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Phylogeny and phylogeography of medicinal leeches (genus *Hirudo*): Fast dispersal and shallow genetic structure

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ABSTRACT

Medicinal leeches (*Hirudo* spp.) are among the best-studied invertebrates in many aspects of their biology. Yet, relatively little is known about their biogeography, ecology and evolution. Previous studies found vast ranges but suggested low genetic diversity for some species. To examine this apparent contradiction, the phylogeny and phylogeography of the widespread *Hirudo verbana*, *Hirudo medicinalis* and *Hirudo orientalis* were investigated in a comparative manner. Populations from across their ranges in Europe, Asia Minor, the Caucasus and Central Asia, were analyzed by various phylogenetic and population genetic approaches using both mitochondrial (COI and 12S) and nuclear DNA sequences (ITS1, 5.8S and ITS2). The populations showed surprisingly little genetic differentiation despite vast ranges. The only clear structure was observed in *H. verbana*. This species is subdivided into an Eastern (southern Ukraine, North Caucasus, Turkey and Uzbekistan) and a Western phylogroup (Balkans and Italy). The two phylogroups do not overlap, suggesting distinct postglacial colonization from separate refugia. Leeches supplied by commercial facilities belong to the Eastern phylogroup of *H. verbana*; they originate from Turkey and the Krasnodar Territory in Russia, two leading areas of leech export. *H. verbana* and *H. medicinalis* have experienced recent rapid population growth and range expansion, while isolation by distance has shaped the genetic setup of *H. orientalis*. The habitat of the latter is patchy and scattered about inhospitable arid and alpine areas of Central Asia and Transcaucasia. Centuries of leech collecting and transport across Europe seem not to have affected the natural distribution of genetic diversity, as the observed patterns can be explained by a combination of historical factors and present day climatic influences.

1. Introduction

The taxonomy of what has until recently been considered as a single species, *Hirudo medicinalis*, has been revised dramatically during the last decade. The changes were initiated by Nesemann and Neubert (1999) who re-established the long-forgotten southern European species *Hirudo verbana* Carena, 1820. Subsequent molecular analyses have validated this change, revealed yet another species – *Hirudo orientalis* Utevsky and Trontelj, 2005 – and provided a phylogenetic hypothesis for the genus *Hirudo* (Trontelj et al., 2004; Trontelj and Utevsky, 2005; Utevsky and Trontelj, 2005; Siddall et al., 2007; Phillips and Siddall, 2009). Several recent studies demonstrated substantial biological differences between *H. medicinalis*, *H. verbana*, and *H. orientalis* (Fig. 1). The three species differ in the biochemical composition of their saliva (Baskova et al., 2008). They have different chromosome numbers (Utevsky et al., 2009) but are able to hybridize, showing varying degrees of postzygotic reproductive isolation (Petrauskienė et al., 2009).

The Western Palearctic medicinal leech species have vast parapatric ranges (Utevsky et al., 2010; Fig. 2). Their biogeographical pattern corresponds with major landscape types and climate belts, but seems not to have been influenced by Pleistocene glaciations and human activity. Unlike several European groups of closely related species and subspecies with a west versus east division of ranges caused by dispersing from different Mediterranean refugia, medicinal leeches show no distributional signature of Pleistocene effects. Their ranges are rather belt-shaped and arranged from the north to the south. *H. medicinalis* is the northernmost species among medicinal leeches. It occupies the deciduous arboreal zone from Britain and southern Norway to the southern Urals and probably as far as the Altai Mountains. *H. verbana* occurs in the Mediterranean and steppe zones from Western Europe to Turkey and Uzbekistan. *H. orientalis* prefers mountainous areas in Transcaucasian countries, Iran and Uzbekistan, whereas *Hirudo troctina* is an Iberian and northwestern African species found in arid landscapes.

Recent molecular studies revealed low genetic diversity in medicinal leeches and a possible bottleneck or selective sweep in

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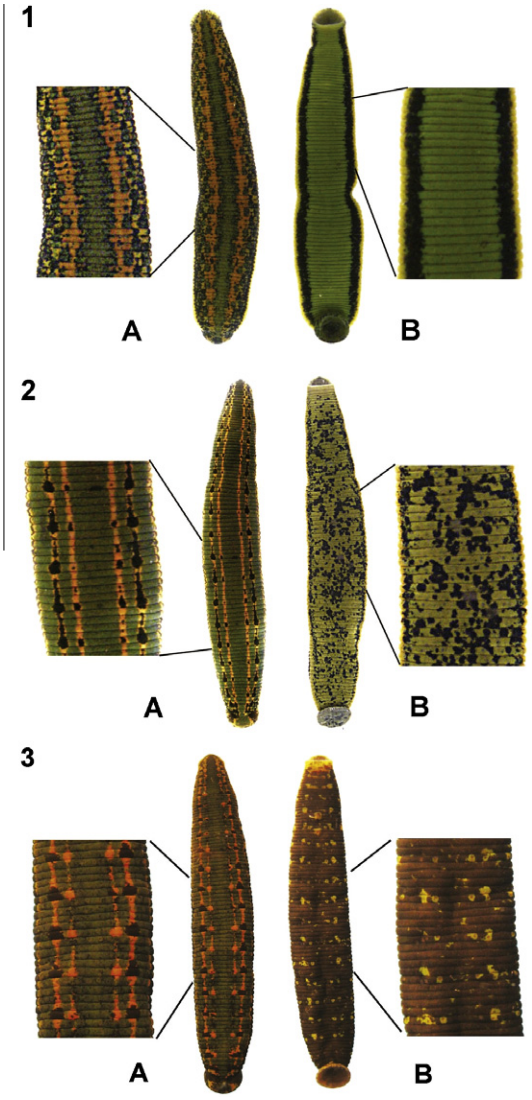

Fig. 1. The three most common species of Western Palearctic medicinal leeches, until recently lumped under the sole taxon *Hirudo medicinalis*: *H. verbana* (1), *H. medicinalis* in its narrow sense (2), and *H. orientalis* (3). Dorsal (A) and ventral (B) coloration of typical individuals is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

H. medicinalis (Trontelj and Utevsky, 2005; Siddall et al., 2007; Petrauskienė et al., 2009). The low diversity and apparent lack of geographic population structure are surprising for species with such large ranges disrupted by several geographical barriers and hydrological divides. However, the small number of populations analyzed so far and their insufficient geographic coverage do not allow generalizations. Moreover, based on mitochondrial COI sequences and polymorphic microsatellite loci, the only genetic structuring found so far was a division between natural and captive-bred populations of *H. verbana*, the species most often used for medical application (Siddall et al., 2007). These results are suggestive of two historical circumstances that might have contributed to the current distribution and population structure, and that we test in this work.

