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A new species of *Hirudo* (Annelida: Hirudinidae): historical biogeography of Eurasian medicinal leeches

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Abstract

Background: Species of *Hirudo* are used extensively for medicinal purposes, but are currently listed as endangered due to population declines from economic utilization and environmental pollution. In total, five species of *Hirudo* are currently described throughout Eurasia, with Turkey being one of the major exporters of medicinal leech, primarily *H. verbana*.

Results: To define the distribution of *Hirudo* spp. within Turkey, we collected 18 individuals from six populations throughout the country. Morphological characters were scored after dorsal and ventral dissections, and Maximum Likelihood (ML) and Bayesian Inference (BI) analyses resolved phylogenetic relationships using mitochondrial cytochrome *c* oxidase subunit I (COI), 12S ribosomal RNA (rRNA), and nuclear 18S rRNA gene fragments. Our results identify a new species of medicinal leech, *Hirudo sulukii* n. sp. in Kara Lake of Adiyaman, Sülüklü Lake of Gaziantep and Segirkan wetland of Batman in Turkey. Phylogenetic divergence (e.g., 10–14 % at COI), its relatively small size, unique dorsal and ventral pigmentation patterns, and internal anatomy (e.g., small and pointed atrium, medium-sized epididymis, relatively long tubular and arc formed vagina) distinguish *H. sulukii* n. sp. from previously described *Hirudo* sp.

Conclusions: By ML and BI analyses, *H. sulukii* n. sp. forms a basal evolutionary branch of Eurasian medicinal leeches. Phylogeographic interpretations of the genus identify a European *Hirudo* “explosion” during the upper Miocene followed by geological events (e.g., Zanclean flood, mountain building) that likely contributed to range restrictions and regional speciation of extant members of the clade.

Background

Hirudinid leeches are parasitic to a variety of vertebrates leading many to regard them with distaste, but their medicinal utility is well established. For centuries, *Hirudo medicinalis* and related species (e.g., *H. verbana*, *H. troctina*) were prescribed to treat virtually every human ailment from arthritis to yellow fever, most without efficacy. In 1830, during their peak usage, a Paris hospital employed more than five million medicinal leeches [30]. Consequently, populations of *H. medicinalis* in Central Europe were depleted, and non-sustainable collecting led to their extinction in many areas. Pollution and habitat drainage further

added to their decline, forcing Europe to import medicinal leeches from the Ottoman Empire (Anatolia), North Africa and Russia [31] to meet demand. By the late 1900's, the advent of “modern” medicine drastically reduced clinical demand for leeches, allowing some threatened populations to rebound.

Leech therapy languished for most of the 20th century, considered “quackery” by mainstream medical practitioners [66], but the discovery of various bioactive compounds in leech saliva [27, 39], and recognition of the leech's superior ability to relieve venous congestion (e.g., [58]), has led to renewed interest in clinical applications. Current fields of employment include reconstructive microsurgery, hypertension, and gangrene treatment [24]. In light of 19th century threats to medicinal leech populations as demand increased, considerable conservation steps were

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implemented to ensure their continued availability. Pursuant to these efforts, much confusion resulted regarding the taxonomic status of different morphological forms [18, 28, 56, 65]. Phylogenetic analysis of nuclear and mitochondrial DNA sequences suggest that the genus *Hirudo* is monophyletic [60], and that species or morphological varieties can be readily identified by coloration patterns. Molecular studies have shown that European medicinal leeches, although usually marketed as *H. medicinalis*, comprise a complex of at least three species: *H. orientalis*, the commonly sold *H. verbana* and the relatively rare *H. medicinalis* [4, 37, 54, 55, 60]. Kutschera and Elliott [36] analyzed the behavior of adult *H. medicinalis*, but could not find differences with respect to its sister taxon *H. verbana*. Morphological and molecular data demonstrate that commercially available medicinal leeches are generally not *H. medicinalis* [35, 56, 60], but rather specimens belonging to the Eastern phylogroup *H. verbana* [61, 62], which is predominantly bred in leech farms and used as a modern 'medicinal' stock.

Turkey is rich in wetlands and known to support at least two species of medicinal leech, *H. medicinalis* and *H. verbana*. Prior to ~2000, it was believed that medicinal leeches from Turkey's wetlands were only *H. medicinalis* [21, 31]. Molecular characterization of Turkish leeches was not performed until the turn of the century, however, and leeches from the Kızılırmak and Yesilirmak Deltas on the Black Sea coast, comprising the majority of leech specimens destined for export, have proven to be to *H. verbana* [4, 51, 55].

Mapped localities of all *Hirudo* species show extensive, belt-shaped ranges extending from east to west. To establish the distribution of *Hirudo* species in Turkey, one of the major exporters of medicinal leeches worldwide, we sampled broadly in three representative localities within the western, eastern and southeastern regions of Turkey. Our data identifies a new species for the genus, *H. sulukii* n. sp., that forms a basal evolutionary branch among European medicinal leeches and sheds light on the evolutionary history of the genus.

Methods

Specimen collection and maintenance

Leech specimens collected throughout Turkey (Kara Lake, Beyaz Cesme Marsh, Uluabat Lake, Segirkan wetland, Balik Lake, Sülüklü Lake) were transported to Firat University, Fisheries Faculty (Elazig, Turkey) and maintained in separate 600 L fiberglass tanks based on collection location. Tank bottoms were elevated with peat soil ~10 cm on one side to create a terrestrial to aquatic continuum. Leeches were fed one adult frog (e.g., *Pelophylax ridibunda*) blood meal per month (others have utilized mammalian blood), and typically survived 2+ years in the laboratory. Specimens were fixed in 70 % ethanol for molecular analysis and some were fixed with 10 % formaldehyde in PBS for dissection. External traits of live specimens were observed by stereomicroscopy. Preserved specimens were dissected dorsally and ventrally, with representative sketches of internal morphology derived directly from the type specimen.

