11

Phylogenetic Analysis of Two Types of Leeches and *Verbana*) Using Mitochondrial (*Medicinalis* Cytochrome *c* Oxidase Subunit I

Najah Sobhi Nayef[2]   Islam Khalid Kamal [1]

[1] Department of Clinical laboratory sciences/

pCollege of Pharmacy/ oUniversity of Mosul

Email: islamkhalid14@gmail.com

[2]Department of Biology/ College of Science/

University of Mosul

Email: najhsbio26@uomosul.edu.iq

ABSTRACT

Medicinal leeches are well-known parasites that have been utilized as therapeutics in medical and surgical applications.

The present study included a comparison between two kinds of medical leeches. The samples were collected from two different regions in Iraq. The first samples of leeches were collected from Khormal village in Sulaymania governorate, and the second from Shaqlawa town in Erbil governorate. The foundation of this research was molecular biology; DNA was extracted from the samples of the two regions using a kit provided by (Geneaid) factory and sent to the National center for Biotechnology Information (NCBI).

NCBI identified the first sample as *Hirudo medicinalis* isolate NI. *Hirudo medicinalis* isolate NI shared the most similarities with *Hirudo medicinalis* isolate PO61. and *Hirudo medicinalis* isolate K067.

NCBI determined that the second sample from the other region, *Hirudo verbana* isolate IN, was the most closely related to *Hirudo verbana* (Accession No. KU672397.1) with 100% sequence similarity and 100% query coverage. Other closely related isolates to *H.verbana* isolate IN were whitmania laevis.

Furthermore, a Random amplified Polymorphic DNA-PCR (RAPD-PCR) was performed to determine the genetic differences between the two types of samples, and the results revealed no discernible differences, which could be attributed to the scarcity of samples.

Finally, phylogenetic trees for the two types were created to show the lines of evolutionary descent of various species.

INTRODUCTION

Leeches are important ectoparasites that inhibit freshwater, marine and land ecosystems. They are mostly renowned due to the sanguivorous habits of some types of Leechs (Davies *et al*., 1981; Young, 1981), as well as. their beneficial medical and surgical use, their clinical uses were widespread in the Roman Empire, which was documented in the second century by the well-known doctor Galenus.

In the 18th and early 19th centuries, France alone used 30-40 million leeches per year, and Russia used 75 million leeches per year. With a documented history of over 2000 years, medicinal leeches have received significant recognition in some aspects of human health. Its blood-sucking ability (hematophagous) has been used clinically for centuries, and this process is known as Hirudotherapy (Darabi-Darestani, 2011). Hirudotherapy is useful in treating venous congestion by alleviating postoperative hematomas (Lent, 1986), as well as improving drainage of tissues after surgical application and enhancing venous circulation, It was discovered that the Leech saliva contains a number of local anesthetics which lessen the sensations felt by the host (Iqbal, *et al.*, 2018) , and thus reduces the chance of the host removing the leech, they also secret very effective anticoagulants to prevent blood thrombosis in the wound created by its mouthparts which slows down the feeding process (Babenko, *et al.*, 2020

; Das, 2014). A vasodilator is also secreted which causes a dilation effect on the vessels proximal to the wound for a better supply (Barzegar, *et al.* 2015 ; Whiteley, 1971).

The studies concerning medical leeches were usually focused on their biology and physiology (Davies, Mcloughlin 1996), their taxonomy, however, was less examined, and the taxonomic classification efforts were usually centered on their morphological characteristics which were often confusing and proved to be unreliable to unambiguously identify morphologically intermediate individuals in certain populations, as well as morphologically similar leech samples (Trontelj, *et al.*, 2004). Recently, modern molecular and cytogenetic methods were employed to revise their taxonomy to obtain more accurate results (Todorov *et al.*, 2016 ; Agnarsson and Kuntner, 2007).

This study aims to evaluate the relationships of two types of leeches with the utilization of mitochondrial sequencing data as well as performing RAPD-PCR test, as well as drawing a phylogenetic tree demonstrating the hierarchical clustering relationships among groups of data of the same origin.

MATERIALS AND METHODS

Sample collection

Between September 2020 and the end of December 2020, samples of medical leeches were collected from Khormal village in Sulaymania and Shaqlawa town in Erbil.

The first group was identified via morphological taxonomy to be *Hirudo medicinalis,* and the second group to be *Hirudo verbana.*

Each group of leeches was placed in a glass container that had been filled to two-thirds with chlorine-free water. The glass container was covered with a porous cloth that was attached to the mouth of the container with a rubber band for ventilation. Depending on the number of leeches in the container, the water in the container was replaced every 3-6 days (Salih, 2017).