First, the wide distribution and lack of genetic diversity could be the result of recent rapid range expansion. Under this hypothesis, leeches dispersed quickly across vast territories of the Western Palearctic. Medicinal leeches might have occupied their wetland habitats as soon as they became available after the glaciers melted. The most likely means of dispersal is by their mammalian terrestrial hosts that can bear attached leeches and transfer them between water bodies (Utevsky et al., 2010). This hypothesis predicts the lack of strong phylogeographic patterns within species, weak differentiation of populations, and low isolation by distance. Most importantly, nucleotide patterns and mismatch distributions should fit expectations of sudden population growth.

The second hypothesis predicts that the extensive exploitation of leeches has left a mark on the genetic structure of medicinal leech populations. Leeches of the genus *Hirudo* have been in medicinal use for centuries (Phillips and Siddall, 2009) and transported over long distances for sale and use in the millions each year (Lukin, 1976). Although the species ranges have probably not been strongly affected by human activity (Utevsky et al., 2010), the impact of transport and overexploitation could still be visible at the genetic level. For example, DNA haplotypes characteristic of southern or eastern regions – where most leeches were collected from – might be found in the surroundings of urban centers where the demand was highest, or where phylogeographic overlap is not expected to occur by natural processes. For these signatures of human influence to be detectable, a certain degree of genetic differentiation has to exist. In a genetically homogeneous population this hypothesis is not testable, and both the first and the second historical scenario could apply.

2. Material and methods

2.1. Sample collection and DNA extraction

Samples of *H. verbana*, *H. medicinalis* and *H. orientalis* were collected from Europe, Turkey, the Caucasus and Central Asia (Table 1, Fig. 3). Altogether, 122 individuals from 32 populations were analyzed. *H. troctina*, used as outgroup, was represented by Moroccan sequences from Trontelj and Utevsky (2005) and a newly sequenced specimen from Lebna Dam, Tunisia. Small pieces (approx. $5 \times 2 \times 1$ mm) of skin and muscle tissue were cut from the lateral part of the body. Care was taken not to reach the digestive system, which often contains blood from unknown host species. Genomic DNA was isolated using the GENE ELUTE Mammalian Genomic DNA minprep kit from Sigma–Aldrich (Steinheim, Germany).

2.2. Amplification and sequencing

Mitochondrial 12S rDNA and cytochrome oxidase subunit one (COI) fragments were amplified as described in Trontelj and Utevsky (2005). PCR products were purified using the Millipore Montage purification system (Bedford, MA, USA). The ribosomal internal transcribed spacers (ITS1 and ITS2) including the 5.8S rRNA gene was amplified using primers described in Hillis and Dixon (1991). PCR was performed by applying 30 cycles of 45 s at 94 °C, 45 s at 48 °C, and 60 s at 72 °C, following a 3 min denaturation step at 94 °C. The purified products were sequenced in both directions with amplification primers under BIG DYE Terminator cycling conditions, purified by ethanol precipitation, and run on an Applied Biosystems 3730xl sequencer by Macrogen (Seoul, Korea). Sequence chromatograms were edited and assembled with the help of ChromasPro 1.32 (Technelysium Pty., Queensland, Australia). The correctness of COI sequences was verified at the amino acid level. The obtained sequences have been assigned GenBank Accession Numbers JN083793–JN083804, JN104641–JN104656 and JN118987–JN119045. Some sequences from previous studies were also used for the reconstruction of phylogenetic trees and networks: AF 099961 (Trontelj et al., 1999); DQ097197 (Phillips and Siddall, 2005); AY763148 – AY763151, AY763154, AY763156 – AY763160, AY763163, AY763164, AY763166, AY763167, AY763170, AY763171, AY768704 (Trontelj and Utevsky, 2005); EF446680 – EF446687, EF446691 –

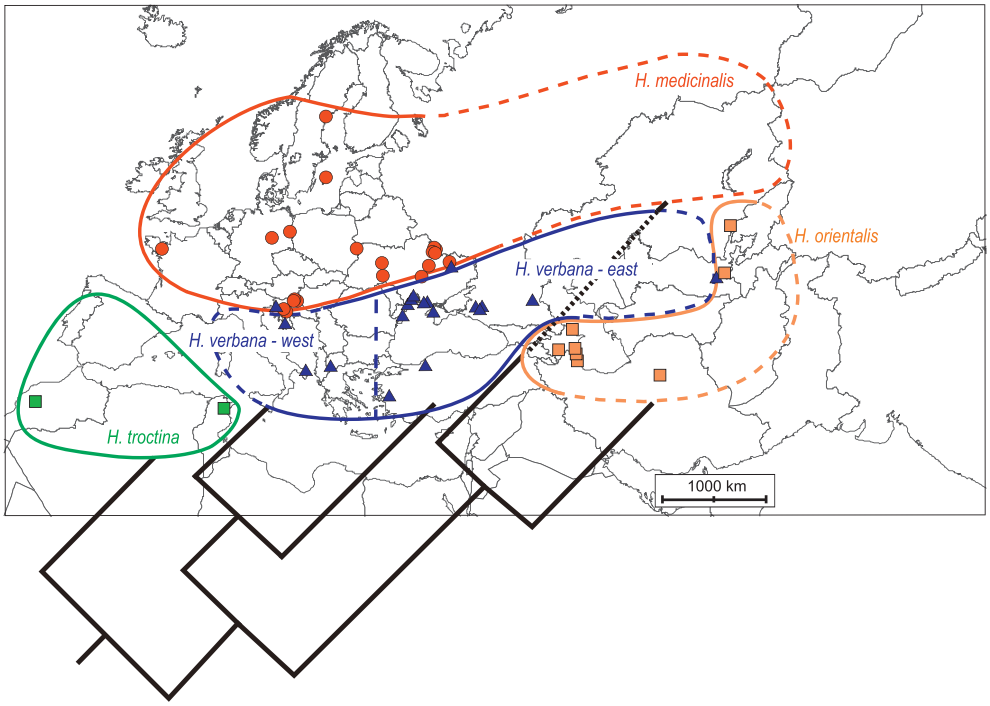

Fig. 2. Approximate ranges of *Hirudo verbana*, *H. medicinalis*, *H. orientalis* and *H. troctina*, and their hypothesized phylogenetic relationships. Geographic locations of analyzed populations are plotted for each species. Range limits are based on Utevsky et al. (2010); dashed lines indicate speculative boundaries. *Hirudo troctina* is used as outgroup and is represented by two populations only.

