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Taxonomic status and distribution of medicinal leeches of the genus *Hirudo* L. (Hirudinea) in Bulgaria

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Abstract: The medicinal leeches of the genus *Hirudo* are among the invertebrates with the best studied biology and physiology, because of their wide application in medicine. However, till the end of the 20th century, there was confusion regarding the taxonomic status of the different morphological forms of these leeches. The prevailing view was that all varieties of the medicinal leeches in Europe represent the same species, i.e. *H. medicinalis.* Recently, the application of molecular methods in studies of the medicinal leeches has led to significant changes in taxonomy of the genus *Hirudo* and proved the existence of four Western Palaearctic species with clearly delineated and non-overlapping ranges. In order to clarify the taxonomic status of the medicinal leeches in Bulgaria, 56 potential sites were monitored during the period 2005-2014, and the recorded specimens were studied, using morphological and karyological methods. The most important external morphological traits of 226 individuals from 16 populations across the country were analysed. Based on the colouration and patterns of dorsum and venter, the medicinal leeches from all populations studied were identified as *H. verbana*. The haploid chromosome number (n=13) confirmed belonging of the studied medicinal leeches to *H. verbana*. Our results may be used as a basis for further monitoring of the medicinal leech populations across the country and assessment of their conservation status in Bulgaria.

Keywords: Medicinal leech, Hirudinea, *Hirudo medicinalis*, *Hirudo verbana*, distribution, population density

Introduction

The medicinal leeches have been known to humans in Egypt, Rome and Greece since ancient times mainly because of their historical use in medicinal phlebotomy (blood-letting) (Elliott, Kutschera 2011). The medicinal leeches have been used widely for draining haematomas from wounds, for removing postoperative occlusions to enhance the success of tissue transplants and the surgical joining of amputated appendages, such as fingers and ears, as well as for stimulating blood flow into the wound and the capillary growth. The leech saliva is a significant source of important bioactive compounds that include anticoagulants, antistatins and other proteolytic inhibitors as hirudin, histamine, hyaluronidase, bdellin, and eglin (Tuszinsky *et al.* 1987, Wells, Coombes 1987, Henriot *et al.* 1990, Sollner *et al.* 1994, Baskova, Zavalova 2001, Salzet 2002, Siddall *et al.* 2007, Elliott, Kutschera 2011). The medicinal leeches have also been used as a model organism in neurobiology (*e.g.* Nicholls, van Essen 1974, Muller *et al.* 1981, Leake 1983, Elliott, Kutschera 2011). The widespread application of the leeches in medicine is probably the main reason for their being among the invertebrates with the best studied biology and physiology (Davies, McLoughlin 1996). The same is not valid with regard to their taxonomy, which has been

substantially revised in recent years through some modern molecular and cytogenetic methods.

Hirudo medicinalis Linnaeus, 1758 was considered as the only European medicinal leech for a long time, in spite of the fact that some other species were described meanwhile. In the 19th and 20th centuries, most researchers accepted the view of Moquin-Tandon (1846) that all varieties of medicinal leeches in Europe represent the same species, namely *H. medicinalis*. One of the neglected species was *H. verbana* Carena, 1820, first described by Carena (1820) from Lago Maggiore (*Lacus Verbanus* in Latin) in northeast Italy, and later regarded as a subspecies of the European medicinal leech (*H. medicinalis* ssp. *officinalis*) (Elliot, Kutschera 2011). In the period from its description till the end of the 20th century, *H. verbana* was "forgotten" by researchers. However, Nesemann, Neubert (1999) re-established the species status of *H. verbana* and separated the European medicinal leeches into the north-western *H. medicinalis* and the south-eastern *H. verbana*. In Bulgaria, as in many other European countries, the findings of medicinal leeches referred mainly to *H. medicinalis*.

