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Dina crnogorensis sp. nov. (Annelida, Hirudinea: Erpobdellidae) – a new leech species from Montenegro

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Abstract
A yellowish spotted and medium-sized new leech of the family Erpobdellidae with a typical annulation of the genus *Dina* was found in a spring in the northeastern Montenegro. In the present study, we used an integrative taxonomic approach that combines morphological and DNA barcoding data to describe a new species, *Dina crnogorensis* **sp. nov**. Morphologically, the new species is most similar to *Dina minuoculata* Grosser, Moritz & Pešić, 2007 and *Dina serbica* Pešić & Grosser, 2022 from the same geographical region, but also to *D. orientalis* Grosser, Nesemann & Pešić, 2011 from the Near and Middle East. Differences in anatomy and the results of species-delimitation analyses based on COI sequences supported species status of the new species.

Key words: Taxonomy, DNA-barcoding, species delimitation, new species, spring.

Introduction

The Western Balkans region is characterized by a complex geological and paleogeographic history that results in an extremely rich and diverse aquatic biota that inhabits this region (Pešić *et al*. 2020). Extensive karstification and erosion processes in the Dinarides, combined with climate aridization that began in the Miocene and significantly intensified during the Pleistocene glaciations, have had a major impact on the hydrology of the Western Balkans (Pešić *et al*. 2020). As a result, such habitat fragmentation was a stimulus for speciation events, but also served as microrefugia for aquatic biota (Pešić *et al*. 2020).

The leech fauna of the Western Balkans shows a high diversity, especially in erpobdellid leeches. Currently, 107 species or subspecies of limnic and terrestrical leeches are known from Europe, of which 50 species have been recorded from the Western Balkans, including 24 taxa of Erpobdellidae (20 of which belong to the Trochetinae) compared to 43 Erpobdellidae (and 36 Trochetinae) for Europe.

The leech fauna of the Western Balkans has long been the subject of research, resulting in a large number of published papers (e.g., Augener 1925, 1926; Sket 1968, 1989; Neubert & Nesemann 1995, 1999;

Grosser *et al*. 2007, 2014, 2015a, b, 2016, 2018; Marinković *et al*. 2019, 2020, 2022; Dmitrović & Pešić, 2020; Pešić & Grosser 2022) and a high number of species originally described from this area. Of the 56 leech taxa described in Europe over the last 60 years, 21 species (three of Glossiphoniidae, two of Piscicolidae, and 16 of Erpobdellidae) have been described from the Western Balkans. All newly described eprbdellid species from Western Balkans belong to the subfamily Trochetinae, half belong to *Dina ohridana*-group which appears to be endemic to the ancient Lake Ohrid in North Macedonia (Sket 1968; Trajanovski *et al*. 2010), while other species prefer spring and running water habitats and include many endemics. The two species previously described, i.e., cave leech *Dina absoloni* Johansson, 1913 (Erpobdellidae) and the land leech *Xerobdella anulata* Autrum, 1958 (Xerobdellidae) in their distribution are also restricted to the Western Balkans.

Based on the data available so far on the diversity of European leeches, it can be noticed that two European leech families have the highest diversity of species (and potentially a high tendency for a speciation): Piscicolidae in eastern, northern and central Europe and Erpobdellidae (especially Trochetinae) in southeastern Europe. This assumption should be verified by additional research that would include the application of molecular techniques. In recent survey we have discovered a new species from the northeastern Montenegro. From the latter area, and the neighboring West Serbia, recently we have discovered two new species (*D. minuoculata* and *D. serbica*), indicating the exceptional diversity of *Dina* leeches in the mountainous areas of the Western Balkans and the need for further research. A new species of the genus *Dina* from northeastern Montenegro will be described in this paper.

Material and Methods

Leeches were collected by tweezers from the underside of hard substratum (stones, wood) and on plants submerged in the water, on banks, as well as on the shore. Material was 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) was checked on the holotype with well developed sexual organs. Measurements were taken with a ruler (we consider the precision of such measurement as sufficient, because they anyway largely depend on the body contraction). Material was examined using a stereomicroscope (Novex), and photographs were taken with a microscope camera (Euromex, VC 3031C). All measurements are given in mm.