DNA extraction

Tissue samples from live specimens were obtained by placing the leech in a 10 % ethanol sedating solution until it was unresponsive to touch. Approximately half of the caudal sucker was removed with a scalpel, and tissue cuttings were immediately processed using the E.Z.N.A.™ Tissue DNA kit (Omega Bio-Tek) following the manufacturer's instructions. Whenever possible, tissue from postmortem specimens was taken from the caudal sucker to avoid contamination from gut contents.

DNA sequence amplification of target genes

Nuclear 18S rRNA, mitochondrial 12S rRNA and partial cytochrome *c* oxidase subunit 1 (COI) DNA fragments were amplified from genomic DNA using the polymerase chain reaction (PCR). All 12S sequences were obtained under conditions described by Borda and Siddall [8]. PCR amplification protocols were conducted as described by Wirchansky and Shain [67] employing primers listed in Table 1. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega, Inc.) according to the manufacturer's protocol.

Table 1 Primers used for PCR amplification and DNA sequencing

Gene	Primer name	Primer sequence	Reference
18S rDNA	C	5'-CGGTAATTCAGTCCCAATAG-3'	Apakupakul et al. (1999) [4]
	Y	5'-CAGACAAATCGCTCCACCAAC-3'	Apakupakul et al. (1999) [4]
12S rDNA	12S-A	5'-AAACTAGGATTAGATACCCTATTAT-3'	Palumbi, 1996 [44]
	12S-B	5'-AAGAGCGACGGGCGATGTGT-3'	Simon et al. [57]
COI	LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	Folmer et al. [20]
	HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Folmer et al. [20]

DNA sequencing and editing

Purified PCR products were shipped to GeneWiz, Inc. (South Plainfield, NJ) for Sanger DNA sequencing using an ABI 3730xl DNA analyzer. Each PCR product was sequenced in both directions using amplification primers, and sequence chromatograms were viewed and manually adjusted in ChromasPro (Technelysium, Queensland, Australia) or BioEdit [26]. Sequence alignments were made with MUSCLE [17] or CLUSTAL W [29, 38]. Accession numbers for all COI, 12S and 18S sequences are listed in Suppl. Data (Table 1).

Phylogeny

Maximum-likelihood (ML) analyses were performed for all DNA comparisons, using the pipeline sequence MEGA 7 [34] to align corresponding sequences from multiple individuals or homologous DNA across species, Gblocks [9] for alignment curation, PhyML [25] for tree building and TreeDyn [11] for tree drawing, as configured in the Phylogeny.fr platform [14]. The aLRT statistical test (approximation of the standard Likelihood Ratio Test; [3]) embedded in PhyML determined branch support values. Default settings were used for all parameters.

Bayesian Inference (BI) analysis was performed on the combined data set (morphological parameters, 18S, 12S, COI in Nexus format) in MrBayes v. 3.2.1x64 [48, 49]. Data were partitioned for 18S and 12S, and by codon position for COI. ModelTest [47] via FindModel was

used to determine the optimal model of evolution for each gene under the Akaike Information Criterion (AIC; [46]). The general time reversible (GTR) model with a gamma distributed rate parameter was used for COI, 12S and 18S. Two analyses were run simultaneously with all parameter sets unlinked by partition for two million generations each, sampling every 100 generations, with a burn-in achieved by <50,000 generations. Setting the burn-in to 500,000 generations left a total of 7413 trees sampled for assessment of posterior probabilities. Gaps were treated as missing data, and default settings were used for all other parameters.

Results

Specimens of *Hirudo* were collected from multiple locations in Turkey (Fig. 1; Tables 2 and 3). These localities are separated by 1312 km (Uluabat Lake to Kara Lake), 1306 km (Uluabat Lake to Beyaz Cesme Marsh) and 289 km (Kara Lake to Beyaz Cesme Marsh). Leeches were typically found in muddy bottoms, as well as underwater and in aquatic/terrestrial vegetation (typically reedbeds), with banks of water proving the most prevalent habitat.

Specimens were scored for morphological characters according to Borda and Siddall [8], Utevsky and Trontelj [65], Klemm [33], Sawyer [53], Neesemann and Neubert [42], Saglam [50] and Govedich et al. [23], Elliott and Dobson [19] (Additional file 1). By these criteria, 10 leeches were identified as *H. verbana*, while

