

Article

High Genetic Diversity of *Hirudo verbana* Carena, 1820 (Annelida: Hirudinea: Hirudinidae) in Romania Confirms That the Balkans Are Refugia Within Refugium

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Abstract: This study presents the first data on the distribution of the Mediterranean medicinal leech *Hirudo verbana* in Romania, as well as the first molecular characterization of the Romanian populations, using mitochondrial DNA markers. In total, 133 specimens from 19 populations in Romania were surveyed for mitochondrial COI and 12S markers. Available GenBank sequences from other localities throughout Europe were included to place the Romanian populations in a broader phylogeographical context. The Romanian *H. verbana* samples belong to 23 COI haplotypes, 4 of which are previously known from Serbia and Ukraine, while 19 haplotypes are herein reported for the first time. For 12S, the number of haplotypes identified was 12, from which only 5 are unique to Romania. The Romanian *H. verbana* cluster within the previously reported eastern phylogroup, known to occur from Serbia to North Caucasus, Turkey, and Uzbekistan. The mismatch distribution analysis and the results of neutrality tests suggest that the Romanian *H. verbana* currently undergoes a population dispersal. This study also revealed that the lower Danube basin is a hotspot of genetic diversity of *H. verbana*, where different lineages from the Balkan and Anatolian glacial refugia mixed in the postglacial period. Species Distribution Models (SDMs) indicated that the main ecological variables influencing the current distribution of *H. verbana* are wetland coverage and elevation. Following the IUCN Red List categories and criteria, the present conservation status of the Mediterranean medicinal leech *H. verbana* in Romania was assessed as Vulnerable (VU). Our study contributes towards a more refined understanding of the genetic diversity of *H. verbana* and of the factors that led to its current geographical distribution in Romania and Europe.

Keywords: medicinal leeches; *Hirudo verbana*; species distribution modeling; habitat; genetic diversity; phylogeography; conservation status; glacial refugia; admixture zone



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1. Introduction

Since ancient times, medicinal leeches (genus *Hirudo* Linnaeus, 1758) have received considerable attention due to their use in bloodletting (phlebotomy) and other therapeutic practices [1–3]. In modern times, studies devoted to their physiology, behavior, or biologically active compounds made them one of the best-studied invertebrate species [4,5]. However, their taxonomy was clarified only quite recently. Despite the great color variability of medicinal leeches, all the varieties were considered as belonging to the same species—*Hirudo medicinalis* Linnaeus, 1758 [6,7]. Consequently, for a long time only this species was recognized to occur in Europe [8]. Only at the end of the 20th century did

Nesemann and Neubert [9] reestablish the long-forgotten south European species *Hirudo verbana* Carena, 1820. Subsequent molecular studies not only confirmed the validity of *H. verbana* and *Hirudo troctina* Johnston, 1816, previously synonymized with *H. medicinalis*, but also permitted the description of an additional species for Transcaucasia—*Hirudo orientalis* Utevsky & Trontelj, 2005 [4,10].

Moreover, genetic studies have also shown that most of the marketed medicinal leeches were not *H. medicinalis* but the closely related *H. verbana* imported from Turkey and southeastern Europe [10,11]. As there is a high demand of *H. verbana* for medical purposes (biomedical research, surgical applications, and pharmacology), wild populations of this species are currently threatened. Reflecting recent taxonomic updates, since 2011, *H. verbana* has been listed separately from *H. medicinalis* in Appendix II of the Convention on International Trade in Endangered Species (CITES; <https://cites.org/eng/app/index.php>, accessed on 6 October 2024), and in 2014 it was included in Annex B of the European Union Regulation of trade of fauna and flora (https://environment.ec.europa.eu/topics/nature-and-biodiversity/wildlife-trade_en, accessed on 6 October 2024). A distinct Natura 2000 species code was assigned to the Mediterranean medicinal leech *H. verbana* in 2016 for separate reporting from *H. medicinalis* under Article 17 of the EU Habitats Directive (Council Directive 92/43/EEC).

Romania is situated in southeastern Europe, at the northeastern edge of the Balkan Peninsula (Figure 1), and is shaped by a diverse array of landscapes, which include the Carpathian Mountains, the Danube River and Delta, the Black Sea, and large plains and hilly areas [12]. The variety of topographic features, combined with its position at the intersection of several biogeographical regions (Alpine, Continental, Steppic, Pannonian, and Black Sea), make Romania one of the most ecologically diverse regions of Europe. Recent studies suggest that the present-day territory of Romania served as an important Pleistocene glacial refugium [13–15].

