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Research Article

SDS-Page profiling and molecular characterization of Hirudin, Eglin C, and Hyaluronidase from the saliva of two local leech species

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Abstract

Leeches of the genera *Hirudo* and *Limnatis* are known hematophagous ectoparasites of vertebrates, including humans. The present study investigated two leech species, *H. oreintalis* and *L. paluda,* during July 2024-June 2025 from thirty-five water bodies of leech collecting from four provinces of Kurdistan Region, Iraq. Morphological identifications of specimens followed by molecular characterizations were dependent on species identification through amplification and sequencing of the mitochondrial (cytochrome c oxidase subunit I, COI locus). The partial gene fragments were sequenced for molecular characterization. These findings confirm the presence of two distinct leech species, *H. oreintalis* and *L. paluda* in the Kurdistan Region. The present work also revealed a comparative study on the saliva contents of both species, their salivary gland contents extracted for further molecular observations, including three proteins, Hirudin, Eglin C, and Hyaluronidase, which were isolated and identified with SDS chromatography. Genes of the mentioned proteins were partially amplified and sequenced, and the nucleotide variances were detected based on MEGA 11, as well as the amino acid sequences were studied with Phyre 2.2 web portal.

Keywords: *Hirudo*, *Limnatis*, COI, Saliva, Proteins.

Introduction

The socioeconomic relevance of human health and livestock farming is significantly challenged by leech infestations. Hematophagous leeches, specifically *Hirudo* and *Limnatis* genera, were strongly related to human health and livestock's low productivity, respectively, especially zoonotic diseases by inadequate healthcare leeching with *Hirudo* as well as wild infestations with *Limnatis* (Kovalenko and Utevsky, 2014; Gebreselassie, 2018). Hematophagous leeches are one of the most prevalent parasites globally. The pandemic parasite-based diseases share a significant problem for both human and animal health systems in most countries (Khan, 2012; Bahmani et al., 2014; Stewart, 2025). For centuries, the genus *Hirudo* has been studied as a biological evolutionary, ecological, and biochemical model organism (Trontelj et al., 1999; Trontelj and Utevsky, 2012). The pathogenic parasite leech *Limnatis* induces allergic reactions in different hosts (both humans and other animals) difficulties related to pain, itching, inflammation, as well as bleeding (especially from soft tissues). Livestock *Limnatis* infestations have not yet received attention from both medicinal and veterinarian health care monitoring systems (Bahmani et al., 2013).

The comparative studies of saliva contents of both hematophagous leech genera, *Hirudo* and *Limnatis*; may help in better understanding of physiological, pathological and parasitological adaptations of these helminths, especially till now new active contents are identify in recent studies, as a Korean research group discovered a natural short antimicrobial peptide, namely: 'Hirunipin-2,' from the salivary glands of *H. nipponia* that has a high potential therapeutic characteristic against super-bacteria (Stewart, 2025). Leeches have a unique salivary gland structure and play a great role in determining their feeding behavior. In concern, leeches' salivary gland contents of three pairs of functional glandular salivary cells in the pharynx (Bilal and Ahmed, 2023). Leech saliva is a very complex bioactive compound, including small molecules, enzymes, peptides, and proteins. Firstly, hirudin, an anticoagulant and other hirudin-like materials, is the most important content, also, anesthetic contents, hence the feeding process is painless in addition to the digestive enzymes for breaking down proteins, facilitating feeding and digestion (Lemke et al., 2013; Bilal and Ahmed, 2023). The present study deals with the intra-genus variance structure of three saliva contents, namely Hirudin, Eglin C, and Hyaluronidase. Hirudin, a naturally dominant anticoagulant protein of the leeches' saliva, has unique properties and functions. It is a highly specific thrombin inhibitor for preventing blood clotting, as well as hirudin-thrombin binding is a very stable complex that dissociates slowly, resulting in prolonged anticoagulation time (Lemke et al., 2013; Stewart, 2025). Hyaluronidase (or hylorodinase) is one of the leech saliva enzymes presents facilitating feeding through breaking down the hyaluronic acid (a key component of connective tissues) making the skin penetration easy as well as it is a good tissue permeability promoter, enhances anticoagulant delivery, like hirudin, causing wound healing, and reducing inflammation the bite wound (Hovingh and Linker, 1999; Lemke et al., 2013).

Eglin is a powerful protein C inhibitor (blocks serine proteases like thrombin, plasmin, and kallikrein) found in the saliva of leeches, prolonging bleeding time. It also has anti-inflammatory properties, hence has a good role in reducing swelling, pain, and promoting healing of the wound (Lemke et al., 2013; Németh et al., 2025). The present investigation concerning the molecular identification of *H. oreintalis* and *L. paluda,* based on the COI partial sequence, rather than a comparative study of three active contents (Hirudin, Eglin, and Hyaluronidase) in saliva of both species, including their biochemical characters with SDS page, as well as molecular investigation through partial sequencing of the previously mentioned proteinic materials expressing genes.

Material and methods

Sample collection and identification

Leech specimens were collected from different sites in Kurdistan Region, exactly (35 stations) (Erbil Province (21 points); Sulaimani Province (seven points); Duhok Province (four points) and Halabja Province (one point), in addition to some *Hirudo* specimens were collected from leeching centers in Erbil, Diyana and Chamchamal that imported from Islamic Republic of Iran. In the present work, two hematophagous leech species were studied. Leeches were collected in shores by monthly surveying and turning rocks logs, vegetation, water debris, macro aquatic invertebrates and frog inspections during May 2024 - June 2025 and transported with the local stream/ river water in a cooled box to Molecular Biology Lab of General Directory of Research Center/ Salahaddin University- Erbil (Sawyer and Sawyer, 2018; Bilal, 2024). Collected specimens undergo morphological and molecular study to confirm their species identification (Sawyer and Sawyer, 2018; Arfuso et al., 2019; Solijonov and Sağlam, 2022; Bilal and Ahmed, 2023; Bilal, 2024; Farzali and Saghlam, 2020; Farzali et al., 2025). For molecular study, leeches' genomic DNA was isolated from the posterior sucker of two specimens for each species using the Beta Bayern tissue DNA preparation Kit (Beta Bayern GmbH, .90453 Bayern, Germany) procedure. The species of collected helminths were identified based on sequencing of Cytochrome Oxidase C Subunit One (COI) with 10 Picomol (pmol) primers (Table 1).

Table 1. A pair of primers used in the COI gene sequence.

Primer code	Sequence 5'-3'	Amplicon size (bp)	PCR Condition
COI-F	5'-GGTCAACAAATCATTAAAGATATTGG-3'	720	95°-5 min; 95°-35 sec, 59°-35 sec, 72°-1 min; 72°-10 min; 4° ∞
COI-R	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'		

Saliva extraction

Leeches belonging to *Hirudo* feed on Agarose Sugar in a glass container to prevent the leech's dehydration, while *Limnatis* feed on toad blood (*Rana rudibanda*) (Malek et al., 2019). Leeches were put to vomit in 8% ethanol, the saliva is obtained by squeezing leeches from the posterior end, enrolled to the anterior end, and collected in an Eppendorf (Alaama et al., 2021).