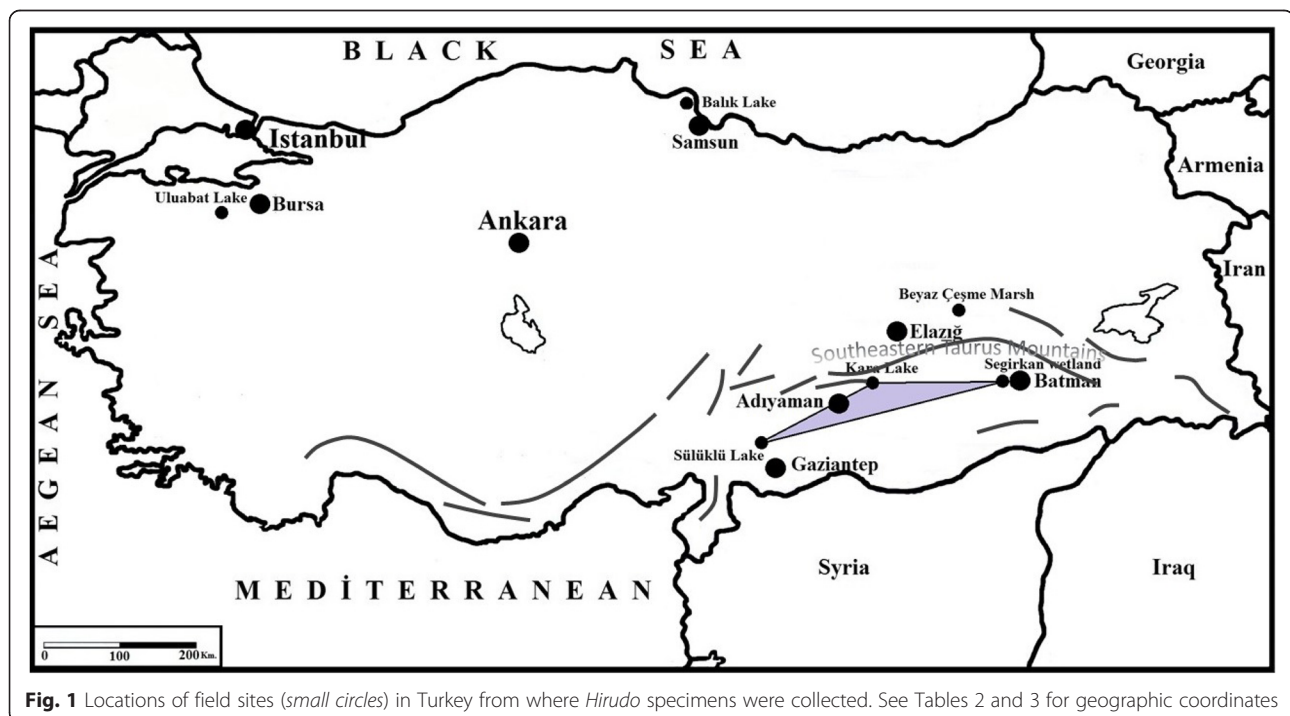


Fig. 1 Locations of field sites (small circles) in Turkey from where *Hirudo* specimens were collected. See Tables 2 and 3 for geographic coordinates

Table 2 Collection field sites in Turkey and specimen descriptions. Depositions in the Academy of Natural Sciences, Philadelphia, PA (ANSP) and Cukurova University Parasitology Museum, Adana, Turkey (CUPM)

Locality	Province	Designation	Catalogue number	Type	Coordinates	Elev.
<i>Hirudo sulukii</i> n. sp.						
Kara Lake	Adiyaman	HS1	CUPM-HIR/2016-1	Para	37°59'35"N 38°48'52"E	1233 m
		HS2	CUPM-HIR/2016-2	Para		
Sülüklü Lake	Gaziantep	HS3	CUPM-HIR/2016-3	Para	37°18'12" N 37°14'53"E	877 m
		HS4	ANSP G1 19489	Para		
		HS5	ANSP G1 19488	Holo		
Segirkan wetland	Batman	HS6	CUPM-HIR/2016-4	Para	37°51'46"N 41°01'00"E	525 m
		HS7	CUPM-HIR/2016-5	Para		

six specimens did not match characters described for any known *Hirudo* species. Specifically, external pigmentation was unique, along with internal distinctions of the epididymis and vagina (see below).

Hirudo sulukii n. sp

Based on morphological and genetic criteria, we formally propose the new species designation, *Hirudo sulukii* n. sp. (LSID: urn:lsid:zoobank.org:act:C338A26A-A205-4894-AB01-AA012293DD25), for leech specimens collected near Adiyaman, Batman and Gaziantep in southeastern Anatolia (Tables 2 and 3). The name “sulukii” is derived from the Turkish word “sülük” in reference to “leech”. Description based on holotype (specimen HS5 from Sülüklü Lake, catalogue ANSP G1 19488 in the Academy of Natural Sciences, Philadelphia, PA, USA). Paratypes deposited in the Academy of Natural Sciences (ANSP G1 19489) and Cukurova University Parasitology Museum, Adana, Turkey (CUPM-HIR/2016-1). Description: adult 64.06 ± 23.06 mm (27–105 mm) mean long, 6.71 ± 2.61 mm (4–12 mm) mean wide, mean width of anterior sucker 3.36 ± 1.10 mm (2–5.2 mm), mean width of posterior sucker 4.53 ± 1.33 mm (2–7 mm) (Fig. 2). Dorsum (Figs. 3 and 4a) pigmentation variably olive green, two orange paramedian stripes thin, two orange paramarginal stripes broad and encompassing black, segmentally-arranged united ellipsoid and elongated spots, dorsal lateral margins of body

with yellow stripes encompassing zigzagged black longitudinal; covered with numerous papillae of body surface; background pigmentation of ventral (Figs. 3 and 4b) surface light greenish and covered with small number irregular dark markings. With classic *Hirudo* arc eyespot pattern [53], containing five pairs bilateral eyespots. Eyespots, five pairs on II, III, IV a1, V a1 and VI a2, forming a parabolic arc (Fig. 5). Number of annuli per somite: I-II-III: one, IV-V: two, VI-VII: three, VIII: four, IX: five (b1, b2, a2, b5, b6). Gonopores situated in furrow between annuli, separated by five annuli, male pore in the furrow XI b5/b6, female pore in the furrow XII b5/b6. Jaws trignathous, monostichodont, papillated.

Male reproductive apparatus notably large, with thick muscular penis sheath terminating in a bulbous prostate, located at ganglion in segment XI. Epididymis medium-sized, spherical, more than twice size of pearlescent-sheened ejaculatory bulb, tightly packed masses of ducting standing upright on either side of the atrium. Testisacs ovoid and larger than ovisacs, located posterior to ganglion in segment XIII. Female reproductive system relatively coiled tubing. The pearlescent-sheened vagina long and upright, evenly bowed tube entering directly into ventral body wall. Oviducts a thin duct forming several coiled and covered with a thick layer of glandular tissue, bi-lobed ovaries. Ovisacs globular ovoid or small bean seed-shaped (Fig. 6).

