Ecologica Montenegrina
ISSN 2336-9744 (online) | ISSN 2337-0173 (print)

Ecologica Montenegrina 51: 1-14 (2022)
This journal is available online at: www.biotaxa.org/em
https://dx.doi.org/10.37828/em.2022.51.1

Article

http://zoobank.org/urn:lsid:zoobank.org:pub:F9A3E295-DFAC-4CDA-8DA7-63D564D05464

Dina serbica, a new species of leeches (Annelida: Hirudinea: Erpobdellidae) from Serbia, based on morphological and molecular evidence

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Received 19 January 2022 | Accepted by *V. Pešić*: 9 February 2022 | Published online 10 February 2022.

Abstract
In the present study we used an integrative taxonomic approach that combines morphological and DNA barcoding data to describe a new species, *Dina serbica* **sp. nov.** from a spring in western Serbia. The average K2P-distance between DNA-barcode sequences of *D. serbica* **sp. nov.** and its closest relative *D. minuoculata* was estimated at 11.96±1.39%. Moreover, the ASAP (assemble species by automatic partitioning) species-delimitation method based on COI sequences from our study and sequences of *Dina* species from GenBank supported the species-status of *D. serbica* **sp. nov.** The first DNA barcoding data for *D. dinarica* (Sket, 1968), *D. montana* (Sket, 1968), *D. minuoculata* Grosser, Moritz & Pešić, 2007 and *D. prokletijaca* Grossser & Pešić, 2016 were uploaded into the Barcode of Life DataSystems database. The study confirmed the efficiency of a standard molecular COI marker in identification of *Dina* leeches, emphasising the need for additional morphological and molecular analysis of species of this genus in the Balkans.

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

Introduction

The genus *Dina* Blanchard, 1892 includes medium to large sized leeches distributed across the Western Palearctic (Neubert and Nesemann 1999), inhabiting different types of running and standing waters. Traditionally, *Dina* leeches have been distinguished by their annulation pattern: the last annulus per somite is widened and subdivided once (*vs*. in *Trocheta* Dutrochet, 1817 the last and often also the first annulus is further subdivided, while *Erpobdella* de Blainville in Lamarck, 1818 has five unsubdivided annuli per somite) (Neubert and Nesemann 1999). Nevertheless, Trontelj and Sket (2000) showed that the annulation pattern is not suitable for the diagnosis of *Dina* and *Trocheta*, and problematized the status of Trochetinae because it does not represent a monophyletic group. Siddal (2002) formally synonymized the genera *Dina* and *Trocheta* (as well as the non-European *Mooreobdella* and *Nephelopsis*) with the genus *Erpobdella*, a concept not widely accepted by other authors who continued to treat *Dina* and *Trocheta* as separate genera (e.g., Grosser *et al*. 2011; Grosser 2015; Ben Ahmed *et al*. 2015; Darabi-Darestani *et al*. 2021).

According to Neubert and Nesemann (1999), the genus consists of three morphologically well-defined groups, i.e., *D. lineata*, *D. ohridana* and *D. absoloni*-group. The latter group includes the stygobiotic *Dina absoloni* Johansson, 1913, which inhabits the southeastern parts of the Dinaric region (Grosser *et al*. 2015b), and *D. latestriata* Neubert and Nesemann, 1995, a species originally described from the shallow littoral zone of Lake Prespa in North Macedonia (Neubert and Nesemann 1995). *Dina ohridana*-complex includes a number of endemic species known from the ancient Lake Ohrid in North Macedonia and adjacent region (Neubert and Nesemann 1999). The phylogenetic relationships, as well as the spatial and temporal speciation of leeches of the latter complex in Lake Ohrid were studied by Trajanovski *et al*. (2010).

Dina lineata-complex is most diverse group and includes more widespread taxa (Neubert and Nesemann 1999). There are currently nine species of *D. lineata*-complex inhabiting the Western Balkan (Sket 1968; Grosser *et al*. 2007, 2014, 2015a, b, 2016; Marinković *et al*. 2021) including some of the recently described species (i.e., *D. minuoculata*, *D. sketi*, *D. prokletijaca*), and all but one (i.e., *D. l. lineata*) of these are distributed over a relatively small area. *Dina minuoculata* Grosser, Moritz & Pešić, 2007 inhabits the northern part of Montenegro and the southwestern part of Serbia (Grosser *et al*. 2015b; Marinković *et al*. 2021); *D. sketi* Grosser & Pešić, 2014 inhabits a small area which cover northwestern Bosnia and Herzegovina (Grosser *et al*. 2014), while *D. prokletijaca* Grosser & Pešić, 2016 is known only from the alpine region of Kosovo (Grosser *et al.* 2016).

So far, phylogenetic relationships within the genus *Dina*, with the exception of the *D. ohridana*-complex, have been largely unexplored and this study provides the first "glimpse" into the molecular diversity of this important limnofaunistic group in the Western Balkans. As a result of an integrative taxonomic approach, we discovered one new species of the genus *Dina* from Serbia, described in the present study.

Material and Methods

Leeched 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 one specimen 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 two paratypes of the new species will be deposited in the Senckenberg Museum Frankfurt (SMF); one paratype specimen is kept in the collection of the first author.

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 and Grainger 2007a, b). In total, ten COI sequences were obtained from specimens collected by the first author and are published in the Barcode of Life Data System (BOLD). For DNA-barcoding and phylogenetic analysis we used also previously published COI sequence data from GenBank. A dataset composed of 75 COI sequences representing COI haplotypes of *Dina dinarica* (n=1), *D. krilata* (n=11), *D. latestriata* (n=4), *D. l. lineata* (n=4), *D. l. lacustris* (n=3), *D. lepinja* (n=8), *D. lyhnida* (n=13), *D. minuoculata* (n=2), *D. montana* (n=2), *D. ohridana* (n=17), *D.* cf. *profunda* (n=4), *D. prokletijaca* (n=2), *D. serbica* **sp. nov**. (n=3) and *D.* cf. *svilesta* (n=1), with *Erbobdella octoculata* (GBAN5615-14) as outgroup taxon (Table 1) was used for delimitation of species boundaries.