The holotype and 5 paratypes of *Dina crnogorensis* **sp. nov.** will be deposited in the invertebrate collection of the Martin-Luther-University Halle-Wittenberg (Martin-Luther-Universität Halle-Wittenberg, Zentralmagazin Naturwissenschaftlicher Sammlungen, Domplatz 4, 06108 Halle/Saale, Germany).

The type material of *Dina minuoculata* was deposited in the invertebrate collection of the Martin-Luther-University (MLU) in 2007. Recently, the type material of *Dina serbica* was transferred from Senckenberg Museum Frankfurt (SMF) to the MLU. This fact that type material of all three speciees is now deposited in the same collection should facilitate future research work.

Molecular analysis

Molecular analyses was conducted at the Canadian Centre for DNA Barcoding (Guelph, Ontario, Canada; (CCDB; http://ccdb.ca/)). In the latter institution the specimens were sequenced for the barcode region of COI following standard invertebrate DNA extraction, amplification and sequencing protocols (Ivanova *et al*. 2007, Ivanova & Grainger 2007a, b). For DNA-barcoding and phylogenetic analysis we used also previously published COI sequence data from GenBank. A dataset composed of 28 COI sequences representing COI haplotypes of *Dina dinarica* (n=1), *D. latestriata* (n=4), *D. l. lineata* (n=4), *D. l. lacustris* (n=3), *D. minuoculata* (n=2), *D. montana* (n =2), *D. prokletijaca* (n=2), *D. serbica* (n=3), *D. sketi* (n=4), and *D. crnogorensis* **sp. nov.** (n = 2) with *Erbobdella octoculata* (GBAN5615-14) as outgroup taxon (Table 1) was used for delimitation of species boundaries. In total, six COI sequences were obtained from specimens collected for the purpose of this study (Table 1) and are published in the Barcode of Life Data System (BOLD).

Table 1. List of newly sequenced specimens of the genus *Dina* used in this study. For the previously published sequences of *Dina* spp. see Pešić & Grosser (2022).

Locality (country, name)	Lat/Long	Voucher code	BOLD/Genbank Acc. nos.
***Dina crnogorensis* sp. nov.**			
Montenegro, Komovi, Trešnjevik, spring	42.7399 N, 19.6805 E	CCDB 38361 H03	DCDDJ087-21/ OM628835
Montenegro, Komovi, Trešnjevik, spring	42.7399 N, 19.6805 E	CCDB 38361 H04	DCDDJ088-21/ OM628834
***Dina sketi* Grosser & Pešić, 2014**			
Bosnia and Herzegovina, Lisina, Crna rijeka basin spring near touristic center Balkana	44.4128 N, 17.0442 E	CCDB 41822 H06	NOVMD090-21
Bosnia and Herzegovina, Lisina, Crna rijeka basin spring near touristic center Balkana	44.4128 N, 17.0442 E	CCDB 41822 H07	NOVMD091-21
Bosnia and Herzegovina, Lisina, Crna rijeka basin, spring near touristic center Balkana	44.4128 N, 17.0442 E	CCDB 41822 H08	NOVMD092-21
Bosnia and Herzegovina, Lisina, Crna rijeka basin spring near touristic center Balkana	44.4128 N, 17.0442 E	CCDB 41822 H09	NOVMD093-21

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 (Kumar *et al*. 2018). MEGAX software was used to calculate Neighbour-Joining (NJ) trees based on K2P distances (standard for barcoding studies) and pairwise deletion of missing data. 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.

In order to assess the genetic differentiation of species we used the online ASAP version (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) with default settings and the K2P distance model. The latter procedure was designated to a list of partitions of species hypotheses using genetic distances, calculated between DNA sequences and ranked by their ASAP-scores: the lower the score, the better the partition (Puillandre *et al*. 2021).

Results

Species delimitation using DNA-barcodes

The final alignment for species delimitation using COI sequence data comprised 658 nucleotide positions (nps) for 27 specimens of the *Dina* spp. and one *Erpobdella octoculata* as outgroup (Table 1). The nucleotide sequences could be translated into amino acid sequences without any stop codons. Neighbour-Joining (NJ) analysis clustered the *Dina* COI sequences into ten clades (Fig. 1). The sequence representing *Dina crnogorensis* **sp. nov.** is reconstructed as a sister clade to *D. serbica*. This sister group relationship was supported with high support values (NY support 91%). The genetic distance between the COI sequences of these two species was estimated at 9.1±1.2 % K2P (Table 2).

