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DNA-Based Study of Medicinal *Leeches (Hirudo verbana*) in Iraq

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***Abstract*—** Leeches of the Hirudinidae family are obligate parasitic organisms in some types of vertebrate animals. The interest in using leeches for medical purposes has been increasing lately. (18) Medicinal leeches were obtained from different clinics & institutes in Baghdad, Iraq, including the Research and Training, Al-Yermok Center, and Rafi Center for Chinese Medicine, during the period from (January to March 2025) . The nine leech samples collected were morphologically examined. Molecular analysis was conducted on only two specimens, using the mitochondrial cytochrome c oxidase subunit I (COI) gene. Molecular analysis revealed a (91%) genetic similarity between the examined samples & the reference sequence of *Hirudo verbana* deposited in GenBank (Accession No. EF446693.1), indicating a close phylogenetic relationship. Phylogenetic tree analysis further supported the clustering of the studied specimens with reference to *H. verbana* isolates. The morphological and molecular findings confirmed that all examined medicinal leeches belonged to *Hirudo verbana*. These results demonstrated the usefulness of the COI gene as a reliable molecular marker for species identification and genetic characterization of medicinal leeches in Iraq.

Key words: *Hirudo verbena*, DNA analysis, Morphology, Iraq

INTRODUCTION

The phylum Annelida, which includes over 22,000 known species, also comprises the leeches as one of its notable subdivisions. The annelids have many vital functions in nature and agriculture, as well as benefiting humans. (1,2).

Classified within the phylum Annelida and class Hirudinea, medicinal leeches are hermaphroditic, segmented worms that act as ectoparasites and rely on blood as their primary food source. Most species inhabit freshwater environments, favoring ponds with soft sediments and abundant littoral plants, fish, amphibians, water birds, reptiles, and mammals (including humans) are among the many aquatic creatures that medicinal leeches feed on by sucking their blood (3,4).

Although over 680 species of leeches are known, merely 15 species are consideredfrom Europe, also occurring in Transcaucasia, Iran, and Uzbekistan, is the most widely recognized. Other notable species include *H. verbena* from Turkey, *H. troctina* in North Africa, *H. nipponia* in Japan, and H. quinquestriata in Australia. In Southeast Asia, species such as Poecilobdella granulosa**,** *Hirudinaria javanica*, and *Hirudinaria*

manillensis are prevalent. *Haementeria officinalis* occurs in Mexico, *Macrobdella decora* in the United States, while *Limnatis nilotica* is the most common medicinal leech in Egypt, and Lebanon (6,7).

Medicinal leeches have been utilized for generations due to their hematophagous behavior. They constitute the taxonomic basis of the class Hirudinea within biological classification. In recent years, these organisms have been widely employed in genetic

Studies have focused on neurophysiology, behavioral patterns, and development (8). The use of leeches for medicinal purposes has been a part of history for a long time, indicating their importance in healthcare (9). As a result of their wide usage in medicines and surgeries, medicinal leeches have received a lot of attention when it comes to studying invertebrates biologically and physiologically. In the recent past, the taxonomic classification of medicinal leeches has been substantially altered using modern approaches like molecular and cytogenetics. (10).

MATERIALS AND MTHODS

Leeches Collection and Preparation

Eighteen medicinal leech specimens were collected. from various clinics and research centers in Baghdad, Iraq between January and March 2025. The centers include the Alhwya Center for Studies, Research and Training, Al-Yermok, and Rafi Center for Chinese Medicine. All the clinics and centers involved in this research have been officially registered under the General Syndicate for Complementary and Herbal Medicine in Iraq.

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and centers involved in this research have been officially registered under the General Syndicate for Complementary and Herbal Medicine in Iraq. Morphological examination was performed on nine specimens, while molecular analysis was conducted on only two specimens because the remaining samples had deteriorated.