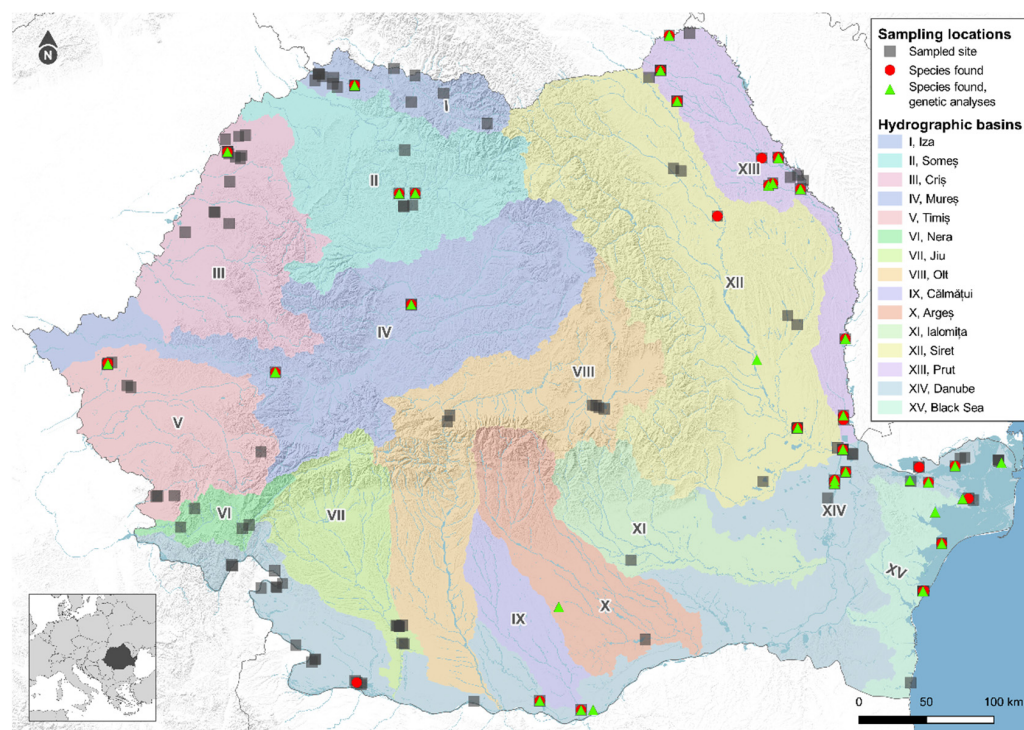


Figure 1. Map of *Hirudo verbana* sampling locations. Gray squares—specimens were actively sought but not found; red circles—specimens were sought and found; green triangles—found specimens were included in the genetic analysis. The main 15 hydrographic basins of Romania are represented in different colors. The inset map in the lower left shows the position of Romania in southeastern Europe.

In Romania, as in other European countries, all reports of medicinal leeches until the end of the 20th century were ascribed to *H. medicinalis* [16–21]. Recent studies [22–24] pointed out that most of the populations of medicinal leeches in Romania belong to *H. verbana*. Since the presence of *H. medicinalis* in Romania was also confirmed more recently [25], all previous records of medicinal leeches must be reconsidered.

Therefore, the main aims of this paper are as follows: (1) to summarize the knowledge about the past and current distribution of the Mediterranean medicinal leech *H. verbana* in Romania, (2) to assess its genetic diversity, and (3) to evaluate the conservation status of the species and its habitats in Romania.

2. Materials and Methods

2.1. Species Sampling and Morphological Examination

Potential suitable habitats for *H. verbana* were surveyed across Romania between June 2014 and August 2023 (Figure 1; Table S1). The scientific literature and museum collections were also reviewed for historical occurrences. The current distribution was documented during surveys carried out as part of national monitoring programs in protected areas [24]. Splash sampling was performed by disturbing the water surface with a hand net for 10 min, thus attracting leeches. Any medicinal leeches seen swimming or attached to the surveyor's boots were collected and retained temporarily in sample bottles partially filled with water from the collection site for in situ counting, measuring, photography, and identification. The leeches were released back at the site of capture, with some specimens being preserved in 96% ethanol for molecular study. Additional specimens were also provided by colleagues from areas not actively surveyed for leeches.

Overall morphology was examined under a Nikon SMZ800 dissecting stereomicroscope (Nikon Corporation, Shinagawa, Tokyo, Japan). The taxonomic identification of medicinal leeches was based primarily on their dorsal and ventral coloration pattern [5].

2.2. DNA Extraction, Amplification, and Sequencing

In total, 133 specimens of *H. verbana*, collected from 19 locations in Romania, were analyzed using two mitochondrial markers. Tissue samples were taken from the caudal sucker to avoid contamination from gut contents. Approximately half of the caudal sucker was removed with a scalpel, and the tissue fragments were processed thereafter. Total genomic DNA was extracted using an Isolate II Genomic DNA Kit (Bioline, London, UK) following the manufacturer's protocol.

For molecular analyses, we obtained sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene fragment and 12S rRNA gene, using universal primers [26] and primers specially developed by Trontelj and Utevsky [4]. The PCR reactions were performed in a total volume of 50 µL containing 10 ng of DNA template, 1X Green GoTaq® Flexi Buffer, 2.5 mM MgCl₂, each dNTP at 0.1 mM, 0.5 µM of each primer, and 1.5 units of GoTaq® DNA polymerase (Promega, Madison, WI, USA). The PCR products were isolated from samples presenting clean and visible bands on 0.5 µg mL^{−1} ethidium bromide-stained agarose gel using a FavorPrep™ Gel/PCR Purification Kit (FAVORGEN® Biotech Corp., Changzhi, Taiwan) following the manufacturer's specifications. DNA sequencing was performed by Macrogen (Seoul, Republic of Korea). The resulting sequences for both genes were edited and aligned in CodonCode Aligner 3.7.1 (CodonCode Corporation, Dedham, MA, USA). In addition, 50 COI and 45 12S sequences of *H. verbana* were retrieved from GenBank (Table S2).

2.3. Phylogeographic and Demographic Analyses

In order to evaluate the phylogenetic relationships between different lineages of medicinal leeches reported in Europe and the populations from Romania, we performed a network analysis using PopART 1.7 (Population Analysis with Reticulate Trees) [27] under a Median-Joining algorithm. The data were analyzed separately because five of the samples

downloaded from GenBank lacked sequences for the 12S marker, making it difficult to use the concatenated sequences.