In the last decade, the application of some molecular methods in studies of the medicinal leeches has led to significant changes in the taxonomy of the genus *Hirudo* Linnaeus, 1758 and proved the existence of four Western Palaearctic species with clearly delineated and non-overlapping ranges (Trontelj *et al.* 2004, Utevsky, Trontelj 2005, Trontelj, Utevsky 2005, Utevsky *et al.* 2009, 2010, Trontelj, Utevsky 2012, Kutschera, Elliott 2014). In accordance with the above finding, Kutschera (2012) noted that these four, well-defined taxa, must be interpreted as a complex of closely related species (the *Hirudo medicinalis* species complex). This species complex includes four taxa: the European medicinal leech (m. l.) *H. medicinalis*, the Mediterranean m. l. *H. verbana*, the Caucasian m. l. *H. orientalis* Utevsky et Trontelj, 2005, and the North African m. l. *H. troctina* Johnson, 1816. The European m. l. *H. medicinalis* is the northernmost species, occupying a vast area from France, Britain and Norway to the Urals. The southern border of its range is far from Bulgaria and passes through France, northern Italy, Hungary, Ukraine and southern Russia. The Mediterranean m. l. *H. verbana* is the second most distributed species in the Western Palaearctic. This is the only species of the genus *Hirudo* subdivided into an Eastern phylogroup (Eastern Europe, Turkey and Central Asia) and a Western phylogroup (Western Balkans and Italy). The border between these phylogroups passes through the Balkan Peninsula (Serbia), and the distribution of the Eastern phylogroup agrees with the Danubian drainage basin, and this one of the Western phylogroup roughly coincides with the Adriatic and Aegean drainage basins (Trontelj, Utevsky 2012, Utevsky, Trontelj 2016). Bulgaria falls entirely within the range of the Eastern phylogroup of *H. verbana*. The third of the Western Palearctic species is the Caucasian m. l. *H. orientalis*, whose habitats are patchy and scattered in the mountainous areas of Central Asia and Transcaucasia. The fourth species is the African m. l. *H. troctina*, which inhabits arid landscapes and has a limited distribution on the Iberian Peninsula and North Africa. The existence of the four species of the genus *Hirudo* has also been proved by differences in their colouration and the biochemical composition of their saliva, as well as by different chromosome numbers (Utevsky, Trontelj 2005, Trontelj, Utevsky 2005, Siddall *et al.* 2007, Baskova *et al.* 2008, Utevsky *et al.* 2009, Elliott, Kutschera 2011, Trontelj, Utevsky 2012, Kutschera, Elliott 2014). Cytogenetic study of three *Hirudo* species from different populations in Azerbaijan, Kazakhstan, Russian Federation, Ukraine and Uzbekistan shows that these species can be differentiated by their haploid number of chromosomes: *H. medicinalis* with n=14, *H. verbana* with n=13 and *H. orientalis* with n=12 (Kovalenko *et al.* 2007, Utevsky *et al.* 2009), thus the validity of the taxon can be corroborated by the chromosome number.

Considering the molecular taxonomy and accepted boundaries of the ranges of the above-mentioned four species, it may be expected that *H. verbana* occurs in Bulgaria. This suggestion was also supported by Jueg (2010), who, on revising the existing collection of leeches at the Museum of Natural History in Berlin, reported the species *Hirudo verbana* Carena, 1820 (determined by Augener, 1925, as *H. medicinalis*), for the first time in Bulgaria. Jueg (2010) assumed that all populations of the medicinal leeches in Bulgaria belong to this species. Recently, Trichkova *et al.* (2013) reported the finding of *H. verbana* in the Asparuhov Val Reservoir (Danube River basin). In all other publications related to the Bulgarian medicinal leeches only *H. medicinalis* has been mentioned (Hristovich 1892, Augener 1925, Valkanov 1926, 1934, 1936, 1957, Arndt 1943, Angelov 1956, Russev 1959, Russev, Marinov 1964, Russev, Kaneva-Abadjieva 1973, Russev, Janeva 1975, 1976, Uzunov *et al.* 1981, 2001, 2011, Kovachev, Uzunov 1986, Janeva 1989, Kovachev *et al.* 1999, Uzunov, Varadinova 2001, Varadinova *et al.* 2009, 2011, 2012, 2013, Vidinova *et al.* 2011). In the context of that apparent contradiction, and as there have been no detailed studies on the taxonomy and distribution of the Bulgarian medicinal

leeches from the genus *Hirudo* so far, by this study we aimed to clarify the taxonomic status and distribution of leeches in the genus *Hirudo* in Bulgaria. For this purpose, various populations of medicinal leeches throughout this country were studied, using morphological and karyological methods.

Material and Methods

From September 2005 till November 2014, we monitored 56 potential sites of medicinal leeches (particularly eutrophic ponds and marshes) throughout Bulgaria, including almost all localities, where medicinal leeches have been reported previously. The localities were in the three main water drainage basins in Bulgaria (the Danube River, Black Sea and Aegean Sea).

The leeches were collected by hands or using a hand-net and were stored in plastic boxes for a short time until their counting and examining on place. The main external morphological traits, such as the colouration of the dorsum and venter, character of pattern on the dorsum, presence or absence of pigmentation on the venter, and presence or absence of a pair of black ventro-lateral stripes were analysed. Once the leeches were identified, counted and photographed, they were released back at the same localities where caught. A single specimen from each locality was stored in 95% ethanol and taken for laboratory analysis.

The collection of Hirudinea at the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, was also examined for preserved comparative material of the genus *Hirudo*.

Seven medicinal leeches from Dragichevo Marsh were collected in the spring of 2015 for the karyological analysis. The specimens were narcotised in 10% ethanol and then dissected on the ventral side to locate testisacs in Carnoy fixative (3:1 95% ethanol and glacial acetic acid). The dissected gonads were squashed in a small drop of 45% acetic acid. The cover slips were removed by dry ice technique. Preparations were stained, using Schiff-Giemsa method to check the number of chromosomes, and analysed, using light/ fluorescent microscopy (Axio Scope A1 – Carl Zeiss Microscope) at 100x magnification and documented with a ProgRes MFcool – Jenoptik AG digital camera.

Summarised information about all localities, in which the medicinal leeches were found or previously reported in literature, is given in Table 1 and Fig. 1, and includes: name of locality, date of finding, altitude, geographic co-ordinates and number of observed individuals. Some of the typical and most suitable habitats for those leeches in Bulgaria are presented in Fig. 2.