The COI sequences obtained from specimens of *Dina sketi* from Bosnia and Herzegovina for the purposes of this study, placed the later species as a sister group (albeit with no considerable support) of a clade consisting of two subclades corresponding to *D. prokletijaca*, and *D. montana*, respectively, two species known from the alpine regions of Montenegro and Kosovo.

The ASAP method allowed to observe barcode gap at about 4-8% K2P distances (Fig. 1A). The applied ASAP procedure identified 10 MOTUs (hypothetical species) at the threshold distance of 3.0 % (K2P) which has the best ASAP-score (2.0) within the available molecular data: (1) *Dina montana*, (2) *D. prokletijaca*, (3) *D. sketi*, (4) merged *D. l. lineata* and *D. l. lacustris*, (5) *D. latestriata* from Prespa Lake, (6) *D.* cf. *latestriata* from Trichonis Lake, (7) *D. minuoculata*, (8) *D. serbica*, (9) *D. dinarica* and (10) *D. crnogorensis* **sp. nov**. At the threshold distance of 8.59 % (K2P) which had the second best ASAP-score

(3.0) but first P-val rank within the available molecular data, *Dina montana*, *Dina prokletijaca* and *D. sketi* were merged in one group.

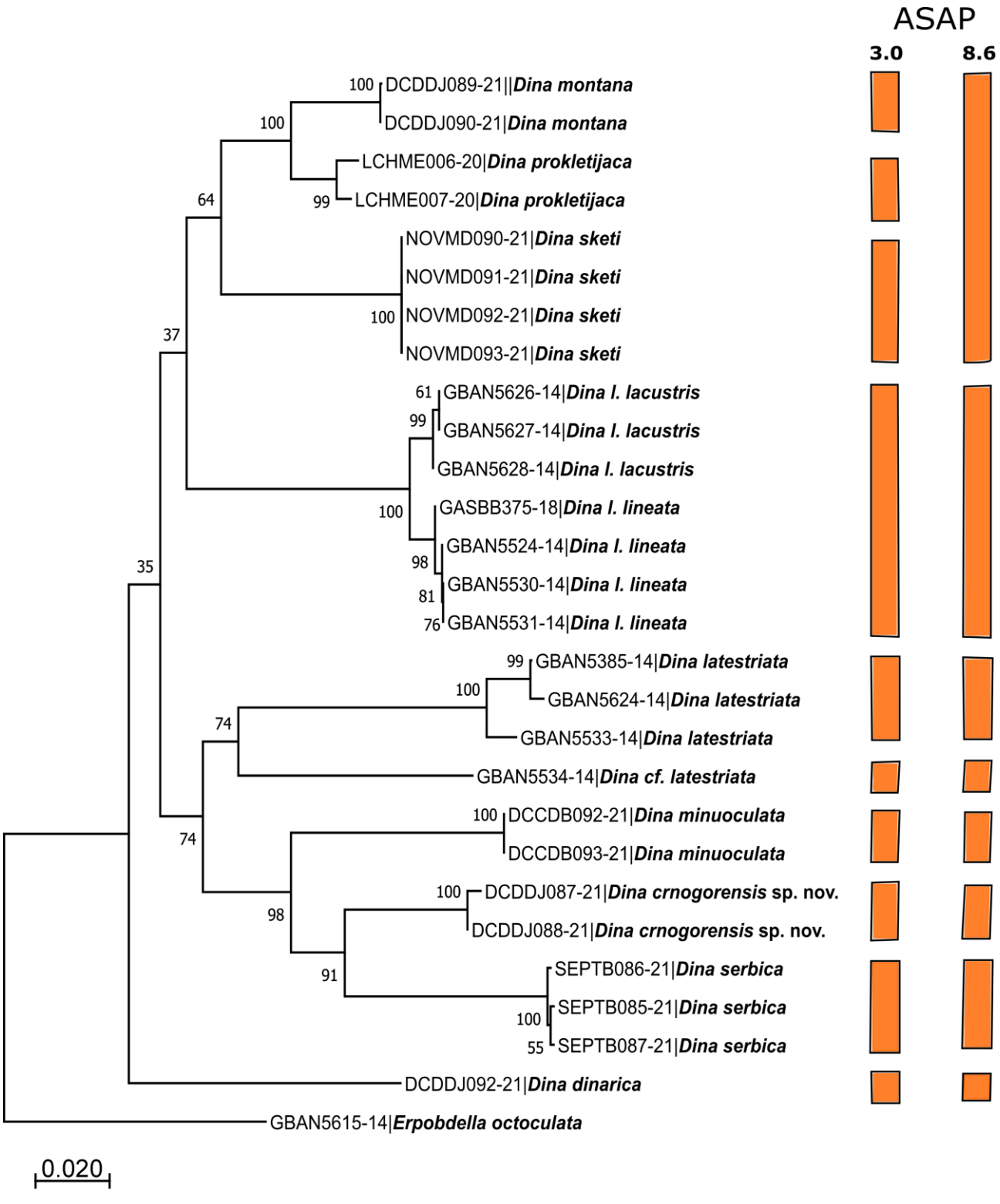

Figure 1. Neighbour-Joining tree of the *Dina* spp. obtained from 28 nucleotide COI sequences. The results of species delimitation by ASAP procedure are indicated by vertical bars.