Molecular study

Saliva proteins separated, detected, and isolated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis SDS-PAGE system (Biobase, China)**.** The glass plates were sprayed with 70% ethanol and dried with sterilized dry cotton to eliminate dust particles. A mixture of 5-6 milliliters of 15% with one millimeter thickness separation gel and 12 wells was used (Cleveland et al., 2002). The wells were filled with 4 µl of leech saliva samples that were heated and denaturized at 95 °C for 10-15 minutes in a 5X loading buffer (with a ratio of 1 part sample/ 4 parts buffer). Six samples revealing each of Hirudin, Eglin C, and Hyaluronidase genes from both genera of leeches were electrophoresed, as well as a 12-band protein marker (SMOBio, USA) (sizes 10 - 245 kDa), at a voltage of 100V for 3-5 hours until the Bromophenol blue dye reached the bottom of the gel. Gel was stained for band visualization by immersing for about 45 minutes in Coomassie staining solution, and de-stained by washing it several times with distilled water and left in a distilled water tank till the color was completely removed. Protein bands and marker bands appeared in blue. A clear, bright, and white image of the gel was obtained on the imaging chamber. The salivary gland genes nucleotide diversity/ differentiation for three genes of Hirudin, Eglin C and Hyaluronidase, were studied with the gene of hirudin accession number X72786, Eglin C gene accession number OL598605, and hyaluronidase gene accession number KJ026763; are selected for designing forward and reverse oligonucleotide primers with Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), with primers used were shown in (Table 2).

Table 2. Mentions the primer's information and its properties.

Gene name	Primer	Oligonucleotide sequences	Product size (bp)	Tm	Reference GenBank
hirudin	Forward	AGTTGTTCGTTGTCTTCCTGG	625	59	X72786
	Reverse	ATCTTCATCTGGGATTTCTTCG			
Eglin C	Forward	CCTCAGTATAATGGTCGTAGTGG	258	58.5	OL598605
	Reverse	TCAGCCAACAACTGGTACG			
hyaluronidase	Forward	GATCGCGGTGACAATTGACGA	540	59	KJ026763
	Reverse	TTCAGTAAGATTGTGTGCGGA			

PCR amplification for the selected partial genes of Hirudin, Eglin C, and Hyaluronidase was done in a total volume of 50 µl of reaction mixture using by Bioresearch PTC-200 Gradient thermocycler.
The initial denaturation was at 95 °C for 5 min, then 35 cycles of denaturation at 95°C for 40 seconds, primer annealing at ٥٨°C for 4٠ seconds, and extension at 72°C for 1 min, and in finally an extra extension at 72°C for 5 min. Electrophoresis was done with a 1.5% agarose gel (Stephen et al., 1997).
DNA sequencing was performed with three repeats of each partial gene for both leech species (18 samples) using by ABI Prism Terminator Sequencing Kit (Applied Biosystem) by Macrogen Molecular Company of Korea. The Finch TV program software is used for editing and basing calls, checking of partial gene chromatograms.
Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied for alignment of Hirudin, Eglin C, and hyaluronidase partial gene sequences for finding out the similarity/ variations of nucleotides as well as helping in finding amino acid variations between the different leeches.

Results

The morphological identification of the collected leeches showed belonging to two hematophagous species, *Hirudo orientalis* and *Limnatis paluda,* according to the morphological and morphometric characteristics, including jaw structure, number and structure, coloring pattern, and rational measurements of body parts. The total body length of the *H. orientalis* was 72.5-108.5 mm (84.6 mm) (Fig. 1C), and the width was 11.7-12.2 mm (11.9 mm). The body color patterns are olive green with thin, orange paramedian deep stripes. The dorsal view of the leech showed a segmental arrangement pairs of rounded quadrangular black dots on its para-marginal dorsal stripes (Fig. 1A). The ventral side showed a regular metameric pattern of light and dark olive-green stripes (Fig. 1B). The five pairs of eyes were clearly located on annuli of II, III, IVa1, Va1, and VIa2 (Fig. 1 A; D). The male gonopore is located in the furrow XI b5/b6, larger and more elevated than that of the female, that located in XII b5/b6; both were open in the clitellum, and five annuli separate both genital openings. **Molecular Identification**

For leech species identification, the genomic DNA was isolated by Bioscience Animal DNA preparation Kit Beta Bayern DNA preparation Kit (Beta Bayern GmbH, .90453 Bayern, Germany). The COI gene primer is used only for parasites and is synthesized by Micro-gene Company (South Korea). The primers yielded a band of 720 bp. The PCR product was electrophoresed and visualized by 1.5% Agarose gel labeled with 100-3000 bp nucleotides. The PCR produces shown in the (Fig. 3).

DNA sequenced, using only forward primers of COI; (5' GGTCAACAAATCATTAAAGATATTGG-3') separately by ABI 3130X genetic analyzer (Applied Biosystem). The PCR products of all samples were used as a source of DNA template for sequence-specific PCR amplification. The sequence of partial genes is aligned by the BLAST program from GenBank (*http://blast.ncbi.nlm.nih.gov/*) and was used to compare our amplified sequences with other stored species of species. The results from the BLAST indicated that the highest query sequence was 100% identity with both *H. orintalis* and *L. paluda* (Table 3). Phylogenetic inferences of both studied leech species, MEGA 11 programs of Phylogenetic analysis based on COI nucleotide sequence, revealed the grouping of the four investigated species of leeches on expected lines. From sequence divergence similarity data and phylogeny constructed, it was revealed that species belonging to respective genera were close to each other. The two samples of leech species grouped each cluster with high similarity to the same Parasite (Fig. 3).

