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ORIGINAL ARTICLE

AN INTEGRATIVE STUDY ON *LIMNATIS PALUDA* (TENNENT, 1859) (CLITELLATA, ARHYNCHOBDELLIDA, PRAOBDELLIDAE): MORPHOLOGICAL CHARACTERS, MOLECULAR DATA, AND CONFIRM OCCURRENCE IN IRAQ

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ABSTRACT

The current study represents a comprehensive morphological and molecular characterization of *Limnatis paluda* (Tennent, 1859) (Clitellata, Arhynchobdellida, Praobdellidae), collected from five freshwater springs in the Taqtaq District of Erbil Province, Kurdistan Region, Iraq. Most prior research on this species has predominantly concentrated on the study of morphological data. DNA sequencing is often advantageous in taxonomy, as it yields information not obtained from morphological studies. The primary objective was to molecularly characterize *L. paluda* using the nuclear 18S rDNA region and the mitochondrial cytochrome c oxidase subunit I (COI) gene by PCR and nucleotide sequencing techniques. The sequences were acquired and compared to existing GenBank sequences. The results support the validation of *L. paluda* in Iraq using both traditional (morphology-based) and modern (molecular-based) techniques. The latter showed a 100% identity percentage of *L. paluda* when compared to the registered sequences in NCBI. The Maximum Likelihood (ML) approach was used to construct the phylogenetic relationship. The results indicate that PCR sequencing is an effective and reliable molecular approach for identifying *L. paluda*, complementing traditional morphological techniques and enhancing the understanding of its biology, it confirming the occurrence of this leech in Iraq.

Keywords**:** COI gene, *Limnatis paluda*, PCR, Phylogeny,18S rDNA.

INTRODUCTION

Leeches, belonging to the phylum Annelida, represent a diverse group of segmented worms comprising over 22,000 species (Mann, 1962; Sawyer, 1986). Among these, approximately 700 species are leeches, with about 100 marine, 90 terrestrial, and the remainder freshwater. As trophic components, leeches play a pivotal role in aquatic ecosystems, influencing ecological balance and nutrient cycling. Morphologically, they are cylindrical or

dorsoventrally flattened segmented worms equipped with anterior and posterior suckers, with the mouth situated within the anterior sucker (Ayhan *et al*., 2024).

The leeches included in the class Clitellata encompass two primary orders: Rhynchobdellae, with a proboscis. For instance, the colossal Amazonian leech *Haementeria ghilianii* (de Filippi, 1849), the duck leech *Theromyzon tessulatum* (Müller, 1774), and the fish leeches (Family Piscicolidae), and Arhynchobdellae leeches without a proboscis (e.g., the horse leech *Haemopis sanguisuga* (Linnaeus, 1758), stinging leech *Haemadipsa picta* Moore, 1929, and the "medicinal" leeches *Acrobdella* spp. and *Hirudo* spp., among others) (Phillips *et al*., 2019). Jawed leeches, members of the suborder Hirudiniformes within Arhynchobdellae, inhabit terrestrial and aquatic environments, feeding predominantly on blood. Some of the species of leeches that act as temporary ectoparasites are the members of families Glossiphoniidae, Praobdellidae, and Haemadipsidae, and these are those leeches that feed upon the blood of various vertebrates, including frogs, reptiles, birds, fish, and mammals, including human beings (Sevim *et al*., 2019).

The genus *Limnatis* Moquin–Tandon, 1827 belongs to the family Praobdellidae, Order Arhynchobdellida (Utevsky *et al*., 2022). It is a group of hematophagous leeches distributed throughout the Middle East and parts of Asia (Raele *et al*., 2015). According to the current classification, there are three species in the genus: *L. paluda*, *L. bacescui* (Manoleli, 1972), and *L. nilotica* (Savigny, 1822) (Nakano *et al*., 2015). *L. paluda* is an aquatic species that typically inhabits lakes and streams (Utevsky *et al*., 2022). Juvenile stages are small and highly motile swimmers that may invade the nasal or oral cavities of humans and animals through contaminated water. Once inside the host, they firmly attach to mucosal surfaces using their terminal suckers and feed on blood and tissues, resulting in local inflammation and blood loss (Solijonov *et al*., 2022; Bantihun *et al*., 2023).