Table 1. List of sequences used in this study.

Locality (country, name)	Lat/Long	Voucher code	BOLD/Genbank Acc. nos.
***Dina dinarica* (Sket, 1968)**			
Montenegro, Cetinje, spring in Tomići village	42.254 N, 18.9902 E	CCDB 38361 H08	DCDDJ092-21/ OM628830
***Dina latestriata* Neubert & Nesemann, 1995**			
Greece, Lake Trichonis, NW shore, S of Panetolio	38.58893 N, 21.46703 E	HM246575	GBAN5534-14
Albania, Lake Prespa	40.76848 N, 20.92642 E	HM246569	GBAN5385-14
Macedonia, Lake Prespa, Golem Grad	40.86655 N, 20.98989 E	HM246600	GBAN5624-14
Greece, Lake Mikri Prespa, Mikrolimni	40.74215 N, 21.10763 E	HM246610	GBAN5533-14
***Dina lyhnida* Šapkarev, 1990**			
Macedonia, Lake Ohrid, in front of Gorica Hill	41.08783 N, 20.79277 E	HM246589	GBAN5569-14
Macedonia, Lake Ohrid, S of Sv. Zaum	40.93950 N, 20.77754 E	HM246595	GBAN5576-14
Macedonia, Lake Ohrid, transect Bulbic	41.1005 N, 20.8003 E– 41.1006 N, 20.7943 E	HM246578	GBAN5578-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246541	GBAN5588-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246546	GBAN5589-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246549	GBAN5590-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246550	GBAN5591-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246563	GBAN5592-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246573	GBAN5593-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246574	GBAN5594-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246526	GBAN5616-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246582	GBAN5595-14
Macedonia, Lake Ohrid, transect in front of Hotel Park	41.0931 N, 20.7953 E– 41.0931 N, 20.7891 E	HM246577	GBAN5610-14
***Dina lepinja* Sket & Šapkarev, 1986**			
Macedonia, Lake Ohrid, in front of Gorica Hill		HM246588	GBAN5568-14
Macedonia, Lake Ohrid, S of Sv. Naum	40.93950 N, 20.77754 E	HM246597	GBAN5575-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246561	GBAN5587-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246544	GBAN5586-14
Macedonia, Lake Ohrid, transect in front of Hotel Park	41.0931 N, 20.7953 E– 41.0931 N, 20.7891 E	HM246539	GBAN5606-14
Macedonia, Lake Ohrid, transect in front of Hotel Park	41.0931 N, 20.7953 E– 41.0931 N, 20.7891 E	HM246543	GBAN5607-14
Macedonia, Lake Ohrid, transect in front of Hotel Park	41.0931 N, 20.7953 E– 41.0931 N, 20.7891 E	HM246556	GBAN5608-14
Macedonia, Lake Ohrid, transect in front of Hotel Park	41.0931 N, 20.7953 E– 41.0931 N, 20.7891 E	HM246571	GBAN5609-14
***Dina krilata* Sket, 1989**			
Albania, Lake Ohrid	40.93412 N, 20.6478 E	HM246629	GBAN5377-14
Albania, Lake Ohrid	40.91579 N, 20.65533 E	HM246625	GBAN5379-14
Albania, Lake Ohrid	40.91579 N, 20.65533 E	HM246626	GBAN5380-14
Macedonia, Lake Ohrid, in Front of Hotel Metropol	41.05635 N, 20.79671 E	HM246593	GBAN5562-14
Macedonia, Lake Ohrid, in front of Gorica Hill	41.08783 N, 20.79277 E	HM246586	GBAN5567-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246538	GBAN5582-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246542	GBAN5583-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246558	GBAN5584-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246572	GBAN5585-14
Macedonia, Lake Ohrid, transect in front of Struga	41.143 N, 20.687 E	HM246531	GBAN5618-14
Macedonia, Lake Ohrid, transect in front of Struga	41.143 N, 20.687 E	HM246532	GBAN5619-14
***Dina l. lacustris* Sket, 1968**			
Macedonia, Ligemii Bogovines	41.95069 N, 20.79578 E	HM246606	GBAN5626-14
Macedonia, Ligemii Bogovines	41.95069 N, 20.79578 E	HM246607	GBAN5627-14
Macedonia, Ligemii Bogovines	41.95069 N, 20.79578 E	HM246611	GBAN5628-14

