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# Integrative taxonomy reveals a new species of the leech genus *Dina* R. Blanchard, 1892 (Annelida, Hirudinida: Erpobdellidae) from the ancient Skadar Lake basin in Montenegro

C. GROSSER<sup>1</sup>, T. REWICZ<sup>2</sup>, M. JOVANOVIĆ<sup>3</sup>, A. ZAWAL <sup>4,5</sup>, & V. PEŠIĆ <sup>3\*</sup>

<sup>1</sup>Bernd-Blindow-Schule Leipzig, Leipzig, Germany, <sup>2</sup>Department of Invertebrate Zoology and Hydrobiology, University of Lodz, Łódź, Poland, <sup>3</sup>Department of Biology, University of Montenegro, Podgorica, Montenegro, <sup>4</sup>Institute of Marine and Environmental Sciences, Center of Molecular Biology and Biotechnology, University of Szczecin, Szczecin, Poland, and <sup>5</sup>Department of Plant Protection, Faculty of Agriculture, University of Tehran, Karaj, Iran

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## Abstract

Leeches of the genus *Dina* are one of the most abundant leech species in running waters in Montenegro. This paper described a new yellowish-spotted and medium-sized leech from the Skadar Lake basin in Montenegro. Phylogenetic analysis based on COI data reveals that *Dina nesemanni* sp. nov. is more closely related to *D. latestriata* from Prespa Lake than to other yellow-spotted leeches known from montane and alpine areas of Montenegro. Morphologically, the new species can be separated from all other yellow-spotted leeches by the peculiar shape of the ovisacs. The new species is a characteristic and possibly an endangered representative of the fauna of highly threatened Mediterranean ponds and swamps in the central and southern part of Montenegro.

<http://zoobank.org/urn:lsid:zoobank.org:pub:973D45A4-D488-4848-89E2-7659D936F06C>

**Keywords:** Taxonomy, DNA barcoding, species delimitation, leeches, new species

## Introduction

The leeches of the genus *Dina* R. Blanchard, 1892 are widely distributed in the Palearctic region, inhabiting different types of running and standing waters (Nesemann & Neubert 1999). So far, 25 species or subspecies of *Dina* are known from Europe, 19 of which inhabit the Western Balkans (Grosser & Pešić 2022). They are among the most abundant leech species in running waters in Montenegro (Grosser et al. 2015; Marinković et al. 2019, 2022). Currently, this genus in Montenegro encompasses four species (Sket 1968; Grosser et al. 2007, 2015, 2016; Grosser & Pešić 2022), i.e., *Dina dinarica* Sket, 1968, *D. montana* Sket, 1968, *D. minuoculata*

Grosser, Moritz & Pešić, 2007, and *D. crnogorensis* Grosser & Pešić, 2022.

Leeches of the genus *Dina* have been distinguished by the ejaculatory ducts forming preatrial loops and their annulation pattern: the last annulus (b6) per somite is widened and often subdivided once (Nesemann & Neubert 1999). Traditionally, members of the subfamily Trochetinae, which in addition to *Dina*, include *Trocheta* Dutrochet, 1817, a genus widely distributed in the Western Palearctic and the monotypic genus *Archaeobdella* Grimm, 1876, known from the Caspian Sea and the eastern Europe (Nesemann & Neubert 1999) are distinguished from *Erpobdella* de Blainville, 1818 by its

\*Correspondence: V. Pešić, Department of Biology, University of Montenegro, Cetinjski put b.b., Podgorica 81000, Montenegro.  
E-mail: [vladopesic@gmail.com](mailto:vladopesic@gmail.com)

annulation pattern (annulus b6 always broadened, the somites subdivided into 5–11 annuli; Nesemann & Neubert 1999). In *Erpobdella*, the somites are homonomously quinqueannulate and each somite is subdivided into five annuli of equal size. Trontelj and Sket (2000) problematized the status of Trochetinae as it does not represent a monophyletic group. Later on, Siddall (2002) formally synonymized the genera *Dina* and *Trocheta* (as well as the non-European *Mooreobdella* and *Nephelopsis*) with the genus *Erpobdella* but did not provide an emended diagnosis of the latter genus. The latter concept was followed by some authors (e.g., Ocegüera-Figueroa et al. 2005, 2011) but was not widely accepted by other, mostly European authors who continued to treat *Dina* as separate genus (e.g., Grosser et al. 2007, 2011, 2015; Trajanovski et al. 2010; Ben Ahmed et al. 2015; Darabi-Darestani 2021; Pešić & Grosser 2022).

Recent studies on *Dina* populations inhabiting the Western Balkans have shown the great potential of DNA barcoding in identifying complexes of species within taxa that require further systematic attention (Grosser et al. 2015; Grosser & Pešić 2022; Pešić & Grosser 2022). So far, molecular studies of the diversity of the genus *Dina* in Montenegro have been limited to the montane and alpine regions of the northern part of Montenegro (Grosser et al. 2007, 2016; Grosser & Pešić 2022), and the central part belonging to the Skadar Lake basin has so far escaped attention. The Skadar lake was created only ca. 1200 years ago by overflowing an already existing marshland (Mazzini et al. 2015; Pešić et al. 2018), but its hydrological system, which amounts to 5,490 km<sup>2</sup>, with numerous springs and ponds, is assumed to be ancient and probably dates back at least to the Pliocene (see Grabowski et al. 2018 for a review).