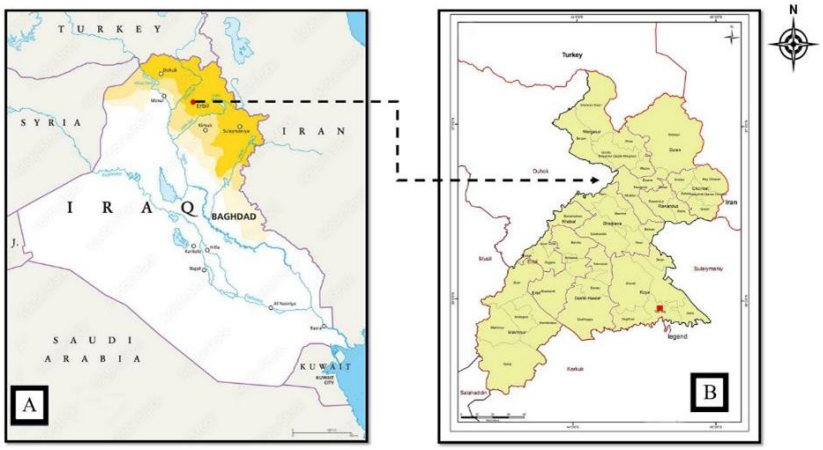
In Iraq, the first record of this genus was *L. nilotica* (Savigny, 1822*),* described in a human case of internal hirudiniasis by Almallah (1968). More recently, *L. paluda* was morphologically identified by Bilal *et al.* (2023) based on salivary gland ultrastructure in specimens collected from Erbil. However, that identification lacked comprehensive morphological characterization and was not supported by molecular data. To date, no study has provided molecular evidence confirming the presence of *L. paluda* in Iraq.

The present study addresses this gap by employing an integrative taxonomic approach to identify *L. paluda* from the Kurdistan Region of Iraq. Detailed morphological examinations were conducted using light microscopy, focusing on external characters (body coloration, sucker morphology, eye arrangement, clitellum, and gonopore position) as well as internal reproductive anatomy. Molecular identification was done to supplement these observations, where PCR amplification and sequencing on the mitochondrial cytochrome c oxidase subunit I (COI) and nuclear 18S rDNA gene markers were conducted. A combination of these approaches increases the level of species-level identification and explains the phylogenetic positioning of *L. paluda* in the Arhynchobdellida group.

Accordingly, this study provides the first combined morphological and molecular characterization of *L. paluda* in Iraq, offering valuable insights into its taxonomy, distribution, and biodiversity status within the region.

MATERIALS AND METHODS

Collection: The survey of different water bodies in the Erbil Province, Kurdistan Region, was carried out from July to October 2024, with a total of 40 leech specimens being collected. Sampling was conducted in five freshwater springs in the Taqtaq District (Latitude: 35.88886°; Longitude: 44.58557 °) (Map. 1, Pl. 1).



Map (1): (A) Map of Iraq that shows the Kurdistan Region (Yellow), (B) Map of Erbil Province shows the specimens collection sites (■).

Plate (1): Sites of specimens collection in Taqtaq District/Erbil Province.

Habitats investigated included ponds, irrigation canals, cow troughs, and specimens collected on the undersides of rocks, submerged vegetation, the banks, and sides of streams. The sample sites were chosen on the basis of reported cases of cattle leech infestations and suitable environmental conditions. Leeches were gathered manually and with aquarium scoops, ensuring little disruption to their natural habitat. Upon collection, the specimens were promptly placed in glass jars containing water from their native environment. To prevent

escape, the jars were sealed using fine, permeable fabric. They were then taken alive to the Zoology Research Laboratory of Salahaddin University.

In the laboratory, the leeches were kept in a glass tank 30 cm wide and 27 cm deep, and the water was non-chlorinated. They were given a meal once per week and could feed on the introduced frogs. The aquarium conditions were exquisitely maintained, and the water was replaced every three days to provide an optimum environment for the leeches (Tubtimon *et al.*, 2014).

Identification: The specimens were identified to the species level based on morphological features using the identification keys provided by Mann (1962), Sawyer (1986), Nakano *et al.* (2015), and Solijonov *et al.* (2022). Diagnostic characters, including body size and shape, coloration patterns, jaw structure, the configuration of the anterior and posterior suckers, and internal reproductive anatomy, were examined. Identification was not verified by a museum but was supported by molecular analysis (COI and 18S rDNA), which confirmed the species as *L. paluda* through BLAST comparison with GenBank references.

External examination: The collected leech specimens were prepared for morphological examination. Some specimens were relaxed by being placed in small to medium jars filled with distilled water, and dropwise additions of 10% ethanol were used until the leech did not respond to the touch. During the next 40-60 minutes, the leeches became limp and would not respond to touch. Under an Olympus stereomicroscope, the Leeches were identified based on their external characteristics, including their general body and mouth shape, sucker form, number and arrangement of eyespots (ocelli), papillae, sensillae, number of annuli per somite (segments), and the location and shape of the male and female gonophores (Sawyer, 1986; Schenková *et al.*, 2021).