Table 3 Collection field sites in Turkey and specimen descriptions. Depositions in the Academy of Natural Sciences, Philadelphia, PA (ANSP) and Cukurova University Parasitology Museum, Adana, Turkey (CUPM)

Locality	Province	Designation	Coordinates	Elev.
<i>Hirudo verbana</i>				
Beyaz Cesme Marsh	Elazig	HV	38°59'51"N 39°55'48"E	1225 m
Uluabat Lake	Bursa	HV1, HV2, HV3, HV4, HV5, HV6, HV7	40°10'23"N 28° 37'26"E	4 m
Balik Lake	Samsun	HV19, HV20a	41°34'48"N 36° 04'30"E	0 m



Remarks

Despite similarities between *Hirudo sulukii* n. sp. and other *Hirudo* species, the former can be distinguished from its closest relatives using internal and external features. *Hirudo sulukii* n. sp. differs from *H. medicinalis* and *H. orientalis* by the form of black spots on the dorsal, paramedian stripes of the body. *Hirudo sulukii* n. sp. has black, segmentally-arranged united ellipsoid and elongated spots, and dorsal lateral margins of body a pair of zigzagged black dorsolateral longitudinal stripes (Fig. 4a). The ventral coloration pattern of *H. sulukii* n. sp. has a variable number of irregular spots (Fig. 4b); *H. orientalis* has black, dorsal rounded or quadrangular spots while *H. medicinalis* has elongated spots. The marginal spots of *H. medicinalis* are fused to form distinct black stripes. The ventral of *H. medicinalis* has an irregular dark mesh-like pattern while that of *H. orientalis* is more regular, formed by segmentally-arranged pairs of light markings on a predominantly black background. *Hirudo verbana* has broad, diffuse paramedian stripes orange in color. The ventral pattern of *H. verbana* is unicolorous greenish to yellow, bounded by a pair of

black ventrolateral stripes. *Hirudo troctina* has a pair of zigzag-shaped, black ventrolateral longitudinal stripes [65]. Hechtel and Sawyer [28] considered external pigmentation to be not only the most useful, but also arguably the best character to distinguish species of *Hirudo*.

In this study we used the approach of Hechtel and Sawyer [28] and Utevsky and Trontelj [65] regarding the size of the epididymis in relation to the ejaculatory duct. The epididymes of *Hirudo sulukii* n. sp. (Fig. 6) and *H. orientalis* are medium-sized. In contrast, the epididymes of *H. verbana* are relatively small, whereas *H. troctina* and *H. medicinalis* have massive epididymes [65]. The vagina of *Hirudo sulukii* n. sp. is relatively long tubular and arc formed (Fig. 6), while in *H. orientalis* the vagina is tubular and evenly curved. The former two species do not show the central swelling and sharp folding typical for *H. verbana*. In *H. medicinalis*, the vagina can have two conditions: straight and tubular, or terminally curved [65]. *Hirudo troctina* has a bulbous vagina [28].

Moquin-Tandon [40] described at least five species of *Hirudo* including *H. verbana* and *H. medicinalis*, but later concluded that they were all varieties of the same leech species. The medicinal leech, *H. sulukii* n. sp., considered here was determined to be morphologically different than all species described by Moquin-Tandon [40, 41].

Phylogenetic analyses

To determine the relationship of specimens to other *Hirudo* species, we subjected them to the comparative analysis of COI (cytochrome c oxidase subunit 1) and 12S rRNA from mitochondria, and nuclear 18S rRNA. Combined COI, 12S and 18S rRNA analysis contained 13 terminals with 1514 aligned characters. Maximum Likelihood of the combined data set yielded five equally parsimonious trees with 500–1000 steps (Fig. 7; Additional file 1); concordant trees were generated independently with COI data (Fig. 8; Additional file 1). Collectively, *H. sulukii* n. sp., formed a basal branch among European medicinal leeches with strong bootstrap support, while resolution among *H. medicinalis*, *H. orientalis* and *H. verbana* lineages was ambiguous, as noted in previous studies [45, 56]. Population structure was shallow among the collected specimens (<2 % divergence at COI; Table 4), suggesting recent invasions into field sites sampled in the current study (see Fig. 1). The Asian species, *H. nipponia*, fell outside the *Hirudo* clade in combined sequence analyses (Fig. 7), suggesting a deep ancestral split with European species, and calling into question the designation of *H. nipponia* within the *Hirudo* phylogroup. Interestingly, *H. nipponia* was equidistant to European *Hirudo* species (~22–25 % at COI), the latter of which were approximately equidistant to each other (i.e., ~10–14 % at COI; Table 4). Inferring a divergence rate of ~2 % per million years at the COI

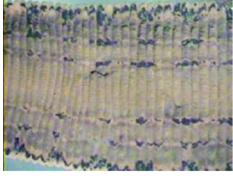
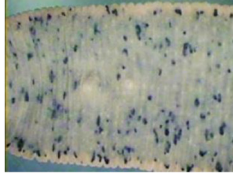

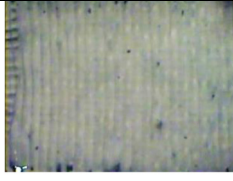

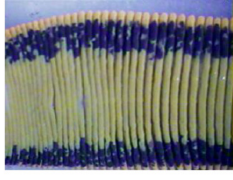
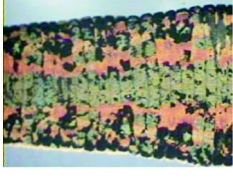


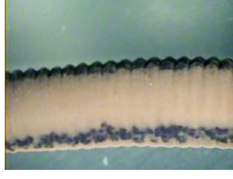
<i>Hirudo</i> specimens	Dorsal view	Ventral view
HS1		
HS2		
HV		
HV1		
HV4		

Fig. 3 Pigmentation patterns of representative *Hirudo* specimens. HS1, 2 – *H. sulukii* n. sp.; HV, HV1, 4 – *H. verbana*. See Tables 2 and 3 for specimen descriptions

locus based on combined geological and molecular data within Oligochaeta [10, 15, 67], we estimate a lower Miocene split between lineages leading to *H. nipponia* and European *Hirudo* sp., and radiation of the latter species during the upper Miocene. Branch patterns of remaining species were consistent with those reported previously [45].