EF446713 (Siddall et al., 2007); EF405599 (Utevsky et al., 2007); EF125043 (Kutschera et al., 2007); HQ333515 – HQ333519 (Kvist et al., 2010).

2.3. Phylogenetic trees and networks

Aligning the sequences using Muscle v3.7 (Edgar, 2004) under default options resulted in unambiguous alignments with very few short gaps. For each gene segment, Modelgenerator (Keane et al., 2006) was used to determine the best fit model of evolution. Rate heterogeneity across sites was approximated by six gamma categories. Models were selected according to the second order Akaike information criterion (AIC2).

Phylogenetic relationships were determined by Bayesian inference using MrBayes v3.1.2. (Ronquist and Huelsenbeck, 2003). Exploratory analyses of alignments obtained in this study showed that single-gene trees differed in the degree of resolution but did not contradict each other in topologies. Hence, subsequent phylogenetic analyses were run on combined mitochondrial and nuclear sequences with mixed models and unlinked model parameters. Searches were performed in two parallel runs with four chains each for two million generations, sampled every 100th generation. After discarding the first 25% of the sampled trees, final topologies were consented following the 50% majority rule. In addition, a maximum likelihood TBR search was performed using PhyML v3.0 (Guindon et al., 2010) under a HKY85 + I + Gamma model and approximate Shimodaira–Hasegawa test values to asses branch support.

Because intraspecific phylogenies remained highly unresolved, with many very similar sequences, some of which may be ancestral, we chose to represent their relationships in the form of networks. We used Network v4.5.1.6 to create median-joining networks (MJNs). This network method is particularly suitable for sequence datasets with incomplete geographical coverage and larger genetic distances (Bandelt et al., 1999). Equal weights were assigned to all positions, and the homoplasy level parameter (ε) was set to zero as low divergence and lack of recombination were expected to result in relatively simple networks. Only mitochondrial DNA haplotypes were used in these analyses.

2.4. Demographic analysis

Demographic history was inferred using mismatch distribution analyses as implemented in DNASP v 5.10 (Librado and Rozas, 2009). Populations that have experienced a rapid expansion in the recent past show unimodal mismatch distributions, while populations at demographic equilibrium have multimodal distributions (Rogers and Harpending, 1992). For major clades, theoretical distributions under the assumption of constant population size and the sudden expansion model were compared to the observed data. The sum of square deviations (SSDs) between the observed and the expected mismatch was used as a test statistic. The raggedness index of the observed distribution (Harpending, 1994) was also computed. This index takes larger values for multimodal distributions commonly found in a stationary population than for unimodal and smoother distributions typical of expanding populations. Harpending's raggedness index, SSD and their *p*-values were computed in Arlequin v3.5 (Excoffier and Lischer, 2010), applying 10,000 bootstrap replicates.

Statistical tests implemented in DNASP were used to detect past population growth under the assumption of selective neutrality. Significantly negative values of Tajima's *D* (Tajima, 1989) can be interpreted as the signature of population expansion (Aris-Brosou and Excoffer, 1996). Fu's F_S statistic is very sensitive to population demographic expansion, which generally leads to large negative F_S values (Fu, 1997). Fu's F_S statistic and Ramos-Onsins and Rozas R_2 statistic have been shown to be the most powerful tests of constant population size (Ramos-Onsins and Rozas, 2002). Haplotype and nucleotide diversity were calculated using DNASP.

Table 1
Provenance of samples analyzed, haplotype ID numbers as used in the network analyses, and numbers of distinct mitochondrial DNA haplotypes (in parentheses) along with their GenBank Accession Numbers.

Provenance	COI		12S	
	Haplotype ID	GenBank Acc. No.	Haplotype ID	GenBank Acc. No.
Hirudo medicinalis				
Germany				
Münchberg	–		1(3), 2(1)	JN118990, JN118991
Saale River, Rotherburg, Halle [b]	–		7(2)	AY763157
France [a]	–		9(1)	DQ097197
Slovenia				
Lendava	–		2(1)	JN118991
Podvinci [b]	–		8(1)	AY763159
Ukraine				
Horila Dolyna, Kharkiv Reg. [b]	–		1(5), 6(1)	JN118990, AY763156
Sheludkivka, Kharkiv Reg.	–		1(3)	JN118990
Vasyshcheve, Kharkiv Reg. [b]	–		6(1)	AY763156
Balakliia, Kharkiv Reg.	–		1(3)	JN118990
Nyzhnya Syrovatka riv., Sumy Reg.	–		1(3)	JN118990
Velyka Chernechchyna, Sumy Reg.	–		1(1), 2(2)	JN118990, JN118991
Mykilske, Sumy Reg.	–		1(2)	JN118990
Svityaz' Lake, Volyn' Reg.	–		1(6)	JN118990
Chornyi Lis, Kirovograd Reg.	–		1(3)	JN118990
Polyana, Khmelnits'k Reg.	–		1(3), 3 (1), 4(2), 5(1)	JN118990, JN118992, JN118993, JN118994
Netechintsi, Khmelnits'k Reg.	–		1(1)	JN118990
TOTAL *H. medicinalis*	–		47	
Hirudo verbana				
North-eastern Ukraine, Kharkiv Reg., Horila Dolyna	1(5)	JN083793	2(5)	JN104650
Crimea, Donuzlav Lake	2(5)	JN083794	6(4)	JN104654
Southern Ukraine, Kherson Reg.				
Mala Kardashinka.	17(1)	JN104644	1(1)	JN104649
Kokhany	1(1)	JN083793	7(1)	JN104655
Shaby	3(2)	JN083795	1(2)	JN104649
South-western Ukraine, Odesa Reg.				
Mayaky	4(1)	JN083796	5(1)	JN104653
Severynivka	1(1), 6(2)	JN083793, JN083798	2(2), 3(3)	JN104650, JN104651
Berezivka	1(2), 3(1), 15(1)	JN083793, JN083795, JN104642	2(2)	JN104650
Vilkove	1(1), 5(1), 7(2), 16(3)	JN083793, JN083797, JN083799, JN104643	1(5), 9(1)	JN104649, JN104656
Russian Federation				
Krasnodar Territory	1(4)	JN083793	1(5)	JN104649
Stavropol Territory	1(2), 14(2)	JN083793, JN104641	2(5)	JN104650
Turkey				
Izmir	8(1), 10(1), 11(2), 12(2), 13(1)	JN083800, JN083801, JN083802, JN083803, JN083804	1(6)	JN104649
Northern Turkey	8(2)	JN083800	1(2)	JN104649
Uzbekistan, Karateri, Samarqand Reg.	1(5)	JN083793	4(5)	JN104652
Commercial facilities (USA) [c]	1(7)	EF446681, EF446682, EF446683, EF446685, EF446686, EF446687, EF446688	-	
Biopharm commercial leeches (USA and UK) [c]	8(1), 9(1)	EF446680, EF446684	–	
Germany, Piethen Kaolin pit, Halle[b]	–		8(1)	AY763160