Groups of leeches were housed in glass containers containing chlorine-free water at approximately two-thirds of the container's capacity. To ensure proper ventilation, a porous cover was placed over the container and fastened with a rubber band. Water renewal was performed every three to six days, according to the density of leeches within the container. Preserved specimens were morphologically identified using an ocular micrometer (Vernier) (11,12) following standard taxonomic keys and references, after careful rinsing under running water (13,14,15) .

A total of non-local medicinal leeches was examined in this section of the study. Specimens were morphologically identified and described based on the diagnostic characteristics. Before proceeding with DNA extraction, the leeches were stored at room temperature in 70% ethanol (v/v) to ensure proper preservation.

Taxonomy

The process followed in isolating DNA from the leech sample through the use of the Geneaid kit included the following procedures:

1. Approximately 0.5 grams of leech tissue was isolated from the growing media and placed into a 1.5 mL Eppendorf tube.
2. This was followed by the addition of 200 µL of the lysozyme solution (0.8 mg/200 mL) to the Eppendorf tube containing the tissue.
3. Mixing of the two was achieved through the use of a vortex mixer.
4. Subsequently, the tube was incubated at 37°C for 30 minutes, with occasional mixing every three minutes.
5. Proteinase K was then added to the tube in an amount of 20 µL, and mixed using a vortex mixer.
6. This step was followed by incubation at 60°C for 10 minutes.
7. Finally, 200 µL of GB buffer was added and mixed using a vortex mixer, and then incubated at 70°C.
8. After adding and manually mixing 200 microliters of absolute ethanol (also known as absolute alcohol), the liquid was moved to a GD tube (gas discharge), which was placed in a collection tube, and centrifuged at 16000g for 30 seconds and remove the filtrate, followed by the addition of 600 microliters of washing solution, centrifugation at the same speed and duration to remove the precipitate, and centrifugation again for three minutes to remove any remaining washing solution.

After moving the GD column to a fresh 1.5 ml tube, 100 microliters of the dissolving solution were added, and the tube was left for three minutes. DNA was then preserved until it was needed after being centrifuged for 30 seconds at a speed of 16,000 g.

DNA Amplification and Sequencing

A fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using primers. The universal forward primer (5′-GGTCAACAAATCATAAAGATATTGG-3′), and reverse primer (5′- TAAACTTCAGGGTGACCAAAAAATCA-3′) were chosen by (17).

The amplification produced an approximately 650 bp fragment. The PCR amplification was performed using a thermocycler under the following conditions: an initial denaturation step at 95°C for 6 min, followed by 35 cycles consisting of denaturation at 95°C for 1 min and 30 s, annealing at 50°C for 1 min and 30 s, and extension at 72°C for 2 min. The amplification process was completed with a final extension step at 72°C for 5 min. The amplified PCR products were then maintained at 4°C until further analysis

Sequencing was done to PCR products. The results were examined using the BLAST Program after gene sequences were compared to those recorded by the National Center for Biotechnology Information (NCBI) (16).

The identified sequences were contrasted with those obtained from the NCBI GenBank nucleotide sequence databases. The neighbor-joining approach was used to create a distance matrix tree (17). Using the MEGA-7 program, bootstrap (1000X) analysis was used to create the phylogenetic tree's topology (18).

RESULT AND DISCUSSION

External Features

The study's leeches were dorsoventrally flattened and tapered at the front end, with a maximum body length of about 14 cm when relaxed. figure A, when contracted, the maximum body length is 4 cm. figure B.