Discussion

Maximum Likelihood and Bayesian Inference analyses yielded trees with concordant topologies and strong support for *H. sulukii* as a basal branch of the European medicinal leeches. Relationships between *H. medicinalis*, *H. verbana* and *H. orientalis* were less conclusive, consistent with confusion regarding their morphological identification [45, 56]. The relatively small size of *H. sulukii*, unique dorsal and ventral pigmentation patterns,

and internal anatomy (e.g., small and pointed atrium, medium-sized epididymis, relatively long tubular and arc formed vagina) are distinguishing features of this previously undescribed leech. Note that *H. sulukii* has thus far been collected only from relatively high elevation field sites (i.e., Kara Lake-Adiyaman 1233 m, Sülüklü Lake-Gaziantep 877 m, and Segirkan wetland- Batman 525 m), and its small size in comparison with other *Hirudo* species may reflect an adaptation to this environment (e.g., reduced foraging season/food supply), as suggested for other annelid species (e.g., [15]).

Previously, only two medicinal leeches were thought to occur in Turkey, *H. verbana* and *H. medicinalis*, while a total of five are currently described throughout Eurasia. The range of *H. verbana* occurs to the south of *H. medicinalis* in an almost parapatric fashion with little overlap [5, 32, 42, 43, 51]. The former is subdivided into

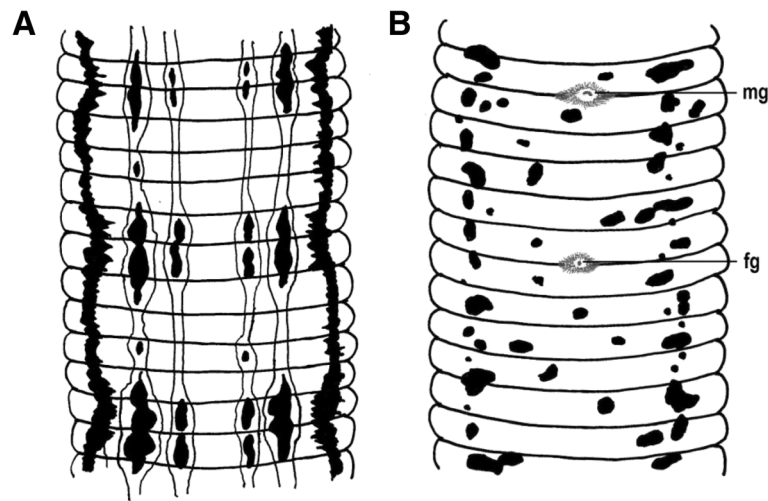


Fig. 4 *Hirudo sulukii* n. sp. Dorsal view (a) and ventral view (b); mg, male gonopore; fg, female gonopore. Based on holotype HS5 from Sülüklü Lake, Turkey (catalogue ANSP G1 19488)

an Eastern (southern Ukraine, North Caucasus, Turkey and Uzbekistan) and Western phylogroup (Balkans and Italy) that do not overlap, suggesting distinct postglacial colonization from separate refugia [61, 64]. Eastern-most records are from Samarqand Province in Uzbekistan [61, 64, 65], resulting in an east-to-west extent of ~4600 km. Leeches supplied by commercial facilities belong to the Eastern phylogroup, originating mostly from Turkey and the Krasnodar Territory in Russia, two leading areas of leech export.

Hirudo medicinalis is distributed from Britain and southern Norway to the southern Urals and probably as far as the Altai Mountains, occupying the deciduous arboreal zone [6, 12, 16, 21, 22, 31, 43, 51, 52, 59, 63, 68]. *Hirudo orientalis* is associated with mountainous areas in the sub-boreal eremial zone and occurs in Transcaucasian countries, Iran and Central Asia, while *H. troctina* has been found in northwestern Africa and Spain in the Mediterranean zone [64]. *Hirudo verbana* and *H. medicinalis* have recently experienced range expansions while *H. orientalis* has remained geographically isolated within arid and alpine areas of Central Asia and Transcaucasia [61].

By molecular clock inference using divergence estimates at the CO1 locus [10, 15, 67], our data suggest a deep, ancestral split between European and Asian (i.e., the lineage leading to *H. nipponia*) medicinal leeches somewhere in the lower Miocene, followed by an “explosion” of *Hirudo* species upon their putative arrival to the European continent during the upper Miocene, 5–10 mya (Fig. 9). The possible misclassification of *H. nipponia* does not affect this evolutionary scenario since it represents a basal, sister branch to the European *Hirudo* phylogroup (see Fig. 8). This evolutionary timeline is supported by tree topologies and relative genetic distance of European *Hirudo* species to each other at the CO1 locus (i.e., 10–14 % divergence; see Table 4). The time frame of these events suggest the presence of an open habitat corresponding with, for example, formation of Levantine land bridges, which may have facilitated mammalian-based, passive dispersal of an ancestral *Hirudo* archetype throughout Europe. Thereafter, tectonic activity at the onset of the Pliocene ~5.3 mya broke the