Table 2. Estimates of genetic distance (K2P) between studied *Dina* spp. The number of base substitutions per site from averaging over all sequence pairs between species are shown. Standard error estimate are shown above the diagonal. This analysis involved 28 nucleotide sequences. There were a total of 668 positions in the final dataset.

Species	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
(1) *D. montana*		0,008	0,016	0,016	0,016	0,013	0,020	0,019	0,018	0,017	0,018
(2) *D. prokletijaca*	0,041		0,015	0,015	0,016	0,012	0,019	0,017	0,017	0,016	0,017
(3) *D. l. lineata*	0,122	0,115		0,005	0,018	0,015	0,019	0,018	0,018	0,017	0,020
(4) *D. l. lacustris*	0,118	0,113	0,016		0,018	0,016	0,020	0,018	0,019	0,018	0,020
(5) *D. dinarica*	0,137	0,129	0,156	0,155		0,017	0,021	0,020	0,019	0,019	0,020
(6) *D. sketi*	0,093	0,084	0,126	0,126	0,142		0,020	0,019	0,018	0,018	0,018
(7) *D. latestriata*	0,178	0,173	0,172	0,176	0,192	0,179		0,017	0,019	0,018	0,019
(8) *D. cf. latestriata*	0,160	0,147	0,158	0,151	0,183	0,164	0,142		0,019	0,018	0,019
(9) *D. minuoculata*	0,158	0,150	0,167	0,165	0,189	0,174	0,162	0,165		0,014	0,014
(10) *D. crnogorensis*	0,154	0,142	0,150	0,157	0,181	0,164	0,157	0,151	0,115		0,012
(11) *D. serbica*	0,166	0,155	0,190	0,195	0,196	0,177	0,169	0,172	0,120	0,091	