Unique haplotypes from our COI and 12S datasets were identified using DnaSP 6 [28]. Some other parameters of genetic diversity like haplotype diversity (H_d), nucleotide diversity (π), Tajima's D , Fu and Li's F_s , and R2 test by Ramos-Onsins and Rozas were calculated only on COI data using DnaSP 6. If the result is negative for the Fu and Li test or for Tajima's D , it means that the population is expanding; if it is close to zero, the population is stable, and if it is positive, there is a population regression. For the R2 test by Ramos-Onsins and Rozas, the interpretation of the results is reversed.

We also used mismatch distribution analysis, a tool designed to study the evolutionary history and dynamics of populations and to detect population expansions or other demographic events (contractions, bottlenecks, or migration events) with specific signatures in the analysis.

To test hypotheses regarding the existence of a cryptic refugium in the Danube River Basin for medicinal leeches, we used the approach described by Petit et al. [29] based on haplotype frequency (h_T) and on both haplotype frequency and the genetic distances between haplotypes (v_T) implemented in PERMUTCpSSR 2.0. Areas with high allelic richness in which $h_T > v_T$ are indicative of refugia, containing high numbers of unique haplotypes, whereas areas with high allelic richness in which $h_T \leq v_T$ indicate an admixture of genetic lineages from two or more different refugia.

2.4. Spatial Analysis and Distribution Modeling

All 15 main hydrographic basins of Romania were considered for spatial analysis and distribution modeling of *H. verbana*. Apart from a small number of rivers that flow directly into the Black Sea, all the other rivers are part of the Danube drainage basin. Geographical coordinates and elevation (in meters) were recorded for each surveyed and sampled location. For points with no elevation data recorded, this value was inferred from the SRTM-derived digital elevation model. Counts and summary statistics for each hydrographic basin were performed in QGIS 3.28.

We used 57 climatic and environmental variables at a spatial resolution of 30 arc-seconds, which represents approximately 1 km² at the equator for these predictions (WorldClim 2.1) [30]. The climatic and environmental variables were generated using weather data from 1970 to 2000 from all weather stations around Romania. Multicollinearity between environmental variables in species distribution models can influence the results, leading to biased predictions by overrepresenting the ecological relevance of correlated variables [31]. To address this, we performed a Variance Inflation Factor (VIF) analysis to test all 19 bioclimatic and 7 climatic variables for multicollinearity, thereby avoiding potential biases in our distribution models [32], using the R package “cat” [33]. This approach allowed us to detect hidden correlations between predictors that are not apparent in pairwise correlations. To mitigate collinearity among the variables, we used a stepwise procedure, retaining only those predictors with a more stringent VIF threshold of <5, which is considered appropriate for managing multicollinearity [31].

To predict suitable areas for the survival of *H. verbana*, we developed a correlative Species Distribution Model (SDM) using MaxEnt 3.4.4, a maximum entropy machine learning algorithm known for its robustness [34]. MaxEnt utilizes presence-only data to construct distribution models, comparing climatic and environmental variables against predictor data from specific areas [35]. The resulting model produces a raster map depicting the relative environmental suitability distribution for the species.

To determine the most parsimonious models, we employed optimal-model selection based on the Area Under the Curve (AUC). This involved constructing models of varying complexity using a regularization multiplier (RM) ranging from 0.5 to 4.0 in 0.5 increments, along with five feature classes (FC), threshold (T), quadratic (Q), hinge (H), linear (L), and product (P), across all possible combinations.

The final distribution maps were generated using all presence locations for *H. verbana* after removing clustered observation points within a 1 km radius to enhance predictive accuracy for distribution estimation [36]. Projected distribution maps were modeled for the entire present distribution range.

3. Results

3.1. Distribution of *Hirudo verbana* in Romania

A total of 225 specimens of *H. verbana* (Figure 2) were found in 39 out of the 115 water bodies and wetlands surveyed for the species (Figure 1; Table S1). *Hirudo verbana* was present in 11 out of the 15 main hydrographic basins (Figure 1). The examination of all specimens of medicinal leeches with unfaded pigmentation from the scientific collections of the “Grigore Antipa” National Museum of Natural History in Bucharest (MGAB 9482/170; MGAB 9063) and the Museum of Zoology of the Faculty of Biology of the Alexandru Ioan Cuza University of Iași revealed that they correspond to *H. verbana* (Table S3).



Figure 2. The dorsal and the ventral coloration patterns of living specimens of *Hirudo verbana* from Satchinez (Romania). Scalebar = 20 mm.

In Romania, *H. verbana* was found only outside of the Carpathian Mountains, in plain and hilly regions, at altitudes ranging from 0 to 268 m a.s.l. (Figure 1; Table S1). Several sites at higher altitudes were also investigated to verify historical records of medicinal leech occurrence. No *H. verbana* were observed above 270 m a.s.l. Instead, only the European medicinal leech *H. medicinalis* occurred at elevations ranging from 248 to 549 m a.s.l. The historical records of medicinal leeches in mountainous areas at altitudes higher than 600 m a.s.l. [37,38] might be due to possible confusion with the horse leech *Haemopsis sanguisuga* (Linnaeus, 1758). The latter is a ubiquitous species frequently found in mountainous regions at altitudes as high as 968 m a.s.l.

The habitats where *H. verbana* are usually found are represented by ditches, canals, dead river arms, floodplain pools, fens, marshes, abandoned fishponds, and slowly running waters (Figure 3). The species prefers muddy substrates rich in detritus, with abundant submerged and emergent vegetation (especially cattail, *Typha* spp.), in the vicinity of which cattle and horses graze. Other ecological requirements include the presence of hosts (toads, frogs, and newts) and gently sloping banks favorable for laying cocoons.