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TABLE 1

***Dina l. lineata* (O. F. Müller, 1774)**			
Finland, Vinnurvanoja metsae	63.8703 N 24.7417 E	-	GASBB375-18
Germany, Mecklenburg-Western Pomerania, Parchim district	53.445 N, 11.910 E	HM246552	GBAN5524-14
Germany, Mecklenburg-Western Pomerania, Parchim district	53.445 N, 11.910 E	HM246583	GBAN5530-14
Germany, Mecklenburg-Western Pomerania, Parchim district	53.445 N, 11.910 E	HM246584	GBAN5531-14
***Dina minuoculata* Grosser, Moritz & Pešić, 2007**			
Montenegro, spring+first order stream in the Tara river canyon (type locality)	42.9862 N, 19.4349 E	CCDB38233 H08	DCCDB092-21/ OM628837
Montenegro, spring+first order stream in the Tara river canyon (type locality)	42.9862 N, 19.4349 E	CCDB38233 H09	DCCDB093-21/ OM628836
***Dina montana* (Sket, 1968)**			
Montenegro, Komovi Mt., Štavna, spring (type locality)	42.7031 N, 19.676 E	CCDB 38361 H05	DCDDJ089-21/ OM628833
Montenegro, Komovi Mt., Štavna, spring (type locality)	42.7031 N, 19.676 E	CCDB 38361 H06	DCDDJ090-21/ OM628832
***Dina ohridana* Sket, 1968**			
Macedonia, Lake Ohrid In Front of Hotel Metropol	41.05635 N, 20.79671 E	HM246592	GBAN5563-14
Macedonia, Lake Ohrid, Camping \`Livadiste\`	41.12643 N, 20.64184 E	HM246567	GBAN5566-14
Macedonia, Lake Ohrid, in front of Hotel Desaret	41.01262 N, 20.80513 E	HM246594	GBAN5572-14
Macedonia, Lake Ohrid, S of Sv. Naum	40.93950 N, 20.77754 E	HM246596	GBAN5577-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1028 N, 20.8019 E– 41.1028 N, 20.7967 E	HM246548	GBAN5596-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1028 N, 20.8019 E– 41.1028 N, 20.7967 E	HM246562	GBAN5597-14
Macedonia, Lake Ohrid, transect in front of Hotel Park	41.0931 N, 20.7953 E– 41.0931 N, 20.7891 E	HM246557	GBAN5611-14
Macedonia, Lake Ohrid, transect in front of Hotel Park	41.0931 N, 20.7953 E– 41.0931 N, 20.7891 E	HM246579	GBAN5612-14
Macedonia, Spring Lake Near Main Springs of Sv. Naum	40.91019 N, 20.74793 E	HM246623	GBAN5631-14
Macedonia, Spring Lake Near Main Springs of Sv. Naum	40.91019 N, 20.74793 E	HM246624	GBAN5632-14
Macedonia, Spring Lake of Sv. Naum	40.91444 N, 20.74226 E	HM246614	GBAN5633-14
Macedonia, Spring Lake of Sv. Naum	40.91444 N, 20.74226 E	HM246615	GBAN5634-14
Macedonia, Spring Lake of Sv. Naum	40.91444 N, 20.74226 E	HM246618	GBAN5635-14
Macedonia, Spring Lake of Sv. Naum	40.91444 N, 20.74226 E	HM246619	GBAN5636-14
Macedonia, Spring Near Old Camping Site of Sv. Naum	40.91208 N, 20.74213 E	HM246631	GBAN5637-14
Macedonia, Spring Near Old Camping Site of Sv. Naum	40.91208 N, 20.74213 E	HM246632	GBAN5638-14
Macedonia, Spring Near Old Camping Site of Sv. Naum	40.91208 N, 20.74213 E	HM246633	GBAN5639-14
***Dina* cf. *profunda* Šapkarev, 1990**			
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246581	GBAN5581-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246540	GBAN5579-14
Macedonia, Lake Ohrid, transect Hyd.Institute - Radozda	41.1031 N, 20.7302 E	HM246547	GBAN5580-14
Macedonia, Lake Ohrid In Front of Hotel Metropol	41.05635 N, 20.79671 E	HM246534	GBAN5603-14
***Dina prokletijaca* Grosser & Pešić, 2016**			
Kosovo*[1], Peje, Lebush, spring Toplla (type locality)	42.6283 N, 20.246 E	KOS2 1|	LCHME006-20/ OM628827
Kosovo*, Peje, Lebush, spring Toplla (type locality)	42.6283 N, 20.246 E	KOS2 2|	LCHME007-20/ OM628840
***Dina serbica* sp. nov.**			
Serbia, spring along the road to Kamena Gora	43.32333 N, 19.57636 E	CCDB 38362 H01	SEPTB085-21/ OM628829

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[1]This designation is without prejudice to positions on status, and in line with UNSCR 1244 and the ICJ Opinion on the Kosovo Declaration of Independence.

TABLE 1

Serbia, spring along the road to Kamena Gora	43.32333 N, 19.57636 E	CCDB 38362 H02	SEPTB086-21/ OM628828
Serbia, spring along the road to Kamena Gora	43.32333 N, 19.57636 E	CCDB 38362 H03	SEPTB087-21/ OM628838
***Dina* cf. *svilesta* Sket, 1989**			
Macedonia, Lake Ohrid In Front of Hotel Metropol	41.05635 N, 20.79671 E	HM246598	GBAN5565-14
***Erpobdella octoculata* (Linnaeus, 1758)**			
Macedonia, Lake Ohrid,transect in front of Hotel Park	41.0931 N, 20.7953 E– 41.0931 N, 20.7891 E	HM246555	GBAN5615-14

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 75 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 COI sequences into nine clades (Fig. 1). The sequence representing *Dina serbica* **sp. nov.** is reconstructed as a sister branch to the clade grouping COI sequences found in *D. minuoculata*. The genetic distance between the COI sequences of these two species was estimated at 11.96±1.39% K2P.

The clade formed by *D. serbica* **sp. nov.** and *D. minuoculata* was placed as the sister group of a clade grouping sequences of *D. latestriata*. Sequences from specimens of the latter species were grouped in two clades. The first highly supported clade contains sequences of the specimens collected from Lake Prespa from where this species was originally described. On the other hand, another published sequence of *Dina latestriata* from Trichonis Lake in Greece was quite distantly related, and formed a separate lineage. The K2P distance of 14.21±1.72% between these two clades is of interspecific level.

A large clade (supported by an NJ bootstrap value of 99%) clustered species of the *Dina ohridana*-group. The range of K2P distances between species of *D. ohridana*-clade varies from 0.39±0.25 % (between *D.* cf. *profunda* and *D. svilesta*) to 2.83±0.67% (between *D. krilata* and *D. lyhnida*).

Dina prokletijaca, in our tree represented by the two specimens from the *locus typicus* (spring Toplla in Kosovo), was placed as the sister clade of *D. montana*. This sister group relationship was supported with a high support value (98%). The genetic distance between these two species was estimated at 4.1±0.81% K2P. The sequences of *Dina lineata* forms a well-supported clade, which is subdivided into two subclades: the first one comprising sequences of nominal *D. l. lineata* from Germany and Finland, and the second which includes topotypes of *D. l. lacustris* from a small glacial lake north of Lake Ohrid. The average K2P genetic distance between these two subspecies was estimated to be 1.6±0.52% K2P. Interestingly, the NY tree revealed *D. lineata*-clade as a sister clade of the *D. ohridana*-group, but with low support (bootstrap value 33%).