Internal examination: For examination of the internal morphology, six specimens were dissected with a sterile lancet and then cleaned with distilled water (Ayhan *et al.*, 2021). Dissections of specimens were pinned with normal saline and performed under a stereomicroscope using fine dissection tools to expose the internal structures, including the pharynx, crop, intestine, and reproductive organs. The male and female reproductive systems were examined once the dorsal midline was dissected and fixed with insect needles on a wax tray. Following an incision along the anterior sucker's ventral midline, the jaws and teeth inside the sucker were visible (Wang *et al.*, 2022).

Photomicrographs: An iPhone 11 Pro Max equipped with an ultra-wide lens featuring a $f/2.4$ aperture and a 120° field of view, a wide lens with a $f/1.8$ aperture, and a 12-megapixel telephoto lens was utilized for picture capture under a stereo and light microscope.

DNA extraction: The fresh leech specimens were submerged in a 10% ethanol until they became insensitive to touch. Using a scalpel, around half of the caudal sucker was cut off, then DNA was extracted using the PureLink® Genomic DNA Kits in accordance with the manufacturer's instructions. Whenever possible, the caudal sucker's tissue was extracted from postmortem specimens to prevent contamination with intestinal contents. The DNA quantity and quality were assessed using a NanoDrop (ND-1000, USA). The genomic DNA purity ranged between (1.7-2) (Ayhan *et al.*, 2024).

DNA amplification and sequencing: The polymerase chain reaction (PCR) was employed to amplify two DNA segments: the mitochondrial cytochrome c oxidase subunit I (COI) gene and the nuclear 18S rDNA region, using primers universally designed for these targets (Borda *et al.*, 2004). The COI gene was amplified using the primer pair LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′), as described by Borda *et al.* (2004). For the 18S rDNA region, the universal primers C1 (5′-ACCCGCTGAATTTAAGCAT-3′) and C3 (5′-CTCTTCAGAGTACTTTTCAAC-3′) were employed, following the protocol outlined by Apakupakul *et al.* (1999).

PCR amplifications were done in the presence of a total reaction volume of 25 µL in a SimpliAmp™ Thermal Cycler (Applied Biosystem, USA. The reaction was prepared with 1 µL of forward and reverse primers (10 µM), 5 µL of genomic DNA (~50 ng), 12.5 µL of Taq PCR Master Mix (Addbio, Korea), and 5.5 µL of deionized water (dH_2O) to obtain the final volume. The amplification protocol followed the conditions described by Phillips *et al.* (2019). In the case of the COI gene, the thermal profile involved an initial denaturation at 95 °C over a period of 5 minutes, followed by 35 cycles comprising denaturation at 95 °C, annealing at 49 °C, and extension at 72 °C, all lasting 30 seconds each, and a final extension at 72 °C lasting 7 minutes. The amplification conditions for 18S rDNA were identical, except for the annealing temperature, which was set at 55 °C (Saglam *et al.*, 2018). PCR products were separated using the electrophoresis method on a 2% agarose gel that had been prepared by dissolving 1g of agarose powder in 50 ml of TBE buffer and adding 3 µL of ethidium bromide to visualize DNA. A total of 5 µL of each PCR product, together with a DNA ladder, was loaded into the wells, and electrophoresis was performed at 100 V and 3 W for approximately 40 minutes. DNA fragments were observed under a UV transilluminator, with the expected amplicon sizes of ~650 bp for COI and ~345 bp for 18S rDNA.

In this study, PCR-amplified DNA was sequenced bidirectionally using the Sanger method on an ABI SeqStudio Genetic Analyzer (Applied Biosystems, USA) at Exogene Genetics Diagnosis. The resulting sequences were analyzed through nucleotide BLAST searches against the NCBI GenBank database and subsequently employed for phylogenetic analysis (Altschul *et al.*, 1990).

Phylogenetic methods: The obtained COI and 18S rDNA sequences (both strands) were verified and edited using BioEdit (Jovanović *et al.*, 2021). A BLAST search was employed on the sequences in the GenBank collection that were similar to the sequences generated out of the specimens analyzed during this study. Subsequently, 18 sequences from representatives of the genus *Limnatis* were downloaded from GenBank and were included for assessment (Tab. 1).

The MEGA X program, version 10.1, was used to compute the differences between DNA sequences (Kumar *et al.*, 2018). It was also employed to compute a Neighbor-Joining (NJ) tree (also based on p-distances) and a Maximum Likelihood (ML) tree provided in MEGA X with an initial NJ tree. In the NJ and ML trees, bootstrapping was done on 1000 replicates (Huelsenbeck *et al.*, 2001; Ronquist *et al.*, 2012).

Table (1): Summary of sequences and phylogenetic clustering of *Limnatis* species based on maximum likelihood analysis.