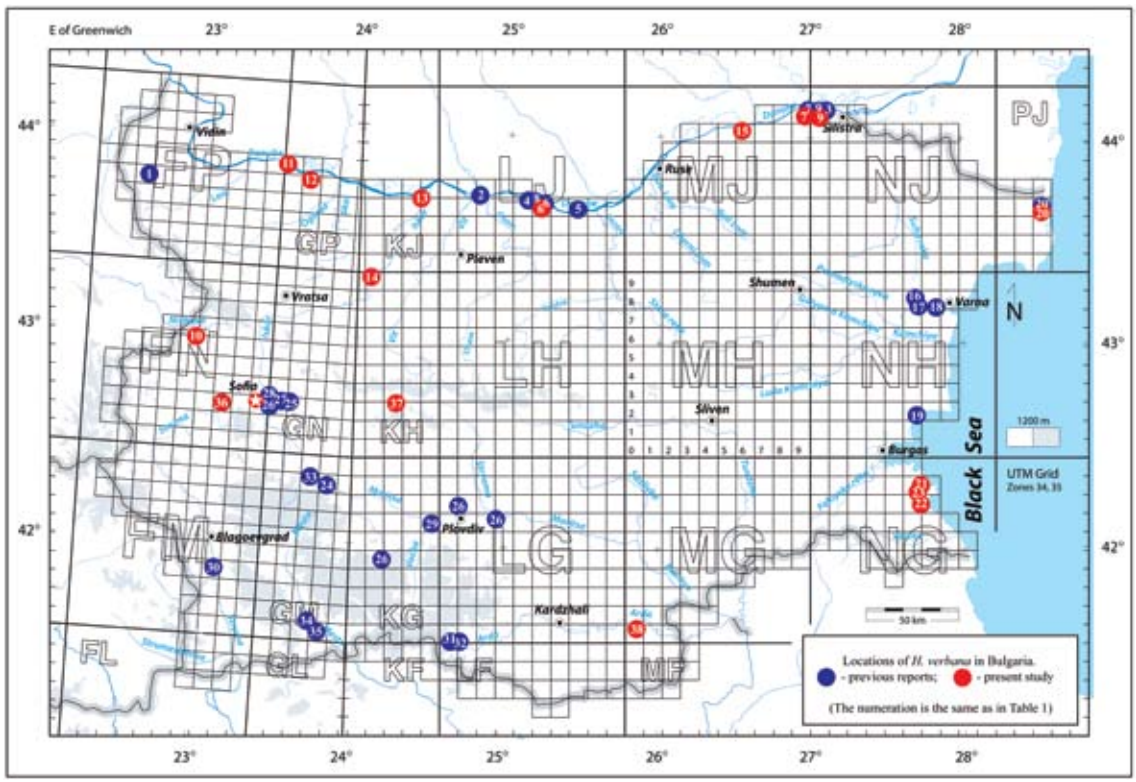

Fig. 1. Localities of medicinal leeches in Bulgaria (the numeration is the same as in Table 1)