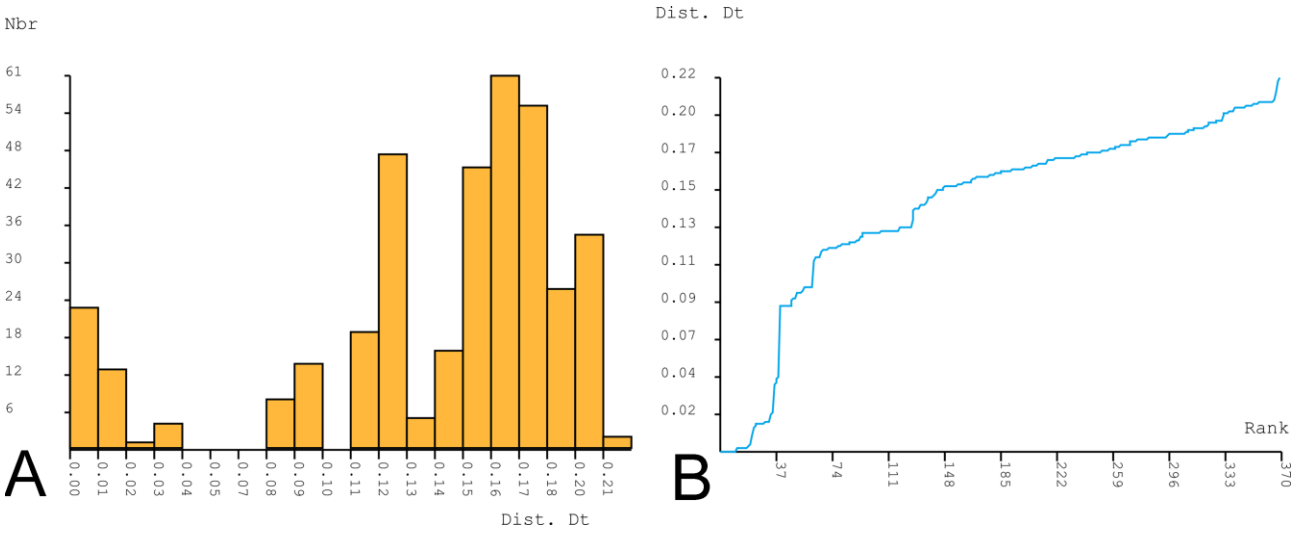

Figure 2. Results of ASAP analysis for COI sequences. (A) Distribution of pairwise differences, (B) Ranked pairwise differences.

Systematics

Annelida Lamarck, 1809
Clitellata Michaelson, 1919
Hirudinea Lamarck, 1818

Family Erpobdellidae R. Blanchard, 1894
Subfamily Trochetinae Perrier, 1897

Genus *Dina* R. Blanchard, 1882

***Dina crnogorensis* Grosser & Pešić sp. nov.**
http://zoobank.org/urn:lsid:zoobank.org:act:ABD1010C-D3D2-40FE-A25B-CAEA27B8A889
Figures 3A, 4, 5A-E

Material examined — Holotype (MLU), sequenced (voucher code: CCDB 38361 H03), Montenegro, northeastern Montenegro, Komovi Mt., Trešnjevik, spring, 42.739948 N, 19.680511 E, 24 June 2021, leg. V. Pešić, body length 52 mm, width 10 mm, dissected. Paratypes: five specimens (body length/width: 34/6.5 mm, 32/6 mm, 31/6 mm, 22/5 mm, 18/4.5 mm; MLU), same data as holotype, one specimen of them sequenced (CCDB 38361 H04). All material is deposited in the zoological collection of the Martin-Luther-University Halle-Wittenberg/Germany (MLU).

Locus typicus — Montenegro, Northeast Montenegro, Dinaric Mountains, Komovi Mt., Trešnjevik, spring, 42.739948 N, 19.680511 E.

Diagnosis — Medium sized leeches; dorsal side dark brownish to black with large bright yellowish spots on each annulus and a pair of dark paramedian stripes; ventral side brigther grayish to light brown; annulation *Dina*-like: quinqueannulate with annulus b6 broadened, genital pores are separated by two annuli (male gonopore in furrow b2/a2, the female in furrow b5/b6); papillae on dorsal and ventral sureface very small; eyes are greatly reduced, no or only a few eyes visible.

Description
Habitus — Medium sized leeches; preserved and contracted adults reach a body length up to 52 mm and a width up to 10 mm (holotype). The body dorso-ventrally flattened, the first third (preclitellar and clitellar region) cylindrical. The caudal sucker only slightly wider than the half of maximum body width. The cranial sucker has a wide but slightly narrowed mouth opening with no or only slightly elongated upper lip. Papillae small and inconspicuous on the dorsal and ventral surface.

Annulation — The annulation typical for the genus *Dina*. The midbody somites quinqueannulate and heteronomously subdivided by clear furrows into annuli b1, b2, a2, b5 and the broadened annulus b6. The annuli do not show a tendency to split; only sometimes, b6 is subdivided by a very shallow furrow on the ventral side; the splitting is mostly not visible on the lateral side, but the tendency to split the annuli increases slightly in the posterior half of the body, but is never obvious.

The male genital pore situated in the furrow b2/a2, the female in the furrow b5/b6; the genital pores separated by two annuli.

Eyes — This new species is characterized by a strong reduction of visible eyes. Only two leeches of the examined material have visible eyes. One specimen (paratype, 34 mm in length) has a single eye that vas poorly visible; in the second specimen (paratype, 18 mm in length) four very small eyes were present, also faintly visible, situated in the anterior transverse row.