Taxa	GenBank	Country	Reference	Bootstrap Support (%)
L. paluda	PV056019.1	Current seq 1 (Iraq)	This study	86%
L. paluda	PV018631.1	Current seq 2 (Iraq)	This study	86%
L. paluda	PQ868999.1	Current seq 3 (Iraq)	This study	80%
L. paluda	PQ553448.1	Current seq 4 (Iraq)	This study	73%
L. paluda	KY989474.2	Iran	(Darabi-Darestani *et al.*, 2021)	80%
L. paluda	MZ318068.1	Uzbekistan	(Utevsky *et al.*, 2022)	97%
L. paluda	MZ318070.1	Azerbaijan	(Utevsky *et al.*, 2022)	97%
L. paluda	MZ318071.1	Uzbekistan	(Utevsky *et al.*, 2022)	97%
L. paluda	MZ318067.1	Uzbekistan	(Utevsky *et al.*, 2022)	97%
L. paluda	MZ318069.1	Uzbekistan	(Utevsky *et al.*, 2022)	97%
L. paluda	MW538548.1	Turkey	GenBank	96%
L. paluda	KY989472.2	Iran	(Darabi-Darestani *et al.*, 2021)	91%
L. paluda	KY989471.2	Iran	(Darabi-Darestani *et al.*, 2021)	89%
L. paluda	KY989473.2	Iran	(Darabi-Darestani *et al.*, 2021)	89%
L. nilotica	MZ318072.1	Morocco	(Utevsky *et al.*, 2022)	95%
L. nilotica	PQ187649.1	Tunisia	GenBank	95%
L. nilotica	PQ187648.1	Tunisia	GenBank	96%
Poecilobdella manillensis (Lesson, 1842)	OP648251.1	China (outgroup)	GenBank	—

RESULTS AND DISCUSSION

In the present study, 40 leech specimens were collected from five freshwater springs in the Taqtaq District. Specimens were scored using morphological characters, and the molecular analysis of the 18S rDNA and COI gene loci showed that all specimens belonged to. *L. paluda*.

External morphology

The leeches exhibited a dorsoventrally flattened body, tapered at the anterior, with a contracted length of approximately 5.5 cm, extending to 12 cm when fully expanded. This observation agreed with prior findings on *L. paluda* morphology (Nakano *et al.*, 2015). The characteristic shape facilitated efficient movement through aquatic environments and attachment to hosts, similar to other *Limnatis* species (Sawyer, 1986).

The specimens were light-green to brown-green on the dorsal side and dark green on the ventral side with an orange to dark-yellow lateral stripe (Pl. 2). In contrast to other species of leeches, *L. paluda* did not have black spots or patterned lines on the dorsal side. Color variation among the populations has been previously reported, with the specimens of the Middle East tending to have either green or dark green on their dorsal pigmentation (Darabi-Darestani *et al.*, 2016; Solijonov *et al.*, 2022) while those from India (Harding, 1927) and Kazakhstan (Nakano *et al.*, 2015) tend to be brownish-red. It is probable that these variations are the combination of genetic and environmental factors.

There were two types of suckers on the body, which included an anterior (oral) sucker and a posterior (caudal) sucker. The oral sucker was rather smaller and yet quite muscular, as it could expand significantly when it was attaching to the host. A median longitudinal furrow was present on the ventral surface, extending toward the buccal ring and leading into a deep oral chamber, which was separated by a transverse muscular septum (velum). The mouth appeared as a small triradiate opening situated centrally within the velum (Pl. 3). The posterior sucker, derived from modifications of the last seven segments, formed a large, disc-shaped structure utilized for attachment and locomotion (Pl. 2). Comparable structural adaptations have been reported in *L. nilotica* and species of *Haemopis* (Arfuso *et al.*, 2019).

The body was comprised of 33 segments, 26 forming the main body and seven contributing to the posterior sucker. Most segments were subdivided into annuli, making true segmentation difficult to distinguish. Most segments had five annuli, although there were fewer at the anterior and posterior ends. Each specimen bore five pairs of eyes arranged in a parabolic arc (Pl. 2), a feature consistent with previous descriptions (Solijonov *et al.*, 2022). Additionally, small sensory papillae (sensilla) were observed on the center annulus of each segment (Pl. 4), although some specimens exhibited incomplete sensilla development, as reported by Nakano *et al.* (2015).

Seventeen pairs of lateral nephridiopores were identified on the ventral surface, except at the anterior and posterior extremes (Pl. 4). The male and female gonopores were separated by five annuli, a characteristic shared with *L. nilotica* (Sawyer, 1986; Saglam *et al*., 2018). Some specimens displayed a well-defined clitellum, confirming reproductive maturity (Pl. 2).

