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Molecular-Genetic Characteristics Of Representatives Of The Genus *Hirudo*: A Review Of Modern Studies

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Abstract

The bite of an infected *Aedes* mosquito (*Aedes aegypti* or, *Aedes albopictus*) can transmit the virus that This review summarizes and analyzes recent molecular genetic studies on the genus *Hirudo*, with particular emphasis on *Hirudo orientalis*. The review is based primarily on scientific literature published between 2004 and 2024, covering molecular identification, genetic diversity, and phylogenetic relationships among species. The analysis demonstrates that identification based solely on morphological characteristics is insufficient for the accurate discrimination of morphologically similar species such as *H. medicinalis*, *H. verbana*, and *H. orientalis*. The application of mitochondrial COI and 12S rRNA markers, together with nuclear ITS1/ITS2 markers, has enabled the identification of genetic differences among these species and supported their recognition as distinct taxa. In particular, molecular studies of *H. orientalis* have revealed the presence of intraspecific genetic diversity and distinct haplotypes, while also contributing significantly to the understanding of its geographic and phylogenetic characteristics. Overall, the application of molecular genetic methods is of considerable importance for the accurate identification of *Hirudo* species, determination of their taxonomic status, investigation of their phylogenetic relationships, and assessment of the genetic diversity of their natural populations. viridae family, are transmitted by the bite of an infected aedes mosquito. Dengue is widespread in over 100 nations worldwide. There is currently no specific treatment for this illness in modern medicine. According to *Ayurveda*, *Dandaka Jwara*, is associated with dengue fever. For this reason, it is often referred to as "break bone fever." Since contemporary science has no cure for dengue fever, *Ayurveda* can be a very helpful tool in controlling the illness. However, a complete understanding of dengue fever from both a modern and an ayurvedic perspective is required.

Keywords: Molecular, review article, *Hirudo*, *Hirudo orientalis*, phylogenetic.

Introduction :

Aquatic ecosystems worldwide are characterised by a rich hydrofauna. However, these groups of organisms face constant pressure from both natural factors and anthropogenic impacts. Consequently, continuous observation and monitoring of aquatic organisms (hydrobionts) are important. A key characteristic of aquatic ecosystems is their interaction with the surrounding landscape and biocenoses. In other words, changes in freshwater bodies—such as pollution from industrial and agricultural activities, the accumulation of heavy metal-rich waste, excess organic matter, and ecological disturbances linked to urbanisation—directly influence the composition of resident organisms, particularly the hirudofauna. Seven main species belonging to the genus *Hirudo* are widely used worldwide for medical purposes:

1. *Hirudo medicinalis* – the classic European medicinal leech, distributed primarily across Northern and Western Europe^[16]
2. *Hirudo verbana* – distributed across Southern and Southeastern Europe; morphologically similar to *H. medicinalis*, yet genetically distinct
3. *Hirudo orientalis* – Distributed in the Caucasus, Iran, and Central Asia; confirmed as a distinct species through molecular studies
4. *Hirudo troctina* – Found primarily in North Africa (Morocco, Algeria, Tunisia)
5. *Hirudo sulukii* – Discovered in Turkey (Aegean and Mediterranean regions); described by N. Sağlam in 2016
6. *Hirudo tianjinensis* – Described as a new species by Wang et al. in 2022 in the Tianjin

region of China

7. *Hirudo nipponia* – Although assigned to a separate genus (*Whitmania*) by some authors, it is also classified within *Hirudo* in certain systematic approaches

Materials And Methods :

Cyclical glacial events have significantly shaped the genetic divergence and biogeographical distribution of certain species within the genus *Hirudo*. Climatic fluctuations, particularly those associated with the Pleistocene epoch, fragmented populations into geographically isolated refugia, promoting genetic differentiation and increasing intraspecific genetic diversity.

Medicinal leeches have long been important in both medicine and zoological systematics. The nineteenth century saw the establishment of fundamental knowledge about their diversity and taxonomic classification through the pioneering studies of Grube and Whitman. Research during

this period focused primarily on external morphological characteristics, including similarities and differences in body form and patterns of geographical distribution. Although these morphological approaches provided an important foundation for early classification of medicinal leeches, relying solely on phenotypic characteristics proved insufficient for accurate taxonomic discrimination and comprehensive classification^[16].