South-western Slovenia [b,c]	18(5)	EF446695, EF446696, EF446699, EF446700, EF446701,	8(1)	AY763160	
Croatia, Island Pag [b,c]	18(1), 19(1)	EF446691, EF446690,	8(1)	AY763160	
Macedonia, Lake Ohrid [b,c]	20(1), 21(1), 22(2), 23 (1)	EF446693, EF446694, AY763150, EF446692,	8(2)	AY763160	
Italy, Lecce [b,c]	21(1), 22(1)	AY763150, EF446698	8(1)	AY763160	
TOTAL *H. verbana*	74		56		
Hirudo orientalis					
Azerbaijan					
Divichi Lake	–		1(3), 3(2)	JN118987, JN118989	
Kyzylagadzh Gulf	–		1(3)	JN118987	
Gajitepe	–		1(1)	JN118987	
Agdam [b]	–		5(1)	AY763163	
Unknown locality [b]	–		4(1)	AY768704	
Uzbekistan					
Dzhumabazar, Samarqand Reg.	–		2(8)	JN118988	
Urgut, Samarqand Reg. [d]	–		2(1)	EF405568	
Kazakhstan, Taskol Lake, Zhambyl Reg.	–		2(4)	JN118988	
Iran (exact place unknown)	–		1(1)	JN118987	
TOTAL *H. orientalis*	–		25		

[a] Phillips and Siddall (2005).
[b] Trontelj and Utevsky (2005).
[c] Siddall et al. (2007).
[d] Utevsky et al. (2007).

2.5. Analysis of geographic population structure

To assess the geographic structure of COI and 12S rDNA haplotypes we used spatial analysis of molecular variance (SAMOVA) as implemented in the SAMOVA software (Dupanloup et al., 2002). Using a simulated annealing procedure, the program searches for the hierarchical configuration of populations among a prespecified number of groups with the highest between-group differentiation (F_{CT}). Configurations containing groups with single populations were not considered (Kurata et al., 2008).

Also, we evaluated the degree to which genetic differentiation of populations could be explained by isolation by distance. Mantel tests were used to determine the significance between genetic and geographical distances in the AIS software (Miller, 2005).

3. Results

3.1. Phylogenetic and phylogeographic relationships

The phylogeny of medicinal leeches is characterized by deep divisions between species, and shallow within-species relationships, largely lacking structure (Fig. 4). Phylogenetic analyses of separate genes led to the same major groups. The genetic variability of COI sequences was highest, while 12S and ITS fragments showed a lower diversity in *H. medicinalis* and *H. verbana*. Conversely, the COI of *H. orientalis* was conserved for all samples sequenced (Table 2). The obtained topologies suggested a sister affinity between *H. medicinalis* and *H. orientalis* as already indicated by foregoing studies (Trontelj and Utevsky, 2005; Baskova et al., 2008), but without statistical support.

Significant intraspecific phylogenetic structure was found only for *H. verbana* that was subdivided into two clades: the first one comprised mainly Italian and western Balkan populations, the second one population from Ukraine to the North Caucasus, Turkey and Uzbekistan (Figs. 3 and 5). We refer to them as Western and Eastern phylogroup, respectively. Commercially obtained specimens, except for Kutschera's et al. (2007) leech, belonged to the Eastern phylogroup. There were also four monophyletic subgroups within this phylogroup (Fig. 4): a south-western/southern Ukrainian clade, an Uzbekistan clade, a clade with Crimean plus one south-western Ukrainian, and a clade containing Krasnodar (south-western Russian), Turkish and commercial [Biopharm and Carolina facilities (Siddall et al., 2007)] leeches.

Median-joining network analyses were performed on separate mtDNA datasets for each species individually. The COI set of *H. verbana* with 23 distinct haplotypes was the most extensive one (Fig. 5). A deep break of ten mutation steps separated western from Eastern populations and was consistent with the Western and Eastern *H. verbana* phylogroups recovered on the combined-data Bayesian tree. In the Eastern phylogroup, a single dominant haplotype (1) was found in individuals from South-western, Southern and North-eastern Ukraine, the Krasnodar and Stavropol territories of the Russian Federation, the Samarqand Region of Uzbekistan, and from commercial facilities except for Biopharm Leeches (UK). A Southern Ukrainian branch consisted of a basal Crimean haplotype and haplotypes from the Kherson and Odesa regions. A Turkish branch included two haplotypes from Biopharm Leeches (UK). All Eastern haplotypes were connected through a maximum of two mutational steps. The median-joining network revealed that the commercial samples either shared the most widespread and common haplotype 1 (COI), or shared another haplotype (8) with some Turkish samples, or had haplotype 9 from the subgroup of Turkish leeches. The Western phylogroup comprised a lineage of Italian and Macedonian (Lake Ohrid) haplotypes. Most of the Western haplotypes were connected through one mutational step

