DNA barcoding for species delimitation of the freshwater leech genus *Glossiphonia* from the Western Balkan (Hirudinea, Glossiphoniidae)

Milica Jovanović‡, Elisabeth Haring§, Helmut Sattmann‖, Clemens Grosser¶, Vladimir Pesic‡

‡ Department of Biology, Faculty of Natural Science and Mathematics, University of Montenegro, Džordža Vašingtona bb, 81000, Podgorica, Montenegro
§ Central Research Laboratories, Natural History Museum Vienna, Burgring 7, 1010, Vienna, Austria
‖ Department of Evolutionary Biology, University of Vienna, Althanstraße 14, 1090, Vienna, Austria
¶ 3rd Zoological Department, Natural History Museum Vienna, Burgring 7, Vienna, Austria
# 4 Bernd-Blindow-Schule Leipzig, Comeniusstraße 17, 04315, Leipzig, Germany

Corresponding author: Milica Jovanović (milicaj@ucg.ac.me)

Academic editor: Yasen Mutafchiev

Received: 23 Mar 2021 | Accepted: 20 Aug 2021 | Published: 15 Sep 2021

Citation: Jovanović M, Haring E, Sattmann H, Grosser C, Pesic V (2021) DNA barcoding for species delimitation of the freshwater leech genus *Glossiphonia* from the Western Balkan (Hirudinea, Glossiphoniidae). Biodiversity Data Journal 9: e66347. [https://doi.org/10.3897/BDJ.9.e66347](https://doi.org/10.3897/BDJ.9.e66347)

Abstract

Glossiphoniid leeches are a diverse group and sometimes abundant elements of the aquatic fauna inhabiting various types of freshwater habitats. In this study, we sampled leeches of the genus *Glossiphonia* from the Western Balkan in order to test the suitability of the mitochondrial cytochrome c oxidase subunit 1 (COI) marker sequence for species delimitation. Morphological analysis revealed the presence of four taxa, *G. complanata* with two subspecies, *G. c. complanata* and *G. c. maculosa*, the latter an endemic of Ohrid Lake, *G. nebulosa* and endemic *G. balcanica*. In total, 29 new barcodes of *Glossiphonia* were sequenced in the course of this study and compared with the available molecular dataset of the latter genus from GenBank/BOLD databases. The applied ASAP distance-based species delimitation method for the analysed dataset revealed an interspecific threshold between 4-8% K2P distance as suitable for species identification purposes of the Western Balkan *Glossiphonia* species. Our study revealed that morphologically identified taxa as *G. nebulosa* and *G. concolor* each consists of more than one clearly different phylogenetic clade. This study contributes to a better knowledge of the taxonomy of glossiphoniid leeches.
leeches and emphasises future work on the revision of this genus using a standard molecular COI marker in species identification.

**Keywords**

DNA barcoding, COI, freshwater leeches, Glossiphoniidae, phylogeny, species delimitation

**Introduction**

Species of the family Glossiphoniidae Vaillant, 1890 are generally small, dorsoventrally flattened leeches, distributed in freshwater ecosystems on all continents except Antarctica (Nesemann and Neubert 1999, Kaygorodova et al. 2020). Representatives of these leeches are normally found feeding on the blood of turtles or amphibians and, as vectors of apicomplexan blood parasites, they play an important role in aquatic ecosystems (Nesemann and Neubert 1999, Siddall et al. 2005, Chiangkul et al. 2021). Some species of the genera *Helobdella* Blanchard, 1896 and *Glossiphonia* Johnson, 1816 feed on the haemolymph of aquatic oligochaetes and snails (Siddall et al. 2005).

Distribution and species boundaries of the leeches of the genus *Glossiphonia*, the most diverse genus of the family, have been studied by several authors by means of the DNA barcode region of the mitochondrial cytochrome c oxidase subunit 1 gene (COI) as a genetic marker (e.g. Siddall et al. 2005, Oceguera-Figueroa and León-Régagnon 2014, Pérez-Flores et al. 2016, Mack and Kvist 2019, Kaygorodova et al. 2020). An integrative approach that combines morphological examination and molecular genetic data had helped to resolve the taxonomic status of some species (Mack and Kvist 2019, Kaygorodova et al. 2020). For example, the molecular studies, conducted by Siddall et al. (2005) and Mack and Kvist (2019) on North American populations previously assigned to *Glossiphonia complanata* (Linnaeus, 1758), reveal the presence of two well-defined species, with *Glossiphonia complanata* restricted to Europe and *Glossiphonia elegans* (Verrill, 1872) living in North America. However, the knowledge on diversity and species delimitation by applying a molecular genetic approach within this leech group is still poorly studied in many parts of their range, especially in the Dinaric Region of the Balkan Peninsula.

At present, all of the European members of the genus *Glossiphonia* have been reported to also inhabit the Western Balkans (Sket 1968, Nesemann and Neubert 1999, Utevsky et al. 2013, Grosser et al. 2015a, Grosser et al. 2015b, Dmitrović and Pešić 2020): the widespread Palearctic *G. complanata*, with two subspecies, the nominal one and *G. complanata maculosa* Sket, 1968 which is known only from Ohrid Lake, *G. concolor* (Apathy, 1988), *G. nebulosa* Kalbe, 1964, *G. paludosa* (Carena, 1824), *G. balcanica* Grosser & Pešić, 2016, a species recently described from Kosovo and Montenegro (Grosser et al. 2016) and *G. pulchella* Sket, 1968 an endemic species known only from the littoral of Lake Ohrid (Sket 1968).
In this study, we applied a standard DNA barcoding marker, a fragment of the COI gene, to analyse specimens of the genus *Glossiphonia* collected recently in various freshwater habitats (lakes, streams and springs) of the Western Balkans (Albania, Bosnia and Hercegovina, Kosovo, Montenegro and North Macedonia). In addition, we analysed the available museum material originating from other European localities, including loci typici of some selected species (e.g. *G. nebulosa*) to obtain reliably identified sequence data. Moreover, we used DNA barcode sequences in both BOLD and GenBank to compare with the sequences obtained in our study. As a result, a dataset, including COI sequences of 29 specimens of *Glossiphonia* spp. plus four sequences representing two other genera (*Helobdella, Placobdella* Blanchard, 1893), was generated in order to contribute to a reference dataset applicable for DNA barcoding studies of the genus *Glossiphonia* in general and, in particular, in the Western Balkans.