By the mid-twentieth century, studies conducted by Epstein and Livanova had focused on the identification and taxonomic

characterisation of leech species occurring throughout the territory of the former USSR. However, advances in molecular genetics led to

new approaches that overcame the limitations of classical morphological methods. Molecular genetic markers enabled more accurate delineation of interspecific boundaries and provided more reliable evidence for species identification and taxonomic assessment. Consequently, molecular approaches have become an essential complement to traditional morphological analyses in the study of leech diversity, taxonomy, and evolutionary relationships ^[5]. For example, despite their considerable morphological similarity, *molecular analyses have shown that H. medicinalis, H. verbana, and H. orientalis* represent distinct species. Studies based on mitochondrial DNA markers, particularly the cytochrome c oxidase subunit I (COI) and 12S rRNA genes, as well as nuclear DNA markers, including the ITS1 and ITS2 regions, have confirmed substantial genetic differentiation among these taxa. These findings are important for accurately identifying and taxonomically distinguishing closely related *Hirudo* species and for more precisely assessing their biogeographical distribution patterns ^[13]. Hybridisation experiments conducted by Petrauskienė and colleagues have demonstrated that artificial cross-breeding among different species of the genus *Hirudo* is possible under controlled laboratory conditions. However, the results revealed that the vast majority of hybrid offspring exhibited reduced viability or were completely non-viable. These findings provide further evidence of reproductive isolation among the species concerned and support their recognition and classification as distinct taxonomic entities. Laboratory experiments involving artificial hybridisation among three

species—*H. medicinalis, H. verbana, and H. orientalis*—have provided evidence of reproductive incompatibility between these taxa. In one experimental cross, female *H. medicinalis* and male *H. verbana* individuals produced cocoons after a defined mating period. However, the offspring that emerged from these cocoons were poorly developed and exhibited markedly reduced viability. Complete mortality of the offspring was observed within eight months, resulting in a reproductive success rate of zero. These findings provide strong evidence of reproductive isolation and genetic incompatibility between the species involved, further supporting their recognition as distinct taxonomic entities ^[12].

Laboratory experiments involving artificial hybridisation among three species—*H. medicinalis, H. verbana, and H. orientalis*—have provided evidence of reproductive incompatibility between these taxa. In one experimental cross, female *H. medicinalis* and male *H. verbana* individuals produced cocoons after a defined mating period. However, the offspring that emerged from these cocoons were poorly developed and exhibited markedly reduced viability. Complete mortality of the offspring was observed within eight months, resulting in a reproductive success rate of zero. These findings provide strong evidence of reproductive isolation and genetic incompatibility between the species involved, further supporting their recognition as distinct taxonomic entities.

Analysis of the hybridisation results provides evidence for genetic barriers among *Hirudo* species, supporting their recognition as distinct species. In modern systematics, reproductive

isolation is a principal criterion for delineating species boundaries. Molecular phylogenetic studies by Siddall and Borda further show that, despite their pronounced morphological similarity, *H. verbana* and *H. medicinalis* represent genetically distinct evolutionary lineages^[13].

Trontelj and Utevsky reported similar findings, suggesting that the reduced viability observed in hybridisation experiments was associated with reproductive isolation. Molecular-genetic analyses later corroborated the polymorphic traits described in early taxonomic accounts of *H. medicinalis*. Studies have shown that variation in colouration and body patterning within this species is not merely random phenotypic variation but is associated with distinct morphotypes. Furthermore, comparative analysis of cytochrome c oxidase subunit I (COI) gene sequences available in the GenBank database has revealed genetic polymorphism in *H. medicinalis*. We applied the same approach to *H. verbana*, showing that this species also exhibits substantial genetic diversity. Collectively, these findings indicate that, despite their morphological similarities, molecular markers can reveal considerable genetic variation within both species^[17-18].

Asian species, particularly *H. nipponia* and *H. tianjinensis*, differ from their European congeners in both morphology and genetics. Molecular analyses based on cytochrome c oxidase subunit I (COI) gene sequences indicate that these species are phylogenetically related to other members of the genus *Hirudo*, while representing distinct evolutionary lineages. These findings suggest that geographic isolation may have driven their

evolutionary divergence, accompanied by independent ecological adaptation^[8].

In recent studies, Hildebrandt and Lemke examined the taxonomic relationships among the three most widespread medicinal leech species in Europe: *Hirudo medicinalis* Linnaeus, 1758, *Hirudo verbana* Carena, 1820, and *Hirudo orientalis* Utevsky & Trontelj, 2005^[7]. The authors noted that although these species' geographical ranges may overlap, their reproductive compatibility under laboratory conditions remains poorly understood. Reports of successful mating under artificial conditions have raised questions regarding the extent of reproductive isolation among these taxa. However, successful mating alone does not necessarily indicate reproductive compatibility; further evidence is needed to determine whether they can produce viable, fertile offspring. Therefore, researchers should evaluate their taxonomic status alongside morphological, molecular-genetic, and reproductive evidence^[6].