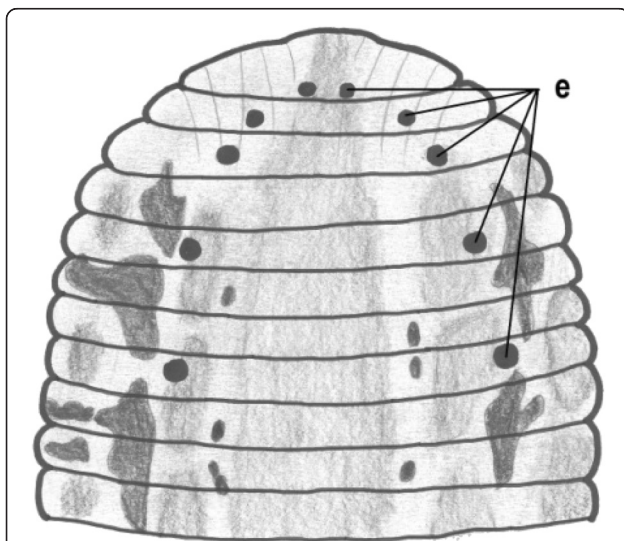


Fig. 5 View of anterior-dorsal and eyes of *Hirudo sulukii* n. sp. e, eyes. Based on holotype HS5 from Sülüklü Lake, Turkey (catalogue ANSP G1 19488)

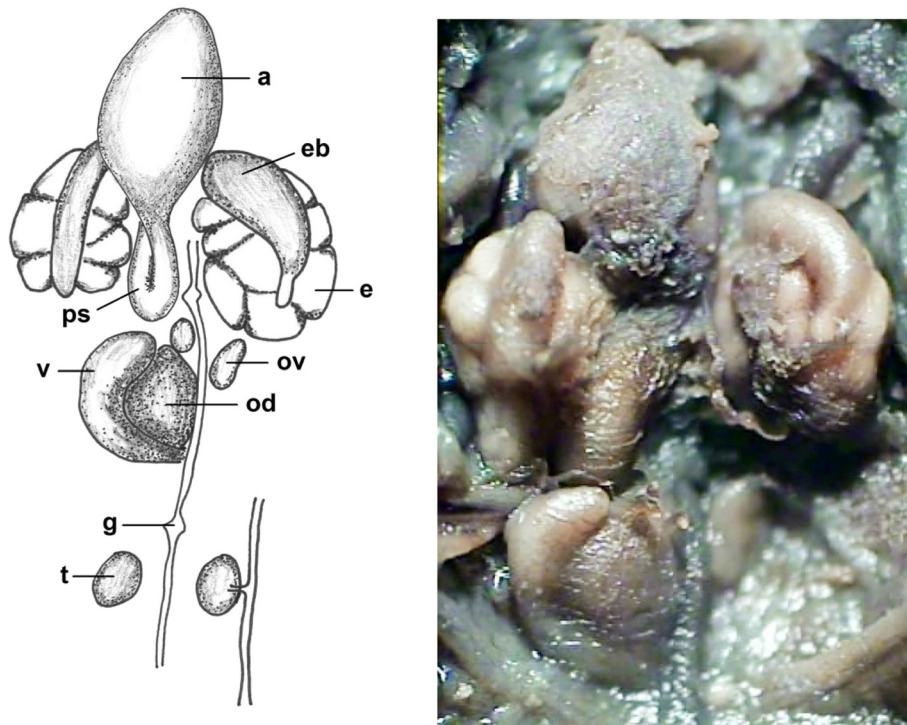


Fig. 6 *Hirudo sulukii* n. sp. Dorsal view of reproductive system. a, Atrium; e, epididymis; eb, ejaculatory bulb; g, ganglion; o, ovisac; od, oviduct; ps, penis sheath; t, testisac, v, vagina. Based on holotype HS5 from Sülüklü Lake, Turkey (catalogue ANSP G1 19488)

land bridge between Morocco and Spain causing the Zanclean Flood that filled the Mediterranean basin, and in combination with mountain building throughout the European continent [7], appears to have restricted panmixia among extant *Hirudo* lineages, leading in part to

their speciation and current geographic ranges. For instance, concurrent with the closing of the Tethys Sea by continental drift of the African and Arabian plates, mountain building events occurred in Southern Turkey forming the Taurus Mountain chain [13]. At present, the

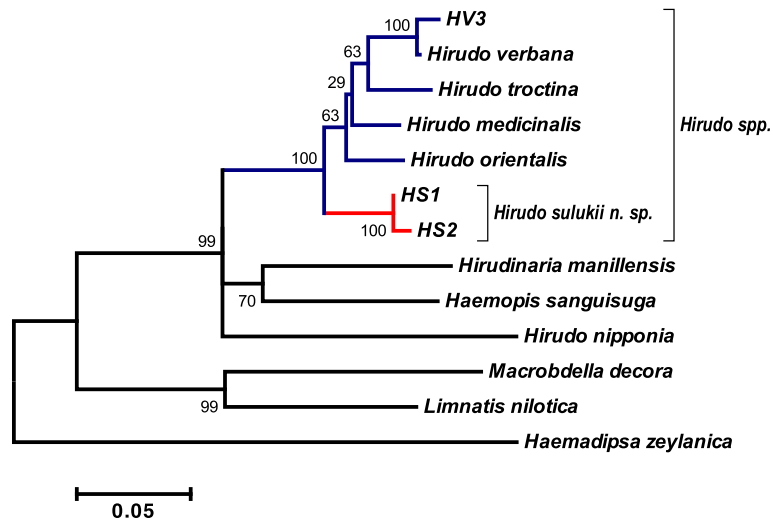


Fig. 7 Maximum Likelihood analysis of a combined COI, 12S and 18S data set (1514 total positions). Bootstrap values are indicated. European *Hirudo* species form a distinct clade with *H. sulukii* n. sp. as a basal member