**Material and Methods**

**Sample collection and morphological analysis**

Glossiphoniid leeches were collected from twenty-two sites in seven countries: Albania, Austria, Bosnia and Hercegovina, Germany, Kosovo, Montenegro and North Macedonia (Fig. 1). 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 preserved in 96% ethanol for further morphological and molecular genetic analysis.

![Distribution map of localities where glossiphoniid leeches were collected. (red dots – present study records, blue dots – records from previous studies where coordinates are available). Note that each point may represent more than one species. Country codes of those countries, from which we had material, are indicated. The map was created using QGIS 2.8.11 software.](Image)
Morphological analysis of 33 individuals was performed using a stereomicroscope (Novex). Leeches were identified to species level according to Nesemann and Neubert (1999) and Grosser et al. (2016). Voucher specimens were deposited in the scientific collection of the Natural History Museum Vienna (NHM).

**Molecular genetic analysis**

DNA analysis was conducted in the Central Research Laboratories of the NHM. Leeches fixed in 96% ethanol were stored at 4°C. Tissue samples from individuals (approx. 2 × 2 × 2 mm) were separated using sterile scalpels and tweezers. DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The final volume of DNA solution was 40 µl.

The present study focused on the COI gene, which was amplified using a polymerase chain reaction (PCR). For all sampled leeches, a 708 bp section was amplified, which contains the standard DNA barcoding region. The universal primers LCO1490, 5′GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198, 5′TAAACTTCAGGGTGAACC AAAAAATCA-3′ (Folmer et al. 1994) were used. The final alignment for the phylogenetic tree reconstructions included 33 sequences and had a length of 658 sites.

Each reaction consisted of 0.5 units of TopTaq DNA polymerase (Qiagen), 2.5 µl 10× TopTaq PCR Buffer, 10 mM of each dNTP, 50 µM of each primer and 1 µl DNA template in a total reaction volume of 25 µl. The PCR cycling protocol included an initial denaturation at 94°C for 3 min, followed by 35 cycles of of denaturation at 94°C for 30 s, annealing for 30 s at 52°C and extension for 1 min at 72°C. The final step was an extension at 72°C for 10 min and a hold at 10°C.

The amplicons were checked by (1%) agarose gel electrophoresis. The QIAquick PCR Purification Kit (Qiagen) was employed to purify amplifications products. Sequencing was performed in both directions at Microsynth (Balgach, Switzerland) using the PCR primers.

**Data analysis**

Sequences (both strands) were checked and edited using BioEdit (Hall 1999). The search in GenBank for sequences similar to the sequences, generated from the studied specimens analysed in the present study, was performed through BLASTn search in the GenBank database (https://blast.ncbi.nlm.nih.gov). Subsequently, 19 published sequences, from representatives of the genus Glossiphonia, were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank) and were included for comparison (listed in Table 1). Differences between DNA sequences (p and K2P distances in %) were calculated with the MEGA X software, version 10.1.7 (Kumar et al. 2018). MEGA X was also used to calculate a Neighbour-Joining (NJ, Saitou and Nei 1987) tree (based on p distances) and Maximum Likelihood (ML) trees (model selected by the BIC criterion (Bayesian Information Criterion) implemented in MEGA X: TN93 + I+ G) with an initial NJ tree and using the Subtree-Pruning-Regrafting - Extensive heuristic search (SPR level 5). Bootstrapping was done with 500 replicates for NJ and ML trees. A Bayesian Inference (BI) tree was
calculated with MrBayes v.3.2.2 (Huelsenbeck and Ronquist 2001, Ronquist et al. 2012) with $10^6$ generations (two runs each with four chains and one heated chain, sampling every hundredth tree). The first 25% of the trees were discarded as burn-in and a 50% majority rule consensus tree was calculated from the remaining trees.

### Table 1.

Taxon names, locality information and accession numbers for the specimens used in phylogenetic analysis and distance estimations. Newly-sequenced taxa are shown in bold font. BOLD accession numbers are given for the sequences produced in the present study, while GenBank accession numbers are provided for published sequences.