However, these findings have prompted debate among researchers regarding the extent of reproductive isolation among medicinal leech species. Studies by Elliott and Kutschera indicate that interspecific crosses conducted under laboratory conditions often produce offspring with low viability and limited ability to survive long-term. These findings suggest reproductive barriers between these taxa and support their recognition as distinct species. Nevertheless, reproductive isolation should be assessed based on offspring viability, fertility, and the ability to establish subsequent generations, alongside molecular-genetic and morphological evidence^[3].

Specimens of *Hirudo* collected from small ponds in Hungary and characterised by an unpigmented, greyish ventral surface were found to differ from the original descriptions provided by Linnaeus. Consequently, these specimens were assigned to *Hirudo verbana* Carena, 1820, also associated in the literature with the name *H. officinalis* Savigny, 1822. Subsequent studies demonstrated that, despite their morphological similarities, *H. medicinalis* and *H. verbana* represent genetically distinct species. Although both species inhabit freshwater environments, evidence of natural interbreeding and offspring produced between them has not been established. These findings prompted more detailed morphological and taxonomic investigations, alongside the determination and comparative analysis of their mitochondrial DNA sequences. The resulting evidence supported their recognition as two distinct species^[10].

Research conducted by K. Darabi-Darestani and colleagues revealed substantial haplotype diversity in *Hirudo orientalis* populations from the Caspian region. The authors suggested that prolonged geographical isolation may explain the observed differences in population genetic structure. The authors also highlighted the role of historical refugia, which may have facilitated the persistence of isolated populations and promoted genetic divergence over evolutionary timescales, as a potential factor contributing to the emergence of this genetic diversity^[11].

Such studies provide valuable insights not only into taxonomic identification but also into evolutionary biology and biogeography. Phylogenetic and population-genetic data enable more accurate

reconstruction of *Hirudo* species' evolutionary history and a better understanding of their distribution patterns and dynamics^[2]. The GenBank database includes the COI gene sequence for *H. verbana* var. *verbana* under accession number EF125043. Hildebrandt and Lemke used this molecular marker to investigate the taxonomic identity of the specimens, and their results support assignment to *H. verbana*. Comparative studies have also examined phylogenetic relationships among the three European medicinal leech species and the North African species *Hirudo troctina* Johnson, 1816, commonly known as the dragon leech. These taxa have been discussed as part of a closely related species group. Research by Kutschera and other investigators has also highlighted genetic polymorphism in *H. verbana*, demonstrating distinct genetic variants within the species^[7]. *Hirudo tianjinensis*, described by Chinese researchers, exhibits morphological and genetic characteristics that distinguish it from other members of the genus *Hirudo*. Its diagnostic external features include five yellow longitudinal stripes on the dorsal surface, dark-brown spots on the ventral side, and a high density of sensory organs. Molecular analyses of the cytochrome c oxidase subunit I (COI) gene indicate a close phylogenetic relationship between *H. tianjinensis* and the Asian species *H. nipponia* Whitman, 1886. We developed an optimised protocol for recombinant production and purification of a novel cysteine-rich anticoagulant (CRA) protein derived from *Hirudo medicinalis* saliva. The yield of purified CRA ranged from 3.7 to 5.5 mg/L, and we assessed levels of potential contaminants, including

bacterial proteins, DNA, and endotoxins. The purified protein was highly pure and a potent anticoagulant. In contrast, a truncated CRA variant lacking the C-terminal region lost its membrane-binding capacity and showed an altered anticoagulant profile, characterised by increased thrombin time effect and reduced prothrombin time and activated partial thromboplastin time effects. These findings highlight CRA's potential as a promising candidate for further investigation as an anticoagulant agent. Accurate species identification is essential for ensuring the species-specific efficacy and quality of medicinal leeches used in medical applications. However, conventional DNA barcoding methods may be ineffective for traditional medicinal preparations because DNA degradation can compromise amplification and analysis of standard barcode regions. This limitation underscores the need to develop mini-barcoding approaches that target shorter DNA fragments, thereby improving the molecular identification of medicinal leech species in degraded biological material. This study aimed to develop and validate mini-barcode markers for the molecular identification of three leech species listed in the Chinese Pharmacopoeia: *Whitmania pigra*, *Whitmania acranulata*, and *Hirudo nipponia*. We designed and validated four novel mini-barcode primer pairs (ND1F1/R1, 12SF1/R1, 16SF1/R1, and COX1F1/R1) using seven morphologically identified specimens. We then evaluated these markers using 16 leech-derived preparations^[9]. We extracted DNA using two approaches: a single-tube extraction method and column-based