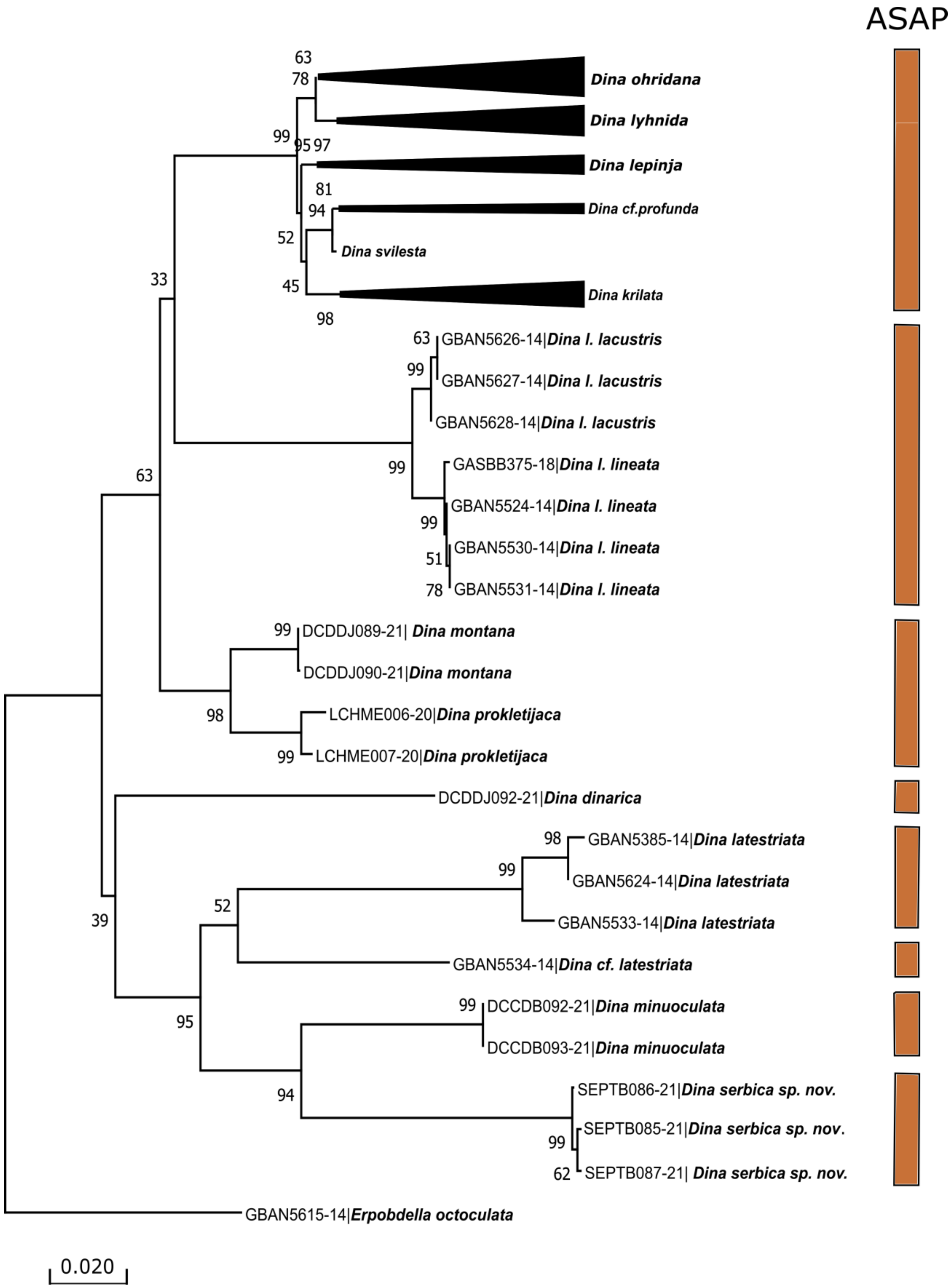

Figure 1. Neighbour-Joining tree of the *Dina* spp., obtained from 75 nucleotide COI sequences. The results of species delimitation by ASAP procedure are indicated by vertical bars.

Another distinct lineage was formed by one sequence of *Dina dinarica*. The level of COI differentiation between *D. dinarica* and the nominal subspecies of *D. lineata* was estimated to 15.61±1.76% K2P divergence indicating long independent history of these taxa.

The mean K2P intraspecific distances within the clades were relatively low, with maximum values found in *D. latestriata* (1.58±0.47%) and *D. prokletijaca* (0.92±0.37%).

The ASAP (assemble species by automatic partitioning) method allowed to observe barcode gap at about 4-7% K2P distances (Fig. 2A). The applied ASAP procedure identified 8 MOTUs (hypothetical species) at the threshold distance of 5.68 % (K2P) which has the best ASAP-score (3.0) within the available molecular data: (1) *H. ohridana*-group, (2) merged *D. montana* (merging *D. prokletijaca*), (3) *D. l. lineata* (merging *D. l. lacustris*), (4) *D. dinarica*, (5) *D.* cf. *latestriata* from Trichonis Lake, (6) *D. latestriata* from Prespa Lake, (7) *D. minuoculata*, and (8) *D. serbica* **sp. nov**.