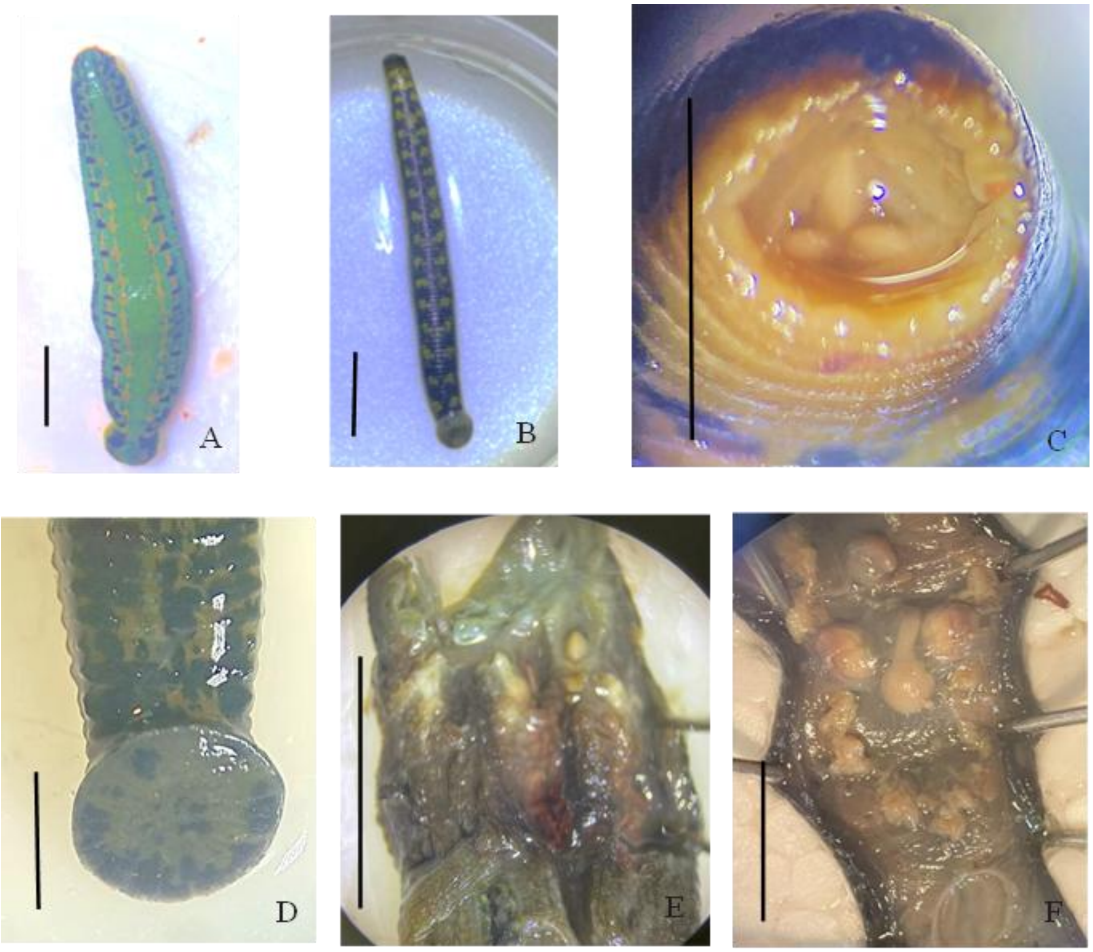


Figure 1. Photomicrographs of *Hirudo orientalis*: A- Whole mount Dorsal view; B- Whole mount Ventral view; C- Mouth and oral sucker (enface view showing the Jaws), D- Posterior haptor, E- Dissected mouth showing

jaws and salivary glands, E- Dissected clitellar region showing male and female reproductive systems. Scale bar= 10 mm.

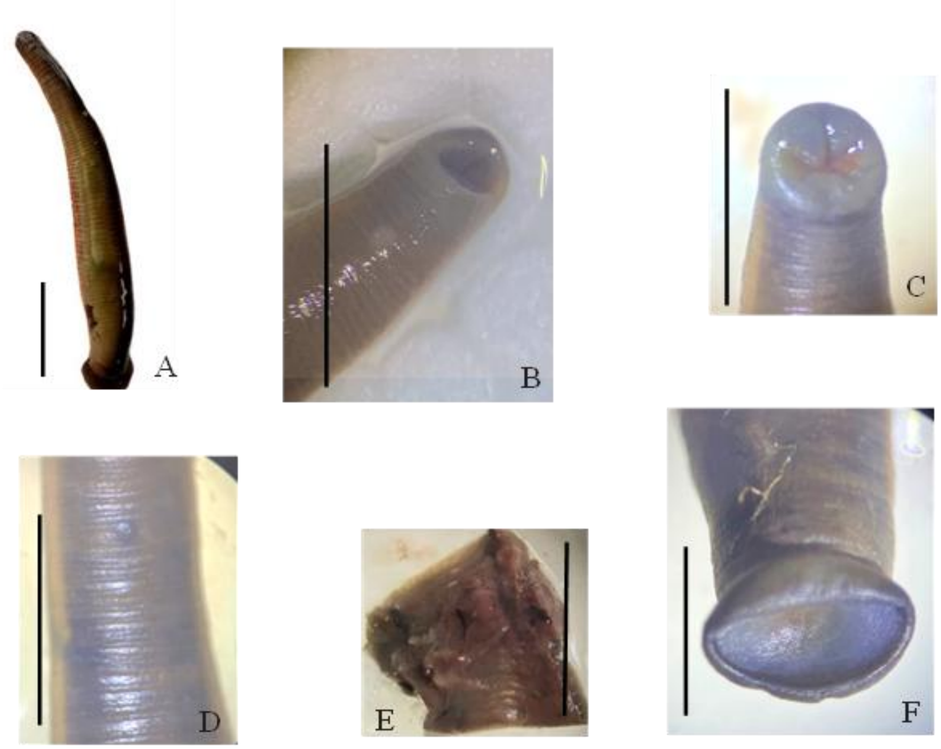


Figure 2. Photomicrographs of *Limnatis paluda*: A- Whole mount Dorsal view; B- Mouth (Ventral view); C- Mouth and oral sucker (Enface view); D- Clitellum (Ventral View) showing male and female gonopores; E- Dissected mouth showing jaws and salivary glands; F- Posterior haptor (Ventral view). Scale bar 10 mm.

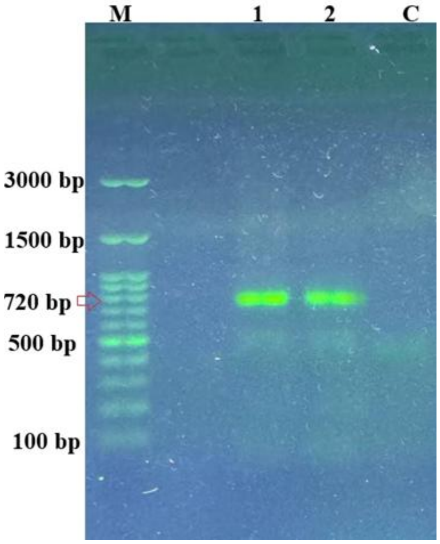


Figure 3. PCR amplification of the partial COI gene from leeches, wells include M; Ladder (3000-100 bp), lane1-2; gene bands with the size of 720 bp amplified, and C indicates a negative control without the band.

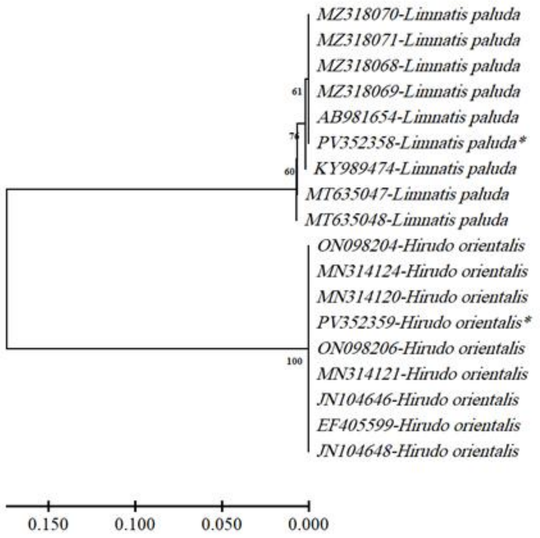


Figure 4. Employing Neighbor joining of the Mega 11 program, shows phylogenetic positioning of two leech samples (*) with similar COI available GenBank sequences.