purification kits. The column-based method yielded higher-quality DNA suitable for subsequent polymerase chain reaction (PCR)

amplification. PCR analysis confirmed that all four candidate mini-barcode markers targeted specific genomic regions and were applicable, with successful amplification from 13 of the 16 preparations. Mini-barcode sequences from morphologically identified *W. pigra* specimens showed > 95% sequence identity to the corresponding reference sequences for ND1, 12S rDNA, 16S rDNA, and COX1 (GenBank accession number EU304459). In contrast, sequence identity for *H. nipponia* and *W. acranulata* specimens was below 85%. Among the leech-derived preparations examined, only the traditional Chinese medicinal product *Maxuekang* exhibited comparatively lower sequence similarity.

Optimal clustering and phylogenetic tree analyses using the Automated Single-Cell Analysis Pipeline (ASAP) identified three distinct clusters corresponding to the morphologically identified species: *W. pigra*, *W. acranulata*, and *H. nipponia*. The analyses also revealed that specimens previously identified as *Hirudo nipponia* in some studies were, in fact, *W. pigra*, highlighting the potential for taxonomic misidentification based on morphology alone. Overall, the findings show that mini-barcoding can substantially improve species-level identification and support quality control of traditional medicinal and commercially available leech-derived products. This approach may support safer, more reliable use of medicinal leeches and the incorporation of molecular species-identification procedures into regulatory and

quality-control standards. According to the traditional taxonomic view, all morphological forms of European medicinal leeches are assigned to a single species, *Hirudo medicinalis*. However, a study using random amplified polymorphic DNA (RAPD) markers showed that *Hirudo verbana*, another species distributed in Europe, is a distinct biological species. Genetic clustering and Principal Coordinates Analysis (PCoA) of eight populations revealed consistent genetic differentiation, indicating that the observed structure was driven primarily by taxonomic divergence rather than geographic variation. Interspecific differences accounted for 60% of the total molecular variation ($\phi_{CT} = 0.60$; $P < 0.001$). Furthermore, several taxon-specific RAPD markers were identified for both species. In contrast, no specific DNA fragments were detected that could link genetic markers to the geographical origins of the sampled populations. These findings provide molecular evidence supporting the recognition of *H. medicinalis* and *H. verbana* as distinct species [14-15].

In molecular systematics studies by Utevsky and Trontelj, they investigated phylogenetic relationships among several *Hirudo* species, including specimens collected in Azerbaijan. The analyses employed both nuclear markers (ITS2 and 5.8S rRNA) and mitochondrial markers (12S rRNA and COI). The results supported the monophyly of the genus *Hirudo*, indicating that its constituent species share a common evolutionary ancestry^[18] (Figure 1.1.).

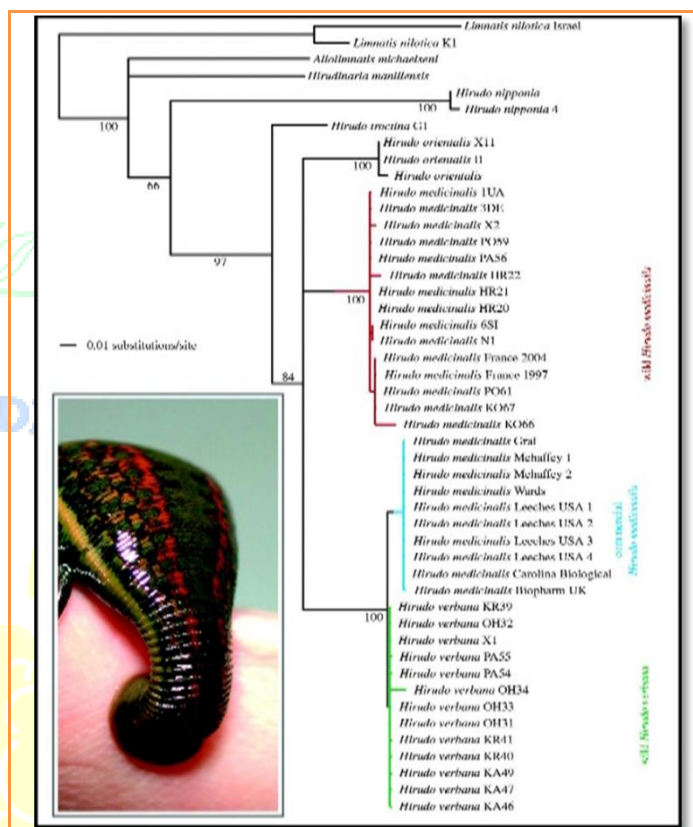


Figure 1.1. Phylogenetic tree of medicinal leeches.