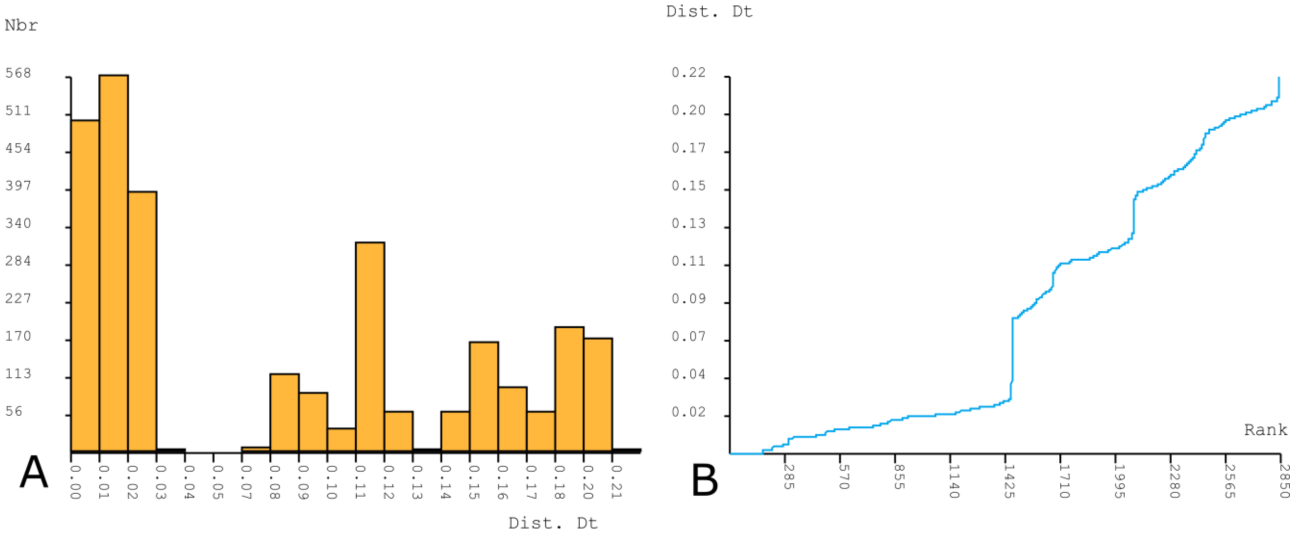

Figure 2. Results of ASAP analysis for COI sequences. (A) Distribution of pairwise differences, (B) Ranked pairwise differences.

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 serbica* sp. nov.**
http://zoobank.org/urn:lsid:zoobank.org:act:5863D4B2-FB65-40B4-A511-AAF022DCA97B
Fig. 3, 4B

Material examined — Holotype (SMF), sequenced (voucher code: CCDB 38362 H03), Serbia, Western Serbia, Kamena Gora, spring on the road to Kamena Gora, 43.32333 N, 19.57636 E, 21 July 2021, leg. Pešić, body length 40 mm, width 6.5 mm, caudal sucker 4.5 mm wide. Paratypes: three specimens (body length/width: 42/7 mm, 42/7 mm, 34/6 mm), same data as holotype, two specimens of them sequenced (CCDB 38362 H01-H02; SMF), one specimen (CCDB 38362 H01) dissected.

Diagnosis — Dorsal surface with numerous yellowish spots. Genital pores separated by two annuli (the male gonopore in the furrow b2/a2, the female in the furrow b5/b6). Atrial cornua short. Ovisacs short extending to annulus a2 of the second somite behind the female genital pore, and curled in their entire course.

Description. *Habitus* — Medium sized leeches; preserved and contracted adults reach a body length of 42 mm (paratype). The body is slightly dorso-ventrally flattened. The caudal sucker is slightly narrower than the maximum body width. The mouth is wide; the upper lip of the oral sucker not elongated (Fig. 3A).

Annulation — Leeches with a typical *Dina*-like annulation. The midbody somites quinqueannulate with annulus b6 only slightly broadened, more clearly broadened in the last half of the body. Annulus b6 not or only very slightly subdivided into annuli c11 and c12, other annuli not subdivided. 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 — The visible eyes reduced. The holotype and two studied paratypes possesses one eye each.

Colour — The colour of living specimens varies brownish to greenish. The dorsal surface of preserved specimens is bright greyish with two dark paramedian longitudinal stripes, along the entire length.

