identification of a broad range of invertebrate taxa. PCR was performed in a total volume of 50 µL, consisting of 1.25 µL of each primer, 25 µL of Add Taq Master Mix (2×; Addbio, South Korea), 5 µL of DNA template (~20 ng/µL), and 17.5 µL of water. The thermal cycling profile applied in this study is given

in Table 2. Successful amplification was confirmed by electrophoresis on 1.5% agarose gel and the resulting amplicons were visualized under UV illumination. PCR products were then submitted to Macrogen Inc. for bidirectional sequencing.

Table 1. Sequences of universal primers for COI barcode region amplification (Folmer et al., 1994).

Primer Name	Sequence (5′→3′)	T_m	GC%
LCO1490	GGTCAACAAATCATAAAGATATTGG	53.6	32
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	59.1	35

Table 2. PCR thermal cycling protocol: steps and conditions.

Steps	Temperature	Time	Cycles
Initial Denaturation	94 °C	5 minutes	1 cycle
Denaturation	94 °C	30 seconds	35 cycles
Annealing	50 °C	30 seconds	
Extension	72 °C	30 seconds	
Final extension	72 °C	7 minutes	1 cycle

Abbreviations: (T_m) melting temperature, (GC%) GC content.

2.3. DNA Sequence Processing and GenBank Submission

Sanger sequencing chromatogram files were visually inspected and trimmed using the SeqTrace 0.9.0 software package (Stucky, 2012) to generate consensus sequences. The consensus sequences were used to search and retrieve close sequence matches using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) available through the National Center for Biotechnology Information (NCBI) for subsequent phylogenetic reconstruction. The sequences generated in this study were submitted to GenBank and their accession numbers are listed in Table 3.

Table 3. List of *Dina* species included in the molecular analyses, their localities, and GenBank accession numbers, including the *D. sivricensis* sequence generated in this study and the outgroup.

Species	Localities	Accession numbers
D. sivricensis	Iraq	PZ144795 (This Study)
D. sivricensis	Türkiye	MG949122
D. sivricensis	Türkiye	MH013409
D. nesemanni	Montenegro	OQ507613
D. nesemanni	Montenegro	OQ507614
D. nesemanni	Montenegro	OQ507615
D. nesemanni	Montenegro	OQ507616
D. lineata	Azerbaijan	ON098208
D. lineata	Azerbaijan	ON098209
D. lineata	Azerbaijan	ON098213
D. lineata	Azerbaijan	ON098216
D. lineata	Iraq	OQ692094
D. lineata	Sweden	PP181987
D. minuoculata	Montenegro	OM628836
D. minuoculata	Montenegro	OM628837
D. serbica	Serbia	OM628828
D. serbica	Serbia	OM628829
D. serbica	Serbia	OM628838
E. ochoterenai	Outgroup	MK208651

2.4. Phylogenetic Analysis

The multiple sequence alignment was performed using ClustalW algorithm implemented in MEGA 11 (Tamura et al., 2021). Phylogenetic analysis was performed by constructing a maximum likelihood (ML) tree, with node support evaluated through 1,000 bootstrap replicates. The best nucleotide substitution model was selected based on Bayesian Information Criterion (BIC) scores (Nei & Kumar, 2000). The maximum likelihood (ML) tree was constructed in MEGA 11, exported in Newick format, visualized and edited in FigTree 1.4.4, and the final layout and figure refinement were completed in Adobe Illustrator version 30 (Adobe, USA) for publication.

To assess evolutionary divergence, mean genetic distances within and between *D. sivricensis* and closely related sequences (excluding the outgroup) were computed using the Kimura 2-parameter model (Kimura, 1980).

3. Results

Systematics:

Phylum: Annelida Lamarck, 1809

Class: Clitellata Michaelsen, 1919

Subclass: Hirudinea Lamarck, 1818

Family: Erpobdellidae Blanchard, 1894

Subfamily: Trochetinae Perrier, 1897

Genus: *Dina* Blanchard, 1892

Dina sivricensis Sağlam, Saunders & Shain, 2025 (Fig. 2)

Figure 2. Representative live specimen of *Dina sivricensis*.

Molecular Phylogenetic Analysis

Twelve leech specimens showing the same morphological features were collected and the COI barcode region was successfully sequenced from one representative specimen identified as *D. sivricensis*, collected from the Zakho district (Table 3). Additionally, 17 COI gene sequences from various *Dina* species were obtained from the NCBI database. Furthermore, a sequence of *Erpobdella ochoterenai* Caballero, 1932 was used as an outgroup for the phylogenetic tree (Table 3).

To confirm the species identity, the obtained COI gene sequence was subjected to BLAST analysis against the GenBank database. The results revealed that the COI gene sequence of *D. sivricensis* isolate PZ144795 showed high similarity to conspecific sequences recently reported from Türkiye (MH013409 and MG949122), with percent identities of 95% and 97%, respectively (Table 3).