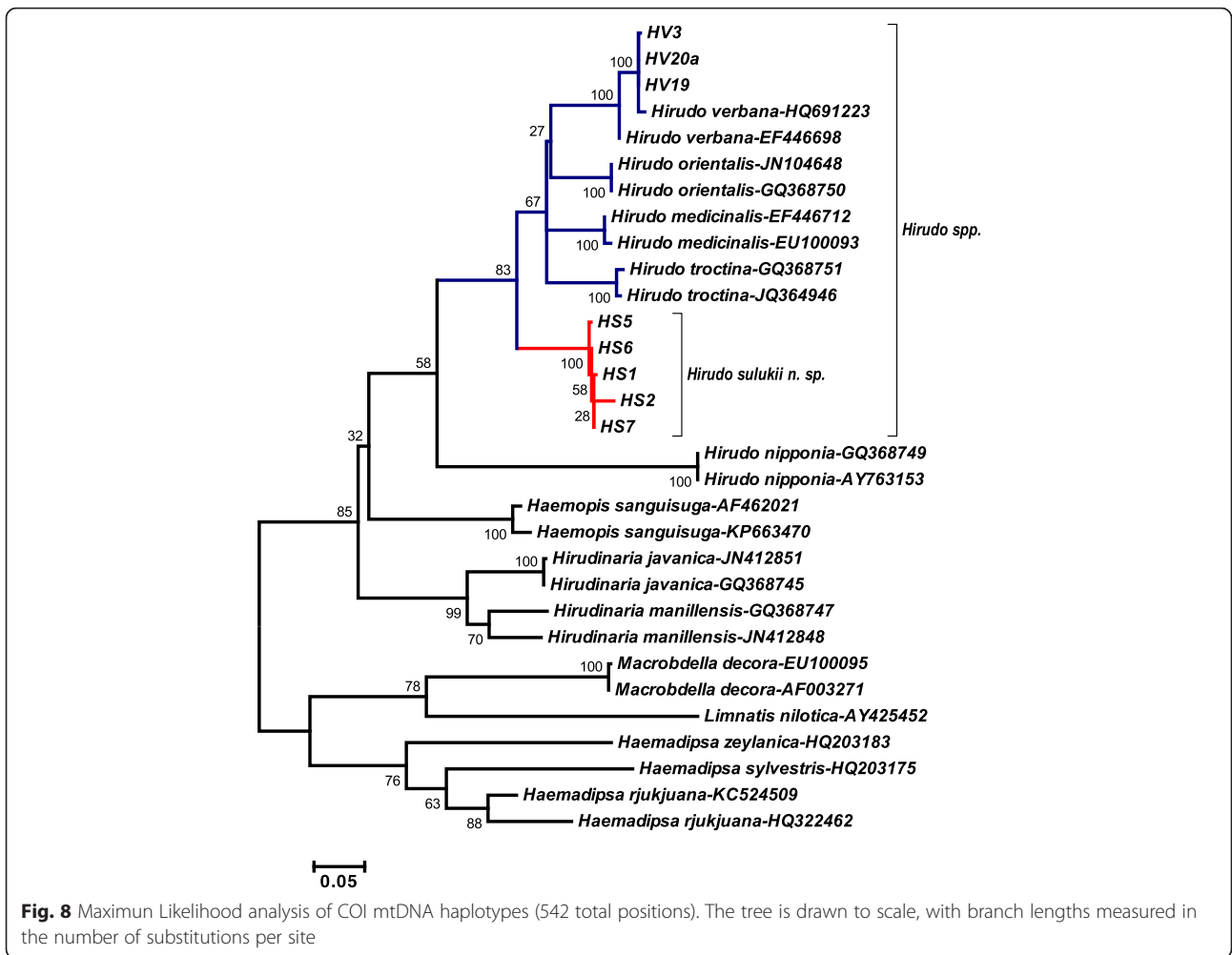
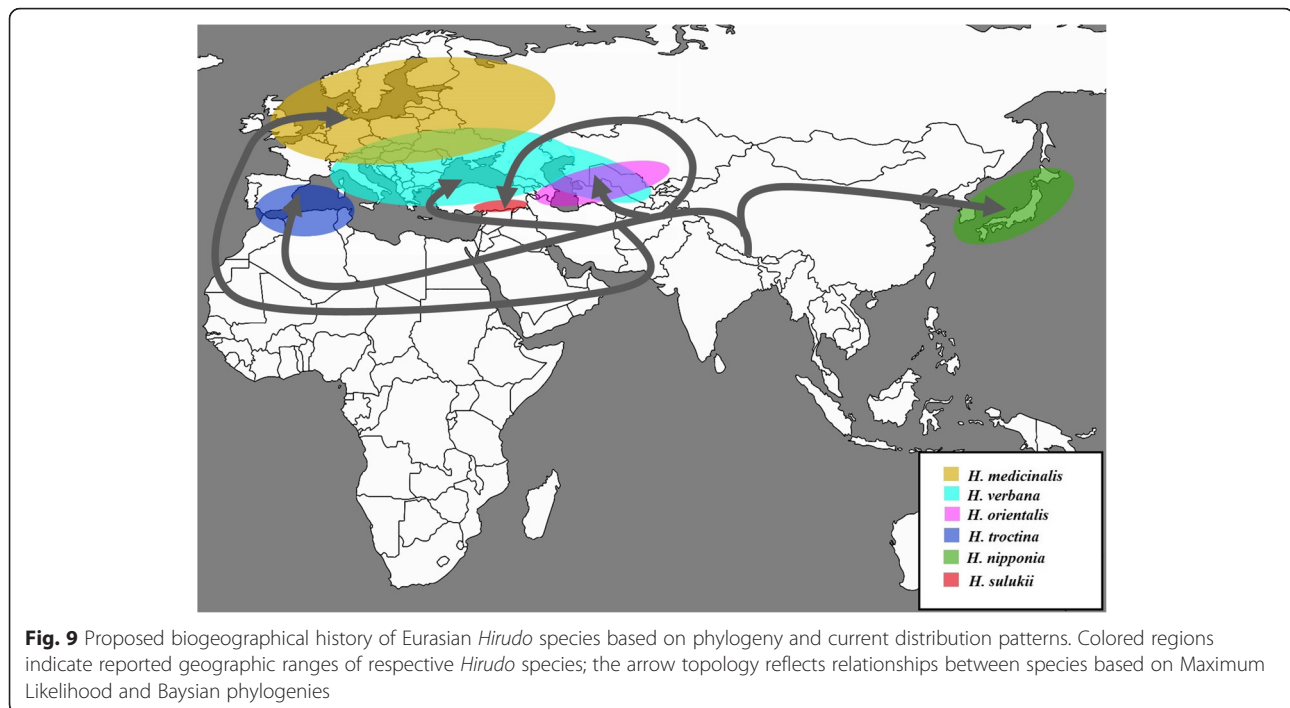