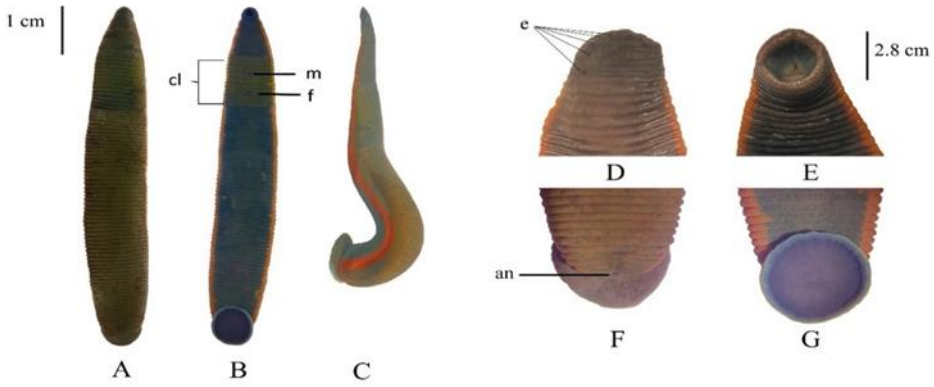


Plate (2): External morphology of *L. paluda*; (A) Dorsal view of the whole body, (B) Ventral view showing the clitellum (cl), male (m), and female (f) gonopores, (C) Lateral view of the body, (D) Dorsal view of the anterior end showing the arrangement of eyes (e), (E) Ventral view of the anterior end showing the oral sucker, (F) Dorsal view of the posterior end showing the anal opening (an), (G) Ventral view of the posterior end showing the caudal sucker.

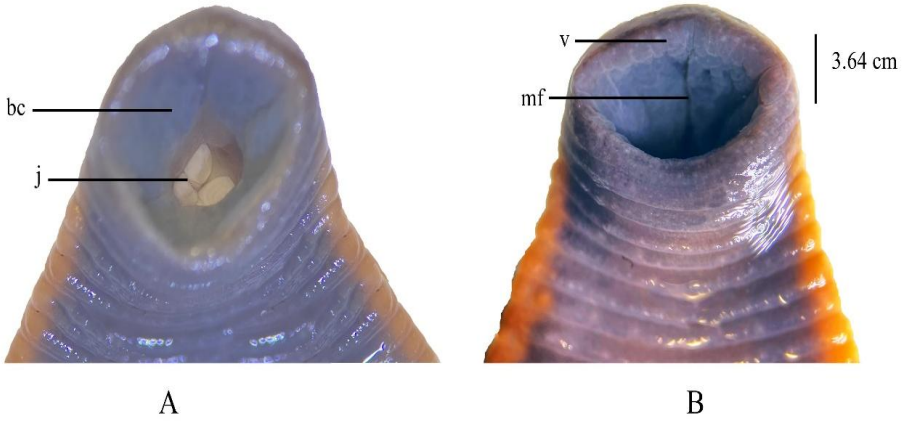


Plate (3): Ventral view of the anterior end (oral sucker) of *L. paluda*; (A) The buccal cavity (bc) is surrounded by three jaws (j), (B) The anterior end shows the median longitudinal furrow (mf) in the ventral surface of the upper lip.

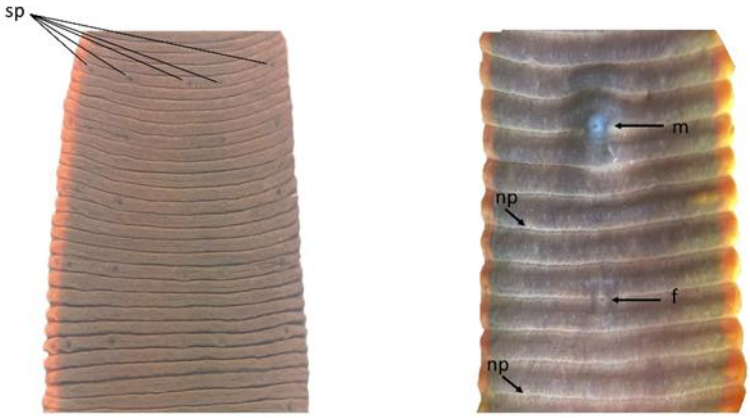


Plate (4): Dorsal and ventral views of the body segments of *L.paluda*; (A) Dorsal view showing sensory papillae (sp), (B) ventral view showing female gonopore (f), male gonopore(m), and nephrediopores (np).

Internal morphology

The mouth opened into the buccal cavity, which contained three jaws with 30 to 45 monostichodont teeth (Pl. 3). The presence of monostichodont dentition aligned with previous descriptions of *L. paluda* (Nakano *et al.*, 2015). Three separated glands appeared as clusters or grapes or strips that extended from each jaw, and spread around the cerebral ganglia, a few anterior to the crop, and located approximately between segments V to XI, muscles and connective tissues surrounded them. The pharynx constricted to form the esophagus, which widened to become a transparent crop. The crop extended through the body and joined the intestine with two large, bulging caeca. The intestine opened into the rectum, with thin, translucent walls, and opened to the exterior via the dorsal anus.