The kit supplied by (Geneaid) was used to extract DNA from leech samples (Shayan *et al*., 2007).

The concentration of DNA in all study samples was adjusted by dilution with TE buffer solution to obtain the concentration required for the PCR reactions, which was 50 nanogram/microliter for each sample.

The Master Reaction mixture for each PCR reaction was made by combining the DNA sample and the initiator of each gene with the ingredients of the Pre-mix inside an Eppendorf 0.2 ml tube supplied by the Biolaps English company.

The reaction volume was set to 20 microliters using distilled water, and the mixture was placed in a Microfuge device for 3-5 seconds to ensure that the reaction constituents have been mixed.

The reaction tubes then were placed in a thermocycler device to perform the multiplier reaction with using a special program for each reaction, and the sample was loaded into Agarose pits that had previously been prepared in 2% conc. with the addition of a volumetric guide (Ladder DNA) supplied by Biolaps within one of the pits.

The samples were then moved to an electrophoresis device for 60-70 minutes before being photographed with a Gel Documentation device.

Bundles from the PCR reaction were harvested from the gel, purified, and sent to be tested for nucleotide sequence using the analysis device provided by the (Geneaid) company (Langridge *et al.,* 1980). The results of the PCR reactions of the previously mentioned samples with the initiators of the generated bundles and gene sequences were read using a 3130 Genetic Analyzer device supplied by the Japanese company Hitachi.

The gene sequences were compared to gene sequences recorded by the National center Biotechnology Information NCBI, and the results were examined using the BLAST Program (Solgi *et al.,* 2021).

Random Amplified Polymorphic DNA (RAPD)

Following the completion of the extraction process and knowing the DNA concentrations, the results of the sequences that identified the strain and species were similar and compared with the results of the National Center Biotechnology Information.

We used the RAPD-PCR technique to identify any additional differences, so four specimens from two types of medical leeches were obtained table (1).

Table: (1) The four specimens used in RAPD-PCR

No.	Species	Locality
1	*Hirudo medicinalis*	Khormal (Sulaimanya)
2	*Hirudo medicinalis*	Khormal (Sulaimanya)
3	*Hirudo verbana*	Shaqlawa (Erbil)
4	*Hirudo verbana*	Shaqlawa (Erbil)

First: Random Primers

Four primers of random amplification of DNA with 10 bp length were used (supplied by American Promega Company) table (2).

Table: (2) The sequence of primers used in RAPD-PCR (of 10 bp length)

No.	Primer	Sequence
1.	UBC-519	5'-ACCGGACACT-3'
2.	UBC-526	5'-AACGGGCACC-3'
3.	UBC-728	5'-GTGGGTGGTG-3'
4.	UBC-735	5'-GGGAGAGGAG-3'

Second: method

1- The best annealing temperature was determined by preparing 50 µl of reaction solution, which included 25 µl of master mix (from the American Promega company), 15 µl of ion free distilled water, 5 µl of primer, and 5 µl of DNA in three different concentrations.

The program was tweaked with graduated temperature (34-40)C° (Gradient annealing temperature). In the thermal polymerization device, 5 tubes were distributed for each concentration of DNA (10 µl for each tube).

The tubes were placed horizontally in the device to achieve a gradual annealing temperature in order to determine the optimal annealing temperature for the primer.

2- After determining the ideal annealing temperature and concentration for the DNA, 25 µl of reaction mixture was prepared in small Eppendorf tubes, comprised of 12.5 µl of master mix, 7 µl ion free distilled water, 2.5 µl of primer, and 3 µl of DNA (0.2 ml).

The tubes were shaken to combine the reaction components before being placed in the thermocycler device to complete the reaction as directed by the program supplied by the Geneaid company: table (3).

Table: (3) The program used in RAPD-PCR

Cycle no.	**Time**	**Temperature C⁰**	**Steps**
1	5 min.	95	Initial Denaturation
	45 sec.	95	Denaturation
35	1 min.	37	Annealing
	1 min.	72	Extension
1	7 min.	72	Final extension
1	5 min.	4	Hold

After the reaction time was completed, 5 µl of each sample was placed in an Agarose gel pit with 3 µl of volumic guide of DNA (DNA ladder-100 bp) for one hour, then it was subjected to an ultra violet source (TM20/U.S.A. Inc. UVP) and photographed with a digital camera (Naji, 2013).