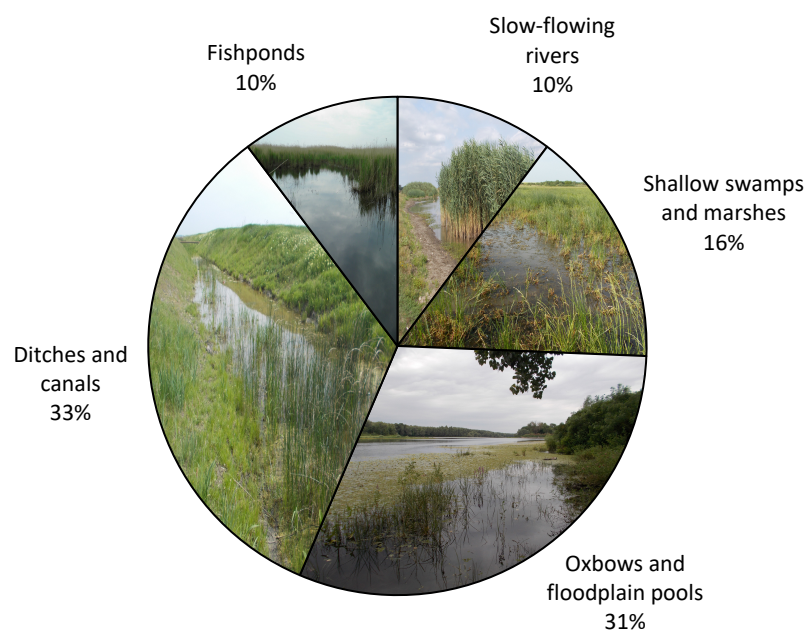


Figure 3. Habitats of *Hirudo verbana* in Romania.

The most abundant populations of *H. verbana* (estimated as CPUE = the number of individuals caught per 10 min sampling) were observed in the wetlands of Moldova, the Danube Delta, and the Balta Mică Natural Park of Brăila (Table S1).

3.2. Phylogenetic and Biogeographic Relationships

3.2.1. Phylogeographic Pattern of *Hirudo verbana* in Europe

The final alignments for COI (183 sequences) and 12S (174 sequences) were 529 bp and 280 bp long, respectively. Due to the lack of available 12S sequences from Serbian *H. verbana* samples, the analysis of the two mitochondrial markers was performed separately.

The COI alignment consisted of 465 monomorphic and 64 polymorphic sites, out of which 46 were parsimony-informative and 18 were singleton mutations. The COI dataset comprised 39 *H. verbana* haplotypes distributed all over Europe, grouped into three main clades: the Eastern European, the Western European, and the Iberian lineages (Figure 4). The Iberian clade is represented in this study by ten samples grouped into two haplotypes. Also, the Western European clade comprises 13 samples distributed across Slovenia, Italy, and North Macedonia, all grouped into two haplotypes.

All Romanian samples belong to the Eastern European clade and are grouped into 23 haplotypes, with 4 shared at the European level and 19 unique to the country. The COI network is star-like, with two common and central haplotypes and several unique haplotypes, separated usually by one or two substitutions (Figure 4b). The pattern is an indication of population expansion. The obtained network shows a low level of divergence between haplotypes from the Eastern European lineage of *H. verbana*.

For Romania, there are some populations/locations where we found unique COI haplotypes: Satchinez marsh, Remetea Oaşului wetland, Leţcani, Valea lui David, and Tălăbasca ponds. Also, there are a few populations with a considerable diversity of haplotypes, like Satchinez (five haplotypes in 10 samples), Băclăneşti (four haplotypes in 7 samples), Vadu (four haplotypes in 4 samples), and Letea (four haplotypes in 10 samples) (Figure 5; Table 1).