We constructed the phylogenetic tree based on molecular analyses of species in the genus *Hirudo*, using mitochondrial markers including the cytochrome c oxidase subunit I (COI) gene and 12S rRNA. The results indicate that, despite their morphological similarities, *H. medicinalis*, *H. verbana*, and *H. orientalis* represent genetically distinct species. In contrast, *H. nipponia* and *H. troctina* exhibit more distant phylogenetic relationships relative to these three species. The clustering of specimens collected from different geographical regions within the same phylogenetic clade suggests shared evolutionary ancestry, although additional evidence is required to establish the role of geographical isolation in their divergence. The phylogenetic position of *H. orientalis* is consistent with its association with the

Caspian region. Overall, molecular marker-based systematics provides an essential framework for resolving taxonomic relationships and improving the accuracy of species identification within the genus *Hirudo*. A 2020 study investigated the genome of *Hirudo medicinalis* and examined gene expression in salivary cells isolated from *H. medicinalis*, *H. orientalis*, and *H. verbana* using RNA sequencing (RNA-seq) and proteomic analyses. The study identified numerous salivary-cell-associated genes, including those encoding previously uncharacterized salivary components. Furthermore, the findings revealed that genes encoding known anticoagulant proteins are expressed not only in salivary cells but also in other cell types. These results provide important insights into the molecular and genetic mechanisms underlying blood feeding in medicinal leeches and contribute to a better understanding of the roles of salivary proteins in regulating the host blood coagulation system.

In a study conducted by Sh. Farzali and N. Saglam, the mitochondrial cytochrome c oxidase subunit I (COI) gene region was analysed in medicinal leeches collected from the Lankaran–Astara region of Azerbaijan. The results revealed 100% sequence identity between the analyzed DNA sequences and those of *Hirudo orientalis* Utevsky & Trontelj, 2005, confirming the molecular identification of the specimens as this species^[4]. A separate study demonstrated that microsatellite profiles differ between *Hirudo medicinalis* and *Hirudo verbana*, with species-specific differentiation detected at four microsatellite loci. Two loci, Hm8 and Hm12, failed to amplify in *H. verbana*, whereas HvA10

and HvH07 failed to amplify in *H. medicinalis*. Among the other European medicinal leech species examined, three of the five successfully amplified loci—Hm2, Hm10, and HvT397—were monomorphic in *H. medicinalis* but polymorphic in *H. verbana*. DNA barcoding further supported these findings, identifying distinct monophyletic groups for the two species and indicating that they represent separate evolutionary lineages, with an average sequence divergence of 8.5% between them^[13].

A study conducted in Iraq examined 19 individuals of the genus *Hirudo*. We amplified the ITS-2, mitochondrial cytochrome c oxidase subunit I (COI), and mitochondrial 12S ribosomal RNA (12S rRNA) markers using polymerase chain reaction (PCR), then sequenced the resulting products.

Comparison of ITS-2

sequences revealed a single genotype in both *Hirudo verbana* and *Hirudo orientalis* samples. In contrast, COI sequence analysis identified four haplotypes in *H. verbana* and two in *H. orientalis*. Analysis of the 12S rRNA marker revealed two haplotypes in *H. verbana*, whereas only one haplotype was detected in *H. orientalis*. Overall, the study provides additional insights into the genetic variation and differentiation of *Hirudo* species used in medical clinics in Iraq.

Conclusion :

Findings from molecular and phylogenetic studies indicate that *Hirudo orientalis* represents a distinct species, genetically differentiated from other medicinal leeches despite their morphological similarities. Evidence concerning its therapeutic potential, capacity to utilize diverse food sources,

and transcriptomic variation further highlights its potential pharmacological and medical significance. These findings underscore the importance of integrating molecular-genetic markers into the taxonomy of *Hirudo*, rather than relying exclusively on morphological characteristics. This integrative approach enables more robust taxonomic identification, supports accurate assessment of the conservation status of natural populations, and provides a scientific basis for developing standardised laboratory breeding protocols and improving the responsible use of medicinal leeches in clinical practice.

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Declaration :

Conflict of Interest : None

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