The excretory system consisted of 17 pairs of metanephridia, starting from segment seven and extending along the lateral body wall. This number was consistent with previous descriptions of the species (Sawyer, 1986). The nervous system featured a ventral nerve cord with cephalic and caudal ganglia. The segmental ganglia housed most neurons, a typical feature of leeches (Elliott *et al.*, 2011).

Male genital organ

The male reproductive apparatus was composed of 8 or 9 pairs of testes contained within small, spherical, brown coelomic test sacs located on either side of the intersegmental. A short, convoluted, white vas efferent arose from each test sac. The vas efferent emptied into the vas deferens. There were two long and longitudinal vasa deferentia, one receiving the vasa efferentia from the test sacs of the left side, the other from the right. Each vas deferens runs anteriorly, then widens to become the epididymis, which is not a bright white, thick, extensively intricate tube. The ejaculatory ducts were convoluted and extended to the anterior

portion of the male atrium. The atrium was continuous with the U-shaped penile sheath, subsequently curved anteriorly into the male gonopore (Pl. 5). The ejaculatory bulb was absent in all examined specimens, consistent with observations by Nakano *et al.* (2015).

Female genital organ

The female reproductive system consisted of paired spheroid ovisacs, each with a short oviduct. Both oviducts merged into a common oviduct, which was slightly folded and descended into a straight, ellipsoid vagina leading to the female gonopore (Pl. 5). This structure was consistent with the reproductive morphology of related *Limnatis* species (Solijonov *et al.*, 2022).

Molecular identification and phylogenetic analysis

Regarding the consequences of the existing study, besides the morphological study, the findings were presented as the first molecular identification and phylogenetic characterization of isolated *L. paluda* in Iraq. The chromatograms were utilized to elucidate and assess the troubleshooting of DNA nucleotide sequences. The chromatogram exhibited four columns with well-defined, uniformly spaced peaks, indicating highly sensitive nucleotide sequencing results. The released sequence lengths for COI were 650 bp, and for 18S rDNA were 345 bp (Diag. 1).

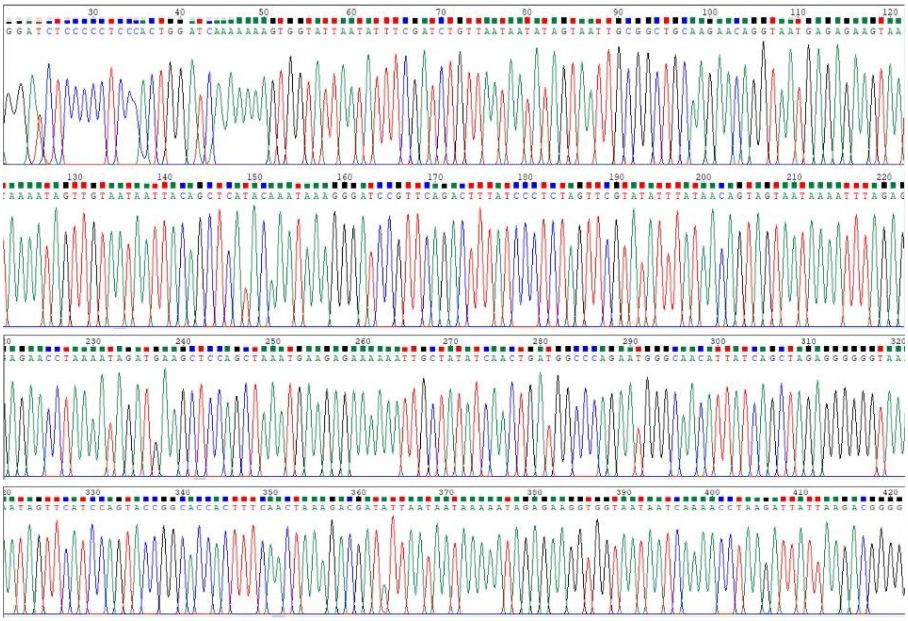


Diagram (1): The chromatogram of PCR products, the sequence result from COI extracts from *L. paluda*. Note the evenly-spaced peaks and the lack of "noise" (the baseline), represented as well-defined colour peaks.

The harvested DNA nucleotide sequences of the isolated leech were input into the Basic Local Alignment Search Tool (BLAST) and then compared with current GenBank DNA sequences. The BLAST results showed a 100% identity percentage with the available *L. paluda* DNA sequences at the National Center for Biotechnology Information (NCBI), as demonstrated in Diagram (2). The stability of the COI gene across populations emphasizes its suitability as a reliable barcoding marker for species-level leech taxonomy (Borda *et al.*, 2011).