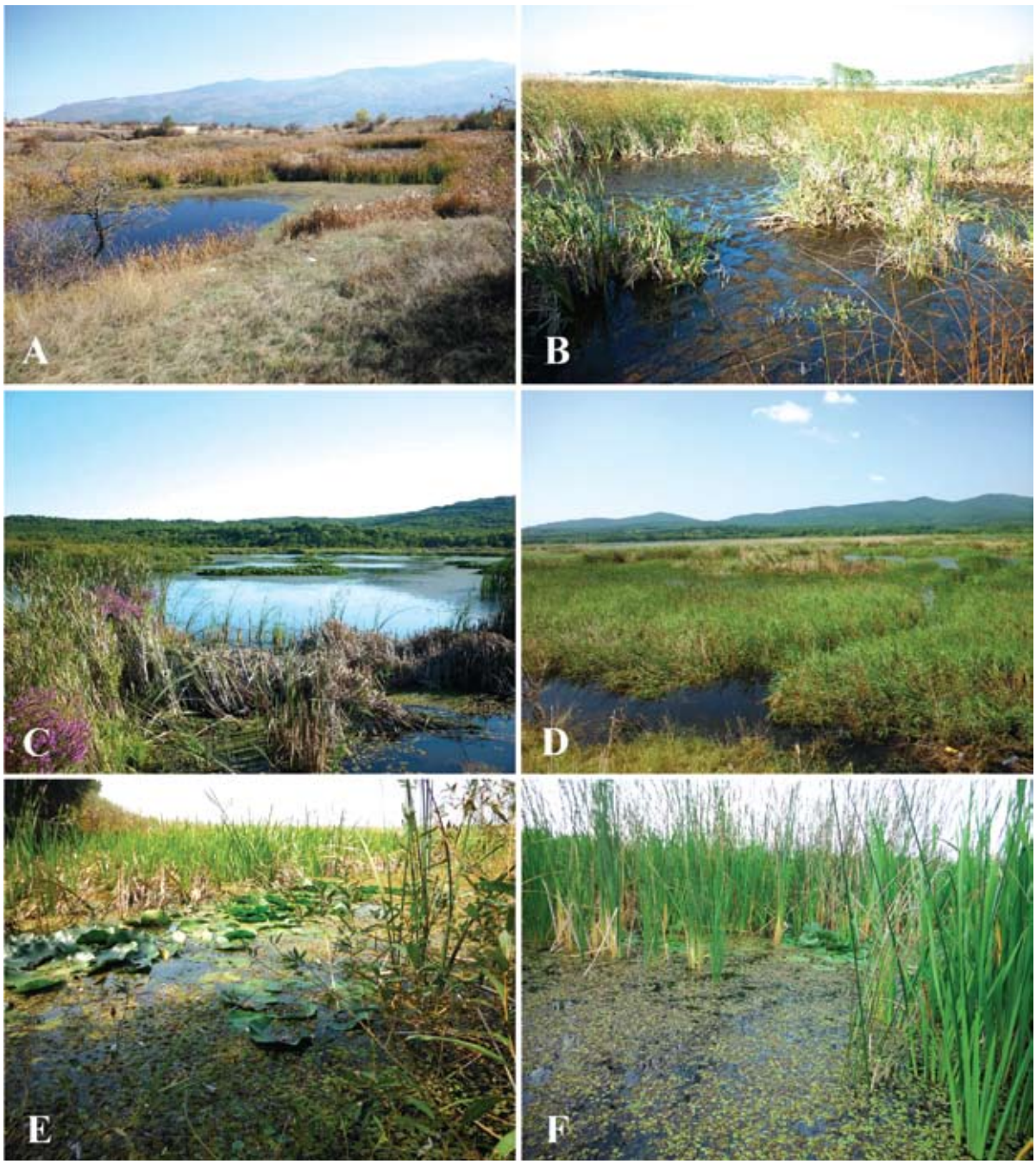

Fig. 2. Typical habitats of *H. verbana* in Bulgaria, characterised by high population density of medicinal leeches: A - Dragichevo Marsh; B – Dragoman Marsh; C - Arkutino Marsh; D - Alepu Marsh; E - Brashlyan Marsh; F - Garvan-Popino Marsh

Results

Medicinal leeches were found in a total of 16 localities, of which 12 are new to Bulgaria (Table 1, Fig. 1). Most of the localities (9) are in the Danube River basin (the Ibisha Reserve on Tsibar Island, Asparuhov Val Reservoir, old riverbed of Iskar River at the village of Bregare and at the town of Cherven Bryag, marshes of Dragoman, Kaikusha, Brashlyan, and Garvan-Popino, Srebarna Lake); four localities are in the Black Sea basin (Orlovo Marsh at Durankulak Lake, and marshes Alepu, Arkutino, and Stamoplo); and three localities are in the Aegean Sea basin (Dragichevo Marsh, the wetlands near the Koprivshtitsa Railway Station, and small reservoir at the village of Madzharovo). The highest altitude, at which the medicinal leeches were found was 958 m a.s.l. (Dragichevo Marsh, Lyulin Mountains), but most of the localities were of low altitude - from 0 to 100 m a.s.l. (Table 1).

The main external morphological traits of 226 individuals from all the localities were analysed (Table 1, Fig. 3). The results showed that individuals of all populations studied had similar colouration and patterns of the dorsum and venter. The basic external characters of the individuals studied were: (1) dorsum with reticulate pattern, with two broad and diffuse paramedian stripes, which were pale-orange in colour; (2) venter was unicolourly greenish to yellow; and (3) lack of ventral pigmentation and presence of a pair of black ventro-lateral stripes (Fig. 3). Based on these characters all recorded individuals were identified as *H. verbana*.

Our review of the collection of the Hirudinea at the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences (IBER, BAS), showed that there is no preserved material of the family Hirudinidae and the genus *Hirudo*, while the material of leeches of the families Glossiphoniidae, Piscicolidae and Erpobdellidae in the same collection is very rich.