A maximum likelihood (ML) phylogenetic tree for *D. sivricensis* was constructed in MEGA; the best-fitting substitution model was T92+G (Tamura, 1992). The tree with the highest log-likelihood value (-2388.43) was selected for interpretation (Fig. 3). In the resulting tree, the *Dina* species from this study were grouped into two clusters. Clade I included *Dina serbica* Pešić & Grosser, 2022, *Dina minuoculata* Grosser, Moritz & Pešić, 2007, *Dina sivricensis*, and *Dina nesemanni* Grosser & Pešić in Grosser, Rewicz, Jovanović, Zawal & Pešić, 2023. Clade II included *Dina lineata*. The COI sequence generated from the representative specimen formed a monophyletic clade with high bootstrap support (100%) alongside its conspecific sequences obtained from Türkiye (MH013409, MG949122). Similarly, the COI gene sequences of *Dina serbica*, *Dina minuoculata*, *Dina nesemanni*, and *Dina lineata* formed well-supported clades, with high bootstrap values ranging from 98 to 100%.

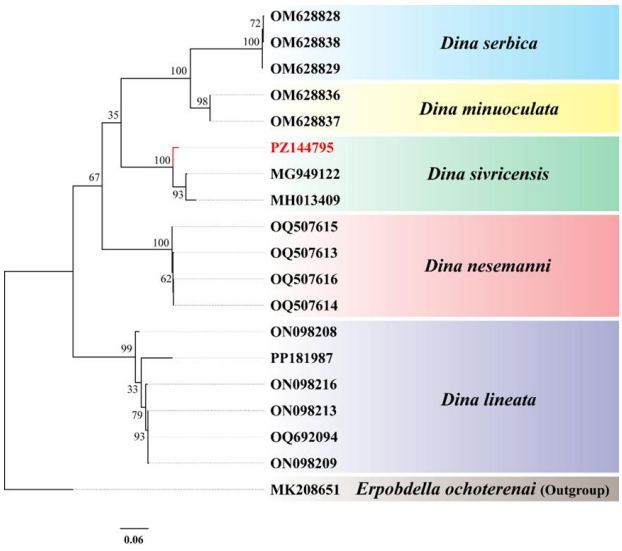

Figure 3. Maximum likelihood phylogenetic tree of *D. sivricensis* inferred from the COI gene. The study sequence is shown in red. Numbers above and below branches indicate bootstrap support values. The scale bar reflects the number of substitutions per nucleotide site.

The results of the evolutionary divergence analysis, determined using the Kimura 2-parameter method (Table 4), showed that there was moderate intraspecific variation between the isolates of *D. sivricensis* from the current study and those from Türkiye (MH013409, MG949122), with a mean intraspecific divergence of 3.44%. This suggests that there is considerable genetic variation within the species. Furthermore, the isolates of *D. sivricensis* from the current study and Türkiye were significantly divergent from *D. nesemanni* (mean interspecific divergences: 15.52%), *D. lineata* (17.93%), *D. minuoculata* (18.80%), and *D. serbica* (20.63%).

Table 4. Estimates of net evolutionary divergence among COI sequences within and between *Dina* species. The values are expressed as mean genetic distances based on the Kimura 2-parameter (K2P) model. The boldface values represent intraspecific divergences.

	Species	1	2	3	4	5
1	*D. sivricensis*	0.0344				
2	*D. nesemanni*	0.1552	0.0017			
3	*D. lineata*	0.1793	0.1806	0.0304		
4	*D. minuoculata*	0.1880	0.1827	0.1732	0.0000	
5	*D. serbica*	0.2063	0.2216	0.2024	0.1310	0.0023

4. Discussion

The molecular characterization of *D. sivricensis* from Zakho District, Duhok Province, Iraq, represents the first record of this species from Iraq and extends its known distribution beyond the type locality in Elazığ, Türkiye (Saglam et al., 2025). The present finding has greatly contributed to the knowledge of the leech fauna of Iraq, which has been enriched with several notable records, including *D. lineata* from the Greater Zab River (Ali & Jawair, 2013), *D. punctata* from the Lesser Zab River (Bilal et al., 2017), and *D. prokletijaca* from Erbil Province (Zewayee & Ali, 2024). In this study, the molecular technique involving the mitochondrial COI marker was successfully employed to confirm the identity of the *D. sivricensis* isolate from Iraq (PZ144795), which showed 95–97% sequence similarity to conspecific Turkish isolates (Saglam et al., 2025). Together with the phylogenetic position of the Iraqi specimen within the *D. sivricensis* clade, these results provide strong molecular support for its taxonomic identification.

The phylogenetic reconstruction further demonstrated that the Iraqi sequence clustered with Turkish *D. sivricensis* sequences in a well-supported monophyletic lineage, with a bootstrap value of 100%. This strong clustering is particularly important because it shows that the Iraqi material is not only morphologically consistent with *D. sivricensis*, but also genetically nested within the same species-level lineage. At the same time, the tree topology clearly separated *D. sivricensis* from other regional congeners, including *D. nesemanni*, *D. minuoculata*, *D. serbica*, and *D. lineata*. The placement of *D. sivricensis* in Clade I, together with several morphologically comparable congeners, also underlines the value of molecular data in resolving species boundaries within *Dina*, where external characters alone may be insufficient.