| Sample ID | Locality (Country/Exact site) | Coordinates | BOLD / GenBank ID | Source |
|-----------|-------------------------------|-------------|-------------------|--------|
| **Glossiphonia verrucata** | | | | |
| ROMIZ I11753 | Unnamed river, Croatia (CRO) | 43.574722°N, 15.818889°E | MK479263 | Mack and Kvist (2019) |
| ROMIZ I11755 | Unnamed river, Croatia (CRO) | 43.574722°N, 15.818889°E | MK479264 | Mack and Kvist (2019) |
| Rio Sadde, Italy (IT) | | | AY962459 | Siddall et al. (2005) |
| Chechuy River, Russia (RUS) | | 58.19640°N, 109.294720°W | MH670857 | Kaygorodova et al. (2020) |
| Lake near Meget, Russia (RUS) | | 52.451440°N, 104.027120°W | MH670858 | Kaygorodova et al. (2020) |
| **Glossiphonia complanata complanata** | | | | |
| BH1_1 | Krupa River near Vrbas, Bosnia and Herzegovina (BH) | 44.616°N, 17.1495°E | LCHME001-20 | This study |
| MN1_1 | Karuč spring, Podgorica, Montenegro (MN) | 42.3585°N, 19.1064°E | LCHME008-20 | This study |
| MN1_2 | Karuč spring, Podgorica, Montenegro (MN) | 42.3585°N, 19.1064°E | LCHME009-20 | This study |
| MN2_1 | River Crnojevića, Cetinje, Montenegro (MN) | 42.3546°N, 19.0178°E | LCHME010-20 | This study |
| MN5_1 | Vitoja spring pool, Podgorica, Montenegro (MN) | 42.3251°N, 19.3634°E | LCHME013-20 | This study |
| MN6_1 | Dobro polje spring, Danilovgrad, Montenegro (MN) | 42.6305°N, 19.0324°E | LCHME014-20 | This study |
| MN7_1 | Mareza spring, Podgorica, Montenegro (MN) | 42.48°N, 19.1822°E | LCHME015-20 | This study |
| MN8_1 | Karuč spring, Podgorica, Montenegro (MN) | 42.3585°N, 19.1064°E | LCHME016-20 | This study |
| AUS_Hir: 2_1 | Kalte Wien, Vienna, Austria (AUS) | 48.2934°N, 16.3915°E | LCHME027-20 | This study |
| Gcomp1 | Stream from the lake Barschsee, Mecklenburg-Vorpommern, Germany, type locality (GER) | 53.9147°N, 11.2815°E | LCHME038-20 | This study |
| Sample ID | Locality (Country/Exact site)                                                                 | Coordinates            | BOLD / GenBank ID    | Source                  |
|-----------|-----------------------------------------------------------------------------------------------|------------------------|----------------------|-------------------------|
| Gcomp2    | Stream from the lake Barschsee, Mecklenburg-Vorpommern, Germany, type locality (GER)          | 53.9147°N, 11.2815°E   | LCHME039-20          | This study              |
| Gcomp3    | Small stream near Jesewitz, Saxony, Germany (GER)                                              | 51.3812°N, 12.6733°E   | LCHME040-20          | This study              |
|           | Durance river, France (FR)                                                                     |                        | MF458715             | Corse et al. (2017)     |
|           | Europe (EU)                                                                                   |                        | AF003277             | Siddall and Burreson (1998) |
|           | Creek, Mecklenburg-Vorpommern, Nordwestmecklenburg district, Germany (GER)                     | 53.81848°N, 10.92799°E | HM246608             | Trajanovski et al. (2010) |
|           | United Kingdom (UK)                                                                            |                        | AY047321             | Light and Siddall (1999) |
| ROMIZ I11750 | Korana river, Croatia (CRO)                                                                | 45.117222°N, 15.592778°E | MK479280           | Mack and Kvist (2019)  |
| ROMIZ I11749 | Korana river, Croatia (CRO)                                                                | 45.117222°N, 15.592778°E | MK479279           | Mack and Kvist (2019)  |
| ROMIZ I11748 | Korana river, Croatia (CRO)                                                                | 45.117222°N, 15.592778°E | MK479278           | Mack and Kvist (2019)  |
| ROMIZ I11717 | Sava river, Slovenia (SLO)                                                                | 46.084444°N, 14.587222°E | MK479277           | Mack and Kvist (2019)  |
| ROMIZ I11743 | Gacka river, Croatia (CRO)                                                                 | 44.851667°N, 15.233611°E | MK479262           | Mack and Kvist (2019)  |

**Glossiphonia complanata maculosa**

| Sample ID | Locality (Country/Exact site)                                                                 | Coordinates            | BOLD / GenBank ID    | Source                  |
|-----------|-----------------------------------------------------------------------------------------------|------------------------|----------------------|-------------------------|
| MAC1_1    | St. Naum spring of Crni Drim, Ohrid Lake, North Macedonia (MAC) – type locality               | 40.9138°N, 20.7433°E   | LCHME020-20          | This study              |
| MAC2_1    | Lagadin, Ohrid Lake, North Macedonia (MAC)                                                    | 41.0422°N, 20.8039°E   | LCHME021-20          | This study              |
| MAC2_2    | Lagadin, Ohrid Lake, North Macedonia (MAC)                                                    | 41.0422°N, 20.8039°E   | LCHME022-20          | This study              |
| MAC3_1    | Peštani, Ohrid Lake, North Macedonia (MAC)                                                     | 41.0095°N, 20.8059°E   | LCHME023-20          | This study              |
| MAC4_1    | Oteševo, Prespa Lake, North Macedonia (MAC)                                                    | 40.9919°N, 20.9322°E   | LCHME024-20          | This study              |
| ALB1a_d   | Pogradec, Ohrid Lake, Albania (ALB)                                                            | 40.9058°N, 20.6556°E   | LCHME029-20          | This study              |
| ALB1a_1   | Pogradec, Ohrid Lake, Albania (ALB)                                                            | 40.9058°N, 20.6556°E   | LCHME030-20          | This study              |
| ALB3b_2   | Tushemisht, Ohrid Lake, Albania (ALB)                                                           | 40.9035°N, 20.7172°E   | LCHME035-20          | This study              |