In this paper, we used morphological data and DNA barcode of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene sequences to describe one new species of the genus *Dina* from central part of Montenegro.

## Materials and methods

Leeches were collected by tweezers from the underside of hard substratum (stones, wood) and on plants submerged in the water. Individuals were immediately preserved in 96% ethanol for further morphological and molecular genetic analysis (see below). The external morphology (i.e., the number and position of eyes,

the annulation, coloration, papillation and the position of genital pores) was examined on several specimens. The characters of sexual organs (location, shape and extension of the genital atrium with the cornua, shape of the ovarian sacks and vasa deferentia) were checked on two paratypes with well-developed sexual organs. Measurements were taken with a ruler (we consider the precision of such measurements sufficient, because the results largely depend on the body contraction). Material was examined using a stereomicroscope (Novex), and photographs were taken with a microscope camera (Euromex, VC 3031C) and a camera (Canon EOS 400D with macro lenses). All measurements are given in millimeter (mm).

The holotype and four paratypes of *Dina nesemanni* **sp. nov.** are deposited in the invertebrate collection of Martin-Luther-Universität Halle-Wittenberg (MLU) in Halle (Saale), Germany; two paratypes are deposited in the collection of the first author, and one paratype is deposited in the collection of the fourth author (V.P.).

## Molecular and DNA barcode analyses

The molecular analysis was conducted at the Canadian Centre for DNA Barcoding (Guelph, Ontario, Canada (CCDB; <http://ccdb.ca/>)). The specimens were sequenced for the barcode region of COI using standard invertebrate DNA extraction (Ivanova et al. 2007), amplification (Ivanova & Grainger 2007a) and sequencing protocols (Ivanova & Grainger 2007b). DNA was extracted from eight specimens of the genus *Dina* listed in Table I. For all other species (Trajanovski et al. 2010; Darabi-Darestani et al. 2021; Grosser & Pešić 2022; Pešić & Grosser 2022), COI sequence data were retrieved from the respective sequence data archives.

In total, we used 43 sequences of *Dina crnogorensis* (n = 2), *D. latestriata* Neubert & Nesemann, 1995 (n = 3), *D. cf. latestriata* (Trichonis Lake) (n = 1), *D. dinarica* (n = 2), *D. farsa* Grosser & Pešić, 2008 (n = 6), *D. lineata lineata* (Müller, 1774) (n = 4), *D. lineata lacustris* Sket, 1968 (n = 2), *D. minuoculata* (n = 2), *D. montana* (n = 2), *D. nesemanni* **sp. nov.** (n = 7), *D. prokletijaca* Grosser & Pešić, 2016 (n = 2), *D. punctata* Johansson, 1927 (n = 2), *D. serbica* Pešić & Grosser, 2022 (n = 3) and *D. sketi* Grosser & Pešić, 2014 (n = 4). *Erpobdella octoculata* (Linnaeus, 1758) from Lake Ohrid was used as the outgroup.

The obtained sequences were edited using Geneious 10.2 (Kearse et al. 2012) and deposited in GenBank (Table I). Simultaneously, the DNA sequences were deposited in the Barcode of Life Data Systems (BOLD) (Ratnasingham & Hebert 2007), to obtain the Barcode Index Numbers (BIN), which group DNA sequences based on the genetic distance, often ascribed as tentative equivalents of species (Ratnasingham & Hebert 2013). Obtaining BINs for sequences deposited in BOLD provides additional verification of species identification and delimitation. Relevant voucher information, photos, and newly generated DNA barcodes are publicly accessible through the dataset DS-DINABLAT (<http://dx.doi.org/10.5883/DS-DINABLAT>) in BOLD.

Sequence comparisons were performed using MUSCLE alignment (Edgar 2004). Intra- and interspecific genetic distances were calculated based on the Kimura 2-parameter model (K2P; Kimura 1980), using MEGA X software (Kumar et al. 2018). The latter software was used to produce Maximum Likelihood (ML) tree (model selected by the BIC (Bayesian Information Criterion) implemented in MEGA X: T92 + G + I) with an initial Neighbour-Joining (NJ) tree and using the Subtree-Pruning-Regrafting - Extensive heuristic search (SPR level 5). The support for tree branches was calculated by the nonparametric bootstrap method (Felsenstein 1985) with 1000 replicates and shown next to the branches. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair.

To visualize patterns of mitochondrial divergence, we constructed neighbour-net networks in SplitStree v.4 (Huson & Bryant 2006) based on COI marker alignments, whereas the relationships within particular species were displayed through a Minimum Spanning Network using PopART (Leigh & Bryant 2015).