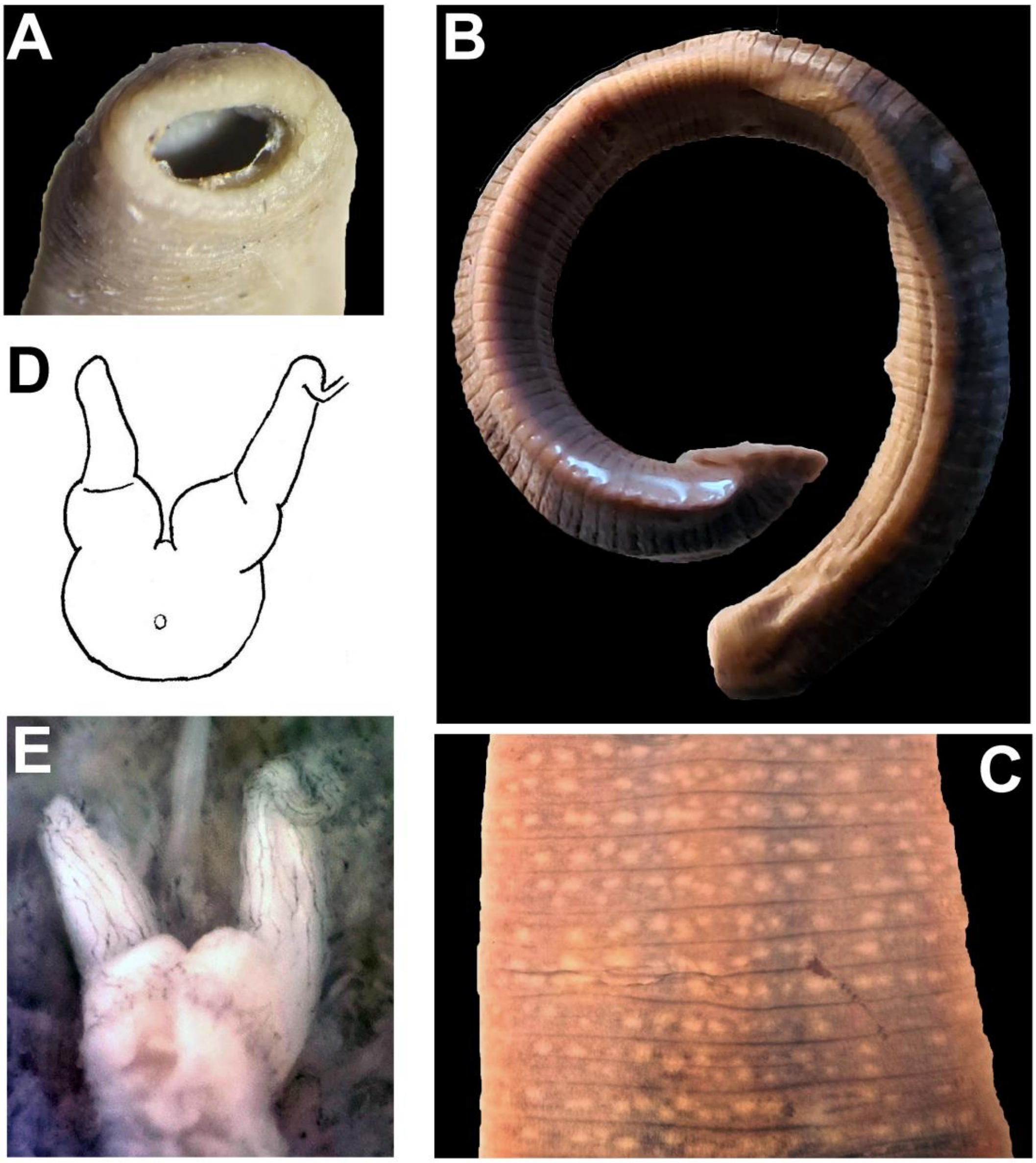

Figure 3. *Dina serbica* **sp. nov**. A – mouth opening, paratype; B – lateral view, paratype; C – colour of the dorsal surface, holotype; E-D – genital atrium, ventral view, paratype.

Each annulus bears a transverse row of bright and numerous yellowish spots (Fig. 3C). Papillae on the dorsal surface, corresponding with the yellowish spots, slightly visible.

Sexual organs — The atrial body small and rounded, cornua short and strong, reaching the border of the somite ahead (approximately to the furrow b6/b1), slightly curved ventrally, ends straight or very slightly curved ventrally, base of the cornua thickened and distinctly offset (Figs. 3E-D). The vas deferens with preatrial loop, strongly coiled and thickened from the third ganglia behind the female genital pore and extending to the next four ganglia; that begins the part with numerous testes (Fig. 4B).

The ovisacs are short and strongly curled in their entire course, extending to annulus a2 of the second somite behind the female genital pore (Fig. 4B).

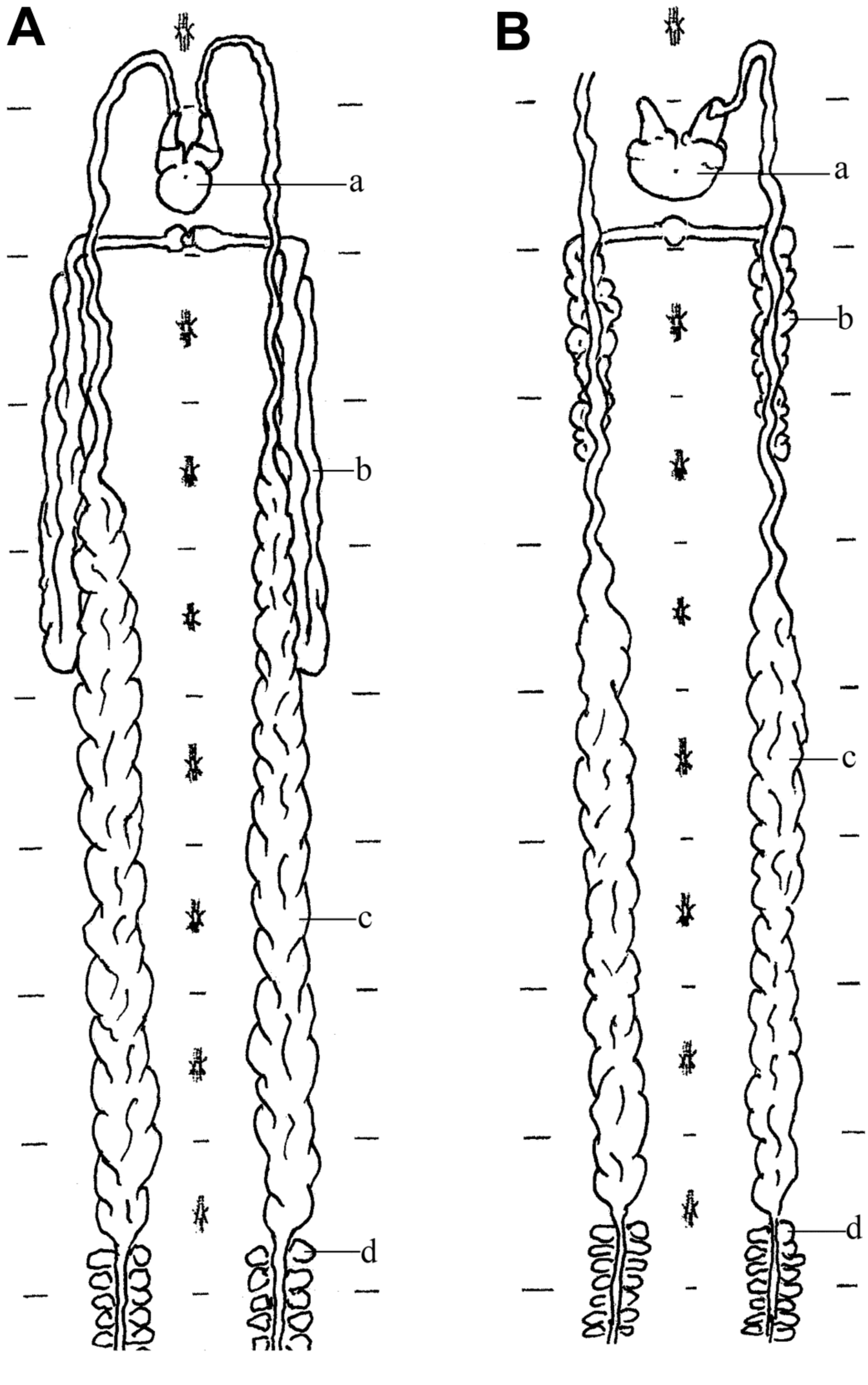

Figure 4. Reproductive system. A – *D. minuoculata* Grosser, Moritz & Pešić, 2007, paratype, a first order stream in the Tara river canyon, Montenegro. B – *Dina serbica* **sp. nov**., paratype, spring along the road to Kamena Gora, Serbia. Abbreviations: a = genital atrium, b = vas deferens, c = ovarian sacks, d = testisacs.