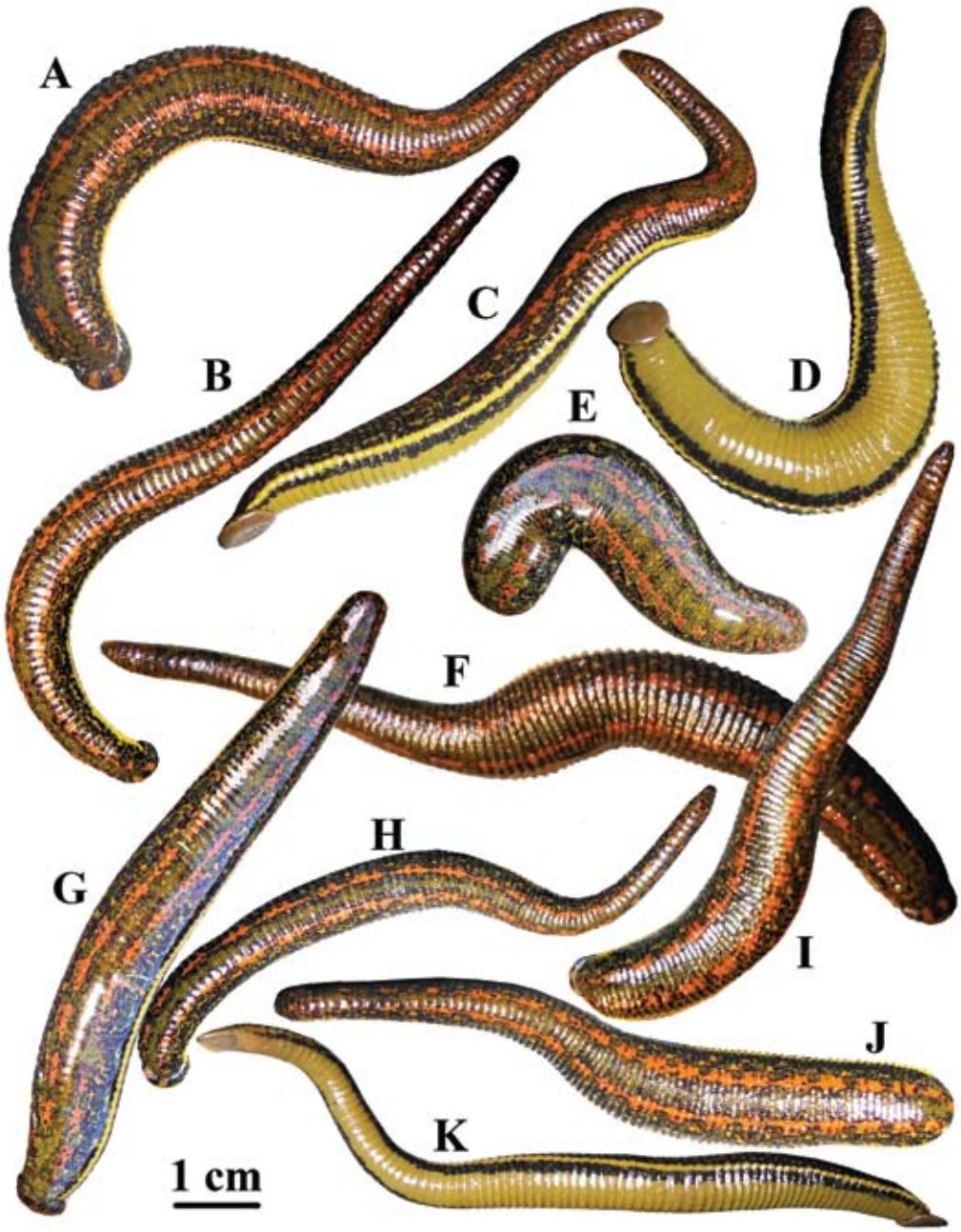

Fig. 3. Views of *H. verbana* caught from different localities, showing characteristic diffuse orange paramedian stripes and reticulate pattern on dorsum, and unicolourly greenish-to-yellowish venter without ventral pigmentation: A – Dragoman; B - Koprivshtitsa Railway Station; C - Kaikusha; D - Dragichevo; E - Srebarna; F - Orlovo Marsh at Durankulak Lake; G - Garvan-Popino; H - Stamoplo; I - Alepu; J-K - Arkutino

Table 1. List of localities of medicinal leeches in Bulgaria reported in the present study and in literature

Localities (No/Name)		Date	Altitude (m a.s.l.)	Latitude	Longitude	Number of individuals observed	Name of the reported species	Author, year
Danube River basin								
1	Rabisha Lake, near the town of Belogradchik	1951-1953	286	N 43.732314°	E 22.592011°	2 ind.	*Hirudo medicinalis*	Angelov 1956; Russev, Janeva 1976
2	The Danube River between rkm 845 and 375	1956-1958	-	-	-	-	*Hirudo medicinalis*	Russev 1959; Russev, Janeva 1976
3	The Danube River, rkm 390	15.06.1961	13	N 44.132328°	E 27.095414°	1 ind.	*Hirudo medicinalis*	Russev, Marinov 1964; Russev, Janeva 1976.
4	Marshes, Belene Island, the Danube River, rkm 568	08.1967	20	N 43.668703°	E 25.222583°	-	*Hirudo medicinalis*	Russev, Kaneva-Abadjieva 1973
5	Marshes, Vardim Island, the Danube River, rkm 543	1964-1967	25	N 43.629750°	E 25.477619°	-	*Hirudo medicinalis*	Russev, Kaneva-Abadjieva 1973
6	Kaikusha Marsh, near the town of Belene	-	20	N 43.599864°	E 25.188090°	-	*Hirudo medicinalis*	Russev, Janeva 1976
		21.07.2011	20	N 43.596047°	E 25.191169°	16 ind.	*Hirudo verbana*	Present study
7	Garvan-Popina Marsh, near the town of Silistra	-	13	N 44.107261°	E 26.910312°	-	*Hirudo medicinalis*	Russev, Janeva 1976
		14.09.2014	13	N 44.096039°	E 26.903001°	19 ind.	*Hirudo verbana*	Present study
8	Murtvoto Blato Marsh, Belene Island	08.2009	19	N 43.674750°	E 25.227522°	-	*Hirudo medicinalis*	Vidinova *et al.* 2011
9	Srebarna Lake, near the town of Silistra	1998-2010	12	N 44.112158°	E 27.069847°	-	*Hirudo medicinalis*	Russev, Janeva 1976; Uzunov *et al.* 2001; Varadinova *et al.* 2009; Varadinova *et al.* 2011; Varadinova *et al.* 2012
		18.09.2014	12	N 44.103236°	E 27.064162°	5 ind.	*Hirudo verbana*	Present study
10	Dragoman Marsh	25.09.2011	703	N 42.934667°	E 22.954969°	8 ind.	*Hirudo verbana*	Present study
11	Ibisha Reserve, Tsibar Island	09.2014	30	N 43.834706°	E 23.511889°	2 ind.	*Hirudo verbana*	Present study
12	Asparuhov Val Reservoir	01.10.2009	97	N 43.738758°	E 23.612456°	1 ind.	*Hirudo verbana*	Trichkova *et al.* 2013
13	Old riverbad of the Iskar River at the village Bregare	19.08.2011	39	N 43.589753°	E 24.338906°	2 ind.	*Hirudo verbana*	Present study