Hirudo medicinalis and Hirudo verbana

The determined sequences were compared to those obtained from the NCBI Gene bank nucleotide sequence databases.

As an outgroup, *Hirudo orientalis* strain HO1 was used. The neighbor-joining method was used to create a distance matrix tree (Saitou and Nei, 1987). The phylogenetic tree's topology was created using bootstrap (1000 X) analysis and the MEGA-7 software (Tamura *et al.*, 2013).

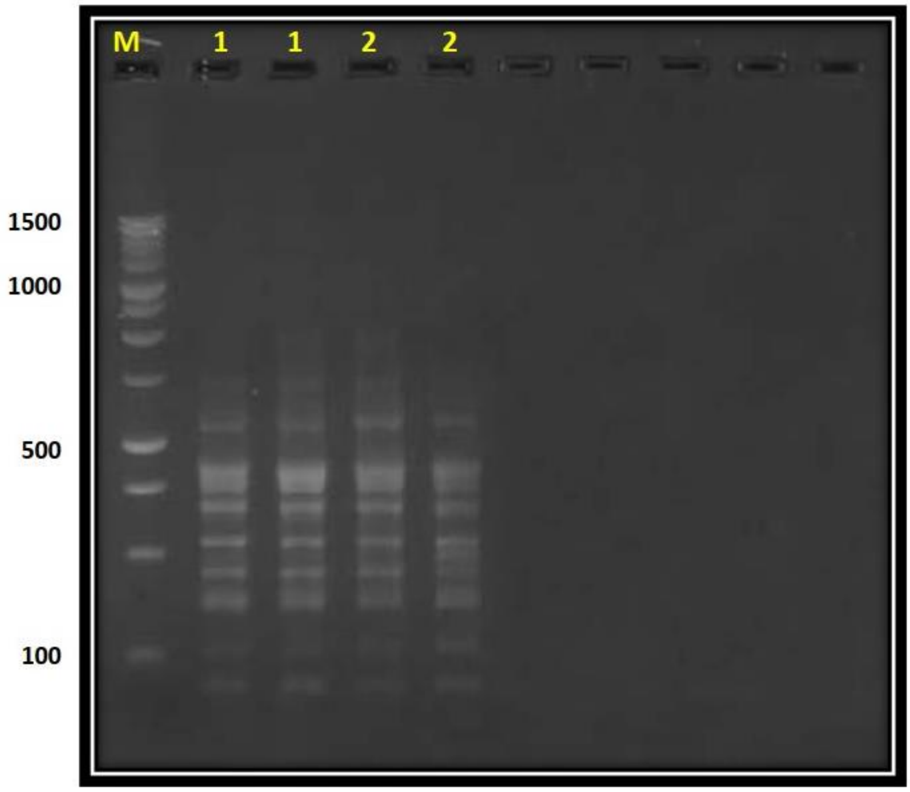
RESULTS AND DISCUSSION

****

After doing extraction of DNA from ten samples of both types of medical leeches and then determining the sequences of nitrogenous bases, RAPD-PCR was done and the results obtained was as follows:

1. Result of RAPD-PCR of medical leech sample using the primer UBC-519 showed many polymorphic bands with the nucleic acid of the medical leech and disappearance of DNA bands with size 100bp and 200bp while the

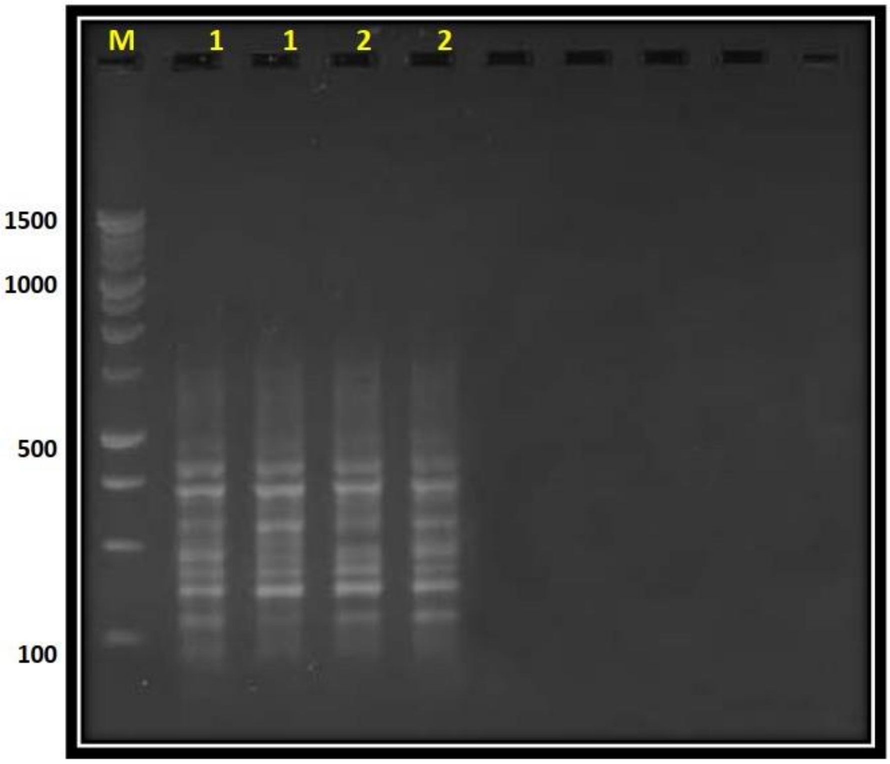
appearance of polymorphic bands with size 250bp,300bp,380bp,400bp,450bp and 550bp.