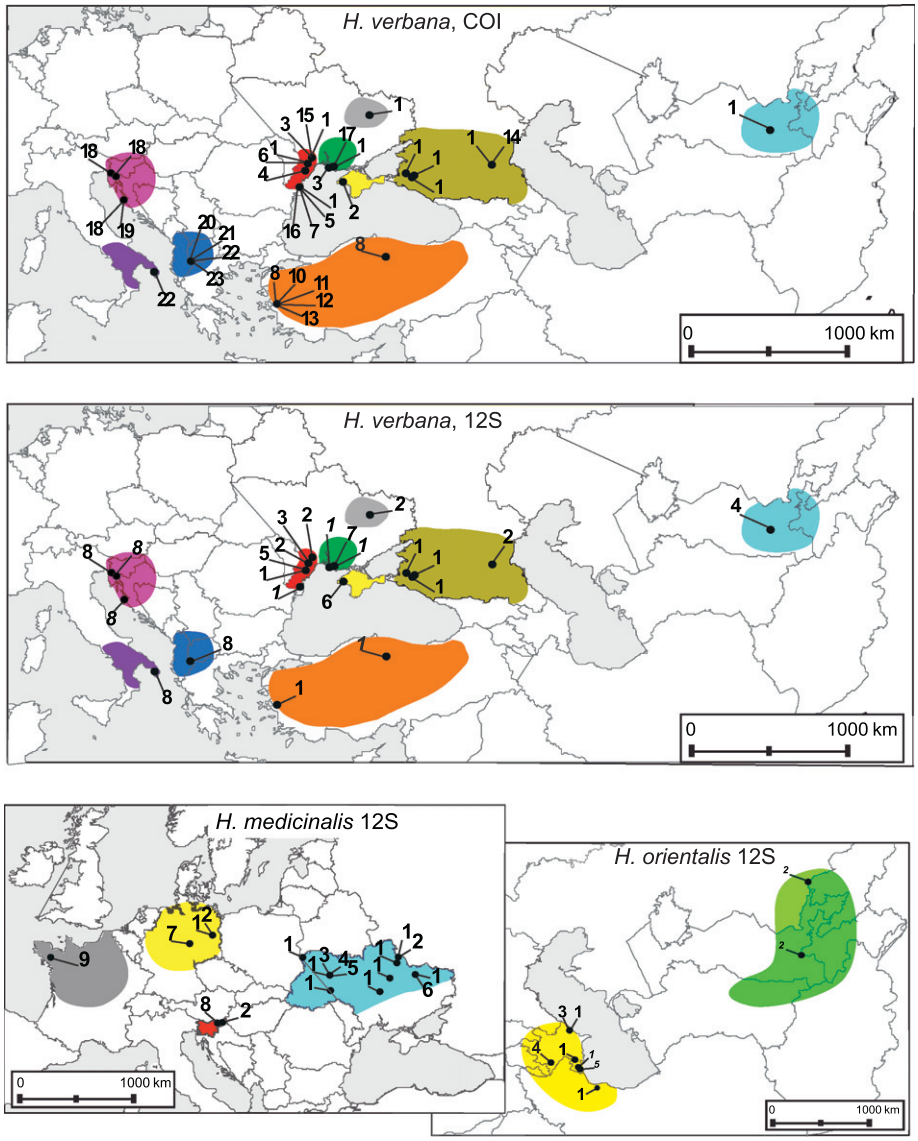

Fig. 3. Geographical distribution of mitochondrial DNA haplotypes used in this study. Haplotype codes are as per Table 1. Colors represent geographic areas where the haplotypes were sampled and are in agreement with the colors on the haplotype networks (Fig. 4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

except for a terminal Ohrid haplotype separated by six steps from its ancestor. The *H. verbana* network built using the 12S dataset comprised nine haplotypes and corroborated the two phylogroups by a divide of six mutation steps. The genealogies within both major branches, however, were less clear and somewhat different from that found in the COI network. Both datasets revealed a relatively high genetic variability in South-western Ukraine.

With *H. medicinalis*, we were largely unsuccessful in amplifying COI sequences. A network (not shown) calculated from GenBank COI sequences abounded with loops, suggesting the lack of sampling and possible sequencing errors. The 12S dataset yielded a star-like network with nine haplotypes, including a widespread haplotype (1) occurring throughout Ukraine and Germany and connected to four less common haplotypes through one mutation step. Four other haplotypes from Ukraine, Slovenia, Germany and France represent older sequences from GenBank that differed from the central haplotype from 6 to 16 mutation steps. These differences might not reflect true genetic distances, but rather be the consequence of less reliable sequencing methods used in the past.

For *H. orientalis*, COI sequences were not variable enough to construct a network. The 12S dataset gave a network consisting of five haplotypes, including two dominant ones: the Caucasian–Iranian one, and the Central Asian one, separated by a single mutation step. The former was connected with other Caucasian haplotypes through one mutation step.

3.2. Geographic genetic structure

In *H. verbana* and based on COI sequences, SAMOVA identified a most fundamental division ($K = 2$) into a western (the Balkans and Italy) and an Eastern (Eastern Europe, Turkey and Uzbekistan) group of populations. This division corresponds with the two phylogroups found by the phylogenetic analysis. On the other hand, 12S and combined mitochondrial matrices failed to provide a structure compatible with any geographical pattern, F_{CT} values being much lower than in the COI analysis. Two deeper COI structurings of Eastern populations received significant F_{CT} values: ($K = 2$) Turkish versus all other populations; and ($K = 3$) Turkish