Table 3. Percentage distribution of samples of leeches' species into the same species according to BLAST to Genbank NCBI of partial COI gene.

Parasite samplesAccession Number	Query Cover %	Identic Number %	Genbank Accession Number	Genbank Parasitic Species Identification
PV352358	100	100	AB981654	*Limnatis paluda*
	100	100	MZ318069	
	100	100	MZ318068	
	100	100	MZ318070	
	100	100	MZ318071	
	100	99.83	KY989474	
	100	99.66	MT635048	
	100	99.66	MT635047	
PV352359	100	100	ON098206	*Hirudo orientalis*
	100	100	MN314121	
	100	100	JN104646	
	100	100	EF405599	
	100	100	JN104648	
	100	100	MN314120	
	100	100	ON098204	
	100	100	MN314124	

Discussion

The results of H. orientalis were came in similar to those of Farzali and Saglam (2020), with a length of 82 mm and a width of 12 mm. They have thin, deep orange-colored paramedian stripes. Dorsal surface with dorsal para-marginal stripes pairs (segmentally arranged) of black quadrangular, rounded dots. The ventral side showed regular metameric pairs of light-colored markings coloration pattern with a larger male gonopore (on XI b5/b6 furrow) than the female gonopore (on XII b5/b6 furrow), which means five annuli separating both genital pores on the clitellum region.

Ahmed and Bilal (2023) mentioned jaws were located in a wide, large preoral chamber located in the anterior sucker, presence of the vellum as a transverse tissue sheet, mouth with a small triradiate opening in the center of the vellum. Three bright white, rigid, triangular, and smooth and rigid jaws were seen (a pair of symmetrical ventrolateral with a single dorsomedial). Worth mentioning, 80 (as average) sharp teeth were noticed on each blade-like jaw (Fig. 1A; B; C). The nasal leech species L. paluda showed a firm muscular, dorsoventrally compressed body, total length 32.4-71.30 mm (42.85 mm), a constant width along the total length, about 3.25-5.90 mm (4.05 mm) (Fig. 2A). The posterior sucker wider than body 5.11-6.56 mm (5.10 mm) (Fig. 2F), with a dark-green with no black patterns, on both sides clear, brighten yellow stripes are seen along all body margins (Fig. 2A; B). The anterior sucker showed three lobes, with three jaws armored with a line of denticles, 2.85-3.95 mm (3.12 mm) located in a narrow depression (Fig. 2B; C; E) and gonopores were seen on ventral side of the clitellum, male ones located on body somite XII, and the female was located on somite XIII (Fig. 2D).

The results of the present study were similar to those of Solijonov and Sağlam (2022) found adults with 90-120 mm length, and 12-18 mm, while young specimens had with length of 40-60 mm and a width of 6-8 mm. Eyes were parabolic, five pairs in number, starting from the II body somite (first pair) and ending on the somite VIa2 (fifth pair). A very large rear sucker with about 15 mm, and the width of large during sticking on the substrate. The dorsal surface with brown-green to light-green without any dark spots or patterned lines, while the ventral surface was dark-green with lateral orange stripes along both plain body sides and Farzali et al. (2025) reported similar morphological and color patterns to that noticed in the present study, like dorsal and ventral coloration, shape and size of the mouth as well as locations of eyes and gonopores. The PCR products for COI of both studied leech species, exactly: *H. orientalis* and *L. paluda* of the present study (eight samples for each species) were sequenced and blasted (Qurey) with available sequences from Genbank (Subjct) and showed 100% identity, hence the species taxa of the studied specimens were insured and depended for further parameters in the present work (Fig. 3, 4 and Table 3). The results showed similar results to that of Utevsky et al. (2009) that studied the chromosomal number and partial sequence of COI and Babenko et al. (2020) studied the partial segment of COI for distinguishing of *H. orientalis* from other species in *Hirudo* genus*;* and Nakano et al. (2015) and Utevsky et al. (2022) identified *L. paluda* basing on the same locus (COI), their studies based on same sizes of amplified segments as well as results of nucleotide sequences alignments submitted to the GenBank were so similar to the present results. The present findings were similar to those of Ben Ahmed et al. (2025), who revealed COI as a useful locus for the identification and description of new species, since they described a new nasal leech, *L. anouarensis,* in Tunisia and distinguished it from *L. nilotica*.

Isolation of protein molecules

Saliva samples from both leech species, *H. oreintalis* and *L. paluda,* were placed and electrophorized on SDS for the detection and identification of the targeted contents (Hirudin, Eglin C, and Hyaluronidase) on 15% one mm thickness separation gel marked with a ladder sized from 10 - 170 KDa. The results showed the presence of the three mentioned targeted contents with molecular weights 7 KDa, 8 KDa, and 91 KDa, respectively (Fig. 5). The present study detected that Hirudin proteomic characterization was so similar to some previous studies concerned this protein, even some of them from other leech species than studied in here; Fritsma (2013) studied in addition to the natural hirudin, an analogue recombinant 7000 Dalton protein DTI, Lepirudin, in the medicinal leech *H. medicinalis* and found the of both proteins similarity in other *Hirudo* species.; Müller et al. (2020) compared the Hirudin characteristics and functional analysis of three medicinal leeches, *H. medicinalis, H. orientalis* and *H. verbana*, and , also compared HLF3 and HLF4 low or no detectable anti-coagulatory activities and found the variability of their functions according to the translated amino acids, hence, it is supporting the present results of variations between the two studied species in the present work, *H. orientalis* and *L. paluda*; Pfordt et al. (2022) compared the structure of hirudin in two hematophagous leeches, *Limnobdella mexicana* and *Haementeria vizottoi*, and described it is identification, molecular and functional characterization and reported different potent rates between both expressed hirudin in platelet aggregation basing on the variability of their molecular constitutes; Lukas et al. (2022) described the typical structure of Hirudin in *Hirudinaria manillensis* including it is molecular identification, and functional characterizations, also compared it to Tandem-Hirudin (TH), and noticed similarity of the both protiens in size, structure and functions with that secret by *Hirudo*; and Liu et al. (2023) measured the anticoagulant activities of five identified Hirudins in *H. manillensis* and the results showed variability of their anti-coagulant activity according to the molecular sequences variability. Ahmed et al. (2024) stated that the leech species *H. troctina* has been used in medical purposes, since the best-known leech saliva-derived bioactive material is hirudin, in addition to hirudin-like factors (HLFs), which are another salivary gland component class that shares similar characteristics to that of hirudin (Fig. 3).