The result of RAPD-PCR using the primer UBC-519

(1: *H. medicinalis*, 2: *H. verbena*)

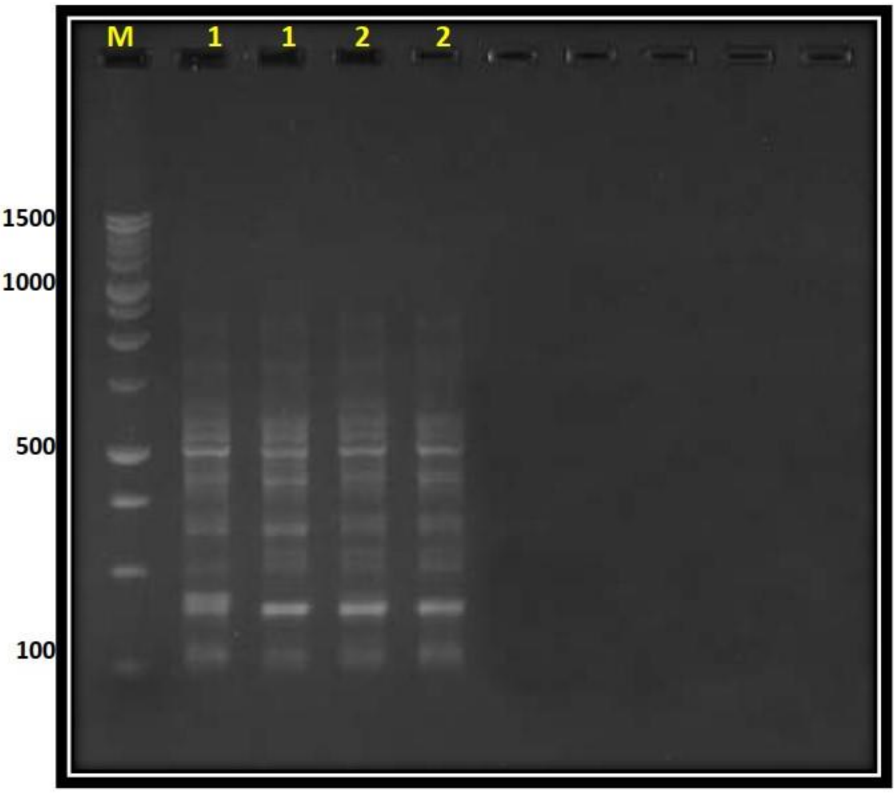
2. Result of RAPD-PCR of medical leech sample using the primer UBC-526 showed the appearance of polymorphic bands in some regions and not in others, DNA bands disappeared with size 100bp, 200bp and 500bp, while polymorphic bands appeared with 220bp,250bp,280bp,300bp,350bp, 400bp and 420bp.