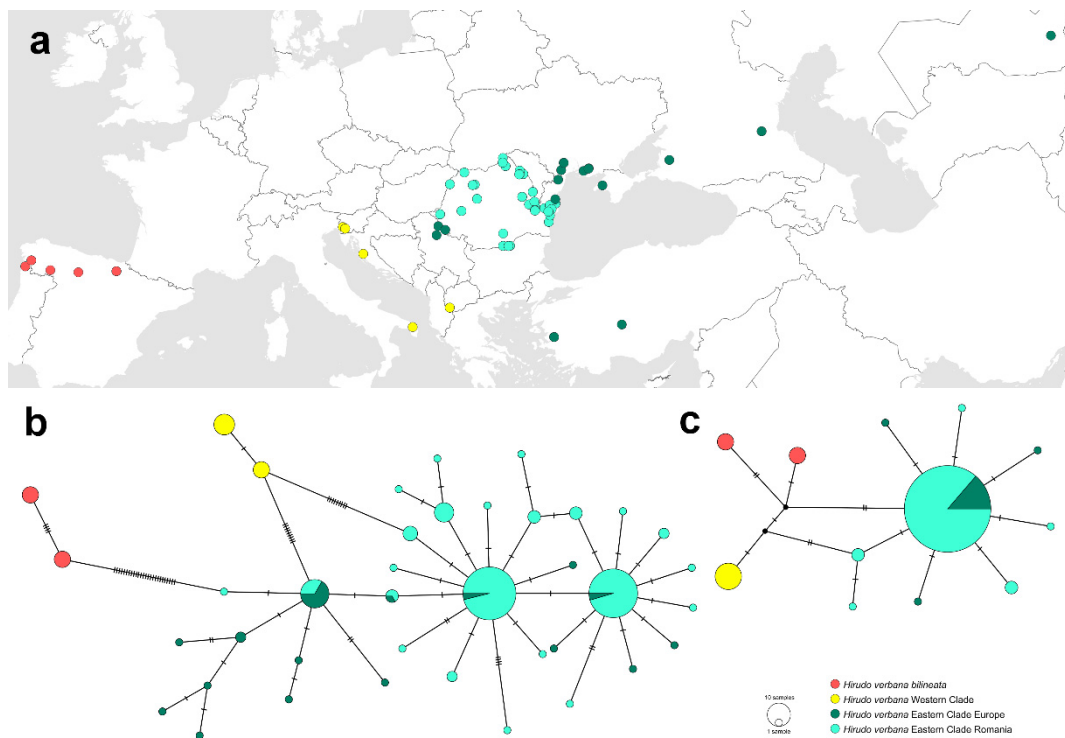


Figure 4. Haplotype networks of *Hirudo verbana* in Europe based on the Median-Joining Network method. (a) Distribution of haplotypes based on geographic position of sequenced populations; (b) COI haplotype network; (c) 12S haplotype network. Hash marks indicate the number of mutations, and the circle size is directly proportional to the frequency of observed haplotypes.

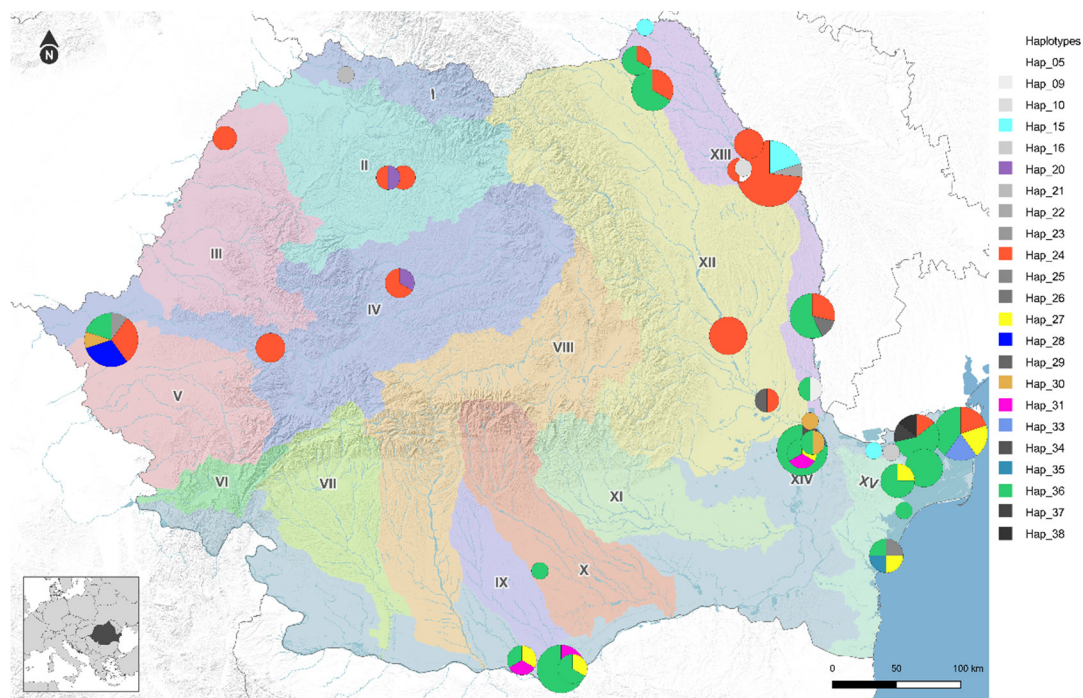


Figure 5. The distribution of *Hirudo verbana* COI haplotypes in Romania. The main 15 hydrographic basins of Romania are represented by the same colors as in Figure 1. Haplotypes found only once are depicted in grayscale. The size of the pie charts is proportional to the number of sequences.

Table 1. Genetic diversity indices for different regions in Romania based on COI sequences. N = number of samples; h = number of haplotypes; Hd = haplotype diversity; π = nucleotide diversity; S = number of variable sites; Tajima's D , Fu's F_s , R2 statistics, neutrality tests.

Population	N	h	Hd	π	S	Tajima's D	Fu's F_s	R2 Statistics
Dorohoi	3	2	0.667	0.00081	1	–	–	–
Buciumeni	5	1	0.000	0.00000	0	–	–	–
Bujoru	8	2	0.429	0.00081	1	0.33350 ($p > 0.10$)	0.536	0.2041
Băclănești	7	4	0.714	0.00162	3	–1.35841 ($p > 0.10$)	–1.798	0.1650
Căptălan	3	2	0.667	0.00126	2	–	–	–
Cristești-Jijia	15	3	0.448	0.00220	4	–0.17792 ($p > 0.10$)	1.263	0.1429
Ilia	3	1	0.000	0.00000	0	–	–	–
Ipotești	6	2	0.533	0.00101	1	0.85057 ($p > 0.10$)	0.625	0.2667
Chiriloaia Lake	3	3	1.000	0.00252	2	–	–	–
Harapu Island	9	3	0.417	0.00084	2	–1.36240 ($p > 0.10$)	–1.081	0.2079
Letea	10	4	0.800	0.00202	3	–0.02107 ($p > 0.10$)	–0.742	0.0450
Murighiol	5	1	0.000	0.00000	0	–	–	–
Pietroșani	3	2	0.667	0.00126	1	–	–	–
Cârja	7	3	0.667	0.00198	3	–0.65405 ($p > 0.10$)	0.110	0.2464
Proboata	3	1	0.000	0.00000	0	–	–	–
Sabangia	4	2	0.500	0.00095	3	–0.61237 ($p > 0.10$)	0.172	0.4330
Satchinez	10	5	0.844	0.00227	3	0.47343 ($p > 0.10$)	–1.803	0.1944
Vadu	4	4	1.000	0.00315	3	0.16766 ($p > 0.10$)	–2.181	0.2003
Viișoara	3	3	1.000	0.00162	2	–	–	–
Romanian <i>H. verbana</i> pop.	133	23	0.757	0.00245	25	–2.06935 ($p < 0.05$)	–19.652	0.0275