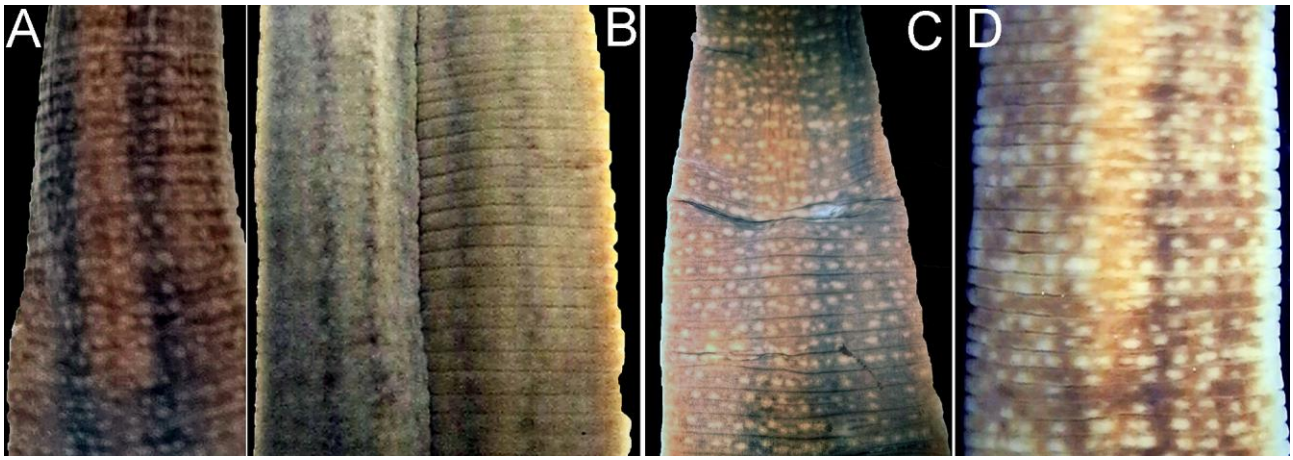

Figure 3. Colour, dorsal view. A — *Dina crnogorensis* **sp. nov.** (holotype); B — *Dina minuoculata* (left paratype, right holotype); C — *Dina serbica* (holotype); D — *Dina orientalis* (paratype).