## Results

### *Species delimitation using DNA-barcodes*

The final alignment for species delimitation using COI sequence data comprised 565 nucleotide positions (nps) for 43 specimens of genus *Dina*, and one specimen of *Erpobdella octoculata* from Lake Ohrid as an outgroup. Fourteen BINs were assigned to 12 species (Figure 1, Figure 2). We observed two BINs within a single species in only two cases: *Dina latestriata* and *D. lineata*; the remaining species follow the pattern of a single BIN equaling to a single species. Due to the limited number of individuals per species, observing geographical patterns in our data is expected difficult. However, we noticed a clear split between the Prespa Lake population (Albania, Greece, North Macedonia; BOLD: ACQ7912) and the Trichonis Lake population (Greece; BOLD: ACQ7911) of *D. latestriata*. A similar spatially split pattern was observed within *D. lineata*, where *D. lineata lineata* from Western and Northern Europe (Germany, Finland; BOLD: ACD1775), and *D. lineata lacustris* from the Balkans (North Macedonia; BOLD: ACQ0952) form two distinct clades. The clade containing specimens from Montenegro is sister to the clade consisting of *D. latestriata* Neubert & Neesemann, 1995 from Prespa Lake (Figure 1).

Number of haplotypes per BIN varies between four (*Dina nesemannii* sp. nov. BOLD: AEC4521), three (*D. latestriata* BOLD: ACQ79112; *D. serbica* BOLD: AEN3738), two (four BINs), and one (seven BINs) (Figure 2). We can mostly observe the pattern that number of haplotypes increasing with the number of sites from where individuals were collected.

The mean genetic distance between COI sequences of studied *Dina* spp. ranged from  $4.1 \pm 0.8\%$  between *D. prokletijaca* and *D. montana* to  $20.7 \pm 2.3\%$  K2P between *D. farsa* and *D. dinarica* (Table II). The mean intraspecific distance within clades was low, ranging from  $0 \pm 0$  (in *D. punctata*, *D. minuoculata*, *D. montana*, *D. dinarica*, *D. sketi*) to  $1.61 \pm 0.43\%$  K25 in *D. latestriata* (Table II).

Table I. List of newly sequenced specimens of the genus *Dina* used in this study.

Locality (country, name)	Lat/Long	Voucher code	BOLD/Genbank Acc. nos.	BOLD BIN
<b><i>Dina nesemannii</i> sp. nov.</b>				
Danilovgrad, Pond Blatina	42.543° N, 19.1228° E	MN11_1	LCHME019-20/OM628839	BOLD:AEC4521
		CCDB 39398 H09	HYDAS188-22/OQ274179	
		CCDB 39398 H10	HYDAS189-22/OQ274178	
Bar, pond near Lake Skadar	42.126° N, 19.258° E	BIOUG57634-D08	TRSKA5839-20/OQ507615	
		BIOUG57634-D07	TRSKA5838-20/OQ507614	
		BIOUG57634-D06	TRSKA5837-20/OQ507613	
		BIOUG57634-D05	TRSKA5836-20/OQ507616	
<b><i>Dina dinarica</i></b>				
Cetinje, Podgor, Smokov Vijenac spring	42.254° N, 18.99° E	BIOUG57634-A01	TRSKA5796-20/OQ507617	BOLD:AEE8690