**Glossiphonia concolor**

| Sample ID | Locality (Country/Exact site)                                                                 | Coordinates            | BOLD / GenBank ID    | Source                  |
|-----------|-----------------------------------------------------------------------------------------------|------------------------|----------------------|-------------------------|
| Gconc1    | Krakower Obersee, Mecklenburg-Vorpommern, Germany (GER)                                       | 53.6074°N, 12.2976°E   | LCHME041-20          | This study              |
| Sample ID | Locality (Country/Exact site) | Coordinates | BOLD / GenBank ID | Source |
|-----------|-----------------------------|-------------|------------------|--------|
| Kila river, Sweden (SWE) | | | AY962458 | Siddall et al. (2005) |
| Ukraine (UKR) | | | KM095097 | Kaygorodova and Mandzyak (2014) |

**Glossiphonia balcanica**

| Gbalc1 | Toplla spring, Dečani, Kosovo (KOS) - type locality | 42.57194°N, 20.29056°E | LCHME036-20 | This study |
| Gbalc2 | Toplla spring, Dečani, Kosovo (KOS) - type locality | 42.57194°N, 20.29056°E | LCHME037-20 | This study |

**Glossiphonia nebulosa**

| Gnebu4 | Berliner Chausssee stream Nieplitz, Berlin, Germany -type locality | 52.1348°N, 12.9449°E | LCHME044-20 | This study |
| Gnebu5 | Yamalo-Nenets Autonomous Okrug, Russia (RUS) | | MN295412 | Bolotov et al. (2019) |
| KOS1_1 | Spring KS 40, Peje, Kosovo (KOS) | 42.6283°N, 20.246°E | LCHME004-20 | This study |
| KOS1_2 | Spring KS 40, Peje, Kosovo (KOS) | 42.6283°N, 20.246°E | LCHME005-20 | This study |
| BH3_1 | Banja Luka, Near castle, Bosnia & Herzegovina (BH) | 44.7657°N, 17.193°E | LCHME002-20 | This study |
| Gnebu1 | Toplla spring, Dečani, Kosovo (KOS) | 42.57194°N, 20.29056°E | LCHME042-20 | This study |
| Gnebu3 | Toplla spring, Dečani, Kosovo (KOS) | 42.57194°N, 20.29056°E | LCHME043-20 | This study |

**Glossiphonia elegans**

| ROMIZ I11505 | Unknown pond, Nopiming, Manitoba, Canada (CAN) | 50.452222°N, 95.5125°W | MK479253 | Mack and Kvist (2019) |
| Lake Bemidji, Beltrami County, Minnesota, USA | | | JQ073869 | Moser et al. (2012) |

**Glossiphonia baicalensis**

| | Lake Baikal, Russia (RUS) | | AY047329 | Light and Siddall (1999) |

**Outgroups**

**Placobdella costata**

| MN4_1 | Oraška jama spring, Danilovgrad, Montenegro (MN) | 42.5309°N, 19.0921°E | LCHME012-20 | This study |
| MN9_1 | Crno oko spring, Podgorica, Montenegro (MN) | 42.4844°N, 19.1542°E | LCHME017-20 | This study |

**Helobdella stagnalis**

| BH3_2 | Near castle, Banja Luka, Bosnia & Herzegovina (BH) | 44.7657°N, 17.193°E | LCHME003-20 | This study |
| MAC4_2 | Oteševo, Prespa Lake, North Macedonia (MAC) | 40.9919°N, 20.9322°E | LCHME025-20 | This study |
In order to assess the genetic differentiation of species within our dataset of 47 Glossiphonia sequences, we used the ASAP procedure 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). The online ASAP version (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) was used, with default settings and the K2P distance model. Besides the ASAP procedure, we also used the species delimitation approach of mPTP by Kapli et al. (2017), which is based on a single-locus coalescent-based method. All sequences, generated in course of the present study, were deposited in BOLD.

Results

Morphological characterisation of collected specimens

Morphological analysis of 29 specimens of the leech genus Glossiphonia from studied area of West Balkans revealed the presence of three species G. complanata, G. balcanica and G. nebulosa (Fig. 2), which could be differentiated morphologically by the following characters: the six-eyed leeches with prominent papillae only on annulus a2 of mid-body somites were assigned to the typical G. complanata complanata. Specimens from Lake Ohrid and Lake Prespa were identified as G. complanata maculosa. Representatives of this subspecies can be separated from the nominal subspecies by the lack of the prominent dorsal papillae, characteristic for G. complanata complanata and by the presence of a brown pigmentation forming an asymmetrical reticulate pattern, often covering completely the body surface (Nesemann and Neubert 1999). Although G. c. maculosa was so far known from Lake Ohrid only, the morphological determination of the specimens from Lake Prespa was straightforward.

Glossiphonia balcanica and G. nebulosa closely resemble one another and can be distinguished by the colour, which is bright brownish in G. balcanica, but more greyish in the specimens of G. nebulosa from the Balkans (see Grosser et al. 2016). Moreover, the dorsal surface of G. balcanica is covered by a few small irregularly arranged papillae and the prominent paramedian papillae located only on annulus a2. Finally, a difference between those two species can be found in the medial fold on the cranial sucker. This medial fold is lacking in G. balcanica specimens, but is prominent in the population of G. cf. nebulosa from Kosovo and only slightly developed in the populations of G. nebulosa from its type locality in Germany (see Grosser et al. 2016 for further discussion).

Molecular genetic analysis

The final alignment comprised 52 sequences and had a length of 658 nucleotide sites. Of the 33 COI sequences generated in the present study, all were included in the final dataset for the phylogenetic analysis.