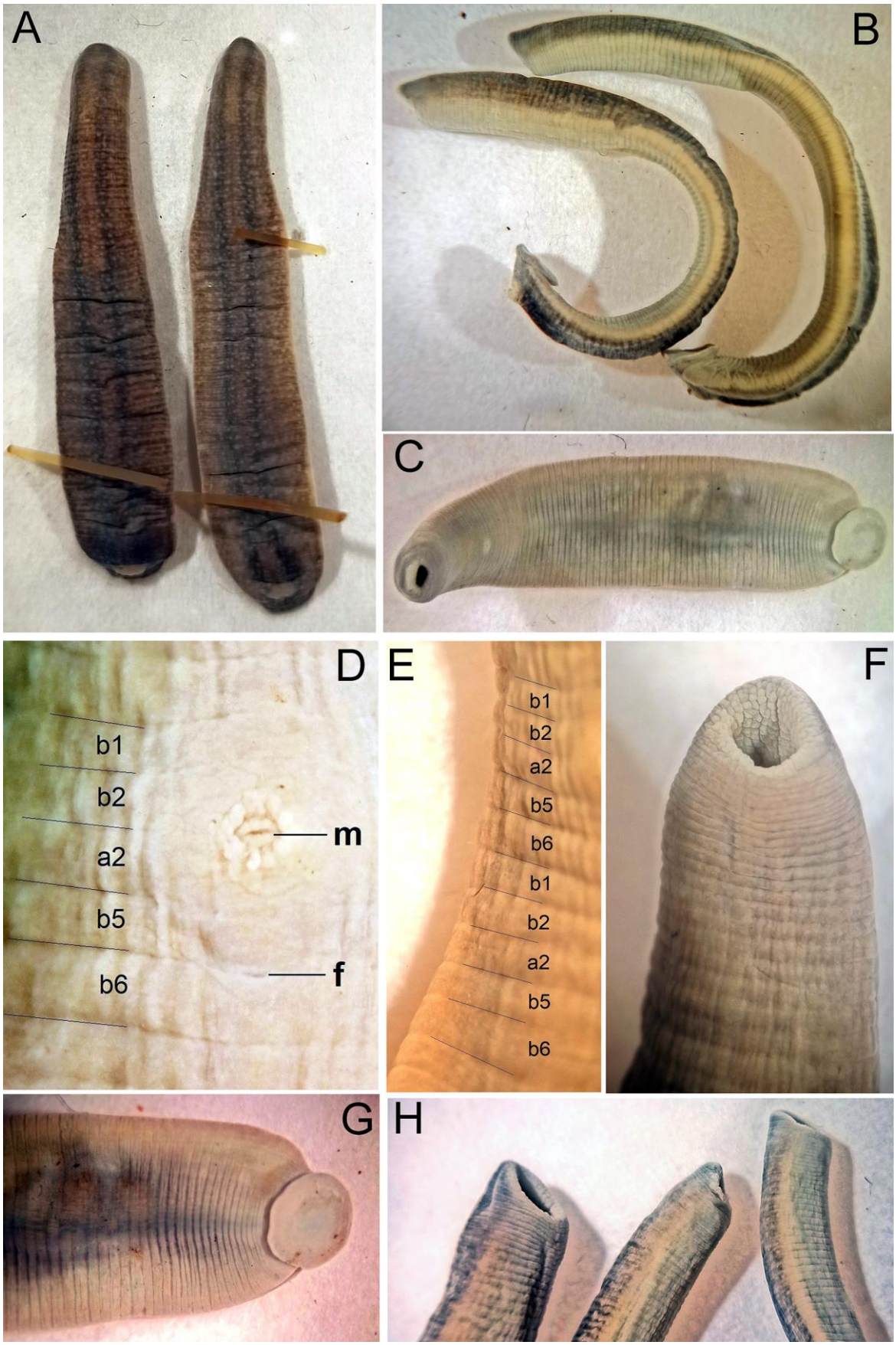

Figure 4. *Dina crnogorensis* **sp. nov.**, external morphology. A — Dorsal view; B — lateral view; C — ventral view; D — position of genital pores; E — annulation; F — caudal sucker; G — cranial sucker, ventral view; H — cranial sucker, lateral view.