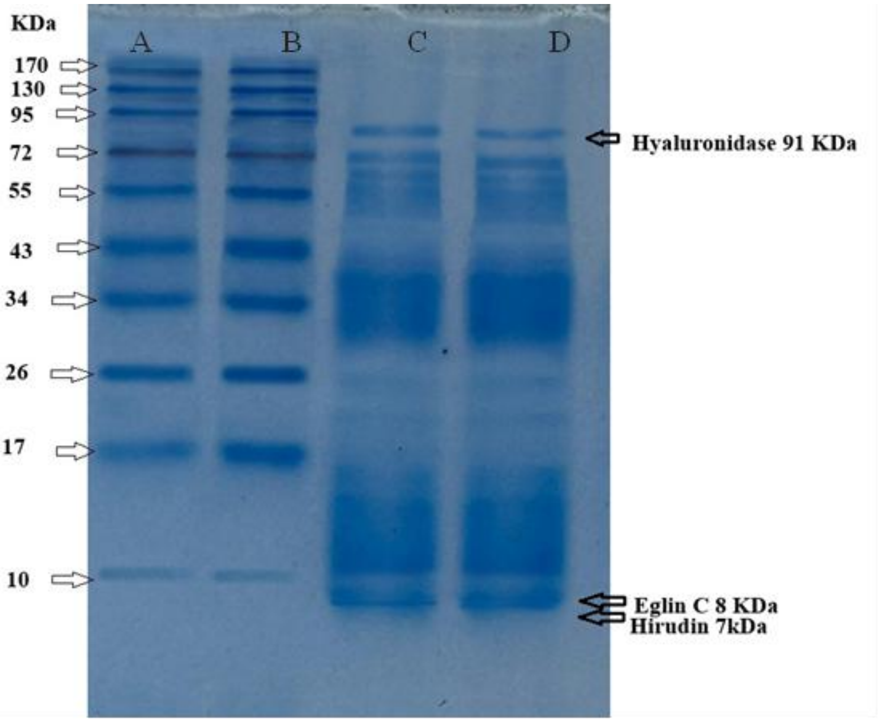


Figure 5. SDS Page of Leech saliva proteins isolation from two different species. A-B- Markers (Ladder), C- *Hirudo oreintalis*, D- *Limnatis paluda*.

Eglin C was revealed in the present study, and the results were fit with that of Betzel et al. (1993) and Németh et al. (2025) in molecular weight, chemical structure, as well as the secondary structure, since they regarded it as a very active small sized leech saliva protein and have role in chymotrypsin inhibition (Fig. 5). Hyaluronidase also is an important leech saliva content and have a great role in detecting of feeding habit, the present study results came in parallel with that of Hovingh and Linker (1999) studied the leech hyaluronoglucuronidase families and reported hyaluronidase I from both hematophagous and macrophage leeches, while hyaluronidae II reported from liquidosomatophagous leeches only, hence, it is concluded that hyaluronidase have great role in detecting of feeding habit. Worth mentioning, Jin et al. (2014) cleared the great enzymatic potential of leech Hyaluronidases and their broad role in medical applications.

Genomic variations detection of studied proteins

The primers of Hirudin band yield as ~625bp, the primer of Eglin C band yield was ~258bp and the primers of Hyaluronidase band yield was 540 bp after PCR product was electrophoresed and visualized by 2% Agarose gel (Fig. 6). For query sequences of the present study alignment NCBI- BLASTX was used, and the GenBank database was intended for new sequence data that was determined and annotated by the submitter. All sequences were uploaded to GenBank and showed 100% identity for each gene from both leech species; accession numbers were taken (Table 4).

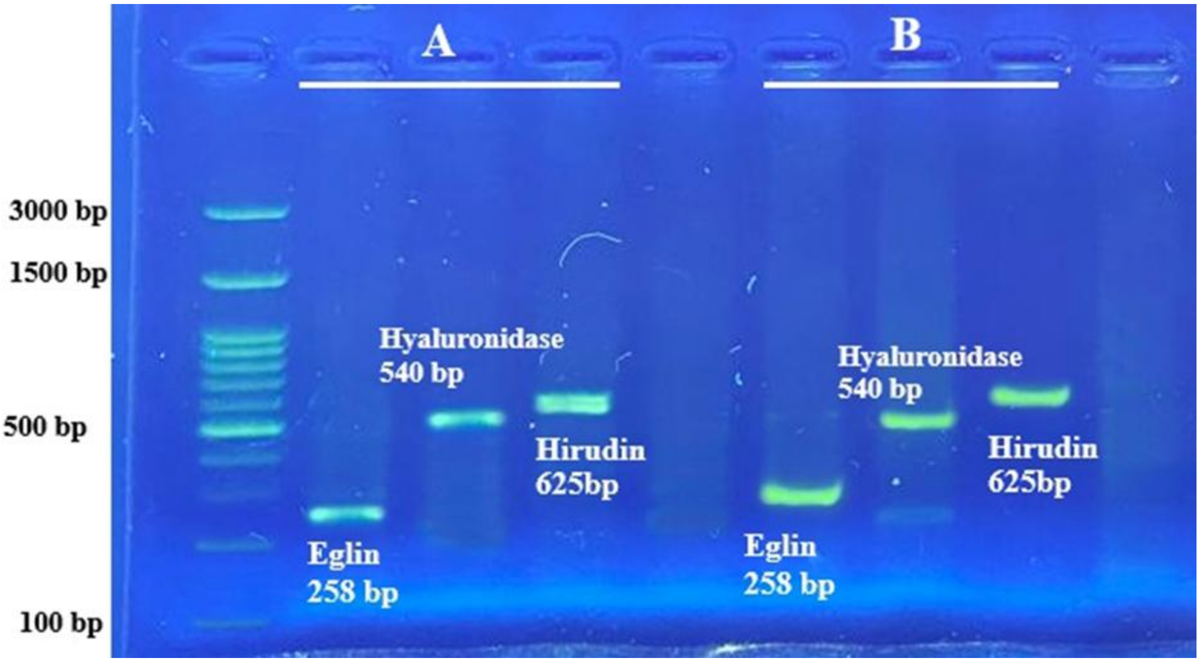


Figure 6. PCR amplification of partial Hirudin, Eglin C, and Hyaluronidase genes for two leech species: A (*Hirudo oreintalis*); B (*Limnatis paluda*), and the first lane is a marker ladder (3000 -100 bp).

Table 4. GenBank accession no. of studied protein genes of both Leech species.

Name	Accession No.	Gene name
Limnatis paluda	PV231193	Eglin C
Hirudo orientalis	PV231194	Eglin C
Limnatis paluda	PV231195	Hirudin
Hirudo orientalis	PV231196	Hirudin
Limnatis paluda	PV231197	Hyaluronidase
Hirudo orientalis	PV231198	Hyaluronidase

For detecting and explanation of inter-genera variant position of nucleotides of each studied partial gene and finding of changed codons amino acid of each Hirudin, Eglin C and Hyaluronidase, a multiple protein sequence alignment analysis of each studied protein gene of *H. orientalis* and *L. paluda* submitted for sequencing, and the total structure as well as the amplified and sequenced parts in the present study were shown in figures 5, 7 and 9 according to phyre 2.2; Protein Data Bank in Europe and Protein Data Bank in Japan and the variation results were shown in figures 8; 10 and 12 as well as tables 5; 6 and 7. Among the two submitted sequences of Eglin C, Hirudin, and Hyaluronidase genes of both *L. paluda* and *H. orientalis*, different amino acids showed variations according to alignment in the program of MEGA version 11, shown in (Fig. 8; Table 5), (Fig. 10; Table 6), and (Fig. 12; Table 7), respectively.