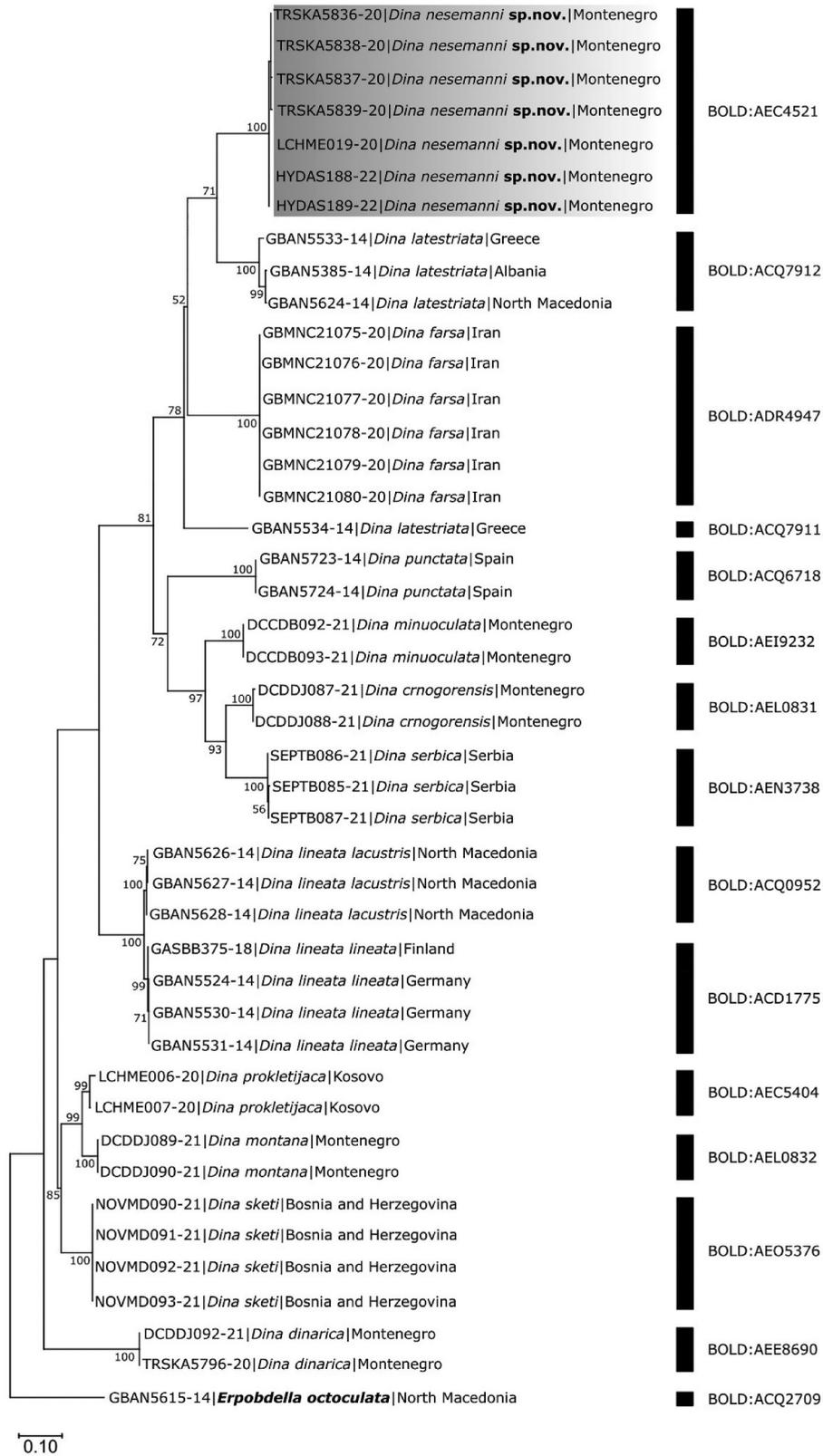


Figure 1. Maximum Likelihood tree (T92 + G + I model) of the *Dina* spp. complex used in our study. Outgroup is given in bold. The results of BIN species delimitation are indicated by vertical bars, and BIN number. New species indicated by shaded area. Only a bootstrap that supported more than 50% is reported.

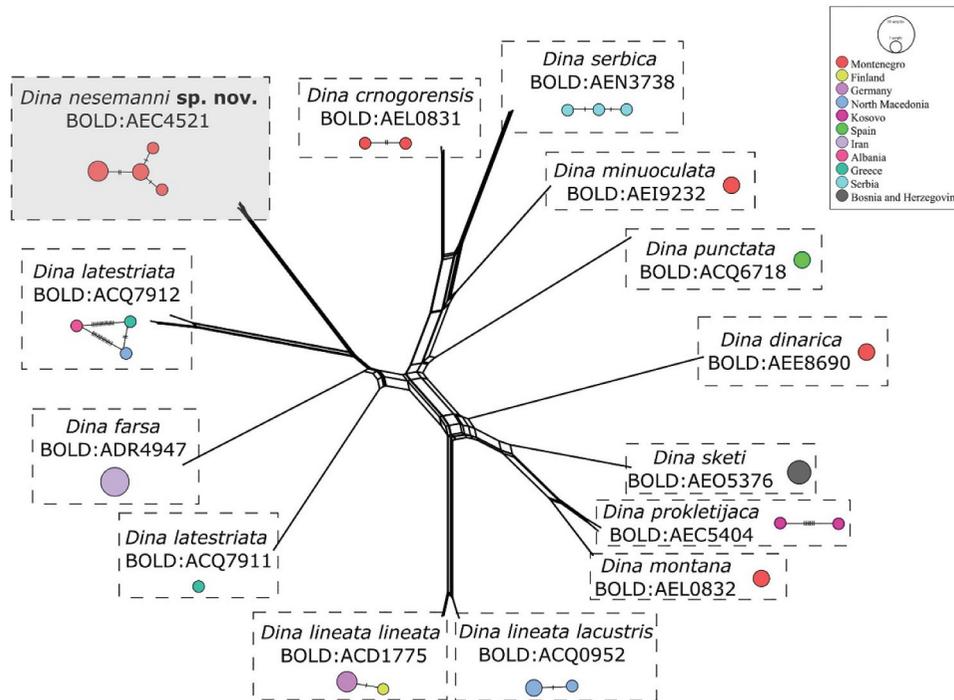


Figure 2. The phylogenetic network was computed with the neighbour-net algorithm and uncorrected p-distances with the Minimum Spanning Networks for the respective species. Each frame represents a single BIN. Each bar in haplotype networks represents a single mutational change, diameter of the circles is proportional to the number of individuals in each haplotype sampled (see open circles with numbers), and the colors correspond to the country of origin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