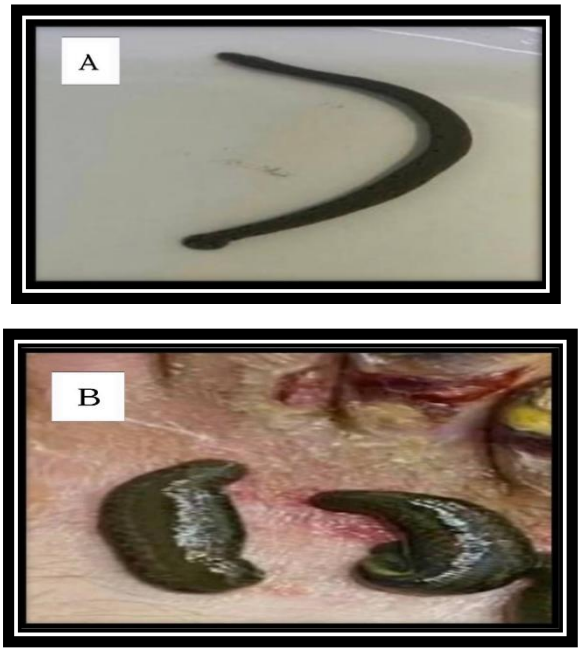

Figure 1. Examined alive Medicinal Leech. A: fully extended. B: contracted.

Molecular Analysis

Genomic DNA Extraction

For molecular investigations, total genomic DNA was extracted from nine individual leeches. Tissue samples were obtained from the body wall muscle located at the caudal sucker (19.20). DNA isolation was carried out using gSYNC™ DNA Extraction Kit, applying the standard procedure recommended for tissue-derived genomic DNA purification.

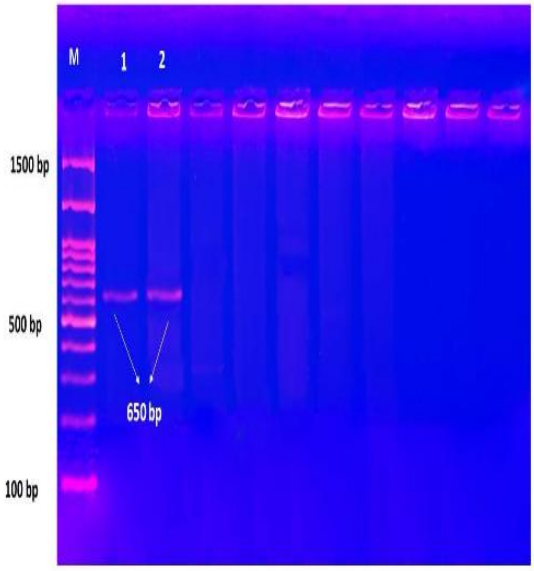

Figure 2. Agarose gel electrophoresis of PCR amplicons from extracted leech DNA, demonstrating a target band of 650 bp.

The diagram illustrates the gel electrophoresis results for the PCR-amplified fragments obtained after extracting genomic DNA from two leeches. The lane labeled M is the marker utilized for the estimation of the size of amplified fragments. There was a clear appearance of a band of about 650 bp in lanes (1) and (2), showing that there was efficient amplification of the gene fragment. However, no visible bands could be seen in the rest of the lanes. This could have been caused by poor DNA quality, low DNA concentration, or inhibitors that interfered with PCR amplification efficiency.