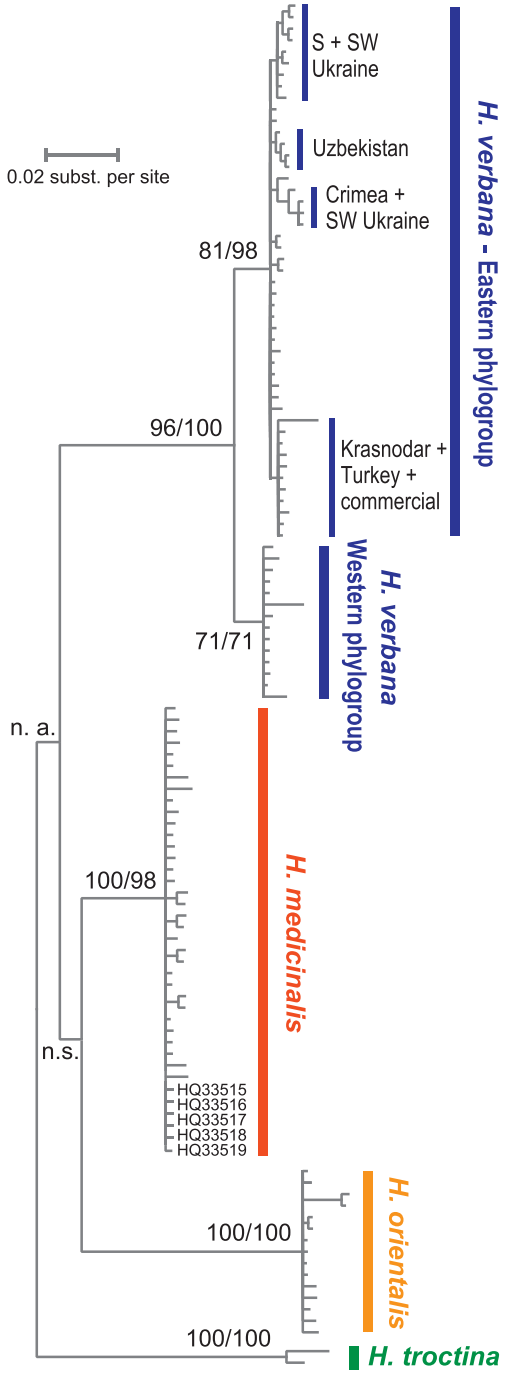

Fig. 4. Phylogenetic relationships between and within Western Palearctic medicinal leech species obtained by Bayesian inference and based on combined COI, 12S rDNA and ITS sequences. Posterior probabilities and approximate Shimodaira–Hasegawa test values from a maximum likelihood analysis are shown for major clades. The tree is rooted at *Hirudo troctina*, following the result of previous phylogenetic analyses with wider taxonomic coverage (Trontelj and Utevsky, 2005).

versus Severynivka (Southwestern Ukraine) plus Shaby (Southern Ukraine) versus all other populations. The western phylogroup was grouped into Slovenian plus Croatian populations versus Italian plus Lake Ohrid populations, albeit with an insignificant F_{CT}. Populations of *H. medicinalis* showed no structure at all. Based on the 12S dataset, *H. orientalis* was found to consist of two groups, a Caucasian and a Central Asian one. However, the F_{CT} value for this grouping was insignificant.

Table 2
DNA sequence diversity of medicinal leeches: number of individuals (n), number of distinct haplotypes (nh), haplotype diversity (H), nucleotide diversity (π), and their standard deviations for mtDNA and nuclear genes (aquaculture individuals are not included).

Species/phylogroup	Locus	n	nh	H	π
Western *H. verbana*	COI	17	4	0.63 ± 0.08	0.0027 ± 0.0012
	12S	6	1	0	0
	ITS	6	1	0	0
Eastern *H. verbana*	COI	51	16	0.82 ± 0.05	0.0032 ± 0.0004
	12S	50	8	0.74 ± 0.04	0.0023 ± 0.0003
	ITS	35	1	0	0
H. medicinalis	COI	21	7	0.76 ± 0.09	0.0034 ± 0.0004
	12S	47	8	0.44 ± 0.09	0.0034 ± 0.0019
	ITS	31	1	0	0
H. orientalis	COI	24	1	0	0
	12S	25	5	0.64 ± 0.07	0.0028 ± 0.0009
	ITS	20	2	0.19 ± 0.11	0.0031 ± 0.0018

3.3. Correlation between geographic and genetic distances

Correlation coefficients between geographic and genetic distances (Mantel's test) and their statistical significances are shown in Table 3. The highest statistically significant correlation was found in *H. orientalis* based on the 12S dataset and suggesting strong isolation by distance. In *H. medicinalis*, the coefficient was intermediate and statistically significant for 12S. The Eastern *H. verbana* showed a lower but still significant correlation with the 12S dataset. In Western *H. verbana*, no isolation by distance was detected. These results suggest that populations of *H. orientalis* experiences much stronger isolation by geographical distance than its congeners.

3.4. Demographic analysis

Demographic analyses were performed on *H. medicinalis* and *H. orientalis* as a whole, whereas for *H. verbana* both major phylogeographic units were analyzed separately to account for possible differences in demographic histories (Table 4). The Western phylogroup of *H. verbana* might have experienced population growth as suggested by a significantly negative Tajima's *D* based on the COI dataset. This was countered by a polymodal mismatch distribution, but both its SSD and raggedness *p*-values were insignificant and did not reject the expansion model. The 12S dataset of the western *H. verbana* contains only one haplotype and cannot be treated by means of demographic analysis. In the Eastern phylogroup of *H. verbana*, both neutrality tests and mismatch distribution conformed to the population growth model. Fu's F_S statistic was significantly negative and Ramos-Onsins and Rozas R_2 was significant, suggesting that populations experienced demographic expansion. Mismatch distribution for both COI and 12S datasets appeared to be unimodal. Insignificant SSD and low values for Harpending's raggedness index further supported a unimodal interpretation of the mismatch distribution in both datasets.

With the 12S data set of *H. medicinalis*, Tajima's *D* was significant and negative suggesting population growth. The mismatch distribution was polymodal but both SSD and raggedness significance values did allow rejecting the expansion model. In the COI dataset, mismatch distribution was unimodal with insignificant values of SSD and Harpending's raggedness that is still in agreement with demographic expansion.