## Taxonomic accounts

**Family:** Erpobdellidae R. Blanchard, 1894

**Genus:** *Dina* R. Blanchard, 1892

***Dina nesemanni* Grosser & Pešić sp. nov.**  
Figures 3, 4A-D

**Materials examined.** Holotype (MLU), Montenegro, Danilovgrad, village Lazine, pond Blatina, 20 April 2022 leg. Pešić & Jovanović, body length 42.5, width 7.5, and the caudal sucker width 4 mm. Paratypes: seven specimens (four specimens deposited in MLU: body length/width/caudal sucker width: 45/8/4.5, 39.5/7/4, 36/6/3.5, 40/7/3.5 mm; dissected; two specimens deposited in collection of the first author: 42/7/4 [CCDB 39398 H09], dissected, 32/6.5/- mm [CCDB 39398 H10], dried), same place data as holotype, leg. Pešić & Jovanović, two specimens sequenced (CCDB 39398 H09, CCDB 39398 H10; Table I); one specimen [MN11\_1], sequenced, deposited in the collection of the

fourth author, same place as holotype, 17 October 2019 leg. Pešić & Jovanović.

**Other material.** Montenegro, Bar, Livari, pond near Lake Skadar, 42.126° N, 19.258° E, 3 June 2018, leg. Zawal, four specimens (sequenced; Table I).

**Locus typicus.** Montenegro, Central Montenegro, Danilovgrad, village Lazine, Blatina Pond, 42.543° N, 19.1228° E.

**Diagnosis.** Medium-sized leeches; basic coloring brownish, ventral side unicolor, dorsal side with an additional dark grey pigmentation with dark paramedian stripes and transverse rows of yellow spots, every fifth row more heavily spotted; annulation *Dina*-like quinqueannulate with annulus b6 broadened, genital pores in the furrows and separated by two annuli, sometimes the female gonopore shifted caudally on annulus; papillae small and inconspicuous; cornua of the genital atrium short, strongly curved to the ventral side with a broadened base and not coiled ends; the strongly coiled and thickened part of the vasa deferentia extends to the posterior end of the sixth or the anterior end of the

Table II. Estimates of average genetic distance (K2P) within and between clades examined species of *Dima* spp. sequence pairs in and between COI haplogroups are shown. Standard error estimates are shown above the diagonal.

Species	Intergroup													
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
(1) <i>D. nesemami</i> <b>sp. nov.</b>														
(2) <i>D. lateseriata</i>	0.0031 ± 0.0016													
(3) <i>D. farsa</i>	0.0161 ± 0.0043	0.017												
(4) <i>D. cf. lateseriata</i> (Trichonis Lake)	0 ± 0	0.139	0.018											
(5) <i>D. punctata</i>	n/c	0.147	0.017	0.018										
(6) <i>D. minuoculata</i>	0 ± 0	0.174	0.141	0.187	0.020									
(7) <i>D. erogorensis</i>	0 ± 0	0.169	0.162	0.171	0.150	0.019								
(8) <i>D. serbica</i>	0.0036 ± 0.0027	0.157	0.170	0.154	0.162	0.112	0.016							
(9) <i>D. l. lineata</i>	0.0024 ± 0.0016	0.192	0.181	0.177	0.176	0.115	0.089	0.014						
(10) <i>D. l. lacustris</i>	0.0009 ± 0.0009	0.166	0.173	0.161	0.183	0.171	0.155	0.196	0.022					
(11) <i>D. prokletijaca</i>	0.0012 ± 0.0012	0.179	0.182	0.154	0.182	0.166	0.161	0.200	0.015	0.020				
(12) <i>D. montana</i>	0.0109 ± 0.0045	0.179	0.182	0.150	0.168	0.147	0.152	0.168	0.118	0.016	0.016			
(13) <i>D. dinarica</i>	0 ± 0	0.173	0.189	0.164	0.164	0.159	0.164	0.175	0.128	0.016	0.016	0.008		
(14) <i>D. sketi</i>	0 ± 0	0.189	0.207	0.188	0.205	0.202	0.195	0.203	0.161	0.159	0.136	0.143	0.017	
		0.170	0.182	0.156	0.164	0.175	0.175	0.186	0.126	0.125	0.079	0.091	0.142	0.018