Table 1. Continued

Localities (No/Name)		Date	Altitude (m a.s.l.)	Latitude	Longitude	Number of individuals observed	Name of the reported species	Author, year
14	Old riverbad of the Iskar River at the town of Cherven Bryag	09.2005	89	N 43.306233°	E 24.063903°	3 ind.	*Hirudo verbana*	Present study
15	Brashlyan Marsh	13.09.2014	16	N 44.006985°	E 26.330309°	27 ind.	*Hirudo verbana*	Present study
Black Sea basin								
16	Around Devnya (without precise locality)	-	-	-	-	-	*Hirudo medicinalis*	Valkanov 1926
17	Beloslav Lake	1934-1936	0	N 43.193092°	E 27.696852°	-	*Hirudo medicinalis*	Valkanov 1936; Valkanov 1957
18	Varna Lake	1934-1936	0	N 43.186864°	E 27.805676°	-	*Hirudo medicinalis*	Valkanov 1936; Valkanov 1957
19	Nesebar Marsh	1934-1936	0	N 42.674596°	E 27.696984°	-	*Hirudo medicinalis*	Valkanov 1936; Valkanov 1957
20	Orlovo Marsh, Durankulak Lake	07.1995-10.1996	0	N 43.679578°	E 28.560172°	-	*Hirudo medicinalis*	Kovachev *et al.* 1999
		12.06.2010; 23.08.2011	0	N 43.679578°	E 28.560172°	8 ind. 19 ind.	*Hirudo verbana*	Present study
21	Alepu Marsh	17.07.2014	2	N 42.354708°	E 27.716097°	6 ind.	*Hirudo verbana*	Present study
22	Stamoplo Marsh	18.08.1995; 17.07.2014	3	N 42.276483°	E 27.752006°	1 ind. 2 ind.	*Hirudo verbana*	Present study
23	Arkutino Marsh	11.09.2011; 27.09.2014	5	N 42.331564°	E 27.725856°	9 ind. 1 ind.	*Hirudo verbana*	Present study
Aegean Sea basin								
24	Kalimana, Chepinska River near the Maritsa River, village of Kovachevo	-	228	N 42.216711°	E 24.193828°	-	*Hirudo medicinalis*	Hristovich 1892; Russev, Janeva 1976
25	Marshes at the village of Novoseltsi (currently the town of Elin Pelin)	12.07.1924	528	N 42.695169°	E 23.470543°	-	*Hirudo medicinalis (verbana)*	Augener 1925; Russev, Janeva 1976; Jueg 2010
26	Around Sofia, Kazichene, Plovdiv, Popovitsa, Batak (without precise localities)	-	-	-	-	-	*Hirudo medicinalis*	Valkanov 1926