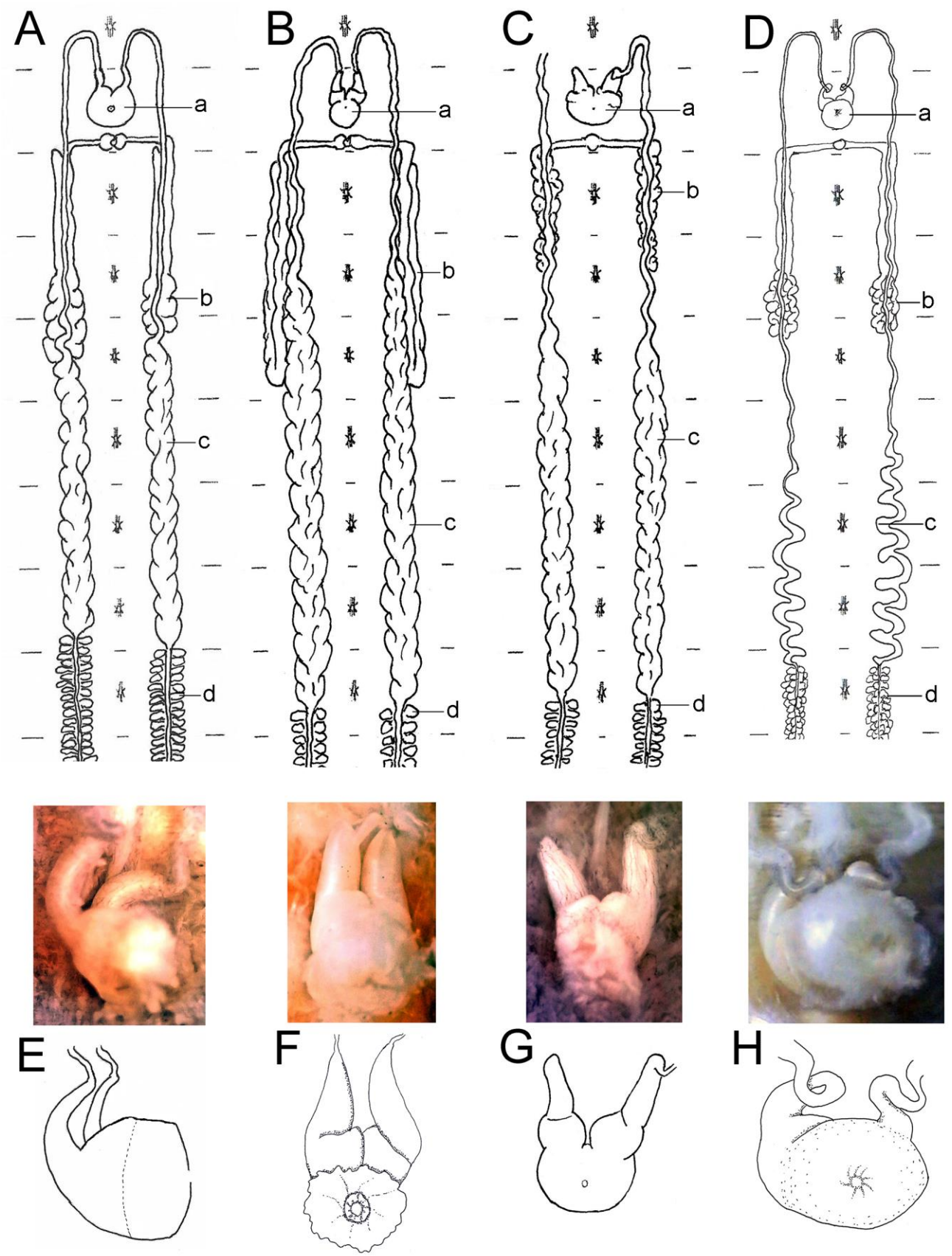

Figure 5. Schematic diagrams of reproduktive system (A-D) and genital atrium (E-H; photographs and drawings, respectively) of selected *Dina* spp. A, E — *Dina crnogorensis* **sp. nov.**, holotype; B, F — *Dina minuoculata*, paratype; C, G — *Dina serbica*, paratype; D, H — *Dina orientalis*, holotype. Abbreviations: a — genital atrium, b — ovarian sacks, c — vas deferens, d — testisacs.

Colour — Dorsal side brownish to blackish with numerous yellowish spots of each annulus in similar number; sometimes there are more spots on annulus a2, than the leech has yellowish transverse rows. Paramedian stripes dark and wide, by yellowish spots with dissolved contours. The anterior part (rarely the posterior part) medially lighter between the stripes. Ventral side brighter.

Sexual organs — Genital atrium: atrium body large; cornua thin and short (reaches to b1/b6), distinctly curved to the ventral side, the ends straight and not coiled. Vas deferens almost straight up to the end of the second somite after the female gonopore, strongly coiled and thickened from the third ganglion to the sixth somite after the female gonopore. Ovisacs in the entire expansion dorsally over the vasa deferentia, anterior part uncoiled, strongly coiled from the second ganglion after the female gonopore up to the third somite.

Etymology — Named after the country from which the type material was collected.

Figure 6. Photograph of the type locality of *Dina crnogorensis* **sp. nov**.: spring at Trešnjevik, Komovi Mt., Montenegro. Photo by V. Pešić

Differential diagnosis — In regard to the external morphology *Dina crnogorensis* **sp. nov.** is most similar to *D. minuoculata* Grosser, Moritz & Pešić, 2007 and *D. serbica* Pešić & Grosser, 2022, from the same geographical region, and *Dina orientalis* Grosser, Nesemann & Pešić, 2011 from the Near and Middle East. All four species share the presence of bright yellowish spots on the dorsal surface of each annulus. The two species from the Western Balkans can be separated from the new species in the shape of ovarian sacks**.** The ovisacs in *D. minuoculata* are not coiled and extend to the end of the third somite after the female genital pore; in *D. serbica* the ovisacs are coiled and short, reaching to the second ganglion after the female genital pore. *Dina orientalis*, a species originally described from Lebanon but also reported from Syria, Turkey and Iran (Grosser *et al.* 2011), resembles the new species in the shape of ovisacs, but differs in vas deferens

which becomes slightly coiled from the fourth (rarely third) ganglia behind the female genital pore and by more numerous (12 – 17) yellowish spots on annuli a2 and b5 and rarer on b6.

In addition to the above-mentioned species, the only yellowish spotted species from the Balkans is *Dina latestriata* Neubert & Nesemann, 1995, which can be easily distinguished by genital pores separated by three annuli (not two annuli as in *D. crnogorensis* sp. nov. and the other mentioned yellowish spotted species).

Distribution – Montenegro; so far only known from the locus typicus, a spring in northeastern Montenegro.

Habitat – The new species was collected in a spring situated in a deciduous forest dominated by the common beech (*Fagus sylvatica* L.).

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