The phylogenetic study evaluated the evolutionary association of the *Limnatis* species, incorporating newly sequenced specimens and previously published sequences. A maximum-likelihood tree was constructed, and *Poecilobdella manillensis* was used as the outgroup to root the tree and put it into an evolutionary context (Diag. 3). The phylogenetic analysis demonstrated a highly supported monophyletic clade of *L. paluda* sequences with 73-99 bootstrap values that could be viewed as statistically strong confidence. Despite this effort, some discrepancies in similarity values were observed. These may reflect natural intraspecific variation within *L. paluda*, which has been reported in other regional populations, e.g. (Utevsky *et al.*, 2022), rather than primer-related errors.

The newly acquired sequences (PQ868999.1, PV056019.1, PV018631.1, and PQ553448.1) were found aligned closely with previously recognized *L. paluda* sequences (KY989474.2, MZ318068.1-MW538548.1), confirming their taxonomic placement within this species. There were also two different subclades of *L. paluda*, indicating a probable intraspecific variation or geographic differentiation.

Two sub-clades per *L. paluda* implied the possibility of either geographic variation or cryptic diversity. The geographic organization of the leech population has also been seen in other publications, whereby the environmental factors and the distribution of the host organisms contributed to the genetic divergence of genes (Borda *et al.*, 2011). This interspecific variation may have been a point of local adaptation or of individuals with specific lineages in *L. paluda*, which should be explored further.

L. nilotica, conversely, formed another, better-supported clade (bootstrap values of 95% and 96%) that had diverged from *L. paluda*. This is the argumentation of the taxonomic disparity of the two species. MZ318072.1, PQ187649.1, and PQ187648.1 sequences in *L. nilotica* are also indicative of a high level of genetic affinity; therefore, their classification was supported. The well-supported bootstrap of the *L. nilotica* clade also provided evidence of its genetic uniqueness, as has been the situation in molecular phylogenetic research of therapeutic leeches (Trontelj *et al.*, 2005). The strong genetic similarity of the sequences of *L. nilotica* showed that the ancestry was very conserved, which might be attributed to a narrow ecological niche or reduced dispersal capabilities.

Score	Expect	Identities	Gaps	Strand
1186 bits(642)	0.0	642/642(100%)	0/642(0%)	Plus/Minus

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Sbjct  162  CCATAAAAAAAAATTATAATCAGCCCATGAGCAGTAACAATAGTATTATAAATTTGATCAC  103

Query  556  TTCCTAAAAATGGCCCTGGTTGAGATAGCTCAATTCGAATAATTATTCTTATAGATGTCC  615
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  102  TTCCTAAAAATGGCCCTGGTTGAGATAGCTCAATTCGAATAATTATTCTTATAGATGTCC  43