In *H. orientalis*, neutrality tests for the 12S data set were statistically insignificant, corroborating the constant population size model. Moreover, the mismatch distribution was profoundly bimodal, even though SSD values and Harpending's raggedness index

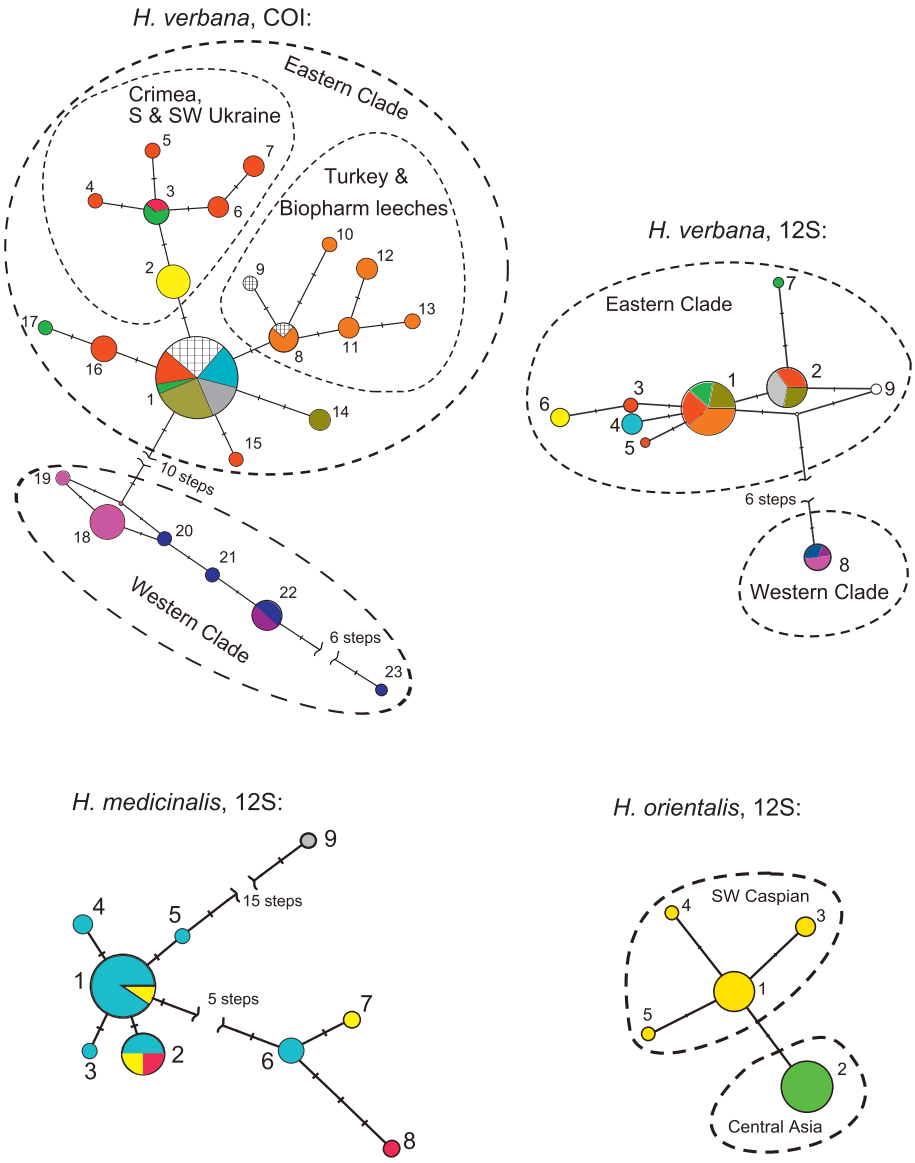

Fig. 5. Median-joining networks of mitochondrial DNA haplotypes. Haplotype numbers correspond to those in Fig. 3 and Table 1, colors to geographical areas in Fig. 3. The mesh pattern represents commercial leeches. Branch lengths are proportional to the number of substitutions, and circle sizes are proportional to the number of individuals with that haplotype.

Table 3
Correlation between genetic and geographical distances (Mantel test).

Species/phylogroup	Correlation coefficient		
	12S	COI	ITS
Western *H. verbana*	–	−0.05	–
Eastern *H. verbana*	0.18*	−0.09	–
H. verbana	0.26*	0.37**	−0.05
H. medicinalis	0.28**	−0.02	–
H. orientalis	0.79**	–	–

* $0.01 < p < 0.05$.
** $p < 0.01$.

were not significant. Altogether, these results suggest that the *H. orientalis* population size did not expand, and the lack of significance may be attributed to a very low genetic diversity found in this species.

4. Discussion

4.1. Historical differentiation

In a recent study, we have demonstrated that the geographical distribution of medicinal leech species can be explained largely by present-day ecological (climatic) parameters (Utevsky et al., 2010). Historical biogeographic patterns at the species level were very weak or non-existing. This lack was surprising, given the extensiveness and ecogeographic heterogeneity of the species ranges. Therefore, with the present study, we still expected to find a historical pattern in the distribution of DNA haplotypes. Surprisingly, a clear phylogeographic structure was supported only for *H. verbana*, and not for the other two wide-ranged medicinal leech species. The structure within *H. verbana* – a large Eastern and a large Western phylogroup – is reminiscent of the typical Western Palearctic Quaternary biogeography (Taberlet et al., 1998; Hewitt, 1999; Schmitt, 2007). Also, the deep genetic divergence and non-overlapping distributions of the two lineages suggest distinct

Table 4
Results of demographic analyses based on mitochondrial DNA sequences (aquaculture individuals are not included).

Species/phylogroup	Western *H. verbana*		Eastern *H. verbana*		*H. medicinalis*		*H. orientalis*	
	COI	12S	COI	12S	COI	12S	COI	12S
Tajima's D test								
D	−1.71	n/a	−1.42	−0.92	0.30	−2.44	n/a	−0.81
p-value	0.025		0.065	0.207	0.658	0.000		0.233
Fu's F_S test								
F_S	0.88	n/a	−8.11	−2.33	−1.38	−2.48	n/a	−0.02
p-value	0.719		0.001	0.100	0.193	0.099		0.512
R_2 test								
R_2	0.16	n/a	0.06	0.08	0.15	0.10	n/a	0.10
p-value	0.559		0.050	0.205	0.580	0.444		0.140
Mismatch analysis								
Curve shape	P	n/a	U	U	U	P	n/a	P
Harpending's raggedness index	0.17	n/a	0.02	0.09	0.19	0.09	n/a	0.12
p-value	0.208	n/a	0.968	0.167	0.060	0.817	n/a	0.336
SSD *p*-value	0.130	n/a	0.906	0.318	0.086	0.620	n/a	0.106

P (polymodal), and U (unimodal) shape of the mismatch distribution curve.
SSD, sum of square deviations between the observed and the expected mismatch.

postglacial colonization histories originating from distinct glacial refugia. However, the northward dispersal of *H. verbana* seems to have been blocked by the range of *H. medicinalis*. The latter species is probably better adapted to the environments of Northern Europe where it can outcompete its congener (Utevsky et al., 2010; Petrauskienė et al., 2011).