The result of RAPD-PCR using the primer UBC-526

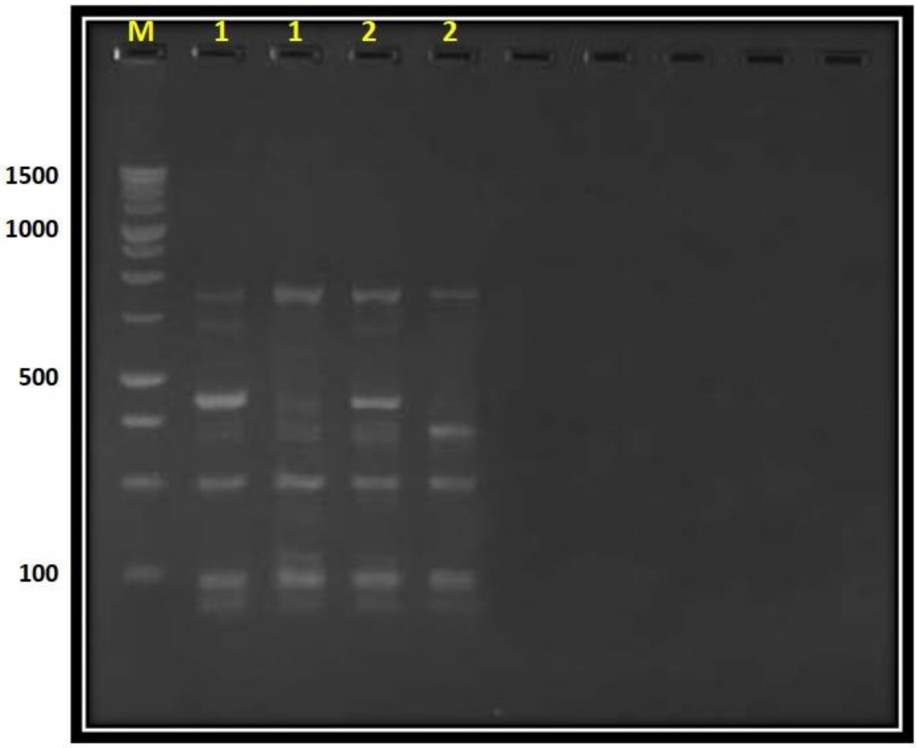
(1: *H. medicinalis*, 2: *H. verbena*)

3. Result of RAPD-PCR of medical leech sample using the primer UBC-728 the reaction showed the disappearance of DNA bands with size 100bp and 200bp while polymorphic bands appeared with size 220bp, 280bp, 370bp, 380bp, 480bp and 550bp.



The result of RAPD-PCR using the primer UBC-728
(1: *H. medicinalis*, 2: *H. verbena*)

4. Result of RAPD-PCR of medical leech sample using the primer UBC-735 the reaction showed disappearance of DNA bands with size 100bp, polymorphic bands appeared in the sample number 1 (*Hirudo medicinalis*) with size 200bp, 300bp and 480bp.



The result of RAPD-PCR using the primer UBC-735

(1: *H. medicinalis*, 2: *H. verbena*)

Bands appeared in the sample number 1 (*Hirudo medicinalis*) with the size 200bp, 300bp and 700bp, the bands appeared in the sample number 2 (*Hirudo verbana*) with size 200bp, 300bp, 480bp and 700bp, the bands in the sample number 2 (*Hirudo verbana*) with size 200bp, 300bp and 400bp.

The primers UBC-519, UBC-526, UBC-728 were able to produce number of bands which were identical in the four samples, while the primer showed disappearance of bands in some samples and their appearance in others and this indicated similarity of the samples with simple difference. The present study based on RAPD molecular markers demonstrates that the *H.verbana* forms a distinct species. Result of sequences of genes were sent to NCBI and the analysis of the results showed discovery of a new strain of *Hirudo medicinalis,* this strain was discovered for the first time and was registered with the code NI as the first letters of the names of the supervisor and the student, this new strain has got the accession number OK383440.1., the other sample of *Hirudo verbana* was determined to be the isolate IN.

Then phylogenetic study was done to determine phylogenetic trees showing the relationship between the collected samples and the related species based on cytochrome C oxidase subunit I (CO-I) region sequences using MEGA-7 software.

The assessment of the cytochrome c oxidase subunit I (CO-I) region from the *Hirudo medicinalis* isolate NI revealed that this section of gene is considered a very preserved sequence region for molecular classification of the *Hirudo medicinalis* species, as the isolate NI clustered with the top 14 *Hirudo medicinalis* hits (Figure 1). *Hirudo medicinalis* isolate NI had the highest sequence similarity to *Hirudo medicinalis* isolate PO61, *Hirudo medicinalis* isolate K067, *Hirudo medicinalis* isolate K066, *Hirudo medicinalis* isolate HMG1, *Hirudo medicinalis* isolate 7349, *Hirudo medicinalis* isolate 7347, *Hirudo medicinalis* isolate 6849. The other isolates, on the other hand, had varying sequence similarities and were assigned to different glades (Table 4 & Figure 1).