Etymology — Named after the (so far eclusive) occurrence of the species in Serbia.

Remarks — The phylogenetic analysis based on COI data placed *Dina serbica* **sp. nov.** as a sister clade to *D. minuoculata* Grosser, Moritz & Pešić, 2007, a species originally described from a first-order stream in the Tara river canyon in North Montenegro (Grosser *et al.* 2007). The average K2P genetic distance between sequences of the specimens of *D. minuoculata* collected from its *locus typicus* and *D. serbica* **sp. nov**. was estimated to be 11.96±1.49% K2P indicating their genetic isolation.

Morphologically, *Dina serbica* **sp. nov**. resembles *D. minuoculata*, in regard to the presence of bright yellowish spots on the dorsal surface of each annulus, the genital pores separated by two annuli and a short atrial cornua. From the latter species, *Dina serbica* **sp. nov**. can be separated in having a short and curled ovarian sacks (*vs*. not curled, and one somite longer in *D. minuoculata*, compare Figs. 4A and -B). Moreover, the reduction of pigmentation of eyes is much further progressed in the new species.

Distribution — Serbia; so far only known from one spring in Western Serbia (Fig. 5).

Habitat — The new species was collected in a rheopsammocrene (seeping rheocrene with sandy-gravel substratum) spring (Fig. 6B) in deciduous forest dominated by the common beech (*Fagus sylvatica* L.).

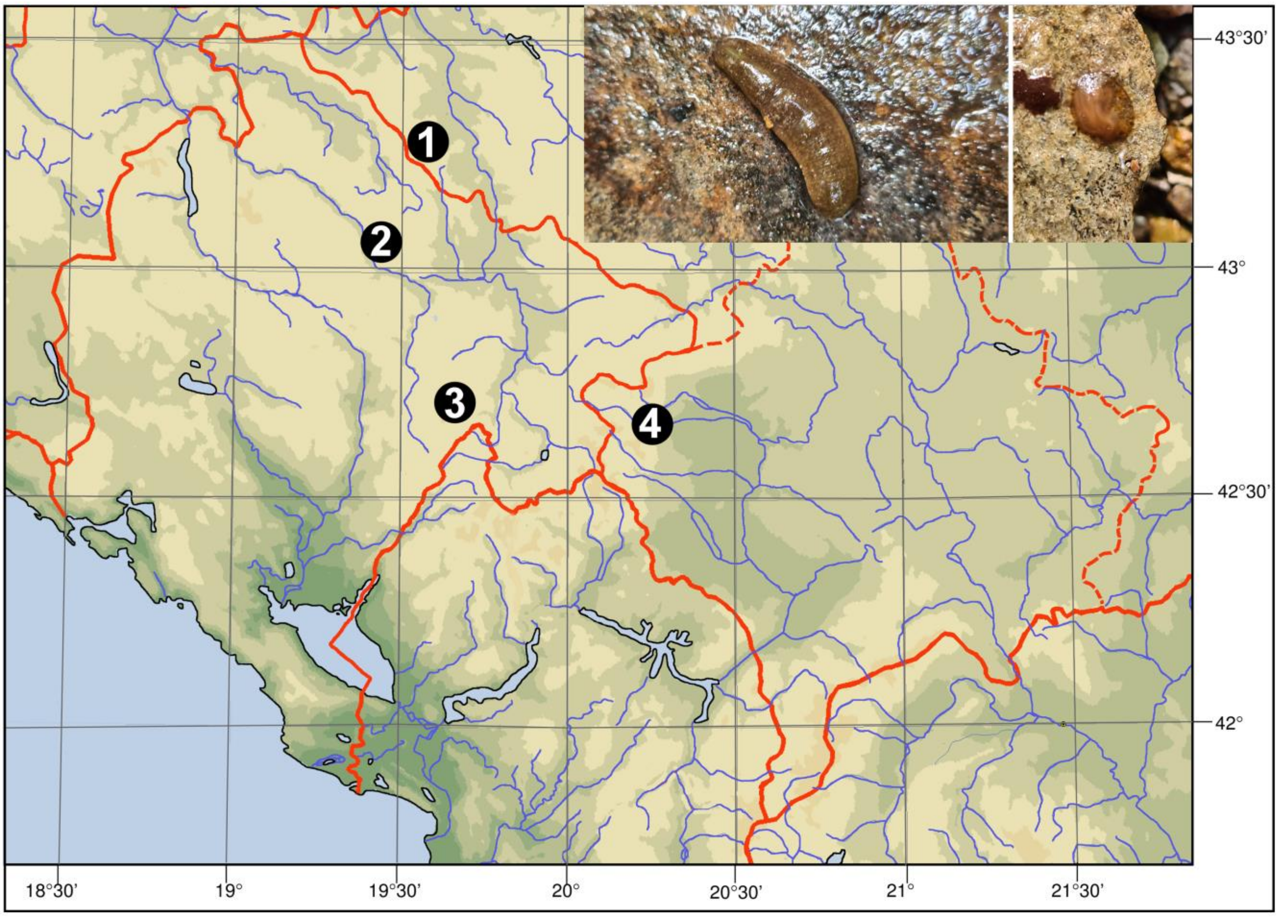

Figure 5. Map of studied area with marked type localities of *Dina serbica* **sp. nov**. (1), *D. minuoculata* (2), *D. montana* (3) and *D. prokletijaca* (4). Inset: Live specimen and coccon of *D. serbica* **sp. nov**. from the type locality (Kamena Gora, Serbia).