The results of the divergence analysis revealed a mean pairwise genetic distance of 3.44% between Iraqi and Turkish populations of *D. sivricensis*. This suggests a moderate level of intraspecific variation in this species, despite the geographic separation between the currently

known populations (Saglam et al., 2025). Regarding leech species classification, these results reinforce the distinctiveness of this species. This distinctiveness stems from the fact that genetic differences with regional congeners were substantially greater: 15.52% to *D. nesemanni*, 17.93% to *D. lineata* and 20.63% to *D. serbica* (Table 4). Such genetic divergences align with those observed in other leech species. For example, in the *Helobdella stagnalis* Linnaeus, 1758 species complex, divergences in the COI gene greater than 10% are indicative of species boundaries (Saglam et al., 2018). Moreover, in the Balkans, where there are cryptic species of *D. serbica* and *D. minuoculata*, a genetic divergence of 11.96% in K2P distance exists. This again highlights the phenomenon of hidden speciation in Erpobdellidae that cannot be identified morphologically (Pešić & Grosser, 2022).

This distinction becomes even clearer when the intraspecific distances in Table 4 are considered. The mean intraspecific divergence was 0.00% in *D. minuoculata*, 0.17% in *D. nesemanni*, and 0.23% in *D. serbica*, whereas *D. lineata* showed a somewhat higher value of 3.04%. The intraspecific divergence observed in *D. sivricensis* (3.44%) is therefore higher than in several of the compared species but still markedly lower than the interspecific distances separating it from its congeners. Taken together, these results are consistent with the interpretation that the Iraqi sample belongs to *D. sivricensis* and suggest the presence of measurable geographic variation within the species.

Such levels of divergence are also consistent with patterns reported for other leech taxa. For example, in the *Helobdella stagnalis* species complex, COI divergences greater than 10% have been considered indicative of species boundaries (Saglam et al., 2018). Moreover, in Balkan representatives of *D. serbica* and *D. minuoculata*, a K2P distance of 11.96% has been reported, highlighting the occurrence of cryptic diversity within Erpobdellidae that cannot always be detected through morphology alone (Pešić & Grosser, 2022). Against this background, the divergence values obtained here fit well within the broader molecular framework currently used to distinguish species-level lineages in freshwater leeches.

The relatively elevated intraspecific divergence observed between the Iraqi and Turkish populations may reflect geographic isolation associated with the mountainous terrain separating the currently known populations. Such geographic barriers may restrict dispersal and gene flow, resulting in population differentiation over time. Although the available molecular and morphological evidence supports the identification of the Iraqi specimen as *D. sivricensis*, the observed level of divergence also warrants further investigation to determine whether it represents geographic population structuring, an early stage of lineage diversification, or the presence of cryptic diversity within the species. Because molecular data are currently available from only a single Iraqi specimen, these possibilities remain speculative and should be tested through broader geographic sampling and the inclusion of additional molecular markers.

The need for integrative taxonomy, integrating molecular and morphological information, is highlighted by the long-standing difficulties in species delimitation in the *Dina* genus. In fact, previous taxonomic characters, such as annulation and pigmentation, have proven to be extremely variable, influenced by various environmental and preservative factors (Nesemann & Neubert, 1994; Pešić & Grosser, 2022; Saglam et al., 2025). In the present study, all 12 collected specimens shared the same general morphological features and one representative specimen was selected for COI sequencing. Although DNA was extracted from additional specimens, successful PCR amplification and high-quality sequencing were achieved for only one individual, while financial limitations prevented further sequencing attempts. Consequently, molecular analyses were restricted to a single representative specimen. This combined approach strengthens the reliability of the identification, even though molecular data were obtained from a single individual. At the same time, the use of only one sequenced specimen means that the extent of genetic variation within the Iraqi population should be regarded as preliminary. Broader sampling from different Iraqi localities will be necessary to determine whether the observed divergence reflects local population structure, wider geographic differentiation, or a more complex phylogeographic pattern.

Although previous taxonomic revisions have suggested that *Dina* species should be considered as synonyms of *Erpobdella*, unique reproductive structures and separate phylogenetic lineages support the continued use of *Dina* as a valid genus (Siddall, 2002; Bilal et al., 2017; Saglam et al., 2025). The present molecular evidence is consistent with this view, as the analyzed Dina species formed recognizable, genetically differentiated lineages in the phylogenetic tree.

Overall, the new record from Duhok fills an important gap in our knowledge of Mesopotamian leeches and expands the documented range of *D. sivricensis* into Iraqi freshwaters. More importantly, it provides the first molecularly supported evidence of the species in Iraq and highlights the value of combining morphology with DNA-based approaches in regional hirudinean surveys. This finding also opens the way for future studies on the diversity, phylogeography, and ecology of Iraqi freshwater leeches, especially in poorly explored river systems from both taxonomic and molecular perspectives.

Ethics committee approval: Ethics committee approval is not required for this study.

Conflict of interest: The authors declare that there is no conflict of interest.

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