Table (4): Most related isolates with their accession numbers that show homology with *Hirudo medicinalis* isolate NI retrieved from NCBI database.

	Query Coverage	E-value	Identity	Accession No.
Hirudo medicinalis	100%	0.0	100%	OK383440.1
Hirudo medicinalis	100%	0.0	100%	KU672396.1
Hirudo medicinalis isolate 6849	100%	0.0	100%	Q333517.1
Hirudo medicinalis isolate 7347	100%	0.0	100%	HQ333516.1
Hirudo medicinalis isolate 7349	100%	0.0	100%	HQ333515.1
Hirudo medicinalis isolate K067	99%	0.0	100%	EF446708.1
Hirudo medicinalis isolate HR20	99%	0.0	99.67%	EF446712.1
Hirudo medicinalis isolate X2	99%	0.0	99.67%	EF446704.1
Hirudo medicinalis isolate HR21	99%	0.0	99.66%	EF446711.1
Hirudo medicinalis isolate HMG1	97%	0.0	100%	KR066919.1
Hirudo medicinalis isolate K066	97%	0.0	99.5%	EF446709.1
Hirudo medicinalis isolate PO61	94%	0.0	100%	EF446705.1
Hirudo medicinalis isolate Hmed_DE	95%	0.0	99.65%	AY763149.1
Hirudo medicinalis isolate PA56	94%	0.0	99.47%	EF446707.1
Hirudo medicinalis isolate Iso2	89%	0.0	98.33%	MT260057.1
Hirudo medicinalis isolate HO5	99%	0.0	92.32%	JN104648.1

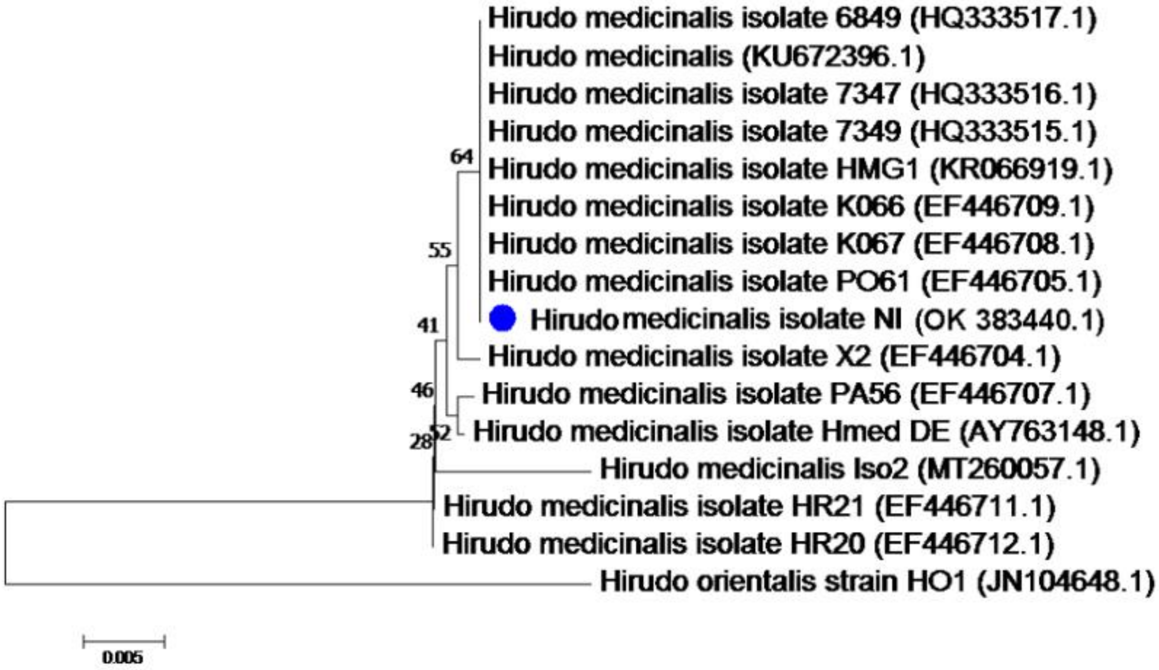


Figure (1): Neighbour-Joining phylogenetic trees showing the relationship between the *Hirudo medicinalis* isolate NI and the related species based on cytochrome C oxidase subunit I (CO-I) region sequences using MEGA-7 software.