Table 5. Explain the numbers and variant positions of nucleotides of each sample are changed with amino acid codons in the Eglin C gene of Leeches.

Leech species	**Gene name**	**Variant position**	**Nucleotide changed**	**Amino acid changed**	**Codon number changed**	**GenBank Reference accession number**
Limnatis paluda	Eglin C	22	T/A	F/Y	7	OL598605
Hirudo orientalis		91	A/C	A/D	50	

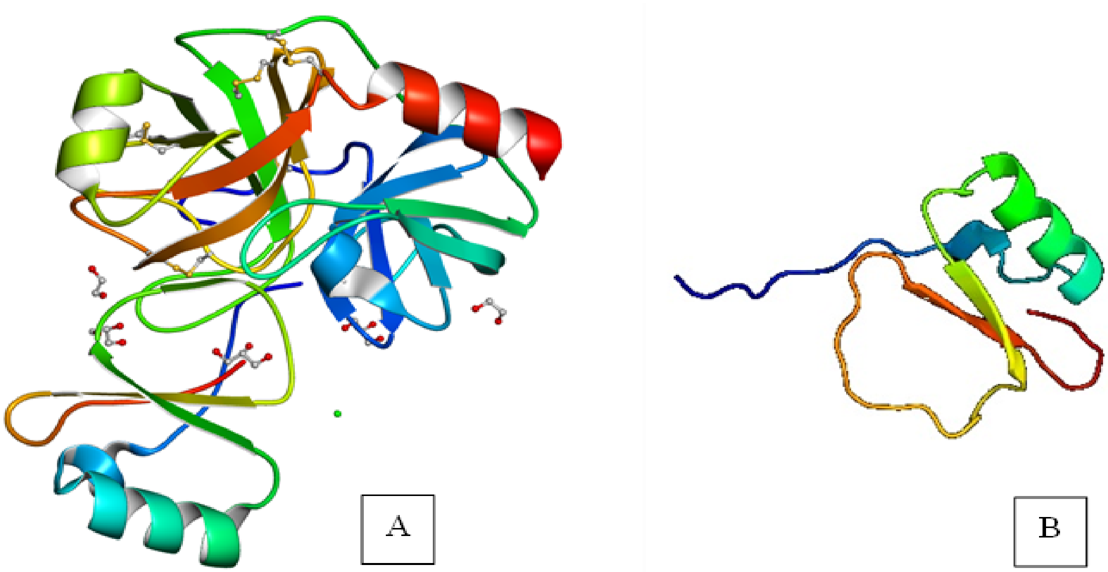
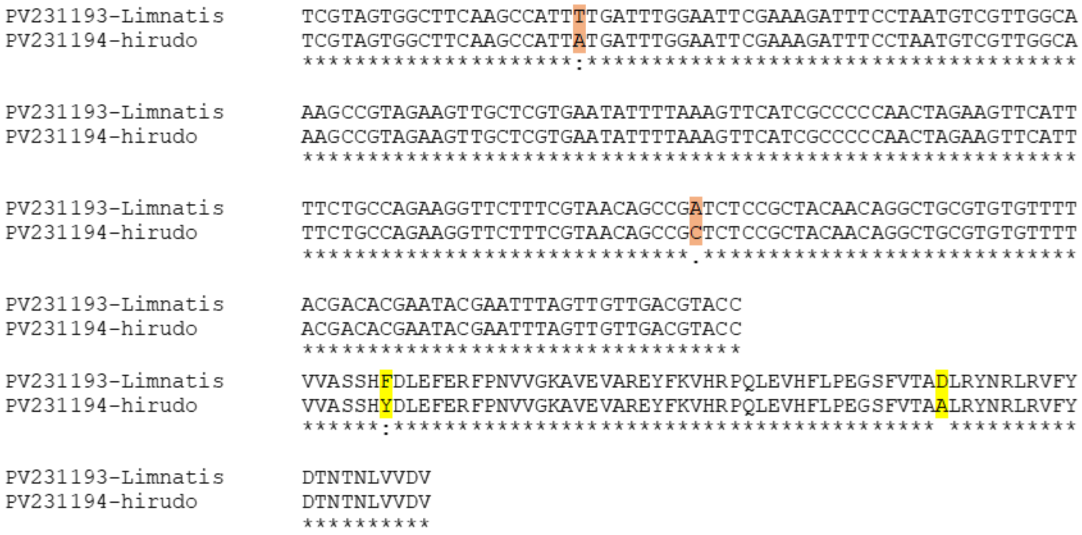


Figure 7. Eglin C structure (according to Phyre 2.2): A- Complete structure; B- The amplified and sequenced part in the present study.



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PV231193-Limnatis      TCGTAGTGGCTTCAAGCCATTTTGATTTGGAATTCGAAAGATTTCCTAATGTCGTTGGCA
PV231194-hirudo        TCGTAGTGGCTTCAAGCCATTATGATTTGGAATTCGAAAGATTTCCTAATGTCGTTGGCA
                       *********************:**************************************

PV231193-Limnatis      AAGCCGTAGAAGTTGCTCGTGAATATTTTAAAGTTCATCGCCCCCAACTAGAAGTTCATT
PV231194-hirudo        AAGCCGTAGAAGTTGCTCGTGAATATTTTAAAGTTCATCGCCCCCAACTAGAAGTTCATT
                       ************************************************************

PV231193-Limnatis      TTCTGCCAGAAGGTTCTTTCGTAACAGCCGATCTCCGCTACAACAGGCTGCGTGTGTTTT
PV231194-hirudo        TTCTGCCAGAAGGTTCTTTCGTAACAGCCGCTCTCCGCTACAACAGGCTGCGTGTGTTTT
                       ******************************.*****************************

PV231193-Limnatis      ACGACACGAATACGAATTTAGTTGTTGACGTACC
PV231194-hirudo        ACGACACGAATACGAATTTAGTTGTTGACGTACC
                       **********************************

PV231193-Limnatis      VVASSHFDLEFERFPNVVGKAVEVAREYFKVHRPQLEVHFLPEGSFVTADLRYNRLRVFY
PV231194-hirudo        VVASSHYDLEFERFPNVVGKAVEVAREYFKVHRPQLEVHFLPEGSFVTAALRYNRLRVFY
                       ******:****************************************** **********

PV231193-Limnatis      DTNTNLVVDV
PV231194-hirudo        DTNTNLVVDV
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Figure 8. Multiple protein sequence alignment analysis of the Eglin C gene of Leeches among two submitted sequences

Table 6. Explain the numbers and variant positions of nucleotides of each sample are changed with amino acid codons in the hirudin gene of Leeches.