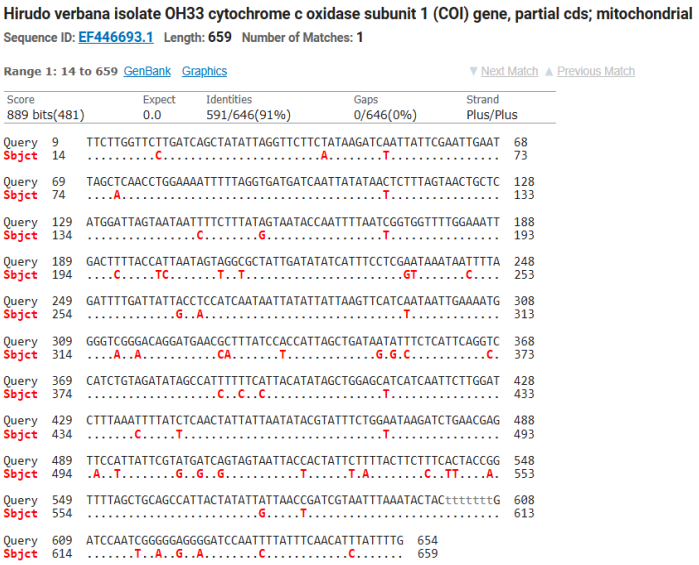

Figure 3. DNA sequencing result of *Hirudo verbena*

The DNA sequence under study matches the mitochondrial gene Cytochrome c oxidase subunit I (COI) in the genus Hirudo, according to BLAST analysis. With a Bit score of 889, an E-value of 0.0, and a 91% sequence identity (591/646 bp) without gaps, the sequence most closely resembled Hirudo verbana isolate OH33 (GenBank accession: EF446693.1). These results reveal a high molecular similarity of the tested sample to the sequence records of the famous medicinal leech species, Hirudo verbana. This high similarity confirms the accuracy of the determination of the species as well as its genetic connection to known members of this species of the medicinal leech. The reason why the COI gene is a reliable molecular marker in the taxonomic study is due to the ability of this gene to distinguish close animal relatives based on nucleotide differences. That is why the COI gene is widely used in molecular taxonomy and DNA barcoding studies.

In molecular taxonomy, species-level confirmation using COI typically requires higher identity values; therefore, this level of similarity may indicate that the sample represents a local genetic lineage not fully represented in genetic databases or possibly a closely related species within the same genus, even though the 91% similarity indicates a clear genetic relationship.

These results align with a number of international investigations concerning the genetic diversity of the genus *Hirudo*. Through molecular analysis, (21) showed that the traditional medicinal leech, which was once thought to be a single species (*Hirudo medicinalis*), actually consists of several genetically distinct species, including *Hirudo verbana*, which is widely used in commercial and medical applications throughout Western Asia and Europe. Later studies have revealed that *H. verbana* has distinct geographic lineages and significant genetic diversity in COI sequences. In a similar vein, (22) verified that *H. verbana*, not *H. medicinalis*, is the species that appears as the closest match in the current BLAST

results, as many medicinal leeches that are commercially available worldwide belong to this species.

the analyzed sequence can be identified as belonging to the genus *Hirudo* and is closely related to the species *Hirudo verbana*. However, to identify the exact species to which the analyzed sequence belongs, further phylogenetic analysis using a number of reference sequences is required and is in accordance with the current recmmendations on molecular taxonomy, which use a combination of BLAST similarity and phylogenetic tree reconstruction to identify a species accurately.

The DNA sequencing technique is regarded as a crucial foundation for recognizing novel strains of living things and highly conserved areas in microorganisms. The sequencing of amplified fragments often uses polymerase chain reaction products to detect the genetic differences in these fragments. Over the last few years, the outcome of the DNA sequencing technique has proven to be highly accurate in detecting genetic changes. (23,24).

Because the genus *Hirudo* is used medicinally, it might be considered the most well-known member of the Hirudinidae family.

The first known species in the genus *Hirudo* is the common European medical leech, *H. medicinalis*. Other species that have been identified include More thorough research has shown that *H. nipponia, H. orientalis, H. sulukii, H. troctina*, and *H. verbena* are legitimate species (25,26).

According to the current study's findings, exterior coloration is thought to be a deceptive feature for identifying medicinal leeches, although color patterns are thought to be variations within a species. Only molecular systematic examinations as vital indication for differentiating *Hirudo* species. According to (27), the fundamental identification feature is the coloration pattern of the dorsal and ventral parts of the body, with the ventral side having greater diagnostic features. A study founded All of these leech specimens belonged to *Hirudo medicinalis*, according to the findings of the morphological analysis and partial 18s rDNA molecular sequencing (28).

Molecular investigation results were highly similar to those reported in previous studies by (17). In contrast, Random Amplified Polymorphic DNA–Polymerase Chain Reaction (RAPD-PCR) analysis did not demonstrate obvious genetic differences among the tested samples.