The 12S alignment consisted of 266 monomorphic and 14 polymorphic sites, out of which 8 were parsimony-informative and 16 were singleton mutations. These resulted in 12 haplotypes, out of which 5 are exclusive to Romania. Most of the Romanian samples were grouped within a common haplotype together with samples from the Eastern European clade of *H. verbana* (Figure 4c).

3.2.2. Demographic Parameters of *Hirudo verbana* in Romania

The genetic diversity indices were calculated for COI in 19 populations (111 individuals) that counted more than three sequenced individuals (Table 1). The results varied considerably across populations. In six populations, we found high values for genetic diversity ($Hd > 0.75$). Moderate values of genetic diversity were found in seven populations, while three populations exhibited low genetic diversity ($Hd < 0.5$). Null values were calculated in four populations (Table 1).

For the entire *H. verbana* population from Romania ($N = 133$), we calculated a high haplotype diversity (Hd) of 0.757 and a nucleotide diversity (π) of 0.00245.

The neutrality tests (Tajima's D and Fu's F_s) had negative values and proved statistically significant ($p < 0.05$) in Romanian populations of *H. verbana*. These results are consistent with the scenario of demographic expansion that took place in the recent history of the population. The mismatch distribution followed an L-shaped outline, which is expected for star-like topologies, indicating the occurrence of a demographic and/or spatial expansion [39,40], with only one exception, namely, the Cristești-Jijia population in which the mismatch distribution is multimodal, suggesting a population subdivision or a complex demographic history.

The calculated values of allelic richness for COI revealed that the haplotype frequency $h_T = 0.730$ (0.0358) is almost equal to the genetic distance between haplotypes $v_T = 0.732$ (0.0747). The levels of total diversity (h_T) and the phylogenetic information (v_T) are relatively high for the Romanian populations of the Mediterranean medicinal leech.

3.3. Spatial Analysis and Distribution Modeling

The number of presence points for *H. verbana* in Romania is very low: only 43 points remained after applying a spatial filter, which limited the prediction model for this area. However, these are the best available data for this species, which is often overlooked. The selected model, based on these 43 distribution points for *H. verbana* in Romania and 57 climatic and environmental variables, had AUC values of 0.972 for the training data and 0.906 for the test data.

The main variables shaping the distribution of *H. verbana* were wetland landscape coverage (34%), elevation (13.6%), and seasonally wet and wet grasslands (7.6%), although no single variable showed extremely high importance.

The predictive models, using all presence points and bioclimatic variables, identified new potentially suitable areas for *H. verbana*. The species is present in all the main Romanian hydrographic basins below 250 m in elevation and has a higher density in the Moldova region and along the Danube River, including the Danube Delta (Figure 6).

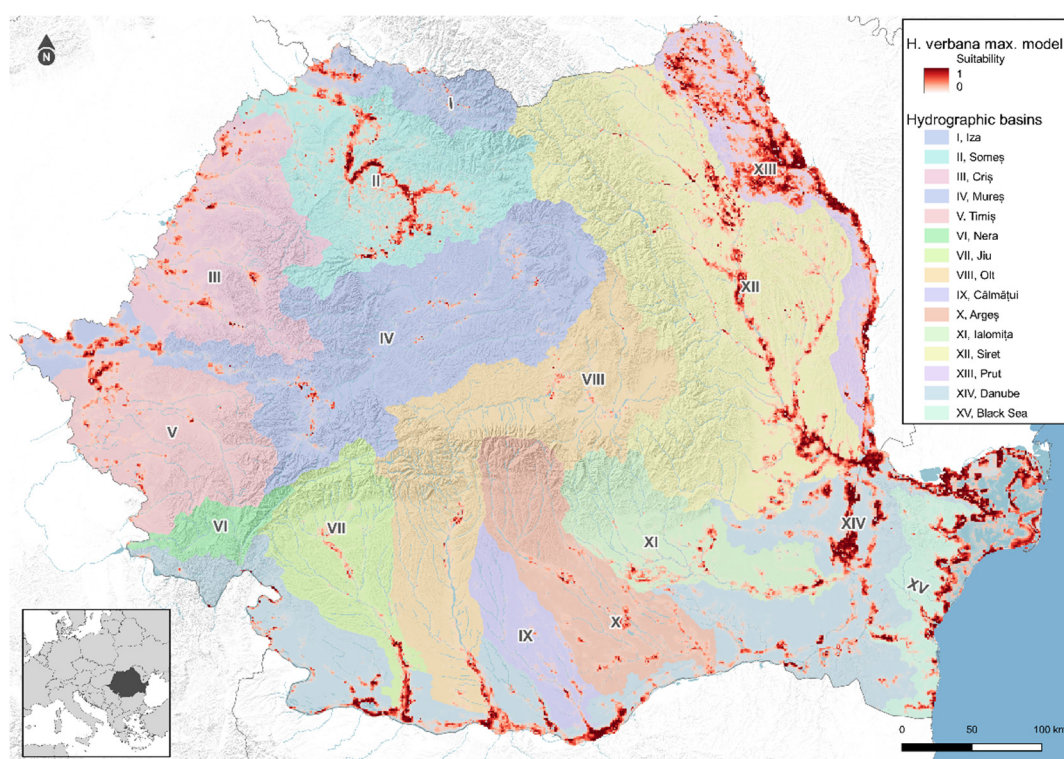


Figure 6. Predicted distribution model for *Hirudo verbana* in Romania using all available presence locations (43 points). Hotter red areas (values close to 1) indicate higher climate and environmental suitability for *H. verbana*. The main 15 hydrographic basins of Romania (inset legend in the upper right) are represented by the same colors as in Figure 1.