Both the ML and NJ trees, based on COI sequences, were in agreement regarding the general topology. The ML tree is shown in Fig. 3 (NJ bootstrap values, as well as posterior
Probability values of the BI analysis are also shown in Fig. 3. Although the BI tree was, in general, very weakly supported and had a slightly different topology (see below), several clades were consistent with the ML and NJ trees. The species of the genus Glossiphonia form a monophylum. Placobdela and Helobdella, which were included as outgroup, form separate sister clades (bootstrap support > 96%).

In both the NJ and ML trees, Glossiphonia COI sequences are clustered into eight clades (Fig. 3). The North American G. elegans forms the sister group to a clade comprising all European members of the genus analysed. Yet, the latter group is supported by low bootstrap support only (ML: 49%). In the BI analysis, this sister group relationship was not found and, in general, the relationships amongst clades were only weakly supported in the BI tree. In the following, the ML tree (Fig. 3) regarding European Glossiphonia is described.

A large clade represents Glossiphonia complanata, which is subdivided into four subclades. Glossiphonia c. maculosa specimens collected from Ohrid Lake (from Albania and North Macedonia) are clustered together (supported by an ML bootstrap value of 97%) and are most closely related to two other G. complanata specimens, one Glossiphonia c. complanata (MK479262), collected from Gacka River, Otočac, Croatia and the other one, a specimen of G. complanata maculosa from Prespa Lake, North Macedonia (LCHME024-20). Thus, with the exception of the Croatian specimen, G. c. maculosa would be an (albeit very weakly supported) monophylum (Subclade 1 in Fig. 3). The range of K2P distances between G. c. maculosa from the Balkan lakes and G. c. complanata (excluding the individual MK479262 from Croatia) was 2.07 ± 0.5%. The remaining (weakly to moderately supported) G. complanata subclades cluster to some extent in a geographic manner: the (predominately more western) Subclade 2 contains seven individuals collected.
from Germany, France and the United Kingdom. Subclade 3 comprises individuals from Austria, Croatia and Slovenia and Subclade 4 contains individuals from Bosnia & Herzegovina, Croatia and Montenegro. Despite bad support values, the BI tree also revealed Subclades 1, 2 and 4.

Glossiphonia balcanica, in our tree represented by two specimens from Kosovo, is the sister clade of *G. complanata*. This sister group relationship was supported with high support values. The third species in our dataset is *G. concolor*, of which we had only one sample from Mecklenburg-Vorpommern, Germany (LCHME041-20). This sequence is very similar to the GenBank sequence of *G. concolor* from Sweden. In contrast, another published sequence (KM095097) from Ukraine is quite distantly related, clustering in the tree (albeit with no considerable support) with a published sequence of *G. baicalensis* (from Lake Baikal, Russia; AY047329). Another distinct lineage is formed by two GenBank sequences of *Glossiphonia verrucata* from Russia.

For the fourth species sequenced in the present study, *G. nebulosa*, we found two subclades, one formed by a specimen from the type locality in Germany (LCHME044-20), together with a specimen from Russia (MN295412). This clade is placed with high support (99%) as the sister group of a clade consisting of *G. nebulosa* specimens from Bosnia and Herzegovina, Croatia and Montenegro.
Herzegovina, as well as Kosovo (in the tree designated as G. cf. nebulosa). Moreover, this subclade contains published sequences assigned to G. verrucata, originating from Croatia (MK479263-64) and Italy (AY96245) and rendering G. verrucata paraphyletic. Due to the position of G. verrucata specimens within the G. nebulosa clade, this species also appears paraphyletic.

Species from the two genera, Placobdela and Helobdella, analysed in this study as outgroup species, formed separate clades with a bootstrap support of 99%. Two specimens of P. costata are identical (BOLD BIN: AEC5178), while the two specimens determined as H. stagnalis are separated by a p-distance of 4.1% and also have separate BINs in BOLD.

Genetic distances and delimitation of species

The mean K2P values between the morphologically determined species of Glossiphonia ranged from 3.17% to 12.69% (Suppl. material 1). The minimal mean K2P distance of 3.17 ± 0.6% was found between G. balcanica from Kosovo and the G. complanata clade. The maximum mean distance of 12.69 ± 1.6% was observed between G. cf. nebulosa clade and the Siberian G. verrucata. A mean distance of 5.07 ± 0.8% separates G. nebulosa (comprising one sequence from Germany and one from Russia) from a clade consisting of specimens from the Balkan Region (including specimens here provisionally assigned to G. cf. nebulosa).

The highest mean intraspecific distances were observed within G. complanata (1.64%, max. 3.0%) and G. cf. nebulosa (1.31%, max. 4.0%), respectively. The mean intraspecific distance within the clade, herein labelled as G. nebulosa, amounted to 0.9% (Suppl. material 1).

For the ASAP analysis, the sequences of G. verrucata from Italy (AY96245) were excluded from the further analysis because of having ambiguous nucleotides (see Kaygorodova et al. (2020) for a discussion). As a result of the ASAP analysis, a barcoding gap at about 4-8% was estimated. The applied ASAP procedure identified 7 MOTUs (hypothetical species) at the threshold distance of 5.46% (K2P) which has the best ASAP-score (3.50) within the available molecular data: G. complanata (merging G. balcanica), G. concolor, G. concolor from Ukraine, G. baicalensis, G. elegans, G. nebulosa and G. verrucata. At the threshold distance of 3.59% (K2P) (but with a poorer ASAP-score of 9.50), ASAP analysis retrieved one more Glossiphonia species (hereafter referred to as G. cf. nebulosa) from the Balkans, morphologically resembling G. nebulosa.