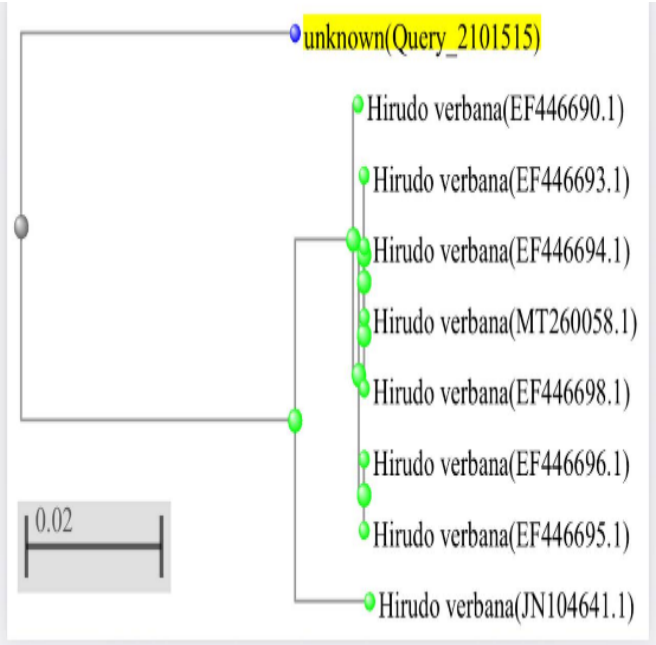

Figure 4. phylogenetic trees showing the relationship between the unknown sequence (Query -2101515) and *Hirudo verbana* isolate based on cytochrome C oxidase subunit I (CO-I) region sequences using MEGA-7 software.

Figure (4) represents a phylogenetic tree that was developed based on mitochondrial COI gene sequences with the help of MEGA-7 software to identify the taxonomic classification of the unknown sample (Query_2101515). Based on the results obtained from the figure, it can be seen that the unknown sequence is very similar to some reference sequences of *Hirudo verbana*. Short branches as well as small genetic distances observed in the phylogenetic tree suggest a close relationship between the unknown sequence and the mentioned species because these two factors are considered to be key indicators of phylogeny. I also concur with this interpretation since it has been recently revealed that COI gene plays a significant role in the DNA barcoding process along with other functions, such as genetic variation and phylogenetic analysis of animal species (29)(30).

Nevertheless, I find it appropriate to express disagreement in using only this type of evidence as a basis for definitive speciation because, according to the latest research, there is the need to reinforce the findings generated from the analysis of the COI marker through other types of molecular indicators along with statistical methods such as bootstrapping due to the presence of a considerable level of intraspecific variation or genetic resemblance of a particular group or among related species that can have an impact on the identification process (31)(32).

CONCLUSION

1. Morphological investigations along with molecular identification confirmed that all medicinal leeches used at the hospitals in Baghdad were Hirudo verbana.

2.It was proved to be efficient for taxonomic identification to employ the COI gene as a molecular marker for getting accurate information about the studied specimens.

Based on DNA sequencing, the genetic similarity of the studied leeches to the reference sequences of the same species was detected with the possibility of the presence of the local variations.

3.It can be stated according to the construction of the phylogenetic tree that there is a close relation between the investigated samples and the strains of Hirudo verbana.

From the point of view of the obtained results, external morphology is insufficient for the differential identification of medicinal leeches due to their strong resemblance.

Recommendations

1.The use of molecular diagnostic methods for leeches, particularly COI gene testing, should be incorporated as one of the primary techniques used along with morphological diagnostics for medicinal leech identification in Iraq.

2.Further research should encompass various provinces in Iraq in order to provide information on genetic diversity and geographical distribution of medicinal leech species.

3.Conducting further genetics studies involving additional molecular markers and drawing additional phylogenetic trees should contribute to the enhancement of the reliability of leech species and lineages identification.

4.A genetic database for medicinal leeches of Iraq must be created and connected to international databases for more effective research and identification purposes.

5.It is necessary to monitor medicinal leeches' quality and source in order to ensure accurate taxonomical identification of medicinal leeches provided in treatment centers.

6.Research into medicinal uses of Hirudo verbana is suggested for application to help the healthcare system.

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N/A

Conflict of Interest

The authors declare no conflict of interest.

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