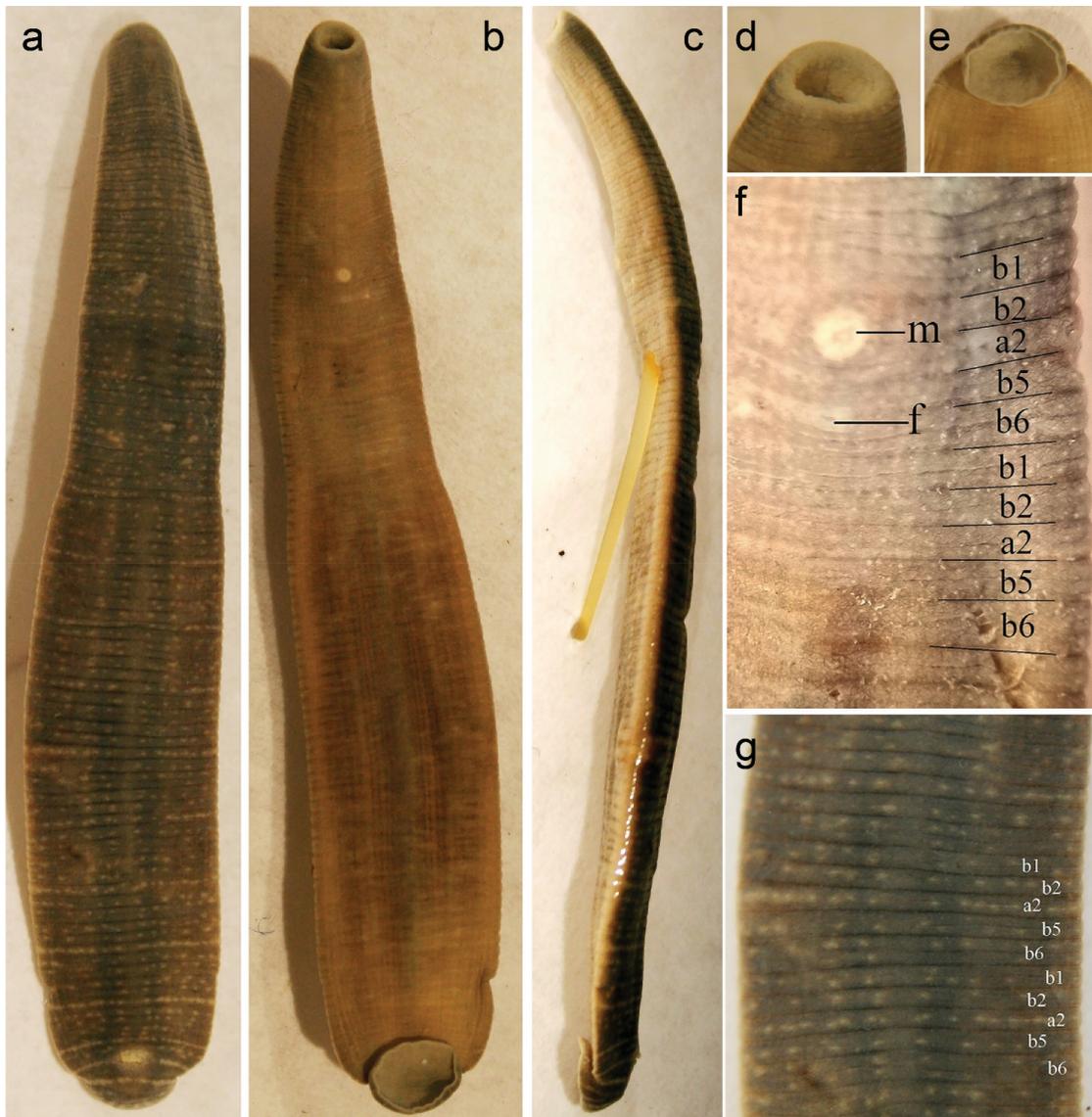


Figure 3. *Dina nesemanni* sp. nov., holotype, external morphology. (a) dorsal view; (b) ventral view; (c) lateral view; (d) cranial sucker; (e) caudal sucker; (f) annulation and gonopore (f, ♀ genital pore, m, ♂ genital pore) position, ventral view; (g) annulation, dorsal view.

seventh somite posterior to the female gonopore; ovisacs run strongly coiled dorsally over the vasa deferentia to the second somite after the female gonopore, from here they change to the ventral side and run slightly windingly along the side of the ventral nerve cord to the third somite posterior to the female gonopore.

### Description

**Habitus.** Medium-sized leeches; preserved and contracted adults reach a body length of up to 45 mm and a width of up to 8 mm (largest paratype). Body dorso-ventrally flattened, slightly in the preclitellar and

clitellar region, stronger in the postclitellar part. The width of the caudal sucker reaches approximately half of the maximum body width. The head region tapering clearly towards the mouth opening. The dorsal part of the cranial sucker is slightly extended. Papillae on the dorsal side small and inconspicuous, corresponding to yellow spots; additional papillae very small, dorsal surface with a texture similar to fine-grained sandpaper.