The cytochrome c oxidase subunit I (CO-I) region of the *Hirudo verbana* isolate IN was found to be a very preserved sequence region for molecular classification of *Hirudo verbana* species and related genera isolates, as the isolate IN was grouped with the top 10 hits of the most related genera (Figure 4-13). *Hirudo verbana* isolate IN had 100% sequence similarity and 100% query coverage to *Hirudo verbana* (Accession No. KU672397.1). *Whitmania laevis*, *Glossiphonia concolor*, and *Whitmania acranulata* were the closest isolates to *Hirudo verbana* isolate IN, with 76.77%, 75.04%, and 77.59% sequence identities and 100%, 98%, and 65% query coverage, respectively. The other isolates, on the other hand, displayed a wide range of sequence similarities and were grouped in different glades (Table 5 & Figure 2).

Table 5: Most related isolates with their accession numbers show homology with *Hirudo verbana* isolate IN retrieved from NCBI database.

Related isolates	Query Coverage	E-value	Identity	Accession No.
Hirudo verbana	100%	0.0	89.14%	KU672397.1
Whitmania laevis	100%	0.0	76.77%	KM655839.1
Glossiphonia concolor	98%	0.0	75.04%	MT628565.1
Whitmania acranulata	65%	0.0	77.59%	KM655838.1
Rossia macrosoma	86%	0.0	73.76%	MW478834.1
Colias croceus	40%	0.0	79.31%	KM592967.1
Colias erate	40%	0.0	79.31%	KP715146.1
Monema flavescens	42%	0.0	78.62%	KU946971.1
Thaumatosmylus hainanus	41%	0.0	78.11%	NC_046578.1
Chrysotus gramineus	41%	0.0	77.47%	MT862388.1

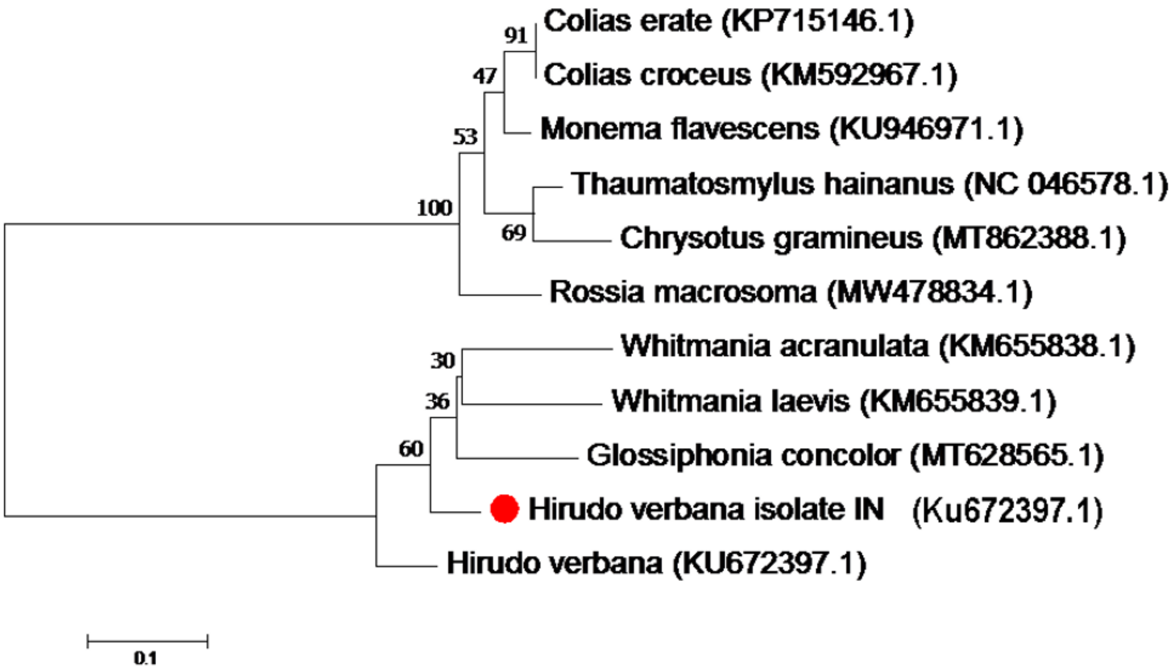


Figure (2): Neighbour-Joining phylogenetic trees showing the relationship between the *Hirudo verbana* isolate IN and the related species based on cytochrome C oxidase subunit I (CO-I) region sequences using MEGA-7 software.

The phenetic grouping and principal coordinate analyses revealed the same fundamental structure, indicating a taxonomic rather than geographic subdivision.