Finally, the mPTP analysis grouped the Glossiphonia COI sequences into six main species also combining G. complanata and G. balcanica. In contrast to the ASAP results, the highly diverged lineages of G. baicalensis and G. concolor were grouped into one species (Fig. 3).
Discussion

Morphological analysis of the examined leeches of the genus *Glossiphonia* from the Western Balkans revealed the presence of three species, *G. complanata*, *G. balcanica* and *G. nebulosa*. Using DNA barcodes, the present study has revealed inconsistency between the past understanding of the taxonomic diversity of the above-listed three species, based exclusively on morphological characters.

*Glossiphonia complanata* was the most abundant species in our study. In the studied area, it is known by two subspecies, the nominal one and *G. complanata maculosa*, known only from Lake Ohrid (Sket 1968). Surprisingly, the results of our study revealed that a published sequence of a specimen of *G. complanata* (MK479262) from Croatia is close to the *G. complanata maculosa* subclade from Ohrid Lake. The latter specimen, reported by Mack and Kvist (2019), was collected from the Gacka River in Otočac, Croatia. The authors emphasised the high genetic variation found between the specimen from Otočac and other specimens of *G. complanata* from Croatia, suggesting that Otočac’s population represent a separately evolving lineage (Mack and Kvist 2019). Future morphological analysis of those specimens should allow us to test if morphology supports the genetic results.

In the course of the present study, only a single *Glossiphonia* specimen was found in Prespa Lake (MAC4_1; LCHME024-20) which was assigned to *G. complanata maculosa*, based on its characteristic colour pattern. Albrecht et al. (2008) emphasised a high degree of isolation of Lake Ohrid endemics with relatively little faunal overlap with the neighbouring Prespa Lake. The COI analysis revealed that the specimen from Lake Prespa clusters with the samples from Ohrid Lake, but is separated by 1.49% mean K2P distance from that *maculosa*-lineage, implying some level of genetic isolation of the populations from these two Balkan Lakes. Yet, more samples, including Croatian localities, should be needed to support this assumption and to assess morphological variation within this clade.

Our results suggest that some specimens, represented by published sequences in our dataset, were probably misidentified. Sequences of two samples from one unnamed river in Croatia (Mack and Kvist 2019) and a sequence from another sample from Rio Sadde, Italy (Siddall et al. 2005), all reported under the name *G. verrucata*, were found within the *G. cf. nebulosa* clade in our study. Already Kaygorodova et al. (2020) showed that the latter sample from Italy is genetically clearly separated from their Siberian leeches that morphologically match *G. verrucata*, a species recently re-described by Jueg and Michalik (2018). The latter species is distributed mainly in the northern Palaearctic (Jueg 2013) with a few records from Central Europe where it was found in the River Danube from Bavaria to Hungary (Nesemann 1997, Nesemann and Neubert 1999). The results of sequence-based species delimitation methods, conducted by Kaygorodova et al. (2020), revealed that specimens from Italy and Russia (Siberia), respectively, represent distinct species. Unfortunately, for the samples from Italy and Croatia, published under the name of *G. verrucata* by Siddall et al. (2005) and Mack and Kvist (2019), respectively, no information on their morphological features used for their identification were provided. The taxonomic
state of this clade (in the present study, summarised under G. cf. nebulosa) deserve further investigation.

Our study revealed that G. nebulosa consists of two phylogenetic clades questioning the status of the populations from Western Balkans. The mean K2P distance of about 4.9% was found between the north-central European clade of G. nebulosa containing a specimen from the type locality of this species in Germany (stream Nieplitz near Berlin) and the clade that encompasses specimens from the Balkans. This might suggest longstanding isolation between populations from north-central Europe and populations from south-eastern Europe. Grosser et al. (2016) had stressed the morphological differences between the populations of G. nebulosa from its type locality and those from Kosovo. To clarify the taxonomical status of the latter populations from the West Balkans, further material should be sampled and studied to cover the distribution ranges of these taxa.

Concerning the species delimitation analyses, the different results of the approaches did not provide convincing conclusions. For example, the mPTP analysis combined G. complanata and G. balcanica into one species which is rather unlikely, comparing the distance between these two lineages (6.14% K2P) with other inter- and intraspecific distances in the genus. Another unexpected result relates to G. complanata and G. balcanica: mean K2P interspecific distance between G. balcanica, an endemic species recently described by Grosser et al. (2016) from Kosovo and the widely distributed G. complanata, amounted to 3.17%. The ASAP procedure, grouped the COI sequences of the latter two species together, which is not consistent with the morphological differences between these two species. Morphologically, G. balcanica is rather different from G. complanata and closely resembles G. cf. nebulosa, with which it lives syntopically at some localities (see Grosser et al. 2016). Phylogenetically, G. balcanica and G. complanata belong to different clades with high support values indicating that the interspecific threshold in ASAP analysis of our analysed dataset might be unrealistic, likely as a result of an underestimation of species diversity (Yu et al. 2017) or estimating a relatively large barcoding gap in species which diverged recently (Kvist et al. 2010). According to Puillandre et al. (2021) ASAP is most effective when species are represented by at least 3–5 sequences. It could be that only having two sequences of G. balcanica vs. the multitude of sequences for G. complanata could have resulted in a merging of the two species. In summary, as recently emphasised by Puillandre et al. (2021), other characters and not just threshold distances with the best score, should be used to select a final species partition, in the sense of integrative taxonomy.