Discussion

The genus *Dina* is the dominant group of erpobdellid leeches in the Dinaric area (Grosser *et al*. 2015a, b; Dmitrović and Pešić 2020; Marinković *et al*. 2019, 2020, 2021). Our study is the first attempt to use DNA barcoding for species delimitation within the latter genus in the Western Balkans.

Figure 6. Photographs of the type localities of selected *Dina* species. A – a first-order stream in the Tara river canyon, Montenegro (*locus typicus* of *D. minuoculata*). B – rheopsammocrene spring along the road to Kamena Gora, Serbia (*locus typicus* of *D. serbica* **sp. nov**.). C – rheocrene spring Toplla, Dečani, Kosovo* (*locus typicus* of *D. prokletijaca*). D – spring at Štavna, Komovi Mt., Montenegro (*locus typicus* of *D. montana*). Photos by V. Pešić (A, D), L. Pešić (B), V. Berlajolli (C).

The use of an integrative approach based on using DNA barcodes allowed us to describe a new species, *D. serbica* **sp. nov**. from western Serbia as well as to determine its similarities with other species of the genus, which would be difficult only on the basis of morphological evidence. For example, our molecular data suggest that *D. serbica* **sp. nov**. and its closest relative *D. minuoculata* is closer to *D. latestriata* than to the *D. lineata*-complex.

Based on morphological evidence, *D. serbica* **sp. nov.** and *D. minuoculata* belongs to the *D. lineata*-group, the latter primarily diagnosed by the position of the male genital pore which is consistently situated in furrow b2/a2: in *D. latestriata* the male genital pore is situated in furrow b1/b2, and male and female gonopores are thus separated by three annuli (Neubert and Nesemann 1999). It should be noted that our results confirmed finding of Trajanovski *et al*. (2010) that *Dina latestriata* sequences cluster in two different clades, likely at the level of a separate species: the first clade comprises specimens of *D. latestriata* from Lake Mikri Prespa (topotypes) and Lake Prespa, while the second clade (here called *D*. cf. *latestriata* sensu Trajanovski *et al*. 2010) include a specimen from the Greek lake Trichonis.

Moreover, result of molecular analysis of this study has revealed a significant mismatch between the past understanding of the taxonomic diversity of the *D. lineata*-complex, and that was revealed by the use of DNA barcodes. *Dina lineata*, the presumably widespread type species, was represented in the studied area by four subspecies, the nominal one, *D. l. lineata*, and *D. l. dinarica*, *D. l. montana* and *D. l. lacustris*, the last three with distribution restricted to the Western Balkans. According to Grosser *et al*. (2015b) most of the Western Balkans records of *D. l. lineata*, a subspecies common in northern Germany, Scandinavia and the Baltic countries, should be ascertained to *D. montana*, and especially to *D. dinarica*. The latter two taxa, orginally desribed as a subspecies of *D. lineata*, recently have been treated as a separate species by Grosser *et al*. (2016). The genetic distances between nominal subspecies of *Dina lineata* and two other species, *D. dinarica* and *D. montana*, are at the interspecific level, 15.55% and 12.04% K2P, respectively. The species status of *D. dinarica* and *D. montana* was supported by the results of NY and ASAP analyzes that confirmed the validity of the decision of Grosser *et al*. (2016) to raise these taxa to the level of separate species.

Dina l. lacustris Sket, 1968 is known only from the alpine lakes Belo and Golemo in Norh Macedonia (Sket 1968). The COI analysis revealed that topotypes of *D. l. lacustris* clusters with the specimens of *Dina l. lineata* from Germany and Finland, but are separated by 1.6±0.52% K2P distance from that nominal subspecies, implying some level of genetic isolation of the population from Macedonia. The applied ASAP analysis based on COI sequences from our study and available sequences of *Dina* species in the GenBank and the BOLD did not support separation within *D. ohridana*-complex, nor the separation of *D. l. lacustris* from the nominal subspecies from Germany and Finland. The obtained results are not consistent with the morphological differences between the species of the *D. ohridana*-complex (see Trajanovski *et al*. 2010 for a discussion).

Moreover, the ASAP procedure combined *D. prokletijaca* and *D. montana* into one species which is rather unlikely, taking into account their morphological differences (see Grosser *et al*. 2016) as well as comparing the distance between these two species (4.1±0.81% K2P) with other inter- and intraspecific distances in the genus.

Estimating a relatively large barcoding gap in species which diverged recently may result in an unrealistic interspecific threshold (Kvist *et al*. 2010) in distance-based species delimitation methods, such as the applied ASAP procedure (Jovanović *et al*. 2021). Trajanovski *et al*. (2010) showed that *Dina ohridana*-group is a relatively young monophyletic complex estimating that its separation from its presumed sister taxon outside the Ohrid Lake (i.e. *Dina lineata*) occurred approximately 8.30±3.60 million years ago. As the latter authors stated, further studies with involving a fast-evolving nuclear markers are needed to clarify the collision between morphological and molecular data.

In this study, we used the ASAP procedure to identify a barcode gap for the selected *Dina* dataset, a method that has not yet been used for erpobdellid leeches. We observed a barcode gap between 3 and 7% K2P genetic distances based on COI sequences used in our study, which corresponds to the values found in some studies on other groups of leeches. For example, Jovanović *et al*. (2001) showed that an interspecific threshold of 5–7% distance is suitable for species identification purposes of Western Balkan glossiphonid leeches.

Overall, the results of this study imply that future studies with using a standard molecular COI marker in species identification are likely to reveal many more species in the genus. Moreover, applying a distance-based species delimitation methods can help to circumscribe species boundaries in complexes with potentially a cryptic species.

Acknowledgements

The first author is indebted to Luka Pešić, his 11-year-old soon, for the help and assistance during the fieldwork, and to Milica Jovanović and Ana Manović (University of Podgorica) for their excellent laboratory work. This study is part of the ”DNA-Eco” scientific project supported by a grant of the Montenegrin Ministry of Science.

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