The taxonomic study found that the two types are genetically related, morphologically distinct, and reproductively isolated. *Hirudo medicinalis* isolate NI was the most closely related to *Hirudo medicinalis* isolates PO61 and K067.

Hirudo verbana isolate IN had 100% sequence similarity and 100% query coverage to *Hirudo verbana* (Accession No. KU672397.1). Whitmania laevis was the most closely related isolate to *Hirudo verbana* isolate IN.

REFERENCES

Agnarsson I. Kuntner M. Taxonomy in a Changing World: Seeking Solutions for a Science in Crisis. Systematic Biology. 2007; 56(3):531-9. doi.org/10.1080/10635150701424546.

Babenko, V.V., Podgorny, O.V., Manuvera, V.A. *et al.* Draft genome sequences of *Hirudo medicinalis a*nd salivary transcriptome of three closely related medicinal leeches. *BMC Genomics* 21, 331 (2020). https://doi.org/10.1186/s12864-020-6748-0

Barzegar A, Azizi A, Faridi P, Mohagheghzadeh A. 2015. Leech therapy in Iranian traditional medicine. Complement Med Res, 22: 50-53.

Darabi-Darestani K. Malek M. Seasonal variation in the occurrence of the Medicinal Leech *Hirudo* Orientalis in Guilan Province, Iran. Aquatic Biology. 2011;11(3):289-94. doi.org/10.3354/ab00310.

Das, B. K. (2014). An overview on *Hirudo*therapy/leech therapy. *Ind Res J Pharm sci*, *1*(1), 34.

Davies r. W., n. j. Mcloughlin 1996. The effects of feeding regime on the growth and reproduction of the medicinal leech *Hirudo medicinalis*. – *Freshwater Biology*, **36**: 563-568.

Davies, R. W., Wrona, F. J., Linton, L., and Wilkialis, J. (1981). Interand intraspecific analyses of the food niches of two sympatric species of Erpobdellidae (Hirudinoidea) in Alberta, Canada. *Oikos* **37:** 105–111.

Iqbal, A., Jan, A., Quraishi, H. A., Sheeraz, M., Shah, A., Rather, S. A., & Raheem, A. (2018). Leech therapy in medical science-A review. *Global J. of Research on Medicinal Plants & Indigenous Medicine*, *7*(6), 75-85.

Langridge, J., Langridge, P., & Bergquist, P. L. (1980). Extraction of nucleic acids from agarose gels. Analytical Biochemistry, 103(1), 264-271.

Lent, C. (1986). New medicinal and scientific uses of the leech. *Nature* **323:** 494.

Naji, S. A. (2013). Genetic and cellular changes in some vegetatively incompatible strains of Mushrooms, *Aspergillus amstelodami,* PhD thesis, Science college, Diyala university. Page 1 149 . (Arabic).

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution*, *4*(4), 406-425.

Salih, A. H. (2017). Leeches in medicine and sport, Electronic edition. Page.(12-14). (Arabic).

Shayan, P., Borji, H., Eslami, A., & Zakeri, S. (2007). Isolation of DNA from a single Helminth using new developed Kit in Iran and its PCR analysis. *Iranian Journal of Parasitology*, *2*(2), 34 39.

Solgi, R., Raz, A., Zakeri, S., Kareshk, A. T., Yousef, A., Jarehan, A., & Djadid, N. D. (2021). Morphological and molecular description of parasitic leeches (Annelida: Hirudinea) isolated from rice field of Bandar Anzali, North of Iran. *Gene reports*, *23*, 101162.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, *30*(12), 2725-2729.

Todorov, Milcho & Grozeva, Snejana & Hubenov, Zdravko & Kenderov, Lyubomir & Trichkova, Teodora. (2016). Taxonomic status and distribution of medicinal leeches of the genus *Hirudo* L. (Hirudinea) in Bulgaria. Acta Zoologica Bulgarica. 68. 171-182.

Trontelj P. Sotler M. Verovnik R. Genetic Differentiation Between Two Species of the Medicinal Leech, *Hirudo medicinalis* and the Neglected H. *Verbana*, Based on Random-Amplified Polymorphic DNA. Parasitology research. 2004; 94(2):118 24.doi.org/10.1007/s00436-004-1181-x.

Whiteley, D. (1971). Invertebrates. David Nichols and Johan Cooke, Oxford, UK

Young, J. O. (1981). A comparative study of the food niches of lake-dwelling triclads and leeches. *Hydrobiologia* **84:** 91–102