4. Discussion

4.1. Taxonomic Status

In the territory of present-day Romania, medicinal leeches have been known since ancient times [41]. The local people and leech merchants distinguished the “black” or “German” medicinal leech (*H. medicinalis*) from the “green” or “Hungarian” medicinal leech (*H. verbana* as *H. officinalis*) [41,42]. Scriban [43,44] recognized five of Moquin-Tandon’s [6] varieties of *Hirudo medicinalis* (*serpentina*, *tesselata*, *signata*, *nigrescens*, and *communis*) as occurring in Romania.

Until the beginning of the 21st century, the taxonomic status of the different color variations of medicinal leeches was uncertain until molecular data showed that there is more than one species involved within the traditionally recognized taxon *H. medicinalis* [3–5,45,46].

Currently, three medicinal leech species are known to occur in Europe—*Hirudo medicinalis*, *H. verbana*, and *H. troctina* [4,47–49]. All three European species can be readily recognized by their dorsal and ventral coloration pattern [4,8]. Though the color varieties depicted by Scriban [43,44] could not be unambiguously assigned to currently recognized species, we suppose that both *H. medicinalis* and *H. verbana* were present in Romania at that time.

4.2. Distribution of *Hirudo verbana* in Romania and Neighboring Countries

Hirudo verbana is a Western Palearctic species, occurring from Central, Southern, and Eastern Europe to Anatolia and Central Asia [48,50–52].

In Romania, we found *H. verbana* only in plain and hilly regions (see Figures 1 and 6). Likewise, Scriban [43] indicated that medicinal leeches occur throughout Romania excluding mountainous areas. Utevsky et al. [48] showed that *H. verbana* is more thermophilic than *H. medicinalis*. Consequently, in Romania, the former is distributed at lower elevations than the latter. In the context of global warming, we might expect a further northward range expansion of *H. verbana*.

Hirudo verbana was also reported from all countries neighboring Romania: Republic of Moldova [53], Ukraine [48,54], Hungary [9], Bulgaria [55], and Serbia [56]. Todorov et al. [55] indicated that in Bulgaria, *H. verbana* is widespread throughout the country, the wetland along the Danube River standing out as the most suitable habitat. Also, the species is abundant in the coastal freshwater lagoons of the Black Sea. For the rest of the country, the species was found in small and scattered swampy eutrophic water basins. The findings of Živić et al. [56] suggest that the distribution of *H. verbana* in Serbia is also fragmented, with habitats that are isolated from one another and generally small in size except for Vojvodina (the Danube River floodplain). However, the extensive and unregulated harvesting of leeches, particularly in the Srem and South Banat Districts, for export to Western European countries has significantly reduced both the area and population size of this species in Serbia [56].

The ecological characteristics and low detectability of *H. verbana*, as well as the limited research performed over time, has resulted in a low number of presence points, most of them coming from targeted field observations. The lack of proper morphological descriptions of the specimens in published scientific papers [16–21] make the historical data unsuitable for assessing the past geographical distribution of the two species of medicinal leeches. The limited number of voucher specimens in museum collections also contribute to this lack of information. Therefore, the distribution of *H. verbana* in Romania was assessed based only on recently collected data. The latter provided a relatively low number of observations, meaning that the prediction model is not very robust. However, it does incorporate the main environmental variables important for the species' ecology, such as wetland area size and altitude. The predicted distribution model was developed using all recent available information on the species' presence across Romania (Figure 6). This preliminary model can be utilized by experts studying this species in the field for further observations and monitoring.

4.3. Phylogenetic and Biogeographic Relationships

Our study is the first aiming to characterize the Romanian populations of *H. verbana*. From the molecular point of view, the mitochondrial DNA diversity can be appreciated as very high. We have identified 19 unique haplotypes for Romania, which is much higher than the number (3) of unique haplotypes found in Serbia by Živić et al. [56].

With one exception, all the analyzed Romanian populations show evidence of a recent expansion. The only population that exhibits two modes under the model of population growth decline or constant population size is the one from Cristești-Jijia. The signs of a stable population indicated by mismatch distribution analysis could be linked to the high number of individuals analyzed from this population (15 individuals).

The haplotype network of *H. verbana* is star-like, which also indicates a population expansion scenario and, probably, a recent population bottleneck. The population expansion is also indicated by the negative results of neutrality tests.

The increased genetic diversity found in the populations of Romania suggests two possible scenarios: the first is the existence of a glacial refugium in the lower Danube Lowland (the former Dacian Basin), and the second is represented by a contact zone of recolonization from multiple refugia. To test these two hypotheses, we used haplotype frequency vs. genetic distance between haplotypes from Romanian populations. The results revealed that the haplotype frequency was almost equal to the genetic distance between haplotypes, which fit the model of a contact zone. However, the possibility of the existence of a cryptic refugium (e.g., Danube River refugium sensu [57]) cannot be ruled out because of the presence of a high number of unique haplotypes [14].