Table 1. Continued

Localities (No/Name)		Date	Altitude (m a.s.l.)	Latitude	Longitude	Number of individuals observed	Name of the reported species	Author, year
27	Marshes at the village of Berievo (currently the village of Ravno Pole), near Sofia	12.07.1924	509	N 42.721855°	E 23.465416°	-	*Hirudo medicinalis*	Arndt 1943; Russev, Janeva 1976
28	Marshes at the village of Kazichene, near Sofia	12.07.1924	544	N 42.657300°	E 23.454258°	-	*Hirudo medicinalis*	Arndt 1943; Russev, Janeva 1976
29	Marshes at the village of Kadievo, near Plovdiv	24.07.1924	175	N 42.132489°	E 24.591019°	-	*Hirudo medicinalis*	Arndt 1943; Russev, Janeva 1976
30	Along the Struma River, downstream of the village of Krupnik	1987-2000	269	N 41.848786°	E 23.144883°	-	*Hirudo medicinalis*	Uzunov, Varadinova 2001
31	The Cherna River downstream of the town of Smolyan	25.06.1969	905	N 41.575597°	E 24.699822°	-	*Hirudo medicinalis*	Russev, Janeva 1975; Russev, Janeva 1976
32	The Cherna River downstream of Raikovo district, town of Smolyan	1969-1972	847	N 41.567040°	E 24.741205°	-	*Hirudo medicinalis*	Janeva 1989
33	Maritsa River downstream of the town of Dolna Banya	1976-1977	590	N 42.312314°	E 23.783133°	-	*Hirudo medicinalis*	Uzunov *et al.* 1981
34	The Mesta River near Bansko, 2.7 km downstream of the confluence with the Iztok River	1978-1981	756	N 41.873030°	E 23.581530°	-	*Hirudo medicinalis*	Kovachev, Uzunov 1986; Uzunov *et al.* 2011
35	The Mesta River	1978-2009	-	-	-	-	*Hirudo medicinalis*	Varadinova *et al.* 2013
36	Dragichevo Marsh	16.08.2011; 26.09.2014	958	N 42.631683°	E 23.156897°	35 ind. 42 ind.	Hirudo verbana	Present study
37	Wetlands near the Koprivshtitsa Railway Station	14.07.2011	857	N 42.711094°	E 24.347975°	12 ind.	*Hirudo verbana*	Present study
38	Small reservoir at the village of Madzharovo, Eastern Rhodopes	03.05.2009	211	N 41.631317°	E 25.859383°	8 ind.	*Hirudo verbana*	Present study

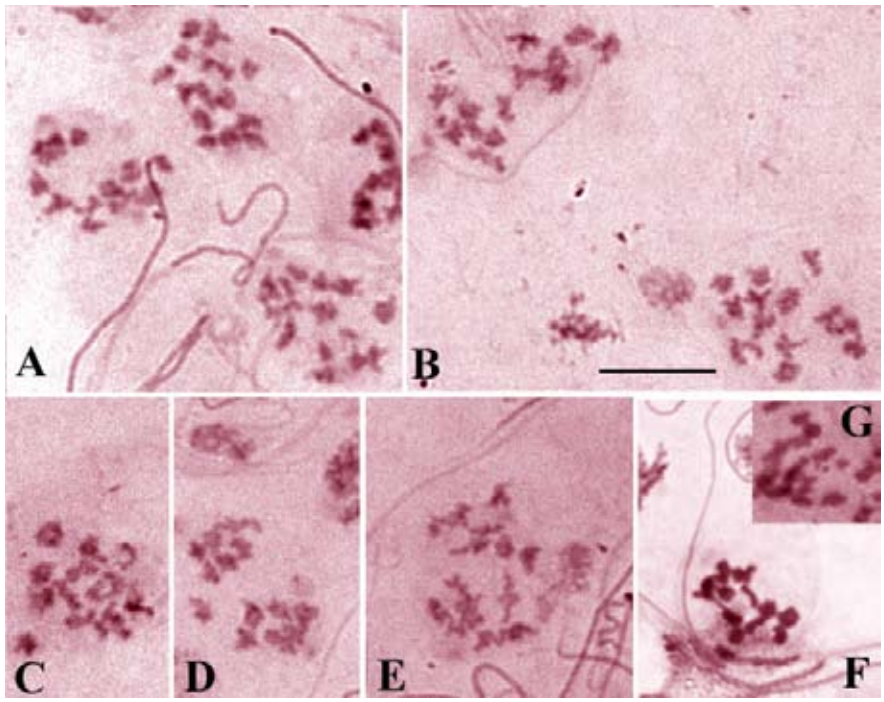

Fig. 4. *Hirudo verbana*: A-E – Diakinesis, F-G – Metaphase I plates. Chromosomes can be easily counted as n=13. Scale-bar (A-G): 10µm

The results of the cytogenetic study showed that the preparations of the leeches' chromosomes were abundant of cells at late prophase stages of spermatogenesis, while the metaphase plates were quite rear and not well spread. All the examined specimens were found to have the same haploid chromosome number, n=13 (Fig. 4), which confirms the species identification as *H. verbana* (Utevsky *et al.* 2009). The chromosomes can be classified as metacentric, submetacentric and acrocentric.

Discussion

The recent molecular systematic investigations of the medicinal leeches have clearly discriminated four European species of the genus *Hirudo*, and showed that each of those species have a specific colouration pattern (Trontelj *et al.* 2004, Utevsky, Trontelj 2005, Siddall *et al.* 2007, Utevsky *et al.* 2009, 2010, Elliott, Kutschera 2011, Trontelj, Utevsky 2012, Kutschera, Elliott 2014). Based on the similar colouration and patterns of dorsum and venter, our study showed that the medicinal leeches from all populations studied belong to only one species. Moreover, the identified morphological characters are typical of *H. verbana*, and distinguish clearly this species from other European leeches of the genus *Hirudo*, in particular from *H. medicinalis* (Utevsky, Trontelj 2005, Siddall *et al.* 2007, Utevsky *et al.* 2009, 2010, Elliott, Kutschera 2011, Trontelj, Utevsky 2012, Kutschera, Elliott 2014). The haploid chromosome number (n=13) of the medicinal leeches from Dragichevo Marsh also confirmed the belonging of the species to *H. verbana*.