Query  616  CAACTATAGCGGCTCATGTCCCTAAAAGAAAGTATAGAGTCC  657
            ||||||||||||||||||||||||||||||||||||||||||
Sbjct  42   CAACTATAGCGGCTCATGTCCCTAAAAGAAAGTATAGAGTCC  1
```

Diagram (2): Pairwise alignment of the *L. paluda* cytochrome c oxidase subunit I (COX1) sequence. The query is the sequence sample, and the subject is the sequence taken from GenBank.

Poecilobdella manillensis was their outgroup position, which supports the monophyly of the *Limnatis* genus and contributes to demonstrating the evolutionary separation observed within the group. The phylogenetic tree visualisation revealed that the *L. nilotica* group was the sister group to clade *L. paluda*, which is the evolutionary divergence between the specified groups. It is a widely applied method for doing phylogenetic studies to establish monophyly and to clarify the taxonomic relationships (Apakupakul *et al.*, 1999). The results confirmed

the monophyletic character of *Limnatis*, which strengthened the classification of the genus as a separate species (within the family of Praobdellidae).

These findings supported the exclusive evolutionary position of *L. paluda* and *L. nilotica*, and the recently sequenced specimens were clustered well with the already known genetic lineages. More molecular markers, especially those of the nuclei, should be used in future research to improve the phylogenetic resolution. Moreover, biogeographic studies of the ranges of *L. paluda* and *L. nilotica* can shed more light on the evolutionary history and possible speciation of these two species. The integration of molecular, ecological, and morphological approaches will be crucial to a thorough understanding of *Limnatis* diversity and evolution.

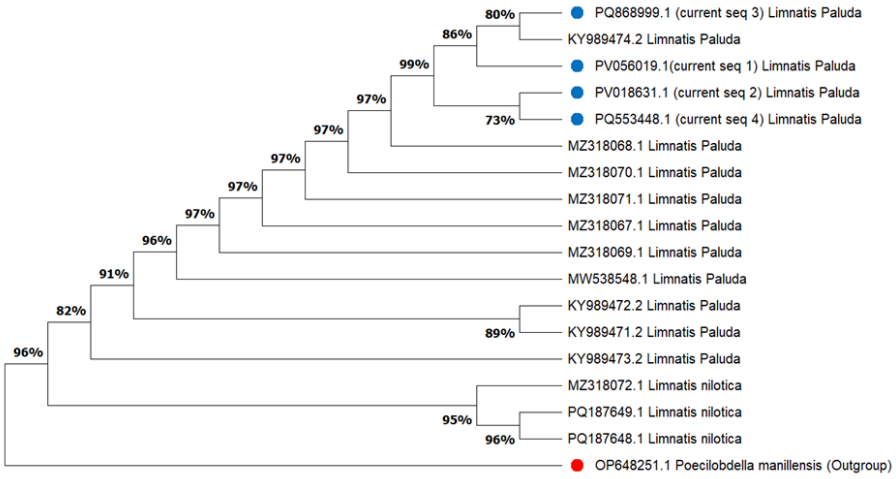

Diagram (3): Maximum Likelihood phylogenetic tree of *Limnatis* species based on mitochondrial gene sequences, showing relationships among newly obtained and reference sequences. The evolutionary history was inferred using the Maximum Likelihood method and the Tamura-Nei model. The tree with the highest log likelihood (-1614.17) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and selecting the topology with the superior log likelihood value. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendant clade is shown next to each internal node in the tree. This analysis involved 18 nucleotide sequences. There was a total of 684 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

CONCLUSIONS

According to the recent findings, this study is recognized as the new, extensive morphological and molecular-based assessment of *L. paluda* in Iraq. The combination of COI

and 18S rDNA gene sequences have been demonstrated to be an efficient molecular marker for establishing species identification, bolstering the accuracy of traditional morphological classification. Given the limitations of morphology alone in differentiating closely related leech species, the integration of DNA-based methods served as a reliable model for distinguishing and enhancing the resolution of their taxonomic status. This combined method provides a good starting point for future research on leech biodiversity and its interactions with hosts in the region's aquatic ecosystems.

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CONFLICT OF INTEREST STATEMENT

It is worth noting that this work is part of a M. Sc. thesis submitted to the Department of Biology, College of Scientific - Salahaddin University, Erbil for the first author.

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دراسة تكاملية لدودة العلق *Limnatis paluda* (Tennant, 1859) (Clitellata, Arhynchobdellida, Praobdellidae) صفاتها المظهرية وبياناتها الجزيئية وتأكيد تواجدها في العراق

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الخلاصة

قدمت التحريات الحالية دراسة تفصيلية للصفات المظهرية والجزيئية للنوع *Limnatis paluda* (Tennant, 1859) ، التابع لصنف Clitellata و رتبة Arhynchobdellida و عائلة Praobdellida ، والذي جُمعَ من خمسة ينابيع مياه عذبة في منطقة طقطق، محافظة أربيل، إقليم كردستان-العراق. ركزت معظم الأبحاث السابقة المتعلقة بهذا النوع من العلق بشكل أساسي على دراسة الصفات المظهرية، وغالبًا ما يكون دراسة تسلسل الحمض النووي مفيدًا في علم التصنيف، لأنه يوفر معلومات لا يتم الحصول عليها من دراسة الصفات المظهرية فقط. كان الهدف الأساسي هو التوصيف الجزيئي لـ *L. paluda* باستخدام منطقة 18S rDNA وجين الوحدة الفرعية الأولى لأوكسيديز السيتوكروم سي للميتوكوندريا I(COI) عن طريق تفاعل البوليميراز المتسلسل وتقنيات تسلسل النيوكليوتيدات. تم الحصول على التسلسلات ومقارنتها بالتسلسلات المسجلة في المركز الوطني لمعلومات البيولوجيا الجزيئية (GenBank) الموجودة. تدعم هذه النتائج التحقق من صحة تواجد *L. paluda* في العراق باستخدام كلٍّ من التقنيات التقليدية (القائمة على الدراسة المظهرية) والحديثة (القائمة على علم الدراسة الجزيئية) ، وقد أظهرت الأخيرة نسبة مطابقة 100% لـ *L. paluda* عند مقارنتها بالتسلسلات المسجلة في بنك الجينات GenBank. واستُخدم أسلوب الاحتمالية القصوى (ML) لبناء العلاقة التطورية. تشير النتائج إلى أن تسلسل تفاعل البوليميراز المتسلسل (PCR) يُعدّ أسلوبًا جزيئيًا فعالًا وموثوقًا به لتشخيص *L. paluda* ، مكملًا لتقنيات دراسة الصفات المظهرية التقليدية ومعززًا فهم حياتية هذا النوع من ديدان العلق وتأكيد تواجدها في العراق.