**Annulation.** Leeches with a typical *Dina*-like annulation; the midbody somites quinqueannulate and heteronomously subdivided into annuli b1, b2, a2, b5, with annulus b6 broadened. The latter annulus is mostly distinct, but occasionally indistinctly

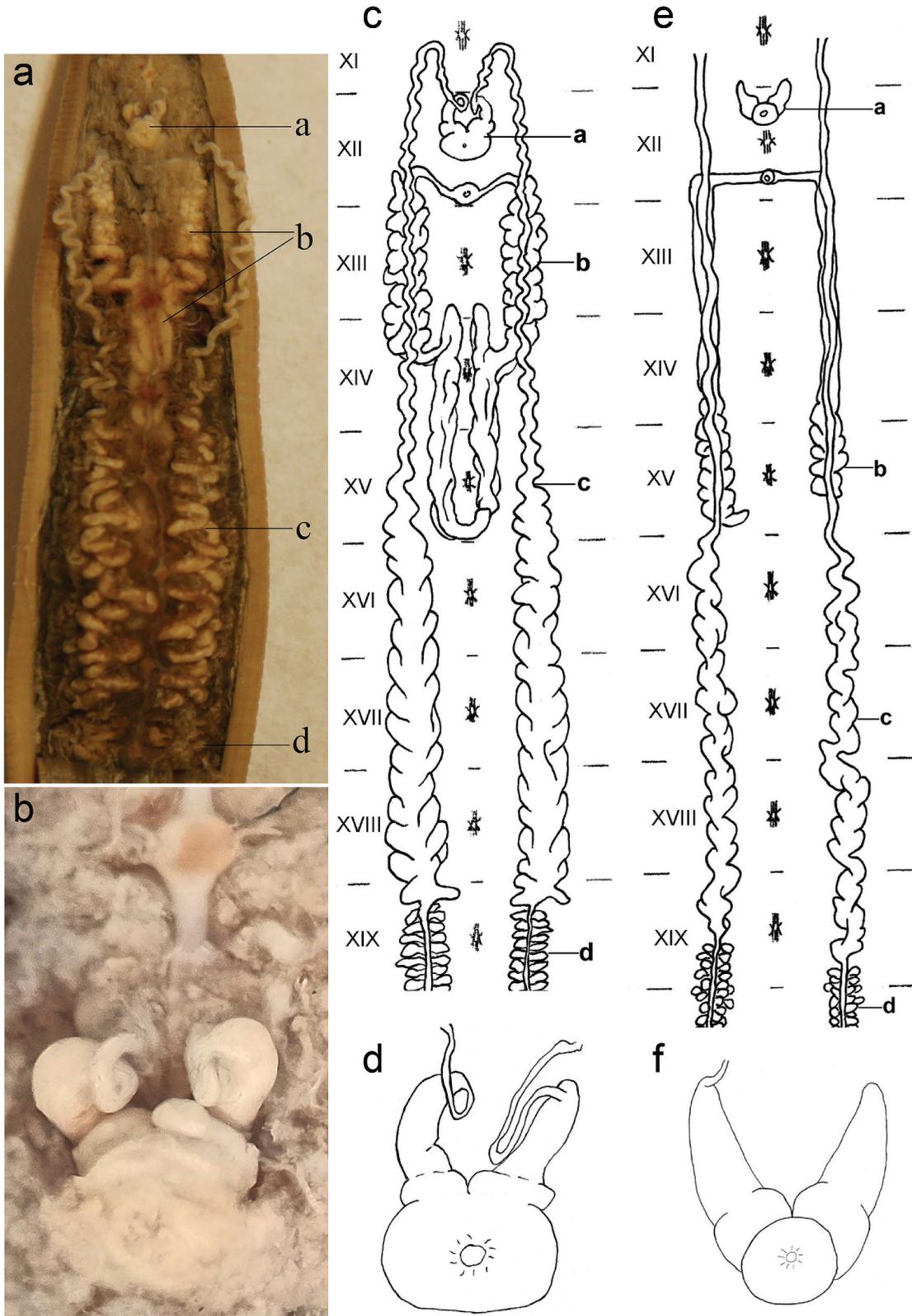


Figure 4. Photographs (a-b) and schematic diagrams (c-f) of reproductive system (a, c, e) and genital atrium (b, d, f). a-d *Dina nesemanni* sp. nov., paratype. (e-f) *D. latestriata*, paratype. Abbreviations: a, genital atrium; b, ovarian sacs; c, vas deferens; d, testisacs. XI–XIX, numbers of somites (according to Nesemann & Neubert 1999).

broadened in some sections. Annulus b6 regularly split into annuli c11 and c12 by a shallow furrow; a tendency to split further annuli more evident in the preclitellar than in the postclitellar region. The male gonopore located in furrow between b2/a2, the female gonopore in furrow between b5/b6 (in holotype female gonopore shifted caudally to c11), and the gonopores are thus separated by two annuli.

*Eyes.* The new species is characterized by a strong reduction of visible eye pigmentation, and only two specimens have one visible eye each.

*Colour.* The basic color brownish; the ventral side unicolor. More or less pronouncedly, the dorsal side shows an additional dark gray pigmentation and yellow spots arranged in a row on each annulus, except on annulus b6 where they are present in two rows with c11 slightly more spotted than c12. Annulus a2 most heavily spotted among the annuli, which creates clear transverse striations at a distance of five annuli on the dorsal side. The dorsal surface typically with a pair of dark paramedian stripes, sometimes an additional pair of paramedian stripes at the edge of the dorsal side, is present (slightly expressed in the holotype, stronger in the paratype). The paramedian stripes delimit a broad median field, which is greyish and less yellow spotted.