Furthermore, the current distribution and population density of *H. verbana* in Bulgaria was ascertained within this study. Although *H. verbana* is widespread throughout the country, the wetlands along the Danube River stand out as the most suitable habitats for that leech. Until the mid-20th century, in this region there were more than 20 relatively large marshes, characterised by extremely rich freshwater fauna and abundant populations of medicinal leeches (Russev, Janeva 1976). From that time on, as part of the measures against malaria in Bulgaria, some large-scale activities for the marsh drainage were implemented and many of those marshes no longer exist. However, our results show that in the marshes on the Danube River islands, as well as in the remaining relatively small marshes along the main river channel, leeches are still abundant. Very high population density of *H. verbana* was registered in the marshes Brashlyan and Garvan-Popina (Table 1). In literature, there were three reports on finding of medicinal leeches reported as *H. medicinalis* in the Danube River (Russev 1959, Russev, Marinov 1964, Russev, Janeva 1976). Most likely those were a result of accidental introductions of single individuals from the adjacent wetlands to the main channel of the river (after flooding or human activities).

During our study, the medicinal leeches were relatively abundant in the swampy freshwater lagoons along the Black Sea coast, where the species was registered at four localities (Table 1, Fig. 1). One of these localities, Orlovo Marsh at Durankulak Lake, was characterised by a very high population density of *H. verbana*. At the other three localities in the southern Black Sea coast (marshes Alepu, Arkutino and Stamoplo) the population density of the species was much smaller and only single specimens were registered. This may be due to the fact that these marshes have been relatively less studied: access to the water body of Arkutino is restricted because of its special conservational status, while Alepu and Stamoplo are highly overgrown with reeds and the access to open water is very difficult.

In the rest of the country, medicinal leeches were found in small local populations, which inhabited mainly old and swampy eutrophic water basins, such as the marshes Dragoman and Dragichevo, with a high quantity of organic matter, muddy bottom and dense aquatic vegetation. Dragichevo Marsh was characterised by a very large amount of *H. verbana* and very good condition of its population. The medicinal leeches have been reported (as *H. medicinalis*) in water bodies that are not typical habitats for them, such as the Cherna River, Maritsa River, Mesta River and Struma River (Russev, Janeva 1975, 1976, Uzunov *et al.* 1981, 2011, Kovachev, Uzunov 1986, Janeva 1989, Uzunov, Varadinova 2001). Finding of these species in such habitats may be explained by the accidental transfer of the leeches, by cattle or humans, from some stagnant basins in the vicinity of those rivers. In our opinion, although medicinal leeches can survive for some time in such conditions, the rivers with swift current are generally not suitable habitats for their successful reproduction and development. Furthermore, the medicinal leeches have high temperature requirements: the optimum temperatures range from 22 to 25°C for living and from 25.5 to 27.5°C for breeding (Kaiser 1954, Elliott 2008, Kutschera, Elliott 2014). They cannot reproduce and survive in many water bodies simply because of the low water temperatures (Elliott, Tullett 1986).

It should be noted that nearly all localities where medicinal leeches have previously been reported from Bulgaria were examined in this study. *H. verbana* was found only in four of them (Srebarna Lake, marshes on Belene Island, Garvan-Popino Marsh and Orlovo Marsh at Durankulak Lake). An important reason for the limited distribution of the medicinal leeches may be that some of the previous water basins, such as Novoseltsi and Nessebar marshes, no longer exist, while others have changed and no longer provide suitable habitats for the species any more. Among the latter, Varna and Beloslav lakes, have experienced an increased shipping traffic and their exposition to sea water was increased by widening and deepening the relevant connecting canals. This led to changes in the physical and chemical conditions of the water in those lakes and to significant water pollution with oil products, thus probably making both lakes unsuitable for medicinal leeches. In the case of Rabisha Lake, it was transformed into a reservoir with a much larger area, which led to significant modification of its water regime; therefore, this water body is no longer a proper habitat for the leeches. On the other hand, our study established 12 new localities of the medicinal leeches in Bulgaria (six in the Danube River basin, three in the Black Sea basin and three in the Aegean Sea basin). Most likely the new findings do not reflect some expansion in the distribution of the species in Bulgaria, but rather may be explained by the insufficient information on the species in the past.

In conclusion, our results from the morphological study of 226 individuals of medicinal leeches from 16 localities throughout Bulgaria, and karyological study of one population in Dragichevo Marsh, show that currently only one species occurs in Bulgaria, and this species has been identified as *H. verbana.* Based on the geographic range of this and other species of the genus *Hirudo* in Europe, it can be assumed that the previous published records of *H. medicinalis* in Bulgaria may also refer to *H. verbana*. Further studies are needed to confirm this assumption. Our results may be used as a basis for future monitoring of the medicinal leech populations across the country and assessment of their conservation status in Bulgaria.

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