The obtained barcode gaps of 4–8% K2P in our dataset of COI sequences is comparable to an interspecific threshold of 5–7% distance that Kaygorodova et al. (2020) accepted as suitable for species identification purposes of Siberian glossiphonid leeches. The results of our study emphasised the importance of molecular genetic methods and of assessing genetic diversity of glossiphonid leeches. Building of the DNA barcode reference library for this group will provide a handy system for better understanding of distribution and species boundaries of this genus in karstic regions of the Western Balkans.
Acknowledgements

Financial support was partly provided within the programme for Scientific & Technological Cooperation in the framework of the bilateral Agreement between the Government of the Republic of Austria and the Government of the Republic of Montenegro. Assistance given by Julia Schindelar and Bárbara Tautscher with the molecular genetic analysis was greatly appreciated. Dejan Dmitrović and Violeta Bërllajolli contributed this study with leech material from Bosnia & Herzegovina and Kosovo. We thank Joachim Händel for providing us with the photograph of *G. nebulosa* and Oliver Maček for help with databases.

Ethics and security

No ethical principles were violated when providing this study.

Conflicts of interest

The authors declare no conflict of interests concerning this study.

References

- Albrecht C, Wolff C, Glöer P, Wilke T (2008) Concurrent evolution of ancient sister lakes and sister species: the freshwater gastropod genus *Radix* in lakes Ohrid and Prespa. Hydrobiologia 615 (1): 157-167. [https://doi.org/10.1007/s10750-008-9555-1](https://doi.org/10.1007/s10750-008-9555-1)
- Bolotov I, Klass A, Kondakov A, Vikhrev I, Bespalaya Y, Gofarov MY, Filippov BY, Bogan A, Lopes-Lima M, Lunn Z, Chan N, Aksenova O, Dvoryankin G, Chapurina Y, Kim SK, Kolosova Y, Konopleva E, Lee JH, Makhrov A, Palatov D, Sayenko E, Spitsyn V, Sokolova S, Tomilova A, Win T, Zubrii N, Vinarski M (2019) Freshwater mussels house a diverse mussel-associated leech assemblage. Scientific Reports 9 (1). [https://doi.org/10.1038/s41598-019-52688-3](https://doi.org/10.1038/s41598-019-52688-3)
- Chiangkul K, Trivalairat P, Kunya K, Purivirojkul W (2021) *Placobdelloides tridens* sp. n., a new species of glossiphoniid leech (Hirudinea: Rhynchobdellida) found feeding on captive *Orlitia borneensis* in Thailand, and an update to the host distribution of *P. siamensis*. Systematic Parasitology 98 (2): 141-154. [https://doi.org/10.1007/s11230-021-09967-3](https://doi.org/10.1007/s11230-021-09967-3)
- Corse E, Meglécz E, Archambaud G, Ardisson M, Martin J, Tougard C, Chappaz R, Dubut V (2017) A from-benchtop-to-desktop workflow for validating HTS data and for taxonomic identification in diet metabarcoding studies. Molecular Ecology Resources 17 (6): e146-e159. [https://doi.org/10.1111/1755-0998.12703](https://doi.org/10.1111/1755-0998.12703)
- Dmitrović D, Pešić V (2020) An updated checklist of leeches (Annelida: Hirudinea) from Bosnia and Herzegovina. Ecologica Montenegrina 29: 10-19. [https://doi.org/10.37828/em.2020.29.2](https://doi.org/10.37828/em.2020.29.2)
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3 (5): 294-9.
• Grosser C, Pešić V, Gligorović B (2015a) A checklist of the leeches (Annelida: Hirudinea) of Montenegro. Ecologica Montenegrina 2 (1): 20-28. https://doi.org/10.37828/em.2015.2.2
• Grosser C, Pešić V, Lazarević P (2015b) A checklist of the leeches (Annelida: Hirudinida) of Serbia, with new records. Fauna Balkana 3: 71-86.
• Grosser C, Pešić V, Berlajolli V, Gligorović B (2016) Glossiphonia balcanica n. sp. and Dina prokletijaca n. sp. (Hirudinida: Glossiphoniidae, Erpobdellidae) - two new leeches from Montenegro and Kosovo. Ecologica Montenegrina 8: 17-26. https://doi.org/10.37828/em.2016.8.2
• Hall T (1999) A BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.
• Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17 (8): 754-755. https://doi.org/10.1093/bioinformatics/17.8.754
• Jueg U (2013) Rote Liste der gefährdeten Egel und Krebsegel Mecklenburg – Vorpommerns, 1. Fassung. Ministerium für Landwirtschaft, Umwelt und Verbraucherschutz Mecklenburg-Vorpommern. 56 pp.
• Jueg U, Michalik P (2018) Lost and found – Fritz Müller’s type material of Glossiphonia verrucata (Fr. Müller, 1844) (Hirudinida, Glossiphoniidae) with notes on the leech fauna of lake Tegel in Berlin (Germany). Evolutionary Systematics 2 (2): 163-168. https://doi.org/10.3897/evolsyst.2.30793
• Kapli P, Lutteropp S, Zhang J, Kobert K, Pavlidis P, Stamatakis A, Flouri T (2017) Multi-rate Poisson Tree Processes for single-species species delimitation under Maximum Likelihood and Markov Chain Monte Carlo. Bioinformatics 33 (11): 1630-1638. https://doi.org/10.1093/bioinformatics/btx025
• Kaygorodova I, Bolbat N, Bolbat A (2020) Species delimitation through DNA barcoding of freshwater leeches of the Glossiphonia genus (Hirudinida: Glossiphoniidae) from Eastern Siberia, Russia. Journal of Zoological Systematics and Evolutionary Research 58 (4): 1437-1446. https://doi.org/10.1111/jzs.12385
• Kaygorodova IA, Mandzyak NB (2014) Molecular phylogeny of Siberian glossiphoniidae (Hirudinea). Molecular Biology 48 (3): 452-455. https://doi.org/10.1134/s0026893314030091
• Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35 (6): 1547-1549. https://doi.org/10.1093/molbev/msy096
• Kvist S, Oceguera-Figueroa A, Siddall M, Erséus C (2010) Barcoding, types and the Hirudo files: Using information content to critically evaluate the identity of DNA barcodes. Mitochondrial DNA 21 (6): 198-205. https://doi.org/10.3109/19401736.2010.529905
• Light J, Siddall M (1999) Phylogeny of the leech family Glossiphoniidae based on mitochondrial gene sequences and morphological data. The Journal of Parasitology 85 (5): 815-823. https://doi.org/10.2307/3285816
• Mack J, Kvist S (2019) Improved geographic sampling provides further evidence for the separation of Glossiphonia complanata and Glossiphonia elegans (Annelida: Clitellata: Glossiphoniidae). Journal of Natural History 53: 335-350. https://doi.org/10.1080/00222933.2019.1590658
• Moser W, Richardson D, Hammond C, Lazo-Wasem E (2012) Molecular characterization of Glossiphonia elegans (Verrill, 1872) (Glossiphoniidae: Hirudinida)
from its type locality, West River, New Haven County, Connecticut, USA. Zootaxa 3195 (1): 287-294. https://doi.org/10.11646/zootaxa.3195.1.4