Fig. 8 Maximun Likelihood analysis of COI mtDNA haplotypes (542 total positions). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site

Table 4 Pairwise distance matrix of *Hirudo* specimens

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 HS1	-												
2 HS2	0.0145	-											
3 HS5	0.0036	0.0182	-										
4 HS6	0.0036	0.0182	0.0036	-									
5 HS7	0.0018	0.0163	0.0054	0.0018	-								
6 HV3	0.1238	0.1373	0.1240	0.1259	0.1237	-							
7 HV19	0.1238	0.1373	0.1240	0.1259	0.1237	0.0018	-						
8 HV20a	0.1238	0.1373	0.1240	0.1259	0.1237	0.0018	0.0000	-					
9 <i>H. verbana</i> -HQ691223	0.1237	0.1372	0.1238	0.1258	0.1235	0.0072	0.0054	0.0054	-				
10 <i>H. medicinalis</i> -EU100093	0.1053	0.1142	0.1054	0.1053	0.1031	0.1059	0.1059	0.1059	0.1101	-			
11 <i>H. orientalis</i> -JN104648	0.1153	0.1285	0.1154	0.1153	0.1130	0.0979	0.0979	0.0979	0.1001	0.0875	-		
12 <i>H. troctina</i> -JQ364946	0.1220	0.1330	0.1221	0.1220	0.1197	0.1097	0.1097	0.1097	0.1139	0.0957	0.0918	-	
13 <i>H. nipponia</i> -AY763153	0.2366	0.2495	0.2363	0.2366	0.2394	0.2342	0.2341	0.2341	0.2393	0.2273	0.2513	0.2202	-

Numbers are divergence values within a 560 bp fragment of the cytochrome c oxidase subunit I (COI) locus



Anatolia region has been isolated completely by the southeastern Taurus Mountains [1], likely isolating *H. sulukii* from other European *Hirudo* populations.

Species of *Hirudo* have had broad applications in medicine, ranging from reconstructive surgeries (e.g., facial, finger reattachment, ear flap) to anticoagulants/analgesics secreted from salivary glands [2, 24]. Thus the discovery of a new *Hirudo* species, particularly a basal member of this phylogroup, has considerable value in the context of medical potential. Specifically, natural variants of known bioactive factors (e.g., hirudin, antistasin, etc.) are logical candidates to explore for their potentially enhanced or novel pharmaceutical properties. The current study has prompted a more systematic survey of *Hirudo* throughout Turkey and surrounding regions with the collective aims of refining the evolutionary history of the genus, facilitating conservation efforts, and identifying species that may expand the repertoire of medicinal applications for this important Hirudinid genus.

Conclusions

By phylogenetic and morphological criteria, specimens collected from Kara Lake of Adiyaman, Sülüklü Lake of Gaziantep and Segirkan wetland of Batman in Turkey comprise a new species, *Hirudo sulukii*. Geographic isolation by the Taurus Mountain chain has likely contained *H. sulukii* within the regional sampling area. By ML and BI analyses, *H. sulukii* n. sp. forms a basal evolutionary branch of Eurasian medicinal leeches, preceded by a

deeper ancestral split with the Asian medicinal leech. *H. nipponia*. Phylogeographic interpretations of the genus identify a European *Hirudo* “explosion” during the upper Miocene followed by geological events (e.g., Zanclean flood, mountain building) that likely contributed to range restrictions and regional speciation of extant members of the European clade.

Additional file

Additional file 1: Supplementary Data Hirudo. (DOCX 311 kb)

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Availability of data and material

Holotype and paratype *Hirudo sulukii* specimens deposited in the Academy of Natural Sciences, Philadelphia, PA, USA (catalogue ANSP G1 19488, 19489). Additional paratypes deposited in Cukurova University Parasitology Museum, Adana, Turkey (catalogue CUPM-HIR/2016-1). All DNA sequences deposited into GenBank (Additional file 1: Table S1).

Authors' contributions

NS collected leech specimens, conducted experimental analyses including specimen dissections, and contributed to writing the manuscript; RS conducted DNA analyses and contributed to writing; SAL assisted with DNA analyses, phylogenies and writing; DHS oversaw experimental analyses and writing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Ethical approval is not required at Rutgers University for research conducted on invertebrates such as medicinal leeches used in our study.

Endnotes

Not applicable

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