The obtained structure differs from the classical cases in that peninsular Italian and West Balkan populations form a homogeneous Western phylogroup of *H. verbana*. Usually, the West Balkan (Illirian) region is considered as a subcenter of the Eastern Pontic Mediterranean refugium, which includes the Balkan Peninsula, Asia Minor and the east coast of the Mediterranean, but not the Apennine Peninsula, which is a separate refugium (Schmitt, 2007). One case that resembles the *H. verbana* Western phylogroup is the Adriatic clade of the chub (*Leuciscus cephalus*) containing western Greek, Albanian and Italian populations (Durand et al., 1999). Areas where the two phylogroups of *H. verbana* got into secondary contact during post-glacial range expansion have not been revealed by our data. Possibly, the Eastern Balkan Peninsula, from which we lacked samples, might be such a suture zone. In the Eastern phylogroup of *H. verbana*, Asia Minor and the Crimean Peninsula emerged as possible sub-centers. South-western Ukraine appears to harbor the highest genetic diversity of *H. verbana*, while the boundaries of its range – North-eastern Ukraine and Uzbekistan – have been colonized by the same widespread haplotype (Fig. 5).

The other two species, *H. medicinalis* and *H. orientalis*, do not display significant historical and/or geographic structure despite the large ranges they occupy. The range of *H. medicinalis* does not contain any impermeable geographic barriers. Thus, signs of past structure could have been overridden by unimpeded gene flow. Extant *H. orientalis* populations, on the other hand, seem to be trapped within isolated habitat patches surrounded by unfavorable arid environments and high mountains. We can speculate that it was able to occupy its current range only as ecological conditions were more suitable for dispersal. In neither species do the inferred patterns point to potential refugia or re-colonization pathways.

4.2. Range expansion

Following the retreat of glaciers and the associated climatic changes after the last glacial maximum, several plant and animal species colonized previously unsuitable habitats and their populations were undergoing rapid expansion (Hewitt, 1996). Such rapid colonizations are expected to result in low levels of genetic diversity as each new founding population represents only a fraction of the ancestral population's genetic diversity (Nichols and Hewitt, 1994; Hewitt, 2000). Despite their large ranges, all three species of medicinal leeches have low genetic diversity, which is in agreement with their fast spreading. Other signs of population growth and fast range expansions are ample and consistent especially among both *H. verbana* phylogroups and *H. medicinalis*. Neutrality tests and mismatch distributions clearly support or at least do not contradict the expansion scenario. A low or even negative correlation between genetic and geographic distances, except in *H. orientalis*, is indicative of high gene flow, which is in agreement with rapid range expansion. Furthermore, a star-like pattern of rarer haplotypes radiating from the common haplotype in the network (e.g., Fig. 5) usually suggests a fast range expansion from a relatively small founding population (Grill et al., 2009).

Isolation by distance is strong in *H. orientalis*. The wetland habitats of this species are ecologically isolated (see above). Neutrality tests and mismatch distribution analyses suggest that the population size has not changed. The lack of phylogenetic and geographic structure and low genetic diversity may still be the consequence of a rapid postglacial colonization of Central Asia and Transcaucasia, followed by trapping of the populations within isolated habitats scattered about today's arid areas.

The question arises, how did these animals disperse so fast and efficiently. They cannot move over land for long distances from pool to pool, and their distribution does not seem to be bound to major basins, like of many other freshwater fauna. They have no resistant eggs or other stadia that would facilitate passive dispersal, yet they seem to be able to bridge long distances of dry, deadly landscape. The answer probably lies in their ectoparasitic feeding behavior. When sucking blood on mammals, they can remain attached for some time, allowing the host to transfer the leech to another suitable habitat patch (Utevsky et al., 2010). Such vectors were in the past most likely large social herbivores, complemented and replaced nowadays by grazing cattle, sheep, and horses.

4.3. Dispersal by humans

Medical use and trade might have played an important role in the dispersal of medicinal leeches. Leeches from the wild used to be, and still are, subject to extensive international trade. Turkey and the Krasnodar Territory of the Russian Federation are the

major regions of contemporary harvesting (Mihailov and Yaroshenko, 2006; Sağlam, 2011). Surprisingly, this century-long practice has not affected species ranges deeply. Nevertheless, the intraspecific genetic structure might be changed due to releasing used leeches. The strongest phylogenetic and geographic structure was found in *H. verbana*. Therefore, alterations of the structure would be most readily detected in this species. However, the phylogeographic groupings of *H. verbana* correlate with major biogeographic limits. Leeches of the Eastern phylogroup have not invaded the range of the Western phylogroup and vice versa in spite of the fact that Eastern leeches have been transported westward. This lack of human induced admixture can only be explained by the inability of the introduced leeches to survive and reproduce. Leeches that have been bred for several generations in aquaculture were reported not to survive in natural conditions (Mihailov and Yaroshenko, 2006). Therefore the observed phylogeographic structure probably reflects the natural distribution of *H. verbana*. The lack of phylogenetic and geographic structure in *H. medicinalis* and *H. orientalis* does not allow any conclusions about the contribution of human activity to their fast dispersal. More sensitive genetic markers, for example microsatellites could help elucidate this question in future.

4.4. Origin of commercial medicinal leeches

Most of commercial and aquaculture specimens belong to the Eastern phylogroup of *H. verbana*, suggesting that commercial facilities obtain leeches from Russia and Turkey, the major leech exporters. The separate clade of exclusively commercial leeches found by Siddall et al. (2007) can be explained by their Eastern origin in combination with the lack of wild leeches from the Eastern phylogroup in that study. According to the results of the network analysis, animals supplied by Biopharm Leeches (UK) originate from Turkey, whereas all other aquaculture leeches come from Eastern Europe, most probably from the Krasnodar Territory, Russian Federation.

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