*Sexual organs.* Genital atrium: atrium body large, slightly flattened dorso-ventrally; cornua short, extending anteriorly to or on annulus b6 of the somite anterior to them, strongly curved to the ventral side, broadened at the base, the ends not coiled. Vasa deferentia with preatrial loops, thin and clearly wavy from the anterior to the third ganglion after the female gonopore, strongly coiled and thickened from the third ganglion to the posterior end of the sixth or anterior end of the seventh somite (to b2) posterior to the female gonopore. Ovisacs are very peculiar in their shape. Viewed from the vagina, the ovisacs initially run anteriorly to annulus b5 or at the level b5/a2. This part is not coiled, but then the ovisacs become strongly coiled and run dorsally across the vasa deferentia to the second somite (to the beginning of the somite or to b2) posterior to the female gonopore. From here, the ovisacs pass ventrally and run slightly curved along the side of the ventral nerve cord, first anteriorly to b6/b5 or across the somite boundary, then posteriorly to the third somite (to b2 or to b6) posterior to the female gonopore; afterward, the ovisacs run back the same way to the anterior on the dorsal side.

**Etymology.** Named after to our colleague Dr Hasko Friedrich Neseemann in appreciation of his comprehensive work on leeches.

**Variability.** The variability between examined specimens was relatively small. Differences were found in the intensity of the dark grayish pigmentation, in the position of the female gonopore (in the furrow b5/c11 or moved to the annulus c11), in the extension of the vasa deferentia (reaching annulus b2 or the posterior end of the sixth somite posterior to the female gonopore) and in the extension of the ovisacs reaching the anterior (annulus b2) or the posterior (annulus b6) of the third somite posterior to the female gonopore.

**Differential diagnosis.** The phylogenetic analysis based on COI data placed *Dina neseemanni* **sp. nov.** as a sister species of *Dina latestriata* Neubert & Neseemann, 1995, a species originally described from Lake Prespa (Neubert & Neseemann 1995). The average K2P genetic distance between these two species was estimated at  $12.9 \pm 1.7\%$  K2P, indicating their significant genetic separation. Morphologically, the two species can be distinguished by the position of gonopores. They are separated by two annuli in *D. neseemanni* **sp. nov.** and three in *D. latestriata*. In both species, the female gonopore is situated in the furrow between b5/b6. The male genital porus is situated in furrow between b2/a2 in *D. neseemanni* **sp. nov.** and in furrow between b1/b2 in *D. latestriata*.

From all other European yellow-spotted *Dina* species, *D. neseemanni* **sp. nov.** can be distinguished by the peculiar shape of the ovisacs, which combines three characteristics, (1) the part of the ovisacs that runs dorsally over the vasa deferentia is strongly coiled, (2) the ovisacs turn to the ventral side and run slightly windingly alongside the ventral nerve cord, (3) the ovisacs run first anteriorly across the somite boundary and then posteriorly on the ventral side. The latter characteristic is not shared by any of the known yellow-spotted leeches. In *D. latestriata*, *D. minuoculata*, *D. serbica*, *D. crnogorensis*, as well in *D. orientalis* from the Near and Middle East (Grosser et al. 2011), the ovisacs run exclusively dorsally over the vasa deferentia (Grosser et al. 2007, 2011; Grosser & Pešić 2022; Pešić & Grosser 2022). In *D. apathyi* Gedroyć, 1916, *D. stschegolewi* (Lukin & Epshtein, 1960), *D. pseudotrocheta* Grosser & Eiseler, 2008, as well as in *D. punctata* Johansson, 1927, and *D. p. mauchi* Neseemann, 1995, the ovisacs also turn to the ventral side and run alongside the



Figure 5. (a) Map of Montenegro with marked localities (1 – Blatina pond, 2 – pond near Lake Skadar). b-c Photographs of sampling sites of *Dina nesemanni* sp. nov. (b) Blatina pond (*locus typicus*); (c) pond near Lake Skadar. Photos by V. Pešić (b) and A. Bańkowska (c).

ventral nerve cord, but they are not strongly coiled in the part that passes over the vasa deferentia on the dorsal side (Grosser 2015).

**Distribution.** Montenegro, the central and southern part of the country, with a Mediterranean climate. Both known localities are permanent ponds irrigated by the nearby water bodies, the river Zeta (Blatine pond) and Skadar Lake (Livari pond), respectively. These lowland ponds are characterized by well-developed emergent vegetation dominated by common reed (Figure 5). We assume that new species is a characteristic but possibly an endangered representative of the fauna of the highly threatened Mediterranean ponds and swamps in the central and southern part of Montenegro.

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No potential conflict of interest was reported by the author(s).

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## Data availability statement

DNA sequences prepared during this study are deposited in the Barcode of Life Data Systems (<http://dx.doi.org/10.5883/DS-DINABLAT>) and GenBank.

## ORCID

A. Zawal  <http://orcid.org/0000-0002-5838-6060>  
V. Pešić  <http://orcid.org/0000-0002-9724-345X>

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