	Gene name	**Variant position**	**Nucleotide changed**	**Amino acid changed**	**Codon number changed**	**GenBank Reference accession number**
Limnatis paluda	Hirudin	40	C/G	Non-coding	Non-coding	X72786
		61	A/T	Non-coding	Non-coding	
Hirudo orientalis		74	C/T	Non-coding	Non-coding	
		385	C/A	Non-coding	Non-coding	
		395	G/T	Non-coding	Non-coding	

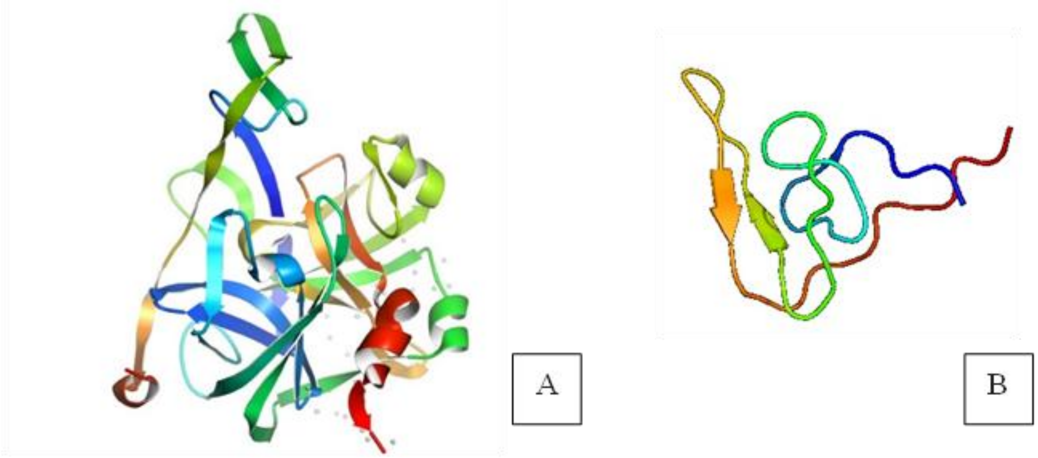

Figure 9. Hirudin structure (according to Phyre 2.2): A- Complete structure; B- The amplified and sequenced part in the present study.

```
PV231195-Limnatis       GGCTGTTTGCATCTGCGTGTCTCAAGCAGGTAAGAAAAACTTAATTTGTCTTTGTAAGAA
PV231196-Hirudo         GGCTGTTTGCATCTGCGTGTCTCAAGCAGGTAAGAAAAAGTTAATTTGTCTTTGTAAGAA
                        *************************************** ********************

PV231195-Limnatis       AGGAAAGTGTTGACTAGAATGTCATGTTTATTTTTGTTTGTTTTGGCCAATCAAATGACC
PV231196-Hirudo         TGGAAAGTGTTGATTAGAATGTCATGTTTATTTTTGTTTGTTTTGGCCAATCAAATGACC
                        :************ **********************************************

PV231195-Limnatis       TTTTTTTTTAAATTCAGTGAGCTACACTGATTGTACGGAATCAGGTCAGAATTATTGTCT
PV231196-Hirudo         TTTTTTTTTAAATTCAGTGAGCTACACTGATTGTACGGAATCAGGTCAGAATTATTGTCT
                        ************************************************************

PV231195-Limnatis       ATGCGTGGTACGTTCGAAATTTACTATTTTTTATGCTTTAAATTCCCCAACAAATGCTGT
PV231196-Hirudo         ATGCGTGGTACGTTCGAAATTTACTATTTTTTATGCTTTAAATTCCCCAACAAATGCTGT
                        ************************************************************

PV231195-Limnatis       TGAATTTTTAGGGAAGTAATGTCTGCGGTGAAGGAAAAAATTGTCAACTGAGCAGTTCTG
PV231196-Hirudo         TGAATTTTTAGGGAAGTAATGTCTGCGGTGAAGGAAAAAATTGTCAACTGAGCAGTTCTG
                        ************************************************************

PV231195-Limnatis       GAAATCAGTGCGTCCATGGGGGTTTGTTTTGGCAATTTATGACAAAAATTTTAATATATC
PV231196-Hirudo         GAAATCAGTGCGTCCATGGGGGTTTGTTTTGGCAATTTATGACAAAAATTTTAATATATC
                        ************************************************************

PV231195-Limnatis       CATACAAGCTACCATAGTTACTCACACGTCTGACGCAAAGAAAATTTATTGGAGTATTTC
PV231196-Hirudo         CATACAAGCTACCATAGTTACTCAAACGTCTGACTCAAAGAAAATTTATTGGAGTATTTC
                        ************************.********* *************************

PV231195-Limnatis       GTAAACCCCTTTTGTTTACCCCCAAAATTGATAATATTGCCCGAGTTACATATTAAATTG
PV231196-Hirudo         GTAAACCCCTTTTGTTTACCCCCAAAATTGATAATATTGCCCGAGTTACATATTAAATTG
                        ************************************************************

PV231195-Limnatis       AAAGATAATGGCAAAATATTATTGATCATTAATATCATCATTTTAATTGCAGAAGGTACT
PV231196-Hirudo         AAAGATAATGGCAAAATATTATTGATCATTAATATCATCATTTTAATTGCAGAAGGTACT
                        ************************************************************

PV231195-Limnatis       CCGAAGCCTAAGAGCCAGACTGAAG
PV231196-Hirudo         CCGAAGCCTAAGAGCCAGACTGAAG
                        *************************

PV231195-Limnatis       AVCICVSQAVSYTDCTESGQNYCLCVGSNVCGEGKNCQLSSSGNQCVHGEGTPKPKSQTE
PV231196-Hirudo         AVCICVSQAVSYTDCTESGQNYCLCVGSNVCGEGKNCQLSSSGNQCVHGEGTPKPKSQTE
                        ************************************************************
```

Figure 10. Multiple protein sequence alignment analysis of the Hirudin gene of leeches among two submitted sequences.

Table 7. Explain the numbers and variant positions of nucleotides of each sample are changed with amino acid codons in the hyaluronidase gene of Leeches

Leech species	Gene name	Variant position	Nucleotide changed	Amino acid changed	Codon number changed	GenBank Reference accession number
Limnatis paluda	hyaluronidase	106	A/T	No changed	36	KJ026763
Hirudo orientalis						