- Nesemann H (1997) Egel und Krebsgei (Clitellata: Hirudinea, Branchiobdellida) Österreichs. Sonderheft der Ersten Vorarlberger Malakologischen Gesellschaft, Rankweil, 104 pp.

- Nesemann H, Neubert E (1999) Clitellata, Branchiobdellata, Acanthobdellata, Hirudinea. In: Schwobel J, Zwig P (Eds) Susswasser fauna von Mitteleuropa. Edition 6, Volume 2. Spectrum Akademischer Verlag, Heidelberg, Berlin, 178 pp.

- Oceguera-Figueroa A, León-Régagnon V (2014) Biodiversidad de sanguijuelas (Annelida: Euhirudinea) en México. Revista Mexicana de Biodiversidad 85: 183-189. https://doi.org/10.7550/rmb.33212

- Pérez-Flores J, Rueda-Calderon H, Kvist S, Siddall ME, Oceguera-Figueroa A (2016) From the worm in a bottle of Mezcal: iDNA confirmation of a leech parasitizing the Antillean Manatee. Journal of Parasitology 102 (5): 553-555. https://doi.org/10.1645/16-46

- Puillandre N, Brouillet S, Achaz G (2021) ASAP: assemble species by automatic partitioning. Molecular Ecology Resources 21 (2): 609-620. https://doi.org/10.1111/1755-0998.13281

- Ronquist F, Teslenko M, van der Mark P, Ayres D, Darling A, Höhna S, Larget B, Liu L, Suchard M, Huelsenbeck J (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61 (3): 539-542. https://doi.org/10.1093/sysbio/sys029

- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4 (4): 406-25. https://doi.org/10.1093/oxfordjournals.molbev.a040454

- Siddall M, Burreson E (1998) Phylogeny of leeches (Hirudinea) based on mitochondrial cytochrome oxidase subunit I. Molecular Phylogenetics and Evolution 9 (1): 156-162. https://doi.org/10.1006/mpev.1997.0455

- Siddall M, Budinoff R, Borda E (2005) Phylogenetic evaluation of systematics and biogeography of the leech family Glossiphoniidae. Invertebrate Systematics 19 (2). https://doi.org/10.1071/iss04034

- Sket B (1968) Poznavanje favne pijavk (Hirudinea) v Jugoslaviji [Zur Kenntnis der Egelfauna (Hirudinea) Jugoslawiens]. Academia Scientiarum et Artium Slovenica Classis IV: Historia Naturalis et Medicina Diss. Ljubljana 9 (4): 127-197.

- Trajanovski S, Albrecht C, Schreiber K, Schultheiß R, Stadler T, Benke M, Wilke T (2010) Testing the spatial and temporal framework of speciation in an ancient lake species flock: the leech genus Dina (Hirudinea: Erpobdellidae) in Lake Ohrid. Biogeosciences 7 (11): 3387-3402. https://doi.org/10.5194/bg-7-3387-2010

- Utevsky S, Utevsky A, Pešić V (2013) First record of Glossiphonia nebulosa (Hirudinida: Glossiphoniidae) from the Skadar Lake in Montenegro. Lauterbornia 76: 123-125.

- Yu G, Rao D, Matsui M, Yang J (2017) Coalescent-based delimitation outperforms distance-based methods for delineating less divergent species: the case of Kurixalus odontotarsus species group. Scientific Reports 7 (1): 16124. https://doi.org/10.1038/s41598-017-16309-1
Supplementary material

Suppl. material 1: Table S1.

Authors: Milica Jovanović, Elisabeth Haring, Helmut Sattmann, Vladimir Pešić
Data type: Table
Brief description: Interspecific mean K2P distances (below diagonal) and mean $p$-distances (above diagonal) and the standard deviations. Diagonal: the ranges of intraspecific genetic divergence are marked in bold font (the mean values are given in parentheses; K2P and $p$-distance gave similar results). The number of specimens considered for each species is indicated in parentheses. In the group of G. cf. nebulosa (n = 8), the three G. verrucata sequences from Italy and Croatia were included for the calculations.

Download file (14.15 kb)