Trontelj and Utevskey [4] found a very surprising genetic uniformity of *H. verbana* across the species' range. Subsequently, the phylogenetic investigation of different populations from the West Atlantic to the Urals and the Caspian Sea made by Trontelj and Utevskey [47] evidenced the presence of two phylogroups (or clades)—the Eastern phylogroup known to occur from Serbia to North Caucasus, Turkey, and Uzbekistan and the Western phylogroup, occurring in the northwestern Balkans and Italy. Finally, Arias et al. [49] indicated the presence of a third phylogroup (clade) present in the Iberian Peninsula. All these phylogroups correspond to the major Mediterranean glacial refugia—Iberic, Apennine, and Balkan [58].

Though we lack genetic data from Albania, Greece, and Bulgaria, the results of our study (Figure 4) suggest that in populations from Romania and southwestern Ukraine, there is an admixture of lineages from western Anatolia as well. In the post-Pleistocene period, these lineages probably migrated from Anatolia to the northeastern Balkans before the Bosphorus and Dardanelles Straits were formed (ca. 7.6–8.2 kya). On the contrary, populations from southern Russia (Krasnodar and Stavropol territories) and eastern Ukraine might originate from the Caucasus (Colchis) glacial refugium, as indicated by the presence of the third major haplotype (Figure 4). However, the northwestern Balkans (from Slovenia and Croatia to Lake Ohrid in North Macedonia) were likely colonized from the Apennine Peninsula due to the establishment of a broad land bridge after the Last Glacial Maximum (LGM, ca. 18–21 kya), when the whole Northern Adriatic dried up completely. The existence of a distinct refugium encompassing the northwestern Balkans and Italy is also indicated by other studies, e.g., [29] for white oaks, [59] for *Triturus* spp., and [60] for freshwater bivalves. The data of Wielstra et al. [59] are especially relevant to our study because medicinal leeches are temporary external parasites on amphibians as well. In this way, they benefit from better dispersal possibilities than other freshwater animals. Also, they have an adaptive resistance outside water for long periods of time, which seems to explain why their distribution does not always follow freshwater networks or basins [48].

In agreement with Gómez and Lunt [61], the Balkan Peninsula itself actually represents not a single refugium but a contact (suture/admixture) zone where the lineages from the Italian and Anatolian refugia also met (“refugia-within-refugia” model). This is a consequence of isolation during the Pleistocene range contractions and subsequent expansions [13,14,52,62]. The lower Danube Lowland (former Dacian Basin) probably served as a distribution corridor for *H. verbana* to Central and Western Europe.

Utevskey et al. [48] did not detect any postglacial colonization pattern for the Western Palearctic species of medicinal leeches. However, such a pattern is revealed in the present study by *H. verbana* alone.

4.4. Conservation Status of the Mediterranean Medicinal Leech

In the historic principalities of Wallachia and Moldavia, the export of medicinal leeches began in the early 1830s, but already in 1835 and 1844, respectively, it became forbidden due to an almost complete depletion of leeches for domestic needs [1,42].

Today, Romania is one of the leading countries regarding the quantity of medicinal leeches regularly exported to Germany, France, Switzerland, Poland, the USA, Uzbekistan,

Lithuania, the Czech Republic, etc. [63]. So far, only the Mediterranean medicinal leech *H. verbana* is commercially exploited in Romania, being mainly collected in the Danube Delta and the Brăila wetlands. Although both wetlands are situated in protected areas, an increasing trend of leech collection has been observed over recent years. This can be attributed to the depletion of fish resources in both areas because of changes in hydrological regimes (land reclamation, damming, desiccation, or drainage works), prompting local people and fishers to start seeking alternative sources of income. Therefore, stricter regulations on the harvest from the wild should apply in those areas (proper regulations for prohibition periods, justification for the sustainability of the allocated yearly quotas, and better control of illegal trade) for an improved protection of this species. Fortunately, the distribution of *H. verbana* exceeds these areas (Figure 6), with good coverage in the main river valleys of the Moldova region where medicinal leeches are not yet commercially exploited.

However, in the western part of Romania (Maramureș, Crișana, and Banat regions), *H. verbana* populations declined dramatically because of degradation and loss of wetland habitats (desiccation, damming, and drainage works), water pollution (intensive use of pesticides in agriculture), reduction in food availability (abandonment of water buffalo breeding and general reduction of amphibian populations), and spread of some invasive species (e.g., the brown bullhead *Ameiurus nebulosus* (Lesueur, 1819) and the black bullhead catfish *Ameiurus melas* (Rafinesque, 1820) that prey upon leeches). Locally, the species is threatened by overcollecting for medicinal use (as in the Danube Delta or the Balta Mică Natural Park of Brăila).

Though this species has not yet been assessed globally for the IUCN Red List of Threatened Species, by applying the IUCN categories and criteria [64], Romanian populations of *H. verbana* were assessed as Vulnerable (VU) (B2b(iii,iv)c(ii)). The same status was mentioned in the *Red Book of Invertebrates from Romania* [65].

Considering the species' conservation status and also its scientific and economic importance, the estimated distribution of *H. verbana* in Romania presented herein could be a valuable starting point for further monitoring as well as for the development and implementation of appropriate conservation measures.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d16120726/s1>, Table S1: Locality data. Details on *Hirudo verbana* populations reported in the present study; Table S2: Genetic sampling. Details on *Hirudo verbana* specimens included in the genetic analysis; Table S3: Museum material. Details on specimens of *Hirudo verbana* found in museum and other scientific collections in Romania.

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