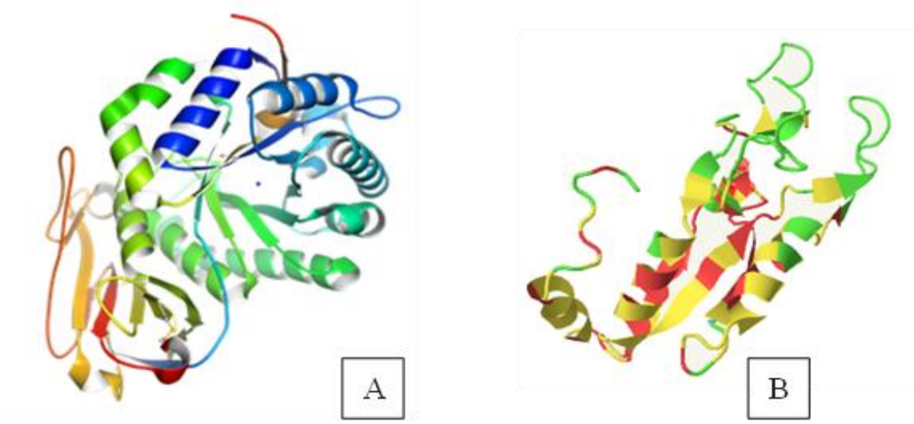

Figure 11. Hyaluronidase structure (according to Phyre 2.2): A- Complete structure; B- The amplified and sequenced part in the present study.

```
PV231197-Limnatis      TATTTTCACCGAAGGGGTTGTGGAGCTTTGTTGACATTACCTCACCGAAATTGTTTAAAC
PV231198-Hirudo        TATTTTCACCGAAGGGGTTGTGGAGCTTTGTTGACATTACCTCACCGAAATTGTTTAAAC
                       ************************************************************

PV231197-Limnatis      TCTTGGAGGGTCTCTCTCCTGGTTACTTCAGGGTTGGAGGAACGTTAGCTAACTGGCTGT
PV231198-Hirudo        TCTTGGAGGGTCTCTCTCCTGGTTACTTCAGGGTTGGAGGAACGTTTGCTAACTGGCTGT
                       **********************************************:*************

PV231197-Limnatis      TCTTTGACTTAGATGAAAATAATAAGTGGAAAGACTATTGGGCTTTTAAAGATAAAACAC
PV231198-Hirudo        TCTTTGACTTAGATGAAAATAATAAGTGGAAAGACTATTGGGCTTTTAAAGATAAAACAC
                       ************************************************************

PV231197-Limnatis      CCGAGACTGCAACAATCACAAGGAGGTGGCTGTTTCGAAAACAAAACAACCTGAAAAAAG
PV231198-Hirudo        CCGAGACTGCAACAATCACAAGGAGGTGGCTGTTTCGAAAACAAAACAACCTGAAAAAAG
                       ************************************************************

PV231197-Limnatis      AGACTTTTGACGACTTAGTCAAACTAACCAAAGGAAGCAAAATGAGACTGTTATTTGATT
PV231198-Hirudo        AGACTTTTGACGACTTAGTCAAACTAACCAAAGGAAGCAAAATGAGACTGTTATTTGATT
                       ************************************************************

PV231197-Limnatis      TAAACGCTGAAGTGAGAACTGGTTATGAAATTGGAAAGAAAATGACATCCACTTGGGATA
PV231198-Hirudo        TAAACGCTGAAGTGAGAACTGGTTATGAAATTGGAAAGAAAATGACATCCACTTGGGATA
                       ************************************************************

PV231197-Limnatis      GCTCGGAAGCTGAAAAATTATTCAAATACTGTGTGTCAAAAGGTTATGGAGATAATATTG
PV231198-Hirudo        GCTCGGAAGCTGAAAAATTATTCAAATACTGTGTGTCAAAAGGTTATGGAGATAATATTG
                       ************************************************************

PV231197-Limnatis      ATTGGGAACTTGGTAATGAACCGGACCATACCTCCGCACACAATCTTACTG
PV231198-Hirudo        ATTGGGAACTTGGTAATGAACCGGACCATACCTCCGCACACAATCTTACTG
                       ***************************************************
PV231197-Limnatis      FSPKGLWSFVDITSPKLFKLLEGLSPGYFRVGGTFANWLFFDLDENNKWKDYWAFKDKTP
PV231198-Hirudo        FSPKGLWSFVDITSPKLFKLLEGLSPGYFRVGGTFANWLFFDLDENNKWKDYWAFKDKTP
                       ************************************************************

PV231197-Limnatis      ETATITRRWLFRKQNNLKKETFDDLVKLTKGSKMRLLFDLNAEVRTGYEIGKKMTSTWDS
PV231198-Hirudo        ETATITRRWLFRKQNNLKKETFDDLVKLTKGSKMRLLFDLNAEVRTGYEIGKKMTSTWDS
                       ************************************************************

PV231197-Limnatis      SEAEKLFKYCVSKGYGDNIDWELGNEPDHTSAHNLT
PV231198-Hirudo        SEAEKLFKYCVSKGYGDNIDWELGNEPDHTSAHNLT
                       ************************************
```

Figure 12. Multiple protein sequence alignment analysis of the hyaluronidase gene of Leeches among two submitted sequences.

The present results of single or two nucleotides in partial genes sequenced in the present study suggest that these two species are various clades descended from a common ancestor; these small variants in the proteinic code may be a result of spontaneous mutation or adaptation to a specific lifestyle (Abdualkader, 2013; Kovalenko and Utevsky, 2014). In general, most mutations are nucleotide substitutions as seen in the present work, that one base is replaced by another one (Fig. 8; 10; 12), and this result support our thinking about adaptative life styles mutations, since changing of a single nucleotide cause changes in the amino acid sequences, which can impact the protein forms and actions (Fan et al., 2025). The anti-inflammatory effects of Eglin C are due to inhibiting specific proteases, which can reduce inflammation, swellings and tissue damages, and can help regulating of inflammatory responses, hence the present study showed two amino acids variation just in the amplified segment (Fig. 8) that suggests more variations all over all gene sequence, which may be the cause of more *Hirudo* ability than *Limnatis* for controlling of swelling after feeding because (Németh et al., 2025).

stated the swelling effects of infestation with *Limnatis,* while no or mild swelling was noticed after leeching with *Hirudo* (Faria et al., 1999; Gideroglu et al., 2003).

As Hirudin is the main anticoagulant natural occurring peptide in leech's saliva, and all Hirudin's have potential effects in anticoagulation make it a valuable compound in leeches' life, since it is a limit factor in their life stay more stable genetically, hence, the present study so similar sequences were noticed between the two studied species as well as in compare to the sequences of the GenBank (Fig. 8; 10; 12) (Fritsma, 2013; Liu et al., 2023). Hyaluronidase, generally, is an active enzyme that breaks down the key component of connective tissue, "hyaluronic acid," that plays a crucial role in skin reactions, healing, and complications (Gideroglu et al., 2003). The present results showed only one amino acid variations between partial gene *H. oreintalis* and *L. paluda* that enhances the idea of better adaptation to the parasitic life style through minimizing of host immune reactions, hence, both species shares best copy of the gene for translating of most adapted protein to the specific host immune system as a mimicry process, especially it is known mentioned leech species have different hosts (Hovingh, and Linker,1999; Khan, 2012).

Conclusion

The present study cleared presence of two hematophagous leeches (*H. oreintalis* and *L. paluda*) in Iraq and improved the validity of COI locus for leech species identification since results showed nucleotides variance, as well as SDS-page is sufficient technique to detection and isolation of leech saliva proteins, the molecular analysis of the Hirudin, Eglin C and Hyaluronidase genes of both leech species revealed active proteomic contents were similar in size but nucleotides variation were noticed in both coding and non-coding regions.

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